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

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

1. Introduction

KNI-272 (I) is a tripeptide mimic whose design was based on the substrate transition state concept of amide hydrolysis by aspartic protease (Fig. 1; Kiso, 1996; Kiso *et al.*, 1999). This peptide potently inhibits HIV protease activity (Mimoto *et al.*, 1992) and displays good pharmacokinetics and an excellent therapeutic index (Kageyama *et al.*, 1993). It is the unusual amino acid allophenylnorstatine [Apns = (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] which has the key structure that mimics the transition state (Mimoto, Imai, Tanaka, Hattori, Takahashi *et al.*, 1991; Mimoto, Imai, Tanaka, Hattori, Kisanuki *et al.*, 1991), although the other unusual amino acids of KNI-272, 5-isoquinolyloxy acetic acid (iQoa), methylthioalanine (Mta), thiazolidine-4-carboxylic acid (Thz) and *tert*-butylamine (^{t}Bu), are also highly optimized to enhance the inhibitory activity and selectivity for HIV protease (Sheha *et al.*, 2000). The P1—P1′ site of aspartic protease (Schechter, 1968) is formed at the hydroxymethylcarbonyl (HMC)–amide bond, between Apns[3] and Thz[4], and both *cis* and *trans* conformers can be presumed for this bond. However, NMR and molecular modeling studies indicate that the *trans* conformer of the HMC–amide bond should predominate (Kato *et al.*, 1994; Ohno *et al.*, 1996), which is consistent with the crystal structure (Doi *et al.*, 2001), the structure of the inhibitor within the KNI-272–HIV protease complex (Baldwin *et al.*, 1995) and the finding that a wide moiety which includes

an HMC group will tend to converge towards a certain conformation (Ohno *et al.*, 1996). Although no strong factor limiting the peptide conformation has been found within the chemical structure of KNI-272, the above results suggest the existence of a stable conformation for the moiety with the HMC group. Since the properties of solvents affect the peptide structures (Karle *et al.*, 1990; Awasthi *et al.*, 2001), the inherent conformational variations of peptides seem to be observed in the crystal structures grown from different solvent systems. To test that hypothesis, the comparison among such crystal structures could be a probe. Therefore, we solved three solvated crystal forms of KNI-272 and compared the stable conformations with previously solved structures.

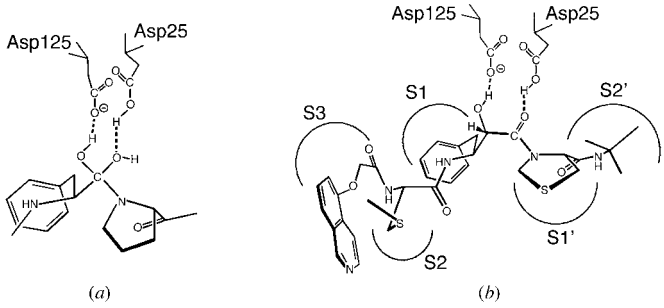


Figure 1
The substrate transition state concept (Kiso, 1996; Kiso *et al.*, 1999). (*a*) Substrate transition state of amide hydrolysis by HIV protease. A water molecule is added to the carbonyl carbon of a substrate activated by catalytic Asp residues of HIV protease. An intermediate takes a tetrahedral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

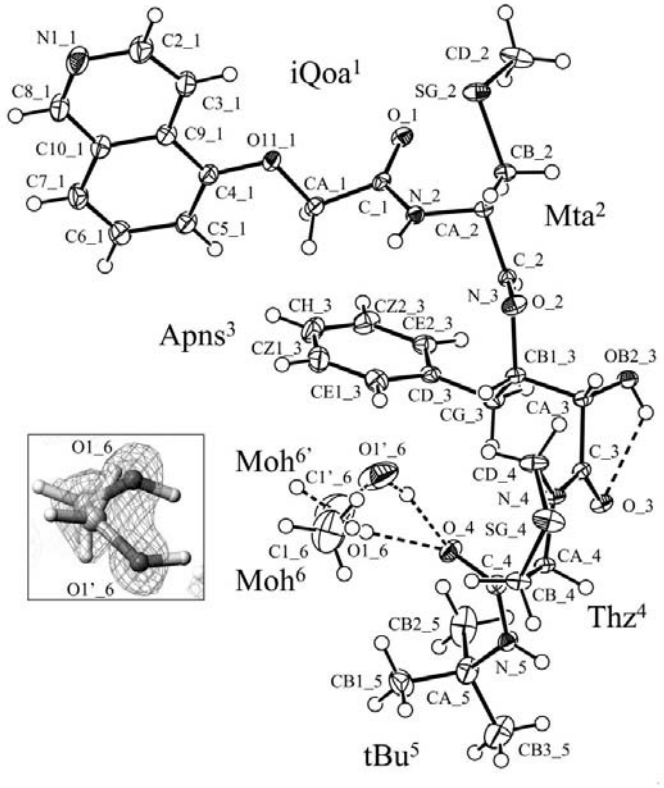
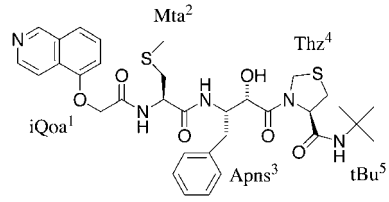


