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New Water-Soluble Prodrugs of HIV Protease Inhibitors Based on *O*→*N* Intramolecular Acyl Migration

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Abstract—To improve the low water-solubility of HIV protease inhibitors, we synthesized water-soluble prodrugs of KNI-272 and KNI-279 which are potent HIV-1 protease inhibitors consisting of an Apns–Thz core structure (Apns; allophenylnorstatine, Thz; thiazolidine-4-carboxylic acid) as an inhibitory machinery. The prodrugs, which contained an *O*-acyl peptidomimetic structure with an ionized amino group leading to the increase of water-solubility, were designed to regenerate the corresponding parent drugs based on the *O*→*N* intramolecular acyl migration reaction at the α-hydroxy-β-amino acid residue, that is allophenylnorstatine. The synthetic prodrugs **3**, **4**, **6**, and **7** improved the water-solubility (>300 mg/mL) more than 4000-fold in comparison with the parent compounds, which is the practically acceptable value as water-soluble drugs. These prodrugs were stable as an HCl salt and in a strongly acidic solution corresponding to gastric juice (pH 2.0), and could be converted to the parent compounds promptly in the aqueous condition from slightly acidic to basic pH at 37 °C, with the suitable migration rate, via a five-membered ring intermediate. Using a similar method, we synthesized a prodrug (**12**) of ritonavir, a clinically useful HIV-1 protease inhibitor as an anti-AIDS drug. In contrast to the prodrugs **3**, **4**, **6**, and **7**, the prodrug **12** was very slowly converted to ritonavir probably through a six-membered ring intermediate, with the $t_{1/2}$ value of 32 h that may not be suitable for practical use.

Introduction

Human immunodeficiency virus type 1 (HIV-1) encodes an aspartic protease that is responsible for the processing of viral precursor proteins such as gag and gag-pol polyproteins to form mature structural proteins and functional enzymes required in the production of infective viral particles.[1–3] Therefore, HIV-1 protease (HIV-1 PR) has been an attractive target for the design of inhibitors for effective antiviral therapy. Recently, many potent protease inhibitors that bind to the active site of HIV-1 PR have been developed based on the strategy of a substrate transition-state mimic, and several compounds are clinically used in combination therapies for AIDS.[4–8] However, the low water-solubility of these anti-AIDS drugs is a serious problem[9–12] causing undesirable pharmaceutical properties such as erratic oral absorption and poor oral bioavailability. Recently, Mimoto et al. reported potent HIV-1 PR inhibitors, KNI-272[13–15] (**1a**) and KNI-279[13] (**2a**), which contain an allophenylnorstatine (Apns) residue as a hydroxymethylcarbonyl (HMC) isostere derived from a natural scissile amino acid sequence 'Phe-Pro' based on the substrate–transition strategy[13–17] (Fig. 1). However, these peptidomimetics as well as other HIV-1 PR inhibitors such as ritonavir[18,19] and amprenavir[20] exhibited significantly low water-solubility in the physiological media.

To overcome this low water-solubility, one effective strategy is to convert the water-insoluble parent drugs into hydrophilic prodrugs by covalently attaching the appropriate solubilizing moieties such as phosphates,[21,22] sugars[23,24] and amines,[25,26] which can eliminate the parent drugs enzymatically or chemically under physiological conditions. The phosphate-type water-soluble prodrugs of HIV-1 PR inhibitors were reported by Thaisrivongs et al.[11] We recently reported distinct water-soluble prodrugs[27] derived from HIV-1 PR inhibitors which contain an Apns core. These prodrugs were designed to enable a chemical regeneration of the parent compound adopting a unique pH sensitive self-cleavable linker.

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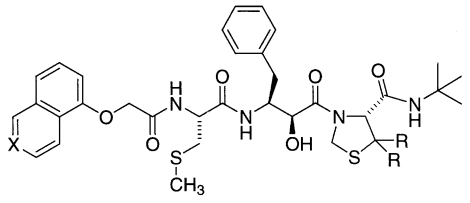
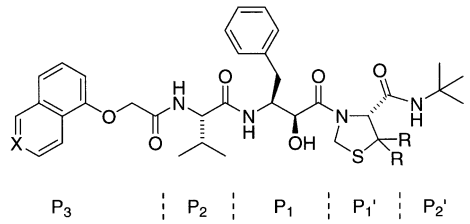


1a X = N, R = H (KNI-272)
1b X = N, R = CH_3 (KNI-227)
1c X = CH, R = H (KNI-217)
1d X = CH, R = CH_3 (KNI-225)



2a X = N, R = H (KNI-279)
2b X = N, R = CH_3
2c X = CH, R = H (KNI-253)
2d X = CH, R = CH_3

Figure 1. Structure of KNI-272 (**1a**), KNI-279 (**2a**) and these analogues.

On the other hand, as shown in Figure 2a, it is well known that an acyl migration reaction is observed between adjacent amino and hydroxyl groups, and the formation of *O*-acylpeptides occurs when the peptides containing β-hydroxyamino acids such as serine and threonine residues are exposed to strong acids.[28] The solubility of these *O*-acylpeptides in aqueous media generally increases by the newly produced amino group, and the reverse reaction to the peptides can be achieved by the pH shift to weak basic condition in aqueous media.[29]

By introducing this pH-dependent reversible group into prodrugs, Hurley et al.[30] reported prodrugs of peptidomimetic inhibitors of renin, which is an aspartic protease responsible for the regulation of blood pressure in the cardiovascular system. This strategy seemed to be applicable to HIV-1 PR inhibitors such as KNI-272 (**1a**) and KNI-279 (**2a**), since the Apns residue in these compounds has both adjacent α-hydroxyl and β-amino groups, and an acyl group connected to hydroxyl group of the Apns residue would be interconvertible through the formation of an energetically favorable five-membered ring intermediate (Fig. 2a). Thus, we designed[31–33] prodrugs **3** and **4** of KNI-272 and -279 based on *O*→*N* intramolecular acyl migration reaction. In this paper, the design and synthesis of a series of prodrugs **3**–**11** (Table 1), which are *O*-acyl derivatives derived from the structures of KNI-272 (**1a**) and KNI-279 (**2a**), are described. We evaluated the solubility and

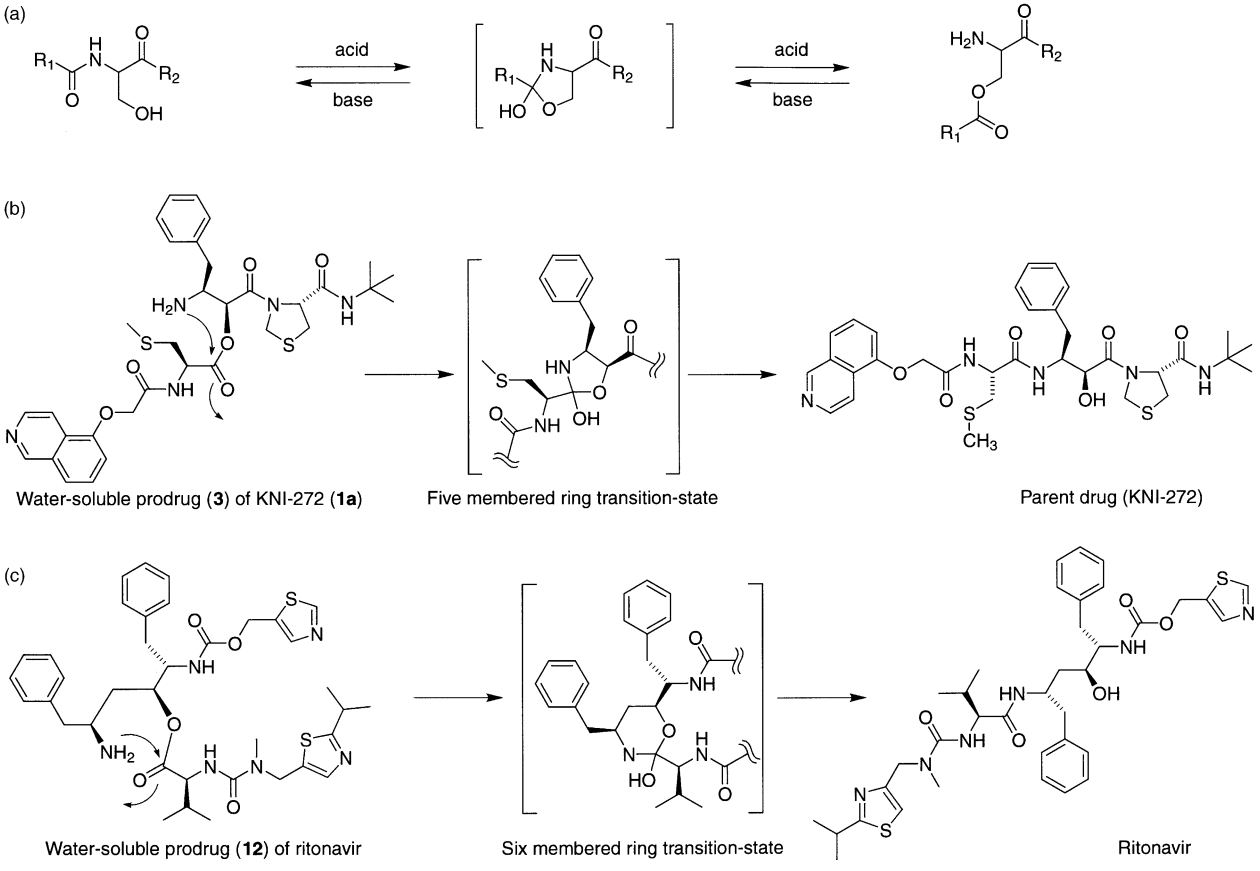
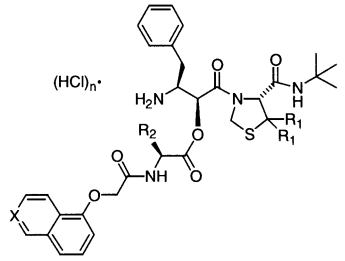


Figure 2. Design of prodrug based in the *O*→*N* acyl migration. (a) *N*→*O* acyl migration and *O*→*N* acyl migration reaction; (b) prodrug of KNI-272; (c) Prodrug of ritonavir.

