

TABLE IV. Structure-Based Dissection of Heat Capacity Changes

	$\Delta C_{p,exp}$ (cal k <sup>-1</sup> mol <sup>-1</sup> )	$\Delta C_{p,solv}$ (cal k <sup>-1</sup> mol <sup>-1</sup> )	$\Delta\Delta C_p$ (cal k <sup>-1</sup> mol <sup>-1</sup> )	$\sigma(\Delta H)$ kcal/mol
KNI-764 → WT	-360 ± 20	-220	140	4.9
KNI-764 → V82F/I84V	-390 ± 15	-251	139	4.9
KNI-577 → WT	-420 ± 20	-196	224	6.3
KNI-577 → V82F/I84V	-380 ± 20	-218	162	5.3

$$\Delta\Delta C_p = \Delta C_{p,solv} - \Delta C_{p,exp}$$

$$\sigma(\Delta H) = \sqrt{RT^2 \Delta\Delta C_p}$$

structure-based calculations presented here, we have applied rigid cutoff criteria derived from a statistical analysis of different protein–ligand complexes<sup>14</sup> (completely buried waters within 6 Å of inhibitor), according to which KNI-764 loses one water molecule against the mutant but KNI-577 does not. If KNI-577 also loses one water molecule, the predicted enthalpy would be  $\approx -2.6$  kcal/mol, which is much closer to the experimental value of  $-2.1$  kcal/mol. It seems that a critical factor required to calculate enthalpy changes from structure is an accurate determination of the number of buried water molecules in a complex. A statistical-based criterion like the one used here may not work in every situation since some water molecules may exchange with the solvent even if they are close to the inhibitor and appear to be completely buried from the solvent in the crystal structure.

The binding of KNI-764 and KNI-577 is favored by entropic contributions to the Gibbs energy of  $-6.7$  and  $-8.5$  kcal/mol respectively. The overall entropy contribution is composed of two major terms, a favorable term associated with the desolvation of the inhibitors and some regions of the protein and an unfavorable term associated with the loss of conformational and rotational/translational degrees of freedom. In our calculations, the binding energy was approximated by assuming that the Gibbs energy associated with the closing of the flaps was very small. This assumption is supported by NMR data<sup>16</sup> indicating that the flaps exist in rapid dynamic equilibrium and that the fully closed conformation is also present in solution. The solvation entropy change was calculated from the changes in solvent accessible surface as described before<sup>16</sup> including a correction term for the new enthalpy parameterization, and is shown in Table III. Conformational entropy changes were calculated as described before<sup>16,19,20,21</sup> and are also shown in Table III. According to this analysis, KNI-764 was correctly predicted to gain solvation entropy with the drug resistant mutant. Again, the error obtained for KNI-577 against the drug-resistant mutation is larger than the one obtained for KNI-764. The overall change in conformational entropy was calculated to be larger for KNI-764 than KNI-577. This is an expected result since KNI-764 has an additional rotatable bond and a larger interaction surface with the protease. For each inhibitor, the conformational entropy changes were predicted to be similar for wild type and mutant proteases within the error of the calculations.

Overall, the structure-based calculations predict the binding thermodynamics of the inhibitor to wild-type protease with higher accuracy than to the mutant (Fig. 6

and Table III). Against the wild type, the error in  $\Delta G$  is 0.2 kcal/mol, whereas against the mutant it is 1.9 kcal/mol and always an overestimation. In our calculations, we have assumed that the conformational energetics of wild type and mutant protein are the same. In fact, this may not be the case. Previously, Todd et al<sup>9</sup> determined that the native conformation of the V82F/I84V mutant of the HIV-1 protease is more stable than the wild type protease and if this difference is due to a higher stability of the open conformation it will lower the affinity of inhibitors by about a factor of ten or 1.4 kcal/mol. If this is indeed the case, the error in the structure-based calculation of  $\Delta G$  will go down to 0.5 kcal/mol and will be close to the one observed for the wild-type protease.

The heat capacity values calculated from the changes in accessible surface areas listed in Table IV underestimate the experimental values, suggesting that other effects in addition to changes in solvation contribute to the observed values. Since linked protonation reactions are negligible, a reasonable alternative is the presence of a significant contribution associated with the change in conformation of the protease between the free and bound states. In the unbound state, the protease exists in a highly dynamic conformational equilibrium that defines a conformational ensemble characterized by significant fluctuations, particularly in the flap region;<sup>18</sup> in the bound state those conformational fluctuations are significantly dampened.<sup>18</sup> Since structural fluctuations are coupled to enthalpy fluctuations, these changes would be reflected in the heat capacity of the protease because heat capacity is proportional to the average square fluctuation of the enthalpy:<sup>22,23</sup>

$$C_p = \frac{\langle \Delta H^2 \rangle - \langle \Delta H \rangle^2}{RT^2} \quad (1)$$

If the differences between the experimental heat capacities and those calculated from the changes in solvation are assumed to be due to diminished structural fluctuations in the bound state, a reduction of  $\approx 5.4$  kcal/mol in the width of the enthalpy distribution of the protease would be expected upon going from the unbound to the bound state. The typical width of the enthalpy distribution for a globular protein of similar molecular weight is on the order of 30 kcal/mol.<sup>23</sup>

While water molecules buried at the protein–ligand interface contribute significantly to the binding enthalpy, they appear not to contribute significantly to the Gibbs energy of binding. The enthalpy gain is compensated by

the entropic loss associated with trapping the water molecules within the protein structure. If the structure-based thermodynamic analysis is performed without considering the water molecules using a procedure described previously,<sup>16</sup> the enthalpy is underestimated by 6-7 kcal/mol; however, the Gibbs energy is calculated reasonably accurately (1.9 kcal/mol standard deviation). If the water molecules are included in the analysis and their entropy loss is considered an adjustable parameter, only a marginal improvement in the calculated Gibbs energy is obtained (1.3 kcal/mol), suggesting that the enthalpic gain is significantly compensated by the entropy loss of immobilizing the water molecules at the protein-ligand interface. Similar observations regarding the role of interface water have been made recently for antigen-antibody complexes.<sup>24</sup>

### CONCLUSIONS

The structural and thermodynamic information presented here describes a mechanism by which an inhibitor can minimize the effects of mutations conferring drug resistance. At the thermodynamic level, the detrimental effects of the mutation on the binding affinity are minimized by the phenomenon of enthalpy/entropy compensation. At the structural level, the thermodynamic response originates from the better adaptability of KNI-764 compared to KNI-577. The better adaptability of KNI-764 is due to the presence of an asymmetric functionality and an additional rotatable bond at the same position where KNI-577 has only one rotatable bond and a symmetric functionality. While inhibitor flexibility is important for adaptability, it must be noted that it should be located at positions facing variable regions of the target and that the asymmetry of the functional group provides a significantly larger number of alternate conformations that are able to establish strong interactions with the target molecule.

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## Design and synthesis of a novel water-soluble A $\beta$ 1-42 isopeptide: an efficient strategy for the preparation of Alzheimer's disease-related peptide, A $\beta$ 1-42, via *O*–*N* intramolecular acyl migration reaction

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**Abstract**—A novel water-soluble isopeptide of Alzheimer's disease-related peptide A $\beta$ 1-42, '26-*O*-acyl isoA $\beta$ 1-42', which could efficiently convert to intact A $\beta$ 1-42 under physiological conditions via *O*–*N* intramolecular acyl migration, was synthesized providing a new system useful for investigation of biological function of A $\beta$ 1-42.  
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Amyloid  $\beta$  peptides (A $\beta$ ) are the main proteinaceous component of the amyloid plaques found in the brains of Alzheimer's disease (AD) patients.<sup>1</sup> Neuritic plaques, pathognomonic features of AD, contain abundant fibrils formed from A $\beta$ , which have been found to be neurotoxic in vivo and in vitro.<sup>2</sup> The predominant forms of A $\beta$  consist of mainly 40- and 42-residue peptides (designated A $\beta$ 1-40 and A $\beta$ 1-42, respectively), which are proteolytically produced from amyloid precursor protein (APP) by enzymatic reactions.<sup>3</sup> Since A $\beta$ 1-42 is suggested to play a more critical role in amyloid formation and the pathogenesis of AD than A $\beta$ 1-40, many studies using synthetic A $\beta$ 1-42 have been carried out to clarify the involvement of A $\beta$ 1-42 in AD.<sup>4</sup>

However, A $\beta$ 1-42 is a highly hydrophobic peptide and forms aggregates in various media. This aggregation is attributed to its intermolecular hydrophobic interaction and hydrogen bond formation among peptide chains, leading to the formation of extended  $\beta$ -sheet structures.<sup>5</sup> Hence, the highly agglutinative potency of A $\beta$ 1-42 results in the synthetic difficulty of this peptide,<sup>5</sup> so-called 'difficult sequence'-containing peptide.<sup>6</sup> Due to

the low solubility and broad elution of A $\beta$ 1-42 under acidic or neutral conditions, the conventional HPLC purification of synthesized A $\beta$ 1-42 in the aqueous TFA–acetonitrile system is too laborious to remove impurities accumulated during the solid-phase peptide synthesis (SPPS). Furthermore, the biological experiments using A $\beta$ 1-42 are problematic because of the large extent of aggregation in a standard storage solution such as dimethylsulfoxide (DMSO).<sup>7</sup> Therefore, the 'in situ' system that can prepare intact A $\beta$ 1-42 in solubilized form under physiological conditions would be a powerful tool in understanding the inherent pathological function of A $\beta$ 1-42. To create such system, (1) a novel propeptide possessing a high solubility during HPLC purification and long-term storage as a solution and (2) a capability of intact A $\beta$ 1-42 production under physiological conditions are required.

*O*–*N* intramolecular acyl migration reaction is a well-known reaction observed in Ser/Thr-containing peptides.<sup>8</sup> In our previous study regarding the synthesis of difficult sequence-containing peptides, we disclosed a novel and efficient method based on this migration reaction of synthesized '*O*-acyl isopeptides'. This method remarkably improved the synthetic yields of difficult sequence-containing pentapeptides.<sup>9</sup> The result also indicated that the branched ester structure in *O*-acyl isopeptides could suppress the unfavorable nature seen in the difficult sequence-containing peptides. Namely, the insertion of the ester bond into peptide chain can

**Keywords:** Water-soluble A $\beta$ 1-42 isopeptide; Alzheimer's disease; A $\beta$ 1-42; *O*–*N* intramolecular acyl migration reaction; Difficult sequence; 26-*O*-acyl isoA $\beta$ 1-42.

