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I. CYCLOPEPTIDE ALKALOIDS
II. PHYCOCYANOBILIPEPTIDES

John Clark Lagarias
(Ph. D. thesis)

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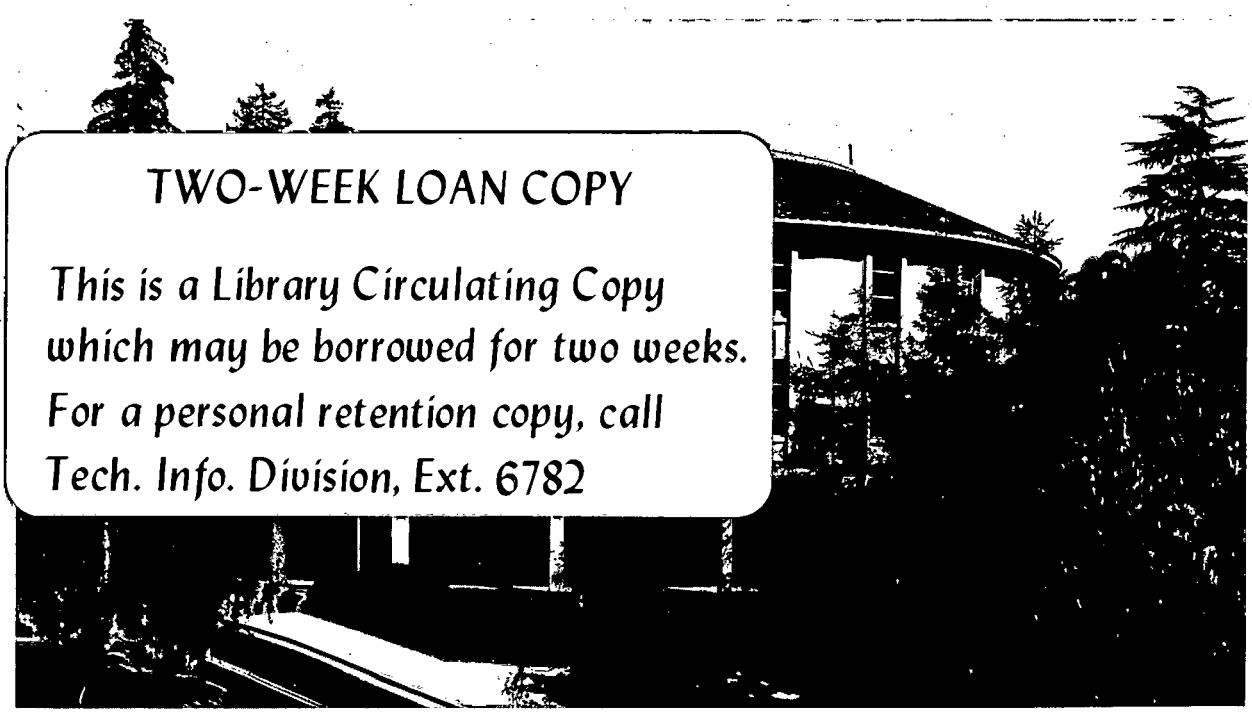

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# I. CYCLOPEPTIDE ALKALOIDS
# II. PHYCOCYANOBILIPEPTIDES

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## ABSTRACT

### I-A. Cyclopeptide Alkaloids. Synthesis of the Ring System and Its Ion Affinity

Several examples of the 14-membered, para-bridged ring system of the cyclopeptide alkaloids have been synthesized via an active ester cyclization. The yield of monomeric cyclopeptide varied from 1 to 33% and was affected by the amino acid substitution pattern and amide conformation of the linear peptide precursors. Both the synthetic models and a naturally occurring cyclopeptide alkaloid, ceanothine B, bind monovalent ($Li^{+}$) and divalent ($Ca^{++}$, $Mg^{++}$) cations.

### I-B. Cyclopeptide Alkaloids. Phencyclopeptines from the Polymorphic Species *Ceanothus integerrimus*

Seven cyclopeptide alkaloids, phencyclopeptines 1-7, have been found distributed among three forms of the shrub *Ceanothus integerrimus*. Chemical degradation, mass spectroscopy, and $^{1}H$ NMR spectroscopy have established structures for these seven compounds, three of which have

have been previously reported. The utility of cyclopeptide alkaloid structure and the distribution for chemotaxonomic assignments is discussed.

I-C. Cyclopeptide Alkaloids. Phencyclopeptines from *Ceanothus sanguineus*

Field desorption mass spectroscopy has identified five phencyclopeptines in the crude alkaloidal extracts of *Ceanothus sanguineus*. A new paired-ion HPLC system for the separation of these alkaloids is discussed. Amino acid analysis, electron impact mass spectroscopy, and $^{1}H$ NMR spectroscopy have established the structures of six phencyclopeptines including two isomeric compounds 5 and 6. The structure of 2 has not been previously reported.

II. Phycocyanobilipeptides. The Structure and Linkage of a Phycocyanobilin Bound to the β-Subunit of C-Phycocyanin

The smallest cyanogen bromide fragment derived from the β-subunit of *Synechococcus* sp 6301 C-phycocyanin, the blue heptapeptide 2, has been investigated by 360 MHz $^{1}H$ NMR spectroscopy. The peptide portion, heptapeptide 3, was synthesized independently and used in comparative spectroscopic analysis. These studies have led to complete assignment of the structure of the peptide-linked phycocyanobilin and elucidation of the nature of the thioether chromophore-peptide linkage.

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## Part I-A.

# CYCLOPEPTIDE ALKALOIDS. SYNTHESIS OF THE RING SYSTEM AND ITS ION AFFINITY

## Introduction

The cyclopeptide alkaloids are natural products which accumulate in the root bark of woody plants, particularly those in the Rhamnaceae family. The widespread occurrence of these compounds in over 25 species of plants makes this an important class of natural product. Although antibiotic, hypotensive, and antitussive properties have been ascribed to the cyclopeptide alkaloids, no definitive pharmacological activity of these compounds has been demonstrated.[1] Their role in the plant is also unknown, although recently cyclopeptide alkaloids have shown photophosphorylation inhibitor activity in spinach chloroplasts.[2] The difficulty of isolating sufficient quantities of these alkaloids, however, and the absence of any method for their synthesis has prevented further biological study of these compounds.

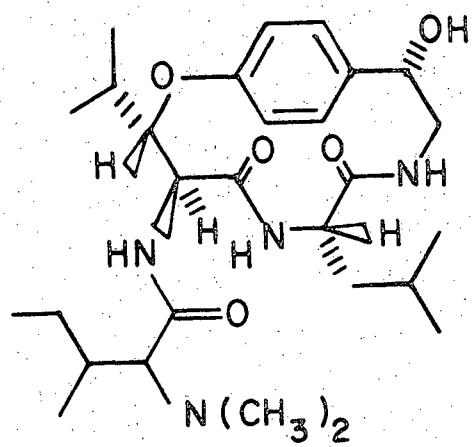
Since the confirmation of the structure of pandamine 1 in 1966,[3] reports of the isolation and structure elucidation of more than seventy cyclopeptide alkaloids have appeared.[1] Although cyclopeptide alkaloids incorporating 13- and 15-membered rings have been discovered, cyclopeptides such as frangulanine 2 with the 14-membered phencyclopeptide nucleus 3 are by far the most common. All the naturally occurring phencyclopeptines contain two amides at the 3,4 and 6,7 positions in the ring, however, variability in the R5 and R9 substituents and in the functionality of the benzylic (1,2) position is considerable.

In the present study our objective has been to develop a synthesis of the phencyclopeptine nucleus which is sufficiently versatile to allow the total synthesis of the natural products. We shall also discuss the evidence and implications of the observation of the specific cation binding of the naturally occurring and synthetic phencyclopeptines.

## Results and Discussion

Our initial experimental approach was designed to develop a general synthetic pathway to the saturated cyclopeptides 4a-c. Successful preparation of these saturated models would then be followed by syntheses directed to compounds with the functionalized benzylic residues found in the natural products (3a,b,c), perhaps via the saturated models as substrates. As a simplification, we chose to omit the nitrogen and alkyl residues on C-8 and C-9, respectively, in our model systems. The exclusion of the β-hydroxy-α-amino acid moiety found in the natural product would eliminate diastereomer separations during the planned synthesis, and the choice of a proline or leucine residue for $R_5$ was made on the basis of convenience.

The cyclopeptide models 4a-e were chosen to test the hypothesis that amide substitution should affect the course of peptide cyclization. These models differ in the degree of substitution of the amide nitrogens in both of the component amino acids. It is commonly accepted that amide resonance stabilizes their planar conformation and that trans conformations are preferred to cis (neglecting hydrogen bonds). The strong trans preference for the amide bond disappears when peptides are N-methylated.[4a] That intramolecular reaction between the ends of the linear peptide is influenced by the amide conformation has been demonstrated in the case



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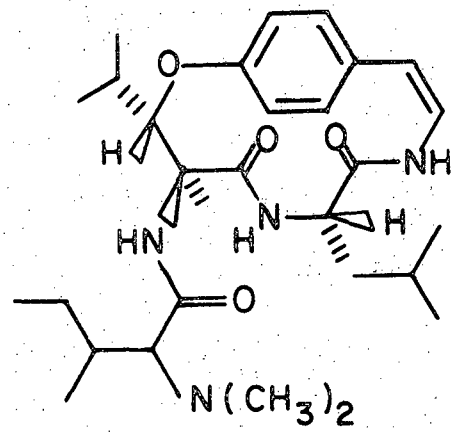

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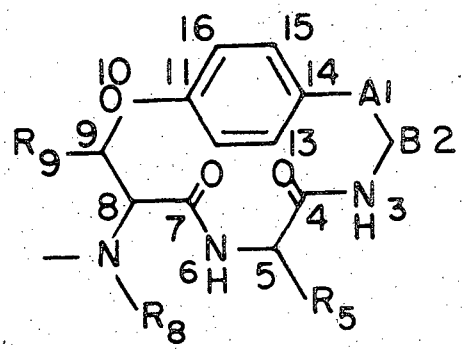

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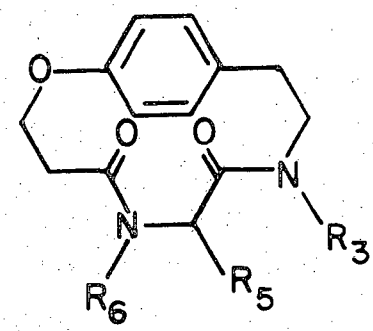
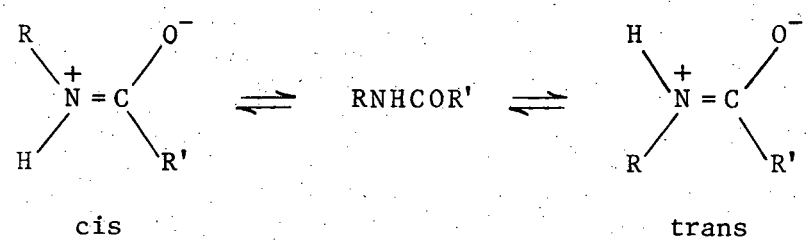


4

A-B components of the cyclopeptide alkaloids

| | | | |
|---|---|---|---|
| 3a, A-B = CH=CH | 4a, $R_3=CH_3$, | $R_5=R_6=(CH_2)_3$ | |
| b, A-B = $COCH_2$ | b, $R_3=CH_3$, | $R_5=(CH_3)_2CHCH_2$, | $R_6=H$ |
| c, A-B = $CH(OH)CH_2$ | c, $R_3=R_6=CH_3$, | $R_5=(CH_3)_2CHCH_2$ | |
| | d, $R_3=H$, | $R_5=R_6=(CH_2)_3$ | |
| | e, $R_3=R_6=H$, | $R_5=(CH_3)_2CHCH_2$ | |

of cyclotripeptide synthesis. Thus 9-membered ring cyclotripeptide can be prepared only when the amides are tertiary (i.e., cyclotrisarcosyl[4a] and cyclotriprolyl[4b]). Attempts to cyclize tripeptides with primary amino acid residues have only lead to the isolation of cyclohexapeptides.[4c] Therefore we chose the five peptide models (4a-e) as our first synthetic goals to test the amide conformational factors.

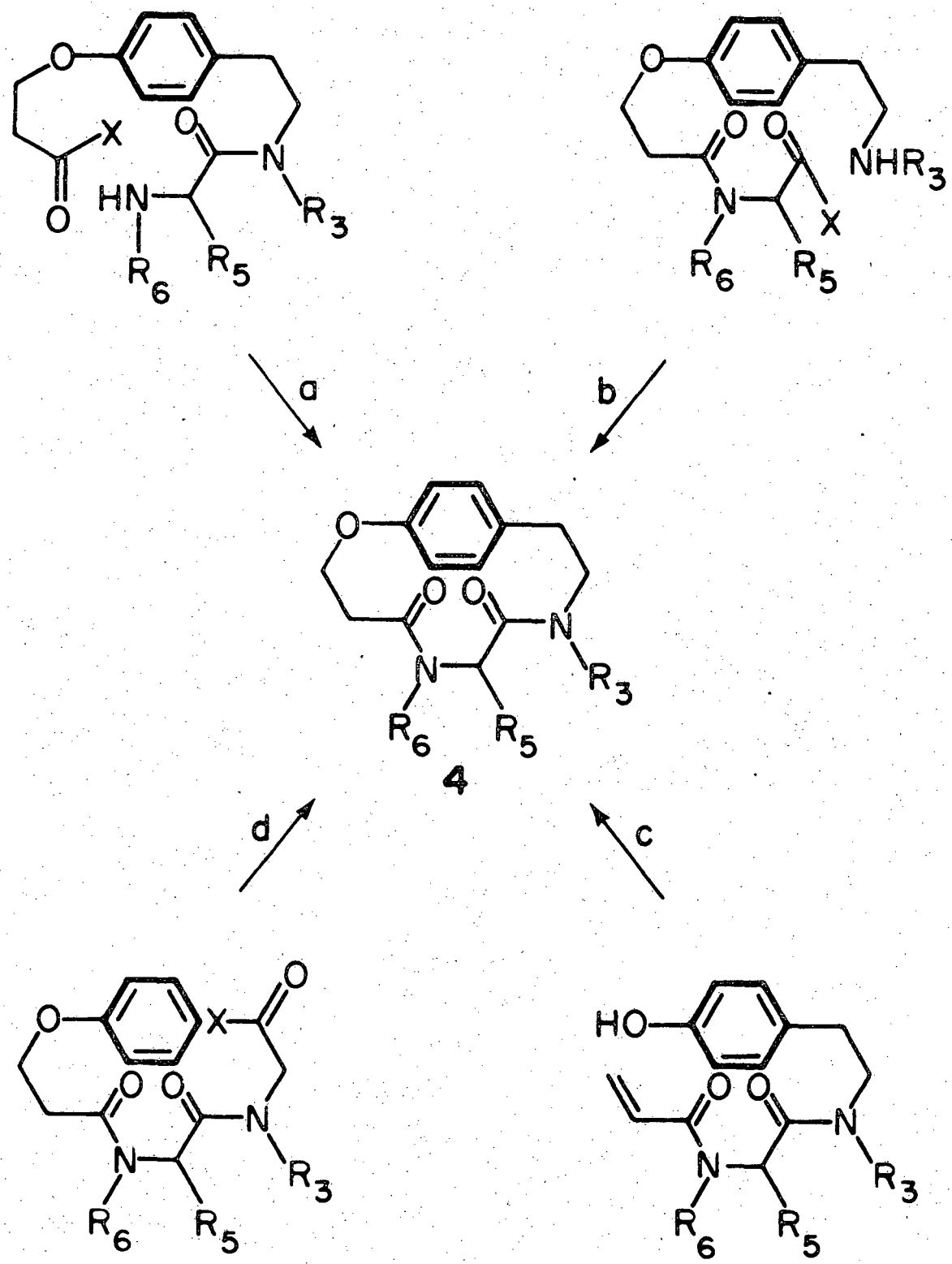



The key reaction of our synthesis of the cyclopeptide alkaloids involves the cyclization step. Initially, we considered four types of ring closure, as illustrated in Scheme I. Among these, a strong choice was high dilution cyclization of an active ester (pathway a), a peptide cyclization successful in the preparation of cyclotripeptides[4a,b] and analogues of the antibiotics actinomycin[5a] and gramicidin.[5b] Intramolecular Michael addition (pathway c), was questionable because of the reversibility of this reaction, especially when forming a strained ring. Cyclization via formation of the 3,4-peptide bond (pathway b) was rejected since this cyclization would require activation of a carbonyl adjacent to a chiral carbon and might lead to racemization of this asymmetric center if forcing conditions were necessary. Final formation of the 1,14-bond by Friedel-Crafts acylation was briefly considered (pathway d); however, reaction conditions necessary to effect this cyclization were considered too vigorous to be compatible with the aryl ether and amide

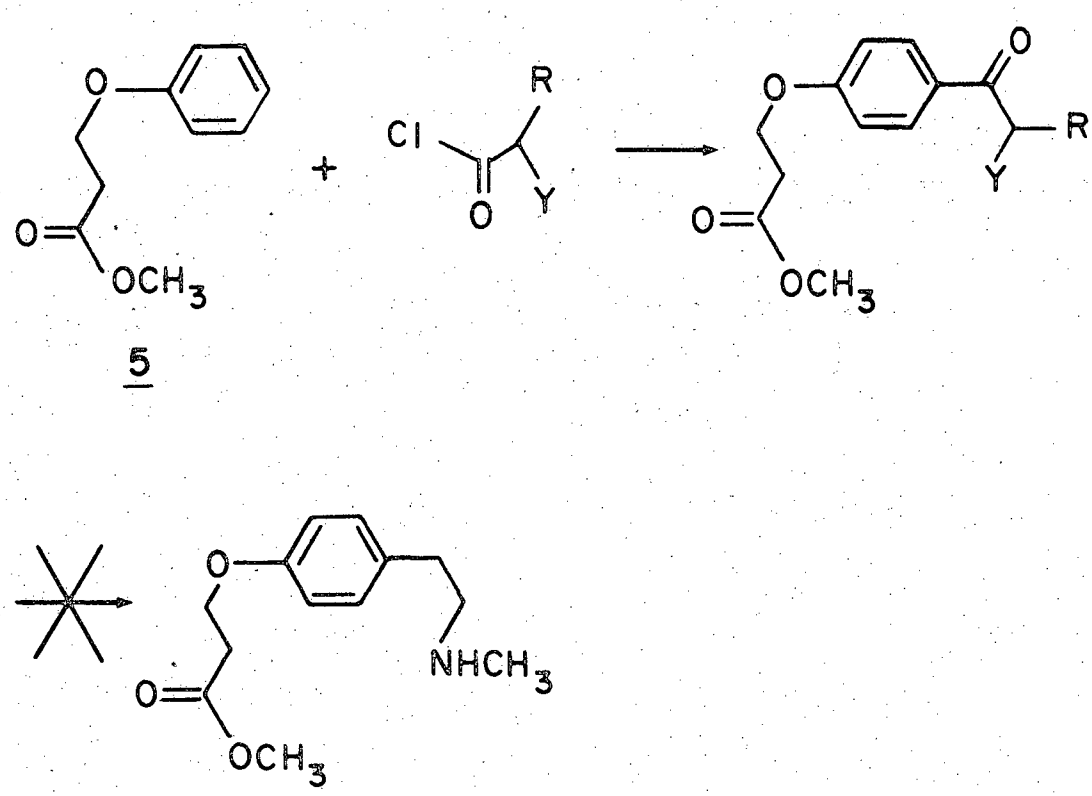
functionalities. For these reasons the 6,7-peptide cyclization of pathway a was our first choice.

Beginning with a 3-phenyloxypropanoate system, our initial synthetic design comprised the early preparation of a para-acylated aryl ether derivative followed by formation of the 3,4-peptide bond and ultimately by the 6,7-peptide cyclization. However, in the first step of this sequence we encountered difficulty in para-acylating aryl ether 5 with α-substituted aliphatic acid derivatives (Scheme II). With trifluoromethane sulfonic-carboxylic anhydride intermediates generated by reaction of carboxylic acid chlorides and silver trifluoromethanesulfonate, exclusive para-acylation of oxygenated aromatics has been reported[6] in some cases. In extending this method, we acylated methyl 3-phenyloxypropanoate (5) with either acetyl chloride or isobutyryl chloride under the reported conditions and obtained greater than 85% yields of isomerically pure para ketones 7a and 7b. However, acylation of 5 with N-trifluoroacetyl-N-methylaminoacetyl chloride (6b) afforded none of the amino ketone. Due to the instability of both aryl ether and ester moieties of 5, no Friedel-Craft procedure successfully effected the desired acylation.

On the other hand, acylation with an α-halogenated acetic acid derivative followed by nucleophilic displacement of the halogen atom with an amine did afford the amino ketone 7d. Unfortunately, the acylation of methyl 3-phenyloxypropanoate (5) via the mixed anhydride formed from trifluoromethane sulfonic acid and chloroacetyl chloride gave a mixture of ortho and para isomers in a 3/2 ratio. A similar isomer mixture was obtained when 5 was acylated with methoxyacetyl chloride by the mixed anhydride procedure. Clearly this acylation method[6] is




Scheme I. Cyclization modes for the preparation of cyclopeptide alkaloids.



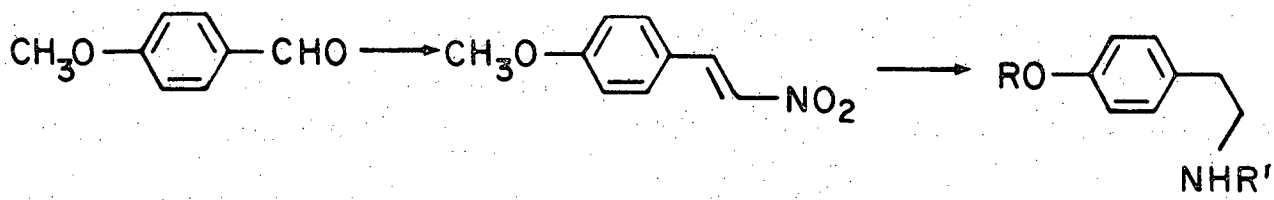

| | |
|---|---|
| 6a, R=Y=H | 7a, R=Y=H |
| b, R=Y=$CH_3$ | b, R=Y=$CH_3$ |
| c, R=H, Y=Br | c, R=H, Y=Br |
| d, R=H, Y=$N(CH_3)COCF_3$ | d, R=H, Y=$NHCH_3 \cdot HCl$ |

Scheme II. Synthetic approach via para acylation of 2-phenyloxypropanoates.

not a general one for exclusive para acylation of oxygenated aromatic compounds. The amino ketone 7d, alternatively, could be prepared in three steps. The previously synthesized ketone 7a was α-brominated with bromine in ether at 0 C. Displacement of the bromide (7c) by methylamine in methanol then afforded a 79% conversion to the amino ketone 7d.

Catalytic reduction of the ketone 7d always stopped at the benzyl alcohol stage and failed to give the desired phenylethylamine, although similar catalytic hydrogenation-hydrogenolysis conversions have been reported.[7] Failure in our case was due to decomposition of the 3-aryloxypropanoic acid moiety in both acid and base. This instability of the 3-aryloxypropanoic acid group necessitated devising a new approach to the cyclopeptide model 4 in which this functionality was introduced near the end of the synthesis.

To overcome these difficulties, we envisioned the preparation of the 9,10 ether linkage after the preparation of the 3,4-peptide bond. The synthesis of the p-hydroxyphenylethylamine system, the key intermediate, proceeded via catalytic reduction of the nitrostyrene 8 in acetic acid. The amine 9, on refluxing in concentrated hydrobromic acid, afforded tyramine hydrobromide 10 in 64% yield. Modifying the trichloroacetaldehyde (chloral) procedure[8] by adding triethylamine led to formylation of tyramine 10. Without the addition of triethylamine, the Schiff's base was the exclusive product of this reaction. The resultant phenol 11 was then converted to the benzyl ether 12b under standard conditions (benzyl chloride in refluxing acetone) and was subsequently reduced with lithium aluminum hydride to the N-methylamine 13b.




| | | | | | |
|---|---|---|---|---|---|
| 8 | 9, R=$CH_3$, | R'=H | 12a, R=CH , | R'=CHO |
| | 10, R=H, | R'=H | 12b, R=$C_6H_5CH_2$, | R'=CHO |
| | 11, R=H, | R'=CHO | 13a, R=$CH_3$, | R'=$CH_3$ |
| | | | 13b, R=$C_6H_5CH_2$, | R'=$CH_3$ |

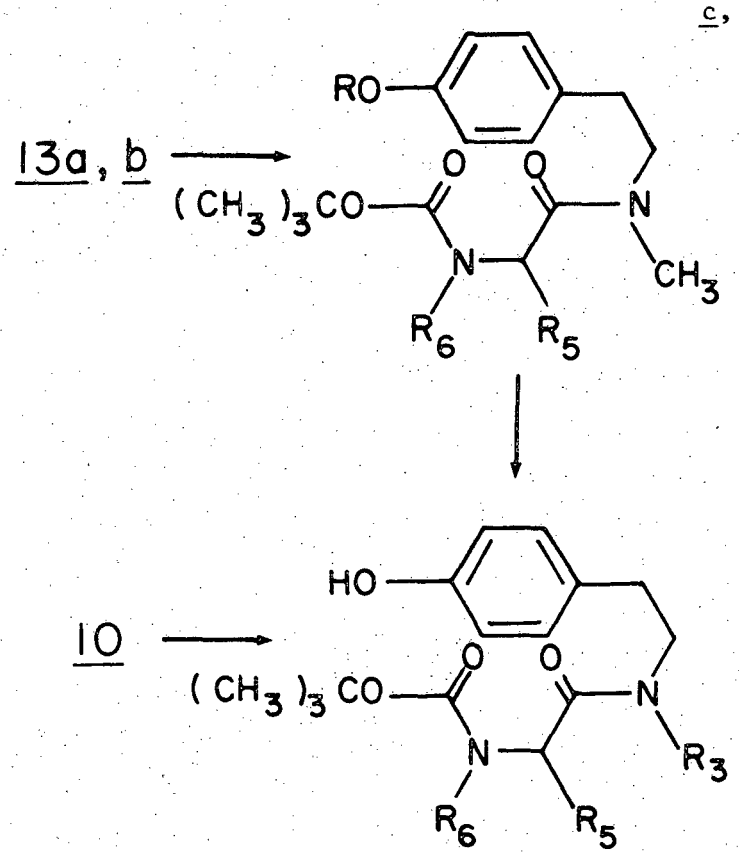
The most effective method for the acylation of amines 10 and 13 with N-tert-butoxycarbonyl amino acids[9] was a mixed anhydride procedure.[10] The yields of peptides 14b and 14c from 13 and peptides 15d and 15e from 10 were greater than 90%. In the case of the preparation of 14a via dicyclohexylcarbodiimide (DCC) coupling, the yield was substantially lower. However, following ether cleavage with $BBr_3$ and subsequent carbamate formation, the pure phenol 15a was obtained. The N-methyl peptides 14b,c were converted in high yield to the phenols 15b,c by hydrogenolysis.

With the phenols (15a-e) in hand, we next considered the alternatives for incorporation of the three carbon propanoate residue (Scheme III). The first attempted alkylation of the phenol (15a) with tert-butyl 3-bromopropanoate or 3-bromopropanoic acid in acetone over potassium carbonate lead to isolation of the corresponding acrylate and starting phenol. Another method investigated to prepare 3-phenoxypropanate systems was the Michael addition of phenols to acrylates.[11] Using p-cresol as a model, we developed conditions for this conversion which gave ether formation in 80% yield. Employing these conditions, however, we observed no reaction of phenol 15a with tert-butyl acrylate.

<u>14a</u>, $R=CH_3$, $R_5=R_6=(CH_2)_3$

<u>b</u>, $R=C_6H_5CH_2$, $R_5=(CH_3)_2CHCH_2$, $R_6=H$

<u>c</u>, $R=C_6H_5CH_2$, $R_5=(CH_3)_2CHCH_2$, $R_6=CH_3$



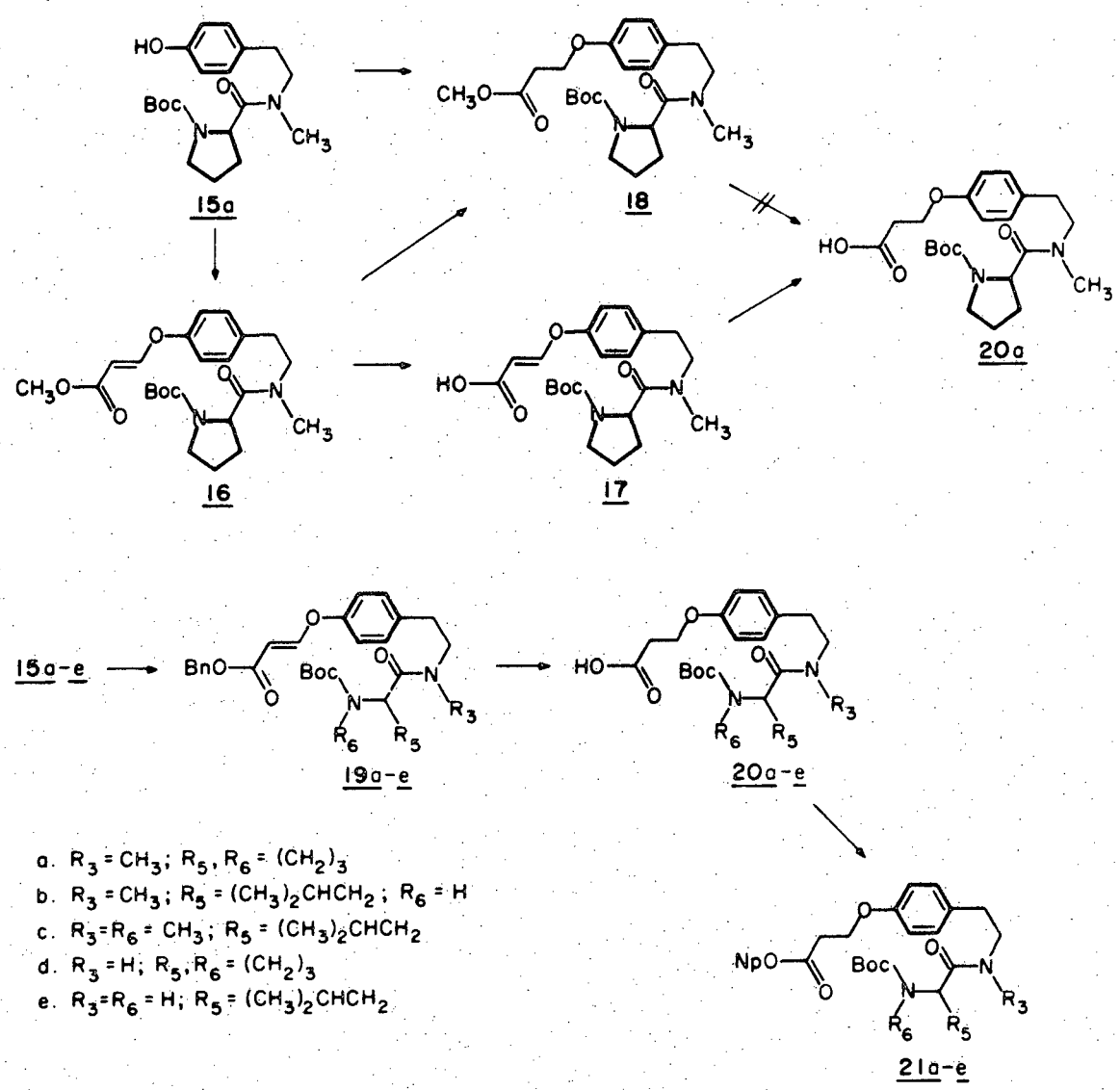

<u>15a</u>, $R_3=CH_3$, $R_5=R_6=(CH_2)_3$

<u>b</u>, $R_3=CH_3$, $R_5=(CH_3)_2CHCH_2$, $R_6=H$

<u>c</u>, $R_3=R_6=CH_3$, $R_5=(CH_3)_2CHCH_2$

<u>d</u>, $R_3=H$, $R_5=R_6=(CH_2)_3$

<u>e</u>, $R_3=R_6=H$, $R_5=(CH_3)_2CHCH_2$

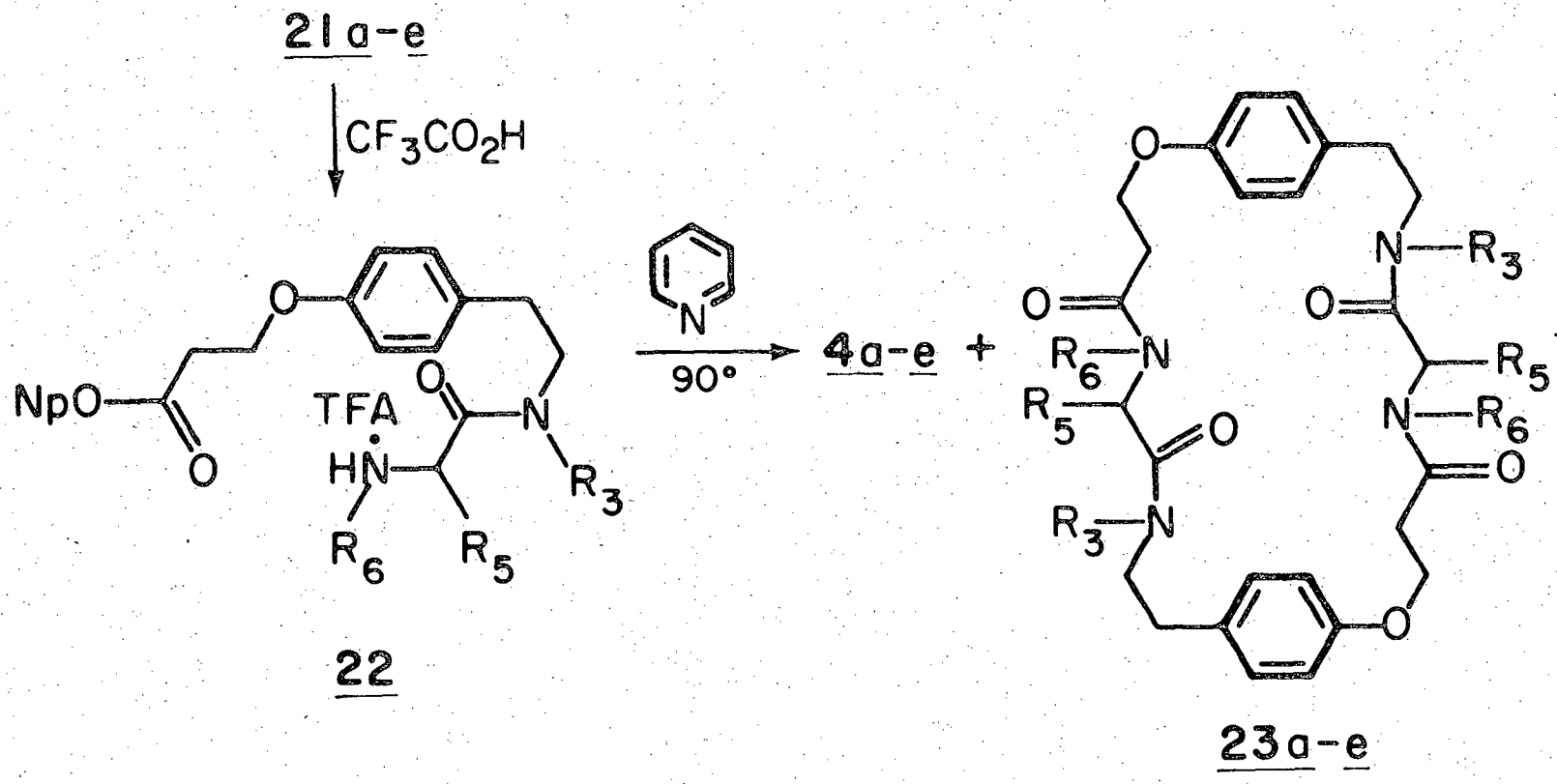



XBL 799-11735

Scheme III. Incorporation of the three-carbon propanoate residue .

