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Synthesis and antiviral property of allophenylnorstatine-based HIV protease inhibitors incorporating D-cysteine derivatives as P_2/P_3 moieties

Ei'ichi Ami,[a] Koichiro Nakahara,[b] Akihiko Sato,[b] Jeffrey-Tri Nguyen,[a] Koushi Hidaka,[a] Yoshio Hamada,[a] Shingo Nakatani,[a] Tooru Kimura,[a] Yoshio Hayashi[a] and Yoshiaki Kiso[a,*]

[a]*Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science and 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan*
[b]*Discovery Research Laboratories, Shionogi Co. Ltd, Settsu, Osaka 566-0022, Japan*

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Abstract—We designed several HIV protease inhibitors with various D-cysteine derivatives as P_2/P_3 moieties based on the structure of clinical drug candidate, KNI-764. Herein, we report their synthesis, HIV protease inhibitory activity, HIV IIIB cell inhibitory activity, cellular toxicity, and inhibitory activity against drug-resistant HIV strains. KNI-1931 showed distinct selectivity against HIV proteases and high potency against drug-resistant strains, surpassing those of Ritonavir and Nelfinavir.
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The human immunodeficiency virus (HIV) encodes an aspartic protease (PR) that is essential for the formation of mature and infectious virions.[1] The HIV PR is regarded as a promising target in the chemotherapy of acquired immunodeficiency syndrome (AIDS).[2] Intensive efforts have been directed to develop potent, orally available, peptidomimetic inhibitors of this enzyme.[3] At the present time, nine PR inhibitors have been approved by the FDA (United States Food and Drug Administration), and several others are now in clinical trials.[4] However, long-term anti-retroviral therapy for HIV infected patients promotes the emergence of resistance mutations of HIV PR, and consequently reduces the clinical efficacy of these inhibitors.[5] Indeed, there is a continuing demand for newer mutant-resistant HIV PR inhibitors. Fortunately, HIV PR variants expressing resistance to inhibitors have also been derived in cell culture and thus, new inhibitors can be evaluated against drug-resistant HIV strains.[6]

We have previously reported highly potent HIV PR inhibitors, KNI-272 (**1**) and KNI-764 (also known as JE-2147, AG-1776, or SM-319777, **2**), that both contain Apns with a hydroxymethylcarbonyl (HMC) isostere for the P_1' position, and for the case of KNI-764, Dmt as an isostere of proline for the P_1' position (Fig. 1).[7] Although KNI-764 is effective against some resistant mutants of the PR, our interest is to improve inhibitory potency against both the wild-type and drug-resistant HIV variants utilizing the 'Apns-Dmt' skeleton. Herein, we report that the introduction of unusual D-amino acids into the P_2/P_3 positions of Apns-based HIV PR inhibitors affords highly effective compounds whose inhibitory potencies surpass those of clinically active drugs such as Ritonavir. We further explore structure–activity relationships (SARs) that would increase HIV PR inhibitory activity, lower cytotoxicity, and high antiviral activities against both wild-type and mutant strains.

Information on the interactions between inhibitor **1** and HIV PR based on X-ray crystallographic data and molecular modeling suggested the feasibility of replacing

Abbreviations: Apns, (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid, allophenylnorstatine; Dmt, (*R*)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid; *m*-CPBA, *m*-chloroperbenzoic acid; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carboiimide hydrochloride; HOBt, 1-hydroxybenzotriazole.

Keywords: HIV protease; Protease inhibitor; Drug-resistant HIV strain; D-Cysteine; Allophenylnorstatine.

* Corresponding author. Tel.: +81 75 595 4635; fax: +81 75 591 9900; e-mail: kiso@mb.kyoto-phu.ac.jp