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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 isopeptides with the newly formed amino group can possess reasonable H<sub>2</sub>O- and MeOH-solubility required in HPLC purification by the formation of salt. Furthermore, from recent research of water-soluble prodrugs<sup>10</sup> and *O*-acyl isopeptides<sup>9</sup> based on *O*–*N* intramolecular acyl migration, it is established that the purified *O*-acyl isoform can completely be converted to the original *N*-acyl form in a short time with no side reaction at pH 7.4.

Based on this background, we conceived the idea that the *O*–*N* intramolecular acyl migration of *O*-acyl isopeptides could be applied to the synthesis of Aβ1-42 via a novel water-soluble isopeptide of Aβ1-42, that is, '26-*O*-acyl isoAβ1-42 (26-AIAβ42, 2)'. This idea would overcome the problems in the synthesis and storage of Aβ1-42 (Fig. 1). Although there are two Ser residues in Aβ1-42 at the positions 8 and 26 with the capability of *O*–*N* intramolecular acyl migration, we selected the Ser<sup>26</sup> for the *O*-acylation, since the adjacent Gly<sup>25</sup> does not epimerize during ester bond formation (Fig. 1).

As depicted in Scheme 1, Fmoc-Ala-*O*-chlorotrityl resin (0.465 mmol/g, 3) was employed and Fmoc-protected amino acids were sequentially coupled using the DIPCDI–HOBT method (2h) after removal of each Fmoc group with 20% piperidine–DMF (20 min) to give peptide resin 4. After Boc-Ser-OH was introduced to 4, the obtained 5 was coupled with Fmoc-Gly-OH at the β-hydroxy group of Ser using the DIPCDI–DMAP method in CH<sub>2</sub>Cl<sub>2</sub> to obtain ester 6. 26-AIAβ1-42-resin (7) was obtained through the coupling of additional amino acid residues by the conventional manner. Finally, 26-AIAβ1-42 (2) was obtained as a major product by the treatment of TFA–*m*-cresol–thioanisole–H<sub>2</sub>O (92.5:2.5:2.5:2.5) for 90 min followed by NH<sub>3</sub>·I–dimethylsulfide for 60 min in TFA–H<sub>2</sub>O (2:1).

In HPLC analysis of crude products (Fig. 2A), 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

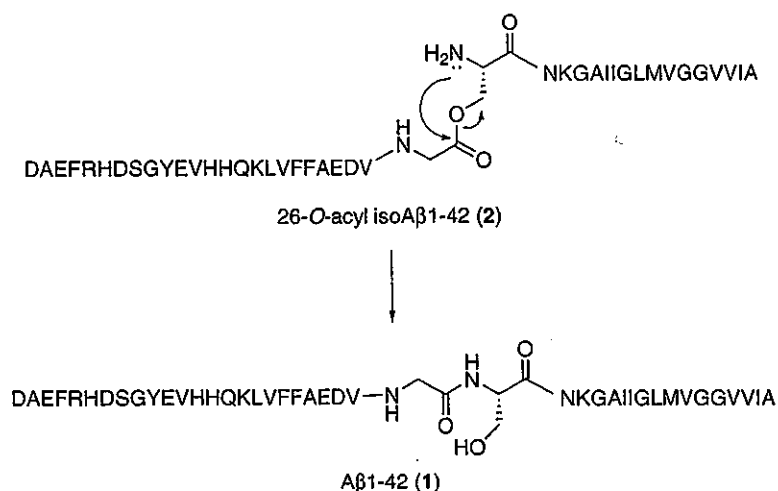
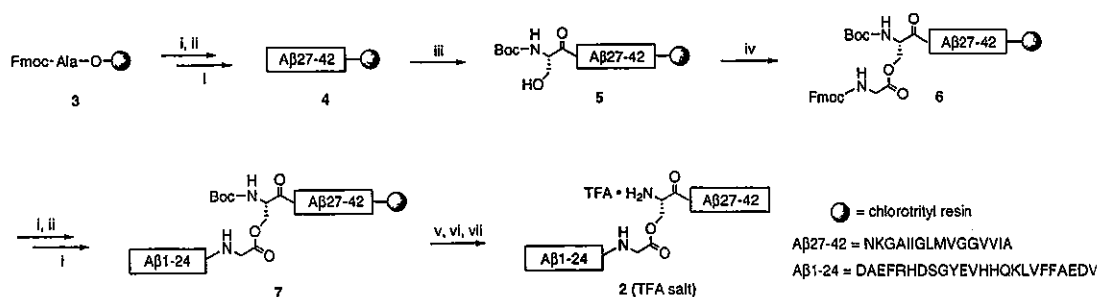
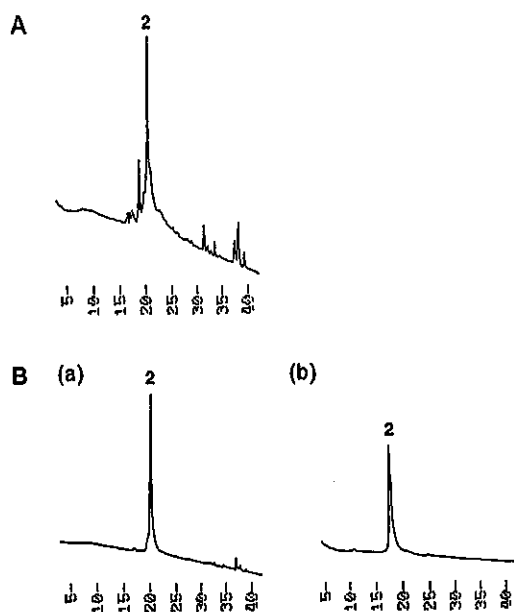


Figure 1. The production of Aβ1-42 (1) via the *O*–*N* intramolecular acyl migration reaction of 26-*O*-acyl isoAβ1-42 (2).



**Scheme 1.** Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-AA-OH (2.5 equiv), DIPCDI (1,3-diisopropylcarbodiimide, 2.5 equiv), HOBT (2.5 equiv), DMF, 2 h; (iii) Boc-Ser-OH (2.5 equiv), DIPCDI (2.5 equiv), HOBT (2.5 equiv), DMF, 2 h; (iv) Fmoc-Gly-OH (3.0 equiv), DIPCDI (3.0 equiv), DMAP (0.2 equiv), CH<sub>2</sub>Cl<sub>2</sub>, 16 h × 2; (v, vi, vii) TFA–*m*-cresol–thioanisole–H<sub>2</sub>O (92.5:2.5:2.5:2.5), 90 min; (vi) NH<sub>3</sub>·I (20 equiv), dimethylsulfide (20 equiv), TFA–H<sub>2</sub>O (2:1), 60 min, 0 °C; (vii) preparative HPLC (a linear gradient of CH<sub>3</sub>CN in 0.1% aqueous TFA).

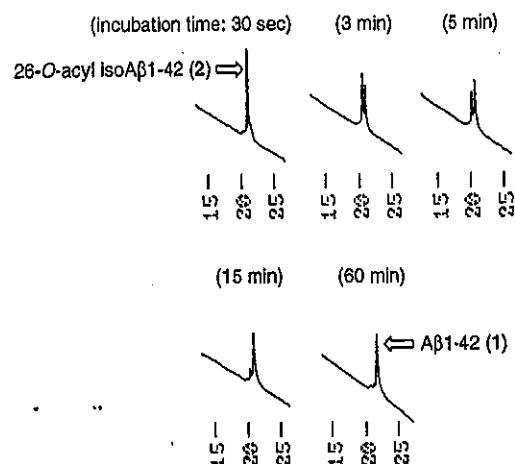


**Figure 2.** HPLC profiles of (A) crude and (B) purified 26-AIA $\beta$ 1-42 (2). Analytical HPLC was performed using a C18 reverse phase column (4.6  $\times$  150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH<sub>3</sub>CN (0–100% CH<sub>3</sub>CN, 40 min for A and B-a, 25–55% CH<sub>3</sub>CN, 60 min for B-b) in 0.1% aqueous TFA at a flow rate of 0.9 mL min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm.

TFA. The crude *O*-acyl isopeptide 2 was dissolved in hexafluoroisopropanol, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH<sub>3</sub>CN. Since 2 was eluted as a narrow single peak, we could easily purified by preparative scale HPLC to give pure 2 (Fig. 2B) as TFA salt with the total isolated yield of 33.6%, calculated from the original loading of chlorotriyl resin.<sup>11</sup> This yield was higher than that obtained in the synthesis of 1 by a standard Fmoc-based SPPS (7.2%). Since 1 was eluted as a broad peak in preparative scale HPLC purification, it was laborious to isolate 1 from impurities as reported.<sup>5</sup> In addition, in the synthesis of 2, no conversion to 1 was observed.

The water-solubility of 2 (TFA salt) was 15 mg mL<sup>-1</sup>, which was 100-fold higher than that of A $\beta$ 1-42 (1, 0.14 mg mL<sup>-1</sup>). Interestingly, the fact that a slight modification of peptide chain by the insertion of one ester bond could drastically increase the solubility of the insoluble original peptide with 42-residues suggests that *O*-acyl isopeptide totally break the secondary structures responsible for the insolubility of the original peptide. As we demonstrated that *O*-acyl isopeptide could suppress the unfavorable nature of difficult sequence-containing pentapeptides in the previous study,<sup>9</sup> the present result in the synthesis of 2 indicates that this method is a powerful strategy for increasing the solubility even in large peptides.

As shown in Figure 3, purified 2 was completely converted to A $\beta$ 1-42 (1) at room temperature in phosphate buffered saline (PBS, pH 7.4) with no side reaction. This migration was rapid with a half-life of 2.6 min, while TFA salt of 2 was stable at 4 °C for at least 30 days as either a solid state or a DMSO solution. Moreover, a slower migration was observed at pH 4.9 (PBS) with a



**Figure 3.** HPLC profiles of the conversion of 26-AIA $\beta$ 1-42 (2) to A $\beta$ 1-42 (1) 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  $\times$  150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH<sub>3</sub>CN (0–100% CH<sub>3</sub>CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm.

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 to produce intact monomer A $\beta$ 1-42 in situ to investigate inherent biological function of A $\beta$ 1-42 in AD. The conversion of 2 (TFA salt) in water for 48 h at room temperature followed by lyophilization yielded A $\beta$ 1-42 (1) quantitatively as TFA salt with the purity of >95%.<sup>12</sup>

In conclusion, we synthesized a novel water-soluble isopeptide of A $\beta$ 1-42, (26-AIA $\beta$ 42, 2), which is a 26-*O*-acyl isopeptide of A $\beta$ 1-42. This *O*-acyl isopeptide has a higher water-solubility than that of A $\beta$ 1-42 (1), and can migrate to intact A $\beta$ 1-42 (1) under various conditions while it is stable under storage conditions. This suggests that the synthesis of A $\beta$ 1-42 via 26-AIA $\beta$ 42 could overcome the solubility problem and give a novel tool for the biological evaluation system, in which 26-AIA $\beta$ 42 can be stored in a solubilized state before the use and rapidly produce the intact A $\beta$ 1-42 in situ during biological experiments.

