Poststatin, a New Inhibitor of Prolyl Endopeptidase

V. Endopeptidase Inhibitory Activity of Poststatin Analogues

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Thirty analogues of poststatin were synthesized, and their inhibitory activities against prolyl endopeptidase, human leukocyte elastase and cathepsin B were measured. The α-ketone was essential and the *S* configuration was preferable to the *R* configuration in the β-substituted-β-amino-α-oxopropionic acid moiety of poststatin analogues for endopeptidase inhibitory activity. The analogue in which the D-leucine residue of poststatin was replaced by L-leucine showed strong inhibitory activity to cathepsin B. Introduction of an aromatic group into the P_4 position and proline into the P_2 position increased inhibitory activity to elastase. Benzyloxycarbonyl-L-homophenylalanyl-(*RS*)-3-amino-2-oxovaleryl-D-leucyl-L-valine was about 6 times more active to prolyl endopeptidase than natural poststatin.

Poststatin (PST), a new inhibitor of prolyl endopeptidase (PEP), was isolated from a culture filtrate of *Streptomyces viridochromogenes* MH534-30F3.[1] The structure was defined as L-Val-L-Val-3-amino-2-oxovaleryl-D-Leu-L-Val.[2] The absolute configuration of the 3-amino-2-oxovaleric acid (named as postine, abbreviated as Pos) moiety was established to be *S*.[3] Total synthesis of PST was achieved by both conventional liquid phase peptide synthesis and solid phase synthesis using (2*R*,3*S*)-3-amino-2-hydroxyvaleric acid[4]. Because prolyl endopeptidase is a serine endopeptidase, proline-containing chloromethyl ketone derivatives or peptide aldehyde analogues such as benzyloxycarbonyl (abbreviated as Z)-Gly-Pro-CH_2Cl or Z-Pro-prolinal were designed, synthesized, and found to show strong inhibitory activity to this enzyme by YOSHIMOTO in 1977[5] and WILK in 1983[6]. In comparison with these compounds, PST contains a unique amino acid, Pos, and includes neither pyrrolidine nor aldehyde groups in its structure. Moreover, PST has D-Leu-Val at the P_1' and P_2' position in its structure, which could be modified or replaced with another suitable structure for the subsite of individual target endopeptidase.

In this paper, we report information on the relationship between structure and inhibitory activity to three enzymes, PEP, human leukocyte elastase and cathepsin B, as representatives of serine and cysteine proteinases.

Chemistry

Poststatin analogues were prepared by the following methods. Liquid phase method A consists of temporary protection by acid sensitive groups (Boc, Z(OMe)) and final deprotection by hydrogenolysis. Liquid phase method B consists of temporary protection by hydrogenation sensitive groups (Z, Z(OMe)) and final deprotection by acid treatment. In both liquid phase methods oxidation of hydroxyl group to ketone was performed by the Pfitzner-Moffatt method[7]. In the solid phase method, Fmoc-strategy with alkoxybenzylester resin was adopted, and the oxidation was performed by the Albright-Goldman method[8].

Structure-activity Relationship

Structures and inhibitory activities are summarized in Table 1. Replacement of Pos moiety by (*S*)-2-aminobutyric acid (**2**) or (2*RS*,3*S*)-3-amino-2-hydroxyvaleric acid (**3**) decreased the inhibitory activity to three enzymes drastically. The epimer of the Pos moiety (**4**) is only about a sixteenth as active as PST for PEP. Replacement of the ethyl side chain of the postine moiety with Me (**5**), Pr (**6**) or benzyl (**7**) suggests that a Me or Et side chain is preferable for PEP inhibition. The analogues **8** and **9** having Me or Et side chain with Z group at the *N*-terminal showed almost the same inhibitory activity to PEP. These data suggest that α-ketone is essential to inhibitory activity, the *S* configuration of the Pos moiety

Table 1. Relationship between structure and endopeptidase inhibitory activity.

No.	Synthetic method	Structure							IC_{50} (μg/ml)		
		P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	PEP	Elast.	Cat-B
1	Natural		Val-	Val-	(*S*)Pos-	D-Leu-	Val		0.030	110	2.1
2	Liquid A		Val-	Val-	(*S*)But-	D-Leu-	Val		>100	>100	>100
3	Natural*		Val-	Val-	(2*RS*,3*S*)H_2Pos-	D-Leu-	Val		>100	>100	>100
4	Solid		Val-	Val-	(*R*)Pos-	D-Leu-	Val		0.47	>100	36
5	Solid		Val-	Val-	(*RS*)Mepos-	D-Leu-	Val		0.050	>100	9.0
(1)	Natural		Val-	Val-	(*S*)Etpos-	D-Leu-	Val		0.030	110	2.1
6	Solid		Val-	Val-	(*RS*)Prpos-	D-Leu-	Val		0.38	>100	1.4
7	Solid		Val-	Val-	(*S*)Bnpos-	D-Leu-	Val		>100	90	0.50
8	Solid	Z-	Val-	Val-	(*RS*)Mepos-	D-Leu-	Val		0.030	40	4.2
9	Solid	Z-	Val-	Val-	(*RS*)Etpos-	D-Leu-	Val		0.034	5.0	1.1
(1)	Natural		Val-	Val-	(*S*)Pos-	D-Leu-	Val		0.030	110	2.1
10	Solid		Val-	Val-	(*RS*)Pos-	Leu-	Val		12	>100	0.040
11	Solid		Val-	Val-	(*RS*)Pos-	Gly-	Val		1.3	>100	0.030
(9)	Solid	Z-	Val-	Val-	(*RS*)Pos-	D-Leu-	Val		0.034	5.0	1.1
12	Solid	Z-	Val-	Val-	(*RS*)Pos-	Leu-	Val		2.2	4.0	0.030
13	Solid		Val-	Pro-	(*RS*)Pos-	D-Leu-	Val		0.30	115	>100
14	Solid	Bz-	Val-	Val-	(*RS*)Pos-	D-Leu-	Val		0.070	7.5	1.4
(9)	Solid	Z-	Val-	Val-	(*RS*)Pos-	D-Leu-	Val		0.034	5.0	1.1
15	Solid	Z-	Val-	Pro-	(*RS*)Pos-	D-Leu-	Val		0.050	2.5	>100
16	Solid	PB-	Val-	Val-	(*RS*)Pos-	D-Leu-	Val		0.040	1.5	1.1
17	Solid	PB-	Val-	Pro-	(*RS*)Pos-	D-Leu-	Val		0.17	0.90	72
18	Solid		Z-	Val-	(*RS*)Pos-	D-Leu-	Val		0.065	>100	1.4
19	Solid		Z-	Pro-	(*RS*)Pos-	D-Leu-	Val		0.020	>100	120
(18)	Solid		Z-	Val-	(*RS*)Pos-	D-Leu-	Val		0.065	>100	1.4
(19)	Solid		Z-	Pro-	(*RS*)Pos-	D-Leu-	Val		0.020	>100	120
20	Liquid B		Z-	Phg-	(*RS*)Pos-	D-Leu-	Val		0.015	40	24
21	Liquid B		Z-	Phe-	(*RS*)Pos-	D-Leu-	Val		0.0070	40	0.64
22	Liquid B		Z-	Hph-	(*RS*)Pos-	D-Leu-	Val		0.0047	34	4.1
23	Liquid B		Z-	Phe-	(*RS*)Pos-	D-Leu-	Val	-OBu^t	0.11	50	>100
24	Liquid B		Z-	Hph-	(*RS*)Pos-	D-Leu-	Val	-OBu^t	0.32	>100	>100
(1)	Natural		Val-	Val-	(*S*)Pos-	D-Leu-	Val		0.030	110	2.1
25	Solid		Val-	Val-	(*RS*)Pos-	D-Leu			1.4	>100	3.7
(9)	Solid	Z-	Val-	Val-	(*RS*)Pos-	D-Leu-	Val		0.034	5.0	1.1
26	Solid	Z-	Val-	Val-	(*RS*)Pos-	D-Leu			0.37	7.0	1.5
(21)	Liquid B		Z-	Phe-	(*RS*)Pos-	D-Leu-	Val		0.0070	40	0.64
27	Liquid B		Z-	Phe-	(*RS*)Pos-	D-Leu			0.12	>100	0.47
28	Liquid B		Z-	Phe-	(*RS*)Pos-	D-Leu-	OBu^t		0.031	11	0.48
29	Liquid B		Z-	D-Phe-	(*RS*)Pos-	D-Leu-	Val		0.038	>100	11
30	Liquid B		Z-	D-Phe-	(*RS*)Pos-	D-Leu			0.80	>100	6.2
31	Liquid B		Z-	D-Phe-	(*RS*)Pos-	D-Leu-	OBu^t		0.19	30	>100

Abbreviations; PEP: Prolyl endopeptidase, Elast.: Elastase (Human leukocyte), Cat-B: Cathepsin B, Liquid A, Liquid B and Solid: Typical procedures are shown in experimental section, Natural*: Derived from natural product, Pos: postine (3-amino-2-oxovaleric acid), But: 2-aminobutyric acid, H_2Pos: dihydropostine (3-amino-2-hydroxyvaleric acid), pos: $-CH(NH_2)COCOOH$, PB: 4-phenylbutyryl, Bn: benzyl, Phg: phenylglycine, Hph: homophenylalanine.

is important and a Me or Et side chain in the postine moiety is preferable for anti-PEP activity.