Table 1. Water-solubility and $O \rightarrow N$ migration rate of prodrugs



3 X = N, R_1 = H, R_2 = CH_2SCH_3, n=2
4 X = N, R_1 = H, R_2 = $CH(CH_3)_2$, n=2
5 X = N, R_1 = H, R_2 = $CH_2CH_2CH_3$, n=2
6 X = N, R_1 = CH_3, R_2 = CH_2SCH_3, n=2
7 X = N, R_1 = CH_3, R_2 = $CH(CH_3)_2$, n=2
8 X = CH, R_1 = H, R_2 = CH_2SCH_3, n=1
9 X = CH, R_1 = H, R_2 = $CH(CH_3)_2$, n=1
10 X = CH, R_1 = CH_3, R_2 = CH_2SCH_3, n=1
11 X = CH, R_1 = CH_3, R_2 = $CH(CH_3)_2$, n=1

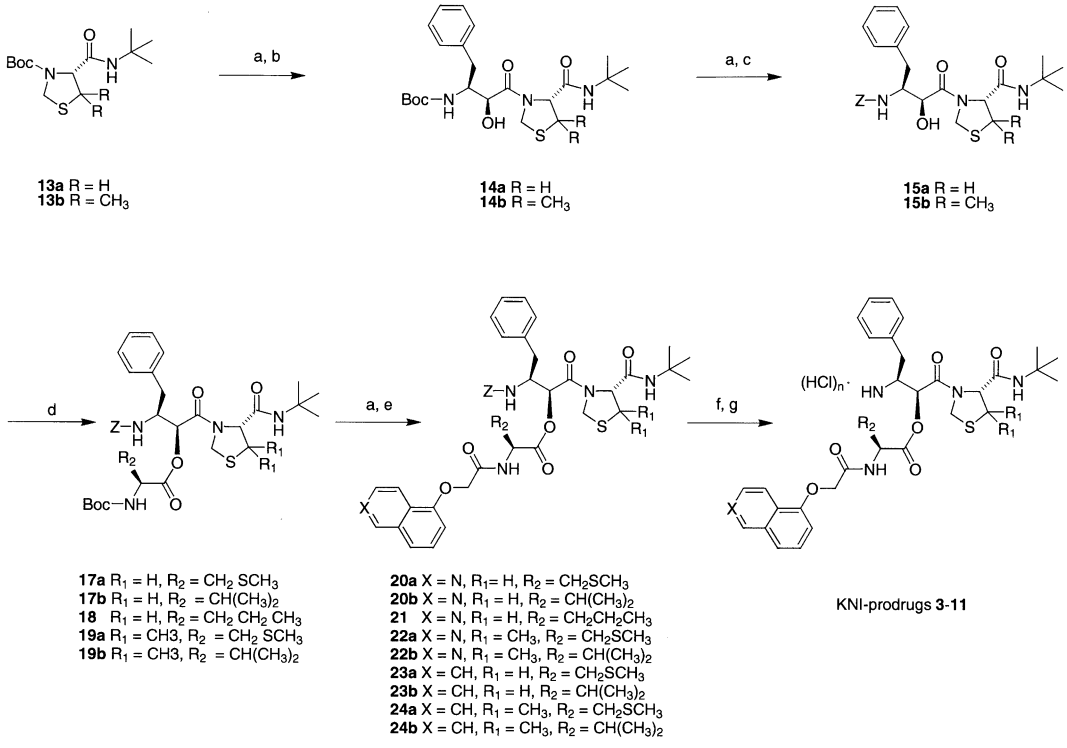
Prodrug	Solubility (mg/mL)		Ratio of solubility	$t_{1/2}$[a](min)			
	Prodrug	Parent drug	(prodrug/parent drug)	pH 4.9	pH 5.5	pH 7.4	pH 8.0
3	>300	0.075	>4000	28	7	<1	<1
4	>300	0.079	>3797	820	290	7.7	5.9
5	—	—	—	—	28	<1	<1
6	>300	0.055	>5455	34	8	<1	<1
7	>300	0.041	>7317	822	305	8.6	6.6
8	3.7	0.0008	4625	35	7.8	<1	<1
9	3	0.0025	1600	800	291	10	8.7
10	4	0.0015	2000	47	8.6	<1	<1
11	2.6	0.002	1300	816	310	22	13

[a]$t_{1/2}$ is the time required for 50% release of parent drugs at 37 °C in PBS (pH 7.4).

the ability of the $O \rightarrow N$ intramolecular acyl migration reaction of these prodrugs to regenerate the parent compounds. For comparison, a prodrug **12** of ritonavir, a clinically approved inhibitor, was also synthesized, since the $O \rightarrow N$ intramolecular acyl migration reaction in the prodrug of ritonavir was expected to proceed through the formation of an energetically less favorable six-membered ring intermediate (Fig. 2c).

Chemistry

The synthesis of prodrugs **3**–**11** is shown in Scheme 1. The Boc group of the starting materials **13a** and **13b**[13,34] was deprotected with 4 N-HCl/dioxane, followed by coupling with Boc-Apns-OH[35,36] using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (EDC·HCl) in the presence of HOBt,[37–40] resulting in protected



Scheme 1. Reagents: (a) 4 N-HCl/dioxane; (b) Boc-Apns-OH, EDC·HCl, HOBt, Et_3N, DMF; (c) Z-OSu, Et_3N, DMF; (d) *N*-Boc-amino acids (**16a**; Boc-Mta-OH, **16b**; Boc-Val-OH, **16c**; Boc-Nva-OH), DCC, DMAP, CH_2Cl_2; (e) iQoa or Noa, EDC·HCl, HOBt, Et_3N, DMF; (f) TFA, dimethylsulfide, anisole; (g) 4 *N*-HCl/EtOAc.

dipeptides **14a** and **14b**. To convert the Boc group to Z group in **14a** and **14b**, the deprotection of the Boc group and subsequent introduction of a Z group using *N*-(benzyloxycarbonyloxy)succinimide (Z-OSu) were performed to give **15a** and **15b**. In the next step, three kinds of Boc-amino acids were introduced to the α-hydroxyl group of Apns in **15a** and **15b** using DCC in the presence of a catalytic amount of DMAP to afford *O*-acyl dipeptides **17**–**19**. Although partial racemization (less than 1–3%) at the α-carbon of the introduced Boc-amino acids was observed during this condensation, the racemized compounds could be removed by preparative HPLC purification at the last step of the synthesis. After the Boc group deprotection of **17**–**19**, condensation with 5-isoquinoyloxyacetic acid (iQoa) or 1-naphtoxyacetic acid (Noa) was carried out to afford the protected prodrugs **20**–**24**. Final deprotection of the Z group in **20**–**24** using TFA-anisole-dimethylsulfide by 'push–pull' mechanism,[41] and subsequent purification by preparative HPLC. Then ion-exchange treatment using 4 N-HCl/EtOAc gave a hydrochloride salt of prodrugs **3**–**11**.

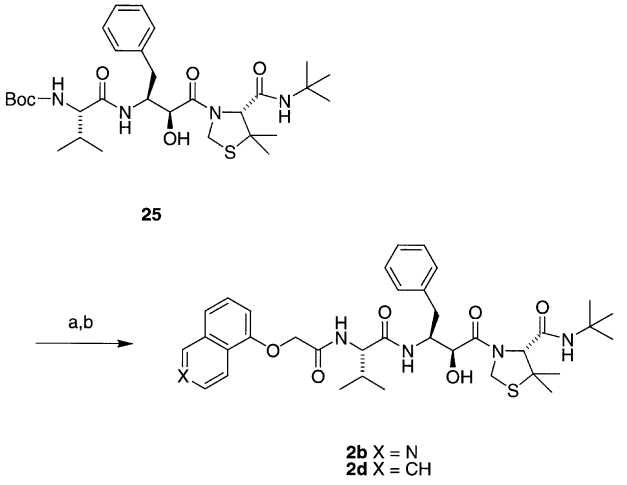
The parent compounds **1a**–**d**, **2a**, and **2c** were synthesized according to Mimoto et al.[13] The parent compounds **2b** and **2d** were synthesized from **25** by a conventional protocol[13] using EDC-HOBt for the amide bond formation (Scheme 2).

The synthesis of ritonavir prodrug **12** is shown in Scheme 3. (2*S*,3*S*,5*S*)-2-Amino-5-Boc-amino-1,6-diphenyl-3-hydroxyhexane (**26**)[42] was coupled with 5-hydroxymethylthiazole-4-nitrophenyl carbonate hydrochloride (**27**)[43] to give the compound **28**, which was condensed *N*-[*N*-[(2-isopropyl-4-thiazole)methyl]-*N*-methyl]carbonyl]-L-valine[44–46] (**29**) using DCC-DMAP to give the ester **30**. The deprotection of **30** using 4N-HCl/dioxane gave the desired ritonavir prodrug **12**.