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11. Spectral data for 2: MALDI-MS (TOF):  $M_{\text{calc}}$ : 4514.04;  $M + H_{\text{found}}$ : 4515.26; HPLC analysis at 230 nm: purity was >96%.
12. Spectral data for 1: MALDI-MS (TOF):  $M_{\text{calc}}$ : 4514.04;  $M + H_{\text{found}}$ : 4515.48; HPLC analysis at 230 nm: purity was >95%; the retention time on HPLC (0–100%  $\text{CH}_3\text{CN}$  for 40 min, 230 nm) of synthesized 1 was identical to that of commercially available A $\beta$ 1-42 (Peptide Institute, Inc., Osaka, Japan).

# Rigid backbone moiety of KNI-272, a highly selective HIV protease inhibitor: methanol, acetone and dimethylsulfoxide solvated forms of 3-[3-benzyl-2-hydroxy-9-(isoquinolin-5-yloxy)-6-methylsulfanylmethyl-5,8-dioxo-4,7-diazanonanoyl]-*N*-*tert*-butyl-1,3-thiazolidine-4-carboxamide

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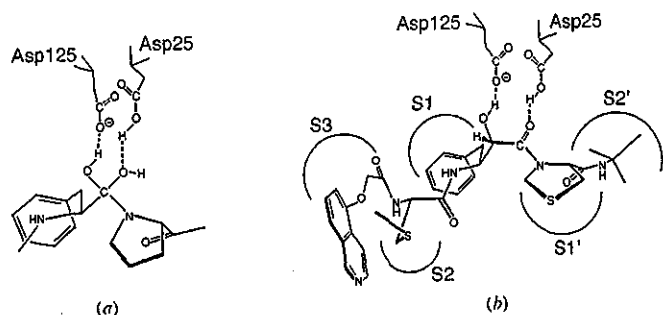
When crystals of kynostatin (KNI)-272, a highly selective HIV protease inhibitor containing allophenylnorstatine [(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid], were grown in three different solvent systems (methanol, acetone and dimethylsulfoxide solutions), the local conformations around the hydroxymethylcarbonyl (HMC) moiety, which mimics the structure of the transition state, were similar in all three forms. The peptide backbones were slightly bent, but their structures differed from typical sheets, turns or helices. Although the isoquinoline ring at the *N*-terminal showed conformational variations, a remarkable similarity was observed in the *C*-terminal region, including the HMC moiety. Moreover, the conformational characteristics of the uncomplexed forms resembled those of the inhibitor within the KNI-272–HIV protease complex. This suggests that the structure of the *C*-terminal region of KNI-272 is rigid or very stable.

## 1. Introduction

KNI-272 (I) is a tripeptide mimic whose design was based on the substrate transition state concept of amide hydrolysis by aspartic protease (Fig. 1; Kiso, 1996; Kiso *et al.*, 1999). This peptide potently inhibits HIV protease activity (Mimoto *et al.*, 1992) and displays good pharmacokinetics and an excellent therapeutic index (Kageyama *et al.*, 1993). It is the unusual amino acid allophenylnorstatine [Apns = (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] which has the key structure that mimics the transition state (Mimoto, Imai, Tanaka, Hattori, Takahashi *et al.*, 1991; Mimoto, Imai, Tanaka, Hattori, Kisanuki *et al.*, 1991), although the other unusual amino acids of KNI-272, 5-isoquinolyloxy acetic acid (iQoa), methylthioalanine (Mta), thiazolidine-4-carboxylic acid (Thz) and *tert*-butylamine (<sup>t</sup>Bu), are also highly optimized to enhance the inhibitory activity and selectivity for HIV protease (Sheha *et al.*, 2000). The P1–P1' site of aspartic protease (Schechter, 1968) is formed at the hydroxymethylcarbonyl (HMC)–amide bond, between Apns<sup>3</sup> and Thz<sup>4</sup>, and both *cis* and *trans* conformers can be presumed for this bond. However, NMR and molecular modeling studies indicate that the *trans* conformer of the HMC–amide bond should predominate (Kato *et al.*, 1994; Ohno *et al.*, 1996), which is consistent with the crystal structure (Doi *et al.*, 2001), the structure of the inhibitor within the KNI-272–HIV protease complex (Baldwin *et al.*, 1995) and the finding that a wide moiety which includes

an HMC group will tend to converge towards a certain conformation (Ohno *et al.*, 1996). Although no strong factor limiting the peptide conformation has been found within the chemical structure of KNI-272, the above results suggest the existence of a stable conformation for the moiety with the HMC group. Since the properties of solvents affect the peptide

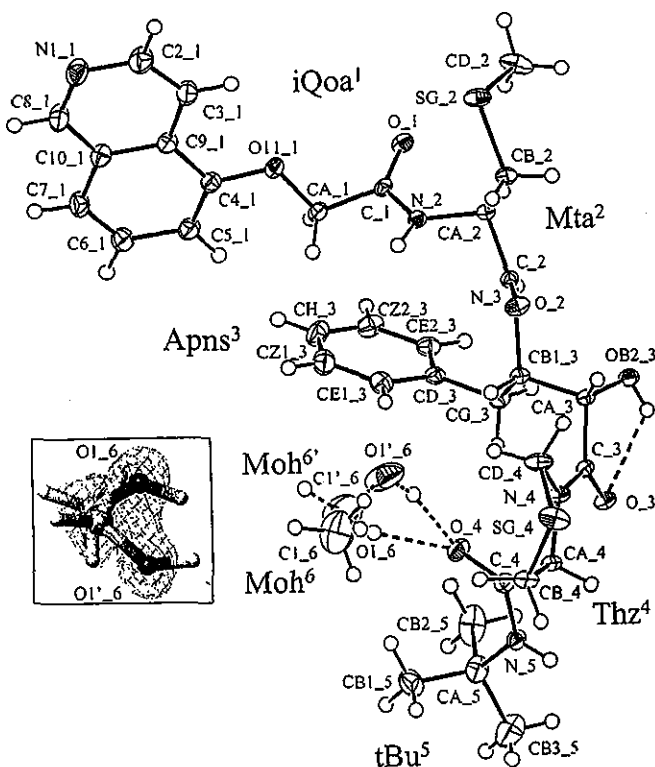
structures (Karle *et al.*, 1990; Awasthi *et al.*, 2001), the inherent conformational variations of peptides seem to be observed in the crystal structures grown from different solvent systems. To test that hypothesis, the comparison among such crystal structures could be a probe. Therefore, we solved three solvated crystal forms of KNI-272 and compared the stable conformations with previously solved structures.



**Figure 1**  
The substrate transition state concept (Kiso, 1996; Kiso *et al.*, 1999). (a) Substrate transition state of amide hydrolysis by HIV protease. A water molecule is added to the carbonyl carbon of a substrate activated by catalytic Asp residues of HIV protease. An intermediate takes a tetrahedral coordination for a brief time and then the C–N bond is broken. (b) Interaction between KNI-272 and HIV protease. Hydrogen bonds with catalytic Asp residues resemble those of the substrate transition state, but in this case the C–N bond is never broken.

## 2. Experimental

KNI-272, which was synthesized as described previously (Mimoto *et al.*, 1992), was dissolved in approximately 60–80% aqueous methanol with warming and the minimum amount of methanol needed to prevent precipitation. The solution was then sealed in a vial at room temperature and crystals of the aqueous methanol form (ME form) were grown for 3–4 d. Crystals of the acetone solvated form (AC form) were grown from KNI-272 acetone solution containing 5–10% hexane. Crystals of the dimethylsulfoxide solvated form (DM form) were grown from KNI-272 dissolved in dimethylsulfoxide, to which was added a small amount of water (5–10%). In this case, the solution was sealed in a vial overnight. All crystals were briefly passed through 100% glycerol on a nylon loop (Hampton Inc.) and then flash-frozen under a nitrogen stream at 90 K. Data collection was carried out on a Bruker SMART APEX CCD area detector using Mo  $K\alpha$  radiation. Intensities were integrated using *SMART* and *SAINT* (Bruker, 1998), and the empirical absorption corrections were applied using *SADABS* (Sheldrick, 1996). The structures were solved using *SHELXS97* (Sheldrick, 1997) and refined with *SHELXL97* (Sheldrick, 1997). H atoms were placed at calculated positions (C–H 0.95–1.00 and N–H 0.88 Å) using isotropic displacement parameters [ $U_{\text{iso}} = 1.5 U_{\text{eq}}$  (C) for methyl H atoms and 1.2  $U_{\text{eq}}$  (parent atom) for all other atoms] and were included in the structure-factor calculations. The H atom in the hydroxyl group of Apns<sup>3</sup> was found in a differential Fourier map and was fixed during the refinements. In the ME form, the hydroxyl H atoms of the disordered methanol molecules were also found in a differential Fourier map and they too were fixed during the refinements. The absolute configurations were determined from the material amino acids and were consistent with the suggested Flack parameters (Flack, 1983). The crystal and experimental data are summarized in Table 1.<sup>1</sup>



**Figure 2**  
Structure of the ME form with displacement ellipsoids drawn at the 50% probability level. Dotted lines represent hydrogen bonds to a disordered methanol molecule. The inset shows the electron density around the solvents at the 0.8 and 2.4  $\sigma$  level.

<sup>1</sup> Supplementary data for this paper are available from the IUCr electronic archives (Reference: OG5001). Services for accessing these data are described at the back of the journal.



Table 1

Crystal and experimental summaries of the ME and AC forms and referenced structures of the MPD form.

MPD represents KNI-272 crystals grown from aqueous 2-methylpentane-2,4-diol solution (Doi *et al.*, 2001).