A method for the three carbon homologation of phenols by Michael addition with propiolate derivatives was successful.[12] Thus we prepared methyl E-3-phenoxypropenoate (16) by addition of phenol 15a to methyl propiolate. If the sodium salt of the phenol was used, prepared with sodium hydride previous to condensation, the Z-isomer was the predominant product. Catalytic hydrogenation of E-3-phenoxypropenoate (16) afforded the propanoate 18, but this product was extremely sensitive to alkali. Attempted hydrolysis of the methyl ester 18 in alcoholic sodium hydroxide lead to rapid and complete β-elimination. In contrast, hydrolysis of methyl 3-phenoxypropenoate (16) with sodium hydroxide was easily accomplished. The resulting acid 17 could be hydrogenated to yield the saturated compound 20a. The general homologation of phenols 15a-e to the corresponding 3-phenoxypropanoic acids which were then converted to their p-nitrophenyl esters 21a-e is diagrammed in Scheme III. The reaction of the phenols 15a-e with benzyl propiolate followed by complete reduction afforded the respective 3-phenoxypropanoic acids 20a-e in high yield. After preparation of p-nitrophenyl esters (ONp) 21a-e with p-nitrophenyl trifluoroacetate in pyridine,[13] the conditions for peptide cyclization were next examined.

Removal of the N-tert-butoxycarbonyl protecting group was accomplished by dissolving the p-nitrophenyl esters 21 in anhydrous trifluoroacetic acid at 0 - 5 C (Scheme IV). By NMR it was clear that this process did not require the presence of a carbonium ion scavenger commonly used during acid catalyzed decomposition of tert-butyl carbamates.[4,5] After evaporation of the excess trifluoroacetic acid, the residual amine salt 21 was dissolved in N,N'-dimethylacetamide and added slowly to pyridine maintained at 90 C. Studies with 21a as the model established acceptable




Scheme IV. Peptide cyclization.

conditions for peptide cyclization (see Experimental Section). Owing to the susceptibility of the 3-phenoxypropanoate system to β-eliminate in alkali, the stability of the p-nitrophenyl esters 21 and the products 4 to these reaction conditions was also tested; both were stable. Using these conditions, the synthesis of each of the cyclopeptide monomers 4a-e on a preparative scale was accomplished. The yields are outlined in Table 1.

In each case, cyclic monomer 4 was separated from the respective dimer 23 by sephadex LH-20 chromatography. The spectral data (UV, CD and $^{13}C$ NMR) manifest the difference between cyclic monomers and dimers, especially with respect to the aromatic chromophore. In the UV, the absorption maxima of the cyclic dimers 23 are shifted to longer wavelengths with a fivefold increase in extinction coefficient relative to the corresponding cyclic monomer 4. In the $^{13}C$ NMR spectra of the cyclic dimers 23, each pair of ortho carbons, C-12, C-16, and C-13, C-15, show a single resonance (Fig. 1). On the other hand, each of the four ortho carbons C-12, C-13, C-15 and C-16 of the cyclic monomers 4 has a unique resonance (Fig. 2). The CD spectra in the 250-300 nm range show the expected larger interaction of the aromatic chromophore with the asymmetric center in the cyclic monomers 4. The differential molar extinction coefficient ($\Delta\varepsilon$) in this region is greater for the monomers than for the dimers.

Contrary to the results of cyclotripeptide synthesis,[4] our data show that the yield of cyclopeptide alkaloid model 4 is independent of the substitution of the amide (N-3, C-4) not involved in the formation of the final peptide bond. Although the linear peptides 21a and 21d

differ by the substitution pattern of one amide, the yields of the cyclic peptides 4a and 4d are similar. The yields of cyclopeptides 4b and 4e are also comparable, but less than that of 4a. Cyclopeptide 4c was obtained in very low yield. Our results show that the reactivity of the free amino group (N-6) in the linear peptide is the major factor affecting the different yields of cyclic monomers. That the rate of acylation of amines is greatly influenced by their degree of substitution is well illustrated by the preparation of N-tert-butoxycarbonylamino acids.[9] The rate of acylation with tert-butoxycarbonylazide decreases in the series proline > leucine ≫ N-methylleucine. The yields of cyclopeptides follow this sequence of decreased reactivity of the nucleophile, with proline (4a and 4d) > leucine (4b and 4e) ≫ N-methylleucine (4c).

The spectral data for the cyclopeptide monomers 4a-d indicates that each macrocycle has a unique geometry. Although the yield of cyclic peptide is independent of the degree of amide substitution in the linear peptide, the configuration of the cyclic product greatly depends on the structure of the amide in the linear peptide.

The ion binding properties of the synthetic peptide, cyclo-[3(4-β-aminoethyl)phenyloxypropanoyl-L-prolyl] (4d) and a natural peptide alkaloid, ceanothine B,[14] were determined by circular dichroism studies in acetonitrile.[15] The cyclopeptide 4d showed selectivity for $Mg^{++}$ and $Ca^{++}$ over $Li^{+}$ and did not interact with $Na^{+}$ and $K^{+}$ (Figs. 3 and 4). Similarly, ceanothine B interacted with $Mg^{++}$ and $Ca^{++}$ and not with $Na^{+}$ (Figs. 5 and 6). Cyclic dimers 23a-e did not exhibit metal complexing when observed by circular dichroism.

It is significant to note that the amino acid components of the cyclopeptide alkaloids contain only hydrophobic residues. Such low molecular weight peptides would probably have a high solubility in the lipid layer of a biomembrane and with respect to their ion affinities, these cyclopeptides could possess ionophoric activity. The high concentration of the cyclopeptide alkaloids in the root bark of plants may indicate an ion solubilizing and transporting function for these alkaloids in plant roots. Also, the reported[2] effect of the cyclopeptide alkaloids on photophosphorylation may be due to alteration of an ion-mediated process. Our results indicate that this class of natural products possesses an affinity for metal ions. The implication that the cyclopeptide alkaloids may function as ionophores in the plant that produces them is clearly suggested by the data presented above.[16]

Our synthetic method can be generalized and modified to include the preparation of cyclopeptides of this type in addition to the synthesis of peptide alkaloids. Functionalization of the benzylic position (C-1) of our model system 4, perhaps via a radical process, will lead to systems found in the natural products 3. By means of a substituted propiolate the positioning of a variety of groups on C-9 can easily be included into our synthetic scheme, as can substituents on the aromatic nucleus. The 3-phenyloxypropenoate 19 may offer a way to incorporate a nitrogen or other substitutents on C-8. Through synthesis of these 14-membered cyclopeptides, 3 or 4, we can answer the question of what variation in structure affects metal complexing ability.

TABLE 1. Isolated yields[a] of cyclopeptides from cyclization of esters 21.

| Ester, 21 | Monomer, 4 | Dimer, 23 | Other neutrals[b] | Total |
|---|---|---|---|---|
| 21-a | 24%(33%)[c] | 22% | 11% | 57% |
| b | 13% | 32% | 35% | 80% |
| c | ~0.4%[d] | 3% | 6% | ~9% |
| d | 24% | 34% | 17% | 75% |
| e | 9% | 15% | 27% | 51% |

[a]After mixed bed ion exchange and sephadex LH-20 chromatography.

[b]Uncharacterized neutral products, consisting in part of oligomers.

[c]This is a GC yield based on 5α-cholestane as internal standard added to the reaction mixture.

[d]Preparative GC followed by high resolution mass spectrometry established the structure of monomer 4c.

Figure Captions - Part I-A

Fig. 1. Fourier transform $^{13}C$ NMR spectra of cyclic dimers in $CDCl_3$ (~0.05 M); 23a, cyclo[3-(4-β-N-methylaminoethyl)phenyloxypropanoyl-L-prolyl]$_2$; 23b, cyclo[3-(4-β-N-methylaminoethyl)phenyloxypropanoyl-L-leucyl]$_2$; 23d, cyclo[3-(4-β-aminoethyl)phenyloxypropanoyl-L-prolyl]$_2$; 23e, cyclo[3-(4-β-aminoethyl)phenyloxypropanoyl-L-leucyl]$_2$.

Fig. 2. Fourier transform $^{13}C$ NMR spectra of cyclic monomers in $CDCl_3$ (~0.05 M); 4a, cyclo[3-(4-β-N-methylaminoethyl)phenyloxypropanoyl-L-prolyl]; 4b, cyclo[3-(4-β-N-methylaminoethyl)phenyloxypropanoyl-L-leucyl]; 4d, cyclo[3-(4-β-aminoethyl)phenyloxypropanoyl-L-prolyl]; 4e, cyclo[3-(4-β-aminoethyl)phenyloxypropanoyl-L-leucyl].

Fig. 3. Circular dichroism spectra of cyclo[3-(4-β-aminoethyl)phenyloxypropanoyl-L-prolyl] (4d), c = $9.4 \times 10^{-4}$ M in $CH_3CN$.

—— · —— = no added salt
—— · —— = $9.4 \times 10^{-3}$ M $NaClO_4$
—— · —— = $8.6 \times 10^{-3}$ M $KPF_6$
..... = $8.3 \times 10^{-3}$ M $LiClO_4$
———— = $9.2 \times 10^{-4}$ M $Mg(ClO_4)_2$
------- = $1.5 \times 10^{-3}$ M $Ca(ClO_4)_2$

Fig. 4. Circular dichroism spectra of cyclo[3-(4-β-aminoethyl)phenyloxypropanoyl-L-prolyl] (4d), c = $9.4 \times 10^{-5}$ M $CH_3CN$.

—— · —— = no added salt
—— · —— = $1.0 \times 10^{-2}$ M $NaClO_4$
———— = $9.3 \times 10^{-3}$ M $Mg(ClO_4)_2$.

Fig. 5. Circular dichroism spectra of ceanothine-B, $c = 1.0 \times 10^{-4}$M in $CH_3CN$.

—— · —— = no added salt

—— . —— = $1.1 \times 10^{-3}$ M $NaClO_4$

———— = $9.2 \times 10^{-4}$ M $Mg(ClO_4)_2$

------ = $1.5 \times 10^{-3}$ M $Ca(ClO_4)_2$

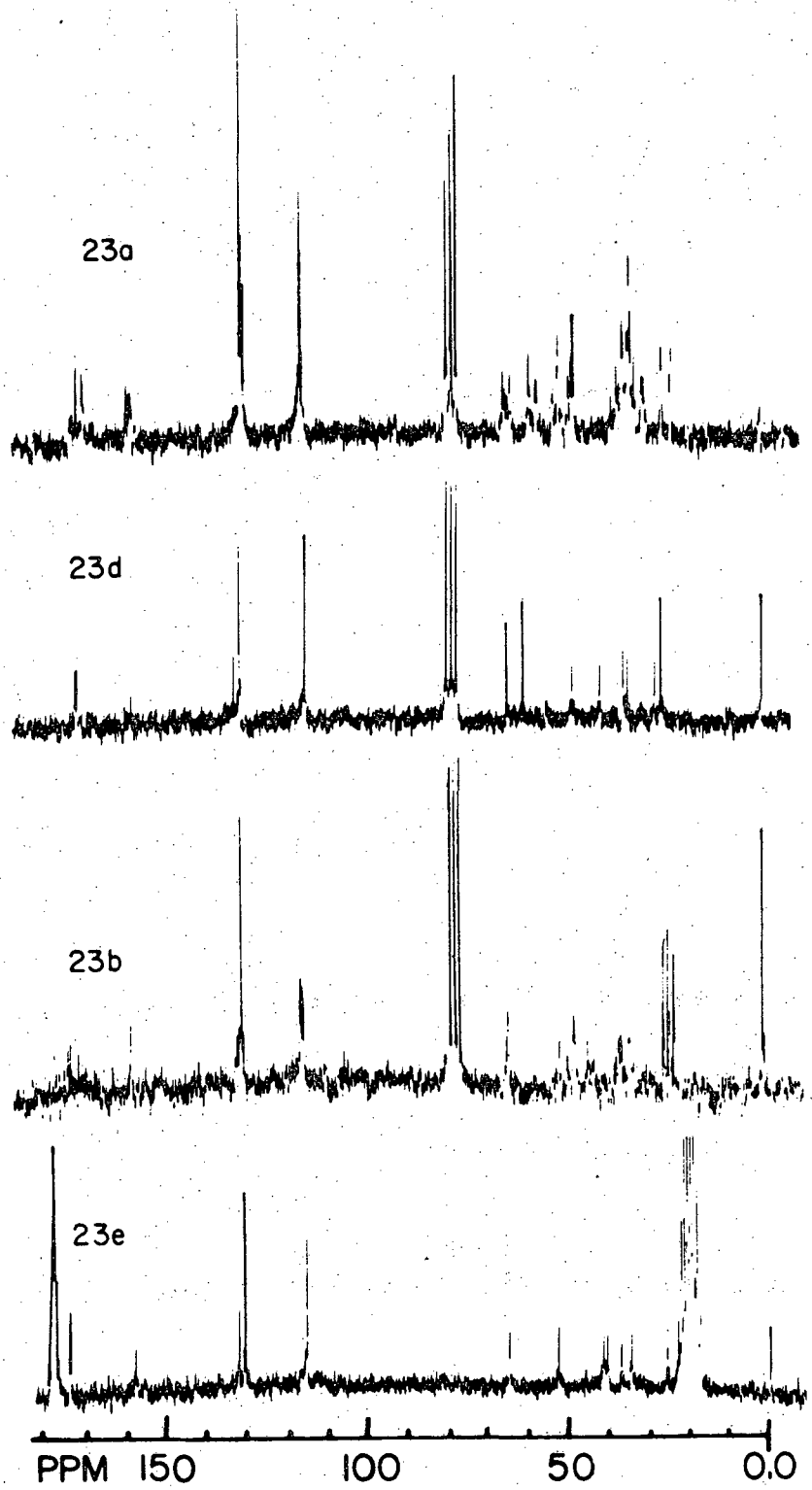
Fig. 6. Circular dichroism spectra of ceanothine-B, $c = 1.0 \times 10^{-5}$ in $CH_3CN$.

—— · —— = no added salt

—— . —— = $1.1 \times 10^{-3}$ M $NaClO_4$

———— = $9.2 \times 10^{-4}$ M $Mg(ClO_4)_2$

------ = $1.5 \times 10^{-3}$ M $Ca(ClO_4)_2$




XBL 799-11770

Fig. 1

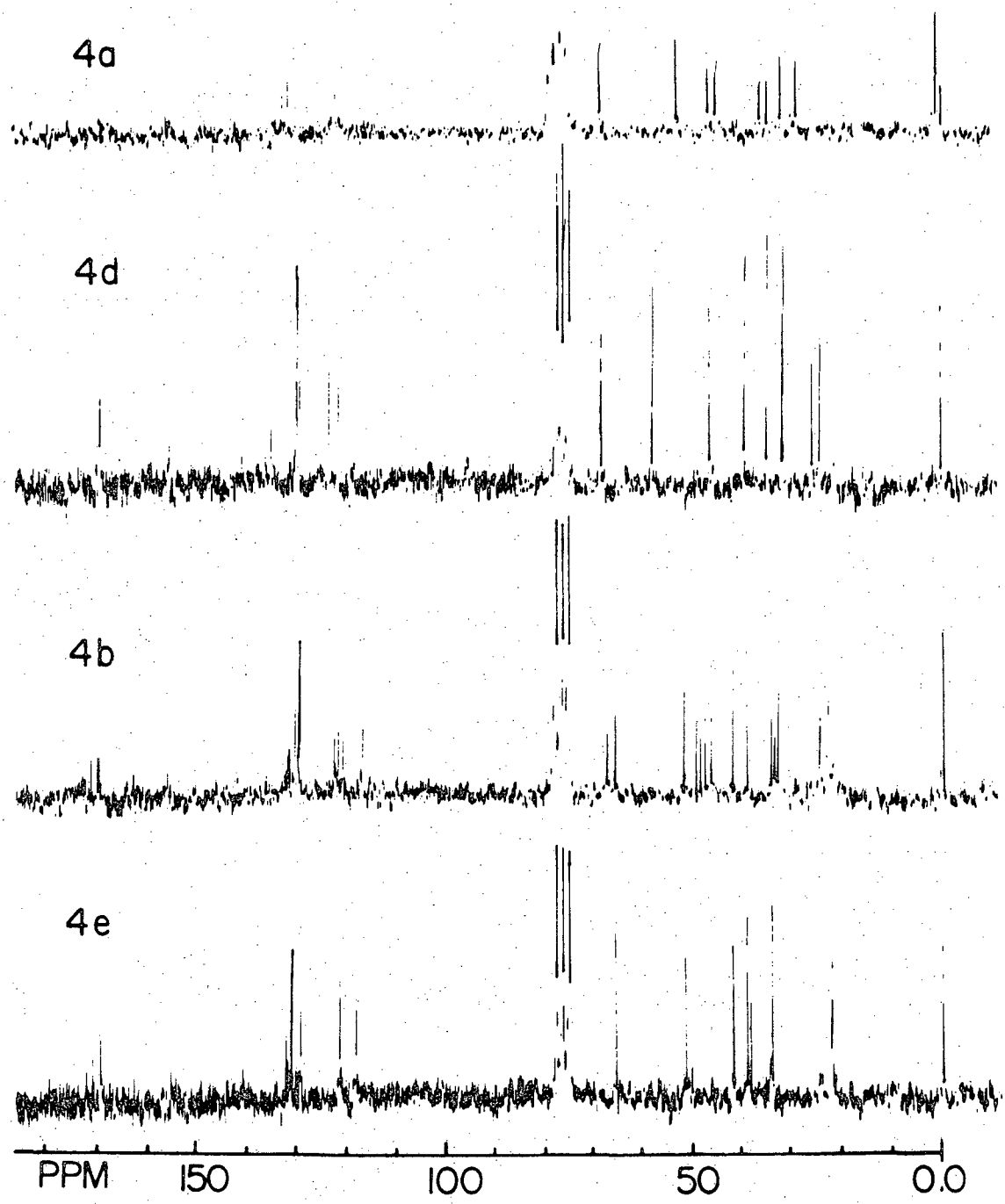



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Fig. 2

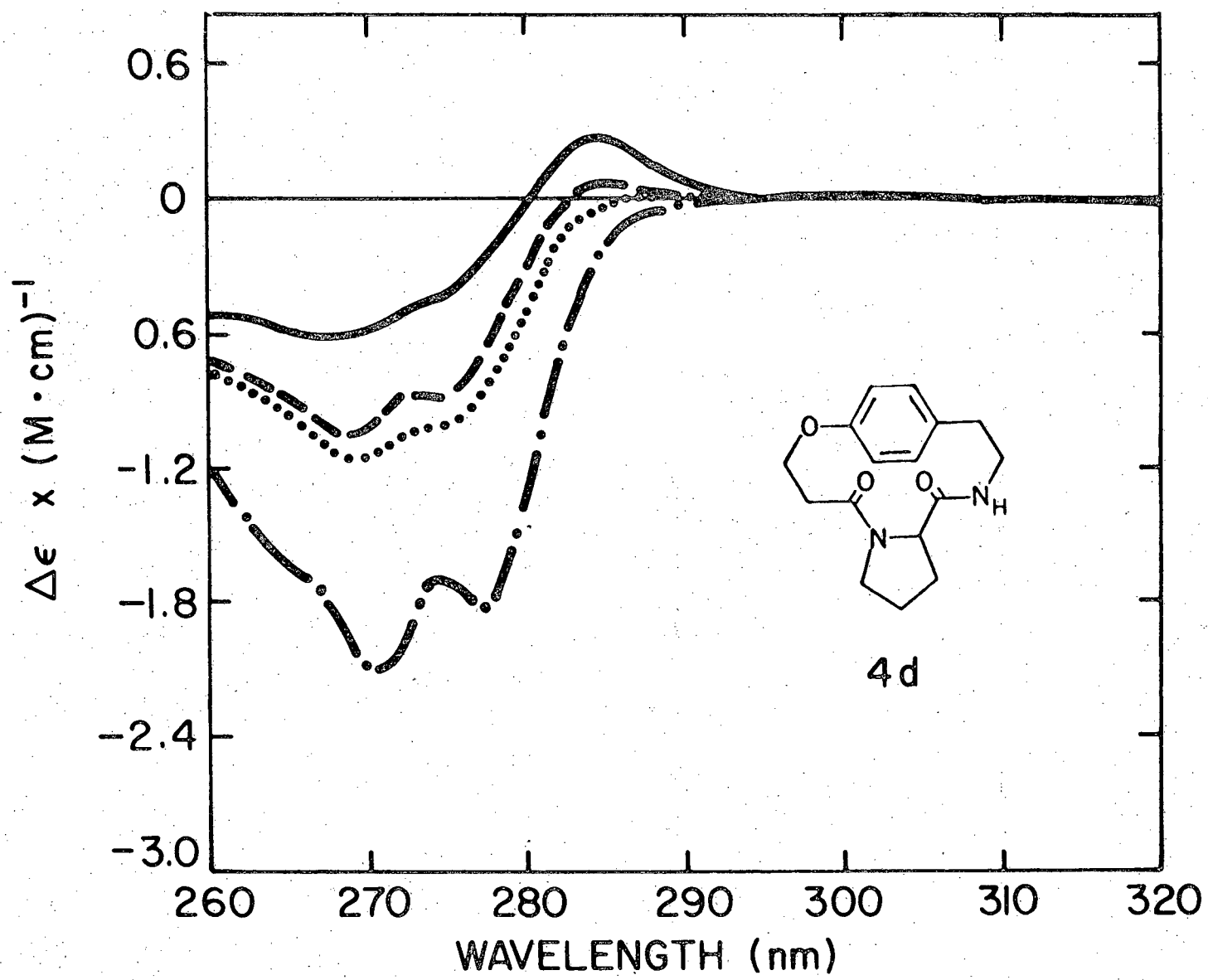


Fig. 3

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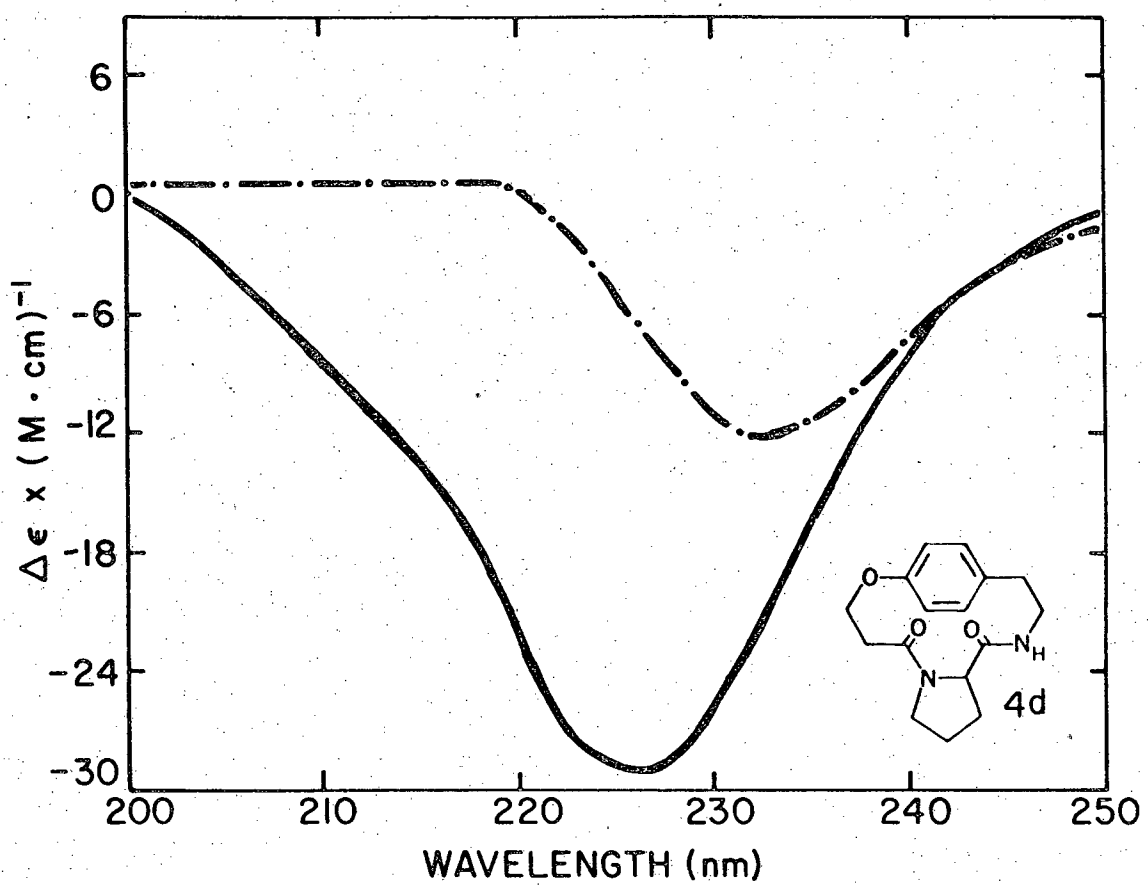
6
0
-6
-12
-18
-24
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Δε x (M · cm)⁻¹
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220
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WAVELENGTH (nm)
O
O
O
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N
H
4d

Fig. 4

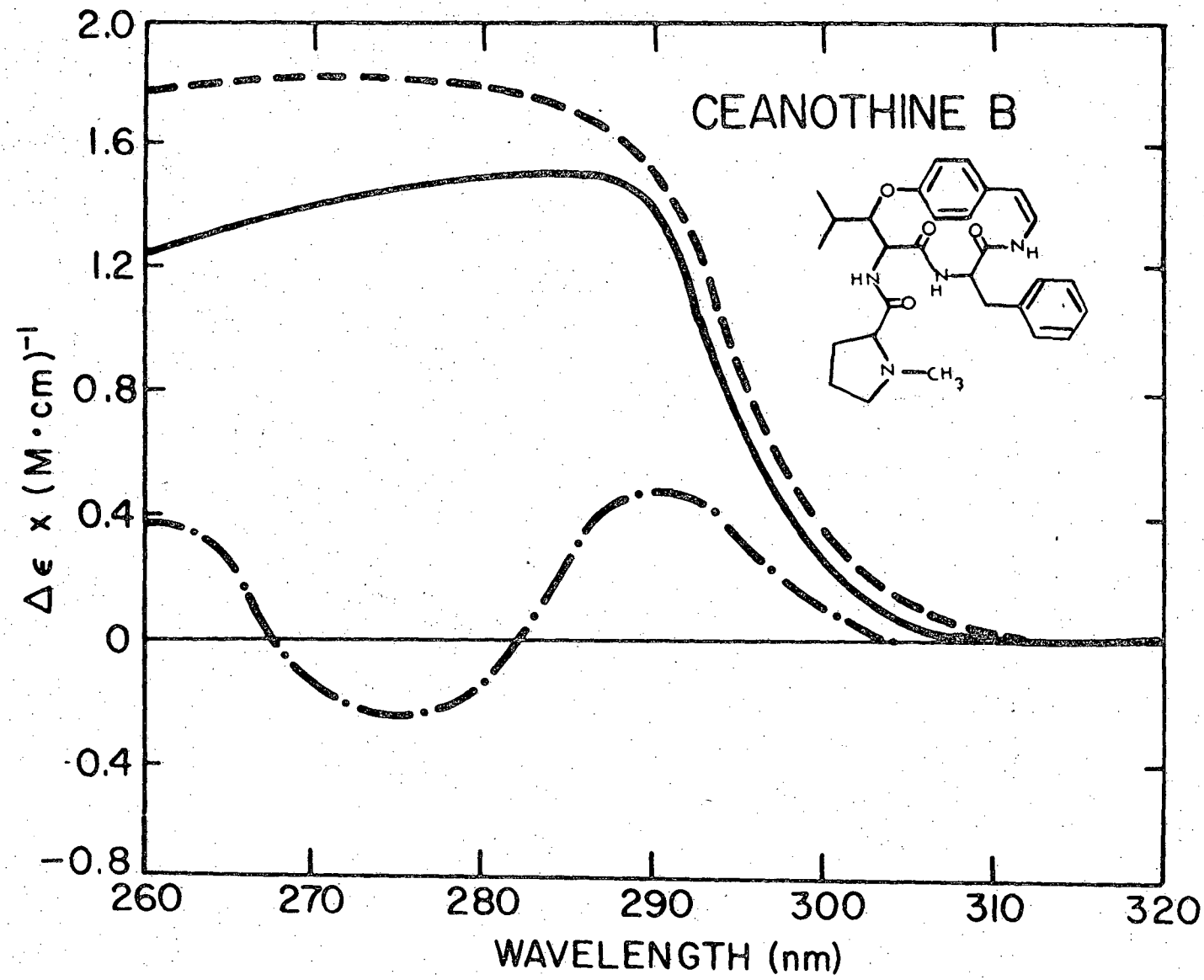


Fig. 5

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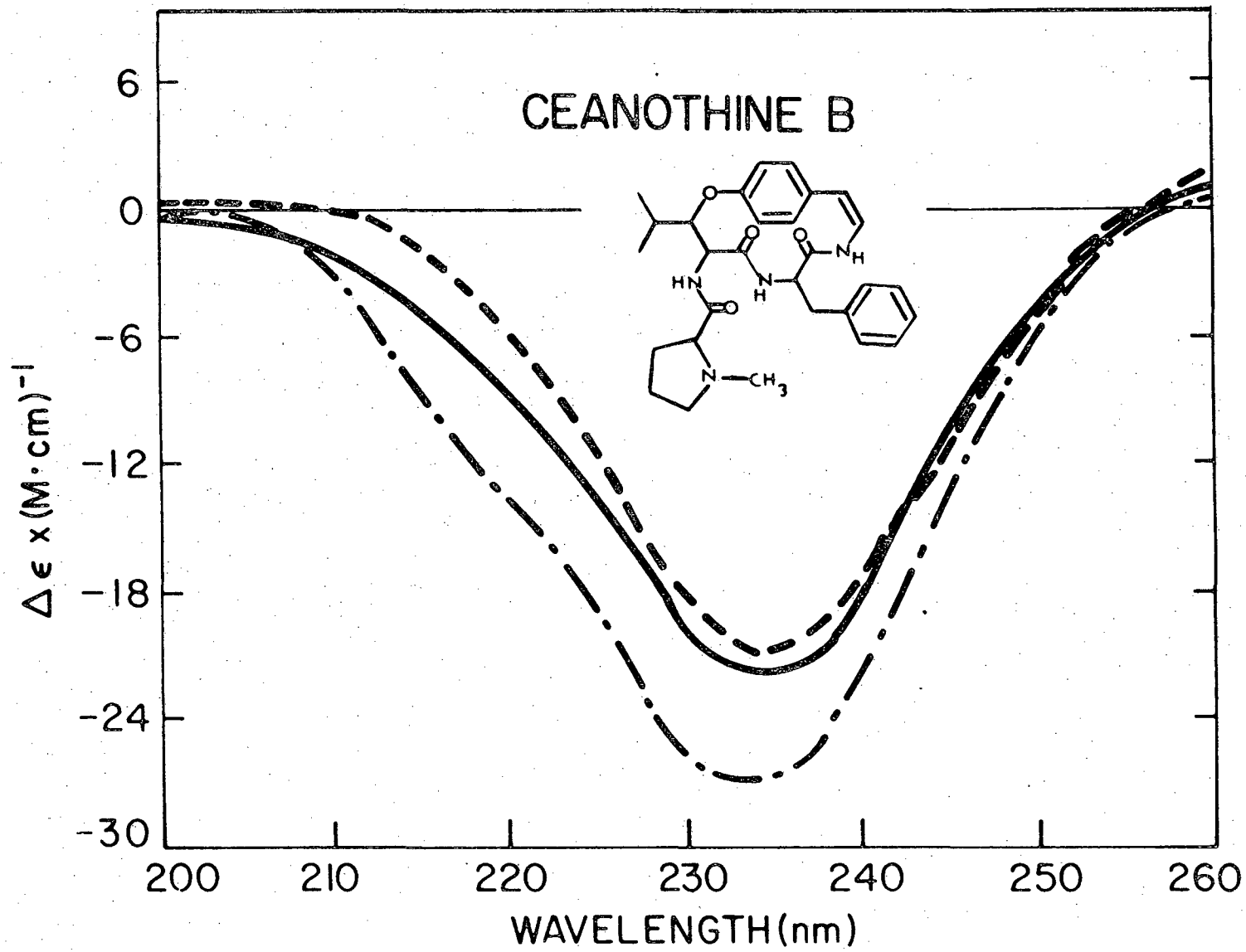


Fig. 6

XBL 799-11741

Acknowledgment

This work (Part 1A) has been published in *The Journal of the American Chemical Society* 100, 8202 (1978), authored by myself, Richard A. Houghten and Henry Rapoport. I am especially grateful for the research direction and encouragement provided by Richard A. Houghten throughout my initial studies of synthetic approaches to the phencyclopeptine nucleus. The success of this investigation is derived in a large part from these inauspicious beginnings. The assistance of Sanford Melzer, an undergraduate research student, in the large-scale preparation of p-tyramine is gratefully acknowledged.

