DISSERTATION

STUDIES ON THE BIOSYNTHESIS OF PARAHERQUAMIDE A AND THE TOTAL SYNTHESIS OF (±) VM55599

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY EMILY M. STOCKING ENTITLED "STUDIES ON THE BIOSYNTHESIS OF PARAHERQUAMIDE A AND THE TOTAL SYNTHESIS OF (±) VM55599" BE ACCEPTED AS FULFILLING, IN PART, REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Adviser

Department Head

Abstract of Dissertation

Studies on the Biosynthesis of Paraherquamide A

Studies that partially elucidate the biosynthetic pathway of the heptacyclic fungal metabolite, Paraherquamide A are presented. Through biosynthetic feeding experiments with stable isotope labeled compounds, it was determined that the carbon skeleton of paraherquamide A consists of three amino acids and two equivalents of dimethylallyl pyrophosphate. L-methionine was determined to be the precursor to C-29, L-tryptophan was determined to be the precursor to the oxindole moiety and L-isoleucine was determined to be the precursor to the β-hydroxy-β-methylproline ring of paraherquamide A. A subsequent feeding experiment with [5-$^{13}C^{3}H_2$]-L-isoleucine indicated that L-isoleucine is converted to β-methyl-proline via a 4-electron oxidation and that the pro-*S* hydrogen of C-5 is retained in the oxidative cyclization. The incorporation of [1-^{13}C]-3(S)-methyl-L-proline also indicated that L-isoleucine undergoes oxidative cyclization prior to coupling to L-tryptophan.

The two dimethylallyl pyrophosphate-derived portions of paraherquamide A were found to arise *via* the well-known mevalonic acid pathway. In addition, it was determined that *P. fellutanum*, a paraherquamide A producing fungus, installs theses two dimethylallyl groups in two stereo-facially distinct manners. The C_5 unit (C-19~C-23) leading to the bicyclo[2.2.2]diazaoctan ring is formed in an entirely non-stereospecific manner, while the C-5 unit (C-24~C-28) leading to the dioxepin ring is installed in a

completely stereospecific manner. Only the latter observation is consistent with the mechanism of known prenylase enzymes.

The first total synthesis of VM55599, a proposed precursor of paraherquamide A, was completed. Feeding experiments with doubly ^{13}C-labeled racemic VM55599, its diastereomer and the oxidized forms of these compounds were performed on *P. fellutanum*. Only the diasteromer of VM55599 (**244a**) was incorporated. The incorporation of the bicyclic intermediate signifies that the dimethylallyl group leading to C-19~C-23 of paraherquamide A is installed prior to the dimethylallyl group leading to C-24~C-28. In addition, the incorporation **244a** indicates that the oxidations of the indole ring leading to the spirooxindole and the dioxepin ring occur after the formation of the bicyclic ring system.

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And last but *definitely* not least…

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I didn't fully see, until the cancer how we fight everyday against the creeping negatives of the world, how we struggle daily against the slow lapping of cynicism. Dispiritedness and disappointment, these were the real perils of life…

-Lance Armstrong

It's Not About the Bike

My Journey back to Life

Table of Contents

List of Abbreviations

Ac_2O	acetic anhydride
AcOH	acetic acid
Bn	benzyl
BOC	tert-butoxycarbonyl
$(BOC)_2O$	di-tert-butyl dicarbonate
BOP reagent	benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate
BSTFA	bis(trimethylsilyl)trifluoroacetamide
Bz	benzoyl
18-C-6	1,4,7,10,13,16-hexaoxacyclooctadecane
CAN	ceric ammonium nitrate
Cbz	benzyloxycarbonyl
CoA	co-enzyme A
Collidine	2,4,6-trimethylpyridine
m-CPBA	meta-chloroperbenzoic acid
CSA	camphorsulfonic acid
DABCO	1,4-diazabicyclo[2.2.2]octane

DBU	1,8-diazobicyclo[5.4.0]undec-7-ene
DCC	1,3-dicyclohexylcarbodiimide
DCU	1,3dicyclohexylurea
DDQ	2,3-dicloro-5,6-dicyano-1,4-benzoquinone
DIBAL	diisobutylaluminum hydride
DKP	diketopiperazine
DMAP	4-*N,N*-dimethylaminopyridine
DMAPP	Dimethylallyl pyrophophate
DME	1,2-dimethoxyethane
DMEA	dimethylethyl amine
DMF	*N,N*-dimethylformamide
DMPU	1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*) Pyrimidinone
DMS	dimethyl sufide
DMSO	dimethyl sulfoxide
Et	ethyl
EtOAc	ethyl acetate
EtOH	Ethanol
HMPA	hexamethylphosphoramide
im	1-imidazolyl
IPP	isopentylpyrophosphate
LDA	lithium diisopropylamine
2,6-lutidine	2,6-dimethylpyridine

Me	methyl
MeOH	methanol
MOM	methoxymethyl
Ms	methanesulfonyl(mesylate)
MTPI	methyl triphenoxyphosphonium iodide
NCS	N-chlorosuccinimide
PMB	p-methoxybenzyl
PPTS	pyridinium p- toluenesulfonate
PTLC	preparative thin layer chromatography
PP	pyrophosphate
i-Pr	isopropyl
Pv	pivaloyl
Py	pyridine
TBDMS	tert-butyldimethylsilyl
TPP	thiamine pyrophophate
t-BDMSCl	tert-butyldimethylsilyl chloride
t-BDMSOTf	tert-butyldimethylsilyl triflouromethanesulfonate
TBAF	tetrabutylammonium fluoride
TBDPS	tert-butyldiphenylsilyl
TBS	tert-butyldimethylsilyl
t-BuOH	tert-butanol
Tf	triflouromethanesulfonate

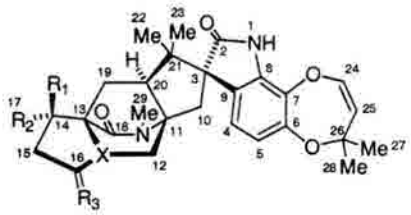
TFAA	triflouroacetic anhydride
TFA	triflouroacetic acid
THF	tetrahydrofuran
THP	tetrahydropyran
TLC	thin layer chromatography
TMS	trimethylsilyl
TMSI	trimethylsilyl iodide
TS	toluenesulfonyl
Z	benzyloxycarbonyl

CHAPTER 1

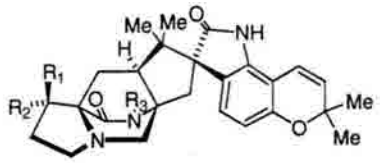
Introduction

1.1 Isolation and Structural Determination

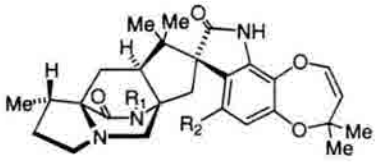
The paraherquamides (**1-13**) are a group of heptacyclic mycotoxins isolated from various *Penicillium sp.* and *Aspergillus sp.* Structurally, the paraherquamides vary with respect to substitution and oxygenation in the proline ring and the prenylated oxindole ring. The first member of this family of mycotoxins discovered was Paraherquamide A (**1**), isolated in 1980 from *Penicillium paraherquei* by Yamazaki and co-workers.[1] Subsequently, paraherquamides A-G (**1-4, 7, 10-11**) were isolated from *Penicillium charlesii (fellutanum)* by a group at Merck.[2] Paraherquamides A (**1**), E (**7**), F (**10**), and G (**11**) were also isolated from a *penicillium sp.* found in the soil of Kemer, Turkey (IMI332995) by a group at Smith-Kline Beecham. Several related compounds, including VM55595 (**12**), VM55596 (**5**), VM55597 (**6**) and VM55599 (**13**), were also isolated from this strain.[3] VM55599 (**13**) is the only member of the family that contains an indole ring in lieu of an oxindole ring. The most recent additions to the paraherquamide family are SB203105 (**8**) and SB200437 (**9**), isolated by the same Smith-Kline Beecham group in 1998 from an *Aspergillus sp.* (IMI337664).[4]



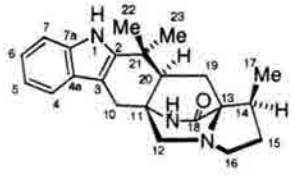
1, (–)-paraherquamide A, R_1 = OH, R_2 = Me, R_3 = H_2, X = N
2, (–)-paraherquamide B, R_1 = H, R_2 = H, R_3 = H_2, X = N
3, (–)-paraherquamide C, R_1 = R_2 = CH_2, R_3 = H_2, X = N
4, (–)-paraherquamide D, R_1 = O, R_2 = CH_2, R_3 = H_2, X = N
5, VM55596, R_1 = OH, R_2 = Me, R_3 = H_2, X = N^+-O^-
6, VM55597, R_1 = OH, R_2 = Me, R_3 = O, X = N



10, paraherquamide F (VM55594), R_1 = H, R_2 = Me, R_3 = Me
11, paraherquamide G (VM54158), R_1 = OH, R_2 = Me, R_3 = Me
12, VM55595, R_1 = H, R_2 = Me, R_3 = H



7, paraherquamide E, (VM54159), R_1 = Me, R_2 = H
8, SB203105, R_1 = Me, R_2 = OH
9, SB200437, R_1 = H, R_2 = H



13, VM55599

Figure 1: Structure of the paraherquamides.

The structure and relative stereochemistry of paraherquamide A (**1**) was established by Yamazaki and co-workers through NMR, IR, UV, MS and single crystal X-ray analysis.[1] Later, the absolute stereochemistry was determined from X-ray analysis of a semi-synthetic paraherquamide derivative (**14**).[5] In 1990, a group at Merck, Sharp & Dohme unambiguously assigned all of the proton and carbon resonances of paraherquamide A (**1**) in the NMR spectra and determined the structure of paraherquamides B-G (**2-4, 7, 10-11**) through NMR and MS analysis.[6] In addition, the relative stereochemistry of paraherquamides D-G (**4**, **7**, **10**, **11**) were assigned via pure absorptive mode 2-D nOe experiments and were in agreement with the published crystal structure of paraherquamide A (**1**) and the brominated dervivative (**14**).[6] The structural assignments for paraherquamides A (**1**), E (**7**, VM54159), F (**10**, VM55594), and G (**11**,

VM55158) were independently corroborated through structural analysis (MS, 1D NMR, 2D NMR and ^{1}H nOe) performed by Blanchflower and co-workers.[3a]

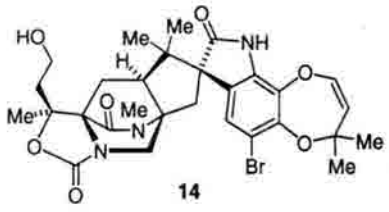


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Figure 2: X-ray crystal analysis of this brominated derivative was used to secure the structure of paraherquamide A.[5]

Blanchflower et al. also isolated and determined the structure of VM55595 (**12**), VM55596 (**5**), VM55597 (**6**) and VM55599 (**13**).[3b] The ^{1}H and ^{13}C chemical shifts as well as ^{1}H nOe data indicated that the relative stereochemistries of VM55595 (**12**), VM55596 (**5**) and VM55597 (**6**) were the same as paraherquamide A (**1**). The structure of VM55599 (**13**) was assigned by 2D ^{1}H COSY NMR and 2D ^{1}H, ^{13}C COLOC NMR. It was determined that VM55599 (**13**) contained an indole ring rather than an oxindole ring and did not possess an oxepin or dioxepin derivative. The relative stereochemistry of VM55599 (**13**) was assigned based on ^{1}H nOe analysis. In their investigation, Blanchflower and co-workers assumed that the stereochemistry of VM55599 (**13**) was the same at C-20 as paraherquamide A (**1**) based on biogenic considerations. The absolute stereochemistry predicted by Blanchflower et al. is the opposite of the depicted stereochemistry above in Figure 1. The absolute stereostructure as drawn is based on further biogenic findings to be discussed in Chapter 3.

1.2 Pharmacology:

Considerable interest was generated by the discovery of paraherquamide because of its potent anthelminthic activity. Helminths are parasitic worms of the intestine such as tapeworm, roundworm and hookworm. Because of the deleterious effects of helminths on livestock (causing sickness or death of the host animal), the search for new anthelminthic drugs is an important endeavor in the field of veterinary medicine. Currently there are three classes of anthelminthics in use: (1) the benzimidazoles (discovered in the 1950's), (2) levamisole/ morentel (discovered in the 1960's), and (3) the ivermectin/ milbemycins (discovered in the 1970's). Unfortunately, as is the case with many drugs, resistance has become a problem. The benzimidazoles and levamisole/ morentel have lost much of their original efficacy because of the resistance built up by the helminths.[7] The ivermectins are also showing signs of inactivity against various strains of helminths.[8] Paraherquamide A has proven to be effective against ivermectin-resistant *Haemonchus contortus* and ivermectin/ benzimidazole-resistant *Trichostronglylus colubriformis*.[9] The efficacy of paraherquamide A against these resistant strains indicates a novel mode of action and caused quite a stir in the field of veterinary medicine. As a result, the pharmacology of paraherquamdie itself was extensively studied and chemically modified analogs were developed.

In vivo evaluations of paraherquamide A were first performed with six-day-old *T. colubriformis* in gerbils. Paraherquamide A was more than 98% effective at dose levels of 1.56 mg/ kg and above and a single oral dose of 200 mg/ kg was well tolerated by the gerbils.[10] Further animal studies were done on sheep and cattle.[10,11] Paraherquamide A proved highly efficacious and had an excellent safety profile in both models. In the sheep

model, paraherquamide displayed a 92% and above efficacy at 0.25 mg/ kg against all but one of the common gastrointestinal nematodes (*Oesophagostomum columbianum*).[10] In cattle, similar results were seen. Paraherquamide A had a > 95% efficacy at 1.0 mg/kg for 7 out of 8 parasites tested. Only *C. puncta* was not well affected (0% efficacy at the 0.5 mg/ kg dosage).[11]

Table 1: Toxicity studies on the paraherquamides.[2, 3]

Compound	*C. elegans* LD_{50} (μL/ ml)	*H. Contortus* MIC_{50} (μL/ ml)	*T. colubriformus* % reduction in fecal egg count[a]
paraherquamide A (**1**)	2.5	31.2	99
paraherquamide B (**2**)	100		
paraherquamide C (**3**)	40		
paraherquamide D (**4**)	160		
VM55596 (**5**)			94[c]
VM55597 (**6**)			0
paraherquamide E (**7**)	6	15.6	99
SB203105 (**8**)			73
SB200437 (**9**)			86[b]
paraherquamide F (**10**)	65	>500	62.5
paraherquamide G (**11**)	20	>500	0
VM55595 (**12**)			0
VM55599 (**13**)			0

[a]Gerbils dosed orally at 4 mg/ kg unless noted. [b]Gerbils dosed orally at 7.7 mg/ kg. [c]Gerbils dosed orally at 2 mg/ kg.

A drawback for the use of paraherquamide as a veterinary medicine arose in the *in vivo* evaluations with the dog model. [12] At doses as low as 0.5 mg/kg, the dogs showed adverse effects (including depression, ataxia and protrusion of the nictitating membrane) within one hour of treatment. In addition, efficacy was < 78% for the four strains of helminths tested at every dosage level. These unfavorable results prompted a toxicity profile comparing paraherquamide to ivermectin and using mice as the animal host.[13] It

was found that the estimated lethal dose to 50% of a group of mice was 14.9 mg/ kg for paraherquamide and 29.5 mg/ kg for ivermectin. It was also determined that the "no effect" doses were 5.6 and 18.0 mg/kg for paraherquamide and ivermectin respectively. Thus, paraherquamide has an acute safety factor that is approximately one-half of ivermectin. Because of its toxicity and its decreased efficacy in dogs, paraherquamide itself has very little potential as a broad-spectrum anthelminthic. However, the results obtained in sheep and cattle indicate it does have potential as a narrow-spectrum anthelminthic for ruminant animals against specific resistant species of helminths.

Many attempts were made to moderate the toxicity and increase the anthelminthic activity of paraherquamide by synthesizing analogs.[14] It was discovered that few modifications could be made without adversely affecting the biological activity of paraherquamide. Although substitution at C-17 is tolerated, the natural stereochemistry at C-14 must be maintained to retain biological activity. Analogs modified in the vinyl ether region (dioxepin ring) also retained activity against the free living nematode model, *Caenorhabditis elegans*, but analogs modified at the N-1 position were significantly less active. Nevertheless, none of the reported analogs displayed greater efficacy than paraherquamide A itself.

1.1 Structurally Related Fungal Metabolites:

Besides the paraherquamides, there are a number of other structurally related fungal metabolites that possess the bicyclo[2.2.2]diazaoctan skeleton (Figure 3). The brevianamides (**15**-**16**) were the first members of this class of compounds discovered.[15] The brevianamides vary from the paraherquamides in that they possess an indoxyl ring in

lieu of an oxindole moiety and do not possess a dioxepin ring. Interestingly, (+)-brevianamide A has the opposite absolute configuration relative to all other members of this class of compounds. In addition, the brevianamides have an *anti*-relationship between the C-20 stereogenic center (paraherquamide numbering) and the prolyl ring, whereas the paraherquamides and all other known members of this class of compounds possess a *syn*-relationship between the C-20 center and the cyclic amino acid residue (Figure 4).

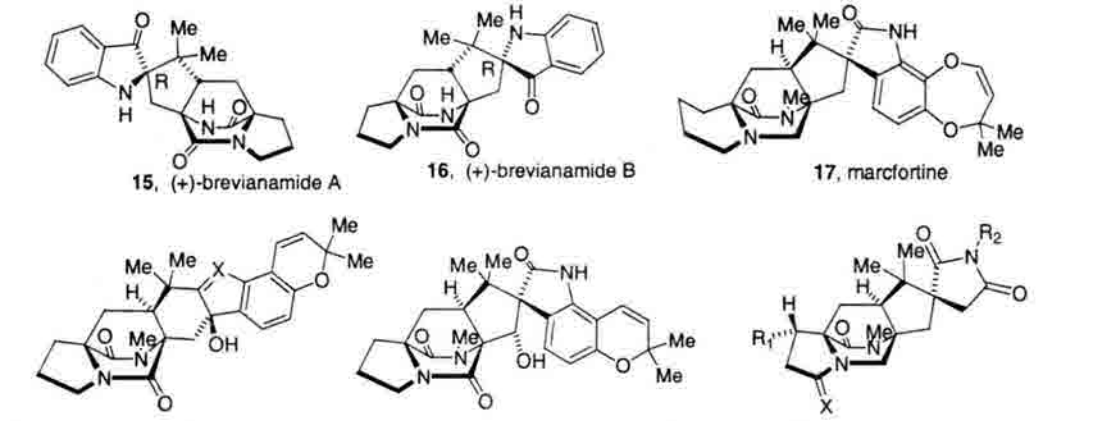


Figure 3: Compounds structurally related to the paraherquamides.

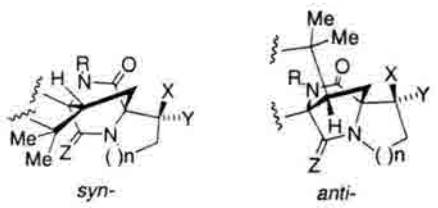


Figure 4: The *syn-/anti-* relationship in the bicyclo[2.2.2]diazaoctan ring system.

Polansky and co-workers isolated marcfortine (**17**) in 1980 from *Penicillium roqueforti*, a fungus used in the production of blue cheese.[16] Structurally, marcfortine is the closest relative of paraherquamide A, varying only in the ring size of the amino acid subunit. Marcfortine contains a pipecolic acid residue, whereas paraherquamide contains a proline ring. Biosynthetic studies showed that lysine is the precursor to the pipecolic acid residue.[17] These studies also showed that tryptophan was the precursor to the oxindole ring, methionine is the precursor to C-29, the N-methyl group, and that the isoprene groups are most likely derived via the mevalonate pathway.

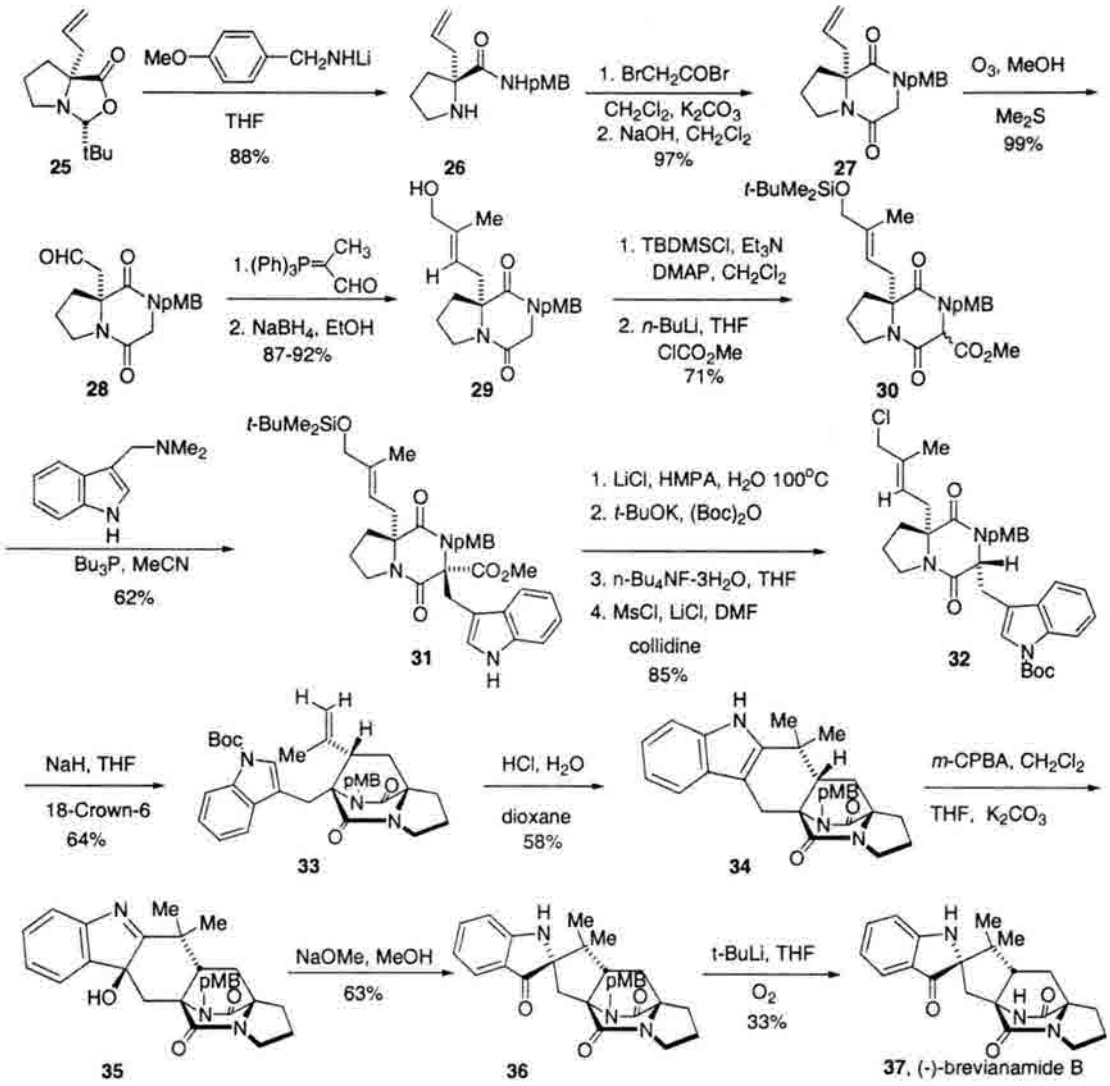
Other fungal metabolites possessing the bicyclo[2.2.2]diazaoctan include aspergamide A (**18**) and aspergamide B (**19**), isolated by Zeek et al. from *Aspergillus ochraceus*;[18] sclerotamide (**20**), isolated from *Aspergillus sclerotiorium*;[19] and the asperparalines (**21**-**24**), isolated from *Aspergillus japonicus*[20] and *Aspergillus sp.* IMI 337664.[4] It is interesting to note that all of these metabolites were isolated outside the *Penicillia*. In addition, the asperparalines are the only members in this class of compounds that are apparently not derived from tryptophan.

1.3 Synthesis:

Brevianamide B was the first member of the bicyclo[2.2.2]diazaoctan class of compounds to be synthesized.[21] The total synthesis, completed by Willams and co-workers in 1988, began with allyated proline derivative **25**, prepared according to the method of Seebach.[22] Reaction of **25** with the lithium salt of *p*-methoxybenzylamine furnished the amide **26**, which was then converted to the diketopiperazine **27** by acylation with bromoacetyl bromide and ring closure under basic conditions. The aldehyde **28**,

formed by ozonolysis of **27**, was converted to the E-allylic alcohol **29** via a Wittig reaction and $NaBH_4$ reduction. Protection of the ether with TBDMSCl followed by carbomethoxylation provided the intermediate **30**.

Scheme 1: Total Synthesis of (-)-Brevianamide B.



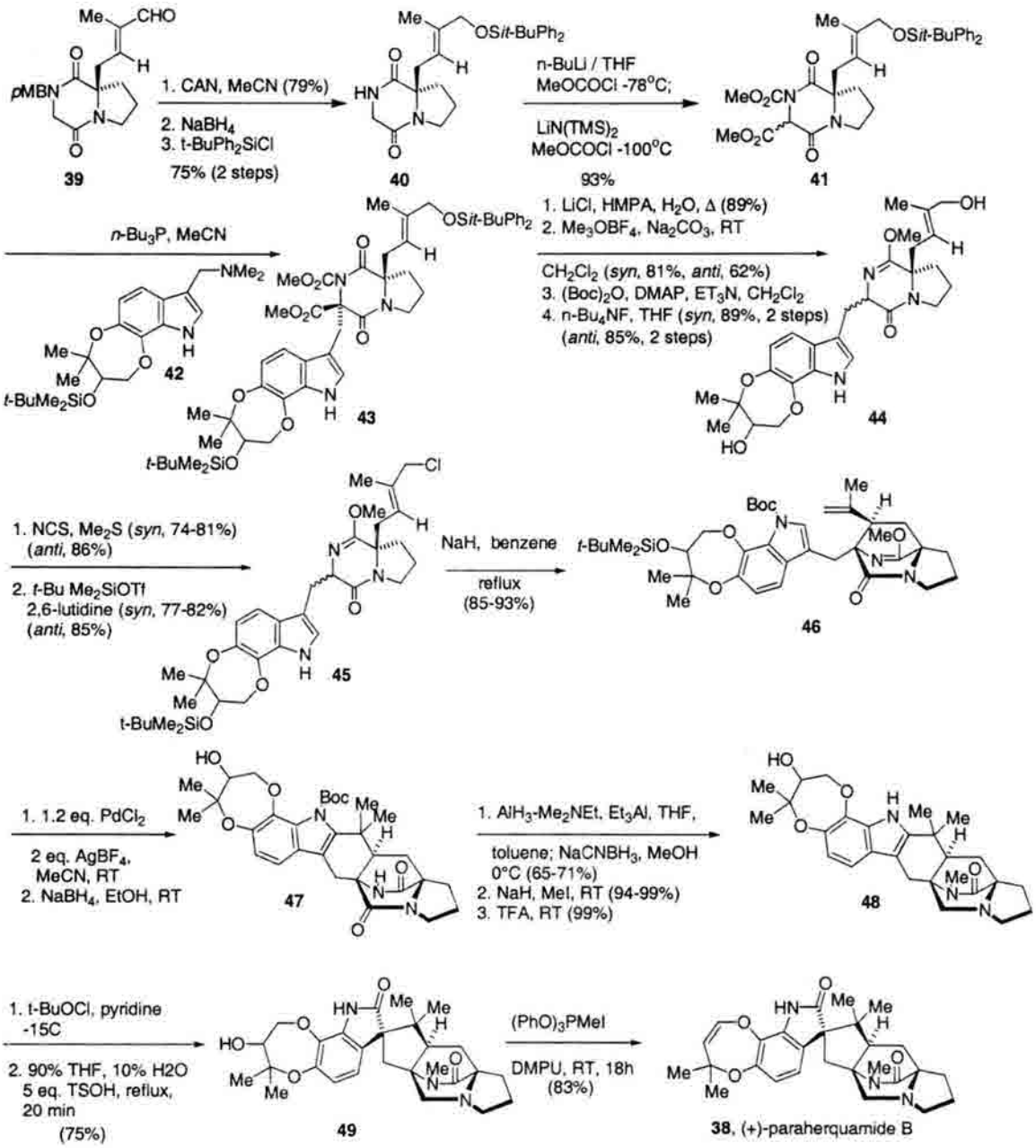
Treatment of **30** with gramine and *n*-tributylphosphine in refluxing acetonitrile provided **31** exclusively as the *syn*-diasteromer.[23] Decarbomethoxylation, N-Boc

protection of the indole nitrogen, removal of the TBDMS protecting group and conversion to the allylic chloride led to the precursor **32**. An intramolecular SN2′ cyclization of **32** was accomplished with 18-crown-6 and sodium hydride in THF to provide the intermediate **33** in a 3-4.9:1 *anti*:*syn* ratio. The hexacyclic intermediate **34** was formed by removal of the N-Boc group and electrophilic cyclization with HCl/dioxane. Stereospecific oxidation with *m*-CPBA provided the hydroxyindolenine **35**, which was treated directly with NaOMe in methanol to give the indoxyl **36**. Removal of the *p*-methoxybenzyl group provided (-)-brevianamide B (**37**) which, except for the optical rotation, was identical to the natural product **16**.

The approach used for the total synthesis of (-)-brevianamide B (**37**) was also used to construct (+)-paraherquamide B (**38**, Scheme 2).[24] The enal **39**, synthesized by the same method used in the synthesis of (-)-brevianamide B (**37**), was treated with ceric ammonium nitrate to remove the *p*-methoxybenzyl group. Reduction of the amide and protection of the resultant alcohol with tert-butyldiphenylsilyl chloride provided **40**. A one pot, two-step carbomethoxylation provided the desired intermediate **41**. Coupling of **41** with the gramine derivative **42** was accomplished in refluxing acetonitrile with 0.5 equivalents of *n*-tributylphophine.[23]

The so-formed indole **43** was decarbomethoxylated with LiCl in wet HMPA and then converted to the lactim ether with Me_3OBF_4. N-Boc protection of the indole and deprotection of both TBS ethers provided the diol **44**. Conversion of the allylic alcohol to the allylic chloride followed by reprotection of the secondary alcohol furnished **45**. Treatment of **45** with sodium hydride in refluxing benzene gave the SN2′ cylization product **46** in good yields.

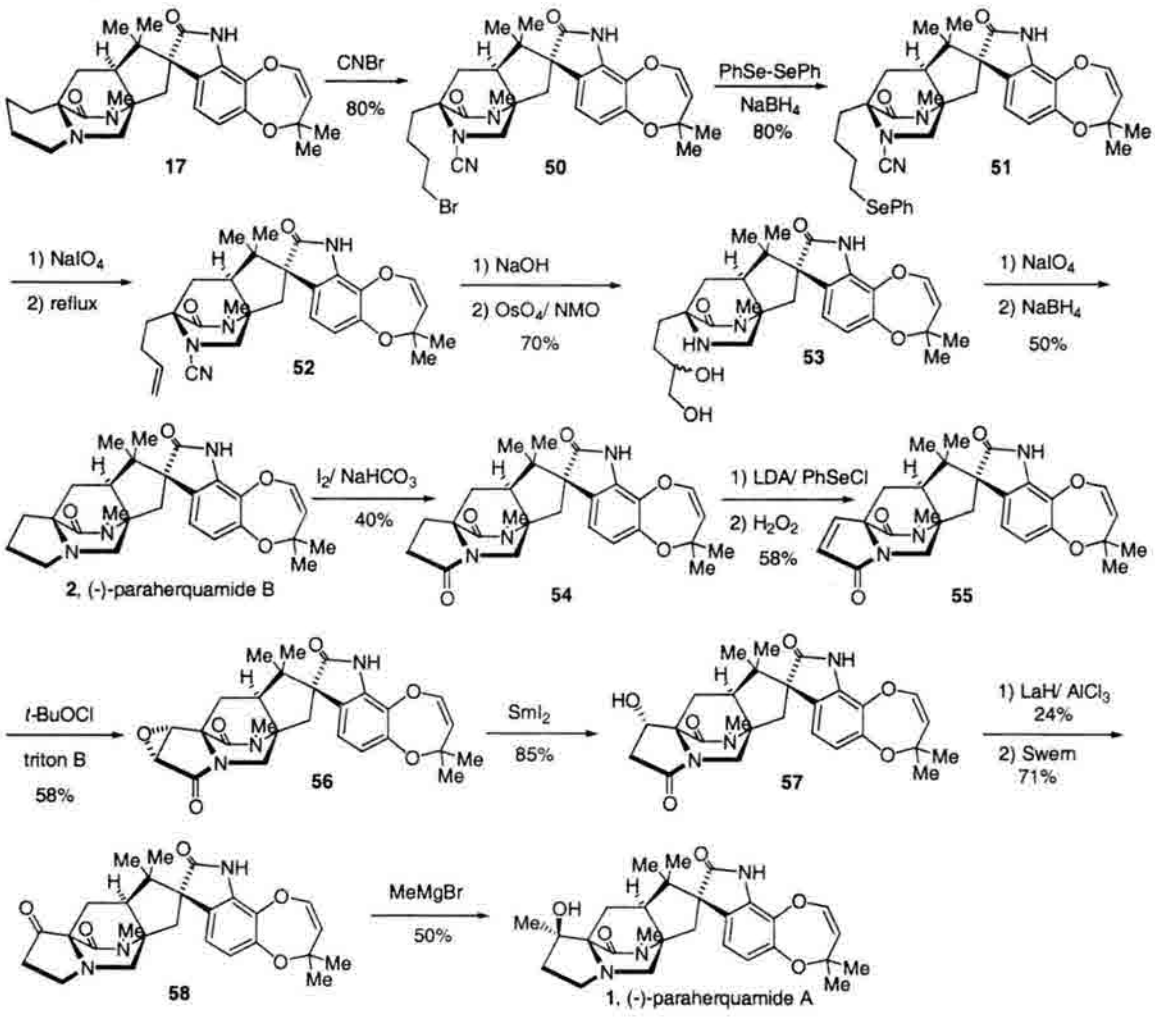
Scheme 2: The Total Synthesis of (+)-Paraherquamide B.[24]



A palladium-mediated Heck-type reaction followed by reduction with $NaBH_4$ provided **47**. Selective reduction of the tertiary amide, N-methylation and removal of

both the N-Boc and TBDMS protecting groups gave the heptacyclic indole **48**. The oxindole **49** was formed by an oxidative pinacole type rearrangement. Finally dehydration of **49** was accomplished with MTPI and DMPU to give (+)-paraherquamide B (**38**) in 83% yield.

Scheme 3: Synthesis of paraherquamide A from marcfortine A.[25]



The first synthesis of (-)-paraherquamide A (**1**) was a semi-synthetic conversion of marcfortine A, which possesses the same absolute configuration as the

paraherquamides, via (-)-paraherquamide B (**2**).[25] The synthesis of (-)-paraherquamide B (**2**) was accomplished in six steps by opening the six-membered pipecolic acid ring of marcfortine A (**17**), oxidative removal of one carbon and ring closure to form the five-membered proline ring (Scheme 3).

The Von Braun reaction of marcfortine with cyanogen bromide provided **50**. Conversion to the selenide **51** was accomplished with diphenyl diselenide and $NaBH_4$. Oxidation of **51** with $NaIO_4$ was followed by elimination of the selenol in refluxing benzene to provide the alkene **52**. Basic hydrolysis of **52** was followed by formation of the diol under osmylation conditions to give **53**. Paraherquamide B (**2**) was formed by cleavage of the diol with $NaIO_4$ and reductive amination with $NaBH_4$.

Oxidation of **2** with $I_2/NaHCO_3$ gave the lactam **54**. Lee and co-workers later described a one-step conversion of marcfortine A (**17**) to the lactam **54** through a platinum-oxygen mediated ring contraction.[26] Treatment of **54** with LDA/ PhSeCl then oxidation with H_2O_2 formed the α, β-unsaturated lactam **55**. Stereoselective epoxidation furnished **56**, which was converted to the alcohol (**57**) with SmI_2. Compound **58** was formed by selective reduction of the prolyl-δ-amide with LAH/ $AlCl_3$, and oxidation to the C-14 ketone. To introduce the methyl group, Lee and co-workers used a modification of the procedure developed by Blizzard and co-workers for the synthesis of a 5-brominated analog of paraherquamide A.[14a] Methylation of **58** with a 3M ethereal solution of methylmagnesium bromide in THF resulted in almost exclusive formation of the desired (natural) isomer of paraherquamide A (**1**) in 50% yield.

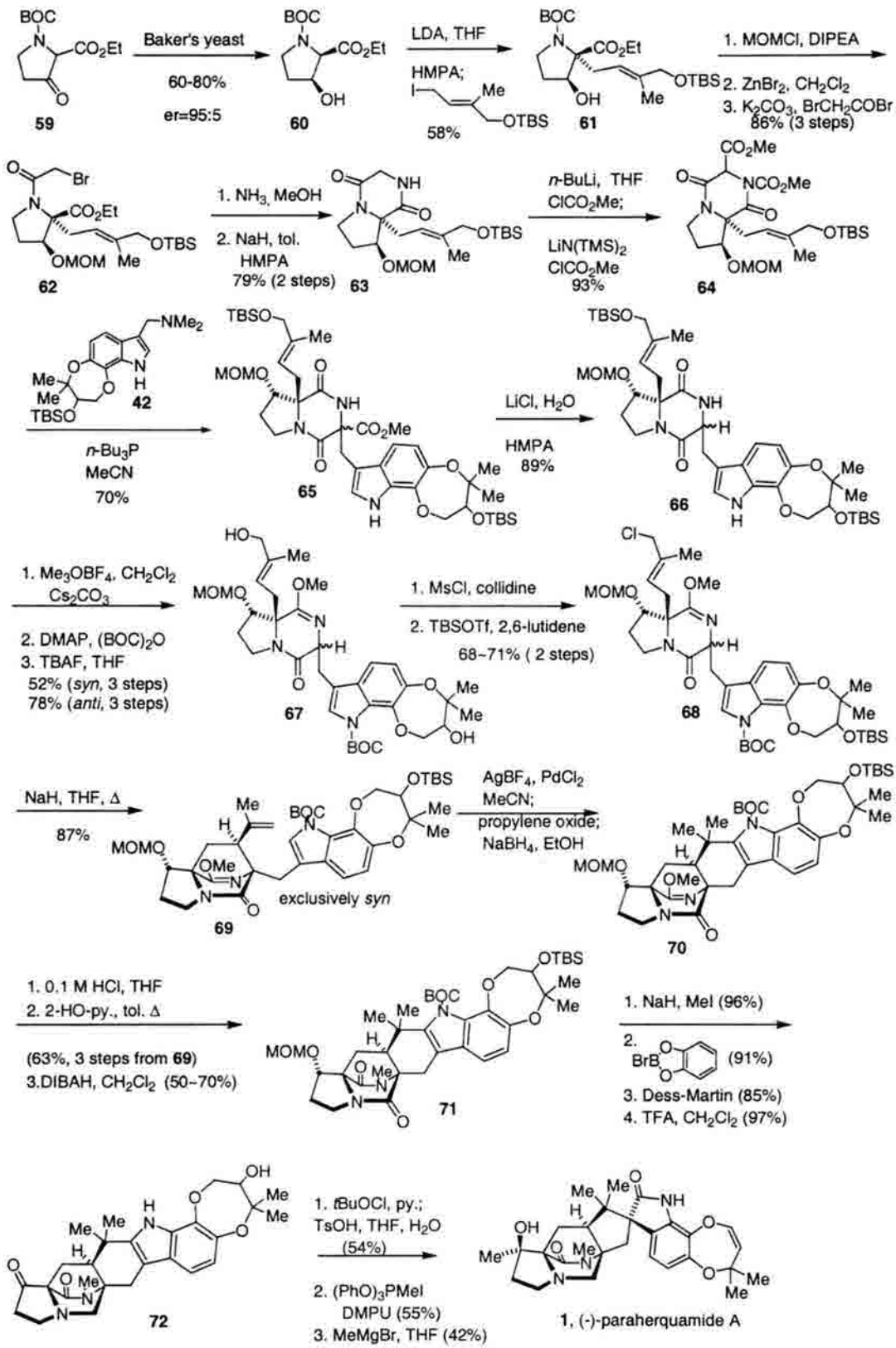
Jeffery Cao and Hidekadzu Tsujishima of the Williams research group recently completed the first total synthesis of paraherquamide A as outlined in Scheme 4.[27] The

Reduction of the β-keto ester **59** with the NADH-dependent alcohol dehydrogenase from baker's yeast provided the optically active β-hydroxy ester **60**. Dianion alkylation of **60** with (*E*)-3-methyl-4-(O-*tert*-butyldimethylsilyl)-2-butene afforded the α-alkylated product **61** with net retention of stereochemistry. The secondary alcohol was protected as the corresponding methoxymethyl (MOM) ether before removal of the N-BOC group with $ZnBr_2$ and subsequent alkylation of the secondary amine with bromoacetylbromide in the presence of K_2CO_3.

Amination of the bromoacetamide **62** was accomplished with methanolic ammonia providing the corresponding glycinamide, which was cyclized directly in the presence of sodium hydride to give the bicyclic diketopiperazine **63**. Double carbomethoxylation was achieved by the addition of n-BuLi and methylchloroformate, which carbomethoxylated the amide nitrogen, followed by the addition of four equivalents of methylchloroformate and five equivalents of $LiN(TMS)_2$ to provide **64**. The presence of the carbomethoxy group at the α-position of **64** allowed Somei coupling with the gramine derivative **42** in the presence of tri(*n*-butyl) phosphine.[23]

Decarbomethoxylation of **65** with LiCl in hot, aqueous HMPA provided **66** as a mixture of diastereomers. Formation of the diol **67** was achieved by protection of the secondary amide of **66** as the corresponding lactim ether followed by N-BOC protection of the indole nitrogen and removal of the silyl ether with tetrabutylammonium fluoride. The allylic alcohol (**67**) was selectively converted to the allylic chloride (**68**) by mesylation in the presence of collidine and the secondary alcohol was re-protected with *tert*-butyldimethylsilyl triflate and 2,6-lutidine.

Scheme 4: Total synthesis of paraherquamide A.[27]



The S_N2' cyclization, which forms the bicyclo[2.2.2]diazaoctan nucleus and sets the stereochemistry at C-20, was accomplished by treating **68** with NaH in refluxing THF and exclusively provided the desired *syn*-isomer. The final, seventh ring of **69** was closed by the addition of 4.68 equivalents of $PdCl_2$ and 3.1 equivalents of $AgBF_4$ in acetonitrile containing propylene oxide as an acid scavenger (to prevent cleavage of the MOM ether). The σ-palladium heptacyclic adduct formed in this reaction was immediately worked up by the addition of ethanol and sodium borohydride, providing the desired 2,3-disubstituted indole **70**.

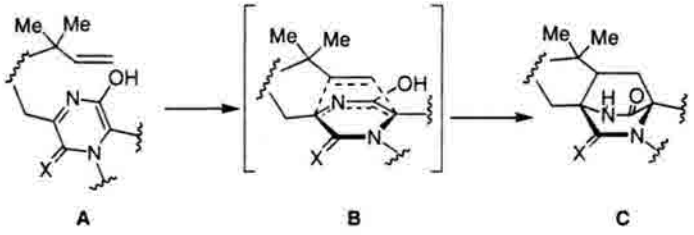
Cleavage of the lactim ether with 0.1M HCl resulted in ring opening of the diketopiperazine ring, which was readily re-cyclized in refluxing toluene containing catalytic 2-hydroxy pyridine. Excess diisobutylaluminum hydride was used to chemoselectively reduce the secondary amide, furnishing **71**. N-Methylation of **71**, removal of the MOM group, oxidation of the secondary alcohol with Dess-Martin periodane and subsequent cleavage of the remaining N-BOC and TBS ether protecting groups produced the ketone **72**.

Oxidative spirocyclization of **72** was attained by treatment with *tert*-butyl hypochlorite to form the labile 3-chloroindolenine followed by a *para*-toluenesulfonic acid mediated pinacol-type rearrangement. The dioxepin ring was formed by dehydration of the secondary alcohol, affording the same penultimate ketone (**58**) obtained by Lee et al. in their synthesis of paraherquamide A. The total synthesis of (-)-parahequamide A (**1**) was finally completed, as previously described, by the addition of methyl Grignard.

1.5 Research Objectives:

The intent of this dissertation is to elucidate the biosynthetic pathway of paraherquamide A. To achieve this objective, the origin of all the carbon atoms in paraherquamide A were determined. This included discovering the source of the unusual non-proteinogenic amino acid, β-methyl-β-hydroxy-proline, and the source of the isoprene moieties (C19-C23 and C24-C28).

Scheme 5: Proposed intramolecular hetero-Diels-Alder cycloaddition of the isoprene moiety across the α-carbons of the amino acid subunits to form the bicyclo[2.2.2] ring system of the paraherquamide family.



As previously mentioned the paraherquamides possess a unique bicyclo[2.2.2]diazaoctan ring. We, and others,[28] have proposed that this ring system is the product of a hetero-Diels-Alder cycloaddition (Scheme 5). Although there are many proposals of the Diels-Alder reaction in nature, there are only a few documented examples of natural products that might arise from enzymatic catalysis of the [4+2] cycloadditon (Chapter 2). Attempts were made to uncover the direct precursors to the proposed biosynthetic Diels-Alder reaction, which may eventually help divine any proteins that are involved in the cycloaddition reaction. In addition, a biomimetic total synthesis of VM55599 was completed to ascertain the feasibility of a Diels-Alder

reaction in this type of system. This synthesis was used to generate isotopically labeled compounds, including VM55599 itself, which were examined as advanced intermediates in the biosynthesis of paraherquamide A.

CHAPTER 2

Natural Products of Diels-Alder Biosynthetic Origin

A Review

2.1 Introduction

The Diels-Alder reaction is a powerful carbon-carbon bond forming reaction for synthetic chemists, allowing facile, stereospecific entry into six-membered ring systems.[29] The structures of various secondary metabolites have led to proposals indicating that Nature might also makes use of this valuable reaction.[30] An intriguing aspect of many of these biosynthetic proposals is the suggestion of enzymatic catalysis for the [4+2] cycloaddition, which would accommodate the observed stereochemistry. In a classical concerted Diels-Alder reaction, the transition state is highly ordered and resembles the product. However, since enzyme catalysis is typically based on stabilizing the transition state, enzymatic catalysis of a Diels-Alder reaction should result in product inhibition. The prospect of a Diels-Alderase is especially enticing to scientists, since it could represent a new mechanism of catalysis in nature.

2.1.1 Biocatalysis of the Diels-Alder Reaction

Until recently, the existence of a Diels-Alder enzyme remained elusive, but Diels-Alder catalysis by biomolecules was not unknown. Hilvert and co-workers first reported antibody catalysis of a Diels-Alder reaction in 1989.[31] Monoclonal antibodies were raised against hapten **1** (Figure 1), which resembles the transition state (**2**) of the Diels-Alder

reaction between tetrachlorothiophene dioxide (**3**) and N-ethyl-maliemide (**4**). By binding the diene **3** and dienophile **4** in a reactive conformation, the antibody catalyzes the Diels-Alder reaction by lowering the entropy of activation. The problem of product inhibition was overcome by extrusion of SO_2. Since the product (**5**) did not resemble **1**, which mimics the transition state (**2**), catalyst turnover was not impeded.

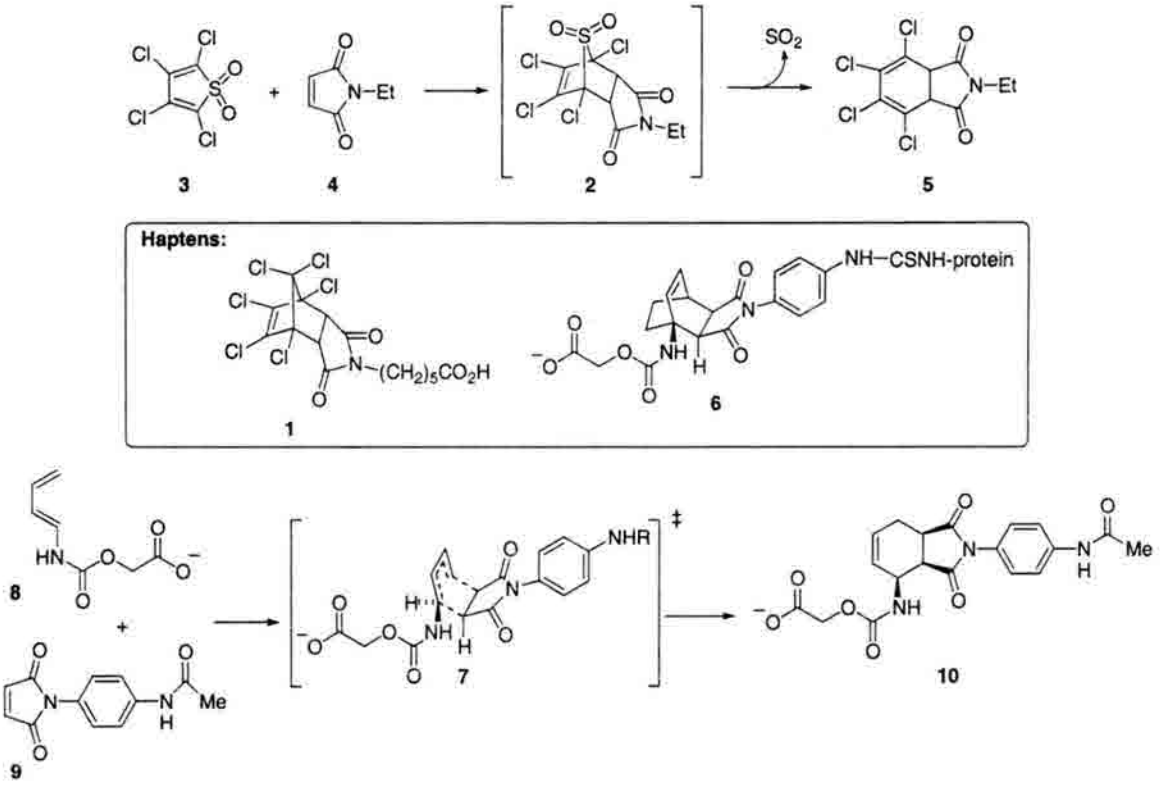


Figure 1: Antibody catalylsis of a Diels-Alder reactions.[31,32]

Braisted and Schultz used an alternative to approach to overcome the difficulty of product inhibition in Diels-Alder antibody catalysis (Figure 1).[32] They used an ethano bridge to lock the cyclohexene ring of the hapten **6** into a conformation resembling the proposed transition state (**7**) for the Diels-Alder reaction bewteen the acyclic diene **8** and

the dienophile **9**. Subsequent structure elucidation of this catalytic antibody revealed the presence of 89 van der Waals interactions and two hydrogen bonds between the antibody and its hapten. Apparently these interactions activate the dienophile and control the relative geometries of the bound substrates.[33]

Another type of biomolecule used to catalyze the Diels-Alder reaction is RNA.[34] However the mechanism of catalysis is radically different from catalytic antibodies. Since RNA Diels-Alderase activity is reliant on base specificity and the coordination of a transition metal, the mode of catalysis is probably akin to Lewis acid catalysis of the Diels-Alder reaction.

The role of protein organization in natural systems and the possible mechanism of catalysis has long been a subject of debate, rekindled by the recent characterization of two naturally occurring potential Diels-Alderases.[35,36] The isolation of theses enzymes also establishes the Diels-Alder reaction as a viable biosynthetic transformation.

2.1.2 Scope of This Review

This review is intended to provide an overview of the natural products from proposed Diels-Alder biogenic origin, both catalyzed and uncatalyzed, and the biosynthetic studies pertaining to these compounds. Although there are countless structures that can formally arise *via* a [4+2] cycloaddition, this review is limited to those natural products that are described in the literature as Diels-Alder cycloadducts. The review is organized into classes of compounds based on their biosynthetic derivations: polyketides (acetate), isoprenoids (mevalonate), phenylpropanoids (shikimic acid), and alkaloids (amino acids). In many cases, this segregation is superficial, since the

compounds are often of mixed biosynthetic origins (i.e. cytochalasans, pycnidone, and the brevianamides).

2.2 Polyketides

Since polyketides are derived from acetate, these compounds are particularly well suited for isotopic labeling studies. Thus, it is hardly surprising that a large portion of the experimental evidence for the Diels-Alder reaction in nature has been obtained in this class of compounds. In fact, both lovastatin and macrophomic acid, for which reported Diels-Alderase enzymes have been isolated,[35,36] are classified as polyketides.

2.2.1 Decalin Polyketides

2.2.1.1 Lovastatin (Mevinolin):

Lovasatin (**11**), also known as mevinolin, has received significant attention in the literature because it is a potent inhibitor of cholesterol biosynthesis in humans. Feeding experiments on the producing fungal strain, *Aspergillus terreus* (ATCC 20542), with [1-^{13}C], [2-^{13}C]-, [1,2-$^{13}C_2$]-, [1-^{13}C, $^{18}O_2$]-, [1-^{13}C, $^{2}H_3$]-, [2-^{13}C-$^{2}H_3$]-acetates established that lovastatin is comprised of an 18-carbon unit and a 4-carbon unit constructed through the head to tail attachment of acetate (Figure 2).[37] Interestingly, the only oxygen atom that could definitively be assigned as a derivative of acetate was O-1′. In addition, it was discovered that the two methyl groups at C-2′ and C-6 are derived from ^{13}C-methionine via S-adenosylmethionine (SAM).

Reexamination of the origin of the oxygen atoms in lovastatin was made possible by the subculture selection of a new strain of *A. terreus* (MF 4845), which increased

production of lovastatin to 200 mg/ L culture.[38] Fermentation of *A. terreus* in the presence of an [$^{18}O_2$] atmosphere showed the oxygen at C-8 was derived from molecular oxygen. A separate feeding experiment with [1-^{13}C, $^{18}O_2$]- acetate indicated the oxygens at C-1, C-11, C-13 and C-15 are derived from acetate. The results of these feeding experiments as well as the previous experiments led to the biosynthetic hypothesis outlined in Figure 2.[37, 38] Vederas and co-workers believed that the enzymes involved in the biosynthesis of lovastatin were similar to those involved in fatty acid biosynthesis.[37] They proposed that condensation of acetate units (via malonate) could produce a triene **12** which would undergo an endo-selective Diels-Alder cycloadditon to the decalin **13**.

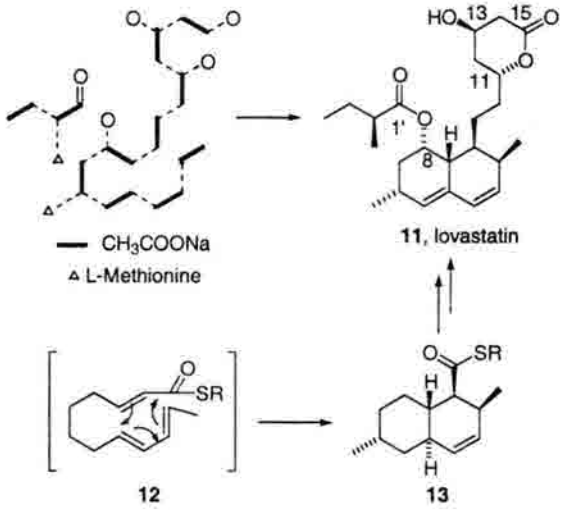
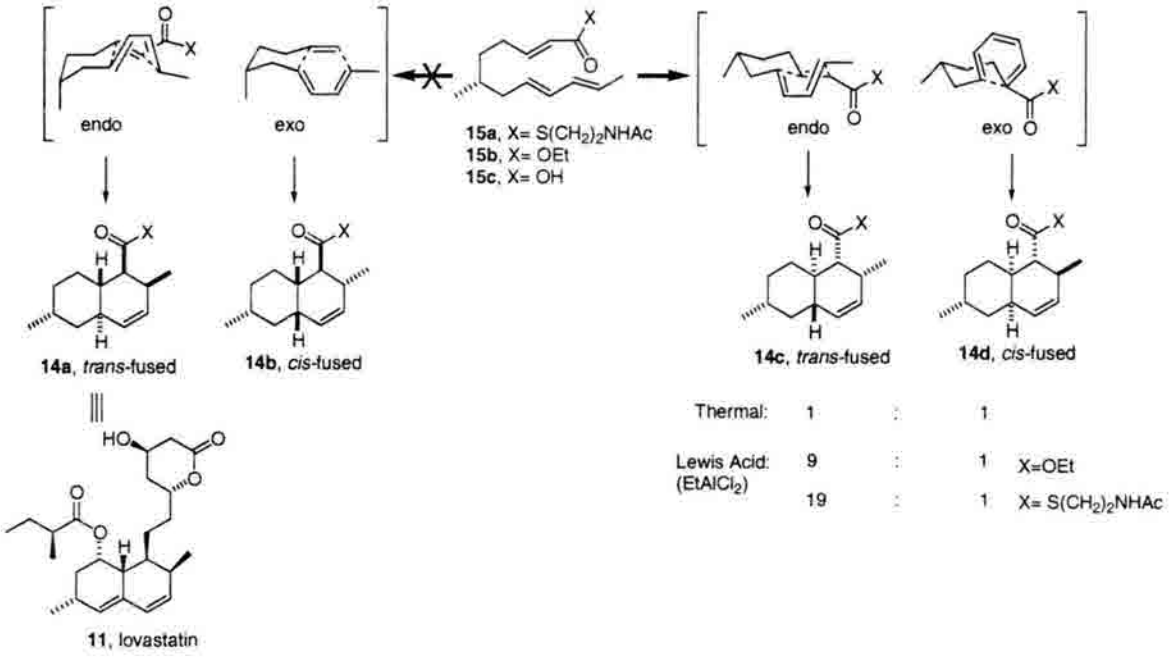


Figure 2: Origin of the carbon atoms of lovastatin (**11**) and proposed biogenesis.[37,38]

The first test of this hypothesis was a synthesis of the analog **14** through laboratory Diels-Alder cyclizations of the N-acetyl cystamine (NAC) thioester (**15a**), ethyl ester (**15b**) and alcohol (**15c**) trienes both thermally (toluene, 160°C, 4 days) and with a Lewis acid catalyst (0.9 eq. $EtAlCl_2$, rt, 3h).[39] As shown in Scheme 1, there was a

1:1 ratio of the *endo*(**14c**):*exo*(**14d**) products in the thermal cyclization, presumably through a chair-like transition state with the side-chain methyl pseudo-equatorial. However, no endo product (**14a**) corresponding to the stereochemistry of lovastatin (**11**) was observed (with a pseudo-axial side chain methyl in the transition state). The Lewis acid-catalyzed Diels-Alder gave the same two products as the thermal reaction but in a 9:1 (endo:exo) ratio for the ethyl ester and a 19:1 ratio for the NAC ester. Absence of the product **14a** in the laboratory cyclizaton suggested the Diels-Alder cyclization in the biogenesis of **11** could be enzymatic.

Scheme 1: Synthesis of the decalin **14** though *in vitro* Diels-Alder cyclization.[39]



To test for Diels-Alder activity *in vivo*, feeding experiments were performed on *A. terreus* (MF4845) with the NAC ester triene (**15a**) under a variety of conditions.[39] The triene was doubly labeled at C-2 and C-11 since β-oxidation could readily degrade the

labeled precursor to shorter synthons (eg. acetate). Incorporation of the intact precursor would lead to adjacent ^{13}C labels perceived as carbon-carbon coupling in the ^{13}C-NMR. However, feeding experiments with [2,11-$^{13}C_2$]-**15a** did not reveal detectable ^{13}C coupling in the ^{13}C NMR spectra (Figure 3). Apparently, the triene **15a** was catabolized before it could undergo cycloaddition.

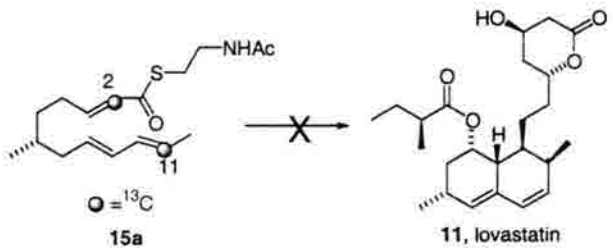
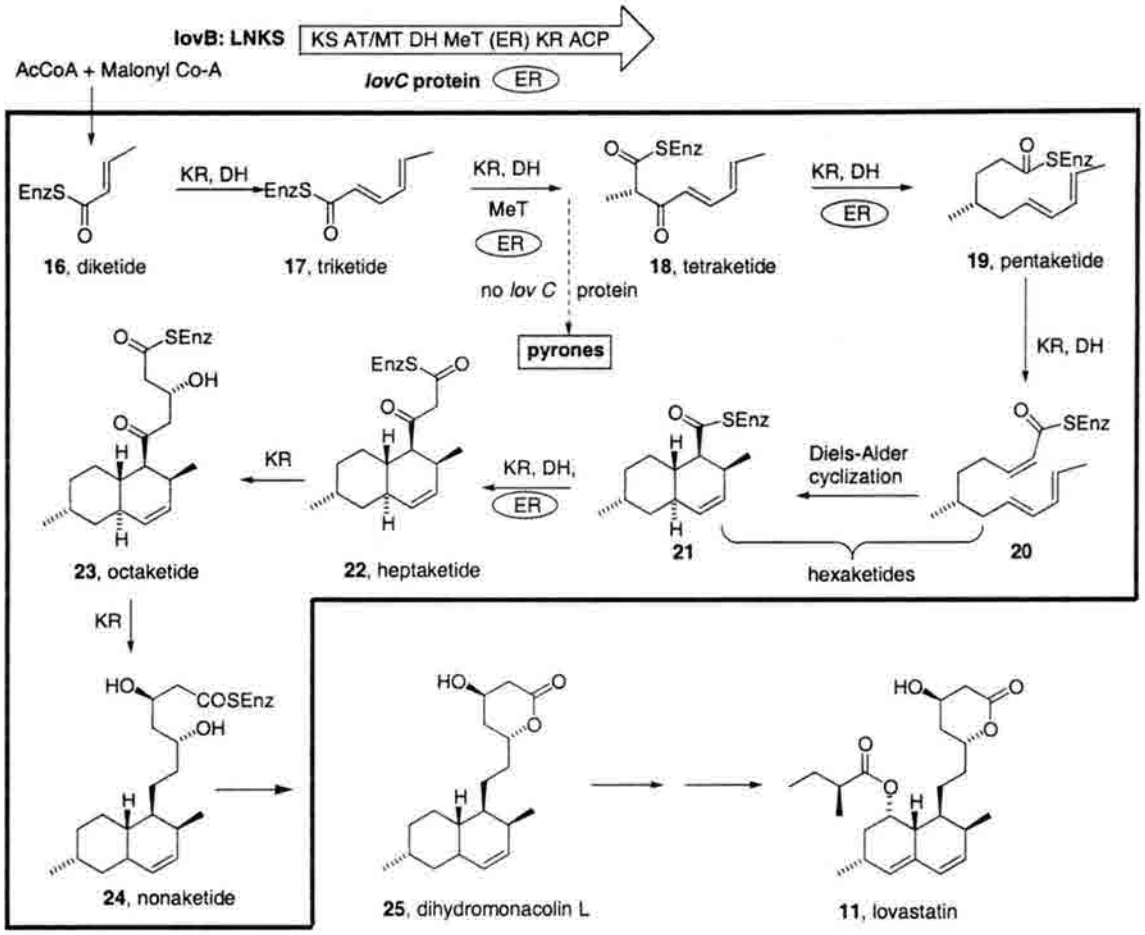


Figure 3: Feeding experiment with [2,11-$^{13}C_2$]-**15a** in *A. terreus* (MF4845).[39]

A complete outline of the proposed biosynthesis of **11** including the role of the *lov B* and *lov C* genes is shown in Scheme 2. Vederas et al. demonstrated that dihydromoncolin L (**25**), a proven intermediate in the biosynthesis of lovastatin (**11**),[40] was formed in a heterologous host, *A. nidulans*, containing the *lov B* and *lov C* genes from *A. terreus*.[41] In addition, expression of the *lov B* protein (lovastatin nonaketide synthase, LNKS) in the absence of *lov C* protein led to truncated pyrones because of the deficiency in enoyl reduction at the tetraketide stage. These results strongly suggest catalytic Diels-Alder activity for LNKS.[41]

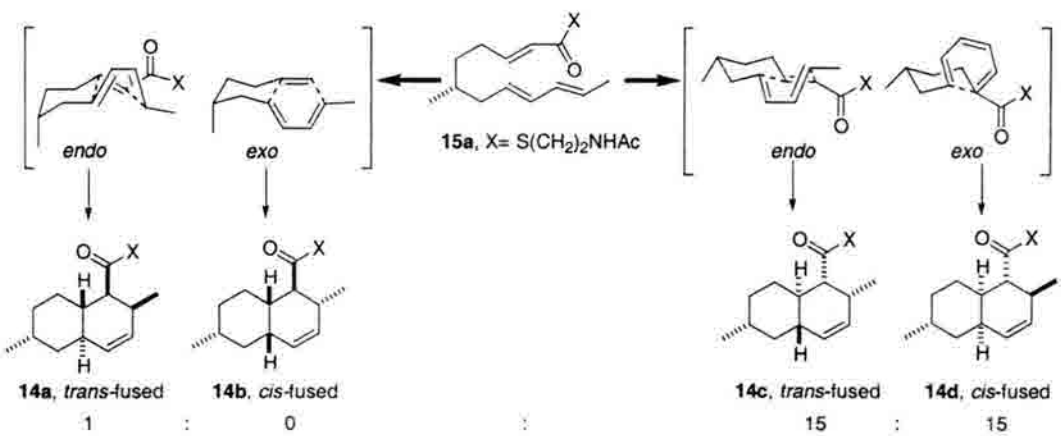
Scheme 2: Proposed biosynthetic pathway for lovastatin. Boxed region shows reactions catalyzed with LNKS and *lov C* protein. The domains for LNKS and *lov C* protein were assigned from sequence homology to other polyketide synthase (PKS) proteins. (Key: KR, ketoreductase; DH, dehydratase, MeT, methyl transferase; ER, enoyl reductase)[35]



The enzymatic activity of LNKS was tested on the NAC ester analog of the proposed cycloaddition precursor. The hexaketide triene NAC ester (**15a**, scheme 3) was added to an aqueous buffered solution containing pure homogenous LNKS protein.[35] The *endo*-Diels-Alder product **14a**, corresponding to the stereochemistry observed in **11**, was obtained along with the non-enzymatic products, **14c** and **14d** (**62a**:**62c**:**62d** ratio 1:15:15). The *trans*-fused *exo*-product **14b** was not observed under any conditions. When **15a** was added to thermally denatured LNKS, adducts **14c** and **14d** were formed,

but **14a** was not detected. Cycloadducts **14c** and **14d** result from a transition state with the C-6 methyl group in a sterically favored pseudo-equatorial arrangement. However, the transition state leading to **14a** requires a more crowded pseudo-axial arrangement of the C-6 methyl group. Thus, it seems the function of LNKS is to bind the substrate in a conformation that resembles the *endo*-transition state that leads to **14a** (analogous to the mode of action of catalytic antibodies). In addition, hydrogen bonding of the carbonyl oxygen within the active site of LNKS would make the dienophile more electron deficient, resembling Lewis acid catalysis of a Diels-Alder reaction. Since the product **14a** was not obtained in the presence of denatured LNKS, the asymmetric induction of the Diels-Alder reaction cannot be caused by non-specific binding of a chiral protein.[42] Therefore, LNKS represents the first naturally occurring Diels-Alderase enzyme to be purified.[35]

Scheme 3: Synthesis of the decalin **14** though an *in-vitro* enzymatic Diels-Alder cyclization.[35]



2.2.1.2 Solanapyrones

Another decalin polyketide thought to arise via a [4+2] cycloaddtion is solanapyrone, a phytoxoin produced by the pathogenic fungus, *Alternaria solani*.[43] A series of feeding experiments with singly and multiply labeled acetate and [methyl-^{13}C]-methionine showed the origin of all the carbons in solanapyrone A (**26**).[44] The results are summarized in Figure 4. An upfield shift of C-13 and C-15 from feeding experiments with [1-^{13}C, ^{18}O]-acetate excluded the possibility that the polyketide is derived by oxidative scission of a longer precursor and indicates that C-15 is the terminal carbon of the polyketide. Feeding experiments with [^{13}C, $^{2}H_3$]-acetate established the fate of the hydrogen atoms and proved that the pro-*S* hydrogen is retained by the polyketide enoyl thiol ester reductase. The stereochemistry at C-1, C-2, C-5 and C-10, the location of the double bond and the stereochemistry of the acetate deuterium at C-5 and C-7 are consistent with a Diels-Alder construction of the decalin ring system.

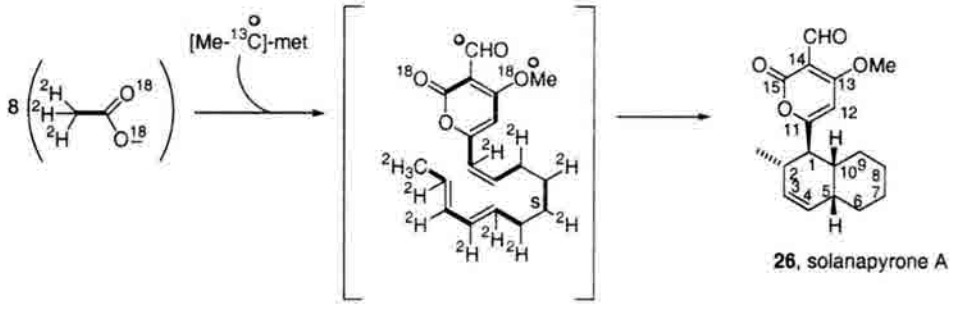


Figure 4: Incorporation of labeled acetate and methionine into solanapyrone A.[44]

The isolation of the minor metabolite solanapyrone D (**27**), a diasteromer of solanapyrone A, gave credence to the Diels-Alder biosynthetic pathway.[45] In the Diels-Alder reaction, rotation about the C-5/C-6 bond could give rise to either the *exo*- or *endo*-

cycloaddition products (Scheme 4). Dreiding models indicate a similar stability of the two transition states (**28** and **29**), but a biomimetic synthesis of solanapyrone A provided a 2:1 product ratio in favor of the *endo*-adduct. [46] Since the *exo*-product is the major isomer observed in the natural system, this was the first indication that the reaction might be enzymatic.

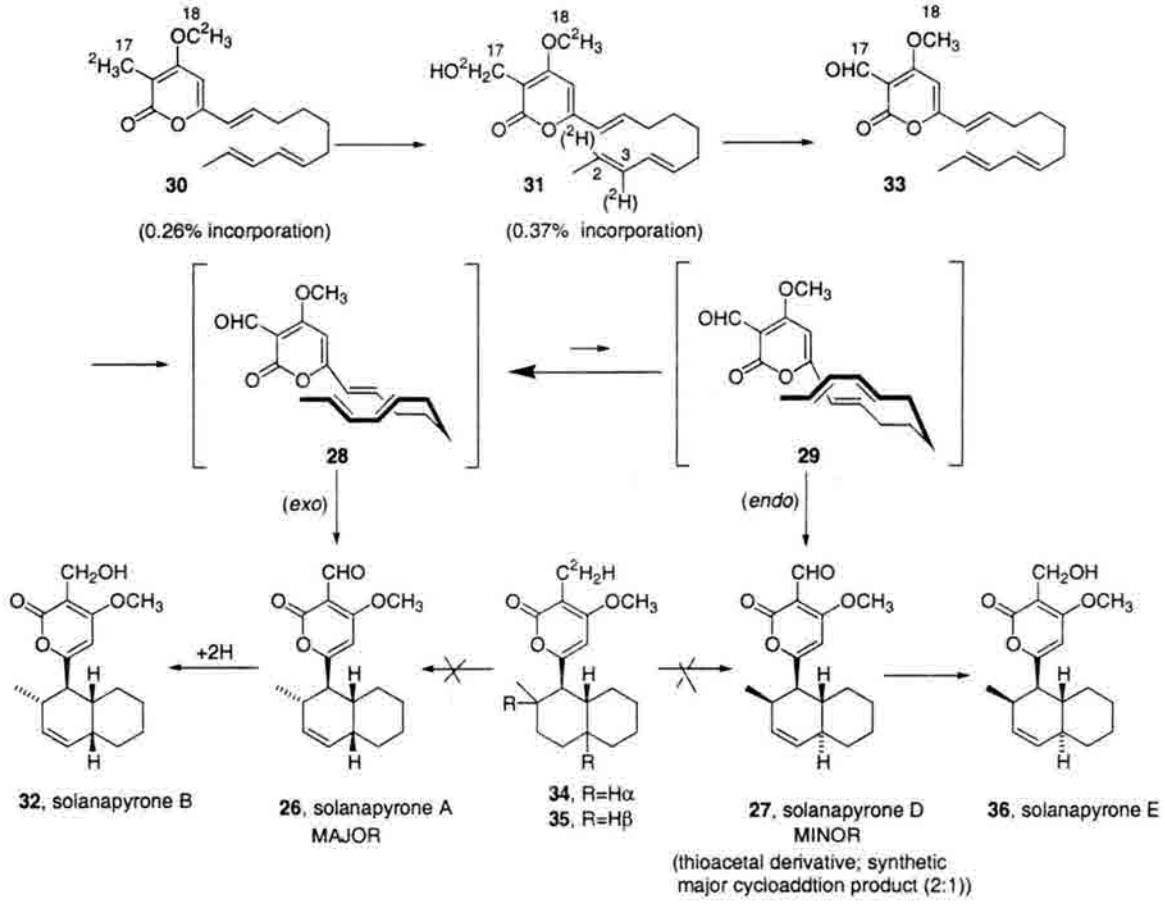
Evidence for the biosynthetic Diels-Alder reaction in solanapyrone biogenesis was obtained when the achiral deuterated trienes, **30** and **31**, were incorporated into solanapyrone A (**26**) and solanapyrone B (**32**) *in vivo*.[47, 48] Incorporation of the [$^2H_5$]-precursor **30** indicated loss of deuterium at C-17. The C17:C18 deuterium integration ratio changed from 2:3 in **28** to a ratio of 1:4.3 for solanopyrone A (**26**) and 1:5.1 for solanapyrone B (**32**). Observation of the same deuterium ratio for solanapyrone A (**26**) and solanapyrone B (**32**) indicates **26** is reduced to **32** and that both triene precursors, **30** and **31**, are oxidized to the same intermediate, presumably the aldehyde **33**. Feeding experiments could not be performed on the C-17 aldehyde (**33**) because it is so reactive, undergoing spontaneous *endo*-cyclization in aqueous conditions.

The reactivity of the aldehyde makes it a likely candidate as a direct precursor to the Diels-alder cyclization, resulting from oxidation of the alcohol at C-17 as shown in Scheme 4. Incorporation of the [$^2H_7$] precursor (**31**) with an essentially unchanged deuterium ratio demonstrated that the diene/ dienophile precursor was incorporated intact. Additionally, the labeled compounds **34** and **35** were not incorporated into **26** or **27**, which shows the Diels-Alder reaction probably occurs after oxidation.

Enzymatic activity was found in a cell-free extract of *A. solani,* which catalyzed the conversion of the C-17 aldehyde triene, prosolanapyrone I (**33**), to solanapyrone A

(**26**) in 25% with a 53:47 ratio of *exo*- to *endo*-cycloaddition.[49] A control with denatured enzyme provided a 3:97 *exo*- to *endo*-cycloaddition ratio with only 10% consumption of starting material. The observed stereoselectivity in the cell-free extract is proof of enzymatic activity.

Scheme 4: Incorporation of deuterated trienes into solanapyrones A (**26**) and D (**27**).[47,48]



Conversion of prosolanapyrone II, the C-17 alcohol triene (**31**), with the crude enzyme preparation was accomplished in 25% yield (19% **26**, 6% **33**) with an *exo*- to *endo*-cycloaddition ratio of 85:15 and an optical purity of 99 ± 4%. The optical purity of the solanapyrone A produced from **33** was 92 ± 8% e.e. Since the optical purity of

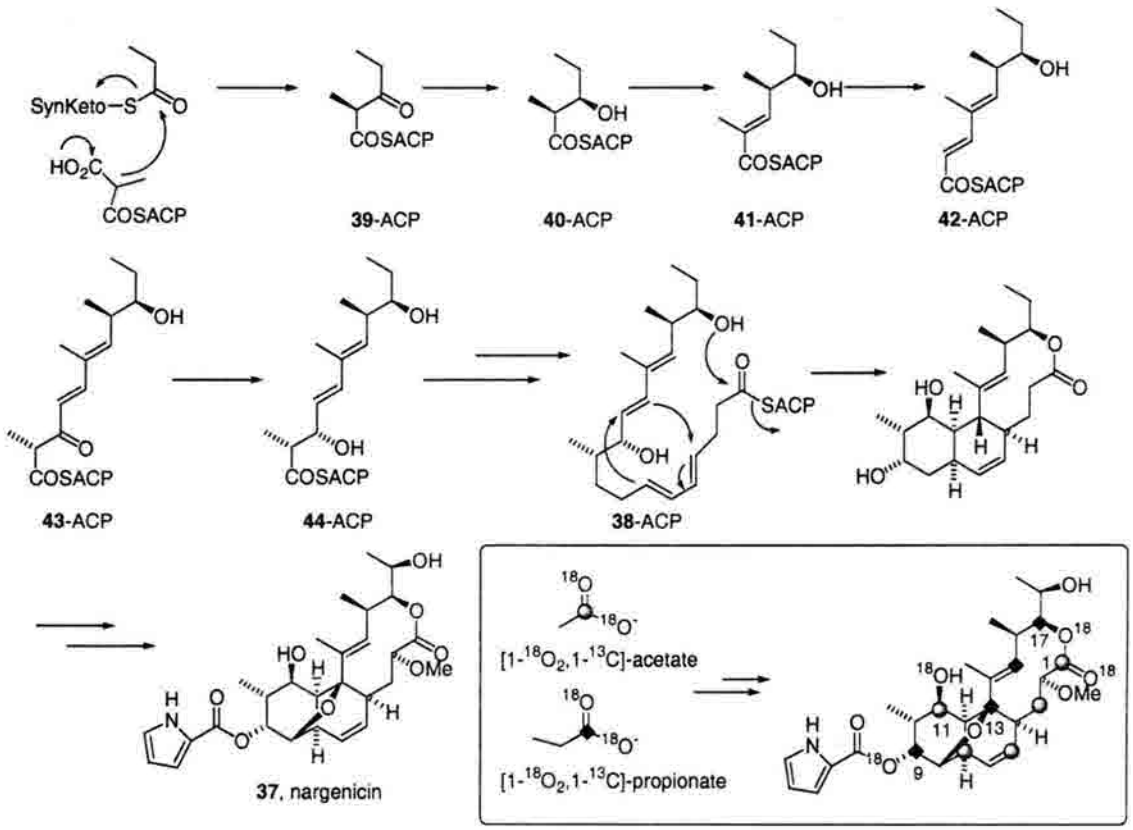
solanapyrone A (**26**) from **33** was less than from **31**, it seems a single enzyme catalyzing the oxidation and cycloaddition is responsible for producing the optically pure solanapyrones found in the natural system. Further proof of this sequence of events was obtained when the enzymatic cycloaddition reaction of **31** was suppressed in the absence of oxygen (argon atmosphere).

2.2.1.3 Nargenicin:

Nargenicin (**37**), a polyketide antibiotic isolated from *Nocardia argentensis*, contains a macrocyclic lactone fused to a *cis*-octalin ring system that is derived from five acetates and four propionates.[50] Feeding experiments with [1-$^{18}O_2$, 1-^{13}C]-acetate and [1-$^{18}O_2$, 1-^{13}C]-propionate indicated that oxygens at C-1 and C-11 are derived from acetate while oxygens at C-9 and C-17 are derived from propionate (Scheme 5, inset). In accord with these results, incubation of *Nocardia argentensis* with ^{13}C-labeled acetate and propionate in an atmosphere of $^{18}O_2$ indicated that the two ether oxygens at C-2 and at C-13 and the hydroxy at C-18 are derived from molecular oxygen.[51]

Since the oxygen at C-13 is not derived from propionate, this implies that the C-4,13 bond is not formed via an aldol-type condensation. Instead, a Diels-Alder cyclization was invoked through the intermediacy of the triene, **38** (Scheme 5). By the incorporation of the ^{13}C and/ or ^{2}H labeled NAC-thioesters of the precursors **40**, **41**, **42**, and **44** (Scheme 5), Cane and co-workers demonstrated that the stereochemistry and level of oxidation are set prior to chain elongation.[52] The incorporation of [$^{13}C_2$]-**44** also further supports the notion that the *cis*-octalin ring system is generated via a Diels-Alder cycloaddition.[52d]

Scheme 5: Proposed biosynthesis of nargenicin.[51,52]



2.2.1.4 Betaenone B:

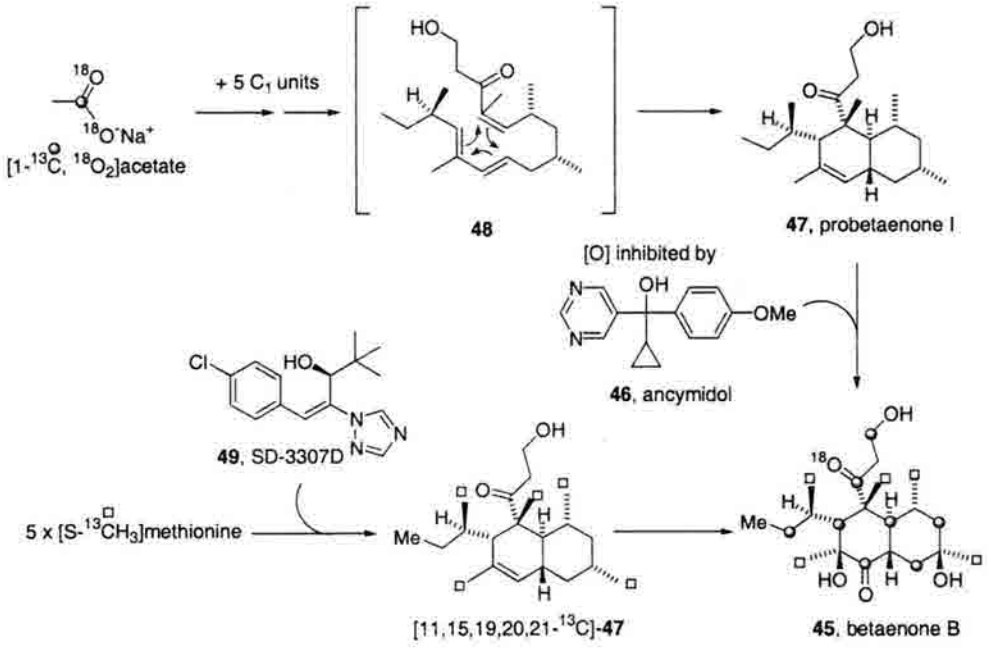
Betaenone B (**45**) is a phytoxin from *Phoma betae* that causese leaf spot disease on the sugar beet. Feeding experiments with [1-^{13}C, $^{18}O_2$]-acetate indicated that only the oxygen at C-16 of betaenone B is derived from acetate (Scheme 9).[53] The absence of an ^{18}O-induced isotopic shift at C-18 from the feeding experiment with [1-^{13}C, $^{18}O_2$]-acetate could indicate that the oxygen is derived from molecular oxygen or it could indicate a "washing out" of the label via exchange with water.

When a P-450 inhibitor, ancymidol (**46**), was added to cultures of *P. betae*, the production of betaenone B (**45**) was suppressed in proportion to the amount of inhibitor added. In addition, a new deoxygenated metabolite, probetaenone I (**47**), was isolated

which was proposed to be a biosynthetic precursor of betaenone B via the intramolecular Diels-Alder reaction of the projected intermediate, **48**.[53]

Probetaenone I (**47**) was later proven to be a precursor to betaenone B (Scheme 6). Separate feeding experiments of *P. betae* with [1-^{14}C]-acetate, [1, 2-^{13}C]-acetate and [S-$^{13}CH_3$]-methionine in the presence of the P-450 inhibitor, SD-3307D (**49**), provided labeled **47**. Subsequent feeding experiments with each labeled probetaenone I (**80**) displayed incorporation into betaenone B (6.02% incorporation from [1-^{14}C]-acetate, 19.1% enrichment from [1, 2-^{13}C]-acetate and 9.6% enrichment from [S-$^{13}CH_3$]-methionine).[54] Synthesis of probetaenone I (**47**) through an intramolecular Diels Alder reaction confirmed the structure and provided credence for the proposed biosynthetic pathway.[55]

Scheme 6: Biosynthetic studies on betaenone B.[53,54]



2.2.2 Macrocyclic Polyketides

2.2.2.1 Cytochalasans:

The cytochalasans are a large family of macrocyclic polyketides that posess cytostatic activity.[56] To date, approximately 60 natural products belonging to this class of mycotoxins have been isolated.[57] Structurally, the cytochalasans are characterized by a highly substituted perhydroisoindole group fused to a macrocyclic ring. The four basic skeletal structures characteristic of the cytochalasans are shown in Figure 5. The majority of the macrocycles are carbocyclic, but the macrocycle can also be a lactone or cyclic carbonate.

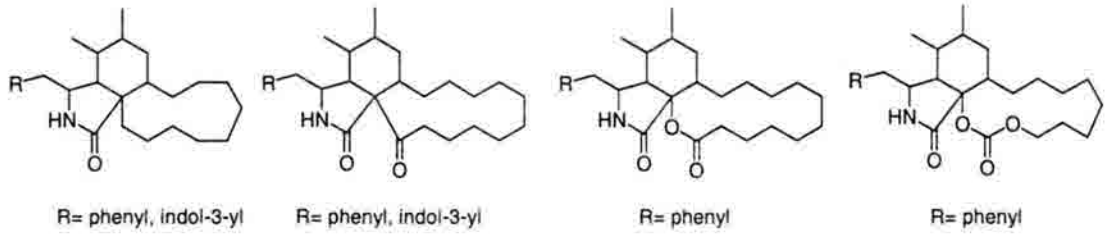
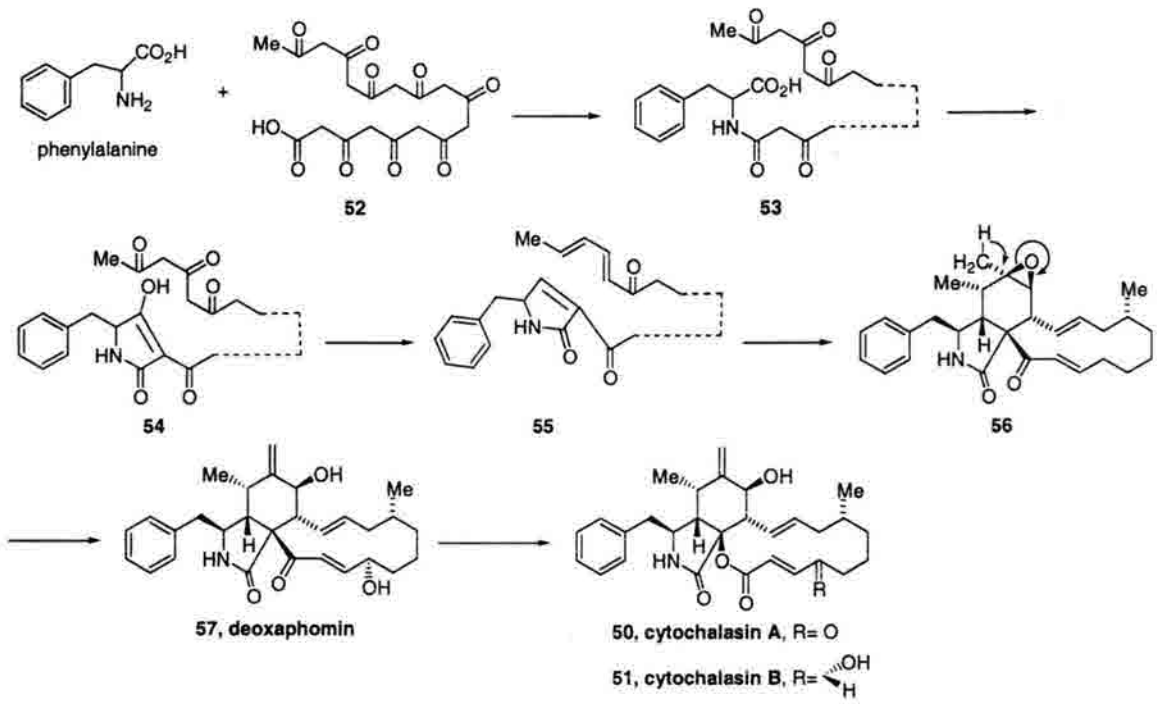


Figure 5: Skeletal structures of the cytochalasans.[56]

Feeding experiments have established acetate,[58,59] propionate,[58,59] methionine,[59,60] phenylalanine[61] and tryptophan[59] as biosynthetic precursors to the cytochalasans. As seen in the proposed biosynthetic scheme for cytochalasin A (**50**) and B (**51**), the perhydroisoindole group is thought to arise via an *endo*-selective intramolecular Diels-Alder reaction (Scheme 7).[56] Incorporation of deoxaphomin (**57**) into cytochalasin B indicates that oxidation to the macrolide occurs after the putative Diels-Alder cyclization and implies a common biosynthetic pathway for the cytochalasans.[62]

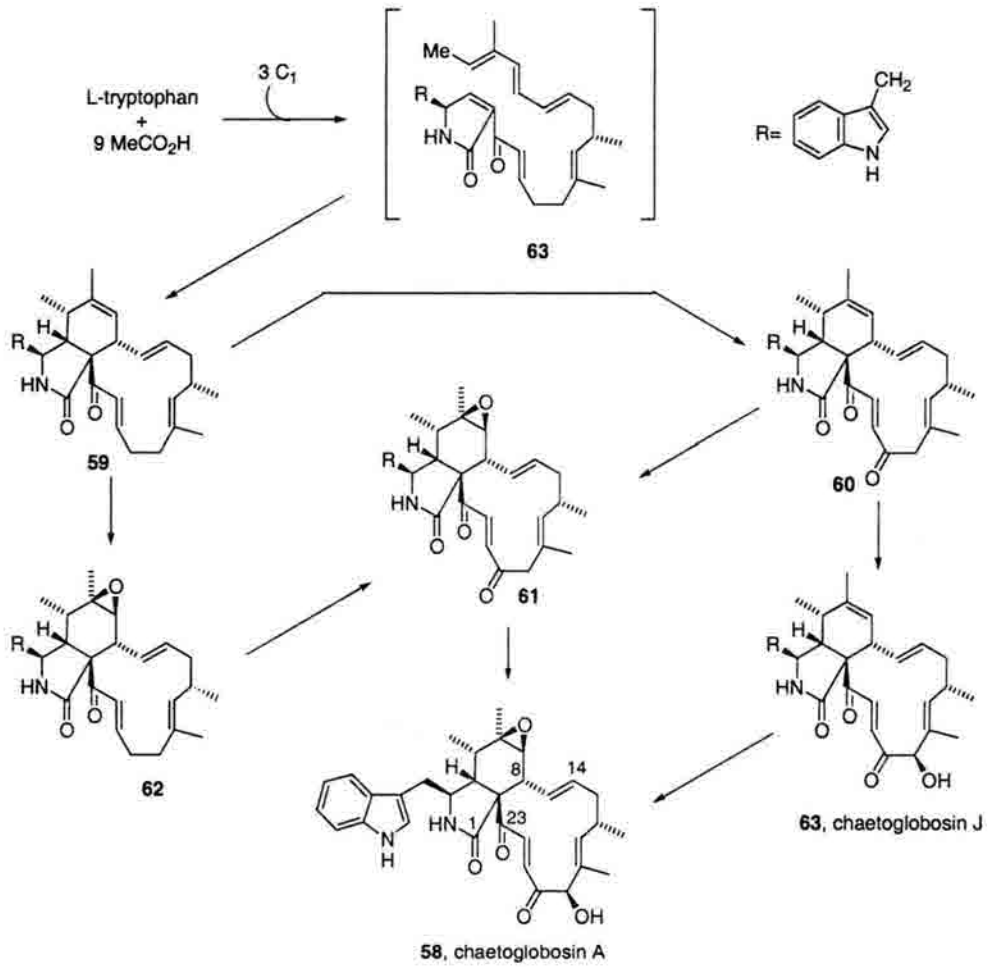
Scheme 7: Proposed biosynthesis of cytochalasin A and B.[56]



Indirect evidence for the Diels-Alder mediated biosynthesis of the cytochalasins was obtained by feeding[63] and inhibition[64] experiments with *Chaetomium subaffine* which produces chaetoglobosin A (**58**). A feeding experiment with [1-^{13}C, $^{2}H_3$]-acetate showed retention of deuterium at C-11, C-8 and C-14.[63] Retention of deuterium at C-8 and C-14 precludes formation of the perhydroisoindole and macrocycle through the proposed anionic carbon-carbon bond formation in which a carbonyl group is located at C-14.[56] A feeding experiment with [1-^{13}C, ^{18}O]-acetate established the acetate origin of the oxygens at C-1 and C-23 while incubation with [$^{18}O_2$] displayed an upfield shift for C-6, C-7 and C-20.[63] An inhibition experiment with a cytochrome *P*-450 inhibitor, metapyrone, led to the formation of the metabolites, **59**, **60**, **61** and **62** (with **59** as the major component of the new metabolites).[64] These results led to the biosynthetic proposal

for chaetoglobosin A outlined in Scheme 8. An intramolecular Diels-Alder reaction of the putative hexaene, **63**, would provide **59**, which could then undergo a stepwise oxidation to provide **58**.

Scheme 8: Proposed biosynthesis of chaetoglobosin A.[64]



The possibility for enzymatic involvment in the proposed Diels-Alder cyclization of the cytochalasans was evidenced by the retro-Diels-Alder reaction of **59** (Figure 6). Instead of forming the expected triene, **63**, pyrolysis (180°C, sealed tube) of **59** produced

equal amounts of starting material and the diasteromer, **65**.[63] The lack of stereoselectivity in the thermal Diels-Alder formation of **59** and **65** supports the hypothesis that an enzyme stablizes the *endo*-transition state in the biological system, which results in exclusive formation of **59**.

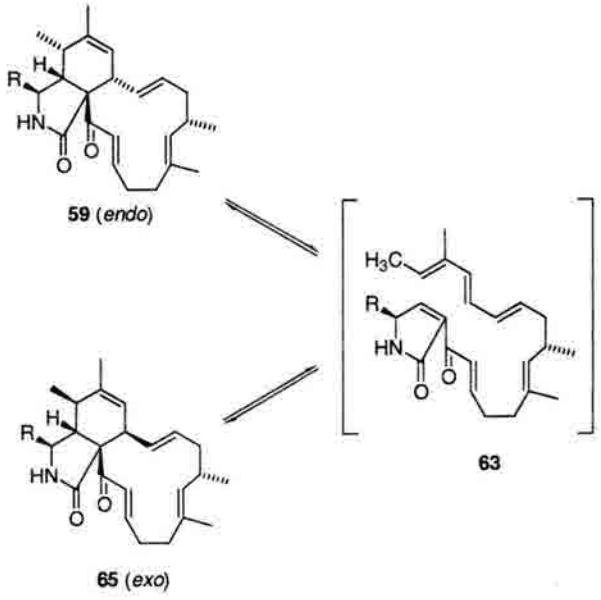


Figure 6: Retro Diels-Alder reaction of chaetoglobosin A precursor **59**.[63]

2.2.2.2 Cochleamycins

The polyketide origin of cochleamycins A (**66**) and B (**68**) were determined from feeding experiments with [1-^{13}C]-acetate, [2-^{13}C]-acetate, [1, 2-^{13}C]-acetate and [3-^{13}C]-propionic acid (Figure 7).[65]

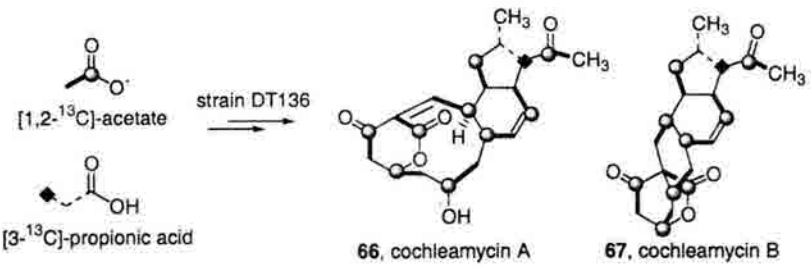
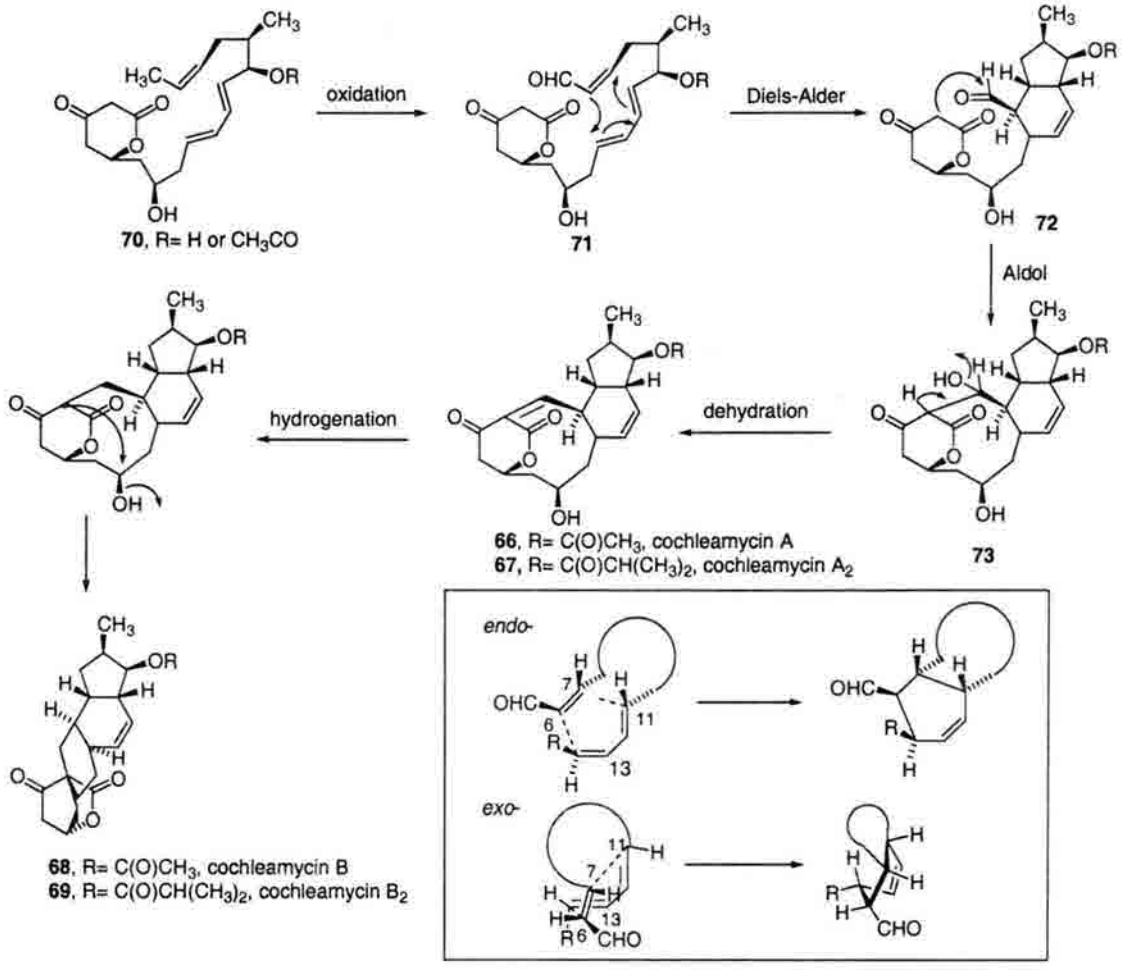


Figure 10: Incorportion of acetate and propionic acid into cochleamycin A and B.[65]

Scheme 9: Proposed biosynthetic pathway of cochleamycins A and B.[65]



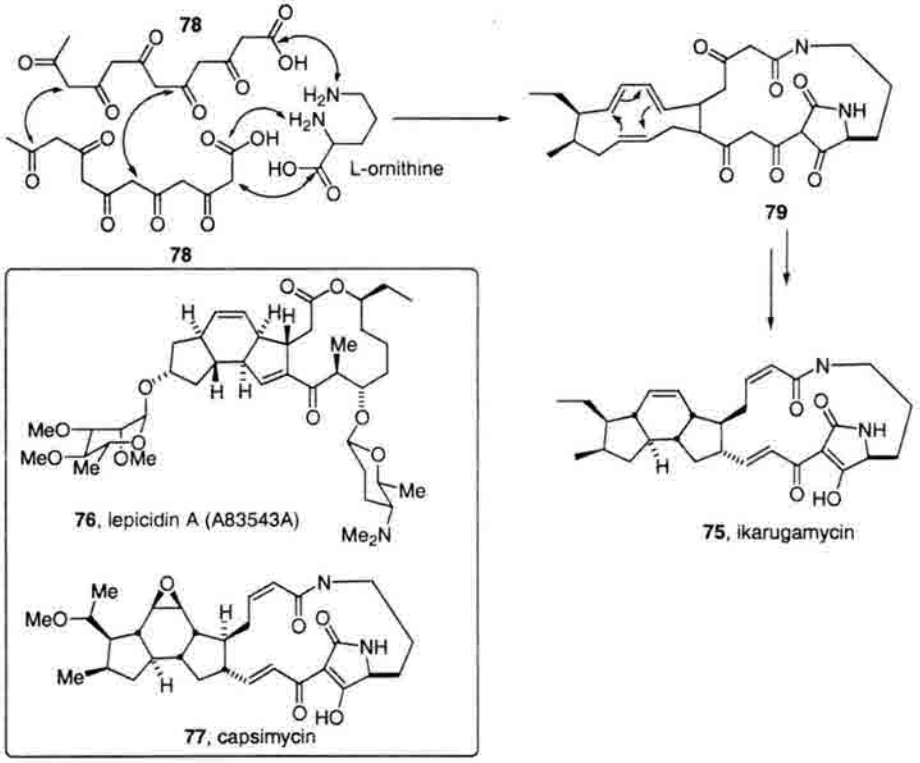
Based on these results, the biosynthesis shown in Scheme 9 was proposed. Starting from the proposed intermediate, **70**, oxidation of the allylic methyl group followed by an intermolecular Diels-Alder reaction and aldol condensation could lead to the formation of **66**. Formation of **68** is thought to arise from reductive trans-annualar cyclization at the C-4 and C-16 positions of **66** accompanied by elimination of the hydroxyl group at C-16. The desired stereochemistry for the intramolecular Diels-Alder reaction at the AB- and BC-ring junctures can be obtained by *endo*-addtion of the *trans*-

olefin at the C-6 postion to the the 11-*trans*, 13-*cis*-diene, or by the *exo*-addition of the *trans*-olefin to the 11-*cis*, 13-*trans*-diene (Scheme 9, inset).

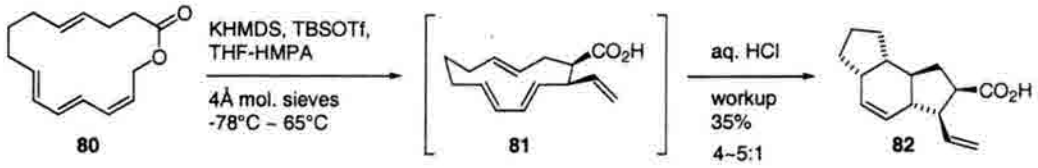
2.2.2.3 Ikarugamycin

Ikarugamycin (**75**) [66] is a member of a small family of macrocyclic antibiotics that possess an unusual perhydro-*as*-indacene ring system. Other members of this family include lepicidin A (**76,** A83543A)[67] and capsimycin (**77**)[68]. The structure and stereochemistry of ikarugamycin were determined by Ito and Hirata in 1972.[66a] They proposed that ikarugamycin was biosynthesized from two hexa-acetate units (**78**) and L-orinthine and that the decahydro-as-indacene skeleton was synthesized via a trans-annular Diels-Alder reaction of **79** (Scheme 10).

Scheme 10: Proposed biosynthesis of ikarugamycin and structures of related natural products lepicidin A and capsimycin.[66,67,68]



Scheme 11: Biomimetic synthesis of perhydro-*as*-indacene ring system of ikarugamycin.[69]



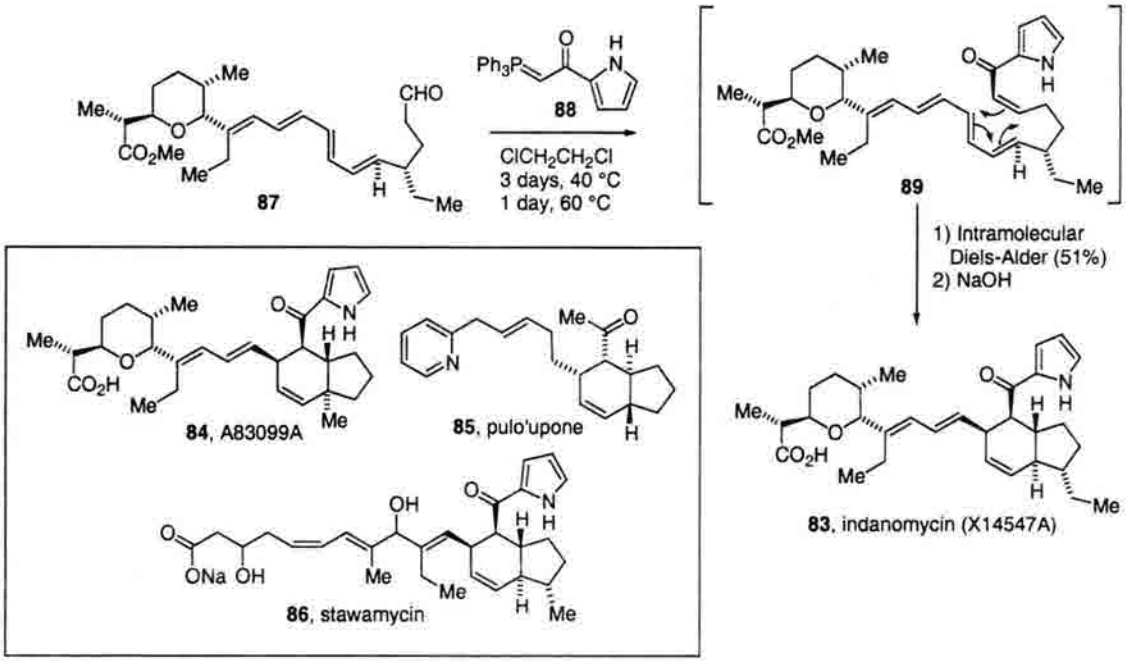
Roush and Works modeled this system with a functionalized cyclodecatriene (Scheme 11).[69] Stereoselective enolate Claisen ring contraction of lactone **80** provided the desired (E, E, E)-cyclododeca-1, 6, 8-triene, **81**, *in situ*. Heating the reaction to 65°C overnight provided a 4~5:1 ratio of the Diels-Alder cycloaddition products, **82** and a second diasteromer. Evans and Black pursued an alternate route for the synthesis of the decahydro-*as*-indacene skeleton of lepicidin A (**76**), utilizing an intramolecular Diels-Alder reaction in conjuction with an aldol reaction.[70]

2.3 Perhydroindane polyketides

Indanomycin (X14547A, **83**)[71] is a member of a small family of polyketides which contain a perhydroindane skeleton. Other members of this family include the antibiotic, A83099A (**84**),[72] the marine natural product, pulo'upone (**85**),[73] and stawamycin (**86**).[74] Roush and co-workers hypothesized that the biosynthesis of indanomycin might involve an intramolecular Diels-Alder reaction via a pentaene intermediate such as **89**.[75] Based on this hypothesis, the total synthesis of indanomycin was completed using an intramolecular [4+2] cycloaddition as the key step (Scheme

12).[75c] The isomerically pure product was obtained in 51% yield with 5 % of a mixture of *cis*-fused products and 5% of the C-10,11 (Z)-olefin isomer.

Scheme 12: Biomimetic total synthesis of indanomycin[75c] and structures of related natural products A88099A,[72] pulo'upone,[73] and stawamycin.[74]



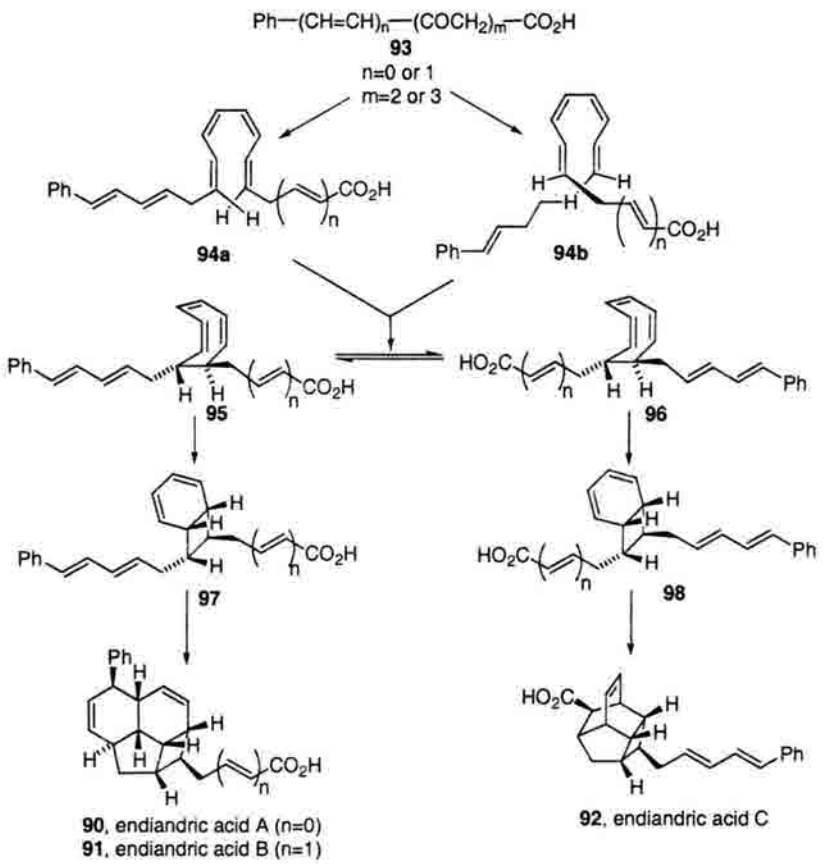
2.2.4 Miscellaneous

2.2.4.1 Endiandric acids:

The endiandric acids are isolates from the leaves of the Australian plant, *Endriandra introrsa* (*Lauraceae*).[76] Since endiandric acid A (**90**) and B (**91**) co-occurs with endiandric acid C (**92**) and because these compounds are isolated in racemic form, Banderanyakae and co-workers postulated a unified biogenesis involving a series of electrocyclizations from an achiral precursor (Scheme 13).[77] They proposed that a polyketide such as **93** might lead to a phenyl polyene acid with a central conjugated tetraene unit. From the all *cis*-tetraene (**94a**) or the *trans*, *cis*, *cis*, *trans*-isomer (**94b**), an

8π conrotatory electrocyclization, followed by a 6π disrotatory electrocyclization and finally an intramolecular Diels-Alder ($4\pi s + 2\pi s$) cycloaddtion would provide endiandric acids A (**90**) and B (**91**) or endiandric acid C (**92**), respectively.

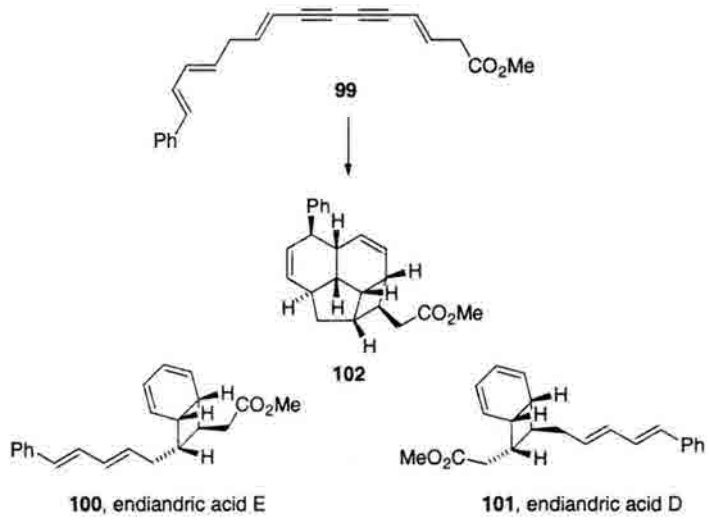
Scheme 13: Biosynthesis of endiandric acid.[77]



Nicolaou and co-workers explored the feasibility of the proposed pathway though a biomimetic synthesis. Step-wise stereocontrolled syntheses of ediandric acids A through D were first completed to determine if the proposed sequence of events was viable.[78] Next, Nicolaou completed a one-pot electrocyclic cascade reaction.[79] Hydrogenation of the acyclic precursor **99** (Scheme 14) with Lindlar's catalyst provided

the methyl esters of endriandric acids E (**100**) and D (**101**), while brief heating of the reaction provided the methyl ester of endriandric acid A (**102**). A chain-elongated version of **99** was used to synthesize endriandric acids B (**91**) and C (**92**) along with the unnatural endriandric acids G-F. These syntheses validate the biosynthetic hypothesis of Banderanyakae et al. and indicate that these electrocyclic reactions are not enzymatically catalyzed. In addition, the syntheses of the unnatural endriandric acids E-G may help in the identification of these compounds in the natural system.

Scheme 14: Biomimetic synthesis of the endiandric acids.[79]



2.2.4.2 Bisorbicillinoids:

The bisorbicillinoids (**103-109**) are a growing family of mycotoxins that are proposed to arise via a common biosynthetic precursor, sorbicillin (**110**), as shown in Figure 8. Bisvertinoquinol (**103**), the first member of this class of compounds to be isolated, was postulated to be a Diels-Alder adduct of two different quinols derived from

the co-metabolites, **110** and 2',3'-dihydrosorbicillin, through enantioselective oxidation of C-5.[80] This route was postulated because of the structure of **103** is consistent with a spontaneous *endo*-selective Diels-Alder reaction. However, from the variations in the sorbyl and dehyrosorbyl sidechains one would expect four Diels-Alder adducts. Since only one optically active bisvertinoquinol type product was observed in the cultures, this suggests chain differentiation occurs after the Diels-Alder reaction and that the product (**103**) is not an artifact of isolation.

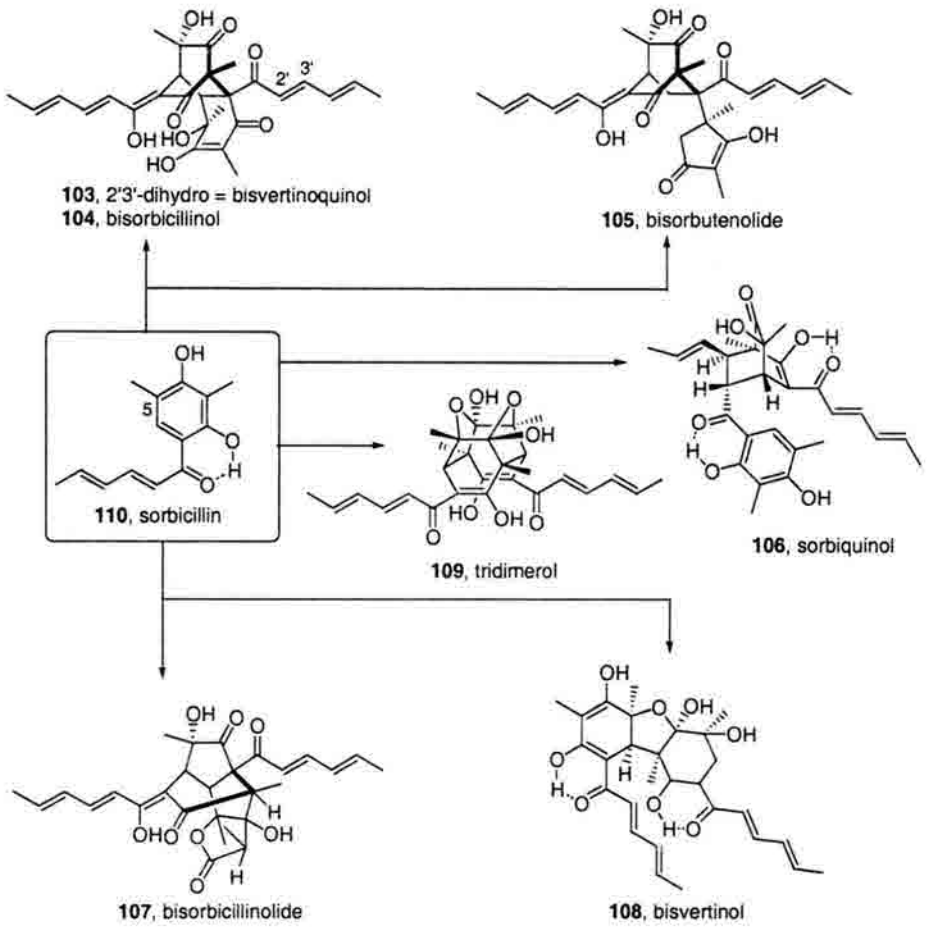
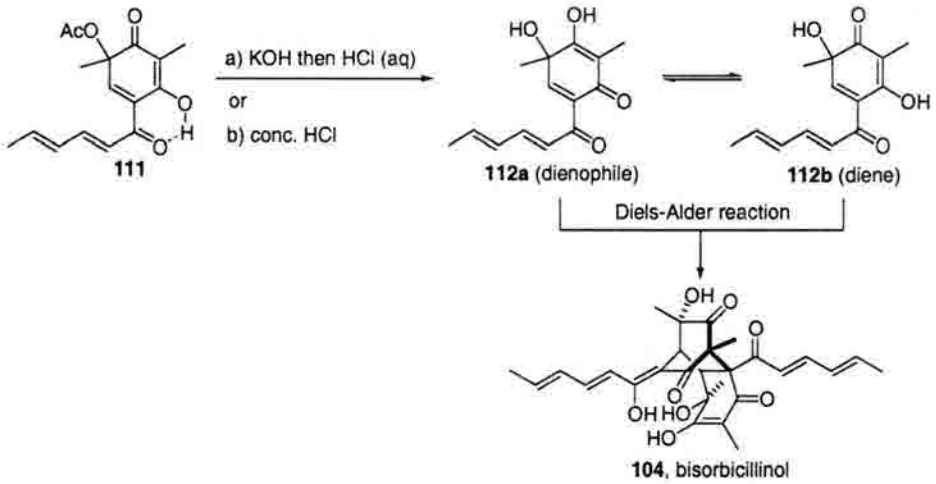


Figure 8: The bisorbicillinoid mycotoxins.[83]

A similar biosynthetic Diels-Alder proposal has been made by Murata and Hirota for bisorbicillinol (**104**), which could then form bisorbutenolide (**105**) through an anionic casade reaction.[81] Sorbiquinol (**106**) has also been postulated to arise from a [4+2] cycloaddition. However, for **106**, the Diels-Alder reaction would occur between the side chain double bond of sorbcillin (**110**) as the dienophile and enantioselectively oxidized sorbicillin as the diene.[82] Alternatively, the biosyntheses of bisorbicillinolide (**107**) and trichodimerol (**109**) can be rationalized as products of an oxidation-Michael-ketalization cascade.[83]

Scheme 15: Biomimetic total synthesis of bisorbicillinol.[83]



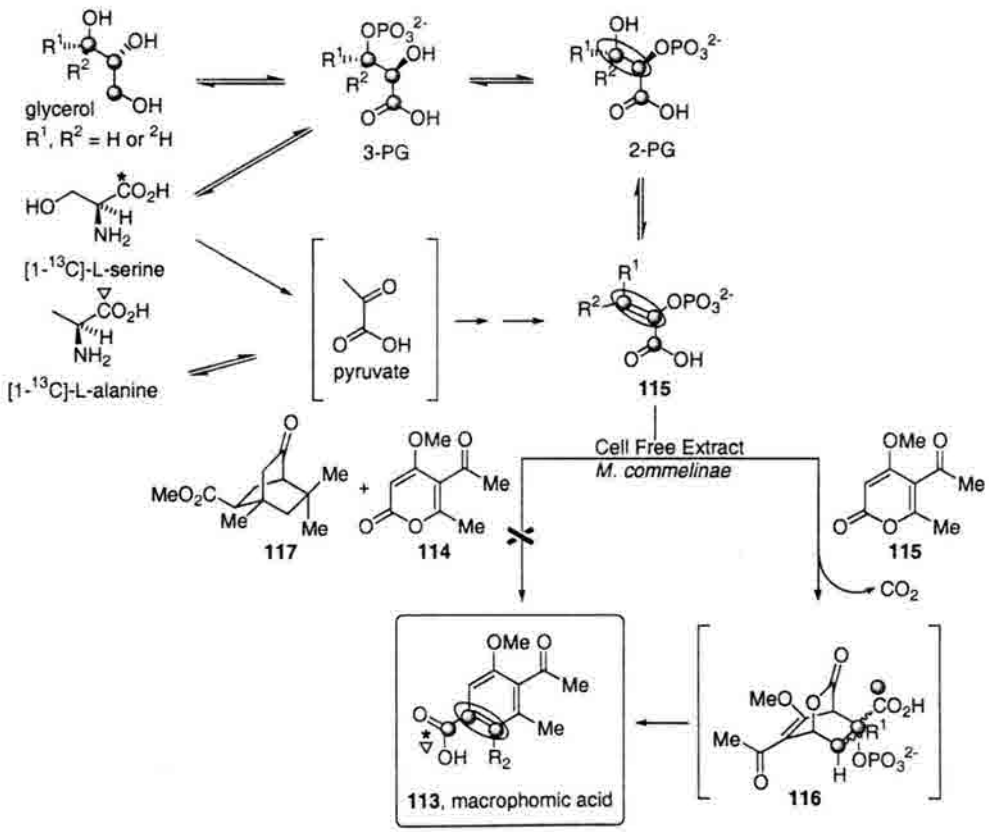
In proof of the proposed biosynthesis, Nicolau and co-workers completed a biomimetic total synthesis of bisorbicillinol (**104**) as shown in Scheme 15.[83] Basic or acidic hydrolysis of the acetoxy functionality of **111** provided the quinols **112a** and **112b** which spontaneously formed the Diels-Alder cycloadduct, **104**. Four stereogenic centers were created in the Diels-Alder reaction with complete regio- and diastereocontrol.

Additionally, the quinol intermediate **112b** has recently been identified as a metabolite of the bisorbicillinoid producing fungus, *Trichoderma* sp. USF-2690.[84]

2.2.4.3 Macrophomic Acid:

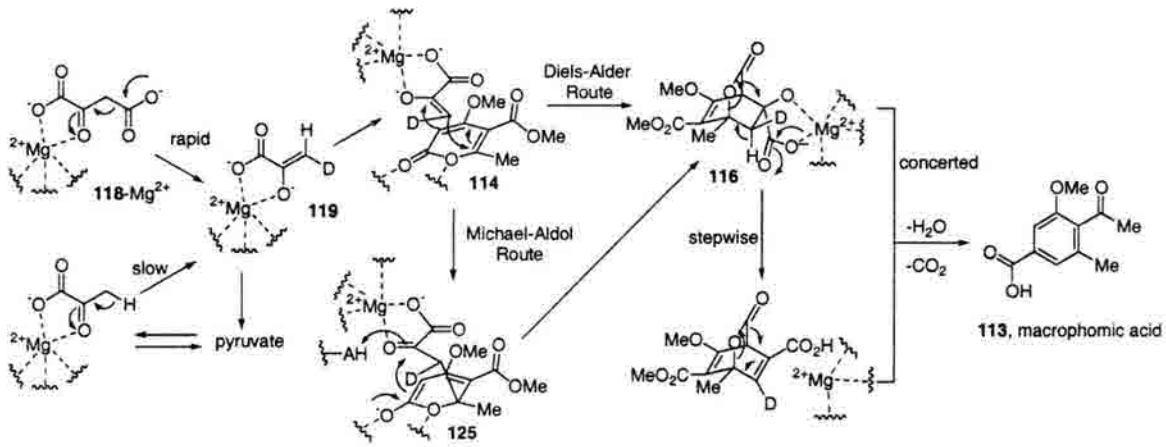
Macrophomic acid (**113**) is a fungal metabolite isolated from *Macrophoma commelinae*. Sakurai et al. established that macrophomic acid is derived from the 2-pyrone **114** with loss of CO_2 and an unidentified C_3 unit.[85] Subsequent work on the biosynthesis of macrophomic acid revealed incorporation of [1-^{13}C]-L-alanine, [1-^{13}C]-L-serine, [U-^{13}C]-glycerol and (1*RS*, 2*S*)-[1-^{2}H]-glycerol and (1*RS*, 2*R*)-[1-^{2}H]-glycerol.[86]

Scheme 16: Original biosynthetic proposal for macrophomic acid.[86]



Based on these experiments, Oikawa et al proposed the biosynthetic pathway outlined in Scheme 16 with phosphoenolpyruvate (**115**) as the C_3-unit leading to **113**. In fact, incubation of a cell-free extract of *M. commelinae* with **114** and **115** led to the enzymatic formation of **113**.[86] The proposed biosynthetic pathway for macrophomic acid entails an inverse-electron-demand Diels-Alder reaction of the pyrone **114** and the dieneophile **115** to provide the intermediate **116**, which is transformed to **127** by successive retro-Diels-Alder and *syn*-elimnation of phosphoric acid. To test this hypothesis, an analog of the putative bicyclic intermediate (**117**) was synthesized and incubated with the cell-free extract of *M. commelinae*, **114** and **115**. The analog **117** was found to inhibit the formation of **113** with an IC_{50} value of 200μM.[86]

Scheme 17: Revised biosynthetic proposal for macrophomic acid.

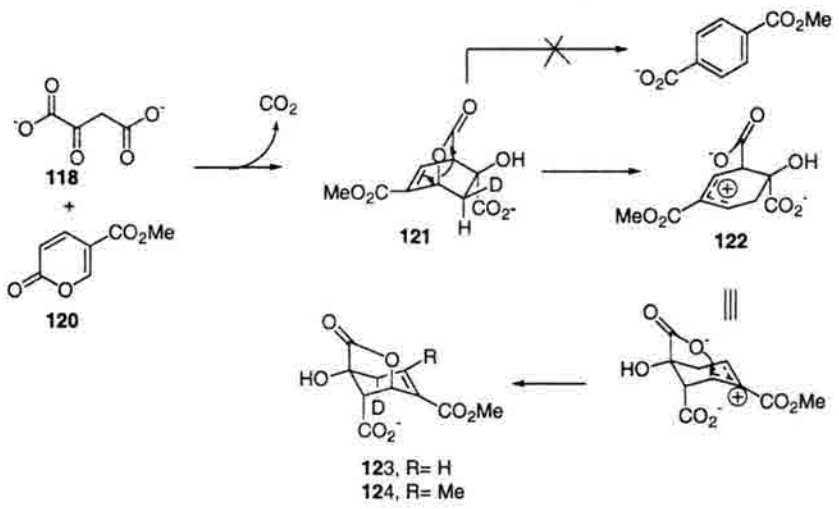


A recent re-examination of the origin of the C_3 unit led to the discovery that oxalacetate (**118**) is a more efficient and direct precursor to macrophomic acid. Using oxalacetate as the sole substrate for the C-3 unit, the Mg^{2+} dependent enzyme macrophomate synthase was isolated and purified.[87,88] This single enzyme, a homodimer

of a 36 kDa protein, was found to catalyze a five-step transformation involving two decarboxylations, two C-C bond forming reactions and a dehydration.[36]

It was believed that the C3-unit might still be an enol pyruvate (**119**), the product of oxaloacetate decarboxyation. To test this, macrophomate synthase was incubated with oxaloacetate in the absence of the 2-pyrone **114** in a lactate dehydrogenase coupled assay.[36] Rapid formation of pyruvate was observed. However in a competion experiment, the 2-pyrone **114** was found to inhibit the conversion of oxaloacetate (**118**) to pyruvate. This indicates that the enzymatic product of oxaloacetate decarboxylation is not hydrolyzed and undergoes further reaction with **114** (Scheme 17).

Scheme 18: Proposed mechanism for the formation of abberant cycloadducts by macrophomate synthase.



Incubation of macrophomate synthase with oxaloacetate (**118**) and 2-pyrones lacking a C-4 substituent, such as methyl coumalate (**120**), result in the formation of abberant bicyclic compounds such as **123** and **124** (Scheme 18).[36,89] The location of the double bond and absence of an oxygen fundtionality at C-5 suggests that the proposed

intermediate **121** undergoes allylic rearrangement of the double bond and subsequent re-lactonization. The abberant cyclo adduct **123** may be formed instead of the benzoate because the lack of a C-4 substituent causes improper interaction between the catalytic residue and the elimination groups or because the C-4 substituent interrupts attack of the carboxylate to the carocation. In either case, the driving force of the rearrangement is probably the reduction of steric energy of **121**.

Deuterium labeling experiments revealed the pro-R position of adduct **124** is retained, which indicates that the first decarboxyation step provides the Z-enolate.[36] The stereochemistry of the decarboxylation is consistent with the known enzyme, phosphoenolpyruvate carboxylase.

Two possible routes, a stepwise Michael-aldol reaction or a concerted Diels-alder reaction, can account for C-C bond formation by macrophomate synthase (Scheme 17).[36] In the Michael-aldol reaction, attack of the enolate on the 2-pyrone **114** would provide the first C-C bond and stabilize the negative charge on the 2-pyrone. Susequently, the enolate could attack the newly formed carbonyl group to afford the bicyclic intermediate **116**. However, an intermediate with a single C-C bond such as **125** has not been observed for reactions catalyzed by macrophomate synthase.

On the other hand, a Diels-Alder cyclization may resemble an antibody-catalyzed Diels-Alder reaction. The bicyclic intermediate **116** in the macrophamoate synthase catalyzed reaction could be stabilized by the groups used for recognition of the enolate and the 2-pyrone **114**. Support for the Diels-Alder proposal can be gleaned from the literature precedent of a [4+2] cycloaddtion of a 2-pyrone and an equivalent of pyruvate enolate.[90] In addition, the high stereospecificity observed in the macrophomate synthase

catalyzed cyclization is typical of Diels-Alder mediated reaction. Nevertheless, more information will be needed to determine if C-C bond forming reactions of macrophomate synthase arise from a concerted Diels-Alder reaction.

2.3 Isoprenoids

2.3.1 Derivatives of Myrcene and trans-β-Ocimene

2.3.1.1 Pervoskone

The terpenes, myrcene and trans-β-ocimene are often used as dienes in the construction of Diels-Alder derived natural products. Perovskone (**126**) is a triterpene isolated from *Pervoskia aborotanoides*. Initially, it was thought that **126** was constructed from an icetexone precursor **127** and geranyl pyrophosphate as shown in Figure 9.[91] Later, a [4+2] cycloaddition route from **128** and trans-β-ocimene **129** was proposed and this route was used to complete the total synthesis (Scheme 19).[92]

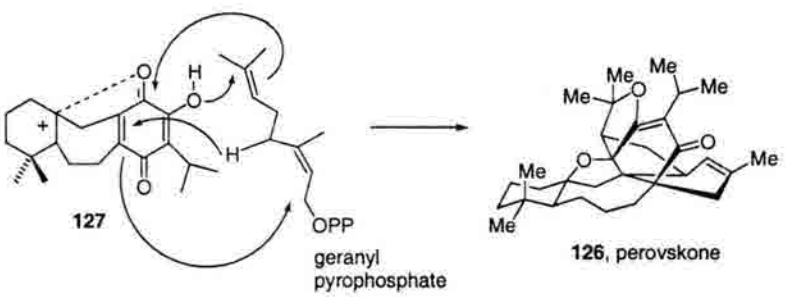
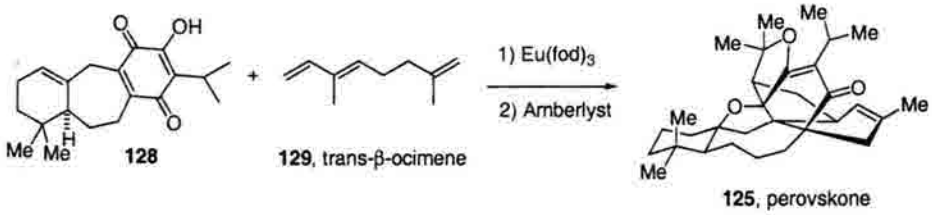


Figure 9: Early biosynthetic proposal for perovskone.[91]

Scheme 22: Biomimetic total synthesis of perovskone.[92]

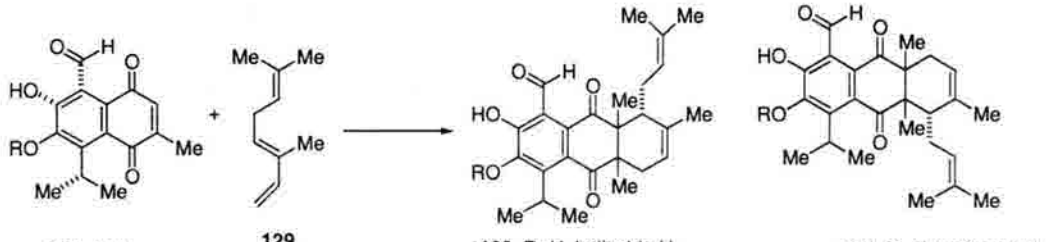


128 **129**, trans-β-ocimene **125**, perovskone

2.3.1.2 Heliocides:

Heliocides H_1 (**130**), H_4 (**131**), B_1 (**132**) and B_4 (**133**) are derived from trans-β-ocimene (**129**) and hemigossypolone (**134**) or its methyl ether derivative (**135**). Heliocides B_1 (**132**) and B_4 (**133**) were synthesized via a [4+2] cycloaddition in a 3:1 ratio, the same ratio observed in the isolation of the natural products (Scheme 20).[93]

Scheme 20: Biomimetic synthesis of heliocides B_1 and B_4.[93]



134, R=H
135, R=Me

129

130, R=H, heliocide H_1
132, R=Me, heliocide B_1

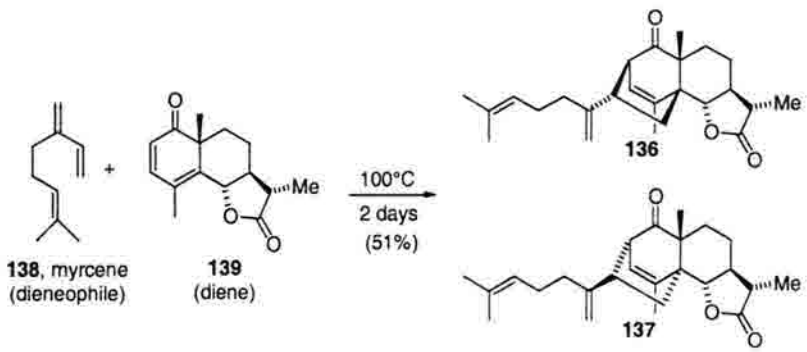
131, R=H, heliocide H_4
133, R=Me, heliocide B_4

2.3.1.3 Eudesmanolides:

The eudesmanolide adducts, **136** and **137**, were isolated from the aerial parts of *Artemisia herba-alba*.[94] They are formally derived via an inverse electron demand Diels-Alder reaction between myrcene (**138**) as the dienophile and the dieneone, **139**, as the

diene. The synthesis of **136** and **137** was accomplished in a 1:1 ratio by heating **138** and **139** to 100°C for 2 days (Scheme 21). Because the conditions required for the synthesis of **136** and **137** are so harsh, it is unlikely that they are artifacts of isolation.

Scheme 21: Biomimetic synthesis of eudesmanolide adducts **136** and **137**.[94]



2.3.2 α-*Exo*-Methylene-γ-Lactones

2.3.2.1 Plagiospirolides

In determining the structure of spiroterpenoids isolated from the Panamanian liverwort *Plagiochila moritziana*, GC-EIMS analysis was carried out on plagiospirolide A (**140**). Diplophyllolide (**141**) and fusicoccadiene (**142**) were detected, possibly resulting from a retro Diels-Alder reaction and both isolates from extracts of *P. moritziana*.[95] Further isolations of *P. moritziana* provided plagiospirolide E (**143**). Again, GC-EIMS provided potential retro-Diels-Alder products, the *P. moritziana* isolate diplophylline (**144**) and the diene (**145**).[96] Since a synthetic Diels-Alder route to the related triterpenes from *Helenium autumnale* required harsh conditions and gave low yields of a mixture of isomers,[97] it is unlikely that **140** is an artifact of isolation. In

addition, since no other diasteromers of **140** were found in *P. moritizana* cultures, it is possible that the putative biosynthetic Diels-Alder reaction is enzymatic.

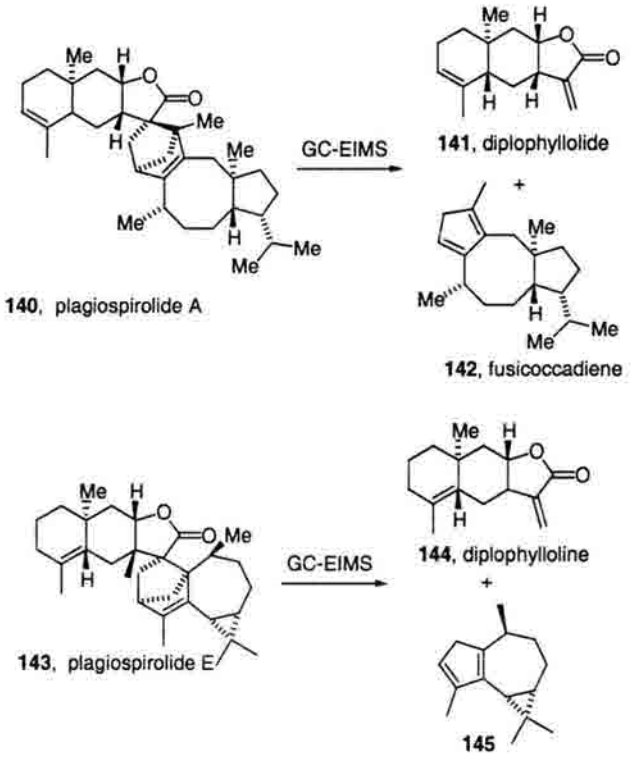


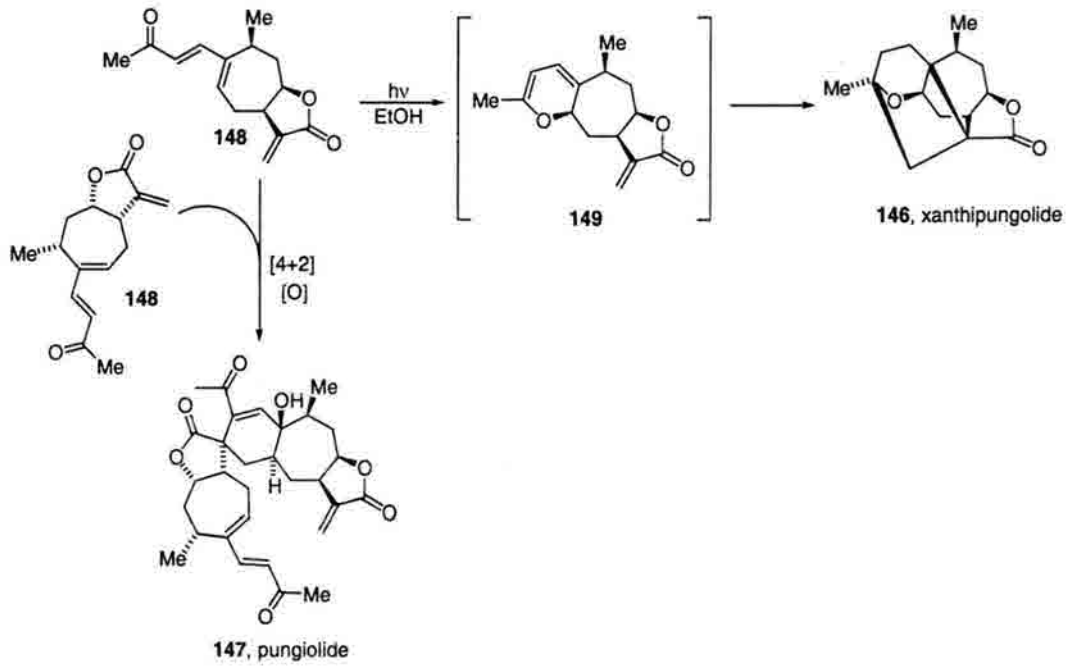
Figure 10: Retro-Diels-Alder reactions on plagiospirolide A and plagiospirolide E.[95,96]

2.3.2.2 Xanthipungliolide, Pungiolide, and Others:

Besides the plagiospirolides, there are a number of Diels-Alder adducts derived from α-*exo*-methylene-γ-lactones. The species *Xanthium pungens* produces both xanthipungolide (**146**) and pungiolide (**147**).[98] Both are proposed biosynthetic derivatives of xanthanolide **148** (Scheme 22). For the biogenesis of **146**, it was proposed that an electrocyclic reaction of **148** forms **149** which is then followed by an intramolecular Diels-Alder reaction. This proposal is supported by the synthesis of **146**

from **148**, accomplished by irradiation of **148** in ethanol. The biosynthesis of the dimer **147** is thought to arise from an intermolecular Diels-Alder of **148** and an oxidation.

Scheme 25: Proposed biosynthesis of xanthipungolide and pungiolide.[98]



Mexicanin F (**150**), from *Helenium mexicanin*, is thought to arise from the co-metabolite mexicanin E (**151**).[99] Upon heating, the dimeric sesquiterpene lactone, absinthin (**152**), gives the monomer, artabisin (**153**).[100] From its fragmentation pattern with CI mass spectrometry, the biogenesis of biennin C (**155**) is thought to occur from an intermolecular cycloaddition of the monomers, **155** and **156**.[101] Ornativolide A (**157**)[102] and fruticolide (**158**)[103] are also [4+2] cycloadducts derived from α-exo-methylene-γ-lactones.

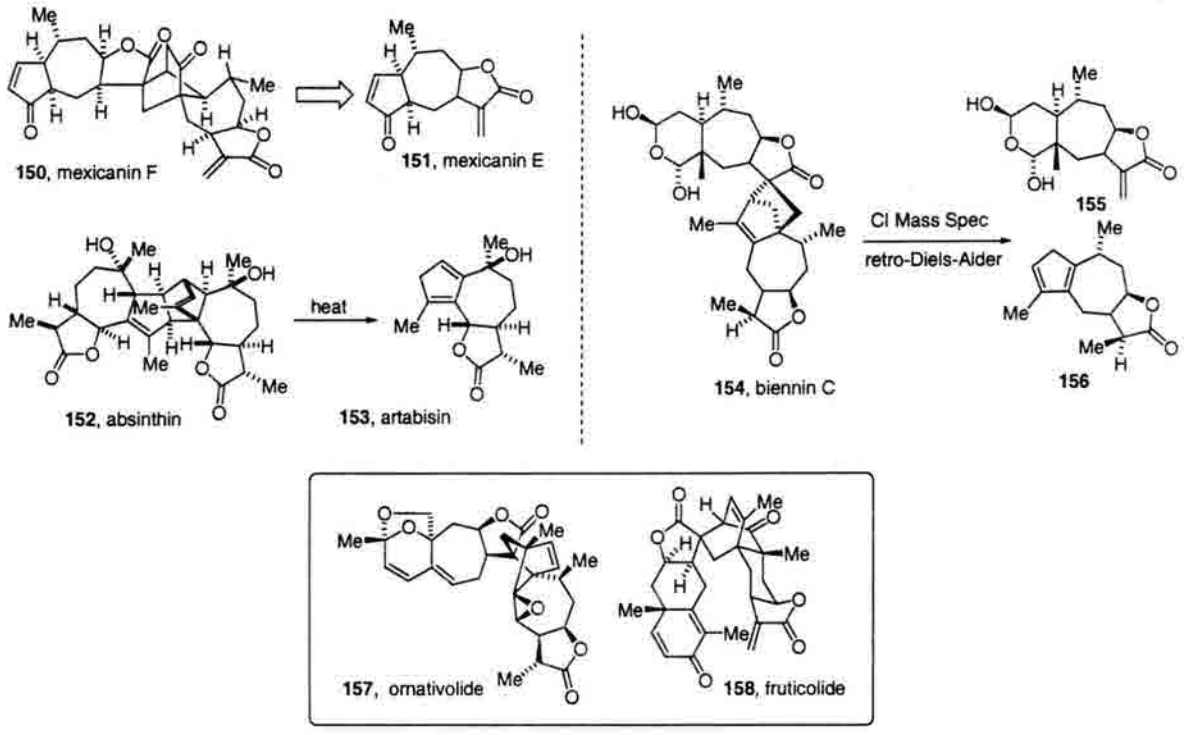


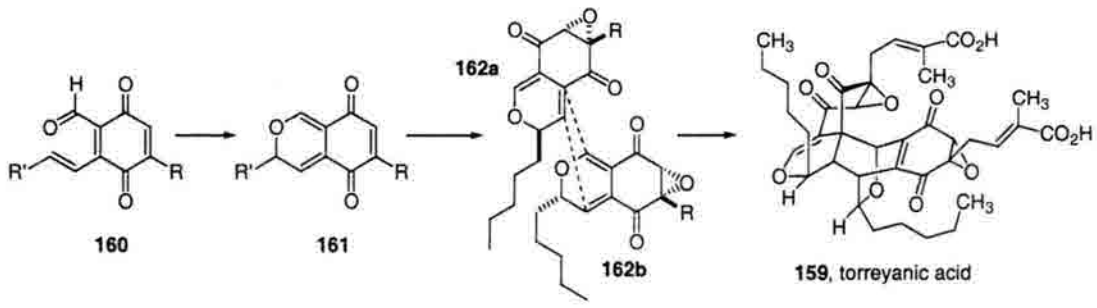
Figure 11: Diels-Alder cycloadducts derived from α-exo-methylene-γ-lactones.[99-103]

2.3.3 Homodimer Terpenoids

2.3.3.1 Torreyanic Acid

Torreyanic acid (**171**) is a cytotoxin isolated from the endophytic fungi, *Pestalotiopsis microspora*.[104] It possesses an unusual dimeric quinione structure that is postulated to arise from a Diels-Alder cycloaddition of two diasteromeric monomers. A proposed biosynthetic pathway might involve the following: a) electrocyclic ring closure of achiral **160** to form racemic **161**, b) enzymatic oxidation to generate the diastereomers **162a** and **162b** and c) a [4+2] cycloaddition to produce **159** (scheme 23). Recently a biomimetic total synthesis of **159** was completed, which employed the [4+2] dimerization of diasteromeric monomers.[105]

Scheme 23: Proposed biosynthesis of torreyanic acid.[104]



2.3.3.2 Longithorone and others:

There are a number of examples of terpenoid homodimers that might arise via a [4+2] cyloaddition. A recent example of a Diels-Alder cylized quinone dimer is longithorone (**163**), isolated from a marine tunicate.[106] Other examples include shizukaol A (**164**),[107] cyclodione (**165**)[108] and maytenone (**166**) shown in Figure 12.[109]

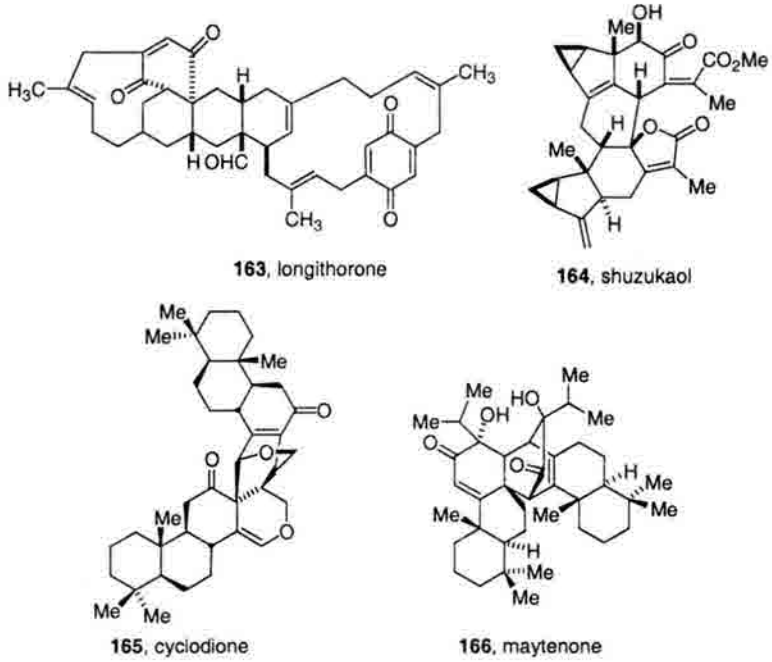
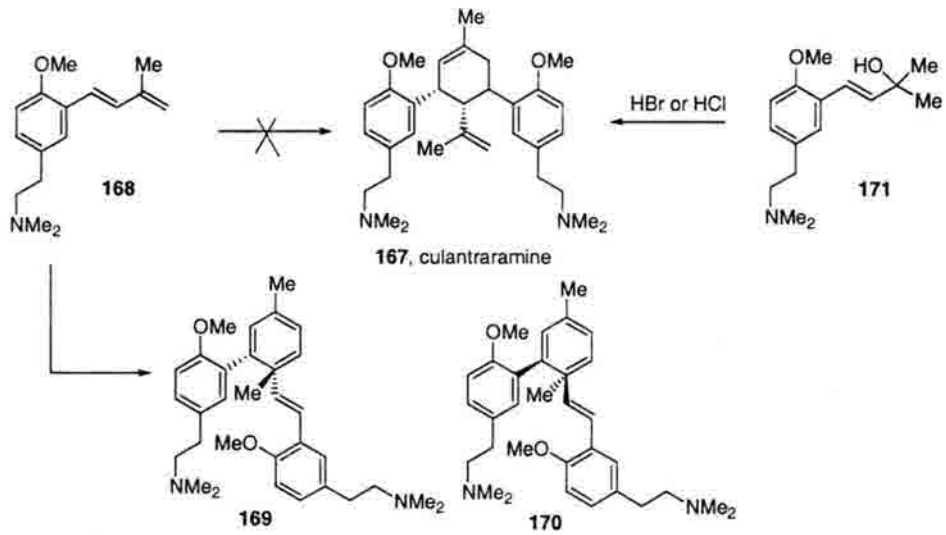


Figure 12: Proposed terpene homodimeric Diels-Alder cycloadducts.[106-109]

2.3.3.3 Culantraramine

Caution needs to be taken when considering the biosynthesis of these dimers. For example, culantraramine (**167**) could be considered as a natural Diels-Alder cycloadduct.[110] However when the proposed precursor, **168**, was allowed to stand in xylene at room temperature for 10 days, the cycloadducts **169** and **170** were obtained, not the natural product **167** (Scheme 24). On the other hand, when **170** was treated with acid, the product **167** was formed at room temperature within 30 minutes. Thus, it seems the biosynthesis of **167** does not occur though a "true" Diels-Alder cycliation, but perhaps through a cation-diene non-synchronous [4+2] cycloaddition.

Scheme 24: Biosynthetic studies on culantraramine.[110]

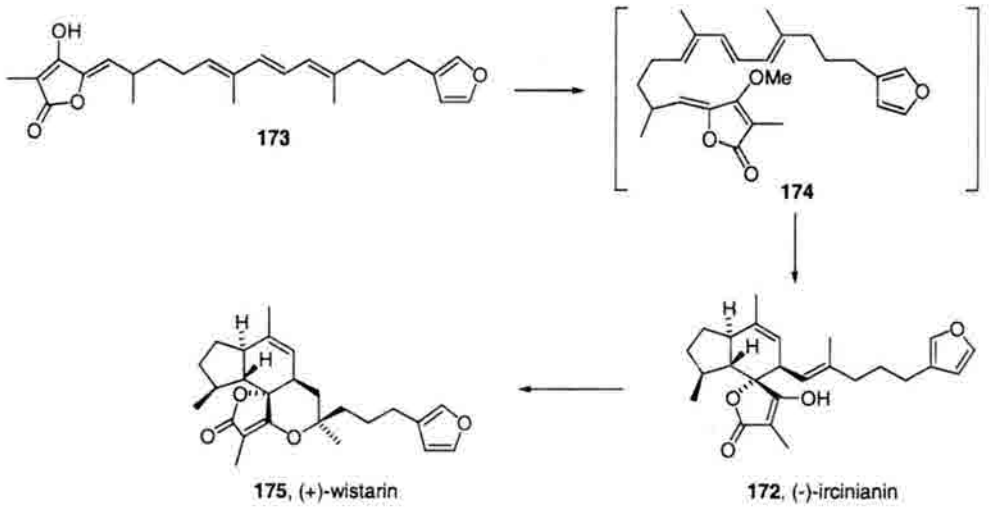


2.3.4 Miscellaneous

2.3.4.1 Ircinianin, Wistarin:

Ircinianin (**172**) is a sesterterpene isolated from the marine sponge *Ircinia wistarii*. It was postulated to arise from a [4+2] cycloaddition of the linear tretraene (**173**).[111] Both the racemate and the (-)-isomer of **172** have been synthesized utilizing this approach.[112] Wistarin (**175**) is a cyclic isomer of **172**. Interestingly, both the (+)- and (-)-isomers of **175** have been isolated, but only one enantiomer of **172** has been isolated.[113]

Scheme 25: Proposed biosynthesis of ircinianin and wistarin.[113]

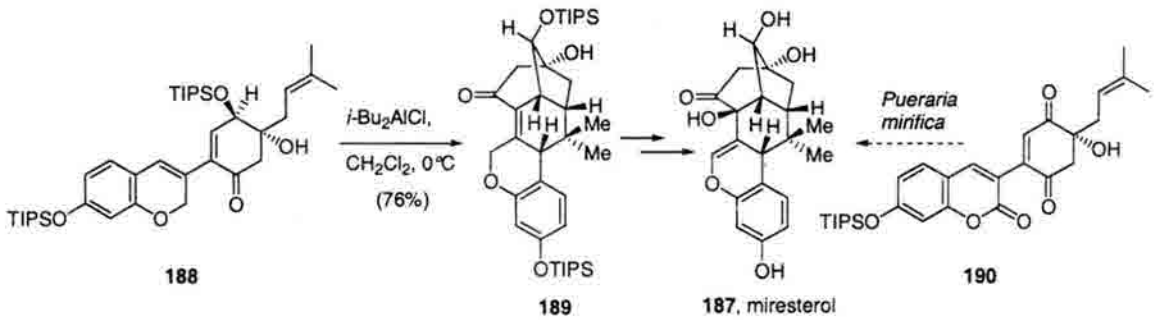


2.3.4.2 Miroesterol:

Miroesterol (**176**) is an estrogenic phenol isolated from the Thai medicinal plant, *Pueraria mirifica*. A key step in the first total synthesis of this compound by Corey and Wu was the Lewis acid catalyzed cyclization of the tricyclic ketone **177** to form **178** (Scheme 26).[114] This reaction can be regarded as a transannular double-cation-olefin

cyclization or as a Lewis acid-catalyzed, inverse electron demand intramolecular Diels-Alder reaction. Interestingly, in the course of the total synthesis of **176**, *ca* 1mg of **179** was also isolated from extracts of *P. mirifica*. It is possible that **179** is a biosynthetic precursor to **176**, in which case **176** may arise via an inverse electron demand Diels-Alder cycloaddition.

