molecular processes arises from the method analysis—determination of K_{eq} for an intermolecular process often requires a concentration titration which, for a small K_{eq}, can be a substantial perturbation in the solvent composition. The analysis of an intramolecular process at a constant concentration precludes complications from changes in solvent activity.[32]

Acknowledgment. We thank the donors of the Petroleum Research Fund, administered by the American Chemical Society (grant 21426-G4), and the University of California Cancer Coordination Research Committee for financial support of this research (to T.A.D.). C.B. is grateful for support as an National Institutes of Health predoctoral trainee (GM07311). The computer hardware was purchased with shared instrument funds from the National Institutes of Health (RR-05690) to T.A.D.

Supplementary Material Available: Proton NMR spectra for all characterized compounds (18 pages). This material is contained in many libraries on microfiche, immediately followed this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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Use of *N*-Fmoc Amino Acid Chlorides and Activated 2-(Fluorenylmethoxy)-5(4*H*)-oxazolones in Solid-Phase Peptide Synthesis.[1] Efficient Syntheses of Highly N-Alkylated Cyclic Hexapeptide Oxytocin Antagonists Related to L-365,209[†]

Debra S. Perlow,* Jill M. Erb, Norman P. Gould, Roger D. Tung, Roger M. Freidinger, Peter D. Williams, and Daniel F. Veber

Department of Medicinal Chemistry, Merck Research Laboratories, West Point, Pennsylvania 19486

Received April 13, 1992

Fmoc amino acid chlorides have been shown to be useful reagents in the solid-phase synthesis of hexapeptides containing up to four sequential secondary amino acids. The oxytocin antagonist cyclo-(D-Phe-Ile-D-Pip-Pip-D-(*N*-Me)Phe-Pro) (1) was prepared in 70% overall yield starting from Boc-L-Pro-*O*-(PAM)-resin. In the synthesis of 1, the high reactivity of Fmoc-L-pipecolic acid chloride used in the di- to tripeptide step averted diketopiperazine formation seen with active ester couplings. The use of Fmoc-amino acid chlorides in the subsequent couplings provided a rapid method for assembly of the linear hexapeptide. The two potent cyclic hexapeptide oxytocin antagonists L-366,682 and L-366,948 were prepared in 45–48% overall yield on a 20 mmol scale using the methodology developed for the synthesis of 1. A particularly difficult coupling was encountered that involved acylation of a sterically hindered N^{δ}-Cbz-piperazic acid *N*-terminus with Fmoc-L-isoleucine. Excess Fmoc-L-isoleucine acid chloride in the presence of tertiary amine base gave only 30% conversion. The efficiency was improved to 76% by utilizing the acid chloride with AgCN in toluene. Further investigation revealed that this combination of reagents produces an activated form of the isoleucine 2-alkoxy-5(4*H*)-oxazolone derivative.

Introduction

Oxytocin (Figure 1) is a peptide hormone that plays a key role in the initiation and maintainence of uterine contractions associated with labor during pregnancy.[2] Recent evidence supports the concept of oxytocin receptor blockade as a new mechanism for treating preterm labor to prevent premature birth.[3] The cyclic hexapeptide L-365,209 (Figure 1), a chemically modified derivative of a natural product isolated from *Streptomyces silvensis*, is a member of a structurally novel class of oxytocin antagonists[4] and has served as the basis for a medicinal chemistry program aimed at improving its potency and aqueous solubility.[5,6] L-365,209 is a cyclic peptide consisting of six amino acids of alternating configuration and containing an N-methylated phenylalanine at position 6 and the unusual amino acid, dehydropiperazic acid, at positions 4 and 5.[7] We became interested in developing a solid-phase peptide synthesis method for the rapid production of L-365,209 analogs in order to define structure–activity relationships for this novel class of antagonist. The presence of four sequential secondary amino acids in L-365,209 poses special problems. In particular, the slower rate of acylation of secondary amino acids requires special carboxyl group activation,[8] and sequences of three or more secondary amino acids are known to be acid labile.[9] These

[†] Dedicated to Professor Ralph F. Hirschmann on the occasion of his 70th birthday.

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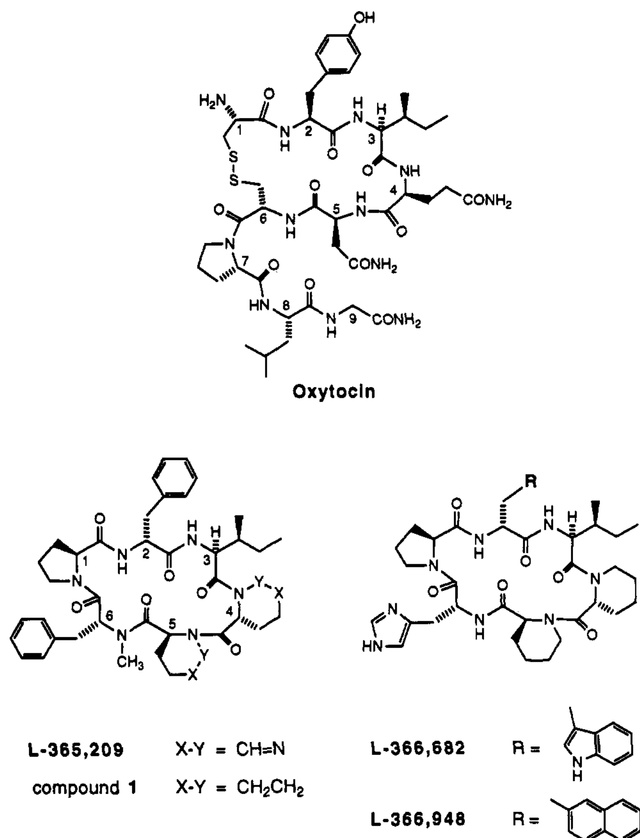
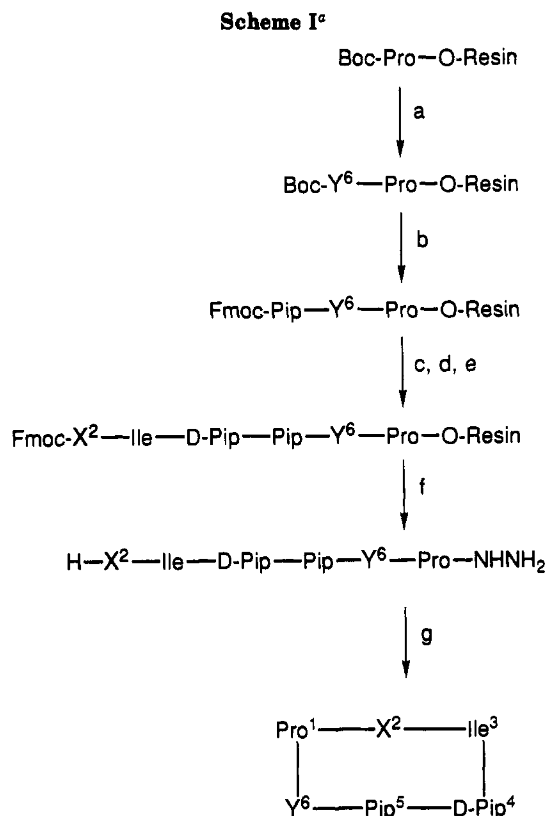


Figure 1.



