14-Nor-9α-hydroxy-α-agarofuran (18). A solution of 0.15 g of 14-nor-10-epieudsm-4-ene-3,9,11-triol[3] was treated with 0.040 g of *p*-toluenesulfonic acid in 10 mL of benzene and 4 mL of THF, as described above. After chromatography on Woelm silica gel there was obtained 0.090 g (64%) of hydroxyagarofuran 18 as a thick oil, which slowly decomposed on standing: NMR δ 0.87, 1.21, 1.34 (s, 3 H each, CH_3), 3.94 (dd, J = 5.8, 10.5 Hz, 1 H, CHOH), 5.38, 5.87 (m, 1 H each, vinyl H).

Oxidation of 0.072 g of this with Jones reagent in the usual manner gave 0.070 g (98%) of 14-nor-9-oxo-α-agarofuran (4), identical in all respects with material obtained previously.[3]

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Registry No. 1, 5956-12-7; 4, 82309-55-5; 7, 60064-95-1; 8, 82309-56-6; 9, 82309-57-7; 10, 82246-77-3; 11, 60113-60-2; 12, 82309-58-8; 13, 82309-59-9; 14, 82246-78-4; 15, 82246-79-5; 16, 82246-80-8; 17, 82390-13-4; 18, 82246-81-9; 11,12-epoxy-10-epieudesm-4-en-3-one, 82309-60-2; diethyl azodicarbonylate, 1972-28-7; 14-nor-10-epieudesm-4-en-3-one, 66428-81-7; 3β,4β-epoxy-14-nordihydroagarofuran, 82246-83-1; 14-nor-10-epieudesm-4-en-3,9,11-triol, 82246-84-2.

Solid-Phase Synthesis of Protected Peptides on a Polymer-Bound Oxime: Preparation of Segments Comprising the Sequence of a Cytotoxic 26-Peptide Analogue

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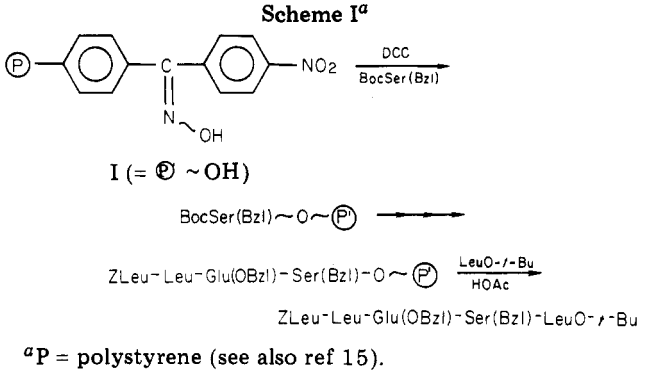
For demonstration of the utility of the *p*-nitrobenzophenone oxime polymer I, protected peptides ranging in length from three to seven amino acids were prepared. These peptides were removed from this support by carboxylic acid catalyzed aminolysis with amino acid or peptide esters. These products were obtained in yields ranging from 16% to 65% and were of high purity. The segments synthesized correspond to the sequence of a cytotoxic 26-peptide analogue of melittin.

We have recently developed the *p*-nitrobenzophenone oxime polymer I as a support for solid-phase peptide synthesis of protected peptide segments.[1] Peptides are displaced from this support with nucleophiles such as hydrazine, yielding peptide hydrazides. Peptides may also be cleaved from this support by nucleophilic displacement with amino acid esters. This reaction is rather slow, but its catalysis by carboxylic acids felicitously allowed synthesis of a number of di- and tripeptides. Furthermore, displacement of dipeptides from I appeared to occur without concomitant racemization when the reaction was catalyzed by carboxylic acids. We now report the application of this approach for the synthesis of longer protected peptides (cf. Scheme I) which might serve as useful intermediates in the segment condensation approach to peptide synthesis. We also show that not only amino acid esters but also peptide esters may be used to cleave peptides from I. This allows segment condensation of peptides assembled on I with previously purified amino compounds.

