Studies of Human Immunodeficiency Virus Type 1 (HIV-1) Protease Inhibitors. III. Structure–Activity Relationship of HIV-1 Protease Inhibitors Containing Cyclohexylalanylalanine Hydroxyethylene Dipeptide Isostere[1)]

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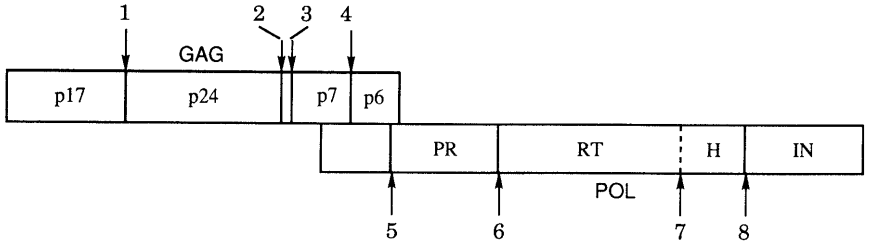
Systematic replacement of the P_4–P_2 subsites of substrate-based human immunodeficiency virus type 1 protease (HIV-1 PR) inhibitors containing cyclohexylalanylalanine hydroxyethylene dipeptide isostere (Cha-ψ[H.E.]-Ala) at positions corresponding to the scissile sites of substrates was carried out. The structure–activity relationship revealed that compounds with the combination of hydrophilic P_3 and β-branched hydrophobic P_2 amino acids generally showed strong inhibitory activity against HIV-1 PR. In particular, compounds 4 (Boc–Orn–Val–Cha-ψ[H.E.]-Ala–NHBu^n; Bu^n = *n*-butyl, K_i = 11 nM) and 6 (Z–Orn–Val–Cha-ψ[H.E.]-Ala–NHBu^n, K_i = 8 nM) exhibited good enzyme selectivity, possessing no significant inhibitory activities toward closely related aspartic proteases, pepsin, cathepsin D, and renin. As a possible model system for evaluating these compounds, anti-retroviral (anti-Mo-MSV/MLV complex (Mo-MSV = Moloney murine sarcoma virus; MLV = murine leukemia virus)) activity was investigated. Both compounds were found to inhibit moderately the focus formation of Mo-MSV/MLV complex in NIH3T3 cells (compound 4, IC_{50} = 1.8 μM; compound 6, IC_{50} = 1.0 μM).

Keywords AIDS; HIV-1 protease inhibitor; hydroxyethylene dipetide isostere; structure–activity relationship; anti-retroviral activity

Human immunodeficiency virus type 1 (HIV-1), a causative virus of the acquired immunodeficiency syndrome (AIDS), encodes an aspartic protease (HIV-1 PR), which is responsible for proteolytic processing of the *gag* and *gag-pol* proteins as shown in Fig. 1.[2)] Inactivation of this protease by site-directed mutagenesis of the catalytic aspartyl residues results in the formation of immature, noninfective virions.[3)] Therefore, HIV-1 PR inhibitors may become a novel type of therapeutic agent for the treatment of AIDS and related diseases.[4)]

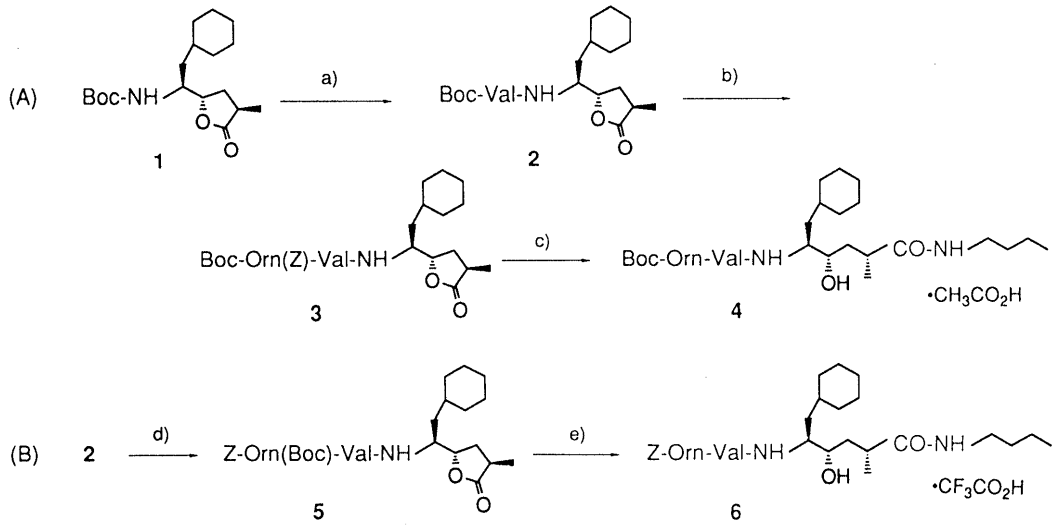
The hydroxyethylene dipeptide isostere mimics the tetrahedral intermediate formed during hydrolysis of a peptide bond.[5)] Many compounds which possess this dipeptide isostere at the scissile bond have been reported to exhibit strong inhibitory activity, especially against aspartic proteases.[6)] In our previous paper, we showed that a cyclohexylalanylalanine hydroxyethylene dipeptide isostere (Cha-ψ[H.E.]-Ala)-containing peptide demonstrated moderate inhibitory activity against HIV-1 PR, as did phenylalanylproline (Phe-ψ[H.E.]-Pro) and phenylalanylalanine (Phe-ψ[H.E.]-Ala) hydroxyethylene dipeptide isostere-containing peptides.[1)] Moreover, a practical synthesis of multigram quantities of the γ-lactone leading to Cha-ψ[H.E.]-Ala could be established by using our new stereocontrolled synthetic route.[7)] We therefore carried out a further structure–activity relationship study at the P_4–P_2 sites with Cha-ψ[H.E.]-Ala-containing peptides, in order to develop still more potent and specific



