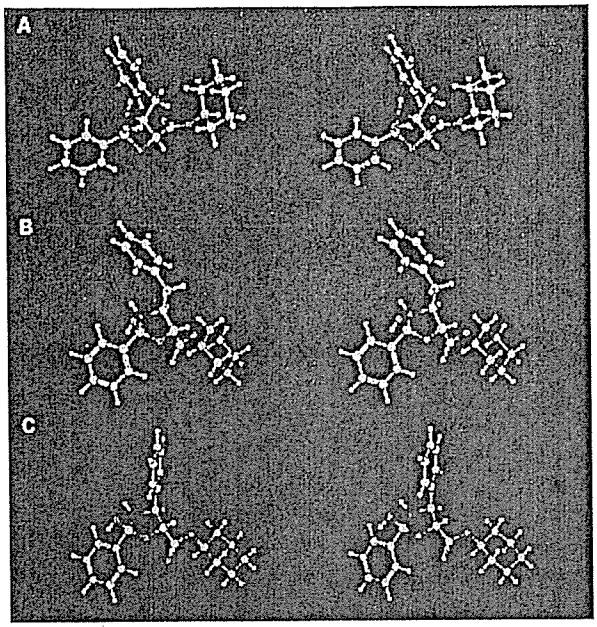


FIGURE 4 Modeling of migration intermediate of **3a–c** using the MMFF94s force field for minimizing the energy (A: **3a**; B: **3b**; C: **3c**).

model compounds, **3a–c**, that are α-hydroxy-β-amino acid derivatives with a bulky cyclohexyl ester structure in place of the taxane ring (Figure 3). Compound **3a** is an Apns derivative with a *2S,3S* configuration, while its diastereomer, **3b**, a phenylnorstatine derivative, and a phenylisoserine derivative, 3c, have a *2R,3S* configuration, as does paclitaxel.

These compounds were dissolved in phosphate-buffered saline (PBS) at pH 7.4 and incubated at 37°C to examine the migration rates. All compounds migrated to their *N*-benzoyl forms **4a–c** at the expected relatively slow rate. A clearly visible influence of stereochemistry on the migration rate was observed. The slowest migration was observed in **3a** ($t_{1/2}$ = 40.5 min) with a *2S,3S* configuration. Migration in **3b** and **3c** with a *2R,3S* configuration showed relatively similar $t_{1/2}$ values of 8.0 and 12.0 min, respectively. The observed difference in migration rates among different stereochemistry can be explained by steric hindrance in the 5-membered ring migration intermediate, as in the slow migration compound **3a**. Substituents at the 2 and 3 positions are fixed in the *syn* conformation at its intermediate (Figure 4A), while substituents of **3b** and **3c** with the *anti* conformation do not face such a steric interaction (Figure 4B and C).

Under acidic aqueous conditions, these compounds remained stable with no migration. The $t_{1/2}$ value of **3c** (the model of paclitaxel prodrug) of 12 min at pH 7.4 appeared appropriate for systemic distribution but not sufficient for metabolism and elimination. From these promising results of the model study, we designed and synthesized the new paclitaxel prodrug isotaxel (2; Figure 2) with improved water solubility. This prodrug, having no additional water-solubilizing auxiliaries and forming no by-product during conversion to the parent drug, is a 2′-*O*-benzoyl isoform of **1**. It was designed to increase water solubility with an ionized 3′-amino group, and allows conversion to **1** via O–N intramolecular acyl migration of the benzoyl group under physiological conditions (Figure 2).[35]

The water solubility of **2** · HCl was determined as 0.45 ± 0.04 mg mL^{-1}, which is 1800-fold higher than paclitaxel (0.00025 ± 0.00004 mg mL^{-1}). To study the kinetics of O–N benzoyl migration, **2** · HCl was dissolved in PBS at various pH and incubated at 37°C. Complete migration was observed at pH 7.4 with a $t_{1/2}$ value of 15.1 ± 1.3 min (Figure 5), and this value is suggested to be appropriate for systemic distribution. On the other hand, slower migration was observed at pH 4.9 with a $t_{1/2}$ value of 252.2 ± 37.7 min and no migration at pH 2.0 after 6 h of incubation (Figure 5). These results indicated that the kinetics of migration from **2** · HCl to parent drug **1** were clearly pH dependent, and since faster migration could be obtained under physiological conditions (pH 7.4) than under acidic conditions, this suggests that the prodrug **2** can be stored in acidic aqueous media. In addition, a solid of **2** · HCl was stably maintained for one month at 4°C with no observation of **1**. Moreover, incubation in 0.035% citric acid saline (pH 4.0) at room temperature showed very slow migration of **2** · HCl (<3% of paclitaxel was released after incubation for 3 h), suggesting a possible condition for the injectable solution in practical clinical use. In addition, the benzoyl ester of **2** was biologically stable since this bond did not cleave in an experiment using porcine liver esterase.

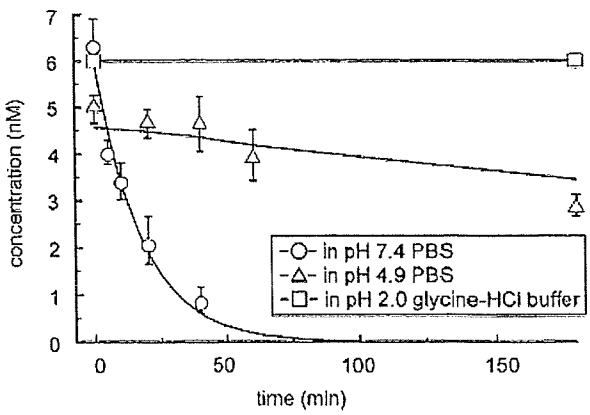


FIGURE 5 Migration of prodrug **2** in different pH conditions at 37°C.

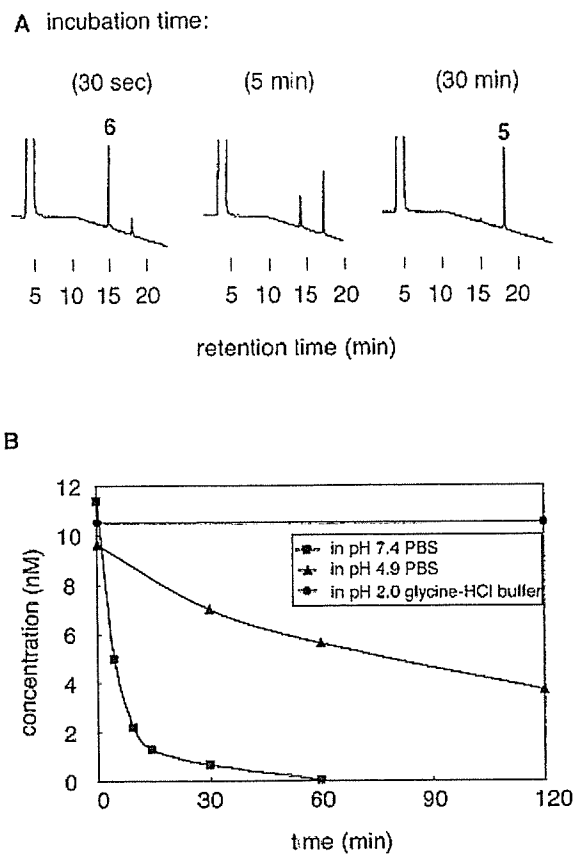


FIGURE 6 (A) HPLC profile of prodrug **6** in PBS (pH 7.4); (B) migration of prodrug **6** in different pH at 37°C.

A taxoid derivative, canadensol **5**, which showed improved potency against cancer with multidrug resistance,[36–38] was selected to evaluate the usage of this water-soluble prodrug strategy with other important taxoids. This taxoid includes the same α-hydroxy-β-amino acid, phenylisoserine, as paclitaxel, but the functional group at the 3′ position differed, with isobutyryl groups in **5** in place of the benzoyl group in **1**. The water-soluble prodrug **6**, which is a 2′-*O*-isobutyryl isoform of **5**, showed 10-fold higher water solubility (2.26 mg mL^{-1}). As shown in Figure 6, complete migration was observed at pH 7.4 with a $t_{1/2}$ value of 4.3 min. In addition, slower migration was observed at pH 4.9 with a value of 82.0 min and no migration at pH 2.0 after 6 h incubation (Figure 6).[39] A solid of **6** · HCl was stable for at least one month at 4°C. Only slight migration of **6** (1% of canadensol was released after 1 h) was observed during incubation in 0.035% citric acid saline (pH 4.0) at room temperature. These findings were quite similar to those obtained in the isotaxel study, suggested that our strategy can be applied to taxoid derivatives. These prodrugs **2** and **6**, 2′-*O*-acyl isoforms of paclitaxel and canadensol, released no additional functional auxiliaries during conversion to the corresponding parent drugs. This would be advantageous in toxicology and medical economics, since the potential side effects caused by reported auxiliaries and the use of detergent for solubilization can be omitted.

