Cyclic Pentapeptide Endothelin A Receptor Antagonists with Attenuated *in Vivo* Clearance

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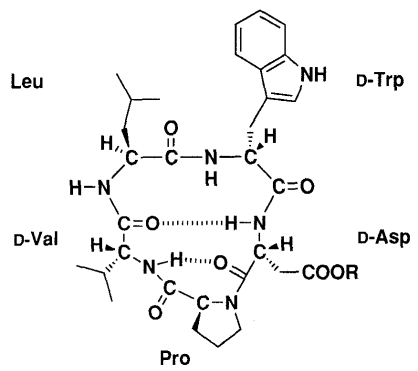
A series of analogues of BQ-123 (1), a potent cyclic pentapeptide endothelin A receptor antagonist, with amino acids linked to the side-chain of the Pro residue *via* an ester linkage was synthesized. All analogues synthesized exhibited potent endothelin A receptor binding affinity similar to that of 1. Of the synthesized analogues, the Lys, Arg and N^{α},N^{ε}-dimethyllysine analogues, 9d—f, exhibited about a three-fold attenuation of *in vivo* clearance compared with 1. In rats, these analogues exhibited a 3-fold-higher plasma concentration and a longer retention time in plasma as compared with those of 1. The attenuated *in vivo* clearance was thought to be a consequence of decreased extraction of the compounds from the blood *via* the hepatic anion transport system, which efficiently extracts 1 from the blood.

Key words endothelin; endothelin antagonist; BQ-123; cyclic pentapeptide; pharmacokinetic evaluation

Endothelin (ET)-1, which was first isolated from the culture medium of porcine aortic endothelial cells, is a potent vasoconstrictor consisting of 21 amino acids.[1] Studies, including a human genomic analysis, have identified two structurally and functionally related isopeptides of ET-1, termed ET-2 and ET-3.[2-4] Several studies have characterized two ET receptor subtypes, ET_A and ET_B, in animal and mammalian systems.[5-7] A third ET receptor subtype, ET_C, was recently cloned from *Xenopus* dermal melanophores,[8] but this subtype has not yet been identified in mammalian tissues. Much effort has been made to identify ET receptor antagonists because such compounds may lead to useful therapeutic agents.[9]

We discovered a cyclic pentapeptide ET_A receptor antagonist, BQ-123 (Fig. 1).[10,11] This compound acts as a potent and selective ET_A receptor antagonist not only *in vitro*, but also *in vivo*, and is expected to be a useful therapeutic agent for the treatment of ET-related human diseases.[12-15] However, the oral absorption of **1** is poor and its plasma concentration after intravenous administration decreases rapidly *in vivo* (Fig. 2), which may limit its usefulness as a therapeutic agent. Namely, **1** may be useful for the treatment of acute disease, but longer-lasting and orally active ET receptor antagonists are desired for the treatment of chronic disease. Compound **2**, the methyl ester of compound **1** (Fig. 1), is orally absorbable but only the parent compound appears in the blood in rats (Fig. 2). We therefore planned to modify **1** to find longer-acting ET receptor antagonists. In general, oligopeptides are susceptible to proteolytic degradation, and are extracted from the blood *via* efficient liver transport systems for excretion in the bile, resulting in a short life-time in blood. Compound **1** is resistant to proteolytic degradation, but **1** is rapidly excreted in the bile: 86% of intravenously given **1** (1 mg/kg) was excreted in the bile in its intact form within 1 h after administration in rats.[16] In addition, **1** was extensively taken up by isolated rat hepatocytes and this uptake was inhibited by metabolic inhibitors, such as rotenone, carbonyl cyanide 4-(trifluo-



1 (BQ-123): R = H
2: R = CH_3

Fig. 1. Structure of BQ-123 (**1**) and Its Methyl Ester (**2**)

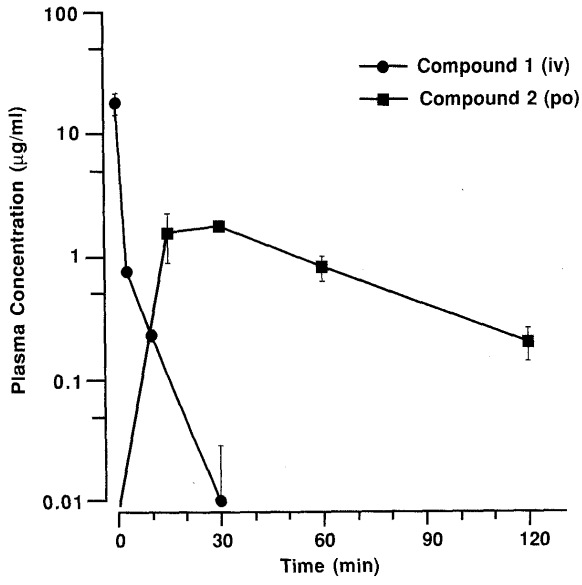


Fig. 2. The Time Course of Plasma Concentration of **1** in Male SD Rats Following a 1-mg/kg Intravenous Dose of **1** or a 50-mg/kg Oral Dose of **2**

The data are mean ± S.D. (n=3).

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romethoxy)phenylhydrazone (FCCP), and organic anions such as dibromosulfophthalein and indocyanine green.[17] These data imply that **1** is efficiently extracted from the blood *via* a hepatic anion transport system. We thought that an analogue not extracted *via* the hepatic anion transport system would show a longer life-time in plasma. Structure–activity studies of cyclic pentapeptide ET_A receptor antagonists revealed that the side-chain of the Pro residue of compound **1** does not bind directly to the ET_A receptor.[11] This suggested that the introduction of a functional group on the side-chain of the Pro residue of **1** might produce ET_A receptor antagonists with various physicochemical properties without loss of ET_A receptor antagonistic activity. We thought that introducing basic functional groups on the Pro side chain would generate potent ET_A receptor antagonists with a longer life-time in plasma, because such compounds would not be extracted from the blood *via* the hepatic anion transport system. In this paper, we describe the synthesis of analogues that have amino acids linked to the side chain of the Pro residue of **1** *via* an ester linkage, and we describe the pharmacokinetic evaluation of these analogues in rats.