To illustrate the utility of this approach, we have prepared segments comprising the sequence of the cytotoxic peptide II, which is an analogue of melittin (Figure 1). The synthesis of this highly active cytotoxin[2,3] by the stepwise Merrifield method has been described. Peptide II was designed with little sequence homology to melittin in the amino terminal 20 residues to demonstrate that these residues serve a purely structural role and may be replaced by a sequence with a high potential to form a predominantly hydrophobic amphiphilic α helix. We would now like to prepare analogues of peptide II with replacements in the C-terminal portion of the chain. We have therefore prepared segments of II which are suitably protected for condensation from the amino toward the carboxyl terminus.[4]

Scheme I[a]



[a] P = polystyrene (see also ref 15).

Results

The C-terminal segment Z-Leu-Leu-Glu(OBzl)-Ser-(Bzl)-Leu-O-*t*-Bu (III; see ref 15 for a list of abbreviations) was prepared by starting from BocSer(Bzl)-I (Scheme I). The subsequent residues were coupled as symmetric anhydrides,[6] and the resulting tetrapeptide was displaced

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Table I. Properties of Synthetic Peptides

compd	yield, %	mp, °C	$[\alpha]_D$,[a] deg
ZLeu-Leu-Glu(OBzl)-Ser(Bzl)-Leu-O-*t*-Bu (III)	60	208-210	-24.0
NPSLeu-Ser(Bzl)-LeuO-*t*-Bu (IV)	65	137-138	-36.8
NPSLeu-Gln-Ser(Bzl)-Leu-Leu-Ser(Bzl)-LeuO-*t*-Bu (V)	46	235-238	-23.3
NPS-Leu-Leu-Gln-Trp(For)-LeuO-*t*-Bu (VI)	64	233	-21.4
BocLys(2ClZ)-Arg(Tos)-Lys(2ClZ)-Arg(Tos)-Gln-GlnNH$_2$ (VII)	30	133-136	-5.8

[a] At concentrations of 1 g/mL in DMF.

MELITTIN

```
                    5                   10
H2N-GLY-ILE-GLY-ALA-VAL-LEU-LYS-VAL-LEU-THR-
                   15                   20
   THR-GLY-LEU-PRO-ALA-LEU-ILE-SER-TRP-ILE-
                   25
   LYS-ARG-LYS-ARG-GLN-GLN-CONH2
```

PEPTIDE II

```
                    5                   10
H2N-LEU-LEU-GLN-SER-LEU-LEU-SER-LEU-LEU-GLN-
                   15                   20
   SER-LEU-LEU-SER-LEU-LEU-LEU-GLN-TRP-LEU-
                   25
   LYS-ARG-LYS-ARG-GLN-GLN-CONH2
```

Figure 1. Amino acid sequences of melittin[13] and peptide II.[2]

from the oxime resin with 1.5 equiv of the acetate salt of LeuO-*t*-Bu. The reaction appeared to be complete within 12 h as judged by the disappearance of the oxime ester absorption at 1775 cm^{-1} in the IR spectrum of an aliquot of the resin. The desired peptide was isolated in 60% yield (based on the attachment of the first amino acid to the resin) by crystallization from methanol. In order to determine the effect of the substitution level of the initial amino acid on the resin on the purity and yield of the final product, we have synthesized this pentapeptide using oxime resins substituted with 0.26, 0.44, and 0.80 mmol of serine/g of resin. In each case chromatographically homogeneous, crystalline products were obtained in 51%, 66%, and 60% yields, respectively. Since the workup procedure was not identical in each case, we do not believe these differences in yields are significant.

The tripeptide NPSLeu-Ser(Bzl)-LeuO-*t*-Bu (IV) was similarly prepared by starting with the BocSer(Bzl)-I polymer. Addition of NPSLeu as its symmetric anhydride and subsequent cleavage of the dipeptide from the resin with the acetate salt of LeuO-*t*-Bu gave a crude product which was crystallized in 65% yield. NPSLeu-Leu-Gln-Trp(For)-LeuO-*t*-Bu (VI) was prepared by an analogous procedure and purified by precipitation from DMF by addition of ethanol/water (4:1), giving a chromatographically pure peptide in 64% yield.