NOVEL AND EFFICIENT SYNTHESIS OF DIFFICULT SEQUENCE-CONTAINING PENTAPEPTIDES THROUGH O–N INTRAMOLECULAR ACYL MIGRATION REACTION OF *O*-ACYL ISOPEPTIDES

Through studies of water-soluble prodrugs of HIV-1 protease inhibitors and taxoids, we conceived the idea that O–N intramolecular acyl migration could be applied to the synthesis of difficult sequence-containing peptides (Figure 7),[40] since the difficult sequences are generally hydrophobic and promote aggregation in solvents during synthesis and purification. Specifically, the synthesis of more hydrophilic "*O*-acyl isopeptides" derived from difficult sequence-containing peptides followed by O–N intramolecular acyl

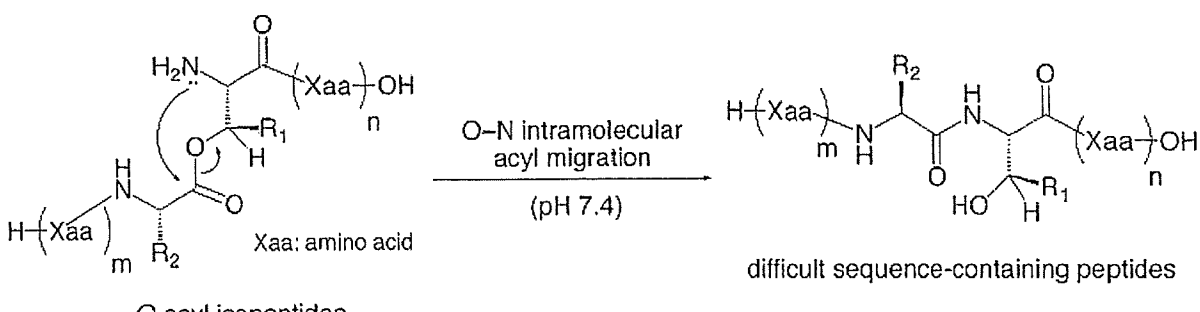
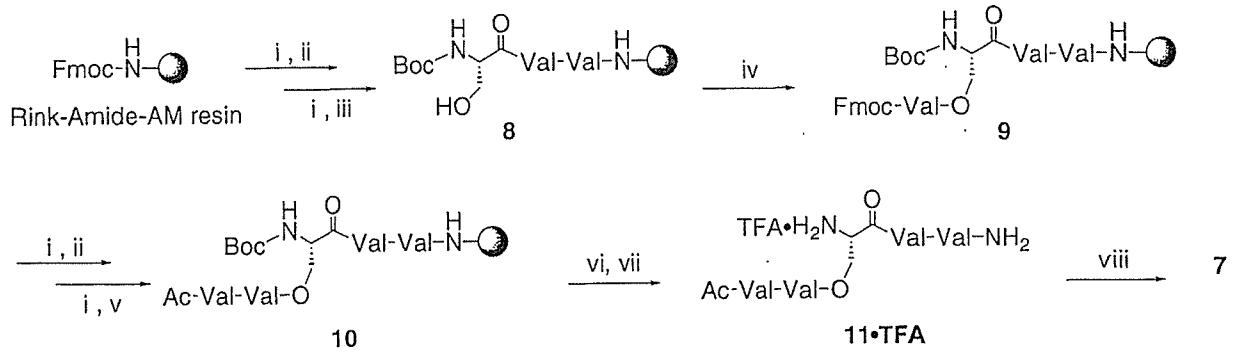


FIGURE 7 The synthetic strategy for difficult sequence-containing peptides via the O–N intramolecular acyl migration reaction of *O*-acyl isopeptide.



SCHEME 1 (Route B) Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc–Val–OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (iii), Boc–Ser–OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (iv) Fmoc–Val–OH (3.0 eq), DIPCDI (3.0 eq), DMAP (0.2 eq), CH_2Cl_2, 16 h × 2; (v) Ac_2O (1.2 eq), TEA (triethylamine, 1.0 eq), DMF, 2 h; (vi), TFA–*m*-cresol–thioanisole–H_2O (92.5:2.5:2.5:2.5), 90 min; (vii), preparative HPLC (a linear gradient of CH_3CN in 0.1% aqueous TFA); (viii), PBS, pH 7.4, 25°C.

migration to the corresponding desired peptide would overcome the solubility problem in HPLC purification. To demonstrate this hypothesis, a model difficult sequence-containing pentapeptide, Ac–Val–Val–Ser–Val–Val–NH_2 **7**, was selected, and its *O*-acyl isopeptide at the Ser residue was synthesized by the Fmoc-based SPPS method (Fmoc: 9-flourenylmethyoxycarbonyl; route B, Scheme 1). As a comparison study, peptide **7** was also synthesized by the standard Fmoc-based SPPS method (route A).

In route A, Rink amide aminomethyl (AM)[41] resin was employed, and the Fmoc-protected amino acids (2.5 eq) were sequentially coupled using the 1,3-diisopropylcarbodiimide (DIPCDI, 2.5 eq)–HOBt (*N*-hydroxybenzotriazole, 2.5 eq) method (2 h)[42] after the removal of each Fmoc group with 20% piperidine/DMF (DMF: dimethylformamide; 20 min). The resulting peptide resin was cleaved with TFA–*m*-cresol–thioanisole–H_2O (TFA: triflouroacetic acid; 92.5:2.5:2.5:2.5)[43,44] for 90 min. An undesired peptide, Fmoc–Val–Val–Ser–Val–Val–NH_2, was obtained at a similar rate to peptide **7**, indicating that the Fmoc group of the pentapeptide–resin was not deprotected during SPPS (Figure 8A). This suggests that the highly hydrophobic nature of Fmoc–peptide–resin prevented the base from accessing the Fmoc group, probably forming insoluble microaggregates on the resin. This result is well supported by a report that

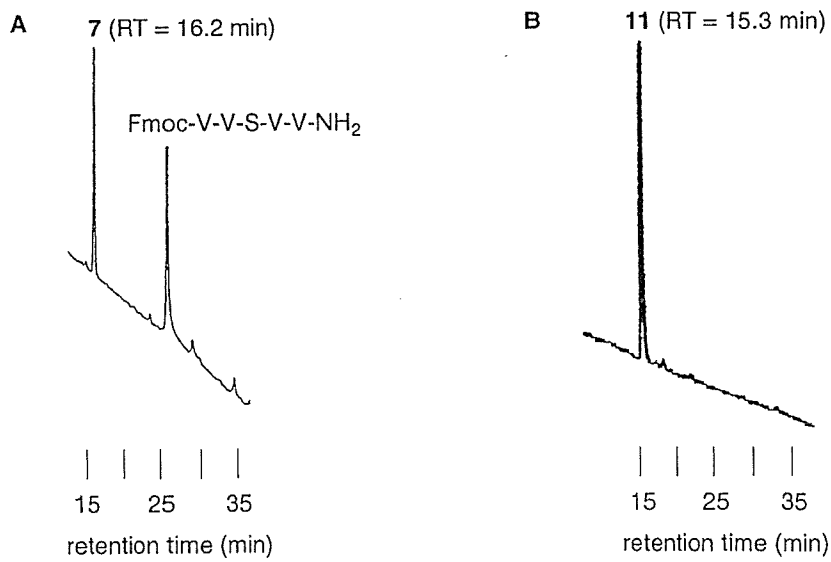


FIGURE 8 (A) HPLC profiles of crude peptide **7** and (B) its *O*-acyl isopeptide **11** (0–100% CH_3CN for 40 min, 230 nm).

undesired aggregation can occur from as early as the fifth residue coupled.[45] Further purification of **7** in preparative scale HPLC was laborious due to the extremely low solubility of the products, the solubility of **7** in H_2O, MeOH, and DMSO being 0.013, 0.051, and 0.64 mg mL^{-1}, respectively. When the DMSO solution of **7** was used for HPLC purification, the overall yield of **7** in route A was only 6.0%.

