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Effect of prime-site sequence of retro-inverso-modified HTLV-1 protease inhibitor

Chiyuki Awahara [a], Tadashi Tatsumi [a], Saki Furuta [b], Gen Shinjoh [b], Hiroyuki Konno [c], Kazuto Nosaka [d], Kazuya Kobayashi [b], Yasunao Hattori [b], Kenichi Akaji [b,*]

[a] Department of Chemistry, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kita-ku, Kyoto 603-8334, Japan
[b] Department of Medicinal Chemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan
[c] Department of Biochemical Engineering, Graduate School of Science and Technology, Yamagata University, Yonezawa, Yamagata 992-8510, Japan
[d] Department of Chemistry, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan

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ABSTRACT

The effects of additional substituents covering the prime-site of retro-inverso (RI)-modified HTLV-1 protease inhibitors containing a hydroxyethylamine isoster were clarified. Stereo-selective construction of the most potent isoster backbone was achieved by the Evans-aldol reaction. Addition of N-acetylated D-amino acid corresponding to the P'_2 site gave an RI-modified inhibitor showing superior inhibitory activity to the previous inhibitor. Inhibitory activities of the newly synthesized inhibitors suggest that partially modified RI inhibitors would interact with HTLV-1 protease in the same manner as the parent hydroxyethylamine inhibitor.

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus etiologically associated with adult T-cell leukemia and a number of other chronic diseases.[1] HTLV-1 protease is an aspartic protease, crucial for processing virus proteins. Thus, HTLV-1 protease is a suitable target for the development of inhibitors for therapeutic use. We have previously reported the synthesis and structure–activity relationship of HTLV-1 protease inhibitors containing a transition-state mimic, hydroxyethylamine dipeptide isoster.[2] The results clearly showed that the configurations at the hydroxyl- and side chain-bearing asymmetric centers of the mimic have marked effects on inhibitory activity. It was also shown that the P_3–P'_1 sequence of the substrate-based inhibitor is a core sequence for potent inhibitory activity.[3] Based on these studies, we found for the first time that retro-inverso (RI) modification of the inhibitor containing the transition-state mimic can retain inhibitory activity.[4]

RI modifications involve a reversion of the peptide sequence accompanied by the replacement of each L-amino acid with the corresponding D-amino acid[5] (Fig. 1a). Fully or partially RI-modified biologically active peptides have been found to retain the properties or biological activity of the parent peptides, since a similar topography of side-chain orientations is retained by RI modification.[6] Similar or improved interactions of RI-modified peptides with proteins or receptors have also been reported.[7] However, few applications of the RI modification to protease inhibitors have been reported. We found that RI modification of HTLV-1 protease inhibitors containing hydroxyethylamine dipeptide isoster **2** showed inhibitory activity, suggesting that the RI-modified inhibitor retained similar topography to the parent inhibitor **1**[4] (Fig. 1b). The relative relationship between the isoster hydroxyl and scissile site substituent in **2** is the same as that in the parent inhibitor **1**, in which the side-chain substituent is supposed to interact with the protease S_1 pocket. The RI-modified inhibitor **2** contains D-amino acids covering the non-prime P_2 to P_5 sites, but it contains no additional prime site amino acids except for the P'_1 site. In this paper, we examined whether the additional D-amino acids or substituents covering the prime site would have effects on inhibitory activity.

2. Results and discussion

In the previous syntheses of RI-modified inhibitors, a stereoselective aldol reaction using a chiral auxiliary, 1-arylsulfonamido-2-indanylrate, [8] was employed to evaluate the most potent configuration at the hydroxyethylamine dipeptide isoster. Thus, all four diastereomers containing different configurations at the isoster hydroxyl site and scissile site substituent were stereo-selectively

* Corresponding author. Tel./fax: +81 75 595 4635.
E-mail address: akaji@mb.kyoto-phu.ac.jp (K. Akaji).

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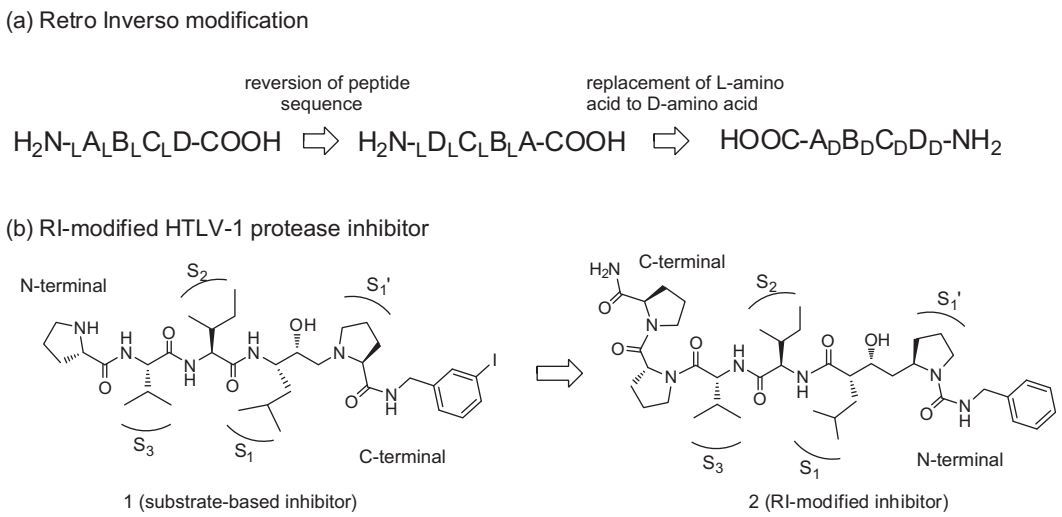


Figure 1. Retro-inverso modification of HTLV-1 protease inhibitors containing a hydroxyethylamine isoster.

