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THE SYNTHESIS OF PEPTIDES AND DEPSIPEPTIDES RELATED TO ESPERIN

JAMES RICHARD HUSMAN

THE SYNTHESIS OF PEPTIDES AND DEPSIPEPTIDES RELATED TO ESPERIN

by

JAMES R. HUSMAN

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This thesis has been examined and approved.

James D. Morrison

Thesis Director, James D. Morrison
Professor of Chemistry

Kenneth K. Andersen

Kenneth K. Andersen, Professor of
Chemistry

Colin D. Hubbard

Colin D. Hubbard, Associate
Professor of Chemistry

Paul R. Jones

Paul R. Jones, Professor of
Chemistry

Samuel C Smith

Samuel C. Smith, Professor of
Animal Sciences

May 6, 1976

Date

This thesis is dedicated to my wife, Julie,

to my son, Daniel,

and to my parents.

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ABSTRACT

THE SYNTHESIS OF PEPTIDES AND
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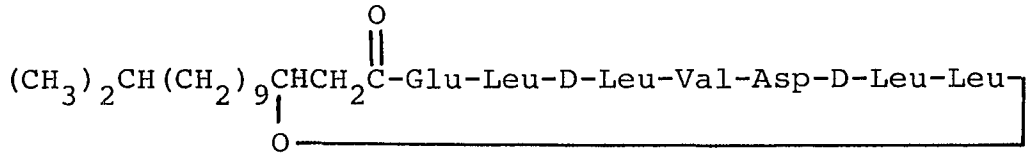
The synthesis and resolution of 3-hydroxy-13-methyltetradecanoic acid is described. A general synthesis of β-hydroxy acids involving the treatment of epoxides with lithium dialkylcuprates was developed.

A stepwise and fragment condensation approach to the peptide sequence corresponding to esperin was carried out. The preparation of depsipeptides useful as synthetic intermediates for esperin synthesis is described.

HISTORICAL

Surfactin

Incompletely characterized hemolytic agents from aerobic sporogenic bacilli were first reported in the 1950's.[1,2] Arima and coworkers succeeded in isolating and identifying a crystalline cyclodepsipeptide which they called surfactin from the culture medium of Bacillus subtilis IAM 1213.[3-10] Bernheimer and Avigad reported a substance with similar properties from B. subtilis which they called subtilysin.[11] Surfactin and subtilysin proved to be identical and the former name has been retained.



Surfactin 1

Surfactin was isolated in yields of 40-50 mg per liter of culture medium after a purification procedure involving precipitation of surfactin calcium salt, conversion to the free acid and chromatography of the crude product on Sephadex G-50 and Sephadex LH-20. After crystallization from 1:1 acetone-water, surfactin was obtained as a white crystalline solid, mp 138-140°, $[\alpha]_D^{\underline{}}$ 40° [c1, $CHCl_3$] and $[\alpha]_D^{\underline{}}$ -39° [c1, CH_3OH].

Surfactin displays a remarkably wide range of physical and biological properties. It is a highly surface active agent, lowering the surface tension of water in dilute basic solution more than twice as much as an equivalent amount of sodium lauryl sulfate.[4] This strong surface activity is presumably the result of the ambiphilic nature of the surfactin structure. The long alkyl portion of the fatty acid and the side chains of the leucine and valine residues are lipophilic while the depsipeptide ring system and the ionizable side chains of aspartic and glutamic acids are hydrophilic. This ambiphilic nature is thought to be a contributing factor to several of surfactin's biological properties.

Buffered solutions of surfactin were found to lyse erythrocytes from several species including rabbit, man, and guinea pig.[11] Hemolytic activity was accentuated by several divalent cations and was inhibited by serum or phospholipids. A quantitative assay for surfactin was developed based on the amount of hemolysis in red cell suspensions.[11]

Surfactin displays bacteriolytic and antibiotic activity toward a number of organisms.[8,9,11] A variety of Gram-positive bacteria protoplasts were lysed; among Gram-negative bacteria, spheroplasts of Escherichia coli were lysed while those of Vibrio comma were not. The growth of Streptococcus pyogenes, Carynebacterium diptheria, and Bacillus megaterium was completely inhibited by surfactin

at concentrations lower than 20 μg/ml.[11] Two other cytolytic toxins of bacterial origin, streptolysin S and staphylococcal δ-toxin, display similar properties.[11] These agents appear to interact in some specific manner with the phospholipids in cell membranes disrupting the semipermeability of the membrane so that osmotic swelling ultimately produces lysis. Surfactin has been shown to inhibit certain membrane related processes, specifically glucose oxidation and alkaline phosphatase synthesis in B. megaterium.[8] While alkaline phosphatase synthesis was disrupted, surfactin had no effect on the activity of the enzyme. Phospholipids from membrane fractions were shown to inhibit the protoplast-bursting effect.[9] A specific interaction between surfactin and membrane phospholipids was thus suggested.

Surfactin displays strong anticoagulant properties.[3] The clotting time, as measured by opacity development, in a system comprised of 0.15% fibrinogen, 5 units/ml of thrombin, and a pH 7.4 tris-sodium chloride buffer at 37° was increased dramatically by low concentrations of surfactin. Initially it was suggested that surfactin extended the range of plasmin, a fibrinolytic enzyme, by altering the enzyme-substrate environment.* However, in vitro studies in the absence of

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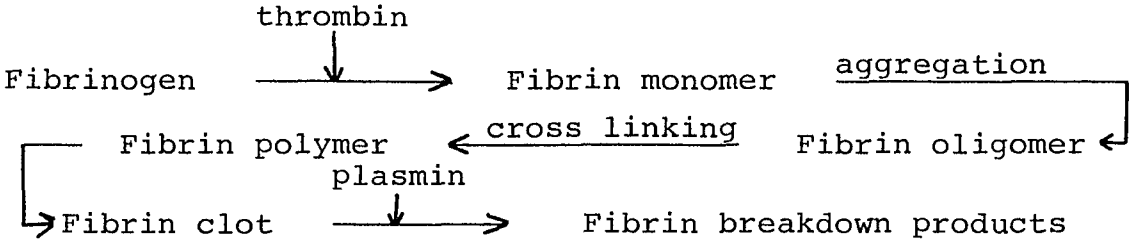
*The blood clotting system is represented in simplified form by the following diagram:



plasmin showed a 50% inhibition of thrombin activity at surfactin concentrations of 30 μg/ml. Since thrombin proteolytic activity toward fibrinogen was not inhibited, it was suggested that surfactin acted by solubilizing fibrin oligomers and thus retarding polymer formation.[4] Other detergents are known to inhibit clotting by a similar mechanism.[12]

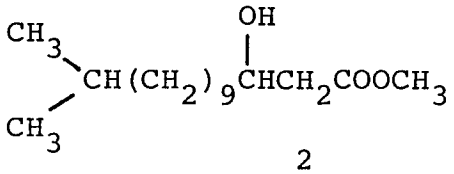
A hypocholesterolemic effect of surfactin has been found in rats and chickens.[13] When orally administered, surfactin lowered significantly both plasma and liver cholesterol levels at a dose of 100 mg/kg/day in rats. No stoichiometric binding of surfactin to cholesterol was observed, in contrast to β-sitosterol, a naturally occurring inhibitor of cholesterol adsorption, which binds cholesterol at a 1:1 molar ratio.[14] ^{14}C-Labeled surfactin was not absorbed in the gastrointestinal tract but was completely excreted in the feces. Since the critical micelle concentration of surfactin is 5-10 μg/ml and the interaction between surfactin and cholesterol begins at approximately 2-20 μg/ml of surfactin regardless of cholesterol concentration, it has been suggested that a physical interaction such as the inclusion of cholesterol molecules into the micelle structure of surfactin is the operative principle.

Surfactin has been shown to inhibit thermal denaturation in proteins.[7] Solutions of bovine serum albumin containing as little as 0.005% surfactin showed substantially

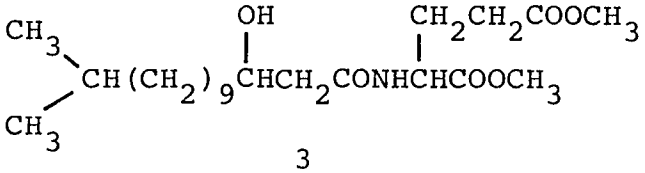
less denaturation than controls containing no surfactin.

Structural studies on surfactin began with the determination of the amino acid composition of the peptide lipid. It was shown to consist of L-aspartic acid, L-glutamic acid, L-valine, L-leucine, D-leucine, and a fatty acid in the proportions 1:1:1:2:2:1.[4] The fatty acid was reported to be a C_{15} hydroxy iso acid.[3] The amino acid sequence was determined by Edman degradation of 13 peptide fragments obtained from partial hydrolysis.[5,15] The configurations of the leucine residues were determined enzymatically, using D-amino acid oxidase from pig kidney, by comparing the amino acid composition of peptide fragments before and after enzyme action. The peptide sequence was determined to be L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu. Successful application of the Edman technique precluded the possibility of ω-carboxyl participation in peptide bonds by the aspartic or glutamic acid residues.

The structure of the fatty acid in surfactin was determined by methanolysis followed by separation of a petroleum ether soluble fraction.[6] Column chromatography of this function gave two products, a fatty acid methyl ester and a dimethyl N-acyl glutamate. Elemental, ir, nmr, and mass spectrometric analyses of these fractions identified the fatty acid ester as methyl-3-hydroxy-13-methyltetradecanoate (2).

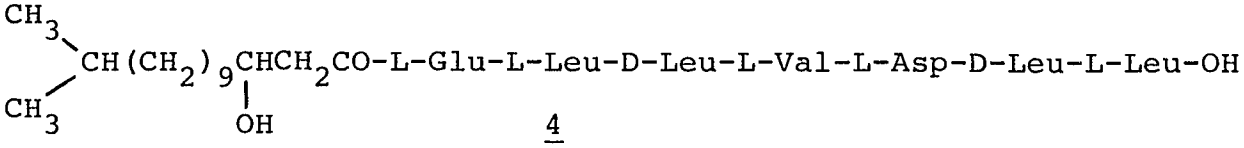


2



3

The dimethyl N-acyl glutamate was shown to have structure 3. This latter fact, together with the fact that surfactin does not react with ninhydrin, confirmed an amide linkage between the fatty acid and glutamic acid. A total of 60% of the theoretical amount of fatty acid was accounted for by these two products. The presence of trace amounts of presumably C_{16} and C_{14} β-hydroxy iso acids was demonstrated by gas chromatographic analysis. Since some separation of homologous or desmethyl variants of the fatty acid may have occurred during the chromatographic purification, the exact composition of the fatty acid residue(s) in surfactin remained open to question. The total primary sequence for surfactin was thereby established as 4.



4

Although surfactin was not acylated by acetic anhydride in pyridine, it was converted by treatment with mild alkali to

a compound which was readily acylated under the same conditions. The presence of a lactone bond was thus suggested but the problem of which acid group was involved in lactone formation remained to be solved.

Surfactin was reduced with lithium borohydride in tetrahydrofuran (only ester bonds are reduced) in three ways: without any pretreatment, after methylation with diazomethane in ether, and after treatment with 0.5 N sodium hydroxide for one hour at room temperature.[10] The products obtained were hydrolyzed and subjected to amino acid analysis. When surfactin was reduced without pretreatment, one of the four leucine residues disappeared; when surfactin was methylated prior to reduction, glutamic and aspartic acid and one of the four leucine residues disappeared; and when the lactone bond was opened with dilute base prior to reduction, no change in amino acid composition was noted. These observations implicated the C-terminal leucine residue as the carboxyl component of the lactone bond. The total structure of surfactin was thus established as 1. This structure was later confirmed by mass spectral analysis.[16]

Esperin

Esperin, a potent antibiotic toward *Mycobacterium tuberculosis* with a structure remarkably similar to surfactin, was first reported in 1950.[17] The exact structure of espirin is still open to question and the literature contains several

ambiguities concerning both esperin and a derivative, esperinic acid. Esperin was reported to be produced from cultures of Bacillus mesentericus but even questions concerning the nature of the producing organism have arisen. These and other questions will be discussed later.

The initial report on esperin included some preliminary structural analysis.[17] Acid hydrolysis of the antibiotic produced a long chain unsaturated fatty acid of C_{14} to C_{16} chain length. Amino acid components, as determined by paper chromatography, consisted of D,L-leucine, L-aspartic acid, and L-valine. No other amino acids were detected. A compound called esperin X, mp 195°, was obtained from esperin by treatment with strong base. Esperin X was reported to show less toxicity and less activity against Mycobacterium tuberculosis than esperin itself. Subsequently, a series of Japanese patents appeared outlining esperin's antibiotic activity.[18,19,20] Growth of M. tuberculosis was inhibited at esperin dilutions of 1:500,000 and the microorganism was reported to "dissolve" at dilutions of 1:100,000.[18] Alkaline hydrolysis of crude esperin followed by acidification of the sodium salt gave a new compound (mp 105°) which inhibited the growth of M. tuberculosis at a dilution of 1:600,000. Based on the manner of preparation, this latter compound would appear to be what has subsequently been referred to as esperinic acid I (mp 195°).[21] The fatty acid component of esperin was isolated by acid hydrolysis and identified by derivatization and chemical modification as trans-2-

tridecenoic acid.[22] No higher or lower homologs were reported. This remained the extent of structural studies until 1959 when Ito and Ogawa published a complete structure for esperin and esperinic acid.[21] This report has generated considerable controversy and will therefore be discussed in detail here.

Esperin was obtained in crystalline form by repeated precipitation from acetone-petroleum ether followed by crystallization from isopropanol-petroleum ether. The purified antibiotic had $[\alpha]^{15}\underline{D}$-24° (c 0.66, CH_3OH) and mp 238°. Esperinic acid, obtained from esperin by treatment with strong base, had $[\alpha]^{15}\underline{D}$-12.5° and mp 195°. The sodium salt of esperinic acid had mp 269°. Based on elemental analysis and a rough determination of molecular weight (730-800 in camphor by micro-Rast method), the empirical formula for esperin was estimated to be $C_{39}H_{67}N_5O_{11}$. Titration data indicated that esperin was a dibasic acid and esperinic acid a tribasic acid. Esperin was determined to contain an ester function, absent in esperinic acid, while esperinic acid contained a hydroxyl moiety, absent in esperin. It was concluded that esperin contained a lactone bond which was opened on treatment with base to give esperinic acid. The amino acid composition of esperin was reexamined by ascending paper chromatography in n-butanol, acetic acid, and water (4:1:2.5). The presence of leucine, valine, aspartic acid, and a new amino acid, glutamic acid, previously not dis-

covered in esperin hydrolysates, was reported. The amino acid ratios were determined by photoelectric transmission densitometer measurements of the paper chromatograms. The reported ratio was leucine 2, valine 1, glutamic acid 1, and aspartic acid 1. The leucine obtained by hydrolysis was racemic whereas the other amino acids were of the L configuration.

The C-terminal amino acid in esperin was identified by two different methods. Treatment of esperin with anhydrous hydrazine resulted in the hydrazinolysis of all peptide bonds (the carboxyl group of the C-terminal amino acid is not attacked).[23] Treatment of the hydrazinolyzed product with 2,4-dinitrofluorobenzene resulted in the formation of bis(dinitrophenyl) derivatives of the hydrazides and an N-dinitrophenyl amino acid from the C-terminal residue (Figure 1). The latter was separated by extraction into aqueous base and identified by paper chromatography as N-(2,4-dinitrophenyl)leucine. This identification was confirmed by Dakin-West degradation of the peptide and the isolation of 3-amino-5-methyl-2-hexanone by paper chromatography (Figure 2).[24] The configuration of the C-terminal residue was determined by treating esperin and esperinic acid with carboxypeptidase. No change in amino acid composition was noted and it was concluded that the C-terminal leucine residue had the D-configuration resulting in resistance to enzyme degradation. This was apparently confirmed

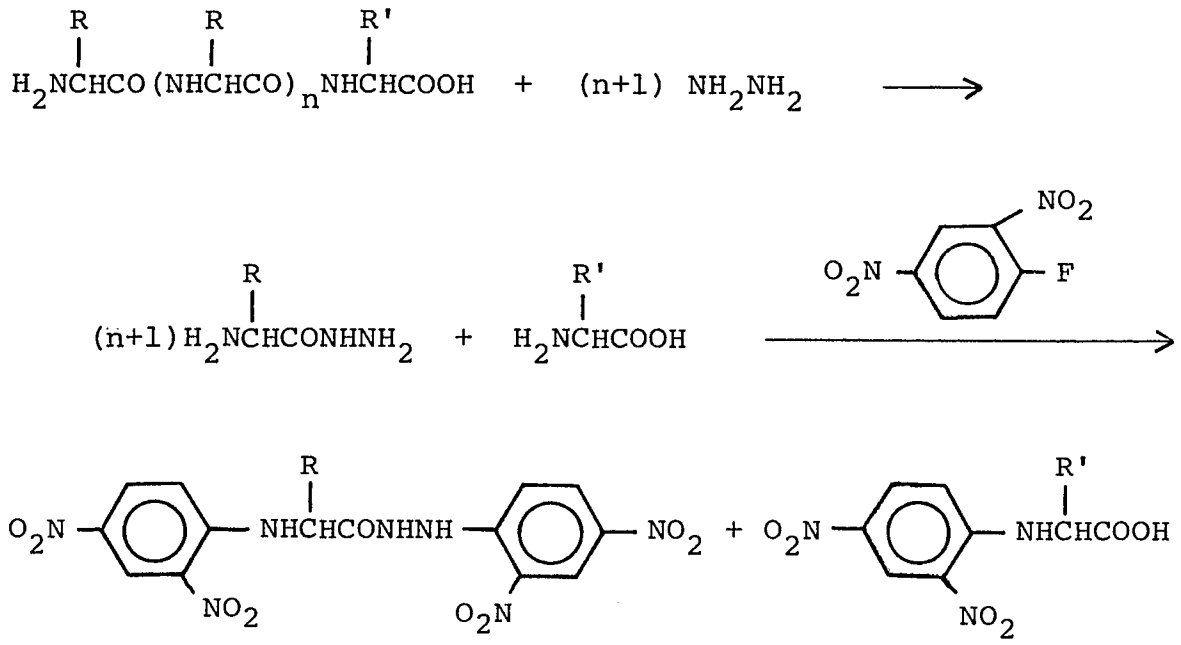


Figure 1. The Identification of C-Terminal Residues by Hydrazinolysis-dinitrophenylation.

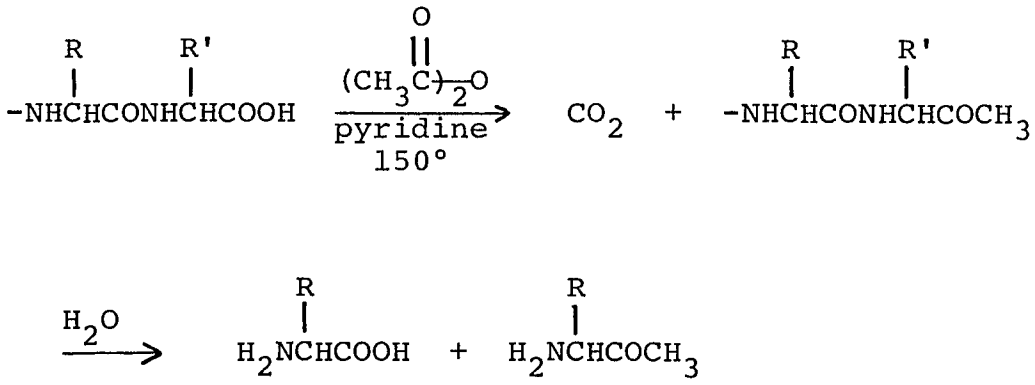
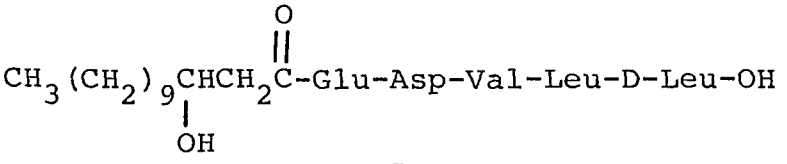


Figure 2. The Identification of C-Terminal Residues by Dakin-West Degradation.

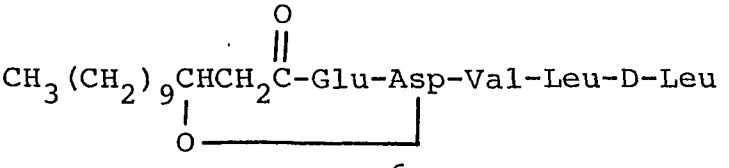
by isolation of L-enriched leucine after Dakin-West degradation of esperin.

The results of partial hydrolysis experiments indicated that the fatty acid residue was linked to glutamic acid. The trans-2-tridecenoic acid isolated earlier was determined to have arisen from dehydration of 3-hydroxytridecanoic acid during the acid hydrolysis. Overlap of isolated peptide hydrolysates indicated the structure of esperinic acid was given by 5.



5

Finally, the position of the lactone bond was determined by hydrazinolysis of esperin followed by a Curtius reaction and hydrolysis (Figure 3). The identification of 2,3-diaminopropionic acid by paper chromatography and the disappearance of aspartic acid in the hydrolysate appeared to confirm the structure of esperin as 6.



6

Compounds with a cyclotridepsipeptide structure had not previously been found in nature.

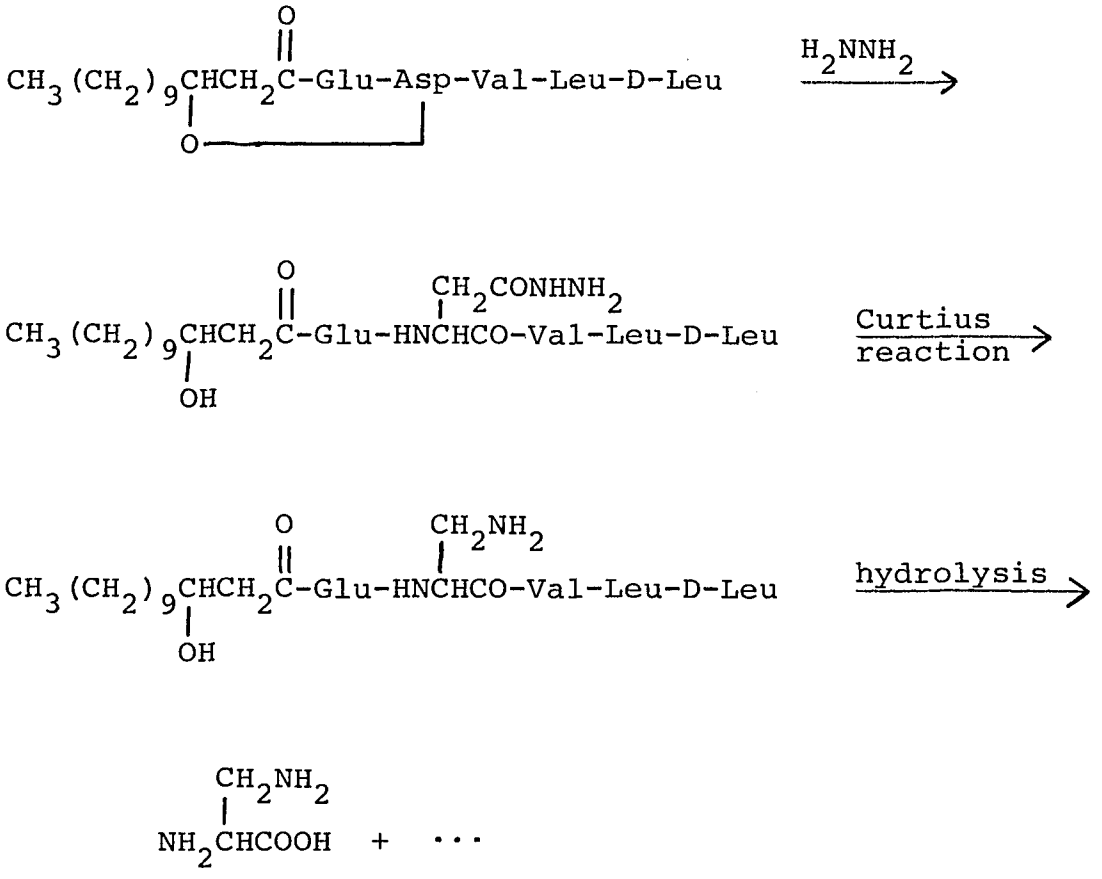


Figure 3. Determination of the Position of the Lactone Bond in Esperin.

This structure remained unchallenged in the literature for seven years until publication of a series of papers by Shemyakin, Ovchinnikov and coworkers giving synthesis-based evidence in opposition to structure 6 for esperin.[25-27] Stating that "certain data of the Japanese authors appeared not very convincing" the Soviet group began by synthesizing 5 with both D- and L-hydroxy acid residues (Chart 1).[25] This product, hereafter referred to as pseudoesperinic acid, had $[\alpha]^{20}\underline{D}$-30° (c 1.6, CH_3OH), mp 181-182° for the L-hydroxy acid derivative and $[\alpha]^{20}\underline{D}$-46° (c 1.6, CH_3OH), mp 181-182° for the D-hydroxy acid derivative. These values are in contrast to those previously reported for esperinic acid: ($[\alpha]^{15}\underline{D}$ 12.5° (c 1.6, CH_3OH and mp 195°).[21] In addition, pseudoesperinic acid was devoid of antibiotic activity against all of the microorganisms tested. The authors concluded that esperinic acid possessed a structure other than 5 and that the reported structure of esperin (6) was therefore also doubtful.

This last supposition was verified in 1968 when the Soviet group reported the total synthesis of 6, hereinafter referred to as pseudoesperin (Chart 2).[27] The cyclization step leading to the intermediate protected cyclotridepsipeptide was successful only with the L-hydroxy acid series. When the same conditions were applied to the D-hydroxy acid derivative, polymer and recovered starting material were the only materials isolated. The physical properties reported for 6 were $[\alpha]^{20}\underline{D}$-30° (c 1, CH_3OH) and mp 238-241° compared

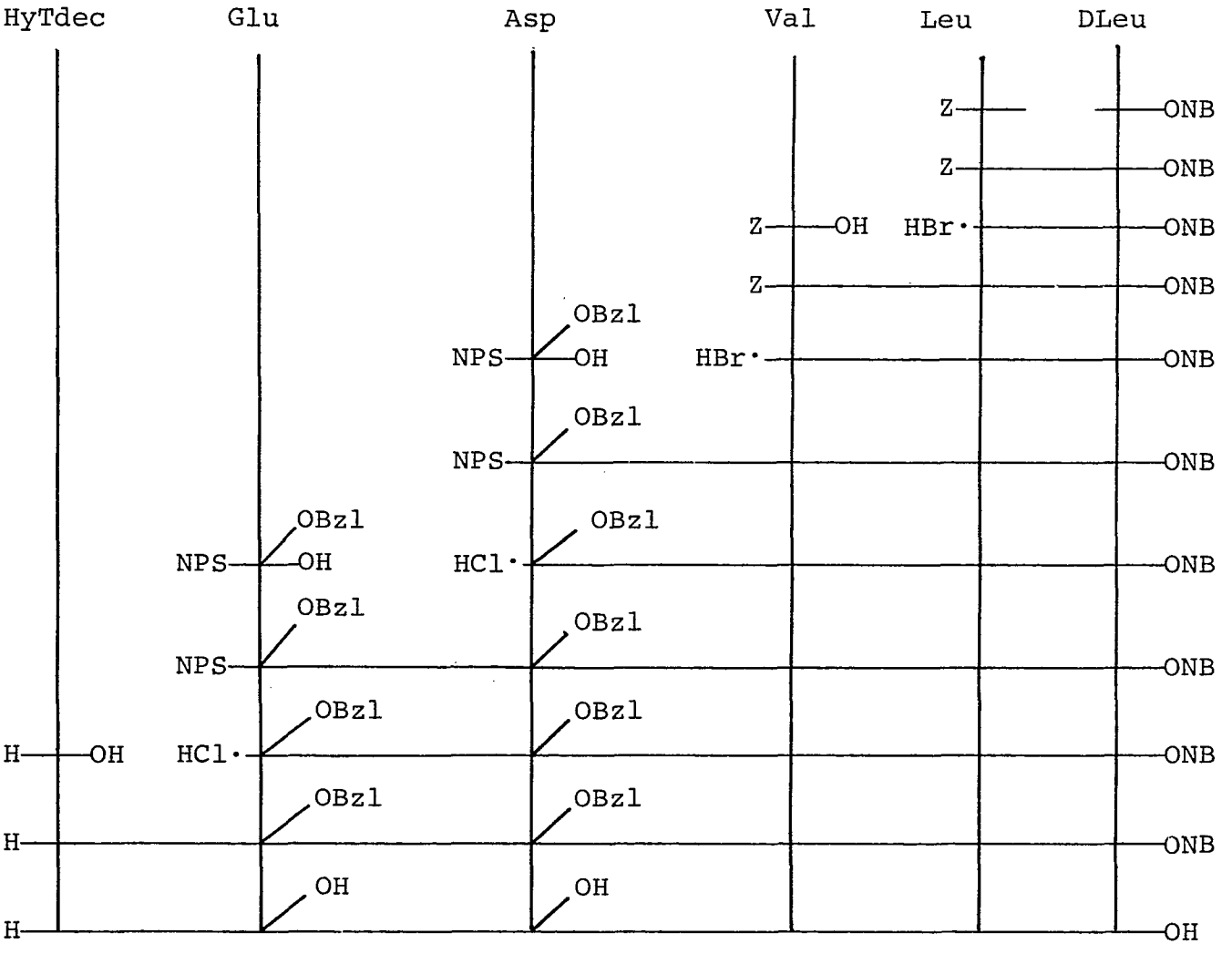


Chart 1. Synthesis of Pseudoesperinic Acid.

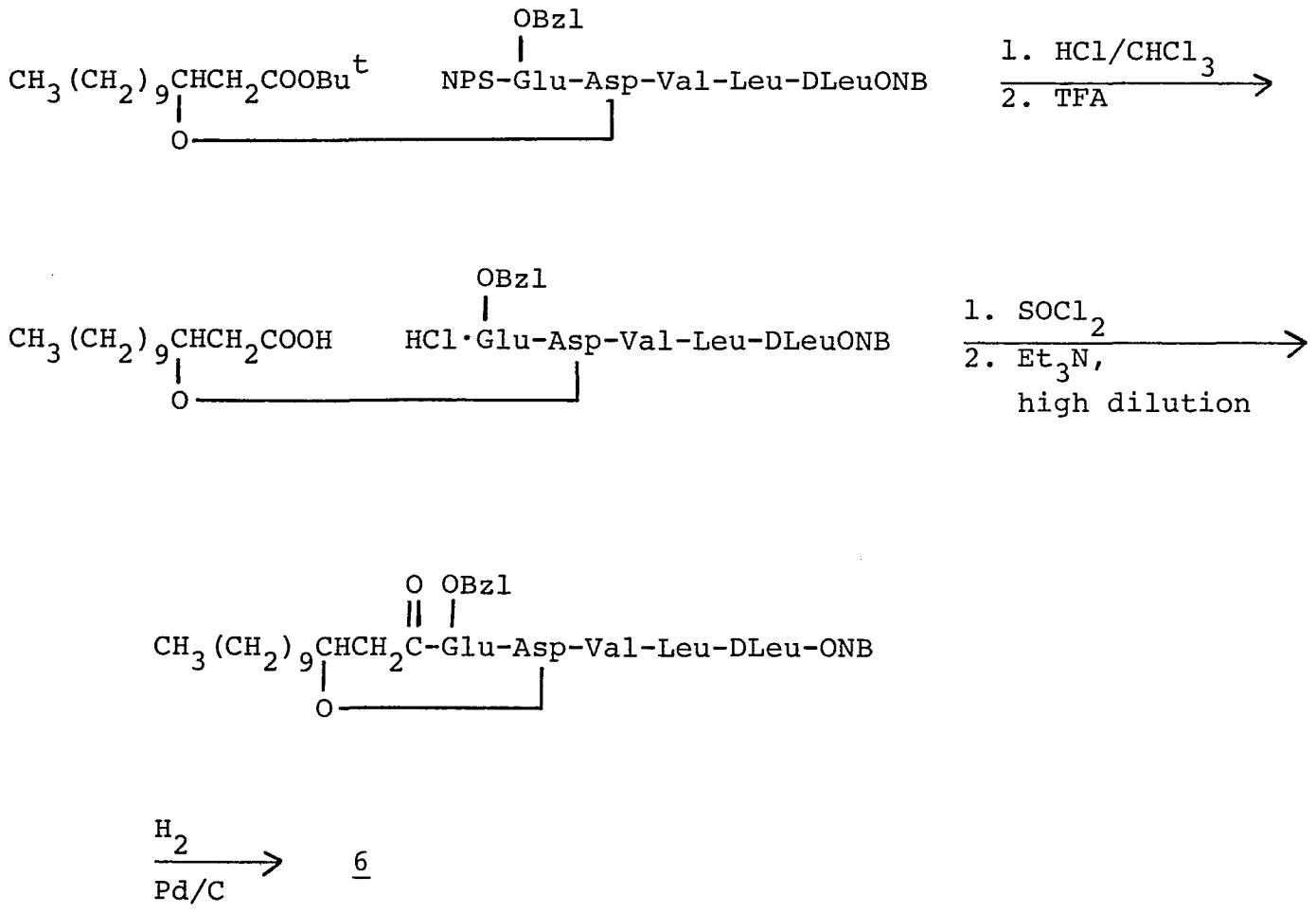


Chart 2. Synthesis of Pseudoesperin.

with $[\alpha]^{20}\underline{D}$-24° (c 0.66, CH_3OH) and mp 238° for natural esperin. The two compounds had different chromatographic behaviors on silica gel and widely different ORD curves:

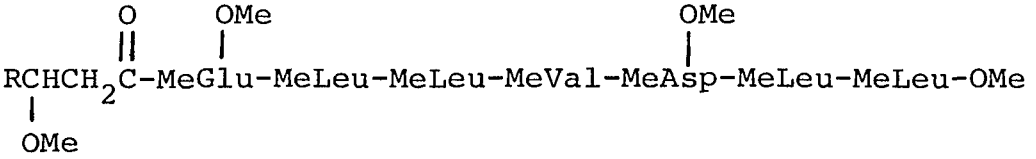
	6	natural esperin
$[\alpha]_{266}$	-940°	-320°
$[\alpha]_{250}$	-2200°	-480°
$[\alpha]_{239}$	-3200°	-740°
$[\alpha]_{215}$	5500°	810°

On treatment with 0.4 N sodium hydroxide 6 was converted to pseudoesperinic acid (5) verifying that the initial structural assignments for the antibiotic and its derivatives were incorrect.

These results led to a reinvestigation of the structure of esperin by mass spectroscopy.[28] The acid hydrolysis product, originally thought to be 2-tridecenoic acid ($C_{12}H_{23}COOH$), was found to consist of at least three homologs: $C_{12}H_{23}COOH$, $C_{13}H_{25}COOH$, and $C_{14}H_{27}COOH$ representing 20, 35, and 45% of the total fatty acid product, respectively. Under hydrolytic conditions which did not result in dehydration, the position of the hydroxyl group was determined by the observation of a major peak at m/e 103 from the ion $^{+}CHOHCH_2COOCH_3$. It was now reported that two different products could be obtained by treatment of esperin with base. Hydrolysis with 1.5-2% sodium hydroxide gave an acid

derivative, previously called esperinic acid and now called esperinic acid I, with [α] D 12.5°. Hydrolysis under milder conditions (0.1 N sodium hydroxide) gave esperinic acid II with [α] D-13°. Esperinic acid I gives a positive Cotton effect in methanol whereas esperinic acid II (and esperin) gives a negative Cotton effect. The authors concluded that the two compounds differed only in the configuration of the hydroxy acid, esperinic acid II having the same configuration as esperin. In support of this conclusion the statement was made that "a similar base-catalyzed inversion has also been observed with the mycolic acids."[29]

The mass spectrum of esperin itself was too complex to provide significant structural information. However, when esperinic acid II (no mp reported) was permethylated by treatment with methylsulfinyl carbanion and methyl iodide, a compound giving a much simplified spectrum was obtained.[30] The spectrum revealed that esperinic acid was an acylheptapeptide and not a pentapeptide derivative as had previously been suggested. The methylation product consisted of three methoxyacyl homologs (7) as well as an additional three unsaturated homologs (8) representing the loss of methanol from the methoxyacyl residue.



