Synthesis of a Novel Esterase-Sensitive Cyclic Prodrug System for Peptides That Utilizes a "Trimethyl Lock"-Facilitated Lactonization Reaction

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Received September 17, 1996⊗

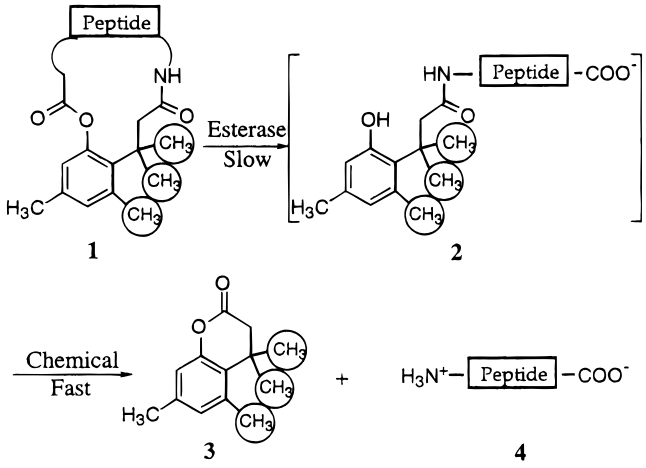
This paper describes a unique strategy for preparing cyclic prodrugs of peptides that have increased metabolic stability and increased cell membrane permeability when compared to the linear peptides. By taking advantage of a unique "trimethyl lock"-facilitated lactonization system, an esterase-sensitive cyclic prodrug of a model hexapeptide H-Trp-Ala-Gly-Gly-Asp-Ala-OH was synthesized by linking the N-terminal amino group to the C-terminal carboxyl group. The key intermediate for both approaches was compound **9** with Boc-Ala attached to the phenol hydroxyl group of the "trimethyl lock" linker through an ester bond, which can then be incorporated into the peptide using a normal coupling reagent for peptide synthesis. The synthesis of the linear peptides was accomplished using both solution-phase and solid-phase approaches with the solution-phase approach having the advantage of using the key intermediate **9** most efficiently. Cyclization using standard high-dilution techniques provided cyclic prodrug **13**. In 90% human plasma, prodrug **13** released the original peptide, as designed, through an apparent esterase-catalyzed hydrolysis of the phenol ester bond.

Introduction

Two of the major obstacles to the development of biologically active peptides as clinically useful therapeutic agents have been their low permeability through biological barriers (*e.g.*, intestinal mucosa, blood−brain barrier) and their metabolic lability.[1] Earlier, we reported that masking the C-terminal and N-terminal polar functional groups of a peptide through cyclization with an (acyloxy)-alkoxy linker can greatly enhance the membrane permeability and metabolic stability of the linear peptide.[2,3] In this paper, we wish to report a new and generally applicable approach to synthesizing esterase-sensitive prodrugs of peptides by taking advantage of a unique "trimethyl lock"-facilitated lactonization system.

Substituted phenol propionic acid derivatives such as **2**, upon unmasking of the hydroxyl group, undergo a facile spontaneous intramolecular cyclization to release the moieties attached to the carboxyl functional group (Scheme 1).[4−6] The facile cyclization reaction is the result of the "trimethyl lock," which was shown earlier to increase the rate of the cyclization reaction on the order of 10^5−10^7.[4−7] The result of such facilitation is that intermediate **2** has a half-life of only approximately 100 s at room temperature in aqueous solution.[8,9] Such systems have been used to develop prodrugs of amines and alcohols[8−10] and redox-sensitive protecting groups of amines.[11] However, the facile lactonization of this system also makes it an attractive target for the development of a unique linker in an esterase-sensitive cyclic prodrug system for peptides and peptide mimetics (Scheme 1). In this approach, the C- and N-terminal ends of a linear peptide can be masked by forming an ester and an amide bond with the phenol hydroxyl and side-chain carboxyl

Scheme 1. Design of an Esterase-Sensitive Prodrug System for Cyclic Derivatization of Peptides



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⊗ Abstract published in *Advance ACS Abstracts*, February 15, 1997.

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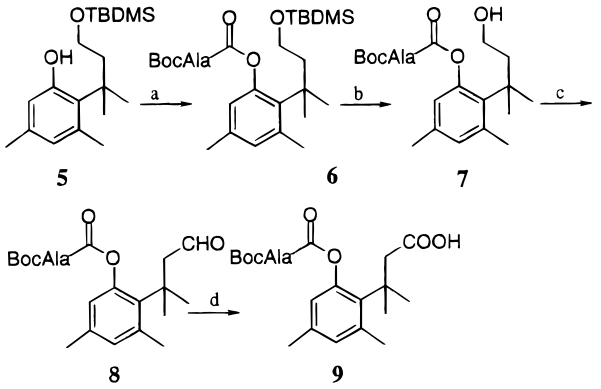
S0022-3263(96)01778-1 CCC: $14.00

