An Abstract of the Thesis of

Rodney L. Eisenberg for the degree of Doctor of Philosophy

in Chemistry presented April 13, 1990

Title: Studies Of The Biosynthesis Of Sarubicin A

Redacted for Privacy

Abstract Approved: Dr. Steven J. Gould

Early studies on the biosynthesis of Sarubicin A, **1**, had suggested the intermediacy of the novel aromatic amino acid 6-hydroxyanthranilic acid, **17**. To investigate the biosynthetic steps beyond **17**, [^{13}C-^{15}N carboxamide] 6-hydroxyanthranilamide, **27a**, was synthesized, and fed to fermentations of *Streptomyces helicus*. Incorporation of **27a** into **1** provided suggestive evidence that **27** is a new product of the shikimate pathway, and provided evidence that amide formation occurs early in the biosynthetic pathway to **1**.

Several possibilities exist for the timing of the hydroxylation and C-glycosylation steps of the quinone ring of **1**. To test the chemical viability of the potential intermediate 5-glycosyl-3,6-dihydroxyanthranilamide, **29**, the model quinione **67** was synthesized and reduced. The reduction product, **68**, was stable in the absence of O_2, suggesting that the proposed C-glycoside **29** is a plausible intermediate.

A model for **29** was synthesized via an ultrasound catalyzed Diels-Alder reaction between the 2,2-dimethyl-5,8-dimethoxy-1,2,3,4-tetrahydro-4-oxoquinazoline-6-carboxaldehyde, **71**, and the diene **72**, to give the pyran derivative **73**. However, attempts to construct the carbon skeleton of **29** with diene **74** were unsuccessful.

Isochorismate synthase was isolated from *Enterobacter aerogenes* 62-1, partially purified, and incubated with chorismic acid, **19**, in $H_2{}^{18}O$. The product, isochorismic acid, **18**, was purified and found to contain ^{18}O at the C-2 hydroxyl, demonstrating that the enzyme-catalyzed reaction proceeds via an SN_2' or addition-elimination type of reaction.

Studies of the Biosynthesis of Sarubicin A

Rodney L. Eisenberg

A Thesis

submitted to

Oregon State University

in partial fulfillment of

the requirements for

the degree of

Doctor of Philosophy

completed April 13, 1990

commencement June 1990

Approved:

Redacted for Privacy

Professor of Chemistry in Charge of major

Redacted for Privacy

Chairman of Department of Chemistry

Redacted for Privacy

Dean of Graduate School

Date thesis is presented April 13, 1990

Table of Contents

List of Figures

List of Tables

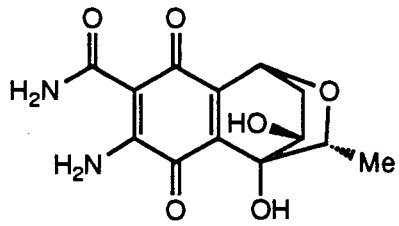
List of Schemes

Studies of the Biosynthesis of Sarubicin A

Introduction

Isolation and Structure Elucidation of Sarubicin A

Sarubicin A, **1**, was first isolated in 1980 independently by two groups, researchers at The UpJohn Company as a metabolite of *Streptomyces helicus*[1] (UC-5837) and by Eckardt in Germany from a *Streptomyces* strain JA 2861.[2] The structure was first elucidated using physical methods including ^{1}H and ^{13}C NMR, mass spectral analysis and UV analysis.[2] Assignments of relative stereochemistry and carbon structure were confirmed by X-ray analysis.[1] The absolute configuration of the bicyclic portion was subsequently established through circular dichroism spectroscopy in 1983.[3] Two total chemical syntheses have since been reported.[4,5]



1

Figure 1. Sarubicin A.

Other antibiotics which have close structural similarities to **1** are illustrated in Figure 2. Granaticin, **2**, a napthoquinone antibiotic, has the 2-oxabicyclo[2.2.2]oct-5-ene structure in common with **1**.[6] The antibiotic G-7063-2, **3**, has the same array of amide and amino groups on the 1,4-benzoquinone skeleton as **1**,[7] as do the paulomycins, **4**.[8]

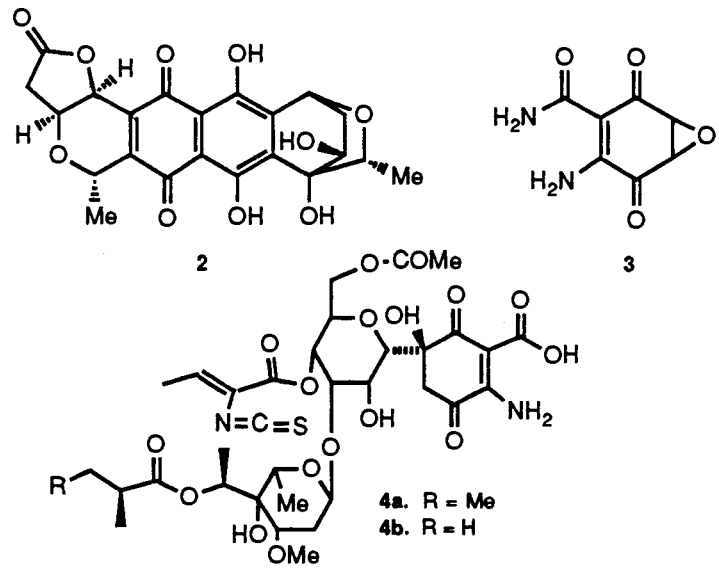


Figure 2. Natural Products Related to Sarubicin A.

Biological Activity of Sarubicin A

Sarubicin A was shown to have antibiotic activity against a few gram positive and gram negative microorganisms by researchers at The Upjohn Company[1] and by Eckardt.[2] It is particularly active against *Streptococcus pneumoniae* (UC-41), exhibiting a MIC of 1.0 μg / mL. Almost no activity was found against yeast or fungi. When the antibiotic was administered to mice it was found to be toxic with an MTD of 5 mg/kg/day with no protection against *S. pneumoniae* at or below this level.

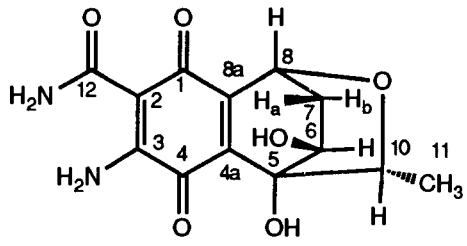
Chemical and Physical Properties of Sarubicin A

Sarubicin A is a red crystalline solid which melts at 194°-195° C.[9] It is soluble in the lower alcohols, acetone, dimethylsulfoxide, dimethylformamide and is partially soluble in water. It can be crystallized from chloroform, or ethyl acetate and is indefinitely stable at room temperature as a crystalline solid . The ultraviolet and visible spectrum in methanol exhibits maxima at 261 nm (ε=13,171) and 472 nm (ε=1,517), and has an optical rotation of +9° (methanol). Assignments for the ^{1}H NMR and ^{13}C NMR spectra for **1** are listed in Table 1.[9]

Table 1: ^{1}H and ^{13}C NMR data for Sarubicin A, **1**.

^{1}H NMR Data (4:1 $CDCl_3$-DMSO-d_6)					^{13}C NMR Data (DMSO-d_6)		
Proton	δ(ppm)	Int.	Mult.	*J* (Hz)	Carbon	δ(ppm)	Mult.
H-8	4.90	1	dd	3.6, 1.9	1	180.3[a]	s
H-7a	1.37	1	dt	14.4, 1.9, 1.8	2	97.7	s
H-7b	2.58	1	m	14.4, 8.0, 3.6	3	154.1	s
H-6	3.80	1	m	8.0, 4.5, 1.8	4	178.8[a]	s
H-10	3.61	1	q	6.0	4a	135.2[b]	s
H-11	0.97	3	d	6.0	5	78.3	s
$CONH_2$	8.68	1	s		6	70.5	d
$CONH_2$	6.64	1	s		7	36.9	t
NH_2	10.70	1	s		8	61.5	d
NH_2	8.00	1	s		8a	147.3[b]	s
(C-5)-OH	5.29	1	s		10	71.6	d
(C-6)-OH	4.82	1	d	4.5	11	16.7	q
					12	169.7	s

a. See ref. 16. b. Carbon resonances for 4a and 8a have not been unambiguously assigned.



1a

Chemical Syntheses of Sarubicin A

Sarubicin A has been synthesized in racemic form by two groups: Yoshii and coworkers,[4] and the Semmelhack group.[5] The first synthesis, by Yoshii, is shown in Scheme 1 and begins from the dimethyl hydroquinone **5**. The key step is the introduction of the *cis*-diol in a stereoselective manner to yield **6**, which is accomplished by catalytic osmylation directed by the neighboring methyl group of **7**. Yields and reagents are given where reported.

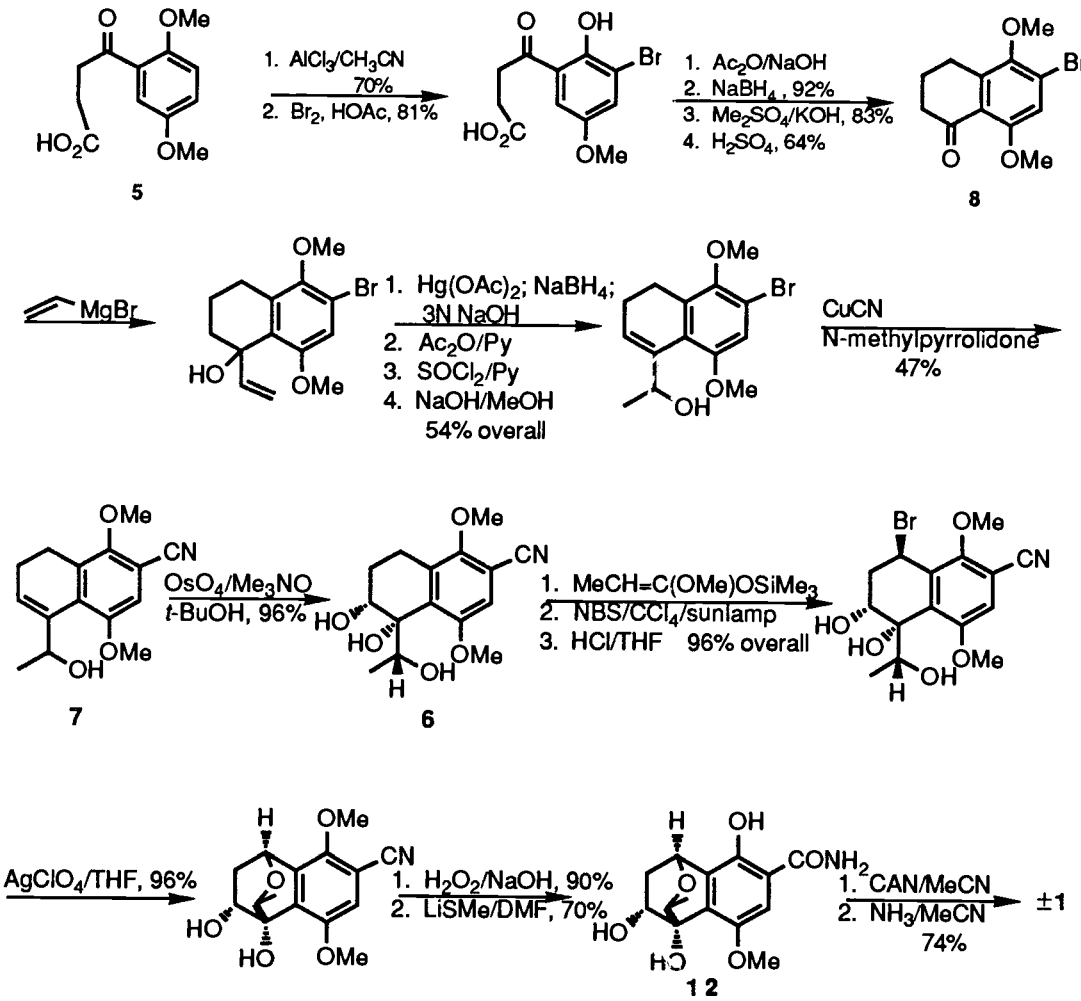
The formal total synthesis by Semmelhack and coworkers is similar, starting from the tetralone **8**. Key steps include a cyanide addition *anti* to an alcohol derived from **8** to produce the required *cis*-diol stereochemistry of **9**. A titanium (IV) mediated methyl addition to the aldehyde **10** provides the proper stereochemistry for **11**. This pathway is illustrated in Scheme 2 and converges with the previous synthesis beginning at **12**.

Previous Biosynthetic Studies of Sarubicin A

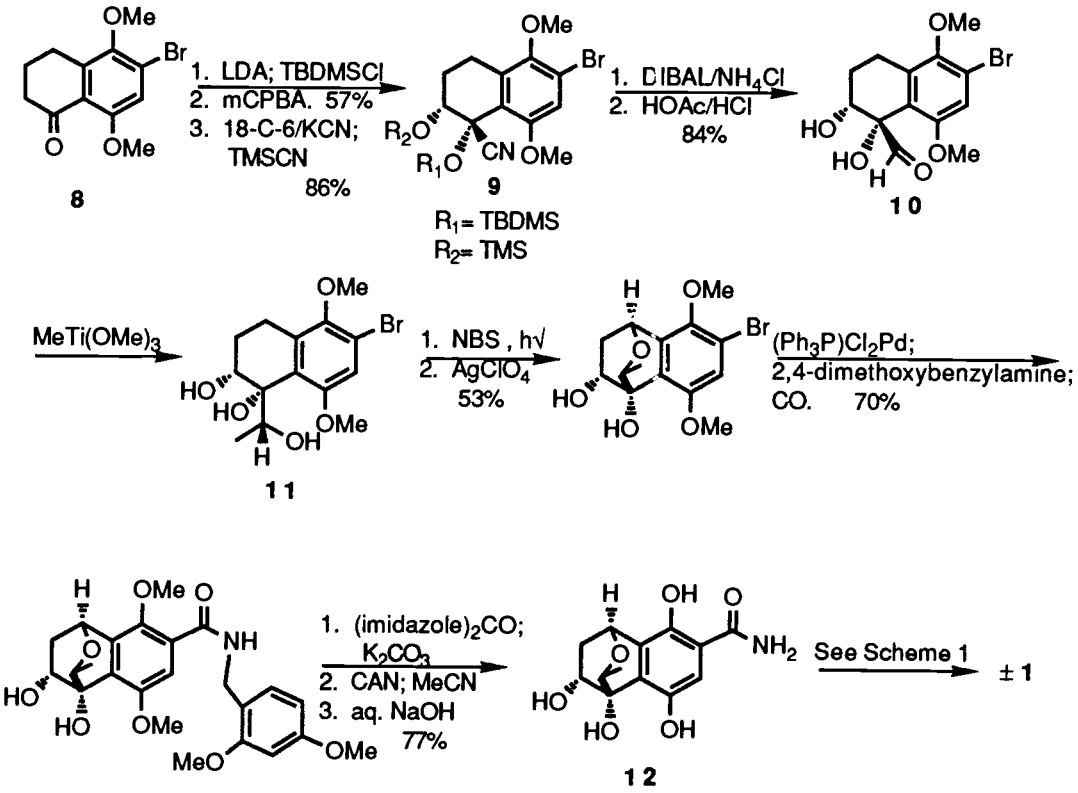
Biogenetic Pathways to Sarubicin A

As a starting point for biosynthetic studies of sarubicin A, two main biogenetic hypotheses were advanced by our group: The aromatic portion of sarubicin A could be derived from an anthranilate derivative via the shikimate pathway, and be condensed with a modified glucose to provide the bicyclic portion or, the carbon skeleton could originate from acetate and thus be a polyketide. These possibilities are shown in Scheme 3.

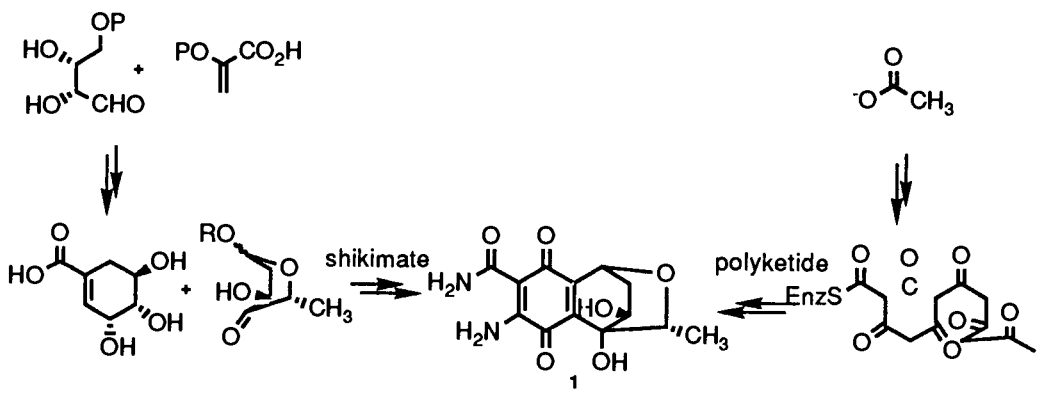
Workers at The Upjohn Company carried out biosynthetic experiments which suggested a shikimate pathway origin for the aromatic portion of **1**, while the bridged tetrahydropyran moiety derived from glucose, **13**.[10] Although never published, the results were provided as a private communication and are summarized in Table 2.



Scheme 1. Synthesis of Sarubicin A by Yoshii.



Scheme 2. Synthesis of Sarubicin A by Semmelhack.



Scheme 3 Biogenetic Pathways for **1**

Table 2. Previous Biosynthetic Experiments of Sarubicin A

Compound Fed.	Incorporation into **1**
[U-^{14}C]-glucose (**13a**)	4%
[1-^{13}C]-glucose (**13b**)	C-8; C-1; C-3
[6-^{13}C]-glucose (**13c**)	C-11 (methyl); C-1; C-3
[U-^{14}C]-shikimic acid (**14a**)	0.5%
anthranilic acid (**15**)	no incorporation
3-hydroxyanthranilic acid (**16**)	no incorporation
tryptophan (ring-labeled)	no incorporation
phenylalanine (ring-labeled)	no incorporation

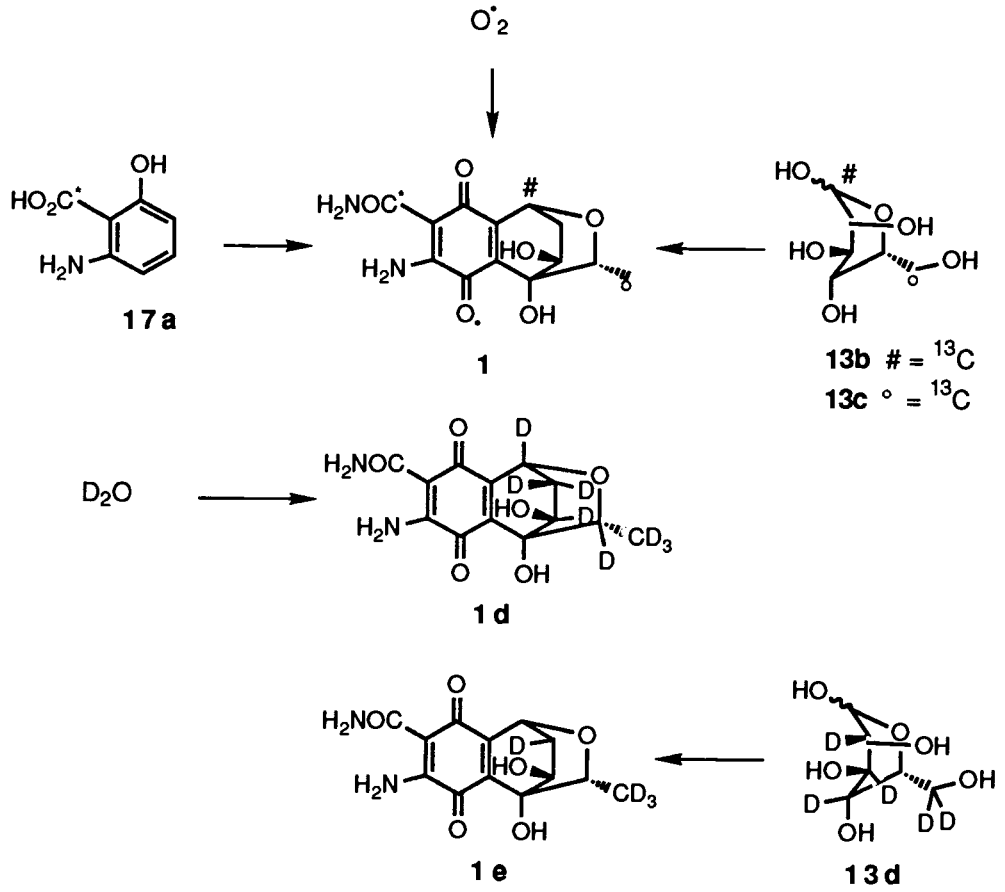
These results effectively ruled out a polyketide biosynthetic pathway. The incorporation of intact glucose was indicated by the high incorporation (4%) of glucose. This was further supported by the labeling of C-8 (**1b**) by [1-^{13}C]-glucose, **13a**, and of the C-11 methyl (**1c**) by [6-^{13}C]-glucose, **13b**, indicating that the tetrahydropyran is derived from glucose. The quinonoid portion was labeled at C-1 and C-3 by both [1-^{13}C] - glucose and [6-^{13}C] - glucose, although at a lower level of enrichment, consistent with a shikimate pathway origin. This was further supported by the incorporation of [U-^{14}C] - shikimic acid, **14a**, into **1**.

Attempts to identify the nature of potential intermediates beyond shikimic acid had been less successful. When anthranilic acid, **15**, or 3-hydroxyanthranilic acid, **16**,[11,12] both established natural products, were fed to *S. helicus*, no incorporation was observed in either case. Similar negative results were obtained from ring-labeled tryptophan and phenylalanine.

Building upon these results, experiments were performed in this laboratory which were designed to identify the origin of the carbinol hydrogens of the bridged tetrahydropyran moiety of **1**.[13] A fermentation in the presence of 10% D_2O was performed, **1d** isolated, and analyzed by ^{2}H NMR spectroscopy. Deuterium enrichment was found at all carbinol positions and allowed identification of the line shapes for each resonance.

[2,3,4,6,6-$^{2}H_5$]-D-glucose, **13d**, was next fed, and the isolated **1e** found to be enriched in the C-11 methyl and H-7_a positions in a 3:1 ratio, respectively. These results were consistent with the work of Floss and coworkers on granaticin using [^{14}C-^{3}H]-glucose:[14] they indicate migration of deuterium from C-4 to C-6 of glucose, retention of configuration at C-2 of glucose when the hydroxyl group is replaced by H, loss of deuterium from H-3 of glucose (a result consistent with a *syn* elimination of water from C-2/C-3) and a subsequent *syn* reduction of the double bond that had been generated.

Subsequent to the above work, efforts were made to determine the origin of the quinone oxygens. A fermentation conducted in the presence of $^{18}O_2$ revealed incorporation of ^{18}O exclusively into the C-4 quinone oxygen of **1**.[15] 6-Hydroxyanthranilic acid, **17**, though not a previously known natural product, was next considered as a possible precursor for the C-1 oxygen. A convenient synthetic route to the putative intermediate was developed[16] and subsequently [^{13}COOH]-6-hydroxyanthranilic acid, **17a**, synthesized. Feeding **17a** to *S. helicus* resulted in the incorporation of ^{13}C label into the carboxamide carbon (C-12) of sarubicin A (**1g**).[16] These findings are summarized in Scheme 4.



Scheme 4. Previous Biosynthetic Experiments of **1**

Aromatic Acids Derived From the Shikimic Acid Pathway

Chorismic acid, **19**, is an important branch point of the shikimic acid pathway. It is a precursor to isochorismic acid, **18**, and also leads to a variety of natural products including anthranilic acid, **15**[17] and *p*-aminobenzoic acid, **20**.[18,19] The shikimate pathway is the primary metabolic pathway which serves as a source of the aromatic amino acids, and has been extensively reviewed.[20,21] Both of the aromatic amino acids **15** and **20** are known to be derived from **19** but not from isochorismic acid, which is less prevalent in nature, but known as a precursor to some natural products.[22] Isochorismic acid has been shown to be involved in the biosynthesis of salicylic acid,[23] **21**, 2,3-dihydroxybenzoic acid,[24] **22**, the

menaquinones, **23**, (vitamins K_2),[25] and enterobactin, **24**.[26,27] These natural products are shown in Figure 3.

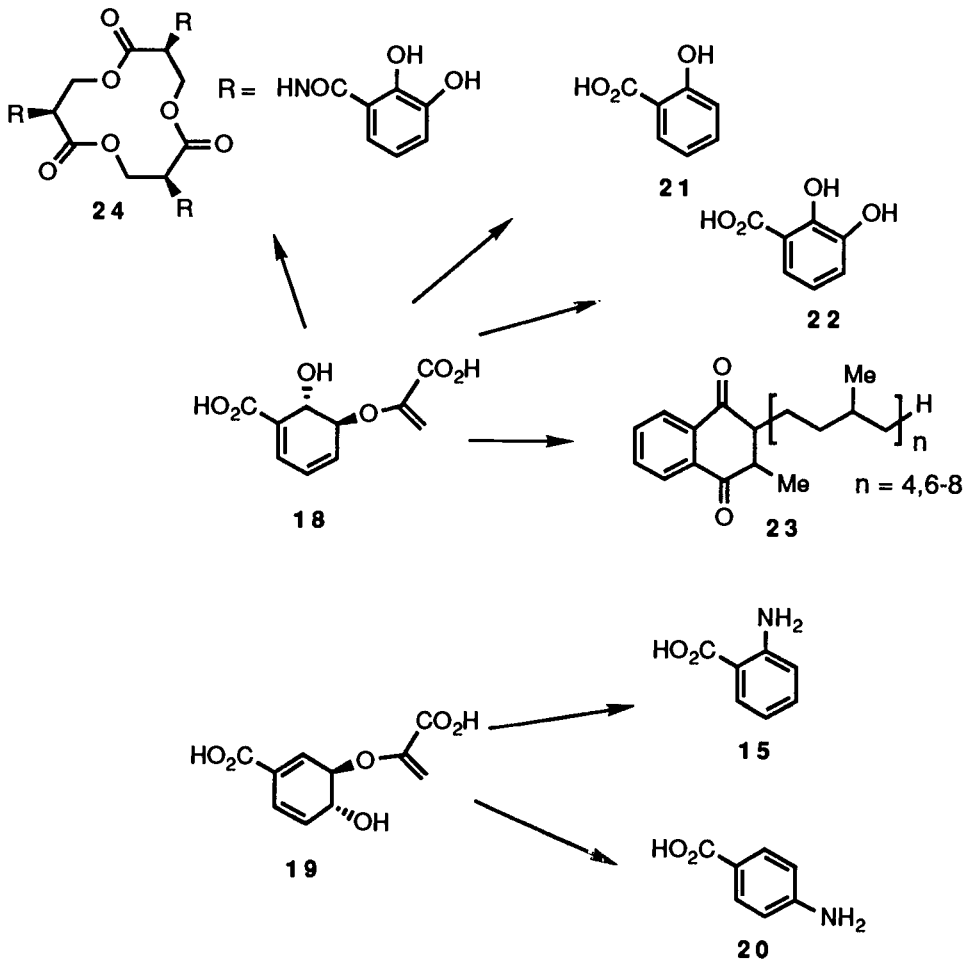


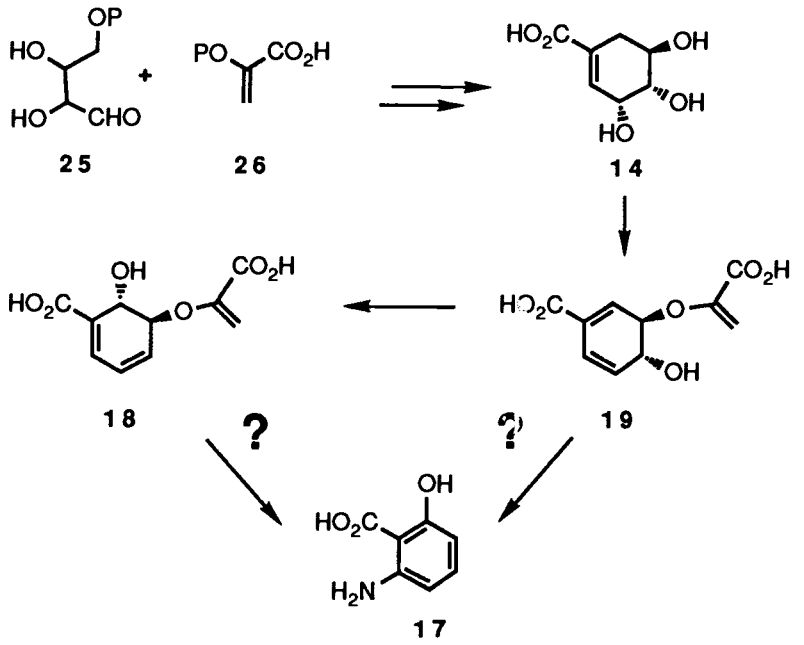
Figure 3. Natural Products From the Shikimate Pathway

Possible Biosynthetic Pathways Leading to 1

The origin of the carbon atoms of **1** have been established by the previous biosynthetic studies: the bridged tetrahydropyran portion is derived from an intact glucose molecule, while the quinonoid moiety

apparently is derived from the shikimate pathway via the previously unknown 6-hydroxyanthranilic acid.

Chorismic acid can be viewed as a precursor to **17**, after being converted to isochorismic acid, **18**, as shown in Scheme 5. Alternatively, isochorismic acid may *not* be involved in the pathway leading to **17**, and therefore chorismic acid, **19**, would be the branch point from the shikimate pathway leading to **17**.



Scheme 5. Possible Origins of **17** From the Shikimate Pathway

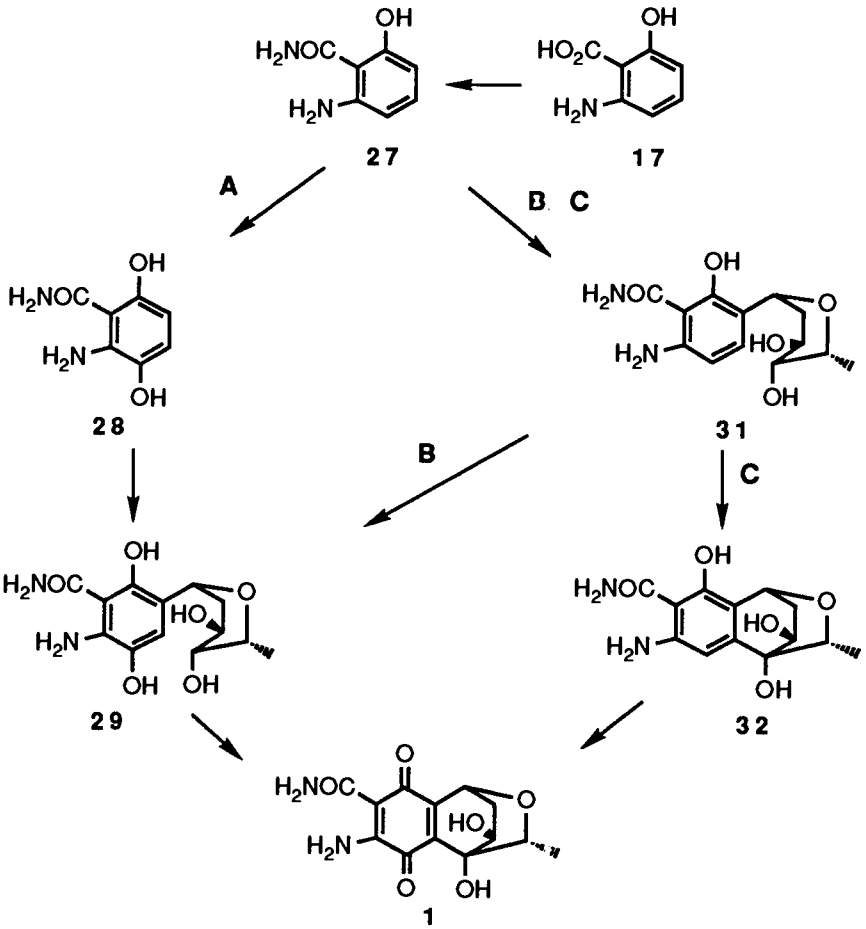
There are several possibilities for the timing of the key steps leading from the novel **17** in the biosynthetic pathway leading to **1**. These include formation of a C-glycoside, introduction of the C-3 phenol (anthranilic acid numbering), intramolecular cyclization of the putative C-glycoside to produce the bicyclic skeleton of **1**, and generation of the carboxamide. Carboxamide formation could conceivably occur at any point along the pathway. There are three main permutations which can be envisioned (for simplicity, conversion of **17** to the corresponding 6-hydroxyanthranilamide, **27**, is assumed to be the first step), and are outlined in Scheme 6:

A. Oxygenation at C-3 of **17** to give a 3,6-dihydroxyanthranilic acid derivative, **28**, followed by C-glycoside formation to yield **29**, and finally cyclization of the aromatic portion with, presumably, a keto-group on the sugar *ortho* to the C-3 phenol to give **1**.

B. C-Glycoside formation producing **31**, then oxygenation of **31** to converge with pathway **A** at **29**, and finally an intramolecular Friedel-Crafts type of cyclization to give **1**.

C. Initially, C-glycosylation to produce **31**, followed by subsequent cyclization at C-4 of the aromatic portion of **31** with the keto sugar would yield **32**, followed then by oxygenation to give **1**.

Pathway **A** seemed plausible based on the notion that hydroxylation of the C-3 position of **17** or a derivative would activate the C-4 position for an intramolecular Friedel-Crafts type of reaction with a keto substituent of the attached modified glucoside. A synthetic investigation into 3,6-dihydroxyanthranilamide, **28**, by Dr. L. R. Hillis in this laboratory revealed it to be unstable, decomposing completely within about 30 minutes.[28] (*vida infra*) This result led to the consideration of pathway **B** which still had the attractive feature of the activating group at C-3 allowing a similar ring closure mechanism.



Scheme 6. Potential Biosynthetic Pathways to **1**

Overview of the Research

The most advanced precursor to **1** suggested to date has been the novel aromatic amino acid **17**. There are two major areas of research which have been carried out, both involving the proposed precursor **17**.

In the first section, studies to address the fate of **1** in the metabolic pathway are discussed. First, the question of the timing of amide formation will be addressed by investigating the intermediacy of 6-hydroxyanthranilamide, **27**, based on the incorporation of ^{13}C-^{15}N carboxamide labeled **27**. Second, attempts to establish the intermediacy of

17 and **27** by the use of radioactive trapping experiments involving whole cell cultures of *S. helicus* will be presented. Third, the chemical stability of the putative advanced intermediates **28** and **29** will be addressed, and a synthetic method outlined which holds much potential for the preparation of putative advanced intermediates containing the 2,6-dideoxy glucose moiety of sarubicin A, possibly ultimately culminating in a biomimetic total synthesis of **1**.

