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Synthesis of a Novel Esterase-Sensitive Cyclic Prodrug of a Hexapeptide Using an (Acyloxy)alkoxy Promoiety

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Synthetic methodology for preparing novel esterase-sensitive cyclic prodrugs of peptides with increased protease stability and cell membrane permeability compared to linear peptides is described. Cyclic prodrug **1** of the hexapeptide H-Trp-Ala-Gly-Gly-Asp-Ala-OH linked by the N-terminal amino group to the C-terminal carboxyl group via an (acyloxy)alkoxy promoiety was synthesized. A convergent synthetic approach involving Boc[[(alaninyloxy)methyl]carbonyl]-*N*-tryptophan (**2**) and H-Ala-Gly-Gly-Asp(OBzl)-OTce (**3**) was used. The key fragment **2** has the promoiety inserted between the Ala and the Trp residues. Fragment **3** was synthesized by a solution-phase approach using standard Boc-amino acid chemistry. These fragments were coupled to produce the protected linear hexapeptide, which after deprotection was cyclized using standard high-dilution techniques to yield cyclic prodrug **1**. In pH 7.4 buffer (HBSS) at 37 °C, cyclic prodrug **1** was shown to degrade quantitatively to the hexapeptide ($t_{1/2}$ = 206 ± 11 min). The rate of hydrolysis of cyclic prodrug **1** was significantly faster in human blood ($t_{1/2}$ = 132 ± 4 min) than in HBSS. Paraoxon, a known inhibitor of esterases, slowed this hydrolysis of cyclic prodrug **1** to a value ($t_{1/2}$ = 198 ± 9 min) comparable to the chemical stability. In human blood, cyclic prodrug **1** was shown to be 25-fold more stable than the linear hexapeptide.

Introduction

The clinical development of orally active peptide drugs has been limited by their unfavorable physicochemical characteristics (e.g., charge, hydrogen-bonding potential, size), which prevent them from permeating biological barriers like the intestinal mucosa, and their lack of stability against enzymatic degradation.[1−13] Even when peptides are administered via the parenteral route (e.g., intravenously), they tend not to gain access to important target areas such as the brain due to their unfavorable physicochemical characteristics and their metabolic lability.[14]

Unfortunately, many of the structural features of a peptide, such as the N-terminal amino group, C-terminal carboxyl group, and side-chain carboxyl, amino, and hydroxyl groups that bestow upon the molecule affinity and specificity for its pharmacological receptor, severely restrict its ability to permeate biological barriers and become a substrate for peptidases. With small organic-based drugs, which exhibit similar structural features and similar unfavorable physicochemical characteristics, prodrug (bioreversibly derivatives of drug) strategies have been successfully employed to transiently (e.g., bioreversibly) alter the drug's physicochemical characteristics and/or its lability to metabolism.[15−25] Unfortunately, the synthesis of peptide prodrugs has been limited due to their structural complexity and the lack of novel methodology.[26]

In this paper, we describe a methodology for the synthesis of novel esterase-sensitive cyclic prodrugs of linear peptides using an (acyloxy)alkoxy promoiety. These cyclic prodrugs were designed to be susceptible to esterase metabolism (slow step), leading to a cascade of chemical reactions and resulting in generation of the linear peptide (Scheme 1). To illustrate the synthetic methodology used to prepare this type of cyclic prodrug, we describe here the preparation of a cyclic prodrug of a model hexapeptide[27] by linking the N-terminal amino group to the C-terminal carboxyl group via an (acyloxy)-alkoxy promoiety. [(Acyloxy)alkoxy]carbamate deriva-

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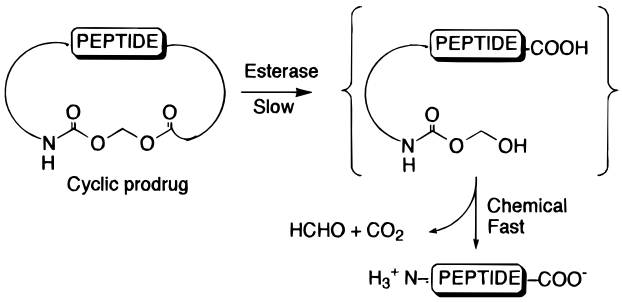
Scheme 1



tives of primary and secondary amines as prodrugs have previously been described in the literature and shown to degrade by a similar mechanism.[28−35] However, to our knowledge, this is the first demonstration of the way in which this promoiety can be used to prepare a cyclic prodrug of a peptide. In separate study,[36] we have shown that cyclic prodrug **1** has enhanced cell membrane permeability and protease stability compared to the linear peptide. In addition, we have been able to show that the enhanced cellular permeability is related to the structural features of the cyclic prodrug.[37]

Results and Discussion

Synthesis of the Cyclic Prodrugs. The synthesis of cyclic prodrug **1** of the linear peptide H-Trp-Ala-Gly-Gly-Asp-Ala-OH was attempted by two different approaches. The first approach involved the synthesis of the linear hexapeptide on Wang resin using standard Fmoc-amino acid chemistry.[38,39] The amino terminal of this peptide was coupled with 1-chloromethyl chloroformate to give the $ClCH_2OCO$-Trp-Ala-Gly-Gly-Asp-Ala-resin. This peptide was cleaved from the resin to give the precursor $ClCH_2OCO$-Trp-Ala-Gly-Gly-Asp-Ala-OH for the final cyclization reaction. However, cyclization of $ClCH_2OCO$-Trp-Ala-Gly-Gly-Asp-Ala-OH under various basic conditions did not yield the desired cyclic prodrug, resulting only in degradation of the precursor peptide.

The alternative approach, which was then pursued, involved insertion of the (acyloxy)alkoxy promoiety between two amino acids in this sequence before the final cyclization step. In the model hexapeptide used in these studies, we decided to insert the promoiety between the Ala and the Trp residues. Therefore, we devised a convergent approach to the synthesis of the cyclic prodrug **1** involving fragments **2** and **3**, as shown in Scheme 2.