Experimental Section

Methyl 3-(4'-Acetylphenyloxy)propanoate (7a). To a suspension of silver trifluoromethanesulfonate (65.1 g, 0.25 mol) in 750 ml of $CH_2Cl_2$ was added a solution of acetyl chloride (19.9 g, 0.25 mol) in 75 ml of $CH_2Cl_2$. After the immediate precipitation of silver chloride, a solution of methyl 3-phenyloxypropanoate (5) (45.6 g, 0.25 mol)[11a] in 75 ml of $CH_2Cl_2$ was introduced. One-half hour later the addition of the same amounts of silver salt and acetyl chloride was repeated. After 1 hour, the mixture was filtered, the filtrate was successively washed with water (3 × 300 ml), sat. $NaHCO_3$ (3 × 300 ml), and sat. NaCl (1 × 300 ml), dried and evaporated. After distillation (Kugelrohr), 53 g (93%) of 7a was obtained: GC (A) $R_t$ at 200°C, 4.3 min; NMR δ 2.55 (s,3H), 2.83 (t,2H, J = 7Hz), 3.73 (s,3H), 4.30 (t, 2H, J = 7Hz), 6.94 (d, 2H, J = 10Hz), 7.76 (d,2H, J = 10Hz). Anal. ($C_{12}H_{14}O_4$): C, H.

Methyl 3-(4'-Bromoacetylphenyloxy)propanoate (7c). Bromine (1.78 g, 11 mmol) was rapidly added to a stirred slurry of ketone 7a (2.48 g, 11 mmol) in 25 ml of $Et_2O$ at 0-5°C. The reaction mixture become homogeneous when allowed to warm to room temperature. After 1 hour, the solution was washed with distilled water (2 × 15 ml), sat. $NaHCO_3$ (15 ml) and sat. NaCl (15 ml), dried and evaporated to afford 2.65 g (80%) of the bromo ketone 7c: mp 68-70°C; GC (A) $R_t$ at 230°C, 2.9 min; NMR δ 2.76 (t, 2H, J = 7Hz), 3.67 (s,3H), 4.27 (t, 2H, J = 7Hz), 4.32 (s,2H), 6.94 (d, 2H, J = 10Hz), 7.76 (d, 2H, J = 10Hz). Anal. ($C_{12}H_{13}O_4Br$): C, H.

Methyl 3-(4'-N-Methylaminoacetylphenyloxy)propanoate (7d). A methanol solution of bromoketone 7c (1.81 g, 6 mmol, in 50 ml) was cooled

to -5°C at which time a 25% (w/w) solution of methylamine in methanol (3.60 g, 30 mmol) was introduced. After 4 hours, 1N HCl (32 ml) was added, and on evaporation and $SiO_2$ chromatography (A), 1.36 g (79%) of the hygroscopic amine hydrochloride 7d was obtained: NMR (DMSO-$d_6$) δ 2.73 (s,2H), 2.90 (t, 2H, J=7Hz), 3.23 (2,3H), 3.70 (s,3H), 4.38 (t, 2H, J = 7Hz), 4.75 (s,2H), 7.20 (d, 2H, J = 10Hz), 7.92 (d, 2H, J = 10Hz).

2-(4'-Methoxyphenyl)ethylamine (9). A solution of p-methoxy-ω-nitrostyrene (8, 50 g, 0.28 mol)[18] in 1.7 L of glacial acetic acid was added over an 8 hour period into 1.7 L of glacial acetic acid containing Pd/C (10%, 17.7 g) and conc. $H_2SO_4$ (46 g, 0.47 mol). Hydrogen was bubbled through the solution with a gas dispersion tube during the addition and for 1 hour afterwards. On subsequent isolation and distillation, the amine 9 (3.3 g, 78%) was isolated: bp 110-112°C (2 mm); NMR δ 1.10 (s,2H), 2.86 (m,4H), 3.70 (s,3H), 6.83 (dd, 4H, J = 8,18Hz).

2-(4'-Hydroxyphenyl)ethylamine Hydrobromide (Tyramine Hydrobromide) 10. A mixture of the amino methyl ether 9 (10.9 g, 72 mmol) in 300 ml 48% HBr was refluxed for 30 minutes. After the solution was cooled in an ice bath, the crystalline precipitate was collected and recrystallized from 95% ethanol to yield 10 g (64%) of 10: mp 243-245°C, NMR δ 3.20 (q, 4H, J = 6Hz), 4.80 (s,phenolic), 7.0 (dd, 4H, J = 8,18Hz).

N-Formyl-2-(4'-hydroxyphenyl)ethylamine (11). While a suspension of tyramine hydrobromide 10 (10 g, 46 mmol) and triethylamine (9.3 g, 92 mmol) in 75 ml $CHCl_3$ was maintained at 0-5°C, a solution of trichloroacetyldehyde (6.76 g, 46 mmol) in 25 ml $CHCl_3$ was added dropwise over a 1 hour period. After refluxing for one-half hour, the resultant solution

was evaporated and the residue recrystallized from water yielding 4.6 g (62%) of the N-formyl derivative 11: mp 97-99.5°C; TLC (B) $R_f$ 0.35 (ninhydrin neg.); NMR δ 2.61 (t, 2H, J = 7Hz), 3.27 (q, 2H, J = 7Hz), 6.61 (d, 2H, J = 8Hz), 6.91 (d, 2H, J = 8Hz), 7.86 (m,1H), 7.92 (s,1H), 9.0 (s,1H). Anal. ($C_9H_{11}NO_2$): C, H, N.

N-Formyl-2-(4'-methoxyphenyl)ethylamine (12a). To a solution of amine 9 (40 g, 0.27 mol) and triethylamine (29.5 g, 0.29 mol) in 250 ml $CHCl_3$ cooled to 0-5°C was added dropwise over a 1 hour period, a solution of trichloroacetaldehyde (43 g, 0.29 mol) in 250 ml $CHCl_3$. Following reflux for 45 minutes, the solution was washed with 5% aq. acetic acid (3 × 250 ml), distilled water (1 × 200 ml), sat. $NaHCO_3$ (1 × 200 ml), dried and evaporated. The residue was distilled to afford 44 g (92%) of the amine 12a: bp 159-161°C (2 mm); GC (A) $R_t$ at 175°C, 9.8 min; NMR δ 2.75 (t, 2H, J = 7Hz), 3.43 (q, 2H, J = 7Hz), 3.73 (s,3H), 6.30 (s,1H), 6.85 (d, 2H, J = 9Hz), 7.05 (d, 2H, J = 9Hz), 8.0 (s,1H). Anal. ($C_{10}H_{13}NO_2$): C, H, N.

N-Formyl-2-(4'-benzyloxyphenyl)ethylamine (12b). A mixture of 11 (4.0 g, 24 mmol), finely powdered, anhydrous $K_2CO_3$ (7.9 g, 57 mmol), and benzyl chloride (3.2 g, 25 mmol) in 100 ml acetone was refluxed for 23 hours. After filtration and evaporation the residue was partitioned between $CHCl_3$ (150 ml) and distilled water (100 ml). The organic phase was successively washed with sat. $NaHCO_3$ (2 × 75 ml), 1N HCl (2 × 75 ml), distilled water (75 ml) and sat. NaCl (75 ml). Following drying and evaporating, 4.7 g (76%) of 12b was isolated: mp 109-110°C; TLC (B) $R_f$ = 0.49; NMR δ 2.73 (t, 2H, J = 7Hz), 3.48 (q, 2H, J = 7Hz), 4.98 (s,2H),

5.70 (m,1H), 6.85 (d, 2H, J = 9Hz), 7.05 (d, 2H, J = 9Hz), 7.33 (m,5H), 8.0 (s,1H). Anal. ($C_{16}H_{17}NO_2$): C, H, N.

N-Methyl-2-(4'-methoxyphenyl)ethylamine (13a). To a rapidly stirred slurry of lithium aluminum hydride (9.10 g, 0.24 mol) in 180 ml THF kept at 0-5°C was added a solution of the N-formyl compound 12a (42.9 g, 0.24 mol) in 100 ml THF during a 50 minute period, then the mixture was refluxed for 30 minutes. After cooling the reaction mixture to 5°C, the excess hydride was destroyed by successive addition of 9 ml water, 9 ml 15% NaOH, and 20 ml water and allowed to stir for an additional 30 minutes. Filtration, evaporation and distillation afforded the N-methylamine 13a (30.5 g, 80%): bp 80-83°C (2 mm); NMR δ 1.20 (s,1H), 2.40 (s,3H), 2.77 (s,4H), 3.62 (s,3H), 6.77 (dd, 4H, J = 8,18Hz). Anal. ($C_{10}H_{15}NO$): C, H, N.

N-Methyl-2-(4'-benzyloxyphenyl)ethylamine (13b). In a manner exactly as above, the amide 12b (4.72 g, 18.5 mmol) was reduced to amine 13b (4.2 g, 94%): bp 136°C (0.1 mm); NMR δ 2.39 (s,3H), 2.74 (m,4H), 4.98 (s,2H), 6.84 (d, 2H, J = 9Hz), 7.06 (d, 2H, J = 9Hz), 7.32 (m,5H). Anal. ($C_{16}H_{19}NO$): C, H, N.

N-Methyl-N,N'-tert-butoxycarbonyl-L-prolyl-2-(4'-methoxyphenyl) ethylamine (14a). A solution of N-tert-butoxycarbonyl-L-proline[9] (24.5 g, 0.11 mol), the amine 13a (18.8 g, 0.11 mol) and DCC (14.3 g, 0.11 mol) in 1.0 L of $CHCl_3$ was stirred for 12 hours. Following removal of the urea by filtration, the solution was washed with 5% acetic acid (2 × 500 ml), distilled water (1 × 500 ml), sat. $NaHCO_3$ (2 × 500 ml), and sat. NaCl (500 ml), dried and evaporated to yield 14a as an oil (30 g, 73%): NMR δ

1.45 (s,9H), 1.85 (m,4H), 2.8 (m,2H), 3.0 (d, 3H, N-$CH_3$), 3.50 (m,4H), 3.73 (s,3H), 4.50 (m,1H), 6.82 (d, 2H, J = 8Hz), 7.05 (d, 2H, J = 8Hz).

N-Methyl-N,N'-tert-butoxycarbonyl-L-leucyl-2-(4'-benzyloxyphenyl) ethylamine (14b). The temperature of a solution of N-tert-butoxycarbonyl-L-leucine[9] (2.77 g, 12 mmol) and N-methyl morpholine (1.16 g, 12 mmol) in 58 ml THF was maintained at -15°C while isobutylchloroformate (1.57 g, 12 mmol) was rapidly added. One minute later, a solution of the N-methylamine 13b (2.77 g, 12 mmol) in 23 ml THF was dripped in during a 2 minute interval while the solution was kept below -15°C. After removal of the cooling bath, the solution was stirred for 4 additional hours, filtered and evaporated. The resulting oil was dissolved in 100 ml ethyl acetate, washed with 1N HCl (3 × 50 ml), sat. $NaHCO_3$ (3 × 50 ml) and sat. NaCl (50 ml), dried and evaporated, yielding 14b as a clear oil (4.80 g, 92%): TLC (B) $R_f$ 0.63; NMR δ 0.92 (dd, 6H, J = 6,12Hz), 1.5 (m,3H), 1.48 (s,9H), 2.77 (m,2H), 2.90 (d, 3H, N-$CH_3$), 3.55 (m,2H), 4.55 (m,1H), 4.98 (s,2H), 5.14 (m,1H), 6.82 (dd, 2H, J = 3,8Hz), 7.05 (d, 2H, J = 8Hz), 7.30 (m,5H). Anal. ($C_{27}H_{38}N_2O_4$): C, H, N.

N-Methyl-N,N'-tert-butoxycarbonyl-N'-methyl-L-leucyl-2(4'-benzyloxyphenyl)ethylamine (14c). The coupling of N-tert-butoxycarbonyl-N-methyl-L-leucine[9,19] (1.86 g, 7.6 mmol) and N-methylamine 13 (1.83 g, 7.6 mmol) was accomplished with the mixed anhydride procedure utilized for the preparation of 14a. The peptide 14c was isolated in 91% yield (3.24 g): TLC (C) $R_g$ 0.56; NMR δ 0.89 (m,6H), 1.45 (m,12H), 2.68 (d, 3H, N-$CH_3$ carbamate), 2.73 (t, 2H, J = 8Hz), 2.89 (m, 3H, N-$CH_3$), 3.43 (m,2H), 3,75 (m,1H), 4.98 (s,2H), 6.83 (d, 2H, J = 8Hz), 7.06

(d, 2H, J = 8Hz), 7.30 (m,5H). Anal. ($C_{28}H_{40}N_2O_4$): C, H, N.

N-Methyl-N,N'-tert-butoxycarbonyl-L-prolyl-2-(4'-hydroxyphenyl) ethylamine (15a). To a benzene solution (20 ml) of the peptide 14a (3.09 g, 8.5 mmol) was added boron tribromide (2.56 g, 10.2 mmol). The resultant heterogeneous mixture was refluxed for 6 hrs. After removal of the solvent, the residue was partitioned between 10% NaOH (50 ml) and $CH_2Cl_2$ (3 × 20 ml). After adjustment of the pH to 9.7, the aqueous layer was washed with $CH_2Cl_2$ (3 × 25 ml) and evaporated to a light yellow oil weighing 1.40 g (67%). That the O-methyl group was completely removed was established by NMR. This oil (1.40 g, 5.6 mmol) was dissolved in 10 ml of dioxane and 10 ml of water, and the pH was maintained at 8.6 with 1N NaOH with a autotitrator. After 2 hrs the pH was adjusted to 2.0, the reaction mixture was extracted with $CH_2Cl_2$ (3 × 25 ml), the $CH_2Cl_2$ was evaporated and the residued chromatographed (B) affording the phenol 15a (1.37 g, 70%) as an oil: TLC (B) $R_f$ 0.2, ninhydrin negative $FeCl_3/K_3Fe(CN)_6$ positive; NMR δ 1.43 (s,9H), 1.8 (m,4H), 2.75 (m,2H), 2.9 (d, 3H, $NCH_3$), 3.2-3.8 (m,4H), 4.58 (m,1H), 6.85 (m,4H), 8.60 (m,1H). Anal. ($C_{19}H_{28}N_2O_4$): C, H, N.

N-Methyl-N,N'-tert-butoxycarbonyl-L-leucyl-2-(4'-hydroxyphenyl) ethylamine (15b). After a slurry of Pd/C (700 mg, 10%) in 25 ml ethanol was treated with $H_2$ at 32 psi for 30 minutes, a solution of benzyl ether 14b (4.77 g, 11 mmol) in 70 ml ethanol was introduced and was hydrogenated at 30 psi for 3 hrs. After filtering, the solution was evaporated to 15b, an oil weighing 3.82 g (100%): NMR δ 0.91 (dd, 6H, J= 6,13Hz), 1.36-1.61 (m,3H), 1.41 (s,9H), 2.78 (m,2H), 2.90 (d, 3H, $NCH_3$), 3.50 (m,2H), 4.52

(m,1H), 5.18 (m,1H), 6.68 (d, 2H, J = 8Hz), 6.93 (d, 2H, J = 8Hz). Anal. ($C_{20}H_{32}N_2O_4$): C, H, N.

N-Methyl-N,N'-tert-butoxycarbonyl-N-methyl-L-leucyl-2-(4'-hydroxyphenyl)ethylamine (15c). In a manner exactly as above, benzyl ether 14c (3.10 g, 6.6 mmol) was converted to phenol 15c (2.5 g, 100%): TLC (C) $R_f$ 0.49, $FeCl_3/K_3Fe(CN)_6$ positive; NMR δ 0.89 (m,6H), 1.44 (s,9H), 1.43-1.45 (m,1H), 1.57 (t, 2H, J = 8Hz), 2.70 (m,5H), 2.90 (m,3H), 3.47 (m,2H), 3.76 (m,2H), 4.59 and 4.80 (m,1H), 5.00 (m,1H), 6.70 (m,2H), 6.95 (m,2H). Anal. ($C_{21}H_{34}N_2O_4$): C, H, N.

N,N'-tert-butoxycarbonyl-L-prolyl-2-(4'-hydroxyphenyl)ethylamine (15d). As a solution of N-tert-butoxycarbonyl-L-proline[9] (7.53 g, 35 mmol) and N-methylmorpholine (3.54 g, 35 mmol) in 175 ml THF was cooled to -15°C isobutylchloroformate (4.78 g, 35 mmol) was rapidly added. After 1 minute a solution of tyramine hydrobromide 10 (7.63 g, 35 mmol) and triethylamine (3.54 g, 35 mmol) in 70 ml of DMF was added over a 2 minute period while the temperature was maintained at -12°C. Four hours after the removal of the cooling bath, the reaction mixture was filtered and evaporated. The residue was dissoved in ethyl acetate (200 ml), washed with 1N HCl (3 × 100 ml), sat. $NaHCO_3$ (3 × 100 ml), and sat. NaCl (1 × 100 ml), dried and evaporated, giving 11.3 g (97%) of pure 15d: NMR δ 1.40 (s,9H), 1.70-2.20 (m,4H), 2.66 (m,2H), 3.25-3.48 (m,4H), 4.18 (m,1H), 6.72 (d, 2H, J = 8Hz), 6.91 (d, 2H, J = 8Hz), 7.86 (m,1H). Anal. ($C_{18}H_{26}N_2O_4$): C, H, N.

N,N'-tert-butoxycarbonyl-L-leucyl-2-(4'-hydroxyphenyl)ethylamine (15e). The coupling of N-tert-butoxycarbonyl-L-leucine[9] (2.31 g, 10 mmol)

and tyramine hydrobromide (10) (2.18 g, 10 mmol) was accomplished exactly as above to give pure 15e as an oil (3.2 g, 89%): NMR δ 0.87 (d, 6H, J = 6Hz), 1.43 (s,9H), 1.5 (m,3H), 2.3 (d, 2H, J = 7Hz), 2.6 (t, 2H, J = 7Hz), 4.54 (m,1H), 6.8 (dd, 4H, J = 8,18Hz). Anal. ($C_{19}N_{30}N_2O_4$): C, H, N.

Benzyl E-3-(4'-β-N,N'-tert-butoxycarbonyl-L-prolyl-N-methylamino-ethyl)phenyloxypropenoate (19a). A mixture of phenol 15a (1.18 g, 3.4 mmol), N-methylmorpholine (0.34 g, 3.4 mmol) and benzyl propiolate (1.09 g, 6.8 mmol) in 20 ml of THF was allowed to stand for 3 hrs at room temperature. After evaporation of the solvent, the residue was dissolved in 60 ml of ethyl acetate, washed with 0.2N HCl (3 × 20 ml), water (20 ml), sat. NaCl (20 ml), dried and evaporated. The resultant oil was chromatographed ($SiO_2$, 100 g, $Et_2O$) to give 1.55 g (90%) of 19a: NMR δ 1.47 (s,9H), 1.6-2.1 (m,4H), 2.63-3.1 (m,2H), 2.95 (s,3H), 3.2-3.75 (m,4H), 4.55 (m,1H), 5.18 (s,2H), 5.58 (d, 1H, J = 12Hz), 6.91 (d, 2H, J = 8Hz), 7.11 (d, 2H, J = 8Hz), 7.38 (s,5H), 7.83 (d, 1H, J = 12Hz). Anal. ($C_{29}H_{36}N_2O_6$): C, H, N.

Benzyl E-3-(4'-β-N,N'-tert-butoxycarbonyl-L-leucyl-N-methylamino-ethyl)phenyloxypropenoate (19b). In a manner analogous to above, phenol 15b (3.90 g, 11 mmol) was converted to 19b (5.3 g, 94%) after chromatography (200 g Sephadex LH-20, methanol): NMR δ 0.92 (dd, 6H, J = 6,12Hz), 1.1-1.8 (m,3H), 1.43 (s,9H), 2.80 (m,2H), 2.91 (d,3H), 3.50 (m,2H), 4.52 (m,1H), 5.12 (m,3H), 5.5 (d, 1H, J = 12Hz), 6.90 (d, 2H, J = 8Hz), 7.13 (d, 2H, J = 8Hz), 7.28 (s,5H), 7.73 (d, 1H, J = 12Hz). Anal. ($C_{30}H_{40}N_2O_6$): C, H, N.

Benzyl E-3-(4'-β-N,N'-tert-butoxycarbonyl-N'-methyl-L-leucyl-N-

methylaminoethyl)phenyloxypropenoate (19c). The acrylate 19c (2.32 g, 70%) was prepared from phenol 15c (2.34 g, 6.2 mmol) as above: TLC $R_f$ 0.27 ($Et_2O$/hexane, 1/1); NMR δ 0.88 (m,6H), 1.43 (m,9H), 1.51 (m,3H), 2.66 (s, 3H, $NCH_3$), 2.77 (t, 2H, J = 7Hz), 2.91 (d, 3H, $NCH_3$), 3.47-3.68 (m,3H), 4.98 (m,1H), 5.10 (s,2H), 5.50 (d, 1H, J = 12Hz), 6.89 (d, 2H, J = 8Hz), 7.11 (d, 2H, J = 8Hz), 7.27 (s,5H), 7.70 (d, 1H, J = 12Hz). Anal. ($C_{31}H_{42}N_2O_6$): C, H, N.

Benzyl E-3-(4'-β-N,N'tert-butoxycarbonyl-L-prolylaminoethyl)phenyloxypropenoate (19d). The conversion of phenol 15d (5.37 g, 16 mmol) to 19d (7.9 g, 99%) was accomplished as above: mp 99-101°C; TLC ($Et_2O$) $R_f$ 0.14; NMR δ 1.41 (s,9H), 1.82 (m,4H), 2.82 (m,2H), 3.30 (m,2H), 3.45 (q, 2H, J = 7Hz), 4.17 (m,1H), 5.11 (s,2H), 5.50 (d, 1H, J = 12Hz), 6.91 (d, 2H, J = 8Hz), 7.11 (d, 2H, J = 8Hz), 7.28 (m,5H), 7.73 (d, 1H, J = 12Hz); $[\alpha]_D^{25}$ -52.6° (c 0.73, $CH_3OH$). Anal. ($C_{28}H_{34}N_2O_6$): C, H, N.

Benzyl E-3-(4'-β-N,N'-tert-butoxycarbonyl-L-leucylaminoethyl)phenyloxypropenoate (19e). As above, phenol 15e (2.9 g, 8.3 mmol) was converted to 19e (3.98 g, 92%), an oil: NMR δ 0.9 (d, 6H, J = 6Hz), 1.4 (s,9H), 3.0 (t, 2H, J = 7Hz), 3.5 (q, 2H, J = 7Hz), 5.0 (m,2H), 5.6 (d, 1H, J = 12Hz), 6.89 (d, 2H, J = 8Hz), 7.11 (d, 2H, J = 8Hz), 7.3 (s,5H), 7.80 (d, 1H, J = 12Hz).

3-(4'-β-N,N'-tert-butoxycarbonyl-L-prolyl-N-methylaminoethyl)phenyloxypropanoic acid (20a). A mixture of 19a (1.51 g, 3.0 mmol) and Pd/C (10%, 100 mg) in 15 ml ethanol was hydrogenated at 37 psi for 1.5 hrs. After filtration and evaporation, 20a (1.25 g, 100%) was obtained: NMR δ 1.43 (s,9H), 1.6-2.2 (m,4H), 2.6-3.1 (m,4H), 2.75 (t, 2H, J = 7Hz), 2.95 (s,3H), 3.28-3.9 (m,4H), 4.2 (t, 2H, J = 7Hz), 4.55 (m,1H), 6.78 (d, 2H,

J = 8Hz), 7.1 (d, 2H, J = 8Hz), 9.5 (s,1H). Anal. ($C_{22}H_{32}N_2O_6$): C, H, N.

3-(4'-β-N,N'-tert-butoxycarbonyl-L-leucyl-N-methylaminoethyl)phenyloxypropanoic acid (20b). With the above procedure, hydrogenation of 19b (1.29 g, 2.5 mmol) afforded the acid 20b (1.03 g, 100%): NMR δ 0.90 (dd, 6H, J = 6,12Hz), 1.1-1.8 (m,3H), 1.41 (s,9H), 2.77 (m,2H), 2.89 (d, 3H, $NCH_3$), 3.5 (m,2H), 4.15 (t, 2H, J = 5Hz), 4.55 (m,1H), 5.48 (m,1H), 6.73 (d, 2H, J = 8Hz), 7.00 (d, 2H, J = 8Hz). Anal. ($C_{23}H_{36}N_2O_6$): C, H, N.

3-(4'-β-N,N'-tert-butoxycarbonyl-N'-methyl-L-leucyl-N-methylaminoethyl)phenyloxypropanoic acid (20c). The conversion of 19c (2.02 g, 3.8 mmol) to the acid 20c (1.65 g, 97%) was accomplished under the above conditions and: UV $\lambda_{max}$ (ε) 277 nm (1585), 283 (1331); NMR δ 0.89 (m,6H), 1.44 (s,9H), 1.53 (m,3H), 2.68 (m,3H), 2.77 (m,2H), 2.91 (m,3H), 3.43 (m,2H), 4.16 (t, 2H, J = 5Hz), 4.98 (m,1H), 6.76 (d, 2H, J = 8Hz), 7.05 (m,2H). Anal. ($C_{24}H_{38}N_2O_6$): C, H, N.

3-(4'-β-N,N'-tert-butoxycarbonyl-L-prolylaminoethyl)phenyloxypropanoic acid (20d). In a manner exactly as above, 19d (5.04 g, 10 mmol), was converted to 20d (4.13 g, 100%): NMR δ 1.43 (s,9H), 1.82 (m,4H), 2.70 (m,2H), 2.77 (t, 2H, J = 7Hz), 3.39 (m,4H), 4.16 (t, 2H, J = 7Hz), 4.22 (m,1H), 6.73 (d, 2H, J = 8Hz), 6.98 (d, 2H, J = 8Hz), 8.90 (m,1H); $[\alpha]_D^{25}$ -56.4° (c 0.87, $CH_3OH$). Anal. ($C_{21}H_{30}N_2O_6$): C, H, N.

3-(4'-β-N,N'-tert-butoxycarbonyl-L-leucylaminoethyl)phenyloxypropanoic acid (20e). The conversion of 19e (3.80 g, 7.4 mmol) to 20e (2.88 g, 92%) was accomplished as above: NMR δ 0.88 (d, 6H, J = 5Hz), 1.41 (s,9H), 1.57 (m,3H), 2.68 (t, 2H, J = 7Hz), 2.74 (t, 2H, J = 7Hz), 3.41

(q, 2H, J = 7Hz), 3.68 and 4.02 (m,1H), 4.17 (t, 2H, J = 7Hz), 5.14 (m,1H), 6.36 (m,1H), 6.75 (d, 2H, J = 8Hz), 6.98 (d, 2H, J = 8Hz). $[\alpha]_D^{25}$ -26.7° (c 1.1, $CH_3OH$). Anal. ($C_{22}H_{34}N_2O_6$): C, H, N.

p-Nitrophenyl 3-(4'-β-N,N'-tert-butoxycarbonyl-L-prolyl-N-methylaminoethyl)phenyloxypropanoate (21a). A mixture of the acid 20a (4.94 g, 12 mmol) and p-nitrophenyl trifluoroacetate[13] (2.64 g, 12 mmol) in 25 ml pyridine was stirred for 4.5 hrs. After evaporation the residue was dissolved in 200 ml of ethyl acetate and washed with 0.3N HCl (3 × 100 ml), sat. $NaHCO_3$ (2 × 100 ml), $H_2O$ (100 ml) and sat. NaCl (100 ml). Chromatography (C) of the residue after evaporation afforded the p-nitrophenyl ester 21a (4.48 g, 70%): NMR δ 1.42 (s,9H), 1.6-2.3 (m,4H), 2.6-3.2 (m,7H), 3.3-3.8 (m,4H), 4.32 (t, 2H, J = 7Hz), 4.55 (m,1H), 6.83 (d, 2H, J = 8Hz), 7.13 (d, 2H, J = 8Hz), 7.28 (d, 2H, J = 10Hz), 8.18 (d, 2H, J = 10Hz). Anal. ($C_{28}H_{35}N_3O_8$): C, H, N.

p-Nitrophenyl 3-(4'-β-N,N'-tert-butoxycarbonyl-L-leucyl-N-methylaminoethyl)phenyloxypropanoate (21b). In a manner exactly as above, 20b (954 mg, 2.2 mmol) was converted to p-nitrophenyl ester 21b (1.04 g, 82%): TLC ($Et_2O$) $R_f$ 0.32; NMR δ 0.94 (dd, 6H, J = 6,12Hz), 1.43 (s,9H), 1.62 (m,3H), 2.77 (t, 2H, J = 7Hz), 2.90 (d, 3H, N-$CH_3$), 3.06 (t, 2H, J = 7Hz), 3.55 (m,2H), 4.31 (t, 2H, J = 7Hz), 4.57 (m,1H), 5.18 (m,1H), 6.80 (d, 2H, J = 8Hz), 7.03 (d, 2H, J = 8Hz), 7.24 (d, 2H, J = 10Hz), 8.20 (d, 2H, J = 10Hz). Anal. ($C_{29}H_{39}N_3O_8$): C, H, N.

p-Nitrophenyl 3-(4'-β-N,N'-tert-butoxycarbonyl-N'-methyl-L-leucyl-N-methylaminoethyl)phenyloxypropanoate (21c). The preparation of the ester 21c from the acid 20c (813 mg, 1.8 mmol) was accomplished as in the

earlier examples. The oil was isolated pure (859 mg, 83%) after chromatography (200 g Sephadex LH-20, methanol): TLC ($Et_2O$) $R_f$ 0.42; NMR δ 0.89 (m,6H), 1.44 (s,9H), 1.52 (m,3H), 2.67 (s,3H), 2.73 (t, 2H, J = 7Hz), 2.89 (d, 3H, N-$CH_3$), 3.03 (t, 2H, J = 7Hz), 3.43 (m,1H), 3.68 (m,1H), 4.28 (t, 2H, J = 7Hz), 4.73 and 4.95 (m,1H), 6.77 (d, 2H, J = 8Hz), 7.06 (d, 2H, J = 8Hz), 7.20 (d, 2H, J=10Hz), 8.18 (d, 2H, J=10Hz), Anal. ($C_{30}H_{41}N_3O_8$): C, H, N.

p-Nitrophenyl 3-(4'-β-N,N'-tert-butoxycarbonyl-L-prolylaminoethyl) phenyloxypropanoate (21d). In an analogous manner, 20d (3.45 g, 8.5 mmol) was converted to p-nitrophenyl ester 21d (3.88 g, 87%) after chromatography (200 g Sephadex LH-20, methanol): NMR δ 1.43 (s,9H), 1.64-2.18 (m,4H), 2.7 (m,2H), 3.05 (t, 2H, J = 7Hz), 3.23-3.52 (m,4H), 4.18 (m,1H), 4.30 (t, 2H, J = 7Hz), 6.80 (d, 2H, J = 8Hz), 7.05 (d, 2H, J = 8Hz), 7.24 (d, 2H, J = 10Hz), 8.19 (d, 2H, J = 10Hz). Anal. ($C_{27}H_{33}N_3O_8$): C, H, N.

p-Nitrophenyl 3-(4'-β-N,N'-tert-butoxycarbonyl-L-leucylaminoethyl) phenyloxypropanoate (21e). As above, 20e (2.70 g, 6.4 mmol) was converted to p-nitrophenyl ester 21e (2.57 g, 74%): mp 116-118°C; TLC (benzene/ethyl acetate), $R_f$ 0.5; NMR δ 0.89 (d, 6H, J = 6Hz), 1.41 (s,9H), 1.61 (m,3H), 2.70 (t, 2H, J = 7Hz), 3.01 (t, 2H, J = 7Hz), 3.43 (m,2H), 3.93 (m,1H), 4.30 (t, 2H, J = 7Hz), 4.75 (m,1H), 6.03 (m,1H), 6.80 (d, 2H, J = 8Hz), 7.04 (d, 2H, J = 8Hz), 7.22 (d, 2H, J = 10Hz), 8.17 (d, 2H, J = 10Hz). Anal. ($C_{28}H_{37}N_3O_8$): C, H, N.

Cyclo[3-(4-β-N-methylaminoethyl)phenyloxypropanoyl-L-prolyl] (4a) and Cyclo[3-(4-β-N-methylaminoethyl)phenyloxypropanoyl-L-prolyl]$_2$ (23a). The p-nitrophenyl ester 21a (719 mg, 1.33 mmol) was dissolved in 10 ml of anhydrous trifluoroacetic acid at 0-5°C. After one hour the solvent

was evaporated to give an oil (1.20 g) which was dissolved in 600 ml of N,N'-dimethylacetamide. The resultant solution was added over a 50 hour period with a metering pump to 600 ml of mechanically stirred pyridine maintained at 90°C. The solution was stirred and heated for an additional 10 hrs, evaporated, the residue was dissolved in methanol and filtered through a mixed bed ion exchange resin. The first 100 ml of eluant was collected and evaporated to give a solid residue (223 mg, 56%) from which, after chromatography (200 g Sephadex LH-20, methanol), three fractions were isolated. Eluted first was 45 g (11%) of cyclic oligomers which was not further purified. Next was the cyclic dimer 23a (88mg, 22%): μmp 251°C (dec); UV $\lambda_{max}$ (ε), 226 nm (21400), 277 (2910), 284 (2510); GC (A) $R_t$ at 275°C, 5.6 min; MS m/e (relative intensity) 604 (0.8), 303 (3), 302 (12), 183 (31), 152 (21), 124 (67), 121 (10), 70 (100), 55 (45); $[\alpha]_D^{25}$ +27.5° (c 0.2, $CH_3OH$); CD-$\Delta E_{max}$ ($\lambda_{max}$ nm), +2.67 (228), -0.11 (268), -0.14 (275.6), -0.14 (283), +0.07 (287); $^1H$ NMR δ 1.36-2.36 (m,8H), 2.5-3.1 (m,12H), 3.0 (s,6H), 3.14-4.27 (m,10H), 6.81 (d, 4H, J = 8Hz), 7.01 (d, 4H, J = 8Hz). Anal. ($C_{34}H_{44}N_4O_6$): C (calculated 67.5, found 66.4), H, N.