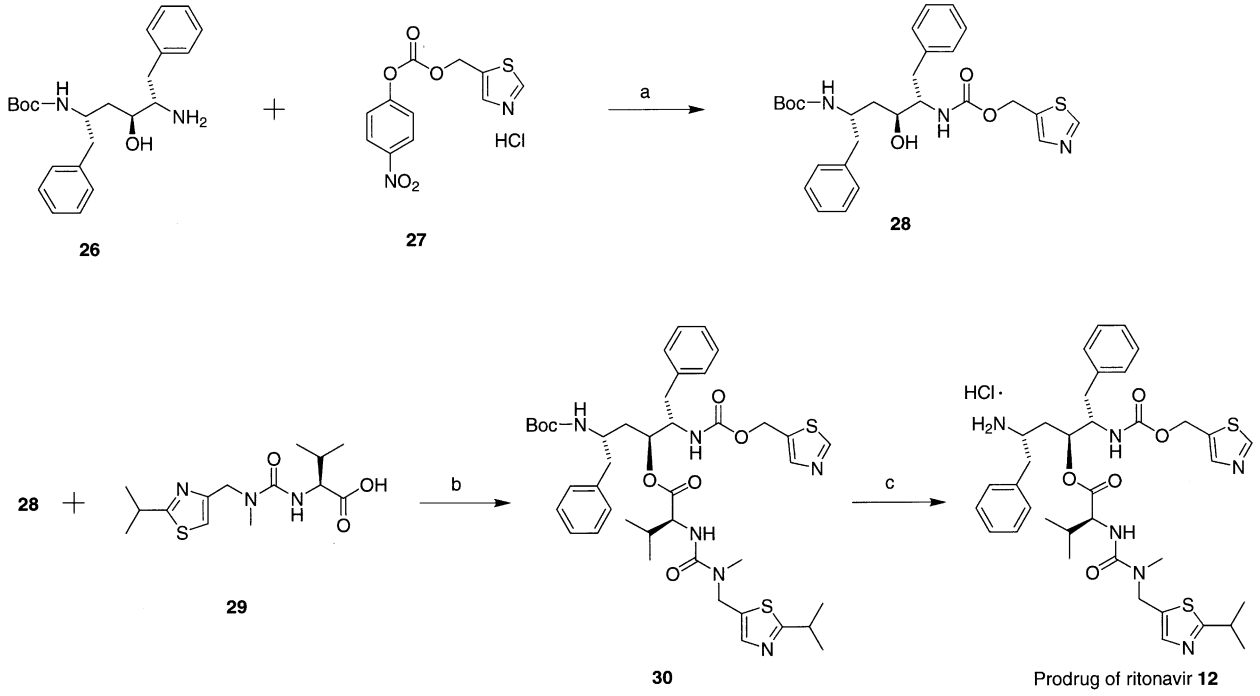
Results and Discussion

Water-solubility of prodrugs

Water-solubility of synthetic prodrugs was determined and compared to that of their parent compounds (Table 1). In all cases, prodrugs exhibited more than 1000-fold higher water-solubility than the corresponding parent drugs, due to the existence of a hydrophilic amine structure in these *O*-acyl structures. Especially, the solubility of compounds **3**, **4**, **6**, and **7** was >300 mg/mL, which is a practicable value as water-soluble pro-



Scheme 2. Reagents: (a) 4 N-HCl/dioxane; (b) iQoa or Noa, EDC·HCl, HOBt, Et_3N, DMF.



Scheme 3. Reagents: (a) THF–DMF; (b) DCC, DMAP, CH_2Cl_2; (c) 4 N-HCl/dioxane.

drugs. However, the compounds **8**–**11** exhibited lower solubility with the values about 2–4 mg/mL, probably resulting from the naphthalene structure at the P3 moiety of the parent compounds. This result demonstrated that the P3 moiety is also important to obtain water-soluble prodrugs, since an isoquinoline structure of the P3 position in **3**, **4**, **6**, and **7** is able to form an HCl salt.

Effects of pH and structures on the *O*→*N* acyl migration of prodrugs

To study the *O*→*N* acyl migration, water-soluble prodrugs **3**–**11** were dissolved in phosphate-buffered saline (PBS) with different pH values ranging from 4.9 to 8.0 and incubated at 37 °C. The migration was monitored by HPLC. Typical HPLC charts for compound **3** (a prodrug of KNI-272 **1a**) in PBS (pH 7.4) are shown in Figure 3 and the time course of the migration in prodrugs **3** and **4** (a prodrug of **2a**) is shown in Figure 4. A time-dependent *O*→*N* acyl migration reaction to parent drugs was observed in both compounds **3** and **4**. The migration rate was dependent on the pH values used. Under acidic conditions, a slower migration was observed than in neutral or basic condition, probably due to the decrease of nucleophilicity of the amino group. Compound **3** showed rapid migration under neutral conditions, that is, 74% of **3** was converted to KNI-272 (**1a**) during the first 30 s after incubation at pH 7.4 with the $t_{1/2}$ value of less than 1 min (Table 1 and Fig. 4). However, the $t_{1/2}$ value was 28 min at pH 4.9. On the other hand, compound **4** exhibited relatively slower migration with $t_{1/2}$ values of 7.7 and 820 min, which were more than 8- and 30-times longer than those of **3** at pH 7.4 and 4.9, respectively. The structural difference of compounds **3** and **4** is in a side chain at the P2 position, and the compound **3**, having a less bulky sulfur atom at this position, showed faster migration. To investigate which factors are more significant for the migration rate, compound **5**, possessing a norvaline (Nva) residue at the P2 position, was synthesized and compared with compounds **3** and **4**. The sulfur atom of methylthioalanine (Mta) is substituted to a methylene group in Nva. A similar rapid migration of **5** as that of **3** was observed at pH 7.4, although compound **5** exhibited

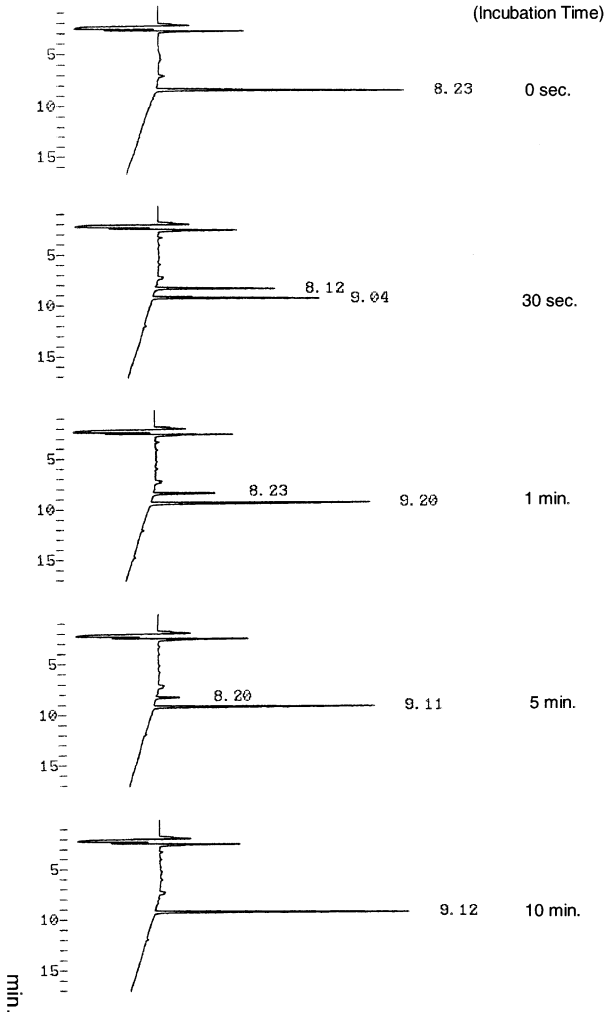


Figure 3. HPLC profiles of the *O*→*N* migration of compound **3** in PBS (pH 7.4) at 37 °C. A peak with a retention time of about 8 min corresponds to compound **3** and a peak with a retention time of about 9 min corresponds to parent compound **1a**.

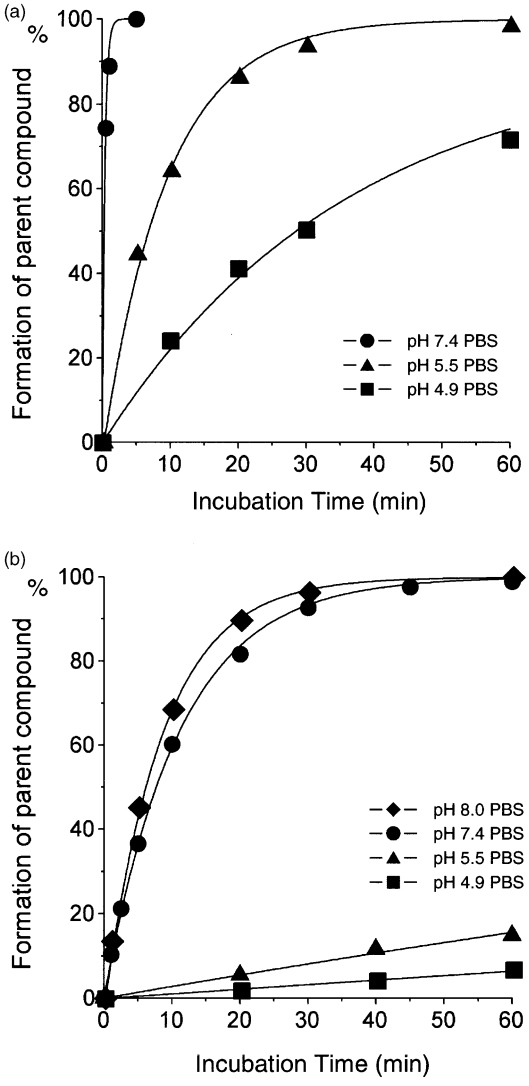


Figure 4. Formation of parent compound in various pH conditions. (a) prodrug **3**; (b) prodrug **4**.

a small increase of $t_{1/2}$ value at pH 5.5. This result indicated that the steric effect is more significant to accelerate the migration, although the existence of an electrostatic sulfur atom at the P2 position may have some effect to prompt migration. Thus, a sterically less hindered side chain is effective for increasing the migration rate, and it is possible to control the migration rate by modifying the side chain of the P2 site.

To investigate other structural factors which influence the migration speed, the P3 and P1′ sites were modified. When the iQoa group at the P3 site was substituted to a more hydrophobic and less electrostatic Noa group in compounds **8** and **9**, almost the same $t_{1/2}$ values were obtained as those of **3** and **4**, respectively, indicating that this modification at the P3 position had no influence on the migration rate. In the P1′ site, it is known that the modification of Thz ($R_1 = H$) to Dmt ($R_1 = CH_3$, 5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid) improves HIV-1 PR inhibitory and antiviral activity. Hence, compounds **6** and **7** with the Dmt residue were synthesized and the effect of Dmt at the P1′ position on the migration speed was evaluated. As shown in Table 1, the $t_{1/2}$ values of **6** and **7** did not show any significant change compared to **3** and **4**, suggesting that this modification is also insignificant and applicable to develop more potent compounds possessing the Apns–Dmt core structure. Compounds **10** and **11** containing both modifications at the P3 and P1′ sites, also did not show any significant change in migration time with **3** and **4**.

From these results, it is suggested that the derivatization of the compounds at a position away from the migration point such as the P3 and P1′ sites will be tolerated, and the strategy for the water-soluble prodrugs using the *N*→*O* acyl migration reaction can be applicable as a general method to increase water solubility of HIV-1 PR inhibitors based on the Apns–Thz and Apns–Dmt core structures.