1	p17 * p24	Ser-Gln-Asn-**TYR** * **PRO**-Ile-Val-Gln
2	p24 * X	Ala-Arg-Val-**LEU** * **ALA**-Glu-Ala-Met
3	X * p7	Ala-Thr-Ile-**MET** * **MET**-Gln-Arg-Gly
4	p7 * p6	Pro-Gly-Asn-**PHE** * **LEU**-Gln-Ser-Arg
5	* PR	Ser-Phe-Asn-**PHE** * **PRO**-Gln-Ile-Thr
6	PR * RT	Thr-Leu-Asn-**PHE** * **PRO**-Ile-Ser-Pro
7	RT51* RNaseH	Ala-Glu-Thr-**PHE** * **TYR**-Val-Asp-Gly
8	RT * IN	Arg-Lys-Ile-**LEU** * **PHE**-Leu-Asp-Gly

Fig. 1. Sites of Scission of the Substrates of HIV-1 PR

PR = protease, RT = reverse transcriptase, H = ribonuclease H, IN = integrase.



a) i) 4N HCl / dioxane, r.t. ii) Boc-Val-OH, DEPC, Et_3N, DMF, r.t. b) i) 4N HCl / dioxane, r.t. ii) Boc-Orn(Z)-OH, DEPC, Et_3N, DMF, r.t. c) i) *n*-butylamine, r.t. ii) Pd / C, H_2, AcOH, MeOH, r.t. d) i) 4N HCl / dioxane, r.t. ii) Z-Orn(Boc)-OH, DEPC, Et_3N, DMF, r.t. e) i) *n*-butylamine, r.t. ii) TFA, r.t.

Chart 1

HIV-1 PR inhibitors.

Chemistry

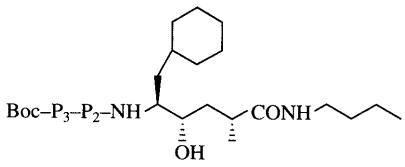
The peptides examined here were prepared using a conventional method of peptide synthesis. Diethylphosphoryl cyanide (DEPC)[8] was employed as a condensation reagent. The ring opening of γ-lactone was conducted at the later stage, because γ-hydroxy carboxamides were prone to cyclize under the acid conditions (HCl/dioxane) used to remove the *tert*-butoxycarbonyl (Boc) group. Two representative synthetic pathways are shown in Chart 1. Most of the peptides were synthesized *via* path A. The peptides that did not require deprotection of the side-chain protecting group were obtained without the hydrogenation in the final step. In the case of compound **13**, the order of the ring opening and deprotection steps was reversed, since Glu(OBzl) was converted into γ-*n*-butylamide during treatment with *n*-butylamine. Moreover, for compounds **29**, **30**, and **31**, deprotection of the *N*-Boc group and acylation, in which acetic anhydride, 3,3-dimethylbutyric acid with DEPC, and succinic anhydride were respectively employed, were conducted before the final sequence c) shown in Chart 1. In the synthesis of compound **6**, path B was chosen. It is noteworthy that trifluoroacetic acid (TFA) treatment (within 5 min at room temperature) suppressed the γ-lactone formation during the N^{δ}-Boc deprotection.[9] All the compounds were characterized, and the data are summarized in Tables I—IV.

Results and Discussion

All of the inhibitors synthesized here were evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) assay and kinetic study as described in a previous report.[10] Initially, inhibitory activity relative to pepstatin A (Iva–Val–Val–Sta–Ala–Sta–OH; Iva = isovaleryl, Sta = statine, $K_i = 1.1\ \mu M$) was determined using the recombinant 55 kDa *gag* substrate and protease. The inhibitory activity shown in the tables represents the concentration that is approximately equipotent with 1 μM pepstatin A. Since we visually judged the remaining amount of 55 kDa *gag* substrate on SDS-PAGE, these values are not considered to be necessarily accurate. Therefore, the inhibition constants (K_i) were determined for the active compounds using the partially purified protease and the synthetic substrate Ac–Ser–Gln–Asn–Tyr–Pro–Ile–Val–NH_2.

