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Design, synthesis, and structure–activity relationships of potent GPIIb/IIIa antagonists: discovery of FK419

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Abstract—The discovery of the non-peptide antiplatelet injectable agent FK419 is reported. Based on the β-turn structure of RGD peptide sequences in the α chain of fibrinogen, which binds the glycoprotein IIb/IIIa (GPIIb/IIIa) on the surface of platelets to induce platelet aggregation, the prototype **2** was designed. After further substituent effects were investigated at the α-position of the carboxylic acid in **2**, we enhanced platelet aggregation inhibition, and discovered the useful feature of reduced prolongation of bleeding time. Finally, the potent platelet aggregation inhibitor FK419 (**3**) could be discovered. FK419 shows a safe feature of reduced prolongation of bleeding time, as well as potent inhibition of platelet aggregation.

1. Introduction

Uncontrolled platelet aggregation and platelet adhesion to the subendothelium by damaged blood vessels causes critical diseases such as myocardial infarction, transient ischemic attack and unstable angina.[1] Platelet aggregation is thought to be induced by the following steps: agonists such as ADP, thrombin, thromboxane A_2, serotonin, and collagen first induce glycoprotein IIb/IIIa (GPIIb/IIIa) on the surface of platelets to adopt an active form. Aggregation of the activated platelet then occurs with fibrinogen mediation.[2] In such processes, even if antiplatelet agents inhibit one of the agonistic pathways responsible for activation of GPIIb/IIIa, platelet aggregation is not necessarily interrupted perfectly, because GPIIb/IIIa may be activated by another agonistic pathway. However, if an agent which antagonizes the GPIIb/IIIa receptor can be developed, it may be a very efficient antiplatelet agent, because GPIIb/IIIa is the obligatory receptor in platelet aggregation. GPIIb/IIIa binds the Arg-Gly-Asp (RGD) sequence in the fibrinogen α chain.[3] On the basis of the β-turn structure[4] of the RGD peptide, many researchers have attempted to find non-peptide small molecules, which have high affinity to GPIIb/IIIa,[5] and Merck researchers discovered the first marketed non-peptide GPIIb/IIIa antagonist, tirofiban (Aggrastate®) as an injectable agent.[6] However, results from in vivo assays, and clinical trials of several development candidates indicated that efficacy, and the side effect of bleeding time prolongation could not be separated.[7] We therefore initiated efforts to find a safer antiplatelet agent with reduced prolongation of bleeding time. In order to stop bleeding, platelets have to bind von Willbrand factor (vWF), which causes platelet adhesion to subendothelium, however, earlier GPIIb/IIIa antagonists are assumed to inhibit platelets binding to vWF.[8] In the course of our study, we proposed that if a GPIIb/IIIa antagonist did not inhibit vWF-platelet binding, that is, platelet adhesion to subendothelium, we may discover a new generation antiplatelet agent with superior safety. We wish to report here the discovery of FK419 (**3**), which has reduced prolongation of bleeding time, as well as potent inhibition of platelet aggregation, and the structure–activity relationships of related compounds.

2. Prototype drug design

Initially, we opted to modify the RGD peptide sequence of fibrinogen. Since the type II′ β-turn structure is

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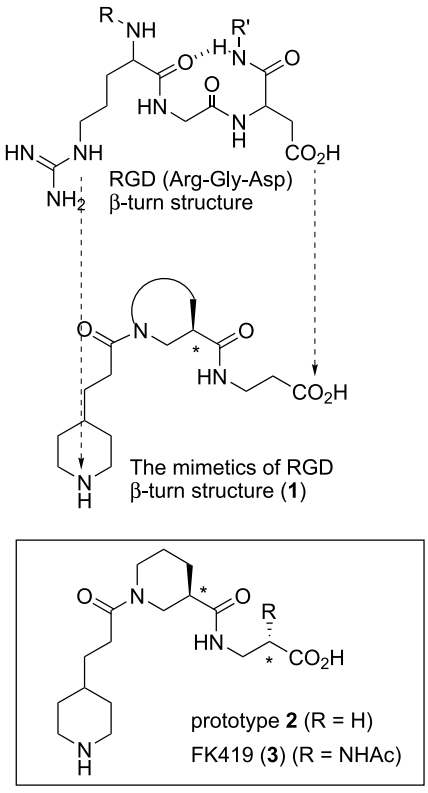


Figure 1. Design of prototype **2** from the structure of RGD β-turn peptide, and the structure of FK419 (**3**).

thought to be present in the RGD sequence of fibrinogen,[9] we designed RGD β-turn mimetics **1** containing *N*-heterocyclic β-amino acids based on the speculation that the ring moiety of **1** could serve as the β-turn of RGD peptide,[10] and the structures of reported GPIIb/IIIa antagonists[11] (Fig. 1). In order for the mimetics **1** to act as GPIIb/IIIa antagonists, incorporation of two functional groups, carboxylic acid and amine, at an appropriate separation and angle must be essential.[12] After the evaluation of the derivatives, which are incorporated in various kinds of ring moieties in **1**,[13–15] we selected piperidine-containing derivative **2** as a prototype.[13,16]

