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Identification of peptidomimetic HTLV-I protease inhibitors containing hydroxymethylcarbonyl (HMC) isostere as the transition-state mimic

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Abstract—Towards the development of chemotherapy for the infection by human T-cell leukemia virus type I (HTLV-I), we have established evaluation systems for HTLV-I protease (PR) inhibitors using both recombinant and chemically synthesized HTLV-I PRs. Newly synthesized substrate-based inhibitors containing hydroxymethylcarbonyl (HMC) isostere showed potent anti-HTLV-I PR activity.

1. Introduction

The human T-cell leukemia virus type I (HTLV-I) is a retrovirus that has been clinically associated with adult T-cell leukemia (ATL)[1] and HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP).[2,3] Estimations in 1997 revealed that between one and two million people were infected with HTLV-I in Japan where the virus is most prevalent in the world.[4] Since the most recent study reported that increased HTLV-I proviral load in central nervous system is a strong predictor for the development of HAM/TSP,[5] effective anti-HTLV-I agents might contribute to suppress the progression of HAM/TSP. However, such chemotherapeutic agents based on the specific anti-HTLV-I activity have not been developed.

HTLV-I encodes a virus-specific aspartic protease (PR) responsible for processing the *gag* and *gag-pro-pol* polyproteins leading to the proliferation of the retrovirus.[6] Since this process is essential for the retroviral replication, it is suggested that HTLV-I PR is one of the major targets to develop the specific anti-HTLV-I agents. The inhibitors containing statine or hydroxyethylamine isostere were already reported.[7] This idea is also supported by the findings that the inhibitors of HIV PR, which is a similar aspartic protease as HTLV-I PR, made a significant contribution to the successful treatments of AIDS.[8] For the last decade, we have developed HIV PR inhibitors, named 'KNI compounds', based on the concept of 'substrate transition-state mimic',[9] and found that an α-hydroxy-β-amino acid derivative, allophenylnorstatine (Apns), which has a hydroxymethylcarbonyl (HMC) isostere (Fig. 1), provided a unique interaction with the active site of HIV PR essentially similar to that of the substrates,[9] and the KNI compounds having Apns exhibited highly potent inhibition of HIV-1 replication. Furthermore, our recent studies using HMC compounds demonstrated effective inhibition of the malaria parasite aspartic protease, plasmepsin II,

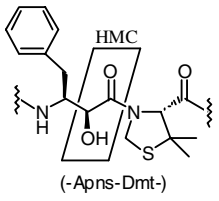

Figure 1. Structure of HMC isostere.

Keywords: Human T-cell leukemia virus type I (HTLV-I); Adult T-cell leukemia (ATL); Aspartic protease; Inhibitor; Hydroxymethylcarbonyl (HMC) isostere; Chemical ligation.