The presence of a D-amino acid just after the postine moiety is significant. Analogues **10**, **11** and **12**, in which D-Leu is replaced by L-Leu or Gly, showed weak inhibitory activity to PEP but strong inhibitory activity to cathepsin B. Therefore, the D configuration is essential for good anti-PEP activity and the L configuration or Gly is preferable for anti-cathepsin B activity.

Although PST shows weak inhibition of elastase, the resemblance of postine to alanine suggests that some analogues of PST might show strong inhibition to the enzyme. Analogues **14**, **9** and **16**, in which a benzoyl, Z or phenylbutyryl group is introduced at the *N*-terminal of PST respectively, showed increased anti-elastase activity. Derivatives **15** and **17**, analogues of **9** and **16** in which P_2 is replaced by Pro, also showed increased anti-elastase activity. On the other hand the analogue **13**, in which the P_2 Val in PST is replaced by Pro showed no significant anti-elastase activity. Analogues **18** and **19**, in which the P_3 Val of PST and analogue **13** are replaced by a Z group, are also inactive to elastase.

Therefore, introducing an aromatic group into P_4 increases anti-elastase activity, and a Pro at P_2 is preferable for elastase inhibition.

Deletion of an amino acid residue at the P_3 position of PST resulted in cyclization between the ketone moiety and the free amino group at the P_2 position followed by spontaneous oxidation to an inactive heterocycle. Thus the presence of some residue at the *N*-terminal of P_2 is necessary, but it is not necessary for it to be an amino acid. Analogues **18**, **19**, **20**, **21** and **22** are active derivatives in which the *N*-terminal of P_2 is blocked by Z groups and P_2 is Val, Pro, phenylglycine (abbreviated as Phg), Phe and homophenylalanine (abbreviated as Hph), respectively. Among them, analogue **22** showed about 6 times more activity to PEP than natural PST.

Analogues **23** and **24** are esters of **21** and **22** and showed diminished activities to PEP and cathepsin B. Therefore modification of the *C*-terminal of P_2' should not be effective for increasing PEP and cathepsin B inhibition. Analogues **29**, **30** and **31**, in which P_2 of analogues **21**, **27** and **28** is replaced by D-Phe, showed weaker activity than that of the Phe analogues. Thus, the configuration of P_2 should be *S*. Deletion of the P_2' Val of PST and analogues **9**, **21** and **29** (**25**, **26**, **27** and **30**) decreased the inhibitory activity to PEP significantly. But esterification of the less active analogue **27** and **30** (**28** and **31**) increased the inhibitory activity to PEP. Therefore, the presence of some residue at the P_2' position is preferable, but it is not necessary that it be an amino acid.

Conclusion

The β-substituted-β-amino-α-oxo-acid moiety of poststatin is essential for the inhibition of serine and cysteine proteinases. Size and stereochemistry of β-substituent is also important for these activities. By comparison of the inhibitory activities to three enzyme, it was demonstrated that the selectivity and magnitude of the inhibitory activity can be modulated by replacement of the β-alkylsubstituent of postine and the P_3-P_2 and P_1'-P_2' amino acids.

Experimental

General Method

Melting points were determined on a micro melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. ^{1}H NMR spectra were recorded at 400 MHz or 270 MHz with a JEOL JNM-GX400 or a JNM-EX270 spectrometer respectively. SI-MS or FAB-MS spectra were measured on a Hitachi M-80H or a JEOL JMS-SX102 mass spectrometer respectively. TLC was carried out on Merck precoated silica gel $60F_{254}$ plates, or precoated RP-$18F_{254}$ plates.

The centrifugal partition chromatography system consisted of a Sanki Engineering Ltd. Model NMF centrifuge operated at 1000 rpm, Model LBP-V pump at flow rate of 2.4 ml/minute, Model UVIS 200 detector operating at 254 nm and Model FCU-V injector. The two phase solvent system composed of 1-BuOH - AcOH - H_2O (500 : 3 : 500) was equilibrated in a separatory funnel at room temperature and the layers separated before use. The upper layer was used as stationary phase and the lower layer was used as mobile phase in descending mode.

Abbreviations used in the following section were defined in the above section and Table 1.

Synthesis

β-Substituted-β-amino-α-hydroxypropionic Acid

3-Amino-2-hydroxybutyric acid (**32**) and 3-amino-2-hydroxyhexanoic acid (**33**) were obtained by an analogous procedure to that described for the preparation of 3-amino-2-hydroxyvaleric acid (H_2Pos).[3]

32: FAB-MS *m/z* 120 $(M+H)^+$; ^{1}H NMR (400 MHz, CD_3COOD) δ 1.34, 1.40 (1.5H, 1.5H, two d, each $J=6.8$ Hz, CH_3), 3.78~3.96 (1H, m, 3-CH), 4.32, 4.50 (0.5H, 0.5H, two d, $J=2.9$ Hz and $J=3.9$ Hz, 2-CH).

33: FAB-MS *m/z* 148 $(M+H)^+$; ^{1}H NMR (400 MHz, CD_3COOD) δ 0.94, 0.96 (1.5H, 1.5H, two t, each $J=7.3$ Hz, CH_3), 1.48 (2H, m, 5-CH_2), 1.70, 1.81 (1H, 1H, two m, 4-CH_2), 3.67, 3.79 (0.5H, 0.5H, two br t, 3-CH), 4.37, 4.47 (0.5H, 0.5H, two br s, 2-CH).

(2*R*,3*S*)-3-Amino-2-hydroxy-4-phenylbutyric acid was synthesized according to the procedure reported by NISHIZAWA *et al.*[9]

9-Fluorenylmethyloxycarbonylation of β-Substituted-β-amino-α-hydroxypropionic Acid

9-Fluorenylmethyloxycarbonyl (abbreviated as Fmoc) amino-2-hydroxybutyric acid, 3-(Fmoc)amino-2-hydroxyhexanoic acid, and 3-(Fmoc)amino-2-hydroxy-4-phenylbutyric acid were synthesized as reported previously.[4]

Typical Solid Phase Method

Fmoc-Val-resin (0.5 g, 0.28 mmol, Kokusan Chemical Works, Ltd.) was placed in the peptide synthesis flask and the solid phase synthesis was carried out with 6.5 ml portions of solvents. A cycle for the incorporation of an amino acid residue into the growing peptide chain was according to the procedure reported by FUJI *et al.*[10] In each cycle, three equivalent of amino acid derivative, three equivalent of 1-hydroxybenzotriazole (abbreviated as HOBt) and three equivalent of diisopropylcarbodiimide were used. To the resultant Boc- or Z-penta (or tetra)peptide resin was added acetic anhydride (0.4 ml) and pyridinium trifluoroacetate (106 mg) in DMSO (6 ml), and the mixture was stirred overnight. The oxidized peptide resin was washed with DMSO (6.5 ml),

thrice with DMF (each 6.5 ml), and thrice with MeOH (each 6.5 ml) and dried over P_2O_5. To liberate the peptide from the resin, the resin was stirred in TFA (6.5 ml) containing 5% (w/v) phenol for 1 hour, and the resin particles removed by filtration. This operation was carried out again, and the resin was washed thrice with TFA (6.5 ml). The combined filtrates and washings were concentrated and gel chromatographed on a column of Sephadex LH-20 with MeOH elution. Evaporation of the active eluate gave the peptide as a slightly colored solid. The product was purified by centrifugal partition chromatography with a two phase solvent system as described above to give corresponding peptide or Z-peptide.

Analogues **4**~**13**, **15**, **18** and **19** were prepared in this way. Analogues **25** and **26** were prepared in a similar manner, using Fmoc-D-Leu-resin instead of Fmoc-Val-resin as a starting material.

4: ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.78~0.99 (27H, m, $CH_3\times9$), 1.43~1.67 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.75 (1H, m, CHa*Hb*(Pos)), 1.83~2.12 (3H, m, β-CH×3(Val)), 4.12 (1H, dd, J=5.9, 8.8 Hz, α-CH(Val)), 4.35 (1H, br dd, α-CH(Val)), 4.52 (1H, m, α-CH(Leu)), 4.97 (1H, ddd, J=4.9, 6.8, 8.3 Hz, CH(Pos)), 8.13 (2H, d, J=8.8 Hz, NH×2(Val)), 8.37 (1H, d, J=6.8 Hz, NH(Pos)), 8.43 (1H, d, J=8.8 Hz, NH(Leu)). The signal related to the α-proton of the *N*-terminal Val overlapped with water in DMSO-d_6.