A prodrug (**12**) of ritonavir[18,19] based on the *O*→*N* acyl migration was also synthesized and the migration was examined in the same manner as that of KNI-series prodrugs. Although ritonavir has a Val residue at the P2 site as compound **4**, prodrug **12** exhibited a $t_{1/2}$ value of 32 h at pH 7.4 (Fig. 5) that was 250-fold longer than that of **4**. This significantly slow migration can be attributed to the fact that the prodrug of ritonavir forms a six-membered ring intermediate in the migration step, while the KNI-series prodrugs form an energetically favorable five-membered ring intermediate,[47] suggesting that this strategy may not be useful for the water-soluble prodrugs via the six-membered ring intermediate in the formation of parent drugs.

Inhibitory activity of synthetic prodrugs

HIV-1 PR inhibitory activity of prodrugs **3**, **4**, **8**, **9** and **12** was determined and shown in Table 2. About 60% of the enzymatic activity was inhibited by 50 nM of prodrugs **3** and **8**. This observed HIV-1 PR inhibitory activity of **3** and **8** is probably due to the activity of their parent compounds formed during incubation in the assay, since the incubation period (5 min) is similar to the $t_{1/2}$ values of these prodrugs under the same incubation conditions (pH 5.5). Furthermore, no inhibitory activity was observed in prodrugs **4** and **9**, which exhibited longer $t_{1/2}$ values than **3** and **8**. These results indicate that the prodrugs have no activity themselves.

On the other hand, when the prodrugs were pre-incubated for 1 h under the same condition (pH 5.5 at 37 °C), most of **3** and **8** was converted to the corresponding parent drugs and exhibited higher inhibitory

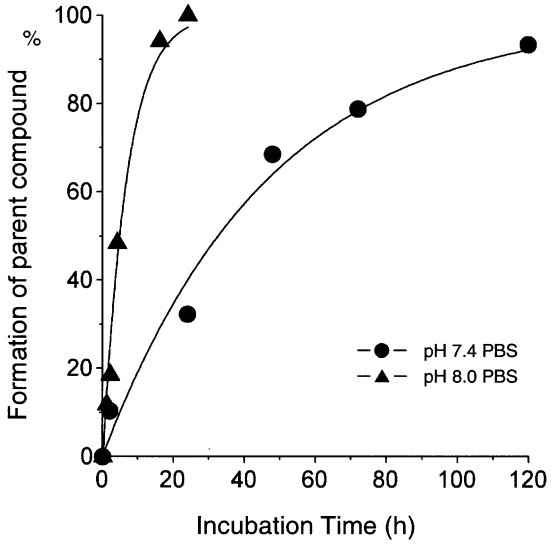


Figure 5. Formation of ritonavir from prodrug **12** in various pH conditions.

Table 2. HIV-1 protease inhibition of prodrugs

Prodrug	Inhibition[a] (%)	Inhibition[b] (%) (pre-incubation)	Conversion[c] (%) (pre-incubation)
3	66.6	95.4	98
4	3.9	23.0	16
8	55.9	93.8	97
9	−0.3	33.3	13
12	1.2	2.7	Not detected[d]

[a]% of HIV-1 protease inhibition in the presence of 50 nM of prodrugs (enzyme and prodrugs were added at the same time).
[b]% of HIV-1 protease inhibition in the presence of 50 nM of prodrugs (prodrugs were pre-incubated for 1 h at 37 °C before enzyme addition).
[c]Formation of parent compounds (%) from prodrugs after 1 h incubation at pH 5.5 at 37 °C.
[d]Ritonavir prodrug (**12**) which was indicated a $t_{1/2}$ value of >1800 min in PBS (pH5.5) at 37 °C, was rarely converted to ritonavir after 5 min (enzyme assay) and 1 h (pre-incubation time) incubation.

activity of more than 90%. In more stable **4** and **9**, less than 20% was converted and exhibited relatively low (20–30%) inhibitory activity. Prodrug **12**, which has an excessively longer $t_{1/2}$ value of >30 h, was hardly converted to ritonavir at pH 5.5 and did not show any inhibitory activity in this enzyme assay. These data suggested that the anti-HIV-1 PR activity was well correlated with the formation of the biologically active parent compounds.

Conclusion

New water-soluble prodrugs of HIV-1 PR inhibitors KNI-272 and KNI-279 based on the *N*→*O* acyl migration reaction were developed, and the ability of these prodrugs was evaluated. The prodrugs **3**, **4**, **6**, and **7** exhibited high solubility in aqueous media and a rapid conversion to the parent drugs under physiological conditions. This suggested that these prodrugs are useful as practical water-soluble prodrugs. This strategy is applicable to other HIV-1 PR inhibitors[13–17,34] with an Apns–Dmt core structure and might be able to overcome the problem of the low water-solubility of HIV-1 PR inhibitors.

Experimental

Reagents and solvents used were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) and Watanabe Chem. Ind., Ltd. (Hiroshima, Japan) without further purification. TLC was performed using Merck Silica gel 60 F_{254} precoated plate. Column chromatography was performed on Merck 107734 silica gel 60 (70–230 mesh). Melting points were measured on Yanagimoto micro melting apparatus. Analytical HPLC was performed using a C18 reverse phase column (4.6×150 mm; YMC Pack ODS AM302) with binary solvent system: linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 1.0 mL/min, detected 230 nm. ^{1}H NMR spectra were obtained on a JEOL AL300 spectrometer with TMS as an internal standard. FAB-MS was performed on a JEOL JMS-SX102A spectrometer equipped with the JMA-DA7000 data system. TOF-MS was performed on a Voyager-DE RP spectrometer (PerSeptive Biosystems, inc.).

Boc-Apns-Thz-NH-Bu^t (14a). To compound **13a**[13,34] (10 g, 28.4 mmol) were added anisole (7.5 mL) and 4 N-HCl/dioxane (110 mL), and stirred for 2 h at room temperature. After the reaction mixture was concentrated in vacuo, ether was added to give precipitate (7.33 g). To the solution of the obtained precipitate (6 g) and Boc-Apns-OH (8.7 g, 29.5 mmol) in DMF (100 mL) were added HOBt (4.5 g, 29.5 mmol), EDC·HCl (5.63 g, 29.5 mmol) and Et_3N (2.7 g, 26.7 mmol) stepwise at 0 °C and stirred overnight at room temperature.[13,34] After removal of the solvent in vacuo, the residue was dissolved in EtOAc, washed sequentially with 5% citric acid, 5% $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was applied to a silica gel column (3.5×18 cm) and eluted with hexane–EtOAc (1:1) to give 11.7 g (94%) of the title compound **14a** as a white solid: mp 79–82 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 (s, 9H), 1.34 (s, 9H), 2.53–2.87 (m, 2H), 3.19 (dd, J=7.5, 11.7 Hz, 1H), 3.32 (br s, 1H), 3.55 (dd, J=6.9, 11.7 Hz, 1H), 3.72 (d, J=6.6 Hz, 1H), 4.04 (m, 1H), 4.50–4.77 (m, 2H), 4.81 (dd, J=6.9, 7.5 Hz, 1H), 4.90–5.15 (m, 2H), 6.30 (br s, 1H), 7.09–7.37 (m, 5H); MS (FAB): m/z 466 $[M+H]^+$. Anal. calcd for $C_{23}H_{35}N_3O_5S$: H, 7.58; C, 59.33; N, 9.02. Found: H, 7.70; C, 59.01; N, 8.86.

Boc-Apns-Dmt-NH-Bu^t (14b). Compound **14b** was prepared from Boc-Dmt-NH-Bu^t (**13b**)[13,34] (6.33 g, 20 mmol) according to the similar procedure described for compound **14a**. Yield 7.97 g (82%); mp 84–87 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.27–1.43 (m, 18H), 1.48–1.60 (m, 6H), 2.60–2.84 (m, 2H), 3.65–3.76 (m, 1H), 3.91 (m, 1H), 4.11 (m, 1H), 4.28–5.08 (m, 5H) 7.07–7.36 (m, 5H); MS (FAB): m/z 494 $[M+H]^+$. Anal. calcd for $C_{25}H_{39}N_3O_5S$: H, 7.96; C, 60.82; N, 8.51. Found: H, 8.05; C, 60.57; N, 8.54.

Z-Apns-Thz-NH-Bu^t (15a). To compound **14a** (3.6 g, 7.75 mmol) was added 4 N-HCl/dioxane (20 mL) and stirred for 2 h at room temperature. After the reaction mixture was concentrated in vacuo, ether was added to give precipitate, which was dissolved in DMF (20 mL) and neutralized with Et_3N (1.09 mL). To this reaction mixture were added Z-OSu (1.93 g, 7.75 mmol) and Et_3N (1.09 mL, 7.75 mmol) stepwise at 0 °C and stirred at room temperature for 4 h. After removal of the solvent in vacuo, the residue was dissolved in EtOAc, washed sequentially with 5% citric acid, 5% $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was applied to a silica gel column (3×18 cm) and eluted with hexane–EtOAc (1:1) to give 3.45 g (89%) of the title compound **14a** as a white solid: mp 69–70 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 (s, 9H), 2.55–2.86 (m, 2H), 3.19 (dd, J=7.6, 11.7 Hz, 1H), 3.314 (m, 1H), 3.52 (dd, J=6.6, 11.7 Hz, 1H), 3.75 (d, 1H), 4.10 (m, 1H), 4.65 (d, J=9.6 Hz, 1H), 4.70–5.10 (m, 5H), 5.26 (d, J=7.8 Hz, 1H), 6.26 (br s, 1H), 7.09–7.42 (m, 10H); MS (FAB): m/z 500 $[M+H]^+$. Anal. calcd for $C_{26}H_{33}N_3O_5S$: H, 6.66; C, 62.50; N, 8.41. Found: H, 6.81; C, 62.31; N, 8.30.