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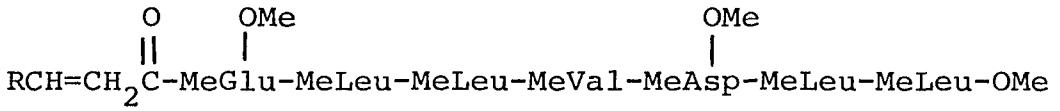
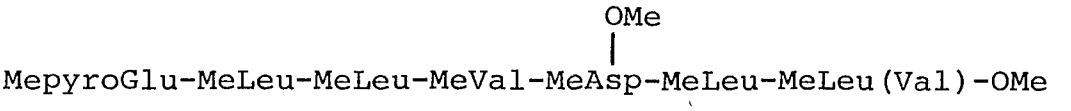


8

R = $C_{12}H_{25}$, $C_{11}H_{23}$, $C_{10}H_{21}$

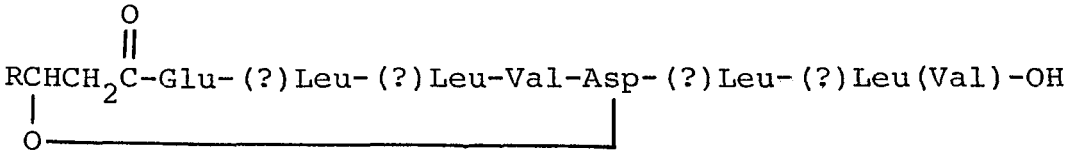
From the relative peak intensities it was concluded that approximately 30% of esperinic acid II contained a C-terminal valine variant. This was confirmed by the mass spectrum of the partial hydrolysis product 9.



m/e	253	380	493	636	793	890 (876)	921 (907)

9

Since the mass spectrometric molecular weight of esperin dimethyl ester (1063 and lower homologs) was consistent with a lactone structure, and since hydrazinolysis, Curtius rearrangement, and hydrolysis of esperin had transferred the aspartic residue to 2,3-diaminopropionic acid[21], the complete composition of esperin was given as 10.



$R = C_{12}H_{25}$ (45%)

$R = C_{11}H_{23}$ (35%)

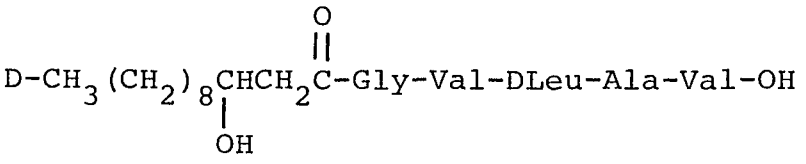
$R = C_{10}H_{21}$ (20%)

Esperin 10

The configurations of the leucine residues and the hydroxy acid remained unspecified since they, of course, could not be determined by mass spectrometry.

Several problems in the assignments of total structures to esperin peptides and esperinic acids I and II immediately became apparent. For example, the configurations of the four leucine residues are not known. All that is known with certainty is that the ratio of D- to L-leucine is 1:1. No information concerning the internal leucylleucine couplet is available, and some of the information concerning the C-terminal couplet is questionable. It will be recalled that the assignment of the D-configuration to the C-terminal leucine was based in large measure on the resistance of esperin to the action of carboxypeptidase. This resistance was taken to imply a C-terminal D-amino acid. Similarly, in studies on the structure of isariin, release of only valine and no other amino acids by the action of carboxypeptidase on isariic acid (11) was used as evidence for a D-leucine

residue at the penultimate position.[31] However, it was subsequently shown by the synthesis of isariic acid that this interpretation was incorrect.[32]



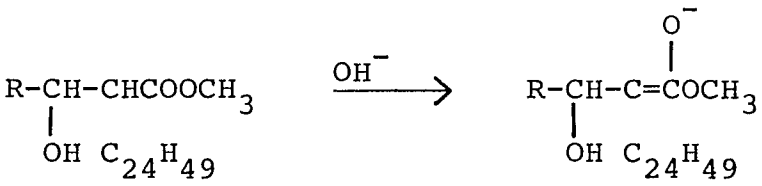
Isariic Acid 11

This confirmed the original supposition of Vining and Taber that the fragment terminating in D-Leu-Ala would be resistant to carboxypeptidase degradation.[33] It is apparent that the same principle may be operative in the case of esperin in which case the C-terminal leucine couplet would have the same configurational order as that found in surfactin. The fact that esperin contains a C-terminal L-valine variant further weakens the assignment of a D-configuration to the C-terminal leucine. Since all of the valine found in esperin was of the L-configuration, at least 30% (the extent of the valine variant composition) of esperin contains an L-amino acid as the C-terminus. It seems extremely unlikely that a configurationally disparate leucine C-terminal variant would be produced in the biosynthesis of the esperin peptides. Finally, the claimed isolation of Leu-D-Leu from partial hydrolysates by the original investigators does not shed any light on the exact leucine configurations since this compound would be indistinguishable from its enantiomer D-Leu-

Leu under the analytical conditions employed. The evidence does suggest, however, that the leucines are found in D,L or L,D pairs and not as D,D or L,L pairs.

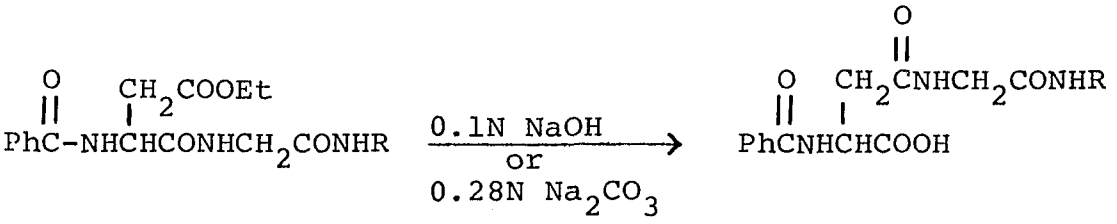
The configuration of the hydroxy fatty acids in esperin has not been determined but all of the β-hydroxy fatty acids occurring in cyclodepsipeptides found thus far have the D-configuration.[34] The related antibiotic viscosin also contains a β-hydroxy fatty acid of the D-configuration.[35] In view of this, the most likely assignment in the case of esperin is D, although the L-isomer cannot be rigorously ruled out.

It will be recalled that the suggested difference between esperinic acid I and esperinic acid II was the configuration of the fatty acid residue. The supporting evidence cited, the base-catalyzed isomerization of mycolic acids[36], apparently involves an epimerization of the asymmetric α-carbon atom via an enolate intermediate:



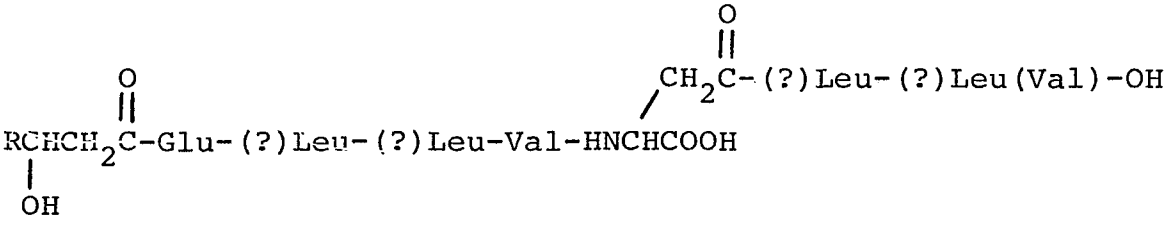
This mechanism is supported by the observation that isomerization occurs only with the esters and not with the free acid.[37] However, a mechanism of this type is not operative in the case of esperin since the asymmetric center concerned is in the β-position. A far more probable explanation for the existence of esperinic acids I and II is that they differ

in the structure of the peptide chain itself. The rearrangement of aspartyl peptide esters upon exposure to alkaline hydrolysis conditions is a well known phenomenon.[38] Battersby and Robinson showed that in the case of benzoyl-DL-aspartyl-α-glycine n-hexylamide β-ethyl ester that the β-isomer is formed predominantly under quite mild conditions[39]:



65%

The succinimido intermediate was isolated by interrupting the reaction before its completion. The possibility of a similar rearrangement during esperin hydrolysis is further supported by the well known resistance of sterically hindered esters to hydrolysis under basic conditions. One of the esperinic acids, or both, may therefore have a β-peptide structure (12).



Esperinic Acid ? 12

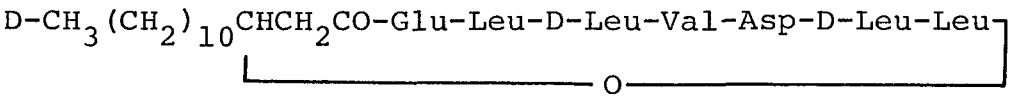
The difference in Cotton effects noted for the esperinic acids is more easily explained by the existence of β-peptide structure than it is by a simple inversion at a single asymmetric center. Indeed, in the case of isariic acid, the L-hydroxy acid derivative gave [α] $\underline{D}$-4.5° ($\underline{c}$ 4, CH_3CH_2OH) and -2.7° ($\underline{c}$ 3, pyridine), whereas the D-hydroxy acid derivative gave [α] $\underline{D}$-5.2° ($\underline{c}$ 5, CH_3CH_2OH) and -9.6° ($\underline{c}$ 5, pyridine). Finally, the mass spectrometric technique employed in the structural determination of esperinic acids I and II would not be expected to distinguish between α- and β-peptides since fragmentation occurs principally at the amide bonds which would result in identical fragments.

As will be recalled, the producing strain of esperin was reported to be Bacillus mesentericus. It now appears that this taxonomic assignment was incorrect (either by accident or design) and it has been suggested that the actual organism was either Bacillus pumilus or Bacillus subtilis.[40,41] The original investigator appears now to have lost the producing strain.[42]

The similarity in the reported structures of esperin and surfactin is quite striking. Neglecting configurations, the amino acid sequences are identical, and as was noted earlier, even the configurations may be the same. Thus the compounds appear to differ in only two ways: the structure of the fatty acid residue is normal in esperin and iso in surfactin and the lactone bond is formed by the third func-

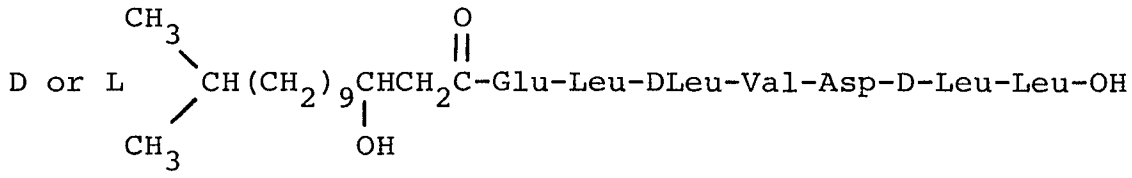
tion of aspartic acid in esperin and by leucine in surfactin. It is not inconceivable that esperin and surfactin do not even differ in this latter respect since an opening and reclosing of the lactone bond could occur during the purification procedures used. This latter possibility is particularly enticing in view of the possible identity of surfactin and esperin producing strains as B. subtilis.

A successful synthesis of norsurfactin (13), an analog of surfactin containing a normal hydroxy acid side chain, has recently been carried out.[43]



Norsurfactin 13

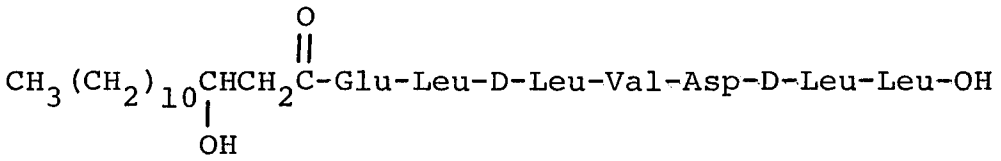
Norsurfactin was shown to possess anticoagulant and hemolytic activity similar to surfactin. This result is not surprising since little change in biological activity would be expected to result from very minor changes in the fatty acid residue. It was felt desirable, however, to prepare sufficient quantities of 3-hydroxy-13-methyltetradecanoic acid to permit resolution of the optical antipodes. Synthesis of the heptapeptide sequence in surfactin would then provide material for an acylation reaction leading to D- or L-surfactinic acid (14).



Surfactinic Acid 14

The synthetic approach to surfactinic acid has the advantage of easily allowing changes in the fatty acid portion of the molecule. This provides a means of determining some structural information concerning esperin.

The problem of establishing a structure for a compound as elusive as esperin is formidable. At least three fatty acid variants exist each of which may occur with the C-terminal valine variant giving six different isomers. Since the natural material is not available, it is impossible to determine which of these compounds or group of compounds is responsible for esperin's potent antibiotic activity. It is not unreasonable to assume, however, especially in view of the results obtained with norsurfactin, that no substantial change in biological activity would result from changes in the fatty acid chain length of one carbon atom. Thus a 3-hydroxytetradecanoic acid derivative was the initial target. Since the peptide sequences, except for configurations, are the same in surfactin and esperin, an initial approach to esperinic acid could be made by acylation of the same heptapeptide to provide 15.



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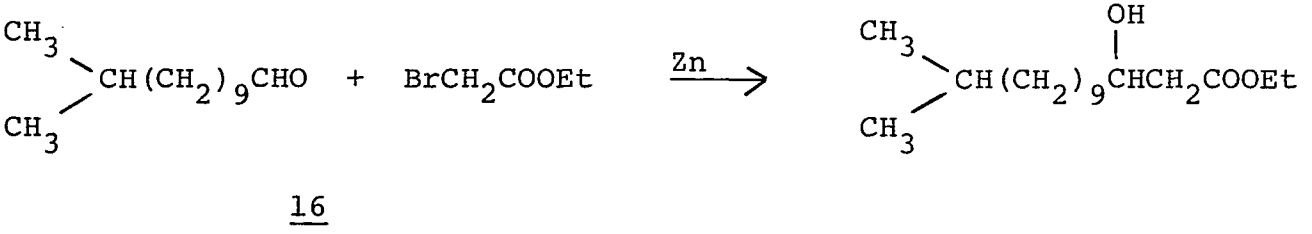
A comparison of physical and biological properties of 15 with those reported for esperinic acid could be used to either confirm or rule out the possibility that surfactin and esperin have identical amino acid sequences. In addition, comparison could be made between the iso and normal chain compounds providing some structure-activity information. An investigation of synthetic routes to esperin was also thought desirable since very few procedures for the synthesis of β-hydroxy acid cyclodepsipeptides have been developed.

RESULTS AND DISCUSSION

β-Hydroxy Acid Synthesis

Previous work on the synthesis of surfactin was limited by the unavailability of the branched chain fatty acid 3-hydroxy-13-methyltetradecanoic acid (17). A synthetic pathway which would provide sufficient hydroxy acid for an optical resolution was required since the configuration of the residue in surfactin was not known with certainty. One of the oldest and most convenient methods for the preparation of β-hydroxy esters (and hence β-hydroxy acids) is the Reformatsky reaction: an aldehyde or ketone reacts with the halozinc enolate formed from ethyl bromoacetate and zinc metal.[44,45] The synthesis of 17 then required the synthesis of the long chain isoaldehyde 16 (Figure 4). Since a large number of synthons for aldehyde functions exist, the principle problem in the synthesis of 16 was the elongation of a short branched chain precursor. Several functionalized multicarbon homologation reactions have recently been introduced involving the use of organoboranes.[46,47] The construction of branched chain functional derivatives, however, is cumbersome and is usually accompanied by low yields. A newer method involving the coupling of Grignard reagents with α,ω-dibromoalkanes in the presence of dilithium tetrachlorocuprate was introduced as an alternative procedure.[48]



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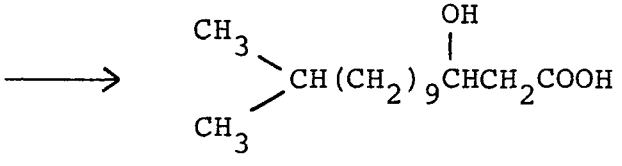


17

Figure 4. The Synthesis of 3-Hydroxy-13-methyltetradecanoic Acid (17).

A more traditional method was employed in the present case: the Kolbe anodic oxidation reaction.[49,50] This reaction developed from research aimed at the generation of organic free radicals by the oxidation of carboxylate ions at a platinum electrode. The principal product of the reaction is the dimerized free radical (Figure 5). This reaction has been applied to a wide range of compounds and was utilized by Corey in the first total synthesis of pentacyclosqualene (Figure 6).[51]

A modification of the reaction using two different carboxylate ions allows its application to the synthesis of unsymmetrical compounds. This crossed-Kolbe reaction has been used to synthesize a wide variety of branched chain fatty acids. In practice, the starting acids are chosen so that the symmetrically coupled products RR and R'R' are easily separable from the desired cross-coupled product RR' (Figure 7).

An improved method for crossed-Kolbe reactions involves the use of one of the acid components in large excess in order to suppress the formation of the symmetrically coupled product from the other acid.[52] In the present study 5-methylhexanoic acid and methyl hydrogen suberate in a ratio of 3:1 were coupled in a large scale crossed-Kolbe reaction resulting in a 51% yield of isoester 18 (Figure 8). This ester was previously prepared in only 15% yield when the coupling was carried out without an excess of the 5-methylhexanoic acid.[53]

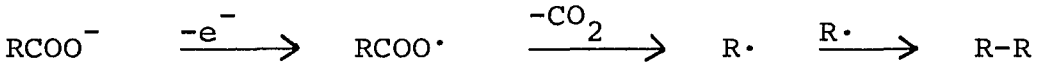
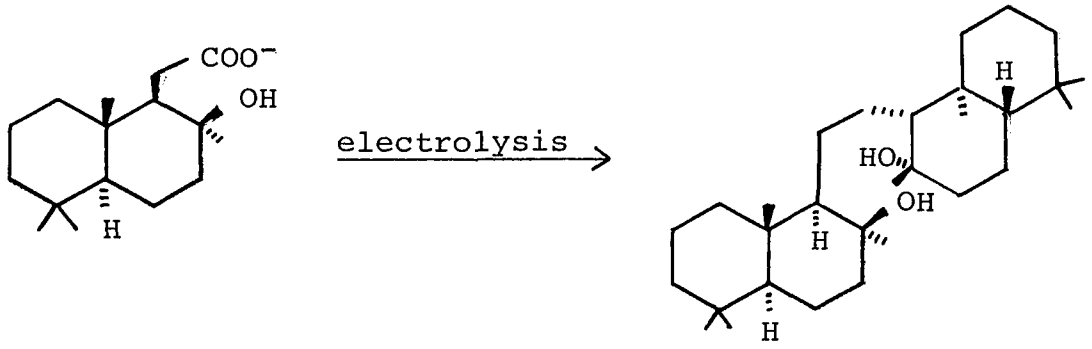
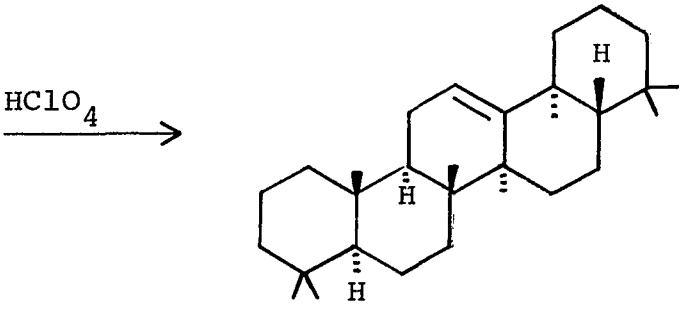


Figure 5. The Kolbe Reaction.





Pentacyclosqualene

Figure 6. The Synthesis of Pentacyclosqualene.

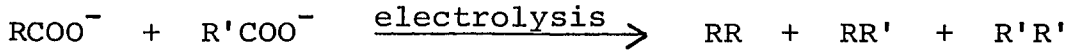
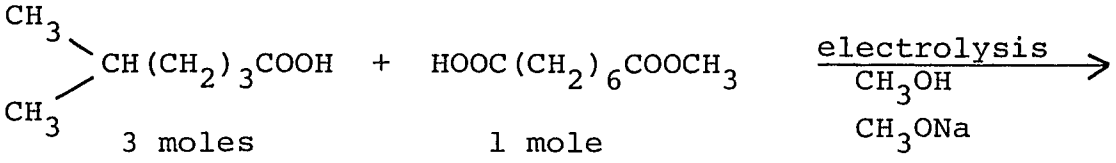
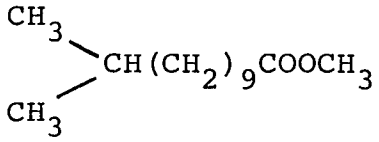


Figure 7. The Crossed Kolbe Reaction.





18

(51%)

Figure 8. The Synthesis of Methyl 11-Methyldodecanoate.

Initially aldehyde 16 was prepared by reduction of this ester to the corresponding alcohol followed by chromium trioxide-pyridine oxidation. While the alcohol was obtained in high purity and good yield, the results of the oxidation reaction were disappointing. An alternate pathway to the aldehyde was therefore devised (Figure 9). Conversion of the ester 18 to the acid was quantitative and the acid chloride was prepared in 91% yield by treatment with oxalyl chloride. While initial studies of the Rosemund reduction of dodecanoyl chloride in toluene at 110° gave only a 46% yield of dodecanol, the aldehyde 16 was produced in 75% yield by running the reaction in xylene at 130°.[54]

An alternate approach to 16 also involving a crossed Kolbe reaction was briefly investigated. It was felt that the coupling of isovaleric acid and undecylenic acid would result in an isoalkene which could be cleaved by ozonolysis to give 16 (Figure 10). The only product isolated from the electrolysis reaction was 12-methyltridecane, the reduced form of the desired product. The reduction of ethylenic double bonds during Kolbe reactions has previously been observed and is particularly troublesome with long electrolysis times since the smooth platinum gradually acquires the characteristics of platinized platinum.[55]

The Reformatsky reaction has traditionally been carried out in benzene or benzene-ether solvents at reflux temperatures. A recent modification of these conditions

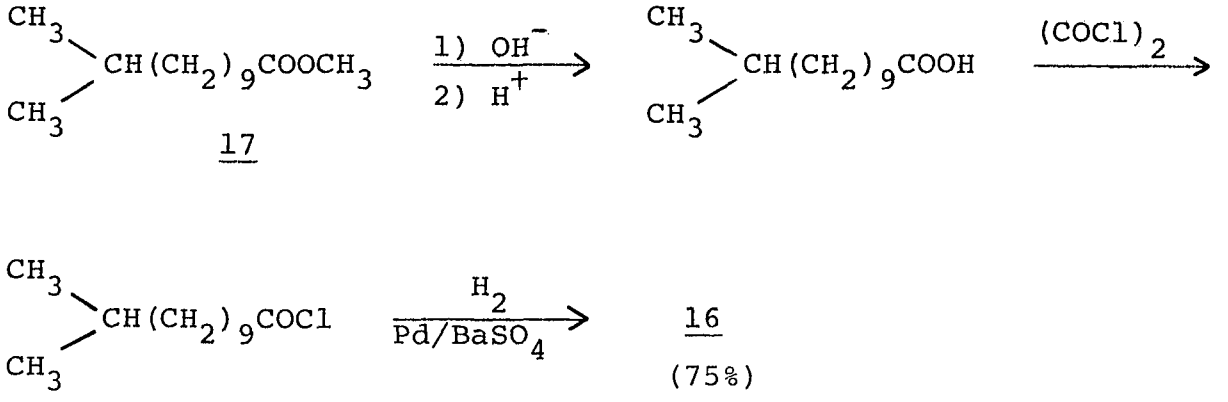


Figure 9. The Synthesis of 11-Methyldodecanal (16).

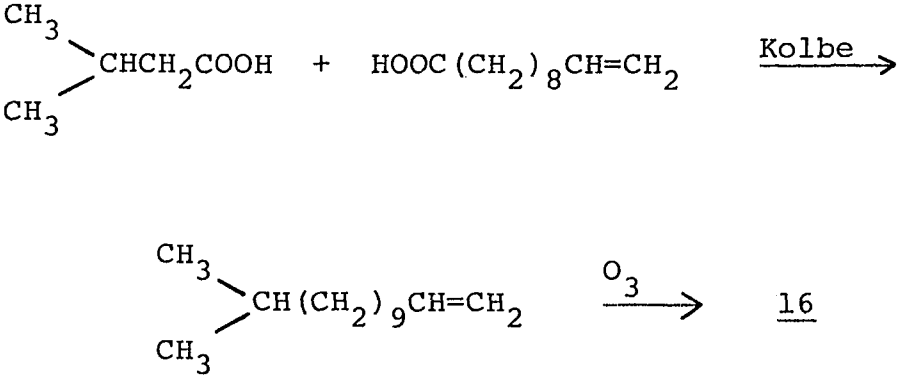


Figure 10. The Attempted Synthesis of 12-Methyl-1-tridecene.

involved the use of a trimethyl borate-tetrahydrofuran solvent combination at room temperature.[56] Side reactions caused by the basicity of the alkoxide products are apparently reduced due to complexation with the solvent. Using this solvent system, and activated zinc dust[57], the β-hydroxy acid 17 was prepared in 45% yield.

Resolution of 17 was first attempted using the method developed by Hardy and coworkers for the resolution of 3-hydroxydodecanoic acid.[58] The (-)-isomer was obtained in optically pure form (11% yield) after four recrystallizations of the D-(+)-amphetamine salt. The (+)-isomer was obtained in 98% ee by four recrystallizations of the (+)-α-phenylethylamine salt (8% yield). By analogy with β-hydroxy fatty acids of known configuration the absolute configurations are probably (-)-D and (+)-L.

A second approach to the production of β-hydroxy acids involving the use of lithium dimethylcuprate was investigated briefly. Herr and coworkers have studied the nucleophilic ring opening of oxiranes by lithium dialkyl cuprates. The cuprates gave alcohol products in good yields[59,60] in contrast to the results obtained with many other organometallic reagents which frequently promote side reactions due to their Lewis acidity or basicity.[61] Very high regiospecificity was observed in the cuprate reactions; from 1,2-epoxybutane and lithium dimethylcuprate, 3-pentanol was prepared in 88% yield and no 2-methylbutanol was observed.

In the present study the high regioselectivity of such reactions was confirmed. From 1,2-epoxyoctane a 97% yield of 2-nonanol was obtained; only a 3% yield of unidentified by-products was observed.

Since carbonyl functions are relatively stable to cuprates, the oxirane reaction has been extended to epoxy esters (Figure 11).[59] While β-hydroxy acids can be obtained from glycidic esters by this reaction, the applicability is limited since only α-substituted β-hydroxy acids can be obtained. A more versatile synthesis of β-hydroxy acids was developed in the present investigation by the ring-opening of methyl 3,4-epoxybutanoate (Figure 12). Two products were obtained in the ratio 83:17 by gas chromatographic analysis. The major product, representing a yield of 50%, was purified by preparative gas chromatography and was identified as methyl 3-hydroxypentanoate. This reaction could be extended to the synthesis of a wide variety of β-hydroxy acids since numerous lithium dialkylcuprates have previously been prepared. The overall reaction may be viewed as the chain extension of an alkyl halide by a four carbon unit incorporating the β-hydroxy moiety.

Peptide Synthesis

Any successful synthesis of biologically active peptides must overcome the problem of racemization arising during the activation of optically active amino acids. If

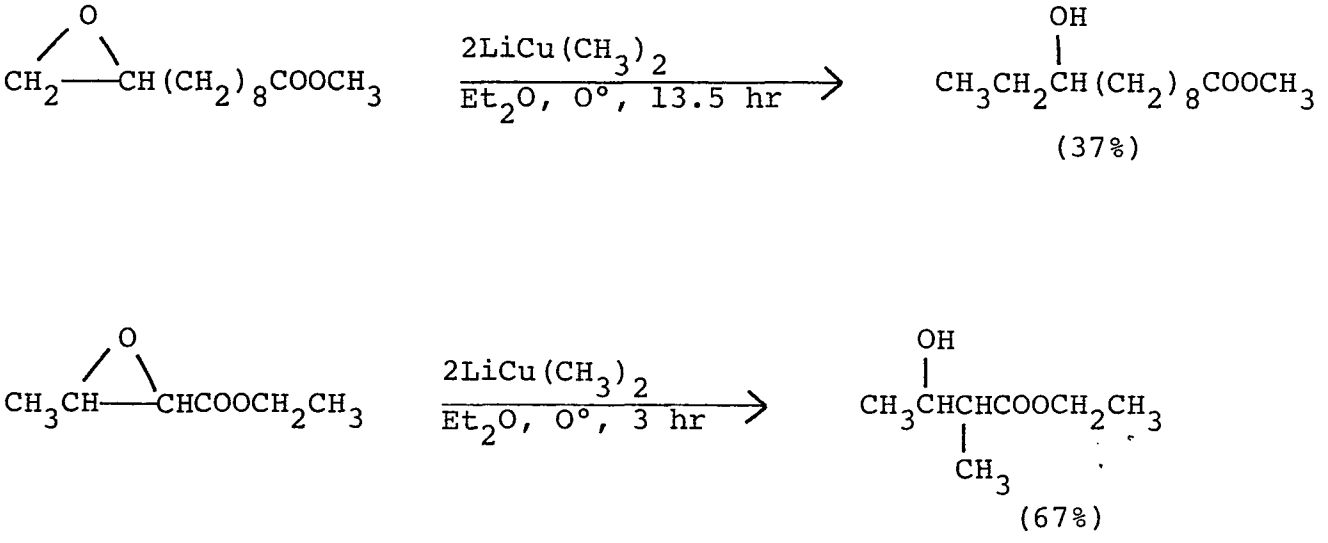


Figure 11. The Reaction of Lithium Dimethylcuprate with Epoxy Esters.[59]

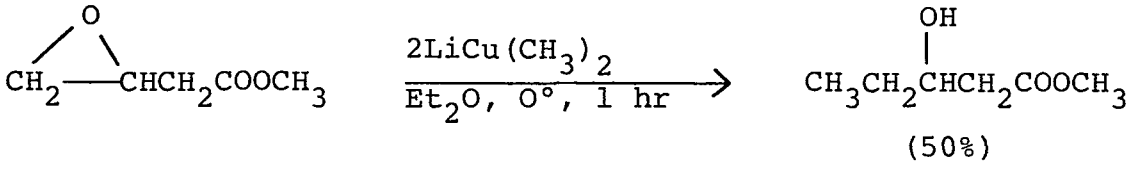


Figure 12. The Reaction of Methyl 3,4-Epoxybutanoate with Lithium Dimethylcuprate.

the fraction of racemization occurring in each coupling step is given as p, the mole fraction, N, of peptide in the final product with the correct configuration of all n residues is given by the expression $N=(1-p)^{n-1}$. The racemization problem, therefore, is extremely serious for higher peptides especially since the separation of diastereomeric peptides differing in the configuration of a single residue is often impossible. In the absence of strong evidence on the absolute minimization of racemization, biological activity cannot be ascribed with complete assurance to the desired product since minor diastereomeric products with potent activity may be present.

Several mechanisms of racemization have been elucidated, the most important of these are (a) the direct abstraction of α-protons from carboxyl-activated amino acids by base, (b) β-elimination reactions of β-hydroxy or β-mercapto amino acids, and (c) the formation of azlactones by carboxyl-activated acyl amino acids or carboxyl-activated peptides (Figure 13).[62] While the acyl groups increase the acidity of the α-proton, urethane-type protecting groups tend to stabilize amino acids toward racemization due to resonance polarization within the protecting group (Figure 14). Azlactone formation promotes racemization because of the acidity of the α-proton in the azlactone. This proton is easily removed by weak bases such as solvent molecules or added free amino peptide. Urethane-type protecting groups also tend to prevent azlactone formation. In the case of

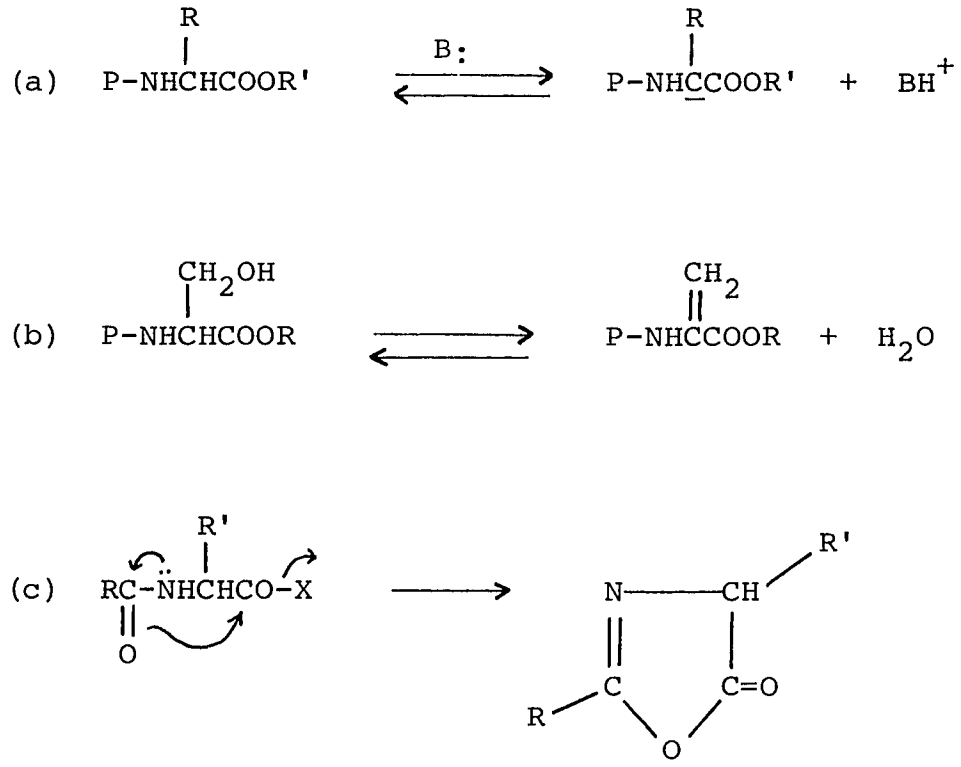


Figure 13. The Principal Mechanisms of Racemization.

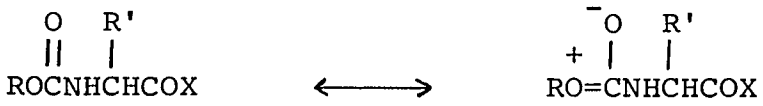


Figure 14. Resonance Stabilization of Urethane-Protected Amino Acids.

tosyl or o-nitrophenylsulfenyl amino acids, of course, azlactone formation is not possible. Peptide synthesis is almost universally carried out by the addition of activated N-protected amino acids to free amino peptides. In this way, the continual activation of the carboxyl groups of the growing peptide chain, with its concomitant racemization, is avoided.

The potential for racemization often directs the choice of fragments in peptide fragment condensation reactions. Thus if glycine is present it is chosen as the point of carboxyl activation since racemization is of no concern. Alternatively, in the absence of glycine, condensation with the carboxyl group of proline or hydroxyproline is chosen since the azlactone mechanism is not applicable.[63] The problem of racemization can thus be reduced to a large extent by the judicious choice of protecting groups and the strategy of synthesis.

