KNI-102, A NOVEL TRIPEPTIDE HIV PROTEASE INHIBITOR CONTAINING ALLOPHENYLNORSTATINE AS A TRANSITION-STATE MIMIC[1)]

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HIV-1 protease inhibitors containing allophenylnorstatine[Apns; (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid]-Pro (*syn* diastereomer) as a transition-state mimic were established to be potent and highly selective. Z-Asn-Apns-Pro-NHBu^t (KNI-102) is the only tripeptide exhibiting substantial anti-HIV activity and may be of minimum size for potent, selective inhibition of HIV protease. Ready availability due to its simple chemical structure and stability should make it valuable for studies of the development of metabolically stable anti-AIDS drugs.

KEYWORDS HIV protease; HIV protease inhibitor; HIV; peptide synthesis; hydroxymethylcarbonyl isostere; transition-state mimic; phenylnorstatine; allophenylnorstatine; AIDS

The human immunodeficiency virus type-1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), codes for a virus-specific aspartic protease responsible for processing the *gag* and *gag-pol* polyproteins and for the proliferation of the retrovirus. The HIV-1 protease functions as a homodimer and can recognize Phe-Pro and Tyr-Pro sequences as the cleavage site, but mammalian aspartic proteases do not have such specificity. These features provided a basis for the rational design of selective HIV protease-targeted drugs for the treatment of AIDS and related complex.[2)]

Several HIV-1 protease inhibitors[3)] have been discovered based on the transition-state analogue concept which was known to be effective in studies of inhibitors of aspartic proteases such as renin and pepsin. These inhibitors contain a critical hydroxyl group as a transition-state mimic which interacts with the catalytically active aspartic acid carboxyl groups of protease, and the stereochemistry of the hydroxyl group[4)] is important for the inhibition.

In the previous paper,[5)] we have described potent and selective HIV-1 protease inhibitors containing a hydroxymethylcarbonyl (HMC) isostere as a transition-state mimic (Fig.1). Unexpectedly, the (2*S*)-hydroxymethylcarbonyl (HMC) inhibitor (KNI-93; **1S**: *syn*[4)] diastereomer) containing allophenylnorstatine (Apns) was more active against HIV-1 protease than the *anti* [4)] diastereomer (KNI-122, **1A**) containing phenylnorstatine (Pns), in contrast to the case of renin inhibitors[6)] which show a preference for the *anti* diastereomer [*e.g.*, cyclohexylnorstatine (Chns)-containing inhibitors; Fig.2].

However, a preferred configuration of the hydroxyl group in a series of HMC inhibitors of HIV protease remains to be established. Also, long-chain peptides are unsuitable for a metabolically stable anti-HIV drug, and it is necessary to minimize the number of natural peptide bonds and reduce the molecular weight. We therefore sought the minimum size required for potent inhibition and the preferred hydroxyl group configuration in HMC-inhibitors, and can now report a novel tripeptide, Z-Asn-Apns-Pro-NHBu^t (KNI-102; **6S**: *syn* diastereomer) which exhibits a potent, selective inhibitory activity against HIV-1 protease (Fig.3, Table 1). In contrast, its *anti* diastereomer, Z-Asn-Pns-Pro-NHBu^t (**6A**) exhibits little inhibitory activity,

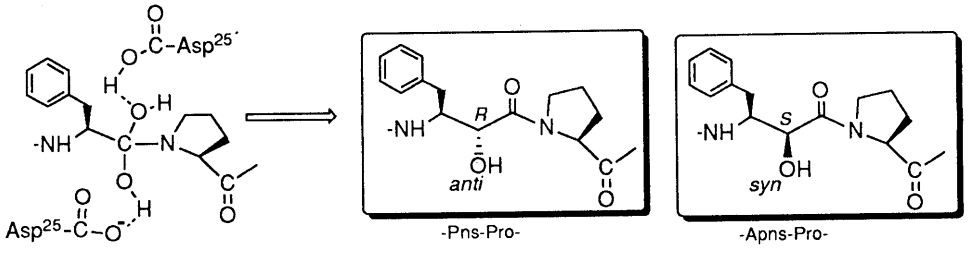

Fig.1. The Phe-Pro Transition State in HIV-1 Protease and P_1-P_1' Pns-Pro and Apns-Pro with the Hydroxymethylcarbonyl (HMC) Isostere Mimicking the Transition State
Pns = phenylnorstatine = (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid; Apns = allophenylnorstatine = (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid.