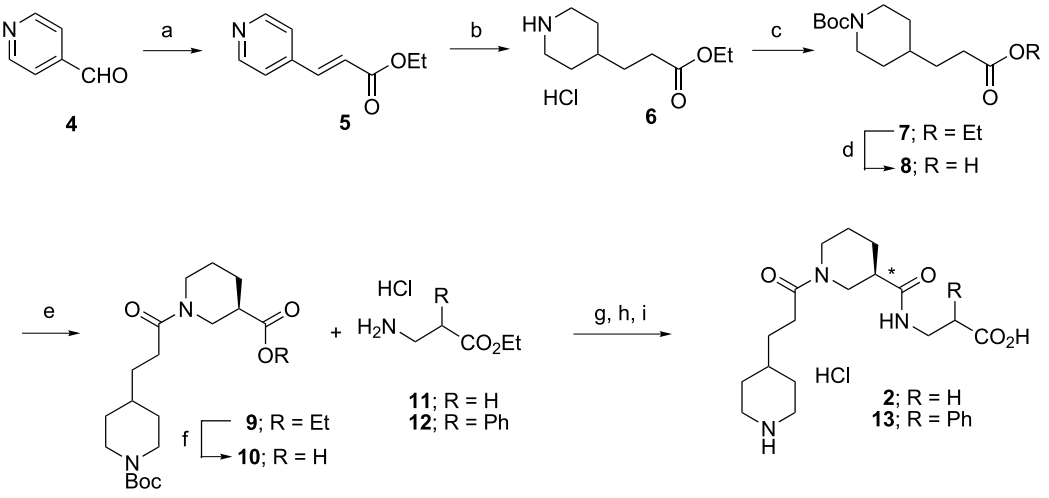
3. Results and discussion

3.1. Chemistry

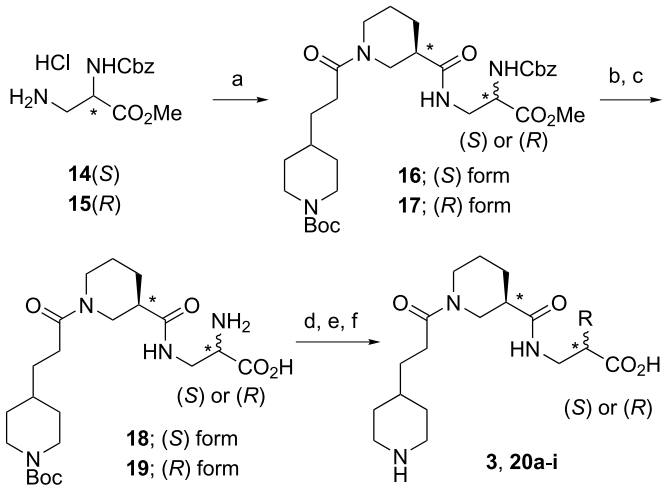
Prototype **2** was synthesized according to Scheme 1. 4-Pyridinecarboxaldehyde (**4**) was treated with triethyl phosphonoacetate and sodium hydride as base to give the α,β-unsaturated ester **5**. The pyridine ring and the α,β-unsaturated bond in **5** were reduced simultaneously under 1 atm of hydrogen atmosphere with catalytic platinum(IV) oxide and 1 equiv of hydrochloric acid in EtOH to give **6** as a hydrochloride. The resulting piperidine ring was *N*-protected with a Boc group to give **7**, and the ester in **7** was hydrolyzed by aqueous sodium hydroxide solution to give **8**. Acid **8** was coupled with ethyl (*R*)-3-piperidinecarboxylate in the presence of EDC and HOBT to give **9**. After hydrolysis of the ethyl ester in **9** with lithium hydroxide, the resulting acid **10** was coupled with β-alanine methyl ester hydrochloride (**11**) in the presence of EDC and HOBT. The ester and Boc groups were cleaved by treatment with lithium hydroxide followed by hydrogen chloride in ethyl acetate, to give **2** as a hydrochloride.

Compound **13** was synthesized according to the procedure for **2** except for using ethyl 3-amino-2-phenylpropanoate hydrochloride (**12**) instead of **11**.

α-Substituted derivatives **3** and **20a–j** were synthesized as illustrated in Scheme 2. α-Substituted β-alanine derivatives **14** and **15** were obtained from *N*-carbobenzyloxy-L-asparagine and *N*-carbobenzyloxy-D-asparagine, respectively, according to the literature procedures.[17] Acid **10** was coupled with amine **14** and **15** using EDC



Scheme 1. Reagents and conditions: (a) $(EtO)_2POCH_2CO_2Et$, NaH, THF, 78%; (b) PtO_2, H_2, HCl, EtOH, 88%; (c) $(Boc)_2O$, Et_3N, CH_2Cl_2, 81%; (d) aq NaOH, THF, EtOH, 91%; (e) ethyl (*R*)-3-piperidinecarboxylate, EDC, HOBT, DMF, 86%; (f) aq LiOH, THF, EtOH, 90%; (g) EDC, HOBT, DMF; (h) aq LiOH; (i) HCl, EtOAc, 89% for **2**, 100% for **13** in three steps.



Scheme 2. Reagents and conditions: (a) **10**, EDC, HOBT, DMF, 93% for **16**, 96% for **17**; (b) Pd–C/H_2, MeOH; (c) aq LiOH, THF, 97% for **18**, 95% for **19** in two steps; (d) acylating reagent, MSA or aq NaOH (see Table 1); (e) HCl, EtOAc; (f) ODS column, then lyophilization (yields for **3**, **20a**–**i**; see Table 1).

Table 1. Acylating reagents used and yields of **3** and **20a**–**i**

Product	R	Acylating reagent	Stereo	Yield, %[a]
20a	NH_2	—	(*S*)	60
20b	NHCOPh-*p*CN	*p*CN-PhCOCl	(*S*)	90[b]
20c	NHCOPh-*p*OMe	*p*MeO-PhCOCl	(*S*)	94[b]
20d	NHCOPh-*p*OMe	*p*MeO-PhCOCl	(*R*)	94[b]
3	NHAc	Ac_2O	(*S*)	82[c]
20e	NHAc	Ac_2O	(*R*)	85[c]
20f	NHCO^{n}Hex	$(^n\text{HexCO})_2O$	(*S*)	90[c]
20g	NHCO^{c}Hex	^{c}HexCOCl	(*S*)	86[b]
20h	NHCO^{t}Bu	^{t}BuCOCl	(*S*)	86[b]
20i	NHCO^{t}Bu	^{t}BuCOCl	(*R*)	79[b]

[a] Isolated yields from **18** or **19**.
[b] 1.1 equiv of acid chloride and 12 equiv of MSA was used.
[c] 2.2 equiv of acid anhydride and 3.0 equiv of aq NaOH solution were used.

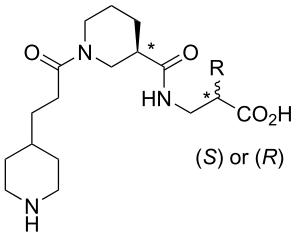
and HOBT to afford **16** and **17**, respectively. The Cbz group was deprotected under 1 atm of hydrogen with catalytic palladium-charcoal, followed by ethyl ester hydrolysis with lithium hydroxide under ice cooling to give α-amino acids **18** and **19**. The amino groups in **18** and **19** were treated with various acylating reagents such as acid chloride or acid anhydride, except in the case of **20a**. Finally, the Boc group at the piperidine ring was deprotected by hydrogen chloride in ethyl acetate. The crude product was purified by ODS column and lyophilized to give **3** and **20a**–**i**. The acylating reagents and yields from **18** or **19** are illustrated in Table 1.

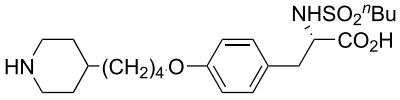
3.2. In vitro assay

Initially, we measured the IC_{50} values for ADP induced human platelet aggregation inhibition (termed IC_{50}A in this letter) of the prototype and optimized compounds (Table 2). The IC_{50}A of tirofiban was also measured as a reference.[6]

Prototype **2** showed moderate efficacy for platelet aggregation inhibition (IC_{50}A = 241 nM). Based on compound **2**, optimization was investigated to improve the efficacy for platelet aggregation inhibition. Namely, various kind of functional groups at the α-position of the carboxylic acid were substituted. When a phenyl group was introduced to the α-position of the carboxylic acid (**13**), IC_{50}A was enhanced compared to **2**. On the other hand, **20a**, which has an amino substituent at the α-position of the carboxylic acid, was found to have a diminished IC_{50}A value. But the α-amide derivatives (**3**, **20b**–**i**) improved IC_{50}A values. These amide bonds correspond to the RGD-*CONH*- amide bond in fibrinogen's α-chain. As a result, **20b**–**d**, which have aromatic amides at the α-position gave greatly enhanced IC_{50}A. So it is speculated that some interaction were generated between the amide carbonyl groups at the α-position of carboxylic acid and the binding site in GPIIb/IIIa. The configuration at the α-position did not affect IC_{50} A values (**20c**,**d**). Even if electron withdrawing groups or electron donating groups were substituted on the aromatic ring, the IC_{50}A values were not affected (**20b**,**c**). Next, aliphatic amide derivatives were examined. The simple acetamide derivative **3** showed good IC_{50}A value (53 nM). Compound **20e**, the epimer of **3**, showed somewhat lower IC_{50}A value than **3**. When the acyl carbon chain was elongated, IC_{50}A was much improved (**20f**).