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A

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        10          20          30          40
MGHHHHHHHH HHSSGHIDDD DKHMPVIPLD PARRPVIKAQ
        50          60          70          80
VDTQTSHPKT IEALLDTGAD MTVLPIALFS SNTPLKNTSV
        90         100         110         120
LGAGGQTQDH FKLTSLPVLI RLPFRTTPIV LTSCLVDTKN
       130         140
NWAIIGRDAL QQCQGVLYLP EAKGPPVIL
```

B

```
        10          20          30          40
PVIPLDPARR PVIKAQVDTQ TSHPKTIEAL LDTGADMTVL
        50          60          70          80
PIALFSSNTP LKNTSVLGAX XQTQDHFKLT SLPVLIRLPF
        90         100         110         120
RTTPIVLTSA LVDTKNNWAI IGRDALQQAQ GVLYLPEAKG

PPVIL
```

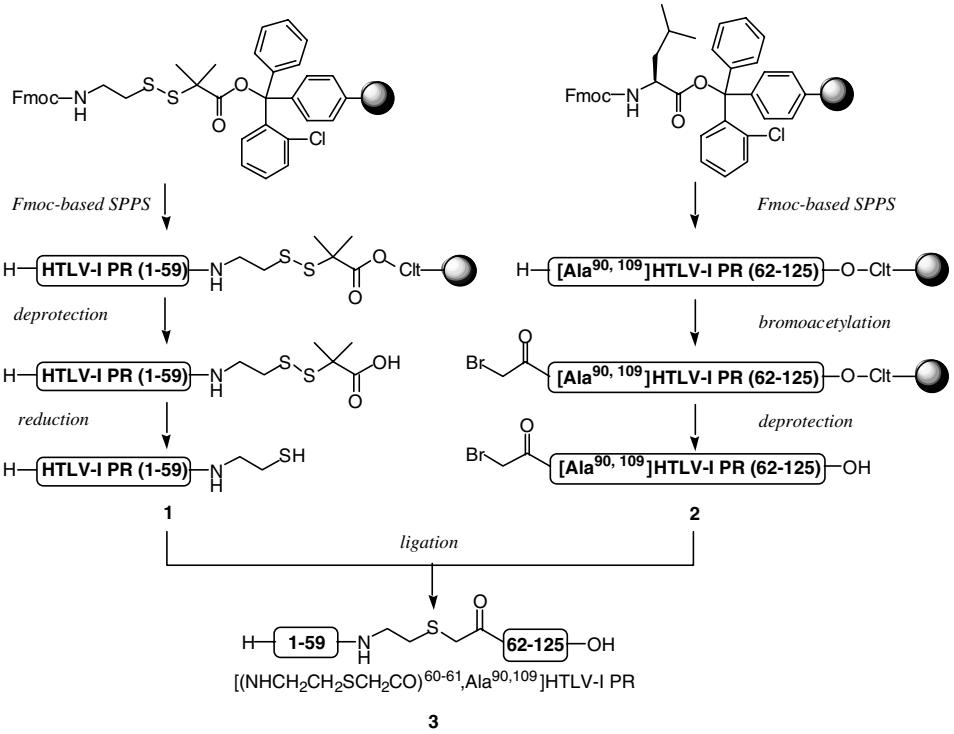
Figure 2. The amino acid sequences of prepared HTLV-I PRs. (A) recombinant HTLV-I PR. Underline: His-tag sequence, boldface: mature HTLV-I PR sequence. (B) synthetic [($NH_2CH_2CH_2$-SCH_2CO)$^{60-61}$, Ala^{90,109}]-HTLV-I PR. Underline: substitution sites from Cys to Ala. XX: thioether linkage (–$NHCH_2CH_2SCH_2CO$–).

suggesting that this motif can be applicable to other aspartic proteases as a universal inhibitory machinery.[10] Based on these backgrounds, to obtain potent HTLV-I PR inhibitors, it would be significant to know whether the existing HIV PR inhibitors with Apns are effective on HTLV-I PR inhibition. Hence, in the present study, we established a screening system for HTLV-I PR inhibitory activity using both recombinant and chemically synthesized HTLV-I PRs and evaluated the existing and newly synthesized compounds having the HMC motif.

2. Expression and synthesis of HTLV-I PRs

Although several studies for the expression of HTLV-I PR in *E. coli*[11] and its chemical synthesis,[12] which are useful for screening HTLV-I inhibitors, were reported, they seem practically difficult in our laboratory. We independently prepared both recombinant (rec) and chemically synthesized (syn) HTLV-I PRs (Fig. 2) by easy ways to establish a screening system. In the preparation of recombinant PR in *E. coli*, the HTLV-I PR gene[13] was inserted down the stream of a decahistidine-containing leader sequence under the control of T7 promoter of pET19b (Novagen). Then, the constructed plasmid was introduced into *E. coli* BL21(DE3)-pLys S cells and the gene expression was induced by the addition of IPTG.[14] The cultured cells were collected by centrifugation and resuspended in lysis buffer A (10 mM Tris containing 100 mM NaH_2PO_4, 1 mM EDTA, 10 mM 2-mercaptoethanol and 8 M urea; pH 8.0). After the bacterial cell lysate was centrifuged, the obtained supernatant was applied to a His-Bind affinity column[15] to give the pure PR. From the SDS-PAGE analysis,[16] the rec-PR showed a single band with a molecular size of approximately 16 kDa.