[a] Key: (i) HCl–dioxane; (ii) Boc-D-(*N*-Me)Phe-OH or Boc-D-(DNP)His-OH, BOP, DIEA, DMF; (b) (i) HCl–dioxane; (ii) Fmoc-Pip-Cl, DIEA, DCM, 5 °C; (c) (i) piperidine–DMF; (ii) Fmoc-AA-Cl, DIEA, DCM, 25 °C; (d) same as step c; (e) Fmoc-D-Phe-Cl, DIEA, DCM, 25 °C; or Fmoc-D-Trp-OH (or Fmoc-D-β-Nal-OH), BOP, DIEA, DMF; (f) (i) piperidine–DMF; (ii) NH_2NH_2, MeOH, 50 °C; (g) (i) i-$C_5H_{11}ONO$, pH 3, −20 °C, DMF; (ii) dilution to 0.003 M, (iii) DIEA to pH 8, DMF; BOP = [(benzotriazol-1-yl)-oxy]tris(dimethylamino)phosphonium hexafluorophosphate; DIEA = diisopropylethylamine; DCM = dichloromethane.

limitations suggested the use of Fmoc-amino acid chlorides (Fmoc-AA-Cl), reagents for peptide synthesis recently described by Carpino and co-workers.[10] Herein, we report an efficient solid-phase synthesis protocol utilizing Fmoc-AA-Cl's for the synthesis of L-365,209 analogs and a modification utilizing an Fmoc-AA-Cl in the presence of AgCN for an especially difficult coupling.

Results and Discussion

An early target for synthesis was compound 1 (Figure 1), the analog of L-365,209, in which the 4- and 5-position dehydropiperazic acids are replaced with the more readily accessible pipecolic acids (Pip). It was anticipated that cyclization of a linear hexapeptide intermediate having a primary amino terminus rather than a secondary amino terminus would maximize cyclization rate and thus minimize the opportunity for epimerization of the activated carboxyl terminus. Previous experience in a different cyclic hexapeptide system had shown that cyclization rate and yield were maximized using an L-amino acid carboxy terminus and a D-amino acid N-terminus.[11] The L-Pro[1]-D-Phe[2] bond was therefore chosen as the point of cyclization.[7]

The synthesis of 1 was thus begun using Boc-L-proline covalently attached as its ester to a PAM[12] resin (Scheme I). A manually operated, motor-driven shaker system was employed for all wash cycles, couplings, and deprotections (see Experimental Section). Following deprotection of the Boc-L-Pro-*O*-resin with HCl in dioxane, Boc-D-(*N*-Me)-Phe-OH was coupled using the BOP reagent[13] (step a). In order to minimize peptide loss from formation of cyclo-(D-(*N*-Me)Phe-Pro), the dipeptide amine salt obtained by deprotection with HCl in dioxane was shaken with Fmoc-L-Pip-Cl (2 equiv) at 5 °C in dichloromethane (DCM) for several minutes, and then diisopropylethylamine (DIEA) was added to obtain a "pH 8" supernatant (estimated by spotting an aliquot on wetted pH indicating paper) (step b). The reaction mixture was shaken for 12 h at 5 °C to ensure complete reaction. When the coupling was attempted using an active ester procedure (BOP reagent) at 5 °C, 60% of the peptide was lost to diketopiperazine formation. The half-life of the neutralized dipeptide resin in DMF at ambient temperature was found to be approximately 15 min as determined by amino acid analysis of resin aliquots withdrawn at several time intervals. Thus, the high reactivity of the Fmoc-AA-Cl and the low temperature contributed to the high yield obtained in step b. All subsequent couplings (steps c–e) were performed at ambient temperature in DCM by adding the Fmoc-AA-Cl (2 equiv) to the amine free base, followed by addition of DIEA to "pH 8". After a coupling time of 1 h for steps c–e, hydrazinolytic removal of the peptide from an aliquot of each resin indicated complete (>99%) conversion to a new component as determined by HPLC analysis. Attempts at cleaving the linear hexapeptide from the resin by transesterification gave low yields of product.

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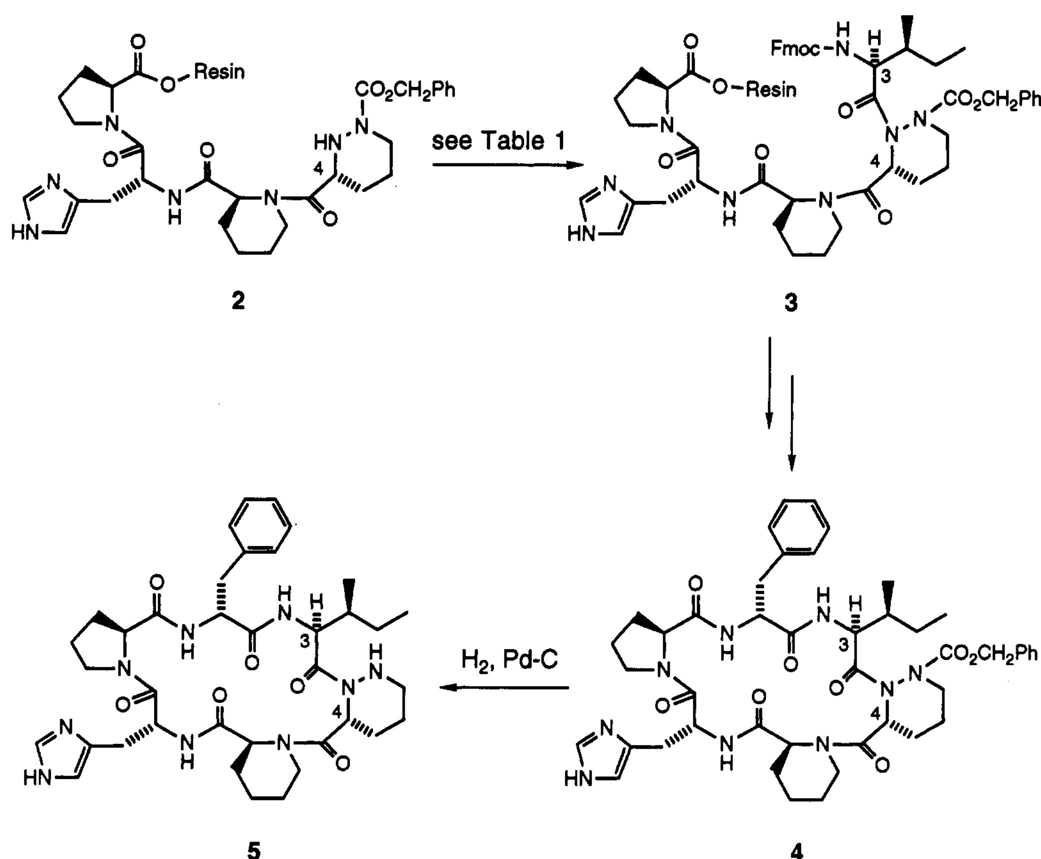
Scheme II



Complete cleavage of the hexapeptide from the resin required relatively vigorous hydrazinolysis conditions (step f). Following aqueous workup to remove excess hydrazine, the hexapeptide hydrazide was obtained in good yield and in greater than 95% purity as determined by HPLC analysis. The linear hexapeptide hydrazide was cyclized in high yield through formation of the acyl azide (step g). The crude product was purified by stirring with mixed bed ion exchange resin to remove acidic and basic impurities, delivering 1 in 70% overall yield and with a purity of 99% as determined by HPLC analysis.