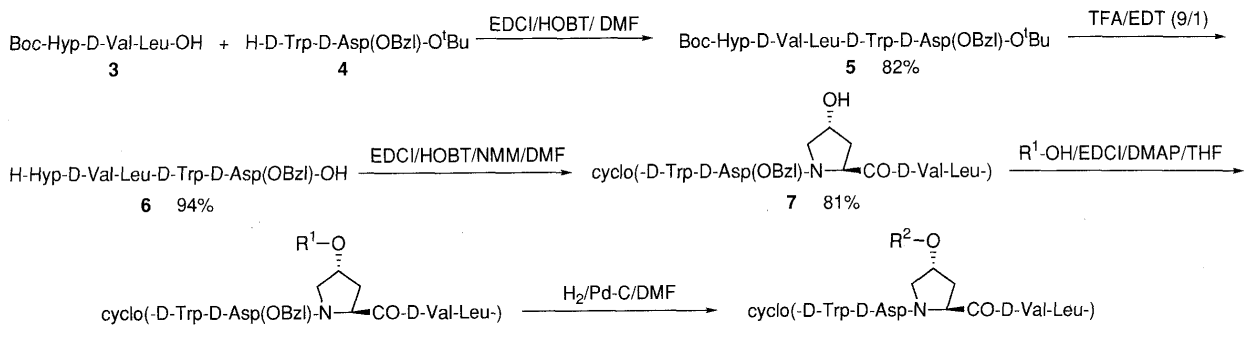
Chemistry

The synthesis of Pro side-chain-modified analogues of **1**, **9a**—**g**, is illustrated in Chart 1. An N-terminal *tert*-butoxycarbonyl (Boc) and C-terminal *tert*-butyl (^{t}Bu)-protected linear pentapeptide **5** was synthesized by fragment condensation of **3** and **4** in 82% yield without racemization at the C-α position of the Leu residue. Deprotection of the Boc and ^{t}Bu protecting groups with trifluoroacetic acid (TFA)/1,2-ethanedithiol (EDT) (9/1) afforded a linear pentapeptide **6**, in which the β-carboxyl group of the D-Asp residue was protected, as a TFA salt in 94% yield. Cyclization of the linear pentapeptide **6** by using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) and 1-hydroxybenzotriazole (HOBT) yielded a cyclic pentapeptide **7**, in which the β-carboxyl group in the D-Asp residue was protected as a benzyl ester, but the hydroxyl group in the Hyp residue was unprotected. Although the TFA salt of **6** was used in the cyclization reaction, neither *N*-trifluoroacetylated linear pentapeptide nor *O*-trifluoroacetylated cyclic pentapeptide was isolated and the desired cyclic pentapeptide **7** was obtained in 81% yield.[18] Esterification of **7** with amino acid derivatives, in which functional groups were protected with benzyloxycarbonyl or nitro groups, by using EDCI and 4-dimethylaminopyridine (DMAP) in THF afforded compounds **8a**—**g**. Deprotection of protecting groups by catalytic hydrogenation gave the desired compounds **9a**—**g**. Usually, the deprotection was performed in DMF without cleavage of the ester bond. However, in the case of the deprotection of **8c** in DMF, a rapid loss of the Orn residue was observed, presumably because of δ-lactam formation. For this reason, **8c** was hydrogenated in acetic acid containing 1 N hydrochloric acid (5%) to yield **9c** as a hydrochloride. Homogeneity and structural integrity were confirmed by HPLC, ^{1}H-NMR and high-resolution FAB-MS. Melting points and high-resolution FAB-MS and HPLC data for **9a**—**g** are listed in Table 1.

Biological Results and Discussion

All analogues synthesized were first evaluated for inhibitory effect on [^{125}I]ET-1-binding to porcine aortic smooth muscle membranes, which are rich in ET_A receptors, according to the reported method.[19] The IC_{50} values are listed in Table 2. All analogues exhibited high ET_A receptor binding affinity, as expected. We then tested the hydrolytic stability of the ester bond in phosphate buffer (pH 7.4); the half-lives are listed in Table 2. Compound **9c** was rapidly hydrolyzed in the buffer and almost all of **9c** was hydrolyzed after 15 min at 37 °C. In contrast, the other analogues were sufficiently stable to chemical hydrolysis, and their pharmacokinetic properties in rats were evaluated.

The pharmacokinetic evaluation of **9a**—**b** and **9d**—**g** was made with reference to **1**. Compounds were intra-

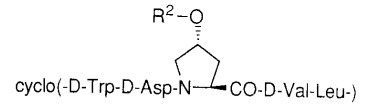


	R^1	yield (%)
8a	Z-Gly-	80
8b	Z-NH-$(CH_2)_5$-CO-	79
8c	Z-Orn(Z)-	80
8d	Z-Lys(Z)-	71
8e	Z-Arg(NO_2)-	41
8f	Z,Me-Lys(Z,Me)-	94
8g	Z-His(Z)-	21

	R^2	yield (%)
9a	Gly-	86
9b	H_2N-$(CH_2)_5$-CO-	84
9c	Orn-	94
9d	Lys-	60
9e	Arg-	82
9f	Me-Lys(Me)-	89
9g	His-	83

Chart 1. Synthesis of Pro Side-Chain Modified Analogues of **1**

Table 1. Data (Melting Points, High-Resolution FAB-MS and HPLC) for the Synthesized Analogues



Compound	R^2	mp, °C	High-resolution FAB-MS (MH^+)		HPLC[a]
			Calcd	Found	t_R (min)
9a	Gly–	215 dec.	684.3357	684.3365	13.2[b]
9b	H_2N-$(CH_2)_5$-CO–	185—195	740.3983	740.3950	9.1
9c[c]	Orn–	212—216	741.3936	741.3913	8.8
9d	Lys–	202—209	755.4092	755.4108	8.9
9e	Arg–	208—215	783.4154	783.4170	16.9[d]
9f	MeLys(Me)–	183—187	783.4405	783.4413	17.7[d]
9g	His–	205—212	764.3731	764.3698	9.4

a) Analytical HPLC was performed on a Spherisorb 3C18 column (4.6 × 150 mm, 3-μm particle size, Phase Separations Co., Ltd.). The solvent system was CH_3CN/H_2O (40/60) with 0.1% TFA, and the flow rate was 0.8 ml/min unless otherwise noted. *b*) A Capcell Pak AG120 column (4.6 × 250 mm, 5-μm particle size, Shiseido Co., Ltd.) was used. The solvent system was CH_3CN/H_2O (35/65) with 0.1% TFA, and the flow rate was 1.0 ml/min. *c*) HCl salt. *d*) The solvent system was CH_3CN/H_2O (35/65) with 0.1% TFA.

