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Design, Synthesis, and Biological Evaluation of Anti-HIV Double-Drugs: Conjugates of HIV Protease Inhibitors with a Reverse Transcriptase Inhibitor through Spontaneously Cleavable Linkers

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Abstract—Based on the prodrug concept as well as the combination of two different classes of anti-HIV agents, we designed and synthesized a series of anti-HIV double-drugs consisting of HIV protease inhibitors conjugated with a nucleoside reverse transcriptase inhibitor in an effort to enhance the antiviral activity. For the conjugation, a series of linkers that conjoins the two different classes of inhibitors has been investigated. Double-drugs using a succinyl amino acid linker were shown to release the parent drugs via spontaneous imide formation at a faster rate compared to compounds using a glutaryl amino acid linker, as expected from the energetically favorable cyclization to the five-membered ring. Among the double-drugs, KNI-1039 (**3b**) with a glutaryl-glycine linker exhibited extremely potent anti-HIV activity compared with that of the individual components. Double-drug **3b** was relatively stable in culture medium, whereas it regenerated active species in cell homogenate. These results suggested that the synergistic enhancement of anti-HIV activities of **3b** may be due to their ability to penetrate into the target cell and subsequent regeneration of two different classes of anti-HIV agents in the cytoplasm.

Introduction

HIV protease (HIV PR) and reverse transcriptase (RT) are major targets for the prevention of HIV proliferation, and inhibitors of these enzymes are widely employed for the chemotherapy of AIDS.[1–3] In order to maintain the efficient antiviral effect and to prevent emergence of a drug-resistant virus, a combination of HIV PR inhibitors and RT inhibitors has become the clinical practice.[4–7] We have studied the structure–activity relationship of dipeptide-based HIV PR inhibitors.[8] Among them, we have obtained several HIV PR inhibitors containing a carboxyl group, which exhibited potent HIV PR inhibition but poor antiviral activity.[8–10]

Bilello et al.[11] have reported that the antiviral efficacy of HIV PR inhibitors depends not only on the enzyme inhibitory activity, but also on their intracellular concentrations closely related to the membrane permeability.[12] The poor antiviral activities of PR inhibitors containing a carboxyl group were probably due to their insufficient cell membrane permeability caused by the presence of the carboxyl group. To improve antiviral activity, we have suggested 'double-drug' strategy that combined an HIV PR inhibitor and a nucleoside RT inhibitor (RTI) in a single molecule.[13–15] The following advantages were envisaged for this strategy: (1) the undesirable physicochemical property such as low membrane permeability would be improved. (2) The conjugation of HIV PR inhibitors with the nucleoside RT inhibitor may facilitate the penetration through the biological membrane mediated by the nucleoside transporter.[16–18] In addition, the nucleosides have the affinity to cell membranes.[19] (3) Once the double-drug escapes extracellular hydrolysis, it would enter the cell wherein the intracellular hydrolysis regenerates the parent inhibitors. They can act on two separate targets and exhibit synergistic anti-HIV efficacy. Based on these premises,

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we had developed potent hybrid-type anti-HIV agents,[13–15] in which the carboxyl group of HIV protease inhibitors was directly esterified with the 5′-hydroxyl group of a nucleoside RT inhibitor, 3′-azido-3′-deoxythimidine (AZT).[20] The anti-HIV activities of these hybrid-type prodrugs were found to be more potent than those of AZT and the parent HIV PR inhibitors. These results suggested that the 'double-drug' would be useful to enhance the anti-HIV efficacy synergistically and improve the physicochemical characteristics. However, this double-drug strategy using direct-esterification was only applicable to the inhibitors containing a carboxyl group.

We have applied the 'double-drug' concept to the dipeptide HIV PR inhibitor KNI-727 (**1**),[8–10] which is devoid of a carboxyl group (Fig. 1). Inhibitor (**1**) exhibited excellent HIV PR inhibitory activity but poor anti-HIV activity. The poor water solubility and the high lipophilic nature of **1** might restrict its penetration across the cell membrane, thus resulting in poor anti-HIV activity. To enhance the anti-HIV activity and improve the physicochemical characteristics of inhibitor **1**, we designed and synthesized the hybrid-type prodrugs of KNI-727 (**1**) conjugated with AZT (Fig. 1).

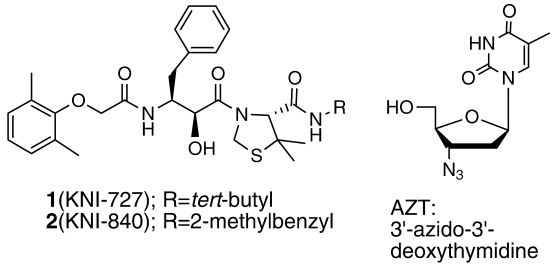
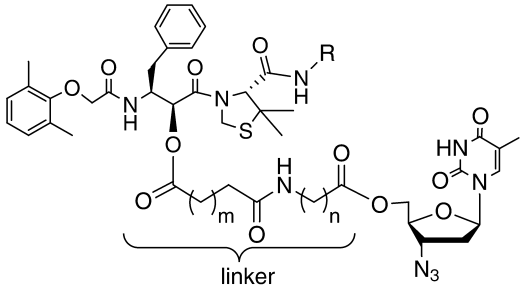


Figure 1. Structure of HIV protease inhibitors (HIV PRI) **1**, **2,** and the nucleoside reverse transcriptase inhibitor (RTI) AZT.



3a; m=1, n=1, R= *tert*-butyl
3b; m=2, n=1, R= *tert*-butyl
3c; m=1, n=2, R= *tert*-butyl
3d; m=2, n=2, R= *tert*-butyl
3e; m=1, n=1, R=2-methylbenzyl
3f; m=2, n=1, R=2-methylbenzyl
3g; m=1, n=2, R=2-methylbenzyl
3h; m=2, n=2, R=2-methylbenzyl

Figure 2. Structure of double-drugs containing spontaneously cleavable linkers.

HIV PR inhibitor **1** contains the hydroxyl group, which is essential for its enzyme inhibitory activity,[8] and nucleoside RT inhibitor AZT also contains the hydroxyl group. Therefore, we connected both the hydroxyl groups of HIV PR inhibitor **1** and RT inhibitor (AZT) through the spontaneously cleavable linker (Fig. 2).

The anti-HIV activities of the resulting double-drugs were found to be more potent than those of AZT and KNI-727 (**1**).[21] These results suggested that the prodrugs would penetrate the cell membrane and exhibit synergistic anti-HIV activity (Fig. 3). In order to confirm whether the double-drugs regenerate the parent drugs inside the cell, the disintegration profile of the prodrugs was determined in various media such as cell homogenate and cell culture. Several disintegrated products were characterized and the in vitro anti-HIV efficacy was evaluated. Furthermore, we synthesized the conjugates of AZT and an HIV PR inhibitor KNI-840 (**2**), which showed more potent HIV PR inhibitory activity compared with inhibitor **1**. In this paper, we describe the design, synthesis and biological evaluation of these anti-HIV double-drugs.

Conjugation Strategy

For the conjugation, a series of linkers connecting the hydroxyl group of KNI-727 (**1**) and AZT was investigated. At first, we incorporated succinic acid as a linker, but the resulting compound **4** (KNI-935) (Fig. 4) was ineffective (EC_{50} = 188.9 nM, HIV-1_{IIIB}/CEM-SS) in the antiviral cell culture assay. Since the succinyl ester linkage was stable towards enzymatic cleavage,[22] the parent drugs were not regenerated inside the cell, and hence the anti-HIV activity was poor.

The essential criteria in the design of prodrugs are that the prodrugs should be stable outside the target cell and after the penetration across the cell membrane, it should regenerate the parent inhibitors.[23] Based on these premises, we studied a series of linkers having the ability to release the parent drugs spontaneously in the physiological environment (Fig. 3).

The intramolecular displacement of benzyl alcohol in β-benzyl-aspartyl peptide with the concomitant formation

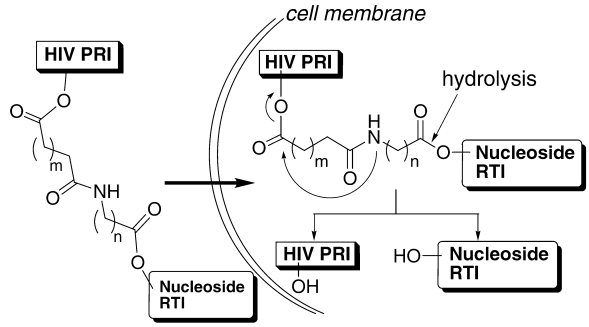


Figure 3. Concept of anti-HIV double-drugs containing spontaneously cleavable linkers.

of aspartylimide derivatives is one of the frequent reactions in peptide synthesis.[24,25] Therefore we tried to utilize this reaction to the design of spontaneously cleavable linker, and employed succinyl amino acid or glutaryl amino acid as a linker. Under mild alkaline conditions, these linkers were expected to release the parent drug KNI-727 (**1**) via intramolecular nucleophilic attack by the amide nitrogen with an imide formation (Fig. 5, path A).[26] The resulting imide fragment would be hydrolyzed and AZT be released. In order to vary the rate of drug release, prodrugs with various linkers such as **3a**–**3d** were synthesized and the stability was analyzed.