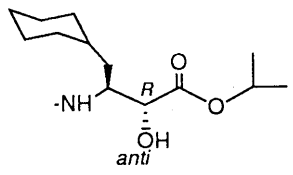

Fig.2. P_1-P_1' Chns Isopropyl Ester in Renin Inhibitors
Cyclohexylnorstatine (Chns)= (2*R*,3*S*)-3-amino-4-cyclohexyl-2-hydroxybutyric acid.

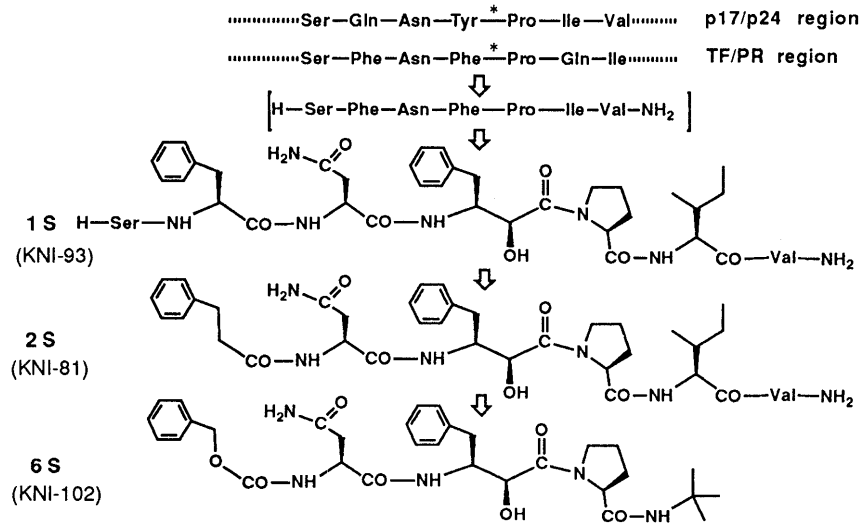

Fig.3. Design of Substrate-Based Inhibitors of HIV Protease

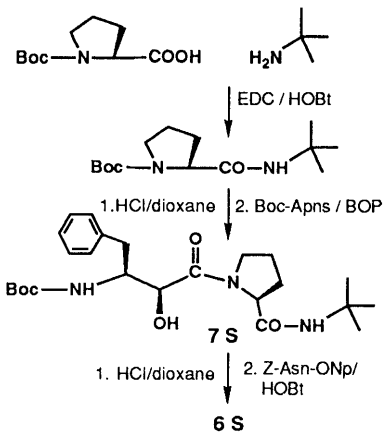

Chart 1. Synthesis of KNI-102 (**6S**)
Boc = t-butoxycarbonyl; BOP = benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate; Z = benzyloxycarbonyl; ONp = *p*-nitrophenyl ester; EDC= 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt=N-hydroxybenzotriazole.

that is, HMC-Pro inhibitors show a preference for the *syn* diastereomer exceptionally.

As previously described,[5] we focused on Phe-Pro scissile site which was a unique structure for HIV-1 protease in the design of substrate-based HIV protease inhibitors (Fig.3), and incorporated the HMC isostere as a transition-state mimic at P_1 site in a heptapeptide amide, Ser-Phe-Asn-Phe-Pro-Ile-Val-NH_2, similar to the TF/PR and p17/p24 sequences. In order to obtain smaller inhibitors, we deleted P_4 Ser and replaced P_3 Phe with the isosteric 3-phenylpropionic acid. Moreover, we replaced P_3 Phe with the isosteric benzyloxycarbonyl group, and deleted P_3' Val and replaced P_2' Ile with the isosteric t-butyl amine. We also designed symmetric-type inhibitors containing HMC structure at the symmetric axis based on the dimeric character of HIV protease.