Peptide esters are also effective amino components for removal of other peptides from the oxime resin. For instance, the heptapeptide NPSLeu-Gln-Ser-Ser(Bzl)-Leu-Leu-Ser(Bzl)-LeuO-*t*-Bu (V) was synthesized by aminolysis of NPSLeu-Gln-Ser(Bzl)-Leu-I with 1.2 equiv each of HCl-Leu-Ser(Bzl)-LeuO-*t*-Bu (prepared from IV by treatment with HCl in ether), diisopropylethylamine (DIEA), and acetic acid. The reaction was 90% complete within 13 h as judged from the infrared spectrum of an aliquot of the resin, and upon subsequent workup the heptapeptide was obtained in 46% yield.

We synthesized the C-terminal hexapeptide of peptide II, BocLys(2ClZ)-Arg(Tos)-Lys(2ClZ)-Arg(Tos)-Gln-GlnNH$_2$ (VII), employing the widely used chloromethyl resin as a support. Removal of this hexapeptide from the resin by ammonolysis with ammonia-saturated trifluoroethanol (TFE) proceeded to only 60% completion after 3 days, and the resulting product was difficult to purify. Chromatography with LH-20 Sephadex and subsequent fractional precipitation from hot methanol gave an analytically pure product in 30% yield.

The physical data for the peptides described above are illustrated in Table I. Satisfactory analytical data (C, H, and N) were obtained for compounds IV–VII. The 270-MHz proton magnetic resonance spectra were consistent with their structures. All the peptides were homogeneous by criteria of chromatography employing silica thin-layer plates and at least two different solvent systems.

Discussion

The oxime ester bond to I serves as an α-carboxylate protecting group which can be activated toward nucleophilic attack by addition of a carboxylic acid such as acetic acid. Thus, by displacing peptides from I with the acetate salts of amino acid and peptide *tert*-butyl esters, it is possible to synthesize peptide esters which would not have been accessible by other solid phase methods. The amino protecting group for segments IV–VI is the NPS moiety.[8] This protection scheme allows selective unmasking[8,9] of the α-amino function for elongation in the N-terminal to C-terminal[4] direction. In cases where elongation in the C to N direction or bilateral elongation is required, an α-amine protecting group such as the (fluorenylmethoxy)-carbonyl[10] or 3-nitro-2-pyridinesulfenyl[11] may be employed. Peptides terminating with these protecting groups have also been prepared by using the oxime resin.[16] Thus the oxime resin allows the rapid synthesis of a versatile array of protected peptide intermediates.

The high level of carboxylate activation inherent to the oxime ester linkage might lead to unwanted intersite reactions if polymers with high loadings of amino acids are employed in the synthesis of peptides on I. However, we found that the pentapeptide III could be synthesized on polymers with relatively high substitution levels (0.80 mmol/g of resin) without greatly affecting the yield or purity of the final product. On the other hand, lower substitution levels (0.2–0.3 mmol/g) are advisable for the synthesis of peptides containing NPSLeu since this amino acid coupled rather slowly to the resin-bound peptides during synthesis of IV–VI. In these cases, intersite ami-

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nolysis reactions decreased the yield and purity of these peptides when highly substituted resins were employed. For instance, IV was obtained in only 30% yield when a resin with a substitution level of 0.8 mmol serine/g of resin was used to prepare this tripeptide.

The coupling of segments (III–XII) is now in progress. The results of these reactions, together with the evaluation of the biological activities of analogues of peptide II with altered C-terminal sequences, will be the subject of a subsequent report.

Experimental Section

Materials and Methods. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 283 infrared spectrophotometer by using KBr pellets. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. The syntheses were carried out with a Beckman Model 990 peptide synthesizer or were done manually. Amino acid analyses were performed with a Beckman Model 121 amino acid analyzer. Elemental analyses were by Galbraith Laboratories, Knoxville, TN. Proton NMR spectra were recorded on a Bruker HS-270 spectrophotometer equipped with a Nicolet data processor system.

Precoated silica gel TLC plates (Merck F-254) were purchased through Scientific Products. The following solvent systems were used: A, chloroform/methanol (95:5); B, chloroform/ethanol (98:2); C, chloroform/acetic acid (95:5); D, chloroform/methanol/acetic acid (93:2:5); E, chloroform/methanol/acetic acid (85:10:5); F, *n*-butanol/acetic acid/water (4:1:1); G, *n*-butanol/acetic acid/water/pyridine (15:3:12:10). Spots were detected by UV or by treatment with ninhydrin after deprotection of the N-blocking group with HCl vapor.