The method of carboxyl activation of the protected amino acid as well as the choice of solvent and reaction conditions has a marked influence on racemization. For many years the azide method was the epitome of a low racemization coupling technique. The most sensitive method of detecting racemization prior to 1970, gas chromatography, failed to detect any racemization. The application of the azide method, however, is not as straightforward as other coupling techniques and its principle use has been for

peptide fragment condensation reactions.

In 1969, König and Geiger introduced the use of 1-hydroxybenzotriazole active esters as a convenient coupling method giving little racemization (Figure 15).[64,65] In a coupling reaction involving N-imidoyl-benzylhistidine the previously observed value of racemization for dicyclohexylcarbodiimide coupling was reduced by 97% with the addition of 1-hydroxybenzotriazole.[66] The reaction was systematically investigated by Kemp employing the isotopic dilution method which is sensitive to at least 0.001% racemization.[67,68] The method was found to give racemization values of 0.08 to 3% for the coupling of Z-Gly-Phe-OH with Gly-OEt. These values compared favorably with those for N-hydroxysuccinimide-dicyclohexylcarbodiimide couplings and for p-nitrophenyl ester reactions. The lowest value of racemization observed for the 1-hydroxybenzotriazole-dicyclohexylcarbodiimide coupling reaction was 0.08% in tetrahydrofuran at 0°. It was envisioned that the synthesis of the peptide sequence of surfactin (and esperin?) could be successfully carried out with a minimum of racemization utilizing this method.

The stepwise synthesis of surfactin derivatives required a suitable choice of amino and carboxyl protecting groups. Both the C-terminal leucine carboxyl group and the third function carboxyls of aspartic and glutamic acid required protection. However, a synthesis of surfactinic or esperinic acid would not necessitate differential

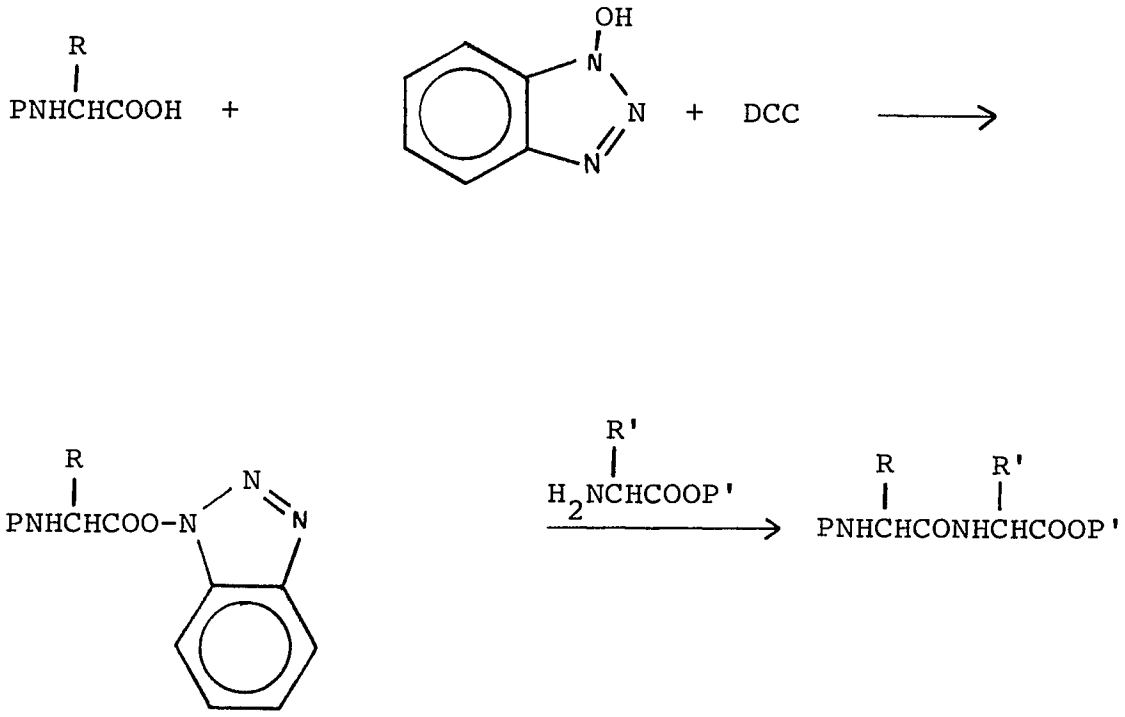


Figure 15. The Use of 1-Hydroxybenzotriazole Active Ester.

protection of the carboxyl groups. The use of methyl or ethyl esters for carboxyl protection was precluded because the base treatment of peptides, particularly those containing aspartic and glutamic acid ω-esters, can give side reactions. Base treatment (saponification) would be required to deprotect methyl or ethyl esters. A suitable alternative was the tert-butyl ester protecting group which was introduced in 1959.[69,70] A tert-butyl ester is stable to hydrogenolysis conditions and is easily removed under mildly acidic conditions. An excellent complementary amino protecting group is the benzyloxycarbonyl group introduced in 1932 by Bergmann and Zervas.[71] This group still remains the most important of the amino protecting groups available to the peptide chemist. Among other methods the benzyloxycarbonyl protecting group can be removed by treatment with 12N hydrochloric acid or, more commonly, by treatment with hydrogen bromide in acetic acid. The mildest conditions for removal, however, involve catalytic hydrogenolysis (Figure 16). The stepwise synthesis of surfactin (and esperin?) peptide utilizing these protecting groups is shown schematically in Chart 3.

Leucine tert-butyl ester hydrochloride (19) was prepared in 59% yield by esterification with isobutylene in dioxane containing a catalytic amount of sulfuric acid.[72] The reported value for the optical rotation of this derivative, $[\alpha]_D^{25}$ 12.4° (c 2, EtOH), was found to be incorrect; the correct value being $[\alpha]_D^{25}$ 18.6° (c 2.00, EtOH).

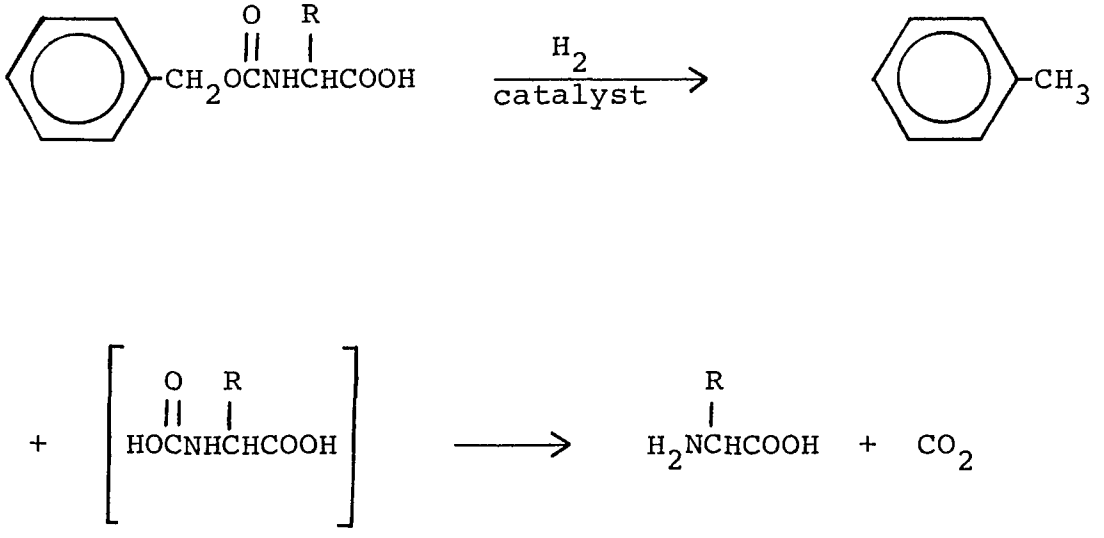


Figure 16. The Catalytic Hydrogenolysis of a Benzyloxycarbonyl Amino Acid.

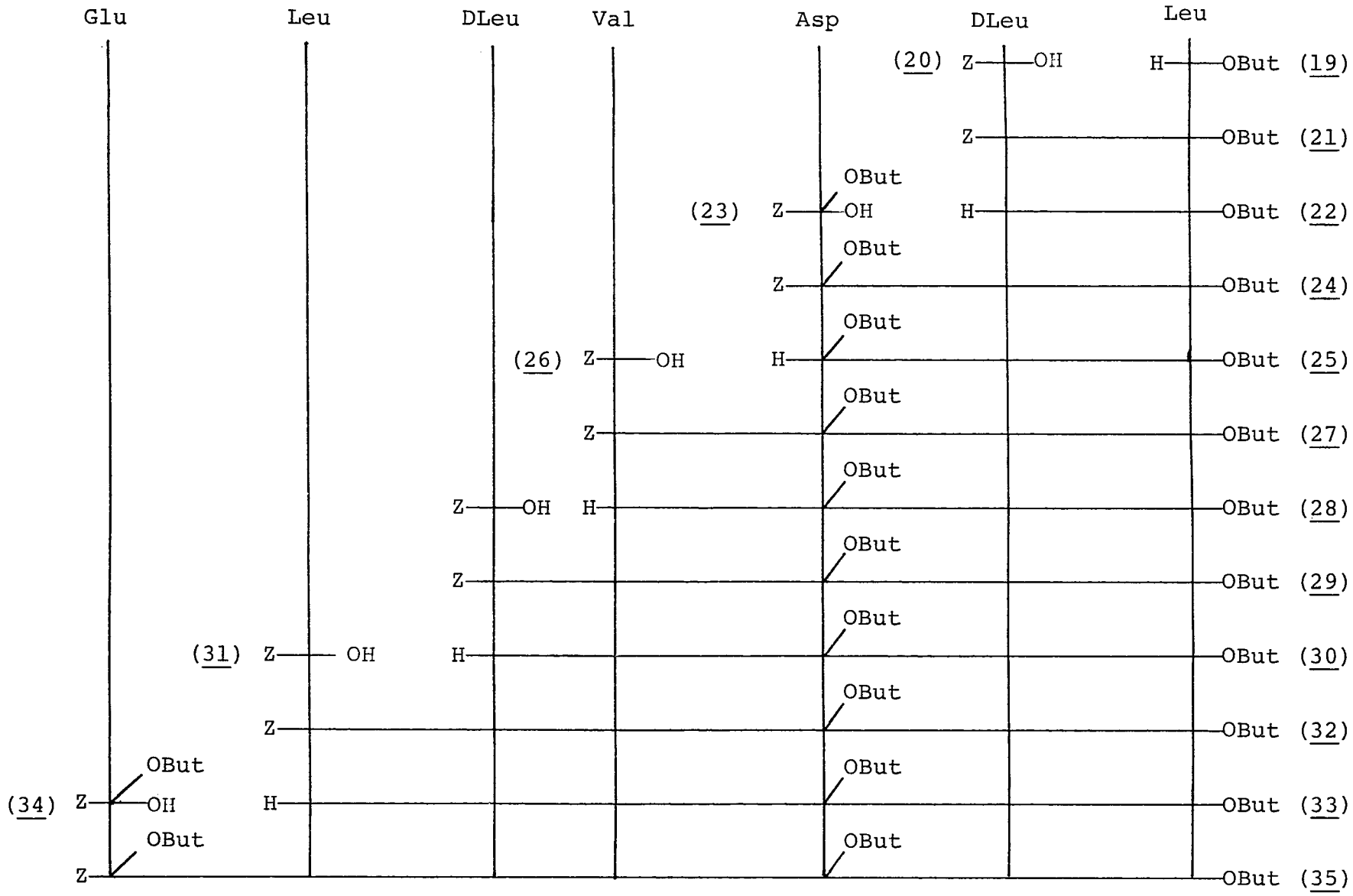


Chart 3. The Stepwise Synthesis of Heptapeptide 35.

Benzyloxycarbonyl D-leucine (20) was prepared in 82% yield by the method of Greenstein and Winitz.[73] Dipeptide 21 was obtained as a viscous oil in 83% yield by the 1-hydroxybenzotriazole-dicyclohexylcarbodiimide (DCC-HOBT) coupling procedure. One equivalent of N-methylmorpholine was added to the reaction mixture to provide free amino leucine tert-butyl ester. Hydrogenolysis of 21 over 5% palladium on carbon gave the free amino dipeptide 22 in quantitative yield as a viscous oil. The product was characterized by derivatization in the form of the hydrogen oxalate salt.

A suitably protected aspartic acid derivative 23 was prepared by the method of Wünsch and Zwick (Figure 17).[74] The fully protected tripeptide (24) was obtained as a white foam in 79% yield (DCC-HOBT coupling). After removal of the benzyloxycarbonyl protecting group by hydrogenolysis (94% yield), the free amino tripeptide was coupled (DCC-HOBT) with benzyloxycarbonylvaline to give the tetrapeptide 27 as a white solid in 53% yield. Again, the benzyloxycarbonyl protecting group was removed by hydrogenolysis (89% yield) and the free amino tetrapeptide was coupled (DCC-HOBT) with benzyloxycarbonyl-D-leucine to give the pentapeptide 29 as a high melting white solid in 80% yield. Removal of the amino protecting group by hydrogenolysis (58% yield) was followed by coupling (DCC-HOBT) with benzyloxycarbonyl-leucine to give hexapeptide 32 as a high melting solid in

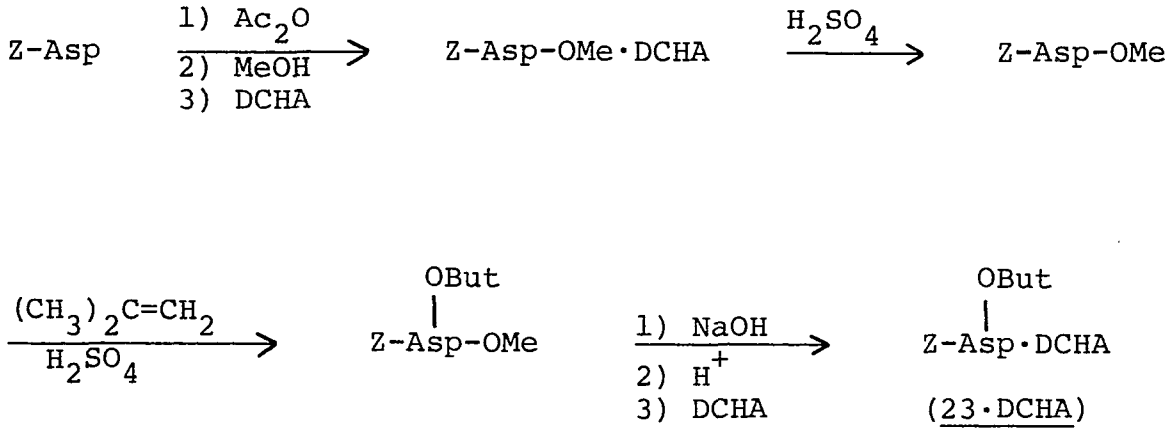


Figure 17. The Preparation of Benzyloxycarbonyl-β-tert-butyl Aspartic Acid Dicyclohexylamine Salt (23·DCHA).

81% yield. An alternative synthesis of this hexapeptide was developed by means of a fragment condensation approach (Chart 4). It was decided to join the fragments in a "three plus three" coupling scheme for two reasons: this approach permits the maximum flexibility in the choice of protecting groups for the C-terminal leucine and aspartic third function carboxyl groups and the tendency toward racemization during fragment condensations is lower for C-terminal amino acids with aliphatic side chains than those with carboxylic acid side chains.[75]

Valine tert-butyl ester (36) was prepared in 63% yield by the method of Roeske.[72] Coupling (DCC-HOBT) of this derivative with benzyloxycarbonyl-D-leucine gave the dipeptide 37 as a wax in 85% yield. Removal of the amino protecting group by hydrogenolysis gave the free amino dipeptide 38 as a gel in 92% yield. This product gave a good elemental analysis and could also be converted to a crystalline hydrogen oxalate salt.

Difficulty was encountered in the preparation of the fully protected tripeptide 39. An initial attempt to couple benzyloxycarbonylleucine with dipeptide 38 (DCC-HOBT) using stoichiometric amounts of the two reactants gave a 53% yield of an impure product with a melting point of 87-90°. A better approach was developed using a 100% excess of benzyloxycarbonylleucine. In this case a 52% yield of pure product was obtained after chromatography on silica gel. This

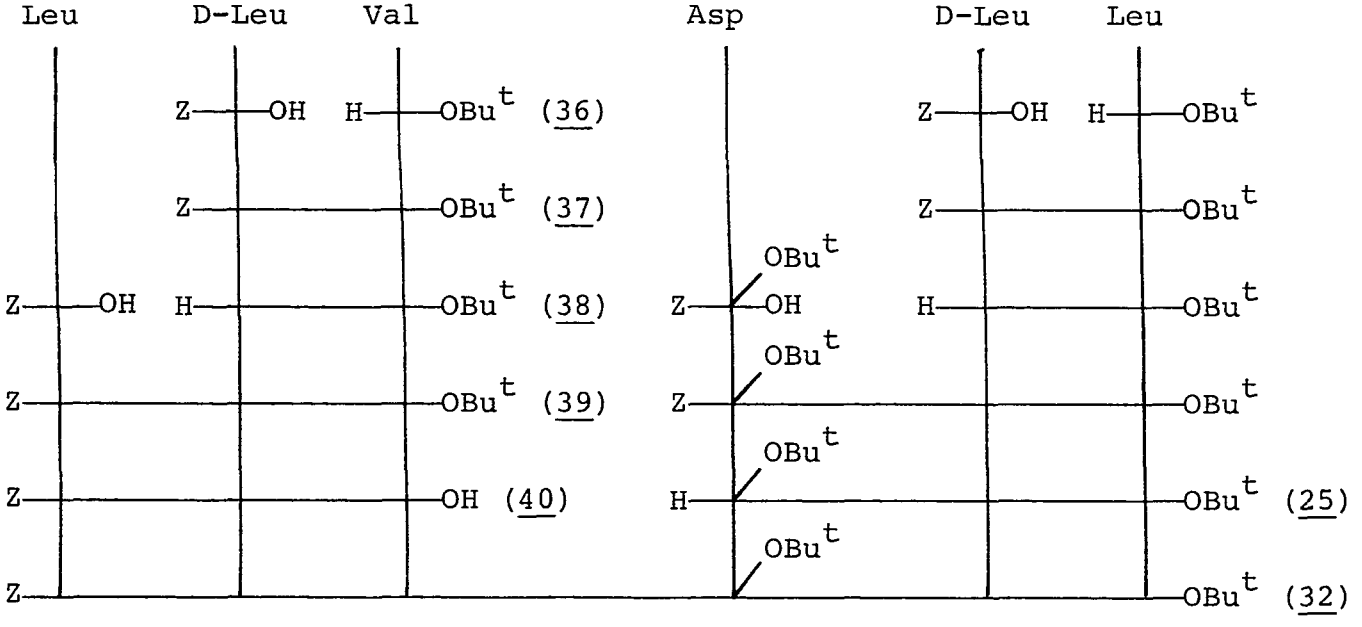


Chart 4. The Synthesis of Hexapeptide 32 by Fragment Condensation.

product had a higher melting point, 104-107°, and gave an excellent amino acid analysis.

Several methods for the removal of the tert-butyl ester protecting group are available including treatment with hydrogen chloride in ethyl acetate[76] or methylene chloride[77] or with p-toluenesulfonic acid in benzene.[78] A frequently employed technique is the treatment of tert-butyl esters with anhydrous trifluoroacetic acid at room temperature.[79] This method is particularly appealing because of its simplicity and because of the stability of the benzyloxycarbonyl protecting group under the conditions employed. The tripeptide tert-butyl ester 39 was treated with anhydrous trifluoroacetic acid for one hour at room temperature. The deprotected tripeptide 40 was obtained as a crystalline solid in the form of its dicyclohexylamine salt in 60% yield.

The fragment coupling was carried out using the DCC-HOBT method. Under these conditions, it was expected that racemization would be held to a minimum. Fully protected hexapeptide was produced in 44% yield. While the product gave excellent values for amino acid analysis, the values obtained from the elemental analysis indicated contamination with N,N'-dicyclohexylurea. Recrystallization of the product from methanol, ethyl acetate, or acetonitrile gave a product which still contained this impurity. This difficulty in separating peptide products from N,N'-dicyclohexylurea is frequently encountered, especially in the preparation

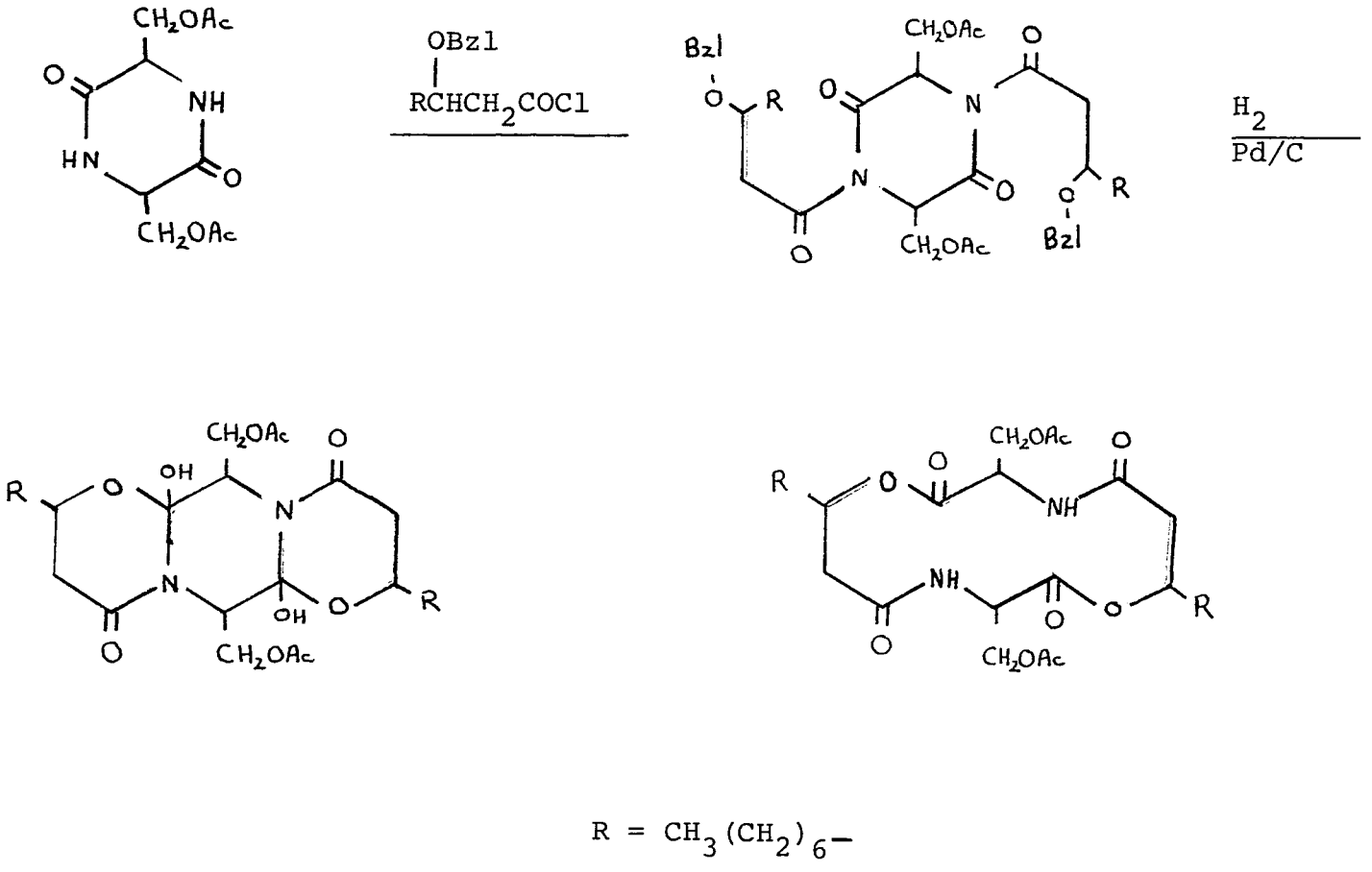
of higher peptides.[80] It was found convenient to remove the benzyloxycarbonyl protecting group by hydrogenolysis and then separate the free amino hexapeptide (33) from N,N'-dicyclohexylurea by fractional crystallization of the latter from methanol solution. In this manner, a quantitative yield of the free amino hexapeptide (33) was obtained.

Due to the low solubility of 33 in tetrahydrofuran it was necessary to depart from the usual DCC-HOBT coupling conditions and to use N,N-dimethylformamide as the reaction solvent. This solvent has been employed for dicyclohexylcarbodiimide mediated coupling reactions in a number of syntheses including an improved synthesis of oxytocin by Bodanszky and du Vigneaud.[81] A 100% excess of dicyclohexylcarbodiimide was used and the reaction time was extended to six hours at 0° and then overnight at room temperature. After filtration of N,N'-dicyclohexylurea, heptapeptide 35 was obtained in 88% yield by precipitation with water. Efforts are currently in progress to purify this derivative by high pressure liquid chromatography. Removal of the benzyloxycarbonyl protecting group by catalytic hydrogenolysis will provide the free amino heptapeptide suitable for acylation by the appropriate β-hydroxy acids. It should be possible to acylate the peptide without protecting the hydroxyl group of the β-hydroxy acid. This technique has been used by Ovchinnikov and coworkers in the synthesis of pseudoesperinic acid[25] and by Hardy and coworkers in the synthesis of isariic acid.[58]

Depsipeptide Synthesis

The strategy involved in the synthesis of a cyclodepsipeptide is determined in large measure by the method of cyclization employed. Shemyakin and coworkers developed a clever but not generally applicable method for the synthesis of cyclodepsipeptides utilizing the hydroxyacyl insertion reaction.[82] This method was applied to the synthesis of the antibiotic serratamolide (Figure 18).[83] This approach is feasible only for the synthesis of symmetrical cyclodepsipeptides or for those containing only one lactone and one lactam function. In all other cases cyclodepsipeptide synthesis involves cyclization by amide bond formation in an aminoacyl hydroxyacid or by ester bond formation in a hydroxyacyl amino acid.

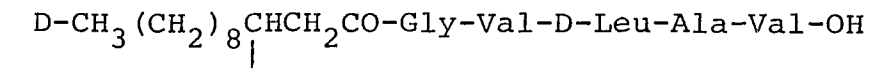
The formation of ester bonds generally requires considerably higher activation of the carboxyl group than is necessary for the formation of amide bonds. Acid chlorides, benzenesulfonic acid anhydrides, or highly activated esters have generally been used.[84] Ring closure by lactonization has been used with success, however, in the synthesis of O-peptides such as actinomycin and its analogs.[85-89] Results with peptolides on the other hand, have been less satisfactory. In the attempted synthesis of isariin, the intermediate isariic acid (41) failed to cyclize even under conditions of high activation such as acetyl chloride-imidazole or 2,4,6-triisopropylbenzene-



$R = CH_3(CH_2)_6-$

Figure 18. The Synthesis of O,O'-Diacetylserratamolide.

sulfonyl chloride.[58] Cyclization by lactone formation was



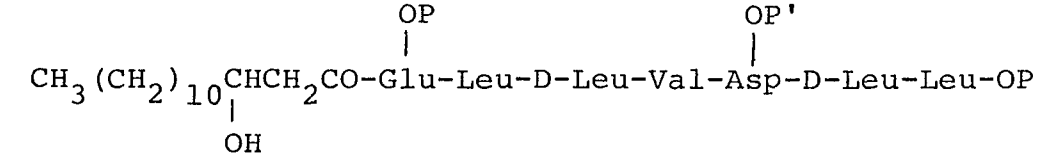
Isariic Acid (41)

also attempted without success in the case of amidomycin[90] and protodestruxin.[91] In the former synthesis the carboxyl component of the open chain compound was valine, as in the case of isariic acid. A synthesis of destruxin B, reported in 1965, involved cyclization by lactone formation although no yield of the cyclized product was reported.[92] In this case, the carboxyl component was β-alanine. A systematic study of the formation of ester bonds between N-protected amino acids and C-protected β-hydroxy acids has shown that steric hindrance, primarily from the amino acid side chain, is the major barrier to ester bond formation.[93]

In the case of esperin, there was reason for optimism concerning the possibility of cyclization via lactonization. The side chain carboxyl group of aspartic acid is free of steric hindrance in the α-position and thus resembles the situation found in destruxin B more closely than, for example, that found in isariin. In addition, the absence of an asymmetric center in the α-position eliminates the problem of racemization during the cyclization step. This is particularly important since the conditions of cyclization, high activation and high dilution with long reaction

times, would be expected to produce significant racemization.

The synthesis of esperin by a lactonization route would involve the preparation of an acyclic intermediate 42.



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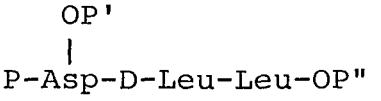
Protecting groups for the third function carboxyl groups of glutamic and aspartic acid and the carboxyl group of leucine would have to be chosen in such a way that the group P' could be removed under conditions which would leave the P groups intact. Cyclization via lactonization would then be followed by removal of the glutamic acid and leucine carboxyl protecting groups P.

Several restrictions on the choice of protecting groups are immediately apparent. Methyl or ethyl esters are precluded for the protection of glutamic acid and leucine since the conditions of their removal, saponification or hydrolysis by hydrochloric acid, would result in the opening of the lactone ring as well. The two principle remaining groups for the protection of carboxyl functions are the tert-butyl ester, previously discussed, and the benzyl ester. The benzyl ester protecting group was introduced to peptide chemistry by Bergmann in 1933.[94] Amino acid benzyl esters are readily prepared by acid catalyzed esterification in refluxing benzene. A variety of methods are available for

the removal of the benzyl ester protecting group including treatment with sodium in liquid ammonia or treatment with hydrogen bromide in acetic acid. By far the most convenient method, however, is catalytic hydrogenolysis. The products are toluene and the free carboxylic acid. A combination of tert-butyl ester and benzyl ester protecting groups, then, could be used to synthesize suitable derivatives such as 42.

An initial study of the preparation of protected tripeptide derivatives 43 was carried out. Since a combi-



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nation of acid labile tert-butyl ester and hydrogenolytically labile benzyl ester was going to be used for carboxyl protection, the choice of the amino protecting group for aspartic acid was limited. This group must be removed selectively in the presence of both of these ester functions. Several highly acid labile amino protecting groups which would seem to be applicable have been introduced to peptide chemistry in recent years. The 2-p-biphenyl-2-propyloxycarbonyl group (Bpoc-), developed by Sieber and Iselin, is considerably more susceptible to acid cleavage than the tert-butyl ester.[95] The Bpoc group can thus be removed selectively in the presence of this ester function.[96] Difficulties in the preparation and storage of Bpoc amino acids

have precluded their wide application. The o-nitrophenylsulfenyl group (NPS) is a suitable alternative. This group was introduced by Zervas[97] and is extremely susceptible to acidolysis. It can be selectively removed in the presence of tert-butyl esters by treatment with two equivalents of hydrogen chloride in organic solvents such as diethyl ether or methanol.[98] The products of the reaction are o-nitrophenylsulfenyl chloride and the peptide hydrochloride (Figure 19).

The synthesis of tripeptide 49 was carried out initially, even though the carboxyl moieties of aspartic acid and leucine have the same protecting group, because of the commercial availability of o-nitrophenylsulfenyl-β-benzylaspartate (Chart 5). The use of the benzyloxycarbonyl protecting group for the amino function of D-leucine was precluded since it cannot be selectively removed in the presence of benzyl esters. Instead, the tert-butyloxycarbonyl group (BOC), introduced by McKay and Albertson, was used.[99] The BOC group is easily cleaved by treatment with acids, usually hydrogen chloride in an organic solvent or trifluoroacetic acid. Hiskey has introduced the use of boron trifluoride etherate as a mild cleavage reagent.[100] The BOC group is introduced by the reaction of amino acids with tert-butyloxycarbonyl azide (BOC-N_3) either by the pH-stat method[101] or by reaction in the presence of bases such as triethyl amine[102] or tetramethylguanidine.[103]

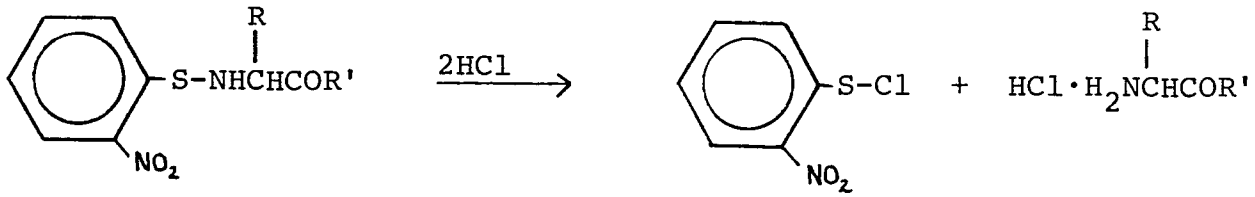


Figure 19. The Removal of the NPS Protecting Group.

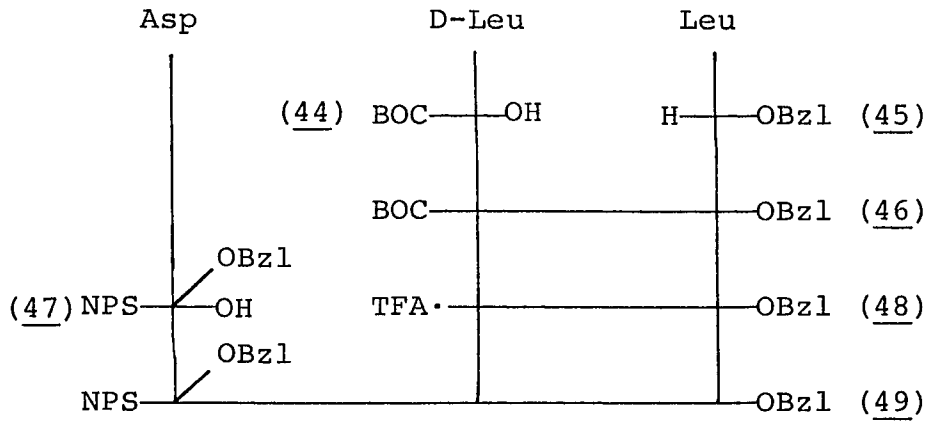


Chart 5. The Synthesis of Tripeptide 49.

tert-Butyloxycarbonyl-D-leucine (44) was prepared in 57% yield by the triethylamine method and was isolated as the crystalline hydrate. Coupling of 44 with leucine benzyl ester by the DCC-HOBT method gave dipeptide 46 as a crystalline solid in 65% yield. The BOC group was removed by treatment with anhydrous trifluoroacetic acid for one hour at room temperature. Formation of the trifluoroacetate salt of the free amino group prevents the formation of a diketopiperazine. Diketopiperazine formation is particularly troublesome in the case of D,L- or L,D-dipeptide esters. The trifluoroacetate 48 was obtained as a crystalline solid in 80% yield. o-Nitrophenylsulfenyl-β-benzylaspartate (47) was coupled with 48 to give the fully protected tripeptide 49 as a bright yellow solid in 78% yield. One equivalent of N-methylmorpholine was added to the reaction mixture to neutralize the trifluoroacetic acid.

