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Synthesis of a Cyclic Charge Transfer Labeled Analogue of the Luteinizing Hormone-Releasing Factor[1]

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The synthesis of

p-Glu-His-Trp-Glu-Tyr-D-Ala-Orn-Nva(Nic$^+$Cl$^-$)-Pro-Gly-NH_2

a cyclic analogue of the luteinizing hormone-releasing factor carrying a charge-transfer label, is described. The linear peptide *p*-Glu-His-Trp-Glu-Tyr-D-Ala-Orn-Orn(TFA)-Pro-Gly-NH_2 was synthesized by the solid-phase method in a 32% overall yield. The side-chain cyclization was carried out in pyridine at high dilution in 65% yield by using 30 equiv of dicyclohexylcarbodiimide and 2 equiv of *N*-hydroxysuccinimide as coupling reagents. Selectivity in the side-chain deprotection of the two ornithine residues was provided by using the benzyloxycarbonyl and the trifluoroacetyl protecting groups.

Introduction

We have undertaken a systematic investigation of the conformation of the luteinizing hormone-releasing factor by using charge-transfer labels[2,3] in order to visualize side chain–side chain interactions. In an attempt to obtain quantitative intramolecular charge-transfer effects, we have prepared a nicotinamidium-labeled cyclic analogue in which the folding of the peptide backbone at the central tetrapeptide sequence is forced by a covalent bond between the side chains of residues at positions 4 and 7. In this paper, we describe the synthesis of [*cyclo*(Glu^4,D-Ala^6,Orn^7),Nva^8(Nic$^+$)]LRF·Cl$^-$. The conformational studies of this and similarly labeled LRF analogues will be reported in a subsequent paper.[4]

Results and Discussion

A combination of the solid phase and the classical peptide synthesis methodologies has been applied to prepare the desired LRF analogues. Schemes I and II outline these syntheses. Selectivity in deprotection of the δ-amino side chains of the two ornithine residues in positions 7 and 8 was provided through the use of the benzyloxycarbonyl group and the trifluoroacetyl group, the latter of which is stable to the hydrogen fluoride treatment employed to cleave the peptide from the resin. This procedure results in a linear peptide in which the ornithine side chain in position 7 is deprotected in preparation for ring closure with the γ-carboxyl side chain of the glutamic acid in position 4. Selective N^{δ}-trifluoroacetylation of orni-

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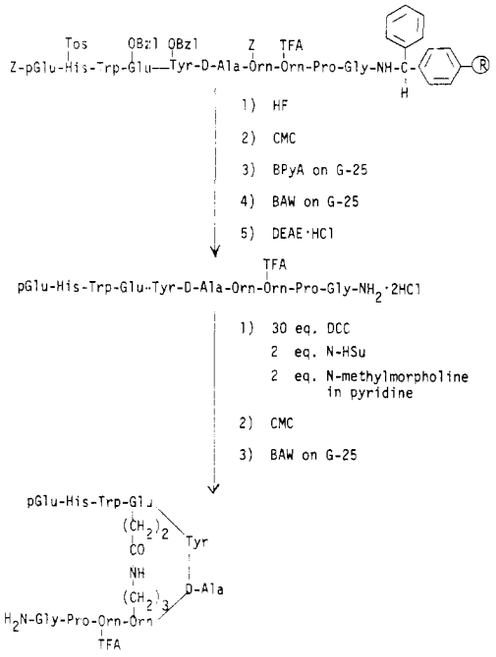
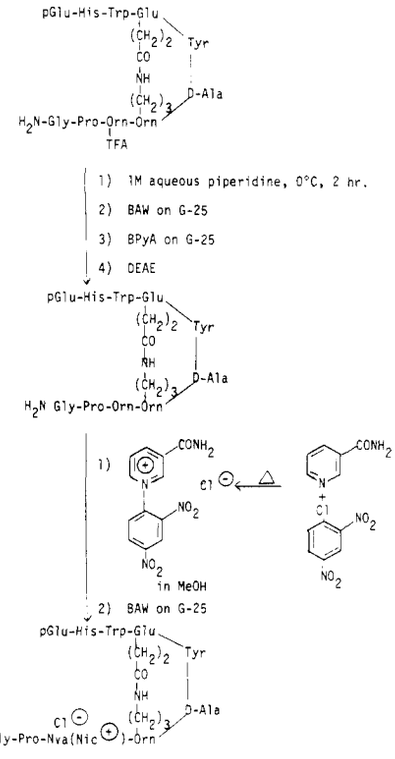
Scheme I. Synthesis of [*cyclo*(Glu⁴,D-Ala⁶,Orn⁷)-Orn⁸(TFA)]LRF