Scheme 3 shows the synthesis of the key intermediate **2** in which the promoiety has been inserted between Boc-Ala and Trp. The synthesis of **2** was begun by reacting 1-chloromethyl chloroformate with *p*-nitrophenol in the presence of *N*-methylmorpholine (NMM) to afford 1-(chloromethyl)-*p*-nitrophenyl carbonate (**4**) in 84% yield. Substitution of the chloride in **4** with iodide was achieved by reaction with NaI to give the iodo compound **5** in 94% yield. Compound **5** was then reacted with the cesium salt of Boc-Ala in DMF to give a mixture of the desired product Boc-(alaninyloxy)methyl *p*-nitrophenyl carbonate (**6**) in 70% yield and the side product *p*-nitrophenyl ester of Boc-alanine (Boc-Ala-OpNP) in 30% yield as determined by NMR. This reaction was shown to afford better yields of **6** when the cesium salt of Boc-Ala rather than the sodium or potassium salts of this amino acid was used. This mixture was difficult to separate, and it was used directly in the next step without further purification. The mixture of compound **6** and Boc-Ala-OpNP was coupled with Trp-OBzl in the presence of NMM and 1-hydroxybenzotriazole (HOBT) in hexamethylphosphoramide (HMPA) to afford the Boc-[[(alaninyloxy)methyl]-carbonyl]-*N*-tryptophan benzyl ester (**7**) and the side product Boc-Ala-Trp-OBzl. Removal of the benzyl group from compound **7** and Boc-Ala-Trp-OBzl was achieved by hydrogenolysis using 10% Pd/C as a catalyst under an H_2 atmosphere in EtOH to give **2** and Boc-Ala-Trp-OH in 96% yield. Compound **2** was purified from Boc-Ala-Trp-OH by preparative reversed-phase HPLC.

Scheme 4 illustrates the solution-phase synthesis of tetrapeptide **3** using standard Boc-amino acid chemistry.[40] However, in using the solution-phase approach it was necessary to temporarily protect the terminal α-carboxyl group during the synthesis of linear tetrapeptide **3**. The key to this solution-phase approach was the selective protection of the α- and *β*-carboxyl groups of the Asp residue. The selective deprotection of the α-carboxyl group of Asp must be carried out under conditions that do not affect the protecting group on the *β*-carboxyl group or the ester bond of the promoiety. Initially, we tried to protect the α-carboxyl group of the Asp residue as the 2,4-dimethoxybenzyl (Dmb) ester[41] and the *β*-carboxyl group as a benzyl ester. However, during removal of the Dmb protecting group in the final step of synthesis of the hexapeptide, the indole ring of the Trp residue was alkylated by the 2,4-dimethoxylbenzyl cation in almost quantitative yield. Different scavengers were used, without success, to suppress this alkylation reaction.[42] Eventually, we successfully used the trichloroethyl (Tce) ester protecting group for the α-carboxyl group of Asp residue, which is quite stable to acidic conditions and can be removed by zinc in AcOH.[43,44] Compounds **9** and **13** were, therefore, used in the synthesis of **3**. Treatment of Boc-Asp(OBzl)-OH with 2,2,2-trichloroethanol in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), HOBT, and NMM gave Boc-Asp(OBzl)-OTce (**8**) in 83% yield. Compound **8** was

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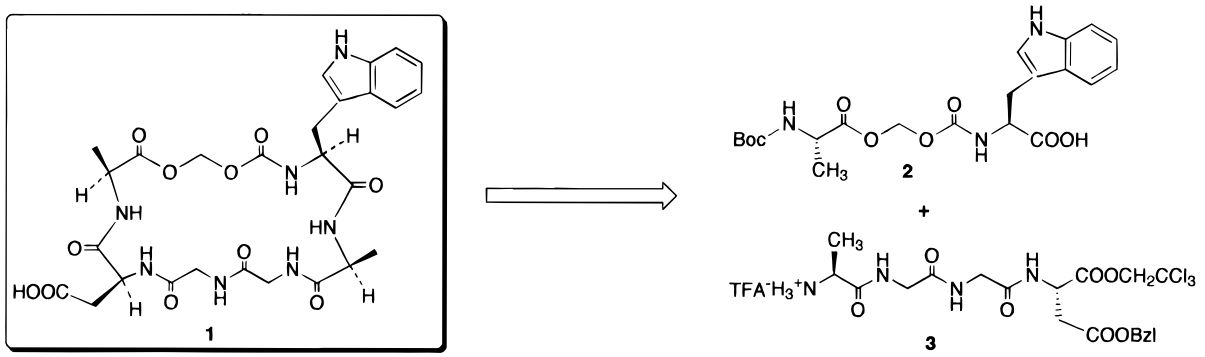
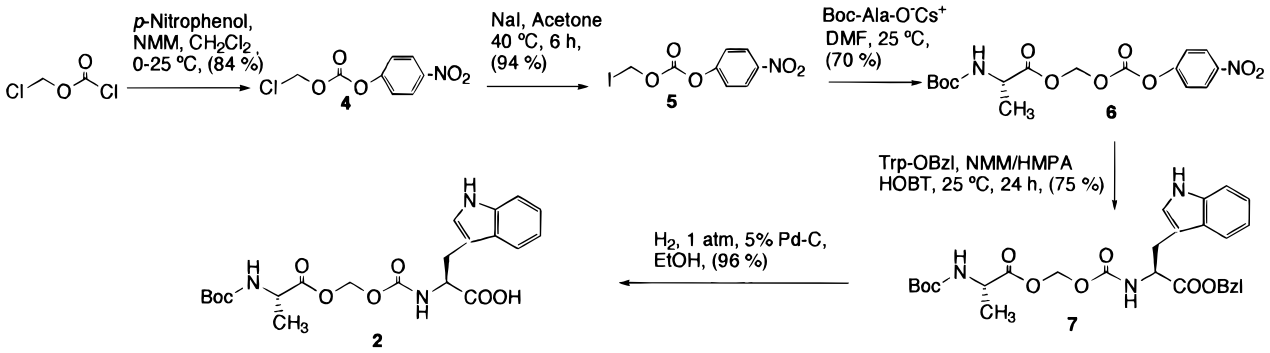
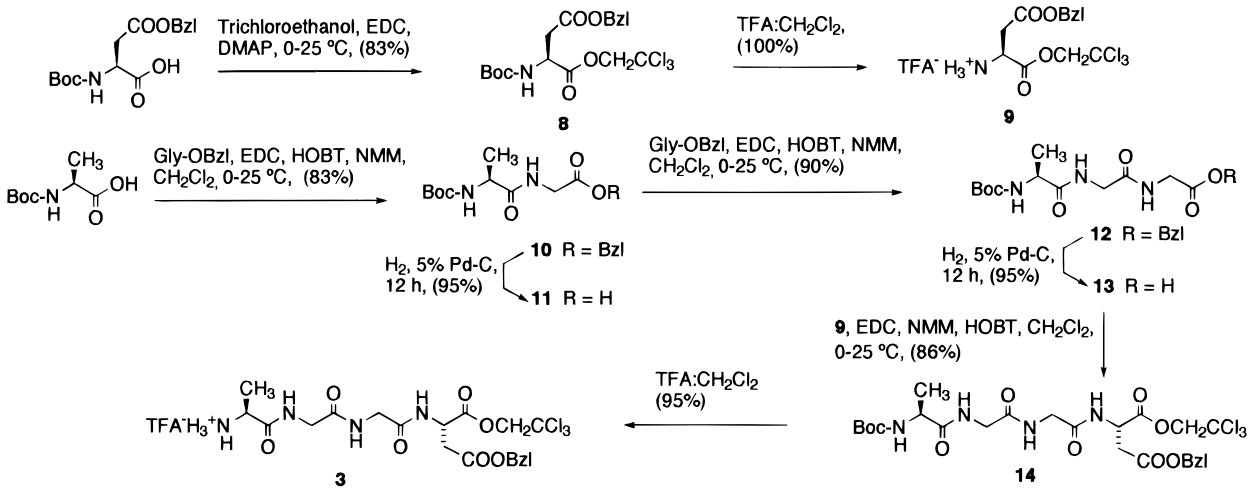
Scheme 2