HIV-1 PR recognizes as substrates the eight sequences shown in Fig. 1. There are numerous reports on the sequences recognized as substrates,[11] but little information about the sequence preferred as an inhibitor. Thus, the inhibitory activity of the eight substrate-based peptides was initially investigated (Table I). Substrate sequence 2 in Fig. 1 seemed to be the most appropriate sequence for Cha-ψ[H.E.]-Ala-containing inhibitors, because a similar Cha-ψ[H.E.]-Val structure is used for a transition state analog of Leu–Val in potent inhibitors of renin, which is an aspartic protease involved in control of blood pressure.[6b] As expected, compound **8**, based on substrate sequence 2, showed strong inhibitory activity. But the most potent inhibitor in this series was compound **9** based on sequence 3, with 4-fold greater potency than compound **8**. This result may be related to the finding by Miller *et al.* that Ac–Thr–Ile–Met–Met–Gln–Arg–NH_2 had the lowest Michaelis constant K_m (1.4 mM) among the hexapeptide substrates they prepared.[12] Next, the correlation between the sequence and activity was examined in detail. The analysis of the substrate sequences revealed that the amino acids at the P_2 and P_3 sites may be categorized into two groups; Asn (substrate sequences 1, 4, 5, 6) or branched amino acids (substrate sequences 2, 3, 7, 8) at the P_2 site, and hydrophobic (substrate sequences 5, 6) or hydrophilic amino acids (substrate sequences 1, 2, 3, 7, 8) at the P_3 site. Compounds **8**, **9**, and **14** exhibited moderate to strong inhibitory activity. This finding suggests that the

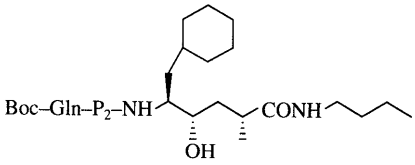
TABLE I. The Substrate-Based HIV-1 PR Inhibitors Containing Cha-ψ[H.E.]-Ala



No.	Sequence[a]		P_3	P_2	Inhibitory activity[b] (μM)	K_i (nM)	Formula	Analysis (%) Calcd (Found) C	H	N	mp (°C)
7	p17*p24	(1)	Gln	Asn	1.0	1050	$C_{31}H_{56}N_6O_8 \cdot 0.25H_2O$	57.69 (57.47	8.83 8.31	13.02 12.82)	211—213
8	p24*X	(2)	Arg	Val	0.1	75	$C_{33}H_{63}N_7O_6$	60.61 (60.43	9.71 9.89	14.99 14.72)	106—108
9	X*p7	(3)	Thr	Ile	0.01	19	$C_{32}H_{60}N_4O_7 \cdot 0.5H_2O$	61.81 (61.82	9.89 9.53	9.01 9.14)	124—127
10	p7*p6	(4)	Gly	Asn	1.0		$C_{28}H_{51}N_5O_7 \cdot 0.25H_2O$	58.56 (58.61	9.04 8.97	12.20 12.17)	189—191
11	*PR	(5)	Phe	Asn	>100		$C_{35}H_{57}N_5O_7$	63.70 (63.52	8.71 8.94	10.61 10.51)	223—226
12	PR*RT	(6)	Leu	Asn	>100		$C_{32}H_{59}N_5O_7$	61.41 (61.10	9.50 9.62	11.19 11.05)	214—216
13	RT51* RNaseH	(7)	Glu ·NH_2	Thr	10		$C_{35}H_{67}N_5O_9 \cdot 1.25H_2O$	58.02 (58.17	9.67 9.49	9.67 9.28)	112—113
14	RT*IN	(8)	Lys ·AcOH	Ile	0.3	700	$C_{36}H_{69}N_5O_8 \cdot H_2O$	60.22 (60.35	9.97 9.84	9.76 9.72)	170—173

a) The number in the parenthesis corresponds to the scission site shown in Fig. 1. *b*) Inhibitory activity is given as the concentration which is equipotent with 1 μM pepstatin A.