Table 2. $IC_{50}A$ values of **2**, **3**, **13**, and **20a–i**



Compd	R	Stereo[a]	IC_{50} (inhibition of ADP induced aggregation), nM[b] ($IC_{50}A$)
2[c]	H	—	241
13[c]	Ph	Racemic	110
20a	NH_2	(*S*)	272
20b	NHCOPh-*p*CN	(*S*)	43
20c	NHCOPh-*p*OMe	(*S*)	32
20d	NHCOPh-*p*OMe	(*R*)	33
3 (FK419)	NHAc	(*S*)	53
20e	NHAc	(*R*)	80
20f	$NHCO^{n}Hex$	(*S*)	26
20g	$NHCO^{c}Hex$	(*S*)	34
20h	$NHCO^{t}Bu$	(*S*)	123
20i	$NHCO^{t}Bu$	(*R*)	37
(Tirofiban)		(*S*)	46

[a] The configuration at the α-position of the carboxylic acid.
[b] Concentration required to reduce ADP-induced human platelet aggregation response by 50%.
[c] As HCl salt.

This substituent effect at the α-position was also observed in the research of tirofiban and its analogues.[6b] When a more bulky acyl substituents were introduced at the α-position (**20g**,**i**), the $IC_{50}A$ values were raised. It was noticeable that much difference in $IC_{50}A$ between the epimer **20h** and **20i** was observed compared to the results with epimer pair of **20c** and **20d**, or **3** and **20e**. Furthermore, in the case of **20h** and **20i**, (*R*) configuration derivative (**20i**) was more potent, but in the case of tirofiban and its enantiomer, (*S*) configuration's tirofiban was more potent.[6b] It was interesting result that the more effective configuration at the α-position of **20i** was contrary to that of tirofiban. From these results, we chose the effective compounds (**3**, **20b–d**,**f**,**g**,**i**) for further evaluation. Next, we measured the IC_{50} values required to inhibit vWF mediated adhesion by 50% (termed $IC_{50}B$). And the ratio of $IC_{50}B/IC_{50}A$ was calculated to evaluate the safety margin in vitro. These results are showed in Table 3.

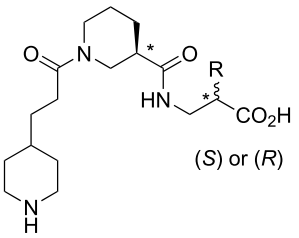
The $IC_{50}B/IC_{50}A$ ratio of prototype **2** (= 1.39) was larger than that of tirofiban (= 0.15), namely prototype **2** may have the possibility of reducing the prolongation of bleeding time. But all of α-aromatic amide derivatives (**20b–d**) showed high $IC_{50}B$ values. And the $IC_{50}B/IC_{50}A$ ratio was decreased compared to the prototype **2**. On the other hand, α-acetamide derivative **3** showed weak $IC_{50}B$ value, so the $IC_{50}B/IC_{50}A$ ratio was obtained superior value (5.45) to prototype **2** and tirofiban. While the elongated amide chain derivative (**20f**) enhanced the $IC_{50}B$ value, the more bulky amide derivatives (**20g**,**i**) lowered $IC_{50}B$ value.

From these in vitro assay results, **3** and **20i** were selected for further study, and they were examined in an in vivo and ex vivo assay, involving measurement of template bleeding time to evaluate the safety margin.

3.3. In vivo and ex vivo assays

Various amounts of **3**, **20i**, and tirofiban were administrated by bolus injection, followed by continuous injection (**3**: 20 μg/kg + 7 μg/kg/h, 100 μg/kg + 30 μg/kg/h, 200 μg/kg + 70 μg/kg/h, and 500 μg/kg + 150 μg/kg/h; **20i**: 3 μg/kg + 3 μg/kg/h, 10 μg/kg + 10 μg/kg/h, 30 μg/kg + 30 μg/kg/h, and 60 μg/kg + 60 μg/kg/h; tirofiban: 39 μg/kg + 1.3 μg/kg/h, 75 μg/kg + 2.5 μg/kg/h, 150 μg/kg + 5 μg/kg/h, and 300 μg/kg + 10 μg/kg/h) to dogs (beagle; $n = 2–4$), respectively. Blood samples were taken at 6 time-points for the platelet aggregation study ex vivo and for measurement of drug concentrations in plasma by HPLC analyses at 30 min intervals after dosing. At the same time as blood sample collection, bleeding time was assessed with an automated spring-loaded device designed to produce a standardized incision applied to the inner side of the upper jowl. Blood from the incision was blotted with filter paper every 30 s until all bleeding had stopped. Prolongation of bleeding time was determined by comparison with the time in the pretreatment period. Drug concentrations in plasma for

Table 3. $IC_{50}B$ values and the $IC_{50}B/IC_{50}A$ ratio of **2**, **3**, **20b–d,f,g,i**



Compd	R	Stereo[a]	IC_{50} (inhibition of vWF mediated adhesion), nM[b] ($IC_{50}B$)	Ratio ($IC_{50}B$)/($IC_{50}A$)
2[c]	H	—	335	1.39
20b	NHCOPh-*p*CN	(*S*)	21	0.49
20c	NHCOPh-*p*OMe	(*S*)	13	0.41
20d	NHCOPh-*p*OMe	(*R*)	21	0.64
3 (FK419)	NHAc	(*S*)	289	5.45
20f	NHCO^{n}Hex	(*S*)	21	0.81
20g	NHCO^{c}Hex	(*S*)	77	2.26
20i	NHCO^{t}Bu	(*R*)	94	2.54
	(Tirofiban)		6.7	0.15

[a] The configuration at the α-position of the carboxylic acid.
[b] Concentration required to inhibit vWF mediated human platelet adhesion by 50%.
[c] As HCl salt.

Table 4. In vivo and ex vivo potency of **3** and **20i**

Compd	Concentration, ng/mL (*C*)[a]	Concentration, ng/mL (*D*)[b]	Ratio (*D*/*C*)
3 (FK419)	46 ± 4.0	770 ± 20	17
20i	31 ± 3.0	244 ± 12	7.8
(tirofiban)	57 ± 1.0	148 ± 5.8	3.0

[a] Concentration required to reduce ADP-induced dog's platelet aggregation response by 50%.
[b] Plasma concentration of drug in dog when bleeding time was 2.5-fold prolonged.

2.5-fold prolongation were calculated (termed *D*). These results and drug concentrations required to reduce ADP-induced dog platelet aggregation by 50% (termed *C*) are illustrated in Table 4. The ratio (*D*/*C*) was calculated to evaluate the safety margin.