Using this methodology, a number of analogs of L-365,209 were produced and were used to study structure–activity relationships in this novel class of oxytocin antagonist.[5] L-366,682 and L-366,948 (Figure 1) were identified as very potent and selective antagonists and were required in larger quantities to support pharmacological testing.[14] The synthesis of L-366,682 and L-366,948 proceeded as described for compound 1 (Scheme I) with the following exceptions. The 2,4-dinitrophenyl protecting group on the imidazole ring of D-His^6 was labile under conditions of Fmoc group removal and was completely removed by four treatments with piperidine–DMF. This necessitated the use of a larger excess of activated Fmoc-AA-Cl in steps c and d, due to acylation of the imidazole. Deacylation of the imidazole occurred on treatment with piperidine in the Fmoc deprotection steps. A single treatment with excess Fmoc-L-Ile-Cl in step d gave a conversion of only 90%. Complete conversion was achieved with a second treatment of reagent. Difficulty was encountered in preparing the acid chloride of Fmoc-D-tryptophan due to its low solubility in DCM, and the

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Table I. Ile^3-D-(N^1-Cbz)Piz^4 Coupling in the Conversion of Tetrapeptide Resin 2 to Pentapeptide Resin 3 (See Scheme II).

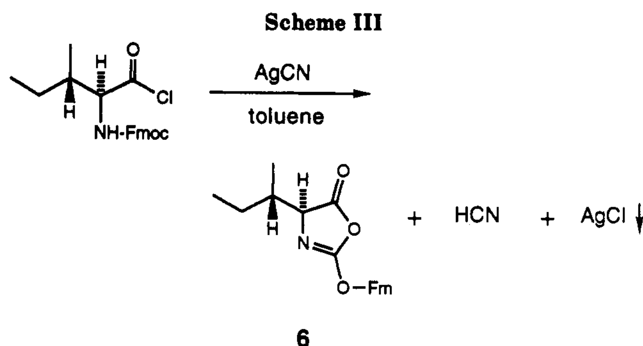
entry[a]	reagents[b] (equiv)	additive[c] (equiv)	solvent[d]	conversn[e] (%)
1	Fmoc-Ile-Cl (2)	DIEA (2)	DCM	10
2	Fmoc-Ile-Cl (10)	DIEA (10)	DCM	30
3	Fmoc-Ile-Cl (10)	none	DCM	50
4	Fmoc-Ile-Cl (10)	pyridine (10)	DCM	49
5	Fmoc-Ile-OH/BOP (2/2)	DIEA (2)	DMF	5
6	Fmoc-Ile-Cl (10)	AgCN (4)	toluene	76
7	Fmoc-Ile-Cl (10)	DIEA (10)	toluene	30
8	oxazolone 6[f] (10)	none	toluene	73
9	oxazolone 6[g] (10)	none	toluene	14
10	oxazolone 6[g] (10)	HCN (1)	toluene	50

[a] All couplings were run at ambient temperature for 12 h. [b] BOP = [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate. [c] DIEA = diisopropylethylamine. [d] DCM = dichloromethane, DMF = dimethylformamide. [e] Estimated by HPLC analysis of the tetra- and pentapeptide hydrazides resulting from hydrazinolysis of the resin after coupling. [f] A solution of oxazolone 6 was prepared by treating Fmoc-Ile-Cl with AgCN in toluene followed by filtration (Scheme III). [g] Prepared by treating Fmoc-Ile-Cl in DCM with aqueous $NaHCO_3$ (ref 14).

coupling was therefore carried out using the BOP reagent in DMF (step e). L-366,682 and L-366,948 were obtained in 45–48% overall yield and were purified by crystallization of the free base.

An especially difficult coupling was encountered in the synthesis of 5 (Scheme II), an analog that contains the unusual amino acid, piperazic acid, at position 4. A variety of coupling conditions were tried as shown in Table I. Acylation of the sterically hindered D-(N^1-Cbz)-piperazic acid[22,23] N-terminus in resin 2 with a 2-fold excess of Fmoc-L-Ile-Cl and DIEA gave only a 10% conversion to the pentapeptide resin 3 (entry 1). A larger excess and

Scheme III



higher concentration of reagents improved the efficiency to only 30% (entry 2). Coupling with the acid chloride in the absence of DIEA improved the conversion to 50% (entry 3), suggesting that the DIEA converts the Fmoc-AA-Cl to a less reactive oxazolone[15] at a rate similar to acylation of the piperazyl amine by the acid chloride. Use of the weaker base, pyridine (entry 4), in an attempt to set up an equilibrium between oxazolone and acid chloride[15] gave the same result as the reaction with no added base. Active ester coupling gave a lower conversion compared to the acid chloride at the same concentration (compare entries 1 and 5). During the course of our earlier solution-phase syntheses of cyclic hexapeptides related to L-365,209 we observed that AgCN in toluene improved the coupling efficiency of Fmoc-AA-Cl's.[16,17] Using this method, 76% conversion of resin 2 to resin 3 was obtained with one treatment (entry 6), and 96% conversion was achieved with two treatments. Improvement in yield due solely to a solvent effect was ruled out (entry 7).

To understand the yield enhancement observed in the AgCN-promoted reaction, several additional experiments were run. Treatment of Fmoc-Ile-Cl with an equimolar amount of AgCN in toluene was found to produce the corresponding 2-(fluorenylmethoxy)-5(4*H*)-oxazolone 6 (Scheme III) with a half-life of approximately 10 min as determined by ^{1}H NMR monitoring of a reaction run in toluene-d_8. The product was identical by ^{1}H and ^{13}C NMR to a sample of 6 prepared by other methods.[15,18] Because acylation of resin 2 (Scheme II) using the conditions of entry 6 in Table I was found to require a minimum of 5 h, it is inferred that the reactive species is related to oxazolone 6. Indeed, treatment of resin 2 with a solution of oxazolone 6 generated by treating Fmoc-Ile-Cl with AgCN in toluene for 1 h followed by removal of the silver salts gave a conversion equal to the in situ method (compare entries 6 and 8). However, treatment of resin 2 with preformed oxazolone 6 prepared by the method of Carpino[15] gave a very low conversion of 14% (entry 9). This same reaction with the addition of HCN (0.1 equiv with respect to oxazolone) improved the conversion by greater than 3-fold (entry 10), suggesting a role for the HCN byproduct produced in the AgCN-promoted reaction. More detailed mechanistic studies of this reaction are currently under investigation.