Eluted last was 4a (97 mg, 24%) obtained after sublimation at 100°C (0.01 mm): μmp 188°C; UV $\lambda_{max}$ (ε) 270 nm (508), 276 (492). GC (A) $R_t$ at 275°C, 3.2 min; MS m/e $C_{17}H_{22}N_2O_3$ requires 302.1630, found 302.1636; $[\alpha]_D^{25}$ +6.3 (c 0.2, $CH_3OH$); CD-$\Delta E_{max}$ ($\lambda_{max}$ nm), +8.72 (222), -1.74 (241), -1.01 (270), -0.97 (275.5); NMR δ 1.74 (m,2H), 1.89 (t, 2H, J = 10Hz), 2.22 (dd, 1H, J = 5,12Hz), 2.57 (m,1H), 2.72 (m,2H), 2.95 (s,3H), 3.02 (m,1H), 3.42 (m,1H), 3.53 (m,1H), 4.24 (dd, 1H, J = 5,12Hz), 4.80 (t, 1H, J = 11Hz), 6.77 (dd, 1H, J = 2,8Hz), 7.09 (m,3H). Anal. ($C_{17}H_{22}N_2O_3$): C, H, N.

Cyclo[3-(4-β-N-methylaminoethyl)phenyloxypropanoyl-L-leucyl] (4b) and Cyclo[3-(4-β-N-methylaminoethyl)phenyloxypropanoyl-L-leucyl]$_2$ (23b). Dissolution of p-nitrophenyl ester 21b (665 mg, 1.2 mmol) in 10 ml anhydrous trifluoroacetic acid at 0-5°C as above, afforded an oil (1.03 g) after evaporation which was dissolved in dimethylacetamide (620 ml) and added dropwise over a 50 hour period to stirred pyridine (600 ml) at 90°C. Stirring was continued for an additional 10 hrs. The pyridine was evaporated, the residue was dissolved in methanol and filtered through a mixed bed ion exchange resin to give an oil (332 mg). The crude product was purified by column chromatography on Sephadex LH-20 in methanol, isolating four fractions:

(1) 95 mg (25%) of cyclic oligomers;

(2) dimer 23b (123 mg, 32%), crystallized from ethanol: μmp 234°C; UV $\lambda_{max}$ (ε) 224 nm (25245), 276 (3234), 283 (2691); MS m/e (relative intensity) 636 (6), 386 (2), 362 (3), 318 (8), 43 (100); CD-$\Delta\varepsilon_{max}$ ($\lambda_{max}$ nm): -8.70 (218), -3.77 (234), -0.48 (278), -0.45 (283); NMR δ 0.83 (m,6H), 0.93 (q, 6H, J = 5Hz), 1.30 (m,4H), 1.47 (m,2H), 2.55 (dq, 4H, J = 7Hz), 2.78 (m,4H), 2.97 (d, 4H, J = 4Hz), 3.00 (m,6H), 4.09 (m,4H), 4.25 (m,2H), 6.20 (m,2H), 6.80 (m,4H), 7.02 (m,4H). Anal. ($C_{36}H_{52}N_4O_6$): C, H, N.

(3) was a mixture of compounds (36 mg, 10%) not further characterized;

(4) cyclic monomer 4b (49 mg, 13%): μmp 119°C after sublimation at 100°C (0.01 mm); UV $\lambda_{max}$ (ε) 226 nm (shoulder, 6052), 275 (690); MS m/e (relative intens.) 319 (4), 318 (17), 276 (6), 275 (36), 44 (100); GC (B) $R_t$ at 230°, 8.6 min; CD-$\Delta\varepsilon_{max}$ ($\lambda_{max}$ nm): +9.84 (230), +0.23 (275), +0.46 (284); NMR δ 0.84 (dd, 4H, J = 4,8Hz), 0.92 (d, 2H, J = 6.5Hz), 1.16 (m,1H), 1.34 (m,2H), 2.25 (dd, 1H, J = 3,8Hz), 2.40 (dd, 0.5H, J = 5,15Hz),

2.63 (m,0.5H), 2.80 (m,2.5H), 2.94 (s,1.5H), 3.04 (s,1.5H), 3.40 (m,0.5H), 3.61 (m,0.5H), 3.95 (q, 0.5H, J = 6.5Hz), 4.21 (dd, 1H, J = 4,11Hz), 4.53 (td, 0.5H, J = 5,9Hz), 4.71 (td, 0.5H, J = 5.6,12Hz), 4.92 (m,1H), 5.62 (m,1H), 6.68 (dd, 0.5H, J = 2.3,8Hz), 6.89 (m,2.5H), 7.17 (m,1H). Anal. ($C_{18}H_{26}N_2O_3$): C, H, N.

Cyclo[3-(4-β-N-methylaminoethyl)phenyloxypropanoyl-N-methyl-L-leucyl] (4c). The conversion of p-nitrophenyl ester 21c (665 mg, 1.2 mmol) to the cyclopeptides was accomplished as described above. After ion exchange, a colorless oil (21 mg) was isolated. Sephadex chromatography (200 g LH-20, $CH_3OH$) afforded two fractions:

(1) 12 mg (3.5%) which was not further characterized;

(2) 8 mg (2.2%) contained three major components by GC (B) $R_t$ at 230°C: 18 min (20%), 21 min (14%), 32 min (56.4%). These products were isolated by preparative GC (3% OV-17, 6' × ¼"). The 18 min component was the desired cyclic peptide 4c (1.6 mg, 0.4%): MS m/e $C_{19}H_{28}N_2O_3$ requires 332.2100, found 332.2091. The other components were not further characterized.

Cyclo[3-(4-β-aminoethyl)phenyloxypropanoyl-L-prolyl] (4d) and Cyclo[3-(4-β-aminoethyl)phenyloxypropanoyl-L-prolyl]$_2$ (23d). The conversion of p-nitrophenyl ester 21d (591 mg, 1.1 mmol) to the cyclopeptides was accomplished exactly as previously described. After ion exchange a light yellow oil (244 mg) was isolated. Sephadex chromatography (200 g LH-20, MeOH) gave three fractions. Fraction 1 was 54 mg (17%) cyclic oligomers. Fraction 2 was cyclic dimer 23d (110 mg, 34%): μmp 221° on crystallization from ethanol; UV $\lambda_{max}$ (ε) 224 nm (25180), 276.5 (3393),

283.5 (2855). MS m/e 576 (0.8), 374 (2), 368 (2), 124 (100), 70 (100); CD-$\Delta\varepsilon_{max}$ ($\lambda_{max}$ nm): -6.9 (224), -0.29 (282), -0.45 (274.5); NMR $\delta$ 1.73 (m,2H), 2.05 (m,4H), 2.52 (m,4H), 2.68 (m,4H), 2.86 (m,2H), 3.32 (m,6H), 3.73 (m,4H), 3.97 (m,2H), 4.58 (d, 2H, J = 7.5Hz), 6.81 (d, 4H, J = 8Hz), 7.01 (d, 4H, J = 8Hz), 7.13 (m,2H). Anal. ($C_{32}H_{40}N_4O_6$): C, H, N. Fraction 3 was cyclic monomer 4d (75 mg, 24%), an oil; UV $\lambda_{max}$ ($\varepsilon$) 223 nm (6198 shoulder), 271 (568), 276 (513); GC (B) $R_t$ at 230°C, 12 min; MS m/e 289 (4), 288 (19), 231 (13), 70 (100); CD-$\Delta\varepsilon_{max}$ ($\lambda_{max}$ nm) -12.42 (232), -2.17 (271), -1.91 (277); NMR $\delta$ 1.55 (m,1H), 1.95 (m,1H), 2.12 (m,2H), 2.19 (dd, 1H, J = 6,13Hz), 2.34 (m,1H), 2.75 (dd, 1H, J = 10,17Hz), 2.89 (m,2H), 3.31 (dd, 1H, J = 10,17Hz), 3.49 (t, 1H, J = 8Hz), 3.80 (m,1H), 4.28 (m,2H), 4.62 (t, 1H, J = 10Hz), 6.36 (m,1H), 6.85 (s,2H), 7.17 (dd, 2H, J = 8,15Hz). Anal. ($C_{16}H_{20}N_2O_3$): C, H, N.

Cyclo[3-(4-β-aminoethyl)phenyloxypropanoyl-L-leucyl] (4e) and Cyclo[3-(4-β-aminoethyl)phenoxypropanoyl-L-leucyl]$_2$ (23e). With the same cyclization procedure, p-nitrophenyl ester 21e (588 mg, 1.1 mmol) was converted to the cyclopeptides. The resulting brown solid was triturated in methanol and filtered. The insoluble portion (40.2 g, 12%) was later identified as cyclic dimer 22e. The methanol filtrate was eluted through a mixed bed ion exchange resin and evaporated to give a solid residue (137 mg). Following Sephadex chromatography (200 g, LH-20, $CH_3OH$), three fractions were isolated. Fraction 1 was 48 mg (15%) of cyclic oligomers, not further characterized. Fraction 2 was cyclic dimer 22e (51 mg, 15%): crystallized from ethanol, µmp 287°C; UV $\lambda_{max}$ ($\varepsilon$) 224.5 nm (19511), 276 (2680), 283 (2267). MS m/e 609 (1), 608 (3), 306 (2), 305 (14), 304 (69), 86 (100); CD-$\Delta\varepsilon_{max}$ ($\lambda_{max}$ nm): -5.65 (229), +0.69 (277), +0.74 (284);

NMR δ 0.93 (m,12H), 1.66 (m,6H), 2.65 (m,8H), 3.26 (m, 2H, J = 7Hz), 3.45 (m, 2H, J = 7Hz), 4.13 (m,2H), 4.23 (m,2H), 4.63 (m,2H), 6.71 (d, 2H, J = 8Hz), 6.97 (d, 2H, J = 8Hz). Anal. ($C_{34}H_{48}N_4O_6$): C, H, N. Fraction 3 was the cyclopeptide 4e (31 mg, 9%): μmp 199°C; UV $\lambda_{max}$ (ε) 226 nm (6127), 276 (734). GC (B) $R_t$ at 230°C, 9.5 min: MS m/e 305 (6), 304 (29), 86 (100), CD-$\Delta\varepsilon_{max}$ ($\lambda_{max}$ nm): +8.12 (226), +0.39 (275), +0.50 (284); NMR δ 0.84 (d, 6H, J = 6Hz), 1.33 (m,3H), 2.31 (m,2H), 2.52 (m,1H), 3.06 (m,2H), 4.00 (dd, 1H, J = 7,14Hz), 4.21 (dd, 2H, J = 6,13Hz), 4.95 (t, 1H, J = 10.5Hz), 5.22 (d, 1H, J = 11Hz), 5.53 (d, 1H, J = 9Hz), 6.87 (dd, 1H, J = 2.4,7Hz), 6.94 (dd, 1H, J = 2.4,7Hz), 7.03 (dd, 1H, J = 2.4,7Hz), 7.09 (dd, 1H, J = 2.4,7Hz). Anal. ($C_{17}H_{24}N_2O_3$): C, H, N.

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16. The recently observed effect of cyclopeptide alkaloids on ion selective mitochondrial swelling may be pertinent in this regard [K.Kawai, Y.Nozawa and Y.Ogihara, *Experientia* 33, 1454 (1977)].

17. All reactions were performed under a nitrogen atmosphere. Solutions were dried over $Na_2SO_4$ and evaporations were done in vacuo with a Berkeley rotary evaporator. Uncorrected melting points were determined on a Thomas-Hoover Capillary MP Apparatus and Kofler Micro Hot Stage (μmp). Both $^1$H-NMR and $^{13}$C-NMR spectra were taken in $CDCl_3$ solution using internal $Me_4Si$ (δ0) on a Varian HR-220 and a TT-23 (with a Brucker WH-90 console equipped with an NIC-80 computer and a Varian 25.14 MHz magnet) respectively. UV spectra were taken in methanol on a Cary 118 instrument. A model AEI-MS12 mass spectrometer with INCOS data system was used for determining mass spectra. The gas chromatography was done on: (a) an F & M Model 402 High Efficiency GC with a 5 ft by 1/8 inch glass column, 3% OV-17 (w/w) on Aeropak 30 (100-120 mesh), and (b) a Hewlett Packard Model 5730A GC with a 3 ft by 1/8 inch glass column and the same liquid phase and solid support. TLC was done on silica (Eastman sheets #6060) and column chromatography used silica gel 60 (EM Reagents) with solvent systems: (a) $CH_3OH$/benzene/acetone, 1/1/1; (b) benzene/acetone, 4/1; and (c) benzene/$Et_2O$, 1/1. Optical rotations were determined on a Bendix Ericsson ETL-NPL Automatic Polarimeter Type 43A. CD spectra were taken in

acetonitrile on a homemade spectrometer [see J.C.Sutherland, L.E.Vickery and M.P.Klein, *Rev. Sci. Instr.* 45, 1089 (1974)]. Ion exchange chromatography was done with a mixed bead resin, BioRex A6501-X8-D, 20-50 mesh, on a column 1.5 × 50 cm. Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley.

The following solvents were routinely distilled prior to use: tetrahydrofuran from sodium benzophenone ketyl, pyridine (predried over NaOH pellets) from BaO, and N,N'-dimethylacetamide from 4A molecular sieves. Spectral grade acetonitrile and analytical reagent grade salts were employed for the ion studies.

18. The p-methoxynitrostyrene was prepared analogously to the process of D.E.Worrall, *Org. Syn.*, Coll. Vol. I, 405 (1932).

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Appendix to Part I-A. Elemental analysis.

| Compound | Molecular formula | Calculated C | Calculated H | Calculated N | Found C | Found H | Found N |
|---|---|---|---|---|---|---|---|
| 4a | $C_{17}H_{22}N_2O_3$ | 67.5 | 7.3 | 9.3 | 67.7 | 7.4 | 9.1 |
| 4b | $C_{18}H_{26}N_2O_3$ | 67.9 | 8.2 | 8.8 | 67.9 | 8.2 | 8.7 |
| 4d | $C_{16}H_{20}N_2O_3$ | 66.6 | 7.0 | 9.7 | 66.5 | 7.0 | 9.7 |
| 4e | $C_{17}H_{24}N_2O_3$ | 67.1 | 8.0 | 9.2 | 66.9 | 8.0 | 9.1 |
| 7a | $C_{12}H_{14}O_4$ | 64.8 | 6.3 | - | 64.7 | 6.1 | - |
| 7c | $C_{12}H_{13}O_4Br$ | 47.9 | 4.4 | - | 48.0 | 4.4 | - |
| 11 | $C_9H_{11}NO_2$ | 65.4 | 6.7 | 8.5 | 65.2 | 6.7 | 8.4 |
| 12a | $C_{10}H_{13}NO_2$ | 67.0 | 7.3 | 7.8 | 67.2 | 7.1 | 7.9 |
| 12b | $C_{16}H_{17}NO_2$ | 75.3 | 6.7 | 5.5 | 75.2 | 6.7 | 5.5 |
| 13a | $C_{10}H_{15}NO$ | 72.7 | 9.1 | 8.5 | 72.9 | 9.0 | 8.7 |
| 13b | $C_{16}H_{19}NO$ | 79.6 | 7.9 | 5.8 | 79.6 | 7.9 | 5.9 |
| 14b | $C_{27}H_{38}N_2O_4$ | 71.3 | 8.4 | 6.2 | 71.5 | 8.3 | 5.9 |
| 14c | $C_{28}H_{40}N_2O_4$ | 71.8 | 8.6 | 6.0 | 72.0 | 8.6 | 5.7 |
| 15a | $C_{19}H_{28}N_2O_4$ | 65.5 | 8.1 | 8.0 | 65.3 | 8.0 | 8.0 |
| 15b | $C_{20}H_{32}N_2O_4$ | 65.9 | 8.8 | 7.7 | 65.8 | 8.7 | 7.5 |
| 15c | $C_{21}H_{34}N_2O_4$ | 66.6 | 9.0 | 7.4 | | | |
| 15d | $C_{18}H_{26}N_2O_4$ | 64.6 | 7.8 | 8.4 | 64.5 | 7.8 | 8.2 |
| 15e | $C_{19}H_{30}N_2O_4$ | 65.1 | 8.6 | 8.0 | 64.8 | 8.4 | 7.9 |
| 19a | $C_{29}H_{36}N_2O_6$ | 68.5 | 7.1 | 5.5 | 68.3 | 7.1 | 5.6 |
| 19b | $C_{30}H_{40}N_2O_6$ | 68.7 | 7.7 | 5.3 | 68.6 | 7.7 | 5.3 |
| 19c | $C_{31}H_{42}N_2O_6$ | 69.1 | 7.9 | 5.2 | 68.9 | 7.9 | 5.1 |
| 19d | $C_{28}H_{34}N_2O_6$ | 68.0 | 6.9 | 5.7 | 67.9 | 6.9 | 5.7 |
| 20a | $C_{22}H_{32}N_2O_6$ | 62.8 | 7.7 | 6.7 | 62.7 | 7.6 | 6.7 |

continued . . .

Elemental analysis (continued)

| Compound | Molecular formula | Calculated C | Calculated H | Calculated N | Found C | Found H | Found N |
|---|---|---|---|---|---|---|---|
| 20b | $C_{23}H_{36}N_2O_6$ | 63.3 | 8.3 | 6.4 | 63.3 | 8.1 | 6.4 |
| 20c | $C_{24}H_{38}N_2O_6$ | 64.0 | 8.5 | 6.2 | 63.9 | 8.6 | 6.0 |
| 20d | $C_{21}H_{30}N_2O_6$ | 62.0 | 7.4 | 6.9 | 61.9 | 7.4 | 6.8 |
| 20e | $C_{22}H_{34}N_2O_6$ | 62.5 | 8.1 | 6.6 | 62.4 | 8.1 | 6.5 |
| 21a | $C_{28}H_{35}N_3O_8$ | 62.1 | 6.5 | 7.8 | 62.1 | 6.5 | 7.8 |
| 21b | $C_{29}H_{39}N_3O_8$ | 62.5 | 7.0 | 7.5 | 62.2 | 7.0 | 7.8 |
| 21c | $C_{30}H_{41}N_3O_8$ | 63.0 | 7.2 | 7.3 | 62.7 | 7.2 | 7.2 |
| 21d | $C_{27}H_{33}N_3O_8$ | 61.5 | 6.3 | 8.0 | 61.6 | 6.4 | 7.9 |
| 21e | $C_{28}H_{37}N_3O_8$ | 61.9 | 6.9 | 7.7 | 61.8 | 6.9 | 7.6 |
| 23a | $C_{34}H_{44}N_4O_6$ | 67.5 | 7.3 | 9.3 | 66.4 | 7.4 | 9.2 |
| 23b | $C_{36}H_{52}N_4O_6$ | 67.9 | 8.2 | 8.8 | 67.7 | 8.0 | 8.7 |
| 23d | $C_{32}H_{40}N_4O_6$ | 66.6 | 7.0 | 9.7 | 66.6 | 7.0 | 9.7 |
| 23e | $C_{34}H_{48}N_4O_6$ | 67.1 | 7.9 | 9.2 | 66.8 | 7.8 | 9.2 |

# Part I-B.

## CYCLOPEPTIDE ALKALOIDS. PHENCYCLOPEPTINES FROM THE POLYMORPHIC SPECIES *CEANOTHUS INTEGERRIMUS*

### Introduction

*Ceanothus integerrimus* ("Deer Brush") is a polymorphic species of the family Rhamnaceae occurring from southern Washington through California into western New Mexico. Although as many as eight varieties of this semi-deciduous shrub have been characterized, only two of the seven found in California are present in significant population. *C. integerrimus* H. and A. var. *integerrimus* inhabits the inner South Coast Range and *C. integerrimus* var. *californicus* (Kell.) G. T. Benson is found in the Sierra Nevada northward through the Cascade and Klamath Ranges.[1]

As part of more comprehensive alkaloid structure studies of Pacific North American Rhamnaceae, we have begun a phytochemical investigation of *Ceanothus integerrimus*. Our investigation of three specimens of this shrub, one of *C. integerrimus* var. *californicus*, and two from different populations of *C. integerrimus* var. *integerrimus* has led to the identification of four new cyclopeptide alkaloids, phencyclopeptines 1-4,[2] in addition to the three previously reported alkaloids 5-7 (Table 2). Employing reversed phase high performance liquid chromatography (HPLC) and mass and $^{1}$H NMR spectroscopy, the distribution of phencyclopeptines among the three plants was determined (Figs. 7, 8 and 9; Tables 2 and 3).

### Results and Discussion

The identification of the HPLC-purified constituents of *C. integerrimus* is based mainly on their characteristic electron impact mass spectra.

According to the fragmentation schemes previously proposed (Fig. 8),[3,4] the mass spectra of the seven alkaloids from the three plants (Table 3) confirm the structural assignments made in Table 2. In addition, the total alkaloid acid hydrolytic products from each plant (Table 4) are consistent with the distribution of phencyclopeptines 1-7 among the three plants shown in Fig. 9. Since tryptophan is destroyed by acid hydrolysis and N-alkylated amino acids are not detected by the usual automatic amino acid analysis due to their low color yield, the failure to detect any other amino acids in the acidic hydrolysate of *C. integerrimus* var. *integerrimus* (Mendocino County) corroborates the observation of only indolic phencyclopeptine components in this plant.

Leucine, isoleucine, and valine and their methylated derivatives were distinguished from one another by mass spectroscopy and $^1$H NMR spectroscopy, and amino acid analysis in some cases. Amino acid analysis of the acid hydrolysates of each HPLC-purified phencyclopeptine confirmed the identity of the ring amino acid ($R_5$) suggested by mass spectroscopy. Fragments produced from the rearrangement of the base peak (BP, a) in the mass spectrum of the phencyclopeptine provided diagnostic evidence for the structure of the N-aklylated amino acid residue, $R_8$ (Table 3).

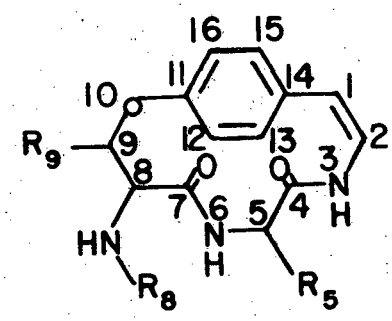
$^1$H NMR spectroscopy furnished the most definitive information regarding the nature of the N-terminal amino acid moiety ($R_8$), since the two methyl groups of isoleucine manifest different multiplicity in their NMR signals, the γ-methyl being a doublet and the δ-methyl a triplet. Both the δ-methyls of leucine and the γ-methyls of valine are two sets of doublets. Furthermore, the chemical shifts of the methyl groups on the N-terminal amino acid ($R_8$) are also diagnostic. In the cases of

phencyclopeptines where $R_9$ is phenyl, a pronounced upfield shift (as much as 0.6 ppm) has been observed in the N-methyl and γ-methyl resonances of the N-terminal amino acids. Such high field resonances do not occur in the spectra of alkaloids which have N-terminal leucine residues since there are no γ-methyl groups. Thus the chemical shift of the leucine δ-methyls in crenatine A 9 occur within the expected range,[5] two doublets at δ 0.78 and 0.83 ppm in $CDCl_3$, whereas the doublet occurring at 0.24 ppm in the spectrum of integerrine 6 is attributable to the γ-methyl of the N-terminal isoleucine residue.

Our observation of unusually high field doublets in the spectra of phencyclopeptines 1, 3, 4, and 7 as well, establishes that the N-terminal amino acids are either derivatives of valine or isoleucine. Such high field resonances were not observed in the $^1H$ NMR spectrum of discarine B 5 and phencyclopeptine 2, in agreement with the literature.[6,7]

It is unusual that only one of the phencyclopeptines, discarine B 5, found in *C. integerrimus* var. *integerrimus* from Santa Cruz County was observed in the extract of the plant of the same species from Mendocino County (Fig. 9). In contrast, the total alkaloidal mixture from var. *integerrimus* of Santa Cruz County and that from var. *californius* contained four common phencyclopeptines, 3, 4, 5 and 7.

TABLE 2. Phenycyclopeptine constituents of *Ceanothus integerrimus*.




| | $R_9$ | $R_5$ | $R_8$ | MW |
|---|---|---|---|---|
| 1, 5-β-Indolylmethyl-8-N-methylvalyl-9-phenylphencyclopeptine | $C_6H_5$ | β-indolyl-$CH_2$ | NMeVal | 579 |
| 2, 5-β-Indolylmethyl-8-N,N-dimethylvalyl-9-isopropylphencyclopeptine | $(CH_3)_2CH$ | β-indolyl-$CH_2$ | $NMe_2Val$ | 559 |
| 3, 5-Benzyl-8-N,N-dimethylisoleucyl-9-phenylphencyclopeptine | $C_6H_5$ | $C_6H_5CH_2$ | $NMe_2Ile$ | 568 |
| 4, 5-Isobutyl-8-N-methylisoleucyl-9-phenylphencyclopeptine | $C_6H_5$ | $(CH_3)_2CHCH_2$ | NMeIle | 520 |
| 5, 5-β-Indolylmethyl-8-N,N-dimethylisoleucyl-9-isopropylphencyclopeptine (Discarine B)[a] | $(CH_3)_2CH$ | β-indolyl-$CH_2$ | $NMe_2Ile$ | 573 |
| 6, 5-β-Indolylmethyl-8-N,N-dimethylvalyl-9-phenylphencyclopeptine (Integerrine)[b] | $C_6H_5$ | β-indolyl-$CH_2$ | $NMe_2Val$ | 593 |
| 7, 5-Isobutyl-8-N,N-dimethylisoleucyl-9-phenylphencyclopeptine (Integerrenine)[c] | $C_6H_5$ | $(CH_3)_2CHCH_2$ | $NMe_2Ile$ | 534 |

continued . . .

TABLE 2 (continued)

| | $R_9$ | $R_5$ | $R_8$ | MW |
|---|---|---|---|---|
| 8, 5-Benzyl-8-N-methylvalyl-9-phenylphencyclopeptine (Integerressine)[d] | $C_6H_5$ | $C_6H_5CH_2$ | $NMe_2Val$ | 554 |

[a]First identified in *Discaria longespina* H. and A.[6]

[b]First identified in *C. integerrimus* H. and A.[8]

[c]First identified in *C. integerrimus* H. and A.[9]

[d]Identified constituent of *C. integerrimus* H. and A;[7] not observed in any of the three plants in the present investigation.

TABLE 3. Mass spectra of the HPLC purified phencyclopeptine components of *C. integerrimus*.

| | Compound | | | | | | |
|---|---|---|---|---|---|---|---|
| Fragment[a] | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| $M^+$ | 579[b] | 559[b] | 568 | 520[b] | 573[b] | 593[b] | 534[b] |
| BP a | 86[b] | 100[b] | 114[b] | 100[b] | 114[b] | 100[b] | 114[b] |
| b | 536[b] | 516 | 511[b] | 463[b] | 516 | 550 | 477[b] |
| c | 215 | 195 | 229 | 215 | 195 | 229 | 229 |
| d | 187 | 167 | 201 | 187 | 167 | 201 | 201 |
| e | 410[c] | 376[c] | 371 | 337 | 376 | - | 337 |
| f | 224[c] | 190 | 224 | 224 | 190 | 224 | 224 |
| g | 494 | - | - | 421 | - | - | - |
| h | 347[c] | 347[c] | 308 | 274 | 347 | 347[c] | - |
| i | 135 | 135 | 135 | 135 | 135 | 135 | 135 |
| j | 451 | 417[c] | 412[c] | 378 | - | - | - |
| k | 317[c] | 283 | 278[c] | 244 | 283 | 317 | 244 |
| l | 289 | 255[c] | 250[c] | 216 | 255 | - | 216 |
| m | 131 | 97[c] | 131 | 131 | 97 | 131 | 131 |
| n | 170[c] | 170 | 131 | 97 | 170 | 170 | 97 |
| o | 159 | 159 | 120 | 86 | 159 | 159 | 86 |
| p | 130 | 130 | 91 | 57 | 130 | 130 | 57 |
| other | 117 | 117 | 98 | 505[d]<br>491[e]<br>477[f] | 117<br>85[g] | 117<br>85[g] | 519[d]<br>505[e]<br>491[f] |

[a]Fragment ions refer to structures in Fig. 8.

[b]High resolution mass spectral data obtained.

[c]Weak ion intensity in some spectra.

[d]$M^+ - 15$

[e]$M^+ - 29$

[f]$M^+ - 43$

[g]Fragments from rearrangement of BP, diagnostic of N-alkylated amino acid N-terminal moiety: m/e 58, MeLeu; 72, $Me_2$Leu; 85, $Me_2$Val and $Me_2$Ile. Taken from Ref. 5.

TABLE 4. Amino acid identification and yields from acid hydrolysis of *C. integerrimus* root bark total alkaloid mixtures.[a]

| Product | *C. integerrimus* var. *integerrimus* Santa Cruz Co. | *C. integerrimus* var. *integerrimus* Mendocino Co. | *C. integerrimus* var. *californicus* |
|---|---|---|---|
| $NH_3$ | 769 | 516 | 590 |
| Ile | - | - | 6 |
| Leu | 352 | - | 190 |
| Phe | 29 | - | 26 |

[a]Crude alkaloid mixtures (250 mg) were hydrolyzed with 1-2 ml 6N HCl containing one drop glacial acetic acid for solubilization in sealed ampules for 24 hrs at 135°C. Yields are reported in nanomoles/250 mg mixture hydrolyzed.

Figure Captions - Part I-B

Fig. 7. HPLC of crude alkaloidal extracts of the polymorphic species *C. integerrimus*. HPLC system employed: LiChrosorb C2 (10 μ, 10 × 150 mm); mobile phase $CH_3CN$/10% aqueous $NH_3$ (9/1, v/v); flow rate, 2 mℓ/min; 35°C, A 254 nm; injection volume, 100 μℓ; c ~ 3 mg/mℓ.

Fig. 8. Electron impact mass spectral fragmentation of phycyclopeptines.

Fig. 9. Phencyclopeptine distribution in *C. integerrimus* var. *integerrimus* H. and A. from Santa Cruz County, *C. integerrimus* var. *integerrimus* H. and A. from Mendocino County, and *C. integerrimus* var. *californicus* (Kell) and G. T. Benson.