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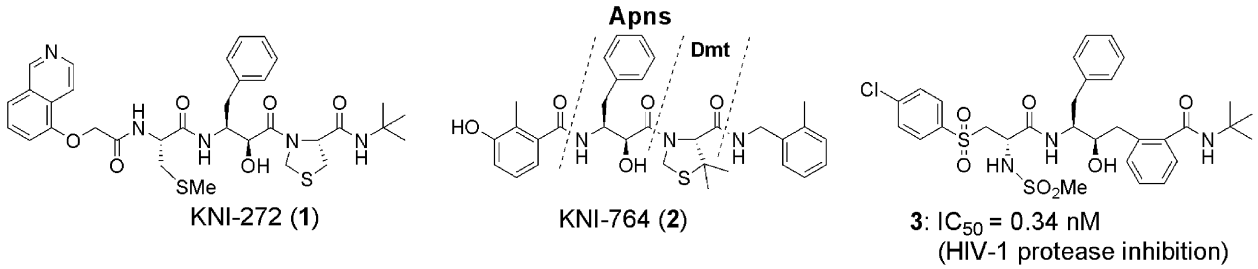
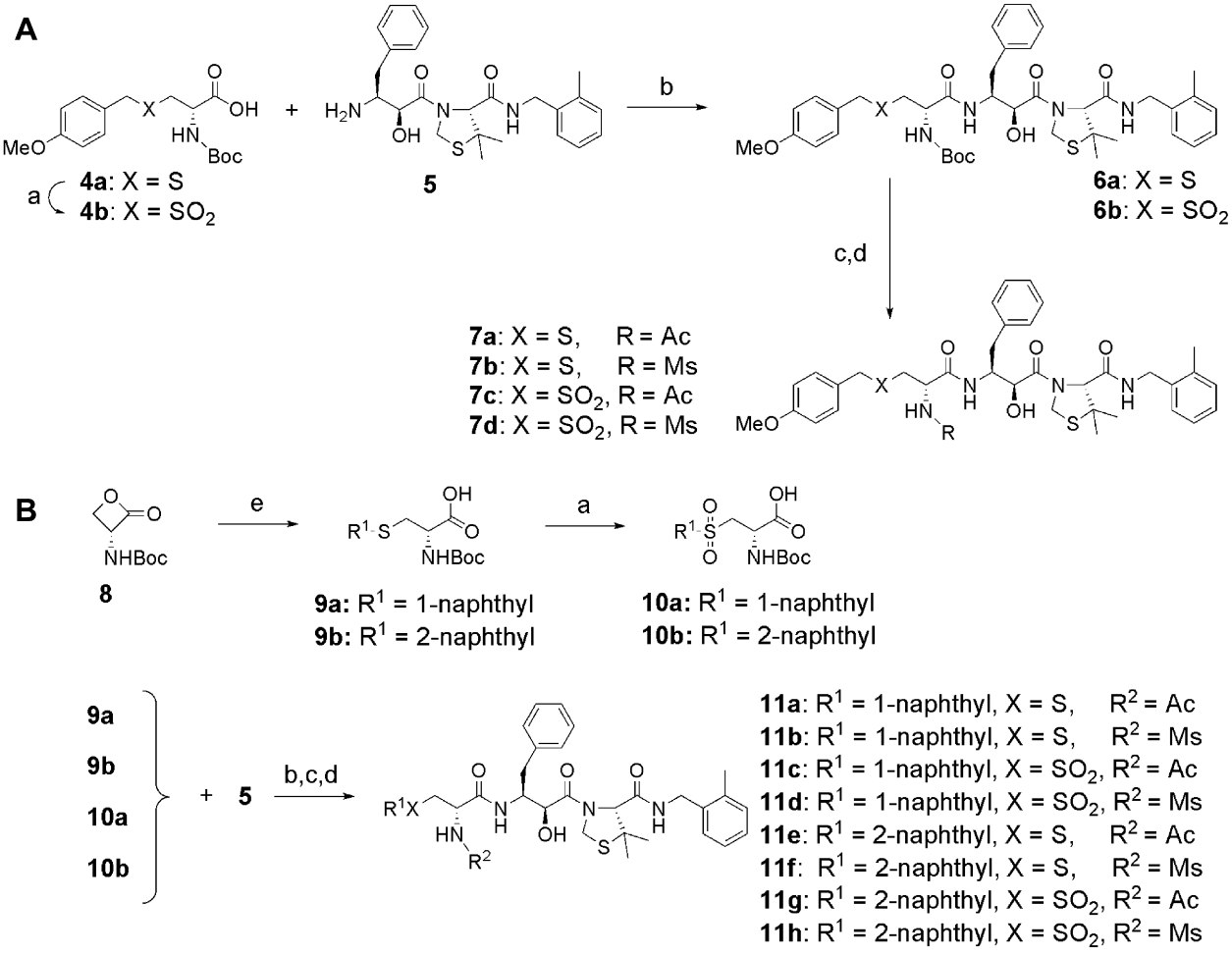


Figure 1. Structures of previously reported HIV protease inhibitors.

the P_2 L-amino acids with D-isomers for more favorable van der Waals interactions.[8] Replacement of an L-amino acid by D-amino acid isomer means that the P_2 and P_3 moieties would be relatively swapping their occupation of the S_2 and S_3 pockets. In Figure 1, Eli Lilly's HIV PR inhibitor **3**, which possesses a P_2 D-amino acid to improve proteolytic stability, is depicted with a different P_2/P_3 orientation to that of inhibitor **1**.[9,10] D-Amino acid substitution has also been applied to different hydroxyethylamine isostere for HIV PR inhibitors with lesser peptidic characters.[5] These reports prompted us to apply various D-cysteine derivatives as P_2/P_3 residues to the Apns-Dmt skeleton.