Z-Apns-Dmt-NH-Bu^t (15b). Compound **15b** was prepared from Boc-Apns-Dmt-NH-Bu^t (**14b**) (1.95 g, 4.53 mmol) according to the similar procedure described for compound **15a**. Yield 1.91 g (92%): mp 79–83 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.27 (s, 9H), 1.40 (s, 3H), 1.49 (s, 3H), 2.53–2.78 (m, 2H), 3.91 (m, 1H), 4.47 (m, 1H), 4.51 (s, 1H), 4.87–5.06 (m, 3H), 5.14 (d, J=7.2 Hz, 1H), 7.09–7.46 (m, 10H); TOF-Ms: m/z 528 $[M+H]^+$. Anal. calcd for $C_{28}H_{37}N_3O_5S$: H, 7.07; C, 63.73; N, 7.96. Found: H, 7.24; C, 63.81; N, 7.91.

Z-Apns(Boc-Mta)-Thz-NH-Bu^t (17a). To a solution of **15a** (700 mg, 1.44 mmol) and Boc-Mta-OH (**16a**) (509 mg, 2.16 mmol) in dichloromethane (9.3 mL) were

added DMAP (25 mg, 0.216 mmol) and a DMF (1.1 mL) solution of DCC (433 mg, 2.16 mmol) at 0 °C and stirred overnight at room temperature. After removal of the solvent in vacuo, the residue was dissolved in EtOAc, washed sequentially with 5% citric acid, 5% $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was applied to a silica gel column (2.5×18 cm) and eluted with hexane–EtOAc (1:1) to give 883 mg (89%) of the title compound **17a** as a white solid: mp 69–72 °C; $[\alpha]_D^{21} = -66.07$ (*c* 0.56, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.31 (s, 9H), 1.43 (s, 9H), 2.13 (s, 3H), 2.86–3.26 (m, 5H), 3.45 (dd, *J* = 5.4, 11.2 Hz, 1H), 4.11 (br s, 1H), 4.32 (m, 1H), 4.50 (m, 1H), 4.57 (m, 1H), 4.76 (t, *J* = 6.4 Hz, 1H), 4.89 (d, *J* = 8.7 Hz, 1H), 5.03 (s, 2H), 5.19–5.62 (m, 3H), 6.21 (br s, 1H), 7.11–7.47 (m, 10H); MS (FAB): *m*/*z* 717 $[M+H]^+$. Anal. calcd for $C_{35}H_{48}N_4O_8S_2 \cdot 1.2H_2O$: H, 6.88; C, 56.92; N, 7.59. Found: H, 6.54; C, 56.88; N, 7.70.

Z-Apns(Boc-Val)-Thz-NH-Bu^t (17b). Compound **17b** was prepared from Z-Apns-Dmt-NH-Bu^t (**15b**) (600 mg, 1.24 mmol) and Boc-Val-OH (**16b**) (391 mg, 1.85 mmol) according to the similar procedure described for compound **17a**. Yield 840 mg (97%); mp 77–80 °C; $[\alpha]_D^{22} = -118.26$ (*c* 0.58, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92–1.10 (m, 6H), 1.30 (s, 9H), 1.42 (s, 9H), 2.22 (m, 1H), 2.78–3.23 (m, 3H), 3.46 (dd, *J* = 6.2, 12 Hz, 1H), 4.02–5.01 (m, 6H), 5.03 (s, 2H), 5.10–5.58 (m, 2H), 6.21 (s, 1H), 7.09–7.42 (m, 10H); MS (FAB): *m*/*z* 699 $[M+H]^+$. Anal. calcd for $C_{36}H_{50}N_4O_8S \cdot 9/4H_2O$: H, 7.43; C, 58.48; N, 7.58. Found: H, 6.70; C, 58.42; N, 7.67.

Z-Apns(Boc-Nva)-Thz-NH-Bu^t (18). Compound **18** was prepared form Z-Apns-Dmt-NH-Bu^t (**15b**) (350 mg, 0.67 mmol) and Boc-Nva-OH (**16c**) (235 mg, 1.00 mmol) according to the similar procedure described for compound **17a**. Yield 443 mg (91%); mp 73–75 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96 (t, 3H), 1.22–1.38 (m, 9H), 1.41 (br.s, 11H), 1.55–1.75 (m, 1H), 1.76–2.00 (m, 1H), 2.73–3.22 (m, 3H), 3.46 (dd, 1H), 4.09 (s, 1H), 4.20–4.43 (m, 2H), 4.61 (d, 1H), 4.79 (t, 1H), 4.84–4.95 (m, 2H), 5.03 (s, 2H), 5.23–5.62 (m, 2H), 6.22 (s, 1H), 7.12–7.42 (m, 10H); TOF-Ms: *m*/*z* 721 $[M+Na]^+$. Anal. calcd for $C_{36}H_{50}N_4O_8S$: H, 7.21; C, 61.87; N, 8.02. Found: H, 7.35; C, 61.86; N, 8.01.

Z-Apns(Boc-Mta)-Dmt-NH-Bu^t (19a). Compound **19a** was prepared from Z-Apns-Dmt-NH-Bu^t (**15b**) (700 mg, 1.33 mmol) and Boc-Mta-OH (**16a**) (468 mg, 1.99 mmol) according to the similar procedure described for compound **17a**. Yield 950 g (94%); mp 121–123 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.30–160 (m, 24H), 2.12 (s, 3H), 2.78–3.19 (m, 4H), 4.05 (br s, 1H), 4.21 (s, 1H), 4.27–4.58 (m, 2H), 4.76–4.94 (m, 2H), 5.04 (s, 2H), 5.26–5.81 (m, 3H), 7.11–7.39 (m, 10H); TOF-Ms: *m*/*z* 745 $[M+H]^+$. Anal. calcd for $C_{37}H_{52}N_4O_8S_2$: H, 7.04; C, 59.65; N, 7.52. Found: H, 7.26; C, 59.81; N, 7.47.

Z-Apns(Boc-Val)-Dmt-NH-Bu^t (19b). Compound **19b** was prepared from Z-Apns-Dmt-NH-Bu^t (**15b**) (700 mg, 1.33 mmol) and Boc-Val-OH (**16b**) (432 mg, 1.99 mmol) according to the similar procedure described for compound **17a**. Yield 920 mg (93%); mp 160–161 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89–1.11 (m, 6H), 1.26–1.62 (m, 24H), 2.20 (m, 1H), 2.80–3.21 (m, 2H), 3.98–4.38 (m, 3H), 4.73–5.11 (m, 5H), 5.39–5.68 (m, 3H), 7.10–7.40 (m, 10H), 6.53 (br s, 1H); TOF-Ms: *m*/*z* 749 $[M+Na]^+$. Anal. calcd for $C_{38}H_{54}N_4O_8S$: H, 7.49; C, 62.79; N, 7.71. Found: H, 7.54; C, 62.70; N, 7.95.

Z-Apns(iQoa-Mta)-Thz-NH-Bu^t (20a). To compound **19a** (747 mg, 1.06 mmol) were added anisole (1.5 mL) and 4N-HCl/dioxane (10 mL) and stirred for 2 h at room temperature. After removal of the solvent in vacuo, the residue was triturated with ether at 0 °C to give precipitate (695 mg). After the reaction mixture was concentrated in vacuo, ether was added to give precipitate (7.33 g). To a solution of the obtained precipitate (400 mg, 0.61 mmol) and iQoa-OH (137 mg, 0.67 mmol) in DMF (5 mL) were added HOBt (103 mg, 0.67 mmol), DCC (139 mg, 0.67 mmol) and Et_3N (85 mg, 0.61 mmol) stepwise at 0 °C and stirred overnight at room temperature. After removal of the solvent in vacuo, the residue was dissolved in EtOAc, washed sequentially with 5% citric acid, 5% $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was applied to a silica gel column (2.5×20 cm) and eluted with hexane–EtOAc (1:2) to give 414 mg (84%) of title compound **20a**: mp 92–95 °C; $[\alpha]_D^{22} = -105.36$ (*c* 0.56, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.31 (s, 9H), 2.11 (s, 3H), 2.72–3.59 (m, 7H), 4.28–5.17 (m, 8H), 5.41–5.70 (m, 2H), 6.19 (s, 1H), 7.01 (d, *J* = 6.9 Hz, 1H), 7.09–7.39 (m, 10H), 7.09–7.39 (m, 10H), 7.52 (dd, *J* = 8.4, 8.4 Hz, 1H), 7.64 (d, *J* = 8.4 Hz, 1H), 8.06 (d, *J* = 5.6 Hz, 1H), 8.57 (d, *J* = 5.6 Hz, 1H), 9.25 (s,1H); MS (FAB): *m*/*z* 802 $[M+H]^+$. Anal. calcd for $C_{41}H_{47}N_5O_8S_2 \cdot 5/4H_2O$: H, 6.05; C, 59.73; N, 8.49. Found: H, 5.98; C, 59.85; N, 8.10.

Z-Apns(iQoa-Val)-Thz-NH-Bu^t (20b). Compound **20b** was prepared from Z-Apns(Boc-Val)-Thz-NH-Bu^t (**19b**) (440 mg, 0.63 mmol) and iQoa-OH (141 mg, 0.69 mmol) according to the similar procedure described for compound **20a**. Yield 372 g (75%); mp 98–102 °C; $[\alpha]_D^{22} = -137.63$ (*c* 0.47, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.73–1.08 (m, 6H), 1.30 (s, 9H), 2.22–2.46 (m, 1H), 2.70–3.28 (m, 3H), 3.33–3.63 (m, 1H), 4.10 (br s, 1H), 4.31 (br s, 1H), 4.53–5.20 (m, 6H), 5.29–5.68 (m, 2H), 6.18 (br s, 1H), 6.92–7.41 (m, 11H), 7.51 (t, *J* = 8.0 Hz, 1H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.89–8.02 (m, 1H), 8.60 (d, *J* = 6.0 Hz, 1H), 9.28 (s, 1H); MS (FAB): *m*/*z* 784 $[M+H]^+$. Anal. calcd for $C_{42}H_{49}N_5O_8S \cdot 1/2H_2O$: H, 6.36; C, 63.61; N, 8.83. Found: H, 6.19; C, 63.66; N, 8.84.