As a result, **3**, **20i**, and tirofiban displayed almost parallel inhibition of platelet aggregation ex vivo in dog (as shown by the value *C*), but have been shown to be different in terms of drug concentration in plasma when bleeding time was 2.5-fold prolonged (value *D*). From the ratio of *D*/*C*, **3** was the safest in terms of reduced prolongation of bleeding time (*D*/*C* = 17). The ratio (*D*/*C*) of **3** was about 6-fold larger than for tirofiban, whereas **20i** was not so good ratio compared to **3**.

4. Conclusions

From the result showed above, compound **3** was selected as a candidate compound, renamed as FK419, which is now under clinical trials.[18] The new injectable GPIIb/IIIa antagonist FK419 has the virtue of reduced prolongation of bleeding time.[19] FK419 is anticipated to be developed as an agent for prevention of cerebrosclerosis, because the risk of brain hemorrhage, which may be caused by antiplatelet agents could be lower. Unfortunately, FK419 is not suitable as an orally active drug because absorption from the intestine is quite poor (bioavailability = 12.0% in dog; fasted). We are currently searching for new orally active antiplatelet agents having the key features of FK419.[20]

5. Experimental

5.1. General

Melting points were determined with a BŰCHI 535. Proton NMR spectra were recorded on a Brucker BIOSPIN AVANCE400 or DPX200. δ Values in parts per million relative to tetramethylsilane are given. IR spectra were recorded with the compound (neat) on a sodium chloride disk or as KBr pellets or Nujol suspension using HITACHI 260-10, or HORIBA FT-710. Mass spectra were recorded with Hewlett Packard 1100LC/MSD. High resolution mass spectra were recorded with Micromass LCT. Results of elemental analysis were recorded with PERKINELMER 2400II.

5.1.1. Ethyl (2*E*)-3-(4-pyridinyl)acrylate (5). To a suspension of sodium hydride (60% dispersion in mineral oil, 39.4 g, 1.02 mol) in THF (500 mL) was added dropwise triethyl phosphonoacetate (230 g, 1.02 mol) in THF (500 mL) over a period of 0.5 h at 15–20 °C. The mixture was stirred for 1 h at the same temperature. To

the reaction mixture was added isonicotinaldehyde (**4**) (100 g, 934 mmol) in THF (40 mL) over a period of 20 min at room temperature, and the mixture was stirred overnight at the same temperature. The resulting mixture was poured into 1 N HCl, and extracted with EtOAc twice. The combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residual solid was triturated with hexane, filtered, washed with hexane, dried in vacuo to give **5** (129 g, 78%) as a solid: ^{1}H NMR (DMSO-d_6) δ 1.28 (t, J = 7.1 Hz, 3H), 4.23 (q, J = 7.1 Hz, 2H), 6.90 (d, J = 16.4 Hz, 1H), 7.64 (d, J = 16.4 Hz, 1H), 7.69 (dd, J = 7.7, 4.5 Hz, 2H), 8.64 (dd, J = 7.7, 4.5 Hz, 2H); MS (APCI) m/z 178 $(M+H)^+$.

5.1.2. Ethyl 3-(4-piperidinyl)propanoate hydrochloride (6). A solution of **5** (123 g, 747 mmol) in EtOH (1500 mL) and 4 N HCl in 1,4-dioxane (187 mL, 748 mmol) was treated with PtO_2 (12.3 g) with bubbling H_2 for 5 h at 45 °C. After the catalyst was filtered off, the filtrate was concentrated in vacuo. The residual solid was triturated with Et_2O, filtered, washed with Et_2O, dried in vacuo to give **6** (146 g, 88%) as a solid: ^{1}H NMR (DMSO-d_6) δ 1.18 (t, J = 7.1 Hz, 3H), 1.20–1.60 (m, 5H), 1.65–1.85 (m, 2H), 2.31 (t, J = 7.3 Hz, 2H), 2.68–2.85 (m, 2H), 3.16–3.22 (m, 2H), 4.05 (q, J = 7.1 Hz, 2H), 8.85–9.55 (br, 2H); MS (APCI) m/z 186 $(M+H)^+$.

5.1.3. *tert*-Butyl 4-(3-ethoxy-3-oxopropyl)-1-piperidinecarboxylate (7). To a suspension of **6** (50 g, 225 mmol) in CH_2Cl_2 (500 mL) were added Et_3N (69 mL, 496 mmol) and di-*tert*-butyl dicarbonate (59 g, 271 mmol) in CH_2Cl_2 (200 mL) under ice cooling. The mixture was stirred for 5 h at room temperature. To this was added water, and extracted with CH_2Cl_2. The organic layer was washed with 10% aqueous $KHSO_4$ solution, water, and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel with $CHCl_3$/MeOH (100:1). The eluate was concentrated in vacuo to give **7** (52 g, 81%) as an oil: IR (neat) 2980, 2960, 2920, 1730, 1690, 1160 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.01–1.19 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H), 1.39 (s, 9H), 1.41–1.72 (m, 5H), 2.32 (t, J = 7.6 Hz, 2H), 2.61–2.72 (m, 2H), 4.04–4.18 (m, 2H), 4.13 (q, J = 7.1 Hz, 2H); MS (APCI) m/z 186 $(M+H-Boc)^+$.

5.1.4. 3-[1-(*tert*-Butoxycarbonyl)-4-piperidinyl]propanoic acid (8). To a solution of **7** (46.9 g, 164 mmol) in THF (200 mL) and EtOH (200 mL) was added dropwise 1 N aqueous NaOH solution (247 mL, 247 mmol), and the mixture was stirred for 2 h at room temperature. Volatiles were evaporated off, and residual aqueous solution was adjusted to pH 4 with 10% aqueous $KHSO_4$ solution, and extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was triturated with the mixture of Et_2O and hexane, collected and dried in vacuo to give **8** (38.5 g, 91%) as a solid: IR (Nujol) 1730, 1660, 1260, 1160 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.84–1.07 (m, 2H), 1.38 (s, 9H), 1.41–1.50 (m, 3H), 1.55–1.65 (m, 2H), 2.22 (t, J = 7.4 Hz, 2H), 2.50–2.80 (m, 2H), 3.88–3.95 (m, 2H), 12.03 (s, 1H); MS (APCI) m/z 158 $(M+H)^+$.

5.1.5. *tert*-Butyl 4-{3-[(3*R*)-3-(ethoxycarbonyl)-1-piperidinyl]-3-oxopropyl}-1-piperidinecarboxylate (9). To a solution of **8** (3.04 g, 11.8 mmol), ethyl (*R*)-3-piperidinecarboxylate (1.86 g, 11.8 mmol) and HOBT (1.60 g, 11.8 mmol) in DMF (20 mL) was added EDC (1.83 g, 11.8 mmol) under ice cooling. The reaction mixture was stirred at room temperature overnight. To the resulting solution was added saturated $NaHCO_3$ followed by extraction with EtOAc. The organic layer was washed with water (×3) and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel with $CHCl_3$/MeOH (100:1). The eluate was concentrated in vacuo to give **9** (4.01 g, 86%) as a viscous oil: IR (Nujol) 1710, 1670, 1620, 1405 cm^{-1}; ^{1}H NMR ($CDCl_3$, rotomer observed) δ 1.00–1.20 (m, 1H), 1.28 (t, J = 7.1 Hz, 3H), 1.45 (s, 9H), 1.48–1.88 (m, 9H), 1.98–2.15 (m, 1H), 2.31–2.51 (m, 3H), 2.62–3.12 (m, 4H), 3.35–3.47 (m, 1/2H), 3.65–3.85 (m, 1H), 4.00–4.22 (m, 4H), 4.56–4.69 (m, 1/2H); MS (APCI) m/z 397 $(M+H)^+$.