Amino acid derivatives were purchased from a variety of sources and were checked for purity by TLC with solvent systems C and D before use. If impurities were detected, the amino acid derivatives were purified to homogeneity by crystallization. All other organic chemicals were purchased from Aldrich. Styrene (1% of divinylbenzene copolymer) was purchased from Bio-Rad (Biobeads SX1). Solvents were purified as previously described.

For larger scale production of the oxime resin I, the previously published procedure[1] gave low substitution levels, and the resulting resin was contaminated with a dark brown impurity. The following procedure gave consistent results and a very clean product with up to 100 g of resin. *p*-Nitrobenzoyl chloride (12 g) and aluminum chloride (12 g) were dissolved in 100 mL of nitrobenzene dried over 3A molecular sieves. The resulting solution was filtered into a dropping funnel. This was slowly added over 30 min to a vigorously refluxing mixture of 70.0 g of Biobeads SX1 and 1.2 L of dichloroethane (distilled from phosphorus pentoxide immediately before use) with efficient stirring. Stirring and heating were continued for 12 h, and the resin was then filtered and washed with dioxane/4 N HCl (3:1; 3 × 1 L), dioxane/water (3:1; 3 × 1 L), DMF (3 × 1 L), and methanol (6 × 0.5 L): yield 76.7 g; substitution level 0.59 mmol/g. The resulting ketone polymer was added to a boiling solution of 70 g of hydroxylamine hydrochloride, 100 mL of pyridine, and 500 mL of ethanol over a 30-min period. Efficient stirring was maintained throughout the above operation and is essential for quantitative conversion of the ketone to the oxime. The mixture was stirred and heated at reflux for another 8 h. The resin was then collected by filtration and washed with methanol/water (3:1; 3 × 0.5 L), DMF (2 × 1 L), and methanol (6 × 0.3 L); yield 70 g.

LeuO-*t*-Bu Acetate (VIII). LeuO-*t*-Bu·HCl (13.3 g) was dissolved in methanol and passed through a 2.5 × 50 cm column of Amberlite IRA400 in the acetate form. Evaporation of the solvent from the eluent gave a solid which was crystallized from methylene chloride/petroleum ether: yield 11.6 g; mp 87–88 °C; $[\alpha]_D$ +9.9° (*c* 1.4, DMF). Anal. Calcd for $C_{12}H_{25}NO_4$: C, 58.27; H, 10.19; N, 5.66. Found: C, 58.32; H, 10.26; N, 5.62.

ZLeu-Leu-Glu(OBzl)-Ser(Bzl)-LeuO-*t*-Bu (III). Boc-Ser(Bzl)-I (3.5 g 3.0 mmol) was deprotected and coupled with the appropriate symmetric anhydrides by using an automated peptide synthesizer and a program previously described.[1] Coupling times were 30 min. The peptide was cleaved from the resin by treatment with a 1.5-fold excess of LeuO-*t*-Bu acetate in methylene chloride for 12 h. The resin was filtered and washed three times with methylene chloride. The filtrate was then washed three times with 5% aqueous citric acid and three times with water and dried over magnesium sulfate. The methylene chloride was evaporated in vacuo to a final volume of 20 mL, and 50 mL of methanol was added. The solvent was again evaporated to about 20 mL, and another 50-mL portion of methanol was added. This process was repeated again, and the peptide crystallized upon standing at 5 °C overnight: yield 1.68 g (60%); mp 208–210 °C; R_f(A) 0.85, R_f(B) 0.20; amino acid analysis Leu 3.00, Glu 1.06, Ser 0.78.

NPSLeu-Ser(Bzl)-LeuO-*t*-Bu (IV). BocSer(Bzl)-I (4.00 g, 1.00 mmol) was deprotected and coupled with 3.00 mmol of the symmetric anhydride of NPSLeu for 15 h. The dipeptide resin was then treated with 3 mmol each of DIEA, AcOH, and LeuO-*t*-Bu hydrochloride dissolved in 40 mL of methylene chloride for 16 h. Workup as for III gave a product which was crystallized twice from ether/petroleum ether: yield 410 mg (65%); mp 137–138 °C; R_f(B) 0.36, R_f(C) 0.50.