Chemistry

Inhibitor **1** was prepared according to the method described previously.[10] Scheme 1 illustrates the synthetic procedure of intermediates **8a,b**, disintegrated product **7b** and KNI-935 (**4**). Compound **1** was coupled with succinic anhydride or glutaric anhydride in THF–ether (1:2) in the presence of dicyclohexylamine (DCHA) to yield the half-ester **8a,b**. Condensation of **8b** and *tert*-butyl ester of glycine by use of benzotriazole-1-yloxy-tris (dimethylamino) phosphonium hexafluorophosphate/1-hydroxybenzotriazole (BOP/HOBt) in the presence of triethylamine, and the following deprotection by trifluoroacetic acid (TFA) afforded **7b**. Compound **4** was prepared by condensation of **8a** and AZT by use of 1,3-dicyclohexylcarbodiimide/dimethylaminopyridine (DCC/DMAP).

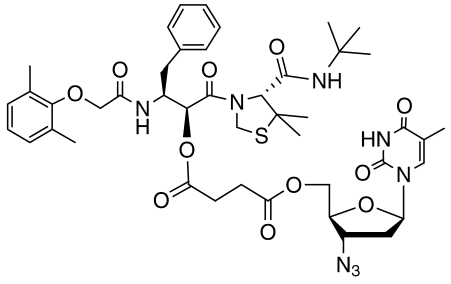


Figure 4. Structure of the conjugate **4** (KNI-935) using succinic acid linker.

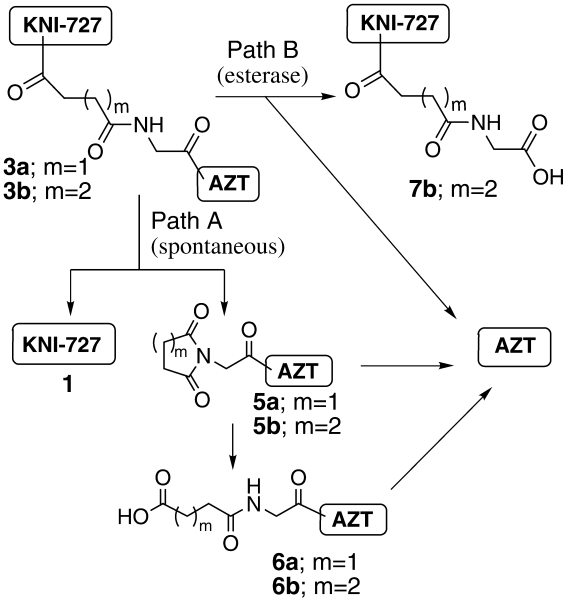


Figure 5. Disintegration pathway of double-drugs to parent inhibitors.

Prodrugs **3a**–**3d** were synthesized as shown in Scheme 2. AZT was coupled with Boc-Gly-OH or Boc-β-Ala-OH using DCC in the presence DMAP, and the following deprotection by 4 N HCl/dioxane afforded **9a,b**. Condensation of **9a,b** with **8a,b** using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC·HCl) in the presence of HOBt resulted in the prodrugs **3a**–**3d**, which were purified by silica gel column chromatography and preparative HPLC using binary solvent system (linear gradient of CH_3CN in 0.1% aqueous TFA). Disintegration products **6a,b** were prepared by coupling **9a** with succinic anhydride or glutaric anhydride in the presence of triethylamine.

Disintegration product **5a** was prepared as shown in Scheme 3. Condensation of compound **10**, which was obtained by the treatment of succinic anhydride with benzyl alcohol, with **9a** afforded compound **11**. Compound **11** was stirred in 50% DMSO in phosphate buffer (pH 7.4) in the presence of triethylamine at 40 °C. The intramolecular cyclization reaction occurred to afford imide **5a**.

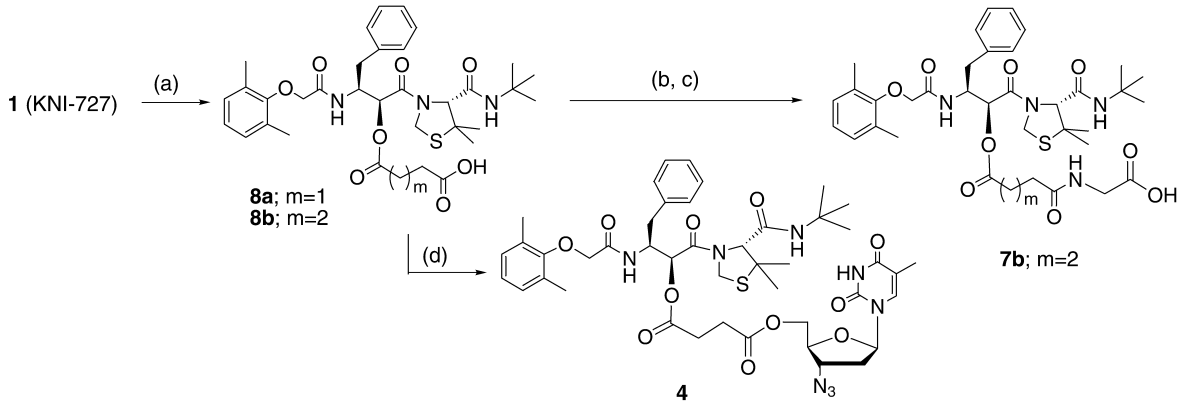
Scheme 4 illustrates the synthetic procedure of prodrugs **3e**–**3h** consisting of another dipeptide HIV PR inhibitor KNI-840 (**2**) and AZT. Dipeptide 2-methylbenzylamide derivative **12** (Apns-Dmt-NH-(2-methyl)Bzl·HCl; Apns = AHPBA = (2*S*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid; Dmt = L-dimethylthioproline = L-1,3-thiazolidine-4-carboxylic acid) and 2,6-dimethylphenoxyacetic acid were prepared according to the methods described previously.[10] Condensation of **12** with 2,6-dimethylphenoxyacetic acid using the EDC-HOBt method gave **2**. The resulting inhibitor **2** was converted to the prodrugs **3e**–**3h** in a manner similar to that described for **3a**–**3d**. All the prodrugs **3e**–**3h** were finally purified by preparative HPLC.

Results and Discussion

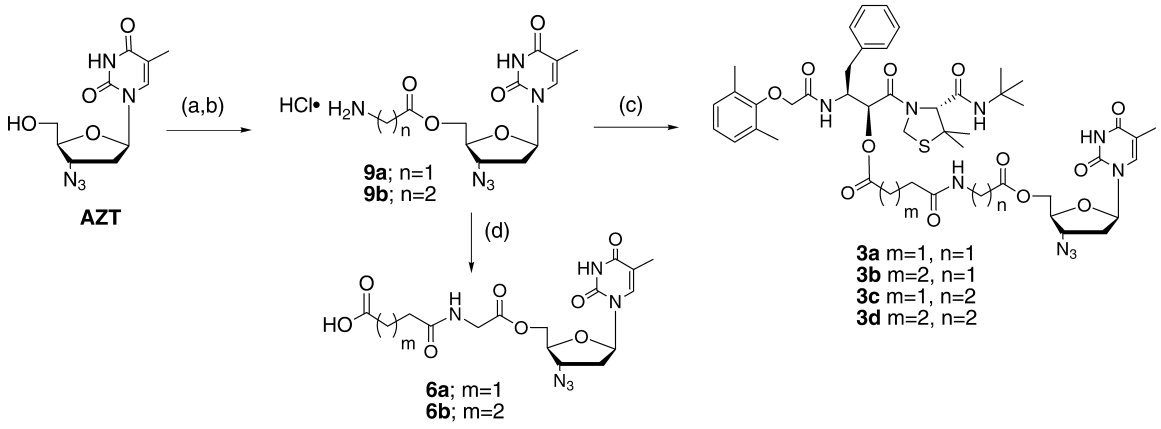
Disintegration studies of prodrugs

The prodrugs were designed to release the parent drug KNI-727 (**1**) spontaneously via intramolecular cyclization through an imide formation. However, there is a possibility of another disintegration pathway such as simple hydrolysis of the ester bond. Therefore, the disintegration profiles in various media were determined by HPLC (Fig. 5).

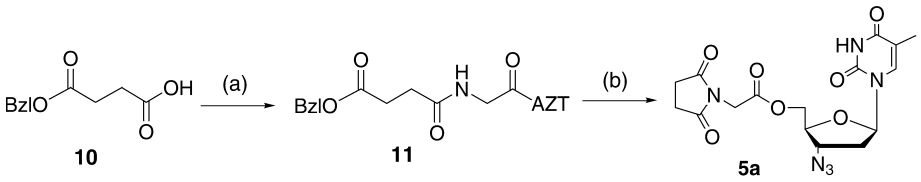
In phosphate buffered saline (pH 7.4, 37 °C), the spontaneous imide formation with the release of KNI-727 (**1**) and several other intermediates was observed for all the prodrugs. The typical HPLC chromatogram and a



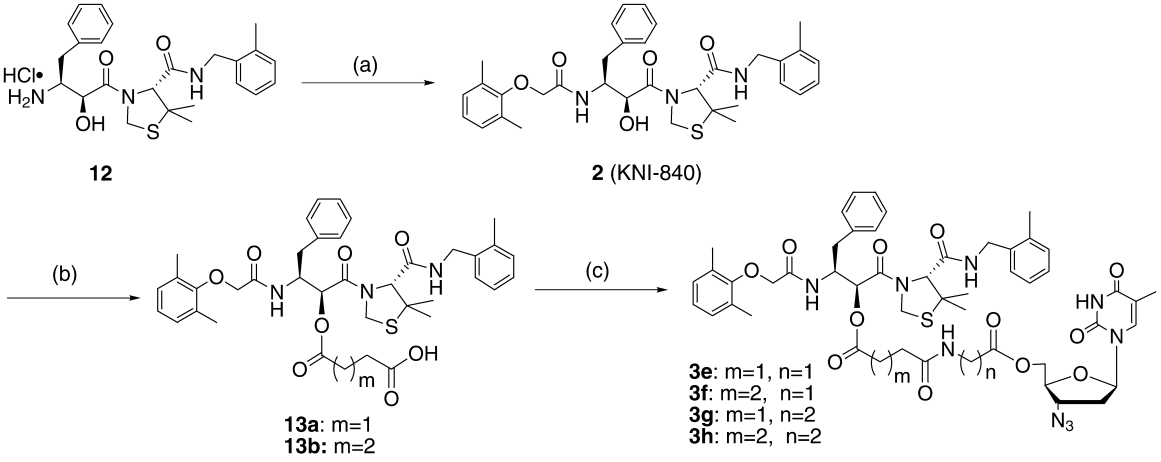
Scheme 1. Reagents and conditions: (a) succinic anhydride or glutaric anhydride, DCHA, THF–ether (1:2); (b) HCl·H-Gly-OBu^t, Et_3N, BOP, HOBt, DMF; (c)TFA; (d) AZT, DCC, DMAP, DMF.