This successful coupling of an NPS-aspartic acid β-benzyl ester to D-leucylleucine dipeptide esters was extended to provide tripeptides with differentiated carboxyl protecting groups (Chart 6). The coupling of 47 with the previously prepared dipeptide ester 22 gave a 67% yield of the fully protected tripeptide 50. The selective removal of the NPS protecting group was accomplished in excellent yield by treatment with 0.3N hydrogen chloride in chloroform. These conditions had been employed by Ovchinnikov and coworkers in the synthesis of pseudoesperin.[27]

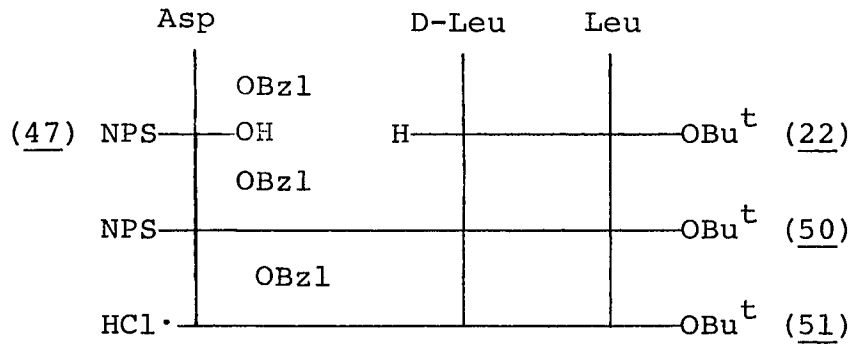


Chart 6. The Synthesis of Tripeptide 51.

Cyclization of hydroxyacyl amino acids, as noted earlier, generally requires a very high degree of activation of the carboxylic acid moiety. This activation step would be carried out on the hydroxyacid 52 obtained by selective deprotection of 42 (Figure 20). In the case where leucine and glutamic acid are protected as the tert-butyl ester and aspartic acid as the benzyl ester (P=Bu^t, P'=Bzl), as envisioned in the synthesis of tripeptide 51, the deprotection step A would involve treatment with anhydrous trifluoroacetic acid and deprotection step C would involve catalytic hydrogenolysis. Restrictions would be placed on the choice of cyclization conditions B, however, because of the presence of tert-butyl esters. The acid lability of this function would preclude the use of the acid chloride method[104], one of the most commonly employed techniques for cyclization. If the protecting groups are reversed (P=Bzl, P'-Bu^t), deprotection step A would be catalytic hydrogenolysis and step C would be treatment with anhydrous trifluoroacetic acid. Cyclization of intermediate 52 could now be carried out by the acid chloride method without the concomitant cleavage of the glutamic acid and leucine carboxyl protecting groups.

Figure 20. The Synthesis of Esperin by Lactonization.

An attempted synthesis of a suitable tripeptide derivative (57) was carried out (Chart 7). A suitably protected aspartic acid derivative (55) required for the synthesis was prepared from benzyloxycarbonyl-β-tert-butylaspartate (Figure 21). Catalytic hydrogenolysis of the benzyloxycarbonyl group gave the free amino derivative, β-tert-butylaspartate, isolated as the acetate salt in 61% yield. The NPS protecting group was introduced by the method of Zervas.[97] The o-nitrophenylsulfenyl chloride and aqueous sodium hydroxide were added in portions to a solution of the aspartic acid ester in basic aqueous dioxane to produce 55. Since free acid NPS derivatives have been found to be unstable during long periods of storage the products are generally isolated and stored as their dicyclohexylamine salts. The dicyclohexylamine salt of 55 was isolated in 48% yield as a bright yellow solid.

The coupling of 55 and the previously prepared dipeptide derivative 48 was carried out by the DCC-HOBT method. The coupling reaction gave only a 29% yield of the fully protected tripeptide 56. A by-product of the reaction, apparently formed by cleavage of the tert-butyl ester protecting group of 56, was tentatively identified by analysis of its nuclear magnetic resonance spectrum. Further difficulties were encountered in the attempted removal of the NPS protecting group from 56. The same conditions employed in the deprotection of NPS-tripeptide

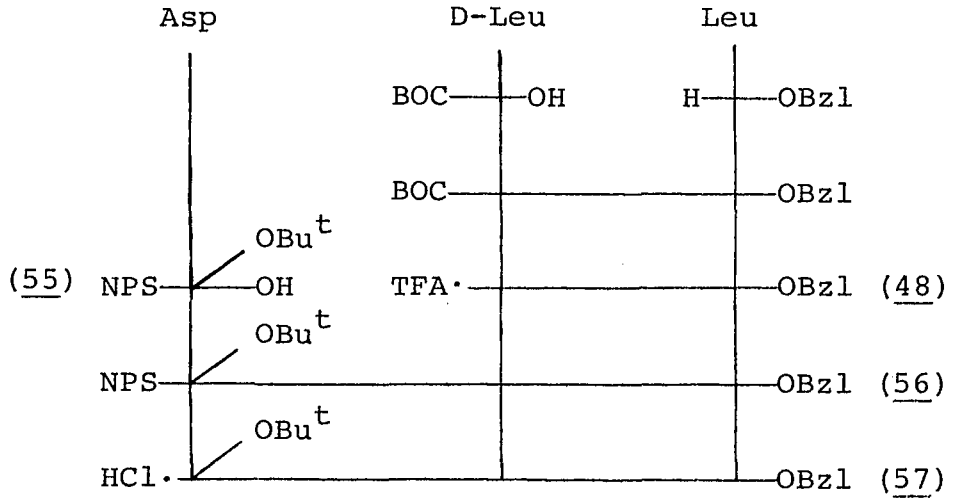


Chart 7. The Synthesis of Tripeptide 57.

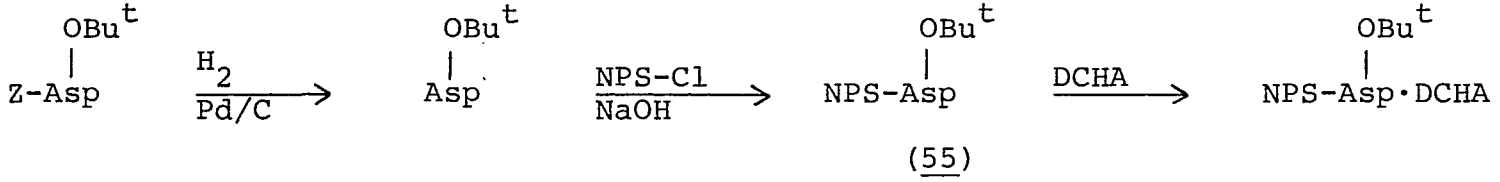


Figure 21. The Preparation of o-Nitrophenylsulfenyl-β-tert-butylaspartate Dicyclohexylamine Salt (used as shown in Chart 7).

50 were used in the attempted synthesis of 57. However, no clean sample of 57 was obtained; only mixtures of several ninhydrin positive compounds. Difficulty in cleaving NPS peptides containing tert-butyl esters had previously been encountered in the synthesis of norsurfactin.[93]

The successful synthesis of tripeptide 51 encouraged further work on the lactonization approach to esperin. One scheme envisioned was the elongation of 51 by the stepwise addition of suitably protected amino acids (Chart 8). This synthesis would require the use of a highly acid labile amino protecting group such as Bpoc or NPS for each amino acid. While the amino protecting group of tripeptide 50 was removed successfully, it was by no means certain that the repeated exposure of the leucine tert-butyl ester protecting group to acidic conditions would not result in some cleavage of this group. It was felt that a fragment condensation approach would have more potential for success (Figure 22). In this manner, the desired combination of side chain and C-terminal carboxyl protecting groups could be incorporated without risking repeated exposure of the tert-butyl ester functions to acidic conditions. Toward this goal a successful synthesis of free amino tetrapeptide 66 was carried out (Chart 9).

The p-nitrobenzyl ester protecting group was introduced by Schwyzer and Sieber.[105] Its principle advantage lies in its resistance to cleavage by hydrogen bromide in

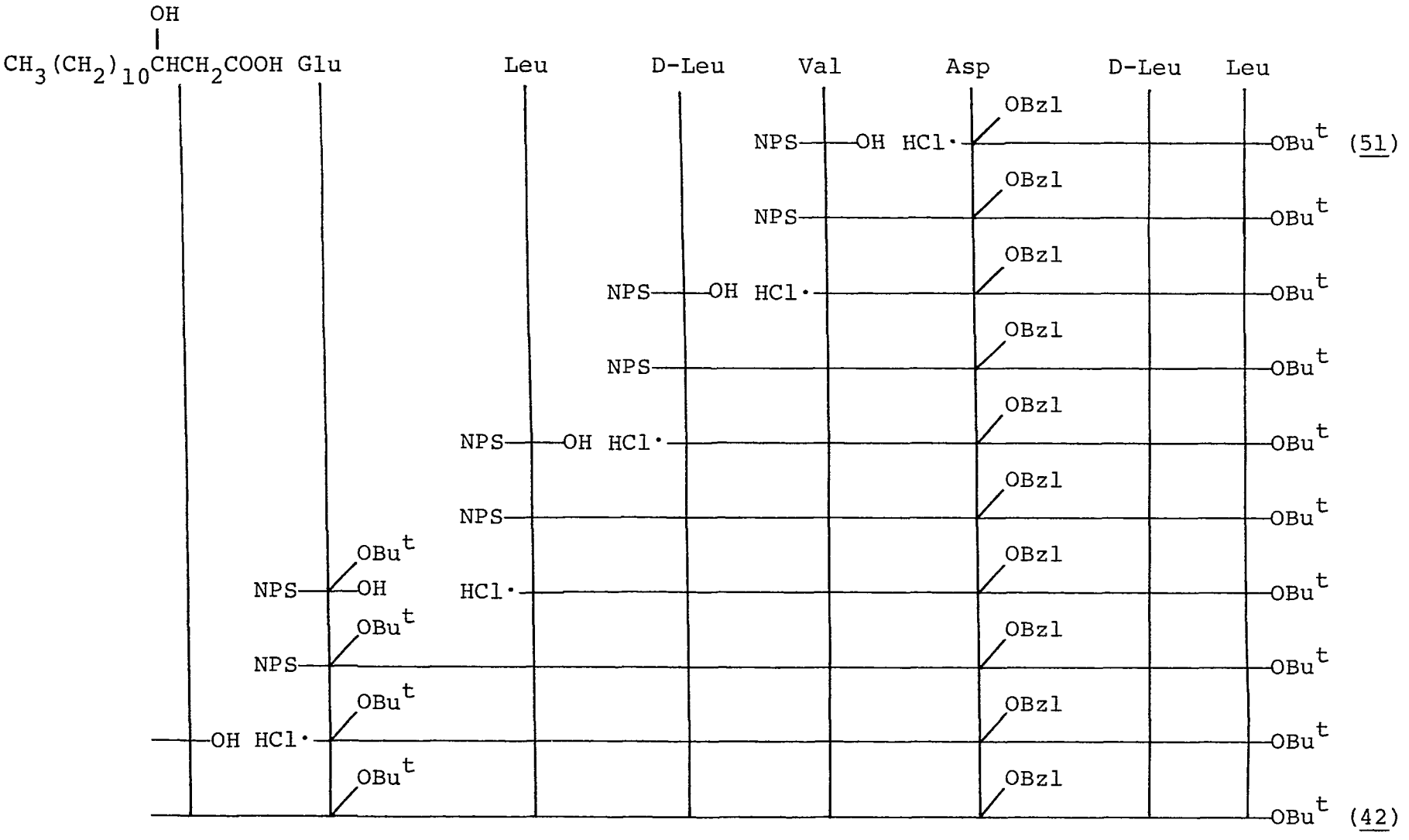


Chart 8. A Scheme for the Stepwise Synthesis of Acylheptapeptide 42 (P=Bu^t, P'=Bzl).

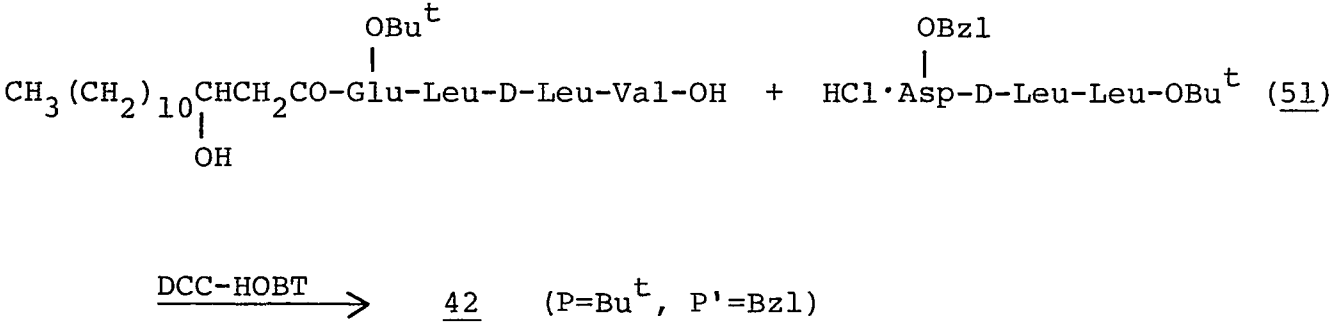


Figure 22. The Fragment Condensation Synthesis of Acylheptapeptide 42. (P=Bu^t, P'=Bzl).

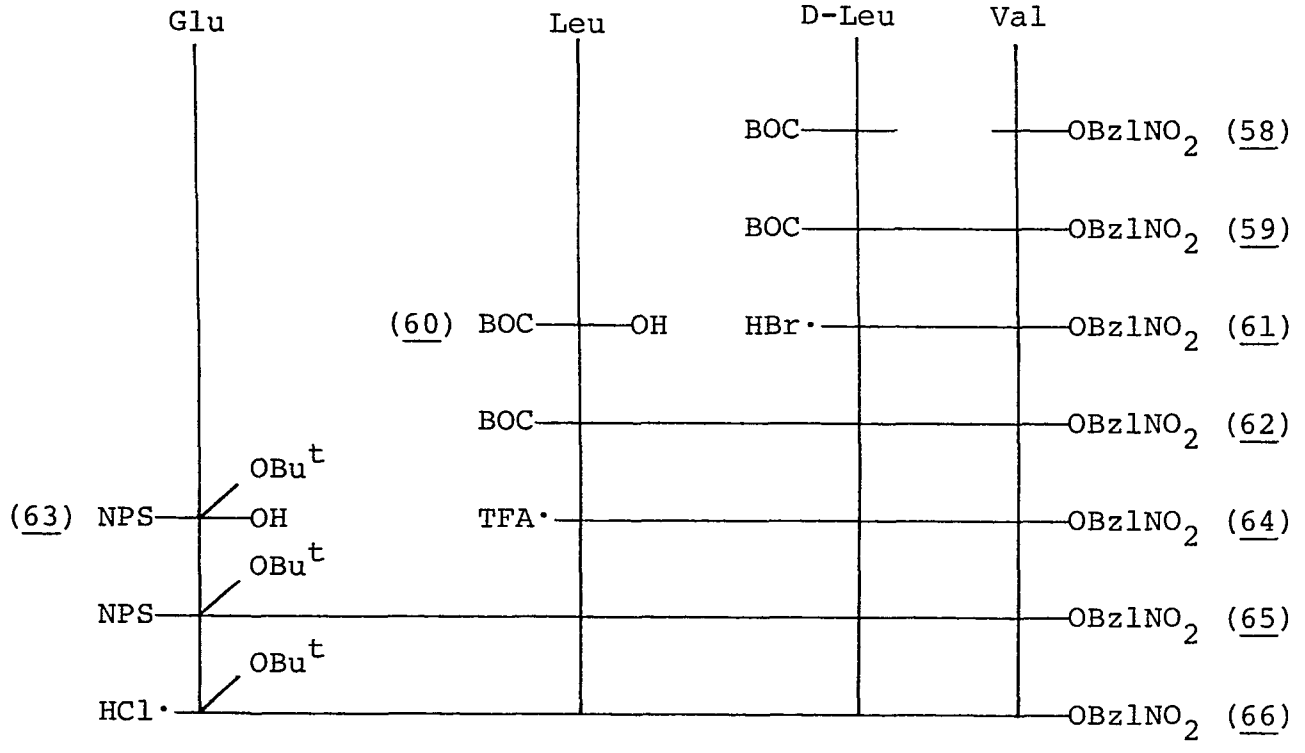


Chart 9. The Synthesis of Tetrapeptide 66.

acetic acid. The ready crystallizability of the p-nitrobenzyl esters offers a further advantage. The group is easily removed by catalytic hydrogenolysis the products being the free acid and p-toluidine. Valine p-nitrobenzyl ester p-toluene sulfonate (58·p-TsOH) was prepared by the method of Mazur and Schlatter in 67% yield.[106] Coupling of 58 with tert-butyloxycarbonyl-D-leucine by the DCC-HOBT method provided the protected dipeptide 59 as a crystalline solid in 82% yield. An initial attempt to remove the BOC protecting group with boron trifluoride etherate gave only a diketopiperazine. However, the BOC group was removed cleanly, in quantitative yield, by the action of hydrogen bromide in acetic acid. Formation of the hydrobromide salt of the free amino group prevented diketopiperazine formation. tert-Butyloxycarbonylleucine (60) was prepared by the method of Ali[103] and was isolated in 97% yield in the form of its hydrate. Coupling of 60 with the dipeptide hydrobromide 61 by the DCC-HOBT method resulted in the formation of the protected tripeptide 62, isolated in 77% yield as a crystalline solid. Treatment of 62 with anhydrous trifluoroacetic acid for one hour at room temperature resulted in a quantitative yield of the tripeptide trifluoroacetate (64), isolated as a foam. The glutamic acid residue was then introduced, carboxyl-protected as the tert-butyl ester and, as in the case of the β-tert-butylaspartyl tripeptide 51, the amino group was protected with NPS. The synthesis of

the protected glutamic acid (63) is shown in Figure 23.

Benzyloxycarbonyl-α-benzylglutamate was prepared by selective alcoholysis of benzyloxycarbonylglutamic acid anhydride and was isolated in 30% yield by fractional crystallization of the dicyclohexylamine salt.[107] After introduction of the tert-butyl ester function (90% yield), the amino and α-carboxyl protecting groups were removed simultaneously by catalytic hydrogenolysis. The NPS protecting group was incorporated by the method of Zervas and the product was isolated in the form of its dicyclohexylamine salt in 61% yield from γ-tert-butylglutamate.[97]

o-Nitrophenylsulfenyl-γ-tert-butylglutamate (63) was coupled with tripeptide 64 by the DCC-HOBT method. Fully protected tetrapeptide 65 was obtained as a crystalline solid in 43% yield after purification by column chromatography. The NPS protecting group was removed by treatment with two equivalents of hydrogen chloride in chloroform. The solid hydrochloride 66 was obtained in 84% yield. This derivative appeared homogeneous by thin layer chromatography in three different solvent systems and gave values for elemental analysis in excellent agreement with those calculated for 66. Work is currently in progress to acylate 66 with β-hydroxytetradecanoic acid. Hydrogenolysis of the resulting acyltetrapeptide would provide a free carboxyl acyltetrapeptide suitable for conversion to acylheptapeptide 42 (P= Bu^t, P'=Bzl).

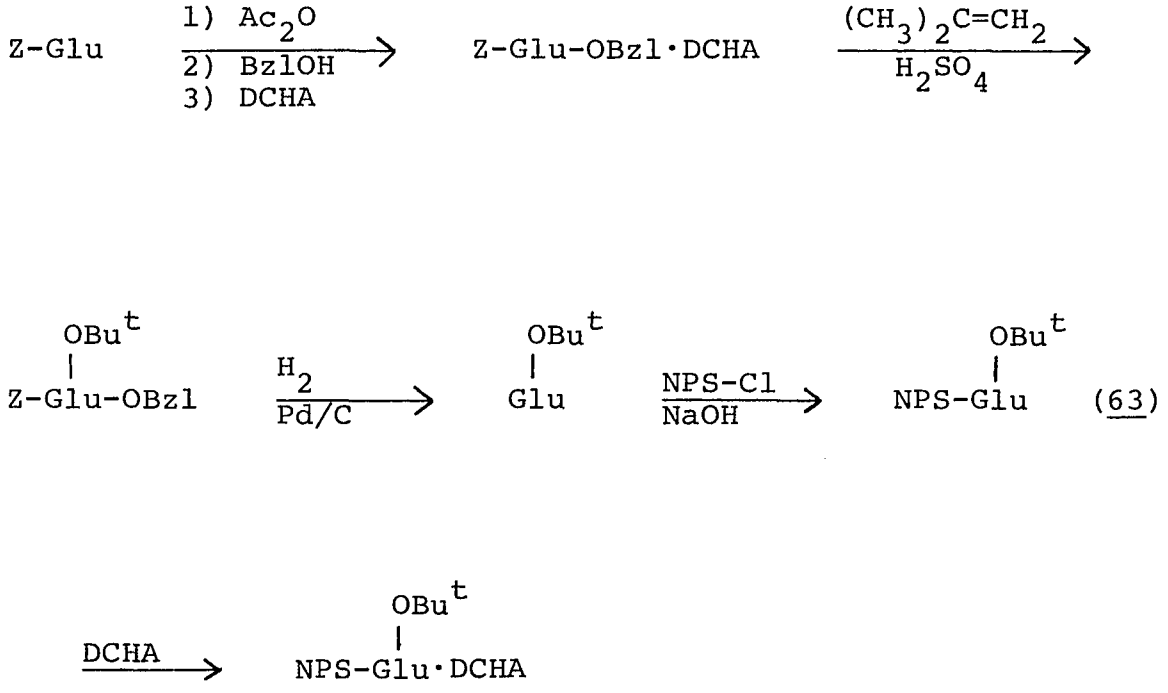


Figure 23. The Preparation of o-Nitrophenylsulfenyl-γ-tert-butylglutamate Dicyclohexylamine Salt (used as shown in Chart 9)

An alternative approach to cyclodepsipeptide formation as mentioned earlier, involves amide bond formation. With the exception of destruxin B, all reported syntheses of naturally occurring peptolides have involved this method. In the syntheses of pseudoesperin, isariin and norsurfactin, three of the four β-hydroxyacid peptolides which have been synthesized (the fourth is serratamolide whose unique synthesis was discussed earlier), ring closure was by amide bond formation between the carboxyl group of the hydroxyacid and the amino group of the peptide chain. This is a particularly well suited point for cyclization since the activated carboxyl moiety which must have a long lifetime under the high dilution conditions required for cyclization does not contain an α-asymmetric center. The danger of racemization is thereby greatly reduced. In the case of esperin, the absence of glycine, proline, or hydroxyproline in the peptide chain strongly suggested the desirability of cyclization at the same point. The synthesis of pseudoesperin by Ovchinnikov and coworkers was carried out in this manner (Figure 24).[27]

The strategy for the synthesis of a molecule such as esperin is complicated by many factors. It was felt, for the reasons mentioned above, that the ring closure step should be attempted between the hydroxyacid carboxyl group and glutamic acid (Figure 25). The glutamic acid and leucine carboxyl protecting groups would have to be removed

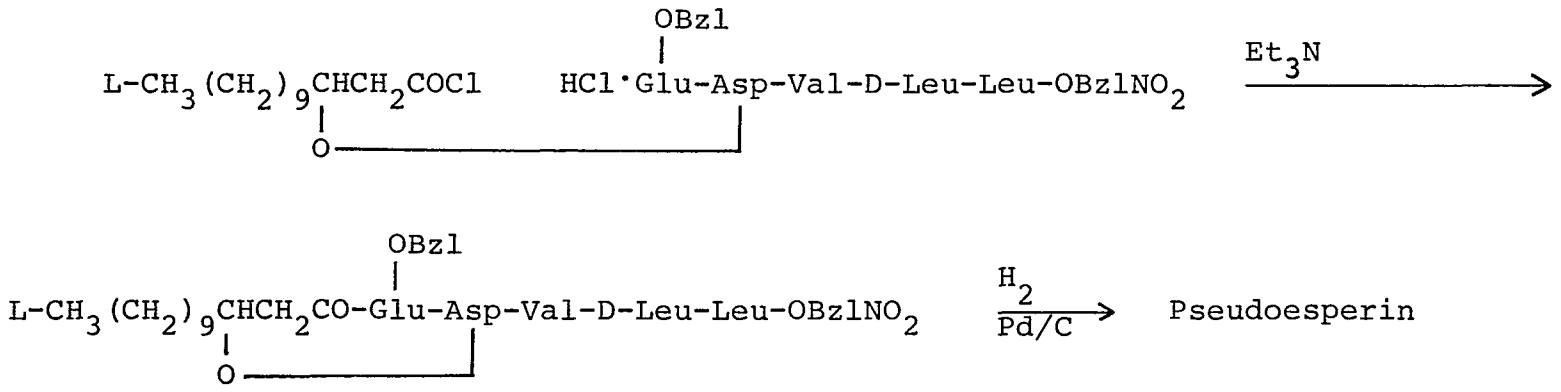


Figure 24. The Synthesis of Pseudoesperin.[27]

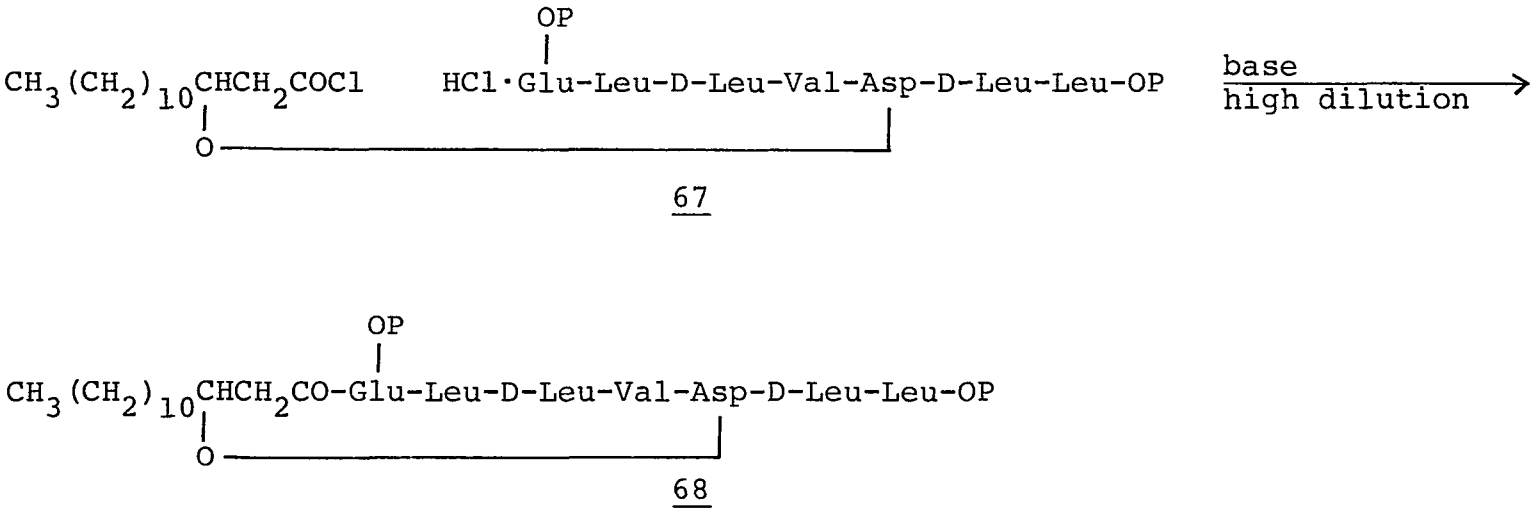
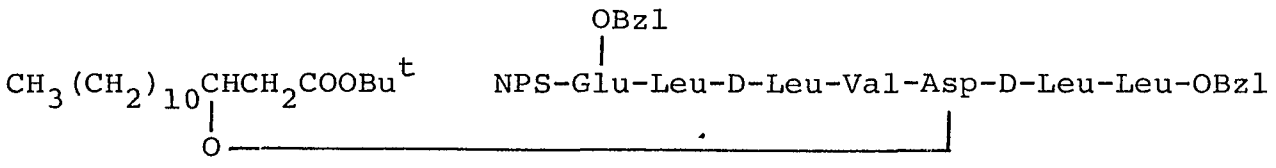


Figure 25. The Cyclization Step in a Proposed Synthesis of Esperin.

after the cyclization step using very mild conditions. The hydrogenolytically labile benzyl ester discussed previously seemed an ideal candidate. Hydrogenolysis conditions should not affect the lactone bond.

With the preferred carboxyl protecting groups selected, the choices for a suitable protecting group for the carboxyl function of the hydroxyacid were considerably narrowed. This group must be removed prior to the formation of the acid chloride 67 under conditions which do not affect the benzyl ester functions. Methyl or ethyl esters are precluded since they cannot be cleanly removed in the presence of benzyl esters. tert-Butyl esters on the other hand, can be removed under mildly acidic conditions which do not affect benzyl esters. Since the amino group of glutamic acid could be free during the formation of the acid chloride 67, an acid sensitive protecting group such as NPS could be used to protect this function. Acidolysis would simultaneously remove both the tert-butyl ester and the NPS protecting group. The initial goal of this phase of the present investigation was therefore, the fully protected peptolide 69. It was felt that the most efficient approach to 69



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would involve the formation of the ester bond between the side chain of aspartic acid and the hydroxyl group of the fatty acid at the earliest possible stage. Again, for reasons of efficiency, a fragment coupling approach was envisioned. A "four plus four" condensation was initially considered (Figure 26). The synthesis of tetrapeptide acid 71, however, was not straightforward. While an attempt to synthesize 71 was subsequently made, initial studies were aimed at a "one plus three plus four" condensation route (Figure 27). The single amino acid component of the 1+3+4 scheme, o-nitrophenylsulfenyl-γ-benzylglutamate, was commercially available. A synthesis of the tripeptide acid component (72) was carried out as shown in Chart 10.

Benzyloxycarbonyl-D-leucine was coupled with valine methyl ester (DCC-HOBT) to give the protected dipeptide 73 in 60% yield. After removal of the amino protecting group with hydrogen bromide in acetic acid (98% yield), the dipeptide hydrobromide was coupled with o-nitrophenylsulfenyl-leucine (DCC-HOBT) to give the NPS tripeptide ester 75 as a yellow solid in 48% yield. The product was homogeneous by thin layer chromatographic analysis and gave an elemental analysis in excellent agreement with the predicted values. Treatment of 75 with two equivalents of sodium hydroxide produced the NPS tripeptide acid 72. The product was recrystallized from diethyl ether to give an 81% yield of yellow solid homogeneous by thin layer chromatographic

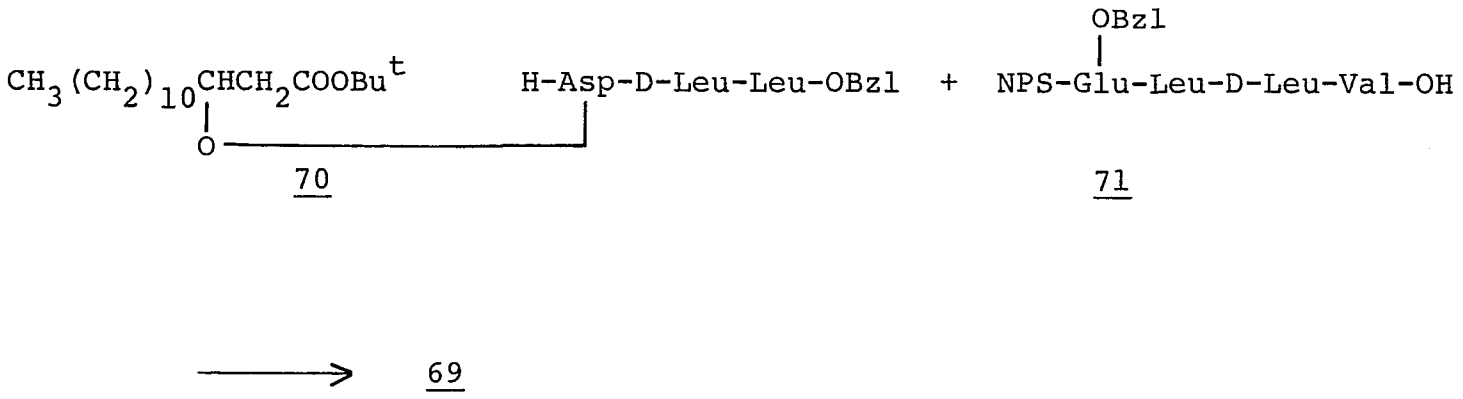


Figure 26. The 4+4 Fragment Condensation Approach to Esperin.

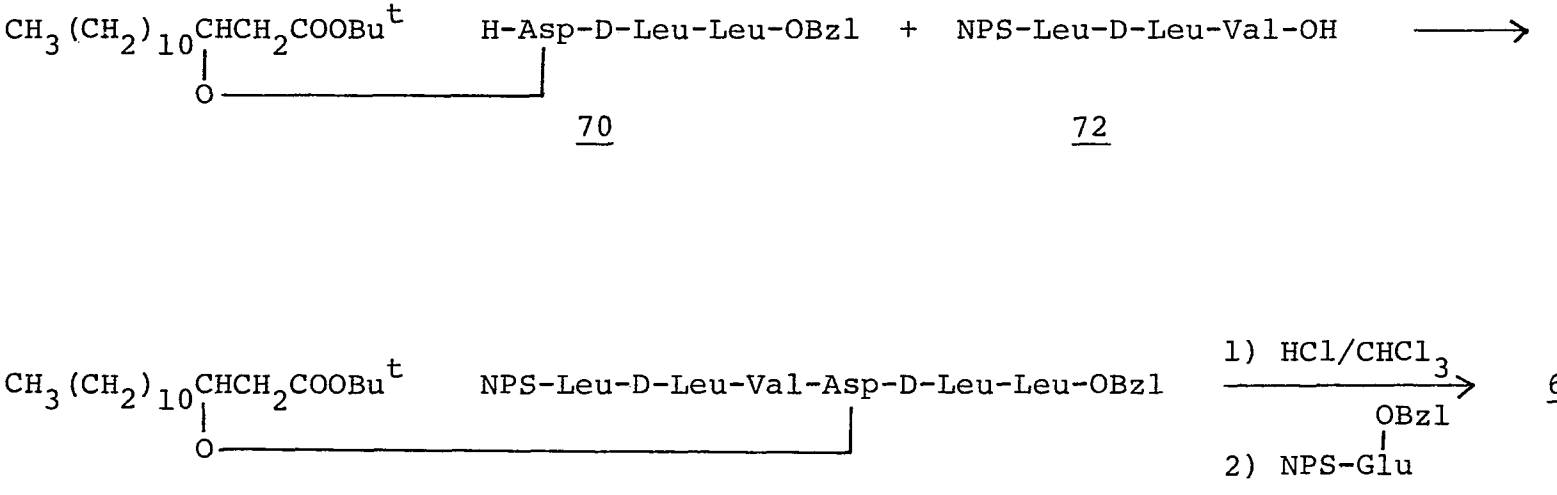


Figure 27. The 1+3+4 Fragment Condensation Approach to Esperin.

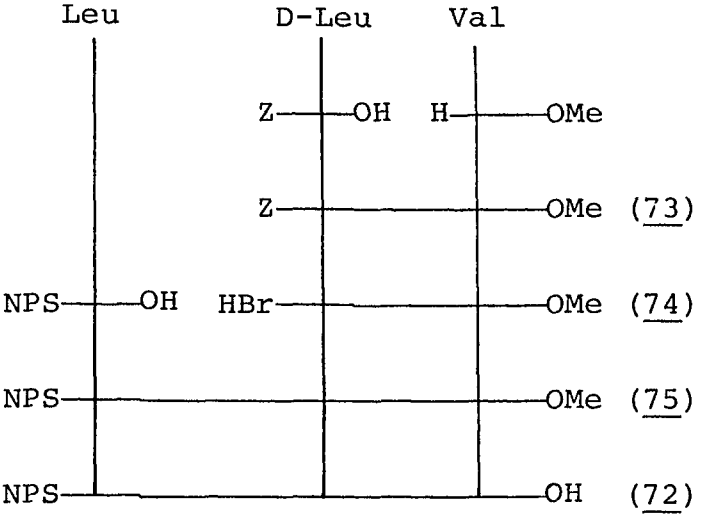
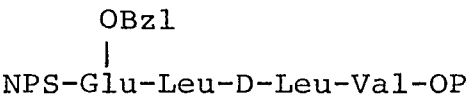


Chart 10. The Synthesis of Tripeptide 72.

analysis in four different solvent systems. The elemental analysis for 72 was in good agreement with the predicted values. With 72 in hand, two of the three fragments required to complete the synthesis in Figure 27 were available.