Scheme II. Synthesis of [*cyclo*(Glu⁴,D-Ala⁶,Orn⁷))Nva⁸(Nic⁺Cl⁻)]LRF



thine was achieved by following the procedure of Schallenberg and Calvin[5] using *S*-ethylthiol trifluoroacetate as the acylating agent. These authors have shown that the trifluoroacetyl group in monoacetylated D,L-lysine and D,L-ornithine was exclusively located on the side chain amino group, and not on the α-amino group. A further confirmation of these results was obtained from the NMR spectrum of the N^{α}-*tert*-butyloxycarbonyl-N^{δ}-trifluoroacetylornithine, which we have prepared. This compound exhibits the characteristic doublet for the α-NH proton signal (Me_2SO-d_6 as solvent) which completely disappears after selective removal of the Boc protecting group with trifluoroacetic acid, while the triplet signal of the δ-NH proton remains unchanged after this treatment.

The N^{α} protection of the N^{δ}-trifluoroacetylornithine with the *tert*-butyloxycarbonyl group was preferentially carried out in dimethyl sulfoxide using triethylamine as base instead of applying the conditions reported by Anfinsen et al.[6] for the synthesis of N^{ϵ}-*tert*-butyloxycarbonyl-*N*-trifluoroacetyllysine. The linear peptide *p*-Glu-His-Trp-Glu-Tyr-D-Ala-Orn-Orn(TFA)-Pro-Gly-NH_2 (Scheme I) was obtained in 32% overall yield after cleavage from the resin with hydrogen fluoride and extensive purifications with ion exchange and successive partition chromatography using basic and acidic eluent mixtures.

The synthesis of the first cyclic LRF analogue for structure–activity relationship studies was carried out in our laboratories.[7] We have further optimized the cyclization conditions. The high dilution method described by Schwyzer et al.[8] and Wieland and Birr[9] was applied and different coupling reagents were tested. With carbonyldiimidazole (30 equiv) in dimethylformamide, we failed to isolate and characterize any cyclic product. Using dicyclohexylcarbodiimide (30 equiv) in dimethylformamide or pyridine, it was shown by thin-layer chromatography (ninhydrin positive spot) and confirmed by NMR that the *N*-acylurea derivative was the main product (3:1 ratio as compared to the desired cyclic compound). However, with 30 equiv of dicyclohexylcarbodiimide and 2 equiv of *N*-hydroxysuccinimide, the *N*-acylurea formation was completely suppressed and a 65% yield of pure cyclic product was obtained. By increasing the *N*-hydroxysuccinimide concentration, we observed a decrease in the yield of cyclization.

Cleavage of the trifluoroacetyl group with aqueous piperidine at 0 °C was followed by thin-layer chromatography; the appearance of a single new spot confirms the homogeneity of the cyclic compound. Furthermore, molecular weight determination by sedimentation equilibrium is consistent with the monomeric nature of the product.

The unprotected cyclic compound was purified by partition chromatography, neutralized on a diethylaminoethylcellulose column (basic form), and treated with 2,4-dinitrophenylnicotinamidium chloride to give the desired charge transfer labeled compound.[3,11]

In a combined in vitro biological assay carried out by Dr. W. Vale at the Salk Institute, La Jolla, Calif.,[12] using LRF as standard (100%), the cyclic compounds described in this paper, together with [(Glu⁴,D-Ala⁶,Orn⁷)]LRF and [Gln⁴,D-Ala⁶,Orn⁷(Ac)]LRF described earlier, exhibit potencies of about 0.1% or less. As we have stated above, we prepared these compounds to analyze the conformational characteristics of LRF and related molecules. It is not surprising that the changes we have made in the LRF sequence lead to inactive compounds.