The second section will detail investigations into the origin of **17** in *S. helicus*. In particular, experiments probing the possible involvement of isochorismic acid into **17** using cell-free extracts of *S. helicus* will be delineated. Isochorismic acid was obtained from partially purified extracts of *Enterobacter aerogenes* containing isochorismate synthase activity, and fully characterized for the first time using 2D NMR techniques. The mechanism of the enzyme-catalyzed conversion of chorismate to isochorismate was studied by the use of isotopically labeled water, and the results will be presented.

Results and Discussion

Advanced Intermediates Beyond 6-Hydroxyanthranilic Acid.

As there are a number of excellent reviews and monographs[29] on biosynthetic methodology available in the literature, a detailed discussion of the techniques of biosynthesis will not be presented here. Discussions of techniques used in this study will be presented as needed in the Results and Discussion session.

This section will detail the preparation of ^{13}C-^{15}N labeled 6-hydroxyanthranilamide, and its incorporation into **1**.

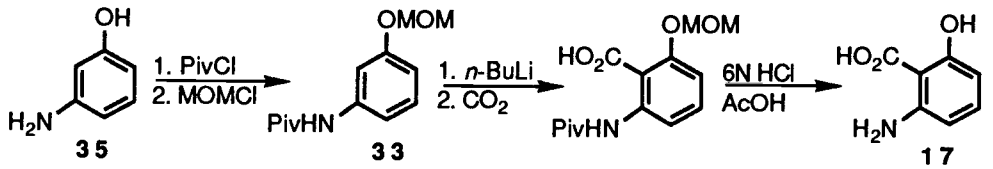
Synthesis of 6-Hydroxyanthranilamide

The pathways presented in Scheme 5 provide for a number of potential intermediates which could be considered as synthetic targets. In order to narrow the possibilities, the timing of the conversion of the acid of **17** to the amide **27** was considered. To test whether this step might be the first one, 6-hydroxyanthranilamide, **17**, was chosen as an initial target for synthesis.

^{15}N-carboxamide labeled **27a** could, in principle, be used, but unambiguous detection of any ^{15}N incorporated into **1** by ^{15}N NMR spectroscopy[30] would be difficult, especially if incorporation levels were low, due to the insensitivity of the ^{15}N nucleus, although it has been used successfully in biosynthetic studies.[31] Detection of ^{15}N by mass spectrometry would likely be equivocal since there is more than one nitrogen present in **1** and clearly defined fragments would have to be identified. A convenient method of indirectly detecting ^{15}N in a ^{13}C NMR spectrum by the use of ^{15}N-^{13}C doubly labeled intermediates has shown much utility in biosynthetic experiments.[32] This method provides a convenient way to discern intact incorporation of a carbon-nitrogen bond in a putative intermediate. In the present case, if bond breakage occurred and the ^{15}N were hydrolyzed, then a simple enrichment of the natural abundance ^{13}C resonance would be observed. However, if ^{13}C-^{15}N bond breakage did not occur, then the natural abundance signal would be

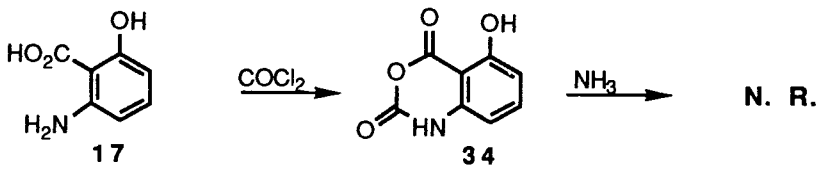
flanked by a doublet from the biosynthetically enriched molecules, caused by the ^{13}C being split by the adjacent ^{15}N. The synthesis of [^{13}C-^{15}N-carboxamide]-6-hydroxyanthranilamide was hence planned with this biosynthetic experiment in mind.

A synthesis of **17** had previously been developed which allowed the introduction of a ^{13}C carboxylic acid via an *ortho*-directed lithiation of **33**, and then quenching with $^{13}CO_2$, as shown in Scheme 7. This had been utilized in a feeding experiment which implicated **17** as a biosynthetic intermediate for **1**.[16]



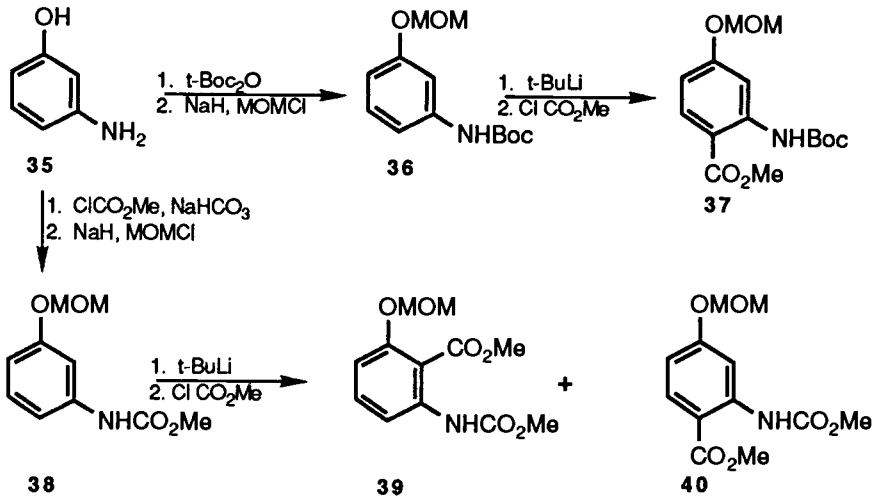
Scheme 7. Previous Synthesis of 6-Hydroxyanthranilic Acid.

Initial efforts to directly form the required amide now focused on attempts to directly convert **17** to **27**. Isatoic anhydrides have been shown to react with ammonium hydroxide as the solvent to afford amides in good yield,[33,34] but these conditions would have been prohibitively expensive for the ^{15}N required. However, it was reasoned that ammonia in stoichiometric amounts in an appropriate solvent might achieve the same result. The isatoic anhydride **34** was prepared from **17** and phosgene, and then treated with ammonia in various solvents and at various temperatures in an attempt to develop an economical procedure. Thus, **34** was dissolved in THF, EtOH, or CH_2Cl_2 and the solution cooled to -78 °C. In each case, the solution was saturated with anhydrous ammonia, the vessel sealed, and heated to 40-80 °C for 2 to 6 hours, depending on the solvent. During the course of these experiments, a deep green color appeared each time. The discoloration could be minimized by exhaustively degassing the solution before introduction of the ammonia, indicating that probable oxidative decomposition was occurring. However, no useful product could be discerned by TLC in any case. Scheme 8 summarizes these results.



Scheme 8. Approach to **27** via an Isatoic Anhydride.

Attempts to develop a more promising route to the amide **27** were next made, utilizing an ether protecting group on the phenol. A nitrogen-protecting group was sought which had the capability of activating and directing *ortho* lithiations. A pivalamide had been an effective directing group in the synthesis of **17**,[16] but this would require too harsh of conditions for removal in the present situation. An initial attempt at introduction of the N-*t*-Boc group onto 3-aminophenol, **35**, using BOC-ON[35] failed. Successful protection of the amino group was realized with di-*t*-butyl-dicarbonate,[36] and the phenol was then protected as the methoxymethyl ether (MOM) to yield **36**. However, lithiation[37,38] of **36** with *t*-butyllithium at -20 °C in THF, followed by quenching of the reaction with methyl chloroformate, gave the undesired 1,2,4-trisubstituted ester **37** in 20% yield as the only observable product. On the assumption that the presence of two bulky groups was at fault, the less bulky methyl urethane **38** was next prepared by reacting **35** with methyl chloroformate in the presence of sodium bicarbonate. Lithiation of **38**, followed by quenching with methyl chloroformate now gave a mixture of the desired isomer **39** (33%), the undesired **40** (6%), and unreacted starting material. These efforts are outlined in Scheme 9.



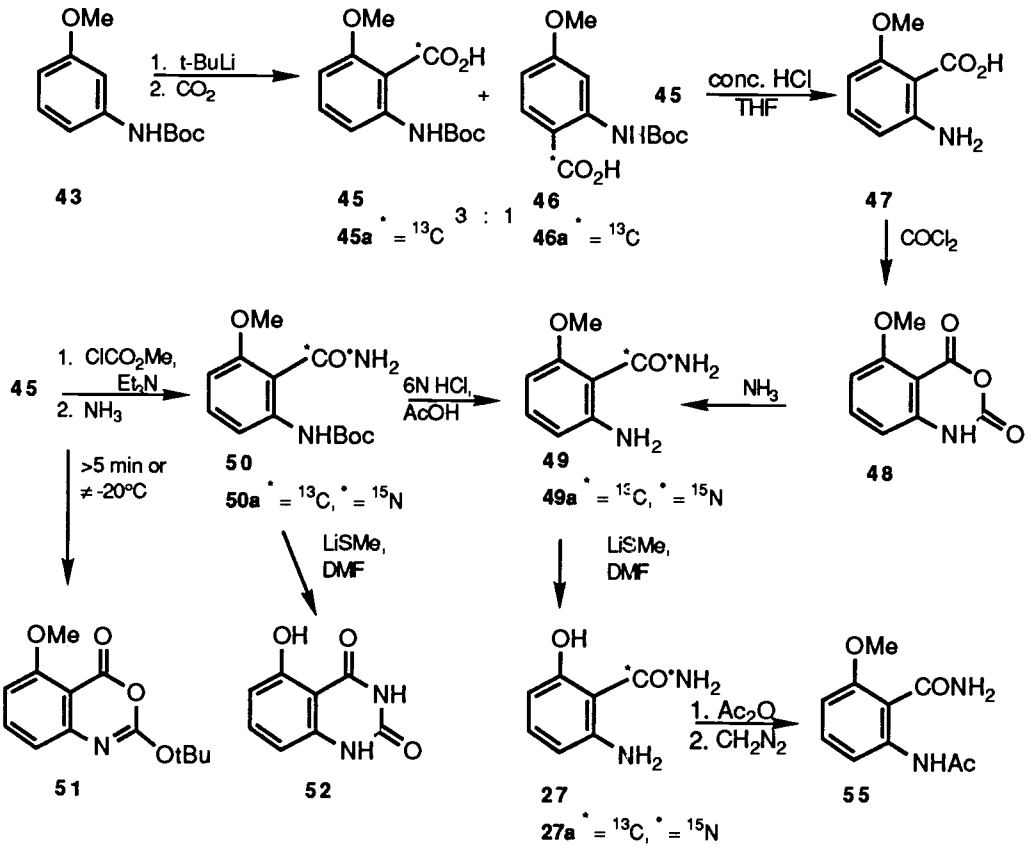
Scheme 9 Approach to 6-Hydroxyanthranilamide, **27**.

The alternative to the less bulky urethane was to replace the bulky MOM group. However, in developing a synthesis of **17** that made use of a methyl ether, this group had been difficult to remove with Lewis or protic acids.[16] However, in a model study, lithium thiomethoxide[39] in DMF was found to smoothly demethylate **41** to give the phenol **42** in 75% yield, as shown in Scheme 10.

Scheme 10. Lithium Thiomethoxide Demethylation Model.

Encouraged by this, N-*t*-Boc-3-anisidine, **43**, was prepared from 3-anisidine, **44**, (98%), lithiated, and quenched with CO_2 to give a 3:1 mixture of **45** and **46** (60% combined yield). This mixture could be separated, but with difficulty, requiring repeated silica column chromatography. Nevertheless, **45** was treated with concentrated HCl in THF, to yield **47**, and then with phosgene to give the isatoic anhydride **48**, but this provided only a very low yield of the carboxamide **49**, even when treated with ammonia in large excess at elevated temperature in a sealed tube in a manner analogous to that described for **34**.

When the mixture of **45** and **46** was reacted at -20 °C with methyl chloroformate/triethylamine to give the mixed anhydride,[40] and then quenched within 5 minutes with anhydrous ammonia, an almost quantitative conversion of **45** to carboxamide **50** was obtained. The acid **46** apparently reacted much more slowly and was subsequently removed from the crude reaction mixture by extraction with aqueous bicarbonate. If the reaction was carried out at 0 °C or -78 °C, or ammonia added much later than five minutes after the chloroformate addition, the major product was the cyclized product **51**. Direct treatment of **50** with lithium thiomethoxide in DMF gave only imide **52**, and attempts at stepwise deprotection at first proved troublesome, as reaction in THF in the presence of concentrated HCl, 3M HCl in ethyl acetate,[41] or trifluoroacetic acid[42] produced either a very slow reaction or no reaction whatsoever. However, the *t*-Boc group was successfully removed with HCl/acetic acid, and subsequent treatment with the thiomethoxide reagent finally gave the target compound **27** in 70% yield. This procedure constitutes a first synthesis of **27**. Scheme 11 summarizes these reactions.



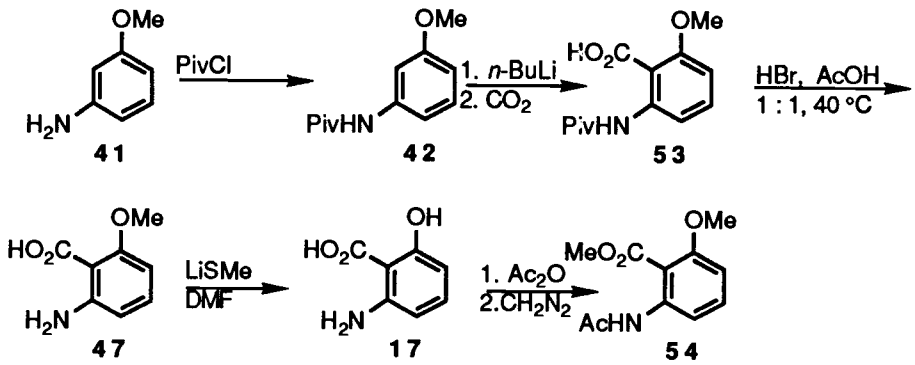
Scheme 11. Synthesis of 6-Hydroxyanthranilamide.

^{13}C-^{15}N-Carboxamide labeled **27a** was next prepared following the described technology (*vida supra*). Thus, **43** was lithiated and quenched with $^{13}CO_2$ generated from $Ba^{13}CO_3$.[43] The mixture of isomers **45a** and **46a** was first treated with $ClCO_2Me$ in the presence of Et_3N followed by $^{15}NH_3$ in THF generated from $^{15}NH_4Cl$[44,45] to yield the doubly labeled amide **50a**. Stepwise deprotection of **50a** with 6N HCl/acetic acid followed by lithium thiomethoxide gave the doubly labelled amide **27a** in 23% overall yield. The carboxamide ^{13}C resonance of **27a** at 170.34 ppm was an enhanced doublet with J_{CN} = 15.1 Hz.

In the original synthesis of **17**, deprotection of the phenolic pivalamide of **17** was erratic, sometimes leading to decarboxylation. Based on the synthesis of **27** reported herein, a more reliable route to **17** was next developed, as shown in Scheme 12. Thus, **42** was lithiated, and quenched with CO_2 to give the pivalamide protected 6-methoxyanthranilic acid, **53**, in

95% yield. Hydrolysis of the pivalamide with HBr/acetic acid at 40° for two days gave 6-methoxyanthranilic acid, **47**, as the HBr salt in 72% yield, whereupon the methyl ether was then cleaved utilizing the thiomethoxide procedure. Chromatography of the crude product on an anion exchange column ultimately gave pure **17** as its acetic acid salt in 56% yield, after exhaustive extraction into ethyl acetate and drying over Na_2SO_4 overnight.

Both **17** and **27** were derivatized to stable, crystalline materials. Following a procedure developed previously,[16] the acid **17** was first N-acetylated with triethylamine/acetic anhydride, followed by treatment with diazomethane to produce the acetamido methyl ester **54**. This could be crystallized from CH_2Cl_2/hexane. In a directly analogous manner the amide **27** was acetylated, then treated with diazomethane to yield the 6-methoxy ether **55**. Crystallization of **55** was accomplished from water in this case.



Scheme 12. Synthesis of 6-Hydroxyanthranilic Acid.

The stage was now set to feed **27a** to *S. helicus* in order to test for incorporation of the intact amide into **1**. Detection of intact incorporation of the C-N amide bond required the acquisition of a high resolution ^{13}C NMR spectrum. A previous ^{13}C NMR study of **1** had utilized deuterium-induced isotope shifts to assign the quinone resonances.[15] However, various samples of **1** had unfortunately exhibited ^{13}C line widths of 5-15 Hz, leading to the conclusion that interfering paramagnetic ions may have been chelated with **1**, thus causing line broadening. Treatment of **1** with Chelex had apparently solved this problem.[15]

However, a series of ^{13}C NMR experiments conducted on **1** now indicated that this troublesome phenomenon was apparently entirely due to concentration effects. The carbonyl resonances at 180.0 and 178.6 ppm are shown in Figure 4 at five concentrations ranging from 0.120 M (35 mg / mL) to 0.038 M (11.25 mg / mL) in DMSO-d_6, each obtained after serial dilution of the original sample. At a concentration of 0.038 M the carbonyl lines of **1** at 180.0 and 178.6 ppm were less than 2 Hz wide at the base. However, increasing the concentration to 0.051 M caused a slight broadening of the resonances. Broadening of these resonances increased dramatically as the concentration was increased until at a concentration of 0.12 M the line widths had increased to at least 15 Hz wide. Therefore the NMR experiment planned was limited to a concentration of 4.5 mg/500μL DMSO-d_6.

Incorporation of 6-Hydroxyanthranilamide into Sarubicin A

Following the previously developed timing for the feeding of precursors, [^{15}N-^{13}C-carboxamide]-**27a** was pulse-fed in 3 aliquots to growing cultures of *Streptomyces helicus* at 24, 48, and 60 hours after inoculation of the production medium.[15] Termination of the fermentation at 72 hours and isolation of the antibiotic yielded 33.0 mg of **1h**. A ^{13}C NMR spectrum of a 4.5 mg sample of **1h** in DMSO-d_6 was obtained. Analysis of the ^{13}C spectrum revealed a ^{15}N-^{13}C coupled doublet (J = 15.0 Hz) centered 1.50 Hz upfield of the natural abundance ^{14}N-^{13}C resonance of **1** at 169.54 ppm. Partial hydrolysis of the ^{13}C-^{15}N amide bond to the acid *in vivo* was indicated by a 1.51 fold enrichment over natural abundance of the ^{14}N-^{13}C carboxamide resonance (referenced to the ^{13}C resonance at 154.1 ppm). This enrichment (4.34 fold ^{15}N-^{13}C) correspond to a 1.29% incorporation of **27a** of which 10.2% was hydrolyzed. The partial spectra of **1h** and **1** are shown in Figure 5.

This feeding experiment established the incorporation of the anthranilamide **27a** and hence placed the timing of the amide formation immediately after acid formation, rather than later in the pathway.

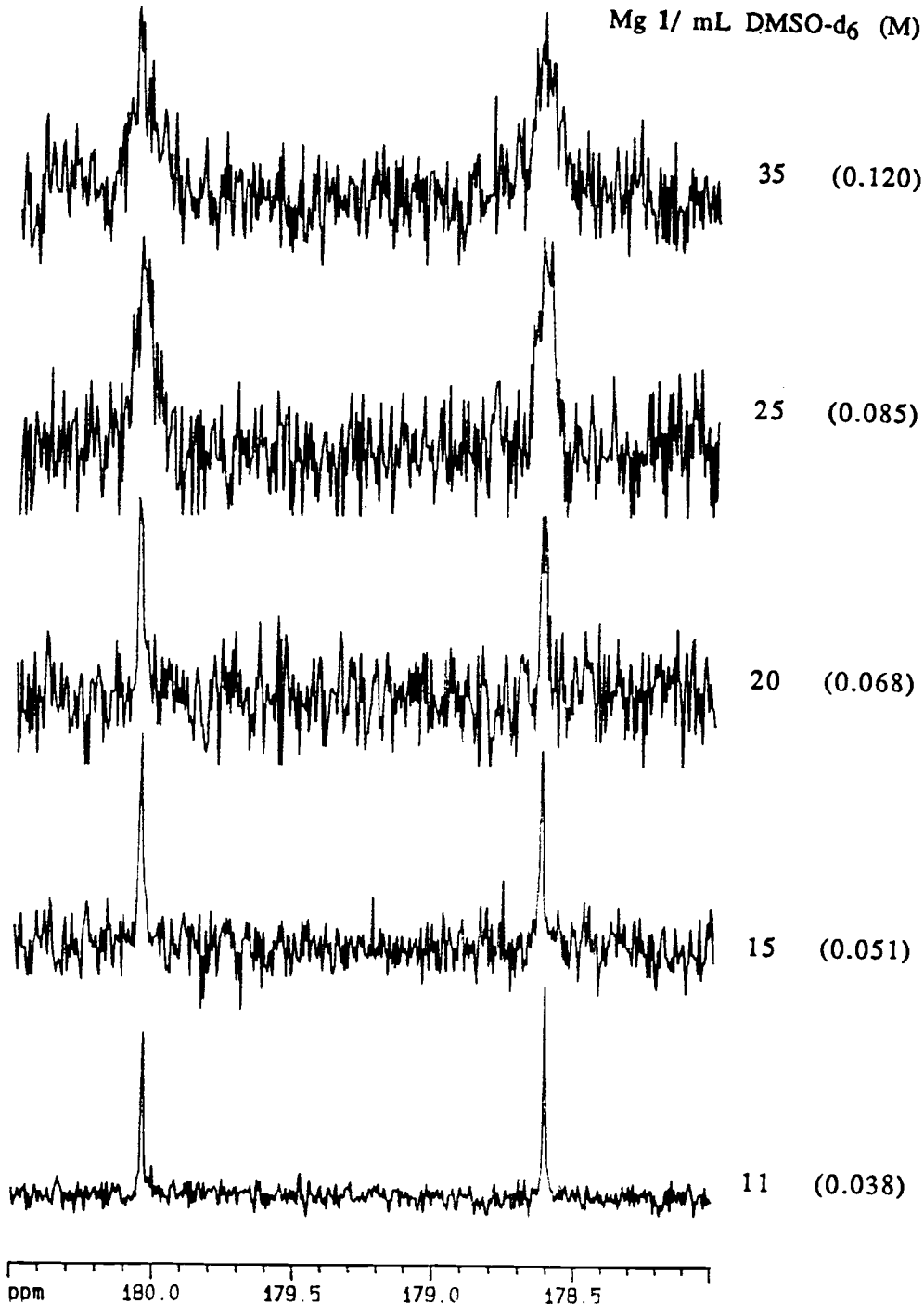


Figure 4 Concentration Effects on the ^{13}C NMR Carbonyl Line Widths of **1**

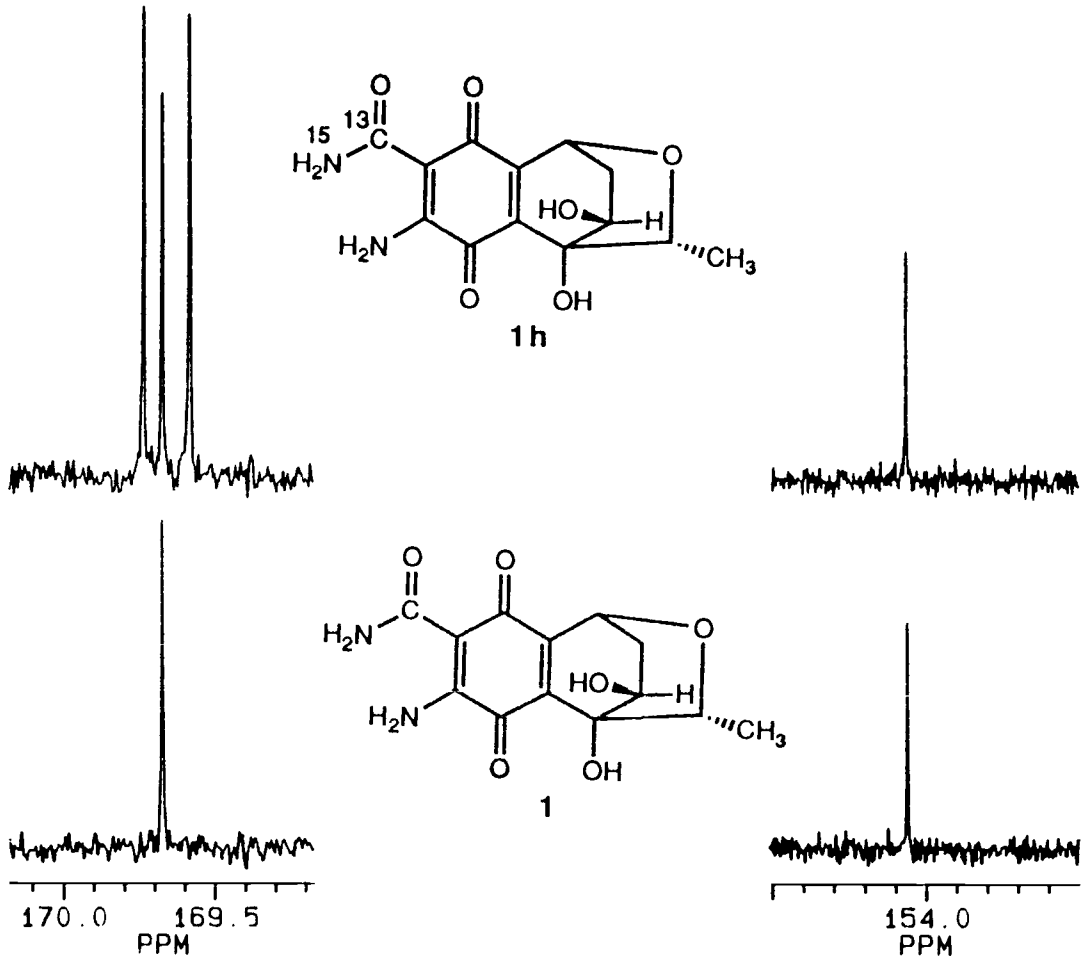


Figure 5. Partial ^{13}C NMR Spectrum of Sarubicin A From Feeding **27a**. .

Radioactive Isotope Trapping Experiments

Description of the Experiment

Both 6-hydroxyanthranilic acid, **17**, and 6-hydroxyanthranilamide, **27**, have been shown to be incorporated into **1**. In order to show that the compound is actually an intermediate, it must be demonstrated that the organism produces the compound under normal antibiotic producing conditions. One method of testing for *in vivo* formation of an intermediate likely to exist only at low concentration is that of an isotope trapping experiment. In this experiment a radioactive precursor known to be incorporated into the antibiotic is fed to growing cultures of the organism. Typically, the label is ^{3}H or ^{14}C and should have a high enough specific activity to ensure that, after dilution and incorporation percentages are taken into account, enough radioactivity remains to be detected. The labeled precursor is fed to the growing cells, and the fermentation terminated shortly thereafter. The cells are broken open and a quantity of the unlabeled compound (carrier) is added to allow easy recovery and purification. If the intermediate is produced by the organism then a small amount of radioactive material should be present which would mix with the added carrier. The material is reisolated from the broth, purified, and crystallized to constant specific molar radioactivity. The radioactivity detected in the recovered material should be at least twice that of background to be considered statistically significant.

In an attempt to verify the *in vivo* production of both **17** and **27**, trapping experiments with these compounds, isolated as stable derivatives, were undertaken. [1-^{14}C]-D-Erythrose[46,47,48] (specific activity of 8 mCi/mmole) was used as the radioactive precursor in both cases.

6-Hydroxyanthranilic Acid

Two 50 mL fermentations of *S. helicus* were inoculated and incubated for 14 hours. [1-^{14}C]-Erythrose was administered, and the fermentations were terminated by sonication at two hours and six hours after the administration of erythrose. In each case, a quantity of authentic **17**

(approximately 65 mg) was added to each flask at the appropriate time prior to the sonication. The mixtures were lyophilized, and then stirred overnight in a mixture of acetic anhydride and triethylamine. After removal of the volatiles at aspirator pressure, the residue was dissolved in methanol and reacted with diazomethane. The solvents were removed and the derivatized **54** purified by silica gel column chromatography. In a control experiment, this methodology had previously resulted in a 30% recovery of **54**. Multiple recrystallizations were performed, and small samples were counted after each recrystallization. With these traps, after four recrystallizations of the derivative, less than 0.04% of the total radioactivity fed remained for the two hour trap, and 0.02% of the total radioactivity fed remained for the six hour trap. The actual disintegrations per minute (DPM) measured were not distinguishable from the background by the fourth recrystallization, so the material was clearly not significantly radioactive.

Table 3. Two Hour 6-Hydroxyanthranilic Acid Trap.

Recrystallization	Specific Activity (dpm/mmole)	Activity Retained
First	9.37×10^5	0.22%
Second	1.24×10^5	0.11%
Third	7.25×10^4	0.06%
Fourth	within background	--

Table 4. Six Hour 6-Hydroxyanthranilic Acid Trap.

Recrystallization	Specific Activity (dpm/mmole)	Activity Retained
First	1.41×10^5	0.03%
Second	within background	--
Third	within background	--

6-Hydroxyanthranilamide

In a manner exactly analogous to that for the trapping experiment described above, two 50 mL fermentations of *S. helicus* were terminated and **27** added. The cells were sonicated and **27** derivatized as its acetamido methoxy ether **55** by a procedure identical to that for **54**. Purification of **55** was accomplished by silica gel column chromatography. After four recrystallizations, the DPM measurements for both the two hour and the six hour trap were within background radioactivity levels - again, clearly not statistically significant.

Table 5. Two Hour 6-Hydroxyanthranilamide Trap.

Recrystallization	Specific Activity (dpm/mmole)	Activity Retained
First	2.19×10^5	0.15%
Second	9.35×10^4	0.06%
Third	7.16×10^4	0.05%
Fourth	within background	--

Table 6. Six Hour 6-Hydroxyanthranilamide Trap.

Recrystallization	Specific Activity (dpm/mmole)	Activity Retained
First	2.21×10^5	0.15%
Second	1.67×10^5	0.12%
Third	6.82×10^4	0.05%
Fourth	within background	--

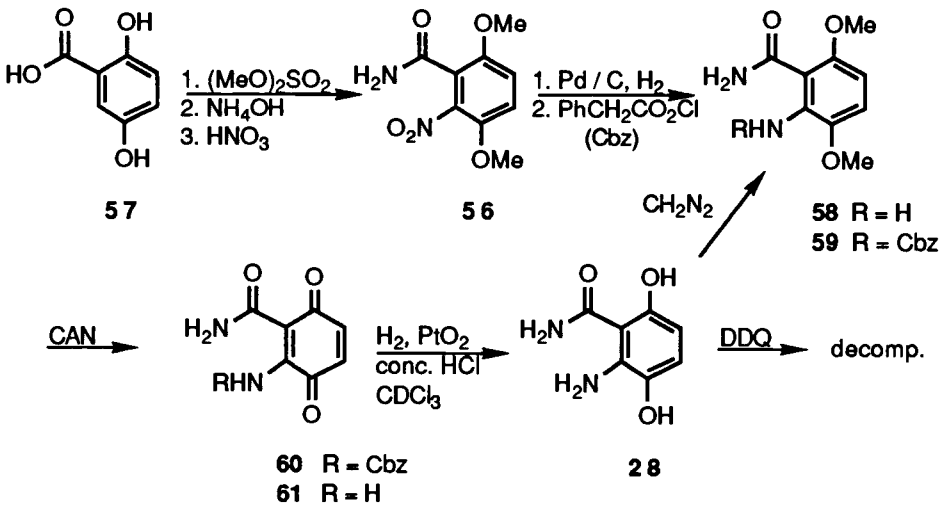
There are several plausible explanations for the lack of radioactivity in these samples. It is possible that the timing of the traps were inappropriate. Biosynthesis of **17** and **27** may not have been occurring at the time of the work-up of the fermentations, and thus, they could not be recovered from the fermentation in radioactive form. It is also possible

that **17** and **27** may not be true intermediates. These possibilities will be discussed later.

Studies of 3,6-Dihydroxyanthranilamides

3,6-Dihydroxyanthranilamide

With the timing of the carboxamide conversion established as the next step beyond 6-hydroxyanthranilic acid formation, attention could now be turned to probing the more advanced stages of the biosynthesis of **1**. In the proposed pathway **A** of Scheme 5, the first step would be hydroxylation of the ring of **27** to give 3,6-dihydroxyanthranilamide, **28**. A previous study by Dr. L. R. Hillis in this laboratory investigated the chemical viability of **28**. Thus, the known 3,6-dimethoxy-2-nitrobenzamide, **56**, was synthesized according to a literature procedure from 2,5-dihydroxybenzoic acid, **57**, as shown in Scheme 13.[49] Reduction of the nitro group and subsequent protection of the amine **58** as the benzyl urethane yielded **59** (88%). Oxidative cleavage of the methoxy protecting groups to the slightly unstable quinone **60** with ceric ammonium nitrate[50] (CAN) was accomplished smoothly (89%), and reduction of **60** to its hydroquinone was expected to produce the putative intermediate **28**. Indeed, when catalytic reduction and concomitant urethane deprotection was carried out in $CDCl_3$ in the presence of a small amount of concentrated HCl the hydroquinone **28** could be detected by ^1H and ^{13}C NMR, but decomposed within 30 minutes. Attempts at *in situ* oxidation to the quinone **61** with 2,3-dichloro-5,6-dicyano benzoquinone or O_2 resulted in decomposition. Attempts to trap **28** as the diacetate with acetic anhydride or acetyl chloride ended in failure. However, the hydroquinone dimethyl ether **58** could be regenerated from **28** by trapping with diazomethane, but only in 8% yield. This at least proved the existence of **28**, but the cumulative results revealed it to be quite unstable.

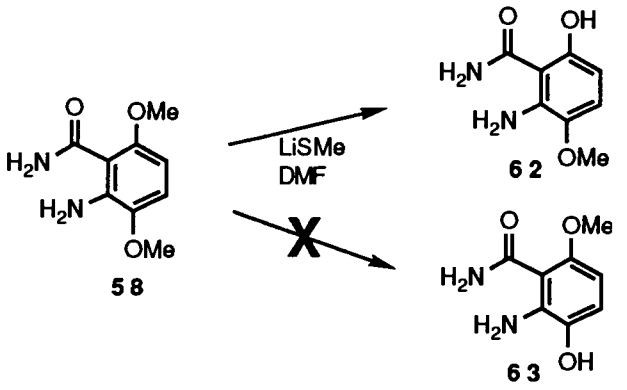


Scheme 13. Synthesis of 3,6-Dihydroxyanthranilamide.