5: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.79~1.08 (24H, m, $CH_3\times8$), 1.33, 1.34 (1.5H, 1.5H, two d, each J=7.3 Hz, CH_3(Mepos)), 1.52~1.79 (3H, m, β-CH_2 (Leu), γ-CH(Leu)), 2.04 (1H, m, β-CH(Val)), 2.18 (2H, m, β-CH×2(Val)), 3.95, 3.97 (0.5H, 0.5H, two d, J=4.9 Hz, 6.8 Hz, α-CH(Val)), 4.36, 4.39 (0.5H, 0.5H, two d, each J=7.8 Hz, α-CH(Val)), 4.39, 4.40 (0.5H, 0.5H, two d, each J=5.4 Hz, α-CH(Val)), 4.61 (1H, m, α-CH(Leu)), 5.17, 5.20 (0.5H, 0.5H, two q, each J=7.3 Hz, CH(Mepos)).

6: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.80~1.07 (27H, m, $CH_3\times9$), 1.37 (2H, m, CH_2(Prpos)), 1.45~1.87 (5H, m, β-CH_2(Leu), γ-CH(Leu), CH_2(Prpos)), 2.03 (1H, m, β-CH(Val)), 2.18 (2H, m, β-CH×2(Val)), 3.93, 3.94 (0.5H, 0.5H, two d, each J=4.9 Hz, α-CH(Val)), 4.40 (2H, m, α-CH×2(Val)), 4.62 (1H, m, α-CH(Leu)), 5.20, 5.25 (0.5H, 0.5H, two br dd, CH(Prpos)).

7: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.76~1.05 (24H, m, $CH_3\times8$), 1.53~1.78 (3H, m, β-CH_2(Leu), γ-CH(Leu)), *ca.* 1.98 (1H, m, obscured by solvent, β-CH(Val)), 2.10 (1H, m, β-CH(Val)), 2.18 (1H, m, β-CH(Val)), 2.87 (1H, dd, J=8.8, 13.9 Hz, PhC*Ha*Hb (Bnpos)), 3.23 (1H, dd, J=4.4, 13.9 Hz, PhCHa*Hb*(Bnpos)), 3.87 (1H, d, J=4.9Hz, α-CH(Val)), 4.36 (1H, d, overlapping, α-CH(Val)), 4.38 (1H, d, J=5.4 Hz, α-CH(Val)), 4.61 (1H, br dd, α-CH(Leu)), 5.50 (1H, m, CH(Bnpos)), 7.11~7.31 (5H, m, Ph).

8: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.79~1.02 (24H, m, $CH_3\times8$), 1.30 (3H, br t, CH_3 (Mepos)), 1.52~1.77 (3H, m, β-CH_2(Leu), γ-CH(Leu)), 1.95~2.10 (2H, m, β-CH×2(Val)), 2.17 (1H, m, β-CH(Val)), 4.04 (1H, m, α-CH(Val)), 4.28~4.40 (2H, m, α-CH×2(Val)), 4.57 (1H, m, α-CH(Leu)), 5.07 (2H, br s, CH_2OCO), 5.12 (1H, m, CH(Mepos)), 7.26~7.40 (5H, m, Ph).

9: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.75~1.05 (27H, m, $CH_3\times9$), 1.50~1.78 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.82~2.10 (3H, m, CHa*Hb*(Pos), β-CH×2(Val)), 2.18 (1H, m, β-CH(Val)), 4.04 (1H, d, J=6.8 Hz, α-CH(Val)), 4.38 (2H, m, α-CH×2(Val)), 4.59 (1H, m, α-CH(Leu)), 5.08 (2H, br s, CH_2OCO), 5.11 (1H, m, CH(Pos)), 7.26~7.41 (5H, m, Ph).

10: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.84~1.07 (27H, m, $CH_3\times9$), 1.55~1.77 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.84~2.11 (2H, m, CHa*Hb*(Pos), β-CH(Val)), 2.19 (2H, m, β-CH×2(Val)), 3.94, 3.96 (0.5H, 0.5H, two d, J=5.4, 4.9 Hz, α-CH(Val)), 4.40, 4.41 (0.5H, 0.5H, two d, J=5.4, 4.9 Hz, α-CH(Val)), 4.44, 4.45 (0.5H, 0.5H, two d, J=6.8, 7.3 Hz, α-CH(Val)), 4.58, 4.63 (0.5H, 0.5H, two dd, J=5.1, 9.5 Hz and J=4.6, 9.5 Hz, α-CH(Leu)), 5.12, 5.15 (0.5H, 0.5H, two t, each J=4.9 Hz, CH(Pos)).

11: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.85~1.10 (21H, m, $CH_3\times7$), 1.51, 1.65 (0.5H, 0.5H, two m, C*Ha*Hb(Pos)), 1.90~2.12 (2H, m, CHa*Hb*(Pos), β-CH(Val)), 2.13~2.30 (2H, m, β-CH×2(Val)), 3.95~4.13 (3H, m, α-CH(Val), CH_2(Gly)), 4.33~4.49 (2H, m, α-CH×2(Val)), 5.07, 5.11 (0.5H, 0.5H, two br dd, CH(Pos)).

12: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.76~1.04 (27H, m, $CH_3\times9$), 1.50~1.77 (5H, m, β-CH_2(Leu), γ-CH(Leu), CH_2(Pos)), 1.80~2.25 (3H, m, β-CH×3(Val)), 4.02 (1H, m, α-CH(Val)), 4.33 (2H, m, α-CH×2(Val)), 4.47, 4.54 (0.5H, 0.5H, two m, α-CH(Leu)), 5.00~5.15 (3H, m, CH(Pos), CH_2OCO), 7.24~7.42 (5H, m, Ph).

13: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.75~1.15 (21H, m, $CH_3\times7$), 1.51~1.83 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.85~2.35 (7H, m, CHa*Hb*(Pos), β-CH_2(Pro), γ-CH_2(Pro), β-CH×2(Val)), 3.50, 3.56 (0.5H, 0.5H, two m, δ-C*Ha*Hb(Pro)), 3.73 (1H, m, δ-CHa*Hb*(Pro), 4.16 (1H, br d, α-CH(Val)), 4.35, 4.39 (0.5H, 0.5H, two br d, α-CH(Val)), 4.57 (1H, m, α-CH(Pro)), 4.72 (1H, m, α-CH(Leu)), 5.08, 5.17 (0.5H, 0.5H, two m, CH(Pos)).

15: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.80~1.06 (21H, m, $CH_3\times7$), 1.50~1.78 (4H, m, β-CH_2 (Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.79~2.29 (7H, m, CHa*Hb*(Pos), β-CH_2(Pro), γ-CH_2(Pro), β-CH×2(Val)), 3.67, 3.80 (1H, 1H, two m, δ-CH_2(Pro)), 4.33, 4.34 (0.5H, 0.5H, two d, each J=7.3 Hz, α-CH(Val)), 4.39 (1H, d, J=5.4 Hz, α-CH(Val)), *ca.* 4.39~4.55 (1H, m, overlapping, α-CH(Pro)), 4.60 (1H, m, α-CH(Leu)), *ca.* 5.04 (1H, m, overlapping, CH(Pos)), 5.06, 5.10 (2H, ABq, J=12.7 Hz, CH_2OCO), 7.26~7.42 (5H, m, Ph).

18: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ

Table 2-1. Physico-chemical data on analogues.