Experimental Section

The N^{α}-*tert*-butyloxycarbonyl amino acid derivatives were obtained from Bachem Fine Chemicals, Inc. Solvents were purchased from Mallinckrodt and were of AR grade. The reagents *N,N*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, trifluoroacetic acid, diisopropylethylamine, and 1,2-ethanedithiol were purchased from Aldrich and were used without further purification. Anisole and piperidine were obtained from Matheson, Coleman and Bell and *S*-ethylthiol trifluoroacetate from the Pierce Chemical Co. *N*-methylmorpholine (Aldrich) was distilled over sodium and stored over molecular sieves. Dimethylformamide was dried over sodium hydroxide and distilled under vacuum over ninhydrin (1 g of ninhydrin per liter of dimethylformamide). Pyridine was dried over barium oxide. Thin-layer chromatography was carried out on precoated silica gel plates (Kodak) using the following solvent systems: 1-butanol/acetic

acid/water (4:1:5 v/v, upper phase) (A), 1-butanol/pyridine/0.1% acetic acid in water (5:3:11 v/v, upper phase) (B), 1-butanol/acetic acid/water (3:1:1 v/v) (C), methyl ethyl ketone/pyridine/water/acetic acid (70:15:18:2 v/v) (D), and chloroform/methanol (1:1 v/v) (E). All intermediates and end products were characterized by NMR spectroscopy. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Amino acid analyses and the testing of the biological activity of the LRF analogues were carried out in Dr. Guillemin's laboratory at the Salk Institute, La Jolla, Calif.

N^{α}-Trifluoroacetyl-L-ornithine. This compound was synthesized according to the procedure of Schallenberg and Calvin:[5] to a solution of L-ornithine monohydrochloride (0.337 g, 2 mmol) in 1 N sodium hydroxide (2 mL) *S*-ethylthiol trifluoroacetate (0.4 mL) was added. The heterogeneous mixture was vigorously stirred for 6 h. A precipitate slowly separated. The reaction mixture was cooled to 0 °C and the solid was collected by filtration and washed with ether. After recrystallization from a water/ethanol mixture (1:1) 250 mg (55%) was obtained: mp 247–249 °C; $[\alpha]^{25}D$ +13.3° (*c* 0.2, 3 N hydrochloride acid), $[\alpha]^{25}D$ +18.9° (*c* 1, dichloroacetic acid) R_f^C 0.42, R_f^D 0.58, R_f^E 0.44 (lit.[13] mp 250–251 °C).

Studies on the Stability of the N^{δ}-Trifluoroacetyl Protecting Group. (A) To HF treatment. Liquid hydrogen fluoride was distilled in a sample of N^{δ}-trifluoroacetylornithine suspended in a little anisole and the mixture was kept at 0 °C for 1 hour, when the hydrogen fluoride was removed under vacuum and ether was added into the residue. The solid material was then filtered and subjected to thin-layer chromatography in systems C, D, and E when no traces of ornithine could be detected.

(B) To Partition Chromatography Solvent Systems. A solution of N^{δ}-trifluoroacetylornithine in the upper phase of 1-butanol/pyridine/0.1% acetic acid in water (5:3:11) was kept at room temperature for 24 h. No decomposition was detected by thin-layer chromatography after this treatment.

(C) To Boc Deprotection Conditions during Solid-Phase Synthesis. A solution of N^{α}-*tert*-butyloxycarbonyl-N^{δ}-trifluoroacetyl-L-ornithine in trifluoroacetic acid was kept at room temperature for 30 min. Thin-layer chromatography of this solution in systems C and D showed only the presence of N^{δ}-trifluoroacetylornithine, which assured the stability of the trifluoroacetyl group during solid-phase synthesis.