Synthesis of 3,6-Dihydroxy-5-Propylanthranilamide

Given the instability of **28**, efforts were directed towards synthesizing a 5-alkyl substituted hydroquinone, as a model system for **29** (Scheme 6). In this view, the C-4 oxygen would be introduced after C-glycosylation (path **B**, Scheme 6), and the resulting pentasubstituted hydroquinone could be potentially more stable than **28**. The hydrogen at C-5 of **58** was found to be readily exchangeable in CD_3OD under neutral conditions, and this indicated that alkylation at C-5 might occur under Friedel-Crafts conditions. This, unfortunately, proved not to be the case. Reaction with various acids ($BF_3 \cdot Et_2O$, TsOH, AgOTf, BCl_3) and the electrophiles dihydropyran, its hydrate,[51] methyl iodide, perbenzoyl glucose, or 1-bromo-perbenzoyl glucose[52] all gave no reaction whatsoever.

Our studies on the synthesis of 6-hydroxyanthranilamide had showed the utility of methyl ether cleavage with the nucleophilic reagent lithium thiomethoxide. Similarly, the 6-methoxy ether of **58** was now found to be selectively cleaved with the thiomethoxide reagent in DMF to yield the stable **62**, as shown in Scheme 14.



Scheme 14. Synthesis of 3-Methoxy-6-Hydroxyanthranilamide, **62**.

Since alkylation of the C-5 position of **62** was planned to be directed by the 6-hydroxy group, an unambiguous proof that the mono-demethylated product was **62**, rather than the undesired isomer 3-hydroxy-6-methoxy anthranilamide **63**, was critical at this stage.

Deuterium-induced isotope shifts in partially exchanged hydroxyl and amino functional groups have been used to assign the ^{13}C spectra of carbohydrates.[53] Similarly, assignment of the quinone carbonyl ^{13}C resonances for **1** were obtained by an isotope shift transmitted through a hydrogen bonded, exchangable hydrogen.[15] In principle, isotope effects resulting from two-bond (β), and three-bond (γ) shifts should be observable. Therefore, this phenomenon could be used to distinguish between **62** and its isomer, **63**.

Accordingly, a sample of **62** was exchanged three times with methanol-d_1, dried thouroghly, and combined with an unexchanged sample in DMSO-d_6. From the deuterium induced isotope shifts of the ^{13}C NMR spectrum thus obtained, (shown in Figure 6) the structure could be assigned to **62** rather than **63**. Partial exchange of the C-5 position with deuterium was observed in the ^1H spectrum of **62**. This allowed assignment of the position *para* to the amino group as evidenced by a broadened C-5 resonance due to ^2H-^{13}C coupling. The isomeric **63** would also be expected to exchange at the *para* position. Two distinct β-isotope shifts were observed 0.044 and 0.053 ppm upfield of the C-6 resonance at 150.8 ppm, arising from the partially exchanged phenol hydrogen and the C-5 deuterium. In contrast to this, the C-3 methoxy-substituted resonance at

140.0 ppm exhibited a very slight broadening, due to the effect of the N-D substitution at C-2. The other isomer, **63**, would be expected to exhibit β-shifts at C-3 (due to the exchanged phenol) and at C-6 (due to deuterium at C-5). A further distinction between a β-shift transmitted through a hetero-atom and one arising from the C-D substitution from the C-5 exchange could be made by increasing the exhange rate between the O-H and N-H bonds. Indeed, when the sample was heated to 320 K, a broadening of the C-6 and C-2 resonances was observed, whereas the C-4 position showed no change. This experiment therefore allowed an unambiguous assignment of the complete ^{13}C spectrum.

Independent confirmation of the ^{13}C assignments was obtained from a ^{1}H-^{13}C long-range correlation experiment (HETCOSY).[54] A 3-bond correlation between the phenol and C-1, and a similar one with H-5 were observed, while the C-3 resonance exhibited correlations with the methoxy hydrogens and H-5. This type of correlation has been observed in the spectra for kinamycin[54] and murayaquinone.[55] The HETCOSY spectrum and assignments are shown in Figure 7.

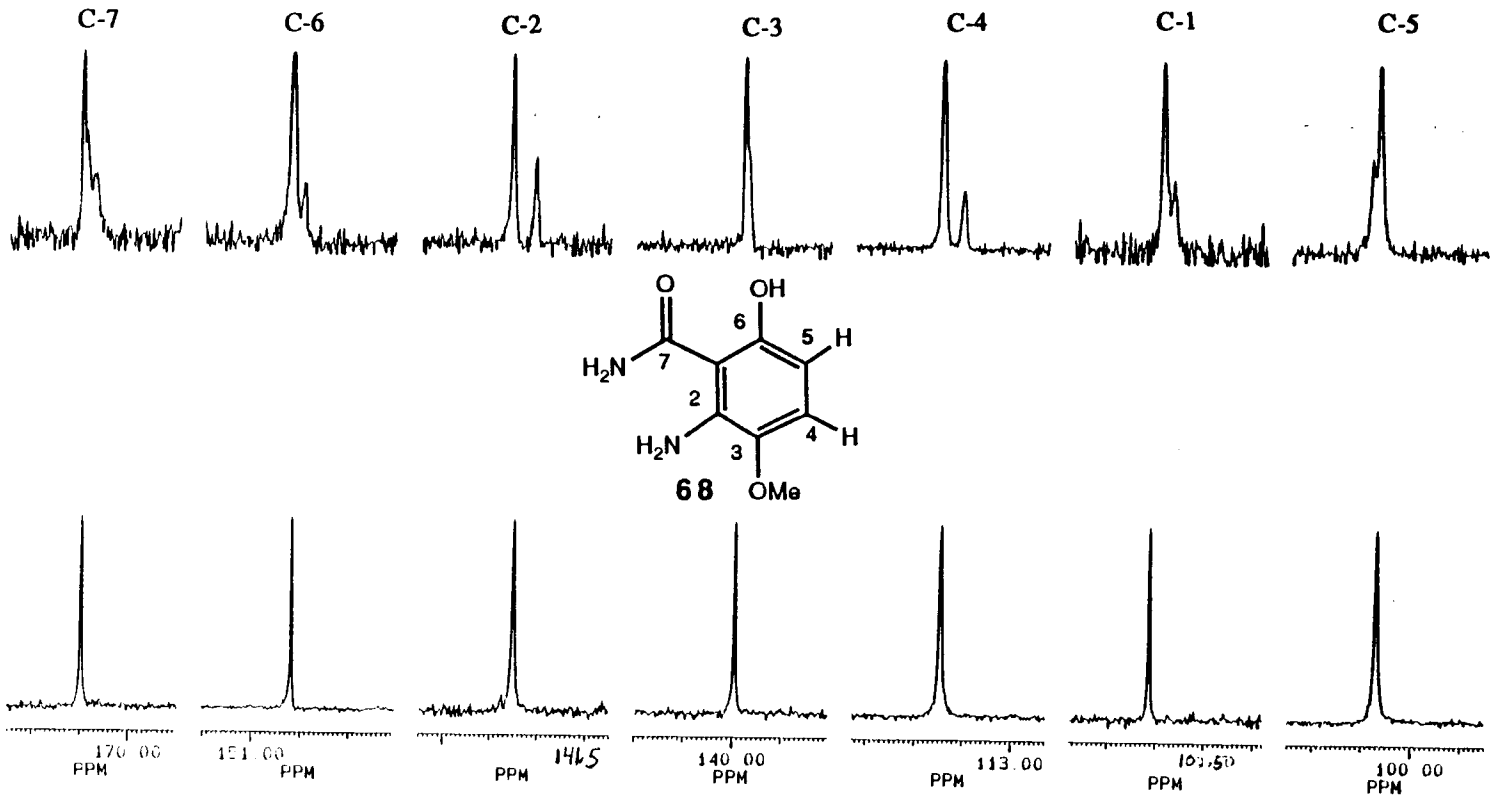


Figure 6. ^{2}H Isotope-Shifted ^{13}C NMR Spectrum of **62**.

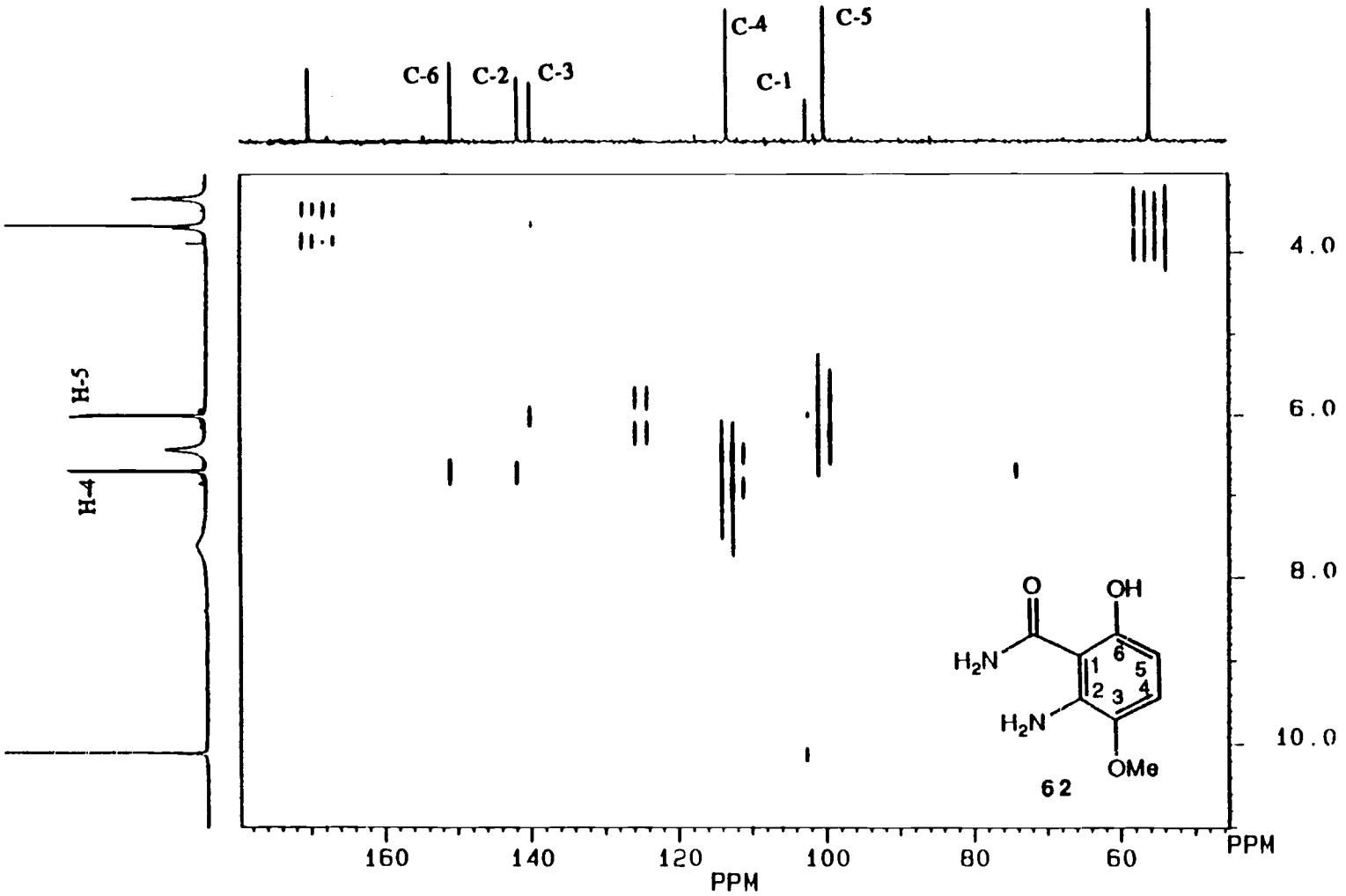
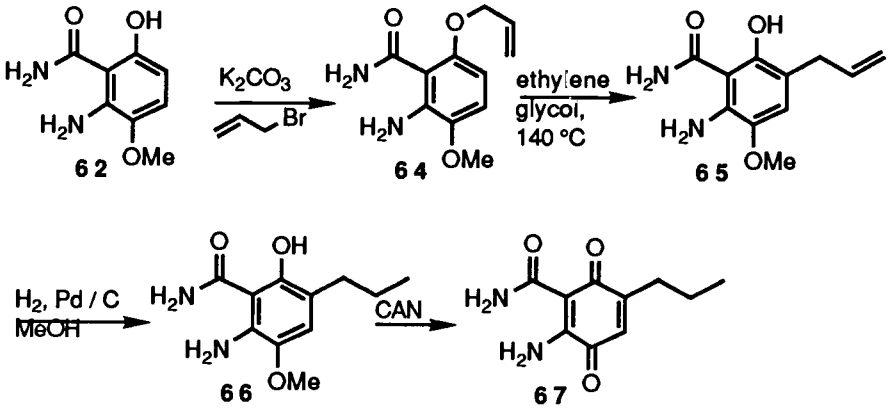


Figure 7. HETCOSY NMR Spectrum of **62**.

With the structure of **62** established, attempts were made to direct alkylation to C-5 utilizing the adjacent phenol. Friedel-Crafts conditions (*vida supra*) were used with **62** but, as before, no reaction occurred. Activation of C-5 via the 6-phenoxide using anionic conditions proved fruitless, as well. Thus, reaction of **62** with potassium hydride followed by methyl iodide in refluxing toluene,[56] as well as reaction under orthoformylation conditions (EtMgBr in THF followed by triethylorthoformate),[57] led only to recovered starting material.

Alkylation of C-5 was finally accomplished by the use of a Claisen rearrangement of the 6-O-allyl ether derived from **62**, as shown in Scheme 15. Thus, **62** was treated with allyl bromide in DMF and anhydrous potassium carbonate to yield **64** (82.9%). Claisen rearrangement of **64** to **65** was accomplished smoothly at 140 °C in ethylene glycol in 95.0% yield using a flask that had been base-washed just before use. Attempts to directly oxidize **65** to the quinone failed, yielding only unidentifiable products. However, the target quinone was successfully prepared by first catalytic reduction of **65** (H_2, Pd/C, 95.5% yield) to the propyl substituted **66**, followed by oxidization to **67** with ceric ammonium nitrate in 97.0% yield.



Scheme 15. Synthesis of Quinone **67**.

Stability of 3,6-Dihydroxy-5-Propylanthranilamide

To test the stability of the hydroquinone derived from **67**, it was catalytically reduced in $CHCl_3$ with PtO_2 under a H_2 atmosphere. This

provided a faint yellow solution from the original deep red color of **67**. After 24 hours, exposure of the yellow solution to O_2 was found to be sufficient to reoxidize to the quinone within seconds, as evidenced by instant reappearance of a deep red color. The product was pure by TLC, which indicated that the hydroquinone **68**, analogous to the proposed intermediate **29**, (Scheme 6), was stable in the absence of O_2 - in marked contrast to that of the tetrasubstituted **28**. However, more direct evidence of the formation of the hydroquinone was sought.

Thus, **67** was dissolved in DMSO-d_6 and sodium dithionite in D_2O was added in an NMR tube. The resulting pale yellow-brown solution was analyzed by ^{13}C NMR spectroscopy after 4 hours. The two quinone carbonyl resonances at 182.9 and 181.9 ppm had disappeared, and a series of six resonances in the range of 160 to 110 ppm were evident, indicating formation of an aromatic compound. The aliphatic region showed at least one impurity, indicating the reduction under these conditions was not as clean as the catalytic reduction conditions. The product was stable for at least a week at room temperature and, after exposure to air, the quinone could be reisolated by extraction into ethyl acetate. The ^{13}C NMR spectra of **67** and the reduction product **68** are compared in Figure 8. This experiment provided direct evidence that the hydroquinone **68** is stable in the absence of O_2.

Synthetic Studies of C-Glycosides

Having demonstrated that **68** was more stable than **29**, methodology was next developed which could be used to provide a series of C-glycosides analogous to **68**. These could be used as putative intermediates for investigations into advanced stages of the biosynthesis of **1**, and provide an entry for a biomimetic total synthesis of **1**, although this will have to await future studies.

There has been considerable interest in the synthesis of C-aryl glycosides in the recent literature.[58] The most straightforward method of forming C-glycoside bonds is by electrophilic attack of a protected, suitably activated sugar derivative on an activated aromatic system.[59] A number of variations on this theme, where the nucleophile is activated as an

organometallic species, have been reported: organolithium derivatives with protected lactones and subsequent reduction of the lactols,[60] glycals with arylpalladium species,[61] and 1,2-anhydro sugars with organocuprates.[62] Other permutations have been activation of an aromatic nucleophile with Group IVa elements (Ti, Zr, Hf) to affect Friedel-Crafts reactions with glycosyl fluorides.[63]

An alternative approach which has shown much utility is the Lewis acid mediated cyclocondensation of aldehydes with appropriate dienes pioneered by Danishefsky.[64] Application of this technology to the construction of a 2,6-dideoxy C-glycoside has been reported.[65] It was expected that adaptation of this methodology to the present work would provide a concise route to the requisite C-glycoside and, potentially, a biomimetic synthesis of **1**.

Since initial attempts to directly alkylate **62** or **58** using acid-catalyzed or phenoxide directing conditions had been unfruitful, lithiation conditions which potentially could allow for the introduction a large variety of electrophiles to **58** were now investigated. A major obstacle to this method was the presence of two primary amino groups of **58**. However, condensation of acetone with the dimethoxy derivative **58** led quantitatively to the acetaminal **69**, which simultaneously protected the aniline and the amide nitrogens while removing two acidic protons. This protection also allowed subsequent bromination of the aromatic ring with no possibility of Beckmann-type rearrangement of the amide to the amine.

Bromination of **69** was carried out uneventfully with Br_2 in a mixture of acetic acid and CH_2Cl_2. Neutralization and reduction of excess Br_2 and any N-bromination products which may have formed was effected with freshly prepared sodium bisulfite in 5% $NaHCO_3$. This provided the 5-bromo substituted **70** in 90.3% yield. The acetaminal protecting group was found to be very labile under the workup conditions, but could be restored merely by heating the crude residue in acetone for 15 minutes, and then crystallizing **70** from the solution.

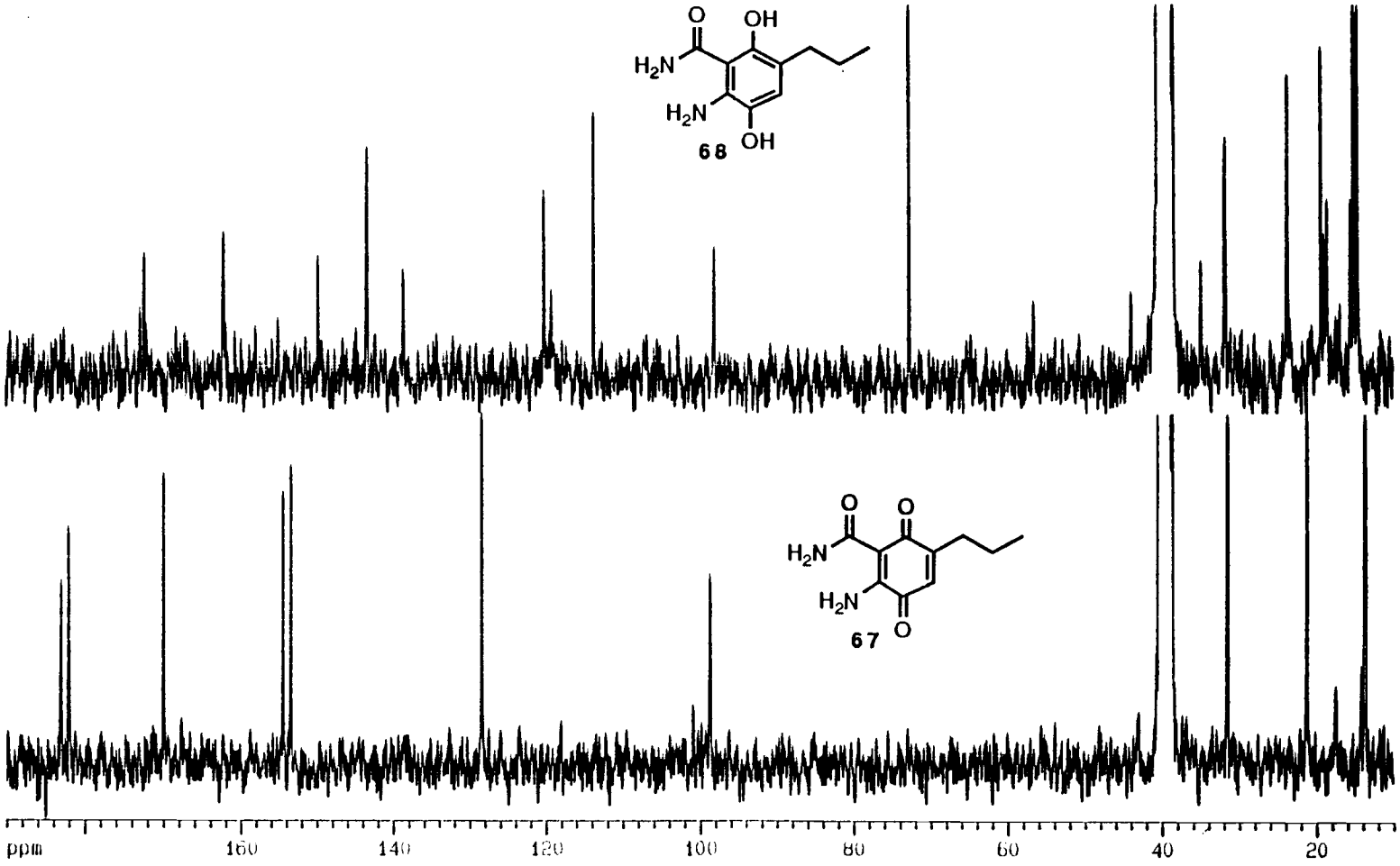
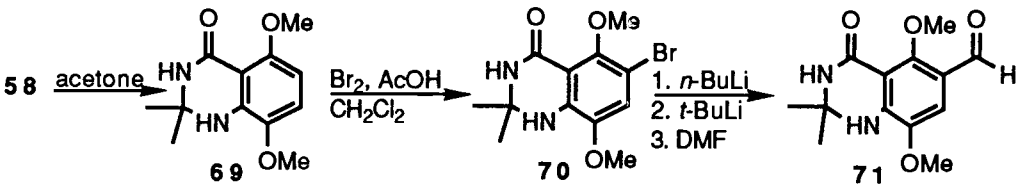


Figure 8. 75 MHz ^{13}C NMR Spectrum of Quinone **67** and Hydroquinone **68**.

Treatment of **70** successively with *n*-BuLi followed by *t*-BuLi at -78 °C generated the trilithio derivative of **70**, which was quenched with anhydrous N,N-dimethylformamide (DMF) after 2.5 hours to give the *p*-aminobenzaldehyde **71** (79%). These efforts are summarized in Scheme 16.



Scheme 16. Synthesis of Aldehyde **71**.

It was anticipated that a cycloaddition reaction between the aldehyde **71** and an appropriate diene could provide the pyran skeleton and the requisite *cis* relative stereochemistry between the methyl group and the C-8 hydrogen at the C-glycoside junction of **1**. Danishefsky and co-workers have demonstrated the feasibility of this approach with the use of the triethylsiloxy diene derived from 3-penten-2-one to construct a 2,6-dideoxy glucose derivative.[62] To outline the scope of the reactivity of this unique *p*-aminobenzaldehyde (**71**) we first attempted a cyclization with the well-known Danishefsky's diene[66] and various Lewis acids including $ZnCl_2$,[64] $BF_3{\cdot}Et_2O$,[66] $Eu(fod)_3$,[67] $TiCl_4$[64], and $MgBr_2$[67] in THF, CH_2Cl_2, benzene, or $CHCl_3$. Unfortunately, no reaction was observed in any case. This may have been due to the intractable insolubility of **71** in all solvents except DMF and DMSO.

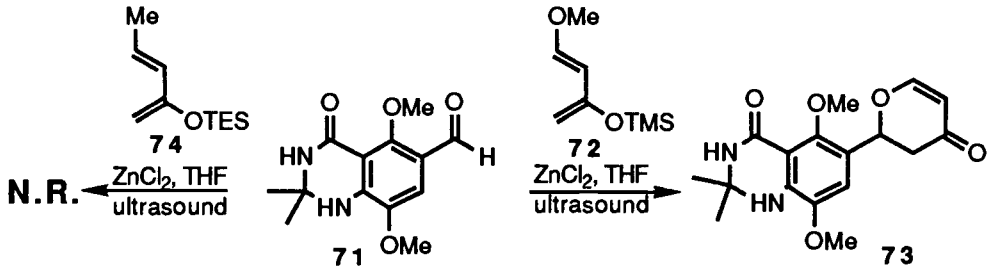
Recently, Snyder and Lee have demonstrated that homo Diels-Alder cycloadditions can be catalyzed by ultrasound.[68] Adapting these conditions to the present situation, a heterogeneous mixture of **71**, diene **72**,[69] and $ZnCl_2$ in THF were sonicated at room temperature (see Scheme 17). The cycloaddition reaction took place over 2 hours to yield the cycloadduct **73** (47%), after deprotection of the silyl enol ether with trifluoroacetic acid. Analysis of the ^1H and ^{13}C spectra confirmed the success of the cycloaddition reaction. Characteristic enone ^1H resonances at 7.49 and 5.46

ppm (J = 6 Hz) as well as the benzyl ^1H at 5.78 ppm were observed, indicated the structure shown for **73**. The ^{13}C NMR spectrum contained an enone carbonyl resonance at 192 ppm.

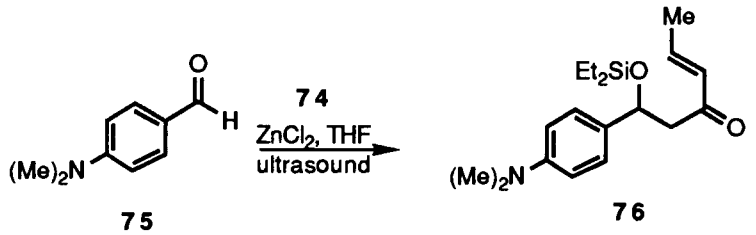
The triethylsiloxy diene **74** was next prepared from 3-penten-2-one according to the literature procedure.[70] Unfortunately, in this case, reaction of **71** with the diene **74** under the same conditions as for the reaction with diene **72** in the presence of $ZnCl_2$ gave no reaction.

A model study was conducted at this point to determine the electronic effect of the amino group on the reactivity of the benzaldehyde. Danishefsky had reported that *o*-methoxybenzaldehyde underwent a smooth cycloaddition with the diene **72** in the presence of $ZnCl_2$.[66] Therefore, *p*-(N,N-dimethylamino)-benzaldehyde, **75**, was next reacted with diene **72** and $ZnCl_2$ in THF, as shown in Scheme 18. While there was no reaction when the mixture was stirred for 2 hours at room temperature without sonication, sonication for 15 minutes yielded a new product. This did not provide the desired cycloaddition product, but rather resulted in an aldol product, apparently followed by a silyl group transfer to yield enone **76** (64%). The ^{13}C NMR spectrum for **76** exhibited an enone carbonyl resonance at 198 ppm, with the double bond resonances at 143.4 (β carbon) and 133.2 ppm (α carbon). A *trans* coupling of 17 Hz between the β- and α-protons of the enone was observed in the ^1H NMR spectrum.

The results obtained with the two *p*-aminobenzaldehyde derivatives **76** and **71** indicate that the amino group is likely deactivating the aldehyde sufficiently to prevent a cycloaddition reaction with the less reactive diene **74**. Protection of the amine of **71** as a urethane or amide may provide both increased reactivity for the aldehyde functionality as well as increased solubility, both of which are desirable conditions for the desired cycloaddition.



Scheme 17. Hetero Diels-Alder Approach to a C-Glycoside.



Scheme 18. *p*-(N,N-DimethylAmino)-benzaldehyde Aldol Reaction.

In an attempt to derivatize the amino functionality of **71**, it was reacted with trifluoroacetic anhydride in triethylamine. However, extensive decomposition resulted, leading to a multitude of spots upon TLC analysis. Similar results were obtained with acetic anhydride/triethylamine conditions. Attempts to derivatize the amino group of **71** with ethyl chloroformate in a mixture of 1 M NaOH and THF gave no reaction, while methyl chloroformate or ethyl chloroformate in triethylamine led to decomposition of the substrate.

Apparently, the presence of the aldehyde of **71** is sufficient to deactivate the hindered amino group enough to prevent reaction with electrophiles.

The synthetic efforts presented here have demonstrated the feasibility of constructing the pyran skeleton of the 5-substituted C-glycoside target. The unusual reactivity of the unique *p*-aminobenzaldehyde **71** has been investigated, showing that even this deactivated aldehyde will react with certain activated dienes under ultrasound-mediated Lewis acid conditions.

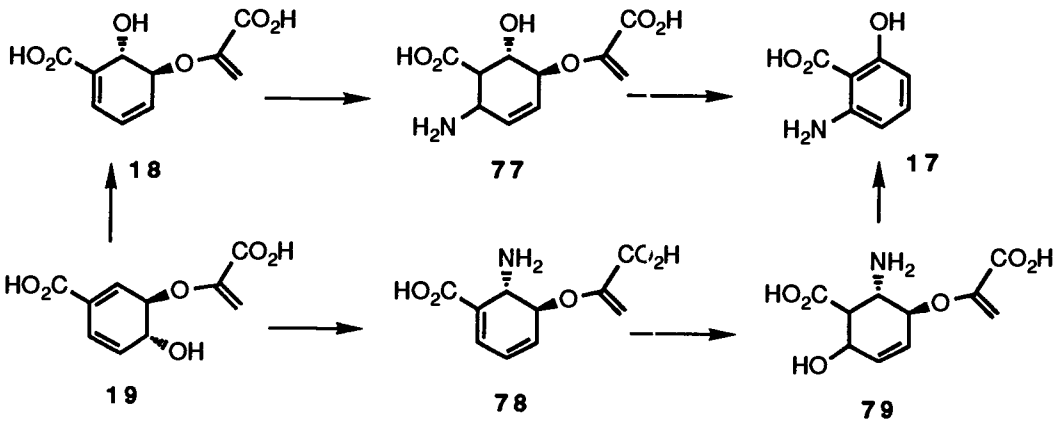
Appropriate derivatization of the amino group could provide increased solubility, and increased reactivity, for the aldehyde, either of which may be sufficient for a successful cycloaddition reaction to occur with the diene **74**. Success in this reaction would set the stage for a very concise biomimetic total synthesis of **1**, as well as provide an entry into potential 5-glycosylated aromatic amino acids. These could be used as potential putative biosynthetic intermediates useful for probing the advanced stages of the biosynthesis of **1**.

The demonstration of the stability of the 5-propyl-substituted hydroquinone **68** provided impetus for further synthetic efforts to construct C-glycosides for biosynthetic experiments of **1**. While these results do not allow a distinction to be made between the putative pathways presented in Scheme 6, the chemical viability of pathways **a** and **b** have been established by the stability of the 5-alkyl-substituted hydroquinone **68**.

Biosynthesis of 17 and 27 From Chorismic and Isochorismic Acid

Possible Biosynthetic Routes to 17.

Isochorismic acid is an attractive possibility as the branch point from the shikimate pathway leading to **1**. Anthranilic acid, **15**, as stated above, has been shown not to be incorporated into **1** when fed to growing cultures of *S. helicus*. If **15** is not an intermediate in the biosynthesis of **1**, then one potential pathway which can be considered would originate from **18**. These alternatives are shown in Scheme 19. Conversion of chorismate to **18**, followed by ammonia addition with either ammonia or glutamine would produce 6-amino-1,6-dihydro-isochorismic acid, **77**. Elimination of pyruvic acid and oxidation would give 6-hydroxyanthranilic acid, **17**. Alternatively, **17** could derive directly from **19** via 2-amino-2-deoxyisochorismic acid, **78**. Addition of H_2O at C-6, to give **79**, elimination of pyruvic acid, and oxidation would yield **17**.

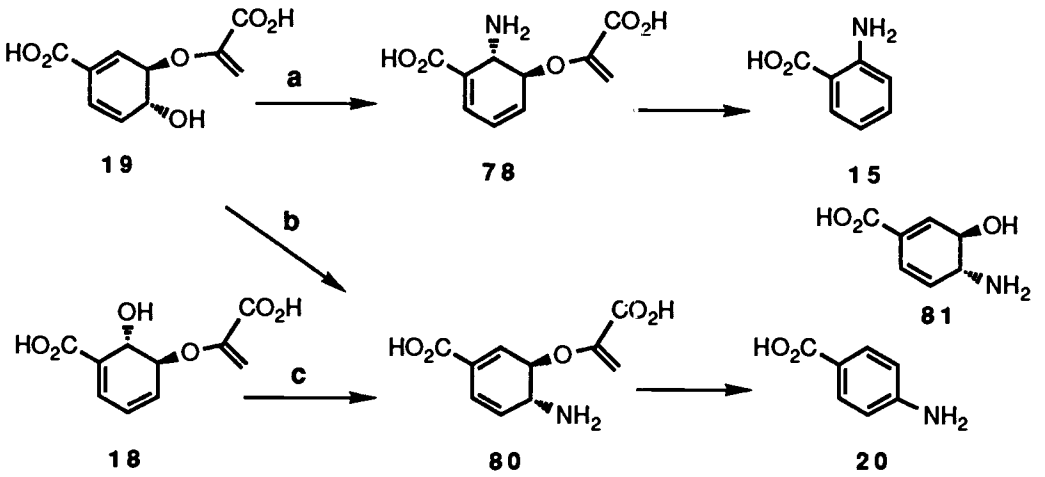


Scheme 19 Possible Biosynthetic Routes to 6-Hydroxyanthranilic Acid

Biosynthetic Formation of Aromatic Amino Acids

Two aromatic amino acids which are similar to **17** that have been studied extensively are anthranilic acid, **15**, and *p*-aminobenzoic acid, **20** (PABA). Two researchers, Dardenne[71] and Haslam,[72] have independently suggested that either: **15** and **20** are both derived directly from chorismate

or, that **15** originated from chorismate, and **20** from isochorismate via analogous addition-elimination reactions utilizing either ammonia or glutamine as the amine source. (see Scheme 20).



Scheme 20 Biosyntheses of *p*-Aminobenzoic Acid and Anthranilic Acid

These postulates have led to the suggestion that 2-amino-2-deoxy-isochorismic acid, **78**, and the analogous 4-amino-4-deoxy-chorismic acid, **80**, are intermediates in the biosyntheses of **15** and **20**, respectively.[73] McCormick and coworkers in 1962 reported that the related amino alcohol **81**, lacking the pyruvate moiety, was produced by a strain of *Streptomyces aureofaciens*,[74] which implicated **78** as an intermediate in the biosynthesis of **15**. Although never isolated from natural sources, **78** has been prepared synthetically and found to be converted to **15** in the presence of anthranilate synthase.[73] Isochorismate is not known to be a precursor to **15**,[75] suggesting that anthranilate is biosynthesized from chorismate, but not from isochorismate, according to pathway **a** as depicted in Scheme 19. Ganem and coworkers synthesized the analogous postulated intermediate for **20**, **80**, and showed that it was indeed an effective substrate for PABA synthase.[76]

PABA synthase, which is known to convert chorismate to **20** has been isolated as well and studied. Berchtold has shown that when PABA synthase is incubated in the presence of synthetic (±)-isochorismic acid no production of **20** takes place.[77] Recently, Leistner has confirmed this

result using PABA synthase obtained from *Streptomyces aminophilus*, *S. griseus*, and *Enterobacter aerogenes*.[18] When PABA synthase was obtained from *S. aminophilus* and *E. aerogenes*, both enzyme preparations were found to be contaminated with isochorismate synthase activity. Incubation with either chorismate or isochorismate produced PABA, with the yield from chorismate much higher. However, when the enzyme source was *S. griseus* there was no contamination of isochorismate activity. In this case, incubation of PABA synthase in the presence of isochorismate produced *no* PABA, while chorismate proved to be an effective substrate.