No.[a]	Rf[b]	Formula	MW	Method[c]	$(M+H)^+$	Fragment Ion[d]
1	0.59	$C_{26}H_{47}N_5O_7$	541.69	FAB (+)	542	498 (M−CO_2+H), 443 (M−(H-Val)+2H), 397 (M−(CO-Val-OH)), 344 (M−(H-Val-Val)+2H), 231 ((Leu-Val-OH)+2H), 199 (H-Val-Val), 171 (M−(CO-Pos-Leu-Val-OH)), 118 ((Val-OH)+2H), 72 (M−(CO-Val-Pos-Leu-Val-OH))
2	0.55	$C_{25}H_{47}N_5O_6$	513.68	SI-MS	514	369 (M−(CO-Val-OH)), 316 (M−(H-Val-Val)+2H), 284 (M−(Leu-Val-OH)), 231 (M−(H-Val-Val-But)+2H), 199 (M−(But-Leu-Val-OH)), 171 (M−(CO-But-Leu-Val-OH))
4	0.55	$C_{26}H_{47}N_5O_7$	541.69	FAB (+)	542	498 (M−CO_2+H), 443 (M−(H-Val)+2H), 344 (M−(H-Val-Val)+2H), 231 ((Leu-Val-OH)+2H), 199 (H-Val-Val), 171 (M−(CO-Pos-Leu-Val-OH)), 72 (M−(CO-Val-Pos-Leu-Val-OH))
5	0.51 0.55[e]	$C_{25}H_{45}N_5O_7$	527.66	FAB (+)	528	429 (M−(H-Val)+2H), 330 (M−(H-Val-Val)+2H), 231 ((Leu-Val-OH)+2H), 199 (H-Val-Val), 171 (M−(CO-Mepos-Leu-Val-OH)), 118 ((Val-OH)+2H), 72 (M−(CO-Val-Mepos-Leu-Val-OH))
6	0.52 0.55[e]	$C_{27}H_{49}N_5O_7$	555.71	FAB (+)	556	457 (M−(H-Val)+2H), 358 (M−(H-Val-Val)+2H), 231 ((Leu-Val-OH)+2H), 216 ((H-Val-Val-NH)+2H), 199 (H-Val-Val), 171 (M−(CO-Prpos-Leu-Val-OH)), 118 ((Val-OH)+2H), 72 (M−(CO-Val-Prpos-Leu-Val-OH))
7	0.47	$C_{31}H_{49}N_5O_7$	603.76	FAB (+)	604	505 (M−(H-Val)+2H), 406 (M−(H-Val-Val)+2H), 231 ((Leu-Val-OH)+2H), 199 (H-Val-Val), 171 (M−(CO-Bnpos-Leu-Val-OH)), 72 (M−(CO-Val-Bnpos-Leu-Val-OH))
8	0.39	$C_{33}H_{51}N_5O_9$	661.79	FAB (+)	662	618 (M−CO_2+H), 528 (M−Z+2H), 429 (M−(Z-Val)+2H), 333 (Z-Val-Val), 330 (M−(Z-Val-Val)+2H), 234 (Z-Val), 231 ((Leu-Val-OH)+2H), 118 ((Val-OH)+2H), 91 (C_7H_7)
9	0.32	$C_{34}H_{53}N_5O_9$	675.82	FAB (+)	676	632 (M−CO_2+H), 542 (M−Z+2H), 443 (M−(Z-Val)+2H), 344 (M−(Z-Val-Val)+2H), 333 (Z-Val-Val), 234 (Z-Val), 231 ((Leu-Val-OH)+2H), 91 (C_7H_7)
10	0.52 0.55[e]	$C_{26}H_{47}N_5O_7$	541.69	FAB (+)	542	498 (M−CO_2+H), 443 (M−(H-Val)+2H), 397 (M−(CO-Val-OH)), 344 (M−(H-Val-Val)+2H), 257 (CO-Leu-Val-OH), 231 ((Leu-Val-OH)+2H), 199 (H-Val-Val), 171 (M−(CO-Pos-Leu-Val-OH)), 118 ((Val-OH)+2H), 72 (M−(CO-Val-Pos-Leu-Val-OH))
11	0.61 0.65[e]	$C_{22}H_{39}N_5O_7$	485.58	FAB (+)	486	387 (M−(H-Val)+2H), 288 (M−(H-Val-Val)+2H), 199 (H-Val-Val), 171 (M−(CO-Pos-Gly-Val-OH)), 72 (M−(CO-Val-Pos-Gly-Val-OH))
12	0.34	$C_{34}H_{53}N_5O_9$	675.82	FAB (+)	676	632 (M−CO_2+H), 542 (M−Z+2H), 443 (M−(Z-Val)+2H), 344 (M−(Z-Val-Val)+2H), 333 (Z-Val-Val), 234 (Z-Val), 231 ((Leu-Val-OH)+2H), 91 (C_7H_7)
13	0.49 0.53[e]	$C_{26}H_{45}N_5O_7$	539.67	FAB (+)	540	441 (M−(H-Val)+2H), 344 (M−(H-Val-Pro)+2H), 70 (pyrrolidinyl)
14	0.45	$C_{33}H_{51}N_5O_8$	645.79	FAB (+)	646	443 (M−(Bz-Val)+2H), 344 (M−(Bz-Val-Val)+2H), 303 (Bz-Val-Val), 231 ((Leu-Val-OH)+2H), 204 (Bz-Val), 176 (M−(CO-Val-Pos-Leu-Val-OH)), 105 (Bz), 91 (C_7H_7)
15	0.37	$C_{34}H_{51}N_5O_9$	673.81	FAB (+)	674	630 (M−CO_2+H), 540 (M−Z+2H), 441 (M−(Z-Val)+2H), 331 (Z-Val-Pro), 234 (Z-Val), 231 ((Leu-Val-OH)+2H), 91 (C_7H_7), 70 (pyrrolidinyl)
16	0.36	$C_{36}H_{57}N_5O_8$	687.88	FAB (+)	688	443 (M−(PB-Val)+2H), 344 (M−(PB-Val-Val)+2H), 345 (PB-Val-Val), 246 (PB-Val), 231 ((Leu-Val-OH)+2H), 147 (PB)
17	0.36	$C_{36}H_{55}N_5O_8$	685.86	FAB (+)	686	441 (M−(PB-Val)+2H), 343 (PB-Pro-Val), 246 (PB-Val), 147 (PB), 91 (C_7H_7), 70 (pyrrolidinyl)
18	0.46	$C_{29}H_{44}N_4O_8$	576.69	FAB (+)	577	533 (M−CO_2+H), 443 (M−Z+2H), 344 (M−(Z-Val)+2H), 234 (Z-Val), 231 ((Leu-Val-OH)+2H), 118 ((Val-OH)+2H), 91 (C_7H_7)
19	0.45	$C_{29}H_{42}N_4O_8$	574.67	FAB (−)	573[f]	465 (M−(BzO)−2H), 439 (M−Z), 368 ((CO-Pos-Leu-Val-OH)−2H), 255 ((CO-Leu-Val-OH)−2H), 229 (Leu-Val-OH), 116 (Val-OH)
20	0.46 0.52[e]	$C_{32}H_{42}N_4O_8$	610.71	FAB (+)	611	567 (M−CO_2+H), 477 (M−Z+2H), 344 (M−(Z-Phg)+2H), 231 ((Leu-Val-OH)+2H), 118 ((Val-OH)+2H), 91 (C_7H_7)
21	0.42 0.47[e]	$C_{33}H_{44}N_4O_8$	624.73	SI-MS	625	581 (M−CO_2+H), 491 (M−Z+2H), 344 (M−(Z-Phe)+2H), 282 (Z-Phe), 231 ((Leu-Val-OH)+2H), 91 (C_7H_7)
22	0.43 0.50[e]	$C_{34}H_{46}N_4O_8$	638.76	FAB (+)	639	595 (M−CO_2+H), 505 (M−Z+2H), 344 (M−(Z-Hph)+2H), 231 ((Leu-Val-OH)+2H), 118 ((Val-OH)+2H), 91 (C_7H_7)
23	0.30	$C_{37}H_{52}N_4O_8$	680.84	SI-MS	681	625 (M−*i*Bu+H), 581 (M−*i*Bu−CO_2+H), 547 (M−Z+2H), 491 (M−*i*Bu−Z+2H), 344 (M−*i*Bu−(Z-Phe)+2H), 231 (M−*i*Bu−(Z-Phe-Pos)+2H)
24	0.41	$C_{38}H_{54}N_4O_8$	694.87	FAB (+)	695	639 (M−*i*Bu+H), 561 (M−Z+2H), 505 (M−*i*Bu−Z+2H), 344 (M−*i*Bu−(Z-Hph)+2H), 231 (M−*i*Bu−(Z-Hph-Pos)+2H), 118 (M−*i*Bu−(Z-Hph-Pos-Leu)+2H), 91 (C_7H_7)
25	0.58 0.64[e]	$C_{21}H_{38}N_4O_6$	442.55	FAB (+)	443	344 (M−(H-Val)+2H), 245 (M−(H-Val-Val)+2H), 199 (H-Val-Val), 171 (M−(CO-Pos-Leu-OH)), 72 (M−(CO-Val-Pos-Leu-Val-OH)), 245 (M−*i*Bu−(Z-Phe)+2H), 132 (M−*i*Bu−(Z-Phe-Pos)+2H), 91 (C_7H_7), 72 (M−(CO-Val-Pos-Leu-Val-OH))

Table 2-2. Physico-chemical data on analogues.