In our initial attempt, we selected commercially available, optically pure, *N*-Boc protected D-cysteine derivatives with an aromatic moiety in the side chain. *N*-Boc protected D-*S*-(*p*-methoxybenzyl)cysteine **4a** and the corresponding sulfonyl derivative **4b**, obtained by *m*-CPBA oxidation of compound **4a**, were used as the starting materials (Scheme 1A). These cysteine derivatives **4a**,**b** were, respectively, coupled with optically pure compound **5**, which is our lead structure with the Apns-Dmt skeleton, using EDC·HCl-HOBt method to afford *N*-Boc tripeptides **6a**,**b** in high yield.[11] Jungheim et al. reported potent HIV PR inhibitors possessing a P_2 acetyl or mesyl moiety, and so we also introduced an acetyl



Scheme 1. Reagents: (a) *m*-CPBA (2 equiv), CH_2Cl_2; (b) HOBt, EDC·HCl, Et_3N, DMF; (c) 4 N HCl/dioxane, anisole; (d) Ac_2O or MsCl, *N*-methylmorpholine, CH_2Cl_2; (e) sodium 1- or 2-naphthalenethiolate, THF.

or mesyl group to the amino group after Boc group cleavage, to afford four analogues, **7a**–**d**, respectively.[10] As for our second group of compounds, utilizing the ring-opening reaction of Vederas' *N*-Boc-D-serine-β-lactone (**8**) with sodium salts of 1- or 2-naphthalenethiol, we obtained **9a**,**b**, respectively, that were further oxidized to corresponding sulfones **10a**,**b** using 2 equiv of *m*-CPBA (Scheme 1B).[12] The respective coupling reactions of compounds **9a**, **9b**, **10a** or **10b** with compound **5** were carried out using a similar procedure as Scheme 1A to afford eight naphthyl analogues **11a**–**h**.

The analogues synthesized in this study (**7a**–**d** and **11a**–**h**) were first tested for HIV PR inhibitory activity. As shown in Table 1, the analogues exhibited mid to high percent inhibitory activity against HIV protease. SAR studies suggested that, in general, 1-naphthyl analogues **11a**–**d** tended to exhibit slightly higher HIV PR inhibition than 2-naphthyl analogues **11e**–**h**, which in turn were more potent than *p*-methoxybenzyl analogues **7a**–**d**. Computer-assisted docking experiments suggested that the aromatic group (R^1) resided near the edge of the active site in the S_3 pocket with no apparent hydrogen bond interactions present (Fig. 2).[13] Inhibition by compounds **7a**–**d** having *p*-methoxybenzyl in P_3 residue was affected by the difference between sulfide and sulfone (X); namely, the sulfide containing analogues **7a**,**b** seemed to exhibit higher inhibitory activity than their corresponding sulfones **7c**,**d**. On the other hand, the general S/SO_2 SAR observed for compounds **11a**–**h** bearing naphthyl groups at the P_3 moiety was opposite, in that sulfide analogues **11a**,**b** and **11e**,**f** exhibited lower HIV PR inhibition than sulfone analogues **11c**,**d** and **11g**,**h**.

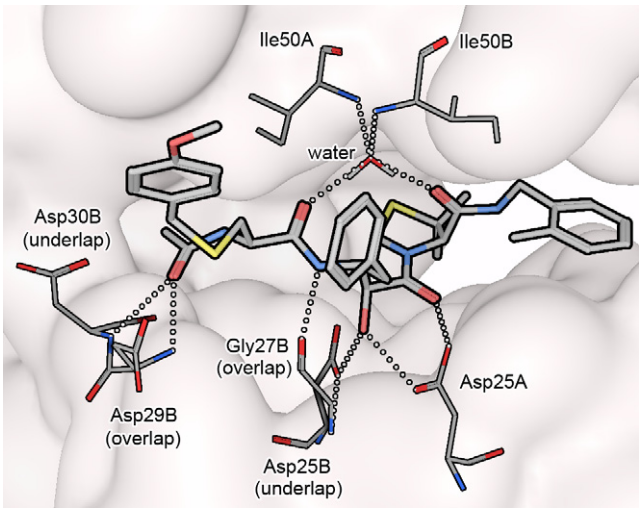
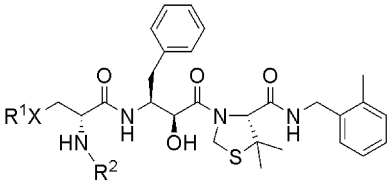