N^{α}-*tert*-Butyloxycarbonyl-N^{δ}-trifluoroacetyl-L-ornithine. N^{δ}-Trifluoroacetylornithine (0.23 g, 1 mmol) was dissolved in dimethyl sulfoxide (5 mL) containing triethylamine (0.28 mL, 2 mmol) and *tert*-butyloxycarbonyl azide (0.2 mL, 1.3 mmol). A further 0.1 mL of *tert*-butyloxycarbonyl azide was added after 8 h and the solution stirred for a total of 24 h at room temperature. The solution was finally diluted with three volumes of water and extracted twice with ether to remove the unreacted *tert*-butyloxycarbonyl azide. The aqueous phase was acidified (pH 2–3) with 1 N sulfuric acid and extracted with ethyl acetate (three times). The combined ethyl acetate extracts were washed to neutral pH with water, dried over magnesium sulfate, and evaporated to dryness. The residue (0.25 g, 76.5% viscous oil) was homogeneous by thin-layer chromatography (R_f^C 0.81, R_f^D 0.82, and R_f^E 0.82) and characterized by NMR spectroscopy (the characteristic α-NH doublet at 7.015 ppm (J = 7.5 Hz, Me_2SO-d_6 as solvent, hexamethyldisiloxane as reference) completely disappears after quantitative cleavage of the N^{α}-*tert*-butyloxycarbonyl protecting group with trifluoroacetic acid).

***p*-Glu-His-Trp-Glu-Tyr-D-Ala-Orn-Orn(TFA)-Pro-Gly-NH_2·CH_3COOH.** The linear compound was synthesized by the solid-phase method using a benzhydrylamine resin[14-15] (18.2 g, 0.16 mequiv/g substitution, 3 mmol) as support. All the amino acids were coupled as the N^{α}-*tert*-butyloxycarbonyl derivatives except pyroglutamic acid, where N^{α}-benzyloxycarbonylpyroglutamic acid was used. The side chains were protected as follows: imidazolyltosylhistidine, glutamic acid γ-benzyl ester, tyrosyl *O*-benzyl ether, and N^{δ}-benzyloxycarbonylornithine for position 7 and N^{δ}-trifluoroacetylornithine for position 8. Dicyclohexylcarbodiimide was used as the coupling reagent in dichloromethane or dimethylformamide/dichloromethane mixture. Completion of the coupling reactions was ensured by use of the ninhydrin test. Cleavage of the N^{α}-*tert*-butyloxycarbonyl group was carried out with a 40% solution of trifluoroacetic acid in dichloromethane containing 1,2-ethanedithiol (2%) and anisole (8%), followed by neutralization with a 10% solution of diisopropylethylamine in dichloromethane. The protected decapeptide was cleaved from the resin by the action of doubly distilled hydrogen fluoride in the presence of anisole for 1 h at 0 °C. The crude peptide (3.3 g) was purified by ion-exchange chromatography on carboxymethylcellulose eluting with an ammonium acetate gradient (0–0.3 M) and by two successive partition chromatographies on Sephadex G-25 in two different systems: 1-butanol/pyridine/0.1% acetic acid in water (5:3:11 v/v) and 1-butanol/acetic acid/water (4:1:5 v/v). The white product (1.24 g, 32%) was homogeneous by thin-layer chromatography in acidic and basic systems (R_f^A 0.23 and R_f^B 0.58) and was characterized by NMR spectroscopy and by quantitative amino acid analyses (hydrolyzates of the final material gave 100% peptide with the ratio: Orn, 2.05; His, 0.94; Trp, 0.96; Glu, 2.07; Pro, 0.96; Gly, 1.00; Ala, 0.99; Tyr, 0.98).

Anal. Calcd for $C_{58}H_{75}N_{16}O_{15}F_3$·$CH_3COOH$·$4H_2O$. C, 50.56; H, 6.15; N, 15.72. Found: C, 50.71; H, 6.13; N, 15.82.