TABLE II. The P_2 Variations of HIV-1 PR Inhibitors Containing Cha-ψ[H.E.]-Ala



No.	P_2	Inhibitory activity[a] (μM)	K_i (nM)	Formula	Analysis (%) Calcd C	H	N	Found C	H	N	mp (°C)
7	Asn	1.0	1050	[b]		[b]			[b]		[b]
15	Val	0.01	56	$C_{32}H_{59}N_5O_7$	61.41	9.50	11.19	61.39	9.29	11.17	209—212
16	Ile	0.1	150	$C_{33}H_{61}N_5O_7 \cdot 0.2H_2O$	61.59	9.62	10.88	61.40	9.39	10.84	222—224
17	Leu	300		$C_{33}H_{61}N_5O_7 \cdot H_2O$	60.24	9.65	10.65	60.18	9.37	10.77	199—204
18	*tert*-Leu	0.03	56.3	$C_{33}H_{61}N_5O_7 \cdot 0.5H_2O$	61.08	9.63	10.79	60.79	9.27	10.93	135—136
19	PhGly[c]	>100		$C_{35}H_{57}N_5O_7 \cdot 0.5H_2O$	62.85	8.74	10.47	63.13	8.57	10.25	212—214
20	Thr	10		$C_{31}H_{57}N_5O_8 \cdot 0.5H_2O$	58.37	9.17	10.98	58.30	9.09	10.95	135—136

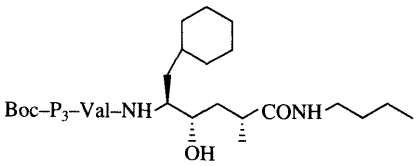
a) Inhibitory activity is given as the concentration which is equipotent with 1 μM pepstatin A. *b*) See Table I. *c*) L-Phenylglycine.

combination of branched and hydrophilic amino acids at the P_2 and P_3 sites, respectively, is preferable to the other combinations.

Further transformation was conducted to optimize each subsite in detail. During the investigation of the P_2 site, the P_4–P_3 site was kept constant as Boc–Gln (Table II). Since branched amino acids were preferred at this site, as shown in Table I, some hydrophobic amino acids were investigated. The peptides containing β-branched amino acids, such as Val, Ile, and *tert*-Leu, were potent inhibitors (**15**, **16**, **18**). Interestingly, the γ-branched amino acid Leu and the relatively bulky amino acid PhGly were not appropriate at this site (**17**, **19**). Moreover, the importance of the hydrophobic substituent was confirmed by the result that the hydrophilic β-branched Thr-containing peptide **20** was a poor inhibitor. Such β-branched amino acid preference in the inhibitors containing Cha-ψ[H.E.]-Ala was identical with the substrate preference reported by Konvalinka *et al.*[11e]

We surveyed the P_3 site to obtain more potent HIV-1 PR inhibitors, with the P_4 and P_2 kept constant as Boc and Val, respectively (Table III). The sequence-based peptide **8** was employed as a reference compound. Orn, Gln, Thr, Nal(1), and AzaTrp at the P_3 site were more or equally preferred (**4**, **15**, **24**, **27**, **28**), compared with Arg. Although Arg(NO_2) and Gly led to a slight decrease

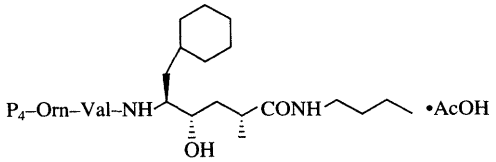
TABLE III. The P_3 Variations of HIV-1 PR Inhibitors Containing Cha-ψ[H.E.]-Ala

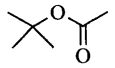
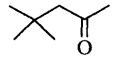
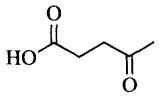
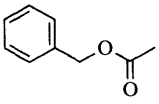


No.	P_3	Inhibitory activity[a] (μM)	K_i (nM)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	mp (°C)
8	Arg	0.1	75	[b]		[b]			[b]		[b]
15	Gln	0.01	56	[c]		[c]			[c]		[c]
21	Arg(NO_2)	0.1	145	$C_{32}H_{61}N_8O_8 \cdot H_2O$	55.29	9.00	15.63	55.18	8.69	15.03	132—134
22	Orn(Z)	10		$C_{40}H_{67}N_5O_8$	64.40	9.05	9.39	64.18	9.31	9.23	130—135
4	Orn·AcOH	0.01	11	$C_{34}H_{65}N_5O_8 \cdot 0.25H_2O$	60.36	9.76	10.35	60.23	9.45	10.43	136—138
23	Lys·AcOH	1.0		$C_{35}H_{67}N_5O_8 \cdot 0.75H_2O$	60.10	9.87	10.01	60.09	9.55	10.09	189—191
24	Thr	0.01	77	$C_{31}H_{58}N_4O_7$	62.18	9.76	9.36	61.84	9.43	9.40	152—154
25	Gly	0.1	172	$C_{29}H_{54}N_4O_6$	62.79	9.81	10.10	62.81	9.60	10.30	161—164
26	Phe	1.0		$C_{30}H_{60}N_4O_6$	67.05	9.38	8.69	66.75	9.05	8.67	125—130
27	Nal(1)[d]	0.01	11.5	$C_{40}H_{62}N_4O_6$	69.13	8.99	8.06	68.86	8.71	8.23	143—154
28	AzaTrp[e]	0.01	67	$C_{37}H_{60}N_6O_6 \cdot 0.5H_2O$	64.04	8.86	12.11	64.22	8.81	12.11	219—222

a) Inhibitory activity is given as the concentration which is equipotent with 1 μM pepstatin A. *b*) See Table I. *c*) See Table II. *d*) 3-(1-Naphthyl)-L-alanine. *e*) L-7-Azatryptophan.