Figure 2. Computer model of KNI-1931 (**7a**) in the active site of HIV protease. Circles represent possible hydrogen bond interactions. Model generated from Molecular Operating Environment software.[13]

Table 1. Synthesized HIV protease inhibitors and their inhibitory activity against HIV protease; activity and cytotoxicity in HIV IIIB protease in MT-4 cells; and activity in drug-resistant NL-432 strain-derived clones



Compound		Structure			HIV protease inhibition		MT-4 cell assay (HIV-1 IIIB)			NL-432 strain and drug-resistant derived clones EC_{50}, nM (fold resistance)			
		R^1	X	R^2	Percent at 1 nM[a]	IC_{50} (nM)	EC_{50} (nM)	LC_{50} (nM)	Selectivity index	NL-432 wild-type	RTV resistant[b]	NFV resistant[c]	RTV & NFV resistant[d]
7a	KNI-1931	*p*-Methoxybenzyl	S	Ac	87	0.18	13	17000	1308	17	18 (1.1)	2.4 (0.14)	14 (0.82)
7b	KNI-1932	*p*-Methoxybenzyl	S	Ms	77	0.26	9.3	10000	1075	16	20 (1.3)	0.67 (0.04)	23 (1.4)
7c	KNI-1990	*p*-Methoxybenzyl	SO_2	Ac	59		102	>27000	>265				
7d	KNI-1986	*p*-Methoxybenzyl	SO_2	Ms	66		203	>26000	>128				
11a	KNI-1964	1-Naphthyl	S	Ac	68		17	8000	471	20	70 (3.5)	6.3 (0.32)	52 (2.6)
11b	KNI-1966	1-Naphthyl	S	Ms	74		6.4	4400	688	9.3	17 (1.8)	0.67 (0.07)	13 (1.4)
11c	KNI-1961	1-Naphthyl	SO_2	Ac	97	0.090	40	16000	400				
11d	KNI-1933	1-Naphthyl	SO_2	Ms	97	0.083	61	19000	311				
11e	KNI-1946	2-Naphthyl	S	Ac	53		18	8300	461	17	65 (3.8)	7.2 (0.42)	32 (1.9)
11f	KNI-1947	2-Naphthyl	S	Ms	67		13	6100	469	17	31 (1.8)	0.80 (0.05)	23 (1.4)
11g	KNI-1965	2-Naphthyl	SO_2	Ac	96	0.086	28	16000	571				
11h	KNI-1969	2-Naphthyl	SO_2	Ms	98	0.090	81	19000	235				
1	KNI-272				40		22	>30000	>1363				
	Ritonavir				90		36	17000	472	80	220 (2.8)	16 (0.20)	162 (2.0)
	Nelfinavir				29		16	11000	688	25	28 (1.1)	308 (12)	67 (2.7)

[a] Percent HIV protease inhibition at 1 nM of the test compound.
[b] Ritonavir-resistant clone from NL-432 strain established by Shionogi Co. Ltd: N37S, G57R, and I84V.
[c] Nelfinavir-resistant clone from NL-432 strain established by Shionogi Co. Ltd: D30N, N37S, and G57R.
[d] Ritonavir- and Nelfinavir-resistant clone from NL-432 strain established by Shionogi Co. Ltd: L19V, V32I, M46L, G57R, L63P, and I85V.

$$- \log(EC_{50}) = 0.499\,(Enz) + 0.905\,(X) - 2.215$$

$n = 12$, $r^2 = 0.85$, $F = 27$, $p < 0.001$

where Enz : normalized log(% HIV protease inhibition at 1 nM of the test compound), ranging from 0 to 1.

X : $SO_2 = 0$; S = 1.

Equation 1. Quantitative structure–activity relationship equation correlating HIV protease inhibition and structural features with cellular antiviral EC_{50}.

As for the P_2 moiety in overall, computer-assisted docking experiments suggested that little differences in size and electrostatic effects existed between an acetyl and mesyl moiety (R^2) in relation to the enzyme's S_2 pocket, and therefore supported the observed general trend that a mesyl group promoted only slightly more potent HIV PR inhibition.[13] The IC_{50} values of inhibitors with >75% inhibition (**7a**,**b**, **11c**,**d**, and **11g**,**h**) were determined. Most interestingly, the HIV PR inhibitory activity for sulfone analogues **11c**,**d** and **11g**,**h** surpassed that for Ritonavir.