The synthesis of tetrapeptide 71 was briefly investigated at this point. If this intermediate were available it would allow the scheme in Figure 26 to be studied using the same fragment (70) needed to complete the scheme in Figure 27. Although the synthesis of 71 would seem straightforward, severe restrictions are placed on the choice of the C-terminal protecting group which must be removed from the fully protected tetrapeptide 76. The removal of the C-



76

terminal protecting group P by catalytic hydrogenolysis is precluded both by the presence of the benzyl ester on the side chain carboxyl group and by the NPS group which is an effective catalyst poison. The base lability of the side chain benzyl ester prohibits the choice of methyl or ethyl esters to protect the C-terminal carboxyl group. Selective hydrazinolysis of methyl or ethyl esters in the presence of benzyl esters has also been shown to be unsuccessful. Finally, the extreme sensitivity of the NPS group to acidolysis excludes the use of the tert-butyl ester protecting

group for the valine residue.

One possible pathway to 71 would involve the simultaneous removal of the amino and C-terminal carboxyl protecting groups followed by the introduction of the NPS group (Figure 28). The last step, however, would be perilous since the NPS group is introduced under relatively strong basic conditions. In the present study, an attempt to synthesize 71 using salt formation protection of the C-terminal carboxyl group was carried out (Chart 11).

Salt formation represents the least elaborate approach to the protection of carboxyl groups.[108] While the approach is simple, problems often arise due to the low solubility of the salt in organic solvents and due to the difficulty of separating the final product from the starting materials. Coupling methods used with salt protection have included the acid chloride method, the mixed anhydride method, and the azide method. Active esters have been employed more frequently recently. With this latter approach in mind, o-nitrophenylsulfenyl-α-pentachlorophenyl-γ-benzylglutamate (77), was prepared in 93% yield by the DCC promoted condensation of pentachlorophenol and o-nitrophenylsulfenyl-γ-benzylglutamate. A suitable tripeptide derivative was obtained by the coupling (DCC-HOBT) of tert-butyloxycarbonyl-leucine with the previously prepared dipeptide ester 38. Fully protected tripeptide 78 was obtained as a crystalline solid in 81% yield. The amino and carboxyl protecting

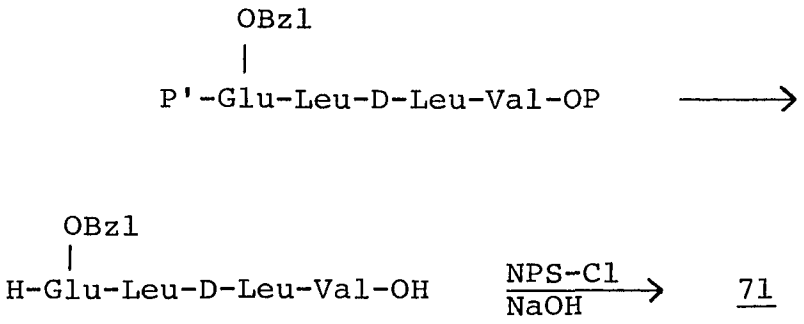


Figure 28. The Synthesis of Tetrapeptide 71 by the NPS-Cl Method.

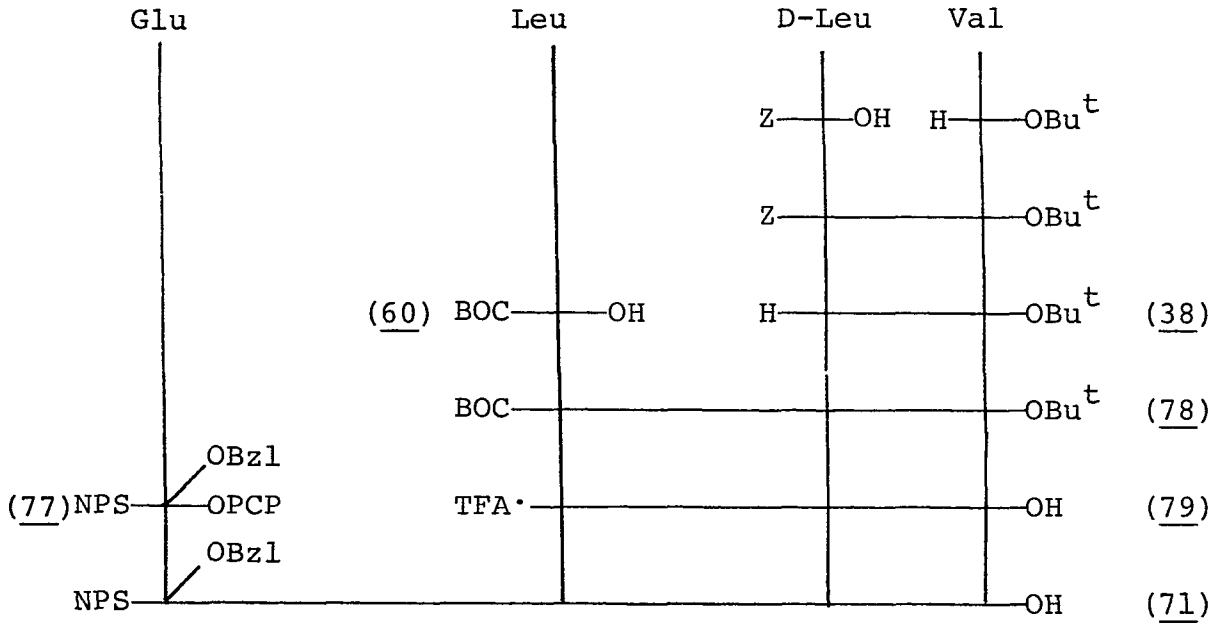


Chart 11. The Synthesis of Tetrapeptide 71 by C-Terminal Salt Protection.

groups were cleaved simultaneously by treatment with anhydrous trifluoracetic acid. Deprotected tripeptide 79 was obtained as a high-melting crystalline solid in 76% yield. The attempted coupling reaction between pentachlorophenyl ester 77 and tripeptide 79 was carried out at pH 8.7 in aqueous pyridine.[109] Aqueous sodium hydroxide was added to the reaction solution periodically in order to keep the pH at a constant value. An attempt to purify the product by dry column chromatography was unsuccessful the only isolated material contained at least four different components.

Failure to accomplish a clean route to 71 excluded the 4+4 scheme (Figure 26) as a route to esperin. The route in Figure 27 remained to be followed, but first fragment 70 had to be prepared. A successful synthesis of a fully protected tetrapeptolide precursor to 70 was successfully carried out. Benzyloxy-α-benzylaspartate was prepared by selective alcoholysis of the anhydride. The α-benzyl ester was isolated by fractional extraction with buffered ammonium hydroxide solution (Figure 29).[110] An exploratory study of ester bond formation using this derivative was carried out with racemic tert-butyl 3-hydroxytetradecanoate.

In the synthesis of isariin, Hardy and coworkers condensed benzyloxycarbonylvaline with tert-butyl 3-hydroxydodecanoate by the action of N,N'-carbonyldiimidazole (CDI) and sodium imidiazolide.[111] A dipeptolide was produced in 59% yield. Other methods of forming this bond which were

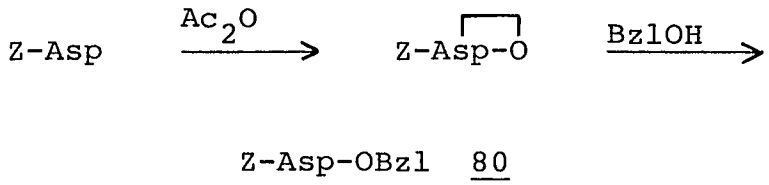
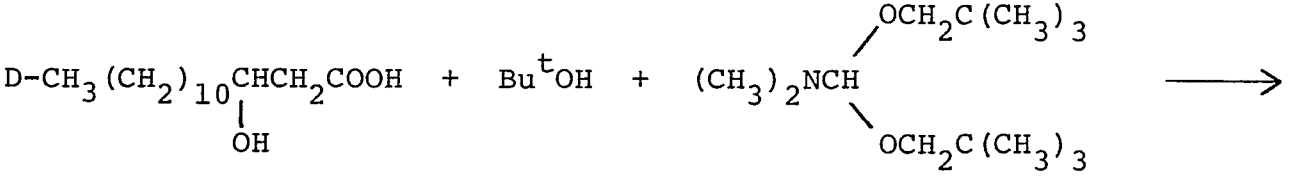


Figure 29. The Preparation of Benzyloxycarbonyl-α-benzylglutamate (80).

investigated, the DCC, mixed anhydride, and benzenesulfonyl chloride methods, were less satisfactory. The CDI method, in modified form, was employed in the synthesis of norsurfactin to couple tert-butyloxycarbonylleucine to p-nitrobenzyl 3-hydroxytetradecanoate.[43] In the latter case, a three fold excess of the free acid was employed, resulting in a virtually quantitative yield of dipeptolide. In the present investigation coupling of tert-butyl 3-hydroxytetradecanoate with a two-fold excess of benzyloxycarbonyl-α-benzylglutamate (80) resulted in an 81% yield of the dipeptolide. The product was isolated as a viscous oil which was homogeneous by thin layer chromatographic analysis.

D-tert-Butyl 3-hydroxytetradecanoate was prepared by N,N-dimethylformamide dineopentyl acetal mediated esterification of the free acid with tert-butanol (Figure 30).[112] The product was obtained in 50% yield after purification by dry column chromatography. The coupling of this optically active β-hydroxyacid with 80 was carried out in the same manner as in the case of the racemic ester (Figure 31). When a 2.5:1 ratio of the aspartic acid derivative to β-hydroxy ester was used, dipeptolide 81 was obtained as a viscous oil in 90% yield. The product appeared homogeneous by thin layer chromatographic analysis in four different solvent systems. The nuclear magnetic resonance spectrum and elemental analysis were in excellent agreement with theory. A second preparation of 81 was carried out in-



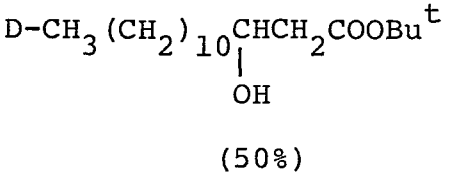


Figure 30. The Preparation of D-tert-Butyl 3-Hydroxytetradecanoate.

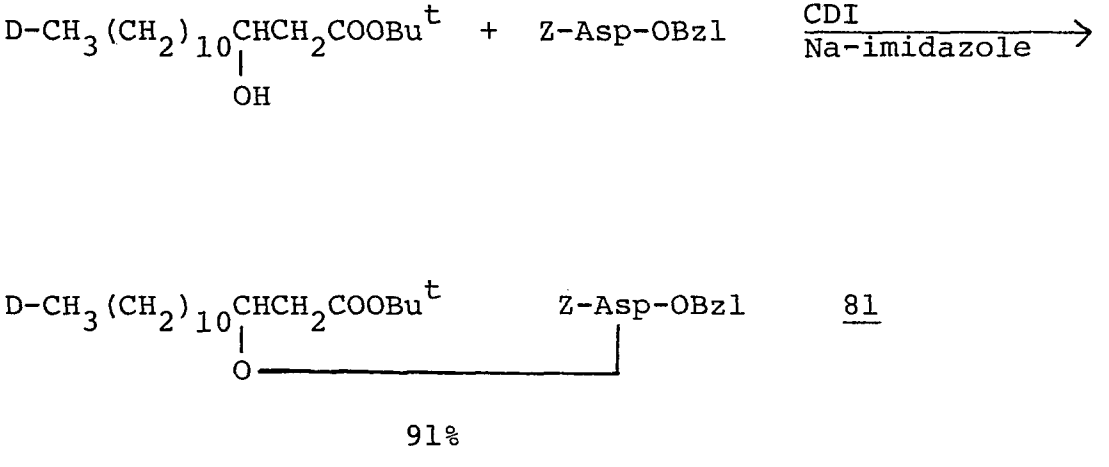


Figure 31. The Preparation of Peptolide 81.

creasing the ratio of aspartic acid derivative to hydroxy ester to 3:1. The yield of purified product was not appreciably increased (91%).

The aspartic acid amino and carboxyl protecting groups were removed simultaneously by catalytic hydrogenolysis (Figure 32). The resulting product 82 was obtained in 74% yield and was homogeneous by thin layer chromatographic analysis in three different solvent systems.

The NPS protecting group was introduced by the previously mentioned method of Zervas.[97] NPS dipeptolide 83 was isolated in 78% yield as a viscous yellow oil. This product was also homogeneous by thin layer chromatographic analysis in three different solvent systems.

Finally, the NPS dipeptolide 83 was coupled with D-leucylleucine benzyl ester trifluoracetate 48 (DCC-HOBT) to give the fully protected tetrapeptolide 84 (Figure 33). This product was purified by dry-column chromatography followed by reverse-phase high pressure liquid chromatography on a C_{18}-Porasil B column. The product was obtained in 24% yield as a yellow solid after crystallization from methanol. The nuclear magnetic resonance spectrum and elemental analysis were in good agreement with the product structure. Current work in progress involves the selective removal of the NPS protecting group from this derivative to provide a suitable substrate for coupling with tripeptide 72 according to the scheme in Figure 27.

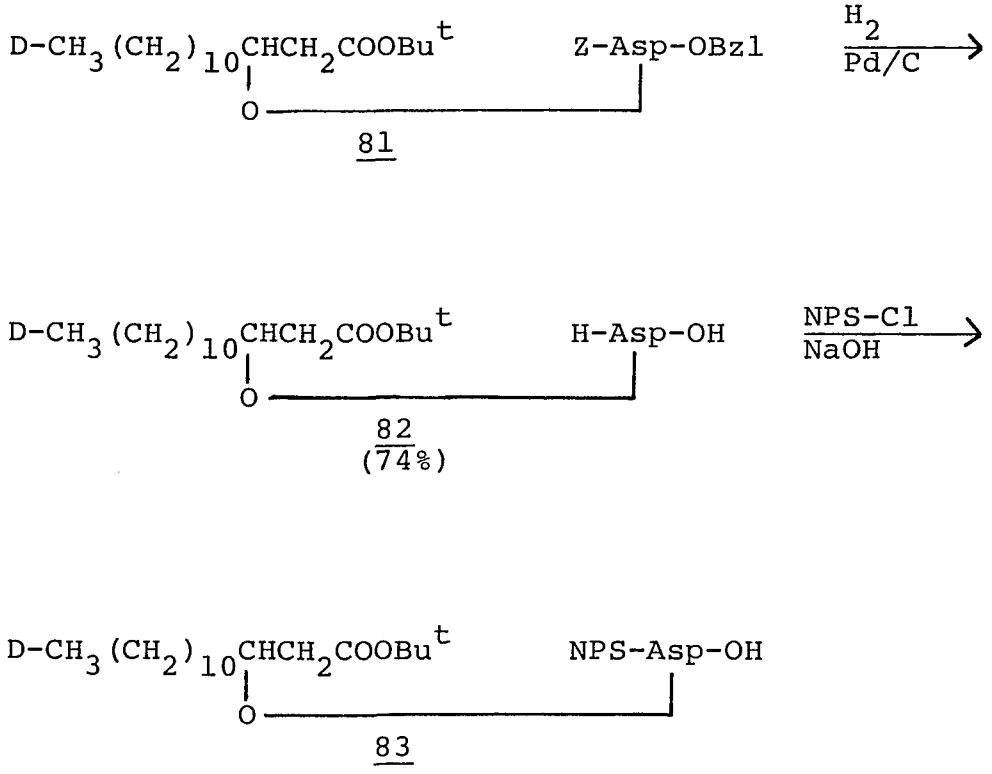
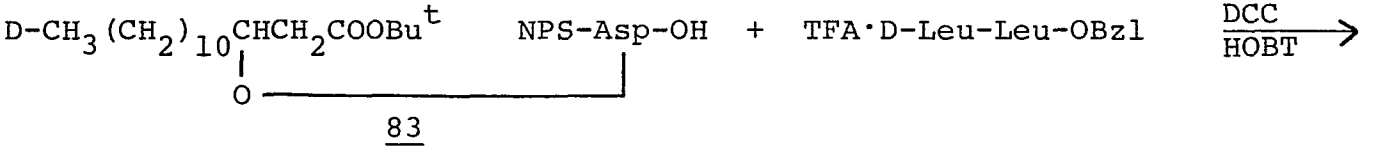
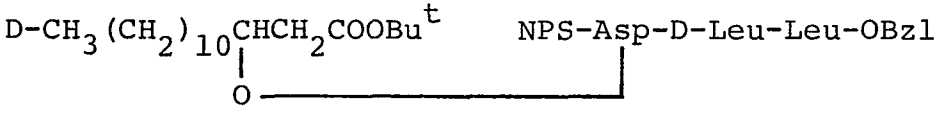


Figure 32. The Synthesis of NPS Dipeptolide 83.



83



84

(24%)

Figure 33. The Preparation of Tetrapeptolide 84.

EXPERIMENTAL

General

Melting Points were obtained using a Thomas-Hoover melting point apparatus and are uncorrected.

Gas-Liquid Partition Chromatography Analyses (glpc) were performed on a Varian Aerograph Model 90-P gas chromatorgraph coupled to a Sargent Welch Model SRG recorder with Disc integrator. Helium was used as a carrier gas at a flow rate of 60 ml/min. Column temperatures are indicated for each compound. The following column systems were used:

A. 3% SE-30 on Varaport 30, 80-100 mesh, 5' x 1/4"

B. 12% STAP on Chromosorb W, 80-100 mesh, 10' x 1/4"

C. 20% Carbowax on Chromosorb W, 80-100 mesh, 10' x 1/4"

D. 12% STAP on Varaport 30, 80-100 mesh, 10' x 3/8"

E. 10% FFAP on Chromosorb W, 80-100 mesh, 5' x 1/4"

Infrared Spectra (ir) were recorded on a Perkin-Elmer 337 grating spectrophotometer and calibrated using the 1601.4 cm^{-1} and 1028 cm^{-1} bands of polystyrene. The spectra of liquids were obtained neat, while those of solids were obtained as mulls.

Nuclear Magnetic Resonance Spectra (nmr) were obtained on a Jeolco Model JNM-MH 100 mHz instrument. All 60 mHz spectra were obtained on a Varian Model A-60 Spectrometer. Chemical shifts are reported relative to tetramethylsilane. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

Optical Rotations were obtained on a Carl Zeiss Photoelectric Precision Polarimeter or a Hansch polarimeter.

Elemental Analyses were performed on an F and M Model 185 carbon, hydrogen and nitrogen analyser at the University of New Hampshire.

Amino Acid Analyses were performed on a Beckman Model 120C amino acid analyzer at the University of New Hampshire.

High Pressure Liquid Chromatography was performed on a Waters ALC/GPC-202 chromatograph equipped with a model 6000 delivery system, USK injector and refractive index and ultraviolet detectors.

Thin Layer Chromatography (tlc) was performed on silica gel (adsorbent A) and basic alumina (adsorbent B) plates in the following solvent systems:

A. Chloroform-methanol-acetic acid (85:10:5)

B. Chloroform-acetone (80:20)

C. n-Butanol-acetic acid-water-pyridine (15:3:12:10)

D. Chloroform-ethanol (80:20)

E. Benzene-ethyl acetate (80:20)

F. Ethyl acetate

G. n-Butanol-acetic acid-water (4:1:1)

H. Acetonitrile-methanol (1:1)

I. Acetonitrile-methanol-water (9:1:1)

Compounds were purchased from commercial sources and used as received. Valine methyl ester hydrochloride and benzyloxycarbonyl-γ-tert-butylglutamate were prepared by Dr. T. L. Ciardelli of these laboratories.

5-Methylhexanoic Acid. Diethylmalonate (201.8 g, 1.26 mol) was added over 30 min to a solution of sodium (28.1 g, 1.22 g-atom) in anhydrous ethanol (625 ml). The solution was warmed to 40° and 1-bromo-3-methylbutane (184 g, 1.22 mol) was added over 30 min. When approximately one-third of the bromide had been added, the solution was heated to reflux in order to initiate the reaction. Heating was then discontinued and the mixture was maintained at reflux by controlling the rate of addition of bromide. The mixture was refluxed 3 hr then allowed to stand at room temperature overnight. The reaction mixture was distilled until the head temperature rose to 100°. Water (250 ml) was added to the pot residue and the organic phase was separated. The aqueous phase was washed with diethyl ether (100 ml). The ether extract and the organic phase from the reaction were combined, dried ($MgSO_4$), concentrated and distilled. The fraction, bp 110-120° (8 mm), was collected (249.3 g). The distillate was added over 30 min to a solution of potassium hydroxide (274 g, 4.88 mol) in water (280 ml). The mixture was heated at 100° for 1 hr. The mixture was treated with water (280 ml) and the solution was distilled until the head temperature rose to 100°. The pot residue was treated with a solution of sulfuric acid (440 g, 4.4 mol) in water (600 ml) and the resulting mixture was refluxed 1 hr and then allowed to stand at room temperature overnight. The

organic phase was separated and the aqueous phase was washed with diethyl ether (100 ml). The ether extract and the organic phase were combined, dried ($MgSO_4$), and concentrated to a yellow oil (195.1 g). The product was distilled. The fraction, bp 109-117° (8 mm), was collected (107.9 g, 68%). Analysis by glpc on column A at 110° showed only one component; nmr (neat) δ 1.07 (d, 6, J=7 Hz, $(C\underline{H}_3)_2CH$), 1.20-1.96 (m, 5, $C\underline{H}\ (C\underline{H}_2)_2$), 2.46 (t, 2, J=7 Hz, $C\underline{H}_2CO$), and 12.36 ppm (s, 1, $COO\underline{H}$).

Methyl Hydrogen Suberate. A mixture of suberic acid (174.2 g, 1.0 mol), concentrated hydrochloric acid (10.5 ml), and methanol (28.8 g, 0.90 mol) was warmed at 80° for 6 hr. The resulting solution was refluxed 3 hr. The product was distilled. The fraction, bp 116-143° (0.6 mm), was collected (58.4 g, 35%); ir (neat) 3150 cm^{-1} (COOH), 1740 cm^{-1} (ester C=O), and 1705 cm^{-1} (acid C=O). Analysis by glpc on column A at 175° indicated the product was approximately 91% monoester plus 9% diester. The product was dissolved in saturated sodium bicarbonate solution (50 ml) and washed with diethyl ether (25 ml). The aqueous phase was acidified with 10% hydrochloric acid and the product was extracted into diethyl ether (50 ml). The ether extract was dried ($MgSO_4$) and concentrated to yield a semi-solid (51.5 g, 30%). Analysis by glpc on column A at 175° indicated that the product was approximately 95% one component; nmr (neat) δ 1.4-2.0 (m, 8, $CH_2(C\underline{H}_2)_4CH_2$), 2.56 (m, 4, $C\underline{H}_2(CH_2)_4C\underline{H}_2$), 3.86 (s, 3, $COOC\underline{H}_3$), and 11.18 ppm

(s, 1, COOH); neutralization equivalent 167.3 (theor. 169.3).

Dimethyl Suberate. Suberic acid (500 g, 2.87 mol) was dissolved in methanol (1.25 liters) containing sulfuric acid (100 ml). The solution was refluxed 12 hr. A portion of the methanol (625 ml) was distilled off and water (1.25 liters) was added. The organic phase was separated and the aqueous phase was washed with diethyl ether (200 ml). The ether extract and the organic phase were combined, washed with water (200 ml), dried ($MgSO_4$), and concentrated. The above experimental procedure was repeated and the combined yield of crude diester was distilled at reduced pressure to give a colorless oil (927.9 g), bp 145-155° (16 mm). A second fraction, bp 155-175° (16 mm), produced an additional 28.7 g of diester after washing with saturated sodium bicarbonate to remove traces of monoester. Analysis by glpc on column A at 155° indicated that the product was 100% diester. Yield (product from two reactions combined): 956.6 g (83%).

Methyl Hydrogen Suberate. Dimethyl suberate (404 g, 2.0 mol) was added over 20 min to a solution of sodium hydroxide (80.0 g, 2.0 mol) in methanol (1 liter). The resulting mixture was allowed to stand at room temperature overnight. About 600 ml of solvent was removed from the pasty product by filtration. The paste was dissolved in water (2 liters) and the resulting solution was acidified with concentrated hydrochloric acid. The oily product was removed and the aqueous phase was washed with diethyl ether

(2 x 250 ml). The organic phases were combined, washed with water (2 x 200 ml), and then saturated sodium chloride (200 ml), dried ($MgSO_4$), and concentrated to a white paste (316.1 g). The crude product was distilled at reduced pressure. After a forerun consisting of the diester (70.9 g) bp 145-175° (16 mm), the monoester was obtained (122.4 g, 33%); bp 147-195° (16 mm); tlc on adsorbent A in solvent B, R_f 0.59. Analysis by glpc on column A at 155° indicated that the product was 97% monoester and 3% diester.

Methyl 11-Methyldodecanoate (18). Methyl hydrogen suberate (519 g, 2.76 mol) and 5-methylhexanoic acid (1080 g, 8.30 mol) were dissolved in methanol (1250 ml) containing 0.22 mol of sodium methoxide (from 5.0 g, 0.22 g-atom of sodium). The solution was electrolyzed between two 10 x 10 cm platinum electrodes. The current was maintained at 1-3 amp and the solution temperature was kept below 30°. The electrode polarity was reversed periodically in order to maintain high current values. The electrolysis was discontinued when the solution became basic to litmus. Total electrolysis time was 223.5 hr. The reaction mixture was acidified by the addition of acetic acid (20 ml). The solution was concentrated and distilled at reduced pressure. Two product-containing cuts were obtained: 423.1 g, bp 77-110° (0.10 mm) and 26.9 g, bp 110-140° (0.10 mm). Analysis by glpc on column A at 130° indicated that the lower boiling fraction contained five significant components, with the

desired ester comprising about 72%, while the second fraction consisted of three components, with the desired ester comprising about 90%. An nmr spectrum of the second fraction confirmed that the desired product was present: δ 1.00 (d, 6, $\underline{J}$=8 Hz, $(C\underline{H}_3)_2CH$), 1.24-1.88 (broad, 17, $C\underline{H}(C\underline{H}_2)_8$), 2.40 (t, 2, $\underline{J}$=8 Hz, $\underline{CH}_2CO$), and 3.78 ppm (s, 3, $COOC\underline{H}_3$). The two fractions were combined and redistilled at reduced pressure. Again, two product-containing cuts were obtained: 226.0 g, bp 82-97° (0.25 mm) and 145.1 g, bp 92-97° (0.25 mm). A lower boiling fraction 65.2 g, bp 50=70° (0.25 mm) was also obtained. Analysis by glpc on column A at 135° indicated that this lowest boiling cut was approximately 93% 2,9-dimethyldecane. The first product-containing cut, bp 82-97° (0.25 mm), consisted of five components with the desired ester comprising approximately 69%. The other components were lower boiling compounds. The second product-containing cut consisted of 100% of the desired ester. Refractionation of the contaminated ester gave a five-component distillate, 78.8 g, bp 45-80° (0.05 mm), with the desired ester comprising 52%. The pot residue, 147.0 g, consisted of three components with the desired ester comprising 91%. The total yield of methyl 11-methyldodecanoate was estimated to be about 320 g (51%).

11-Methyldodecanol. A solution of methyl 11-methyldodecanoate (37.7 g, 0.165 mol) in anhydrous diethyl ether (80 ml) was added dropwise to a solution of lithium aluminum

hydride (42.5 ml of a 3.9 M solution in diethyl ether, 0.165 mol) in anhydrous diethyl ether (80 ml) at 0°. The resulting solution was stirred 2 hr at room temperature then refluxed 30 min. Water (6.5 ml) was added dropwise, followed by a 15% sodium hydroxide solution (6.5 ml), and then more water (19.5 ml). The mixture was filtered and the solid was washed thoroughly with diethyl ether. Concentration of the combined filtrate and washings gave a colorless oil (31.6 g, 96%). Analysis by glpc on column E at 200° indicated that the product was approximately 90% one component; ir (neat) 3345 cm^{-1} (OH).

11-Methyldodecanal (16). Chromium trioxide (36.0 g, 0.36 mol) was added in several portions over 5 min to a solution of pyridine (57.0 g, 0.72 mol) in freshly distilled methylene chloride (900 ml). A solution of 11-methyldodecanol (12.0 g, 0.06 mol) in methylene chloride (25 ml) was added and the resulting mixture was stirred 15 min. The supernate was decanted from the tarry residue which was washed thoroughly with diethyl ether (200 ml). The supernate and washings were combined and concentrated. The residue was treated with diethyl ether (250 ml) and filtered. The filtrate was washed with 0.2 N sodium hydroxide (25 ml), water (25 ml), 5% hydrochloric acid (25 ml), water (25 ml), saturated sodium chloride, dried ($MgSO_4$), and concentrated. The product still contained pyridine and was therefore dissolved in diethyl ether (50 ml), washed

with 5% hydrochloric acid (2 x 25 ml), water (25 ml), dried ($MgSO_4$), and concentrated to a clear oil (10.8 g). Analysis by glpc on column A at 150° indicated a 10 component mixture with the major fraction comprising approximately 66%. The crude product was distilled. The fraction bp 83-85° (0.7 mm) was collected (2.54 g, 21%). Analysis by glpc on column A at 150° indicated that the product was approximately 94% 11-methyldodecanal by comparison with a known sample.

Dodecanoyl Chloride. A solution of dodecanoic acid (20.0 g, 0.10 mol) and thionyl chloride (23.7 g, 0.20 mol) was refluxed for 6 hr. The solution was concentrated and the crude product was distilled at reduced pressure. A total of 20.8 g (96%) of colorless liquid, bp 90-92° (0.10 mm), was collected: ir 1800 cm^{-1} (C=O).

Dodecanal. A 5% palladium on barium sulfate catalyst and a quinoline-sulfur catalyst regulator solution were prepared according to the method of Mosettig.[113] The reduction apparatus was dried in an oven overnight. Dodecanoyl chloride (17.5 g, 0.08 mol), 5% palladium on barium sulfate (2.0 g) and 0.20 ml of quinoline-sulfur regulator solution were added to sodium-dried toluene (55 ml). The mixture was heated to 100° and treated with a stream of dry hydrogen gas. The hydrochloric acid by-product was collected by bubbling the exhaust gas through water. The progress of the reaction was monitored by titration of the hydrochloric acid produced with 5N sodium hydroxide using phenolphthalein

as indicator. After 8 hr a total of 12 ml of 5N sodium hydroxide had been neutralized. The addition of 1.0 g of 5% palladium catalyst and 0.10 ml of regulator did not increase the rate of hydrogen chloride evolution appreciably. After 8.5 hr the evolution was very slow and the reaction was stopped. A total of 12.3 ml (77% of theoretical) of 5N sodium hydroxide had been neutralized. The reaction mixture was treated with decolorizing carbon and filtered through celite. The filtrate was concentrated and the crude product was distilled at reduced pressure. The fraction, bp 77-80° (0.10 mm), was collected as a clear oil (5.91 g): ir 2715 cm^{-1} (aldehyde CH), 1725 cm^{-1} (aldehyde C=O), no other carbonyl band appeared in the ir. A second fraction, 0.88 g, bp 94-105° (0.5 mm) was collected: ir 2720 cm^{-1} (aldehyde CH), 1725 cm^{-1} (aldehyde C=O), 1800 cm^{-1} (acid chloride C=O). The pot residue gave an ir spectrum consistent with a mixture of aldehyde, acid chloride and free acid. Total yield of aldehyde was approximately 45%.

11-Methyldodecanoic Acid. Methyl 11-methyldodecanoate (268 g, 1.17 mol) was dissolved in methanol (330 ml) and the resulting solution was added slowly to a solution of potassium hydroxide (154 g, 2.34 mol) in methanol (1.5 liters). The resulting mixture was stirred two days at room temperature. The mixture was concentrated to one-half its original volume on a steam bath, dissolved in water (1.5 liters), and washed with diethyl ether (200 ml). The

aqueous phase was acidified to Congo red with concentrated hydrochloric acid. The oily product was extracted with diethyl ether and the aqueous phase was washed with diethyl ether (200 ml). The organic phases were combined, dried ($MgSO_4$), and concentrated to a white solid (240.0 g, 96%): nmr ($CDCl_3$) δ 0.87 (d, $\underline{J}$=6 Hz, $(C\underline{H}_3)_2CH$), 1.10-1.88 (m, 17, $C\underline{H}(C\underline{H}_2)_8$), 2.33 (t, 2, $\underline{J}$=8 Hz, $C\underline{H}_2C{=}O$), and 11.67 ppm (s, 1, $COO\underline{H}$).

11-Methyldodecanoyl Chloride. 11-Methyldodecanoic acid (4.28 g, 0.02 mol) was treated with oxalyl chloride (7.35 g, 0.05 mol). The solution was stirred at room temperature for 25 min and then heated at reflux for 1.5 hr. The solution was concentrated and the product was distilled at reduced pressure. A total of 4.24 g (91%) of a colorless liquid, bp 86-88° (0.50 mm) was obtained: ir 1790 cm^{-1} (C=O); nmr δ 1.34 (d, 6, $\underline{J}$=5 Hz, $(C\underline{H}_3)_2CH$), 1.52-2.36 (m, 17, $C\underline{H}(C\underline{H}_2)_8CH_2$), and 3.27 ppm (t, 2, $\underline{J}$=8 Hz, $C\underline{H}_2COCl$).

11-Methyldodecanal (16). 11-Methyldodecanoyl chloride (8.69 g, 0.037 mol), 5% Pd on $BaSO_4$ (2.5 g) and quinoline-sulfur catalyst poison (0.25 ml) were added to dry xylene (40 ml). The mixture was heated to 130° and treated with dry hydrogen gas. The hydrochloric acid by-product was collected by bubbling the exhaust gas through water. The hydrochloric acid was titrated with 5N sodium hydroxide using phenolphthalein as indicator. Hydrogen chloride evolution ceased after 2.5 hr; a total of 6.65 ml (89% of

theoretical) of base was neutralized. The mixture was filtered, concentrated, and distilled to give a colorless liquid (5.54 g, 75%), bp 83-84° (0.35 mm): ir 1725 (C=O) and 2710 cm^{-1} (aldehyde CH). Analysis by glpc on column A at 150° indicated that the distillate was approximately 95% one component.

Attempted Preparation of 12-Methyl-1-tridecene. A solution of undecylenic acid (92.1 g, 0.50 mol) and 3-methylbutanoic acid (204 g, 2.0 mol) in methanol (500 ml) containing sodium methoxide (from 0.05 g-atom of sodium) was electrolyzed between two 10 x 10 cm platinum electrodes at 0.5-2.5 amp for five days. The electrodes were periodically removed and cleaned in order to prevent build-up of waxy materials. The reaction was terminated after five days even though the solution was still acidic. The solution was concentrated and distilled at reduced pressure. After a small forerun, a clear oil, bp 107-120° (12 mm) was collected (59 g). Analysis by glpc on column A at 120° indicated that this product was approximately 92% one component plus about 8% of one other compound; nmr δ 1.08-1.32 (m, 9, $C\underline{H}_3$-), 1.65 ppm (s, 21, $C\underline{H}(C\underline{H}_2)_{10}$). The nmr spectrum showed a lack of vinyl protons and was consistent with the structure of the reduced form of the desired product or 12-methyltridecane.