Leu-Ser(Bzl)-LeuO-*t*-Bu·HCl (IX). Peptide IV (0.55 mmol) was dissolved in 8 mL of ether, and 0.35 mL of 4 N HCl in dioxane (1.4 mmol) was added. The mixture was immediately placed in a −10 °C freezer. After 30 min the precipitated hydrochloride salt was filtered and washed with cold ether and petroleum ether: yield 285 mg (96%); mp 181–182 °C; R_f(E) 0.23, R_f(F) 0.59.

NPSLeu-Gln-Ser(Bzl)-Leu-Leu-Ser(Bzl)-LeuO-*t*-Bu (V). BocLeu-I (1.00 g, 0.5 mmol) was sequentially coupled with the symmetric anhydrides of BocSer(Bzl), BocGln, and NPSLeu. Coupling times were 2 h except for NPSLeu, which coupled more slowly and was allowed to react for 12 h. The resulting resin was treated with a 1.2-fold molar excess of IX, DIEA, and AcOH in 10 mL of methylene chloride for 13 h. An aliquot of the resin was filtered and examined by IR spectroscopy at this time, and it appeared that the aminolysis had gone to about 90% completion. The resin was then washed several times with methanol to remove the reactants and some impurities. The peptide was then eluted from the resin with warm DMF, the filtrate was evaporated to a small volume at room temperature in vacuo, and methanol was added, giving a solid: yield 269 mg (46%); mp 235–238 °C; R_f(A) 0.40, R_f(D) 0.40; amino acid analysis Ser 1.79, Glu 1.02, Leu 4.00.

NPSLeu-Leu-Gln-Trp(For)-LeuO-*t*-Bu (VI). The synthesis was initiated with 2.5 g (0.77 mmol) of BocTrp(For)-I, and the appropriate amino acids were coupled as usual.[1] Cleavage of the peptide from the resin was accomplished with a 3-fold excess of LeuO-*t*-Bu acetate in DMF/methylene chloride (1:1) for 4 h. The peptide was washed from the resin with DMF, and evaporation of the filtrate gave an oil which solidified after trituration with ethyl acetate/ether (1:1); yield 0.54 g (76%). This product appeared to be contaminated with a small amount of LeuO-*t*-Bu (TLC), so it was purified by precipitation from DMF by addition of ethanol/water (4:1). The peptide was crystallized from methanol: yield 450 mg (64%); mp 233 °C; R_f(A) 0.30, R_f(E) 0.84; amino acid analysis Leu 3.0, Glu 1.0.

BocLys(2ClZ)-Arg(Tos)-Lys(2ClZ)-Arg(Tos)-Gln-GlnNH$_2$ (VII). Chloromethylated polystyrene was esterified with BocGln by using the method of Gisin,[14] resulting in a substitution level of 0.38 mmol/g. The resulting polymer (1.50 mmol) was coupled with the symmetric anhydrides of BocGln, AocArg(Tos), and BocLys(2-ClZ) according to the following protocol:[12] (1) wash, methylene chloride (4x); (2) wash, TFA/methylene chloride (4:6; 1x); (3) deprotect, TFA/methylene chloride (4:6; 1 × 20 min); (4) wash, methylene chloride (2x); (5) wash, *i*-PrOH (1x); (6) wash, methylene chloride (3x); (7) neutralize, 5% DIEA in methylene chloride (1 × 2 min); (8) wash, methylene chloride (2x); (9) neutralize, 5% DIEA (1 × 2 min); (10) wash, methylene chloride (5x); (11) couple, 3 equiv of symmetric anhydride in methylene chloride (1 × 20 min); (12) couple, 10% trifluoroethanol, 1 equiv