	ME	AC	DM
Formula	C <sub>33</sub> H <sub>41</sub> N <sub>5</sub> O <sub>6</sub> S <sub>2</sub>	C <sub>33</sub> H <sub>41</sub> N <sub>5</sub> O <sub>6</sub> S <sub>2</sub>	C <sub>33</sub> H <sub>41</sub> N <sub>5</sub> O <sub>6</sub> S <sub>2</sub>
Solvation	CH <sub>4</sub> O	2C <sub>3</sub> H <sub>8</sub> O	C <sub>2</sub> H <sub>6</sub> SO
<i>M<sub>r</sub></i>	699.87	783.98	745.96
System	Monoclinic	Monoclinic	Monoclinic
Space group	<i>P</i> 2 <sub>1</sub>	<i>P</i> 2 <sub>1</sub>	<i>C</i> 2
<i>a</i> (Å)	10.5077 (9)	10.6395 (7)	29.390 (6)
<i>b</i> (Å)	13.288 (1)	13.2130 (9)	12.882 (3)
<i>c</i> (Å)	13.515 (1)	14.692 (1)	10.609 (2)
$\beta$ (°)	101.924 (1)	98.931 (1)	103.373 (3)
<i>V</i> (Å <sup>3</sup> )	1846.3 (3)	2040.4 (2)	3907.6 (13)
<i>Z</i>	2	2	4
<i>T</i> (K)	90.0 (2)	90.0 (2)	90.0 (2)
<i>D<sub>x</sub></i> (g cm <sup>-3</sup> )	1.259	1.276	1.268
<i>F</i> (000)	744	836	1584
Wavelength (Å)	0.7107	0.7107	0.7107
$\mu$ (mm <sup>-1</sup> )	0.196	0.187	0.241
No. of reflections (obs)	16 068	23 703	12 220
<i>R</i> <sub>int</sub>	0.0191	0.0161	0.0344
No. of reflections (used)	8028	8940	7717
$\theta_{\max}$ (°)	27.1	27.1	26.7
Flack parameter	-0.01 (4)	0.01 (3)	0.09 (6)
<i>R</i>	0.0333	0.0286	0.0443
<i>wR</i>	0.0835	0.0729	0.1136
Goodness-of-fit	1.026	1.034	0.992
( $\Delta/\sigma$ ) <sub>max</sub>	0.011	0.007	0.009
Fraction of $\theta_{\max}$	0.998	0.997	0.988
$\Delta\rho_{\max}$ (e Å <sup>-3</sup> )	0.338	0.285	0.835
$\Delta\rho_{\min}$ (e Å <sup>-3</sup> )	-0.301	-0.218	-0.475

### 3. Results and discussion

The structures of the ME, AC and DM forms are shown in Figs. 2, 3 and 4, respectively. In the ME form, electron densities persist in the solvent region (Fig. 2, inset) and are interpreted as being a methanol molecule disordered to two sites (Moh<sup>6</sup> and Moh<sup>6</sup>). Both the disordered methanol molecules are located at the positions close to the thiazolidine ring (Thz<sup>4</sup>) and hydrogen-bonded to the carbonyl oxygen of Thz<sup>4</sup>: O1<sub>6</sub>···O<sub>4</sub> 2.842 (4) and O1'<sub>6</sub>···O<sub>4</sub> 2.706 (4) Å. The AC form contains two acetone molecules (Ace<sup>6</sup> and Ace<sup>7</sup>). Ace<sup>6</sup> interacts with the amide nitrogen of Mta<sup>2</sup> [N<sub>2</sub>···O1<sub>6</sub> 3.019 (2) Å], but no hydrogen bond is formed with Ace<sup>7</sup> (Fig. 3). Ace<sup>6</sup> is sandwiched between the Thz<sup>4</sup> and the isoquinoline (iQoa<sup>1</sup>) rings, and the hydrophobic interactions among these residues seem to contribute to the peptide conformation of the AC form. A dimethylsulfoxide molecule (Dms<sup>6</sup>) is present within the DM form (Fig. 4) and is hydrogen-bonded to the amide nitrogen of Mta<sup>2</sup>: N<sub>2</sub>···O1<sub>6</sub> 2.832 (3) Å. This interaction pattern is similar to that of Ace<sup>6</sup> in the AC form. The relative disposition of Dms<sup>6</sup> for the peptide is also similar to that of Ace<sup>6</sup>, but the isoquinoline ring is not folded to Dms<sup>6</sup>. Apparently KNI-272 is 'hygroscopic' for the solvents used, so that its crystallization is accompanied by solvation and the properties of solvents affect the conformations of KNI-272.

Within the substrate-HIV protease complex, at least one water molecule is used for the hydrolysis of the amide bond and should therefore be located in close proximity to the

substrate (Kiso, 1996). NMR analysis has shown that water molecules bind between KNI-272 and HIV protease, stabilizing the complex structure (Wang, Freedberg, Wingfield *et al.*, 1996). It thus appears that the hygroscopicity of KNI-272 supports the structure mimicking the substrate transition state within the complex.

According to the substrate transition state concept (Kiso, 1996; Kiso *et al.*, 1999), the HMC group of the Apns<sup>3</sup> residue should assume a conformation that mimics the substrate of HIV protease (Fig. 1), which is a homodimeric protein with a symmetric catalytic center (Pearl & Taylor, 1987). It is known, moreover, that when the asymmetric KNI-272 is bound, catalytic Asp residues assume different ionization states that contribute to a structure that mimics the transition state (Wang, Freedberg, Yamazaki *et al.*, 1996). This means that the structure around the HMC group is the most important when considering the interaction between KNI-272 and the catalytic center of the protease. The bond distances around the HMC moiety of the Apns<sup>3</sup> residue clearly distinguish the CA<sub>3</sub>-OB<sub>2\_3</sub> hydroxyl bond from the C<sub>3</sub>-O<sub>3</sub> carbonyl bond (Table 2), and no trend toward tautomerism between the hydroxyl and carbonyl groups is seen in any of the crystal forms. Such clear localization of the hydroxyl and carbonyl groups would provide support for the structure mimicking the transition state within the complex. Bond rotation is chemically permitted for CA<sub>3</sub>-C<sub>3</sub>, but the conformations of the hydroxyl and carbonyl groups are synperiplanar in the three forms: OB<sub>2\_3</sub>-CA<sub>3</sub>-C<sub>3</sub>-O<sub>3</sub> -20.9 (2), -24.1 (2) and -24.6 (4)° for the ME, AC and MD forms, respectively. A hydrogen bond is formed between OB<sub>2\_3</sub> and O<sub>3</sub>, making a five-membered ring at the HMC group, which contributes to the stability of the synperiplanar conformations.

Similar geometry was previously found around the HMC group of KNI-272 crystals grown from aqueous 2-methylpentane-2,4-diol solution (MPD form; Doi *et al.*, 2001). It is noteworthy that geometries found at the HMC structure are similar despite the different physical properties of the respective solvent systems (*e.g.* polarity and dielectric constant). Within the structure of the KNI-272-HIV protease complex (the 1HPX form; Baldwin *et al.*, 1995), the HMC group interacts with two Asp residues and there are many contacts between KNI-272 and the protein. Nevertheless, the geometry of HMC in the 1HPX form is surprisingly similar to those of the uncomplexed structures, which implies that the local conformation of the HMC moiety in single crystals reflects the active conformation of the inhibitor-enzyme complex.

The peptide backbones of all the forms are slightly bent but cannot be classified as a typical sheet, turn or helix (Table 3). For comparison, the ME, AC, DM and MPD forms of KNI-272 were fitted to the 1HPX form using the four C $\alpha$  atoms of residues 2-5 (Fig. 5). Conformational variation is observed in the isoquinoline ring of the iQoa<sup>1</sup> residue, which would reflect the molecule-molecule contacts within the crystal and solvent environments. Similar variation is observed for the side chains of Mta<sup>2</sup> and Apns<sup>3</sup>, but their spatial distributions are significantly smaller than that of iQoa<sup>1</sup>. This suggests that the

**Table 2**  
Selected geometry around the HMC moiety of the Apns residue.

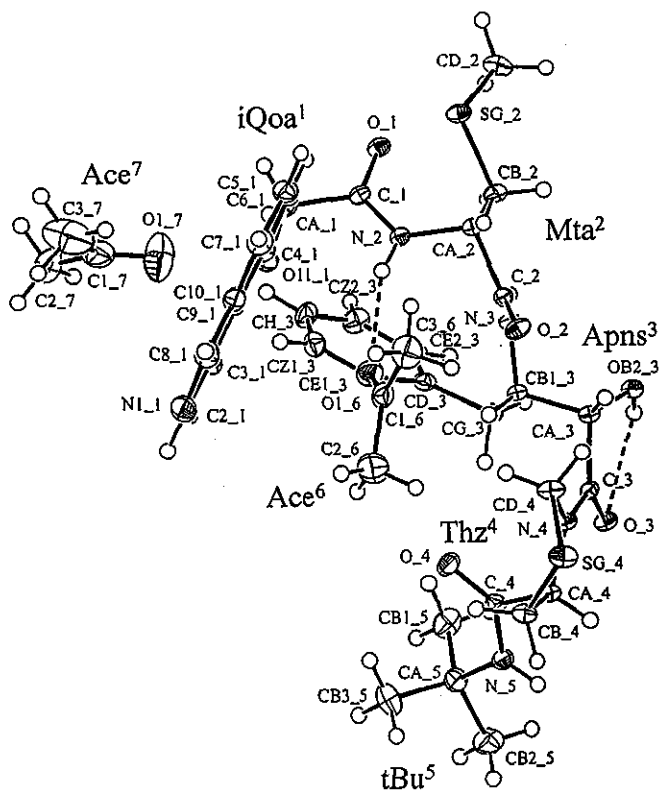
MPD represents KNI-272 crystals grown from aqueous 2-methylpentane-2,4-diol solution (Doi *et al.*, 2001). 1HPX represents the structure of the inhibitor within the HIV protease-KNI-272 complex (Baldwin *et al.*, 1995).