On the other hand, in route B (Scheme 1), Boc–Ser–OH (2.5 eq) was coupled to the H–Val–Val–NH–resin to obtain **8**, and subsequent coupling with Fmoc–Val–OH (3.0 eq) to the β-hydroxyl group of Ser was performed using the DIPCDI (3.0 eq)–DMAP (4-dimethylaminopyridine, 0.2 eq) method in CH_2Cl_2 to obtain ester **9**. *O*-Acyl isopeptide **11** · TFA was obtained as a major product through the coupling of another Val residue, its *N*-acetylation and TFA treatment (Figure 8B). Subsequently, preparative HPLC purification of crude **11** was successfully achieved based on its excellent solubility in H_2O and MeOH (59.6 and 126.6 mg mL^{-1}, respectively). This result indicates that the protected peptide resin **10** is efficiently synthesized with no interference from the difficult sequences. Specifically, the branched ester structure could modify the property of the "difficult sequence" as well as improve its solubility. In addition, since H–Ser–Val–Val–NH_2 was not formed as a by-product: (1) the esterification of the secondary hydroxyl group of Ser was successfully completed on the solid support, (2) the formed ester bond was stable in both piperidine and TFA treatments; and (3) diketopiperazine was not formed when the last Fmoc group was removed, corresponding to a report that diketopiperazine formation did not occur in similar elongation of the peptide chain from the secondary hydroxyl group.[46] Although slight racemization (0.8%) of the esterified Val residue occurred in the DIPCDI–DMAP method, the racemized product could be removed by HPLC purification. Finally, **11** · TFA was dissolved in PBS (pH 7.4) and completely converted to the corresponding parent peptide **7** with a half-life of 112 min via O–N intramolecular acyl migration at room temperature with no side reaction. Compound **11** · TFA was stable at 4°C for at least 30 days as a solid state. As depicted in Figure 9, **7** was clearly formed as a white precipitate from **11** and migration was completed after 16 h. The resultant precipitate was centrifuged and washed with water and methanol to give high purity **7**. Consequently, the overall yield of **7** in route B was 40.6%.

This result indicates remarkable improvement of the synthetic yield of a difficult sequence-containing

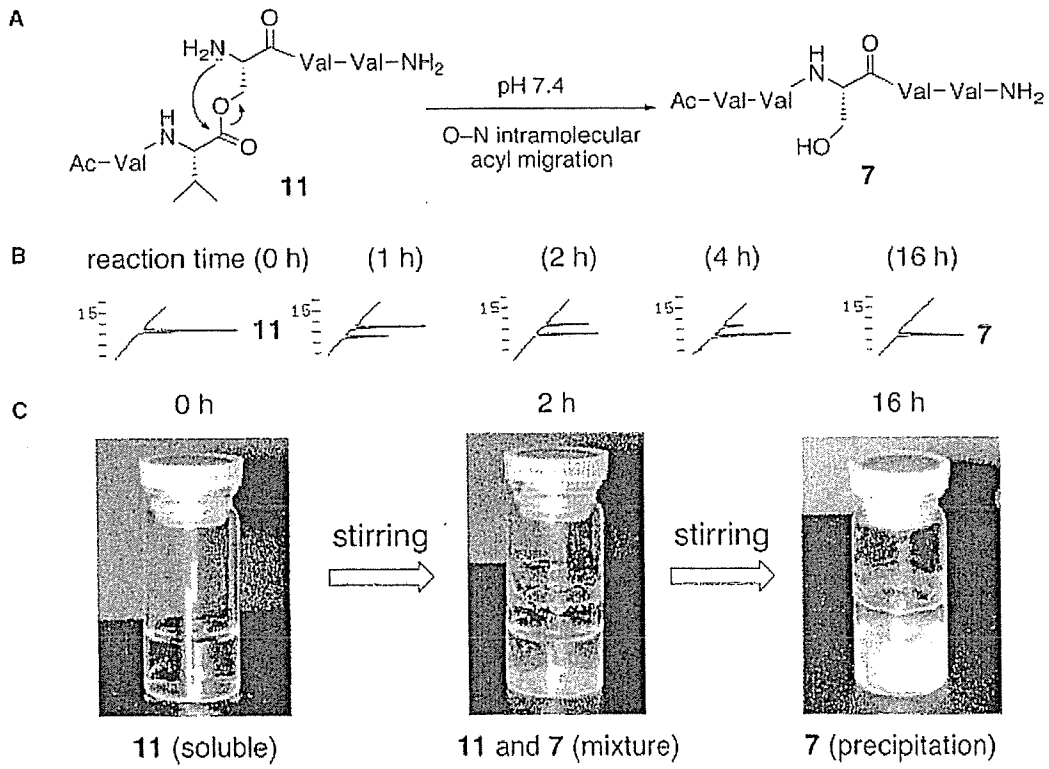


FIGURE 9 (A) Conversion of *O*-acyl isopeptide **11** to **7** via O–N intramolecular acyl migration in PBS (pH 7.4, 25°C), (B) its periodical HPLC profile, and (C) photographs.

pentapeptide. The branched ester structure in *O*-acyl isopeptide could suppress the unfavorable nature seen in the difficult sequence-containing peptide. Specifically, insertion of the ester bond into the peptide chain can probably disrupt the secondary structure formed by the inherent peptide chain, leading to the improvement of coupling and deblocking efficacy during SPPS. In addition, *O*-acyl isopeptide, with the newly formed and ionized amino group, can achieve reasonable H_2O and MeOH solubility required in HPLC purification by salt formation. Furthermore, the result indicated that the purified *O*-acyl isoform was completely converted to the original *N*-acyl form in a short time with no side reaction at pH 7.4. These results suggest that synthesis via the *O*-acyl isopeptide is advantageous for synthesizing difficult sequence-containing peptides. However, it is necessary to evaluate whether our observation in small model peptides is applicable to larger difficult sequence-containing peptides. Therefore, we focused next on one of the large, difficult sequence-containing peptides, amyloid β peptide (Aβ) 1–42, which is significant in both peptide chemistry and medical science.

A NOVEL WATER-SOLUBLE Aβ1–42 ISOPEPTIDE: AN EFFICIENT STRATEGY FOR THE PREPARATION OF THE ALZHEIMER'S DISEASE-RELATED PEPTIDE, Aβ1–42, VIA O–N INTRAMOLECULAR ACYL MIGRATION

Amyloid β peptides (Aβs) are the main proteinaceous component of the amyloid plaques found in the brains of Alzheimer's disease (AD) patients.[47] Neuritic plaques, pathognomonic features of AD, contain abundant fibrils formed from Aβs, which have been found to be neurotoxic in vivo and in vitro.[48] The predominant forms of Aβs mainly consist of 40- and 42-residue peptides (designated Aβ1–40 and Aβ1–42, respectively), which are proteolytically produced from amyloid precursor protein (APP) by enzymatic reactions.[49] Since Aβ1–42 is thought to play a more critical role in amyloid formation and in the pathogenesis of AD than Aβ1–40, many studies using synthetic Aβ1–42 have been carried out to clarify the involvement of Aβ1–42 in AD.[50–53]

However, Aβ1–42 is a difficult sequence-containing peptide with a highly hydrophobic nature and forms aggregates in various media.[54–59] Due to its low solubility and broad elution under acidic or neutral conditions, the conventional HPLC purification of synthesized Aβ1–42 in the aqueous TFA–acetonitrile system is too laborious to remove impurities accumulated during SPPS. Furthermore, biological experiments using Aβ1–42 are problematic due to the large extent of aggregation in a standard storage solution such as dimethylsulfoxide (DMSO).[60] Therefore, an "in situ" system that could prepare intact Aβ1–42 in a soluble form under physiological conditions would be a powerful tool in understanding its inherent pathological function. To create such a system, (1) a novel propeptide possessing high solubility during HPLC purification and long-term storage as a solution and (2) the capability of intact Aβ1–42 production under physiological conditions are required.

Based on a finding in the synthesis of a small difficult sequence-containing peptide, we applied the O–N intramolecular acyl migration method to the synthesis of Aβ1–42 via a novel water-soluble isopeptide of Aβ1–42, i.e., "26-*O*-acyl isoAβ1–42 (26-AIAβ1–42, **13**)."[61] This overcomes the problems in the synthesis and storage of Aβ1–42 (Figure 10). Although there are two Ser residues in Aβ1–42 at positions 8 and 26 with the capability of O–N intramolecular acyl migration, we selected the Ser[26] for