Completion of the synthesis of the cyclic hexapeptide 4 unexpectedly yielded two isomers in a ratio of 3:2 that were separated by preparative HPLC. Removal the Cbz group from each of these isomers gave products that were found to be identical by HPLC, TLC, NMR, and FAB mass spectroscopy (5). A possible explanation is that two stable rotomeric forms of 4 were isolated, perhaps cis–trans isomers about the hindered Ile^3-(N^δ-Cbz)Piz^4 amide bond.[19] Upon removal of the Cbz group the rotational barrier about the Ile^3-Piz^4 bond is altered such that 5 is isolated as a single compound.

In summary, Fmoc amino acid chlorides have proven to be effective reagents for use in the the solid-phase synthesis of highly N-alkylated peptides as exemplified by the 70% overall yield obtained for cyclic hexapeptide 1. The potent oxytocin antagonists L-366,682 and L-366,948 were prepared efficiently on a 20 mmol scale using this methodology. We have also shown that an activated form of the 2-(fluorenylmethoxy)-5(4*H*)-oxazolone derivative of isoleucine, obtained by treatment of Fmoc-Ile-Cl with AgCN, performs well in a case where steric hinderance limits the coupling efficiency of other protocols.

Experimental Section

General. Fmoc-L-isoleucine, Fmoc-D-phenylalanine, Fmoc-D-tryptophan, N^α-Boc-N^{im}-DNP-D-histidine were purchased from Bachem Inc. D-β-napthylalanine (D-β-Nal) was purchased from Synthetek, Inc. and protected using the TMSCl/Fmoc-Cl procedure of Bolin et al.[20] Boc-D-(*N*-methyl)phenylalanine was prepared by the method of Benoiton and Cheung.[21] Pipecolic acid (Pip) was resolved and protected as described below. D-(N^δ-Cbz)-Piperazic acid (D-(N^δ-Cbz)-Piz) was prepared using a slight variation on the method of Hassal and co-workers.[22,23] The Boc-L-Pro-PAM-resins were purchased from Applied Biosystems Inc. and Bachem Inc. All solvents were reagent grade and stored over 4A molecular sieves (DCM = dichloromethane, DMF = dimethylformamide, DIEA = diisopropylethylamine). Tetrahydrofuran (THF) was distilled from CaH_2–$NaBH_4$ under inert atmosphere. Dioxane was dried and freed of peroxides by passage through a column of activity I neutral alumina.

The motorized shaker apparatus and reaction vessels were custom designed and produced in house with the following specifications. The reaction vessels for a 1 mmol scale measured 3 cm × 9 cm with a female ground glass joint on top to accommodate a drying tube and a fritted disk and stopcock on the bottom for draining by vacuum. The vessel used for the 20 mmol scale was of the same design measuring 9 cm × 22 cm. The motorized shaker apparatus allows for a 90° shake of the reaction vessel at the rate of 32 shakes/min.

Analytical HPLC was run on a Spectra Physics SP4270/8800 using a Vydac 0.5 cm × 15 cm C_{18} reversed-phase column. Gradient conditions are as follows. Mobile phase A: 0.1% trifluoroacetic acid in water. Mobile phase B: 0.1% trifluoroacetic acid in acetonitrile. AT t = 0 min, A:B = 95:5; at t = 15 min, A:B = 0:100; flow rate = 2.0 mL/min. UV detection at 215 nm. TLC plates were purchased from Analtech, Inc. "Uniplate" silica gel GF (10 × 20 cm, 250 μm). Determination of reaction pH was estimated by spotting an aliquot on wetted E. Merck pH sticks. ^{1}H NMR spectra were measured at 300 MHz, and ^{13}C NMR spectra were measured at 75.4 MHz on a Varian XL-300 with $(CH_3)_4Si$ as an internal standard. Optical rotations were measured on a Perkin-Elmer 241 polarimeter at ambient temperature using a 1-mL capacity cell.

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N-[(9-Fluorenylmethoxy)carbonyl]-L-pipecolic Acid. Pipecolic acid was resolved by crystallization with an equimolar amount of tartaric acid from ethanol.[24] To an ice-cold stirred suspension of L-Pip D-(−)-tartrate salt (20.0 g; 71.7 mmol) in DCM (250 mL) under an atmosphere of nitrogen was added chlorotrimethylsilane (63.7 mL; 0.504 mol) followed by DIEA (88.0 mL; 0.505 mol). The solution was stirred at 0 °C for 30 min and then warmed to ambient temperature for 2 h. The solution was cooled to 0 °C prior to the addition of 9-fluorenylmethyl chloroformate (20.7 g, 80.0 mmol). After 15 h at 5 °C the solution was washed with 5% aqueous HCl (3 × 250 mL) and evaporated to an oil which was redissolved in ether (500 mL) and extracted with aqueous $NaHCO_3$ (4 × 300 mL). The combined aqueous layers were carefully acidified with 3 N HCl, and the product was extracted into ether (4 × 250 mL). The combined ether extracts were washed with water (250 mL) and brine (250 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The product was crystallized from ether (19 g; 75% of theoretical). Alternatively, the free amino acid could be obtained by ion-exchange chromatography as follows. L-Pipecolic acid D-(−)-tartaric acid salt (2.69 g) was dissolved in water (20 mL) and passed through a column packed with 16 mL of Biorad AG 4-X4 quaternary ammonium anion exchange resin. After elution with water and evaporation under reduced pressure, L-pipecolic acid was crystallized from ethanol and water to give 1.18 g, $[\alpha]_D = -18.5°$ (H_2O, $c = 1$). The free amino acid was then protected using the TMSCl/Fmoc-Cl procedure of Bolin and co-workers.[20] TLC: R_f = 0.32 (80:20 $CHCl_3$–CH_3OH). HPLC: retention time = 9.72 min, purity = 99.9%. $[\alpha]_D$: −21.7° (CH_3OH, $c = 1$). ^1H NMR ($CDCl_3$, 3:2 mixture of rotamers): δ 7.75 (m, 2 H), 7.56 (m, 2 H), 5.02 (d, J = 4.2 Hz, 0.6 H), 4.73 (d, J = 5.1 Hz, 0.4 H), 4.37 (m, 2 H), 4.05 (m, 1 H), 3.12 (m, 0.6 H), 2.97 (m, 0.4 H), 2.29 (m, 1 H), 1.55 (m, 6 H).

N-[(9-Fluorenylmethoxy)carbonyl]-D-pipecolic Acid. The title compound was obtained from D-Pip L-(+)-tartrate salt in a manner identical to the procedure for the L isomer given above, $[\alpha]_D = +21.4°$ (CH_3OH, $c = 1$).