The enzymic reaction of **19** to **18** was, as the above result suggests, found to be reversible. When isochorismate synthase from either of the above sources was incubated with either chorismate or isochorismate, an equilibrium mixture consisting of approximately 80% chorismate and 20% isochorismate was formed. This observation could explain why isochorismate acts as an apparent substrate for PABA synthase only when isolated from sources contaminated with isochorismate synthase activity.

These studies indicate that both of the intermediates **15** and **20** originate directly from **19**, and that **18** is not involved in either biosynthetic pathway. Isochorismate synthase activity has never been reported in a *Streptomyces* species before the enzyme was found in *S. aminophilus*.

6-Hydroxyanthranilic acid differs from anthranilic acid and *p*-aminobenzoic acid by the presence of a 6-hydroxyl group, analogous to salicylic acid. Salicylic acid is known to be derived from isochorismate in some organisms.[23] Both pathways represented in Scheme 19 are therefore viable as possible biosynthetic pathways leading to **17**.

In the next section, studies investigating the intermediacy of chorismic acid and isochorismic acid in the biosynthesis of 6-hydroxyanthranilic acid and 6-hydroxyanthranilamide utilizing cell-free extracts of *S. helicus* are presented. Chorismic acid was prepared biosynthetically from the organism *Enterobacter aerogenes* 62-1, and isochorismic acid was enzymatically prepared from chorismic acid with an enzyme preparation containing isochorismate synthase activity (*vida infra*).

Studies of *S. Helicus*

Preparation of Cell-Free Extracts of *S. helicus*.

A series of cell-free extracts of *S. helicus* were prepared from fermentation broths at various times after inoculation. Sarubicin A was detectable 24 hours after inoculation as evidenced by a red color appearing in the incubating broths. The cell-free extracts were prepared by two different methods as described in the Experimental section (Table 15): Thus, flasks containing 200 mL production broths were prepared, inoculated with seed medium, and incubated under standard conditions for sarubicin A production. At the appropriate time, a flask was removed from the incubator, and the cell-free extracts prepared.

Method A Cell-Free Extracts

Incubations were performed on each of the extracts obtained using chorismate as a substrate. Extracts were prepared at 24, 36, 48 and 60 hours after inoculation of the production broths in buffer a (described in Table 15, in the Experimental section). With these extracts, in no case could any isochorismate be detected by HPLC, detected by UV at 278 nm, at 30 or 60 minutes (elution time 12.7 minutes). By comparing the peak areas of chorismate, detected by UV at 278 nm as well, (elution time 25.8 minutes) at 0 and 60 minutes, it was found that the chorismate concentration decreased by no more than 15% over 60 minutes. Prephenate, an ever-present impurity in small amounts, eluted under these conditions at 21.5 minutes and was detectable by UV under these conditions as well.

Due to the apparent lack of activity in these extracts, efforts were next made to develop conditions which would yield active cell-free extracts.

Method B Cell-Free Extracts

Cell-free extracts were prepared at 24, 30, and 38 hours after initiation of the fermentation. The buffers used are described in Table 15 of the Experimental section. Incubations were performed using either

chorismate or isochorismate as a substrate. When the fresh 24 hour extract in buffer a was incubated with chorismate no significant decrease in chorismate concentration was observed, and no isochorismate was detected.

However, when a 24 hour extract in buffer c was incubated, an 88% decrease in the concentration of chorismate was observed over 60 minutes, but still, no isochorismate could be detected. The enzyme chorismate mutase was apparently present in the 24 hour extract as indicated by an increase of 100% in the amount of prephenate over 60 minutes over that of the control experiment. In the control experiment, the chorismate concentration did not decrease an appreciable amount over 60 minutes, and prephenate peak area did not change relative to chorismate.

When fresh cell free extracts in buffer d were incubated, no detectable metabolism of **19** could be discerned.

When a 30 hour extract in buffer c was incubated, a 58% decrease in chorismate concentration could be observed over 60 minutes. However, no significant chorismate mutase activity could be detected.

This indicated that there was significant enzyme activity which could metabolize chorismate in both 24 and 30 hour extracts. Similar experiments with the 38 hour extract in buffer c revealed no significant decrease in the amount of chorismate over a 60 minute incubation period.

If the 24 hour cell-free extract in buffer c were left at 0 °C for 30 hours, and then incubated with chorismate, the ability to metabolize chorismate was completely lost. Similarly, if the extract was frozen at - 80 °C for a week, then rethawed, and incubated with chorismate, almost all of the activity was lost. Apparently, the presence of Mg^{2+} and DTT (buffer c) stabilize the cell-free extract for at least a short time.

Isochorismate was next tested as a substrate. Thus, the fresh cell-free extracts of *S. helicus* prepared at 24, 30, and 38 hours were incubated in the presence of isochorismate under the same conditions as for the experiments with chorismate. No decrease in isochorismate concentration was observed in any of the extracts in buffer a and buffer d, leading to the conclusion that these cell-free extracts were inactive.

When identical incubations using isochorismate as a substrate were performed with the 24 and 30 hour cell-free extracts in buffer c,

approximately 10% of the isochorismate disappeared, but no chorismate could be detected.

With a cell-free extract now available which could metabolize chorismate, the enzymatic conversion of chorismate to 6-hydroxyanthranilic acid was next considered.

6-Hydroxyanthranilic Acid and Amide Assays

Efforts were next directed towards developing a reliable, direct assay for **17** and **27**. In principle they could be separated and detected directly by HPLC using UV detection, since both absorb at 235 nm. Numerous attempts were made to develop an HPLC protocol which would allow identification of each of the compounds. When eluted on a C-18 reverse-phase HPLC column with an aqueous solution consisting of 15% methanol with 0.15 M phosphoric acid, **17** and **27** emerged at 4.0 min and 6.6 min respectively. The detection limit for both compounds was approximately 1 nanomole with the UV detector set at 235 nm. However, when a cell-free extract sample was analyzed, a large number of peaks were evident at elution times of up to 10 minutes, obscuring any potential **17** and **27**. A decrease of the methanol concentration to 5% yielded elution times of 11.2 and 13.4 minutes for **17** and **27**, respectively, but the cell-free extract eluted material up for to 15 minutes. If the acid component was changed to 0.1 M acetic acid, there was no change in elution times for either **17** or **27**. However, adjusting the pH to 3.5 from 2.1 (1 M NaOH) caused **17** to elute at 18.2 minutes, but the cell-free extract eluted material beyond 20 minutes, obscuring any potential **17** that could be present. The amide **27** did not elute before 45 minutes in this case.

Efforts toward detecting derivatives of **17** and **27** were Next made. Conveniently, the previously prepared N-acetyl, O-methyl derivatives **54** and **55** could be separated and detected at 254 nm on a C-18 column eluting with an aqueous solution of 10% methanol and 0.1% trifluoroacetic acid. Elution times were 14.8 minutes and 30.0 minutes for **55** and **54**, respectively.

A procedure was developed for the derivatization and detection of **54** and **55**, beginning with small amounts of **17** and **27**. A 750 μL incubation

mixture containing cell-free extract of *S. helicus* was prepared (*vida infra*) and 1.3 μmoles each of **17** and **27** added. The mixture was acidified with concentrated HCl to pH 1 and the mixture centrifuged to remove any precipitated protein, and then extracted with ethyl acetate. Removal of the solvent gave a residue which was reacted with acetic anhydride/triethylamine followed by treatmen with diazomethane. After filtration through a small silica gel plug, and elution with ethyl acetate, the eluant was concentrated, and the residue dissolved in methanol and analyzed by HPLC. A peak corresponding to **55** was evident, but **54** could not be detected.

If the incubation mixture was lyophilized, and the crude lyophilate derivatized similarily, then both **54** and **55** could be detected by HPLC. This procedure showed that small amounts of both **17** and **27** could be derivatized from a cell-free mixture and detected.

Incubation conditions to test for production of **17** and/or **27** by cell-free extracts of *S. helicus* were:

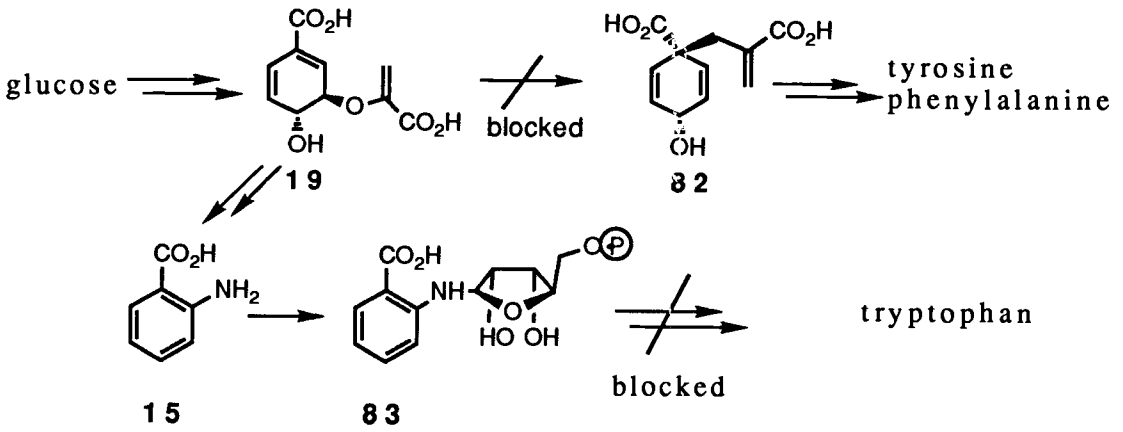
Each of the cell-free extracts which had been prepared were incubated at 30 °C for 2 hours, and then the mixture lyophilized and derivatized as described. Unfortunately, in no case could either **54** and **55** be detected by HPLC.

The results obtained from the cell-free extracts indicate that *S. helicus* is capable of metabolizing chorismate, but that the enzyme activity is very unstable, as evidenced by activity only being found in the extracts prepared after 24 hours and 30 hours. The apparent lack of ability of these extracts to convert either **19** or **18** to **17** or **27** could have several plausible explanations. First, it is clear that both the timing of the preparation of the cell-free extracts, and the method of preparation are critical to preserving any enzyme activity. Second, it is possible that enzymes responsible for the conversion of either chorismate or isochorismate to **17** or **27** are not stable to the conditions employed. The fact that **19** was significantly metabolized by the 24 hour preparation in buffer II, but no isochorismate could be detected, may indicate that **18** is not a substrate for the biosynthesis of **17**.

Enterobacter Aerogenes 62-1

The mutant organism *E. aerogenes* 62-1 (formerly *Aerobacter aerogenes* 62-1; also referred to as *Klebsiella pneumoniae*) has a variety of useful properties which could be exploited for studies of the biosynthesis of **1**. It is a convenient source of **19** which could be used as a potential precursor to **17** and **27** since assays for isochorismate synthase require **19** as a substrate. *E. aerogenes* also serves as a source of isochorismate synthase, and therefore isochorismate could be enzymatically produced and used as potential substrate. Isolation of this enzyme provides an opportunity to probe the mechanistic details of the enzyme-catalyzed isomerization of **19** to **18** as well.

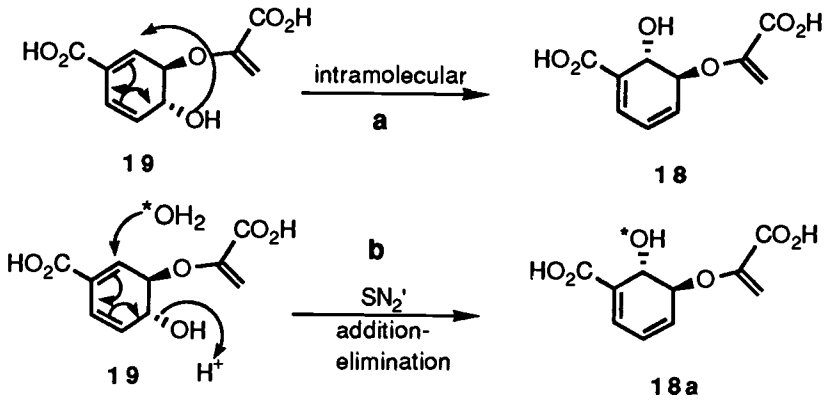
Isochorismate synthase was first detected and subsequently isolated[75] from *Enterobacter aerogenes* 62-1. The organism is an auxotrophic mutant which lacks chorismate mutase and N-phosphoribosylanthranilate synthetase activities. It is therefore unable to convert chorismic acid to prephenic acid or produce N-phosphoribosyl anthranilic acid, an intermediate in the biosynthesis of tryptophan. Reactions leading to the vitamins K and folic acid from chorismic acid are quantitatively insignificant.[78] Due to these metabolic blocks, shown in Scheme 21, *E. aerogenes* accumulates chorismate into the medium and thus can be used as a convenient source of chorismic acid.[79,80]



Scheme 21. Metabolic Blocks of *Enterobacter aerogenes* 62-1

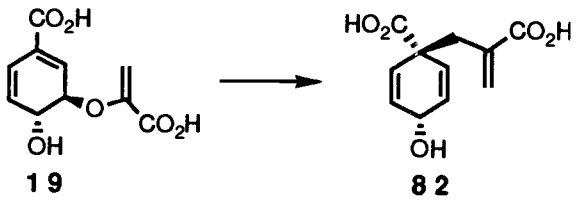
Mechanism of Isochorismate Synthase

Isochorismate synthase, the enzyme catalyzing the conversion of **19** to **18**, has been identified and studied.[25,75] However, little is known about the step-by-step details of the isomerization reaction. Two potential mechanisms were recognized by us, and they are shown in Scheme 10. In pathway **a**, the isomerization reaction could be viewed as an *intramolecular* 1,5-sigmatropic oxygen shift. This pathway would require that the hydroxyl oxygen atom of **19** be retained in the product **18**. Another possible pathway, in which the C-2 hydroxyl would be derived from water via an SN_2' or addition-elimination mechanism analogous to that postulated for the formation of anthranilate from **19** is outlined in pathway **b** of Scheme 22.



Scheme 22. Isochorismate Synthase Mechanisms

There are few if any chemical reactions in nature which are known to be truly concerted, thermal rearrangements. One possible exception is the conversion of **19** to prephenic acid, **82**. In contrast to most enzyme catalyzed reactions, the conversion of **19** to **82** proceeds smoothly in the absence of enzyme thus allowing a direct comparison with the enzyme catalyzed reaction.



Scheme 23 Chorismate Mutase Reaction

Both the non-enzyme catalyzed, and the *in vitro* enzyme catalyzed Claisen rearrangement of chorismate to prephenate, **82**, have been studied extensively.[81,82,83,84,85,86] The enzyme increases the reaction rate by a factor of 1.9 x 10^6.[82] Both the enzyme-catalyzed[83] and the non-enzymic[87] reactions have been shown to proceed through the more stable pseudo-chair conformation.[81,82] The non-enzymic reaction was found to proceed by an unsymmetrical mechanism by the presence of a secondary isotope effect at C-5, but not at C-9, the bond-making site, of **82**, indicating bond-breaking to be the rate determining step.[85] On the other hand, the *in vitro* enzymic reaction showed no isotope effect whatsoever, suggesting that the RDS occurred before the isotopically sensitive rearrangement.[87] Calculations on the chair and boat conformers suggest that the enzyme would only provide a rate enhancement of 10^3 if it were only reducing the entropy of activation, leading to the conclusion that the enzyme must also lower the enthalpy of reaction, presumably by an initial nucleophilic displacement of the enolpyruvyl group.[87] Further support for this was recently provided by the finding that an antibody which catalyzed the reaction only increase the rate by about 10^4.[83]

In order to determine the fate of the C-4 hydroxyl of **18**, and subsequently to distinguish between pathway **a** and pathway **b** in Scheme 20, an enzyme-mediated conversion of **19** to **18** in the presence of $H_2{}^{18}O$ was planned. If the reaction followed path **a**, then no incorporation of ^{18}O into either **19** or **18** would occur. Conversely, if water were involved in the reaction, as in pathway **b**, then incorporation of ^{18}O into the C-2 hydroxyl would be expected for **18** and the C-4 position of **19**. Therefore, both the substrate and product should be labeled with ^{18}O if pathway **b** were followed.

A convenient, unambiguous method of analysis for the incorporation of ^{18}O into either **19** or **18** could be that of indirect detection of ^{18}O by ^{13}C NMR. The stable isotope ^{18}O, (which itself is transparent to NMR, when it is directly attached to a ^{13}C isotope) causes a small upfield shift in the ^{13}C resonance. This phenomenon was first discovered independently by Van Etten[89] and Vederas[90] and has since been exploited in a number of biosynthetic experiments.[91]

To carry out these studies, the following was performed. Chorismic acid was produced in large (gram) quantities with *E. aerogenes*. This allowed an assay for isochorismate synthase to be developed. Isochorismate synthase was next fractionated in order to concentrate the protein enough to enzymatically produce enough isochorismate to characterize by ^{13}C NMR. With a procedure to produce enough isochorismate, the enzyme-catalyzed mechanism was then studied via an incubation in the presence of $H_2{}^{18}O$.

Production of Chorismic Acid by *E. aerogenes*

Following the published protocol,[79] *E. aerogenes* 62-1 was grown in 1 L volumes, the cells isolated via centrifugation, transferred to the production medium and shaken for 16 hours at 30 °C. The cells were removed by centrifugation, and the supernatant made alkaline (10M NaOH, 5 mL/L). Application to a column of Dowex 1-X4 (200-400 mesh, Cl^- form) under pressure proved to be difficult as a maximum flow rate of only 5 mL/minute could be obtained. The flow rate could be improved to about 10 mL/minute by the application of a vacuum to the column. Chorismate was eluted from the column with 1 M NH_4Cl (pH 8.5). Fractions containing chorismate were pooled, acidified to pH 1.5 with 6 N HCl and extracted exhaustively with ether. After removal of the solvent, several crystallizations of the oil (400 mg) were attempted. Crystallization attempts from ether/ petroleum ether (b.p. 60-80 °C); ethyl acetate/petroleum ether (b.p. 60-80 °C), ether/hexane; or ethyl acetate/hexane all proved unsuccessful. A solvent system reported by Knowles[84] was found to be effective. Thus, ether : CH_2Cl_2 : hexane (1:1:2) with cooling to -78 °C yielded 360 mg of chorismic acid as very fine white crystals. Crystalline chorismic acid was found to be stable for up to 6 months if stored at -78 °C. Storage at

-20 °C resulted in significant amounts of rearrangement to prephenic acid over a six-month period.

A more time efficient procedure was developed for the production of chorismic acid by the use of a larger fermentation volume utilizing a New Brunswick Scientific Microferm fermenter. A 10 liter volume of growth medium was inoculated with a 100 mL broth, identical to the growth medium, and incubated at 30 °C for 8 hours with stirring at 200 rpm, and aeration at 10 liters/minute. The cells were collected by centrifugation in less than 1 hour with a Sharples™ centrifuge and immediately resuspended in the production medium (see Experimental Section for details). The mixture was incubated under identical conditions as for the growth medium for 16 hours and the cells removed by centrifugation. The supernatant was made alkaline with 10 M NaOH and applied to a Dowex 1-X4 (100-200 mesh, Cl^- form) at a rate of 50 mL / minute. Elution of chorismate and isolation in an identical manner as for the 2 liter volume yielded 5.02 g of crystalline chorismic acid.

Assay conditions for Isochorismic Acid

Gibson had originally isolated enough **18** from an enzymic reaction with isochorismate synthase to establish its identity.[75] However, this method was limited in that large quantities (1600 mL, 1.0 mg protein / mL) of partially purified enzyme from *E. aerogenes* were required to obtain approximately 20 milligrams of isochorismate from 1.5 grams of chorismate. The reported purification process for the separation of isochorismate from chorismate involved several steps: Paper electrophoresis at pH 3, followed by chromatography on an anion exchange column, subsequent extraction into ether and concentration *in vacuo*. Therefore, a new, more direct, method for the isolation of **18** from a much smaller incubation volume was desired.

The assay for **18** was carried out in 25 mmoles Tris-HCl buffer, pH 8.0, in the presence of 5 mmoles Mg^{2+}, variable amounts of enzyme preparations, and 10 μmoles of chorismate in a total volume of 1 mL. Isochorismate was detected by either:

a) Decomposition to salicylic acid. This assay was based on the observation by Gibson[75] that isochorismate, upon heating in pH 7 buffer at 100 °C, formed approximately 25% salicylic acid, which could be detected by fluorescence spectroscopy. The major product detected was 3-carboxyphenylpyruvic acid (75%). Chorismic acid produced no salicylic acid under these conditions.

b) HPLC detection. Both **19** and **18** have similar ultraviolet spectra: Both absorb at 278 nm (**19** ε = 2630,[92] **18** ε = 13,000). An HPLC separation protocol for **18** and **19** utilizing a C-8 reversed phase column and an isocratic solvent system consisting of 9 : 1 water : methanol and 0.15 M phosphoric acid with UV detection at 278 nm as developed by Leistner was initially used.[93]

Under these conditions (C-8 analytical column, 4.6 x 250 mm, flow 1.0 mL/minute) **18** eluted at 12.7 min, and **19** at 25.8 min. Enzyme incubation samples could be directly assayed by removing an aliquot, typically 5 µL, and directly analyzing by HPLC. Conversion of **19** to **18** was estimated by:

a) comparing relative peak heights obtained from a strip chart recorder or,

b) measuring and comparing peak areas using an integrator. An integrator was not available until relatively late in these studies.

Production and Fractionation of Isochorismate Synthase

Isochorismate synthase was fractionated by the following procedure. *E. aerogenes* was grown as described[75] in the presence of limiting aromatic amino acids tryptophan, L-phenylalanine, and tyrosine, each 10^{-4} M. Cell growth in the absence of Fe^{2+} was required to derepress isochorismate synthase.

Initially, the cells were grown in one-liter quantities in two-liter flasks with shaking at 37 °C for 16 hours and isolated from the medium via centrifugation at 5000 x g for 30 minutes. The yield was approximately 5 grams of cells/liter. Since only one-liter quantities could be centrifuged at a time, the production of large quantities of cells was rather laborious.

In an effort to increase the time efficiency of cell production, the conditions were adapted to the use of a Microferm™ fermenter. The growth

medium constitution was unchanged except that the fermentation volume was 10 liters, and stirring at 200 rpm with aeration at ten liters/minute at 37 °C for sixteen hours provided a cell yield similar to the fermentation with the one liter shaker flasks. Isolation of the cells from the medium could be accomplished efficiently with the use of a Sharples™ centrifuge to yield 76 grams of cells in less than one hour of centrifugation time from a ten liter fermentation.

Suspension of the cells in sodium phosphate buffer (0.01 M, pH 7.0, 2 mL/g cells), followed by sonication in an ice bath, and then centrifugation of the cell debris at 38,400 x g yielded a crude cell-free extract. Following the reported procedure,[75] the crude extract was applied to a DEAE cellulose column. Isochorismate synthase was eluted with 0.1 M NaCl in 0.01 M sodium phosphate buffer (pH 7.0). The column fractions were assayed by HPLC and the active fractions pooled to provide a partially purified enzyme preparation (270 mL). Protein determinations were carried out by the method of Lowry.

Attempts to isolate enough of **18** to characterize by ^{1}H NMR using the partially purified enzyme preparation obtained from the DEAE column were unfortunately not successful. When a 4 mL final volume containing 2.0 mg of protein and 22 μmoles (5 mg) of chorismate was incubated for 2 hours, and then assayed by HPLC, a new peak appeared with an elution time of 19.0 min, and a peak height ratio of 1.75 was obtained. The substrate and product were separated from the aqueous mixture by acidification with 10M HCl, and then extraction into ether, followed by removal of the volatiles under aspirator pressure at 20 °C. However, attempts to isolate **18** by HPLC were not successful. When the eluant containing the material eluting at 12.7 minutes was lyophilized, the non-volatile phosphoric acid remained, apparently decomposing any **18** present to uncharacterizable products. Extraction of the residue into ether to remove the acid followed by evaporation of the solvent under reduced pressure gave less than 1 milligram of material. Analysis of the ^{1}H NMR spectrum of the residue in DMSO-d_6 revealed no characterizable products. (Complex multiplets were observed at 0.8-1.5 and 7.8 ppm). Recovery of **19** was not attempted at this stage.

Although unambiguous proof had not yet been obtained that the new compound which appeared in the incubation mixture was **18**, it was at this point assumed to be so. This was based at this point upon the observation that the compound, when obtained directly from the HPLC column, exhibited a UV maximum at 278 nm.

To compare the effect of varying amounts of enzyme concentration on the amount of the presumed **18** formed from **19**, the following study was done. In a total volume of 1.0 mL of Tris-HCl (25 μmoles, pH 8.0), Mg^{2+} (2.3 μmoles), and **19** (2 μmoles), the amount of enzyme was varied from 0.08 mg to 0.32 mg protein. The relative amounts of isochorismate were expressed as peak height ratios (**18 : 19**). Table 7 summarizes the results. From the results obtained, it can be seen that increasing the enzyme concentration relative to the concentration of **19** increases the efficiency of the conversion of **19** to **18**.

Table 7. Effect of Enzyme Concentration on Isochorismate Production

Protein (mg)	chorismate (μmoles)	Peak height ratio (**18 : 19**)
0.08	2	0.667
0.16	2	1.71
0.24	2	2.46
0.32	2	2.91

a. Incubation was for 2 hours at 37 °C. The reaction was terminated by addition of 100 μL of 10 M HCl.

Ammonium Sulfate Precipitation

Isochorismate synthase was further concentrated and fractionated by ammonium sulfate precipitation of the material obtained from the DEAE column. Fractions obtained were from 0-30%, 30-46%, 46-62%, 62-77%, and 77-99% ammonium sulfate saturation. Each precipitate was dissolved in sodium phosphate buffer 0.01 M, pH 7.0, 5 - 8 mL) and assayed for enzyme activity. The intermediate fractions from 30-62% saturation contained most

of the activity and were pooled to yield 76 milligrams of protein (12 mL, 7.6 milligrams/mL), a 5.9 % recovery of enzyme. All further studies were performed without any further purification of the enzyme. The purification is summarized in Table 8.

Table 8. Fractionation of Isochorismate Synthase

Pur. stage	Protein conc. (mg/mL)	Protein (mg)	%protein	Sp. Act.[a]
Whole cells (68 g)				
Cell free extract	7.3	1825	100	
DEAE column	0.71	182	10.0	1×10^{-4}
$(NH_4)_2SO_4$	13.5	108	5.9	3×10^{-2}

a. Specific activity is defined as µmoles product/mg protein/60 min.

The purification procedure was repeated to yield 134 mg of protein (18.5 mg / mL, 5.1% of the total protein) from 76 g of cells.

With concentrated enzyme now available, it was possible to conduct the incubation using much higher concentrations of enzyme and the substrate **19**. A series of experiments designed to determine optimum conditions for the production of **18** were carried out. First, the effect of increasing concentrations of **19** was determined. All the reactions were performed at 37 °C in a total volume of 4 mL and pH 8.0. Table 9 summarizes the effect of increasing concentrations of **19**.

Table 9. Effect of Chorismate Concentration on Production of 18

Conditions: Volume: 4 mL; protein: 3.4 mg (13.5 mg/mL); Tris-HCl: 50 μM; Mg^{2+}: 5 μM; pH 8.0; Incubation time: 2 hours, at 37 °C. Assay by HPLC (C-8 Econosphere column)

Chorismate (mg)	Peak height ratio (**18 : 19**)
3.0	1.61
5.0	0.74
7.0	0.54
10.0	0.32

A second series of incubations were performed with the chorismate concentration held constant and the amount of enzyme varied. The results are summarized in Table 10.

Table 10. Effect of Protein Concentration on Production of 18

Conditions: Volume: 4 mL; protein: 18.5 mg / mL); Tris-HCl: 50 μM; Mg^{2+}: 5 μm; pH 8.0; Incubation time: 2 h, at 37 °C. Assay by HPLC (C-8 Econosphere column)

Protein (mg)	Peak height ratio (**18 : 19**)
2.0	1.30
5.0	2.12
8.0	2.30
10.0	3.55

The results from the two sets of experiments above indicated that an acceptable ratio of protein to **19** would be 1:1 g/g in order to obtain enough **18** in a single incubation to obtain ^{13}C NMR data.

In this instance the HPLC column used was an Alltech Econosphere C-8 (5 μm) reversed-phase column (4.6 x 250 mm) eluting with the same

conditions as before. Under these conditions **19** eluted at 30.0 minutes, prephenate at 23.2 minutes, and **18** at 19.1 minutes.

Attempts to isolate **18** from an enzyme mixture proved to be troublesome. In a typical experiment, a 4 mL incubation, containing 15 mg each of enzyme and the substrate **19** was acidified to pH 1 (conc. HCl), extracted exhaustively with ether, and the solvent removed under reduced pressure at 20 °C. Separation of **18** from **19** was accomplished by repeated injections and collection of the respective compounds. Extraction of **18** from the eluant into ether yielded 2 mg of a residue which was analyzed by ^1H NMR in DMSO-d_6. Unfortunately, the spectrum obtained was almost completely devoid of **18** and otherwise uninterpretable.

At this point a serious problem with the C-8 Econosphere HPLC column became evident. While analyzing samples for the above experiments the elution time for **19** changed from 30.0 minutes to 25.4 min while using 500 mL of solvent consisting of 10% methanol and 0.15 M H_3PO_4 in H_2O. The pH of this solvent system was 1.8, and apparently was causing significant hydrolysis of the C-8 stationary phase. In an attempt to rectify this problem, the H_3PO_4 concentration was reduced to 0.1% to give a pH of 1.9. While this had no effect upon the resolution of the peaks, degradation of the column was not halted. Passage of 2.5-3 liters of solvent through the column was sufficient to degrade the column until the elution time for **19** was less than 5 minutes. The pH of the solvent system could be increased to pH 2.1 by the replacement of H_3PO_4 with 0.1% trifluoroacetic acid (TFA). While this had no effect on elution times, column degradation was not appreciably halted. This problem of column degradation was not observed while using the Lichrosorb column (*vida supra*).

It was obvious by this time that a new method for the isolation of **18** was necessary. The isolation problem was solved by changing the acid component of the HPLC solvent system from the non-volatile phosphoric acid to the volatile TFA. Collection of **18** and **19** followed by lyophilization of the solvents yielded pure compounds.

Confirmation that the compound assumed to be isochorismate was indeed **18** was provided by the ^1H NMR spectrum obtained in DMSO-d_6, which was identical to that reported by Gibson.[75] Unambiguous assignment of the ^1H spectrum of **18** will be provided below.

Efforts were next directed toward increasing the amount of **18** isolated from the incubation mixture. A successful isolation of **18** was obtained by incubating four mL of a mixture containing 330 μmoles Tris, 100 μmoles $MgCl_2$, and 110 μmoles (25 mg) of chorismate, and 25 mg protein (18.5 mg/mL), pH 8.0 (1M NaOH). Chorismate and isochorismate were isolated by HPLC yielding 3.3 mg of **18** and 7.7 mg of **19**.

Table 11 summarizes the efforts towards the isolation of **18**.

Table 11. Isochorismate Isolation Conditions

Substrate (mg)					Product (mg)	
Protein	**19**	volume (mL)	Tris (μM)	Mg^{2+} (μM)	**19**	**18**
11.5	12.0	2.0	40	12	3.7	1.2
11.5	20.0	2.0	64	40	6.7	1.3
25.0	25	4.0	82	25	7.0	2.5
30.0	30.0	4.0	100	25	7.7	3.3
35.0	35.0	4.0	115	33	12.0	5.2

Isochorismate and Chorismate NMR Studies

Stability of Chorismic Acid and Isochorismic Acid

Unambiguous assignment of the ^{13}C resonances for C-4 of **19** and C-2 of **18** were essential for this study. While the ^{13}C spectrum of chorismate[94] has been published, only the ^{1}H NMR spectrum for isochorismate has been reported.

The ^{1}H and ^{13}C NMR spectra of **18** were initially obtained in DMSO-d_6. Both **19** and **18** have been reported to to be unstable at room temperature. Chorismate rearranges to prephenic acid in 16 hours in aqueous solution at pH values from 3 to 10.[85] In DMSO-d_6, **19** has been reported to primarily eliminate water rather than undergo a Claisen rearrangement.[85] Isochorismate has been reported to rearrange to 3'-carboxyphenylpyruvic acid via an analogous Claisen rearrangement followed by elimination of water, but the half-life of the reaction has never been reported.[75,95]

Berchtold, in addition has reported that **18** is unstable, but did not report the rate of decomposition.[96] The fate of **19** in DMSO had not been reported. Therefore the stability of **18** in DMSO-d_6 was determined.

^1H spectra **18** were obtained at 0, 3, 6, 9, 19, 24, 48, 55, and 72 hours at 294 K, and decomposition of **18** followed by comparison of the integration of the H-5 resonance at 6.99 ppm and a new aromatic resonance appearing at 7.45 ppm. No attempt was made to characterize or isolate any of the decomposition products from **18**. The ^1H spectra obtained at 0 and 72 hours are compared in Figure 9, and reveal that under these conditions, decomposition of **19** was only about 40% complete after 72 hours.

A ^{13}C NMR spectrum was obtained between 9 and 19 hours, and the line widths found to be 2-4 Hz wide. Since the expected ^{13}C-^{18}O isotope shift of a secondary alcohol is only 1-3 Hz, it was clear that another solvent was necessary.

Similar results were found when the ^1H NMR spectrum for **19** was obtained in DMSO-d_6, although very poor resolution was observed in concentrations more than 4 mg/500μL. At a concentration of 8 mg/500 μL the ^{13}C spectrum of **19** revealed line widths of 3-7 Hz.

The stability of **19** was therefore studied in deuterated dimethylformamide (DMF-d_7). Chorismate was found to decompose to an aromatic compound, presumably due to the elimination of water as evidenced by a four proton spin system appearing between 7.8 and 7.3 ppm. The concentration of the aromatic compound increased to approximately equimolar proportions with **19** within 16 hours. Analysis of the ^{13}C spectrum of **19**, obtained between 0 and 1 hours revealed line widths of 1 Hz or less.