Scheme 2. Synthetic Approach to the Intermediate with Boc-Ala Attached to the Linker[a]



[a] Key: (a) BocAla-OpNP, DMAP/DCM, reflux; (b) HOAc/THF/H_2O; (c) PCC; (d) $KMnO_4$.

groups, respectively, of the linker, which is also referred to as the pro-moiety (Prom) (Scheme 1). To demonstrate the feasibility of this approach, we synthesized the cyclic prodrug of a model hexapeptide (H-Trp-Ala-Gly-Gly-Asp-Ala-OH) by linking the N-terminal amino group to the C-terminal carboxyl group via this "trimethyl lock" linker. The cyclic prodrug was shown to be 70 times more able to permeate an *in vitro* cell culture model of the intestinal mucosa than was the parent linear peptide.[12] As it was designed, the parent peptide was shown to be released by an apparent esterase-catalyzed cleavage of the phenol ester linkage (Scheme 1) in 90% human plasma.[12]

Results and Discussion

Due to the facile cyclization of the system described, the incorporation of the "linker" into the cyclic system requires first the protection of the phenol hydroxyl group. Therefore, the key intermediate for successful synthesis of cyclic prodrug **13** was compound **9** with Boc-Ala attached to the phenol hydroxyl group of the "linker" through an ester bond (Scheme 2). The synthesis of **9** started with commercially available 3,5-dimethylphenol. The *tert*-butyldimethyl silyl (TBDMS)-protected diol **5** was prepared by a literature procedure[9] in three steps with over 90% yield for each step. Reaction of activated *p*-nitrophenol (pNP) ester of the *N*-Boc-alanine with **5** in the presence of 4-(*N*,*N*-dimethylamino)pyridine (DMAP) in refluxing dichloromethane (DCM) gave **6** in 80% yield. Treatment of the TBDMS ether **6** with acetic acid in the presence of water and THF yielded the primary alcohol **7**, which was converted in a two-step oxidation with pyridinium chlorochromate (PCC) and $KMnO_4$ to the key intermediate **9**. It should be noted that the Boc-alanyl group of **7** has a tendency to migrate to the primary hydroxyl group, presumably due to the high steric hindrance of the phenol hydroxyl position. This migration occurs more readily during column chromatography. Therefore, compound **7** was used for the oxidation without purification.

Because solid-phase peptide synthesis normally allows for faster synthesis of a peptide than does the solution-phase approach, we first employed the solid-phase approach to the synthesis of the linear peptide. This synthesis involves the attachment of β-benzyl-protected aspartic acid as the first amino acid to Wang's resin[13] through activation of the carboxyl group directly with diisopropylcarbodiimide (DIC). On the basis of the substitution density of the resin (0.95 mmol/g) and the weight increase after the coupling of protected aspartic acid, about 50% loading was achieved. Synthesis of the linear pentapeptide attached to the resin **10** (Scheme 3) was accomplished using standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry, and the ninhydrin test was used to determine whether the coupling was completed for each step.[14,15] The "trimethyl lock" linker **9** was attached as the last protected amino acid using BOP ((benzotriazoyloxy)tris(dimethylamino)phosphonium hexafluorophosphate) as the activating reagent to give **11a**. The cleavage of the peptide from the solid support with 50% trifluoroacetic acid (TFA) in DCM gave the linear peptide **12** in about 50% yield. This linear peptide was used directly for the cyclization reaction without purification. Cyclization of **12** with BOP-Cl [*N*,*N*-bis(2-oxo-3-oxazolidinyl)phosphinic acid] as the activating reagent[16] gave the cyclized product in about 8% yield, which was then converted to the final product **13** through catalytic hydrogenation. It should be noted that although using more than 1 equiv of an amino acid is a common practice in solid-phase peptide synthesis, it results in a significant waste of the key intermediate **9**. Because the preparation of **9** represents the most time-consuming process, we were interested in finding a synthetic approach to the final product, which would allow for a more efficient use of **9**. Therefore, we also studied the solution-phase approach.

In the solution-phase approach, linear pentapeptide **14** was prepared using standard Boc-amino acid chemistry.[15,17] The key to this solution-phase approach was the selective protection of the α- and β-carboxyl groups of the Asp residue. We successfully used the trichloroethyl (Tce) ester protecting group for the α-carboxyl group of Asp residue. Compound **14** was treated with 50% TFA in DCM to give **15** in quantitative yield. The pentapeptide **15** was reacted with **9** in the presence of 1-(3-(dimethylamino)propyl)ethylcarbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt), and *N*-methylmorpholine (NMM) to give the fully protected linear hexapeptide **11b** in 68% yield. The protecting groups were removed using zinc/HOAc (acetic acid) and 50% TFA/DCM to provide **12** in 50% overall yield. Compound **12** was purified by preparative reversed-phase HPLC. Cyclization was then accomplished by a standard high-dilution technique using BOP-Cl as an activating reagent[16] in the presence of NMM and DMAP to afford the cyclic peptide with Asp-β-benzyl protection in 15% yield. Hydrogenolysis of the protected cyclic peptide provided the desired cyclic prodrug **13** in quantitative yield.