Scheme 29: Total synthesis of miresterol.[114]



188 **189** **187**, miresterol **190**

2.3.4.3 Pycnidione and others:

Pycnidione (**180**),[115] eupenifeldin (**181**)[116] and epolone (**182**)[117] are a group of recently isolated fungal metablites that possesses identical tropolone rings attached to a sesquiterpene backbone. Biosynthetically, these compounds are proposed to arise via a hetero Diels-Alder reaction of the C-11 hydroxylated humelene **183** and quinone methide tropolone **184** (Figure 13). The quinone methide **184** may in turn be generated from dehydration of the dihydroxy species **185**. Cai and co-workers suggested epolone B (**182**) might be a biosynthetic precursor to pycnidione (**180**) though a second hetero Diels-Alder reaction.[115]

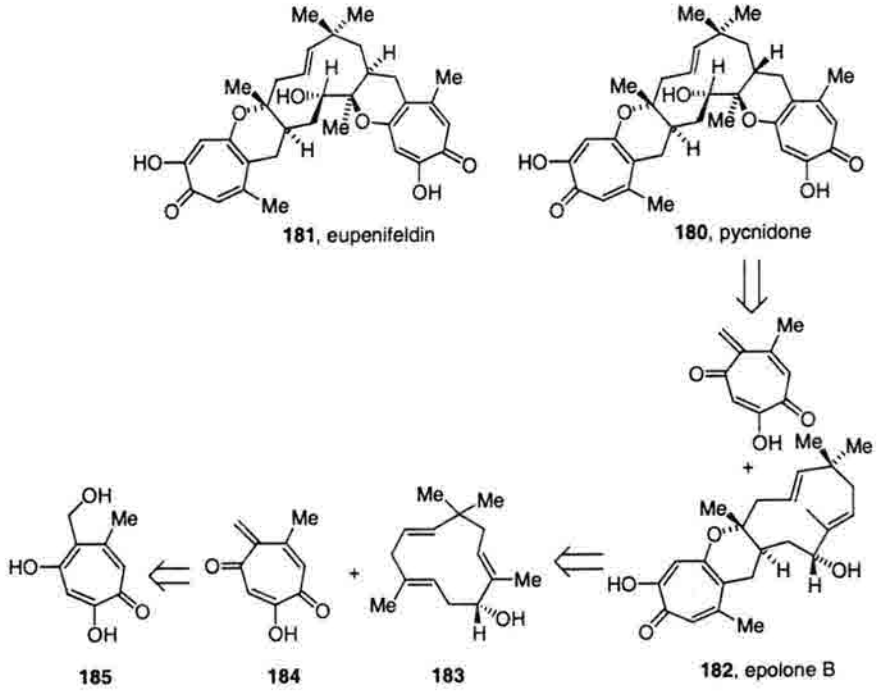
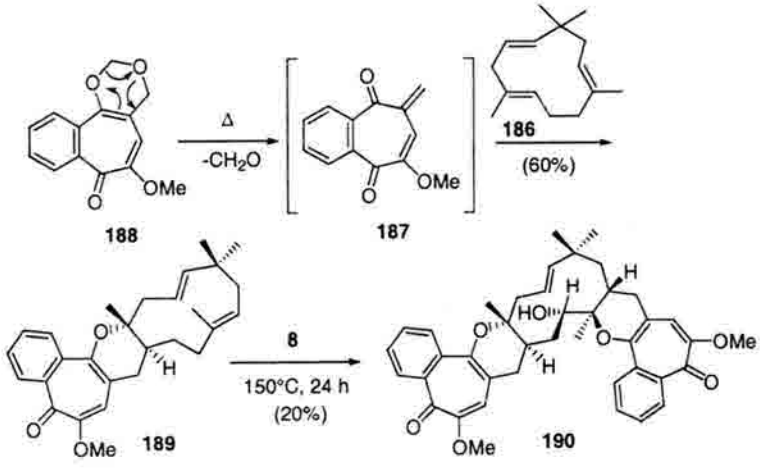


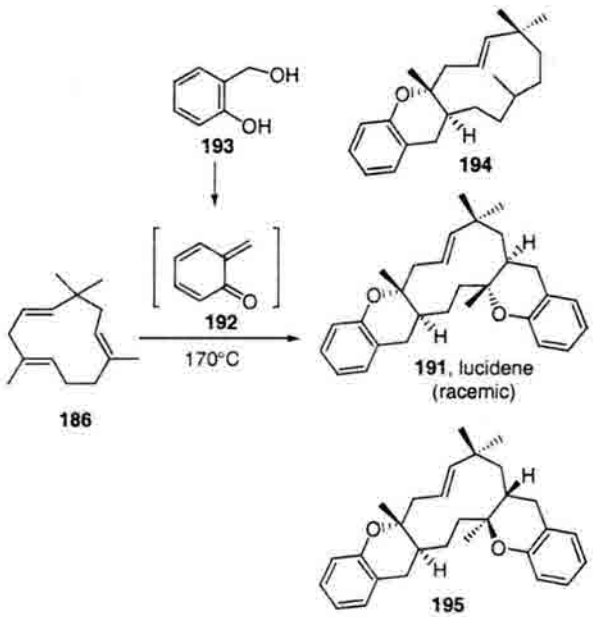
Figure 13: Proposed biosynthesis of epolone B and pycnidione.[118]

Scheme 27: Biomimetic synthesis of epolone B.[118]



To determine whether or not a hetero Diels Alder reaction is biosynthetically viable for epolone B, a model study was performed using humulene (**186**) and the benzotropolone **187** (Scheme 27). The benzotropolone **187** was formed from a thermal retro-Diels Alder reaction of **188**.[118] *In situ* trapping with humulene (**186**) afforded the Diels Alder cycloadduct **189**, analogous to epolone B (**182**). Addition of an execess of **187** at 150°C gave **190** as a 1:1 mixture of diastereomers. Since the Diels-Alder cycloadducts (**180-182**) are enatiomerically pure, there is a possibility that the addition of the tropolone is enzymatically catalyzed in the natural system.

Scheme 28: Biomimetic total synthesis of lucidene.[120]



A structurally similar compound, lucidene (**191**) has been isolated in racemic form from the root bark of *U. Lucida*.[119] It has been proposed as the product of a double [4+2] cycloaddtion of *o*-benzo-quinone methide (**192**) and α-humelene (**186**), which is

also a co-metabolite. A biomimetic synthesis provided the natural product **191** as well as the mono adduct **194** and isolucidene (**195**).[120] Unlike, compounds **180-182**, lucidene (**191**) is not optically active, thus it most likely arises from a non-enzymatic Diels-Alder reaction.

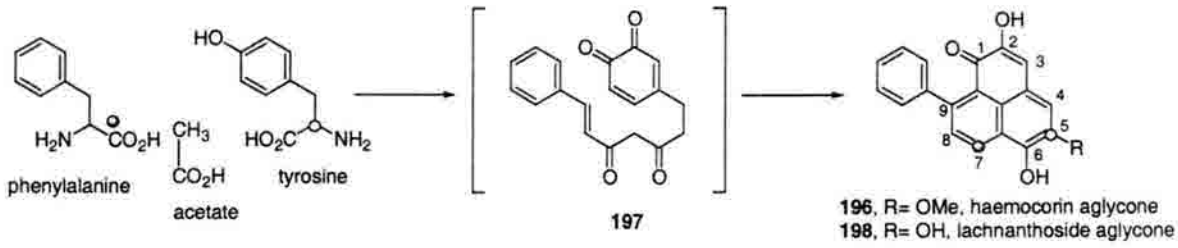
2.4 Phenylpropanoids

2.4.1 Intramolecular cycloadducts

2.4.1.1 Phenylphenalenones:

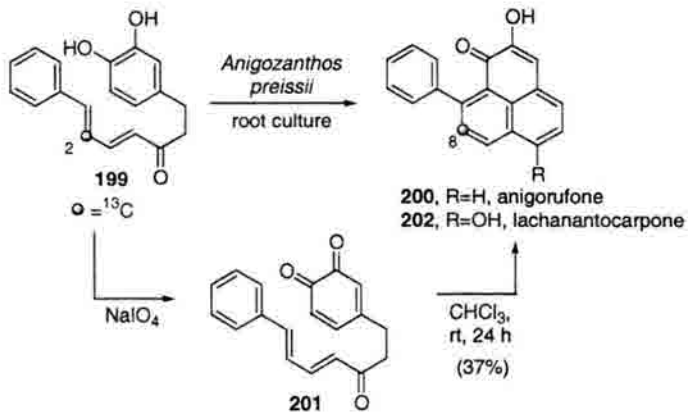
Phenylphenalenones are characteristic pigments found in the monocotyledon family, tinctoria. An early study on the biosynthesis of these compounds indicated that [2-^{14}C]-tyrosine was incorporated specifically into C-5 of haemocorin aglycone (**196**).[121] A biosynthetic pathway was proposed that involves condensation of one molecule each of phenylalanine and tyrosine (or the metabolic equivalent) with one molecule of acetic acid and loss of a carboxyl group to provide a diarylheptanoid intermediate (**197**). This intermediate could then cyclize, possibly through a Diels-Alder cycloaddition, to provide the phenylphenalenone ring system. Further evidence for this pathway was the specific incorporation of [1-^{13}C]phenylalanine into C-7 of lachnanthoside aglycone (**198**).[122] Although phenylalanine and tyrosine were found to be precursors to the phenylphenalenones, other shikimate-derived phenylpropanoids, such as cinnamic acid and coumaric acid have also been determined as precursors.[123, 124]

Scheme 29: Proposed biosyntheis of the phenylphenalenones.[125]



It was not until 1995 that experimental evidence for the intermediacy of a diarylheptanoid in phenylphenalenone biosynthesis was obtained. Höschler and co-workers showed that the Diels-Alder precursor, [2-^{13}C]1-phenyl-7(3,4-dihydroxyphenyl)hepta-1,3-dien-3-one (**199**), was specifically incorporated into anigorufone (**200**) from feeding experiments with the cultured roots of *Angiozanthos preissii*.[124] An earlier synthetic study showed that after oxidation with $NaIO_4$, unlabeled **199** was converted to lachananthocarpone (**202**) spontaneously at room temperature via an intermolecular Diels-Alder cycloaddition.[125] Thus, the Diels-Alder cyclization leading to the phenylphenalenone ring system appears to be non-enzymatic.

Scheme 30: Synthesis/ biosynthesis of phenylphenalenones.[124,125]



2.4.1.2 Brombyins:

The brombyins are novel napthelene derivatives from the Australian tree, *Brombya platynema*.[126] Although the metabolites **203** and **204** could be biogenetically derived from the oxidative coupling of two cinnamic acid residues (9-2', 7-7', **205**, Figure 14), this seems unlikely because of the perhydrogenated nature of one of the six-membered rings and because of the lack of optical activity. Instead, the isolation of the intermediate **206** led to the hypothesis of the linkage of a single C_6C_3 moiety (**207**) with an acetate chain to give an intermediate such as **208** (Figure 14). A Diels-Alder cyclization of the intermediate **209** could lead to two racemic products corresponding to **203** and **204**.

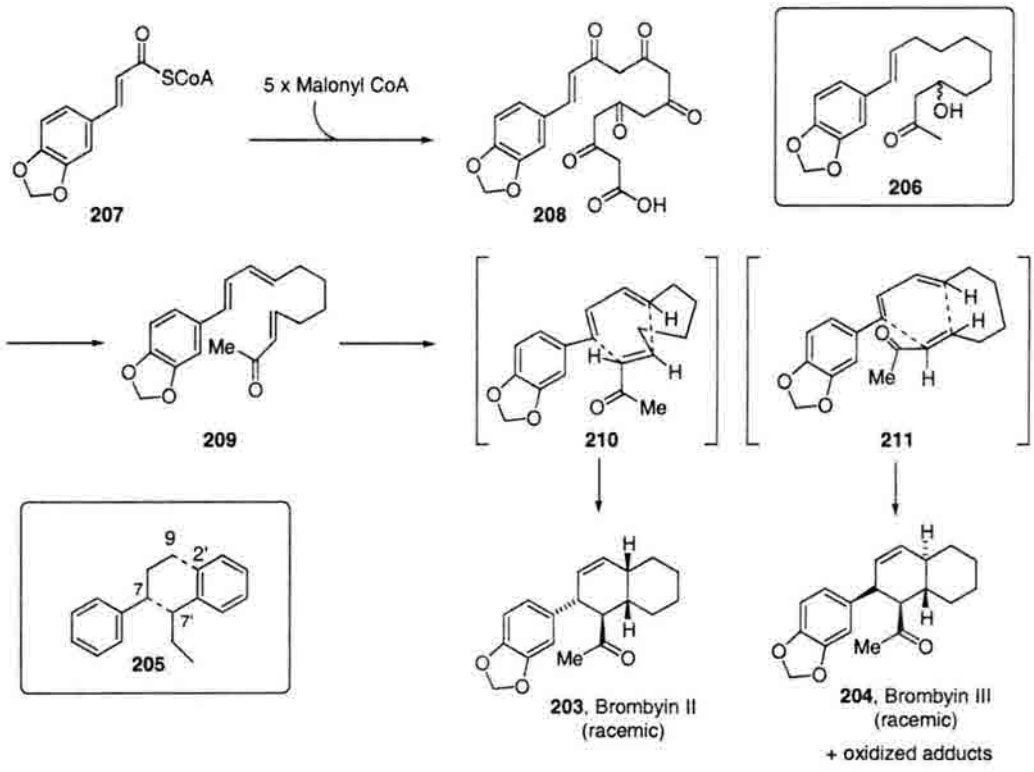


Figure 14: Proposed Biosynthesis of the brombyins.[126]

2.4.2 Intermolecular Dimeric Cycloadducts

2.4.2.1 Dimeric Coumarins:

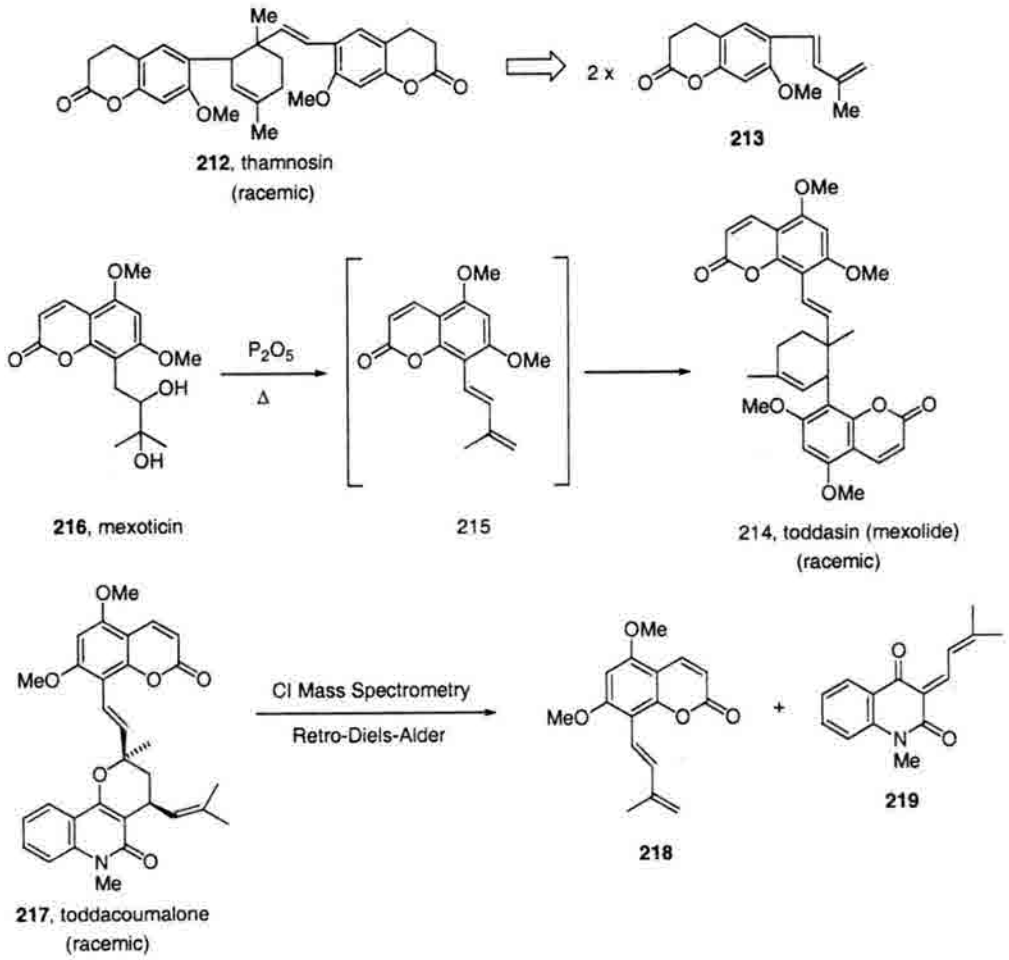


Figure 15: Dimeric coumarin Diels-Alder cylcoadducts.[127-130]

Another group of phenylpropanoid Diels-Alder adducts is represented by the dimeric coumarins (Figure 15). The first dicoumarin discovered, thamnosin (**212**), was postulated to arise from the Diels-Alder cycloaddition of the monomer **213**.[127] Later, the dicoumarin toddasin (**214**, mexolide) was isolated from two different sources, *Toddalia asiatica* and *Murraya exotica*.[128,129] Electron impact mass spectrometry of **214** led to the

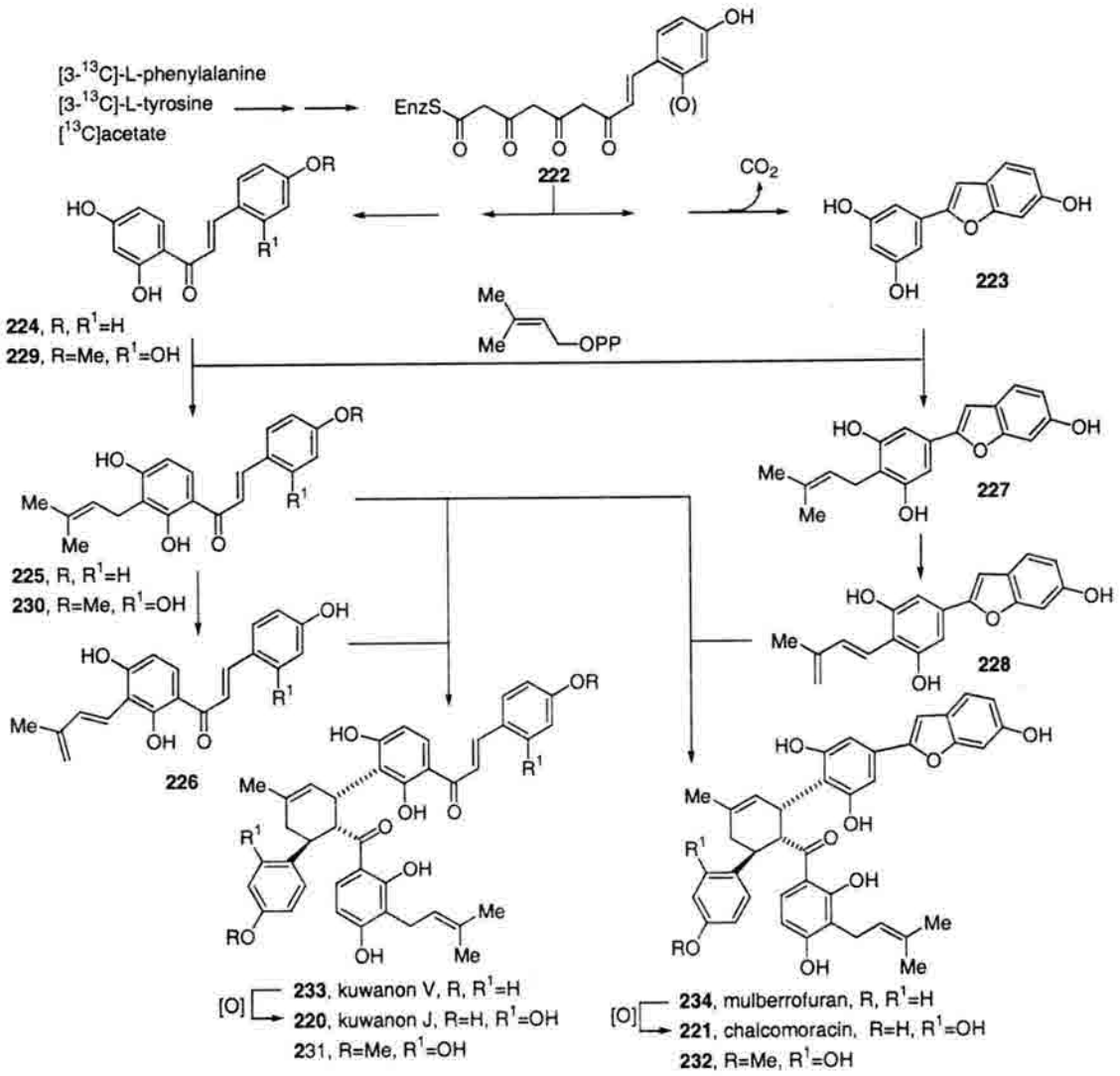
formation of the retro-Diels-Alder fragment, **215**.[128] Treatment of mexoticin (**216**), a co-metabolite of **214** in *Murraya exotica*, with P_2O_5 in refluxing xylenes led to the formation of **214**, presumably through the dehydration product **215**.[129] Toddacoumalone (**217**) was the first example of a mixed coumarin dimer. When a CI mass spectrum of **217** was acquired, protonated ions corresponding to the coumarin (**218**) and the quinolone (**219**) were obtained.[130]

2.4.2.2 Kwanon J and Chalcomoracin:

Other phenylpropanoids that are reportedly derived from a Diels-Alder cyclization are metabolites from *Morus alba* L., kuwanon J (**220**) and chalcomoracin (**221**). Selection of callus cultures from *M. alba* with a high productivity of the Diels-Alder type adducts (~100 times more of **220** and **221** than the intact plant) allowed biosynthetic studies of these compounds.[131] Feeding experiments with [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]-acetate revealed that **220** and **221** are formed from the condensation of two cinnamoylpolyketide-derived skeletons (Scheme 31).

The arylbenzofuran skeleton of **220** is apparently formed from a novel type of cyclization of the cinnamoylpolyketide **222** followed by decarboxylation to give **223**.[131] Interestingly, feeding experiments with [^{13}C]-acetate did not provide the expected labeling pattern for the prenyl groups of **220** and **221** indicating that the isoprene groups are not derived via the usual mevalonate pathway.[131,132] Incorporation of [3-^{13}C]-L-phenylalanine and [3-^{13}C]-L-tyrosine into both halves of **220** and **221** suggest a common biosynthetic route to the cinnamoylpolyketide skeleton through *p*-coumarate.[133]

Scheme 31: Proposed biosynthesis of kuwanon J (**220**) and chalcomoracin (**221**).[134]



Addition of the O-methylated chalcone, **229**, to *M. alba* cell cultures resulted in the formation of **230** as well as the O-methyl derivatives of kuwanon J (**231**) and chalcomoracin (**232**).[134] The structure of metabolite **230** indicates that prenylation occurs after aromatization of the cinnamoylpolyketide. Subsequent addition of **230** to *M. alba* cell cultures resulted in the formation of **231** and **232.** This strongly suggests that, in the natural system, one molecule of the prenylated chalcone is recognized as the dienophile

(**225**) while another prenylated chalcone, after dehydrogenation, acts as the diene (**226** or **228**). The fact that **231** and **232** are optically active and possess the same stereochemistry as **220** and **221** suggest that the Diels-Alder reaction is enzymatic.[134] Close examination of the Diels-Alder type adducts of [2-^{13}C]-acetate feeding experiments with *M. alba* revealed that the adducts, kuwanon V (**233**) and mulberrofuran (**234**) had a higher enrichment factor (24 and 22%) than either kuwanon J (**220**) or chalcomoracin (**221**) (4 and 17%).[135] This suggests that **233** and **234** give rise to **220** and **221** by hydroxylation.

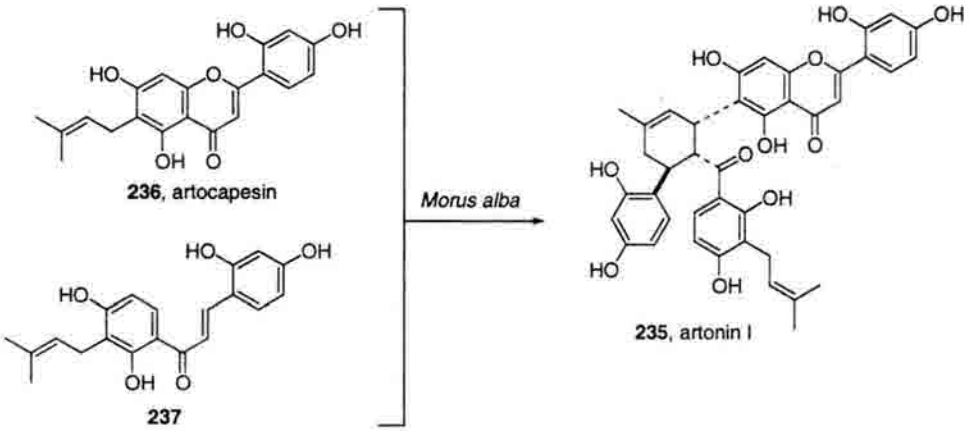


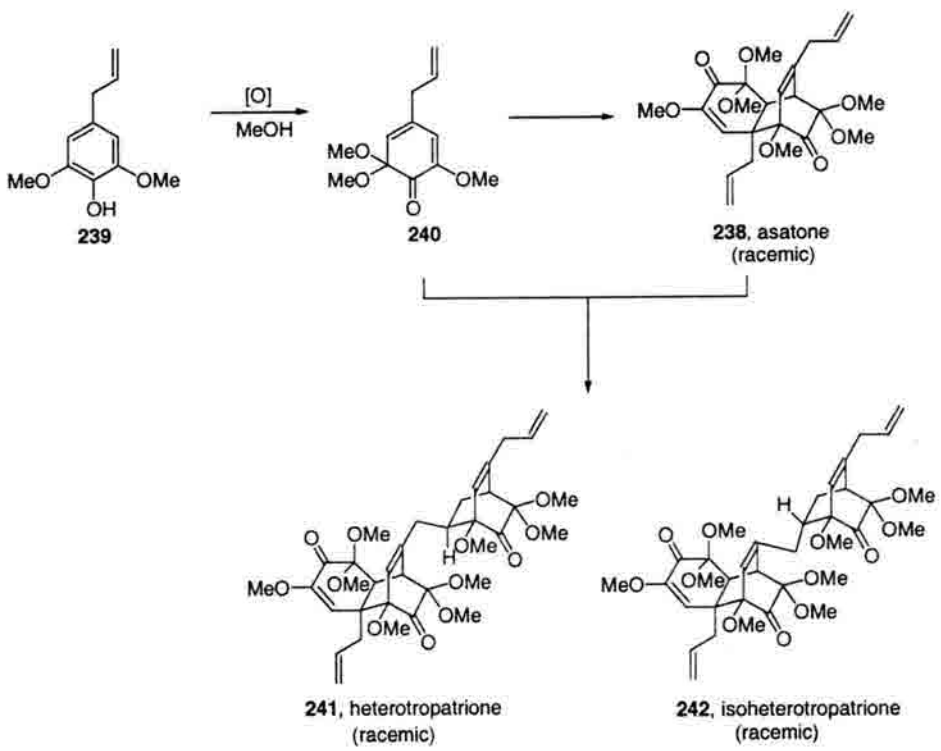
Figure 16: *In vivo* Diels-Alder mediated synthesis of artonin.[136]

The *M alba* cell cultures were also used to determine the structure of the Diels-Alder adduct, artonin (**235**) (Figure 16).[136] Since artonin is only a minor metabolite from the root bark of *Artocarpus heteropyllus* (0.7 mg, 3.5 x 10^{-6} yield), structural determination was difficult. The co-occurrence of artocarpesin (**236**) in the same plant led to the proposed biogenesis and structure shown in Figure 13. To confirm the proposal, **236** was added to an *M. alba* cell culture. Work-up provided an abberant metabolite (8 mg) that was identical to **235** by mass spectrum and ^{1}H NMR. These results indicate that

in the *M. alba* cultures, **236** reacted as a diene and **237**, which is produced in the cells, acted as a dienophile in formation of the cycloadduct.

2.4.2.3 Asatone

Scheme 32: Biosynthetic studies on asatone.[138,139]



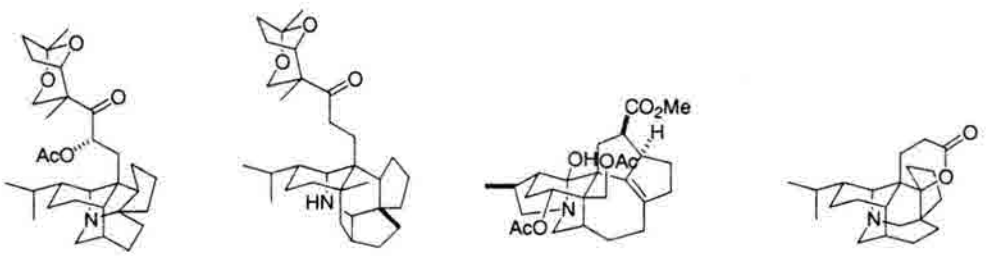
Asatone (**238**) is a neolignan isolated from the stems and rhizomes of *Asarum taitonese* Hayata.[137] The base peak in the mass spectrum of **238** was observed at half the molecular weight, which is consistent with a retro-Diels-Alder fragmentation. The skeleton of **238** is comprised of two C_6C_3 units. Biosynthetically, these C_6C_3 units can be envisioned as enzymatically oxidized 2,6-dimethoxy-4-allylphenol (**239**). The optically inactive dienone (**240**) can then dimerize to provide **238** through an intermolecular Diels-Alder reaction (Scheme 32). In fact, anodic oxidation of **239** in methanol provided a

mixture of **240** and **238**. Upon standing at room temperature, **240** was quantitatively converted into asatone (**238**).[138] The related lignans, heterotropatrione (**241**) and isoheterotropatrione (**242**) were postulated to be the Diels Alder adducts of **238** and **240**.[139]

2.5 Alkaloids

2.5.1 Daphniphyllum Alkaloids

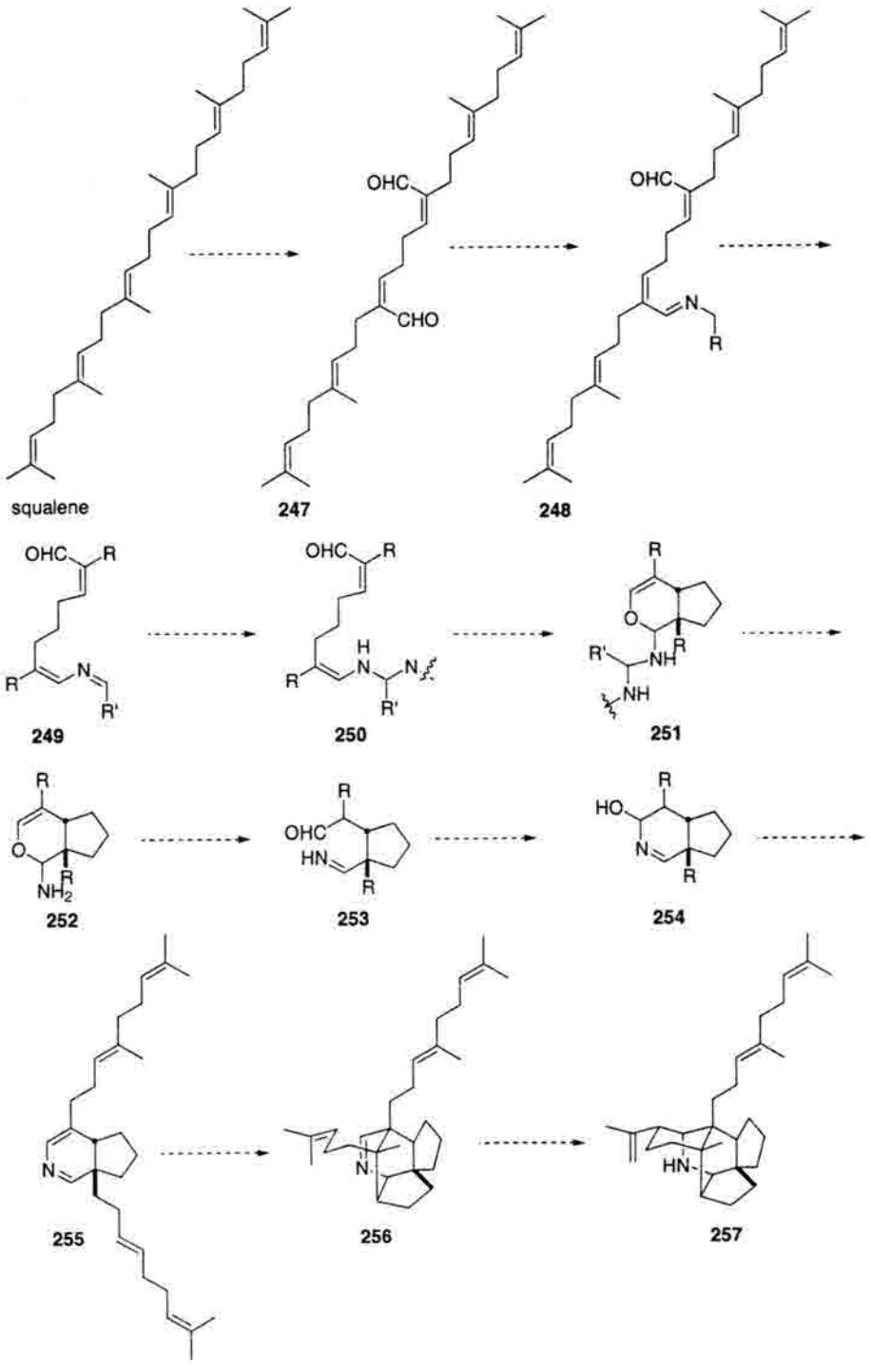
The daphniphyllum alkaloids are a growing class of polycyclic natural products that were first isolated in 1909 from the deciduous tree Yuzurha (*Daphniphyllum macropodum*). The four different skeletal classes of daphniphyllum alkaloids are represented by daphnipylline (**243**), secodaphnipylline (**244**), yuzurimine (**245**) and daphnilactone A (**246**) show in Figure 17. Early work on the biosynthesis of daphniphylline (**243**) established its mevalonate origin via a squalene-like intermediate.[140] Later, Heathcock and co-workers devised a biosynthetic proposal for the construction of the complex polycyclic ring systems of the daphniphyllum alkaloids through a Diels-Alder cyclization (Scheme 33).[141,142]



243 **244** **245** **246**

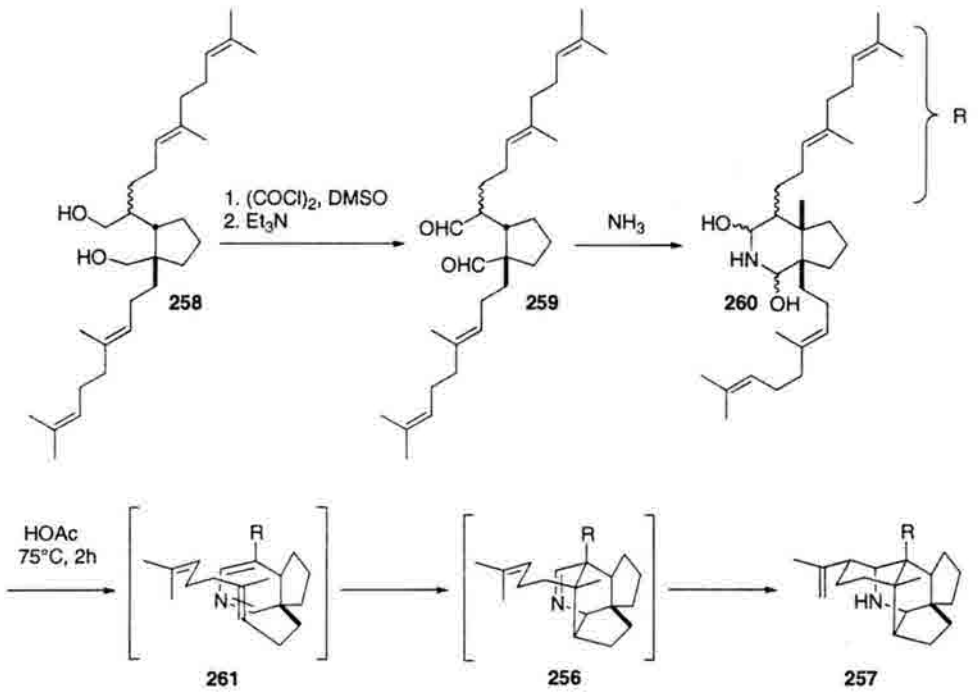
Figure 17: Representative structures of the daphniphylum alkaloids.

Scheme 33: Proposed biosynthesisof the daphnipyllum alkaloids.[144]



Heathcock proposed that the squalene derived dialdehyde **247** might condense with pyridoxamine to provide the azadiene **248**. A prototropic shift of **248** would give **249**, which upon nucleophilic addition of an amine (possibly from lysine) would furnish the enamine **250**. An intramolecular enamine/enal cyclization of **250** would afford a bicyclic dihydropyran dervative **251**. A process of proton mediated addition and elimination would, according to Heathcock's proposal, provide the dihydropyridine derivative **255**. A catalyzed intramolecular Diels-Alder reaction of **255** would give the tetrahydropyridine **256**. Subsequent ene-like cyclization of **256** would give the pentacyclic compound proto-daphniphylline (**257**), a proposed precursor to daphniphylline.

Scheme 34: Biomimetic total synthesis of proto-daphniphylline (**257**).



To explore the proposed biosynthesis, Heathcock and co-workers completed a biomimetic total synthesis of proto-daphniphylline as shown in Scheme 34.[143] The synthesis is a one pot procedure that Heathcock used to synthesize five daphniphyllum alkaloids.[143,144] Oxidation of the 1,5-diol **258** to the dialdehyde **259** was accomplished through a Swern Oxidation. The crude reaction mixture was treated with ammonia followed by Acetic Acid and ammonium acetate to provide the azadiene **261**. An intramolecular Diels Aler reaction furnished the imine **256**. Heating the acetic acid solution of the imine then facilitates an intra-molecular aza-Prins cyclization gave proto-daphniphylline (**257**).

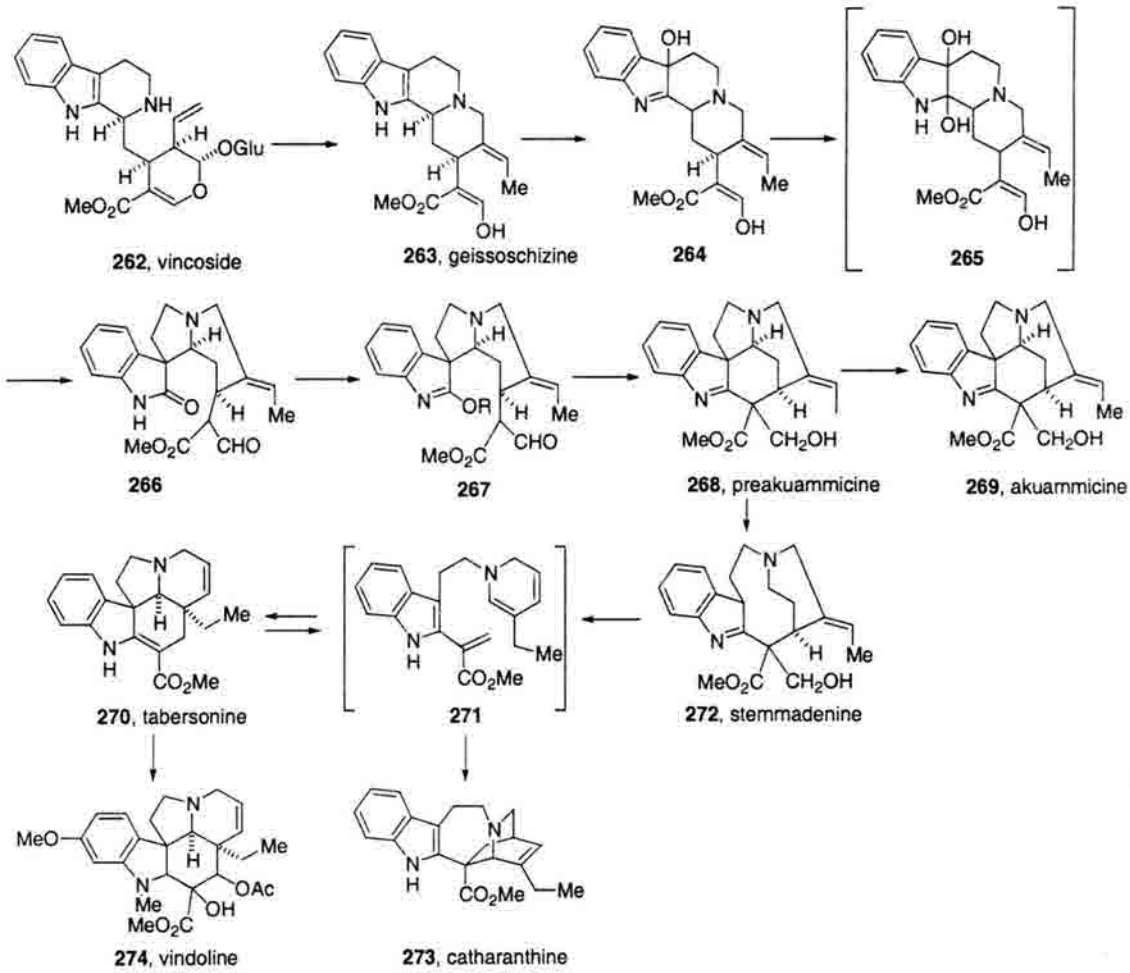
2.5.2 Indole Alkaloids

2.5.2.1 Iboga/ Aspidosperma Alkaloids

The iboga and aspidosperma alkaloids are perhaps the most well known examples of potential Diels-Alder derived natural products, and yet there is still no definitive proof for this biosynthetic pathway. By 1970, Ian Scott had elucidated a significant portion of the biosynthetic pathway through hydroponic feeding experiments with *Vinca rosea* shoots.[145] These results along with chronological isolation studies led to the proposed biosynthetic proposal outlined in Scheme 35. The intermediacy of **277** was invoked to explain the incorporation of stemmadenine (**272**) into both catharanthine (**273**, iboga skeleton) and in vindoline (**274**, aspidosperma skeleton). Scott proposed that heterolytic ring opening and concommitant dehydration of stemmadenine (**272**) would lead to the formation of dehydrosecodine (**271**), which could undergo two possible [4+2]

cycloadditions. If the 2-dehydropyridine system of **271** behaves as a dienophile, then vindoline (**274**) is produced. If, on the other hand the 2-dehydropyridine ring of **271** reacts as a diene, then catharanthine (**273**) is formed. However, with the exception of biomimetic syntheses,[146] no direct biosynthetic evidence for this postulate has been obtained.

Scheme 35: Proposed biosynthesis of the iboga and aspidosperma alkaloids.[145]



2.5.2.2 Manzamine Alkaloids:

The manzamines are a growing group of cytotoxic marine sponge alkaloids that possess unusual polycyclic diamine skeletons. Among this group are manzamine A (**275**) and B (**276**), [147] ircinals A (**277**) and B (**278**),[148] ircinols A (**279**) and B (**280**),[149] keramaphidin B (**281**),[150] xestocyclamine (**282**)[151] and ingenamine (Figure 18).[152] The ircinals **277** and **278** were proposed to be biosynthetic precursors to the manzamines **275** and **276**.[148] In fact, **277** was chemically transformed to **275** through a Pictet-Spengler cyclization with tryptamine and subsequent DDQ oxidation.[148] Ircinols **279** and **280** are antipodes of the alcoholic forms of **277** and **278** and represent the first alkaloids in this class of compounds to possess the opposite absolute configuration to the manzamines.[147] Keramaphidin B (**281**) was also postulated as a manzamine biosynthetic precursor via formation of an ircinal from hydrolysis the N-2/C-3 bond of the imino form of **281**.[150]

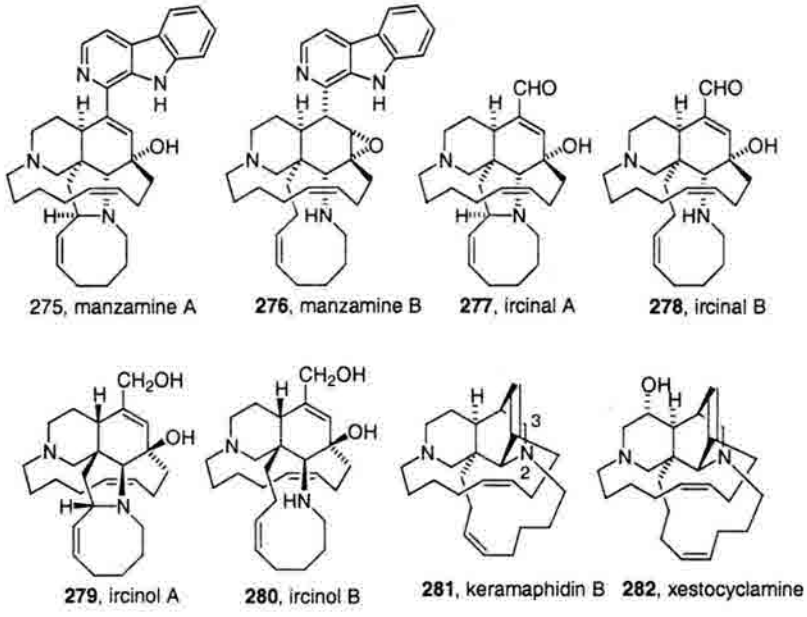
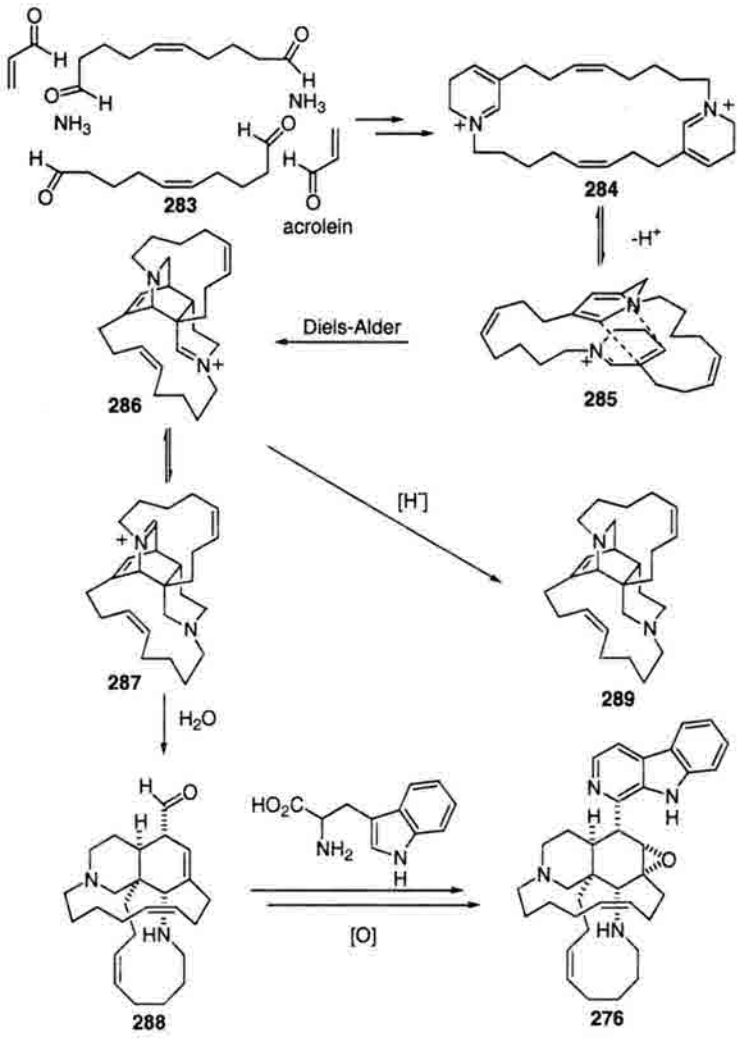


Figure 20: Structures of the manzamine alkaloids.

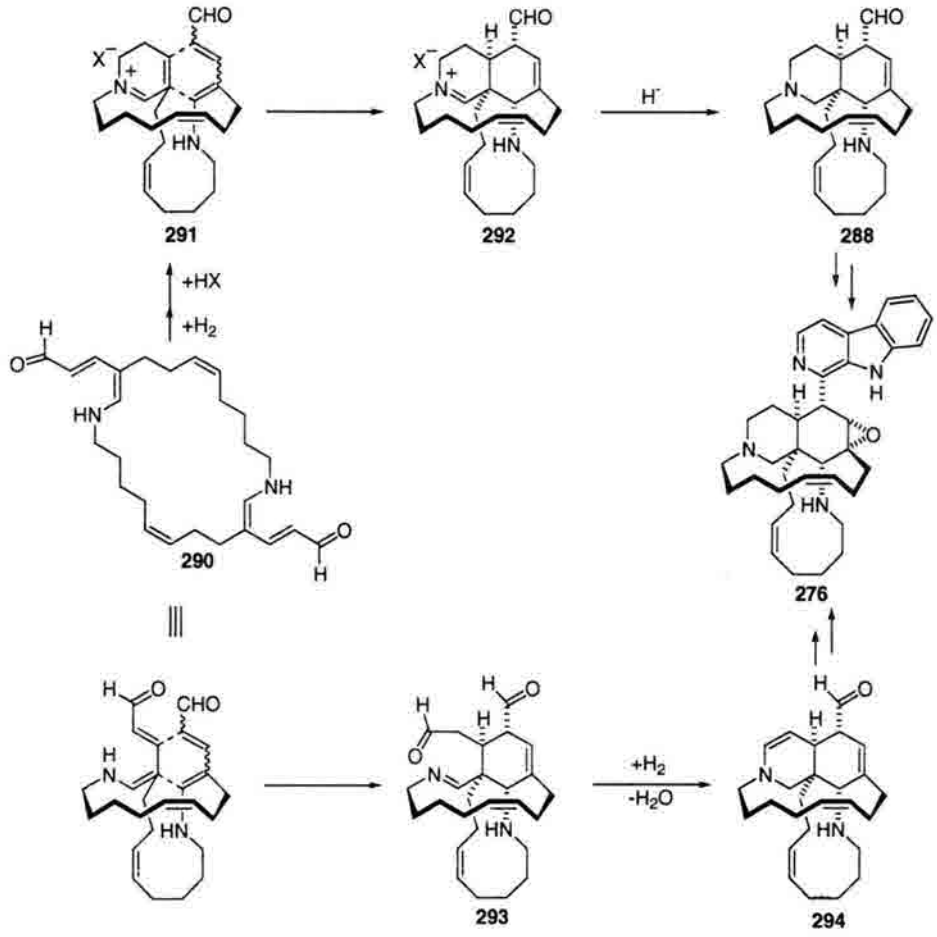
Scheme 36: Baldwin's proposed biosynthesis of manzamine B.[155]



In 1992, Baldwin and co-workers outlined a biogenesis for the manzamines (Scheme 36).[153] Manzamine B (**276**) was envisioned to be derived from four building blocks: ammonia, a C-10 unit (**283**), a C-3 unit (acrolein) and tryptophan. The key step for the proposed biogenesis is an intramolecular *endo* Diels-Alder cycloaddition of a partially reduced bis-pyridium species (**284→285→286**). The intermediacy of **284** is supported by the isolation of the bispyridium macrocycles, cyclostellettamines A-F from the marine sponge *Stelletta maxima*.[154] Later, Baldwin et al. expanded their proposal to

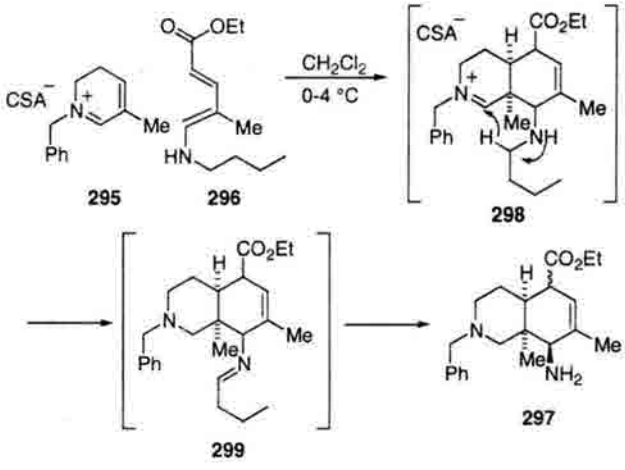
include keramaphidin B (**281**).[155] Baldwin completed a biomimetic total synthesis of **281** by dissolving the proposed intermediate **284** in a methanol/ TRIS buffer solution followed by reduction with $NaBH_4$ to provide a small amount of **281**.[156] The low yield of **289** is a reflection of the inclination of intermediate **285** to disproportionate. *In vivo*, a Diels-Alderase could limit the conformational mobility of the substrate, which would not only minimize the change in entropy toward the transition state but would also prevent the problem of disproportionation.

Scheme 37: Kaiser's proposed biosynthesis of manzamine B.[157a]



Marazano and co-workers proposed an alternate route for the biosythesis of the manzamines in 1998.[157] They suggested that the biomimetic Diels-Alder reaction could involve a substituted 5-amino-2,4-pentadienal as the diene thereby avoiding the problem of disproportionation and implementing less ring strain during the [4+2] cycloadditon. In this proposal, the building blocks of malondialdehyde (instead of acrolein), ammonia, and an appropriate unsaturated di-aldehyde generate the macrocycle **290**.

Scheme 38: Model study for the proposed biosynthetic Diels-alder cycloaddtion of the manzamine alkaloids.[157b]



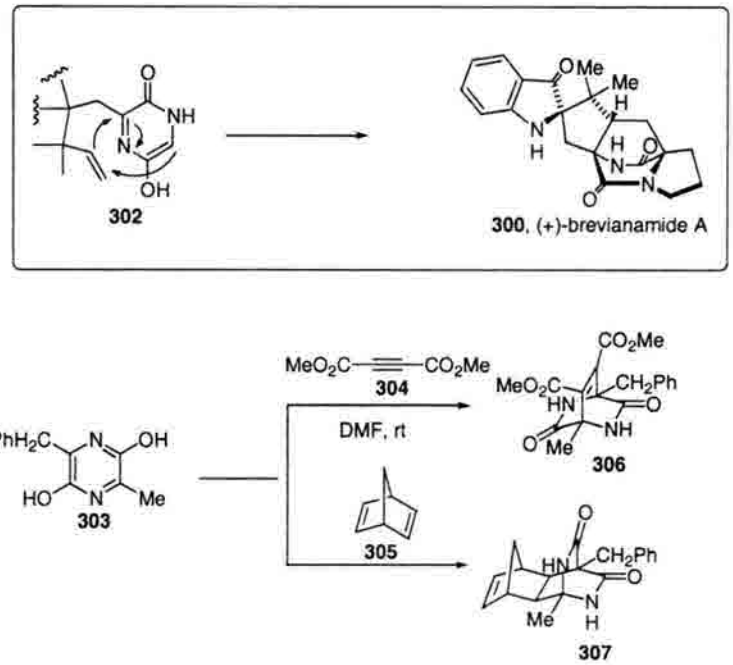
From **290**, two possible routes were proposed. Analogous to the Baldwin model, reductive cyclization of **290** would lead to the dihydropyridinium species **291**. Diels-Alder cycloaddition followed by reduction of **292** would directly produce Baldwin's proposed manzamine precursor, aldehyde **288**. Alternatively, direct cyclization of **290** to give **293**, followed by reduction of the resulting imine function and cyclization would provide the enamine **294**, which presumably could lead to manzamine B (**276**). The

occurrence of the proposed enamine **292** is justified by the isolation of the manzamine dimer, kauluamine, isolated from an Indonesian sponge.[158]

To test the first of his postulated biosynthetic routes, Marazano and co-workers performed a model synthesis as shown in Scheme 38. However, the cycloaddition of the salt **295** with **296** only produced the amino ester **297**. Presumably intramolecular hydride transfer occurred to give **298** followed by hydrolysis of the resultant imine **299**.

2.5.2.3 The Brevianamides

Scheme 39: Proposed biosynthesis of the brevianamide A and model study.



Birch and Wright first isolated the fungal metabolite brevianamide A (**300**) from *Penicillium brevicompactum* as well as other minor metabolites including brevianamide B (**301**).[159] In 1970, Sammes proposed that the unique bicyclo[2.2.2]diazoctan ring system of brevianamide A originated from a hetero Diels-Alder [4+2] cycloaddition

reaction.[160] He tested this hypothesis by treating a model dihydroxy pyrazine **303** with dimethyl acetylenedicarboxylate (**304**) and with norbornadiene (**305**) to provide the Diels-Alder cycloadducts **306** and **307**.

Early radio-labeling/ feeding experiments indicated that tryptophan, proline, and mevalonic acid were precursors to brevianamide A (Figure 21).[161] This same study also revealed the incorporation of *cyclo*-L-tryptophyl-L-proline (**308**, brevianamide F) into brevianamide A. From these results, it was postulated that the reverse prenylated intermediate deoxybrevianamide E (**309**) was a biosynthetic precursor. Deoxybrevianamide E (**309**) is an isolate from the austamide (**310**)-producing fungus, *Aspergillus ustus*, but it has not been isolated from brevianamide-producing cultures. In 1993 Williams and co-workers performed feeding experiments with [8-$^{3}H_2$]-**309** that proved deoxybrevianamide E was a biosynthetic precursor to **300** and **301**.[162]

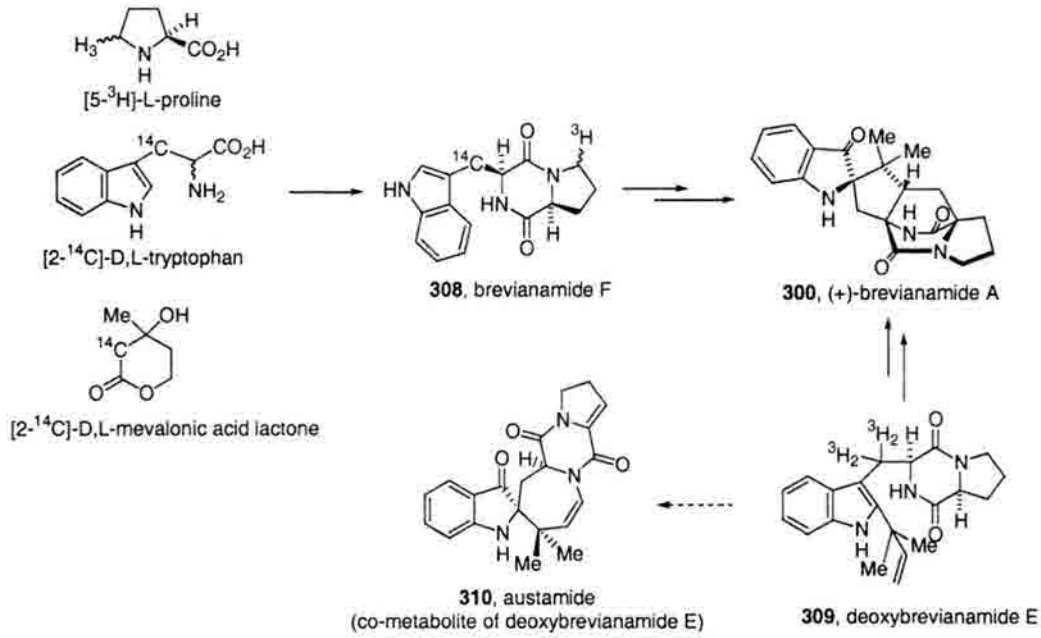
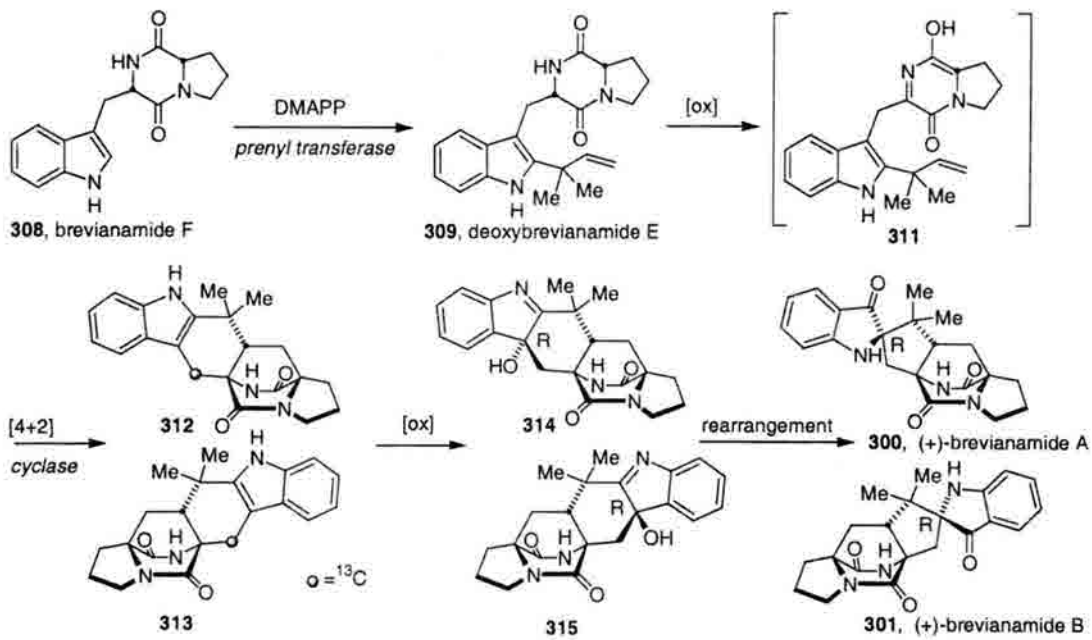


Figure 21: Biosynthetic studies on the brevianamides.[161,162]

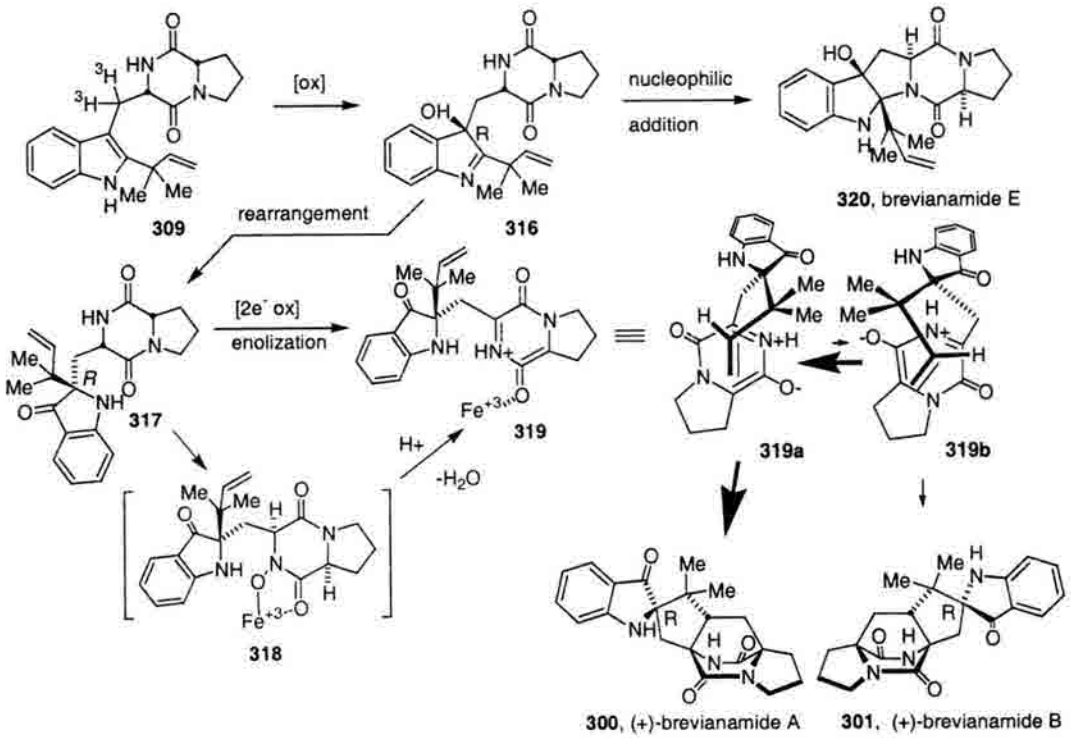
The total synthesis of **301**, completed by Williams and co-workers, revealed an enantiomorphic relationship between **300** and **301** with respect to the bicyclo[2.2.2]diazaoctan.[163] Based on these results, the biogenesis outlined in Scheme 40 was postulated. According to this proposal to-electron oxidation of **309** would yield the diene **311**, which would suffer intramolecular Diels-Alder cycloaddition to form the hexacyclic compounds **312** and **313**. Oxidation and a pinacol-type rearrangement would provide **300** and **301**. Feeding experiments were performed with *P. brevicompactum* using the proposed ^{13}C-labeled intermediates **312** and **313**, yet no detectable incorporation was observed.[162] Although this result does not rigorously exclude the intermediacy of **312** or **313**, it did lead to the proposal of an alternate biosynthesis of **300** and **301**.[162]

Scheme 40: Proposed biogenesis of the brevianamides.[163]



In the new proposal (Scheme 41), **309** would undergo oxidation to the hydroxyindolenine **316** and pinacol rearrangement to the indoxyl **317** *before* forming the requisite azadiene **319** through 2e- oxidation and enolization. The intermediacy of **316** is supported by the isolation of the co-metabolite, brevianamide E (**320**), which can be envisioned as shunt metabolite via nucleophilic addition. In fact, [8-$^{3}H_2$]-**309** was incorporated into **320** in high radiochemical yield (38.5% specific incorporation).[161] The natural products **300** and **301** would arise from a diels-Alder cycloaddition of the azadiene **319**. For this hypothesis to be valid, **319** must form a major conformer **319a** that forms **300** and a minor conformer **319b** that forms **301**.

Scheme 41: Revised biosynthetic proposal for the brevianamides.[162]



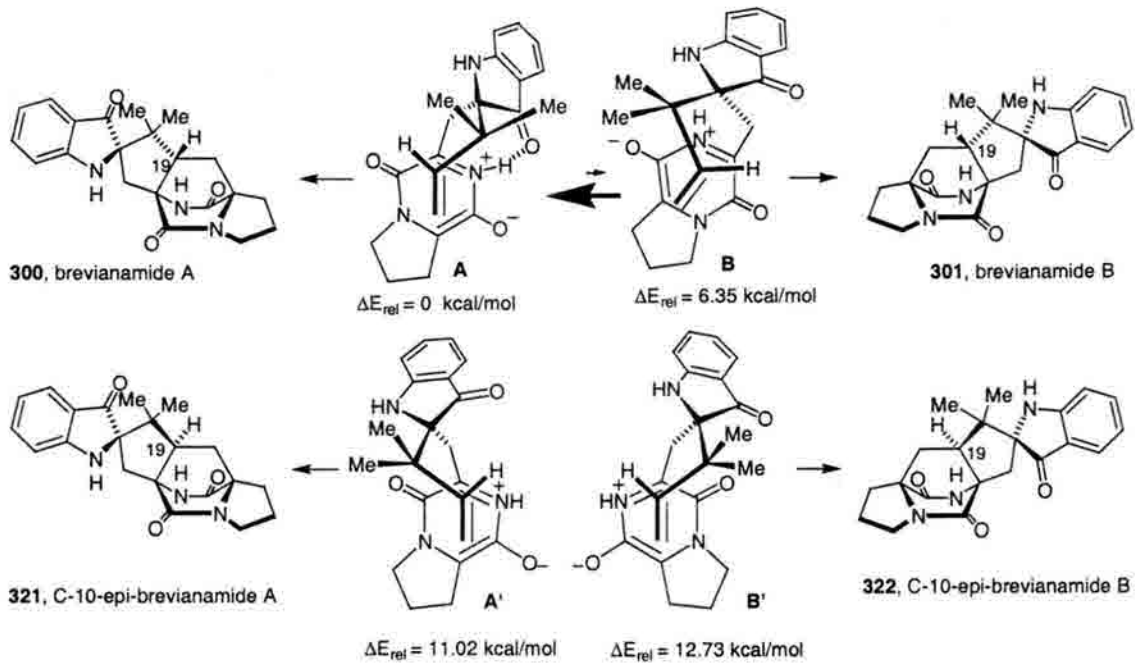
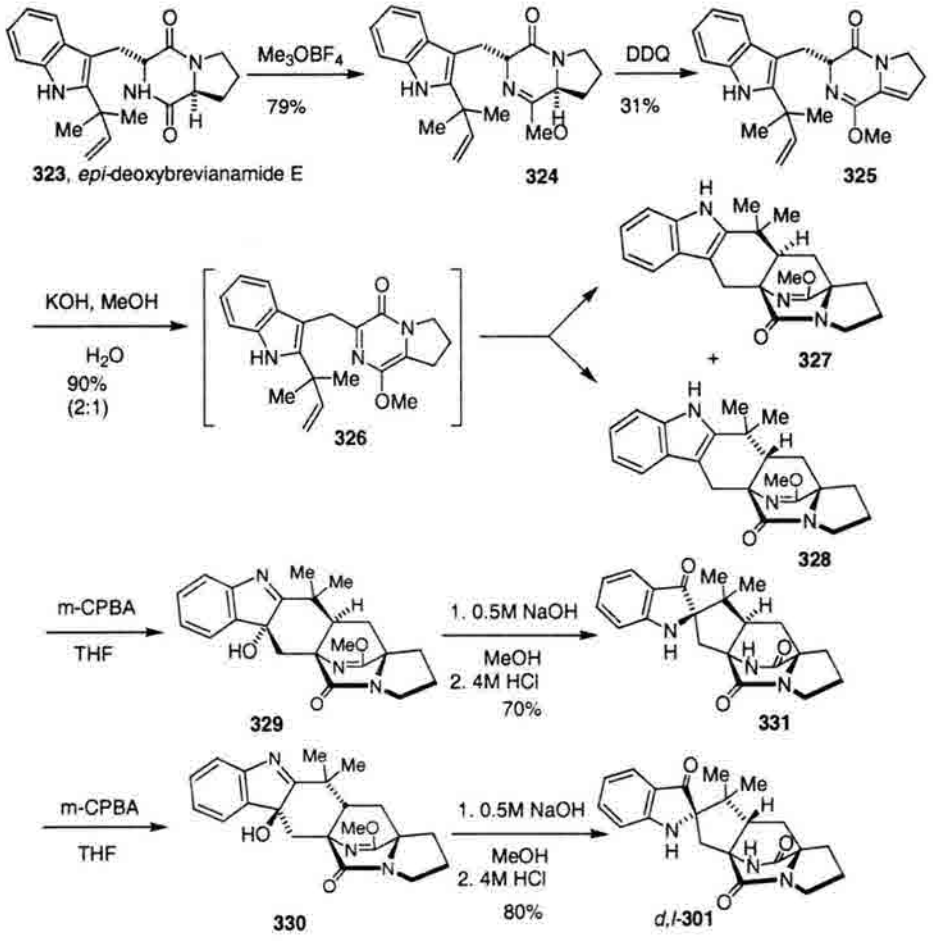


Figure 22: Theoretical studies on the Diels-Alder cyclization of the brevianamides.[164]

Ab initio calculations were carried out to determine if there was a conformational preference for the azadiene **319** (Figure 22).[164] The potential energy barriers for the diastereomeric transtion state structures **A**, **B**, **A'** and **B'** were calculated (6-31G*/3-21G). The potential energy barrier for **A**, was determined to be 38.68 kcal/mol and the potential energy barriers for **B**, **A'**, and **B'** were higher by 6.35, 11.02, and 12.73 kcal/ mol respectively. While the transition state structures **A** and **B** lead to the observed biosynthetic products **300** and **301**, the transition structures **A'** and **B'** lead to stereoisomers **321** and **322**, which are unknown as natural products. The positioning of the vinyl group in relation to the azadiene system may cause the difference in energy between the four transition states and the difference between **A** and **B** could be caused by the ability or inability to form a hydrogen bond between the hydrogen of the amine group and the oxygen of the carbonyl group. This *ab initio* study is consistent with the observed

product ratios of **300** and **301** and supports the proposal of an intramolecular Diels-Alder cycloaddition of the proposed biosynthetic key intermediate **270**. However, the issue of enzymatic catalysis remains unsolved.

Scheme 41: Biomimetic synthesis of brevianamide B.[166]



Although the biogenesis of the brevianamides was first postulated to occur though a Diels-Alder cycloaddition in 1970,[160] there was very little published data on the reactivity of the putative azadienic system until recently.[165] To expore the feasibility of a [4+2] construction of the bicyclo[2.2.2]diazaoctan, a biomimetic total synthesis of

brevianamide B was completed (Scheme 42).[166] Treatment of *epi*-deoxybreviamamide E (**323**) with trimethyloxonium tetrafluoroborate provided the lactim ether **324**. Subsequent oxidation with DDQ gave the azadiene **325**, which cyclized spontaneously upon tautamerization under basic conditions to give the racemic cycloadducts **327** and **328**.

Separate diastereoselective oxidations of **327** and **328** with *m*-CPBA provided the hydroxyindolenines **329** and **330**. Finally, base-catalyzed pinacol-type rearrangements and removal of the lactim ethers provided racemic C-19-epi-brevianamide A (**331**) and racemic brevianamide B (**301**). This study demonstrates that the core bicyclo[2.2.2] diazaoctan most likely occurs via an intramolecular Diels-Alder cyclization. However, there is complete facial exclusivity in the biosynthetic system, while this is not the case for the laboratory cyclization. These results raise the possibility of protein organization of the transition state structure, but leaves uncertainty as to the oxidation state of the indole moiety.

2.6 Summary:

A number of examples have been presented which indicate that Nature utilizes the Diels-alder reaction to generate a complex array of natural products. In many cases, such as in the endiandric acids,[77] lucidene[120] and asatone,[138] these reactions are non-enzymatic and give rise to racemic products. For the natural products that are enatiomerically pure, there is growing evidence that the Diels-Alder reactions can be enzymatically catalzyed. For most of the presented compounds, the evidence is circumstantial. For example, the biomimetic laboratory cyclizations of epolone B[118] was not stereoselective, indicating that there may be some protein organization in the natural system. However, the

enzymatic activity observed for cell-free extracts of *A. solani*[49] and for the isolated enzymes lovastatin nonaketide synthase[35] and macrophomate synthase[36] provide definitive proof for the existence of a Diels-Alderase enzyme.

CHAPTER 3

Origin of the β-Methyl-Proline Ring in Paraherquamide A

3.1 The β-methyl-proline ring in natural products

There are a number of natural products that contain the non-proteinogenic amino acid β-methyl-proline (Figure 5). Some of these are the previously mentioned alkaloids, paraherquamide A (**1**), VM55599 (**13**) and asperparaline (**21**). Another group of compounds that contain the β-methyl-proline ring are the peptide antibiotics, bottromycin (**73**), scytonemin A (**74**),[167] and roseotoxin B (**75**).[168]

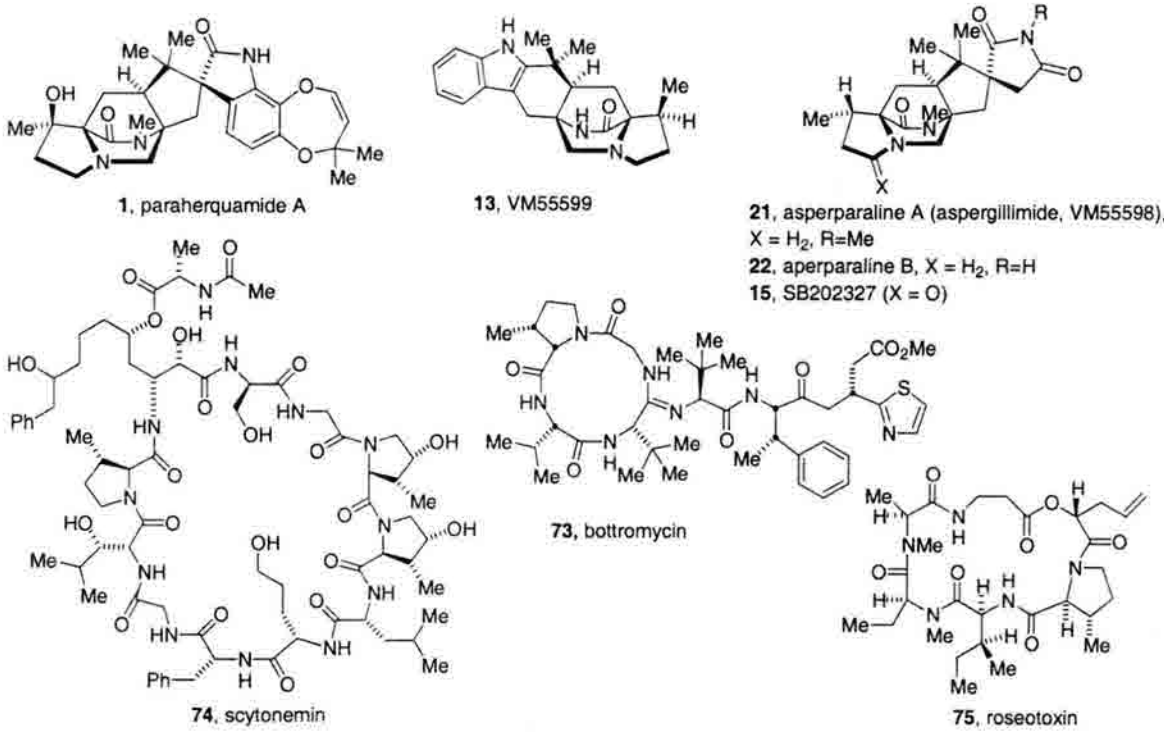


Figure 5: Compounds containing the β-methyl proline ring.

Arigoni and co-workers studied the biosynthesis of the β-methylproline residue in the cyclic peptide antibiotic, bottromycin (**73**), a natural substance isolated from *Streptomyces bottropenis* (Figure 5). Their studies revealed incorporation of [*methyl*-^{13}C]-L-methionine (**76**) into bottromycin at the 3(*R*)-methyl group of the β-methyl proline ring of bottromycin with a specific incorporation of 20-25%.[169] Methylation of the β-position of proline could arise *via* methyl transfer from S-adenosylmethionine to a 2,3-dehydroproline derivative followed by reduction. However, the ETH group has demonstrated that the mechanism for this conversion is a B_{12}-type radical coupling mechanism without loss of the prolyl α-proton.

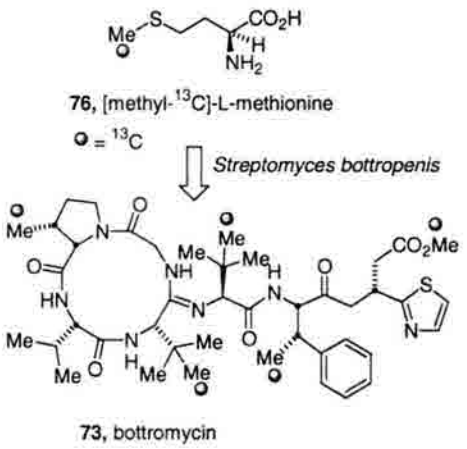


Figure 6: Origin of the methyl group of β-methyl proline in bottromycin.[169]

3.2 Incorporation of primary amino acids

Based on the previous biosynthetic studies on marcfortine and brevianamide, it seems plausible that the oxindole ring of paraherquamide is derived from L-tryptophan.

The results of the biosynthesis of marcfortine also indicate that C-29, the N-methyl group, is derived through methyl transfer from L-methionine via *S*-adenosyl methionine (SAM). The biosynthetic pathways of brevianamide and bottromycin suggest that the β-methyl-β-hydroxy proline of paraherquamide is derived from proline itself (or perhaps glutamic acid) and is methylated by SAM. To test these possibilities, feeding experiments were performed on *Penicillium fellutanum* using [1-^{13}C]-L-tryptophan (**77**),[170] [*methyl*-^{13}C]-L-methionine (**76**)[170] and [1-^{13}C]-L-proline (**78**). The results are depicted in Figure 7 and the percentage of incorporation, as calculated from ^{13}C-NMR and electrospray mass spectra, are reported in Table 2.

Table 2: Incorporation of labeled amino acids into paraherquamide A

Compound	mmol	**1** (mmol)	Enhancement of ^{13}C peak	Incorporation by ^{13}C NMR	Incorporation by ES$^+$ MS
77	0.152	0.041	9.4% (C-12)	2.5 %	1.2 %
76	0.164	0.049	2.2% (C-29)	0.66 %	0.4 %
78	0.149	0.011	ND[a] (C-18)	ND[a]	ND[a]
79a	0.155	0.059	8.9 % (C-18)	3.7 %	3.0 %
79a	0.157	0. 028	16.7% (C-18)	3.3 %	2.2%

[a] ND=Not Detectable

Incorporation of [1-^{13}C]-L-tryptophan (**77**) occurred, as expected, with an enrichment of 9.4% at C-12 and 2.5% incorporation of the precursor into paraherquamide, as determined from the ^{13}C-NMR. Incorporation of [*methyl*-^{13}C]-L-methionine (**76**) was only observed at C-29 (2.2% enhancement, 0.6% incorporation by NMR) but not at C-17. In addition, the feeding experiments with [1-^{13}C]-L-proline (**78**) did not show any incorporation within detectable limits of ^{13}C-NMR or electrospray mass

spec. Although the non-incorporation of methionine and proline does not rigorously exclude them from being precursors to the β-methyl-β-hydroxy proline ring of paraherquamide A, it does decrease the likelihood. Therefore, an alternate pathway was explored.

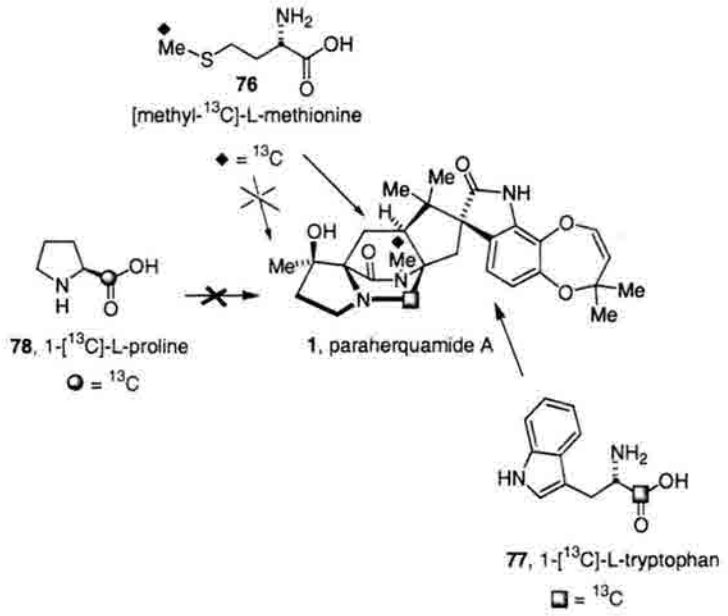


Figure 7: *P. fellutanum* feeding experiments with methionine, tryptophan and proline

Close examination of the β-methyl-proline ring revealed that if hydroxylation occurs with retention, then the 3-position would possess *S*-stereochemistry, which is the same stereochemistry found in L-isoleucine. Oxidative cyclization of the terminal methyl group of L-isoleucine could provide the requisite β-methyl-proline ring. Feeding experiments were performed on *P. fellutanum* cultures using 90% enriched [1-^{13}C]-L-isoleucine (**79a**). Inspection of the ^{13}C-NMR revealed 8.9-16.7 % enrichment at C-18 and 3.3-3.7% incorporation of **79a** into paraherquamide A.[170] The ^{13}C-NMR of paraherquamide A from the feeding experiment with [1-^{13}C]-L-isoleucine (**79a**) is shown in Figure 8.

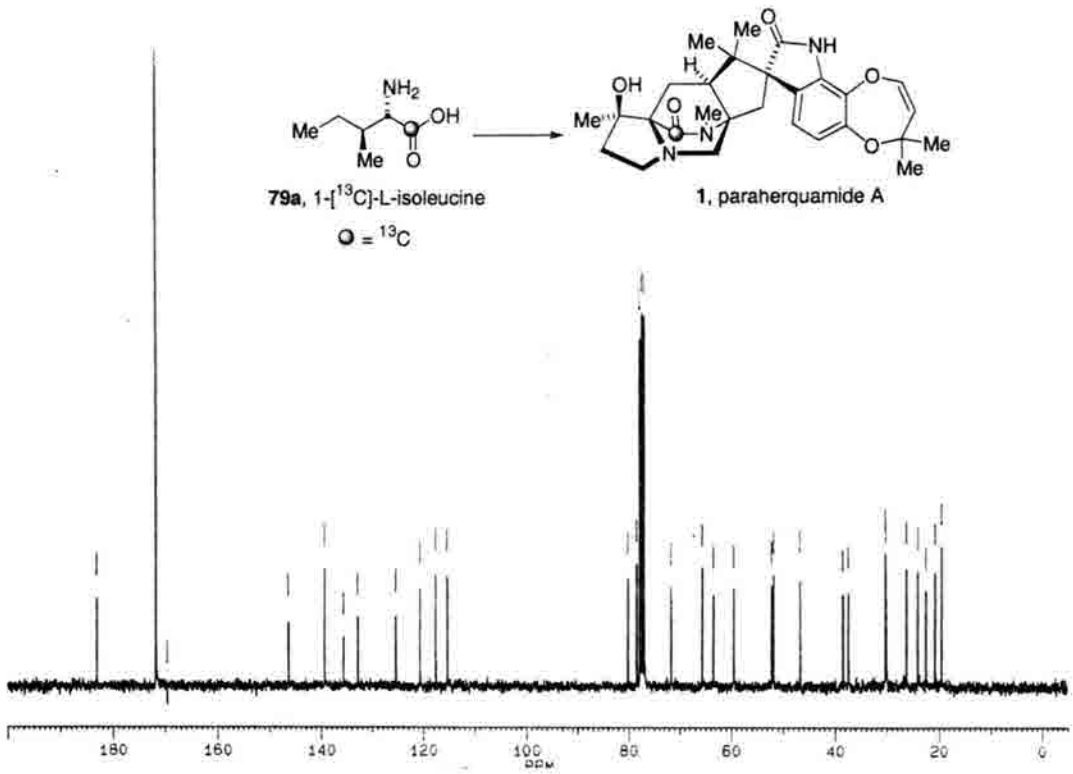


Figure 8: ^{13}C spectrum of paraherquamide A from feeding experiment with L-isoleucine.[174]

3.3 Mechanism of Oxidative Ring Closure of L-isoleucine

The oxidative cyclization of L-isoleucine (**79**) appears to be a unique biosynthetic transformation. However, there are other types of oxidative cyclizations of L-isoleucine. For example, L-isoleucine has been postulated to undergo oxidative cyclization onto the amino terminus to form polyoximic acid (**80**, Figure 9).[171]

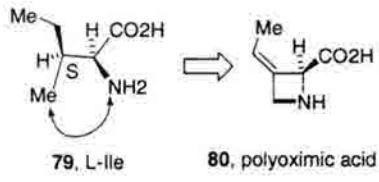


Figure 9: Possible oxidative cyclization of polyoximic acid.[171]

It has also been shown from feeding experiments with *Pseudomonas syringae* pv. *glycinea* that L-isoleucine (**79**) is the precursor to coronamic acid (**81**) via L-*allo*-isoleucine (**82**).[172] Feeding experiments with [6-^{13}C, 6-$^{2}H_3$]-D,L-isoleucine mixed with [6-^{13}C,6-$^{2}H_3$]-D,L-*allo*-isoleucine showed retention of two deuterons at C-6, the cyclopropane methylene. This indicates that during the cyclization, C-6 cannot possess an oxidation state higher than an alcohol. However, neither the C-6 alcohol of isoleucine or *allo*-isoleucine nor the γ-lactone derivatives were incorporated. These results along with the fact that the C-3 tritium label of [L-^{14}C, 3-^{3}H]-L-allo-isoleucine is retained in the biosynthesis of coronamic acid led to the proposed biosynthesis shown in Figure 28.[172]

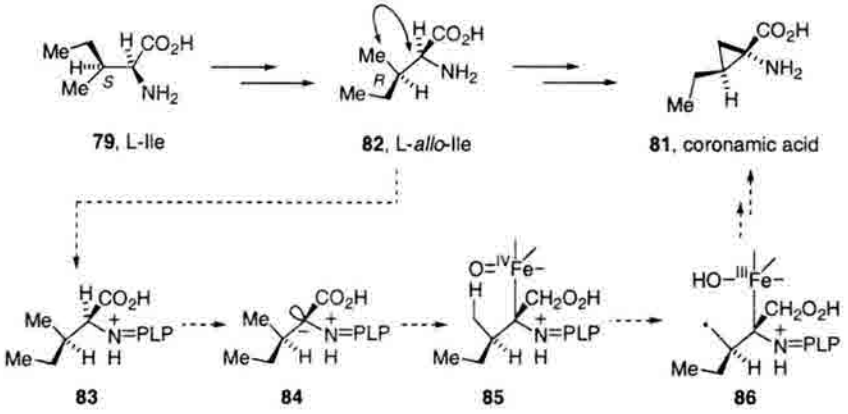
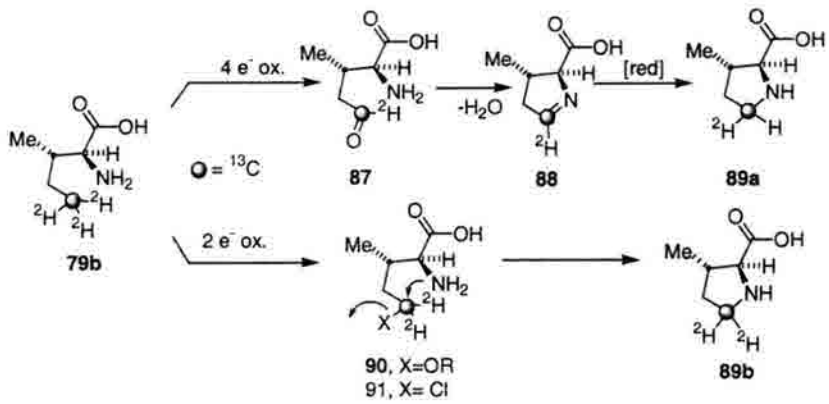


Figure 28: Proposed biosynthetic conversion of L-*allo*-isoleucine to coronamic acid.[172]

It is postulated that the reaction of L-*allo*-isoleucine (**82**) with pyridoxal phosphate (PLP, **83**) would provide a Schiff base, which after deprotonation, would yield the α-carbanion (**84**). The carbanion could then react with a ferryl species, forming the intermediate (**85**). Hydrogen abstraction from the C-6 methyl group by the iron-oxo

group would provide a carbon radical (**86**) that would collapse to give a PLP adduct of coronamic acid.

Scheme 6: Possible Biosynthetic Pathway for the conversion of L-isoleucine to 3(S)-methyl-L-proline



There are several possible mechanisms for the oxidative cyclization of L-isoleucine to β-methyl-proline. Two plausible pathways are depicted in Scheme 6. One pathway involves 4-electron oxidation of the distal sidechain methyl group of L-isoleucine (**79**) to the aldehyde (**87**). Cyclization and loss of water would provide the imine **88**, which could then be reduced to form β-methyl-proline (**89**). Another reasonable pathway involves 2-electron oxidation of the distal sidechain methyl group to the alcohol (**90**, R=H) or to the chlorinated intermediate **91**. The chlorinated intermediate could be displaced directly via nucleophilic attack to give β-methyl proline (**89**). The alcohol would probably be converted to a better leaving group, such as a phosphate, before nucleophilic displacement to provide β-methyl proline.

Although the chlorination of L-isoleucine in a biological system seems, at first, unlikely, there is precedent for such a transformation. Victorin C (**92**), a macrocyclic peptide, contains a dichlorinated leucine moiety (Figure 29). Feeding experiments with ^{2}H-labeled-α-keto-isovalerate showed that the methine proton of the isopropyl group is not lost during the biosynthesis of 5,5-dichloroleucine (**93**).[173] These results are consistent with a radical chlorination in the biosynthetic pathway of victorin C. A similar chlorination could be invoked for the oxidative cyclization of L-isoleucine.

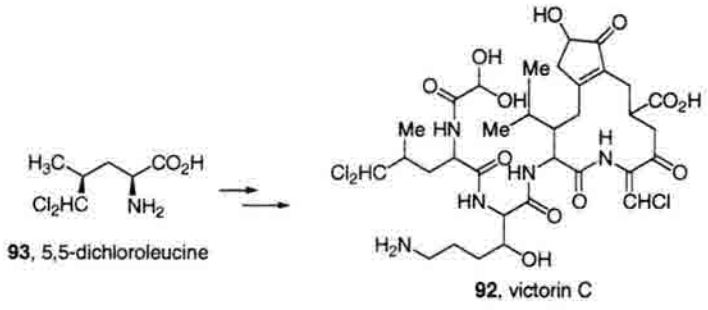
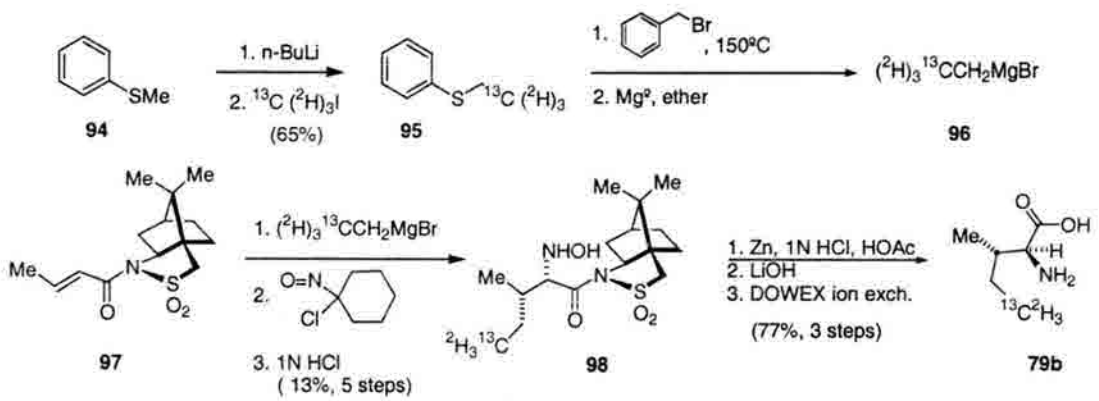


Figure 29: Incorporation of chlorinated leucine into victorin C

To distinguish between a 4-electron and 2-electron oxidative cyclization, [5-^{13}C, 5-$^{2}H_3$]-L-isoleucine (**79b**) was synthesized and fed to cultures of *P. fellutanum*.[174] In the event of a 4-electron oxidation, only one deuterium would be retained (**89a**), whereas in a 2-electron oxidation, two deuterons would be preserved (**89b**, Scheme 6).

Synthesis of [5-^{13}C,5-$^{2}H_3$]-L-isoleucine (**79b**) was accomplished using the procedure developed by Oppolzer and co-workers for the synthesis of unlabeled L-isoleucine (Scheme 7).[175] However, since 2-[$^{13}C^{2}H_3$]-ethylmagnesiumbromide is not commercially available, it was synthesized from [$^{13}C^{2}H_3$]-iodomethane. Reaction of

thioanisole (**94**) with n-BuLi followed by the addition of [$^{13}C^{2}H_3$]-iodomethane provided $^{13}C^{2}H_3$-ethylphenylsulfide (**95**). The 2-[$^{13}C^{2}H_3$]-ethylbromide distilled from the reaction of **95** with benzylbromide at 150°, was added dropwise as an ethereal solution to activated Mg° to form the Grignard reagent, **95**. Successive treatment of the N-crotonoylborane-10, 2-sultam (**97**) with 2-[$^{13}C^{2}H_3$]-ethylmagnesiumbromide (**96**) followed by 1-chloro-1-nitrosocyclohexane and 1 N aqueous HCl at –78 °C provided the 1,4-addition/electrophilic amination product (**98**). N,O-hydrogenolysis of the hydroxylamine **98** with Zn° powder in 1N HCl/ AcOH followed by saponification of the sultam with LiOH in THF/H_2O and DOWEX ion exchange provided [5-^{13}C,5-$^{2}H_3$]-L-isoleucine (**79b**).



Scheme 7: Synthesis of [5-^{13}C,5-$^{2}H_3$]-L-isoleucine (**79b**).

A feeding experiment with *Penicillium fellutanum* using [5-^{13}C, 5-$^{2}H_3$]-L-isoleucine (**79b**) followed by isolation and purification of paraherquamide A revealed 0.34% incorporation of the labeled amino acid as calculated from the electrospray mass spectrum. Because the ^{13}C peaks of interest were partially buried under neighboring

signals, ^{13}C NMR could not be used directly to determine the percentage of incorporation. Instead, the specific incorporation at C-16 was calculated from a volume integral of the deuterium-shifted signal in the ^{1}H-^{13}C HSQC from the feeding experiment (see below). The integral of the prolyl methine proton, C-14, was used as an internal standard and was set at 1.00. The integral of the deuterium-shifted H-16b from the labeled compound was determined to be 1.52. Thus the percentage of [16-$^{13}C^{2}H^{1}H$]-paraherquamide A produced in the feeding experiment is 1.52% and the specific incorporation of [5-^{13}C, 5-$^{2}H_3$]-L-isoleucine is 0.21%. It is also noteworthy that the incorporation observed for the feeding experiments with [5-^{13}C, 5-$^{2}H_3$]-L-isoleucine (**79b**) was much lower than the feeding experiments with [1-^{13}C]-L-isoleucine (**79a**). Presumably, the difference in incorporation efficiency is due to a deuterium isotope effect. Parry and co-workers observed a similar phenomenon in their biosynthetic studies of coronamic acid.[172]

Close inspection of the ^{13}C-spectrum of paraherquamide from feeding experiments with [5-^{13}C, 5-$^{2}H_3$]-L-isoleucine (Figure 12c) revealed a triplet at 51.6 ppm. This indicates that, in the labeled compound, C-16 is coupled to a single deuterium atom. However, the triplet was partially obscured by neighboring ^{13}C-signals that complicated interpretation of the spectrum. To resolve this problem, DEPT experiments were performed (Figure 12).

The triplet from the labeled paraherquamide is seen in both the DEPT 135 and DEPT 90 spectra, indicating a $^{13}C^{2}H^{1}H$ pattern for C16 in the labeled compound. In the event of a $^{13}C^{2}H_2$ pattern, the triplet would not appear in either DEPT spectrum. From these experiments, it was determined that cyclization of L-isoleucine occurs though a 4-

electron oxidation of the terminal methyl group such as the putative intermediate **87** (Scheme 6) followed by cyclization and diastereoselective 2-electron reduction to give **89a**.

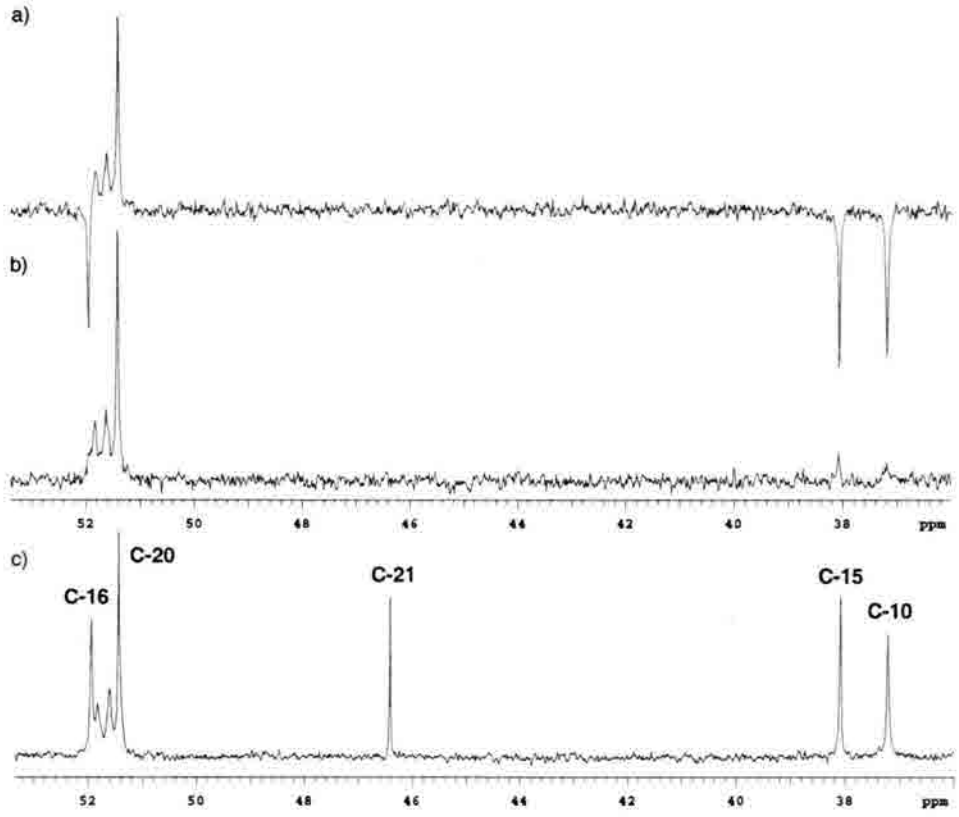


Figure 12: a) DEPT 135 experiment with CH up and CH_2 down, b) DEPT 90 experiment with only CH's shown, c) partial ^{13}C spectrum (100 MHz) of Paraherquamide A from the feeding experiment with 5-[$^{13}C^{2}H_3$]-L-ile.

To determine if the *pro-R* or *pro-S* hydrogen was retained in the oxidative cyclization, CW-selective proton decoupling experiments were performed as shown in Figure 13. The ^{13}C signals were decoupled from protons H-16a (*pro-S*) at 3.21 ppm (Figure 13c) and H-16b (*pro-R*) at 2.22 ppm (Figure 13b) respectively.[176] When H-16a is decoupled (Figure 13c), the triplet (seen in Figure 13a) for the deuterium-coupled ^{13}C-

labeled C-16 becomes complex suggesting that $^{13}C^{2}H$ is coupled to H-16b. In addition, the signal of C-16 (CH_2) and C-20 are split indicating, as expected, that these signal are coupled to protons. When H-16b is decoupled (Figure 13b), the triplet from the deuterium-coupled ^{13}C signal is not affected. Therefore, it was determined that H-16a (3.21 ppm) is the deuteron, H-16b (2.22 ppm) is the proton and the *pro-S* hydrogen is retained in the oxidative cyclization.

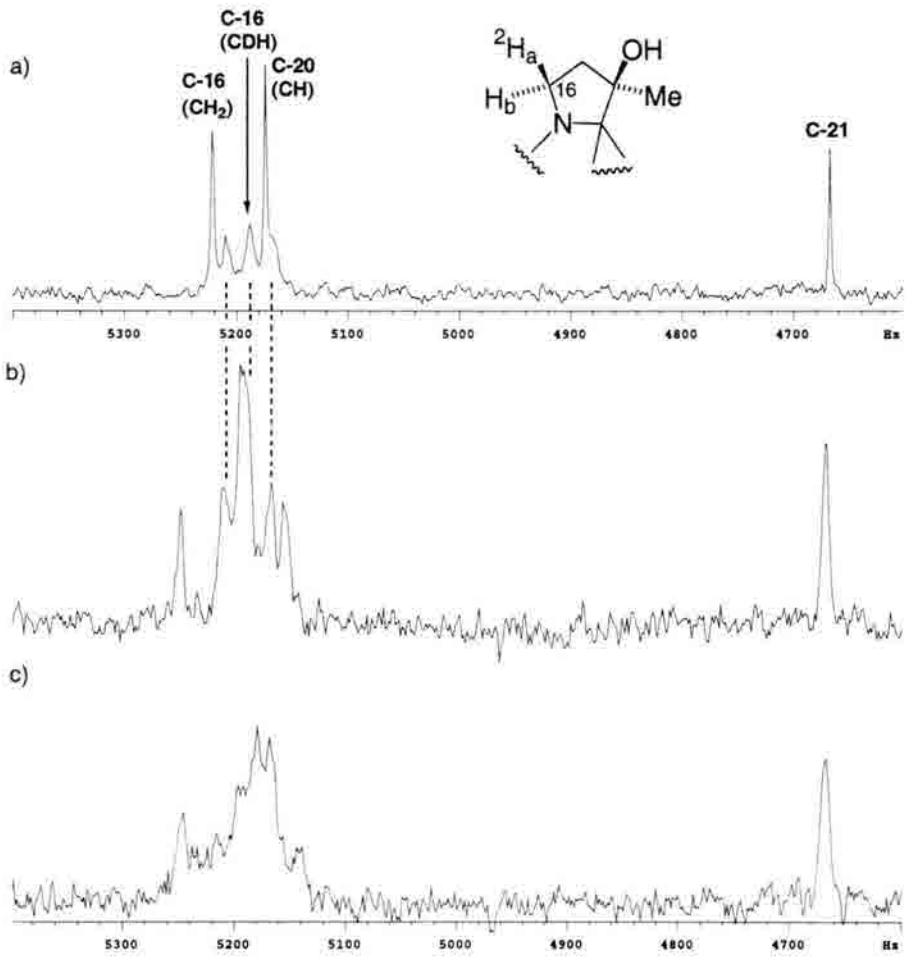


Figure 13: a) Partial ^{13}C spectrum (100 MHz) of paraherquamide A from the feeding experiment with 5-[$^{13}C^{2}H_3$]-L-Ile, b) partial ^{13}C spectrum (100 MHz) of paraherquamide A from the experiment with H-16b at 2.22 ppm decoupled, c) partial ^{13}C spectrum of paraherquamide A (100 MHz) from the experiment with H-16a at 3.21 ppm decoupled.

These results were corroborated by comparison of the HSQC of paraherquamide A from the 5-[$^{13}C^{2}H_3$]-L-Ile feeding experiment with the HSQC from unlabelled paraherquamide A (Figure 14). A deuterium isotopic shift was observed for the proton of the $^{13}C^{2}H^{1}H$ methylene in the HSQC of paraherquamide from the feeding experiment. Since a shifted ^{13}C filtered ^{1}H signal is observed at 2.22 ppm (H-16b), but not at 3.21 ppm (H-16a), this experiment proves H-16a is the deuteron and H-16b is the proton. These results imply that reduction occurs on the same face of the proline ring as the methyl group, C-17.

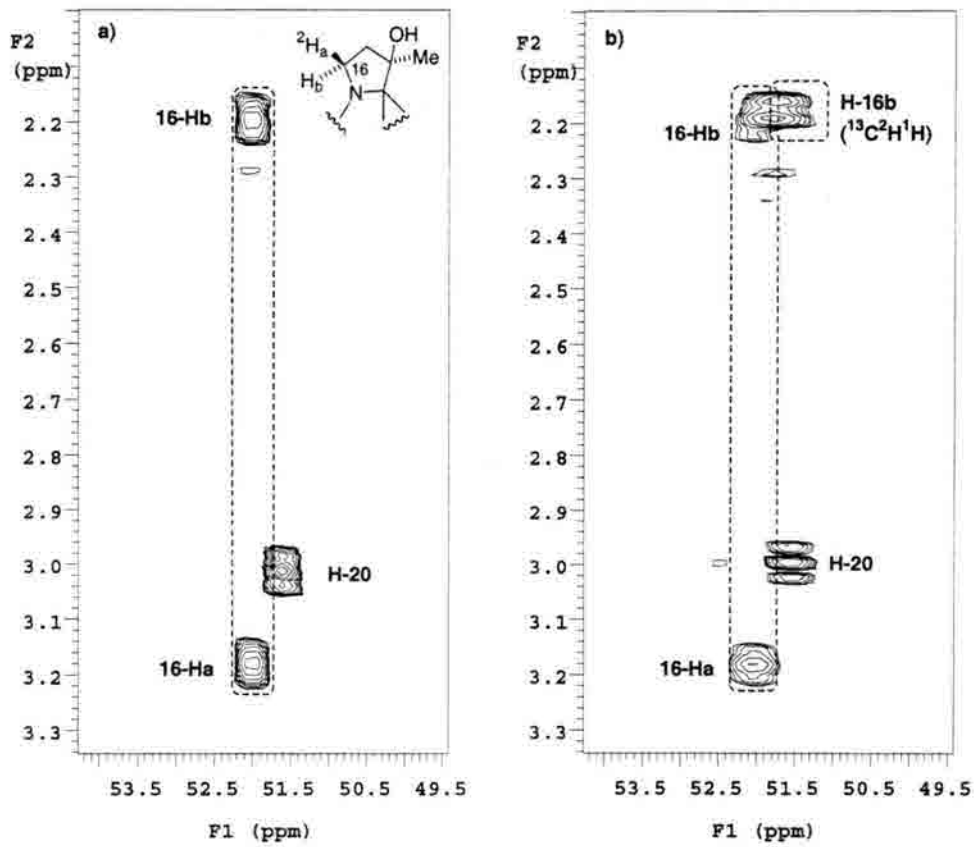


Figure 14: a) HSQC of unlabelled paraherquamide A, b) HSQC of paraherquamide A from the feeding experiment with 5-[$^{13}C^{2}H_3$]-L-Ile

3.4 Temporal aspects of the oxidative cyclization of L-isoleucine

Although it was clear that L-isoleucine was the biosynthetic precursor to the β-methyl-proline ring, the timing of the oxidative cyclization remained ambiguous. Does cyclization of L-isoleucine occur while coupled to L-tryptophan, or prior to that? The doubly labeled dipeptides, [1-^{13}C]-L-isoleucyl-[1-^{13}C]-L-tryptophan (**99**); [1-^{13}C]-L-tryptophanyl-[1-^{13}C]-L-isoleucine (**100**) and 2,5-[$^{13}C_2$]-*cyclo*-L-tryptophan-L-isoleucine (**101**) were synthesized and fed to *P. fellutanum* to test the former possibility (Figure 33, Table 3).[170] Within the limits of detection, incorporation was not observed by ^{13}C NMR or electrospray mass spectroscopy for the ^{13}C-labeled diketopiperazine (**101**). For the dipeptides **99** and **100,** incorporations at C-18 (1.2-1.8%) and at C-12 (0.4-0.9%) were observed by ^{13}C NMR. However, the ^{13}C-NMR spectra of paraherquamide from these feeding experiments did not provide compelling evidence for site-specific incorporation of both labels from the intact dipeptides. The observed unequal levels of incorporation are more consistent with hydrolysis of the dipeptides and re-incorporation of the individual amino acids, presumably coupled with additional metabolic degradation and reconstitution of ^{13}C-enriched building blocks. The mass spectra of paraherquamide A isolated from these feeding experiments also indicate that the doubly labeled metabolites are not incorporated intact.

Results of the dipeptide feeding experiments indicated that oxidative cyclization of L-isoleucine must precede coupling to L-tryptophan. A synthesis of [^{13}C]-β-methyl proline needed to be developed to determine if indeed this was the case. Although there

are syntheses of β-methylproline in the literature,[177] the synthesis of the labeled compound was hindered by the expense and availability of ^{13}C-labeled synthons.

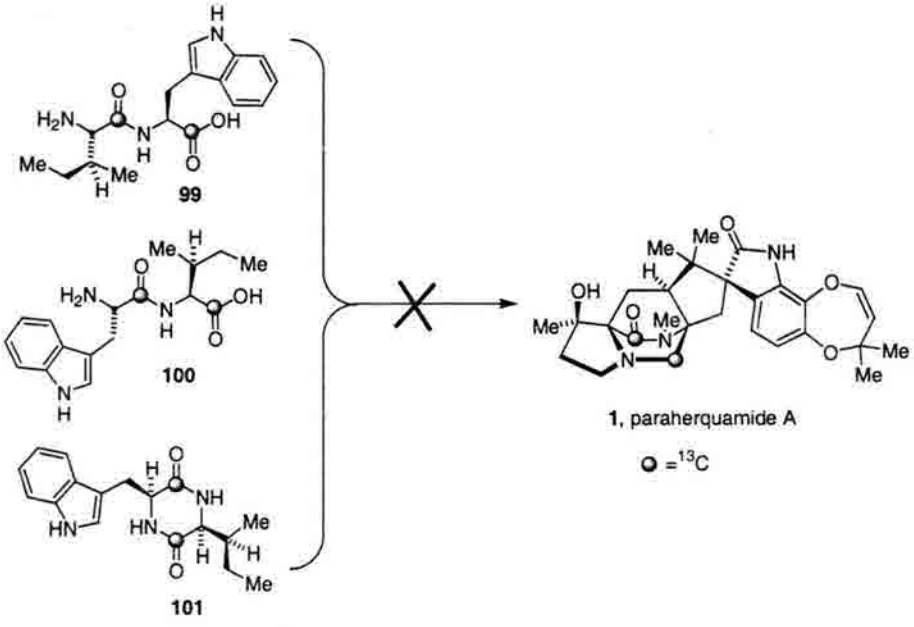


Figure 15: Feeding experiments with the dipeptides of L-isoleucine and L-tryptophan.[170]

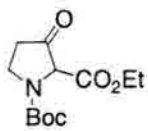
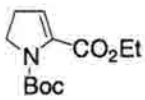
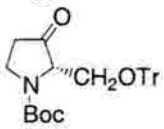
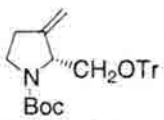
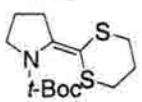
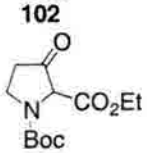
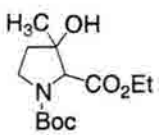
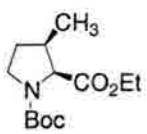
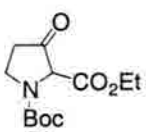
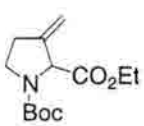
Table 3: Incorporations of labeled precursors into paraherquamide A

Compound	mmol	1 (mmol)	Enhancement of ^{13}C peak	Incorporation by ^{13}C NMR	Incorporation by ES^+ MS
99	0.081	0.019	7.7% (C-18)	1.8 % (C-18)	1.6 % (M+H+1)
			1.7% (C-12)	0.4 % (C-12)	0.3 % (M+H+2)
99	0.028	0.023	1.5% (C-18)	1.3 % (C-18)	0.77 % (M+H+1)
			1.2% (C-12)	0.8 % (C-12)	0.25 % (M+H+2)
100	0.120	0.017	8.4% (C-18)	1.2 % (C-18)	0.5 % (M+H+1)
			3.5% (C-12)	0.5 % (C-12)	0.08 % (M+H+2)
100	0.091	0.014	4.5% (C-18)	1.7 % (C-18)	1.5 % (M+H+1)
			5.8% (C-12)	0.9 % (C-12)	0.3 % (M+H+2)
101	0.043	0.042	ND[a] (C-18)	ND[a] (C-18)	0.61% (M+H+1)
			ND[a] (C-12)	ND[a] (C-12)	0 % (M+H+2)
89c	0.169	0.041	60.2% (C-18)	14.6% (C-18)	10.1% (M+H+1)

[a] ND=Not detectable

Several early synthetic attempts to make β-methyl proline are shown in Table 4. The first attempts at the synthesis of the β-methyl proline ring were based on the β-keto ester (**59**) used for the total synthesis of paraherquamide A (**1**).[178] It was envisioned that a Wittig reaction with ^{13}C-labeled triphenylphosphonium methyl iodide followed by reduction would provide the desired β-methyl proline. This proposal had the advantages of a relatively inexpensive ^{13}C-synthon in [^{13}C]-iodomethane and introduction of the isotopic label late in the synthesis. Unfortunately, this route was unsuccessful, most likely because of the enolizable nature of the ketone.

Table 4: Early endeavors to synthesize 3(S)-methyl-L-proline.

Key Intermediate	Reaction Condidtions	Product/ Yield	Key Intermediate	Reaction Condidtions	Product/ Yield
 **59**	$PPh_3CH_3^+ I^-$ n-BuLi	N.R.	 **105**	$(CH_3)_2CuLi$	N.R.
 **102**	$PPh_3CH_3^+ I^-$ n-BuLi	 **103**, (0-15%)	 **106**	LDA MeI	N.R.
 **59**	CH_3MgBr $CeCl_3$ THF, 0°C	N.R.	 **107**	$SOCl_2$, Pyridine H_2 Pd/C	 **108**, (36-54%)
 **59**	5 eq. CH_2I_2 Zn^0, $TiCl_4$	 **104**, (~30%)			

Attempts to circumvent this problem by reducing the ester to the alcohol (**102**) was only sucessful on one occasion. The use of cerium trichloride in a Grignard reaction

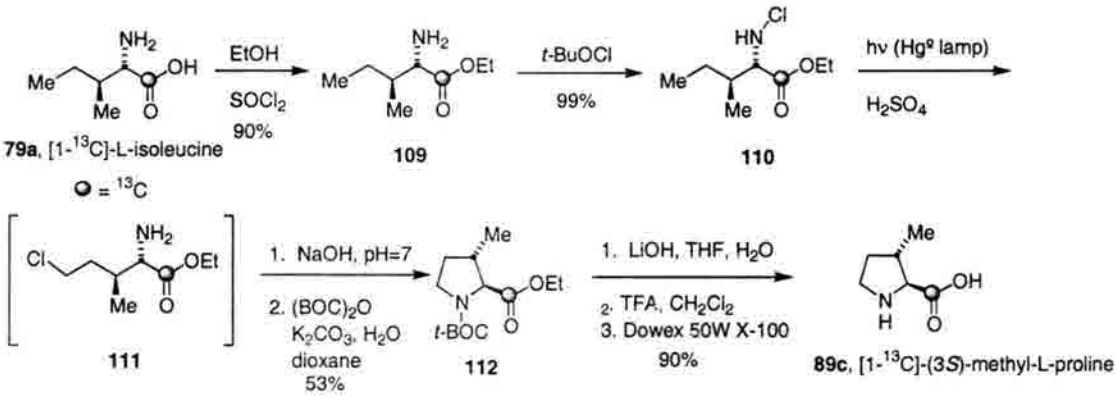
was also fruitless. Generation of the 3-*exo*-methylene proline (**104**) from the β-keto ester (**59**) was finally accomplished in 30% yield using five equivalents of diiodomethane. This synthesis, however, was not appropriate for the isotopically labeled compound because of the large excess of the expensive ^{13}C-CH_2I_2 that would be required.

Other synthetic attempts utilizing the inexpensive ^{13}C-iodomethane such as cuprate addition to the α.β-unsaturated ester (**105**) and alkylation of the 2-amino ketene *S,S*-acetal homoenolate equivalent (**106**)[179] were also unsuccessful. Some success was achieved in forming the β-methyl proline from dehydration and reduction of the β-hydroxy, β-methyl proline (**107**), generated from glycine ethyl ester and methylvinyl ketone. The advantage of this synthesis was that the labeled starting material, ^{13}C-glycine, is relatively inexpensive. The drawback however is that the undesired *cis*-product (**108**) is obtained. However, the *cis*-compound could be epimerized with LDA.

Re-examination of the literature revealed a route to β-methyl proline by Lavergne et al. (Scheme 8), which could stereospecifically form the desired ^{13}C-labeled-3(*S*)-methyl-L-proline (**89c**) from L-isoleucine using the Hoffman-Löffler-Freytag reaction.[180] Esterification of [1-^{13}C]-L-isoleucine (**79a**) with thionyl chloride in absolute ethanol was followed by treatment of the resultant [1-^{13}C]-L-isoleucine ethyl ester (**109**) with *t*-butylhypochlorite affording the N-chlorinated derivative **110**. The Hoffman-Löffler-Freytag intermediate **111** was obtained after exposure of **110** to a mercury lamp in 85% H_2SO_4. Upon neutralization, this intermediate suffered spontaneous cyclization to give the desired labeled β-methyl-proline ethyl ester. The reaction mixture was N-BOC protected to facilitate separation of the desired product from any unreacted [1-^{13}C]-L-isoleucine ethyl ester. Hydrolysis of the ethyl ester of **112** with LiOH in aqueous THF

followed by removal of the *t*-BOC group with TFA and ion exchange chromatography (Dowex 50WX2-100) afforded the desired labeled 1-[^{13}C]-3(*S*)-methyl-L-proline (**89c**) as the free amino acid.

Scheme 8: Hoffman-Löeffler-Freytag synthesis of [1-^{13}C]-3(S)-methyl-L-proline.



A feeding experiment on *P. fellutanum* with [1-^{13}C]-3(*S*)-methyl-L-proline (**89c**) gave paraherquamdie A showed that 60.2 % enrichment at C-18 with 14.6% incorporation of the labeled precursor (Figure 16). A significantly higher level of incorporation was observed for [1-^{13}C]-3(*S*)-methyl-L-proline (**89c**) than for [1-^{13}C]-L-isoleucine (**79a**) (14.6% *versus* 3.7%). This is consistent with the postulate that 3(*S*)-methyl-L-proline is biosynthesized from L-isoleucine and is therefore farther along the biosynthetic pathway to paraherquamide A. In addition, 3(*S*)-methyl-L-proline is a non-proteinogenic amino acid that is most likely used exclusively in the production of paraherquamide A; whereas L-isoleucine may be consumed in the biosynthesis of proteins and other primary and/or secondary metabolites in the fungus. Therefore, 3(*S*)-methyl-L-proline should be converted more efficiently into paraherquamide A (**1**).

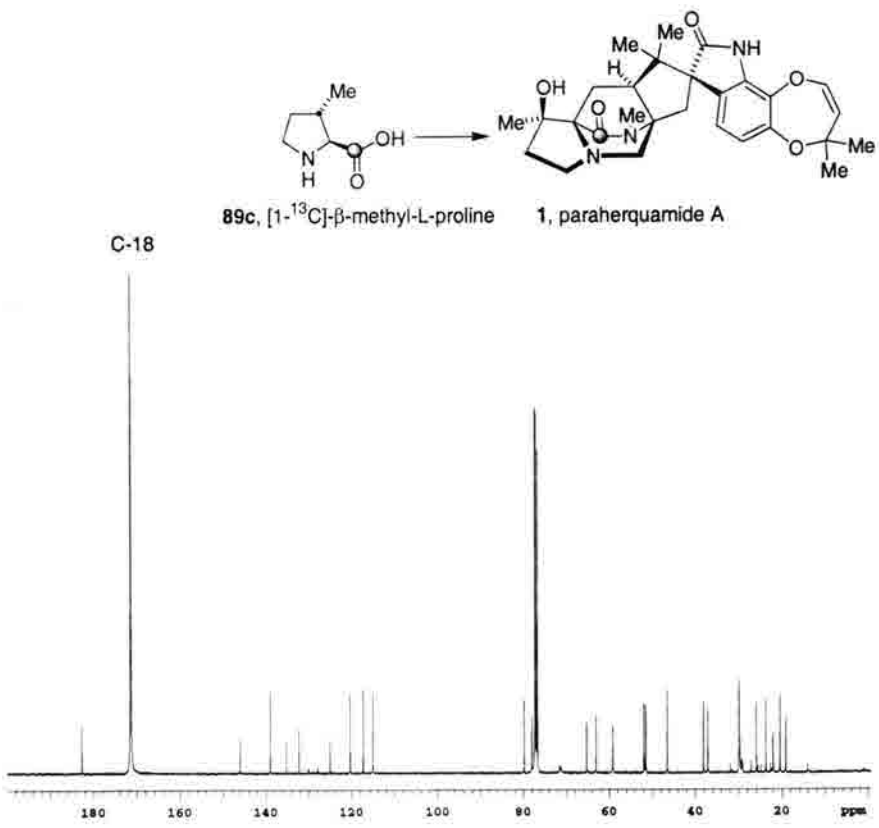


Figure 34: ^{13}C NMR spectrum of Paraherquamide A from feeding experiment with [1-^{13}C]-3(*S*)-methyl-L-proline.

CHAPTER 4

The Isoprene Units

4.1 Origin of the Isoprenyl groups

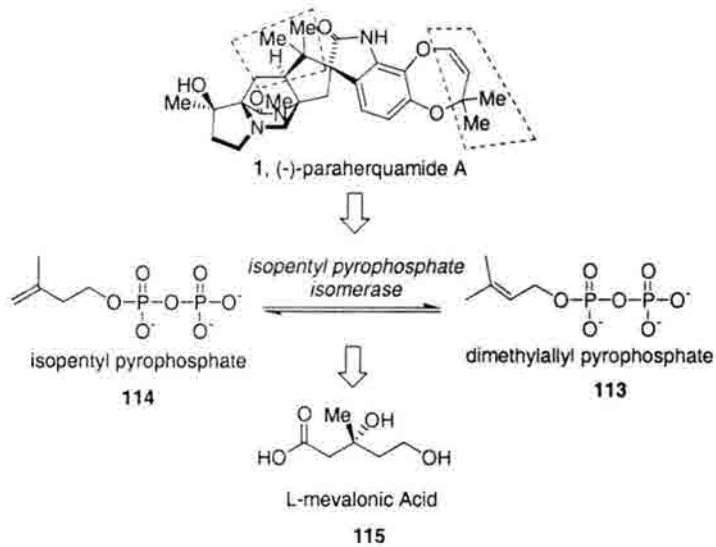
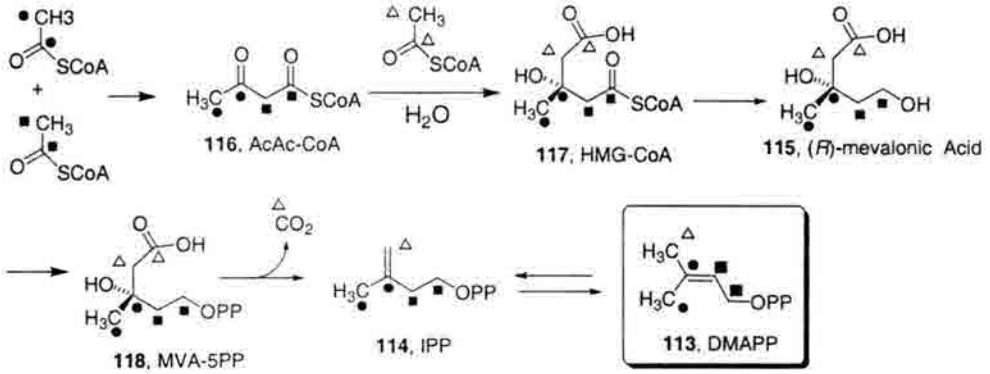


Figure 17: Possible mevalonate origin of the isoprenoid portions of paraherquamide A

In addition to the amino acid residues, paraherquamide (**1**) contains two five-carbon isoprene units. One isoprene group (C-19~C-23) is involved in forming the bicyclo[2.2.2] nucleus and a second isoprene fragment (C-23~C-28) is oxidatively added to a phenolic hydroxyl group of the tryptophan core. Until recently, it would be safe to assume that the isoprene units in paraherquamide originate from dimethylallyl pyrophoshate (**113**, DMAPP) or its tautomer, isopentenyl pyrophosphate (**114**, IPP), *via*

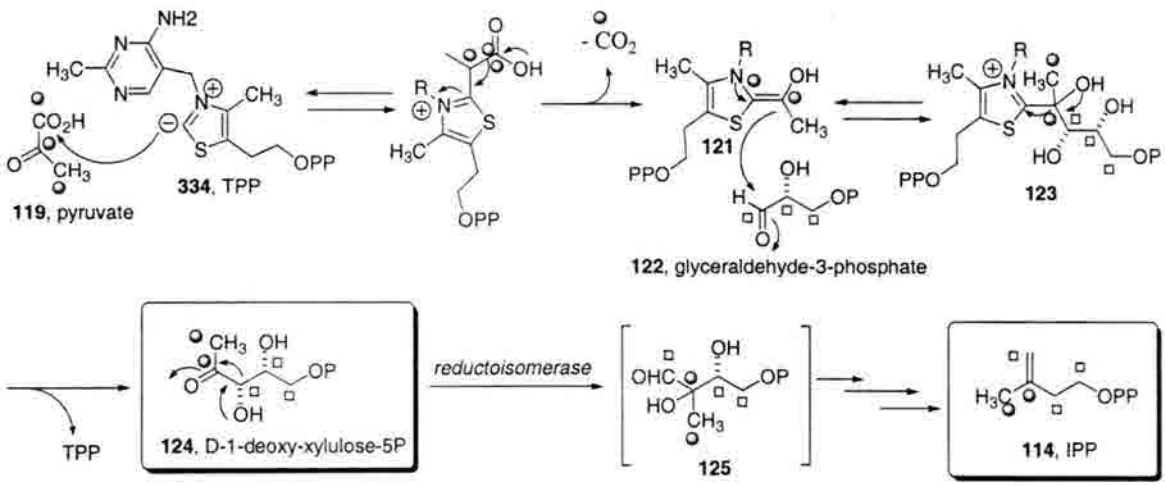
the well-known mevalonic acid (**115**) pathway (Figure 17). In this pathway, two equivalents of acetyl CoA condense *via* a Claisen-type condensation to form acetoacetyl CoA (**116**). A third equivalent of acetyl CoA is then added through an aldol-type reaction to give, after hydrolysis of one thioester, hydroxymethylglutaryl CoA (**117**). A net four-electron reduction of **117** produces mevalonic acid (**115**), which is subsequently phosphorylated to give mevalonic acid 5-pyrophosphate (**118**). Finally, IPP (**114**) is produced by phosphorylation of the tertiary alcohol followed by decarboxylation with concomitant loss of inorganic phosphate. The isotopic labeling pattern that would be observed in DMAPP derived from [1,2-$^{13}C_2$]-acetate is shown in Scheme 9. As depicted, coupling should be observed between C-1 and C-2 and between C-3 and C-4 of DMAPP. However, no coupling should be observed for C-5 due to loss of $^{13}CO_2$. Cane and co-workers have demonstrated that the *E*-methyl group of DMAPP is derived from C-2 of mevalonic acid (i.e., the methyl group of mevalonic acid becomes C-4 of DMAPP).[181]

Scheme 9: Isotopic-labeling pattern of dimethylallylpyrophosphate derived from [1,2-$^{13}C_2$]-acetate via the mevalonic acid pathway.



In the last few years several reports have emerged showing that isoprenoids can be formed through an alternate 1-deoxy-D-xylulose pathway. This has been shown in bacteria,[182] the green algae *Scenedesmus obliquus*[183] and, in higher plants like *Taxus chinensis*,[184] barley (*Hordeum vulgare* L.), duckweed (*Lemna giba* L.), carrot (*Daucus carota* L.),[185] and *Ginkgo biloba*.[186] In these cases, the biosynthesis of several cytoplasmic sterols proceeds *via* the acetate/mevalonate pathway, while the plastidic isoprenoids are synthesized *via* the 1-deoxy-D-xylulose pathway. The 1-deoxy-D-xylulose pathway, as proposed by Arigoni and Rohmer, is outlined in Scheme 10.[187] The condensation of pyruvate (**119**) with thiamine pyrophosphate (**120**, TPP) followed by carboxylation would provide the enamine **121**. A head to head condensation of **121** with glyceraldehyde 3-phosphate (**122**) provides the intermediate **123**. Ketalization and attendant loss of TPP provides 1-deoxy-D-xylulose (**124**). Reductoisomerase catalyzes the 1,2-ketol rearrangement to **125**, which is subsequently reduced to provide IPP (**114**). Scheme 10 also shows the labeling pattern that would be observed in IPP from a feeding experiment with [U-$^{13}C_6$]-D-glucose.

Scheme 10: 1-Deoxy-D-xylulose pathway and labeling pattern from [U-$^{13}C_6$]-D-glucose.



The predicted ^{13}C-labelling patterns of for the isoprenyl groups of paraherquamide A are shown for both the deoxy-xylulose and mevalonic acid pathways in Figure 17. In the case of the mevalonic acid pathway, the same results should be observed from feeding experiments with [1,2-$^{13}C_2$]-acetate and [U-$^{13}C_6$]-glucose. Coupling of adjacent carbons for the isoprene derived carbons would be observed except for one of each of the geminal dimethyl groups, which would not show any coupling. In the deoxyxylulose pathway, feeding experiments with [1,2-$^{13}C_2$]-acetate would not show any specific incorporation, since pyruvate and glyceraldehyde-3-phosphate are the precursors. Feeding experiments with [U-$^{13}C_6$]-glucose, however, should exhibit long-range coupling of C-1 and C-2 with C-4 of the isoprene units, since they are derived from the same synthon, glyceraldehyde-3-phoshate.

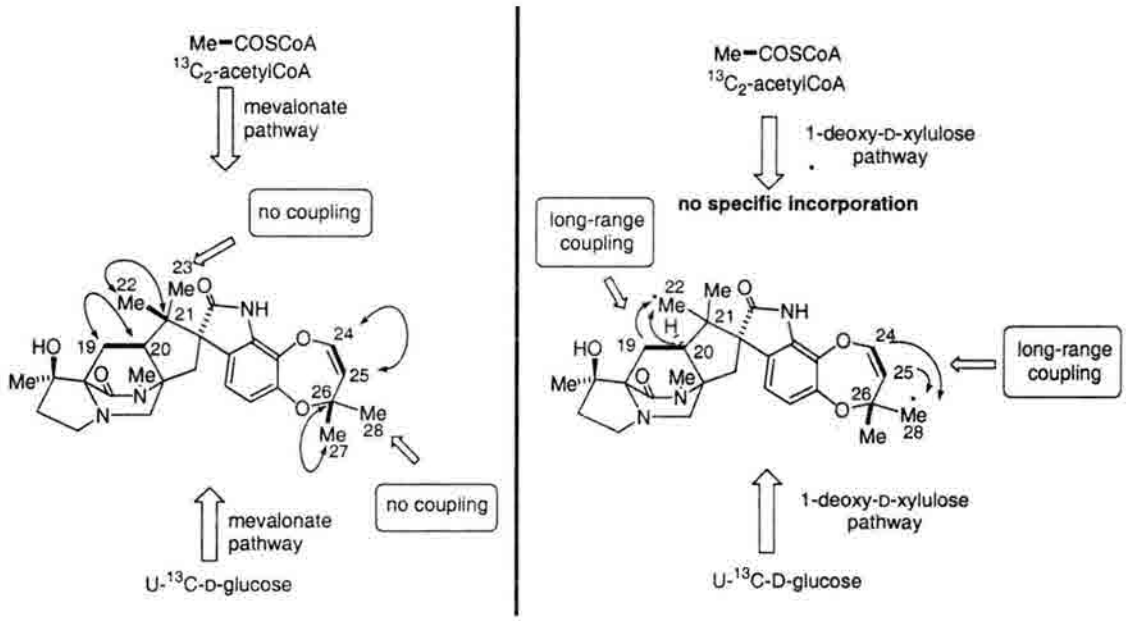


Figure 17: Predicted labeling pattern for the isoprenyl groups of paraherquamide A for the mevalonate and 1-deoxy-D-xylulose pathways

Feeding experiments were performed with [1,2-$^{13}C_2$]-acetate and [U-$^{13}C_6$]-D-glucose on cultures of *Penicillium fellutanum*.[188] The paraherquamide A from each of these feeding experiments was isolated and purified. Incorporation was observed for both feeding experiments. Since [1,2-$^{13}C_2$]-acetate fed to *Penicillium fellutanum* was incorporated into both of these isoprene units as intact C-2 units, this unambiguously confirms that the paraherquamide A-producing fungi constructs the primary isoprene units *via* the classical mevalonic acid pathway (Tables 5 and 6). In retrospect, this result is hardly surprising since the related mycotoxins, brevianamide and marcfortine, are also known derivatives of the mevalonic acid pathway. With respect to the carbons that form the two C_5 units, C-19 to C-23, and C-24 to C-28, the results of the feeding experiment with [$^{13}C_2$]-acetate were essentially the same as with [U-^{13}C]-glucose.

Table 5. Specific incorporations, chemical shifts and coupling constants for the C_5 carbon atoms of paraherquamide A (**1**) in the feeding experiment with [$^{13}C_2$]-acetate.

Paraherquamide Carbon #	δ	$J_{C\text{-}C}$ **(Hz)**	**% ^{13}C at each C[a]**	**% ^{13}C specifically incorporated at each position from intact C_2 units**
19	22.2	35	4.6%	32
20	51.4	34	4.2%	32
21	46.4	36	4.4%	42
22	20.5	36	4.4%	31
23	23.7	36	3.3%	41
24	138.9	79	5.1%	37
25	115.1	79	3.2%	34
26	79.8	40	3.6%	31
27	29.9	40	2.9%	39
28	29.8	–	3.9%	0

[a] % ^{13}C inclusive of natural abundance ^{13}C.

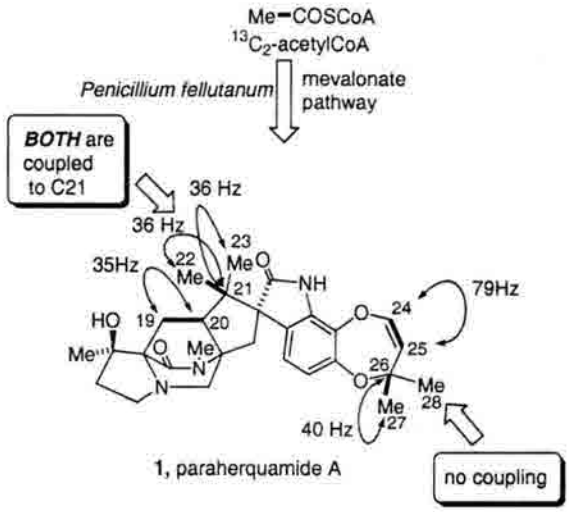


Figure 18: Labeling pattern of paraherquamide A from feeding experiments with [$^{13}C_2$]-acetate in *P. fellutanum*

Table 6. Specific incorporations, chemical shifts and coupling constants for the C_5 carbon atoms of paraherquamide A (**1**) in the feeding experiment with [U-$^{13}C_6$]-glucose.

Paraherquamide Carbon #	δ	$J_{C\text{-}C}$ (Hz)	% ^{13}C at each C[a]	% ^{13}C specifically incorporated at each position from intact C_2 units
19	22.2	34	2.1%	41
20	51.4	34	3.1%	32
21	46.4	36	2.6%	36
22	20.5	36	3.2%	14
23	23.7	36	2.9%	21
24	138.9	81	4.0%	35
25	115.1	79	3.5%	37
26	79.8	40	3.5%	37
27	29.9	40	2.1%	40
28	29.8	–	2.8%	0

[a] % ^{13}C inclusive of natural abundance ^{13}C.

As seen in Tables 5 and 6 and in Figure 18, in the C-5 unit comprising C-24~C-28 of the dioxepin ring, the expected mevalonic acid pathway labeling pattern is observed.

Coupling constants show that C-24 and C-25 are coupled, C-26 is coupled to C-27 and C-28, at δ 29.80, shows enhancement with respect to the- control spectrum, but no coupling. In the C_5 fragment involved in the formation of the [2.2.2] diazaoctan, C-19~C-23, the observed couplings indicate that C-19 is coupled to C-20, while C-21 is coupled to C-22 *and* C-23, but not to both simultaneously. The labeling pattern for C-19~C-23 contradicts the expected labeling pattern and indicates that at some point in the biosynthesis, the geminal methyl groups become equivalent.

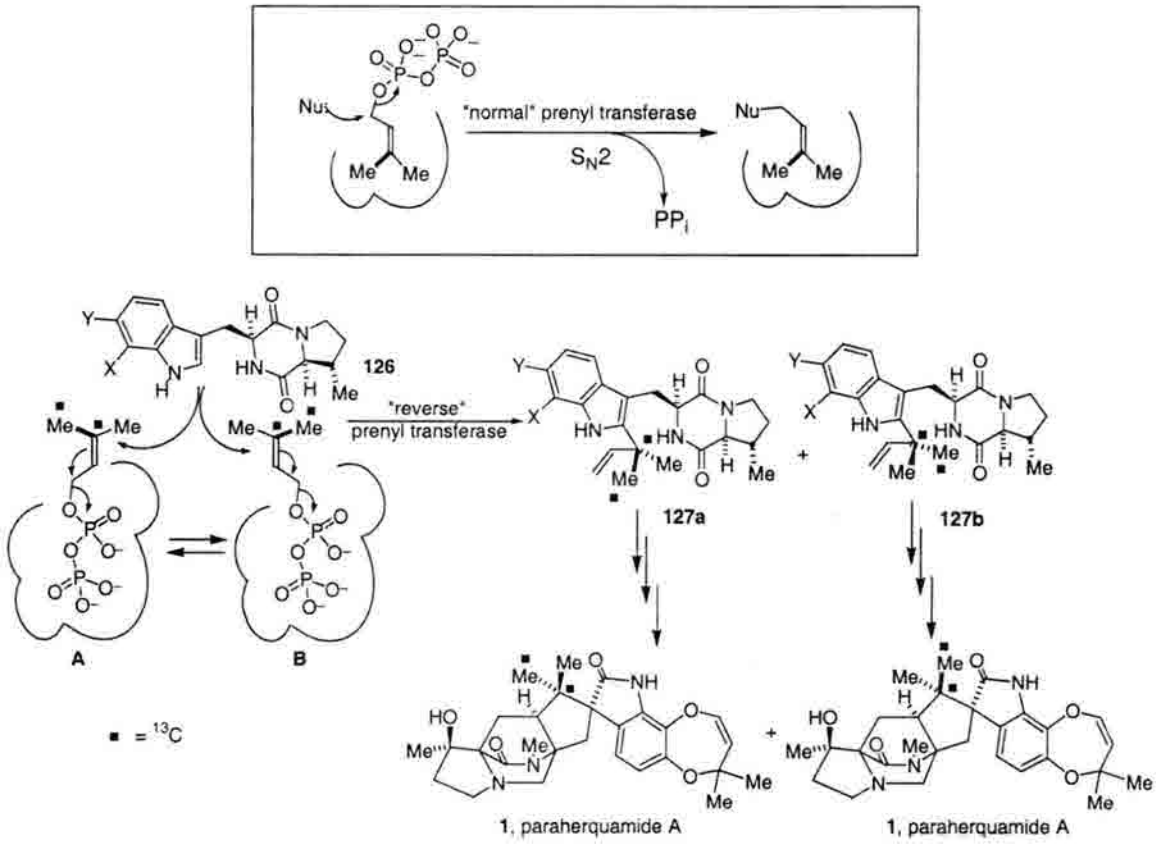


Figure 19: Proposed mechanism of reverse prenylation in paraherquamide A

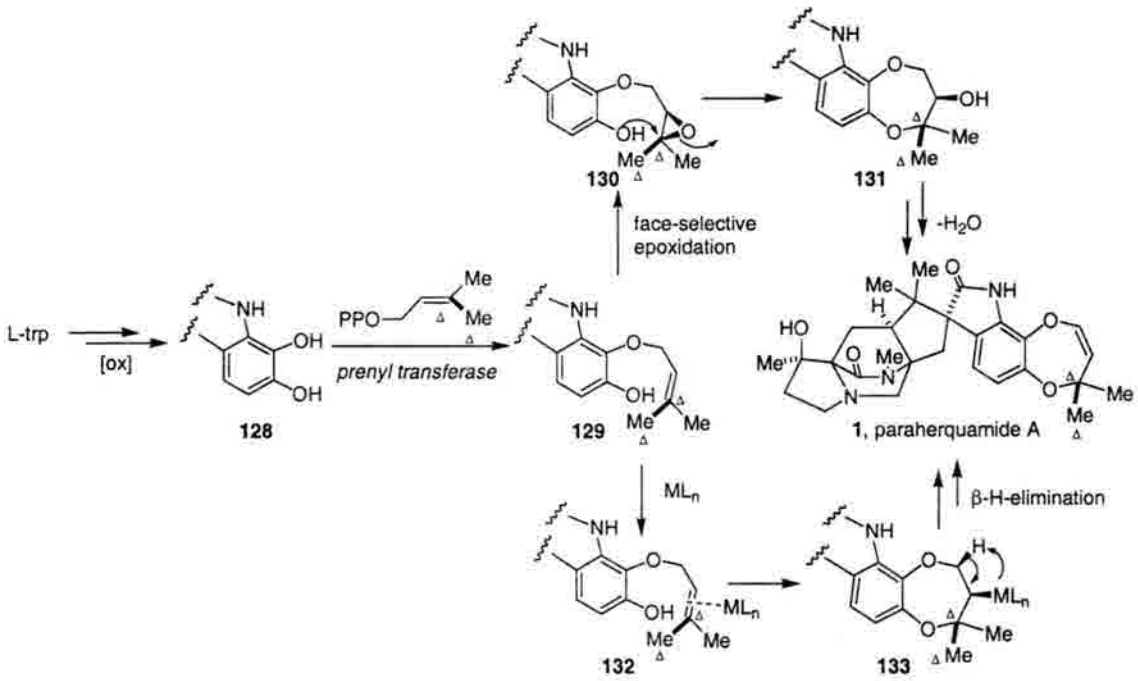
The unanticipated coupling of both methyl groups, C-22 and C-23, to C21 may possibly be explained by the mode of attachment of the isoprene group. As previously

mentioned C-19~C-23 is thought to be involved in the formation of the [2.2.2]diazaoctan ring system as displayed in Scheme 5 (Chapter 1). It is believed that the isoprene group is attached in a *reverse* manner to either the indole or oxindole and undergoes a hetero-Diels-Alder cycloaddition with the oxidized piperazine ring. "Normal" prenylation reactions are catalyzed by prenyl transferases (Figure 19, inset). The isoprene group is located in a hydrophobic pocket of the enzyme active site, while the pyrophosphate tail is in the hydrophilic environment. Nucleophilic displacement of the pyrophosphate with Walden inversion at carbon would provide prenylation while maintaining the stereochemical integrity of the methyl groups of DMAPP.

It is possible that an antithetic enzyme may catalyze the "reverse" prenylation in the biosynthesis of paraherquamide A. In this case, the hydrophilic diphosphate portion of DMAPP is buried in an enzyme active site, "upside down" in relation to "normal" prenyl transferases. The hydrophobic isoprene portion would then be subject to a facially indiscriminate S_N' attack as shown in Figure 19. The isoprene group could be presented to the hypothetical generic indole substrate **126** in a conformationally flexible (**A** ⇌ **B**) disposition with respect to the tryptophan-derived substrate. This would result in loss of stereochemical integrity of the geminal methyl groups in the prenylated products **127a** and **127b**. The subsequent oxidative ring-closing reactions should not affect the stereochemical integrity of the relevant quaternary centers once they have been set in the key C-C bond-forming reverse prenylation reaction.

Since the geminal methyl groups of the dioxepin moiety for paraherquamide A (**1**) are clearly differentiated, it is quite likely that this isoprene group (C-24~C-28) is introduced in the molecule *via* direct alkylation with DMAPP by a normal prenyl

transferase (to give **129**, Scheme 11). Prenylation would be followed by a net stereospecific oxidative addition to the olefinic π-system. Of several possibilities, a plausible mechanism for the formation of this ring system is *via* face-selective epoxidation of the olefin (to give **130**, Scheme 11) followed by *a completely stereospecific ring-opening of the epoxide* and dehydration (**130** → **131** → **1**).



Scheme 11: Possible mechanisms for the stereospecific introduction of the C-24-C28 isoprene unit of paraherquamide A

Alternatively, face-selective complexation of a transition metallo-protein to the olefinic π–system (**132**), followed by stereospecific intramolecular nucleophilic addition (to give **133**) and reductive elimination to the enol-ether would yield the dioxepin moiety of paraherquamide A (**1**). We are aware of no biosynthetic precedent for the latter

possibility and the former (*via* **130**) therefore appears to be the most likely. Whatever the mechanism for the construction of this interesting ring system, the C-O bond-forming reaction in the construction of the dioxepin is fashioned without loss of stereochemical integrity.

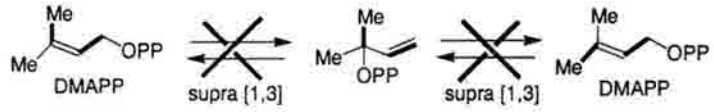


Figure 20

Another possible explanation for the observed retention and loss of stereochemical integrity of the respective geminal methyl groups in paraherquamide A (**1**) is that the methyl groups of the DMAPP in C-19~C-23 are scrambled *via* a dimethyl vinyl carbinol-type intermediate (Figure 20) derived from DMAPP. However, this would necessarily provide stereochemically scrambled isotopomers of DMAPP to the cells' cytosolic pool. Unless there are two pools of *completely compartmentalized* DMAPP in the biosynthesis of paraherquamide A (**1**), scrambling would also be expected in the isoprene unit constituting C-24~C-28. In one of these pools the stereochemical integrity of DMAPP would be sacrificed through a dimethyl vinyl carbinol-type of intermediate that is then used *exclusively* for the assembly of the C-19~C-23 unit. In the other pool, DMAPP would retain the normal stereochemical integrity of the mevalonate pathway and is then used *exclusively* for fashioning the dioxepin moiety C-24~C-28. According to Ockam's razor (principle of mechanistic economy) this possibility seems to be highly unlikely. Paraherquamide A (**1**) is therefore unique in that the mode of construction of each quaternary center derived from isoprene building blocks is distinct: one center is

formed in a completely stereospecific manner and the other is formed in an entirely non-stereospecific manner (Figure 21).

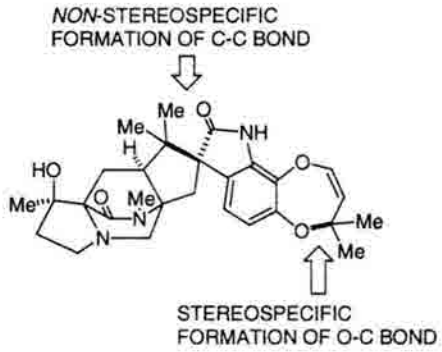
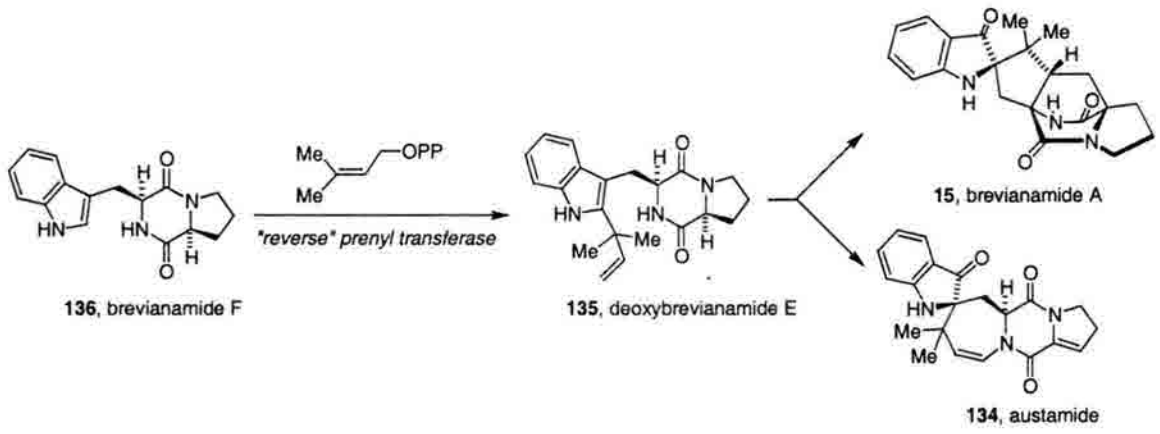


Figure 21

4.2 Further Studies on "Reverse" Prenylation in Related Natural Products

To further probe the possible existence of a reverse prenylase enzyme, we investigated the stereochemical integrity of the reverse prenyl groups in the related yet simpler natural products, brevianamide A (**15**) and austamide (**135**).[188b] The biosynthesis of both austamide (**134**), a metabolite of *Aspergillus ustus*, and brevianamide A (**15**), a metabolite of *Penicillium brevicompactum* and several related *Penicillium* sp., is thought to proceed through the intermediacy of deoxybrevianamide E (**135**), as shown in Scheme 12. Thus, "reverse" prenylation of *cyclo*-L-Trp-L-Pro (**136**, brevianamide F) produces deoxybrevianamide E (**135**), which is converted by distinct modes of oxidative cyclization into brevianamides A (**15**) and B (**16**) in *Penicillium* sp., or into austamide (**134**) in *Aspergillus ustus*. In brevianamide A (**15**), the reverse isoprene unit, analogous to C19-C23 in paraherquamide A (**1**), is believed to undergo a net oxidative cyclization across the two amino acid α-carbons culminating in the formation of the

bicyclo[2.2.2]diazaoctan. In austamide (**134**), the reverse isoprene unit is thought to proceed through an alternate mode of oxidative cyclization onto the tryptophanyl amide nitrogen atom to form the unsaturated 7-membered ring.



Scheme 12: Intermediacy of deoxybrevianamide E in the biosynthesis of the fungal metabolites, brevianamide and austamide.

The intermediacy of deoxybrevianamide E (**135**) in the biosynthesis of brevianamides A and B was confirmed through incorporation of [8-$^{3}H_2$]-deoxybrevianamide E in cultures of *Penicillium brevicompactum* as reported by Williams and co-workers. In the case of austamide (**134**), deoxybrevianamide E is a co-metabolite isolated from cultures of *Aspergillus ustus*. However, since no feeding experiments testing its intermediacy have been performed to date, the involvement of **135** in the biosynthesis of austamide is based solely on circumstantial evidence.

Feeding experiments were performed with [$^{13}C_2$]-acetate in cultures of *Penicillium brevicompactum*, (ATCC: 9056) and with *Aspergillus ustus*, (ATCC: 36063), which produces austamide (**134**).[188b] Incorporation of intact C-2 units was observed in both austamide (**134**) and brevianamide A (**15**), as shown in Tables 7 and 8, respectively. Thus, as with paraherquamide A (**1**), the isoprene units are derived *via* the mevalonic acid

pathway as evidenced by the significant levels of incorporation of the labeled acetate units into the isoprene moieties. Of further significance, the two geminal methyl groups derived from the reverse isoprene units in both austamide (**134**) and brevianamide A (**15**) exhibit coupling to the quaternary carbon to which they are bonded.

Table 7. Specific incorporations, chemical shifts and coupling constants for the C_5 carbon atoms of austamide (**134**) in the feeding experiment with [$^{13}C_2$]-acetate.

Austamide C#	**δ**	**$J_{C\text{-}C}$ (Hz)**	**% ^{13}C at each C[a]**	**% ^{13}C specifically incorporated at each position from intact C_2 units**
18	42.1	35	4.9%	42%
19	128.7	76	5.0%	39%
20	125.5	76	5.5%	34%
21	26.1	35	5.5%	24%
22	23.5	35	5.3%	32%

[a] % ^{13}C inclusive of natural abundance ^{13}C.

In austamide (**134**), as expected, C-19 shows coupling to C-20, while C-21 and C-22 both show coupling to C-18 with coupling constants of ~35Hz (Table 7, Figure 41). The coupling between methyl groups, C-21 and C-22, and the adjacent quaternary carbon, C-18, can be clearly seen in the INADEQUATE spectrum shown in Figure 42. As seen visually by the intensity of coupled ^{13}C-signals in the ^{13}C-spectrum and from the calculated values shown in Table 7, the percentage of ^{13}C incorporated at C-21 and C-22 as C_2 units is approximately the same. Therefore, no facial bias is observed in the mode of reverse prenylation in the biosynthesis of austamide (**134**).

In an INADEQUATE experiment, cross peaks represent connectivity between adjacent carbons.[189] This type of experiment is not used frequently because of the

extremely low sensitivity inherent to this technique. In this instance, however, since the austamide (**134**) sample has been enriched with $^{13}C_2$ units, the INADEQUATE is an excellent way to show which carbons arises from intact C_2 units. Besides the coupling observed between C-18 to C-21 and C-22, C-14 and C-15 give strongly coupled signals in the INADEQUATE spectrum, as shown in Figure 22. The high level of incorporation of [$^{13}C_2$]-acetate in the proline ring can be traced to the fact that L-proline is derived from L-glutamate, which in turn arises from α-ketoglutarate, a product of the condensation of acetyl-CoA in the citric acid cycle.[190]

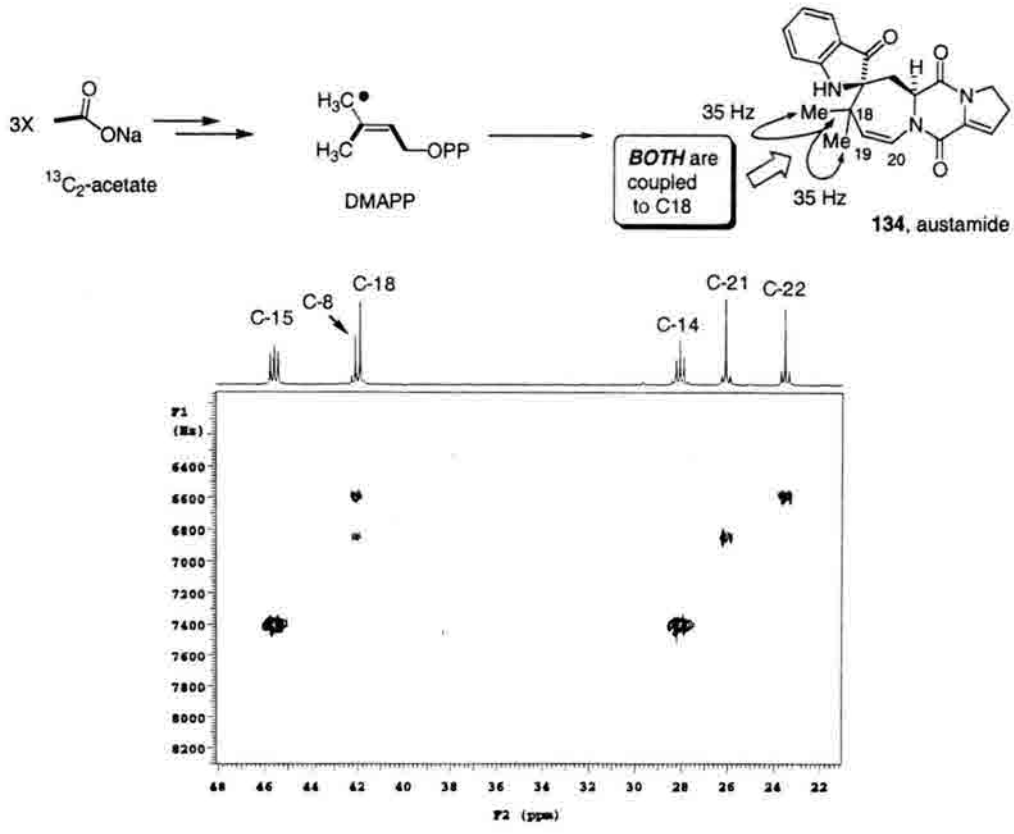


Figure 22: INADEQUATE spectrum of austamide from feeding experiment with [$^{13}C_2$]-acetate on *A. ustus*

An initial feeding experiment with [$^{13}C_2$]-acetate in *Penicillium brevicompactum* gave very high levels of specific incorporation of [$^{13}C_2$]-acetate into brevianamide A (**15**) with concommitant couplings generated between different intact C_2 units in the carbons arising from DMAPP in the same molecule. As a result, C-18 and C-19 showed coupling not only to the carbons that come from intact C_2 units, but also to contiguous labeled carbons that most likely do not arise from intact $^{13}C_2$-units (Figure 23). From the splitting pattern seen in the ^{13}C-spectrum, it was apparent that C-19 was coupled to C-20 (d, J=37 Hz), but that C-19 was also coupled to C-20 and C-18 concurrently (dd, J=37, 37 Hz). The resonance for C-18 also showed multiple couplings.

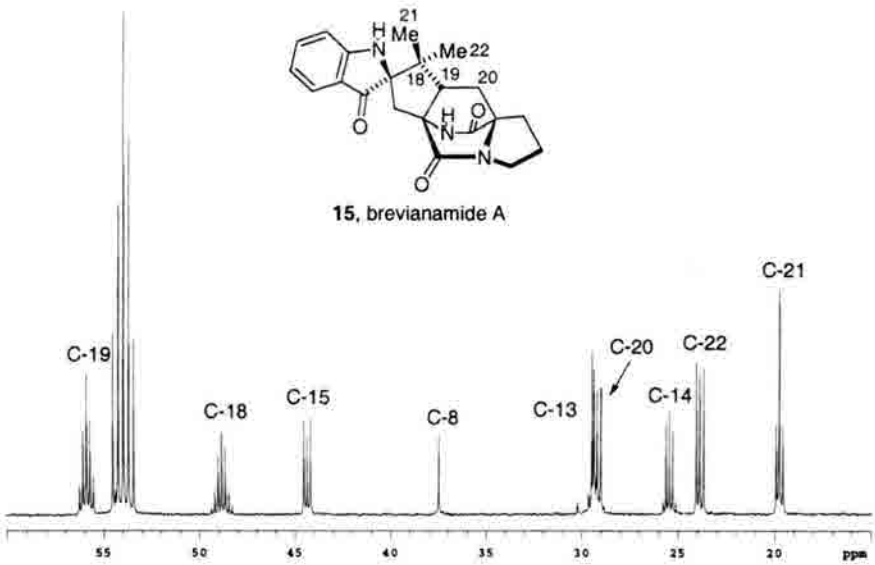


Figure 23: First feeding experiment with [$^{13}C_2$]-acetate in *P. brevicompactum*

In order to avoid the complications that these simultaneous incorporations of two or more C_2 units introduced in the ^{13}C spectrum of the resulting brevianamide A (**15**), a second feeding experiment was performed in which 200 mg of [$^{13}C_2$]-acetate were used

together with 1000 mg of unlabeled acetate. This lowered the probability of simultaneous incorporation of two labeled acetate units in the same molecule of brevianamide A (**15**), thereby simplifying the ^{13}C spectrum of this metabolite resulting from a feeding experiment. In effect, the resulting brevianamide A (**15**) showed a much simpler ^{13}C spectrum (Figure 43), with lower incorporation of labeled acetate (Table 8), and only doublets for the carbon couplings. As expected, C-18 (d, *J*=37 Hz) exhibits couplings to C-21 (d, *J*=37 Hz) and C-22 (d, *J*=37 Hz) (Figure 6), while C-19 (d, *J*=37 Hz) is coupled only to C-20 (d, *J*=37 Hz).