Z-Apns(iQoa-Nva)-Thz-NH-Bu^t (21). Compound **21** was prepared from Z-Apns(Boc-Nva)-Thz-NH-Bu^t (**20**) (220 mg, 0.31 mmol) and iQoa-OH (70.4 mg, 0.35 mmol) according to the similar procedure described for compound **20a**. Yield 238 mg (97%); mp 72–76 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89–1.06 (m, 3H), 1.31 (s, 9H), 1.36–1.61 (m, 2H), 1.65–2.12 (m, 2H), 2.72–3.28 (m, 3H), 3.45 (dd, *J* = 5.3, 12.0 Hz, 1H), 4.02–4.48 (m, 1H), 4.53–4.88 (m, 4H), 4.93 (d, *J* = 9.6 Hz, 2H), 5.02 (s,

2H), 5.38–5.66 (m, 2H), 6.16 (s, 1H), 6.90–7.40 (m, 12H), 7.50 (t, $J=8.1$ Hz, 1H), 7.64 (d, $J=8.4$ Hz, 1H), 8.00 (d, $J=5.8$ Hz, 1H), 8.60 (d, $J=5.8$ Hz, 1H), 9.25 (s, 1H); TOF-Ms: m/z 784 $[M+H]^+$. Anal. calcd for $C_{42}H_{49}N_5O_8S_2\cdot 1/4H_2O$: H, 6.33; C, 63.98; N, 8.88. Found: H, 6.58; C, 63.98; N, 8.64.

Z-Apns(iQoa-Mta)-Dmt-NH-Bu^t (22a). Compound **22a** was prepared from Z-Apns(Boc-Mta)-Dmt-NH-Bu^t (**19a**) (410 mg, 0.59 mmol) and iQoa-OH (131 mg, 0.64 mmol) according to the similar procedure described for compound **20a**. Yield 414 g (85%); mp 88–92 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (s, 9H), 1.42–1.61 (m, 6H), 2.01–2.18 (m, 3H), 2.73–3.30 (m, 4H), 4.07 (br s, 1H), 4.22 (s, 1H), 4.35 (br s, 1H), 4.45–5.17 (m, 8H), 5.48–5.68 (m, 2H), 5.78–5.81 (m, 1H), 6.56 (s, 1H), 6.85–7.04 (m, 1H), 7.09–7.37 (m, 10H), 7.41–7.57 (m, 1H), 7.58–7.71 (m, 1H), 7.98–8.10 (m, 1H), 8.51–8.62 (m, 1H), 9.23 (s, 1H); TOF-Ms: m/z 831 $[M+H]^+$. Anal. calcd for $C_{43}H_{51}N_5O_8S_2\cdot 1/4H_2O$: H, 6.19; C, 62.22; N, 8.44. Found: H, 6.37; C, 61.99; N, 8.21.

Z-Apns(iQoa-Val)-Dmt-NH-Bu^t (22b). Compound **22b** was prepared from Z-Apns(Boc-Val)-Dmt-NH-Bu^t (**19b**) (440 mg, 0.65 mmol) and iQoa-OH (141 mg, 0.69 mmol) according to the similar procedure described for compound **20a**. Yield 422 mg (86%); mp 91–95 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88–1.09 (m, 6H), 1.33 (s, 9H), 1.41–1.60 (m, 6H), 2.22–2.42 (m, 1H), 2.76–2.97 (m, 1H), 3.02–3.21 (m, 1H), 4.08 (br s, 1H), 4.19–4.39 (m, 1H), 4.52–5.11 (m, 7H), 5.41–5.75 (m, 2H), 6.51 (br s, 1H), 6.81–7.02 (m, 2H), 7.04–7.36 (m, 11H), 7.50 (t, $J=8.0$ Hz, 1H), 7.63 (d, $J=8.0$, 1H), 7.89–8.00 (m, 1H), 8.53–8.65 (m, 1H), 9.25 (s, 1H); TOF-Ms: m/z 813 $[M+H]^+$. Anal. calcd for $C_{44}H_{53}N_5O_8S\cdot 1/4H_2O$: H, 6.60; C, 64.70; N, 8.58. Found: H, 6.71; C, 64.83; N, 8.32.

Z-Apns(Noa-Mta)-Thz-NH-Bu^t (23a). Compound **23a** was prepared from Z-Apns(Boc-Mta)-Thz-NH-Bu^t (**17a**) (430 mg, 0.61 mmol) and Noa-OH (131 mg, 0.65 mmol) according to the similar procedure described for compound **20a**. Yield 434 mg (89%); mp 80–82 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.31 (s, 9H), 2.10 (s, 3H), 2.87–3.28 (m, 5H), 3.40–3.58 (m, 2H), 4.12 (m, 1H), 4.32 (m, 1H), 4.54–5.18 (m, 7H), 5.40–5.72 (m, 2H), 6.16 (br s, 1H), 6.79 (d, $J=7.5$ Hz, 1H), 7.08–7.48 (m, 11H), 7.49–7.66 (m, 3H), 7.81–7.88 (m, 1H), 8.27–8.36 (m, 1H); MS(FAB): m/z 801 $[M+H]^+$. Anal. calcd for $C_{42}H_{48}N_4O_8S_2$: H, 6.04; C, 62.98; N, 6.99. Found: H, 6.21; C, 62.83; N, 7.03.

Z-Apns(Noa-Val)-Thz-NH-Bu^t (23b). Compound **23b** was prepared from Z-Apns(Boc-Val)-Thz-NH-Bu^t (**17b**) (425 mg, 0.59 mmol) and Noa-OH (134 mg, 0.66 mmol) according to the similar procedure described for compound **20a**. Yield 476 g (97%); mp 81–83 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92–1.11 (m, 6H), 1.30 (s, 9H), 2.20–2.46 (m, 1H), 2.70–3.27 (m, 3H), 3.36–3.70 (m, 2H), 4.08 (br s, 1H), 4.30 (br s, 1H), 4.53–5.12 (m, 7H), 5.23–5.67 (m, 2H), 6.16 (br s, 1H), 6.78 (d, $J=7.5$ Hz, 1H), 7.03–7.41 (m, 11H), 7.44–7.62 (m, 3H), 7.78–7.91 (m, 1H), 8.12–8.28 (m,1H); MS(FAB): m/z 783 $[M+H]^+$. Anal. calcd for $C_{43}H_{50}N_4O_8S\cdot 3/4H_2O$: H, 6.52; C, 64.85; N, 7.03. Found: H, 6.42; C, 64.99; N, 7.05.

Z-Apns(Noa-Mta)-Dmt-NH-Bu^t (24a). Compound **24a** was prepared form Z-Apns(Boc-Mta)-Dmt-NH-Bu^t (**19a**) (437 mg, 0.59 mmol) and Noa-OH (131 mg, 0.65 mmol) according to the similar procedure described for compound **20a**. Yield 434.6 mg (89%); mp 79–83 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (s, 9H), 1.40–1.62 (m, 6H), 2.05–2.16 (m, 3H), 2.78–3.29 (m, 4H), 4.06 (br s, 1H), 4.22 (s, 1H), 4.34 (br s, 1H), 4.44–5.18 (m, 7H), 5.49–5.89 (m, 2H), 6.51–6.87 (m, 1H), 7.09–7.42 (m, 11H), 7.48–7.59 (m, 3H), 7.69–7.90 (m, 1H), 8.23–8.39 (m, 1H); TOF-Ms: m/z 830 $[M+H]^+$. Anal. calcd for $C_{44}H_{52}N_4O_8S_2$: H, 6.32; C, 63.75; N, 6.76. Found: H, 6.51; C, 63.49; N, 6.66.

Z-Apns(Noa-Val)-Dmt-NH-Bu^t (24b). Compound **24b** was prepared from Z-Apns(Boc-Val)-Dmt-NH-Bu^t (**19b**) (425 mg, 0.59 mmol) and Noa-OH (134 mg, 0.66 mmol) according to the similar procedure described for compound **20a**. Yield 459 g (94%); mp 77–82 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.88 (m, 6H), 1.32 (s, 9H), 1.39–1.59 (m, 6H), 2.23–2.39 (m, 1H), 2.84–2.97 (m, 1H), 3.00–3.22 (m, 1H), 3.99–4.12 (m, 1H), 4.19–4.52 (m, 2H), 4.58–5.12 (m, 6H), 5.39–5.80 (m, 2H), 6.47–6.82 (m, 1H), 7.04–7.40 (m, 10H), 7.48–7.61 (m, 3H), 7.78–7.89 (m, 1H), 8.13–8.27 (m, 1H); TOF-Ms: m/z 811 $[M+H]^+$. Anal. calcd for $C_{45}H_{54}N_4O_8S$: H, 6.71; C, 66.64; N, 6.91. Found: H, 6.93; C, 66.62; N, 6.68.

Apns(iQoa-Mta)-Thz-NH-Bu^t·2HCl (3). To compound **20a** (100 mg, 0.125 mmol) was added dimethylsulfide (0.366 mL, 5 mmol), anisole (136 μL, 1.25 mmol) and TFA (4.8 mL, 62.3 mmol) at −5 °C and stirred for 1 h at 0–10 °C and overnight at room temperature. After the reaction mixture was concentrated in vacuo at room temperature, ether was added to give precipitate (85 mg). This crude product was purified using preparative HPLC (YMC 120A ODS; 20×250 mm) eluted in a linear gradient with 30–80% MeCN in 0.1% aqueous TFA over 30 min at a flow rate of 1 mL/min and the desired fraction was lyophilized to give a white powder as a TFA salt. To a solution of this powder in EtOAc was added 4 N-HCl/EtOAc at 0 °C to form a crystal and separation by centrifuge gave the 25 mg (29%) of desired compound **24** as a white fluffy powder: mp 166–170 °C; $[\alpha]_D^{23}=-131.87$ (c 0.46, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.26 (s, 9H), 2.13 (s, 3H), 2.78–3.19 (m, 5H), 3.40 (dd, $J=7.6$, 11.6 Hz, 1H), 3.99 (br s, 1H), 4.69 (d, $J=8.8$ Hz, 1H), 4.76 (t, $J=6.9$ Hz, 1H), 4.81–5.05 (m 4H), 5.64 (br s, 1H), 7.19–7.46 (m, 6H), 7.68 (t, $J=8.0$ Hz, 1H), 7.82 (d, $J=8.0$ Hz, 1H), 7.89 (s, 1H), 8.24 (br s, 1H), 8.32 (d, $J=8.0$ Hz, 1H), 8.58 (d, $J=6.0$ Hz, 1H), 8.78 (d, $J=8.1$ Hz, 1H) 9.46 (s, 1H); TOF-MS: m/z 668 $[M+H]^+$. Anal. calcd for $C_{33}H_{43}Cl_2N_5O_6S_2$: H, 5.82; C, 53.50; N, 9.46. Found: H, 5.95; C, 53.41; N, 9.67.