No.[a]	Rf[b]	Formula	MW	Method[c]	$(M+H)^+$	Fragment Ion[d]
26	0.35	$C_{29}H_{44}N_4O_8$	576.69	FAB (+)	577	533 (M−CO_2+H), 443 (M−Z+2H), 344 (M−(Z-Val)+2H), 333 (Z-Val-Val), 245 (M−(Z-Val-Val)+2H), 91 (C_7H_7)
27	0.44	$C_{28}H_{35}N_3O_7$	525.6	FAB (+)	526	482 (M−CO_2+H), 392 (M−Z+2H), 282 (Z-Phe), 245 (M−(Z-Phe)+2H), 91 (C_7H_7)
28	0.33	$C_{32}H_{43}N_3O_7$	581.71	FAB (+)	582	526 (M−*i*Bu+H), 482 (M−*i*Bu−CO_2+H), 448 (M−Z+2H), 392 (M−*i*Bu−Z+2H), 245 (M−*i*Bu−(Z-Phe)+2H), 132 (M−*i*Bu−(Z-Phe-Pos)+2H), 91 (C_7H_7)
29	0.48	$C_{33}H_{44}N_4O_8$	624.73	FAB (+)	625	581 (M−CO_2+H), 491 (M−Z+2H), 344 (M−(Z-Phe)+2H), 282 (Z-Phe), 231 ((Leu-Val-OH)+2H), 91 (C_7H_7)
30	0.52	$C_{28}H_{35}N_3O_7$	525.60	FAB (+)	526	482 (M−CO_2+H), 392 (M−Z+2H), 282 (Z-Phe), 245 (M−(Z-Phe)+2H), 91 (C_7H_7)
31	0.32 0.48[e]	$C_{32}H_{43}N_3O_7$	581.71	FAB (+)	582	526 (M−*i*Bu+H), 482 (M−*i*Bu−CO_2+H), 448 (M−Z+2H), 392 (M−*i*Bu−Z+2H), 245 (M−*i*Bu−(Z-Phe)+2H), 132 (M−*i*Bu−(Z-Phe-Pos)+2H), 91 (C_7H_7)

[a] The numbering of analogues is defined in Table 1. [b] TLC was carried out on Merck precoated RP-18F_{254} plates using a solvent system of 5% AcOK containing 1% citric acid : MeCN (3 : 2) for analogues **1, 2, 4** and **25**, and using a solvent system of 5% AcOK containing 1% citric acid : MeCN (13 : 7) for analogues **5**～**19**. TLC was carried out on Merck precoated silica gel 60F_{254} plates using a solvent system of $CHCl_3$: MeOH : AcOH (93 : 7 : 2) for analogues **20**～**22**, **26**, **27**, **29** and **30**, and using a solvent system of CH_2Cl_2 : MeOH (20 : 1) for analogues **23**, **24**, **28** and **31**. [c] FAB-MS; xenon gas, acceleration voltage, 10 kV. SI-MS; xenon gas, acceleration voltage, 3 kV. Analogues **25** and **26** were measured in a 3-nitrobenzyl alcohol matrix, and all the other analogues were measured in a glycerol matrix. [d] *i*Bu stands for isobutylene. Other abbreviations are defined in Table 1. [e] Diastereomer could be separated. [f] This peak is $[M-H]^-$, and the fragment ions in analogue **19** are also shown as negative ions.

0.75～1.05 (21H, m, $CH_3\times7$), 1.48～1.78 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.81～2.12 (2H, m, CHa*Hb*(Pos), β-CH(Val)), 2.18 (1H, m, β-CH(Val)), 4.10 (1H, m, α-CH(Val)), 4.37, 4.38 (0.5H, 0.5H, two d, each J=5.4 Hz, α-CH(Val)), 4.59 (1H, m, α-CH(Leu)), 5.00～5.18 (3H, m, CH_2OCO, CH(Pos)), 7.23～7.44 (5H, m, Ph).

19: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.76～1.00 (15H, m, $CH_3\times5$), 1.46～2.25 (10H, m, β-CH_2(Leu), γ-CH(Leu), CH_2(Pos), β-CH_2(Pro), γ-CH_2(Pro), β-CH(Val)), 3.43, 3.50 (2H, two m, δ-CH_2(Pro)), 4.14～4.37 (2H, m, α-CH×2 (Val, Pro)), 4.49 (1H, m, α-CH(Leu)), 4.90～5.17 (3H, m, CH(Pos), CH_2OCO), 7.22～7.45 (5H, m, Ph).

25: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.80～1.08 (21H, m, $CH_3\times7$), 1.52～1.79 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.81～2.12 (2H, m, β-CH(Val), CHa*Hb*(Pos)), 2.18 (1H, m, β-CH(Val)), 3.91 (1H, d, J=5.4 Hz, α-CH(Val)), 4.36 (1H, d, J=6.8 Hz, α-CH(Val)), 4.43, 4.46 (0.5H, 0.5H, two t, each J=4.6 Hz, α-CH(Leu)), 5.10, 5.12 (0.5H, 0.5H, two dd, J=4.4, 7.8 Hz and 4.4, 8.3 Hz, CH(Pos)).

26: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.80～1.00 (21H, m, $CH_3\times7$), 1.54～1.77 (4H, m, β-CH_2 (Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.80～2.14 (3H, m, β-CH×2(Val), CHa*Hb*(Pos)), 4.01, 4.02 (0.5H, 0.5H, two d, each J=6.8 Hz, α-CH(Val)), 4.32 (1H, d, J=6.8 Hz, α-CH(Val)), 4.44 (1H, m, α-CH(Leu)), 5.02～5.16 (3H, m, CH_2OCO, CH(Pos)), 7.27～7.40 (5H, m, Ph).

MS data and Rf values for these synthetic compounds and the following analogues of PST synthesized by the liquid phase method are shown in Table 2.

Typical Liquid Phase Method (Liquid B)

Z-D-Leu-L-Val-OBu^t (**34**)

To an ice-cold solution of Z-D-leucine (1.000 g, 3.77 mmol), L-valine *t*-butyl ester hydrochloride (0.719 g, 3.43 mmol) and HOBt (0.927 g, 6.86 mmol) in CH_2Cl_2 (17 ml) was added triethylamine (0.530 ml, 3.79 mmol) and 97% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (abbreviated as EDC) perchlorate (1.265 g, 4.80 mmol), and the resulting solution was chilled in an ice bath for 2 hours. Stirring was continued for 15 hours at room temperature. The solution was washed with 4% aq $NaHCO_3$, water, 1% aq citric acid and water (each 7 ml) and dried (Na_2SO_4). After removal of the solvent, the product was purified by silica gel column chromatography with CH_2Cl_2-MeOH (400 : 3) to give **34** as crystals, 1.101 g (87.9%): mp 85～86°C; $[\alpha]_D^{28}$ +31.1° (*c* 1.1, $CHCl_3$); FAB-MS *m/z* 421 $(M+H)^+$, 365, 321, 287, 231, 118, 91, 57; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87, 0.91 (3H, 3H, d, d, J=6.8 Hz, CH_3(Val)), 0.95 (6H, d, J=6.4 Hz, CH_3(Leu)), 1.46 (9H, s, Bu^t), 1.53 (1H, ddd, J=4.8, 4.8, 4.8Hz, β-C*Ha*Hb(Leu)), 1.59～1.79 (2H, m, β-CHa*Hb*(Leu), γ-CH(Leu)), 2.15 (1H, m, β-CH(Val)), 4.24 (1H, br, α-CH(Leu)), 4.41 (1H, dd, J=8.8, 4.4 Hz, α-CH(Val)), 5.11, 5.13 (2H, ABq, J=12.0 Hz, CH_2OCO), 5.16 (1H, br, NH), 6.47 (1H, br, NH), 7.27～7.40 (5H, m, Ph).

Z-*erythro*-(2*RS*,3*RS*)-H_2Pos-D-Leu-L-Val-OBu^t (**35**)

To a solution of **34** (1.619 g, 3.85 mmol) in MeOH (14 ml) was added palladium-black catalyst (40.0 mg). The mixture was hydrogenated at room temperature in a hydrogen atmosphere for 16 hours. The catalyst was

filtered off, and evaporation of the solvent gave D-Leu-L-Val-OBu^t (**36**; 1.100 g, 3.84 mmol) as a clear oil. To the **36** was added *Z-erythro*-(2*RS*,3*RS*)-H_2Pos (1.132 g, 4.24 mmol) and HOBt (1.041 g, 7.70 mmol) in DMF (5 ml). To the mixture was added EDC·HCl (1.033 g, 5.39 mmol) under ice cooling, and the mixture was stirred in an ice bath for 2 hours and at room temperature for 6 hours. The mixture was diluted with EtOAc (50 ml) and washed with 4% aq $NaHCO_3$ (40 ml), saturated aq NaCl (20 ml), 1% aq citric acid (20 ml) and saturated aq NaCl (20 ml), and dried (Na_2SO_4). After removal of the solvent, the product was purified by silica gel column chromatography with CH_2Cl_2-MeOH (100:1~200:3) to give **35** as a solid, 2.020 g (98.0%): FAB-MS *m/z* 536 $(M+H)^+$, 480 (M−isobutylene (abbreviated as *i*Bu)+H)$^+$, 402 $(M-Z+2H)^+$, 346 $(M-i\text{Bu}-Z+2H)^+$, 231 $(M-i\text{Bu}-(Z\text{-}H_2\text{Pos})+2H)^+$, 118 $(M-i\text{Bu}-(Z\text{-}H_2\text{Pos-Leu})+2H)^+$, 91 $(C_7H_7)^+$.