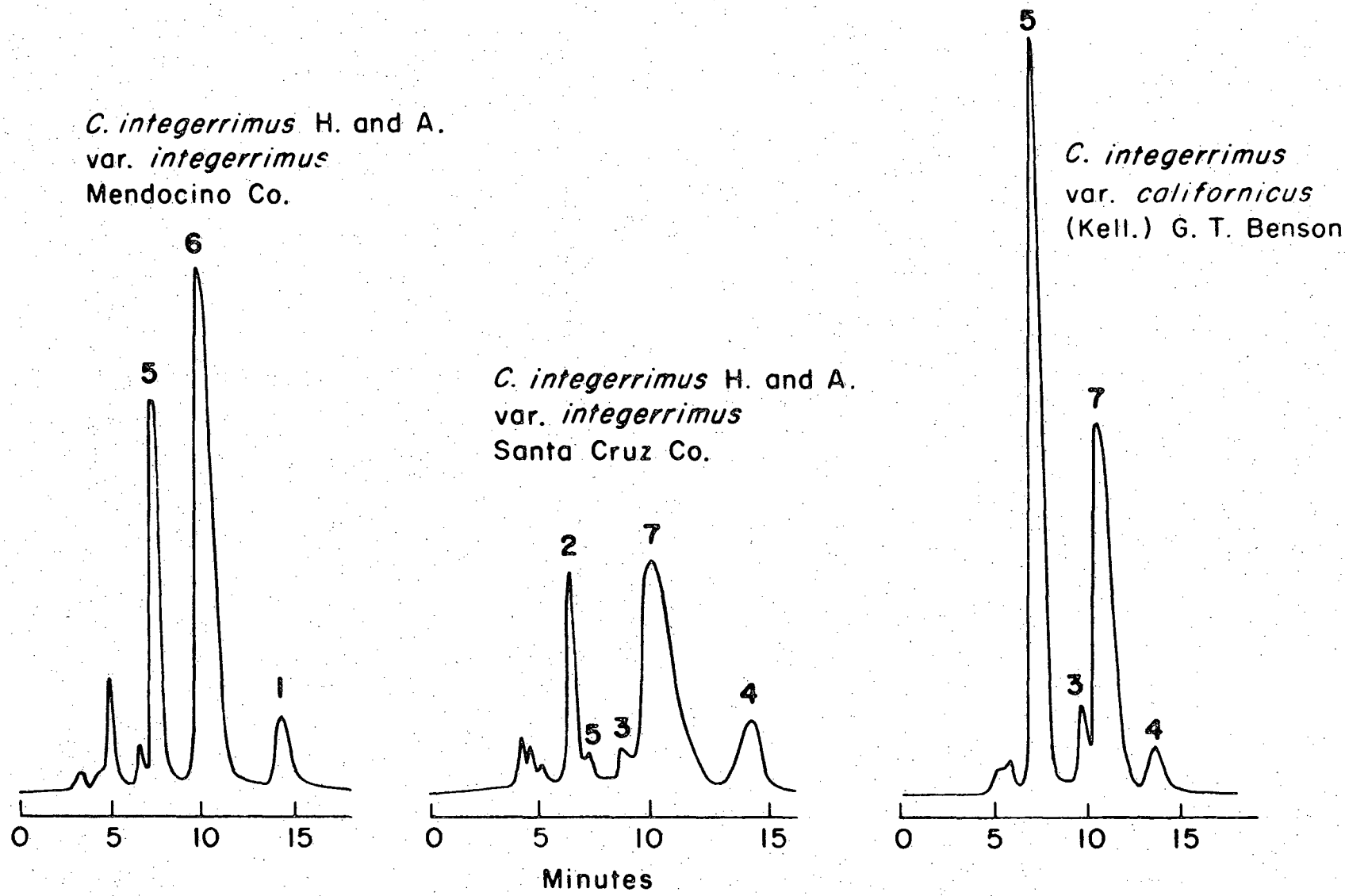
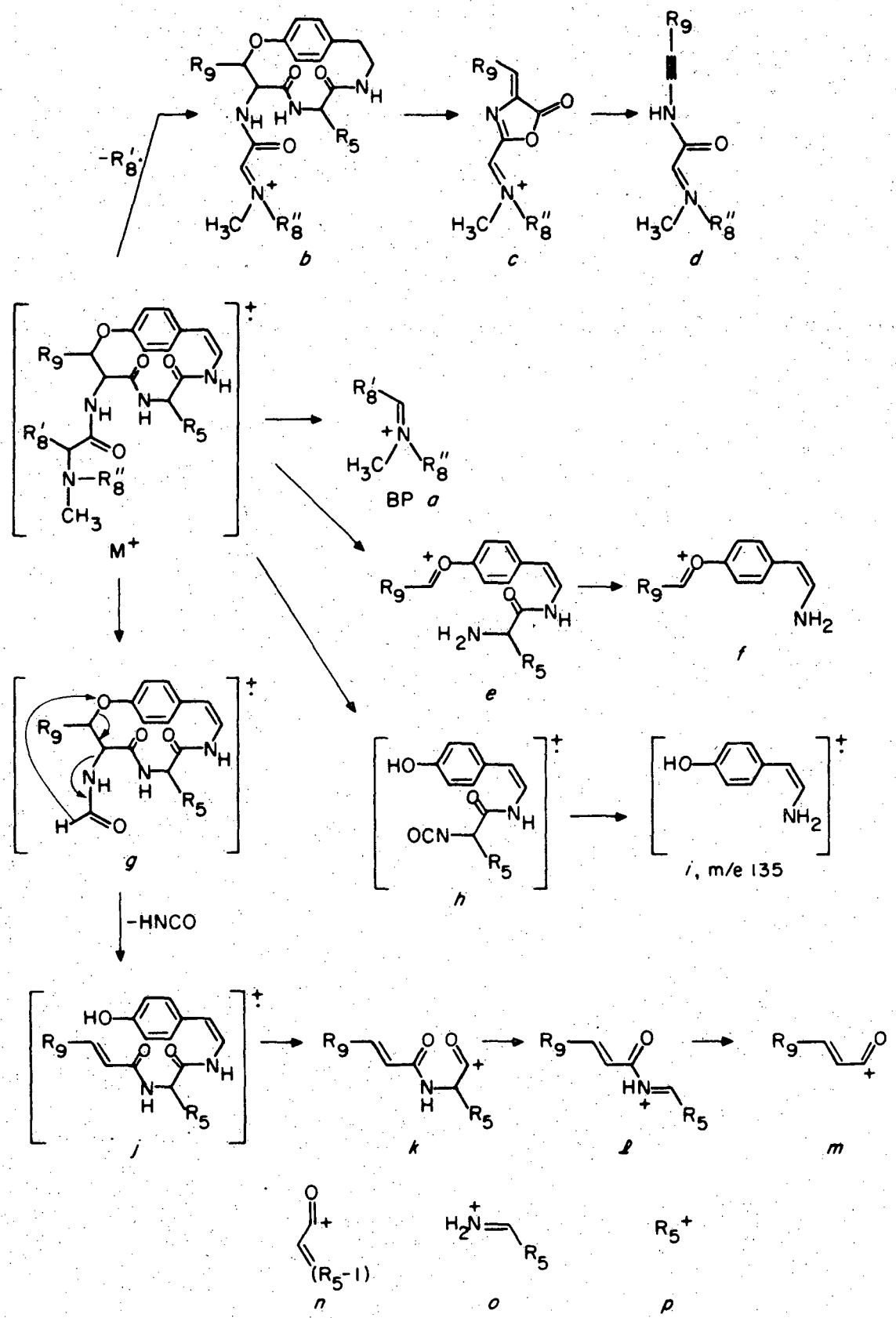

C. integerrimus H. and A.
var. integerrimus
Mendocino Co.
6
5
1
0
5
10
15
C. integerrimus H. and A.
var. integerrimus
Santa Cruz Co.
2
7
5
3
4
0
5
10
15
Minutes
5
C. integerrimus
var. californicus
(Kell.) G. T. Benson
7
3
4
0
5
10
15

Fig. 7




XBL 799-11737

Fig. 8

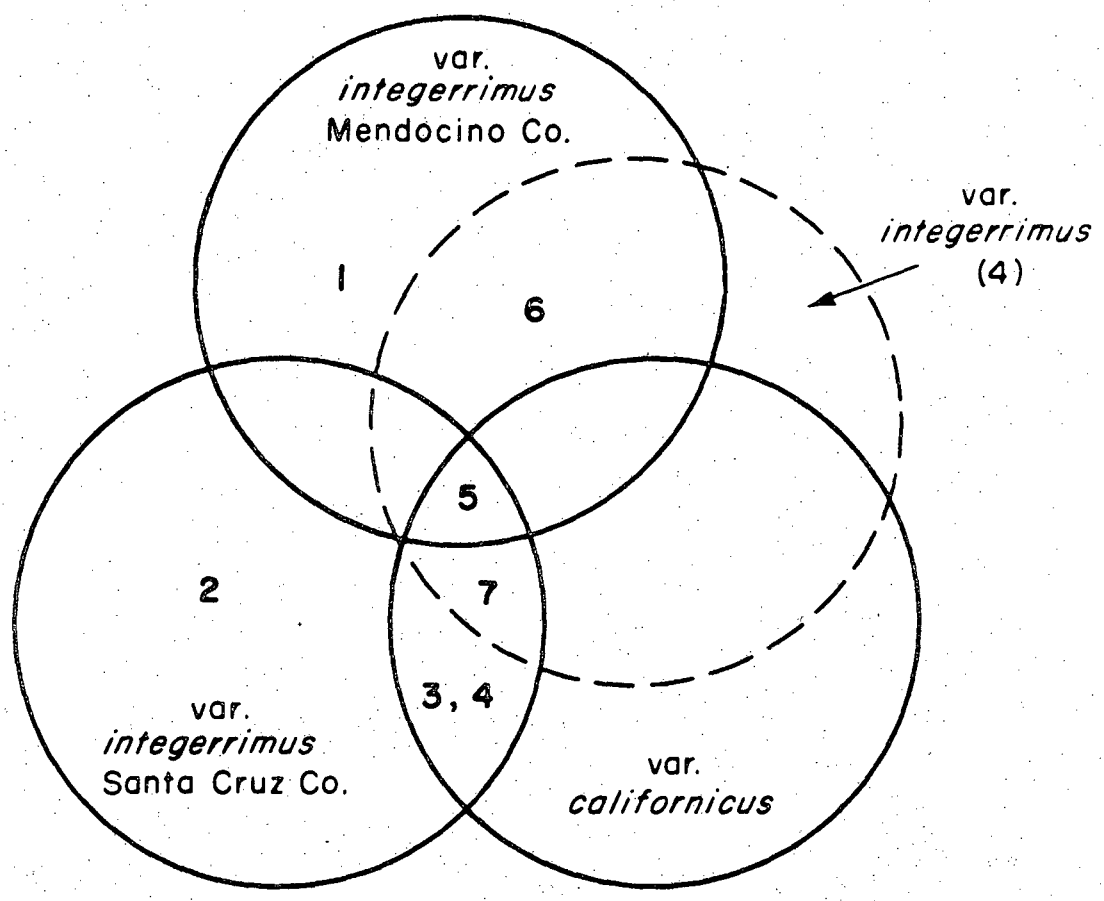
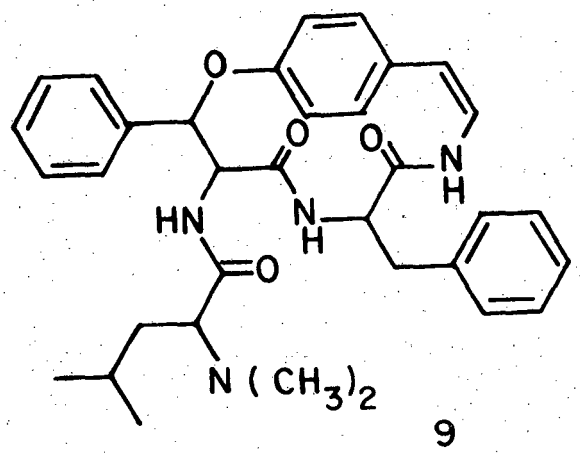

var.
integerrimus
Mendocino Co.
var.
integerrimus
(4)
1
6
5
2
7
3, 4
var.
integerrimus
Santa Cruz Co.
var.
californicus

Fig. 9




9

Integerressine 8 has been reported as the major alkaloid of *C. integerrimus* var. *integerrimus* roots, integerrenine 7 as a minor alkaloid, and integerrine 6 as a trace component.[8,9] Our results are different from this reported estimation. In the extract of *C. integerrimus* var. *integerrimus* from Santa Cruz County, integerrine 7 was the major alkaloid whereas integerrine 6 and interressine 8 were absent. On the other hand, integerrine 6 was the major constituent of *C. integerrimus* var. *integerrimus* obtained from Mendocino County.

Conservative botanical opinion has been that the polymorphic forms of *C. integerrimus* may represent responses to varying amounts of moisture and therefore should be included in a single species *C. integerrimus* H. and A.[1] It is possible that qualitative differences in alkaloid composition between plants from different populations of *C. integerrimus* may similarly reflect the response of the plants to local environmental conditions.

The phytochemical investigation of *C. integerrimus* also poses a difficult challenge to both the botanist and the chemist because interspecific hybridization within the genus Ceanothus is widespread. Thus

the variation in the alkaloidal characters could be representative of the degree of interspecific hybridization in Ceanothus. This concept might explain the disparities among the alkaloid contents of the three examples of *C. integerrimus* var. *integerrimus* examined in this investigation and those observed by others.[8,9] Furthermore, the reported association of nitrogen-fixing actinomycetes with the roots of Ceanothus[10] as well as with other plants which produce phencyclopeptines may implicate the symbionts in the production of cyclopeptide alkaloids. These intriguing possibilities further complicate the phytochemical investigation of *C. integerrimus* and should be addressed in future studies of Ceanothus.

The chemotaxonomic utility of the phencyclopeptines must rely upon the examination of many plants from each different population of *C. integerrimus*. The procedure outlined here, involving standard isolation, HPLC purification, and mass spectral identification, provides a quick and objective means upon which to base plant taxonomic and evolutionary relationships.

Acknowledgment

This work (Part 1-B) has appeared in print in *The Journal of Natural Products* 42, 220 (1979), co-authored by Dane Goff, Frederick K. Klein and Henry Rapoport. Dane Goff, an undergraduate research assistant, contributed significantly in the development of high-pressure liquid chromatographic systems. For collecting the plant specimens and crude alkaloid extractions I thank Frederick K. Klein. I am also indebted to Dr. L. R. Heckard, University of California, Berkeley, and Dr. Malcolm A. Nobs, Carnegie Institute of Washington, Stanford, California, for plant identification and enlightening discussions of basic plant taxonomy.

## Experimental Section[11]

### Plant Material[12]

Root bark of *C. integerrimus* var. *integerrimus* was obtained from its type locality in the Santa Cruz Mountains of California and from a population in the North Coast Ranges of Mendocino County, California, while root bark of *C. integerrimus* var. *californicus* came from its type locality in the Sierra Nevada Mountains of Calaveras County, California. Counting annuli revealed the plant from Santa Cruz county was 11 years old, the one from Mendocino county was 16 years old, and the var. *californicus* was considerably older. Herbarium voucher specimens were submitted to the University Herbarium, University of California, Berkeley.

### Extraction Procedure

Plant material (500 g), frozen in liquid nitrogen, was ground to a fine powder in a Waring blender and extracted with 0.1N HCl (2 × 2 liters) over a period of 8-12 hrs at room temperature. After filtration, the extracts were combined, adjusted to pH 10 with sat. NaOH, and extracted with $CH_2Cl_2$ (2 × 1 liter). The combined $CH_2Cl_2$ layers were concentrated to 100 ml, and extracted with 0.1N HCl (5 × 20 ml) or until further acid extracts were alkaloid free. The combined acid extracts were made alkaline with sat. $Na_2CO_3$ to pH 10, extracted with $CH_2Cl_2$ (5 × 50 ml), and evaporated, affording the following alkaloidal yields: *C. integerrimus* var. *integerrimus* (Santa Cruz county), 0.09%; *C. integerrimus* var. *californicus*, 0.33%; *C. integerrimus* var. *integerrimus* (Mendocino county), 0.14% (root bark).

## HPLC Isolation of Phencyclopeptines

Semi-preparative HPLC was performed on a LiChrosorb C2 column (10 μ, 10 × 150 mm or 10 × 250 mm, E. M. Merck). The crude alkaloidal mixtures were dissolved in 1/1 methanol/acetonitrile at a concentration of 3 mg/mℓ, and injection volumes ranged from 10-250 μℓ. The mobile phase was a mixture of acetonitrile and 0.0015% (v/v) aq. ammonia with the aqueous ammonia comprising 10-30%, the flow rate was usually 2 mℓ/min, and the temperature was maintained at 40°C. Alkaloidal components were detected at 254 nm. Figure 7 shows a tpyical HPLC tracing for the alkaloid mixtures from each plant variety; 10-20 injections provided sufficient material of each component for structural analysis. Fractions were evaporated in vacuo and dried under high vacuum immediately after collection.

## Yields of Phencyclopeptine Components

*C. integerrimus* var. *integerrimus* (Santa Cruz County). Of the eight components separated by HPLC shown in Fig. 7, five showed mass spectral patterns characteristic of the phencyclopeptine nucleus. These components were obtained in the following relative yield: 7 (70%), 2 (16%), 4 (10%), 3 (4%), and 5 (trace).

*C. integerrimus* var. *integerrimus* (Mendocino County). Three of seven components contained the phencyclopeptine nucleus 6 (70%), 5 (15%), and 1 (15%).

*C. integerrimus* var. *californicus*. Four phencyclopeptines were identified by mass spectroscopy in relative amounts as follows: 7 (45%), 5 (45%), 4 (5%), and 3 (5%).

Structures of Phencyclopeptine Components

5-β-Indolylmethyl-8-N-methylvalyl-9-phenylphencyclopeptine (1). $C_{34}H_{37}N_5O_4$; μmp > 350°C; MS: $M^+$ $C_{34}H_{37}N_5O_4$ requires 579.2845, found 579.2788, M-43 $C_{31}H_{30}N_5O_4$ requires 536.2298, found 536.2302, BP $C_5H_{12}N$ requires 86.0970, found 86.0970 (see Fig. 8 for complete mass spectra); amino acid analysis after acid hydrolysis: no amino acids observed; $^1H$ NMR, high field region: δ 0.27 (d, 3H, J=6.9Hz val-γ-$CH_3$), 0.54 (d, 3H, J=6.9Hz val-γ-$CH_3$).

5-β-Indolylmethyl-8-N,N-dimethylvalyl-9-isopropylphencyclopeptine (2). $C_{32}H_{41}N_5O_4$; μmp 233°; MS: $M^+$ $C_{32}H_{41}N_5O_4$ requires 559.3158, found 559.3146, M-43 m/e 516, BP $C_6H_{14}N$ requires 100.1126, found 100.1130 (see Fig. 8); $^1H$ NMR, high field region: δ 0.84 (d, 3H, J=6.8Hz, $(C\underline{H}_3)_2CH$), 0.93 (d, 3H, J=6.8Hz, $(C\underline{H}_3)_2CH$), 0.96 (d, 3H, J=6.9Hz, val-γ-$CH_3$), 1.18 (d, 3H, J=6.9Hz val-γ-$CH_3$).

5-Benzyl-8-N,N-dimethylisoleucyl-9-phenylphencyclopeptine (3). $C_{34}H_{40}N_4O_4$; μmp > 350°; MS: $M^+$ m/e 568, M-57, $C_{30}H_{31}N_4O_4$ requires 511.2345, found 511.2332, BP $C_7H_{16}N$ requires 114.1282, found 114.1279 (see Fig. 8); $^1H$ NMR, high field region: δ 0.18 (d, 3H, J=6.9Hz ileu-γ-$CH_3$), 0.80 (t, 3H, J=6.9Hz, ileu-δ-$CH_3$).

5-Isobutyl-8-N-methylisoleucyl-9-phenylphencyclopeptine (4). $C_{30}H_{40}N_4O_4$; μmp 213°; MS: $M^+$ $C_{30}H_{40}N_4O_4$ requires 520.3049, found 520.3053, M-57 $C_{26}H_{31}N_4O_4$ requires 463.2345, found 463.2356, BP $C_6H_{14}N$ requires 100.1126, found 100.1131 (see Fig. 8); amino acid analysis after acid hydrolysis: 1.0 leucine; $^1H$ NMR, high field region: δ 0.57 (d, 3H,

J = 6.9Hz, ileu-γ-$CH_3$), δ 0.66 (m, 6H, ileu-δ-$CH_3$ and leu(C5)-δ-$CH_3$), 0.76 (d, 3H, J=6.5Hz, leu-(C5)-δ-$CH_3$).

5-β-Indolylmethyl-8-N,N-dimethylisoleucyl-9-isopropylphencyclopeptine (Discarine B) (5). $C_{33}H_{43}N_5O_4$; μmp 233°, Ref. 6, mp 235-236°; MS: $M^+$ $C_{33}H_{43}N_5O_4$ requires 573.3315, found 573.3297, M-57 m/e 516, BP $C_7H_{16}$ requires 114.1282, found 114.1284 (see Fig. 8), $^1H$ NMR (identical to Ref. 6,7), high field region: δ 0.82 (d, 3H, J=6.7Hz, ileu-γ-$CH_3$), 0.90 (t, 3H, J=7.5Hz, ileu-δ-$CH_3$), 0.91 (d, 3H, J=6.8Hz, $(\underline{CH}_3)_2CH$), 1.18 (d, 3H, J=6.8Hz, $(\underline{CH}_3)_2CH$).

5-β-Indolylmethyl-8-N,N-dimethylvalyl-9-phenylphencyclopeptine (Integerrine) (6). $C_{35}H_{39}N_5O_4$; μmp 246°, Ref. 8, mp 258°; MS: $M^+$ $C_{35}H_{39}N_5O_4$ requires 593.3002, found 593.2924, M-43 m/e 550, BP $C_6H_{14}N$ requires 100.1126, found 100.1127 (see Fig. 8); $^1H$ NMR, high field region: δ 0.16 (d, 3H, J=6.8Hz, val-γ-$CH_3$), 0.70 (d, 3H, J=6.8Hz, val-γ-$CH_3$).

5-Isobutyl-8-N,N-dimethylisoleucyl-9-phenylphencyclopeptine (Integerrenine) (7). $C_{31}H_{42}N_4O_4$; μmp 259°, Ref. 9, mp 278°; MS: $M^+$ $C_{31}H_{42}N_4O_4$ requires 534.3205, found 534.3200, M-57 $C_{27}H_{33}N_4O_4$ requires 477.2502, found 477.2515, BP $C_7H_{16}N$ requires 114.1282, found 114.1283 (see Fig. 8); $^1H$ NMR (identical to Ref. 9), high field region: δ 0.36 (d, 3H, J=6.7Hz, ileu-γ-$CH_3$), 0.78 (d, 3H, J=6.5Hz, leu-δ-$CH_3$), 0.85 (d, 3H, J=6.5Hz, leu-δ-$CH_3$), 0.86 (t, 3H, J=7.3Hz, ileu-δ-$CH_3$).

References and Notes for Part I-B

1. M.Van Rensselaer and H.E.McMinn, Ceanothus, (Santa Barbara Botanic Garden, Santa Barbara, California, 1942), pp.179-183.

2. We propose the name phencyclopeptine to represent the fundamental para-bridged 14-membered ring nucleus most common in this large class of widely occurring alkaloids. This basic nucleus and the numbering system shown in Table 2 allow individual alkaloids to be simply and unambiguously designated. Thus we can avoid the multitude of trivial names based on botanical anagrams that have no structural significance.

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9. R.Tschesche, J.Rheingans, H.-W.Fehlhaber and G.Legler, *Chem. Ber.* 100, 3924 (1967).

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11. HPLC was performed with a Spectra Physics Model SP3500B Chromatograph and a model 748 oven, [Santa Clara, California]. UV absorbance was monitored with an Altex Model 151 Dual Wavelength Detector, [Altex Scientific Inc., Berkeley, California]. HPLC grade solvents from Burdick and Jackson Laboratories [Muskegon, Michigan], and water purified with a Milli-Q system, [Millipore Corp., Bedford, Massachusetts] were used for HPLC. Uncorrected melting points were determined on a Kofler Micro Hot Stage (μmp). A model AEI-MS12 mass spectrometer [AEI Scientific Apparatus Ltd., Manchester, England] with INCOS data system was used for determining low resolution mass spectra. High resolution mass spectra were obtained with a Consolidated Electrodynamics CEC-110B instrument. Amino acid analyses were performed on a Beckman 120C Chromatograph [Fullerton, California]. Unless otherwise indicated, $^1$H NMR spectra were taken in $CDCl_3$ solution ($CHCl_3$ at 7.21 ppm) at 22°C on a homemade spectrometer based on a Bruker 63 kG magnet operating at 270 MHz with a Nicolet 1180 data system. Evaporations were done in vacuo with a Buchi rotary evaporator.

12. Identification of plant materials was performed by Dr. L.R.Heckard, University of California, Berkeley, California, and Dr. M.A.Nobs, Carnegie Institution of Washington, Stanford, California. All three plants were collected in the months of May and June.

13. Compounds of the same structure isolated from different plants had the same mp's and $^1$H NMR spectra.

# Part I-C.

# CYCLOPEPTIDE ALKALOIDS. PHENCYLOPEPTINES FROM *CEANOTHUS SANGUINEUS*

## Introduction

*Ceanothus sanguineus* Pursh., a species of the family Rhamnaceae, commonly known as Redstem Ceanothus for its red-purple branches, inhabits wooded slopes, open hills, flats and ledges from Northern California northward into British Columbia and eastward into Idaho and Montana.[1] In the present report, as part of a systematic study of the alkaloids of California *Ceanothus* species, we describe the identification of six cyclopeptide alkaloids from crude extracts of the root bark of this shrub. Before chromatographic separation, however, the number and nominal masses of the constituents of the crude acidic extract were ascertained by field desorption (FD) mass spectrometry.[2] This composite FD mass spectrum of the alkaloidal mixture revealed the presence of five major components with molecular ions m/e 504, 520, 534, 559, and 573 (Fig. 10). After high performance liquid chromatography (HPLC) (Fig. 11), the structures of phencyclopeptines 1-6, including those of the two isomers with molecular weight of 534 (Table 5), were established by electron impact (EI) mass spectrometry, $^{1}$H NMR spectroscopy, and amino acid analysis. Of the six components, five have been previously reported[3,7] while 2 is a new compound.

## Discussion

In contrast to mass spectrometry methods used in previous phytochemical investigations of Ceanothus, we have employed field desorption (FD) mass spectrometry as a means to rapidly determine the alkaloid

composition in crude extracts.[2] The FD analysis of crude alkaloidal mixtures from *Ceanothus sanguineus* revealed parent ions of five phencyclopeptines (Fig. 10). Given this result, the development of a chromatographic system to resolve all five components became our initial goal.

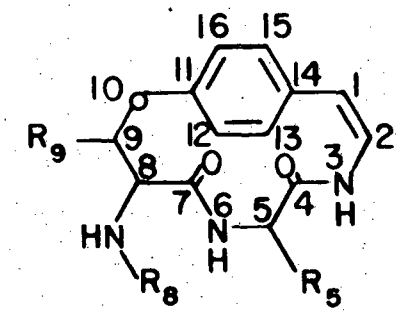
When the reversed phase HPLC system using 0.001% $NH_4OH$ (aq.)/acetonitrile mixtures[4] proved unsuccessful, a paired-ion, reverse phase HPLC system using 0.01N perfluorobutyric acid/acetonitrile mixtures was devised (Fig. 11, system 1). The use of perfluorobutyric acid decreased column degradation and tailing of peaks observed with the alkaline eluants. In addition, this paired-ion proved to be superior to the sulfonic acids due to its high volatility and ease of removal. A silica HPLC system was employed to separate phencyclopeptines 2 and 3 which co-chromatographed in system 1 (Fig. 11, system 2).

The structural assignments of the HPLC-purified phencyclopeptine components of *Ceanothus sanguineus* are based primarily on their characteristic electron impact mass spectra (Table 6). With the exception of the tryptophan-containing phencyclopeptines 2 and 3, the assignment of the ring amino acid residue ($R_5$) is based on acidic hydrolysis of the purified phencyclopeptine followed by amino acid analysis. Amino acid analysis reveals that the two isomeric phencyclopeptines, frangufoline 5 and adouetine Y' 6, which are inseparable by reversed phase HPLC (Fig. 11, system 1), are present in a ratio of 1 to 2, respectively. Further evidence for the mixture of these two isomers is provided by $^1H$ NMR spectroscopy and thin-layer chromatography. As shown in Fig. 12 the two doublets at 0.59 and 0.64 ppm have been assigned to the leucine δ-methyl groups of frangufoline 5, in agreement with the reported values of 0.60

and 0.65 ppm.[8] The assignments of the doublet at 0.38 ppm and the multiplet at 0.69 ppm to the isoleucine γ- and δ-methyl groups of adouetine-Y' 6 are consistent with the literature.[9] Integration of these signals confirms the 1 to 2 ratio of leucine to isoleucine indicated by amino acid analysis. The two large doublets at 1.01 and 1.27 (Fig. 12), assigned to the $R_9$ methyls of both 5 and 6, are consistent with the literature values of 0.99 and 1.25 ppm for 5[8] and 0.99 and 1.23 ppm for 6.[10] Furthermore, silica tlc ($CHCl_3$) of this mixture of isomers reveals two spots with $R_f$ 0.40 and 0.33, which agrees well with the reported chromatographic data for these two phencyclopeptines.[9]

In an earlier report describing the isolation and characterization of alkaloids from *Ceanothus integerrimus*, the chemotaxonomic utility of the phencyclopeptines was discussed. In the future, the use of Field Desorption/Collision Induced Dissociation (FD/CID) mass spectrometry employing the linked B/E scan[2] may provide a useful and rapid approach to the analysis of individual phencyclopeptines in crude plant extracts without the need for chromatographic separation.

TABLE 5. Phencyclopeptines of *Ceanothus sanguineus*




| | $R_5$ | $R_8$ | $R_9$ | MW |
|---|---|---|---|---|
| 1, 5-Benzyl-8-N-(N'-methylprolyl)-9-isopropyl phencyclopeptine (Ceanothine B)[a] | $CH_2C_6H_5$ | NMePro | $CH(CH_3)_2$ | 504 |
| 2, 5-sec-Butyl-8-N(N'-methylphenylalanyl)-9-isopropylphencyclopeptine | $CH(CH_3)CH_2CH_3$ | NMePhe | $CH(CH_3)_2$ | 520 |
| 3, 5-β-Indolylmethyl-8-N-(N',N'dimethyl-valyl)-9-isopropylphencyclopeptine[b] | -β-indolyl-$CH_2$ | $NMe_2$Val | $CH(CH_3)_2$ | 559 |
| 4, 5-β-Indolylmethyl-8-N-(N',N'-dimethyl isoleucyl)-9-isopropylphencyclopeptine (Discarine B)[c] | -β-indolyl-$CH_2$ | $NMe_2$Ile | $CH(CH_3)_2$ | 573 |
| 5, 5-Isobutyl-8-N-(N',N'-dimethylphenyl-alanyl)-9-isopropylphencyclopeptine (Frangufoline)[d] | $CH_2CH(CH_3)_2$ | $NMe_2$Phe | $CH(CH_3)_2$ | 534 |
| 6, 5-sec-Butyl-8-N-(N',N'-dimethylphenyl-alanyl)-9-isopropylphencyclopeptine (Adouetine Y' or Myrianthine B)[e] | $CH(CH_3)CH_2CH_3$ | $NMe_2$Phe | $CH(CH_3)_2$ | 534 |

[a] First identified in *Ceanothus americanus L.*[2]
[b] First identified in *Ceanothus integerrimus* H. and A.[4]
[c] First identified in *Discaria longespina* H. and A.[5]
[d] First identified in *Rhamnus frangula L.*
[e] First identified in *Waltheria americana L.*[7]

TABLE 6. Low resolution mass spectra of HPLC purified components of *Ceanothus sanguineus*.[a]

| Fragment | Compound 1 | 2 | 3 | 4 | 5,6 |
|---|---|---|---|---|---|
| $M^+$ | 504 | 520 | 559 | 573 | 534 |
| BP a | 84 | 134 | 100 | 114 | 148 |
| b | 461 | 429 | 516 | 516 | 443 |
| c | 195 | 181 | 195 | 195 | 195 |
| d | 167 | 153 | 167 | 167 | 167 |
| e | 337 | 303 | 376 | 376 | 303 |
| f | 190 | 190 | 190 | 190 | 190 |
| g | 421 | 387 | 460 | 460 | 387 |
| h | 308 | 274 | 347 | 347 | 274 |
| i | 135 | 135 | 135 | 135 | 135 |
| j | 378 | 344 | 417 | 417 | 344 |
| k | 244 | 210 | 283 | 283 | 210 |
| l | 216 | 182 | 255 | 255 | 182 |
| m | 97 | 97 | 97 | 97 | 97 |
| n | - | - | 170 | 170 | - |
| o | 120 | 86 | 159 | 159 | 86 |
| p | 91 | - | 130 | 130 | - |
| other | - 489[b] 475[c] | - 463[d] 133[e] | - | - | 519[b] 370[f] 398[g] 133[h] |

[a]Fragment ions according to fragmentation scheme in Ref. 4.
[b]$M^+ - 15$
[c]$M^+ - 29$
[d]$M^+ - C_4H_9$
[e]a-H
[f]g-OH
[g]b-$NH(CH_3)_2$

Figure Captions - Part I-C

Fig. 10. Field desorption mass spectrum of the crude acidic, alkaloid extract of *Ceanothus sanguineus* (emitter current 16 mA).

Fig. 11. HPLC of crude alkaloidal mixtures from *C. sanguineus*. HPLC systems employed: 1) LiChrosorb RP-18 (10 μ, 10 × 250 mm); mobile phase 0.015 M perfluorobutyric acid/$CH_3CN$ (6/4 v/v); flow rate 1.6 mℓ/min; 25°C; A 254 nm, injection volume 10 μℓ; C = 3.8 mg/mℓ. 2) Waters Porasil (10 μ, 3.2 × 280 mm); mobile phase $CHCl_3$/hexane/triethylamine (95/5/0.1 v/v); flow rate 1.6 mℓ/min; 41°C; A 280 nm; injection volume 10 μℓ, c = 3.5 mg/mℓ.

Fig. 12. Expanded view of high field region of $^1H$ NMR of 2:1 myrianthine-B 6 and frangulanine 5 mixture.