Through comparison of the two methods, it can be seen that the solution-phase approach allows for somewhat more efficient use of the key intermediate **9**. In the solid-phase approach, the yield of the linear peptide **12** calculated from the key intermediate **9** was 25%, and in the solution-phase approach the yield was 38%. The

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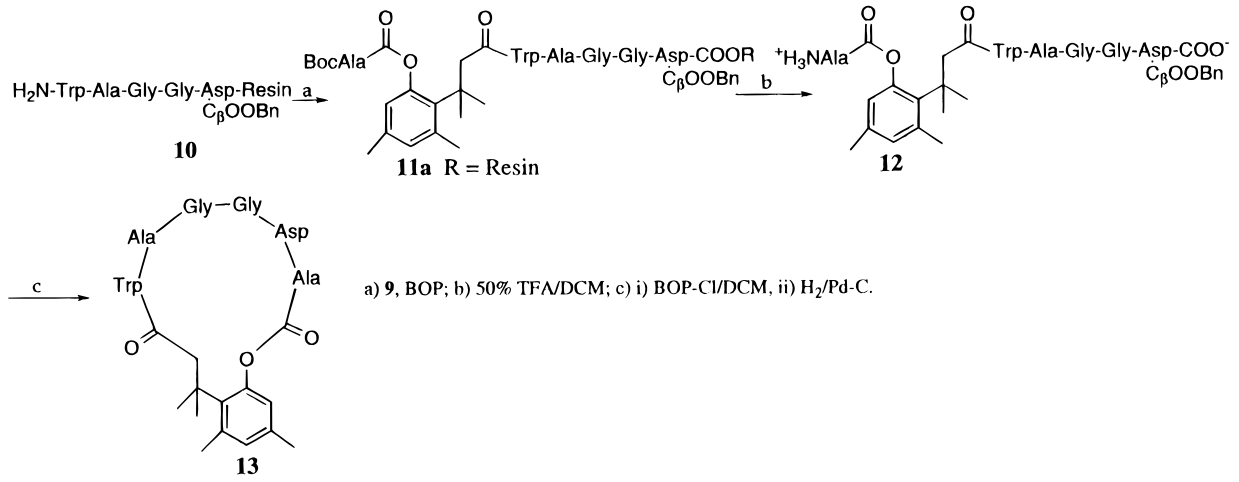
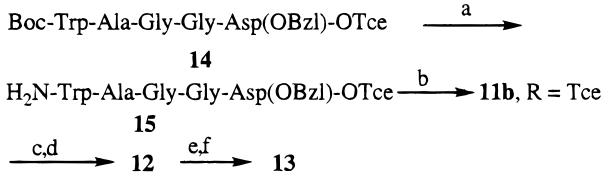
Scheme 3. Synthetic Approach to the Cyclic Prodrug of the Model Hexapeptide



Scheme 4. Solution-Phase Synthetic Approach to Cyclic Prodrug 13[a]



[a] (a) TFA/DCM, 50%; (b) **9**, EDC, HOBt, NMM; (c) Zn/HOAc; (d) TFA/DCM, 50%; (e) BOP-Cl, NMM, DMAP; (f) H_2Pd-C.

overall low yield (8−15%) for the cyclization was probably due to (1) possible oligomerization even under high-dilution conditions and (2) low recovery from preparative HPLC purification due to the small amount of sample that was used. One could certainly use even higher dilution conditions to minimize the possible oligomerization problem.

Prodrug **13** was designed to release its original peptide through an esterase-catalyzed hydrolysis of the phenol ester bond (Scheme 1). The stability of cyclic prodrug **13** was evaluated in 90% human plasma and was compared to its chemical degradation in a physiological buffer system (Hanks' balanced salt solution (HBSS), pH 7.4).[12] In HBSS, cyclic prodrug **13** degraded slowly ($t_{1/2}$ = 1795 ± 289 min) but quantitatively to the hexapeptide. The rate of degradation of cyclic prodrug **13** was significantly faster in 90% human plasma ($t_{1/2}$ = 508 ± 24 min), and it could be slowed ($t_{1/2}$ = 1729 ± 245 min) substantially by inclusion of paraoxon, a known esterase inhibitor, in the incubation mixture. In 90% human plasma, only trace amounts of the hexapeptide were observed when cyclic prodrug **13** was incubated in the presence or absence of paraoxon because of the instability of this natural peptide ($t_{1/2}$ = 3.7 min) to peptidases in this biological medium. The relative membrane permeabilities of cyclic prodrug **13** and the linear hexapeptide were evaluated using monolayers of Caco-2 cells, an *in vitro* cell culture model of the intestinal mucosa.[18] The apparent permeability coefficient (P_{app}) of the linear hexapeptide was estimated to be less than 0.17×10^{-8} cm/s, whereas cyclic prodrug **13** exhibited a P_{app} value of 12.1×10^{-8} cm/s. The low permeation of the linear model hexapeptide was due in part to its high susceptibility to enzymatic degradation by peptidases present in Caco-2 cell monolayers ($t_{1/2}$ = 14 min). However, even when the linear hexapeptide was stabilized to peptidase-catalyzed metabolism, using a cocktail of potent peptidase inhibitors, it was still substantially less able to permeate the Caco-2 cellular barrier than was the cyclic prodrug **13**.[3]