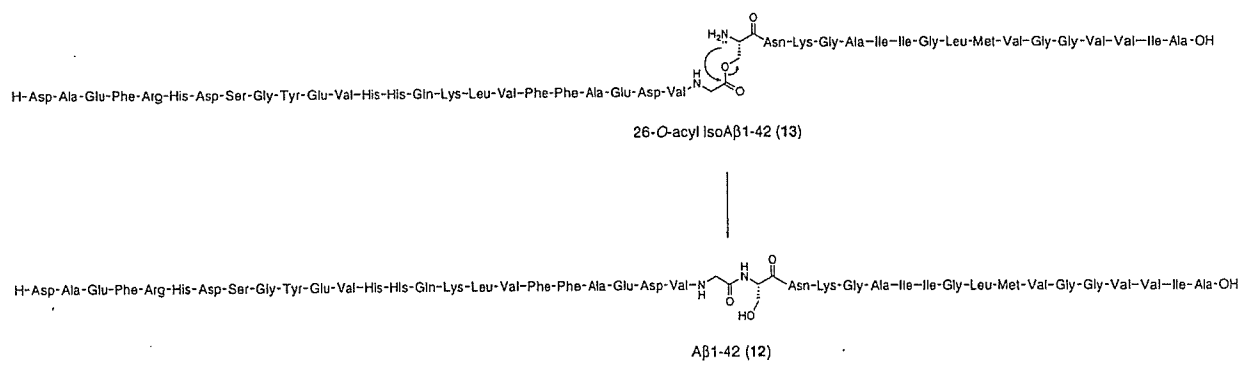
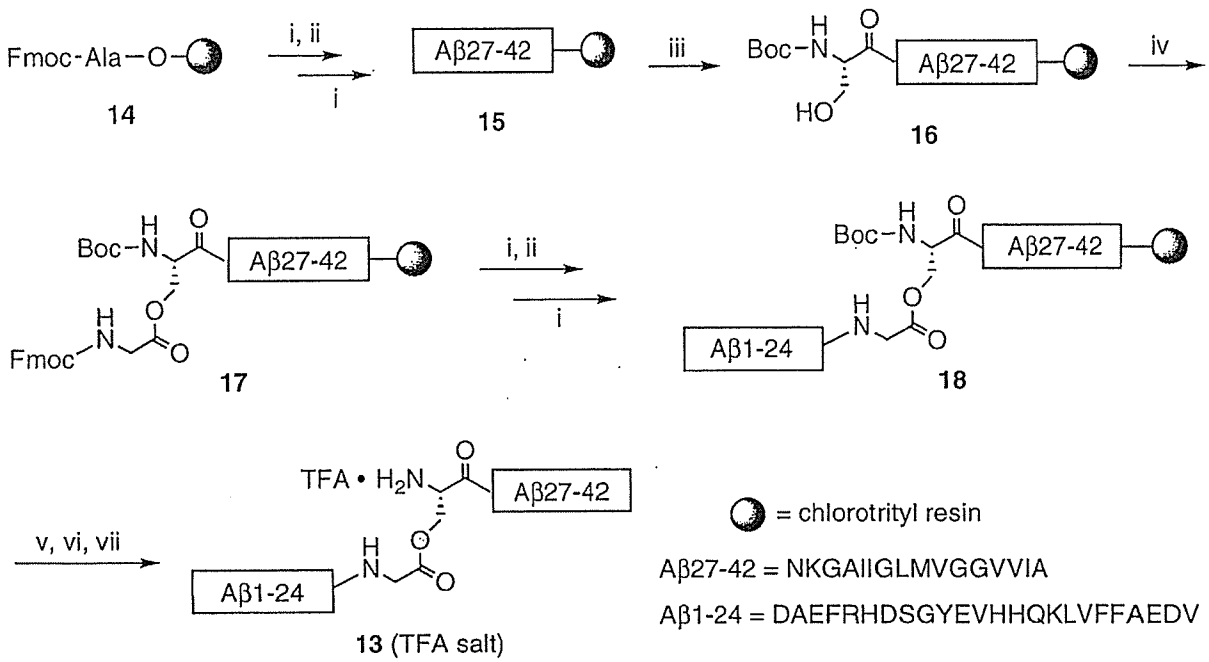


FIGURE 10 The production of Aβ1–42 (**12**) via the O–N intramolecular acyl migration reaction of 26-*O*-acyl isoAβ1–42 (**13**).



SCHEME 2 Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii), Fmoc–AA–OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (iii), Boc–Ser–OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (iv), Fmoc–Gly–OH (3.0 eq), DIPCDI (3.0 eq), DMAP (0.2 eq), CH_2Cl_2, 16 h × 2; (v), TFA–*m*-cresol–thioanisole–H_2O (92.5:2.5:2.5:2.5), 90 min; (vi), NH_3I (20 eq), dimethylsulfide (20 eq), TFA:H_2O (2:1), 60 min, 0°C; (vii), preparative HPLC (a linear gradient of CH_3CN in 0.1% aqueous TFA).

O-acylation, since the adjacent Gly^{25} does not epimerize during ester bond formation (Figure 10).

As depicted in Scheme 2, Fmoc–Ala–*O*-chlorotrityl resin (0.465 mmol/g, **14**) was employed and Fmoc-protected amino acids were sequentially coupled using the DIPCDI–HOBt method (2 h) after the removal of each Fmoc group with 20% piperidine–DMF (20 min) to give peptide resin **15**. After Boc–Ser–OH

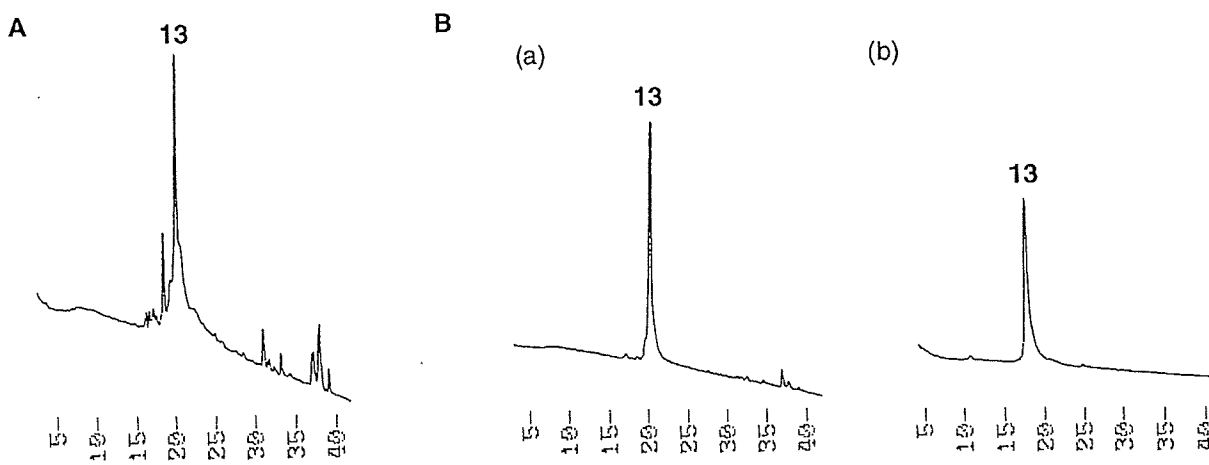


FIGURE 11 HPLC profiles of (A) crude and (B) purified 26-AIAβ1–42 (13). Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min for A and B-a, 25–55% CH_3CN, 60 min for B-b) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40°C), detected at 230 nm.

was introduced to **15**, the obtained **16** was coupled with Fmoc–Gly–OH at the β-hydroxy group of Ser using the DIPCDI–DMAP method in CH_2Cl_2 to obtain ester **17**. The compound 26-AIAβ1–42–resin (**18**) was obtained through the coupling of additional amino acid residues in the conventional manner. Finally, 26-AIAβ1–42 (**13**) was obtained as a major product by treatment with TFA–*m*-cresol–thioanisole–H_2O (92.5:2.5:2.5:2.5) for 90 min followed by NH_3I–dimethylsulfide for 60 min in TFA:H_2O (2:1).

In HPLC analysis of crude products (Figure 11A), Aβ1–25 (DAEFRHDSGYEVHHQKLVFFAEDVG) was not observed as a by-product, although a very low rate (1.6%, HPLC yield) of Aβ26-42 (SNKGAIIGLMVGGVVIA) was detected. This indicates that (1) the esterification of the β-hydroxy group of Ser was successfully completed on the solid support and (2) the formed ester bond between Gly and Ser was stable in both piperidine and TFA. The crude *O*-acyl isopeptide **13** was dissolved in hexafluoroisopropanol, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH_3CN. Since **13** was eluted as a narrow single peak, it could easily be purified using preparative scale HPLC to give pure **13** (Figure 11B) as TFA salt with a total isolated yield of 33.6%, calculated from the original loading of chlorotrityl resin. This yield was higher than that obtained in the synthesis of **12** by standard Fmoc-based SPPS (7.2%). Since **12** was eluted as a broad peak in preparative scale HPLC purification, it was laborious to isolate **12** from impurities as reported.[56–59] In addition, in the synthesis of **13**, no conversion to **12** was observed.