Scheme 2. Reagents and conditions: (a) Boc-Gly-OH or Boc- β-Ala-OH, DCC, DMAP, DMF; (b) 4 N HCl/dioxane; (c) **8a** or **8b,** EDC·HCl, HOBt, DMF; (d) succinic anhydride or glutaric anhydride, Et_3N, DMF.



Scheme 3. Reagents and conditions: (a) **9a**, Et_3N, EDC·HCl, HOBt, DMF; (b) Et_3N, DMSO, phosphate buffer, 40 °C.



Scheme 4. Reagents and conditions: (a) 2,6-dimethylphenoxyacetic acid, Et_3N, HOBt, EDC·HCl, DMF; (b) succinic anhydride or glutaric anhydride, DCHA, THF–ether (1:2); (c) **9a** or **9b**, Et_3N, HOBt, BOP, DMF.

kinetic profile for the disintegration of prodrug **3a** are shown in Figures 6 and 7, respectively. All of the peaks in the chromatograms were unambiguously assigned by mass spectrometry. At first, prodrug **3a** released compound **1** through the imide formation, and the resulting **5a** disintegrated to AZT directly or indirectly via **6a** (path A, Fig. 5).

The drug release rate varied depending on the linkers (Table 1). The rate was indicated as the $t_{1/2}$, which is the time required for 50% release of KNI-727 (**1**) in PBS (pH 7.4, 37 °C). Prodrug **3a** conjugated by the succinylglycine linker had the faster releasing rate ($t_{1/2} = 0.7$ h) compared with **3b** (KNI-1039) containing glutarylglycine linker ($t_{1/2} = 12$ h), as expected from energetically favorable cyclization to the five-membered ring. The $t_{1/2}$ of the prodrugs (**3c, 3d**) containing β-alanine in the linker were longer than those of prodrugs (**3a, 3b**) containing glycine.

In order to confirm whether the double-drugs regenerate the parent drugs inside the cell, the disintegration behavior of the KNI-1039 (**3b**), which exhibited potent anti-HIV activity, was examined in a culture medium (RPMI-1640 containing 10% of heat-inactivated fetal calf serum) and a Molt-4 cell homogenate. In the culture medium, the prodrug **3b** disintegrated through path A and the kinetic profile was approximately the same as in PBS, pH 7.4 (Fig. 8A). In Molt-4 cell homogenate, the disintegration pattern of **3b** was different from that observed in PBS, and compound **7b**, KNI-727 (**1**) and AZT were observed (Fig. 8B). In this medium, the esterase-mediated hydrolysis (path B) as well as spontaneous cleavage (path A) as shown in Figure 5 would occur. The resulting compound **7b** was stable, and was not converted to the parent drug **1**.

In order to examine the sensitivity of the KNI-1039 (**3b**) to the esterase-mediated hydrolysis, the stability in the presence of carboxyesterase was determined. Porcine liver esterase (PLE) was used in our study, since this esterase has been widely used in similar studies and also the results correlated well with those of plasma studies.[15,27–29] The prodrug **3b** was completely hydrolyzed within 2 h resulting in compound **7b** and AZT, but the parent drug KNI-727 (**1**) was not regenerated. Consequently, the ester linked to AZT would be readily hydrolyzed, but that of KNI-727 (**1**) was resistant to hydrolysis with the esterase. These results suggested that prodrug KNI-1039 (**3b**) would regenerate the parent drug KNI-727 (**1**) through the spontaneous intramolecular

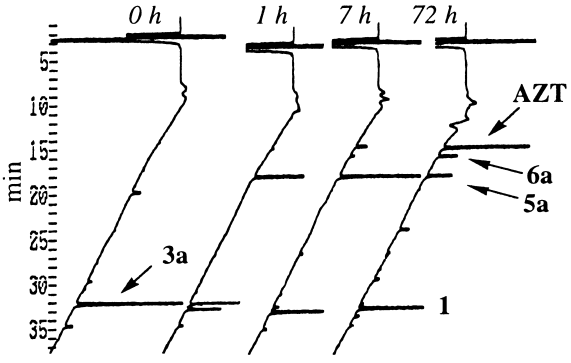


Figure 6. Typical HPLC chromatograms of the disintegration of prodrug **3a** in PBS (pH 7.4, 37 °C), at 0, 1, 7 h and 72 h.

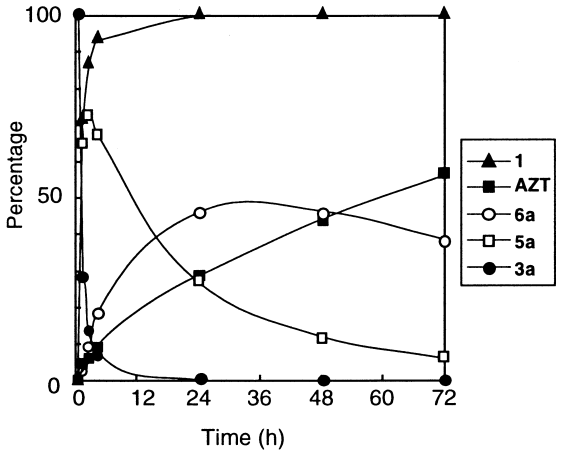


Figure 7. Kinetic profile of disintegration of prodrug **3a** and the appearance of parent drugs in PBS, pH 7.4 at 37 °C.

Table 1. Biological activities and disintegration rate of 'double-drugs'

Compound	m	n	R	HIV protease inhibition (%)[a]	EC_{50}HIV-1_{LAI} Molt-4 (nM)	Relative potency	$t_{1/2}$[b] (h)
3a (KNI-1038)	1	1	*tert*-Butyl	37	5.3	1.2	0.7
3b (KNI-1039)	2	1	*tert*-Butyl	55	0.1	62	12
3c (KNI-1046)	1	2	*tert*-Butyl	7	1.0	6.2	1.9
3d (KNI-1047)	2	2	*tert*-Butyl	12	3.4	1.8	20
3e	1	1	2-Methylbenzyl	38	11.4	0.5	–
3f (KNI-1043)	2	1	2-Methylbenzyl	15	0.8	7.8	–
3g	1	2	2-Methylbenzyl	7	23.1	0.3	–
3h	2	2	2-Methylbenzyl	28	30.0	0.2	–
AZT	—	—	—	—	6.2	1.0	–
1 (KNI-727)	—	—	—	100 (92)	92.0	0.07	–
2 (KNI-840)	—	—	—	100 (97)	168.9	0.04	–

[a]% of HIV-1 protease inhibition in the presence of 5 μM or 50 nM (in parentheses) of inhibitors.
[b]$t_{1/2}$ is the time required for 50 release of parent drug (**1**) at 37 °C in phosphate buffered saline (pH 7.4).

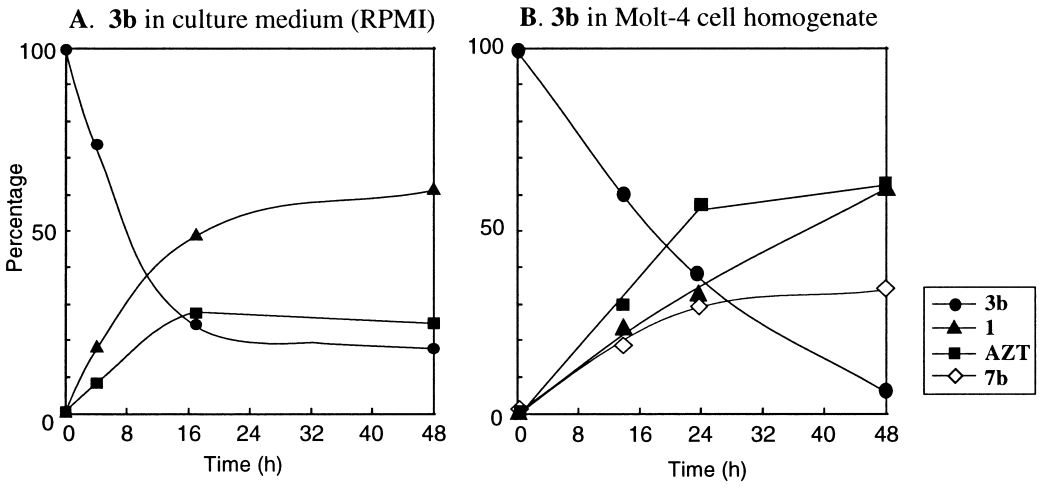


Figure 8. Disintegration profiles of prodrug **3b** (KNI-1039) in cell culture medium (RPMI-1640 containing 10% of heat-inactivated fetal calf serum) and Molt-4 cell homogenate at 37 °C.

cyclization reaction, and AZT by the hydrolysis inside the cell.