These peptides except for **6S** and **6A** were synthesized by the efficient solid-phase method.[5,7] Compound **6S** was conveniently synthesized by the solution method in a stepwise manner (Chart 1). After the acidolytic removal of the Boc group, Boc-Apns was condensed by the means of BOP reagent without the protection of the hydroxy group to give **7S** in almost quantitative yield. After the coupling reaction of Z-Asn-ONp at the last stage, pure **6S** was obtained readily. Compound **6A** was prepared by the same procedure. The protease inhibitory activities of HMC isostere-containing peptides (Table I) were examined by using the chemically synthesized [$Ala^{67,95}$]-HIV-1 protease[5] and the synthetic substrate, Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2.[8]

Compound **2S** (KNI-81) containing Apns, in which P_4 Ser was deleted and P_3 Phe was replaced by the isosteric 3-phenylpropionic acid, exhibited substantial inhibitory activity, and was more active than the *anti* diastereomer (**2A**). Replacement of P_2 Asn with Ser[9] (compound **3S**) decreased the inhibitory activity, but compound **3S** was also more active than the *anti* diastereomer (**3A**). Furthermore, incorporation of phenylacetyl group at P_3 site (compounds **4A** and **4S**)

Table I. Protease Inhibitory Activities of HMC Compounds (IC_{50}, nM)

No	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	HIV Protease	Pepsin
1A (KNI-122)	Ser-	Phe-	Asn-	Pns-	Pro-	Ile-	Val-NH_2[5]	100	>10,000
1S (KNI-93)	Ser-	Phe-	Asn-	Apns-	Pro-	Ile-	Val-NH_2[5]	5	>10,000
2A		Pp-	Asn-	Pns-	Pro-	Ile-	Val-NH_2[5]	3,000	>10,000
2S (KNI-81)		Pp-	Asn-	Apns-	Pro-	Ile-	Val-NH_2	468	>10,000
3A		Pp-	Ser-	Pns-	Pro-	Ile-	Val-NH_2[5]	>10,000	N.D.
3S		Pp-	Ser-	Apns-	Pro-	Ile-	Val-NH_2	1,594	N.D.
4A		Pa-	Ser-	Pns-	Pro-	Ile-	Val-NH_2	>10,000	N.D.
4S		Pa-	Ser-	Apns-	Pro-	Ile-	Val-NH_2	5,041	N.D.
5A		Pp-	Asn-	Chns-	Pro-	Ile-	Val-NH_2[5]	>10,000	N.D.
5S		Pp-	Asn-	Achns-	Pro-	Ile-	Val-NH_2	1,999	N.D.
6A		Z-	Asn-	Pns-	Pro-$NHBu^t$			>10,000	N.D.
6S (KNI-102)		Z-	Asn-	Apns-	Pro-$NHBu^t$			89	>100,000
8A		Val-	Val-	Pns-	Phe-	Val-	Val-NH_2[5]	350	4,000
8S		Val-	Val-	Apns-	Phe-	Val-	Val-NH_2	2,600	>10,000

Pp = 3-phenylpropionyl; Pa = phenylacetyl; Achns = allocyclohexylnorstatine = (2*S*,3*S*)-3-amino-4-cyclohexyl-2-hydroxybutyric acid; Bu^t=t-butyl; N.D. = not determined.