The water solubility of **13** (TFA salt) was 15 mg mL^{-1}, 100-fold higher than that of Aβ1–42 (**12**, 0.14 mg mL^{-1}). Interestingly, as a slight modification of the peptide chain by inserting one ester bond could drastically increase the solubility of the insoluble original peptide with 42 residues, this suggests that the *O*-acyl isopeptide totally breaks the secondary structures responsible for the insolubility of the original peptide. As we demonstrated that the *O*-acyl isopeptide could suppress the unfavorable nature of

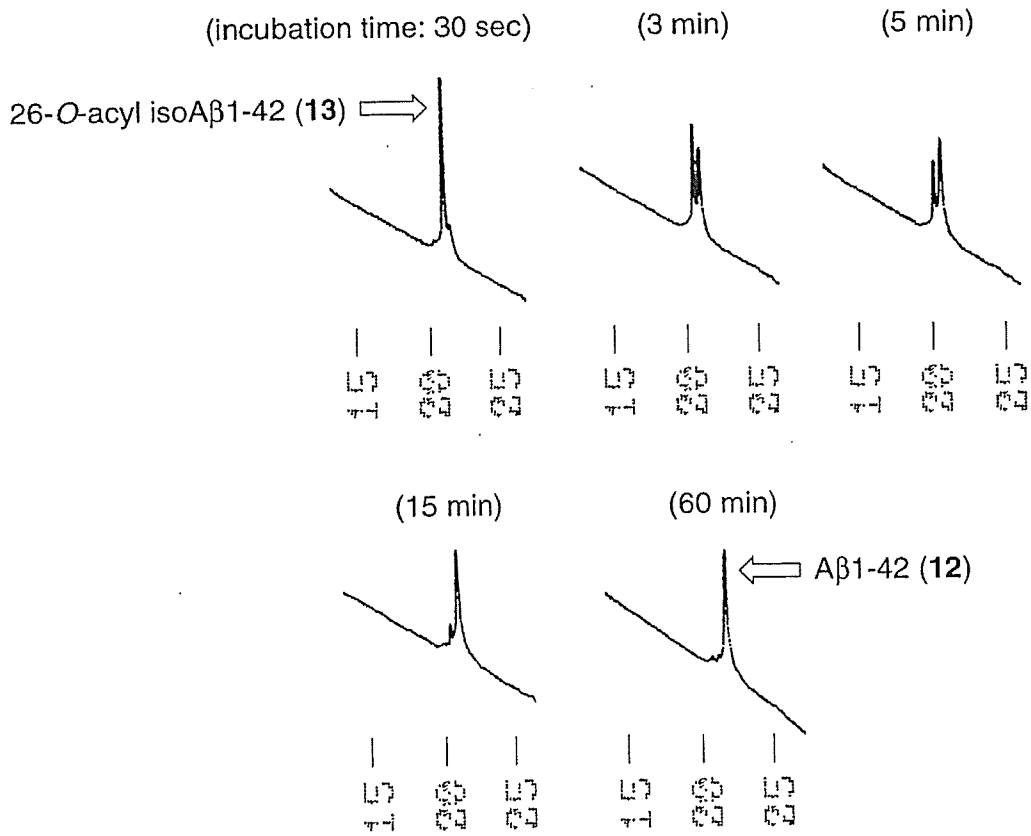


FIGURE 12 HPLC profiles of the conversion of 26-AIAβ1–42 (**13**) to Aβ1–42 (**12**) via O–N intramolecular acyl migration in PBS (pH 7.4, 25°C). Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40°C), detected at 230 nm.

difficult sequence-containing pentapeptides in the previous study,[40] this result in the synthesis of **13** indicates that this method is a powerful strategy for increasing the solubility of even large peptides.

As shown in Figure 12, purified **13** was completely converted to Aβ1–42 (**12**) at room temperature in PBS (pH 7.4) with no side reaction. This migration was rapid with a half-life of 2.6 min, while the TFA salt of **13** was stable at 4°C for at least 30 days in either a solid state or a DMSO solution. Moreover, slower migration was observed at pH 4.9 (PBS) with a half-life of 3 h and no migration at pH 3.5 (acetate buffer) after incubation for 3 h. This rapid migration under physiological conditions enables the production of an intact monomer, Aβ1–42, in situ to investigate the inherent biological function of Aβ1–42 in AD. The conversion of **13** (TFA salt) in water for 48 h at room temperature followed by lyophilization yielded Aβ1–42 (**12**) quantitatively as TFA salt with >95% purity.

This result demonstrates that this O–N acyl migration strategy via *O*-acyl isopeptides is applicable for the synthesis of large peptides. In particular, it is noteworthy that only one insertion of the isopeptide structure into the sequence of 42-residue peptide can suppress the unfavorable nature of its difficult sequence. Therefore, the O–N acyl migration strategy can be applied to larger difficult sequence-containing peptides than Aβ1–42 as a general method. In addition, rapid migration of *O*-acyl isopeptides to intact Aβ1–42 under physiological conditions (pH 7.4) was observed while it was stable under storage conditions. Hence, our strategy not only overcomes the solubility problem in the synthesis of Aβ1–42, but also provides a novel tool for the biological evaluation system in AD research, in which 26-*O*-acyl isoAβ1–42 can be stored in a solubilized form before use and rapidly produces intact Aβ1–42 in situ during biological experiments.

PERSPECTIVE FOR THE FUTURE

Half a century has passed since the N–O acyl migration of Ser/Thr-containing peptides in strong acids treatment was reported.[1–3] This classical side reaction has recently been revived as a powerful key reaction in the field of modern medicinal chemistry in the development of sophisticated prodrugs. In addition, this reaction was introduced again in peptide chemistry for the synthesis of difficult sequence-containing peptides, which are a major drawback in automated solid-phase peptide synthesis. These applications contribute to medical science to understand the function and mechanism of bioactive peptides and provide new prodrug candidates. In addition, this strategy, based on the economical reaction in aqueous media, will develop further in 21st century science such as organic chemistry by providing new water-soluble substrates required in green chemical reactions.

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

Hikoichiro Maegawa, Tooru Kimura, Yasuhiro Arii, Yasuko Matsui, Soko Kasai, Yoshio Hayashi and Yoshiaki Kiso*

Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan

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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,

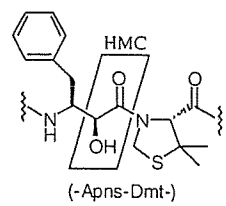

Figure 1. Structure of HMC isostere.

Keywords: Human T-cell leukemia virus type I (HTLV-I); Adult T-cell leukemia (ATL); Aspartic protease; Inhibitor; Hydroxymethylcarbonyl (HMC) isostere; Chemical ligation.
* Corresponding author. Tel.: +81 75 595 4635; fax: +81 75 591 9900; e-mail: kiso@mb.kyoto-phu.ac.jp