	ME	AC	DM	MPD	1HPX
CB1_3–CA_3	1.548 (2)	1.548 (2)	1.545 (4)	1.529	1.53
CA_3–OB2_3	1.409 (2)	1.407 (2)	1.409 (3)	1.412	1.38
OB2_3–H	0.855	0.874	0.848	0.757	0.97
CA_3–C_3	1.533 (2)	1.525 (2)	1.528 (4)	1.534	1.52
C_3–O_3	1.236 (2)	1.228 (2)	1.233 (3)	1.240	1.23
N_3–CB1_3–CA_3–OB2_3	–65.2 (2)	–67.2 (1)	–72.1 (3)	–65.1	–75
CB1_3–CA_3–C_3–O_3	101.9 (2)	98.5 (1)	97.5 (3)	107.9	89
OB2_3–CA_3–C_3–O_3	–20.9 (2)	–24.1 (2)	–24.6 (4)	–14.2	–33
OB2_3···O_3	2.693 (2)	2.704 (1)	2.693 (3)	2.669	2.68
OB2_3–H···O_3	2.187	2.359	2.200	2.269	2.22
∠OB2_3–H···O_3	117.7	103.8	117.0	114.2	108

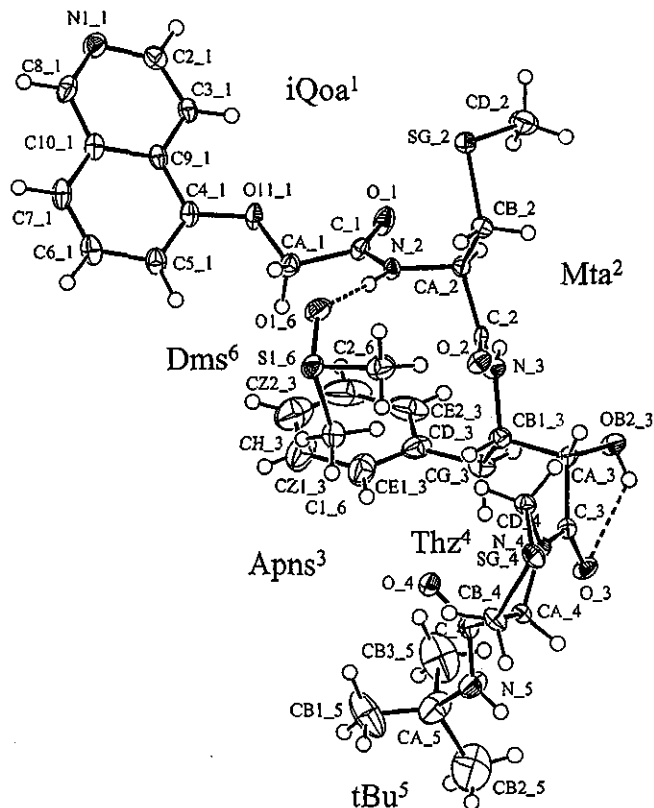
conformation of iQoa<sup>1</sup> is flexible and reflects the specific environment of the KNI-272 molecule, and that this flexibility is favorable for stabilizing the inhibitor molecule on the enzyme. In the 1HPX form, the isoquinoline ring is located on the protein surface, bending into the cavity of the active center, which would delay the release of the inhibitor from the protease. By contrast, the backbones of the Mta<sup>2</sup>–Apns<sup>3</sup>–Thz<sup>4</sup>–tBu<sup>5</sup> moieties are very similar to one another (Fig. 5),

with torsion angles ( $\varphi_3'$ ,  $\psi_3$ ,  $\varphi_4$ ,  $\psi_4$  and  $\varphi_5$ ) that are indicative of the similarity of the backbone conformations (Table 3). In the MPD form, the hydrating waters (disordered to three sites) are located beside the phenyl ring of Apns<sup>3</sup>, forcing the side chain to swing, thereby causing slight shifts in the CB1\_3 position related to the  $\varphi_3$  angle. In addition, the Thz<sup>4</sup> ring of the 1HPX form shows a 'puckering' that differs slightly from the other forms (*e.g.* the  $\chi_4$  angle). Water molecules 301 and 607 are located beside Thz<sup>4</sup> within the complex and seem to affect the 'puckering' of the Thz<sup>4</sup> ring. Analysis of molecular dynamics using NOE restraints has shown there to be three predominant families of KNI-272 structures (conformations A, B and C) with conformational variations at iQoa<sup>1</sup> (Ohno *et al.*, 1996). Of those, conformation A was similar to the 1HPX form. In particular, the backbone of Apns<sup>3</sup>–Thz<sup>4</sup>–tBu<sup>5</sup> of conformation A showed remarkable similarity to that of the 1HPX form. These similarities suggest the existence of a stable conformation of the backbone of the Mta<sup>2</sup>–Apns<sup>3</sup>–Thz<sup>4</sup>–tBu<sup>5</sup> moiety. In other words, this moiety is conformationally restricted and reflects the active form in the complex.

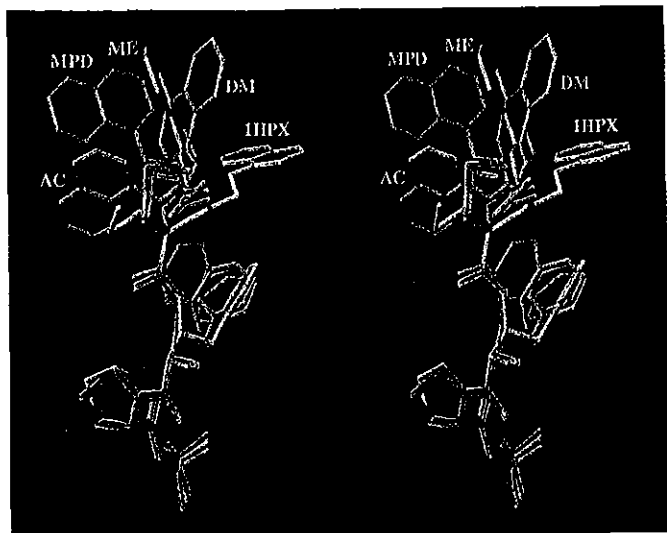
The findings summarized above suggest that KNI-272 is pre-organized to the active form so that, except for the iQoa<sup>1</sup>



**Figure 3**  
Structure of the AC form with displacement ellipsoids drawn at the 50% probability level. Dotted lines represent hydrogen bonds.



**Figure 4**  
Structure of the DM form with displacement ellipsoids drawn at the 50% probability level. Dotted lines represent hydrogen bonds.



**Figure 5**  
Stereoview of the superimposition for four KNI-272 molecules. Molecular fitting was carried out for the four C $\alpha$  atoms of residues 2–5 with an r.m.s.d. of 0.03–0.08 Å. H atoms are omitted for clarity. The colors cyan, yellow, green, orange and gray represent the ME, AC, DM, MPD and IHPX forms, respectively. The side chain of Apns<sup>3</sup> was disordered to two sites in the MPD form, but only one part is drawn for clarity. This figure is produced using *TRUBO-FRODO* (Roussel *et al.*, 2002).

residue, effective and selective binding to HIV protease is achieved without any large conformational changes. However, we still do not know what the key factor is. From studies of HIV protease inhibitors (Mimoto, Kato *et al.*, 1999; Mimoto, Hattori *et al.*, 2000), it seems that a certain combination of unusual amino acids, Apns, Thz and 'Bu, mediates the actual restrictions for five rotatable bonds in the regions ( $\varphi_3$ ,  $\varphi_3'$ ,  $\psi_3$ ,  $\varphi_4$  and  $\psi_4$ ) by steric hindrance. The basic skeleton of the Mta<sup>2</sup>–Apns<sup>3</sup>–Thz<sup>4</sup>–'Bu<sup>5</sup> moiety would therefore be very important for an HIV protease inhibitor having an HMC group. It has also been adapted to the recently developed water-soluble HIV protease inhibitor prodrugs, which also show potent activity (Sohma *et al.*, 2003).

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## O–N Intramolecular Acyl Migration Reaction in the Development of Prodrugs and the Synthesis of Difficult Sequence-Containing Bioactive Peptides\*

**Abstract:** *N*–*O* intramolecular acyl migration in Ser- or Thr-containing peptides is a well-known side reaction in peptide chemistry. It results in the mutual conversion of ester and amide bonds. Our medicinal chemistry study focused on the fact that the *O*-acyl product can be readily converted to the original *N*-acyl form under neutral or slightly basic conditions in an aqueous buffer and the liberated ionized amino group enhances the water solubility of *O*-acyl products. Because of this, we have developed a novel class of “*O*–*N* intramolecular acyl migration”-type water-soluble prodrugs of HIV-1 protease inhibitors. These prodrugs released the parent drugs via a simple chemical mechanism with no side reaction. In this study, we applied this strategy to important cancer chemotherapeutic agents, paclitaxel and its derivatives, to develop water-soluble taxoid prodrugs, and found that these prodrugs, 2'-*O*-isoform of taxoids, showed promising results with higher water solubility and proper kinetics in their parent drug formation by a simple pH-dependent chemical mechanism with *O*–*N* intramolecular acyl migration. These results suggest that this strategy would be useful in toxicology and medical economics.

After the successful application of *O*–*N* intramolecular acyl migration in medicinal chemistry, this concept was recently used in peptide chemistry for the synthesis of “difficult sequence-containing

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\*This paper is dedicated to the memory of Murray Goodman. We will always remember his contributions to peptide science and to peptide societies all over the world, his enthusiasm for life and science encouraging all of us, and his warm, joyous, and humane personality.

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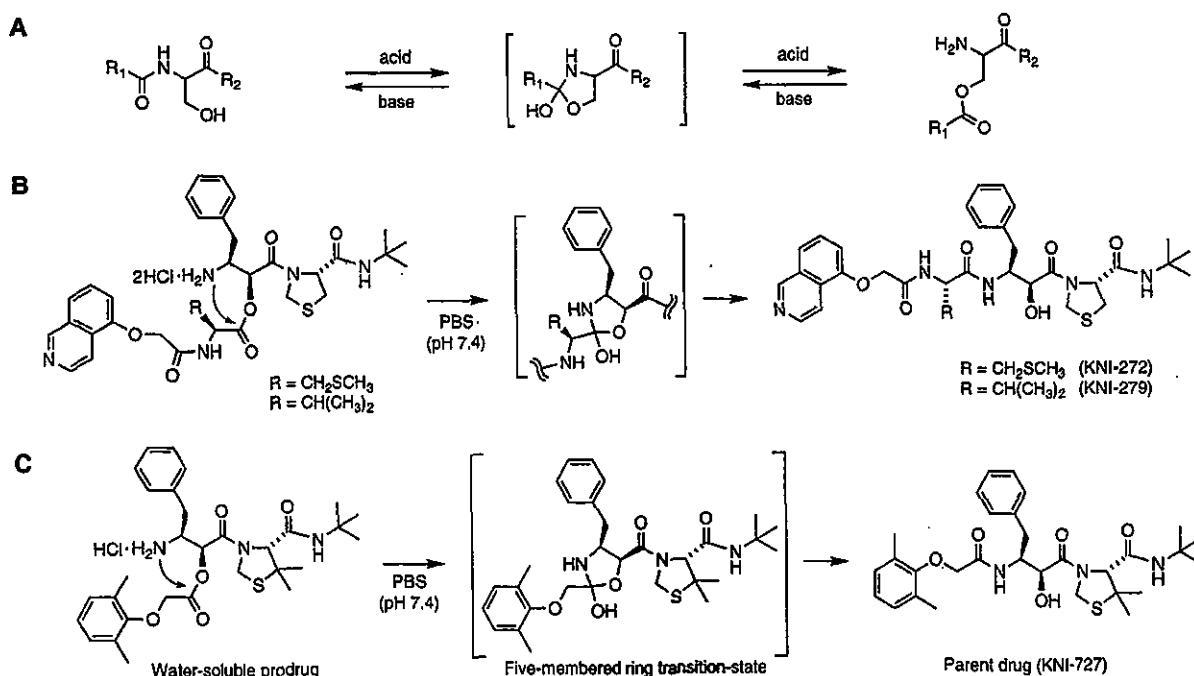
peptides." The strategy was based on hydrophilic O-acyl isopeptide synthesis followed by the O-N intramolecular acyl migration reaction, leading to the desired peptide. In a model study with small, difficult sequence-containing peptides, synthesized "O-acyl isopeptides" not only improved the solubility in various media and efficiently performed the high performance liquid chromatography purification, but also altered the nature of the difficult sequence during SPPS, resulting in the efficient synthesis of O-acyl isopeptides with no complications. The subsequent O-N intramolecular acyl migration of purified O-acyl isopeptides afforded the desired peptides as precipitates with high yield and purity. Further study of the synthesis of a larger difficult sequence-containing peptide, Alzheimer's disease-related peptide (A $\beta$ 1-42), surprisingly showed that only one insertion of the O-acyl group drastically improved the unfavorable nature of the difficult sequence in A $\beta$ 1-42, and achieved efficient synthesis of 26-O-acyl isoA $\beta$ 1-42 and subsequent complete conversion to A $\beta$ 1-42 via the O-N intramolecular acyl migration reaction of 26-O-acyl isoA $\beta$ 1-42. This suggests that our new method based on O-N intramolecular acyl migration is an important method for the synthesis of difficult sequence-containing bioactive peptides. © 2004 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 76: 344-356, 2004