[$NHCH_2CH_2SCH_2CO^{60-61}$, Ala^{90,109}]-HTLV-I PR was synthesized using stepwise Fmoc-based solid phase peptide synthesis (SPPS) followed by thioether forming ligation (Fig. 2, Scheme 1).[17] Briefly, a mercaptoethylamide peptide segment, HTLV-I PR (1-59)-$NHCH_2CH_2SH$ **1**, was prepared starting from an Fmoc-AEDI-O-Clt-resin.[18,19] The SPPS was achieved by an ABI 431A synthesizer with a standard DCC-HOBt protocol, and then the



Scheme 1. Synthetic scheme for an HTLV-I PR analog **3** using thioether-forming ligation.

peptide segment containing a linker moiety was cleaved by HF–dimethyl sulfide–*m*-cresol (3:6:1) (0 °C, 1 h). The treatment of the resultant with dithiothreitol (DTT) in 6 M guanidine·HCl containing 200 mM Tris (pH 8.5) gave the desired peptide as the main product. The crude peptide **1** was purified by RP-HPLC and characterized by amino acid analysis of its hydrolysate and MALDI-TOF MS.[20] Another segment, $BrCH_2CO$-[$Ala^{90,109}$]-HTLV-I PR (62–125) **2** was also prepared by the conventional SPPS, and bromoacetylation of the N-terminal amino group was carried out using bromoacetic acid and 1,3-diisopropylcarbodiimide.

Obtained protected peptide resin was deprotected and cleaved from resin with HF–*m*-cresol (0 °C, 1 h), followed by the purification using RP-HPLC, and characterization by amino acid analysis and MALDI-TOF MS.[21]

The ligation of the two segments **1**, **2** was carried out in 6 M guanidine·HBr-containing 200 mM Tris (pH 8.5) at room temperature under an Ar atmosphere for 3 h with vigorous stirring (Fig. 3). Then, the treatment with DTT reduced the undesirable disulfide dimer of peptide segment **1**, which was removed by the gel filtration with Hiload 16/60 superdex 75 prep grade in 8 M urea-containing 0.5 M KH_2PO_4. The purified protein was characterized as [$NHCH_2CH_2SCH_2CO^{60-61}$, $Ala^{90,109}$]-HTLV-I PR **3** by amino acid analysis and MALDI-TOF MS[22] and showed a single peak on RP-HPLC analysis.

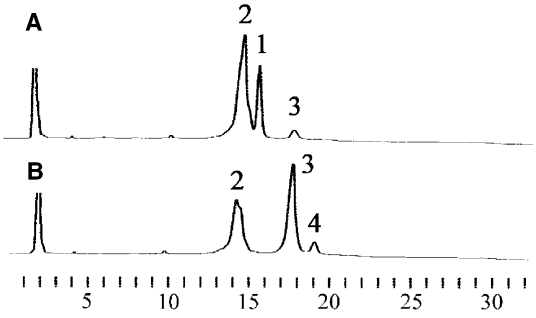


Figure 3. HPLC profiles of ligation reaction of peptide **1** (1.0 equiv) and **2** (1.5 equiv) at 0 h (A) and 3 h (B). The peaks **3** and **4** show [$NHCH_2CH_2SCH_2CO^{60-61}$, $Ala^{90,109}$]-HTLV-I PR and disulfide dimer of peptide **1**, respectively. HPLC was performed using a C18 reverse phase column with a liner gradient of 35–55% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate of 0.9 mL/min with a detection at UV 230 nm.