***p*-Glu-His-Trp-Glu-Tyr-D-Ala-Orn-Orn(TFA)-Pro-Gly-NH_2·CH_3COOH.** The linear decapeptide (197.8 mg) was converted to the hydrochloride salt on a cellex-D diethylaminoethylcellulose (HCl form) column. After lyophilization, the peptide (191 mg, 0.14 mmol) was dissolved in a dimethylformamide/pyridine mixture (20 mL, 1:1 v/v) containing *N*-hydroxysuccinimide (32 mg, 0.28 mmol) and *N*-methylmorpholine (36.2 μL, 0.28 mmol). The solution was added into a solution of dicyclohexylcarbodiimide (880 mg, 4.2 mmol) in pyridine (200 mL) over a period of 5 days at room temperature. The reaction mixture was further kept at 40 °C for an additional 4 days. After evaporation of the pyridine under reduced pressure, the product was precipitated with peroxide-free ether. Trituration of the solid material three times with ether removed the unreacted dicyclohexylcarbodiimide. The crude compound (183 mg, 96%) was purified by partition chromatography on Sephadex G-25 in 1-butanol/acetic acid/water (4:1:5 v/v), by ion-exchange chromatography on carboxymethylcellulose eluting with an ammonium acetate gradient (0–0.1 M), and once more by partition chromatography using the same solvent system. The white cyclic product (120.5 mg, 65%) showed a single, ninhydrin negative, spot on thin-layer chromatography (R_f^A 0.32 and R_f^B 0.75) and gave the correct amino acid analysis with the ratio: Orn, 2.06; His, 1.04; Trp, 1.09; Glu, 2.06; Pro, 1.14; Gly, 1.00; Ala, 1.01; Tyr, 0.95.

Anal. Calcd for $C_{58}H_{73}N_{16}O_{14}F_3$·$\frac{1}{2}CH_3COOH$·$4H_2O$: C, 51.45; H, 6.08; N, 16.27; F, 4.14. Found: C, 51.49; H, 6.06; N, 16.29; F, 4.14.

A few milligrams of the final material were dissolved in 1 M aqueous piperidine at 0 °C and the cleavage of the TFA group was followed each minute by thin-layer chromatography (systems A, B, C, D, E). A single new spot appeared up to the point of the full deprotection.

Molecular Weight Determination. The molecular weight of the cyclic compound was determined by sedimentation equilibrium using a Spinco Model R analytical centrifuge equipped with a TRLC temperature unit. Centrifugation was carried out at 20 °C at an angular velocity of 60 000 rpm for 50 h, when we confirmed that equilibrium had been reached. The cyclic peptide was dissolved in 0.1 M KCl (10^{-3} M concentration of the peptide). A 12-mm double-sector cell with sapphire windows and Kel-F double-sector centerpiece was used. The partial specific volume (0.732 mL/g) was estimated from the amino acid composition.[16] The molecular weight was found to be 951, compared with a value of 1275 calculated from the primary structure of the cyclic monomer.

***p*-Glu-His-Trp-*cyclo*(Glu-Tyr-D-Ala-Orn)-Orn-Pro-Gly-NH_2.** *p*-Glu-His-Trp-*cyclo*(Glu-Tyr-D-Ala-Orn)-Orn(TFA)-Pro-Gly-NH_2 (146 mg) dissolved in 1 M aqueous piperidine (7 mL) was maintained at 0 °C for 2 h. Acetic acid was added to stop the reaction and the solution was then lyophilized. The crude, completely deprotected peptide was purified by partition chromatography on Sephadex G-25 on 1-butanol/pyridine/0.1% acetic acid in water (5:3:11 v/v) to give 136.3 mg (96%) of pure, white compound: R_F^A 0.08 and R_f^B 0.47. Amino acid analysis gave the ratio: Orn, 1.93; His, 0.95; Trp, 0.90; Glu, 1.96; Pro, 0.93; Gly, 1.00; Ala, 1.01; Tyr, 0.96.