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Conclusions

We have described a strategy for preparing a cyclic prodrug of a model hexapeptide via the N- and C-terminal ends by utilizing a unique "trimethyl lock"-facilitated lactonization system. However, it should be feasible to use this methodology to cyclize other biologically active peptides by linking the C-terminal carboxyl group to a side-chain amino (*e.g.*, Lys, Arg) or hydroxyl (*e.g.*, Ser, Thr, Tyr) group or linking a side-chain carboxyl group (*e.g.*, Asp, Glu) to a side-chain amino (*e.g.*, Lys, Arg) or hydroxyl (*e.g.*, Ser, Thr, Tyr) group. The application of this methodology to biologically active peptides (*e.g.*, opioid peptides) and peptide mimetics [*e.g.*, Arg-Gly-Asp (RGD) analogs] is currently under investigation in our laboratories.

Experimental Section

General Methods. ^1H NMR spectra were recorded on either a 500 MHz or a 300 MHz instrument. High-performance liquid chromatography (HPLC) was conducted using a dual pump system with a UV detector. All starting materials were purchased from Aldrich Chemical Co., Sigma, Fluka Chemicals, or Bachem Bioscience, Inc., and used as received. Wang's resin for solid-phase peptide synthesis was obtained from Bachem Bioscience, Inc., with 0.95 mmol/g substitution.

1-*O*-(*tert*-Butyldimethylsilyl)-3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (5). To a mixture of 11.63 g of 3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanol[9] and 9.3 g of TBDMS chloride in 70 mL of methylene chloride cooled in an ice bath was added dropwise a solution of 30 mL of methylene chloride and 31 mL of triethylamine during a period of 1 h with stirring. The reaction mixture was then warmed to room temperature and stirred for 1 d. Then solvent was evaporated, and the residue was dissolved in 250 mL of methylene chloride and washed with water (20 mL × 4). After the mixture was dried over $MgSO_4$ for 1 h, solvent evaporation gave 18.09 g (100%) of a white solid product, the NMR spectrum of which is identical with that reported in the literature.[9]

1-*O*-(*tert*-Butyldimethylsilyl)-3-(2′-Boc-alanyl-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (6). To a mixture of 5.18 g of the TBDMS-diol **5**, 5 g of Boc-Ala-OpNP, and 2 g of DMAP was added 20 mL of anhydrous methylene chloride.

After the mixture was refluxed for 7 h, another 0.4 g of DMAP was added, and the reaction was refluxed for an additional 7 h. After solvent evaporation, the residual was dissolved into 200 mL of methylene chloride and washed with saturated $NaHCO_3$ (20 mL × 4), 1% HCl (20 mL × 2), and brine (20 mL × 2). After the mixture was dried over Na_2SO_4 and the solvent evaporated, the residue was purified by column chromatography (silica gel) using a gradient of 5−10% ethyl acetate in hexane to give a colorless oil, which later solidified to give a white solid product (6.87 g, 84%). ^{1}H-NMR ($CDCl_3$, δ): 6.80 (1 H, s, aromatic H), 6.50 (1 H, s, aromatic H), 5.18 (1 H, m, NH), 4.51 (1 H, m, NHC*H*), 3.47 (2 H, t, J = 7.6 Hz, OCH_2), 2.51 (3 H, s, $PhCH_3$), 2.21 (3 H, s, $PhCH_3$), 2.01 (2 H, m, OCH_2CH_2), 1.54 (3 H, d, J = 7.3 Hz, $CHCH_3$), 1.45 (15 H, s, $OC(CH_3)_3$, $C(CH_3)_2$), 0.83 (9 H, s, $SiC(CH_3)_3$, −0.04 (6 H, s, $Si(CH_3)_2$). CIMS (NH_3) (rel intensity): 494 (M + 1, 1), 438 (4), 394 (9). HRMS: calcd for $C_{27}H_{48}NO_5Si$ 494.3302, found 494.3316. Anal. Calcd for $C_{27}H_{47}NO_5Si$: C, 65.68; H, 9.59; N, 2.84. Found: C, 65.90; H, 9.91; N, 2.76.