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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
```

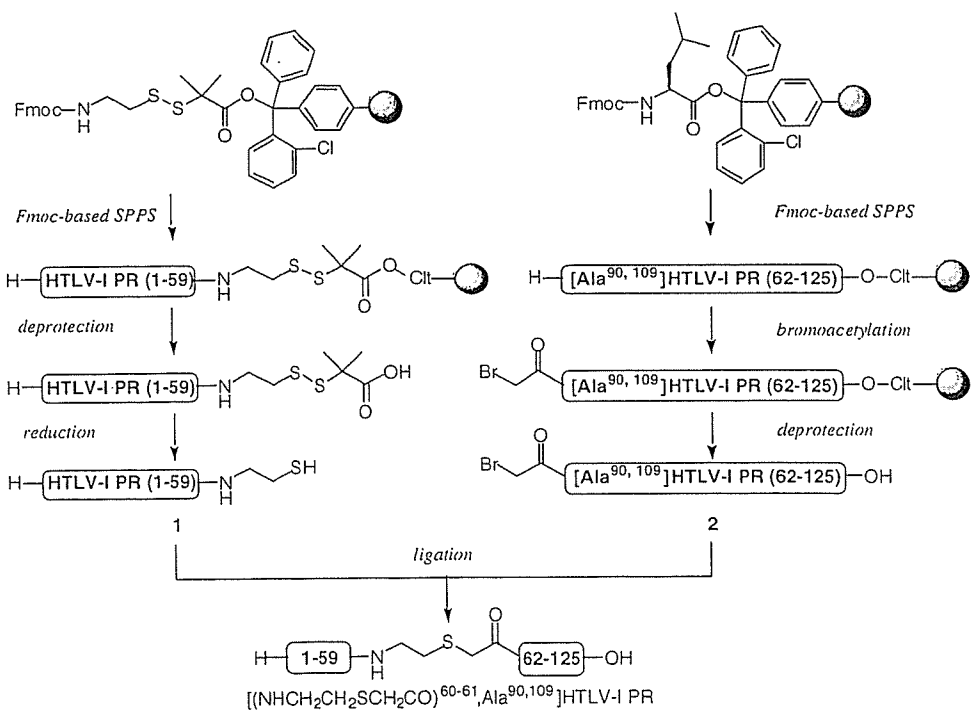
Figure 2. The amino acid sequences of prepared HTLV-I PRs. (A) recombinant HTLV-I PR. Underline: His-tag sequence, boldface: mature HTLV-I PR sequence. (B) synthetic [$(NH_2CH_2CH_2SCH_2CO)^{60-61}$, $Ala^{90,109}$]-HTLV-I PR. Underline: substitution sites from Cys to Ala. XX: thioether linkage ($-NHCH_2CH_2SCH_2CO-$).

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.

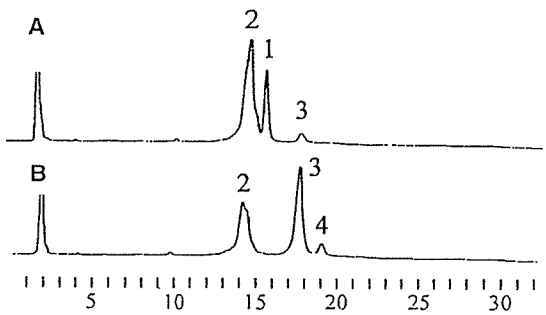


Figure 3. HPLC profiles of ligation reaction of peptide **1** (1.0 equiv) and **2** (1.5 equiv) at 0 h (A) and 3 h (B). The peaks **3** and **4** show [$NHCH_2CH_2SCH_2CO^{60-61}$, $Ala^{90,109}$]-HTLV-I PR and disulfide dimer of peptide **1**, respectively. HPLC was performed using a C18 reverse phase 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′	10	—	39	—	97
6 (KNI-577)	Bz(3-OH, 2-Me)-Apns-Dmt-NHBu′	38	—	57	—	88
7 (KNI-727)	Pac(diMe)-Apns-Dmt-NHBu′	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′, *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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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).
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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).
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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.
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Short Rapid Communication

Search for Substrate-Based Inhibitors Fitting the S_2' Space of Malarial Aspartic Protease Plasmepsin II

AIKO KISO,[a] KOUSHI HIDAKA,[a] TOORU KIMURA,[a] YOSHIO HAYASHI,[a] AZIN NEZAMI,[b] ERNESTO FREIRE[b] and YOSHIAKI KISO[a]*

[a] Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan
[b] Department of Biology and The Johns Hopkins Malaria Research Institute, The Johns Hopkins University, Baltimore, Maryland 21218, USA

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Abstract: Plasmepsin (Plm) has been identified as an important target for the development of new antimalarial drugs, since its inhibition leads to the starvation of *Plasmodium falciparum*. A series of substrate-based dipeptide-type Plm II inhibitors containing the hydroxymethylcarbonyl isostere as a transition-state mimic were synthesized. The general design principle was provision of a conformationally restrained hydroxyl group (corresponding to the set residue at the P_2' position in native substrates) and a bulky unit to fit the S_2' pocket. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimalarial drug; aspartic protease inhibitor; plasmepsin; transition-state mimic; allophenylnorstatine

INTRODUCTION

Malaria parasites use hemoglobin as a source of nutrients during their growth and maturation in erythrocytes. The prevention of this process is thought to be a key target for the development of a new drug against malaria. Recently, it became clear that malaria parasites encode several proteases that are essential components of their hemoglobin-degradation pathway [1]. For example, in the food vacuole of *Plasmodium falciparum*, four aspartic proteases, plasmepsin (Plm) I, II, and IV and histo-aspartic protease (HAP), whose amino acid sequences are highly conserved, are involved in the pathway [2,3]. Since it is reported that the inhibition of Plm I and II which initiate the hemoglobin-degradation leads to starvation of the parasites [4], these enzymes are the targets for the development of new antimalarial drugs and several inhibitors, including our compounds [5,6], have already been reported [7–12].

Our previous study on the development of new antimalarial drugs focused on Plm II as a target enzyme for structure-based drug design, since Plm II can be efficiently expressed in *E. coli*, and its high-resolution structure has been determined by x-ray crystallography [8]. Our HIV protease (PR) inhibitor study developed a series of substrate-based peptidomimetic inhibitors containing an allophenylnorstatine [Apns; (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] with a hydroxymethylcarbonyl (HMC) isostere as an ideal

*Correspondence to: Yoshiaki Kiso, Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan; e-mail: kiso@mb.kyoto-phu.ac.jp
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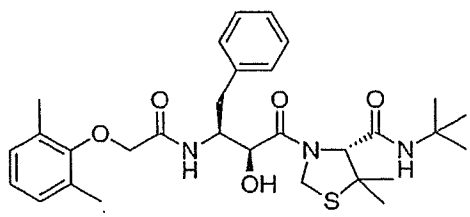
Contract/grant sponsor: Ministry of Education, Science and Culture of Japan.

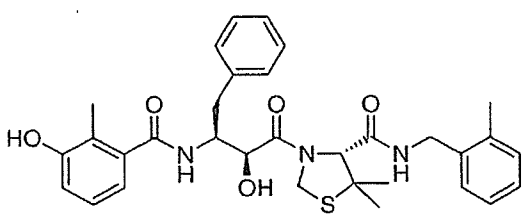
Table 1 Substrate Cleavage Sites (*) of Plasmepsin II and HIV-1 Protease

Enzyme	Cleavage site	- P_4	P_3	P_2	P_1*P_1'	P_2'	P_3'	P_4' -
Plasmepsin II	α33/34	- Glu	Arg	Met	Phe*Leu	Ser	Phe	Pro -
	α108/109	- Leu	Leu	Val	Thr*Leu	Ala	Ala	His -
	α136/137	- Ser	Thr	Val	Leu*Thr	Ser	Lys	Tyr -
	β32/33	- Gly	Arg	Leu	Leu*Val	Val	Tyr	Pro -
HIV-1 protease	p17/p24	- Gln	Arg	Gly	Tyr*Pro	Ile	Val	Gln -
	p24/p1	- Ala	Arg	Val	Leu*Ala	Glu	Ala	Met -
	p1/p9	- Ala	Thr	Ile	Met*Met	Gln	Arg	Gly -
	p9/p6	- Pro	Gly	Asn	Phe*Leu	Gln	Ser	Arg
	TF/PR	- Ser	Phe	Asn	Phe*Pro	Gln	Ile	Thr
	PR/RT	- Thr	Leu	Asn	Phe*Pro	Ile	Ser	Pro
	RT/RN	- Ala	Glu	Thr	Phe*Tyr	Val	Asp	Gly
	RN/IN	- Arg	Lys	Ile	Leu*Phe	Leu	Asp	Gly

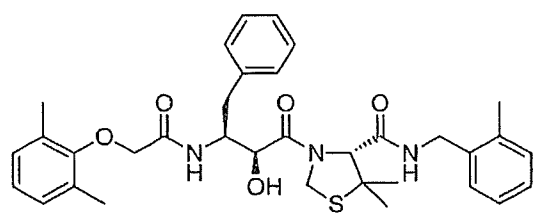
TF, transframe protein; PR, protease; RT, reverse transcriptase; RN, ribonuclease H; IN, integrase.



KNI-727
K_i 0.07 μM (Plm II)
K_i 1.3 μM (Cat D)
Selectivity 22.4
EC_{50} 10 μM



KNI-764
K_i 0.03 μM (Plm II)
K_i 0.2 μM (Cat D)
Selectivity 6.7
EC_{50} 5.7 μM



KNI-840
K_i 0.02 μM (Plm II)
K_i 0.08 μM (Cat D)
Selectivity 4.1
EC_{50} 10.7 μM

Figure 1 Structures of KNI compounds.