Table 2. Endothelin A Receptor Binding Inhibitory Activity and Hydrolytic Stability of Synthesized Analogues

Compound	ET_A receptor binding inhibition IC_{50} (nM)[a]	Hydrolytic stability $t_{1/2}$ (h)[b]
9a	27	2.7
9b	24	170
9c	22	≪0.25
9d	28[c]	2.3
9e	13	2.1
9f	20	2.4
9g	14	3.8

a) Porcine aortic smooth muscle membranes. Values represent one IC_{50} determination unless otherwise noted. *b*) In phosphate buffer (pH 7.4). *c*) $n=3$ IC_{50} determinations.

venously infused into male Sprague–Dawley rats ($n=3$) over a period of 30 min (0.1 $mg \cdot kg^{-1} \cdot min^{-1}$). The plasma concentration of the compounds was measured at 2, 5, 15 and 30 min after the infusion was started, and at the same time points and 60 min after the infusion was stopped. Table 3 summarizes the plasma concentration at 30 min after the initiation of infusion, the area under the plasma concentration time curve (*AUC*), the terminal phase half-life ($t_{1/2}$), the total clearance (CL_{tot}) and the steady-state distribution volume (V_{dss}) data. The highest plasma concentration was obtained with the Lys analogue (**9d**), and the time courses of the plasma concentrations of **9d** and **1** are illustrated in Fig. 3. Compound **9d** showed an *AUC* value three times greater, a half-life three times longer and a total clearance three times lower than those of **1**. The Arg and N^{α},N^{ε}-dimethyllysine (MeLys(Me)) analogues (**9e** and **9f**, respectively) also showed improved pharmacokinetic properties similar to those of **9d**. In contrast, the analogues with Gly and ε-aminohexanoic acid (**9a** and **9b**, respectively) exhibited pharmacokinetic parameters similar to those of **1**. The introduction of His (**9g**) did not improve the pharmacokinetic properties. These results imply that the introduction of at least two strongly basic functional groups on the side chain of the Pro residue of **1** affords endothelin antagonists with attenuated *in vivo* clearance.

Compound **1** is excreted mainly into the bile in its intact form by efficient extraction from the blood *via* a hepatic anion transport system, as mentioned in the introductory section. Compounds **9d—f** are cationic at physiological pH, while **1** is anionic under the same conditions. The cationic compounds could not be extracted by the same transport system that extracts anionic compounds such as **1**. The attenuation in clearance of **9d—f** is thought to be a result of the reduced hepatic extraction of the compounds from the blood. However, it may be due not only to reduced extraction by the transport system, but also to changes in the volume of distribution or the serum protein binding of the compounds. We therefore checked the V_{dss} of the compounds. The difference in V_{dss} between the original compound **1** and the analogues is not significant (Table 3). We then examined the serum protein binding rate of **1** and **9d** because, in general, compounds with a very high serum protein binding rate often have reduced *in vivo* clearance. The serum protein binding of **1** and **9d** amounted to 15.4±3.8% ($n=3$) and 18.9±3.6% ($n=3$), respectively. The low serum protein binding of both compounds did not seem to affect their clearance. These observations strongly suggest that the attenuated *in vivo* clearance of **9d—f** is brought about by decreased extraction of the compounds from the blood *via* the hepatic anion transport system. A detailed study on the hepatic anion transport system will be reported elsewhere.

In conclusion, the introduction of amino acids on the side-chain of the Pro residue of **1** *via* ester linkage produced ET_A receptor antagonists as potent as **1**. Of the synthesized analogues, the Lys, Arg and Me-Lys(Me) analogues, **9d—f**, exhibited about a three-fold attenuation of *in vivo* clearance, which is thought to be brought about by decreased extraction of the compounds from the blood *via* the hepatic anion transport system.

Experimental

Melting points were determined on a Yanaco MP-S3 melting point apparatus without correction. IR spectra were obtained with a Horiba FT-IR FT-200 spectrometer. ^{1}H-NMR spectra were recorded on a Varian VXR-300 spectrometer or a JEOL JNM-EX400 spectrometer. Chemical

Table 3. Pharmacokinetic Evaluation of **1**, **9a—b**, and **9d—g** after Intravenous Infusion in SD rats[a)]

Compound	Plasma concentration[b)] ($\mu g \cdot ml^{-1}$)	AUC ($\mu g \cdot ml^{-1} \cdot min$)	Half-life $t_{1/2}$ (min)	CL_{tot} ($ml \cdot min^{-1} \cdot kg^{-1}$)	V_{dss} ($l \cdot kg^{-1}$)
1	2.16±0.30	66.4± 9.2	4.4±1.5	45.8±6.6	0.13±0.01
9a	2.02±0.23	59.4± 8.1	3.3±0.6	51.1±3.3	0.16±0.03
9b	3.52±0.44	101±16	11.5±2.0	37.2±6.0	0.21±0.05
9d	6.59±0.43	221±13	14.0±2.1	13.6±0.8	0.18±0.02
9e	5.83±0.18	215±11	16.4±1.5	14.0±0.7	0.23±0.01
9f	5.99±0.68	215±22	18.8±1.8	14.1±1.5	0.25±0.01
9g	1.79±0.19	53.5± 5.4	4.8±0.1	56.5±5.5	0.24±0.01

a) Values represent mean±S.D. ($n=3$). *b*) At 30 min after the initiation of infusion.