3-Hydroxy-13-methyltetradecanoic Acid (17). Under nitrogen 11-methyldodecanal (38.0 g, 0.19 mol) was dissolved in a solution of trimethyl borate (50 ml, distilled from

calcium hydride just before use) and tetrahydrofuran (50 ml, distilled from lithium aluminum hydride just before use). Activated zinc dust[57] (12.5 g, 0.09 g-atom) was added and then ethyl bromoacetate (32.1 g, 0.19 mol) was injected through a septum in several portions over 15 min. The exothermic reaction was kept under control by the intermittent application of an ice bath. The mixture was stirred at room temperature for 6 hr. A solution of glycerine (50 ml) and concentrated ammonium hydroxide (50 ml) was added, the organic phase was separated, and the aqueous phase was washed with diethyl ether (3 x 100 ml). The organic phases were combined, dried ($MgSO_4$), and concentrated to a clear, viscous oil (47.7 g). The crude product was dissolved in 95% ethanol (50 ml) and a solution of potassium hydroxide (15.1 g of 85% potassium hydroxide, 0.23 mol) in 95% ethanol (125 ml) was added slowly over a period of 15 min. The mixture was stirred at room temperature for 3 hr. The mixture was dissolved in water (700 ml) and the solution was extracted with diethyl ether (2 x 100 ml). The aqueous phase was cooled in an ice bath and then acidified (litmus) with 10% hydrochloric acid. The oily product was separated. The residual aqueous phase was washed with diethyl ether (2 x 100 ml). The ether extract and the oily product were combined, dried ($MgSO_4$), and concentrated to a viscous yellow oil. Pentane (20 ml) was added and the solution was refrigerated. The white waxy plates which precipitated were collected

by filtration and washed with cold pentane to afford 25.9 g of product. Concentration of the pentane wash gave 4.8 g of a semi-solid material. Recrystallization of the latter from methanol-water gave 1.3 g of white soap-like crystals, mp 48-50°. The solid fractions were combined and recrystallized from benzene-pentane. A first crop of 17.8 g white solid, mp 103.0-104.5° was obtained: nmr ($CDCl_3$) δ 0.86 (d, 6, J=6 Hz, $(C\underline{H}_3)_2CH$), 1.26 (s, 20, $C\underline{H}(C\underline{H}_2)_9C\underline{H}$), 2.52 (m, 2, $C\underline{H}_2COOH$), 4.25 (broad s, 1, $CHO\underline{H}$), and 8.38 ppm (broad s, 1, $COO\underline{H}$); ir 3480 (CHOH), 3210 (bonded COOH), and 1715 cm^{-1} (C=O); tlc on adsorbent A in solvent D, R_f 0.19. The mother liquor was concentrated and a viscous oil (5.2 g) was obtained. The oil was treated with sodium hydroxide (1.6 g, 40 mmol) in ethanol (15 ml). The mixture was stirred overnight and then poured into water (75 ml). The solution was washed with diethyl ether (50 ml), and acidified (litmus) with 10% hydrochloric acid. The product was dissolved in diethyl ether, washed with water (25 ml), dried ($MgSO_4$) and concentrated to a pale-yellow solid (4.48 g), mp 47-49°: ir 3485 (CHOH), 2800-3200 (COOH), and 1715 cm^{-1} (C=O); 60 mHz nmr ($CDCl_3$) δ 0.87 (d, 6, J=6 Hz, $(C\underline{H}_3)_2CH$), 1.27 (m, 19, $(C\underline{H}_2)_9CHOH$), 2.43 (m, 2, $C\underline{H}_2COO$), 4.02 (m, 1, $C\underline{H}OH$), and 7.28 ppm (s, 2, $CHO\underline{H}$, and $COO\underline{H}$).

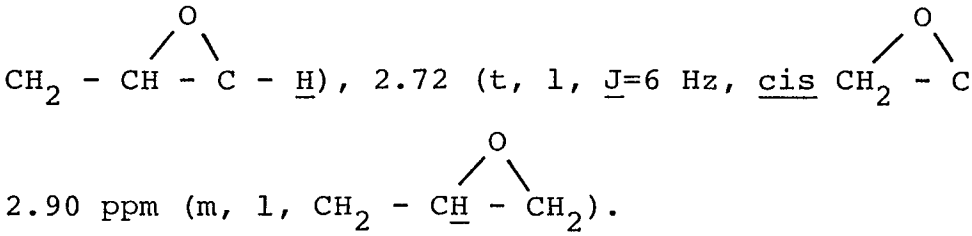
Resolution of D,L-3-Hydroxy-13-methyltetradecanoic Acid. A solution of D-amphetamine (8.77 g, 65 mmol) in diethyl ether (14 ml) was added to a stirred solution of

D,L-3-hydroxy-13-methyltetradecanoic acid (16.8 g, 65 mmol) in diethyl ether (336 ml). The resulting mixture was allowed to stand at room temperature overnight. The mixture was heated to boiling and sufficient ethanol was added to effect solution. The solution was allowed to stand at room temperature overnight. The fibrous precipitate was collected and dried. Yield: 7.37 g; $[\alpha]_D^{21}$ -3.0° (c 2.06, $CHCl_3$). Four recrystallizations from 2:1 diethyl ether-ethanol gave a constant rotation value of $[\alpha]_D^{22}$ -4.9° (c 2, $CHCl_3$). The salt was decomposed with 5% hydrochloric acid and the hydroxy acid was extracted with diethyl ether. The ether phase was washed with water (5 ml), dried (Na_2SO_4), and concentrated to a white solid (1.88 g). Recrystallization from 5:1 pentane-benzene gave (-)-3-hydroxy-13-methyltetradecanoic acid as brilliant white plates (1.83 g, 11%): $[\alpha]_D^{22}$ -14.0° (c 2.02, $CHCl_3$); mp 45-47°.

Partially resolved (+)-3-hydroxy-13-methyltetradecanoic acid ($[\alpha]_D^{25}$ 7.4° (c 2.00, $CHCl_3$)), obtained by decomposition of the concentrated mother liquor from the (-)-acid resolution, (7.70 g, 30 mmol), was dissolved in diethyl ether (75 ml). The solution was warmed and treated with a solution of (+)-α-phenylethylamine (3.61 g, 30 mmol, 95% ee) in diethyl ether (20 ml). The solution was cooled at -15° for two days. The white crystalline product was collected and dried (10.6 g, 94%); $[\alpha]_D^{22}$ 10.9° (c 2.10, $CHCl_3$). Four recrystallizations from 2:1 diethyl ether-

ethanol gave a constant rotation of $[\alpha]_D^{22}$ 19.7° (c 2, $CHCl_3$). The salt was decomposed with 5% hydrochloric acid and the hydroxy acid was extracted into diethyl ether. The ether phase was washed with water (5 ml), dried (Na_2SO_4), and concentrated to a white solid (1.25 g). Recrystallization from 5:1 pentane-benzene gave (+)-3-hydroxy-13-methyltetradecanoic acid as fine white plates (1.20 g, 8%): $[\alpha]_D^{24}$ 13.8° (c 2.10, $CHCl_3$); mp 45-48°.

1,2-Epoxyoctane. A solution of m-chloroperbenzoic acid (11.0 g, 0.054 mol) in methylene chloride (120 ml, distilled from phosphorous pentoxide) was added to a solution of 1-octene (6.06 g, 0.054 mol) in methylene chloride (75 ml) over a period of 10 min while maintaining the reaction temperature below 25°. The solution was then stirred at room temperature for 45 min. A 10% solution of sodium thiosulfite was added until the solution gave a negative test with starch-iodide paper. The solution was washed with saturated sodium bicarbonate (2 x 50 ml), water (50 ml), saturated sodium chloride (50 ml) and dried ($MgSO_4$). Solvent was removed by distillation at atmospheric pressure and the product was distilled at reduced pressure. The fraction, bp 73-74° (28 mm) (3.98 g), was collected. Analysis by glpc on column B at 170° indicated that the distillate was approximately 89% of the desired epoxide and 11% starting alkene; nmr δ 1.14 (t, 3, J=5 Hz, CH_3), 1.54 (m, 8, $(CH_2)_4$), 1.64 (m, 2, $CH_2CH(O)CH_2$-, 2.46 (q, 1, J=3 Hz, J'=6 Hz, trans

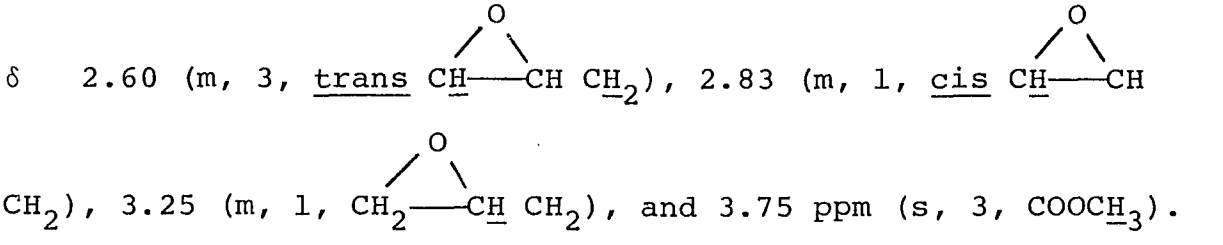


3-Nonanol. A suspension of cuprous iodide (7.62 g, 40 mmol) in anhydrous diethyl ether (10 ml), under nitrogen, was cooled to 0° and a solution of freshly prepared methyllithium in anhydrous diethyl ether (92 ml of a 0.868 m solution, 80 mmol) was added over a period of 2 min. The mixture was stirred at 0° for 10 min and then a solution of 1,2-epoxyoctane (2.56 g, 20 mmol) in anhydrous ether (15 ml) was added dropwise over 5 min. The mixture was stirred 2 hr at 0°, allowed to warm to room temperature, and then poured into cold saturated ammonium chloride (300 ml). The organic phase was separated and the aqueous phase was washed with diethyl ether (100 ml). The organic phases were combined, dried ($MgSO_4$), and concentrated to a viscous oil (2.49 g, 86%). Analysis by glpc on column B at 170° indicated that the product was approximately 97% one component; nmr (neat) δ 1.22 (m, 6, CH_3), 1.60 (m, 12, $CH_2CH(CH_2)_5$), 3.68 (m, 1, CH), and 4.82 ppm (s, 1, OH, exchangeable with D_2O); ir 3355 cm^{-1} (OH).

Methyl 3-Butenoate.[114] Vinyl acetic acid (43.0 g, 0.50 mol) was added to methanol (98 ml) containing anhydrous hydrogen chloride (7.5 g). The solution was refluxed 1 hr then stirred at room temperature overnight. The solution was poured slowly into water (275 ml). The organic phase

was separated and the aqueous phase was washed with diethyl ether (50 ml). The organic phases were combined, dried (Na_2SO_4), and distilled. The fraction, bp 108-111° was collected as a clear oil (33.4 g, 60%). Analysis by glpc on column C at 130° indicated that the product was approximately 90% one component plus traces of two unknown impurities.

Methyl 3,4-Epoxybutanoate. A solution of m-chloroperbenzoic acid (4.47 g, 0.022 mol, technical grade) in methylene chloride (50 ml) was added over 10 min to a solution of methyl 3-butenoate (2.00 g, 0.02 mol) in methylene chloride (30 min). The resulting solution was stirred at room temperature for 4 days. The mixture was poured into 50 ml of saturated sodium bicarbonate. The organic phase was separated, washed successively with saturated sodium bicarbonate (25 ml), water (25 ml), dried ($MgSO_4$), and concentrated to a clear oil (1.73 g). Analysis by glpc on column C at 185° indicated a six component mixture with the major product comprising approximately 81%. The oil was distilled. The fraction, bp 84-86° (32 mm), was collected; glpc analysis on column C at 185° indicated that the product was approximately 91% one component. Yield: 1.25 g (49%); nmr



δ 2.60 (m, 3, trans CH——CH CH_2), 2.83 (m, 1, cis CH——CH CH_2), 3.25 (m, 1, CH_2——CH CH_2), and 3.75 ppm (s, 3, $COOCH_3$).

Methyl 3-Hydroxypentanoate. A suspension of cuprous iodide (3.81 g, 20 mmol) in anhydrous diethyl ether (5 ml) was cooled to 0° and a solution of methyllithium in anhydrous diethyl ether (43 ml of a 0.938 M solution, 40 mmol) was added over a period of 2 min. The mixture was stirred for 10 min at 0° and then a solution of methyl 3,4-epoxybutanoate (1.16 g, 10 mmol) in anhydrous diethyl ether (10 ml) was added over a period of 5 min. The resulting mixture was stirred 1 hr at 0°, allowed to warm to room temperature, and was then poured into 200 ml of saturated aqueous ammonium chloride. The product was extracted into diethyl ether and the aqueous phase was washed with diethyl ether (50 ml). The organic phases were combined, dried ($MgSO_4$), and concentrated to an oil (0.48 g). Saturation of the aqueous phase from the above washings with sodium chloride and extraction with diethyl ether (50 ml) in the same fashion afforded 0.30 g of oil. Analysis by glpc of the crude product on column A indicated two products in the approximate ratio of 83:17. The major component was identified as methyl 3-hydroxypentanoate by nmr analysis of a sample purified by preparative gas chromatography on column D at 165°: nmr δ 0.92 (t, 3, $\underline{J}$=8 Hz, $C\underline{H}_3CH_2$), 1.45 (m, 2, $CH_3C\underline{H}_2$), 2.35 (m, 2, $C\underline{H}_2COO$), 3.30 (s, 1, $CHO\underline{H}$), 3.54 (s, 3, $COOCH_3$), and 3.76 ppm (m, 1, $C\underline{H}OH$).

Leucine t-Butyl Ester Hydrochloride (19).[72] Leucine (6.55 g, 50 mmol) was suspended in dioxane (100 ml) containing sulfuric acid (10 ml) and the mixture was cooled to -78°.

Isobutylene (100 ml) was added and the vessel was sealed and stirred at room temperature for 24 hr. The reaction mixture was poured into diethyl ether (500 ml) and 1N sodium hydroxide (500 ml). The aqueous phase was separated and washed with diethyl ether (50 ml). The organic phase and ether washing were combined, dried ($MgSO_4$), concentrated to about 25 ml and diluted with hexane (100 ml). The hexane solution was treated with dry hydrogen chloride until no further precipitation of hydrochloride was noticed. The white solid was collected and recrystallized from ethyl acetate to give white needles (6.4 g, 59%): mp 161.5-163°; $[\alpha]_D^{25}$ 18.6 (c 2.00, EtOH); lit.[72] mp 166-167°; $[\alpha]_D^{20}$ 12.4° (c 2, EtOH).

Benzyloxycarbonyl-D-leucine (20).[73] Sodium bicarbonate (21.0 g, 0.25 mol) and D-leucine (13.1 g, 0.10 mol) were suspended in water (125 ml) and benzyloxycarbonyl-chloride (18.7 g, 0.11 mol) was added dropwise over 15 min. The mixture was stirred at room temperature overnight. After four hours an additional portion of benzyloxycarbonyl chloride (1 ml) was added. The reaction mixture was washed with 50 ml of diethyl ether. The aqueous phase was acidified (Congo red) by the addition of 10% hydrochloric acid. The product was extracted with ethyl acetate (50 ml) and the aqueous phase was washed with ethyl acetate (50 ml). The organic phases were combined, washed with 2N citric acid (50 ml), water (50 ml), saturated sodium chloride (50 ml), dried

($MgSO_4$), and concentrated to a viscous, colorless oil (21.7 g, 82%): $[\alpha]_D^{22}$ 16.7° (c 1.97, EtOH); lit.[115] $[\alpha]_D^{20}$ -16.4 (c, 1.83, EtOH) for the L-isomer; nmr ($CDCl_3$) δ 0.92 (d, 6, J=5 Hz, $(CH_3)_2CH$), 1.60 (m, 3, $CH_2CH(CH_3)_2$), 4.26 (m, 1, NHCH), 4.98 (s, 2, $-CH_2Ph$), 5.44 (d, 1, J=9 Hz, NHCH), 7.14 (s, 5, Ph-), and 9.92 ppm (s, 1, COOH).

Benzyloxycarbonyl-D-leucylleucine-tert-butyl Ester (21). A mixture of benzyloxycarbonyl-D-leucine (5.31 g, 20 mmol), leucine tert-butyl ester hydrochloride (4.47 g, 20 mmol), and 1-hydroxybenzotriazole monohydrate (5.40 g, 35 mmol) in tetrahydrofuran (80 ml) was cooled to -5°. N-Methylmorpholine (2.02 g, 20 mmol) and dicyclohexylcarbodiimide (4.54 g, 22 mmol) were added and the mixture was stirred 2 hr at -5° and 1 hr at room temperature. The mixture was filtered and the filtrate was concentrated. The viscous oily residue was filtered through a 3.5 x 15 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluant. Concentration of the eluate (350 ml) yielded a viscous oil (7.2 g, 83%): $[\alpha]_D^{25}$ 0° (c EtOH); tlc on adsorbent A in solvent B, R_f 0.80.

D-Leucylleucine-tert-butyl Ester (22). Benzyloxycarbonyl-D-leucylleucine-tert-butyl ester (6.07 g, 14.0 mmol) was dissolved in a solution of methanol (10 ml), water (4 ml) and acetic acid (4 ml). The solution was deoxygenated with nitrogen and 5% palladium on carbon (0.40 g) was added. The mixture was hydrogenated at 20 psi for 3 hr at room temperature. The mixture was filtered through celite and concentra-

ted. A diethyl ether solution of the crude product was washed successively with saturated sodium bicarbonate (2 x 25 ml), water (25 ml), dried ($MgSO_4$) and filtered through celite. The solution was concentrated *in vacuo* to a yellow, viscous oil (4.07 g, 97%): $[\alpha]_D^{25}$ 17.8° (*c* 5.14, $CHCl_3$); tlc on adsorbent A in solvent B, R_f 0.40; on adsorbent A in solvent C, R_f 0.84.

Anal. Calcd for $C_{16}H_{32}N_2O_3$: C, 63.96; H, 10.74; N, 9.33. Found: C, 64.32; H, 10.42; N, 9.33.

D-Leucylleucine-*tert*-butyl Ester Hydrogen Oxalate Hemihydrate. Anhydrous oxalic acid (0.30 g, 3.3 mmol) was dissolved in diethyl ether (2 ml) and the resulting solution was added to a solution of *D*-leucylleucine *tert*-butyl ester (1.00 g, 3.3 mmol) in diethyl ether (1.5 ml). The resulting mixture was refrigerated and filtered to afford 0.82 g (62%) of white needles: mp 141-142°; $[\alpha]_D^{25}$ -41.9° (*c* 1.5, MeOH).

Anal. Calcd for $C_{18}H_{34}N_2O_6 \cdot 0.5H_2O$: C, 54.11; H, 8.83; N, 7.01. Found: C, 53.92; H, 8.86; N, 6.92.

Benzyloxycarbonyl-α-methylaspartate Dicyclohexylamine Salt.[116] Benzyloxycarbonylaspartic acid (100.5 g, 0.393 mol) was covered with freshly distilled acetic anhydride (70 ml) and the mixture was stirred at 45°. The mixture was concentrated and then treated with methanol (400 ml). The resulting solution was refluxed for 4 hr. The solution was concentrated and the product was dissolved in diethyl ether (400 ml). The product was extracted into

saturated sodium bicarbonate (750 ml). The aqueous phase was acidified (litmus) with 10% hydrochloric acid and the resulting oil was extracted with diethyl ether (150 ml). The aqueous phase was washed with diethyl ether (100 ml). The organic phases were combined and dried ($MgSO_4$). Dicyclohexylamine (74.9 g, 0.413 mol) was added dropwise to the ether solution. The crude product separated as a white solid. The product was collected and recrystallized from 2:1 ethanol-pentane to afford a white crystalline solid (83.9 g, 46%). A second recrystallization from 4:1 ethanol-pentane gave white crystals (45.1 g): mp 160-161°; $[\alpha]_D^{25}$ 5.3°, (c 1.00, EtOH). A second crop (30.2 g) was obtained by concentration of the mother liquor; mp 160-161°, $[\alpha]_D^{25}$ 4.6° (c 1.00, EtOH). Lit.[116] mp 159-160°, $[\alpha]_D^{20}$ 4.9 ± 0.5° (c 1, EtOH).

Benzyloxycarbonyl-α-methylaspartate.[116] Benzyloxycarbonyl-α-methylaspartate dicyclohexylamine salt (75.3 g, 0.16 mol) was suspended in water (500 ml) and 10% sulfuric acid (80 ml) was added slowly with stirring. The resulting oil was extracted with diethyl ether (100 ml) and the aqueous phase was washed with diethyl ether (50 ml). The ether phases were combined, dried ($MgSO_4$), and concentrated. The resulting oil was crystallized from diethyl ether-pentane to give white crystals (40.7 g, 91%): mp 90.0-90.5°; $[\alpha]_D^{25}$ -17.3°, (c 2.00, EtOH); tlc on adsorbant A in solvent B, R_f 0.57, ninhydrin negative; lit.[116] mp 88-89°; $[\alpha]_D^{20}$ -16.1 (c 2, EtOH).

Benzyloxycarbonyl-α-methyl-γ-tert-butylaspartate.[116] Benzyloxycarbonyl-α-methylaspartate (20.2 g, 0.072 mol) was dissolved in methylene chloride (145 ml) and the resulting solution was cooled to -78°. Sulfuric acid (1.0 ml) was added followed by isobutylene (80 ml). The reaction vessel was stoppered and the solution was stirred at room temperature for five days. Excess isobutylene was carefully vented and the solution was treated with saturated sodium bicarbonate (100 ml). The organic phase was separated and washed with water (2 x 100 ml), dried ($MgSO_4$), and concentrated to a pale yellow oil (23.6 g, 97%): $[\alpha]_D^{25}$ -13.8° (c 2.55, EtOH); nmr ($CDCl_3$) δ 1.31 (s, 9, -C)CH_3)$_3$), 2.65 (d, 2, J=5 Hz, -CH_2C=O), 3.52 (s, 3, -OCH_3), 4.44 (m, 1, CH), 4.92 (s, 2, -CH_2O-), 5.86 (m, 1, J=8 Hz, -NH-), and 7.10 ppm (s, 5, Ph-); lit.[116] $[\alpha]_D^{20}$ -13.2 ± 0.5° (c 2.0, EtOH).

Benzyloxycarbonyl-β-tert-butylaspartate Dicyclohexylamine Salt (23). Benzyloxycarbonyl-α-methyl-β-tert-butylaspartate (36.4 g, 0.108 mol) was dissolved in 90% aqueous dioxane (150 ml) and 1N sodium hydroxide solution (125 ml) was added dropwise over 40 min. The solution was stirred for 5 min. A 10% hydrochloric acid solution (30 ml) was added dropwise while cooling the reaction mixture in an ice bath. The solution was concentrated, treated with 2N citric acid (50 ml) and extracted with diethyl ether (3 x 100 ml). The product was extracted from the combined ether fractions with saturated sodium

bicarbonate (3 x 100 ml). The aqueous portions were combined, washed with diethyl ether (2 x 50 ml), and acidified with 2N citric acid. The oily product was extracted with diethyl ether (50 ml) and the aqueous phase was washed with diethyl ether (100 ml). The ether phases were combined, washed with water (100 ml), and dried ($MgSO_4$). The ether solution was cooled in an ice bath and dicyclohexylamine (18.0 g, 0.099 mol) was added dropwise over 20 min. The mixture was refrigerated overnight. The solid product was collected, washed with diethyl ether and dried to give white crystals (45.1 g, 83%): mp 129.5-130.5°; $[\alpha]_D^{24}$ 12.7° (c 2.01, EtOH); tlc on adsorbent A in solvent B, R_f 0.04; on adsorbent A in solvent C, R_f 0.84; lit.[116] mp 125.5-126.5°; $[\alpha]_D^{20}$ 13.1 ± 0.5° (c 2, EtOH).

Benzyloxycarbonyl-β-tert-butyl-aspartyl-D-leucyl-leucine-tert-butyl Ester (24). Benzyloxycarbonyl-β-tert-butylaspartate (9.57 g, 29.6 mmol) and D-leucylleucine-tert-butyl ester were dissolved in tetrahydrofuran (180 ml), 1-hydroxybenzotriazole monohydrate (8.00 g, 52.3 mmol) was added and the resulting solution was cooled to 0°. A solution of dicyclohexylcarbodiimide (6.10 g, 29.6 mmol) in tetrahydrofuran (20 ml) was added in one portion. The mixture was allowed to stir overnight while warming to room temperature. The mixture was filtered and the filtrate was concentrated. The solid residue was treated with ethyl acetate (50 ml) and filtered. The filtrate was washed

successively with saturated sodium bicarbonate (25 ml), 2N citric acid (25 ml), saturated sodium bicarbonate (25 ml), 2N citric acid (25 ml), water (2 x 25 ml) and saturated sodium chloride (25 ml). The solution was dried ($MgSO_4$) and concentrated. The product was filtered through a 3.5 x 14 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. A total of 450 ml was collected which on concentration gave a pale yellow, viscous oil (16.0 g); tlc on adsorbent A in solvent A, R_f 0.82; on adsorbent A in solvent B, three spots R_f 0.87, R_f 0.77 and one spot at the origin. All three spots were ninhydrin negative. After treatment with 1:1 hydrobromic acid-acetic acid only the spot at R_f 0.87 was ninhydrin positive. The crude product was chromatographed on a 4.4 x 18 cm column of silica gel (40-140 mesh) using 5:1 chloroform-acetone as eluent. A total of 350 ml was collected which on concentration afforded a white foam (14.2 g, 79%): $[\alpha]_D^{20}$ 37.2° (c 2.23, $CHCl_3$); tlc on adsorbent A in solvent A, R_f 0.93; on adsorbent A in solvent B, R_f 0.76; on adsorbent A in solvent C, R_f 0.82; on adsorbent B in solvent A, R_f 0.86; on adsorbent B in solvent B, 0.70; amino acid analysis: Leu 2.06, Asp 0.94.

Anal. calcd for $C_{32}H_{51}N_3O_8$: C, 63.44; H, 8.48; N, 6.93. Found: C, 63.31; H, 8.65; N, 7.10.

β-tert-Butylaspartyl-D-leucylleucine-tert-butyl Ester (25). Benzyloxycarbonyl-β-tert-butylaspartyl-D-leucylleucine-tert-butyl ester (10.2 g, 16.8 mmol) was

dissolved in methanol (100 ml) containing 4 ml of acetic acid and 4 ml of water. The solution was hydrogenated over 5% palladium on carbon (1.0 g) at 20 psi for 14 hr. The reaction mixture was filtered and concentrated. A diethyl ether solution of the product was washed successively with saturated sodium bicarbonate (3 x 25 ml), water (25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated to a viscous, pale-yellow oil (7.40 g, 94%): $[\alpha]_D^{22}$ 34.6° (c 1.48, $CHCl_3$).

Anal. Calcd for $C_{24}H_{45}N_3O_6$: C, 61.11; H, 9.61; N, 8.90. Found: C, 61.08; H, 9.65; N, 8.77.

Benzyloxycarbonylvalyl-β-tert-butylaspartyl-D-leucyl-leucine-tert-butyl Ester (27). Benzyloxycarbonylvaline (4.75 g, 18.9 mmol), β-tert butylaspartyl-D-leucylleucine-tert-butyl ester (8.91 g, 18.9 mmol) and 1-hydroxybenzotriazole monohydrate (5.11 g, 33.4 mmol) were dissolved in tetrahydrofuran (80 ml). The solution was cooled to 0° and a solution of dicyclohexylcarbodiimide (4.29 g, 20.8 mmol) in tetrahydrofuran (5 ml) was added in one portion. The mixture was allowed to stir overnight while warming to room temperature. The mixture was filtered and the filtrate was concentrated. The residue was treated with ethyl acetate (25 ml) and filtered. The filtrate was washed successively with saturated sodium bicarbonate (25 ml), 2N citric acid (25 ml), water (25 ml), saturated sodium chloride (2 x 25 ml), dried ($MgSO_4$), and concentrated. The crude product

was filtered through a 3.5 x 18 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. A total of 200 ml of ethyl acetate was collected which was concentrated to a white solid (13.0 g, 97%). The product was recrystallized from 2:1 pentane-ethyl acetate to give a white solid (7.1 g, 53%): mp 167-172°; $[\alpha]_D^{22}$ 9.8° (c 3.00, $CHCl_3$); tlc on adsorbent A in solvent A, R_f 0.68; on adsorbent A in solvent C, R_f 0.93; on adsorbent A in solvent D, R_f 0.35.

Anal. Calcd for $C_{37}H_{60}N_4O_9$: C, 63.04; H, 8.57; N, 7.94. Found: C, 63.14; H, 8.52; N, 8.20.

Valyl-β-tert-butylaspartyl-D-leucylleucine tert-Butyl Ester (28). Benzyloxycarbonylvalyl-β-tert-butyl-aspartyl-D-leucylleucine tert-butyl ester (4.53 g, 6.4 mmol) was dissolved in a solution of methanol (42 ml), acetic acid (2.8 ml) and water (2.8 ml). The solution was hydrogenated over 5% palladium on carbon (3.0 g) at 20 psi for 72 hr. The mixture was filtered and concentrated. An ethyl acetate solution of the oily product was washed with saturated sodium bicarbonate (2 x 25 ml), water (25 ml), dried ($MgSO_4$) and concentrated to a white foam (3.36 g, 92%): $[\alpha]_D^{25}$ -0.4° (c 2.00, $CHCl_3$); tlc on adsorbent A in solvent A, R_f 0.72; on adsorbent A in solvent B, R_f 0.40; on adsorbent A in solvent C, R_f 0.85. All spots were ninhydrin positive.

Anal. Calcd for $C_{29}H_{54}N_4O_7$: C, 61.02; H, 9.54; N, 9.82. Found: C, 61.12; H, 9.42; N, 9.81.

Benzyloxycarbonyl-D-leucylvalyl-β-tert-butylaspartyl-D-leucylleucine tert-Butyl Ester (29). A solution of benzyloxycarbonyl-D-leucine (1.71 g, 6.45 mmol), valyl-β-tert-butylaspartyl-D-leucylleucine tert-butyl ester (3.68 g, 6.45 mmol), and 1-hydroxybenzotriazole monohydrate (1.98 g, 12.9 mmol) in tetrahydrofuran (32 ml) was cooled to 0°. A solution of dicyclohexylcarbodiimide (1.46 g, 7.09 mmol) in tetrahydrofuran was added and the resulting mixture was stirred 1 hr at 0° and 2 days at room temperature. The mixture was filtered and concentrated. An ethyl acetate solution of the crude product was washed with saturated sodium bicarbonate (25 ml), 2N citric acid (25 ml), saturated sodium chloride (2 x 25 ml), dried ($MgSO_4$), and concentrated to a white solid (5.22 g), mp 175-182°. The product was recrystallized from ethyl acetate-pentane to yield 3.90 g of white crystals: mp 192-194°; $[\alpha]_D^{22}$ 21.9° (c 2.00, $CHCl_3$). A second crop, 0.31 g, mp 176-178°, $[\alpha]_D^{22}$ 16.8° (c 2.00, $CHCl_3$), was obtained on concentration of the mother liquor; tlcs on both materials were identical: on adsorbent A in solvent A, R_f 0.79; on adsorbent A in solvent B, R_f 0.63; on adsorbent A in solvent C, R_f 0.93. In all cases the spot was negative to ninhydrin until treatment with 1:1 hydrobromic acid-acetic acid. Yield: 4.21 g (80%).

Anal. Calcd for $C_{43}H_{71}N_5O_{10}$: C, 63.13; H, 8.75; N, 8.56. Found: C, 63.24; H, 8.70; N, 8.78.

D-Leucylvalyl-β-tert-butylaspartyl-D-leucylleucine-tert-butyl Ester (30). Benzyloxycarbonyl-D-leucylvalyl-β-tert-butylaspartyl-D-leucylleucine-tert-butyl ester (3.57 g, 4.37 mmol) was dissolved in a solution of methanol (35 ml), acetic acid (2.3 ml), and water (2.3 ml). The peptide was hydrogenated over 5% palladium on carbon (2.0 g) at 20 psi for 72 hr. The reaction mixture was filtered and concentrated to a clear viscous oil (3.75 g). An ethyl acetate solution of the product was washed with saturated sodium bicarbonate (2 x 25 ml), water (25 ml), dried ($MgSO_4$), and concentrated to a white foam (2.60 g). The product was dissolved in 3:1 pentane-ethyl acetate and refrigerated. A white solid (0.33 g) was obtained which gave two spots on tlc on adsorbent A in solvent B, R_f 0.12 and R_f 0.40. The mother liquor was concentrated to a white foam (1.73 g, 58%): mp 163-168°; tlc on adsorbent A in solvent A, R_f 0.65; on adsorbent A in solvent B, R_f 0.12; on adsorbent A in solvent C, R_f 0.87; on adsorbent A in solvent D, R_f 0.74; in all cases the spot was ninhydrin positive; $[\alpha]_D^{22}$ -19.0° (c 2.01, MeOH).

Anal. Calcd for $C_{35}H_{65}N_5O_8$: C, 61.46; H, 9.58; N, 10.24. Found: C, 61.05; H, 9,81; N, 9.97.

Benzyloxycarbonylleucine (31).[73] Sodium bicarbonate (21.0 g, 0.25 mol) and leucine (13.1 g, 0.10 mol) were suspended in water (125 ml) and benzyloxycarbonyl chloride (18.7 g, 0.11 mol) was added dropwise over 15 min. The mixture was stirred at room temperature overnight. The

solution was washed with diethyl ether (50 ml) and acidified to Congo red by the addition of 10% hydrochloric acid. The product was extracted with ethyl acetate (50 ml) and the aqueous phase was washed with ethyl acetate (50 ml). The organic phases were combined, washed with 2N citric acid (50 ml), water (50 ml), saturated sodium chloride (50 ml), dried ($MgSO_4$), and concentrated *in vacuo* to a clear, viscous oil (19.1 g, 72%): $[\alpha]_D^{22}$ -17.5° (*c* 2.00, EtOH); lit.[115] $[\alpha]_D$ -16.4°; tlc on adsorbent A in solvent A, R_f 0.73; on adsorbent A in solvent D, R_f0.79; in both cases the spot was ninhydrin negative until treatment with 1:1 hydrobromic acid-acetic acid; nmr ($CDCl_3$) δ 0.95 (d, 6, *J*=5 Hz, $CH(C\underline{H}_3)_2$), 1.67 (m, 3, $(CH_3)_2C\underline{H}C\underline{H}_2$), 4.49 (m, 1, NHC*H*), 5.23 (s, 2, $C\underline{H}_2$-Ph), 5.72 (d, 1, *J*=9 Hz, N*H*CH), 7.70 (s, 5, Ph-), and 10.25 ppm (s, 1, COO*H*).

Benzyloxycarbonylleucyl-D-leucylvalyl-β-*tert*-butylaspartyl-D-leucylleucine *tert*-Butyl Ester (32). Benzyloxycarbonylleucine (0.74 g, 2.79 mmol), D-leucylvalyl-β-*tert*-butylaspartyl-D-leucylleucine *tert*-butyl ester (1.91 g, 2.79 mmol), and 1-hydroxybenzotriazole monohydrate (0.85 g, 5.58 mmol) were dissolved in tetrahydrofuran (15 ml) and the resulting solution was cooled to 0°. Dicyclohexylcarbodiimide (0.63 g, 3.07 mmol) was added and the mixture was stirred overnight while warming to room temperature. The mixture was treated with hot ethyl acetate (200 ml) and filtered. The filtrate was concentrated and a chloroform solution (200 ml) of the solid residue was washed successively with

saturated sodium bicarbonate (30 ml), 2N citric acid (30 ml), water (30 ml), dried ($MgSO_4$), and concentrated. The solid residue was dissolved in chloroform (150 ml) and again successively washed with saturated sodium bicarbonate (50 ml), 2N citric acid (50 ml), water (50 ml), dried ($MgSO_4$), decolorized twice (norit), and concentrated to a pale yellow solid (2.36 g, 91%). The product was filtered through a 3.5 x 10 cm column of basic alumina (Brockmann Activity I) using chloroform as eluent. Concentration of 400 ml of eluate gave a white solid (2.09 g, 81%). Recrystallization from ethyl acetate gave white plates (1.32 g), mp 207-210°. A second crop (0.57 g), mp 200-206°, was obtained on concentration of the mother liquor: tlc on adsorbent A in solvent A, R_f 0.68; on adsorbent A in solvent D, R_f 0.84; and on adsorbent A in solvent C, R_f 0.95; $[\alpha]_D^{21}$ 35.0° (c 2.04, $CHCl_3$).