5.1.6. (3*R*)-1-{3-[1-(*tert*-Butoxycarbonyl)-4-piperidinyl]-propanoyl}-3-piperidinecarboxylic acid (10). To a solution of **9** (3.09 g, 10.1 mmol) in THF (10 mL) and EtOH (10 mL) was added dropwise 3.0 M aqueous LiOH solution (10 mL, 30.0 mmol) under ice cooling and the mixture was stirred for 1 h at room temperature. The resulting solution was adjusted to pH 3 with 10% aqueous $KHSO_4$ solution, and extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was triturated with hexane, collected, and dried in vacuo to give **10** (3.34 g, 90%) as a solid: IR (KBr) 2931, 2862, 1732, 1716, 1697, 1686, 1456, 1279, 1248 cm^{-1}; ^{1}H NMR (DMSO-d_6 rotomer observed) δ 0.84–1.10 (m, 2H), 1.38 (s, 9H), 1.38–1.76 (m, 8H), 1.82–2.01 (m, 1H), 2.20–2.45 (m, 3H), 2.59–2.76 (m, 2H), 2.89–3.09 (m, 1H), 3.28–3.40 (m, 1H), 3.69–3.98 (m, 3+1/2H), 4.31–4.44 (m, 1/2H), 11.75–12.82 (br, 1H); MS (APCI) m/z 269 $(M+H-Boc)^+$.

5.1.7. 3-[({(3*R*)-1-[3-(4-Piperidinyl)propanoyl]-3-piperidinyl}carbonyl)amino]propanoic acid hydrochloride (2). To a solution of **10** (1.35 g, 3.66 mmol), β-alanine ethyl ester hydrochloride (**11**) (560 mg, 3.66 mmol), and HOBT (495 mg, 3.66 mmol) in DMF (10 mL) was added EDC (570 mg, 3.66 mmol) under ice cooling. The reaction mixture was stirred at room temperature overnight. To the resulting solution was added saturated $NaHCO_3$ solution, and extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel with $CHCl_3$/MeOH (100/1). The eluate was concentrated in vacuo to give the desired intermediate (1.70 g, 100%) as an oil: IR (neat) 2920, 2850, 1730, 1670, 1620, 1535, 1160, 860 cm^{-1}; ^{1}H NMR ($CDCl_3$, rotomer observed) δ 1.00–1.21 (m, 2H), 1.27 (t, J = 7.1 Hz, 3H), 1.45 (s, 9H), 1.52–1.77 (m, 7H), 1.83–2.09 (m, 2H), 2.17–2.39

(m, 3H), 2.48–2.73 (m, 4H), 3.16–3.68 (m, 4+1/2H), 3.83–3.96 (m, 1/2H), 4.02–4.25 (m, 2+1/2H), 4.16 (q, J = 7.1 Hz, 2H), 4.36–4.49 (m, 1/2H), 6.23–6.36 (m, 1/3H), 6.55–6.66 (m, 2/3H); MS (APCI) m/z 468 $(M+H)^+$. To a solution of this material (900 mg, 1.72 mmol) in THF (8 mL) and EtOH (8 mL) was added $LiOH·H_2O$ (240 mg, 5.77 mmol) solution in H_2O (8 mL) under ice cooling. The reaction mixture was stirred at room temperature for 1 h. The resulting solution was washed with Et_2O. The pH of the aqueous layer was adjusted to 3 with 10% aqueous $KHSO_4$ solution, and extracted with EtOAc. The organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give the carboxylic acid intermediate (850 mg, 100%) as an oil: IR (neat) 1720, 1610, 1430, 1160 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.84–1.09 (m, 2H), 1.32–1.83 (m, 9H), 1.38 (s, 9H), 2.26–2.40 (m, 5H), 2.55–2.75 (m, 3H), 2.84–3.27 (m, 4H), 3.71–3.98 (m, 3H), 4.11–4.38 (m, 1H), 7.90–8.02 (m, 1H); MS (APCI) m/z 440 $(M+H)^+$. To a solution of this carboxylic acid (910 mg, 2.07 mmol) in EtOAc (9 mL) was added 4 N HCl solution in EtOAc (5.2 mL, 21 mmol) under ice cooling. The reaction mixture was stirred at room temperature for 2 h. The resulting precipitate was collected, washed with Et_2O, and dried under vacuum to give **2** (690 mg, 89%) as a white solid: IR (Nujol) 1710, 1620, 1450 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.21–1.65 (m, 7H), 1.62–1.83 (m, 3H), 2.29–2.41 (m, 4H), 2.56–3.07 (m, 4H), 3.15–3.26 (m, 4H), 3.70–3.85 (m, 1H), 4.13–4.37 (m, 4H), 7.97–8.10 (m, 1H), 8.60–8.76 (br, 1H), 8.91–9.03 (br, 1H); HRMS (ESI) m/z calcd for $C_{17}H_{30}N_3O_4$ $(M+H)^+$: 340.2236, found: 340.2227; $[\alpha]_D^{25}$ −24.3 (c 1.0, MeOH).

5.1.8. 2-Phenyl-3-[({(3*R*)-1-[3-(4-piperidinyl)propanoyl]-3-piperidinyl}carbonyl)amino] propanoic acid hydrochloride (13). This compound was prepared from **10** and ethyl 3-amino-2-phenylpropanoate hydrochloride (**12**) using a procedure similar to that employed for the preparation of **2** (100%): IR (Nujol) 1724, 1635, 1616 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.05–1.85 (m, 11H), 2.02–2.60 (m, 4H), 2.60–3.06 (m, 3H), 3.10–3.27 (m, 2H), 3.33–3.68 (m, 3H), 3.70–3.90 (m, 2H), 4.10–4.40 (m, 1H), 7.18–7.40 (m, 5H), 7.95–8.20 (m, 1H), 8.60–9.20 (m, 2H); HRMS (ESI) m/z calcd for $C_{23}H_{34}N_3O_4$ $(M+H)^+$: 416.2549, found: 416.2535.