Table 8. Specific incorporations, chemical shifts and coupling constants for the C_5 carbons of brevianamide A (**15**) in the feeding experiment with [$^{13}C_2$]-acetate.

Brevianamide A (16) Carbon atom #	**δ**	**$J_{C\text{-}C}$ (Hz)**	**% ^{13}C at each C[a]**	**% ^{13}C specifically incorporated at each position from intact C_2 units**
18	48.9	37	3.9%	30%
19	55.9	37	3.5%	28%
20	29.2	37	4.1%	29%
21	19.8	37	4.0%	13%
22	23.9	37	3.9%	22%

[a] % ^{13}C inclusive of natural abundance ^{13}C

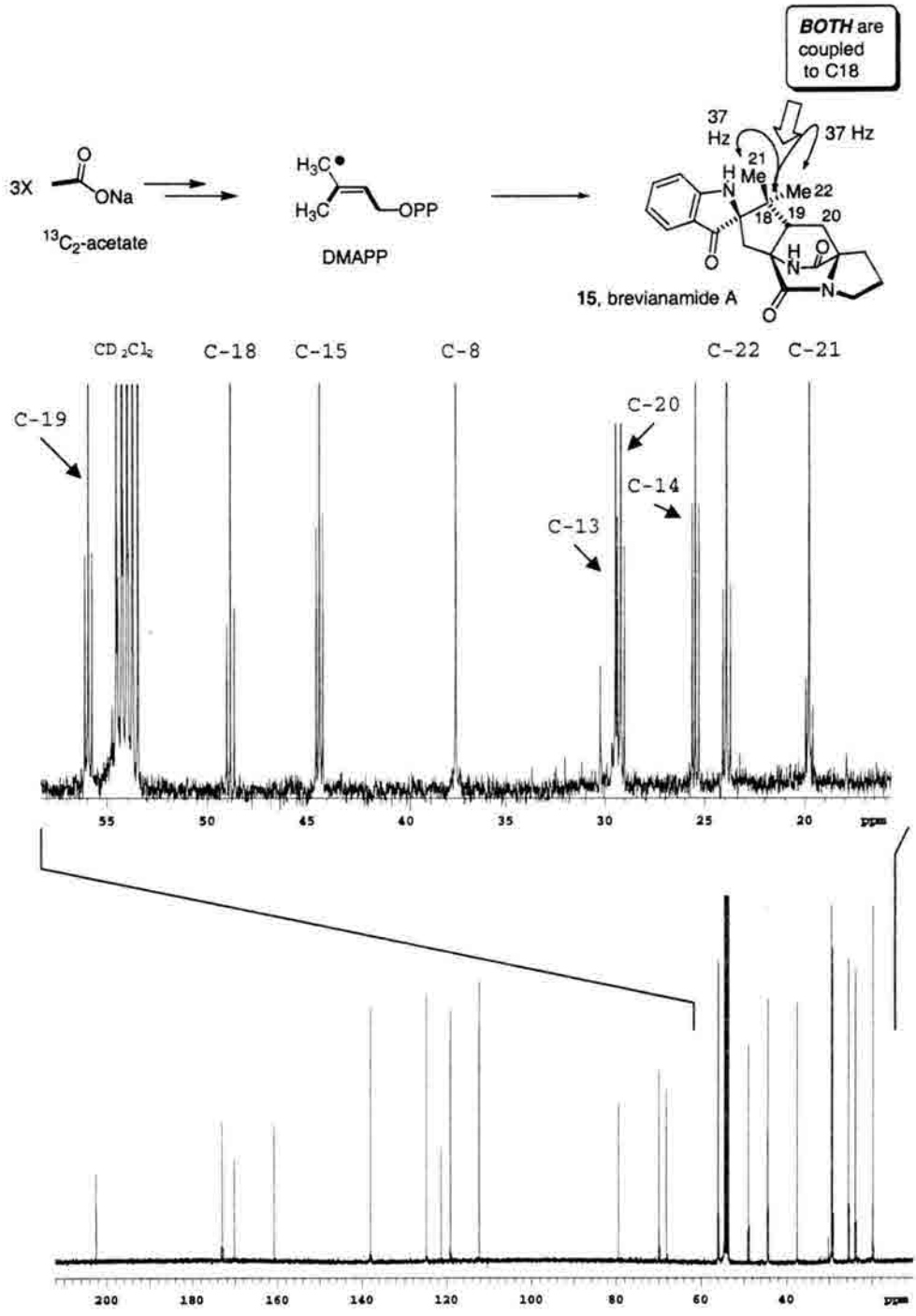


Figure 24: ^{13}C-spectrum of brevianamide A from feeding experiment with [$^{13}C_2$]-acetate in *P. brevicompactum.*

Another noteworthy feature of the ^{13}C NMR spectrum of brevianamide A (**15**) is that C-22, which we have assigned by ^{1}H NMR nOe experiments,[191] shows a higher percentage of specifically incorporated ^{13}C from intact C_2 units (22%) than C-21 (13%). Within experimental error, those values agree with the measured value for C-18 (30% of ^{13}C atoms specifically incorporated from intact C_2 units; see Table 8): C-18 is coupled to either C-21 or C-22, but not to both of them simultaneously. This indicates that, although there is loss of stereochemical integrity of the methyl groups derived from DMAPP in the biosynthesis of brevianamide A (**15**), there is *some degree* of stereofacial bias in the attachment of the reverse prenyl group to *cyclo*-L-Trp-L-Pro (**136**) in the biosynthetic formation of deoxybrevianamide E (**135**), the key reverse-prenylated precursor.

4.3 Studies on the Mechanism of "Reverse" Prenylation

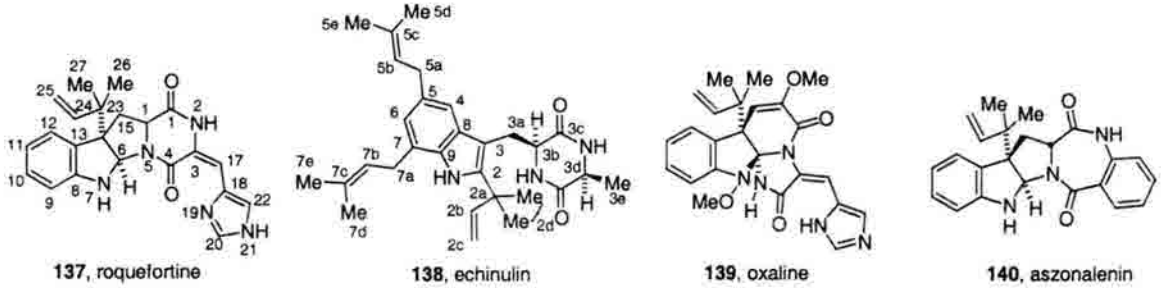


Figure 25: Structure of some reverse prenylated indole alkaloids

It is interesting to note that deoxybrevianamide E is not the only "reverse" prenylated natural indole alkaloid to have been described in the literature. Several other natural substances such as roquefortine (**137**, Figure 25), isolated from *Penicillium roqueforti*, and some members of the echinulin family (**138**), isolated from *Aspergillus*

amstelodami, as well as oxaline (**139**) and aszonalenin (**140**) and numerous other alkaloids have been described that also contain the "reverse" prenyl group.[192] The irregular structure of the "reverse" isoprene group in these and other metabolites has led to considerable speculation as to the mechanism of reverse prenylation in the biosynthesis of these substances.

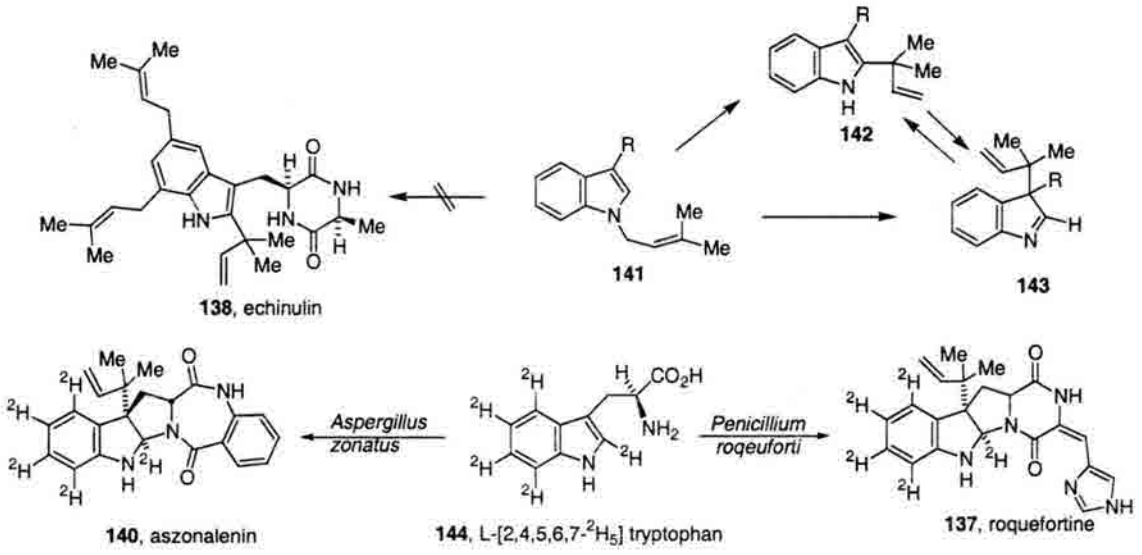


Figure 26: Possible mode of reverse prenylation in echinulin; incorporation of deuterated tryptophan in roquefortine and aszonalenin.

One possible mode of reverse prenylation that has been advanced for the biosynthesis of both roquefortine (**137**) and the echinulins (**138**) involves an aza-Claisen-type rearrangement from an N-prenylated indole (**141** → **142** *via* **143**), as shown in Figure 26. This was first proposed by Barrow *et al.* in 1979 for the biosynthesis of roquefortine.[193] Thus, aza-Claisen rearrangement of **141** would yield the reverse prenylated 3-indolenine **143**, which could subsequently suffer 1,2-migration of the dimethylvinyl carbon substituent followed by loss of the C-2 hydrogen atom of the indole

nucleus to yield **142**. Separate experimental observations to probe this mode of reverse prenylation has cast some doubt on this type of mechanism for both roquefortine and the echinulins.[194,195,196]

In the case of roquefortine (**137**), Bhat *et al.* showed retention of deuterium from C-2 of L-[2,4,5,6,7-$^{2}H_5$]tryptophan (**144**) at C-6 of **137**, thus questioning the intermediacy of **141** in the biosynthesis of this metabolite.[194] It is of further significance that Gorst-Allman *et al.* observed partial scrambling (~2:1 ratio) of the ^{13}C-label derived from [$^{13}C_2$]-acetate in the geminal carbons of roquefortine (**137**).[195] To the best of our knowledge, this was the first reported experimental data that revealed a non-face-selective reverse prenylation. The same group also inferred the aza-Claisen pathway as a possible mechanism to accommodate these observations.[195]

In the instance of the echinulins, Grundon *et al.* concluded that the aza-Claisen type mechanism may not be operative in this system due to the lack of incorporation of the tritiated N-prenyl precursors 1-([1-^{3}H]-3,3-dimethylallyl)-L-tryptophan and *cyclo*-L-alanyl-1-([1-^{3}H]-3,3-dimethylallyl)-L-tryptophan (corresponding to **142**).[196]

To further explore the mechanism of reverse prenylation, feeding experiments were performed on the austamide-producing fungi, *Aspergillus ustus*. As previously mentioned, it is believed that deoxybrevianamide E is a precursor in the biosynthesis of austamide. There are several routes by which brevianamide F (**136**) can be converted to deoxybrevianamide E (**135**). Some of these routes are shown in Figure 27.

Due to synthetic considerations, the two easiest routes to investigate involve direct normal prenylation of either the diketopiperazine nitrogen or the indole nitrogen. Prenylation of the diketopiperazine nitrogen would provide (**145**), which could undergo

subsequent intramolecular S_N2' displacement to afford deoxybrevianamide E (**135**). Alternatively, indole prenylation and aza-Claisen rearrangement of (**147**) followed by 1,2-migration of the dimethylvinyl carbon substituent and loss of the C-2 hydrogen atom of the indole nucleus would provide deoxybrevianamide E (**135**). Both potential intermediates, **145** and **147**, were synthesized as outlined in Scheme 13 and Scheme 14 and then fed to cultures of *Aspergillus ustus*.

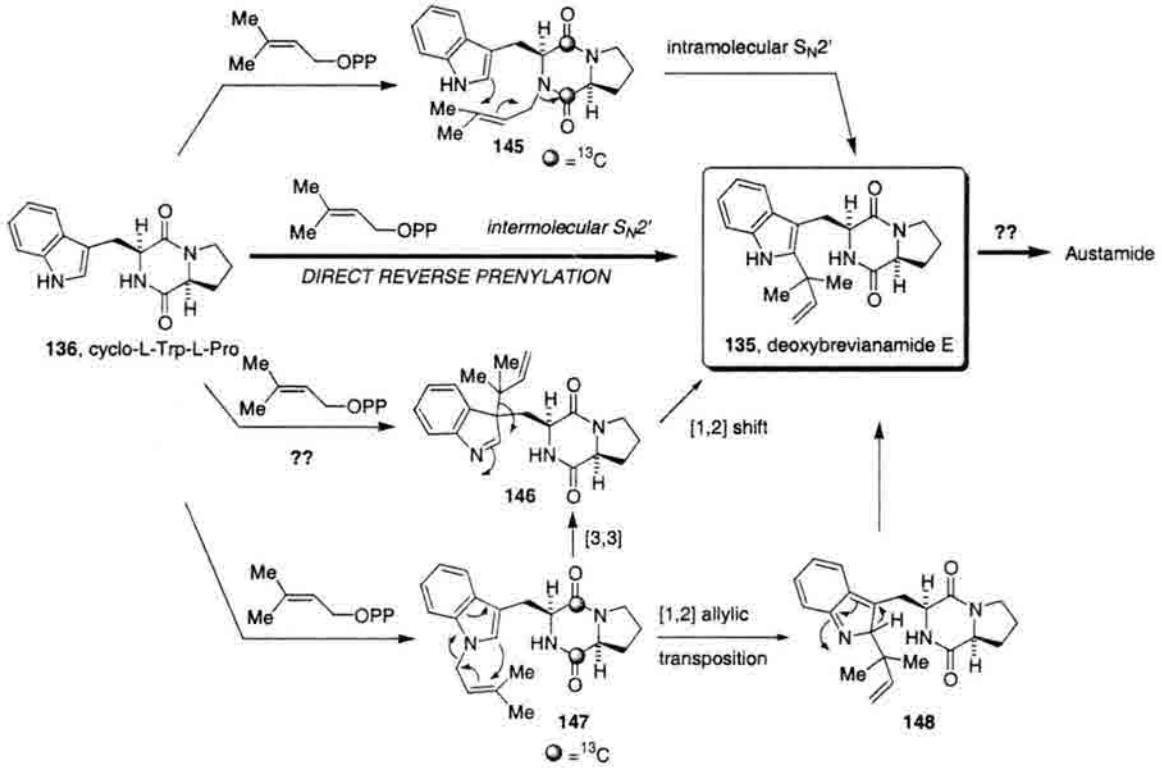
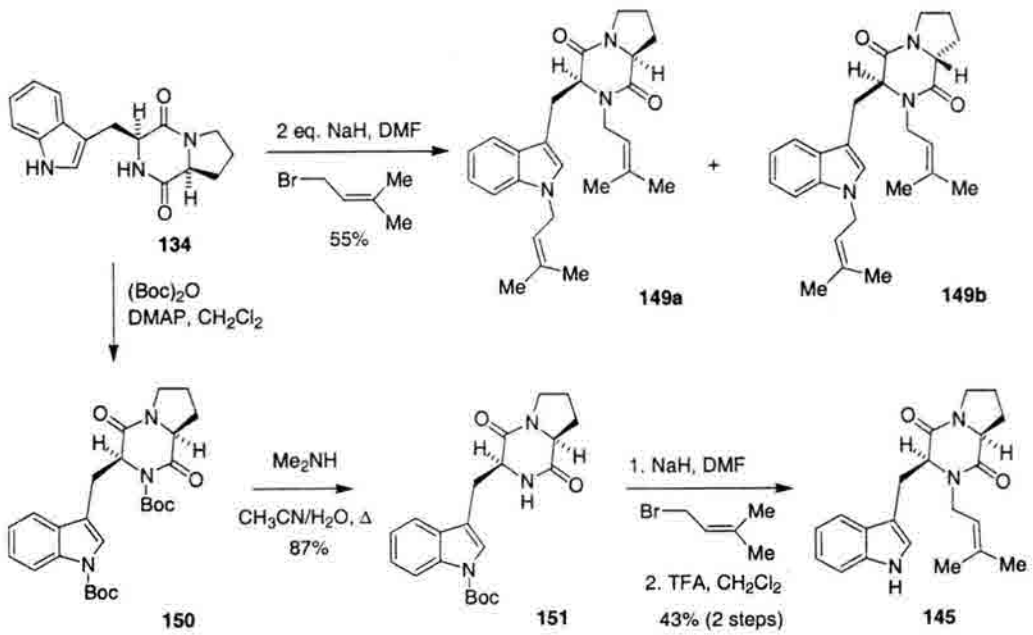


Figure 27: Possible modes of reverse prenylation of deoxybrevianamide E leading to austamide.

It was hoped that selective prenylation could be achieved directly, based on the difference of pKa of the indole and amide nitrogens. However, treatment of **134** with one

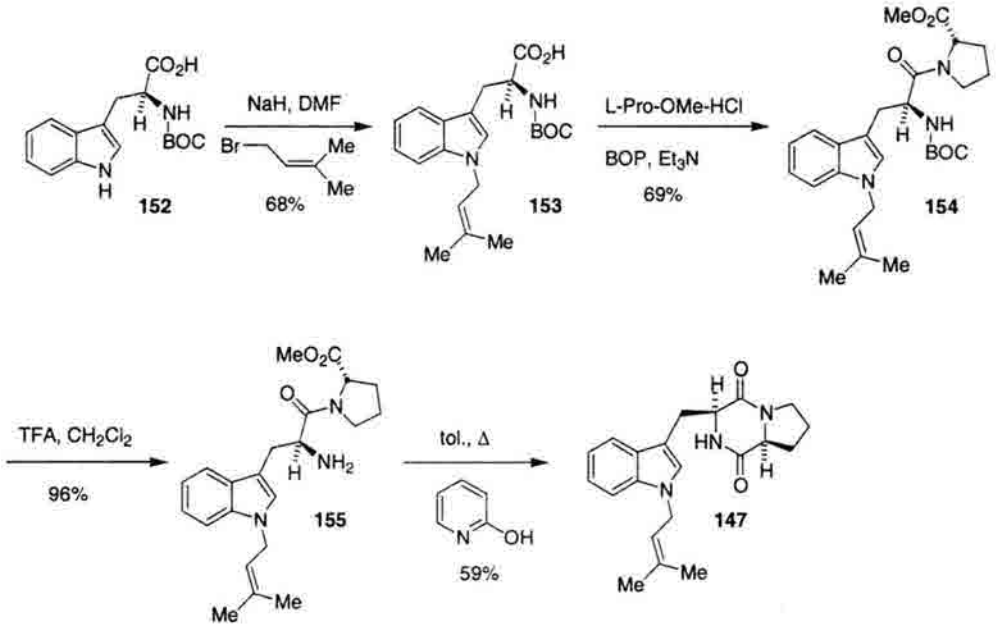
equivalent of sodium hydride and one equivalent of prenyl bromide provided a mixture of both monoprenylated species as well as the diprenylated compound **149**. Instead, the selective installation of the isoprene unit on the amide nitrogen was accomplished by conversion of **134** to the di-N-*t*-BOC substrate **150** (90%). Selective removal of the amide-derived N-*t*-BOC group was accomplished by treatment of **150** with dimethylamine in water at reflux temperature, which provided **151** in 87% yield. Treatment of **151** with NaH in DMF in the presence of prenyl bromide (57%) followed by cleavage of the *t*-BOC group from the indole nitrogen with TFA provided **145** in 76% yield.

Scheme 13: N-prenylation of *cyclo*-L-trp-L-pro at the diketopiperazine nitrogen



Sammes, et al. previously reported the simple indole N-prenylated compound **147** by a five-step procedure starting with N-CBz-L-tryptophan,.[197] An alternate, four-step route from N-BOC-L-tryptophan (**152**) was devised as shown in Scheme 14. Prenylation of **152** with prenyl bromide in the presence of sodium hydride in DMF yielded the desired N-prenylated substance **153** in 68% yield. Peptide coupling of L-proline methyl ester to **153** gave the dipeptide **154** in 69% yield. Cleavage of the N-BOC group with trifluoroacetic acid in methylene chloride (96%) followed by cyclization of the incipient amino methyl ester (**155**) with 2-hydroxypyridine in hot toluene provided **147** in 59% yield.

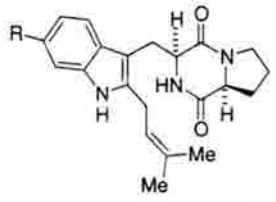
Scheme 14: N-prenylation of *cyclo*-L-trp-L-pro at the indole nitrogen



Feeding experiments were performed using the doubly ^{13}C-labeled compounds **145** and **147** on cultures of *A. ustus*. Incorporation into austamide was not observed for either proposed intermediate. However, both of these compounds were extremely

insoluble in the culture media. Even in the presence of DMSO, they were only sparingly soluble, which probably affected the uptake of precursor. In addition, it was later discovered that the sodium hydride in the prenylation step caused epimerization at the α-carbon of the amino acids.

In order to fully investigate the mechanism of reverse prenylation, these feeding experiments should be repeated after addressing the problems of solubility and epimerization. In addition, the intermediacy of deoxybrevianamide E (**135**) needs to be established in the biosynthesis of austamide and the direct reverse prenylation at both C-2 and C-3 of the indole should be investigated (Figure 27).



156, tryprostatin A, R=OMe
157, tryprostatin B, R=H

Figure 28: Structure of tryprostatins A and B

On a side note, the compounds **149a**, **149b**, **145** and **147** were investigated as potential cell cycle inhibitors.[198] The disposition of the dimethylallyl moiety at the 2-position of the indole in cell cycle inhibitors tryprostatins A (**156**) and B (**157**) suggested that the display of the isoprene group at either the indole nitrogen or the tryptophanyl amide nitrogen might closely mimic the display of this side chain in the natural products. Since **149a**, **149b**, **145** and **147** can be considered as analogs of tryprostatin B (**147**, Figure 28), their biological activities were evaluated. The effects of these compounds on

cell cycle control and microtubule assembly were examined and the results are shown in Table 9 and Figure 29.

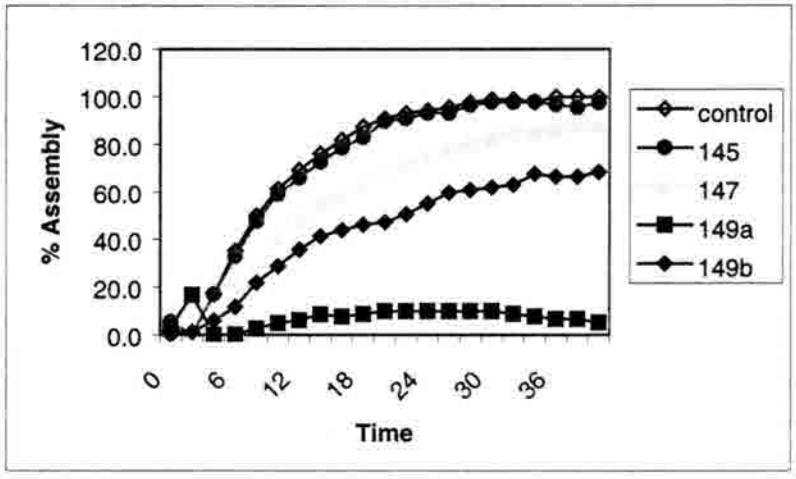


Figure 29: The effect of compounds **145**, **147** and **149a** and **149b** on microtubule assembly.[198]

Table 9. Biological activity of tryprostatin analogs.

compound	concentration (μM)	cell cycle	cell proliferation %	*in vitro* microtubule assembly %
145	500	slightly		
	250	toxic	176	98.5*
		no effect		
147	500	slightly		
	250	toxic	154	90.1*
		no effect		
149a	50	toxic	ND[b]	4.6*
	25	no effect		93.3*
149b	250	toxic	ND	36.6 ± 11.0**
	100	arrest	84	
	50	no effect	143	

* Results are the mean of two independent assays.
** Results are the mean +/- S.D. (n = 3 experiments)

Compounds **149a** and **149b** were the most toxic compounds of the four analogs evaluated. Compound **149b** completely inhibited cell proliferation at 100 μM but this inhibition was not cell cycle dependent. Compound **149b** also inhibited microtubule assembly strongly (64% inhibition at 250 μM). Compound **149a** is the most potent compound of those tested displaying 4.6% microtubule assembly at 50 μM as compared to 36.6% for **149b** at five-fold higher concentration (250 μM). In addition, substance **149a** was highly cytotoxic to cells down to 50 μM concentration (see Table 9 and Figure 48). Compounds **145** and **147** slightly inhibited cell proliferation at concentrations above 500 μM but neither of these derivatives inhibited microtubule assembly *in vitro.*

4.3 Possible "Reverse" Prenylated Intermediates in the Biosynthesis of Paraherquamide A

The basic premise of our proposal for a hetero-Diels-Alder mediated bicyclo[2.2.2]diazaoctan formation is the intermediacy of a "reverse" prenylated metabolite. Since brevianamide F (**136**) and deoxybrevianamide E (**135**) are proven intermediates in the biosynthesis of brevianamide A (**15**) and brevianamide B (**16**), we assumed that the reverse prenylation occurs at a similar point in the biosynthesis of paraherquamide A (**1**). However, there are actually several different stages at which the reverse addition of the isoprene unit to the indole system might occur. Determining the initial "reverse" prenylated intermediate will give an indication of the temporal sequence of events in forming the complex polycyclic ring system of paraherquamide (**1**). In addition, the identification of such an intermediate may help in the isolation of the proposed "reverse" prenylase enzyme.

A few of the many possible sequence of events for reverse prenylation of the indole moiety in paraherquamide biosynthesis are outlined in Figure 30. In analogy to what has been established experimentally for the structurally related brevianamides, reverse prenylation of the indole nucleus might occur after formation of the diketopiperazine **158** to give **159**. Alternatively, either L-tryptophan itself or the dipeptides L-tryptophanyl-3(*S*)-methyl-L-proline or 3(*S*)-methyl-L-prolyl-L-tryptophan might be prenylated giving rise to potential intermediates **160~162**. These possibilities needed to explored through feeding experiments with the doubly isotopically labeled compounds **158**, **159**, **160**, **161**and **162**.

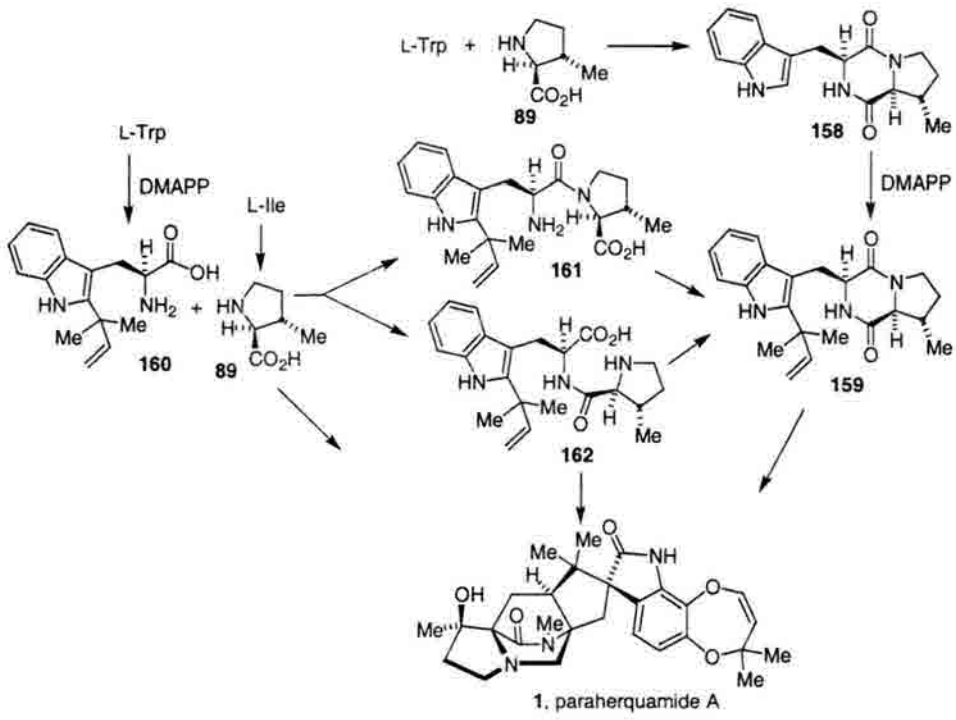
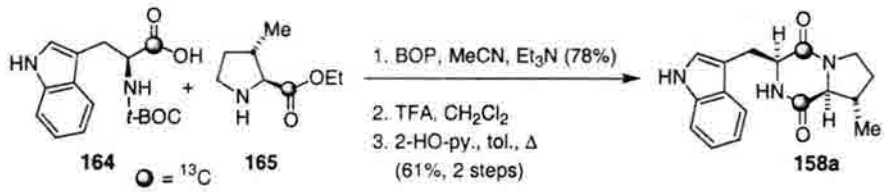


Figure 30: Possible reverse prenylated intermediates leading to the 2.2.2[bicyclo] diazaoctan of paraherquamide A

The diketopiperazine, **158**, was synthesized as outlined in Scheme 15. Coupling of the N-BOC-protected [1-^{13}C]-L-tryptophan (**163**) to [1-^{13}C]-3(*S*)-methyl-L-proline

ethyl ester (**164**) was accomplished in good yield using the BOP reagent. Removal of the BOC group with TFA and cyclization in refluxing toluene containing a catalytic amount of 2-hydroxy pyridine afforded **158a** in 48% yield over the three steps. Unfortunately, the diketopiperazine **158a** was very insoluble in water, which made the feeding experiments more challenging. Feeding experiments were first performed using DMSO to solubilize the proposed precursor. However, under these conditions **158a** was still not entirely soluble and the DMSO seemed to have a deleterious effect on the production of paraherquamide. Eventually, after testing many detergents, it was determined that TWEEN 80 substantially increased the solubility of **158a** in the culture broth without inhibiting production of paraherquamide A. Nonetheless, even under these conditions, incorporation of the proposed intermediate **158a** was not observed within the limits of detection.

Scheme 15: Synthesis of [$^{13}C_2$]-*cyclo*-L-tryptophan-3(*S*)-methyl-L-proline



The lack of incorporation of **158a** does not necessarily exclude it as a possible intermediate. The reason for non-incorporation may simply be due to poor uptake of the compound by the cells. However, these results do cast some doubt on the intermediacy of **158a**, and so the alternate intermediates outlined in Figure 16 were explored. A common feature of the remaining intermediates **159**, **160**, **161** and **162**, is the reverse

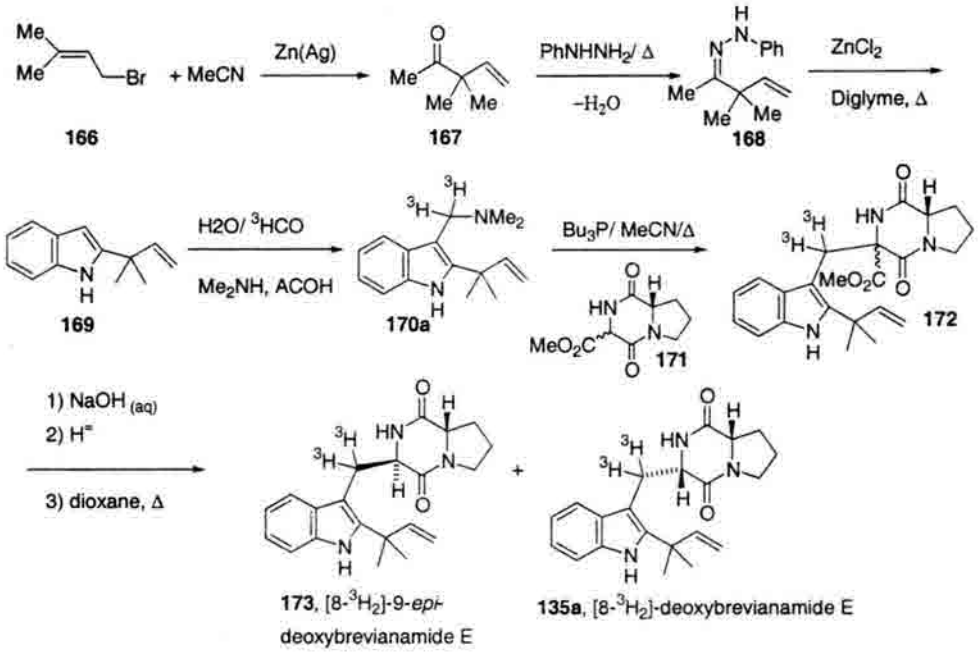
prenyl group at the 2-position of the indole in the tryptophan moiety. Syntheses of these intermediates, especially the diketopiperazine **159**, could be achieved from published procedures for the synthesis of deoxybrevianamide E. [23, 199,200]

Until recently, only two syntheses of deoxybrevianamide E have been published; the first, by Saxton et al. in 1975,[199] confirmed the structure and a second, higher yielding synthesis by Kametani et al., in 1980, was employed to elucidate the relative and absolute stereochemistry of brevianamide E.[23,199] Neither of the published routes to deoxybrevianamide E are stereocontrolled and both afford mixtures of deoxybrevianamide E and C-9-*epi*-deoxybrevianamide E.

For our studies on the biosynthesis of potential reverse prenylated metabolites, we required a more efficient synthesis of this substance that was readily amenable to the incorporation of stable isotopes. Work previously performed in the Williams' group utilized a modification of the Kametani synthesis[199] of deoxybrevianamide E in the synthesis of [8-$^3H_2$]-deoxybrevianamide E (Scheme 16).[161]

This synthesis commences with a Reformatsky-type reaction between prenylbromide (**166**) and acetonitrile in the presence of a zinc/silver amalgam to provide **167**, which is consequently converted to the phenylhydrazone **168**. The indole **169** was synthesized from **168** using the Fischer indole synthesis. At this point, the tritium-label was introduced with [^{3}H]-labeled formaldehyde and dimethylamine to produce the gramine derivative **170a**. Somei coupling of **170a** with the diketopiperazine **171** gave **172** as a mixture of diastereomers. Subsequent hydrolysis and decarboxylation provided [8-$^3H_2$]-deoxybrevianamide E (**135a**) and [8-$^3H_2$]-9-*epi*-deoxybrevianamide E (**173**) in a 1:14 ratio with **173** as the major product.

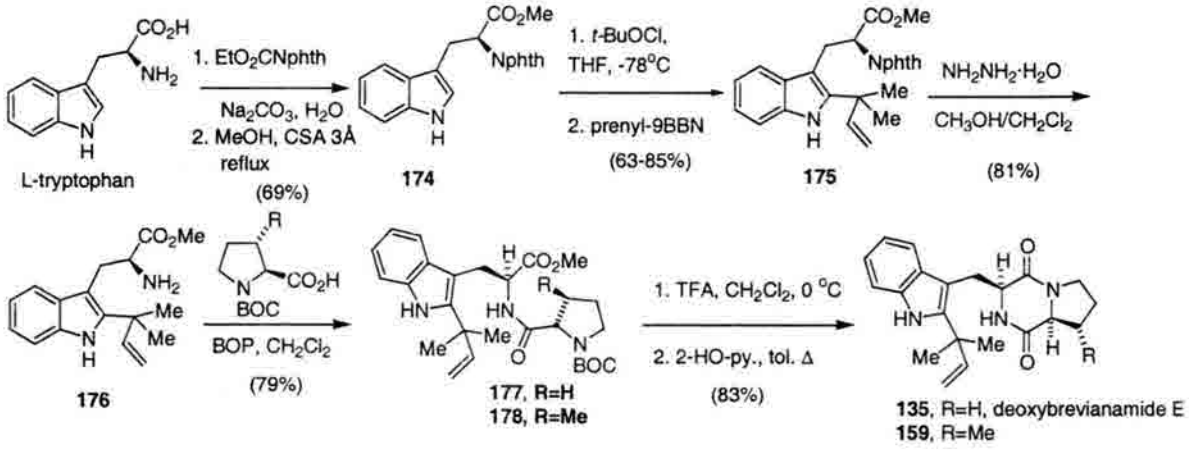
Scheme 52: Synthesis of [8-$^3H_2$]-deoxybrevianamide E (**135a**).[161]



The main problem with this synthesis is the final decarbomethoxylation reaction since non-stereospecific protonation of the incipient enolate gives deoxybrevianamide E (**135a**) as the minor product. While the minor amount of tritium-labeled deoxybrevianamide E produced by this procedure was used in a biosynthetic study of brevianamide A, this approach seemed impractical for the production of substantial quantities of ^{13}C-labeled "reverse" prenylated intermediates we would require. Therefore, a new and more efficient stereocontrolled synthesis of deoxybrevianamide E was developed based on the reverse prenylation technique made available by Danishefsky, et al.[201,202] This synthesis does not produce any of the diastereomeric C-9-*epi*-deoxybrevianamide E and is readily amenable to the incorporation of stable isotopes in both amino acid subunits for double labeling experiments.

As shown in Scheme 17, L-tryptophan is converted into the corresponding N-phthalimido methyl ester derivative **174** by sequential treatment with N-carboethoxyphthalimide followed by camphorsulfonic acid in methanol. Thus, treatment of **174** with *tert*-butylhypochlorite in THF at low temperature provided the corresponding labile 3-chloroindolenine that was prenylated with prenyl-9-BBN in high overall yield to afford **175**.[201]

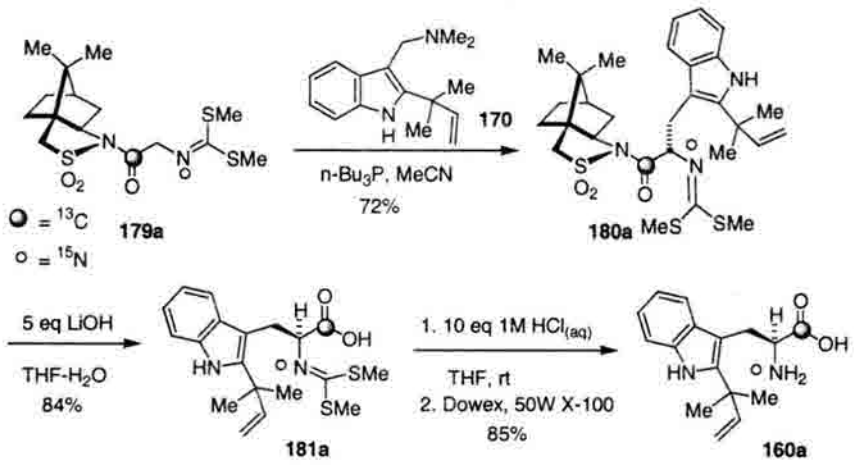
Scheme 17: Synthesis deoxybrevianamide E *via* the Danishefsky method.



Cleavage of the phthalimido group of **175** with hydrazine hydrate provided **176** in 81% yield. Coupling of this substance with N-BOC-L-proline with the BOP reagent in dichloromethane furnished the dipeptide **177** in 79% yield. Removal of the BOC group with TFA and cyclization to deoxybrevianamide E (**135**) was accomplished by refluxing the incipient aminomethyl ester in toluene in the presence of 2-hydroxypyridine in 83% yield in just eight steps from L-tryptophan. The same approach was also used to prepare the methylated derivative **159**.

Although this route allows facile entry into all of the proposed intermediates, the ^{13}C-label would necessarily be derived from labeled L-tryptophan, which is very expensive. Therefore another route to stable-isotope labeled 2-dimethylallyl-L-tryptophan (**160**) was developed starting from the labeled Oppolzer sultam glycinate (**179**) as shown in Scheme 18.[203] This route allows access to either the singly-labeled ^{13}C-tryptophan derivative or the doubly labeled ^{13}C, ^{15}N-derivative.

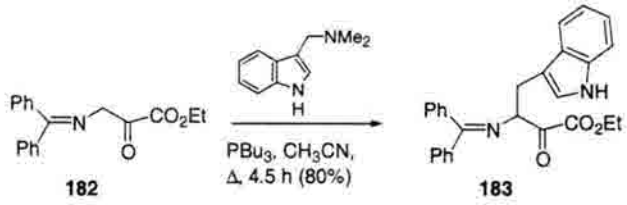
Scheme 18: Synthesis [^{15}N, 1-^{13}C]-2-dimethylallyl-L-tryptophan



In the case of 2-dimethylallyl-tryptophan itself, the doubly ^{13}C, ^{15}N labeled material was chosen to explore the possibility that the tryptophanyl moiety was oxidatively deaminated at some point in the biosynthesis (specifically in this case, formation of the azadiene system for the proposed IMDA cyclization reaction). The retention of the ^{15}N-label, or loss thereof, could therefore provide significant mechanistic insight into the coupling and cyclization with 3(*S*)-methyl-L-proline.

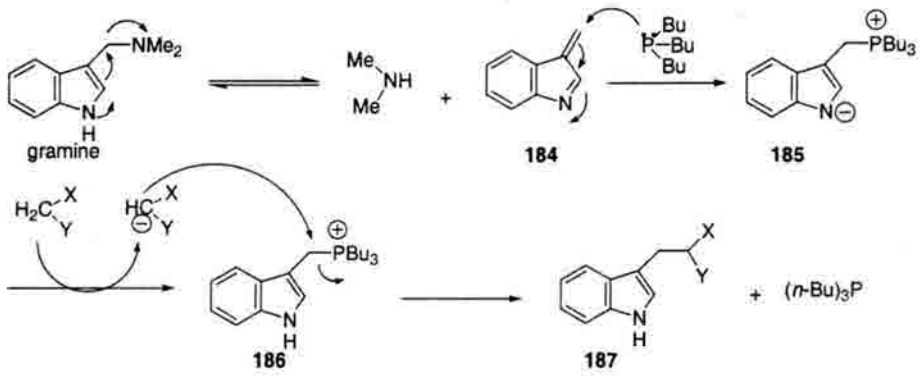
The synthesis of **160a** was accomplished *via* a Somei-type coupling reaction of **179a** with **170** and 0.3 equivalents of PBu_3 in refluxing acetonitrile over a period of 8 hours (Scheme 18).[199b] The sultam-moiety of **180a** was hydrolyzed in a 2:1 mixture of THF and water with 5 equivalents of LiOH over a period 24 hours. Purification of **181a** was accomplished by flash column chromatography, and allowed recovery of the chiral sultam. The N-dithiomethylmethylene group was removed with 10 equivalents of 1N HCl in THF at room temperature over 24 hours. The solvent was removed *in vacuo* and the HCl salt was submitted to Dowex ion exchange chromatography to provide the free amino acid, **160a**.

Scheme 19: Synthesis N-Diphenylmethylene-D,L-tryptophan ethyl ester.



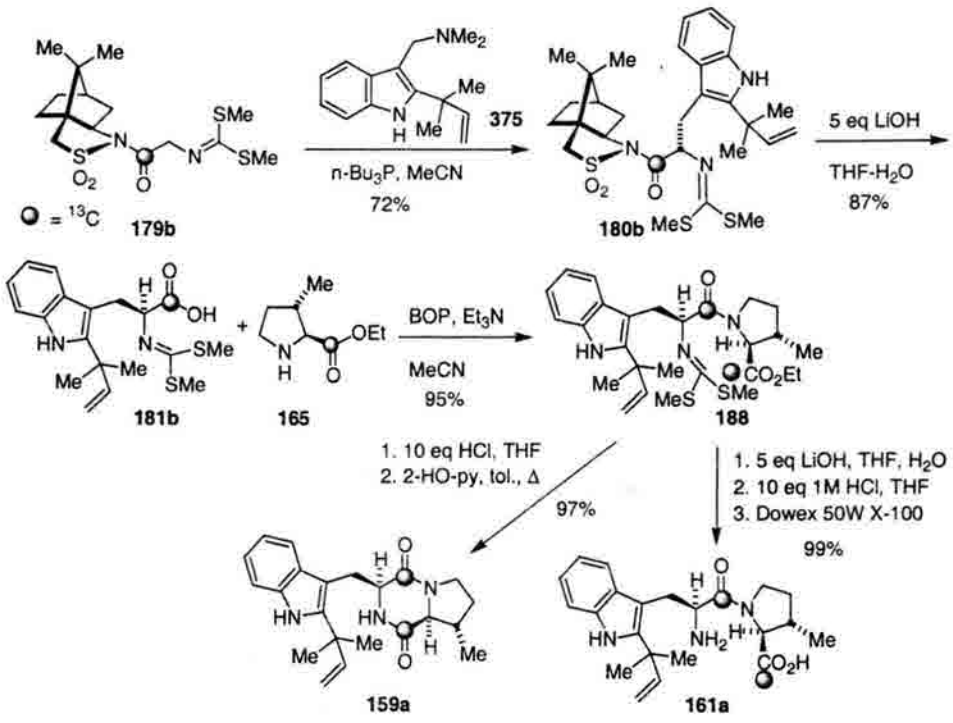
The concept of a Somei coupling reaction to generate the 2-dimethylallyl-L-tryptophan directly was first envisioned by Jeffery Cao through his investigations on the total synthesis of paraherquamide A.[204] Based on the methodology of O'Donnell and co-workers[205] for the synthesis of racemic α-amino acids from the Schiff base **182**,[206] Cao was able to develop a synthesis of racemic tryptophan (Scheme 19). The synthesis of **183** was accomplished through the Somei coupling of gramine with the benzophenone imine of glycine ethyl ester.

Scheme 20: Proposed mechanism for the Somei coupling reaction.



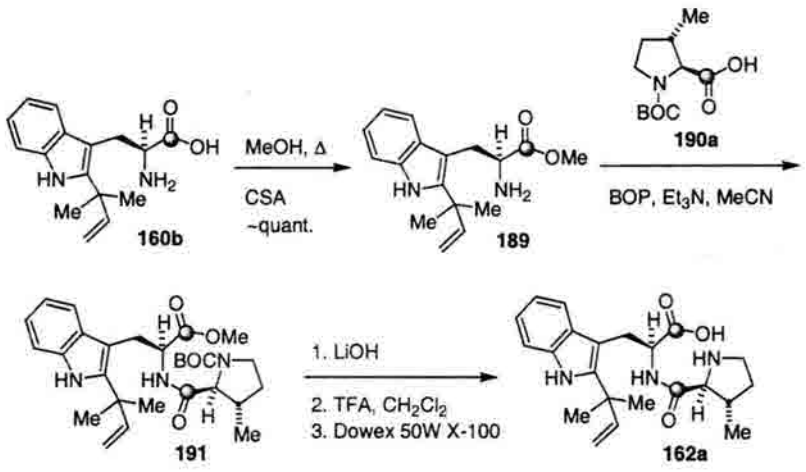
A proposed mechanism of the Somei reaction is shown in Scheme 20. In refluxing acetonitrile, gramine equilibrates with dimethylamine and the indolenine **184**. Attack of the indolenine **185** by the catalyst, tributylphosphine (PBu_3), generates the anion **185**. Proton abstraction by **185** produces a carbanion intermediate from the carbon acid H_2CXY. Nucleophilic attack of ^-CHXY at the α-carbon of **186** forms the coupling product **187** and regenerates the catalyst, PBu_3. For this catalytic cycle to propagate, the anion **185** must be a stronger base than the anion ^-CHXY. The pKa for deprotonation of indole is 16.97 and the pKa for the imine **182** is 18.8.[207] These pKa's are close enough under the reaction conditions, refluxing CH_3CN, that the racemic protected tryptophan **183** is produced in 80% yield. It was believed that this reaction might also work for the Oppolzer sultam **179**, which should have a similar pKa to the Schiff's base **182**. It is interesting to note that the attempted Somei coupling of gramine itself with the Oppolzer sultam was not successful.

Scheme 21: Syntheses of ^{13}C-labeled proposed "reverse" prenylated intermediates **159a** and **161a**



The doubly labeled diketopiperazine of 2-dimethylallyl-L-tryptophan and 3(*S*)-methyl-L-proline (**159a**) was prepared from the ^{13}C-labeled sultam **179b** according to the procedure outlined in Scheme 21. The intermediate, **180b**, was prepared in the same manner as **180a** in 86% yield. After removal of the sultam with LiOH, coupling to **165** was achieved in 95% yield utilizing the BOP reagent. Removal of the N-dithiomethylmethylene protecting group followed by cyclization in refluxing toluene with a catalytic amount of 2-hydroxy-pyridine afforded the diketopiperazine (**159a**) in excellent yield. Alternatively, the dipeptide, L-tryptophanyl-3(*S*)-methyl-L-proline (**161a**), was prepared by deprotection of **188** with LiOH followed by 1M HCl and Dowex ion exchange in essentially quantitative yield.

Scheme 22: Synthesis of [$^{13}C_2$]-3(*S*)-methyl-L-prolyl-L-tryptophan (**162a**).



The dipeptide, [$^{13}C_2$]-3(*S*)-methyl-L-prolyl-L-tryptophan (**162a**), was synthesized according to the procedure outlined in Scheme 22. [1-^{13}C]-2-dimethylallyl-L-tryptophan, **160b**, was synthesized in the same manner as **160b** in 77% yield. Protection of the carboxylic acid of **397** as the methyl ester followed by coupling to 1-[^{13}C]-N-BOC-3(*S*)-methyl-L-proline (**190a**) with BOP reagent provided the dipeptide, **191**. Deprotection of the methyl ester with LiOH followed deprotection of the BOC group with TFA and ion exchange with DOWEX provided the dipeptide, **162a**, in 69 % yield.

Feeding experiments were performed on *P. fellutanum* with the potential ^{13}C-labeled intermediates, **158a**, **159a**, **160a**, **161a** and **162a.** The results are shown in Table 10. Somewhat surprisingly, *none* of these potential precursors were incorporated intact. However, by ^{13}C NMR, the dipeptides, **161a** and **162a** showed incorporation in 0.44-0.92% respectively at C-18, but no incorporation at C-12. This suggests that the dipeptides are catabolized and that 3(*S*)-methyl-L-proline is re-incorporated, but the reverse prenylated L-tryptophan is not. As previously mentioned, the lack of

incorporation does not rigorously exclude the intermediacy of proposed intermediates in the biosynthesis of paraherquamide A. However, since the dipeptides **161a** and **162a** were catabolized, this implies that the compounds **161a** and **162a** were taken *inside* the cell. Since these compounds were not subject to transport limitations, the intermediacy of the reverse prenylated tryptophan (**160a**) and the dipeptides **161a** and **162a** in the biosynthesis of paraherquamide A (1) is unlikely.

Table 10: Results of feeding experiments with reverse prenylated tryptophanyl derivatives

Compound	mmol	1 (mmol)	Enhancement of ^{13}C peak	Incorporation by ^{13}C NMR
158a	0.042	0.028	ND[a]	ND[a]
159a	0.059	0.035	ND[a]	ND[a]
160a	0.098	0.026	ND[a]	ND[a]
161a	0.040	0.010	1.8% (C-18)	0.44% (C-18)
162a	0.031	0.015	1.9% (C-18)	0.92% (C-18)

[a] ND=Not Detectable

CHAPTER 5

Total Synthesis of (±) VM55599: Implications in the Elucidation of the Biosynthetic Pathway of Paraherquamide A

5.1 Proposed Intermediacy of VM55599 in Paraherquamide Biosynthesis

The fungal metabolite, VM55599 (**13**), was the first documented example of a hexacyclic indole alkaloid in the paraherquamide family. This type of compound has long been postulated as a biosynthetic precursor to the brevianamide, paraherquamide and marcfortine families.[3,17,28] Blanchflower and co-workers assigned the structure and relative stereochemistry of VM55599 based on extensive NMR studies.[3] In particular, H-20 and C-12 were deemed to be *trans*-diaxial based on the strong nOe from H-22 to H-12a (Figure 31). Since VM55599 was isolated from a paraherquamide producing *Penicillium sp.* (IMI 332995), these workers assumed that C-20 was *S*, the same absolute stereochemistry found in paraherquamide. They concluded that the stereochemistry of C-14 was *R*, inverted from the C-14 *R* configuration of paraherquamide itself. If VM55599 was a biosynthetic precursor paraherquamide, it would indicate that oxidation of the prolyl ring would have to occur with inversion of stereochemistry.

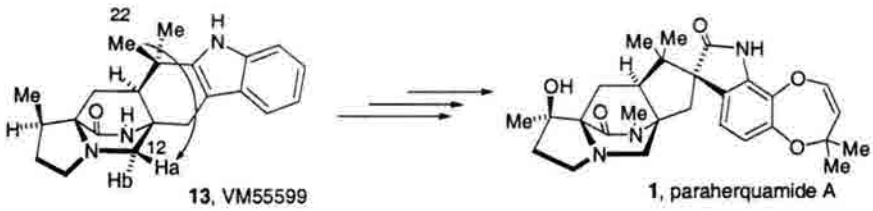


Figure 31: nOe and proposed structure of VM55599, a putative intermediate in the biosynthesis of paraherquamide A.

As discussed in Chapter 3, L-isoleucine was determined to be the biosynthetic precursor to paraherquamide A. If the absolute stereochemistry of VM55599 is as predicted by Blanchflower and co-workers, then VM55599 would have to be derived from *allo*-L-isoleucine (Figure 32). If on the other hand, L-isoleucine is the precursor to the β-methyl proline ring of VM55599, then the absolute configuration of the bicyclo[2.2.2]diazaoctan portion of VM55599 would be *enantiomorphic* to that of paraherquamide A. In either case, the potential intermediacy of VM55599 in the biosynthesis of the paraherquamides is suspect.

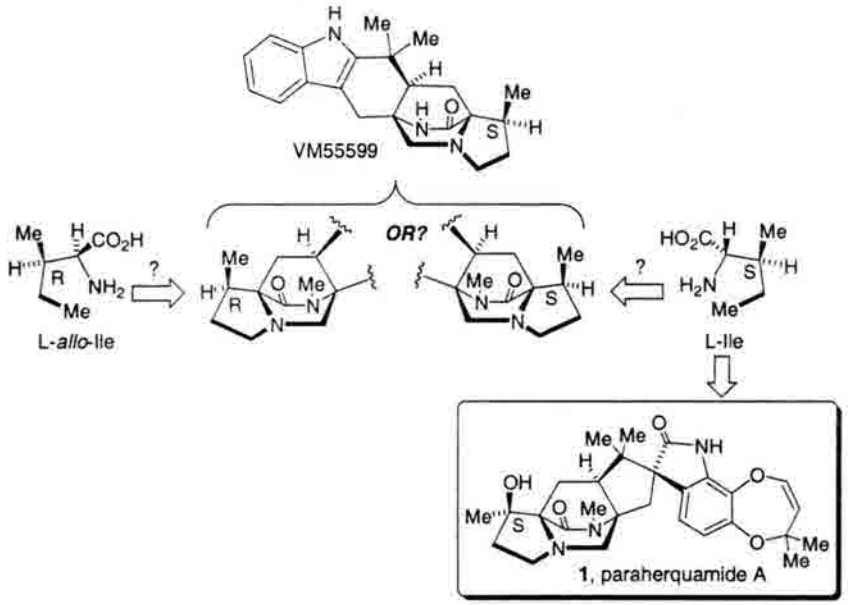
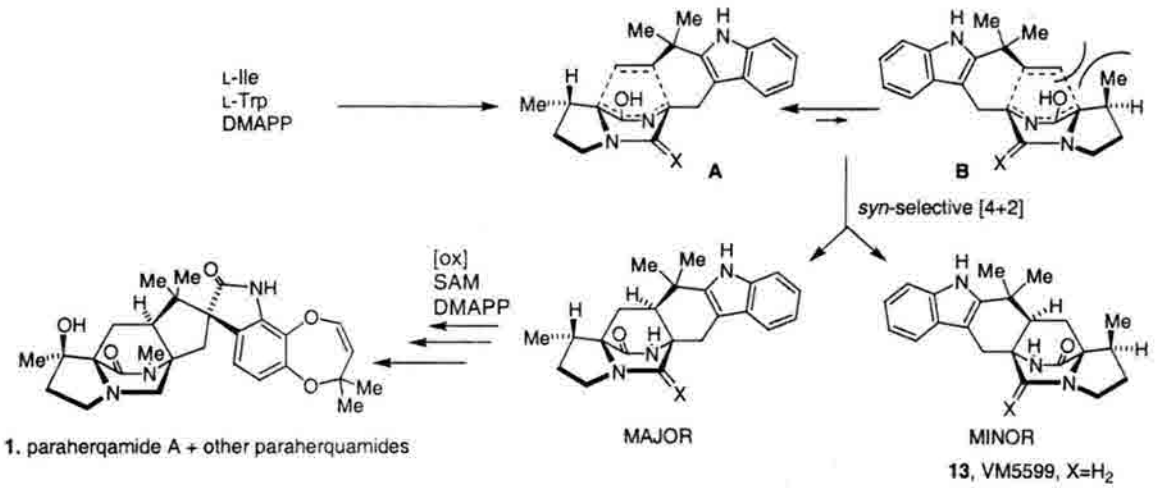


Figure 32: Possible biogenic origin of the β-methyl proline ring of VM55599.

The presence of VM55599 in paraherquamide-producing fungal cultures can be explained by the proposed unified biogenesis outlined in Scheme 58. Each metabolite would result from the intramolecular [4+2] cycloaddition of a common azadiene from two of four possible diastereomeric transition structures **A** and **B**. If cycloaddition occurs with the methyl group of the β-methylproline ring *anti-* to the isoprene unit (**A**, Scheme 58), then an intermediate would be formed, leading to all of the paraherquamides containing a β-methylproline moiety. This is presumed to be a major pathway. A minor shunt pathway, would involve cycloaddition from the more hindered face of the azadiene system (see **B**, Scheme 2) with the methyl group of the β-methylproline ring *syn-* to the isoprene, providing the cycloadduct corresponding to VM55599 (**13**).

Scheme 23: Proposed unified biogenesis for paraherquamide A and VM55599



It is interesting to note that the cyclization of the isoprenyl olefin across the azadiene ring system could proceed *via* four distinct diasteromeric transition-state

structures **a**, **b**, **c** or **d** (Figure 33) resulting in the four corresponding cycloadducts **A**, **B**, **C** or **D**. Thus far, however, cycloadducts **C** and **D** leading to C-20-*epi* metabolites have been not detected from paraherquamide-producing fungi.

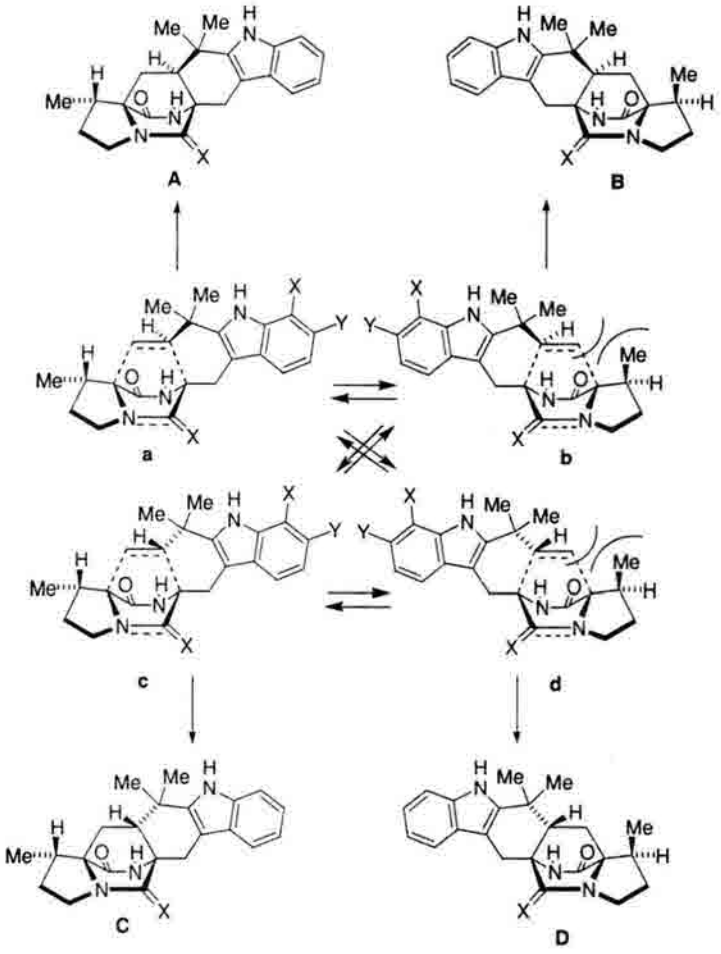


Figure 33: All four possible diastereomeric transition states/ cycloadducts from the hetero-Diels-Alder reaction leading to VM55599 and paraherquamide A

Another option for the biogenesis of paraherquamide is that the proposed Diels-Alder precursor is oxidized from the indole to the oxindole before cyclization (Figure 34). In this case, VM55599 could be a shunt metabolite resulting from [4+2]

cycloaddition prior to oxidation. To distinguish between these possible biosynthetic pathways, an efficient synthesis of VM55599 needed to be developed. Ideally, the synthesis would be amenable to isotopic labeling and would allow for the synthesis of the hexacyclic diastereomer that is the proposed precursor to paraherquamide A.

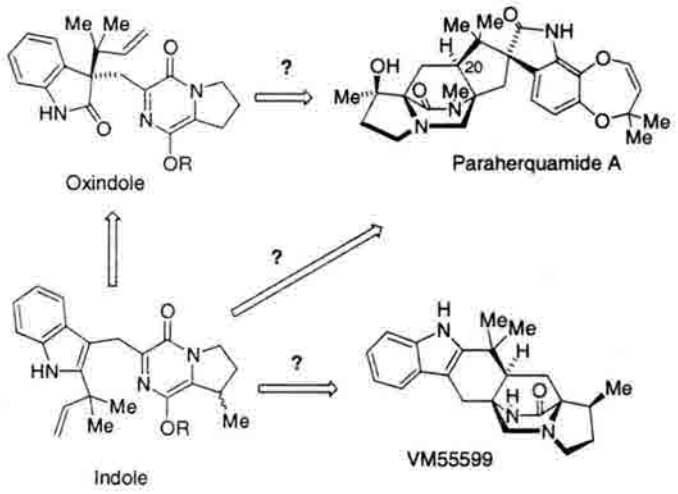


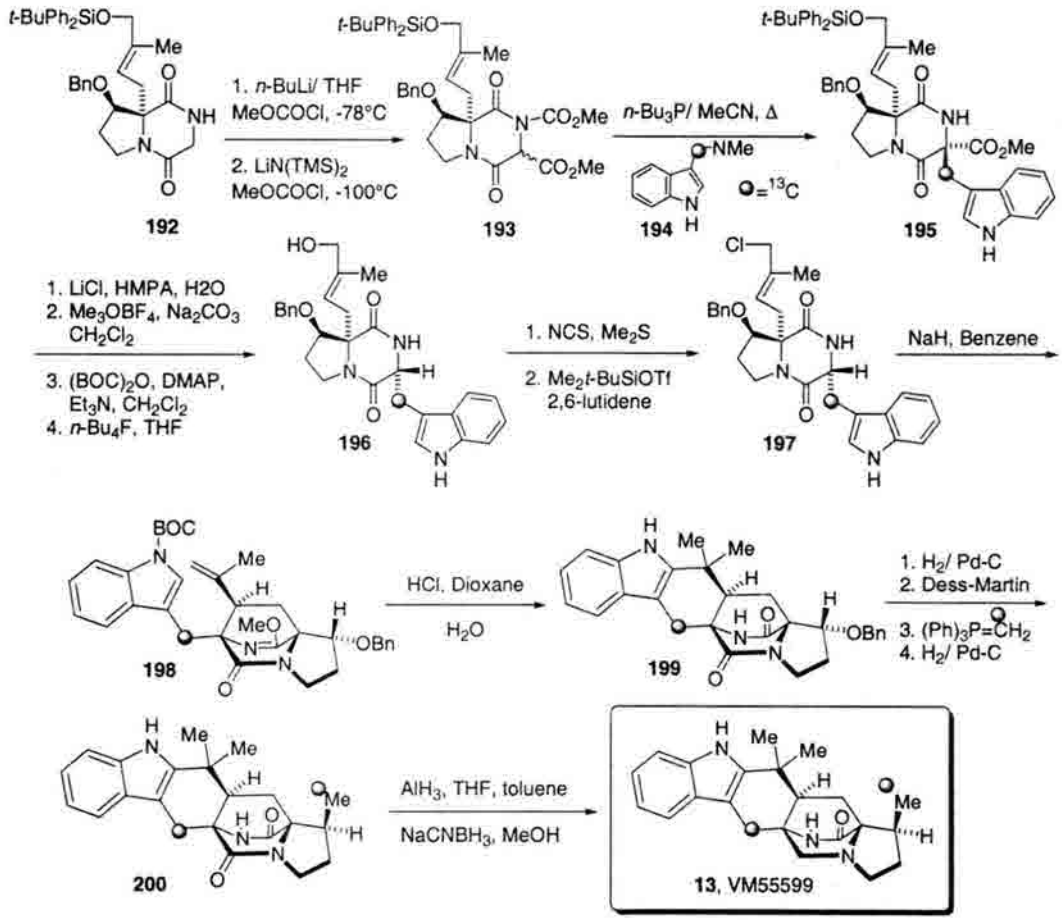
Figure 34: Possible oxidation states of reverse prenylated intermediate prior to [4+2] cycloaddtion

5.2 Total Synthesis of VM55599

Initially the synthesis of VM55599 was envisioned as an extension of the synthetic methodology developed for the total synthesis of paraherquamdie A and B (Scheme 24). The synthesis of VM55599 would begin from a similar optically active precursor (**192**) developed for the synthesis of paraherquamide A (Scheme 4, chapter 1). Somei coupling with [^{13}C]-gramine (**194**) would allow introduction of the first isotopic label in the tryptophanyl portion of VM55599. The crucial step of this reaction, as in the synthesis of paraherquamide A, is the stereoselective S_N2' cyclization (**198** → **199**). The β-methyl proline moiety would be synthesized from **199** by debenzylation, oxidation,

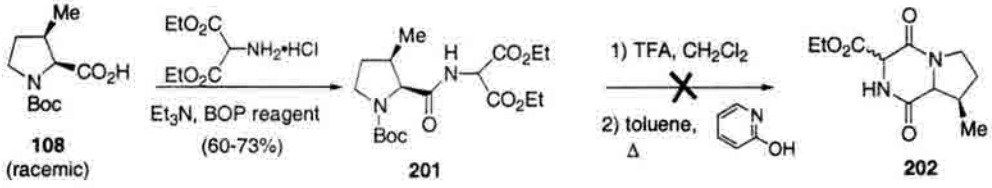
olefination and stereospecific reduction, which is expected to occur from the less hindered face, to provide **200**. The second isotopic label could be introduced at this juncture (**199** → **200**) *via* the Wittig reaction. The advantage of this route is that it would provide a single diastereomer with the same absolute stereochemistry as natural (-)-paraherquamide A. Direct comparison of the synthetic compound with the natural VM55599 would ascertain the absolute chemistry of VM55599. The disadvantage of this route is that it is somewhat lengthy, including 13 linear steps after introduction of the first isotopic label.

Scheme 24: First proposed synthesis of VM55599



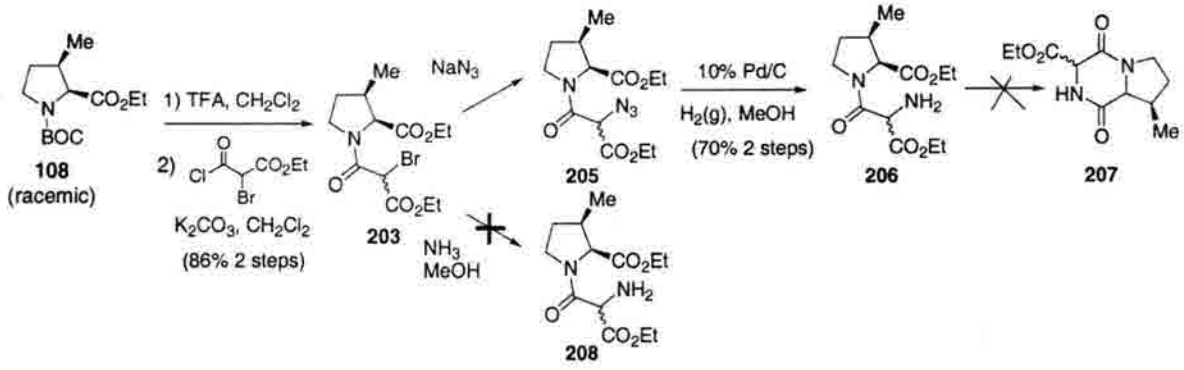
The development of the biomimetic model synthesis for brevianamide (Chapter 2, Scheme 41) led to a Diels-Alder approach for the synthesis of VM55599. It was thought that the exact same synthesis used in the model study could be employed; with the exception that β-methyl proline would be used in lieu of proline. One advantage this synthesis possesses is the potential production of all eight of the possible stereoisomers including VM55599 and the proposed progenitor of paraherquamide A.

Scheme 25: Attempted VM55599 synthesis from the model study



Since the methylated stereocenter would be lost in a subsequent oxidation reaction, it was unnecessary to use the optically pure 3(*S*)-methyl-L-proline in this synthesis. Racemic *cis*-β-methyl proline (**108**), obtained from early synthetic attempts at β-methyl-L-proline (Chapter 3, Table 4), condensed smoothly with diethylaminomalonate in fair yield (Scheme 25). Unfortunately, cyclization attempts were unsuccessful under a range of conditions. Since diketopiperazines usually form quite readily, it was surprising that the dipeptide **201** would not cyclize. It is possible that the presence of the methyl group caused enough conformational constraint to prevent the formation of **202**. Ensuing cyclization attempts with the *trans*-β-methyl proline were also unsuccessful.

Scheme 26: Attempted alternate routes to the cursory diketopiperazine for the synthesis of VM55599

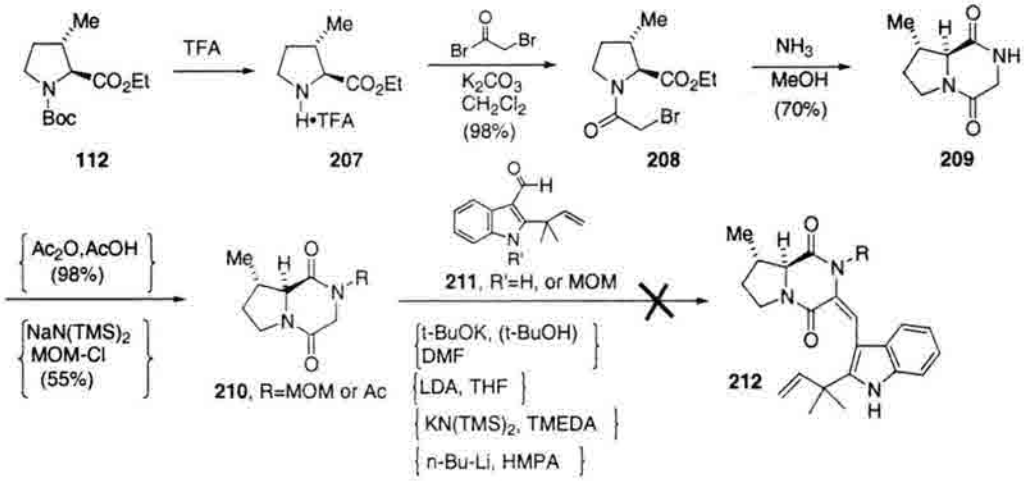


To circumvent any possible deleterious steric interactions, the cyclization of the diketopiperazine was attempted in the opposite direction (Scheme 26). After deprotection of the N-BOC group with TFA, **108** was acylated with 2-bromo-ethylmalonyl chloride to provide **203** in excellent yield. Attempts to aminate **203** directly with methanolic ammonia were unsuccessful. However, formation of **205** by treatment of **203** with sodium azide, followed by reduction with H_2 and Pd^0/C, provided the requisite amine **206**. Again, cyclization attempts were unfruitful. In this case, acidity of the hydrogen α- to the free amine seemed to complicate the cyclization

The presence of the ester at the α-position of the diketopiperazine was necessary for the Somei coupling reaction, and yet it was proving problematic in the cyclization reaction. Thus, a different tactic was undertaken to couple the "reverse" prenylated indole to the diketopiperazine. Based on the work of Nakatsuka [208] and Fukuyama,[209] an aldol condensation of the protected diketopiperazine **210** and the aldehyde **211** was tried (Scheme 27). Attempted aldol condensation with the N-protected diketopiperazine **210** only produced the deprotected diketopiperazine **209** in the case of N-acylated-**210** and only starting material in the case of N-MOM-protected-**210**. Protection of the indole

nitrogen to prevent formation of the indolenine and the use of different bases had no effect on the outcome of the reaction. The use of different bases had no effect on the reaction.

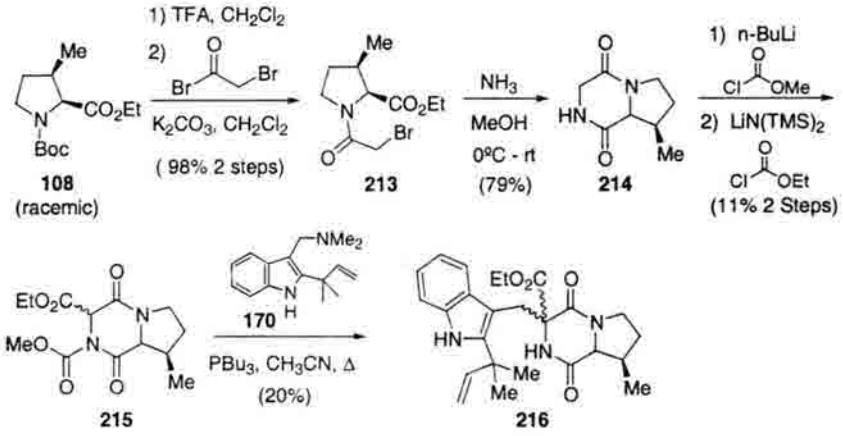
Scheme 27: Attempted aldol condensations for the synthesis of VM55599



The Somei coupling reaction had been successful in the model study, so its usage was re-investigated for the synthesis of VM55599. To avoid problems in the cyclization reaction, the α-ethyl ester was introduced after formation of the diketopiperazine. A similar strategy had been employed for the synthesis of paraherquamide A. The diketopiperazine **214** was synthesized in excellent yield from racemic *cis*-β-methyl proline (**108**), bromoacetyl bromide and ammonia. Acylation of the secondary amine with methylchloroformate and *n*-butyl lithium followed by acylation at the α-position with methylcholoroformate and lithium hexamethyldisilamide provided **215** in poor yield (11%, 2 steps). Coupling of **215** with the gramine derivative **170** proceeded in 20%

yield. The low yields obtained in the acylation and the coupling step made this synthetic strategy for VM55599 unacceptable since it is not conducive to isotopic labeling.

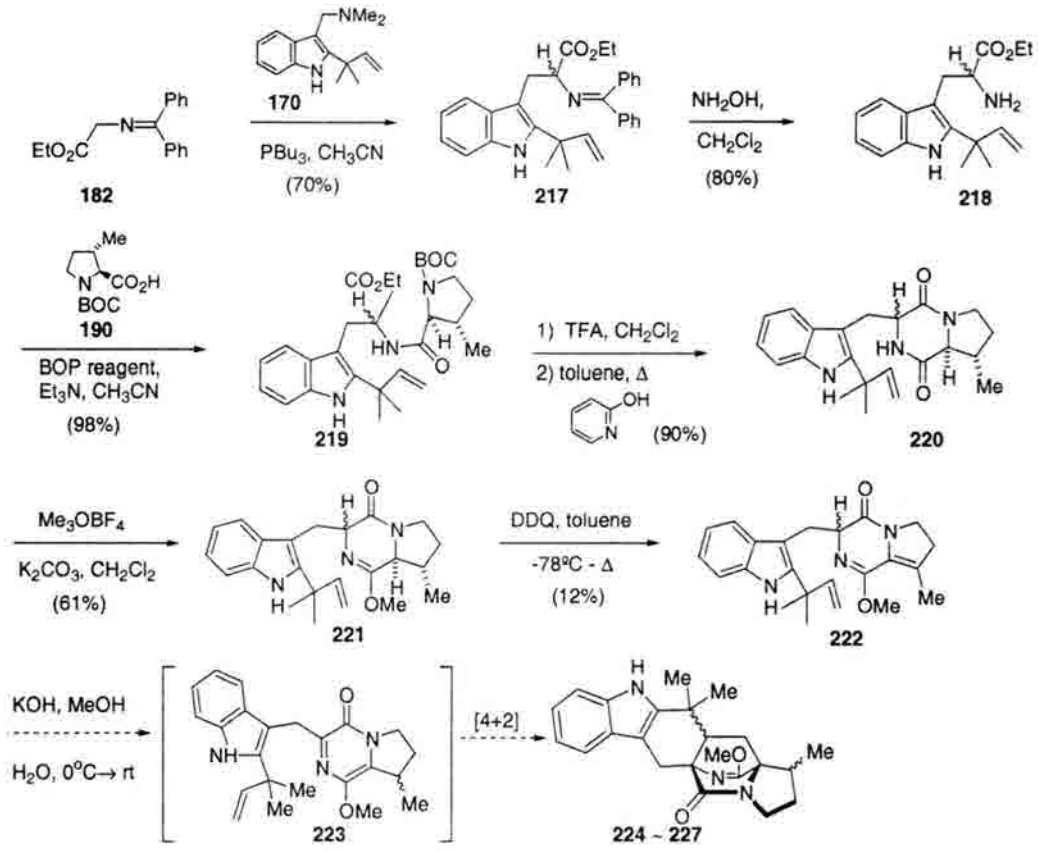
Scheme 28: Somei coupling strategy modeled after the total synthesis of paraherquamide.



Other synthetic methodology arising from the total synthesis of paraherquamide A (Scheme 19, Chapter 4) led to the idea that the Somei coupling of the gramine derivative **170** could be performed on the benzophenone imine of glycine ethyl ester (Scheme 29). The so-produced tryptophanyl moiety would then be subject to normal amino acid coupling techniques. Coupling of the imine **182** with the gramine derivative **170** in refluxing acetonitrile proceeded smoothly in 70% yield. Deprotection of the benzophenone imine was accomplished by stirring **217** with excess hydroxylamine in methylene chloride. Ensuing BOP reagent mediated coupling to N-BOC-3(S)-methyl-L-proline (**190**) provided **219** in excellent yields. Deprotection of the BOC group with TFA and cyclization in refluxing toluene with catalytic 2-hydroxypyridine supplied the diketopiperazine **220** in 90% yield. The lactim ether **221** was synthesized in 61% yield

with trimethyloxonium-tetrafluoroborate and K_2CO_3 in methylene chloride. The aza-diene **222** required for tautomerization and the intramolecular Diels-Alder reaction was formed by DDQ oxidation of **221** in 12 % yield. Again, the low yield obtained in this step would be problematic for isotopic labeling.

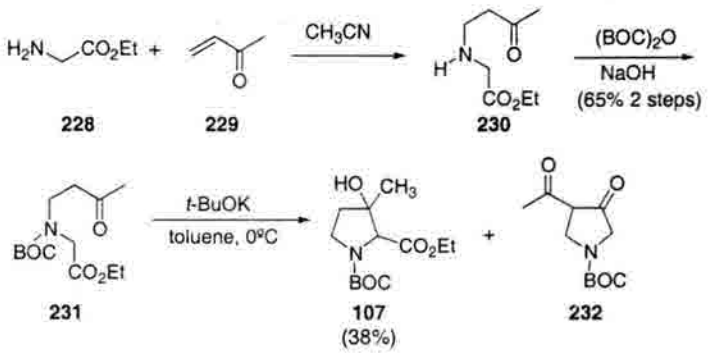
Scheme 29: Attempted synthesis of VM55599 from glycine



Introducing the correct oxidation state of the prolyl ring early in the synthesis obviated the low yield obtained in the oxidation of **221**. This was accomplished by using β-methyl-β-hydroxy proline (**107**), developed in early synthetic studies for β-methyl-proline (Table 4, Chapter 3). The synthesis of **107** was accomplished in three steps

(Scheme 30) by the Michael addition of glycine ethyl ester (**228**) and methylvinyl ketone (**229**). N-BOC-protection of **230** to give **231** and Dieckman cyclization provided the product **107.** The undesired regioisomer **232** is readily removed though base extraction.

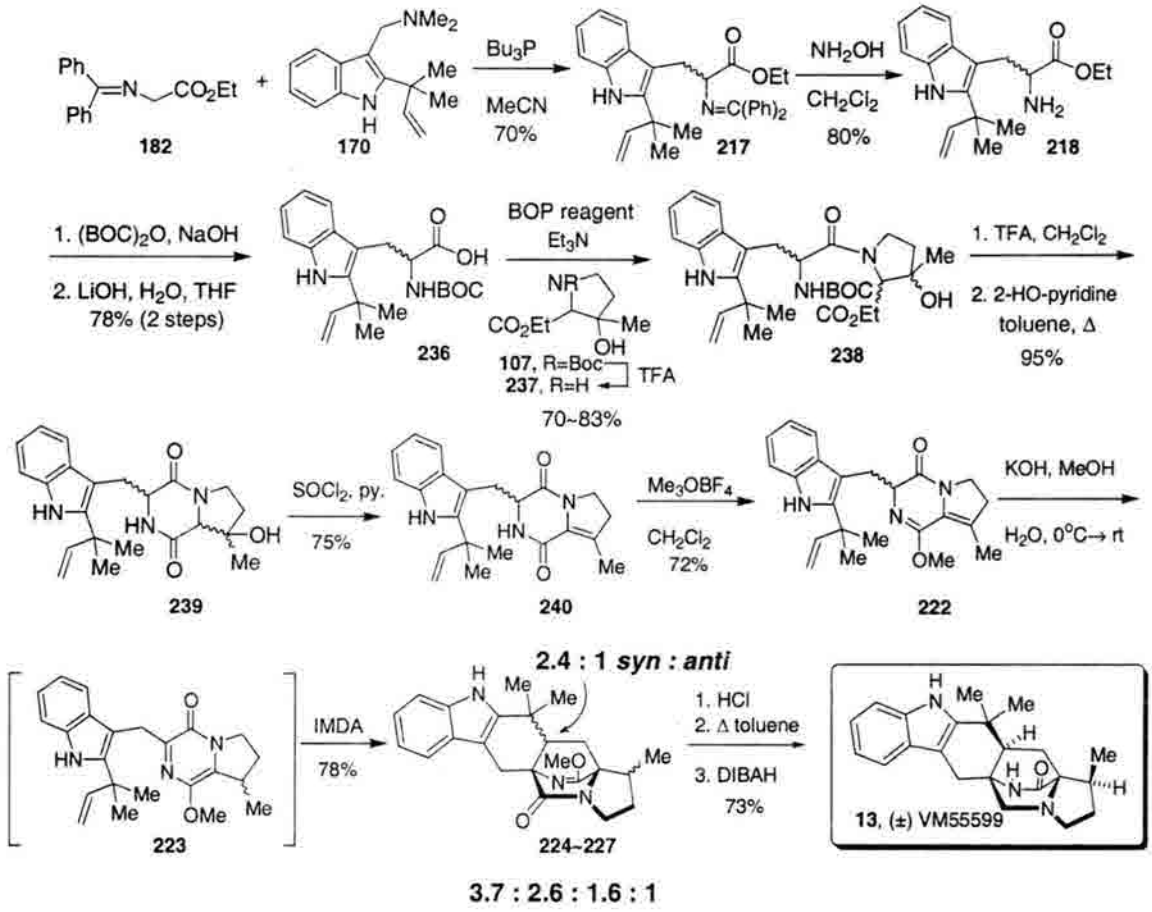
Scheme 30: Synthesis of β-hydroxy-β-methyl proline



The total synthesis of VM55599 is outlined below in Scheme 31. As previously shown, the benzophenone imine of glycine ethyl ester (**182**) was condensed with the dimethylallylated gramine derivative **170** in the presence of tri-*n*-butylphosphine in acetonitrile to furnish the tryptophan derivative **217**he amino ethyl ester **218** in high yield. At this point, a different protecting strategy needed to be employed since **107** was subject to a retro-aldol reaction under the basic conditions required to remove the ester functionality. For this reason, **218** was re-protected with a BOC group and the ethyl ester was removed by basic hydrolysis to furnish the acid **236** in 78% yield over two steps. BOP reagent mediated coupling of acid **236** with racemic β-methyl-β-hydroxyproline ethyl ester (**237**) provided the desired dipeptide **238** in 70~83% yield as a mixture of stereoisomers. The BOC groups were cleaved with TFA and the resulting amino ethyl

esters were cyclized to a mixture of the corresponding diastereomeric piperazinediones (**239**) in the presence of 2-hydroxypyridine in refluxing toluene in excellent yield.

Scheme 31: Total synthesis of (±)-VM55599



Treatment of **239** with thionyl chloride in pyridine furnished the unsaturated DKP **240** in 75% yield. Subsequent treatment of **240** with trimethyloxonium tetrafluoroborate in dichloromethane provided the azadiene **222** in 72% yield. The azadiene **222** was subjected to KOH in aqueous methanol to effect tautomerization to the labile incipient azadiene **223**. Spontaneous intramolecular Diels-Alder cycloaddition at room temperature

to afforded a mixture of all four possible racemic cycloadducts **224~227** in 78% combined yield in a 3.7: 2.6: 1.6: 1 ratio, respectively.

The Diels-Alder cycloadducts (**224~227**) were separable by PTLC on silica gel and their relative stereochemistry was assigned by extensive ^{1}H NMR nOe studies (Tables 11-14). The *syn*-stereochemistry at C-20 for **224** and **225** was assigned based on the nOe between H-20 (using the VM55599 numbering system) and the OMe of the lactim ether. The *anti*-stereochemistry assignment of C-20 for both **225** and **227** was made based on the nOe between H-23 and the OMe. The assignment of stereochemistry at C-14 for **443** and **445** was deduced from the nOe between H-17 and H-19/19′. Everett et al. also observed this nOe in the original VM55599 isolation paper. For **444** and **446**, the stereochemical assignment of C-14 was inferred from the nOe between H-14 and H-19/19′.

Table 11: nOe data for compound **224** (precursor to VM55599)

Proton	**nOe (%)**
N-1H	4(2.7), 22(1.1), 23(0.8)
4	N-1(0.8), 7(0.3),
10′	7(1.7), 10(20.2), 22(0.2), 23(1.71)
10	7(1.0), 10'(21.2), 24(0.1), 20(1.71)
14	14(3.2), 15(16.4), 16'(1.4), 17(0.9), 24(0.2)
15′	14(1.5), 15'(38.7), 16(1.0), 16'(3.0), 17(1.8), 20(4.1), 23(0.5)
15, 19, 19′	14(1.5), 15'(38.7), 16(7.4), 16'(0.3), 17(1.8), 20(4.1), 23(0.5)
16′	4(0.2), 14(1.2), 15'(2.8), 16(15.6)
16	4(0.3), 15'(1.0), 15(2.9), 23(0.4)
17	N-1(5.4), 24(0.2)
20	10(1.3), 19, 19'(3.0), 22(1.0, 23(0.1), 24(0.1)
22	14(7.7), 15' (1.6), 15, 19, 19'(13.7), 20(6.2), 23(1.2)
23	N-1(2.3), 16(1.3), 19,19'(7.7), 20(0.5), 22(1.7)
24(OMe)	---

Table 12: nOe data for compound **225**

Proton	nOe (%)
N-1H	4(1.6), 22(1.1), 23(0.5)
4	N-1(1.3), 5(3.0)
10′	7(1.5), 10, (19.6),
10	7(1.5), 19(20.2), 20(1.7), 24(0.1)
14	15'(1.9), 16'(1.1), 19(3.8), 22(1.2)
15′, 19′	14(3.9), 15(17.9), 16(0.7), 16'(3.7), 17(0.8), 19(16.4), 22(0.3)
15, 19	14(2.3), 16(1.7), 16'(0.3), 17(0.2), 19'(16.0), 20(0.8), 22(0.4), 23(0.8)
16'	10'(0.2), 14(5.7), 15'(3.0), 15(3.9), 16(0.7)
16	15(0.7), 16'(16.0), 17(0.2), 19(3.4)
17	14(2.3), 15'(3.0), 15(3.9), 16(0.7), 24(0.3)
20	10(1.3), 19'(3.5), 19(0.6), 22(1.0), 23(0.1), 24(0.04)
22	N-1(5.6), 19'(2.2), 19(4.8), 20(6.0), 23(0.5)
23	N-1(2.7), 16(2.1), 19(8.8), 20(1.0), 22(2.2)
24(OMe)	17(0.3)

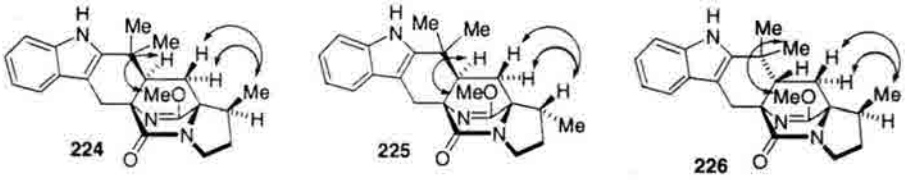
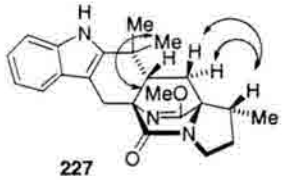


Table 13: nOe data for compound **226**

Proton	nOe (%)
N-1H	--
4	N-1(1.5)
10′	7(1.6), 10'(13.3), 16'(15), 20(0.5)
10	7(0.7), 10'(17.0), 20(1.4)
14	24(0.01), 15'(1.8), 23(0.1)
15′	16'(0.9), 16(1.4), 14(2.9), 15(14.9), 17(0.2)
15	15'(15.8), 16(1.4), 16'(4.0), 17(1.3)
16′, 24(OMe)	15'(0.4), 16(18.2), 17(0.4), 19'(0.8), 19(3.6), 22(0.7)
16	10(1.8), 14(1.2), 15'(2.5), 16'(13.4), 19(0.3),
17	15'(0.1), 16(4.3), 19(1.7), 19'(5.0)
19′	17(0.6), 19(15.4), 20(5.9), 22(0.3)
19	20(1.0), 19'(19.1), 22(1.0), 17(1.2), 23(2.2)
20	10(0.7), 19'(2.9), 19(0.4), 22(1.1)
22	N-1(4.8), 19'(0.7), 19(1.9), 20(4.6), 22(0.9)
23	N-1(12.3), 19(7.2), 22(1.1), 24(0.6)

Table 14: nOe data for compound **227**

Proton	nOe (%)
N-1H	---
4	5(1.0)
10a	7(1.2), 10'(18.0)
10b	7(0.5), 10(15.1), 20(1.3)
14	10'(0.9), 17(0.3), 19'(1.0), 19(2.6), 23(0.8)
15'	14(2.1), 15(16.2), 16'(2.1), 17(0.3)
15	15'(11.9), 16(1.9), 16'(1.3), 17(0.3)
16'	15'(1.0), 16(13.8)
16	15'(0.8), 15(1.5), 16'(11.9), 17(0.1)
17	14(4.9), 15'(1.6), 15(0.9), 24(0.3)
19'	14(4.6), 19(7.9), 20(5.8), 23(0.3)
19	19'(13.3), 20(8.3)
20	16'(0.8), 16(0.2), 19'(1.4), 19(4.0), 22(0.9)
22	N-1(3.2), 20(4.5), 19'(2.1), 23(0.9)
23	N-1(2.7), 19'(10.6), 22(0.9), 24(1.2)
24(OMe)	23(0.4)

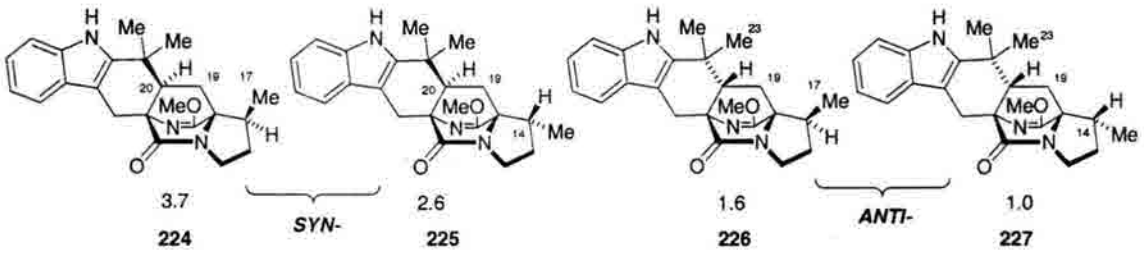


227

The structures of all four cycloadducts (**224~227**) depicting their relative stereochemistries are shown in Figure 35. The *syn-*: *anti-* relationship at the C-20 stereogenic center was 2.4: 1 and is consistent with the results reported on the model system that lacked the methyl group in the proline ring.**Error! Bookmark not defined.** Of interest, was the unexpected observation that the major products (**224** and **226**) in each diastereomeric subset displayed the methyl group in the β-methylproline ring *syn-* to the bridging isoprene unit (see Figure 35). The diastereoselectivity in this regard was 1.47: 1 favoring the methyl group disposed *syn-* to the bridging isoprene moiety. Although it is

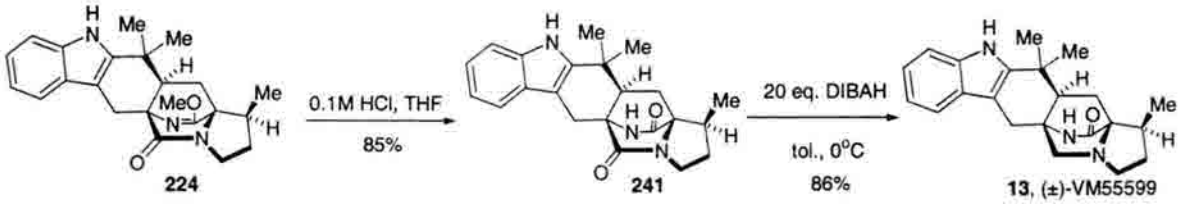
reasonable to expect modest diastereoselectivity for this Diels- Alder cycloaddition, based on the slight steric bias that might be exerted by the methyl group in the proline ring, we anticipated a modest preference for cycloadducts that displayed the methyl group *anti-* to the bridging isoprene moiety.

Figure 35: Structures/ diastereomeric ratios of cycloaddtion products



Cycloadduct **224** was converted into racemic VM55599 as depicted in scheme 33. Treatment of **224** with dilute HCl effected cleavage of the lactim ether to the corresponding secondary amide **241** in 85% yield. Selective reduction of **241** with excess DIBAH[210] (20 equivalents) provided VM55599 in 86% yield whose ^{1}H and ^{13}C NMR spectra matched the published data. The synthetic material was consequently utilized to guide re-isolation of natural VM55599 from cultures of *Penicillium* sp. IMI332995 (obtained from the International Mycological Institute). Only 0.4 mg of VM55599 was isolated from 12L of solid culture (Czapek DOX plates), while 400 mg of paraherquamide was isolated from the same culture. The synthetic and natural specimens were found to have identical ^{1}H NMR spectra and TLC mobility, thereby confirming the assignment (Figure 36).

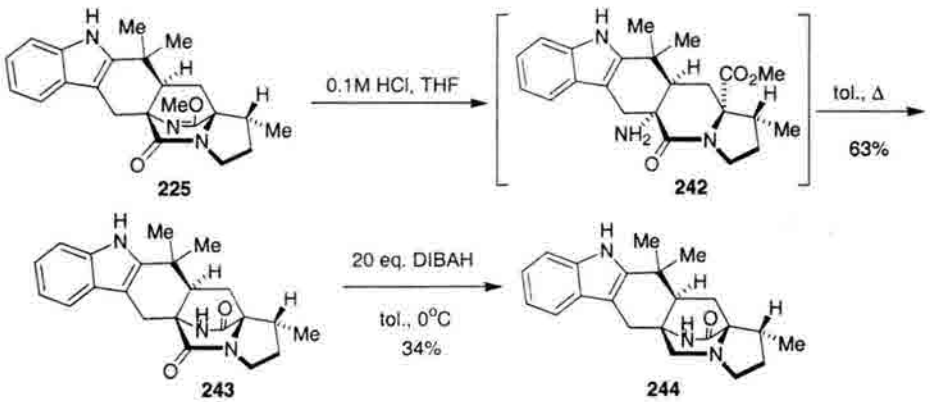
Scheme 33: Conversion of cycloadduct **224** to (±) VM55599



224 **241** **13**, (±)-VM55599

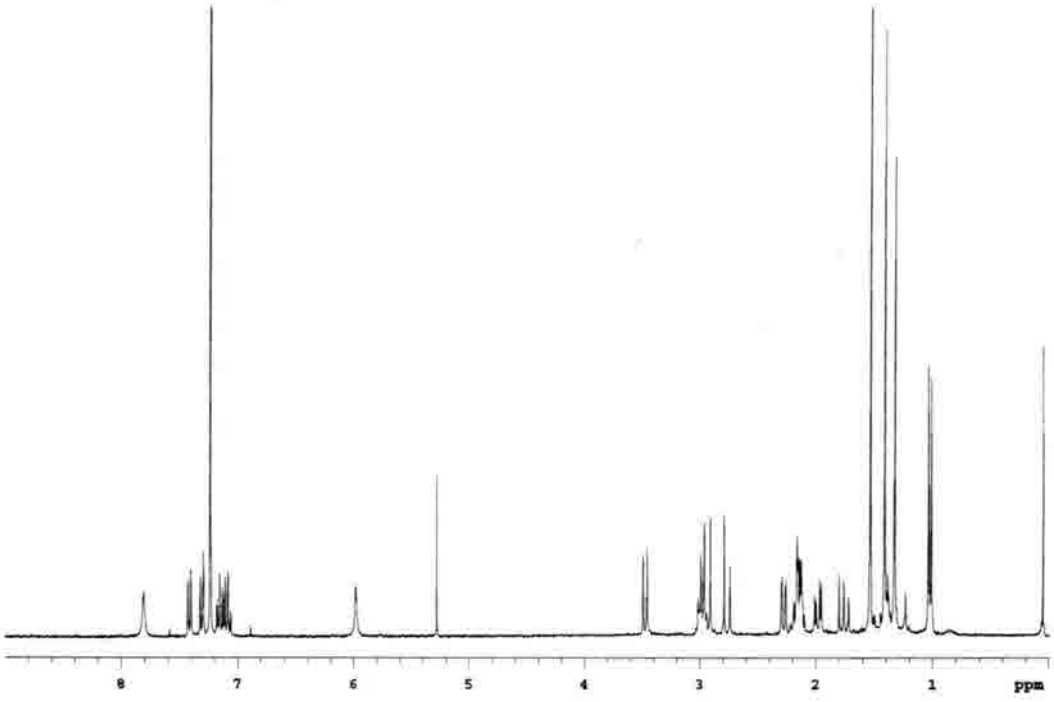
To further confirm the structural assignment of VM55599, the three other cycloadducts **225~227** were similarly converted into the corresponding C-14 and/or C-20 epimers of VM55599 (**244**, **246** and **249**) as shown in Schemes 34~36.[211] It was interesting to observe that, in the case of cycloadducts **225** and **227**, cleavage of the lactim ether with dilute HCl led to the production of the ring-opened amino esters **242** and **247**, respectively. These were readily cyclized to the corresponding bicyclo[2.2.2]-containing secondary amides **243** and **248**, respectively, by simply heating these substances in toluene at reflux temperature overnight.

Scheme 34: Conversion of cycloadduct **225** to a VM55599 diastereomer



225 **242** **243** **244**

a)



b)

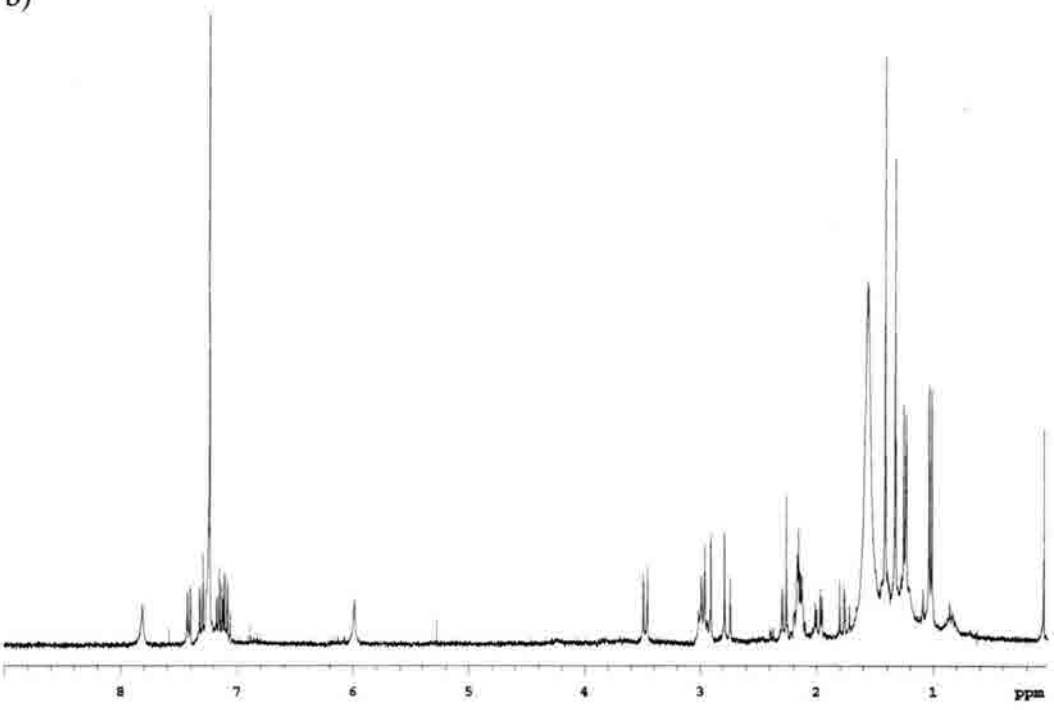
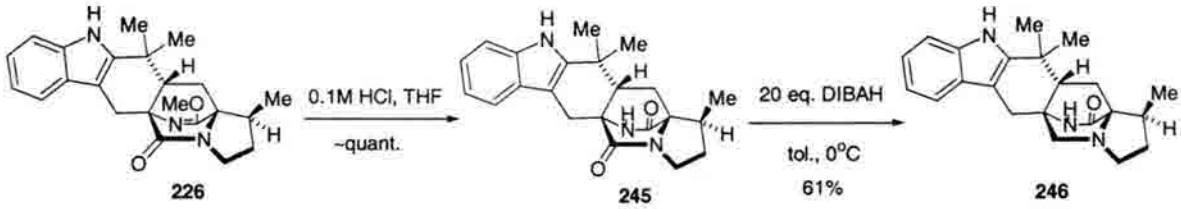


Figure 36: ^{1}H NMR spectra (300 MHz) in $CDCl_3$ of a) Synthetic VM55599, b) Natural VM55599.

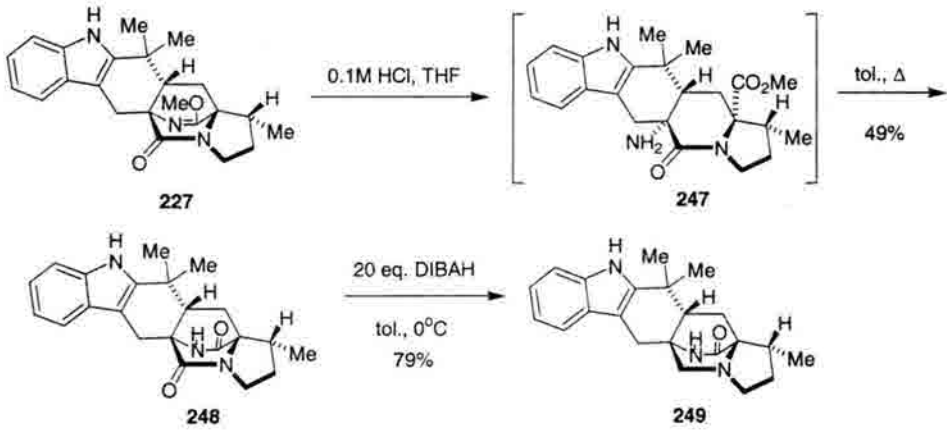
Scheme 35: Conversion of cycloadduct **226** to a VM55599 diastereomer



226 **245** **246**

In contrast, the lactim ethers of both cycloadducts **224** and **226** could be cleaved to the corresponding bicyclo[2.2.2]-containing substrates without attendant ring-opening to the corresponding amino esters. It would appear that there is $A^{(1,3)}$-type strain in compounds **225** and **227** caused by compression between the methyl group disposed on the β-face of the proline ring and the lactim ether methoxy group that is relieved upon ring-opening to **242** and **247**, respectively. In substrates **224** and **226**, where the methyl group in the proline ring is on the α-face, the opportunity for $A^{(1,3)}$-type strain is obviated by the *anti*-relationship between the lactim ether group and the methyl group.

Scheme 36: Conversion of cycloadduct **227** to a VM55599 diastereomer



227 **247**

248 **249**

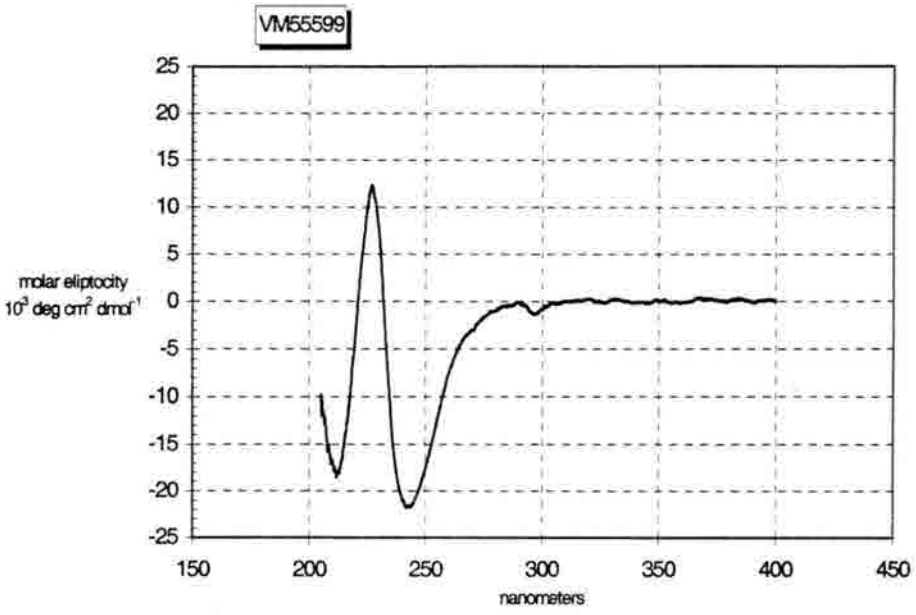
DIBAH reduction of the tertiary amides of compounds **243**, **245** and **248** gave the corresponding diastereomers of VM55599 (**244**, **246** and **249**, respectively). The NMR spectra of the VM55599 diastereomers **244**, **246** and **249** were fully consistent with the assigned structures and significantly, all were distinctly different from the spectra for natural VM55599.

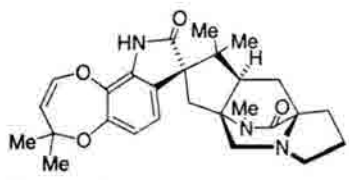
5.3 Determination of the absolute stereochemistry of VM55599

NOe data on the synthetic racemic VM55599 analog (**224**) only provided information regarding the relative configuration of VM55599. To ascertain whether the absolute configuration of the bicyclo[2.2.2]diazaoctan portion or C-14 of the prolyl ring of VM55599 is enantiomorphic to paraherquamide A, the absolute stereochemistry needs to be determined. It was hoped that a CD spectrum of the natural VM555599 would help solve the absolute stereochemistry (Figure 54).[212] The CD spectrum of natural VM55599 is similar to the CD spectrum of synthetic (+)-paraherquamide B (**38**)[24], which possesses a bicyclo[2.2.2]diazaoctan ring system that is enantiomorphic to natural paraherquamide A (**1**). However, because of the difference in chromophores, i.e. indole versus oxindole, the absolute stereochemistry cannot be assigned on this data alone.

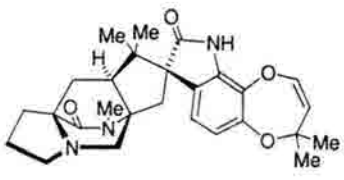
A definitive assignment of the absolute stereochemistry of VM55599 could be made from an X-ray structure of the appropriate derivative, either a heavy atom derivative or a derivative possessing known stereochemistry. Since only a small amount of natural VM55599 was isolated from cultures, derivatives needed to be made from the synthetic material. The enantiomers of synthetic (±)-VM55599 were separated on a Regis (R, R) Whelk-O2 Kromasil column with an elutant of 7.5% *i*-PrOH/Hexanes. Two

Figure 54: CD spectra of VM55599 and paraherquamide B

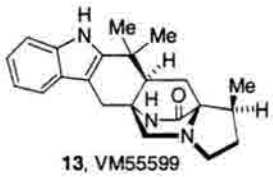




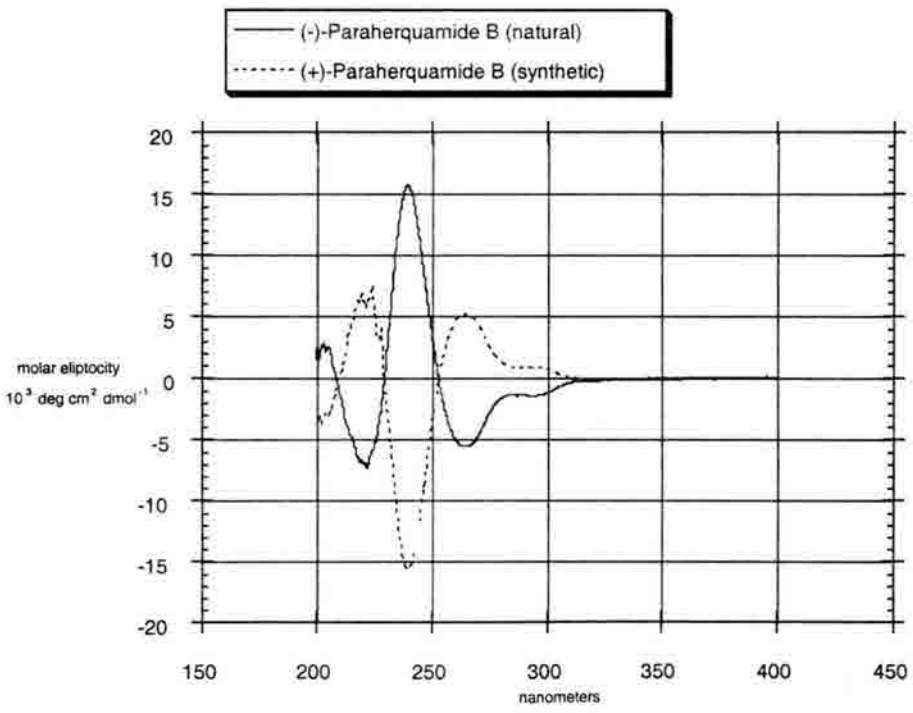
38, synthetic (+)-paraherquamide B



2, natural (-)-paraherquamide B

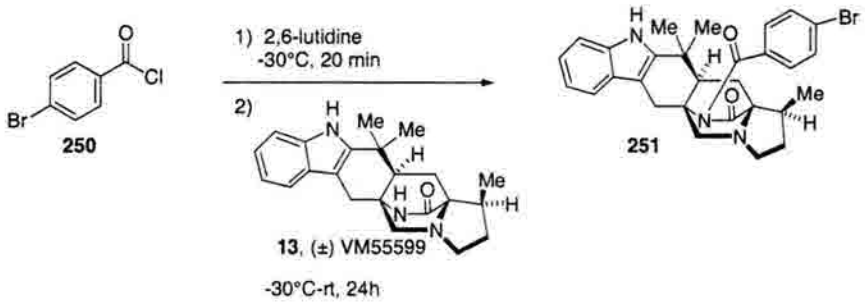


13, VM55599



peaks were separated at 37 minutes and at 54 minutes. The peak at 37 minutes was identified as natural VM55599 by comparison of retention time and co-elution with the natural product.

Scheme 37: Synthesis of brominated analog of VM55599



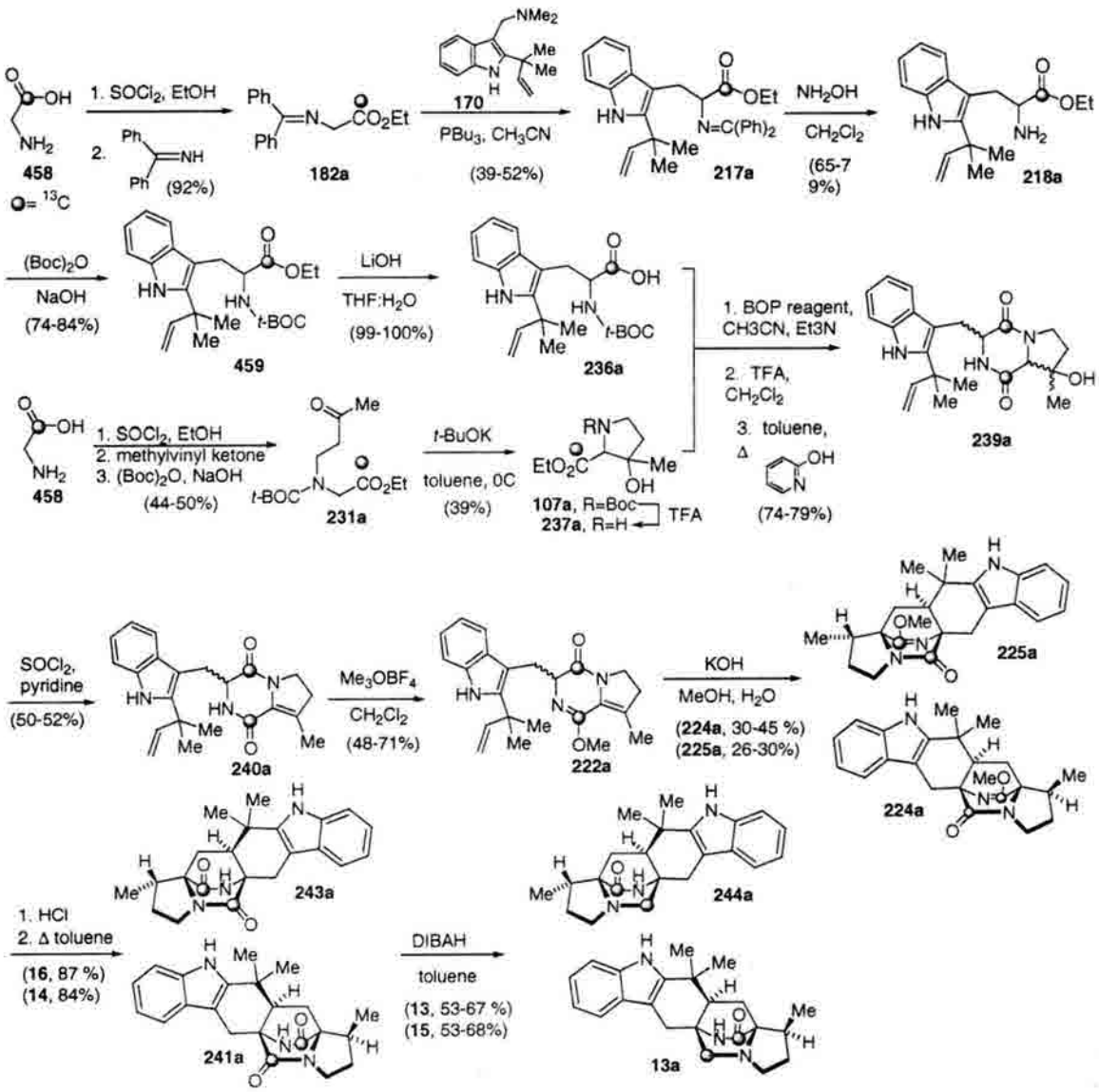
Several different attempts were made to derivatize VM55599. N-Acylation with camphanyl chloride and NaH was unsuccessful, most likely because of steric hinderance. However N-acylation with *p*-bromo-benzoylchloride (**250**) to make the brominated-derivative (**251**) was accomplished as outlined in Scheme 37.[213] Unfortunately, all attempts to crystallize this compound were futile.

5.4 Synthesis/ feeding experiments of ^{13}C- VM55599 and analogs

To determine if VM55599 (**13**) is an intermediate in the biosynthesis of paraherquamide or if it is a shunt metabolite, a feeding experiment with the ^{13}C-labeled (±)-VM55599 (**13a**) was completed. The oxidized form of $^{13}C_2$-VM55599 (**241a**) as well as the alleged ^{13}C-labeled paraherquamide progenitors (**243a** and **244a**) were also examined as potential pathway metabolites in *Penicillium fellutanum*. The ^{13}C-labeled compounds were synthesized according to our previously described total synthesis of

racemic VM55599 (Scheme 38). The advantage of this synthesis is that all four candidate precursors are accessible from a single synthesis. In addition, both of the ^{13}C-labels in the product are derived from relatively inexpensive ^{13}C-glycine.

Scheme 38: Synthesis of ^{13}C-labeled VM55599 and analogs for feeding experiments with *P. fellutanum*



Because the racemic cycloadducts (**13a**, **241a**, **243a**, and **244a**) possess the unfortunate property of being insoluble in water, the detergent TWEEN 80 was used to increase the solubility of these substances in the culture broth. Feeding experiments were performed on *P. fellutanum* (ATCC:20841) using all four potential precursors followed by isolation and purification of paraherquamide A. Within the limits of detection by ^{13}C NMR and mass spec, no incorporation was observed for VM55599 ((±)-**13a**) or its oxidized counterpart (±)-**241a**. In addition, no incorporation was observed for the diketopiperazine (±) **243a**.

However, for the C-14 epimer of VM55599 (±)-**244a**, significant incorporation was observed by ^{13}C NMR at C-12 (0.72%) and C-18 (0.87%) of paraherquamide A. From analysis of the electrospray mass spectrum, incorporation was determined to be 0.72% for the intact doubly labeled material. Although the incorporation levels at C-12 and C-18 were unequal by ^{13}C-NMR, no mono-^{13}C-labeled paraherquamide A, from catabolism of (±)-**244a**, was detected in the mass spectrum. The slight incorporation differences observed at C12 and C18 are probably due to an error from integration of the ^{13}C-NMR peaks, since the C18 peak was broadened (Figure 38).

The lack of incorporation of the diketopiperazine (±)-**243a** raises interesting questions concerning the timing of the reduction of prolyl-derived carbonyl group. The incorporation of compound (±)-**244a** in significant isotopic yield, indicates that the formation of the bicyclo[2.2.2] diazaoctan occurs at the stage of the non-oxidized tryptophanyl moiety (i.e., indolyl). This requires oxidations of the indole ring to form both the catechol-derived dioxepin and spirooxindole to occur *after* the formation of this intermediate. It appears that the dioxepin-derived isoprenylation and the S-

adenosylmethionine-mediated N-methylation reactions occur late in the pathway. These results also cast considerable doubt on the intermediacy of VM55599 and its oxidized precursor, as being intermediates in paraherquamide biosynthesis and provide additional circumstantial evidence that VM55599 is a minor shunt metabolite. Finally, these results document, for the first time, the intermediacy of an advanced metabolite **244a** containing the core structural elements of the paraherquamide framework prior to a series of oxygenation reactions.

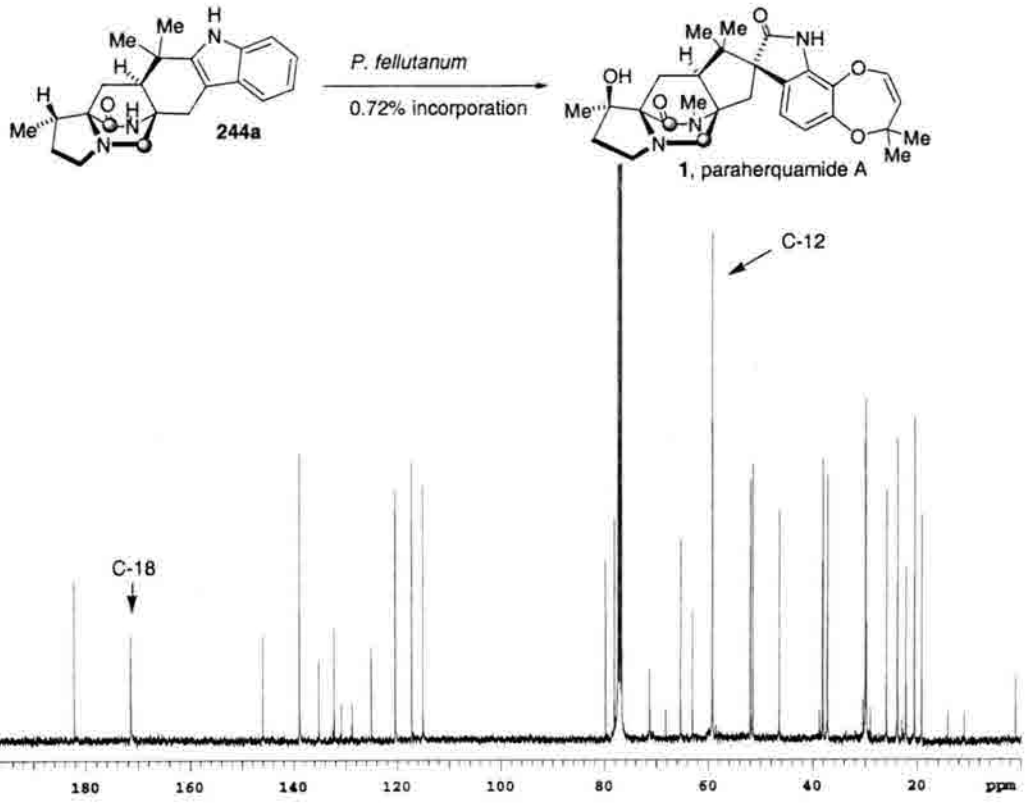


Figure 38: ^{13}C-NMR from feeding experiment with doubly labeled cycloadduct **244a**

CHAPTER 6

Conclusion:

An Overview of the Biosynthesis of Paraherquamide A

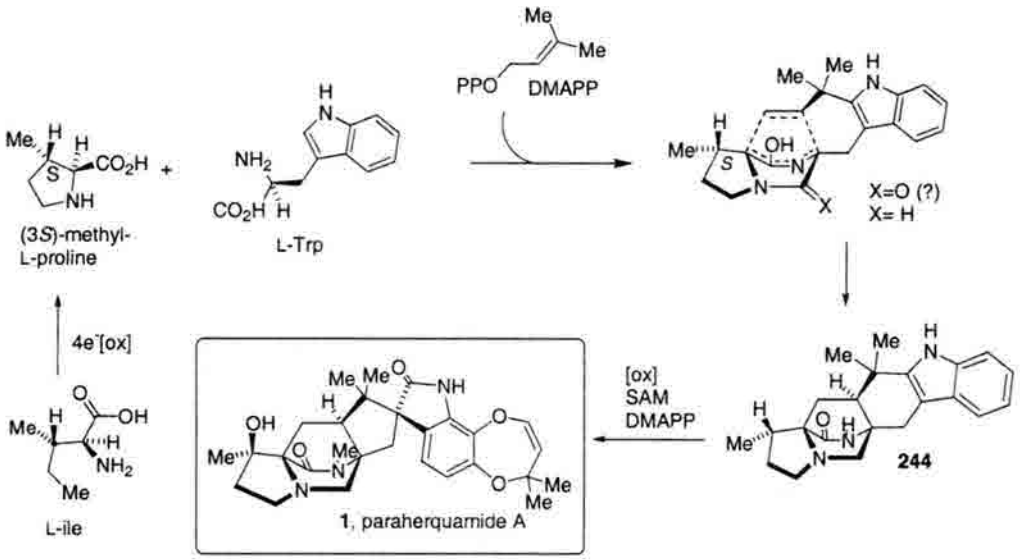
6.1 Known Aspects of Paraherquamide A Biosynthesis

This work has revealed the origin of the carbon atoms of paraherquamide A. The proteinogenic amino acids, which give rise to paraherquamide, were determined to be L-methionine, L-tryptophan and L-isoleucine. L-isoleucine was found to be the source for the unusual non-proteinogenic amino acid, β-methyl-β-hydroxy-proline. Feeding experiments with [5-^{13}C,5-^{2}H$_3$]-L-isoleucine indicate that the L-isoleucine is converted to β-methyl-β-hydroxy-proline via a 4-electron oxidation with retention of the *pro-S* hydrogen at C-5. It was also determined that L-isoleucine is converted to β-methyl-proline before it is coupled to tryptophan or a trytophanyl derivative.

The mevalonate origin of the isoprene moieties (C-19~C-23 and C-24~C-28) of paraherquamide A was also uncovered, along with an unusual finding. It was discovered that *P. fellutanum* constructs each quaternary center derived from dimethylallyl pyrophosphate by distict mechanisms: the quaternary center in the dioxepin ring is formed in a completely stereospecific manner and the quaternary center in the bicyclo[2.2.2]diazaoctan is formed in an entirely non-stereospecific manner. It is believed that a "reverse" prenylase enzyme, which is thought to be antithetic to a normal prenylase enzyme, may catalyze the attachment of the "reverse" prenyl group. Unfortunately, no reverse prenylated intermediates that precede the Diels-Alder reaction in the biosynthesis of paraherquamide A were identified.

Lastly, the hexacyclic intermediate **244** was established as a precursor to paraherquamide A. This intermediate may be the result of a hetero-Diels-Alder cycloaddition, although further studies need to be done to confirm this. The incorporation of **244** indicates that the first equivalent of DMAPP is used in the formation of the bicyclo[2.2.2]diazaoctan. This result also indicates that the bicyclo[2.2.2] dizaoctan ring system is formed before oxidation of the indole ring, leading to the catechol-derived dioxepin and the spirooxindole. Finally, the incorporation of **461** indicates that oxidation of the proline ring and N-methylation also occur *after* formation of the bicyclo ring system. The complete sequence of events, as they are now known, is depicted in Scheme 39.

Scheme 39: Biogenesis of paraherquamide A.



CHAPTER 7

Experimental

7.1 General synthetic considerations

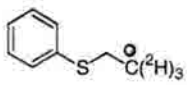
^{13}C-labeled and ^{13}C, ^{15}N-labeled Oppolzer's glycine template and $^{13}CD_3$-iodomethane were obtained from the NIH Stable Isotopes Resource at Los Alamos National Laboratory. All other reagents were commercial grade and used without purification unless otherwise noted. ^1H NMR spectra and ^{13}C NMR spectra were obtained on a Bruker AC300P 300 MHz NMR spectrometer, a Mercury 300 or Inova 400 Varian NMR spectrometer at the Colorado State University Chemistry Department. NMR spectra were taken in $CDCl_3$ (^1H, 7.24 ppm; ^{13}C 77.0 ppm), CD_3OD (^1H, 4.87 ppm, ^{13}C, 49.15 ppm), d_6-DMSO (^1H, 2.50 ppm, ^{13}C, 39.51 ppm) and D_2O (^1H, 4.8 ppm) obtained from Cambridge Isotope Labs. Electrospray mass spectra were obtained using a FisonsVG AutoSpec. Exact masses were obtained using a VG Quattro-SQ. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR spectrometer. EM Science Silica Gel 60 was used for column chromatography and PTLC were performed with EM Science precoated TLC plates (Kiselgel 60, F_{254}, 0.25 mm).

7.2 Chemical Synthesis Experimentals

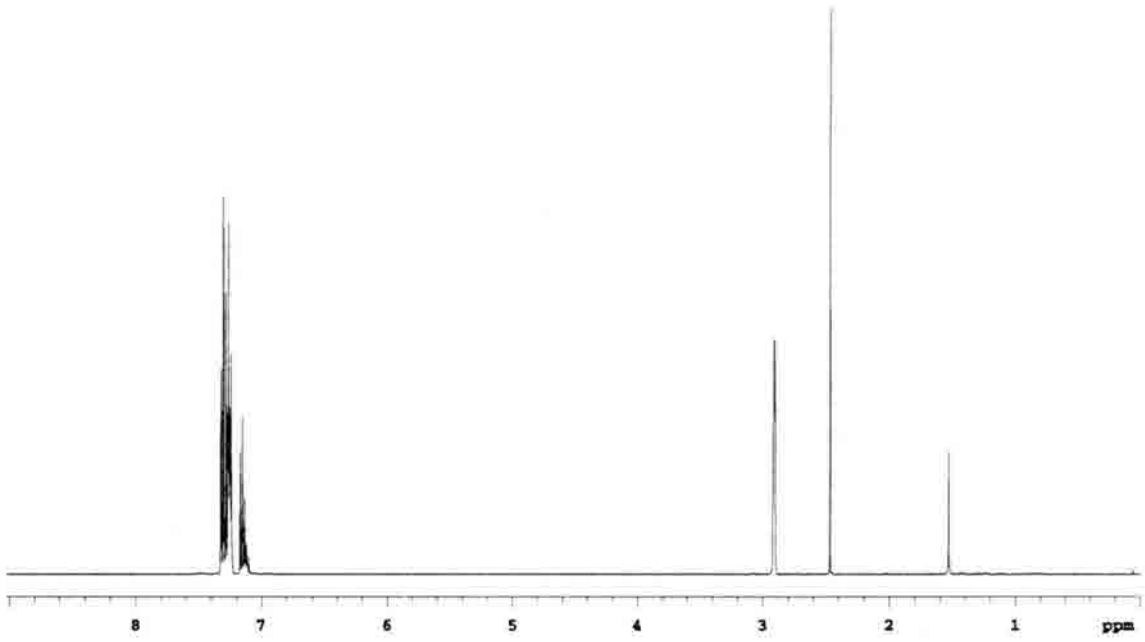
[2-$^{13}C^{2}H_3$]-ethylphenylsulfide (95):

To a stirring solution of thioanisole (4.2 mL, 36.1 mmol) in THF at 0°C, 19.18 mL of a 2.5 M solution on n-BuLi was added dropwise. The resultant mixture was slowly brought to room temperature and stirred for 12h at which time the mixture was again cooled to 0°C and $^{13}C^{2}H_3$-methyl iodide (4.59 g, 39.7 mmol) was added dropwise to the reaction. The reaction mixture was slowly brought to room temperature and stirred for 4h. A saturated solution of $NH_4Cl_{(aq)}$ was added and the mixture was extracted three times with EtOAc. The combined organic extracts were washed with saturated $NH_4Cl_{(aq)}$, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The remaining oil was distilled under reduced pressure to afford [2-$^{13}C^{2}H_3$]-ethylphenylsulfide as an oil. Yield: 3.28 g, 23.5 mmol, 65%.

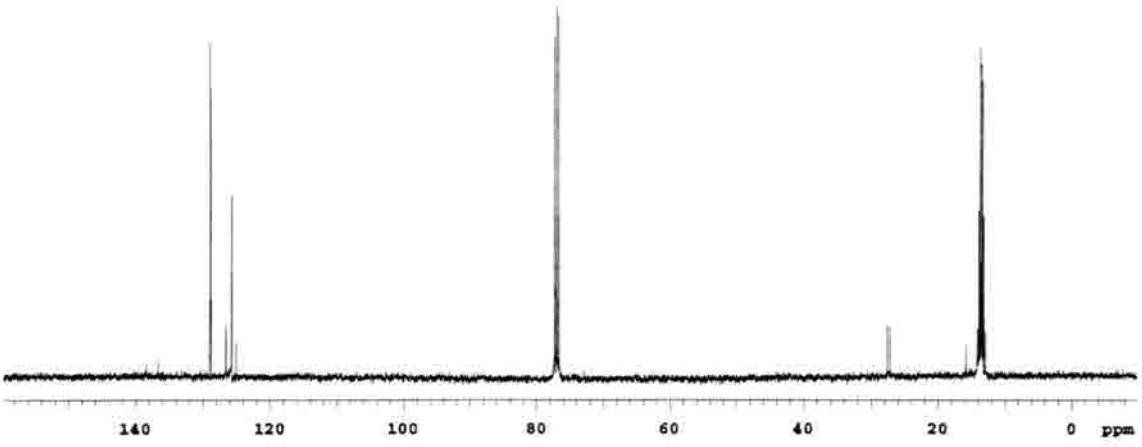
^{1}H NMR (400 MHz, $CDCl_3$): δ 2.91 (2H, s), 7.13-7.32 (5H, m). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.5 (septet, $J_{C-^{2}H}$= 20.0 Hz), 27.3 (d, J_{c-c}= 35.3 Hz), 125.7, 128.8, 129.0. HRMS (EI+): Calcd for $C_7{}^{13}C_1H_7{}^{2}H_3S_2$: 142.0725; found 142.0720 (M+).



95



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems1001_sm

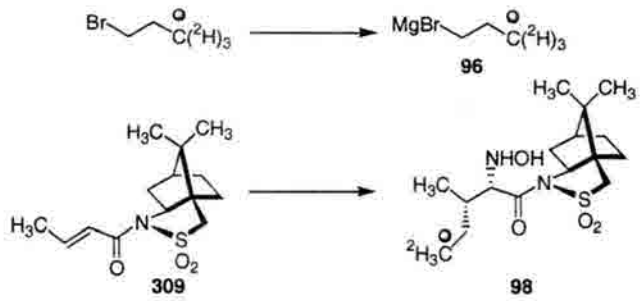


^{13}C NMR, 100 MHz, $CDCl_3$, filename: 1001_sm_13C

95

2-[$^{13}C^{2}H_3$]-bromoethane:

A mixture of [2-$^{13}C^{2}H_3$]-ethylphenylsulfide (810 mg, 5.69 mmol) and benzyl bromide (1.35 mL, 11.26 mmol) was heated to150°C and 2-[$^{13}C^{2}H_3$]-bromoethane was distilled off through a short path distillation apparatus over a period of 9 hours. The crude material was used immediately in the next reaction without further purification. Yield: 557 mg, 4.9 mmol, 88%.



96

309

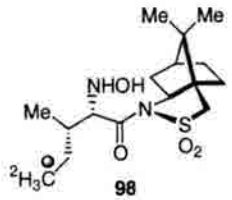
98

(2*S*, 2'*S*, 3'*S*)-N-[2'-(Hydroxyamino)-3'methyl-5'$^{13}C^{2}H_3$-pentanoyl] borane-10,2-sultam (98):

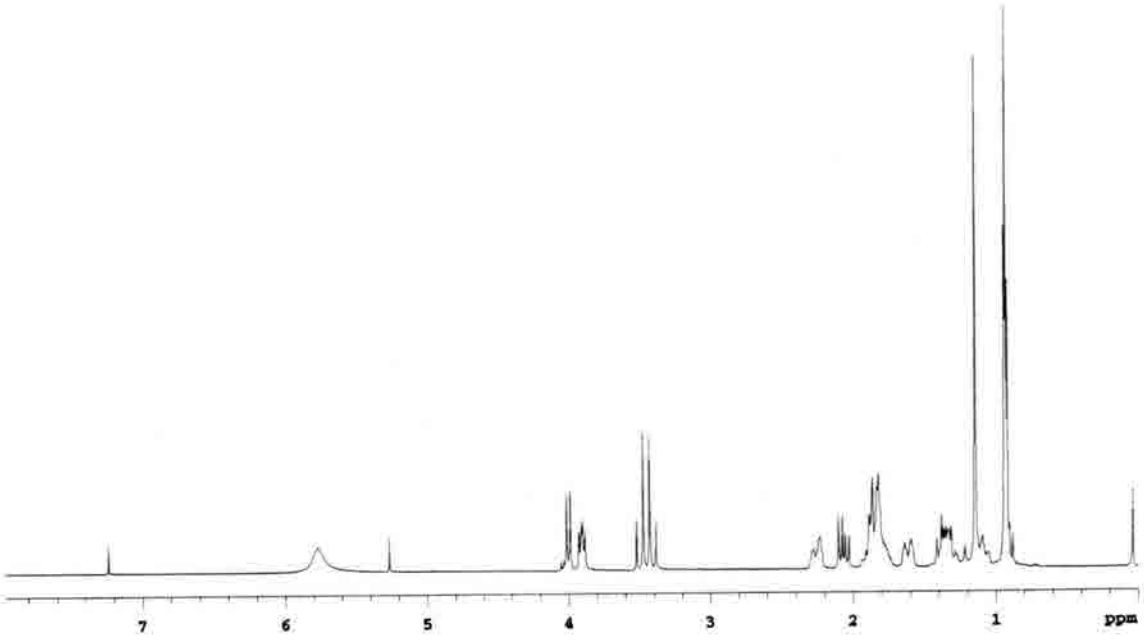
An ethereal solution (2 mL) of dibromoethane (61μL, 0.71 mmol) was added dropwise to Mg° turnings (105 mg, 4.32 mmol in a two neck flask fitted with a reflux condenser. When the bubbling subsided, an ethereal solution (1.2 mL) of 2-[$^{13}C^{2}H_3$]-bromoethane (370 mg, 3.27 mmol) was added dropwise. The mixture was allowed to react until bubbling subsided and most of the magnesium was consumed (~30 minutes). The Grignard reagent, **96**, was directly added via cannula to a 0.07 M solution of **97**[214] in THF at –78°C. The reaction was stirred at –78°C for a total of 36 h at which time a 1M THF

solution of 1-chloro,1-nitrosocyclohexane[215] was added dropwise. After stirring an additional 1.5 h, 1 M $HCl_{(aq)}$ was added to the reaction, the cooling bath was removed and stirring was continued for 30 minutes before removing the THF *in vacuo*. An additional 20 mL of 1M $HCl_{(aq)}$ was added and the mixture was extracted three times with hexanes. The aqueous layer was made basic (pH=10) by the addition of solid $NaHCO_3$ and extracted five times with dichloromethane. After drying over anhydrous Na_2SO_4 and evaporating *in vacuo*, the crude product (**25**) was purified by flash column chromatography using a 1:10 mixture of diethylether and dichloromethane. Yield: 124 mg, 0.355 mmol, 15%.

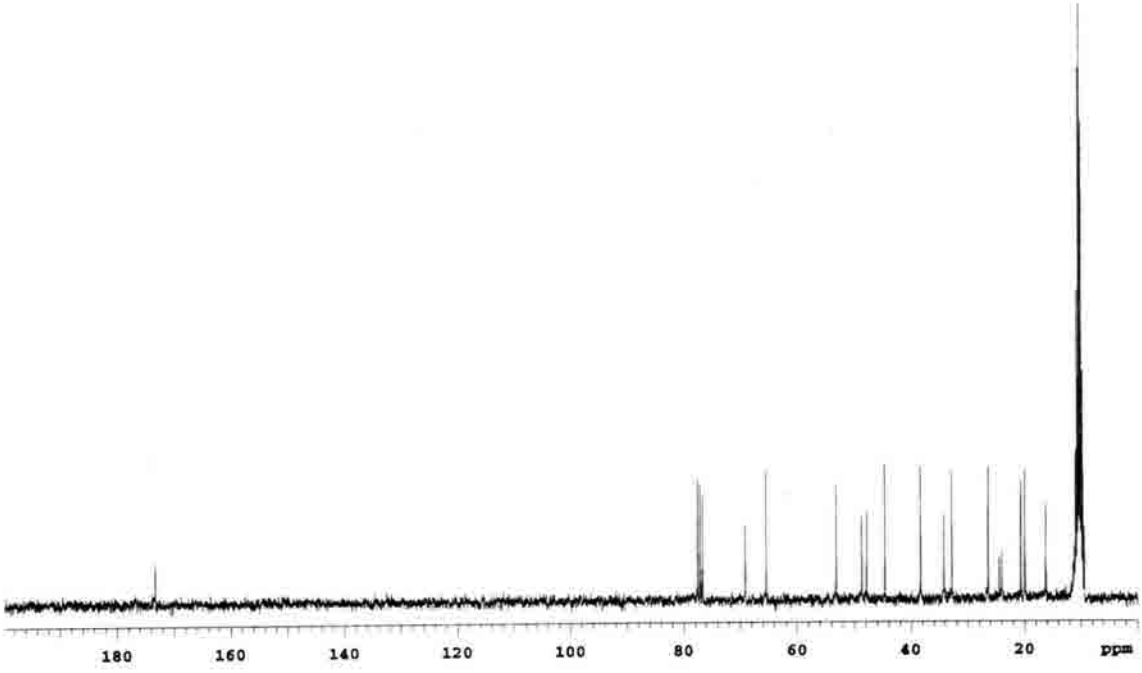
^{1}H NMR (300 MHz, $CDCl_3$): δ 0.92 (3 H, d, J = 7.0 Hz), 0.93 (3H, s), 1.10 (1H, m), 1.15 (3H, s), 1.36 (2 H, m), 1.62 (1H, d, J = 13.2 Hz), 1.90-1.76 (4H, m), 2.06 (1H, dd, J = 7.7, 13.9 Hz), 2.25 (1H, m), 3.41 (1H, d, J = 13.9 Hz), 3.50 (1H, d, J = 13.6 Hz), 3.91 (1H, dd, J = 4.7, 7.7 Hz), 4.00 (1H, d, J = 7.7 Hz), 5.77 (2H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 10.2 (septet, J_{C-^2H} = 18.8 Hz), 16.2, 19.6, 20.6, 24.2 (d, J_{C-C} = 34.9 Hz), 26.4, 32.8, 34.2, 38.3, 44.6, 47.8, 48.6, 53.1, 65.4, 69.0 (d, J_{C-C} =4.0 Hz). HRMS (FAB+): Calcd for $C_{15}{}^{13}C_1H_{26}{}^2H_3N_2O_4S_1$: 349.2054; found 349.2056 (M+H).



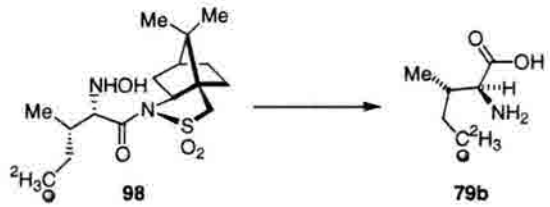
98



^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems1002_2



^{13}C NMR, 75 MHz, $CDCl_3$, filename: 1002_2_13C

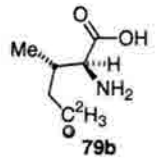


L-[5-^{13}C,5-$^{2}H_3$]isoleucine (**79b**):

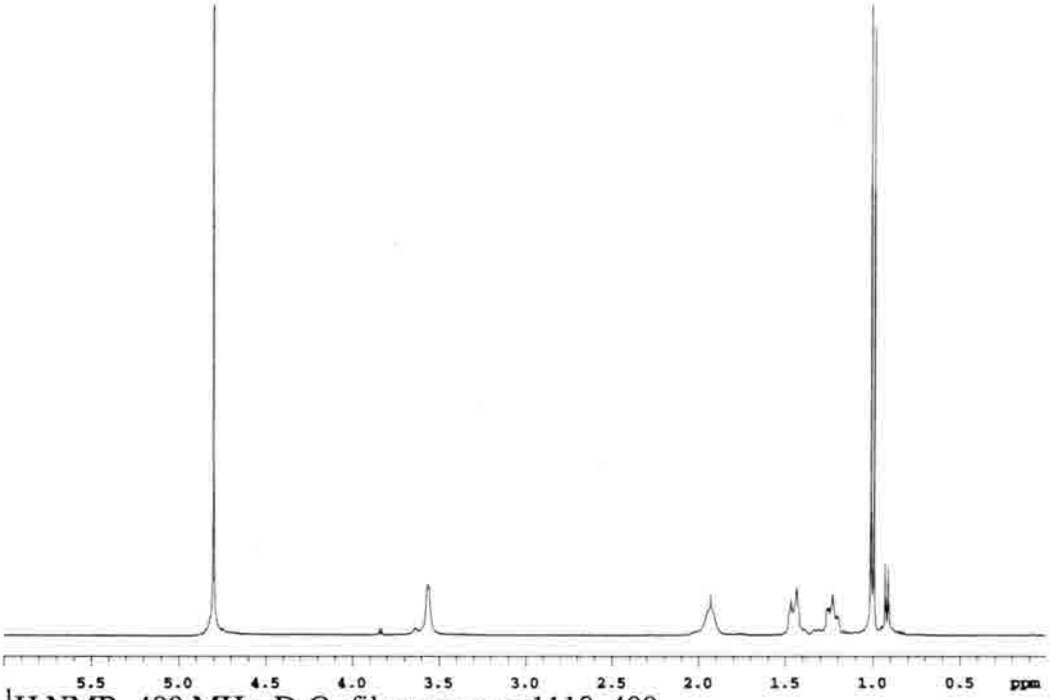
A mixture of **98** (119 mg, 0.34 mmol) and activated Zn° powder (887 mg) was stirred in a 2:1 solution of HCl: AcOH for 2 days at 0°C. The mixture then was filtered through glass wool, the solvent removed *in vacuo* and the residue made basic by the addition of aqueous $NaHCO_3$. The aqueous solution was extracted three times with dichloromethane. Drying over anhydrous Na_2SO_4 and concentration *in vacuo* afforded (2*S*, 2'*S*, 3'*S*)-N-[2'-amino-3'methyl-5'$^{13}C^{2}H_3$-pentanoyl] borane-10,2-sultam as a solid (102 mg, 0.31 mmol, 90%). Without further purification, (2*S*, 2'*S*, 3'*S*)-N-[2'-amino-3'methyl-5'$^{13}C^{2}H_3$-pentanoyl] borane-10,2-sultam (82 mg, 0.247 mmol) was saponified by stirring in a 1:4 mixture of 1N LiOH in THF (2.5 mL) at room temperature over 24 hours. The THF was removed under reduced pressure and the residue was partitioned between dichloromethane and water. The aqueous layer was brought to pH=7, evaporated to dryness and re-suspended in de-ionized water before loading onto a DOWEX (50W X2-100) column. The column was rinsed with several portions of water (until the wash remained clear upon the addition of $AgNO_3$) and then was eluted with 2% $NH_4OH_{(aq)}$. Removal of most of the $NH_4OH_{(aq)}$ *in vacuo* followed by lyophilization provided **297** as a colorless white solid.[216] Yield: 28.5mg, 0.21 mmol, 85%.

^{1}H NMR (400 MHz, D_2O): 1.00 (3H, d, *J*= 7.0 Hz), 1.23 (1H, m), 1.45 (1H, m), 1.93 (1H, bs), 3.56 (1H, bs). ^{13}C NMR (400 MHz, D_2O): 10.4 (septet, $J_{C,^2H}$ = 18.5 Hz), 15.0,

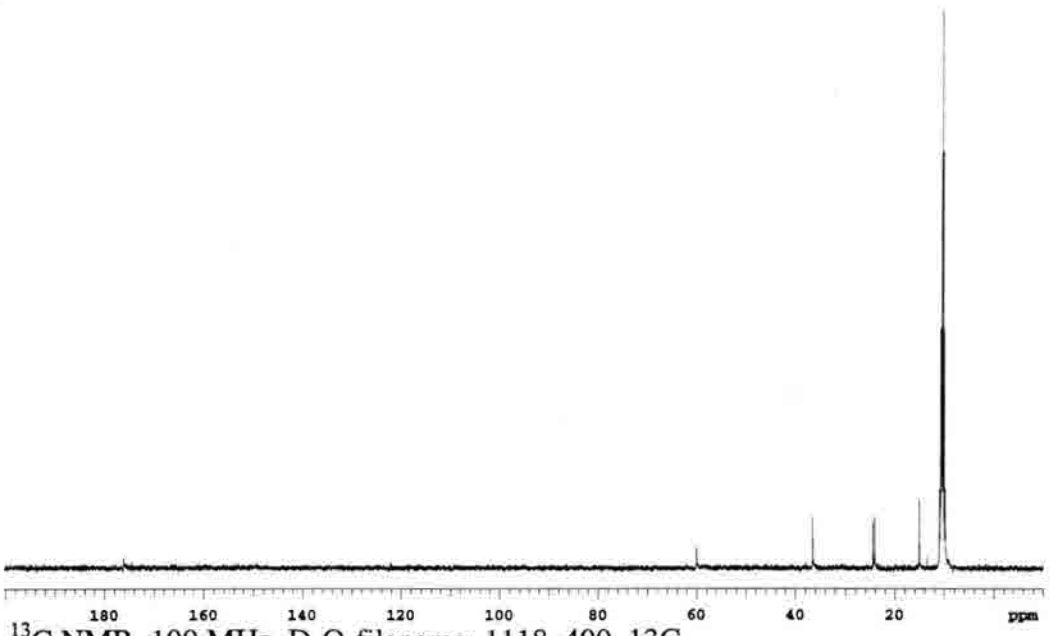
24.2 (d, $J_{C\text{-}C}$ = 35 Hz), 36.5, 60.0, 176.1. HRMS (FAB+): Calcd for $C_5{}^{13}C_1H_{11}{}^2H_3N_1O_2$: 136.1246. Found 136.1247 (M+H). $[\alpha]_D{}^{25}$= +11.82° (c=0.11, H_2O).



79b



^{1}H NMR, 400 MHz, D_2O, filename: ems1118_400

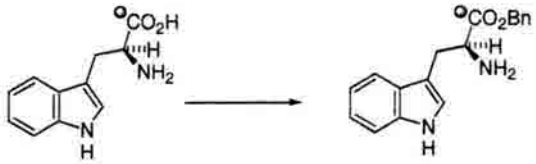


^{13}C NMR, 100 MHz, D_2O, filename: 1118_400_13C

N-Benzyloxycarbonyl-[1-^{13}C]-L-Isoleucine:

[1-^{13}C]-L-isoleucine (32.1 mg, 0.244 mmol) was dissolved in distilled, deionized (dd) H_2O (75 μL) and 5M NaOH (50 μL). The reaction mixture was cooled to 0°C while stirring. Benzylchloroformate (38.5 μL, 0.270 mmol) and 2M NaOH (135 μL) were added dropwise alternately under an argon atmosphere. The rate of addition was controlled to maintain a temperature between 0°C and 10°C. After addition was complete, the reaction was brought to room temperature slowly and stirred for an additional 2 h. The reaction mixture was then adjusted to pH 10 with saturated aqueous Na_2CO_3 and extracted four times with diethyl ether. The aqueous layer was acidified with 2M HCl to pH 3 and extracted into diethyl ether four times. The ethereal layer was dried over anhydrous magnesium sulfate, filtered, and the solvent removed *in vacuo* to provide a colorless oil. Yield: 57.0 mg, 88%.

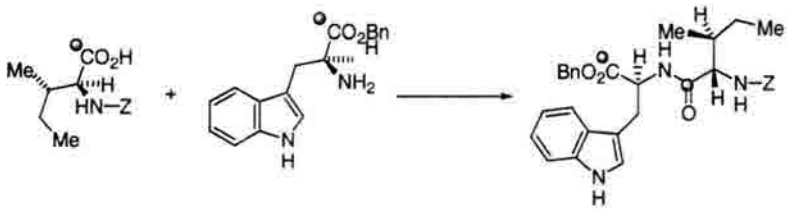
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 0.91 (3H, t, J = 7.3 Hz), 0.92 (3H, d, J = 6.6 Hz), 1.27 (1H, m), 1.49 (1H, m), 1.84 (1H, m), 4.00 (1H, dd, J = 6.9, 6.9 Hz), 5.08 (2H, s), 6.68 (1 H, bs), 7.36 (5H, s), 11.1 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.8, 15.7, 25.1, 38.0, 58.5 (d, $J_{C\text{-}C}$ = 55 Hz), 67.8, 128.3, 128.5, 128.6, 136.3, 156.5, 177.3. IR (NaCl, CH_2Cl_2): 3313, 3047, 2966, 1714, 1520, 1455, 1415, 1339, 1092, 739, 697 cm^{-1}. $[\alpha]_D^{25}$ = + 6° (c = 0.0125, CH_2Cl_2). [217]



[1-^{13}C]-L-Tryptophan Benzyl Ester:

[1-^{13}C]-L-tryptophan (44 mg, 0.214 mmol) and *p*-toluenesulfonic (102 mg, 0.536 mmol) acid monohydrate benzyl alcohol (250 μL), and dry toluene (15 mL) were refluxed under an argon atmosphere in a Dean Stark apparatus half filled with activated 4 Å molecular sieves for 12 h. The toluene was evaporated under reduced pressure and the residue was covered with a layer of EtOAc. The solution was then made basic by the addition of saturated sodium carbonate. The aqueous layer was separated from the organic layer and extracted 3 more times with EtOAc. After drying with anhydrous sodium sulfate, the solvent was removed *in vacuo*. Yield: 34 mg, 54%. An analytical sample of unlabeled material was recrystallized from EtOAc and hexanes to provide a cream colored solid, mp 74 - 75°C.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.71 (2H, bs, D_20 exch), 3.08 (1H, dd, J = 7.3, 14.4 Hz), 3.27 (1H, dd, J = 5, 14.4 Hz), 3.87 (1H, bs), 5.12 (2H, s), 6.91 (1 H, d , J = 2 Hz), 7.10 (1H, ddd, J =1, 7, 7 Hz) , 7.18 (1H, ddd, J = 1, 7, 7 Hz), 7.25 (1H, d J = 6 Hz), 7.26, (1H, m), 7.32 (2H, d, J = 6 Hz), 7.32 (2H, t, J = 4 Hz), 7.60 (1H, d, J = 7 Hz), 8.21 (1H, bs, D_20 exch). ^{13}C NMR (75 MHz, $CDCl_3$): δ 31.0, 55.4 (d, $J_{C\text{-}C}$ = 60 Hz), 66.9, 77.4, 111.3, 111.4, 119.0, 122.4, 123.1, 127.8, 128.5, 128.5, 128.8, 135.9, 136.5, 175.6. IR (NaCl, CH_2Cl_2): 3389, 3161, 3047, 1731, 1586, 1451, 1177, 742, 695 cm $^{-1}$. $[\alpha]_D^{25}$ = +30.8° (c = 0.0065, CH_2Cl_2).

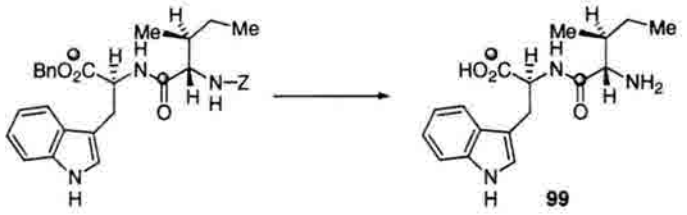


[$^{13}C_2$]-N-Benzyloxycarbonyl-L-Isoleucyl-L-Tryptophan-Benzyl ester:

L-[1-^{13}C]tryptophan benzyl ester (34.0 mg , 0.115 mmol) was added to L-[1-^{13}C]-N-benzyloxycarbonyl-isoleucine (35.0 mg, 0.131 mmol) dissolved in acetonitrile (5 mL). Triethylamine (24 μL, 0.172 mmol) and BOP reagent (50.92 mg, 0.115 mmol) were added to the flask. After stirring for 2 h at room temperature, brine (10 mL) was added to the reaction mixture. The solution was extracted three times with EtOAc. The combined EtOAc layers were extracted with 2 M HCl, water, 0.5 M $NaHCO_3$, and brine successively. The EtOAc fraction was dried over $MgSO_4$, filtered and evaporated to dryness. The crude product was purified by radial chromatography using a 3% mixture of methanol in CH_2Cl_2. Yield: 51.8 mg, 86%. An analytical sample of unlabeled material was recrystallized from EtOAc and hexanes to provide a white solid, mp 152 - 153°C.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.81 (3H, t J = 7 Hz), 0.86 (3H, d, J = 7 Hz), 1.04 (1H, m), 1.27 (1H, m), 1.79 (1H, m), 3.27 (1H, dd, J = 5, 15 Hz), 3.34 (1H, dd, J = 5, 15 Hz), 4.04 (1 H, dd, J = 6, 10 Hz), 4.98 (1H, ddd, J = 6, 6, 8 Hz), 5.08 (4H, s), 5.35 (1H, d, J = 9 Hz, D_20 exch), 6.43 (1H, d, J = 8 Hz, D_20 exch), 6.75 (1H, bs), 7.08 (1H, t, J = 8 Hz), 7.18 (1H, ddd, J = 1, 8, 8 Hz), 7.29 (11H, m), 7.50 (1H, d, J = 8 Hz), 7.96 (1H, bs, D_20 exch). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.6, 15.5, 24.9, 27.9, 37.9, 53.1 (d, $J_{C\text{-}C}$ = 61 Hz), 59.8 (d, $J_{C\text{-}C}$ = 52 Hz), 67.2, 67.5, 109.7, 111.5, 118.7, 120.0, 122.5, 123.4, 127.7, 128.3, 128.4, 128.7, 128.7, 128.8, 135.4, 136.3, 156.4, 171.1, 171.6. IR (NaCl, CH_2Cl_2):

3319, 3045, 2946, 1710, 1655, 1523, 1452, 1348, 1227, 1101, 1029, 738, 694 cm $^{-1}$. $[\alpha]_D^{25} = +12.7°$ (c = 0.01, CH_2Cl_2).