Anal. Calcd for $C_{49}H_{82}N_6O_{11}$: C, 63.20; H, 8.88; N, 9.02. Found: C, 63.36; H, 8.44; N, 9.52.

Valine tert-Butyl Ester Hydrochloride (36).[72] Valine (29.3 g, 0.25 mmol) was added to a solution of sulfuric acid (48 ml) in dioxane (480 ml) and the resulting solution was cooled to -78°. Condensed isobutylene (480 ml) was added, the vessel was sealed and the solution was stirred at room temperature for 24 hr. The reaction vessel was vented and the solution was poured into 0.25N sodium hydroxide (2 liters) and diethyl ether (300 ml). The organic phase was separated and the aqueous phase was

washed with diethyl ether (2 x 100 ml). The organic phases were combined, dried ($MgSO_4$), and concentrated to a volume of about 80 ml. Diethyl ether (200 ml) was added and a stream of dry hydrogen chloride was bubbled through the solution. The hydrochloride was collected by filtration (21.8 g). The mother liquor was concentrated, the residue was dissolved in pentane (100 ml) and treated with dry hydrogen chloride gas. A second crop of hydrochloride (7.47 g) was collected. By repeating this process, two additional crops of 4.41 g and 1.44 g were obtained. The solids were combined and recrystallized from ethyl acetate to give white needles (33.2 g, 63%): mp 146.5-147°; $[\alpha]_D^{24}$ 21.6° (c 2.12, EtOH); lit.[72] mp 147-149°, $[\alpha]_D^{25}$ 20.5° (c EtOH).

Benzyloxycarbonyl-D-leucylvaline tert-Butyl Ester (37). N-Methylmorpholine (5.06 g, 0.05 mol) was dissolved in tetrahydrofuran (150 ml) and valine tert-butyl ester hydrochloride (10.5 g, 0.05 mol) was added. The resulting mixture was stirred for 20 min. Benzyloxycarbonyl-D-leucine (13.3 g, 0.05 mol) and 1-hydroxybenzotriazole monohydrate (15.3 g, 0.10 mol) were added and the mixture was cooled to 0°. A solution of dicyclohexylcarbodiimide (11.35 g, 0.055 mol) in tetrahydrofuran (10 ml) was added and the mixture was stirred overnight while warming to room temperature. The mixture was filtered and concentrated. The residue was dissolved in ethyl acetate (50 ml) and refrigerated overnight. The mixture was filtered, washed successively with saturated sodium bicarbonate (30 ml), 2N citric acid (30 ml),

water (30 ml), saturated sodium chloride (30 ml), dried ($MgSO_4$), and concentrated to a viscous, yellow oil (22.4 g). The crude product was filtered through a 3.8 x 25 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. A total of 400 ml of eluate was collected. A small amount of N,N'-dicyclohexylurea separated upon refrigeration. The mixture was filtered and the filtrate was concentrated to 100 ml. Refrigeration again afforded a small amount of N,N'-dicyclohexylurea which was filtered off. The filtrate was concentrated to a viscous, yellow oil (17.9 g, 85%) which solidified to a hard wax after standing 2 days: tlc on adsorbent A in solvent A, R_f 0.80; on adsorbent A in solvent C, R_f 0.90; on adsorbent A in solvent D, R_f 0.82. The spots were ninhydrin negative until treatment with 1:1 hydrobromic acid-acetic acid. $[\alpha]_D^{20}$ 12.6° (c 5.36, EtOH); nmr ($CDCl_3$) δ 0.89 (m, 12, $(C\underline{H}_3)_2CH$), 1.42 (s, 9, $(C\underline{H}_3)_3C$), 1.42-2.24 (m, 4, $C\underline{H}_2C\underline{H}(CH_3)_2$ and CH $C\underline{H}$ $(CH_3)_2$), 4.20 (m, 2, $C\underline{H}CO$), 5.00 (s, 2, $C\underline{H}_2Ph$), 5.18 (d, 1, J=9 Hz, $CHCON\underline{H}$), 6.42 (d, 1, J=9 Hz, $Ph\text{-}CH_2OCON\underline{H}$), and 7.16 ppm (s, 5, Ph).

Anal. Calcd for $C_{23}H_{36}N_2O_5$: C, 65.69; H, 8.63; N, 6.66. Found: C, 65.59; H, 8.62; N, 6.81.

D-Leucylvaline tert-Butyl Ester (38). Benzyloxycarbonyl-D-leucylvaline tert-butyl ester (17.5 g, 0.42 mol) was dissolved in methanol (100 ml) containing four drops of acetic acid. The compound was hydrogenated at 1 atm over 5% palladium on carbon (2.0 g). The reaction was monitored

by tlc on adsorbent A in solvent D. Starting material at R_f 0.70, ninhydrin negative, was completely absent after 12 hr. A new ninhydrin positive spot appeared at R_f 0.63 along with a trace of ninhydrin positive material at the origin. The mixture was filtered and concentrated to a viscous oil (12.7 g). An ethyl acetate solution of the crude product was stirred with saturated sodium bicarbonate (25 ml) for 12 hr. The organic phase was separated, washed with water (2 x 25 ml), dried ($MgSO_4$), and concentrated *in vacuo* to a pale yellow gel (11.0 g, 92%): tlc on adsorbent A in solvent A, R_f 0.32; on adsorbent A in solvent C, R_f 0.89; on adsorbent A in solvent D, R_f 0.63. In each case, the spot was ninhydrin positive. $[\alpha]_D^{27}$ 31.9° (*c* 3.13, $CHCl_3$).

Anal. Calcd for $C_{15}H_{30}N_2O_3$: C, 62.90; H, 10.56; N, 9.78. Found: C, 62.77; H, 10.51; N, 9.72.

D-Leucylvaline *tert*-Butyl Ester Hydrogen Oxalate Hemihydrate. Anhydrous oxalic acid (30 mg, 0.35 mmol) in diethyl ether (2 ml) was added to a solution of D-leucylvaline *tert*-butyl ester (100 mg, 0.35 mmol) in diethyl ether (2 ml). The resulting mixture was refrigerated 2 hr and then filtered. A white solid was obtained (0.12 g, 92%), mp 133-134°. An analytical sample, mp 133-134° was obtained by recrystallization from ethanol-diethyl ether, $[\alpha]_D^{25}$ -33.7° (*c* 1.56, MeOH).

Anal. Calcd for $C_{17}H_{32}N_2O_7 \cdot 0.5H_2O$: C, 52.96; H, 8.63; N, 7.27. Found: C, 52.78; H, 8.27; N, 7.30.

Benzyloxycarbonylleucyl-D-leucylvaline-tert-butyl Ester (39). Method A. Benzyloxycarbonylleucine (1.33 g, 5.0 mmol), D-leucylvaline tert-butyl ester (1.43 g, 5.0 mmol), and 1-hydroxybenzotriazole monohydrate (1.53 g, 10.0 mmol) were dissolved in tetrahydrofuran (15 ml) and the resulting solution was cooled at 0°. A solution of dicyclohexylcarbodiimide (1.03 g, 5.5 mmol) in tetrahydrofuran (2 ml) was added and the resulting mixture was stirred 1 hr at 0° and 4 hr at room temperature. The mixture was filtered and the filtrate was concentrated. An ethyl acetate solution of the crude product was washed successively with saturated sodium bicarbonate (2 x 25 ml), 2N citric acid (2 x 25 ml), water (25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated to a viscous oil. The crude product was filtered through a 2.8 x 15 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. The product was concentrated in vacuo to a white foam (2.26 g). The foam was triturated under pentane (10 ml) and refrigerated two days. A white solid (1.52 g, 53%) was collected by filtration, mp 86-90°. An analytical sample was prepared by chromatography on silica gel (150-230 mesh) using benzene ethyl acetate (4:1 v/v) as eluent: mp 97-98°; nmr ($CDCl_3$) δ 0.94 (m, 18, $(C\underline{H}_3)_2CH$), 1.44 (s, 9, $C(C\underline{H}_3)_3$), 1.4-2.3 (m, 7, $(CH_3)_2C\underline{H}CH$, $(CH_3)_2C\underline{H}C\underline{H}_2$), 4.44 (m, 3, $C\underline{H}C{=}O$), 5.16 (s, 2, $C\underline{H}_2Ph$), 5.49 (d, 1, J=7 Hz, N$\underline{H}$), 6.83 (m, 2, N$\underline{H}$), and 7.43 ppm (s, 5, Ph-); $[\alpha]_D^{25}$ 21.5° (c 2.00, $CHCl_3$).

Anal. Calcd for $C_{29}H_{47}N_3O_6$: C, 65.26; H, 8.88; N, 7.87. Found: C, 65.31; H, 9.14; N, 7.87.

Method B. Benzyloxycarbonylleucine (2.00 g, 7.54 mmol), D-leucylvaline tert-butyl ester (1.08 g, 3.77 mmol), and 1-hydroxybenzotriazole monohydrate (2.31 g, 15.5 mmol) were dissolved in tetrahydrofuran (20 ml) and the solution was cooled to 0°. Dicyclohexylcarbodiimide (1.71 g, 8.30 mmol) in tetrahydrofuran (2 ml) was added and the resulting mixture was stirred 1 hr at 0° and 3 hr at room temperature. The mixture was filtered and the filtrate was concentrated. An ethyl acetate solution of the crude product was washed successively with saturated sodium bicarbonate (2 x 20 ml), 2N citric acid (20 ml), water (20 ml), saturated sodium chloride (20 ml), dried ($MgSO_4$), and concentrated to a viscous, yellow oil (3.25 g). The oil was filtered through a 2.8 x 20 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. Concentration of eluate (150 ml) gave a clear, viscous oil (2.39 g). The crude product was purified by chromatography on a 2 x 30 cm column of silica gel (150-230 mesh) using benzene-ethyl acetate (4:1) as eluent. The separation was monitored by tlc on adsorbent A in solvent E. The early fractions contained an unknown impurity with R_f 0.55. Fractions containing a component with R_f 0.21 were combined and concentrated in vacuo to a white foam (1.26 g, 63%). The product was triturated under pentane and filtered to give a white solid (1.04 g, 52%):

mp 104-107°; $[\alpha]_D^{24}$ 20.2 (c 2.02, $CHCl_3$); amino acid analysis: Val, 1.00; Leu, 1.99.

Benzyloxycarbonylleucyl-D-leucylvaline (40). Benzyloxycarbonylleucyl-D-leucylvaline tert-butyl ester (0.53 g, 1.0 mmol) was treated with anhydrous trifluoroacetic acid (2 ml) and the resulting solution was stirred 1 hr at room temperature and then concentrated in vacuo. The product was dissolved in ethyl acetate and concentrated in vacuo twice. Yield: 0.49 g (100%) of a white foam; tlc on adsorbent A in solvent A, R_f 0.51; on adsorbent A in solvent D, R_f 0.66; 60 mHz nmr ($CDCl_3$) δ 0.89 (m, 18, $(CH_3)_2CH$), 1.58 (m, 7, $CHCH(CH_3)_2$, $CH_2CH(CH_3)_2$), 4.00-4.95 ($CHC{=}O$), 5.09 (s, 2, CH_2Ph), and 7.30 ppm (s, 5, Ph-). The product was converted to the dicyclohexylamine salt without further purification.

Benzyloxycarbonylleucyl-D-leucylvaline Dicyclohexylamine Salt. Benzyloxycarbonylleucyl-D-leucylvaline (0.47 g, 0.98 mmol) was dissolved in diethyl ether (2 ml) and treated with dicyclohexylamine (0.18 g, 0.98 mmol) in diethyl ether (1 ml). The mixture was cooled at -20° overnight. The product was collected by filtration and recrystallized from ethanol-diethyl ether to give white crystals (0.39 g), mp 201-202°. A second crop (0.12 g), mp 186-190°, was obtained by concentration of the mother liquor. Yield: 0.51 g (78%): $[\alpha]_D^{22}$ 13.5 (c 1.55, MeOH).

Anal. Calcd for $C_{37}H_{62}N_4O_6$: C, 67.44; H, 9.48; N, 8.50. Found: C, 67.44; H, 9.21; N, 8.68.

Benzyloxycarbonylleucyl-D-leucylvalyl-β-tert-butyl-aspartyl-D-leucylleucine tert-Butyl Ester (32). A solution of benzyloxycarbonylleucyl-D-leucylvaline (1.87 g, 3.92 mmol), β-tert-butylaspartyl-D-leucylleucine-tert-butyl ester (1.85 g, 3.92 mmol) and 1-hydroxybenzotriazole monohydrate (1.20 g, 7.83 mmol) in tetrahydrofuran (15 ml) was cooled to 0° and treated with a solution of dicyclohexylcarbodiimide (0.89 g, 4.31 mmol) in tetrahydrofuran (2 ml). The resulting mixture was stirred 1 hr at 0° and overnight at room temperature. The mixture was concentrated. The solid residue was treated with hot acetonitrile (50 ml) and filtered. The filtrate was concentrated and the solid residue was filtered through a 3.0 x 30 cm column of basic alumina (Brockmann Activity I) using 1:1 methanol-ethyl acetate as eluent. Concentration of 200 ml of eluate gave a white solid. The crude product was treated with hot acetonitrile (75 ml) and filtered. The filtrate was refrigerated overnight to give a white solid (1.62 g, 44%): mp 215-217°; tlc on adsorbent A in solvent A, R_f 0.71; on adsorbent A in solvent G, R_f 0.88 (streak); on adsorbent A in solvent H, R_f 0.88; on adsorbent A in solvent I, two spots R_f 0.93 (ninhydrin positive after treatment with 1:1 hydrobromic acid-acetic acid), and R_f 0.78 (N,N'-dicyclohexylurea): $[\alpha]_D^{25}$ +41.2° (c 2.13, $CHCl_3$); amino acid analysis: Leu 4.02; Val 0.98; Asp 1.00. Recrystallization of the sample from acetonitrile or methanol did not result in the separation of the product from dicyclohexylurea. The

sample was hydrogenolyzed without further purification.

Leucyl-D-leucylvalyl-β-tert-butylaspartyl-D-leucyl-leucine tert-butyl Ester (33). Benzyloxycarbonylleucyl-D-leucylvalyl-β-tert-butylaspartyl-D-leucylleucine tert-butyl ester (1.11 g, 1.2 mmol) was dissolved in a solution of methanol (40 ml), acetic acid (10 drops), and water (1 ml). The hexapeptide was hydrogenated at 1 atmosphere over 5% palladium on carbon (0.25 g) for 2 hr. The mixture was filtered through celite and the celite was washed with hot methanol (25 ml). The filtrate and washing were combined and concentrated. The solid residue was dissolved in methanol (30 ml) and cooled at -5° for 2 days. Crystals of N,N'-dicyclohexylurea (0.11 g, mp 230-231°) were filtered off and the filtrate was concentrated to a white solid (0.92 g). The crude product was recrystallized from methanol-diethyl ether to give a white solid (0.89 g, 98% based on 1.00 g of starting material): mp 157-162°; $[\alpha]_D^{24}$ -20.3° (c 1.11, MeOH).

Anal. Calcd for $C_{41}H_{76}N_6O_9 \cdot 3H_2O$: C, 57.86; H, 9.71; N, 9.88. Found: C, 57.50; H, 9.04; N, 9.76.

Benzyloxycarbonyl-γ-tert-butylglutamylleucyl-D-leucyl-valyl-β-tert-butylaspartyl-D-leucylleucine tert-Butyl Ester (35). Benzyloxycarbonyl-γ-tert-butylglutamate dicyclohexylamine salt (1.50 g, 2.90 mmol) was suspended in 50% aqueous ethanol and treated with Dowex 50W-X8 ion exchange resin (H+ form). The mixture was stirred for 30 min and filtered. The

filtrate was concentrated, the residue was extracted with ethyl acetate (50 ml), washed with water (3 x 25 ml), dried ($MgSO_4$), and concentrated *in vacuo* to give *34* as a colorless oil.

A portion of the above oil (0.55 g, 1.63 mmol) was dissolved in N,N-dimethylformamide (5 ml) and the solution was cooled to 0°. Dicyclohexylcarbodiimide (0.67 g, 3.26 mmol) was added, the resulting mixture was stirred 3 min and a solution of free amino hexapeptide *33* (1.30 g, 1.63 mmol) in N,N-dimethylformamide (10 ml) was added in several portions over 2 min. The mixture was stirred 6 hr at 0° and overnight at room temperature. Acetic acid (0.25 ml) was added, the mixture was stirred for 30 min and then poured into water (150 ml). The precipitate was collected by filtration and dried *in vacuo* over P_2O_5 to give a white solid (1.61 g, 88%): mp 170-175°; tlc on adsorbent A in solvent A, R_f 0.60; on adsorbent A in solvent D, R_f 0.73, with a second very weak spot at R_f 0.65 which developed only after treatment with sulfuric acid followed by heating at 100°; on adsorbent A in solvent E, R_f 0.16; on adsorbent A in solvent G, R_f 0.99; spots were developed by treatment with 1:1 hydrobromic acid-acetic acid followed by ninhydrin; $[\alpha]_D^{25}$ -3.0° (*c* 1.90, MeOH).

tert-Butyloxycarbonyl-D-leucine Monohydrate (44). D-Leucine (13.1 g, 0.10 mol) and triethylamine (42 ml) were dissolved in water (150 ml) and a solution of tert-butyloxycarbonyl azide in dioxane (150 ml) was added over 10 min. The resulting mixture was stirred 2 hr during which time solution occurred. The solution was concentrated and the residue was dissolved in water (100 ml). The solution was washed with diethyl ether (50 ml) and acidified (litmus) with 10% hydrochloric acid. The product was extracted into ethyl acetate (100 ml), washed with water (25 ml), dried ($MgSO_4$), and concentrated to a viscous, yellow oil (18.1 g). The addition of a few ml of water caused the oil to solidify. The crude product was recrystallized from methylene chloride-pentane to give white plates (14.1 g, 57%): mp 86-88°; $[\alpha]_D^{21}$ 24.4° (c 2.00, HOAc); lit.[117] mp 84°, $[\alpha]_D$ 25.2° (c 2, AcOH).

tert-Butyloxycarbonyl-D-leucylleucine Benzyl Ester (46). tert-Butyloxycarbonyl-D-leucine monohydrate (2.49 g, 10 mmol) was dissolved in diethyl ether (50 ml) and dried over magnesium sulfate (1 hr) then over calcium sulfate (1 hr). The mixture was filtered and the filtrate was concentrated to a clear oil. The oil was dried in vacuo over phosphorous pentoxide overnight. The tert-butyloxycarbonyl-D-leucine was dissolved in tetrahydrofuran (30 ml) and leucine benzyl ester p-toluenesulphonate (3.94 g, 10 mmol) and N-methylmorpholine (1.01 g, 10 mmol) were added. The

mixture was stirred 15 min and then 1-hydroxybenzotriazole monohydrate (3.06 g, 20 mmol) was added. The resulting solution was cooled to 0° and then dicyclohexylcarbodiimide (2.27 g, 11 mmol) in tetrahydrofuran (5 ml) was added. The reaction mixture was stirred 1 hr at 0° and 1 hr at room temperature. The mixture was filtered and the filtrate was cooled to 0° overnight. Additional white solid was filtered off. The filtrate was concentrated and an ethyl acetate solution of the crude product was washed successively with saturated sodium bicarbonate (25 ml), 2N citric acid (25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$) and concentrated to a semi-solid. The crude product was filtered through a 2.8 x 15 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. Concentration of a 250 ml fraction and recrystallization of the residue from ethyl acetate-pentane gave white crystals (2.75 g, 63%), mp 95-96.0°. A second crop (0.05 g, 2%), mp 93-94.5°, was obtained by concentration of the mother liquor. Analysis by tlc on adsorbent A in solvent A, R_f 0.76; on adsorbent A in solvent D, R_f 0.88; $[\alpha]_D^{25}$ 20.5° (c 2.04, $CHCl_3$).

Anal. Calcd for $C_{24}H_{38}N_2O_5$: C, 66.33; H, 8.81; N, 6.45. Found: C, 66.23; H, 8.89; N, 6.46.

D-Leucylleucine Benzyl Ester Trifluoroacetate (48). tert-Butyloxycarbonyl-D-leucylleucine benzyl ester (0.43 g, 1.0 mmol) was treated with anhydrous trifluoroacetic acid (8.5 ml). The resulting solution was stirred 1 hr at room

temperature and was then concentrated. The product was dissolved in diethyl ether (5 ml) and treated with pentane (5 ml). The product crystallized on refrigeration as fine white crystals (0.30 g, 80%): mp 125-127°; $[\alpha]_D^{25}$ -34.1° (c 2.08, EtOH); tlc on adsorbent A in solvent A, R_f 0.27; on adsorbent A in solvent D, R_f 0.78.

Anal. Calcd for $C_{21}H_{31}F_3N_2O_5$: C, 56.23; H, 6.97; N, 6.25. Found: C, 56.31; H, 7.00; N, 6.39.

o-Nitrophenylsulfenyl—β-benzylaspartyl-D-leucylleucine Benzyl Ester (49). N-(o-Nitrophenylsulfenyl)-β-benzylaspartate (0.20 g, 0.53 mmol) was dissolved in tetrahydrofuran (4 ml) and the solution was cooled to 0°. D-Leucylleucine benzyl ester trifluoroacetate (0.24 g, 0.53 mmol), 1-hydroxybenzotriazole monohydrate (0.16 g, 1.0 mmol), and dicyclohexylcarbodiimide (0.12 g, 0.58 mmol) were added to the solution. The mixture was stirred 1 min and was then treated with a solution of N-methylmorpholine (0.05 g, 0.53 mmol) in tetrahydrofuran (1 ml). The mixture was stirred 1 hr at 0° and then overnight at room temperature. The mixture was filtered and concentrated. The crude product was chromatographed on a 2.8 x 10 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. Concentration of the eluate gave a yellow solid (0.29 g, 78%). Recrystallization from ethyl acetate-pentane afforded an analytical sample: mp 124-127°; $[\alpha]_D^{25}$ -0.4° (c 2.35, DMF).

Anal. Calcd for $C_{26}H_{44}N_4O_8S$: C, 62.41; H, 6.40; N, 8.09. Found: C, 62.46; H, 6.38; N, 8.07.

o-Nitrophenylsulfenyl-β-benzylaspartyl-D-leucyl-leucine tert-Butyl Ester (50). o-Nitrophenylsulfenyl-β-benzylaspartic acid dicyclohexylamine salt (1.00 g, 1.79 mmol) was treated with 2N citric acid (30 ml). The free o-nitrophenylsulfenyl-β-benzylaspartic acid was extracted into ethyl acetate. The organic phase was dried ($MgSO_4$) and concentrated. The oil was dissolved in tetrahydrofuran (9 ml) and D-leucylleucine tert-butyl ester (0.54 g, 1.79 mmol) and 1-hydroxybenzotriazole monohydrate (0.55 g, 3.58 mmol) were added. The solution was cooled to 0° and a solution of dicyclohexylcarbodiimide (0.41 g, 1.97 mmol) in tetrahydrofuran (2 ml) was added. The mixture was stirred 1 hr at 0° and then 1 hr at room temperature. The mixture was filtered and the filtrate was concentrated to a yellow foam (1.05 g). An ethyl acetate solution of the product was washed with saturated sodium bicarbonate (20 ml), 2N citric acid (20 ml), dried ($MgSO_4$) and concentrated. The crude product was filtered through a 3.5 x 12 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. A total of 300 ml eluate was collected which on concentration gave a yellow foam (0.79 g, 67%): tlc on adsorbent A in solvent B, R_f 0.77; on adsorbent A in solvent D, R_f 0.83; on adsorbent A in solvent E, R_f 0.36. An analytical sample was obtained by crystallization from ethyl acetate-pentane: mp 124-127°; $[\alpha]_D^{25}$ -1.4° (c 1.17, DMF).

Anal. Calcd for $C_{33}H_{46}N_4O_8S$: C, 60.16; H, 7.04; N, 8.51. Found: C, 60.20; H, 7.05; N, 8.27.

β-Benzylaspartyl-D-leucylleucine tert-Butyl Ester Hydrochloride (51). o-Nitrophenylsulfenyl-β-benzylaspartyl-D-leucylleucine tert-butyl ester (0.50 g, 0.76 mmol) was dissolved in chloroform (3 ml) at 0°. A solution of hydrogen chloride in chloroform (5.0 ml of a 0.303N solution, 1.52 mmol) was added and the mixture was stirred 15 min at 0°. The mixture was concentrated, the residue was triturated under pentane and filtered to give a fine white solid (0.40 g, 97%): tlc on adsorbent A in solvent A, R_f 0.40; on adsorbent A in solvent B, R_f 0.69; and on adsorbent A in solvent D, R_f 0.72. All spots were ninhydrin positive. Recrystallization from ethyl acetate gave fine white crystals (0.28 g): mp 175-176°; $[\alpha]_D^{21}$ 1.9° (c 2.45, EtOH).

Anal. Calcd for $C_{27}H_{44}ClN_3O_6$: C, 59.82; H, 8.18; N, 7.75. Found: C, 59.81; H, 7.95; N, 7.69.

β-tert-Butylaspartate Acetate. Benzyloxycarbonyl-β-tert-butylaspartate (3.23 g, 0.01 mol) was dissolved in a solution of methanol (100 ml) and acetic acid (1 ml). The solution was hydrogenated at 20 psi over 5% palladium on carbon (0.8 g) overnight. The mixture was filtered and the filtrate was concentrated to a white solid. The product was triturated under diethyl ether and filtered to give a white solid (1.52 g, 61%): mp 208-209°; tlc on adsorbent A in solvents A and D showed only one spot, remaining at the origin and ninhydrin positive; $[\alpha]_D^{20}$ 8.0° (c 2.37 for Asp(OBu^t); lit.[118] for Asp(OBu^t), mp 189-190°,

$[\alpha]_D^{23}$ 8.5 ± 1° (c 1.02, 90% HOAc).

o-Nitrophenylsulfenyl-β-tert-butylaspartate Dicyclohexylamine Salt (55·DCHA). β-tert-Butylaspartate acetate (3.74 g, 0.015 mol) was dissolved in dioxane (38 ml) containing 2N sodium hydroxide (15 ml). o-Nitrophenylsulfenyl chloride (3.13 g, 0.016 mol) was added in ten equal portions over 15 min while 2N sodium hydroxide (9 ml) was added dropwise over the same period. The pH was maintained at 8-10 by controlling the rate of addition of base. The resulting mixture was stirred 10 min and then treated with water (150 ml). The mixture was filtered and the solid was washed with water. The filtrate and washings were combined and acidified to pH 5 with 0.5N sulfuric acid. The product was extracted with diethyl ether (2 x 75 ml). The organic phases were combined, washed with water (75 ml), dried ($MgSO_4$) and concentrated. The product was dissolved in diethyl ether (25 ml) and treated with dicyclohexylamine (3 ml) in diethyl ether (25 ml). The product separated as yellow crystals (2.34 g, mp 160-161°) after standing four days at -15°. Concentration of the mother liquor and the addition of diethyl ether gave 3.48 g of impure solid, mp 145-149°. Recrystallization from methanol-diethyl ether gave yellow crystals (1.43 g, mp 155-157°). Yield: 3.77 g (48%); $[\alpha]_D$ -62.7° (c 2.00, $CHCl_3$); tlc on adsorbent A in solvent A, R_f 0.60 and on adsorbent A in solvent D, R_f 0.65; lit.[119] mp 160-162°; $[\alpha]_D^{20}$ -76.5° (c 0.5, DMF).

o-Nitrophenylsulfenyl-β-tert-butylaspartyl-D-leucyl-leucine Benzyl Ester (56). o-Nitrophenylsulfenyl-β-tert-butylaspartate (1.24 g, 3.6 mmol) and D-leucylleucine benzyl ester trifluoroacetate (1.62 g, 3.6 mmol) were dissolved in tetrahydrofuran (15 ml) and the solution was cooled to -10°. 1-Hydroxybenzotriazole monohydrate (1.11 g, 7.2 mmol) was added followed by dicyclohexylcarbodiimide (0.82 g, 40 mmol). The resulting mixture was stirred 10 min at -10° and then N-methylmorpholine (0.37 g, 3.6 mmol) in tetrahydrofuran (2 ml) was added. The mixture was stirred 1 hr at -10° and then at room temperature overnight. The mixture was filtered and the filtrate was concentrated in vacuo. The oily residue was chromatographed on a 2.8 x 20 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. Concentration of eluate (75 ml) gave a viscous oil which was crystallized from ethyl acetate-pentane to give a bright yellow rosettes (0.60 g, 25%), mp 153-155°. An additional 0.10 g (4%) of product was obtained by chromatography of the concentrated mother liquor on a 2.0 x 15 cm column of silica gel (150-230 mesh) using benzene-ethyl acetate (4:1, v/v) as eluent. Analysis by nmr ($CDCl_3$) δ 0.97 (m, 12, $(CH_3)_2CH$), 1.51 (s, 9, $C(CH_3)_3$), 1.58 (m, 6, $CH_2CH(CH_3)_2$), 2.91 (m, 2, $CH_2C{=}O$), 5.24 (s, 2, CH_2Ph), 7.47 (s, 5, Ph-), and 7.1-8.6 ppm (m, 4, o-O_2NPh-S-); $[\alpha]_D^{25}$ -1.6° (c 2.01, EtOH).

Attempted Preparation of β-tert-Butylaspartyl-D-leucylleucine Benzyl Ester Hydrochloride (57). o-Nitrophenylsulfenyl-β-tert-butyl aspartyl-D-leucylleucine benzyl ester (0.50 g, 0.76 mmol) was dissolved in chloroform (3 ml) and the solution was cooled to 0°. A solution of anhydrous hydrogen chloride in chloroform (4.75 ml of a 0.320N solution, 1.52 mmol) was added and the resulting solution was stirred 15 min at 0°. The solution was concentrated and the oily product was triturated under pentane. The pale yellow solid was collected by filtration (0.44 g). The product was dissolved in ethyl acetate and precipitated as a gel by the addition of diethyl ether. The gel was collected by filtration and dried in vacuo over phosphorous pentoxide to give a pale yellow solid (0.20 g); tlc on adsorbent A in solvents D, E, and F showed the product was a mixture of at least two ninhydrin positive components.

Valine p-Nitrobenzyl Ester p-Toluene Sulfonate (58). Valine (23.4 g, 0.20 mol) was suspended in chloroform (600 ml) and p-nitrobenzyl alcohol (146 g, 0.956 mol) and p-toluene sulfonic acid monohydrate (114 g, 0.60 mol) were added. The mixture was refluxed for 5.5 hr. Solvent was returned to the pot after filtration through a bed of anhydrous calcium sulfate by means of a Soxhlet apparatus. The reaction solution was concentrated and the residue was treated with diethyl ether (500 ml). The mixture was filtered and the brown solid was recrystallized from iso-

propanol to give light brown needles (57.3 g, 67%): mp 211-212°; $[\alpha]_D^{25}$ 14.7° (c 1.00, pyridine); tlc on adsorbent A in solvent D, R_f 0.69, ninhydrin positive.

tert-Butyloxycarbonyl-D-leucylvaline p-Nitrobenzyl Ester (59). tert-Butyloxycarbonyl-D-leucine monohydrate (3.42 g, 13.7 mmol) and 1-hydroxybenzotriazole monohydrate (4.20 g, 27.4 mmol) were dissolved in tetrahydrofuran (30 ml). Valine p-nitrobenzyl ester p-toluene sulfonate (5.82 g, 13.7 mmol) was added and the resulting mixture was cooled to 0°. N-Methylmorpholine (1.39 g, 13.7 mmol) was added followed by a solution of dicyclohexylcarbodiimide (3.11 g, 15.1 mmol) in tetrahydrofuran (3 ml). The mixture was stirred for 2 hr at 0° and then overnight at room temperature. The mixture was filtered and the filtrate was concentrated. The product was filtered through a 3.0 x 17 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. Concentration of 125 ml of eluate gave a pale yellow solid (6.33 g). The product was recrystallized from ethyl acetate-petroleum ether (bp 30-60°) to give a tan solid (5.25 g, 82%): mp 111-112°; $[\alpha]_D^{24}$ 20.9° (c 2.19, HOAc); nmr ($CDCl_3$) δ 0.91 (m, 12, $(CH_3)_2CH$), 1.42 (s, 9, $(CH_3)_3C$), 1.51 (m, 3, $(CH_3)_2CHCH_2$), 2.11 (m, 1, $(CH_3)_2CHCH$), 4.05 (m, 1, NHCH), 4.47 (NHCH), 4.75 (d, 1, J=8 Hz, NHCH), 5.14 (s, 2, $CH_2Ph\text{-}NO_2$), 6.60 (d, 1, J=9 Hz, NHCH), and 7.35 and 8.35 (ABq, 4, J=8 Hz, $PhNO_2$).

Anal. Calcd for $C_{23}H_{35}N_3O_7$: C, 59.34; H, 7.58; N, 9.03. Found: C, 59.49; H, 7.55; N, 9.08.

D-Leucylvaline p-Nitrobenzyl Ester Hydrobromide (61). tert-Butyloxycarbonyl-D-leucylvaline p-nitrobenzyl ester (5.10 g, 11 mmol) was treated with saturated hydrogen bromide in acetic acid (11 ml). The resulting solution was stirred for 15 min at room temperature and concentrated in vacuo. The orange foam was dissolved in ethyl acetate and concentrated in vacuo. This process was repeated twice to give an orange foam (4.89 g, 100%). The product was extremely hygroscopic and was converted to tripeptide 62 without further purification.

tert-Butyloxycarbonylleucine Monohydrate (60).[103] A suspension of leucine (11.59 g, 0.088 mol) and tetramethylguanidine (20.4 g, 0.178 mol) in dimethylformamide (190 ml) was cooled to 0° and tert-butyloxycarbonylazide (19.2 g, 0.134 mol) was added dropwise over a period of 20 min. The resulting solution was stirred 1 hr at 0° and five days at room temperature. The solution was concentrated and the crude product was stirred with 75 ml of 2N citric acid and 150 ml of ethyl acetate. The organic phase was separated and the aqueous phase was washed with ethyl acetate (2 x 30 ml). The ethyl acetate phases were combined, washed with water (3 x 35 ml), saturated sodium chloride (35 ml), dried ($MgSO_4$), and concentrated. The resulting oil solidified with the addition of a small amount of water. The solid was recrystallized from pentane-methylene chloride to give large white plates (15.7 g), mp 86-88°. A second crop

(5.8 g), mp 86-88°, was obtained by concentration of the mother liquor. Yield: 21.5 g (97%); $[\alpha]_D^{21}$ -25.3° (c 2.00, HOAc); lit.[120] mp 67-72°, $[\alpha]_D$ -24° (c 2, HOAc).

tert-Butyloxycarbonylleucyl-D-leucylvaline p-Nitrobenzyl Ester (62). tert-Butyloxycarbonylleucine monohydrate (2.73 g, 11 mmol), D-leucylvaline p-nitrobenzyl ester hydrobromide (4.89 g, 11 mmol), and 1-hydroxybenzotriazole monohydrate (3.36 g), were dissolved in tetrahydrofuran (30 ml). The solution was cooled to 0° and dicyclohexylcarbodiimide (2.49 g, 12 mmol) was added. A solution of N-methylmorpholine (1.11 g, 11 mmol) in tetrahydrofuran was added dropwise over a period of 8 min. The mixture was stirred 2 hr at 0° and then overnight at room temperature. The mixture was filtered and the filtrate was concentrated. The solid residue was treated with hot ethyl acetate (50 ml) and filtered. The filtrate was washed successively with saturated sodium bicarbonate (2 x 25 ml), 2N citric acid (2 x 25 ml), water (25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated. The product was filtered through a 2.8 x 15 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluant. Concentration of 125 ml of eluate gave a solid residue. The product was recrystallized from ethyl acetate-petroleum ether (bp 30-60°) to give white crystals (4.87 g, 77%): mp 158-159°; $[\alpha]_D^{25}$ 1.8° (c 2.11, HOAc); tlc on adsorbent A in solvent A, R_f 0.73; on adsorbent A in solvent D, R_f 0.86.