Figure 2
Structure of the ME form with displacement ellipsoids drawn at the 50% probability level. Dotted lines represent hydrogen bonds to a disordered methanol molecule. The inset shows the electron density around the solvents at the 0.8 and 2.4 σ level.



2. Experimental

KNI-272, which was synthesized as described previously (Mimoto *et al.*, 1992), was dissolved in approximately 60–80% aqueous methanol with warming and the minimum amount of methanol needed to prevent precipitation. The solution was then sealed in a vial at room temperature and crystals of the aqueous methanol form (ME form) were grown for 3–4 d. Crystals of the acetone solvated form (AC form) were grown from KNI-272 acetone solution containing 5–10% hexane. Crystals of the dimethylsulfoxide solvated form (DM form) were grown from KNI-272 dissolved in dimethylsulfoxide, to which was added a small amount of water (5–10%). In this case, the solution was sealed in a vial overnight. All crystals were briefly passed through 100% glycerol on a nylon loop (Hampton Inc.) and then flash-frozen under a nitrogen stream at 90 K. Data collection was carried out on a Bruker SMART APEX CCD area detector using Mo $K\alpha$ radiation. Intensities were integrated using *SMART* and *SAINT* (Bruker, 1998), and the empirical absorption corrections were applied using *SADABS* (Sheldrick, 1996). The structures were solved using *SHELXS*97 (Sheldrick, 1997) and refined with *SHELXL*97 (Sheldrick, 1997). H atoms were placed at calculated positions (C—H 0.95–1.00 and N—H 0.88 Å) using isotropic displacement parameters [U_{iso} = 1.5 U_{eq} (C) for methyl H atoms and 1.2 U_{eq} (parent atom) for all other atoms] and were included in the structure-factor calculations. The H atom in the hydroxyl group of Apns^3 was found in a differential Fourier map and was fixed during the refinements. In the ME form, the hydroxyl H atoms of the disordered methanol molecules were also found in a differential Fourier map and they too were fixed during the refinements. The absolute configurations were determined from the material amino acids and were consistent with the suggested Flack parameters (Flack, 1983). The crystal and experimental data are summarized in Table 1.[1]

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

Table 1
Crystal and experimental summaries of the ME and AC forms and referenced structures of the MPD form.

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

	ME	AC	DM
Formula	$C_{33}H_{41}N_5O_6S_2$	$C_{33}H_{41}N_5O_6S_2$	$C_{33}H_{41}N_5O_6S_2$
Solvation	CH_4O	$2C_3H_6O$	C_2H_6SO
M_r	699.87	783.98	745.96
System	Monoclinic	Monoclinic	Monoclinic
Space group	$P2_1$	$P2_1$	$C2$
a (Å)	10.5077 (9)	10.6395 (7)	29.390 (6)
b (Å)	13.288 (1)	13.2130 (9)	12.882 (3)
c (Å)	13.515 (1)	14.692 (1)	10.609 (2)
β (°)	101.924 (1)	98.931 (1)	103.373 (3)
V (Å^3)	1846.3 (3)	2040.4 (2)	3907.6 (13)
Z	2	2	4
T (K)	90.0 (2)	90.0 (2)	90.0 (2)
D_x (g cm^{-3})	1.259	1.276	1.268
$F(000)$	744	836	1584
Wavelength (Å)	0.7107	0.7107	0.7107
μ (mm^{-1})	0.196	0.187	0.241
No. of reflections (obs)	16 068	23 703	12 220
R_{int}	0.0191	0.0161	0.0344
No. of reflections (used)	8028	8940	7717
θ_{max} (°)	27.1	27.1	26.7
Flack parameter	–0.01 (4)	0.01 (3)	0.09 (6)
R	0.0333	0.0286	0.0443
wR	0.0835	0.0729	0.1136
Goodness-of-fit	1.026	1.034	0.992
$(\Delta/\sigma)_{max}$	0.011	0.007	0.009
Fraction of θ_{max}	0.998	0.997	0.988
$\Delta\rho_{max}$ (e Å^{-3})	0.338	0.285	0.835
$\Delta\rho_{min}$ (e Å^{-3})	–0.301	–0.218	–0.475