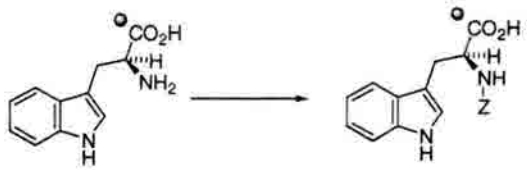


99

[$^{13}C_2$]-L-Isoleucyl-L-Tryptophan (99):

[$^{13}C_2$] -Z-L-isoleucine-L-tryptophan benzyl ester (15.4 mg, 0.028 mmol) was suspended with 20% $Pd(OH)_2$/ C (10 mg) in 2 mL of 95% ethanol. After bubbling argon followed by $H_2(g)$ through the solution, it was placed under $H_2(g)$ atmosphere with an $H_2(g)$ balloon. The reaction was determined to be complete by TLC after 16 h. The reaction mixture was filtered through a pad of celite and evaporated to dryness. Yield: 9 mg, 100%. A pinkish solid was obtained by dissolving the dipeptide in deionized, distilled water and lyophilizing, mp 90°C (decomp).

^{1}H NMR (300 MHz, CD_3OD): δ 0.85 (3H, t, J = 7 Hz), 0.91 (3H, d, J = 7 Hz), 1.10 (1H, m), 1.42 (1H, m), 1.81 (1H, m), 3.19 (1H, dd, J = 7.3, 14.7 Hz), 3.36 (1H, dd, J = 5, 16 Hz), 3.52 (1 H, d, J = 5 Hz), 4.61 (1H, dd, J = 5, 7 Hz), 6.97 (1H, ddd, J = 1, 8, 8 Hz), 7.04 (1H, ddd, J = 1, 8, 8 Hz), 7.11 (1H, bs), 7.28 (1H, d, J = 8 Hz), 7.62 (1H, d, J = 7 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 11.9, 15.5, 25.3, 29.3, 38.5, 57.0 (d, $J_{C\text{-}C}$ = 56 Hz), 59.8 (d, $J_{C\text{-}C}$ = 47 Hz), 112.2, 112.3, 119.7, 119.8, 124.5, 129.4, 138.1, 170.4, 177.6. IR (NaCl, CH_3OH): 3404, 3025, 2966, 1652, 1593, 1456, 1393, 742 cm^{-1}. $[\alpha]_D^{25} = +2.2°$ (c = 0.005, CH_3OH).



N-Benzyloxycarbonyl-L-[1-^{13}C]Tryptophan:

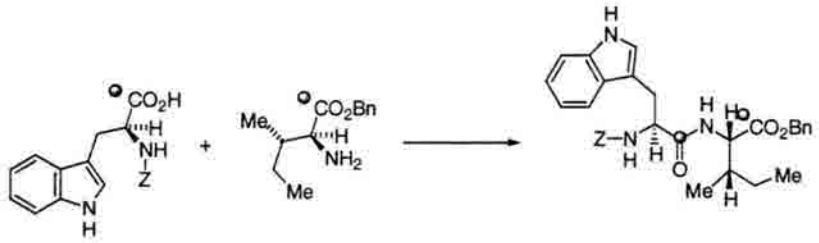
L-[1-^{13}C]tryptophan (50.0 mg, 0.244 mmol) was protected in the same manner described for N-benzyloxycarbonyl-L-[1-^{13}C]isoleucine. Yield: 83.0 mg, 100%.

^{1}H NMR (300 MHz, $CDCl_3$): δ 3.33 (2H, m), 4.75 (1H, dd, J = 5, 12 Hz), 5.08 (1H, d, J = 12 Hz), 5.14 (1H, d, J = 12 Hz), 5.37 (1 H, d, J = 8 Hz, D_2O exch), 6.90 (1 H, s), 7.08 (1H, t, J = 7 Hz) , 7.19 (1H, t, J = 7 Hz), 7.33, (6H, s), 7.57 (1H, d, J = 8 Hz), 8.09 (1H, bs, D_20 exch) 10.22 (1H, bs, D_20 exch). ^{13}C NMR (100 MHz, $CDCl_3$): δ 27.6, 54.5 (d, J_{C-C} = 60 Hz), 67.1, 77.4, 109.9, 111.3, 118.6, 119.8, 122.2, 123.1, 127.6, 128.2, 128.3, 128.5, 136.0, 156.0, 176.5. IR (NaCl, CH_3OH): 3414, 2962, 1704, 1515, 1455, 1260, 1020, 795 cm^{-1}. $[\alpha]_D^{25}$ = -4.0° (c = 0.01, CH_3OH).

L-[1-^{13}C]Isoleucine Benzyl Ester:

L-[1-^{13}C]isoleucine (32.2 mg, 0.244 mmol), *p*-toluenesulfonic acid monohydrate (51 mg, 0.268 mmol), benzyl alcohol (100 μL), and dry toluene (15 mL) were refluxed for 24 h under an argon atmosphere through an addition funnel half filled with activated 4Å molecular sieves. The toluene was evaporated under reduced pressure and the residue was covered with a layer of CH_2Cl_2. The solution was then made basic by the addition of saturated sodium carbonate. The aqueous layer was separated from the organic layer and extracted 3 more times with CH_2Cl_2. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo* to give a colorless oil. Yield: 34.4 mg, 63%.

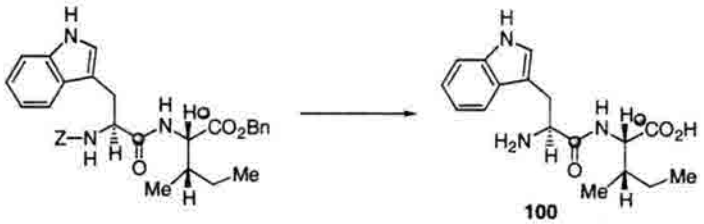
^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (3H, t, J = 7.3 Hz), 0.93 (3H, d, J = 6.7 Hz), 1.19 (1H, m), 1.42 (1H, m), 1.55 (2H, bs, D_20 exch), 1.78 (1H, m), 3.40 (1H, d, J = 5 Hz), 5.14 (1H, d, J = 12 Hz), 5.18 (1H, d, J = 12 Hz), 7.36 (5H, m). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.8, 16.0, 24.8, 39.3, 59.4 (d, $J_{C\text{-}C}$ = 57 Hz), 66.7, 128.5, 128.7, 136.0, 175.7. IR (NaCl, CH_2Cl_2): 3385, 2962, 1732, 1456, 1167 cm^{-1}. $[\alpha]_D^{25}$ =+ 16.2° (c = 0.015, CH_2Cl_2).



[$^{13}C]_2$-N-Benzyloxycarbonyl-L-Tryptophyl-L-Isoleucine-Benzyl Ester:

The coupling of N-benzyloxycarbonyl-L-[1-^{13}C]tryptophan (53.8 mg, 0.242 mmol) and L-[^{13}C]isoleucine benzyl ester (81.4 mg, 0.239 mmol) was performed as described for [$^{13}C]_2$-L-Z-isoleucyl-L-tryptophan-benzyl ester. Yield: 123.0 mg, 99 %. An analytical sample of unlabeled material was recrystallized from EtOAc and hexanes to provide a white solid, mp 128 - 129°C.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.71 (3H, d, J = 7.1 Hz), 0.79 (3H, t, J = 7.3 Hz), 0.94 (1H, m), 1.23 (1H, m), 1.75 (1H, m), 3.15 (1H, dd, J = 7.8, 14.5 Hz), 3.33 (1H, dd, J = 5, 14.5 Hz), 4.51 (2H, m), 5.08 (2H, d, J = 3 Hz), 5.13 (2H, s), 5.6 (1H, d, J = 6 Hz, D_20 exch), 6.22 (1H, d, J = 8 Hz, D_20 exch), 6.96 (1H, bs), 7.10 (1H, t, J = 7 Hz), 7.18 (1H, ddd, J = 1, 7, 7 Hz), 7.35 (11H, m), 7.69 (1H, d, J = 7 Hz), 8.05 (1H, bs, D_2O exch). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.7, 15.4, 25.2, 28.8, 38.0, 55.7 (d, $J_{C\text{-}C}$ = 55 Hz), 56.9 (d, $J_{C\text{-}C}$ = 61 Hz), 67.1, 67.2, 110.6, 111.4, 119.0, 120.0, 122.5, 123.6, 127.5, 128.3, 128.4, 128.6, 128.7, 128.8, 135.6, 136.5, 156.2, 171.3, 171.3. IR (NaCl, CH_2Cl_2): 3308, 3044, 2946, 1710, 1656, 1518, 1452, 1337, 1221, 1139, 738, 694 cm^{-1}. $[\alpha]_D^{25}$ = + 1.0° (c = 0.01, CH_2Cl_2).



100

[$^{13}C_2$]-L-Tryptophyl-L-Isoleucine (100):

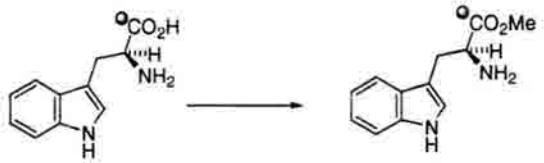
[$^{13}C_2$]-L-Z-tryptophyl-L-isoleucine benzyl ester (48 mg, 0.0881 mmol) and 20 % $Pd(OH)_2/C$ (15 mg) were suspended in 2 mL of 95% ethanol. After bubbling argon through the reaction mixture, it was placed under $H_2(g)$ atmosphere with an H_2 (g) balloon. After 16 h the reaction was complete by TLC. The reaction mixture was filtered through a pad of celite and evaporated *in vacuo* . Yield: 30 mg, 100%. A white solid was obtained by dissolving the dipeptide in distilled deionizedwater and lyophilizing, mp 171°C (decomp).

^{1}H NMR (300 MHz, CD_3OD): δ 0.90 (3H, t, J = 7 Hz), 0.92 (3H, d, J = 7 Hz), 1.14 (1H, m), 1.55 (1H, m), 1.88 (1H, m), 3.17 (1H, dd, J = 8, 15 Hz), 3.44 (1H, dd, J = 5, 15 Hz), 4.15 (1H, dd, J = 6, 6 Hz), 4.27 (1H, d, J = 5 Hz), 7.04 (1H, ddd, J = 1, 8, 8 Hz) , 7.11 (1H, ddd, J = 1, 7, 7 Hz), 7.21 (1H, bs), 7.35 (1H, d, J = 8 Hz), 7.70 (1H, d, J = 8 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 12.3, 16.5, 18.2, 26.3, 29.3, 39.2, 55.4 (d, $J_{C\text{-}C}$ = 53 Hz), 60.0 (d, $J_{C\text{-}C}$ = 60 Hz), 108.7, 112.6, 119.4, 120.3, 122.9, 125.8, 128.6, 138.4, 170.2, 175.9. IR (NaCl, CH_3OH): 3272, 3075, 2962, 1665, 1592, 1457, 1404, 742 cm $^{-1}$. $[\alpha]_D^{25}$ = + 6.6° (c = 0.005, CH_3OH).

N-*t*-butoxycarbonyl-[1-^{13}C]-L-isoleucine:

NaOH (0.5 mL of a 1.0 M solution) was added to L-[1-^{13}C]isoleucine (32.2 mg, 0.244 mmol, 90% ^{13}C) dissolved in dioxane (1.0 mL) and dd H_2O (0.5 mL). The solution was cooled in an ice bath while stirring and di-tert-butyl-dicarbonate (62 μL, 0.270 mmol) was added. The reaction mixture was brought to room temperature and allowed to stir for one hour. The dioxane was then evaporated under reduced pressure and the residue was covered with a layer of EtOAc and acidified to pH 2-3 with $KHSO_4$. The aqueous layer was separated from the organic layer and extracted two more times with EtOAc. The EtOAc fractions were pooled, dried over $MgSO_4$ and evaporated under reduced pressure to leave a colorless oil. Yield: 51.6 mg, 92%.

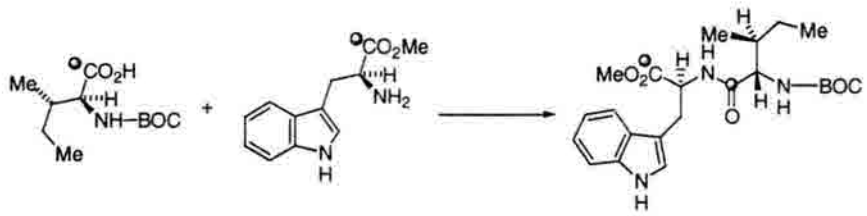
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 0.88 (3H, t, J = 7.7 Hz), 0.91 (3H, d, J = 6.6 Hz), 1.22 (1H, m), 1.42 (9H, s), 1.46 (1H, m), 1.80 (1H, m), 3.92 (1H, dd, J = 7.7, 7.7 Hz), 6.04 (1H, bs), 10.29 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.6, 15.5, 24.4, 28.3, 37.8, 57.8 (d, $J_{C\text{-}C}$ = 58 Hz), 80.0, 155.7, 177.2. IR (NaCl, CH_2Cl_2): 3326, 3110, 2971, 2587, 1715, 1663, 1509, 1454, 1401,1365, 1248, 1165 cm^{-1}. $[\alpha]_D^{25}$= + 5.4° (c = 0.012, CH_2Cl_2).



L-[1-^{13}C]-Tryptophan Methyl Ester:

L-[1-^{13}C]-tryptophan (50 mg, 0.244 mmol, 98% ^{13}C) and DL-10-camphorsulfonic acid (115 mg, 0.495 mmol) were dissolved in 20 mL of absolute methanol. The solution was refluxed through an addition funnel containing 3 Å molecular sieves for 24 h under an argon atmosphere. The methanol was evaporated off under reduced pressure and the residue was covered with a layer of EtOAc. The solution was made basic by the addition of saturated sodium carbonate. The aqueous layer was separated from the organic layer and extracted 3 more times with EtOAc. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo* to leave a yellow oil. Yield: 41.3 mg, 78 %.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.83 (2H, bs, D_20 exch), 3.07 (1H, dd, J = 7.6, 14.4 Hz), 3.31 (1H, dd, J = 4.7,14.4), 3.73 (3H, s), 3.86 (1H, dd, J = 5, 7 Hz), 7.01 (1H, d, J = 2 Hz), 7.14 (1H, ddd, J = 1, 7, 7 Hz), 7.20 (1H, ddd, J = 1, 7, 7 Hz), 7.34 (1H, d, J = 8 Hz), 7.63 (1H, d, J = 8 Hz), 8.52 (1H, bs, D_20 exch). ^{13}C NMR (75 MHz, $CDCl_3$): δ 30.9, 52.2, 55.1 (d, $J_{C\text{-}C}$ = 59 Hz), 111.0, 111.4, 118.8, 119.6, 122.2, 123.2, 127.6, 136.4, 175.9. IR (NaCl, CH_2Cl_2): 3387, 3162, 2936, 1732, 1586, 1448, 1201, 1099, 1007, 742 cm $^{-1}$. $[\alpha]_D^{25}$ = + 20.4° (c = 0.01, CH_2Cl_2).

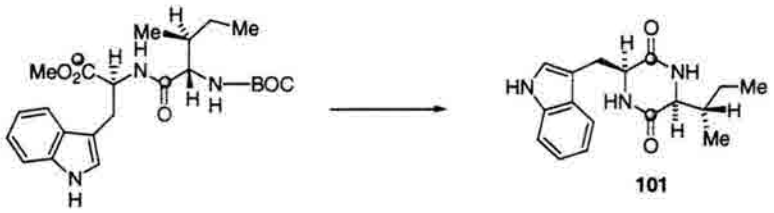


[$^{13}C_2$]-L-N-*t*-Butoxycarbonyl-Isoleucyl-L-Tryptophan-Methyl Ester:

L-[^{13}C]tryptophan methyl ester (41.3 mg, 0.180 mmol) was added to (41.4 mg, 0.179 mmol) of L-[1-^{13}C]-N-*t*-butoxycarbonyl-isoleucine and dissolved in acetonitrile (2.75 mL). One equivalent of triethylamine (25 μL, 0.180 mmol) and BOP reagent (79.5 mg, 0.180 mmol) were added to the flask. After stirring for 2 h at room temperature, brine (10 mL) was added to the reaction mixture. The solution was extracted three times with EtOAc and the combined EtOAc layers were extracted with 2 M HCl, water, 0.5 M $NaHCO_3$, and brine successively. The organic layer was dried over $MgSO_4$, filtered and evaporated to dryness. The crude product was purified by chromatotron using a 9:1 mixture of CH_2Cl_2/ CH_3OH. Yield: 60.0 mg, 80%. An analytical sample of unlabeled material was recrystallized from EtOAc and hexanes to provide a white crystalline solid, mp 142-143°C.

^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 0.82 (3H, t, J = 7.3 Hz), 0.85 (3H, d, J = 7.7 Hz), 1.10 (1H, m), 1.41 (9 H, s), 1.44 (1H, m), 1.72 (1H, m), 2.89 (1H, bs), 3.17 (1H, dddd, J = 6.6, 15.0, 15.0, 15.0 Hz), 3.59 (3H, s), 3.90 (1H, dd, J = 6.6, 8.8 Hz), 4.66 (1H, dd, J = 7.3, 14.3 Hz), 6.03 (1H, d, J = 9.5 Hz), 6.99 (1H, ddd, J = 1.1, 7.0, 6.08 (1H, ddd, J = 1.1, 7.0, 7.0), 7.12 (1H, d, J = 2.2 Hz), 7.35 (1H, d, J = 7.7 Hz), 7.51 (1H, d, J = 7.7 Hz), 7.70 (1H, d, J = 7.0 Hz, 10.47 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ

11.6, 15.5, 24.8, 27.9, 28.5, 37.7, 52.7 (d, $J_{C\text{-}C}$ = 62 Hz), 59.3 (d, $J_{C\text{-}C}$ = 60 Hz), 80.0, 109.8, 111.5, 118.6, 119.8, 122.4, 123.3, 127.7, 136.4, 155.9, 171.5, 172.2. IR (NaCl, CH_2Cl_2): 3305, 2967, 1657, 1514, 1442, 1360, 1248, 1165 cm^{-1}. $[\alpha]_D^{25}$= + 35.3° (c = 0.01, CH_2Cl_2).



[$^{13}C]_2$-Cyclo-L-Isoleucine-L-Tryptophan (101):

[$^{13}C_2$]-N-boc-L-isoleucine-L-tryptophan-methyl ester (59 mg, 0.141 mmol) was dissolved in CH2Cl2 (10 mL). The solution was cooled to 0°C and trifluoroacetic acid (1 mL) was added. The reaction was stirred at 0°C under an argon atmosphere for 5 h. The solvent and most of the TFA were removed *in vacuo*. The residue was dissolved in dry toluene and refluxed under an argon atmosphere for 1 hour. The toluene was evaporated *in vacuo* and the crude product was purified by flash column chromatography using a 9:1 mixture of CH_2Cl_2/ CH_3OH to provide a white solid, mp 163°C (decomp). Yield: 37 mg, 87%.

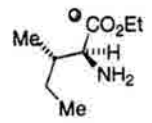
^{1}H NMR (400 MHz, $CDCl_3$ + 5 drops CD_3OD): δ 0.76 (3H, t, J = 7 Hz), 0.87 (3H, d, J = 7 Hz), 0.91 (1H, m), 1.09 (1H, m), 1.83 (1H, m), 3.03 (1H, ddd, J = 1, 9, 13 Hz), 3.50 (1H, ddd, J = 3, 3, 14 Hz), 3.80 (1H, m), 4.21 (1H, m), 7.04 (1H, s), 7.06 (1H, t , J = 7 Hz), 7.13 (1H, t , J = 7 Hz), 7.32 (1H, d, J = 8 Hz), 7.56 (1H, d, J = 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$ + 5 drops CD_3OD): δ 11.5, 14.9, 23.4, 30.4, 38.1, 54.9 (d, $J_{C\text{-}C}$ = 51 Hz), 59.8 (d, $J_{C\text{-}C}$ = 52 Hz), 108.8, 111.3, 118.4, 119.6, 122.2, 132.8, 126.8, 136.4, 166.9, 167.9. IR (NaCl, CH_3OH): 3319, 3198, 3045, 2957, 1666, 1457, 1326, 1095, 733 cm^{-1}. $[\alpha]_D^{25}$= - 31.5° (c = 0.0065, CH_3OH).

109

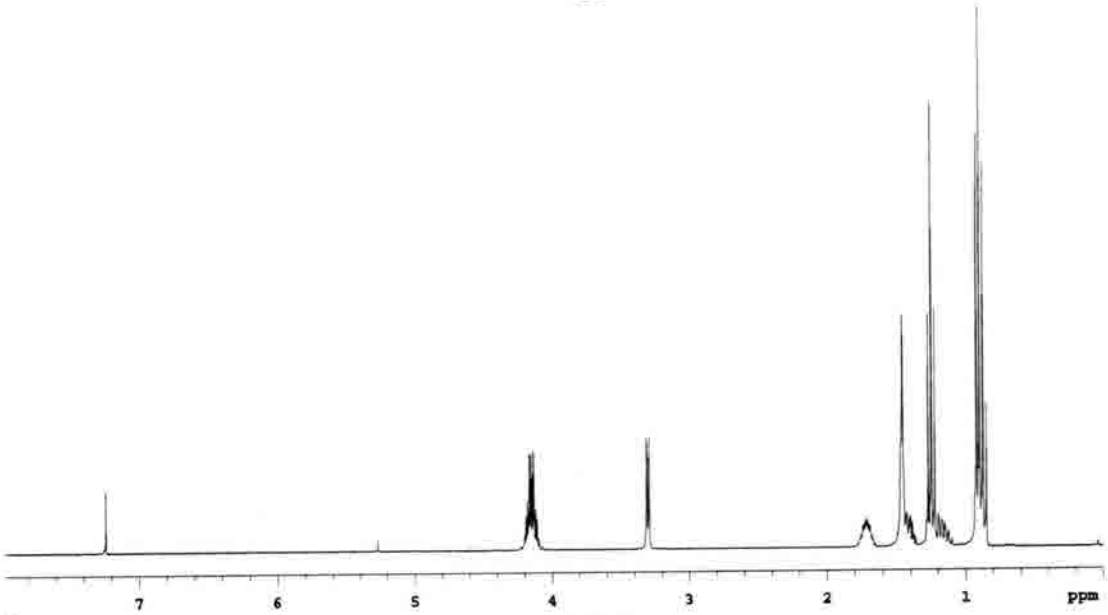
Synthesis of L[1-^{13}C]isoleucine ethyl ester (109):

To a suspension of L-[1-^{13}C]isoleucine (500 mg, 3.8 mmol) in 23 mL of absolute ethanol at 0°C was added 7 eq. of thionyl chloride (1.93 mL, 26.5 mmol) dropwise. The mixture was brought to room temperature and stirred until all the solids dissolved and then refluxed for an additional 10 hours. The reaction mixture was concentrated under reduced pressure, re-dissolved in water and made basic (pH= 9-10) by the addition of 20% aqueous ammonia. The aqueous layer was extracted with EtOAc four times. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford a colorless oil. To remove residual EtOAc, the product was re-dissolved in CH_2Cl_2 and again concentrated under reduced pressure. Due to the volatility of the product, it should not be placed on a high vacuum line. Yield: 545 mg, 3.4 mmol, 90%.

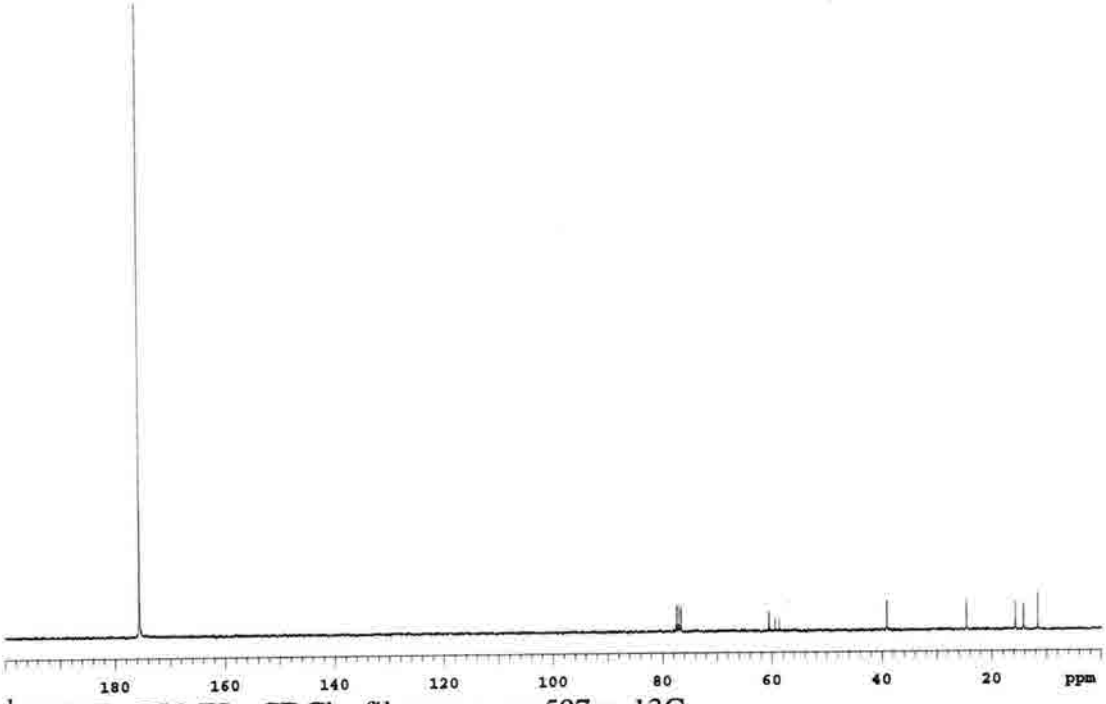
^{1}H NMR (300 MHz, $CDCl_3$): δ 0.87 (3H, t, J = 7.3 Hz), 0.91 (3H, d, J = 6.6 Hz), 1.15 (1H, m), 1.25 (3H, t, J = 7.0 Hz), 1.40 (1H, m), 1.45 (2H, bs, D_20 exch), 1.71 (1H, m), 3.30 (1H, d, J = 5.1 Hz), 4.15 (2H, m). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.6, 14.2, 15.6, 24.6, 39.2, 59.0 (d, J_{c-c}= 57 Hz), 60.4, 175.5. HRMS (FAB$^+$) calcd for C_7 $^{13}C_1H_{18}O_2N_1$ (M+H) 161.1371, found 161.1373.



109



^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems756_2



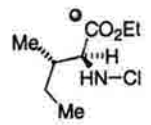
^{1}H NMR, 75 MHz, $CDCl_3$, filename: ems597cr_13C

109 110

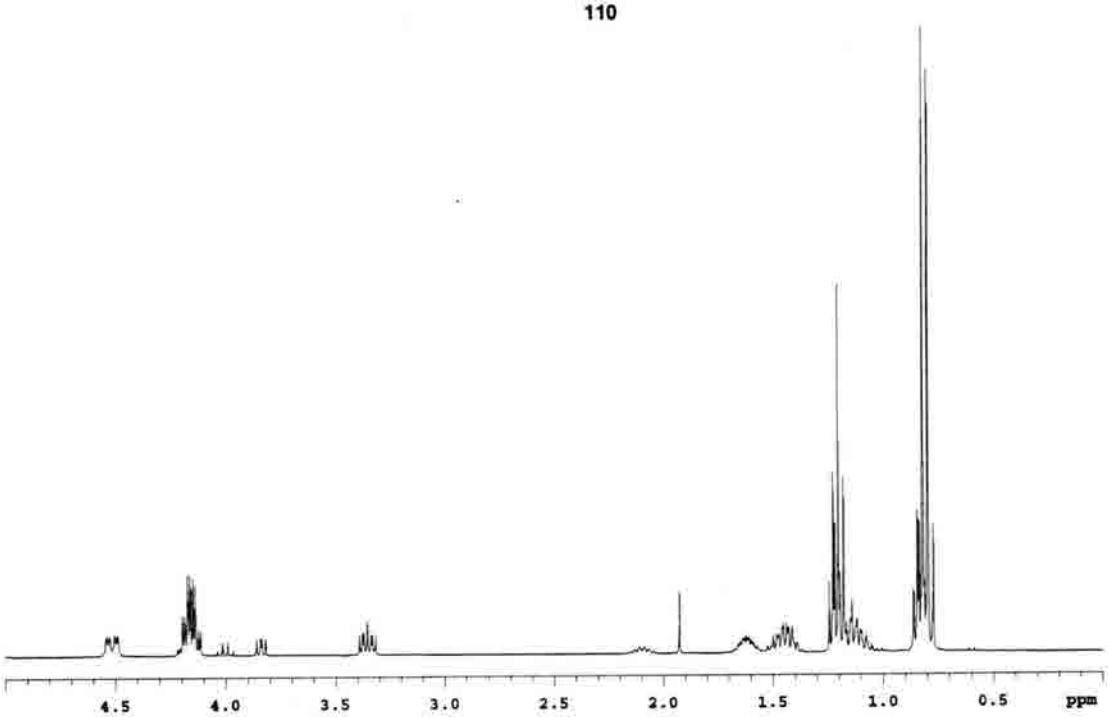
N-Cl-[1-^{13}C]- L-isoleucine ethyl ester (110):

To a solution of [1-^{13}C]-L-isoleucine ethyl ester (**109**, 360.5 mg, 2.2 5 mmol) in benzene (2.8 mL) at 0°C was added one equivalent of freshly prepared *t*-butyl hypochlorite (228 μL, 2.25 mmol). The reaction mixture was kept below 5°C and stirred in the dark for 1.5 hours. The mixture was washed with 1 mL of 0.1 N HCl, and water (3 times, 1 mL aliquots) before drying over anhydrous Na_2SO_4 and concentrating under reduced pressure at room temperature to afford a yellow oil. The product was carried on to the next step without further purification. Yield: 424 mg, 2.18 mmol, 97%.

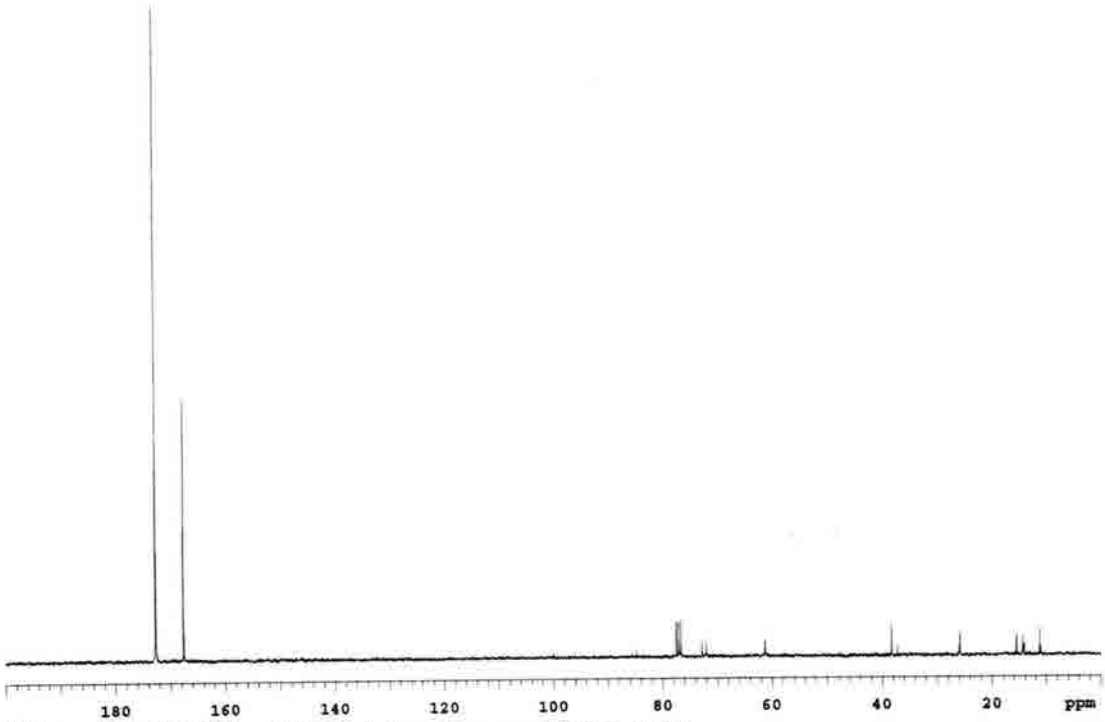
^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (3H, t, J = 7.3 Hz), 0.92 (3H, d, J = 5.1 Hz), 1.21 (1H, m), 1.30 (3H, t, J = 7.7 Hz), 1.53 (1H, m), 1.72 (1H, m), 3.45 (1H, dd, J = 6.6, 11.4 Hz), 4.25 (2H, q, J = 7.0 Hz), 4.61 (1H, d, J = 11.4 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.2, 14.3, 15.4, 5.8, 38.2, 61.2, 72.3 (d, $J_{C\text{-}C}$= 57 Hz), 172.7.



110



^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems599cr



^{13}C NMR, 75 MHz, $CDCl_3$, filename: ems599cr_13C

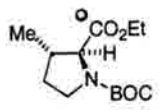
110 112

N-Boc-L-[1-^{13}C]-3(*S*)-methyl-proline ethyl ester (112):

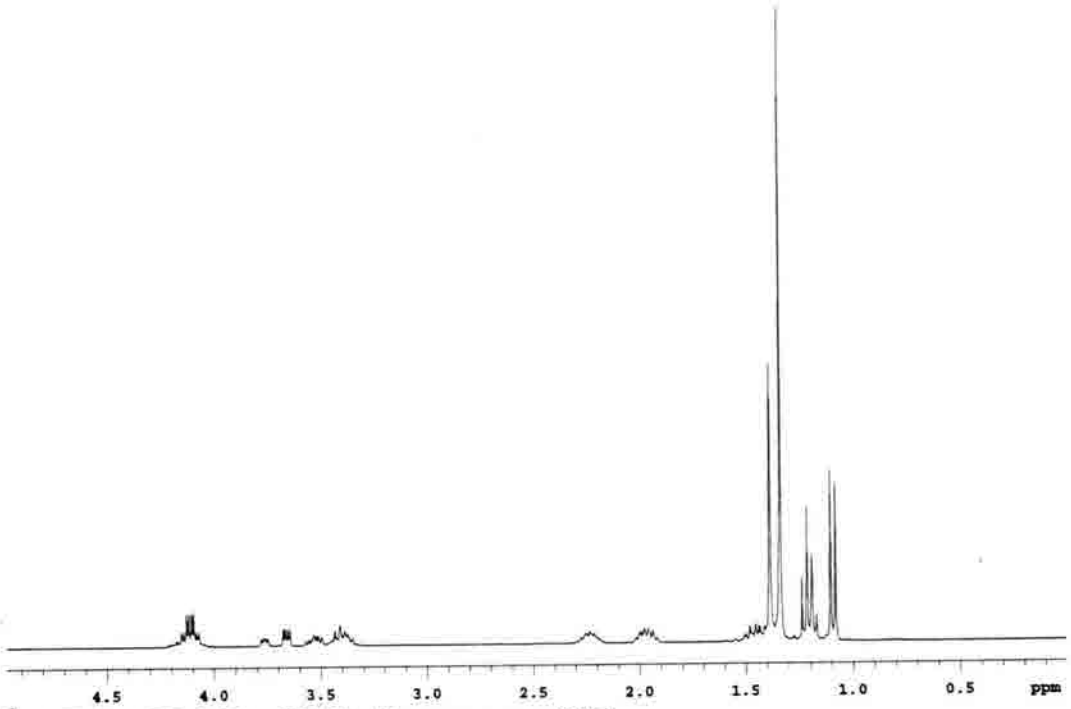
A solution of N-Cl-[1-^{13}C]-L-isoleucine ethyl ester (424 mg, 2.18 mmol) in 85% H_2SO_4 (14.5 mL) at 0°C was prepared in a photochemical reaction vesssel fitted with a quartz immersion well and Hg° lamp. After purging the system with argon for 15 minutes, the reaction mixture was then irradiated with a mercury lamp for 40 hrs under an atmosphere of argon while maintaining the temperature between 0-5°C. The reaction was tested for completion by taking an aliquot (5 drops) of the reaction mixture and treating with 5 mL of a 5% KI solution (in 1:1 water, acetone). When a clear pale yellow color was obtained the reaction was complete. After addition of 100 mL of an ice/water mixture, the reaction was carefully neutralized with aqueous 10 M NaOH at 0°C. The reaction mixture was concentrated under reduced pressure,the residue was taken up in absolute ethanol and the solids filtered off. Evaporaion of the ethanolic solution under reduced pressure left crude [1-^{13}C]-3(*S*)-methyl-L-proline ethyl ester (298 mg, 1.87 mmol, 86%). The crude product was dissolved in 7.48 mL of a 1:1 mixture of dioxane and water and cooled to 0°C. Di-*tert*-butyl-dicarbonate (449 mg, 2.06 mmol, 1.1 eq) and solid K_2CO_3 (258 mg, 1.87 mmol, 1 eq.) were added. The reaction was slowly brought to room temperature and stirred for a total of 8 hrs. The dioxane was removed under reduced pressure, the reaction was lowered to a pH of 2 with 10% aqueous $KHSO_4$, and extracted 4 times with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed in vacuo to afford **112** as an oil. Yield: 255 mg, 0.99 mmol (45% from of N-Cl-[1-^{13}C]-L-

isoleucine ethyl ester). N-Boc-[1-^{13}C]-L-isoleucine ethyl ester was also recovered (50.3 mg, 0.19 mmol, 9% from of N-Cl-[1-^{13}C]-L-isoleucine ethyl ester).

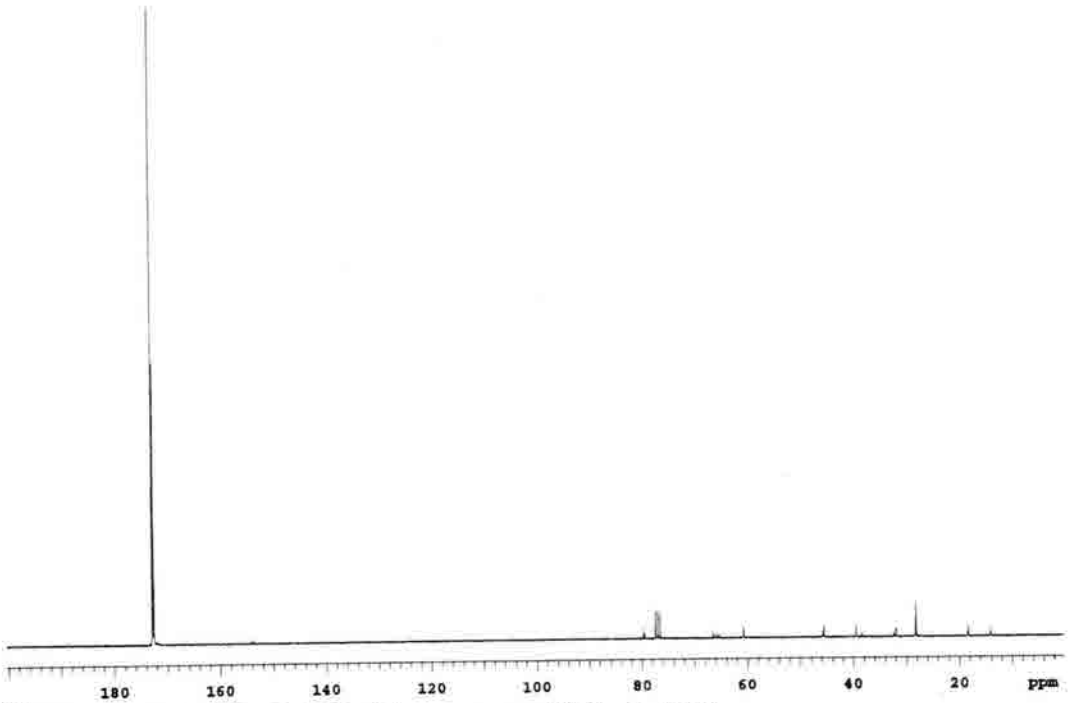
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.13 (3H, d, J = 7.0 Hz), 1.24 (3H, t, J = 7.0 Hz), 1.40 (9H, s), 1.52 (1H, m), 2.01 (1H, m), 2.27 (1H, m), 3.33 (1H, m), 3.49 (1H, m), 3.72(1H, d, J = 5.5 Hz), 4.15 (2H, m). ^{13}C NMR (75 MHz, d_6-DMSO, 120°C): δ 13.2, 17.4, 27.4, 31.0, 38.7, 44.9, 59.4, 65.4, 78.2, 152.6, 171.3. IR (NaCl, CH_2Cl_2): 2974, 2933, 2877, 1747, 1704, 14769, 1455, 1397, 1366, 1278, 1249, 1174, 1148, 1115, 1032 cm^{-1}. $[\alpha]_D^{25}$=-44.1° (c = 1.24, CH_3OH).



112



^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems602b



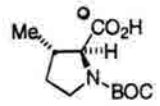
^{13}C NMR, 75 MHz, $CDCl_3$, filename: ems602b_2_13C

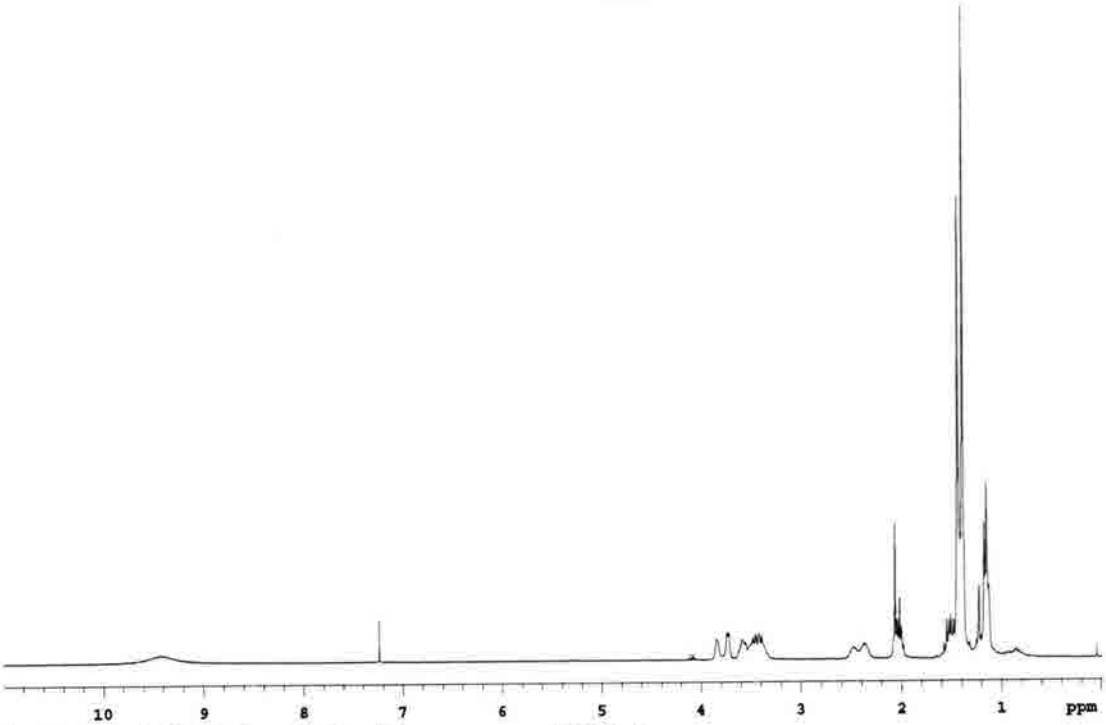
112

N-Boc-[1-^{13}C]-3(*S*)-methyl-L-proline:

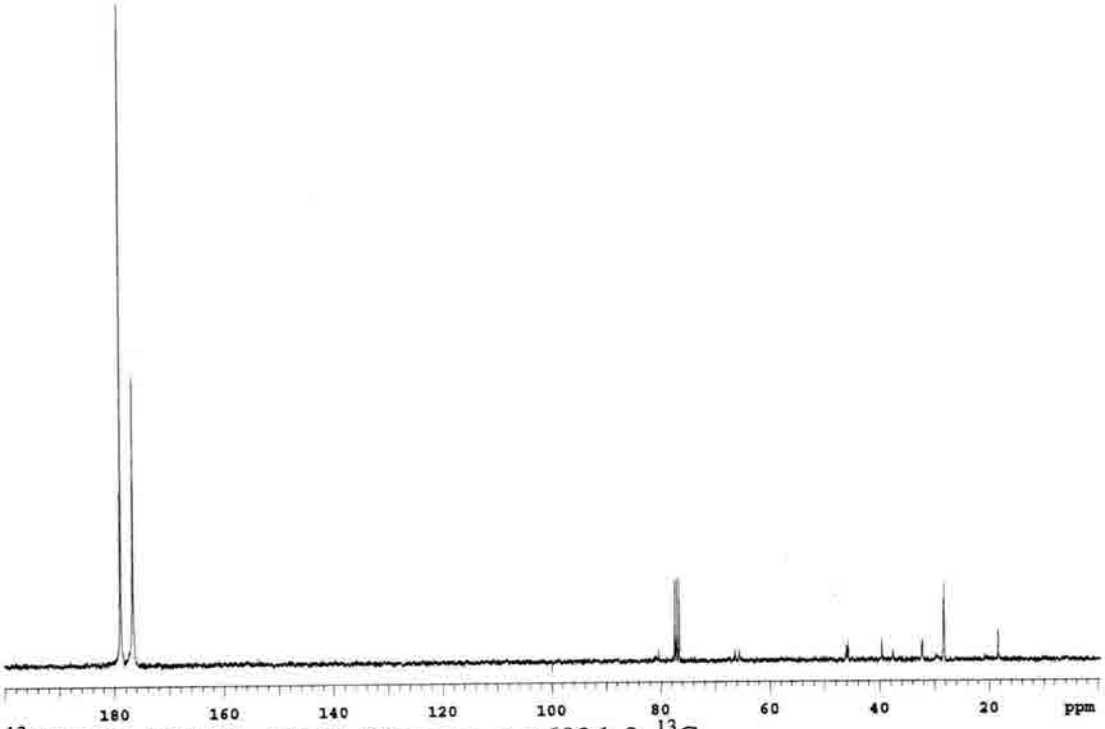
N-Boc-[1-^{13}C]-3(S)-methyl-L-proline ethylester (**112**, 62 mg, 0.24 mmol) was dissolved in 5 mL of anhydrous methanol. NaOH (48 mg, 1.2 mmol, 5 eq.) was added and the solution was refluxed for 2 hours. The methanol was removed in vacuo and 0.1 M HCl was added to achieve a pH=2. This mixture was extracted 3 times with EtOAc and the combined organic layers were dried over anhydrous $NaSO_4$ and evaporated under reduced pressure to afford N-Boc-[1-^{13}C]-3(*S*)-methyl-L-proline as a solid. Yield: 50 mg, 0.217 mmol, 90%. An analytical sample of unlabeled material was recrystallized from EtOAc and hexanes to provide an orangish solid, mp 147-148 °C.

^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.12 (3H, d, J = 7.0 Hz), 1.40 (9H, s) 1.48 (1H, m), 1.99 (1H, m), 2.28 (1H, m), 3.32 (1H, m), 3.47 (1H, m), 3.66 (1H, d, J = 5.5 Hz), 11.77 (1H, bs). ^{13}C NMR (MHz, d_6-DMSO, 120°C): δ 17.7, 27.5, 31.0, 38.7, 44.8, 65.4, 152.8, 172.6. IR (NaCl, CH_2Cl_2): 3438 (broad), 3106 (broad), 2965, 2604 (broad), 1694, 1413, 1252, 1157 cm^{-1}. $[\alpha]_D^{25}$= -135° (c=0.1, CH_2Cl_2). HRMS (FAB$^+$) calcd for C_{10} $^{13}C_1H_{20}O_4N_1$ (M+H) 231.1426, found 231.1420.

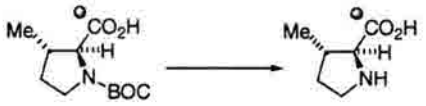




^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems602d_3



^{13}C NMR, 75 MHz, $CDCl_3$, filename: ems602d_3_^{13}C

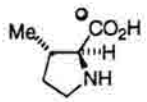


89c

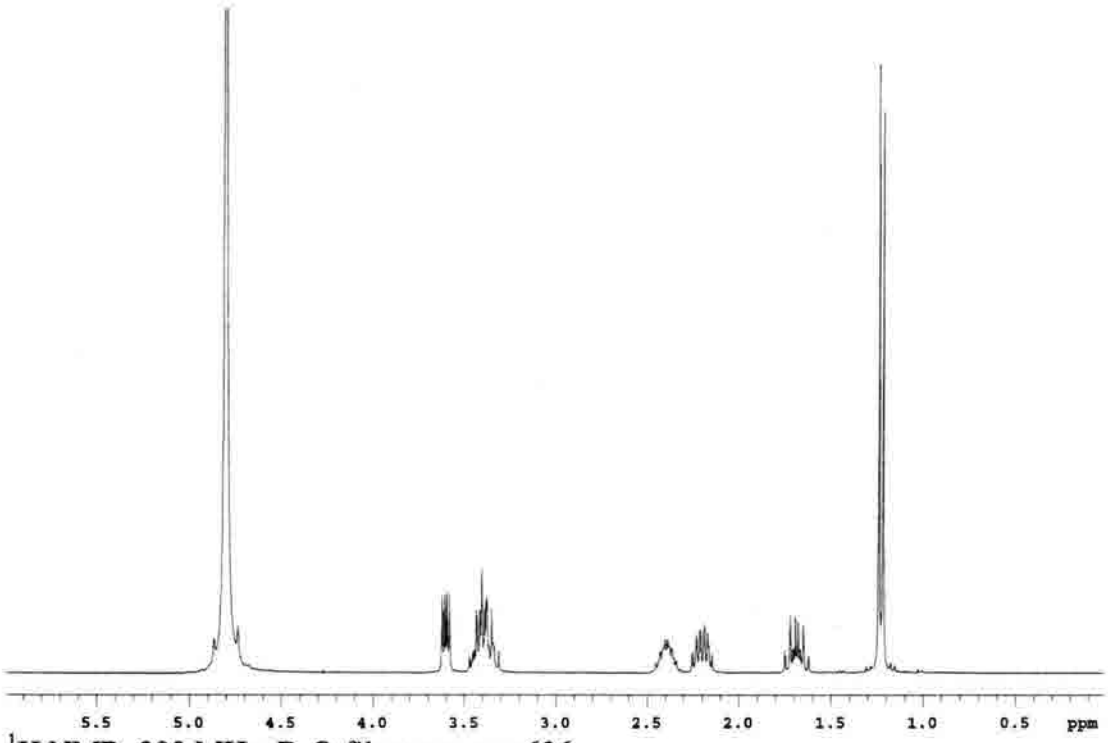
[1-^{13}C]-3(*S*)-methyl-L-proline:

N-Boc-[1-^{13}C]-3(*S*)-methyl-L-proline was taken up in 0.5 mL of CH_2Cl_2 and placed under argon with stirring at 0°C. To this solution, TFA, (348 uL, 4.34 mmol, 20 eq.) was added slowly. The solution was allowed to come to room temperature while continuing to stir over 3 hours. The CH_2Cl_2 and excess TFA were removed *in vacuo*. The crude reaction mixture was loaded onto a DOWEX (50W X2-100) ion exchange resin column and washed with 500 mL of water (until the pH of the wash was 7). The free amino acid was then eluted with 2% NH_4OH. The NH_4OH solution was evaporated under reduced pressure. The residue was then taken up in a small amount of distilled deionized water and lyophilized to leave **89c** as an off-white amorphous solid. Yield: 28 mg, 0.215 mmol, 99%.

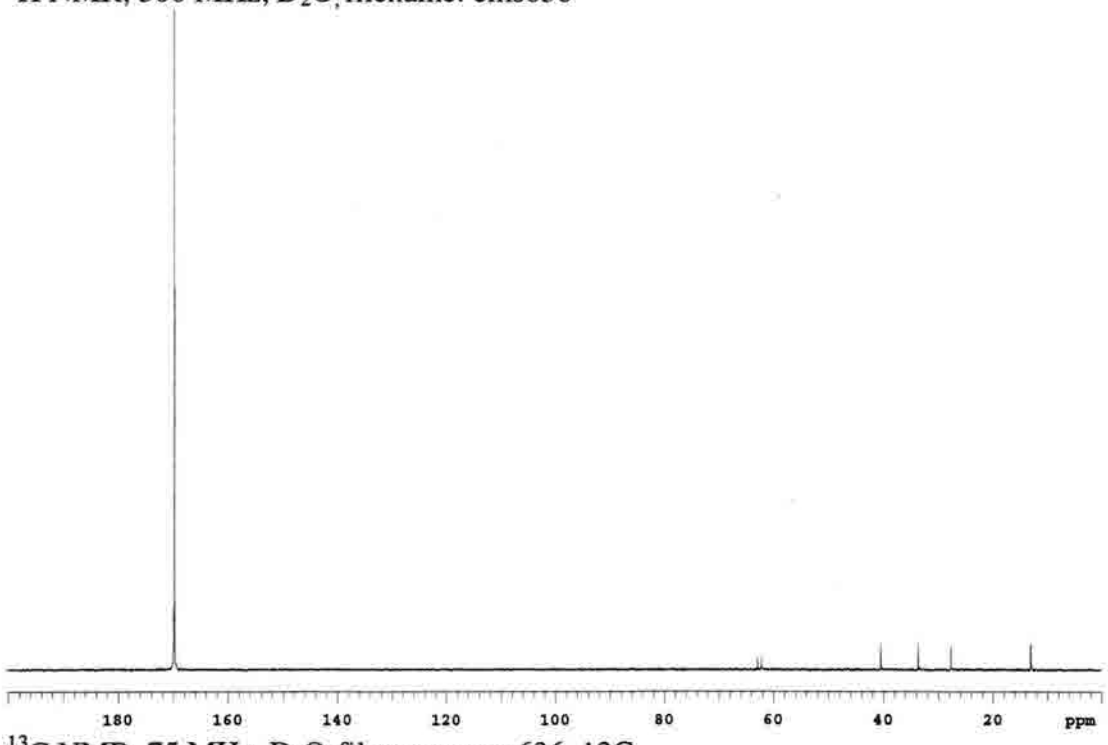
^{1}H NMR (300 MHz, D_2O): δ 1.23 (1H, d J = 7.0 Hz), 1.68 (1H, m), 2.20 (1H, m), 2.39 (1H, m), 3.39 (2H, m), 3.60 (1H, dd, J = 4.0, 6.7 Hz). ^{13}C NMR (75 MHz, D_2O): δ 13.1, 27.7, 33.7, 40.5, 62.6 (d, $J_{c\text{-}c}$= 53 Hz), 169.8. Enhanced ^{13}C peak. IR (NaCl, CH_3OH): 3389-2284, 2958, 1556, 1434, 1378, 1299, 1282, 1212, 1122, 1030, 961 cm^{-1}. $[\alpha]_D^{25}$= -27° (c = 0.1, H_2O). (Literature: $[\alpha]_D^{25}$= - 29± 2 (c = 0.1, H_2O)) HRMS (FAB$^+$) calcd for $C_5\,^{13}C_1H_{12}O_2N_1$ (M+H) 131.0902, found 131.0907.



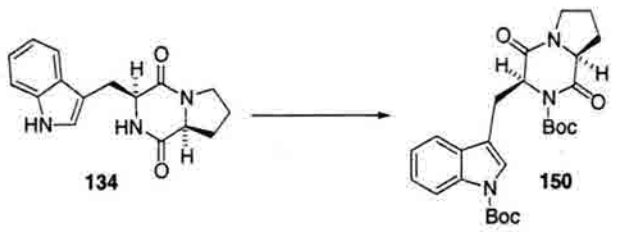
89c



^{1}H NMR, 300 MHz, D_2O, filename: ems636



^{13}C NMR, 75 MHz, D_2O, filename: ems636_13C



N,N'-Di-BOC-*cyclo*-L-tryptophan-L-proline (150).

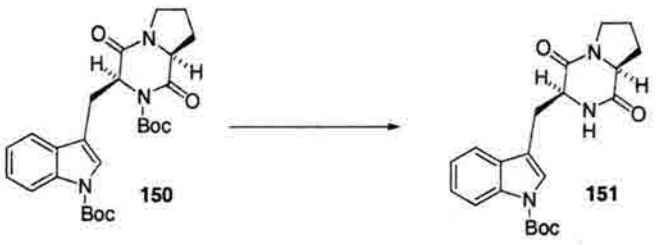
Crystalline *cyclo*-L-Trp-L-Pro (**134**) (1.5 g, 5.298 mmol) was suspended in 10 mL of dry CH_2Cl_2 under an Ar atmosphere. To this suspension, dimethylaminopyridine (DMAP) (64.7 mg, 0.53 mmol, 0.1 equiv) and triethylamine (1.072 g, 10.60 mmol, 1.48 mL, 2 equiv) were added. The mixture was cooled to –18 °C (ice-salt bath), and di*tert*-butyldicarbonate (2.312 g, 10.60 mmol, 2 equiv) was added in one portion. The mixture was stirred at that temperature for 30 min., for 2h at 0°C, and 2 h at room temperature. The reaction was diluted with 100 mL of dichloromethane, and washed with 1% aq. $KHSO_4$ soln. (2 x 50 mL), then with 5% $NaHCO_3$ aq. soln. (50 mL), and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 2.63 g of crude reaction product, which was separated by silica gel column chromatography, using toluene-EtOAc 2:1 as eluent to give 2.30 g of **150** as a viscous colorless oil (90% yield). In dmso-d_6 at 80°C, this compound appears as a mixture of two predominant rotamers in a ~2:1 proportion that interconvert slowly on the nmr time scale. For that reason, both ^1H and ^{13}C spectra include the signals for both major and minor rotamers.[218]

Major rotamer: ^1H NMR ($CDCl_3$, 25°C, 400 MHz): δ 0.11 (1H, m), 1.25 (1H, m), 1.48 (1H, m), 1.63 (9H, s), 1.56 (9H, s), 1.77 (1H, m), 3.09 (1H, ddd, J = 4.4, 10.3, 10.3 Hz), 3.46 (1H, dd, J = 5.0, 14.7 Hz), 3.56 (1H, dd, J = 2.9, 14.7 Hz), 3.59 (1H, m), 3.84 (1H, dd, J = 6.2, 11.1 Hz), 4.93 (1H, dd, J = 2.6, 4.7 Hz), 7.19 (1H, t, J = 7.3 Hz), 7.25 (1H,

bs), 7.27 (1H, t, J = 7.0 Hz) 7.47 (1H, d, J = 7.9 Hz), 8.08 (1H, d, J = 7.9 Hz). ^{13}C NMR ($CDCl_3$, 25°C, 100 MHz): δ 20.6, 27.6, 28.9, 28.9, 44.7, 60.2, 60.5, 83.7, 84.1, 113.7, 115.0, 119.6, 122.6, 124.7, 126.2, 130.2, 134.1, 149.3, 151.0, 164.0, 165.8.

Minor rotamer: ^{1}H NMR ($CDCl_3$, 25°C, 400 MHz): δ 1.40 (9H, s), 1.45(1H, m), 1.64 (9H, s), 1.84 (2H, m), 2.11 (1H, ddd, J = 5.9, 5.9, 10.9 Hz), 3.13 (1H, dd, J = 6.5, 9.4 Hz), 3.31 (3H, m, H-8, H-8'), 3.48 (1H, m), 5.03 (1H, dd, J = 5.6, 5.6 Hz), 7.22 (1H, ddd, J = 0.9, 7.3, 7.3 Hz), 7.29 (1H, ddd, J = 1.2, 7.3, 7.3 Hz), 7.57 (1H, bs), 7.52 (1H, d, J = 7.6 Hz), 8.10 (1H, d, J = 7.9 Hz). ^{13}C NMR ($CDCl_3$, 25°C, 100 MHz): δ 21.9, 27.6, 27.9, 28.1, 29.2, 45.2, 59.2, 61.1, 84.0, 84.3, 114.3, 115.3, 118.9, 122.7, 124.8, 125.3, 129.9, 135.4, 150.1, 149.4, 164.9, 167.3.

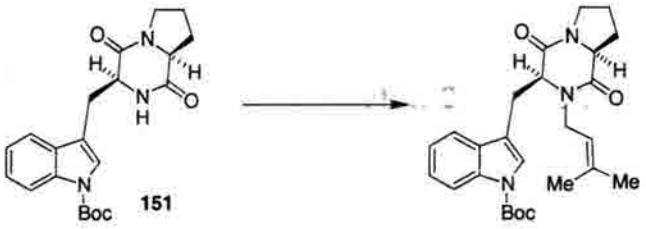
IR (neat, NaCl): 2980, 2934, 2878, 1777, 1731, 1667, 1457, 1455, 1369, 1323, 1292, 1257, 1155, 1084, 1018, 965, 917, 852, 768, 748, 732 cm^{-1}. HRMS (FAB+) Calcd for $C_{26}H_{33}N_3O_6$: 483.2369. Found 483.2368 (M+). Optical Rotation: $[\alpha]_D$ = +77.21 (CH_2Cl_2, c = 1.18).



N-BOC-*cyclo*-L-tryptophan-L-proline (151).

To a stirred suspension of N,N'-di-BOC-cyclo-L-Trp-L-Pro (**151**) (2.00 g, 4.135 mmol) in 100 mL of MeCN under an Ar atmosphere was added dimethylamine (40% aq. soln., 3.0 mL). The solution was heated to reflux for 90 min. The solvent was removed under reduced pressure, and the residue was separated by means of silica gel column chromatography, using hexane-EtOAc-MeOH (4:5:1) as eluent to give 1.48 g of **151** as a colorless glass (93% yield). This fraction was further purified using column chromatography, with CH_2Cl_2-MeOH 25:1 as eluent to give 1.39 g of pure **151** as a colorless glass (87% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.51 (1H, m), 1.58 (9H, s), 1.71 (1H, m), 1.83 (1H, m), 2.09 (1H, ddd, *J*=6.4, 6.4, 11.8 Hz), 3.13 (1H, dd, *J*=14.8, 4.8 Hz), 3.15-3.25 (3H, m, H-8, H-12), 3.51 (1H, ddd, *J*=8.8, 8.8, 12.0 Hz), 4.19 (1H, ddd, *J*=6.4, 4.4, 4.4 Hz), 7.11 (1H, br. s, H-10), 7.17 (1H, ddd, *J*=7.6, 7.6, 1.2 Hz), 7.25 (1H, ddd, *J*=7.6, 7.6, 1.2 Hz), 7.44 (1H, s), 7.51 (1H, d, *J*=7.6 Hz), 8.05 (1H, br d, *J*=8.0 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 21.9, 28.3, 29.1, 30.5, 45.5, 58.0, 58.2, 84.2, 114.7, 115.4, 119.3, 122.9, 124.9, 125.5, 130.1, 135.6, 169.4, 149.7, 165.6. IR (neat, NaCl): 3236, 2979, 2927, 2876, 1732, 1664, 1452, 1370, 1332, 1308, 1256, 1228, 1159, 1109, 1085, 1017, 865, 765, 747, 729, 699 cm^{-1}. HRMS (FAB+) Calcd for $C_{21}H_{25}N_3O_4$: 383.1845. Found 383.1842 (M+). Optical rotation: $[\alpha]_D$ = +4.45 (CH_2Cl_2, c = 0.88).

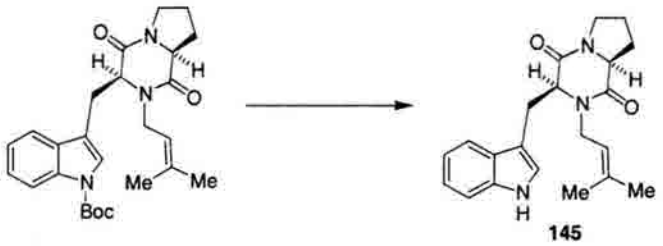


N-BOC-N'-prenyl-*cyclo*-L-tryptophan-L-proline.

To a stirred solution of compound **151** (925 mg , 2.412 mmol) in 4 mL of dry DMF under an Ar atmosphere at 0°C, NaH was added (58 mg of a 60% oil suspension, 2.412 mmol, 1 equiv). The mixture was stirred at 0°C for 30 min., and prenyl bromide (719 mg, 4.83 mmol, 2 equiv, 502 □L) was added. The reaction mixture was stirred for 1 h at 0°C, and then 2 h at room temperature. The reaction was quenched then with 5% aqueous $NaHCO_3$ (50 mL) and extracted with CH_2Cl_2 (2 x 50 mL). The organic phases were combined, washed with brine (2 x 50 mL), and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 340 mg of crude reaction product, which was separated by means of radial silica gel chromatography, using toluene-EtOAc 1:1 to 2:3 as eluent to give 640 mg of N-BOC-N'-prenyl-*cyclo*-L-tryptophan-L-proline as a colorless oil (57% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.27 (1H, m), 1.57 (3H, br. s), 1.61 (9H, s), 1.68 (3H, br. s), 1.64 (2H, m), 1.73 (1H, m), 1.99 (1H, ddd, *J*=6.4, 6.4, 12.4 Hz), 2.76 (1H, dd, *J*=11.2, 6.8 Hz), 3.09 (1H, m), 3.12 (1H, dd, *J*=14.8, 5.2 Hz), 3.32 (1H, dd, *J*=14.8, 4.4 Hz), 3.44 (1H, ddd, *J*=8.8, 8.8, 11.6 Hz), 3.55 (1H, dd, *J*=14.8, 8.8 Hz), 4.19 (1H, dd, *J*=4.8, 4.8 Hz), 4.38 (1H, dd, *J*=14.8, 6.0 Hz), 5.08 (1H, br dd, *J*=7.2, 7.2 Hz), 7.18 (1H, m), 7.24 (1H, m), 7.31 (1H, s), 7.47 (1H, d, *J*=7.6 Hz), 8.05 (1H, br d, *J*=7.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 17.7, 21.6, 25.7, 27.1, 28.0, 29.0, 41.6, 44.9, 57.9, 61.4, 83.9,

114.2, 115.1, 118.4, 119.0, 122.5, 124.7, 125.2, 129.8, 135.2, 137.6, 149.2, 165.3, 166.8. IR (neat, NaCl): 3448, 3309, 3114, 3052, 2977, 2932, 2886, 1732, 1667, 1453, 1371, 1335, 1256, 1157, 1085, 1018, 854, 768, 748, 701 cm^{-1}. HRMS (FAB+) Calcd for $C_{26}H_{34}N_3O_4$: 452.2549. Found 452.2532 (M+H). Optical rotation: $[\alpha]_D$ = +29.7 (CH_2Cl_2, c = 1.33).



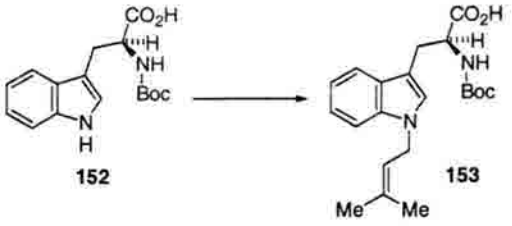
145

N'-Prenyl-*cyclo*-L-tryptophan-L-proline (145).

To a reaction vessel containing N-BOC-N'-prenyl-*cyclo*-L-tryptophan-L-proline obtained as described above (355 mg, 0.762 mmol) was added TFA (2 mL) under an Ar atmosphere at 0°C for 2h with magnetic stirring. The TFA was then removed under reduced pressure at 0°C and the resulting residue was dissolved in 50 mL of CH_2Cl_2. The solution was washed with 5% aqueous Na_2CO_3 (25 mL) and brine (25 mL), and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 250 mg of the crude reaction product, which was separated by means of radial silica gel chromatography, using CH_2Cl_2-MeOH 50:1 as eluent to give 213 mg of **145** as a colorless oil (76% yield).

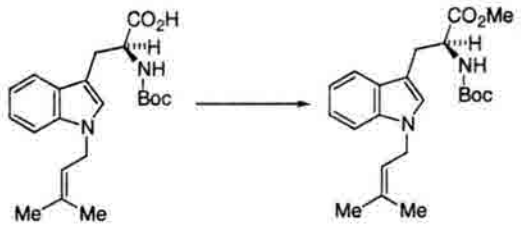
^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.14 (1H, m), 1.56 (1H, m), 1.67 (3H, br. s), 1.70 (1H, m), 1.73 (3H, br. s), 1.89 (1H, br. ddd, J=6.4, 6.4, 12.8 Hz), 2.18 (1H, dd, J=10.8, 6.4 Hz), 2.97 (1H, ddd, J=2.0, 9.2, 12.0 Hz), 3.19 (1H, dd, J=14.8, 4.8 Hz), 3.42 (1H, ddd, J=8.4, 8.4, 12.0 Hz), 3.49 (1H, dd, J=14.8, 3.2 Hz), 3.60 (1H, dd, J=14.4, 8.8 Hz), 4.22 (1H, dd, J=4.0, 4.0 Hz), 4.55 (1H, dd, J=14.4, 5.6 Hz), 5.13 (1H, br dd, J=7.2, 7.2 Hz), 6.92 (1H, d, J= 2.4 Hz), 7.08 (1H, dd, J= 7.5, 7.5 Hz), 7.14 (1H, dd, J= 7.5, 7.5 Hz), 7.30 (1H, d, J=7.5 Hz), 7.55 (1H, d, J=7.5 Hz), 8.41 (1H, br s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 17.9, 21.6, 25.8, 27.1, 29.2, 41.2, 44.8, 57.9, 61.7, 109.3, 111.1, 118.4, 118.9, 119.7,

122.4, 123.9, 127.2, 136.0, 137.9, 165.6, 167.5. IR (neat, NaCl): 3281, 3069, 2979, 2027, 2879, 1667, 1659, 1651, 1637, 1455, 1338, 1256, 1252, 1215, 1154, 1105, 1010, 971, 921, and 741 cm^{-1}. HRMS (FAB+) Calcd for $C_{21}H_{26}N_3O_2$: 352.2025. Found 352.2020 (M+H). Optical rotation: $[\alpha]_D = +70.1$ (CH_2Cl_2, c = 0.83).



N-Prenyl-N'-BOC-L-tryptophan (153).

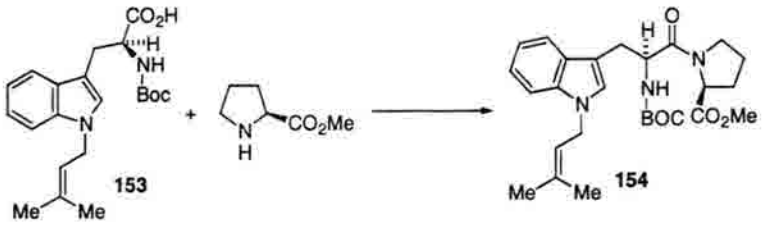
To a stirred solution of N-BOC-L-Trp (**152**) (2.045 g, 6.72 mmol) under an argon atmosphere in 10 mL of dry DMF at 0°C (ice bath), was added NaH (733 mg of a 55% oil suspension, 16.80 mmol, 2.5 equiv). The mixture was stirred at 0°C for 10 min., and prenyl bromide (1.502 g, 10.08 mmol, 1.5 equiv, 1.17 mL) was then added. The reaction mixture was stirred for 1 h at 0°C, then for 2 h at room temperature. The reaction was quenched with 50 mL of water and washed with 25 mL of hexane. The hexane wash was discarded; the aqueous layer was acidified with 1M aqueous $NaHSO_4$ until the pH=3. The mixture was extracted with CH_2Cl_2 (3 x 25 mL); the organic layers were combined, washed with water (2 x 25 mL), and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 2.04 g of crude reaction product **153** as a yellowish oil, which was used directly in the following reaction without further purification (68% yield). Attempts to purify this substance through crystallization from several solvents were unsuccessful. Considering that this compound was difficult to purify, a small portion was transformed into its methyl ester in the following reaction for characterization.



N-Prenyl-N'-BOC-L-tryptophan methyl ester.

To a stirred solution of crude **153** (133 mg, 0.358 mmol) in 1 mL of MeOH at 0°C, was added a solution of $TMSCHN_2$ (2M solution in hexane) via syringe until the N_2 evolution ceased and the yellow color was persistent. Removal of the solvent under reduced pressure gave a residue that was purified by means of silica gel column chromatography, using toluene-EtOAc (16:1) as eluent to yield 73 mg of pure ester (53% yield).

^1H NMR ($CDCl_3$, 400 MHz): δ 1.42 (9H, s), 1.75 (3H, bs), 1.80 (3H, bs), 3.25 (2H, bs), 3.66 (3H, s), 4.62 (3H, m), 5.04 (1H, d, J = 7.6 Hz), 5.33 (1H, m), 6.88 (1H, s), 7.08 (1H, dd, J = 8.0, 8.0 Hz), 7.18 (1H, dd, J = 8.0, 8.0 Hz), 7.28 (1H, d, J = 8.0 Hz), 7.51 (1H, d, J = 8.0 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 18.0, 25.6, 27.9, 28.3, 43.9, 52.1, 54.2, 79.7, 108.5, 109.5, 118.9, 119.1, 119.8, 121.5, 126.0, 128.4, 136.1, 136.3, 155.2, 172.8. IR (neat, NaCl): 3391, 3048, 2976, 2931, 1746, 1714, 1613, 1503, 1468, 1391, 1366, 1251, 1209, 1167, 1060, 1014, 856, 778, 739 cm^{-1}. Optical rotation: $[\alpha]_D$ = +26.0 (CH_2Cl_2, c = 1.23). HRMS (FAB+) Calcd for $C_{22}H_{30}N_2O_4$: 386.2206. Found 386.2202 (M+).

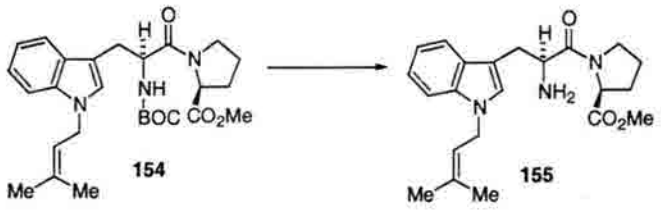


N-Prenyl-N'-BOC-L-tryptophyl-L-proline methyl ester (154).

To a stirred solution of crude **153** (1.144 g, 3.071 mmol if 100% pure) in 10 mL of THF was added proline methyl ester hydrochloride (1.017 g, 6.142 mmol, 2 equiv). The resulting mixture was cooled to 0°C, and Et_3N (684 mg, 6.756 mmol, 2.2 equiv, 938 µL) was added dropwise via syringe over 5 min. To this mixture was added 1-hydroxybenzotriazole (415 mg, 3.071 mmol, 1 equiv) followed by the addition of DCC in small portions (665 mg, 3.22 mmol, 1.05 equiv). The ice bath was removed, and the temperature was allowed to reach 25°C. The reaction was complete after 6 h at that temperature (TLC analysis). The reaction was worked-up by first filtering off the DCU, followed by washing with Et_2O (5 x 10 mL) and combining the filtrate with the washings. The solvents were removed under reduced pressure, and the residue was taken up in 100 mL EtOAc. The resulting solution was washed with 5% aqueous $NaHCO_3$ (25 mL), 10% aqueous citric acid solution, again with 5% aqueous $NaHCO_3$ (25 mL), and brine (25 mL), and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 1.47 g of the crude reaction product, which was separated by means of silica gel column chromatography, using hexane-EtOAc 1:1 as eluent to give 1.018 g of pure **154** as a colorless glass (69% yield).

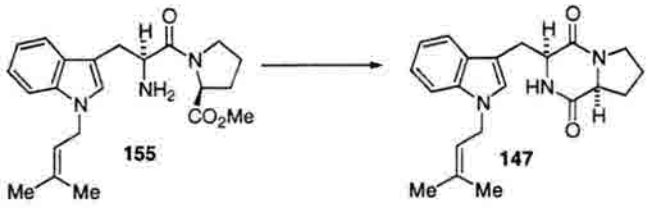
^{1}H NMR (400 MHz, dmso-d_6, 80°C): δ 1.28 (9H, s), 1.69 (3H, br. s), 1.70-1.89 (3H, m), 1.77 (3H, bs), 2.13 (1H, m), 2.89 (1H, dd, J = 14.4, 8.0 Hz), 3.02 (1H, dd, J = 14.4, 5.6

Hz), 3.32 (1H, ddd, J = 9.6, 6.4, 6.4 Hz), 3.57 (1H, m), 4.33 (1H, dd, J = 8.8, 5.2 Hz), 4.45 (1H, m), 4.65 (2H, d, J = 6.8 Hz), 5.31 (1H, dd, J =7.2, 7.2 Hz), 6.48 (1H, bs), 6.99 (1H, dd, J = 8.0, 8.0 Hz), 7.09 (1H, dd, J = 8.0, 8.0 Hz), 7.13 (1H, s), 7.32 (1H, d, J = 8.0 Hz), 7.53 (1H, d, J=8.0 Hz). ^{13}C NMR (100 MHz, dmso-d_6, 80°C): δ 18.3, 25.2, 25.8, 27.8, 28.8, 29.2, 44.1, 47.1, 52.2, 59.3, 78.8, 110.1, 110.3, 119.0, 119.2, 119.2, 121.1, 121.6, 127.6, 128.7, 135.8, 136.6, 171.2, 172.9. IR (neat, NaCl): 3433, 3306, 3051, 2975, 2932, 2867, 1747, 1731, 1644, 1496, 1455, 1366, 1251, 1171, 1098, 1054, 1014, 859, 739 cm^{-1}. HRMS (FAB+) Calcd for $C_{27}H_{38}N_3O_5$: 484.2811. Found 484.2803 (M+H). Optical rotation: $[\alpha]_D$ = -17.6 (CH_2Cl_2, c = 1.23).



N-Prenyl-L-tryptophyl-L-proline methyl ester (155).

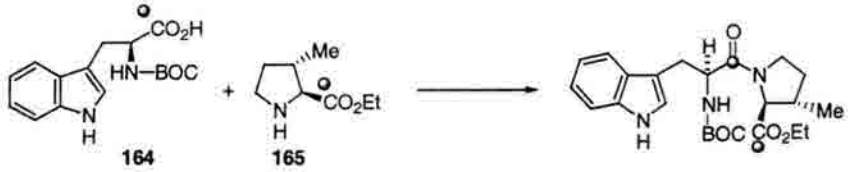
To a stirred solution of compound **154** (821 mg, 1.698 mmol) in 2 mL of dry CH_2Cl_2 at 0°C was added 2 mL of TFA under an Ar atmosphere. The mixture was allowed to stir for 6h. The solvent was removed under reduced pressure at 0°C and the resulting residue was taken up in 100 mL of EtOAc. The solution was washed with 5% aqueous Na_2CO_3 (25 mL), brine (25 mL), and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 514 mg of crude reaction product, which, by TLC analysis was found to contain a significant amount of the diketopiperazine **147**. For this reason, together with the fact that the purification of this crude mixture proved to be very difficult, the crude product was used directly in the next step. (96% crude yield).



N-Prenyl-*cyclo*-L-tryptophyl-L-proline (147).[219]

To a stirred solution of crude compound **155** (350 mg, 0.767 mmol) in 10 mL of dry toluene at 0°C under an Ar atmosphere was added 2-hydroxypyridine (0.1 eq., 0.077 mmol, 0.7 mg). The resulting solution was refluxed for 6 h. The toluene was then removed under reduced pressure and the resulting residue was purified by means of silica gel column chromatography, using the toluene-EtOAc-MeOH (12:10:1) as eluent to give 151 mg of the pure diketopiperazine **147** (59% yield).

^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.82 (3H, bs), 1.88 (3H, bs), 1.88-1.95 (1H, m), 1.97-2.10 (2H, m), 2.35 (1H, m), 2.98 (1H, dd, *J* = 15.0, 10.6 Hz), 3.56-3.70 (2H, m), 3.78 (1H, ddd, *J* = 15.0, 3.7, 0.7 Hz), 4.09 (1H, dd, *J* = 7.5, 7.5 Hz), 4.39 (1H, dd, *J* = 10.6, 3.7 Hz), 4.70 (2H, d, *J* = 7.0 Hz), 5.41 (1H, ddqq, *J* = 7.0, 7.0, 1.5, 1.5 Hz), 5.88 (1H, bs), 7.16 (1H, dd, *J* = 7.5, 7.5 Hz), 7.05 (1H, s), 7.27 (1H, dd, *J* = 7.5, 7.5 Hz), 7.37 (1H, d, *J* = 7.5 Hz), 7.61 (1H, d, *J* = 7.5 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 18.3, 22.8, 25.9, 27.0, 28.5, 44.3, 45.6, 54.9, 59.4, 108.5, 110.2, 118.9, 119.7, 119.8, 122.4, 126.8, 127.6, 136.8, 136.9, 165.8, 169.6. IR (neat, NaCl): 3361, 3244, 3053, 2975, 2923, 2874, 1777, 1668, 1546, 1466, 1374, 1312, 1218, 1168, 1129, 1014, 919, 847, 738, 700 cm^{-1}. HRMS (FAB+) Calcd for $C_{21}H_{25}N_3O_2$: 352.2025. Found 252.2021 (M+H). Optical rotation: $[\alpha]_D$ = -107.9 (CH_2Cl_2, c = 0.80).



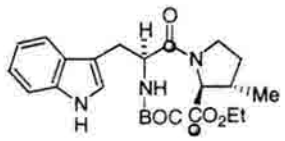
164 **165**

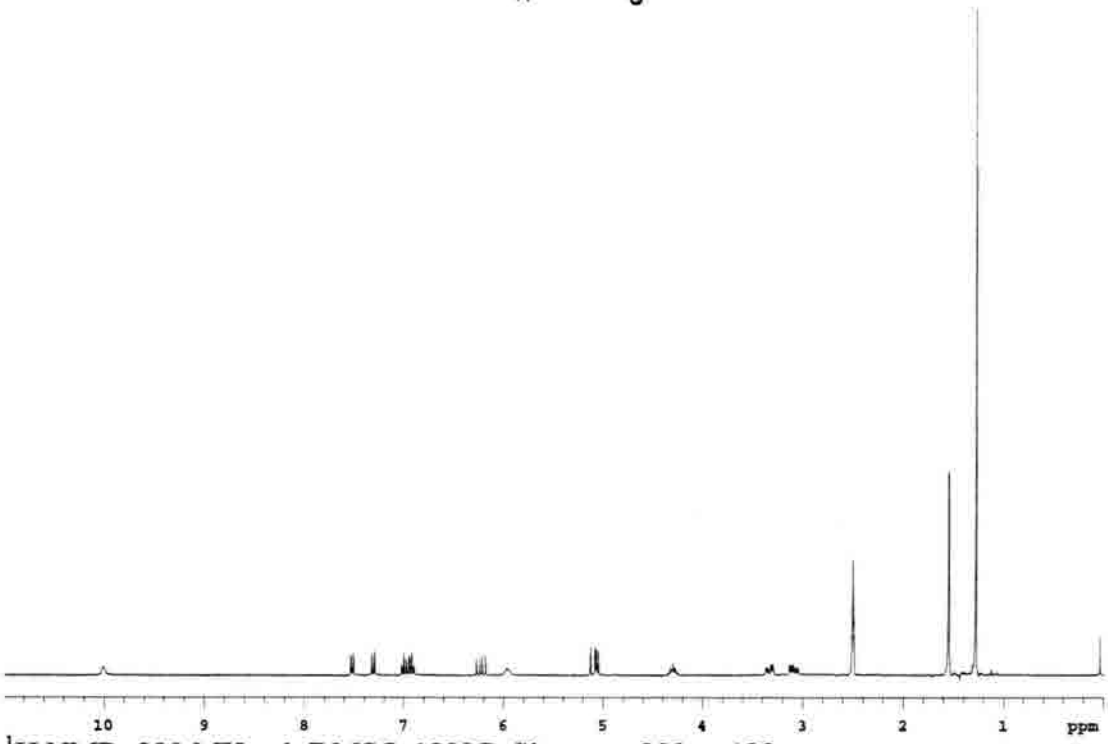
[$^{13}C_2$]-N-Boc-L-tryptophan-3(*S*)-methyl-L-proline ethyl ester:

[1-^{13}C]-L-tryptophan (60 mg, 0.292 mmol) was dissolved in 1.17 mL of a 1:1 mixture of dioxane and water and cooled to 0°C under an inert atmosphere. Di-*tert*-butyl-dicarbonate (76.1 μL, 0.331 mmol, 1.1 eq) and anhydrous K_2CO_3 (40.3 mg, 0.292 mmol, 1 eq.) were added to the stirring solution. The reaction mixture was brought to room temperature and stirred for 3 hrs. The dioxane was removed under reduced pressure, the reaction mixture was lowered to pH=2 with 10% aqueous $KHSO_4$, and extracted 4 times with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo* to afford N-Boc-L-[1-^{13}C]tryptophan (**164**). In a separate reaction vessel, N-Boc-3(*S*)-methyl-L-proline ethyl-ester (**165**, 75.4 mg, 0.292 mmol) was taken up in 0.5 mL of CH_2Cl_2 and placed under argon with stirring at 0°C. To this solution, TFA (469 uL, 5.84 mmol, 20 eq.) was added slowly. The solution was allowed to come to room temperature while continuing to stir over 3 hours. The CH_2Cl_2 and excess TFA were removed *in vacuo* to leave TFA salt of **165** as an oil. The crude protectected amino acids were combined with one equivalent of BOP reagent (129 mg, 0.292 mmol) and 2 equivalents of triethylamine (81.4 μL, 0.584 mmol) in 438 mL of dry acetonitrile. The reaction was stirred for 3.5 hours at room temperature under an argon atmosphere. A saturated aqueous solution of NaCl was added and the mixture was extracted three times with EtOAc. The combined organic extracts were dried over anhydrous $NaSO_4$ and

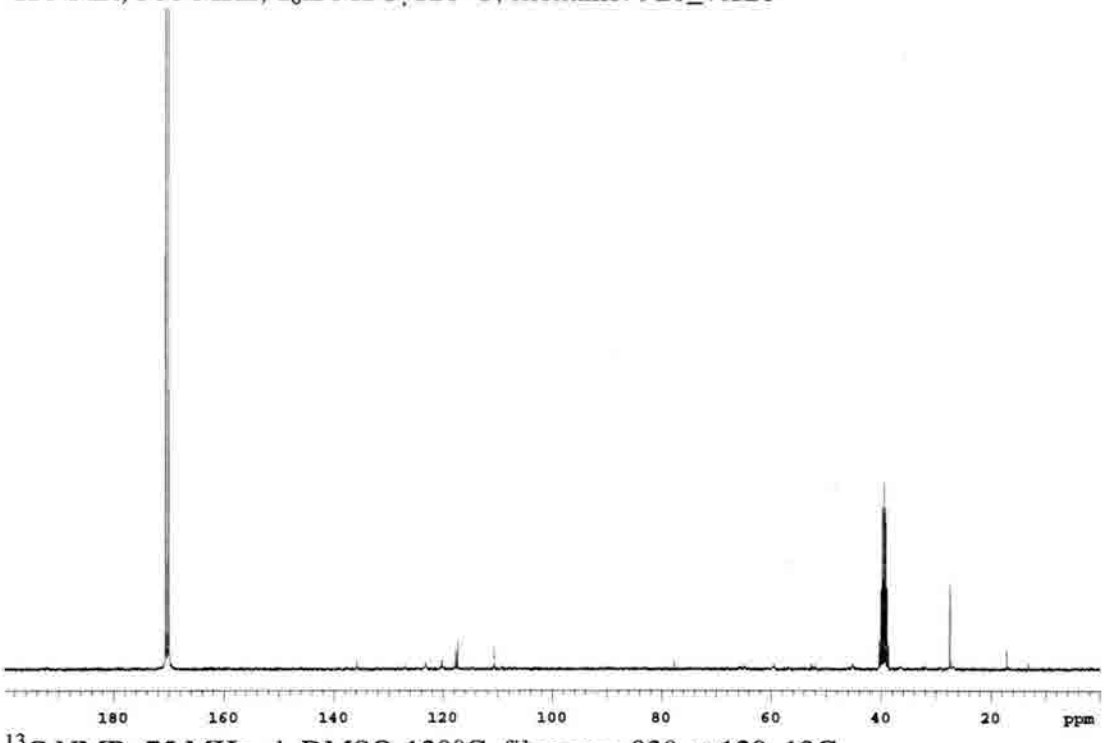
concentrated under reduced pressure. The crude product was purified via flash column chromatography (eluted with 50% EtOAc/ hexanes) to provide [$^{13}C_2$] N-Boc-L-tryptophan-L-3(*S*)-methyl-proline ethyl ester as a foam. An analytical sample of unlabeled material was recrystallized from EtOAc and hexanes to provide a white solid, melting point 152.5 °C. Yield: 101.8 mg, 0.229 mmol, 78% 2 steps.

^{1}H NMR (300 MHz, d_6-DMSO, 120°C) [220]: δ 1.12 (3H, bs), 1.21 (3H, t, *J*= 7.3 Hz), 1.34 (10H, s), 1.57 (1H, m), 2.08 (1H, bs), 2.23 (1H, bs), 2.87-3.37 (4H, m), 3.86 (1H, m), 4.13 (2H, dd, *J*= 7.0, 14.3 Hz), 4.54 (1H, bs), 6.11 (1H, bs), 7.00 (1H, dd, *J*= 1.1, 7.0 Hz), 7.08 (1H, dd, *J*= 1.1, 7.0 Hz), 7.35 (1H, d, *J*= 8.0), 7.56 (1H, d, *J*= 8.0), 10.48 (1H, bs). ^{13}C NMR (75 MHz, d_6-DMSO, 120°C)[220]: δ 13.2, 17.1, 26.8, 32.1, 36.4, 45.2, 52.4 (d, J_{CC}= 55 Hz), 59.5, 65.3 (d, J_{CC}= 60 Hz), 77.7, 109.2, 110.6, 117.3, 117.7, 120.2, 123.1, 127.0, 135.8, 154.1, 170.0, 170.5. IR (NaCl, CH_2Cl_2): 3323, 3057, 2966, 2926, 2873, 1738, 1711, 1648, 1510, 1455, 1391, 1366, 1351, 1297, 1255, 1170, 1098, 1030, 955, 863, 742 cm^{-1}. $[\alpha]_D^{25}$= -18.9° (c=1.2, CH_2Cl_2). HRMS (FAB$^+$) calcd for $C_{22}{}^{13}C_2H_{34}O_5N_3$ (M+H) 446.2566, found 446.2574.

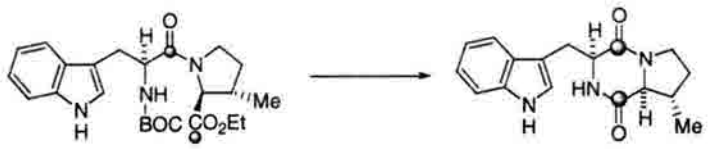




^{1}H NMR, 300 MHz, d_6-DMSO, 120°C, filename: 920_vt120



^{13}C NMR, 75 MHz, d_6-DMSO, 120°C, filename: 920_vt120_13C



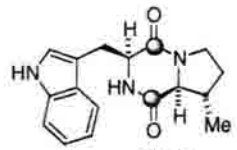
158a

[$^{13}C_2$]-*cyclo*- L-tryptophan-L-3(*S*)-methyl-proline (158a):

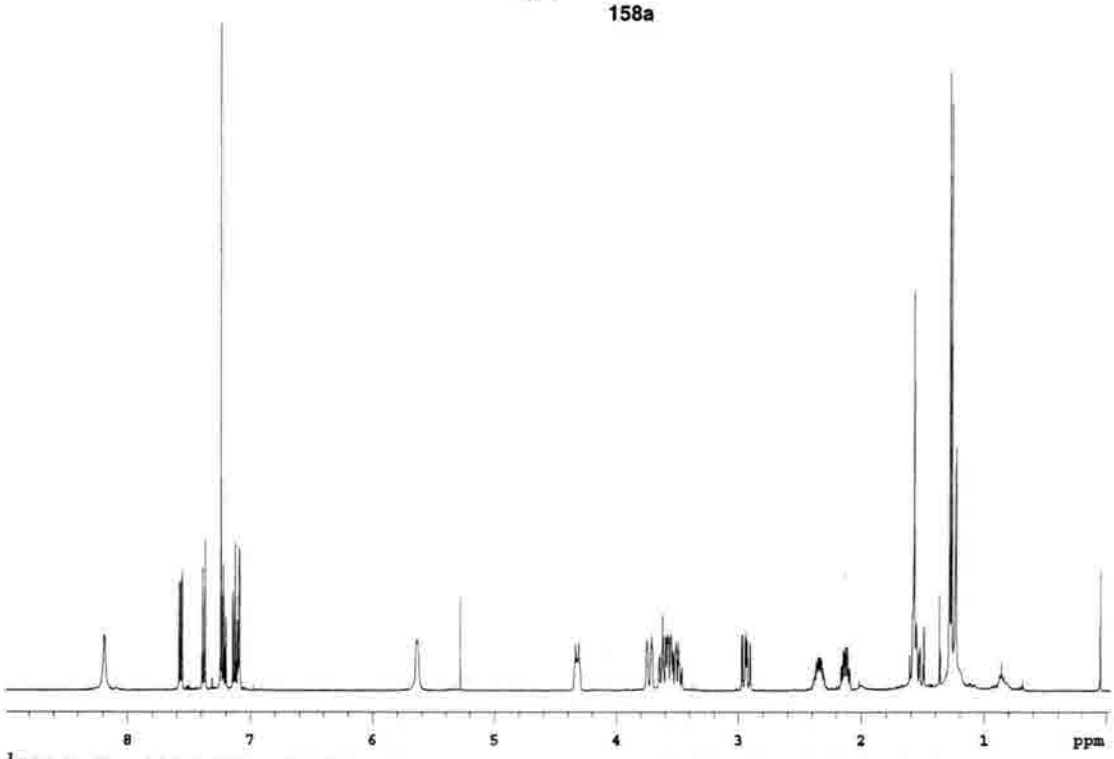
To a stirring solution of [$^{13}C_2$]-N-Boc-L-tryptophan-3(*S*)-methyl-L-proline ethyl ester (102 mg, 0.229 mmol) at 0°C under an inert atmosphere, TFA (353 μL, 4.58 mmol, 20 eq.) was slowly added. The solution was brought to room temperature and was stirred for 3 hours. The CH_2Cl_2 and excess TFA were removed *in vacuo*. The residue was taken up in aqueous 10% $NaCO_3$ and extracted 3 times with EtOAc. The combined organic extracts were dried over anhydrous $NaSO_4$ and concentrated under reduced pressure. The crude product was dissolved in 1.5 mL of toluene, 2-hydroxy pyridine (4.4 mg, 0.046 mmol, 0.2 eq) was added, and the solution refluxed under an argon atmosphere for 4 hours. The toluene was removed under reduced pressure and the product purified by flash column chromatography (eluted with 2% CH_3OH/ CH_2Cl_2) to provide [$^{13}C_2$]-*cyclo*-L-tryptophan-3(*S*)-methyl-L-proline (**158a**) as a white solid. Yield: 42 mg, 0.140 mmol, 61%.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.27 (3H, d, *J*= 6.6 Hz), 1.53 (1H, m), 2.13 (1H, m), 2.34 (1H, m), 2.94 (1H, ddd, *J*= 2.3, 10.9, 14.8 Hz), 3.55 (3H, m), 3.73 (1H. d, *J*= 14.7 Hz), 4.32 (1H, m), 5.63 (1H d, *J*= 4.3 Hz), 7.09 (1H, d, *J*= 2.0 Hz), 7.12 (1H, ddd, *J*= 0.8, 7.8, 7.8 Hz), 7.22 (1H, ddd, *J*= 1.2, 8.2, 8.2 Hz), 7.38 (1H, d, *J*= 8.2 Hz), 7.57 (1H, d, *J*= 8.5 Hz), 8.19 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.2, 26.8, 31.6, 37.1, 44.1, 54.2 (d, J_{CC}= 52 Hz), 64.0 (d, J_{CC}= 52 Hz), 109.9(d, J_{CC}= 29 Hz), 111.5, 118.5, 120.0, 122.8,

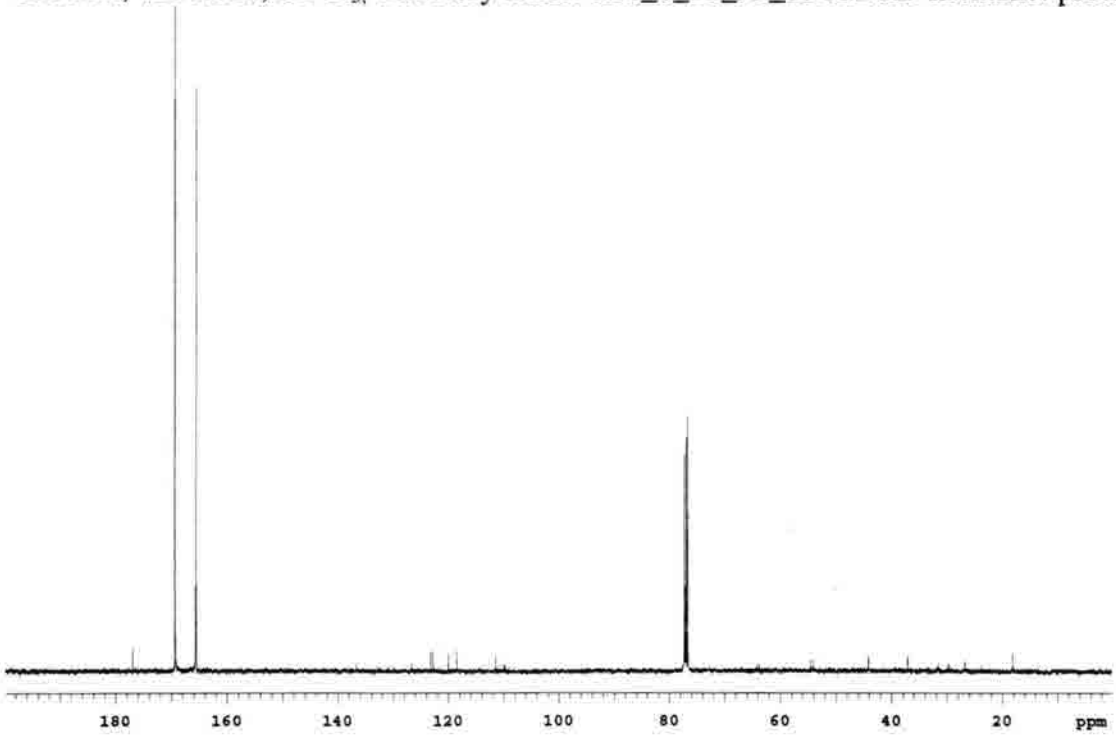
123.2, 126.7, 136.6, 165.6, 169.4. IR (NaCl, CH_2Cl_2): 3389, 3054, 2922, 2851, 1632, 1612, 1454, 1415, 1301, 1102, 737, 693, 673 cm^{-1}. HRMS (FAB^+) calcd for $C_{15}{}^{13}C_2H_{20}O_2N_3$ (M+H) 300.1623, found 300.1617. Optical Rotation: $[\alpha]_D^{25}$= -92.9° (c=1.2, 5% CH_3OH/CH_2Cl_2).



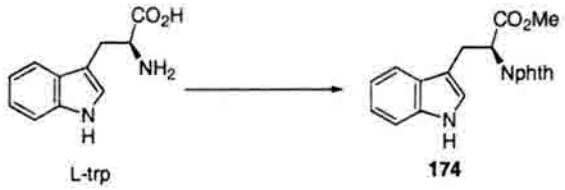
158a



^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems_6_23_99_13C2DKP filename: proton



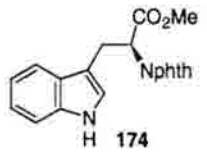
^{13}C NMR, 400 MHz, $CDCl_3$, filename: ems_6_23_99_13C2DKP



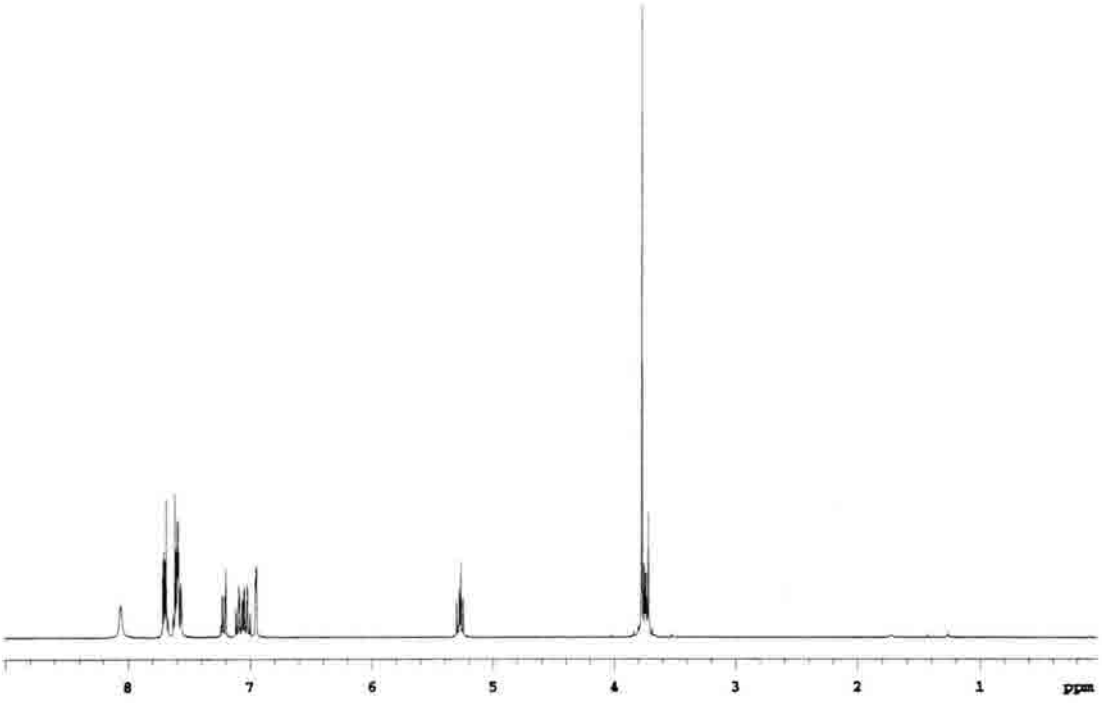
N-phthalamido-L-tryptophan methylester (174):

L-tryptophan (4.08 g, 20mmol), N-carboethoxyphthalimide (4.38 g, 20 mmol) and sodium carbonate (2.12 g, 20 mmol) were stirred in 125 mL of H_2O at room temperature for 6 hours under an atmosphere of argon. The solution was extracted with ether (2 X 25 mL) then acidified to pH=2 with 10% $KHSO_{4\,(aq)}$ and extracted with ethyl acetate (3 X 30). The combined organic layers were washed with a saturated solution of $NaCl_{\,(aq)}$ and then dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure to leave a yellow foam, 6.87 g (crude). A portion of the N-phthalamido-L-tryptophan (1 g, 2.99 mmol) were refluxed in 50 mL of anhydrous methanol with D,L-camphorsulfonic acid (243 mg, 1.05 mmol) for 24 hours though an addition funnel containing 3 Å molecular sieves under an argon atmosphere. The methanol was removed under reduced pressure and the residue was covered with a layer of ethyl acetate and washed with saturated $Na_2CO_{3\,(aq)}$ three times. The aqueous layer was separated from the organic and then extracted three more times with ethyl acetate. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo* and the product purified by flash column chromatography (10:5:1 dichloromethane/ hexane/ ether) to give **379** (776 mg, 2.23 mmol, 69% from L-trp).

^{1}H NMR (300 MHz, $CDCl_3$): δ 3.73 (1H, d, J = 5.9 Hz), 3.74 (1H, d, J = 9.5 Hz), 3.77 (3H, s), 5.27 (1H, dd, J = 6.6, 9.5 Hz), 6.95 (1H, d, J = 2.6 Hz), 7.02 (1H, ddd, J = 1.1, 7.3, 7.3 Hz), 7.09 (1H, ddd, J =1.1, 7.0, 7.0 Hz), 7.22 (1H, d, J = 6.6 Hz), 7.58 (1H, d, J = 7.7 Hz), 7.60(1H, dd, J = 2.9, 5.5 Hz), 7.61 (1H, d, J = 2.9 Hz), 7.69 (1H, d, J = 2.9 Hz), 7.70 (1H, dd, J = 2.9, 5.5 Hz), 8.06 (1H, bs). ^{13}C NMR (75 MHz, CD_3OD): δ 25.8, 53.6, 53.9, 111.9, 112.7, 119.4, 120.5, 123.0, 123.6, 124.4, 128.1, 132.6, 135.0, 137.1, 1687.6, 170.7. IR (neat, NaCl): 3417, 3059, 2954, 1747, 1731, 1714, 1614, 1455, 1392, 1258, 1196, 1105, 1010, 911, 881, 792, 718, 648, 616, 532 cm^{-1}.



174



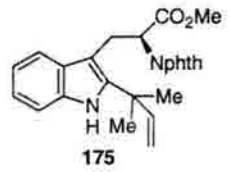
^{1}H NMR,300 MHz, $CDCl_3$, Filename: ems635

174 175

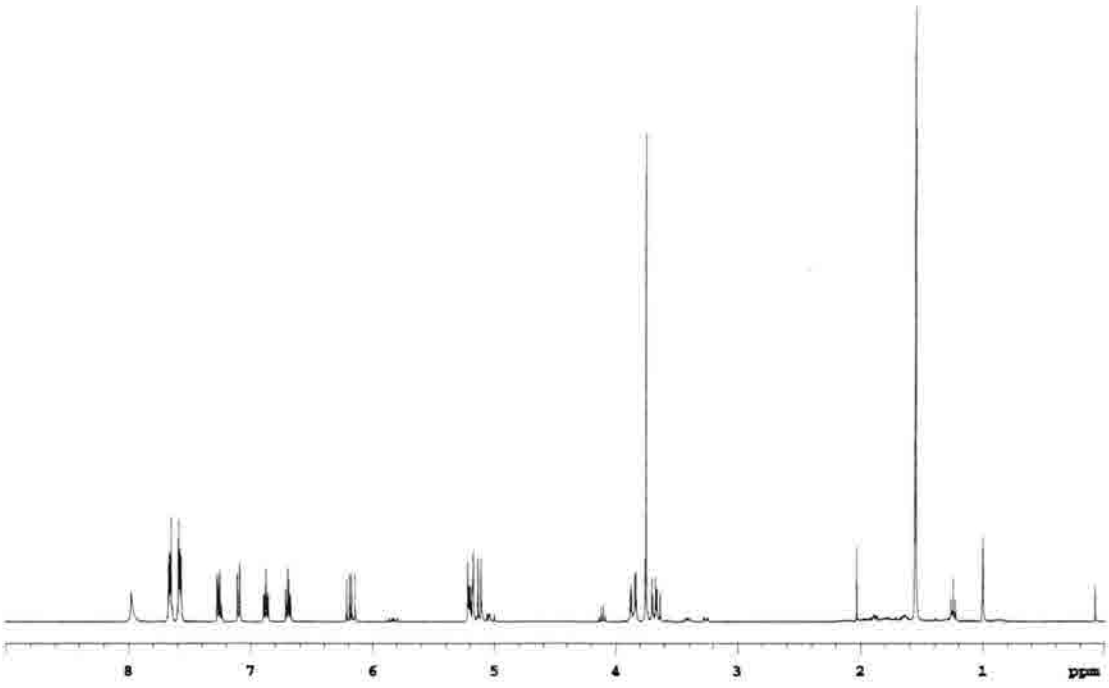
N-phthalamido-2-dimethylallyl-L-tryptophan methylester (175):

A solution **174** (744 mg, 2.13 mmol) in THF (25 mL) was cooled to -78°C under an argon atmosphere. Freshly prepared *tert*-butyl-hypochlorite (767 μL) was added dropwise and the mixture was stirred for 2 hours while maintaining a temperature of -78°C. Upon the dropwise addition of a THF solution of prenyl 9-BBN (0.5 M) at –78 °C, the reaction mixture turned yellow. The reaction was brought to room temperature and stirred overnight. The solvent was evaporated under reduced pressure and the product purified via flash column chromatography (4:1 hexanes/ EtOAc) to provide **175** in 63-85% yield (555 mg, 1.43 mmol).

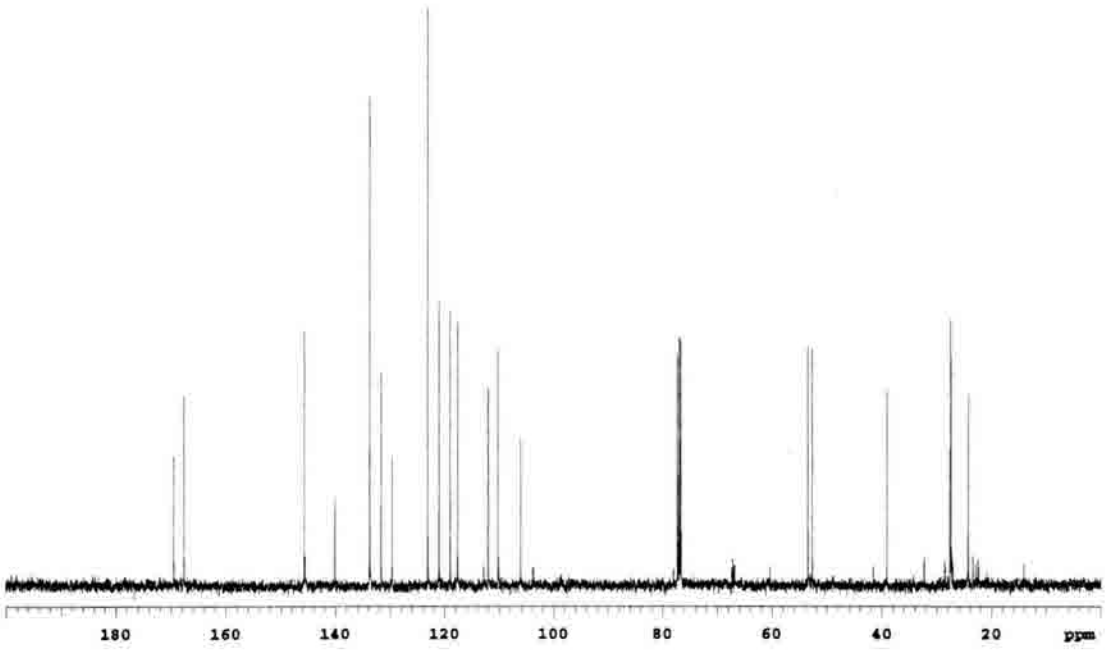
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.55(6H, s), 3.67 (1H, dd, *J* = 11.3, 15.2 Hz), 3.76 (3H, s), 3.86 (1H, dd, *J* = 3.9, 15.2 Hz), 5.12 (1H, dd, *J* = 0.8, 10.5 Hz), 5.19 (1H, dd, *J* = 3.9, 11.3 Hz), 5.20 (1H, dd, *J* = 0.8, 17.6 Hz), 6.18 (1H, dd, *J* = 10.5, 17.6 Hz), 6.70 (1H, ddd, *J* = 0.8, 7.8, 7.8 Hz), 6.88 (1H, ddd, *J* = 1.2, 7.8, 7.8 Hz), 7.10 (1H, d, *J* = 7.8 Hz), 7.27 (1H, d, *J* = 7.8 Hz), 7.58 (1H, dd, *J* = 3.1, 5.5 Hz), 7.59 (1H, d, *J* = 3.1 Hz), 7.65 (1H, dd, *J* = 3.1, 5.5 Hz), 7.66 (1H, d, *J* = 3.1 Hz), 7.98 (1H, bs). ^{13}C NMR (100 MHz, CD_3OD): δ 24.8, 27.4, 27.6, 39.1, 106.1, 110.2, 112.0, 117.6, 119.0, 121.0, 123.1, 129.6, 131.7, 133.8, 133.9, 140.1, 145.7, 167.7, 169.6. HRMS (FAB+): Calcd for $C_{25}H_{24}N_2O_4$: 416.1736. Found 416.1739 (M+H).



175



^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems640_pure_set, filename: proton



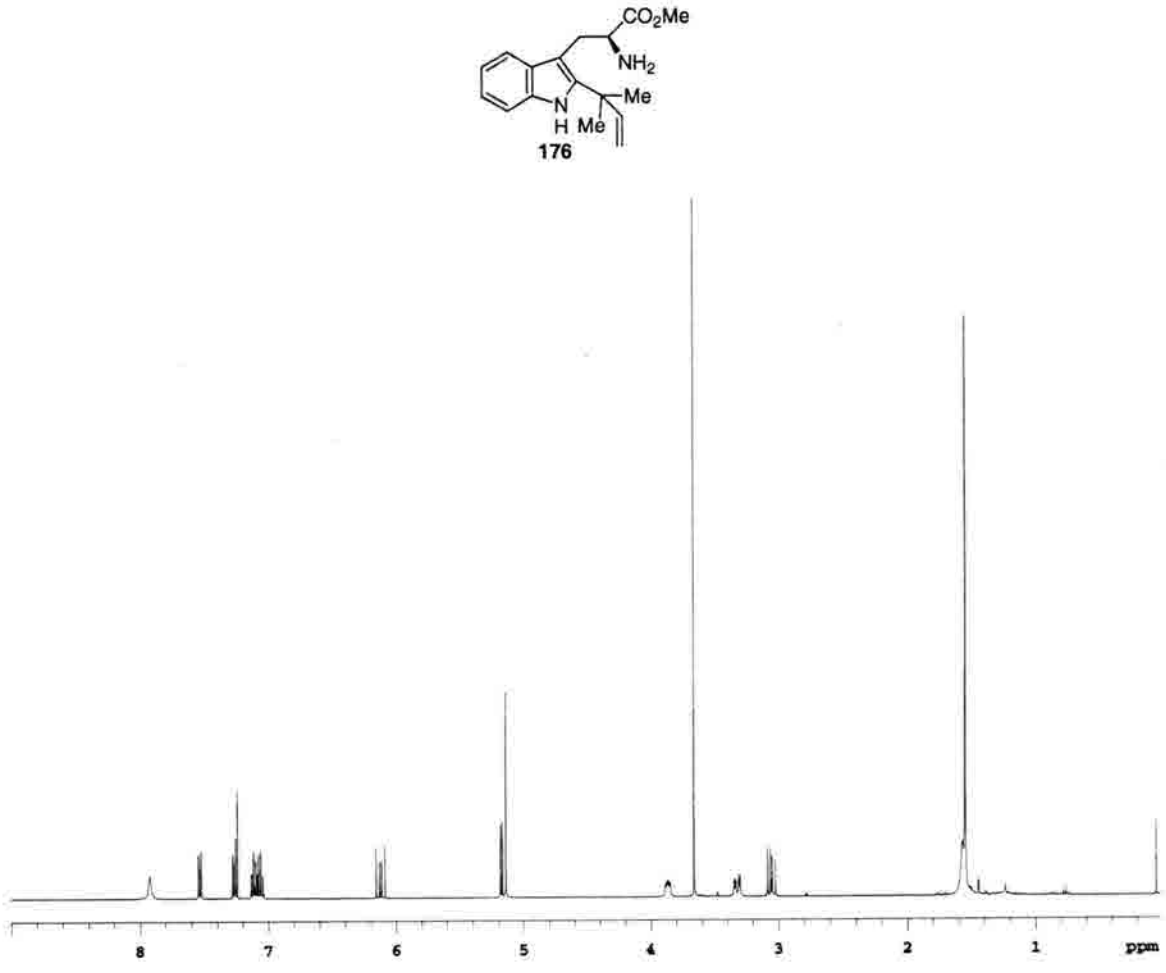
^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems640_pure_set, filename: carbon

175 176

2-dimethylallyl-L-tryptophan methylester (176):

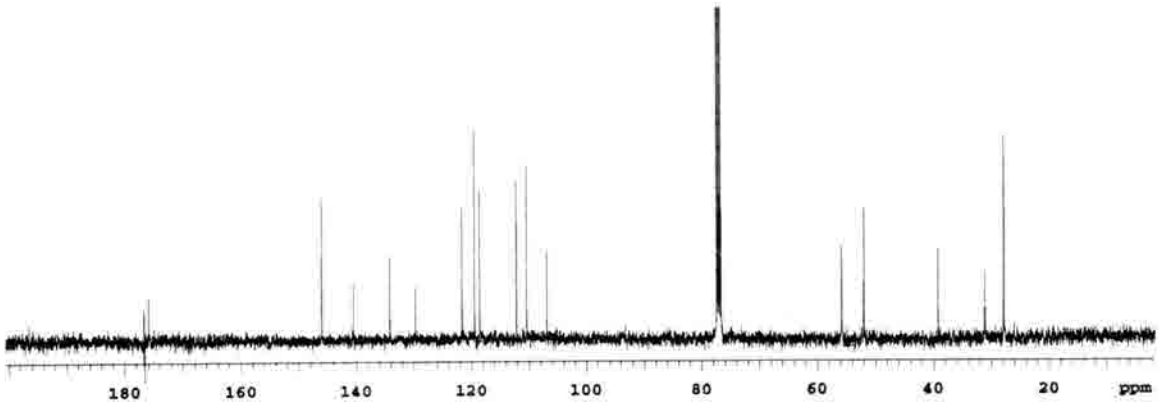
A solution of **175** (1.01 g, 2.42 mmol) in a 1:3 solution of CH_3OH/ CH_2Cl_2 (24 mL) was stirred in an ice bath under an atmoshphere of argon for 30 minutes. (The presence of oxygen can cause diimide formation of the hydrazine and reduction of the double bond.) Hydrazine hydrate (411 μl, 8.48 mmol) was added and the reaction was stoppered and sealed with Teflon tape. The reaction was stirred at room temperature for 24 hours over which time a white precipitate formed. The solvent was evaporated, 1M HCl was added, and the aqueous solution was extracted three times with ethyl acetate. The organic layers were discarded. The aqueous solution was cooled in an ice bath, made basic by the addition of saturated $Na_2CO_{3\,(aq)}$, and extracted three times with ethyl acetate. The combined organic layers from the basic extraction were dried over anhydrous Na_2SO_4 and concentrated to provide **176** (561 mg, 1.96 mmol) in 81% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.55 (3H, s), 1.55 (3H, s), 1.57 (2H, bs), 3.06 (1H, dd, *J* = 9.4, 14.5 Hz), 3.32 (1H, dd, *J* = 5.0, 14.4 Hz), 3.66 (3H, s), 3.86 (1H, dd, *J* = 5.0, 9.4 Hz), 5.15 (1H, dd, *J* = 1.2, 10.5 Hz), 5.16 (1H, dd, *J* = 0.8, 17.6 Hz), 6.12 (1H, dd, *J* = 10.2, 17.6 Hz), 7.06 (1H, ddd, *J* = 1.2, 7.0, 7.0 Hz), 7.12 (1H, ddd, *J* = 1.6, 6.6, 6.6 Hz), 7.27 (1H, d, *J* = 7.8 Hz), 7.54 (1H, d, *J* = 7.8 Hz), 7.92 (1H, bs). ^{13}C NMR (100 MHz, CD_3OD): δ 18.1, 25.9, 27.8, 27.9, 31.5, 38.9, 44.0, 54.6, 63.9, 104.4, 110.4, 110.8, 112.6, 117.8, 119.9, 122.0, 128.9, 134.4, 141.4, 145.6, 165.9, 169.2. HRMS (FAB+): Calcd for $C_{17}H_{23}N_2O_2$: 287.1760. Found 287.1760 (M+H).



176

^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems799b_400, filename: proton

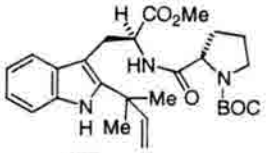


^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems799b_400, filename: carbon

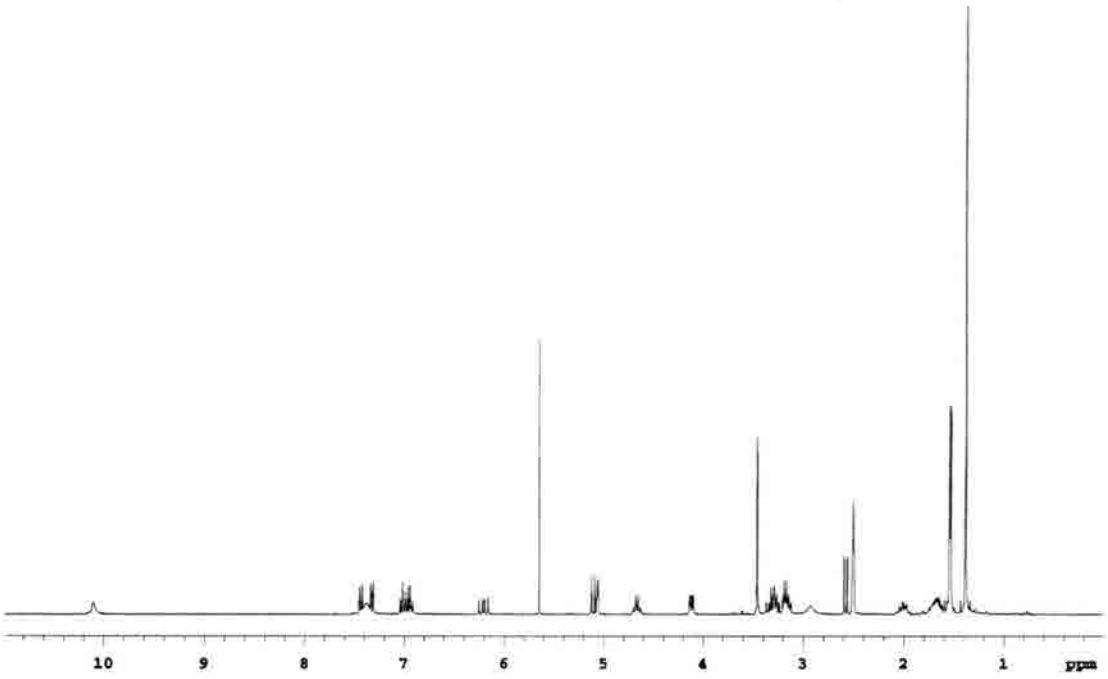
N-*tert*-butoxycarbonyl-L-proline-2-dimethylallyl-L-tryptophan methylester (177):

To a solution of **176** (77 mg, 0.269mmol) in dry acetonitrile (4.1 mL) was added N-Boc-L-proline, 58 mg, 0.269 mmol), BOP reagent (119 mg, 0.269 mmol), and Et_3N (41 μL, 0.296 mmol). The reaction mixture was stirred at room temperature under an inert atmosphere for 4 h. A saturated aqueous solution of NaCl was added and the reaction was extracted four times with EtOAc. The combined organic layers were washed with 2M HCl, water, 10% $NaHCO_3$ (aq.), water and brine successively. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product was purified by flash silica gel column chromatography using 40% EtOAc/Hex to give **177** (103 mg, 0.213 mmol) in 79% yield.

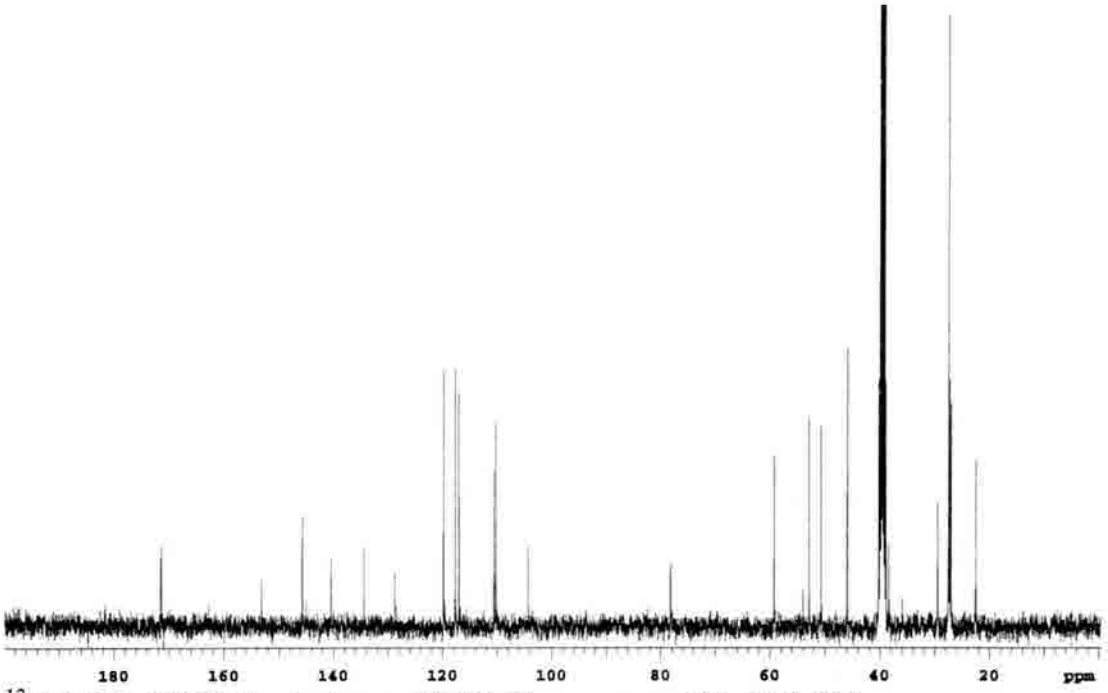
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.38 (9H, s), 1.53 (3H,s), 1.55 (3H,s), 1.67 (3H, m), 2.00 (1H, m), 2.92 (1H, bs), 3.61 (2H, m), 3.29 (2H, m), 3.46 (3H, d, J = 1.09 Hz), 4.12 (1H, dd, *J* = 3.3, 8.3 Hz), 4.66 (1H, dd, *J* = 8.1, 15.8 Hz), 5.07 (1H, d, *J* = 10.6 Hz), 5.09 (1H, d, *J* = 17.6 Hz), 6.20 (1H, ddd, *J* = 1.5, 10.6, 17.6 Hz), 6.94 (1H, t, *J* = 7.0 Hz), 7.02 (1H, t, *J* = 7.0 Hz), 7.33 (1H, d, *J* = 7.7 Hz), 7.44 (1H, d, *J* = 8.0 Hz) 10.10 (1H, bs). ^{13}C NMR (100 MHz, d_6-DMSO, 120°C): δ 22.5, 27.0, 27.2, 27.3, 27.5, 29.4, 38.3, 45.9, 50.6, 52.8, 59.2, 78.2, 104.3, 110.2, 110.6, 117.0, 117.7, 119.8, 128.7, 134.4, 140.5, 145.7, 153.1, 171.4, 171.5. IR (neat, NaCl): 3334, 3058, 2974, 2878, 1745, 1693, 1514, 1462, 1392, 1366, 1249, 1198, 1164, 1124, 1009, 919, 852, 742 cm^{-1}. HRMS (FAB+): Calcd for $C_{27}H_{38}N_3O_5$: 484.2811. Found 484.2796 (M+H).



177



^{1}H NMR, 300 MHz, d_6-dmso, 120°C, filename: ems852_dmso_120

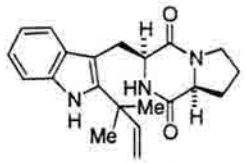


^{13}C NMR, 100 MHz, d_6-dmso, 120°C, filename: ems852_13C_VT

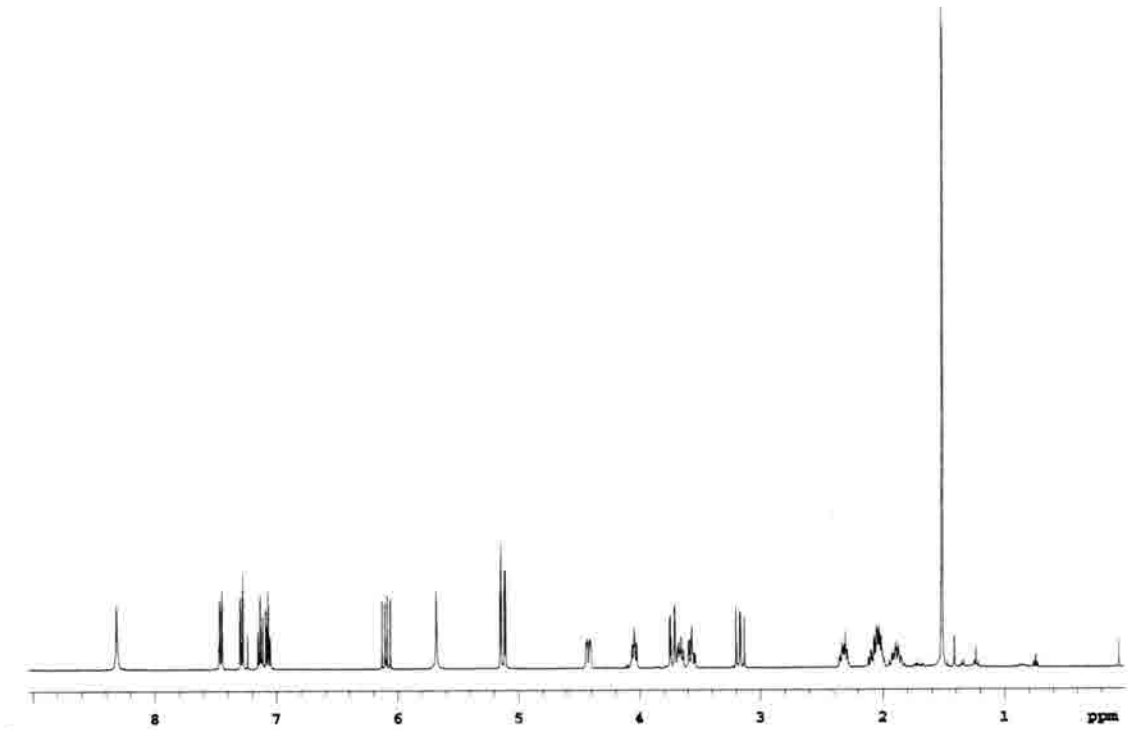
Synthetic Deoxybrevianamide E (135):

To a stirring solution of **135** (121 mg, 0.250 mmol) in CH_2Cl_2 (1 mL) at 0°C under an inert atmosphere, TFA (401 μL, 4.58 mmol) was slowly added. The solution was brought to room temperature and was stirred for 3 hours. The CH_2Cl_2 and excess TFA were removed *in vacuo*. The residue was taken up in aqueous 10% $NaCO_3$ and extracted 3 times with EtOAc. The combined organic extracts were dried over anhydrous $NaSO_4$ and concentrated under reduced pressure. The crude product was dissolved in 1.25 mL of toluene, 2-hydroxy pyridine (4.8 mg, 0.05 mmol) was added, and the solution refluxed under an argon atmosphere for 8 hours. The toluene was removed under reduced pressure and the product purified by flash column chromatography (eluted with 2% CH_3OH/ CH_2Cl_2) to give **135** as a solid (73 mg, 0.207 mmol, 83%).

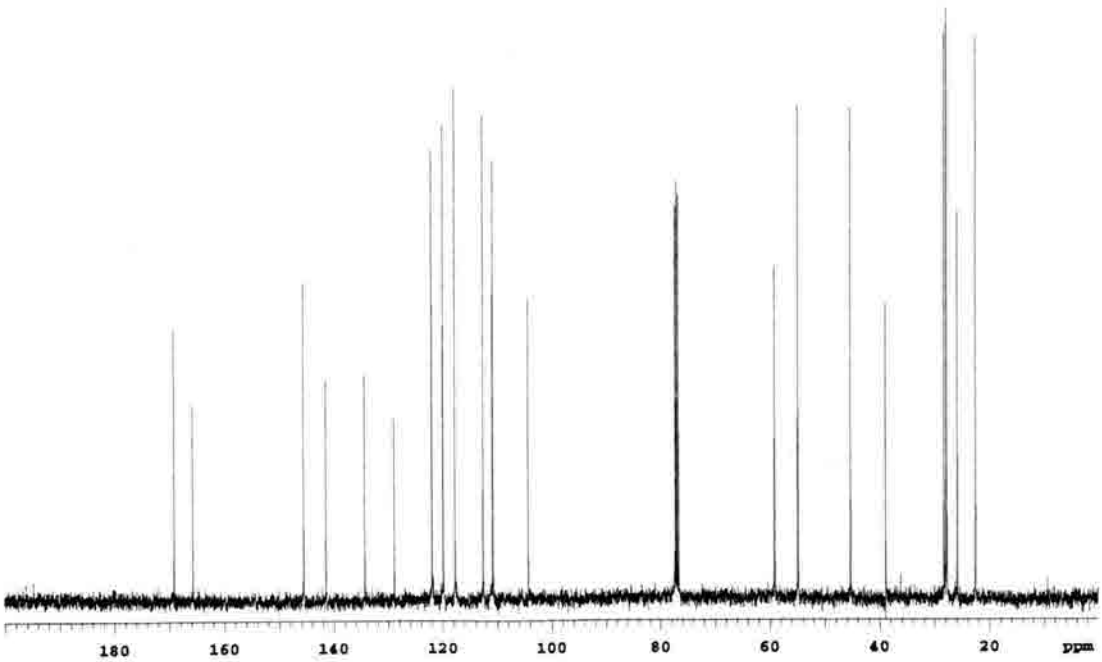
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.51 (3H, s), 1.52 (3H, s), 1.88 (1H, m), 2.05 (2H, m), 2.31 (1H, m), 3.17 (1H, dd, *J* = 11.7, 15.2 Hz), 3.56 (1H, m), 3.66 (1H, m), 3.73 (1H, dd, *J* = 4.3, 15.6 Hz), 4.04 (1H, dd, *J* = 7.4, 7.4 Hz), 4.42 (1H, dd, *J* = 2.7, 11.7 Hz), 5.13 (1H, d, *J* = 10.9 Hz), 5.13 (1H d, *J*= 16.8 Hz), 5.68 (1 H, bs), 6.10 (1H, dd, *J* = 10.6, 17.6 Hz), 7.07 (1H, ddd, *J*= 0.8, 7.0, 7.0 Hz), 7.14 (1H, ddd, *J*= 0.8, 7.0, 7.0 Hz), 7.29 (1H, d, *J*= 7.8 Hz), 7.46 (1H, d, *J* = 7.8 Hz), 8.31 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.0, 25.8, 27.8, 27.9, 28.2, 39.0, 45.2, 54.8, 59.3, 104.3, 110.8, 112.6, 117.7, 120.1, 122.2, 129.1, 134.5, 141.6, 145.6, 165.8, 169.3.



135, deoxybrevianamide E



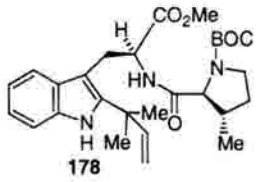
^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems860_set, filename: proton



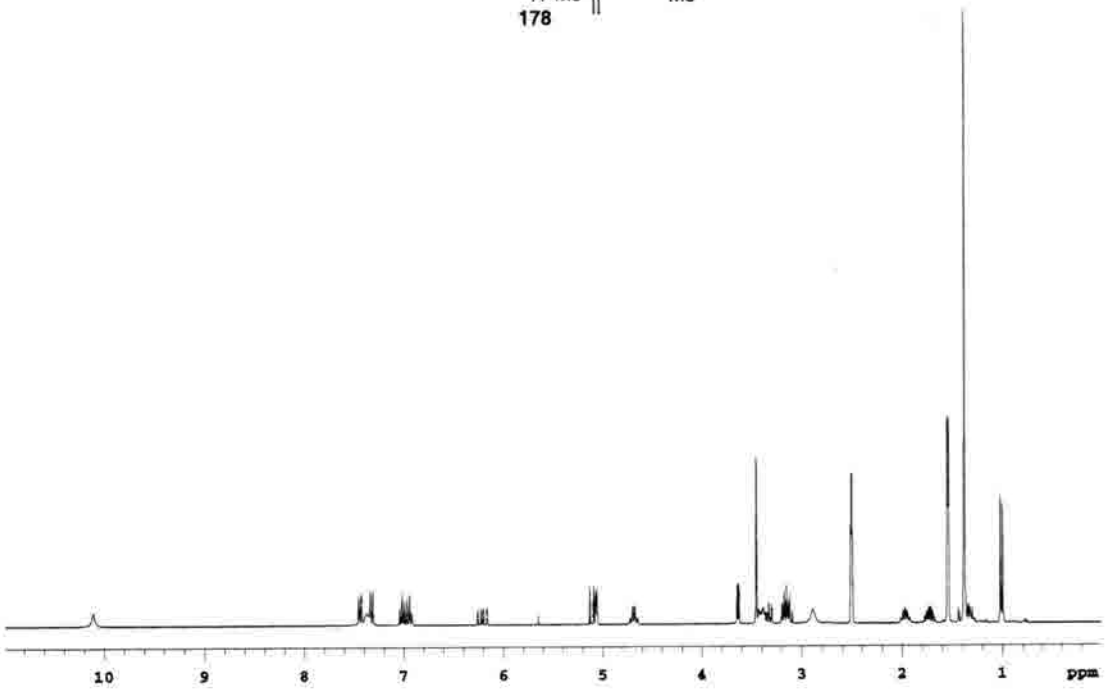
^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems860_set, filename: carbon

To a solution of **176** (55 mg, 0.192 mmol) in dry acetonitrile (2.9 mL) was added N-Boc-(3*S*)–methyl-L-proline (41 mg, 0.192 mmol), BOP reagent (85 mg, 0.192 mmol), and Et_3N (29 μL, 0.211 mmol). The reaction mixture was stirred at room temperature under an inert atmosphere for 4 h. A saturated aqueous solution of NaCl was added and the reaction was extracted four times with EtOAc. The combined organic layers were washed with 2M HCl, water, 10% $NaHCO_3$ (aq.), water and brine successively. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product was purified by flash silica gel column chromatography using 40% EtOAc/Hex to give (**178**) (81 mg, 85%) as a colorless glass.

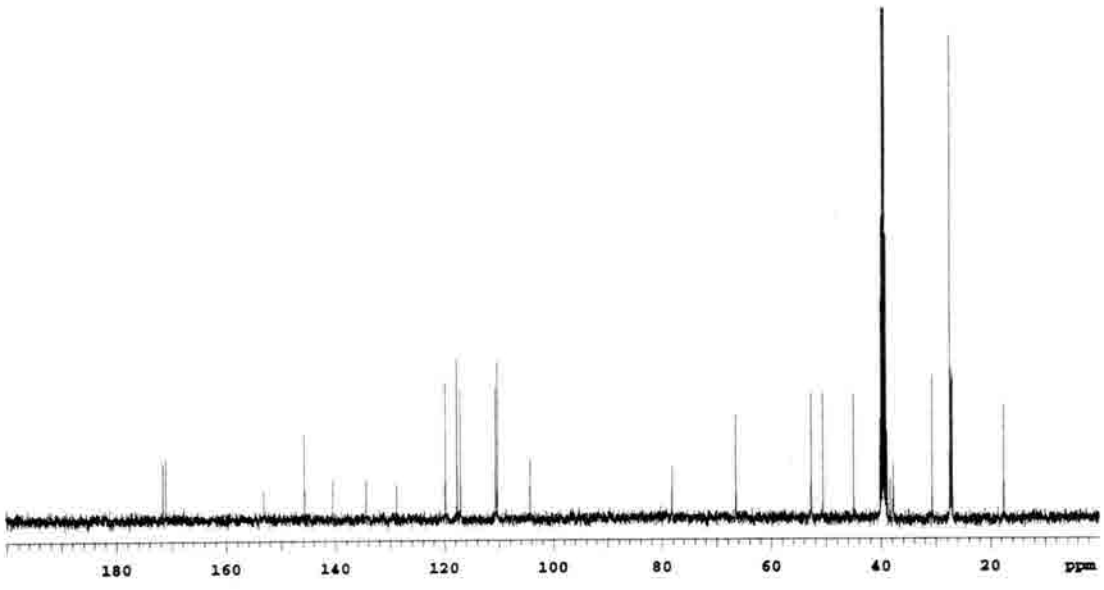
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.01 (3 H, d, *J* = 7.0 Hz), 1.33 (1H, m), 1.38 (9H, s), 1.54 (3H, s), 1.55 (3H, s), 1.72 (1H, m), 1.97 (1H, m), 2.89 (1H, bs), 3.15 (2H, m), 3.36 (2H, m), 3.45 (3H, d, *J* = 1.5 Hz), 3.64 (1H, d, *J* = 5.5 Hz), 4.69 (1H, dd, *J* = 7.3, 14.7 Hz), 5.08 (1H, d, *J* = 10.2 Hz), 5.10 (1H, d, *J* = 17.6 Hz), 6.21 (1H, ddd, *J* = 1.1, 10.6, 17.6 Hz), 6.95 (1H, t, *J* = 7.3 Hz), 7.02 (1H, t, *J* = 7.0 Hz), 7.33 (1H, d, *J* = 8.0 Hz), 7.45 (1H, d, *J* = 8.0 Hz), 10.15 (1H, bs). ^{13}C NMR (100 MHz, d_6-DMSO, 120°C): δ 17.6, 27.0, 27.2, 27.3, 27.5, 30.7, 37.7, 45.0, 50.6, 52.7, 66.4, 78.2, 104.2, 110.2, 110.6, 116.9, 117.7, 119.8, 128.8, 134.4, 140.4, 145.7, 153.1, 171.0, 171.6. Calcd for $C_{28}H_{40}N_3O_5$: 498.2968. Found 498.2957 (M+H).



178



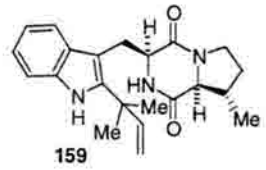
^{1}H NMR, 300 MHz, d_6-dmso, 120°C, filename:ems851_dmso_120



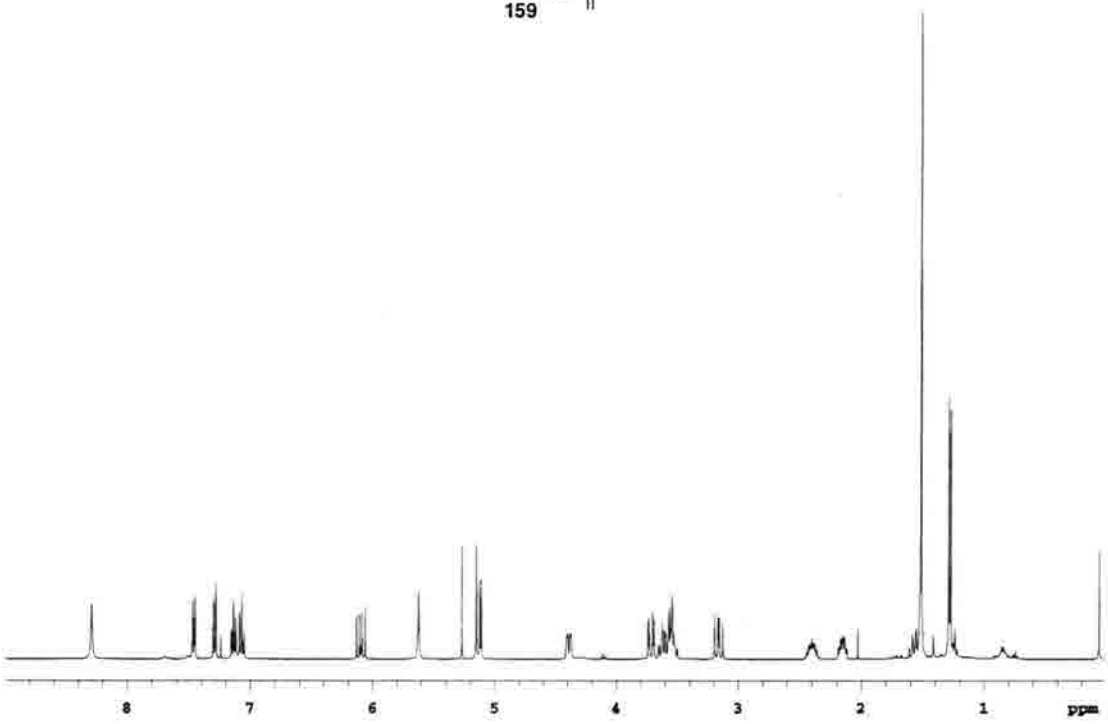
^{13}C NMR, 100 MHz, d_6-dmso, 120°C, filename:ems851_13C_VT

To a stirring solution of **178** (110 mg, 0.22 mmol) at 0°C under an inert atmosphere, TFA (353 uL, 4.4 mmol) was slowly added. The solution was brought to room temperature and was stirred for 3 hours. The CH_2Cl_2 and excess TFA were removed *in vacuo*. The residue was taken up in aqueous 10% $NaCO_3$ and extracted 3 times with EtOAc. The combined organic extracts were dried over anhydrous $NaSO_4$ and concentrated under reduced pressure. The crude product was dissolved in 1.25 mL of toluene, 2-hydroxy pyridine (4.2 mg, 0.044 mmol) was added, and the solution refluxed under an argon atmosphere for 8 hours. The toluene was removed under reduced pressure and the product purified by flash column chromatography (eluted with 2% CH_3OH/ CH_2Cl_2) to provide **159** as a solid (63 mg, 0.17 mmol, 79%).

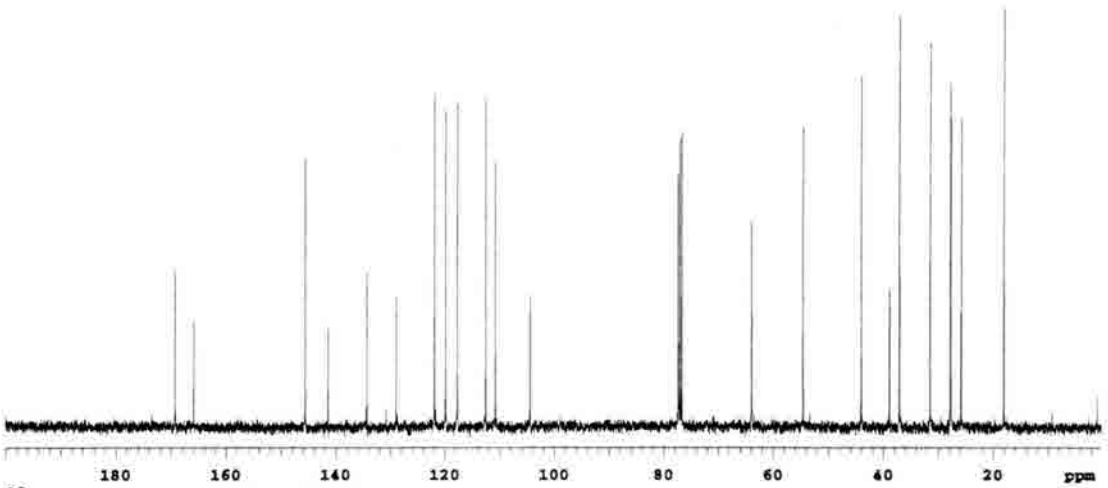
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.28 (3H, d, J = 6.4 Hz), 1.51 (6H, s), 1.55 (1H, m), 2.15 (1H, m), 2.40 (1H, m), 3.52 (1H, dd, J = 11.7, 15.2 Hz), 3.57 (3H, m), 3.72 (1H, dd, J = 3.9, 15.2 Hz), 4.39 (1H, dd, J = 3.1, 11.7 Hz), 5.13 (1H, d, J = 16.8 Hz), 5.13 (1H d, J = 10.6 Hz), 5.62 (1 H, bs), 6.09 (1H, dd, J = 10.2, 17.6 Hz), 7.07 (1H, t, J= 7.0 Hz), 7.14 (1H, ddd, J= 0.8, 8.2, 8.2 Hz), 7.29 (1H, d, J = 8.2 Hz), 7.46 (1H, d, J = 7.8 Hz), 8.29 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): d 18.1, 25.9, 27.8, 27.9, 31.5, 37.1, 38.9, 44.0, 54.6, 63.9 104.4, 110.8, 112.6, 117.9, 122.0, 128.9, 134.4, 141.4, 145.6, 165.8, 169.2.



159



^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems859_set, filename: proton



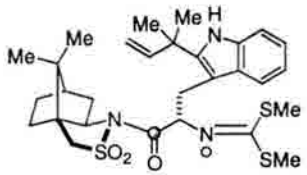
^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems859_set, filename: carbon

[2-^{15}N], [1-^{13}C]-2-Amino-N-bismethylsulfanyl-ethene-3-[2-(1,1-dimethyl-allyl)-1*H*-indol-3-yl]-1-(10,10-dimethyl-3,3-dioxo-3λ^{6}-thia-4-aza-tricyclo[5.2.1.0^{255,255}]dec-4-yl)-propan-1-one (180):

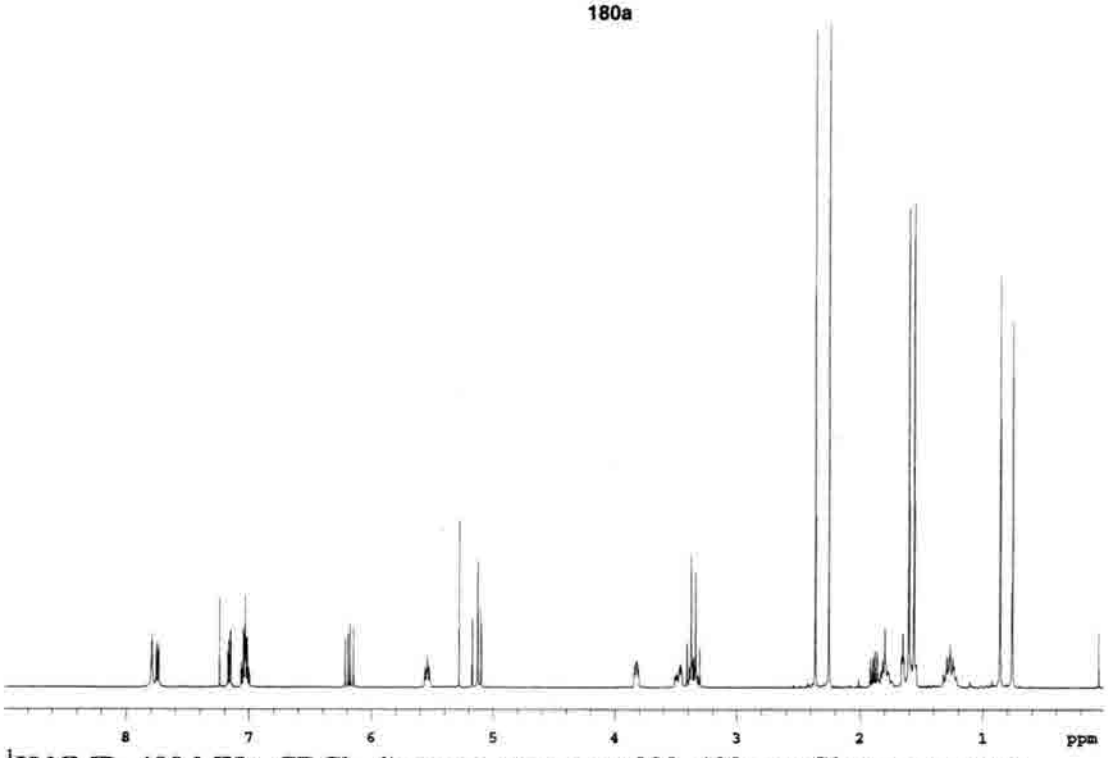
The ^{13}C, ^{15}N-labeled Oppolzer sultam glycinate (**179a**, 330 mg, 0.887 mmol), the gramine derivative (**170**, 255 mg, 1.05 mmol, 1.2 eq.) and tri-*n*-butylphosphine (66 μL, 0.263 mmol, 0.3 eq.) were refluxed in acetonitrile (7.9 mL, 0.11 M) under argon for 8 h. After cooling to room temperature, the solvent was concentrated under reduced pressure and the crude product was purified by flash silica gel column chromatography (20% EtOAc/hex.) to yield 361 mg, 0.630 mmol (72%) of (**180a**).

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.76 (3H, s), 0.86 (3H, s), 1.27 (2H, m), 1.55 (1H, m), 1.56 (3H, s), 1.61 (3H, s), 1.65 (1H, dd, *J*= 3.9, 3.9 Hz), 1.80 (2H, m), 1.89 (1H, dd, *J*= 7.8, 13.7 Hz), 2.25 (3H, s), 2.36 (3H, s), 3.32 (1H, d, *J*= 13.7 Hz), 3.36 (1H, m), 3.39 (1H, d, *J*= 14.0 Hz), 3.48 (1H, m), 3.83 (1H, dd, *J*= 5.0, 7.0 Hz), 5.11 (1H, dd, *J*= 1, 10.6 Hz), 5.15 (1H, dd, *J*= 0.8, 17.6 Hz), 5.54 (1H, m), 6.19 (1H, dd, *J*= 10.6, 17.6 Hz), 7.01(1H, ddd, *J*= 1.2, 7.0, 7.0 Hz), 7.05 (1H, ddd, *J*= 1.6, 7.0, 7.0 Hz), 7.16 (1H, dd, *J*= 1.6, 6.6 Hz), 7.74 (1H, dd, *J*= 2.0, 7.0 Hz), 7.79 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.8 (d, $J_{N\text{-}C}$ = 5 Hz), 15.9, 19.8, 20.4, 26.4, 27.6, 27.7, 29.8, 32.8, 38.2, 39.5, 44.6, 47.5, 48.1, 53.2, 65.3, 66.7 (d, $J_{C\text{-}C}$=58 Hz), 105.7 (d, $J_{N\text{-}C}$ = 3 Hz), 109.6, 111.6, 118.9, 120.1, 121.2, 129.9, 134.1, 140.6, 146.5, 161.7, 171.9 (d, $J_{N\text{-}C}$=3 Hz). IR (NaCl, CH_2Cl_2): 3411,

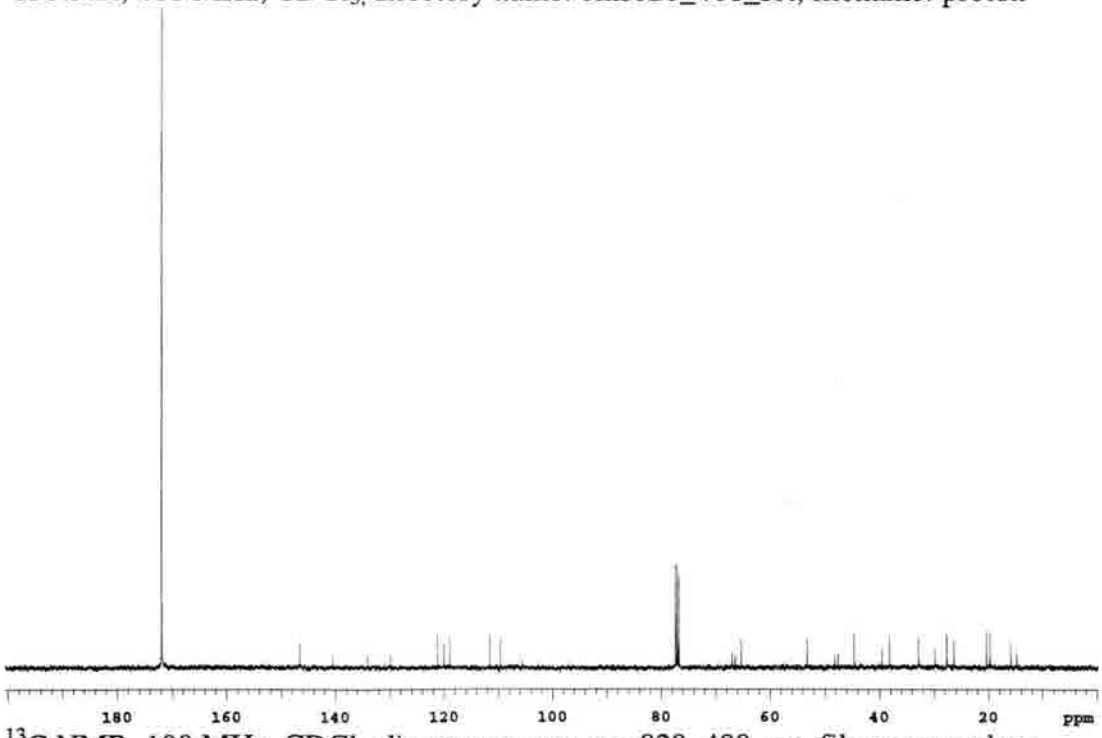
3081, 3055, 2961, 2926, 2883, 1653, 1558, 1541, 1457, 1335, 1207, 1133, 1049, 1009, 912, 733 cm^{-1}. Optical rotation: $[\alpha]_D$ = +7.5° (CH_3OH, c = 0.53). HRMS (FAB^+) calcd for $C_{28}{}^{13}C_1H_{40}N_1{}^{15}N_1O_3S_3$: 576.2236. Found 576.2243 (M+H).



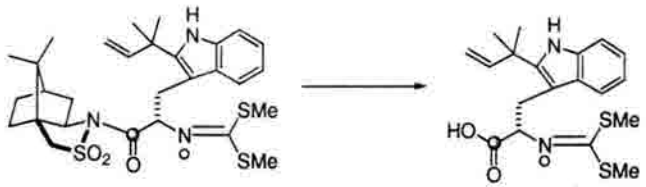
180a



^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems820_400_set, filename: proton



^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems820_400_set, filename: carbon

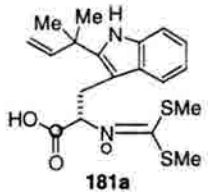


180a 181a

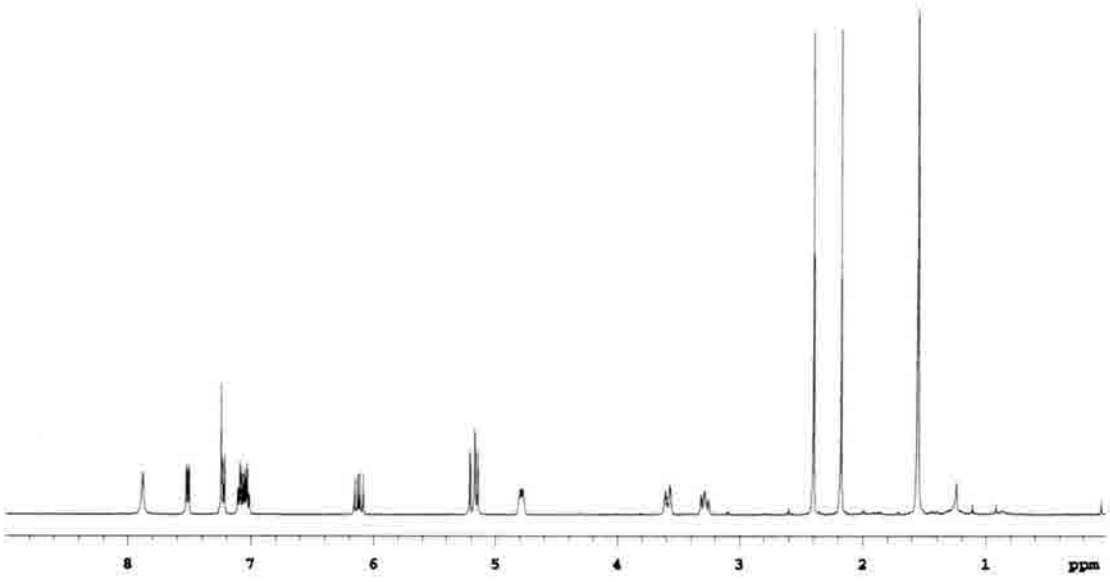
[^{15}N],[1-^{13}C]-N-bismethylsulfanyl-ethene-2-dimethylallyl-L-tryptophan (181a):

Solid LiOH (14 mg, 0.59 mmol, 5.0 eq) was added to a THF/water (2:1) solution of **180a** (67.5 mg, 0.118 mmol, 0.03 M). The mixture was stirred at ambient temperature for 24 hours. The THF was removed under reduced pressure and the remaining aqueous layer was acidified to pH=2 with 10% aqueous $KHSO_4$ before extracting with dichloromethane three times. The product was purified by flash column chromatography (eluted with 2% CH_3OH/ CH_2Cl_2) to provide 37.5 mg, 84% yield, of **181a**.