Both protein solutions were concentrated using a centrifugal filter (Centricon Plus-20, Millipore) followed by the replacement with 7.5 M guanidine·HCl and dialysis with a large excess amount of 20 mM PIPES, pH 7.0, containing 2 mM DTT, 1 mM EDTA, 150 mM NaCl and 10% glycerol at 25 °C for 16 h to fold into the matured protein structures with the PR activity.[11c] Then, the solution was concentrated again using the same centrifugal filter to a protein concentration greater than 5 μM, and this enzyme solution was used for the HTLV-I PR inhibition assay.

3. Synthesis of HTLV-I PR inhibitors

Based on an HTLV-I cleavage site, octapeptides **10**–**13** with the HMC motif were newly designed and synthesized manually using a conventional Fmoc-based SPPS. The peptide resins were cleaved by TFA–*m*-cresol–thioanisole–H_2O (85:5:5:5), and the octapeptides were purified by RP-HPLC and characterized by MALDI-TOF MS before biological evaluation. Synthesis of **5**–**9** was previously reported.[23–25]

4. Results and discussion

Both the recombinant and synthetic HTLV-I PR analogs specifically hydrolyzed synthetic peptide substrates, APQVL*PVMHP (p19/24), KTKVL*VVQPK (p24/15)

Table 1. Inhibition of HTLV-I PRs and HIV-1 PR by HMC compounds

Compound	Structure	Inhibition (%)				
	P4 P3 P2 P1 P1′ P2′ P3′ P4′	rec-HTLV-I PR		syn-HTLV-I PR		HIV-1 PR[b]
		At 100 μM	At 5 μM	At 100 μM	At 5 μM	At 50 nM
5 (KNI-272)	iQoa-Mta-Apns-Thz -$NHBu^t$	10	—	39	—	97
6 (KNI-577)	Bz(3-OH, 2-Me)-Apns-Dmt-$NHBu^t$	38	—	57	—	88
7 (KNI-727)	Pac(diMe)-Apns-Dmt-$NHBu^t$	43	—	32	—	96
8 (KNI-764)	Bz(3-OH, 2-Me)-Apns-Dmt-NHBzl(Me)	31	—	79	—	96
9 (KNI-840)	Pac(diMe)-Apns-Dmt-NHBzl(Me)	19	—	11	—	98
10 (KNI-10159)	H-Pro-Gln-Val-Anst -Pro- Val-Met-His-OH	93	43	—	58	3
11 (KNI-10160)	H-Pro-Gln-Val-Anst -Dmt-Val-Met-His-OH	100	63	—	76	37
12 (KNI-10161)	H-Pro-Gln-Val-Apns-Pro- Val-Met-His-OH	94	54	—	59	6
13 (KNI-10162)	H-Pro-Gln-Val-Apns-Dmt-Val-Met-His-OH	100	66	—	80	58
Ritonavir	—[a]	20	—	20	—	100
Pepstatin A	Iva-Val-Val- Sta -Ala- Sta -OH	—	17	—	15	23

[a]See Ref. 8. iQoa, isoquinolyloxyacetyl; Mta, methylthioalanine; Thz, 1,3-thiazolidine-4-carboxylic acid; $NHBu^t$, *tert*-butylamide; Bz(3-OH, 2-Me), 3-hydroxy-2-methylbenzoyl; Pac(diMe), 2,6-dimethylphenoxyacetyl; NHBzl(Me), 2-methylbenzylamide; Iva, isovaleryl; Sta, (3*S*,4*S*)-statine; Anst, allonorstatine, [(2*S*,3*S*)-3-amino-2-hydroxy-5-methylhexanoic acid]; Apns, allophenylnorstatine, [(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid]; Dmt, (*R*)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid. The modified p19/24, APQVL*NphVMHPL, was used as a substrate.