TABLE IV. The P_4 Variations of HIV-1 PR Inhibitors Containing Cha-ψ[H.E.]-Ala



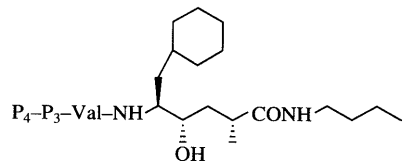
No.	P_4	Inhibitory activity[a] (μM)	K_i (nM)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	mp (°C)
4		0.01	11	[b]		[b]			[b]		[b]
29		10		$C_{31}H_{59}N_5O_7$	60.66	9.69	11.41	60.55	9.58	11.71	232—234
30		0.3		$C_{35}H_{67}N_5O_7 \cdot 2H_2O$	59.29	10.09	9.88	59.00	9.47	9.78	207—211
31		1.0		$C_{33}H_{61}N_5O_9$	58.99	9.15	10.42	58.88	8.67	10.60	174—176
6[c]		0.01	8.0	$C_{37}H_{60}N_5O_8F_3 \cdot 0.5H_2O$	57.80	8.00	9.11	57.75	7.59	9.15	130—138

a) Inhibitory activity is given as the concentration which is equipotent with 1 μM pepstatin A. *b*) See Table III. *c*) TFA salt.

in potency (**21**, **25**), replacement by Orn(Z), Lys and Phe made the inhibitors much less potent (**22**, **23**, **26**). These results revealed an intriguing fact: basically, hydrophilic amino acids are preferred, and hydrophobic amino acids are excluded at this site as well as in the case of the substrate-based peptides shown in Table I. However, a conflict arose in that hydrophilic Lys diminished the inhibitory activity and hydrophobic Nal(1) improved it. Our interpretation of this is that the optimal range of hydrophilic substituents at this site is rather narrow, so that Lys, with a one-carbon-longer side chain than Orn's, disrupts the interaction between the protease and inhibitor. On the other hand, bulkier hydrophobic Nal(1) and heterocyclic AzaTrp can fit the S_3 site, probably due to a conformational change or good matching with another hydrophobic pocket at this site.

Since Orn–Val was found to be one of the best choices at the P_3 and P_2 sites, slight changes at the P_4 site were

TABLE V. Enzyme Specificity



No.	P_4	P_3	HIV-1 protease		Pepsin IC_{50} (M)	Cathepsin D IC_{50} (M)	Renin IC_{50} (M)
			Inhibitory activity (M)[a]	K_i (M)			
4	Boc	Orn·AcOH	1×10^{-8}	1.1×10^{-8}	2.7×10^{-6}	2.1×10^{-5}	—
6	Z	Orn·TFA	1×10^{-8}	8.0×10^{-9}	3.1×10^{-6}	9.6×10^{-5}	5.6×10^{-6}
15	Boc	Gln	1×10^{-8}	5.6×10^{-8}	1.8×10^{-7}	1.7×10^{-7}	—
24	Boc	Thr	1×10^{-8}	7.7×10^{-8}	6.0×10^{-7}	1.4×10^{-6}	—
27	Boc	Nal	1×10^{-8}	1.2×10^{-8}	8.0×10^{-7}	1.5×10^{-6}	—

a) Inhibitory activity is given as the concentration which is equipotent with 1 μM pepstatin A.

examined next (Table IV). Compounds **29**, **30**, and **31** were less potent than compound **4**. This fact suggests that the *tert*-butyl and oxygen in the Boc group are important and the succinyl group is not appropriate for demonstrating inhibitory activity. On the other hand, conversion of the Boc into the benzyloxycarbonyl (Z) group slightly improved the K_i value. These results imply that this site needs both a hydrophobic moiety and an oxygen atom that can form hydrogen bonding.

The enzyme specificity was investigated for the compounds with strong inhibitory activity (Table V). The inhibitory activity of compounds **4**, **6**, **15**, **24**, and **27** toward closely related aspartic proteases, such as pepsin, cathepsin D, and renin, was examined. Although compounds **4** and **6** showed preferential inhibitory activity against HIV-1 PR, compounds **15**, **24**, and **27** inhibited pepsin and cathepsin D moderately to strongly. In particular, compound **15** showed IC_{50}s of 0.18 and 0.17 μM against pepsin and cathepsin D, respectively. This result suggests that the polarity at the P_3 site influences the enzyme specificity; a highly hydrophilic P_3 amino acid was the most selective in the series of the inhibitors containing Cha-ψ[H.E.]-Ala.