**Keywords:** O-N intramolecular acyl migration reaction; water-soluble prodrug; paclitaxel; difficult sequence-containing peptide; O-acyl isopeptide; A $\beta$ 1-42; 26-O-acyl isoA $\beta$ 1-42

## INTRODUCTION

N-O intramolecular acyl migration is well known as a side reaction of Ser- or Thr-containing peptides.<sup>1-5</sup>  $\beta$ -Hydroxyl groups are acylated by the N-O shift under strong acidic conditions through a five-membered ring intermediate (Figure 1A). However, the resulting O-acyl isopeptides can be readily converted to original N-acyl compounds under neutral or slightly

basic conditions in aqueous buffer. Therefore, after deprotection of protected Ser/Thr-containing peptides with strong acid, this O-N intramolecular acyl migration reaction is conventionally manipulated to obtain the desired peptides. On the other hand, the solubility of O-acyl isopeptides in aqueous media generally increases with the newly produced and ionized amino group, although these isopeptides have had limited attention in peptide synthesis so far. In addition, it was



**FIGURE 1** (A) Mechanism of N-O and O-N intramolecular acyl migration reaction; (B) O-N intramolecular acyl migration-type water-soluble prodrugs of KNI-272 and -279; (C) water-soluble prodrug of KNI-727.

predicted that the intramolecular acyl migration reaction would also occur in a compound with *N*-acylated  $\alpha$ -hydroxy- $\beta$ -amino acids, which has a similar 2-aminoethanol structure to  $\beta$ -hydroxy- $\alpha$ -amino acids. Utilizing these features, we have developed a novel class of "O-N intramolecular acyl migration"-type water-soluble prodrugs of HIV-1 protease inhibitors such as KNI-272, -279, and -727.<sup>6-11</sup> These inhibitors contain an  $\alpha$ -hydroxy- $\beta$ -amino acid residue, allophenylnorstatine [Apns, (2*S*, 3*S*)-3-amino-2-hydroxy-4-phenylbutanoic acid], which has a hydroxymethylcarbonyl (HMC) isostere derived from a natural scissile amino acid sequence "Phe-Pro."<sup>12-20</sup> As depicted in Figures 1B and C, these "O-N intramolecular acyl migration"-type water-soluble prodrugs of HIV-1 protease inhibitors, which are *O*-acyl isoforms of the parent drugs, increase water solubility and are easily converted to the corresponding *N*-acyl forms, i.e., parent drugs via O-N intramolecular acyl migration. This migration can be precisely controlled by pH and is completed in a short time with no side reactions under physiological conditions (pH 7.4). Hurley et al. also reported a study on the O-N-acyl migration of renin inhibitors.<sup>21</sup> From these findings, a new prodrug concept based on O-N intramolecular acyl migration was established, and the sparingly water-soluble drugs with an *N*-acyl-2-aminoethanol structure are generally potential targets for this water-soluble prodrug strategy.

Paclitaxel (**1**), one of the most important chemotherapeutic agents with promising antitumor activity,<sup>22</sup> has poor water solubility (0.00025 mg/mL). This is one of its major drawbacks, since the need to coinject with a detergent, Cremophor EL<sup>®</sup>, is thought to cause hypersensitivity reactions.<sup>23</sup> In this study, to solve this problem, we applied the O-N intramolecular acyl migration-type water-soluble prodrug strategy to paclitaxel, focusing on its *N*-benzoylphenylisoserine residue, an  $\alpha$ -hydroxy- $\beta$ -amino acid derivatives. This prodrug, designated "isotaxel," which is a 2'-*O*-isoform of paclitaxel, was synthesized and its potential as a prodrug was evaluated. Furthermore, we synthesized the water-soluble prodrug of other taxoid to understand the generalization of this strategy.

In the development of automated solid-phase peptide synthesis (SPPS), most peptides with ~50 amino acids are routinely prepared with no difficulty. However, the synthesis of specific sequences, so-called difficult sequence-containing peptides, are still problematic and the peptides often have low yield and purity in SPPS<sup>24-29</sup> (for a review, see Ref. 30). It is known that the difficult sequences are generally hydrophobic and promote aggregation in solvents during synthesis and purification. This aggregation is attrib-

uted to intermolecular hydrophobic interaction and a hydrogen-bond network among resin-bound peptide chains, resulting in the formation of extended secondary structures such as  $\beta$ -sheets.<sup>24,25</sup> To solve this problem, Mutter et al. developed building blocks, so-called pseudo-prolines, which are dipeptide derivatives, including Ser/Thr-derived oxazolidines or Cys-derived thiazolidine.<sup>26-28</sup> Sheppard and Johnson et al. also reported a building block, 2-hydroxy-4-methoxybenzyl (Hmb), a protecting group for the backbone amide nitrogen<sup>29</sup> (for a review, see Ref. 30). These special building blocks were designed to disrupt the secondary structure formed by interchain hydrogen bonding. However, in these approaches, prior modification of Fmoc-amino acids by two to six steps of solution-phase synthesis is required, and strong acids are required to remove the building blocks. Therefore, the development of novel methods using conventional amino acid derivatives are of great significance in the synthesis of difficult sequence-containing peptides. Since it is known that the difficult sequences are generally hydrophobic and promote aggregation in solvents during synthesis and purification, we applied the concept of O-N intramolecular acyl migration, namely, our idea was based on the synthesis of the hydrophilic *O*-acyl isopeptide followed by the O-N intramolecular acyl migration reaction leading to the desired peptide.

In this article, we review our application of the O-N intramolecular acyl migration reaction to the development of water-soluble prodrugs of taxoids and the development of a novel synthetic method for difficult sequence-containing peptides. In particular, in the latter case, we first evaluated the viability of our synthetic strategy through the synthesis of difficult sequence-containing small model peptides, and then applied it to the synthesis of a natural difficult sequence-containing large peptide, Alzheimer's disease-related peptide A $\beta$ 1-42. We successfully synthesized these "*O*-acyl isopeptides," which has not only improved solubility in various media and achieved efficient high performance liquid chromatography (HPLC) purification, but also changed the unfavorable nature of the difficult sequence during SPPS, resulting in the efficient synthesis of *O*-acyl isopeptides. The subsequent O-N intramolecular acyl migration of purified *O*-acyl isopeptides afforded the desired peptides in high yield and purity. It is interesting to note that only one *O*-acyl group insertion drastically improved the nature of the difficult sequence in A $\beta$ 1-42 with 42 amino acid residues. Therefore, this new method will contribute to the synthesis of larger difficult sequence-containing peptides.

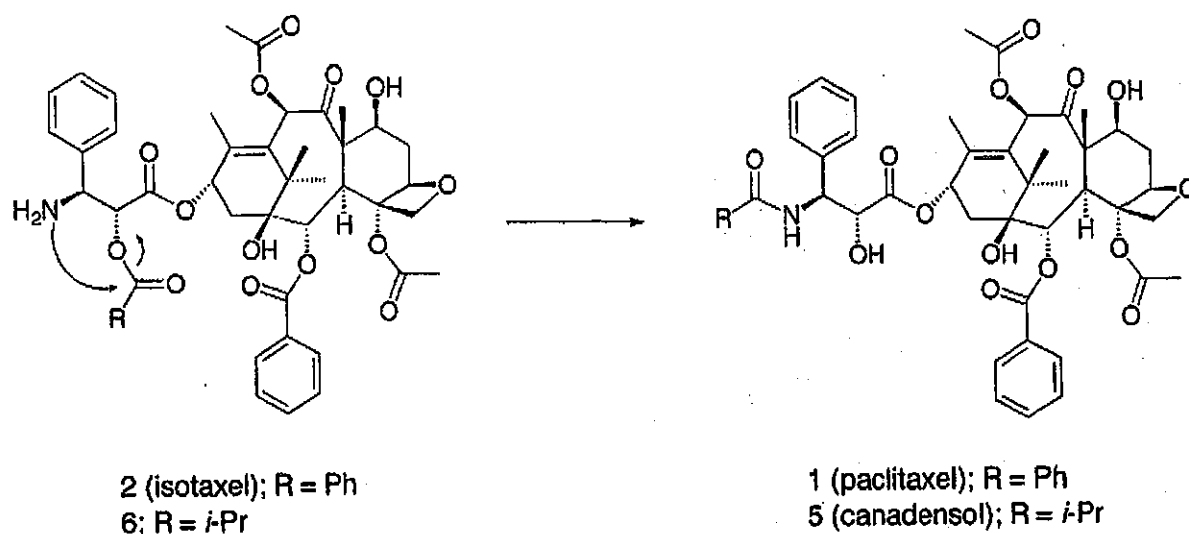


FIGURE 2 The O-N acyl migration of isotaxel 2 to paclitaxel 1 and 6 to canadensol 5.

### DESIGN AND SYNTHESIS OF ISOTAXEL: A NOVEL WATER-SOLUBLE PACLITAXEL PRODRUG WITH NO AUXILIARY AND NO BY-PRODUCT

To improve the poor water solubility of paclitaxel 1 (0.00025 mg/mL), many water-soluble prodrugs with modified hydroxyl groups at the C-2' and/or C-7 positions with solubilizing moieties have been reported, and some are currently undergoing clinical evaluation.<sup>31-33</sup> However, none of these applications are presently in clinical use and the released auxiliary moieties may have some unfavorable effects *in vivo*.<sup>34</sup> These factors suggest that novel approaches for water-soluble prodrugs of 1 are needed.