Biological activities

The enzyme inhibitory potencies of these prodrugs against HIV PR and RT were evaluated as reported previously.[10,30] All of the prodrugs **3a**–**3h** exhibited no HIV-1 RT inhibitory activity and poor HIV PR inhibitory activity, whereas the parent HIV PR inhibitor KNI-727 (**1**) showed potent enzyme inhibition. Since both of the hydroxyl groups of the HIV PR inhibitor and the RT inhibitor are essential for their enzyme inhibitory activity,[8,31] masking the hydroxyl moieties of these inhibitors results in poor enzyme inhibitory activity.

The anti-HIV activities of the prodrugs were evaluated against HIV-1 strain LAI in Molt-4 cells.[32] All of the prodrugs **3a**–**3d** showed more potent anti-HIV activity than that of the parent inhibitor KNI-727 (**1**). Interestingly, the anti-HIV activities of prodrugs seemed to be affected by the structure of linkers. We suppose that the differences of the activities were due to the stability of the prodrugs. Prodrug **3a** (KNI-1038) containing a succinylglycine linker showed a similar antiviral activity to that of AZT, which might be due to the instability of compound **3a** ($t_{1/2}=0.7$ h). Prodrug **3a** might considerably disintegrate outside the cell. On the other hand, prodrug **3b** (KNI-1039) containing glutarylglycine linker exhibited remarkable anti-HIV activity ($EC_{50}=0.1$ nM, therapeutic index >2000) that was 920 and 62 times more potent than those of KNI-727 (**1**) and AZT, respectively. These results suggested that the conjugates would penetrate the cell membrane and then the two classes of enzyme inhibitors were generated, which could attack different targets in the infected cell, thus exhibiting synergistic anti-HIV activity. The $t_{1/2}$ of **3c** was longer than that of **3a**, and the anti-HIV activity of **3c** was 5 times more potent than that of **3a**. Prodrug **3d** exhibited longer half-life ($t_{1/2}=20$ h), but its antiviral activity was poor. This is probably due to the high stability of **3d**, which prevents the disintegration to its parent compounds.

As mentioned above, in Molt-4 homogenate, prodrug KNI-1039 (**3b**) disintegrated via spontaneous cleavage (path A) as well as esterase-mediated hydrolysis (path B). Therefore, the disintegrated compounds **5a, 6b** and **7b** were synthesized and evaluated for biological activity. It was difficult to synthesize **5b,** which is the disintegration material of prodrug **3b**. Therefore, **5a** was used instead of **5b**. All the compounds except for **7b** did not show the RT and HIV PR inhibitory activity. Compound **7b** exhibited moderate HIV PR inhibitory activity (5 μM inhibition = 99%, 50 nM inhibition = 17%), but the antiviral activity was low ($EC_{50}=3800$ nM). The antiviral activity of **5a** was almost the same ($EC_{50}=13$ nM) as that of AZT ($EC_{50}=15$ nM),[33] and **6b** was 2 times less potent ($EC_{50}=34$ nM) than AZT. These results support our hypothesis, that is the prodrug KNI-1039 (**3b**) penetrates across the cell membrane and then the regenerated active species inhibit different targets with synergistic effect. The antiviral efficacy of prodrugs depends on many factors, such as enzyme inhibition, cell membrane permeability, extracellular stability and intracellular disintegration, and the correlation between them is very complicated.

We also applied this double-drug strategy to another dipeptide HIV PR inhibitor KNI-840 (**2**), which exhibited more potent HIV PR inhibitory activity (50 nM inhibition = 97%) compared with KNI-727 (**1**) but poor antiviral activity (Table 1). Prodrugs **3e**–**3h** consisting of KNI-840 (**2**) and AZT showed better antiviral activity than parent HIV PR inhibitor **2**, but less potent than AZT except for **3f**. Prodrug **3f** containing a glutarylglycine linker exhibited the most potent anti-HIV activity in **3e**–**3h**, and the activity was 7.8 times more potent than that of AZT.

Conclusion

Based on the 'double-drug' strategy, we synthesized and evaluated a series of prodrug-type anti-HIV agents conjugating HIV PR inhibitors to a nucleoside-type RT

inhibitor using spontaneously cleavable linkers. Among the prodrugs studied, KNI-1039 (**3b**), a 'double-drug' of an HIV PR inhibitor KNI-727 (**1**) conjugated with a RT inhibitor AZT by a glutarylglycine linker, exhibited remarkable anti-HIV activity, which was 920 and 62 times more potent than those of KNI-727 (**1**) and AZT, respectively. Prodrug **3b** was relatively stable in culture medium, whereas it regenerated active species in cell homogenate. These results suggested that the double-drug KNI-1039 (**3b**) penetrates across the cell membrane and then the regenerated active species inhibit different targets with synergistic effect. We believe that this 'double-drug' approach in the field of medicinal chemistry would pave the way for the development of more potent drugs.

Experimental

Reagents and solvents were used as purchased from Wako Pure Chemical Ind Ltd. (Osaka, Japa), nacalai tesque (Kyoto, Japan), and Aldrich Chemical Co. Inc (Milwaukee, WI) without further purification. Column chromatography was performed on Merck 107734 silica gel 60 (70–230 mesh). TLC was performed using Merck Silica gel $60F_{254}$ precoated plates. Melting points were measured on a Yanagimoto micro melting apparatus without correction. 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 (0–100%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min, detected at UV 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20×250 mm; YMC Pack ODS SH343-5) with binary solvent system: linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 5.0 mL/min, detected at UV 230 nm. ^{1}H NMR spectra were obtained on a JEOL 300 MHz or Varian 400 MHz spectrometer with TMS as an internal standard. FAB–MS was performed on a JEOL JMS-SX102A spectrometer equipped with the JMA-DA7000 data system. Optical rotation was determined with a Horiba SEPA-300 polarimeter.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-(2,6-dimethylphenoxyacetyl)-amino-2-succinyloxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (2,6-dimethylphenoxyacetyl-Apns(succinyl)-Dmt-NHBu^t, 8a). To the solution of protease inhibitor **1** (KNI-727) (500 mg, 0.90 mmol) in THF–ether (1:2) were added succinic anhydride (108 mg, 1.08 mmol) and DCHA (0.215 mL, 1.08 mmol), and stirred for 18 h at room temperature. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (30 mL), washed with 10% citric acid and saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Purification of the product by silica gel column chromatography ($CHCl_3$–MeOH) and reprecipitation from *n*-hexane gave 440 mg of the title compound as a white solid. Yield 75%: mp 108–112 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.23 (d, J = 8.6 Hz, 1H), 7.64 (s, 1H), 7.38 (d, J = 6.8 Hz, 2H), 7.28–7.18 (m, 3H), 7.00 (d, J = 7.7 Hz, 2H), 6.94–6.89 (m, 1H), 5.34 (d, J = 3.9 Hz, 1H), 5.08 (d, J = 8.6 Hz, 1H), 4.90 (d, J = 8.6 Hz, 1H), 4.52 (s, 1H), 4.50 (br m, 1H), 4.21 (d, J = 14.1 Hz, 1H), 3.99 (d, J = 14.1 Hz, 1H), 2.98–2.89 (m, 2H), 2.75–2.55 (m, 4H, partially covered by DMSO peaks), 2.14 (s, 6H), 1.47 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); MS (FAB) m/z = 656 $[M+H]^+$. Anal. calcd for $C_{34}H_{41}N_3O_5S$: C, 62.27; H, 6.92; N, 6.41. Found: C, 61.98; H, 6.94; N, 6.37.

(*R*)-*N*-*tert*-Butyl-3-[(2*S*,3*S*)-3-(2,6-dimethylphenoxyacetyl)-amino-2-glutaryloxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (2,6-dimethylphenoxyacetyl-Apns(glutaryl)-Dmt-NHBu^t, 8b). Compound **8b** was prepared from glutaric anhydride and compound **1** in a manner similar to that described for compound **8a**. Yield 60%: mp 112–113 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.12 (d, J = 9.0 Hz, 1H), 7.66 (s, 1H), 7.35 (d, J = 6.6 Hz, 2H), 7.25–7.15 (m, 3H), 7.01–6.89 (m, 3H), 5.36–5.33 (m, 1H), 4.97 (d, J = 8.7 Hz, 1H), 4.93 (d, J = 8.7 Hz, 1H), 4.52 (s, 1H), 4.48 (br m, 1H), 4.18 (d, J = 14.1 Hz, 1H), 3.97 (d, J = 14.1 Hz, 1H), 2.91–2.88 (m, 2H), 2.77–2.74 (m, 2H), 2.30–2.20 (m, 4H), 2.13 (s, 6H), 1.48 (s, 3H), 1.46 (s, 3H), 1.26 (s, 9H); HRMS (FAB): m/z 670.3170 for $[M+H]^+$ (calcd 670.3162 for $C_{35}H_{48}N_3O_8S$).