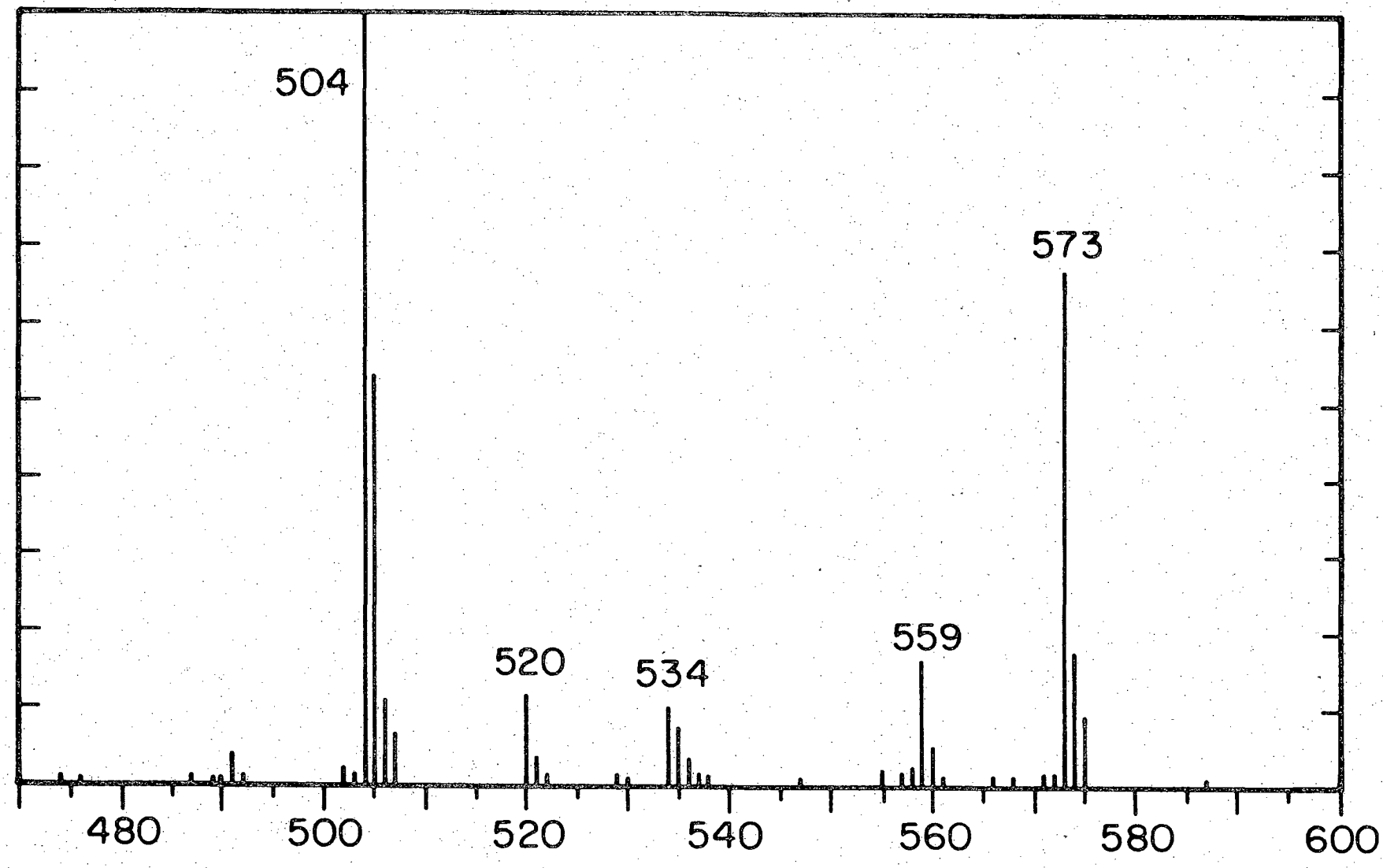



Fig. 10

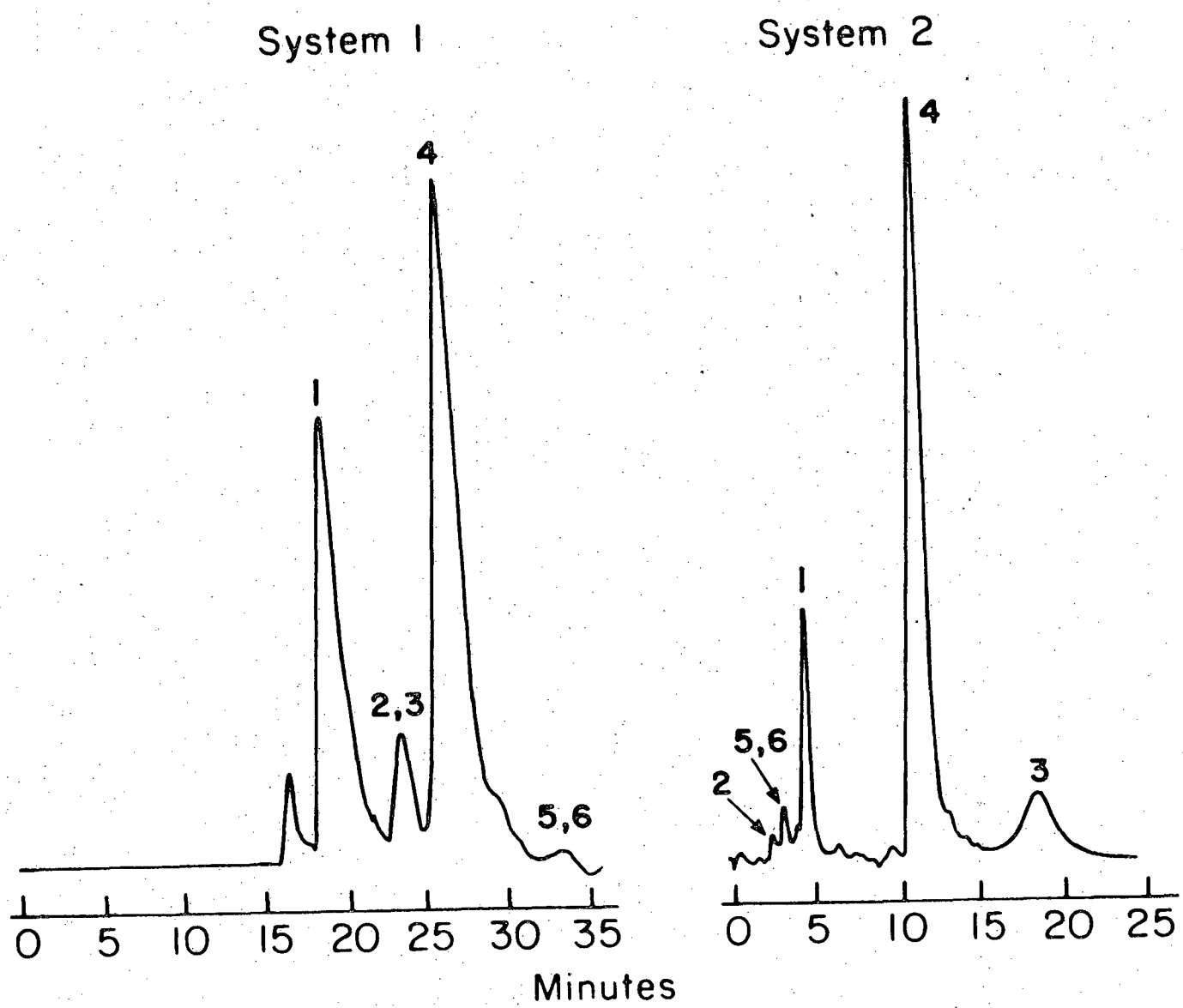



Fig. 11

XBL 799-11739

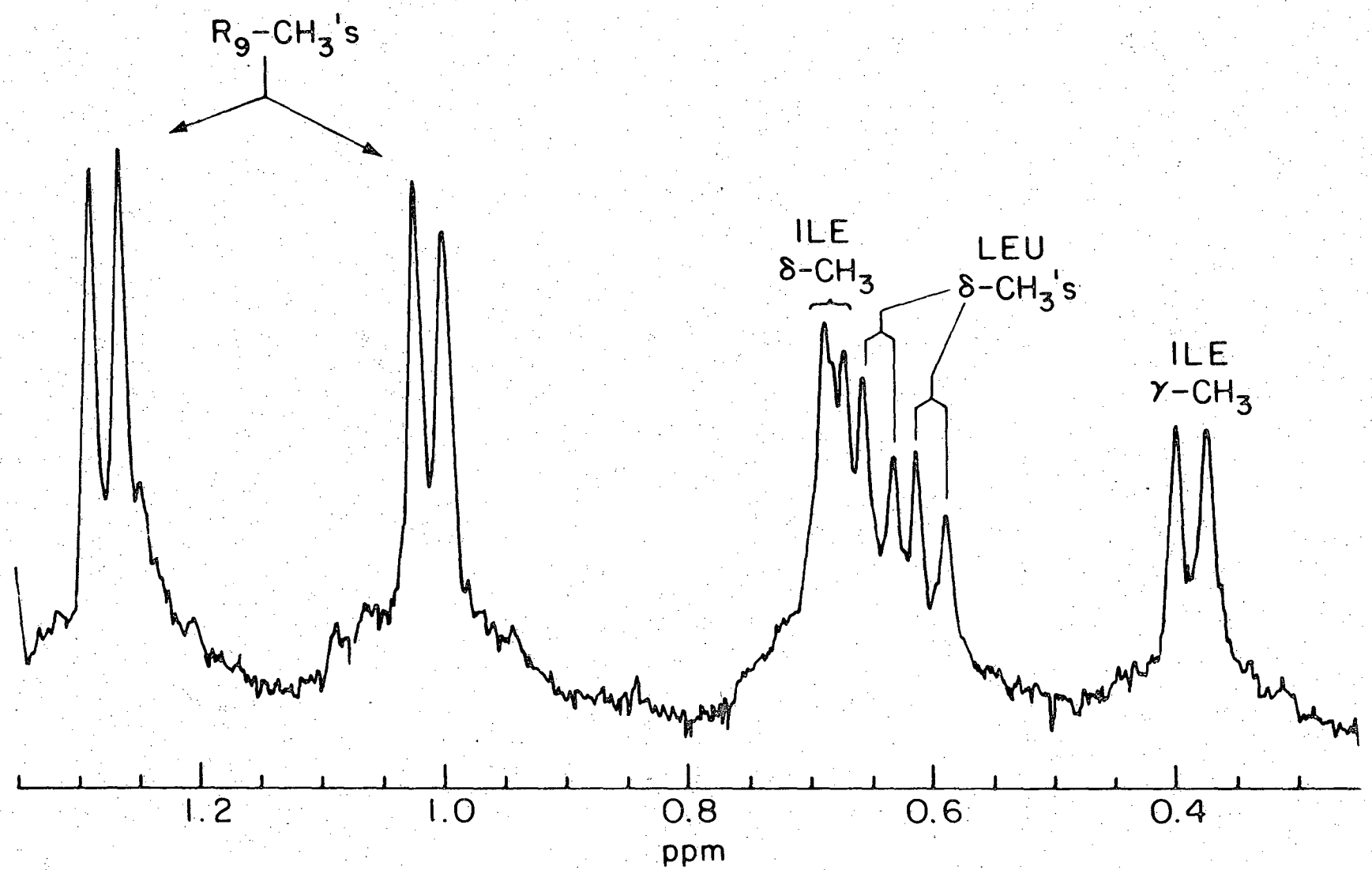



Fig. 12

## Experimental Section

### Plant Material

Root bark of *C. sanguineus* was collected from plants in Klamath National Wilderness on Hawkinsville Road, 5.5 miles east of Hawkinsville, on Humbug Mountain.

### Extraction Procedures

The extraction procedure, as described in an earlier report[4] was used. The total crude alkaloid yield was 0.22% of the root bark.

### Field Desorption Mass Spectrometry

As shown in Fig. 10 the FD mass spectrum of the crude alkaloidal mixture reveals five major components with nominal masses as follows: m/e 504, 520, 534, 559 and 573.

### HPLC Isolation of Phencyclopeptines

Preparative HPLC was performed with two systems (Fig. 11).

System one used a LiChrosorb RP-18 column (10μ, 10 × 250 mm, E. M. Merck). The crude alkaloidal mixture was dissolved in methanol and filtered through a 5 micron teflon filter. Injection volumes ranged from 100 to 200 μℓ at a concentration of 3-4 mg/mℓ. The mobile phase was a 65/35 (v/v) mixture of 0.015M aqueous perfluorobutyric acid and acetonitrile. The chromatography was done at room temperature with a flow rate of 1 to 2.5 mℓ/min. Alkaloidal components were detected at 254 nm. Figure 11 shows a typical HPLC tracing. The collected fractions were evaporated in vacuo and the residues were dissolved in 1.5M $NH_4OH$ (4 mℓ) and extracted with $CH_2Cl_2$ (3 × 1.5 mℓ). The combined organic layers were extracted with $H_2O$ (1 × 1.5 mℓ) and then dried under a stream of nitrogen.

The second system used a Waters Porasil column (10μ, 3.2 × 250 mm). The crude alkaloidal mixtures were dissolved in $CHCl_3$ and chromatography was performed at 37-41°C with a mobile phase of $CHCl_3$/hexane/triethylamine, 95/5/0.1. Alkaloids were detected at 280 nm as shown in Fig. 11. Fractions collected were immediately evaporated in vacuo and dried under high vacuum. Relative weight percents of *C. sanguineus* phencyclopeptines (HPLC System 2) were 1 (37%), 2 (3%), 3 (12%), 4 (43%), 5 and 6 (6%).

Structures of Phencyclopeptines from *C. Sanguineus*

5-Benzyl-8-N-(N'-methylprolyl)-9-isopropylphencyclopeptine (Ceanothine B) (1). $C_{29}H_{36}N_4O_4$; μmp 225°, Ref. 11, mp 238.5 - 240.5°; MS: $M^+$ $C_{29}H_{36}N_4O_4$ requires 504.2736, found 504.2795, BP $C_5H_{10}N$ requires 84.0813, found 84.0809; amino acid analysis 110°C/16 hrs: phenylalanine (1.0); HPLC-system, retention time in minutes: 1, 19.5; 2, 4.5 (Fig. 11); $^1H$ NMR ($CDCl_3$) - 0.91 (d, 3H, J=6.7Hz, R9-$CH_3$), 1.24 (d, 3H, J=6.7Hz, R9-$CH_3$), 1.63 (m, 1H, R9-CH), 1.7-1.9 (m, 2H, R8-γ1 $CH_2$ and β1 $CH_2$), 1.98 (s, 3H, N-$CH_3$), 2.1 - 2.2 (m, 1H, R8-γ2 $CH_2$), 2.2 - 2.3 (m, 1H, R8-β2 $CH_2$), 2.68 (dd, 1H, 4.3Hz, 10.6Hz, R8-δ1 $CH_2$), 2.85 (dd, 1H, J=8.2 and -14.7Hz, R5-$CH_2$), 3.01 (m, 1H, R8-δ2 $CH_2$), 3.08 (dd, 1H, J=4.2 and -14.7 Hz, R5-$CH_2$), 4.3 - 4.4 (m, C5-H), 4.34 (dd, 1H, J=7.0 and 10.0Hz, C8-H), 4.93 (dd, 1H, 2.0 and 7.0Hz, C9-H), 5.99 (d, 1H, J=6.9Hz, C1-H), 6.39 (d, 1H, J=7.4Hz, R8-αCH), 6.4 - 6.5 (m, N3-H), 6.66 (m, 1H, C2-H), 7.0 - 7.3 (m, 9H, aromatic, R5-Ø, C12,13,15,16-H's), 7.75 (d, 1H, J=10.0Hz, C8-NH). This NMR spectrum is identical with that obtained from an authentic sample of Ceanothine B from *Ceanothus americanus* Linn.[3]

5-sec-Butyl-8-N-(N'-methylphenylalanyl)-9-isopropylphencyclopeptine (2). $C_{30}H_{40}N_4O_4$; µmp 229°; MS: $M^+$ m/e 520, M-91 $C_{23}H_{33}N_4O_4$ requires 429.2502, found 429.2506, BP $C_9H_{12}N$ requires 134.0970, found 134.0966; amino acid analysis 110°C/16 hrs: isoleucine (1.0); HPLC-1, 24.0; 2, 2.5 (Fig. 11).

5-β-Indolylmethyl-8-N-(N',N'-dimethylvalyl)-9-isopropylphencyclopeptine (3). $C_{32}H_{41}N_5O_4$; µmp 229°, Ref. 4, µmp 233°; MS: $M^+$ m/e 559, M-2H $C_{32}H_{39}N_5O_4$ requires 557.3002, found 557.2957, M-43 m/e 516, BP $C_6H_{14}N$ requires 100.1126, found 100.1125; amino acid analysis 110°C/16 hrs: no amino acids observed; HPLC-1, 24.0; 2, 18.7 (Fig. 11).

5-β-Indolylmethyl-8-N(N',N'-dimethylisoleucyl)-9-isopropylphencyclopeptine (Discarine B) (4). $C_{33}H_{43}N_5O_4$; µmp 233°, Ref. 4, µmp 233°; MS: $M^+$ $C_{33}H_{43}N_5O_4$ requires 573.3315, found 57.3264, M-57 $C_{29}H_{34}N_5O_4$ requires 516.2611, found 516.2644, BP $C_7H_{16}N$ requires 114.1283, found 114.1281; amino acid analysis 110°C/16 hrs: tryptophan (low recovery); HPLC-1, 26.7; 2, 11.1 (Fig. 11); $^1H$ NMR identical with previously reported spectra.[4,12]

5-Isobutyl-8-N-(N',N'-dimethylphenylalanyl)-9-isopropylphencyclopeptine (Frangufoline) (5) and 5-sec-Butyl-8-N-(N',N'-dimethylphenylalanyl)-9-isopropylphencyclopeptine (Adouetine Y') (6).[13] $C_{31}H_{42}N_4O_4$; MS: $M^+$ M/e 534, M-91 $C_{24}H_{35}N_4O_4$ requires 443.2648, found 443.2649, BP $C_{10}H_{14}N$ requires 148.1126, found 148.1118; µmp 261°, Ref. 6, mp 289-290° for 5, and Ref. 9, 244° for 6; amino acid analysis 110°C/16 hrs: ile/leu, 2/1; $^1H$ NMR, high field region: δ 0.38 (d, 2H, J=6.7Hz, ile-γ-$CH_3$), 0.60 (d, 1H, 6.7Hz, leu-δ-$CH_3$), 0.64 (d, 1H, 6.7Hz leu-δ-

$CH_3$), 0.68 (m, 2H, ile-δ-$CH_3$), 1.01 (d, 3H, J=6.4Hz, R9-Me), 1.27 (d, 3H, J=6.7Hz, R9-Me); TLC Analtech Silica Gel G (250 μ)-eluant, $CHCl_3/Et_2O/MeOH$, 45/15/1, $R_f$ 0.40 (5) and 0.33 (6), Ref. 12, $R_f$ 0.66 (5), 0.58 (6); Ref. 14, $R_f$ 0.44 (5), 0.35 (6), HPLC-1, 33.5; 2, 3.2 (Fig. 11).

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# Part II.

# PHYCOCYANOBILIPEPTIDES. THE STRUCTURE AND LINKAGE OF A PHYCOCYANOBILIN BOUND TO THE β-SUBUNIT OF C-PHYCOCYANIN

## Introduction

The intensely blue protein, C-phycocyanin, is a major light harvesting component of the photosynthetic apparatus of cyanobacteria (blue-green algae) and of certain red algae.[1] Composed of two distinct polypeptide chains, α and β subunits,[2] the monomer of C-phycocyanin contains three distinct covalently bound prosthetic groups known as phycocyanobilins — one on the α-chain and two on the β-chain.[3] Since the initial work in the 1930's,[4] intensive study of the phycocyanobilins has not yet provided the unambiguous assignment of the structure of the natural prosthetic groups nor the precise nature of the covalent linkage to the apoprotein.[5,6]

The methodology most widely used to examine the structure of these pigments has entailed the cleavage of the chromophore-protein linkages of C-phycocyanin through treatment under various conditions.[7-14] From these studies a number of degradation products and phycobilins released from the protein have been characterized spectroscopically, including the "blue pigment" 1 whose dimethyl ester has been synthesized recently.[15] It is clear, however, that all of these pigments, while derived from the various native prosthetic groups, are products whose nature is dependent on the cleavage conditions[5] and the potential of the latter for introducing artifacts.

The second experimental approach, which requires the chromophore-

protein linkage to be maintained, has been applied to the determination of the amino acid sequences about the sites of attachment of the three phycocyanobilins.[16-19] From such analyses of proteolytically prepared oligopeptides from C-phycocyanin, chromophore-protein linkages involving the side chains of serine,[16] aspartic acid,[17] cysteine[17-19] and tyrosine[19] have been proposed. Two recent studies of highly purified chromopeptides from the C-phycocyanins of *Mastigocladus laminosus*[18] and *Synechococcus* sp. 6301[20] however, have unambiguously established the linkage of a cysteine residue to each of the three phycocyanobilins. Although substantial evidence implicates a thioether linkage,[17,18,20-22] direct proof of the structure of any proposed chromoprotein linkage in C-phycocyanin is lacking.

In the present study, we report the structure determination of one of the three peptide-bound phycocyanobilins, namely $\beta_1$-phycocyanobiliheptapeptide 2 obtained from the cyanogen bromide cleavage of *Synechococcus* sp. 6301 C-phycocyanin.[20,23] A comparison of the $^1$H-NMR spectra of this chromopeptide 2 with those of synthetic peptide 3 was undertaken to permit the direct assignment of the structure of the $\beta_1$-phycocyanobilin as well as that of the chromoprotein linkage.

Chromopeptide 2, derived from residues 79-85 of the β-chain,[20] was chosen as the substrate to demonstrate a new methodology for the analysis of the prosthetic groups of biliproteins. Our approach required that the conditions used in obtaining the substrate for structural investigation should not alter the native structure of the prosthetic group or its linkage to the protein. The cyanogen bromide cleavage procedure satisfied these requirements and in addition provided a good

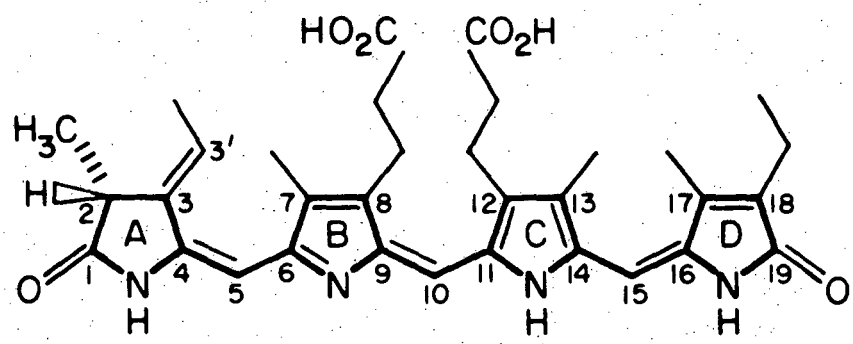
yield of the chromopeptide 2. Furthermore, the development of an effective high performance liquid chromatographic (HPLC) system for the separation of this type of chromophore-linked peptide assured the homogeneity of the cleavage product. Using this methodology and the comparative $^{1}H$ NMR spectroscopic analysis described in this report, the direct proof of the structure of an intact phycocyanobilin and its thioether linkage has now been realized.

## Results and Discussion

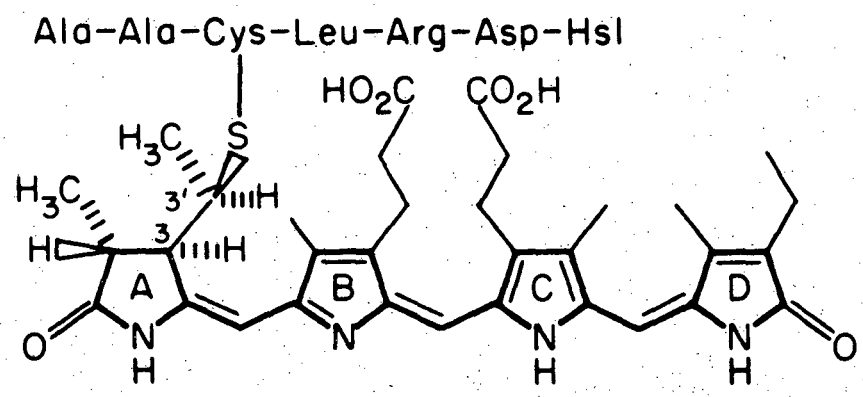
To facilitate the interpretation of the $^{1}H$-NMR spectrum of the chromopeptide 2, authentic heptapeptide 3 was necessary and it was obtained in quantity by synthesis. We chose first to prepare the protected heptapeptide 4,[24] which subsequently could be deblocked with acid to afford 3.

The synthesis of the protected heptapeptide 4 was accomplished with the repetitive mixed anhydride method (Scheme I).[25] This process was chosen in preference to the solid phase method[26] as the intermediate peptides (6-10) could be highly purified after each condensation step. Furthermore, the deblocked peptides Leu-Arg-Asp-Hsl (11) and Cys(SSEt)-Leu-Arg-Asp-Hsl (12) which were obtained from intermediates 8 and 9 respectively, were necessary for the interpretation of the NMR spectra of heptapeptides 2 and 3.

The lactone of homoserine (Hsl) proved to be an effective protecting group for the C-terminal residue. It was formed rapidly as the crystalline trifluoroacetic acid salt upon dissolution of L-homoserine in anhydrous trifluoroacetic acid, and throughout the synthesis of the heptapeptide 4 the homoserine lactone moiety remained quite stable.[27] With one exception the yields of the purified peptide after each condensation were >90%.




1




2

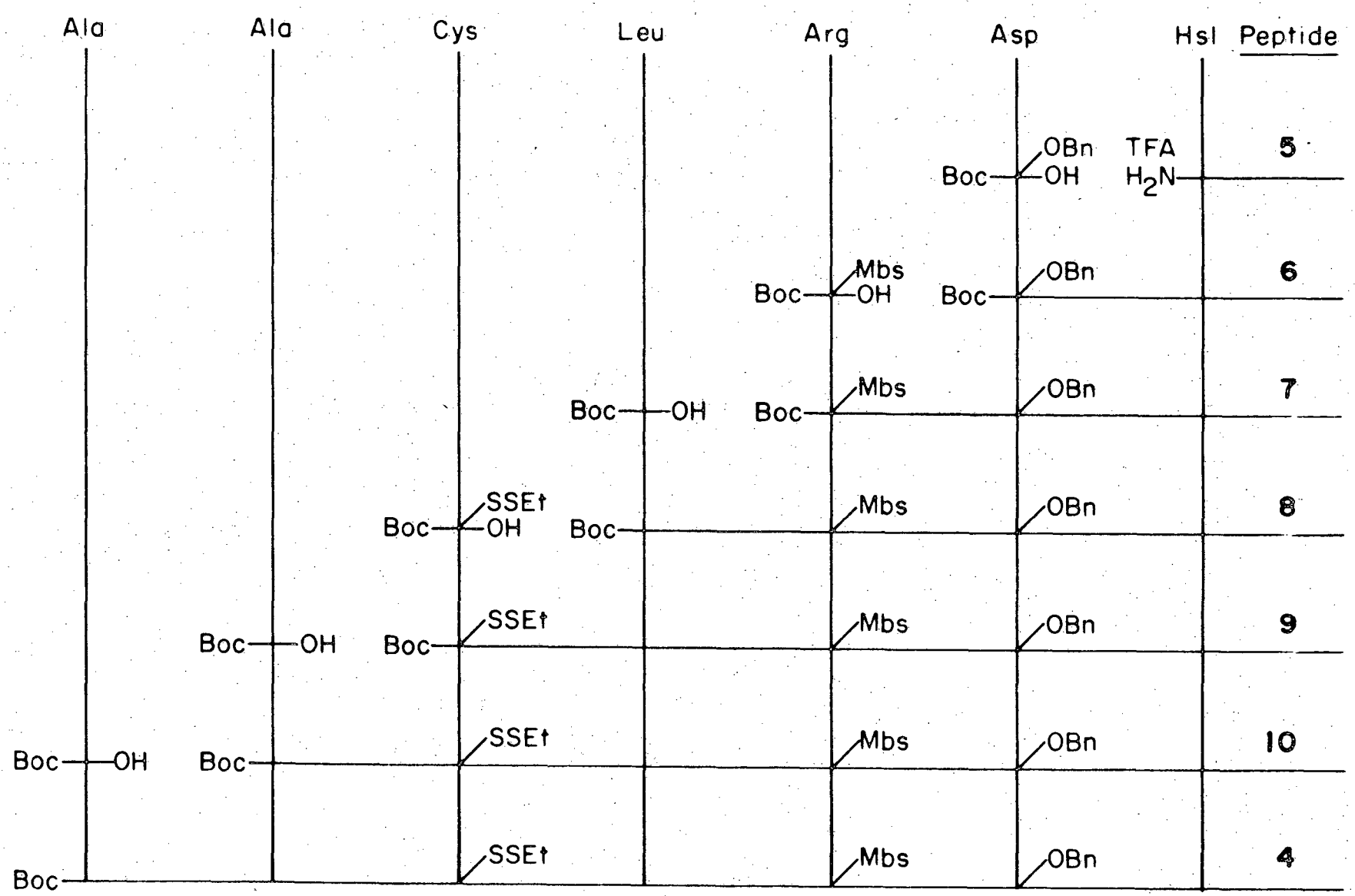
BocNH-Ala-Ala-Cys(SSEt)-Leu-Arg(Mbs)-Asp(Bn)-Hsl

4

↓ HF/EtSH

Ala-Ala-Cys(SSEt)-Leu-Arg-Asp-Hsl

3




Scheme I. Repetitive excess mixed anhydride synthesis of the protected heptapeptide 4.

This exception was the low yield of tripeptide 7 for which the cyclization of the mixed anhydride intermediate of the arginine derivative was responsible. Since this side product can be easily separated from the desired tripeptide, the use of excess arginine derivative should improve this conversion.

For the synthesis of the hexa- and heptapeptides 10 and 4, the t-Boc group was removed with 4N HCl in anhydrous dioxane. This method was preferred because of the reported instability of the ethyl disulfide in anhydrous trifluoroacetic acid.[28] Final removal of the protecting groups was accomplished with anhydrous hydrogen fluoride for one-half hour at 0°C.[29] Ethyl mercaptan, instead of anisole, served to scavenge the carbonium ion and to prevent acid-catalyzed disulfide interchange. Despite this precaution, a complex mixture of side products accompanied the heptapeptide 3 which, after HPLC purification (Fig. 13) was isolated in 30% yield. The side reactions encountered during the HF cleavage probably involve the disulfide moiety. Similar reaction of the protected tetrapeptide 8 with HF/EtSH provided pure material without need for further purification; however, the pentapeptide 9 also gave a complex mixture of products which required purification by HPLC.

HPLC proved to be an effective tool for the analysis and purification of the peptide mixtures. Using reversed-phase C18 chromatography, all of the synthetic oligopeptides could be separated. The protected peptides 4-10 were eluted with 60/40 $CH_3CN/H_2O$ and detected at 225 nm; they showed increased retention times with increased size. The deblocked peptides were detected at 190 nm using a gradient eluent containing a mixture of acidic phosphate buffer and acetonitrile (see Experimental

Section). With the latter system both the chromopeptide 2 (Fig. 14) and the synthetic heptapeptide 3 (Fig. 13) were purified on a preparative scale for $^1$H NMR spectroscopy.

In order to determine the chromophore structure and mode of linkage to the peptide, the 360 MHz $^1$H NMR spectra of the chromopeptide 2 and the synthetic peptide 3 (Figs. 15 and 16), in $D_2O$ and in pyridine-$d_5$, were compared.

Complete assignment of the $^1$H-NMR spectra of the synthetic heptapeptide 3 has involved extensive spin decoupling experiments and comparative NMR studies with synthetic tetra- and pentapeptide, 11 and 12. Parallel analysis of the NMR spectra of chromopeptide 2 reveals the expected similarity of the resonant frequencies of the corresponding amino acid residues of 3 (Tables 7 and 8).

In $D_2O$, minor differences in the spectra of the two heptapeptides are evident in the resonances attributed to the cysteine, leucine and arginine residues (Fig. 15, Table 7). The position of the α-hydrogens of these three amino acids in 2 are displaced 0.10 - 0.20 ppm upfield from those in synthetic peptide 3. Smaller (0.1 ppm) upfield shifts are also observed in some of the resonances of the leucine and arginine side chains of chromopeptide 2. Little difference in the chemical shifts of the N-terminal alanyl, C-terminal homoserine lactone, and aspartic acid side chains of the two peptides in $D_2O$ is evident. These upfield shifts in the spectrum of chromopeptide 2 can arise from the local magnetic ring current field of the bound phycocyanobilin and from phycocyanobilin-induced conformational change in the peptide backbone. In pyridine-$d_5$, the similarity of the spectra of the two heptapeptides 2 and 3 is even more

striking with respect to the peptide moieties (Fig. 16, Table 8). Only the resonances attributed to the alpha and beta hydrogens of cysteine and the alpha hydrogen of the N-terminal alanine are shifted significantly (0.10 ppm upfield) from their assigned frequencies in the spectrum of 3. The chemical shifts of the other amino acid residues of 2 and 3 fall within 0.03 ppm of each other in pyridine-$d_5$.

The observation that the differences in the spectra of the two heptapeptides in both solvents are the largest for amino acid residues nearest the cysteine is consistent with the proposed thioether linkage of phycocyanobilin to the peptide chain.[17,18,20-22] Moreover, the essential identity in chemical shifts of the arginine δ-methylenes and aspartic acid β-methylenes in the two peptides in both $D_2O$ and pyridine-$d_5$ discounts the possibility of a second linkage involving the side chain of these amino acids.

From the previous spectral analysis of the peptide moiety of 2 the assignment of the remaining resonances to the phycocyanobilin prosthetic group can be made. Shown in Table 9, the $^1$H-NMR spectral assignments of the bilin moiety of 2 show great similarity with those reported for the blue pigment 1.[10] In particular the resonances attributed to the aromatic methyls of C-7, 13 and 17, olefinic methines of C-5, 10 and 15, the ethyl group of C-18, and the propionic acid methylenes at C-8 and 12 are salient features of the spectra of both pigments (1 and 2).

The complex spin system of ring A of 2 was deciphered with a number of double resonance experiments (Figs. 17 and 18). Two broad doublets at 1.21 and 1.44 ppm in the spectrum of chromopeptide 2 in $D_2O$ (Fig. 17) have been assigned to the C-2 and C-3' methyl groups, respectively. The doublet

at 1.21 ppm is coupled with a multiplet at 2.69 ppm (Fig. 17a), while irradiation of a multiplet at 3.58 ppm collapses the doublet at 1.44 ppm to a singlet (Fig. 17b). In pyridine-$d_5$, the two doublets at 1.39 ppm and 1.48 ppm are coupled with multiplets at 2.70 ppm and 3.52, respectively (Fig. 18a,b).

Converse double resonance experiments have provided conclusive evidence for the proposed thioether linkage to ring A of chromopeptide 2. Irradiation of the higher field, methyl doublets, 1.21 ppm in $D_2O$ and 1.39 ppm in pyridine-$d_5$, effects a change in the corresponding multiplets at 2.69 ppm (Fig. 17c) and 2.70 ppm (Fig. 18c). Due to overlapping resonances at 2.69 ppm in the $D_2O$ spectrum of 2 the precise nature of this change cannot be ascertained. However, the analogous irradiation of the doublet at 1.39 ppm in the pyridine-$d_5$ spectrum of 2 clearly brings about the collapse of the multiplet at 2.70 ppm to a doublet with $J = 4.3$ Hz (Fig. 18c).

When the lower field methyl doublets at 1.44 ppm in $D_2O$ and 1.48 ppm in pyridine-$d_5$ are irradiated, the collapse of resonances at 3.58 ppm (Fig. 17d) and 3.52 ppm (Fig. 18d) is observed. In this case, the effect of these decoupling irradiations on the multiplet at 3.52 ppm in pyridine-$d_5$ (Fig. 18d) is obscured by overlapping resonances, whereas the multiplet at 3.58 ppm in $D_2O$ becomes a doublet (Fig. 17d).

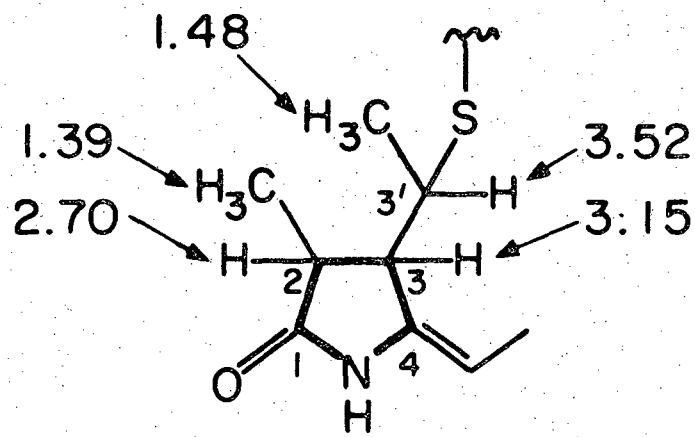
The results of these decoupling experiments confirmed the presence of two alpha substituted ethyl groups, $CH_3CH(X)$-, each adjacent to a methine. Upon irradiation of a multiplet at 3.15 ppm in the pyridine-$d_5$ spectrum of 2, a change in the multiplicity of the resonances at 2.70 ppm (C-2-H) and 3.52 ppm (C-3'-H) was observed. Owing to the presence of overlapping signals, the precise effect of this irradiation on the multiplet

at 3.52 ppm could not be determined, however, the collapse of the multiplet at 2.70 ppm to a quartet (J = 7.2 Hz) was clearly discernible (Fig. 18e). These data show that the two ethyl moieties which include C-2 and C-3 are adjacent to the same methine hydrogen, C-3-H, δ 3.15 in pyridine-$d_5$.