3. Results and discussion

The structures of the ME, AC and DM forms are shown in Figs. 2, 3 and 4, respectively. In the ME form, electron densities persist in the solvent region (Fig. 2, inset) and are interpreted as being a methanol molecule disordered to two sites (Moh[6] and Moh[6′]). Both the disordered methanol molecules are located at the positions close to the thiazolidine ring (Thz[4]) and hydrogen-bonded to the carbonyl oxygen of Thz[4]: O1_6···O_4 2.842 (4) and O1′_6···O_4 2.706 (4) Å. The AC form contains two acetone molecules (Ace[6] and Ace[7]). Ace[6] interacts with the amide nitrogen of Mta[2] [N_2···O1_6 3.019 (2) Å], but no hydrogen bond is formed with Ace[7] (Fig. 3). Ace[6] is sandwiched between the Thz[4] and the isoquinoline (iQoa[1]) rings, and the hydrophobic interactions among these residues seem to contribute to the peptide conformation of the AC form. A dimethylsulfoxide molecule (Dms[6]) is present within the DM form (Fig. 4) and is hydrogen-bonded to the amide nitrogen of Mta[2]: N_2···O1_6 2.832 (3) Å. This interaction pattern is similar to that of Ace[6] in the AC form. The relative disposition of Dms[6] for the peptide is also similar to that of Ace[6], but the isoquinoline ring is not folded to Dms[6]. Apparently KNI-272 is 'hygroscopic' for the solvents used, so that its crystallization is accompanied by solvation and the properties of solvents affect the conformations of KNI-272.

Within the substrate–HIV protease complex, at least one water molecule is used for the hydrolysis of the amide bond and should therefore be located in close proximity to the substrate (Kiso, 1996). NMR analysis has shown that water molecules bind between KNI-272 and HIV protease, stabilizing the complex structure (Wang, Freedberg, Wingfield *et al.*, 1996). It thus appears that the hygroscopicity of KNI-272 supports the structure mimicking the substrate transition state within the complex.

According to the substrate transition state concept (Kiso, 1996; Kiso *et al.*, 1999), the HMC group of the Apns[3] residue should assume a conformation that mimics the substrate of HIV protease (Fig. 1), which is a homodimeric protein with a symmetric catalytic center (Pearl & Taylor, 1987). It is known, moreover, that when the asymmetric KNI-272 is bound, catalytic Asp residues assume different ionization states that contribute to a structure that mimics the transition state (Wang, Freedberg, Yamazaki *et al.*, 1996). This means that the structure around the HMC group is the most important when considering the interaction between KNI-272 and the catalytic center of the protease. The bond distances around the HMC moiety of the Apns[3] residue clearly distinguish the C*A*_3—O*B*2_3 hydroxyl bond from the C_3—O_3 carbonyl bond (Table 2), and no trend toward tautomerism between the hydroxyl and carbonyl groups is seen in any of the crystal forms. Such clear localization of the hydroxyl and carbonyl groups would provide support for the structure mimicking the transition state within the complex. Bond rotation is chemically permitted for C*A*_3—C_3, but the conformations of the hydroxyl and carbonyl groups are synperiplanar in the three forms: O*B*2_3—C*A*_3—C_3—O_3 −20.9 (2), −24.1 (2) and −24.6 (4)° for the ME, AC and MD forms, respectively. A hydrogen bond is formed between O*B*2_3 and O_3, making a five-membered ring at the HMC group, which contributes to the stability of the synperiplanar conformations.