2,6-Dimethylphenoxyacetyl-Apns(glutaryl-Gly-OH)-Dmt-NHBu^t (7b). To a solution of **8b** (100 mg, 0.149 mmol) in DMF (2 mL) was added HCl·H-Gly-OBu^t (25 mg, 0.149 mmol), triethylamine (50 μL, 0.358 mmol), HOBt (46 mg, 0.298 mmol) and BOP (93 mg, 0.209 mmol) at 0 °C, and the mixture was stirred for 18 h at rt. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (30 mL), washed with 10% citric acid and saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$–MeOH) and reprecipitated from *n*-hexane. The product was redissolved in trifluoroacetic acid (2 mL) and stirred for 1 h. The reaction mixture was concentrated in vacuo, and the residue was dissolved in EtOAc (30 mL), washed with saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The product was obtained as a colorless oil (51 mg). Yield 47%: TLC R_f 0.10 ($CHCl_3$:MeOH = 10:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.26 (d, J = 8.7 Hz, 1H), 8.16 (t, J = 6.0 Hz, 1H), 7.67 (s, 1H), 7.38 (d, J = 6.6 Hz, 2H), 7.27–7.16 (m, 4H), 7.01–6.89 (m, 1H), 5.33 (d, J = 3.9 Hz, 1H), 5.11 (d, J = 8.7 Hz, 1H), 4.92 (d, J = 8.7 Hz, 1H), 4.55 (s, 1H), 4.49 (br m, 1H), 4.52 (d, J = 14.1 Hz, 1H), 4.00 (d, J = 14.1 Hz, 1H), 3.72 (d, J = 5.7 Hz, 2H), 2.98–2.84 (m, 2H), 2.45 (t, J = 7.4 Hz, 2H), 2.21 (t, J = 7.4 Hz, 1H), 2.13 (s, 6H), 1.85–1.78 (m, 2H), 1.47 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); MS (FAB): m/z 727 for $[M+H]^+$.

2,6-Dimethylphenoxyacetyl-Apns(succinyl-AZT)-Dmt-NHBu^t (4, KNI-935). To a solution of **8a** (100 mg, 0.152 mmol) in DMF (2 mL) was added AZT (45 mg, 0.167 mmol), DCC (35 mg, 0.167 mmol) and DMAP (4 mg, 0.030 mmol) at 0 °C, and the mixture was stirred for 18 h at rt. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (30 mL), washed with 10% citric acid, 5% $NaHCO_3$ and saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in

vacuo. Purification of the product by silica gel column chromatography ($CHCl_3$–MeOH) and reprecipitation from *n*-hexane gave 52 mg of the title compound as a white solid. Yield 38%: mp 64–65 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.30 (s, 1H), 8.21 (d, J = 8.4 Hz, 1H), 7.64 (s, 1H), 7.45 (d, J = 1.2 Hz, 1H), 7.38 (d, J = 7.2 Hz, 2H), 7.27–7.16 (m, 3H), 7.01–6.89 (m, 3H), 6.14–6.09 (m, 1H), 5.35 (d, J = 3.9 Hz, 1H), 5.08 (d, J = 8.7 Hz, 1H), 4.89 (d, J = 8.7 Hz, 1H), 4.52 (s, 1H), 4.51–4.41 (m, 2H), 4.32–4.15 (m, 3H), 4.01–3.96 (m, 2H), 2.98–2.84 (m, 2H), 2.77–2.64 (m, 4H), 2.45–2.28 (m, 2H), 2.13 (s, 6H), 1.79 (s, 3H), 1.46 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); HRMS (FAB): m/z 905.3880 for $[M+H]^+$ (calcd 905.3868 for $C_{44}H_{57}N_8O_{11}S$).

Gly-AZT-HCl (9a). To a solution of Boc-Gly-OH (175 mg, 1.00 mmol) in DMF (2 mL) were added AZT (267 mg, 1.00 mmol), DCC (206 mg, 1.00 mmol) and DMAP (12 mg, 0.1 mmol) at 0 °C, and the mixture was stirred at rt for 18 h. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (30 mL), washed with 10% citric acid, 5% $NaHCO_3$ and saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Purification of the product by silica gel column chromatography ($CHCl_3$–MeOH) and reprecipitation from *n*-hexane gave the Boc protected title compound as a white solid. The resulting product was redissolved in 4 N HCl–dioxane at 0 °C, and was added anisole (95.3 μL, 0.87 mmol), and the mixture was stirred at rt for 1 h. After removal the solvent in vacuo, the residue was precipitated from ether to give 260 mg of the title compound as a white solid. Yield 79%: mp 80–83 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.37 (s, 1H), 8.52 (br s, 3H), 7.54 (s, 1H), 6.17–6.09 (m, 1H), 4.63–4.56 (m, 1H), 4.46–4.36 (m, 2H), 4.00–3.95 (m, 1H), 3.83 (d, J = 8.4 Hz, 2H), 2.57–2.48 (m, 1H), 2.40–2.30 (m, 1H), 1.82 (s, 3H); HRMS (FAB): m/z 325.1255 for $[M+H]^+$ (calcd 325.1260 for $C_{12}H_{17}N_6O_5$).

β-Ala-AZT.HCl (9b). Compound **9b** was prepared from Boc-β-Ala-OH and AZT in a manner similar to that described for compound **9a**. Yield 89%: mp 77–80 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.37 (s, 1H), 8.04 (br s, 3H), 7.48 (d, J = 0.9 Hz, 1H), 6.15–6.07 (m, 1H), 4.55–4.49 (m, 1H), 4.31–4.30 (m, 2H), 4.01–3.96 (m, 1H), 3.03 (t, J = 7.0 Hz, 2H), 2.77–2.71 (m, 2H), 2.53–2.44 (m, 1H), 2.53–2.44 (m, 1H), 2.39–2.30 (m, 1H), 1.81 (s, 3H); HRMS (FAB): m/z 339.1413 for $[M+H]^+$ (calcd 339.1417 for $C_{13}H_{19}N_6O_5$).

Succinyl-Gly-AZT (6a). To a solution of **9a** in DMF was added triethylamine (0.232 mL, 1.668 mmol) and succinic anhydride (61 mg, 0.62 mmol) at 0 °C, and the mixture was stirred for 18 h at rt. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (50 mL), washed with saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Purification of the product by preparative HPLC gave 108 mg of the title compound as white foam. Yield 46%: TLC R_f 0.31 ($CHCl_3$–MeOH–H_2O = 8:3:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.4 (s, 1H), 8.36 (t, J = 5.7 Hz, 1H), 7.46 (s, 1H), 6.15–6.05 (m, 1H), 4.49–4.43 (m, 1H), 4.36–4.19 (m, 2H), 4.00–3.94 (m, 1H), 3.90–3.86 (m, 2H), 2.46–2.28 (m, 6H), 1.85 (s, 3H); HRMS (FAB): m/z 425.1425 for $[M+H]^+$ (calcd 425.1421 for $C_{16}H_{21}N_6O_8$).

Glutaryl-Gly-AZT (6b). Compound **6b** was prepared from glutaric anhydride and **9a** in a manner similar to that describe for compound **6a**, and obtained as a TFA salt. Yield 37%: TLC R_f 0.17 ($CHCl_3$:MeOH = 10:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.1 (br s, 1H), 11.4 (s, 1H), 8.33 (t, J = 5.8 Hz, 1H), 7.95 (s, 1H), 7.47 (s, 1H), 6.16–6.11 (m, 1H), 4.49–4.42 (m, 1H), 4.39–4.19 (m, 2H), 4.00–3.95 (m, 1H), 3.86 (t, J = 5.4 Hz, 2H), 2.46–2.30 (m, 2H), 2.27–2.14 (m, 4H), 1.80 (s, 3H), 1.74–1.67 (m, 2H); MS (FAB): m/z 438 for $[M+H]^+$.

2,6-Dimethylphenoxyacetyl-Apns(succinyl-Gly-AZT)-Dmt-NHBu^t (3a, KNI-1038). To a solution of **8a** (200 mg, 0.31 mmol) in DMF was added compound **9a** (155 mg, 0.37 mmol), HOBt (98 mg, 0.77 mmol), triethylamine (0.105 mL, 0.64 mmol) and BOP (202 mg, 0.46 mmol) at 0 °C, and the solution was stirred for 18 h at rt. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (50 mL), washed with 5% $NaHCO_3$, 10% citric acid and saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Purification of the product by silica gel column chromatography ($CHCl_3$–MeOH) and preparative HPLC, and reprecipitation from *n*-hexane gave 231 mg of the title compound as a white solid. Yield 78%: mp 113–117 °C; $[\alpha]_D^{32}$ −4.69° (*c* 0.06, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.34 (s, 1H), 8.45 (t, J = 5.6 Hz, 1H), 8.21 (d, J = 8.4 Hz, 1H), 7.64 (s, 1H), 7.45 (s, 1H), 7.38 (d, J = 7.2 Hz, 2H), 7.27–7.16 (m, 3H), 7.00–6.89 (m, 3H), 6.15–6.11 (m, 1H), 5.34 (d, J = 3.6 Hz, 1H), 5.08 (d, J = 8.7 Hz, 1H), 4.89 (d, J = 8.7 Hz, 1H), 4.52 (s, 1H), 4.50–4.41 (m, 2H), 4.32–4.14 (m, 3H), 4.06–3.94 (m, 2H), 3.90–3.87 (m, 2H), 2.98–2.84 (m, 2H), 2.74–2.53 (m, 4H), 2.45–2.28 (m, 2H, partially covered by DMSO peaks), 2.13 (s, 6H), 1.79 (s, 3H), 1.47 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); HRMS (FAB): m/z 962.4090 for $[M+H]^+$ (calcd 962.4068 for $C_{46}H_{60}N_9O_{12}S$). Anal. calcd for $C_{46}H_{59}N_9O_{12}S{\cdot}H_2O{\cdot}CF_3COOH$: C, 52.69; H, 5.71; N, 11.52. Found: C, 52.44; H, 5.74; N, 11.15.