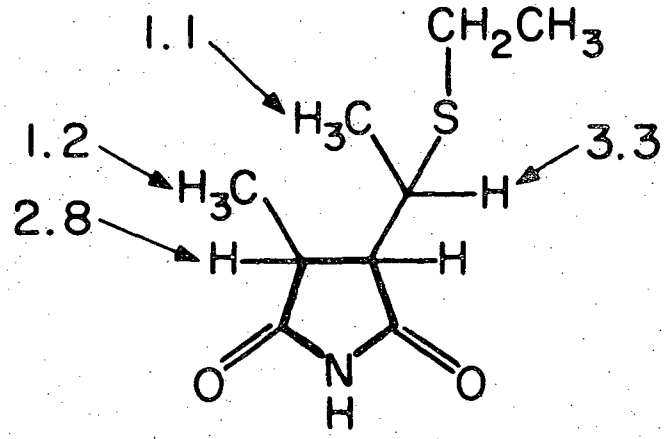
The assignment of the higher field methyl doublet (1.21 ppm in $D_2O$ and 1.39 ppm in pyridine-$d_5$) to the C-2 methyl group is based on comparison with model compounds. In a recent study on bilin-protein linkages, the synthesis and NMR spectrum (in DMSO-$d_6$) of the thioether-containing succinimide 13 were reported,[30] and the chemical shifts of methines C-2 and C-3' were 2.8 and 3.3 ppm, respectively. The analogous resonances of the C-2 and C-3' methines of the $\beta_1$-phycocyanobilin in 2, 2.69 and 3.58 ppm in $D_2O$ (2.70 and 3.52 ppm in pyridine-$d_5$) respectively, similarly reflect the assignment of the thioether methine resonance to the lower field value. Since the lower field resonance is coupled with the doublet at 1.44 ppm in $D_2O$ (1.48 ppm in pyridine-$d_5$) this doublet has been assigned to the C-3'-methyl, alpha to the sulfur (Table 9).

The assignment of the stereochemistry of the thioether-linkage shown in 2 requires three assumptions. If one assumes that (a) the absolute stereochemistry of C-2 is R;[31] (b) the geometry of ring A is trans;[32] and (c) the cleavage of 1 from C-phycocyanin involves a concerted trans-periplanar elimination requiring the relative stereochemistry at C-3 and C-3' to be RR or SS,[33] then the assignment of the RRR stereochemistry at C-2, 3 and 3' can be made. Although this stereochemical assignment for the $\beta_1$-phycocyanobilin-peptide linkage is probable, the stereochemical questions have not been unambiguously answered.

Our proposed structure 2 is consistent with the observation that




**2**, partial (δ in pyridine-$d_5$)

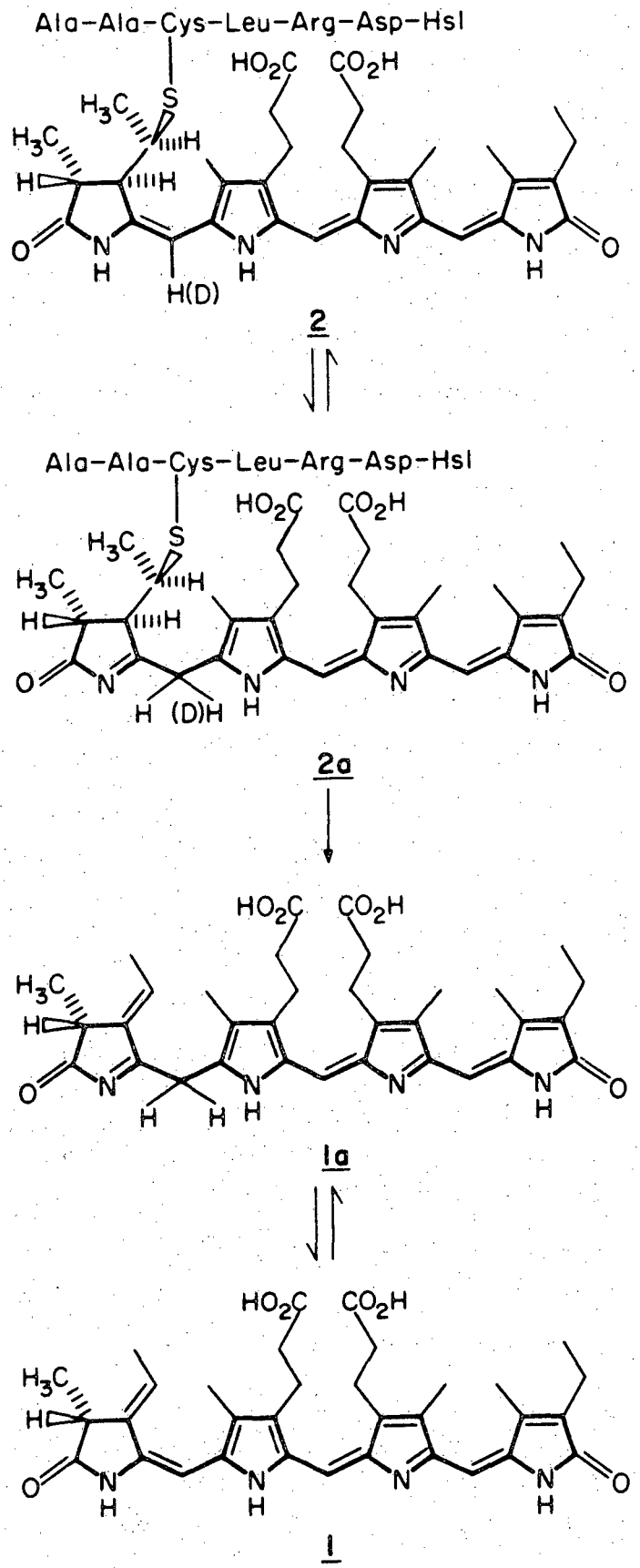



**13** (δ in DMSO-$d_6$)

the blue pigment 1 is the major pigment obtained from C-phycocyanin under a variety of conditions.[13] As shown in Scheme II, a tautomerization-β-elimination mechanism could explain these experimental results. The deuterium exchange of the C-5-methine observed in the NMR spectrum of 2 provides clear evidence for the equilibrium between tautomers 2 and 2a. This tautomerization would increase the acidity of the C-3 hydrogen of 2a (pyrrolinone form) through conjugation with the C-1 carbonyl, and thus promote the β-elimination of the peptide. Presumably a rapid equilibrium between the resulting pigment 1a and the blue pigment 1 would then be established.[34] Due to the additional double bond (C3-C3'), it is plausible to assume the position of this equilibrium favors the blue pigment 1. Whether this pigment can indeed be released from the chromopeptide 2 under the same conditions used to liberate it from the intact protein[13] remains to be determined.

A large difference in the temperature dependence of the $^1$H-NMR spectra of the synthetic peptide 3 and the chromopeptide 2 in $D_2O$ was observed. While the spectrum of 3 was relatively unaffected by changes in temperature between 15° and 40°C, the effect on the spectrum of the chromopeptide 2 was significant. In contrast to 3, at 15°C many of the resonances of the chromopeptide 2 were so broad that much of the fine structure was not discernible. This was especially the case for proton resonances of the $\beta_1$-phycocyanobilin group of 2. Although the line width and resolution greatly improved at 40°C, many of the resonances due to the prosthetic group of 2 remained broad.

These temperature effects on the NMR spectrum of 2 in $D_2O$ can be interpreted to represent interconversions between different molecular




Scheme II. Proposed tautomerization and trans-periplanar β-elimination of $\beta_1$-phycocyanobilinheptapeptide 2.

conformations or aggregations of the chromopeptide. The observed line broadening in $D_2O$ may be attributed to a large number of interconverting prototropic forms and geometric isomers of the bilin chromophore. These interconversions would result in a concomitantly large number of magnetically nonequivalent environments for individual protons of the phycocyanobilin. Since the line width depends on the rate of interconversion between such isomers, the temperature dependence of the NMR spectrum can thus be explained.

Concentration dependence in the circular dichroism (CD) spectrum of 2 in $D_2O$ (Fig. 19) suggests that dimerization of the phycocyanobilin moiety (perhaps through $\pi$-$\pi$ complex formation) is occurring at the concentration at which the NMR spectrum was obtained (2.4 mM in $D_2O$). Comparative analysis of the NMR spectra of 2 and 3 in $D_2O$ also shows that the phycocyanobilin prosthetic group effects a conformational change in the peptide backbond of 2. The influence of the bilin moiety is particularly evident in amino acid residues closest to the thioether linkage as discussed earlier. On the other hand, the great similarity in the NMR spectra of 2 and 3 in the aromatic solvent pyridine-$d_5$[35] shows that the chromopeptide 2 exists as a monomeric species in this solvent. This observation is consistent with a $\pi$-$\pi$ interaction in $D_2O$ suggested by CD data. The exact nature of the complex formation in $D_2O$ warrants further investigation which may shed light on the orientation and interaction of the three bilins within native C-phycocyanin.

The NMR and electronic absorption spectral data indicate that chemical modification of the chromopeptide has not occurred during the cyanogen bromide cleavage. A comparison of the absorption spectrum of

C-phycocyanin and chromopeptide 2 in 30% acetic acid (Fig. 20) shows an insignificant difference in the spectrum of the phycocyanobilin prosthetic group after cleavage. The difference in absorption maxima, $\lambda_{max}$ is 655 nm for 2 and 660 nm for denatured C-phycocyanin, is small considering the presence of three phycocyanobilins attached to different peptide sequences in C phycocyanin.[3,20] Thus we conclude that the conditions of the cyanogen bromide cleavage leave the phycocyanobilin group unmodified.

Comparative NMR analysis of the two heptapeptides 2 and 3 shows no evidence for a second chromopeptide linkage (see earlier discussion). The conditions used for the isolation of chromopeptide 2 limit any second linkage to one that would be cleaved by cyanogen bromide in 70% formic acid at 25°C for 18 hrs and by subsequent chromotographic procedures. These limitations rule out both an ester linkage involving seryl[16] or tyrosyl[19] hydroxyl groups, and an amide linkage to the propionic acid side chains of the $\beta_1$-phycocyanobilin moiety. The proposed[17] aspartic acid-enol ester linkage is of doubtful stability even in the native protein. It also requires ring A to exist as a less stable (relative to pyrrole) tautomeric pyrrolenine. For the $\beta_1$-phycocyanobilin prosthetic group, the thioether linkage appears to be the only linkage to the protein.

In this report we present a methodology for the analysis of one of three phycocyanobilins of C-phycocyanin. This approach, which involves the isolation, HPLC purification, and spectroscopic analysis of small chromopeptides, will be applied to the study of the other two peptide-linked phycocyanobilins of C-phycocyanin. In this way, the structures and linkages of all the pigment prosthetic groups associated with an individual biliprotein should be established. Such data hopefully will

provide the fundamental structural information for further studies of the arrangement of the bilin prosthetic groups within the protein which are responsible for the light-harvesting effectiveness of algal biliproteins.

TABLE 7. 360 MHz $^1$H NMR assignments for the peptide moieties of chromopeptide 2 and synthetic peptide 3 in $D_2O$ at 40°C.

| Chemical shift[a] 2 | 3 | Number of H's | Multiplicity, J, Hz | Assignment |
|---|---|---|---|---|
| 4.07 | 4.07 | 1 | q, 7.2 (6.8)[c] | ala(1) αCH[d] |
| 1.51 | 1.53 | 3[b] | d, 7.2 (6.8) | ala(1) $\beta CH_3$ |
| 4.27 | 4.37 | 1 | q, 7.2 (6.9) | ala(2) αCH |
| 1.29 | 1.37 | 3 | d, 7.2 (6.9) | ala(2) $\beta CH_3$ |
| 4.50 | 4.67 | 1 | dd, 6.8, 7.6 | cys αCH |
| 3.02 | 3.00 | 1[b] | dd, 7.6, -13.9 | cys $\beta_1 CH$ |
| 3.11 | 3.11 | 1 | dd, 6.8, -13.9 | cys $\beta_2 CH$ |
| 4.26 | 4.41 | 1 | dd | leu αCH |
| 1.51 | 1.61 | 3[b] | m | leu $\beta CH_2$, γCH |
| 0.77 | 0.87 | 3 | d, 5.3 (4.1) | leu $\delta_1 CH_2$ |
| 0.83 | 0.92 | 3 | d, 5.3 (4.1) | leu $\delta_2 CH_2$ |
| 4.15 | 4.30 | 1 | dd, 6.0, 7.8 | arg αCH |
| 1.64 | 1.81 | 1[b] | m | arg $\beta_1 CH_2$ |
| 1.74 | 1.81 | 1[b] | m | arg $\beta_2 CH_2$ |
| 1.54 | 1.61 | 2[b] | m | arg $\gamma_2 CH_2$ |
| 3.11 | 3.19 | 2 | t, 6.8 | arg $\delta CH_2$ |
| 4.63 | 4.70 | 1 | dd, 5.9, 7.8 | asp αCH |
| 2.77 | 2.80 | 1 | dd, 7.8, -16.8 | asp $\beta_1 CH_2$ |
| 2.87 | 2.92 | 1 | dd, 5.9, -16.8 | asp $\beta_2 CH_2$ |
| 4.58 | 4.63 | 1 | dd, 9.4, 10.4 | hsl αCH |
| 2.30 | 2.32 | 1 | m | hsl $\beta_1 CH_2$ |
| 2.59 | 2.60 | 1 | m | hsl $\beta_2 CH_2$ |
| 4.37 | 4.38 | 1 | m | hsl $\gamma_1 CH_2$ |
| 4.53 | 4.54 | 1 | m | hsl $\gamma_2 CH_2$ |

[a]The chemical shift values are in ppm from sodium 2,2-dimethyl-2-silipenta-5-sulfonate (DSS) and $CHCl_3$ was used as the internal chemical shift marker. The chemical shift of 10 mM $CHCl_3$ in $D_2O$ is 7.66 ppm relative to DSS.

[b]Overlapping resonances were observed.

[c]The coupling constants in parenthesis represent values obtained for the chromopeptide 2 which differed from those of heptapeptide 3.

[d]Ala(1) designates the N-terminal solution.

TABLE 8. 360 MHz $^1$H NMR assignments for the peptide moieties of chromopeptide 2 and synthetic peptide 3 in D5-pyridine at 25°C.

| Chemical shift (ppm)[a] 2 | 3 | Number of H's | Multiplicity J, Hz | Assignment[b] |
|---|---|---|---|---|
| 4.57 | 4.70 | 1 | m | ala(1) αCH |
| 1.80 | 1.84 | 3 | d, 6.3(5.5) | ala(1) $\beta CH_3$ |
| 4.92-5.00 | 4.94-5.04 | 4[c] | m | ala(2) αCH |
| 1.50 | 1.53 | 3 | d, 7.0(7.4) | ala(2) $\beta CH_3$ |
| 5.14 | 5.26 | 1 | m | cys αCH |
| 3.42 | 3.49 | 1 | dd, 7.8, -13.6 | cys $\beta_1 CH_2$ |
| 3.54 | 3.64 | 1 | dd, 4.9, -13.6 | cys $\beta_2 CH_2$ |
| 4.92-5.00 | 4.94-5.04 | 4[c] | m | leu αCH |
| 1.89-2.00 | 1.89-2.00 | 7[c] | m | leu $\beta CH_2$ |
| 1.90 | 1.90 | 7[c] | m | leu γCH |
| 0.77 | 0.75 | 3 | d, 5.7 | leu $\delta_1 CH_3$ |
| 0.77 | 0.78 | 3 | d, 4.3 | leu $\delta_2 CH_3$ |
| 4.92-5.00 | 4.94-5.04 | 4[c] | m | arg αCH |
| 1.89-2.00 | 1.89-2.00 | 7[c] | m | arg $\beta CH_2$ |
| 1.89-2.00 | 1.89-2.00 | 7[c] | m | arg $\gamma CH_2$ |
| 3.33 | 3.30 | 2 | m | arg $\delta CH_2$ |
| 5.56 | 5.56 | 1 | m | asp αCH |
| 3.25 | 3.27 | 1 | dd, 7.3, -17.0 | asp $\beta_1 CH_2$ |
| 3.41 | 3.39 | 1 | dd, 5.9, -17.0 | asp $\beta_2 CH_2$ |
| 4.92-5.00 | 4.94-5.04 | 4[c] | m | hsl αCH |
| 2.23 | 2.20 | 1 | m | hsl $\beta_1 CH_2$ |
| 2.43 | 2.46 | 1 | m | hsl $\beta_2 CH_2$ |
| 4.09 | 4.09 | 1 | m | hsl $\gamma_1 CH_2$ |
| 4.29 | 4.29 | 1 | m | hsl $\gamma_2 CH_2$ |

[a]The chemical shift values are in ppm from TMS and were determined from a residual proton of pyridine (7.81 ppm from TMS at 25°C).

[b]The NH's were not assigned

[c]Multiple resonances occurred at these frequencies.

TABLE 9. 360 MHz $^1$H NMR assignments of the bilin moiety of chromopeptide 2 in $D_2O$ and pyridine-$d_5$ and the blue pigment 1 (BP) in pyridine-$d_5$.

| Chemical Shift[a] | | | Number of H's | Multiplicity J, Hz | Assignment |
|---|---|---|---|---|---|
| 2($D_2O$) | 2(pyr-$d_5$) | 1(pyr-$d_5$)[b] | | | |
| 1.02 | 1.23 | 1.11 | 3 | t, 7.4 | 18-$CH_2\underline{CH_3}$ |
| 1.21 | 1.39 | 1.34 | 3 | d, 7.3 | 2-$CH_3$ |
| 1.44 | 1.48 | 1.58 | 3 | d, 7.1 | 3'-$CH_3$ |
| 2.11 | 2.02 | 1.89 | 3 | s | 7,13,17-$CH_3$ |
| 2.15 | 2.07 | 1.95 | 3 | s | |
| 2.17 | 2.12 | 2.01 | 3 | s | |
| 2.23 | 2.48 | 2.34 | 2 | q | 18-$\underline{CH_2}CH_3$ |
| 2.69 | 2.83,2.85 | 2.70 | 4 | m | 8,12-$\underline{CH_2}CH_2$ |
| 2.69 | 2.70 | 2.70 | 1 | dq | 2-H |
| 3.11 | 3.09,3.17 | 2.97 | 4 | m | 8,12-$CH_2\underline{CH_2}$ |
| c | 3.15 | -- | 1 | m | 3-H |
| 3.58 | 3.52 | 6.17 | 1 | m | 3'-H |
| 5.94 | 5.87 | 5.71 | 1[d] | s | 5-H |
| 6.32 | 6.08 | 5.92 | 1 | s | 15-H |
| 7.41 | 7.29 | 7.09 | 1 | s | 10-H |

[a]The chemical shifts in $D_2O$ and pyridine-$d_5$ are reported in ppm from DSS and TMS respectively as described in Tables 7 and 8.

[b]From Ref. 10.

[c]The chemical shift attributed to the C3-methine was not determined in $D_2O$.

[d]Slow exchange of the C5-methine with $D_2O$ was reponsible for the reduced size of the signal.

Part II - Figure Captions

Fig. 13. Reversed phase HPLC (system C) of heptapeptide 3 as the crude reaction product and after purification.

Fig. 14. Reversed phase HPLC (system D) of crude chromopeptide 2.

Fig. 15. (a) The 360 MHz $^1H$ NMR spectrum of chromopeptide 2 at 40°C (2.4 mM, $D_2O$). (b) The 360 MHz $^1H$ NMR spectrum of synthetic heptapeptide 3 at 40°C (2.55 mM, $D_2O$).

Fig. 16. (a) The 360 MHz $^1H$ NMR spectrum of chromopeptide 2 at 25°C (2 mM, pyridine-$d_5$). (b) The 360 MHz $^1H$ NMR spectrum of synthetic heptapeptide 3 at 25°C (1 mM, pyridine-$d_5$).

Fig. 17. The partial 360 MHz $^1H$ NMR spectrum of the chromopeptide 2 taken at 40°C in $D_2O$: (a) after irradiation of the multiplet at 2.69 ppm; (b) after irradiation of the multiplet at 3.58 ppm; (c) after irradiation of the doublet at 1.21 ppm; (d) after irradiation of the multiplet at 1.44 ppm.

Fig. 18. The partial 360 MHz $^1H$ NMR spectrum of the chromopeptide 2 taken at 25°C in pyridine-$d_5$. Lower spectra: coupled. Upper spectra: (a) after decoupling the multiplet at 2.70 ppm; (b) after irradiation of multiplet at 3.52 ppm; (c) after irradiation of doublet at 1.39 ppm; (d) after decoupling the doublet at 1.48 ppm; (e) after irradiation of the multiplet at 3.15 ppm.

Fig. 19. The circular dichroism spectrum of the chromopeptide 2 in $D_2O$:

-------- = $5.7 \times 10^{-5}$ M,

———— = $2.21 \times 10^{-3}$ M.

Fig. 20. Absorption spectra in 25% HOAc of HPLC purified chromopeptide 2 ( ------- is $2.2 \times 10^{-5}$ M) and C-phycocyanin from *Synechococcus* sp. 6301 ( ———— is $5.7 \times 10^{-6}$ M).

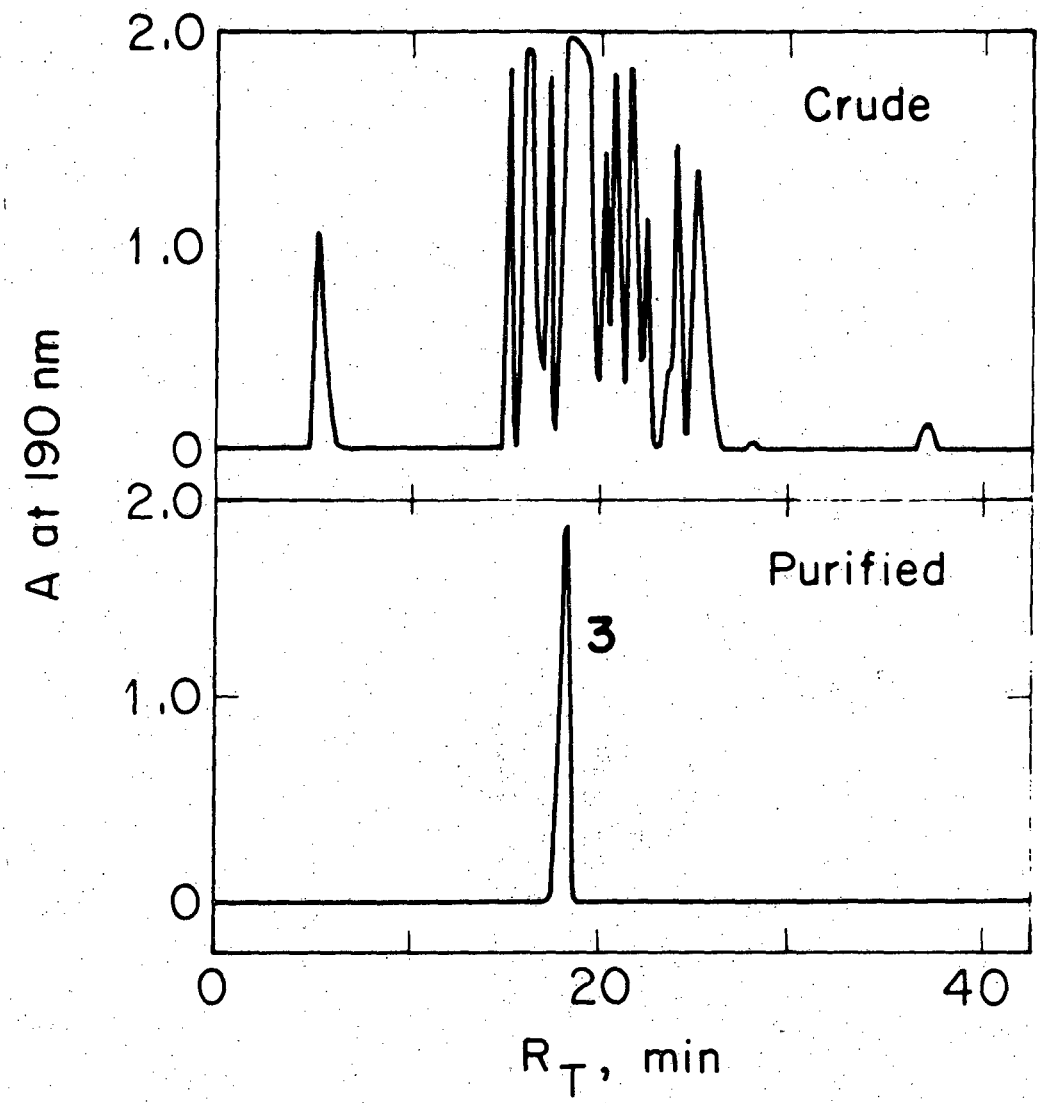



Fig. 13

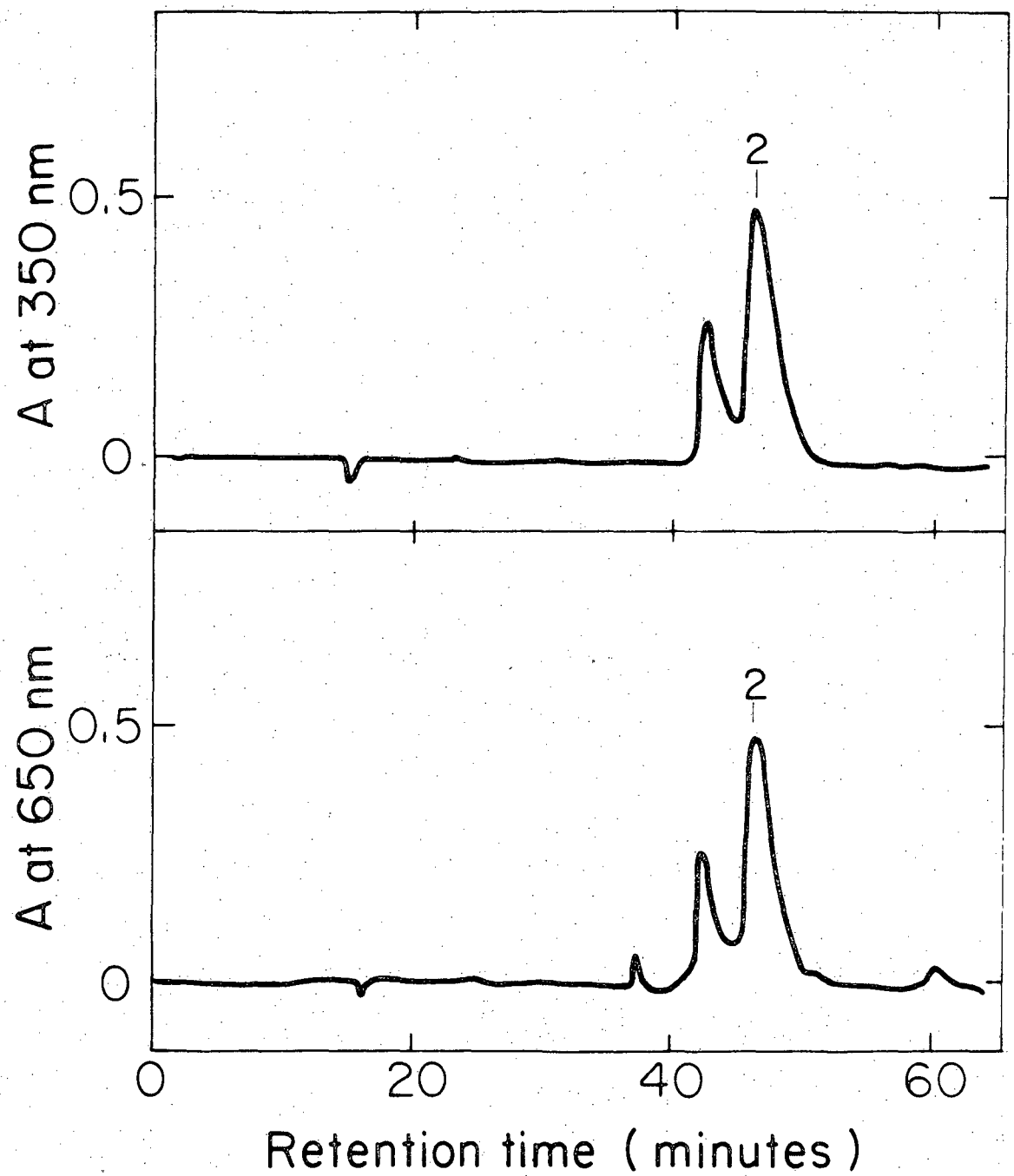



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Fig. 14

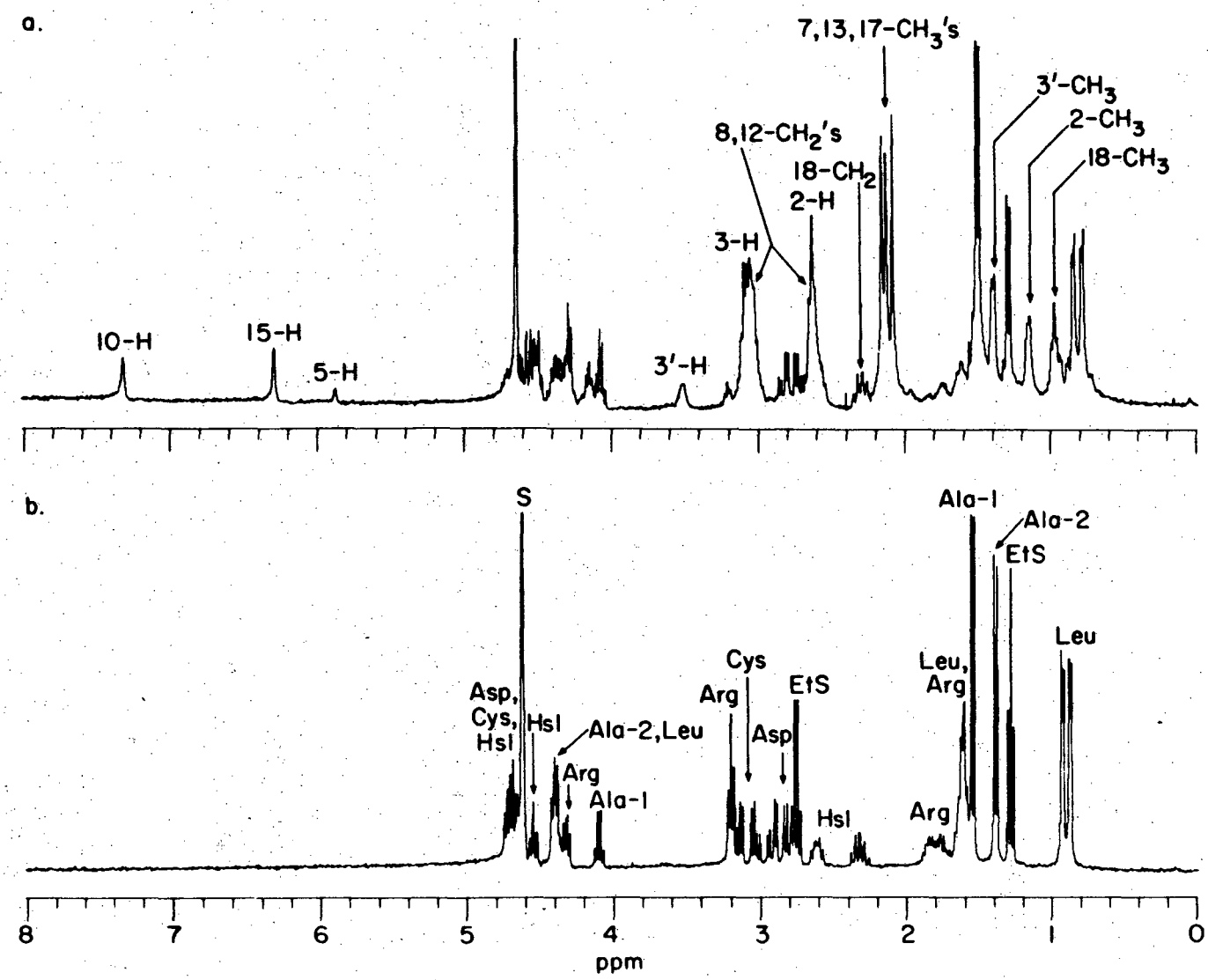


Fig. 15

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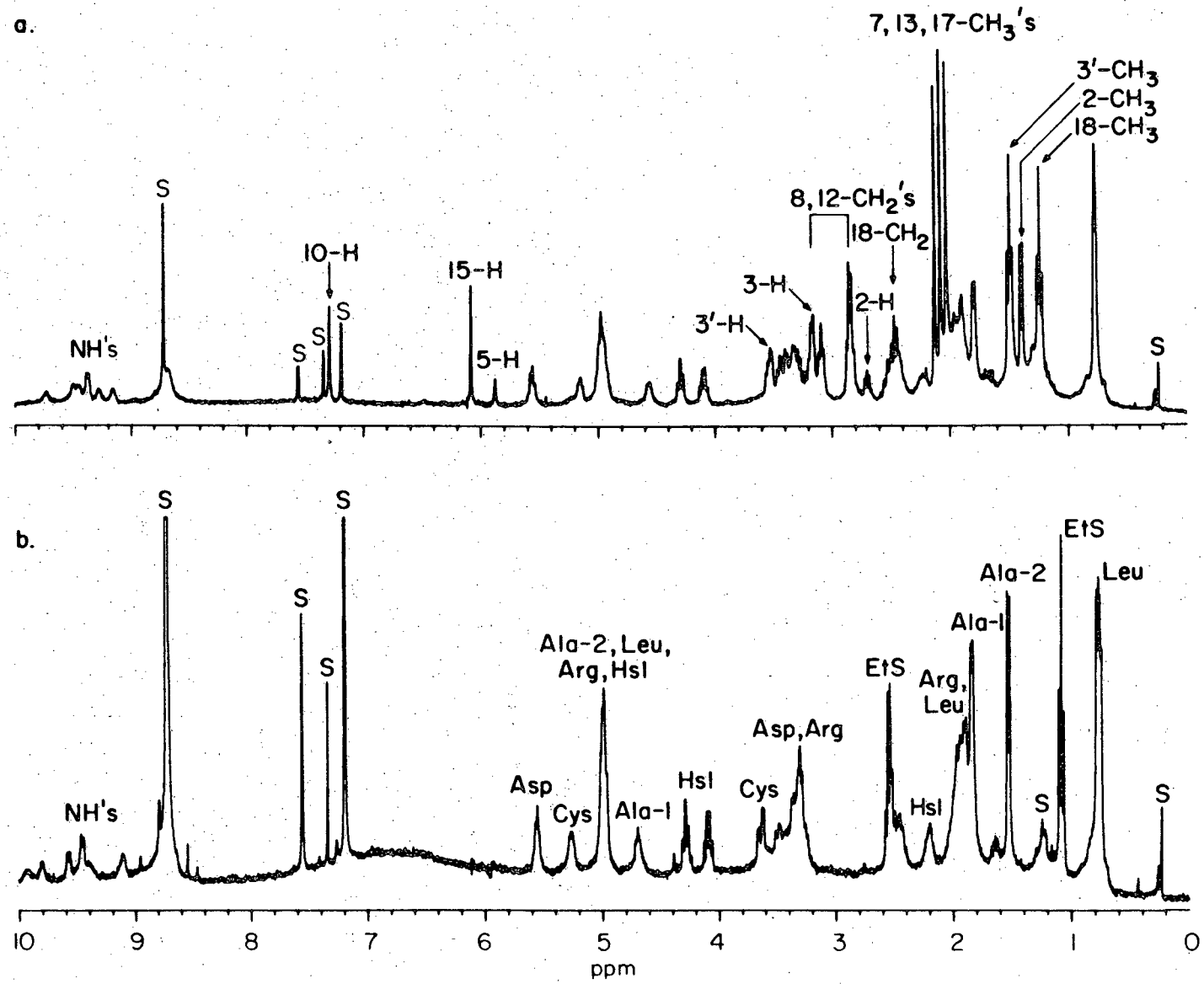


