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Novel Insight into Inhibitor Binding of Highly Symmetric HIV-1 Protease

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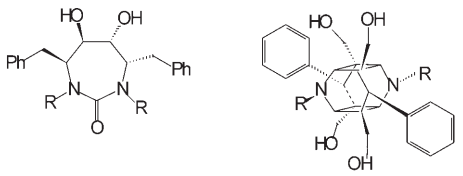
Retroviral proteases such as HIV protease belong to the family of aspartic proteases. They consist of two identical polypeptide chains with a centered active site cavity.[1] The crystal structure of HIV protease proved to have a complete C_2-symmetry of this active site cavity with enzyme subsites S1/S1′ and S2/S2′.[2] Peptide derived inhibitors of HIV protease have been developed from the natural HIV peptide substrate origin or from known inhibitors of other aspartic proteases. Some approaches have been carried out to develop nonpeptidic HIV-1 protease inhibitors which promised better oral bioavailability compared to the peptidic compounds.[3,4] The class of cyclic ureas **1** followed the principle of C_2-symmetry which was thought to be favorable with respect to a symmetric binding of these compounds to the symmetrically arranged enzyme subsites.[5] The



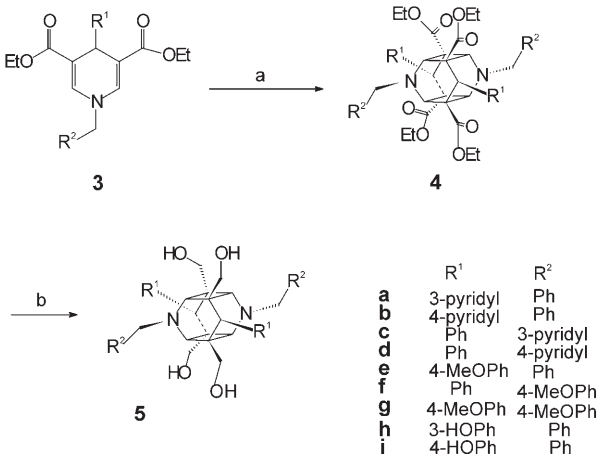
1 **2**

introduction of a hydroxy group into the original *N*-benzyl functions R which bind to the S2/S2′ subsites increased activity and in the case of structurally related azacyclic ureas the *N*-benzyl substitution patterns have been extended to corresponding methoxy functions. Both functional groups showed hydrogen bonding to the amide functions of the protein backbone.[5] However, these changes in the substitution patterns of the aromatic *N*-benzyl residues R in cyclic urea template **1** did not increase the poor bioavailability of the early structures.[6] The substituents which bind to the S1/S1′ region remained unchanged with both being benzylic substituents.

We developed highly symmetrically built nonpeptidic HIV protease inhibitors **2** which possess totally C_2-symmetric properties. As we failed to cocrystallize HIV protease and one of our early compounds with NH, *N*-acyloxy, and *N*-alkyl substituents so far, we decided to approach to the improvement of inhibitor affinities to the target enzyme by analyzing structure–activity relationships with respect to the cyclic urea type. We used the best compound of our early derivatives with *N*-benzyl substitution in compound **2**, **H17**,[4] as a template to introduce functional groups within the aromatic phenyl substituents which may serve as hydrogen bond acceptor functions, that is, methoxy groups and nitrogen atoms, as well as hydrogen bond donor functions such as hydroxy groups for the enzyme subsites. With the determined affinity constants K_i, the binding mode to the enzyme subsites will be discussed in comparison to the binding mode of the urea type.

1,4-Dihydropyridines **3** have been prepared either by a cyclocondensation reaction of an aromatic aldehyde, ethyl propiolate, and an amine compound in acetic acid[4] or in the case of the *N*-picolyl derivative **3d** by an alkylation reaction of the NH 1,4-dihydropyridine via the generated 1,4-dihydropyridine anion. Cage dimers **4** have been given by a solution dimerization reaction of the 1,4-dihydropyridine monomers on excitation of the 1,4-dihydropyridine chromophore at wavelengths $\lambda > 270$ nm by [2+2] cyclocondensation reactions. Alcoholic target compounds **5** were yielded by ester group reductions at low temperatures with lithium aluminum hydride. The hydroxyphenyl derivatives **5h** and **i** have been given from the benzyl protected precursors. In the case of compound **5h** the removal of the protection group succeeded by the use of lithium aluminum hydride, whereas in the case of derivative **5i**, thioanisole in trifluoroacetic acid has been used.[7]

In the first series of novel HIV protease inhibitors **5 a–d** we introduced a pyridyl residue into our cage dimeric skeleton by the replacement of the original phenyl moieties R^1 and R^2 of the monomeric adducts **3** (Scheme 1). Such a pyridyl function with the free nitrogen electron pair promised hydrogen bonding possibilities to the protein backbone of either the S1/S1′ or the S2/S2′ subsites of the enzyme. In the series of cyclic ureas the S2/S2′ subsites proved to accommodate lipophilic substituents with possibilities to increase binding affinities by the introduction of hydrogen bonding meta substituents whereas para substituents of the residues R in cyclic urea template **1** were less promising.[5] We decided to place the pyridyl nitro-



Scheme 1. General synthesis of target compounds **5**. A) $h\nu$, $\lambda > 270$ nm, THF, 6 weeks, 25 °C; B) $LiAlH_4$ (2 equiv), THF, 24 h, −8 °C.

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gen atom both into the meta and the para position of the phenylic moieties in compounds **5a** and **b** with R^1 = 3-pyridyl and 4-pyridyl, respectively.