[b]HIV-1 PR inhibition was determined by monitoring the fluorescence change (305 nm, λ_{ex} = 275 nm) associated with the cleavage of the fluorogenic substrate, H-Lys-Ala-Arg-Val-Tyr*Nph-Glu-Ala-Nle-NH_2. Nph, *p*-nitrophenylalanine.

Table 2. K_i values for pepstatin A and **13**

Inhibitor	K_i (rec PR)[a] (μM)	K_i (syn PR)[a] (μM)
Pepstatin A	14.6 ± 3.8	36.5 ± 5.0
13 (KNI-10162)	3.9 ± 0.7	2.0 ± 0.5

[a] Values represent the mean of three experiments ± SEM.

and APQVL*NphVMHPL (the modified p19/24) (* indicate scissile bond),[26,27] and pepstatin A, which is known as a typical aspartyl PR inhibitor, showed a moderate inhibitory activity with K_i values of 14.6 and 36.5 μM against rec- and syn-HTLV-I PRs, respectively (Tables 1 and 2).[28,29] These K_i values were almost equal to that of previously reported recombinant HTLV-I PR ($K_i = 17$ μM).[11a] These results suggested that the both rec- and syn-HTLV-I PRs are enzymatically similar to the native HTLV-I PR even they have modifications in their structures and the screening system using these PRs is effective to evaluate HTLV-I PR inhibitors.

The inhibitory activities of the HMC compounds are summarized in Tables 1 and 2.[28,29] Compounds **5**–**9**, which were developed as potent HIV PR inhibitors, showed weak inhibitory activity against the HTLV-I PRs. Ritonavir, which is a clinically used HIV PR inhibitor, also showed weak activity. These results indicated that HIV PR inhibitors were not effective for the inhibition of HTLV-I PR, suggesting that the recognition of inhibitors is different between the two viral proteases despite the relatively similar natural substrate sequences. However, this result is compatible with the findings that the most synthetic HIV-1 PR substrates were not cleaved by HTLV-I PR, except for IRKIL*FLDG from the RT/IN site.[11c] Hence, to obtain effective HMC compounds with a potent anti-HTLV-I PR activity, we designed and synthesized new inhibitors **10**–**13** consisting of the P4–P4′ sites of the HTLV-I PR cleavage sequence at the p19/24 (MA/CA) site in the *gag* region. These octapeptides showed a potent inhibitory activity with 50% to 70% inhibition at 5 μM and a relatively decreased anti-HIV PR activity. The K_i values of **13** were 3.9 and 2.0 μM against rec- and syn-HTLV-I PRs, respectively (Table 2), which were at least 3-fold more potent than pepstatin A. The similar anti-HTLV-I PR activity shown among these peptides was probably due to the conservative modification only at the P1 and P1′ sites. These results suggest that the HMC motif functions as the inhibitory machinery of HTLV-I PR, but the recognition of the other P and P′ sites are also important for the effective interaction leading to the potent HTLV-I PR inhibition, since weak inhibitors **6**–**9** also possess the same Apns-Dmt core structure as potent compound **13**. Extensive modifications focusing on the size of the molecule and each side chain structure based on the present octapeptides are underway.

In conclusion, we have established an evaluation system for HTLV-I PR inhibitors using both recombinant and chemically synthesized HTLV-I PRs and found that newly synthesized substrate-based inhibitors with the HMC motif showed potent anti-HTLV-I PR activity.

Acknowledgements

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References and notes

1. Hinuma, Y.; Nagata, K.; Hanaoka, M.; Nakai, M.; Matsumoto, T.; Kinoshita, K.; Shirakawa, S.; Miyoshi, I. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6476.
2. Osame, M.; Usuku, K.; Izumo, S.; Ijichi, N.; Amitani, H.; Igata, A.; Matsumoto, M.; Tara, M. *Lancet* **1986**, *1*, 1031.