Scheme 3



Scheme 4



treated with 50% TFA in DCM to give **9** in quantitative yield. The tripeptide Boc-Ala-Gly-Gly-OH (**13**) was synthesized using standard Boc-amino acid chemistry with EDC and HOBT as coupling reagents. Coupling between the tripeptide **13** and Tce ester **9** in the presence of EDC and HOBT gave the tetrapeptide **14** in 86% yield; it was then treated with 50% TFA in DCM to yield **3** in 95% yield.

Scheme 5 shows the assembly of fragments **2** and **3** for the synthesis of cyclic prodrug **1**. Tetrapeptide **3** was reacted with Boc-[[(alaninyloxy)methyl]carbonyl]-*N*-tryptophan (**2**) in the presence of EDC, HOBT, and NMM to give the fully protected linear hexapeptide **15** in 70% yield. Both of the protecting groups on **15** were removed by treatment first with zinc in AcOH to remove the Tce protecting group and then with 50% TFA in DCM to remove the Boc protecting group, affording **16** in 60% overall yield. The linear hexapeptide **16** was purified by preparative reversed-phase HPLC. Cyclization of **16** under high dilution conditions using EDC and HOBT as condensation reagents did not yield the desired cyclic prodrug **17**. Cyclization was then accomplished by the standard high-dilution technique using *N*,*N*-bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl) as an activating reagent[45] in the presence of NMM and 4-(*N*,*N*-(dimethylamino)pyridine (DMAP) to give cyclic peptide **17** in 20% yield after HPLC purification. The low yield for the cyclization reaction may be due to the possible formation of oligomers even under high dilution conditions and the low recovery during purification by preparative HPLC. Hydrogenolysis of cyclic peptide **17** to remove the Bzl protecting group from the Asp residue was achieved with 10% Pd/C as a catalyst under an H_2 atmosphere in EtOH to yield the desired cyclic prodrug **1** in quantitative yield. The crude cyclic prodrug **1** was

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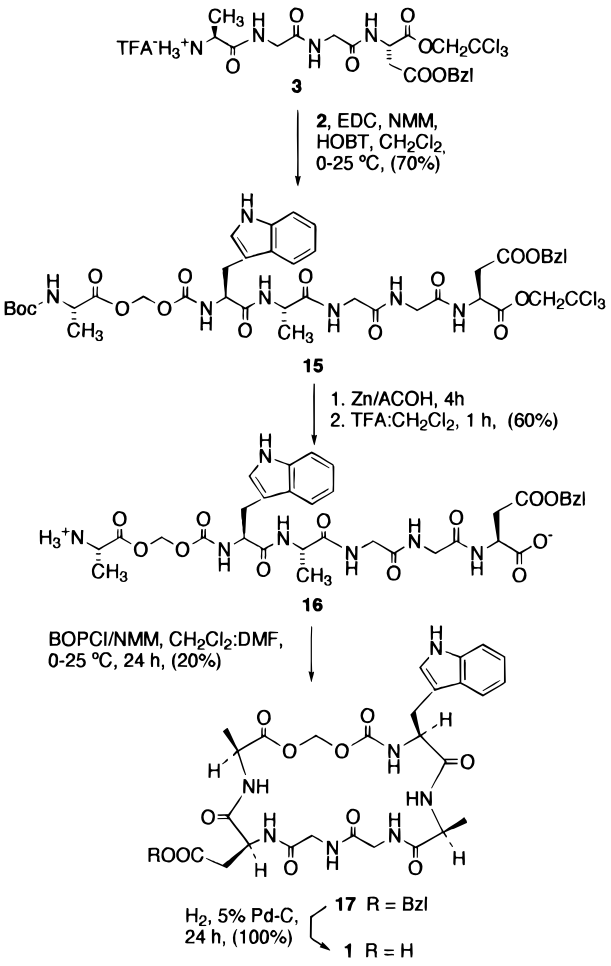
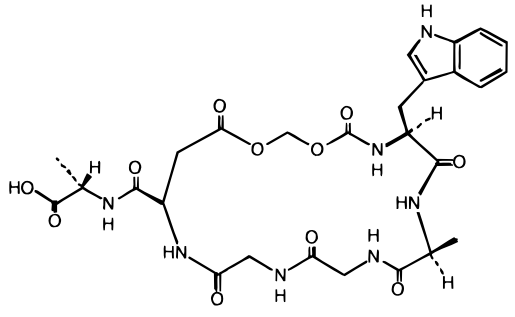
Scheme 5



purified by preparative reversed-phase HPLC and was analyzed by analytical reversed-phase HPLC.