Fig. 16

XBL 799-11738

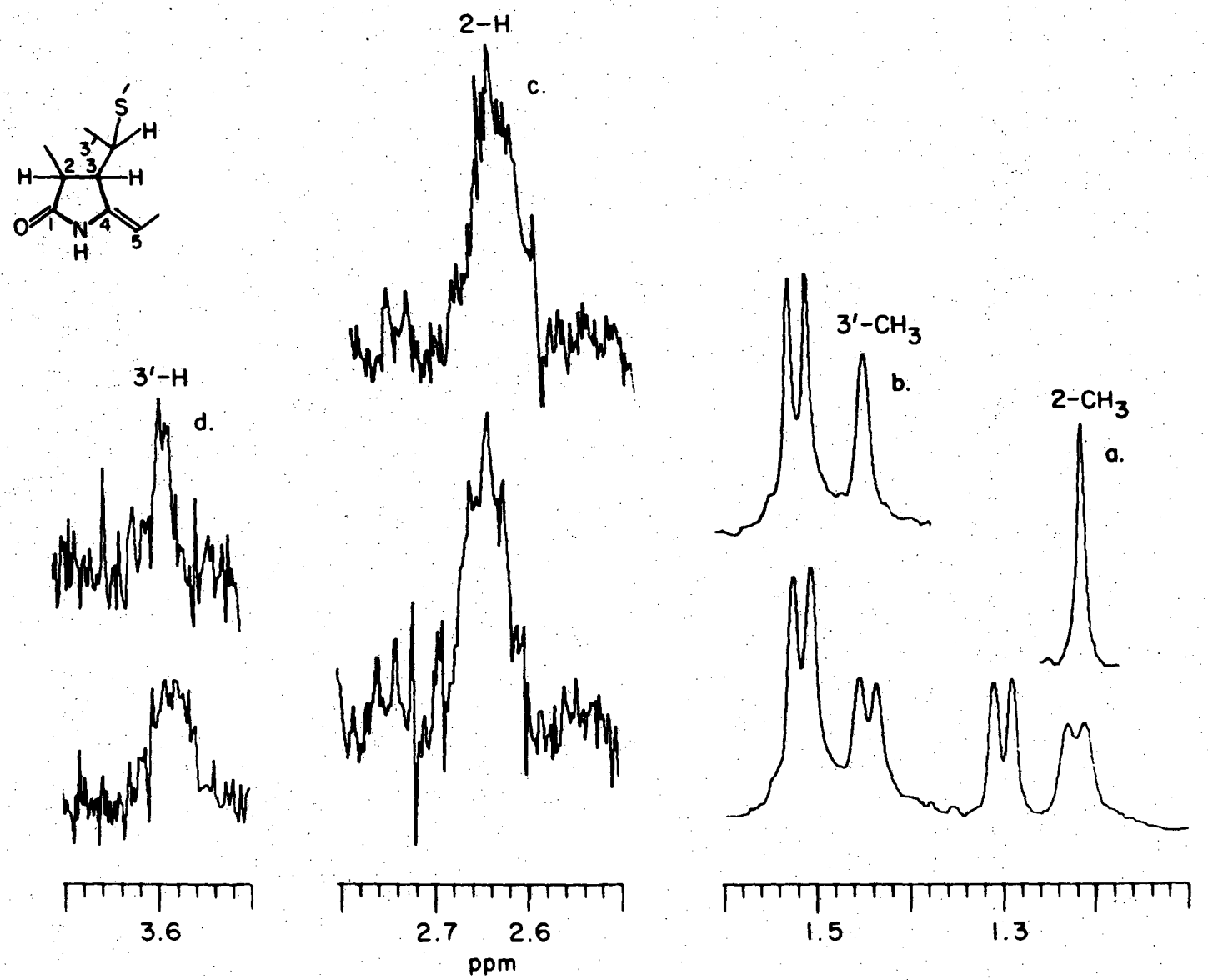


Fig. 17

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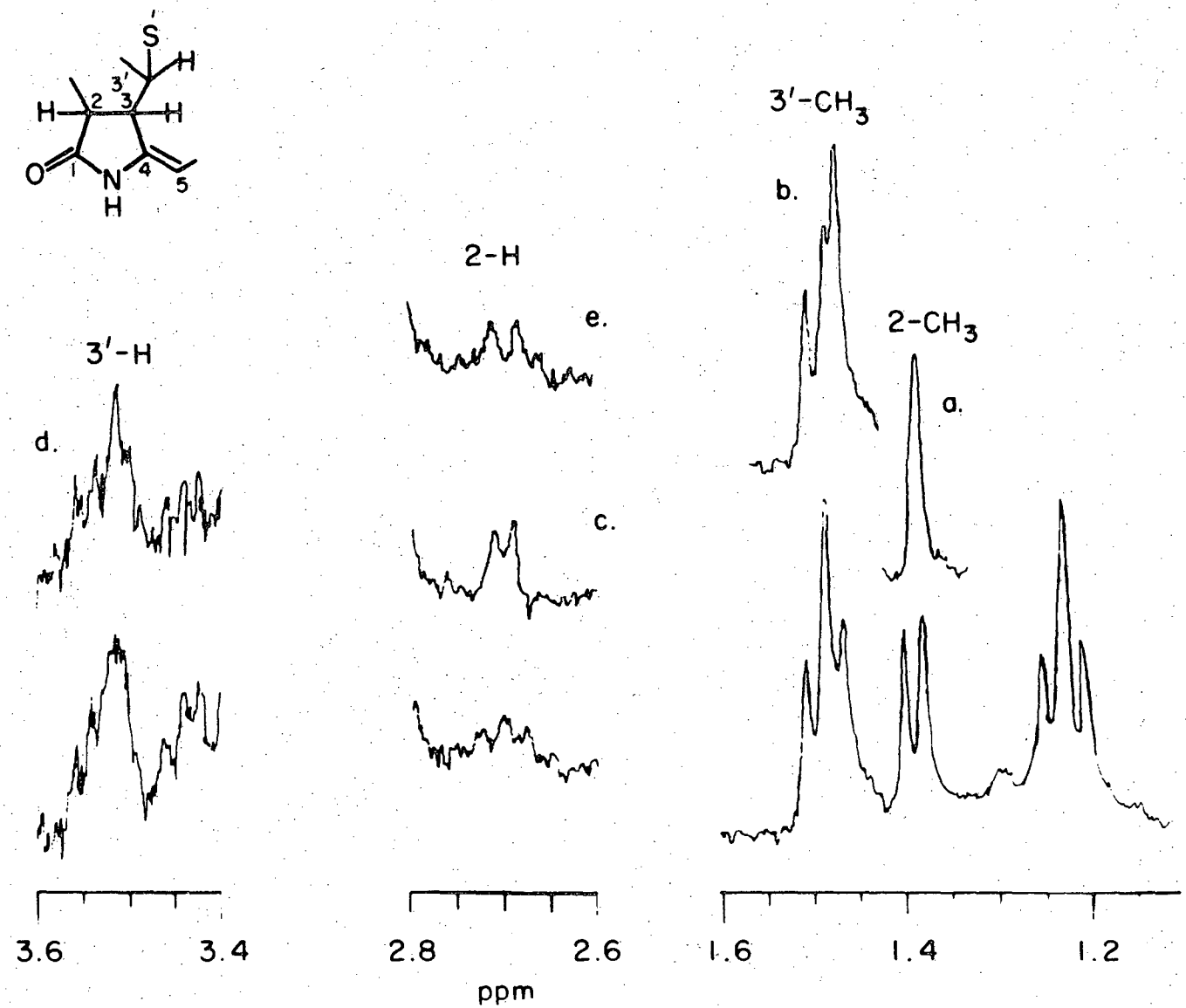


Fig. 18

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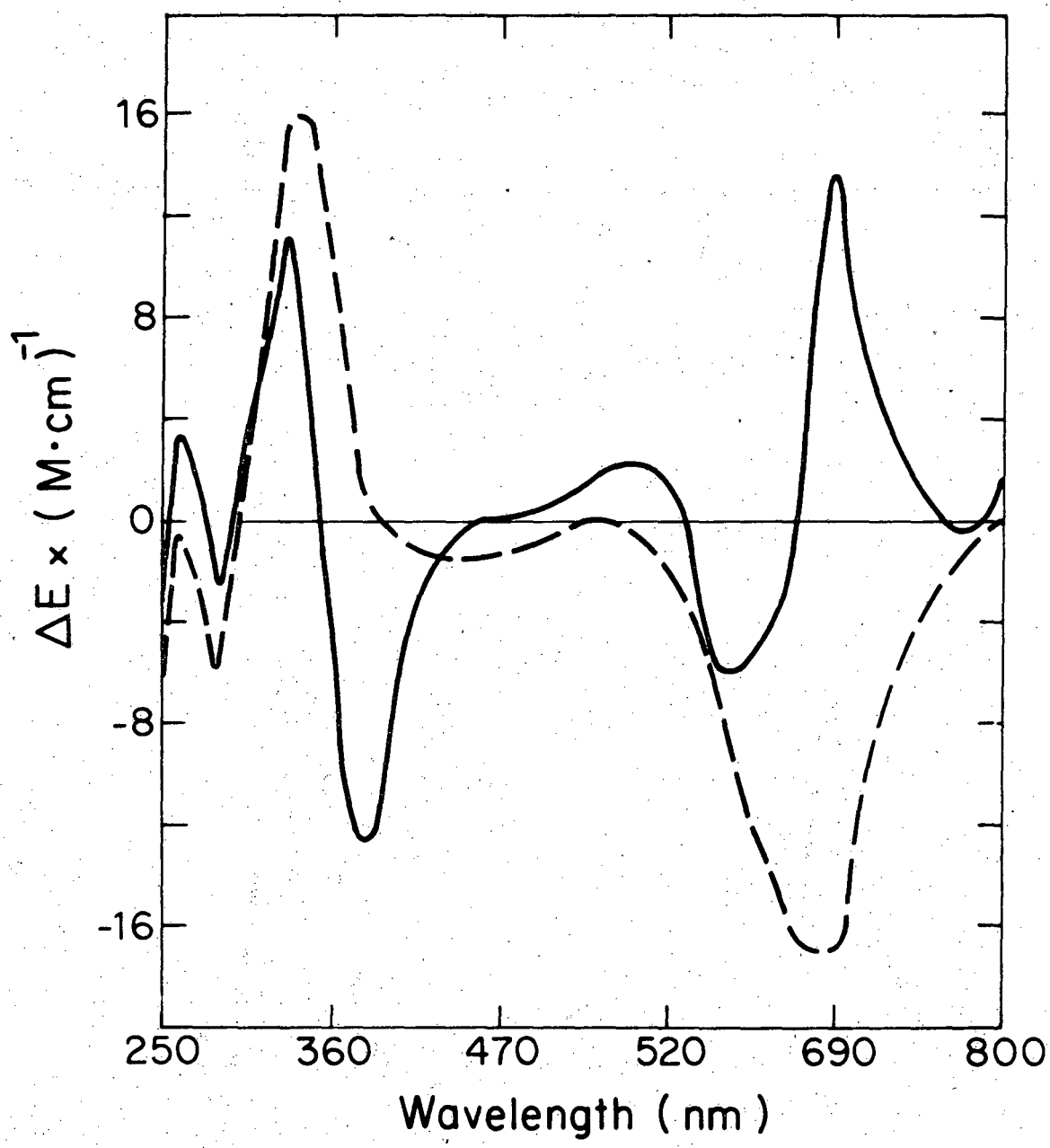


XBL 799-11733

Fig. 19

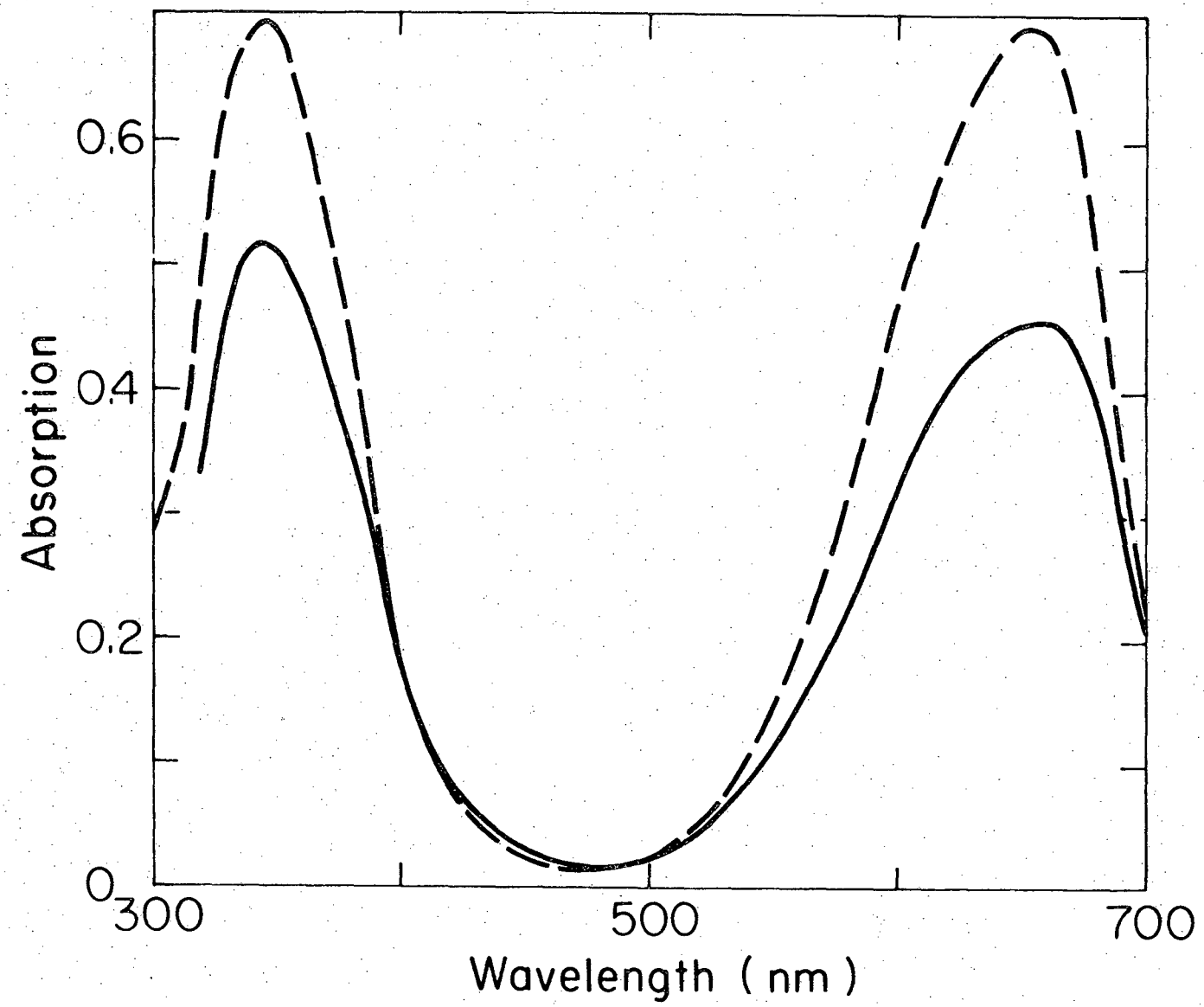


Fig. 20

XBL 799-11740

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## Experimental Section

### Methods

All reactions were performed under $N_2$. Solutions were dried over $Na_2SO_4$ and evaporations were done *in vacuo* with a Buchi Rotary Evaporator. Uncorrected mp's were determined on a Thomas-Hoover Capillary MP Apparatus and a Kofler Micro Hot Stage (μmp). $^1$H NMR spectra of the protected peptides 5-9 were taken in $CDCl_3$ solution using internal $Me_4Si$ (δ 0.0) on a homemade spectrometer[36] based on a Bruker 63 KG magnet operating at 270 MHz with a Nicolet 1180 data system. The 360 MHz $^1$H NMR spectra of the deblocked peptides 3, 11, and 12 and chromopeptide 2 in $D_2O$ and in pyridine-$d_5$ were taken at the Stanford Magnetic Resonance Laboratory. TLC was done on silica gel 60 (EM Reagents) with the following solvent systems: A (BuOH/HOAc/$H_2O$, 4/1/5, upper phase); B (benzene/acetone, 2/1); C (benzene/$Et_2O$, 1/1); D (benzene/acetone, 1/1). HPLC was done using a Spectra Physics 8000 instrument equipped with a Schoeffel 770 variable wavelength detector. The following column and condition systems were used: A [10 μ Spherisorb ODS (3 × 250 mm), isocratic (60/40, $CH_3CN/H_2O$), Flow rate (F) 0.5 mℓ/min]; B [10 μ Spherisorb ODS (3 × 250 mm), isocratic (25/75, $CH_3CN$/0.01M $KH_2PO_4$, pH 2.1), F 0.5 mℓ/min]; C [10 μ LiChrosorb RP 18 (10 × 250 mm), gradient (t = 0: 0.01M $KH_2PO_4$, pH 2.1, to 15 min; 25/75, $CH_3CN$/0.01M $KH_2PO_4$, pH 2.1, remaining isocratic to 30 min), F 2.0 mℓ/min]; D [10 μ LiChrosorb RP 18 (9 × 500 mm, all glass system),[37] same eluent as C, F 0.8 mℓ/min]. Absorption spectra were taken on a Cary 118 Spectrometer and are baseline corrected. CD spectra were taken on a homemade spectrometer.[38] Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley. Amino acid analyses were performed on a Beckman 120C Analyzer.

Materials

The following solvents were routinely distilled immediately prior to use: THF and dioxane from sodium benzophenone ketyl and dimethylacetamide from Linde 4A molecular sieves at reduced pressure. HPLC grade solvents from Burdick and Jackson Laboratories and water purified with a Milli-Q System [Millipore Corp.] were used for HPLC. Amino acid derivatives of L-alanine and L-aspartic acid were purchased from Sigma Chemical Company.

360 MHz $^1$H NMR Sample Preparation

After HPLC purification (systems C and D), the peptide solutions were concentrated to ~3 mℓ and desalted with Sephadex G10 (15 × 500 mm), eluting with 25% HOAc. Following lyophilization, the peptide residues were dissolved in 0.01M TFA-d in 99.8% $D_2O$ and lyophilized again. The solutions for NMR analysis were prepared in 99.996% $D_2O$ or 99.96% pyridine-$d_5$ [Aldrich Chemical Co.] in a dry box [Vacuum Atmosphere Corp.] under argon.

$\beta_1$-Phycocyanobiliheptapeptide (2). Pure *Synechococcus* sp. 6301 C-phycocyanin was prepared as previously described.[20] The protein preparation was dialyzed exhaustively against deionized glass-distilled water and lyophilized. Lyophilized phycocyanin (450 mg, ~12 μmoles) was dissolved in 14.5 mℓ of water, formic acid (98-100%, 34.5 mℓ) was slowly added to this solution with constant swirling, and then cyanogen bromide (600 mg, 5.7 mmol) was added and the mixture swirled for several minutes to dissolve it. Reaction was allowed to proceed in the dark, under nitrogen, for 18 hrs at room temperature. The reaction mixture was then

applied directly to a column of Sephadex G50 (Fine, 5 × 53 cm), equilibrated and developed with a 30% v/v aqueous acetic acid. The appropriate blue fractions were pooled, concentrated and purified by HPLC (system D, Fig. 14). The bilin-carrying heptapeptide 2 was obtained in a yield of 60%, as determined from the absorbance at 650 nm ($\varepsilon_M$ = 32,000 $M^{-1}$ $cm^{-1}$, Fig. 20); $^1H$ NMR ($D_2O$ and pyridine-$d_5$, Figs. 15 and 16); amino acid analysis and sequence analysis as in Ref. 20.

α-L-amino-γ-butyrolactone(L-homoserine lactone) (5). L-Homoserine was quantitatively converted to the lactone salt within 24 hrs at room temperature in anhydrous TFA. Evaporation of the solvent afforded a white solid: mp 122-125°C; $[\alpha]_D^{25}$ -33.9° (c 1.31, TFA). Anal. ($C_6H_8NO_4F_3$): C, H, N.

Peptide Synthesis — General Procedure

N-tert-Butoxycarbonyl-$O^{\beta}$-benzyl-L-aspartyl-L-homoserine lactone (6). N-tert-Butoxycarbonyl-$O^{\beta}$-benzyl-L-aspartic acid (3.23 g, 10 mmol) and N-methylmorpholine (1.01 g, 10 mmol) were dissolved in 50 ml THF.[39] After cooling to -15°C, isobutyl chloroformate (1.37 g, 10 mmol) was rapidly introduced, and one minute later a solution of L-homoserine lactone trifluoroacetic acid salt (2.15 g, 10 mmol) and triethylamine (1.01 g, 10 mol) in 25 ml THF was added. The cooling bath was then removed and the mixture was stirred for an additional 1.5 hrs, filtered and evaporated. The residue was dissolved in 100 ml ethyl acetate, washed with $H_2O$ (3 × 50 ml), sat. $NaHCO_3$ (3 × 50 ml), 0.3 N HCl (3 × 50 ml), sat. NaCl (1 × 50 ml), dried and evaporated to give the dipeptide 6 as an oil weighing 3.87 g (95%): TLC (B) $R_f$ 0.55; HPLC (A), RT 4.1 min; $^1H$ NMR

(see Supplementary Materials). Anal. ($C_{20}H_{26}N_2O_7$): C, H, N.

$N^{\alpha}$-tert-Butoxycarbonyl-$N^{G}$-p-methoxybenzenesulfonyl-L-arginyl-$O^{\beta}$-benzyl-L-aspartyl-L-homoserine lactone (7). Dipeptide 6 (3.63 g, 9.0 mmol) was dissolved in 40 ml anhydrous TFA containing 0.5 ml anisole at 0°C. After one-half hour, the solvent was evaporated and the oily residue was crystallized by triturating with $Et_2O$, affording 3.62 g, 96% yield of the TFA salt, mp 138-140°C. Following the synthetic procedure outlined above, the TFA salt (2.88 g, 6.9 mmol) and $N^{\alpha}$-tert-butoxycarbonyl-$N^{G}$-p-methoxybenzenesulfonyl-L-arginine (3.05 g, 6.9 mmol)[40] were condensed. After chromatography (B, 450 g $SiO_2$, 4 × 74 cm), pure tripeptide 7 (2.79 g, 55%) was obtained: mp 78-81°C; HPLC (A), RT 4.0 min; $^1H$ NMR (see Supplementary Materials). Anal. ($C_{33}H_{44}N_6O_{11}S$): C, H, N, S,

N-tert-Butoxycarbonyl-L-leucyl-$N^{G}$-p-methoxybenzenesulfonyl-L-arginyl-$O^{\beta}$-benzyl-L-aspartyl-L-homoserine lactone (8). Removal of the t-Boc protecting group from tripeptide 7 was accomplished with TFA/anisole at 0°C. Condensation of the tripeptide salt (2.42 g, 2.8 mmol) with N-tert-butoxycarbonyl-L-leucine (0.684 g, 2.96 mmol)[41] as described above, afforded tetrapeptide 8 as a glass weighing 2.35 g (99%): μmp 98°C; HPLC (A), RT 4.7 min; TLC (D), $R_f$ 0.4; $^1H$ NMR (see Supplementary Materials). Anal. ($C_{39}H_{55}N_7O_{12}S$): C, H, N.

N-tert-Butoxycarbonyl-S-ethylmercapto-L-cysteinyl-L-leucyl-$N^{G}$-p-methoxybenzenesulfonyl-L-arginyl-$O^{\beta}$-benzyl-L-aspartyl-L-homoserine lactone (9). Deblocking of the tetrapeptide 8 with TFA/anisole afforded the crystalline TFA salt after trituration with $Et_2O$. This salt (2.2 g, 1.9 mmol) was condensed with N-tert-butoxycarbonyl-S-ethylmercapto-L-

cysteine (0.57 g, 2.0 mmol)[41,42] to give 9 as a glass weighing 1.89 g (97%): µmp 108°C; HPLC (A), RT 6.3 min; TLD (D), $R_f$ 0.43; $^1H$ NMR (see Supplementary Materials). Anal. ($C_{44}H_{64}N_8O_{13}S_3$): C, H, N, S.

N-tert-Butoxycarbonyl-L-alanyl-S-ethylmercapto-L-cysteinyl-L-leucyl-$N^G$-p-methoxybenzenesulfonyl-L-arginyl-$O^\beta$-benzyl-L-aspartyl-L-homoserine lactone (10). The pentapeptide 9 (1.78 g, 1.8 mmol) was dissolved in a premixed solution of 7 ml 4N HCl in dioxane and 2.5 ml ethyl mercaptan. After 30 minutes, 40 ml $Et_2O$ was added and the resulting solid was filtered and triturated with ethyl acetate. A solution of the dihydrochloride salt (1.45 g, 1.5 mmol) thus obtained and triethylamine (0.30 g, 3.0 mmol) in 7.5 ml dimethylacetamide was added to a mixture of the preformed mixed anhydride of N-tert-butoxycarbonyl-L-alanine (0.29 g, 1.6 mmol) at -15°C as before. After purification, the hexapeptide 10 (1.5 g, 94%) was obtained: mp 131-145°C; HPLC (A), RT 7.2 min; TLC (D) $R_f$ 0.36; $^1H$ NMR (in acetone-$d_6$; see Supplementary Materials). Anal. ($C_{47}H_{69}N_9O_{14}S_3$): C, H, N.

N-tert-Butoxycarbonyl-L-alanyl-L-alanyl-S-ethylmercapto-L-cysteinyl-L-leucyl-$N^G$-p-methoxybenzenesulfonyl-L-arginyl-$O^\beta$-benzyl-L-aspartyl-L-homoserine lactone (4). Removal of the t-Boc group of 10 with HCl/dioxane/EtSH was accomplished as above. The heptapeptide 4 was prepared in 98% yield (1.26 g) from the hexapeptide dihydrochloride salt (1.31 g, 1.2 mmol). Recrystallization of the crude product from acetone gave analytically pure material: µmp 167°C; HPLC (A), RT 7.8 min. Amino acid analysis: [1] 6N HCl 110°C/16 hrs: amino acid, experimental value (calculated number of residues) — ala 2.00 (2), 1/2 cys 0.57 (0.50),

leu 0.98 (1), arg 1.01 (1), asp 1.01 (1), hse/hsl 0.95 (1); [2] $HCO_3H$/ 0°C/4 hrs followed by 6N HCl/110°C/16 hrs: ala 2.00 (2), cys-acid 1.00 (1), leu 0.90 (1), arg 0.92 (1), asp 0.93 (1), hse/hsl 0.88 (1). Anal. ($C_{50}H_{74}N_{10}O_{15}S_3$): C, H, N, S.

HF Deblocking — General Procedure

L-Alanyl-L-alanyl-S-ethylmercapto-L-cysteinyl-L-leucyl-L-arginyl-L-aspartyl-L-homoserine lactone (3). The blocked heptapeptide 4 (27.0 g, 23 mmol) and ethyl mercaptan (300 μℓ, 4.0 mmol) were dissolved in 1.5 ml anhydrous hydrogen fluoride freshly distilled from $CoF_3$. After one-half hour stirring at 0-5°C, the HF was removed in vacuo. The residue was dissolved in 2 ml $H_2O$, washed with EtOAc (3 × 2 ml) and lyophilized. Following HPLC purification (system B, Fig. 13) and desalting with sephadex G10, the heptapeptide 3 was obtained as the di-TFA salt in 30% yield: μmp 173°C; HPLC (B), RT 20.9 min; $^1H$ NMR ($D_2O$, see Fig. 15).

L-Leucyl-L-arginyl-L-aspartyl-L-homoserine lactone (11). The tetrapeptide 8 (11.3 mg, 13.3 μmol) was deblocked in an analogous manner. The crystalline tetrapeptide salt (10 mg, 100%) of 11 thus obtained was chromotographically and spectroscopically pure: μmp 114°C; HPLC (B), RT 11.2 min; TLC (A), $R_f$ 0.17; $^1H$ NMR ($D_2O$, see Supplementary Materials).

S-Ethylmercapto-L-cysteinyl-L-leucyl-L-arginyl-L-aspartyl-L-homoserine lactone (12). The deblocking of the pentapeptide 9 (33 mg, 34 mol) was accomplished as described previously and the crude product (15.6 mg, 53%) was purified by HPLC: HPLC (B), RT 18.5 min; mp 123°C; $^1H$ NMR ($D_2O$, see Supplementary Materials).

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23. The chromopeptide 2 obtained by CNBr cleavage of C-phycocyanin and the subject of this report corresponds to the peptide CNBr-2 of Ref. 20.

24. We have used the following abbreviations for structural simplicity: SSEt to represent the S-ethylmercapto protecting group for the cysteine SH; MBs to represent the p-methoxybenzenesulfonyl protecting group for the arginyl guanidine moiety; Bn for benzyl; Hse and Hsl to represent L-homoserine and its lactone; TFA for trifluoroacetic acid.

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27. Ring opening of homoserine lactone was found to be quite difficult even in aqueous bicarbonate solutions. The $^1$H NMR study of deblocked peptides 4, 11 and 12 in neutral and acidic $D_2O$ showed no evidence for the free hydroxy acid even after 4 hrs at 40°C.

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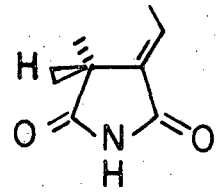
31. The assignment of R stereochemistry for C-2 made in Ref. 22 has been based on the isolation of optically active E-2-ethylidene-3-methylsuccinimide (i) from the chromic acid degradation of C-phycocyanin. However, the actual optical activity data for the succinimide cleavage product obtained in this way are unavailable in the literature. In another study, levorotatory enantiomer of succinimide i has been reported as resulting from the chromate degradation of the blue pigment 1 [G.Knobloch, Doctoral Thesis, Technical University, Braunschweig, (1972)]. By comparison with the authentic (+) and (-) isomers [H.Brockman, Jr. and G.Knobloch, *Chem. Ber.* 106, 803 (1973)], the assignment of absolute stereochemistry at C-2 has been made. Since the blue pigment 1 may not be representative of all three phycocyanobilin moieties of C-phycocyanin, the assignment of absolute stereochemistry at C-2 based on the optical activity of succinimide cleavage products obtained from 1 or from the native biliprotein requires the demonstration of the stereochemical identity of all three phycocyanobilins.



i

32. Before the present study, no direct experimental evidence had appeared regarding the geometry of ring A of phycocyanobilin. Based on biosynthetic analogy with other tetrapyrrolic pigments with trans-dihydro rings, trans-stereochemistry for ring A of

phycocyanobilin has been proposed (Ref. 22). The coupling constant of 4.3 Hz, which we observe for $^3J_{2H-3H}$ of 2, closely agrees with the value of this coupling constant in trans-succinimide models, the cis coupling constant being somewhat larger (Refs. 22 and 30). However, only when the appropriate bilitriene models with cis- and trans-dihydro ring A are compared, can be validity of the stereochemical assignment be ascertained.

33. In Refs. 22 and 30 a concerted trans-periplanar elimination mechanism is demonstrated for model succinimides. The authors argue that the obtention of only E-2-ethylidene-3-methyl-succinimide from chromic acid-treated C-phycocyanin, contaminated with none of the Z isomer, suggests that the same mechanism must be occurring with the biliprotein. This requires the relative stereochemistry of C-3 and C-3' be RR or SS.

34. In a preliminary study of C-phycocyanin, we have observed that in addition to the blue pigment 1, other pigments are obtained from the methanol-treated biliprotein.

35. The slight upfield shifts of the N-terminal alanine residue of 2 has been interpreted to represent internal salt formation with one of the propionic acid side chains of the $\beta_1$-phycocyanobilin chromophor in pyridine-$d_5$. The difference in the chemical shifts of the homologous C-8 and C-12 methylenes of 2 (Table 9) support this hypothesis.

36. The 270 MHz spectrometer was designed and constructed by Willy C. Shih of the Laboratory of Chemical Biodynamics, U.C.Berkeley.

37. When a brilliantly blue solution of chromopeptide 2 was passed through a stainless steel Millipore filter, a multi-hued solution emerged. Thus an all-glass and teflon system was devised to avoid the presumed metal-catalyzed decomposition of the chromopeptide 2.

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39. For the syntheses of the penta-, hexa- and heptapeptides (9, 10 and 4, respectively), N,N-dimethylacetamide was used instead of THF because of its better solvent properties.

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Appendix to Part II: Elemental analyses.

| Compound | Molecular formula | Calculated | | | | Found | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | C | H | N | S | C | H | N | S |
| 5 | $C_6H_8NO_4F_3$ | 33.50 | 3.75 | 6.51 | - | 33.22 | 3.95 | 6.46 | - |
| 6 | $C_{20}H_{26}N_2O_7$ | 59.10 | 6.45 | 6.89 | - | 58.84 | 6.42 | 6.73 | - |
| 7 | $C_{33}H_{44}N_6O_7S$ | 54.08 | 6.05 | 11.47 | 4.38 | 53.79 | 6.01 | 11.20 | 3.99 |
| 8 | $C_{39}H_{55}N_7O_{12}S$ | 55.37 | 6.55 | 11.59 | - | 54.97 | 6.49 | 11.49 | - |
| 9 | $C_{44}H_{64}N_8O_{13}S_3$ | 52.36 | 6.39 | 11.10 | 9.53 | 52.54 | 6.27 | 11.07 | 9.96 |
| 10 | $C_{47}H_{69}N_9O_{14}S_3$ | 52.25 | 6.44 | 11.67 | - | 52.19 | 6.29 | 11.60 | - |
| 4 | $C_{50}H_{74}N_{10}O_{15}S_3$ | 52.16 | 6.48 | 12.17 | 8.36 | 51.86 | 6.52 | 12.04 | 8.09 |

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