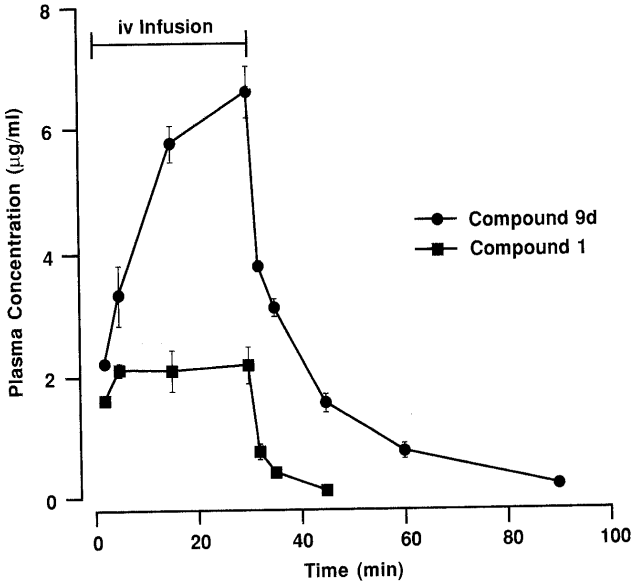


Fig. 3. The Time Course of Plasma Concentration of **1** and **9d** in Male SD Rats Following a 3-mg/kg Intravenous Infusion (0.1 mg/kg/min for 30 min) of **1** and **9d**

The data are mean±S.D.

shifts were reported in parts per million (ppm) downfield from tetramethylsilane (TMS), and coupling constants (J) in hertz (Hz). (Note: In the description of the NMR spectra, the designation "br s" used alone indicates a broad signal of undetermined multiplicity.) FAB-MS were recorded on a JEOL JMS-DX-300 spectrometer in either a glycerol or 3-nitrobenzyl alcohol matrix using xenon as a target gas. High-resolution mass spectra were determined on the same instrument.

Compound **3** was prepared according to the reported method.[11)]

H-D-Trp-D-Asp(OBzl)-O^{t}Bu (4) 1-Hydroxybenzotriazole monohydrate (HOBT, 447 mg, 2.91 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI·HCl, 559 mg, 2.91 mmol) were added to a solution of D-Asp(OBzl)-O^{t}Bu (740 mg, 2.65 mmol) and Boc-D-Trp-OH (806 mg, 2.65 mmol) in CH_2Cl_2 (20 ml) at 0 °C. After having been stirred at 0 °C for 1 h and then at room temperature for 2 h, the mixture was diluted with CH_2Cl_2, washed with 30 ml each of saturated $NaHCO_3$, 10% citric acid and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with hexane–EtOAc (1:1) to give Boc-D-Trp-D-Asp(OBzl)-O^{t}Bu (1.45 g, 97%) as a white amorphous solid. An aliquot (792 mg, 1.40 mmol) of the above-mentioned product was dissolved in formic acid (12 ml) and the resulting solution was stirred at room temperature for 1 h. Volatile materials were evaporated and the residue was taken up with EtOAc (60 ml). This solution was washed with saturated $NaHCO_3$ (30 ml×2), dried over $MgSO_4$ and concentrated under reduced pressure to give **4** as a colorless oil: TLC Rf ($CHCl_3$:MeOH:AcOH=10:1:1) 0.27. FAB-MS m/z: 466 (MH^+).

Boc-Hyp-D-Val-Leu-D-Trp-D-Asp(OBzl)-O^{t}Bu (5) HOBT (230 mg, 1.50 mmol) and EDCI·HCl (287 mg, 1.50 mmol) were added to a mixture of **3** (556 mg, 1.25 mmol) and **4** (610 mg, 1.31 mmol) in DMF (10 ml) at 0 °C, and the resulting mixture was stirred at 0 °C for 1 h and then at room temperature for 4 h. It was then partitioned between EtOAc (100 ml) and water (50 ml). The organic layer was washed with 50 ml each of saturated $NaHCO_3$, 10% citric acid and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by chromatography on silica gel (E. Merck, Lobar, Lichroprep, Si 60, size B) with hexane–EtOAc (1:3) to give **5** (907 mg, 82%) as a white amorphous solid: TLC Rf (EtOAc) 0.41. FAB-MS m/z: 892 (MH^+).

***cyclo*(-D-Trp-D-Asp(OBzl)-Hyp-D-Val-Leu) (7)** A solution of **5** (770 mg, 0.86 mmol) in TFA (10 ml) and 1,2-ethanedithiol (1 ml) was stirred at room temperature for 1 h and then was evaporated *in vacuo*. The residue was triturated with ethyl ether (30 ml) to give the TFA salt of **6** (693 mg, 94%) as a white powder. Compound **6** (690 mg, 0.81 mmol) was dissolved in DMF (15 ml), and this solution was neutralized with *N*-methylmorpholine (NMM, 90 μl, 0.81 mmol), then added at 0 °C to a solution of EDCI·HCl (234 mg, 1.22 mmol) and HOBT (187 mg, 1.22 mmol) in DMF (60 ml) over a period of 1 h. After the addition, the resulting mixture was stirred at 0 °C for 3 h and then at room temperature overnight. It was concentrated under reduced pressure and the residue was taken up with water (50 ml). This solution was extracted with EtOAc (30 ml×2). The combined organic extracts were washed with 50 ml each of saturated $NaHCO_3$, 10% citric acid and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with EtOAc to give **7** (474 mg, 81%) as a white amorphous solid: TLC Rf (EtOAc) 0.45. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.73 (3H, d, J=6.2 Hz), 0.76 (3H, d, J=6.2 Hz), 0.85 (3H, d, J=6.8 Hz), 0.96 (3H, d, J=6.8 Hz), 1.20—1.94 (5H, m), 2.39 (1H, d, J=4.6 Hz), 2.42 (1H, dd, J=4.6, 16.5 Hz), 2.72—2.83 (1H, m), 2.88 (1H, dd, J=9.5, 16.5 Hz), 3.26 (1H, dd, J=5.1, 14.9 Hz), 3.35—3.54 (3H, m), 3.65—3.77 (1H, m), 3.87 (1H, t, J=9.6 Hz), 4.52—4.64 (1H, m), 4.68—4.78 (1H, m), 4.89 (1H, dd, J=3.7, 8.5 Hz), 5.00 and 5.24 (each 1H, ABq, J=12.3 Hz), 5.14—5.28 (1H, m), 6.11 (1H, d, J=8.1 Hz), 6.56 (1H, d, J=6.2 Hz), 7.01 (1H, d, J=1.6 Hz), 7.09 (1H, dt, J=1.6, 7.3 Hz), 7.14 (1H, d, J=8.9 Hz), 7.20 (1H, dt, J=1.6, 7.3 Hz), 7.29—7.44 (6H, m), 7.56 (1H, dd, J=1.6, 7.3 Hz), 7.63 (1H, d, J=9.6 Hz), 8.12 (1H, d, J=1.6 Hz). FAB-MS m/z: 717 (MH^+).