Using the same methodology as described above, we also synthesized cyclic prodrug **18** of the hexapeptide H-Trp-Ala-Gly-Gly-Asp-Ala-OH by linking the N-terminal amino group of the Trp residue to a side-chain C-terminal carboxyl group of the Asp residue via the (acyloxy)alkoxy linker. However, cyclic prodrug **18** was found to be very unstable and, therefore, could not be evaluated in human blood stability studies and cellular permeability studies.[46]



18

Biological Results

To be effective, a prodrug should degrade chemically and/or enzymatically to the parent drug *in vivo*. Cyclic prodrug **1** was designed to undergo esterase-mediated hydrolysis of the ester bond of the carbamate moiety followed by two fast chemical degradation steps to release the linear hexapeptide (Scheme 1). The stability of cyclic prodrug **1** was evaluated in human blood and was compared to its chemical degradation in Hanks' balanced salt solution (HBSS), pH 7.4. In this physiological buffer system, cyclic prodrug **1** degraded quantitatively to the linear hexapeptide with an apparent half-life ($t_{1/2}$) of 206 ± 11 min. The rate of hydrolysis of cyclic prodrug **1** was significantly faster in human blood ($t_{1/2}$ = 132 ± 4 min) than in HBSS (Figure 1). This facilitated hydrolysis in human blood was inhibited ($t_{1/2}$ = 198 ± 9 min) when paraoxon, a known inhibitor of serine-dependent esterases, was included in the incubation mixture. In human blood, cyclic prodrug **1** was 25-fold more stable than the linear hexapeptide. These data suggest that cyclic prodrug **1** is converted to the linear hexapeptide by an esterase-mediated reaction in human blood.

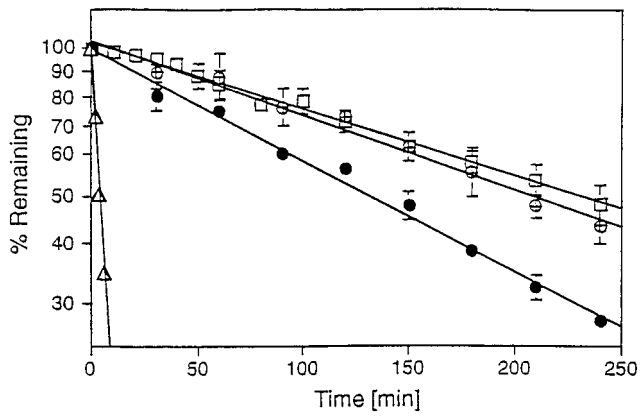


Figure 1. Stability of the linear hexapeptide (△) and cyclic prodrug **1** in human blood (in the presence (⊕) and absence (●) of paraoxon) and in Hanks' balanced salt solution (HBSS), pH 7.4 (□). Disappearance of the cyclic prodrug was monitored by HPLC over a time period of 180 min. Data points are averages ± SD of triplicate.

In separate studies,[36] we evaluated the relative cellular permeability properties of cyclic prodrug **1** and the linear hexapeptide. Transport studies were conducted using monolayers of Caco-2 cells, an *in vitro* cell culture model of the intestinal mucosa.[47] In comparison with the linear hexapeptide, cyclic prodrug **1** was found to be at least 76 times more able to permeate this model of the intestinal mucosa.

In separate studies,[37] we determined the major solution conformation of cyclic prodrug **1** using NMR, CD, and molecular dynamic simulations that indicate that the cyclic prodrug **1** exhibits one major conformer in solution and has a well-defined secondary structure. The solution conformation of cyclic prodrug **1** appears to be compact due to intramolecular hydrogen bonding and the presence of a β-turn. The increased ability of cyclic prodrug **1** to permeate membranes could be due to reductions in the average hydrodynamic radius of the molecule, thus increasing paracellular flux, and/or to an increase in passive diffusion via the transcellular route because of a reduction in the hydrogen-bonding potential of the cyclic prodrug.

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Conclusion

In conclusion, we have described a methodology for preparation of an (acyloxy)alkoxy-linked cyclic prodrug of a model hexapeptide via the N- and C-terminal ends. 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 by 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 cyclic prodrug prepared in this study was shown to degrade chemically to the linear hexapeptide. Human blood increased the rate of hydrolysis of the cyclic prodrug to the hexapeptide, suggesting the presence of an esterase in blood capable of hydrolyzing the [(acyloxy)-alkoxy]carbamate linkage. In separate studies,[36] we have demonstrated that this cyclic prodrug showed improved peptidase stability and a greater ability to permeate cell membranes as compared to the parent peptide. The application of this methodology to biologically active peptides (*e.g.*, opioid peptides) is currently under investigation in our laboratory.

Experimental Section

General Methods. ^{1}H NMR spectra were recorded on 500 and 300 MHz instruments. Chemical shifts are expressed in parts per million (δ) relative to tetramethylsilane (TMS) with either TMS or residual solvent as an internal reference. Abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; b, broad. High-resolution mass spectra (HRMS) were obtained using VG Analytical ZAB double-focusing spectrometer. All other starting materials were purchased from Aldrich Chemical Co., Sigma Chemical Co., Fluka Chemicals, or Bachem Bioscience, Inc. and used as received.

HPLC was conducted using a Rainin HPLX pumping system with a Dynamax UV detector. Data are reported as follows: column type, eluent, flow rate, and retention time. The desired product was purified by a preparative reversed-phase HPLC system using a C-18 column (12 μm, 300 Å, 25 cm × 21.4 mm i.d., flow rate 5 mL/min) eluting with a gradient of solvent A, 0.1%TFA/H_2O:5%ACN, and solvent B, ACN. The gradient method used for the preparative HPLC was started from 20% of solvent B and ended with 100% of solvent B over 65 min. The desired peptide was analyzed by analytical reversed-phase HPLC using a C-18 column (5 μm, 300 Å, 25 cm × 4.6 mm i.d., flow rate 1 mL/min) eluting with a gradient of solvent A, 0.1%TFA/H_2O:5%ACN, and solvent B, ACN. The total HPLC analysis run was 18 min. The solvent gradient used for the analytical HPLC was started with a gradient change of solvent B from 10% to 50% for over 14 min; then, the composition of solvent B was increased to 100% in 2 min followed by elution at 100% solvent B for 2 min. Finally, a gradient change back to 10% solvent B was done over 2 min.