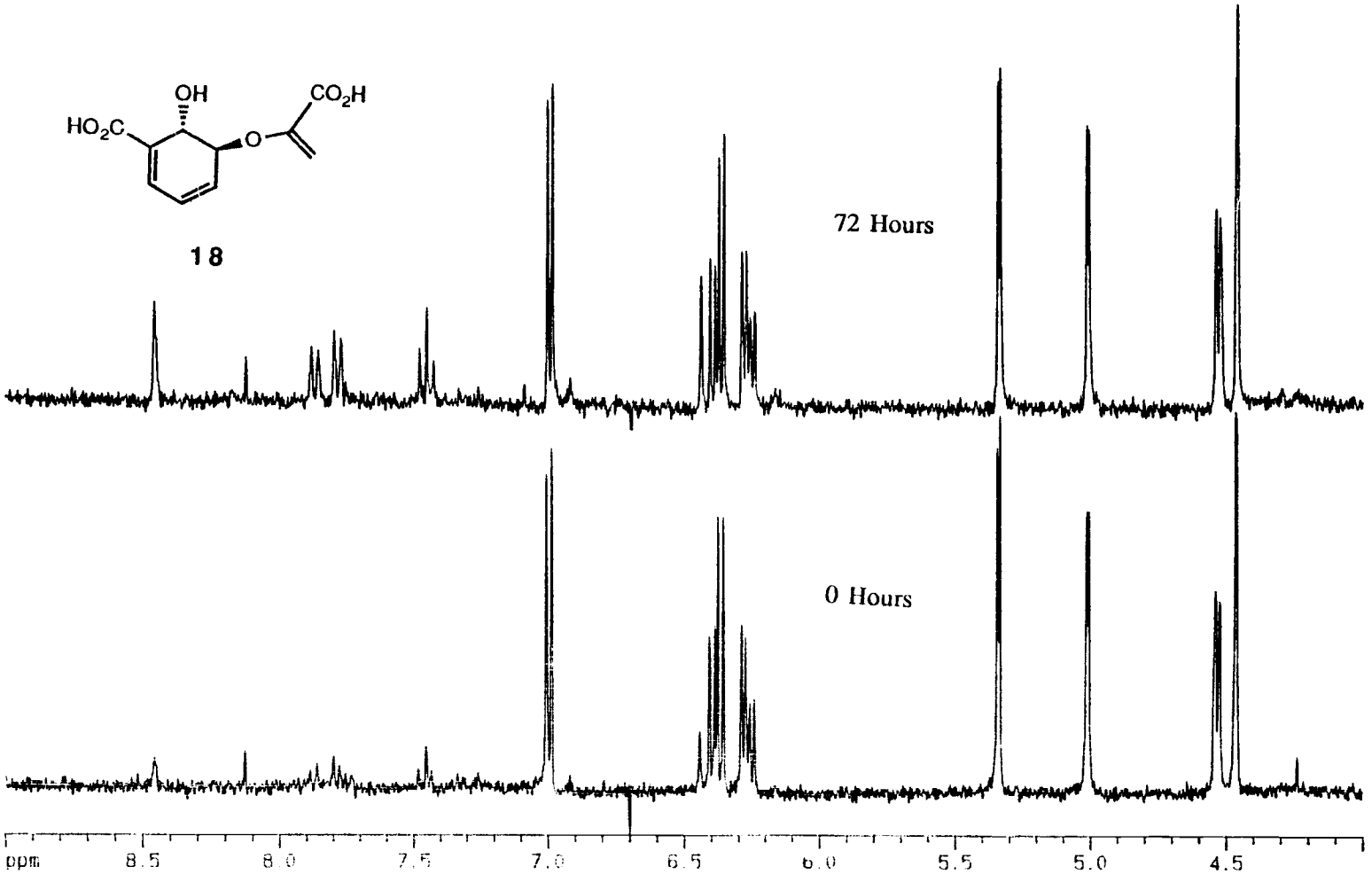


Figure 9 Isochorismate ^1H NMR Spectrum at 0 and 72 Hours

Assignment of the NMR Spectra of 18 and 19

Assignment of the ^{1}H spectrum of **19** reported by Gibson,[75] was confirmed by a 2-dimensional ^{1}H-^{1}H correlation experiment (COSY). Analysis of the COSY NMR spectrum, shown in Figure 10, revealed the resonance at 5.05 ppm, assigned to H-3, to be coupled to resonances at 4.63 (H-4), 6.83 (H-2), and a weak coupling to 6.04 ppm (H-5), while the H-4 resonance at 4.63 ppm was coupled to 5.05 (H-3), 6.04 (H-5), and 6.30 (H-6). These couplings are fully consistent with the assignments shown in Figure 10.

With the complete ^{1}H spectrum of **19** assigned, the unambiguous assignment of the ^{13}C spectrum was now possible. Although a 1-bond ^{1}H-^{13}C 2-dimensional correlation experiment (HETCOR) would have allowed an unambiguous assignment for both C-3 and C-4 of **19**, the long range ^{1}H-^{13}C experiment HETCOSY, which reveals 1-bond as well as longer range correlations (typically 3-bond), was the experiment of choice. Although all of the expected 3-bond couplings were not observed, enough were observed to allow all of the ^{13}C resonances to be assigned. The HETCOSY spectrum and assignments of the correlations for **19** are shown in Figure 11.

Since the ^{13}C NMR spectrum obtained for isochorismate in DMSO-d_6 exhibited resonances of 2-4 Hz wide, as stated above, ^{1}H and ^{13}C spectra were obtained in DMF-d_7. In this solvent, the H-2 and H-3 resonances were found to have overlapped to give a two proton multiplet at 4.72 ppm. These two resonances were separated (4.46 ppm and 4.95 ppm) when the ^{1}H spectrum was obtained in DMSO-d_6. Since the C-2 and C-3 resonances had to be unambiguously distinguished from each other, the resolution of the H-2 and H-3 ^{1}H resonances was critical. However, a comparison of the ^{13}C spectra of **18** in DMSO-d_6 and DMF-d_7 revealed only slight differences in chemical shifts. The C-2 and C-3 resonances in the former solvent were 62.12 and 74.17 ppm while those in the latter solvent were 63.83 and 75.64 ppm. Thus, ^{13}C assignments in DMF-d_7 for C-2 and C-3 could be confidently made from experiments performed in DMSO-d_6.

Assignment by Gibson[75] of the ^{1}H NMR spectrum of **18** in DMSO-d_6 was confirmed by a COSY NMR experiment and is shown in Figure 12. It was of particular importance to distinguish between the H-2 and H-3

resonances so that the ^{13}C NMR spectrum could be unambiguously assigned. Analysis of the ^{1}H resonance at 4.52 ppm (a doublet of doublets J = 4.95, 0.81 Hz) revealed a 5 Hz coupling to the vinylic resonance at 6.25 and a 0.9 Hz coupling to the resonance at 4.46, while the broadened singlet at 4.46 ppm was showed a correlation to the 4.52 resonance and a weak W-coupling to the 6.25 resonance. This confirmed the assignment of H-2 at 4.46 ppm and H-3 at 4.52 ppm.

A HETCOR NMR experiment was next performed to assign the protonated portion of the ^{13}C spectrum. For the purposes of this study the unambiguous assignment of the two oxygen-substituted positions at C-2 and C-3 was most critical. A one bond correlation between the most upfield ^{13}C resonance at 62.1 ppm and the ^{1}H resonance at 4.46, and a similar correlation between the ^{13}C and ^{1}H resonances at 74.2 and 4.52 ppm respectively were sufficient to unambiguously assign the C-2 and C-3 carbons as 62.1 and 74.2 ppm respectively. The COSY and HETCOR NMR spectra and correlation assignments for **18** are contained in Figure 12 and Figure 13 respectively.

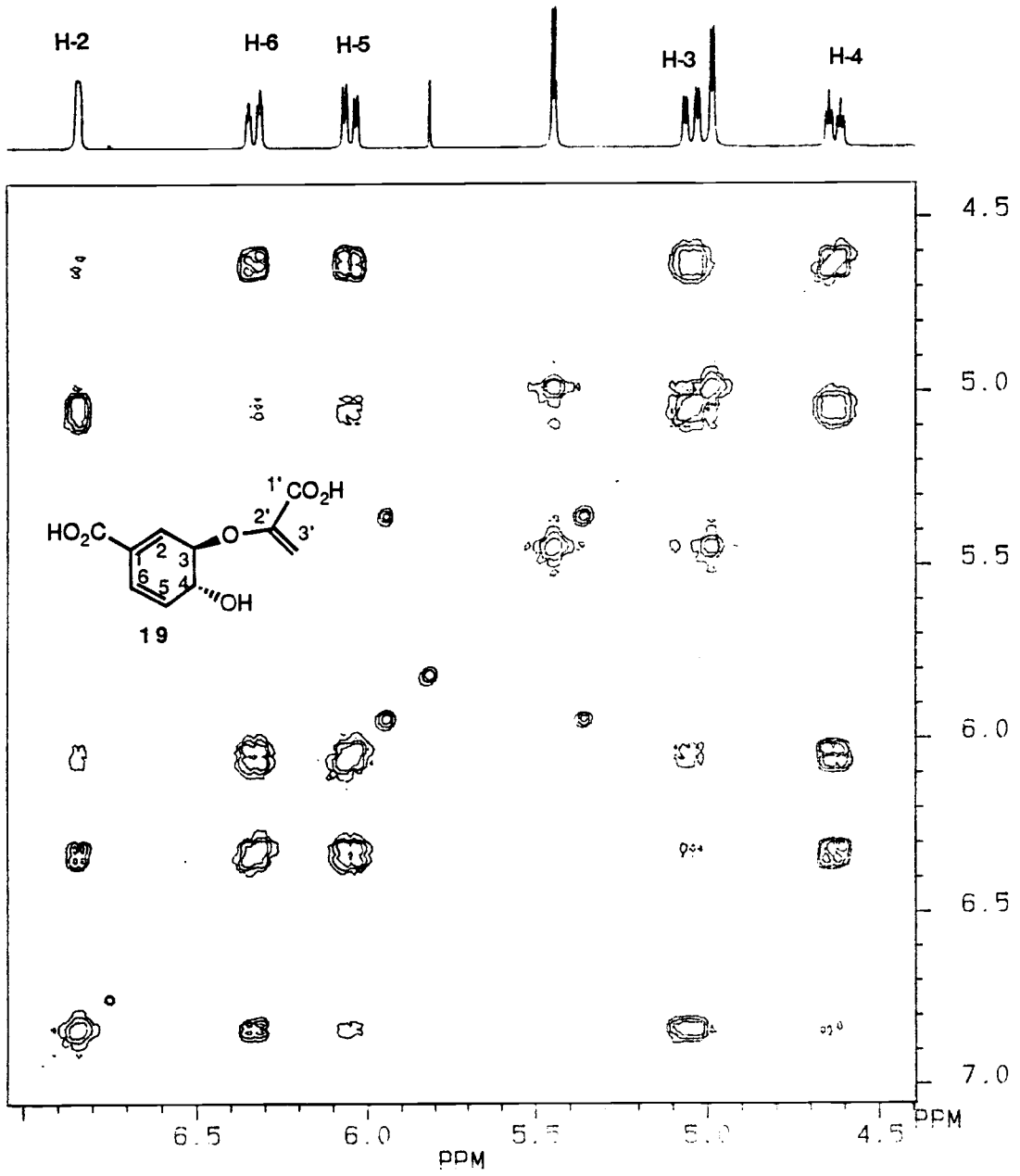


Figure 10. COSY NMR Spectrum of Chorismic Acid

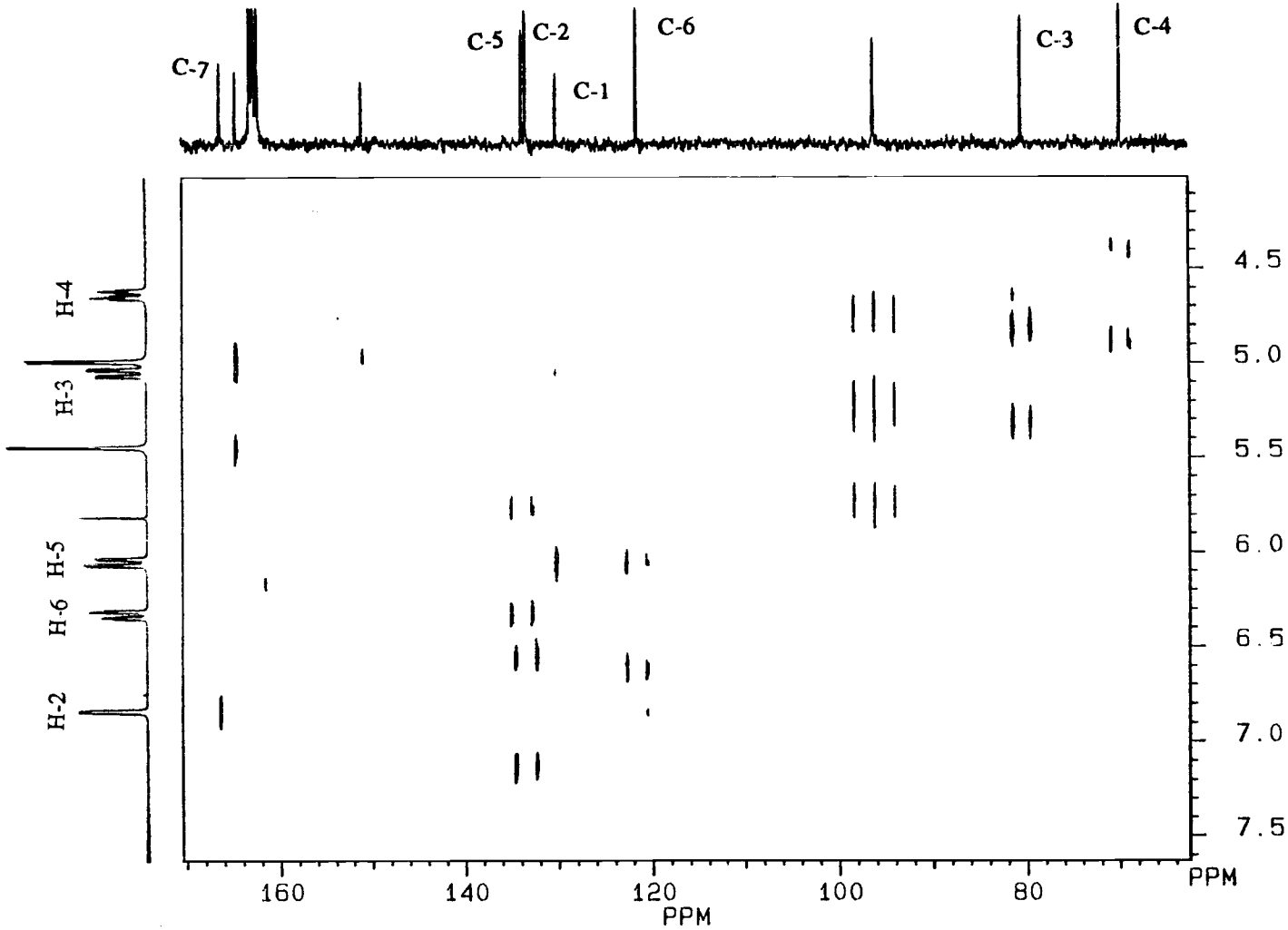


Figure 10. HETCOSY NMR Spectrum of Chorismic Acid

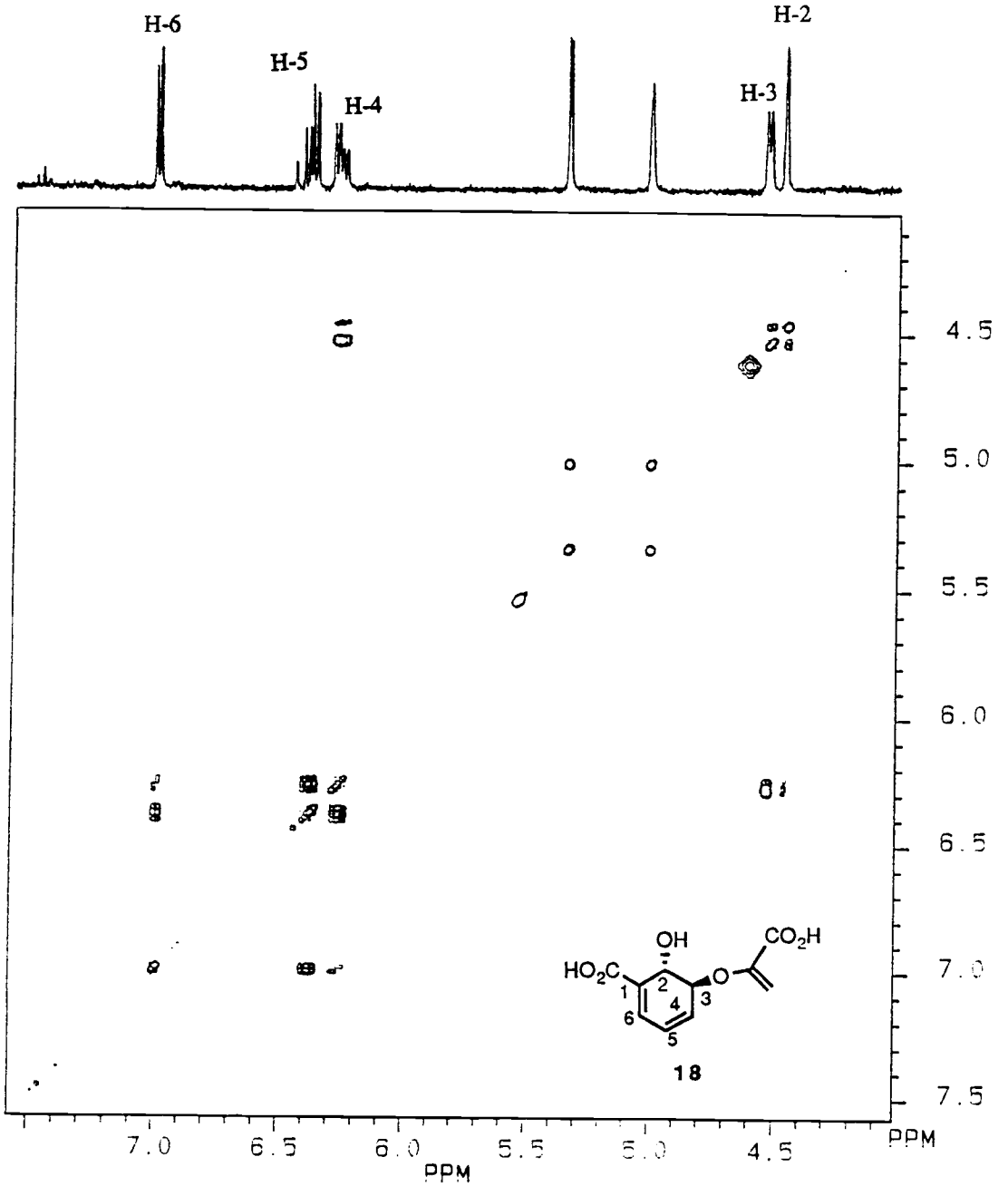


Figure 11. COSY NMR Spectrum of Isochorismic Acid.

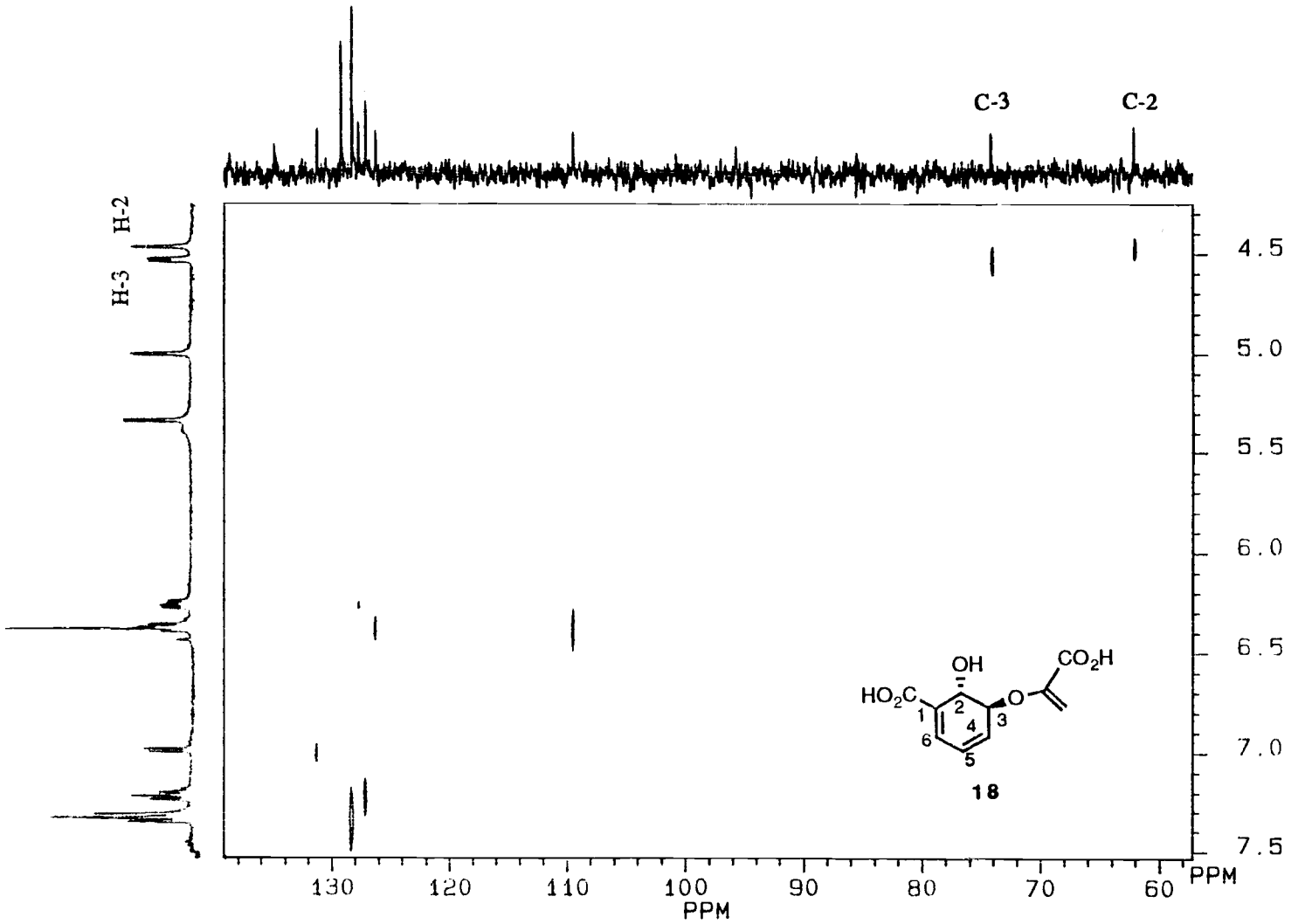


Figure 12. HETCOR NMR Spectrum of Isochorismic Acid

Isochorismate Synthase Incubation In $H_2{}^{18}O$

To distinguish between the two mechanistic possibilities for the conversion of **19** to **18** proposed in Scheme 20, an incubation of isochorismate synthase with **19** in the presence of $H_2{}^{18}O$ was planned. The use of 50% $H_2{}^{18}O$ would show both a ^{13}C-^{16}O and a ^{13}C-^{18}O resonance, thus providing an internal standard for ^{13}C analysis in the event of labeling of either **19** or **18**. Due to the cost of $H_2{}^{18}O$ ($120/gram) the volume of the labeled incubation had to be limited, and a total volume of 4 mL was used.

Experimental conditions utilizing 35 mg of **19**, which were shown to yield 5.2 mg **18** and 12.0 mg **19**, were used for the labeling experiment. Thus, Tris (basic form), $MgCl_2 \cdot H_2O$, and **19** were dissolved in 2 mL of 98-99% $H_2{}^{18}O$ and the pH adjusted to 8.1 with 1 M NaOH. Enzyme preparation (18.5 mg/mL, 1.89 mL) was added and the volume made up to 4 mL with unlabeled H_2O. Incubation for two hours followed by HPLC isolation as described (*vida supra*) provided 12.9 mg of **19** and 5.5 mg of **19**. The ^{13}C spectra in DMF-d_7 were obtained for **19** and **18** from the labeling experiment. These were compared with spectra for unlabeled sample obtained under identical conditions. The isochorismate obtained showed an approximate 50% incorporation of ^{18}O only at C-2. This was shown by a 1.31 Hz upfield-shifted peak from the natural abundance C-2 peak, approximately the same intensity as the natural abundance peak. In contrast to this result, the chorismate ^{13}C NMR spectrum revealed no discernable incorporation of ^{18}O at C-4 or elsewhere. This unexpected result indicated that the reaction was proceeding in the forward direction with incorporation of water from the solvent, but that the reverse reaction from **18** to **19** was not occurring. The spectra for **19** and **18** are shown in Figure 14 and Figure 15 respectively.

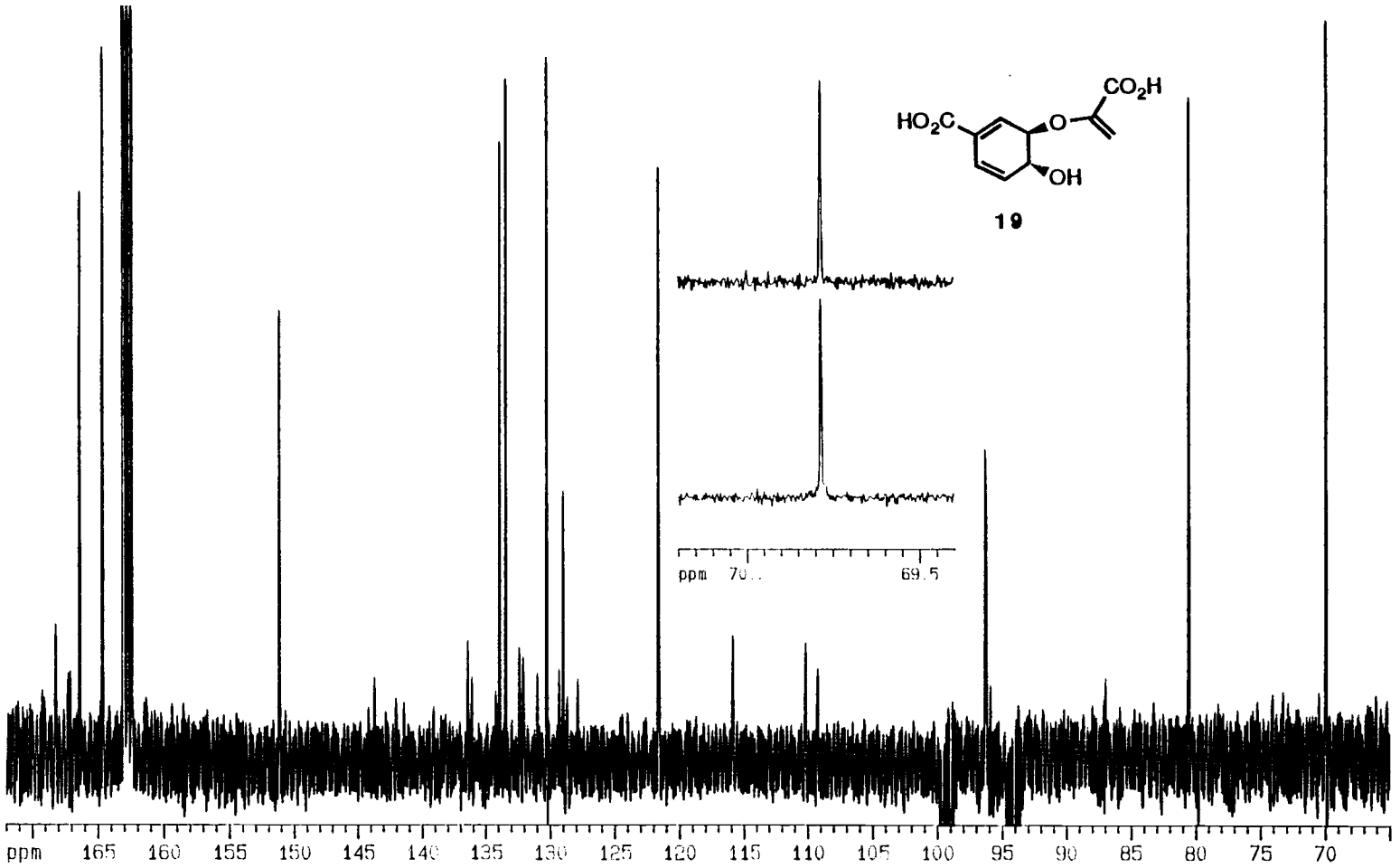


Figure 13. ^{13}C NMR Spectrum of **19** From $H_2{}^{18}O$ Experiment

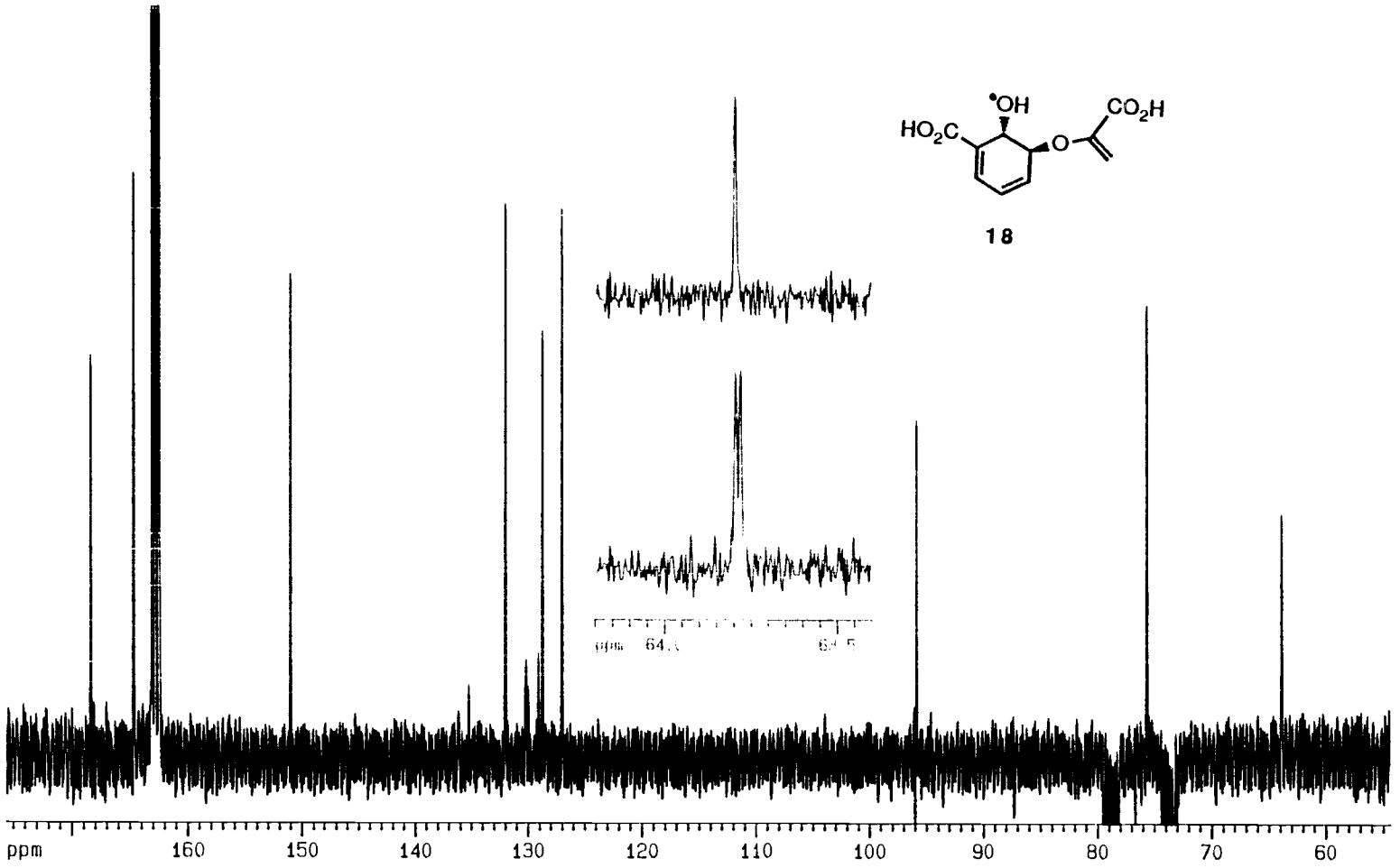


Figure 14. ^{13}C NMR Spectrum of **18** From $H_2{}^{18}O$ Experiment

The apparently anomalous lack of the reverse reaction could have at least three possible explanations:

a) The enzymic reaction is not reversible.

b) An isotope shift at C-4 of **19** was too small to be discerned.

c) Equilibrium was not achieved under the conditions of the experiment.

Leistner[18] has shown that when enzyme preparations containing isochorismate synthase activity are presented with either **18** or **19**, an 80:20 mixture of **19** to **18** results, thus demonstrating the reversibility of the enzyme-catalyzed reaction. In view of the 1.31 Hz ^{18}O-induced shift observed with **18**, and the 0.75 Hz line width of the C-4 resonance of **19** isolated from the labeling reaction, the second possibility is unlikely. Therefore, the third possibility is most likely.

To confirm possibility c, **19** and **18** were each incubated with enzyme at different concentrations and at different stages of purity and the relative peak areas from HPLC analyses were compared. Amounts of each component were estimated by determining standard curves of both **19** and **18** relating HPLC peak areas to amounts injected from 0.0125 to 0.500 μg of each compound. Thus, a series of incubations were performed each containing: Tris-HCl (48 μM); Mg^{2+} (4.5 μM); **19** or **18**, (90 μg); and varying amounts of protein. When protein obtained from ammonium sulfate precipitation (460 μg) was incubated for 2 hours with chorismate, the **18:19** ratio was 0.45. An identical incubation with isochorismate produced **18:19** of 0.64. When the amount of protein was reduced to 125 μg, approximating the relative enzyme : substrate ratio used for the labeling reaction, **18:19** ratio with **19** as substrate was 0.54. However, when presented with **18**, the ratio was 1.28. These results, summarized in Table 12, reveal that a large excess of protein is required to reach an equilibrium. Under the conditions of the labeled $H_2{}^{18}O$ experiment equilibrium was not reached even though the reaction is clearly reversible.

Table 12. Enzyme Reversibility

Protein source	protein (μg)	Substrate[b]	**18 : 19** ratio[c]
$(NH_4)_2SO_4$ ppt	460	**19**	0.45
$(NH_4)_2SO_4$ ppt	460	**18**	0.64
$(NH_4)_2SO_4$ ppt	125	**19**	0.54
$(NH_4)_2SO_4$ ppt	125	**18**	1.28
DEAE column	25	**19**	0.39
DEAE column	25	**18**	2.64
Crude extract	180	**19**	0.032
Crude extract	180	**18**	1.27

a. Final volume 200 μL containing Tris-HCl (48 μM) pH 8.0, Mg^{2+} (4.5 μM), substrate (90 μg), and protein; incubation for 2 hours at 37 °C; 5 μL injected. b. 90 μg substrate used. c. estimated from peak areas developed from standard curves.