***cyclo*(-D-Trp-D-Asp(OBzl)-Hyp(*Z*-Lys(*Z*))-D-Val-Leu) (8d)** EDCI (32 mg, 0.17 mmol) was added to a mixture of **7** (40 mg, 0.056 mmol), N^{α},N^{ε}-bisbenzyloxycarbonyllysine (*Z*-Lys(*Z*), 70 mg, 0.17 mmol) and 4-dimethylaminopyridine (2 mg, 0.01 mmol) in THF (1 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 2 h and then at room temperature overnight. It was partitioned between EtOAc (50 ml) and water (30 ml). The organic layer was washed with 30 ml each of saturated $NaHCO_3$, 10% citric acid and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$:MeOH=30:1) to give **8d** (44 mg, 71%) as a white amorphous solid: TLC Rf ($CHCl_3$:MeOH=30:1) 0.23. FAB-MS m/z: 1113 (MH^+).

***cyclo*(-D-Trp-D-Asp-Hyp(Lys)-D-Val-Leu) (9d)** A mixture of **8d** (41 mg, 0.037 mmol) and 10% Pd–C (10 mg) in DMF (2 ml) was stirred overnight under atmospheric pressure of hydrogen. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was triturated with ethyl ether to give **9d** (17 mg, 60%) as a white powder: mp 202—209 °C. TLC Rf (BuOH:AcOH:H_2O=4:1:1) 0.22. IR (KBr) cm^{-1}: 3270, 3058, 2960, 1741 (C=O), 1658, 1546, 1440, 1390, 1218, 742. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 0.60 (3H, d, J=6.3 Hz, Leu-H$_{\delta 1}$), 0.72 (3H, d, J=6.3 Hz, Leu-H$_{\delta 2}$), 0.82 (3 H, d, J=6.8 Hz, D-Val-H$_{\gamma 1}$), 0.86 (3H, d, J=6.8 Hz, D-Val-H$_{\gamma 2}$),

0.92—1.06 (1H, m, Leu-H$_\gamma$), 1.06—1.79 (9H, m, Leu-H$_\beta$+D-Val-H$_\beta$ +Lys-H$_\beta$+Lys-H$_\gamma$+Lys-H$_\delta$), 1.83—1.98 (1H, m, Hyp-H$_{\beta1}$), 1.98—2.10 (1H, m, D-Asp-H$_{\beta1}$), 2.40—2.95 (5H, m, D-Asp-H$_{\beta2}$+Lys-H$_\varepsilon$+Hyp-H$_{\beta2}$+D-Trp-H$_{\beta1}$), 3.09—3.19 (1H, m, Hyp-H$_{\delta1}$), 3.23—3.41 (2 H, m, Lys-H$_\alpha$+D-Trp-H$_{\beta2}$), 3.65—3.78 (1H, m, Hyp-H$_{\delta2}$), 3.91—4.06 (1H, m, Leu-H$_\alpha$), 4.06—4.18 (1H, m, D-Val-H$_\alpha$), 4.18—4.31 (1H, m, D-Trp-H$_\alpha$), 4.79—4.89 (1H, m, Hyp-H$_\alpha$), 4.92—5.07 (1H, m, D-Asp-H$_\alpha$), 5.10—5.22 (1H, m, Hyp-H$_\gamma$), 6.95 (1H, t, J=7.4 Hz, D-Trp-H$_5$), 7.03 (1H, t, J=7.4 Hz, D-Trp-H$_6$), 7.12 (1H, d, J=2.1 Hz, D-Trp-H$_2$), 7.30 (1H, d, J=7.4 Hz, D-Trp-H$_7$), 7.51 (1H, d, J=7.4 Hz, D-Trp-H$_4$), 7.51 (1H, d, J=7.3 Hz, D-Asp-NH), 7.67 (1H, d, J=10.1 Hz, D-Val-NH), 8.64 (1H, d, J=5.4 Hz, Leu-NH)), 8.73 (1H, d, J=8.4 Hz, D-Trp-NH), 10.79 (1H, d, J=2.1 Hz, D-Trp-H$_1$). FAB-MS m/z: 755.4108 (Calcd for $C_{37}H_{54}N_8O_9$+H: 755.4092).

***cyclo*(-D-Trp–D-Asp–Hyp(Gly)–D-Val–Leu) (9a)** Yield 86%, mp 215 °C (dec.). TLC *Rf* (BuOH : AcOH : H$_2$O=4 : 1 : 1) 0.45. IR (KBr) cm^{-1}: 3247, 2962, 1749 (C=O), 1652, 1556, 1403, 1224, 1099, 1060, 742. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.61 (3H, d, J=6.4 Hz, Leu-H$_{\delta1}$), 0.72 (3H, d, J=6.4 Hz, Leu-H$_{\delta2}$), 0.82 (3H, d, J=6.6 Hz, D-Val-H$_{\gamma1}$), 0.85 (3H, d, J=6.6 Hz, D-Val-H$_{\gamma2}$), 0.94—1.05 (1H, m, Leu-H$_\gamma$), 1.10—1.27 (2H, m, Leu-H$_\beta$), 1.68—1.80 (1H, m, D-Val-H$_\beta$), 1.80—1.95 (1H, m, Hyp-H$_{\beta1}$), 2.12—2.22 (1H, m, D-Asp-H$_{\beta1}$), 2.60—2.96 (4H, m, D-Asp-H$_{\beta2}$+Hyp-H$_{\beta2}$+Hyp-H$_{\delta1}$+D-Trp-H$_{\beta1}$), 3.10—3.90 (4H, m, Hyp-H$_{\delta2}$+D-Trp-H$_{\beta2}$+Gly-H$_\alpha$), 4.00—4.18 (2H, m, Leu-H$_\alpha$+D-Val-H$_\alpha$), 4.20—4.30 (1H, m, D-Trp-H$_\alpha$), 4.81—4.95 (2H, m, Hyp-H$_\alpha$+D-Asp-H$_\alpha$), 5.15—5.25 (1H, br s, Hyp-H$_\gamma$), 6.95 (1H, t, J=7.7 Hz, D-Trp-H$_5$), 7.03 (1H, t, J=7.7 Hz, D-Trp-H$_6$), 7.13 (1H, br s, D-Trp-H$_2$), 7.30 (1H, d, J=7.7 Hz, D-Trp-H$_7$), 7.42 (1H, d, J=9.3 Hz, D-Asp-NH), 7.52 (1H, d, J=7.7 Hz, D-Trp-H$_4$), 7.67 (1H, d, J=11.2 Hz, D-Val-NH), 8.65 (1H, d, J=4.5 Hz, Leu-NH), 8.74 (1H, d, J=7.3 Hz, D-Trp-NH), 10.79 (1H, br s, D-Trp-H$_1$). FAB-MS m/z: 684.3365 (Calcd for $C_{33}H_{45}N_7O_9$+H: 684.3357).