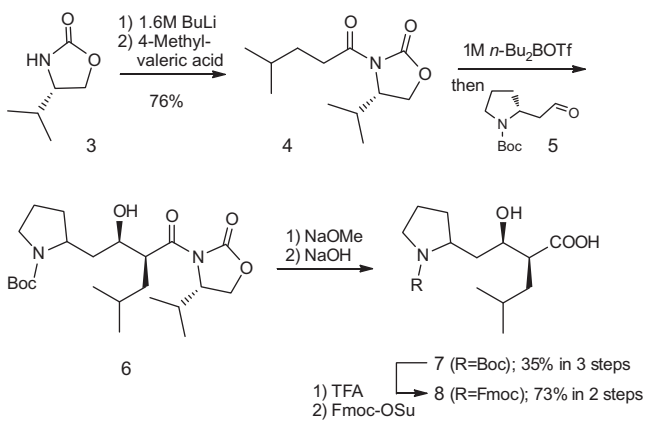
prepared by the combination of the indanylrate auxiliary and L- or D-amino acid. Based on these results, in this study, we focused on an isoster having the most potent *syn*-configuration. The necessary key intermediate **7** was stereo-selectively synthesized employing a diastereo-selective aldol reaction using Evans oxazolidin-2-one auxiliary[9] (Scheme 1). Thus, 4-methylvaleric acid was condensed with oxazolidine-2-one **3** according to the literature procedure to yield desired product **4**. The oxazolidinone **4** was treated with slightly less equivalent of *n*-Bu_2BOTf and then with a known aldehyde **5**[4] to give the desired product **6** as a single diastereomer. Without further purification, the amide **6** was esterified with NaOMe followed by hydrolysis with NaOH to yield N-Boc protected carboxylic acid derivative **7** having the desired dipeptide isoster backbone with 35% yield (in three steps). Use of an equivalent or slightly excess equivalent of *n*-Bu_2BOTf lowered the yield to 17–0%. Thiazolidine-2-one auxiliary[10] instead of the conventional Evans auxiliary gave no desired product in this case.

Condensation of the necessary D-amino acid derivatives for the synthesis of RI-modified HTLV-1 protease inhibitors was carried out by conventional Fmoc-based solid phase peptide synthesis (SPPS) (Scheme 2). For the SPPS, Boc protecting group of the dipeptide isoster **7** was first converted to Fmoc derivative **8**. Starting from commercially available Rink amide resin (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin)[11], each D-amino acid derivative (2.6 equiv) corresponding to the P_2 to P_5 sites was successively introduced by the combination of 20% piperidine/DMF treatment and DIPCDI-HOBt-mediated coupling (25 °C, 2 h) to yield peptide resin **9**. Completion of each coupling reaction was confirmed by the ninhydrin test. The dipeptide isoster **8** (2.6 equiv) was introduced by DIPCDI-HOAt[12]-mediated coupling (25 °C, 24 h), and the N^{α}-Fmoc of the product was removed by treatment with 20% piperidine/DMF to give an intermediate **10**. Acylation of the terminal amino group on the resin was conducted using an excess amount of carboxylic acid or chloride derivative to give the N^{α}-modified peptide resin. Each peptide resin thus synthesized was then treated with TFA (25 °C, 2 h) to cleave each inhibitor from the resin. The crude product obtained by precipitation with ether was purified by reverse-phase preparative HPLC to yield the desired derivative **11**–**18** (overall yield, 7–29%). The homogeneity of each synthesized compound was confirmed by analytical HPLC,[13] TOF-MS, and ^{1}H NMR.

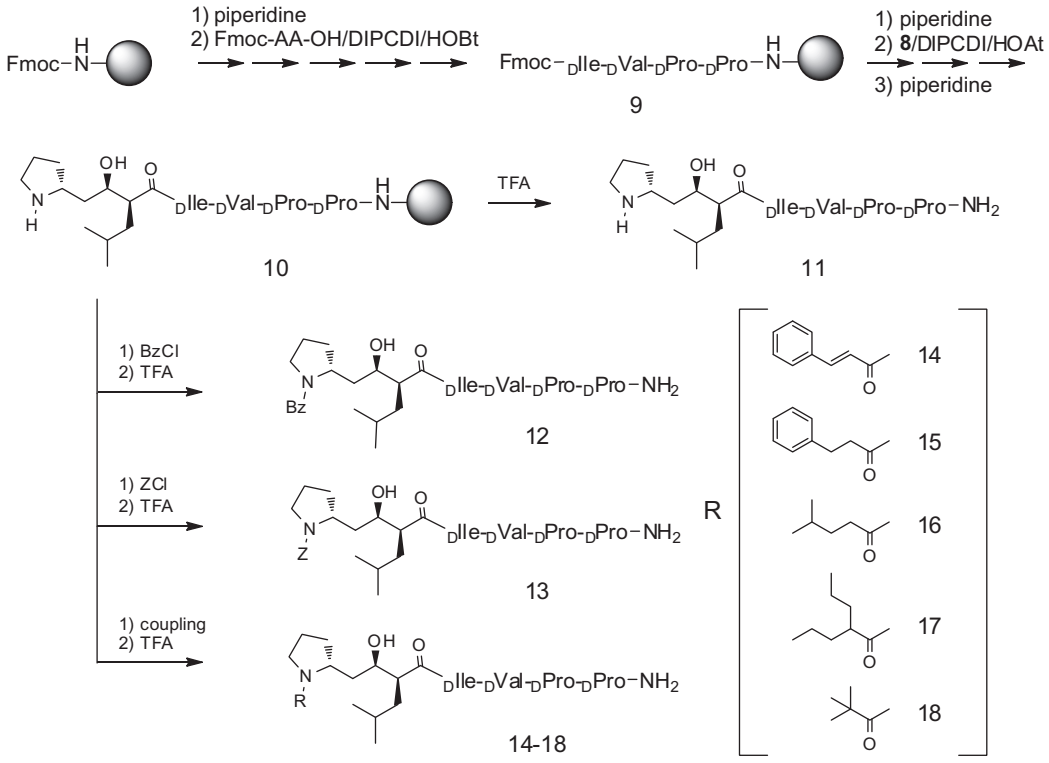
For further elongation to the P'_2 and P'_3 sites, each D-amino acid derivative was condensed to **10** using DIPCDI-HOBt as above without difficulty (Scheme 3). Each peptide resin was then treated with TFA (25 °C, 2 h) to yield the corresponding derivative **20**–**22**. The crude products isolated as above were purified by preparative HPLC to give a single peak on analytical HPLC.[13]