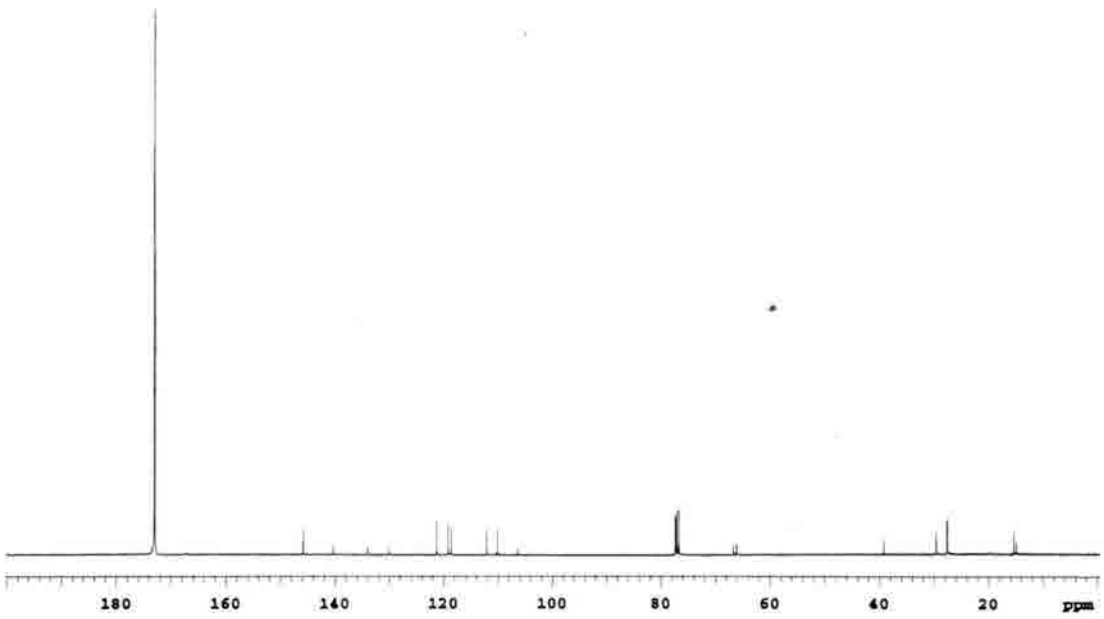
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.55 (3H, s), 1.56 (3H, s), 2.18 (3H, s), 2.40 (3H, s), 3.29 (1H, m), 3.59 (1H, dddd, *J*= 2.7, 3.5, 3.5, 14.8 Hz), 4.78 (1H, m), 5.16 (1H, d, *J*= 10.5 Hz), 5.18 (1H d, *J*= 17.6 Hz), 6.12 (1H, dd, *J*= 10.5, 17.2 Hz), 7.03 (1H, ddd, *J*= 0, 7.8, 7.8 Hz), 7.09 (1H, ddd, *J*= 1.2, 7.0, 7.0 Hz), 7.23 (1H, d, *J*= 7.0 Hz), 7.52 (1H, d, *J*= 7.8 Hz), 7.88 (1H, bs), 10.02 (1H, very bs). ^{13}C NMR (300 MHz, CDCl3): δ 14.9 ($J_{N\text{-}C}$ = 5 Hz), 15.4, 27.6, 27.7, 29.6, 39.2, 66.4 (d, $J_{C\text{-}C}$ = 58 Hz), 106.4, 110.1, 112.1, 118.6, 119.1, 121.3, 130.0, 134.1, 140.4, 145.8, 170.0, 173.0 ($J_{N\text{-}C}$= 3 Hz). IR (NaCl, CH_2Cl_2): 3374, 3054, 2923, 2852, 1667, 1547, 1461, 1428, 1307, 1242, 1009, 913, 742 cm^{-1}. $[\alpha]_D^{25}$= +16.4° (c=0.495, CH_2Cl_2). HRMS (FAB$^+$) calcd for $C_{18}{}^{13}C_1H_{25}N_1{}^{15}N_1O_2S_2$: 379.1361. Found 379.1353 (M+H).



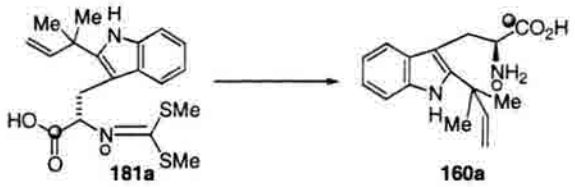
181a



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems1150_400



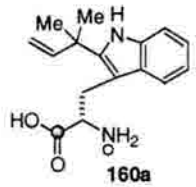
^{13}C NMR, 100 MHz, $CDCl_3$, filename: 1150_400_13C



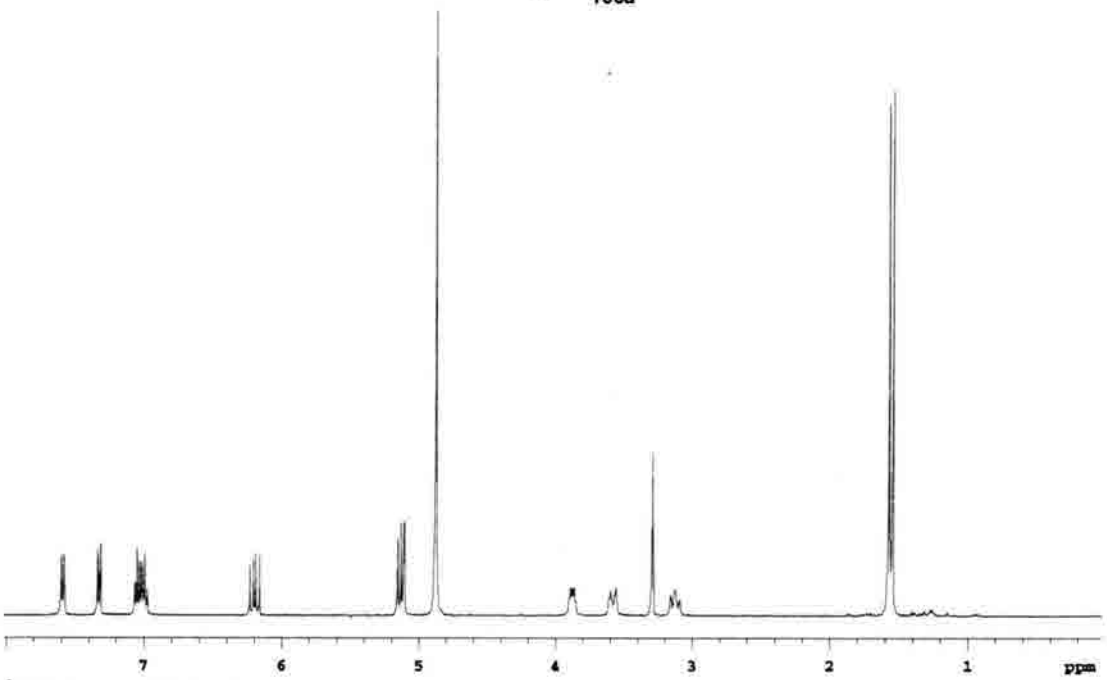
N-[^{15}N]-1-[^{13}C]-2-dimethylallyl-L-tryptophan (160a):

181a (37.5 mg, 0.099 mmol) was stirred with 1 M HCl (0.99 mL, 10 eq) in THF (1.8 mL, 0.05 M) at room temperature for 24 h. The THF was removed under reduced pressure and the remaining aqueous layer was extracted with diethyl ether. The diethyl ether layer was washed twice with a small amount of water and the combined aqueous layers were extracted two times with diethyl ether. The aqueous layer was evaporated under reduced pressure, re-dissolved in a small amount of distilled water and loaded onto a DOWEX (50W X2-100) column. The column was eluted several times with distilled water before eluting with approximately 200 mL of 3% aqueous NH_4OH. The elutant was evaporated *in vacuo* and then re-suspended in a small amount of de-ionized water lyophillized to give an off-white solid (**160a**) in 59% yield (16 mg 0.058 mmol).

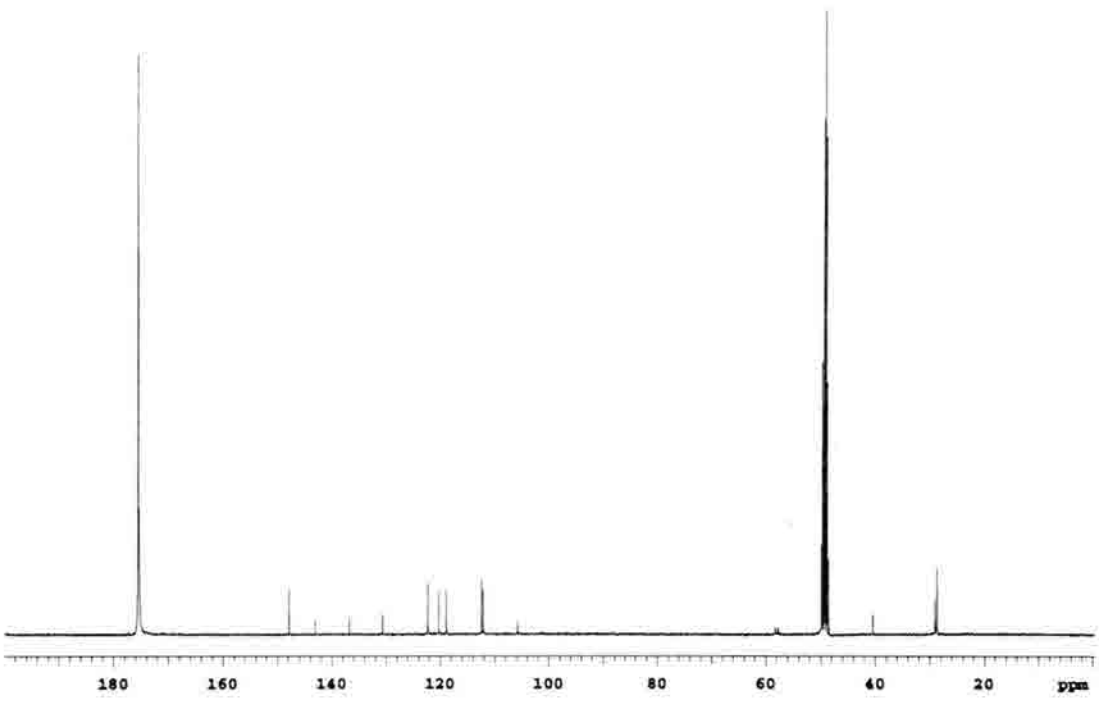
^{1}H NMR (400 MHz, CD_3OD): δ 1.55 (3H, s), 1.57 (3H, s), 3.12 (1H, m), 3.58 (1H, m), 3.88 (1H, m), 4.11 (1H, dd, *J*= 1.2, 10.5 Hz), 5.14 (1H, dd, *J*= 0.8, 17.6 Hz), 6.19 (1H, dd, *J*= 10.9, 17.6 Hz), 6.99 (1H, ddd, *J*= 0, 7.0, 7.8 Hz), 7.05 (1H, ddd, *J*= 0.8, 7.0, 7.8 Hz), 7.32 (1H, d, *J*= 8.2 Hz), 7.59 (1H, d, *J*= 7.8 Hz). ^{13}C NMR (75 MHz, CD_3OD): 28.7, 29.0, 40.4, 58.0 (dd, $J_{N\text{-}C}$ = 5 Hz, $J_{C\text{-}C}$= 54 Hz), 105.7 ($J_{N\text{-}C}$= 3 Hz), 112.1, 112.4, 118.9, 120.3, 122.3, 130.7, 136.8, 143.1, 147.9, 175.4. IR (neat, NaCl): 3689-1738 (broad), 3349, 2966, 2924, 1631, 1538, 1461, 1381, 1334, 1302, 919, 745 cm^{-1}. HRMS (FAB+): Calcd for $C_{15}{}^{13}C_1H_{21}N_1{}^{15}N_1O_2$, 275.1607. Found 275.1594 (M+H). Optical Rotation $[\alpha]_D$ =+7.5° (CH_3OH, c = 0.53).



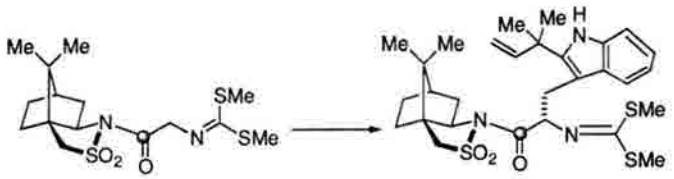
160a



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems904_400_CD3OD



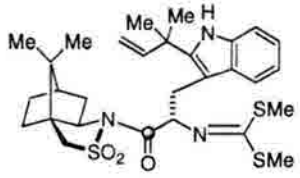
^{13}C NMR, 100 MHz, $CDCl_3$, filename: ems904_400_CD3OD_13C



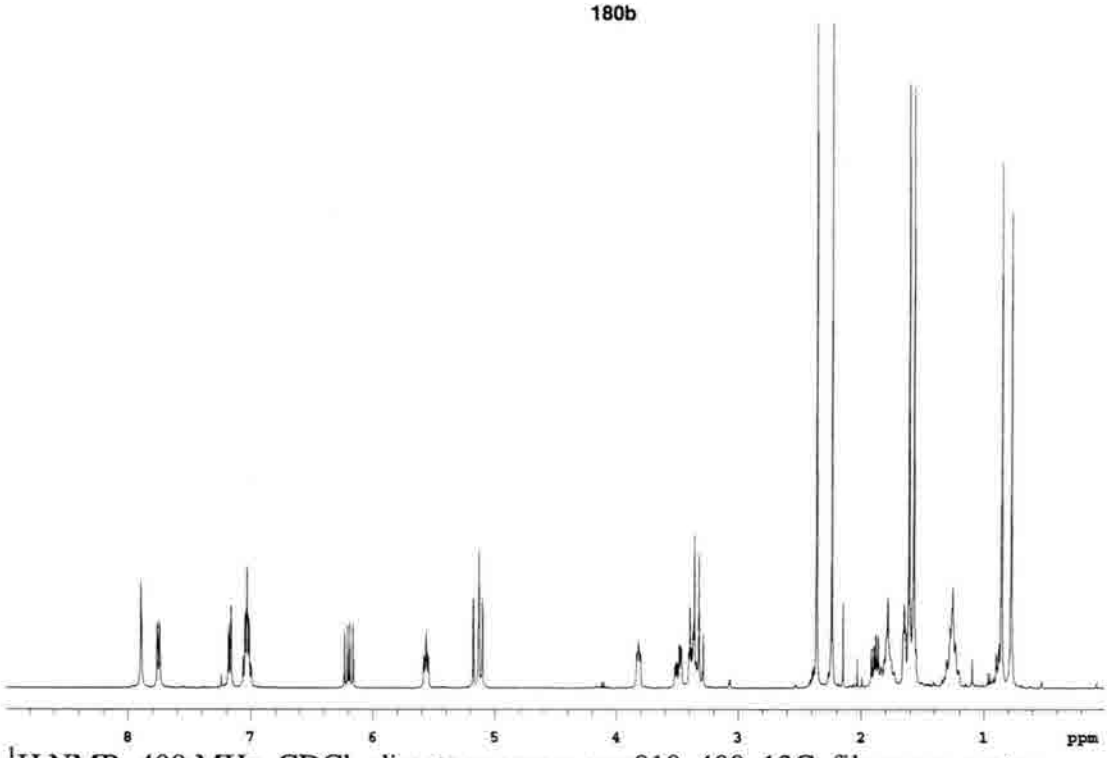
179b 180b

Preparation of **180b** (190 mg, 0.508 mmol) from **179b** was accomplished in the same manner as **180a**. Yield: 249 mg, 0.435 mmol, 86%.

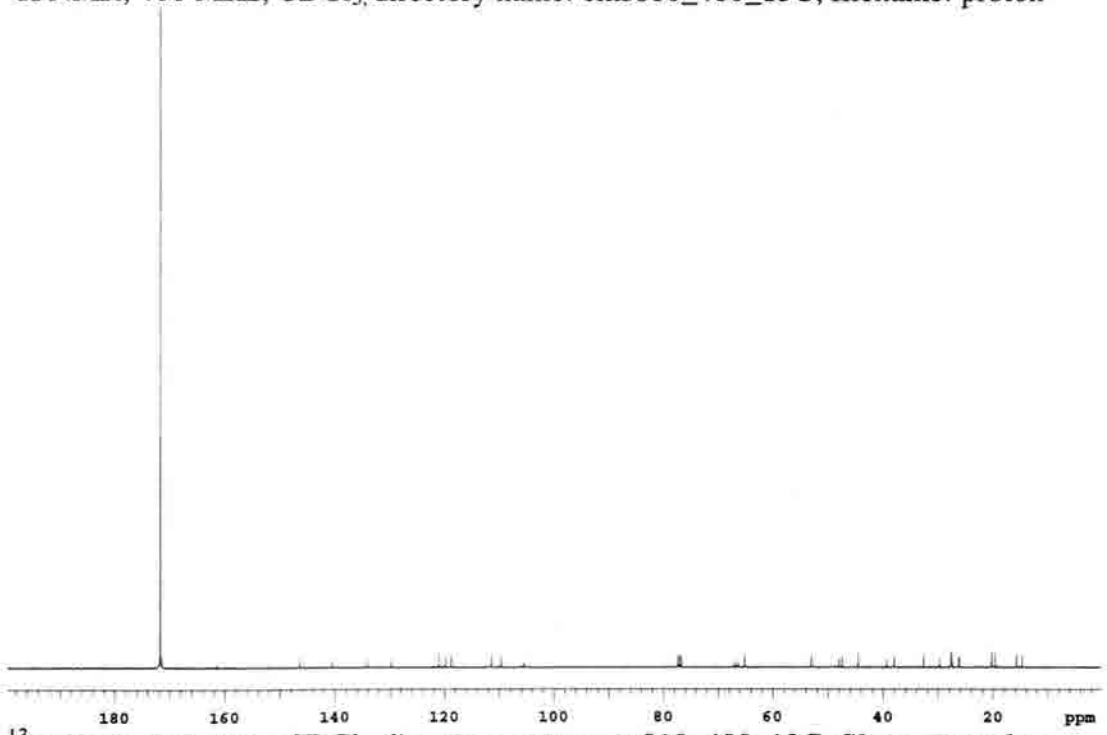
^{1}H NMR (400 MHz, $CDCl_3$): δ 0.78 (3H, s), 0.85 (3H, s), 1.25 (2H, m), 1.55 (1H, m), 1.57 (3H, s), 1.61 (3H, s), 1.65 (1H, dd, *J*= 3.5, 3.5 Hz), 1.78 (2H, m), 1.89 (1H, dd, *J*= 7.8, 13.7 Hz), 2.24 (3H, s), 2.36 (3H, s), 3.30 (1H, d, *J*= 13.7 Hz), 3.37 (1H, m), 3.38 (1H, d, *J*= 13.7 Hz), 3.49 (1H, ddd, *J*= 1.6, 6.5, 14.0 Hz), 3.83 (1H, dd, *J*= 4.7, 7.4 Hz), 5.11 (1H, d, *J*= 10.9 Hz), 5.15 (1H, d, *J*= 18.8 Hz), 5.56 (1H, dd, *J*= 7.8, 7.8 Hz), 6.20 (1H, dd, *J*= 10.6, 17.6 Hz), 7.03 (2H, m), 7.17 (1H, d, *J*= 8.2 Hz), 7.75 (1H, d, *J*= 7.0 Hz), 7.89 (1H, bs). ^{13}C NMR (100 MHz, CDCl3): δ 14.7, 15.7, 19.6, 20.3, 26.2, 27.5, 27.6, 29.7, 32.6, 38.0, 39.4, 44.5, 47.4, 48.0, 53.0, 65.1, 66.6 (d, J_{CC}= 57 Hz), 105.5, 109.5, 111.4, 118.7, 119.9, 121.0, 129.7, 134.1, 140.5, 146.4, 161.4 (d, J_{CC}= 6 Hz), 173.5. IR (NaCl, CH_2Cl_2): 3411, 3081, 3055, 2961, 2926, 2883, 1653, 1558, 1541, 1457, 1335, 1207, 1133, 1049, 1009, 912, 733 cm^{-1}. HRMS (FAB+): Calcd for $C_{28}{}^{13}C_1H_{40}N_3O_3S_3$ 575.2265. Found 575.2264 (M+H).



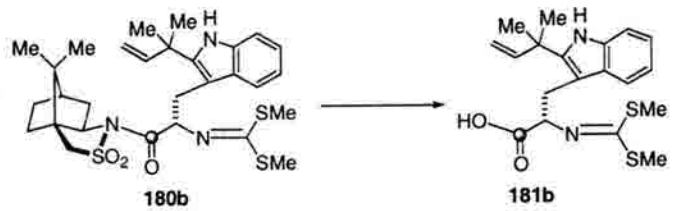
180b



^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems810_400_13C, filename: proton

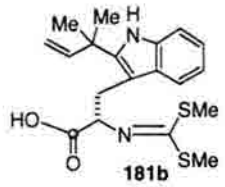


^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems810_400_13C, filename: carbon

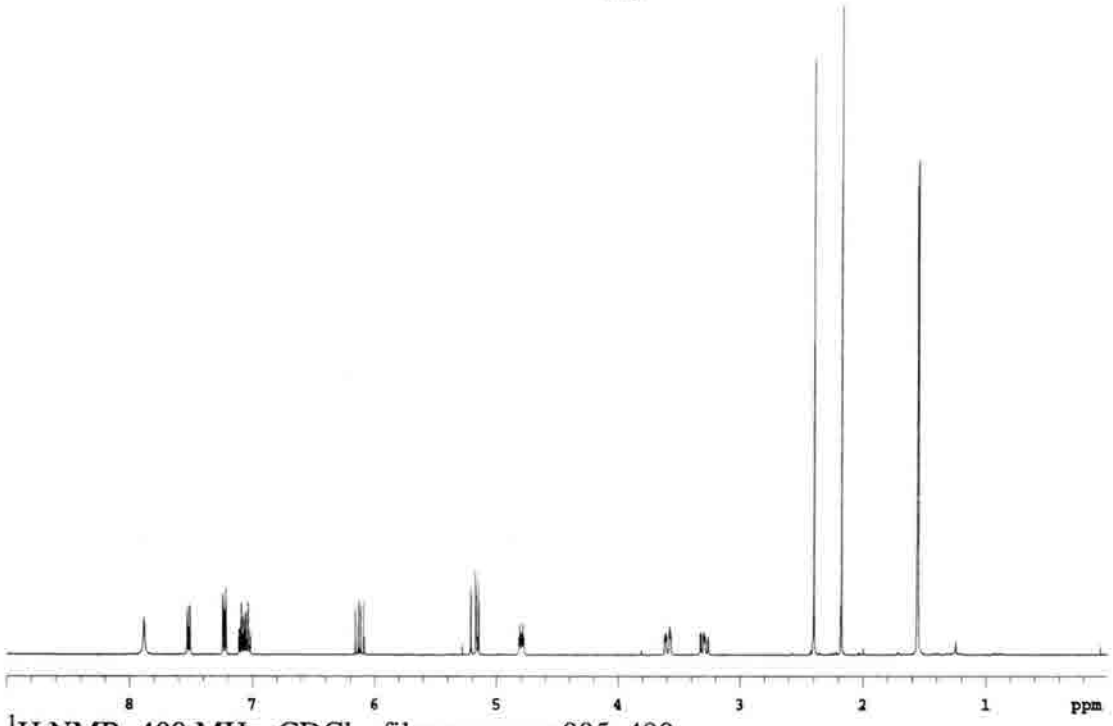


181b was prepared from **180b** (40 mg, 0.070 mmol) in the same manner as **181a**. Yield: 23 mg, 0.061 mmol, 87%.

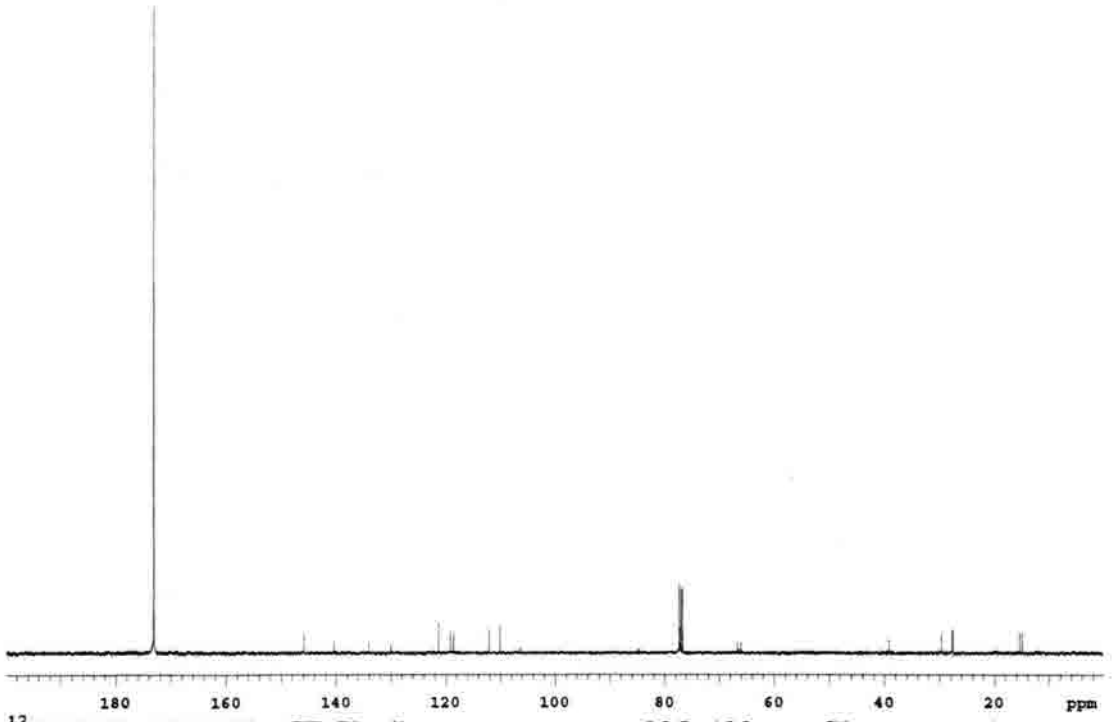
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.55 (3H,s), 1.56 (3H, s), 2.18 (3H, s), 2.40 (3H, s), 3.29 (1H, ddd, *J*= 2.7, 10.1, 14.4 Hz), 3.37 (1H, ddd, *J*= 2.7, 2.7, 14.8 Hz), 4.79 (1H. ddd, *J*= 3.9, 3.9, 8.2 Hz), 5.16 (1H, dd, *J*= 1.2, 10.5 Hz), 5.19 (1H dd, *J*= 0, 17.6 Hz), 6.12 (1H, dd, *J*= 10.5, 17.1 Hz) 7.03 (1H, ddd, *J*= 1.2, 8.2, 8.2 Hz), 7.09 (1H, ddd, *J*= 1.2, 7.1, 7.1 Hz), 7.23 (1H, d, *J*= 8.2 Hz), 7.52 (1H, d, *J*= 7.4 Hz), 7.88 (1H, bs), 10.02 (1H, very bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.9, 15.4, 27.6, 27.7, 29.6, 39.2, 66.4 (d, J_{CC}= 56 Hz), 66.4, 106.4, 110.1, 112.1, 118.6, 119.1, 121.3, 130.0, 134.1, 140.4, 145.8, 173.5. Enhanced ^{13}C peak. IR (NaCl, CH_2Cl_2): 3374, 3054, 2923, 2852, 1667, 1547, 1461, 1428, 1307, 1242, 1009, 913, 742 cm^{-1}. $[\alpha]_D^{25}$= +16.4° (c=0.495, CH_2Cl_2). HRMS (FAB^+) calcd for $C_{18}{}^{13}C_1H_{25}N_2O_2S_2$ 378.1391. Found 378.1378 (M+H).



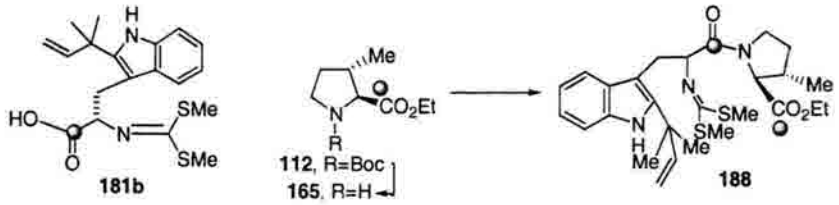
181b



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems905_400



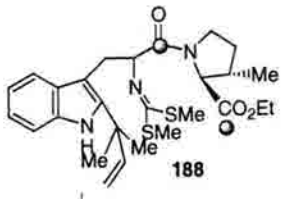
^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems905_400_set, filename: carbon



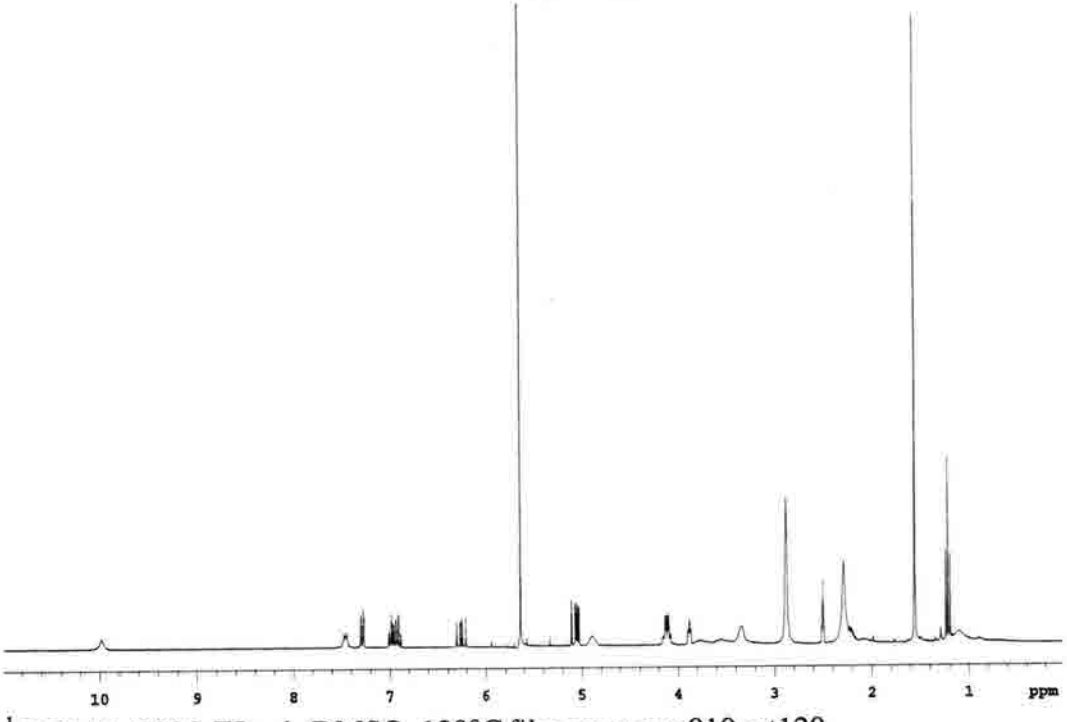
N-Boc-β-Methyl-L-proline ethyl-ester (**112**, 57.4 mg, 0.222 mmol) was taken up in 0.5 mL of CH_2Cl_2 and placed under argon with stirring at 0°C. TFA (469 uL, 5.84 mmol, 20 eq.) was then added slowly. The solution was brought to room temperature and was stirred an additional 3 hours. The CH_2Cl_2 and excess TFA were removed *in vacuo* to leave the L-[^{13}C]-3(*S*)-methyl-proline ethyl ester TFA salt as an oil. To this compound, **181b** (84mg, 0.222 mmol) was added along with with one eq. of BOP reagent (98 mg, 0.222 mmol) and 2 eq. of triethylamine (68 μL, 0.488 mmol) in 3.4 mL of dry acetonitrile. The reaction was stirred for 3.5 hours at room temperature under an argon atmosphere. A saturated aqueous solution of NaCl was added and the mixture was extracted three times with EtOAc. The combined organic extracts were dried over anhydrous $NaSO_4$ and concentrated under reduced pressure. The crude product was purified via flash column chromatography (eluted with 4% CH_3OH/ CH_2Cl_2) to provide **188**. Yield: 106.8 mg, 0.206 mmol, 93% 2 steps.

^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.10 (3H, bs), 1.21 (3H, t, *J*= 7.3 Hz), 1.56 (6H, s), 2.65 (1H, bs), 2.28 (2H, m), 2.88 (6H, s), 3.38 (2H, bs), 3.56 (1H, bs), 3.76 (1H, bs), 3.88(1H, dd, *J*= 4.8, 4.8 Hz), 4.12 (2H, dddd, *J*= 2.9, 7.0, 7.0, 7.0 Hz), 4.90 (1H, bs), 5.05 (1H, dd, *J*= 1.1, 10.6 Hz), 5.08 (1H dd, *J*= 1.1, 17.6 Hz), 6.26 (1H, dd, *J*= 10.3, 17.2 Hz), 6.91 (1H, ddd, *J*= 1.1, 7.3, 7.3 Hz), 6.99 (1H, ddd, *J*= 1.1, 7.0, 7.0 Hz), 7.29 (1H, d, *J*= 7.7 Hz), 7.46 (1H, d, *J*= 7.3 Hz), 9.98 (1H, bs). ^{13}C NMR (75 MHz, d_6-DMSO,

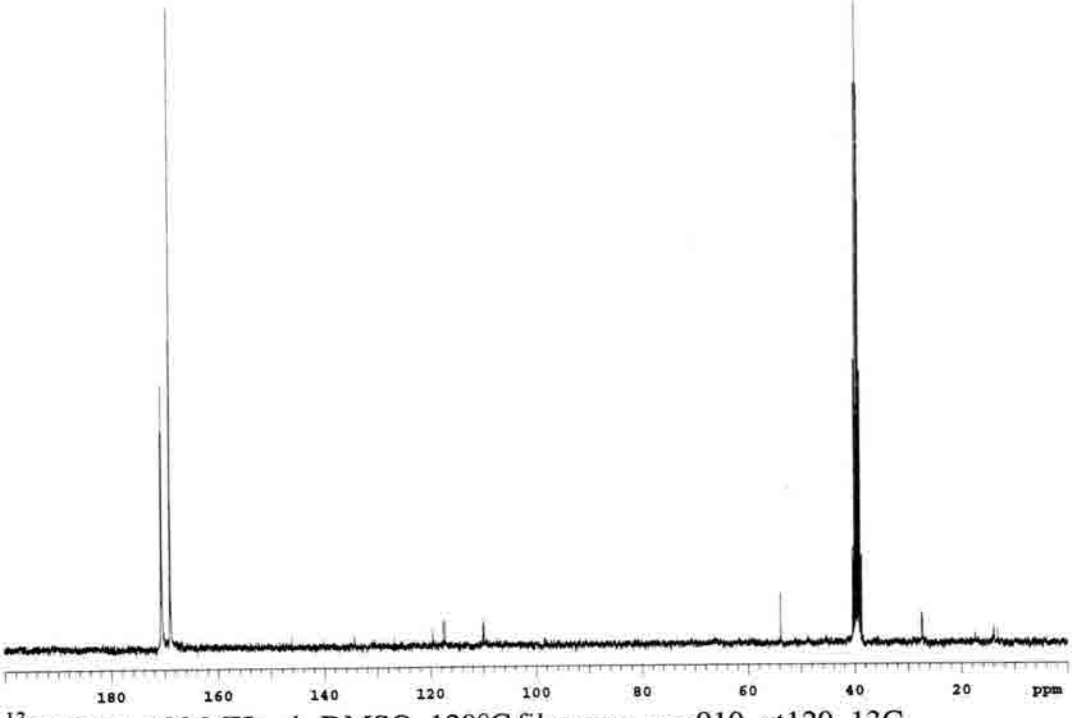
120°C): δ 13.2, 13.9, 17.4, 27.3, 27.4, 28.1, 32.0, 38.4, 45.2, 59.5, 67.0 (d, J_{CC}= 57.7 Hz), 109.9, 110.1 117.3, 117.6, 119.6, 129.1, 134.4, 140.2, 146.1, 167.0, 169.0, 170.7. IR (NaCl, CH_2Cl_2): 3319, 3052, 2966, 2927, 2874, 1699, 1598, 1577, 1460, 1429, 1312, 1235, 1174, 1154, 1032, 1008, 912, 742 cm^{-1}. HRMS (FAB^+) calcd for $C_{25}{}^{13}C_2H_{38}N_3O_3S_2$ 518.2422. Found 518.2422 (M+H). Optical Rotation: $[\alpha]_D{}^{25}$= -74.9° (c=0.595, CH_2Cl_2).



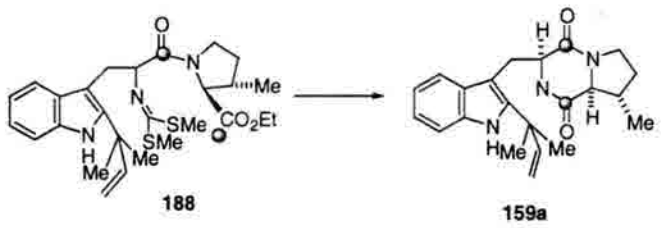
188



^{1}H NMR, 300 MHz, d_6-DMSO, 120°C filename: ems910_vt120



^{13}C NMR, 100 MHz, d_6-DMSO, 120°C filename: ems910_vt120_13C

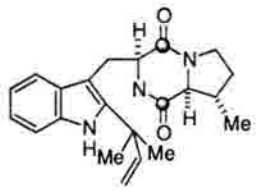


188 159a

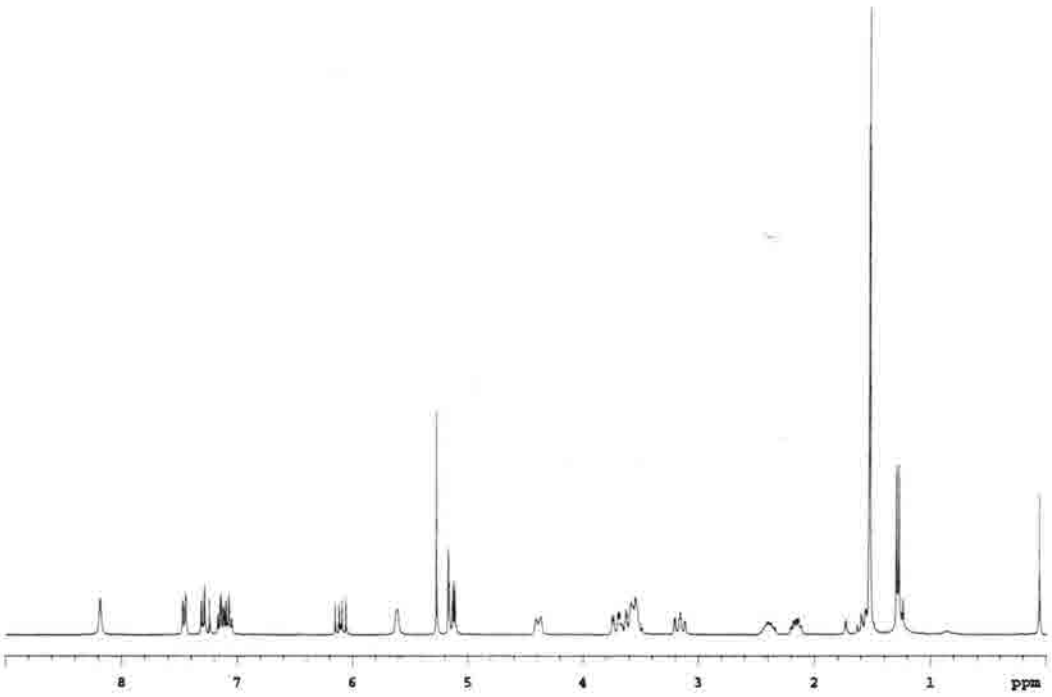
[$^{13}C_2$]-*cyclo*-2-dimethylallyl-L-tryptophan-3(*S*)-methyl-L-proline (159a): Compound **188**, (57 mg, 0.113 mmol) was stirred with 1 M HCl (1.13 mL, 10 eq) in THF (2.26 mL, 0.05 M) at room temperature for 24 h. An aqueous solution of 10% $NaCO_3$ was added until basic and the reaction was extracted 3 times with EtOAc. The combined organic extracts were dried over anhydrous $NaSO_4$ and concentrated under reduced pressure. The crude product was dissolved in 2.0 mL of toluene, 2-hydroxy pyridine (2.0 mg, 0.023 mmol, 0.2 eq) was added, and the solution refluxed under an argon atmosphere for 4 hours. The toluene was removed under reduced pressure and the product purified by flash column chromatogratphy (eluted with 4% CH_3OH/ CH_2Cl_2) to provide **159a** as a white solid. Re-crystallized from ethyl acetate and hexane. Yield: 40 mg, 0.109 mmol, 97%.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.28 (3H, d, *J*= 6.24), 1.54 (6H, s), 2.16 (1H, m), 2.39 (1H, m), 3.15 (1H, ddd, *J*=1.6, 11.3, 14.4 Hz), 3.54 (2H, m), 3.61 (1H, ddd, *J*= 1.9, 9.4, 9.4 Hz), 3.68 (1H, dd, *J*= 3.9, 15.2 Hz), 4.38 (1H, d, *J* = 11.7 Hz), 5.16 (1H, dd, *J* = 0, 17.9 Hz), 5.17 (1H dd, *J*= 0, 10.9 Hz), 5.6 (1 H, bs), 6.26 (1H, dd, *J*= 10.9, 17.9 Hz), 7.08 (1H, t, *J*= 7.4 Hz), 6.99 (1H, t, *J*= 7.0 Hz), 7.30 (1H, d, *J*= 8.2 Hz), 7.46 (1H, d, *J*= 8.2 Hz), 8.02 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.2, 26.0, 27.9, 28.0, 31.6, 37.2, 39.0, 44.1, 54.8 (d, J_{CC}= 52.2 Hz), 64.0 (d, J_{CC}= 53.7 Hz), 104.7, 110.8, 112.8, 117.9, 120.1, 122.2, 129.1, 134.3, 141.4, 145.6,165.9, 169.3. IR (NaCl, CH_2Cl_2): 3364, 3051,

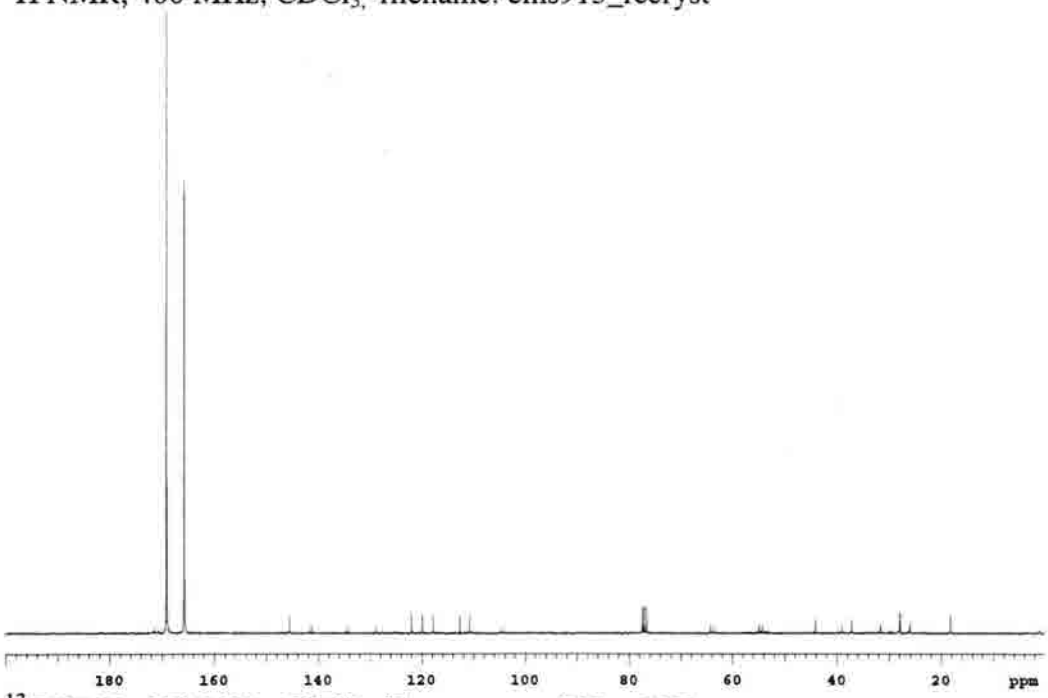
2969, 2867, 1633, 1461, 1403, 1321, 1305, 1252, 1223, 1009, 921, 743 cm^{-1}. $[\alpha]_D^{25}$= -30° (c=0.07, CH_2Cl_2). HRMS (FAB^+) calcd for $C_{20}{}^{13}C_2H_{27}N_3O_2$ 367.2170. Found 367.2185 (M+H).



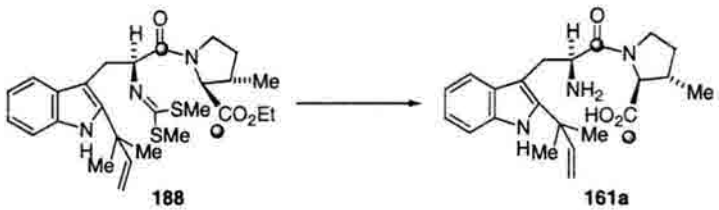
159a



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems913_recryst



^{13}C NMR, 100 MHz, $CDCl_3$, filename: ems913__13C_recryst

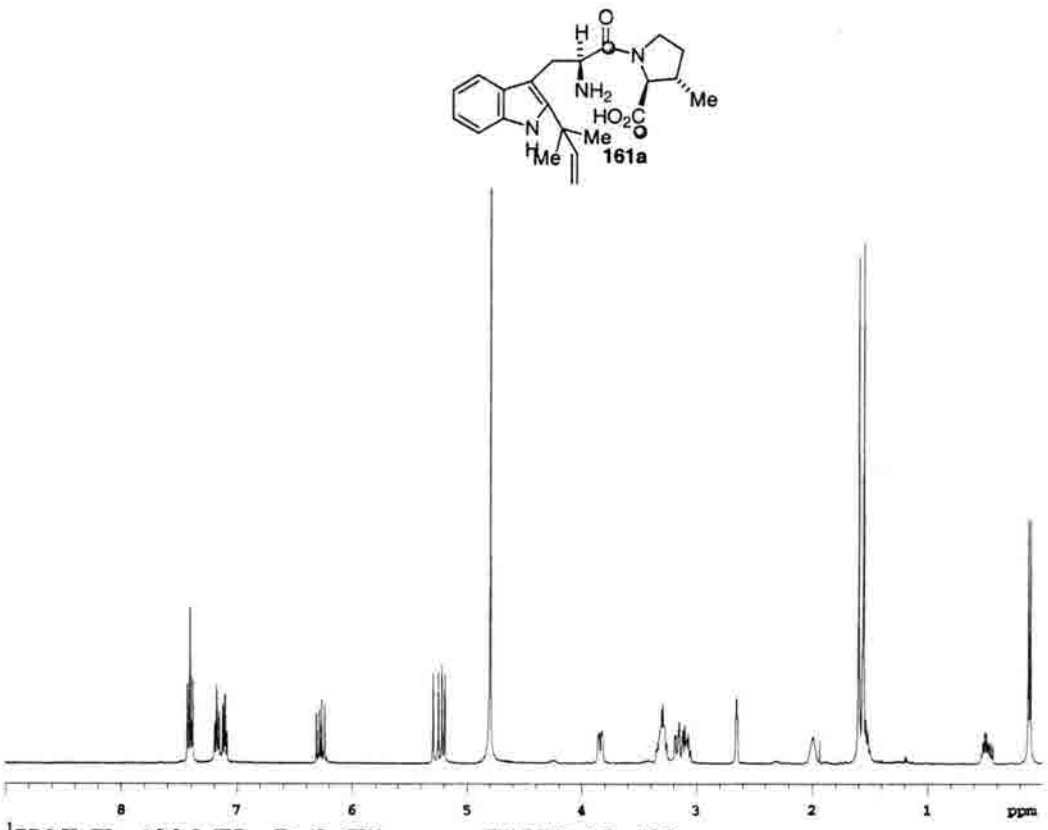


188 161a

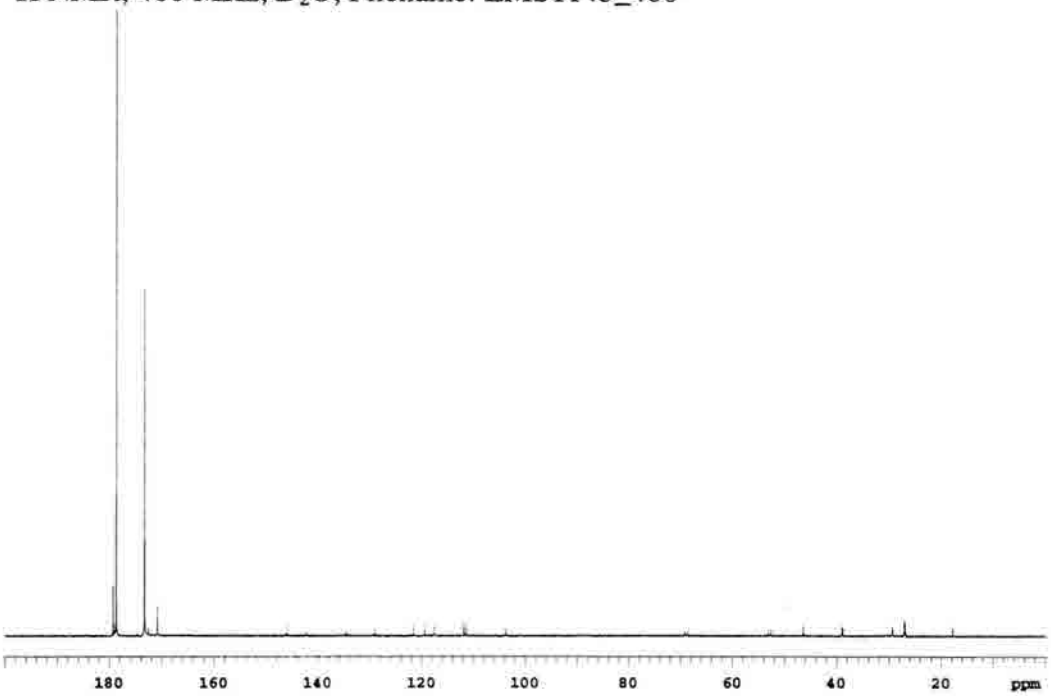
[$^{13}C_2$]-2-dimethylallyl-L-tryptophyl-3(*S*)-methyl-L-proline (161a): To a 0.03M THF/H_2O (2:1) solution of **188** (29.5 mg, 0.057 mmol), was added 5 equivalents of LiOH (6.8 mg, 0.285 mmol). The mixture was stirred at room temperature for 24 hours. The THF was removed *in vacuo* and the residue was acidified to pH=2 with 10% aqueous $KHSO_4$ before extracting 3 times with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was re-suspended in THF (1.14 mL), 1N HCl was added (0.57 mL), and the mixture was stirred for 24 hours at room temperature. The THF and excess HCl were removed under reduced pressure. The residue was loaded onto a suitably prepared DOWEX ion exchange column, then desalted with de-ionized water and eluted with 2% aqueous NH_4OH. The elutant was evaporated *in vacuo* and then re-suspended in a small amount of de-ionized water lyophillized to give a white solid (22 mg, 0.057 mmol, 100%).

^{1}H NMR (400 MHz, D_2O): δ 0.12 (3H, d, *J*= 6.6), 0.49 (1H, m), 1.53 (1H, m), 1.56 (3H, s), 1.60 (3H,s), 2.00 (1H, m), 2.63 (1H, dd, *J*= 0, 3.0 Hz), 3.08 (1H, m), 3.14 (1H, dd, *J*= 0, 14.4 Hz), 3.30 (2H, m), 3.84 (1H, dd, *J*= 0, 9.4 Hz), 5.21 (1H dd, *J*= 0, 10.9 Hz), 5.27 (1H, dd, *J*= 0.8, 17.2 Hz), 6.28 (1H, dd, *J* = 10.9, 17.2 Hz), 7.10 (1H, t, *J*= 7.4 Hz), 7.18 (1H, t, *J*= 7.0 Hz), 7.40 (1H, d, *J*= 8.2 Hz), 7.42 (1H, d, *J*= 9.0 Hz). ^{13}C NMR (100 MHz, D_2O): δ 17.8, 26.9, 27.1, 29.3, 29.4, 38.9, 39.0, 46.4, 52.8 (d, J_{CC}= 52 Hz), 68.9 (d, J_{CC}=

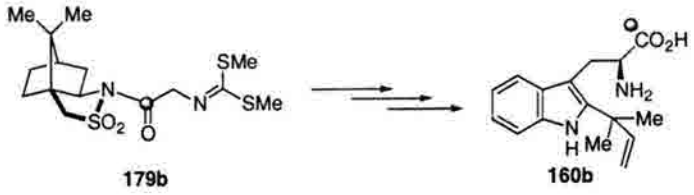
54 Hz), 103.8, 111.4, 111.9, 117.6, 119.4, 121.6, 129.0, 134.5, 142.2, 146.0, 173.3, 178.7. IR (NaCl, CH_2Cl_2): 3566-1600 (broad), 3421, 3355, 3056, 2965, 2930, 2873, 1558, 1458, 1362, 921, 743 cm^{-1}. $[\alpha]_D^{25}$= +32° (c=0.1, H_2O). HRMS (FAB^+) calcd for $C_{20}{}^{13}C_2H_{30}N_3O_3$ 386.2354. Found 386.2347 (M+H).



^{1}H NMR, 400 MHz, D_2O, Filename: EMS1145_400



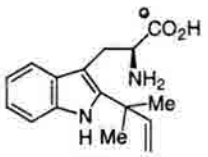
^{13}C NMR, 100 MHz, D_2O, Filename: EMS1145_400_13C



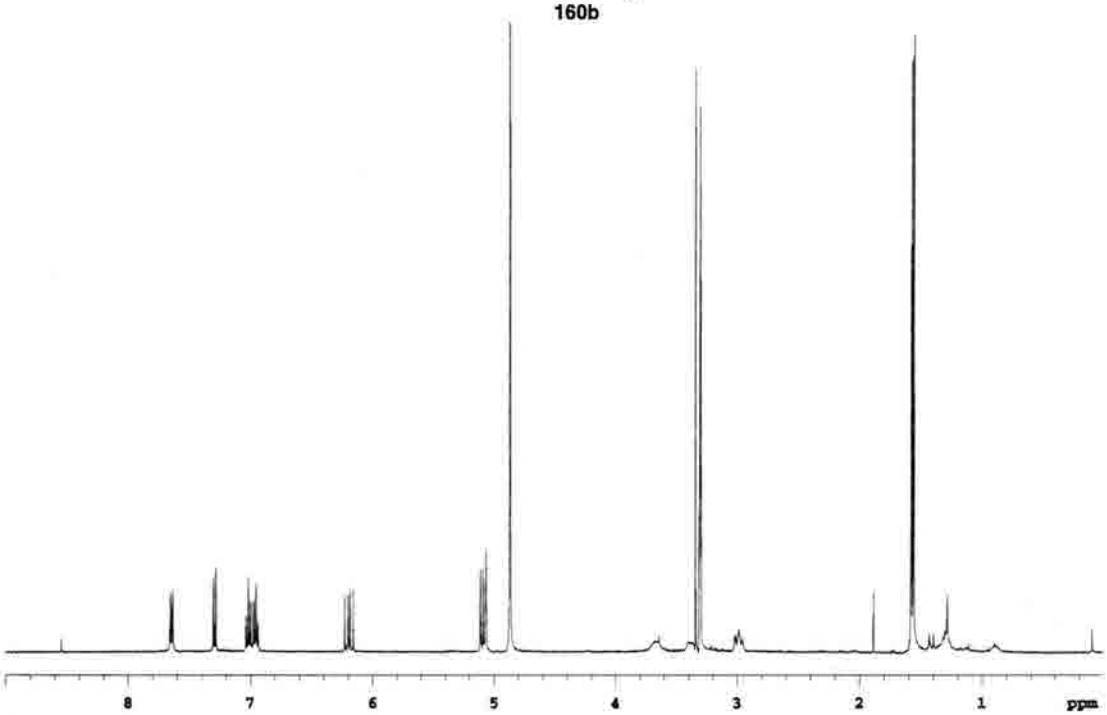
1-[^{13}C]-2-dimethylallyl-L-tryptophan (160b):

160b was synthesized from **179b** by the procedure described for **160a**. Yield: 66% from **179b**.

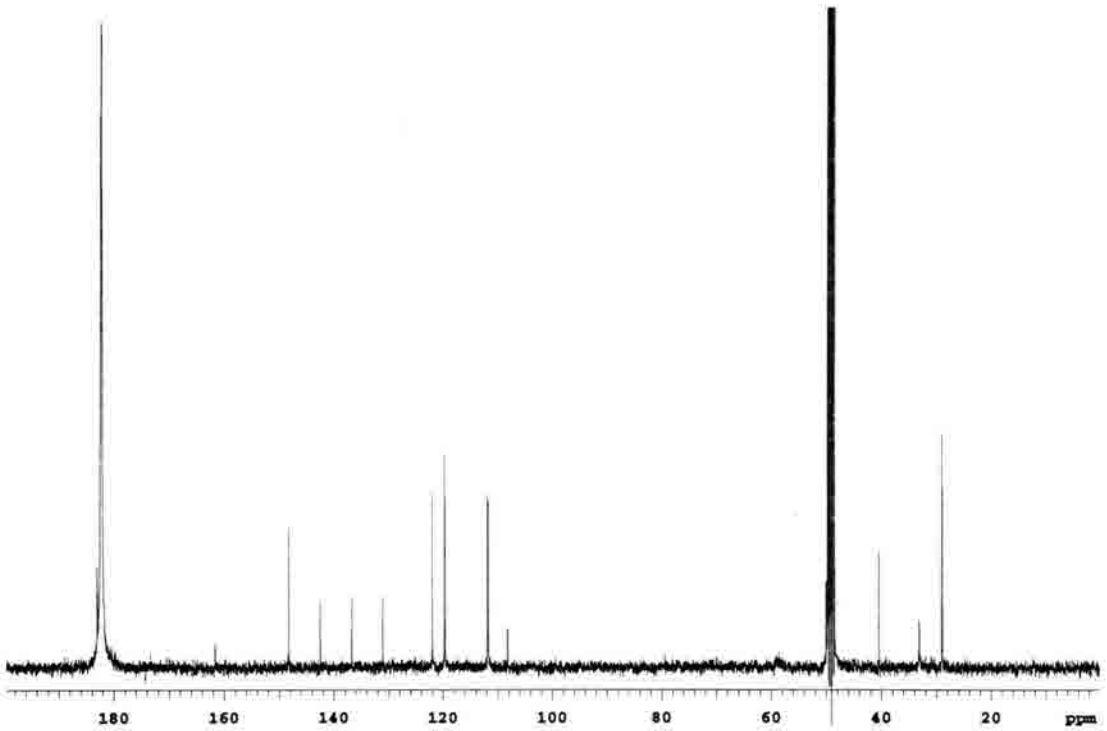
^{1}H NMR (400 MHz, CD_3OD): δ 1.57 (3H, s), 1.59 (3H, s), 2.99 (1H, dd, J = 0, 12.5 Hz), 3.37 (1H, m), 3.68(1H, m), 5.08 (1H, d, J = 0, 10.5 Hz), 5.09 (1H, dd, J = 0, 17.5 Hz), 6.20 (1H, dd, J = 10.5, 17.5 Hz), 7.028 (1H, t, J = 7.0 Hz), 7.03 (1H, t, J = 7.0 Hz), 7.30 (1H, d, J = 7.8 Hz), 7.65 (1H, d, J = 7.7 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 28.9, 28.0, 33.1, 40.5, 58.8 ($J_{C\text{-}C}$ = 55 Hz) 108.2, 111.8, 111.9, 119.7, 119.8, 122.0, 131.1, 136.7, 142.5, 148.3, 182.3. IR (neat, NaCl): 3349, 2966, 2924, 1631, 1538, 1461, 1381, 1334, 1302, 919, 745 cm^{-1}. $[\alpha]_D^{25}$= +11.5° (H_2O, c = 0.23). HRMS (FAB+): Calcd for $C_{15}{}^{13}C_1H_{21}N_2O_2$: 274.1637. Found 274.1643 (M+H).



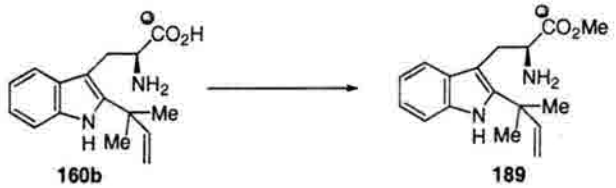
160b



^{1}H NMR, 400 MHz, $CDCl_3$, Filename: EMS1144_400



^{13}C NMR, 100 MHz, $CDCl_3$, Filename: EMS1144_300_2_13C



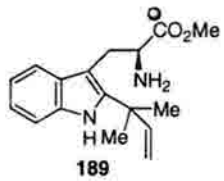
160b 189

1-[^{13}C]-2-dimethylallyl-L-tryptophan methylester (189):

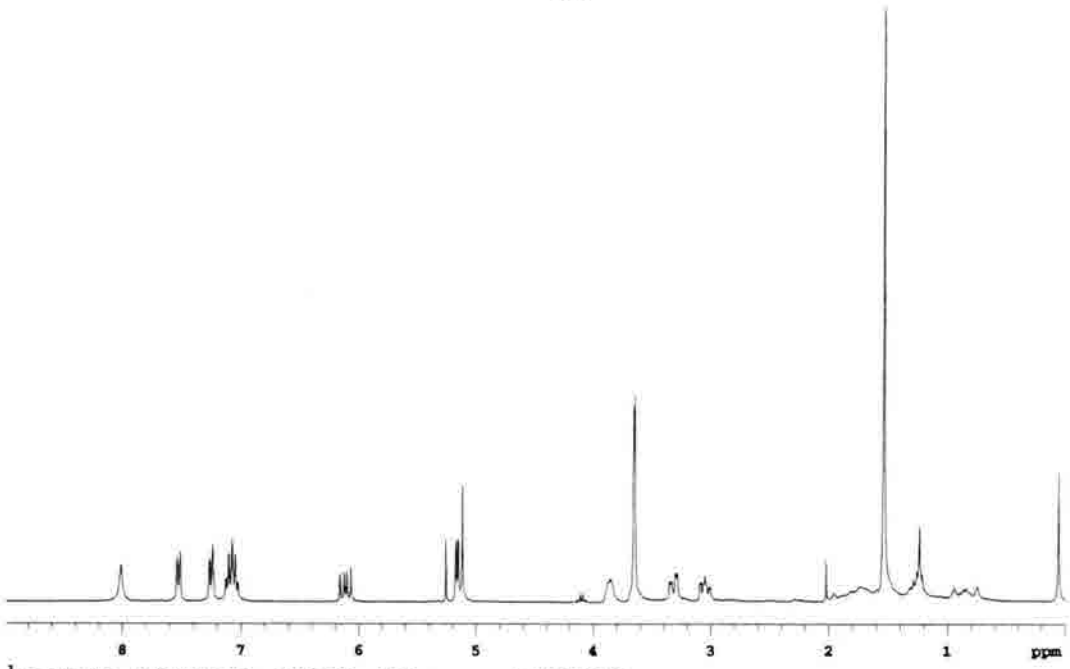
1-[^{13}C]-2-dimethylallyl-L-tryptophan (**160b,** 51 mg, 0.187 mmol) and D, L-camphorsulfonic acid (115 mg, 0.497 mmol, 2.0 eq.) was dissolved in 2.5 mL of anhydrous methanol and refluxed though an addition funnel containing 3 Å molecular sieves under an argon atmosphere for 24 hours. The methanol was removed under reduced pressure and the residue was covered with a layer of ethyl acetate. The solution was made basic by the addition aqueous saturated sodium carbonate. The aqueous layer was separated from the organic and then extracted three more times with ethyl acetate. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo* to leave **189** (53.5 mg, 0.186 mmol, 99%) as a yellowish oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.62 (6 H, s), 1.72 (2H, bs), 3.12 (1H, dd, J = 9.5, 14.3 Hz), 3.39 (1H, dd, J =5.1, 14.7 Hz), 3.73 (3H, s), 3.94 (1H, dd, J = 4.8, 9.5 Hz), 5.22 (1H, dd, J = 1.1, 10.6 Hz), 5.23 (1H, dd, J =1.1, 17.6 Hz), 6.19 (1H, dd, J =10.3, 17.6 Hz), 7.12 (1H, ddd, J =1.1, 7.0 Hz), 7.18 (1H, dd, J = 1.1, 7.0 Hz), 7.33 (1H, dd, J = 0.7, 7.0 Hz), 7.6 (1H, dd, J = 0.7, 7.7 Hz), 7.98 (1H, bs). ^{13}C NMR (100 MHz, CD_3OD): δ 27.8, 27.9, 31.2, 39.1, 52.0, 51.9 (d, J_{CC}= 54 Hz), 55.4, 56.1, 106.7, 110.4, 112.0, 118.4, 119.2, 121.4, 129.6, 134.1, 140.4, 145.9, 180.0.). IR (neat, NaCl): 3396, 3284, 3081, 3056, 2962, 2924, 2853, 1699, 1653, 1457, 1436, 1260, 1199, 1154, 1095, 1018, 919, 862, 799, 742 cm^{-1}. HRMS (FAB+): Calcd for $C_{15}{}^{13}C_1H_{21}N_2O_2$: 274.1637. Found 274.1643 (M+H).

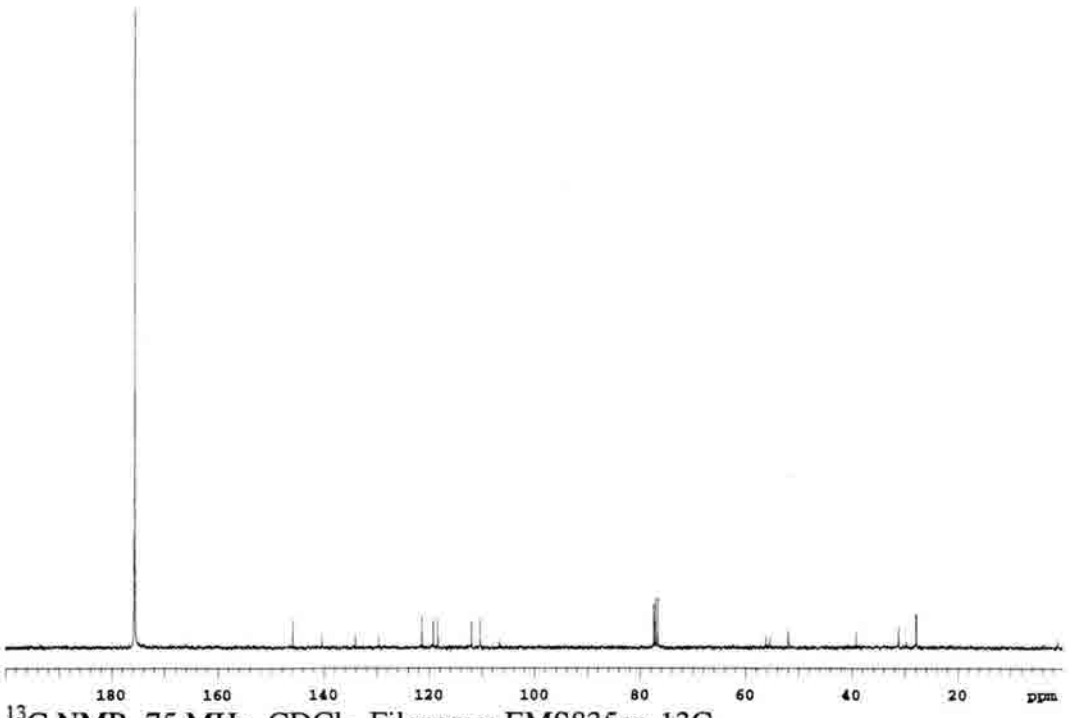
Optical Rotation: $[\alpha]_D^{25} = -16.5°$ (CH_2Cl_2, c = 0.085).



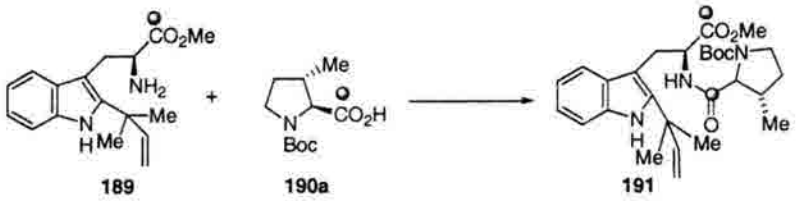
189



^{1}H NMR, 300 MHz, $CDCl_3$, Filename: EMS836cr



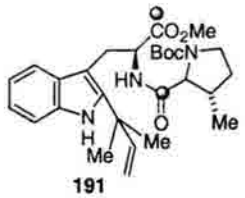
^{13}C NMR, 75 MHz, $CDCl_3$, Filename: EMS835cr_13C



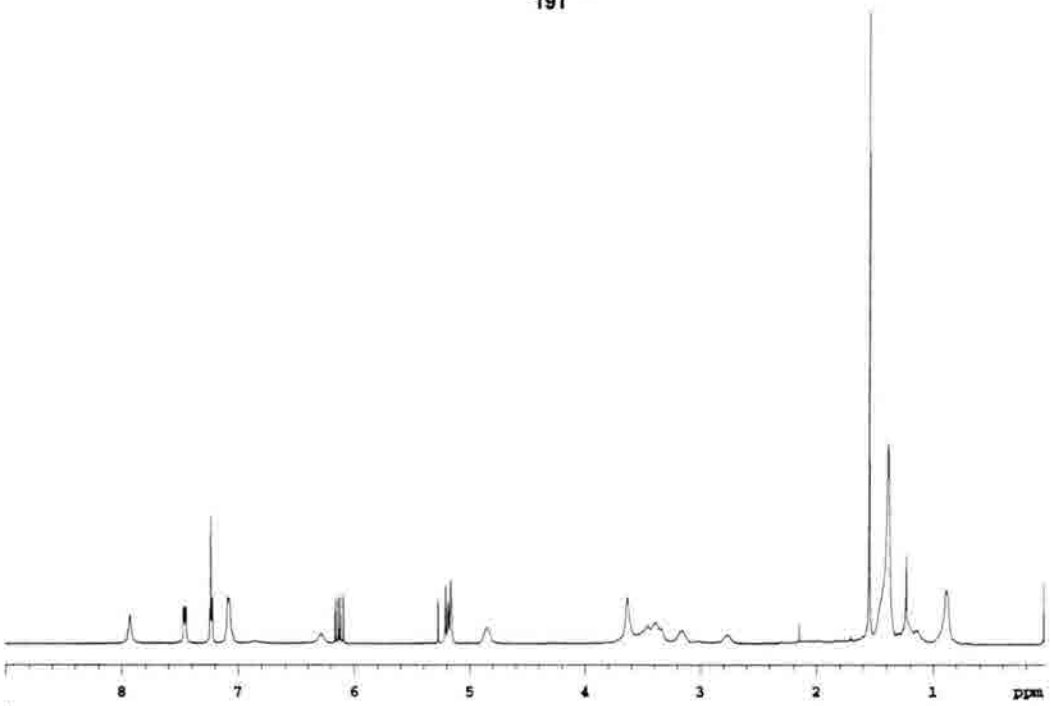
N-Boc-β-methylproline (**190a**, 42.5 mg, 0.185 mmol, 1.0 eq.) was mixed with **189** (53 mg, 0.185 mmol, 1.0 eq.), BOP reagent (82 mg, 0.185 mmol, 1.0 eq.), and Et_3N (28 μL, 0.2035 mmol, 1.1 eq.) in dry acetonitrile (2.8 mL) and stirred at room temperature under an inert atmosphere for 4 h. A saturated aqueous solution of NaCl was added and the reaction was extracted four times with EtOAc. The combined organic layers were washed with 2M HCl, water, 10% $NaHCO_3$ (aq.), water and brine successively. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product was purified by flash silica gel column chromatography using 40% EtOAc/Hex to give **191** (69.5 mg, 75%) as a colorless glass.

^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.00 (3 H, d, J = 7.0 Hz), 1.33 (1H, m), 1.38 (9H, s), 1.54 (3H,s), 1.55 (3H,s), 1.72 (1H, m), 1.97 (1H, m), 2.89 (1H, bs), 3.12 (1H, m), 3.18 (1H, dd, J = 0, 8.4 Hz), 3.35 (2H, m), 3.45 (3H, s), 3.64 (1H, d, J = 5.5 Hz), 4.73 (1H, dd, J = 7.3, 14.6 Hz), 5.03 (1H, d, J = 10.3 Hz), 5.10 (1H, d, J = 17.6 Hz), 6.21 (1H, ddd, J = 1.5, 10.6, 17.6 Hz), 6.94 (1H, t, J = 7.3 Hz), 7.01 (1H, t, J = 7.0 Hz), 7.33 (1H, d, J = 8.0 Hz), 7.37 (1H, bs), 7.47 (1H, d, J = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.4, 27.5, 27.6, 28.1, 29.67, 31.5, 39.1, 45.7, 52.1, 52.9 (d, $J_{C\text{-}C}$ = 63 Hz), 67.9 (d, $J_{C\text{-}C}$ = 53 Hz), 80.5, 105.2, 110.4, 112.4, 117.6, 119.9, 121.7, 130.1, 134.0, 140.6, 145.6, 154.6, 172.2, 172.7. IR (neat, NaCl): 3337, 2968, 2929, 2875, 1703, 1625, 1504, 1462, 1435,

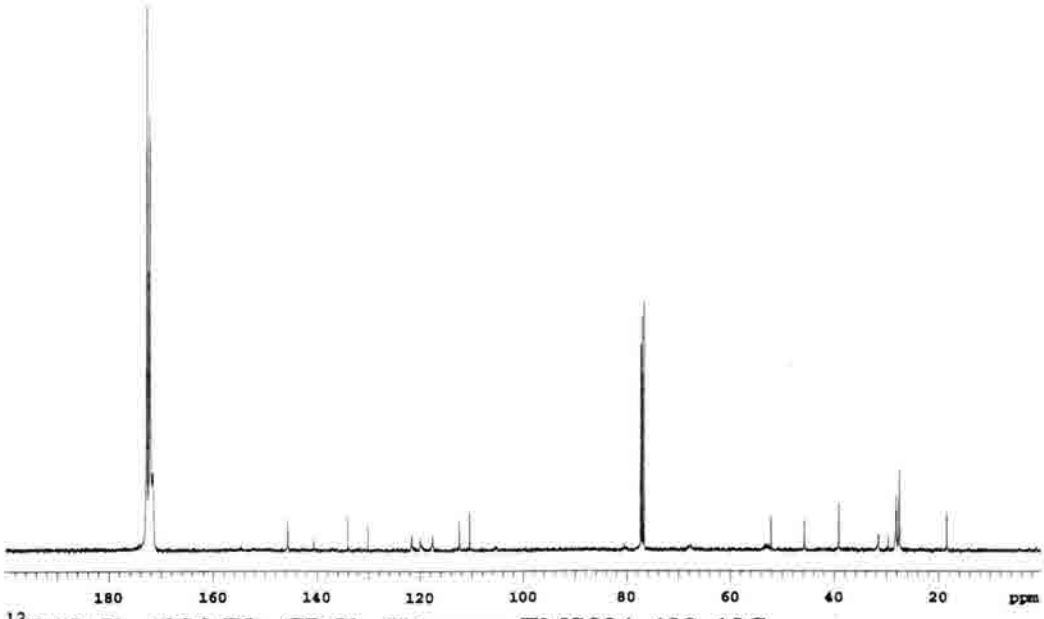
1392, 1365, 1248, 1167, 1151, 1120 cm^{-1}. $[\alpha]_D^{25}$= -29.1° (H_2O, c = 0.34). HRMS (FAB+): Calcd for $C_{26}{}^{13}C_2H_{40}N_3O_5$: 500.3035. Found 500.3040 (M+H).



191



^1H NMR, 400 MHz, $CDCl_3$, Filename: EMS824_400

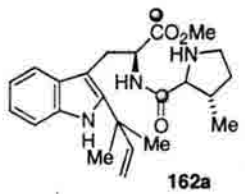


^{13}C NMR, 100 MHz, $CDCl_3$, Filename: EMS824_400_13C

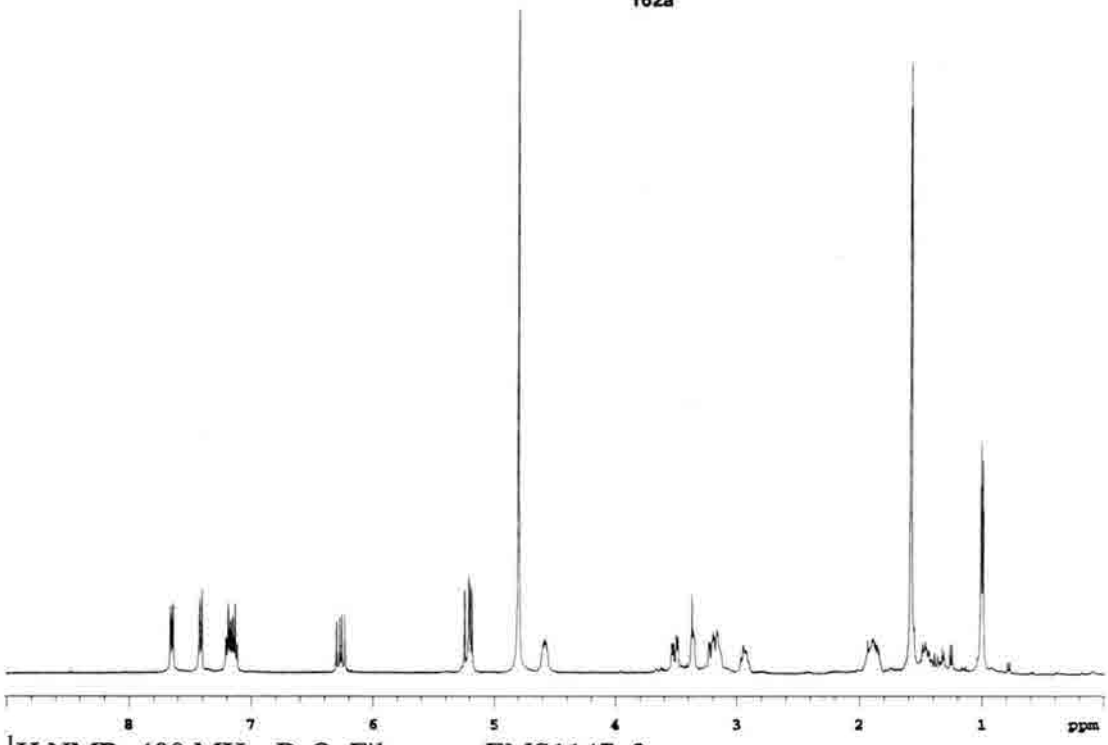
[$^{13}C_2$]-2-dimethylallyl-L-tryptophyl-3(*S*)-methyl-L-proline (162a): To a 0.03M THF/H_2O (2:1) solution of **191** (23 mg, 0.046 mmol), was added 5 equivalents of 1N LiOH (230 μL, 0.23 mmol). The mixture was stirred at room temperature for 24 hours. The THF was removed *in vacuo* and the residue was acidified to pH=2 with 10% aqueous $KHSO_4$ before extracting 3 times with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was re-suspended in CH_2Cl_2 (0.5 mL), TFA was added (74 μL, 0.92 mmol, 20 eq.), and the mixture was stirred for 24 hours at room temperature. The CH_2Cl_2 and excess TFA were removed under reduced pressure. The residue was dissolved in water and extracted with EtOAc three times the aqueous layer evaporated to dryness. The residue was re-suspended in water, loaded onto a suitably prepared DOWEX ion exchange column, then desalted with de-ionized water and eluted with 2% aqueous NH_4OH. The elutant was evaporated *in vacuo* and then re-suspended in a small amount of de-ionized water lyophillized to give a white solid (12.3 mg, 0.032 mmol, 69%).

^{1}H NMR (400 MHz, D_2O): δ 1.00 (3H, d, *J*= 6.2), 1.46 (1H, m), 1.58 (3H, s), 1.58 (3H,s), 1.88 (2H, m), 2.94 (1H, m), 3.19 (2H, m), 3.35 (1H, m), 3.35 (1H, dd, *J*= 5.5, 14.8 Hz), 4.59 (1H, m), 5.20 (1H, dd, *J*= 0.8, 10.5 Hz), 5.23 (1H, dd, *J*= 0, 17.5 Hz), 6.27 (1H, dd, *J*= 10.5, 17.5 Hz), 7.13 (1H, t, *J*= 7.0 Hz), 7.19 (1H, t, *J*= 7.0 Hz), 7.42 (1H, d, *J*= 8.2

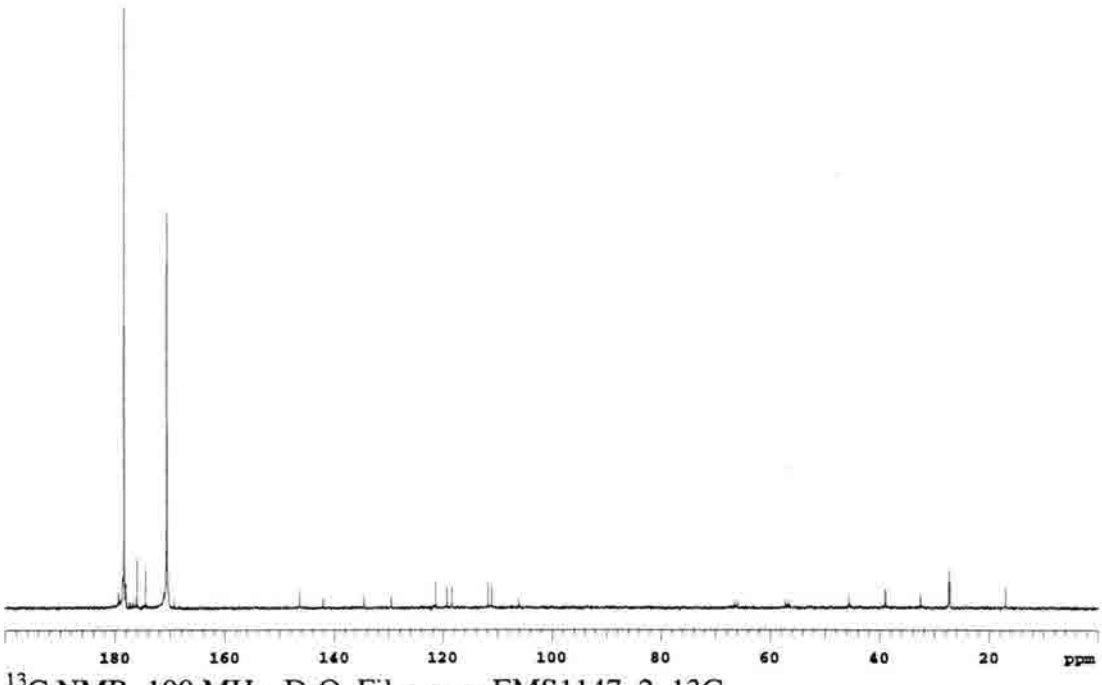
Hz), 7.65 (1H, d, J= 7.8 Hz). ^{13}C NMR (100 MHz, D_2O): δ 17.0, 27.2, 27.3, 32.5, 38.9, 39.0, 45.6, 56.9 (d, $J_{C\text{-}C}$= 54 Hz), 66.19 (d, $J_{C\text{-}C}$= 52 Hz), 106.1 (d, $J_{C\text{-}C}$ = 5 Hz), 111.1, 111.8, 118.4, 119.4, 121.4, 129.6, 134.6, 142.0, 146.3, 170.6, 178.4. IR (NaCl, CH_2Cl_2): 3630-1700 (broad), 3292, 3056, 2964, 2964, 2929, 2872, 1558, 1540, 1457, 1362, 921, 743 cm^{-1}. $[\alpha]_D^{25}$= +8.0° (c=0.1, H_2O). HRMS (FAB^+) calcd. for $C_{20}{}^{13}C_2H_{30}N_3O_3$ 386.2354. Found 386.2359 (M+H).



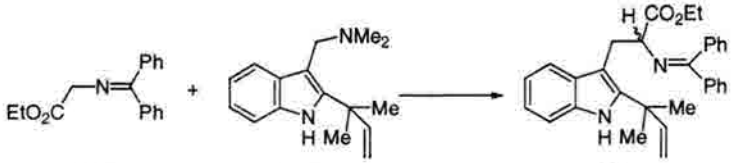
162a



^{1}H NMR, 400 MHz, D_2O, Filename: EMS1147_2



^{13}C NMR, 100 MHz, D_2O, Filename: EMS1147_2_13C

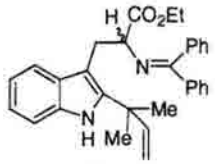


182 170 217

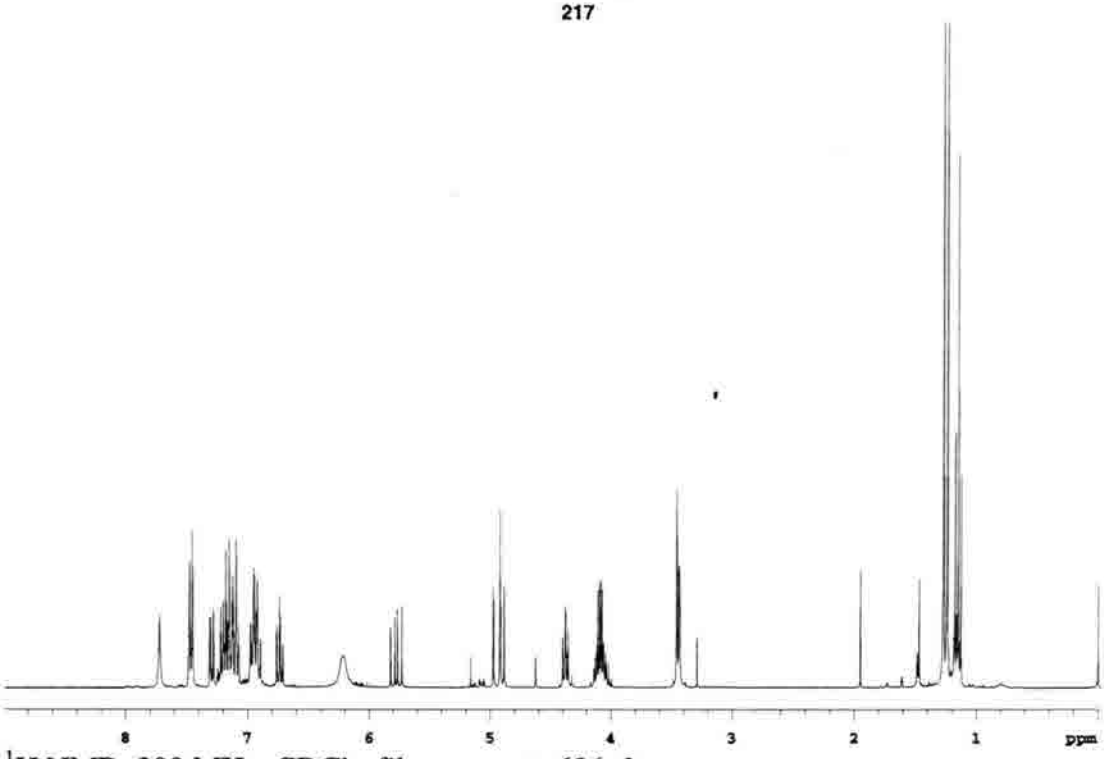
N-(diphenylmethylene)2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan ethyl ester (217):

N-(diphenylmethylene)-glycine-ethylester (**182**), (3.2 g, 12.0 mmol) and the gramine derivative (**170**)[13] (3.2 g, 13.2 mmol) were stirred in acetonitrile (110 mL) under argon until the solids dissolved. Tri-*n*-butylphosphine, (1.5 mL, 6 mmol) was added and the mixture was brought to reflux temperature for 8 h. After cooling to room temperature, the solvent was concentrated under reduced pressure and the crude product was purified by flash silica gel column chromatography (15% EtOAc/hexanes) to yield 3.78 g (70%) of **217** as an oil.

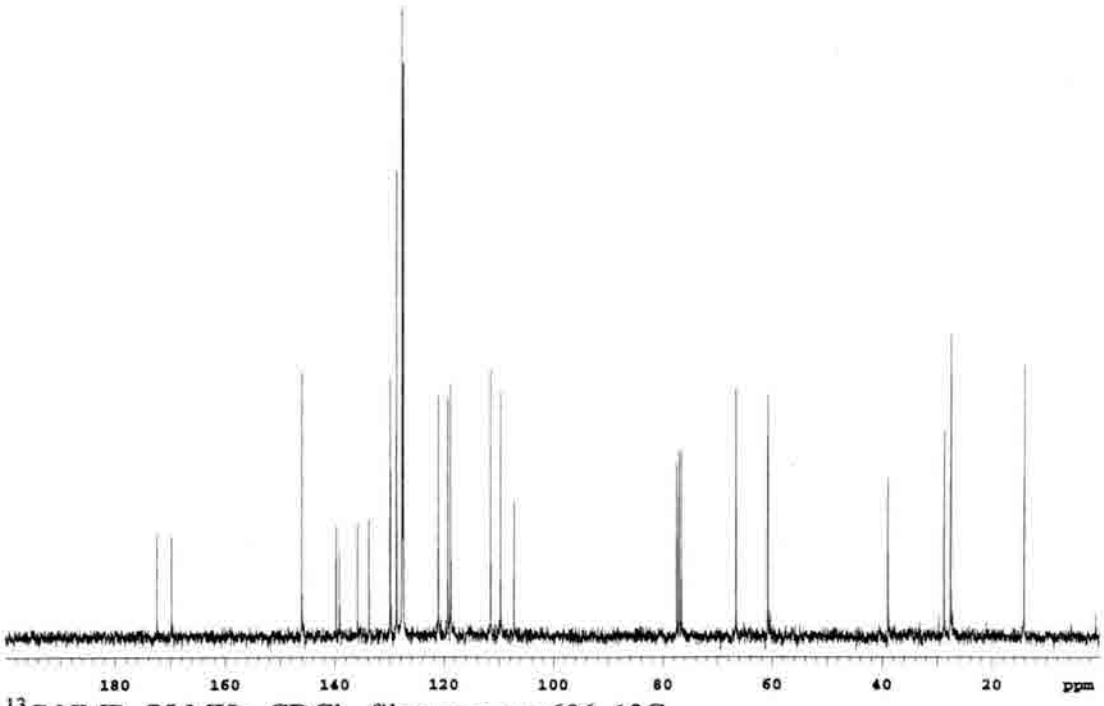
^{1}H NMR (300 MHz, $CDCl_3$): δ 1.29 (3H, t, *J*= 7.0 Hz), 1.38 (3H, s), 1.41 (3H, s), 3.59 (2H, dd, *J*= 1.1, 7.0 Hz), 4.23 (2H, m), 4.52 (1H, dd, *J*= 6.2, 7.3 Hz), 5.04 (1H, dd, *J*= 1.1, 10.6 Hz), 5.09 (1 H, dd. *J*= 1.3, 17.2 Hz), 5.92 (1H dd, *J*= 17.2, 10.3 Hz), 6.39 (2H, bs), 6.87 (1H, ddd, *J*= 1, 8.3, 8.3 Hz), 7.06 (1H, ddd, *J*= 1.1, 7.5, 7.5 Hz), 7.09 (2H, dd, *J*= 1.1, 7.5, 7.5 Hz), 7.30 (5H, m), 7.45 (1H, d, *J*= 8.0 Hz), 7.60 (2H, m), 7.86 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.1, 27.5, 27.5, 28.7, 38.9, 60.8, 66.6, 107.2, 109.7, 111.5, 118.8, 119.3, 121.1, 127.5, 127.6, 127.7, 128.77, 129.9, 130.0, 133.8, 135.9, 139.2, 139.8, 146.0, 169.7, 172.4. I.R. (NaCl neat): 3405, 3057, 2972, 1731, 1621, 1597, 1575, 1489, 1462, 1446, 1286, 1245, 1185, 1069, 1029, 917, 781, 742, 697 cm^{-1}. HRMS (FAB+): Calcd for $C_{31}H_{33}N_2O_2$: 465.2542. Found 465.2541 (M+H).



217



^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems686_2



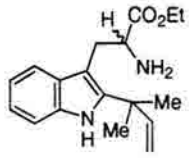
^{13}C NMR, 75 MHz, $CDCl_3$, filename: ems686_13C

217 218

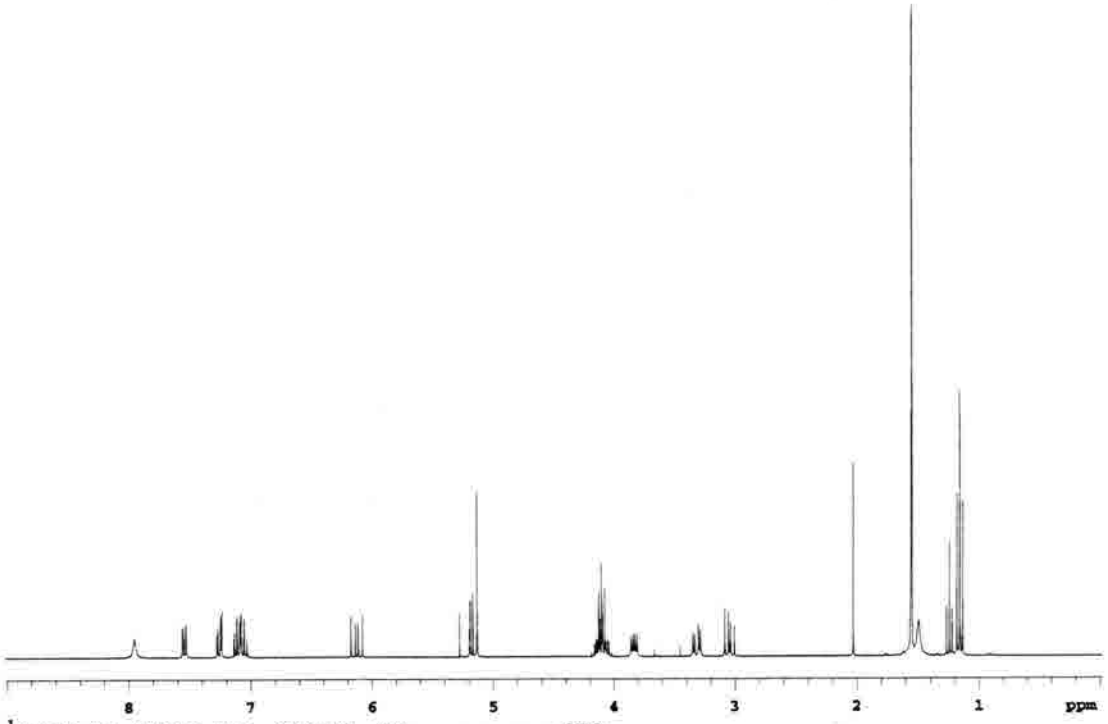
2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan ethyl ester (218):

Compound **217**, (1.92 g, 4.27 mmol) was stirred with NH_2OH•HCl (2.25 g, 32.45 mmol) and solid Na_2CO_3 (3.21 g, 30.31 mmol) in CH_2Cl_2 (17 mL) at room temperature under argon for 24 h. The solution was acidified to pH 3 with 10% $NaHSO_4$ (aq) and the organic layer was separated from the aqueous phase. The aqueous layer was extracted three more times with EtOAc before it was made basic with 10% Na_2CO_3 (aq) and extracted three times with EtOAc. The combined organic layers from the basic extract were dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure to give 1.03 g (80%) of **218** as a colorless oil.

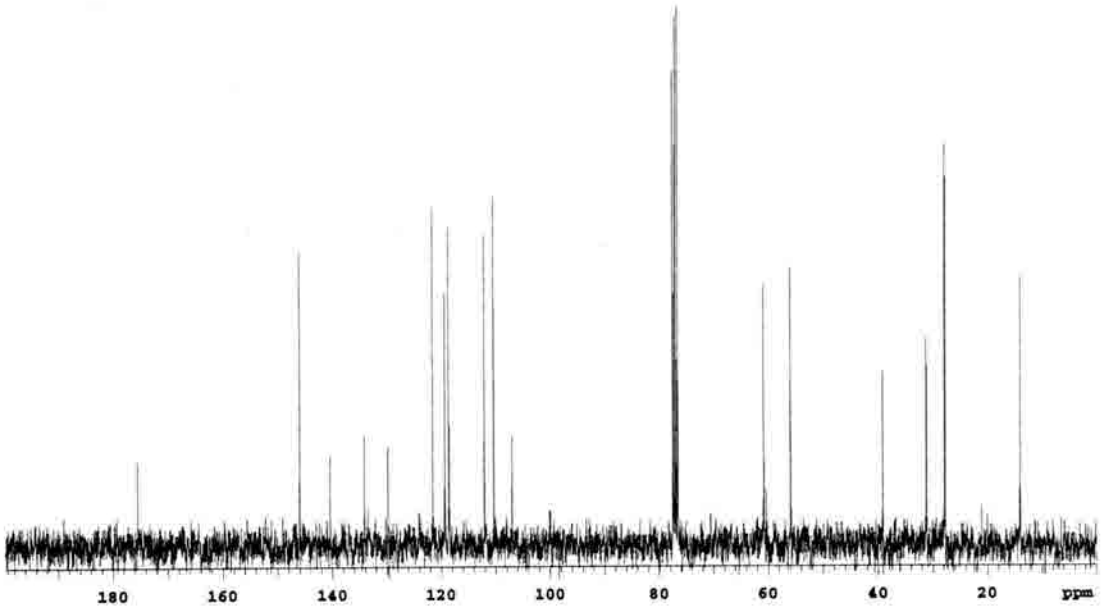
^{1}H NMR (300 MHz, $CDCl_3$): δ 1.19 (3H, t, *J*= 7 Hz), 1.52 (2H, bs), 1.58 (6H, s), 3.08 (1H, dd, *J*= 14.3, 9.5Hz), 3.35 (1H, dd, *J*= 14.7, 5 Hz), 3.86 (1H, dd, *J*= 9.5, 5.1Hz), 4.13 (2H, m), 5.18 (1 H, dd, *J*= 0.7, 10.6 Hz), 5.20 (1 H, dd, *J*= 0.7, 17.2 Hz), 6.16 (1H, dd, *J*= 17.2, 10.6 Hz), 7.08 (1H, t, *J*= 7 Hz), 7.14 (1H, t, *J*= 7 Hz), 7.29 (1H, d, *J*= 7 Hz), 7.58 (1H, d, *J*= 7 Hz), 7.98 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.0, 27.8, 27.9, 31.2, 39.1, 55.9, 60.8, 107.0, 110.3, 112.1, 118.6, 119.3, 112.5, 129.8, 134.1, 140.4, 146.0, 175.5. IR (NaCl neat) 3399, 3243, 3081, 3056, 2973, 1733, 1638, 1617, 1580, 1462, 1300, 1282, 1195, 1105, 1029, 917, 859, 743 cm $^{-1}$. HRMS (FAB+): Calcd for $C_{18}H_{25}N_2O_2$: 301.1916. Found 301.1919 (M+H).



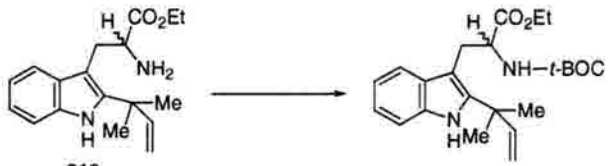
218



^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems670



^{13}C NMR, 75 MHz, $CDCl_3$, filename: ems670_13C

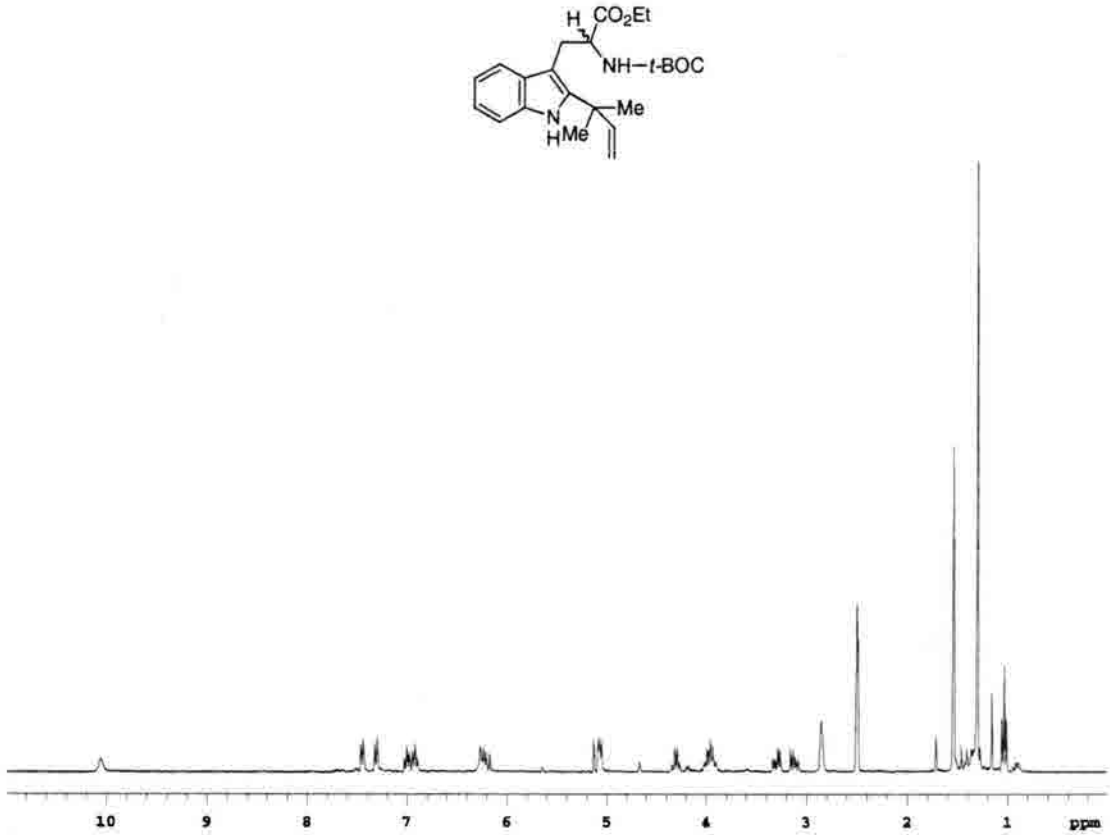


218

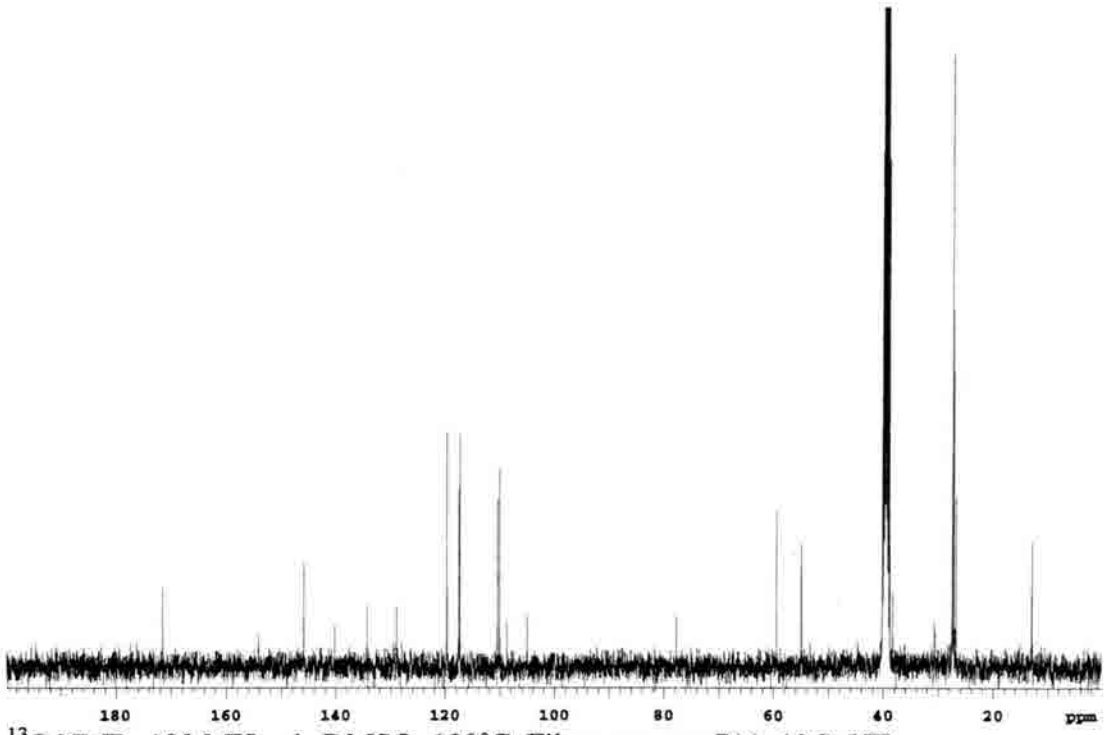
N-[(1,1-dimethylethoxy)carbonyl]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan ethyl ester:

218 (1.57 g, 5.23 mmol) was stirred with 1 equivalent of 0.5 M NaOH, di-*tert*-butyl-pyrocarbonate (1.25 g, 5.75 mmol) in dioxane (5.23 mL) at room temperature for 3 h. The dioxane was removed under reduced pressure and the solution was brought to pH=2 with the addition of aqueous 10% $KHSO_4$. The aqueous layer was extracted 3 times with EtOAc and dried over anhydrous Na_2SO_4. After removing the solvent under reduced pressure, the product was purified by flash silica gel column chromatography using 30% EtOAc/hexane to yield 1.843 g (88%) of the product as an oil.

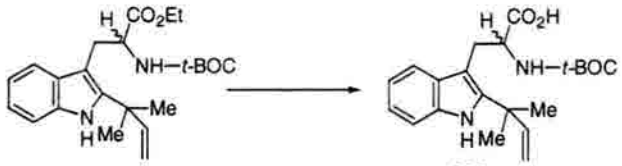
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.04 (3H, t, *J*= 6.96 Hz), 1.31 (9H, s), 1.55 (6H, s), 3.12 (1H, dd, *J*= 7.7, 14.3 Hz), 3.30 (1H, dd, *J*= 6.8, 14.8 Hz), 3.6 (2H,m), 4.30 (1H, dd, *J*= 7.7, 15.6 Hz), 5.07 (1H, dd, *J*= 0, 10.3 Hz), 5.10 (1H, dd, *J*= 0, 17.6 Hz), 6.22 (1H, dd, *J*= 10.7, 17.6 Hz), 6.26 (1H, bs), 6.92 (1H, t, *J*= 7.3 Hz), 7.00 (1H, t, *J*= 7.0 Hz), 7.31 (1H, d, *J*= 8.0 Hz), 7.45 (1H, d, *J*= 7.7 Hz), 10.06 (1H, bs). ^{13}C NMR (100 MHz, d_6-DMSO, 116°C): δ 12.9, 26.8, 27.3, 27.5, 30.7, 38.3, 54.9, 59.4, 77.7, 105.0, 108.7, 110.0, 110.5, 117.4, 117.5, 119.7, 128.9, 134.4, 140.3, 145.9, 154.2, 171.5. IR (NaCl neat): 3376, 3083, 3057, 2976, 2933, 1697, 1503, 1462, 1376, 1167, 1021, 917, 861, 742 cm^{-1}. HRMS (FAB+): Calcd for $C_{23}H_{32}N_2O_4$: 400.236208. Found 400.236330 (M^+).



^{1}H NMR, 300 MHz, d_6-DMSO, 120°C, Filename: ems711_dmso_120



^{13}C NMR, 100 MHz, d_6-DMSO, 120°C, Filename: ems711_13C_VT

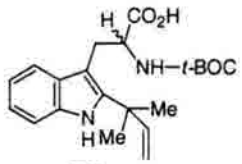


236

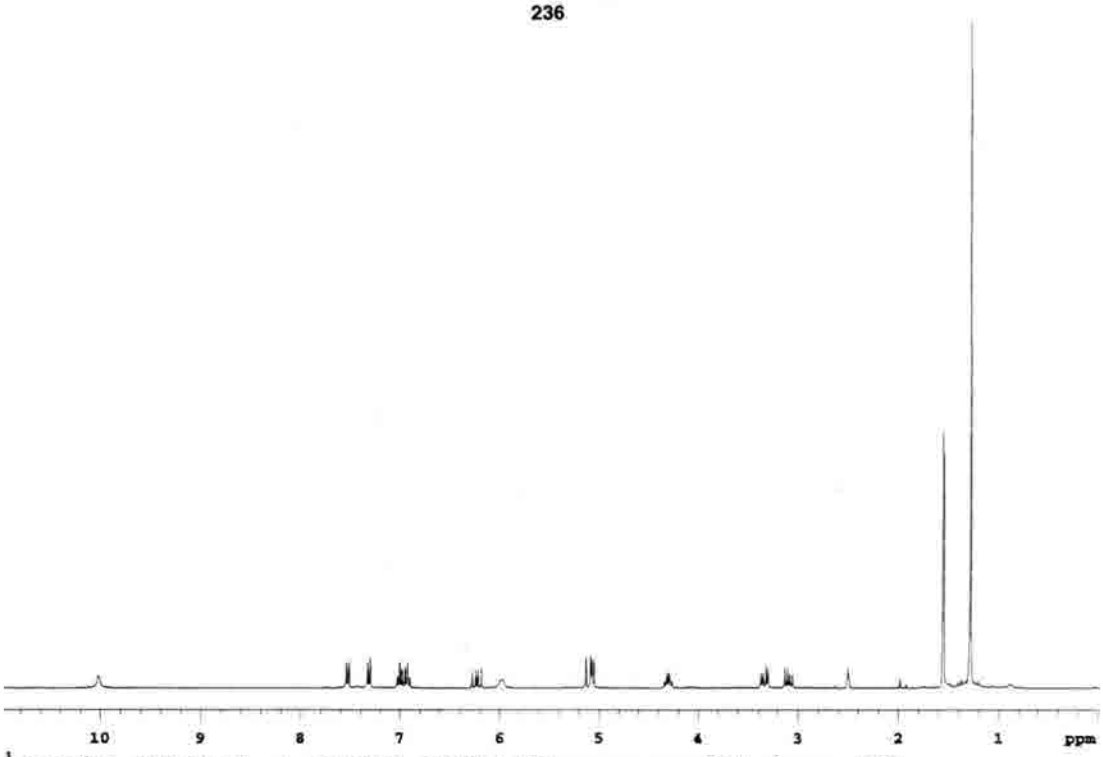
N-[(1,1-dimethylethoxy)carbonyl]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan (236):

Racemic N-Boc-prenyl-tryptophan-ethylester, (1.97 g, 4.60 mmol) was stirred with LiOH (589 mg, 25 mmol) in a THF:H_2O solution (2:1) (16 mL) overnight. The solution was acidified with 10% $KHSO_4$(aq) and extracted 3 times with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was concentrated under reduced pressure to afford 1.63 g (89%) of **236** as an oil. The product was deemed sufficiently pure to use directly for the next step without further purification.

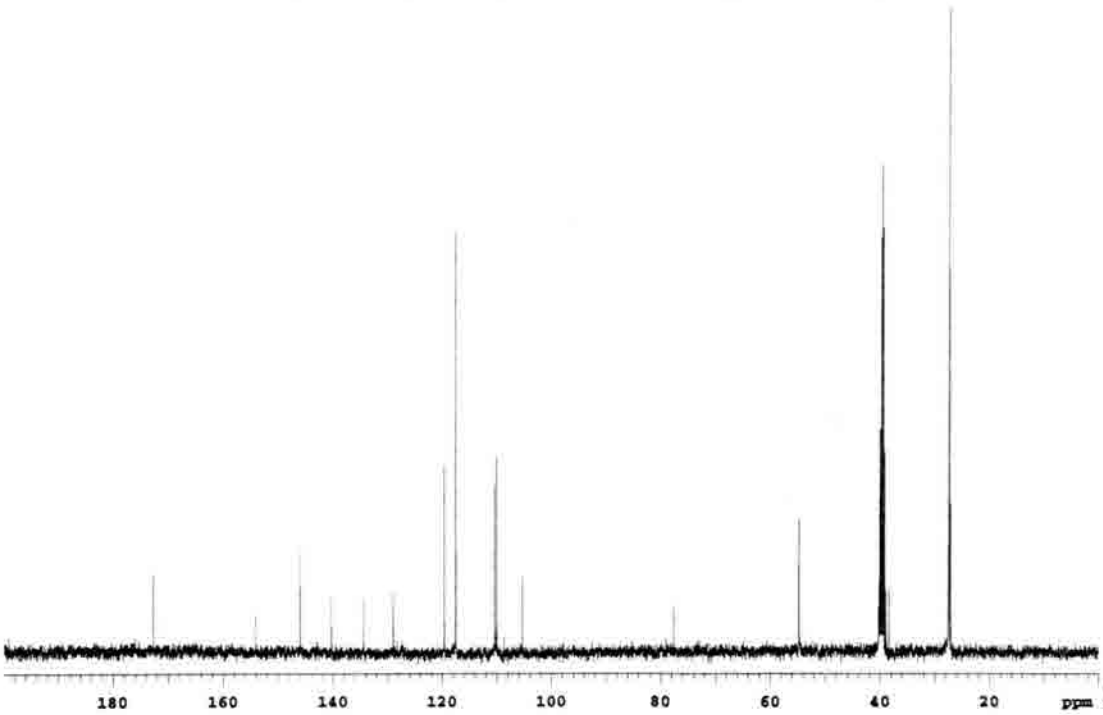
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.28 (9H, s), 1.55 (3H, s), 1.56 (3H, s), 3.09 (1H, dd, *J*= 8.1, 14.7 Hz), 3.46 (1H, dd, *J*= 6.2, 14.7), 4.30 (1H, dd, *J*= 8.1, 14.7 Hz), 5.06 (1H, dd, *J*= 1.1, 10.6 Hz), 5.10 (1H, dd, *J*= 0, 17.2 Hz), 5.98 (1H, d, *J*= 5.9 Hz), 6.22 (1H, dd, *J*= 10.6, 17.2 Hz), 6.92 (1H, ddd, *J*=1.1, 7.7, 7.7 Hz), 7.00 (1H, ddd, *J*=0.8, 7.7, 7.7 Hz), 7.31 (1H, d, *J*= 7.7 Hz), 7.53 (1H, d, *J*= 7.7 Hz), 10.02 (1H, bs), 11.51 (1H, very bs). ^{13}C NMR (100 MHz, d_6-DMSO, 120°C): δ 27.1, 27.3, 27.4, 38.3, 54.6, 77.6, 105.4, 110.1, 110.4, 117.5, 119.6, 129.0, 134.4, 140.3, 146.0, 154.2, 172.7. IR (NaCl neat) 3368-2563, 3368, 3087, 3053, 2974, 2926, 1712, 1502, 1460, 1394, 1367, 1245, 1164, 1054, 1010, 919, 742 cm $^{-1}$. HRMS (FAB+): Calcd for $C_{21}H_{28}N_2O_4$: 372.2049. Found 372.2052 (M+).



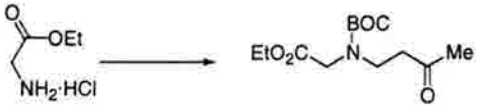
236



^{1}H NMR, 300 MHz, d_6-DMSO, 120°C, Filename: ems854_dmso_120



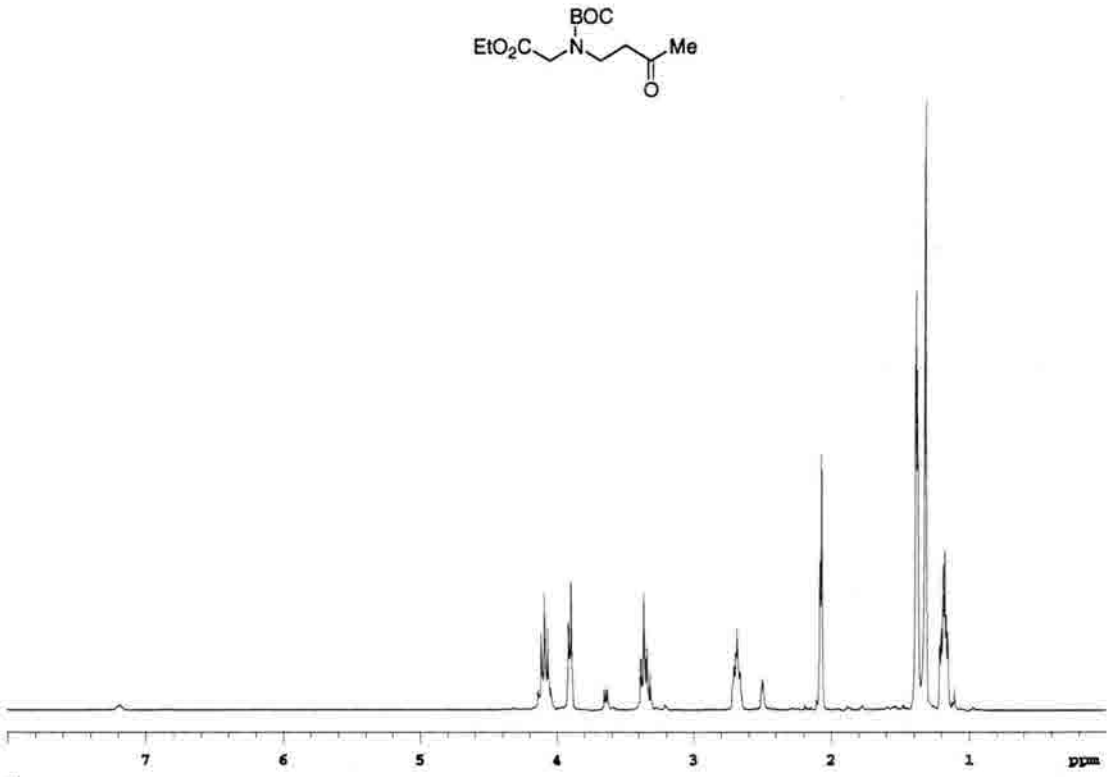
^{13}C NMR, 100 MHz, d_6-DMSO, 120°C, Filename: ems854_13C_VT



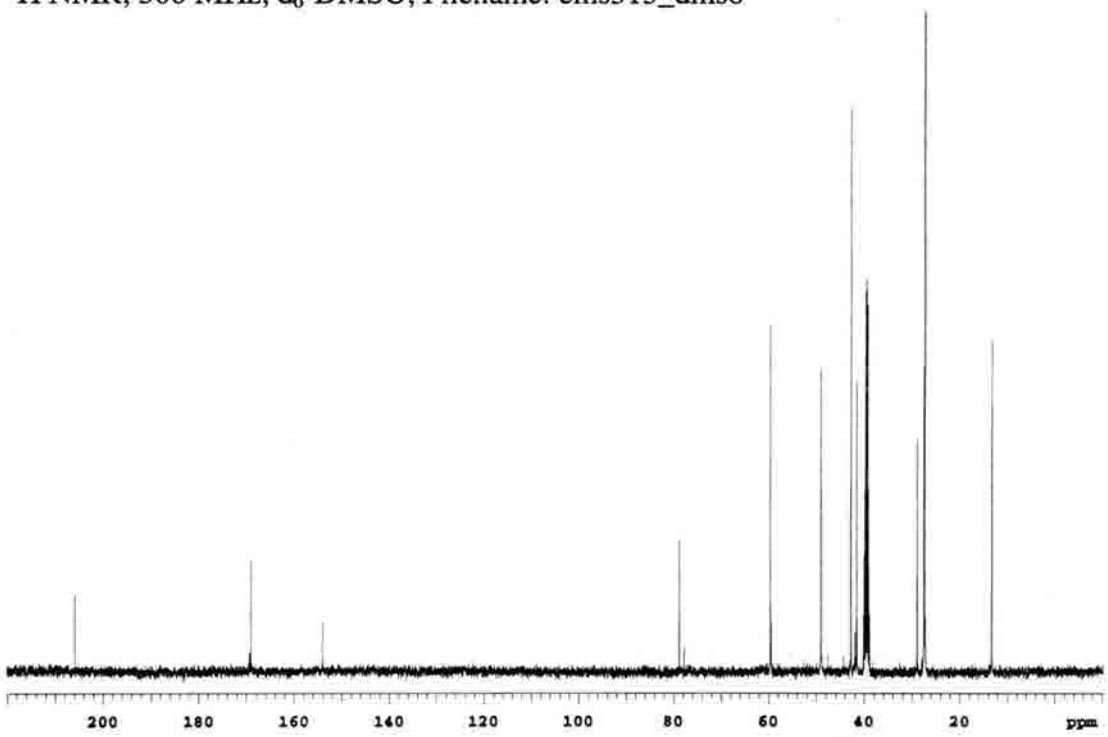
N-[1,1-dimethyethoxy) carbonyl]-N-(3-oxobutyl)-glycine ethyl ester:

The hydrochloride salt of glycine ethyl ester was neutralized by the addition of 1 equiv. of aqueous 10% Na_2CO_3 and extracting 5 times with CH_2Cl_2. After drying the organic layer over anhydrous Na_2SO_4 the solvent was removed under reduced pressure, and the crude free amine was obtained. Glycine ethyl ester, (9.06 g, 87.8 mmol) was stirred with methyl vinyl ketone (7.28 mL, 1.0 equiv.) in acetonitrile (88 mL) at room temperature, under argon in the absence of light. After 3 h, the solvent was removed under reduced pressure and the flask was placed under vacuum for 1 h. The free amine decomposes fairly rapidly upon standing, and it was found best to proceed directly to the next step without further purification. To a solution of the adduct obtained above, (13.76g, 79.4 mmol) at 0°C in dioxane (160 mL), was added di-*tert*-butyl-pyrocarbonate (17.3 g, 1.0 equiv., 79.4 mmol), 1M NaOH solution (79.4 mL) and deionized water (79.4 mL). The reaction was allowed to stir under argon, in the absence of light, at room temperature overnight. The reaction was worked up by adding saturated NaCl solution (100 mL), extracting 3 times with EtOAc, drying over anhydrous Na_2SO_4 and evaporating of the solvent under reduced pressure. The crude product was purified by Kugelrohr distillation; the product distilled at 102 °C at 1 mm Hg affording 15.64 g of the product as an oil (65% for the two steps).

^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.23 (3 H, ddd, *J*=1.6, 7.3, 7.3 Hz), 1.41 (9H, s), 2.10 (3H, s), 2.70 (2H, t, *J*= 6.6 Hz), 3.44 (2H, ddd, *J*=1.5, 6.6, 6.6 Hz), 3.9 (2H, s), 4.14 (2H, dddd, *J*= 1.5, 7.0, 7.0, 7.0 Hz). ^{13}C NMR (100 MHz, d_6-DMSO, 120°C): δ 13.3, 27.4, 27.6, 28.9, 41.6, 42.8, 49.0, 59.6, 78.8, 154.0, 169.1, 205.9. IR (NaCl, neat): 3611, 3398, 2978, 2936, 1748, 1698, 1462, 1397, 1367, 1250, 1162, 1129, 1029, 894, 866, 778 cm^{-1}. HRMS (FAB+) Calcd. for $C_{13}H_{24}NO_5$: 274.165448. Found 274.165921 (M+H).



^{1}H NMR, 300 MHz, d_6-DMSO, Filename: ems315_dmso



^{13}C NMR, 100 MHz, d_6-DMSO, 120°C, Filename: ems315_13C_dmso

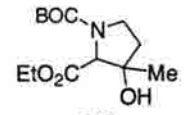
107

3-hydroxy-3-methyl-1,2-pyrrolidinedicarboxylic acid 1-(1,1-dimethylethyl) 2-ethyl ester (107):

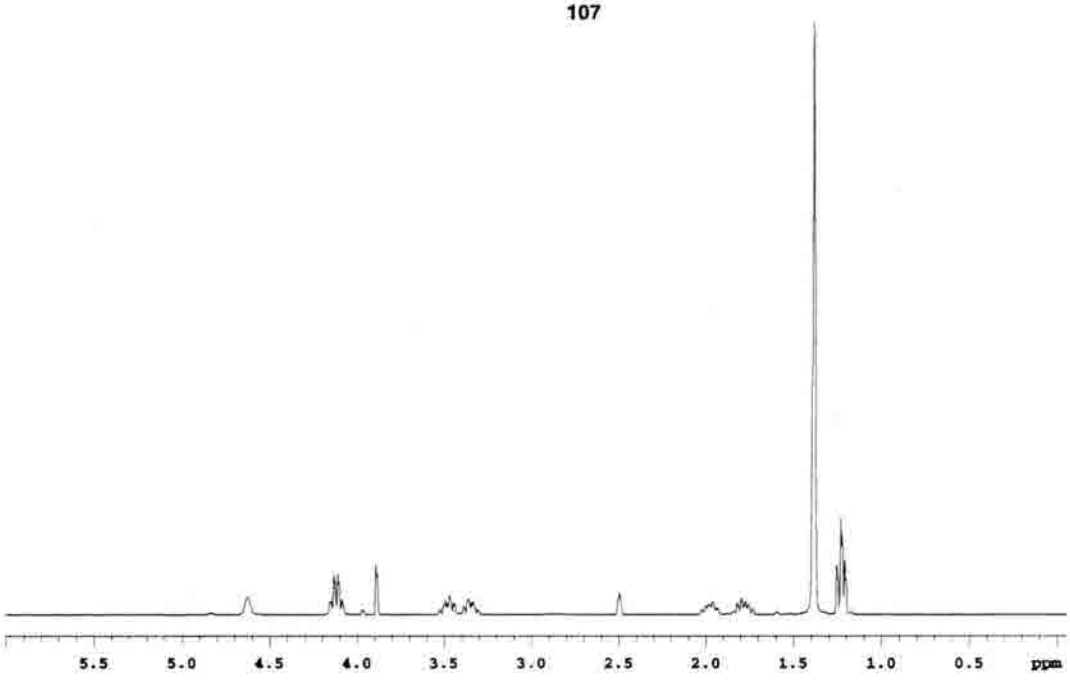
A solution of the N-Boc-protected compound obtained above, (5g, 18.29 mmol) in toluene (100 mL), was cooled to 0°C. Solid potassium *tert*-butoxide (2.05 g, 1.0 equiv.) was added portionwise, and the solution was stirred under argon for 45 min. at 0°C. The reaction was quenched by the addition of ice cold 10% aqueous $KHSO_4$ (pH=2-3). The organic layer was separated from the aqueous layer and the aqueous layer was extracted 3 times with CH_2Cl_2. The combined organic fractions were washed with pH = 7 phosphate buffer and brine successively. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give a yellowish oil. The oil was re-suspended in CH_2Cl_2 and extracted 3 times with pH = 10 Na_2CO_3 buffer. The combined aqueous extracts were extracted two more times with CH_2Cl_2. The organic layers were washed with brine, dried over anhydrous Na_2SO_4 and evaporated to give 1.89g (38%) of the product as an oil which solidified to an off-white amorphous solid upon standing. In some instances it was necessary to purify by flash column chromatography (10-20% EtOAC/ hexanes).

^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.23 (3H, ddd, *J*=1.0, 7.0, 7.0 Hz), 1.40 (12H, s), 1.77 (1H, m), 1.98(1H, m), 3.36 (1H, m), 3.48 (1H, m), 3.89 (1H, d, *J*= 2.2 Hz), 4.12 (2H, dddd, *J*= 2.2, 7.0, 7.0, 7.0 Hz), 4.62 (1H, bs). ^{13}C NMR (100 MHz, d_6-DMSO, 116°C): δ 13.5, 26.6, 27.4, 43.8, 59.1, 68.4, 78.2, 152.8, 168.9. IR (NaCl, neat): 3446, 3093, 2977, 2935,m 2900, 1743, 1681, 1456, 1403, 1367, 1161, 1097, 1033, 926, 860,

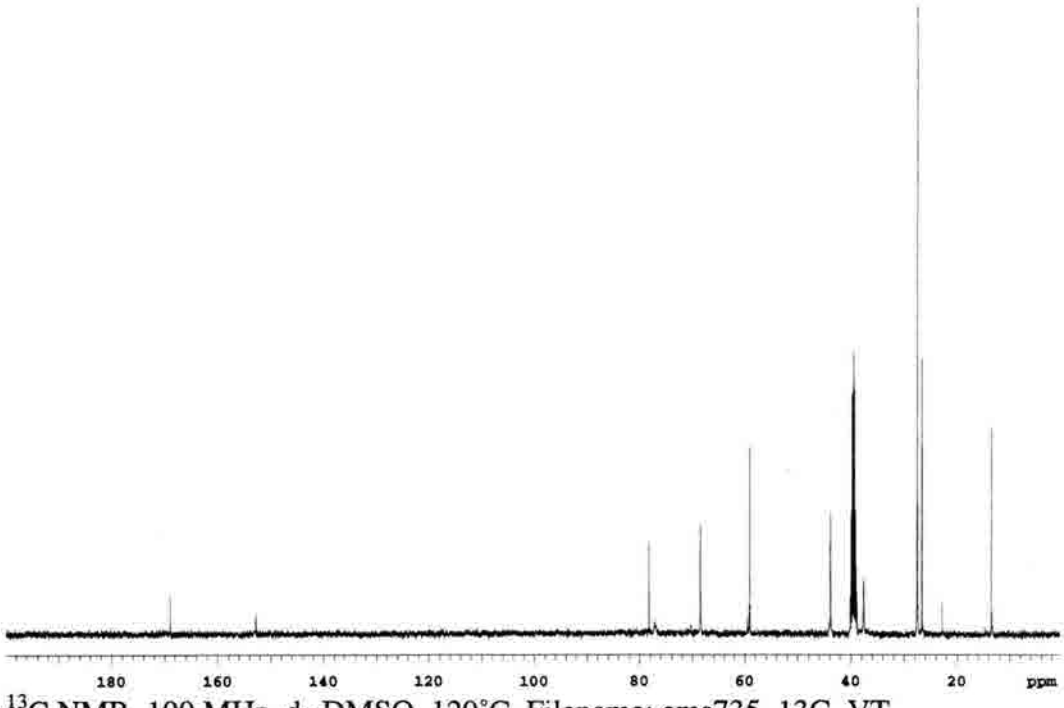
774, 739 cm[1]. HRMS (FAB+): Calcd for $C_{13}H_{24}N_1O_5$: 274.165448. Found 274.166420 (M+H).



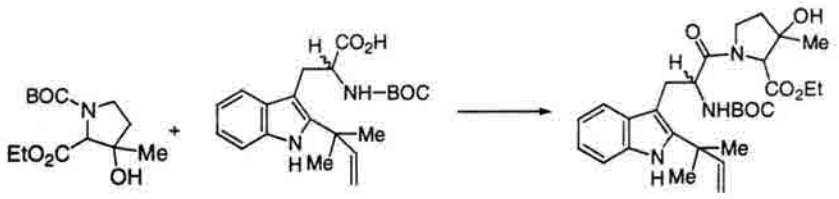
107



^{1}H NMR, 300 MHz, d_6-DMSO, 120°C, Filename: ems735_dmso_120



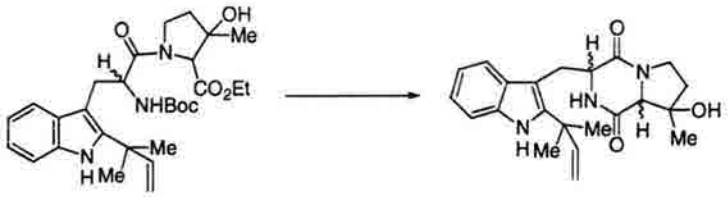
^{13}C NMR, 100 MHz, d_6-DMSO, 120°C, Filename: ems735_13C_VT



107 236 238

1-[N-[(1,1-dimethylethoxy)carbonyl]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophyl]-3-hydroxy-3-methyl-D,L-proline ethyl ester (238):

Compound **107** (1.21 g, 4.43 mmol) was stirred with TFA (6.8 mL), in CH_2Cl_2 (7 mL) at 0°C. The reaction was allowed to come to room temperature and stir for an additional 3 h. A saturated solution of $NaHCO_3$ was added until the solution became basic, and the organic layer was separated from the aqueous phase. The aqueous layer was extracted 3 more times with EtOAc and the combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. Compound **237** (β-hydroxy-β-methylproline ethyl ester) was mixed with compound **236** (1.65 g, 4.43mmol), BOP (1.96 g, 4.43 mmol), and Et_3N (1.24 mL) in acetonitrile (67 mL) at room temperature for 4 h. A saturated aqueous solution of NaCl was added and the reaction was extracted four times with EtOAc. The combined organic layers were washed with 2M HCl, water, 10% $NaHCO_3$ (aq.), water and brine successively. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product was partially purified by flash silica gel column chromatography using 4% MeOH/CH_2Cl_2; the slightly impure mixture of diastereomers (**238**) (1.88g, 80%) were directly carried on to the next step without further purification.



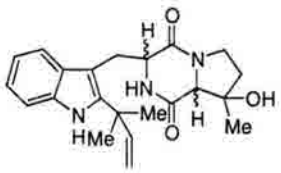
238 239

3-[[2-(1,1-dimethyl-2-propenyl)-1H-indol-3-yl]methyl]-hexahydro-8-hydroxy-8-methyl-pyrrolo[1,2a]pyrazine-1,4-dione (239):

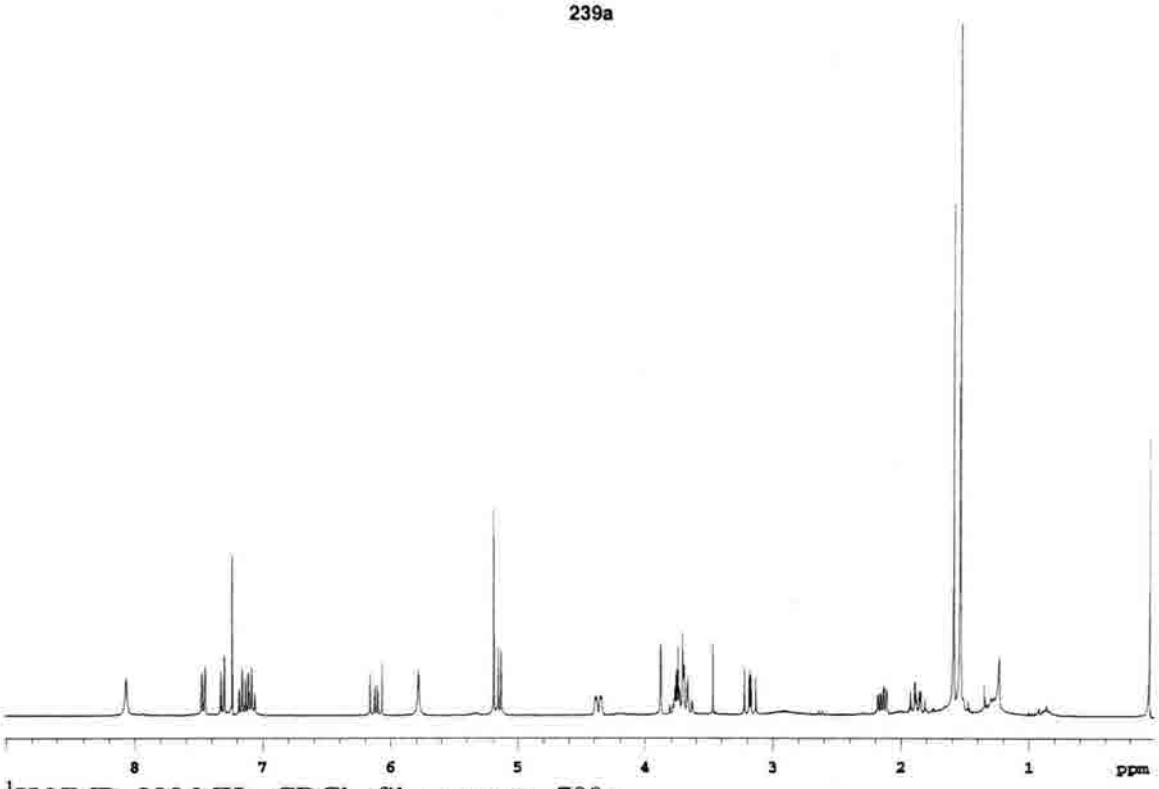
To a solution of **238** (527 mg, 1.00 mmol) in CH_2Cl_2 (2 mL) at 0°C was added TFA (1.6 mL). The ice bath was removed and the mixture was allowed to come to room temperature and stir for an additional 3 h. A saturated solution of $NaHCO_3$ was added until the solution became basic, and the organic layer was separated from the aqueous phase. The aqueous layer was extracted 3 more times with EtOAc and the combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The crude free amine was then dissolved in toluene (5 mL) with 2-hydroxypyridine (19 mg) and the solution was refluxed overnight under argon and the solvent was removed under reduced pressure. The four diastereomers could be partially separated using PTLC, but in practice the mixture of products were purified as a mixture of diasteromers *via* radial silica gel chromatograhy using an elutant of 2% MeOH/CH_2Cl_2 to afford 361 mg (95%) of **239** as a mixture of diastereomers (solid). Data for two of the diastereomers, **239a** (higest R_f) and **239d** (lowest R_f) are descibed below (relative stereochemistry not assigned); diastereomers **239b** and **239c** could not be seperated.

(239a) ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.57 (6H, s), 1.62 (3H, s), 1.89 (1H, m), 2.18 (1H, ddd, *J*=7.3, 7.3, 13.5 Hz), 2.95 (1H, bs), 3.21, (1H, dd *J*=9.7, 15.4 Hz), 3.74 (3H, m),

3.91 (1H, d, *J*=1.5 Hz), 4.40 (1H, dd, *J*=2.2, 11.3 Hz), 5.19 (1H, dd, *J*=0, 17.2 Hz), 5.20 (1H, dd, *J*=0, 11.0 Hz), 5.81 (1H, bs), 6.15 (1H, dd, *J*=10.9, 16.8 Hz), 7.12 (1H, ddd, *J*=1, 7.3, 7.3 Hz), 7.19 (1H, ddd, *J*=1.5, 7.3 , 7.3 Hz), 7.34 (1H, d, *J*= 8.0 Hz), 7.50 (1H, d, *J*=7.7 Hz), 8.10 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.9, 26.1, 27.8, 27.9, 36.9, 39.0, 43.3, 54.5, 65.8, 77.8, 104.5, 110.9, 112.9, 117.8, 120.1, 122.2, 128.0, 132.2, 141.5, 145.6, 166.0, 168.0. I.R. (NaCl neat) 3360, 3054, 2968, 2924, 1666, 1651, 1462, 1434, 1302, 1262, 1138, 1105, 1010, 919, 734 cm $^{-1}$. HRMS (FAB+): Calcd. for $C_{22}H_{28}N_3O_3$: 382.213067. Found 382.212574 (M+H). R_f 0.75 (eluted twice with 2% MeOH/CH_2Cl_2).

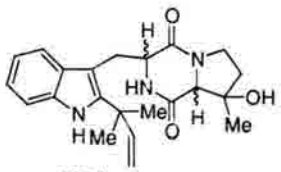


239a

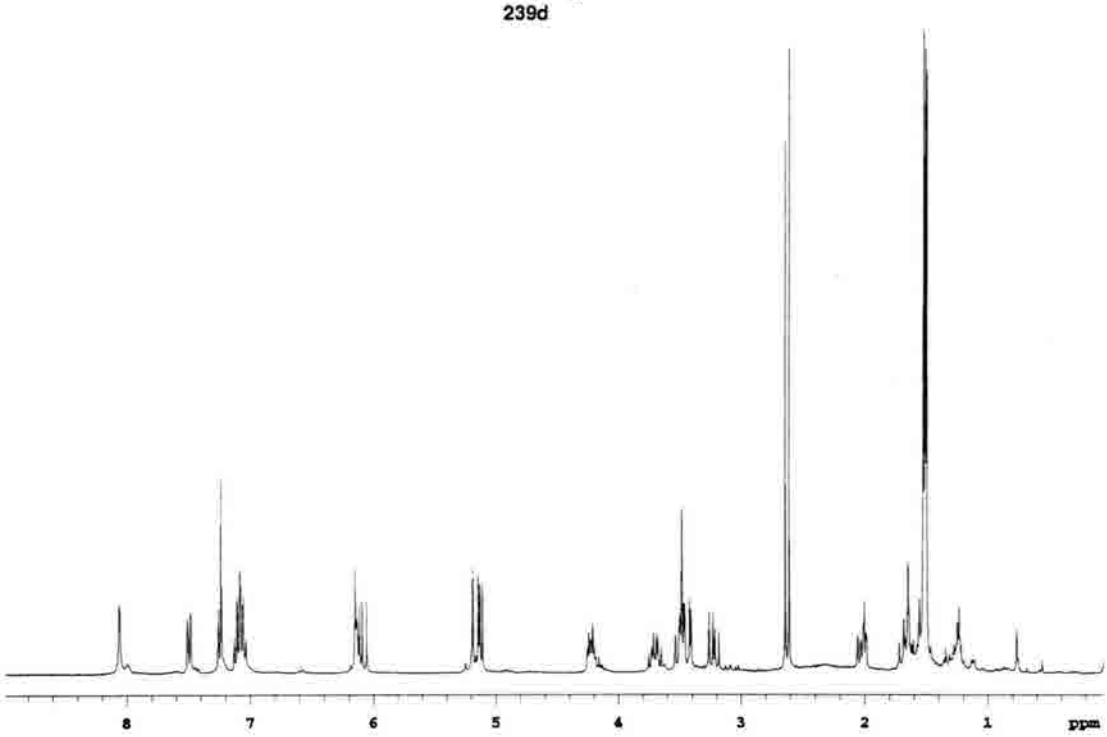


^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems700a

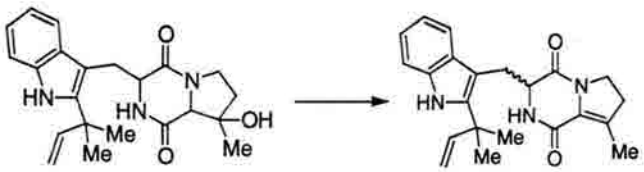
(239d) ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.54 (3H, s), 1.55 (3H, s), 2.04 (2H, m), 2.66 (3H, d, *J*=9.2 Hz), 3.25 (1H, dd, *J*=14.3, 9.5), 3.46 (1H, dd, *J*=14.6, 3.6), 3.53 (3H, m), 3.74 (1H, m), 4.25 (1H, m), 5.16 (1H, dd, *J*=1.1, 10.6 Hz), 5.19 (1H, dd, *J*=0.8, 17.2 Hz), 6.12 (1H, dd, *J*=10.6, 17.6 Hz), 6.18 (1H, bs), 7.12 (2H, m), 7.28 (1H, dd, *J*=1.5, 6.6 Hz), 7.53 (1H, dd, J=1.5, 7.0 Hz), 8.09 (1H, bs). ^{13}C NMR (300 MHz, $CDCl_3$): δ 25.7, 27.7, 28.0, 36.6, 36.9, 39.1, 43.3, 58.2, 65.0, 77.9, 105.1, 110.5, 111.9, 118.4, 119.8, 121.9, 128.9, 134.2, 141.4, 146.0, 166.2, 167.6. I.R. (NaCl neat) 3340, 3084, 3044, 2970, 2924, 1671, 1658, 1461, 1447, 1372, 1327, 1198, 1138, 1009, 987, 732 cm $^{-1}$. HRMS (FAB+): Calcd for $C_{22}H_{28}N_3O_3$: 382.213067. Found 382.211498 (M+H). R_f 0.43 (eluted twice with 2% MeOH/CH_2Cl_2).



239d



^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems700D

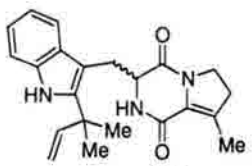


239 240

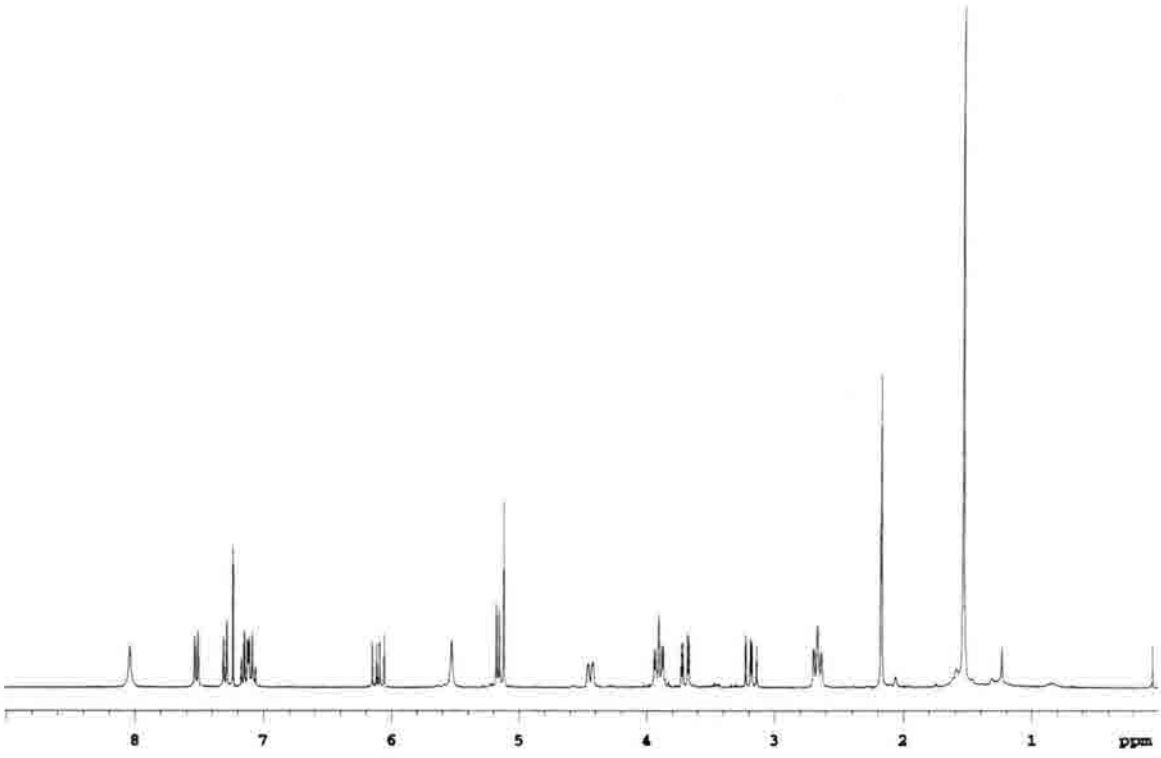
(3R,S)-3-[[2-(1,1-dimethyl-2-propenyl)-1H-indol-3-yl]methyl]-2,3,6,7-tetrahydro-8-methyl-pyrrolo[1,2-a]pyrazine-1,4-dione (240):

Compound **239**, (535 mg, 1.40 mmol) was cooled to 0°C in THF (5.6 mL) under an argon atmosphere. Two equivalents of pyridine, 226 μL, 2.0 equiv.), was added and the solution was stirred for ~15 min. $SOCl_2$ (112 μL, 1.1 equiv.), was added and the mixture was allowed to come to room temperature over 3 h. Water was added to the reaction mixture which was then extracted three times with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product was purified by flash silica gel column chromatography using 2% MeOH/CH_2Cl_2 to afford 381 mg (75%) of compound **240** as a glass.

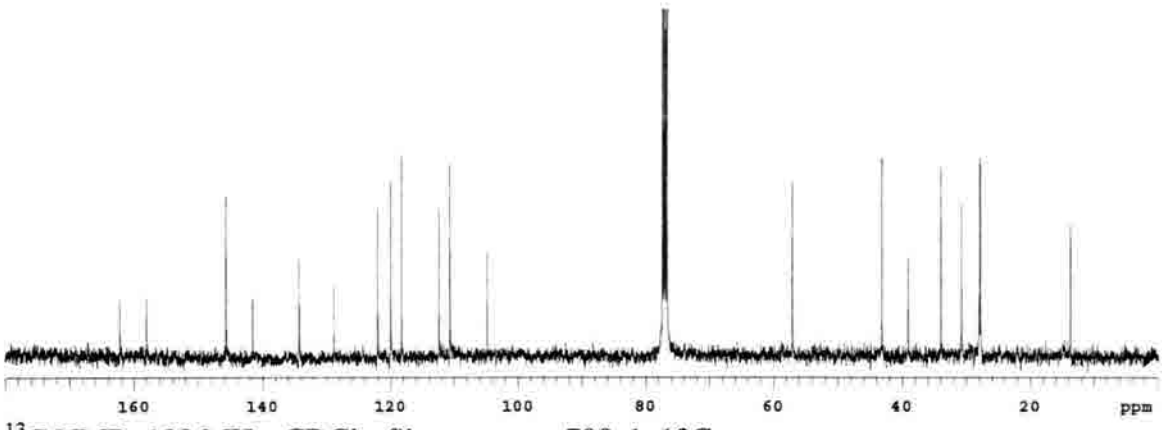
^{1}H NMR (300 MHz, $CDCl_3$): δ 1.56 (6H, s), 2.02 (3H,s), 2.69 (2H, dd, J=9.2, 9.2 Hz), 3.21 (1H, dd, *J*=14.6, 11.3 Hz), 3.72 (1H, dd, *J*=14.7, 3.3 Hz), 3.93 (2H, ddd, *J*=3.0, 11.7, 11.7 Hz), 4.47 (1H, d, *J*=10.6 Hz), 5.17 (1H, dd, *J*= 0, 10.26 Hz), 5.18 (1H, dd, *J*=0, 17.2 Hz), 5.55 (1H, bs), 6.13 (1H, dd, *J*=10.6, 17.6 Hz), 7.11 (1H, m ddd, *J*=1.1, 7.3, 7.3 Hz), 7.18 (1H, ddd, *J*=0.7, 7.3, 7.3 Hz), 7.32 (1H, d, *J*=7.7 Hz), 7.55 (1H, d, *J*=7.7 Hz), 8.07 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.7, 26.8, 28.0, 30.8, 33.9, 39.1, 43.1, 57.0, 104.8, 110.7, 112.4, 118.3, 120.0, 122.0, 128.9, 134.3, 134.3, 141.5, 145.7, 158.1, 162.2. I.R. (NaCl neat) 3344, 3047, 2968, 1682, 1645, 1440, 1324, 1251, 1114, 1007, 917, 744 cm^{-1}. HRMS (FAB+): Calcd for $C_{22}H_{26}N_3O_2$: 364.2025. Found 364.2032 (M+H). R_f 0.2 (eluted with 2% MeOH/CH_2Cl_2)



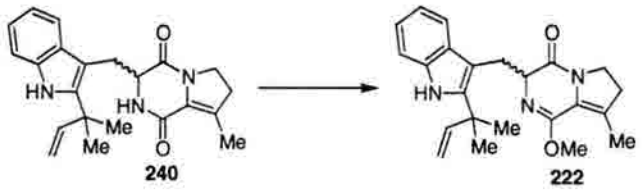
240



^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems706

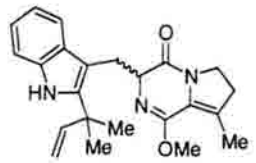


^{13}C NMR, 100 MHz, $CDCl_3$, filename: ems708a1_13C

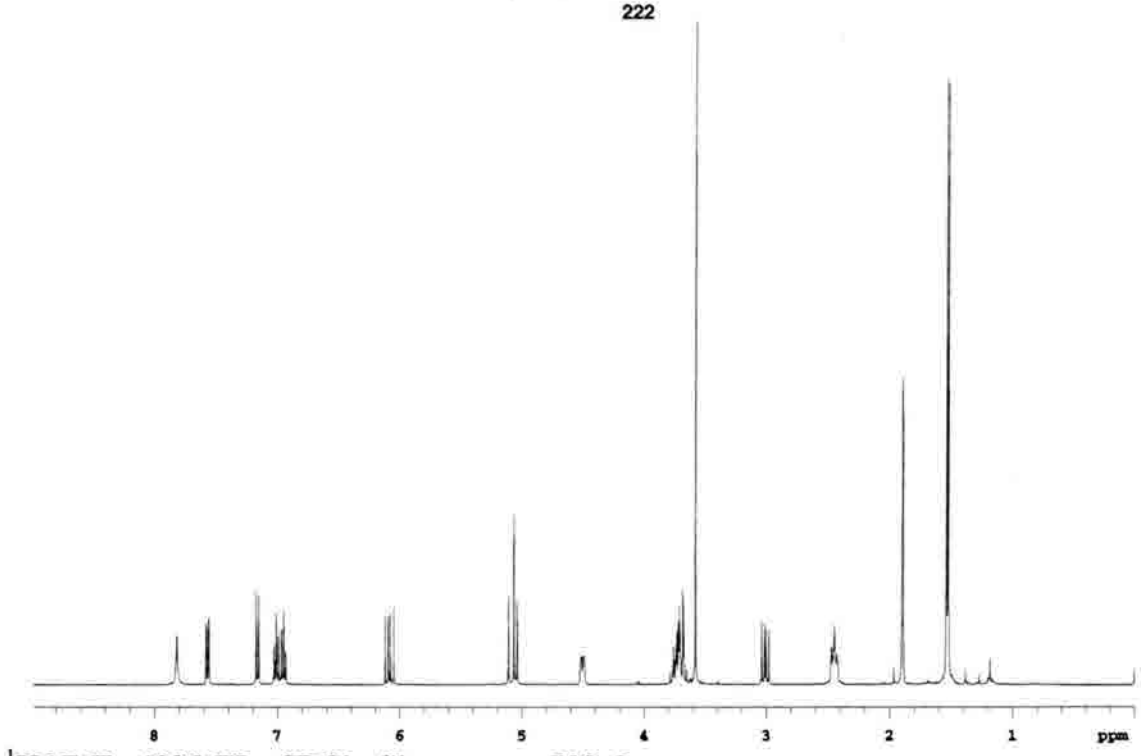


(3R,S)-3-[[2-(1,1-dimethyl-2-propenyl)-1H-indol-3-yl]methyl]-6,7-dihydro-1-methoxy-8-methyl-pyrrolo[1,2-a]pyrazine-4(3H)-one (222): A solution of **240**, (257 mg, 0.7 mmol) was stirred with $(CH_3)_3OBF_4$ (314 mg, 2.12 mmol, 3.0 equiv.), anhydrous K_2CO_3 (5 eq., 489 mg, 3.54 mmol, 5.0 equiv.) in CH_2Cl_2 (7 mL) for 7 h at ambient temperature under an argon atmosphere. The reaction was poured into ice water and extracted with CH_2Cl_2 three times. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated under reduced pressure and purified by silica gel column chromatography (eluted with 50% EtOAc/hexanes) to yield 192 mg (72%) of the azadiene **222** as a brittle foam. R_f 0.4 (eluted with 50% EtOAc/hexanes).

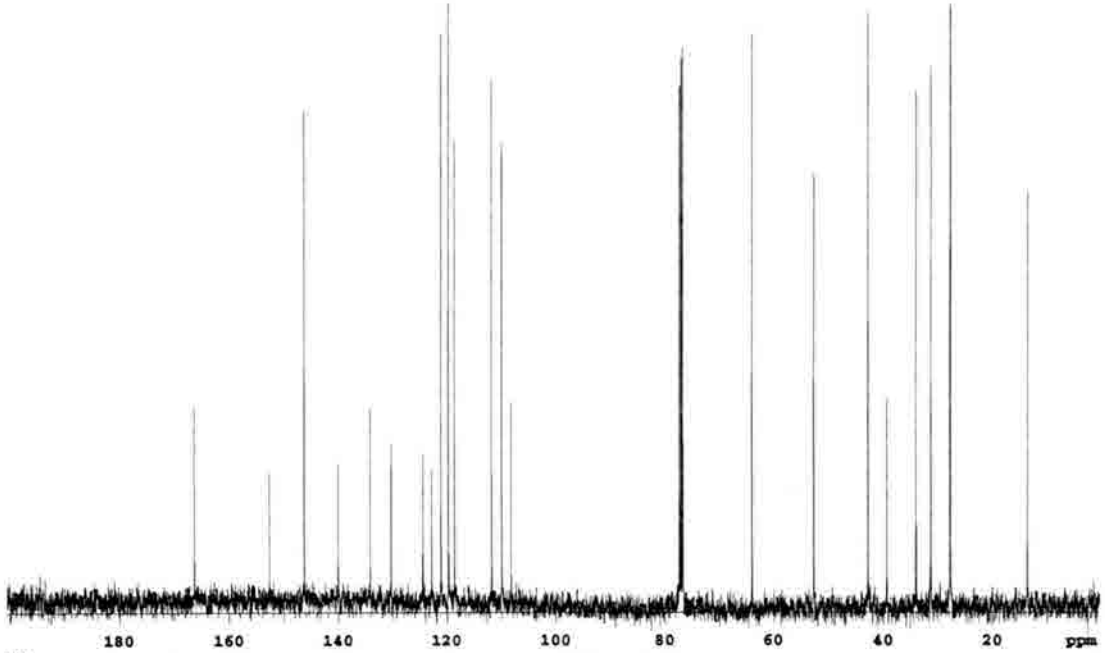
^{1}H NMR (300 MHz, $CDCl_3$): δ 1.61 (3H, s), 1.62 (3H, s), 1.99 (3H, s), 2.55 (2H, m), 3.09 (1H, dd, *J*= 9.2, 14.3 Hz), 3.66 (3H, s), 3.79 (3H, m), 4.59 (1H, d, *J*=7.0 Hz), 5.15 (1H, dd, *J*=1.1, 10.3 Hz), 5.18 (1H, dd, *J*=1.1, 17.2 Hz), 6.15 (1H, dd, *J*=10.3, 17.2 Hz), 7.04 (1H, ddd, *J*=1.1, 8.1, 8.1 Hz), 7.15 (1H, ddd, *J*=1.1, 7.0, 7.0 Hz), 7.26 (1H, d, *J*=8.1 Hz), 7.65(1H, d, *J*= 7.7 Hz), 7.86 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.7, 27.7, 27.8, 31.4, 34.0, 39.3, 42.8, 52.7, 64.0, 108.1, 109.9, 111.8, 118.6, 119.7, 121.1, 122.8, 124.5, 130.3, 134.1, 140.0, 146.2, 152.6, 166.3. I.R. (NaCl neat): 3345, 2962,2924, 1676, 1634, 1456, 1335, 1304, 1242, 1051, 917, 741 cm^{-1}. HRMS (FAB+): Calcd. for $C_{23}H_{28}N_3O_2$: 378.2177. Found 378.2182 (M+H).