1-Chloromethyl *p*-Nitrophenyl Carbonate (4). A solution of 1-chloromethyl chloroformate (3.2 mL, 36 mmol) was added dropwise into an ice-cold reaction mixture of *p*-nitrophenol (5 g, 36 mmol) and *N*-methylmorpholine (3.6 mL, 36 mmol) in $CHCl_3$ (50 mL). The reaction mixture was stirred at 0 °C for 1 h and at ambient temperature for 24 h. After being stirred overnight, the reaction mixture was successively washed with 10% aqueous citric acid (2 × 20 mL), H_2O (2 × 50 mL), saturated aqueous $NaHCO_3$ (2 × 40 mL), H_2O (2 × 50 mL), and saturated aqueous NaCl (20 mL). The $CHCl_3$ layer was dried over anhydrous Na_2SO_4, decanted, and evaporated to give pure 1-chloromethyl *p*-nitrophenyl carbonate (**4**) (6.9 g, 84%), which is a light yellow oil. The purity of compound **4** was determined by ^{1}H- and ^{13}C-NMR (see the Supporting Information). ^{1}H-NMR ($CDCl_3$, δ): 5.85 (2H, s), 7.42 (2H, d, J = 8 Hz), 8.30 (2H, d, J = 9 Hz). The NMR spectrum of **4** is identical to that reported in the literature.[48]

1-Iodomethyl *p*-Nitrophenyl Carbonate (5). NaI (5 g, 30 mmol) was added in one portion to a solution of 1-chloromethyl carbonate (**4**) (6.9 g, 30 mmol) in acetone (50 mL), and the reaction mixture was stirred at 50 °C for 24 h. After being stirred overnight, the solvent was evaporated and the residue was redissolved in Et_2O (100 mL). The Et_2O layer was successively washed with 10% aqueous Na_2SO_3 (2 × 20 mL), H_2O (2 × 50 mL), and saturated aqueous NaCl (20 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to afford 1-iodomethyl *p*-nitrophenyl carbonate (**5**) (9.1 g, 94%) as a pure yellow oil. The purity of compound **5** was determined by ^{13}C-NMR (see the Supporting Information). ^{1}H-NMR ($CDCl_3$, δ): 6.03 (2H, s), 7.39 (2H, d, J = 9 Hz), 8.24 (2H, d, J = 9 Hz). The NMR spectrum of **5** is identical to that reported in the literature.[48]

Boc-[[(alaninyloxy)methyl]carbonyl]-*N*-tryptophan Benzyl Ester (7). The cesium salt of Boc-Ala was prepared by reacting Boc-Ala (2 g, 10 mmol) with Cs_2CO_3 (1.7 g, 5.2 mmol) in CH_3OH (30 mL). After the reaction mixture was stirred for 1 h, the solvent was removed under reduced pressure to afford the cesium salt of Boc-Ala as a white powder, which was used directly in the next reaction. A solution of the cesium salt of Boc-Ala (0.96 g, 3 mmol) in DMF (20 mL) was added slowly to an ice-cold stirred solution of iodomethyl *p*-nitrophenyl carbonate (**5**) (1 g, 3 mmol) in DMF (50 mL) followed by addition of Boc-Ala (2 g, 10 mmol) over a period of 2 h. After the reaction mixture was stirred for 24 h at room temperature, the solvent was removed under reduced pressure to afford the oily residue, which was then dissolved in EtOAc (100 mL) and washed with 10% $NaHCO_3$ (2 × 20 mL), H_2O (2 × 50 mL), and saturated aqueous NaCl (20 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solid was decanted out. The EtOAc was evaporated under reduced pressure to give a mixture of Boc-(alaninyloxy)methyl *p*-nitrophenyl carbonate (**6**) (70% yield) and the side product Boc-Ala-OpNP (30% yield) as determined by NMR. This mixture was difficult to separate and was used directly in the next step without any further purification. ^{1}H of compound **6** ($CDCl_3$, δ): 1.28−1.30 (12H, br), 4.23 (1H, br), 5.43 (1H, d, J = 7.4 Hz), 5.81 and 5.74 (2H, dd, J = 5.5 Hz), 7.27 (2H, d, J = 9 Hz), 8.08 (2H, d, J = 7.3 Hz).

NMM (0.12 mL, 1.05 mmol) was added to a stirred solution of a mixture of Boc-(alaninyloxy)methyl *p*-nitrophenyl carbonate (**6**) (0.4 g, 1.04 mmol) Boc-Ala-OpNP (0.096 g, 0.31 mmol), TrpOBzl·HCl (0.35 g, 1.05 mmol), and HOBT (0.142 g, 1.05 mmol) in HMPA (50 mL). After being stirred for 24 h at room temperature, the reaction mixture was diluted with CH_2Cl_2 (200 mL) and washed with cold 10% NaOH (2 × 50 mL) and H_2O (2 × 100 mL). The CH_2Cl_2 layer was separated, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to yield a mixture (pale yellow oil) of Boc-[[(alaninyloxy)methyl]carbonyl]-*N*-tryptophan benzyl ester (**7**) (75% yield) and the side product Boc-Ala-Trp-OBzl (25% yield). This mixture was used in the next step because of the difficulty of separating the components. ^{1}H-NMR of compound **7** ($CDCl_3$, δ): 1.35−1.43 (3H, br), 1.44 (9H, s), 3.30 (2H, d, J = 5.4 Hz), 4.70 (1H, br), 4.89−4.98 (1H, br), 5.04 (2H, s), 5.69 (2H, br), 6.82 (2H, br),7.07−7.31 (8H, br), 7.50 (1H, d, J = 7.4 Hz); MS (FAB) m/z 539 ($M^+ + 1$).