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of DIEA (1 × 10 min); (13) wash, methylene chloride (3x); (14) wash, ethanol/methylene chloride (1:2; 3x). Recoupling was performed after each coupling of AocArg(Tos). The resulting hexapeptide resin (3.0 g, 0.89 mmol based on the substitution level of the first amino acid attached to the resin) was suspended in 50 mL of trifluoroethanol, which had been saturated at 0 °C with ammonia freshly distilled from sodium. The suspension was sealed in a pressure bottle and shaken for 3 days at room termperature. The flask was then cooled to 0 °C and the trifluoroethanol removed by filtration. The peptide resin was washed several times with methanol, and then the peptide was eluted from the resin with warm DMF. Evaporation of the solvent and trituration of the residue with ether gave 850 mg of peptide (60%). This product was dissolved in 7 mL of DMF, applied to a 2.5 × 80 cm column of LH-20 Sephadex, and eluted with DMF. The major peak corresponding to the appropriate molecular weight was collected, and the solvent was removed under reduced pressure; yield 737 mg (52%). The peptide was still heterogeneous by criteria of TLC, so the product was dissolved in hot methanol, and upon cooling a homogeneous product precipitated: yield 0.42 g (30%); mp 133-136 °C; R_f(F) 0.38, R_f(G) 0.80.

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Registry No. III, 82190-46-3; IV, 82190-47-4; V, 82198-68-3; VI, 82190-48-5; VII, 82198-69-4; VIII, 38024-17-8; IX, 82190-49-6; Leu-O-*t*-Bu·HCl, 2748-02-9; NPSLeu symmetric anhydride, 82190-50-9; BocSer(Bzl) symmetric anhydride, 64817-69-2; BocGln symmetric anhydride, 68623-39-2; BocGln, 13726-85-7; AocArg(Tos) symmetric anhydride, 76802-47-6; BocLys (2-ClZ) symmetric anhydride, 70889-80-4; *p*-nitrobenzoyl chloride, 122-04-3; melittin, 37231-28-0.

Synthesis of Peptide Alkaloids. 5.[1,2] New Method for Synthesis of Ansa Peptides. Amino Acids and Peptides. 34

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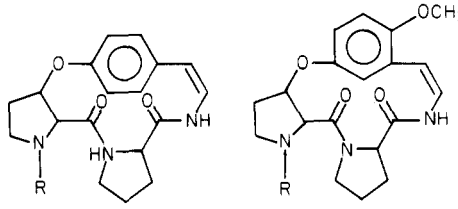
Fourteen-membered para-ansa copounds and 13-membered meta-ansa compounds have been synthesized by catalytic hydrogenation of the pentafluorophenyl esters of ω-(*Z*)-amino carboxylic acids in 50% and 80% yields, respectively.

Reports on the isolation and structure elucidation of about 100 cyclopeptide alkaloids[3] have appeared during the last 15 years. These compounds typically contain a para or meta ansa bridge consisting of an enamine function and dipeptide of a β-hydroxy amino acid. This structural characteristic is illustrated by zizyphine G (1) or A (2),



1 2

containing a 13- or 14-membered ring, respectively. The synthesis of zizyphine A has been published in a preliminary report.[4]

The cyclic alkaloid peptides have been found in plants of the Rhamnaceae and Sterculiaceae families only. Presumably, the biosynthesis of the β-phenoxy unit happens by radical or ionic addition of the phenol group of tyrosin to the double bond of an α,β-dehydro amino acid. This assumption is supported by the isolation of a "linear" peptide alkaloid from Rhamnaceae containing a free phenolic group and a dehydro amino acid residue. It is interesting that "linear" peptide alkaloids have recently been found in marine sponges.[5] Some of these peptide alkaloids are active against lower fungi and gram-positive bacteria.[3] Discarine B is a specific inhibitor of energy-transfer reactions in chloroplasts.[6] Experimental evidence points to their function as ionophores in plants.[7,8]

Several approaches to the synthesis of peptide alkaloids have been published. The synthesis of analogous but larger 17-membered dihydro ring systems have been described.[9] The ring closure of model systems containing a β-phenoxy carboxylic acid instead of a β-phenoxy amino acid residue was studied.[8,10] The azido and *p*-nitrophenyl ester methods were used for ring formation. In synthesizing peptide alkaloids, the three main difficulties are forming the styrylamino unit, forming the (*S*,*S*)-β-phenoxy amino acid, and the ring closure. A practical solution to the last problem will be given here.

The shortest bridge in a para-ansa compound which can be formed by ring closure of a para-substituted aromatic compound in satisfactory yield contains 10 sp^3-hybridized members.[11] In peptide alkaloids with a 14-membered ring

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