3-(2′-Boc-alanyl-4′,6′-dimethylphenyl)-3,3-dimethylpropanol (7). To 5.0 g of the Boc-Ala-TBDMS-diol **6** were added 19 mL of THF, 19 mL of H_2O, and 58 mL of HOAc. The reaction was stirred at rt for 1 h. Then solvent was evaporated to give a colorless oily residue (4.8 g). This product was used directly for the oxidation without purification because of the potential for rearrangement during column chromatography. ^{1}H-NMR ($CDCl_3$, δ): 6.79 (1 H, s, aromatic), 6.51 (1 H, s, aromatic), 5.35 (1 H, m, NH), 4.47 (1 H, m, NHC*H*), 3.49 (2 H, m, $HOCH_2$), 2.49 (3 H, s, $PhCH_3$), 2.20 (3 H, s, $PhCH_3$), 2.04 (2 H, m, $HOCH_2CH_2$), 1.54 (3 H, d, J = 7.4 Hz, $CHCH_3$), 1.46 (6 H, s, $C(CH_3)_2$), 1.42 (9 H, s, $C(CH_3)_3$).

3-(2′-Boc-alanyl-4′,6′-dimethylphenyl)-3,3-dimethylpropionic Acid (9). To a solution of 4.38 g of PCC in 200 mL of DCM was added dropwise a solution of alcohol **7** (3.90 g) in 200 mL of DCM. The reaction color changed from orange to black during the addition. The solution was then stirred at rt for 1 h. After filtration through Celite, solvent evaporation, and subsequent purification with a short silica gel column, 3.08 g of an oily product was obtained (79%). The oily product was then dissolved in 40 mL of acetone and added dropwise to a solution of 1.42 g of $KMnO_4$ in 40 mL of acetone−H_2O (1:1). The reaction mixture was stirred at rt for 17 h. Acetone was then evaporated from the reaction mixture, and the residue was filtered through Celite. After filtration, the pH (about 8) was adjusted to 3 with dilute HCl. The mixture was then extracted with ethyl acetate (100 mL × 3), and the combined organic extracts were dried over $MgSO_4$ overnight. Solvent evaporation gave a white solid product (2.9 g, 88%). ^{1}H-NMR ($CDCl_3$, δ): 6.72 (1 H, s, aromatic), 6.44 (1H, s, aromatic), 5.09 (1 H, m, NH), 4.30 (1 H, m, NHC*H*), 2.74 (2 H, b, CH_2COO), 2.44 (3 H, s, $PhCH_3$), 2.13 (3 H, s, $PhCH_3$), 1.46 (9 H, m, $CHCH_3$, $C(CH_3)_2$), 1.35 (9 H, s, $C(CH_3)_3$). FABMS: 394 (M + 1), 338, 294. HRMS: calcd (M + 1) 394.2230, found 394.2231. Anal. Calcd for $C_{21}H_{31}NO_6$: C, 64.10; H, 7.94; N, 3.56. Found: C, 63.92; H, 8.10; N, 3.30.

Synthesis of Linear Peptide 12. A typical synthesis used Wang's alkoxy resin (Bachem Bioscience, 0.95 mmol/g, 100−200 mesh). Fmoc-protected amino acids were used for solid-phase peptide synthesis unless otherwise indicated. For a scale of 0.5 g of resin, Asp (β-benzyl-aspartic acid) (4.5 equiv) was preactivated with DIC for 10−20 min before being added to the resin together with 1 equiv of DMAP. The coupling reaction was carried out for 2.5 h and then washed with DMF (20 mL × 4) and DCM (30 mL × 3). The coupling reaction was repeated using 2.3 equiv of Asp using the same conditions. For subsequent coupling reactions, about 4 equiv of the amino acids were used with DIC/HOBt as the activating reagents, and reactions were carried out for about 2 h. The coupling reaction was monitored using a ninhydrin test as well as by the weight of the resin and was repeated if the ninhydrin test indicated incomplete reaction after 2 h. Deprotection was accomplished using 20% piperidine in DMF in 10−15 min. The coupling of compound **9** (1−1.5 equiv) was accomplished using BOP as the activating reagent in the presence of triethylamine. The final cleavage of the peptide from the resin was accomplished by treatment with 50% TFA/DCM at rt for 30 min. Solvent evaporation under reduced pressure gave the final linear peptide, which was directly used for cyclization without purification. ^{1}H-NMR (CD_3OD, δ): 7.54 (1 H, d, aromatic), 7.31 (6 H, m, aromatic), 7.10 (1 H, m, aromatic), 7.02 (1 H, m, aromatic), 6.98 (1 H, b, aromatic), 6.81 (1 H, b, aromatic), 6.58 (1 H, b, aromatic), 5.10 (2 H, s, $PhCH_2$), 4.83 (1 H, t, α-H), 4.54 (1 H, t, α-H), 4.37 (1 H, m, α-H), 4.15 (1 H, m, α-H), 3.88 (2 H, b, Gly-α-H), 3.61 (2 H, b, Gly-α-H), 3.17−2.89 (4 H, m, Asp-CH_2, Trp-CH_2), 2.63 (2 H, s, $(CH_3)_2CCH_2CO$), 2.43 (3 H, s, $PhCH_3$), 2.19 (3 H, s, $PhCH_3$), 1.71 (3 H, d, Ala-CH_3), 1.46 (6 H, s, $C(CH_3)_2$), 1.21 (3 H, d, Ala-CH_3). FABMS: calcd for $C_{45}H_{55}N_7O_{11}$ 869, found 870.5 (M + 1).