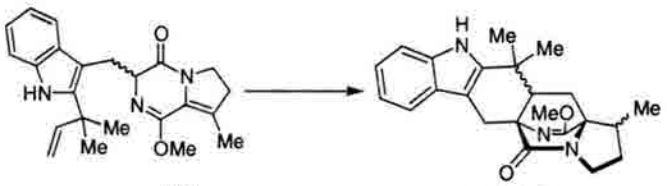
222



^{1}H NMR, 400 MHz, $CDCl_3$, filename ems720_2



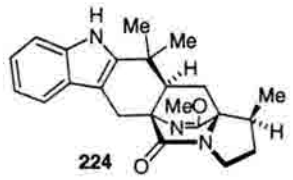
^{13}C NMR, 100 MHz, $CDCl_3$, filename ems720_13C_2



222 224-227

2,3,11,12,12a,13-hexahydro-14-methoxy-1,12,12-trimethyl-5H,6H-5a,13a-(nitrilometheno)-1H-indolizinno[7,6-b]carbazol-5-one (224-227):

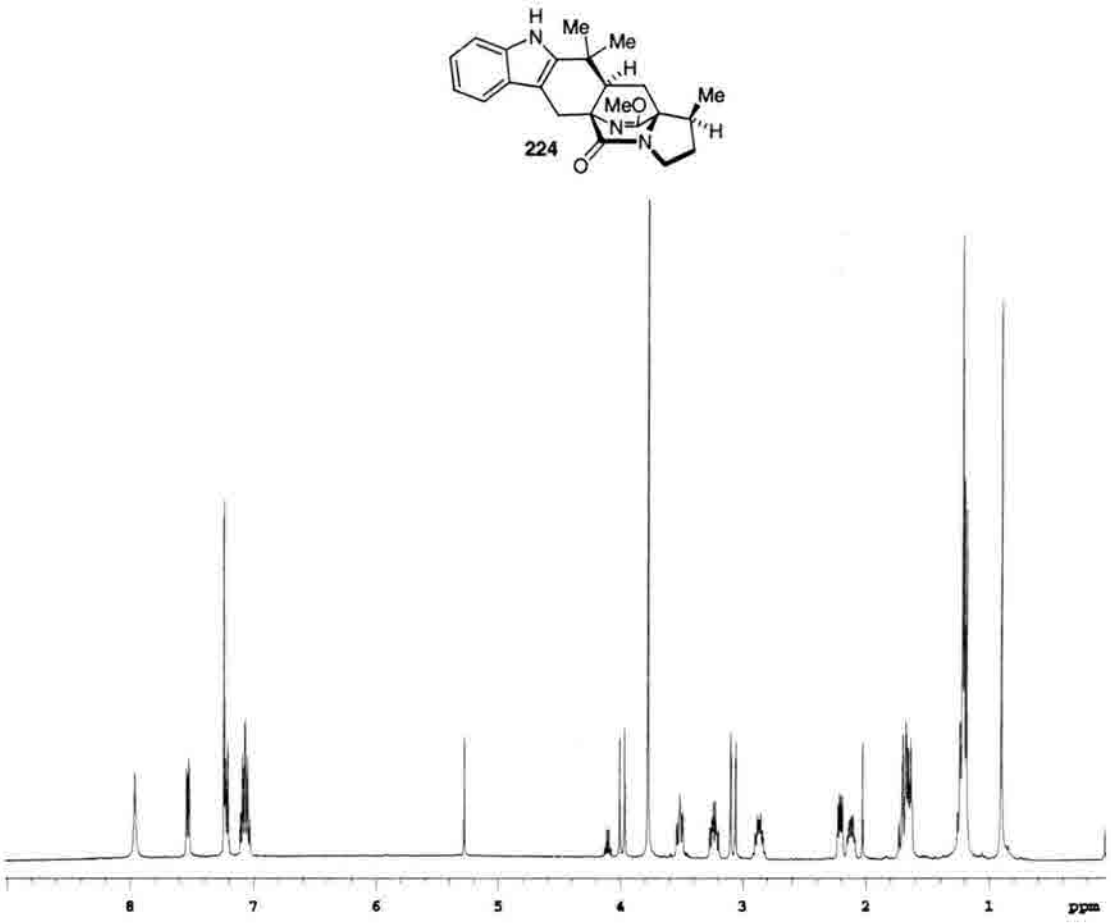
Azadiene **222** (264 mg, 0.70 mmol), was stirred in MeOH (47 mL) and 20% KOH (aq) (12.6 mL) under an argon atmosphere at 0°C. The reaction mixture was allowed to come to room temperature and continued to stir for 10 h. When the reaction was complete as indicated by TLC analysis, phosphate buffer (pH=7) was added until the solution was neutral. The aqueous phase was extracted 3 times with EtOAc. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The mixture of diastereomers could be partially separated by flash silica gel column chromatography (eluted with 2~4% $MeOH/CH_2Cl_2$) however, cycloadducts **225** and **226** had to be separated by successive PTLC (eluted with 2% $MeOH/CH_2Cl_2$). The order of elution was (from fastest mobility to slowest mobility): **227**, **226**, **225**, and finally **224**. Yield: **227**, 23 mg; **226**, 37 mg; **225**, 62 mg; **224**, 85 mg; (78% combined yield). Data for each is as follows:



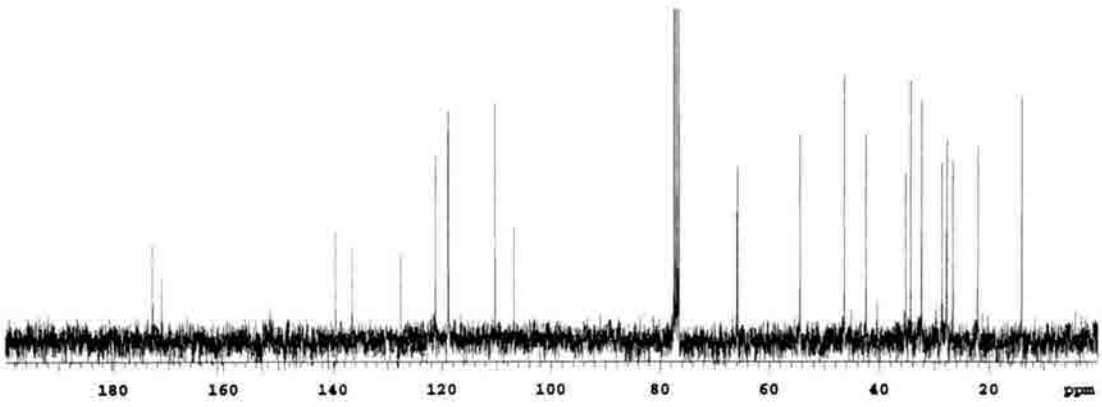
224

(1S,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-14-methoxy-1,12,12-trimethyl-5H,6H-5a,13a-(nitrilometheno)-1H-indolizino[7,6-b]carbazol-5-one (224):

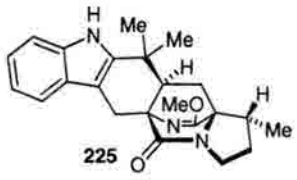
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.08 (3H, s), 1.22 (3H, d, *J*= 7.0 Hz), 1.31 (3H, s), 1.64-1.78 (3H, m), 2.16 (1H, m), 2.28 (1H, dd, *J*= 9.2, 5.5 Hz), 2.90 (1H, m), 3.12 (1H, d, *J*= 15.7 Hz); 3.26 (1H, m), 3.56 (1H, m), 3.81 (3H, s), 4.03 (1H, d, *J*= 15.7 Hz), 7.12 (2H, m), 7.27 (1H, d, *J*= 7 Hz), 7.57 (1H, d, *J*= 7 Hz), 7.74 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.02, 22.1, 26.6, 27.7, 28.6, 32.4, 34.3, 35.2, 42.4, 46.4, 54.4, 65.7, 66.0, 106.8, 110.2, 118.8, 118.9, 121.2, 127.6, 136.5, 139.5, 171.1, 172.8. IR (NaCl, neat): 3306, 2969, 1668, 1651, 1455, 1428, 1345, 1310, 1194, 995, 740, 714 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{28}N_3O_2$: 378.2181. Found 378.2176 (M+H). R_f 0.40 (eluted twice with 2% MeOH/CH_2Cl_2).



^{1}H NMR, 400 MHz, $CDCl_3$, filename ems837d_400



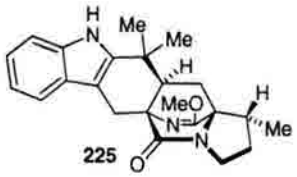
^{13}C NMR, 100 MHz, $CDCl_3$, filename ems721d_13C



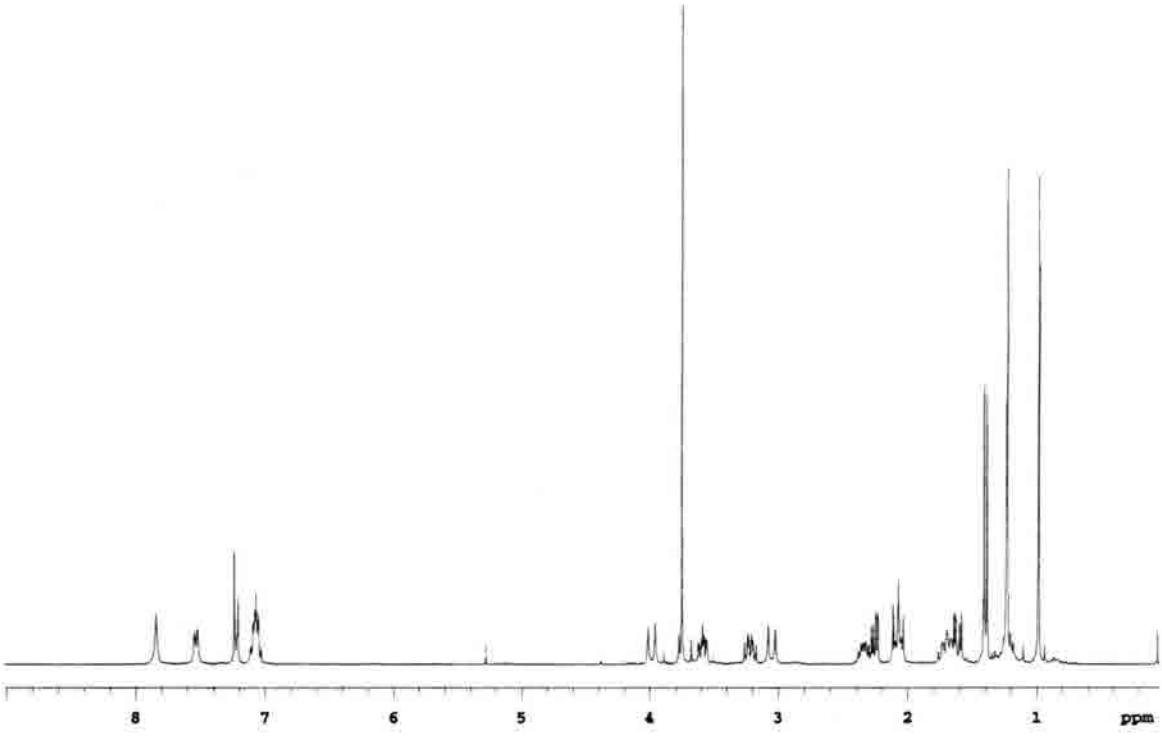
225

(1R,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-14-methoxy-1,12,12-trimethyl-5H,6H-5a,13a-(nitrilometheno)-1H-indolizino[7,6-b]carbazol-5-one (225):

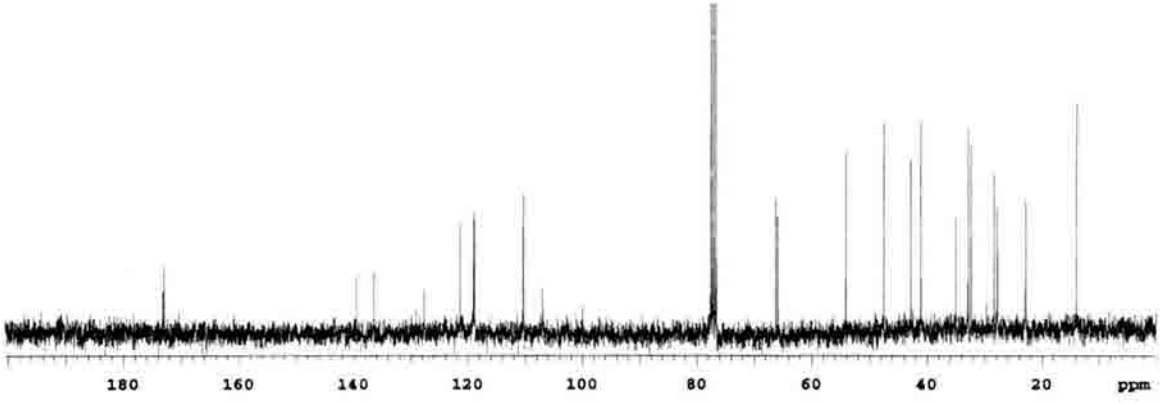
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.02 (3H, s), 1.26 (3H, s), 1.43 (3H, d, *J*= 7.0 Hz), 1.73 (1H, m), 1.86 (1H, dd, *J*= 10.6, 12.5 Hz), 2.11 (1H, dd, *J*= 4.8, 12.5 Hz), 2.11 (1H, m), 2.29 (1H, dd, *J*= 10.6, 4.8 Hz), 2.38 (1H, m), 3.12 (1H, d, *J*= 16.1 Hz); 3.25 (1H, m), 3.62 (1H, m), 3.65 (3H, s), 4.06 (1H, d, *J*= 16.1 Hz), 7.15 (2H, m), 7.31 (1H, d, *J*= 7 Hz), 7.61 (1H, d, *J*= 7 Hz), 7.73 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.95, 22.9, 27.8, 28.4, 32.3, 32.9, 35.0, 41.1, 42.8, 47.5, 54.0, 65.8, 66.2, 106.9, 110.3, 118.8, 119.0, 121.9, 127.6, 136.46, 139.55, 172.9, 173.1. IR (NaCl, neat): 3306, 3047, 2951, 1167, 1633, 1462, 1435, 1372, 1311, 1185, 980, 743 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{28}N_3O_2$: 378.2181. Found 378.2163 (M+H). R_f 0.49 (eluted twice with 2% MeOH/CH_2Cl_2).



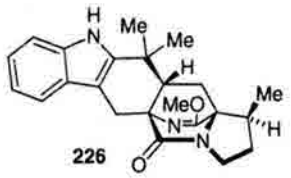
225



^{1}H NMR, 300 MHz, $CDCl_3$, filename ems721c



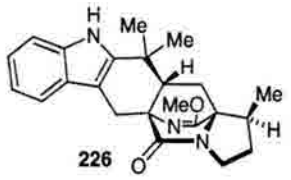
^{13}C NMR, 75 MHz, $CDCl_3$, filename ems721c_13C



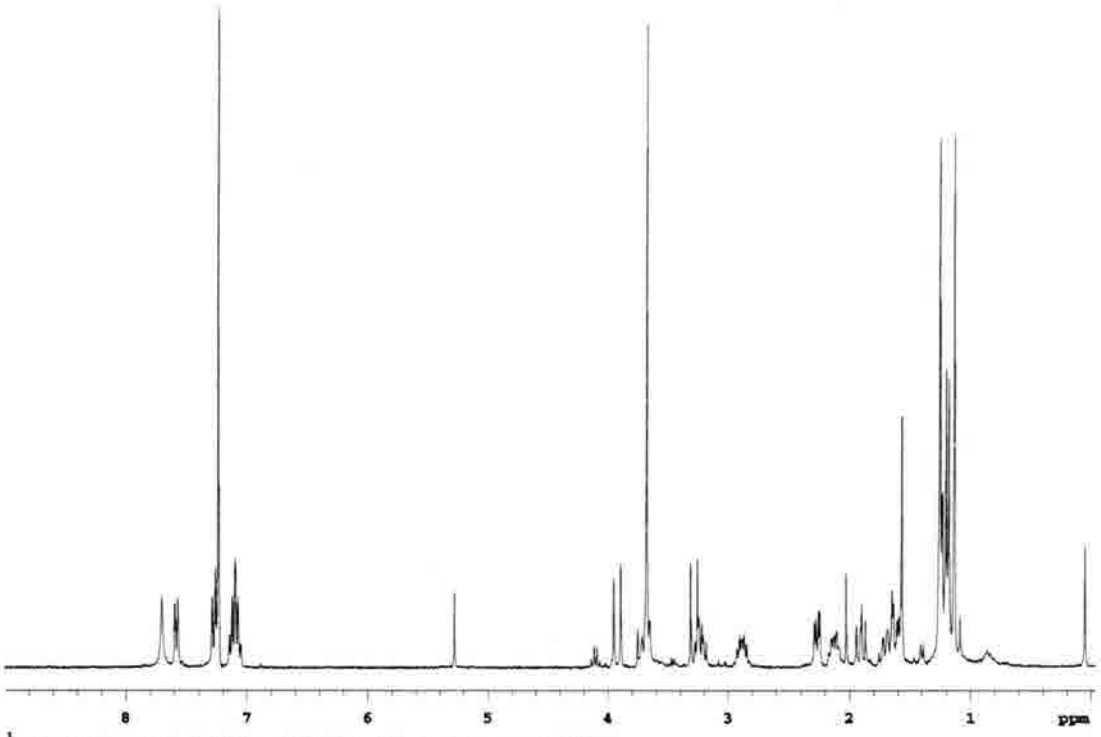
226

(1S,5aR,12aS,13aS)-rel-2,3,11,12,12a,13-hexahydro-14-methoxy-1,12,12-trimethyl-5H,6H-5a,13a-(nitrilometheno)-1H-indolizino[7,6-b]carbazol-5-one (226):

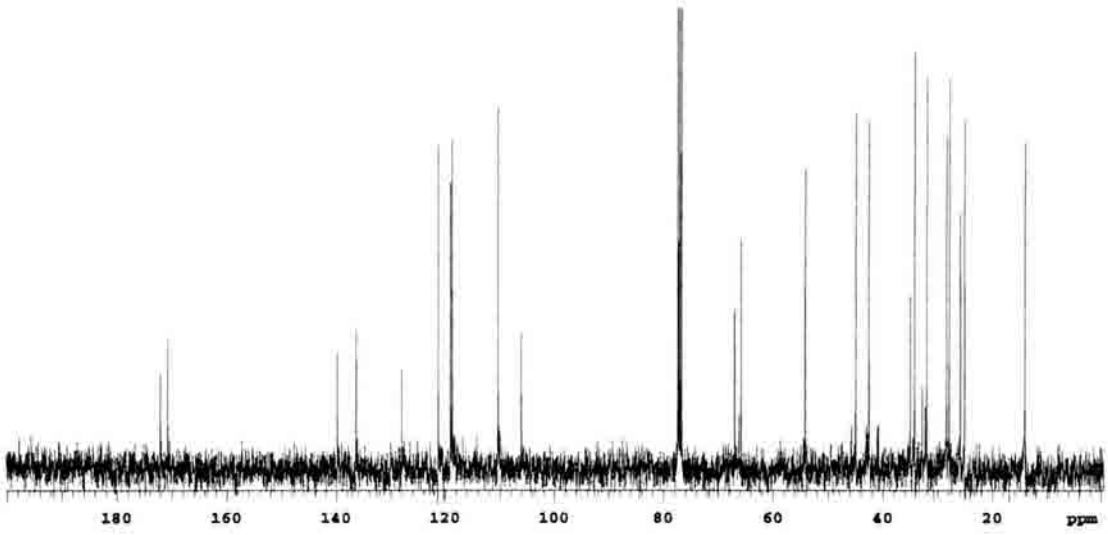
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.13 (3H, s), 1.19 (3H, d, *J*= 7.0 Hz), 1.25 (3H, s), 1.68 (1H, m), 1.88 (1H, dd, *J*= 9.8, 12.9 Hz), 1.97 (1H, dd, *J*= 3.9, 12.9 Hz), 2.12 (1H, m), 2.23 (1H, dd, *J*= 9.8, 3.9 Hz), 2.88 (1H, m), 3.22 (1H, m), 3.29 (1H, d, *J*= 17.2 Hz), 3.65 (3H, s), 3.68 (1H, m), 3.92 (1H, d, *J*= 17.2 Hz), 7.09 (2H, m), 7.27 (1H, d, *J*= 7 Hz), 7.57 (1H, d, *J*= 7 Hz), 7.94 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 25.2, 25.9, 27.9, 28.4, 32.0, 34.2, 35.0, 42.5, 45.0, 54.2, 65.8, 66.9, 106.1, 110.3, 118.8, 119.4, 121.3, 128.0, 136.4, 139.8, 170.8, 172.1. IR (NaCl, neat, cm^{-1}): 3407, 3312, 3053, 2964, 1667, 1455, 1427, 1345, 1306, 1230, 1180, 1042, 995, 815, 738. HRMS (FAB+) Calcd for $C_{23}H_{28}N_3O_2$: 378.2181. Found 377.2109 (M-H). R_f 0.55 (eluted twice with 2% MeOH/CH_2Cl_2).



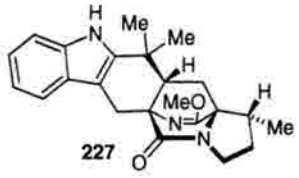
226



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems837b



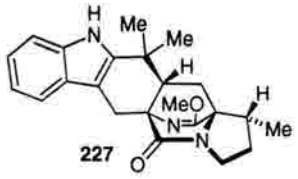
^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems748b_set, filename: carbon



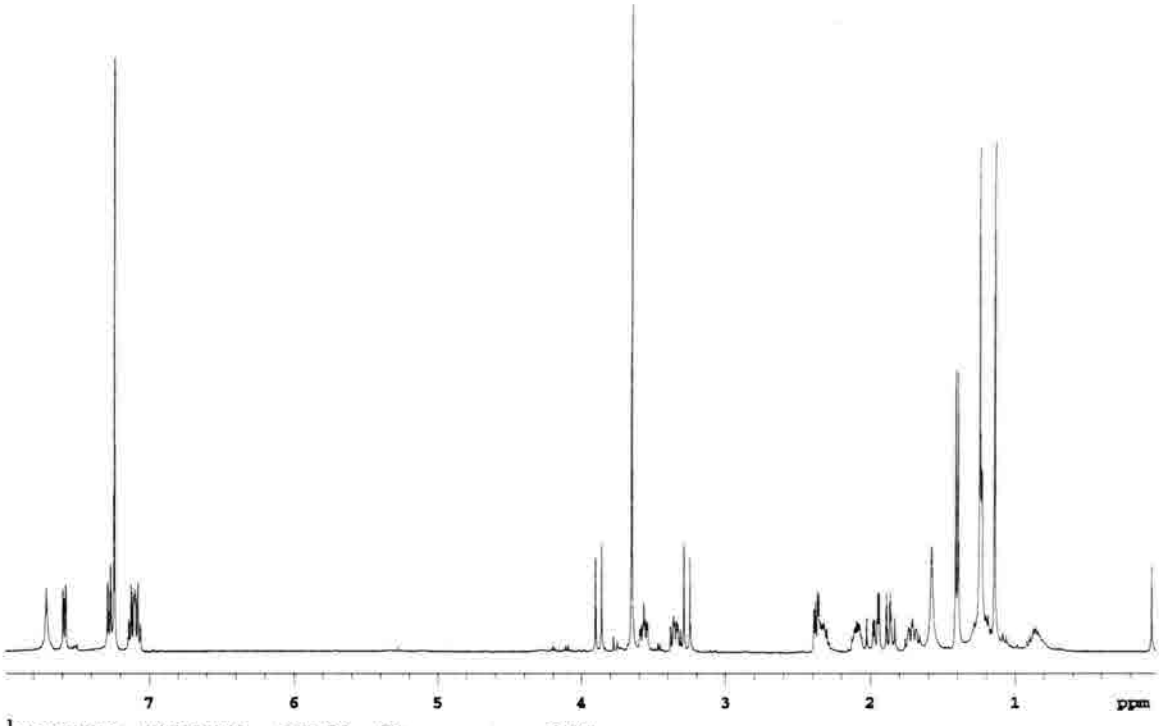
227

(1R,5aR,12aS,13aS)-rel-2,3,11,12,12a,13-hexahydro-14-methoxy-1,12,12-trimethyl-5H,6H-5a,13a-(nitrilometheno)-1H-indolizino[7,6-b]carbazol-5-one (227):

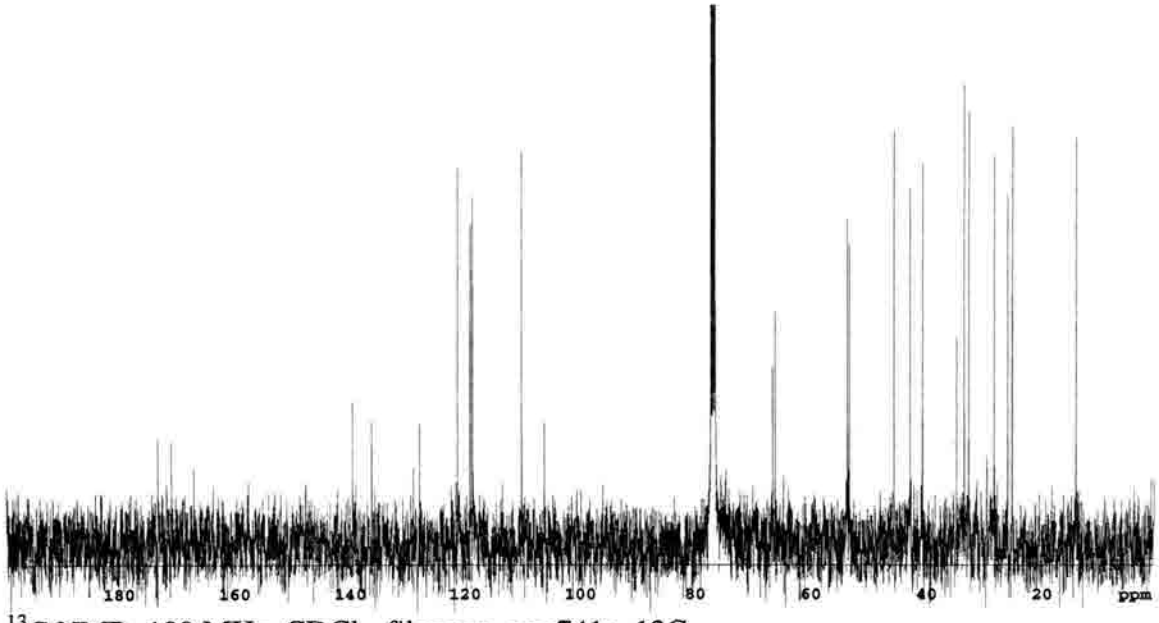
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.15 (3H, s), 1.19 (3H, d, *J*= 7.0 Hz), 1.25 (3H, s), 1.71(1H, m), 1.86 (1H, dd, *J*= 9.9, 13.2 Hz), 1.97 (1H, dd, *J*= 4.4, 13.2 Hz), 2.10 (1H, m), 2.33 (1H, m), 2.38 (1H, dd, *J*= 9.9, 4.4 Hz), 3.27 (1H, d, *J*= 17.2 Hz), 3.35 (1H, m), 3.57 (1H, m), 3.65 (3H, s), 3.88 (1H, d, *J*= 17.2 Hz), 7.10 (2H, m), 7.28 (1H, d, *J*= 7 Hz), 7.59 (1H, d, *J*= 7 Hz), 7.70 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 25, 26.1, 28.4, 32.9, 33.6, 34.9, 40.7, 43.0, 45.8, 53.8, 66.2, 66.7, 106.4, 110.3, 118.9, 119.2, 121.5, 128.0, 136.4, 139.7, 171.1, 173.4. IR (NaCl, neat): 3407, 3310, 2958, 2918, 1660, 1626, 1461, 1423, 1307, 1283, 1008, 741 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{28}N_3O_2$: 378.2181. Found 378.2173, (M+H). R_f 0.57 (eluted twice with 2% MeOH/CH_2Cl_2).



227



^{1}H NMR, 400 MHz, $CDCl_3$, filename ems837a



^{13}C NMR, 100 MHz, $CDCl_3$, filename ems741a_13C

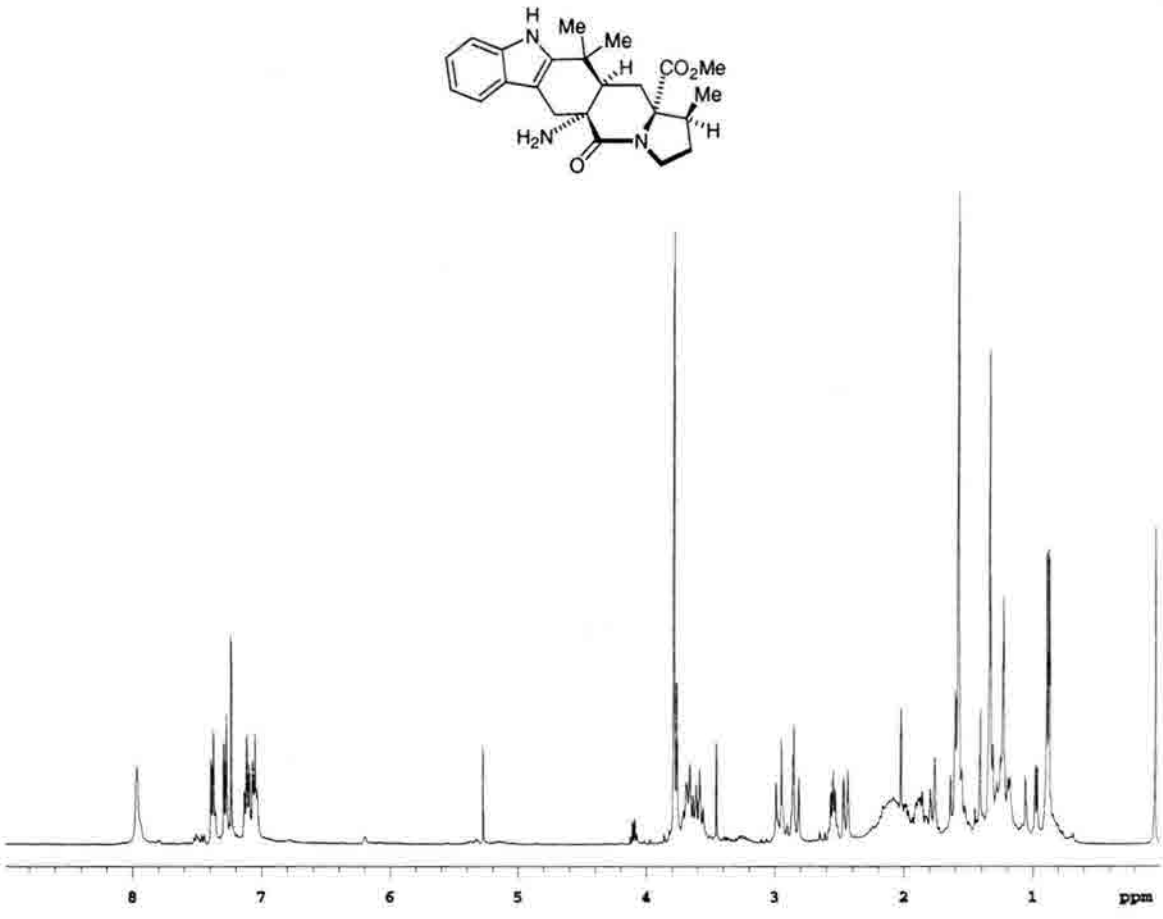
***General procedure for lactim ether deprotection of cycloadducts* 224~227.**

One equivalent of the lactim ether cycloadduct was stirred in THF (0.025 M) at 0°C. To this solution was added 0.1M HCl (3.0 equiv.) and the reaction was stirred 5~15 minutes until starting material was no longer detected by TLC analysis. The reaction was netralized with pH=7 phosphate buffer and extracted three times with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. In the case of cycloadducts **225** and **227**, the ring opened products **242** and **247** were obtained. In the case of cycloadduct **224**, a small percentage of conversion to the corresponding ring-opened amine methyl ester was sometimes observed. These ring-opened amine methyl esters were re-cyclized by refluxing in toluene (0.025 M) overnight. The corresponding piperazinedione products (**241**, **243**, **245** and **248**) were purified using flash silica gel column chromatography (eluted with 2~4% $MeOH/CH_2Cl_2$). Data for each piperazinedione products follows.

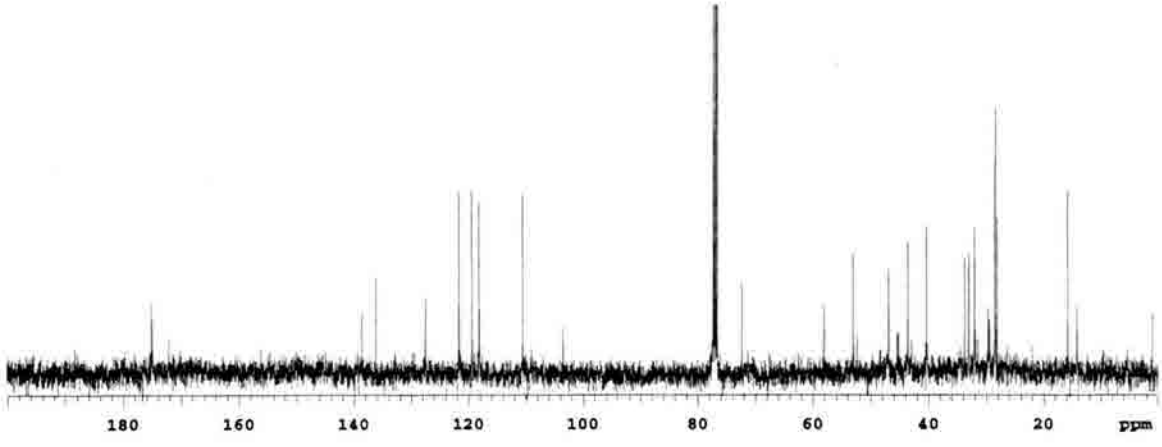
(1S,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazole-5,14-dione (241) from 224:

Yield: 16.5 mg (85%). In this instance, a small percentage of the ring-opened amine methyl ester was observed by ^{1}H nmr analysis and TLC. The mixture of ring-opened product and the desired piperazinedione was refluxed overnight in toluene and purifed by PTLC.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.08 (3H, s), 1.20 (3H, d, *J*= 6.6 Hz), 1.33 (3H, s), 1.67 (1H, m), 1.91 (1H, dd, *J*= 5.0, 13.4 Hz), 2.03 (1H, dd, *J*= 10.1, 13.4 Hz), 2.16 (1H, m), 2.25 (1H, dd, *J*= 3.9, 10.1 Hz), 2.60 (1H, d, *J*= 15.4 Hz), 3.01 (1H, m), 3.28 (1H, m), 3.58 (1H, m), 3.87 (1H, d, *J*= 15.4 Hz) 5.84 (1H, bs), 7.11 (2H, m), 7.27 (1H, d, *J*= 7.3 Hz), 7.50 (1H, d, *J*= 7.7 Hz), 7.72 (1H, bs). ^{13}C NMR (100 MHz, d_6-DMSO) δ 13.8, 21.3, 23.8, 24.8, 28.1, 31.3, 34.2, 34.7, 42.5, 48.2, 59.2, 66.9, 103.4, 110.7, 117.5, 118.2, 120.6, 126.5, 136.5, 140.7, 168.3, 173.4. IR (NaCl, neat): 3313, 3066, 2960, 2913, 1681, 1455, 1404, 1257, 1187, 1088, 1022, 795, 734, 693 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{25}N_3O_2$: 363.1946. Found 363.194254 (M^+). R_f 0.40 (eluted twice with 4% MeOH/CH_2Cl_2).

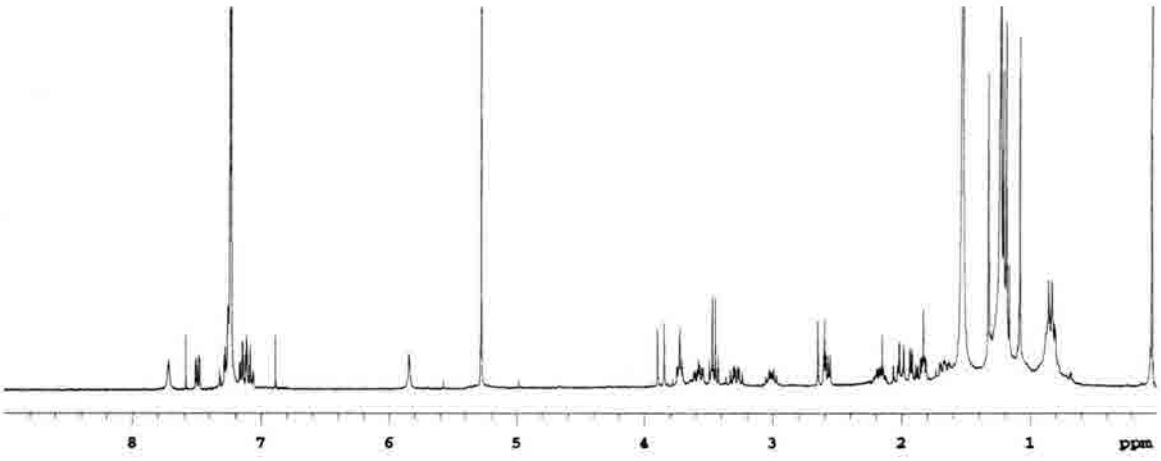


^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems782ii, filename: proton

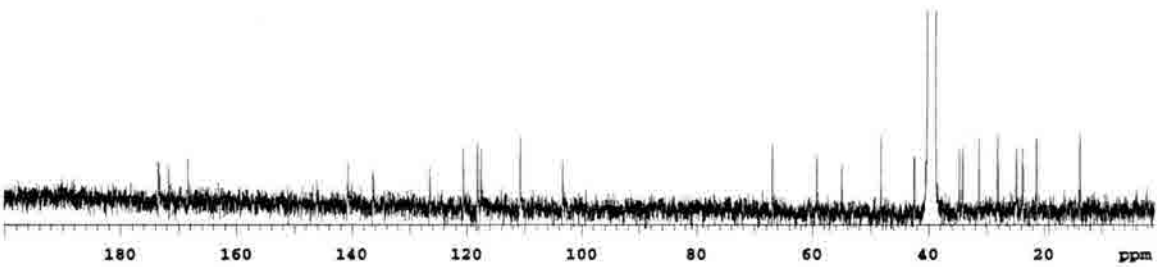


^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems782ii, filename: carbon

241



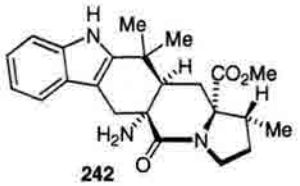
^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems857



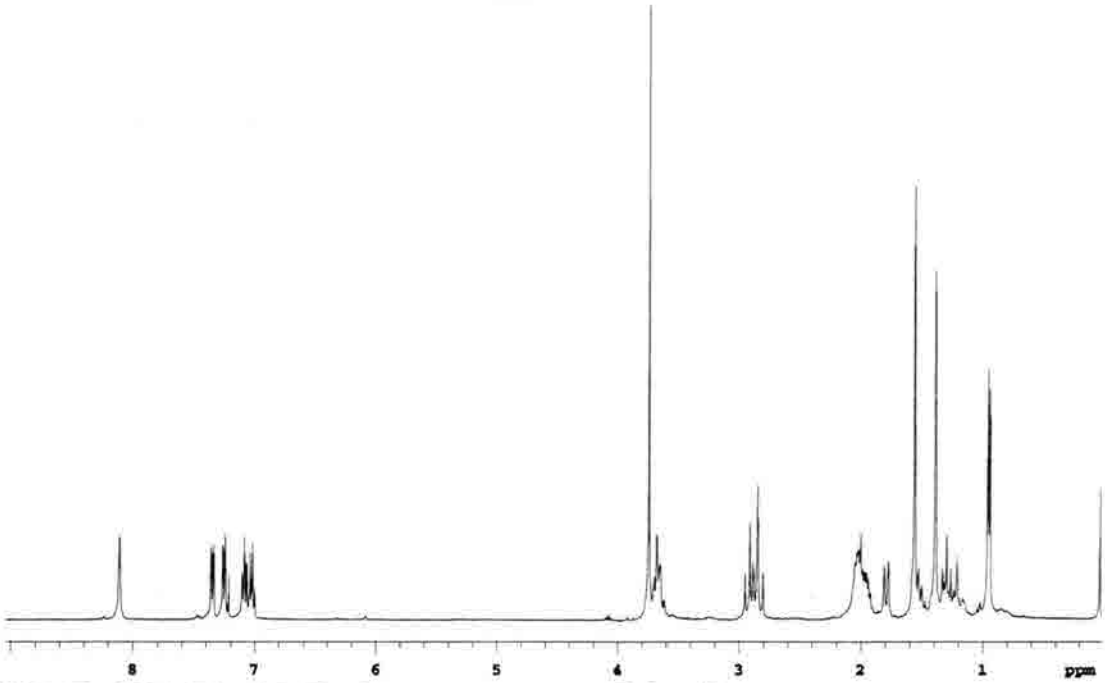
^{13}C NMR, 100 MHz, d_6-DMSO, filename ems782_13C_dmso

(1R,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazole-5,14-dione (243) from 225 *via* 242: Yield: 26 mg (63%).

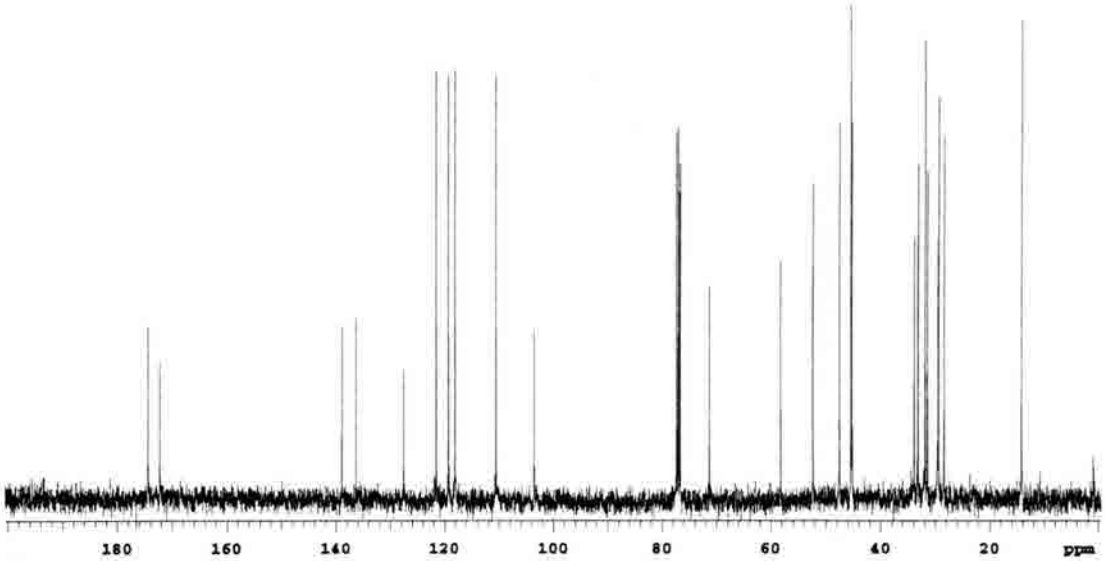
Data for **242**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.04 (3H, d, *J*= 7.0 Hz), 1.34 (3H, s), 1.46 (3H, s), 1.58 (2H, bs), 1.66 (1H, m), 1.90 (1H, dd, *J*= 9.8, 14.1 Hz), 2.02 (1H, m), 2.05 (1H, dd, *J*= 9.4, 9.4), 2.18 (1H, m), 3.03 (1H, dd, *J*= 9.0, 14.1 Hz), 3.13 (1H, d, *J*= 16.4 Hz), 3.15 (1H, d, *J*= 16.4 Hz), 3.64 (3H, s), 3.65 (1H, m), 3.73 (1H, m), 7.08 (1H, ddd, *J*= 7.8, 7.8, 1.2), 7.14 (1H, ddd, *J*= 7.8, 7.8, 1.2), 7.30 (1H, d, *J*= 7.8 Hz), 7.52 (1H, d, *J*= 7.8 Hz), 7.84 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 25.3, 28.2, 29.2, 30.7, 34.1, 35.0, 45.1, 45.2, 47.6, 51.9, 57.1, 68.5, 105.4, 110.5, 118.5, 119.4, 121.8, 128.1, 136.5, 139.8, 172.4, 173.3. IR (NaCl, neat): 3301, 2961, 1732, 1637, 1451, 1220, 735 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{30}N_3O_3$: 396.2287. Found 396.2287 (M+H). R_f 0.30 (eluted twice with 4% MeOH/CH_2Cl_2).



242



^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems783ii, filename: proton

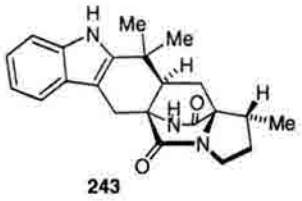


^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems783ii, filename: carbon

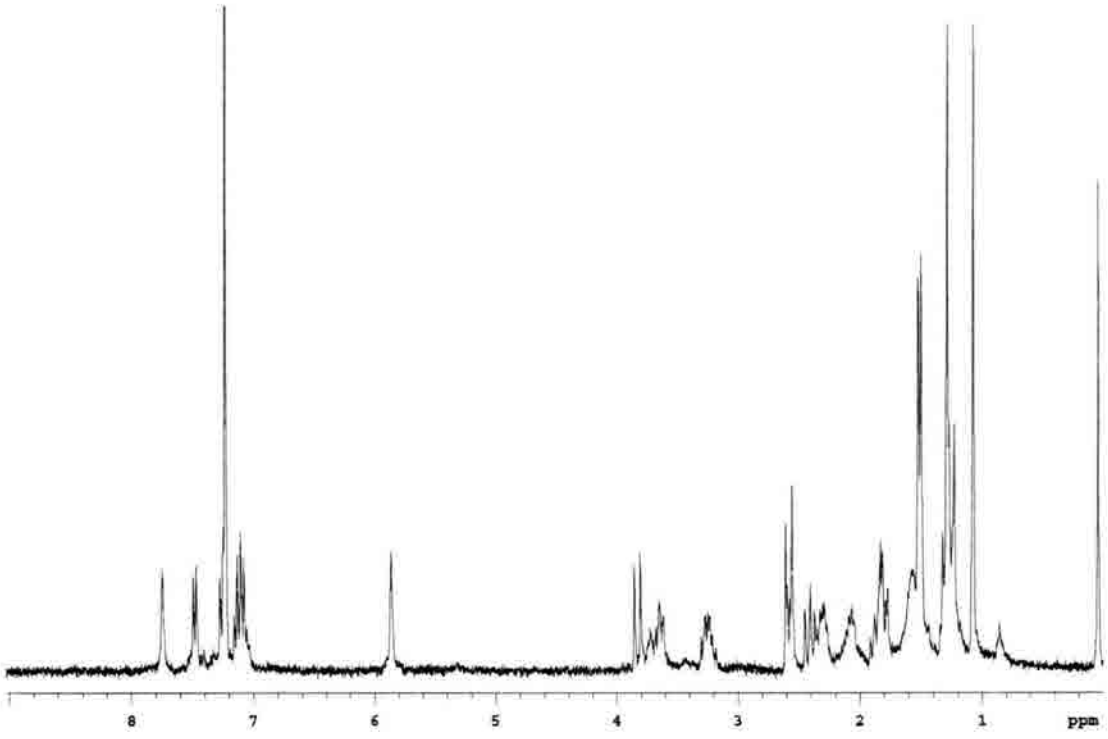
242 243

The ring-opened amine methyl ester **242** was cyclized to the piperazinedione **243** as described above.

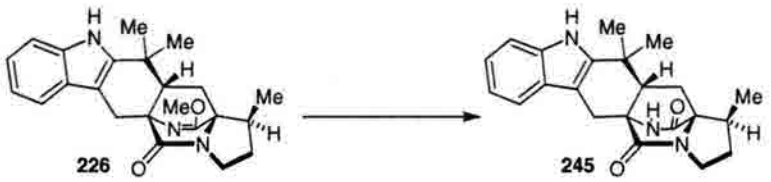
Data for **243**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.07 (3H, s), 1.29 (3H, s). 1.52 (3H, d, *J*= 7 Hz), 1.80 (1H,dd, *J*= 5.0, 13.5 Hz), 1.85 (1H, m), 2.09 (1H, m), 2.30 (1H, m), 2.41 (1H, dd, *J*= 10.5, 13.5 Hz), 2.58 (1H, dd, *J*= 5.0, 10.5 Hz), 2.59 (1H, d, *J*=15.2 Hz), 3.25 (1H, m), 3.65 (1H, m), 3.83 (1H, d, *J*= 15.2 Hz), 5.95 (1H, bs), 7.08 (1H, ddd, *J*= 1.2, 7.8, 7.8 Hz), 7.13 (1H, ddd, *J*= 0.8, 7.8, 7.8 Hz), 7.26 (1H, d, *J*= 7.8 Hz), 7.48 (1H, d, *J*= 7.8 Hz), 7.78 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$ + 1 drop d_6-DMSO): δ 12.9, 22.2, 24.5, 28.0, 29.4, 31.0, 34.5, 41.5, 43.0, 49.3, 59.8, 66.6, 103.9, 110.5, 118.6, 119.7, 121.0, 126.7, 136.4, 139.9, 169.9, 173.5. IR (NaCl, neat): 3664, 3326, 2960, 1677, 1453, 1258, 1092, 799, 703 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{26}N_3O_2$: 364.2025. Found 364.2023 (M+H). R_f 0.30 (eluted twice with 4% MeOH/CH_2Cl_2).



243

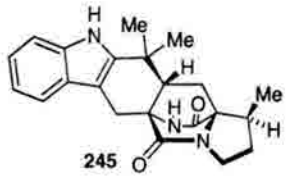


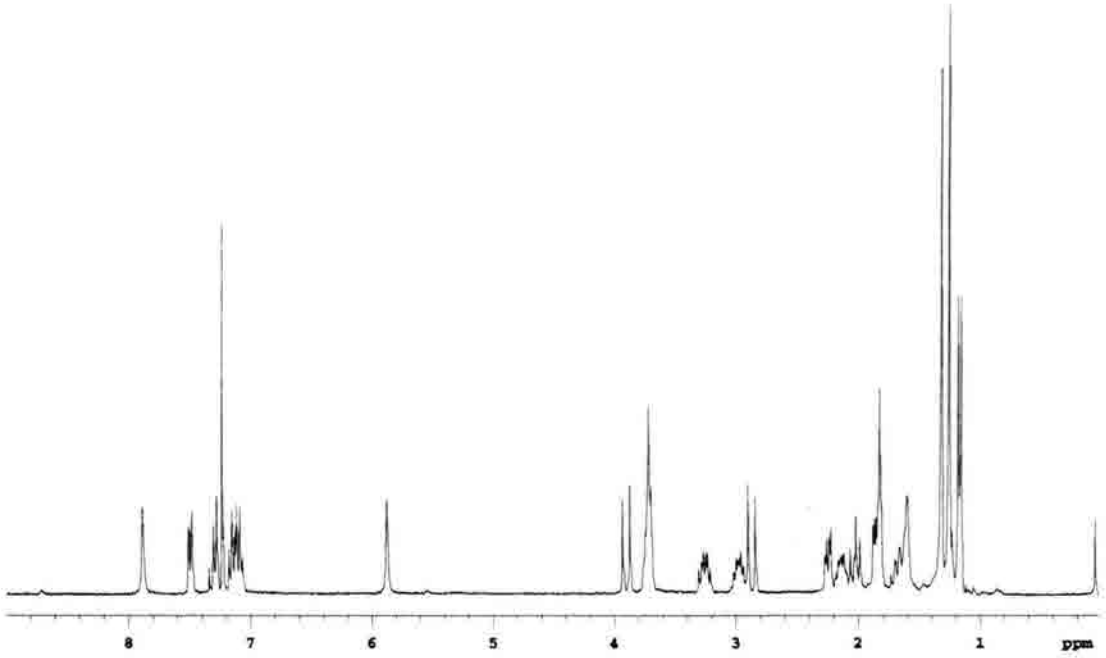
^{1}H NMR, 300 MHz, $CDCl_3$, filename: ems764



(1S,5aR,12aS,13aS)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazole-5,14-dione (245) from cycloadduct 226. Yield: 14.5 mg, (100%).

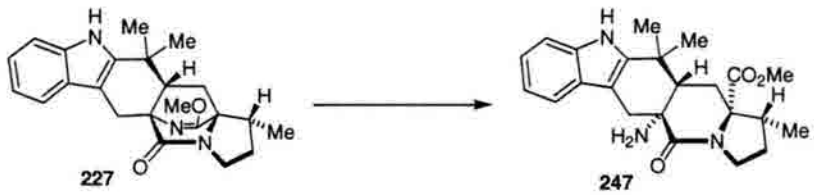
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17 (3H, d, *J*= 7.0 Hz), 1.26 (3H, s), 1.33 (3H, s), 1.66 (1H, m), 1.85 (1H, dd, *J*= 3.9, 13.7 Hz), 2.02 (1H, dd, *J*= 10.1, 13.3 Hz), 2.14 (1H, m), 2.25 (1H, dd, *J*= 3.9, 10.1), 2.89 (1H, d, *J*= 17.9), 2.98 (1H, m), 3.73 (1H, m), 3.90 (1H, d, *J*= 17.6 Hz), 5.90 (1H, bs), 7.09 (1H, m), 7.15 (1H, m), 7.30 (1H, d, *J*= 8 Hz), 7.50 (1H, d, *J*= 7.8Hz), 7.89 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 23.8, 25.2, 27.3, 29.0, 31.9, 34.5, 34.6, 42.9, 44.9, 61.2, 68.2, 103.7, 110.67, 118.36, 119.7, 122.1, 127.2, 136.4, 139.6, 169.2, 173.1. IR (NaCl, neat): 3298, 2962, 1682, 1455, 1399, 1302, 1231, 742 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{25}N_3O_2$: 363.1946. Found 363.1949 (M^+). R_f 0.40 (eluted twice with 4% $MeOH/CH_2Cl_2$).



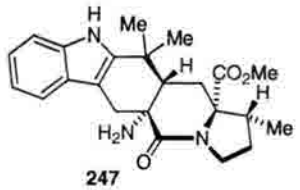


^{1}H NMR, 300 MHz, $CDCl_3$, filename ems768

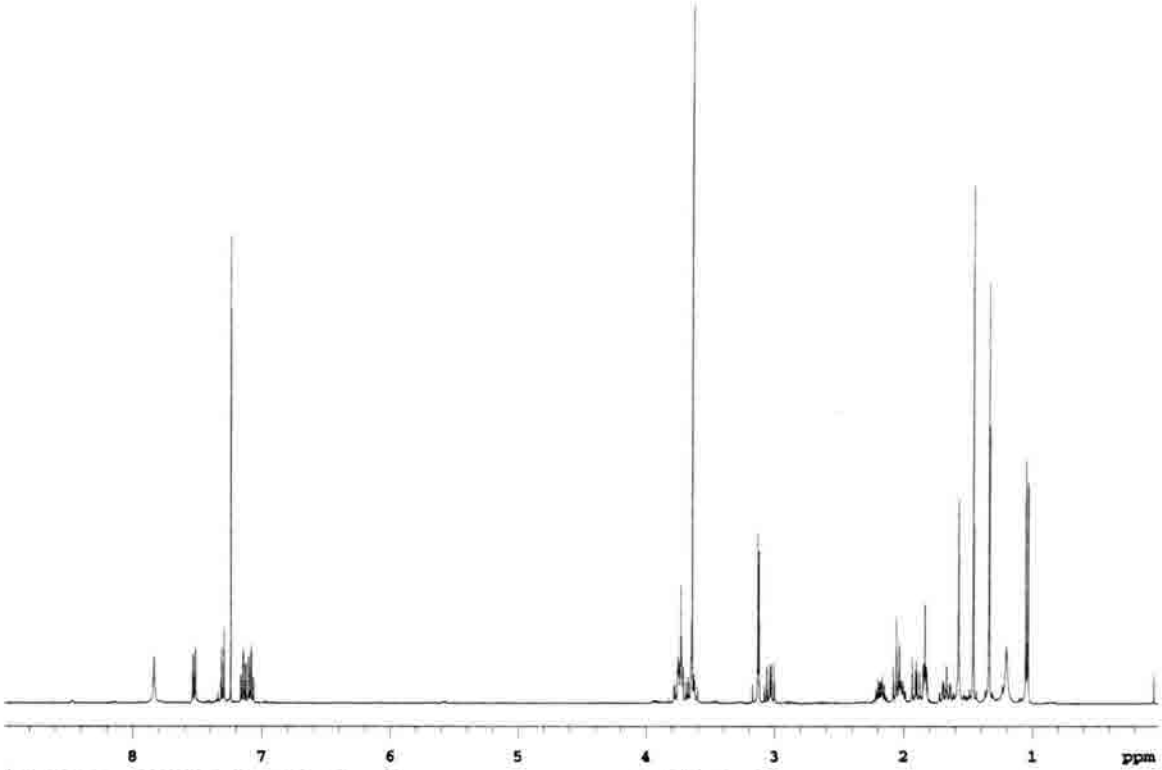
(1R,5aR,12aS,13aS)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazole-5,14-dione (248) from cycloadduct 227 *via* 247. Yield: 4.7 mg, (49%).



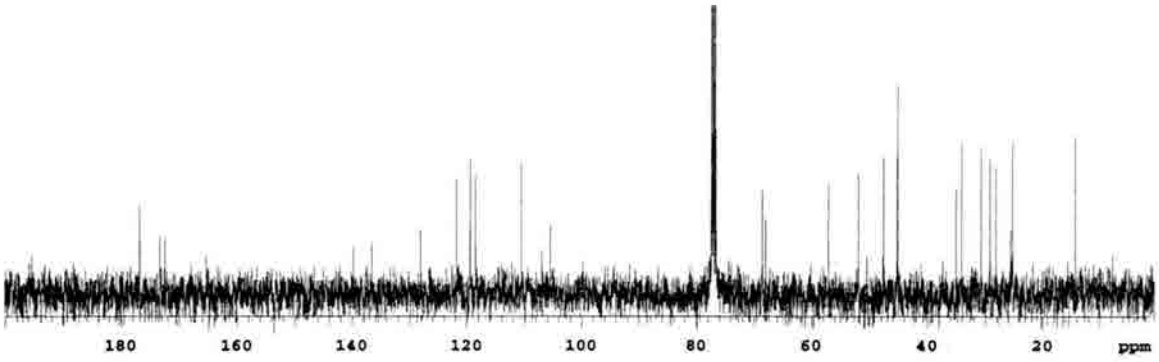
Data for **247**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.95 (3H, d, *J*= 6.6 Hz), 1.28 (1H, d, *J*= 13.7 Hz), 1.31 (1H, d, *J*= 13.3 Hz), 1.39 (3H, s), 1.53 (1H, m), 1.56 (3H, s), 1.76 (1H, d, *J*= 13.3 Hz), 2.00 (3H, m), 2.82 (1H, d, *J*= 16.4 Hz), 2.88 (1H, d, J=13.7 Hz), 2.93 (1H, d, *J*= 16.4 Hz), 3.68 (2H, m), 3.74 (3H, s), 7.02 (1H, m), 7.08 (1H, m), 7.25 (1H, d, *J*= 7.8 Hz), 7.35 (1H, d, *J*= 7.8 Hz), 8.11 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 28.4, 29.4, 31.5, 31.9, 33.2, 33.9, 45.2, 45.4, 47.5, 52.3, 58.2, 71.3, 103.5, 110.6, 118.1, 119.3, 121.5, 127.6, 136.3, 138.9, 172.2, 174.4. IR (NaCl, neat): 3352, 2964, 1725, 1681, 1455, 1392, 1262, 1115, 1020, 808, 761, 732 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{30}N_3O_3$: 396.2287. Found 396.2281 (M+H). R_f 0.6 (eluted with 4% MeOH/CH_2Cl_2).



247



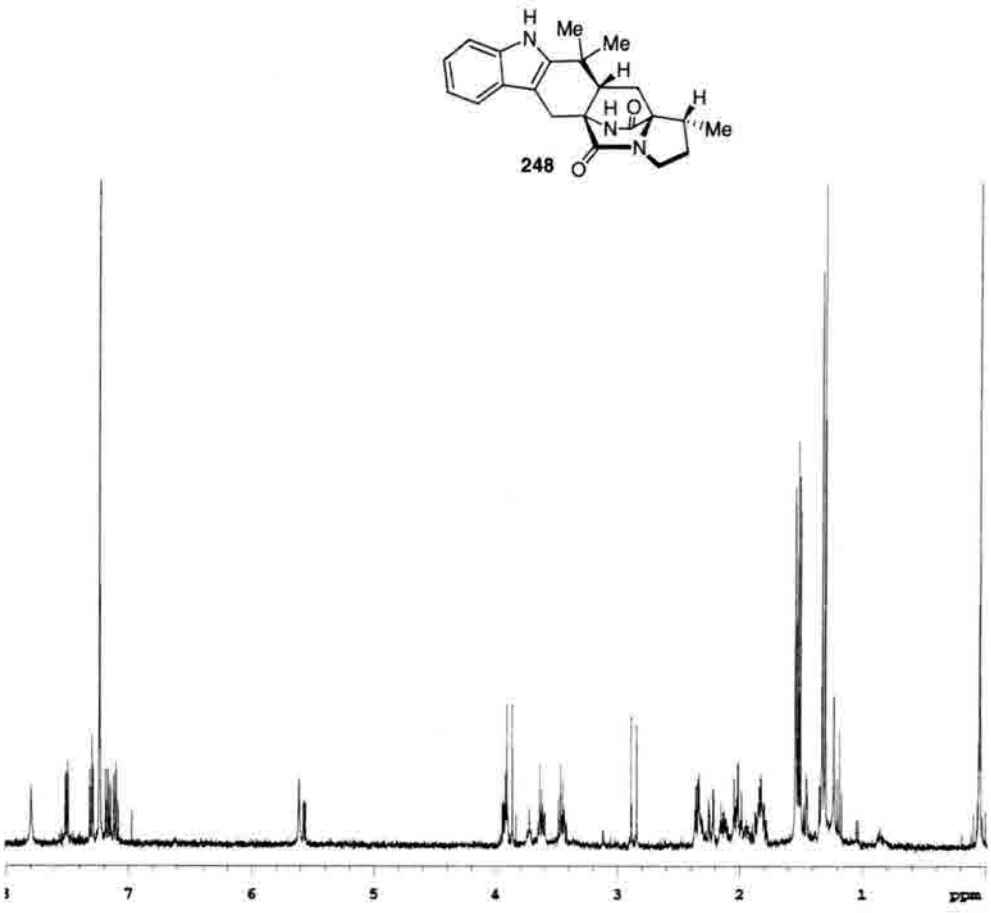
^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems784ii_2, filename: proton



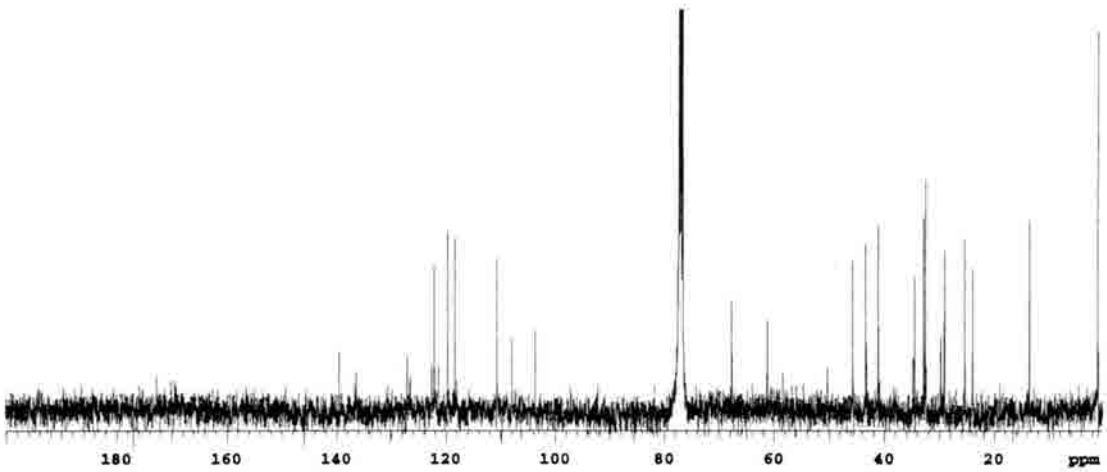
^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems784ii_2, filename: carbon

The ring-opened amine methyl ester **247** was cyclized to the piperazinedione **248** as described above. Data for **248**:

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.29 (3H, s), 1.32 (3H, s), 1.50 (3H, d, *J*= 7.0 Hz), 1.82 (1H, m), 1.99 (1H, dd, *J*= 10.5, 13.6), 2.10 (1H, m), 2.22 (1H, dd, *J*= 3.5, 13.6), 2.31 (2H, m), 2.85 (1H, d, *J*= 17.9 Hz), 3.44 (1H, m), 3.61 (1H, m), 3.87 (1H, d *J*= 17.9 Hz), 5.73 (1H, bs), 7.10 (1H, ddd, *J*= 1.2, 7.4, 7.4 Hz), 7.16 (1H, ddd, *J*= 1.6, 7.8, 7.8 Hz), 7.30 (1H, d, *J*= 7.8 Hz), 7.50 (1H, d, *J*= 7.4 Hz), 7.85 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.6, 23.9, 25.4, 29.0, 32.5, 32.8, 34.5, 41.1, 43.3, 45.7, 61.2, 67.7, 103.7, 110.7, 118.4, 119.7, 122.2, 127.1, 136.4, 139.6, 170.2, 172.8. IR (NaCl, neat): 3228, 2925, 1684, 1670, 1570, 1453, 1406, 1291, 871, 737 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{25}N_3O_2$: 363.1946. Found 363.1953 (M^+). R_f 0.5 (eluted with 4% MeOH/CH_2Cl_2).



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems858

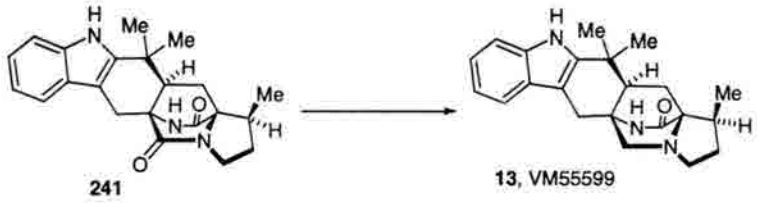


^{13}C NMR, 100 MHz, $CDCl_3$, filename: ems858_13C

Racemic VM55599 (**13**) and diastereoisomers (**244**, **246**, **249**):

*General procedure for DIBAH reduction of **241**, **243**, **245** and **248**:*

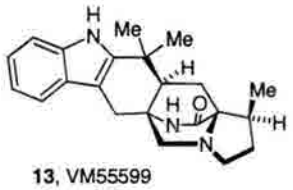
The cycloadduct (**241**, **243**, **245** or **248**) (0.005 M in toluene) was stirred at 0°C under an atmosphere of argon and DIBAH, (20 equiv. as a 1.0 M solution in toluene) was added. The reaction was allowed to come to room temperature and stirred for 24 h. The reaction was again cooled to 0°C and $Na_2SO_4 \cdot 10\ H_2O$ was added slowly until bubbling subsided. The mixture was stirred an additional 30 min. and then filtered through a fritted funnel. The solid residue was rinsed with ethyl acetate and the combined filtrates were evaporated under reduced pressure. The product was isolated *via* flash silica gel column chromatography or PTLC using 2% MeOH/CH_2Cl_2.



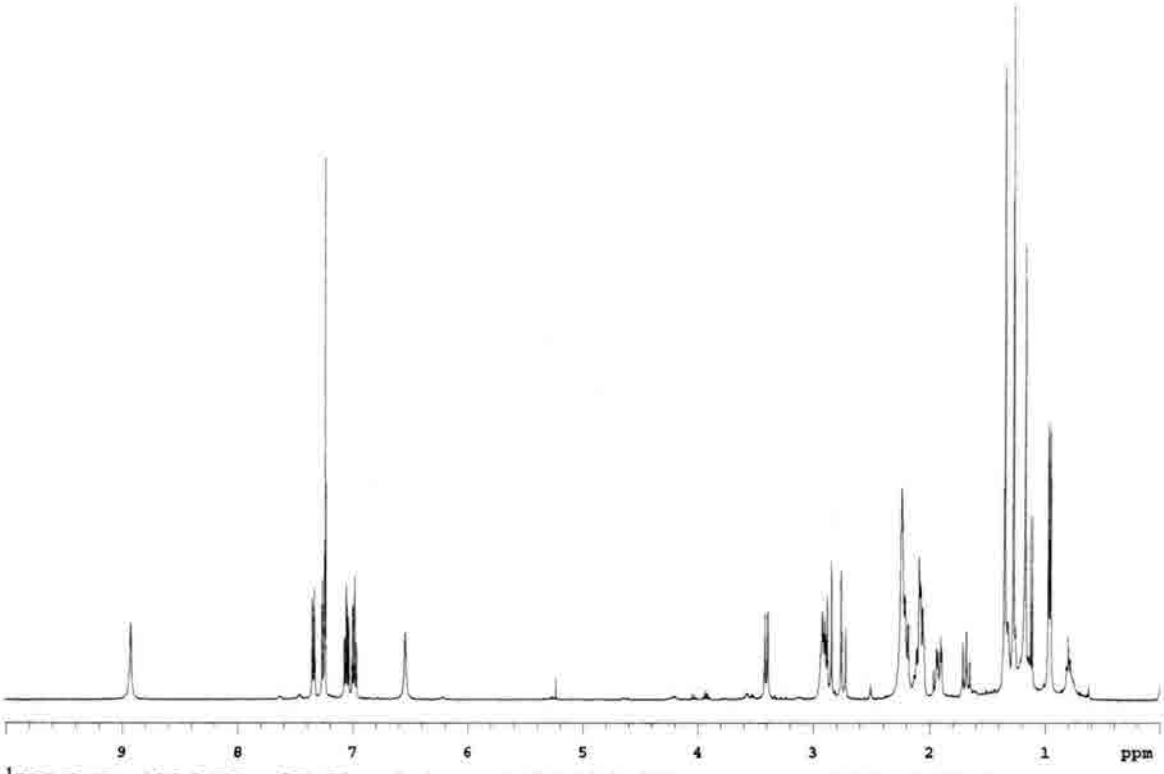
(±)-VM55599 (13):

Yield: 13.5 mg, (86%); obtained as an amorphous powder.

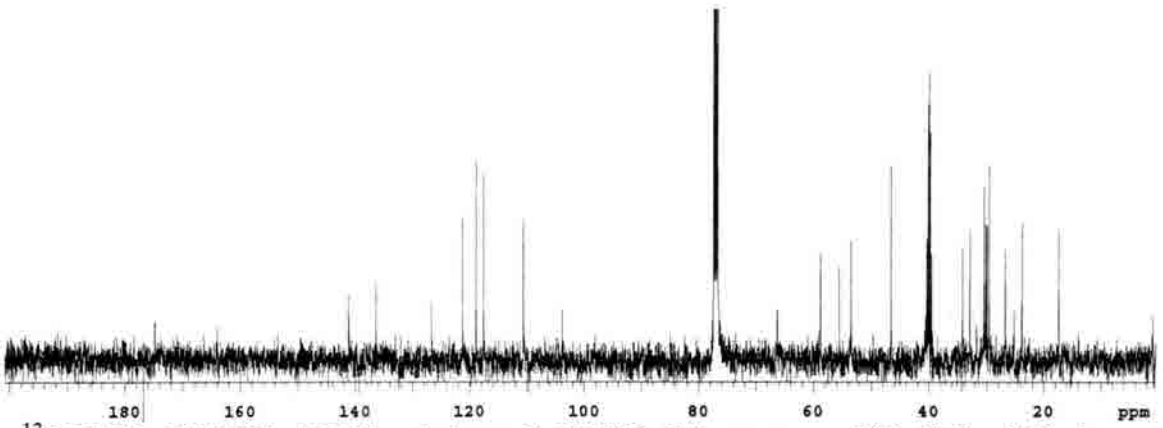
^{1}H NMR (400 MHz, $CDCl_3$ + 1 drop d_6-DMSO): δ 1.00 (3H, d, *J*= 7.0 Hz), 1.31 (3H, s), 1.37 (1H, m), 1.39 (3H, s), 1.73 (1H, dd, *J*= 11.7, 13.2 Hz), 2.01 (1H, dd, *J*= 4.3, 13.2 Hz), 2.13 (3H, m), 2.24 (1H, dd, *J*= 1.6, 10.1 Hz), 2.76 (1H, d, *J*= 15.2 Hz), 2.90 (1H, d, J=15.2 Hz), 2.96 (2H, m), 3.45 (1H, d, *J*= 10.1 Hz), 6.28 (1H, bs), 7.09 (1H, ddd, *J*= 0.8, 7.8, 7.8 Hz), 7.16 (1H, ddd, *J*= 1.2, 7.0, 7.0 Hz), 7.33 (1H, d, *J*= 7.3 Hz), 7.43 (1H, d, *J*= 7.8 Hz), 8.44 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$ + 1 drops d_6-DMSO): δ 17.5, 24.0, 26.8, 30.1, 30.2, 30.5, 33.0, 34.2, 46.6, 53.6, 55.7, 58.9, 66.4, 104.1, 110.6, 117.7, 119.2, 121.5, 126.8, 136.4, 141.1, 174.8. I.R. (NaCl, neat): 3303, 3048, 2920, 1650, 1454, 1296, 779, 734, 695 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{28}N_3O$: 350.2232. Found 350.2235 (M+H). R_f 0.38 (eluted with 4% MeOH/CH_2Cl_2). This synthetic compound was identical to natural VM55599 (obtained from *Penicillium* sp. IMI332995) by TLC (silica gel, eluted with 4% MeOH/CH_2Cl_2), ^{1}H nmr and ^{13}C nmr.



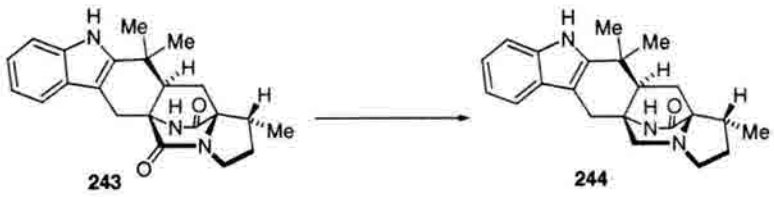
13, VM55599



^{1}H NMR, 400 MHz, $CDCl_3$ + 2 drops d_6-DMSO, Filename: ems795_cdcl3_dmso



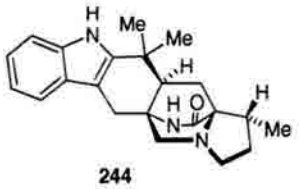
^{13}C NMR, 100 MHz, $CDCl_3$ + 2 drops d_6-DMSO, Filename: ems795_13C_cdcl3_dmso



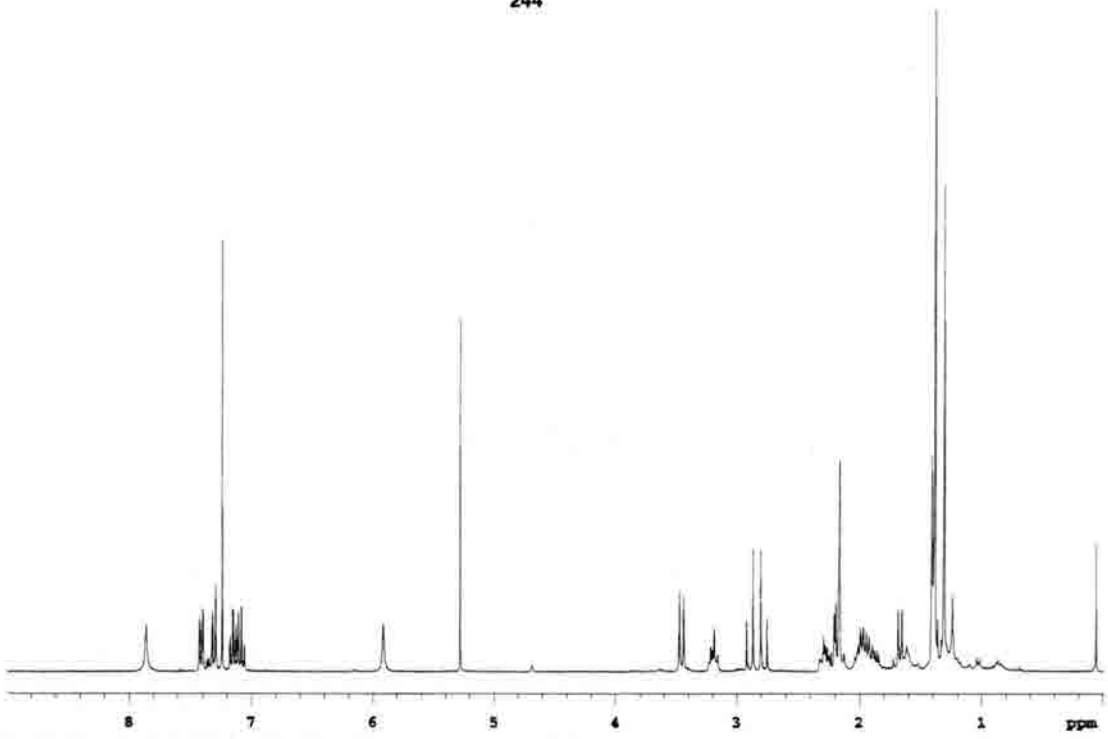
(1R,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazol-14-one (244):

Yield: 2.4 mg, (54%); obtained as an amorphous powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.30 (3H, s), 1.38 (3H, s), 1.39 (3H, d, *J*= 7.3 Hz), 1.66 (1H, m), 1.91 (3H, m), 2.19 (3H, m), 2.28 (1H, m), 2.78 (1H, d, *J*= 15.2 Hz), 2.89 (1H, d, *J*= 15.2 Hz), 3.19 (1H, m), 3.45 (1H, d, *J*= 10.2 Hz), 5.48 (1H, bs), 7.08 (1H, ddd, *J*=1.2, 7.4, 7.4 Hz), 7.15 (1H, ddd, *J*=1, 7.8, 7.8 Hz), 7.30 (1H, d, *J*= 7.8 Hz), 7.40 (1H, d, *J*=7.4 Hz), 7.80 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.0, 24.0, 29.9, 30.1, 30.5, 30.6, 34.0, 40.4, 46.3, 53.8, 56.7, 59.8, 65.5, 104.6, 110.6, 117.9, 119.5, 121.8, 126.9, 136.3, 140.8, 173.7. IR (NaCl, neat): 3305, 3060, 2924, 1667, 1455, 1368, 1261, 1109, 1014, 801, 741, 706 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{28}N_3O$: 350.2232. Found 350.2235 (M+H). R_f 0.40 (eluted with 4% MeOH/CH_2Cl_2).



244

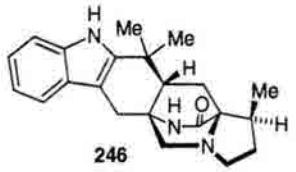


^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems793

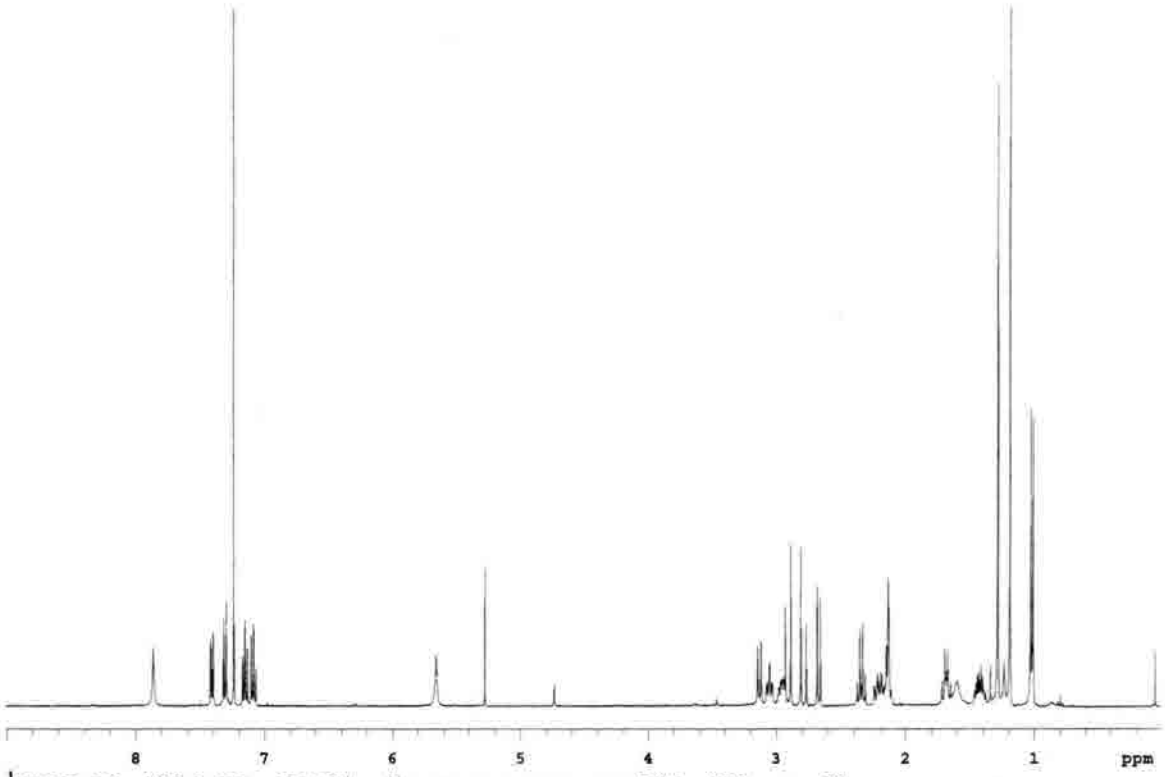
(1S,5aR,12aS,13aS)-(±)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazol-14-one (246):

Yield: 8.5 mg, (61%); obtained as an amorphous powder.

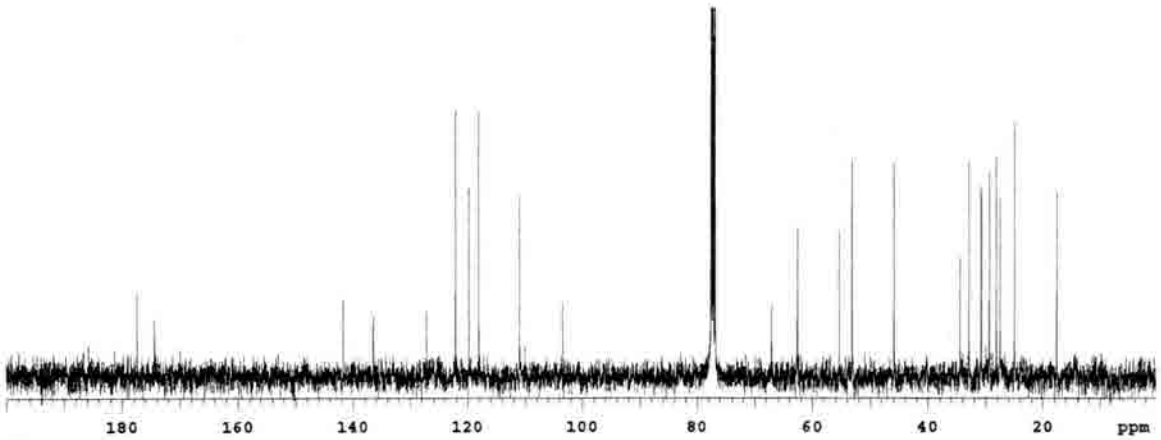
^{1}H (300 MHz, $CDCl_3$): δ 1.02 (3H, d, *J*= 7.3 Hz), 1.19 (3H, s), 1.29 (3H, s), 1.42 (1H, m), 1.69 (1H, m), 2.14 (2H, m), 2.22 (1H, m), 2.35 (1H, dd, *J*= 8.6, 17.2 Hz), 2.67 (1H, d, *J*= 10.2 Hz), 2.79 (1H, d, *J*= 17.2 Hz), 2.92 (1H, d, *J*= 17.2 Hz), 2.96 (1H, m), 3.06 (1H, ddd, *J*= 2.5, 8.6, 8.6 Hz), 3.14 (1H, d, *J*= 10.6 Hz), 5.67 (1H, bs), 7.08 (1H, ddd, *J*=1.2, 7.4, 7.4 Hz), 7.15 (1H, m), 7.31 (1H, dd, *J*= 1.1, 7.1 Hz), 7.41 (1H, dd, *J*=1.0, 7.7 Hz), 7.87 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.4, 24.8, 27.3, 28.0, 29.2, 30.6, 32.7, 34.3, 45.7, 53.0, 55.2, 62.4, 66.9, 103.24, 110.8, 117.9, 119.6, 121.9, 127.0, 136.3, 141.5, 174.2. I.R. (NaCl, neat): 3281, 3060, 2960, 1668, 1462, 133, 1123, 1009, 740, 702 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{28}N_3O$: 350.2232. Found 350.2233 (M+H). R_f 0.17 (eluted with 4% $MeOH/CH_2Cl_2$).



246



^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems792_400_set, filename: proton



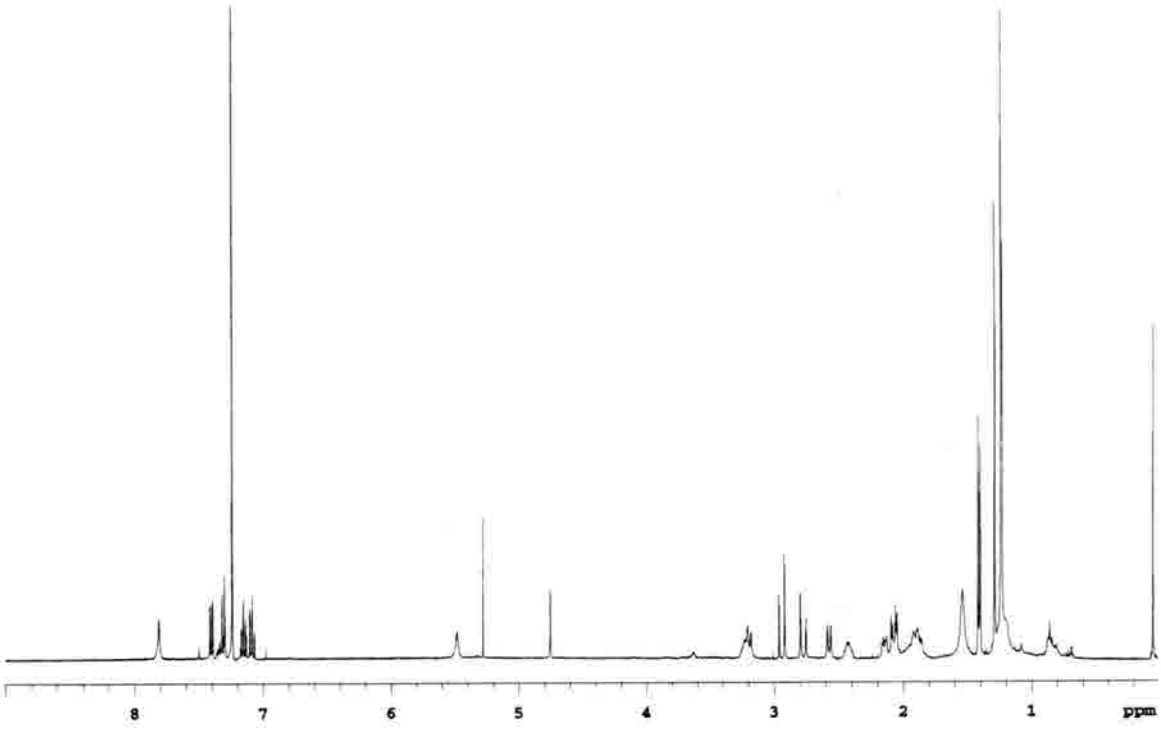
^{13}C NMR, 100 MHz, $CDCl_3$, directory name: ems792_400_set, filename: carbon

(1R,5aR,12aS,13aS)-(±)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazol-14-one (248):

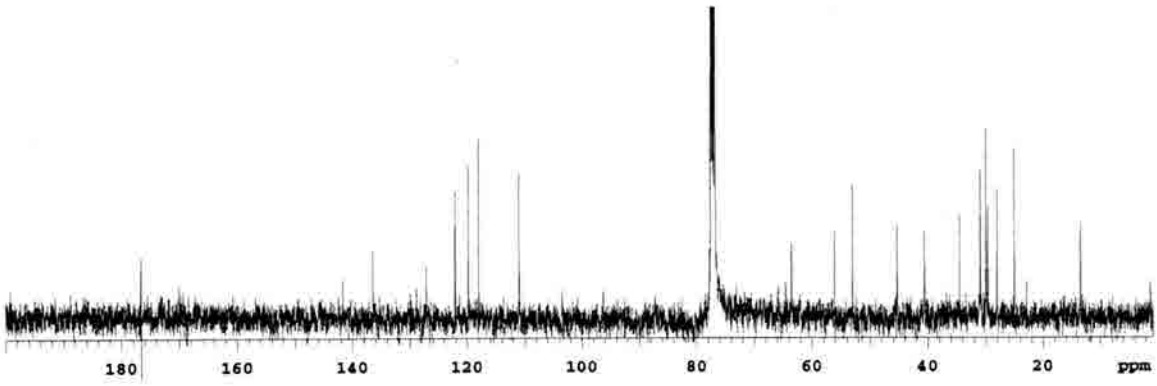
Yield: 19 mg, (79%); obtained as an amorphous powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.24 (3H, s), 1.29 (3H, s), 1.41 (3H, d, *J*= 6.6 Hz), 1.89 (3H, m), 2.07 (1H, dd, *J*= 4, 13 Hz), 2.07 (1H, m), 2.14 (1H, m), 2.43 (1H, m), 2.57 (1H, d, J = 10.5 Hz), 2.77 (1H, d, J = 17.4 Hz), 2.94 (1H, d, *J*= 17.4 Hz), 3.19 (1H, d, *J*= 10.5 Hz), 3.22 (1H, m), 5.48 (1H, bs), 7.09 (1H, ddd, *J*= 0.8, 7.4, 7.4 Hz), 7.15 (1H, ddd, *J*= 1.2, 7.6, 7.6 Hz), 7.31 (1H, d, *J*= 7.8 Hz), 7.40 (1H, d, *J*= 7.8 Hz), 7.81 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$ + 1 drop d_6-DMSO): δ 11.9, 24.5, 25.3, 27.5, 27.9, 28.8, 29.5, 34.4, 37.8, 43.8, 52.7, 54.6, 59.2, 69.5, 101.5, 110.9, 117.8, 119.1, 121.6, 136.5, 140.6, 176.8. IR (NaCl, neat): 3213, 2959, 1694, 1454, 1259, 1022, 797, 702 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{28}N_3O$: 350.2232. Found 350.2235 (M+H). R_f 0.23 (eluted with 4% MeOH/CH_2Cl_2).

248



^{1}H NMR, 400 MHz, $CDCl_3$, directory name: ems794_400_set, filename: proton



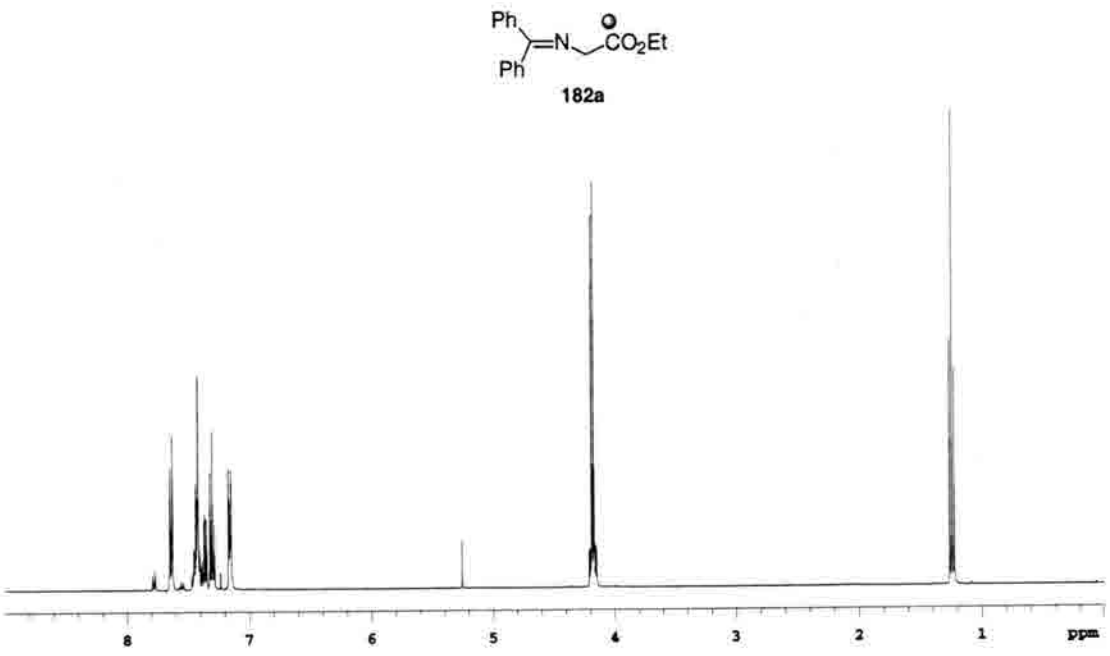
^{13}C NMR, 100 MHz, $CDCl_3$, filename: ems794_13C_400

458 182a

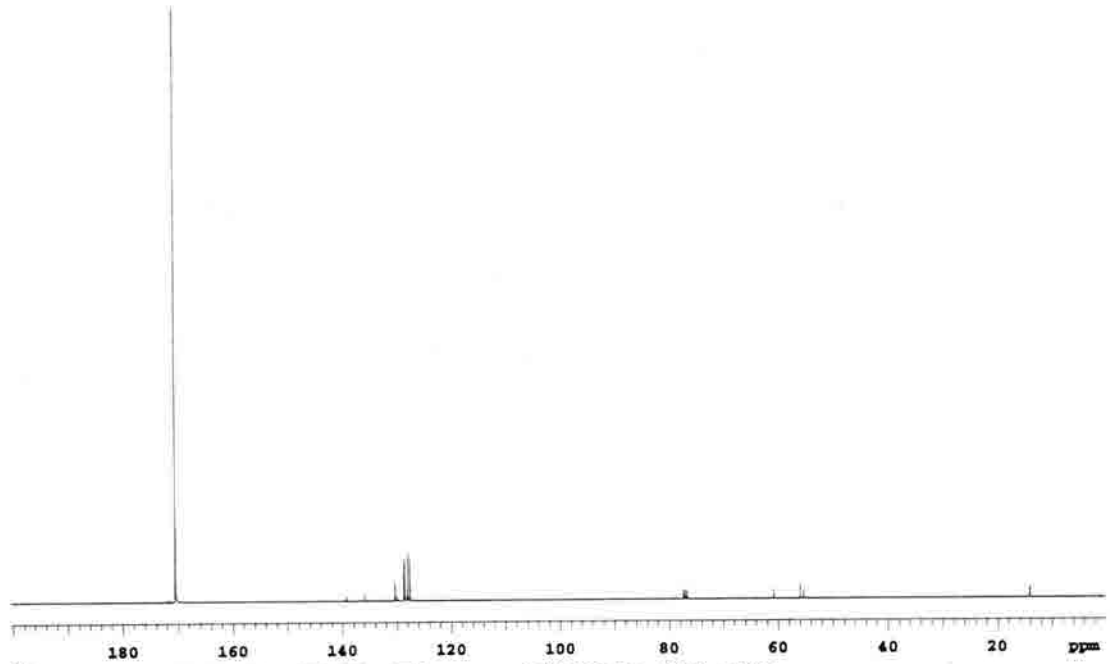
[^{13}C]-N-(diphenylmethylene)glycine ethylester (182a):

Thionyl chloride (7.9 mL, 107.8 mmol) was added to a stirring solution of 1-[^{13}C]-glycine (**458**, 1.17 g, 15.4 mmol) in 94 mL of absolute EtOH at 0°C. The solution was brought to room temperature and stirred for an additional 24 hours. The solvent was removed under reduced pressure and the product was placed under vacuum overnight to give 1-[^{13}C]-glycine ethylester hydrochloride. To this product, dichloromethane (31 mL) followed by distilled benzophenone imine (2.58 mL, 15.4 mmol) was added at room temperature. The resultant mixture was stirred for 24 hours at room temperature. The mixture was filtered and washed with dichloromethane. The combined filtrates were evaporated under reduced pressure. The product was purified by flash column chromatography (10% EtOAc/ Hexanes) to afford N-(diphenylmethylene)glycine ethylester (**182a**) as a yellowish-white solid. Yield: 3.82 g, 14.2 mmol, 92%.

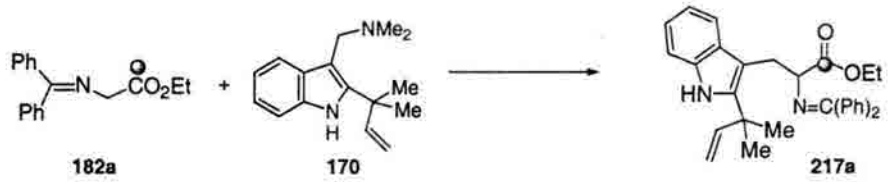
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.25 (3H, t, *J*= 7.0 Hz), 4.18 (2H, dddd, *J*= 3.0, 7.4, 7.4, 7.4 Hz), 4.19 (2H, d, *J*= 7.0 Hz), 7.16 (2H, m), 7.46-7.29 (6H, m), 7.65 (2H, m). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 55.6 (d, $J_{C\text{-}C}$ = 63 Hz), 60.7, 127.6, 128.0, 128.6, 128.7, 128.7, 130.4, 135.9, 139.2, 159.5, 170.5. HRMS (FAB+): Calcd for $C_{16}{}^{13}C_1H_{18}NO_2$: 269.1371. Found 269.1364 (M+H).



^{1}H NMR, 400 MHz, $CDCl_3$, Filename: EMS952_400



^{13}C NMR, 100 MHz, $CDCl_3$, Filename: EMS952_400_13C

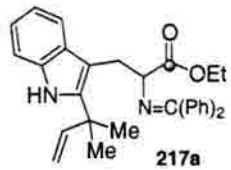


182a 170 217a

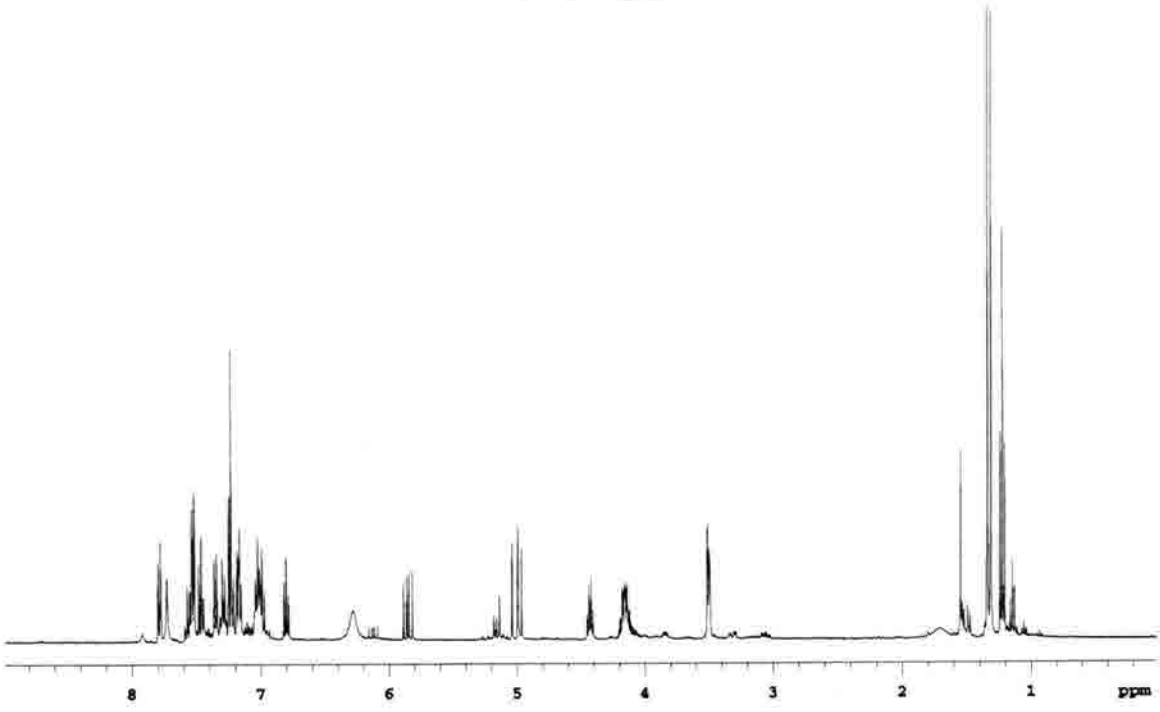
[^{13}C]-N-(diphenylmethylene)2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan ethyl ester (217a):

Synthesized in the same manner as the unlabeled compound, **217**, as previously described. Yield 39-52% as an oil.

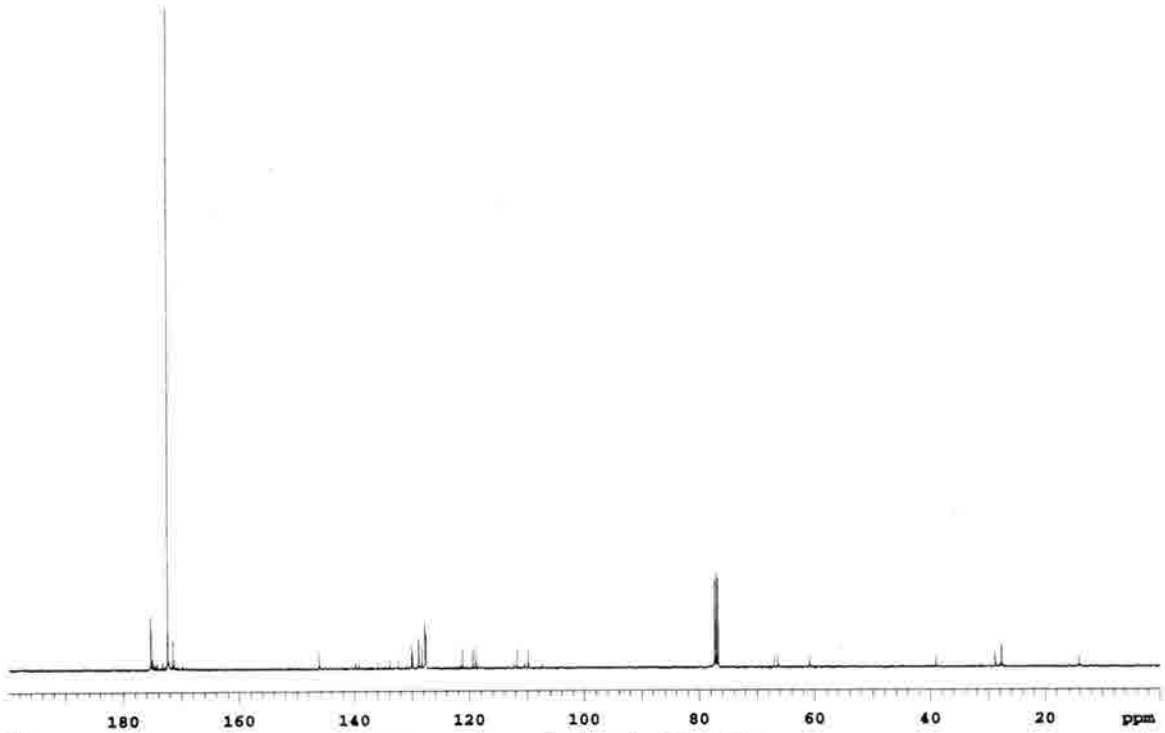
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.23 (3H, t, *J*= 7.2 Hz), 1.32 (3H, s), 1.35 (3H, s), 3.51 (2H, dd, *J*= 2.4, 6.7 Hz), 4.16 (2H, m), 4.43 (1H, dd, *J*= 5.7, 12.9 Hz), 4.98 (1H, dd, *J*= 1.1, 10.6 Hz), 5.02 (1 H, dd. *J*= 1.3, 18.3 Hz), 5.86 (1H dd, *J*= 10.6, 18.3 Hz), 6.28 (2H, bs), 6.81 (1H, dd, *J*= 8.1, 8.1 Hz), 7.00 (1H, dd, *J*= 8.0, 8.0 Hz), 7.03 (2H, dd, *J*= 7.5, 7.5 Hz), 7.24 (5H, m), 7.36 (1H, d, *J*= 8.0 Hz), 7.56 (2H, m), 7.73 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 27.5, 27.6, 28.7, 39.0, 60.8, 66.6 (d, $J_{C\text{-}C}$ = 61 Hz), 107.4, 109.7, 111.6, 118.9, 119.4, 121.2, 127.5, 127.7, 127.8, 128.3, 128.8, 129.9, 130.0, 132.4, 133.8, 135.9, 139.2, 139.9, 146.0, 169.7, 172.4. HRMS (FAB+): Calcd for $C_{30}{}^{13}C_1H_{33}N_2O_2$: 466.2576 Found 466.2577 (M+H).



217a



^{1}H NMR, 400 MHz, $CDCl_3$, Filename: EMS895_400

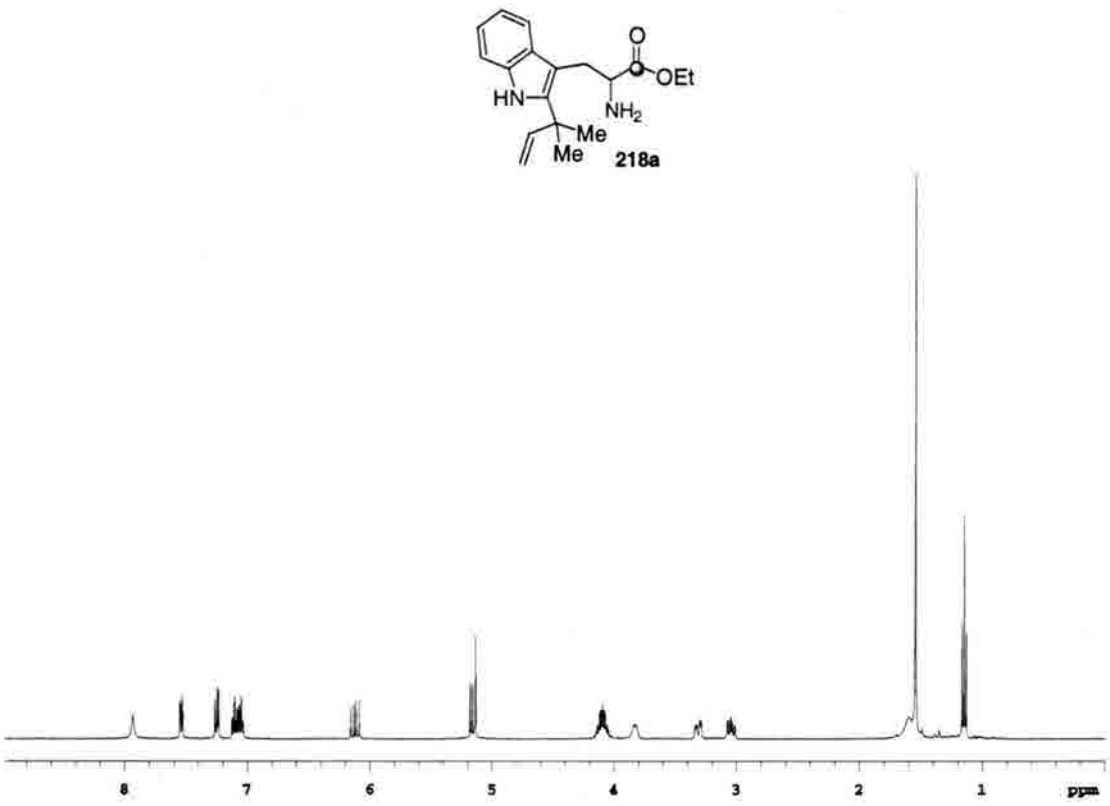


^{13}C NMR, 100 MHz, $CDCl_3$, Filename: EMS895_400_13C

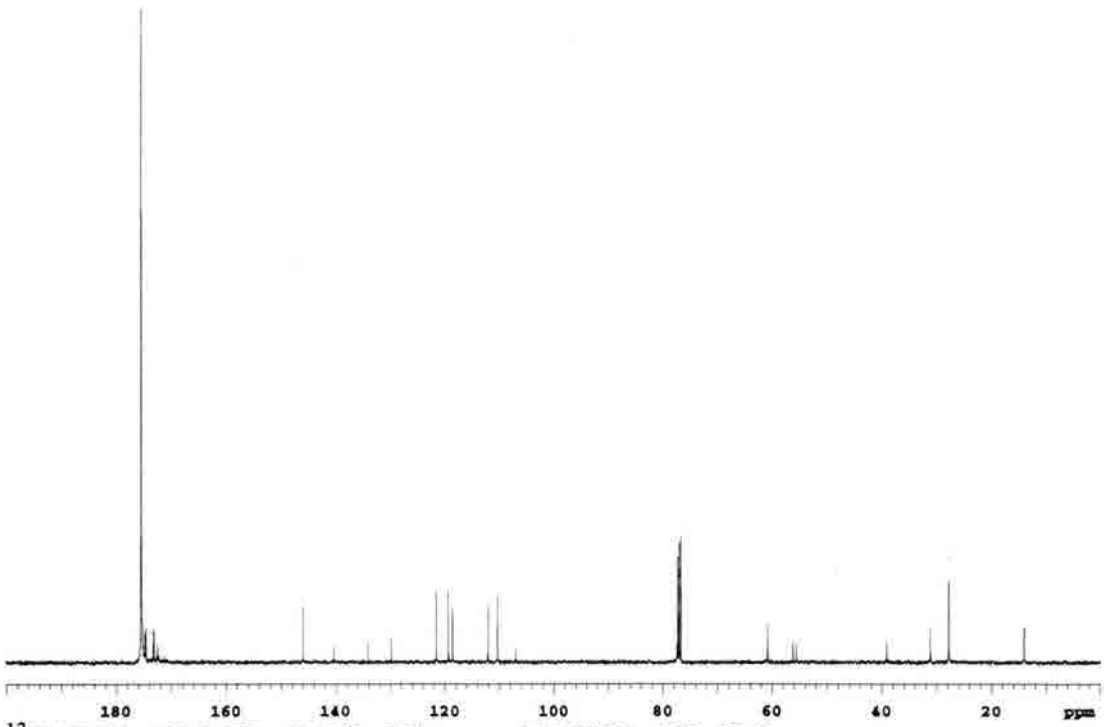
[^{13}C]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan ethyl ester (218a):

Prepared in the same manner as the unlabeled compound, **218**, as previously described. Yield: 65-79%.

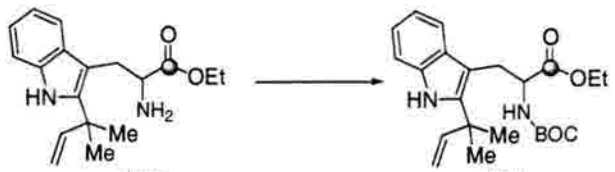
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.16 (3H, t, *J*= 7.0 Hz), 1.55 (6H, s), 1.60 (2H, bs), 3.05 (1H, ddd, *J*= 3.7, 9.6, 13.6 Hz), 3.31 (1H, dd, *J*= 5.0, 14.7 Hz), 3.86 (1H, ddd, *J*= 2.0, 5.0, 14.4 Hz), 4.13 (2H, m), 5.15 (1 H, d, *J*= 10.5 Hz), 5.16 (1 H, d, *J*= 17.3 Hz), 6.13 (1H, dd, *J*= 10.5, 17.3 Hz), 7.06 (1H, ddd, *J*= 1.2, 7.7, 7.7 Hz), 7.11 (1H, ddd, *J*= 1.1, 7.0, 7.0 Hz), 7.26 (1H, d, *J*= 7.8 Hz), 7.55 (1H, d, *J*= 7.8 Hz), 7.94 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.0, 27.8, 27.9, 31.2, 39.2, 55.9 (d, $J_{C\text{-}C}$ = 60 Hz), 60.8, 107.0, 110.3, 112.1, 118.6, 119.4, 112.5, 129.8, 134.2, 140.4, 146.0, 175.5. HRMS (FAB+): Calcd for $C_{17}{}^{13}C_1H_{25}N_2O_2$: 302.1950. Found 302.1946 (M+H).



^{1}H NMR, 400 MHz, $CDCl_3$, Filename: EMS973_400



^{13}C NMR, 100 MHz, $CDCl_3$, Filename: EMS973_400_13C

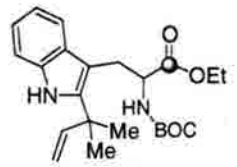


218a 459

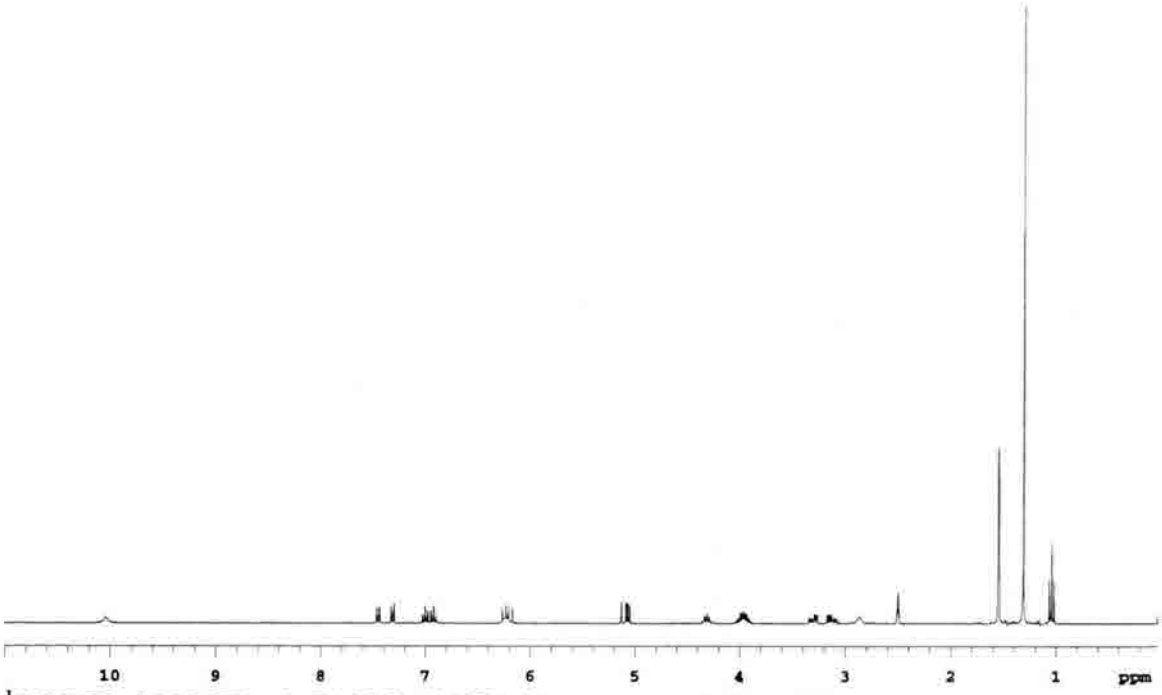
[^{13}C]-N-[(1,1-dimethylethoxy)carbonyl]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan ethyl ester (**459**):

Followed the same procedure as described for the unlabeled compound. Yield: 74-84% as an oil.

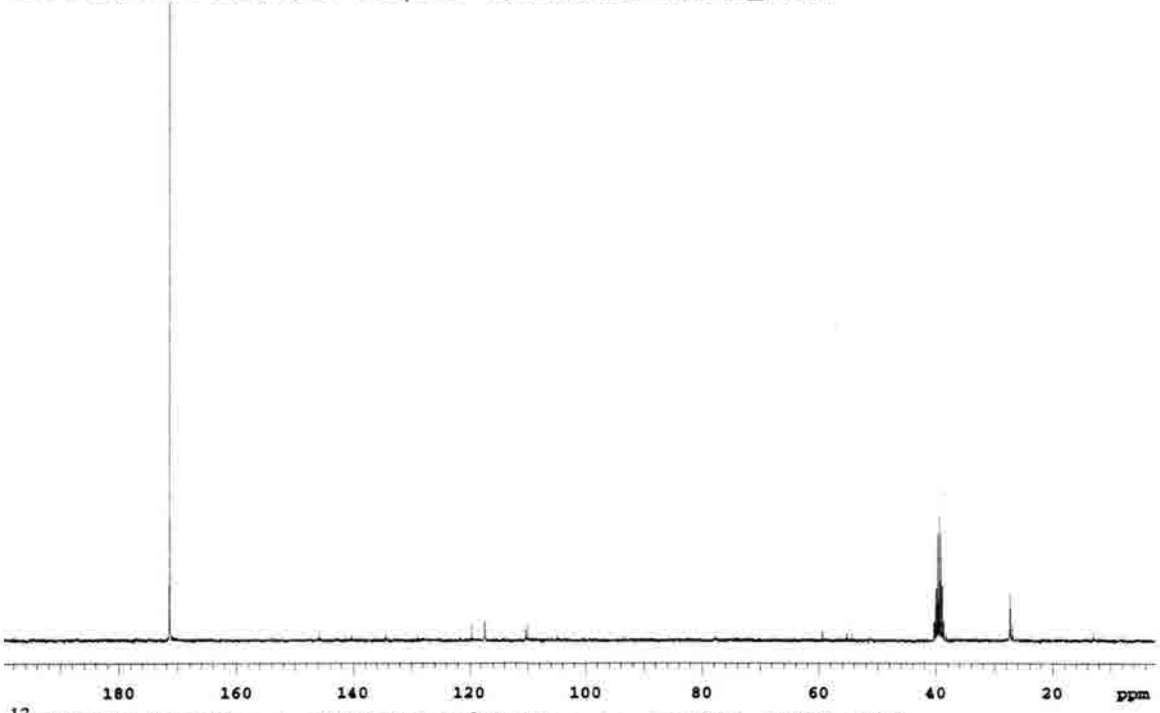
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.04 (3H, t, *J*= 7.0 Hz), 1.31 (9H, s), 1.54 (3H, s), 1.55 (3H, s), 3.12 (1H, ddd, *J*= 4.9, 7.8, 14.6 Hz), 3.30 (1H, ddd, *J*= 2.6, 6.8, 14.6 Hz), 3.97 (2H, m), 4.31 (1H, dd, *J*= 7.8, 14.6 Hz), 5.07 (1H, dd, *J*= 1.0, 10.6 Hz), 5.10 (1H, dd, *J*= 1.0, 17.4 Hz), 6.22 (1H, dd, *J*= 10.6, 17.4 Hz), 6.23 (1H, bs), 6.92 (1H, ddd, *J*= 1.2, 8.1, 8.1 Hz), 7.00 (1H, ddd, *J*= 1.2, 7.7, 7.7 Hz), 7.32 (1H, d, *J*= 8.0 Hz), 7.45 (1H, d, *J* =7.7 Hz), 10.04 (1H, bs). ^{13}C NMR (100 MHz, d_6-DMSO, 116°C): δ 12.9, 26.9, 27.2, 27.4, 30.7, 38.3, 54.8 (d, $J_{C\text{-}C}$ = 62 Hz), 59.4, 77.7, 104.9, 110.0, 110.4, 117.3, 117.5, 119.7, 128.9, 134.4, 140.3, 145.8, 154.1, 171.4. HRMS (FAB+): Calcd for $C_{22}{}^{13}C_1H_{32}N_2O_4$: 401.2396. Found 401.2408 (M^+).



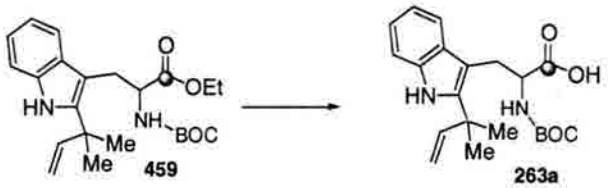
459



^{1}H NMR, 300 MHz, d_6-DMSO, 120 °C, filename: ems940_vt120



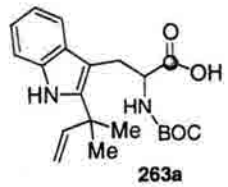
^{13}C NMR, 75 MHz, d_6-DMSO, 120 °C, filename: ems940_vt120_13C



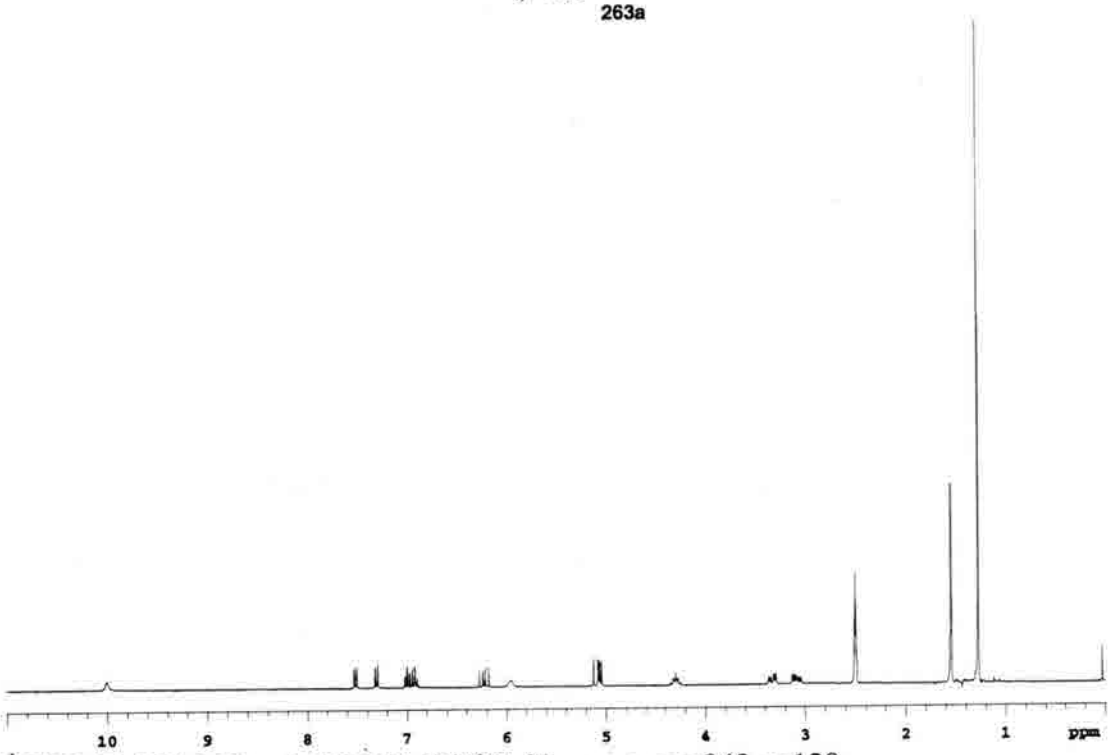
[^{13}C]-N-[(1,1-dimethylethoxy)carbonyl]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan (263a):

Followed the procedure described for **263**. Yield 99-100%.

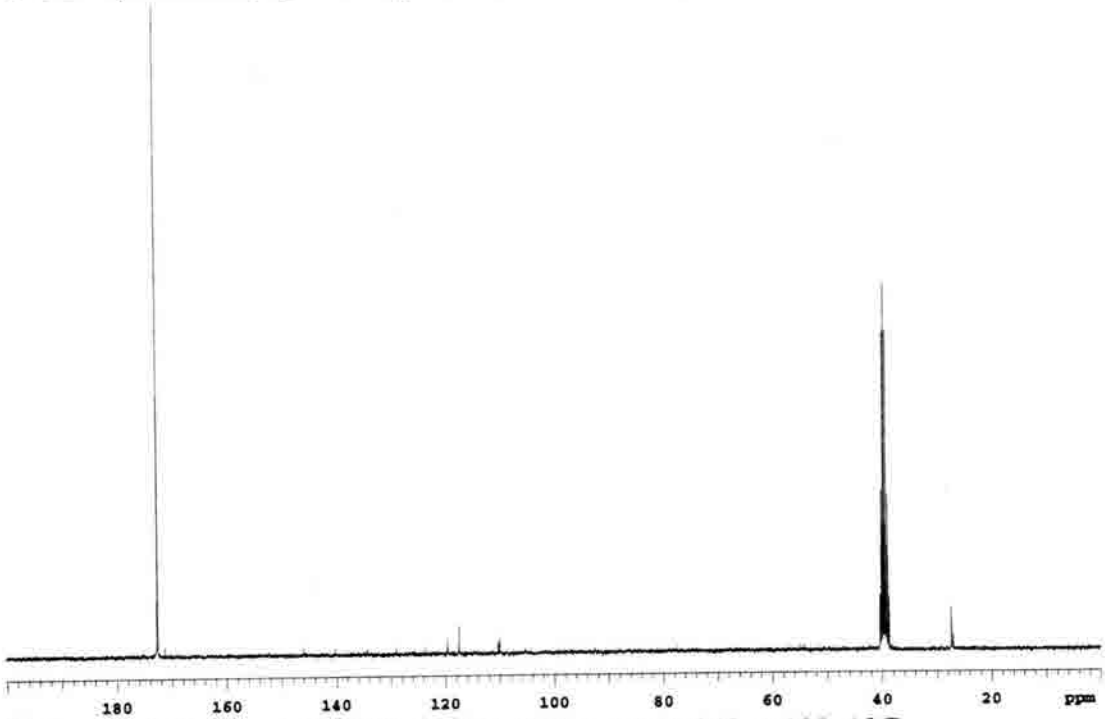
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.28 (9H, s), 1.55 (3H, s), 1.56 (3H, s), 3.09 (1H, dd, *J*= 8.1, 14.7 Hz), 3.46 (1H, dd, *J*= 6.2, 14.7), 4.30 (1H, dd, *J*= 8.1, 14.7 Hz), 5.06 (1H, dd, *J*= 1.1, 10.6 Hz), 5.10 (1H, dd, *J*= 0, 17.2 Hz), 5.98 (1H, d, *J*= 5.9 Hz), 6.22 (1H, dd, *J*= 10.6, 17.2 Hz), 6.92 (1H, ddd, *J*= 1.1, 7.7, 7.7 Hz), 7.00 (1H, ddd, *J*= 0.8, 7.7, 7.7 Hz), 7.31 (1H, d, *J*= 7.7 Hz), 7.53 (1H, d, *J*= 7.7 Hz), 10.02 (1H, bs), 11.51 (1H, very bs). ^{13}C NMR (75 MHz, d_6-DMSO, 120°C): δ 27.1, 27.3, 27.4, 38.3, 54.6 (d, $J_{C\text{-}C}$= 59 Hz), 77.5, 105.4, 110.0, 110.4, 117.5, 119.6, 123.6, 129.0, 134.4, 140.3, 146.0, 154.2, 172.7. HRMS (FAB+): Calcd for $C_{20}{}^{13}C_1H_{28}N_2O_4$: 373.2083. Found 373.2091 (M+).



263a



^{1}H NMR, 300 MHz, d_6-DMSO, 120 °C, filename: ems942_vt120



^{13}C NMR, 75 MHz, d_6-DMSO, 120 °C, filename: ems942_vt120_13C

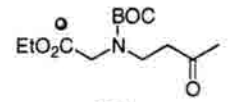
458, 1-[^{13}C]-glycine **231a**

[^{13}C]-N-[1,1-dimethyethoxy) carbonyl]-N-(3-oxobutyl)-glycine ethyl ester (**231a**):

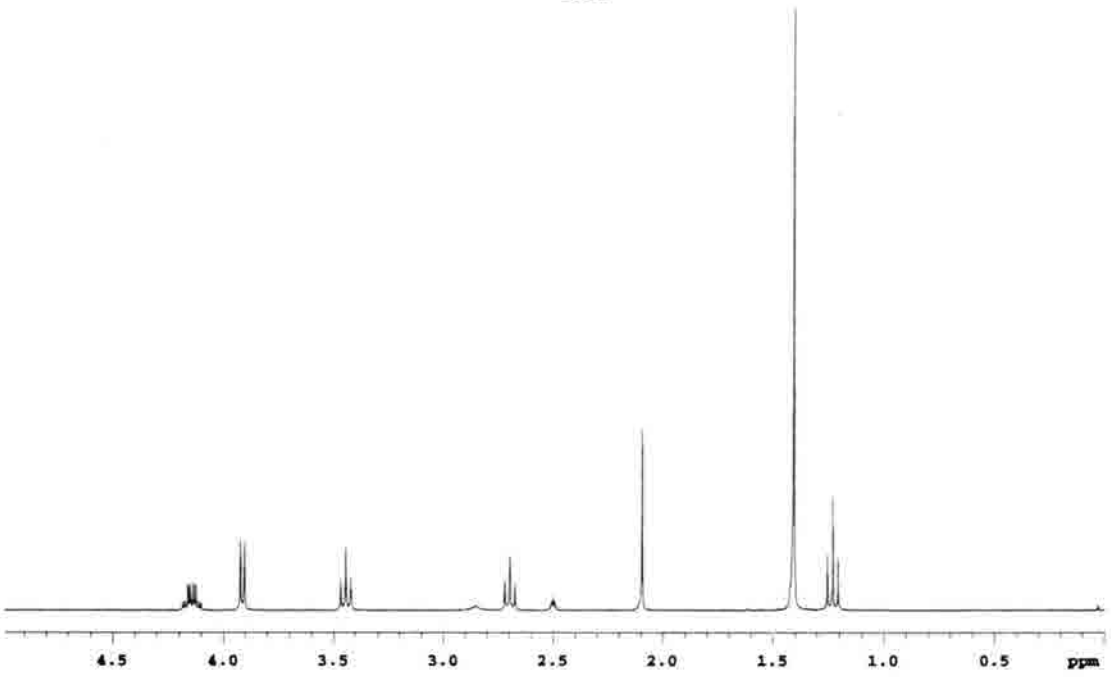
Thionyl chloride (14.1 mL, 193.3 mmol) was added to a stirring solution of 1-[^{13}C]-glycine (2.1 g, 27.6 mmol) in 160 mL of absolute EtOH at 0°C. The solution was brought to room temperature and stirred for an additional 24 hours. The solvent was removed under reduced pressure and the product was placed under vacuum overnight to give 1-[^{13}C]-glycine ethylester hydrochloride. From this point, the same procedure was followed as described for the synthesis of the unlabeled compound.

Yield: 3.36 g, 12.3 mmol, 44% from 1-[^{13}C]-glycine.

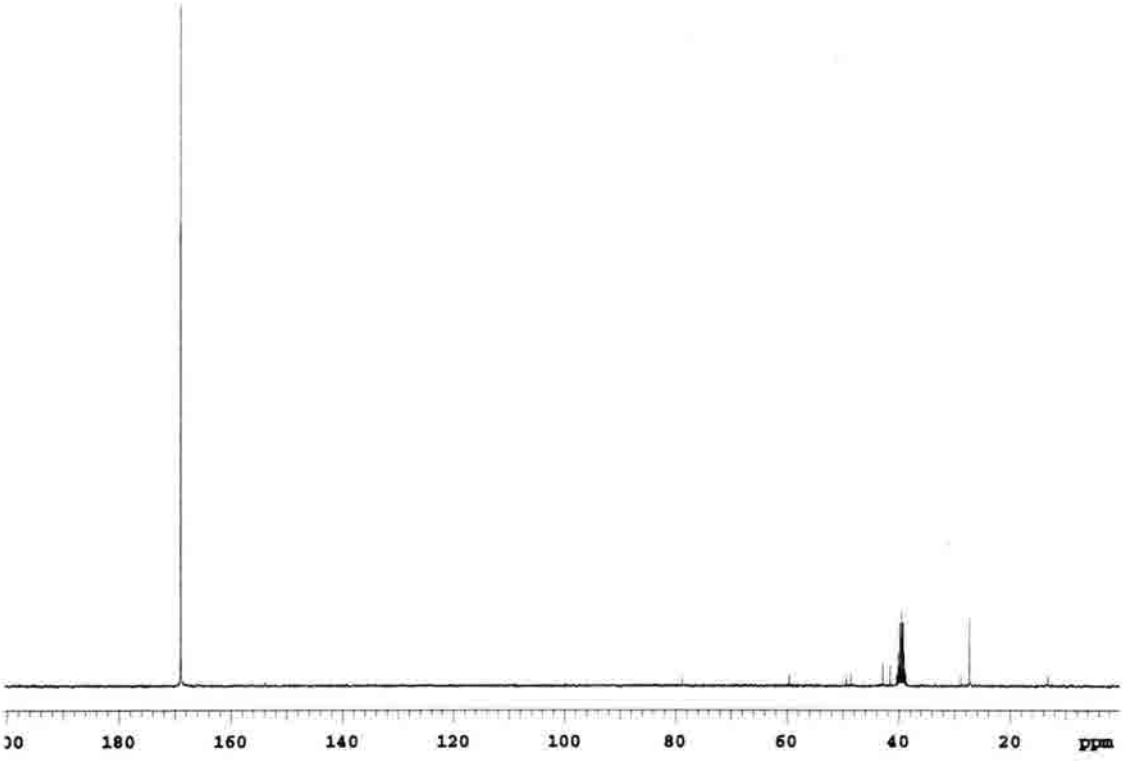
^{1}H NMR (300 MHz, d_6-DMSO, 120°C): δ 1.23 (3H, t, *J*= 7.0 Hz), 1.41 (9H, s), 2.10 (3H, s), 2.70 (2H, t, *J*= 6.7 Hz), 3.44 (2H, t, *J*= 6.7 Hz), 3.92 (2H, d, *J*= 5.4 Hz), 4.14 (2H, dddd, *J*= 3.6, 7.0, 7.0, 7.0 Hz). ^{13}C NMR (75 MHz, d_6-DMSO, 120°C): δ 13.2, 27.3, 28.8, 41.5, 42.8, 48.9 (d, $J_{C\text{-}C}$ = 62 Hz), 59.6, 78.8, 154.0, 169.0, 205.8. HRMS (FAB+) Calcd. for $C_{12}{}^{13}C_1H_{24}NO_5$: 275.1688. Found 275.1678 (M+H).



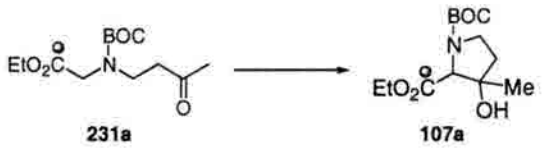
231a



^{1}H NMR, 300 MHz, d_6-DMSO, 120 °C, filename: ems926_vt120



^{13}C NMR, 75 MHz, d_6-DMSO, 120 °C, filename: ems926_vt120_13

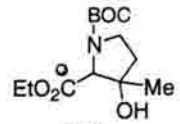


231a 107a

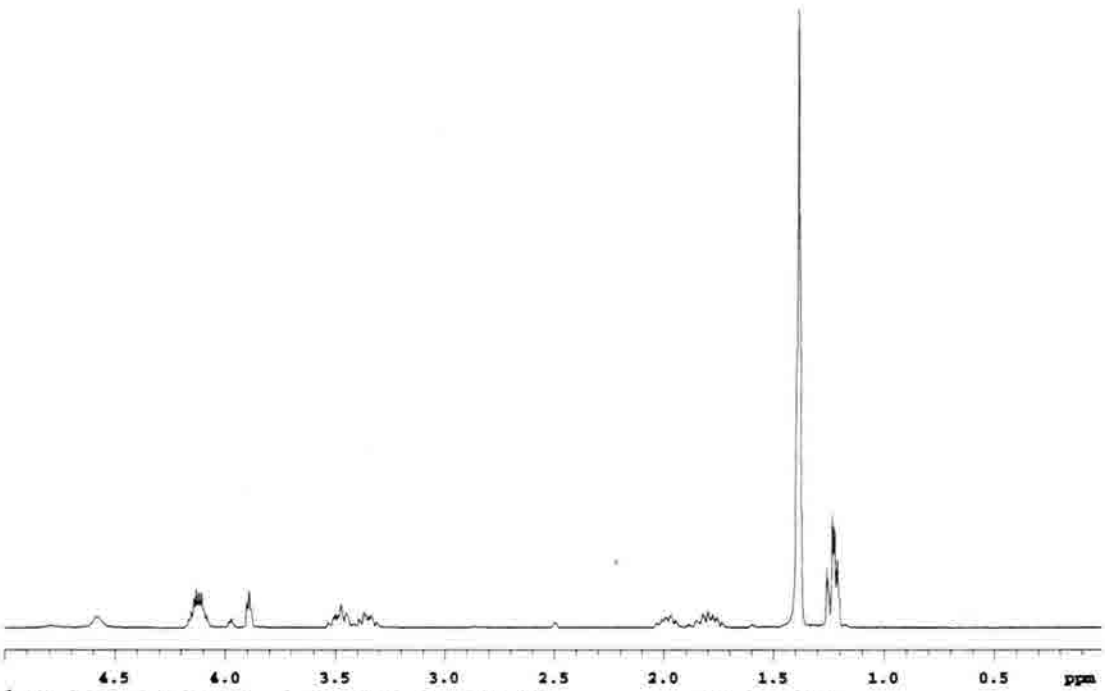
[^{13}C]-3-hydroxy-3-methyl-1,2-pyrrolidinedicarboxylic acid 1-(1,1-dimethylethyl) 2-ethyl ester (107a):

The same procedure was followed as described for the synthesis of **107**. Purification was accomplished by flash column chromatography (30% EtOAc/ Hexanes). Yield: 39% as an off-white amorphous solid.

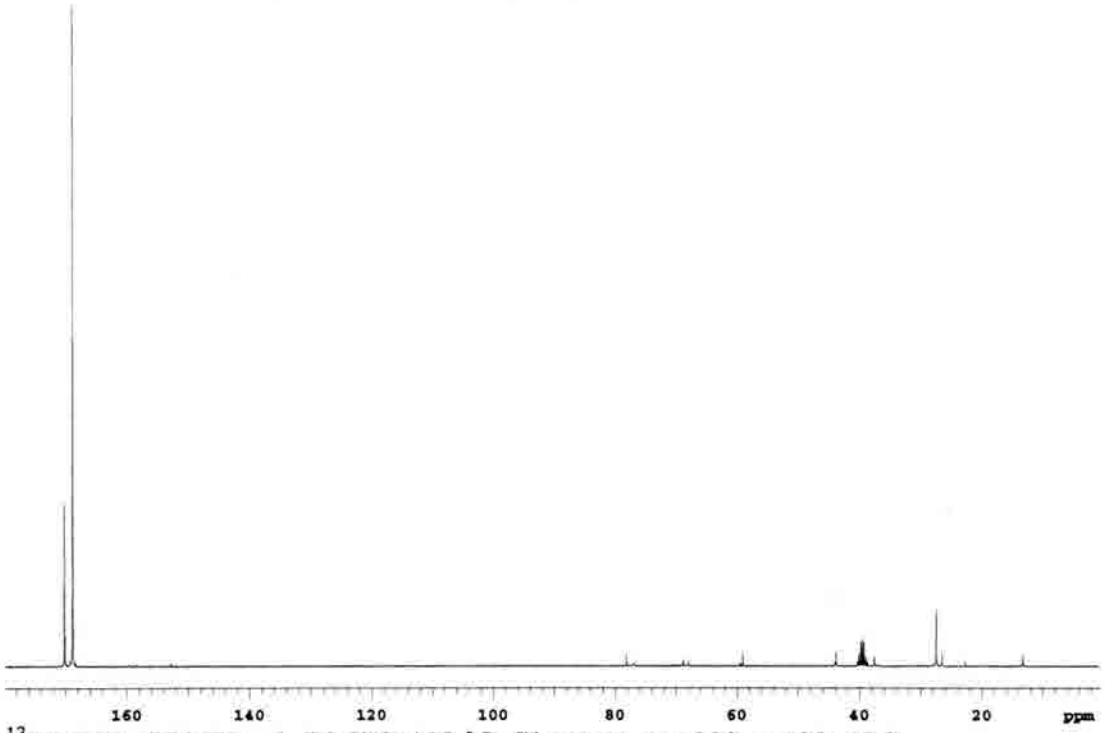
^{1}H NMR (300 MHz, d_6-DMSO, 120°C)[221]: δ 1.22 (3H, t, *J*= 7.0 Hz), 1.39 (12H, s), 1.80 (1H, m), 1.98 (1H, m), 3.36 (1H, m), 3.48 (1H, m), 3.89 + 3.98 (1H, d, *J*= 3.6 Hz), 4.12 (2H, m), 4.58 + 4.79 (1H, bs). ^{13}C NMR (75 MHz, d_6-DMSO, 120°C): δ 13.2 + 13.4, 22.7, 26.5, 27.4, 37.6, 43.8 + 44.0, 59.0 + 59.5, 68.4 + 70.2 (d, $J_{C\text{-}C}$ = 64 Hz), 76.9 + 78.1, 152.8, 168.9 + 170.2. HRMS (FAB+): Calcd for $C_{12}{}^{13}C_1H_{24}N_1O_5$: 275.1688 Found 275.1687 (M+H).



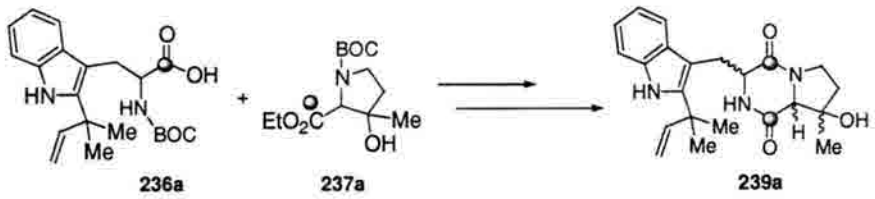
107a



^{1}H NMR, 300 MHz, d_6-DMSO, 120 °C, filename: ems938_vt120



^{13}C NMR, 75 MHz, d_6-DMSO, 120 °C, filename: ems938_vt120_13C



236a 237a 239a

[$^{13}C_2$]-3-[[2-(1,1-dimethyl-2-propenyl)-1H-indol-3-yl]methyl]-hexahydro-8-hydroxy-8-methyl-pyrrolo[1,2a]pyrazine-1,4-dione (239a):

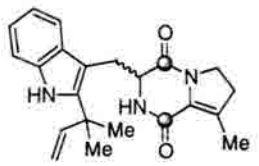
Followed previously described procedure for **239**. Yield: 74-79% as a mixture of diastereomers from N-Boc-dimethylallyl-trp.

239a 240a

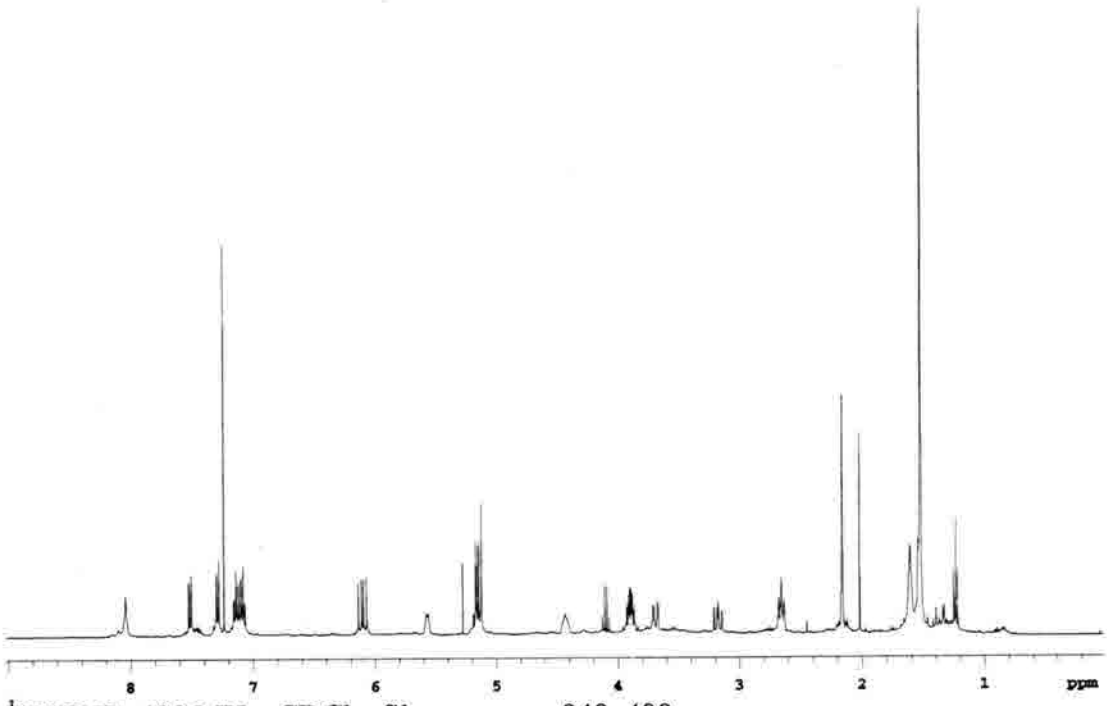
[$^{13}C_2$]-(3R,S)-3-[[2-(1,1-dimethyl-2-propenyl)-1H-indol-3-yl]methyl]-2,3,6,7-tetrahydro-8-methyl-pyrrolo[1,2-a]pyrazine-1,4-dione (240a):

Followed previously described procedure for **240**. Yield: 50-52%.

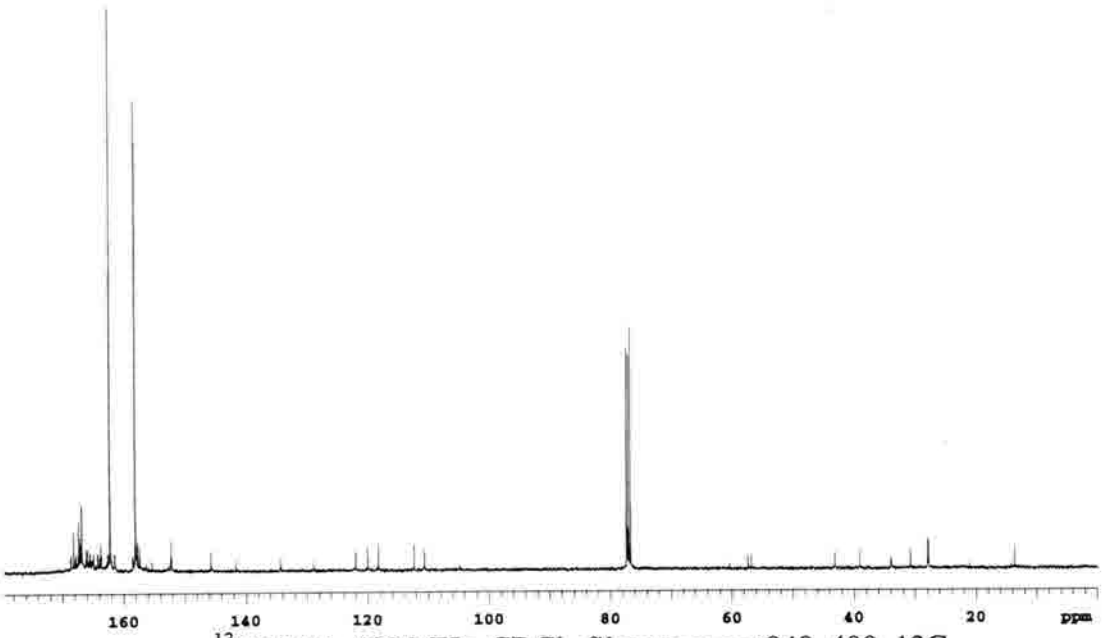
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.53 (6H, s), 2.17 (3H,s), 2.66 (2H, dd, *J*= 9.3, 9.3 Hz), 3.18 (1H, ddd, *J*= 2.3, 11.4, 13.7), 3.72 (1H, ddd, *J*= 1.3, 3.4, 14.5 Hz), 3.90 (2H, ddd, *J*= 1.1, 3.3, 14.4 Hz), 4.43 (1H, m), 5.14 (1H, dd, *J*= 0, 10.5 Hz), 5.15 (1H, dd, *J*= 0, 17.7 Hz), 5.55 (1H, d, *J*= 7.0 Hz), 6.13 (1H, dd, *J*= 10.5, 17.7 Hz), 7.08 (1H, ddd, *J*= 1.1, 7.3, 7.3 Hz), 7.14 (1H, ddd, *J*= 0.7, 7.3, 7.3 Hz), 7.29 (1H, d, *J* = 7.7 Hz), 7.52 (1H, d, *J*= 7.7 Hz), 8.04 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.7, 27.8, 27.9, 30.8, 33.9, 39.0, 43.1, 57.0 (d, $J_{C\text{-}C}$ = 52 Hz), 104.8, 110.7, 112.4, 118.3, 120.0, 122.0, 128.9, 134.3, 134.3, 141.5, 145.7, 158.1, 162.2. HRMS (FAB+): Calcd for $C_{20}{}^{13}C_2H_{26}N_3O_2$: 366.2092. Found 366.2091 (M+H).



240a



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems948_400

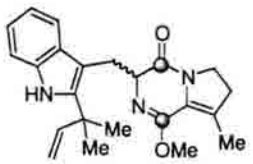


^{13}C NMR, 400 MHz, $CDCl_3$, filename: ems948_400_13C

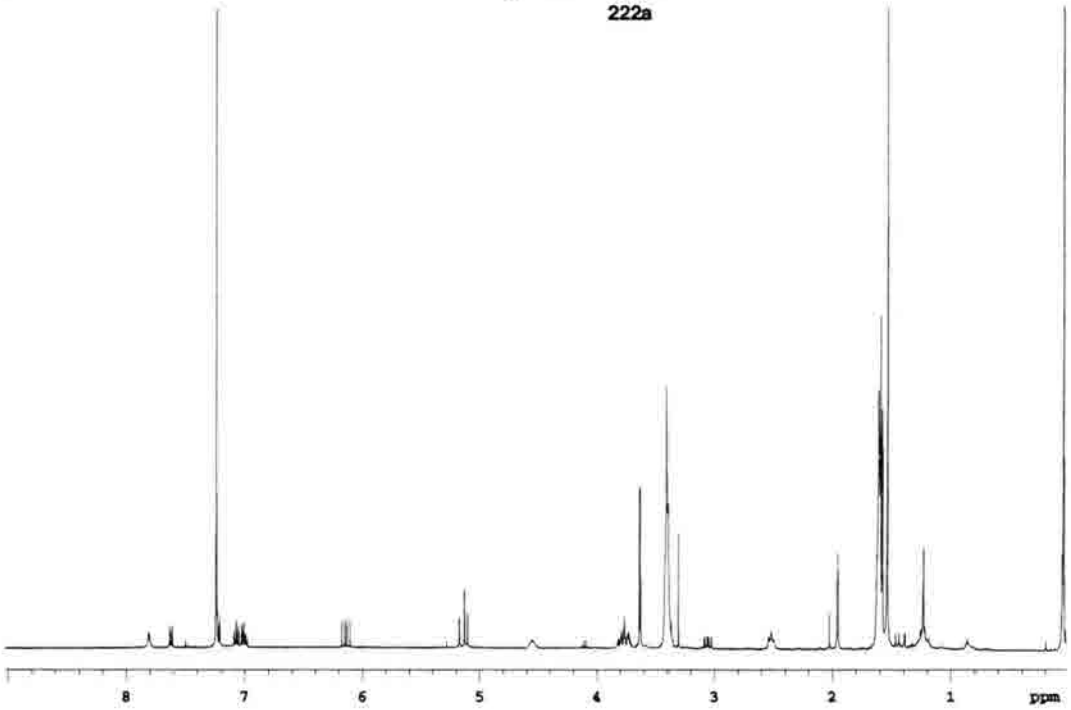
[$^{13}C_2$]-(3R,S)-3-[[2-(1,1-dimethyl-2-propenyl)-1H-indol-3-yl]methyl]-6,7-dihydro-1-methoxy-8-methyl-pyrrolo[1,2-a]pyrazine-4(3H)-one (222a):

Followed the procedure previously described for **222**. Yield: 48-71%.

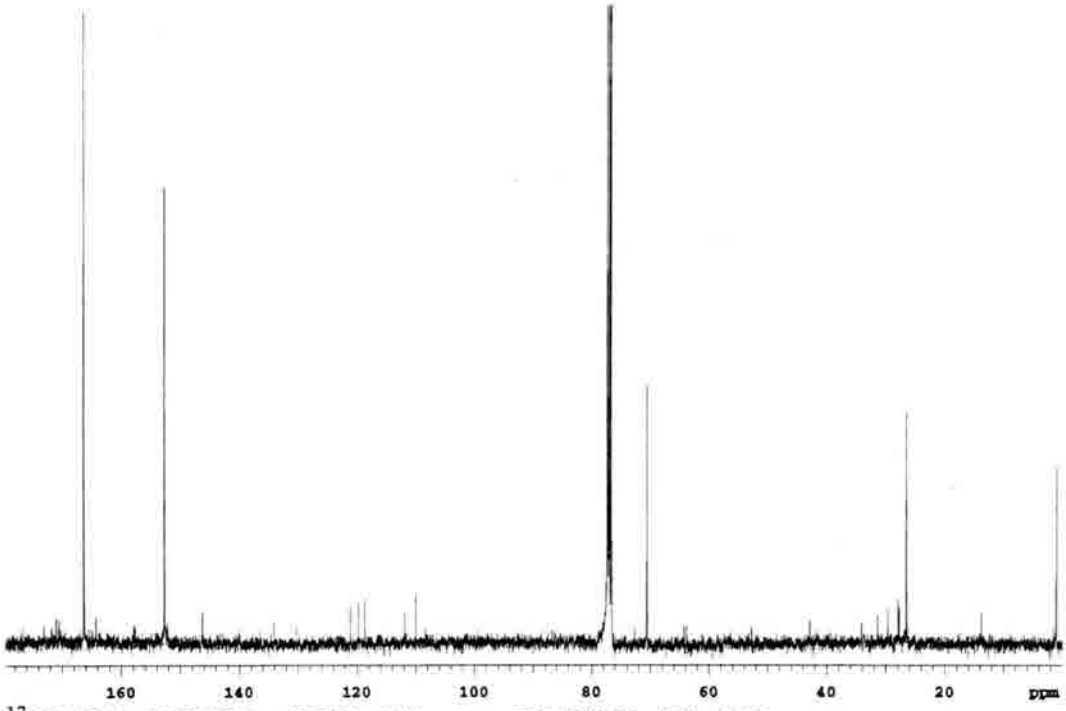
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.61 (3H, s), 1.62 (3H, s), 1.99 (3H, s), 2.55 (2H, m), 3.05 (1H, ddd, *J*= 1.7, 9.3, 14.4 Hz), 3.63 (3H, d, *J*= 3.8 Hz), 3.76 (3H, m), 4.55 (1H, d, *J*= 7.0 Hz), 5.12 (1H, dd, *J*= 1.2, 10.4 Hz), 5.15 (1H, dd, *J*= 1.1, 17.2 Hz), 6.14 (1H, dd, *J*= 10.5, 17.5 Hz), 7.01 (1H, ddd, *J*= 1.1, 8.1, 8.1 Hz), 7.22 (1H, ddd, *J*= 1.1, 7.0, 7.0 Hz), 7.22 (1H, d, *J*= 8.2 Hz), 7.62 (1H, d, *J*= 7.7 Hz), 7.81 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.7, 27.5, 27.9, 29.7, 31.4, 34.1, 42.8, 52.7, 64.0 (d, $J_{C\text{-}C}$ = 48 Hz), 108.1, 110.0, 111.9, 118.7, 119.8, 121.2, 122.8, 124.5, 130.4, 134.1, 140.1, 146.3, 152.7, 166.3. HRMS (FAB+): Calcd. for $C_{21}{}^{13}C_2H_{28}N_3O_2$: 380.2249. Found 380.2249 (M+H).



222a



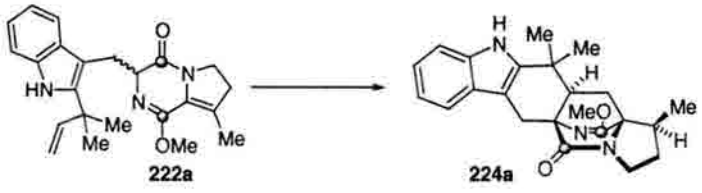
^{1}H NMR, 400 MHz, $CDCl_3$, Filename: EMS949_400



^{13}C NMR, 100 MHz, $CDCl_3$, Filename: EMS949_400_13C

[$^{13}C_2$]-2,3,11,12,12a,13-hexahydro-14-methoxy-1,12,12-trimethyl-5H,6H-5a,13a-(nitrilometheno)-1H-indolizinno[7,6-b]carbazol-5-one (474):

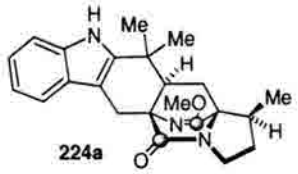
Followed same procedure as described for **224~227**. Yield: 77-88% combined yield for all 4 diastereomers.