5.1.9. *tert*-Butyl 4-[3-((3*R*)-3-{[((2*S*)-2-{[(benzyloxy)carbonyl]amino}-3-methoxy-3-oxopropyl)amino]carbonyl}-1-piperidinyl)-3-oxopropyl]-1-piperidinecarboxylate (16). To a solution of **10** (20.0 g, 54.3 mmol), **14** (17.2 g, 59.7 mmol), and HOBT (5.07 g, 59.7 mmol) in DMF (10 mL) was added EDC (10.9 mL, 59.7 mmol) dropwise under ice cooling. The reaction mixture was stirred at 4 °C overnight. To the resulting solution was added water followed by extraction with EtOAc twice. The organic layer was washed with saturated aqueous $NaHCO_3$ solution, water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel with EtOAc/hexane (1:1). The eluate was concentrated in vacuo to give **16** (30.5 g, 93%) as a colorless oil: IR (neat) 3307, 2974, 2933, 2855, 1724, 1689, 1535, 1434 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.95–1.15 (m, 2H), 1.20–1.80 (m, 9H), 1.46 (s, 9H), 2.15–2.50 (m, 4H), 2.56–2.67 (m, 2H), 3.15–3.45 (m, 3H), 3.45–3.75 (m, 2H), 3.69 (s, 3H), 3.95–4.20 (m, 4H), 4.25–4.55 (m, 1H), 5.12 (s, 2H), 6.41 (d, J = 8.8 Hz, 3H), 7.25–7.45 (m, 5H); MS (APCI) m/z 503 $(M+H-Boc)^+$.

5.1.10. *tert*-Butyl 4-[3-((3*R*)-3-{[((2*R*)-2-{[(benzyloxy)carbonyl]amino}-3-methoxy-3-oxopropyl)amino]carbonyl}-1-piperidinyl)-3-oxopropyl]-1-piperidinecarboxylate (17). This compound was prepared from **10** and **15** using a procedure similar to that employed for the preparation of **16** (96%): IR (neat) 3309, 2974, 2935, 2860, 1726, 1689, 1535, 1435 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.80–1.15 (m, 2H), 1.15–1.90 (m, 9H), 1.38 (s, 9H), 2.00–2.80 (m, 6H), 2.80–4.00 (m, 6H), 3.61 (s, 3H), 4.10–4.45 (m, 2H), 5.04 (s, 2H), 7.36 (s, 5H), 7.64 (d, J = 8.1 Hz, 1H), 7.95–8.15 (m, 1H); MS (ESI) m/z 625.3 $(M+Na)^+$.

5.1.11. (2*S*)-2-Amino-3-{[((3*R*)-1-{3-[1-(*tert*-butoxycarbonyl)-4-piperidinyl]propanoyl}-3-piperidinyl)carbonyl]amino}propanoic acid (18). To a solution of **16** (9.70 g, 16.1 mmol) in MeOH (200 mL) was added 10% palladium on charcoal (1.94 g). The reaction mixture was stirred under H_2 atmosphere at room temperature for 3 h. The catalyst was filtered off, and washed with MeOH. The filtrate was concentrated in vacuo. The residue was dissolved with THF (100 mL). To this was added 1 N aqueous LiOH solution (48.3 mL, 48.3 mmol) dropwise under ice cooling, and stirred for further 0.5 h at 5 °C. The pH of the resulting solution was adjusted to 7 with 20% aqueous $KHSO_4$ solution, and evaporated under vacuum to remove THF. The residual aqueous solution was purified by ODS column chromatography with $MeCN/H_2O$ (3:7). The eluate was concentrated in vacuo, and lyophilized to give **18** (7.08 g, 97%) as an amorphous solid: IR (KBr) 1691, 1649, 1427, 1244, 1165 cm^{-1}; ^1H NMR (D_2O) δ 0.90–1.22 (m, 3H), 1.45 (s, 9H), 1.48–1.60 (m, 2H), 1.61–1.80 (m, 4H), 1.80–2.10 (m, 1H), 2.35–2.55 (m, 4H), 2.70–2.88 (m, 2H), 2.95 (dd, J = 13.0, 10.6 Hz, 1H), 3.12–3.38 (m, 1H), 3.62 (dd, J = 14.9, 6.8 Hz, 1H), 3.77 (dd, J = 14.9, 3.4 Hz, 1H), 3.82–4.10 (m, 3H), 3.90 (dd, J = 6.8, 3.4 Hz, 1H), 4.15–4.40 (m, 1H); MS (APCI) m/z 455 $(M+H)^+$.

5.1.12. (2*R*)-2-Amino-3-{[((3*R*)-1-{3-[1-(*tert*-butoxycarbonyl)-4-piperidinyl]propanoyl}-3-piperidinyl)carbonyl]amino}propanoic acid (19). This compound was prepared from **17** using a procedure similar to that employed for the preparation of **18** (95%): ^1H NMR (D_2O) δ 0.90–1.22 (m, 3H), 1.45 (s, 9H), 1.46–1.60 (m, 2H), 1.65–1.85 (m, 4H), 1.85–2.10 (m, 1H), 2.40–2.55 (m, 4H), 2.70–2.90 (m, 2H), 2.98 (dd, J = 12.8, 10.6 Hz, 1H), 3.12–3.35 (m, 1H), 3.53–3.67 (m, 1H), 3.76 (dd, J = 3.7, 2.2 Hz, 1H), 3.82–3.95 (m, 2H), 4.18–4.38 (m, 1H); MS (ESI) m/z 455.3 $(M+H)^+$.

5.1.13. (2*S*)-2-Amino-3-[({(3*R*)-1-[3-(4-piperidinyl)propanoyl]-3-piperidinyl}carbonyl)amino] propanoic acid (20a). To a solution **18** (200 mg, 0.44 mmol) in EtOAc (4 mL) was added 4 N HCl solution in EtOAc (1.10 mL, 4.40 mmol) under ice cooling. The reaction mixture

was stirred at room temperature for 2 h. The resulting precipitate was collected and washed with Et_2O and dried under vacuum to give a crude solid. It was dissolved in water, and the pH of the solution was adjusted to 7.0 with saturated aqueous $NaHCO_3$ solution. The resulting solution was purified by ODS column chromatography with MeCN/H_2O (3:97). The eluate was concentrated in vacuo, and lyophilized to give **20a** (94 mg, 60%) as an amorphous solid: IR (KBr) 1631, 1566, 1444, 1228, 1084 cm^{-1}; ^1H NMR (D_2O) δ 1.26–1.90 (m, 9H), 1.90–2.08 (m, 3H), 2.35–2.58 (m, 3H), 2.75–3.10 (m, 3H), 2.75–3.10 (m, 3H), 3.12–3.60 (m, 6H), 3.78–4.05 (m, 1H) 4.12–4.38 (m, 1H); HRMS (ESI) *m/z* calcd for $C_{17}H_{31}N_4O_4$ $(M+H)^+$: 355.2345, found: 355.2331.