Z-L-Homophenylalanine (Z-L-Hph (**37**))

The starting L-homophenylalanine ethyl ester hydrochloride was derived from L-Hph which was prepared from DL-Hph according to the optical resolution procedure reported by MIYAZAWA *et al.*[11] To an ice-cold solution of L-homophenylalanine ethyl ester hydrochloride (1.22 g, 5.01 mmol) in $CHCl_3$ (10 ml) was added triethylamine (1.54 ml, 11.0 mmol) and benzyl chloroformate (0.96 ml, 6.01 mmol), and the mixture was stirred for in an ice bath for 20 minutes and at room temperature for 2 hours. The mixture was washed with 1 N HCl (10 ml) and saturated aq NaCl (10 ml). Evaporation of the solvent gave Z-L-homophenylalanine ethyl ester as a solid. To an ice-cold solution of Z-L-homophenylalanine ethyl ester in MeOH (5 ml) was added 1 N NaOH (5.5 ml), and the mixture was stirred for 2 hours at room temperature. After evaporation of the solvent, water (10 ml) was added and the mixture washed twice with ether (each 10 ml). The mixture was acidified with 1 N HCl (6.6 ml), extracted thrice with AcOEt (each 10 ml) and the extract dried (Na_2SO_4). After removal of the solvent, the product was purified by silica gel column chromatography with $CHCl_3$-MeOH-AcOH (200:2:1) to give **37** as crystals, 0.760 g (48.4%): mp 103~104°C; $[\alpha]_D^{26}$ −15.3° (*c* 1.0, MeOH); FAB-MS *m/z* 314 $(M+H)^+$, 270, 224, 180, 91; ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.03, 2.22 (1H, 1H, m, m, β-CH_2), 2.71 (2H, t, J=7.9 Hz, γ-CH_2), 4.45 (1H, br dt, α-CH), 5.13 (2H, s, CH_2OCO), 5.33 (1H, br d, J=8.3 Hz, NH), 7.00~7.52 (10H, m, Ph×2).

Z-L-Hph-(2*RS*,3*RS*)-H_2Pos-D-Leu-L-Val-OBu^t (**38**)

To a solution of **35** (329.7 mg, 0.616 mmol) in MeOH (3 ml) was added palladium-black catalyst (5.2 mg). The mixture was hydrogenated at room temperature in a hydrogen atmosphere for 26.5 hours. The catalyst was filtered off, and evaporation of the solvent gave (2*RS*,3*RS*)-H_2Pos-D-Leu-L-Val-OBu^t (**39**; 247.1 mg, 0.615 mmol). To **39** (150.2 mg, 0.374 mmol) was added Z-L-Hph (128.9 mg, 0.411 mmol) and HOBt (102.8 mg, 0.761 mmol) in DMF (2 ml). To the mixture was added EDC·HCl (100.4 mg, 0.524 mmol) under ice cooling, and the mixture was stirred in an ice bath for 6 hours. The mixture was diluted with EtOAc (20 ml) and washed with 4% aq $NaHCO_3$ (12 ml), water (8 ml), 1% aq citric acid (8 ml) and saturated aq NaCl (8 ml) and dried (Na_2SO_4). Evaporation of the solvent gave **38**, 259.8 mg (99.7%) as a solid: FAB-MS *m/z* 697 $(M+H)^+$, 641 $(M-i\text{Bu}+H)^+$, 607 $(M-C_7H_7+2H)^+$, 563 $(M-Z+2H)^+$, 507 $(M-i\text{Bu}-Z+2H)^+$, 402 $(M-(Z\text{-Hph})+2H)^+$, 346 $(M-i\text{Bu}-(Z\text{-Hph})+2H)^+$, 231 $(M-i\text{Bu}-(Z\text{-Hph-}H_2\text{Pos})+2H)^+$, 91 $(C_7H_7)^+$.

Z-L-Hph-(*RS*)-Pos-D-Leu-L-Val-OBu^t (**24**)

A mixture of **38** (259.8 mg, 0.373 mmol), pyridinium trifluoroacetate (36.9 mg, 0.191 mmol), dicyclohexylcarbodiimide (abbreviated as DCC; 232.0 mg, 1.124 mmol), anhydrous DMSO (2.0 ml) and benzene (2.0 ml) was stirred at room temperature for 6.5 hours. The reaction mixture was diluted with EtOAc (10 ml), and the undissolved material was removed by filtration. The filtrate was washed with water (10 ml) and dried (Na_2SO_4). After evaporation of the solvent, the product was purified by silica gel column chromatography with CH_2Cl_2-MeOH (100:1) to give **24** as a white solid. This solid was chromatographed on a column of Sephadex LH-20 with MeOH elution. Evaporation of the active eluate gave **24** as a solid, 211.8 mg (81.8%): FAB-MS (Table 2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.77~1.03 (15H, m, CH_3×5), 1.43, 1.45 (4.5H, 4.5H, two s, Bu^t), 1.50~2.25 (8H, m, β-CH_2×3 (Hph, Pos, Leu), γ-CH(Leu), β-CH(Val)), 2.68 (2H, m, γ-CH_2(Hph)), 4.21 (1H, m, α-CH(Hph)), 4.39, 4.31 (0.5H, 0.5H, two dd, J=4.4, 8.0 Hz and J=4.8, 8.4 Hz, α-CH(Val)), 4.49 (1H, m, α-CH(Leu)), 5.02~5.55 (4H, m, CH_2OCO, CH(Pos), NH), 6.38~6.73 (total 2H, four br d, NH), 7.08~7.42 (11H, m, Ph×2, NH).

Z-L-Hph-(*RS*)-Pos-D-Leu-L-Val (**22**)

A solution of **24** (209.2 mg, 0.301 mmol) in TFA (3 ml) was stirred at room temperature for 2 hours. The solution was evaporated, and the residue was coevaporated twice with toluene (each 2 ml). The product was purified by silica gel column chromatography with $CHCl_3$-MeOH-AcOH (600:5:2) to give **22** as an amorphous solid, 166.1 mg. This solid was chromatographed on a column of Sephadex LH-20 with MeOH elution. Evaporation of the active eluate gave **22** as an amorphous solid, 112.4 mg (58.5%): FAB-MS (Table 2); ^{1}H NMR (400 MHz, CD_3OD) δ 0.72~1.05 (15H, m, CH_3×5), 1.20~2.25 (8H, m, β-CH_2×3 (Hph, Pos, Leu), γ-CH(Leu), β-CH(Val)), 2.69 (2H, m, γ-CH_2(Hph)), 3.91~4.20 (3H, m, α-CH×2(Hph, Val)), 4.35~4.61 (1H, m, α-CH(Leu)), 4.82~5.16 (3H, m, CH_2OCO, CH(Pos)), 7.00~7.61 (10H, m, Ph×2). The methine proton of the Pos residue was observed at *ca.* 4 ppm because the ketone moiety of the Pos residue formed a hemiketal in CD_3OD.

The analogues **20**, **21**, **23** and **29** were prepared in a similar manner. The analogues **27**, **28**, **30**, and **31** were prepared in a similar manner, using D-leucine *t*-butyl ester hydrochloride instead of L-valine *t*-butyl ester hydrochloride as starting material.

20: ^{1}H NMR (400 MHz, CD_3CN) δ 0.70~1.00 (15H, m, $CH_3\times5$), 1.40~1.90 (5H, m, β-CH_2(Leu), γ-CH (Leu), CH_2(Pos)), 2.13 (1H, m, β-CH(Val)), 4.28 (1H, m, α-CH(Val)), 4.46 (1H, m, α-CH(Leu)), 4.98~5.12 (3H, m, CH_2OCO, CH(Pos)), 5.30 (1H, br d, α-CH(Phg)), 6.44 (1H, br, NH(Phg)), 6.92, 6.95 (0.5H, 0.5H, two d, J=8.3, 10.7 Hz, NH(Val)), 7.14 (1H, d, J=6.8 Hz, NH(Pos)), 7.20~7.47 (10H, m, Ph×2), 7.48, 7.56 (0.5H, 0.5H, two d, each J=8.3 Hz, NH(Leu)).

21: ^{1}H NMR (400 MHz, CD_3CN) δ 0.65~1.02 (15H, m, $CH_3\times5$), 1.30~1.91 (5H, m, β-CH_2(Leu), γ-CH(Leu), CH_2(Pos)), 2.14 (1H, m, β-CH(Val)), 2.85 (1H, m, β-C*Ha*Hb(Phe)), 3.11 (1H, m, β-CHa*Hb*(Phe)), 4.32 (1H, dd, J=5.6, 8.5 Hz, α-CH(Val)), 4.43 (1H, m, α-CH(Phe)), 4.52 (1H, m, α-CH(Leu)), 4.83~5.13 (3H, m, CH_2OCO, CH(Pos)), 6.03, 6.08 (0.5H, 0.5H, two d, each J=8.6 Hz, NH(Phe)), 7.03, 7.09 (0.5H, 0.5H, two d, each J=8.5 Hz, NH(Val)), *ca.* 7.10~7.40 (11H, m, overlapping, Ph×2, NH(Pos)), 7.61, 7.63 (0.5H, 0.5H, two d, each J=5.7Hz, NH(Leu)).