Cyclic Peptide 13. To 94 mg of linear peptide **12** was added 10 mL of anhydrous DMF, 240 mL of anhydrous DCM, 100 μL of NMM, and 158 mg of BOP-Cl. The reaction solution was stirred for 34 h. The reaction mixture was then washed with water (20 mL × 2) and dried over $MgSO_4$ for 2 h. Purification with HPLC (reversed phase, 70% methanol in water) afforded 7 mg of a pure cyclic peptide with benzyl protection of the β-carboxyl group of Asp. ^{1}H-NMR ($CDCl_3$, δ): 6.5−7.6 (19 H, NH, aromatic), 5.14 (2 H, m, $PhCH_2$), 4.82 (1 H, m, α-H), 4.52 (1 H, m, α-H), 4.38 (1 H, m, α-H), 3.66−4.03 (5 H, m, α-H), 2.88−3.11 (4 H, Asp-CH_2, Trp-CH_2), 2.71 (1 H, d, $(CH_3)_2CCH_2$), 2.51 (3 H, s, CH_3), 2.40 (1 H, d, $(CH_3)_2$-CCH_2), 2.17 (3 H, s, CH_3), 1.5−1.7 (9 H, m, CH_3), 1.13 (3 H, d, CH_3). FABMS: calcd for $C_{45}H_{53}N_7O_{10}$ 851, found 852.7 (M + 1). HRMS: calcd (M + 1) 852.3932, found 852.3953.

To 7 mg of the aforementioned cyclic peptide with benzyl protection of the β-carboxyl group of aspartic acid was added 20 mL of absolute ethanol and 3 mg of Pd−C (10%). The reaction was stirred at rt under 1 atm of hydrogen for 22 h. Filtration of the reaction mixture and washing of the Pd/C with methanol (10 mL × 4) followed by solvent evaporation gave a colorless residue, which was washed with chloroform. After drying under vacuum, 3.5 mg of product was obtained. ^{1}H-NMR (CD_3OD, δ): 6.59−7.51 (7 H, aromatic), 4.84 (1 H, m, α-H), 4.40−4.62 (2 H, m, α-H), 3.60−4.23 (5 H, m, α-H), 2.78−3.20 (4 H, m, Asp-CH_2, Trp-CH_2), 2.44 (1 H, d, $(CH_3)CCH_2$), 2.36 (3 H, s, CH_3), 2.18 (3 H, s, CH_3), 2.04 (1 H, d, $(CH_3)CCH_2$), 1.10−1.70 (12 H, m, CH_3). FABMS: 784 (M + Na), 762 (M + 1). HRMS: calcd for $C_{38}H_{47}N_7O_{10}$ (M + 1) 762.3463, found 762.3481.

Boc-Asp(OBn)-OTce. Boc-Asp(OBn)-OH (1 g, 3 mmol), 2,2,2-trichloroethanol (0.4 mL, 3 mmol), and DMAP (0.18 g, 1.5 mmol) were dissolved in DCM (30 mL) and cooled to 0 °C. To this cooled solution was added EDC (0.57 g, 3 mmol), and the reaction mixture was stirred at 0 °C for 3 h and then at ambient temperature for 21 h. The precipitate was filtered out, and the filtrate was diluted with EtOAc (100 mL). The EtOAc layer was successively washed with saturated $NaHCO_3$ (2 × 20 mL), H_2O (2 × 50 mL), and saturated aqueous NaCl (20 mL). The EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield Boc-Asp-(OBn)-OTce (1.13 g, 83%) as a yellow oil. ^{1}H-NMR ($CDCl_3$, δ): 1.45 (9H, s), 2.93 and 3.15 (2H, dd, J = 17, 4.5 Hz), 4.67 and 4.75 (2H, dd, J = 12.3 Hz), 4.7−4.75 (1H, m), 5.13 (2H, s), 5.56 (1H, d, J = 9 Hz), 7.34−7.38 (5H, m). MS (FAB) m/z: 454 (M^+ + 1). Anal. Calcd for $C_{18}H_{22}NO_6Cl_3$: C, 47.68; H, 4.86; N, 3.09. Found: C, 48.20; H, 4.70; N, 3.20. HMRS: calcd for $C_{18}H_{22}NO_6Cl_3$ 454.0591, found 454.0594.

H-Asp(OBn)-OTce. TFA (5 mL) was added to a stirred solution of Boc-Asp(OBn)-OTce (1 g, 2.2 mmol) in DCM (5 mL). The reaction mixture was stirred at room temperature for 45 min. Volatile compounds in the reaction mixture were removed using a rotary evaporator under vacuum. The residue was triturated and washed with anhydrous Et_2O, and the solid was isolated by decantation. The solid H-Asp(OBn)-OTce (0.78 g, 100%) was dried under vacuum to remove the residual Et_2O and was used in the next step without further purification. FABMS: calcd for $C_{13}H_{14}Cl_3NO_4$ 353, found 354 (M^+ + 1).