Anal. Calcd for $C_{29}H_{46}N_4O_8$: C, 60.19; H, 8.01; N, 9.68. Found: C, 60.15; H, 8.02; N, 9.60.

Leucyl-D-leucylvaline p-Nitrobenzyl Ester Trifluoroacetate (64). tert-Butyloxycarbonylleucyl-D-leucylvaline p-nitrobenzyl ester (4.82 g, 8.3 mmol) was treated with anhydrous trifluoroacetic acid (25 ml). The resulting solution was stirred 1 hr at room temperature and then concentrated in vacuo to a white foam (4.94 g, 100%): $[\alpha]_D^{25}$ 8.0° (c 2.00, MeOH).

Anal. Calcd for $C_{26}H_{39}F_3N_4O_8$: C, 52.69; H, 6.63; N, 9.46. Found: C, 52.56; H, 6.49; N, 9.52.

Benzyloxycarbonyl-α-Benzylglutamate Dicyclohexylamine Salt. This compound was prepared according to the method of Weygand and Hunger.[107] A yield of 16.9 g (30%) of crystalline solid was obtained: mp 166-167°; $[\alpha]_D^{21}$ -12.5° (c 3.05, MeOH); lit.[107] mp 163-164°; $[\alpha]_D$ -13.1° (c 3.22, MeOH).

Benzyloxycarbonyl-α-benzyl-γ-tert-butylglutamate. A suspension of benzyloxycarbonyl-α-benzylglutamate dicyclohexylamine salt (15.2 g, 27.5 mmol) in 50% aqueous ethanol (300 ml) was treated with Dowex 50W-X8 ion exchange resin (100 ml, H+ form). The mixture was stirred for 30 min and filtered. The filtrate was concentrated and the residue was extracted with ethyl acetate (3 x 50 ml). The extracts were combined and washed successively with 2N citric acid (25 ml), water (25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated to a viscous oil.

The above oil was dissolved in methylene chloride (75 ml) containing sulfuric acid (0.3 ml). Condensed isobutylene (50 ml) was added, the reaction vessel was sealed, and the solution was stirred 64 hr at room temperature. The solution was cooled to -78°, treated with saturated sodium bicarbonate (25 ml), and allowed to warm to room temperature as the excess isobutylene boiled off. The solution was concentrated and an ethyl acetate solution of the product was washed successively with saturated sodium bicarbonate (3 x 40 ml) and saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated *in vacuo* to a clear, viscous oil (10.2 g, 90%). The product was converted to γ-*tert*-butylglutamate without further purification.

γ-*tert*-Butylglutamate. Benzyloxycarbonyl-α-benzyl-γ-*tert*-butylglutamate (10.2 g, 23.9 mmol) was dissolved in methanol (30 ml) and hydrogenolyzed over 5% palladium on carbon (0.5 g) at 1 atmosphere for 30 hr. The mixture was filtered through celite and the filtrate was concentrated. The oily residue was triturated under diethyl ether and the resulting solid was collected by filtration (4.52 g, 93%): mp 174-175°; $[\alpha]_D^{25}$ 9.4° (*c* 2.03, H_2O); lit.[98] mp 182°; $[\alpha]_D^{20}$ 9.8° (*c* 2, H_2O).

o-Nitrophenylsulfenyl-γ-*tert*-butylglutamate Dicyclohexylamine Salt (63·DCHA). This compound was prepared according to the method of Zervas and Hamaladis.[98] A yield of 7.17 g (61%) of crystalline solid was obtained: mp 175-

176°; $[\alpha]_D$ 0° (c 4.00, MeOH); lit.[98] mp 179-180°; $[\alpha]_D^{20}$ -25.0 (c 4, MeOH).

o-Nitrophenylsulfenyl-γ-tert-butylglutamylleucyl-D-leucylvaline p-Nitrobenzyl Ester (65). A suspension of o-nitrophenylsulfenyl-γ-tert-butylglutamate dicyclohexylamine salt (1.88 g, 3.5 mmol) in tetrahydrofuran (30 ml) was treated with leucyl-D-leucylvaline p-nitrobenzyl ester trifluoroacetate (2.07 g, 3.5 mmol) and the resulting solution was concentrated. The residue was treated with ethyl acetate (15 ml) and the mixture was filtered. The filtrate was concentrated, dissolved in tetrahydrofuran (30 ml) and the solution was cooled to 0°. The solution was treated with 1-hydroxybenzotriazole monohydrate (1.07 g, 7.0 mmol) followed by dicyclohexylcarbodiimide (0.79 g, 3.9 mmol). The resulting mixture was stirred overnight while gradually warming to room temperature. The mixture was filtered and the filtrate was concentrated. An ethyl acetate solution of the product was washed successively with saturated sodium bicarbonate (3 x 15 ml), 2N citric acid (2 x 15 ml), water (15 ml), saturated sodium chloride (15 ml), dried ($MgSO_4$), and concentrated to a yellow oil. The crude product was chromatographed on a 2.8 x 32 cm column of silica gel (150-230 mesh) using chloroform-ethanol (80:20, v/v) as eluent. The fast-moving yellow band was collected and concentrated to a viscous yellow oil (1.60 g). The oil was rechromatographed on a 2.8 x 30 cm column of silica gel (70-230 mesh) using benzene-ethyl acetate (80:20, v/v) as eluent. The

product was collected as a slow-moving yellow band which was concentrated to a yellow foam. Crystallization of the foam from ethyl acetate-pentane gave a yellow solid (1.23 g, 43%): mp 75-77°; nmr ($CDCl_3$) δ 0.95 (m, 18, $(C\underline{H}_3)_2CH$), 1.43 (s, 9, $C(C\underline{H}_3)_3$), 5.24 (s, 2, $C\underline{H}_2PhNO_2$), 7.0-8.1 (m, 4, $\underline{o}$-NO_2Ph-S), and 7.50 and 8.17 (ABq, 4, $\underline{p}$-NO_2Ph-); tlc on adsorbent A in solvent D, R_f 0.82; on adsorbent A in solvent E, R_f 0.56; $[\alpha]_D$ -12° (c 2.05, MeOH).

Anal. Calcd for $C_{32}H_{56}N_6O_{11}S$: C, 57.33; H, 6.91; N, 10.29. Found: C, 57.13; H, 6.75; N, 9.90.

γ-tert-Butylglutamylleucyl-D-leucylvaline p-Nitrobenzyl Ester Hydrochloride (66). o-Nitrophenylsulfenyl-γ-tert-butylglutamylleucyl-D-leucylvaline p-nitrobenzyl ester (1.00 g, 1.22 mmol) was treated with 0.339N hydrogen chloride in anhydrous chloroform (7.20 ml, 2.44 mmol) at 0°. The resulting solution was stirred at 45 min at 0°, concentrated in vacuo, and the resulting foam was triturated under pentane and filtered. The pale yellow solid (0.90 g) was triturated under pentane for 24 hr and collected by filtration. The solid (0.83 g) was dissolved in methanol and concentrated in vacuo. The residue was triturated under pentane a third time, collected by filtration and dried in vacuo to a white solid (0.71 g, 84%): tlc on adsorbent A in solvent A, R_f 0.30; on adsorbent A in solvent D, R_f 0.74; on adsorbent A in solvent E, R_f 0.10. $[\alpha]_D$ 15.9° (c 1.00, MeOH).

Anal. Calcd for $C_{33}H_{54}ClN_5O_9$: C, 56.60; H, 7.77; N, 10.00. Found: C, 56.58; H, 7.87; N, 9.96.

Benzyloxycarbonyl-D-leucylvaline Methyl Ester (73). Benzyloxycarbonyl-D-leucine (10.6 g, 40 mmol), valine methyl ester hydrochloride (6.70 g, 40 mmol), and N-methylmorpholine (4.04 g, 40 mmol) were added to tetrahydrofuran (120 ml) and the resulting mixture was cooled to 0°. Dicyclohexylcarbodiimide (9.07 g, 44 mmol) was added and the mixture was stirred overnight while warming to room temperature. The mixture was filtered and the filtrate was concentrated. An ethyl acetate solution of the crude product was washed successively with saturated sodium bicarbonate (25 ml), 2N citric acid (25 ml), saturated sodium bicarbonate (25 ml), 2N citric acid (25 ml), water (25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated. The crude product was filtered through a 3.5 x 16 cm column of basic alumina (Brockmann Activity I) using ethyl acetate as eluent. A total of 300 ml of eluate was collected. The solution was concentrated and the semi-solid mass was crystallized from ethylacetate-pentane to give a white solid (5.96 g): mp 102-103°; $[\alpha]_D^{21}$ 31.9° (c 2.00, $CHCl_3$). The mother liquor was concentrated and the oily residue was triturated under pentane. A semi-solid mass formed which failed to crystallize on standing one week. Analysis by tlc on adsorbent A in solvent D gave identical results for both the solid sample and the semi-solid: R_f 0.84, nin-

hydrin negative until treatment with 1:1 hydrobromic acid-acetic acid. Yield: 9.06 g (60%).

Anal. Calcd for $C_{20}H_{30}N_2O_5$: C, 63.47; H, 7.99; N, 7.39. Found: C, 63.48; H, 7.98; N, 7.40.

D-Leucylvaline Methyl Ester Hydrobromide (74). Benzyloxycarbonyl-D-leucylvaline methyl ester (5.80 g, 15.3 mmol) was treated with saturated hydrogen bromide in acetic acid (15.3 g, saturated at 0°). The resulting solution was stirred 30 min at room temperature. Anhydrous diethyl ether (25 ml) was added and the solution was cooled at 5°. The white solid product was extremely hygroscopic and was therefore not filtered off. The mixture was dried in vacuo to give a pale yellow foam (4.91 g, 98%). A portion of the product (2.15 g) was dissolved in ethyl acetate, dried ($MgSO_4$), and precipitated by the addition of pentane. The pale orange solid was collected and dried (1.78 g, 83%): mp 171-173° (dec); $[\alpha]_D^{24}$ -24.6° (c 1.8, MeOH).

o-Nitrophenylsulfenylleucyl-D-leucylvaline Methyl Ester (75). o-Nitrophenylsulfenylleucine dicyclohexylamine salt (4.65 g, 10 mmol) was suspended in diethyl ether (25 ml) and stirred with 2N citric acid (25 ml) until all of the solid dissolved. The ether phase was separated, washed with 2N citric acid (10 ml), water (10 ml), dried ($MgSO_4$), and concentrated to give o-nitrophenylsulfenylleucine as a yellow solid (2.70 g). A portion of the solid (2.42 g, 8.5 mmol) was dissolved in tetrahydrofuran (25 ml) and the

solution was cooled to 0°. The solution was treated with 1-hydroxybenzotriazole monohydrate (2.60 g, 17.0 mmol) followed by D-leucylvaline methyl ester hydrobromide (2.76 g, 8.5 mmol). The solution was stirred 1 min and then dicyclohexylcarbodiimide (1.93 g, 9.4 mmol) was added followed in 1 min by a solution of N-methylmorpholine (0.86 g, 8.5 mmol) in tetrahydrofuran (5 ml). The resulting mixture was stirred 1 hr at 0° and 1 hr at room temperature. The mixture was filtered and the filtrate was concentrated. An ethyl acetate solution of the product was washed with saturated sodium bicarbonate (30 ml) and then cooled at 5° overnight. A small amount of N,N'-dicyclohexylurea was filtered off and the solution was washed successively with saturated sodium bicarbonate (25 ml), 2N citric acid (25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated to a yellow solid (4.30 g, 99%). The product was recrystallized from ethyl acetate-pentane to give yellow crystals (0.27 g, mp 190-191°). A second crop was obtained on concentration of the mother liquor (1.80 g, mp 178-181°). Yield: 2.07 g (48%). Analysis by tlc on adsorbant A in solvent C, R_f 0.98; on adsorbant A in solvent F, R_f 0.88; nmr ($CDCl_3$) 0.94 (m, 18, $(CH_3)_2CH$), 3.64 (s, 3, $COOCH_3$), 7.14 and 7.52 (m, 2, meta-o-NO_2-Ph-S), 7.86 and 8.12 ppm (d, J=8 Hz, 2, ortho-o-NO_2-Ph-S); $[\alpha]_D^{25}$ 4.3° (c 2.02, EtOH).

Anal. Calcd for $C_{24}H_{38}N_4O_6S$: C, 56.45; H, 7.50; N, 10.97. Found: C, 56.46; H, 7.53; N, 10.75.

o-Nitrophenylsulfenylleucyl-D-leucylvaline (72). o-Nitrophenylsulfenylleucyl-D-leucylvaline methyl ester (0.37 g, 0.7 mmol) was dissolved in aqueous dioxane (90%, 10 ml) and treated with sodium hydroxide (0.72 ml of 2.00N NaOH, 1.4 mmol). The resulting mixture was stirred 40 min then poured into 50 ml of ice water containing hydrochloric acid (0.36 ml of 3.95N HCl, 1.4 mmol). The precipitated product was collected and dried *in vacuo* over phosphorous pentoxice to give 0.37 g of yellow solid, mp 87-90° (dec). Recrystallization from diethyl ether gave a yellow solid (0.29 g, 81%): mp 100-101° (dec); tlc on adsorbent A in solvent A, R_f 0.60; on adsorbent A in solvent C, R_f 0.84; on adsorbent A in solvent D, R_f 0.31; and on adsorbent A in solvent F, R_f 0.12; $[\alpha]_D^{25}$ -2.67° (*c* 2.10, EtOH).

Anal. Calcd for $C_{23}H_{36}N_4O_6S$: C, 55.62; H, 7.31; N, 11.28. Found: C, 55.97; H, 7.86; N, 11.17.

o-Nitrophenylsulfenyl-α-pentachlorophenyl-γ-benzylglutamate (77). o-Nitrophenylsulfenyl-γ-benzylglutamate (3.2 g, 8.2 mmol) (obtained from the dicyclohexylamine salt by treatment with 2N citric acid) was dissolved in ethyl acetate (20 ml) and the resulting solution was cooled to 0°. A solution of dicyclohexylcarbodiimide (1.65 g, 8.0 mmol) in diethyl ether (2 ml) was added, the mixture was stirred 3 min and then a solution of pentachlorophenol (2.08 g, 7.8 mmol) in ethyl acetate (3 ml) was added dropwise over a period of 2 min. The mixture was stirred at 0° for 30 min then at room temperature overnight. The resulting

mixture was cooled to -10° and filtered. The filtrate was concentrated and the resulting yellow oil was chromatographed on a 4.5 x 17 cm column of silica gel (70-230 mesh) using chloroform-ethanol (80:20, v/v) as eluent. The first 150 ml of eluate was collected and concentrated to give a yellow foam (4.1 g, 93%): tlc on adsorbent A in solvent A, R_f 0.83; on adsorbent A in solvent C, R_f 0.89; on adsorbent B in solvent D, R_f 0.83. A portion of the foam (3.33 g) was crystallized from diethyl ether to give yellow crystals (1.46 g): mp 84-86°; $[\alpha]_D^{25}$ -58.0° (c 1.04, EtOAc); lit.[121] mp 84-85°; $[\alpha]_D^{20}$ -55.8° (c 1, EtOAc).

tert-Butyloxycarbonylleucyl-D-leucylvaline tert-Butyl Ester (78). tert-Butyloxycarbonylleucine (0.98 g, 4.2 mmol), D-leucylvaline tert-butyl ester (1.20 g, 4.2 mmol), and 1-hydroxybenzotriazole monohydrate (1.29 g, 8.4 mmol) were dissolved in tetrahydrofuran (20 ml) and the resulting solution was cooled to 0°. Dicyclohexylcarbodiimide (0.95 g, 4.6 mmol) was added and the mixture was stirred 1 hr at 0° and overnight at room temperature. The mixture was filtered and the filtrate was concentrated. The semi-solid residue was treated with ethyl acetate (50 ml) and refrigerated. The mixture was filtered and the filtrate was washed successively with saturated sodium bicarbonate (2 x 25 ml), 2N citric acid (2 x 25 ml), water (25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated to a white solid (2.15 g). The crude product was filtered through a 2.8 x 20 cm column of basic alumina (Brockmann Activity I)

using ethyl acetate as eluent. Concentration of the eluate gave a white solid (1.89 g, 90%). The product was recrystallized from ethyl acetate-pentane to give a white solid (1.70 g, 81%): mp 123-124°; tlc on adsorbent A in solvent A, R_f 0.71; on adsorbent A in solvent D, R_f 0.92; and on adsorbent A in solvent E, R_f 0.35; $[\alpha]_D^{25}$ 22.6° (c 2.10, $CHCl_3$).

Anal. Calcd for $C_{26}H_{49}N_3O_6$: C, 62.49; H, 9.88; N, 8.41. Found: C, 62.48; H, 9.65; N, 8.51.

Leucyl-D-leucylvaline Trifluoroacetate (79). tert-Butyloxycarbonylleucyl-D-leucylvaline tert-butyl ester (1.50 g, 3.0 mmol) was treated with anhydrous trifluoroacetic acid (15 ml) and the resulting solution was stirred 1 hr. The solution was concentrated and the solid product was dissolved in ethanol and concentrated in vacuo three times. The product was recrystallized from ethyl acetate to give a white solid (0.75 g), mp 229-230°. A second crop (0.32 g), mp 228-229°, was obtained by concentration and refrigeration of the mother liquor. Yield: 1.07 g (76%); $[\alpha]_D^{22}$ 38.1° (c 1.50, H_2O); nmr (D_2O) δ 1.06 (m, 18, CH_3), and 1.80 ppm (m, 7, $CH_2CH(CH_3)_2$).

Anal. Calcd for $C_{19}H_{34}N_3O_6 \cdot 0.5H_2O$: C, 48.91; H, 7.56; N, 9.01. Found: C, 48.80; H, 7.98; N, 9.14.

Attempted Preparation of o-Nitrophenylsulfenyl-γ-benzylglutamylleucyl-D-leucylvaline (71).[109] A solution of leucyl-D-leucylvaline trifluoroacetate hemihydrate (0.47 g, 1.0 mmol) in 50% aqueous pyridine (14 ml) was adjusted to pH 8.9 by the addition of 5N sodium hydroxide (350 µl).

o-Nitrophenylsulfenyl-α-pentachlorophenyl-γ-benzylglutamate (0.64 g, 1.0 mmol) was added in 10 equal portions over 2 hr. After one-half of the active ester had been added, additional pyridine (5 ml) was added to the reaction solution. The pH of the solution was maintained between 8.6-8.8 by the periodic addition of 5N sodium hydroxide. After stirring 5 hr the mixture became homogeneous. The solution was concentrated *in vacuo* at 50° and the oily residue was treated with water (25 ml). The aqueous phase was neutralized (litmus) by the addition of 10% hydrochloric acid. The product was extracted with ethyl acetate (25 ml), washed successively with water (25 ml) and saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated *in vacuo* to a viscous yellow oil (0.82 g). A portion of this oil (0.57 g) was subjected to dry-column chromatography on a 3.0 x 30 cm column of silica gel using ethyl acetate as eluent. The upper yellow section of the column was extracted with methanol. The mixture was filtered and concentrated. An ethyl acetate solution of the residue was dried ($MgSO_4$) and concentrated to a yellow solid (0.22 g): tlc on adsorbant A in solvent A, 4 spots visible; on adsorbant A in solvent C, 3 spots visible.

Benzyloxycarbonyl-α-benzylaspartate (80).[110] Benzyloxycarbonylaspartic acid (13.0 g, 49.0 mmol) and acetic anhydride (9 ml, 95 mmol) were mixed and heated at 45° for 4 hr. The solid product was broken up and dried *in vacuo*. The solid was treated with freshly distilled benzyl alcohol

(10.8 g, 0.10 mol) in a sealed vessel. The solution was heated at 100° for 4 hr. The resulting yellow oil was dissolved in diethyl ether (140 ml). The ether solution was extracted with 5% ammonium hydroxide (30 ml). The aqueous phase was separated and acidified to Congo red with 15% hydrochloric acid. The oily product was extracted into diethyl ether, dried ($MgSO_4$), and concentrated to a viscous, yellow oil (11.5 g). The crude product was dissolved in diethyl ether and extracted successively with 75, 15 and 10 ml of ammonium chloride-buffered 0.5% ammonium hydroxide (40 g of NH_4Cl per liter of 0.5% NH_4OH). The ether phase contained the α-benzyl ester. The aqueous phases were combined, acidified to Congo red with 10% hydrochloric acid and the resulting oil was extracted into diethyl ether (125 ml). This ether solution was extracted successively with 50, 10 and 10 ml of the buffer solution. The ether phase was combined with that from the first series of extractions. The aqueous phases were combined and acidified to Congo red with 10% hydrochloric acid. The resulting oil was extracted into diethyl ether (125 ml) and this solution was extracted successively with 25, 15, and 10 ml of buffer. The extraction procedure was repeated a fourth time in the same manner with 20, 10, and 5 ml of buffer. All of the ether phases were combined and extracted with 5% ammonium hydroxide (2 x 25 ml). The aqueous phase was acidified to Congo red and the resulting oil was extracted into diethyl ether (50 ml).

The ether solution was dried ($MgSO_4$) and concentrated to a white solid (5.5 g). Recrystallization from ether-petroleum ehter gave white crystals (4.36 g, 25%): mp 84-86°; $[\alpha]_D^{20}$ -9.4° (c 5.05, HOAc); lit.[110] mp 84-85°; $[\alpha]_D$ -9.66° (c 5.59, HOAc).

tert-Butyl O-(N-Benzyloxycarbonyl-α-benzylaspartyl)-3-hydroxytetradecanoate. Benzyloxycarbonylaspartic acid α-benzyl ester (0.71 g, 2.0 mmol) was dissolved in anhydrous tetrahydrofuran (3 ml) and the solution was cooled to 0°. N,N'-Carbonyldiimidazole (0.34 g, 2.1 mmol) was added, the solution was stirred 15 min and then a solution of tert-butyl-3-hydroxytetradecanoate (0.30 g, 1.0 mmol) in tetrahydrofuran (2 ml) was added followed by 0.25 ml of sodium-imidazole catalyst solution (1 g of imidazole and 0.1 g sodium in 15 ml of anhydrous tetrahydrofuran). The solution was stirred at 0° for 30 min and at room temperature five days. Analysis by glpc on column A at 180° indicated no change in composition after 65 hr; tert-butyl-3-hydroxytetradecanoate was still present. An additional equivalent of benzyloxycarbonylaspartic acid α-benzyl ester (0.36 g, 1.0 mmol) and N,N'-carbonyldiimidazole (0.16 g, 1.0 mmol) in dry tetrahydrofuran (2 ml) cooled to 0° was added. The solution was stirred 4 more days at room temperature. Analysis by glpc indicated a small amount of starting material was still present. The solution was concentrated and an ethyl acetate solution of the product was washed with saturated sodium bi-

carbonate (20 ml), water (20 ml), saturated sodium bicarbonate (20 ml), water (20 ml), saturated sodium chloride (20 ml), dried ($MgSO_4$), and concentrated to a viscous yellow oil (0.79 g). Analysis by tlc on adsorbant A in solvent D, R_f 0.73 and 0.87, both spots were ninhydrin negative until treatment with 1:1 hydrobromic acid-acetic acid. The spot at R_f 0.73 was identified as benzyloxycarbonylaspartic acid α-benzyl ester by tlc comparison with a known sample. The crude product was dissolved in ethyl acetate (25 ml), treated with saturated sodium bicarbonate (50 ml), and stirred overnight. The organic phase was washed with water (25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated to a viscous, yellow oil (0.52 g, 81%): tlc on adsorbent A in solvent D, R_f 0.90, ninhydrin positive after treatment with 1:1 hydrobromic acid-acetic acid; nmr ($CDCl_3$) δ 0.87 (t, $C\underline{H}_3(CH_2)_{10}$), 1.24 (m, $(C\underline{H}_2)_{10}$), 1.38 (s, $C(C\underline{H}_3)$, 2.36 (m, $CHC\underline{H}_2COOBu^t$), 2.86 (m, $C\underline{H}_2COOCH$), 4.60 (m, $C\underline{H}COOBzl$), 5.02 and 5.08 (both s, $C\underline{H}_2Ph$), 6.00 (m, $N\underline{H}CH$), and 7.20 ppm (s, Ph).

Anal. Calcd for $C_{37}H_{53}NO_8$: C, 69.45; H, 8.35; N, 2.19. Found: C, 69.47; H, 8.44; N, 2.30.

D-tert-Butyl β-Hydroxytetradecanoate. N,N-Dimethylformamide dineopentyl acetal (20 ml, 72 mmol) was added to a solution of D-β-hydroxytetradecanoic acid (4.89 g, 20 mmol) in freshly distilled tert-butanol (55 ml) under nitrogen. The resulting mixture was stirred 10 min at room

temperature and 20 min at 70°. The solution was concentrated at 70° *in vacuo*. A diethyl ether solution (75 ml) of the crude product was washed successively with water (2 x 5 ml), saturated sodium bicarbonate (2 x 50 ml), 2N citric acid (2 x 50 ml), saturated sodium chloride (2 x 50 ml), dried (Na_2SO_4), and concentrated *in vacuo* at 60° to a pale yellow oil (3.74 g). Analysis by glpc on column A at 175° indicated that the major component comprised about 85% of the mixture. A portion of the crude product was purified by dry-column chromatography on a 3.0 x 50 cm column of silica gel (Brockmann Activity III) using benzene as eluent. The product was extracted with diethyl ether. The extract was dried ($MgSO_4$) and concentrated to a pale yellow oil (2.23 g). Overall estimated yield: 50%. Analysis by glpc on column A at 175° indicated that the oil was at least 97% one component: $[\alpha]_D^{25}$ -16.0° (*c* 2.35, $CHCl_3$).

tert-Butyl *O*-(N-Benzyloxycarbonyl-β-benzylaspartyl)-*D*-β-hydroxytetradecanoate (81). Method A. N,N'-Carbonyldiimidazole (1.35 g, 8.3 mmol) was added to a solution of benzyloxycarbonyl-α-benzylasparate (2.97 g, 8.3 mmol) in dry tetrahydrofuran (2 ml) under nitrogen. The resulting solution was stirred 30 min. *D*-*tert*-Butyl β-hydroxytetradecanoate (1.00 g, 3.3 mmol) in tetrahydrofuran (1 ml) was added followed by 1 ml of a solution of sodium (0.2 g) and imidazole (2.0 g) in tetrahydrofuran (25 ml). The resulting solution was stirred under nitrogen for five days. The

solution was concentrated and a solution of the oily residue in ethyl acetate was washed successively with water (10 ml), 2N citric acid (2 x 10 ml), and water (10 ml). The solution was stirred over saturated sodium bicarbonate overnight, washed with water (2 x 25 ml), dried ($MgSO_4$) and concentrated to a viscous oil (2.96 g). The crude product was purified by chromatography on silica gel (70-230 mesh) using ethyl acetate as eluant. Concentration in vacuo gave the product as a viscous oil (1.92 g, 90%): tlc on adsorbent A in solvent A, R_f 0.86; on adsorbent A in solvent C, R_f 0.97; on adsorbent A in solvent D, R_f 0.85; and on adsorbent A in solvent E, 0.68; $[\alpha]_D^{25}$ 8.76° (c 5.02, $CHCl_3$); nmr ($CDCl_3$) δ 0.90 (t, 3, J=6 Hz, $CH_3(CH_2)_{10}$), 1.32 (m, 20, $(CH_2)_{10}$), 1.46 (s, 9, $C(CH_3)_3$), 2.45 (m, 2, CH_2COOBu^t), 3.00 (m, 2, CH_2COOCH), 4.82 (m, 1, CHCOOBzl), 5.26 (s, 2, CH_2-Ph), 5.32 (s, 2, CH_2-Ph), 6.27 (d, 1, J=9 Hz, NH), and 7.56 ppm (s, 10, Ph).

Anal. Calcd for $C_{37}H_{53}NO_8$: C, 69.45; H, 8.35; N, 2.19. Found: C, 69.22; H, 8.48; N, 2.38.

Method B. N,N-Carbonyldiimidazole (4.40 g, 27.2 mmol) was added to a solution of benzyloxycarbonyl-α-benzyl-aspartate (9.70 g, 27.2 mmol) in dry tetrahydrofuran (7 ml). The mixture was stirred 30 min, an additional 8 ml of tetrahydrofuran was added and the mixture was stirred 45 min. A solution of D-tert-butyl β-hydroxytetradecanoate (2.72 g, 9.1 mmol) in tetrahydrofuran (2 ml) was added followed by 2 ml of a solution of sodium (0.2 g) and imidazole (2.0 g)

in tetrahydrofuran (25 ml). The resulting solution was stirred for five days. The solution was concentrated *in vacuo*. An ethyl acetate solution of the crude product was washed successively with water (25 ml), 2N citric acid (25 ml), saturated sodium bicarbonate (2 x 25 ml), and then stirred over saturated sodium bicarbonate (50 ml) overnight. The organic phase was separated, washed with water (2 x 25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated to a viscous oil (10.6 g) which solidified on standing. The crude product was purified by column chromatography on silica gel (70-320 mesh) using ethyl acetate as eluent. The product was obtained as a viscous, clear oil (5.26 g, 91%): tlc on adsorbent A in solvent A, R_f 0.86; on adsorbent A in solvent C, R_f 0.94; on adsorbent A in solvent D, R_f 0.94; on adsorbent A in solvent E, R_f 0.71; and on adsorbent A in solvent G, R_f 0.99; $[\alpha]_D^{25}$ 9.4° (*c* 5.28, $CHCl_3$).

Peptolide (82). Peptolide 81 (5.13 g, 8.02 mmol) was dissolved in a solution of methanol (40 ml) and acetic acid (3 drops). The compound was hydrogenolized over 5% palladium on carbon (1.0 g) at 1 atm for 3 hr. The mixture was filtered and the filtrate was concentrated *in vacuo* to a white foam (3.00 g, 89%). The product was crystallized from N,N-dimethylformamide to give white sticky solid (2.50 g, 74%): tlc on adsorbent A in solvent A, R_f 0.16; on adsorbent A in solvent C, R_f 0.71; on adsorbent A in solvent

G, R_f 0.47, all spots were ninhydrin positive; $[\alpha]_D^{25}$ -12.2° (c 2.12, $CHCl_3$); (60 mHz) nmr ($CDCl_3$ δ 0.87 (t, 3, J=6 Hz, $CH_3(CH_2)_{10}$), 1.27 (m, 20, $(CH_2)_{10}$), 1.43 (s, 9, $(CH_3)_3C$), 2.45 (m, 2, $OCHCH_2$), and 2.09 ppm (m, 2, $CHCH_2COOCH$).

Anal. Calcd for $C_{22}H_{41}NO_6$: C, 63.58; H, 9.95; N, 3.37. Found: C, 63.45; H, 9.99; N, 3.51.

Peptolide (83). Peptolide 82 (250 mg, 0.60 mmol) was suspended in N,N-dimethylformamide (10 ml) and the mixture was cooled to 10° and treated alternately with small portions of o-nitrophenylsulfenylchloride (125 mg, 0.66 mmol) and 1N sodium carbonate (1.26 ml, 1.26 mmol) over a period of 30 min. The resulting mixture was stirred 2 hr at room temperature then poured into water (125 ml) and refrigerated overnight. The mixture was filtered. The filtrate was acidified (litmus) with 10% hydrochloric acid and the oily product was extracted with ethyl acetate (2 x 25 ml). The ethyl acetate solution was washed successively with 2N citric acid (10 ml), water (2 x 25 ml), saturated sodium chloride (25 ml), dried ($MgSO_4$), and concentrated in vacuo to a viscous, yellow oil (0.36 g). The crude product was chromatographed on a 2.2 x 15 cm column of silica gel (150-230 mesh) using chloroform-ethanol (4:1, v/v) as eluent. The product was collected as a fast-running yellow band. Concentration of the eluate in vacuo gave a viscous, yellow oil (0.27 g, 78%): tlc on adsorbent A in solvent A, R_f 0.57; on adsorbent A in solvent C, R_f 0.82; on adsorbent A in

solvent E, R_f 0.0; nmr ($CDCl_3$) δ 0.90 (t, 3, J=6 Hz, $CH_3(CH_2)_{10}$), 1.29 (m, 20, $CH_3(CH_2)_{10}$), 1.47 (s, 9, $(CH_3)_3C$), 2.56 (m, 2, CH_2COOBu^t), 2.92 (m, 2, CH_2COOCH), 3.88 (m, 1, CHCOOH), 5.34 (m, 1, CHOCO), and 7.25-8.49 ppm (m, 5, o-NO_2PhS); $[\alpha]_D^{22}$ -14° (c 2.2, $CHCl_3$).

Peptolide (84). A solution of peptolide 83 (187 mg, 0.329 mmol), D-leucylleucine benzyl ester trifluoroacetate (147 mg, 0.329 mmol) and 1-hydroxybenzotriazole monohydrate (101 mg, 0.657 mmol) in tetrahydrofuran (2 ml) was cooled to 0°. Dicyclohexylcarbodiimide (75 mg, 0.36 mmol) was added and the resulting mixture was stirred 10 min. N-Methylmorpholine (33 mg, 0.33 mmol) was added and the mixture was stirred 1 hr at 0° and 2 hr at room temperature. The mixture was filtered and the filtrate was concentrated. An ethyl acetate solution of the crude product was washed successively with saturated sodium bicarbonate (2 x 5 ml), 2N citric acid (5 ml), water (5 ml), saturated sodium chloride (5 ml), dried ($MgSO_4$) and concentrated. The crude product was dissolved in diethyl ether (3 ml) and cooled at 5° overnight. A small amount of white solid (11 mg, mp 228-230°) was removed by filtration. The filtrate was concentrated and the crude product was purified by dry-column chromatography on a 2 x 20 cm column of silica gel (Brockmann Activity III) using ethyl acetate as eluent. The fast-running yellow band was extracted from the column with methanol. The extract was concentrated, the residue was dissolved in ethyl acetate, dried

($MgSO_4$), and concentrated to a yellow foam (0.16 g): $[\alpha]_D^{22}$ 6.5° (c 2.15, $CHCl_3$); tlc on adsorbent A in solvent E, R_f 0.40 (three faster running impurities were also present). A portion of the product (0.14 g) was further purified by high pressure liquid chromatography on a 4' x 3/8" column of C_{18}-Porasil B (Waters Associates). A total of 60 mg was obtained. The product crystallized from methanol to provide a pale yellow solid: mp 57-59°; (60 mHz) nmr ($CDCl_3$) δ 0.95 (m, 15, $CH_3(CH_2)_{10}$, $(CH_3)_2CH$), 1.20-2.00 (m, 26, $(CH_2)_{10}$), $CHCH_2CH(CH_3)_2$), 1.45 (s, 9, $(CH_3)_3C$), 2.63 (m, 2, CH_2COOBu^t), 3.05 (m, 2, CH_2COOCH), 5.40 (s, 2, CH_2Ph), 7.28 (s, 5, Ph), and 6.8-8.4 ppm (m, 4, O-NO_2PhS-).

Anal. Calcd for $C_{47}H_{72}N_4O_{10}S$: C, 63.77; H, 8.20; N, 6.33. Found: C, 63.70; H, 7.86; N, 6.13.

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