The inhibitory activities of RI-modified inhibitors thus obtained were examined using a synthetic mutant HTLV-1 protease and substrate dodecapeptide according to our previous procedure.[14] Cleavage of the substrate by a mutant protease in the presence of the RI-modified inhibitor was monitored by analytical HPLC. The rate of cleavage was estimated from the decrease in the substrate during incubation. The inhibitory activity of each RI-modified inhibitor was evaluated using the corresponding IC_{50} value obtained from the sigmoidal dose–response curve (Table 1). The experiment was repeated three times and obtained IC_{50} values below 600 μM were in good agreement in three experiments, although the values over 1 mM gave rise to apparent deviation.

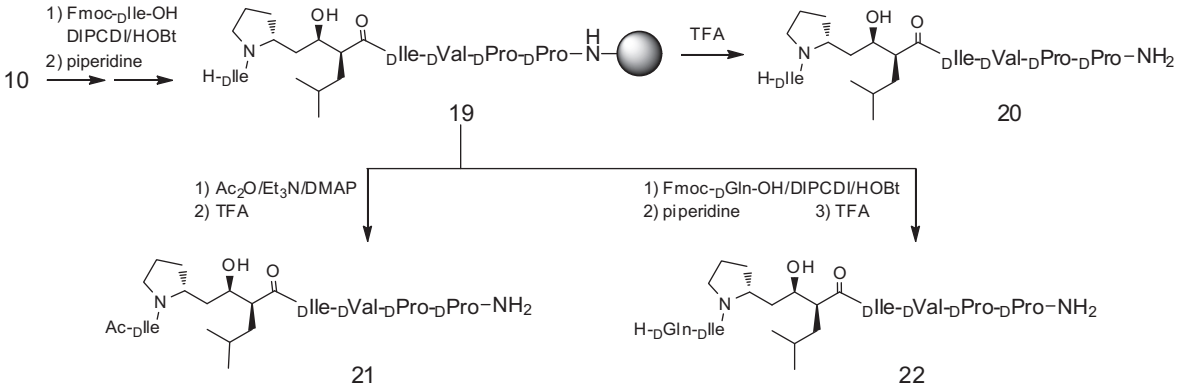
In contrast to our previous RI inhibitor **2** (IC_{50} = 160 μM), compound **11** having an N-terminal imino group without the acyl group showed no inhibitory activity. N-benzoylated compound **12** showed weak inhibitory activity (IC_{50} = 1.1 mM), and insertion of two atoms between the aromatic ring and carbonyl carbon recovered inhibitory activities to nearly the same level as the previous compound **2**. The effect was not correlated with the atom species (compounds **13** to **15**, IC_{50} = 160 to 380 μM), which suggests that the effect may be attributed to the planar aromatic ring suitably situated away from the N-terminus. Substitution with hydrophobic aliphatic groups instead of the aromatic ring decreased the inhibitory activities; substitution with a normal or secondary alkyl group maintained moderate inhibitory activity



Scheme 1.



Scheme 2.



Scheme 3.

(compounds **16** and **17**, IC_{50} = 520 and 420 μM), but a tertiary alkyl group marked lowered the activity (compound **18**, IC_{50} = 1 mM).

Further elongation of the corresponding D-amino acids toward the P'_2 and P'_3 sites marked lowered inhibitory activity when the compounds had an N-terminal amino group (compounds **20** and **22**, IC_{50} = 1.5 and 2.0 mM). Thus, the presence of a terminal ionic group, regardless of the prime site position, showed strong negative effects on the interactions of the inhibitor and HTLV-1 protease. Acylation of the P'_2 site D-Ile increased the inhibitory activity to yield compound **21** (IC_{50} = 110 μM) which was 1.5 times more potent than the previous RI inhibitor **2**. These results suggest that the small aliphatic or aromatic substitution situated three atom distances from the P'_1 site imino group in the RI-modified inhibitor can interact with the protease S'_2 pocket as with conventional substrate-based statin-type inhibitor or hydroxymethylcarbonyl (HMC)-type inhibitors. In the crystal structures of HTLV-1 protease complexed with those inhibitors, [15] the hydrophobic interactions at these prime-sites were clearly demonstrated.