Conclusion

The positive incorporation of **27a** into **1** suggests the intermediacy of the novel amide **27**. This would place the timing of the conversion of amide formation early in the biosynthetic sequence. This is supported by the observation that the amide formation from the acid **17** is apparently reversible, as evidenced by incorporation of intact amide **27a** and some hydrolysis to, presumably, the acid **17**. However, the unsuccessful attempts to trap the N-acetyl-methoxy derivative of the amide and the corresponding methyl ester of the acid **17** from growing cells or to detect them in cell-free extracts of *S. helicus* make this conclusion somewhat tentative.

There are a number of possibilities which could explain the negative results obtained from the trapping experiments and the cell-free experiments utilizing chorismate and isochorismate. First, it is possible that the timing of the trapping experiments was incorrect, and that biosynthesis of both **17** and **27** were not occurring at that time. Second, it is possible that the conditions used for generating cell-free extracts

destroyed the enzyme activity necessary for the biosynthetic production of **17** and **27**. The observation that the cell-free extracts prepared from *S. helicus* were unstable, losing their ability to consume chorismate within eight hours, lends credence to this possibility. Third, **17** and **27** may be produced within an enzyme complex and are only transient intermediates, and are thus not produced in large enough quantities to be detectable by the methods employed. Fourth, the possibility exists that **17** and **27** may not be involved in the biosynthesis of **1**. However, the positive incorporations observed for both **17** and **27**, would make this possibility seem the most unlikely one at this point.

The observation that cell-free extracts of *S. helicus* could consume chorismate but not isochorismate leaves open the possibility that the branch point from the shikimate pathway is at chorismate rather than isochorismate. One possibility is shown in pathway **b** of Scheme 18 which leads through the intermediate **78**, which has been implicated in anthranilate biosynthesis.

Of the four major biosynthetic steps which must occur after formation of the acid **17**, the incorporation of **27a** suggests the first step to be amide formation. While the demonstration of the chemical instability of 3,6-dihydroxyanthranilamide, **28**, does not at all rule out the possibility of the next step being hydroxylation of the aromatic ring, the demonstrated stability of the 5-propyl-3,6-dihydroxyanthranilamide **68** underscores the viability of either C-glycosylation or hydroxylation as being the next step. The synthetic methodology developed toward constructing a C-glycoside provides an entry into a series of C-glycosides which could be used to delineate the order of the three remaining biosynthetic steps beyond amide formation which must occur leading to **1**. This synthetic work also provides a potential entry into a concise biomimetic total synthesis of **1**.

The enzyme isochorismate synthase, which catalyzes the conversion of chorismate to isochorismate, was partially purified from the organism *E. aerogenes* 62-1 and the mechanism of the isomerization found to be consistent with an addition-elimination mechanism involving the addition of water from the solvent.

Experimental

General Procedures. ^{1}H NMR spectra were taken on a Bruker AM 400 or AC 300 spectrometer; ^{13}C NMR spectra were obtained at 100.6 or 75.4 MHz on the AM 400 or AC 300 spectrometers, and were either broadband or Waltz decoupled. IR spectra were recorded on a Nicolet 5DXB FTIR spectrometer. Low resolution mass spectra were taken on a Varian MAT CH-7 spectrometer with a System Industries 150 data system. High resolution mass spectra were taken on a Kratos MS 50 TC spectrometer. Ultraviolet spectra were obtained on an IBM 942(UV/vis spectrophotometer. Elemental analyses were performed by Desert Analytics (Tucson, AZ). Melting points were obtained on a Büchi melting point apparatus and are uncorrected. Sonications were carried out with a Branson 2000 Water Bath Sonicator. Tetrahydrofuran (THF), benzene, and ether were distilled from potassium or sodium benzophenone ketyl just prior to use. N,N-Dimethylformamide (DMF), methylene chloride, and dimethyl sulfoxide (DMSO), were distilled from CaH_2 and stored over 3Å sieves except for methylene chloride which was distilled just prior to use. Acetone was distilled from P_2O_5. Ethyl acetate and hexane were distilled prior to use. All other solvents and reagents were used as obtained unless otherwise noted. Flash chromatography was carried out on silica gel (EM Reagents, Kieselgel 60, 230-400 mesh) or Silicar CC-4 (Mallinkrodt). Analytical thin layer chromatography (TLC) was carried out on precoated Kieselgel 60 F_{254} (0.25 mm plates) and visualized by long and/or short wave UV. Ion exchange resins were purchased from Sigma Chemical Company (St. Louis, MO) and were converted to the necessary ionic form according to the manufacturer's recommendations.

Isatoic anhydride 34 from 6-hydroxyanthranilic acid, 17. To a solution of **17** (50 mg, 0.264 mmol) in dioxane (10 mL) was added phosgene (1.93M in toluene, 0.27 mL, 0.528 mmol) and powdered $NaHCO_3$ (110 mg, 1.32 mmol). The mixture was stirred at 50° C for 1 h until no starting material was visible by TLC (20% methanol/ethyl acetate). The mixture was filtered and concentrated *in vacuo* to a white powder which was carefully washed with cold methanol to yield 44 mg (94%) of **34** as a

white powder: mp. >300 °C; IR (KBr) 3330-3022, 1765, 1703, 1639, 1516, 1386 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) δ 10.67 (br s, 1 H), 10.12 (br s, 1 H), 7.63 (t, J = 8.0 Hz, 1 H), 6.73 (d, J = 8.0 Hz, 1 H), 6.71 (d, J = 8.0 Hz, 1 H); ^{13}C NMR (acetone-d_6, 100.6 MHz) δ 165.12, 161.91, 146.76 142.44, 139.39, 111.30, 106.06, 98.32.

N-(*t*-Butoxycarbonyl)-3-[(Methoxymethyl)oxy]-Aniline, 36. To a solution of 3-aminophenol (4.00 g, 36.7 mmol) in THF (200 mL) was added di-t-butyl dicarbonate (9.67 g, 44.3 mmol) in THF (75 mL). The solution was stirred at reflux for 24 h, evaporated *in vacuo* to give a brown oil. This was dissolved in ethyl acetate, washed with cold 0.5 M HCl, H_2O, and then saturated $NaHCO_3$, followed by sat. brine. Drying of the solution with Na_2SO_4 and removal of the volatiles *in vacuo* yielded a brown oil. After crystallization from CH_2Cl_2/hexane 7.34 g (96%) of light tan crystals were obtained.

To a mixture of NaH (0.80 g, 33.2 mmol) in THF (200 mL) at 0° C was added the N-t-BOC protected 3-aminophenol (3.30 g, 15.8 mmol). After stirring for 1 h at 0° C chloromethyl methyl ether (1.32 mL, 17.3 mmol) was added and the reaction allowed to come to room temperature overnight. The solution was diluted with ethyl acetate, washed with 5% aqueous NaOH, water, and sat. brine. After drying over Na_2SO_4 the solvent was evaporated *in vacuo*. The residue was passed through a flash column (4.5 X 15 cm) eluting with 5% ethyl acetate/CH_2Cl_2 to obtain a light yellow oil (2.56 g, 64%): IR (neat) 3340, 2978, 1730, 1709, 1537, 1393, 1151 cm^{-1}; ^1H NMR (acetone-d_6, 300 MHz) δ 8.41 (br s, 1 H), 7.36 (m, 1 H), 7.14 (m, 2 H), 6.65 (m, 1 H), 5.15 (s, 2 H), 3.41 (s, 3 H), 1.46 (s, 9 H); ^{13}C NMR: (acetone-d_6, 75.4 MHz) δ 158.73, 153.26, 141.74, 130.14, 112.39, 110.61, 107.22, 94.91, 79.91, 55.92, 28.44; MS (70 ev) *m/z* 253, M^+, (97.3), 197 (97.3), 153 (44.8), 121 (48.7), 57 (100), 45 (95.5); Anal. Calc. for $C_{13}H_{19}NO_4$: C, 61.63; H, 7.56; N, 5.53. Found: C, 61.76; H, 7.62; N, 5.43.

N-(Methoxycarbonyl)-3-[(Methoxymethyl)oxy]-Aniline, 38. To a solution of 3-aminophenol (5.00 g, 45.9 mmol) in ethyl acetate was added 8% $NaHCO_3$ (150 mL) followed by methyl chloroformate (3.89 mL, 50.5 mmol). The 2-phased solution was stirred overnight. After the phases were separated, the organic layer was washed with 1N HCl, and then H_2O,

followed by sat. brine, and then dried over $MgSO_4$. Concentration of the organic layer *in vacuo* yielded a white solid, which was crystallized from CH_2Cl_2/hexanes to yield 7.53 g (94.8%) of colorless crystals: mp. 95°-95.5 °C; IR (KBr) 3407, 3300, 1697, 1541, 1455, 1225 cm^{-1}; ^1H NMR (acetone-d_6, 300 MHz) δ 8.57 (br s, 1 H), 8.32 (s, 1 H), 7.21 (t, J = 2.2 Hz, 1 H), 7.09 (t, J = 8.2 Hz, 1 H), 6.97 (ddd, J = 8.1, 2.2, 1.7 Hz, 1 H), 6.51 (dd , J = 8.2, 2.2, 1.1 Hz, 1 H), 3.68 (s, 3 H); ^{13}C NMR (acetone-d_6, 75.4 MHz) δ 158.66, 154.75, 141.29, 130.28, 110.42, 110.27, 106.21, 51.99; MS (FAB$^+$) *m/z* 168 (M^++1); Anal. Calc. for $C_8H_9NO_3$: C, 57.47; H, 5.43; N, 8.38. Found: C, 57.33; H, 5.41; N, 8.38.

To a suspension of NaH (1.37 g, 57.0 mmol) in dry THF under an argon atmosphere at O °C was added the 3-[N-methoxycarbonyl]-aminophenol in THF. After 1 h at O °C the mixture was quenched with chloromethyl methyl ether (2.25 mL, 29.6 mmol), and allowed to stir at room temperature overnight. The heterogeneous mixture was diluted with ethyl acetate, and extracted with 5% NaOH. The organic layer was washed with water and then sat. brine, dried over Na_2SO_4, and then concentrated *in vacuo* to give a light yellow oil, which was pure by TLC. A small amount was subjected to Kügelrohr distillation to yield a colorless oil: IR (neat) 3326, 2954, 1738, 1716, 1610, 1543, 1151 cm^{-1}; ^1H NMR (CD_3CN, 400 MHz,) δ 7.79 (br s, 1 H), 7.22 (d, J = 1.8 Hz, 1 H), 7.19 (t, J = 8.5 Hz, 1 H), 7.03 (dd, J = 8.5, 1.8 Hz, 1 H), 6.69 (dd, J = 8.5, 1.8 Hz, 1 H), 5.14 (s, 2 H), 3.69 (s, 3 H), 3.41 (s, 3 H); ^{13}C NMR (CD_3CN, 100.6 MHz) δ 158.74, 155.13, 141.13, 130.66, 112.90, 111.60, 107.58, 95.24, 56.30, 52.66; MS (70 ev) *m/z* 211, M^+, (100), 179 (67.9), 45 (97.6); Anal. Calc. for $C_{10}H_{13}NO_4$: C, 56.85; H, 6.21; N, 6.63. Found: C, 56.87; H, 6.37; N, 6.15.

Lithiations: General procedure for urethane protected anilines: The protected aniline was dissolved in dry THF under an Argon atmosphere and cooled to -78°C. *t*-BuLi was added and the canary yellow solution stirred for 15 min then warmed to -20° and stirred for an additional 2-2.5 h whereupon the appropriate electrophile was added and the reaction allowed to warm slowly to room temperature over 6 h. The work-up procedures are described below.

Methyl N-(*t*-Butoxycarbonyl)-4-[(Methoxymethyl)oxy]-Anthranilate, 37. To a stirring solution of 35 (100.0 mg, 0.395 mmol) in

dry THF was added *t*-BuLi (1.7 M. 0.79 mL, 1.34 mmol). The canary yellow solution was stirred as described and quenched by the addition of methyl chloroformate (0.30 mL, 0.395 mmol). After dilution with ethyl acetate, the organic layer was washed with water and then sat. brine, then dried over Na_2SO_4. Concentrated of the volatiles *in vacuo* afforded a light yellow oil. Silica chromatography eluting with 21% Et_2O/hexanes yielded in order of elution: **37** (27 mg, 22%) as a colorless oil followed by the starting material (47 mg, 47% recovered). Physical data for **37**: IR (neat) 3300, 2978, 1732, 1689, 1587, 1264, 1155 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) δ 10.52 (br s, 1 H), 8.18 (d, J = 2.5 Hz, 1 H), 7.96 (d, J = 9.0 Hz, 1 H), 6.73 (dd, J = 9.0, 2.5 Hz, 1 H), 5.28 (s, 2 H), 3.88 (s, 3 H), 3.45 (s, 3 H), 1.53 (s, 9 H); ^{13}C NMR (acetone-d_6, 100.6 MHz) δ 168.96, 163.06, 153.15, 144.97, 133.50, 109.84, 108.68, 105.96, 94.73, 81.02, 56.39, 52.41, 28.37; MS (70 ev) *m/z* 311, M^+ (15), 212 (80), 180 (100), 57 (75).

Methyl 6-[(Methoxymethyl)Oxy]-N-(Methylcarbonyl)-Anthranilate, 39. Methyl urethane **38** (73.5 mg, 0.348 mmol) was treated as above with *t*-BuLi (1.7M, 0.70 mL, 1.184 mmol). The resulting solution was quenched with methyl chloroformate (0.027 mL, 0.348 mmol). The mixture was diluted with ethyl acetate, washed with water, followed by brine, and dried over Na_2SO_4. Concentration of the solvent at reduced pressure yielded a yellow oil which was fractionated with a Chromatotron plate (1 mm silica plate) eluting with 3% ethyl acetate/ $CHCl_3$ to yield in order of elution: the 1,2,4 trisubstituted ester **40** (5.6 mg, 6%) followed by **39** (27.9 mg, 33.0%) each as a clear, colorless oil. **39**: IR (neat) 3360, 2955, 1742, 1694, 1599, 1274, 1024 cm^{-1}; ^1H NMR (acetone-d_6, 300 MHz) δ 8.64 (br s, 1 H), 7.72 (d, J = 8.4 Hz, 1 H), 7.40, (t, J = 8.4 Hz, 1 H), 6.93 (dd, J = 8.3, 1.1 Hz; 1 H), 5.23 (s, 2 H), 3.89 (s, 3 H), 3.71 (s, 3 H), 3.45 (s, 3 H); ^{13}C NMR (acetone-d_6, 75.4 MHz) δ 168.18, 156.89, 154.56, 139.39, 132.82, 114.62, 111.00, 95.69, 56.36, 52.64, 52.49; MS (70 ev) *m/z* 269 (50), 193 (100), 176 (30), 161 (71), 135 (18), 107 (15). HRMS Calc. for $C_{12}H_{15}NO_6$: 269.08995. Found: 269.08990. **40**: ^1H NMR (acetone-d_6, 400 MHz) δ 10.69 (br s, 1 H), 8.17 (d, J = 2.4 Hz, 1 H), 7.98 (d, J = 8.9 Hz, 1 H), 6.77 (dd, J = 8.9, 2.4 Hz, 1 H), 5.29 (s, 2 H), 3.89 (s, 3 H), 3.76 (s, 3 H), 3.45 (s, 3 H).

N-(*t*-Butoxycarbonyl)-3-Anisidine, 43. To a solution of 3-anisidine (5.95 mL, 52.9 mmol) in THF (250 mL) was added di-*t*-

butyldicarbonate (12.0 g, 55.0 mmol) in THF (30 mL). The solution was stirred for 48 h at 50 °C. The solvent was evaporated, and the oily red residue crystallized from hexane to yield 11.09 g (94%) of white crystals: mp 57°-58 °C; IR (neat) 3340, 2980, 1728, 1706, 1607, 1238 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) δ 8.37 (br s, 1 H), 7.28 (m, 1 H), 7.16 (t, J = 8 Hz, 1 H), 7.08 (dd, J = 8, 2 Hz, 1 H), 6.57 (dd J = 8, 2 Hz, 1 H), 3.76 (s, 3 H), 1.48 (s, 9 H); ^{13}C NMR (acetone-d_6, 100.6 MHz) δ 161.14, 153.60, 141.82, 130.18, 111.30, 108.44,. 104.91, 79.91, 55.34, 28.48; MS (FAB$^+$) *m/z* 224, M$^+$+1 (60), 168 (100). Anal. Calc. for $C_{12}H_{17}NO_3$: C, 64.54; H, 7.69; N, 6.29. Found: C, 64.36; H, 7.69; N 6.29.

N-(*t*-Butoxycarbonyl)-6-Methoxyanthranilic Acid, 45. To a stirring solution of **43** (5.00 g, 22.4 mmol) in dry THF was added *t*-BuLi (1.40M. 40 mL, 54.9 mmol). The canary yellow solution was stirred (*vida supra*) then quenched as follows: Carbon dioxide was passed through $CaSO_4$ and bubbled into the solution for 2 h, while stirring at room temperature. After stirring an additional 6 h at room temperature, the solution was extracted twice with 5% NaOH. The aqueous layer was acidified with solid citric acid, and extracted three times with ethyl acetate. The combined organic extracts were washed with brine, dried over Na_2SO_4 and evaporated to a red-yellow residue (3.62 g, 60% 3:1 **45:46** by ^1H NMR). The mixture was used without further purification. Physical data for **45**: IR (KBr) 3377, 2957, 1698, 1593, 1552, 1383 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz,) δ 10.38 (br s, 1 H), 8.08 (d J = 8.0 Hz, 1 H), 7.51 (t J = 8.0 Hz, 1 H), 6.88 (d J = 8.0 Hz, 1 H), 4.06 (s, 3 H), 1.51 (s, 9 H); ^{13}C NMR (DMSO-d_6, 100.6 MHz) δ 170.56, 157.26, 152.27, 137.59, 128.52, 118.37, 110.77, 106.13, 79.03, 55.75, 28.05; MS (FAB$^+$) *m/z* 268, (M$^+$+1) (4.4), 194 (30.8), 176 (56.7), 161 (100); **16**: ^1H NMR (DMSO-d_6, 400 MHz) δ 10.70 (br s, 1 H), 8.10 (d, J = 2.0 Hz, 1 H), 8.01 (d J = 8.0 Hz, 1 H), 6.65 (dd, J = 8.0, 2.0 Hz, 1 H), 3.88 (s, 3 H), 1.52 (s, 9 H).

N-(*t*-Butoxycarbonyl)-6-Methoxyanthranilamide, 50. To a stirring mixture of acids **45** and **46** (595 mg; 445 mg **45**, 1.67 mmol) in CH_2Cl_2 at -20°C was added sequentially Et_3N (0.90 mL, 6.7 mmol) and then, after 10 min, the methyl chloroformate (0.70 mL, 8.9 mmol). The cloudy

solution was stirred for 5 min, and then NH_3 was bubbled into the solution for 5 min. After stirring for another 10 min at room temperature, the cloudy mixture was washed with $NaHCO_3/H_2O$, sat. brine, and dried over Na_2SO_4. Removal of the volatiles at reduced pressure yielded a yellow-brown oil. Chromatography on silica gel (2.5 x 16 cm) eluting with 5% $CH_3OH/CHCl_3$ yielded a light tan solid residue of **50** (406 mg, 91% from **45**) after removal of the solvent. The residue was crystallized from CH_2Cl_2/hexanes: mp. 145°-146°C; IR (KBr) 3475, 2984, 1707, 1600, 1160, 1027 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz,) δ 11.22 (br s, 1 H), 8.02 (d, J = 8.2 Hz, 1 H), 7.91 (br s, 1 H), 7.37 (t, J = 8.2 Hz, 1 H), 7.11 (br s, 1 H), 6.76 (d, J = 8.2 Hz, 1 H), 3.94 (s, 3 H), 1.48 (s, 9 H); ^{13}C (acetone-d_6, 100.6 MHz,) δ 170.19, 159.30, 153.45, 143.49, 132.98, 112.60, 109.18, 105.61, 80.13, 56.52, 28.41; MS (FAB $^+$) *m/z* 267 (M^++1), 194 (65), 167 (100); HR MS Calc. for $C_{13}H_{18}N_2O_4$: 267.13457. Found: 267.13447.

6-Methoxyanthranilamide, 49. The amide **50** (300 mg, 1.13 mmol) was stirred in acetic acid (2.0 mL) and 6N HCl (2.0 mL) at room temperature for 2 h. After the reaction was neutralized carefully with saturated $NaHCO_3$, it was extracted three times with ethyl acetate. The organic layer was washed with sat. brine, dried over Na_2SO_4, and concentrated *in vacuo* to yield 177 mg (95%) of a light yellow powder. A small amount was crystallized from $CHCl_3$/hexanes to yield light yellow crystals: mp. 151.5°-152 °C; IR (KBr) 3465, 1640, 1607, 1400, 1257, 1140 cm^{-1}; ^1H NMR (CD_3CN, 400 MHz) δ 7.54 (br s, 1 H), 7.08 (t, J = 8.1 Hz, 1 H), 6.35-6.20 (br s, 2 H), 6.32 (d, J = 8.1 Hz, 1 H), 6.24 (d, J = 8.1 Hz, 1 H), 6.07 (br s, 1 H), 3.83 (s, 3 H); ^{13}C NMR (CD_3CN, 100.6 MHz) δ 171.10, 160.47, 153.38, 133.01, 111.05, 104.62, 99.43, 56.56; MS (70 ev) *m/z* 166 (98.1), 149, (100), 122 (67.5), 107 (64.4); Anal. Calc. for $C_8H_{10}N_2O_2$: C, 57.82, H, 6.07; N, 16.86. Found: C 57.52; H, 5.84; N, 16.57.

6-Hydroxyanthranilamide, 27. To a solution of methoxy amide **49** (100 mg, 0.60 mmol) in DMF (4 mL) was added LiSMe (97 mg, 1.79 mmol) and the mixture stirred at 80°C for 6 h. The DMF was removed under high vacuum, whereupon the oily residue was dissolved in methanol (5 mL) and diluted with ethyl acetate. The solution was washed with saturated NH_4Cl,

the water followed by sat. brine. After drying the organic layer over Na_2SO_4, and concentrating *in vacuo*, a light tan powder was obtained. The residue was chromatographed on Silicar CC-4 (1.5 x 10 cm) eluting with 5% $MeOH/CHCl_3$ to yield 64 mg (70%) of a light tan powder, after removal of the solvent. An unidentified aromatic impurity (10%) was present, as evidenced by a 3 proton aromatic system in the ^1H NMR spectrum: 7.75 (t, J = 8.0 Hz), 7.10 (d, J = 8.0 Hz), 6.85 (d, J = 8.0 Hz). A small sample (10 mg) was purified by HPLC, (Alltech Econosphere C-18 reversed phase column, (4.6 x 250 mm, 5 μm) connected to a precolumn packed with C-18,30-40 μm, material) eluting at 1.0 mL/min with 85:15 H_2O/MeOH, and 0.15 M H_3PO_4. Detection was by UV at 254 nm. Elution of **27** occurred at 6.6 min, while the impurity apparently decomposed during the separation. The aqueous eluant containing **27** was neutralized with solid $NaHCO_3$, and then extracted three times with ethyl acetate. The organic layers were washed with sat. brine, and then dried with Na_2SO_4, followed by evaporation of the solvent at reduced pressure to yield a light brown powder (8 mg): mp 134°-135 °C; IR (KBr) 3324, 1640, 1616, 1577, 1242 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 9.73 (s, 1 H), 7.57 (br s, 2 H), 6.88 (t, J = 8.0 Hz, 1 H), 6.64 (br s, 2 H), 6.16 (d, J = 8.0 Hz, 1 H), 6.04 (d, J = 8.0 Hz, 1 H); ^{13}C NMR (acetone-d_6, 100.6 MHz) δ 170.35, 157.62, 152.09, 131.35, 107.77, 102.54, 102.53; UVmax (c = 8.223 x 10^{-5} M, MeOH) 330 nm (5350), 233 nm (23000), 211 (19000); MS: (70 ev)*m/z* 152 (67.8), 135 (100), 107 (46.9), 79 (29.8); HRMS calc. for $C_7H_8N_2O_2$: 152.05864. Found: 152.05820.

N-(2,2-Dimethyl-1-oxo-propyl)-6-Methoxyanthranilic Acid. The reactions were carried out as previously described.[17] To a solution of *m*-anisidine (10.5 mL, 93.5 mmol) in ethyl acetate was added 5% $NaHCO_3$ (75 mL) followed by pivaloyl chloride (12.15 mL, 95 mmol). The 2-phase solution was stirred overnight, and then separated. The organic layer was washed with 1N HCl, H_2O, and then sat. brine. Drying of the organic layer with $MgSO_4$ followed by concentration of the solvent at reduced pressure yielded a yellow residue, which was then crystallized from CH_2Cl_2/hexanes to yield 16.24 g (89.5%) of **41** as fluffy white crystals: mp. 126°-126.5 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.39 (t, J = 2.2 Hz, 1 H), 7.35 (br s, 1

H), 7.19 (t, J = 8.0 Hz, 1 H), 6.94 (ddd, J = 7.8, 2.2 1.1 Hz, 1 H), 6.66 (ddd, J = 8.1, 2.2, 1.1 Hz, 1 H), 3.80 (s, 3 H), 1.31 (s, 9 H); ^{13}C NMR (($CDCl_3$, 100.6 MHz) δ 176.61, 160.11, 139.26, 129.49, 111.76, 110.34, 105.22, 55.25, 39.63, 27.56.

To a solution of **12** (5.70 g, 27 54 mmol) in dry THF under Ar at 0°C was added *n*-BuLi (2.4M 25.2 mL, 60.58 mmol). After stirring for 2 h at O °C the yellow solution was quenched by bubbling CO_2 through the solution for 3 h. The mixture was extracted twice with 5% NaOH, acidified to pH 3 with 6N HCl, and extracted 3 times with ethyl acetate. The organic layer was washed with sat. brine, dried over Na_2SO_4 and concentrated to a yellow oil. Crystallization was accomplished from MeOH/H_2O to yield 6.67 g (96.5%) of the protected amino acid as pale yellow crystals: mp. 115°-115.5°C; IR (KBr) 3255-3120, 2975, 1700, 1685, 1652, 1559, 1507, 1473, 1384, 1161 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 11.97 (br s, 1 H), 11.33 (br s, 1 H), 8.37 (dd, J = 8.4, 0.9 Hz, 1 H), 7.53 (t, J = 8.4 Hz, 1 H), 6.95 (d, J = 8.1 Hz, 1 H), 4.07 (s, 3 H), 1.28 (s, 9 H); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 177.61, 169.29, 159.95, 143.50, 134, 87, 114.42, 106.98, 106.93, 57.38, 40.86, 27.67; MS (70 ev) *m/z* 251 (M^+, 24), 194 (20), 176 (442), 149 (100), 61 (28), 57 (51); Anal. Calc. for $C_{13}H_{17}NO_4$: C, 62.15; H, 6.77; N, 5.58. Found: C, 61.83; H, 6.69; N, 5.92.

6-Methoxyanthranilic acid, 47. The N-pivaloyl protected **53** (1.50 g, 5.98 mmol) was dissolved in acetic acid (7 mL), followed by 48% HBr (7 mL), and then stirred under N_2 for 3 days at 40 °C. The volatiles were removed at 40 °C under high vacuum and the product crystallized from MeOH/Et_2O to yield 1.22 g (82.3%) of **47** (as the hydrobromide salt) as light brown crystals: mp. 157°-157.5 °C; IR (KBr) 3200-3000, 1700, 1621, 1550, 1473, 1285, 1223, 811 cm^{-1}; ^1H NMR (CD_3OD, 300 MHz) δ 7.61 (t, J = 8.5 Hz, 1 H), 7.25 (d, J = 8.5 Hz, 1 H), 7.07 (d, J = 8.5 Hz, 1 H), 3.93 (s, 3 H); ^{13}C NMR (CD_3OD, 75.4 MHz) δ 167.75, 160.84, 134.70, 133.84, 116.61, 116.44, 113.22, 57.17; HRMS Calc. for $C_8H_{10}NO_3$ (M^++1): 168.06610. Found: 168.06659.

6-Hydroxyanthranilic acid, 17. The acid **47** (495 mg, 2.00 mmol) was dissolved in DMF (11 mL) and LiSMe (650 mg, 12.00 mmol) added. After heating at 95°C for 24 h, the DMF was removed *in vacuo*. The residue was taken up in 5% NaOH (25 mL). This was washed with ethyl acetate then applied to a Dowex 1X-4 (200-400 mesh, 1.5 x 8 cm). The column was washed

with water (30 mL), and eluted with 1M NH_4Cl pH 8.0 (1 ml fractions at 1 ml/min). The starting material (25 mg) was eluted within 8 fractions. The column was next washed with water (30 mL), then eluted with 0.5N HCl. The fractions containing the amino acid were pooled and extracted exhaustively with ethyl acetate, then dried over Na_2SO_4 overnight. Concentration yielded 235 mg (55.3%) of **17**, as the acetic acid salt: mp 144°- 144.5 °C; IR (KBr) 3300, 1680, 1670, 1635, 1555, 1440, 1180 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 7.03 (t, J = 8.0 Hz, 1 H), 6.19 (dd J = 8.0, 1.1 Hz, 1 H), 6.00 (br d J = 8.1 Hz, 1 H), 1.90 (s, 3 H); ^{13}C (CD_3OD, 100.6 MHz) δ 172.87, 172.01, 162.63, 150.53, 134.47, 107.09, 103.33, 98.42, 21.04; MS (70 ev) *m/z* 153, M^+ (70), 135 (100), 107 (95), 79 (79); HRMS Calc. for $C_7H_7NO_3$: 153.04261. Found: 153.04260.

Methyl [(N-Acetyl)-6-Methoxy]-Anthranilate, 54. The 6–hydroxyanthranilic acid derivative, **54**, was prepared as described in the literature[17] mp: 118°-119 °C (Lit. 118°-119 °C).

N-Acetyl-6-Methoxyanthranilamide, 55.
6-Hydroxyanthranilamide, **27**, was dissolved in acetic anhydride (2 mL) and Et_3N (2 mL) and stirred at RT overnight. The solution was distilled under vacuum and the residue dissolved in methanol (5 mL). Freshly prepared ethereal diazomethane was added until bubbling ceased, and the mixture was stirred for 1 h. Evaporation of the volatiles *in vacuo* yielded a white powder, which was crystallized from water to yield **55** as colorless crystals: mp 174°-174.5 °C; UV (c = MeOH); ^1H NMR ($CDCl_3$, 400 MHz) δ 12.09 (br s, 1 H), 8.32 (d, J = 8.0, 1 H), 7.85 (br s, 1 H), 7.40 (t, J = 8.0, 1 H), 6.70 (d, J = 8.0, 1 H), 6.26 (br s, 1 H), 3.94 (s, 3 H), 2.19 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 170.06, 169.14, 158.19, 142.59, 132.96, 114.35, 107.51, 105.80, 56.25, 25.53; MS (70 ev) *m/z* 208 (89), 176 (27), 166 (78), 149 (100), 122 (28), 107 (18); Anal. Calc. for $C_{10}H_{12}N_2O_3$: C, 57.67; H, 5.81; N, 13.46. Found: C, 57.59; H, 5.82, N, 13.33.

Labeled Syntheses

[$^{13}CO_2H$]-N-(*t*-Butoxycarbonyl)-6-Methoxyanthranilic Acid, 45a. Into a flask was placed $Ba^{13}CO_3$ (99% ^{13}C, 4.44 g, 22.42 mmol) and fitted with a dropping funnel filled with conc. H_2SO_4 which was in turn

fitted with a vacuum adapter and a U-tube filled with $CaSO_4$. This was fitted to another flask charged with the urethane **43** (5.00 g, 22.42 mmol) in anhydrous THF (175 mL). The apparatus was purged with N_2 and the urethane solution cooled to -78 °C. *t*-BuLi (1.40 M, 40 mL, 55 mmol) was added and the solution stirred for 15 min, then warmed to -20 °C, and stirred for 2.5 h. The yellow solution was frozen with liquid N_2 and evacuated to 0.25 mm Hg, and the system closed to the vacuum pump. Generation of $^{13}CO_2$ was accomplished by careful addition of H_2SO_4 to the flask containing the $Ba^{13}CO_3$. When CO_2 evolution had ceased, the THF solution was allowed to warm until just thawed, and then refrozen. The mixture was next allowed to warm to room temperature over 6 h. Workup of the reaction mixture was carried out in an identical fashion as for the unlabeled material to yield 3.27 g (54.7% overall) of a 3:1 mixture of isomers **45a** and **46a** (by ^1H NMR). TLC analysis and ^1H NMR data were identical with that of unlabeled **45** and **46** described above. The mixture was used without any further purification.

[^{13}C-^{15}N-Carboxamide]-N-(*t*-Butoxycarbonyl)-6-Methoxyanthranilamide, 50a.

Generation of $^{15}NH_3$ from $^{15}NH_4Cl$. A flask fitted with a reflux condensor was filled with $^{15}NH_4Cl$ (99% ^{15}N, 415 mg, 7.46 mmol), dissolved in water (1 mL) and purged with N_2. NaOH (3 g) in water (5 mL) was added and the mixture heated to 110 °C. The $^{15}NH_3$ generated was swept with N_2 for 35 min through solid NaOH and into an anhydrous THF solution (25 mL) which was cooled to -78 °C.

Synthesis of **50a**. To a 3:1 mixture of acids **45a** and **46a** (2.00 g, 1.50 g **45a**, 5.6 mmol) in dry THF (60 mL), cooled to -20°C, was added Et_3N (3.12 mL, 22.4 mmol) followed by $ClCO_2Me$ (2.31 mL, 29.84 mmol). After stirring for exactly 5 min the previously prepared $^{15}NH_3$ solution, which was warmed to -42 °C, was transferred via canula into the solution containing the activated acids, and the resulting white slurry stirred at -20 °C for 30 min, and then at RT for an additional 20 min. The product **50a** was isolated as described for **50** to yield from the silica gel column 952 mg (63.5%) of a light tan powder: mp 145°-146°C; The ^1H NMR and ^{13}C NMR spectra were identical to that described for **50** except that the now intense carboxamide

resonance at δ 170.19 was split into a doublet J = 15.1 Hz centered on 170.17 ppm.

[^{13}C-^{15}N-Carboxamide]-6-Methoxyanthranilamide, 49a. The doubly labelled **50a** (300 mg, 1.12 mmol) was dissolved in glacial acetic acid (2 mL) and 6N HCl (2 mL), and then stirred at room temperature for 2h. Workup of the mixture was accomplished as for **49** to give 178 mg (94.7%) of a white powder: mp 151°-152°C; MS (70 ev) *m/z* 168 (83), 150 (100), 136 (4), 123 (34), 108 (38). The rest of the physical data were identical with **49** except the carboxamide ^{13}C resonance was a doublet of J = 15.1 Hz centered on 171.08 ppm.