In order to evaluate the anti-viral activity of the potent and selective HIV-1 PR inhibitors **4** and **6**, we examined the anti-Mo-MSV/MLV complex activity (Mo-MSV = Moloney murine sarcoma virus; MLV = murine leukemia virus) as a model experiment. The focus formation of Mo-MSV was reported to be inhibited by the aspartic protease inhibitor, pepstatin A.[13] Furthermore, Ruprecht *et al.* utilized murine retroviruses for *in vivo* evaluation of candidate anti-AIDS drugs, and reported that chronic 3′-azide-3′-deoxythymidine (AZT) treatment of mice infected with Rauscher murine leukemia virus complex (Ra-MLV) suppressed mouse viremia when started soon after inoculation.[14] On the other hand, the protease of Mo-MLV belongs to the aspartic protease family[15] and cleaves several amide bonds including the characteristic Tyr/Phe–Pro as a homodimer, like HIV-1 PR.[16] Thus, the inhibitors possessing strong inhibitory activity against HIV-1 PR were expected to inhibit the proliferation step of MSV/MLV complex. In fact, both compounds exhibited moderate anti-Mo-MSV/MLV complex activity. The concentrations of compounds **4** and **6** necessary to inhibit the focus formation by 50% (IC_{50}) were 1.8 and 1.0 μM, respectively. We are now investigating the anti-HIV activity of these compounds.

In conclusion, a structure–activity relationship study of HIV-1 PR inhibitors containing Cha-ψ[H.E.]-Ala was carried out. The peptides with β-branched hydrophobic and hydrophilic amino acids at the P_2 and P_3 sites, respectively, generally showed strong inhibitory activity against HIV-1 PR. Of the inhibitors examined here, compounds **4** (K_i 11 nM) and **6** (K_i 8 nM), which exhibited good enzyme selectivity, were further investigated. Both compounds possessed moderate anti-Mo-MSV/MLV complex activity (compound **4**, $IC_{50} = 1.8$ μM; compound **6**, $IC_{50} = 1.0$ μM).

Experimental

Melting points were determined with a Yanagimoto melting point apparatus and are uncorrected. Infrared (IR) spectra were measured on a Nic 5SXC FT IR spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-GX 270 FT NMR instrument. Chemical shifts are expressed in ppm relative to tetramethylsilane with tetramethylsilane as an internal reference. Mass spectra (MS) were measured on a JEOL JMS-D 300 mass spectrometer. Column chromatography was carried out on Kieselgel 60 F_{254} (Merck, 70—230 mesh). The organic solutions were dried over anhydrous Na_2SO_4 before vacuum evaporation.

(3*R*,5*S*,1′*S*)-5-{1′-(*N*-*tert*-Butoxycarbonyl-L-valyl)amino-2′-cyclohexyl}ethyl-3-methyldihydrofuran-2(3*H*)-one (2) (3*R*,5*S*,1′*S*)-5-(1′-*tert*-Butoxycarbonylamino-2′-cyclohexyl)ethyl-3-methyldihydrofuran-2(3*H*)-one (600 mg, 1.84 mmol) was added to 4 N HCl/dioxane solution (5 ml), and this solution was stirred for 30 min at room temperature. The solvent was removed *in vacuo*, and the residue was evaporated with benzene. The resultant residue was dried *in vacuo* for 2 h. This solid was dissolved in *N*,*N*-dimethylformamide (DMF, 5 ml), and then Boc–Val–OH (439 mg, 2.02 mmol), 93% DEPC (0.35 ml, 2.02 mmol), and triethylamine (0.56 ml, 3.86 mmol) were added at 0 °C. The mixture was stirred at room temperature for 4 h, then the solvent was removed *in vacuo*. The residue was extracted with ethyl acetate (AcOEt) and the organic layer was washed with 5% citric acid, 5% $NaHCO_3$, and brine. Drying followed by evaporation and purification by silica gel chromatography (*n*-hexane : AcOEt = 4 : 1) afforded **2** (646 mg, 83%) as colorless crystals. mp 155—156 °C. $[\alpha]_D^{25}$ −45.4° (c = 0.53, $CHCl_3$). *Anal.* Calcd for $C_{23}H_{40}N_2O_5$: C, 65.06; H, 9.50; N, 6.60. Found : C, 64.69; H, 9.35; N, 6.56. IR (KBr): 1770, 1695, 1651 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.78—1.95 (32H, m) including 0.90 (3H, d, J = 6.6 Hz), 0.98 (3H,

d, J=7.3 Hz), 1.25 (3H, d, J=7.9 Hz) and 1.45 (9H, s), 2.19—2.37 (2H, m), 2.63—2.78 (1H, m), 3.94 (1H, dd, J=5.9, 7.9 Hz), 4.25—4.35 (1H, m), 4.50—4.57 (1H, m), 4.88 (1H, br d, J=7.9 Hz), 6.20 (1H, br d, J=7.9 Hz). MS m/z: 425 ($M^+ + 1$), 351, 325, 251, 172, 126, 116, 72, 57.