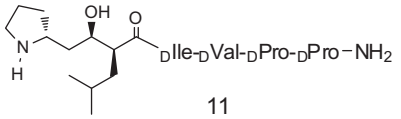
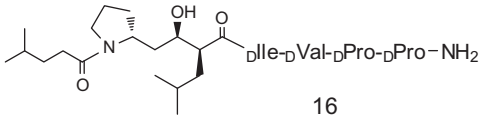
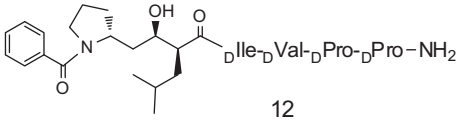
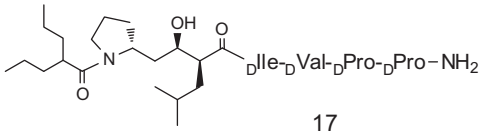
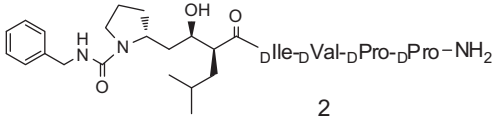
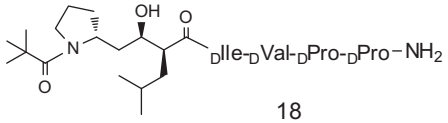
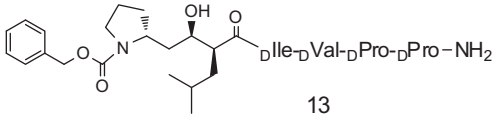
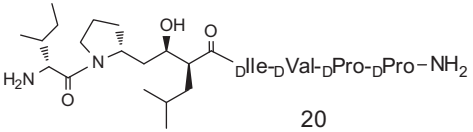
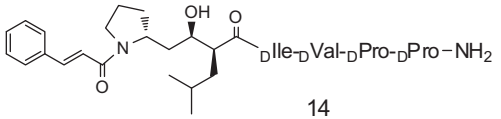
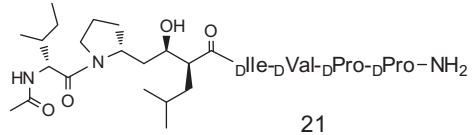
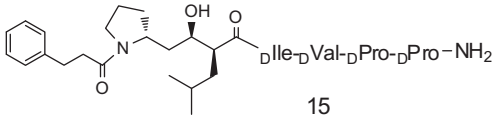
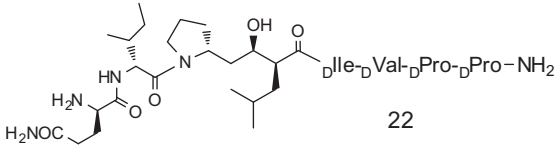
Thus, prime-site modification of the RI-modified HTLV-1 protease inhibitor based on hydrophobicity can improve inhibitory activity, although only moderately. The results suggest that an appropriately RI-modified inhibitor interacts with the protease in a similar manner as the substrate-based inhibitor. Based on the similar topography at side-chain orientations of the RI-modified inhibitor, further optimization of the side-chain structures is now underway by replacing the D-Ile to D-allo-Ile. Replacements of D-Pro to D-Methyl Pro to suppress the main-chain *cis*/*trans* conversion at the Pro residue are also underway.

3. Experimental

3.1. General

^1H and ^{13}C NMR spectra were measured with a Bruker AM-300 FT-NMR spectrometer in $CDCl_3$ or CD_3OD at 300 and 75 MHz, respectively. Chemical shifts were relative to tetramethylsilane as

Table 1

compound	IC_{50} (μM)	compound	IC_{50} (μM)
 11	no inhibition	 16	520
 12	1100	 17	420
 2	160	 18	1000
 13	380	 20	1500
 14	290	 21	110
 15	160	 22	2000

an internal standard. The coupling constants were given in Hz. Mass spectra were obtained on JMS-SX 102A and Bruker Daltonics autoflex mass spectrometers. Optical rotations were determined with a HORIBA SEPA-300 polarimeter. Preparative HPLC was performed using a COSMOSIL 5C18-ARII column (10 × 250 mm) with a linear gradient of CH_3CN containing 0.1% TFA in 0.1% aqueous TFA at a flow rate of 3.0 mL/min on a HITACHI ELITE LaChrom system (OD, 220 nm). For analytical HPLC, a COSMOSIL 5C18-ARII column (4.6 × 150 mm) was employed with a linear gradient of CH_3CN containing 0.1% TFA in 0.1% aqueous TFA at a flow rate of 1.0 mL/min on a HITACHI ELITE LaChrom system (OD, 220 nm).

3.2. (*S*)-4-Isopropyl-3-(4-methylpentanoyl)oxazolidin-2-one 4

A solution of *n*-BuLi (1.6 mL, 2.6 mmol, 1.6 M in hexane) was added dropwise to a stirred solution of **3** (310 mg, 2.4 mmol) in THF (5.0 mL) at −78 °C. The mixture was stirred at this temperature for 30 min. In a separate flask containing 4-methylvaleric acid (0.30 mL, 2.4 mmol) and THF (5.0 mL) were added Et_3N (0.40 mL, 2.9 mmol) and isobutylchloroformate (0.34 mL, 2.6 mmol) at 0 °C. After stirring for 30 min, the lithio-(4*S*)-4-isopropyl-2-oxazolidinone was added at 78 °C and stirred for 1 h. The reaction was quenched with saturated aqueous $NaHCO_3$. The organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (hexane/EtOAc = 6:1) to yield **4** (800 mg, 76%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (d, J = 6.9 Hz, 3H), 0.91–0.94 (m, 9H), 1.52–1.65 (m, 3H), 2.31–2.42 (m, 1H), 2.80–2.91 (m, 1H), 2.94–3.05 (m, 1H), 4.20 (dd, J = 9.0, 3.3 Hz, 1H), 4.27 (t, J = 8.6 Hz, 1H), 4.41–4.46 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.6, 17.9, 22.2, 22.3, 27.6, 28.3, 33.2, 33.5, 58.3, 63.2, 154.0, 173.5. $[\alpha]_D^{28}$ +81 ($CHCl_3$, c 1.5).