[^{13}C-^{15}N Carboxamide]-6-Hydroxyanthranilamide, 27a. To a solution of doubly labelled methoxy amide **49a** (160 mg, 0.95 mmol) in DMF (4 mL) was added LiSMe (205 mg, 3.81 mmol), and the mixture stirred at 80°C for 6 h. The mixture was worked up in an identical manner to that of **27** to yield, after silica gel column chromatography, 90 mg (61.5%) of a white powder. This was used without further purification for the feeding experiment. The physical data (IR, ^{1}H NMR, ^{13}C NMR) were identical to **27** except for the ^{13}C carboxamide resonance was a doublet of J = 15.1 Hz centered at 170.34 ppm. MS (70 ev) *m/z* 154 (62), 136 (100), 108 (62), 79 (44). An unidentified impurity (10% by ^{1}H NMR analysis) was present as in the unlabeled material.

Methyl (2,5-Dimethoxy)-benzoate, 57. To a solution of 2,5-dihydroxybenzoic acid (29.00 g, 188.0 mmol) in ethanol (65 mL) and 50% aqueous KOH (25 mL) was added alternately dimethyl sulfate (145 mL, 1530 mmol) and 50% aqueous KOH (150 mL) while maintaining the temperature at 50-60 °C. After addition was complete the mixture was heated to 95 °C for 5 min to hydrolyze any excess dimethyl sulfate. The mixture was cooled to 0 °C, diluted with water and extracted with ether. The organic layer was washed with 1N NaOH, water, brine, dried over $MgSO_4$ and concentrated *in vacuo* to yield a light yellow, clear oil. Distillation (106-107 °C, 0.7 mm Hg) produced 33.03 g (89.9%) of **57**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.31 (d, J = 3.0, Hx, 1 H), 7.00 (dd, J = 8.9, 3.0 Hz, 1 H), 6.90 (d, J = 8.9 Hz, 1 H), 3.87 (s, 3 H), 3.84 (s, 3

H), 3.77 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 166.44, 153.42, 152.98, 120.49, 119.49, 115.92, 113.83, 56.70, 55.76, 52.00; MS (70 ev) *m/z* 196, M^+ (100), 181 (58), 165 (72), 150 (30), 129 (31), 86 (61); HRMS Calc. for $C_{10}H_{12}O_4$: 196.07356. Found: 196.07350.

2,5-Dimethoxybenzamide. Methyl 2,5-Dimethoxybenzoate (15.55 g, 80.0 mmol) was dissolved in concentrated NH_4OH (95 mL) and stirred overnight at room temperature. The white mixture was cooled to 0°C, filtered, the white residue washed with cold water, and crystallized from hot water to give 13.79 g (96.0%) of white needles of the amide: mp: 141-142 °C; ^1H NMR (acetone-d_6, 400 MHz) δ 7.87 (br s, 1 H), 7.75 (d, J = 3.2 Hz, 1 H), 7.02 (dd, J = 9.0, 3.2 Hz, 1 H), 6.92 (d, J = 9.0 Hz, 1 H), 6.44 (br s, 1 H), 3.92 (s, 3 H), 3.82 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 166.91, 153.77, 152.14, 121.31, 120.00, 115.60, 112.99, 56.43, 55.78; MS (70 ev) *m/z* 181 (100), 165 (25), 135 (44); HRMS Calc. for $C_9H_{11}NO_3$: 181.07393. Found: 181.07393.

2,5-Dimethoxy-6-Nitrobenzamide, 56. To 2,5-dimethoxybenzamide (13.53 g, 74.75 mmol) was added (precooled to -25°C) 70% HNO_3 (240 mL). The mixture was allowed to stir to 15 °C over 50 min whereupon the yellow slurry was poured into cold water, and filtered. Washing of the yellow residue with water followed by crystallization from boiling acetone to yield 15.36 g (91.8%) of **56** as yellow needles: mp 225-226 °C; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.90 (br s, 1 H), 7.75 (br s, 1 H), 7.33 (d, J = 9.3 Hz, 1 H), 7.28 (d, J = 9.3 Hz, 1 H), 3.82 (s, 3 H), 3.80 (s, 3 H); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 163.44, 149.33, 144.07, 139.11, 120.88, 115.43, 115.14, 57.09, 56.76; IR (KBr) 3382, 1654, 1531, 1370, 1270, 1054 cm^{-1}; MS (70 ev) *m/z* 226 (100), 179 (38.9), 150 (62.5), 135 (38.2), 120 (92.8), 95 (67.2), 76 (73.7); Anal. Calc. for $C_9H_{10}N_2O_5$: C, 47.79; H, 4.46; N, 12.38. Found: C, 47.49; H, 4.38; N, 12.26.

3,6-Dimethoxyanthranilamide, 58. To a solution of 2,5-dimethoxybenzamide, **56**, (2.91 g, 12.9 mmol) in methanol (250 mL) was added 10% Pd/charcoal (100 mg), and the solution stirred under hydrogen for 3 h, until H_2 uptake had ceased. The catalyst was filtered, and the clear, colorless solution evaporated *in vacuo* to yield a purple-white powder. Crystallization from CH_2Cl_2/hexanes gave 2.43 g (96.5%) of **58** as colorless

crystals: mp 129-130 °C; ^{1}H NMR (CD_3OD, 400 MHz) δ 6.81 (d, J = 8.9 Hz, 1 H), 6.20 (d, J = 8.9 Hz, 1 H), 3.81 (s, 3 H), 3.79 (s, 3 H); ^{13}C NMR (DMSO-d_6, 100.6 MHz) δ 169.50, 152.35, 141.42, 141.18, 111.73, 104.83, 96.25, 55.88, 55.72; IR ($CHCl_3$) 3530, 3400, 3000, 1640, 1610, 1570, 1240 cm^{-1}; MS (70 ev) *m/z* 196 (100), 179 (28.1), 164 (66.4), 136 (31.5), 89 (15.8), 61 (26.2); Anal. Calc. for $C_9H_{12}N_2O_3$: C, 55.10; H, 6.12; N, 14.29. Found: C, 55.33; H, 5.95; N, 14.17.

6-Hydroxy-3-Methoxyanthranilamide, 62. To a solution of **58** (1.60 g, 8.2 mmol) in dry DMF (70 mL) was added lithium thiomethoxide (1.81 g, 8.21 mmol), and the solution stirred at 80 °C for 14 h. The volatiles were removed at aspirator pressure, and the residue dissolved in methanol, then diluted with ethyl acetate, Saturated NH_4Cl was added immediately, and the phases separated. Washing of the organic layer with sat. brine, followed by drying over Na_2SO_4,and concentration of the volatiles *in vacuo* yielded a green oil. Crystallization from ethyl acetate produced 1.28 g (86.0%) of a light green powder: mp 158-159 °C; IR (KBr) 3400, 3180, 1641, 1618, 1576, 1453, 1406, 1223, 1133, 823 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.11 (s, 1 H), 7.49 (br s, 2 H), 6.69 (d, J = 8.6 Hz, 1 H), 6.42 (br s, 2 H), 6.00 (d, J = 8.6 Hz, 1 H), 3.68 (s, 1 H); ^{13}C NMR (DMSO-d_6, 100.6 MHz) δ 170.19, 150.83, 141.70, 139.99, 113.30, 102.63, 100.14, 56.05; MS (70 ev) *m/z* 182 (85), 165 (97), 150 (100), 122 (29), 94 (25); HRMS Calc. for $C_8H_{10}N_2O_3$: 182.06920. Found: 182.06920.

3-Methoxy-6-(2-Propenoxy)-Anthranilamide, 64. To a solution of **62** (1.73 g, 9.51 mmol) in DMF (100 mL) was added powdered K_2CO_3 (2.36 g, 17.10 mmol) followed by allyl bromide (0.812 mL, 10.46 mmol). The green mixture was stirred for 3 h at room temperature. Dilution of the mixture was accomplished with ethyl acetate, followed by washing with sat. $NaHCO_3$, and then sat. brine. The organic layer was dried over Na_2SO_4 and concentrated to a dark oil. Crystallization from CH_2Cl_2/hexanes yielded 610 mg of **65** as brown crystals. Passage of the mother liquor through a silicar column eluting with 1:3 ethyl acetate/CH_2Cl_2, followed by crystallization, gave an additional 285 mg. The combined yield was 854 mg (82.9%): mp 119-120 °C; IR (KBr) 3400, 3000, 1651, 1634, 1557, 1404, 1257 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.92 (br s, 1 H), 6.69 (d, J = 8.5 Hz, 1 H), 6.54 (br s, 1 H), 6.09 (d, J = 8.5 Hz, 1 H), 6.05 (m, 1 H), 5.42 (ddd, J = 16.9, 1.4, 1.2 Hz, 1 H), 5.32 (ddd, J

= 10, 1.4, 1.2 Hz, 1 H), 4.55 (dt, J = 6, 1.2 Hz, 2 H), 3.82 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 170.61, 152.14, 143.21, 142.31, 132.69, 118.63, 111.59, 103.42, 97.79, 70.43, 55.99; MS (FAB^+) m/z 223 (M^++1); UVmax (c = 2.97 x 10^{-5} M, MeOH) 343.2 nm (4,280), 254.4 (sh, 6673), 233.6 (14,593), 209.6 (16851); HRMS Calc. for $C_{11}H_{14}N_2O_3$: 222.10052. Found: 222.10050.

6-Hydroxy-3-Methoxy-5-(2-Propenyl)-Anthranilamide, 65. The allyl ether **64** (400 mg, 1.80 mmol) was dissolved in ethylene glycol (45 mL) and heated to 145 °C for 20 min whereupon the dark brown solution was poured into CH_2Cl_2 and 100 ml water added. The aqueous phase was extracted with CH_2Cl_2 3 times, the organics were combined, washed with water, brine, and then dried over Na_2SO_4. Removal of the solvent *in vacuo* provided a single compound as a brown cake in 99% yield which was greater than 95% pure by ^1H NMR analysis. Attempts at crystallization led to some decomposition to an unidentifiable product. This was therefore used without further purification: mp 86°-88 °C; ^1H NMR ($CDCl_3$, 300 MHz) δ 11.75 (s, 1 H), 7.12 (br s, 2 H), 6.79 (s, 1 H), 6.00 (m, 1 H), 5.10 (m, 1 H), 5.05 (m, 1 H), 4.12 (br s, 2 H), 3.81 (s, 3 H), 3.35 (dt, J = 6.5, 1.2 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 172.92, 153.10, 141.26, 136.88, 133.61, 118.47, 117.47, 115.48, 105.15, 56.73, 33.67; MS (FAB^+) *m/z* 223 (M^++1); HRMS Calc. for $C_{11}H_{14}N_2O_3$: 222.10052. Found: 222.10052.

6-Hydroxy-3-Methoxy-5-Propylanthranilamide, 66. The crude allyl anthranilamide **65** (400 mg, 1.80 mmol) was dissolved in methanol (30 mL) and a catalytic amount of 10% Pd/C added in a base-washed flask. The flask was placed on a hydrogenation apparatus at 1 atmosphere and stirred for 6 h, until hydrogen uptake had ceased. Filtration of the catalyst and removal of the solvent *in vacuo* provided a brown cake (395 mg). Decolorization by passage through a short Silicar column and crystallizization from CH_2Cl_2/hexanes yielded 375 mg (95.5%) of colorless crystals: mp 115-116 °C. IR ($CHCl_3$) 3480, 2962, 1646, 1625, 1459, 1229 cm^{-1}; ^1H NMR (CD_2Cl_2, 300 MHz) δ 11.96 (s, 1 H), 7.05 (br s, 2 H), 6.82 (s, 1 H), 4.04 (br s, 2 H), 3.80 (s, 3 H), 2.51 (t, J = 7.8 Hz, 2 H), 1.55 (tq, J = 7.8, 7.3 Hz, 2 H), 0.92 (t, J = 7.3 Hz, 3 H); ^{13}C NMR (CD_2Cl_2, 75.4 MHz) δ 173.40, 153.86, 141.48, 133.41, 121.64, 118.26, 105.43, 57.16, 31.97, 23.30, 14.09; MS (70 ev) *m/z* 224 (36.3), 207 (93.4), 192 (80.7), 178 (100), 163 (24.6), 135 (17), 107 (10);

UVmax (c = 1.52 x 10^{-4} M, MeOH) 337.6 nm (3,676) 241.6 (12,478), 262.4 (sh, 6,336), 205.6 (11,565); Anal. Calc. for $C_{11}H_{16}N_2O_3$: C, 58.91; H, 7.19; N, 12.49. Found: C, 59.16; H, 7.12; N, 12.17.

2-Amino-3-Carboxamido-5-Propyl-1,4-Benzoquinone, 67. To a solution of the phenol **66** (100 mg, 0.446 mmol) in acetonitrile (25 mL) was added ammonium cerium (IV) nitrate (492 mg, 0.897 mmol) in water (5 mL) over 30 s. The intense orange solution was diluted with ethyl acetate, washed 2 times with sat. brine, and then dried over Na_2SO_4. Removal of the solvents under reduced pressure yielded a darl. red solid which was passed through a silicar column, eluting with 60:4C ethyl acetate/hexanes. The fractions containing the quinone were combined, and the solvent removed *in vacuo* to yield 90 mg (97.0%) of a deep red solid: mp 139-140 °C; IR ($CHCl_3$) 3488, 3424, 1647, 1590, 1551, 1345 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.25 (br s, 2 H), 6.61 (s, 1 H), 4.10 (br s, 2 H), 2.45 (t, J = 7.0 Hz, 2 H), 1.56 (qt, J =7.4 Hz, 7.0; 2 H)), 0.97 (t, J = 7.4 Hz, 3 H); ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 182.94, 181.91, 169.69, 154.26, 153.07, 128.34, 98.55, 31.50, 21.15, 13.68; UVmax (c = 9.57 x 10^{-5} M, MeOH) 447 nm (1,065), 325.0 (1,055), 264 (8,807), 208 (10,300); MS (70 ev) *m/z* 208 (11.6), 163 (100), 148 (23.4), 106 (19.2), 68 (35.1); HRMS Calc. for $C_{10}H_{12}N_2O_3$: 208.08486. Found: 208.08480.

Reduction and Reoxidation of Quinone 67.

In $CHCl_3$. The quinone **67** (8.0 mg 0.0385 mmol) was dissolved in $CHCl_3$ and methanol (1.0 mL each) and PtO_2 (0.5 mg) added to the intense red solution. The flask was purged with H_2, and stirred for 30 min. The light yellow mixture that resulted was stored under H_2 for 48 h. A small aliquot was removed for TLC analysis after 2 h. App ication to silica gel resulted in the appearance of a red spot, indicating reoxidation of the substrate. The quinone was apparent (rf 0.38, 2:1 ethyl acetate/$CHCl_3$) as the only spot. At 48 h, the vessel was purged with O_2, and the dirty yellow solution instantly turned red. Filtration and concentration yielded the quinone **67**.

With $Na_2S_2O_4$ in DMSO-d_6/D_2O For NMR Analysis of 68. To a solution of **67** in DMSO-d_6 (500 μL) in an A purged 5 mm NMR tube was

added $Na_2S_2O_4$ (14.0 mg, 0.0804 mmol) dissolved in D_2O (100 μL). The tube was sealed, vortexed and a light yellow solution resulted. At 4 h a ^{13}C NMR (75.4 MHz) spectrum was obtained. The solution was kept at room temperature for 1 week, during which ^{13}C NMR spectra were obtained. After 7 d, the solution was poured into ethyl acetate. A red solution resulted, which was washed with sat. brine, and dried over Na_2SO_4. Concentration of the solution *in vacuo* yielded the quinone **67**, which was contaminated with a small amount of origin material and a faint spot at lower R_f when analyzed by TLC. (R_f 0.50, impurity R_f 0.44, 3:1 ethyl acetate/hexane). ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 172.29, 162.20, 149.73, 143.32, 120.09, 113.61, 98.02, 31.7, 23.6, 14.61, (impurities: 72.61, 19.25, 15.07).

2,3-Dihydro-5,8-Dimethoxy-2,2-Dimethylquinazolin-4-(1H)-One, 69. The amide **58** (2.35 g, 12.0 mmol) was dissolved in acetone, (30 mL) *p*-toluenesulfonic acid (5 mg) added, and the solution was stirred overnight at room temperature. The solvent was removed under reduced pressure and the white powder crystallized from $CHCl_3$/acetone/hexane to yield 2.80 g (99.0%) of white crystals: mp: 194.5-195.5 °C; IR (KBr) 3410, 3250, 2941, 1660, 1642, 1517, 1250, 1107, 798 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.79 (d, J = 8.9 Hz, 1 H), 6.22 (d, J = 8.9 Hz, 1 H), 6.16 (br s, 1 H), 4.75 (br s, 1 H), 3.86 (s, 3 H), 3.81 (s, 3 H), 1.52 (s, 6 H); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 163.04, 154.73, 140.29, 138.59, 113.83, 103.75, 99.42, 66.37, 56.12, 55.93, 29.18; MS (70 ev) *m/z* 236 (34), 221 (100), 206 (13), 191 (23), 180 (14), 164 (10), 150 (15), 122 (8), 91 (15); UVmax (c = 7.66 x 10^{-5} M, MeOH) 352.0 nm (3,786), 278.4 (3,603), 235.2 (15,131), 216.8 (22,220); HRMS Calc. for $C_{12}H_{16}N_2O_3$: 236.11618. Found: 236.11620.

6-Bromo-2,3-Dihydro-5,8-Dimethoxy-2,2-Dimethylquinazolin-4-(1H)-One, 70. The acetaminal **69** was dissolved in CH_2Cl_2 (35 mL) and Br_2 in acetic acid (6.49 M, 1.50 mL, 9.74 mmol) added over 2 min. Complete reaction could be observed by the appearance of a dark color when the Br_2 was in excess. The brown solution was stirred for an additional 5 min, then poured slowly into a saturated solution of $Na_2S_2O_3$ and saturated $NaHCO_3$. Extraction of the aqueous mixture into CH_2Cl_2, followed by washing of the organic layer with water, sat. brine, and then

drying over $MgSO_4$ yielded a dark oil after re noval of the solvent *in vacuo*. Analysis of the mixture by ^1H NMR revealed partial hydrolysis of the acetaminal group during workup, so the residue was dissolved in acetone (10 mL), *p*-toluenesulfonic acid (5 mg) added and the solution stirred overnight. Hexane (5 mL) was added and the solution was then cooled slowly to -78 °C to yield 2.72 g (90.3%) of white crystals: mp 197-198 °C; IR (KBr) 3200, 2930, 1670, 1598, 1506, 1356, 1228, 1071, 994 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.95 (br s, 1 H), 7.08 (s, 1 H), 6.28 (br s, 1 H), 3.78 (s, 3 H), 1.36 (s, 6 H); ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 160.51, 150.23, 143,06, 138.67, 116.66, 108.02, 102.32, 66.58, 61.00, 56.14, 28.25; MS (70 ev) *m/z* 314 (23), 299 (100), 286 (25), 271 (28), 230 (22), 221 (20), 91 (78); UVmax (c = 2.68 x 10^{-5} M, MeOH) 356.8 nm (3,055), 299.2 (3,502), 236.0 (21,052), 224.0 nm (23,027); HRMS calc. for $C_{12}H_{15}BrN_2O_3$: 314.02665. Found: 314.02650.

2,2-Dimethyl-5,8-Dimethoxy-1,2,3,4-Tetrahydro-4-Oxoquinazoline-6-Carboxaldehyde, 71. To a solution of **70** (1.25 g, 3.97 mmol) in dry THF over Ar cooled to -78 °C was added tetramethylethylenediamine (1.80 mL, 11.92 mmol), followed by *n*-butyllithium (1.6 M in hexanes 5.5 mL, 8.74 mmol). After 10 min, *t*-butyllithium (1.7 M in pentane, 7.0 mL, 11.92 mmol) was added and the canary-yellow heterogeneous mixture stirred at -78 °C for 2.5 h. The mixture was quenched with N,N-dimethylformamide (3.1 mL, 40 mmol) and the mixture allowed to stir to room temperature overnight. The mixture was carefully quenched by the addition of ethanol (5 mL). The mixture was diluted with ethyl acetate and washed with saturated $NaHCO_3$, follwed by water, and then sat. brine. Drying over Na_2SO_4 and evaporation *in vacuo* yielded a yellow powder. Trituration with acetone (3 mL) gave a yellow powder (830 mg, 79.1%) which was analytically pure: mp 275.5-277 °C; IR (KBr) 3255, 3180, 2970, 1675, 1655, 1613, 1522, 1405, 1230, 993; ^1H NMR (DMSO-d_6, 300 MHz) δ 10.03 (s, 1 H), 8.05 (br s, 1 H), 7.38 (br s, 1 H), 7.08 (s, 1 H), 3.82 (s, 6 H), 1.42 (s, 6 H); ^{13}C NMR (DMSO-d_6, 75.4 MHz) δ 186.78, 160.87, 160.43, 144.98, 142.34, 117.02, 108.28, 104.42, 66.83, 63.86, 55.72, 28.62; MS (70 ev) *m/z* 264, M^+ (32), 249 (100), 206 (36), 192 (9), 150 (8), 91 (31); UVmax (c = 4.40 x 10^{-5} M, MeOH) 352.8 nm (10,860), 320.8 (11,860), 277.2 (7,160), 240.4 (16,060);

Anal. Calc. for $C_{13}H_{16}N_2O_4$: C, 59.08; H, 6.10; N, 10.60; Found: C, 59.16; H, 6.03; N, 10.53.

6-(1,2-Dihydro-4-Oxo-4H-Pyran-2-yl)-2,3-Dihydro-5,8-Dimethoxy-2,2-Dimethylquinazolin-4-(1H)-One, 73. To a heterogeneous mixture of **71** (50.0 mg, 0.189 mmol) in anhydrous THF under Ar in a thick-walled conical vial was added 7[illegible] (123 μL, 90%, 0.568 mmol) followed by $ZnCl_2$ (1.0 M in THF, 190 μL, 0.189 mmol). The mixture was sonicated in a water bath at room temperature for 1 h until **71** had dissolved and was no longer detected by TLC (ethyl acetate R_f 0.18) whereupon the homogeneous solution was treated with trace TFA, and poured into a saturated $NaHCO_3$ solution. The aqueous phase was extracted 3 times with ethyl acetate, and the organic layer washed with sat. brine and then dried over Na_2SO_4. Filtration and evaporation of the solvent *in vacuo* yielded a brown powder, which was purified via silica gel chromatography (1.0 X 8 cm) eluting with ethyl acetate yielded 40 mg (64%) of a yellow powder, which was analytically pure by TLC (ethyl acetate, R_f 0.14): IR (KBr) 3200, 2870, 1662, 1656, 1616, 1517, 1509, 1279, 1250, 1222, 1069 cm^{-1}; ^1H NMR (CD_2Cl_2, 300 MHz) δ 7.49 (dd, J = 6.03, 0.80 Hz, 1 H), 6.94 (s, 1 H), 6.37 (br s, 1 H), 5 78 (dd, J = 14.86, 3.25 Hz, 1 H), 5.46 (dd, J = 6.03, 1.37 Hz, 1 H), 4.95 br s, 1 H), 3.86 (s, 3 H), 3 79 (s, 3 H), 2.88 (dd, J = 16.84, 14.87, 1 H), 2.50 (ddd, J = 16.85, 3.30, 1.35, 1 H), 1.53 (s, 6 H); ^{13}C NMR (CD_2Cl_2, 75.4 MHz) δ 192.31, 163.86, 162.21, 152.35, 142.90, 139.60, 119.74, 111.52, 107.25, 107.09, 76.40, 67.32, 63.04, 56.26, 43.36, 29.35, 29.25; UVmax (c = 4.67 x 10^{-5} M, MeOH) 353.6 nm (5,911), 240.0 (24,632), 222.4 (24,546); HRMS (FAB^+) Calc. for 333.14503; Found: 333.14502.

1-[4-(N,N-Dimethylamino)phenyl]-1-Triethylsiloxy-4-Hexene-3-One, 76. A small, thick-walled conical vial was filled with *p*-N,N-dimethylaminobenzaldehyde (50.0 mg 0.335 mmol) and the triethylsiloxy diene **74** (132 mg, 0.670 mmol). After purging with N_2, $ZnCl_2$ (1.0 M in THF, 0.50 mL, 0.503 mmol) was added, and the reaction stirred for 30 min. Periodic analysis by TLC (2:1 ethyl acetate/hexanes R_f 0.51, diene 0.69) showed only starting material. Therefore, the mixture was sonicated for 20 min in a water bath at 20 °C. The reaction was monitored by TLC, and

the reaction was terminated when no more starting material could be detected by dilution with 5% $NaHCO_3$ followed by ethyl acetate. Washing of the organic layer with sat. brine, drying with $MgSO_4$, and concentration of the solution *in vacuo* yielded a dark oil. This was purified by passage through a small silica gel column eluting with 2:1 ethyl acetate/hexanes to yield, after concentration of the volatiles, 74.1 mg (64%) of a light yellow oil: IR (Neat) 2953, 1688, 1675, 1616, 1522, 1350, 1073, 743 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.22 (d, J = 8.48, 1 H), (6.83 dq, J = 17.37, 6.92 Hz, 1 H), 6.68 (d, J = 8.45 Hz, 1 H), 6.12 (dq, J = 17.39, 1.50 Hz, 1 H), 5.13 (dd, J = 8.60, 4.10 Hz, 1 H), 3.07 (dd, J = 16.69, 8.77 Hz, 1 H), 2.94 (s, 6 H), 2.59 (dd, J = 14.69, 4.30 Hz, 1 H), 1.88 (dd, J = 6.92, 1.51 Hz, 1 H), 0.82 (t, J = 7.72 Hz, 9 H), 0.49 (q, J = 7.75 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 198.58, 150.46, 143.36, 133.17, 133.02, 127.03, 112.43, 71.98, 51.30, 40.76, 18.39, 6.86, 4.98; UVmax (c = 4.32 x 10^{-5} M, MeOH) 356 nm (1,300), 292 (4,511), 256 (25,585), 208 (29633); HRMS (FAB^+) Calc. for 348.23603. Found: 348.23602.

Biosynthetic Experimental

General

Sterilization of culture media was performed in a AMSCO general purpose steam powered autoclave at 121 °C for either 30 min (200 mL to 1000 mL) or 45 min (10 L). Fermentations were carried out in a Lab-Line model 3595 incubator shaker at 28-32 °C and 225-250 rpm. Incubations for agar petri plates and slants were done in VWR model 1520 incubators. Sterile transfers were carried out in an EdgeGARD™ hood manufactured by the Baker Company, Inc. (Sanford, ME). Centrifugation of samples were accomplished in a IEC B-20A centrifuge (Needham Heights, MA), and sonications were done using a Heat-Systems-Ultrasonics, Inc. model W-225R cell disrupter (Farmingdale, NY).

Radioactivity measurements were made using glass vials in a Beckman model LS 7800 Liquid Scintillation Counter on samples dissolved in 10 mL of Beckman Redi-Solv™ MP. Microgram samples were weighed on a Cahn Model 29 Automatic Electrobalance. Protein concentrations were determined according to the method of Lowry.[97]

Culture Maintenance.

Streptomyces helicus was maintained at 4 °C on agar petri plates composed of a yeast malt extract media (Table 7).

Table 13. Maintenance Medium for *S. helicus*.

Yeast Extract	4.0 g
Malt Extract	10.0 g
Glucose	4.0 g
Distilled Water	1000 mL
pH Adjustment (2% KOH)	pH 7.3
Agar	20.0 g

These petri plates contained about 25 mL each of this preparation and were inoculated from sporulated soil by the following method: Inoculation of a plate was accomplished by suspending a loop of sporulated soil sample in 10 mL sterilized water containing a small amount of Triton-X-100, vortexing vigourously, and spreading 0.10 mL of the suspension onto the plate. The 5 plates typically prepared each time by this method were incubated at 28 °C for 10 days until a thick grey sporulation was visible and were then stored at 4 °C. The petri plates had a shelf life of 7-12 months when prepared in this manner.

Production of Sarubicin A

The following procedures were used to generate NMR quality sarubicin A and are representative of the procedures used for biosynthetic feeding experiments.

Seed media and production media used for production of Sarubicin A are listed in Table 8.[98]

Table 14. Culture Conditions for the Production of Sarubicin A

Seed Medium		Minimal Salts		Production Medium	
Pharmamedia	5.0 g	$MgSO_4 \cdot 7H_2O$	10.0 g	Dextrin	1 g
Glucose	5.0 g	$MnSO_4 \cdot H_2O$	0.25 g	$(NH_4)SO_4$	0.20 g
Tap water	200 mL	$ZnSO_4 \cdot 7H_2O$	0.80 g	$CaCO_3$	1 g
pH (1N NaOH)	7.2	$FeSO_4 \cdot 7H_2O$	0.30 g	distilled water	200 mL
		$CoCl_2 \cdot 6H_2O$	0.10 g	Mineral Salts	0.20 mL
		distilled water	50 mL	No pH adjustment.	

Seed cultures (200 mL) were prepared and autoclaved in 1 L flasks. After cooling to room temperature, the media were inoculated by a sterile loop transfer of about 1 cm^2 of the grey growth from a previously prepared plate, and the flask then placed in an incubator shaker at 32 °C for 24 h. Sterile, 1 L flasks containing 200 mL of the production medium were

inoculated with 10 mL of the seed medium, and then incubated for 72 h at 28-29 °C. Sarubicin A production could be monitored visually as evidenced by the appearance of a red color in the broth beginning about 30 h, which then turned brick red by about 65 h. Production typically reached a maximum at about 70-72 h and could be followed by UV analysis of a 0.5 mL aliquot (1 : 1 dilution with water) at 472 nm.

Isolation and purification of sarubicin A from the broth was accomplished as follows:

After 72 h, the broth was adjusted to pH 3 (6N HCl), and vacuum-filtered to remove mycelia and media particulates. The red aqueous filtrate was saturated with $(NH_4)_2SO_4$ (260 g / L) and extracted with 4 500-mL portions of ethyl acetate. The extracts were dried (Na_2SO_4), filtered and concentrated, and sarubicin A was isolated by chromatography on a Silicar CC-4 (1.8 x 10 cm). Elution with initially CH_2Cl_2 (25 - 35 mL) followed by 5% methanol/CH_2Cl_2 yielded typically 100-200 mg of sarubicin A after removal of the solvents at aspirator pressure. Crystallization was accomplished from $CHCl_3$.

Feeding of [^{13}C-^{15}N-carboxamide]-6-Hydroxyanthranilamide, 4a, to *Streptomyces Helicus*, and Sarubicin A isolation. The seed medium for the seed culture was prepared by suspending Pharmamedia (2.5 g) and glucose, (2.5 g) in tap water (100 mL) in a 500 mL flask. The pH was adjusted to 7.2 with 1N NaOH and the mixture autoclaved at 121 °C for 20 min. The seed medium was inoculated via loop transfer with spores of *S. helicus* and shaken at 225 rpm and 29 °C for 24 h. Meanwhile, the production medium was prepared by dissolving glucose, 5.0 g, $(NH_4)_2SO_4$, 1.0 g, $CaCO_3$, 5.0 g, trace salts, 1.0 mL, and deionized water to 1.0 L. The trace salts consisted of $MgSO_4 \cdot 7H_2O$, 200 g, $MnSO_4 \cdot H_2O$, 5.0 g, $ZnSO_4 \cdot H_2O$, 10.0 g, $FeSO_4 \cdot 7H_2O$, 6.0 g, $CoCl_4 \cdot 6H_2O$, 2.0 g, and deionized water to 1L. To each of 4 200 mL volumes in 1 L autoclaved flasks were added 10 mL of seed medium. The doubly labeled **27a** (80.0 mg, 467 μmoles) was dissolved in water (25 mL) and the pH adjusted to 3 to facilitate dissolution. To each of 3 flasks was added the solution of **27a** (2.5 mL) at 24 h, followed by identical aliquots at 36 h and 48 h. The fourth flask was a control and identical volumes of water (pH 3) was added at the appropriate times. The fermentation was stopped at

72 h by the addition of conc. HCl to the combined flasks containing **27a**. After filtering, the filtrate was saturated with $(NH_4)_2SO_4$ (250 g) and extracted with ethyl acetate (250 mL) 4 times. The combined extracts were washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was chromatographed on Silicar CC-4 (1.8 x 10 cm) eluting initially with CH_2Cl_2 (50 mL) and then with 1-5% MeOH in CH_2Cl_2 to yield, after concentration, a dark red residue. Crystallization from $CHCl_3$ yielded 33.0 mg (95.5 μmoles) of **1h** as bright orange crystals. An unidentifiable impurity (15% by ^1H NMR) was also present.

^{13}C NMR Studies of 1h. A 4.5 mg sample of **1c** was analyzed by Waltz decoupled ^{13}C NMR (DMSO-d_6, 100.6 MHz, SW 25000 Hz, SI = TD = 64 K, AQ = 1.31 s, NS = 25640, PW = 36°). An unlabeled sample was run under identical conditions for standardization. (NS = 20746). The partial spectra are shown in Figure 5.

Trapping Experiments

General Trapping Conditions. Five 50 mL fermentation broths were each inoculated with 2.5 mL of a 24 h seed broth in the usual manner. After 14 h, 5.5 μCi of [1-^{14}C] erythrose (1.22 X 10^7 dpm) was added to four of the five flasks. The fifth was left as a control for 72 h to monitor production of **1**. Two of the fermentations were terminated at 16 h and the other two at 20 h.

The appropriate carrier was added to the fermentation broth whereupon the cells were immediately sonicated at 0 °C for 8 min (100% power, continuous) and the mixtures lyophilized. To each flask was added 25 mL each of Ac_2O and Et_3N and the mixture stirred overnight at room temperature. The volatiles were removed *in vacuo* under high vacuum and the residue triturated two times in hot ethyl acetate, then filtered. The combined extracts were concentrated and dissolved in MeOH (50 mL). Freshly prepared CH_2N_2 was added until bubbling ceased, then the mixture was allowed to stir overnight. The mixtures were then concentrated to dryness, and the respective derivatives isolated and purified as described (*vida infra*). The specific activities of each isolated compound during the recrystallizations are tabulated in the Results and Discussion section.

6-Hydroxyanthranilic Acid, 17.

2 Hour Trap. Authentic **17** (65.3 mg, 345 μmol) was added to a flask at 16 h and the vessel treated as described. Purification of **54**, the acetamido methoxy methyl ester of **17** was accomplished via a silica flash column (11 X 1 cm, 5% Et_2O/CH_2Cl_2) to yield 23.1 mg (104 μmol) of **54** as a white powder. The total radioactivity remaining prior to crystallization was 1.29 X 10^5 dpm, 1.06% of the total activity fed. Successive recrystallizations from CH_2Cl_2/hexane yielded a radioactivity count not significantly above background.