Boc-[[(alaninyloxy)methyl]carbonyl]-*N*-tryptophan (2). A mixture of Boc-[[(alaninyloxy)methyl]carbonyl]-*N*-tryptophan benzyl ester (**7**) (0.2 g, 0.37 mmol) and Boc-Ala-Trp-OBzl (0.043 g, 0.09 mmol) was dissolved in absolute EtOH (25 mL), and 10% Pd−C (0.03 g) was added. The reaction mixture was stirred under an H_2 atmosphere using a hydrogen balloon on the top of the round-bottom flask. After 10 h, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure to yield Boc-[[(alaninyloxy)methyl]carbonyl]-*N*-tryptophan (**2**) and Boc-Ala-Trp-OH, both in 96% yield. The desired product **2** was purified from the side product Boc-Ala-Trp-OH by a preparative reversed-phase HPLC system using a C-18 column (12 μm, 300 Å, 25 cm × 21.4 mm i.d., flow rate 5 mL/min). The peptide **2** was analyzed by analytical reversed-phase HPLC using a C-18 column (5 μm, 300 Å, 25 cm × 4.6 mm i.d., flow rate 1 mL/min) with a retention time of 15.26

(48) Jose, A. *European Pat. Appl.* 0167451, 1985.

min. ^{1}H-NMR ($(CD_3)_2CO$, δ): 1.30 (3H, d, J = 6.9 Hz), 1.39 (9H, s), 3.19−3.43 (2H, m), 4.16 (1H, d, J = 6.2 Hz), 4.53−4.54 (1H, br), 4.78 (1H, br), 5.62−5.74 (2H, br), 7.03 (1H, t, J = 6.8 Hz), 7.10 (1H, t, J = 6.8 Hz), 7.38 (1H, d, J = 7.5 Hz).7.63 (1H, d, J = 7.8 Hz); MS (FAB) m/z 449 ($M^+ + 1$). Anal. Calcd for $C_{21}H_{27}N_3O_8$: C, 56.13; H, 6.01; N, 9.35. Found: C, 56.19; H, 6.09; N, 9.29. HRMS: calcd for $C_{21}H_{27}N_3O_8$ 449.1798, found 449.1804.

Boc-Asp(OBzl)-OTce (8). Boc-Asp(OBzl)-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 CH_2Cl_2 (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 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-(OBzl)-OTce (**8**) (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.

TFA·H-Asp(OBzl)-OTce (9). TFA (5 mL) was added to a stirred solution of Boc-Asp(OBzl)-OTce (**8**) (1 g, 2.2 mmol) in CH_2Cl_2 (5 mL). The reaction mixture was stirred at room temperature for 45 min. Volatile compounds in the reaction mixture were removed by rotary evaporation under vacuum. The residue was triturated and washed with anhydrous Et_2O, and the solid was isolated by decantation. The solid TFA·H-Asp(OBzl)-OTce (**9**) (0.78 g, 100%) was dried under vacuum to remove the residual Et_2O and was used in the next step without further purification. MS (FAB) m/z 354 ($M^+ + 1$).

Boc-Ala-Gly-OBzl (10). EDC (1 g, 5.3 mmol) was added to a cold stirred solution (0 °C) of Boc-alanine (1 g, 5.3 mmol), HCl·Gly-OBzl (1.07 g, 5.3 mmol), and 0.71 g of HOBT (0.71 g, 5.3 mmol) in 50 mL of CH_2Cl_2. To this solution was added NMM (0.5 mL, 5.3 mmol), and the reaction mixture was stirred at 0 °C for 4 h and at ambient temperature for 24 h. The reaction mixture was concentrated under reduced pressure, and the residue was dissolved in EtOAc (100 mL). The EtOAc layer was successively washed with 10% aqueous citric acid (2 × 20 mL), H_2O (40 mL), saturated $NaHCO_3$ (2 × 20 mL), H_2O (40 mL), and saturated aqueous NaCl (25 mL). The EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was recrystallized from EtOAC/petroleum ether to give a colorless solid of Boc-Ala-Gly-OBzl (**10**) (1.48 g, 83%), which was determined by ^{1}H-NMR to have high purity. ^{1}H-NMR ($CDCl_3$, δ): 1.35 (3H, d, J = 6 Hz), 1.42 (9H, s), 4.05 (2H, d, J = 5 Hz), 4.25 (1H, m), 5.15 (2H, s), 5.28 (1H, d, J = 5 Hz), 7.02 (1H, br), 7.33 (5H, s). MS (FAB) m/z: 337 ($M^+ + 1$). HRMS: calcd for $C_{17}H_{24}N_2O_5$ 337.1774, found 337.1763.

Boc-Ala-Gly-OH (11). To a solution of Boc-Ala-Gly-OBzl (**10**) (1g, 3 mmol) in absolute EtOH (50 mL) was added 10% Pd−C (100 mg) in one portion. The reaction mixture was stirred for 24 h under a H_2 atmosphere using a balloon on the top of the round-bottom flask. After being stirred for 24 h, the reaction mixture was filtered to remove the Pd−C, and the solvent was removed under reduced pressure to give Boc-Ala-Gly-OH (**11**) (0.74 g, 95%). The purity of compound **11** was determined by ^{1}H-NMR (see the Supporting Information). ^{1}H-NMR ($CDCl_3$, δ): 1.33 (3H, d, J = 7 Hz), 1.41 (9H, s), 3.97 (2H, d, J = 5.6 Hz), 4.2 (1H, m), 6.18 (1H, br), 7.50 (1H, br). MS (FAB) m/z: 247 ($M^+ + 1$). HRMS: calcd for $C_{10}H_{18}N_2O_5$ 247.1277, found 247.1294.