General Procedure for the Preparation of Fmoc Amino Acid Chlorides.[10] N-[(9-Fluorenylmethoxy)carbonyl]-L-pipecolic Acid Chloride. Fmoc-L-Pip (712 mg; 2.03 mmol) was dissolved in dry DCM (5 mL) under an atmosphere of nitrogen and cooled to 0 °C. Oxalyl chloride (0.35 mL; 4.0 mmol) was added followed by DMF (0.016 mL; 0.2 mmol), and the reaction was stirred for 1 h during which time gas evolution was observed. The solvent was removed by evaporation under reduced pressure, and the residue was taken up in dry DCM (5 mL) and evaporated under reduced pressure (two times) to yield a foam. Dissolving a small sample in dry methanol to form the methyl ester indicated greater than 98% conversion to the acid chloride by HPLC analysis; retention time = 11.30 min (methyl ester), 9.71 min (acid). ^1H NMR ($CDCl_3$): δ 7.77 (d, J = 7.3 Hz, 2 H), 7.57 (m, 2 H), 7.41 (t, J = 7.1 Hz, 2 H), 7.33 (m, 4 H), 5.18 (m, 1 H), 4.48 (m, 5 H), 4.23 (t, J = 6.8 Hz, 1 H), 2.11 (m, 1 H), 1.46 (m, 1 H), 1.03 (d, J = 6.8 Hz, 2 H), 0.96 (t, J = 7.3 Hz, 3 H).

General Procedure for Hydrazinolysis of Resin Aliquots. A 20-mg sample of N-deprotected resin was dried for 24 h in vacuo and then stirred with 1:1 MeOH–hydrazine (1 mL) for 30 min at ambient temperature. The volatiles were then removed under reduced pressure at 50 °C. The residue was slurried in MeOH (0.5 mL), and the resin beads were allowed to settle. A 0.010-mL sample of the supernatant was analyzed by HPLC (see above for conditions).

Cyclo-(D-Phe-Ile-D-Pip-Pip-D-(N-Me)Phe-Pro) (1). Step a: Boc-D-(N-Me)Phe-Pro-O-(PAM)-resin. Boc-Pro-O-(PAM)-resin (1.32 g, 1.0 mmol) with a loading of 0.76 mequiv of nitrogen/g was placed in a shaker flask and swelled for 2 h in 20 mL of DCM. The resin was then treated with 20 mL of the following: DCM wash (3×, 2 min each), 4 N HCl–dioxane deblocking (2×, 15 min each), DCM wash (3×, 2 min each), DMF wash (3×, 2 min each), 10% DIEA–DMF neutralization (2×, 2 min each), and DMF wash (2×, 2 min each). Boc-D-(N-Me)Phe (0.558 g, 2.0 mmol) in 15 mL of 1:1 DCM–DMF was then added followed by DIEA (0.350 mL, 2.0 mmol). The resin was shaken for 5 min when solid BOP reagent[13] (0.884 g, 2.0 mmol) was added. The resin was shaken for 10 min, and more DIEA (0.04 mL) was added to bring the supernatant to pH 8. The reaction was shaken for an additional 15 h at ambient temperature. To complete the cycle, the supernatant was drained and the resin was washed for 2 min each with 20 mL of the following: DMF (2×); DCM; CH_3OH; DCM; CH_3OH; DCM (3×). An aliquot of the resin was N-deprotected and hydrazinolyzed as described above (HPLC t_R = 3.60 min).

Step b: Fmoc-Pip-D-(N-Me)Phe-Pro-O-(PAM)-resin. The resin from the previous step was treated with 20 mL of the following: 4 N HCl–dioxane deblocking (2×, 15 min each) and DCM wash (6×, 2 min each). The resin was then cooled to 5 °C in a cold room, and then a 0 °C solution of Fmoc-Pip-Cl (2.0 mmol) in DCM (20 mL) was added and the mixture was shaken for 5 min. DIEA (0.35 mL, 2.0 mmol) was added, the reaction mixture was shaken for 2 min, and the supernatant was adjusted to pH 8 by the addition of more DIEA (0.1 mL). The resin was shaken for 15 h at 5 °C. To complete the cycle, the supernatant was drained and the resin was washed for 2 min each with 20 mL of the following: DCM (3×), CH_3OH, DCM, CH_3OH, and DCM (3×). An aliquot of the resin was N-deprotected and hydrazinolyzed as described above (HPLC t_R = 4.81 min).

Steps c–e: Fmoc-D-Phe-Ile-D-Pip-Pip-D-(N-Me)Phe-Pro-O-(PAM)-resin. Coupling of the remaining three amino acids was accomplished by cycling the resin through the following protocol. The resin was treated with 20 mL of the following: DMF wash (3×, 2 min each), 20% piperidine–DMF deblocking (2×, 15 min each), DMF wash (3×, 2 min each), and DCM wash (3×, 2 min each). The Fmoc amino acid chloride (2 mmol) in DCM (20 mL) was shaken with the resin at ambient temperature for 5 min, and then DIEA (2 mmol) was added. The mixture was shaken for 10 min, and if neccessary more DIEA was added to bring the supernatant to pH 8. The resin was shaken for 2 h at ambient temperature. The supernatant was drained, and the resin was washed for 2 min each with 20 mL of the following: DCM (4×), CH_3OH, DCM, CH_3OH, and DCM (3×). An aliquot of resin at each stage was N-deprotected and hydrazinolyzed as described above (HPLC t_R = 5.93 min (tetrapeptide), 6.52 min (pentapeptide), 7.75 min (hexapeptide).

Step f: H-D-Phe-Ile-D-Pip-Pip-D-(N-Me)Phe-Pro-$NHNH_2$. The resin was treated with 20 mL of the following: DMF wash (3×, 2 min each) and 20% piperidine–DMF deblocking (2×, 15 min each). The resin was washed for 2 min each with 20 mL of the following: DMF (3×), DCM (4×), CH_3OH, DCM, and CH_3OH. The resin was dried at 0.5 Torr for 24 h. The dried resin (1.5 g) had a nitrogen value of 4.97% from combustion analysis, indicating a peptide loading of 0.59 mmol/g. The resin was stirred with 1:1 methanol–hydrazine (60 mL) under nitrogen at ambient temperature for 1 h and at 50 °C for 30 min. The excess hydrazine and methanol were removed under reduced pressure at 50 °C, and methanol (60 mL) was added and evaporated under reduced pressure three times. The residue was then suspended in methanol (60 mL) and filtered. The filtrate was evaporated under reduced pressure, and the residue was dried in vacuo for 15 h. The residue was dissolved in n-butanol (100 mL) and extracted with water (3 × 50 mL) to remove traces of hydrazine. The n-butanol layer was evaporated under reduced pressure, and methanol (100 mL) was added and evaporated under reduced pressure. The hydrazide (HPLC t_R = 7.75 min, purity = 95%) was dried in vacuo for 15 h (579 mg; 75% of theoretical).