transition-state mimic [13–18]. Since the substrate recognition profiles of Plm I and II [19] are similar to that of HIV-1 PR (Table 1), and Phe^{33}-Leu^{34} in the α-chain which is a primary cleavage site in hemoglobin-degradation exhibits a similar structure to the Apns-containing scaffold, it was hypothesized that HIV-1 PR inhibitors would be effective against Plms, so the inhibitory activity of 12 selected HIV PR inhibitors were evaluated against Plm II [5]. As predicted, these substrate

transition-state mimic compounds are capable of inhibiting Plm II. Among them, KNI-727 [14–16], –764 [17–18] and –840 [16] which contain Apns-Dmt [Dmt = dimethylthioproline; (*R*)-5,5-dimethyl-1,3-thiazolidinecarboxylic acid] at the P_1-P_1' positions exhibited potent Plm II inhibitory activity with K_i values of 0.07, 0.03 and 0.02 μM, respectively (Figure 1). The Apns-Dmt scaffold is based on the substrate Phe-Leu (P_1-P_1') scissile site transition state; that is, Apns is the Phe-transition state mimic and Dmt is the conformational constrained cyclized Leu-mimic. Interestingly, KNI-727 also exhibited a 23-fold higher potency against Plm II compared with highly homologous human cathepsin D (Cat D). Moreover, these three compounds also exhibited antimalarial activity in cultures of red blood cells infected with *P. falciparum* with EC_{50} values of 5–10 μM (Figure 1) [5]. This result suggests that Plm II inhibitors based on the HMC-type substrate transition-state mimic are potential antimalarial drugs. In further screening, KNI-10006, which has an (1*S*,2*R*)-1-amino-2-indanol at the P2′ position, was found to inhibit Plm II with a remarkably high affinity [6–20].

This paper describes the SAR of Plm II inhibitors based on the substrate transition-state mimic. Sixteen compounds (**4a–p**) were designed and synthesized in which the P_2' position of KNI-727 is modified (Figure 2). Since native substrates contain a Ser residue at the P_2' position (Table 1), a hydroxyl group was introduced with a fixed conformation and a bulky unit to fit the space of the S_2' pocket of Plm II. The enzyme inhibitory activities of these synthetic compounds were evaluated.

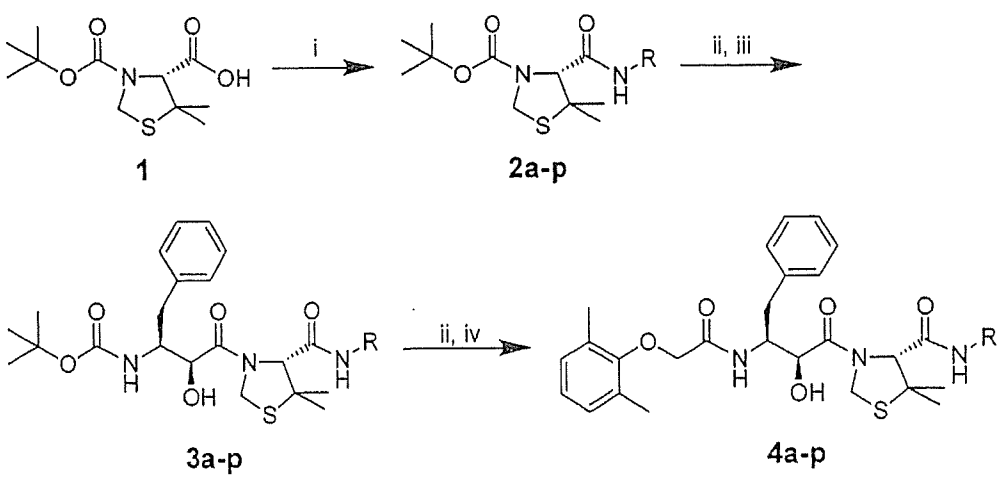
MATERIALS AND METHODS

Synthesis

The dipeptidic compounds (**4a–p**) were prepared by the usual Boc strategy in the liquid phase (Scheme 1). Coupling of Boc-Dmt-OH [17] with the free amino group of the P_2' ligands yielded compounds **2a–p**. The Boc group was deprotected with 4N HCl/dioxane, followed by coupling with Boc-Apns-OH [17] using EDC in the presence of HOBt, resulting in protected compounds **3a–p**. Finally, deprotection of **3a–p** and coupling with 2,6-dimethylphenoxyacetic acid using BOP yielded the desired dipeptide-type analogs, **4a–p**. Compounds **4i** and **4j** were separated by column chromatography from a *cis*-mixture, but the configurations of each diastereomer have not been established yet.

Enzyme Inhibition Assay

Recombinant HIV-1 PR was purchased from Bachem AG, Bubendorf, Switzerland. HIV PR substrate [H-Lys-Ala-Arg-Val-Tyr-Phe(*p*-NO_2)-Glu-Ala-Nle-NH_2] was synthesized by a conventional solid-phase method. The determination of HIV-1 PR inhibitory activity of the test compounds at 50 nM was based on the inhibition of cleavage of the HIV PR substrate by using recombinant HIV-1 PR (2 μg/ml) in 50 mM MES-NaOH (pH 5.5) containing 2.5 mM DTT, 1 mM EDTA-2Na, 0.2% Nonidet P-40 and 15% glycerol. After incubation for 15 min at 37 °C, the reaction was terminated by the addition of 1N HCl, and the amount of *N*-terminal cleavage fragment produced was measured by HPLC.



Scheme 1 Reagents and conditions: (i) R-NH_2, TEA, BOP, DMF, rt, 18 h; (ii) anisole, 4N-HCl/dioxane, rt, 1 h (iii) Boc-Apns-OH, HOBt, EDC, TEA, DMF, rt, 18 h; (iv) TEA, 2,6-dimethylphenoxyacetic acid, BOP, DMF, rt, 18 h.

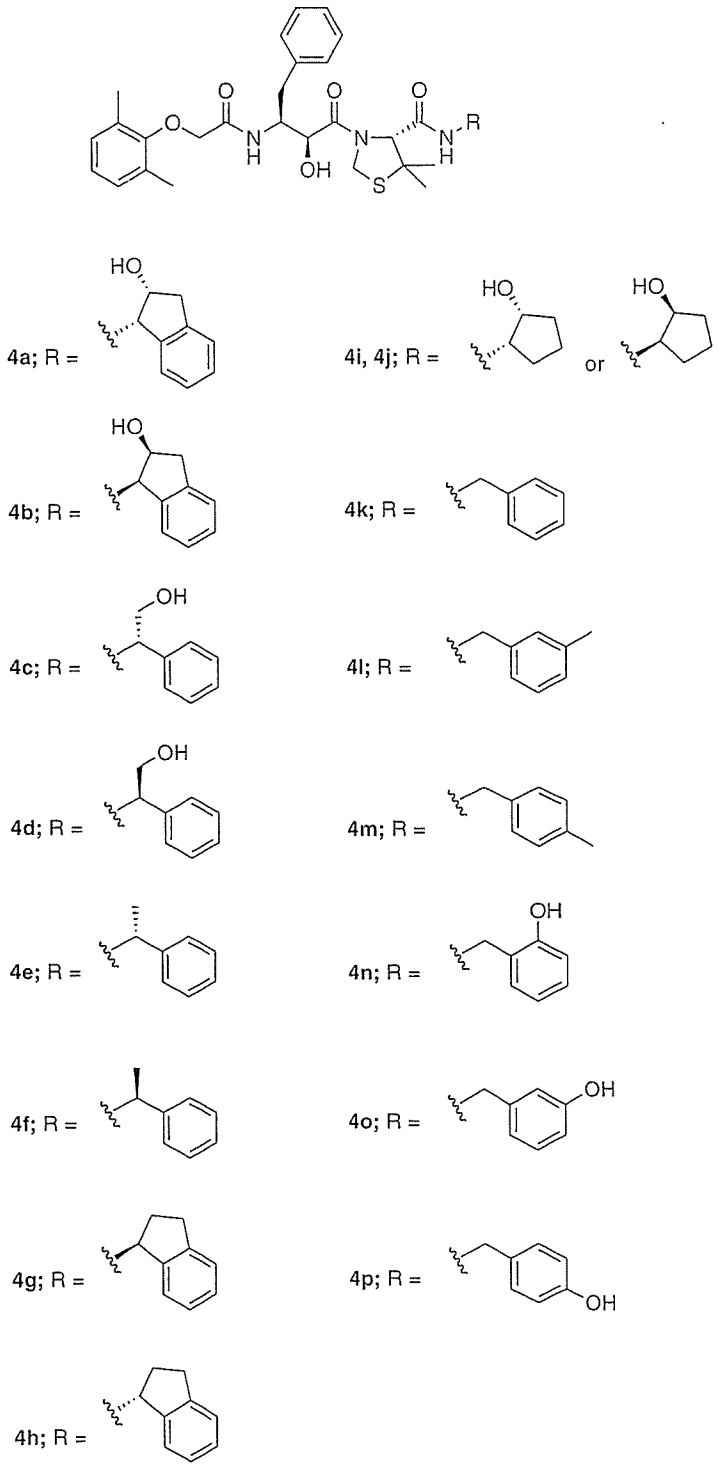


Figure 2 Structures of synthetic KNI-727 analogs.

Inhibition constants (K_i) against Plm II and Cat D were obtained as described previously [5]. Briefly, the rate of substrate hydrolysis at 25 °C was measured using 400 nM protease in 10 mM sodium formate (pH 4.0), 163 μM chromogenic substrate [H-Ala-Leu-Glu-Arg-Thr-Phe-Phe(p-NO_2)-Ser-Phe-Pro-Thr-OH] which was purchased from California Peptide Research Inc., Napa, CA and 2%

DMSO with increasing amounts of inhibitor. K_i were estimated by fitting the data to standard equations for tight binding competitive inhibitors.

RESULTS AND DISCUSSION

As shown in Table 2, the previously reported compound **4a** [6], which has (1*S*,2*R*)-1-amino-2-indanol at the P_2' position, exhibited remarkably potent inhibitory activity with a K_i value of 0.5 nM. This activity is 140-fold more potent than KNI-727. On the other hand, compound **4b**, which has the opposite configuration of **4a**, exhibited a lower inhibitory activity, suggesting that the stereochemistry at the aminoindanol part of **4a** is favorable in the interaction with the S_2' pocket of Plm II. Compounds **4c** and **4d** have a phenylglycinol with a hydroxyl group, which is more flexible than that of aminoindanol at the P_2' position, but these compounds exhibited a lower inhibitory activity than **4a**. In order to understand the significance of the hydroxyl group and the aminoindanol structure in the potent enzyme inhibition, aminoindan was introduced at the P_2' position. The inhibitory activities of the resultant compounds **4g** and **4h** were relatively low. Aminocyclopentanol was also introduced at the P_2' position, in which the phenyl ring in **4a** was removed. However, compounds **4i** and **4j** showed a lower potency. From these results, it is suggested that the tight binding observed in the aminoindanol structure at the P_2' position is due to the effect of both the hydrophobic indan structure and its spatially arranged hydroxyl group which allows for proper interaction with Plm II.