3.3. (*R*)-*tert*-Butyl 2-((2*R*,3*S*)-2-hydroxy-3-((*S*)-4-isopropyl-2-oxooxazolidine-3-carbonyl)-5-methylhexyl)pyrrolidine-1-carboxylate 6

To a solution of **4** (800 mg, 3.5 mmol) in CH_2Cl_2 (5.0 mL) were added *n*-Bu_2BOTf (800 mg, 2.9 mmol) and DIEA (0.82 mL, 4.6 mmol) at 0 °C. After the mixture had been stirred for 30 min, the reaction mixture was cooled to 78 °C, and **5** (390 mg, 1.8 mmol) in CH_2Cl_2 (5.0 mL) was added. After the resulting mixture had been stirred for 30 min, the temperature was raised up to room temperature. The reaction mixture was cooled to 0 °C and quenched with saturated aqueous NH_4Cl and 30% H_2O_2. The mixture was extracted with EtOAc and the whole was washed with saturated aqueous $NaHCO_3$, water, and brine. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was roughly purified by silica gel column chromatography (hexane/EtOAc = 2:1) to yield **6**. This compound was used next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (d, J = 6.6 Hz, 3H), 0.90–0.94 (m, 9H), 1.37–1.62 (m, 5H), 1.45 (s, 9H), 1.82–2.00 (m, 4H), 2.31–2.41 (m, 1H), 3.31 (brt, J = 6.2 Hz, 2H), 3.71–3.73 (m, 1H), 4.17–4.32 (m, 3H), 4.46–4.51 (m, 1H), 5.04 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.6, 17.9, 21.9, 23.4, 26.5, 28.3, 28.5, 31.1, 37.2, 39.6, 45.3, 46.4, 53.7, 58.9, 62.9, 69.2, 79.7, 154.0, 156.5, 175.1.

3.4. (*S*)-2-((*R*)-2-((*R*)-1-(*tert*-Butoxycarbonyl)pyrrolidin-2-yl)-1-hydroxyethyl)-4-methylpentanoic acid 7

To a solution of the crude compound **6** in MeOH (5.0 mL) was added NaOMe (300 mg, 5.5 mmol) at 15 °C and stirred for 16 h. The reaction was quenched with saturated aqueous NH_4Cl. The mixture was extracted with EtOAc and the whole was washed with 1 M HCl, saturated aqueous $NaHCO_3$, water, and brine. The combined organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was roughly purified by silica gel column chromatography (hexane/EtOAc = 4:1). The crude product, without further purification, was dissolved in MeOH/1 M NaOH (1:1, 10 mL) and stirred for 16 h. The reaction was quenched with 1 M HCl. The mixture was extracted with EtOAc, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH/AcOH = 200:2.5:0.5) to yield **7** (210 mg, 35%, 3 steps) as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (d, J = 6.6 Hz, 3H), 0.93 (d, J = 6.3 Hz, 3H), 1.31–1.53 (m, 2H), 1.47 (s, 9H), 1.56–1.75 (m, 3H), 1.83–2.00 (m, 4H), 2.58–2.64 (m, 1H), 3.27–3.36 (m, 2H), 3.73–3.75 (m, 1H), 4.15–4.20 (m,1H). ^{13}C NMR (75 MHz,$CDCl_3$): δ 21.7, 23.3, 26.2, 28.4, 31.2, 36.6, 39.5, 46.7, 48.5, 53.6, 68.3, 80.5, 157.1, 176.8. LRMALD-IMS, m/z 352.19 for $[M+Na]^+$ (calcd for $C_{17}H_{31}NNaO_5$ 352.21). $[\alpha]_D^{28}$ 11 ($CHCl_3$, c 1.5).

3.5. (*S*)-2-((*R*)-2-((*R*)-1-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)pyrrolidin-2-yl)-1-hydroxyethyl)-4-methylpentanoic acid 8

To a solution of carboxylic acid **7** (210 mg, 0.64 mmol) in CH_2Cl_2 (5.0 mL) was added TFA (5.0 mL) and stirred for 1 h. The solvent was removed under reduced pressure. The crude product was dissolved in DMF (5.0 mL) and then Et_3N (0.27 mL, 1.9 mmol) and Fmoc-OSu (320 mg, 0.95 mmol) were added to the solution. After the mixture had been stirred for 3 h, the reaction was quenched with saturated aqueous NH_4Cl and AcOEt. The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH/AcOH = 200:2.5:0.5) to yield **8** (210 mg, 73%) as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (d, J = 6.0 Hz, 3H), 0.92 (d, J = 6.3 Hz, 3H), 1.29–1.41 (m, 1H), 1.44–1.55 (m, 2H), 1.59–1.75 (m, 3H), 1.90–2.00 (m, 3H), 2.58–2.64 (m, 1H), 3.37 (t, J = 6.5 Hz, 2H), 3.69–3.72 (m, 1H), 4.21–4.26 (m, 2H), 4.40 (dd, J = 10.2, 6.6 Hz, 1H), 4.49 (dd, J = 10.5, 6.9 Hz, 1H), 7.32 (t, J = 7.5 Hz, 2H), 7.40 (t, J = 7.2 Hz, 2H), 7.59 (d, J = 7.2 Hz, 2H), 7.76 (d, J = 7.5 Hz, 2H). ^{13}C NMR (75 MHz,$CDCl_3$): δ 21.9, 23.6, 23.8, 26.4, 31.4, 36.9, 39.6, 46.7, 47.5, 48.7, 54.7, 67.8, 68.5, 120.2, 125.1, 125.2, 127.3, 128.0, 141.55, 141.57, 143.9, 144.0, 157.5, 177.3. HRFABMS, m/z 452.2435 for $[M+H]^+$ (calcd for $C_{27}H_{34}NO_5$ 452.2437). $[\alpha]_D^{22}$ 2.8 ($CHCl_3$, c 0.24).