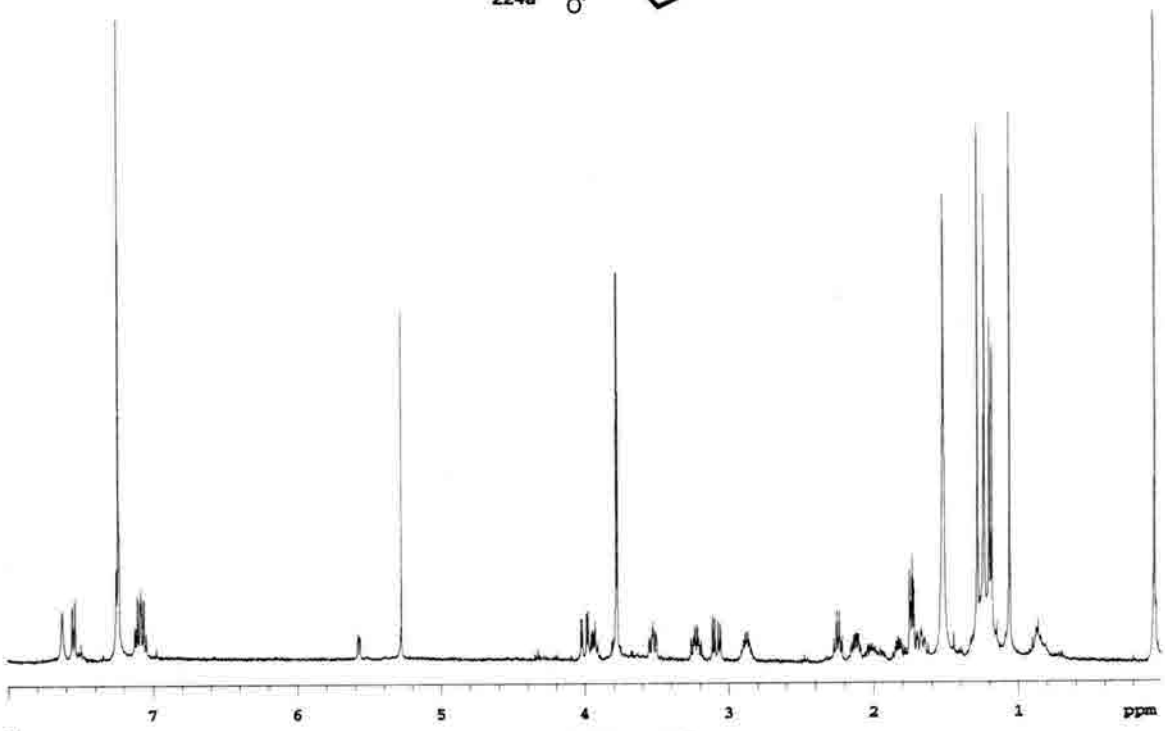
[$^{13}C_2$]-(1S,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-14-methoxy-1,12,12-trimethyl-5H,6H-5a,13a-(nitrilometheno)-1H-indolizino[7,6-b]carbazol-5-one (224a):

Yield: 30-45% from **222a**.

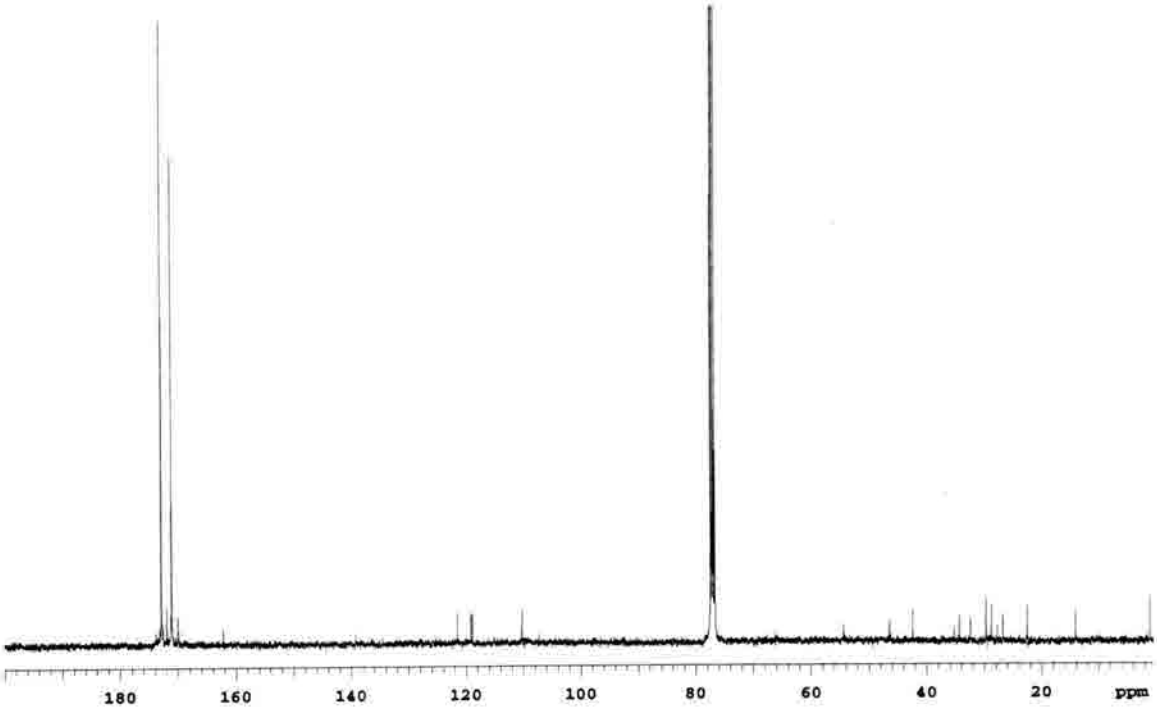
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.06 (3H, s), 1.19 (3H, d, *J*= 7.0 Hz), 1.28 (3H, s), 1.62-1.88 (3H, m), 2.12 (1H, m), 2.24 (1H, dd, *J*= 7.6, 15.2 Hz), 2.88 (1H, m), 3.09 (1H, dd, *J*= 6.2, 15.9 Hz), 3.23 (1H, ddd, *J*= 7.0, 10.6, 10.6 Hz), 3.53 (1H, m), 3.81 (3H, d, *J*= 3.5 Hz), 4.00 (1H, dd, *J*= 4.4, 16.0 Hz), 7.08 (2H, m), 7.25 (1H, d, *J*= 7.0 Hz), 7.55 (1H, d, *J*= 7.7 Hz), 7.63 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.0, 22.5, 26.7, 27.7, 28.7, 32.4, 34.3, 35.3, 42.4, 46.4, 54.4, 65.7 (d, $J_{C\text{-}C}$ = 53 Hz), 66.0 (d, $J_{C\text{-}C}$= 48 Hz), 107.3, 110.3, 118.8, 119.2, 121.5, 127.6, 139.3, 162.2, 171.1, 172.8. IR (HRMS (FAB+) Calcd for $C_{21}{}^{13}C_2H_{28}N_3O_2$: 380.2249. Found 380.2239 (M+H).



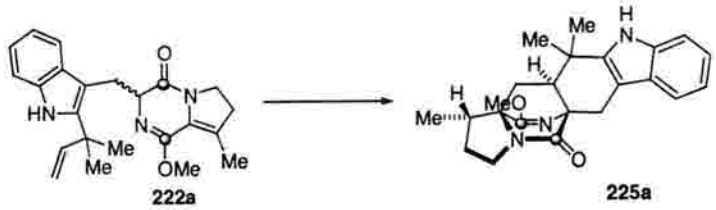
224a



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems966D_400



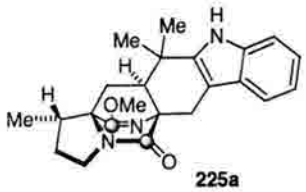
^{13}C NMR, 400 MHz, $CDCl_3$, filename: ems966D_400_13C



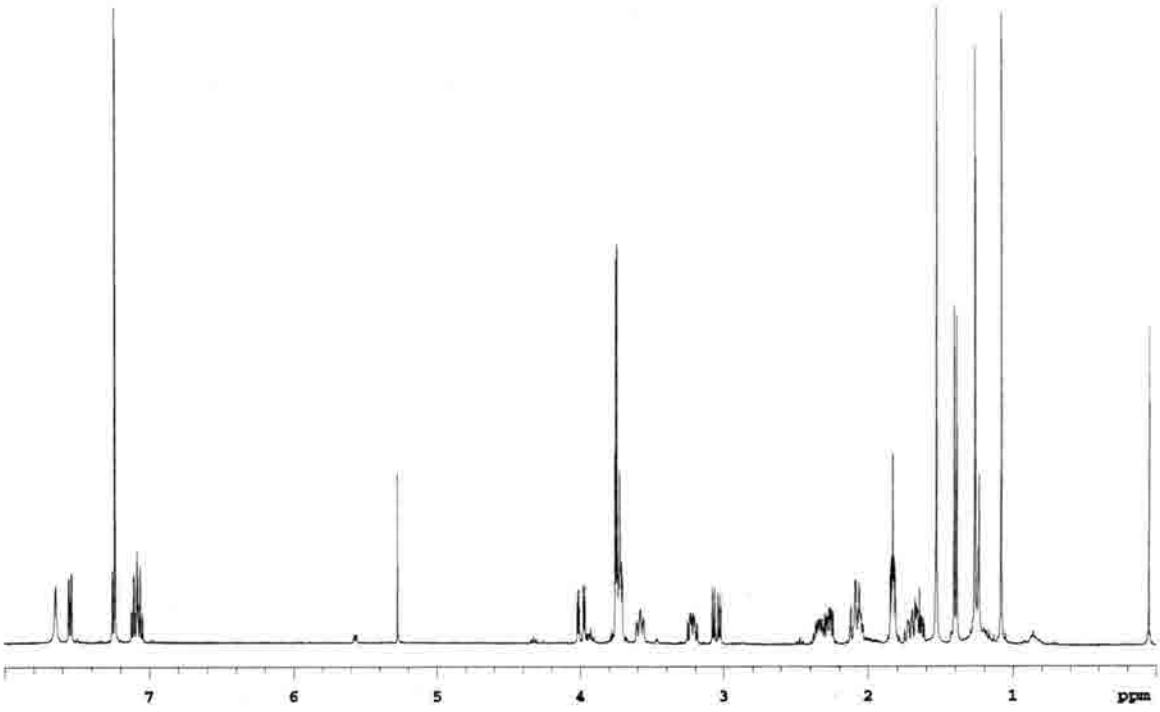
[13C$_2$]-(1R,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-14-methoxy-1,12,12-trimethyl-5H,6H-5a,13a-(nitrilometheno)-1H-indolizino[7,6-b]carbazol-5-one (225a):

Yield: 26-30% from **222a**.

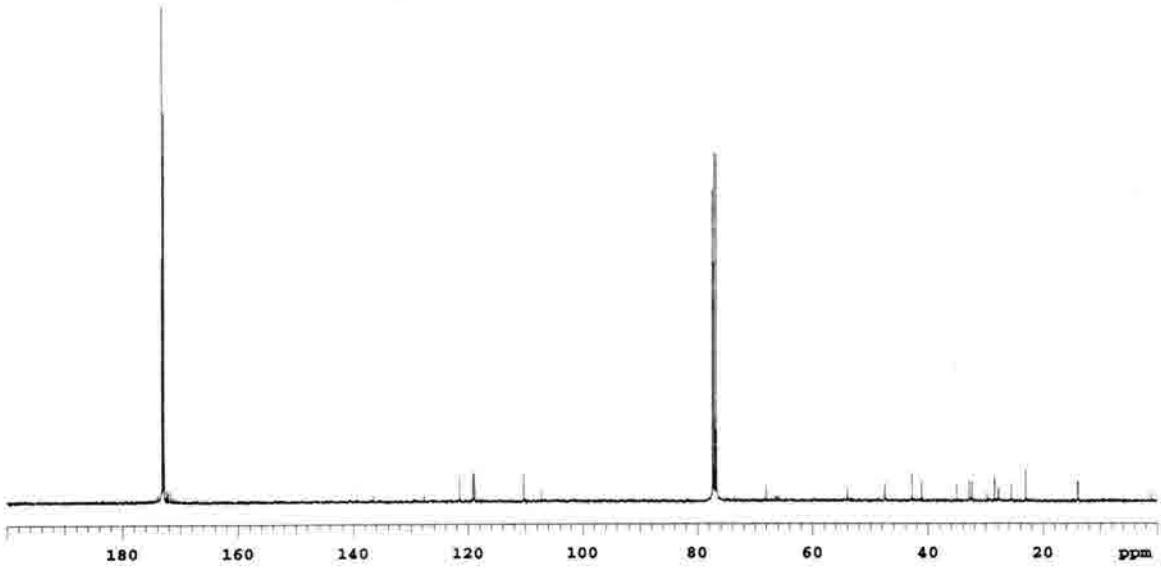
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.08 (3H, s), 1.26 (3H, s), 1.39 (3H, d, *J*= 7.0 Hz), 1.64 (1H, m), 1.87 (1H, m), 2.07 (1H, m), 2.09 (1H, dd, *J*= 11.1, 13.0 Hz), 2.29 (1H, m), 2.32 (1H, m), 3.04 (1H, dd, *J*= 6.4, 16.1 Hz); 3.22 (1H, m), 3.58 (1H, m), 3.65 (3H, d, *J*= 3.8 Hz), 4.06 (1H, dd, *J*= 4.6, 16.2 Hz), 7.08 (2H, m), 7.24 (1H, d, *J*= 7.7 Hz), 7.54 (1H, d, *J*= 7.7 Hz), 7.65 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.9, 23.0, 27.8, 28.4, 32.4, 32.9, 35.1, 41.1, 42.8, 47.5, 54.0, 65.8 (d, $J_{C\text{-}C}$ = 48 Hz), 66.2 (d, $J_{C\text{-}C}$= 51 Hz), 107.2, 110.3, 118.9, 119.2, 121.4, 127.7, 136.5, 139.4, 172.8, 173.1. HRMS (FAB+) Calcd for $C_{21}{}^{13}C_2H_{28}N_3O_2$: 380.2249. Found 378.2239 (M+H).



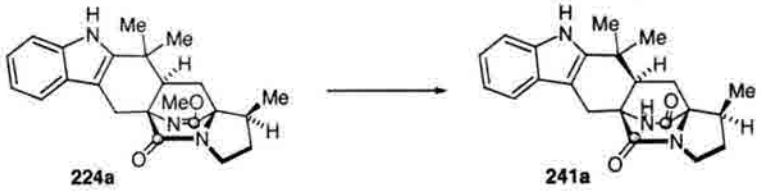
225a



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems966C_400

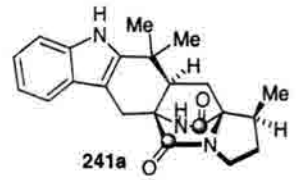


^{13}C NMR, 400 MHz, $CDCl_3$, filename: ems966C_400_13C

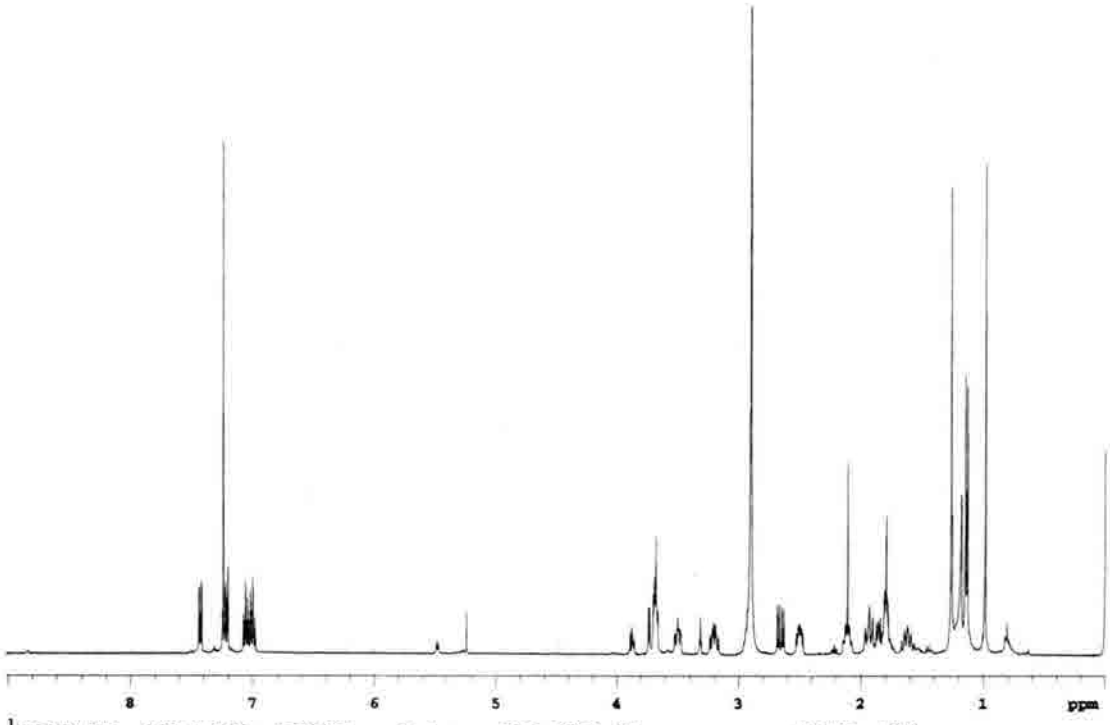


[$^{13}C_2$]-(1S,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazole-5,14-dione (241a) from 224a: Followed same procedure described for the synthesis of **447**. Yield: 87%

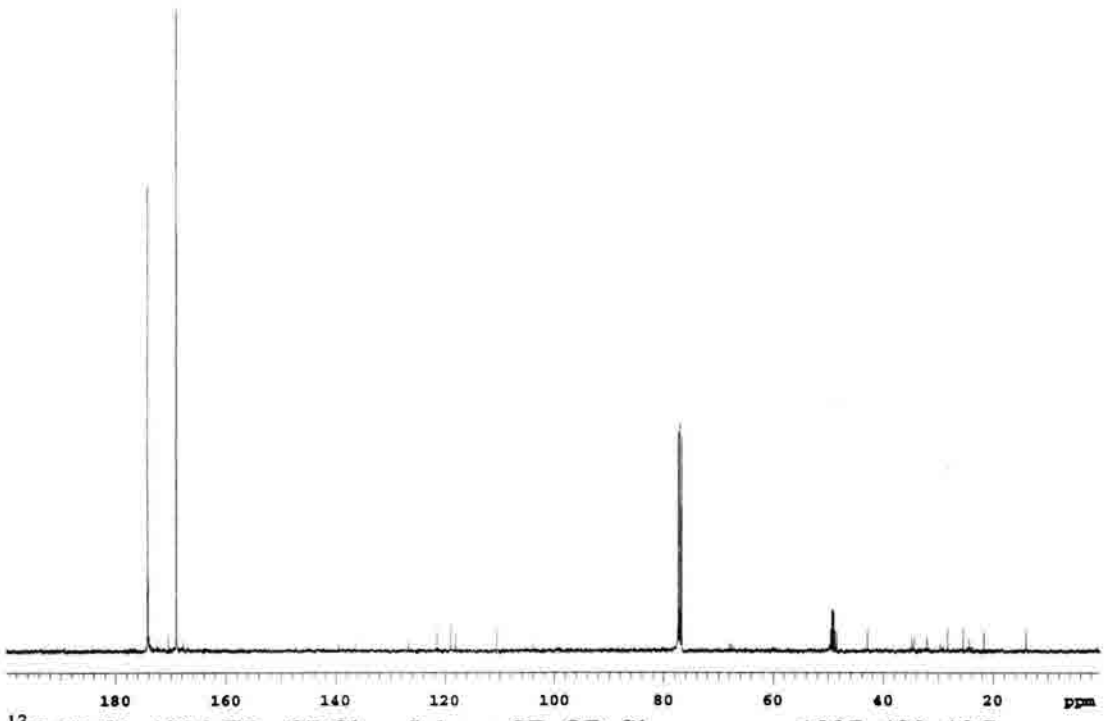
^{1}H NMR (400 MHz, $CDCl_3$ + 5 drops CD^3OD): δ 0.98 (3H, s), 1.14 (3H, d, *J*= 7.0 Hz), 1.26 (3H, s), 1.63 (1H, m), 1.86 (1H, dd, *J*= 5.0, 13.4 Hz), 1.93 (1H, m), 2.11 (1H, m), 2.50 (1H, m), 2.65 (1H, dd, *J*= 6.8, 15.4 Hz), 2.93 (1H, m), 3.20 (1H, m), 3.58 (1H, m), 3.72 (1H, dd, *J*= 4.6, 15.4 Hz) 7.00 (1H, ddd, *J*= 1.1, 7.7, 7.7 Hz), 7.06 (1H, ddd, *J*= 1.1, 7.7, 7.7 Hz), 7.21 (1H, dd, *J*= 0.8, 7.8 Hz), 7.43 (1H, d, *J*= 7.7 Hz). ^{13}C NMR (100 MHz, $CDCl_3$ + 5 drops CD^3OD) δ 13.9, 21.6, 24.4, 25.5, 28.3, 32.0, 34.3, 34.8, 42.8, 48.4, 59.9 (d, $J_{C\text{-}C}$= 45 Hz), 67.7 (d, $J_{C\text{-}C}$= 43 Hz), 103.9, 110.5, 118.1, 118.9, 121.4, 126.7, 136.4, 139.5, 169.0, 174.2. HRMS (FAB+) Calcd for $C_{20}{}^{13}C_2H_{25}N_3O_2$: 365.2014. Found 365.2014 (M+H).



241a



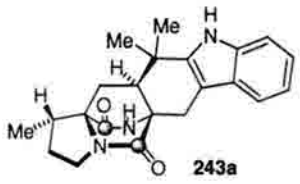
^{1}H NMR, 400 MHz, $CDCl_3$ + 5 drops CD_3OD, filename: ems1007_400



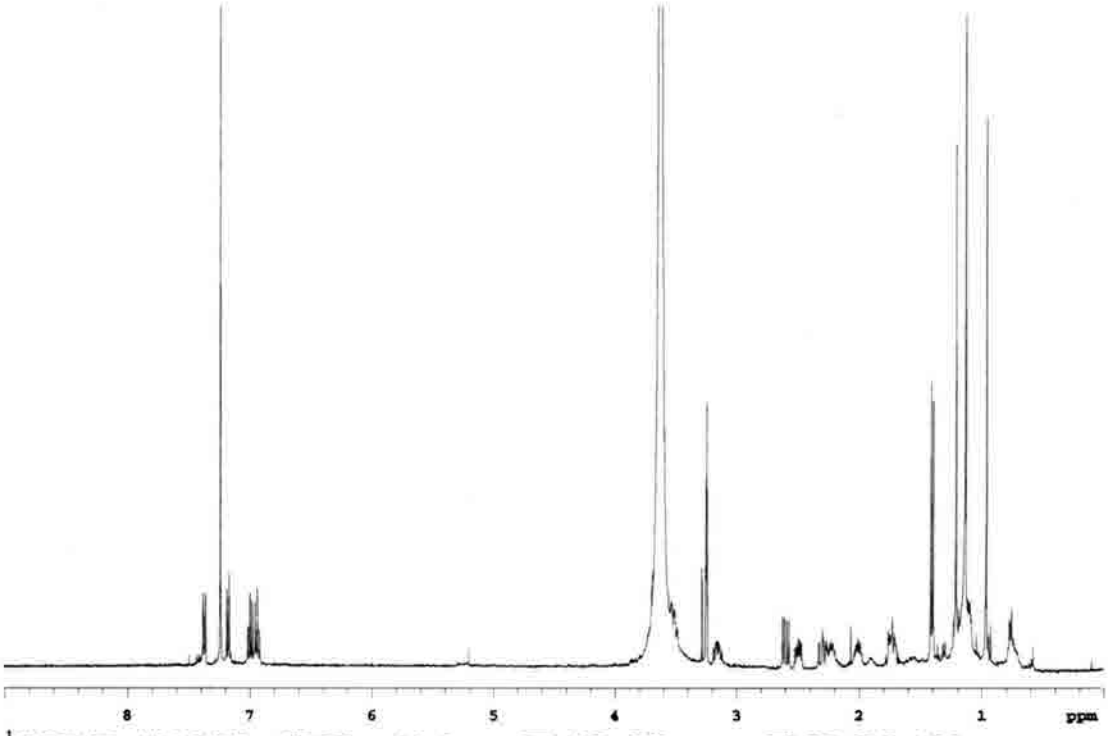
^{13}C NMR, 400 MHz, $CDCl_3$ + 5 drops CD_3OD, filename: ems1007_400_13C

[$^{13}C_2$]-(1R,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazole-5,14-dione (243a) from 225a: Followed same procedure described for the synthesis of **243**. Yield: 84%.

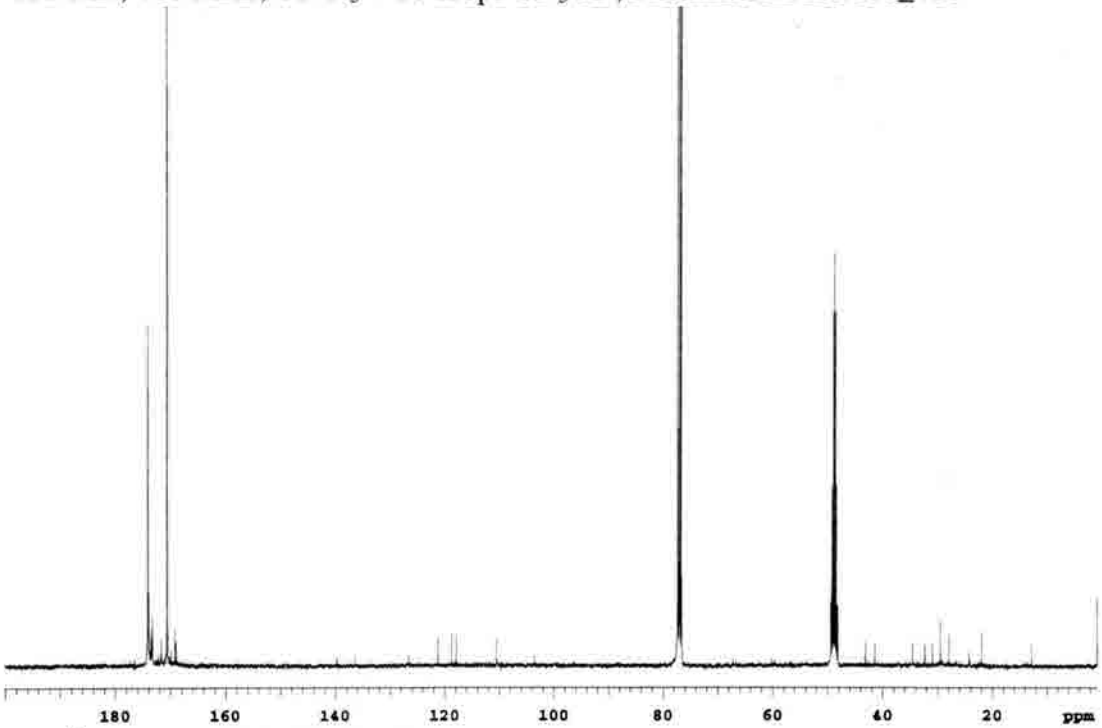
^{1}H NMR (400 MHz, $CDCl_3$): δ 0.97 (3H, s), 1.22 (3H, s). 1.41 (3H, d, *J*= 7.0 Hz), 1.74 (2H, m), 2.00 (1H, m), 2.23 (1H, m), 2.41 (1H, ddd, *J*= 2.0, 10.5, 13.5 Hz), 2.50 (1H, m), 2.60 (1H, dd, *J*= 6.9, 15.4 Hz), 3.25 (1H, m), 3.65 (1H, m), 3.83 (1H, d, *J*= 15.2 Hz), 6.94 (1H, ddd, *J*= 1.2, 7.8, 7.8 Hz), 7.00 (1H, ddd, *J*= 1.2, 7.0, 7.0 Hz), 7.18 (1H, d, *J*= 7.0 Hz), 7.38 (1H, d, *J*= 7.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$ + 10 drops CD_3OD): δ 12.8, 21.9, 24.2, 27.9, 29.5, 30.9, 32.3, 34.5, 41.4, 43.1, 59.9 (d, $J_{C\text{-}C}$= 51 Hz), 67.0 (d, $J_{C\text{-}C}$= 49 Hz), 103.6, 110.5, 117.8, 118.7, 121.2, 126.5, 136.5, 139.7, 170.6, 174.1. HRMS (FAB+) Calcd for $C_{20}{}^{13}C_2H_{26}N_3O_2$: 365.2014. Found 365.2019 (M+H).



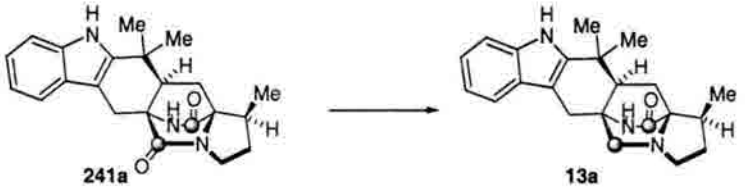
243a



^{1}H NMR, 400 MHz, $CDCl_3$ + 10 drops CD_3OD, Filename: EMS1008_400



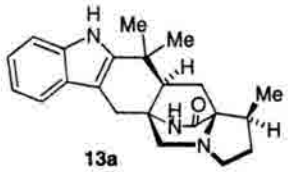
^{13}C NMR, 100 MHz, $CDCl_3$ + 10 drops CD_3OD, Filename: EMS1008_400_13C



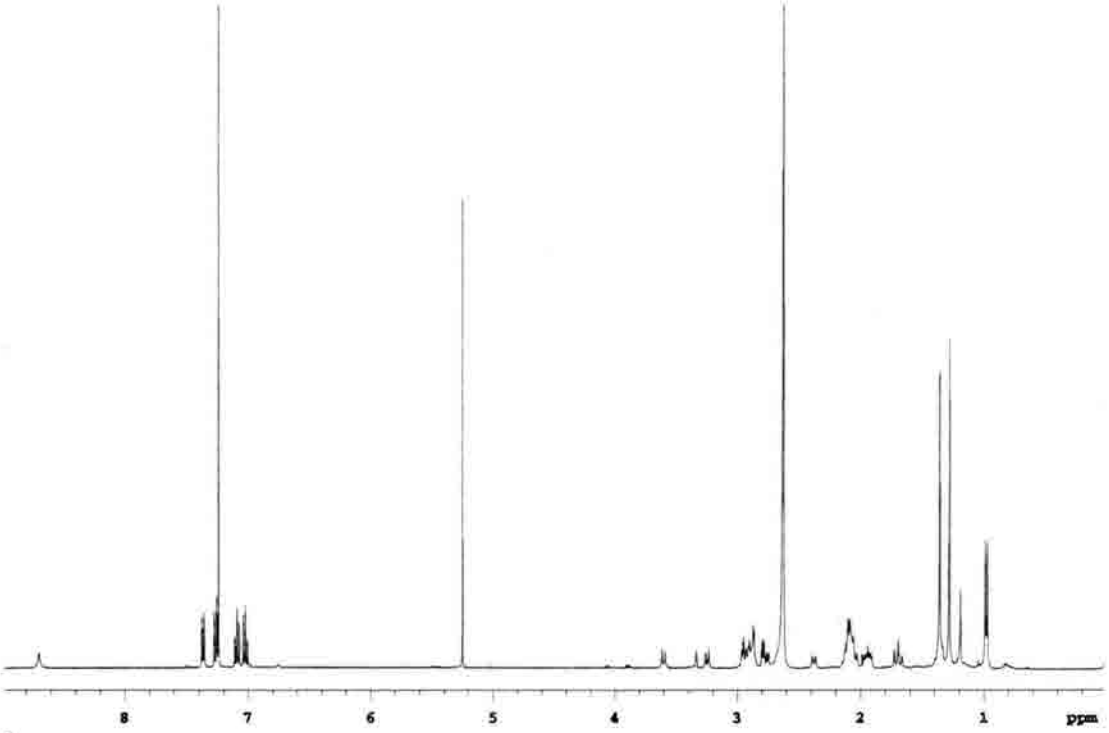
(±)-[12,18-$^{13}C_2$]-VM55599 (13a):

Followed same procedure described for the synthesis of **13**. Yield: 53-67%.

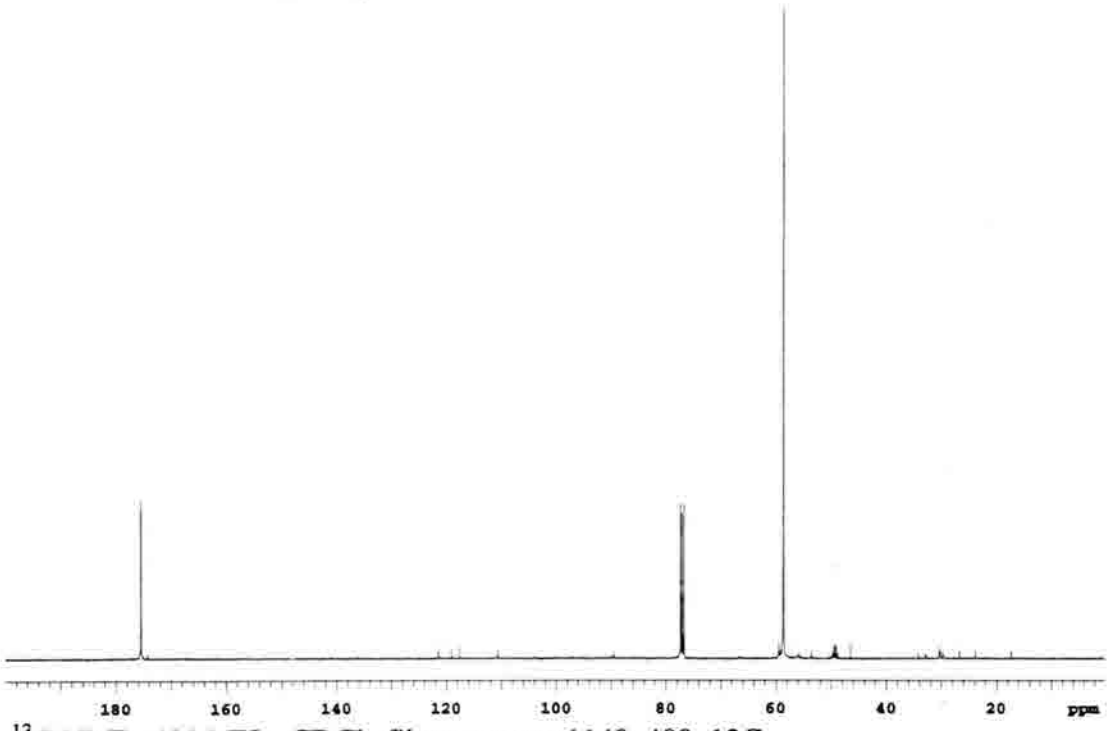
^{1}H NMR (400 MHz, $CDCl_3$ + 1 drop d_6-DMSO): δ 0.98 (3H, d, *J* = 7.0 Hz), 1.28 (3H, s), 1.34 (1H, m), 1.35 (3H, s), 1.69 (1H, dd, *J* = 12.4, 12.4 Hz), 1.94 (1H, m), 2.08 (3H, m), 2.64 (1H, m), 2.77 (1H, dd, *J*= 7.2, 15.2 Hz), 2.91 (3H, m), 3.60 (1H, dd, *J*= 10.1. 142.9 Hz), 6.28 (1H, bs), 7.01 (1H, ddd, *J*= 0.8, 7.8, 7.8 Hz), 7.08 (1H, ddd, *J*= 1.2, 7.0, 7.0 Hz), 7.26 (1H, d, *J*= 7.8 Hz), 7.36 (1H, d, *J*= 7.3 Hz), 8.69 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$ + 4 drops CD_3OD): δ 17.3, 23.8, 26.7, 29.7, 30.2, 30.4, 32.9, 34.2, 46.5, 53.6, 55.6 (d, $J_{C\text{-}C}$ = 34 Hz), 58.6, 66.3 (d, $J_{C\text{-}C}$ = 43 Hz), 103.9, 110.6, 117.6, 119.1, 121.5, 126.7, 136.3, 140.9, 175.5. HRMS (FAB+) Calcd for $C_{20}{}^{13}C_2H_{28}N_3O$: 352.2299. Found 352.2298 (M+H).



13a



^{1}H NMR, 400 MHz, $CDCl_3$, filename: ems1149_400



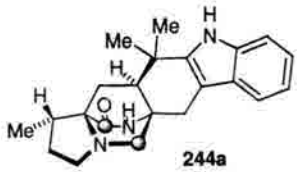
^{13}C NMR, 400 MHz, $CDCl_3$, filename: ems1149_400_13C

(1R,5aR,12aR,13aS)-rel-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5H,6H-5a,13a-(iminomethano)-1H-indolizino[7,6-b]carbazol-14-one (244a):

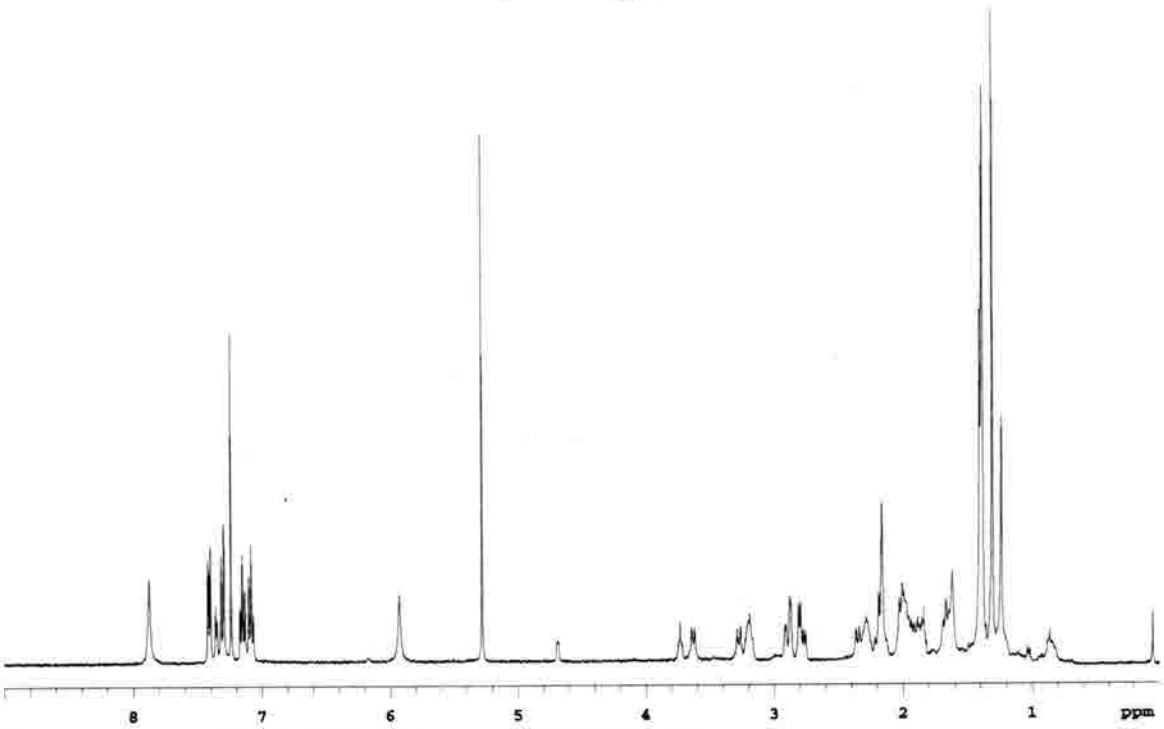
Followed the same procedure as described for the unlabeled compound **244**.

Yield: 53-68%

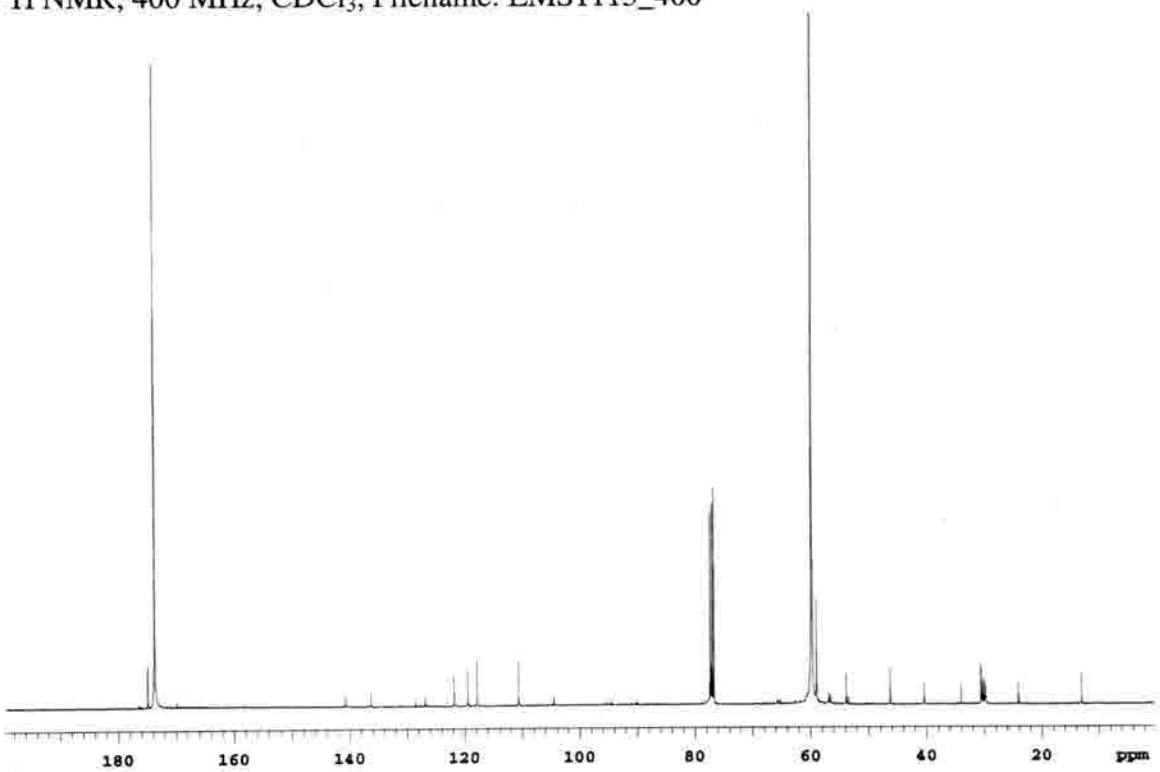
^{1}H NMR (400 MHz, $CDCl_3$): δ 1.30 (3H, s), 1.38 (3H, s), 1.40 (3H, d, J = 7.0 Hz), 1.65 (1H, m), 1.93 (3H, m), 2.19 (3H, m), 2.28 (1H, m), 2.78 (1H, dd, *J*= 6.5, 15.2 Hz), 2.89 (1H, dd, *J*= 3.8, 15.2 Hz), 3.19 (1H, m), 3.45 (1H, dd, *J*= 10.2, 142.9 Hz), 5.92 (1H, bs), 7.08 (1H, dd, *J*= 7.4, 7.4 Hz), 7.15 (1H, dd, *J*= 7.2, 7.2 Hz), 7.30 (1H, d, *J*= 7.8 Hz), 7.40 (1H, d, *J*= 7.2 Hz), 7.87 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.0, 24.0, 29.8, 30.1, 30.4, 30.5, 34.0, 40.4, 46.3, 53.8, 56.7(d, $J_{C\text{-}C}$= 34 Hz), 59.8, 65.4 (d, $J_{C\text{-}C}$= 46 Hz), 104.5, 110.6, 117.9, 119.5, 121.8, 126.9, 136.3, 140.8, 173.7. HRMS (FAB+) Calcd for $C_{20}{}^{13}C_2H_{28}N_3O$: 352.2299. Found 352.2299 (M+H).



244a



^{1}H NMR, 400 MHz, $CDCl_3$, Filename: EMS1113_400



^{13}C NMR, 100 MHz, $CDCl_3$, Filename: EMS1113_400_13C

7.3 General Biosynthetic Considerations

[1-^{13}C]-L-tryptophan, [*methyl*-^{13}C]-L-methionine and [1-^{13}C]-L-proline [1-^{13}C]-L-isoleucine (90% enrichment) were obtained from the NIH Stable Isotopes Resource at Los Alamos National Laboratory. [$^{13}C_2$]-Acetic acid, sodium salt, 99% atom % ^{13}C, and [U-$^{13}C_6$]-D-glucose 99% atom % ^{13}C were obtained from Aldrich Chemical Co. With the exception of *Penicillium sp.* IMI IMI332995, obtained from the International Mycological Institute, all fungal spores were purchased from the American Type Culture Collection and re-hydrated according to ATCC specifications.

The ^{13}C spectra of labeled and unlabeled samples obtained for each of the fungal metabolites were obtained under comparable conditions (*i.e.* sample concentration, number of scans, etc.). ^{13}C NMR spectra for paraherquamide A (**1**) taken in $CDCl_3$, austamide (**263**), taken in $CDCl_3$ (^{1}H, 7.24 ppm; ^{13}C 77 ppm) and brevianamide A (**15**), taken in CD_2Cl_2 (^{1}H, 5.32 ppm; ^{13}C 54 ppm) were obtained on an INOVA 400 Varian NMR with a dual full band console at the Chemistry Central Instrument Facility at Colorado State University. The pulse sequence consisted of a 1.3 second relaxation delay, a pulse angle of 45°, an acquisition time of 0.64 seconds and a spectral window of 25157 Hz. ^{13}C data was obtained with continuous WALTZ 16 composite pulse decoupling. The ^{13}C data was processed using –0.634 Hz sq. sine bell correction and -0.634 Hz sine bell shift with a digital resolution of 1.54 Hz/pt. The ^{13}C spectra were acquired until a satisfactory signal-to-noise ratio was obtained (*ca.* 16 h). The resulting ^{13}C spectra were thoroughly phased and the baseline carefully corrected to obtain a satisfactory, reproducible integral.

For the acetate and glucose feeding experiments, ^{13}C NMR spectra of paraherquamide A (**1**) were obtained on a Bruker AMX 500 MHz NMR at Los Alamos National Laboratory and taken in $CDCl_3$ (^1H, 7.24 ppm; ^{13}C 77 ppm). The pulse sequence included a 4.0 second relaxation delay, an acquisition time of 0.79 seconds and a spectral window of 41667 Hz. ^{13}C data was obtained with continuous WALTZ 16 composite pulse decoupling. The ^{13}C data was processed using 2 Hz exponential line broadening with a digital resolution of 2.54 Hz/pt.

The INADEQUATE spectrum for austamide and the HSQC experiment for paraherquamide A were obtained on an INOVA 400 Varian NMR at the Chemistry Central Instrument Facility at Colorado State University. All mass spectra were obtained on a Fisons VG Quattro SQ at the Chemistry Central Instrument Facility at Colorado State University. Samples were dissolved in 1:1 water/acetonitrile without pH adjustment and measured with positive ion electrospray (20 scans, 8 seconds/scan) with a cone voltage of 25 volts.

7.4 Biosynthesis Experimentals

7.4.1 Procedure for completing feeding experiments

Spores from the respective fungi suspended in sterile water or in a 15% aqueous glycerol solution were spread onto fungus specific sterile agar slants; 50 μL of the suspension was used per slant. The slants were placed in an incubator at 25 °C for 10-12 days. For the production of paraherquamide A (**1**), *Penicillium fellutanum*, ATTC: 20841, was initially grown on sterile malt extract agar slants (20g D-glucose, 20g malt extract, 1g peptone and 20 g agar per liter of distilled, de-ionized water). The austamide (**134**)

producing *Aspergillus ustus*, ATCC: 36063 was also grown on sterile malt extract agar slants. For the production of brevianamide A (**15**), *Penicillium brevicompactum*, ATCC: 9056 was grown on sterile Czapek Dox slants (3.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$ • 7 H_2O, 0.5 g KCl, 0.01 g $FeSO_4$ • 7 H_2O, 30 g D-glucose, 15.0 g agar per liter of distilled de-ionized water).

The spores from eight slants were shaken into four 6-liter flasks containing 600 mL of sterile glucose corn steep liquor (40g glucose and 22g corn steep liquor per one liter of distilled de-ionized water). The inoculated flasks were placed in an incubator at 25°C for 6 days. The glucose corn steep liquor was removed leaving a disk of the fungus. The undersides of the disks, the mycelia cells, were rinsed with 100 mL of sterile water.

Sterile trace element solution (35 mM $NaNO_3$, 5.7 mM K_2HPO_4, 4.2 mM $MgSO_4$, 1.3 mM KCl, 36 μM $FeSO_4$•$7H_2O$, 25 μM $MnSO_4$•H_2O, 7 μM $ZnSO_4$•$7H_2O$, 1.5 μM $CuCl_2$•$2H_2O$) containing the labeled precursor was placed into each of two flasks (see table 11 for the volume and molarity of each solution). Two control flasks were also set up, each containing sterile trace element solution. The flasks were put into the incubator at 25 °C for 10 days and swirled daily to ensure even distribution of the isotopically labeled precursor.

A detergent had to be used to dissolve the water-insoluble proposed precursors. The water-insoluble compounds were dissolved in a 10% solution of absolute ethanol/ chloroform. TWEEN 80 (0.2 ml) was added to the dissolved precursor. The samples were then sonicated at 40°C under a sterile stream of argon until the solvent was completely removed. Sterile trace element solution was added to the each precursor/ TWEEN 80 residue and the mixture was sonicated for ~15 minutes to aid in micelle formation.

Table 11:

Proposed Precursor	Mmol precursor	Molarity (M)	Volume (mL)	Mmol **1**Produced
77	0.152	7.60×10^{-4}	100	0.0405
76	0.161	8.04×10^{-4}	100	0.0486
79a	0.155	7.75×10^{-4}	100	0.0527
101	0.043	4.30×10^{-4}	50	0.0420
Control	---	---	100	0.0222
79a	0.157	7.85×10^{-4}	100	0.0283
Control	---	---	100	0.0193
79b	0.120	5.32×10^{-4}	112.5	0.0166
Control	---	---	112.5	0.03529
99	0.081	5.45×10^{-4}	75	0.0194
100	0.120	6.00×10^{-4}	100	0.0170
Control	---	---	100	0.0182
99	0.028	7.85×10^{-4}	50	0.0231
100	0.0914	7.85×10^{-4}	75	0.0141
Control	---	--	75	0.0277
78	0.149	7.45×10^{-4}	100	0.0109
Control	---	---	100	0.0182
89c	0.169	8.45×10^{-4}	100	0.0405
Control	---	---	100	0.0101
158a	0.042	2.12×10^{-4}	100	0.0284
159a	0.059	2.97×10^{-4}	100	0.0347
Control	---	---	100	0.0217
160a	0.098	9.80×10^{-4}	100	0.0263
161a	0.040	2.00 x 10-4	100	0.0103
162a	0.031	1.56 x 10-4	100	0.0146
Control	---	---	100	0.0123
13a	0.0464	2.32×10^{-4}	100	0.0394
241a	0.0621	3.11×10^{-4}	100	0.0284
244a	0.0546	2.73×10^{-4}	100	0.0260
243a	0.0629	3.15×10^{-4}	100	0.0334
Control	---	---	---	0.02170

The aqueous media was decanted off and stored at 4°C with 1-2 mL of chloroform. The mycelia cells from each flask were harvested, combined with the cells

from the duplicate experiment and pulverized with 500 mL of methanol in an Oster blender. The methanol suspensions of mycelia cells were placed in a shaker for at room temperature for 24 hours. Ten grams of Celite was added to each suspension before filtering through Whatman #2 paper. The filtrate was stored at 4 °C. The residual mycelia and Celite were re-suspended in methanol, placed in the shaker for an additional 42 hours and re-filtered.

The methanol solutions from both filtrations were combined and evaporated *in vacuo*. For paraherquamide A, the aqueous solution from each feeding experiment was added to the corresponding methanolic residue and the mixture was acidified to pH 4 with 12 mL of glacial acetic acid. The acidic solution was extracted 4 times with 150 mL portions of ethyl acetate. The organic layer was discarded. The aqueous layer was brought to pH 9-10 by the addition of 50 mL of 5M NaOH. The aqueous layer was then extracted 4 times with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous sodium carbonate and evaporated to dryness. For brevianamide A and austamide, the aqueous solution from each feeding experiment was added to the corresponding methanolic residue, taken a pH of 7-8 and extracted 4 times with ethyl acetate. The combined organic layers were washed successively with 10% aqueous Na_2CO_3 solution and brine, then dried over anhydrous sodium sulfate and evaporated to dryness. The fungal metabolites were purified *via* radial chromatography and thin layer chromatography on silica gel using a gradient elution of 4-10% methanol in methylene chloride. This was followed by preparative TLC on silica gel pre-coated plates with methylene chloride-methanol (10:1 for paraherquamide A (**1**); 25:1 for austamide (**134**)) or methylene chloride-acetone mixtures (2:1 for brevianamide A (**15**)).

7.4.2 Calculation of the percentage of incorporation by ^{13}C-NMR

Gated-decoupled experiments were precluded by the small amounts of these metabolites and the intrinsic lower sensitivity of this kind of experiment; for that reason, standard ^{13}C spectra were measured for both the metabolite from control experiments and from feeding experiments. The relative abundance of ^{13}C in each carbon of paraherquamide A (**1**), brevianamide A (**15**), and austamide (**134**) resulting from feeding experiments was determined through comparison of the integration for that peak with the total integration in the standard ^{13}C spectrum.

First, the ratio of the integral of the labeled peak to the added integrals of the rest of the peaks was obtained. Then, the ratio of the integral of the corresponding peak in the unlabeled spectrum to the added integrals in the rest of the unlabeled spectrum was found. The total percentage of ^{13}C at the labeled position was found by dividing the ratio of integrals obtained from the labeled paraherquamide A by the ratio from the unlabeled paraherquamide A and multiplying by 1.1%, the natural abundance of ^{13}C. The percentage of ^{13}C enrichment at the labeled position was obtained by subtracting 1.1% from the total percentage of ^{13}C. The percentage of precursor incorporated was determined by multiplying the percentage of ^{13}C enrichment by the mmol of paraherquamide A produced and then dividing this number by the mmol of isotopically enriched precursor.

For the feeding experiments with [1,2-^{13}C]-acetate and [U-$^{13}C_6$]-glucose, multiply labeled metabolites were obtained. For these instances, when the relative integrations of the ^{13}C-spectrum from the isotopically enriched metabolite was compared to the relative integration for each carbon signal in compounds from control experiments, it was

possible to calculate the relative abundance of ^{13}C for each position with respect to the rest of carbon signals. The total abundance of ^{13}C in each metabolite was then determined by calculating the increase in intensity for the peaks corresponding to the isotopomers with one, two, three, and four ^{13}C atoms in the mass spectra for each of the metabolites resulting from feeding experiments, relative to the intensity of the peak that corresponds to molecules with ^{12}C atoms only. The ^{13}C abundance for each position was finally calculated taking into account the relative abundance of ^{13}C (from the ^{13}C NMR spectrum) and the average ^{13}C abundance for the metabolite (from the MS) isolated from the feeding experiment.

7.4.3 Calculation of the percentage of incorporation from the mass spectra

The percentage of ^{13}C-enrichment in the fungal metabolites from isotopically labeled biosynthetic precursors was calculated according to the method Lambert et al.[222] These calculations are based on the comparison of the mass spectrum of the labeled material to the mass spectrum of the unlabelled material. For these experiments, electrospray mass spectroscopy was used, thus the base peak in the mass spectrum was the M+H peak. The percentage of precursor incorporated was determined by multiplying the percentage of ^{13}C-enrichment by the mmol of paraherquamide A produced and then dividing this number by the mmol of isotopically enriched precursor.

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[210] *Stereocontrolled total synthesis of (+/-)-gelsemine* Fukuyama, T.; Liu, G., *Pure & Appl. Chem.*, **1997**, *69*, 501-505.

[211] Conditions for the conversion of compounds **13-15** to the VM55599 diasteromers **19**, **21** and **24** were not optimized.

[212] Special thanks to R.W. Woody of the Department of Biochemistry at Colorado State University for the use of his spectrophotometer and helpful discussions.

[213] The brominated analog was synthesized by Professor Juan Sanz-Cervera.

[214] *Assymetric Dihydroxylations of β-Substituted N-(α,β-enoyl)-borane-10,2-Sultams* Oppolzer, W.; Barras, J.-P., *Helv. Chim Acta* **1987**, *70*, 1666.

[215] *A Productive Method for the Preparaton of Geminal Chloronitroso Compounds* Diekmann, H.; Luttke, W., *Angew. Chem. Int. Ed.*, **1968**, *7*, 387-388.

[216] A small amount of *allo*-L-isoleucine was detected by ^{1}H nmr.

[217] Variable temperature ^{1}H and ^{13}C nmr data is reported for unlabeled compound. ^{13}C-labeled compound was identical by TLC mobility and ^{1}H and ^{13}C nmr at 22°C.

[218] The two rotamers can actually be separated by successive elution of 1:1 hexanes/ ethyl acetate by silica gel PTLC. The two compounds were found to be rotamers and not diastereomers since, each rotamer was converted to compound **151** by treatment with dimethylamine. The product (**151**) from each rotamer displayed identical TLC mobility, ^{1}H NMR and ^{13}C NMR spectra.

[219] The relative stereochemistry of this compound was determined by extensive ^{1}H nmr nOe studies.

[220] The observed peaks were still broad at 120°C in d_6-DMSO.

[221] Some peaks reported in duplicate because coalescence of rotamers was incomplete. Relegated to VT NMR at 120°C.

[222] J.B. Lambert, H.B. Shurvell, D.A. Lightner, R.G. Cooks, *Organic Structural Spectroscopy*, Prentice Hall, New Jersey, **1998**, pp. 447-448.

Appendix 1. Publications

Studies on the Biosynthesis of Paraherquamide A. Origin of the β-Methylproline Ring

Emily M. Stocking, Juan F. Sanz-Cervera,† and Robert M. Williams*

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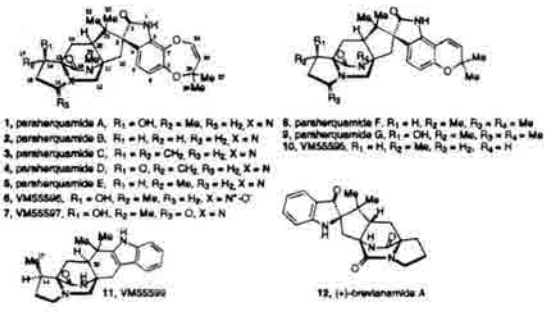
The paraherquamides are potent anthelmintic alkaloids isolated from various *Penicillium* sp.[1] These substances have attracted considerable attention due to their molecular complexity, intriguing biogenesis, and potential as antiparasitic drugs.[2] The most potent member of this family is paraherquamide A (**1**), which contains the unusual amino acid, β-methyl-β-hydroxyproline. The paraherquamides differ with respect to substitution and oxygenation in the proline ring and the prenylated oxindole ring; paraherquamide B (**2**) is the simplest member of the paraherquamide family, being comprised of the amino acids proline, tryptophan, and two isoprene units. Other members of this class include paraherquamides C-G, VM55596, VM55597, and VM55595. Recently, a Smith-Kline Beecham group reported the isolation and structural elucidation of the simpler indole alkaloid VM55599 (**11**) which, like compounds **5**−**10**, contains the unusual amino acid β-methylproline. As part of a program directed primarily at elucidating the biosynthetic mechanism of formation of the unique core bicyclo[2.2.2] ring system that is common to all of these alkaloids,[3] we have initiated studies on the biosynthesis of the unusual, functionalized proline derivatives that constitute the paraherquamide family. Herein, we report the biosynthetic incorporation of primary amino acid building blocks that constitute the core framework of this class of alkaloids.



Birch and associates carried out preliminary studies on the biosynthesis of the structurally related alkaloid brevianamide A (**12**), a substance that contains the core bicyclo[2.2.2] ring system.[4] These workers found that [15-^{3}H, 8-^{14}C]*cyclo*-L-tryptophan-L-proline (**13**), [3-^{14}C]-L-tryptophan, and [5-^{3}H]-L-proline were biosynthetically incorporated into brevianamide A in significant radiochemical yield. From these studies it seemed plausible that the monoketopiperazine ring system in paraherquamide A (**1**) might arise from tryptophan and proline. For paraherquamide A, C-14 methylation might arise via methyl transfer from *S*-adenosylmethionine to a 2,3-dehydroproline derivative followed by reduction. Close examination of the absolute stereochemistry of paraherquamide A, which possesses the (*S*)-absolute stereochemistry at C-14, led us to speculate that the methylated proline may instead be derived from L-isoleucine, and this possibility was experimentally tested.

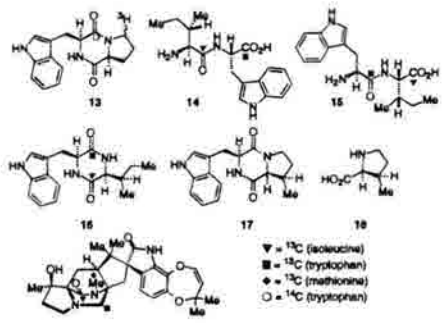


Figure 1.

To determine the primary metabolic building blocks that comprise the bicyclo[2.2.2] monoketopiperazine ring system of paraherquamide A, feeding experiments were performed on *Penicillium fellutanum* (ATCC: 20841) using [1-^{13}C]-L-tryptophan, [*methyl*-^{13}C]-L-methionine, and [1-^{13}C]-L-isoleucine (Figure 1). The position of ^{13}C incorporation in paraherquamide A was determined using ^{13}C NMR, and the percentage of the labeled amino acid incorporated was determined using ^{13}C NMR.[5] The [1-^{13}C]-L-tryptophan was incorporated, as expected (2.5%), with the label at C-12. The [methyl-^{13}C]methionine was not incorporated in the β-methylproline ring, but rather, only at C-29, the *N*-methyl position of the monoketopiperazine ring (0.6%). Feeding of [1-^{13}C]-L-isoleucine to *P. fellutanum* (ATCC: 20841), followed by harvesting the cells and isolation of paraherquamide A, revealed that the labeled L-isoleucine was incorporated into the monoketopiperazine ring system in high isotopic yield (3.3−3.7%) with the label at C-18. After determining the primary amino acid building blocks of the

† On leave from the Department of Organic Chemistry of the University of Valencia, Spain.

(1) (a) Yamazaki, M.; Okuyama, E. *Tetrahedron Lett.* **1981**, *22*, 135. (b) Ondeyka, J. G.; Goegelman, R. T.; Schaeffer, J. M.; Kelemen, L; Zitano, L. *J. Antibiot.* **1990**, *43*, 1375. (c) Liesch, J. M.; Wichmann, C. F. *J. Antibiot.* **1990**, *43*, 1380. (d) S. E.; Banks, R. M.; Everett, J. R.; Manger, B. R.; Reading, C. *J. Antibiot.* **1991**, *44*, 492. (e) Blanchflower, S. E.; Banks, R. M.; Everett, J. R.; Reading, C. *J. Antibiotics* **1993**, *46*, 1355.

(2) (a) Blizzard, T. A.; Mrozik, H.; Fisher, M. H.; Schaeffer, S. M. *J. Org. Chem.* **1990**, *55*, 2256. (b) Blizzard, T. A.; Marino, G.; Mrozik, H.; Fisher, M. H.; Hoogsteen, K.; Springer, J. P. *J. Org. Chem.* **1989**, *54*, 2657. (c) Blizzard,T. A.; Margiatto, G.; Mrozik, H.; Schaeffer, J. M.; Fisher, M. H. *Tetrahedron Lett.* **1991**, *32*, 2437. (d) Blizzard, T. A.; Margiatto, G.; Mrozik, H.; Schaeffer, J. M.; Fisher, M. H. *Tetrahedron Lett.* **1991**, *32*, 2441. (e) Blizzard, T. A.; Rosegay, A.; Mrozik, H.; Fisher, M. H. *J. Labelled Compd. Radiopharm.* **1989**, *28*, 461. (f) Shoop, W. L.; Egerton, J. R.; Eary, C. H.; Suhayda, D. *J. Parasitol.* **1990**, *76*, 349. (g) Shoop, W. L.; Michael, B. F.; Haines, H. W.; Eary, C. H. *Vet. Parasitol.* **1992**, *43*, 259. (h) Shoop, W. L.; Haines, H. W.; Eary,C. H.; Michael, B. F. *Am. J. Vet. Res.* **1992**, *53*, 2032. (i) Ostlind, D. A.; Mickle, W. G.; Ewanciw, D. V.; Andriuli, F. J.; Campbell, W. C.; Hernandez, S.; Mochales, S.; Munguira, E. *Res. Vet. Sci.* **1990**, *48*, 260. (j) Schaeffer, J. M.; Blizzard, T. A.; Ondeyka, J.; Goegelman, R.; Sinclair, P. J.; Mrozik, H. *Biochem. Pharmacol.* **1992**, *43*, 679.

(3) (a) Williams, R. M.; Kwast, E.; Coffman, H,; Glinka, T. *J. Am. Chem. Soc.* **1989**, *111*, 3064. (b) Sanz-Cervera, J. F.; Glinka, T.; Williams, R. M. *J. Am. Chem. Soc.* **1993**, *115*, 347. (c) Sanz-Cervera, J. F.; Glinka, T.; Williams, R. M. *Tetrahedron* **1993**, *49*, 8471.

(4) (a) Baldas, J.; Birch, A. J.; Russell, R. A. *J. Chem. Soc. Perkin Trans. 1*, **1974**, 50. (b) Birch, A. J.; Wright, J. J. *Tetrahedron* **1970**, *26*, 2329. (c) Birch, A. J.; Wright, J. J. *Tetrahedron* **1972**, *28*, 2999.

(5) For a leading reference on the method employed, see: Leete, E.; Bodem, G. B. *J. Am. Chem. Soc.* **1976**, *98*, 6321. These values were further corroborated by electrospray mass spectroscopy (see supporting information).

S0002-7863(96)01322-4 CCC: $12.00

paraherquamide A ring system, we attempted to further establish the structure of possible isoleucine/tryptophan conjugates on this pathway.

We have previously proposed a common biosynthetic route to the brevianamides and paraherquamides involving prenylation of *cyclo*-L-tryptophan-L-proline, oxidation of the indole followed by an oxidative [4 + 2] cycloaddition to provide the core bicyclo[2.2.2] ring system.[3] Since L-isoleucine forms the β-methylproline ring of paraherquamide A, *cyclo*-L-Trp-L-β-methylproline (**17**) or *cyclo*-L-Trp-L-Ile (**16**) are plausible precursors. There are numerous possible sequences of events that might occur in the formation of the final β-methylproline ring system. Formation of the dipeptides NH_2-L-Ile-L-Trp-COOH (**14**) or NH_2-L-Trp-L-Ile-COOH (**15**) and dehydration to *cyclo*-L-Trp-L-Ile (**16**) followed by oxidation of the terminal carbon of L-Ile and cyclization to form the β-methylproline moiety would result in *cyclo*-L-Trp-L-β-methylproline (**17**). Another possibility involves oxidation of the L-Ile followed by cyclization and reduction to afford the L-β-methylproline (**18**) followed by coupling to L-Trp to give *cyclo*-L-Trp-L-β-methylproline (**17**). Many other possibilities exist that would involve formation of the β-methylproline ring at a later stage.

We have investigated the simplest of these possibilities: doubly labeled NH_2-[1-^{13}C]-L-Ile-[1-^{13}C]-L-Trp-COOH (**14**); NH_2-[1-^{13}C]-L-Trp-[1-^{13}C]-L-Ile-COOH (**15**), and [2,5-$^{13}C_2$]-*cyclo*-L-Trp-L-Ile (**16**) were synthesized and fed to *P. fellutanum*. After the cells were grown and harvested as described above, 1.2−1.8% incorporation at C-18 and 0.4−0.9% incorporation at C-12 were evidenced by ^{13}C NMR. The ^{13}C NMR spectra of the paraherquamide A so produced did not provide compelling evidence for site-specific incorporation of both labels from the intact dipeptides.[5] This low level of incorporation is more consistent with dipeptide hydrolysis, reincorporation of the individual amino acids presumably coupled with additional metabolic degradation, and reconstitution of ^{13}C-enriched building blocks. Moreover, the mass spectra of the paraherquamide A isolated from these feeding experiments did not show the isotopic peak pattern expected from incorporation of the intact doubly labeled metabolites. Rather, the M + 2 peaks (via electrospray as protonated M + 1 molecular ions) were more intense than the M + 3 peaks (protonated M + 2 molecular ions), thus confirming that the double label had not been incorporated.

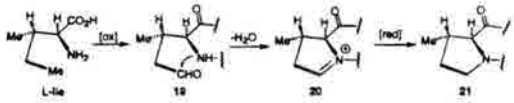


Figure 2.

Oxidative cyclization of the nitrogen atom onto the C-5-methyl group of isoleucine appears to be a unique biosynthetic transformation.[6,7] A reasonable pathway, depicted in Figure 2, would involve four-electron oxidation of the distal side chain methyl group to aldehyde **19** followed by cyclization and loss of water to produce iminium **20**; subsequent reduction (or in the case of VM55597, oxidation) of **20** furnishes the β-methylproline derivative **21**.

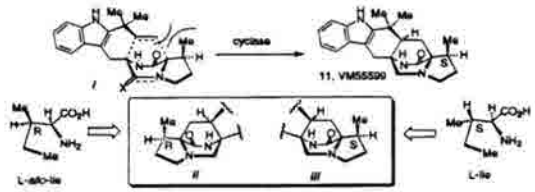


Figure 3.

Another interesting implication of the [1-^{13}C]-L-isoleucine incorporation into paraherquamide A involves the stereochemistry of the related metabolite VM55599 (**11**) isolated from the paraherquamide-producing mold *Penicillium* sp. IMI332995. Since paraherquamide A and VM55599 both possess the bicyclo[2.2.2] monoketopiperazine ring system, it seems plausible that these substances arise via a related or common [4 + 2] cycloaddition.[3] The relative stereochemistry of VM55599 was assigned[1e] by extensive ^{1}H NMR nOe studies, and, using the assumption that the absolute stereochemistry at C-20 is *S* (the same as that found in the paraherquamides), the stereochemistry at C-14 was assigned as *R*, which is the *opposite* to that found in paraherquamides A (**1**), E (**5**), F (**8**), G (**9**), VM55596 (**6**), VM55597 (**7**), and VM55595 (**10**). On the bais of the findings reported here, the side chain stereochemistry of L-isoleucine is preserved in the biosynthesis of paraherquamide A with hydroxylation at C-14 proceeding with net *retention* of configuration. If L-isoleucine is also the precursor to the β-methylproline ring of VM55599, it must follow that the bicyclo[2.2.2] ring system of this compound must be *enantiomorphic* to that of the paraherquamides (see ii and iii, Figure 3). Alternatively, VM55599 may be derived from L-*allo*-isoleucine; this would result in the (*R*)-stereochemistry at C-14 and would accommodate the same bicyclo[2.2.2] ring system absolute stereochemistry as paraherquamide A (compare **1** and ii). Finally, since the methyl group of the β-methylproline ring is *syn* to the isoprene unit comprising the bicyclo[2.2.2] ring system, this implies that the cyclization to form this system occurs from the more hindered face of the azadiene system (see i, Figure 3). The stereochemical paradox posed by VM55599 raises numerous interesting questions concerning the biogenesis of these substances: is VM55599 a biosynthetic precursor to the paraherquamide family members **6**, **7**, and **9** or is VM55599 a minor shunt metabolite with the opposite absolute stereochemistry of the bicyclo[2.2.2] ring system? The mechanism of formation of the bicyclo[2.2.2] ring system in both series continues to pose[3] an interesting stereochemical and enzymological phenomenon.

Efforts are underway to resolve the stereochemical puzzle posed by VM55599 and to fully establish the sequence of events that result in modeling of the substituted proline derivatives[8] that comprise this alkaloid family.

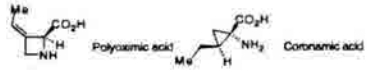
Acknowledgment. This work was supported (in part) by the NIH (Grant CA 43969), NSF (CHE 9320010), the PRF-AC, and the NIH Division of Research Resources Grant (RR-02231 to C.J.U.). Mass spectra were obtained on instruments supported by the NIH Shared Instrumentation Grant GM49631. J.F.S.-C. thanks the University of Valencia for financial support. Dr. Chris Rithner (CSU) is acknowledged for NMR technical assistance.

Supporting Information Available: Experimental procedures and spectral data for all new compounds employed in this study including methods of isotopic incorporation (8 pages). See any current masthead page for ordering and Internet access instructions.

JA961322Z

(6) (a) Herbert, R. B. *The Biosynthesis of Secondary Metabolites*; Chapman and Hall: London, 1981. Proline biosynthesis proceeds via reductive amination of glutamate semialdehyde which can be derived from either glutamate or ornithine; see: (b) Hermann, K. M., Somerville, R. L., Eds. *Amino Acids: Biosynthesis and Genetic Regulation*; Biotechnology Series; Addison Wesley: London, 1983.

(7) Related examples of biosynthetic oxidative cyclizations of the isoleucine side chain methyl group can be found in polyoximic acid and (via *allo*-isoleucine) coronamic acid; see, respectively: (a) Hanessian, S.; Fu, J-M.; Tu, Y.; Isono, K. *Tetrahedron Lett.* **1993**, *34*, 4153. (b) Parry, R. J.; Mhaskar, S. V.; Lin, M. T.; Walker, A. E.; Mafoti, R. *Can. J. Chem.* **1994**, *72*, 86.

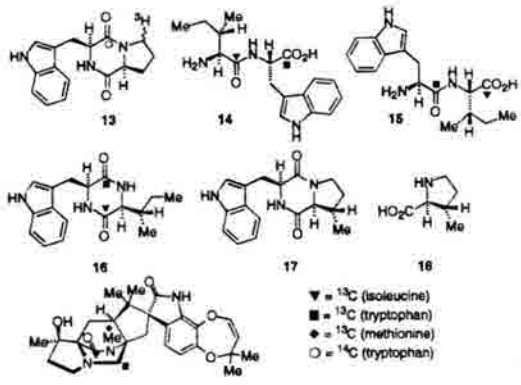


(8) We are currently pursuing the synthesis of ^{13}C-labeled β-methylproline; see: Kollnitsch, J.; Scott, A. N.; Doldouras, G. A. *J. Am. Chem. Soc.* **1966**, *88*, 3624.

Additions and Corrections

Studies on the Biosynthesis of Paraherquamide A. Origin of the β-Methylproline Ring [*J. Am. Chem. Soc.* **1996**, *118*, 7008−7009]. EMILY M. STOCKING, JUAN F. SANZ-CERVERA, ROBERT M. WILLIAMS,* AND CLIFFORD J. UNKEFER

The correct stereochemistry for compounds (**16**) *cyclo*-L-tryptophan-L-isoleucine, (**17**) *cyclo*-L-tryptophan-L-β-methylproline, and (**18**) L-β-methylproline in Figure 1 is shown below. The authors are indebted to Dr. Jeremy Everett for bringing this error to our attention.



JA9754158

Electron Transfer from C_{76} (C_{2v}') and C_{78} (D_2) to Radical Cations of Various Arenes: Evidence for the Marcus Inverted Region [*J. Am. Chem. Soc.* **1997**, *119*, 5744−5745]. DIRK M. GULDI* AND KLAUS-DIETER ASMUS

The correct notation for the C_{76} and C_{78} isomers throughout the paper should be C_{76} (D_2) and C_{78} (C_{2v}').

JA975412V

Facile Metathetical Exchange between Carbon Dioxide and the Divalent Group 14 Bisamides M[N(SiMe$_3$)$_2$]$_2$ (M = Ge and Sn) [*J. Am. Chem. Soc.* **1996**, *118*, 10912−10913]. LAWRENCE R. SITA,* JASON R. BABCOCK, AND RIMO XI

After publication, we have become aware of prior work by Wannagat and co-workers in which they describe a related metathetical exchange process that occurs between carbon dioxide and NaN(SiMe$_3$)$_2$ to produce, along with 1,3-bis-(trimethylsilyl)carbodiimide (85% yield), a mixture of products arising from secondary reactions [Wannagat, U.; Kuckertz, H.; Krüger, C.; Pump, J. *Z. Anorg. Allg. Chem.* **1964**, *333*, 54−60]. We regret this omission, however, it does not affect the findings or conclusions of the present work in which the synthetic utility of this form of heterocumulene metathesis is demonstrated for subvalent Group 14 compounds.

JA975414F

Chemical Structure of Blepharismin, the Photosensor Pigment for *Blepharisma japonicum* [*J. Am. Chem. Soc.* **1997**, *119*, 5762−5763]. GIOVANNI CHECCUCCI, RICHARD K. SHOEMAKER, ELISABETTA BINI, RONALD CERNY, NENGBING TAO, JAE-SEOK HYON, DOMENICO GIOFFRE, FRANCESCO GHETTI, FRANCESCO LENCI, AND PILL-SOON SONG*

Page 5762: Richard Shoemaker's initial appeared incorrectly as S. in the journal.

Page 5762: The ring system of the structure shown in Figure 1 should be named benzodianthrone, instead of naphthodianthrone. The ring system linked by the bond 8-8′ is dihedrally twisted, and the lack of a CD signal may be attributable to a racemic mixture of the two possible enantiomers. We thank Prof. Heinz Falk for pointing out the correct naming and asymmetry of the blepharismin structure.

JA975413N

Computer Software Reviews

Axum Version 5.0 for Windows. MathSoft, Inc.: 101 Main Street, Cambridge, Massachusetts, 02142; (617) 577-1017. $199.95.

Axum 5.0 is a technical graphics and data analysis program. This version comes on eleven 3.5 in. 1.4 megabyte disks and is compatible with the DOS/Windows 3.x, Windows NT, and Windows '95 operating systems. MathSoft recommends a minimum 486-computer with 16 megabytes of memory and 14−20 megabytes of disk space depending on the installation procedure chosen. A math co-processor is also recommended but is not required. Hence, Axum is a reasonable choice for high-end academic research or industrial computers but will be more demanding of older, microlab academic computers.

Unlike many available software packages that also render technical graphics and perform data analysis, Axum is geared toward scientific applications rather than business applications. This is reflected throughout the program, including the ability to change the precision of the data with a single click on an icon and the inclusion of many mathematical functions, scientifically-important graph options, and a programing language to perform sophisticated data analysis. An important weakness of the program is the lack of an easy procedure within the program to insert complex characters into labels or titles (for example, Greek characters or mathematical symbols), although the availability of tool bar buttons for fast addition of superscripts and subscripts to text is an improvement over previous versions of Axum. Additional features of the program include the ability to cut and paste comments, equations, and other figures (such as a chemical model) from any Windows-compatible word processor or graphics program into an Axum graph, in addition to the expected ability to cut and paste graphs generated with Axum into word processor programs.

In general, Axum is icon-driven and most commands are easily executed by using a tool bar that is similar to those used in most word processors and other graphics programs written for Windows and Macintosh platforms. As in older versions of Axum, the keyboard can be used to execute most commands by using combinations of the ALT, CTRL, and SHIFT keys. This is cumbersome, however, and the program works much more smoothly with the mouse.

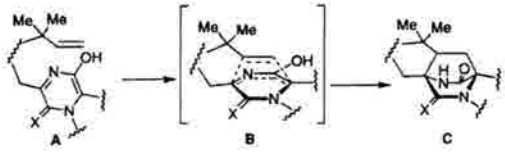
Data can be imported in the usual ASCI format or can be imported directly from several popular spreadsheets like Excel, Quattro Pro, Paradox, and Lotus. Data can also be deleted or copied to new data sheets or new locations within the same data sheet by dragging or cutting and pasting individual data cells or entire data columns.

To make a graph, the user must click on the icon specifying a two-dimensional plot or a three-dimensional plot. This causes a menu of

Reverse versus Normal Prenyl Transferases in Paraherquamide Biosynthesis Exhibit Distinct Facial Selectivities**

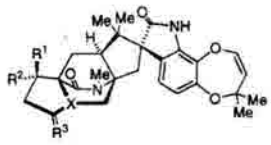
Emily M. Stocking, Juan F. Sanz-Cervera,* and Robert M. Williams*

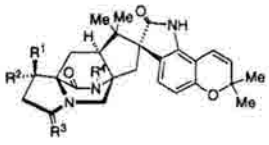
The paraherquamides (**1**–**10**)[1] are a group of fungal metabolites that, together with sclerotamide (**11**),[2] marcfortine (**12**),[3] asperparaline A (**14**, also aspergillimides, **15**),[4] and the brevianamides (**16**, brevianamide A; **17**, brevianamide B),[5] have recently attracted much attention due to the range of interesting biological activities that this family displays, including anthelmintic, paralytic, and insecticidal activities.[1–5] These substances are the consequence of mixed biogenetic origins, being derived from the oxidative polycyclization of amino acids and isoprene units. Most interesting in this regard is the emerging body of evidence that supports the notion that the common bicyclo[2.2.2] core structural motif is formed by a biosynthetic intramolecular [4+2] cycloaddition of the isoprene-derived olefin across an azadiene moiety derived from a preformed, oxidized piperazinedione [**A** → **B** → **C**, Eq. (1)].[6, 7]

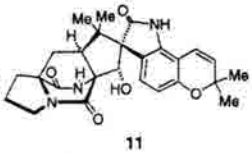


(1)

Although [4+2] cycloadditions are used extensively in synthetic organic chemistry, such pericyclic reactions are quite rare in Nature, and in only a few cases has experimental evidence been obtained to support the intermediacy of a Diels–Alder type of cycloaddition.[7, 8] Our research efforts have thus focused on the biogenesis of these compounds, with particular emphasis on the key cycloaddition step.

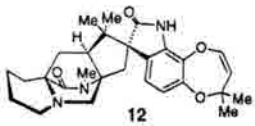






11

1 A: R^1 = OH, R^2 = Me, R^3 = H_2, X = N
2 B: R^1 = H, R^2 = H, R^3 = H_2, X = N
3 C: R^1 = R^2 = CH_2, R^3 = H_2, X = N
4 D: R^1 = O, R^2 = CH_2, R^3 = H_2, X = N
5 E: R^1 = H, R^2 = Me, R^3 = H_2, X = N
6 VM55596: R^1 = OH, R^2 = Me, R^3 = H_2, X = N^+-O^-
7 VM55597: R^1 = OH, R^2 = Me, R^3 = O, X = N
8 F: R^1 = H, R^2 = Me, R^3 = R^4 = Me
9 G: R^1 = OH, R^2 = Me, R^3 = R^4 = Me
10 VM55595: R^1 = H, R^2 = Me, R^3 = H_2, R^4 = H



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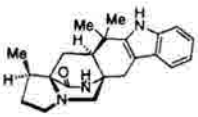
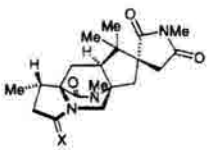
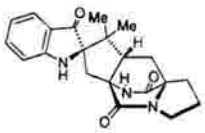


13 VM55599



14 (X = H_2)
15 (X = O)



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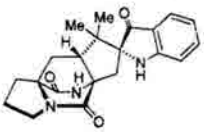

17

In this family of metabolites, the dimethylallyl group involved in forming the bicyclo[2.2.2] nucleus is apparently introduced by reaction of dimethylallyl pyrophosphate (DMAPP) with the tryptophan unit, resulting in a net *reverse prenylation* at the 2-position of the indole ring. In metabolites **1**–**12**, there is a second isoprene fragment that is oxidatively added to a phenolic hydroxy group of the tryptophan core. Until recently, it would have been safe to assume that the isoprene moieties are biosynthesized by the well-known mevalonic acid pathway. However, in the last few years several reports have clearly shown that in some organisms isoprenoids are not formed through this pathway, but rather through the 1-deoxy-D-xylulose pathway. This has been shown not only in bacteria,[9] but also in the green algae *Scenedesmus obliquus*[10] and, somewhat surprisingly, in higher plants like *Taxus chinensis*,[11] barley (*Hordeum vulgare* L.), duckweed (*Lemna giba* L.), carrot (*Daucus carota* L.),[12] and *Ginkgo biloba*.[13] In these cases, it has been shown that the biosynthesis of several cytoplasmic sterols proceeds by the acetate/mevalonate pathway, while the plastidic isoprenoids are synthesized by the new 1-deoxy-D-xylulose pathway.

In the course of isotopic labeling studies aimed at examining the origin of the isoprene units in the paraherquamide structure, we discovered an unexpected stereochemical distribution of the methyl groups derived from DMAPP. We

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Supporting information for this article is available on the WWW under http://www.wiley-vch.de/home/angewandte/ or from the author.

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carried out feeding experiments with [U-$^{13}C_6$]-glucose and [$^{13}C_2$]-acetate which aid in distinguishing between the two pathways using ^{13}C NMR spectroscopy since all the resonances from the ^{13}C NMR spectrum of paraherquamide A have been unequivocally assigned (with the exception of C27 and C28).[1d] The different labeling patterns to be expected from these two pathways are shown in Figure 1.

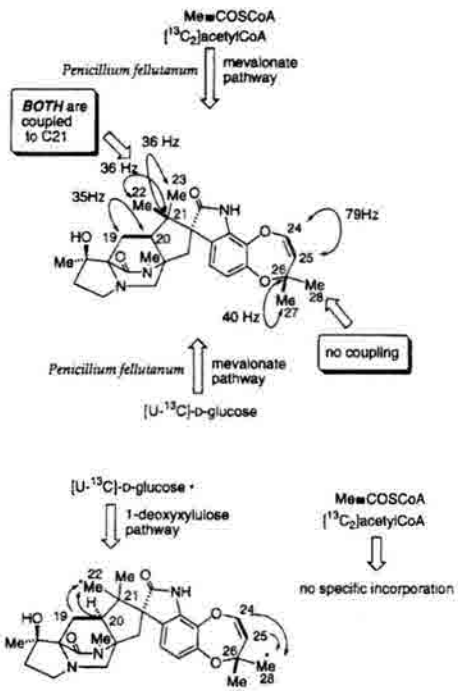

Figure 1. ^{13}C labeling in paraherquamide A corresponding to a feeding experiment with [U-$^{13}C_6$]-D-glucose and [$^{13}C_2$]-acetate by the mevalonate (top) and 1-deoxy-D-xylulose (bottom) pathways. Thick lines represent intact acetate units and arrows represent couplings expected and observed in the ^{13}C NMR spectrum.

An initial feeding experiment was carried out on *Penicillium fellutanum* with [U-$^{13}C_6$]-glucose; harvesting the resulting paraherquamide A provided a labeling pattern in which most signals appeared split around the singlet corresponding to the uncoupled carbon atom, as one would expect from the catabolism of glucose. In this case we focused our attention on the carbon atoms that form the two C_5 units: C19 to C23, and C24 to C28. The ^{13}C NMR spectrum of paraherquamide A[1d] isolated from a control experiment was used for comparison purposes. Although the specific incorporation was low, the couplings are clearly visible in the ^{13}C NMR spectrum of the resulting paraherquamide A (Table 1).

In order to confirm that acetate, and not 1-deoxy-D-xylulose-5-phosphate, is the key intermediate in the biogenesis of the two C_5 units, a second feeding experiment was carried out, this time using [$^{13}C_2$]-acetate. Specific incorporation of intact C_2 units was observed, in agreement with the mevalonic acid pathway (Figure 1). Regarding the carbon

Table 1. Specific incorporations, chemical shifts, and coupling constants for the C_5 carbon atoms of paraherquamide A in the feeding experiment with [U-$^{13}C_6$]-glucose.

C	δ	$J_{C,C}$ [Hz]	% ^{13}C in each position	% ^{13}C specifically incorporated as intact C_2 units in each positon	% ^{13}C in the [U-$^{13}C_6$] glucose used, specifically incorporated in each position
19	22.17	34	1.4	41	0.0075
20	51.42	34	1.9	32	0.0052
21	46.40	36	1.5	36	0.0061
22	20.47	36	1.8	14	0.0018
23	23.71	36	1.9	21	0.0029
24	138.94	81	2.3	35	0.0059
25	115.05	79	2.0	37	0.0065
26	79.81	40	2.0	37	0.0064
27	29.93	40	2.2	40	0.0075
28	29.80	–	1.7	0	0.0037

atoms that form the two C_5 units, C19 to C23, and C24 to C28, the results of the feeding experiment with [$^{13}C_2$]-acetate were essentially the same as those with [U-^{13}C]-glucose. In both cases, the signal for C-28 at $\delta = 29.80$ showed enhancement with respect to the control spectrum, but no splitting. In the first C_5 fragment, the observed couplings mean that C19 is coupled to C20, while C21 is coupled to C22 or C23, but not to both simultaneously. For the second C_5 unit, the coupling constants show that C24 and C25 are coupled, while C26 is coupled to C27. In this case, C28 shows no coupling.

The non-mevalonic pathway would cause incorporation of [U-$^{13}C_6$]-glucose in such a way that a long-range coupling would be observed between C19 and one of the methyl groups (C22 or C23) as well as C20 and the same methyl group (C22 or C23), due to the fact that in this case three carbon atoms would come from one glucose molecule, while the other two would come from a different one. However, in our feeding experiment with [U-$^{13}C_6$]-glucose no long-range coupling between C22 and C19/C20 or C28 and C24/C25 was observed. This result is consistent with the mevalonic acid pathway, in which two pairs of carbon atoms come from two glucose molecules, while the third carbon atom comes from a third glucose molecule, thus precluding long-range couplings. The lack of observed long-range couplings, together with the observed incorporation of [1,2-$^{13}C_2$]-acetate thus indicate that the operative pathway for the formation of both C_5 units is the mevalonic pathway, as the labeling pattern observed is consistent with that expected for such a mechanism (Figure 1). The electrospray ionization mass spectra of the isolated **1** from both feeding experiments agree with the incorporation of intact C_2 units from acetate.[14]

It is significant that in the C_5 fragment formed by C24 to C28, C28 shows no coupling with C26, while C27 does. This means that the methyl groups in DMAPP are not equivalent in the biosynthesis of this metabolite. In contrast, in the other C_5 fragment, formed by C19 to C23, both methyl groups show coupling with C20, although not simultaneously. This unexpected result must be interpreted to mean that the *reverse* prenyl transferase presents the olefinic π system of DMAPP in a manner in which both faces of the π system are susceptible to attack by the 2-position of the indole moiety.

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The simplest explanation is to invoke binding of the DMAPP in an "upside down" orientation relative to "normal" prenyl transferases which permits a facially nonselective S_N' attack on the π system (Figure 2). In this situation, the pyrophosphate group is likely anchored in the enzyme active site with the hydrophobic isopropenyl moiety being presented in a conformationally flexible ($\mathbf{A} \rightleftharpoons \mathbf{B}$) disposition with respect to the tryptophan-derived substrate. This is in contrast to the normal mode of prenyl transfer where, the nucleophilic displacement at the pyrophosphate-bearing methylene carbon atom occurs with inversion of configuration at carbon with the hydrophobic tail of DMAPP buried in the enzyme active site.[15]

In contrast, the methyl groups in the other C_5 unit (dioxepin moiety) are clearly differentiated; therefore, it is quite likely that this C_5 group (C24 to C28) is introduced in the molecule by direct alkylation with DMAPP by a normal prenyl transferase followed by a stereospecific net oxidative addition to the olefinic system. A plausible mechanism, of several possibilities, for the formation of this ring system, is by face-selective epoxidation followed by ring-opening and dehydration. Significantly, since this isoprene unit is introduced without loss of stereochemical integrity, Ockham's razor can be invoked for there not being a mechanism for scrambling the DMAPP via a dimethyl vinyl carbinol-type intermediate which would necessarily provide stereochemically scrambled isoprene equivalents to the cell's cytosolic pool of DMAPP.

The results of these experiments clearly indicate that the C_5 units in **1** are introduced in stereofacially distinct manners. Since it has been established that prenylation of the indole moiety in the biosynthesis of the related brevianamides occurs in a fashion analogous to that postulated for intermediates **19**[7d] (Figure 2) and that this is also a reasonable expectation in the paraherquamide biosynthesis, the prenyl transferase that installs this C_5 unit must display DMAPP to the 2-position of the indole in a π facially indiscriminate manner. The work described herein demonstrates the first case where *both* a non face-selective *and* a face-selective addition to the trisubstituted olefinic portion of a DMAPP-derived moiety has occurred within the same molecule.[16]

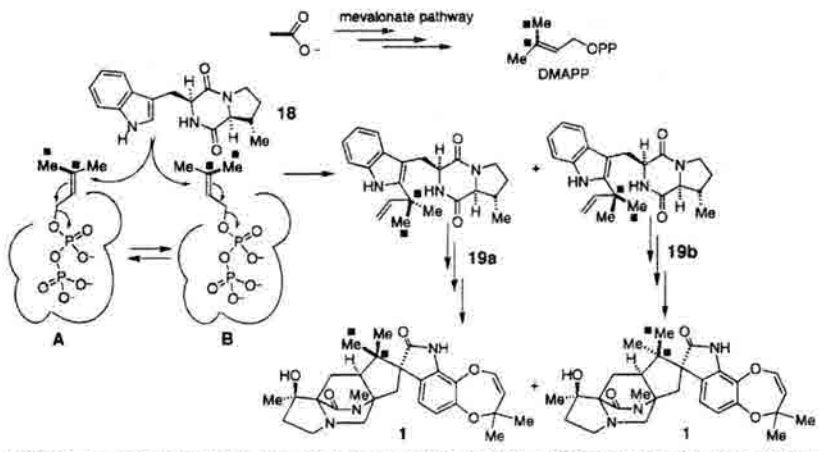

Figure 2. A possible biosynthetic sequence that may explain why C22 and C23 are rendered equivalent in the biosynthesis of paraherquamide A. Thick bonds labeled with ■ represent one intact C2 unit from acetate, incorporated in C3/C5 of individual DMAPP molecules, and in C21, C22, and C23 of **1**. For the sake of clarity and simplicity, the labels that would appear in other positions are not represented (see Figure 1).

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[14] For the control paraherquamide A ($M_w = 493$), the isotopic peaks for the protonated molecular ion appear at *m/z* (%): 494.30 (100), 495.30 (32.65), 496.30 (6.49), and 497.27 (1.13). In contrast, for **1** isolated from the [U-$^{13}C_6$]-glucose feeding experiment, the molecular ion peaks appear at: *m/z* (%): 494.27 (100), 495.27 (45.27), 496.27 (18.04), 497.27 (6.87), 498.26 (2.94), 499.25 (1.45), and 500.22 (0.79). For **1** isolated from the [$^{13}C_2$]-acetate feeding experiment, the molecular ion peaks appear at: *m/z* (%): 494.25 (100), 495.24 (47.42), 496.24 (17.76), 497.23 (6.66), and 498.19 (3.38). The increased intensities of the protonated $M+2$ (*m/z* 496) and $M+4$ (*m/z* 498) peaks agree with the incorporation of the acetate units.

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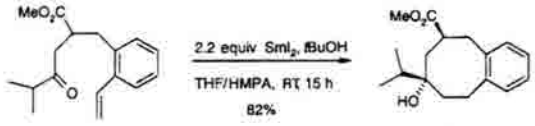
A New Samarium Diiodide Induced Reaction: Intramolecular Attack of Ketyl Radical Anions on Aryl Substituents with Formation of 1,4-Cyclohexadiene Derivatives**

Chimmanamada U. Dinesh and Hans-Ulrich Reissig*

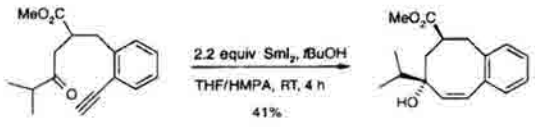
Dedicated to Professor Edward Piers on the occasion of his 60th birthday

Samarium diiodide was introduced by Kagan et al.[1] as a selective one electron transfer reagent in organic chemistry. Several variants of SmI_2-induced cyclization reactions[2] have attained synthetic importance owing to their high stereoselectivity and ability to undergo sequential reactions.[3] Frequently, ketyl radical anions generated by the electron transfer are the reactive species which add to a multiple bond offered at an appropriate distance. We now report that these ketyl radical anions can attack an aryl moiety in an intramolecular fashion and, after a second electron transfer, lead to 1,4-cyclohexadiene derivatives. This reaction has not yet been observed in samarium chemistry to the best of our knowledge, and it should gain considerable synthetic importance owing to its high diastereoselectivity.

Motivated by the examples reported by Molander and McKie,[4] we recently developed a synthesis of benzannulated cyclooctane derivatives.[5] Our previously unpublished example **1**→**2** (HMPA = hexamethyl phosphoramide) demonstrates that this samarium diiodide promoted transformation proceeds with high diastereoselectivity and in very good yield in spite of the sterically demanding isopropyl group. To explore further mechanistic details of the 8-*endo* cyclization and to obtain new options for functionalizations of the newly generated eight-membered ring, alkyne derivatives such as **3** were subjected to the general reaction conditions. The expected benzannulated cyclooctene **4** was isolated in 41% yield as a single diastereomer. To our knowledge this is the first successful 8-*endo*-*dig* cyclization of a samarium ketyl.[6]



1 **2**



3 **4**

Since they were more readily accessible,[7] the disubstituted alkynes **5**–**7** were first subjected to samarium diiodide cyclization conditions. However, we obtained products derived from neither an 8-*endo*-*dig* cyclization nor from the more likely 7-*exo*-*dig*-reaction, but surprisingly cyclohexadienes **11**–**13** which were formed by attack on aryl substituents. Examples **8** and **9** demonstrate that the alkyne units are not required for the cyclization, as samarium diiodide now affords cyclization products **14** and **15** or the lactone **16**. The cyclohexanone moiety in **6**–**8** allows smooth cyclization;[8] however, the steric hindrance in isopropyl derivative **10** seems to be too high. Under the conditions applied mainly starting material was recovered and no product was formed. The fact that **1** and **3** were transformed into bicyclic **2** and **4**, and that the precursor **10** was essentially inert, supports the reversibility of the first electron transfer to the carbonyl group. The subsequent steps are responsible for the productivity of the sequence.

Particularly remarkable is the high diastereoselectivity of the reaction, since in all examples only one diastereomer could be detected. The relative configuration at the two newly generated asymmetric centers of **14** was proven by X-ray analysis.[9] This showed not only the *cis* arrangement of the bridgehead hydrogen atom with respect to the hydroxyl group, but also the location of R^2 and of the methoxycarbonyl

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Total Synthesis of VM55599. Utilization of an Intramolecular Diels−Alder Cycloaddition of Potential Biogenetic Relevance

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Abstract: The total synthesis of VM55599, a natural metabolite of *Penicillium* sp. IMI332995, has been achieved via an intramolecular Diels−Alder cycloaddition of a reverse isoprene moiety across an azadiene system. The diastereoselectivity of the intramolecular Diels−Alder cycloaddition has biogenetic implications and is discussed in the context of the biogenetic relationship of VM55599 to the paraherquamides.

Introduction

VM55599 is a minor secondary metabolite of *Penicillium* sp. IMI332995.[1] This substance was co-isolated with several known members of the paraherquamide family including paraherquamide A, VM54158 (paraherquamide G), VM54159 (paraherquamide E), and VM55594 (paraherquamide F) by Everett et al.[1] In addition, this *Penicillium* strain was found to produce several new metabolites in the paraherquamide family including VM55595, VM55596, and VM55597 (Figure 1).[1] The relative stereochemistry of VM55599 was assigned by ^{1}H NMR/NOE data but the absolute stereochemistry remains unknown; the absolute stereostructure depicted below is a prediction based on biogenetic considerations to be discussed below. Of particular interest in this regard is the stereochemical disposition of the methyl group in the β-methylproline ring which was assigned as being *syn* to the bridging isoprene moiety. In all other known members of the paraherquamide family, the methyl group in the β-methylproline ring is disposed *anti* to the bridging isoprene moiety.

VM55599,[1] the paraherquamides,[2] brevianamides,[3] marcfortines,[4] and most recently, the sclerotamides,[5] are indolic secondary metabolites isolated from various fungi and have attracted considerable attention due to their molecular complexity, intriguing biogenesis,[6] and some members, most notably the paraherquamides, display potent antiparasitic activity.[7] These

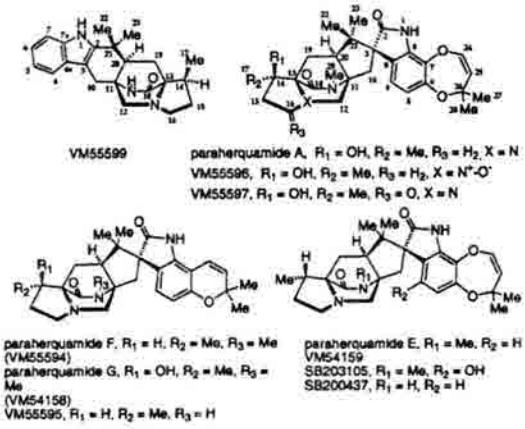

Figure 1.

alkaloids share the unusual bicyclo[2.2.2] ring system that has been proposed to arise via the [4 + 2] cycloaddition of the isoprene moiety across the α-carbons of the amino acid units.[6c,d,8,9] Previous work on the biosynthesis of these substances invoked a facial divergence in the Diels−Alder cyclization which sets the relative *syn*-/*anti*-stereochemical relationship at this stereogenic center.[10,11] Specifically, the cyclization of the isoprenyl olefin across the azadiene ring system can proceed via four distinct diastereomeric transition structures **a**, **b**, **c**, or **d** (Figure 2), resulting in the four corresponding cycloadducts **A**, **B**, **C**, or **D**. Cycloadduct **B** corresponds to VM55599, and cycloadduct **A** is the putative structure leading to paraherqua-

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mide; cycloadducts **C** and **D** lead to C-20-*epi* metabolites thus far not detected in paraherquamide-producing fungi.

In addition, recent theoretical work on an indoxyl-based Diels–Alder cyclization pathway supported the observed isomer distribution of the brevianamides in *Penicillium brevicompactum* which produces brevianamide A as the major metabolite and brevianamide B as the minor metabolite, both of which possess the *anti*-relationship.[12] As part of a program directed primarily at elucidating the biosynthetic mechanism of formation of the unique bicyclo[2.2.2] ring system, particularly with respect to the question of possible enzymatic catalysis of this reaction, we report here the first total synthesis of VM55599 using an intramolecular Diels–Alder cyclization reaction that may be of biogenetic relevance.[13]

Results and Discussion

The synthesis of VM55599 was accomplished as shown in Scheme 1. The benzophenone imine **1** of glycine ethyl ester was condensed with the dimethylallylated gramine derivative **2**[14] in the presence of tri-*n*-butylphosphine[15] in acetonitrile to furnish the tryptophan derivative **3** in 70% yield. Cleavage of the benzophenone imine with hydroxylamine provided the amino ethyl ester **4** in high yield. Subsequent *t*-BOC protection and basic hydrolysis of the ethyl ester furnished the acid **5** in 78% yield over two steps. Coupling of acid **5** with racemic β-methyl-β-hydroxyproline ethyl ester with BOP reagent[16] provided the desired dipeptide **7** in 70–83% yield. The BOC group was cleaved with TFA, and the resulting amino ethyl ester was cyclized to the corresponding piperazinedione **8** in the presence of 2-hydroxypyridine in refluxing toluene in excellent yield.

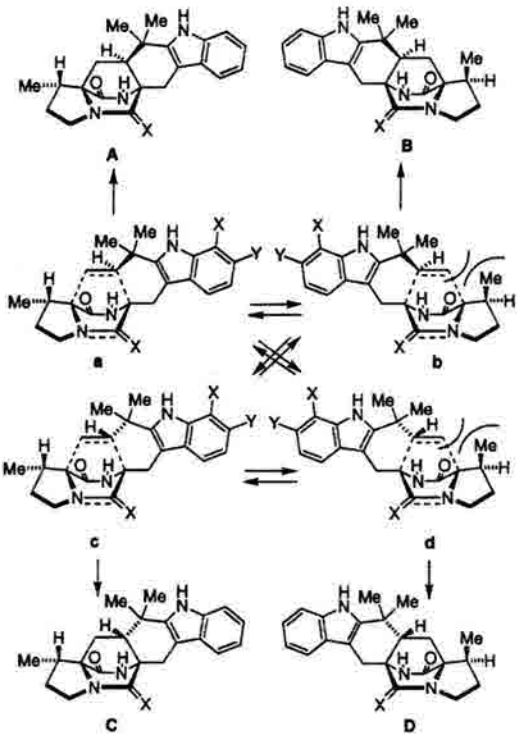

Figure 2.

Treatment of **8** with thionyl chloride in pyridine furnished the unsaturated substance **9** in 75% yield. Subsequent treatment of **9** with trimethyloxonium tetrafluoroborate in dichloromethane provided the azadiene **10** in 72% yield. Treatment of azadiene **10** with KOH in aqueous methanol effected tautomerization to the labile incipient azadiene **11** which spontaneously suffered intramolecular Diels–Alder cycloaddition at room temperature to give a mixture of all four possible racemic cycloadducts **12**–**15** in 78% combined yield in a 3.7:2.6:1.6:1 ratio, respectively.

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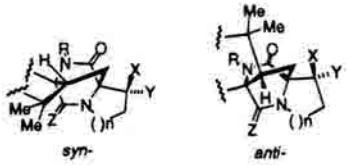
(10) The *syn*/*anti* relationship refers to the relative stereochemistry between the C-20 stereogenic center (VM55599 numbering) and the cyclic amino acid residue (proline, β-methylproline, or pipecolic acid):



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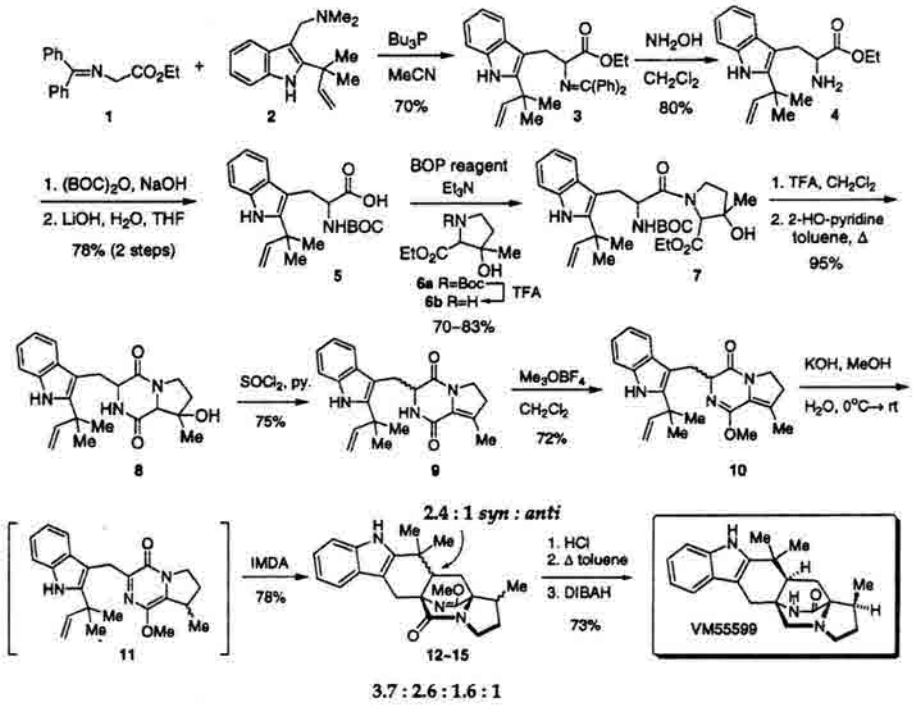
(14) This substance was prepared according to ref 6d.

(15) (a) Kametani, T.; Kanaya, N.; Ihara, M. *J. Chem. Soc., Perkin Trans 1* **1981**, 959. (b) Somei, M.; Karasawa, Y.; Kaneko, C. *Heterocycles* **1981**, *16*, 941.

(16) BOP = (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (purchased from Aldrich Chemical Co.).

Scheme 1



The Diels–Alder cycloadducts **12**–**15** were separable by PTLC on silica gel, and their relative stereochemistry was assigned by ^{1}H NMR NOE studies.[17] The *syn*-stereochemistry at C-20 for **12** and **13** was assigned based on the NOE between H-20 (using the VM55599 numbering system) and the OMe of the lactim ether. The *anti*-stereochemistry assignment of C-20 for both **14** and **15** was made based on the NOE between H-23 and the OMe. The assignment of stereochemistry at C-14 for **12** and **14** was deduced from the NOE between H-17 and H-19/19′. This NOE was also observed by Everett et al. in the original VM55599 isolation paper.[1] For **13** and **15**, the stereochemical assignment of C-14 was inferred from the NOE between H-14 and H-19/19′.

The structures of all four cycloadducts **12**–**15** depicting their relative stereochemistries are shown in Figure 3. The *syn/anti* relationship[10] at the C-20 stereogenic center was 2.4:1 and is consistent with results reported earlier from this laboratory on a simpler system lacking the methyl group in the proline ring.[13] Of significant interest was the unexpected observation that the major products (**12** and **14**) in each diastereomeric subset displayed the methyl group in the β-methylproline ring *syn* to the bridging isoprene unit (see Figure 3). The diastereoselectivity in this regard was 1.47:1 favoring the methyl group disposed *syn* to the bridging isoprene moiety. Although it is reasonable to expect modest diastereoselectivity for this Diels–Alder cycloaddition, purely on the basis of the slight steric bias expected to be exerted by the methyl group in the proline ring, we anticipated a modest preference for cycloadducts that displayed the methyl group *anti* to the bridging isoprene moiety.

Confirmation of the structure for cycloadduct **12** was secured through conversion into racemic VM55599. Thus, treatment of

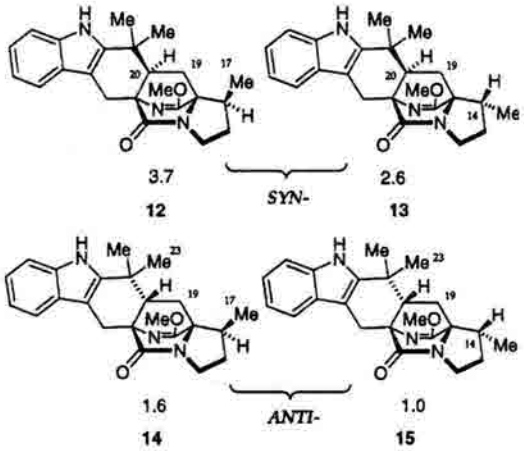


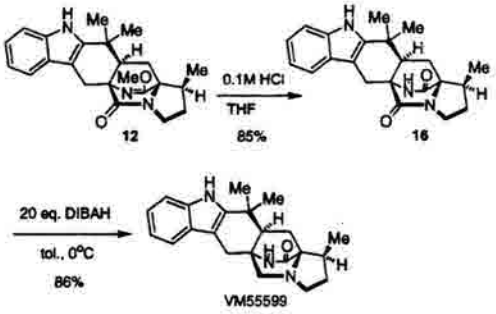
Figure 3.

12 with dilute HCl effected cleavage of the lactim ether to the corresponding secondary amide **16** in 85% yield (Scheme 2). Selective reduction of this substance with excess DIBAH[18] (20 equiv) provided VM55599 in 86% yield whose ^{1}H and ^{13}C NMR spectral characteristics matched those published.[1] The synthetic material was subsequently utilized to guide reisolation of natural VM55599 from cultures of *Penicillium* sp. IMI332995 (obtained from the International Mycological Institute) grown in our laboratory. The synthetic and natural specimens were found to have identical ^{1}H NMR spectra and TLC mobility thereby confirming the assignment (see Supporting Information).

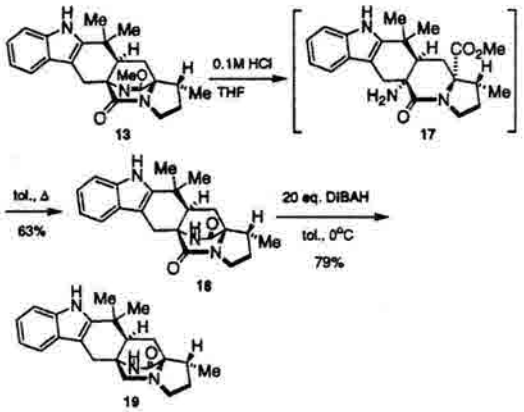
(17) Complete NOE data on cycloadducts **12**–**15** can be found in Supporting Information.

(18) We thank Prof. Tohru Fukuyama for suggesting the use of excess DIBAH for this transformation; see Fukuyama, T.; Liu, G. *Pure Appl. Chem.* **1997**, *69*, 501.

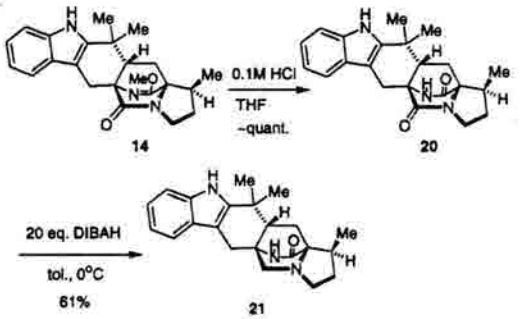
Scheme 2



Scheme 3



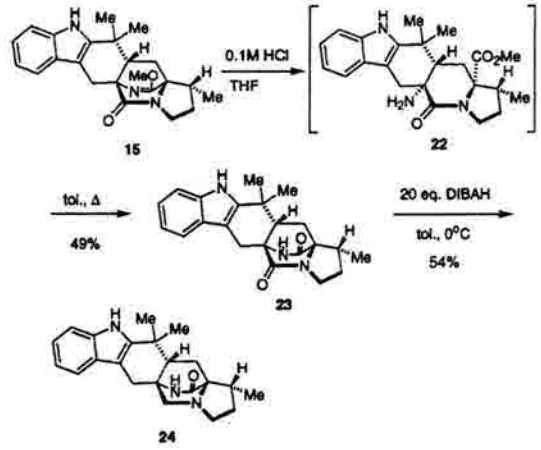
Scheme 4



To further confirm this assignment, the three other cycloadducts **13**−**15** were similarly converted into the corresponding C-14 and/or C-20 epimers of VM55599 (**19**, **21**, and **24**) as shown in Schemes 3−5.[19] It was interesting to observe that, in the case of cycloadducts **13** and **15**, cleavage of the lactim ether with dilute HCl led to the production of the ring-opened amino esters **17** and **22**, respectively. These were readily cyclized to the corresponding bicyclo[2.2.2]-containing secondary amides **18** and **23**, respectively, by simply heating these substances in toluene at reflux temperature overnight. In contrast, the lactim ethers of both cycloadducts **12** and **14** could be cleaved to the corresponding bicyclo[2.2.2]-containing substrates without attendant ring-opening to the corresponding amino esters. It would appear that there is $A^{(1,3)}$-type strain in compounds **13** and **15**

(19) Conditions for the conversion of compounds **13**−**15** to the VM55599 diasteromers **19**, **21**, and **24** were not optimized.

Scheme 5



caused by compression between the methyl group disposed on the β-face of the proline ring and the lactim ether methoxy group that is relieved upon ring-opening to **17** and **22**, respectively. In substrates **12** and **14**, where the methyl group in the proline ring is on the α-face, the opportunity for $A^{(1,3)}$-type strain is obviated by the *anti*-relationship between the lactim ether group and the methyl group. Subsequent DIBAH reduction of the tertiary amides of compounds **18**, **20**, and **23** gave the corresponding diastereomers of VM55599 (**19**, **21**, and **24**, respectively).

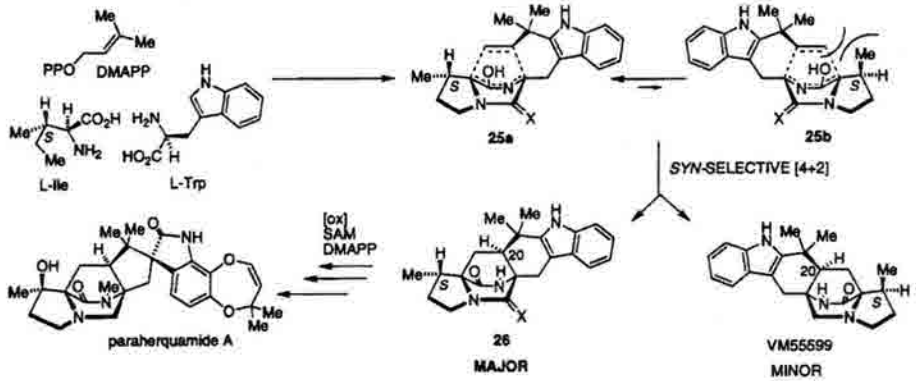
The NMR spectra of the VM55599 diastereomers **19**, **21**, and **24** were fully consistent with the assigned structures, and significantly, all were distinctly different from the spectra for natural VM55599 (see Supporting Information).[1]

A significant implication of these observations concerns the biogenesis and absolute stereochemistry of VM55599. In particular, it should first be noted that natural paraherquamide derivatives containing a non-hydroxylated β-methylproline residue, such as VM55594, VM55595, VM54159, SB203105,[20] and SB200437,[20] all display the methyl group at the β-position of the proline residue *anti* to the bridging isoprene moiety. In stark contrast, VM55599 is the only member of the paraherquamide family thus far isolated that displays the methyl group at the β-position of the proline residue *syn* to the bridging isoprene moiety. We previously demonstrated that the β-methyl-β-hydroxyproline ring of paraherquamide A is biosynthetically derived from L-isoleucine.[21] Enzymatic hydroxylation of the (*S*)-β-methylproline ring in paraherquamide A biosynthesis must therefore occur with net *retention* of stereochemistry leaving the methyl group *anti* to the bridging isoprene moiety. One possible scenario constituting a unified biogenesis of paraherquamide A (and congeners) and VM55599 is depicted in Scheme 6. Since Paraherquamide A and VM55599 both possess the bicyclo[2.2.2] monoketopiperazine ring system and are coproduced by the same fungi, it is tempting to speculate that these substances arise via a related or common [4 + 2] cycloaddition. Thus, if a similar Diels−Alder cyclization, whether it be uncatalyzed or enzyme-catalyzed, is operating in the biosynthetic construction of these metabolites, the isoprene unit must approach the azadiene from the *same* face as the methyl group in the proline ring for VM55599 (**25b**, Scheme

(20) Banks, R. M.; Blanchflower, S. E.; Everett, J. R.; Manger, B. R.; Reading, C. *J. Antibiot.* **1997**, *50*, 840.

(21) Stocking, E.; Sanz-Cervera, J. F.; Williams, R. M.; Unkefer, C. J. *J. Am. Chem. Soc.* **1996**, *118*, 7008.

Scheme 6



6), whereas in paraherquamide A, Diels Alder cyclization must occur from the face *opposite* to the methyl group (**25a**, Scheme 6). In both cases, the diastereofacial selectivity of the Diels–Alder reaction must give the *syn*-relative stereochemistry at C-20 (VM55599 numbering). It is interesting to note that metabolites possessing the *anti*-relative stereochemistry at C-20 have not yet been isolated from paraherquamide-producing fungi. Since VM55599 is a very minor metabolite of *Penicillium* sp. IMI332995,[22] it seems reasonable that a *syn*-selective Diels–Alder reaction gives, via conformer **25a**, cycloadduct **26** as a major product that is then further metabolized to the paraherquamide family. The minor cycloaddition product (via conformer **25b**), after adjustment of the oxidation state at C-12, would furnish VM55599 as a dead-end shunt metabolite with the absolute stereochemistry depicted (predicted).

It is therefore quite interesting that the diastereofacial bias of the Diels–Alder cycloaddition on synthetic azadiene **11** gives a slight preponderance (1.47:1) of cycloaddition from the *same* face as the methyl group in the β-methylproline ring and modest selectivity (2.4:1) favoring the (C-20) *syn*-relative stereochemistry. These results indicate that the *intrinsic* facial bias of this type of Diels–Alder cycloaddition is modest at best and that the biological system may be subject to protein organization of the precyclization conformers.[9]

Conclusion

This study confirms the structural and relative stereochemical assignment made for VM55599[1] and further demonstrates that the core bicyclo[2.2.2] ring system common to this family of alkaloids very likely arises by a biosynthetic intramolecular Diels–Alder cyclization from a preformed dioxopiperazine[23] that subsequently undergoes oxidation to an azadiene species. Finally, the C-20-*epi*-metabolites (with the *anti*-stereochemistry corresponding to the brevianamides) have not yet been detected from paraherquamide-producing fungi, and there have been no reports on the isolation of similarly epimeric metabolites from the brevianamide-producing *Penicillium* sp. Thus, in each biosynthetic system, there appears to be complete facial exclusivity in the construction of the bicyclo[2.2.2] ring nucleus with respect to the relative stereochemistry set at C-20; such is not the case for the laboratory cycloaddition reported here. It is also important to stress that the laboratory cyclization described here occurs spontaneously at room temperature in water, which indicates that the fundamental thermodynamics of this cyclization are amenable to cytosolic constraints. Thus, "catalysis" of this type of cycloaddition may not be required biosynthetically, but the predisposition of the precyclization conformers (ie., **25a/25b**) leading to the observed paraherquamide stereoisomers may be a manifestation of incidental protein organization.[9,24] Uncertainties as to the oxidation state of the putative azadiene moiety in the biosynthetic system still exist and are the subject of ongoing investigations in these laboratories.

(22) Our own work with *Penicillium* sp. IMI 332995 provided ca. 0.5 mg of VM55599 and ~250 mg of paraherquamide A (~1:500 ratio) from 12 L of surface culture media.

(23) For relevant work, see (a) Dunkerton, L. V.; Chen, H.; McKillican, B. P. *Tetrahedron Lett.* **1988**, *29*, 2539. (b) Fabre, J. L.; Farge, D.; James, C.; Lave, D. *Tetrahedron Lett.* **1985**, *26*, 5447.

Experimental Section

***N*-(Diphenylmethylene)-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan Ethyl Ester (3).** *N*-(Diphenylmethylene)glycine ethyl ester (**1**) (3.2 g, 12.0 mmol) and the gramine derivative **2**[14] (3.2 g, 13.2 mmol) were stirred in acetonitrile (110 mL) under argon until the solids dissolved. Tri-*n*-butylphosphine, (1.5 mL, 6 mmol) was added, and the mixture was brought to reflux temperature for 8 h. After being cooled to room temperature, the solvent was concentrated under reduced pressure, and the crude product was purified by flash silica gel column chromatography (15% EtOAc/hex) to yield 3.78 g (70%) of **3** as sticky yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.29 (3H, t, J = 7.0 Hz), 1.38 (3H, s), 1.41 (3H, s), 3.59 (2H, dd, J = 1.1, 7.0 Hz), 4.23 (2H, m), 4.52 (1H, dd, J = 6.2, 7.3 Hz), 5.04 (1H, dd, J = 1.1, 10.6 Hz), 5.09 (1 H, dd, J = 1.3, 17.2 Hz), 5.92 (1H dd, J = 10.3, 17.2 Hz), 6.39 (2H, bs), 6.87 (1H, ddd, J = 1, 8.3, 8.3 Hz), 7.06 (1H, ddd, J = 1.1, 7.5, 7.5 Hz), 7.09 (2H, dd, J = 1.1, 7.5, 7.5 Hz), 7.30 (5 H, m), 7.45 (1H, d, J = 8.0 Hz), 7.60 (2H, m), 7.86 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.1, 27.50, 27.52, 28.7, 38.9, 60.8, 66.6, 107.2, 109.7, 111.5, 118.8, 119.3, 121.1, 127.5, 127.6, 127.7, 128.7, 129.9, 130.0, 133.8, 135.9, 139.2, 139.8, 146.0, 169.7, 172.4. IR (NaCl neat): 3405, 3057, 2972, 1731, 1621, 1597, 1575, 1489, 1462, 1446, 1286, 1245, 1185, 1069, 1029, 917, 781, 742, 697 cm^{-1}. HRMS (FAB+): Calcd for $C_{31}H_{33}N_2O_2$: 465.2542. Found 465.2541 (M + H).

2-(1,1-Dimethyl-2-propenyl)-D,L-tryptophan Ethyl Ester (4). Compound **3** (1.92 g, 4.27 mmol) was stirred with NH_2OH·HCl (2.25 g, 32.45 mmol) and anhydrous Na_2CO_3 (3.21 g, 30.31 mmol) in CH_2Cl_2 (17 mL) at room temperature under argon for 24 h. The solution was acidified to pH 3 with 10% $KHSO_4$ (aq), and the organic layer was separated from the aqueous phase. The aqueous layer was extracted three more times with EtOAc before it was made basic with 10% Na_2CO_3 (aq) and extracted three times with EtOAc. The combined organic layers from the basic extract were dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure to give 1.03 g (80%) of **4** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.19 (3H, t, J = 7 Hz), 1.52 (2H, bs), 1.58 (6H, s), 3.08 (1H, dd, J = 14.3, 9.5 Hz),

(24) For an analogous system, see Oikawa, H.; Katayama, K.; Suzuki, Y.; Ichihara, A. *J. Chem. Soc., Chem. Commun.* **1995**, 1321.

3.35 (1H, dd, J = 14.7, 5.1 Hz), 3.86 (1H, dd, J = 9.5, 5.1 Hz), 4.13 (2H, m), 5.18 (1 H, dd, J = 0.7, 10.6 Hz), 5.20 (1 H, dd, J = 0.7, 17.2 Hz), 6.16 (1H, dd, J = 10.6, 17.2 Hz), 7.08 (1H, t, J = 7 Hz), 7.14 (1H, t, J = 7 Hz), 7.29 (1H, d, J = 7 Hz), 7.58 (1H, d, J = 7 Hz), 7.98 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.0, 27.8, 27.9, 31.2, 39.1, 55.9, 60.8, 107.0, 110.3, 112.1, 118.6, 119.3, 112.5, 129.8, 134.1, 140.4, 146.0, 175.5. IR (NaCl neat) 3399, 3243, 3081, 3056, 2973, 1733, 1638, 1617, 1580, 1462, 1300, 1282, 1195, 1105, 1029, 917, 859, 743 cm^{-1}. HRMS (FAB+): Calcd for $C_{18}H_{25}N_2O_2$: 301.191603. Found 301.191898 (M + H).

N-[(1,1-Dimethylethoxy)carbonyl]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan Ethyl Ester. Compound **4** (1.57 g, 5.23 mmol) was stirred with 1 equiv of 0.5 M NaOH and di-*tert*-butyl pyrocarbonate (1.25 g, 5.75 mmol) in dioxane (5.23 mL) at room temperature for 3 h. The dioxane was removed under reduced pressure, and the solution was brought to pH = 2 with the addition of aqueous 10% $KHSO_4$. The aqueous layer was extracted three times with EtOAc and dried over anhydrous Na_2SO_4. After removing the solvent under reduced pressure, the product was purified by flash silica gel column chromatography using 30% EtOAc/hexane to yield 1.843 g (88%) of the product as an oil. ^{1}H NMR (300 MHz, DMSO-d_6, 120 °C): δ 1.04 (3H, t, J = 7.0 Hz), 1.31 (9H, s), 1.55 (6H, s), 3.12 (1H, dd, J = 7.7, 14.3 Hz), 3.30 (1H, dd, J = 6.8, 14.8 Hz), 3.96 (2H, m), 4.30 (1H, dd, J = 7.7, 15.6 Hz), 5.07 (1H, dd, J = 0, 10.3 Hz), 5.10 (1H, dd, J = 0, 17.6 Hz), 6.22 (1H, dd, J = 10.7, 17.6 Hz), 6.26 (1H, bs), 6.92 (1H, t, J = 7.3 Hz), 7.00 (1H, t, J = 7.0 Hz), 7.31 (1H, d, J = 8.0 Hz), 7.45 (1H, d, J = 7.7 Hz), 10.06 (1H, bs). ^{13}C NMR (100 MHz, DMSO-d_6, 116 °C): δ 12.9, 26.8, 27.3, 27.5, 30.7, 38.3, 54.9, 59.4, 77.7, 105.0, 108.7, 110.0, 110.5, 117.4, 117.5, 119.7, 128.9, 134.4, 140.3, 145.9, 154.2, 171.5. IR (NaCl neat) 3376, 3083, 3057, 2976, 2933, 1697, 1503, 1462, 1376, 1167, 1021, 917, 861, 742 cm^{-1}. HRMS (FAB+): Calcd for $C_{23}H_{32}N_2O_4$: 400.236208. Found 400.236330 (M$^+$).

N-[(1,1-Dimethylethoxy)carbonyl]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan (5). N-[(1,1-Dimethylethoxy)carbonyl]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophan ethyl ester (1.97 g, 4.60 mmol) was stirred with LiOH (589 mg, 25 mmol) in a THF:H_2O solution (2:1) (16 mL) overnight. The solution was acidified with 10% $KHSO_4$(aq) and extracted three times with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, and the solvent was concentrated under reduced pressure to afford 1.63 g (89%) of **5** as an amorphous solid. The product was deemed sufficiently pure to use directly for the next step without further purification. ^{1}H NMR (300 MHz, DMSO-d_6, 120 °C): δ 1.28 (9H, s), 1.55 (3H, s), 1.56 (3H, s), 3.09 (1H, dd, J = 8.1, 14.7 Hz), 3.46 (1H, dd, J = 6.2, 14.7), 4.30 (1H, dd, J = 8.1, 14.7 Hz), 5.06 (1H, dd, J = 1.1, 10.6 Hz), 5.10 (1H, dd, J = 0, 17.2 Hz), 5.98 (1H, d, J = 5.9 Hz), 6.22 (1H, dd, J = 10.6, 17.2 Hz), 6.92 (1H, ddd, J = 1.1, 7.7, 7.7 Hz), 7.00 (1H, ddd, J = 0.8, 7.7, 7.7 Hz), 7.31 (1H, d, J = 7.7 Hz), 7.53 (1H, d, J = 7.7 Hz), 10.02 (1H, bs), 11.51 (1H, very bs). ^{13}C NMR (100 MHz, DMSO-d_6, 120 °C): δ 27.1, 27.3, 27.4, 38.3, 54.6, 77.6, 105.4, 110.1, 110.4, 117.5, 119.6, 129.0, 134.4, 140.3, 146.0, 154.2, 172.7. IR (NaCl neat) 3368−2563, 3368, 3087, 3053, 2974, 2926, 1712, 1502, 1460, 1394, 1367, 1245, 1164, 1054, 1010, 919, 742 cm^{-1}. HRMS (FAB+): Calcd for $C_{21}H_{28}N_2O_4$: 372.2049. Found 372.2052 (M$^+$).

Synthesis of 6a. N-[(1,1-Dimethyethoxy)carbonyl]-N-(3-oxobutyl)-glycine Ethyl Ester. The hydrochloride salt of glycine ethyl ester was neutralized by the addition of 1 equiv of aqueous 10% Na_2CO_3 and extracting five times with CH_2Cl_2. After drying the organic layer over anhydrous Na_2SO_4, the solvent was removed under reduced pressure, and the crude free amine was obtained. Glycine ethyl ester (9.06 g, 87.8 mmol) was stirred with methyl vinyl ketone (7.28 mL, 1.0 equiv) in acetonitrile (88 mL) at room temperature under argon in the absence of light. After 3 h, the solvent was removed under reduced pressure, and the flask was placed under vacuum for 1 h. The free amine decomposes fairly rapidly upon standing, and it was found best to proceed directly to the next step without further purification. To a solution of the adduct obtained above (13.76 g, 79.4 mmol) at 0 °C in dioxane (160 mL) were added di-*tert*-butyl pyrocarbonate (17.3 g, 1.0 equiv, 79.4 mmol), 1 M NaOH solution (79.4 mL), and deionized water (79.4 mL). The reaction was allowed to stir under argon, in the absence of light, at room-temperature overnight. The reaction was worked up by adding saturated NaCl solution (100 mL), extracting three times with EtOAc, drying over anhydrous Na_2SO_4, and evaporating of the solvent under reduced pressure. The crude product was purified by Kugelrohr distillation; the product distilled at 102 °C at 1 mmHg affording 15.64 g of the product as an oil (65% for the two steps). ^{1}H NMR (300 MHz, DMSO-d_6, 120 °C): δ 1.23 (3H, ddd, J = 1.6, 7.3, 7.3 Hz), 1.41 (9H, s), 2.10 (3H, s), 2.70 (2H, t, J = 6.6 Hz), 3.44 (2H, ddd, J = 1.5, 6.6, 6.6 Hz), 3.92 (2H, s), 4.14 (2H, dddd, J = 1.5, 7.0, 7.0, 7.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6, 120 °C): 13.3, 27.4, 27.6, 28.9, 41.6, 42.8, 49.0, 59.6, 78.8, 154.0, 169.1, 205.9. IR (NaCl, neat): 3611, 3398, 2978, 2936, 1748, 1698, 1462, 1397, 1367, 1250, 1162, 1129, 1029, 894, 866, 778 cm^{-1}. HRMS (FAB+) Calcd. for $C_{13}H_{24}NO_5$: 274.165448. Found 274.165921 (M + H).

3-Hydroxy-3-methyl-1,2-pyrrolidinedicarboxylic Acid 1-(1,1-Dimethylethyl) 2-Ethyl Ester (6a). A solution of the N-Boc-protected compound obtained above (5 g, 18.29 mmol) in toluene (100 mL) was cooled to 0 °C. Solid potassium *tert*-butoxide (2.05 g, 1.0 equiv) was added portionwise, and the solution was stirred under argon for 45 min at 0 °C. The reaction was quenched by the addition of ice cold 10% aqueous $KHSO_4$ (pH = 2−3). The organic layer was separated from the aqueous layer, and the aqueous layer was extracted three times with CH_2Cl_2. The combined organic fractions were washed with pH = 7 phosphate buffer and brine successively. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give a yellowish oil. The oil was resuspended in CH_2Cl_2 and extracted three times with pH = 10 Na_2CO_3 buffer. The combined aqueous extracts were extracted two more times with CH_2Cl_2. The organic layers were washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give 1.89 g (38%) of the product as an off-white amorphous solid. ^{1}H NMR (300 MHz, DMSO-d_6, 120 °C): δ 1.23 (3H, ddd, J = 1.0, 7.0, 7.0 Hz), 1.40 (12H, s), 1.77 (1H, m), 1.98 (1H, m), 3.36 (1H, m), 3.48 (1H, m), 3.89 (1H, d, J = 2.2 Hz), 4.12 (2H, dddd, J = 2.2, 7.0, 7.0, 7.0 Hz), 4.62 (1H, bs). ^{13}C NMR (100 MHz, DMSO-d_6, 116 °C): δ 13.5, 26.6, 27.4, 37.6, 43.8, 59.1, 68.4, 78.2, 152.8, 168.9. IR (NaCl, neat·): 3446, 3093, 2977, 2935,m 2900, 1743, 1681, 1456, 1403, 1367, 1161, 1097, 1033, 926, 860, 774, 739 cm^1. HRMS (FAB+): Calcd for $C_{13}H_{24}N_1O_5$: 274.165448. Found 274.166420 (M + H).

1-[N-[(1,1-Dimethylethoxy)carbonyl]-2-(1,1-dimethyl-2-propenyl)-D,L-tryptophyl]-3-hydroxy-3-methyl-D,L-proline Ethyl Ester (7). Compound **6a** (1.21 g, 4.43 mmol) was stirred with TFA (6.8 mL) in CH_2Cl_2 (7 mL) at 0 °C. The reaction was allowed to come to room temperature and stir for an additional 3 h. A saturated solution of $NaHCO_3$ was added until the solution became basic, and the organic layer was separated from the aqueous phase. The aqueous layer was extracted three more times with EtOAc, the combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. Compound **6b** (β-hydroxy-β-methylproline ethyl ester) was mixed with compound **5** (1.65 g, 4.43 mmol), BOP reagent (1.96 g, 4.43 mmol), and Et_3N (1.24 mL) in acetonitrile (67 mL) at room temperature for 4 h. A saturated aqueous solution of NaCl was added, and the reaction was extracted four times with EtOAc. The combined organic layers were washed with 2 M HCl, water, 10% $NaHCO_3$ (aq), water, and brine successively. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product was partially purified by flash silica gel column chromatography using 4% MeOH/CH_2Cl_2; the slightly impure mixture of diastereomers **7** (1.88 g, 80%) were directly carried on to the next step without further purification.

3-[[2-(1,1-Dimethyl-2-propenyl)-1*H*-indol-3-yl]methyl]hexahydro-8-hydroxy-8-methylpyrrolo[1,2-*a*]pyrazine-1,4-dione (8). To a solution of **7** (527 mg, 1.00 mmol) in CH_2Cl_2 (2 mL) at 0 °C was added TFA (1.6 mL). The ice bath was removed, and the mixture was allowed to come to room temperature and stir for an additional 3 h. A saturated solution of $NaHCO_3$ was added until the solution became basic, and the organic layer was separated from the aqueous phase. The aqueous layer was extracted three more times with EtOAc, the combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The crude free amine was then dissolved in toluene (5 mL) with 2-hydroxypyridine (19 mg), the solution was refluxed overnight under argon, and the solvent was removed under reduced pressure. The four diastereomers could be partially separated

using PTLC, but in practice the mixture of products was purified as a mixture of diasteromers via radial silica gel chromatography using an elutant of 2% MeOH/CH_2Cl_2 to afford 361 mg (95%) of **8** as a mixture of diastereomers (solid). Data for two of the diastereomers, **8a** and **8d**, are descibed below (relative stereochemistry not assigned); diastereomers **8b** and **8c** could not be separated.

8a: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.57 (6H, s), 1.62 (3 H, s), 1.89 (1H, m), 2.18 (1H, ddd, J = 7.3, 7.3, 13.5 Hz), 2.95 (1H, bs), 3.21, (1H, dd J = 9.7, 15.4 Hz), 3.74 (3H, m), 3.91 (1H, d, J = 1.5 Hz), 4.40 (1H, dd, J = 2.2, 11.3 Hz), 5.19 (1H, dd, J = 0, 17.2 Hz), 5.20 (1H, dd, J = 0, 11.0 Hz), 5.81 (1H, bs), 6.15 (1H, dd, J = 10.9, 16.8 Hz), 7.12 (1H, ddd, J = 1.1, 7.3, 7.3 Hz), 7.19 (1H, ddd, J = 1.5, 7.3, 7.3 Hz), 7.34 (1H, d, J = 8.0 Hz), 7.50 (1H, d, J = 7.7 Hz), 8.10 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.9, 26.1, 27.8, 27.9, 36.9, 39.0, 43.3, 54.5, 65.8, 77.8, 104.5, 110.9, 112.9, 117.8, 120.1, 122.2, 128.0, 132.2, 141.5, 145.6, 166.0, 168.0. IR (NaCl neat) 3360, 3054, 2968, 2924, 1666, 1651, 1462, 1434, 1302, 1262, 1138, 1105, 1010, 919, 734 cm^{-1}. HRMS (FAB+): Calcd. for $C_{22}H_{28}N_3O_3$: 382.213067. Found 382.212574 (M + H). R_f 0.75 (eluted twice with 2% MeOH/CH_2Cl_2).

8d: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.54 (3 H, s), 1.55 (3H, s), 2.04 (2H, m), 2.32 (1H, bs), 2.66 (3H, d, J = 9.2 Hz), 3.25 (1H, dd, J = 9.5, 14.3), 3.46 (1H, dd, J = 14.6, 3.6), 3.53 (2H, m), 3.74 (1H, m), 4.25 (1H, m), 5.16 (1H, dd, J = 1.1, 10.6 Hz), 5.19 (1H, dd, J = 0.8, 17.2 Hz), 6.12 (1H, dd, J = 10.6, 17.6 Hz), 6.18 (1H, bs), 7.12 (2H, m), 7.28 (1H, dd, J = 1.5, 6.6 Hz), 7.53 (1H, dd, J = 1.5, 7.0 Hz), 8.09 (1H, bs). ^{13}C NMR (300 MHz, $CDCl_3$): δ 25.7, 27.7, 28.0, 36.6, 36.9, 39.1, 43.3, 58.2, 65.0, 77.9, 105.1, 110.5, 111.9, 118.4, 119.8, 121.9, 128.9, 134.2, 141.4, 146.0, 166.2, 167.6. IR (NaCl neat) 3340, 3084, 3044, 2970, 2924, 1671, 1658, 1461, 1447, 1372, 1327, 1198, 1138, 1009, 987, 732 cm^{-1}. HRMS (FAB+): Calcd for $C_{22}H_{28}N_3O_3$: 382.213067. Found 382.211498 (M + H). R_f 0.43 (eluted twice with 2% MeOH/CH_2Cl_2).

(3*R*,*S*)-3-[[2-(1,1-Dimethyl-2-propenyl)-1*H*-indol-3-yl]methyl]-2,3,6,7-tetrahydro-8-methylpyrrolo[1,2-*a*]pyrazine-1,4-dione (9). Compound **8** (535 mg, 1.40 mmol) was cooled to 0 °C in THF (5.6 mL) under an argon atmosphere. Pyridine (226 μL, 2.0 equiv) was added, and the solution was stirred for ~15 min. $SOCl_2$ (112 μL, 1.1 equiv) was added, and the mixture was allowed to come to room temperature over 3 h. Water was added to the reaction mixture which was then extracted three times with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product was purified by flash silica gel column chromatography using 2% MeOH/CH_2Cl_2 to afford 381 mg (75%) of compound **9** as a glass. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.56 (6H, s), 2.02 (3H, s), 2.69 (2H, dd, J = 9.2, 9.2 Hz), 3.21 (1H, dd, J = 11.3, 14.6 Hz), 3.72 (1 H, dd J = 3.3, 14.7 Hz), 3.93 (2H, ddd, J = 3.0, 11.7, 11.7 Hz), 4.47 (1H, d, J = 10.6 Hz), 5.17 (1H, dd, J = 0, 10.26 Hz), 5.18 (1H, dd, J = 0, 17.2 Hz), 5.55 (1H, bs), 6.13 (1H, dd, J = 10.6, 17.6 Hz), 7.11 (1H, ddd, J = 1.1, 7.3, 7.3 Hz), 7.18 (1H, ddd, J = 0.7, 7.3, 7.3 Hz), 7.32 (1H, d, J = 7.7 Hz), 7.55 (1H, d, J = 7.7 Hz), 8.07 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.7, 26.8, 28.0, 30.8, 33.9, 39.1, 43.1, 57.0, 104.8, 110.7, 112.4, 118.3, 120.0, 122.0, 128.9, 134.30, 134.33, 141.5, 145.7, 158.1, 162.2. IR (NaCl neat) 3344, 3047, 2968, 1682, 1645, 1440, 1324, 1251, 1114, 1007, 917, 744 cm^{-1}. HRMS (FAB+): Calcd for $C_{22}H_{26}N_3O_2$: 364.2025. Found 364.2032 (M + H). R_f 0.2 (eluted with 2% MeOH/CH_2Cl_2).

(3*R*,*S*)-3-[[2-(1,1-Dimethyl-2-propenyl)-1*H*-indol-3-yl]methyl]-6,7-dihydro-1-methoxy-8-methylpyrrolo[1,2-*a*]pyrazin-4(3*H*)-one (10). A solution of **9** (257 mg, 0.7 mmol) was stirred with $(CH_3)_3OBF_4$ (314 mg, 2.12 mmol, 3.0 equiv) and anhydrous K_2CO_3 (5 equiv, 489 mg, 3.54 mmol, 5.0 equiv) in CH_2Cl_2 (7 mL) for 7 h at ambient temperature under an argon atmosphere. The reaction was poured into ice water and extracted with CH_2Cl_2 three times. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated under reduced pressure, and purified by silica gel column chromatography (eluted with 50% EtOAc/hexanes) to yield 192 mg (72%) of the azadiene **10** as a brittle foam. R_f 0.4 (eluted with 50% EtOAc/hexanes). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.61 (3H, s), 1.62 (3H, s), 1.99 (3H, s), 2.55 (2H, m), 3.09 (1H, dd, J = 9.2, 14.3 Hz), 3.66 (3H, s), 3.79 (3H, m), 4.59 (1H, d, J = 7.0 Hz), 5.15 (1H, dd J = 1.1, 10.3 Hz), 5.18 (1H, dd J = 1.1, 17.2 Hz), 6.15 (1H, dd, J = 10.3, 17.2 Hz), 7.04 (1H, ddd, J = 1.1, 8.1, 8.1 Hz), 7.15 (1H, ddd, J = 1.1, 7.0, 7.0 Hz), 7.26 (1H, d, J = 8.1 Hz), 7.65 (1H, d, J = 7.7 Hz), 7.86 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.7, 27.7, 27.8, 31.4, 34.0, 39.3, 42.8, 52.7, 64.0, 108.1, 109.9, 111.8, 118.6, 119.7, 121.1, 122.8, 124.5, 130.3, 134.1, 140.0, 146.2, 152.6, 166.3. IR (NaCl neat) 3345, 2962,2924, 1676, 1634, 1456, 1335, 1304, 1242, 1051, 917, 741 cm^{-1}. HRMS (FAB+): Calcd. for $C_{23}H_{28}N_3O_2$: 378.217697. Found 378.218152 (M + H).

2,3,11,12,12a,13-Hexahydro-14-methoxy-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(nitrilometheno)-1*H*-indolizino[7,6-*b*]carbazol-5-one (12−15). Azadiene **10** (264 mg, 0.70 mmol) was stirred in MeOH (47 mL) and 20% KOH (aq) (12.6 mL) under an argon atmosphere at 0 °C. The reaction mixture was allowed to come to room temperature and continued to stir for 10 h. When the reaction was complete as indicated by TLC analysis, phosphate buffer (pH = 7) was added until the solution was neutral. The aqueous phase was extracted three times with EtOAc. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The mixture of diastereomers could be partially separated by flash silica gel column chromatography (eluted with 2−4% MeOH/CH_2Cl_2); however, cycloadducts **13** and **14** had to be separated by successive PTLC (eluted with 2% MeOH/CH_2Cl_2). The order of elution was (from fastest mobility to slowest mobility): **15**, **14**, **13**, and finally **12**. Yield: **15**, 23 mg; **14**, 37 mg; **13**, 62 mg; **12**, 85 mg (78% combined yield). Data for each is as follows:

(1*S*,5a*R*,12a*R*,13a*S*)-*rel*-2,3,11,12,12a,13-Hexahydro-14-methoxy-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(nitrilometheno)-1*H*-indolizino[7,6-*b*]carbazol-5-one (12). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.08 (3H, s), 1.22 (3H, d, J = 7.0 Hz), 1.31 (3H, s), 1.64−1.78 (3H, m), 2.16 (1H, m), 2.28 (1H, dd, J = 9.2, 5.5 Hz), 2.90 (1H, m), 3.12 (1H, d, J = 15.7 Hz); 3.26 (1H, m), 3.56 (1H, m), 3.81 (3H, s), 4.03 (1H, d, J = 15.7 Hz), 7.12 (2H, m), 7.27 (1H, d, J = 7 Hz), 7.57 (1H, d, J = 7 Hz), 7.74 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.02, 22.1, 26.6, 27.7, 28.6, 32.4, 34.3, 35.2, 42.4, 46.4, 54.4, 65.7, 66.0, 106.8, 110.2, 118.8, 118.9, 121.2, 127.6, 136.5, 139.5, 171.1, 172.8. IR (NaCl, neat): 3306, 2969, 1668, 1651, 1455, 1428, 1345, 1310, 1194, 995, 740, 714 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{28}N_3O_2$: 378.2181. Found 378.2176 (M + H). R_f 0.40 (eluted twice with 2% MeOH/CH_2Cl_2).

(1*R*,5a*R*,12a*R*,13a*S*)-*rel*-2,3,11,12,12a,13-Hexahydro-14-methoxy-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(nitrilometheno)-1*H*-indolizino[7,6-*b*]carbazol-5-one (13). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.02 (3H, s), 1.26 (3H, s), 1.43 (3H, d, J = 7.0 Hz), 1.73 (1H, m), 1.86 (1H, dd, J = 10.6, 12.5 Hz), 2.11(1H, dd, J = 4.8, 12.5 Hz), 2.11 (1H, m), 2.29 (1H, dd, J = 10.6, 4.8 Hz), 2.38 (1H, m), 3.12 (1H, d, J = 16.1 Hz); 3.25 (1H, m), 3.62 (1H, m), 3.65 (3H, s), 4.06 (1H, d, J = 16.1 Hz), 7.15 (2H, m), 7.31 (1H, d, J = 7 Hz), 7.61 (1H, d, J = 7 Hz), 7.73 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.9, 22.9, 27.8, 28.4, 32.3, 32.9, 35.0, 41.1, 42.8, 47.5, 54.0, 65.8, 66.2, 106.9, 110.3, 118.8, 119.0, 121.9, 127.6, 136.5, 139.6, 172.9, 173.1. IR (NaCl, neat): 3306, 3047, 2951, 1167, 1633, 1462, 1435, 1372, 1311, 1185, 980, 743 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{28}N_3O_2$: 378.2181. Found 378.2163 (M + H). R_f 0.49 (eluted twice with 2% MeOH/CH_2Cl_2).

(1*S*,5a*R*,12a*S*,13a*S*)-*rel*-2,3,11,12,12a,13-Hexahydro-14-methoxy-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(nitrilometheno)-1*H*-indolizino[7,6-*b*]carbazol-5-one (14). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.13 (3H, s), 1.19 (3H, d, J = 7.0 Hz), 1.25 (3H, s), 1.68 (1H, m), 1.88 (1H, dd, J = 9.8, 12.9 Hz), 1.97 (1H, dd, J = 3.9, 12.9 Hz), 2.12 (1H, m), 2.23 (1H, dd, J = 9.8, 3.9 Hz), 2.88 (1H, m), 3.22 (1H, m), 3.29 (1H, d, J = 17.2 Hz), 3.65 (3H, s), 3.68 (1H, m), 3.92 (1H, d, J = 17.2 Hz), 7.09 (2H, m), 7.27 (1H, d, J = 7 Hz), 7.57 (1H, d, J = 7 Hz), 7.94 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 25.2, 25.9, 27.9, 28.4, 32.0, 34.2, 35.0, 42.5, 45.0, 54.2, 65.8, 66.9, 106.1, 110.3, 118.8, 119.4, 121.3, 128.0, 136.4, 139.8, 170.8, 172.1. IR (NaCl, neat, cm^{-1}): 3407, 3312, 3053, 2964, 1667, 1455, 1427, 1345, 1306, 1230, 1180, 1042, 995, 815, 738. HRMS (FAB+) Calcd for $C_{23}H_{28}N_3O_2$: 378.2181. Found 377.2109 (M − H). R_f 0.55 (eluted twice with 2% MeOH/CH_2Cl_2).

(1*R*,5a*R*,12a*S*,13a*S*)-*rel*-2,3,11,12,12a,13-hexahydro-14-methoxy-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(nitrilomethano)-1*H*-indolizino[7,6-*b*]carbazol-5-one (15). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.15 (3H, s), 1.25 (3H, s), 1.41 (3H, d, J = 7.0 Hz), 1.71 (1H, m), 1.86 (1H, dd, J = 9.9, 13.2 Hz), 1.97 (1H, dd, J = 4.4, 13.2 Hz), 2.10 (1H, m), 2.33 (1H, m), 2.38 (1H, dd, J = 9.9, 4.4 Hz), 3.27 (1H, d, J = 17.2 Hz), 3.35 (1H, m), 3.57 (1H, m), 3.65 (3H, s), 3.88 (1H, d, J = 17.2 Hz), 7.10 (2H, m), 7.28 (1H, d, J = 7 Hz), 7.59 (1H, d, J = 7 Hz), 7.70 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 25.3, 26.1, 28.4, 32.9, 33.6, 34.9, 40.7, 43.0, 45.8, 53.8, 66.2, 66.7, 106.4, 110.3, 118.9, 119.2, 121.5, 128.0, 136.4, 139.7, 171.1, 173.4. IR (NaCl, neat): 3407, 3310, 2958, 2918, 1660, 1626, 1461, 1423, 1307, 1283, 1008, 741 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{28}N_3O_2$: 378.2181. Found 378.2173, (M + H). R_f 0.57 (eluted twice with 2% MeOH/CH_2Cl_2).

General Procedure for Lactim Ether Deprotection of Cyclo Adducts (12−15). One equivalent of the lactim ether cycloadduct was stirred in THF (0.025 M) at 0 °C. To this solution was added 0.1 M HCl (3.0 equiv), and the reaction was stirred 5−15 min until starting material was no longer detected by TLC analysis. The reaction was netralized with pH = 7 phosphate buffer and extracted three times with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. In the case of cycloadducts **13** and **15**, the ring opened products **17** and **22** were obtained. In the case of cycloadduct **12**, a small percentage of conversion to the corresponding ring-opened amine methyl ester was sometimes observed. These ring-opened amine methyl esters were recyclized by refluxing in toluene (0.025 M) overnight. The corresponding piperazinedione products (**16**, **18**, **20**, and **23**) were purified using flash silica gel column chromatography (eluted with 2−4% MeOH/CH_2Cl_2). Data for each is as follows:

(1*S*,5a*R*,12a*R*,13a*S*)-*rel*-2,3,11,12,12a,13-hexahydro-1,12,12-trimethyl-5*H*,6H-5a,13a-(iminomethano)-1*H*-indolizino[7,6-*b*]carbazole-5,14-dione (16) from 12. Yield: 16.5 mg (85%). In this instance, a small percentage of the ring-opened amine methyl ester was observed by ^{1}H NMR analysis and TLC. The mixture of ring-opened product and the desired piperazinedione was refluxed overnight in toluene and purifed by PTLC. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.08 (3H, s), 1.20 (3H, d, J = 6.6 Hz), 1.33 (3H, s), 1.67 (1H, m), 1.91 (1H, dd, J = 5.0, 13.4 Hz), 2.03 (1H, dd, J = 10.1, 13.4 Hz), 2.16 (1H, m), 2.25 (1H, dd, J = 3.9, 10.1 Hz), 2.60 (1H, d, J = 15.4 Hz), 3.01 (1H, m), 3.28 (1H, m), 3.58 (1H, m), 3.87 (1H, d, J = 15.4 Hz) 5.84 (1H, bs), 7.11 (2H, m), 7.27 (1H, d, J = 7.3 Hz), 7.50 (1H, d, J = 7.7 Hz), 7.72 (1H, bs). ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.8, 21.3, 23.8, 24.8, 28.1, 31.3, 34.2, 34.7, 42.5, 48.2, 59.2, 66.9, 103.4, 110.7, 117.5, 118.2, 120.6, 126.5, 136.5, 140.7, 168.3, 173.4. IR (NaCl, neat): 3313, 3066, 2960, 2913, 1681, 1455, 1404, 1257, 1187, 1088, 1022, 795, 734, 693 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{25}N_3O_2$: 363.1946. Found 363.1943 (M$^+$). R_f 0.40 (eluted twice with 4% MeOH/CH_2Cl_2).

(1*R*,5a*R*,12a*R*,13a*S*)-*rel*-2,3,11,12,12a,13-Hexahydro-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(iminomethano)-1*H*-indolizino[7,6-*b*]carbazole-5,14-dione (18) from 13 via 17. Yield: 26 mg (63%). Data for **17**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.95 (3H, d, J = 6.6 Hz), 1.28 (1H, d, J = 13.7 Hz), 1.31 (1H, d, J = 13.3 Hz), 1.39 (3H, s), 1.53 (1H, m), 1.56 (3H, s), 1.76 (1H, d, J = 13.3 Hz), 2.00 (3H, m), 2.82 (1H, d, J = 16.4 Hz), 2.88 (1H, d, J = 13.7 Hz), 2.93 (1H, d, J = 16.4 Hz), 3.68 (2H, m), 3.74 (3H, s), 7.02 (1H, m), 7.08 (1H, m), 7.25 (1H, d, J = 7.8 Hz), 7.35 (1H, d, J = 7.8 Hz), 8.11 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 28.4, 29.4, 31.5, 31.9, 33.2, 33.9, 45.2, 45.4, 47.5, 52.3, 58.2, 71.3, 103.5, 110.6, 118.1, 119.3, 121.5, 127.6, 136.3, 138.9, 172.2, 174.4. IR (NaCl, neat): 3352, 2964, 1725, 1681, 1455, 1392, 1262, 1115, 1020, 808, 761, 732 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{30}N_3O_3$: 396.2287. Found 396.2281 (M + H). R_f 0.30 (eluted twice with 4% MeOH/CH_2Cl_2). The ring-opened amine methyl ester **17** was cyclized to the piperazinedione **18** as described above. Data for **18**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.07 (3H, s), 1.29 (3H, s), 1.52 (3H, d, J = 7 Hz), 1.80 (1H,dd, J = 5.0, 13.5 Hz), 1.85 (1H, m), 2.09 (1H, m), 2.30 (1H, m), 2.41 (1H, dd, J = 10.5, 13.5 Hz), 2.58 (1H, dd, J = 5.0, 10.5 Hz), 2.59 (1H, d, J = 15.2 Hz), 3.25 (1H, m), 3.65 (1H, m), 3.83 (1H, d, J = 15.2 Hz), 5.95 (1H, bs), 7.08 (1H, ddd, J = 1.2, 7.8, 7.8 Hz), 7.13 (1H, ddd, J = 0.8, 7.8, 7.8 Hz), 7.26 (1H, d, J = 7.8 Hz), 7.48 (1H, d, J = 7.8 Hz), 7.78 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$ + 1 drop DMSO-d_6): δ 12.9, 22.2, 24.5, 28.0, 29.4, 31.0, 34.5, 41.5, 43.0, 49.3, 59.8, 66.6, 103.9, 110.5, 118.6, 119.7, 121.0, 126.7, 136.4, 139.9, 169.9, 173.5. IR (NaCl, neat): 3664, 3326, 2960, 1677, 1453, 1258, 1092, 799, 703 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{26}N_3O_2$: 364.2025. Found 364.2023 (M + H). R_f 0.30 (eluted twice with 4% MeOH/CH_2Cl_2).