Step g: Cyclo-(D-Phe-Ile-D-Pip-Pip-D-(N-Me)Phe-Pro) (1). The hydrazide (579 mg, 0.737 mmol) was dissolved in dry, degassed DMF (6 mL) under an atmosphere of nitrogen and cooled to −15 °C. To the stirred solution was added 5 N HCl–THF (0.69 mL, 3.5 mmol), and the reaction was further cooled to −25 °C. Isoamyl nitrite (0.105 mL, 0.774 mmol) was added in small portions over a period of 1 h, monitoring for the disappearance of excess isoamyl nitrite by spotting aliquots on acidified starch–KI paper. When a positive starch–KI test persisted for 30 min, addition of isoamyl nitrite was stopped. HPLC analysis indicated complete conversion to the acyl azide (hydrazide retention time = 7.75 min, acyl azide retention time = 9.29 min). The reaction was then diluted with dry, degassed DMF (150 mL) which was precooled under nitrogen to −25 °C. DIEA (0.67 mL, 3.8 mmol) was added to the stirred solution to pH 8. The reaction temperature was maintained at −20 °C for 24 h. A single product was detected by HPLC analysis

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(retention time = 11.16 min). The solution was evaporated under reduced pressure to an oil which was purified by stirring for 1 h with 10 mL of Bio-Rad analytical-grade mixed-bed resin AG 501-X8 in 30 mL of 3:1 DMF–water. The resin was removed by filtration, and the filtrate solvents were evaporated under reduced pressure. Compound 1 was obtained as a white solid by lyophilization from dioxane (520 mg; 70% of theoretical). TLC: R_f = 0.32 (98:2:0.2 $CHCl_3$–CH_3OH–NH_4OH). HPLC: t_R = 11.14 min, purity = 99%. ^1H NMR ($CDCl_3$): δ 7.87 (d, J = 7.1 Hz, 1 H), 7.67 (d, J = 8.6 Hz, 1 H), 7.23 (m, 10 H), 5.48 (t, J = 7.6 Hz, 1 H), 5.30 (d, J = 4.4 Hz, 2 H), 4.53 (m, 3 H), 4.00 (m, 1 H), 3.83 (m, 1 H), 3.49 (m, 2 H), 2.83 (s, 3 H), 2.15 (m, 1 H), 0.81 (m, 5 H). FAB MS: m/z = 741 ($M + H^+$). Anal. Calcd for $C_{42}H_{56}N_6O_6$, 1.35 H_2O: C, 65.91; H, 7.73; N, 10.98. Found: C, 65.95; H, 7.60; N, 10.84.

Cyclo-(D-Trp-Ile-D-Pip-Pip-D-His-Pro) (L-366,682). Step a: Boc-D-(DNP)His-Pro-*O*-(PAM)-resin. Boc-Pro-*O*-(PAM)-resin (33 g, 20 mmol) with a loading of 0.61 mequiv of nitrogen/g was placed in a shaker flask and swelled for 2 h by the addition of 250 mL of DCM. The resin was then treated with 200 mL of the following: DCM wash (3×; 2 min each), 4 N HCl–dioxane deblocking (2×; 15 min), DCM wash (3×; 2 min each), DMF wash (3×; 2 min each), 10% DIEA–DMF neutralization (2×; 2 min each), DMF wash (2×, 2 min each). The resin was then shaken for 5 min with a solution of Boc-D-(DNP)His (17.6 g, 40.0 mmol) in 200 mL of 1:1 DCM–DMF and DIEA (10.5 mL, 40.0 mmol). Solid BOP reagent[11] (17.6 g, 40.0 mmol) was added to the flask. After the reaction mixture was shaken for 10 min, more DIEA (1.0 mL) was added to bring the supernatant to pH 8, and the reaction was shaken for 15 h at ambient temperature. The supernatant was drained, and the resin was washed for 2 min each with 200 mL of the following: DMF (2×); DCM; CH_3OH; DCM; CH_3OH; and DCM (3×). An aliquot of the resin was N-deprotected and hydrazinolyzed as described above (HPLC t_R = 1.8 min).

Step b: Fmoc-Pip-D-(DNP)His-Pro-*O*-(PAM)-resin. The resin was treated with 200 mL of the following: 4 N HCl–dioxane deblocking (2×, 15 min each) and DCM wash (6×, 2 min each). The resin was cooled to 5 °C in a cold room and to it was added a 0 °C solution of Fmoc-Pip-Cl (50 mmol) in DCM (200 mL). The mixture was shaken for 5 min, and then DIEA (10.5 mL, 60.0 mmol) was added. The mixture was shaken for 2 min, and the supernatant was adjusted to pH 8 by the addition of more DIEA (3 mL). The resin was then shaken for 15 h at 5 °C. The supernatant was drained, and the resin was washed for 2 min each with 200 mL of the following: DCM (3×), CH_3OH, DCM, CH_3OH, and DCM (3×). An aliquot of the resin was N-deprotected and hydrazinolyzed as described above (HPLC t_R = 3.05 min).

Step c: Fmoc-D-Pip-Pip-D-His-Pro-*O*-(PAM)-resin. The resin was treated with 200 mL of the following: DMF wash (3×, 2 min each); 20% piperidine–DMF deblocking (2×, 15 min each); DMF wash (3×, 2 min each); DCM wash (3×, 2 min each). To the resin was added a solution of Fmoc-D-Pip-Cl (50 mmol) in DCM (200 mL) at ambient temperature. The mixture was shaken for 5 min, and then DIEA (8.7 mL; 50.0 mmol) was added. After 15 min of shaking, the supernatant was adjusted to pH 8 by the addition of more DIEA (2.3 mL, 13.0 mmol), and the reaction mixture was shaken for 15 h. The supernatant was drained, and the resin was washed for 2 min each with 200 mL of the following: DCM (4×); CH_3OH; DCM; CH_3OH; and DCM (3×). An aliquot of the resin was N-deprotected and hydrazinolyzed as described above (HPLC t_R = 2.74 min).

Step d: Fmoc-Ile-D-Pip-Pip-D-His-Pro-*O*-(PAM)-resin. The previous deprotection-coupling cycle was repeated using Fmoc-Ile-Cl (50 mmol). The resin was washed for 2 min each with 200 mL of the following: DCM (2×), DMF (2×), and DCM (2×). Hydrazinolysis of an aliquot of resin indicated 90% conversion to the pentapeptide, and thus the resin was treated with more Fmoc-Ile-Cl (50 mmol) in DCM (200 mL) and DIEA (60 mmol to obtain a pH 8 supernatant). Greater than 99% coupling efficiency was achieved after the second treatment as determined by N-deprotection and hydrazinolysis of a resin aliquot (HPLC t_R = 4.64 min). The supernatant was drained, and the resin was washed for 2 min each with 200 mL of the following: DCM (4×); CH_3OH; DCM; and CH_3OH.

Step e: Fmoc-D-Trp-Ile-D-Pip-Pip-D-His-Pro-*O*-(PAM)-resin. The resin was treated with 200 mL of the following: DCM wash (3×, 2 min each), DMF wash (3×, 2 min each), 20% piperidine–DMF deblocking (2×, 15 min each), and DMF wash (3×, 2 min each). Fmoc-D-Trp (21.3 g, 70 mmol) and DIEA (13 mL, 75 mmol) in 200 mL of DMF were added to the resin, and the mixture was shaken for 5 min. Solid BOP reagent[11] (31 g, 70 mmol) was added, and after the mixture was shaken for 15 min, the supernatant was adjusted to pH 8 by the addition of more DIEA (0.5 mL). The resin was shaken for 15 h, the supernatant was drained, and the resin was washed for 2 min each with 200 mL of the following: DCM (4×); CH_3Oh; DCM; CH_3OH; and DCM (3×). An aliquot of the resin was N-deprotected and hydrazinolyzed as described above (HPLC t_R = 6.24 min).