3.6. RI-peptide resin 10

The deprotected peptide resin **10** was constructed by Fmoc-based solid phase peptide synthesis using Fmoc-NH-SAL-resin (0.47 mmol/g, 66 mg, 0.037 mmol). Piperidine (20%) in DMF was added to the resin. After agitating for 20 min, the resin was washed with DMF. To this resin were added Fmoc-D-AA-OH (0.099 mmol), HOBt·H_2O (13 mg, 0.099 mmol), DIPCDI (15 μL, 0.099 mmol), and DIEA (17 μL, 0.099 mmol) in DMF. The mixture was agitated for 2 h and was washed with DMF. Completion of the coupling reaction was confirmed using Kaiser ninhydrin test. Following piperidine treatments and couplings of Fmoc-AA-OH were repeated as above. Finally, coupling of **8** (44 mg, 0.099 mmol) was carried out with HOAt (13 mg, 0.099 mmol), DIPCDI (15 μL, 0.099 mmol), and DIEA (17 μL, 0.099 mmol). The mixture was agitated for 18 h, and the resin was washed with DMF. After agitating with piperidine (20%) in DMF for 20 min, the product resin **10** was washed with DMF.

3.7. RI-peptide 11

TFA was added to the dried resin **10** and the mixture was agitated for 2 h. The mixture was filtered, and TFA was removed under reduced

pressure. Ether was added and the precipitate was purified by preparative HPLC to yield **11** (5.0 mg, 22% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.90–1.06 (m, 18H), 1.17–1.27 (m, 1H), 1.50–1.59 (m, 3H), 1.63–1.79 (m, 3H), 1.87 (m, 3H), 1.95–2.16 (m, 8H), 2.18–2.38 (m, 3H), 2.47–2.53 (m, 1H), 3.46–3.89 (m, 4H), 3.94–4.00 (m, 1H), 4.17–4.30 (m, 1H), 4.38–4.51 (m, 2H), 4.53 (dd, J = 8.1, 5.1 Hz, 1H), 8.04 (d, J = 8.7 Hz, 1H), 8.20 (d, J = 8.1 Hz, 1H). LRMALDIMS, m/z 635.26 for $[M+H]^+$ (calcd for $C_{33}H_{59}N_6O_6$ 635.45).

3.8. RI-peptide 12

To **10** (0.037 mmol) in CH_2Cl_2/DMF (1:1) were added benzoyl chloride (10 μL, 0.075 mmol) and Et_3N (15.6 μL, 0.112 mmol). The mixture was agitated for 30 min, and the resin was washed with DMF. After the resin was dried under reduced pressure, TFA was added and the mixture was agitated for 2 h. The mixture was filtered, and TFA was removed under reduced pressure. Ether was added and the precipitate was purified by preparative HPLC to yield **12** (2.8 mg, 10% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.86–0.92 (m, 12H), 0.95 (d, J = 6.6 Hz, 3H), 0.99 (d, J = 6.6 Hz, 3H), 1.16–1.34 (m, 2H), 1.46–1.65 (m, 5H), 1.67–1.89 (m, 4H), 1.95–2.14 (m, 8H), 2.18–2.29 (m, 2H), 2.41–2.48 (m, 1H), 3.06–3.09 (m, 1H), 3.46–3.58 (m, 3H), 3.62–3.75 (m, 3H), 3.78–3.87 (m, 1H), 3.89–4.01 (m, 1H), 4.20–4.28 (d, J = 9.3 Hz, 2H), 4.41–4.46 (m, 2H), 7.44–7.53 (m, 5H). LRMALDIMS, m/z 761.30 for $[M+Na]^+$ (calcd for $C_{40}H_{62}N_6NaO_7$ 761.46).

3.9. RI-peptide 13

To **10** (0.037 mmol) in CH_2Cl_2/DMF (1:1) were added benzyl chloroformate (10 drops from a syringe), Et_3N (15 drops from a syringe), and catalytic amount of DMAP. The mixture was agitated for 30 min, and the product was isolated and purified as above to yield **13** (3.8 mg, 13% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.86–0.90 (m, 12H), 0.94 (d, J = 6.6 Hz, 3H), 0.99 (d, J = 6.6 Hz, 3H), 1.18–1.25 (m, 1H), 1.46–1.72 (m, 6H), 1.82–2.16 (m, 12H), 2.19–2.39 (m, 3H), 3.39–3.44 (m, 1H), 3.61–3.72 (m, 3H), 3.79–3.87 (m, 1H), 3.90–3.97 (m, 1H), 4.13 (brs, 1H), 4.22–4.25 (m, 1H), 4.41–4.47 (m, 2H), 4.65 (dd, J = 8.3, 5.3 Hz, 1H), 5.13 (s, 2H), 7.23–7.37 (m, 5H), 8.02–8.08 (m, 2H). LRMALDIMS, m/z 791.77 for $[M+Na]^+$ (calcd for $C_{41}H_{64}N_6NaO_8$ 791.47).

3.10. RI-peptide 14

To **10** (0.037 mmol) in CH_2Cl_2/DMF (1:1) were added *trans*-cinnamic acid (15 mg, 0.093 mmol), HOBt·H_2O (14 mg, 0.092 mmol), DIPCDI (15 μL, 0.093 mmol), and DIEA (16 μL, 0.093 mmol). The mixture was agitated for 2 h, and the product was isolated and purified as above to yield **14** (8.2 mg, 29% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.85–0.99 (m, 18H), 1.17–1.27 (m, 1H), 1.43–1.67 (m, 6H), 1.71–1.86 (m, 2H), 1.94–2.16 (m, 10H), 2.18–2.24 (m, 2H), 2.38–2.45 (m, 1H), 3.53–3.84 (m, 6H), 3.92–3.95 (m, 1H), 4.23–4.26 (m, 1H), 4.41–4.49 (m, 3H), 4.63–4.67 (m, 1H), 6.95 (d, J = 15.6 Hz, 1H), 7.36–7.42 (m, 3H), 7.57–7.67 (m, 3H), 8.07 (d, J = 8.4 Hz, 1H). LRMALDIMS, m/z 787.74 for $[M+Na]^+$ (calcd for $C_{42}H_{64}N_6NaO_7$ 787.47).