Boc-Ala-Gly-Gly-Asp(OBn)-OTce. To a cooled (0 °C) stirred solution of Boc-Ala-Gly-Gly-OH (1.6 g, 5.13 mmol), Asp-(OBn)-OTce (2.4 g, 5.13 mmol), HOBt (0.69 g, 5.13 mmol), and NMM (0.5 mL, 5.13 mmol) in DCM (100 mL) was added EDC (0.99 g, 5.16 mmol) in one portion. The reaction mixture was stirred at 0 °C for 4 h and at ambient temperature for 24 h.

The reaction mixture was diluted with DCM (250 mL) and washed successively with 10% aqueous citric acid (2 × 50 mL), H_2O (100 mL), saturated $NaHCO_3$ (2 × 50 mL), H_2O (100 mL), and saturated aqueous NaCl (50 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to furnish Boc-Ala-Gly-Gly-Asp(OBn)-OTce (2.8 g, 86%) as pale yellow oil. ^{1}H-NMR (DMSO-d_6, δ): 1.17 (3H, d, J = 7.2 Hz), 1.37 (9H, s), 2.85 and 2.97 (2H, dd, J = 17, 6.2 Hz), 3.71−3.79 (3H, br), 3.98 (2H, br), 4.86 (2H, d, J = 4.3 Hz), 5.12 (2H, s), 7.01 (1H, d, J = 6.5 Hz), 7.33−7.37 (5H, br), 8.07 (2H, d, J = 6 Hz), 8.54 (1H, d, J = 7.8 Hz). FABMS: calcd for $C_{25}H_{33}N_4O_9Cl_3$ 638, found 645 (M^+ + Li), 661 (M^+ + Na).

H-Ala-Gly-Gly-Asp(OBn)-OTce. Boc-Ala-Gly-Gly-Asp-(OBn)-OTce (0.29 g, 0.44 mmol) was dissolved in DCM (5 mL) and the solution was cooled to 0 °C. To this clear solution was added TFA (5 mL), and the reaction mixture was stirred at room temperature for 1 h. Volatile compounds in the reaction mixture were removed using a rotary evaporator under vacuum. The residue was triturated and washed with anhydrous Et_2O, and the solid was isolated by decantation. H-Ala-Gly-Gly-Asp(OBn)-OTce (0.27 g, 95%) was dried under vacuum to remove the residual Et_2O and was used in the next step without further purification. ^{1}H-NMR (DMSO-d_6, δ): 1.36 (3H, d, J = 7.2 Hz), 2.85 and 2.98 (2H, dd, J = 17.0, 6.0 Hz), 3.70−4.01 (6H, m), 4.86 (2H, d, J = 4.1 Hz), 5.12 (2H, s), 7.36−7.37 (5H, br), 8.09 (2H, br), 8.24 (1H, br), 8.64 (1H, d, J = 7.2 Hz). FABMS: calcd for $C_{20}H_{25}N_4O_7Cl_3$ 538, found 538 (M^+).

Boc-Trp-Ala-Gly-Gly-Asp(OBn)-OTce (14). To a cooled (0 °C) stirred solution of H-Ala-Gly-Gly-Asp(OBn)-OTce (2.2 g, 5.13 mmol), HOBt (0.69 g, 5.13 mmol), Boc-Trp-OH (1.56g, 5.13 mmol), and NMM (0.5 mL, 5.13 mmol) in DCM (100 mL) was added EDC (0.99 g, 5.16 mmol) in one portion. The reaction mixture was stirred at 0 °C for 4 h and at ambient temperature for 24 h. The reaction mixture was diluted with DCM (250 mL) and washed successively with 10% aqueous citric acid (2 × 50 mL), H_2O (100 mL), saturated $NaHCO_3$ (2 × 50 mL), H_2O (100 mL), and saturated aqueous NaCl (50 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to furnish Boc-Trp-Ala-Gly-Gly-Asp(OBn)-OTce (3.6 g, 86%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$, δ): 1.21 (3H, d, J = 7.2 Hz), 1.43 (9H, s), 2.99 and 3.17 (2H, m), 3.23 (2H, d, J = 6.8 Hz), 3.52−3.59 (1H, m), 3.73−3.89 (1H, m), 3.97 (2H, d, J = 5.9 Hz), 4.23−4.27 (1H, m), 4.41−4.48 (1H, m) 4.75 (1H, d, J = 11.8 Hz), 4.41 (1H, d, J = 11.8 Hz), 5.02−5.09 (1H, m), 5.14 (2H, s), 6.25 (1H, m), 6.49 (1H, m), 7.04−7.40 (9H, m), 7.64 (1H, d, J = 7.4 Hz). Anal. Calcd for $C_{36}H_{43}N_6O_{10}Cl_3$: C, 52.43; H, 5.22; N, 10.19. Found: C, 52.34; H, 5.05; N, 9.94.