Cellular antiviral activity, toxicity, and selectivity index (SI) for these analogues were determined against HIV strain IIIB in MT-4 cells by MTT assay (Table 1).[14] Inhibitors **7a**,**b** were identified as the most promising candidates due to their high SIs as a result of both low effective concentration (EC_{50}) and high lethal concentration (LC_{50}). Indeed, inhibitor **7a**,**b**'s low cytotoxicity profiles would enable larger therapeutic windows than current clinical drugs (cf. SI: **7a**, 1308; **7b**, 1075; Ritonavir, 472; Nelfinavir, 688). Well-fitted, multiple normalized collinear, quantitative SAR equations were derived to correlate enzymatic inhibition and structural features with cellular assay results. Inclusion of the R^1 and R^2 moieties as descriptors did not greatly contribute to the overall equation. The R^1 and R^2 moiety descriptors were subsequently excluded as minor determinants, for similar reasons formerly explained for HIV PR inhibition, to form a simplified and more statistically valid equation (Eq. 1, $F = 27$, $p < 0.001$) that associated high cellular activity with high HIV PR inhibition, and sulfide analogues ($r^2 = 0.85$, $n = 12$). Whether the compound is an S or SO_2 analogue is a greater determinant of activity than percent HIV PR inhibition (64% vs. 36% contribution to the equation, respectively).

Compounds **7a**,**b** and **11a**,**b**,**e**,**f** were selected, based on their favorable EC_{50} values (less than 20 nM), for further evaluation of antiviral activity against drug-resistant HIV variants. The compounds exhibited fairly potent activities against wild-type NL-432 strain over those of Ritonavir and Nelfinavir. The effectiveness of compounds **7a**,**b** and **11b**,**f** against Ritonavir-resistant strains surpassed those of Ritonavir and Nelfinavir. Moreover, the evaluated compounds' potencies exceeded those of Ritonavir and Nelfinavir in Nelfinavir-resistant as well as Ritonavir/Nelfinavir-resistant variants. In terms of potencies against wild-type and variants, compounds **7a**,**b** and **11b** were the top three most potent. Also taking into account fold resistance (insusceptibility to mutations) and selectivity index, compound **7a** was identified as the overall most promising HIV PR inhibitor from the current study. Computer-assisted docking experiments on inhibitor **7a**, based on the X-ray crystallographic data for compounds **1** (PDB 1HPX) and **2** (PDB 1MSM), revealed that a water molecule could mediate the interactions between Ile50A and Ile50B of the dimer in the hairpin regions of HIV PR's flaps and the inhibitor; and compound **7a**'s transition-state mimic HMC moiety could interact with the catalytic Asp25A and Asp25B; while Gly27B could form a hydrogen bond with Apns' amide proton (Fig. 2).[13] As for the focal point of this study, the carbonyl oxygen from the P_2 acetyl moiety could interact with the backbone of Asp29B and Asp30B via hydrogen bond interactions. Although our calculations did not predict hydrogen bond interactions with the P_3 moiety, the *p*-methoxybenzyl moiety was believed to reside in the S_3 pocket.

In summary, Apns-based HIV PR inhibitors, containing various D-cysteine derivatives as P_2/P_3 moieties, presented in this work form a promising new series of highly potent inhibitors. Considering the compounds' potency against a spectrum of drug-resistant variants and their favorable cytotoxicity profiles, as well as the preliminary nature of this short communication, a more complete study is currently underway. Among the compounds in the current series, KNI-1931 (**7a**) demonstrated distinct SI as an HIV therapeutic agent, and high resistant profiles against clinically used drug-resistant strains.

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13. Computer-assisted docking experiments were performed with Molecular Operating Environment software (revision 2006.08.04) using PDB 1MSM (a complex of **1** and HIV PR) as the base coordinates. **2** (PDB 1HPX, a complex of **2** and HIV PR) was superposed onto **1**, and a chimera was created, in which P_2'–P_1 originated from **2**, while P_2–P_3 were from **1**. P_2/P_3 were modified to form the desired inhibitor. Ideal ionization states for Asp25A and Asp25B were decided after examining different permutations. An interacting water molecule (present in both PDB 1MSM and 1HPX) was added. Inhibitor and PR residues with hydrogen bond interactions to inhibitor were made flexible. Energy minimization (force field MMFF94x) was performed after each step.
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