Step f: H-D-Trp-Ile-D-Pip-Pip-D-His-Pro-$NHNH_2$. The resin was treated with 200 mL of the following: DMF wash (3×, 2 min each) and 20% piperidine–DMF deblocking (2×, 15 min each). The resin was washed for 2 min each with 200 mL of the following: DMF (3×), DCM (4×), CH_3OH, DCM, and CH_3OH. The resin was dried at 0.5 Torr for 24 h. Amino acid analysis of the dried resin (49 gm) indicated a peptide loading of 0.3 mmol/g. The resin was stirred with 1:1 methanol–hydrazine (600 mL) under an atmosphere of nitrogen at ambient temperature for 1 h and at 50 °C for 1 h. The excess hydrazine and methanol were removed under reduced pressure at 50 °C, and methanol (600 mL) was added and evaporated under reduced pressure three times. The residue was suspended in methanol (600 mL) and filtered. The filtrate was evaporated under reduced pressure, and the resulting glass was dried in vacuo for 15 h. The glass was dissolved in n-butanol (400 mL) and extracted with water (3 × 150 mL) to remove traces of hydrazine. The n-butanol layer was then evaporated under reduced pressure, and methanol (200 mL) was added and evaporated under reduced pressure. The hydrazide (HPLC retention time = 6.24 min, purity = 92%) was dried in vacuo for 15 h (10.63 g, 68% of theoretical).

Step g: Cyclo-(D-Trp-Ile-D-Pip-Pip-D-His-Pro) (L-366,682). The hydrazide (10.63 g, 13.5 mmol) was split into two equal batches. Each batch (6.75 mmol) was dissolved in dry, degassed DMF (100 mL) and cooled under an atmosphere of nitrogen to −15 °C. A solution of 4.52 M HCl–THF (7.5 mL, 0.34 mmol) was added, and the reaction was further cooled to −25 °C. Isoamyl nitrite (0.92 mL; 6.8 mmol) was added in small portions over 1 h, monitoring for the disappearance of excess isoamyl nitrite by spotting aliquots on starch–KI paper. When a slight excess (positive test) persisted for 30 min, the addition was stopped. HPLC analysis indicated complete conversion to the acyl azide (retention time = 7.46 min). The reaction was diluted with dry, degassed DMF (1 L) that had been precooled under nitrogen to −25 °C. The solution was brought to pH 8 by the addition of DIEA (5.9 mL). The reaction temperature was maintained at −20 °C for 24 h. A new product was detected by HPLC analysis (t_R = 7.58 min). The two batches were combined at this point, and the solvent was evaporated under reduced pressure. The resulting oil was partitioned between $CHCl_3$ and 10% aqueous $NaHCO_3$. A precipitate formed at the interface on standing and was filtered and dried to give the title compound (7.25 g, purity = 97% by HPLC analysis; 48% of theoretical). Crystallization from MeOH gave material that was essentially homogeneous by HPLC analysis. TLC: R_f = 0.42 (80:20:1 $CHCl_3$–CH_3OH–NH_4OH). HPLC: t_R = 7.58 min, purity = 99.9%. ^1H NMR (DMSO-d_6): δ 8.19 (m, 2 H), 7.57 (d, J = 7.8 Hz, 1 H), 7.50 (s, 1 H), 7.30 (d, J = 8.0 Hz, 1 H), 7.14 (d, J = 1.3 Hz, 1 H), 6.98 (m, 4 H), 6.72 (s, 1 H), 5.26 (d, J = 4.1 Hz, 1 H), 5.03 (d, J = 1.3 Hz, 1 H), 4.75 (m, 1 H), 4.54 (m, 1 H), 4.22 (m, 1 H), 3.97 (m, 1 H), 3.70 (m, 1 H), 3.36 (m, 1 H), 0.74 (m, 5 H). FAB MS: m/z = 756 ($M + H^+$). Mp: 165–180 °C dec. Anal. Calcd for $C_{40}H_{53}N_9O_6$, 1.8 H_2O: C, 60.94; H, 7.24; N, 15.99. Found: C, 60.90; H, 6.98; N, 16.12.

Cyclo-(D-β-Nal-Ile-D-Pip-Pip-D-His-Pro) (L-366,948). This compound was produced in a manner similar to L-366,682, only Fmoc-D-β-naphthylalanine was substituted for Fmoc-D-tryptophan in step e. The crude product was dissolved in $CHCl_3$ (1 L) and extracted with water (2 × 1 L), and the organic layer was evaporated under reduced pressure to give an oil. Crystallization from EtOAc–MeOH gave the title compound as a white solid (6.80 g, 45% of theoretical). TLC: R_f = 0.55 (80:20:1 $CHCl_3$–CH_3OH–NH_4OH). HPLC: t_R = 8.62 min, purity = 99.7%. ^1H NMR (DMSO-d_6): δ 8.35 (d, J = 8.0 Hz, 1 H), 8.18 (d, J = 8.6 Hz, 1 H), 7.85 (d, J = 8.0 Hz, 1 H), 7.80 (d, J = 8.6 Hz, 2 H), 7.72 (s, 1 H), 7.46 (m, 5 H), 6.92 (d, J = 8.0 Hz, 1 H), 5.27 (d, J = 4.6 Hz,

1 H), 5.03 (d, J = 4.0 Hz, 1 H), 4.74 (dd, J = 5.7, 4.3 Hz, 1 H), 4.62 (dd, J = 5.6, 4.3 Hz, 1 H), 4.50 (t, J = 8.6 Hz, 1 H), 4.21 (m, 1 H), 3.94 (d, J = 13.4 Hz, 1 H), 3.72 (d, J = 8.57 Hz, 1 H), 3.05 (m, 2 H), 2.78 (m, 3 H), 0.68 (d, J = 6.4 Hz, 2 H), 0.49 (t, J = 7.7 Hz, 3 H). FAB MS: m/z = 767 (M + H$^+$). Mp: 220–230 °C dec. Anal. Calcd for $C_{42}H_{54}N_8O_6$, 1.0 EtOAc, 2.5 H_2O: C, 61.39; H, 7.50; N, 12.45. Found: C, 61.36; H, 7.17; N, 12.69.

H-D-(N^δ-Cbz)Piz-Pip-D-His-Pro-*O*-(PAM)-resin (2). The title resin was prepared on a 1 mmol scale, using steps a and b to obtain the tripeptide resin as described above for L-366,682. The tripeptide resin was treated with 20 mL of the following: DMF wash (3×, 2 min each), 20% piperidine–DMF deblocking (2×, 15 min each), DMF wash (3×, 2 min each), and DCM wash (3×, 2 min each). D-(N^α-Fmoc, N^δ-Cbz)-Piz-Cl[22,23] (1.21 g, 2.5 mmol) was dissolved in 20 mL of DCM and shaken with the resin at ambient temperature for 5 min, and then DIEA (2 mmol) was added. The mixture was shaken for 10 min, and the pH of the supernatant was checked. No further DIEA was required to obtain a pH 8 solution. The resin was shaken for 2 h at ambient temperature. The supernatant was drained, and the resin was washed for 2 min each with 20 mL of the following: DCM (4×), CH_3OH, DCM, CH_3OH, and DCM (3×). The resin was N-deprotected by treatment with 20 mL of the following: DMF wash (3×, 2 min each), 20% piperidine–DMF deblocking (2×, 15 min each), and DCM wash (3×, 2 min each) to give resin 2. An aliquot of the resin was hydrazinolyzed as described above (HPLC t_R = 4.32 min).