Boc-Ala-Gly-Gly-OBzl (12). Boc-Ala-Gly-OH (**11**) (0.5 g, 2 mmol), HCl·Gly-OBzl (0.4 g, 2 mmol), and HOBT (0.27 g, 2 mmol) were dissolved in CH_2Cl_2 (40 mL) and cooled to 0 °C. To this solution were added EDC (0.39 g, 2 mmol) and NMM (0.2 mL, 2 mmol), and the reaction mixture was stirred for 2 h at 0 °C and at ambient temperature for 24 h. The reaction mixture was concentrated under reduced pressure, and the residue was dissolved in EtOAc (100 mL). The EtOAc solution was successively washed with 10% aqueous citric acid (2 × 30 mL), H_2O (50 mL), saturated $NaHCO_3$ (2 × 40 mL), H_2O (50 mL), and saturated aqueous NaCl (50 mL). The EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to furnish Boc-Ala-Gly-Gly-OBzl (**12**) (0.71 g, 90%) as a yellow oil. Compound **4** was determined to be pure by ^{1}H-NMR (see the Supporting Information). ^{1}H-NMR ($CDCl_3$, δ): 1.28 (3H, d, J = 7.2 Hz), 1.31 (9H, s), 3.85−4.15 (5H, m), 5.06 (2H, s), 5.45 (1H, br), 7.25 (5H, br), 7.41 (1H, br). MS (FAB) m/z: 394 ($M^+ + 1$). HRMS: calcd for $C_{19}H_{27}N_3O_6$ 394.1960, found 394.1978.

Boc-Ala-Gly-Gly-OH (13). To a solution of Boc-Ala-Gly-Gly-OBzl (**12**) (0.5 g, 1.3 mmol) in 50 mL of absolute EtOH was added in one portion 10% Pd−C (50 mg). The reaction mixture was stirred under a H_2 atmosphere for 24 h. After being stirred for 24 h, the reaction mixture was filtered to remove the Pd−C, and the solvent was removed under reduced pressure to give Boc-Ala-Gly-Gly-OH (**13**) (0.37 g, 95%). ^{1}H-NMR ($CDCl_3$, δ): 1.39 (3H, d, J = 7.2 Hz), 1.43 (9H, s), 3.99−4.15 (5H, m), 5.03 (1H, br), 6.91−7.03 (2H, br); MS (FAB) m/z 325 (M^++Na). HRMS: calcd for $C_{12}H_{21}N_3O_6$ 304.1533, found 304.1509. Anal. Calcd for $C_{12}H_{21}N_3O_6$: C, 47.52; H, 6.93; N, 13.86. Found: C, 47.18; H, 6.58; N, 13.48.

Boc-Ala-Gly-Gly-Asp(OBzl)-OTce (14). To a cooled (0 °C) stirred solution of Boc-Ala-Gly-Gly-OH (**13**) (1.6 g, 5.13 mmol), TFA·Asp(OBzl)-OTce (**9**) (2.4 g, 5.13 mmol), HOBT (0.69 g, 5.13 mmol), and NMM (0.5 mL, 5.13 mmol) in CH_2Cl_2 (100 mL) was added in one portion EDC (0.99 g, 5.16 mmol). The reaction mixture was stirred at 0 °C for 4 h and at ambient temperature for 24 h. The reaction mixture was diluted with CH_2Cl_2 (250 mL) and washed 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-(OBzl)-OTce (**14**) (2.8 g, 86%) as a 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 Hz and 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). MS (FAB) m/z: 645 (M^+ + Li), 661 (M^+ + Na). Anal. Calcd for $C_{25}H_{32}N_5O_7Cl_3$: C, 48.47; H, 5.17; N, 11.31. Found: C, 48.45; H, 5.09; N, 11.43.

TFA·H-Ala-Gly-Gly-Asp(OBzl)-OTce (3). Boc-Ala-Gly-Gly-Asp(OBzl)-OTce (**14**) (0.29 g, 0.44 mmol) was dissolved in CH_2Cl_2 (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 by rotary evaporation under vacuum. The residue was triturated and washed with anhydrous Et_2O, and the solid was isolated by decantation. TFA·H-Ala-Gly-Gly-Asp(OBzl)-OTce (**3**) (0.27 g, 95%) was dried under vacuum to remove the residual Et_2O and was used in the next step without further purification. The purity of this compound was determined by ^{1}H-NMR (see the Supporting Information). ^{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). MS (FAB) m/z: 528 ($M^+ + 1$).

Boc-Ala-(OCH_2OCO)-Trp-Ala-Gly-Gly-Asp(OBzl)-OTce (15). EDC (0.09 g, 0.44 mmol) was added to a cooled (0 °C) stirred solution of Boc-[[(alaninyloxy)methyl]carbonyl]-*N*-tryptophan (**2**) (0.2 g, 0.44 mmol), Ala-Gly-Gly-Asp(OBzl)-OTce (**3**) (0.29 g, 0.44 mmol), HOBT (0.6 g, 0.44 mmol), and NMM (0.09 mL, 0.89 mmol) in CH_2Cl_2 (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 CH_2Cl_2 (250 mL) and washed 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-(OCH_2OCO)-Trp-Ala-Gly-Gly-Asp(OBzl)-OTce (**15**) (0.3 g, 70%). ^{1}H-NMR (DMSO-d_6, δ): 1.15 (3H, d, J = 7.7 Hz), 1.23 (3H, d, J = 6.8 Hz), 1.35

(9H, s), 2.80−3.2 (4H, m), 4.85 (2H, s), 5.11 (2H, s), 5.48−5.63 (2H, m), 6.93−6.99 (1H, m), 7.02−7.07 (1H, m), 7.14 (1H, s), 7.29−7.40 (7H, m), 7.66 (1H, d, J = 7.5 Hz), 7.79 (1H, m), 8.02−8.10 (2H, m), 8.28 (1H, d, J = 6.6 Hz), 8.56 (1H, d, J = 7.5 Hz), 10.78 (1H, s). MS (FAB) m/z: 994 (M^+ + Na). HRMS: calcd for $C_{41}H_{51}N_7O_{14}Cl_3$ 970.2560, found 970.2539.