(3*R*,5*S*,1′*S*)-5-{1′-(*N*$^{\alpha}$-tert-Butoxycarbonyl-*N*$^{\delta}$-benzyloxycarbonyl-L-ornithyl-L-valyl)amino-2′-cyclohexyl}ethyl-3-methyldihydrofuran-2(3*H*)-one (3) Compound **2** (212 mg, 0.50 mmol) was added to 4 N HCl/dioxane solution (2 ml), and this solution was stirred for 30 min at room temperature. The solvent was removed *in vacuo*, and the residue was evaporated with benzene. The resultant residue was dried *in vacuo* for 2 h. This solid was dissolved in DMF (3 ml), and then Boc-Orn(Z)-OH (183 mg, 0.50 mmol), 93% DEPC (95 μl, 0.55 mmol), and triethylamine (146 μl, 1.05 mmol) were added at 0 °C. The mixture was stirred at room temperature for 3 h, then the solvent was removed *in vacuo*. The residue was extracted with AcOEt, and the organic layer was washed with 5% citric acid, 5% $NaHCO_3$, and brine. Drying followed by evaporation and purification by precipitation from *n*-hexane–diethylether (3 : 1, v/v) afforded **3** (286 mg, 85%) as a white solid. mp 93—97 °C. $[\alpha]_D^{25}$ −49.6° (c=0.90, $CHCl_3$). *Anal.* Calcd for $C_{36}H_{56}N_4O_8$: C, 64.26; H, 8.39; N, 8.33. Found : C, 63.89; H, 8.39; N, 8.09. IR (KBr): 1771, 1702, 1649 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.76—1.95 (36H, m) including 0.92 (3H, d, J=6.8 Hz), 0.97 (3H, d, J=6.8 Hz), 1.22 (3H, d, J=7.3 Hz) and 1.45 (9H, s), 2.27—2.41 (2H, m), 2.65—2.80 (1H, m), 3.15—3.38 (2H, m), 4.08—4.32 (3H, m), 4.47—4.53 (1H, m), 5.01—5.18 (3H, m), 5.25—5.32 (1H, m), 6.33 (1H, br d, J=8.8 Hz), 6.84 (1H, br d, J=7.3 Hz), 7.28—7.37 (5H, m). MS m/z: 672 (M^+), 572, 448, 392, 348, 320, 293, 221, 204, 160, 126, 91, 72.

(2*R*,4*S*,5*S*)-5-(*N*$^{\alpha}$-*tert*-Butoxycarbonyl-L-ornithyl-L-valyl)amino-6-cyclohexyl-4-hydroxy-2-methylhexanoic Acid *n*-Butylamide Acetate (4) Compound **3** (140 mg, 0.21 mmol) was dissolved in *n*-butylamine (2 ml), and this solution was left for 3 d at room temperature. The solvent was removed *in vacuo*, and the residue was evaporated with chloroform. Purification by precipitation from diethylether afforded (2*R*,4*S*,5*S*)-5-(*N*$^{\alpha}$-*tert*-butoxycarbonyl-*N*$^{\delta}$-benzyloxycarbonyl-L-ornithyl-L-valyl)-amino-6-cyclohexyl-4-hydroxy-2-methylhexanoic acid *n*-butylamide (155 mg, 100%) as a white solid. A mixture of this compound, acetic acid (20 mg), and 10% Pd/C (20 mg) in MeOH (10 ml) was stirred under a hydrogen atmosphere for 2 h at room temperature. The catalyst was filtered off and the filtrate was concentrated. The residue was precipitated from diethylether to afford **4** (115 mg, 82%) as a white solid. mp 136—138 °C. $[\alpha]_D^{25}$ −54.2° (c=0.21, MeOH). *Anal.* Calcd for $C_{32}H_{61}N_5O_6 \cdot C_2H_4O_2 \cdot 0.25H_2O$: C, 60.36; H, 9.76; N, 10.35. Found: C, 60.23; H, 9.45; N, 10.43. IR (KBr): 3317, 1691, 1642 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.80—1.81 (47H, m) including 1.10 (3H, d, J =6.8 Hz) and 1.45 (9H, s), 2.03—2.16 (1H, m), 2.50—2.61 (1H, m), 2.87—2.94 (2H, m), 3.15 (2H, t, J=6.8 Hz), 3.42—3.48 (1H, m), 3.87—3.95 (1H, m), 4.06—4.13 (1H, m), 4.20 (1H, d, J=6.8 Hz). MS m/z: 611 (M^+), 440, 382, 286, 268, 212, 172, 126, 115, 72, 57.