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

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

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

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

	ME	AC	DM	MPD	1HPX
*CB*1_3—C*A*_3	1.548 (2)	1.548 (2)	1.545 (4)	1.529	1.53
*CA*_3—O*B*2_3	1.409 (2)	1.407 (2)	1.409 (3)	1.412	1.38
O*B*2_3—H	0.855	0.874	0.848	0.757	0.97
C*A*_3—C_3	1.533 (2)	1.525 (2)	1.528 (4)	1.534	1.52
C_3—O_3	1.236 (2)	1.228 (2)	1.233 (3)	1.240	1.23
N_3—*CB*1_3—C*A*_3—O*B*2_3	−65.2 (2)	−67.2 (1)	−72.1 (3)	−65.1	−75
*CB*1_3—C*A*_3—C_3—O_3	101.9 (2)	98.5 (1)	97.5 (3)	107.9	89
O*B*2_3—C*A*_3—C_3—O_3	−20.9 (2)	−24.1 (2)	−24.6 (4)	−14.2	−33
O*B*2_3···O_3	2.693 (2)	2.704 (1)	2.693 (3)	2.669	2.68
O*B*2_3—H···O_3	2.187	2.359	2.200	2.269	2.22
∠O*B*2_3—H···O_3	117.7	103.8	117.0	114.2	108

conformation of iQoa[1] is flexible and reflects the specific environment of the KNI-272 molecule, and that this flexibility is favorable for stabilizing the inhibitor molecule on the enzyme. In the 1HPX form, the isoquinoline ring is located on the protein surface, bending into the cavity of the active center, which would delay the release of the inhibitor from the protease. By contrast, the backbones of the Mta[2]–Apns[3]–Thz[4]–^{t}Bu[5] moieties are very similar to one another (Fig. 5), with torsion angles ($\varphi3'$, $\psi3$, $\varphi4$, $\psi4$ and $\varphi5$) that are indicative of the similarity of the backbone conformations (Table 3). In the MPD form, the hydrating waters (disordered to three sites) are located beside the phenyl ring of Apns[3], forcing the side chain to swing, thereby causing slight shifts in the CB1_3 position related to the $\varphi3$ angle. In addition, the Thz[4] ring of the 1HPX form shows a 'puckering' that differs slightly from the other forms (*e.g.* the $\chi4$ angle). Water molecules 301 and 607 are located beside Thz[4] within the complex and seem to affect the 'puckering' of the Thz[4] ring. Analysis of molecular dynamics using NOE restraints has shown there to be three predominant families of KNI-272 structures (conformations *A*, *B* and *C*) with conformational variations at iQoa[1] (Ohno *et al.*, 1996). Of those, conformation *A* was similar to the 1HPX form. In particular, the backbone of Apns[3]–Thz[4]–^{t}Bu[5] of conformation *A* showed remarkable similarity to that of the 1HPX form. These similarities suggest the existence of a stable conformation of the backbone of the Mta[2]–Apns[3]–Thz[4]–^{t}Bu[5] moiety. In other words, this moiety is conformationally restricted and reflects the active form in the complex.

The findings summarized above suggest that KNI-272 is pre-organized to the active form so that, except for the iQoa[1]

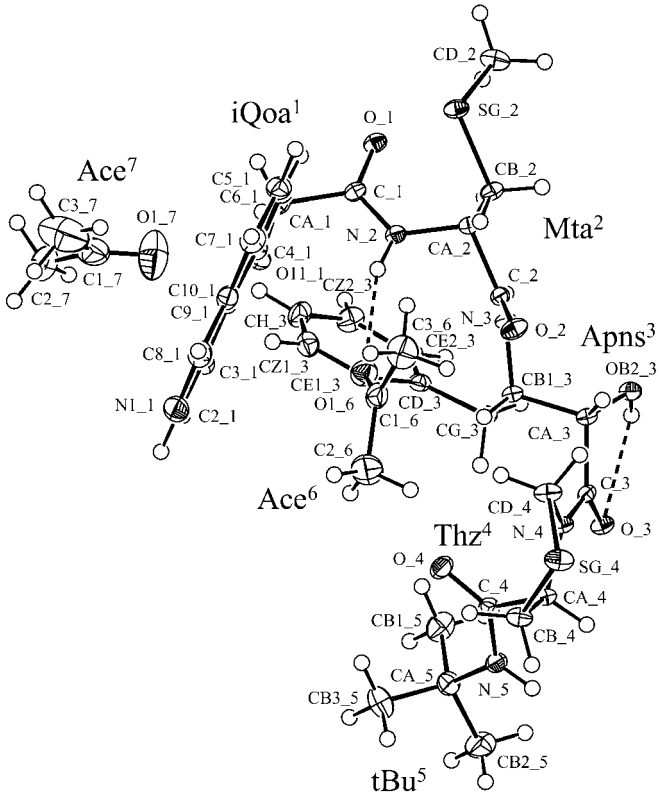


Figure 3
Structure of the AC form with displacement ellipsoids drawn at the 50% probability level. Dotted lines represent hydrogen bonds.