Table 2 Inhibitory Activity Against Plm II, Cat D and HIV-1 PR

	Compound	Plm II K_i (nM)	Cat D K_i (nM)	HIV-1 PR % inhibition (50 nM)
4a	(KNI-10006)	0.5	2	98
4b	(KNI-10007)	71	806	21
4c	(KNI-10043)	22	53	16
4d	(KNI-10044)	532	1040	0
4e	(KNI-10030)	11	111	41
4f	(KNI-10063)	542	nd	85
4g	(KNI-10026)	15	105	97
4h	(KNI-1269)	99	nd	71
4i	(KNI-2017a)	108	nd	89
4j	(KNI-2017b)	709	nd	41
4k	(KNI-10046)	96	260	90
4l	(KNI-10053)	41	36	58
4m	(KNI-10052)	572	1321	55
4n	(KNI-10047)	55	252	77
4o	(KNI-10048)	99	771	89
4p	(KNI-10054)	273	1642	84

nd, not determined.

The compounds (**4l–p**) with benzylamine derivatives exhibited lower inhibitory activities than KNI-840 which is introduced with *o*-methylbenzylamine. This result indicates that the inhibitory activity decreases in the order *o*– > *m*– > *p*-substitutions at the phenyl ring regardless of the presence of the methyl or hydroxyl group in these positions. In the case of the methyl substitution, a similar tendency was previously reported in HIV-1 PR inhibitors [17], suggesting that the recognition mechanism at the P_2' site of both Plm II and HIV-1 PR is similar.

In comparison with the Cat D inhibition, the selectivity of Plm II ($K_{i\ \text{Cat D}}/K_{i\ \text{Plm II}}$) did not improve in a series of modifications at the P_2' position (selectivity <11). Compound **4a** was also found to be a potent Cat D inhibitor (K_i = 2 nM). The replacement of *t*-butylamine in KNI-727 to aminoindanol in **4a** at the P_2' position contributed to a 650-fold increase in the Cat D inhibitory potency. This result suggests that a similar interaction induced by the hydroxyl group of aminoindanol enhances the binding affinity to Cat D, resulting in a decrease of the selectivity towards Plm II. This observed low selectivity, however, would not be a serious problem in future clinical use because the usual malaria treatment does not require a long time compared with the therapy of HIV infection.

As shown in Table 2, several compounds that exhibited potent Plm II inhibitory activity also exhibited potent inhibitory activities against HIV-1 PR. This is also attributed to the similarity in native substrate recognition between Plm II and HIV-1 PR. In particular, compound **4a** showed a high inhibitory activity against both Plm II and HIV-1 PR. This result suggests that the active conformations of **4a** in Plm II and HIV-1 PR may be similar. Therefore, a conformation of **4a** was obtained from the crystal structure of KNI-764 bound to HIV-1 PR (PDB entry, 2KZK) as a starting conformation. Then, its modeled structure bound to the active site of Plm II was generated adopting the conformation to the crystal structure of a complex of pepstatin A and Plm II (PDB entry, 1SME). The energy minimization with a MMFF94s force field was performed using the

Molecular Operating Environment modeling package (MOE 2003.02, Chemical Computing Group, Inc., Montreal, Canada). As shown in Figure 3, the indan moiety is fitted in the S_2' pocket of Plm II while the hydroxyl group of aminoindanol interacts with a hydroxyl group of Tyr192 by forming favorable hydrogen bonds. This suggests that the hydroxyl group of aminoindanol probably has the same role as the carbonyl group of Ser at the P_2' position in the substrate. A similar orientation of aminoindanol is shown in the case of indinavir in HIV-1 protease (PDB entry, 2BPX). However, the binding mode of aminoindanol in compound **4a** is different from the proposed model of a 1,2-dihydroxyethylene compound by Hallberg's group [12]. This is due to the fact that our modeled orientation of the phenyl group of compound **4a** is based on the active conformation of KNI-764 in HIV-1 PR.

Compound **4a** which exhibited the best activity against Plm II among all the test compounds also inhibited Plm I, IV and HAP [6]. This result suggests that compound **4a** might be able to inhibit the degradation of hemoglobin in the food vacuole. The activity of compound **4a** was also evaluated in *P. falciparum*-infected erythrocyte cultures. There was a significant reduction in the potency of compound **4a** in this assay (IC_{50} = 6.8 μM). This decrease can be explained by the fact that Plm inhibitors must penetrate four membranes (i.e. erythrocyte, parasitophorous vacuolar, parasite plasma, and food vacuole membranes) to reach their target. The excessive hydrophobicity of compound **4a** due to a dimethylphenoxyacetyl group at the P_2 position may cause a lowered cell penetration, hence suppressing its intrinsic activity.

In conclusion, novel Plm II inhibitors were designed and synthesized based on the substrate transition state. The Apns-Dmt scaffold was a Phe-Leu (P_1-P_1') transition-state mimic. Since native substrates contain a Ser residue at the P_2' position, a hydroxyl group was introduced with a fixed conformation and a bulky unit to fit the space of the S_2' pocket of Plm II. From the SAR at the P_2' position, (1*S*,2*R*)-1-amino-2-indanol was the best P_2' ligand (compound **4a**, KNI-10006) of the synthetic compounds, and both the hydroxyl group and the indan structure of the aminoindanol are important for its tight binding. Based on these observations, further modifications are under way to develop improved potential antimalarial compounds.

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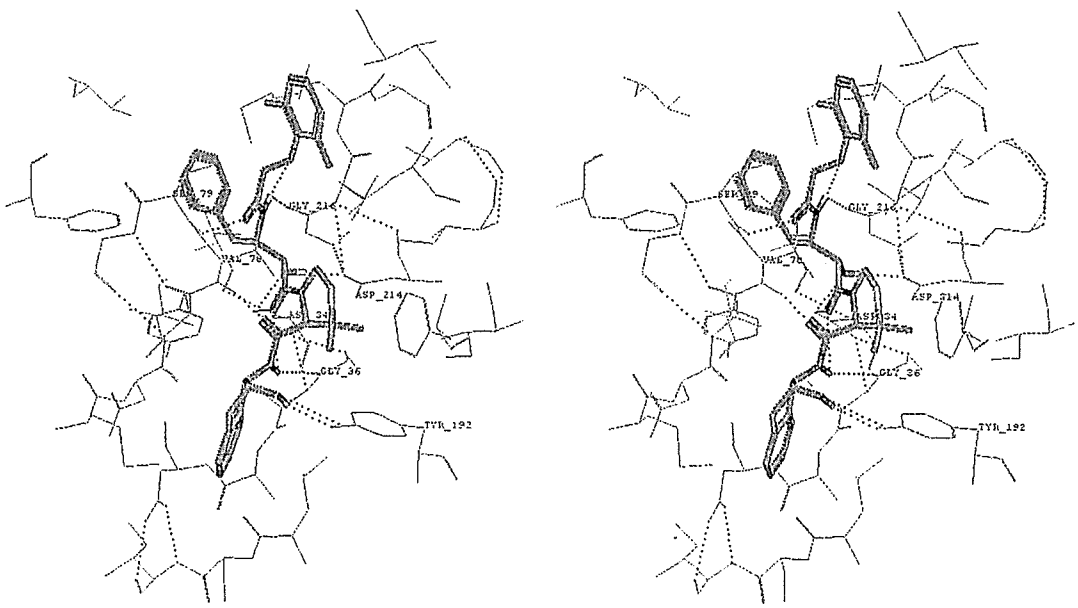

Figure 3 Modeled structure of compound **4a** bound to the active site of Plm II. Residues in the active site of Plm II are represented in light blue. Hydrogen bonds are represented with dotted line.