6 Hour Trap. Authentic **17** (62.1 mg 328 μmol) was added to a fermentation flask and the broth worked up in the usual manner. Separation yielded 40.3 mg (181 μmol) of the derivative **54** as a viscous oil which resisted all attempts at crystallization. Therefore the oil was filtered through a silica gel column to yield 20.1 mg (90.1 μmol) of **54** which could now be crystallized from CH_2Cl_2/hexane.

6-Hydroxyanthranilamide, 27.

2 Hour Trap. Authentic **27** (67.2 mg, 323 μmol) was added to the fermentation at 16 h and the broth worked up in the usual fashion. The acetamido methoxy derivative **55** was purified by passage through a silica flash column (1 X 11 cm, 7% MeOH/$CHCl_3$) to yield 17.1 mg (82.2 μmol) of **55**. To this was added 32.8 mg of unlabelled **55** to increase the mass for recrystallizations. Crystallizations were accomplished from hot water.

6 Hour Trap. Authentic **27** (64.1 mg, 308 μmol) was added to a fermentation at 20 h and the broth worked up as described. Purification as for the 2 h trap yielded 17.8 mg (85.6 μmol) of **55** to which was added 20.1 mg to increase the mass for recrystallizations.

Cell-Free Studies of *S. helicus*

Preparation of Cell-Free Extracts To assay for isochorismate synthase activity in *S. helicus*, a cell-free preparation was necessary. A series of cell-free extracts were prepared from production fermentations at various times after inoculation of the medium with *S. helicus*. Sarubicin A can be detected in the broth at 24 hours.

Method A: extracts were prepared at 24, 36, 48 and 60 hours after inoculation of the production broths. The cells were washed two times with 0.10 M sodium phosphate buffer, pH 7.0, by suspending the cells in buffer, and the buffer removed by centrifugation, and then suspended in 0.01 M phosphate buffer (10-15 mL). Sonication of the mixture 10 times for 30 seconds each at 70 watts with cooling intervals of 2 to 3 minutes, followed by centrifugation at 47500 times g for 45 min, provided the cell-free extract.

Method B: Extracts were prepared at 24, 30, and 38 hours. Cells were isolated by centrifugation and washed successively with 1 M KCl, 0.8 M NaCl, to remove any proteases present outside the cells, and then with 0.01 M sodium phosphate buffer. They were washed once more with one of the buffers in Table 15, and then suspended in 10 mL of the final wash buffer. Buffers used are included in Table 15. The cells were sonicated 2 times for 15 seconds at 100 % power at 0° C with a 3 minute cooling period in between sonications. Then the cell debris was removed by centrifugation as in Method A. The cell-free extracts thus obtained were either used within 4 hours, or frozen in aliquots (0.5 mL) at -80 °C.

Assay For Isochorismate in *S. helicus* Cell Free Extracts

Assays for isochorismate synthase activity in *S. helicus* were carried out in the following incubation mixture: a solution consisting of Tris-HCl (ph 8.0), 15.6 µmoles, and Mg^+, 1.5 µmoles; either chorismate or isochorismate, 0.40 µmoles; and cell-free extract, 200 µL, in a total volume of 470 µL at pH 8.0. Incubations were performed at 37 °C for 60 minutes. Aliquots (10 µL) were removed at 0, 30 and 60 minutes for HPLC analysis as described in the Isochorismate Synthase Isolation section. Control

experiments for the active extracts were performed under identical conditions, except that the incubation mixture was boiled for 10 minutes before the addition of chorismate.

The cell-free extracts prepared are listed in Table 15.

Table 15. Cell-Free Extracts of *S. helicus*

Time (hours)	Buffer	protein (mg/mL)	Method
21	a	0.35	A
21	d	0.35	A
24	a	0.56	B
24	c	0.56	B
26	c	0.70	B
30	a	0.60	B
30	c	0.61	B
36	a	0.63	A
38	a	0.65	B
38	c	0.63	B
48	a	0.65	A
60	a	0.70	A

Buffer systems included: a) 10 mM phosphate buffer, pH 7.0; b) 10 mM phosphate buffer, pH 7.0 with 10 mM Mg^{2+}; c) 10 mM phosphate buffer, pH 7.0, 10 mM Mg^{2+}, 1.0 mM dithiothreitol. d) 50 mM Tris-HCl, pH 8.0.

Detection of 54 and 55 in *S. helicus* Cell Free Extracts

Incubation conditions to test for production of **17** and/or **27** by cell-free extracts of *S. helicus* were: cell-free extract: 250 µL; Tris-HCl, 15 µmoles, or sodium phosphate, 10 µmoles; Mg^{2+}, 10 µmoles; glutamine, 40 µmoles; $(NH_4)_2SO_4$, 40 µmoles; NAD^+, 10 µmoles; $NADP^+$, 10 µmoles; NADH, 10 µmoles; NADPH, 10 µmoles; ATP, 10 µmoles; pyridoxal 5'-phosphate, 10 µmoles; chorismate or isochorismate, 5 µmoles; final volume, 1000 µL.

The final pH was 8.0 in the case of the Tris buffer, and pH 7.1 for the phosphate buffer. Fresh cell-free extracts were used within four hours of preparation.

Each of the cell-free extracts which had been prepared were incubated at 30 °C for 2 hours, and then the mixture lyophilized and derivatized as described.

Derivatization and Detection of 54 and 55

HPLC Conditions. Conveniently, the previously prepared N-acetyl, O-methyl derivatives of 6-hydroxyanthranilic acid **54** and 6-hydroxyanthranilamide **55**, could be separated and detected at 254 nm using an Alltech econosphere 5 μm, C-18 reversed-phase HPLC column (4.6 x 250 mm), equipped with a precolumn(4.6 x 2.0 cm, 30 μm C-18 material) eluting with an aqueous solution of 10% methanol and 0.1% trifluoroacetic acid at 1.0 mL/min. The HPLC detector was set at 254 nm. Elution times were 14.8 minutes and 30.0 minutes for **55** and **54** respectively, and the detection limit was 1 nanomole for each compound.

A 1000 μL incubation mixture containing cell-free extract of *S. helicus* was prepared (*vida infra*) and 0.2 mg (1.3 μmoles) each of **17** and **27** added. The mixture was acidified with concentrated HCl to pH 1, and then centrifuged to remove any precipitated protein. Lyophilization of the solvent gave a residue, which was reacted with acetic anhydride (2.0 mL) in the presence of triethylamine (2.0 mL) followed by treatment with freshly prepared ethereal diazomethane (25 mL). After filtration through a small silica gel plug, and elution with ethyl acetate, the eluant was concentrated, and the residue dissolved in methanol (200 μL). An aliquot (5 μL) was analyzed by HPLC. A peak corresponding to **55** eluted at14.8 min, followed by **54** at 30.0 min.

Isochorismate Synthase

Growth conditions for *E. aerogenes*.

Maintenance of *E. aerogenes*. Cultures of *E. aerogenes* were maintained on brain heart infusion agar (BHI) (Difco) prepared as follows: BHI agar (5.6 g) was dissolved in deionized water (100 mL) and heated to

boiling until the agar has dissolved. The solution was autoclaved at 121 °C for 20 min and then poured into a sterile slant tube and allowed to cool for 4 h. The agar was inoculated with *E. aerogenes* via sterile loop transfer and incubated overnight at 30 °C then stored at 4 °C. Subcultures were prepared monthly.

Production of Chorismic Acid The procedure used is a modification of the reported conditions.[90]

Growth Medium. A salts base was prepared which consisted of: $MgSO_4 \cdot 7H_2O$ (10 g), citric acid monohydrate (100 g), KH_2PO_4 (500 g), $NaNH_4HPO_4$ (175 g) dissolved in water (670 mL). To a solution of the salts base (200 mL) was added Difco yeast extract (20 g), Difco casamino acids (20 g), and DL-tryptophan (410 mg) and made up to 10 L with water and the solution autoclaved at 121 °C for 45 min. After cooling, a sterile 16% glucose solution (100 mL) was added.

Accumulation Medium. Na_2HPO_4 (120 g), KH_2PO_4 (13.6 g), glucose (180 g), NH_4Cl (27 g) were dissolved in water (10 L) and $MgCl_2$ (0.05 M, 20 mL), and L-tryptophan (0.01 M, 10 mL) added. The mixture was not sterilized.

An aliquot of the growth medium (100 mL) was removed and inoculated with *E. aerogenes* by sterile loop transfer and shaken at 30 °C for 8 h at 250 rpm and then transferred to the 10 L growth medium. Cells were grown at 30 °C for 6 h in a Microferm™ 14 L fermentor with stirring at 400 rpm and aeration at a rate of 10 L / min. The cells were collected by centrifugation with a Sharples™ centrifuge, immediately resuspended in the accumulation medium, and incubated for 16 h under the same conditions as for the growth of the cells.

Isolation of Chorismic Acid, 19. After removal of the cells from the accumulation medium via centrifugation, the supernatant was made alkaline by the addition of 10 M NaOH (50 mL) and applied to a Dowex 1-X4 (100-200 mesh, Cl^- form, 5 x 10 cm) at a rate of 50 mL / minute. The operations were carried out at 4 °C. Chorismate was eluted after washing the column with water (200 mL) with 1 M NH_4Cl, (adjusted to pH 8.5 with

conc. NH_4OH). Fractions were collected at 1.5 mL / min and monitored by UV (280 nm). Fractions containing chorismate were pooled, acidified to pH 1.5 (conc. HCl), extracted eight times with ether, the organic phase dried over $MgSO_4$ and concentrated at 20 °C at aspirator pressure. The faint yellow solid (6.25 g) was crystallized from 1 : 1 : 2/ether : CH_2Cl_2 : hexane with cooling to -78 °C to yield a white powder (5.95 g) which was stored at -80 °C. ^1H NMR (DMF-d_7 400 MHz) δ 6.83 (d, J = 1.98 Hz, 1 H), 6.33 (dt, J = 9.99, 1.84 Hz, 1 H), 6.05 (dd, J = 10.08, 3.02 Hz, 1 H), 5.44 (d, J = 2.48 Hz, 1 H), 5.04 (dd, J = 10.44, 2.92 Hz, 1 H), 4.99 (d, J = 2.47 Hz, 1 H), 4.63 (dt, J = 10.43, 2.58 Hz, 1 H); ^{13}C NMR (DMF-d_7 100.6 MHz) δ 166.38, 164.66, 151.07, 133.81, 133.39, 130.18, 121.54, 96.22, 69.84; UV max (c = 4.425 x 10^{-4} M, H_2O) 278 nm (2750).

Production and Isolation of Isochorismate Synthase

Growth of Cells. In 9.5 L of water was dissolved KH_2PO_4 (136 g), $(NH_4)_2SO_4$ (20 g), $MgSO_4 \cdot H_2O$ (2.0 g), $CaCl_2$ (0.10 g), L-phenylalanine (0.33 g), L-tyrosine (0.36 g), L-tryptophan (0.41 g), and the solution adjusted to pH 7.4 (2.5 M KOH). After autoclaving the solution at 121 °C for 45 min a sterile glucose solution (50 g in 500 mL water) was added. An aliquot (100 mL) was removed and inoculated with *E. aerogenes* via sterile loop transfer, and then incubated at 37 °C with shaking at 250 rpm for 8 h. The seed culture was transferred to the 10 L volume and the cells grown at 37 °C with stirring at 400 rpm and aeration at 10 L/min for 15 h. At this point, the cells were collected by centrifugation and washed with 0.05 M sodium phosphate buffer, pH 7.0 to yield 76 g of cells. After suspension in phosphate buffer (0.01 M, 250 mL), the cells were sonicated 20 times for 30 s at 70 watts, with 2-3 min cooling between sonications in an ice bath, and the cell debris removed by centrifugation (0 °C, 45 min at 38400 x g) to yield the crude cell-free extract (250 mL, 7.3 mg / mL) A DEAE cellulose column (Whatman DE 52 preswollen, 160 g, 5 x 20 cm) was prepared and equilibrated according to the manufacterer's instructions with sodium phosphate buffer (0.01 M), and the crude cell-extract, which was diluted with 350 mL phosphate buffer, was applied to the DEAE column at 4 mL/min. The column was washed with buffer (150 mL), and isochorismate synthase eluted with 0.01 M phosphate buffer which was 0.1 M in NaCl. Column

fractions collected (16 mL each) were monitored by UV (254, and 280 nm), and those containing protein were assayed for isochorismate as described below. Fractions containing isochorismate synthase activity were pooled (255 mL, 0.71 mg / mL).

$(NH_4)_2SO_4$ Precipitation. Protein obtained from the DEAE column was fractionally precipitated with $(NH_4)_2SO_4$ in five fractions. Thus, the DEAE eluant (200 mL) was brought to 30% saturation with $(NH_4)_2SO_4$ and the precipitate centrifuged (47500 x g, 45 min). Fractions at 46%, 62%, 77%, and 99% $(NH_4)_2SO_4$ saturation were similarly obtained. Assay by HPLC revealed most of the activity in the 46% and 62% fractions. These were dissolved in phosphate buffer (0.01 M) and pooled (8.0 mL, 13.5 mg / mL). The preparation was used without any further purification.

Assay for Isochorismate Synthase Activity

Assays for isochorismic acid were performed by either of two methods:

a) Decomposition to salicylic acid. Gibson observed that upon heating in pH 7 buffer at 100 °C, isochorismate decomposed to approximately 25% salicylic acid which could be detected by fluorescencs spectroscopy (excitation 300 nm, emission 410 nm).[72] The sample to be assayed (0.100 mL) (100 µmol isochorismate maximim) was added to 0.10 M sodium phosphate buffer, pH 7.0 (1.500 mL) and the fluorescence recorded. After heating to 100 °C for 10 min, the sample was cooled to room temperature and the fluorescence recorded again. Isochorismate concentrations could be estimated by comparison of the net fluorescence increase with a standard curve developed using authentic salicylic acid. Chorismate did not produce any salicylic acid under these conditions.

b) Direct HPLC analysis. Both chorismate and isochorismate absorb at 278 nm (**19** ε = 2630, **18** ε = 13000).[75] A modification of the HPLC solvent system developed by Leistner[94] was used for this assay. An Alltech analytical column packed with Lichrosorb C-8 reversed phase material (4.6 x 250 mm, 5 µm) with a C-8 guard column attached (4.6 x 100 mm, 30-40 µm) was used. Elution with an isocratic solvent system consisting of 9 : 1 water :

methanol and 0.1% trifluoroacetic acid at a flow rate of 1.0 mL / min provided **18** at 12.7 min followed by **19** at 25.8 min. Detection was by UV with the detector set at 278 nm. Samples were assayed by direct injection of 5-10 μL of the assay mixture.

Incubation Conditions For Isochorismate Synthase Assays.

Assay mixtures consisted of a final volume of 200 μL containing 10 μmol Tris, 1 μmol Mg^{2+}, 0.5 μmol chorismate, and 25 μL of protein solution (final pH 8.0) which were incubated at 37 °C for 1 to 2 h. The assay was typically by HPLC.

Enzymatic Synthesis and Isolation of Isochorismic Acid. Tris base (55.4 mg, 458 μmol), $MgCl_2 \cdot H_2O$ (27.0 mg, 133 μmol), and **19** (35.0 mg, 155 μmol) were dissolved in 2.0 mL H_2O and the pH adjusted to 8.2 (1 M NaOH, 30 μL). Enzyme from the $(NH_4)_2SO_4$ precipitation (1.89 mL, 35.0 mg protein) was added to give a final volume of 4.0 mL, pH 8.0. Incubation of the mixture was performed at 37 °C for 2 h, whereupon the reaction was terminated by the addition of conc. HCl (300 μL), and then saturated with NaCl. Extraction of the acidified mixture 5 times with an equal volume of ether and then 3 times with ethyl acetate, followed by evaporation of the combined organic phases at aspirator pressure and 20 °C, yielded a colorless oil. The residue was immediately dissolved in methanol (100 μL), and then diluted with water (800 μL) and filtered through a 5 μm membrane. Separation of **18** from **19** was accomplished with a semi-prep HPLC column (Whatman C-8, 10 μm Partisil™, 10 x 250 mm) eluting with 95 : 5 water : methanol, and 0.1% trifluoroacetic acid at 3.0 mL / min. Collection of **18**, eluting at 8.0 min, followed by **19** eluting at 16.0 min by repeated injections (250 μL) and lyophilization of the respective samples yielded **18** (5.5 mg) and **19** (12.9 mg). Physical data for **18**: ^1H NMR (DMSO-d_6 400 MHz) δ 6.99 (d, J = 5.56 Hz, 1 H), 6.37 (dd, J = 9.55, 5.61 Hz, 1 H), 6.25 (dd, J = 9.40, 4.91 Hz, 1 H), 5.33 (d, J = 2.65 Hz, 1 H), 5.00 (d, J = 2.65 Hz, 1 H), 4.52 (dd, J = 4.95, 0.81 Hz, 1

H), 4.46 (br s, 1 H); ^{13}C NMR (DMSO-d_6 100.6 MHz) δ 167.50, 163.87, 149.41, 131.35, 130.53, 127.77, 126.34, 95.72, 74.17, 62.12; UV max (c = 4.425 x 10^{-4} M, H_2O) 278 nm (12800).

NMR data for **18** was obtained in DMF-d_7 as well.

18 ^1H NMR (DMF-d_7 400 MHz) δ 7.10 (d, J = 5.35 Hz, 1 H), 6.45 (dt, J = 9.40, 5.61 Hz, 1 H), 6.34 (dd, J = 9.22, 5.60 Hz, 1 H), 5.44 (d, J = 2.33 Hz, 1 H), 5.09 (d, J = 2.43 Hz, 1 H), 4.72 (m, 2 H); ^{13}C NMR (DMF-d_7 100.6 MHz) δ 168.23, 164.62, 150.83, 131.91, 131.79, 128.54, 126.89, 95.85, 75.64, 63.83.

19: ^1H NMR (DMF-d_7 400 MHz) δ 7.10 (d, J = 5.35 Hz, 1 H), 6.45 (dt, J = 9.40, 5.61 Hz, 1 H), 6.34 (dd, J = 9.22, 5.60 Hz, 1 H), 5.44 (d, J = 2.33 Hz, 1 H), 5.09 (d, J = 2.43 Hz, 1 H), 4.72 (m, 2 H); ^{13}C NMR (DMF-d_7 100.6 MHz) δ 168.23, 164.62, 150.83, 131.91, 131.79, 128.54, 126.89, 95.85, 75.64, 63.83.

$H_2{}^{18}O$ Enzymatic Synthesis of Isochorismate.

The enzymatic reaction was performed exactly as above except that $H_2{}^{18}O$ (2 mL, 98 - 99% ^{18}O) was used in place of $H_2{}^{16}O$ to give a final ratio of 50% ^{18}O labeled water. Incubation followed by isolation and HPLC purification **19** and **18** yielded **19** (11.9 mg) and **B** (4.6 mg). A ^{13}C NMR was obtained for **19** and **18**:

18: 4.6 mg was dissolved in 380 µL DMF-d_7. The ^{13}C NMR data for **18** was obtained under the following conditions: SW = 100.6 MHz; 01 = 54821 Hz; SI = TD = 16K; sweep width = 12195 Hz; PW = 3.0, RD = 0; AQ = 2.69 s; decoupler offset 8100 Hz; NS, 16323. A Gaussian multiplication was performed on the raw data (LB = -0.50, GB = 0.40) before Fourier transformation.

19: 11.9 mg was dissolved in 400 µL DMF-d_7. The ^{13}C NMR data for **19** was obtained under the following conditions: SF 100.6 MHz; 01, 55201 Hz; SI = TD = 16K; SW = 10869 Hz; PW = 3.0, RD = 0; AQ = 3.02 s; decoupler offset 8100 Hz; NS, 4342. A Gaussian multiplication was performed on the raw data (LB = -0.50, GB = 0.40) before Fourier transformation.

Bibliography

1. Slechta, L.; Chidester, C. G.; Reusser, F. *J Antibiot.* **1980**, 917.

2. Reinhardt, G.; Bradler, G.; Eckardt, K., Tresselt, D.; Ihn, W. *J. Antibiot.* **1980**, 787.

3. Eckardt, K.; Tresselt, D.; Ihn, W,; Kajtar, M.; Angyanán, J.; Radics, L. Hollósi, M. *J. Antibiot.*, **1983**, 976.

4. Takeuchi, Y.; Sudani, M.; Yoshii, E. *J. Org. Chem.*, **1983**, *48*, 4152.

5. Semmelhack, M.F.; Appapillai, Y.; Sato, T. *J. Am. Chem. Soc.*, **1985**, *107*, 4577.

6. Brufani, M.; Dobler, M. *Helv. Chim. Acta.* **1968**, *51*, 1269.

7. Noble, M.; Noble, D.; Sykes, R. B. *J. Antibiot.*, **1977**, *30*, 455.

8. Wiley, P. F.; Mizsak, S. A.; Baczynskyj, L.; Argoudelis, A. D.; Duchamp, D. J.; Watt, W. *J. Org. Chem.*, **1986**, *51*, 2493.

9. Tresselt, D.; Eckardt, K.; Ihn, W.; Radics, L.; Reinhardt, G. *Tetrahedron* **1981**, *37*, 1961.

10. Schlecta L., Private communication

11. a) Brockmann, H.; Lackner, H. *Chem. Ber.* **1967**, *100*, 353.
    b) Weissbach, H.; Redfield, G. B.; Beaven, V.; Katz, E. *J. Biol. Chem.* **1965**, *240*, 4377.
    c) Katz, E.; Weissbach, H. *J. Biol. Chem.* **1963**, *238*, 666.

12. Gerber, N. N. *Can. J. Chem.* **1968**, *46*, 790

13. Gould, S.J.; Tann, C.-H.; Prabhakaran, P.C.; Hillis, L.R. *Bioorg. Chem.*, **1988**, *16*, 258.

14. Snipes, C.E., Chang, C.-J., Floss, H. G. *J. Am. Chem. Soc.* **1979**, *101*, 701.

15. Hillis, L.R.; Gould, S. J. *J. Am. Chem. Soc.* **1985**, *107*, 4593.

16. Hillis, L.R.; Gould, S. J. *J. Org. Chem.*, **1985**, *50*, 718.

17. Gibson, F. *Biochem. J.*, **1964**, *90*, 248.

18. Johanni, M.; Hofmann, P.; Leistner, E. *Arch. Biochim. Biophys.* **1989**, *271*, 495.

19. Gil, J. A.; Naharro, G.; Villanueva, J. R.; Martin, J. F. *J. Gen. Microbiol.* **1985**, *131*, 1279.

20. Haslam, E. *The Shikimate Pathway*; Halsted Press: New York, 1974.

21. Luckner, M. *Secondary Metabolism in Microorganisms, Plants, and Animals*; Springer- Verlag: New York, 1984, pp 259-272.

22. Young, I. G.; Batterham, T.; Gibson, F. *Biochim. Biophys. Acta.* **1968**, *165*, 567.

23. Marshall, B. J.; Ratledge, C. *Biochim. Biophys. Acta.* **1971**, *230*, 643.

24. Young, I. G.; Cox, G. B.; Gibson, F. *Biochim. Biophys. Acta.* **1967**, *141*, 319.

25. Weische, A.; Johanni, M.; Leistner, E. *Arch. Biochim. Biophys.* **1987**, *256*, 212.

26. Young, I. G.; SLangman, L.; Luke, R. K. J.; Gibson, F. *J. Bacteriol.* **1971**, *106*, 51.

27. Nahlik, M. S.; Fleming, T. P.; McIntosh, M. A. *J. Bacteriol.* **1987**, *169*, 4163.

28. Eisenberg, R. L.; Hillis, L. R.; Gould, S. J. Manuscript in preparation.

29. a) Herbert, R. B. *The Biosynthesis of Secondary Metabolites*; 2nd ed., Chapman and Hall: New York, 1981, pp. 1-28.
b) *Specialist Periodical Reports on Biosynthesis*, The Chemical Society, London.
c) Luckner, M. *Secondary Metabolism in Microorganisms, Plants, and Animals*, 2nd ed.; Springer-Verlag: Berlin, 1984.

30. Philipsborn, W.; Müller, R. *Angew. Chem. Int. Ed. Eng.* **1986**, *25*, 383.

31. Booth, H.; Bycroft, B. W.; Corbett, K.; Maloney, A. P. *J. Chem. Soc. Chem. Commun.* **1977**, 624-626.

32. Gould, S. J.; Chang, C. C.; Darling, D. S.; Roberts, J. D.; Squillacote, M. *J. Am. Chem. Soc.*, **1980**, *102*, 1707.
b) Gould, S. J.; Chang, C. C. *J. Am. Chem. Soc.*, **1978**, *100*, 1624.
c) Haber, A.; Johnson, R. D.; Rinehart, Jr., K. L.; *J. Am. Chem. Soc.* **1977**, *99*, 3541.
d) Burton, G.; Nordlöv, H.; Hosozawa, S.; Matsumoto, H.; Jordan, P. M.; Fagerness, P. E.; Pryde, L. M.; Scott, A. I. *J. Am. Chem. Soc.* **1979**, *101*, 3114.
e) Leete, E.; McDonell, J. A. *J. Am. Chem. Soc.* **1981**, *103*, 658.

33. Saá, C.; Guitián, E.; Castedo, L.; Suau, R.; Saá, J. M. *J. Org. Chem.*, **1986**, *51*, 2781.

34. Sellstedt, J. H.; Guinosso, C. J.; Begany, A. J.; Bell, S. C.; Rosenthale, M. *J. Med. Chem.* **1975**, *18*, 926.

35. Itoh, M. *Tetrahedron Lett.* **1975**, 4393.

36. *J. Am. Chem. Soc.* **1985**, *107*, 8066.

37. Muchowski, J. M.; Venuti., *J. Org. Chem.*, **1980**, *45*, 4798.

38. a) Beak, P.; Snieckus, V. *Acc. Chem. Res.* **1982**, *15*, 306.
b) Beak, P.; Zajdel, W. J.; Reitz, D. B. *Chem Rev.* **1984**, *84*, 471.

39. Kelly, T. R.; Dali, H. M.; Tsang, W.-G. *Tetrahedron Lett.* **1977**, 3859.

40. Fukuyama, T.; Li, L.; Laird, A. A.; Frank, R. F. *J. Am. Chem. Soc.* **1987**, *109*, 1587.

41. Stahl, G. L.; Walter, R.; Smith, C. W. *J. Org.Chem.* **1978**, *43*, 2285.

42. Lundt, B. F.; Johansen, N. L.; Volund, A.; Markussen, *Int. J. Pept. Protein. Res.* **1978**, *12*, 258.

43. Pomerant, C.; Fink, J. *J. Labeled. Cmpnds* **1979**, *16*, 278.

44. Lambert, J. B.; Oliver, W. L.; Roberts, J. D *J. Am. Chem. Soc.* **1965**, *87*, 5085.

45. Ott, D. G. *Syntheses With Stable Isotopes of Carbon, Nitrogen, and Oxygen* John Wiley & Sons: New York, 1981, p. 79.

46. a) Serriani, A. S.; Nunez, H. A.; Barker, R. *Carbohydrate Res.* **1979**, 71.
b) Serriani, A. S.; Clark, E. L.; Barker, R. *ibid.* **1979**, 79.

47. [1-^{14}C]-D-Erythrose was kindly provided by W. R. Erickson.

48. [1-^{14}C]-D-Erythrose has been shown to be efficiently incorporated (4%) into sarubicin A. Unpublished results by Dr. L. R. Hillis in this laboratory.

49. Rees, C. W., West, D. E. *J. Chem. Soc. (C)* **1970**, 583.

50. Jacob, P. III, Callery, P. S., Shulgin, A. T., Castagnoli, N. Jr. *J. Org. Chem.*, **1976**, *41*, 3627.

51. Woods, G. F. *Organic Synth. Coll. Vol III*, **1955**, 470.

52. The sugar derivatives were kindly provided by Dr. Karl DeJesus.

53. a) Pfeffer, P. E.; Valentine, K. M.; Parrish, F. W.; *J. Am. Chem. Soc.*, **1979**, *101*, 1265.
b) Newmark, R. A.; Hill, J. R. *Org. Magn. Reson.* **1980**, *13*, 40.
c) Christofides, J. C.; Davies, D. B. *J. Chem. Soc. Chem. Commun.* **1983**, 324.
d) Reuben, J. *J. Am. Chem. Soc.* **1983**, *105*, 3711.
e) Christofides, J. C.; Davies, D. B. *J. Am. Chem. Soc.* **1983**, *105*, 5099.

54. Sato, Y.; Geckle, M.; Gould, S. J. *Tetrahedron Lett.*, **1985**, *26*, 4019.

55. Sato, Y.; Kohnert, R.; Gould, S. J. *Tetrahedron Lett.* **1986**, *27*, 143.

56. Joullié M. *J. Am. Chem. Soc.* **1987**, *109*, 5099.

57. a) Casnati, G, Crisafulli, M; Ricca, A. *Tetrahedron Lett.* **1965**, 243.
b) Mori, K.; Takechi, S. *Tetrahedron*, **1985**, *41*, 3049.

58. For a recent review, see the special issue of *Carbohydr. Res.* on C-glycoside synthesis: *Carbohydr. Res.* **1987**, *171*.

59. See for example: Eade, R. A.; Pham, H. P. *Aust. J. Chem.* **1979**, *32*, 2483.

60. a) Czernicki, S.; Ville, G. *J. Org. Chem.* **1989**, *54*, 610.
b) Kawasaki, M.; Matsuda, F.; Terashima, S. *Tetrahedron Lett.* **1985**, 2673.

61. Czerneck, S.; Dechavanne, V. *Can. J. Chem.* **1983**, *61*, 533.

62. Bellosta, V.; Czernicki, S. *J. Chem. Soc., Chem. Commun.* **1989**, 199.

63. Matsumoto, T.; Katsuki, M.; Suzuki, K. *Tetrahedron Lett.* **1989**, *30*, 833.

64. Danishefsky, S. J.; Larson, E.; Askin, D.; Kato, N. *J. Am. Chem. Soc.* **1985**, *107*, 1246.

65. Danishefsky, S. J., Uang, B. J., Quallich, G. *J. Am. Chem. Soc.* **1985**, *107*, 1285.

66. Danishefsky, S.; Kerwin, J. F.; Kobayashi, S. *J. Am. Chem. Soc.* **1982**, *104*, 358.

67. Bednarski, M.; Danishefsky, S. *J. Am. Chem. Soc.* **1983**, *105*, 3716.

68 Lee, J.; Snyder, J. K. *J. Am. Chem. Soc.* **1989**, *111*, 1522.

69. Danishefsky, S.; Kitahara, T. *J. Am. Chem. Soc.* **1974**, *96*, 7807.

70. Danishefsky, S. J.; Uang, B. J.; Quallich, G. *J. Am. Chem. Soc.* **1985**, *107*, 1285.

71. Dardenne, G. A.; Larsen, P. O.; Wieczorkowska, *Biochim. Biophys. Acta.* **1975**, *381*, 416.

72. Reference 20. p. 107

73. a) Ganem, B.; Teng, C-Y. *J. Am. Chem. Soc.* **1984**, *106*, 2463.
b) Policastro, P. P.; Au, K. G.; Walsh, C. T.; Berchtold, G. A. *J. Am. Chem. Soc.* **1984**, *106*, 2443.

74. McCormick, J. D.; Reichenthal, J.; Hirsch, Ul; Sjolander, N. O. *J. Am. Chem. Soc.* **1962**, *84*, 3711.

75. Young, I. G.; Batterham, T. J.; Gibson, F. *Biochim. Biophys. Acta.* **1969**, *177*, 389.

76. Teng, C-Y.; Ganem, B.; Doktor, S. Z.; Nichols, B. P.; Bhatnagar, R. K.; Vining, L. C. *J. Am. Chem. Soc.* **1985**, *107* 5008.

77. Busch, F. R.; Berchtold, G. A. *J. Am. Chem. Soc.* **1983**, *105*, 3346.

78. a) Gibson, M. I.; Gibson, F. *Biochem. J.* **1964**, *90*, 248.
b) Cotton, R. G. H.; Gibson, F. *Biochim. Biophys. Acta.* **1965**, *100*, 76.

79. Gibson, F. *Biochem. Prep.* **1968**, *12*, 94.

80. Gibson, F. *Biochim. Biophys. Acta.*. **1964**, *90*, 256.

81. Andrews, P. R.; Hadden, R. C. *Aust. J. Chem.* **1979**, *32*, 65.

82. Andrews, P. R.; Smith, G. D.; Young, I. G. *Biochemistry* **1973**, *12*, 3492.

83. Sogo, S. G.; Widlanski, T. S.; Hoare, J. H.; Grimshaw, C. E.; Berchtold, G. A.; Knowles, J. R. *J. Am. Chem. Soc.* **1984**. *106*, 2701.

84. Addadi, L.; Jaffe, E. K.; Knowles, J. R. *Biochemistry*, **1983**, *22*, 4494.

85. Knowles, J. R.; Copley, S. D. *J. Am. Chem. Soc.* **1987**, *109*, 5008.

86. Pawlak, J. L.; Padykala, R. E.; Kronis, J. D.; Aleksejczyk, R. A.; Berchtold, G. A. *J. Am. Chem. Soc.* **1989**, *111*, 3374.

87. Knowles, J. R.; Copley, S. D. *J. Am. Chem. Soc.* **1985**, *107*, 5306.

88. Jackson, D. Y.; Jacobs, J. W.; Sugasawara, R.; Reich, S. H.; Bartlett, P. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 4841.

89. a) Risley, J. M.; Van Etten, R. L.; *J. Am. Chem. Soc.* **1980**, *102*, 6699.
b) Risley, J. M.; Van Etten, R. L.; *J. Am. Chem. Soc.* **1980**, *102*, 4609.
c) Risley, J. M.; Van Etten, R. L.; *J. Am. Chem. Soc.* **1979**, *101*, 252.

90. a) Vederas, J. C. *J. Am. Chem. Soc.* **1980**, *102*, 374.
b) Vederas, J. C.; Nakashima, T. T. *J. Chem. Soc. Chem. Commun.* **1980**, 183.

91. Vederas, J. C. *Nat. Prod. Rep.* **1987**, *4*, 277 and references cited within.

92. Edwards, J. M.; Jackman, L. M. *Aust. J. Chem.* **1965**, *18*, 1227.

93. Weische, A.; Johanni, M.; Leistner, E. *Arch. Biochim. Biophys.* **1987**, *256*, 212.

94. Ife, R. J.; Ball, L. F.; Lowe, P.; Haslam, E. *J. Chem. Soc. Perk. Trans. 1* **1976**, 1776.

95. Young, I. G.; Batterham, T.; Gibson, F. *Biochim. Biophys. Acta.* **1968**, *165*, 567.

96 Berchtold, G. A.; Busch, F. R. *J. Am. Chem. Soc.* **1983**, *105*, 3346.

97. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.*, **1951**, *193*, 265.

98. Culture media and fermentation conditions were obtained from L. Schlecta. See also ref 15.