***cyclo*(-D-Trp–D-Asp–Hyp(-CO-(CH$_2$)$_5$-NH$_2$)–D-Val–Leu) (9b)** Yield 84%, mp 185—195 °C. TLC *Rf* (BuOH : AcOH : H$_2$O=4 : 1 : 1) 0.56. IR (KBr) cm^{-1}: 3276, 3054, 2960, 1730 (C=O), 1658, 1537, 1390, 1263, 1234, 1178, 1103, 744. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.61 (3H, d, J=6.4 Hz, Leu-H$_{\delta1}$), 0.72 (3H, d, J=6.4 Hz, Leu-H$_{\delta2}$), 0.82 (3H, d, J=6.6 Hz, D-Val-H$_{\gamma1}$), 0.86 (3H, d, J=6.6 Hz, D-Val-H$_{\gamma2}$), 0.93—1.05 (1H, m, Leu-H$_\gamma$), 1.11—1.25 (2H, m, Leu-H$_\beta$), 1.24—1.80 (7H, m, D-Val-H$_\beta$+CO-CH$_2$-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_2$), 1.88—2.00 (1H, m, Hyp-H$_{\beta1}$), 2.02 (1H, dd, J=2.7, 15.4 Hz, D-Asp-H$_{\beta1}$), 2.23—2.42 (2H, m, CO-CH$_2$-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_2$), 2.60—2.95 (5H, m, CO-CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH$_2$+D-Asp-H$_{\beta2}$+Hyp-H$_{\beta2}$+D-Trp-H$_{\beta1}$), 3.17 (1H, dd, J=3.9, 12.5 Hz, Hyp-H$_{\delta1}$), 3.24 (1H, dd, J=3.4, 15.7 Hz, D-Trp-H$_{\beta2}$), 3.91 (1H, d, J=12.5 Hz, Hyp-H$_{\delta2}$), 4.01—4.15 (2H, m, Leu-H$_\alpha$+D-Val-H$_\alpha$), 4.20—4.30 (1H, m, D-Trp-H$_\alpha$), 4.84 (1H, dd, J=6.1, 8.5 Hz, Hyp-H$_\alpha$), 4.90—5.00 (1H, m, D-Asp-H$_\alpha$), 5.11—5.20 (1H, m, Hyp-H$_\gamma$), 6.95 (1H, t, J=7.4 Hz, D-Trp-H$_5$), 7.03 (1H, t, J=7.4 Hz, D-Trp-H$_6$ 7.11 (1H, d, J=1.7 Hz, D-Trp-H$_2$), 7.30 (1H, d, J=7.4 Hz, D-Trp-H$_7$), 7.38 (1H, d, J=9.3 Hz, D-Asp-NH), 7.52 (1H, d, J=7.4 Hz, D-Trp-H$_4$), 7.80 (1H, d, J=9.8 Hz, D-Val-NH), 8.58 (1H, d, J=5.9 Hz, Leu-NH), 8.72 (1H, d, J=8.3 Hz, D-Trp-NH), 10.79 (1H, d, J=1.7 Hz, D-Trp-H$_1$). FAB-MS m/z: 740.3950 (Calcd for $C_{37}H_{53}N_7O_9$+H: 740.3983).

***cyclo*(-D-Trp–D-Asp–Hyp(Orn)–D-Val–Leu) Dihydrochloride (9c)** Yield 94%, mp 212—216 °C. TLC *Rf* (BuOH : AcOH : H$_2$O=4 : 1 : 1) 0.45. IR (KBr) cm^{-1}: 3382, 3257, 2960, 1734 (C=O), 1654, 1540, 1457, 1220, 1095, 1056, 744. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.60 (3H, d, J=6.3 Hz, Leu-H$_{\delta1}$), 0.73 (3H, d, J=6.3 Hz, Leu-H$_{\delta2}$), 0.83 (3H, d, J=6.4 Hz, D-Val-H$_{\gamma1}$), 0.88 (3H, d, J=6.4 Hz, D-Val-H$_{\gamma2}$), 0.94—1.27 (3H, m, Leu-H$_\gamma$+Leu-H$_\beta$), 1.36—2.00 (6H, m, D-Val-H$_\beta$+Orn-H$_\beta$+Orn-H$_\gamma$+Hyp-H$_{\beta1}$), 2.34—2.39 (1H, m, D-Asp-H$_{\beta1}$), 2.64—2.95 (6H, m, D-Asp-H$_{\beta2}$+Orn-H$_\delta$+Hyp-H$_{\beta2}$+Hyp-H$_{\delta1}$+D-Trp-H$_{\beta1}$), 3.16—3.52 (2H, m, Hyp-H$_{\delta2}$+D-Trp-H$_{\beta2}$), 3.93—4.02 (1H, m, Leu-H$_\alpha$), 4.04—4.16 (2H, m, Orn-H$_\alpha$+D-Val-H$_\alpha$), 4.18—4.27 (1H, m, D-Trp-H$_\alpha$), 4.90—5.10 (2H, m, Hyp-H$_\alpha$+D-Asp-H$_\alpha$), 5.28—5.38 (1H, m, Hyp-H$_\gamma$), 6.95 (1H, t, J=7.5 Hz, D-Trp-H$_5$), 7.04 (1H, t, J=7.5 Hz, D-Trp-H$_6$), 7.14 (1H, br s, D-Trp-H$_2$), 7.20—7.42 (2H, m, D-Trp-H$_7$+D-Val-NH), 7.49 (1H, d, J=7.5 Hz, D-Trp-H$_4$), 7.84 (1H, d, J=9.3 Hz, D-Asp-NH), 8.75 (1H, d, J=4.9 Hz, Leu-NH), 8.80 (1H, d, J=7.8 Hz, D-Trp-NH), 10.83 (1H, br s, D-Trp-H$_1$). FAB-MS m/z: 741.3913 (Calcd for $C_{36}H_{52}N_8O_9$+H: 741.3936).