(3*R*,5*S*,1′*S*)-5-{1′-(*N*$^{\alpha}$-Benzyloxycarbonyl-*N*$^{\delta}$-*tert*-butoxycarbonyl-L-ornithyl-L-valyl)amino-2′-cyclohexyl}ethyl-3-methyldihydrofuran-2(3*H*)-one (5) The title compound **5** was prepared as described above for **3** using Z-Orn(Boc)-OH instead of Boc-Orn(Z)-OH, to yield a white solid (48 mg, 80%). mp 191—193 °C. $[\alpha]_D^{25}$ −41.8° (c =0.19, $CHCl_3$). *Anal.* Calcd for $C_{36}H_{56}N_4O_8$: C, 64.26; H, 8.39; N, 8.33. Found : C, 64.00; H, 8.46; N, 8.34. IR (KBr): 1777, 1710, 1642 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.75—1.95 (36H, m) including 0.88 (3H, d, J=7.3 Hz), 0.92 (3H, d, J=7.3 Hz), 1.24 (3H, d, J=7.3 Hz) and 1.43 (9H, s), 2.19—2.38 (2H, m), 2.65—2.78 (1H, m), 3.05—3.22 (2H, m), 4.18—4.31 (3H, m), 4.49—4.58 (1H, m), 4.89—4.98 (1H, m), 5.11 (2H, s), 5.80 (1H, d, J=7.3 Hz), 6.56 (1H, br d, J=9.2 Hz), 6.80 (1H, br d, J=6.6 Hz), 7.30—7.40 (5H, m). MS m/z: 672 (M^+), 572, 491, 464, 409, 365, 320, 284, 226, 212, 170, 126, 91, 72, 57.

(2*R*,4*S*,5*S*)-5-(*N*$^{\alpha}$-Benzyloxycarbonyl-L-ornithyl-L-valyl)amino-6-cyclohexyl-4-hydroxy-2-methylhexanoic Acid *n*-Butylamide Trifluoroacetate (6) Compound **5** (45 mg, 0.067 mmol) was dissolved in *n*-butylamine (2 ml), and this solution was left for 3 d at room temperature. The solvent was removed *in vacuo*, and the residue was evaporated with chloroform. Purification by precipitation from diethylether afforded (2*R*,4*S*,5*S*)-5-(*N*$^{\alpha}$-benzyloxycarbonyl-*N*$^{\delta}$-*tert*-butoxycarbonyl-L-ornithyl-L-valyl)amino-6-cyclohexyl-4-hydroxy-2-methylhexanoic acid *n*-butylamide (50 mg, 100%) as a white solid. TFA (5 ml) and anisole (0.2 ml) were added to this compound, and the solution was stirred for 5 min at room temperature. The solvent was removed *in vacuo* at room temperature, and the residue was precipitated from diethylether to afford **6** (43 mg, 84%) as a white solid. mp 130—138 °C. $[\alpha]_D^{25}$ −46.4° (c=0.50, MeOH). *Anal.* Calcd for $C_{35}H_{59}N_5O_6 \cdot C_2HF_3O_2 \cdot 0.5H_2O$: C, 57.80; H, 8.00; F. 7.41; N, 9.11. Found: C, 57.75; H, 7.59; F, 7.15; N, 9.15. IR (KBr): 3301, 1676, 1637 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.70—2.01 (36H, m) including 0.96 (3H, d, J=6.8 Hz), 2.38—2.47 (1H, m), 2.69—2.81 (2H, m), 2.96—3.04 (2H, m), 3.25—3.33 (1H, m), 3.73—3.83 (1H, m), 4.17 (1H, dd, J=6.5, 8.8 Hz), 4.58 (1H, br d, J=5.4 Hz), 5.03 (2H, s), 7.28—7.47 (6H, m), 7.51—7.77 (5H, m). MS m/z: 645 (M^+), 627, 446, 348, 329, 302, 286, 222, 172, 126, 91, 72.

Assay for Anti-retroviral Activity NIH3T3 cells were grown on a 35 mm petri dishes using Dulbecco's modified Eagle's medium supplemented with 10% v/v fetal calf serum. The cells (1.5×10^5) were placed in each dish, and incubated at 37 °C overnight under an atmosphere containing 5% carbon dioxide. The next day, the dishes were seeded with Mo-MSV/MLV complex (prepared in Dulbecco's modified Eagle's medium) at a potency of 50 foci/dish. Polybrene™ (hexadimethrine bromide), previously prepared as a stock aqueous solution at 2 mg/ml, was added to a final concentration of 10 μg/ml, and incubation was continued for another 6 h. Then, the medium was replaced with fresh medium containing the peptide sample to be tested.

The dish was further incubated for 4 d. Foci formation of transformed cells was determined microscopically.

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