TFA·H-Ala-(OCH$_2$OCO)-Trp-Ala-Gly-Gly-Asp(OBzl)-OH (16). To a stirred solution of Boc-Ala-(OCH$_2$OCO)-Trp-Ala-Gly-Gly-Asp(OBzl)-OTce (**15**) (0.2 g, 0.2 mmol) in AcOH (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 CH_2Cl_2 (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 solution was strirred at room temperature for 2 h, CH_2Cl_2 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 give TFA·H-Ala-(OCH$_2$OCO)-Trp-Ala-Gly-Gly-Asp(OBzl)-OH (**16**) (0.10 g, 60%). The product was further purified by preparative reversed-phase HPLC using a C-18 column (12 μm, 300 Å, 25 cm × 21.4 mm i.d., flow rate 5 mL/min). Peptide **16** was analyzed by analytical reversed-phase HPLC using a C-18 column (5 μm, 300 Å, 25 cm × 4.6 mm i.d., flow rate 1 mL/min); the retention time of linear peptide **16** is 13.03 min. ^{1}H-NMR (DMSO-d_6, δ): 1.24 (3H, d, J = 7.2 Hz), 1.29 (3H, d, J = 7.2 Hz), 2.70−2.95 (2H, m), 3.15−3.19 (2H, m), 3.71−3.82 (4H, m), 4.35 (1H, m), 4.62 (1H, m), 5.10 (2H, s), 5.68 (2H, m), 6.95−7.20 (3H, br), 7.36 (5H, br), 7.68 (1H, br), 7.85 (1H, br), 8.12 (2H, br), 8.31 (4H, br). MS (FAB) m/z 740 (M^+ + 1). HRMS: calcd for $C_{34}H_{42}N_7O_{12}$ 740.2891, found 740.2912.

Cyclic Hexapeptide Prodrug 1. A solution of linear peptide **16** (0.138 g, 0.186 mmol) and NMM (0.2 mL, 1.98 mmol) in CH_2Cl_2 (100 mL) was added dropwise to a cooled (0 °C), stirred solution of BOP-Cl (0.236 g, 0.93 mmol) and DMAP (0.023 g, 0.186 mmol) in 50 mL of CH_2Cl_2 over 2 h. After the reaction mixture was stirred for 3 days at room temperature, the solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2 (100 mL). The organic layer was successively washed with 10% aqueous citric acid (2 × 20 mL), H_2O (50 mL), saturated $NaHCO_3$ (2 × 20 mL), H_2O (50 mL), and saturated aqueous NaCl (20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give crude cyclic peptide prodrug **17.** The crude product was purified by preparative reversed-phase HPLC using a C-18 column (12 μm, 300 Å, 25 cm × 21.4 mm i.d., flow rate 5 mL/min). After HPLC purification, compound **17** was isolated in 20% yield (0.027 g). Cyclic peptide **17** was analyzed by analytical reversed-phase HPLC using a C-18 column (5 μm, 300 Å, 25 cm × 4.6 mm i.d., flow rate 1 mL/min). The retention time of cyclic peptide **17** is 14.3 min. MS (FAB) m/z: 722 (M^+ + 1).

To a solution of cyclic hexapeptide **17** (0.027 g, 0.037 mmol) in absolute EtOH (15 mL) was added in one portion 10% Pd−C (10 mg). The reaction mixture was stirred under a H_2 atmosphere for 24 h. The reaction mixture was filtered to remove the Pd−C, and the solvent was then removed under reduced pressure to give cyclic prodrug **1** (0.023 g, 100%). The crude product was purified by preparative reversed-phase HPLC using a C-18 column (12 μm, 300 Å, 25 cm × 21.4 mm i.d., flow rate 5 mL/min). Cyclic peptide **1** was analyzed by analytical reversed-phase HPLC using a C-18 column (5 μm, 300 Å, 25 cm × 4.6 mm i.d., flow rate 1 mL/min) with a retention time of 11.2 min. ^{1}H-NMR (D_2O, δ): 0.98 (3H, d, J = 7.2 Hz), 1.37 (3H, d, J = 7.2 Hz), 2.65−2.85 (2H, m), 3.10−3.45 (2H, m), 3.65−4.40 (8H, m), 5.34 (1H, d, J = 6.3 Hz), 5.95 (1H, d, J = 6.6 Hz), 7.11 (1H, t, J = 7.2 Hz) 7.20 (1H, t, J = 7.2 Hz) 7.27 (1H, s), 7.46 (1H, d, J = 6.3 Hz), 7.53 (1H, d, J = 7.8 Hz). MS (FAB) m/z: 632 (M^+ + 1). HRMS: calcd for $C_{27}H_{33}N_7O_{11}$ 632.2316, found 632.2319.

Blood Hydrolysis. Cyclic prodrug **1** was incubated with human blood (Topeka Blood Bank, Topeka, KS) at ~24 μM for 4 h in a temperature-controlled (37.0 ± 0.5 °C) shaking water bath (60 rpm). At various time points, 20 μL samples were removed and residual enzyme activity quenched by adding 150 μL of a freshly prepared 6 N guanidinium hydrochloride solution in Hanks' balanced salt solution containing 0.01% (v/v) phosphoric acid. A portion (150 μL) of this mixture, which exhibited a pH-value around pH 3, was transferred to an Ultrafree-MC 5000 NMWL filter unit (Millipore, Bedford, MA) and centrifuged at 7500 rpm (5000*g*) for 60 min (4 °C). Aliquots (50 μL) of the filtrate were diluted with mobile phase and analyzed by HPLC. Recovery for the prodrug was ≥97%. Chromatographic analyses were carried out on a Shimadzu LC-10A gradient system (Shimadzu, Inc., Tokyo, Japan) consisting of LC-10AD pumps, a SCP-6 controller, and a RF-535 fluorescence detector connected to a LCI-100 integrator (Perkin-Elmer, Norwalk, CT). Samples were injected on a Dynamax C-18 reversed-phase column (5 μm, 300 Å, 25 cm × 4.6 mm i.d., Rainin Instruments, Woburn, MA) equipped with a guard column. The fluorescence of the eluent was monitored at emission λ = 345 nm (excitation λ = 285 nm). Gradient elution was performed at a flow rate of 1 mL/min from 0.1−74.0% (v/v) acetonitrile in water using trifluoroacetic acid (0.1%, v/v) as the ion-pairing agent. Under these conditions, the retention times of the linear model hexapeptide and the cyclic prodrug **1** were 7.7 and 11.8 min, respectively.

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Supporting Information Available: Copies of NMR spectra (16 pages). This material is contained in libraries on microfiche, immediately follows 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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