Fmoc-Ile-D-(N^δ-Cbz)Piz-Pip-D-His-Pro-*O*-(PAM)-resin (3). Resin 2 was treated with a toluene solution (20 mL) of Fmoc-Ile-Cl (3.53 g, 10 mmol), and the mixture was shaken for 5 min. To the resin mixture was then added AgCN (0.5 g, 3.7 mmol) in portions until a neutral pH was obtained. After being shaken for 12 h, the resin was washed for 2 min each with 20 mL of the following: toluene (2×); DCM (2×); and toluene (2×). The resin was then retreated with Fmoc-Ile-Cl (10 mmol) in toluene (20 mL). No additional AgCN was required to give a neutral pH. The mixture was shaken for 12 h. An aliquot of resin was N-deprotected and hydrazinolyzed as described above (HPLC t_R = 4.65 min) and indicated a conversion of 96%. The cycle was completed by washing the resin for 2 min each with 20 mL of the following: DCM (4×); CH_3OH; DCM; and CH_3OH.

H-D-Phe-Ile-(Cbz)-D-Piz-Pip-D-His-Pro-*O*-(PAM)-resin. Resin 3 was treated with 20 mL of the following: DMF wash (3×, 2 min each), 20% piperidine–DMF deblocking (2×, 15 min each), DMF wash (3×, 2 min each), and DCM wash (3×, 2 min each). During the piperidine–DMF washes, the silver salts dissolved and were thus conveniently removed. To the resin was added a solution of Fmoc-D-Phe-Cl (2 mmol) in DCM (20 mL), and the mixture was shaken for 5 min. To the resin mixture was then added DIEA (2 mmol). The mixture was shaken for 10 min, and the supernatant was checked for pH. No further DIEA was needed to obtain a pH 8 solution. The resin was shaken for 2 h at ambient temperature. The supernatant was drained, and the resin was washed for 2 min each with 20 mL of the following: DCM (4×), CH_3OH, DCM, CH_3OH, and DCM (3×). The resin was N-deprotected by treatment with 20 mL of the following: DMF wash (3×, 2 min each), 20% piperidine–DMF deblocking (2×, 15 min each), and DCM wash (3×, 2 min each).

Cyclo-(D-Phe-Ile-D-(N^δ-Cbz)Piz-Pip-D-His-Pro) (4). The hydrazide was generated (800 mg) and cyclized as described above for compound 1 (HPLC t_R = 6.88 min). Two major products were detected by HPLC analysis in a ratio of 3:2. Preparative HPLC separated the isomers: **4a** (80 mg; HPLC t_R = 8.41 min, purity = 99%) and **4b** (60 mg; HPLC t_R = 8.78 min, purity = 99%).

Cyclo-(D-Phe-Ile-D-Piz-Pip-D-His-Pro) (5). Each isomer from above (**4a** and **4b**) was N-deprotected by stirring with 10% Pd/C (25 mg) in 1% acetic acid in ethanol (5 mL) under 1 atm of hydrogen for 16 h at ambient temperature. The catalyst was removed by filtration, the solvent was removed under reduced pressure, and the residue from each reaction was purified by preparative HPLC. Spectroscopic characterization of the product obtained from each reaction indicated that they were identical. HPLC: t_R = 7.70 min, purity 99%. ^{1}H NMR (DMSO-d_6): δ 8.73 (s, 1 H), 8.32 (d, J = 8.0 Hz, 1 H), 7.60 (d, J = 7.1 Hz, 1 H), 7.35 (d, J = 8.5 Hz, 1 H), 7.22 (m, 6 H), 5.16 (m, 3 H), 4.95 (d, J = 5.2 Hz, 1 H), 4.70 (m, 1 h), 4.40 (dd, J = 7.3, 7.0 Hz, 1 H), 4.18 (m, 1 H), 3.75 (m, 1 H), 3.59 (m, 1 H), 0.72 (m, 6 H). FAB MS: m/z = 718 (M + H$^+$). Anal. Calcd for $C_{37}H_{51}N_9O_6$, 1.65 CF_3CO_2H: C, 52.38; H, 5.96; N, 13.64. Found: C, 52.44; H, 5.95; N, 13.94.

2-[(9-Fluorenylmethyl)oxy]-4(*S*)-(1(*S*)-methylpropyl)-5-(4*H*)-oxazolone. Method A. To a rapidly stirred solution of Fmoc-Ile-Cl (353 mg, 1.00 mmol) in dry toluene (20 mL) was added AgCN (146 mg, 1.1 mmol). The reaction was stirred for 30 min, filtered through washed Celite, and evaporated under reduced pressure.

Method B. The method of Carpino and co-workers was utilized.[14] To a rapidly stirred solution of Fmoc-Ile-Cl (353 mg, 1.00 mmol) in dry DCM (20 mL) at 0 °C was added a solution of saturated aqueous $NaHCO_3$. After 20 min, the DCM layer was separated, dried over Na_2SO_4, filtered, and evaporated under reduced pressure.

The ^{1}H and ^{13}C NMR spectra of the oxazolones produced by the two methods were identical with the exception of a small peak at 108.7 ppm, ascribed to a trace of HCN, observed in the ^{13}C NMR spectrum of the AgCN derived oxazolone. ^{1}H NMR ($CDCl_3$): δ 7.75 (d, J = 7.5 Hz, 2 H), 7.60 (m, 2 H), 7.40 (t, J = 7.4 Hz, 2 H), 7.30 (t, J = 7.4 Hz, 2 H), 4.56 (m, 2 H), 4.36 (t, J = 7.5 Hz, 1 H), 4.19 (d, J = 4.4 Hz, 1 H), 1.92 (m, 1 H), 1.46 (m, 1 H), 1.29 (m, 1 H), 0.97 (d, J = 6.9 Hz, 2 H), 0.90 (t, J = 7.4 Hz, 3 H). ^{1}H NMR (toluene-d_8): δ 7.50 (d, J = 6.7 Hz, 2 H), 7.40 (dd, J = 13.2, 6.2 Hz, 4 H), 7.15 (m, 4 H), 4.29 (m, 2 H), 4.04 (t, J = 7.6 Hz, 1 H), 3.68 (d, J = 4.7 Hz, 1 H), 1.68 (m, 1 H), 1.15 (m, 1 H), 0.84 (d, J = 6.8 Hz, 2 H), 0.73 (t, J = 7.4 Hz, 3 H). ^{13}C NMR (toluene-d_8): δ 174.9, 158.7, 143.5 (2 C), 141.8 (2 C), 128.2 (2 C), 127.5 (2 C), 120.4 (2 C), 72.0, 70.2, 46.7, 37.7, 25.2, 15.3, 11.6.

Acknowledgment. We are pleased to acknowledge the contributions of M. Tang and D. G. Kolodin (amino acid analysis), J. P. Moreau (elemental analysis), and J. Kaysen (manuscript preparation). We also thank Dr. P. S. Anderson for his encouragement and support.