In the O-N intramolecular acyl migration-type water-soluble prodrugs of HIV-1 protease inhibitors with

Apns, the observed O-acyl migration of prodrugs such as KNI-272 (Figure 1B) under physiological conditions was so rapid ( $t_{1/2} < 1 \text{ min}$ )<sup>9</sup> that it might prove difficult for paclitaxel, which has a similar  $\alpha$ -hydroxy- $\beta$ -amino acid residue, i.e., (2*R*, 3*S*)-phenylisoserine, to undergo a systemic distribution after injection. However, we also observed that O-benzoyl group migration in a prodrug of Bz-Apns-Thz-NHBu<sup>t</sup> (KNI-565; Thz: thiazolidine-4-carboxylic acid) was relatively slow,<sup>10</sup> possibly due to the less electrophilic nature of the carbonyl carbon and the steric effect of the phenyl ring. Hence, we decided to perform a model study to create a new type of water-soluble paclitaxel prodrug (Figure 2).

To examine the effect of the benzoyl group and stereochemistry of  $\alpha$ -hydroxy- $\beta$ -amino acids on the kinetics of O-N acyl migration, we designed three

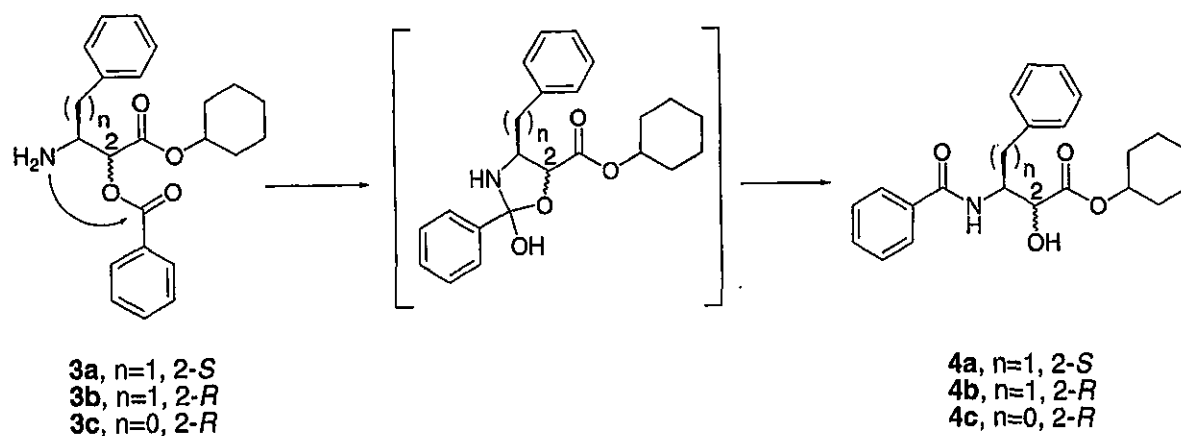
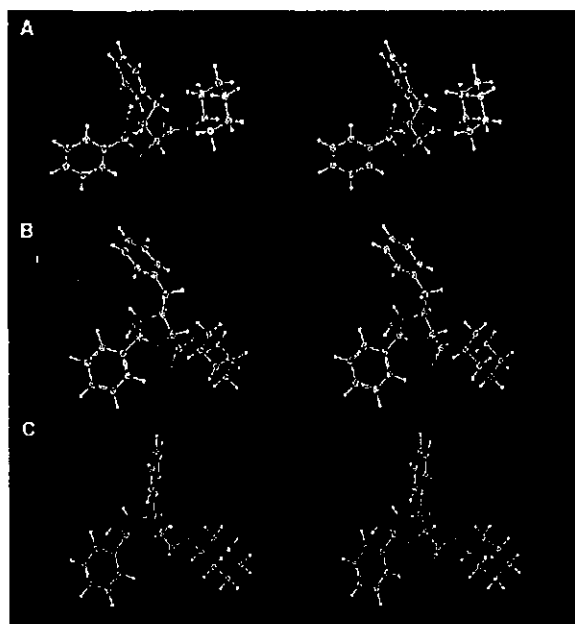


FIGURE 3 The O-N acyl migration in model compounds, designed to examine the effect of the benzoyl group and stereochemistry of  $\alpha$ -hydroxy- $\beta$ -amino acids on the kinetics.



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

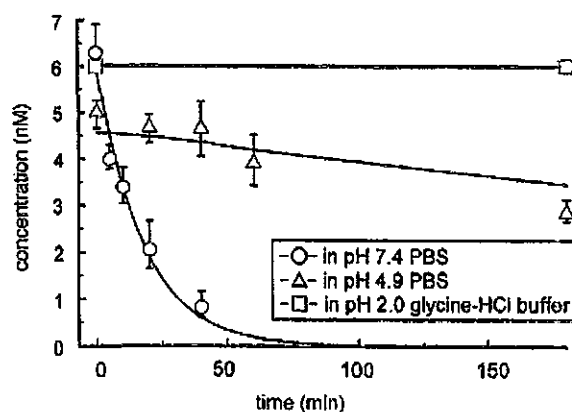
model compounds, 3a–c, that are  $\alpha$ -hydroxy- $\beta$ -amino acid derivatives with a bulky cyclohexyl ester structure in place of the taxane ring (Figure 3). Compound 3a is an Aps derivative with a 2*S*,3*S* configuration, while its diastereomer, 3b, a phenylnorstatine derivative, and a phenylisoserine derivative, 3c, have a 2*R*,3*S* 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 2*S*,3*S* configuration. Migration in 3b and 3c with a 2*R*,3*S* 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

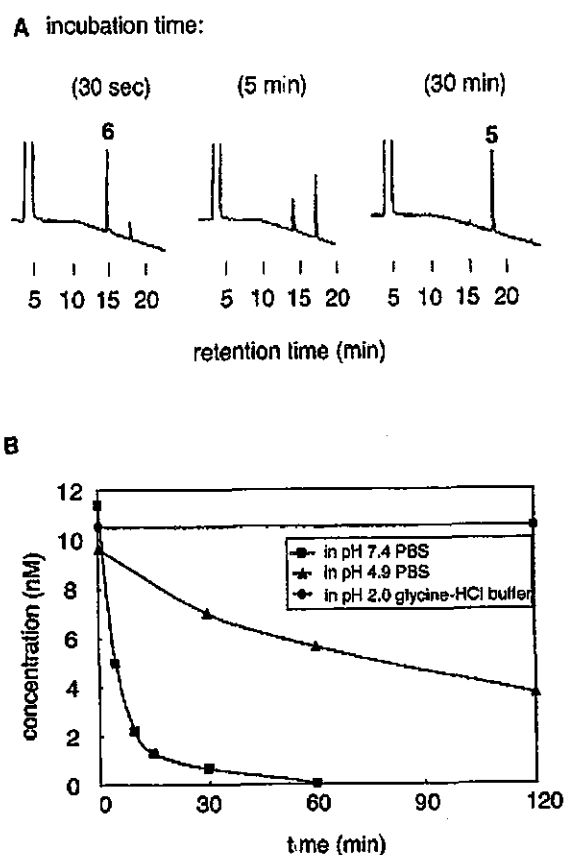
not sufficient for metabolism and elimination. From these promising results of the model study, we designed and synthesized the new paclitaxel prodrug isotaxel (2; Figure 2) with improved water solubility. This prodrug, having no additional water-solubilizing auxiliaries and forming no by-product during 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).<sup>35</sup>

The water solubility of 2 · HCl was determined as  $0.45 \pm 0.04$  mg mL<sup>-1</sup>, which is 1800-fold higher than paclitaxel ( $0.00025 \pm 0.00004$  mg mL<sup>-1</sup>). 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  $15.1 \pm 1.3$  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  $252.2 \pm 37.7$  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.





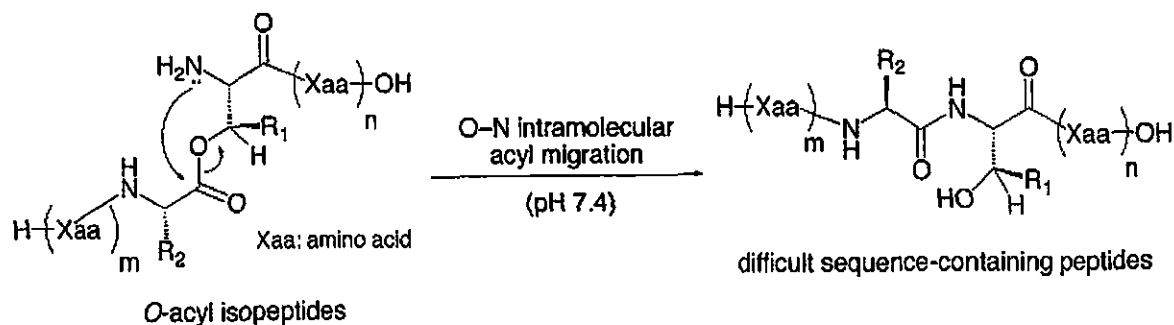
**FIGURE 6** (A) HPLC profile of prodrug **6** in PBS (pH 7.4); (B) migration of prodrug **6** in different pH at 37°C.

A taxoid derivative, canadensol **5**, which showed improved potency against cancer with multidrug resistance,<sup>36-38</sup> was selected to evaluate the usage of this water-soluble prodrug strategy with other important taxoids. This taxoid includes the same  $\alpha$ -hydroxy- $\beta$ -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

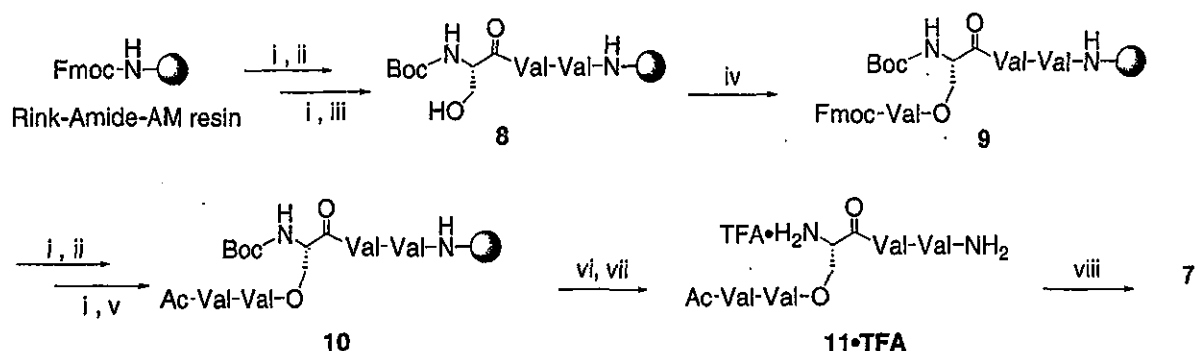
2'-*O*-isobutyryl isoform of **5**, showed 10-fold higher water solubility ( $2.26 \text{ mg mL}^{-1}$ ). As shown in Figure 6, complete migration was observed at pH 7.4 with a  $t_{1/2}$  value of 4.3 min. In addition, slower migration was observed at pH 4.9 with a value of 82.0 min and no migration at pH 2.0 after 6 h incubation (Figure 6).<sup>39</sup> 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),<sup>40</sup> 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.