H-Trp-Ala-Gly-Gly-Asp(OBn)-OTce (15). Boc-Trp-Ala-Gly-Gly-Asp(OBn)-OTce (0.29 g, 0.44 mmol) was dissolved in DCM (5 mL), and the solution was cooled to 0 °C. To this clear solution was added TFA (5 mL), and the reaction mixture was stirred at room temperature for 1 h. Volatile compounds in the reaction mixture were removed using a rotary evaporator under vacuum. The residue was triturated and washed with anhydrous Et_2O, and the solid was isolated by decantation. H-Trp-Ala-Gly-Gly-Asp(OBn)-OTce (0.3 g, 95%) was dried under vacuum to remove the residual Et_2O and was used in the next step without further purification. FABMS: calcd for $C_{31}H_{35}N_6O_8Cl_3$ 724, found 725 (M^+ + 1).

Boc-Ala-(Prom)-Trp-Ala-Gly-Gly-Asp(OBn)-OTce (11b). EDC (0.09 g, 0.44 mmol) was added into a cooled (0 °C) and stirred solution of Boc-Ala-(Prom)-OH (0.17 g, 0.44 mmol), H-Trp-Ala-Gly-Gly-Asp(OBn)-OTce (0.32 g, 0.44 mmol), HOBt (0.6 g, 0.44 mmol), and NMM (0.09 mL, 0.89 mmol) in DCM (50 mL). The mixture was stirred for 2 h at 0 °C and for 30 h at ambient temperature. The reaction mixture was diluted with DCM (250 mL) and washed successively with 10% aqueous citric acid (2 × 50 mL), H_2O (100 mL), saturated $NaHCO_3$ (2 × 50 mL), H_2O (100 mL), and saturated aqueous NaCl (50 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to furnish an oil of Boc-Ala-(Prom)-Trp-Ala-Gly-Gly-Asp(OBn)-OTce (0.33 g, 68%). The peptide was analyzed by analytical reversed-phase HPLC using a C-18 column (5 μm, 300 Å, 25 cm × 4.6 mm, flow rate 1 mL/min) eluting with a gradient starting from 20% of solvent A (0.1% TFA/H2O:5%ACN v/v) to 100% of solvent B (ACN) in 20 min. The retention time of the peptide is 16.85 min. ^{1}H-NMR ($CDCl_3$, δ): 0.99 (3H, d, J = 6.7 Hz), 1.38 (9H, s), 1.49−1.72 (9H, m), 2.11 (3H, s), 2.43−2.71 (2H, m), 2.98−3.17 (4H, m), 3.71−3.82 (1H, m), 3.92−4.02 (4H, m), 4.11−4.18 (1H, m), 4.33 (1H, t, J = 6.8 Hz), 4.53−4.71 (3H, m), 5.13 (2H, s), 6.39 (2H, s), 6.71 (2H, s), 6.95−7.53 (9H, m), 8.32 (1H, s). FABMS: calcd for $C_{52}H_{64}N_7O_{13}Cl_3$ 1099, found 1100 (M + 1).

H-Ala-(Prom)-Trp-Ala-Gly-Gly-Asp(OBn)-OH (12). To a stirred solution of Boc-Ala-(Prom)-Trp-Ala-Gly-Gly-Asp-(OBn)-OTce (0.22 g, 0.2 mmol) in HOAc (50 mL) was added Zn dust (1 g) over 1 h. After the reaction mixture was stirred for 24 h at room temperature, the insoluble material was filtered out, and the filtrate was concentrated under reduced pressure to give an oily residue. The resulting residue was dissolved in DCM (10 mL), and the solution was cooled to 0 °C. To this clear solution were added TFA (5 mL), phenol (2 g), and ethanedithiol (0.2 mL). After the mixture was stirred at room temperature for 2 h, DCM was evaporated and the residue was triturated with anhydrous Et_2O. The solid was washed with anhydrous Et_2O, isolated by decantation, and dried under reduced pressure to afford H-Ala-(Prom)-Trp-Ala-Gly-Gly-Asp(OBn)-OH (0.09 g, 50%). The product was purified further by preparative reversed-phase HPLC using a C-18 column (12 μm, 300 Å, 25 cm × 21.4 mm, flow rate 5 mL/min) eluting with a gradient starting from 30% of solvent A (0.1% TFA/H2O:5%ACN v/v) to 100% of solvent B (ACN) in 70 min. The peptide was analyzed by analytical reversed-phase HPLC using a C-18 column (5 μm, 300 Å, 25 cm × 4.6 mm, flow rate 1 mL/min) eluting with a gradient starting from 20% of solvent A (0.1% TFA/H2O:5%ACN v/v) to 100% of solvent B (ACN) in 20 min. The retention time of the peptide is 14.97 min.

Acknowledgment. Financial support was provided by Glaxo, Inc., The United States Public Health Service (DA09315), and the Swiss National Science Foundation.

JO961778Z