***cyclo*(-D-Trp–D-Asp–Hyp(Arg)-D-Val–Leu) (9e)** Yield 82%, mp 208—215 °C. TLC *Rf* (BuOH : AcOH : H$_2$O=4 : 1 : 1) 0.39. IR (KBr) cm^{-1}: 3297, 2962, 2873, 1737 (C=O), 1664, 1546, 1440, 1392, 1216, 1103, 743. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.60 (3H, d, J=6.6 Hz, Leu-H$_{\delta1}$), 0.72 (3H, d, J=6.6 Hz, Leu-H$_{\delta2}$), 0.83 (3H, d, J=6.8 Hz, D-Val-H$_{\gamma1}$), 0.87 (3H, d, J=6.8 Hz, D-Val-H$_{\gamma2}$), 0.97—1.05 (1H, m, Leu-H$_\gamma$), 1.05—2.05 (7H, m, Leu-H$_\beta$+D-Val-H$_\beta$ +Arg-H$_\beta$+Hyp-H$_{\beta1}$+D-Asp-H$_{\beta1}$), 2.40—2.55 (2H, m, Arg-H$_\gamma$), 2.55—2.70 (1H, m, D-Asp-H$_{\beta2}$), 2.70—2.92 (2H, m, D-Trp-H$_{\beta1}$+Hyp-H$_{\beta2}$), 2.95—3.07 (1H, m, Hyp-H$_{\delta1}$), 3.13—3.55 (3H, m, D-Trp-H$_{\beta2}$+Arg-H$_\delta$), 3.60—3.68 (1H, m, Hyp-H$_{\delta2}$), 3.93—4.05 (1H, m, Leu-H$_\alpha$), 4.05—4.16 (1H, m, D-Val-H$_\alpha$), 4.16—4.28 (1H, m, D-Trp-H$_\alpha$), 4.75—4.83 (1H, m, Arg-H$_\alpha$), 4.83—4.96 (1H, m, Hyp-H$_\alpha$), 4.96—5.06 (1H, m, D-Asp-H$_\alpha$), 5.06—5.12 (1H, m, Hyp-H$_\gamma$), 6.84 (1H, br s, Arg-δ-NH), 6.95 (1H, t, J=7.5 Hz, D-Trp-H$_5$), 7.03 (1H, t, J=7.5 Hz, D-Trp-H$_6$), 7.12 (1H, br s, D-Trp-H$_2$), 7.30 (1H, d, J=7.5 Hz, D-Trp-H$_7$), 7.45—7.60 (2H, m, D-Asp-NH+D-Trp-H$_4$), 7.64 (1H, d, J=10.3 Hz, D-Val-NH), 8.63 (1H, d, J=4.9 Hz, Leu-NH), 8.70 (1H, d, J=8.3 Hz, D-Trp-NH), 10.78 (1H, br s, D-Trp-H$_1$). FAB-MS m/z: 783.4170 (Calcd for $C_{37}H_{54}N_{10}O_9$+H: 783.4154).

***cyclo*(-D-Trp–D-Asp–Hyp(Me-Lys(Me))–D-Val–Leu) (9f)** Yield 89%, mp 183—187 °C. TLC *Rf* (BuOH : AcOH : H$_2$O=4 : 1 : 1) 0.27. IR (KBr) cm^{-1}: 3305, 3052, 2960, 1727 (C=O), 1658, 1535, 1502, 1440, 1390, 1178, 744. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.60 (3H, d, J=6.3 Hz, Leu-H$_{\delta1}$), 0.73 (3H, d, J=6.3 Hz, Leu-H$_{\delta2}$), 0.83 (3H, d, J=6.8 Hz, D-Val-H$_{\gamma1}$), 0.87 (3H, d, J=6.8 Hz, D-Val-H$_{\gamma2}$), 0.92—1.05 (1H, m, Leu-H$_\gamma$), 1.05—1.76 (9H, m, Leu-H$_\beta$+D-Val-H$_\beta$+Me-Lys(Me)-H$_\beta$+Me-Lys(Me)-H$_\gamma$+Me-Lys(Me)-H$_\delta$), 1.88 (1H, dd, J=9.0, 15.1 Hz, Hyp-H$_{\beta1}$), 2.01 (1H, dd, J=3.1, 15.5 Hz, D-Asp-H$_{\beta1}$), 2.22 (3H, s, Me-Lys(Me)-N-CH$_3$), 2.41 (3H, s, Me-Lys(Me)-N-CH$_3$), 2.52—2.73 (3 H, m, D-Asp-H$_{\beta2}$+Me-Lys(Me)-H$_\varepsilon$), 2.77—2.91 (2H, m, Hyp-H$_{\beta2}$+D-Trp-H$_{\beta1}$), 3.02—3.12 (2H, m, Hyp-H$_{\delta1}$+Me-Lys(Me)-H$_\alpha$), 3.28—3.37 (1H, m, D-Trp-H$_{\beta2}$), 3.63 (1H, d, J=12.7 Hz, Hyp-H$_{\delta2}$), 3.94—4.03 (1H, m, Leu-H$_\alpha$), 4.10 (1H, t, J=9.2 Hz, D-Val-H$_\alpha$), 4.17—4.26 (1H, m, D-Trp-H$_\alpha$), 4.85 (1H, dd, J=5.4, 9.0 Hz, Hyp-H$_\alpha$), 4.97—5.07 (1H, m, D-Asp-H$_\alpha$), 5.13—5.21 (1H, m, Hyp-H$_\gamma$), 6.95 (1H, t, J=7.6 Hz, D-Trp-H$_5$), 7.03 (1H, t, J=7.6 Hz, D-Trp-H$_6$), 7.12 (1H, d, J=2.0 Hz, D-Trp-H$_2$), 7.30 (1H, d, J=7.6 Hz, D-Trp-H$_7$), 7.51 (1H, d, J=7.6 Hz, D-Trp-H$_4$), 7.59 (1H, d, J=9.2 Hz, D-Val-NH), 7.61 (1H, d, J=9.8 Hz, D-Asp-NH), 8.64 (1H, d, J=5.4 Hz, Leu-NH), 8.73 (1H, d, J=8.3 Hz, D-Trp-NH), 10.79 (1H, br s, D-Trp-H$_1$). FAB-MS m/z: 783.4413 (Calcd for $C_{39}H_{58}N_8O_9$+H: 783.4405).