23: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.60~1.01 (15H, m, $CH_3\times5$), 1.43, 1.44 (4.5H, 4.5H, two s, OBu^t), 1.46~2.02 (5H, m, β-CH_2(Leu), γ-CH(Leu), CH_2(Pos)), 2.16 (1H, m, β-CH(Val)), 2.87~3.28 (2H, m, β-CH_2(Phe)), 4.24~4.53 (3H, m, α-CH×3(Val, Leu, Phe)), 4.88~5.34 (3H, m, CH_2OCO, CH(Pos)), 5.45, 5.56 (0.5H, 0.5H, two br, NH(Phe)), 6.23, 6.34 (1H, two br d, NH(Pos)), 6.41, 6.62 (0.5H, 0.5H, two d, each J=8.4 Hz, NH(Val)), 7.10~7.40 (11H, m, Ph×2, NH(Leu)).

29: ^{1}H NMR (270 MHz, DMSO-d_6) δ 0.70~1.00(15H, m, $CH_3\times5$), 1.35~1.90 (5H, m, β-CH_2(Leu), γ-CH(Leu), CH_2(Pos)), 2.06 (1H, m, β-CH(Val)), 2.71, 2.95 (0.5H, 0.5H, two br d, β-C*Ha*Hb(Phe)), 2.76, 3.00 (0.5H, 0.5H, two br d, β-CHa*Hb*(Phe)), 4.15 (1H, dd, J=5.6, 8.9 Hz, α-CH(Val)), 4.37 (1H, m, α-CH(Phe)), 4.52 (1H, m, α-CH(Leu)), 4.83~5.06 (3H, m, CH_2OCO, CH(Pos)), 7.10~7.40 (10H, m, Ph×2), 7.49, 7.52 (0.5H, 0.5H, two d, each J=4.0 Hz, NH(Phe)), 8.18, 8.27 (0.5H, 0.5H, two d, each J=8.9 Hz, NH(Val)), 8.39, 8.44 (0.5H, 0.5H, two d, each J=7.1 Hz, NH(Pos)), 8.53, 8.56 (0.5H, 0.5H, two d, each J=4.8 Hz, NH(Leu)), 12.60 (1H, br, COOH).

27: ^{1}H NMR (270 MHz, DMSO-d_6) δ 0.70~1.02 (9H, m, $CH_3\times3$), 1.40~1.92 (5H, m, β-CH_2 (Leu), γ-CH(Leu), CH_2(Pos)), 2.74, 2.98 (1H, 1H, two m, β-CH_2(Phe)), 4.28 (1H, m, α-CH(Leu)), 4.39 (1H, m, α-CH(Phe)), 4.93, 4.94 (2H, ABq, overlapping, CH_2OCO), *ca.* 4.97 (1H, m, overlapping, CH(Pos)), 7.10~7.42 (10H, m, Ph×2), 7.48, 7.52 (0.5H, 0.5H, two d, each J=8.9 Hz, NH(Phe)), 8.41 (1H, d, J=7.3 Hz, NH(Pos)), 8.88, 8.94 (0.5H, 0.5H, two d, each J=8.3 Hz, NH(Leu)), 12.72 (1H, br, COOH).

28: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.69, 0.78 (1.5H, 1.5H, two t, each J=7.8 Hz, CH_3(Pos)), 0.93, 0.94 and 0.95, 0.96 (3H, 3H, (two d)×2, J=6.8, 6.8 Hz, J=6.4, 6.4 Hz, $CH_3\times2$(Leu)), 1.46, 1.47 (4.5H, 4.5H, two s, OBu^t), *ca.* 1.50~1.75 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.85, 1.94 (1.5H, 1.5H, two m, CHa*Hb* (Pos)), 3.01, 3.08 (0.5H, dd, J=7.6, 14.0 Hz, 0.5H, br dd, J=6.8, 14.0 Hz, β-C*Ha*Hb(Phe)), 3.04, 3.13 (0.5H, dd, J=7.6, 14.0 Hz, 0.5H, br dd, J=6.8, 14.0 Hz, β-CHa*Hb*(Phe)), 4.44 (2H, m, α-CH×2 (Leu, Phe)), 5.09, 5.11 (2H, ABq, J=12.0 Hz, CH_2OCO), 5.22, 5.24 (0.5H, 0.5H, two ddd, each J=5.0, 7.8, 7.8 Hz, CH(Pos)), 5.32 (1H, br d, NH(Phe)), 6.33, 6.36 (0.5H, 0.5H, two br d, NH(Pos)), 7.13~7.40 (11H, m, Ph×2, NH(Leu)).

30: ^{1}H NMR (270 MHz, DMSO-d_6) δ 0.73~1.00 (9H, m, $CH_3\times3$), 1.40~1.90 (5H, m, β-CH_2(Leu), γ-CH(Leu), CH_2(Pos)), 2.74, 2.97 (1H, 1H, two m, β-CH_2(Phe)), 4.28 (1H, m, α-CH(Leu)), 4.38 (1H, m, α-CH(Phe)), 4.93, 4.94 (1H, 1H, two s, CH_2OCO), *ca.* 4.97 (1H, m, overlapping, CH(Pos)), 7.13~7.40 (10H, m, Ph×2), 7.49, 7.52 (0.5H, 0.5H, two d, each J=8.6 Hz, NH(Phe)), 8.38, 8.43(0.5H, 0.5H, two d, J=7.3, 6.9 Hz, NH(Pos)), 8.88, 8.91 (0.5H, 0.5H, two d, J=8.3, 8.2 Hz, NH(Leu)), 12.68 (1H, br, COOH).

31: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.70, 0.78 (1.5H, 1.5H, two t, each J=7.6 Hz, CH_3(Pos)), 0.94(6H, d, J=5.6 Hz, $CH_3\times2$(Leu)), 1.46, 1.48 (4.5H, 4.5H, two s, OBu^t), 1.50~1.73 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.86, 1.95 (0.5H, 0.5H, two m, CHa*Hb* (Pos)), 2.69~3.18 (2H, m, β-CH_2(Phe)), 4.44 (2H, m, α-CH×2(Leu, Phe)), 5.09 (2H, s, CH_2OCO), 5.23 (1H, ddd, J=5.0, 7.6, 7.6 Hz, CH(Pos)), 5.34 (1H, br d, NH(Phe)), 6.28, 6.37 (0.5H, 0.5H, two br d, each J=7.6 Hz, NH(Pos)), 7.10~7.40 (11H, m, Ph×2, NH(Leu)).

Acylation of Poststatin Analogues

To 4-phenylbutyric anhydride (67.3 mg, 0.217 mmol) prepared from 4-phenylbutyric acid and DCC was added poststatin (28.4 mg, 0.0524 mmol) in MeOH (2 ml) and triethylamine (50 μl, 0.357 mmol), and the mixture was stirred for 30 minutes at room temperature. The mixture was acidified with AcOH (200 μl) and was chromatographed on a column of Sephadex LH-20 with 5% AcOH-MeOH elution. Evaporation of the active eluate gave *N*-(4-phenylbutyryl)poststatin (**16**), 29.9 mg (83.0%): FAB-MS (Table 2); ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.72~1.05 (27H, m, $CH_3\times9$), 1.40~1.77 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb (Pos)), 1.78~1.94 (3H, m, CHa*Hb*(Pos), CH_2CH_2CO), 1.95~ 2.37 (5H, m, β-CH×3(Val), CH_2CH_2CO), 2.58, 2.60 (1H, 1H, two t, each J=7.3 Hz, $PhCH_2$), 4.23~4.45 (3H, m, α-CH×3(Val)), 4.46~4.67 (1H, m, α-CH(Leu)), 5.11 (1H, m, CH(Pos)), 7.18 (3H, m, aromatic protons), 7.27 (2H, m, aromatic protons).

The analogues **14** and **17** were prepared in a similar manner from poststatin and analogue **13** respectively.

14: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.80~1.08 (27H, m, $CH_3\times9$), 1.50~1.80 (4H, m, β-CH_2(Leu), γ-CH(Leu), C*Ha*Hb(Pos)), 1.85~2.12 (2H,

m, CHa*Hb*(Pos), β-CH(Val)), 2.19 (2H, m, β-CH × 2(Val)), 4.35~4.47 (2H, m, α-CH × 2(Val)), 4.52 (1H, d, J=9.3 Hz, α-CH(Val)), 4.65 (1H, m, α-CH(Leu)), 5.14 (1H, m, CH(Pos)), 7.46 (2H, t, J=7.3 Hz, aromatic protons), 7.55 (1H, t, J=7.3 Hz, aromatic proton), 7.85 (2H, d, J=7.3 Hz, aromatic protons).