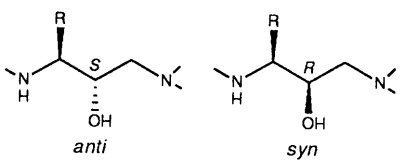

Fig. 4. Hydroxyethylamine Isostere

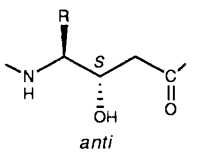

Fig.5. Statine-Type Isostere

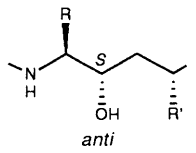

Fig.6. Hydroxyethylene Isostere

decreased the potency, and also in this case, the *syn* configuration of the hydroxyl group was preferred. Replacement of P_1 Apns with allocyclohexylnorstatine (Achns)[10] (compound **5S**) reduced the inhibitory potency, and also in this case, the Achns-containing inhibitor was more active than the Chns-containing inhibitor (**5A**), in contrast to the case of renin inhibitors.[6] The Apns-containing tripeptide **6S** (KNI-102), in which P_3 Phe was replaced by the isosteric benzyloxycarbonyl group, P_3' Val deleted and P_2' Ile replaced by the isosteric t-butylamine, exhibited a higher activity compared to the pentapeptide **2S**. It was surprising that such a small compound as **6S** was more potent than a longer compound **2S**. On the other hand, the Pns-containing tripeptide **6A** exhibited little activity even at a concentration of 5μM.

Thus it became clear that the *syn* configuration of the hydroxyl group was preferred in both cases of low molecular-weight and long-chain HMC-Pro inhibitors of HIV protease. In contrast, symmetric-type inhibitors containing P_1' Phe showed a preference for the *anti* diastereomer (**8A**), in agreement with the case of renin inhibitors.[6] This difference of HMC-Pro inhibitors from symmetric-type inhibitors may be based on the unique conformation of the proline residue at P_1' site.

In the case of hydroxyethyl-Pro-type inhibitors of HIV protease (Fig.4),[11,12] the preference shifted from the *syn* diastereomer in short-chain inhibitors to the *anti* diastereomer in long inhibitors, in contrast to the series of HMC-Pro inhibitors. The discrepancy between the HMC-Pro inhibitors and the hydroxyethyl-Pro inhibitors seems to be due to the conformational difference between the constrained peptide bond and the relatively flexible methylene-amine bond. A preference for the *syn* hydroxyl group shown in this series of HMC-Pro inhibitors is exceptional among various inhibitors of aspartic proteases such as HIV protease, renin and pepsin, which implies the uniqueness of the HMC-Pro structure.

As shown in Table I, HMC-Pro inhibitors of HIV protease did not practically inhibit pepsin but the symmetric-type compound containing P_1' Phe (**8A**) did inhibit pepsin,[5] which showed that Apns-Pro inhibitors of HIV protease were selective. Especially, the tripeptide containing Apns-Pro (**6S**; KNI-102) was a potent and highly selective HIV protease inhibitor, causing no inhibition of pepsin even at a concentration of 80μM. Since dipeptides Boc-Pns-Pro-$NHBu^t$ and Boc-Apns-Pro-$NHBu^t$ (**7S**) exhibited little inhibitory activity (IC_{50} > 10,000 nM; not shown in Table I), the tripeptide, KNI-102 (**6S**) was identified to be of minimum size required for potent inhibition. None of four peptide bonds in KNI-102 are natural type, and KNI-102 exhibits substantial anti-HIV activity whereas long-chain peptides have little antiviral activity,[13,14] which implies that the tripeptide is relatively stable in the cell.

In conclusion, Apns-Pro inhibitors (*syn* diastereomer) of HIV-1 protease were established to be potent and highly selective. KNI-102 is the only tripeptide exhibiting substantial anti-HIV activity and may be of minimum size for potent, selective inhibition of HIV protease. Ready availability due to its simple chemical structure and stability should make it valuable for studies of the development of metabolically stable anti-AIDS drugs.

REFERENCES AND NOTES

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4) In the renin inhibitors, the 2*R* hydroxy group was preferred in norstatine-type compounds (Fig.2), while the 3*S* hydroxy group was favored in the case of the statine-type compounds (Fig.5). This discrepancy between norstatine- and statine-type compounds arises only from the difference in priority for designating the absolute configuration because of the presence of additional C2 carbon atom in statine. This fact caused some confusions in discussing the stereostructure-activity relationships of statine, norstatine and hydroxyethyl (Fig.4 and 6) analogues. Therefore, in order to clarify the stereochemical relation between the critical hydroxy group and the side chain group of P_1 L-amino acid derivative, we define *syn* and *anti* for the configuration of the hydroxyl group relating to the side chain group in the study of protease inhibitors mimicking the substrate transition state.
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