***p*-Glu-His-Trp-*cyclo*(Glu-Tyr-D-Ala-Orn)-Nva(Nic$^+CH_3$-COO^-)-Pro-Gly-NH_2·CH_3COOH.** The acetate salt of *p*-Glu-His-Trp-*cyclo*(Glu-Tyr-D-Ala-Orn)-Orn-Pro-Gly-NH_2 (136.3 mg) was neutralized through a DEAE-cellulose (cellex-D) column. After lyophilization, the decapeptide free base (121 mg, 0.1026 mmol) was dissolved in absolute, distilled methanol (6 mL) and treated with a solution of 2,4-dinitrophenylnicotinamidium chloride (33.8 mg, 0.1041 mmol) in methanol (1.5 mL) slowly over 5–6 h at room temperature. The reaction mixture was stirred overnight, the methanol evaporated to a small volume, and the product precipitated with ether. The solid material was triturated three times with ether and purified by two successive partition chromatographies on Sephadex G-25 in 1-butanol/acetic acid/water (4:1:5 v/v) to give a pure yellow compound (114.9 mg, 81%), which was homogeneous by thin-layer chromatography in acidic and basic systems (R_f^A 0.00 and R_f^B 0.36) and was characterized by NMR spectroscopy and by quantitative amino acid analysis which gave the ratio: Orn, 1.02; His, 1.00; Trp, 0.86; Glu, 1.96; Pro, 0.97; Gly, 1.00; Ala, 1.01; Tyr, 0.92. The product was converted to its bisacetate salt by neutralization on a DEAE-cellulose column and acidification

with acetic acid.

Anal. Calcd for $C_{62}H_{78}N_{17}O_{14}\cdot CH_3COO^-\cdot CH_3COOH\cdot 6H_2O$: C, 52.41; H, 6.47; N, 15.74. Found: C, 52.57; H, 6.63; N, 15.72.

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Registry No.—N^{α}-Trifluoroacetyl-L-ornithine, 5123-49-9; N^{α}-*t*-Boc-N^{α}-trifluoroacetyl-L-ornithine, 63865-89-4; *tert*-butyloxycarbonyl azide, 1070-19-5; *p*-Glu-His-Trp-Glu-Tyr-D-Ala-Orn-Orn(TFA)-Pro-Gly-$NH_2\cdot CH_3CO_2H$, 63865-91-8; *p*-Glu-His-Trp-*cyclo*(Glu-Tyr-D-Ala-Orn)-Orn(TFA)-Pro-Gly-$NH_2\cdot CH_3CO_2H$, 63904-16-5; *p*-Glu-His-Trp-*cyclo*(Glu-Tyr-D-Ala-Orn)-Orn(TFA)--Pro-Gly-$NH_2\cdot 2HCl$, 63865-92-9; *p*-Glu-His-Trp-*cyclo*(Glu-Tyr-D-Ala-Orn)-Orn-Pro-Gly-NH_2, 63865-93-0; *p*-Glu-His-*cyclo*(Trp-Glu-Tyr-D-Ala-Orn)-Nva($Nic^+CH_3COO^-$)-Pro-Gly-$NH_2\cdot CH_3CO$-OH, 63904-76-7; *p*-Glu-His-*cyclo*(Trp-Glu-Tyr-D-Ala-Orn)-Nva(Nic^+Cl^-)-Pro-Gly-NH_2, 63865-97-4; Z-*p*-Glu-His(Tos)-Trp-Glu(OBzl)-Tyr(OBzl)-D-Ala-Orn(Z)-Orn(TFA)-Pro-Gly-NH_2, 63915-17-3; 2,4-dinitrophenylnicotinamidium chloride, 53406-00-1.