3.11. RI-peptide 15

To **10** (0.037 mmol) in CH_2Cl_2/DMF (1:1) were added 3-phenylpropanoic acid (15 mg, 0.091 mmol), HOBt·H_2O (14 mg, 0.092 mmol), DIPCDI (15 μL, 0.093 mmol), and DIEA (16 μL, 0.093 mmol). The mixture was agitated for 2 h, and the product was isolated and purified as above to yield **15** (6.0 mg, 21% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.86–0.91 (m, 12H), 0.94 (d, J = 6.6 Hz, 3H), 0.99 (d, J = 6.9 Hz, 3H), 1.16–1.27 (m, 1H), 1.33–1.70 (m, 7H), 1.78–2.13 (m, 11H), 2.19–2.32 (m, 2H), 2.35–2.42 (m, 1H), 2.64 (t, J = 7.2 Hz, 1H), 2.93 (t, J = 7.5 Hz, 1H), 3.37–3.49 (m, 2H), 3.59–3.71 (m, 2H), 3. 83 (dd, J = 16.7, 7.1 Hz, 1H), 3.90–3.97 (m, 1H), 4.16–4.28 (m, 1H), 4.24 (d, J = 9.0 Hz, 1H), 4.30–4.39 (m, 1H), 4.41–4.45 (m, 2H), 4.65 (dd, J = 8.0, 5.0 Hz, 1H), 7.17–7.37 (m, 5H), 8.09 (br d, J = 8.4 Hz, 1H). LRMALDIMS, m/z 789.38 for $[M+Na]^+$ (calcd for $C_{42}H_{66}N_6NaO_7$ 789.49).

3.12. RI-peptide 16

To **10** (0.037 mmol) in CH_2Cl_2/DMF (1:1) were added isovaleryl chloride (20 drops from a syringe), Et_3N (25 drops from a syringe), and catalytic amount of DMAP. The mixture was agitated for 30 min, and the product was isolated and purified as above to yield **16** (2.4 mg, 9% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.84–1.00 (m, 24H), 1.16–1.27 (m, 2H), 1.39–1.47 (m, 2H), 1.51–1.70 (m, 4H), 1.81–1.85 (m, 1H), 1.96–2.14 (m, 11H), 2.22–2.31 (m, 4H), 2.37–2.42 (m, 1H), 3.06–3.09 (m, 1H), 3.46–3.55 (m, 3H), 3.62–3.72 (m, 2H), 3.79–3.87 (m, 1H), 3.91–3.98 (m, 1H), 4.24 (d, J = 9.0 Hz, 2H), 4.35–4.48 (m, 2H), 4.44 (d, J = 8.1 Hz, 1H). LRMALDIMS, m/z 741.36 for $[M+Na]^+$ (calcd for $C_{38}H_{66}N_6NaO_7$ 741.49).

3.13. RI-peptide 17

To **10** (0.037 mmol) in CH_2Cl_2/DMF (1:1) were added 2-propylpentanoic acid (14 μL, 0.088 mmol), HOBt·H_2O (13 mg, 0.085 mmol), DIPCDI (14 μL, 0.088 mmol), and DIEA (15 μL, 0.088 mmol). The mixture was agitated for 2 h, and the product was isolated and purified as above to yield **17** (2.0 mg, 7% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.85–0.98 (m, 24H), 1.19–1.50 (m, 10H), 1.55–1.62 (m, 4H), 1.69–1.73 (m, 1H), 1.76–1.88 (m, 1H), 1.93–2.16 (m, 10H), 2.20–2.35 (m, 2H), 2.38–2.45 (m, 1H), 2.60–2.68 (m, 1H), 3.48–3.61 (m, 3H), 3.63–3.72 (m, 2H), 3.67 (d, J = 8.7 Hz, 1H), 3.79–3.87 (m,1H), 3.91–3.98 (m, 1H), 4.16–4.27 (m, 1H), 4.38–4.47 (m, 3H), 4.65 (dd, J = 8.1, 4.8 Hz, 1H), 7.99–8.10 (m, 2H). LRMALDIMS, m/z 783.47 for $[M+Na]^+$ (calcd for $C_{41}H_{72}N_6NaO_7$ 783.54).

3.14. RI-peptide 18

To **10** (0.037 mmol) in CH_2Cl_2 were added trimethylacetyl chloride (10 drops from a syringe), Et_3N (15 drops from a syringe), and catalytic amount of DMAP. The mixture was agitated for 2 h, and the product was isolated and purified as above to yield **18** (4.3 mg, 16% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.85–0.92 (m, 12H), 0.94 (d, J = 6.6 Hz, 3H), 0.99 (d, J = 6.9 Hz, 3H), 1.15–1.33 (m, 1H), 1.26 (s, 9H), 1.37–1.47 (m, 3H), 1.53–1.68 (m, 4H), 1.79–1.88 (m, 1H), 1.90–2.16 (m, 10H), 2.19–2.33 (m, 2H), 2.35–2.43 (m, 1H), 3.48–3.56 (m, 2H), 3.62–3.73 (m, 2H), 3.79–3.87 (m, 2H), 3.91–3.98 (m, 1H), 4.24 (d, J = 9.0 Hz, 1H), 4.36–4.47 (m, 3H), 4.65 (dd, J = 8.3, 5.0 Hz, 1H), 8.08 (br d, J = 8.4 Hz, 1H). LRMALDIMS, m/z 741.36 for $[M+Na]^+$ (calcd for $C_{38}H_{66}N_6NaO_7$ 741.49).