5.1.14. (2*S*)-2-[(4-Cyanobenzoyl)amino]-3-[({(3*R*)-1-[3-(4-piperidinyl)propanoyl]-3-piperidinyl} carbonyl)amino]propanoic acid (20b). To a suspension of **18** (204 mg, 0.45 mmol) in MeCN (5 mL) was added *N*-(trimethylsilyl)acetamide (MSA; 700 mg, 5.3 mmol), and the reaction mixture was stirred at 40 °C for 30 min, and then cooled to 0 °C. To this solution was added *p*-cyanobenzoyl chloride (80 mg, 0.48 mmol) under ice cooling, and the reaction mixture was stirred for a further 0.5 h at room temperature. The pH of the resulting solution was adjusted to 2.5 with 20% aqueous $KHSO_4$ solution, and extracted with EtOAc, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was dissolved in EtOAc (6 mL). To this was added 4 N HCl solution in EtOAc (2.1 mL, 8.4 mmol) under ice cooling. The reaction mixture was stirred at room temperature for 2 h. The resulting precipitate was collected, and washed with Et_2O and dried under vacuum, dissolved in water, and the pH of the solution adjusted to 7 with saturated aqueous $NaHCO_3$ solution. The solution was purified by ODS column chromatography with MeCN/H_2O (1:4). The eluate was concentrated in vacuo, and lyophilized to give **20b** (191 mg, 90%) as an amorphous solid: IR (KBr) 1641, 1630, 1610, 1444, 1390 cm^{-1}; ^1H NMR (D_2O) δ 1.25–1.62 (m, 7H), 1.62–1.82 (m, 2H), 1.82–2.02 (m, 3H), 2.25–2.52 (m, 3H), 2.70–3.12 (m, 3H), 3.23 (dd, J = 13.2, 10.6 Hz, 1H), 3.35–3.48 (m, 2H), 3.56–3.83 (m, 3H), 4.05–4.22 (m, 1H), 4.58–4.78 (m, 1H), 7.92 (d, J = 1.6 Hz, 4H); HRMS (ESI) *m/z* calcd for $C_{25}H_{34}N_5O_5$ $(M+H)^+$: 484.2560, found: 484.2577; Anal. Calcd for $C_{25}H_{33}N_5O_5{\cdot}2H_2O$: C, 57.79; H, 7.17; N, 13.47. Found: C, 57.65; H, 7.21; N, 13.42.

5.1.15. (2*S*)-2-[(4-Methoxybenzoyl)amino]-3-[({(3*R*)-1-[3-(4-piperidinyl)propanoyl]-3-piperidinyl}carbonyl)amino]propanoic acid (20c). This compound was prepared from **18** and *p*-methoxybenzoyl chloride using a procedure similar to that employed for the preparation of **20b** (94%): IR (KBr) 1644, 1640, 1633, 1622, 1608, 1502 1254, 1180, 1022, 850 cm^{-1}; ^1H NMR (D_2O) δ 1.20–1.60 (m, 7H), 1.60–1.75 (m, 2H), 1.75–2.00 (m, 3H), 2.15–2.30 (m, 1H), 2.30–2.50 (m, 2H), 2.65–2.85 (m, 1H), 2.85–3.05 (m, 2H), 3.20 (dd, J = 13.4, 10.4 Hz, 1H), 3.40–3.48 (m, 2H), 3.55–3.85 (m, 3H), 3.90 (s, 3H), 4.05–4.20 (m, 1H), 4.55–4.71 (m, 1H), 7.09 (d, J = 8.8 Hz, 1H), 7.11 (d, J = 9.1 Hz, 1H), 7.79 (d, J = 8.8 Hz, 1H), 7.80 (d, J = 9.1 Hz, 1H); HRMS (ESI) *m/z* calcd for $C_{25}H_{37}N_4O_6$ $(M+H)^+$: 489.2713, found: 489.2713.

5.1.16. (2*R*)-2-[(4-Methoxybenzoyl)amino]-3-[({(3*R*)-1-[3-(4-piperidinyl)propanoyl]-3-piperidinyl}carbonyl)amino]propanoic acid (20d). This compound was prepared from **19** and *p*-methoxybenzoyl chloride using a procedure similar to that employed for the preparation of **20b** (98%): IR (KBr) 1647, 1608, 1254, 1180, 1024, 850 cm^{-1}; ^1H NMR (D_2O) δ 1.25–1.60 (m, 7H), 1.60–1.78 (m, 2H), 1.78–2.00 (m, 3H), 2.27–2.50 (m, 3H), 2.70–3.20 (m, 3H), 3.22 (dd, J = 13.6, 10.3 Hz, 1H), 3.30–3.49 (m, 2H), 3.53–3.88 (m, 2H), 3.91 (s, 3H), 4.05–4.30 (m, 1H), 4.60–4.72 (m, 1H), 7.10 (d, J = 9.0 Hz, 2H), 7.81 (d, J = 9.0 Hz, 2H); HRMS (ESI) *m/z* calcd for $C_{25}H_{37}N_4O_6$ $(M+H)^+$: 489.2713, found: 489.2721.

5.1.17. (2*S*)-2-(Heptanoylamino)-3-[({(3*R*)-1-[3-(4-piperidinyl)propanoyl]-3-piperidinyl}carbonyl)amino]propanoic acid (20f). To a suspension of **18** (200 mg, 0.44 mmol) in THF (4 mL) were added 1 N aqueous NaOH solution (1.45 mL, 1.45 mmol) and heptanoic anhydride (0.26 mL, 0.97 mmol) under ice cooling. After 15 min, the reaction mixture was stirred at room temperature for further 1 h. The resulting mixture was washed with Et_2O, and the pH of the aqueous layer was adjusted to 2.0 with 20% $KHSO_4$ solution, and extracted with a mixed solvent of EtOAc/THF (1:1), dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was dissolved in EtOAc (10 mL). To this was added 4 N HCl solution in EtOAc (1.1 mL, 4.4 mmol) under ice cooling. The reaction mixture was stirred at room temperature for 2 h. The resulting precipitate was collected, and washed with Et_2O, and dried under vacuum, dissolved in water, and the pH of the solution was adjusted to 7 with saturated aqueous $NaHCO_3$ solution. The solution was then purified by ODS column chromatography with MeCN/H_2O (1:3). The eluate was concentrated in vacuo, and lyophilized to give **20f** (184 mg, 90%) as an amorphous solid: IR (KBr) 3430, 3413, 1648, 1456, 1392 cm^{-1}; ^1H NMR (D_2O) δ 0.86 (t, J = 6.5 Hz, 3H), 1.20–1.80 (m, 17H), 1.80–2.05 (m, 3H), 2.29 (t, J = 7.2 Hz, 2H), 2.42–2.55 (m, 2H), 2.73–3.06 (m, 3H), 3.07–3.34 (m, 1H), 3.35–3.50 (m, 3H), 3.66 (ddd, J = 13.8, 4.2, 3.0 Hz, 1H), 3.77–3.98 (m, 1H), 4.12–4.38 (m, 1H), 4.39 (dd, J = 8.9, 4.2 Hz, 1H); HRMS (ESI) *m/z* calcd for $C_{24}H_{43}N_4O_5$ $(M+H)^+$: 467.3233, found: 467.3231.