References and Notes

(1) Abbreviations used in the text are: LRF, luteinizing hormone-releasing factor (also called luliberin); Z, benzyloxycarbonyl; TFA, trifluoroacetyl; Bzl, benzyl; Tos, toluenesulfonyl; HF, hydrogen fluoride; CMC, carboxymethylcellulose; BPyA, 1-butanol/pyridine/0.1% acetic acid in water (5:3:11); BAW, 1-butanol/acetic acid/water (4:1:5); G-25, Sephadex G-25 fine; DEAE-cellulose, diethylaminoethylcellulose; DCC, dicyclohexylcarbodiimide; *N*-HSu, *N*-hydroxysuccinimide; Nic^+, nicotinamidium; Ac, acetyl; Me_2SO, dimethyl sulfoxide; Boc, *tert*-butyloxycarbonyl.
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Synthesis of Tentoxin and Related Dehydro Cyclic Tetrapeptides[1,2]

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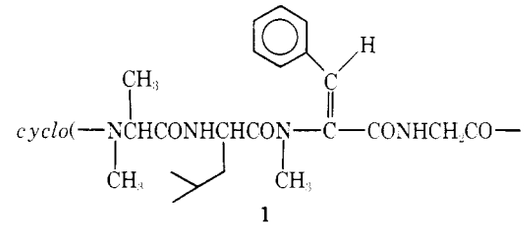
Three methods are reported for synthesizing the dehydro cyclic tetrapeptide tentoxin, *cyclo*(-L-MeAla-L-Leu-MePhe[(*Z*)Δ]-Gly-), a plant toxin that inhibits chloroplast coupling factor 1. Boc-MeAla-Leu-Phe(3-SBzl)-Gly-OMe was prepared by solid-phase synthesis, oxidized to the sulfoxide, dehydrosulfenylated, and *N*-methylated to give Boc-MeAla-Leu-MePhe[(*Z*)Δ]-Gly-OMe. Boc-MeAla-Leu-MePhe(3-SBzl)-Gly-OMe was prepared stepwise in solution from *erythro*-Boc-MePhe(3-SBzl) and converted by dehydrosulfenylation to the dehydro tetrapeptide which was also prepared in good yield by coupling Boc-MeAla with H-Leu-MePhe[(*Z*)Δ]-Gly-OMe. The synthesis of the cyclic tetrapeptides (-X-Leu-MePhe[(*Z*)Δ]-Gly-), where X = L-MeAla, D-MeAla, L-Pro, D-Pro, L-Me[2,3-^{3}H]Ala, L-*N*-[^{13}C-Me]MeAla, and D-*N*-[^{13}C-Me]MeAla, was achieved using the trichlorophenyl ester method. Saponification of Boc-*N*-methyldehydrophenylalanyl peptides led to hydantoin formation with loss of *tert*-butyl alcohol.

The cyclic tetrapeptide, tentoxin, *cyclo*(*N*-methyl-L-alanyl-L-leucyl-*N*-methyl-(*Z*)-dehydrophenylalanylglycyl) (**1**) is a phytotoxin produced by the plant pathogenic fungus *Alternaria tenuis*.[3] When applied to germinating seedlings tentoxin causes chlorosis in some species but has little apparent effect on others.[3,4] This selectivity has been linked to the presence of a single tentoxin binding site on chloroplast coupling factor 1 (CF_1), a key protein involved in ATP synthesis.[5] CF_1 from sensitive species bind tentoxin strongly ($K_{assn} = 10^8$), while CF_1 from insensitive species binds tentoxin weakly ($K_{assn} \leq 10^4$). Tentoxin is the only inhibitor of CF_1 reported to exhibit such species specificity.

Tentoxin contains two structural features not commonly found in peptides, the 12-membered cyclic tetrapeptide ring system and the α,β-unsaturated amino acid, *N*-methyldehydrophenylalanine, MePhe[(*Z*)Δ].[2,6] Although several naturally occurring biologically active cyclic tetrapeptides have been identified in nature recently,[7] and a number of peptides containing dehydro residues have been reported,[8] tentoxin



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remains the only peptide isolated that contains both structural features. We report here methods to synthesize tentoxin and several tentoxin analogues that are required for biochemical and conformational studies in progress.

Results and Discussion

(I) Synthesis of Linear Tetrapeptides. The synthesis of peptides containing dehydro amino acid residues may be complicated by the chemical reactivity of the double bond. The simplest unit, dehydroalanine, rapidly adds anhydrous

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