2,6-Dimethylphenoxyacetyl-Apns(glutaryl-Gly-AZT)-Dmt-NHBu^t (3b, KNI-1039). Compound **3b** was prepared from compound **8b** and **9a** in a manner similar to that described for compound **3a**. Yield 25%: mp 94–96 °C; $[\alpha]_D^{32}$ −4.77° (*c* 0.06, MeOH); ^{1}H NMR (400 MHz, DMSO- d_6) δ 11.43 (s, 1H, AZT-NH), 8.33 (t, J = 5.9 Hz, 1H, Gly-NH), 8.24 (d, J = 8.6 Hz, 1H, AHPBA-NH), 7.66 (S, 1H, *tert*-butyl-NH), 7.44 (d, J = 1.1 Hz, 1H, AZT-6H), 7.37 (d, J = 7.0 Hz, 2H, aromatic), 7.25–7.15 (m, 3H, aromatic), 6.98 (d, J = 7.1 Hz, 2H, aromatic), 6.92–6.88 (m, 1H, aromatic), 6.13–6.10 (m, 1H, AZT-1′-H), 5.31 (d, J = 3.7 Hz, 1H, AHPBA-2-CH), 5.09 (d, J = 8.9 Hz, 1H, phenoxyacetyl-2-CH_2), 4.90 (d, J = 8.9 Hz, 1H, phenoxyacetyl-2-CH_2), 4.53 (s, 1H, Dmt-4-CH), 4.51–4.46 (m, 1H, AHPBA-3-CH), 4.45–4.41 (m, 1H, AZT-3′-H), 4.31–4.24 (m, 2H, AZT-5′-H), 4.20 (d, J = 14.2 Hz, 1H, Dmt-2-CH_2), 3.98 (d, J = 14.2 Hz, 1H, Dmt-2-CH_2), 3.96–3.90 (m, 1H, AZT-4′-H), 3.87–3.84 (m, 2H, Gly-CH_2), 2.97–2.84 (m, 2H, AHPBA-4-CH_2), 2.46–2.40 (m, 1H, AZT-2′-H), 2.45 (t,

$J = 7.4$ Hz, 2H, glutaryl-α-CH_2), 2.35–2.27 (m, 1H, AZT-2′-H), 2.22 (t, $J = 7.3$ Hz, 2H glutaryl-γ-CH_2), 2.11 (s, 6H, 2,6-dimethylphenoxyacetyl-CH_3), 1.84–1.80 (m, 2H, glutaryl-β-CH_2), 1.78 (d, $J = 0.9$ Hz, 3H, AZT-5-CH_3), 1.46 (s, 3H, Dmt-5-CH_3) 1.39 (s, 3H, Dmt-5-CH_3), 1.23 (s, 9H, *tert*-butyl-CH_3); HRMS (FAB): *m/z* 976.4229 for $[M+H]^+$ (calcd 976.4224 for $C_{47}H_{62}N_9O_{12}S$). Anal. calcd for $C_{47}H_{64}N_9O_{12}S \cdot H_2O \cdot CF_3COOH$: C, 53.11; H, 5.82; N, 11.38. Found: C, 53.17; H, 5.91; N, 10.88.

2,6-Dimethylphenoxyacetyl-Apns(succinyl-β-Ala-AZT)-Dmt-NHBu^t (3c, KNI-1046). Compound **3c** was prepared from compounds **8a** and **9b** in a manner similar to that described for compound **3a**. Yield 54%: mp 98–100 °C; $[\alpha]_D^{33}$ −7.50° (*c* 0.09, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.35 (s, 1H), 8.25–8.20 (m, 1H), 8.05 (t, $J = 5.4$ Hz, 1H), 7.64 (s, 1H), 7.44 (s, 1H), 7.38 (d, $J = 7.2$ Hz, 2H), 7.27–7.16 (m, 3H), 7.01–6.89 (m, 3H), 6.14–6.10 (m, 1H), 5.34 (d, $J = 3.9$ Hz, 1H), 5.08 (d, $J = 8.7$ Hz, 1H), 4.89 (d, $J = 8.7$ Hz, 1H), 4.51 (s, 1H), 4.50–4.42 (m, 2H), 4.25–4.14 (m, 3H), 4.03–3.95 (m, 2H), 3.29–3.24 (m, 2H), 2.97–2.84 (m, 2H), 2.71–2.51 (m, 4H), 2.42–2.28 (m, 4H, partially covered by DMSO peaks), 2.13 (s, 6H), 1.79 (s, 3H), 1.46 (s, 3H), 1.40 (s, 3H), 1.24 (s, 9H); HRMS (FAB): *m/z* 976.4244 for $[M+H]^+$ (calcd 976.4224 for $C_{47}H_{62}N_9O_{12}S$).

2,6-Dimethylphenoxyacetyl-Apns(glutaryl-β-Ala-AZT)-Dmt-NHBu^t (3d, KNI-1047). Compound **3d** was prepared from compounds **8b** and **9b** in a manner similar to that described for compound **3a**, and obtained as a TFA salt. Yield 83%: mp 93–96 °C; $[\alpha]_D^{31}$ −57.0° (*c* 0.02, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.36 (s, 1H), 8.25 (d, $J = 8.7$ Hz, 1H), 7.94 (t, $J = 5.3$ Hz, 1H), 7.67 (s, 1H), 7.45 (s, 1H), 7.38 (d, $J = 7.2$ Hz, 2H), 7.27–7.16 (m, 3H), 7.01–6.89 (m, 3H), 6.14–6.10 (m, 1H), 5.33 (d, $J = 3.3$ Hz, 1H), 5.09 (d, $J = 9.0$ Hz, 1H), 4.90 (d, $J = 9.0$ Hz, 1H), 4.54 (s, 1H), 4.50–4.43 (m, 2H), 4.30–4.13 (m, 3H), 4.06–3.94 (m, 2H), 3.29–3.16 (m, 2H), 2.98–2.84 (m, 2H), 2.64–2.54 (m, 2H), 2.44–2.28 (m, 4H, partially covered by DMSO peaks), 2.18–2.10 (m, 2H), 2.13 (s, 6H), 1.79 (s, 3H), 1.80–1.76 (m, 2H), 1.47 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); HRMS (FAB): *m/z* 990.4404 for $[M+H]^+$ (calcd 990.4380 for $C_{48}H_{64}N_9O_{12}S$). Anal. calcd for $C_{48}H_{63}N_9O_{12}S \cdot H_2O \cdot CF_3COOH$: C, 54.39; H, 5.84; N, 11.42. Found: C, 54.86; H, 6.39; N, 10.52.

3-(Benzyloxycarbonyl)propionic acid (10). To a solution of succinic anhydride (1.0 g, 10 mmol) in DMF was added benzyl alcohol (0.94 mL, 9.09 mol) and DCHA (2.39 mL, 11 mmol) at 0 °C, and the mixture was stirred for 18 h at rt. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (30 mL) and washed with saturated NaCl. The organic layer was extracted with saturated $NaHCO_3$. The aqueous phase was acidified with citric acid to pH 3–4, and extracted with EtOAc (30 mL). The organic layer was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated in vacuo. The residue was obtained as a white solid (1.77 g, 94%) and was used without further purification in the next step: mp 52–53 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.22 (s, 1H), 7.35–7.32 (m, 5H), 5.10 (s, 2H), 2.60–2.52 (m, 4H, partially covered by DMSO peaks); MS (FAB): *m/z* 209 $[M+H]^+$.

Bzl-succinyl-Gly-AZT (11). To a solution of **10** (127 mg, 0.61 mmol) in DMF was added **9a** (200 mg, 0.55 mmol), triethylamine (77 μL, 0.55 mmol), HOBt (93 mg, 0.61 mmol) and EDC·HCl (117 mg, 0.61 mmol) at 0 °C, and the mixture was stirred for 18 h at rt. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (50 mL), washed with saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Purification of the product by silica gel column chromatography ($CHCl_3$–MeOH) gave 108 mg of the title compound as a colorless oil. Yield 46%: TLC R_f 0.34 ($CHCl_3$–MeOH = 10:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.35 (s, 1H), 8.42–8.38 (t, $J = 5.9$ Hz, 1H), 7.45 (d, $J = 1.2$ Hz, 1H), 7.39–7.28 (m, 5H), 6.15–6.05 (m, 1H), 5.07 (s, 2H), 4.49–4.42 (m, 1H), 4.38–4.17 (m, 2H), 4.00–3.94 (m, 1H), 3.90–3.86 (m, 2H), 2.59–2.43 (m, 5H), 2.38–2.28 (m, 1H), 1.79 (d, $J = 0.6$ Hz, 3H); MS (FAB): *m/z* 515 $[M+H]^+$.