5.1.18. (2*S*)-2-(Acetylamino)-3-[({(3*R*)-1-[3-(4-piperidinyl)-propanoyl]-3-piperidinyl}carbonyl)amino]propanoic acid (3). This compound was prepared from **18** and acetic anhydride using a procedure similar to that employed for the preparation of **20f** (82%). Purified amorphous product was crystallized from H_2O/EtOH (0.5:99.5): mp >210 °C; IR (KBr) 1633, 1624, 1444, 1396 cm^{-1}; ^1H NMR (D_2O) δ 1.30–2.10 (m, 11H), 2.03 (s, 3H), 2.30–2.60 (m, 3H), 2.70–3.10 (m, 3H), 3.10–3.55 (m, 4H), 3.69 (dt, J = 13.9, 4.2 Hz, 1H), 3.80–4.05 (m, 1H), 4.10–4.35 (m, 1H), 4.38 (dd, J = 8.4, 4.2 Hz, 1H); HRMS (ESI) *m/z* calcd for $C_{19}H_{33}N_4O_5$ $(M+H)^+$: 397.2456, found: 397.2438; Anal. Calcd for $C_{19}H_{32}N_4O_5{\cdot}3H_2O$: C, 50.65; H, 8.50; N, 12.44. Found: C, 50.51; H, 8.54; N, 12.33; $[\alpha]_D^{26}$ −11.8 (*c* 1.00, MeOH).

5.1.19. (2*R*)-2-(Acetylamino)-3-[({(3*R*)-1-[3-(4-piperidinyl)-propanoyl]-3-piperidinyl}carbonyl)amino]propanoic acid (20e). This compound was prepared from **19** and acetic anhydride using a procedure similar to that employed for the preparation of **20f** (85%). Purified amorphous solid was crystallized from H_2O/EtOH (0.5:99.5): mp >124.0–124.5 °C; IR (KBr) 1666, 1627, 1599, 1402, 1144 cm^{-1}; ^1H NMR (D_2O) δ 1.30–2.10 (m, 11H), 2.03 (s, 3H), 2.30–2.65 (m, 3H), 2.80–3.10 (m, 3H), 3.10–3.50 (m, 4H), 3.69 (dt, J = 13.9, 4.2 Hz, 1H), 3.80–4.00 (m, 1H), 4.10–4.35 (m, 1H), 4.38 (dd, J = 8.4, 4.2 Hz, 1H); HRMS (ESI) m/z calcd for C_{19} $H_{33}N_4O_5$ $(M+H)^+$: 397.2456, found: 397.2451; Anal. Calcd for $C_{25}H_{33}N_5O_5{\cdot}3H_2O$: C, 50.65; H, 8.50; N, 12.44. Found: C, 50.88; H, 8.51; N, 12.49; $[\alpha]_D^{26}$ −45.9 (*c* 1.00, MeOH).

5.1.20. (2*S*)-2-[(Cyclohexylcarbonyl)amino]-3-[({(3*R*)-1-[3-(4-piperidinyl)propanoyl]-3-piperidinyl}carbonyl)amino]-propanoic acid (20g). This compound was prepared from **18** and cyclohexanecarbonyl chloride using a procedure similar to that employed for the preparation of **20b** (86%): IR (KBr) 1643, 1637, 1633, 1523, 1469, 1441, 1255, 1225 cm^{-1}; ^1H NMR (D_2O) δ 1.20–1.80 (m, 18H), 1.80–2.05 (m, 3H), 2.15–2.60 (m, 4H), 2.73–3.15 (m, 3H), 3.20–3.35 (m, 1H), 3.35–3.52 (m, 3H), 3.66 (dt, J = 14.0, 3.9 Hz, 1H), 3.78–3.95 (m, 1H), 4.20–4.34 (m, 1H), 4.38 (dd, J = 8.5, 4.4 Hz, 1H); HRMS (ESI) m/z calcd for $C_{24}H_{41}N_4O_5$ $(M+H)^+$: 465.3077, found: 465.3077.

5.1.21. (2*S*)-2-[(2,2-Dimethylpropanoyl)amino]-3-[({(3*R*)-1-[3-(4-piperidinyl)propanoyl]-3-piperidinyl}carbonyl)amino]-propanoic acid (20h). This compound was prepared from **18** and pivaloyl chloride using a procedure similar to that employed for the preparation of **20b** (86%): IR (KBr) 1631, 1541, 1390, 1230 cm^{-1}; ^1H NMR (D_2O) δ 1.19 (s, 9H), 1.33–1.89 (m, 8H), 1.90–2.05 (m, 3H), 2.42–2.56 (m, 3H), 2.83–3.18 (m, 3H), 3.20–3.58 (m, 4H), 3.62–3.74 (m, 1H), 3.80–3.95 (m, 1H), 4.10–4.30 (m, 1H), 4.30–4.38 (m, 1H); HRMS (ESI) m/z calcd for $C_{22}H_{39}N_4O_5$ $(M+H)^+$: 439.2920, found: 439.2942.

5.1.22. (2*R*)-2-[(2,2-Dimethylpropanoyl)amino]-3-[({(3*R*)-1-[3-(4-piperidinyl)propanoyl]-3-piperidinyl}carbonyl)amino]propanoic acid (20i). This compound was prepared from **19** and pivaloyl chloride using a procedure similar to that employed for the preparation of **20b** (79%): IR (KBr) 1631, 1541, 1390, 1228 cm^{-1}; ^1H NMR (D_2O) δ 1.19 (s, 9H), 1.28–1.88 (m, 8H), 1.90–2.05 (m, 3H), 2.30–2.60 (m, 3H), 2.75–3.25 (m, 3H), 3.28–3.49 (m, 2H), 3.53 (dd, J = 8.1, 5.2 Hz, 1H), 3.61 (dd, J = 8.5, 4.0 Hz, 1H), 3.52–3.98 (m, 1H), 4.12–4.28 (m, 1H), 4.30–4.44 (m, 2H); HRMS (ESI) m/z calcd for $C_{22}H_{39}N_4O_5$ $(M+H)^+$: 439.2920, found: 439.2903.

5.2. Platelet aggregation study[21]

Platelet aggregation assays were performed using NBS HEMA TRACER 801, an eight channel aggregometer (Nikobioscience, Tokyo, Japan). Light transmittance of platelet poor plasma (PPP) was calibrated as 100%. Venous blood from healthy male volunteers was collected onto sodium citrate. Platelet rich plasma (PRP) was prepared by rapid centrifugation of whole blood. PRP was incubated for 2 min in the aggregometer at 37 °C. ADP (2.5 μM for humans and 20–40 μM for dogs) was added as an agonist at which the full response of platelet aggregation was obtained, and the change in light transmittance was monitored with a PL500 recorder (Yokogawa, Japan). Percent inhibition of aggregation in the drug-treated sample was calculated by comparison with the aggregation in absence of drug or in the pretreatment period.

5.3. Platelet adhesion to vWF coated plate

Platelets were washed with modified HEPES-Tyrode's buffer (129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 10 mM HEPES, 5.5 mM glucose, 0.1% bovine serum albumin (BSA), pH 7.4) containing 1 μM PGE 1. After washing, platelets were suspended in modified HEPES-Tyrode's buffer containing 1.0 mM $CaCl_2$, and platelet count was adjusted. Adhesion assay protocol was performed as follows. Ninety-six-well microtiter plates were coated with 1 μg/well of von Willebrand factor. The plates were then blocked with 1% BSA. After the plates were washed with buffer, washed platelets, which were activated by 20 μM ADP for 10 min were added to each well in the presence of drugs or buffer, and incubate for 30 min at 37 °C. The plates were then washed three times with buffer. The number of adhered cells was determined by the acid phosphatase activity of cells at 410 nm using a microplate reader.

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