3.15. RI-peptide 20

To **10** (0.037 mmol) in CH_2Cl_2/DMF (1:1) were added Fmoc-D-Ile-OH (35 mg, 0.099 mmol), HOBt·H_2O (15 mg, 0.099 mmol), DIPCDI (15 μL, 0.099 mmol), and DIEA (17 μL, 0.099 mmol). The mixture was agitated for 2 h. After the resin was washed with DMF, piperidine (20%) in DMF was added to the resin. After agitating

for 20 min, the deprotected resin was washed with DMF to yield **19**. The resin was dried under reduced pressure, and then TFA was added and the mixture was agitated for 2 h. The mixture was filtered, and TFA was removed under reduced pressure. Ether was added to the crude product to form the precipitate. The precipitate was washed with ether and purified by preparative HPLC to yield **20** (7.5 mg, 27% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.85–1.02 (m, 21H), 1.06 (d, J = 6.9 Hz, 3H), 1.15–1.30 (m, 2H), 1.44–1.69 (m, 7H), 1.74–1.77 (m, 1H), 1.81–1.90 (m, 1H), 1.93–2.16 (m, 11H), 2.19–2.25 (m, 2H), 2.38–2.42 (m, 1H), 3.52–3.61 (m, 3H), 3.64–3.73 (m, 2H), 3.78–3.87 (m, 1H), 3.90–3.97 (m, 1H), 4.02 (d, J = 6.3 Hz, 1H), 4.22–4.30 (m, 1H), 4.36–4.50 (m, 3H), 4.66 (dd, J = 8.0, 5.0 Hz, 1H), 8.07–8.10 (br d, J = 8.4 Hz, 1H). LRMALDIMS, m/z 748.27 for $[M+H]^+$ (calcd for $C_{39}H_{70}N_7O_7$ 748.53).

3.16. RI-peptide 21

To the deprotected resin **19** prepared as above, acetic anhydride (2.5 μL, 0.025 mmol), Et_3N (5.1 μL, 0.037 mmol), and catalytic amount of DMAP were added. The mixture was agitated for 10 min, and the product was isolated and purified as above to yield **21** (3.1 mg, 11% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.85–0.96 (m, 21H), 0.99 (d, J = 6.9 Hz, 3H), 1.16–1.24 (m, 2H), 1.38–1.45 (m, 3H), 1.53–1.69 (m, 4H), 1.78–1.87 (m, 3H), 1.93–2.13 (m, 10H), 1.98 (s, 3H), 2.19–2.27 (m, 2H), 2.37–2.43 (m, 1H), 3.06–3.09 (m, 1H), 3.50–3.56 (m, 2H), 3.59–3.72 (m, 3H), 3.77–3.84 (m, 2H), 3.93–3.96 (m, 1H), 4.22 (br d, J = 9.0 Hz, 1H), 4.28–4.31 (m, 1H), 4.41–4.45 (m, 2H), 4.51 (br d, J = 9.6 Hz, 1H), 4.65 (dd, J = 8.1, 5.1 Hz, 1H). LRMALDIMS, m/z 812.32 for $[M+Na]^+$ (calcd for $C_{41}H_{71}N_7NaO_8$ 812.53).

3.17. RI-peptide 22

To the deprotected resin **19** prepared as above were added Fmoc-D-Gln-OH (36 mg, 0.098 mmol), $HOBt{\cdot}H_2O$ (15 mg, 0.098 mmol), DIPCDI (15 μL, 0.098 mmol), and DIEA (17 μL, 0.098 mmol). The mixture was agitated for 2 h. After the resin was washed with DMF, piperidine (20%) in DMF was added to the resin. After agitating for 20 min, the deprotected resin was washed with DMF. After the resin was dried under reduced pressure, TFA was added and the mixture was agitated for 2 h. The product was isolated and purified as above to yield **22** (5.8 mg, 16% from Fmoc-NH-SAL-resin) as a white powder. ^{1}H NMR (300 MHz, CD_3OD): δ 0.84–1.05 (m, 24H), 1.16–1.29 (m, 2H), 1.39–1.74 (m, 8H), 1.84–1.88 (m, 2H), 1.94–2.16 (m, 12H), 2.19–2.33 (m, 2H), 2.36–2.54 (m, 3H), 3.38–3.44 (m, 1H), 3.60–3.72 (m, 3H), 3.79–3.86 (m, 2H), 3.89–3.94 (m, 1H), 3.97–4.01 (m, 1H), 4.21–4.28 (m, 1H), 4.35–4.49 (m, 4H), 4.66 (dd, J = 8.1, 4.8 Hz, 1H), 8.02 (br d, J = 8.4 Hz, 1H), 8.13 (br d, J = 8.7 Hz, 1H). LRMALDIMS, m/z 876.29 for $[M+H]^+$ (calcd for $C_{44}H_{78}N_9O_9$ 876.59).

3.18. Measurement of inhibitory activity

Enzyme assays were carried out using the synthetic HTLV-1 protease mutant[14] at an enzyme concentration of 5.6 nM. The reaction mixture (0.5 M sodium acetate buffer, pH 5.6, containing 10% glycerol, 10 mM DTT and 4 M NaCl) was analyzed on a Cosmosil 5C18-ARII column (4.6 × 150 mm), employing a linear gradient of CH_3CN (10–40%, 30 min) in aq 0.1% TFA. Each IC_{50} value was obtained from a sigmoidal dose–response curve obtained from the decrease of the substrate in the reaction mixture. Each experiment was repeated 3 times.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.02.050.

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