Succinimidylmethylcarbonyl-AZT (5a). To a solution of **11** (160 mg, 0.31 mmol) in DMSO (3 mL) was added phosphate buffer (pH 7.4) (3 mL) and triethylamine (43 μL, 0.31 mmol), and the mixture was stirred for 18 h at 40 °C. After removal of the solvent in vacuo, the residue was the residue was dissolved in EtOAc (50 mL), washed with saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Purification of the product by preparative HPLC gave 38 mg of the title compound as white foam. Yield 30%: TLC R_f 0.56 ($CHCl_3$–MeOH = 10:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.36 (s, 1H), 7.44 (d, $J = 1.1$ Hz, 1H), 6.15–6.05 (m, 1H), 4.51–4.45 (m, 1H), 4.39–4.17 (m, 4H), 4.02–3.97 (m, 1H), 2.78–2.71 (m, 4H), 2.54–2.27 (m, 2H), 1.80 (s, 3H); HRMS (FAB): *m/z* 407.1320 for $[M+H]^+$ (calcd 407.1315 for $C_{16}H_{19}N_6O_7$).

(*R*)-*N*-(2-Methylbenzyl)-3-[(2*S*,3*S*)-3-(2,6-dimethylphenoxyacetyl)amino-2-hydroxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (2,6-dimethylphenoxyacetyl-Apns-Dmt-NH-(2-Me)Bzl, 2, KNI-840). To a solution of 2-methylbenzylamide derivative **12** (400 mg, 0.84 mmol) in DMF was added 2,6-dimethylphenoxyacetic acid (166 mg, 0.92 mmol), HOBt (141 mg, 0.92 mmol) and EDC·HCl (177 mg, 0.92 mmol) at 0 °C, and the mixture was stirred for 18 h at rt. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (50 mL), washed with 5% $NaHCO_3$, 10% citric acid and saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Purification of the product by silica gel column chromatography ($CHCl_3$–MeOH) and reprecipitation from *n*-hexane gave 450 mg of the title compound as a white solid. Yield 89%: mp 80–81 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.38 (t, $J = 5.6$ Hz, 1H), 8.15 (d, $J = 8.7$ Hz, 1H), 7.32–6.90 (m, 12H), 5.52 (d, $J = 7.2$ Hz, 1H), 4.98 (d, $J = 9.6$ Hz, 1H), 4.95 (d, $J = 9.6$ Hz, 1H), 4.51 (s, 1H), 4.48–4.46 (m, 1H), 4.41 (d, $J = 14.4$ Hz, 1H), 4.39 (d, $J = 14.4$ Hz, 1H), 4.18 (dd, $J = 14.7$, 6.6 Hz, 1H), 4.00 (d, $J = 14.4$ Hz, 1H), 2.84–2.73 (m, 2H), 2.26 (s, 3H), 2.15 (s, 6H), 1.51 (s, 3H), 1.36 (s, 3H) ; MS (FAB): *m/z* 604 $[M+H]^+$. Anal.

calcd for $C_{34}H_{41}N_3O_5S$: C, 67.64; H, 6.84; N, 6.96. Found: C, 67.36; H, 6.94; N, 7.05.

(*R*)-*N*-(2-Methylbenzyl)-3-[(2*S*,3*S*)-3-(2,6-dimethylphenoxyacetyl)amino-2-succinyloxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (2,6-dimethylphenoxyacetyl-Apns(succinyl)-Dmt-NH-(2-Me)Bzl, 13a). Compound **13a** was prepared from compound **2** and succinic anhydride in a manner similar to that described for compound **8a**. Yield 82%: mp 69–70 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.40 (t, J = 5.4 Hz, 1H), 8.26 (d, J = 8.8 Hz, 1H), 7.34–6.87 (m, 12 H), 5.40 (d, J = 3.7 Hz, 1H), 5.06 (d, J = 8.8 Hz, 1H), 4.93 (d, J = 8.8 Hz, 1H), 4.61–4.49 (m, 1H), 4.53 (s, 1H), 4.41–4.34 (m, 1H), 4.38 (dd, J = 15.2, 6.3 Hz, 1H), 4.22–4.12 (m, 1H), 4.17 (d, J = 14.4 Hz, 1H), 4.04 (d, J = 14.4 Hz, 1H), 2.97–2.82 (m, 2H), 2.73–2.61 (m, 2H), 2.57–2.50 (m, 2H), 2.25 (s, 3H), 2.14 (s, 6H), 1.48 (s, 3H), 1.36 (s, 3H); HRMS (FAB): m/z 704.3008 for $[M+H]^+$ (calcd 704.3006 for $C_{38}H_{46}N_3O_8S$).

(*R*)-*N*-(2-Methylbenzyl)-3-[(2*S*,3*S*)-3-(2,6-dimethylphenoxyacetyl)amino-2-glutaryloxy-4-phenylbutanoyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxamide (2,6-dimethylphenoxyacetyl-Apns(glutaryl)-Dmt-NH-(2-Me)Bzl, 13b). Compound **13b** was prepared from compound **2** and glutaric anhydride in a manner similar to that described for compound **8a**. Yield 60%: mp 74–76 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.13 (br s, 1H), 8.42 (t, J = 5.6 Hz, 1H), 8.27 (d, J = 8.4 Hz, 1H), 7.35–6.90 (m, 12H), 5.37 (d, J = 3.3 Hz, 1H), 5.08 (d, J = 8.9 Hz, 1H), 4.96 (d, J = 8.9 Hz, 1H), 4.60–4.52 (br m, 1H), 4.56 (s, 1H), 4.39 (dd, J = 15.0, 6.0 Hz, 1H), 4.21–4.12 (m, 1H), 4.17 (d, J = 14.4 Hz, 1H), 4.05 (d, J = 14.4 Hz, 1H), 2.97–2.85 (m, 2H), 2.47–2.44 (m, 2H), 2.34–2.28 (m, 2H), 2.25 (s, 3H), 2.14 (s, 6H), 1.85–1.75 (m, 2H), 1.49 (s, 3H), 1.36 (s, 3H); MS (FAB): m/z 718 $[M+H]^+$. Anal. calcd for $C_{39}H_{47}N_3O_8S$: C, 65.25; H, 6.60; N, 5.85. Found: C, 64.98; H, 6.77; N, 5.80.

2,6-Dimethylphenoxyacetyl-Apns(succinyl-Gly-AZT)-Dmt-NH-(2-Me)Bzl (3e). Compound **3e** was prepared from compounds **13a** and **9a** in a manner similar to that described for compound **3a**. Yield 50%: mp 107–109 °C; $[\alpha]_D^{27}$ + 8.08° (c 0.05, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.35 (s, 1H), 8.45 (t, J = 5.9 Hz, 1H), 8.39 (t, J = 5.4 Hz, 1H), 8.24 (d, J = 8.7 Hz, 1H), 7.45 (d, J = 0.9 Hz, 1H), 7.34–7.15 (m, 6H), 7.11–6.90 (m, 6H), 6.13 (t, J = 6.6 Hz, 1H), 5.40 (d, J = 3.6 Hz, 1H), 5.06 (d, J = 8.9 Hz, 1H), 4.93 (d, J = 8.9 Hz, 1H), 4.62–4.53 (m, 1H), 4.53 (s, 1H), 4.48–4.17 (m, 5H), 4.18 (d, J = 14.4 Hz, 1H), 4.03 (d, J = 14.4 Hz, 1H), 3.99–3.94 (m, 1H), 3.88 (dd, J = 5.7, 2.7 Hz, 2H), 2.97–2.85 (m, 2H), 2.74–2.62 (m, 2H), 2.58–2.53 (m, 2H, partially covered by DMSO peaks), 2.45–2.41 (m, 1H), 2.37–2.27 (m, 1H), 2.25 (s, 3H), 2.13 (s, 6H), 1.79 (s, 3H), 1.48 (s, 3H), 1.36 (s, 3H); HRMS (FAB): m/z 1010.4097 for $[M+H]^+$ (calcd 1010.4082 for $C_{50}H_{60}N_9O_{12}S$).

2,6-Dimethylphenoxyacetyl-Apns(glutaryl-Gly-AZT)-Dmt-NH-(2-Me)Bzl (3f, KNI-1043). Compound **3f** was prepared from compound **13b** and **9a** in a manner similar to that described for compound **3a**. Yield 69%: mp 98–100 °C; $[\alpha]_D^{27}$ −2.50° (c 0.04, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.34 (s, 1H, AZT-NH), 8.41 (t, J = 5.6 Hz, 1H, benzylamine-NH), 8.35 (t, J = 5.9 Hz, 1H, Gly-NH), 8.25 (d, J = 8.4 Hz, 1H, AHPBA-NH), 7.45 (d, J = 0.9 Hz, 1H, AZT-6H), 7.35–7.15 (m, 6H, aromatic), 7.12–6.89 (m, 6H, aromatic), 6.13 (m, 1H, AZT-1′-H), 5.38 (d, J = 3.9 Hz, 1H, AHPBA-2-CH), 5.09 (d, J = 9.0 Hz, 1H, phenoxyacetyl-2-CH_2), 4.95 (d, J = 9.0 Hz, 1H, phenoxyacetyl-2-CH_2), 4.63–4.52 (m, 1H, AHPBA-3-CH), 4.55 (s, 1H, Dmt-4-CH), 4.48–4.15 (m, 6H, AZT-3′-H, benzylamine-CH_2, AZT-5′-H, Dmt-2-CH_2), 4.10–3.92 (m, 2H, Dmt-2-CH_2, AZT-4′-H), 3.88–3.85 (m, 2H, Gly-CH_2), 2.97–2.85 (m, 2H, AHPBA-4-CH_2), 2.46–2.41 (m, 3H, partially covered by DMSO peaks, glutaryl-α-CH_2, AZT-2′-H), 2.37–2.32 (m, 1H, AZT-2′-H), 2.28–2.21 (m, 2H, glutaryl-γ-CH_2), 2.25 (s, 3H, benzylamine-CH_3), 2.13 (s, 6H, 2,6-dimethylphenoxyacetyl-CH_3), 1.86–1.81 (m, 2H, glutaryl-β-CH_2), 1.79 (s, 3H, AZT-5-CH_3), 1.49 (s, 3H, Dmt-5-CH_3), 1.36 (s, 3H, Dmt-5-CH_3); HRMS (FAB): m/z 1024.4235 for $[M+H]^+$ (calcd 1024.4224 for $C_{51}H_{62}N_9O_{12}S$). Anal. calcd for $C_{51}H_{61}N_9O_{12}S\cdot H_2O\cdot CF_3COOH$: C, 55.06; H, 5.58; N, 10.90. Found: C, 55.34; H, 5.67; N, 10.48.