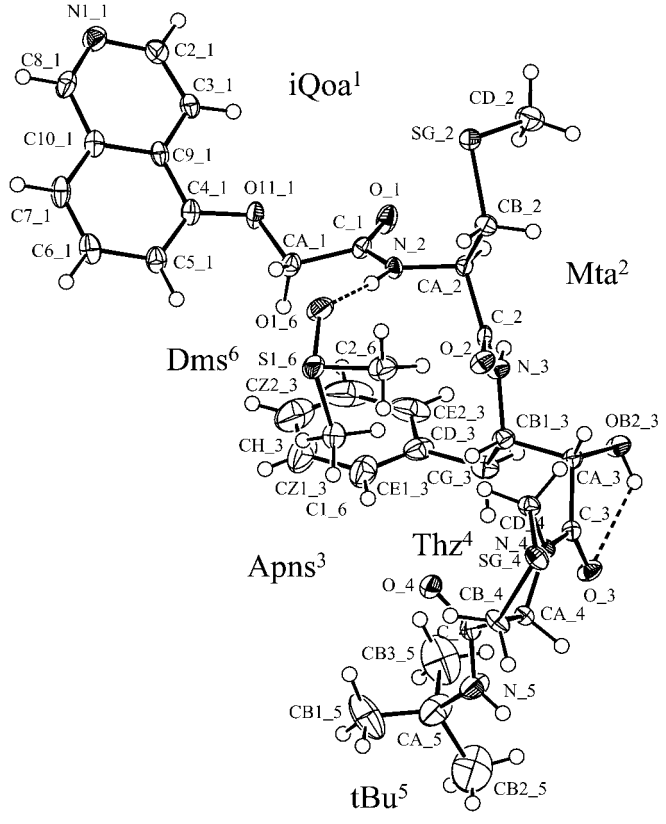


Figure 4
Structure of the DM form with displacement ellipsoids drawn at the 50% probability level. Dotted lines represent hydrogen bonds.

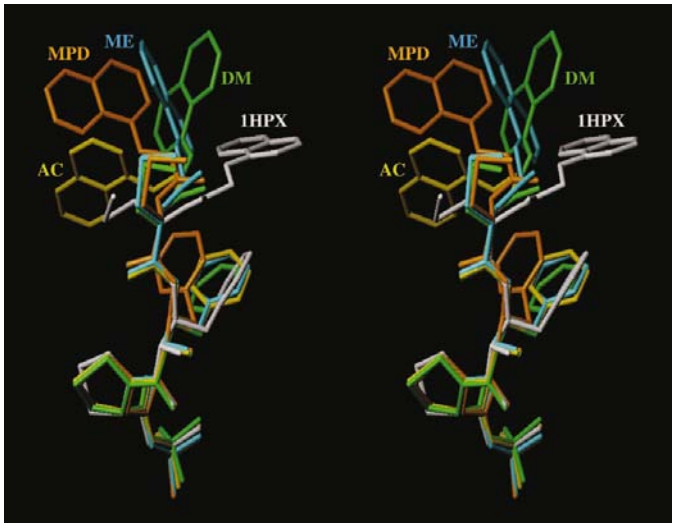


Figure 5
Stereoview of the superimposition for four KNI-272 molecules. Molecular fitting was carried out for the four Cα atoms of residues 2–5 with an r.m.s.d. of 0.03–0.08 Å. H atoms are omitted for clarity. The colors cyan, yellow, green, orange and gray represent the ME, AC, DM, MPD and 1HPX forms, respectively. The side chain of Apns^3 was disordered to two sites in the MPD form, but only one part is drawn for clarity. This figure is produced using *TRUBO-FRODO* (Roussel *et al.*, 2002).

residue, effective and selective binding to HIV protease is achieved without any large conformational changes. However, we still do not know what the key factor is. From studies of HIV protease inhibitors (Mimoto, Kato *et al.*, 1999; Mimoto, Hattori *et al.*, 2000), it seems that a certain combination of unusual amino acids, Apns, Thz and ^{t}Bu, mediates the actual restrictions for five rotatable bonds in the regions ($\varphi3$, $\varphi3'$, $\psi3$, $\varphi4$ and $\psi4$) by steric hindrance. The basic skeleton of the Mta^2–Apns^3–Thz^4–^{t}Bu^5 moiety would therefore be very important for an HIV protease inhibitor having an HMC group. It has also been adapted to the recently developed water-soluble HIV protease inhibitor prodrugs, which also show potent activity (Sohma *et al.*, 2003).

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