3. Gessain, A.; Barin, F.; Vernant, J. C.; Gout, O.; Maurs, L.; Calender, A.; De Thé, G. *Lancet* **1985**, *2*, 407.
4. Uchiyama, T. *Annu. Rev. Immunol.* **1997**, *15*, 15.
5. Osame, M. *J. Neurovirol.* **2002**, *8*, 359.
6. Oroszlan, S.; Luftig, R. B. *Curr. Top. Microbiol. Immunol.* **1990**, *157*, 153.
7. (a) Ménard, A.; Leonard, R.; Rlido, S.; Geoffre, S.; Picard, P.; Berteau, F.; Precigoux, G.; Hospital, M.; Guillemain, B. *FEBS Lett.* **1994**, *346*, 268; (b) Akaji, K.; Teruya, K.; Aimoto, S. *J. Org. Chem.* **2003**, *68*, 4755.
8. Flexner, C. N. *Eng. J. Med.* **1998**, *338*, 1281.
9. Kiso, Y. *Biopolymers* **1996**, *40*, 235.
10. Nezami, A.; Luque, I.; Kimura, T.; Kiso, Y.; Freire, E. *Biochemistry* **2002**, *41*, 2273.
11. (a) Kobayashi, M.; Ohi, Y.; Asano, T.; Hayakawa, T.; Kato, K.; Kakinuma, A.; Hatanaka, M. *FEBS Lett.* **1991**, *293*, 106; (b) Ding, Y. S.; Owen, S. M.; Lal, R. B.; Ikeda, R. A. *J. Virol.* **1998**, *72*, 3383; (c) Louis, J. M.; Oroszlan, S.; Tözsér, J. *J. Biol. Chem.* **1999**, *274*, 6660.
12. (a) Hrusková-Heidingsfeldová, O.; Bláha, I.; Urban, J.; Strop, P.; Pichová, I. *Leukemia* **1997**, *11*, 45; (b) Teruya, K.; Kawakami, T.; Akaji, K.; Aimoto, S. *Tetrahedron Lett.* **2002**, *43*, 1487.
13. The gene was kindly provided by Prof. A. Adachi of Tokushima University.
14. The cell was cultured in M9ZB medium containing 100 μg/mL ampicillin to the optical density at 600 nm of 0.6, and then the expression of the HTLV-I PR was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The inducing culture was incubated at 37 °C for 3 h.
15. The supernatant was incubated with 2 mL of Ni-NTA agarose (Qiagen) for 1 h at 25 °C with shaking. The Ni-NTA agarose was packed into an empty column and washed three times with 5 mL of buffer B (buffer A, pH 6.3). The protein was eluted with buffer C (buffer A, pH 4.5).
16. Laemmli, U. K. *Nature* **1970**, *227*, 680.
17. Englebretsen, D. R.; Garnham, B. G.; Bergman, D. A.; Alewood, P. F. *Tetrahedron Lett.* **1995**, *36*, 8871.
18. (a) Méry, J.; Brugidou, J.; Derancourt, J. *Pept. Res.* **1992**, *5*, 233; (b) Méry, J.; Granier, C.; Juin, M.; Brugidou, J. *Int. J. Peptide Protein Res.* **1993**, *42*, 44.
19. Kiso, Y.; Kimura, T.; Fujiwara, Y.; Nishizawa, N.; Matsumoto, H.; Kishida, M.; Akaji, K.; Takaku, H. *Peptides Frontiers Peptide Sci.* **1999**, *23*, 333.
20. Standard amino acid analysis of peptide **1**: Asx (7.4), Thr (5.6), Ser (2.6), Glx (2.9), Gly (2.1), Ala (6.0), Val (4.5), Met (1.0), Ile (3.4), Leu (7.2), Phe (1.0), Lys (3.0), His (0.9), Trp (0.9), Arg (1.8), Pro (7.2). MALDI-TOF MS of

peptide **1** (*m/z* 6319.89 ($M+H^+$), calcd for $C_{286}H_{465}N_{73}O_{83}S_2$: 6319.41).