2,6-Dimethylphenoxyacetyl-Apns(succinyl-β-Ala-AZT)-Dmt-NH-(2-Me)Bzl (3g). Compound **3g** was prepared from compounds **13a** and **9b** in a manner similar to that described for compound **3a**. Yield 65%: mp 94–96 °C; $[\alpha]_D^{31}$ + 6.80° (c 0.05, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.35 (s, 1H), 8.39 (t, J = 5.7 Hz, 1H), 8.23 (d, J = 8.7 Hz, 1H), 8.05 (t, J = 5.7 Hz, 1H), 7.44 (d, J = 1.2 Hz, 1H), 7.34–7.15 (m, 6H), 7.11–6.89 (m, 6H), 6.14–6.10 (m, 1H), 5.40 (d, J = 3.9 Hz, 1H), 5.06 (d, J = 8.7 Hz, 1H), 4.93 (d, J = 8.7 Hz, 1H), 4.61–4.52 (m, 1H), 4.52 (s, 1H), 4.49–4.43 (m, 1H), 4.37 (dd, J = 15.0, 6.3 Hz, 1H), 4.30–4.15 (m, 3H), 4.18 (d, J = 14.4 Hz, 1H), 4.03 (d, J = 14.4 Hz, 1H), 4.00–3.95 (m, 1H), 3.28 (dd, J = 12.3, 6.3 Hz, 2H), 2.98–2.85 (m, 2H), 2.73–2.58 (m, 2H), 2.54–2.27 (m, 6H, partially covered by DMSO peaks), 2.25 (s, 3H), 2.14 (s, 6H), 1.79 (s, 3H), 1.48 (s, 3H), 1.36 (s, 3H); HRMS (FAB): m/z 1024.4227 for $[M+H]^+$ (calcd 1024.4239 for $C_{51}H_{62}N_9O_{12}S$).

2,6-Dimethylphenoxyacetyl-Apns(glutaryl-β-Ala-AZT)-Dmt-NH-(2-Me)Bzl (3h). Compound **3h** was prepared from compounds **13b** and **9b** in a manner similar to that described for compound **3a**. Yield 65%: mp 97–99 °C; $[\alpha]_D^{31}$ −3.92° (c 0.05, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.32 (s, 1H), 8.39 (t, J = 5.9 Hz, 1H), 8.24 (t, J = 8.3 Hz, 1H), 7.92 (t, J = 5.1 Hz, 1H), 7.44 (d, J = 0.9 Hz, 1H), 7.34–7.15 (m, 6H), 7.12–6.89 (m, 6H), 6.14–6.09 (m, 1H), 5.38 (d, J = 3.5 Hz, 1H), 5.07 (d, J = 8.7 Hz, 1H), 4.94 (d, J = 8.7 Hz, 1H), 4.62–4.54 (m, 1H), 4.55 (s, 1H), 4.49–4.34 (m, 2H), 4.29–4.17 (m, 3H), 4.17 (d, J = 14.5 Hz, 1H), 4.04 (d, J = 14.5 Hz, 1H), 3.99–3.95 (m, 1H), 3.35–3.26 (m, 2H), 2.97–2.84 (m, 2H), 2.53–2.15 (m, 8H, partially covered by DMSO peaks), 2.25 (s, 3H), 2.13 (s, 6H), 1.82–1.76 (m, 2H), 1.79 (s, 3H), 1.49 (s, 3H), 1.36 (s, 3H); HRMS (FAB): m/z 1038.4382 for $[M+H]^+$ (calcd 1038.4395 for $C_{52}H_{64}N_9O_{12}S$).

Disintegration studies of prodrugs in PBS buffer. The disintegration profile of the prodrugs **3a**–**3d** were determined in phosphate buffered saline (PBS, pH 7.4). To 12 mL of PBS (pH 7.4) was added 120 μL of prodrug solution (0.5 mM in DMSO) and the mixture was incubated at 37 °C in a water bath. At different points of time, 1 mL of the samples was withdrawn and directly analyzed by HPLC. HPLC was performed using C18 reverse phase column (4.6×150 mm; YMC Pack ODS AM302) with binary solvent system: linear gradient of CH_3CN (0–100%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min, detected at UV 230 nm.

Disintegration studies of prodrugs in the presence of esterase. Porcine liver esterase (PLE; carboxylic-ester hydrolase; EC 3.1.1.1; E-2884) was obtained from Sigma Chemical Co. (St Louis, MO) as a suspension in a 3.2 M $(NH_4)_2SO_4$ solution (pH 8). 3.2 μL of this suspension (containing 3750 units of enzyme per mL) was diluted with 12 mL of PBS (pH7.4). In this solution, the disintegration profile of the prodrug **3b** was determined in a manner similar to that described above.

Disintegration studies of conjugates in cell culture medium. To 50 μL of cell culture medium (RPMI-1640 containing 10% of heat-inactivated fetal calf serum) was added 0.5 μL of a solution of prodrug **3b** (10 mM in DMSO) and the mixture was incubated at 37 °C. At different points of time, 10 μL of the samples was withdrawn and added to 100 μL of cold MeOH. The solution of the samples were stirred vigorously and filtered through a membrane filter (0.45 μm), and 75 μL of the filtrate was analyzed by HPLC in a manner similar to that described above.

Disintegration studies of conjugates in Molt-4 cell homogenate. Molt-4 cells were suspended to 2 mL of ice-cold Hank's balanced salt solution (HBSS), and was homogenized on ice using a 15 mL Wheaton glass homogenizer (15 strokes, pestle/wall clearance 0.64–0.76 mm). The cell debris and nuclei were removed at 4 °C by centrifugation for 10 min at 10,000 rpm (9000*g*) using a Marathon 21K/BR centrifuge (Herle AG, Gosheim, FRG). To 50 μL of Molt-4 homogenate was added 0.5 μL of a solution of prodrug (10 mM in DMSO) and the mixture was incubated at 37 °C. At different points of time, 10 μL of the samples was withdrawn and added to 100 μL of cold MeOH. The solution of the samples were stirred vigorously and filtered through a membrane filter (0.45 μm), and 75 μL of the filtrate was analyzed by HPLC in a manner similar to that described above.

HIV PR inhibition. HIV PR inhibitory activity of the test compounds was determined based on the inhibition of cleavage of the HIV PR substrate (H-Lys-Ala-Arg-Val-Tyr-Phe(*p*-NO_2)-Glu-Ala-Nle-NH_2) by using recombinant HIV-1 PR. HIV PR substrate was synthesized by solid-phase methods. Recombinant HIV-1 protease was purchased from Bachem AG, Bubendorf-Switzerland. In the inhibition assay, 25 μL of 200 mM 2-(*N*-morpholino-)ethanesulfonic acid (MES)–NaOH buffer, pH 6.0, containing 2 mM dithiothreitol (DTT) and 2 mM EDTA was mixed with 5 μL of prodrug (50 μM or 500 nM) dissolved in DMSO and 10 μL of HIV-1 protease (2 μg/mL) in 50 mM MES–NaOH, pH 6.0 containing 2.5 mM DTT, 1 mM EDTA-2Na, 0.2% Nonidet P-40, and 15% glycerol. The enzyme reaction was initiated by addition of 10 μL of a 1.0 mM substrate solution in the above-described assay buffer. After incubation for 15 min at 37 °C, the reaction was terminated by addition of 75 μL of 1 N HCl, and the N-terminal cleavage fragment (H-Lys-Ala-Arg-Val-Tyr-OH) was separated by reverse-phase HPLC on a C18 column (3.0×75 mm; YMC Pack ODS AS-3E7) with linear gradient of CH_3CN (6–16%, 5 min) in 0.1% aqueous TFA at a flow rate of 1.0 mL/min, and determined the quantity by monitoring fluorescence intensity (Ex, 275 nm; Em, 305 nm).

HIV RT inhibition. The prodrugs dissolved in DMSO were mixed with standard RT solution containing 20 mU/mL recombinant HIV-RT, and assayed with non-RI RT assay kit (Asahikasei, Japan).[30]

Antiviral activity. Antiviral activity of test compounds was determined based on inhibition of HIV-1 IIIB-induced cytopathic effect in Molt-4 cells in vitro. HIV-1_{LAI} (100 TCID50) and each 4-fold diluted prodrug were inoculated to 5000/well Molt-4 cells in a microplate and cultured for 7 days. EC_{50} values were calculated from the ratios of surviving cells, which were quantitated by WST-8 assay kit[32] (Dojin Lab., Kumamoto, Japan).

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