(1*S*,5a*R*,12a*S*,13a*S*)-*rel*-2,3,11,12,12a,13-Hexahydro-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(iminomethano)-1*H*-indolizino[7,6-*b*]carbazole-5,14-dione (20) from cycloadduct 14. Yield: 14.5 mg, (100%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17 (3H, d, J = 7.0 Hz), 1.26 (3H, s), 1.33 (3H, s), 1.66 (1H, m), 1.85 (1H, dd, J = 3.9, 13.7 Hz), 2.02 (1H, dd, J = 10.1, 13.3 Hz), 2.14 (1H, m), 2.25 (1H, dd, J = 3.9, 10.1), 2.89 (1H, d, J = 17.9 Hz), 2.98 (1H, m), 3.26 (1H, m) 3.73 (1H, m), 3.90 (1H, d, J = 17.6 Hz), 5.90 (1H, bs), 7.09 (1H, m), 7.15 (1H, m), 7.30 (1H, d, J = 8 Hz), 7.50 (1H, d, J = 7.8 Hz), 7.89 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 23.8, 25.2, 27.3, 29.0, 31.9, 34.5, 34.6, 42.9, 44.9, 61.2, 68.2, 103.7, 110.7, 118.4, 119.7, 122.1, 127.2, 136.4, 139.6, 169.2, 173.1. IR (NaCl, neat): 3298, 2962, 1682, 1455, 1399, 1302, 1231, 742 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{25}N_3O_2$: 363.1946. Found 363.1949 (M$^+$). R_f 0.40 (eluted twice with 4% MeOH/CH_2Cl_2).

(1*R*,5a*R*,12a*S*,13a*S*)-*rel*-2,3,11,12,12a,13-Hexahydro-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(iminomethano)-1*H*-indolizino[7,6-*b*]carbazole-5,14-dione (23) from Cycloadduct 15 via 22. Yield: 4.7 mg, (49%). Data for **22**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.04 (3H, d, J = 7.0 Hz), 1.34 (3H, s), 1.46 (3H, s), 1.58 (2H, bs), 1.66 (1H, m), 1.90 (1H, dd, J = 9.8, 14.1 Hz), 2.02 (1H, m), 2.05 (1H, dd, J = 9.4, 9.4), 2.18 (1H, m), 3.03 (1H, dd, J = 9.0, 14.1 Hz), 3.13 (1H, d, J = 16.4 Hz), 3.15 (1H, d, J = 16.4 Hz), 3.64 (3H, s), 3.65 (1H, m), 3.73 (1H, m), 7.08 (1H, ddd, J = 7.8, 7.8, 1.2), 7.14 (1H, ddd, J = 7.8, 7.8, 1.2), 7.30 (1H, d, J = 7.8 Hz), 7.52 (1H, d, J = 7.8 Hz), 7.84 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 25.3, 28.2, 29.2, 30.7, 34.1, 35.0, 45.1, 45.2, 47.6, 51.9, 57.1, 68.5, 105.4, 110.5, 118.5, 119.4, 121.8, 128.1, 136.5, 139.8, 172.4, 173.3. IR (NaCl, neat): 3301, 2961, 1732, 1637, 1451, 1220, 735 cm^{-1}. HRMS (FAB+) Calcd for $C_{23}H_{30}N_3O_3$: 396.2287. Found 396.2282 (M + H). R_f 0.6 (eluted with 4% MeOH/CH_2Cl_2). The ring-opened amine methyl ester **22** was cyclized to the piperazinedione **23** as described above. Data for **23**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.29 (3H, s), 1.32 (3H, s), 1.50 (3H, d, J = 7.0 Hz), 1.82 (1H, m), 1.99 (1H, dd, J = 10.5, 13.6 Hz), 2.10 (1H, m), 2.22 (1H, dd, J = 3.5, 13.6 Hz), 2.31 (2H, m), 2.85 (1H, d, J = 17.9 Hz), 3.44 (1H, m), 3.61 (1H, m), 3.87 (1H, d J = 17.9 Hz), 5.73 (1H, bs), 7.10 (1H, t, J = 7.4 Hz), 7.16 (1H, t, J = 7.8 Hz), 7.30 (1H, d, J = 7.8 Hz), 7.50 (1H, d, J = 7.4 Hz), 7.85 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.6, 23.9, 25.4, 29.0, 32.5, 32.8, 34.5, 41.1, 43.3, 45.7, 61.2, 67.7, 103.7, 110.7, 118.4, 119.7, 122.2, 127.1, 136.4, 139.6, 170.2, 172.8. IR (NaCl, neat): 3228, 2925, 1684, 1670, 1570, 1453, 1406, 1291, 871, 737 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{25}N_3O_2$: 363.1946. Found 363.1953 (M$^+$). R_f 0.5 (eluted with 4% MeOH/CH_2Cl_2).

Racemic VM55599 and Diastereoisomers (18, 19, 21). General Procedure for DIBAH Reduction of 16, 18, 20, and 23. The cycloadduct (**16**, **18**, **20**, or **23**) (0.005 M in toluene) was stirred at 0 °C under an atmosphere of argon, and DIBAH (20 equiv as a 1.0 M solution in toluene) was added. The reaction was allowed to come to room temperature and stirred for 24 h. The reaction was again cooled to 0 °C and Na_2SO_4·$10H_2O$ was added slowly until bubbling subsided. The mixture was stirred an additional 30 min and then filtered through a fritted funnel. The solid residue was rinsed with ethyl acetate, and the combined filtrates were evaporated under reduced pressure. The product was isolated via flash silica gel column chromatography or PTLC using 2% MeOH/CH_2Cl_2.

(±)-VM55599. Yield: 13.5 mg, (86%); obtained as an amorphous powder. ^{1}H NMR (400 MHz, $CDCl_3$ + 1 drop DMSO-d_6): δ 1.00 (3H, d, J = 7.0 Hz), 1.31 (3H, s), 1.37 (1H, m), 1.39 (3H, s), 1.73 (1H, dd, J = 11.7, 13.2 Hz), 1.96 (1H, dd, J = 4.3, 13.2 Hz), 2.13 (3H, m), 2.24 (1H, dd, J = 1.6, 10.1 Hz), 2.76 (1H, d, J = 15.2 Hz), 2.90 (1H, d, J = 15.2 Hz), 2.96 (2H, m), 3.45 (1H, d, J = 10.1 Hz), 6.28 (1H, bs), 7.04 (1H, ddd, J = 0.8, 7.8, 7.8 Hz), 7.11 (1H, ddd, J = 1.2, 7.0, 7.0 Hz), 7.29 (1H, d, J = 7.3 Hz), 7.39 (1H, d, J = 7.8 Hz), 8.40 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$ + 1 drops DMSO-d_6): δ 17.5, 24.0, 26.8, 30.1, 30.2, 30.5, 33.0, 34.2, 46.6, 53.6, 55.7,

58.9, 66.4, 104.1, 110.6, 117.7, 119.2, 121.5, 126.8, 136.4, 141.1, 174.8. I. R. (NaCl, neat): 3303, 3048, 2920, 1650, 1454, 1296, 779, 734, 695 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{28}N_3O$: 350.2232. Found 350.2235 (M + H). R_f 0.38 (eluted with 4% MeOH/CH_2Cl_2). This synthetic compound was identical to natural VM55599 (obtained from *Penicillium* sp. IMI332995) by TLC (silica gel, eluted with 4% MeOH/CH_2Cl_2), ^1H NMR, and ^{13}C NMR.

(1*R*,5a*R*,12a*R*,13a*S*)-*rel*-2,3,11,12,12a,13-Hexahydro-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(iminomethano)-1*H*-indolizino[7,6-*b*]carbazol-14-one (19). Yield: 19 mg (79%) obtained as an amorphous powder. ^1H NMR (400 MHz, $CDCl_3$): δ 1.30 (3H, s), 1.38 (3H, s), 1.39 (3H, d, J = 7.3 Hz), 1.66 (1H, m), 1.91 (3H, m), 2.19 (3H, m), 2.28 (1H, m), 2.78 (1H, d, J = 15.2 Hz), 2.89 (1H, d, J = 15.2 Hz), 3.19 (1H, m), 3.45 (1H, d, J = 10.2 Hz), 5.91 (1H, bs), 7.08 (1H, ddd, J = 1.2, 7.4, 7.4 Hz), 7.15 (1H, ddd, J = 1, 7.8, 7.8 Hz), 7.30 (1H, d, J = 7.8 Hz), 7.40 (1H, d, J = 7.4 Hz), 7.86 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.0, 24.0, 29.9, 30.1, 30.5, 30.6, 34.0, 40.4, 46.3, 53.8, 56.7, 59.8, 65.5, 104.6, 110.6, 117.9, 119.5, 121.8, 126.9, 136.3, 140.8, 173.7. IR (NaCl, neat): 3305, 3060, 2924, 1667, 1455, 1368, 1261, 1109, 1014, 801, 741, 706 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{28}N_3O$: 350.2232. Found 350.2235 (M + H). R_f 0.40 (eluted with 4% MeOH/CH_2Cl_2).

(1*S*,5a*R*,12a*S*,13a*S*)-(±)-*rel*-2,3,11,12,12a,13-Hexahydro-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(iminomethano)-1*H*-indolizino[7,6-*b*]carbazol-14-one (21). Yield: 8.5 mg, (61%); obtained as an amorphous powder. ^1H (300 MHz, $CDCl_3$): δ 1.02 (3H, d, J = 7.3 Hz), 1.19 (3H, s), 1.29 (3H, s), 1.42 (1H, m), 1.69 (1H, m), 2.14 (2H, m), 2.22 (1H, m), 2.35 (1H, dd, J = 8.6, 17.2 Hz), 2.67 (1H, d, J = 10.2 Hz), 2.79 (1H, d, J = 17.2 Hz), 2.92 (1H, d, J = 17.2 Hz), 2.96 (1H, m), 3.06 (1H, ddd, J = 2.5, 8.6, 8.6 Hz), 3.14 (1H, d, J = 10.6 Hz), 5.67 (1H, bs), 7.08 (1H, ddd, J = 1.2, 7.4, 7.4 Hz), 7.15 (1H, m), 7.31 (1H, dd, J = 1.1, 7.1 Hz), 7.41 (1H, dd, J = 1.0, 7.7 Hz), 7.87 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.4, 24.8, 27.3, 28.0, 29.2, 30.6, 32.7, 34.3, 45.7, 53.0, 55.2, 62.4, 65.9, 103.2, 110.8, 117.9, 119.6, 121.9, 127.0, 136.3, 141.5, 174.2. IR (NaCl, neat): 3281, 3060, 2960, 1668, 1462, 133, 1123, 1009, 740, 702 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{28}N_3O$: 350.2232. Found 350.2233 (M + H). R_f 0.17 (eluted with 4% MeOH/CH_2Cl_2).

(1*R*,5a*R*,12a*S*,13a*S*)-(±)-*rel*-2,3,11,12,12a,13-Hexahydro-1,12,12-trimethyl-5*H*,6*H*-5a,13a-(iminomethano)-1*H*-indolizino[7,6-*b*]carbazol-14-one (24). Yield: 2.4 mg (54%) obtained as an amorphous powder. ^1H NMR (400 MHz, $CDCl_3$): δ 1.24 (3H, s), 1.29 (3H, s), 1.41 (3H, d, J = 6.6 Hz), 1.89 (3H, m), 2.07 (1H, dd, J = 4, 13 Hz), 2.07 (1H, m), 2.14 (1H, m), 2.43 (1H, m), 2.57 (1H, d, J = 10.5 Hz), 2.77 (1H, d, J = 17.4 Hz), 2.94 (1H, d, J = 17.4 Hz), 3.19 (1H, d, J = 10.5 Hz), 3.22 (1H, m), 5.48 (1H, bs), 7.09 (1H, ddd, J = 0.8, 7.4, 7.4 Hz), 7.15 (1H, ddd, J = 1.2, 7.6, 7.6 Hz), 7.31 (1H, d, J = 7.8 Hz), 7.40 (1H, d, J = 7.8 Hz), 7.81 (1H, bs). ^{13}C NMR (100 MHz, $CDCl_3$ + 1 drop DMSO-d_6): 11.9, 24.5, 25.3, 27.5, 27.9, 28.8, 29.5, 34.4, 37.8, 43.8, 52.7, 54.6, 59.2, 69.5, 101.5, 110.9, 117.8, 119.1, 121.6, 136.5, 140.6, 176.8. IR (NaCl, neat): 3213, 2959, 1694, 1454, 1259, 1022, 797, 702 cm^{-1}. HRMS (FAB+) Calcd for $C_{22}H_{28}N_3O$: 350.2232. Found 350.2235 (M + H). R_f 0.23 (eluted with 4% MeOH/CH_2Cl_2).

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Supporting Information Available: General experimental considerations, tables of complete ^1H NMR NOE data for cycloadducts **12**–**15**, and ^1H NMR spectra of natural and synthetic VM55599. This material is available free of charge via the Internet at http://pubs.acs.org.

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Synthesis and Evaluation of Microtubule Assembly Inhibition and Cytotoxicity of Prenylated Derivatives of *cyclo*-L-Trp-L-Pro

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Abstract—The synthesis of three isoprenylated derivatives of *cyclo*-L-Trp-L-Pro is described. These substances have been evaluated for cytotoxic activity in rat normal fibroblast 3Y1 cells and have also been evaluated in vitro for the inhibition of microtubule assembly.

Introduction

The tryprostatins,[1] cyclotryprostatins[2] and spirotryprostatins[3] (Fig. 1) are a class of prenylated fungal metabolites produced from *Aspergillus fumigatus*. Osada et al. have demonstrated that these substances are cell cycle inhibitors interfering with the G2/M phase progression in G2/M synchronous cultures of tsFT210 cells.[1–5] The primary target of tryprostatin A and cyclotryprostatins A and B are microtubules which induce M-phase specific inhibition and microtubule disassembly. Cell cycle inhibitors are considered to be promising candidates as anticancer drugs and have also received a considerable amount of attention recently as probes of the cell cycle.[4]

The tryprostatin family of secondary metabolites are the consequence of several modes of isoprenylation of the tryptophan moiety of the simple cyclic dipeptide progenitor *cyclo*-L-Trp-L-Pro. The structurally most interesting and complex members of this family are the spirotryprostatins which, curiously, display among the weakest biological activity of this family of cell cycle inhibitors.[5,6] On the other hand, the very simple substance tryprostatin A has been shown to inhibit tau or MAP2-dependent microtubule assembly. The presence of the methoxy group on the aromatic ring reduces the cytotoxicity while enhancing the specificity against microtubule disruptive activities. For example, the IC_{50} of tryprostatin A was determined by MTT assay to be 400 μM whereas tryprostatin B exhibited an IC_{50} value of 4 μM. The interesting profile of cell cycle inhibitory activity displayed by the tryprostatin family has prompted us to investigate the synthesis of some very simple and readily prepared analogues of tryprostatin B.

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Results and Discussion

Synthesis of tryprostatin B analogues

The disposition of the dimethylallyl moiety at the 2-position of tryprostatins A and B that is essential for biological activity, suggested that the display of the isoprene group at either the indole nitrogen or the tryptophyl amide nitrogen, might closely mimic the display of this side-chain in the natural products. We therefore designed and synthesized compounds **5**, **7** and **10**, which can be viewed as analogues of tryprostatin B and evaluated their biological activities. The simple tryprostatin B analogues targeted in this study (compounds **5**, **7** and **10**) were prepared as shown in Schemes 1 and 2.

The simple indole *N*-prenylated compound **5** has been previously reported by Sammes et al. by a five-step procedure starting with *N*-CBz-L-tryptophan.[7] We have devised an alternate, four-step route from *N*-Boc-L-tryptophan (**1**) as shown in Scheme 1. Dimethylallylation of **1** with prenyl bromide in the presence of sodium hydride in DMF yielded the desired *N*-prenylated substance **2** in 68% yield. Peptide coupling of L-proline methyl ester to **2** gave the dipeptide **3** in 69% yield.

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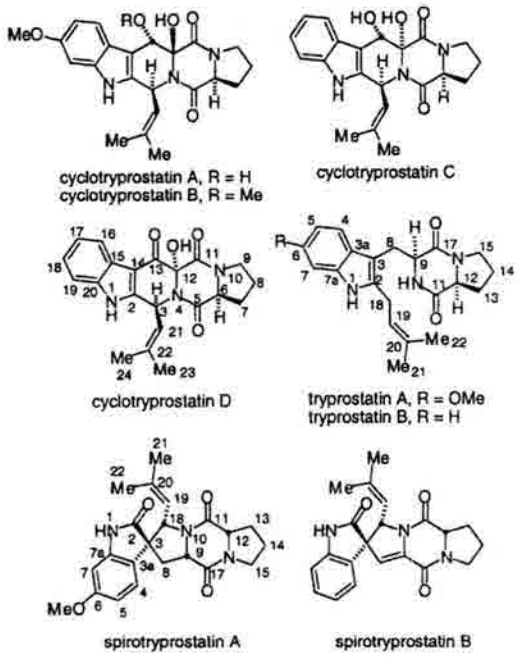
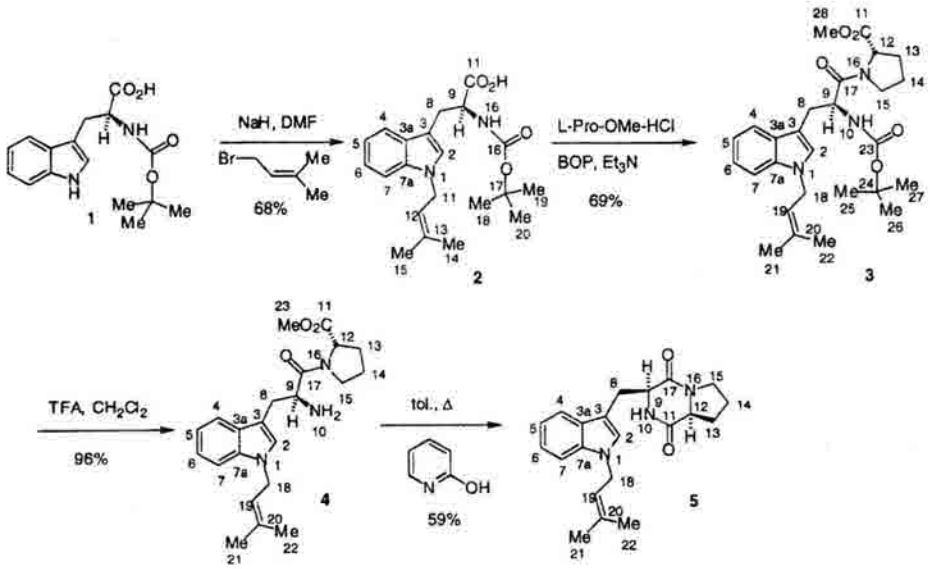


Figure 1.

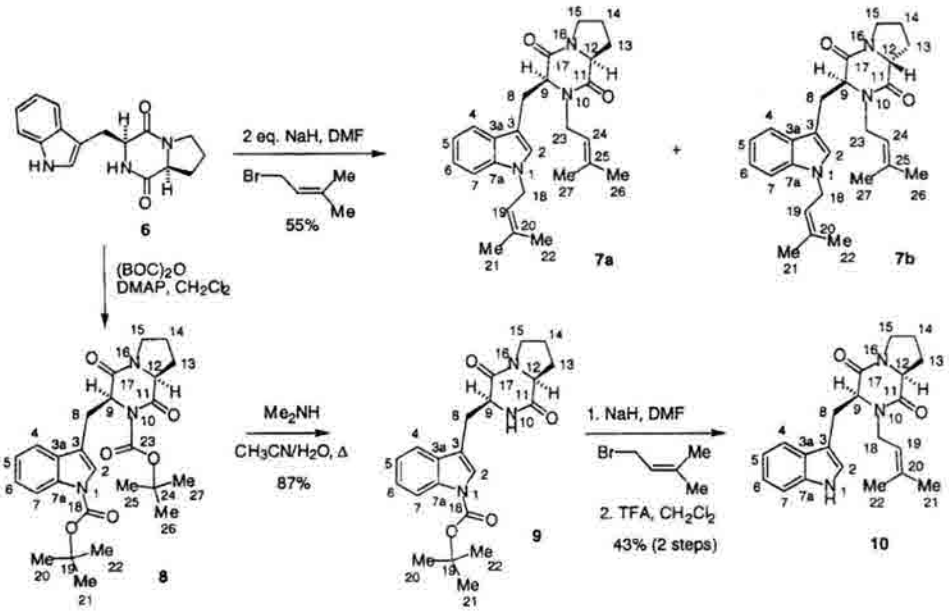
Cleavage of the *t*-Boc group with trifluoroacetic acid in methylene chloride (96%) followed by cyclization of the incipient amino methyl ester (**4**) with 2-hydroxypyridine in hot toluene provided **5** in 59% yield.

The synthesis of compounds **7** and **10** was conducted as shown in Scheme 2 using *cyclo*-L-Trp-L-Pro (**6**, also known as brevianamide F) as the key starting material. Simply treating **6** with 2 equivalents of sodium hydride in DMF in the presence of prenyl bromide furnished **7a** (*syn*-) in 55% isolated, purified yield. In addition, approximately 7% of the *anti*-epimer **7b** was detected by ^{1}H NMR and isolated.[8] The optical purity of compound **7b** was determined by acid hydrolysis (6N HCl, 110 °C, 24 h) and thin layer chromatography (TLC) recovery of proline (silica gel, eluted with EtOH). Chiral high-performance liquid chromatography (HPLC) analysis (Chiralcel WH) of the proline recovered from the hydrolysis reaction indicated a 29:71 ratio of L-Pro:D-Pro. The relative and absolute stereochemical assignment for **7b** was established through independent synthesis of *cyclo*-L-Trp-D-Pro followed by prenylation with 2 equivalents of sodium hydride in DMF in the presence of prenyl bromide that furnished an authentic specimen of **7b**. Thus, base-catalyzed epimerization of the proline residue of **7a** occurs to a more significant extent than that for the tryptophyl residue under the basic conditions of the alkylation reaction. Subjecting the *syn*-compound (**7a**) to the hydrolysis conditions (6N HCl, 110 °C, 24 h) followed by TLC isolation and chiral HPLC analysis as above, revealed a 83:17 ratio of L-Pro:D-Pro. Thus, the optical integrity (83:17 er) of **7a** was partially compromised under the basic prenylation conditions. Despite extensive effort, we were unable to resolve the individual enantiomers of either **7a** or **7b** by chiral HPLC. Both the *syn*- (**7a**) and *anti*- (**7b**) isomers of this diprenylated substance were subjected to cell cycle inhibition and cytotoxicity evaluation.

For the selective installation of the isoprene unit on the amide nitrogen, we found that conversion of **6** to the di-*N*-*t*-Boc substrate **8** (90%) followed by selective removal of the amide-derived *N*-*t*-Boc group could be accomplished by treatment of **8** with dimethylamine in water at reflux temperature which provided **9** in 87% yield.[9] Treatment of **9** with NaH in DMF in the presence of



Scheme 1.



Scheme 2.

Table 1. Biological activity of tryprostatin analogues

Compound	Concentration (μM)	Cell cycle	Cell proliferation (%)	In vitro microtubule assembly (%)[a]
5	500	Slightly toxic		
	250	No effect	176	98.5[c]
7a	50	Toxic	ND[b]	4.6[c]
	25	No effect		93.3[c]
7b	250	Toxic	ND	36.6±11.0[d]
	100	Arrest	84	
	50	No effect	143	
10	500	Slightly toxic		
	250	No effect	154	90.1[c]

[a]250 μM of tryprostatin A exhibited 71.4% assembly in this study.
[b]This compound was toxic to cells at the indicated concentration; thus, cell proliferation percent could not be obtained.
[c]Results are the mean of two independent assays.
[d]Results are the mean ±S.D. (n = three experiments).

prenyl bromide (57%) followed by cleavage of the *t*-Boc group from the indole nitrogen with TFA provided **10** in 76% yield.

Biological activity. The effects of compounds **5**, **7** and **10** on cell cycle control and microtubule assembly were examined and the results are shown in Table 1 and Figure 2.

Compounds **7a** and **7b** were the most toxic compounds of the four tryprostatin B analogues evaluated. Compound **7b** completely inhibited cell proliferation at 100 μM but this inhibition was not cell cycle dependent. Compound **7b** also inhibited microtubule assembly strongly (64% inhibition at 250 μM). Compound **7a** is the most potent compound of those tested displaying 4.6% microtubule assembly at 50 μM as compared to 36.6% for **7b** at 5-fold higher concentration (250 μM). In addition, substance **7a** was highly cytotoxic to cells down to 50 μM concentration (see Table 1 and Fig. 2). Compounds **5** and **10** slightly inhibited cell proliferation at concentrations above 500 μM but neither of these derivatives inhibited microtubule assembly in vitro.

The striking similarity between the biological activity of compounds **7a** and **7b** and tryprostatin B indicate that the corresponding methoxy derivatives should display decreased toxicity and enhanced selectivity for inhibition of the cell cycle similar to that of tryprostatin A. It should also be noted that *cyclo*-L-Trp-L-Pro (brevianamide F, **6**) was completely inactive in the cell cycle inhibition and microtubule assembly assays at 250 μM concentration (data not shown). This clearly indicates the significance of the display of the isoprene moiety as an obligate functional array for the expression of biological activity in this family of alkaloids. The syntheses of the methoxy-substituted substances and related tryprostatin analogues are under investigation and their biological activities will be reported in due course.

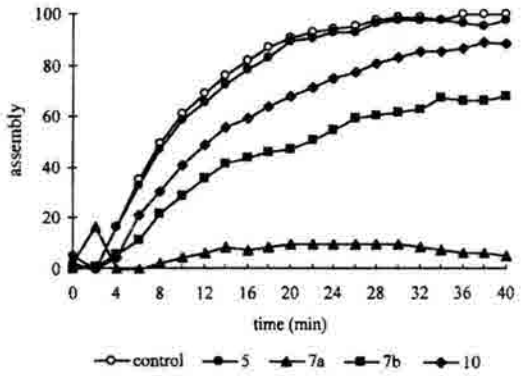


Figure 2. Microtubule assembly assay as determined by turbidity. The concentration of 7a was 50 μM; all other compounds were assayed at 250 μM.

Experimental

Cell culture and proliferation assay

Rat normal fibroblast 3Y1 cells[10] were grown in Dulbecco's modified MEM culture medium supplemented with 10% fetal calf serum under a humidified atmosphere containing 5% CO_2.

Exponentially growing 3Y1 cells were treated with compounds **5**, **7** and **10** for 24 h. The distribution of DNA content was determined by flow cytometry and relative cell numbers (cell number at 24 h per initial cell number at 0 h × 100) were counted. MTT assay is a colorimetric assay using 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide. The cell viability was determined by this assay with minor modifications.[11]

Preparation of microtubule and turbidity assay (in vitro microtubule assembly assay)

Calf brain microtubule protein was prepared by two cycles of assembly–disassembly[12] and stored at −80 °C in Mes buffer (100 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), 1 mM EGTA and 0.5 mM $MgCl_2$ at pH 6.8. Protein concentrations were determined by using the Dc Protein Assay (BioRad, Hercules, CA). Microtubule assembly was monitored by the turbidity assay as described previously.[13] In brief, microtubule protein (2.0 mg/mL in Mes buffer) was incubated at 37 °C and the change in absorbance at 350 nm was monitored over time. To examine the effect of compounds **5**, **7** and **10** on polymerization, the microtubule protein was preincubated with 1% DMSO containing various concentrations of each compound at 0 °C and polymerization was initiated with the addition of 1 mM GTP and with warming to 37 °C. The distribution of DNA content was determined as previously described.[14]

Synthesis of compounds 5, 7 and 10

***N*-Prenyl-*N'*-Boc-L-tryptophan (2).** To a stirred solution of *N*-Boc-L-Trp (**1**) (2.045 g, 6.72 mmol) under an Ar atmosphere in 10 mL of dry DMF at 0 °C (ice bath), was added NaH (733 mg of a 55% oil suspension, 16.80 mmol, 2.5 equiv). The mixture was stirred at 0 °C for 10 min and prenyl bromide (1.502 g, 10.08 mmol, 1.5 equiv, 1.17 mL) was then added. The reaction mixture was stirred for 1 h at 0 °C, then for 2 h at rt. The reaction was quenched with 50 mL of water and washed with 25 mL of hexane. The hexane wash was discarded; the aqueous layer was acidified with 1 M aqueous $NaHSO_4$ until the pH = 3. The mixture was extracted with CH_2Cl_2 (3×25 mL); the organic layers were combined, washed with water (2×25 mL) and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 2.04 g of crude reaction product **2** as a yellowish oil, which was used directly in the following reaction without further purification (68% yield). Attempts to purify this substance through crystallization from several solvents were unsuccessful. Considering that this compound was difficult to purify, a small portion was transformed into its methyl ester in the following reaction for characterization.

***N*-Prenyl-*N'*-Boc-L-tryptophan methyl ester.** To a stirred solution of crude **2** (133 mg, 0.358 mmol) in 1 mL of MeOH at 0 °C, was added a solution of $TMSCHN_2$ (2 M solution in hexane) via syringe until the N_2 evolution ceased and the yellow color was persistent. Removal of the solvent under reduced pressure gave a residue that was purified by means of silica gel column chromatography, using toluene:EtOAc (16:1) as eluent to yield 73 mg of pure ester (53% yield). Optical rotation: $[\alpha]_D = +26.0$ (CH_2Cl_2, *c* 1.23). ^1H NMR ($CDCl_3$, 400 MHz): δ 7.51 (1H, d, $J = 8.0$ Hz, H-7), 7.28 (1H, d, $J = 8.0$ Hz, H-4), 7.18 (1H, dd, $J = 8.0$, 8.0 Hz, H-6), 7.08 (1H, dd, $J = 8.0$, 8.0 Hz, H-5), 6.88 (1H, s, H-2), 5.33 (1H, m, H-19), 5.04 (1H, d, $J = 7.6$ Hz, H-10), 4.62 (3H, m, H-9, H-11, H-11'), 3.66 (3H, s, H-21), 3.25 (2H, brs, H-8, H-8'), 1.80 (3H, br. s, H-21), 1.75 (3H, brs, H-22), 1.42 (9H, s, H-18, H-19, H-20). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.76 (s, C-11), 155.21 (s, C-16), 136.31 (s, C-7a*), 136.14 (s, C-13*), 128.36 (s, C-3a), 126.00 (d, C-2), 121.52 (d, C-6**), 119.84 (d, C-12), 119.05 (d, C-5**), 118.85 (d, C-4), 109.5 (s, C-7), 108.52 (s, C-3), 79.70 (s, C-17), 54.23 (d, C-9), 52.13 (q, C-21), 43.95 (t, C-11), 28.29 (q, C-25, C-26, C-27), 27.91 (t, C-8), 25.63 (q, C-14), 17.99 (q, C-15) (assignments for signals with the same superscript may be interchanged). IR (neat, NaCl): 3391, 3048, 2976, 2931, 1746, 1714, 1613, 1503, 1468, 1391, 1366, 1251, 1209, 1167, 1060, 1014, 856, 778, 739 cm^{-1}. HRMS (FAB+) calcd for $C_{22}H_{30}N_2O_4$: 386.2206; found 386.2202 (M+).

***N*-Prenyl-*N'*-Boc-L-tryptophyl-L-proline methyl ester (3).** To a stirred solution of crude **2** (1.144 g, 3.071 mmol if 100% pure) in 10 mL of THF was added proline methyl ester hydrochloride (1.017 g, 6.142 mmol, 2 equiv). The resulting mixture was cooled to 0 °C and Et_3N (684 mg, 6.756 mmol, 2.2 equiv, 938 μL) was added dropwise via syringe over 5 min. To this mixture was added 1-hydroxybenzotriazole (415 mg, 3.071 mmol, 1 equiv) followed by the addition of DCC in small portions (665 mg, 3.22 mmol, 1.05 equiv). The ice bath was removed, and the temperature was allowed to reach 25 °C. The reaction was complete after 6 h at that temperature (TLC analysis). The reaction was worked up by first filtering

off the DCU, followed by washing with Et_2O (5×10 mL) and combining the filtrate with the washings. The solvents were removed under reduced pressure and the residue was taken up in 100 mL EtOAc. The resulting solution was washed with 5% aqueous $NaHCO_3$ (25 mL), 10% aqueous citric acid solution, again with 5% aqueous $NaHCO_3$ (25 mL), and brine (25 mL), and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 1.47 g of the crude reaction product, which was separated by means of silica gel column chromatography, using hexane:EtOAc 1:1 as eluent to give 1.018 g of pure **3** as a colorless glass (69% yield).

Optical rotation: $[\alpha]_D = -17.6$ (CH_2Cl_2, *c* 1.23). ^{1}H NMR (400 MHz, DMSO-d_6, 80 °C): δ 7.53 (1H, d, *J* = 8.0 Hz, H-4), 7.32 (1H, d, *J* = 8.0 Hz, H-7), 7.13 (1H, s, H-2), 7.09 (1H, dd, *J* = 8.0, 8.0 Hz, H-6*), 6.99 (1H, dd, *J* = 8.0, 8.0 Hz, H-5*), 6.48 (1H, br s, H-10), 5.31 (1H, br dd, *J* = 7.2, 7.2 Hz, H-19), 4.65 (2H, d, *J* = 6.8 Hz, H-18, H-18′), 4.45 (1H, m, H-9), 4.33 (1H, dd, *J* = 8.8, 5.2 Hz, H-12), 3.57 (1H, m, H-15), 3.32 (1H, ddd, *J* = 9.6, 6.4, 6.4 Hz, H-15′), 3.02 (1H, dd, *J* = 14.4, 5.6 Hz, H-8), 2.89 (1H, dd, *J* = 14.4, 8.0 Hz, H-8′), 2.13 (1H, m, H-13), 1.70–1.89 (3H, m, H-13′, H-14, H-14′), 1.77 (3H, br s, H-21**), 1.69 (3H, brs, H-22**), 1.28 (9H, s, H-25, H-26, H-27). ^{13}C NMR (100 MHz, DMSO-d_6, 80 °C): δ 172.88 (s, C-11*), 171.16 (s, C-17*), 136.60 (s, C-23**), 135.77 (s, C-7a**), 128.74 (s, C-20**), 127.60 (s, C-3a), 121.60 (d, C-2), 121.10 (d, C-5), 119.16 (d, C-19), 119.16 (d, C-6), 118.99 (d, C-4), 110.30 (d, C-7), 110.13 (s, C-3), 78.83 (s, C-24), 59.32 (d, C-9), 52.20 (d, C-12), 47.11 (t, C-15), 44.11 (t, C-18), 29.19 (t, C-13), 28.78 (s, C-25, C-26, C-27), 27.83 (t, C-8), 25.85 (q, C-21), 25.21 (t, C-14), 18.36 (q, C-22) (assignments for signals with the same superscript may be interchanged). IR (neat, NaCl): 3433, 3306, 3051, 2975, 2932, 2867, 1747, 1731, 1644, 1496, 1455, 1366, 1251, 1171, 1098, 1054, 1014, 859, 739 cm^{-1}. HRMS (FAB+) calcd for $C_{27}H_{38}N_3O_5$: 484.2811; found 484.2803 (M + H).

***N*-Prenyl-L-tryptophyl-L-proline methyl ester (4).** To a stirred solution of compound **3** (821 mg, 1.698 mmol) in 2 mL of dry CH_2Cl_2 at 0 °C was added 2 mL of TFA under an Ar atmosphere. The mixture was allowed to stir for 6 h. The solvent was removed under reduced pressure at 0 °C and the resulting residue was taken up in 100 mL of EtOAc. The solution was washed with 5% aqueous Na_2CO_3 (25 mL), brine (25 mL) and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 514 mg of crude reaction product, which, by TLC analysis was found to contain a significant amount of the diketopiperazine **4**. For this reason, together with the fact that the purification of this crude mixture proved to be very difficult, the crude product was used directly in the next step (96% crude yield).

***N*-Prenyl-*cyclo*-L-tryptophyl-L-proline (5).**[15] To a stirred solution of crude compound **4** (350 mg, 0.767 mmol) in 10 mL of dry toluene at 0 °C under an Ar atmosphere was added 2-hydroxypyridine (0.1 equiv, 0.077 mmol, 0.7 mg). The resulting solution was refluxed for 6 h. The toluene was then removed under reduced pressure and the resulting residue was purified by means of silica gel column chromatography, using the toluene:EtOAc: MeOH (12:10:1) as eluent to give 151 mg of the pure diketopiperazine **5** (59% yield).

Optical rotation: $[\alpha]_D = -107.9$ (CH_2Cl_2, *c* 0.80). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.61 (1H, d, *J* = 7.5 Hz, H-4), 7.37 (1H, d, *J* = 7.5 Hz, H-7), 7.27 (1H, dd, *J* = 7.5, 7.5 Hz, H-6*), 7.16 (1H, dd, *J* = 7.5, 7.5 Hz, H-5*), 7.05 (1H, s, H-2), 5.88 (1H, br s, H-10), 5.41 (1H, ddqq, *J* = 7.0, 7.0, 1.5, 1.5 Hz, H-19), 4.70 (2H, d, *J* = 7.0 Hz, H-18, H-18′), 4.39 (1H, dd, *J* = 10.6, 3.7 Hz, H-12), 4.09 (1H, dd, *J* = 7.5, 7.5 Hz, H-9), 3.78 (1H, ddd, *J* = 15.0, 3.7, 0.7 Hz, H-8), 3.56–3.70 (2H, m, H-15, H-15′), 2.98 (1H, dd, *J* = 15.0, 10.6 Hz, H-8′), 2.35 (1H, m, H-13), 1.97–2.10 (2H, m, H-13′, H-14), 1.88–1.95 (1H, m, H-14′), 1.88 (3H, br s, H-21), 1.82 (3H, brs, H-22). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.56 (s, C-17*), 165.81 (s, C-11*), 136.96 (s, C-7a**), 136. 84 (s, C-20**), 127.61 (s, C-3a), 126.76 (d, C-2), 122.36 (d, C-5), 119.84 (d, C-19), 119.66 (d, C-6), 118.85 (d, C-4), 110.16 (d, C-7), 108.45 (s, C-3), 59.43 (d, C-9), 54.87 (d, C-12), 45.60 (t, C-15), 44.31 (t, C-18), 28.51 (t, C-13), 27.03 (t, C-8), 25.90 (q, C-21), 22.83 (t, C-14), 18.31 (q, C-22) (assignments for signals with the same superscript may be interchanged). IR (neat, NaCl): 3361, 3244, 3053, 2975, 2923, 2874, 1777, 1668, 1546, 1466, 1374, 1312, 1218, 1168, 1129, 1014, 919, 847, 738, 700 cm^{-1}. HRMS (FAB+) calcd for $C_{21}H_{25}N_3O_2$: 352.2025; found 252.2021 (M + H).

***N,N*-Diprenyl-*cyclo*-L-tryptophan-L-proline (7).**[15] To a stirred solution of *cyclo*-L-Trp-L-Pro (brevianamide F, **6**) (247 mg, 0.872 mmol) in 9 mL of dry DMF under an Ar atmosphere, at 0 °C, was added NaH (77 mg of a 60% oil suspension, 1.919 mmol, 2.2 equiv). The mixture was stirred at 0 °C for 15 min and prenyl bromide (650 mg, 4.36 mmol, 5 equiv, 508 μL) was added. The reaction mixture was stirred for 1 h at 0 °C and then 2 h at rt. The reaction was then quenched with 5% aqueous $NaHCO_3$ (50 mL) and extracted with dichloromethane (2×50 mL). The organic phases were combined, washed with brine (2×50 mL), and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 340 mg of crude reaction product, which was separated by means of radial chromatography on silica gel, using hexane:EtOAc 1:1 as eluent, to give 247 mg of slightly impure product as a slightly yellowish oil (68% yield). Pure **7a** was obtained through column chromatography, using toluene:EtOAc 3:2 as eluent to give 200 mg of **7a** as a colorless oil (55% yield). $R_f = 0.21$. Approximately 7% of the *trans*-epimer, **7b**, was detected by ^{1}H NMR. This compound was purified by PTLC using toluene:EtOAc 3:2 as eluent. $R_f = 0.25$.

(7a) Optical rotation: $[\alpha]_D = -21.5$ ($CHCl_3$, *c* 0.019). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.57 (1H, d, *J* = 7.7 Hz, H-4), 7.20 (1H, d, *J* = 8.1 Hz, H-7), 7.13 (1H, ddd, *J* = 7.0, 7.0, 1.1 Hz, H-6*), 7.04 (1H, ddd, *J* = 7.7, 7.7, 1.1 Hz, H-5*), 6.77 (1H, d, *J* = 2.4 Hz, H-2), 5.26 (1H, ddqq, *J* = 7.0, 7.0, 1.5, 1.5 Hz, H-24), 5.17 (1H, ddqq, *J* = 7.0, 7.0, 1.5, 1.5 Hz, H-19), 4.81 (1H, dd, *J* = 14.7, 5.9 Hz, H-18), 4.59 (2H, d, *J* = 7.0 Hz, H-23, H-23′), 4.29 (1H, dd,

J=4.0, 4.0 Hz, H-9), 3.53–3.68 (3H, m, H-8, H-12, H-18′), 3.36 (1H, ddd, J=6.6, 9.2, 16.1 Hz, H-15), 3.15 (1H, dd, J=14.7, 4.4 Hz, H-8′), 2.89 (1H, ddd, J=4.8, 10.6, 10.6 Hz, H-15′), 1.79 (3H, br s, H-26**), 1.75 (6H, brs, H-27,** H-21), 1.71 (3H, brs, H-22**), 1.66† (1H, m, H-13), 1.27 (1H, m, H-14), 0.68 (1H, m, H-14′),-0.29 (1H, m, H-13′). (†This signal was partly obscured under the methyl group singlets.) ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.41 (s, C-17*), 164.62 (s, C-11*), 138.20 (s, C-7a**), 136. 14 (s, C-25**), 135.60 (s, C-20**), 128.03 (s, C-3a), 127.22 (d, C-2), 121.63 (d, C-5***), 119.77 (d, C-24), 119.69 (d, C-4), 119.00 (d, C-6***), 117.90 (d, C-19), 109.14 (d, C-7), 106.83 (s, C-3), 59.54 (d, C-9), 59.14 (d, C-12), 44.22 (t, C-15), 43.98 (t, C-23), 40.25 (t, C-18), 28.14 (t, C-13), 26.97 (t, C-8), 25.74 (q, C-26****), 25.52 (q, C-21****), 20.49 (t, C-14), 18.02 (q, C-27*****), 17.93 (q, C-22*****) (assignments for signals with the same superscript may be interchanged). IR (neat, NaCl): 3286, 3051, 2969, 2931, 1654, 1464, 1458, 1375, 1363, 1357, 1313, 1265, 1194, 1106, 1032, 1014, 978, 843, 741 cm^{-1}. HRMS (FAB+) calcd for $C_{26}H_{34}N_3O_2$: 420.2651; found 420.2648 (M+H).

The optical integrity of this sample was determined by subjecting the sample to acid hydrolysis (6N HCl, 110 °C, 24 h) followed by evaporation to dryness. The sample was re-suspended in 1 mL of water and the pH was adjusted to neutrality with dilute NaOH. The sample was concentrated to approximately 200 μL and loaded onto an analytical silica gel TLC plate (20×20 cm) and eluted with ethanol. The proline band was scraped off the plate and washed from the powdered silica gel with ethanol. The ethanol was evaporated, dissolved in 1 mL of water and passed through a Dowex 50WX2-100 (H^+) ion-exchange column and eluted with water followed by 2% NH_4OH. The NH_4OH eluate was evaporated and the residue was dissolved in a small volume of water for HPLC analysis. Chiral HPLC analysis was performed on a Chiralcel WH column, eluted with the following solvent system: 28 mL of solvent A (A=*n*-butanol:acetone, 1:1)+12 mL of solvent B (water: acetic acid, 23:7). Co-injection with authentic L-proline and D-proline revealed a ratio of 83:17, L-proline:D-proline. Authentic L-proline was subjected to the same TLC isolation and chiral HPLC analysis procedure as above; chiral HPLC analysis revealed that no racemization attended this procedure.

(7b) Optical rotation: $[\alpha]_D$=+9.1 (CH_2Cl_2, c 0.44). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.53 (1H, d, J=7.7 Hz, H-4), 7.24 (1H, d, J=8.1 Hz, H-7), 7.15 (1H, ddd, J=7.0, 7.0, 1.1 Hz, H-6), 7.06 (1H, m, H-5), 6.83 (1H, s, H-2), 5.24 (1H, m, H-24), 5.14 (1H, m, H-19), 4.62 (2H, d, J=14.7, 5.9 Hz, H-23, H-23′), 4.52 (1H, dd, J=5.9, 14.7 Hz, H-18), 4.19 (1H, dd, J=4.0, 4.0 Hz, H-9), 3.56 (1H, dd, J=8.8, 14.7 Hz, H-18′), 3.42 (2H, m, H-8, H-15), 3.18 (1H, dd, J=4.8, 15.0 Hz, H-8′), 3.15 (1H, ddd, J=2.2, 9.5, 11.5 Hz, H-15′), 2.24 (1H, dd, J=6.2, 10.98 Hz, H-12), 1.90(1H, ddd, J=5.9, 5.9, 11.8 Hz, H-13), 1.80 (3H, br s, H-26*), 1.72 (6H, br. s, H-27,* H-21*), 1.70† (1H, m, H-14), 1.65 (3H, br. s, H-22*), 1.58 (1H, m, H-13′), 1.19 (1H, m, H-14′). (†This signal was partly obscured under the methyl group singlets.) ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.22 (s, C-17*), 165.74 (s, C-11*), 137.70 (s, C-7a**), 136.43 (s, C-25**), 136.43 (s, C-20**), 128.02 (s, C-3a), 127.26 (d, C-2), 121.9 (d, C-5***), 119.9 (d, C-24), 119.3 (d, C-4), 119.07 (d, C-6***), 118.57 (d, C-19), 109.47 (d, C-7), 107.98 (s, C-3), 61.84 (d, C-9), 57.90 (d, C-12), 44.78 (t, C-15), 44.08 (t, C-23), 41.16 (t, C-18), 29.12 (t, C-13), 27.04 (t, C-8), 25.84 (q, C-26****), 25.64 (q, C-21****), 21.66 (t, C-14), 18.02 (q, C-27*****), 17.85 (q, C-22*****) (assignments for signals with the same superscript may be interchanged). IR (neat, NaCl): 3047, 2971, 2920, 2878, 1658, 1447, 1371, 1291, 1207, 979, 743 cm^{-1}.

An independent synthesis of **7b** was conducted following exactly the same procedure as described for the synthesis of **7a** and **7b** from brevianamide F as described below. The *cyclo*-L-trp-D-pro was obtained in 66% overall yield from *N*-*t*-BOC-D-proline. The dialkylation was conducted as described above with prenyl bromide yielding **7b** in 45% yield. $[\alpha]_D$=+33.5 (CH_2Cl_2, c 0.275). Subjecting this sample to the hydrolysis conditions described above followed by chiral HPLC analysis of the recovered proline, revealed a ~85:15 ratio of D-proline: L-proline. Subjecting brevianamide F (*cyclo*-L-Trp-L-Pro) to the acidic hydrolysis conditions, TLC separation of the proline produced and chiral HPLC analysis revealed exclusive formation of L-proline.

***cyclo*-D-Proline-L-tryptophan.** *N*-*t*-Boc-D-proline (73 mg, 0.34 mmol), was stirred with L-tryptophan methyl ester (97 mg, 0.34 mmol), BOP reagent (173 mg, 0.34 mmol) and Et_3N (52 μL, 1.1 equiv) in acetonitrile (5.1 mL) at rt for 3 h. A saturated aqueous solution of NaCl was added and the reaction was extracted four times with EtOAc. The combined organic layers were washed with 2N HCl, water, 10% $NaHCO_3$ (aq), water and brine successively. The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The product was partially purified by flash silica gel column chromatography using 4% MeOH in CH_2Cl_2 as eluant and carried forward in the next step without further characterization. The resultant dipeptide, *N*-*t*-Boc-D-Pro-L-Trp-OMe, was dissolved in CH_2Cl_2 (0.5 mL) and cooled to 0 °C. Trifluoroacetic acid (0.5 mL) was added, the ice bath was removed and the mixture was stirred for an additional 3 h. A saturated solution of $NaHCO_3$ was added until the solution became basic and the organic layer was separated from the aqueous phase. The aqueous layer was extracted three more times with EtOAc and the combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The crude free amine was then dissolved in toluene (1.25 mL) with 2-hydroxypyridine (4 mg) and the solution was refluxed overnight under argon. The solvent was removed under reduced pressure and the diketopiperazine was purified by flash column chromatography using 4% MeOH/CH_2Cl_2 as the eluant. Precipitation from ethyl acetate and hexanes gave an amorphous white powder. Yield: 63 mg, (66%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.41 (1H, m), 1.70 (1H, m), 1.81 (1H, m), 2.07 (1H, m), 2.83 (1H, dd, J=6.6, 10.6 Hz), 3.15 (1H, m) 3.18 (1H, dd, J=4.0, 14.6× Hz), 3.39 (1H, dd, J=6.2, 14.6 Hz), 3.54 (1H,

ddd, J=8.8, 8.8, 12.1 Hz), 4.23 (1H, ddd, J=4.0, 4.0, 6.6 Hz), 5.92 (1H, bs), 7.04 (1H, d, J=2.2 Hz), 7.11 (1H, ddd, J=1.1, 8.0, 8.0 Hz), 7.18 (1H, ddd, J=1.1, 8.0, 8.0 Hz), 7.34 (1H, d, J=8.0 Hz), 7.60 (1H, d, J=8.0 Hz), 8.17 (1H, bs). ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.6, 29.0, 30.8, 45.1, 57.9, 58.4, 109.4, 111.2, 118.8, 119.9, 122.5, 124.1, 126.9, 136.1, 165.3, 169.3. I.R. (NaCl neat) 3260, 2924, 2884, 1651, 1455, 1338, 1302, 1104, 1010, 743 cm^{-1}. $[\alpha]_{D25}$ = +50.9° (*c* 0.23, CH_2Cl_2). HRMS (FAB+) calcd for $C_{16}H_{18}N_3O_2$: 284.1399; found 284.1389.

***N,N'*-Di-Boc-*cyclo*-L-tryptophan-L-proline (8).**[15] Crystalline *cyclo*-L-Trp-L-Pro (brevianamide F, **6**) (1.5 g, 5.298 mmol) was suspended in 10 mL of dry CH_2Cl_2 under an Ar atmosphere. To this suspension, dimethylaminopyridine (DMAP) (64.7 mg, 0.53 mmol, 0.1 equiv) and triethylamine (1.072 g, 10.60 mmol, 1.48 mL, 2 equiv) were added. The mixture was cooled to −18 °C (ice-salt bath), and di-*tert*-butyldicarbonate ($(Boc)_2O$) (2.312 g, 10.60 mmol, 2 equiv) was added in one portion. The mixture was stirred at that temperature for 30 min for 2 h at 0 °C and 2 h at rt. The reaction was diluted with 100 mL of dichloromethane and washed with 1% aq $KHSO_4$ soln (2×50 mL), then with 5% $NaHCO_3$ aq soln (50 mL) and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave 2.63 g of crude reaction product, which was separated by silica gel column chromatography, using toluene: EtOAc 2:1 as eluent to give 2.30 g of **8** as a viscous colorless oil (90% yield).

$[\alpha]_D$ = +77.21 (CH_2Cl_2, *c* 1.18). IR (neat, NaCl): 2980, 2934, 2878, 1777, 1731, 1667, 1457, 1455, 1369, 1323, 1292, 1257, 1155, 1084, 1018, 965, 917, 852, 768, 748, 732 cm^{-1}. HRMS (FAB+) calcd for $C_{26}H_{33}N_3O_6$: 483.2369; found 483.2368 (M+).

In DMSO-d_6 at 80 °C, this compound appears as a mixture of two predominant rotamers in a ~2:1 proportion that interconvert slowly on the NMR time scale. For that reason, both ^{1}H and ^{13}C spectra include the signals for both major and minor rotamers.[16] ^{1}H NMR ($CDCl_3$, 25 °C, 400 MHz): major rotamer: δ 8.08 (1H, d, J=7.9 Hz, H-7), 7.47 (1H, d, J=7.9 Hz, H-4), 7.27 (1H, t, J=7.0 Hz, H-6) 7.25 (1H, bs, H-2), 7.19 (1H, t, J=7.3 Hz, H-5), 4.93 (1H, dd, J=2.6, 4.7 Hz, H-9), 3.84 (1H, dd, J=6.2, 11.1 Hz, H-12), 3.59 (1H, m, H-15), 3.56 (1H, dd, J=2.9, 14.7 Hz, H-8), 3.46 (1H, dd, J=5.0, 14.7 Hz, H-8′) 3.09 (1H, ddd, J=4.4, 1,.3, 10.3 Hz, H-15′), 1.77 (1H, m, H-13), 1.63 (9H, s, H-20, H-21, H-22), 1.56 (9H, s, H-25, H-26, H-27), 1.48 (1H, m, H-14), 1.25 (1H, m, H-14′). 0.11 (1H, m, H-13′); minor rotamer: δ 8.10 (1H, d, J=7.9 Hz, H-7), 7.52 (1H, d, J=7.6 Hz, H-4), 7.57 (1H, bs, H-2), 7.29 (1H, ddd, J=1.2, 7.3, 7.3 Hz, H-6), 7.22 (1H, ddd, J=0.9, 7.3, 7.3 Hz, H-5), 5.03 (1H, dd, J=5.6, 5.6 Hz, H-9), 3.48 (1H, m, H-15), 3.31 (3H, m, H-8, H-8′, H-15′), 3.13 (1H, dd, J=6.5, 9.4 Hz, H-12), 2.11 (1H, ddd, J=5.9, 5.9, 10.9 Hz, H-13), 1.84 (2H, m, H-13′, H-14), 1.64 (9H, s, H-20, H-21, H-22), 1.45(1H, m, H-14′), 1.40 (9H, s, H-25, H-26, H-27). ^{13}C NMR ($CDCl_3$, 25 °C, 100 MHz): major rotamer: δ 165.85 (s, C-17*), 164.02 (s, C-11*), 150.98 (s, C-18**), 149.30 (s, C-23**), 134.14 (s, C-7a*), 130.16 (s, C-3a*), 126.16 (d, C-2), 124.73 (d, C-6), 122.58 (d, C-5), 119.64 (d, C-4), 115.01 (d, C-7), 113.71, (s, C-3), 84.07 (s, C-19), 83.73 (s, C-24), 60.53 (d, C-12), 60.21 (d, C-9), 44.73 (t, C-15), 28.89 (t, C-8), 28.89 (t, C-13, q, C-20, C-21, C-22***), 27.59 (q, C-25, C-26, C-27***), 20.57 (t, C-14) (*assignments for signals with the same superscript may be interchanged) minor rotamer: δ 167.25 (s, C-17*), 164.95 (s, C-11*), 150.11 (s, C-18**), 149.35 (s, C-23**), 135.38 (s, C-7a*), 129.90 (s, C-3a*), 125.25 (d, C-2), 124.81 (d, C-6), 122.73 (d, C-5), 118.94 (d, C-4), 115.27 (d, C-7), 114.29, (s, C-3), 84.34 (s, C-19), 83.96 (s, C-24), 61.13 (d, C-9), 59.20 (d, C-12), 45.18 (t, C-15), 29.16 (t, C-13), 28.09 (q, C-20, C-21, C-22***), 27.95 (t, C-8), 27.65 (q, C-25, C-26, C-27***), 21.95 (t, C-14) (*assignments for signals with the same superscript may be interchanged).

***N*-Boc-*cyclo*-L-tryptophan-L-proline (9).**[15] To a stirred suspension of *N,N'*-di-Boc-*cyclo*-L-Trp-L-Pro (**8**) (2.00 g, 4.135 mmol) in 100 mL of MeCN under an Ar atmosphere was added dimethylamine (40% aq soln, 3.0 mL). The solution was heated to reflux for 90 min. The solvent was removed under reduced pressure and the residue was separated by means of silica gel column chromatography, using hexane:EtOAc:MeOH (4:5:1) as eluent to give 1.48 g of **9** as a colorless glass (93% yield). This fraction was further purified using column chromatography, with CH_2Cl_2:MeOH 25:1 as eluent to give 1.39 g of pure **9** as a colorless glass (87% yield).

Optical rotation: $[\alpha]_D$ = +4.45 (CH_2Cl_2, *c* 0.88). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.05 (1H, br d, J=8.0 Hz, H-7), 7.51 (1H, d, J=7.6 Hz, H-4), 7.44 (1H, s, H-2), 7.25 (1H, ddd, J=7.6, 7.6, 1.2 Hz H-6), 7.17 (1H, ddd, J=7.6, 7.6, 1.2 Hz, H-5), 7.11 (1H, brs, H-10 (N-H)), 4.19 (1H, ddd, J=6.4, 4.4, 4.4 Hz, H-9), 3.51 (1H, ddd, J=8.8, 8.8, 12.0 Hz, H-15), 3.15–3.25 (3H, m, H-8, H-12, H-15′), 3.13 (1H, dd, J=14.8, 4.8 Hz, H-8′), 2.09 (1H, ddd, J=6.4, 6.4, 11.8 Hz, H-13), 1.83 (1H, m, H-14), 1.71 (1H, m, H-13′), 1.58 (9H, s, H-20, H-21, H-22), 1.51 (1H, m, H-14′). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.44 (s, C-17*), 165.56 (s, C-11*), 149.68 (s, C-18), 135.57 (s, C-7a*), 130.05 (s, C-3a*), 125.48 (d, C-2), 124.90 (d, C-6), 122.89 (d, C-5), 119.30 (d, C-4), 115.39 (d, C-7), 114.71, (s, C-3), 84.16 (s, C-19), 58.16 (d, C-9), 57.97 (d, C-12), 45.45 (t, C-15), 30.49 (t, C-13), 29.10 (q, C-20, C-21, C-22), 28.34 (t, C-8), 21.86 (t, C-14) (assignments for signals with the same superscript may be interchanged). IR (neat, NaCl): 3236, 2979, 2927, 2876, 1732, 1664, 1452, 1370, 1332, 1308, 1256, 1228, 1159, 1109, 1085, 1017, 865, 765, 747, 729, 699 cm^{-1}. HRMS (FAB+) calcd for $C_{21}H_{25}N_3O_4$: 383.1845; found 383.1842 (M+).

***N*-Boc-*N'*-prenyl-*cyclo*-L-tryptophan-L-proline.**[15] To a stirred solution of compound **9** (925 mg, 2.412 mmol) in 4 mL of dry DMF under an Ar atmosphere at 0 °C, NaH was added (58 mg of a 60% oil suspension, 2.412 mmol, 1 equiv). The mixture was stirred at 0 °C for 30 min and prenyl bromide (719 mg, 4.83 mmol, 2 equiv, 502 μL) was added. The reaction mixture was stirred for 1 h at 0 °C and then 2 h at rt. The reaction was then quenched with 5% aqueous $NaHCO_3$ (50 mL)

Reverse Prenyl Transferases Exhibit Poor Facial Discrimination in the Biosynthesis of Paraherquamide A, Brevianamide A, and Austamide

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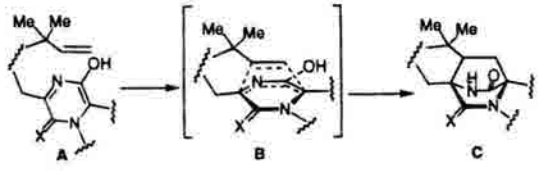
Abstract: The mode of attachment of dimethylallyl pyrophosphate (DMAPP) in the biosynthesis of the indole alkaloids paraherquamide A, austamide, and brevianamide A has been studied. Feeding experiments on *Penicillium fellutanum*, *Penicillium brevicompactum*, and *Aspergillus ustus* using [$^{13}C_2$]-acetate showed isotopic scrambling of the geminal methyl groups originating from C-2 of the indole ring precursors in paraherquamide A, brevianamide A, and austamide biosynthesis. The labeling patterns suggest that the methyl groups of dimethylallyl pyrophosphate become equivalent during the biosyntheses; a non-face-selective S_N' mechanism has been invoked to account for these observations.

Introduction

The indole alkaloids are a large, structurally and biologically diverse group of compounds.[1] Among the classes of indole alkaloids, the paraherquamides (**1**−**10**, Figure 1)[2] comprise a family of fungal metabolites that, together with sclerotamide (**11**),[3] marcfortine A (**12**),[4] asperparaline A (**14**, also named aspergillimide, and **15**, SB202327),[5] and the brevianamides (**16** and **17**),[6] have recently attracted much attention due to their anthelmintic, paralytic, and insecticidal activities.[2−6] Also fascinating in this family are the pathways which lead to the biosynthesis of these complex and structurally unique substances. These secondary metabolites are the consequence of mixed biogenetic origins, being derived from the oxidative polycyclization of amino acids and isoprene units. Especially interesting in this regard is the emerging body of evidence that supports the notion that the bicyclo [2.2.2] core structural motif common to **1**−**17** is formed by a biosynthetic intramolecular [4+2] cycloaddition of the isoprene-derived olefin across an azadiene moiety derived from a preformed, oxidized piperazinedione (**A** → **B** → **C**), as shown in Scheme 1.[7,8] Such pericyclic reactions are quite rare in nature, and in only a few cases has experimental evidence been obtained to support the intermediacy of a Diels−Alder-type of cycloaddition.[8,9] Our research efforts have thus focused on the biogenesis of these compounds, with particular emphasis on the key cycloaddition step.

Scheme 1



In the case of these metabolites, the intriguing possibility of a Diels−Alder cycloaddition necessitates the formation of a "reverse" prenylated intermediate in which dimethylallylpyrophosphate (DMAPP) suffers electrophilic attack not at C-1, the phosphorylated end, but at C-3, which bears the geminal methyl groups; this is formally an S_N2' process. The existence of this type of intermediate was first alluded to in 1971 when deoxybrevianamide E (**20**) was isolated from the austamide-producing (**21**) cultures of *Aspergillus ustus* by Steyn.[10] Further

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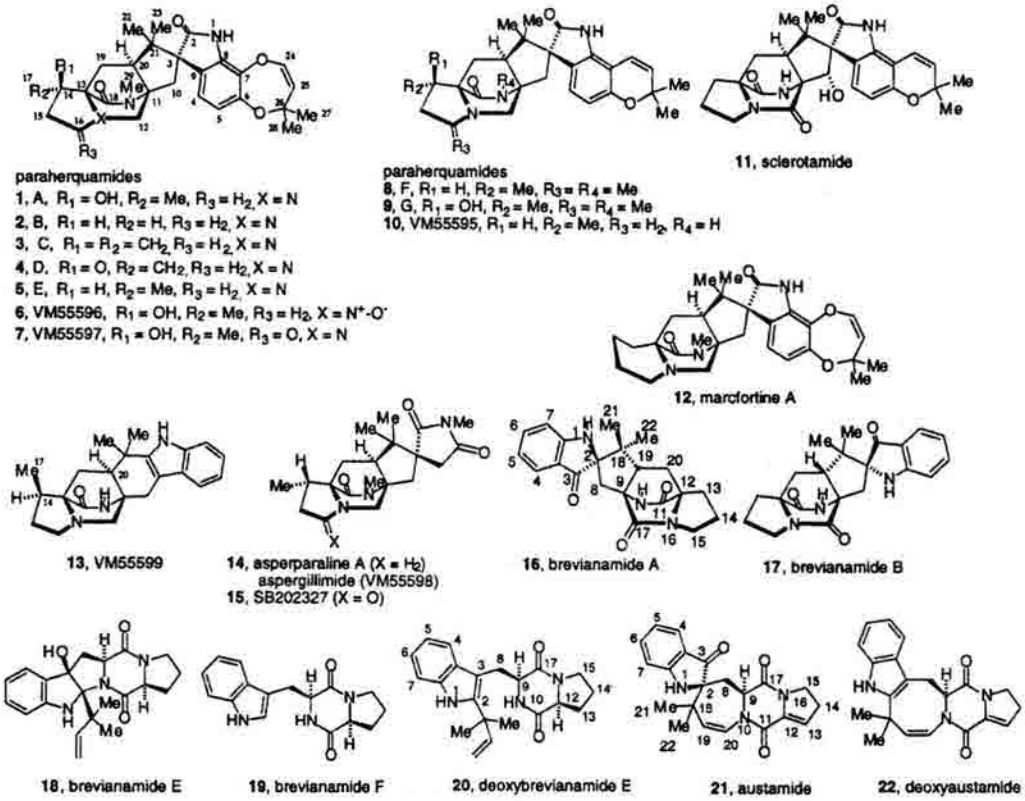

Figure 1.

evidence for a "reverse-prenylated" intermediate was reported by Williams and co-workers, who were able to experimentally demonstrate significant incorporation of synthetic tritiated deoxybrevianamide E (**20**) into brevianamide A (**16**), brevianamide B (**17**), and brevianamide E (**18**) in cultures of *Penicillium brevicompactum*.[8d,e]

It is interesting to note that deoxybrevianamide E, as a potential simple progenitor of the family of alkaloids displayed in Figure 1, is not the only "reverse" prenylated natural indole alkaloid to have been described in the literature. Several other natural substances such as roquefortine (**23**, Figure 2), isolated from *Penicillium roqueforti*, and some members of the echinulin family (**24**), isolated from *Aspergillus amstelodami*, as well as oxaline (**25**) and aszonalenin (**26**) and numerous other alkaloids have been described that also contain the "reverse" prenyl group.[11] The irregular structure of the "reverse" isoprene group in these and other metabolites has led to considerable speculation as to the mechanism of reverse prenylation in the biosynthesis of these substances.[1]

One possible mode of reverse prenylation that has been advanced for the biosynthesis of both roquefortine and the echinulins involves an aza-Claisen-type rearrangement from an *N*-prenylated indole (**27** → **28** via **29**), as shown in Scheme 2. This was first proposed by Barrow et al. in 1979 for the biosynthesis of roquefortine.[12] Thus, aza-Claisen rearrangement of **27** would yield the reverse prenylated 3-indolenine **29**, which could subsequently suffer 1,2-migration of the dimethylvinyl carbon substituent followed by loss of the C-2 hydrogen atom of the indole nucleus to yield **28**. Separate experimental observations to probe this mode of reverse prenylation have cast some doubt on this type of mechanism for both roquefortine and the echinulins.[1,13-15]

In the case of roquefortine, Bhat et al. showed retention of deuterium from C-2 of L-[2,4,5,6,7-$^2H_5$]tryptophan (**30**) at C-6 of roquefortine, thus questioning the intermediacy of **28** in the biosynthesis of this metabolite.[13]

It is of further significance that Gorst-Allman et al. observed partial scrambling (~2:1 ratio) of the ^{13}C-label derived from

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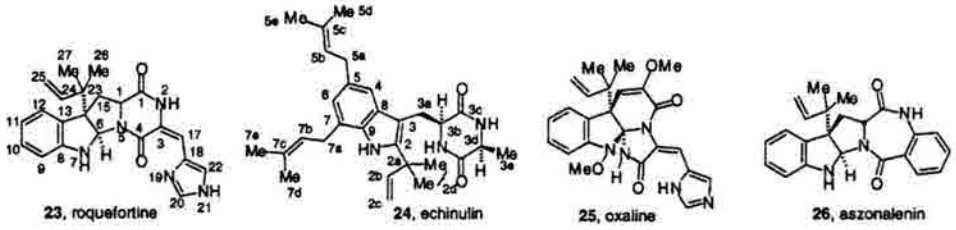

Figure 2.

[$^{13}C_2$]-acetate in the geminal carbons of roquefortine. To the best of our knowledge, this was the first reported experimental data that revealed a nonface-selective reverse prenylation. The same group also inferred the aza-Claisen pathway as a possible mechanism to accommodate these observations.[15]

In the instance of the echinulins, Grundon et al. concluded that the aza-Claisen type mechanism may not be operative in this system due to the lack of incorporation of the tritiated *N*-prenyl precursors 1-([1-^{3}H]-3,3-dimethylallyl)-L-tryptophan and *cyclo*-L-alanyl-1-([1-^{3}H]-3,3-dimethylallyl)-L-tryptophan (corresponding to **27**).[14]

Another possibility for the attachment of the "reverse" prenyl group, and perhaps the simplest explanation, is a direct formal S_N2' mechanism. This was first proposed by Bhat et al. for attachment of the dimethylallyl moiety at C-3 of the indole in roquefortine;[13] the direct S_N' mechanism was also postulated by our group with regard to the biosynthesis of the reverse prenyl unit in paraherquamide A (**1**).[16]

During the course of our investigations into the biosynthesis of paraherquamide A (**1**), we initially set out to determine if the isoprene units of paraherquamide were derived via the well-known mevalonic acid pathway,[17] or via the more recently discovered deoxyxylulose pathway.[18] Through feeding experiments with U-^{13}C-glucose and [$^{13}C_2$]-acetate we found that the isoprene units of paraherquamide A (**1**) arise from the classical mevalonic acid pathway, but we also found an unexpected stereochemical distribution of the isotopically enriched geminal methyl groups derived from DMAPP.[16] Through an analysis of the coupling of intact C_2 units by ^{13}C NMR spectroscopy, we determined that the stereochemical integrity of DMAPP is maintained in the assembly of the dioxepin moiety. On the other hand, we observed a scrambling of the ^{13}C labels for the geminal methyl groups at C-22 and C-23, which indicates a loss of stereochemical integrity during the biosynthetic construction of this quaternary center. This unanticipated result indicates that the reverse prenylation of the indole ring occurs via a nonface-selective mechanism. We have subsequently explored the stereochemical integrity of the reverse prenyl groups in the related yet simpler natural products, brevianamide A (**16**) and austamide (**21**), and report these results here along with full details of the previously communicated paraherquamide A (**1**) studies.[16]

Results and Discussion

The biosynthesis of both austamide (**21**), a metabolite of *Aspergillus ustus*, and brevianamide A (**16**), a metabolite of *Penicillium brevicompactum* and several related *Penicillium* sp., is thought to proceed through the intermediacy of deoxybrevianamide E (**20**), as shown in Scheme 3.[1,8,10] Thus, "reverse" prenylation of *cyclo*-L-Trp-L-Pro (**19**, brevianamide F) produces deoxybrevianamide E, which is converted by distinct modes of oxidative cyclization into brevianamides A and B in *Penicillium* sp., or into austamide (**21**) in *Aspergillus ustus*. The intermediacy of deoxybrevianamide E in the biosynthesis of brevianamides A and B has been confirmed through incorporation of tritium-

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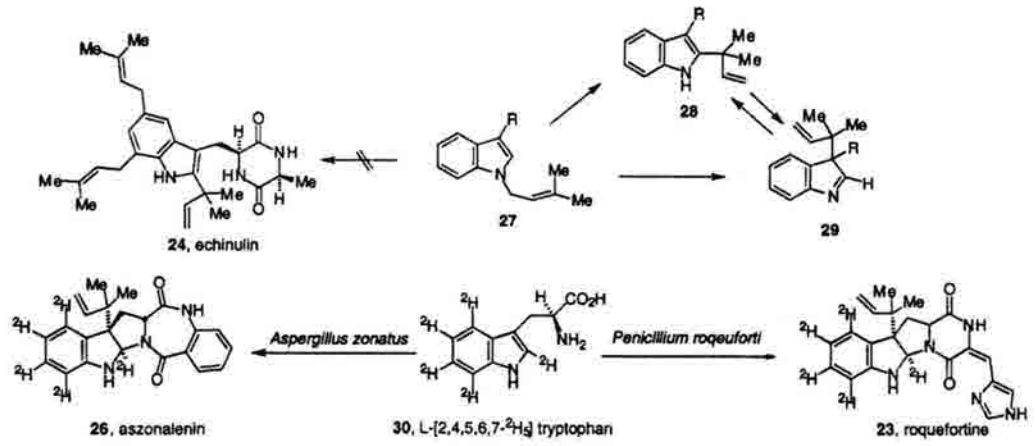
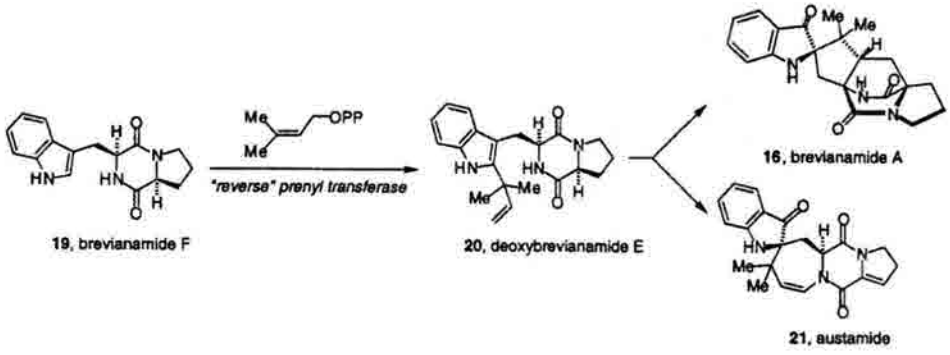
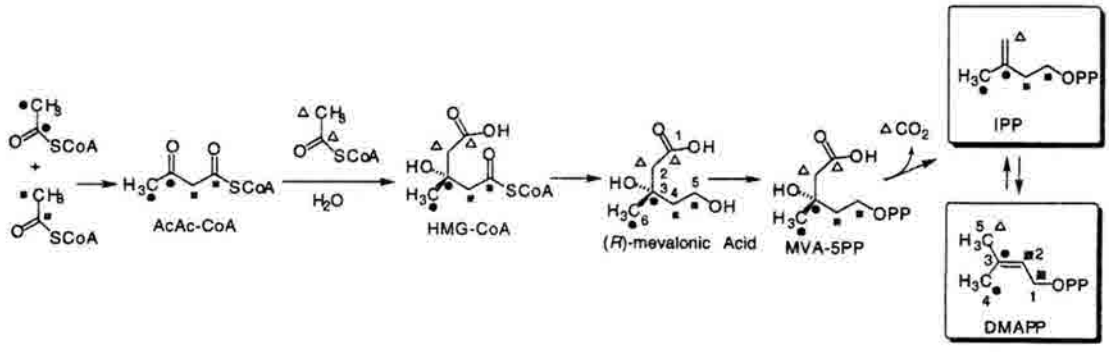
Scheme 2



Scheme 3



Scheme 4. The Classical Mevalonic Acid Pathway Showing the Labeling Pattern via 1,2-Doubly Labeled Acetate

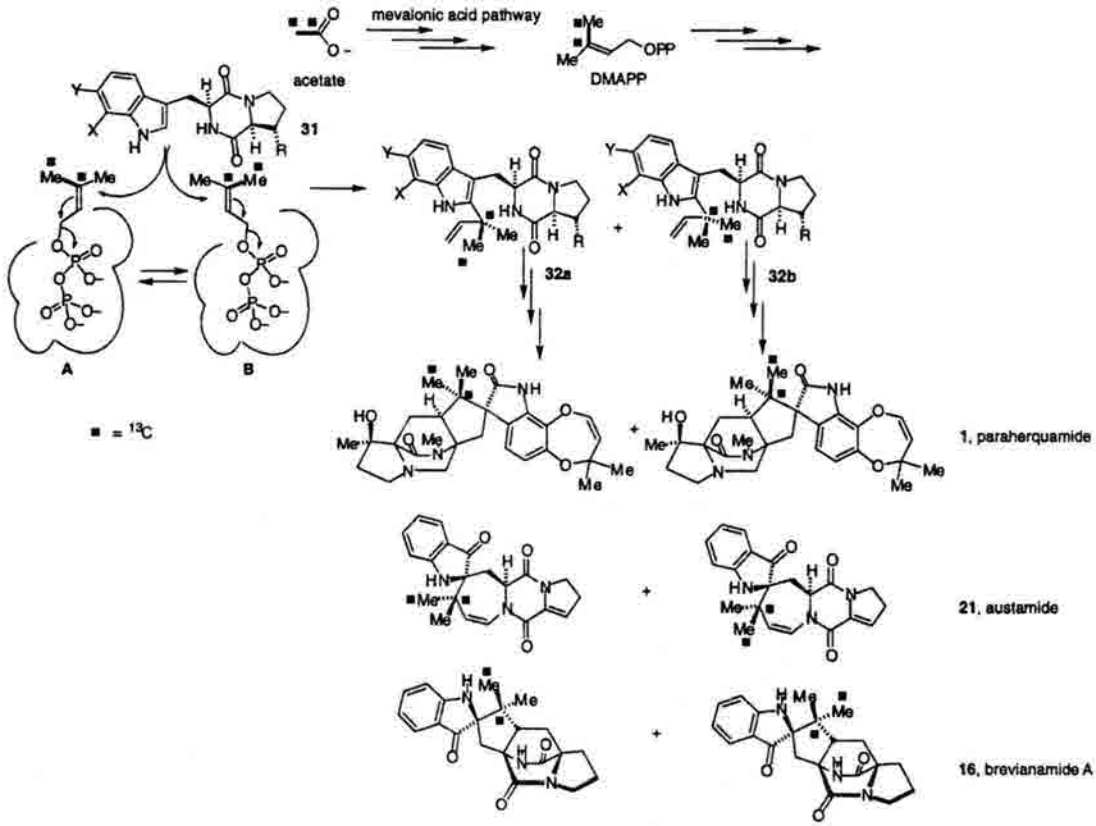


labeled deoxybrevianamide E in cultures of *Penicillium brevicompactum* as reported from this laboratory.[8d,e] In the case of austamide (**21**), deoxybrevianamide E is a co-metabolite that has been isolated along with deoxyaustamide (**22**) from cultures of *Aspergillus ustus*. The involvement of this substance in this pathway is presently based only on this circumstantial evidence.[10]

DMAPP arising from the mevalonic acid pathway is derived from three C-2 units followed by loss of a C-1 unit, as shown in Scheme 4. Thus, incorporation of [$^{13}C_2$]-acetate into isoprenylated metabolites should exhibit coupling between C-1 and C-2 and between C-3 and C-4 of DMAPP; however, no coupling should be observed between C-3 and C-5 since it has been demonstrated that the *E*-methyl group of DMAPP is derived from C-2 of mevalonic acid (i.e., the methyl group of mevalonic acid becomes C-4 of DMAPP; see Scheme 5).[17]

Paraherquamide A (**1**) contains two isoprene units, one comprising C-24 to C-28 in the dioxepin ring, and one comprising C-19 to C-23, which constitutes the bicyclo[2.2.2] ring system. Since [$^{13}C_2$]-acetate fed to *Penicillium fellutanum* was incorporated into both of these isoprene units as intact C-2 units, this unambiguously confirms that the paraherquamide A-producing fungi constructs the primary isoprene units via the classical mevalonic acid pathway (Tables 1 and 2). With respect

Scheme 5. A Possible Biosynthetic Sequence that May Explain How the Geminal Methyl Groups Are Rendered Equivalent in the Biosynthesis of Paraherquamide A (**1**), Austamide (**21**), and Brevianamide A (**16**)[a]



[a] The two black squares represent one intact C_2 unit from acetate, incorporated in C-3/C-5 of individual DMAPP molecules, and in C-21, C-22, and C-23 of **1** and C-18, C-21, and C-22 for both **16** and **21**. For the sake of clarity and simplicity, the labels that would appear in other positions are not represented.

Table 1. Specific Incorporations, Chemical Shifts, and Coupling Constants for the C_5 Carbon Atoms of Paraherquamide A (**1**) in the Feeding Experiment with [$^{13}C_2$]-Acetate

paraherquamide C no.	δ	J_{C-C} (Hz)	% ^{13}C at each C[a]	% ^{13}C specifically incorporated at each position from intact C_2 units
19	22.2	35	4.6	32
20	51.4	34	4.2	32
21	46.4	36	4.4	42
22	20.5	36	4.4	31
23	23.7	36	3.3	41
24	138.9	79	5.1	37
25	115.1	79	3.2	34
26	79.8	40	3.6	31
27	29.9	40	2.9	39
28	29.8		3.9	0

[a] % ^{13}C inclusive of natural abundance ^{13}C.

Table 2. Specific Incorporations, Chemical Shifts, and Coupling Constants for the C_5 Carbon Atoms of Paraherquamide A (**1**) in the Feeding Experiment with [U-$^{13}C_6$]-Glucose[19]

C no.	chemical shift (ppm)	$J_{^{13}C-^{13}C}$ (Hz)	% ^{13}C in each position[a]	% ^{13}C specifically incorporated at each position from intact C_2 units
19	22.2	34	2.1	41
20	51.4	34	3.1	32
21	46.4	36	2.6	36
22	20.5	36	3.2	14
23	23.7	36	2.9	21
24	138.9	81	4.0	35
25	115.1	79	3.5	37
26	79.8	40	3.5	37
27	29.9	40	2.1	40
28	29.8		2.8	0

[a] Percent ^{13}C is inclusive of natural abundance ^{13}C.

to the carbons that form the two C_5 units, C-19 to C-23, and C-24 to C-28, the results of the feeding experiment with [$^{13}C_2$]-acetate were essentially the same as those with [U-^{13}C]-glucose.[16] In both feeding experiments, the signal for C-28 at δ 29.80 showed enhancement with respect to the control spectrum, but no splitting. In the first C_5 fragment (C-19−C-23), the observed couplings mean that C19 is coupled to C-20, while C-21 is coupled to C-22 and C-23, but not to both simultaneously. For the second C_5 unit (C-24−C-28), the coupling constants show that C-24 and C-25 are coupled, while C-26 is coupled to C-27. In this case, C-28 shows no coupling.

It is significant that in the C_5 fragment comprised of carbons C-24 to C-28, carbon C-26 is coupled to C-27, but C-28 shows no coupling (Figure 3). This clearly establishes that the methyl groups in DMAPP are not equivalent in the biosynthesis of the dioxepin ring of this metabolite. In contrast, in the other C_5 fragment, comprised of carbons C-19 to C-23, both methyl groups show coupling with C-21, although not simultaneously.

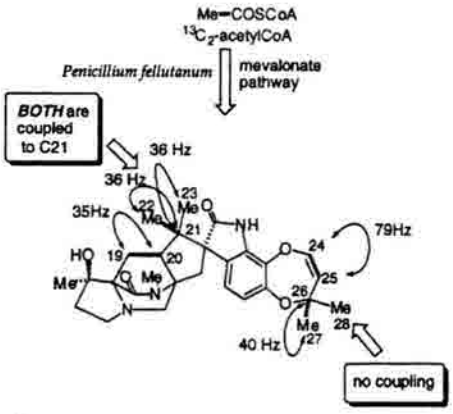


Figure 3.

Table 3. Specific Incorporations, Chemical Shifts, and Coupling Constants for the C_3 Carbon Atoms of Austamide (**21**) in the Feeding Experiment with [$^{13}C_2$]-Acetate

austamide C no.	δ	J_{C-C} (Hz)	% ^{13}C at each C[a]	% ^{13}C specifically incorporated at each position from intact C_2 units
18	42.1	35	4.9	42
19	128.7	76	5.0	39
20	125.5	76	5.5	34
21	26.1	35	5.5	24
22	23.5	35	5.3	32

[a] % ^{13}C inclusive of natural abundance ^{13}C.

Table 4. Specific Incorporations, Chemical Shifts, and Coupling Constants for the C_3 Carbons of Brevianamide A (**16**) in the Feeding Experiment with [$^{13}C_2$]-Acetate

brevianamide A (**16**) C no.	δ	J_{C-C} (Hz)	% ^{13}C at each C[a]	% ^{13}C specifically incorporated at each position from intact C_2 units
18	48.9	37	3.9	30
19	55.9	37	3.5	28
20	29.2	37	4.1	29
21	19.8	37	4.0	13
22	23.9	37	3.9	22

[a] Percent of ^{13}C is inclusive of natural abundance ^{13}C.

These data clearly demonstrate that the stereochemical integrity of DMAPP is maintained in the formation of the quaternary C–O bond of the dioxepin ring, whereas the stereochemical integrity of the DMAPP that forms the bicyclo[2.2.2] nucleus is sacrificed at some stage in the biosynthesis.

To further examine the mode of attachment of reverse prenyl groups in the simpler monoprenylated metabolites brevianamide A (**16**) and austamide (**21**), feeding experiments were performed with [$^{13}C_2$]-acetate in cultures of *Penicillium brevicompactum* (ATCC: 9056), which produces brevianamide A (**16**), and with *Aspergillus ustus* (ATCC: 36063), which produces austamide (**21**). In brevianamide A (**16**), the reverse isoprene unit, analogous to C-19–C-23 in paraherquamide A (**1**), undergoes a net oxidative cyclization across the two amino acid α-carbons culminating in the formation of the bicyclo[2.2.2] ring system.[8] In austamide (**21**), the reverse isoprene unit suffers an alternative mode of oxidative cyclization to the tryptophyl amide nitrogen atom and thus becomes the unsaturated seven-membered ring.

Incorporation of intact C-2 units was observed for both austamide (**21**) and brevianamide A (**16**), as shown in Tables 3 and 4, respectively. Thus, as with paraherquamide A (**1**), the isoprene units are derived via the mevalonic acid pathway due to the significant levels of incorporation of the labeled acetate units into the isoprene moieties. Significantly, the two geminal methyl groups derived from the reverse isoprene units in both austamide (**21**) and brevianamide A (**16**) exhibit coupling to the quaternary carbon to which they are bonded (Figure 4).

In austamide (**21**), as expected, C-19 shows coupling to C-20. C-21 and C-22 both show coupling to C-18 with coupling constants of ~35 Hz (Table 3, Figure 4). The coupling between both methyl groups, C-21 and C-22, and the adjacent quaternary carbon, C-18, can be clearly seen in the INADEQUATE spectrum shown in Figure 5.

In an INADEQUATE experiment, cross-peaks represent connectivity between adjacent carbons.[20] This type of experiment is not used frequently because of the extremely low sensitivity inherent to this technique. In this instance, however, since the austamide (**21**) sample has been enriched with $^{13}C_2$ units, the INADEQUATE is an excellent way to show which carbons arise from intact C_2 units. Besides the coupling observed between C-18 to C-21 and C-22, C-14 and C-15 give strongly coupled signals in the INADEQUATE spectrum, as shown in Figure 5. The high level of incorporation of [$^{13}C_2$]-acetate in the proline ring can be traced to the fact that L-proline is derived from L-glutamate, which in turn arises from α-ketoglutarate, a product of the condensation of acetyl-CoA in the citric acid cycle.[21]

As seen visually by the intensity of coupled ^{13}C-signals in the ^{13}C-spectrum (Figure 5) and from the calculated values shown in Table 3, the percentage of ^{13}C incorporated at C-21 and C-22 as C_2 units is approximately the same. There is thus essentially no facial bias in the mode of reverse prenylation in the biosynthesis of austamide (**21**).

An initial feeding experiment with [$^{13}C_2$]-acetate in *Penicillium brevicompactum* gave very high levels of specific incorporation of [$^{13}C_2$]-acetate into brevianamide A (**16**) with concomitant couplings generated between different intact C_2 units in the carbons arising from DMAPP in the same molecule. As a result, C-18 and C-19 showed coupling not only to the carbons that come from intact C_2 units but also to contiguous labeled carbons that most likely do not arise from intact $^{13}C_2$ units. From the splitting pattern seen in the ^{13}C spectrum, it was apparent that C-19 was coupled to C-20 (d, J = 37 Hz), but that C-19 was also coupled to C-20 and C-18 concurrently (dd, J = 37, 37 Hz). The resonance for C-18 also showed multiple couplings.

To avoid the complications that these simultaneous incorporations of two or more C_2 units introduced in the ^{13}C spectrum of the resulting brevianamide A (**16**), a second feeding experiment was performed in which 200 mg of [$^{13}C_2$]-acetate were used together with 1000 mg of unlabeled acetate. This lowered the probability of simultaneous incorporation of two labeled acetate units in the same molecule of brevianamide A (**16**), thereby simplifying the ^{13}C spectrum of this metabolite resulting from a feeding experiment. In effect, the resulting brevianamide A (**16**) showed a much simpler ^{13}C spectrum (Figure 6), with lower incorporation of labeled acetate (Table 4), and only doublets for the carbon couplings. As expected, C-18 (d, J = 37 Hz) exhibits couplings to C-21 (d, J = 37 Hz) and C-22 (d,

(19) It should be noted that the values published in ref 17 for the glucose feeding experiments were calculated incorrectly and the corrected values now appear in Table 2.

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(21) Stryer, L. *Biochemistry*; W. H. Freeman and Company: New York, 1988; p 579.

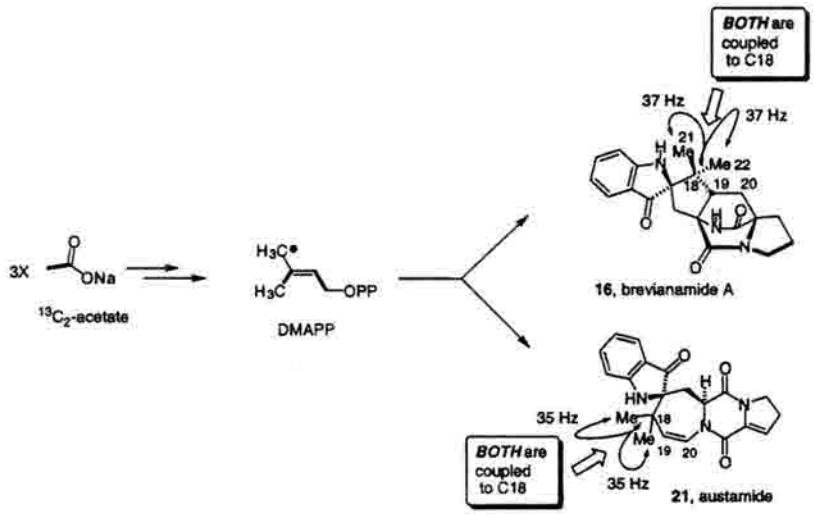


Figure 4.

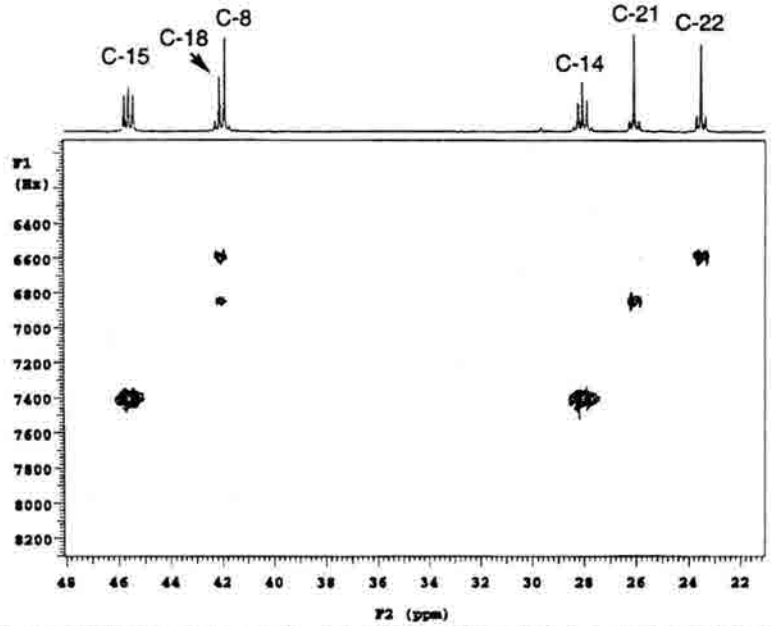


Figure 5. Portion of the INADEQUATE spectrum of austamide (**21**) derived from a [$^{13}C_2$]-acetate feeding experiment exhibiting the coupling between the geminal methyl groups and C-18. The corresponding portion of the ^{13}C-spectrum is shown at the top.

J = 37 Hz) (Figure 6), while C-19 (d, 37 Hz) is coupled only to C-20 (d, J = 37 Hz).

Another noteworthy feature of the ^{13}C NMR spectrum of brevianamide A (**16**) is that C-22, which we have assigned by ^{1}H NMR nOe experiments,[22] shows a higher percentage of specifically incorporated ^{13}C from intact C_2 units (22%) than C-21 (13%). Within experimental error, those values agree with the measured value for C-18 (30% of ^{13}C atoms specifically incorporated from intact C_2 units; see Table 4): C-18 is coupled to either C-21 or C-22, but not to both of them simultaneously. This indicates that, although there is loss of stereochemical integrity of the methyl groups derived from DMAPP in the biosynthesis of brevianamide A (**16**), there is *some degree* of stereofacial bias in the attachment of the reverse prenyl group to *cyclo*-L-Trp-L-Pro (brevianamide F) in the biosynthetic formation of deoxybrevianamide E, the key reverse-prenylated precursor.

The unusual lack of stereospecificity observed in the construction of the quaternary center at the indole 2-position in all of these metabolites can be interpreted to mean that the isoprene unit destined for C-2 of the indole ring in all of these metabolites is attached via a "reverse" prenyl transferase, which presents the olefinic π-system of DMAPP so that both faces of the π-system are susceptible to attack by the 2-position of the indole (Scheme 5).

(22) We have determined through ^{1}H NMR spectroscopy that both H-21 and H-19 in brevianamide A (**16**) show mutual nOe effects. This result unequivocally establishes the stereochemistry shown.

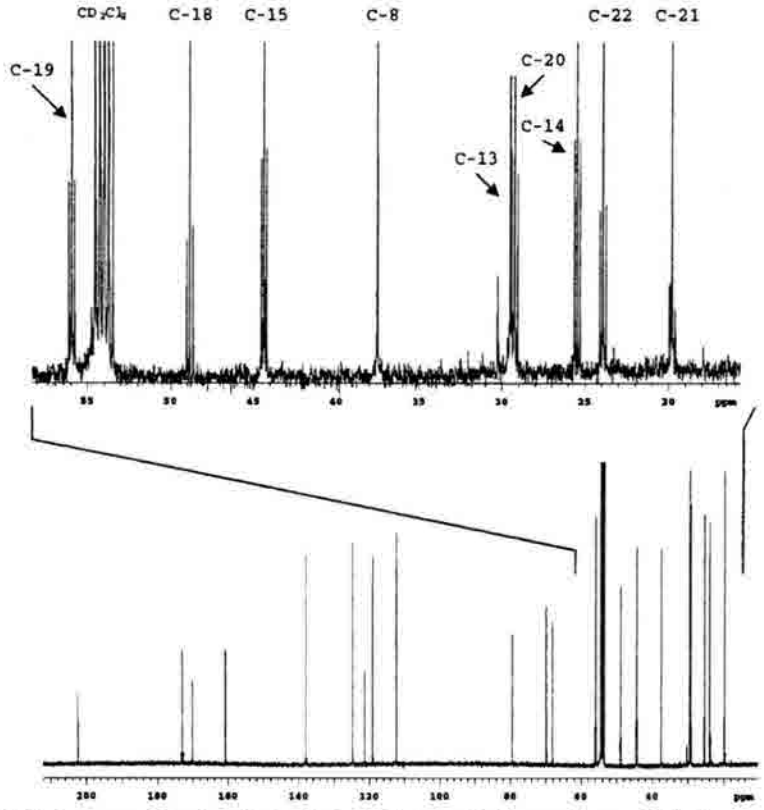


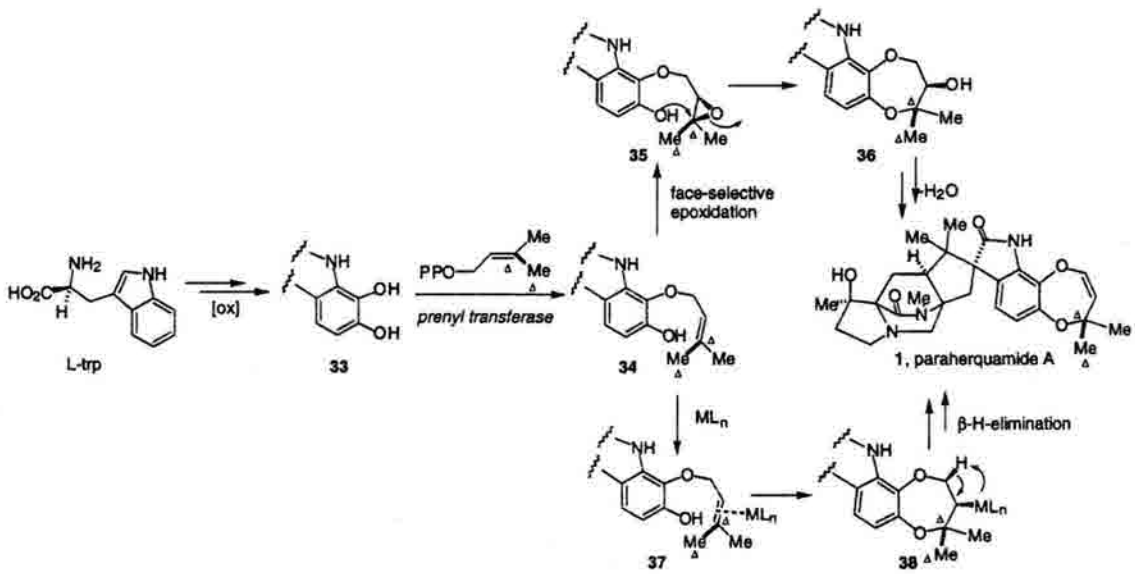
Figure 6. 100 MHz ^{13}C NMR spectrum of brevianamide A (**16**) in CD_2Cl_2 from a feeding experiment with [$^{13}C_2$]-acetate diluted with unlabeled acetate in a 1:5 proportion.

If the hydrophilic diphosphate portion of DMAPP is buried in an enzyme active site, "upside down" in relation to "normal" prenyl transferases, the hydrophobic isoprenyl moiety would be susceptible to a facially indiscriminate S_N' attack as shown in Scheme 5. In this instance, the isoprene group would be presented to the hypothetical generic indole substrate **31** in a conformationally flexible (**A** ⇌ **B**) disposition with respect to the tryptophan-derived substrate. This would result in loss of stereochemical integrity of the geminal methyl groups in the prenylated products **32a** and **32b**. The subsequent oxidative ring-closure reactions to each family of structural types would not be expected to affect the stereochemical integrity of the relevant quaternary centers once they have been set in the key C−C bond-forming reverse prenylation reactions.

In contrast, the methyl groups in the other C_5 unit constituting the quaternary carbon of the dioxepin moiety for paraherquamide A (**1**) are clearly differentiated. Thus, it is quite likely that this C_5 group (carbons 24 to 28) is introduced in the molecule via direct alkylation with DMAPP by a normal prenyl transferase (to give **34**, Scheme 6), followed by a net stereospecific oxidative addition to the olefinic π-system. Of several possibilities, a plausible mechanism for the formation of this ring system is via face-selective epoxidation of the olefin (to **35**, Scheme 6) followed by *a completely stereospecific ring-opening of the epoxide* and dehydration (**35** → **36** → **1**). Alternatively, face-selective complexation of a transition metallo-protein to the olefinic π-system (**37**) followed by stereospecific intramolecular nucleophilic addition (to give **38**) and reductive elimination to the enol-ether would yield the dioxepin moiety of paraherquamide A (**1**). We are aware of no biosynthetic precedent for the latter possibility and the former (via **35**), therefore, appears to be the most likely. Whatever the mechanism for the construction of this interesting ring system, the C−O bond-forming reaction in the construction of the dioxepin is fashioned without loss of stereochemical integrity.

Another possible explanation for the observed retention and loss of stereochemical integrity of the respective geminal methyl groups in paraherquamide A (**1**) is that the methyl groups of the DMAPP in C-19−C-23 are scrambled via a dimethyl vinyl carbinol-type intermediate derived from DMAPP. However, this would necessarily provide stereochemically scrambled isotopomers of DMAPP to the cells' cytosolic pool and scrambling would also be expected in the isoprene unit constituting C-24−C-28 unless there are two pools of *completely compartmentalized* DMAPP in the biosynthesis of paraherquamide A (**1**). In one of these pools the stereochemical integrity of DMAPP would be sacrificed through a dimethyl vinyl carbinol-type of intermediate that is then used *exclusively* for the assembly of the C-19−C-23 unit. In the other pool, DMAPP would retain the normal stereochemical integrity of the mevalonate pathway and is then used *exclusively* for fashioning the dioxepin moiety C-24−C-28. This possibility seems to be highly unlikely. Paraherquamide A (**1**) is therefore unique in that the mode of construction of each quaternary center derived from isoprene building blocks is distinct: one center is formed in a completely stereospecific manner and the other is formed in an entirely nonstereospecific manner.

Scheme 6



Conclusion

Isotopic enrichment experiments with [$^{13}C_2$]-acetate have shown that the isoprene units in the secondary metabolites paraherquamide A (**1**), brevianamide A (**16**), and austamide (**21**) all arise via the classical mevalonate pathway. In all three systems, we have observed a loss of stereochemical integrity at the isoprene-derived quaternary center attached to the 2-position of the indole ring. In the biosynthesis of paraherquamide A (**1**), the geminal methyl groups from the isoprene unit which antecedes the bicyclo[2.2.2] ring system shows approximately equal incorporation of ^{13}C from intact C-2 units, which indicates that these methyl groups become essentially equivalent at some point in the biosynthesis. The isoprene unit that constitutes the dioxepin ring displays retention of stereochemical integrity and infers a completely net face-selective biosynthetic addition reaction to the olefinic π-system derived from DMAPP via the tryptophyl ring hydroxyl group. In the biosynthesis of austamide (**21**), there are also approximately equal levels of specific ^{13}C enrichment from intact C_2 units at the isoprene derived geminal methyl groups. Brevianamide A (**16**), on the other hand, exhibits significant but incomplete loss of stereochemical integrity in the construction of the reverse prenyl unit. In this regard, it is most interesting to note that *both* brevianamide A (**16**) and austamide (**21**) are apparently fashioned from the *same* reverse-prenylated precursor, namely, deoxybrevianamide E (**20**). While *Penicillium* sp. and *Aspergillus* sp. are very similar genera of fungi genetically, it would appear that the reverse prenylases in each organism that construct deoxybrevianamide E display distinct levels of facial discrimination in the transfer of DMAPP to the 2-position of the indole. Studies to clarify these subtleties and to further elucidate the generality of the poor facial discrimination in reverse prenylations are in progress in these laboratories.

Experimental Section

Materials and Methods. [$^{13}C_2$]-Acetic acid, sodium salt, 99% atom % ^{13}C, and [U-$^{13}C_6$]-D-glucose 99% atom % ^{13}C were obtained from Aldrich Chemical Co. The ^{13}C spectra of labeled and unlabeled samples obtained for each of the fungal metabolites were obtained under comparable conditions (i.e. sample concentration, number of scans, etc.). ^{13}C NMR spectra of paraherquamide A (**1**) were obtained on a Bruker AMX 500 MHz NMR at Los Alamos National Laboratory and taken in $CDCl_3$ (^1H, 7.24 ppm; ^{13}C 77 ppm). The pulse sequence included a 4.0 s relaxation delay, an acquisition time of 0.79 s, and a spectral window of 41667 Hz. ^{13}C data were obtained with continuous WALTZ 16 composite pulse decoupling. The ^{13}C data were processed using 2 Hz exponential line broadening with a digital resolution of 2.54 Hz/pt. ^{13}C NMR spectra for both austamide (**21**), taken in $CDCl_3$ (^1H, 7.24 ppm; ^{13}C 77 ppm), and brevianamide A (**16**), taken in CD_2Cl_2 (^1H, 5.32 ppm; ^{13}C 54 ppm), were obtained on an INOVA 400 Varian NMR with a dual full band console at the Chemistry Central Instrument Facility at Colorado State University. The pulse sequence consisted of a 1.3 s relaxation delay, a pulse angle of 45°, an acquisition time of 0.64 s, and a spectral window of 25157 Hz. ^{13}C data were obtained with continuous WALTZ 16 composite pulse decoupling. The ^{13}C data were processed using a −0.634 Hz square sine bell correction and a −0.634 Hz sine bell shift with a digital resolution of 1.54 Hz/pt. The ^{13}C spectra were acquired until a satisfactory signal-to-noise ratio was obtained (ca. 16 h). The resulting ^{13}C spectra were thoroughly phased and the baseline carefully corrected to obtain a satisfactory, reproducible integral. The INADEQUATE spectrum for austamide was obtained on an INOVA 400 Varian NMR using an inadqt pulse sequence at the Chemistry Central Instrument Facility at Colorado State University. All mass spectra were obtained on a Fisons VG Quattro SQ at the Chemistry Central Instrument Facility at Colorado State University. Samples were dissolved in 1:1 water/acetonitrile without pH adjustment and measured with positive ion electrospray (20 scans, 8 s/scan) with a cone voltage of 25 V.

General Procedure for Isotopic Enrichment Experiments. Spores from the respective fungi suspended in a 15% aqueous glycerol solution were spread onto fungus specific sterile agar slants; 50 μL of the suspension was used per slant. The slants were placed in an incubator at 25 °C for 10−12 days. The spores from eight slants were shaken into four 6-L flasks containing 600 mL of sterile glucose corn steep liquor (40 g of glucose and 22 g of corn steep liquor per 1 L of distilled deionized water). The inoculated flasks were placed in an incubator at 25 °C for 6 days. The glucose corn steep liquor was removed leaving a disk of the fungus. The undersides of the disks, the mycelia cells, were rinsed with 100 mL of sterile water.

One hundred milliliters of sterile trace element solution (35 mM $NaNO_3$, 5.7 mM K_2HPO_4, 4.2 mM $MgSO_4$, 1.3 mM KCl, 36 μM $FeSO_4 \cdot 7H_2O$, 25 μM $MnSO_4 \cdot H_2O$, 7 μM $ZnSO_4 \cdot 7H_2O$, 1.5 μM $CuCl_2 \cdot$

$2H_2O$) containing 250 mg of [$^{13}C_2$]-sodium acetate (except for brevianamide A, where 200 mg of [$^{13}C_2$]-sodium acetate plus 1 g of unlabeled acetate were used) was placed into each of two flasks (four flasks in the case of brevianamide A) containing the fungus. Two control flasks were also set up, each containing 100 mL of sterile trace element solution. The flasks were put into the incubator at 25 °C for 10 days and swirled daily to ensure even distribution of the [$^{13}C_2$]-sodium acetate.

The aqueous media was decanted off and stored at 4 °C with 1−2 mL of chloroform. The mycelia cells from each flask were harvested, combined with the cells from the duplicate experiment, and pulverized with 500 mL of methanol in an Oster blender. The methanol suspensions of mycelia cells were placed in a shaker at room temperature for 24 h. Ten grams of Celite was added to each suspension before filtering through Whatman No. 2 paper. The filtrate was stored at 4 °C. The residual mycelia and Celite were re-suspended in methanol, placed in the shaker for an additional 42 h, and re-filtered.

The methanol solutions from both filtrations were combined and evaporated in vacuo. For paraherquamide A, the aqueous solution from each feeding experiment was added to the corresponding methanolic residue and the mixture was acidified to pH 4 with 12 mL of glacial acetic acid. The acidic solution was extracted 4 times with 150 mL portions of ethyl acetate. The organic layer was discarded. The aqueous layer was brought to pH 9−10 by the addition of 50 mL of 5 M NaOH. The aqueous layer was then extracted 4 times with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous sodium carbonate, and evaporated to dryness. For brevianamide A and austamide, the aqueous solution from each feeding experiment was added to the corresponding methanolic residue, taken at a pH of 7−8, and extracted 4 times with ethyl acetate. The combined organic layers were washed successively with 10% aqueous Na_2CO_3 solution and brine, then dried over anhydrous sodium sulfate and evaporated to dryness. The fungal metabolites were purified via radial chromatography and thin-layer chromatography on silica gel using a gradient elution of 4−10% methanol in methylene chloride. This was followed by preparative TLC on silica gel precoated plates with methylene chloride−methanol (10:1 for paraherquamide A (**1**); 25:1 for austamide (**21**)) or methylene chloride−acetone mixtures (2:1 for brevianamide A (**16**)).

Paraherquamide A (1): *Penicillium fellutanum* (ATTC: 20841) was initially grown on sterile malt extract agar slants (20 g of glucose, 1 g of peptone, and 20 g of agar per liter of distilled, deionized water). Yield of paraherquamide A (**1**): 18 mg, 0.036 mmol, from the control experiment, 15 mg, 0.030 mmol, from the [$^{13}C_2$]-acetate experiment, and 34 mg, 0.070 mmol from the U-$^{13}C_6$-glucose feeding experiment. The incorporation levels were as follows: 0.35% total incorporation and 0.07% specific total incorporation (intact C-2 units) of [$^{13}C_2$]-acetate into paraherquamide A (**1**) was observed.

Austamide (21): *Aspergillus ustus* (ATCC: 36063) was initially grown on sterile malt extract agar slants. Yield of austamide (**21**): 15 mg, 0.041 mmol, from the control experiment, and 14 mg, 0.039 mmol, from the [$^{13}C_2$]-acetate experiment. The incorporation levels were as follows: 0.57% total incorporation and 0.19% specific total incorporation (intact C-2 units) of [$^{13}C_2$]-acetate into austamide (**21**) was observed.

Brevianamide A (16): *Penicillium brevicompactum* (ATCC: 9056) was grown on sterile Czapek Dox slants (3.0 g of $NaNO_3$, 1.0 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 30 g of D-glucose, 15.0 g of agar per liter of distilled deionized water). [$^{13}C_2$]-Acetate (200 mg) plus 1 g of unlabeled acetate were used in the feeding experiment. Yield of brevianamide A (**16**): 17 mg, 0.047 mmol, from the control experiment, and 15 mg, 0.041 mmol, from the [$^{13}C_2$]-acetate experiment. The incorporation levels were as follows: 2.19% total incorporation and 0.32% specific total incorporation (intact C-2 units) of [$^{13}C_2$]-acetate into brevianamide A (**16**) was observed.

Determination of Isotopic Enrichment. Gated-decoupled experiments were precluded by the small amounts of these metabolites and the intrinsic lower sensitivity of this kind of experiment; for that reason, standard ^{13}C spectra were measured for the metabolite from both control experiments and from feeding experiments. The relative abundance of ^{13}C in each carbon of paraherquamide A (**1**), brevianamide A (**16**), and austamide (**21**) resulting from feeding experiments was determined through comparison of the integration for that peak with the total integration in the standard ^{13}C spectrum. When this relative integration was compared to the relative integration for each carbon signal in compounds from control experiments, it was possible to calculate the relative abundance of ^{13}C for each position with respect to the rest of carbon signals. The total abundance of ^{13}C in each metabolite was then determined by calculating the increase in intensity for the peaks corresponding to the isotopomers with one, two, three, and four ^{13}C atoms in the mass spectra for each of the metabolites resulting from feeding experiments, relative to the intensity of the peak that corresponds to molecules with ^{12}C atoms only. The ^{13}C abundance for each position was finally calculated taking into account the relative abundance of ^{13}C (from the ^{13}C NMR spectrum) and the average ^{13}C abundance for the metabolite (from the MS) isolated from the feeding experiment.[23]

Acknowledgment. Financial support from the National Institutes of Health (to R.M.W., grant CA 70375, Shared Instrumentation Grant GM49631 for mass spectroscopy) and the Dirección General de Investigación Científica y Técnica of Spain (to J.F.S.-C., Proyecto PB98-1438) is gratefully acknowledged. NMR spectra for paraherquamide A (**1**) were obtained on a Bruker AMX 400 MHz NMR at Los Alamos National Laboratory courtesy of Clifford J. Unkefer. We would also like to thank Professor Rodney Croteau and Professor J. Alberto Marco for helpful discussions.

Supporting Information Available: Complete details of the calculations of ^{13}C incorporation into paraherquamide A (**1**), austamide (**21**), brevianamide A (**16**), and a control sample (Trp-OMe) are provided (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(23) The method used for the incorporation calculations was applied to three known dilutions of L-[10-^{13}C]-L-tryptophan methyl ester (99% enriched; carboxyl group is labeled). Comparison of the percentage of ^{13}C enrichment obtained from these calculations with the actual percentage of enrichment gave an average error of 20% (most likely arising from signal integration of the ^{13}C and mass spectra peaks; see Supporting Information).

Studies on the Biosynthesis of Paraherquamide: Concerning the Mechanism of the Oxidative Cyclization of L-Isoleucine to β-Methylproline

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Paraherquamide A (**1**) is one member of a group of heptacyclic fungal metabolites (**1**−**13**, Figure 1) with potent anthelminthic activity isolated from various *Penicillium* sp.[1] Among its unusual structural features, paraherquamide A contains a β-methyl-β-hydroxy proline moiety. A previous report from this laboratory on the biosynthesis of paraherquamide A demonstrated that the prolyl ring is formed via a heretofore-unknown oxidative cyclization of the terminal methyl group of L-isoleucine onto the α-amino group.[2,3]

There are several possibilities of how the oxidative cyclization of L-isoleucine to β-methylproline can occur; two reasonable prospects are shown in Scheme 1. One pathway involves 4-electron oxidation of the distal side-chain methyl group to aldehyde **15** followed by cyclization and loss of water to produce the imine **16**; subsequent reduction of **16** (or in the case of VM55597, oxidation) furnishes the β-methylproline ring, **17**. Another reasonable pathway is oxidation of the terminal methyl group to an alcohol (**18**, R = H) followed, for example, by phosphorylation and nucleophilic displacement of the phosphate group. One other possibility is chlorination of the distal side chain methyl group to **19** followed by nucleophilic displacement to give **20**. Precedent for the latter pathway was reported by Arigoni and Looser, where chlorinated leucine moieties in the natural product victorin C were observed.[4] To help distinguish between 2e⁻ (via **18** or **19**) and 4e⁻ (via **15**) oxidation mechanisms in these putative pathways, L-[5-^{13}C,5-^{2}H$_3$]isoleucine was synthesized and fed to cultures of *P. fellutanum* (ATCC: 20841). Depending on the net oxidation state change, the labeling pattern shown in **17** or **20** should be observed for the 4e⁻ and 2e⁻ pathways in the isolated paraherquamide A, respectively.

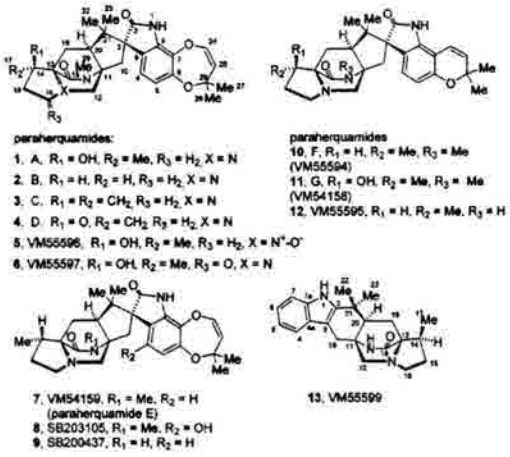
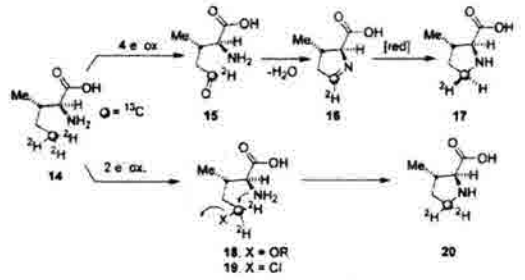
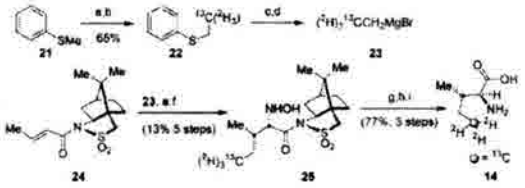


Figure 1.

Scheme 1. Possible Pathways for the Oxidative Cyclization of L-Isoleucine to β-Methylproline



Scheme 2. Synthesis of l-[5-^{13}C,5-^{2}H$_3$]Isoleucine[a]



[a] Conditions: (a) *n*-BuLi, (b) ^{13}C(^{2}H)$_3$I, (c) benzyl bromide, 150 °C, (d) Mg^0, (e) 1-chloro-1-nitrosocyclohexane, (f) 1 N HCl, (g) Zn^0, 1 N HCl/AcOH, (h) LiOH, (i) DOWEX ion exchange.

L-[5-^{13}C,5-^{2}H$_3$]Isoleucine was synthesized by using the procedure developed by Oppolzer and co-workers for the synthesis of unlabeled L-isoleucine (Scheme 2).[5,6] However, since 2-[^{13}C^{2}H$_3$]-ethylmagnesiumbromide is not commercially available, it was synthesized from ^{13}C^{2}H$_3$-iodomethane.[7] Reaction of thioanisole

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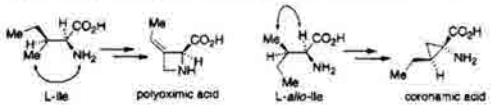
[‡] Los Alamos National Laboratory.
[§] Universidad de Valencia.

(1) (a) Yamazaki, M.; Okuyama, E.; Kobayashi. M.; Inoue, H. *Tetrahedron Lett.* **1981**, *22*, 135−136. (b) Ondeyka, J. G.; Goegelman, R. T.; Schaeffer, J. M.; Kelemen, L.; Zitano, L. *J. Antibiot.* **1990**, *43*, 1375−1379. (c) Liesch, J. M.; Wichmann, C. F. *J. Antibiot.* **1990**, *43*, 1380−1386. (d) Blanchflower, S. E.; Banks, R. M.; Everett, J. R.; Manger, B. R.; Reading, C. *J. Antibiot.* **1991**, *44*, 492−497. (e) Blanchflower, S. E.; Banks, R. M.; Everett, J. R.; Reading, C. *J. Antibiot.* **1993**, *46*, 1355−1363.

(2) Stocking, E. M.; Sanz-Cervera, J. F.; Williams, R. M.; Unkefer, C. J. *J. Am. Chem. Soc.* **1996**, *118*, 7008−7009.

(3) There are other known oxidative cyclizations of L-isoleucine. The β-methyl group of L-isoleucine has been postulated to undergo oxidative cyclization onto the amino terminus to form the heterocycle, polyoximic acid. (Hanessian, S.; Fu, J.-M.; Tu, Y.; Isono, K. *Tetrahedron Lett.* **1993**, *43*, 4153−4156.) In L-*allo*-isoleucine, the β-methyl group is know to undergo oxidative cyclization onto the α-carbon to form coronamic acid. (Pany, R. J.; Mhaskar, S. V.; Lin, M. T.; Walker, A. E.; Mafoti, R. *Can. J. Chem.* **1994**, *72*, 86−99.)



(4) (a) Looser, M. Ph.D. Thesis, ETH, 1989 (D. Arigoni). (b) An alternative pathway for β-methylproline biosynthesis has been discovered in the biosynthesis of the antibiotic bottromycin in *Streptomyces bottropenis* involving an *S*-adenosylmethionine-based β-methylation of proline, see: Kellenberger, J. L. Ph.D. Thesis, ETH, 1997 (D. Arigoni).

(5) (a) Oppolzer, W.; Tamura, O. *Tetrahedron Lett.* **1990**, *31*, 991−994. (b) Oppolzer, W.; Tamura, O.; Deerberg, J. *Helv. Chim. Acta* **1992**, *75*, 1965−1973.

(6) Yields shown reflect nonoptimized reaction conditions.

(7) The synthesis of this substance will be reported elsewhere, but is also commercially available.

10.1021/ja005655e CCC: $20.00
Published on Web 00/00/0000 PAGE EST: 1.9

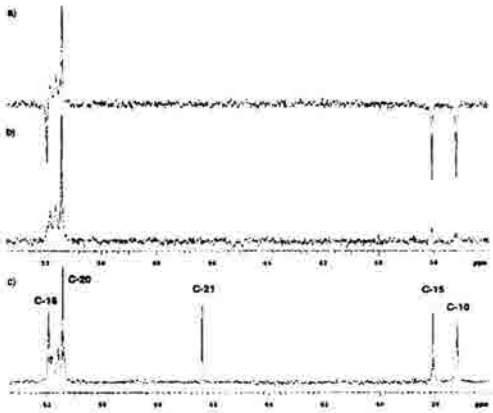


Figure 2. (a) DEPT 135 experiment with CH up and CH_2 down, (b) DEPT 90 experiment with only CH's shown, and (c) partial ^{13}C spectrum (100 MHz) of **1** from the feeding experiment with 5-[$^{13}C^2H_3$]-L-ile.

(**21**) with *n*-BuLi followed by the addition of [$^{13}C^2H_3$]-iodomethane provided $^{13}C^2H_3$-ethylphenylsulfide (**22**). The 2-[$^{13}C^2H_3$]-ethylbromide, distilled from the reaction of **22** with benzylbromide at 150 °C, was added dropwise as an ethereal solution to activated Mg^0 to form the Grignard reagent, **23**. Successive treatment of the *N*-crotonoylborane-10,2-sultam (**24**) with 2-[$^{13}C^2H_3$]-ethylmagnesiumbromide followed by 1-chloro-1-nitrosocyclohexane and 1 N aqueous HCl at −78 °C provided the 1,4-addition/electrophilic amination product (**25**). *N,O*-Hydrogenolysis of the hydroxylamine, **25**, with Zn^0 powder in 1 N HCl/AcOH, followed by saponification of the sultam with LiOH in THF/H_2O and DOWEX ion exchange, provided L-[5-^{13}C,5-$^2H_3$]isoleucine (**14**).

A feeding experiment in *Penicillium fellutanum* with L-[5-^{13}C, 5-$^2H_3$]isoleucine (**14**), followed by isolation and purification of the paraherquamide A produced, revealed 0.32% incorporation of the labeled amino acid.[8] Close inspection of the ^{13}C-spectrum (Figure 2c) revealed a triplet at 51.6 ppm, which would indicate that, in the labeled compound, C-16 is coupled to a single deuterium atom. However, as seen in Figure 2c, the triplet was partially obscured by neighboring ^{13}C-signals, which complicated interpretation of the spectrum. To resolve this problem, DEPT experiments were performed.

As seen in Figure 2, the DEPT spectra unambiguously assigns a $^{13}C^2H^1H$ pattern to C-16 in the labeled paraherquamide A. In both the DEPT 135 and DEPT 90 spectra, the triplet is seen. In the event of a $^{13}C^2H_2$ pattern, the triplet would not appear in either DEPT spectrum. *From these experiments, it was determined that cyclization of L-isoleucine occurs though a 4e⁻ oxidation of the terminal methyl group such as via the putative intermediate* ***15*** *followed by cyclization and diastereoselective 2e⁻ reduction to give* ***17****.*

To determine if the *pro-R* or *pro-S* hydrogen was retained in the oxidative cyclization, CW-selective proton decoupling experiments were performed as shown in Figure 3. The ^{13}C signals were decoupled from protons H-16a (*pro-S*) at 3.21 ppm (Figure 3c) and H-16b (*pro-R*) at 2.22 ppm (Figure 3b), respectively.[9] When H-16a is decoupled (Figure 3c), the expected triplet splitting

(8) Determined from the ES mass spectrum. Specific incorporation at C-16 is 0.21% as determined by NMR. We have shown good incorporation of 1-[^{13}C]-L-Ile in the past (see ref 2), but the deuterium isotope effect is expected to have an effect on the rate of biosynthesis of β-methylproline. This can account for both the low percentage of incorporation and the relatively low yield of **1** isolated in this experiment.

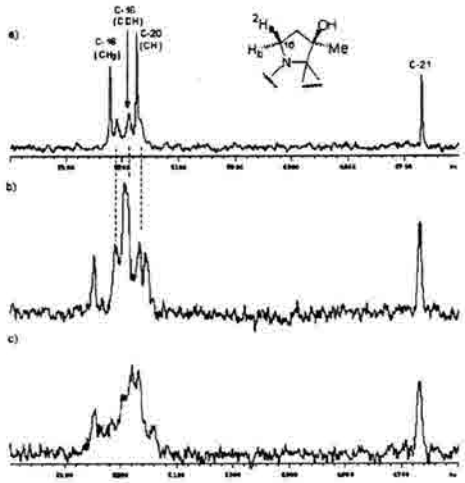


Figure 3. (a) Partial ^{13}C spectrum (100 MHz) of paraherquamide A (**1**) from the feeding experiment with 5-[$^{13}C^2H_3$]-L-ile, (b) partial ^{13}C spectrum (100 MHz) of **1** from the experiment with H-16b at 2.22 ppm decoupled, and (c) partial ^{13}C spectrum of **1** (100 MHz) from the experiment with H-16a at 3.21 ppm decoupled.

pattern (seen in Figure 3a) for the deuterium coupled ^{13}C-labeled C-16 becomes complex suggesting that $^{13}C^2H$ is coupled to H-16b. When H-16b is decoupled (Figure 3b), the triplet from the deuterium coupled ^{13}C signal is not affected. Therefore, it was determined that H-16a (3.21 ppm) is the deuteron, H-16b (2.22 ppm) is the proton and the *pro-S* hydrogen is retained in the oxidative cyclization.[10] *This result implies that reduction occurs on the same face of the proline ring as the methyl group, C-17.*

In summary, the results described provide the first mechanistic glimpse of the events likely to be involved in the biosynthesis of the β-methylproline moiety of the paraherquamide family of anthelmintic agents. Efforts to elucidate the exact nature of the enzymatic oxidizing and reducing species involved in the conversion of L-isoleucine to β-methylproline are under investigation in these laboratories.

Acknowledgment. This work was supported by the National Institutes of Health (Grant CA70375 to R.M.W.), the National Stable Isotopes Resource, NIH Division of Research Resources (RR 02231 to R.A.M. and L.A.S.), the ACS Division of Organic Chemistry Fellowship (to E.M.S., sponsored by SmithKline Beecham), and the Pharmacia-Upjohn Company Fellowship (to E.M.S.). Mass spectra were obtained on instruments supported by the National Institutes of Health Shared Instrumentation Grant GM49631. We also wish to thank Dr. Chris Rithner of the Central Instruments Facility at Colorado State University for assistance with several NMR experiments.

Supporting Information Available: Full experimental procedures for the synthesis of L-[5-^{13}C,5-$^2H_3$]isoleucine, method for the determination of the percentage of isotopic incorporation, and HSQC spectrum of **1** from the feeding experiment with $^{13}C^2H_3$-L-ile (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Assignments of H-16a and H-16b are based on the assignments of Blanchflower and co-workers (refs 1d and 1e).

(10) This result was confirmed through a HSQC experiment. The ^{13}C signal from the deuterium-coupled C-16 shows connectivity with the proton at 2.22 ppm, H-16b, but not with the proton at 3.21 ppm, H-16a.

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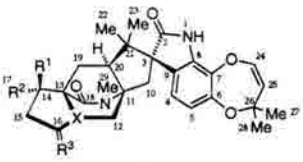

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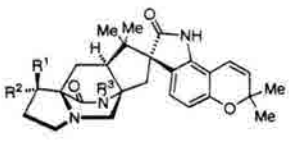

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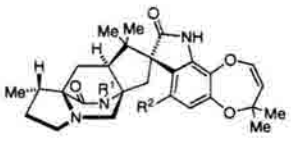

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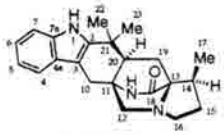


13, VM55599

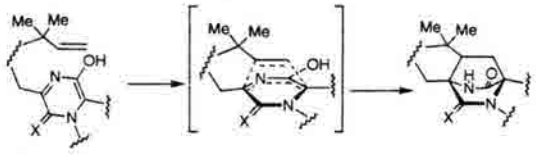
Figure 1. **1**, paraherquamide A: $R^1 = OH$, $R^2 = Me$, $R^3 = H_2$, X = N; **2**, paraherquamide B: $R^1 = H$, $R^2 = H$, $R^3 = H_2$, X = N; **3**, paraherquamide C: $R^1 = R^2 = CH_2$, $R^3 = H_2$, X = N; **4**, paraherquamide D: $R^1 = O$, $R^2 = CH_2$, $R^3 = H_2$, X = N; **5**, VM55596: $R^1 = OH$, $R^2 = Me$, $R^3 = H_2$, $X = N^+O^-$; **6**, VM55597: $R^1 = OH$, $R^2 = Me$, $R^3 = O$, X = N; **7**, paraherquamide E (VM54159): $R^1 = Me$, $R^2 = H$; **8**, SB203105: $R^1 = Me$, $R^2 = OH$; **9**, SB200437: $R^1 = H$, $R^2 = H$; **10**, paraherquamide F (VM55594): $R^1 = H$, $R^2 = Me$, $R^3 = Me$; **11**, paraherquamide G (VM54158): $R^1 = OH$, $R^2 = Me$, $R^3 = Me$; **12**, VM55595: $R^1 = H$, $R^2 = Me$, $R^3 = H$.

Studies on the Biosynthesis of Paraherquamide: Synthesis and Incorporation of a Hexacyclic Indole Derivative as an Advanced Metabolite**

Emily M. Stocking, Juan F. Sanz-Cervera, and Robert M. Williams*

The paraherquamides (Figure 1),[1] along with the brevianamides,[2] marcfortines,[3] and sclerotamides[4] are indolic fungal metabolites that share the common structural feature of an unusual bicyclo[2.2.2]diazaoctane core. It has been postulated that the bicyclo[2.2.2]diazaoctane ring system arises through an intramolecular hetero Diels–Alder cycloaddition of the isoprene moiety across the α-carbons of the amino acid subunits, as shown in Scheme 1.[5]

In 1993, Everett and co-workers isolated a very minor metabolite that also possesses the bicyclo[2.2.2]diazaoctane core, VM55599 (**13**, Figure 1), from *Penicillium* sp. (IMI 332995) which produces paraherquamide A.[6] Based on



Scheme 1. Proposed formation of the bicyclo[2.2.2]diazaoctane ring system in the paraherquamides.

the structural similarities of these co-metabolites, Everett et al. speculated that VM55599 might be a biosynthetic precursor to paraherquamide A.[6] The relative stereochemistry of VM55599 as shown in Figure 1 was assigned by ^{1}H NMR spectroscopy with nuclear Ovenhauser enhancements but the absolute configuration of this substance remains unknown. The stereochemistry of the methyl group in the β-methylproline ring was assigned as being *syn* to the bridging isoprene moiety. In all other known members of the paraherquamide family, the methyl group in the β-methylproline ring is disposed *anti* to the bridging isoprene moiety. If VM55599 was indeed a precursor to paraherquamide A, then oxidation of the β-methylproline ring would have to occur with inversion of stereochemistry at the C-14 center that bears the methyl group.

Previous studies from this laboratory on the biosynthesis of paraherquamide A demonstrated that L-isoleucine is the precursor to the β-methyl-β-hydroxy proline ring of paraherquamide A.[7] The relative disposition of the methyl group in the prolyl ring is retained in the biosynthetic conversion of L-isoleucine into paraherquamide A and, thus, the hydroxylation at C-14 occurs with net retention. These findings bring into question the potential intermediacy of VM55599 in the biosynthesis of the paraherquamides. Furthermore, if L-isoleucine is also the precursor to VM55599, then the absolute stereochemistry of this metabolite must be that depicted in

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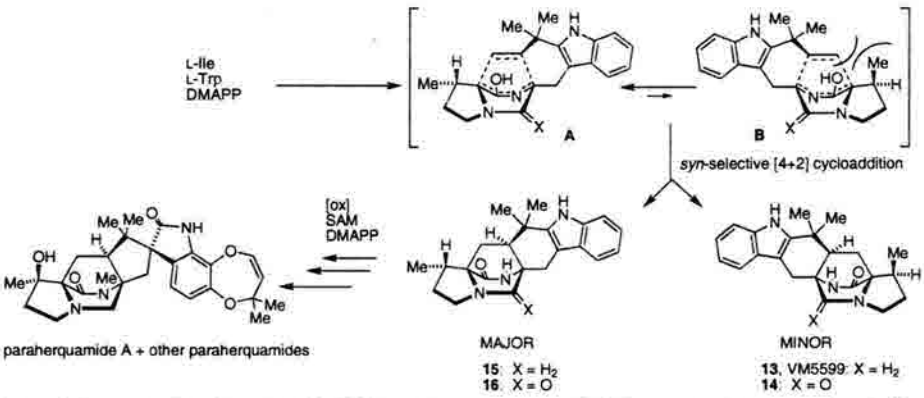


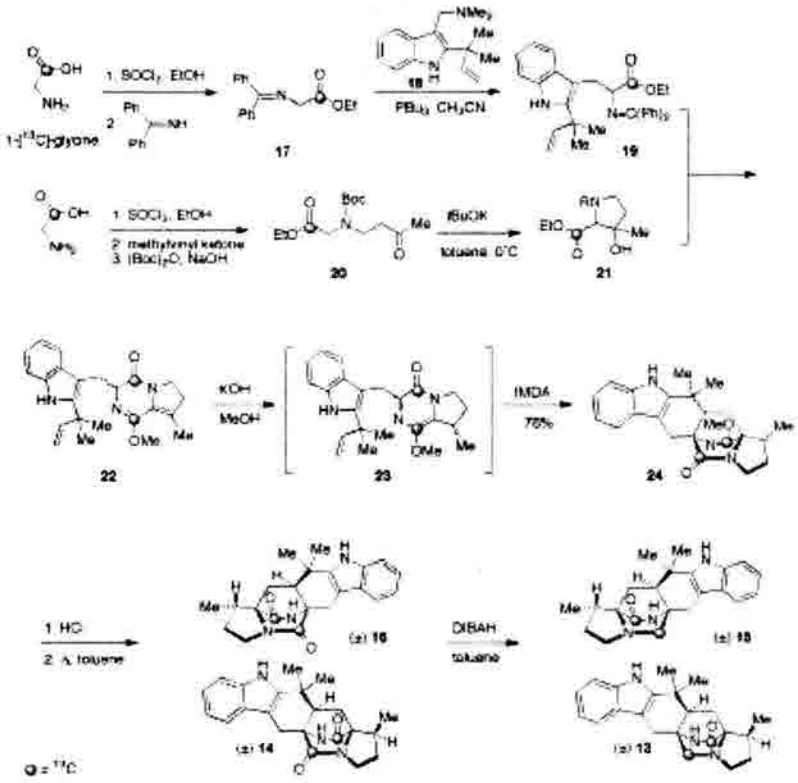
Scheme 2. Proposed unified biogenesis of VM55599 and the paraherquamides. DMAPP = dimethylallylpyrophosphate, SAM = *S*-(5′-adenosyl)-L-methionine chloride.

Figure 1 wherein the absolute configuration of the bicyclo[2.2.2]diazaoctane portion of this molecule is *enantiomorphic* to that of paraherquamide A.[8]

A unified biogenesis of VM55599 and the paraherquamides has been previously suggested as shown in Scheme 2.[9] Every metabolite would result from the intramolecular [4+2] cycloaddition of a common azadiene through two of the four possible diastereomeric transition structures. If cycloaddition occurs with the methyl group of the β-methylproline ring *anti* to the isoprene unit (as in **A**, Scheme 2), then the intermediate **15** would be formed and would lead to all of the paraherquamides containing a β-methylproline moiety. This is presumed to be the major pathway. A minor shunt pathway would involve cycloaddition from the more hindered face of the azadiene system (see **B**, Scheme 2) with the methyl group of the β-methylproline ring *syn* to the isoprene unit; VM55599 would thus result. To test this hypothesis, we have synthesized racemic doubly ^{13}C-labeled putative cycloadducts (**13**–**16**) and have examined these substances as potential pathway metabolites in *Penicillium fellutanum*.

The proposed ^{13}C-labeled compounds were synthesized according to our previously described synthesis of racemic VM55599 (Scheme 3).[9c, 10] The advantage of this strategy is that all four candidate precursors are accessible from a single synthesis. In addition, both of the ^{13}C labels in the product are derived from relatively inexpensive ^{13}C-glycine.

The racemic cycloadducts (**13**–**16**) possessed the unfortunate property of being insoluble in water so rendering the planned feeding experiments challenging. To circumvent this problem, the detergent TWEEN 80 was added and was found



Scheme 3. Synthesis of the ^{13}C-labeled compounds **13**–**16**. Boc = *tert*-butoxycarbonyl, IMDA = *N*-(carboxymethyl)glycine, DIBAH = diisobutylaluminum hydride.

to increase the miscibility of these substances in the culture broth without inhibiting production of paraherquamide A. Feeding experiments were performed on *P. fellutanum* (ATCC20841) with all four potential precursors, followed by isolation and purification of paraherquamide A. Within the limits of detection by ^{13}C NMR spectroscopy and mass spectrometry no incorporation was observed for VM55599 ((±)-**13**) or its oxidized counterpart (±)-**14**. In addition, no incorporation was observed for the diketopiperazine (±)-**16**. However, for the C-14 epimer of VM55599 ((±)-**15**), significant incorporation was observed by ^{13}C NMR spectroscopy at C-12 and C-18 of paraherquamide A. From analysis of the electrospray mass spectrum, incorporation was determined to be 0.72% for the intact doubly labeled material.[11] ^{13}C-Monolabeled paraherquamide A, from catabolism of (±)-**15**, was not detected in the mass spectrum. The implications of these observations are considerable.

Since the diketopiperazine (±)-**16** was not incorporated, this raises interesting questions concerning the timing of the reduction of the prolyl-derived carbonyl group. The incorporation of compound (±)-**15** in significant isotopic yield, indicates that the formation of the bicyclo[2.2.2]diazaoctane occurs at the stage with the nonoxidized tryptophyl moiety (that is, the indolyl group). This mandates that oxidations of the indole ring to form both the catechol-derived dioxepin and spirooxindole occur *after* the formation of this intermediate. It thus follows that the dioxepin-derived isoprenylation and the *S*-adenosylmethionine-mediated *N*-methylation reactions occur late in the pathway. These results also cast considerable doubt on the intermediacy of VM55599[6] and its oxidized precursor **14** in the paraherquamide biosynthesis and provide additional circumstantial evidence that VM55599 is a minor shunt metabolite. Finally, the present work documents the intermediacy of an advanced metabolite **15**, which contains the core structural elements of the paraherquamide framework, prior to a series of oxygenation reactions. Efforts to elucidate the exact sequence of biosynthetic reactions immediately preceding and following the formation of **15** are currently under way in these laboratories.

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[8] Elucidation of the absolute stereochemistry of VM55599 is in progress. The absolute stereostructures for compounds **13**–**16** (which are racemic) are depicted with the absolute configurations presumed to be those of the natural metabolites.

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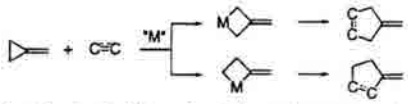
Novel [3+2] Cycloaddition of Alkylidenecyclopropanes with Aldehydes Catalyzed by Palladium

Itaru Nakamura, Byoung Ho Oh, Shinichi Saito, and Yoshinori Yamamoto*

A metal-catalyzed cycloaddition between methylenecyclopropane and a carbon–carbon multiple bond can proceed through two different reaction pathways to give regioisomeric [3+2] carbocycles (Scheme 1).[1–3] The research groups of



Scheme 1. Metal-catalyzed cycloaddition of methylenecyclopropane and a carbon–carbon multiple bond.

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Appendix 2. Research Proposal

Total Synthesis of Okaramine B

Emily Stocking
Colorado State University
Department of Chemistry

Abstract:

The first total synthesis of the insecticidal compound Okaramine B has been proposed. A convergent synthesis will be performed which joins the two structurally unique portions of the molecule: an azetidine tryptophanyl derivative and an azocinindole derivative. Conditions for the construction of both portions of the molecule are described. The key steps in this synthesis are the zirconium mediated formation of the azetidine ring and formation of the azocine ring via an intra-molecular reaction of an α-aminoalkylstannane nucleophile with an in situ-generated 3-chloroindolenine.

The okaramines (figure 1) are a group of indole alkaloids isolated from Penicillium simplicium that possess insecticidal activity.[1] Okaramine B (**1**), which displays the most potent insecticidal activity within this group, contains the unusual functionalities of an azetidine moiety and an azocinindole ring. Thus far, all structure activity relationships for the okaramines are based on isolated okaramine congeners (table 1). The conformation of the azocine ring, the presence of the azetidine ring and the oxidation state at C-2 and C-12 all seem to contribute to the activity of the okaramines. Although tremendous progress has been achieved in determining the structure activity relationship of the okaramines with isolates alone, an efficient total synthesis of okaramine B needs to be developed in order to fully understand the importance of the

different functionalities. In addition, a convergent total synthesis would allow the development of analogs to test for insecticidal activity.

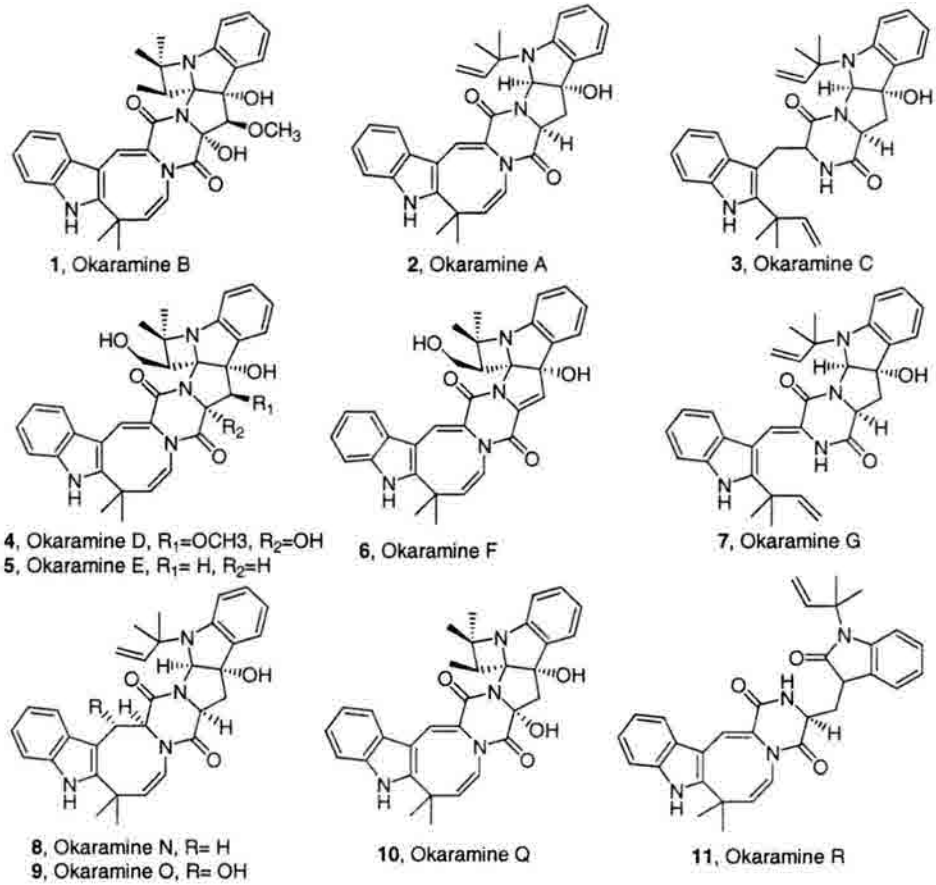


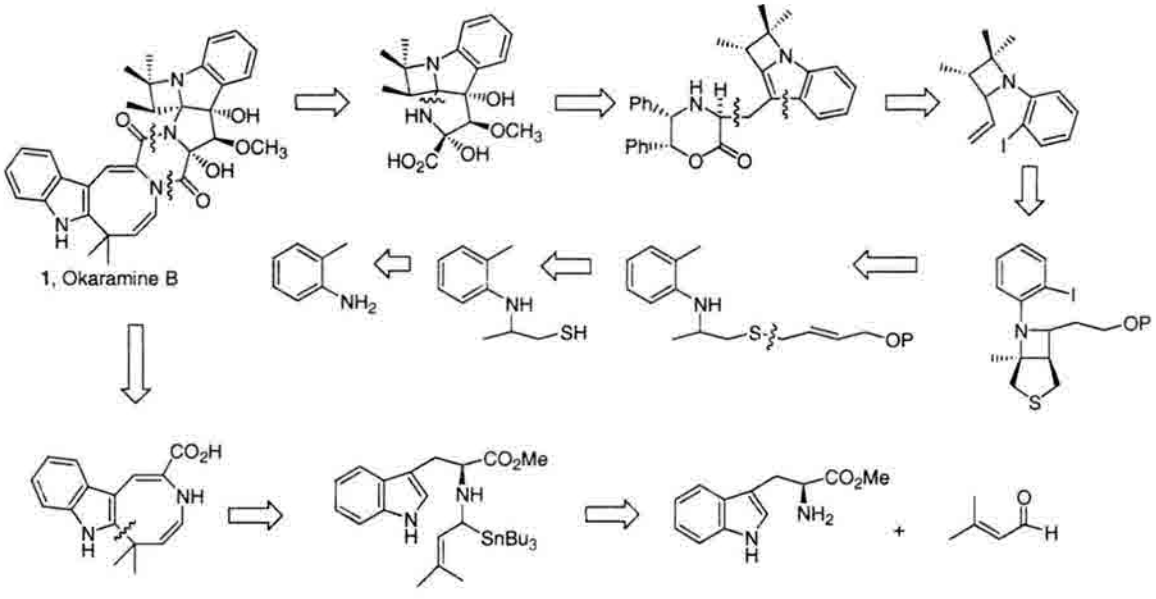
Figure 1

Table 1:

Okaramines	LD_{50} μg/g diet	Okaramines	LD_{50} μg/g diet
B (**1**)	0.2	G (**7**)	40
A (**2**)	8	N (**8**)	>100
C (**3**)	8	O (**9**)	>100
D (**4**)	20	Q (**10**)	8
E (**5**)	>100	R (**11**)	>100
F (**6**)	>100		

The retrosynthetic plan is outlined in Scheme 1. Okaramine B is essentially a highly derivatized cyclic dipeptide of L-tryptophan. Thus a natural place to begin the

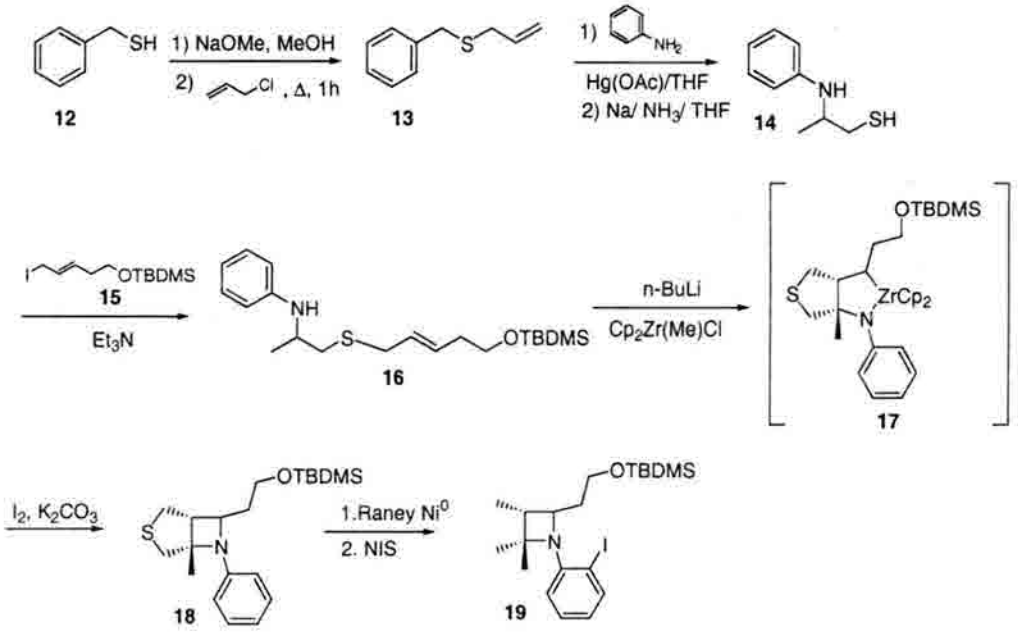
retro-synthetic analysis is the amide linkages of the diketopiperzine ring. Breaking these bonds provides two segments, the azocine tryptophan derivative and the azetidine tryptophan derivative. The key features of the azetidine derivative sysnthesis are: 1) use of the Williams' lactone to control the stereochemistry of the oxidation, 2) formation of the indole ring using the Heck reaction, 3) zirconium mediated formation of the azetidine ring. The azocine moiety can be synthesized from commercially available 3-methyl-2-butenal and tryptophan methyl ester via intra-molecular nucleophilic attack at the 2-position of the indole using a methodology developed by Schkeryantz et al.[2]



In forming the azetidine ring, an intramolecular zirconium mediated cyclization will be used. While this type of cyclization does work inter-molecularly (which would allow introduction of the azetidine later in the synthesis), intra-molecular cyclization allows regioselective control and exclusive formation of the *cis*-diastereomer. Introducing the azetidine early in the synthesis should not be problematic. With the exception of mineral acid, which makes the protonated azetidine ring subject to nucleophilic attack and ring cleavage, the azetidine ring is stable under most conditions.[3] The thio-ether was chosen for the intra-molecular cyclization because de-sulfurization can easily be accomplished with Raney Ni°. Formation of the azetidine ring will be accomplished as outlined in scheme 2. The intermedate, **14**, will be synthesized according to the procedure of Barluenga et al.[4] Alkylation of the thiol, **14**, with the

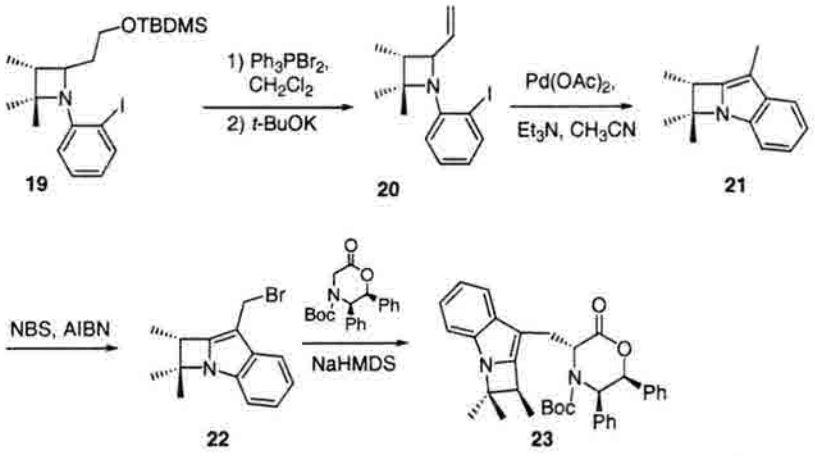
iodide, **15**,[5] can be accomplished in the presence of a mild base such as triethylamine. The synthesis of the azetidine, **18**, via the zirconacycle, **17**, was modeled after the azetidine synthesis described by Barluenga et al.[6] In his report, Barluenga describes the synthesis of a *cis*-5,4 fused ring system using zirconocene methyl chloride. He does not use a thioether, but other reports have shown that sulfur functionalities are tolerated by zirconocenes.[7] After desulfurization with Raney Ni°, iodination will be accomplished with N-iodosuccinamide (NIS) presumably with a statistical ratio of 2:1 (ortho: para) to give **19**.

Scheme 2:

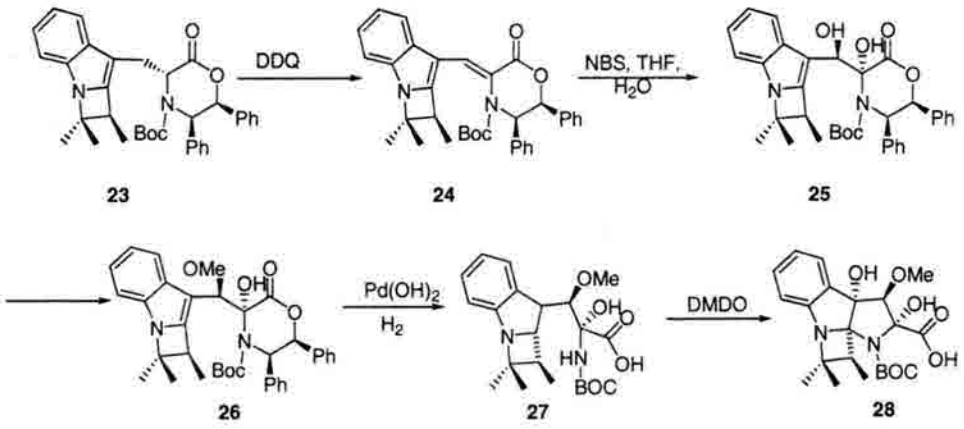


The TBDMS ether, **19** (scheme 3), will be converted directly to the bromide with triphenylphosphine dibromide.[8] The bromide should easily be converted to the alkene, **20**, under basic conditions. This alkene can then be transformed to the indole, **21**, via an intramolecular Heck reaction.[9] Selective bromination at the 3-methyl position of the indole can be accomplished with NBS and AIBN.[10] Coupling to the Williams' lactone will be achieved with NaHMDS.[11] Since the synthesis is racemic to this point, the diastereomers of **23** will need to be separated.

Scheme 3:

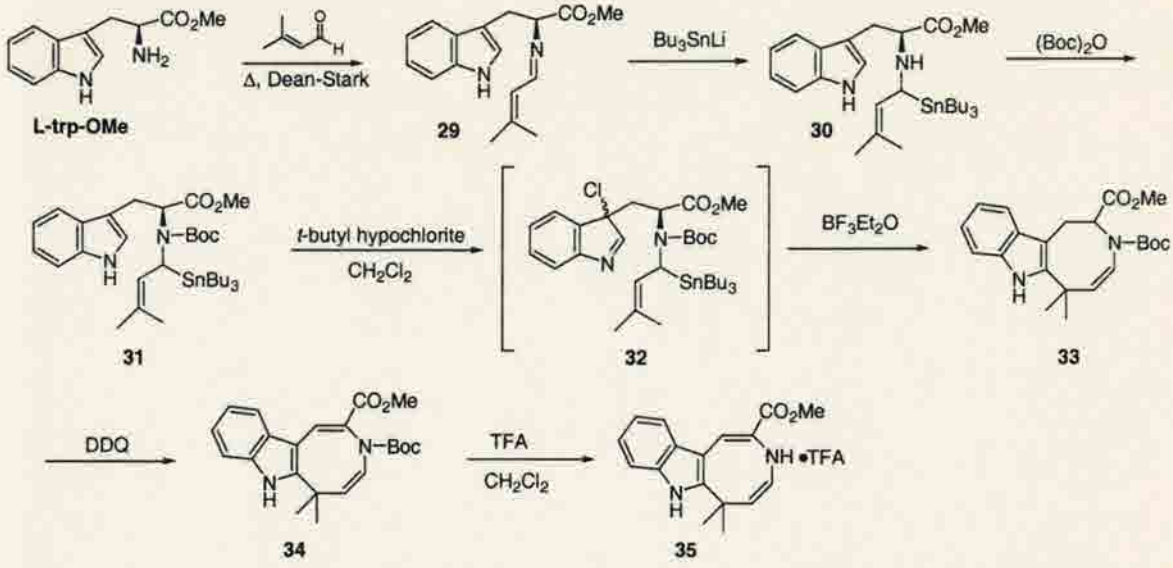


In the synthesis of Fumitremorgin, Kadato et al. reported the formation of an α, β-*trans*-diol of a tryptophanyl derivative.[12] The same approach will be applied to the synthesis of the *trans*-diol of Okaramine B. Oxidation of **23** with DDQ should afford **24**. If the mechanism is as Kadato et al. predict, then bromine should attack from the less hindered α-side to form a bromonium ion which is then ring opened by attack of H_2O at the β-position in a diaxial orientation. The *trans*-bromohydrin should then undergo spontaneous solvolysis to give the *trans*-diol, **25**. Hydrogenolysis of the Williams' lactone with $Pd(OH)_2$/ H_2 should provide the BOC-protected amino acid, **27**. Treatment with dimethyldioxirane (DMDO) ought to give **28**.[2] Using DMDO toward the total synthesis of gypsetin, Danishefsky et al. obtained a 1:1 ratio of diastereomers with N-Boc-2-dimethylallyl-L-trp-OMe as a substrate. In this case, however, it is hoped that the methoxy group in the β-position will direct the oxidation and provide the desired stereoselectivity. Alternatively, **28** can be formed from the 3-hydroxyindolenine with C_6Cl_6O in dichloromethane/water.[13] However, the stereoselectivity of this reaction has not been explored.

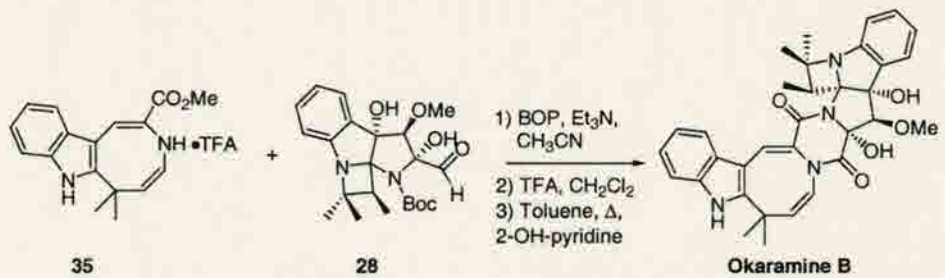


The azocinindole ring system of Okaramine B is unique. Only two other compounds posess a similar ring system, cycloechinulin from *Aspergillus ochraceus*[14] and 10, 20-Dehydro[12, 13-dehydroprolyl]-2-(1', 1'-dimethylallyl-tryptophanyl) diketopiperazine from *Aspergillus ustus*.[15] The synthesis for tetrahydroaustamide (also produced by *Aspergillus ustus*) developed by Hutchison et al.[16] could easily be applied to the synthesis of the azocinindole ring of Okaramine B. However, the recent work done in Danishefsky's laboratories on the synthesis of 2-dimethylallyl-tryptophanyl derivatives from tryptophan evoke an alternative approach.[2] Cyclization of 2-dimethylallyl-L-tryptophan itself could provide the incorrect regio-selectivity (formation of the 7 membered ring). Instead, a cyclization from intra-molecular nucleophilic displacement at the 2-position of the indole (through the 3-chloroindolenine) will be pursued.

Tryptophan methylester will be condensed with 3-methyl-2-butenal to provide the desired imine (29).[17] Addition of the deprotonated tributylstannane to **29** should provide the α-aminoalkylstannane **30.**[18] The N-Boc protected stannane, **31**, will be subjected to tert-butyl hypochlorite followed by BF_3•OEt to provide the azocinindole ring **33**.[2] While this technique has been used on a number of nucleophiles, including tri-*n*-butyl-allylstannane, it has not been attempted intra-molecularly. This synthesis will provide an interesting and useful extension of Danishefsky's methodology. The azocinindole, **33**, can be oxidized with DDQ to afford the α, β-unsaturated azocine, **34**.[10] Deprotection of the N-Boc group with TFA in dichloromethane should provide the intermediate, **35**, which is ready to be coupled to **28**.



Coupling of the tryptophanyl derivatives can be accomplished with BOP reagent, although DCC or EDCI may also be used. Once the amino acids are coupled, the N-Boc group will be removed with TFA and the dipeptides will be cyclized in refluxing toluene with catalytic 2-hydroxy pyridine to provide Okaramine B.



In summary, the first total synthesis of Okaramine B has been proposed. The convergent nature of this synthesis will allow the development of analogs for testing for insecticidal activity and provide substrates to study structure/ activity relationships.

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So long, and thanks for all the fish.

-Douglas Adams

So Long, and Thanks for all the Fish

The Fourth in a Trilogy