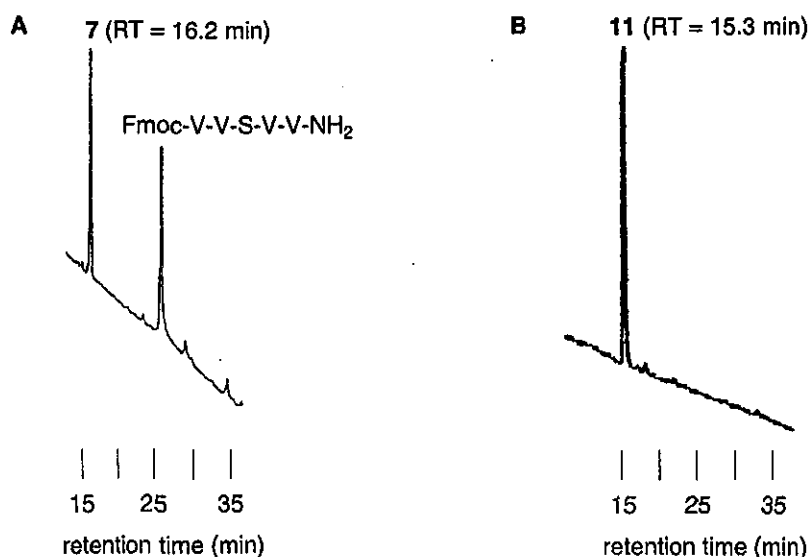


**SCHEME 1** (Route B) Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-Val-OH (2.5 eq), DIPCDI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (iii), Boc-Ser-OH (2.5 eq), DIPCDI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (iv) Fmoc-Val-OH (3.0 eq), DIPCDI (3.0 eq), DMAP (0.2 eq),  $\text{CH}_2\text{Cl}_2$ , 16 h  $\times$  2; (v)  $\text{Ac}_2\text{O}$  (1.2 eq), TEA (triethylamine, 1.0 eq), DMF, 2 h; (vi), TFA-*m*-cresol-thioanisole- $\text{H}_2\text{O}$  (92.5:2.5:2.5:2.5), 90 min; (vii), preparative HPLC (a linear gradient of  $\text{CH}_3\text{CN}$  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<sub>2</sub> **7**, was selected, and its *O*-acyl isopeptide at the Ser residue was synthesized by the Fmoc-based SPPS method (Fmoc: 9-fluorenylmethoxycarbonyl; 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)<sup>41</sup> 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)<sup>42</sup> 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- $\text{H}_2\text{O}$  (TFA: trifluoroacetic acid; 92.5:2.5:2.5:2.5)<sup>43,44</sup> for 90 min. An undesired peptide, Fmoc-Val-Val-Ser-Val-Val-NH<sub>2</sub>, 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%  $\text{CH}_3\text{CN}$  for 40 min, 230 nm).

undesired aggregation can occur from as early as the fifth residue coupled.<sup>45</sup> 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<sub>2</sub>O, MeOH, and DMSO being 0.013, 0.051, and 0.64 mg mL<sup>-1</sup>, 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<sub>2</sub> 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<sub>2</sub>Cl<sub>2</sub> 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<sub>2</sub>O and MeOH (59.6 and 126.6 mg mL<sup>-1</sup>, 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<sub>2</sub> 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.<sup>46</sup> 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

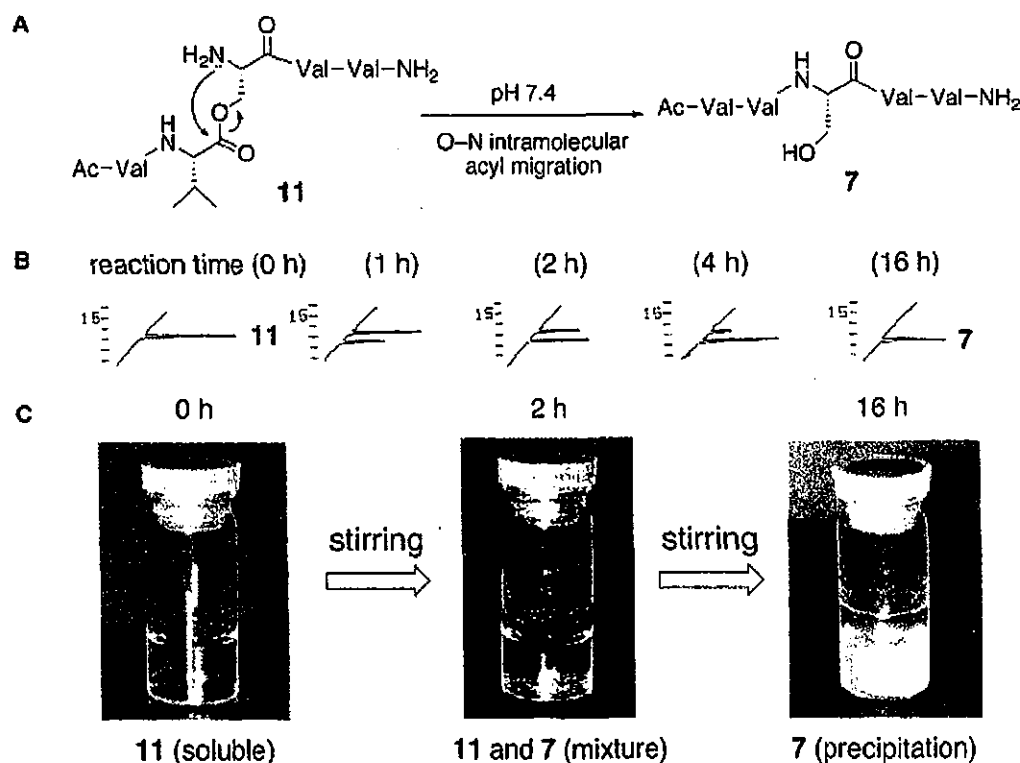


FIGURE 9 (A) Conversion of *O*-acyl isopeptide **11** to **7** via *O*-*N* intramolecular acyl migration in PBS (pH 7.4, 25°C), (B) its periodical HPLC profile, and (C) photographs.

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<sub>2</sub>O 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  $\beta$  peptide (A $\beta$ ) 1–42, which is significant in both peptide chemistry and medical science.

#### A NOVEL WATER-SOLUBLE A $\beta$ 1–42 ISOPEPTIDE: AN EFFICIENT STRATEGY FOR THE PREPARATION OF THE ALZHEIMER'S DISEASE-RELATED PEPTIDE, A $\beta$ 1–42, VIA O–N INTRAMOLECULAR ACYL MIGRATION

Amyloid  $\beta$  peptides (A $\beta$ s) are the main proteinaceous component of the amyloid plaques found in the brains of Alzheimer's disease (AD) patients.<sup>47</sup> Neuritic plaques, pathognomonic features of AD, contain abundant fibrils formed from A $\beta$ s, which have been found to be neurotoxic *in vivo* and *in vitro*.<sup>48</sup> The

predominant forms of A $\beta$ s mainly consist of 40- and 42-residue peptides (designated A $\beta$ 1–40 and A $\beta$ 1–42, respectively), which are proteolytically produced from amyloid precursor protein (APP) by enzymatic reactions.<sup>49</sup> Since A $\beta$ 1–42 is thought to play a more critical role in amyloid formation and in the pathogenesis of AD than A $\beta$ 1–40, many studies using synthetic A $\beta$ 1–42 have been carried out to clarify the involvement of A $\beta$ 1–42 in AD.<sup>50–53</sup>

However, A $\beta$ 1–42 is a difficult sequence-containing peptide with a highly hydrophobic nature and forms aggregates in various media.<sup>54–59</sup> Due to its low solubility and broad elution under acidic or neutral conditions, the conventional HPLC purification of synthesized A $\beta$ 1–42 in the aqueous TFA–acetonitrile system is too laborious to remove impurities accumulated during SPPS. Furthermore, biological experiments using A $\beta$ 1–42 are problematic due to the large extent of aggregation in a standard storage solution such as dimethylsulfoxide (DMSO).<sup>60</sup> Therefore, an “*in situ*” system that could prepare intact A $\beta$ 1–42 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 A $\beta$ 1–42 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 A $\beta$ 1–42 via a novel water-soluble isopeptide of A $\beta$ 1–42, i.e., “26-*O*-acyl isoA $\beta$ 1–42 (26-AIA $\beta$ 1–42, 13).”<sup>61</sup> This overcomes the problems in the synthesis and storage of A $\beta$ 1–42 (Figure 10). Although there are two Ser residues in A $\beta$ 1–42 at positions 8 and 26 with the capability of O–N intramolecular acyl migration, we selected the Ser<sup>26</sup> for

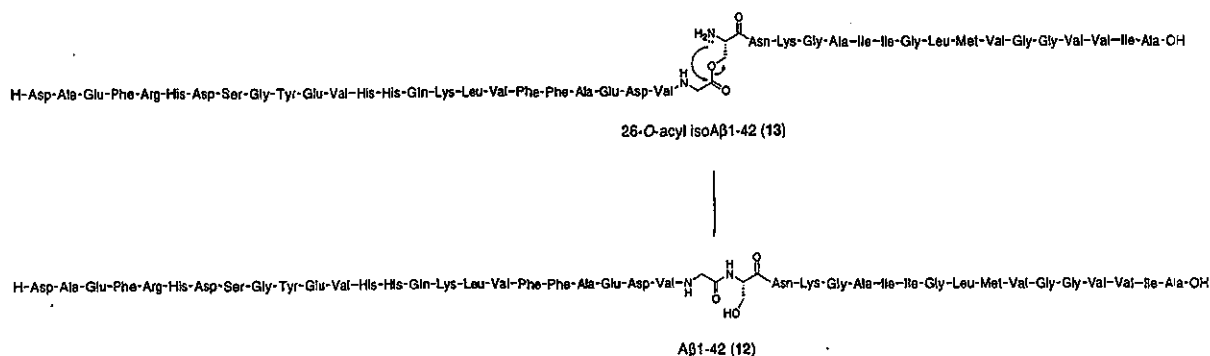


FIGURE 10 The production of A $\beta$ 1–42 (12) via the O–N intramolecular acyl migration reaction of 26-*O*-acyl isoA $\beta$ 1–42 (13).