17: ^{1}H NMR (400 MHz, CD_3CN+CD_3COOD) δ 0.72~1.05 (21H, m, CH_3 × 7), 1.40~2.37 (15H, m, β-CH_2(Leu), γ-CH(Leu), CH_2(Pos), β-CH_2(Pro), γ-CH_2(Pro), $PhCH_2CH_2CH_2$, β-CH × 2(Val)), 2.58 (2H, t, J=7.6 Hz, $PhCH_2$), 3.62 (1H, m, δ-C*Ha*Hb(Pro)), 3.80, 3.87 (0.5H, 0.5H, two m, δ-CHa*Hb*(Pro)), 4.23~4.66 (4H, m, α-CH × 4 (Val × 2, Pro, Leu)), 5.06, 5.31 (0.5H, 0.5H, two m, CH(Pos)), 7.18 (3H, m, aromatic protons), 7.26 (2H, m, aromatic protons).

Synthesis of Analogue **2** (Liquid A)

(*S*)-2-(*p*-Methoxybenzyloxycarbonyl)aminobutyric Acid (Z(OMe)-(*S*)-But, **40**)

40 was prepared from (*S*)-2-aminobutyric acid by the method described.[12] The product was purified by silica gel column chromatography with $CHCl_3$-MeOH-AcOH (300:1:1) to give **40** as a white solid (94.4%): mp 57~58°C; $[\alpha]_D^{26}$ −12.4° (*c* 1.1, MeOH); FAB-MS *m/z* 266 $(M-H)^-$; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.95 (3H, t, J=7.4 Hz, CH_3), 1.73, 1.92 (1H, 1H, m, m, β-CH_2), 3.79 (3H, s, CH_3O), 4.35 (1H, br ddd, α-CH), 5.04 (2H, br s, CH_2OCO), 5.27 (1H, br d, J=7.9 Hz, NH), 6.87 (2H, m, aromatic protons), 7.92 (2H, m, aromatic protons), 7.80 (1H, br, COOH).

Z(OMe)-(*S*)-But-D-Leu-L-Val-OBzl (**41**)

A solution of Boc-D-Leu-L-Val-OBzl[4] (200.2 mg, 0.476 mmol) in TFA (2 ml) was stirred at room temperature for 40 minutes. The solution was evaporated, and the residue was coevaporated twice with toluene (each 2 ml). To the residue was added **40** (130.0 mg, 0.486 mmol) and HOBt (96.7 mg, 0.716 mmol) in DMF (2 ml). *N*-methylmorpholine (54 μl, 0.482 mmol) and 97% EDC·$HClO_4$ (156.7 mg, 0.594 mmol) was added under ice cooling, and the mixture was stirred in an ice bath for 2 hours and at room temperature overnight. The mixture was diluted with CH_2Cl_2 (20 ml) and washed with 1% aq citric acid, water, saturated aq $NaHCO_3$ and water (each 5 ml) and dried (Na_2SO_4). After removal of the solvent, the product was purified by silica gel column chromatography with CH_2Cl_2-MeOH (200:3) to give **41** as a solid, 206.3 mg (76.0%): SI-MS *m/z* 570 $(M+H)^+$, 526, 480, 450, 121; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (3H, d, J=6.8 Hz, CH_3), 0.87~1.00 (12H, m, CH_3 × 4), 1.45~1.80 (4H, m, β-C*Ha*CHb(But), β-CH_2(Leu), γ-CH(Leu)), 1.88 (1H, m, β-CHaC*Hb*(But)), 2.18 (1H, m, β-CH(Val)), 3.79 (3H, s, CH_3O), 4.04 (1H, br ddd, α-CH(But)), 4.51 (1H, dd, J=4.6, 8.5 Hz, α-CH(Val), 1H, overlapping, α-CH(Leu)), 4.99, 5.05 (2H, ABq, J=11.4 Hz, CH_2OCO), 5.06, 5.16 (2H, ABq, J=11.8 Hz, CH_2OCO), 5.22 (1H, br d, J=6.6 Hz, NH(But)), 6.44 (1H, br d, J=6.9 Hz, NH), 6.81 (1H, br d, J=8.5 Hz, NH), 6.84~6.90 (2H, m, aromatic protons), 7.23~7.38 (7H, m, aromatic protons).

Boc-L-Val-(*S*)-But-D-Leu-L-Val-OBzl (**42**)

Crude **42** was obtained in a manner similar to that described in the preparation of **41** by coupling the trifluoroacetate salt of deprotected **41** (0.329 mmol) with Boc-Val (75.3 mg, 0.347 mmol). The product was purified by silica gel column chromatography with CH_2Cl_2-MeOH (50:1) to give **42** as a white solid, 152.4 mg (76.7%): SI-MS *m/z* 605 $(M+H)^+$, 549, 505, 459, 450, 415, 231, 91; ^{1}H NMR (400 MHz, $CDCl_3$) 0.80~1.02 (21H, m, CH_3 × 7), 1.43 (9H, s, Boc), 1.55~1.75 (4H, m, β-C*Ha*Hb(But), β-CH_2(Leu), γ-CH(Leu)), 1.96 (1H, m, β-CHa*Hb*(But)) 2.03~2.23 (2H, m, β-CH × 2(Val)), 4.01 (1H, br t, α-CH(Val)), 4.38 (1H, br ddd, α-CH(But)), 4.49 (1H, dd, J=5.2, 8.8 Hz, α-CH(Val)), 4.61 (1H, m, α-CH(Leu)), *ca.* 5.09 (1H, overlapping, NH(Val)), 5.10, 5.16 (2H, ABq, J=12.6 Hz, CH_2OCO), 6.72 (1H, br d, J=7.6 Hz, NH(Leu)), 6.86 (1H, br d, NH(But)), 7.04 (1H, br d, J=8.8 Hz, NH(Val)), 7.29~7.40 (5H, m, Ph).

Z-L-Val-L-Val-(*S*)-But-D-Leu-L-Val-OBzl (**43**)

Crude **43** was obtained in a manner similar to that described in the preparation of **41** by coupling the trifluoroacetate salt of deprotected **42** (0.232 mmol) with Z-Val (61.8 mg, 0.246 mmol). The product was purified by silica gel column chromatography with CH_2Cl_2-MeOH (80:1) to give **43** as crystals, 152.5 mg (89.1%): mp 237~238°C; $[\alpha]_D^{24}$ +27.5° (*c* 0.4, $CHCl_3$); SI-MS *m/z* 738 $(M+H)^+$, 630, 604, 531, 505, 418, 406, 333, 234, 208, 91; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.70~1.00 (27H, m, CH_3 × 9), 1.40~1.78 (4H, m, β-C*Ha*Hb(But), β-CH_2(Leu), γ-CH(Leu)), 1.79~1.97 (2H, m, β-CH(Val), β-CHa*Hb*(But)), 1.97~2.16 (2H, m, β-CH × 2(Val)), 4.40~4.60 (2H, m, α-CH × 2), 4.70~5.45 (7H, br, α-CH × 3, CH_2OCO × 2), 6.30 (1H, br, NH), 7.10~7.38 (11H, m, Ph × 2, NH), 7.52 (1H, br, NH), 7.92 (1H, br, NH), 8.61 (1H, br, NH).

L-Val-L-Val-(*S*)-But-D-Leu-L-Val (**2**)

To a solution of **43** (133.4 mg, 0.181 mmol) in AcOH-MeOH-H_2O (12:6:2, v/v (ml)) was added palladium-black catalyst (7.0 mg). The mixture was hydrogenated at room temperature in a hydrogen atmosphere for 6.5 hours. The catalyst was filtered off, and evaporation of the solvent gave an oily product. The product was diluted with water (60 ml), and the mixture was adsorbed on a column of Diaion HP-20 (5ml, wet volume). After washing with water (35 ml), **2** was eluted with 50% (v/v) aq MeOH. Evaporation of the eluate gave **2** as a white powder, 93.0 mg (100%); mp 247~255°C (dec); $[\alpha]_D^{23}$ −5.4° (*c* 1.0, MeOH); SI-MS *m/z* 514 $(M+H)^+$, 369, 316, 284, 231, 199; ^{1}H NMR (270 MHz, CD_3OD+D_2O) δ 0.74~1.16 (27H, m, CH_3 × 9), 1.52~1.94 (5H, m, β-CH_2(But), β-CH_2(Leu), γ-CH(Leu)), 1.95~2.31 (3H, m, β-CH × 3(Val)), 3.82

(1H, d, $J=5.6$ Hz, α-CH(Val)), 4.13 (1H, d, $J=5.6$ Hz, α-CH(Val)), 4.22 (1H, d, $J=8.3$ Hz, α-CH(Val)), 4.32 (1H, dd, $J=6.1, 7.8$ Hz, α-CH), 4.42 (1H, br t, α-CH).

Synthesis of Analogue **3**

The analogue **3** was synthesized from poststatin according to the procedure described in the previous paper.[2)]

Enzyme Assay

PEP, elastase and catepsin B were prepared and the inhibitory activities were measured by the procedure described in the previous papers.[1,2)]

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