21. Standard amino acid analysis of peptide **2**: Asx (5.2), Thr (3.9), Ser (1.2), Glx (5.9), Gly (3.4), Ala (5.3), Val (4.7), Ile (4.0), Leu (10.0), Tyr (0.9), Phe (1.4), Lys (3.0), His (0.7), Arg (2.5), Pro (5.9). MALDI-TOF MS of peptide **2** (*m/z* 7199.02 (M^+), calcd for $C_{328}H_{531}BrN_{86}O_{90}$: 7199.16).
22. Standard amino acid analysis of peptide **3**: Asx (10.0), Thr (11.1), Ser (5.2), Glx (9.2), Gly (5.3), Ala (11.0), Val (8.5), Met (0.9), Ile (6.6), Leu (17.2), Tyr (1.0), Phe (3.8), Lys (5.9), His (1.8), Trp (0.5), Arg (4.5), Pro (12.5). MALDI-TOF MS of peptide **3** (*m/z* 13399.9 ($M+H^+$), calcd for $C_{607}H_{998}N_{161}O_{174}S_2$: 13399.6).
23. Mimoto, T.; Imai, J.; Kisanuki, S.; Enomoto, H.; Hattori, N.; Akaji, K.; Kiso, Y. *Chem. Pharm. Bull.* **1992**, *40*, 2251.
24. Mimoto, T.; Kato, R.; Takaku, H.; Nojima, S.; Terashima, K.; Misawa, S.; Fukazawa, T.; Ueno, T.; Sato, H.; Shintani, M.; Kiso, Y.; Hayashi, H. *J. Med. Chem.* **1999**, *42*, 1789.
25. Matsumoto, H.; Kimura, T.; Hamawaki, T.; Kumagai, A.; Goto, T.; Sano, K.; Hayashi, Y.; Kiso, Y. *Bioorg. Med. Chem.* **2001**, *9*, 1589.
26. Daenke, S.; Schramm, H. J.; Bangham, C. R. M. *J. Gen. Virol.* **1994**, *75*, 2233.
27. The activities of rec- and syn-HTLV-I PRs were determined by measuring the rate of hydrolysis of the synthetic substrates, APQVL*PVMHP (p19/24), KTKVL*VVQPK (p24/15) and APQVL*NphVMHPL (the modified p19/24). The enzyme reactions contained 2 μM protease (as a dimer) and 20–200 μM substrates in 200 mM sodium citrate buffer, pH 5.3, containing 1 mM DTT, 1 M NaCl, 5 mM EDTA and 6% glycerol, which were incubated at 37 °C for various periods, and then terminated by the addition of 20% TCA. The produced peptide fragments were measured by HPLC using a C18 column with a linear gradient of 4–28% (for p19/24), 0–18% (for p24/15) and 5–35% (for the modified p19/24) CH_3CN containing 0.1% TFA for 12, 14 and 15 min, respectively, monitored at 215 nm. The concentration of each fragment was calculated with a standard curve.
28. All inhibitors were dissolved in DMSO to make a 5 mM stock solution. Final concentrations in the inhibitor assay were 2.0 μM HTLV-I PR, 200 μM substrate (the modified p19/24), 200 mM sodium citrate buffer, pH 5.3, containing 1 mM DTT, 1 M NaCl, 5 mM EDTA, 6% glycerol and 2% DMSO containing 5 or 100 μM inhibitor at 37 °C for 6 h. The residual protease activity was analyzed by the same way.
29. Inhibition constants (K_i) were determined by the method of Dixon.[30] The reactions were incubated at 37 °C for 30 min. The modified p19/24, APQVL*NphVMHPL, was used as a substrate.
30. Dixon, M. *Biochem. J.* **1953**, *55*, 170.