Compounds **4**–**11** were prepared according to the similar procedure described for compound **3** starting from the corresponding intermediates **20**–**24**.

Apns(iQoa-Val)-Thz-NH-Bu^t·2HCl (4). 32% yield from **20b**; mp 164–170 °C; $[\alpha]_D^{22} = -124.77$ (*c* 0.55, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.89–1.03 (m, 6H), 1.27 (s, 9H), 2.22–2.38 (m, 1H), 2.70–3.11 (m, 3H), 3.41 (dd, *J* = 7.4, 11.9 Hz, 1H), 4.62–4.75 (m, 2H), 4.80 (t, *J* = 7.0 Hz, 1H), 4.86 (d, *J* = 9.2 Hz, 1H), 4.94 (br s, 2H), 5.57 (br s, 1H), 7.17–7.46 (m, 6H), 7.65 (t, *J* = 8.0 Hz, 1H), 7.77 (d, *J* = 8.0 Hz, 1H), 7.90 (s, 1H), 8.08–8.29 (m, 3H), 8.48 (d, *J* = 8.7 Hz, 1H), 8.56 (d, *J* = 5.7 Hz, 1H), 9.41 (s, 1H); TOF-MS: *m*/*z* 650 $[M+H]^+$. Anal. calcd for $C_{34}H_{45}Cl_2N_5O_6S$: H, 6.28; C, 56.50; N, 9.69. Found: H, 6.24; C, 56.44; N, 9.84.

Apns(iQoa-Mta)-Dmt-NH-Bu^t·2HCl (5). 26% yield from **22a**; mp 164–170 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.27 (s, 9H), 1.41 (s, 3H), 1.49 (s, 3H), 2.12 (s, 3H), 2.80–3.22 (m, 4H), 3.82 (br s, 1H), 4.49 (s, 2H), 4.83–5.09 (m, 5H), 5.72 (s, 1H), 7.13–7.42 (m, 6H), 7.53 (d, *J* = 7.8 Hz, 1H), 7.77–7.92 (m, 2H), 7.99 (t, *J* = 7.8 Hz, 1H), 8.40–8.81 (m, 5H), 9.06 (d, *J* = 8.7 Hz, 1H), 9.79 (s, 1H); TOF-MS: *m*/*z* 696 $[M+H]^+$. Anal. calcd for $C_{35}H_{47}Cl_2N_5O_6S_2 \cdot 3/2H_2O$: H, 6.33; C, 52.82; N, 8.79. Found: H, 6.25; C, 53.19; N, 8.23.

Apns(iQoa-Val)-Dmt-NH-Bu^t·2HCl (6). 48% yield from **22b**; mp 158–162 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.99–1.01 (m, 6H), 1.28 (s, 9H), 1.41 (s, 3H), 1.50 (s, 3H), 2.23–2.38 (m, 1H), 2.83 (dd, *J* = 9.8, 14.4 Hz, 1H), 3.05 (dd, *J* = 3.0, 14.4 Hz, 1H), 3.89 (br s, 1H), 4.55 (s, 2H), 4.69 (dd, *J* = 5.0, 8.8 Hz, 1H), 4.82–5.05 (m, 4H), 5.51 (d, *J* = 2.7 Hz, 1H), 7.22–7.43 (m, 6H), 7.69 (t, *J* = 8.0 Hz, 1H), 7.78–7.88 (m, 2H), 8.12–8.28 (m, 3H), 8.52–8.64 (m, 2H), 9.49 (s, 1H); TOF-MS: *m*/*z* 678 $[M+H]^+$. Anal. calcd for $C_{36}H_{49}Cl_2N_5O_6S \cdot 2H_2O$: H, 6.79; C, 54.96; N, 8.90. Found: H, 6.84; C, 54.86; N, 8.58.

Apns(Noa-Mta)-Thz-NH-Bu^t·HCl (7). 54% yield from **23a**; mp 174–177 °C; $[\alpha]_D^{30} = -103.1$ (*c* 0.485, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.27 (s, 9H), 2.14 (s, 3H), 2.80–3.19 (m, 5H), 3.57 (s, 1H), 3.84 (br s, 1H), 4.62–5.02 (m, 6H), 5.72 (br s, 1H), 6.96 (d, *J* = 7.5 Hz, 1H), 7.19–7.58 (m, 9H), 7.78–7.95 (m, 2H), 8.20–8.51 (m, 3H), 8.77 (d, *J* = 8.7 Hz, 1H); TOF-MS: *m*/*z* 667 $[M+H]^+$. Anal. calcd for $C_{34}H_{43}ClN_4O_6S_2 \cdot 7/4H_2O$: H, 6.38; C, 55.57; N, 7.62. Found: H, 6.23; C, 55.99; N, 7.14.

Apns(Noa-Val)-Thz-NH-Bu^t·HCl (8). 30% yield from **23b**; mp 121–126 °C; $[\alpha]_D^{30} = -83.76$ (*c* 0.485, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.88–1.03 (m, 6H), 1.24 (s, 9H), 2.20–2.36 (m, 1H), 2.83–3.15 (m, 3H), 3.57 (s, 1H), 3.83 (br s, 1H), 4.67–4.98 (m, 6H), 5.70 (br s, 1H), 6.91 (d, *J* = 7.5 Hz, 1H), 7.20–7.58 (m, 9H), 7.80–7.95 (m, 2H), 8.17–8.24 (m, 1H), 8.36 (br s, 3H), 8.49–8.58 (m, 1H); TOF-MS: *m*/*z* 649 $[M+H]^+$. Anal. calcd for $C_{35}H_{45}ClN_4O_6S \cdot H_2O$: H, 6.85; C, 58.28; N, 7.77. Found: H, 6.48; C, 58.57; N, 7.27.

Apns(Noa-Mta)-Dmt-NH-Bu^t·HCl (9). 33% yield from **24a**; mp 132–136 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.27 (s, 9H), 1.41 (s, 3H), 1.49 (s, 3H), 2.14 (s, 3H), 2.84–3.17 (m, 4H), 3.87 (br s, 1H), 4.50 (s, 1H), 4.88–5.04 (m, 5H), 5.64 (d, *J* = 2.7 Hz, 1H), 6.96 (d, *J* = 7.5 Hz, 1H), 7.18–7.60 (m, 6H), 7.78–7.92 (m, 2H), 8.27–8.48 (m, 2H), 8.89 (d, *J* = 8.7 Hz, 1H); TOF-MS: *m*/*z* 696 $[M+H]^+$. Anal. calcd for $C_{36}H_{47}ClN_4O_6S_2 \cdot 3/2H_2O$: H, 6.64; C, 57.01; N, 7.38. Found: H, 6.38; C, 57.19; N, 7.00.

Apns(Noa-Val)-Dmt-NH-Bu^t·HCl (10). 37% yield from **24b**; mp 171–174 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.88–1.01 (m, 6H), 1.28 (s, 9H), 1.41 (s, 3H), 1.50 (s, 3H), 2.22–2.38 (m, 1H), 2.85 (dd, *J* = 9.8, 14.3 Hz, 1H) 3.06 (dd, *J* = 3.0, 14.3 Hz, 1H), 3.89 (br s, 1H), 4.55 (s, 1H), 4.71 (dd, *J* = 4.8, 9.0 Hz, 1H), 4.78–5.01 (m, 4H), 5.51 (br s, 1H), 6.89 (d, *J* = 7.8 Hz, 1H), 7.20–7.42 (m, 6H), 7.44–7.57 (m, 3H), 7.81 (s, 1H), 7.83–7.90 (m, 1H), 8.08–8.28 (m, 3H), 8.43 (d, *J* = 9.0 Hz, 1H); TOF-MS: *m*/*z* 677 $[M+H]^+$. Anal. calcd for $C_{37}H_{49}ClN_4O_6S \cdot 2H_2O$: H, 7.12; C, 59.30; N, 7.48. Found: H, 6.98; C, 59.57; N, 7.24.

Apns(iQoa-Nva)-Thz-NH-Bu^t·2HCl (11). 35% yield from **21**; mp 154–159 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.90 (t, *J* = 7.2 Hz, 3H), 1.27 (s, 9H), 1.32–1.52 (m, 2H), 1.69–1.96 (m, 2H), 2.82–3.10 (m, 4H), 3.42 (dd, *J* = 7.2, 11.4 Hz, 1H) 3.79 (br s, 1H), 4.61–4.82 (m, 3H), 4.95 (d, *J* = 9.0 Hz, 1H), 5.01 (s, 2H), 5.76 (s, 1H), 7.18–7.56 (m, 7H), 7.80 (t, *J* = 8.3 Hz, 1H), 7.91–8.06 (m, 2H), 8.43–8.60 (m, 4H), 8.64 (d, *J* = 6.6 Hz, 1H), 9.00 (d, *J* = 8.7 Hz, 1H), 9.72 (s, 1H); TOF-Ms: *m*/*z* 650 $[M+H]^+$. Anal. calcd for $C_{34}H_{45}Cl_2N_5O_6S \cdot 2H_2O$: H, 6.51; C, 53.82; N, 9.23. Found: H, 6.36; C, 53.56; N, 9.03.

iQoa-Val-Apns-Dmt-NH-Bu^t (2b). To compound **25** (300 mg, 0.53 mmol) were added anisole (0.15 mL) and 4 N-HCl/dioxane (5 mL), and stirred for 2 h at room temperature. After the reaction mixture was concentrated in vacuo, *n*-hexane was added to give precipitate at 0 °C. To a solution of the obtained precipitate (140 mg, 0.28 mmol) and iQoa-OH (62.4 mg, 0.31 mmol) in DMF (2 mL) were added HOBt (47 mg, 0.31 mmol), EDC-HCl (59 mg, 0.31 mmol) and Et_3N (39 μL, 0.28 mmol) stepwise at 0 °C and stirred overnight at room temperature. After removal of the solvent in vacuo, the residue was dissolved in EtOAc, washed sequentially with 5% citric acid, 5% $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was applied to a silica gel column (2.5×18 cm) and eluted with $CHCl_3$–MeOH (20:1) to give 140 mg (78%) of title compound **2b**; mp 122–125 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.68–0.96 (m, 6H), 1.30 (s, 9H), 1.42–1.63 (m, 6H), 1.99–2.20 (m, 1H), 2.63–2.92 (m, 2H), 3.99 (br s, 1H), 4.18–4.40 (m, 2H), 4.40–4.80 (m, 4H), 4.85 (m, 1H), 6.85–7.28 (m, 7H), 7.46–7.58 (m, 1H), 7.60–7.69 (m, 1H), 7.94 (d, *J* = 5.4 Hz, 1H), 8.61 (t, *J* = 6.2 Hz, 1H), 9.26 (d, *J* = 5.0 Hz, 1H); TOF-MS: *m*/*z* 678 $[M+H]^+$. Anal. calcd for $C_{36}H_{47}N_5O_6S \cdot 1/2H_2O$: H, 7.04; C, 62.95; N, 10.20. Found: H, 7.04; C, 62.91; N, 10.09.