***cyclo*(-D-Trp–D-Asp–Hyp(His)–D-Val–Leu) (9g)** Yield 83%, mp 205—212 °C. TLC *Rf* (BuOH : AcOH : H$_2$O=4 : 1 : 1) 0.46. IR (KBr) cm^{-1}: 3272, 2960, 1749 (C=O), 1652, 1540, 1457, 1218, 1097, 1054, 744. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.60 (3H, d, J=6.6 Hz, Leu-H$_{\delta1}$), 0.72 (3H, d, J=6.6 Hz, Leu-H$_{\delta2}$), 0.83 (3H, d, J=6.8 Hz, D-Val-H$_{\gamma1}$), 0.87 (3H, d, J=6.8 Hz, D-Val-H$_{\gamma2}$), 0.94—1.27 (3 H, m, Leu-H$_\beta$+Leu-H$_\gamma$), 1.64—1.79 (2H, m, D-Val-H$_\beta$+Hyp-H$_{\beta1}$), 2.28—2.39 (1H, m, D-Asp-H$_{\beta1}$), 2.63—3.03 (6H, m, D-Asp-H$_{\beta2}$+Hyp-H$_{\beta2}$+D-Trp-H$_{\beta1}$+Hyp-H$_{\delta1}$+His-H$_\beta$), 3.05—3.60 (2H, m, D-Trp-H$_{\beta2}$+Hyp-H$_{\delta2}$), 3.93—4.03 (1H, m, Leu-H$_\alpha$), 4.07—4.17 (2H, m, His-H$_\alpha$+D-Val-H$_\alpha$), 4.17—4.30 (1H, m, D-Trp-H$_\alpha$), 4.87 (1H, dd, J=4.1, 8.5 Hz, Hyp-H$_\alpha$), 4.95—5.05 (1H, m, D-Asp-H$_\alpha$), 5.18—5.28 (1H, m, Hyp-H$_\gamma$), 6.90 (1H, s, His-H$_5$), 6.95 (1H, t, J=7.5 Hz, D-Trp-H$_5$), 7.04 (1H, t, J=7.5 Hz, D-Trp-H$_6$), 7.14 (1H, br s, D-Trp-H$_2$), 7.31 (1H, d, J=7.5 Hz, D-Trp-H$_7$), 7.36 (1H, d, J=11.2 Hz, D-Val-NH), 7.50 (1H, d, J=7.5 Hz, D-Trp-H$_4$), 7.66 (1H, s, His-H$_2$), 7.73 (1H, d, J=9.3 Hz, D-Asp-NH), 8.71 (1H, d, J=4.9 Hz, Leu-NH), 8.77 (1H, d, J=7.8 Hz, D-Trp-NH), 10.81 (1H, br s, D-Trp-H$_1$). FAB-MS m/z: 764.3698 (Calcd for $C_{37}H_{49}N_9O_9$+H: 764.3731).

Biological Methods. Pharmacokinetic Studies in Rats Male Sprague–Dawley rats aged 7—10 weeks were used in the study. Three rats were used in each study group. The rats were allowed free access to water and food. A cannula was placed in the carotid artery for blood sampling, and another cannula was placed in the femoral vein for intravenous administration. Test compounds (3 mg/kg of body weight) dissolved in saline were infused through the femoral vein at a rate of 0.1 mg/kg/min (2.2 ml/h) over a period of 30 min. Blood (100 μl) was withdrawn from the carotid artery through the cannula *via* a heparinized capillary at 2, 5, 15 and 30 min after the infusion was started, and at the same time points and 60 min after the infusion was stopped. Blood samples were centrifuged, and the plasma was deproteinized with EtOH containing 0.1% TFA. The supernatants were subjected to HPLC analysis to determine the plasma concentrations of test compounds. The analytical HPLC conditions are shown in Table 1. The effluent was monitored with a fluorescence detector (Ex. 287 nm, Em. 348 nm). The *AUC* was calculated using the log trapezoidal method and extrapolated to infinity.

The half-life ($t_{1/2}$) of the terminal phase was calculated by linear regression analysis. Total clearance (CL_{tot}) is given by the following equation:

$$CL_{tot} = \text{dose}/AUC$$

Serum Protein-Binding Studies Determination of the serum protein binding ratio of **1** and **9d** was performed by the ultracentrifugal filtration method. Rat serum was adjusted to pH 7.4 with 1 N HCl prior to use. The test compounds (10 μM) were dissolved in the rat serum (500 μl) and incubated for 15 min. A 40-μl aliquot was removed and deproteinized with 200 μl of EtOH. The supernatant was analyzed by HPLC to determine the total concentration of the test compound. The remaining sample was placed in an ultrafiltration apparatus (UFC3LGC, Nippon Millipore, Tokyo) and centrifuged. The filtrate was deproteinized with EtOH, and the supernatant was analyzed by HPLC to determine the unbound concentration of the test compound. The control experiment was performed using 10 mM phosphate buffer (pH 7.4)–saline instead of the serum to check the absorption of the ligand on the filtration membrane.

Hydrolysis Studies The test compounds (10 μM) were dissolved in 0.1 M phosphate buffer (pH 7.4) and incubated at 37 °C. At 0, 15, 30, 60 and 120 min, 50-μl aliquots were removed and quenched with EtOH (200 μl) and 0.3% aqueous TFA (50 μl). The amount of the compound left was determined by HPLC analysis. The integrated areas and time were fitted to a first-order decay curve, and the half-life ($t_{1/2}$) was calculated by linear regression analysis.

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