Noa-Val-Apns-Dmt-NH-Bu^t (2d). 70% yield from **25**; mp 105–108 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.62–

0.73 (s, 3H), 0.80–0.95 (s, 3H), 1.30 (s, 9H), 1.46–1.62 (m, 6H), 2.11 (m, 1H), 2.59–2.82 (m, 2H), 3.73–3.98 (m, 1H), 4.10–4.38 (m, 2H), 4.40–5.18 (m, 5H), 6.39 (br s, 1H), 6.80 (d, $J=7.5$ Hz, 1H), 6.82–7.20 (m, 6H), 7.35–7.46 (m, 1H), 7.48–7.63 (m, 3H), 7.79–7.92 (m, 1H), 8.15–8.28 (m, 1H); TOF-MS: m/z 677 $[M+H]^+$. Anal. calcd for $C_{37}H_{48}N_4O_6S$: H, 7.15; C, 65.65; N, 8.28. Found: H, 7.17; C, 65.42; N, 8.40.

(2*S*,3*S*,5*S*)-5-[*N*-[(*tert*-butyloxy)carbonyl]amino]-2-[*N*-[(5-thiazolyl)methoxy]carbonyl]amino]-1,6-diphenyl-3-hydroxyhexane] 28. To a solution of [(2*S*,3*S*,5*S*)-2-amino-5-[*N*-[(*tert*-butyloxy)carbonyl]amino]-1,6-diphenyl-3-hydroxyhexane] **26**[42] (1.0 g, 2.6 mmol) in THF–DMF (1:1, 10 mL) was added 4-nitrophenyl (5-thiazolyl)methyl carbonate hydrochloride **27**[43] (0.91 g, 2.8 mmol) and stirred overnight at room temperature. After the reaction mixture was concentrated under reduced pressure, the resulting residue was dissolved in EtOAc, washed sequentially with 5% citrate, 5% $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was applied to a silica gel column (2.5×20 cm) and eluted with *n*-hexane–EtOAc (1:1) to give 1.1 mg (81%) of title compound **28**: mp 72–81 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38 (s, 9H), 1.60 (m, 2H), 2.72 (d, $J=6.6$ Hz, 2H), 2.85 (d, $J=7.5$ Hz, 2H), 3.49 (br s, 1H), 3.64 (br s, 1H), 3.71–3.98 (m, 2H), 4.53 (br s, 1H), 5.16 (d, $J=9.0$ Hz, 1H), 5.23 (s, 2H), 7.00–7.37 (m, 10H), 7.82 (s, 1H), 8.78 (s, 1H); TOF-MS: m/z 526 $[M+H]^+$. Anal. calcd for $C_{28}H_{35}N_3O_5S$: H, 6.71; C, 63.98; N, 7.99. Found: H, 6.74; C, 63.78; N, 7.80.

(2*S*,3*S*,5*S*)-3-[*O*-[*N*-[*N*-methyl-*N*-[(2-isopropyl-4-thiazolyl)methyl]amino]carbonyl]-L-valyl]oxy]-5-[*N*-[(tert-butyloxy)carbonyl]amino]-2-[*N*-[(5-thiazolyl)methoxy]carbonyl]amino]-1,6-diphenyl-3-hydroxyhexane] 30. To a solution of compound **28** (600 mg, 1.14 mmol) and *N*-[*N*-methyl-*N*-[(2-isopropyl-4-thiazolyl)methyl] amino]-carbonyl]-L-valine **29**[44–46] (537 mg, 1.71 mmol) in CHCl3 (2 mL) were added DMAP (27 mg, 0.22 mmol) and DCC (400 mg, 1.94 mmol) at 0 °C and the reaction mixture was stirred overnight at room temperature. After the reaction mixture was concentrated under reduced pressure, the resulting residue was dissolved in EtOAc, washed sequentially with 5% citrate, 5% $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated in vacuo. The residue was recrystallized from hexane to give 250 mg (27%) of the title compound as a solid: mp 72–76 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.01 (d, $J=6.9$ Hz, 3H), 1.07 (d, $J=6.9$ Hz, 3H), 1.36 (s, 15H), 1.68 (m, 2H), 2.28 (dq, $J=6.9$, 11.2 Hz, 1H), 2.72 (br s, 4H), 2.95 (s, 3H), 3.25 (heptalet, $J=6.9$ Hz, 1H), 3.70–4.60 (m, 6H), 4.90–5.28 (m, 4H), 6.41 (br s, 1H), 6.97 (s, 1H), 7.00–7.40 (m, 10H), 7.78 (s, 1H), 8.77 (s, 1H); TOF-MS: m/z 844 $[M+Na]^+$. Anal. calcd for $C_{42}H_{56}N_6O_7S_2$: H, 6.87; C, 61.44; N, 10.24. Found: H, 6.92; C, 61.21; N, 10.06.

(2*S*,3*S*,5*S*)-5-amino-3-[*O*-[*N*-[*N*-methyl-*N*-[(2-isopropyl-4-thiazolyl)methyl]amino]carbonyl]-L-valyl]oxy]-2-[*N*-[(5-thiazolyl)methoxy]carbonyl]amino]-1,6-diphenyl-3-hydroxyhexane] hydrochloride (Ritonavir prodrug) 12. To compound **30** (200 mg, 0.24 mmol) was added anisole (0.15 mL) and 4 N-HCl/dioxane (2 mL) and the reaction mixture was stirred for 3 h at 0 °C. After the reaction mixture was concentrated in vacuo, ether was added to form precipitate at 0 °C. The resulting precipitate was applied to a silica gel column (2×18 cm) and eluted with EtOAc–MeOH (10:1) to give 148.5 mg (81%) of the title compound **12** as a powder: mp 121–125 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92–1.05 (m, 6H), 1.30 (d, $J=6.9$ Hz, 6H), 1.68–1.83 (m, 1H), 1.89–2.03 (m, 1H), 2.18–2.32 (m, 1H), 2.67–3.07 (m, 5H), 3.23 (heptalet, $J=6.9$ Hz, 1H), 3.32–3.52 (m, 1H), 3.90–4.07 (m, 2H), 4.40–4.58 (m, 2H), 4.92–5.37 (m, 11H), 6.68 (d, $J=7.0$ Hz, 1H), 7.06–7.30 (m, 11H), 7.42 (d, $J=9.3$ Hz, 1H), 7.85 (s, 1H), 8.16 (br s, 3H), 9.10 (s, 1H); TOF-MS: m/z 722 $[M+H]^+$. Anal. calcd for $C_{37}H_{49}ClN_6O_5S_2 \cdot 14/3H_2O$: H, 6.99; C, 52.81; N, 9.98. Found: H, 6.56; C, 52.86; N, 9.61.

Water-solubility of prodrugs and parent compounds

Water-solubility of the prodrugs and parent compounds was determined by HPLC analysis. HPLC was performed using C18 reverse phase column (YMC AM-302 ODS; 4×150 mm) with a binary solvent system at a flow rate of 1 mL/min, detected at UV 230 nm. An excessive amount of the prodrugs or the parent drugs was suspended in pure water under supersonification for 5 min at rt, and filtered by centrifugal membrane filtration of 0.45 μm pore size. Water-solubility was calculated from each peak area of the above solution by HPLC compared with the sample, the concentration of which is already known. The conversion of prodrugs to the parent drugs was not observed during the experiment, since the compound solution was slightly acidic due to an HCl salt.

Determination of the reaction rate of *O*→*N* acyl migration of synthetic prodrugs

The migration rate of prodrugs of KNI-compounds and ritonavir determined by RP-HPLC. 5 μL of methanol solutions (1 mg/mL) of prodrugs was poured into 500 μL PBS buffer (pH 4.9, 5.5, 7.4, 8.0) and incubated at 37 °C. At different points of time, 200 μL of the mixture was analyzed directly by HPLC.

HPLC was performed using C18 reverse-phase column (YMC AM-302 ODS; 4×150 mm) with a binary solvent system at a flow rate of 1 mL/min, detected at UV 230 nm. Prodrugs **3**–**7** were flowed out by linear gradient of MeCN 25–80% (15 min) and prodrugs **8**–**11** were flowed out by linear gradient of MeCN 30–100%(15 min) in 0.1% aqueous TFA. Because ritonavir prodrug and it's parent compound showed similar retention times under the above eluent condition, ritonavir prodrug was flowed out by linear gradient of MeCN 40–80% (15 min) in 0.1 M ammonium acetate buffer (pH 7.5). Half-life of prodrugs was calculated from peak areas at each incubation time.

HIV-1 PR inhibition

HIV-1 PR inhibitory activity of prodrugs **3**, **4**, **8**, **9** and **12** was determined based on the previously reported

method[34,48,49] using a substrate S10[50–52] [H-Lys-Ala-Arg-Val-Tyr-Phe(*p*-NO_2)-Glu-Ala-Nle-NH_2] and recombinant HIV-1 PR (NY5-type sequence).

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