Affinity constants indicated that the meta positioning of the nitrogen in the 3-pyridyl substituted compound **5a** is more favorable than the para positioning in compound **5b** (Table 1).

Table 1. HIV protease affinity constants of cage dimers.[a]

Compd	K_i [μM]	Compd	K_i [μM]
5a	1.8 ± 0.4	**5b**	4.7 ± 1.0
5c	25 ± 0.9	**5d**	47 ± 16
5e	25 ± 9.0	**5f**	5.7 ± 1.0
5g	0.7 ± 0.9	**5h**	0.6 ± 0.1
5i	8.3 ± 2.0	**H17**	7.8 ± 2.0

[a] Mean of two determinations.

The same sensitivity of the hydrogen bonding substitution in either the meta or the para position of the aromatic R^1 residue was also found for corresponding hydroxyphenyl residues R^1 with the meta substitution in the 3-hydroxyphenyl residue in derivative **5h** and a resulting affinity constant of 0.6 μM, being more favorable than the para substitution in the 4-hydroxyphenyl residue with a resulting affinity constant of 8.3 μM in derivative **5i**. The differences in activity from the meta to the para substituted aromatic residues suggested a binding to the S2/S2′ subsites of the enzyme because we have comparable substitution patterns of our aromatic residues as in the corresponding cyclic urea compounds with meta as well as para substituted phenyl residues R which bind to the S2/S2′ subsites and unsubstituted benzyl substituents which bind to the S1/S1′ subsites.[5]

To further investigate the binding mode of the derivatives we carried out docking studies with the energetically minimized starting structures of constructed compounds **5a** and **5b** and compared it with the X-ray structure of azacyclic urea A-98881 (Figure 1B–D). The interaction of the 3-OCH_3/4-OH substituents of A-98881 with the backbone NH groups of Asp29, Asp30, Asp29′, and Asp30′ (S2/S2′ subsites) is shown in Figure 1B. The inhibitor's hydroxy oxygens in the S2/S2′ subsites make hydrogen bonds to the backbone NH groups of Asp30/30′. The distances between the methoxy oxygens and the backbone NH functions of Asp29/29′ are slightly longer with 3.3 and 3.4 Å but still suggestive of significant hydrogen bonding interactions. In the docking results, the central cage dimeric 1,4-dihydropyridine unit of all inhibitors has an orientation between the active site aspartates, Asp25/25′, and the flap Ile50/50′ residues which is similar to the binding mode observed for the cyclic urea A-98881.

Surprisingly, we found an increased binding strength of **5a** that can be explained by the establishment of two hydrogen bonds between the pyridine nitrogens and two water molecules which are bound to the carboxylate and backbone NH functions of Asp29/Asp29′. These two water molecules have been already recognized as binding partners for HIV protease inhibitors.[8] Only for **5a** (and not for **5b**) the nitrogen atoms are in close proximity to the two water molecules affiliated with Asp29/29′ (Figure 1C and 1D). Thus, compound **5a** shows an unexpected subsite binding different from the cyclic ureas. It is interesting to note, that in the case of compound **5i**, the docking solution suggested that the 3-hydroxy groups replace the affiliated waters and make direct hydrogen bonds to Asp29/29′ resulting in an increasing of the inhibitor affinity (docking solution not shown). Also, the meta position is necessary to establish the hydrogen bonds towards Asp29/29′.

We then replaced the *N*-benzyl substituent in our target compounds by a picolyl substituent. We positioned the nitrogen atom again in both the meta and para position of the aromatic residue in compound **5c** and **d** with R^2 = 3-pyridyl and 4-pyridyl, respectively. The determined affinity constants to HIV protease were surprising. Again we found similar differences in the enzyme affinities between the meta nitrogen positioning in compound **5c** and the para positioning in compound **5d** with each being less active compared to the compounds with the pyridyl residue positioning in compound **5a** and **5b**, respectively. To closer investigate the binding mode of these compounds of lowered activities compared to compounds **5a** and **5b** we again docked the minimized structures to the enzyme subsites of HIV protease (Figure 1E).

Interestingly, we found a binding mode different from the pyridyl substituted derivatives with a binding of the picolyl residues to the S1/S1′ subsites of the enzyme. The comparably lowered affinities of **5c** and **5d** may result from the lack of a hydrogen bond to the backbone region of Asp29/29′ and Asp30/30′ (distance greater than 4.5 Å). So the picolyl residues with the additional methylene functions are preferably found within the S1/S1′ binding pocket without showing hydrogen bonding.

So the results of the docking studies show that corresponding positioned hydrogen bond acceptor functions within aromatic residues do not result in similar binding properties to the enzyme subsites.

To further investigate the subsite directed inhibitor binding properties of HIV protease we evaluated the effect of an introduced methoxy function as potential hydrogen bond acceptor function into the para position of both the phenyl and the *N*-benzyl function of the cage dimeric skeleton **2**. Methoxy functions in azacyclic ureas such as in A-98881 were reported to cause increased inhibitor activities by binding to the S2/S2′ enzyme subsite whereas the benzylic substituents bind to the S1/S1′ subsite.[4]

The introduction of the methoxy function into the phenyl substituent R^1 in compound **5e** led to a decrease in inhibitory activity compared to the unsubstituted phenyl derivative **H17** with a K_i value of 7.8 μM.[4] The decrease in activity suggests a binding of the aromatic residue of compound **5e** to the S1/S1′ region of the enzyme, because if the methoxyphenyl function would bind to the S2/S2′ subsite an increase in activity would have been expected compared to the unsubstituted phenyl derivative **H17**. Docking studies confirmed this assumption with a binding of the methoxyphenyl residue to the S1/S1′ subsite as shown in Figure 1F.

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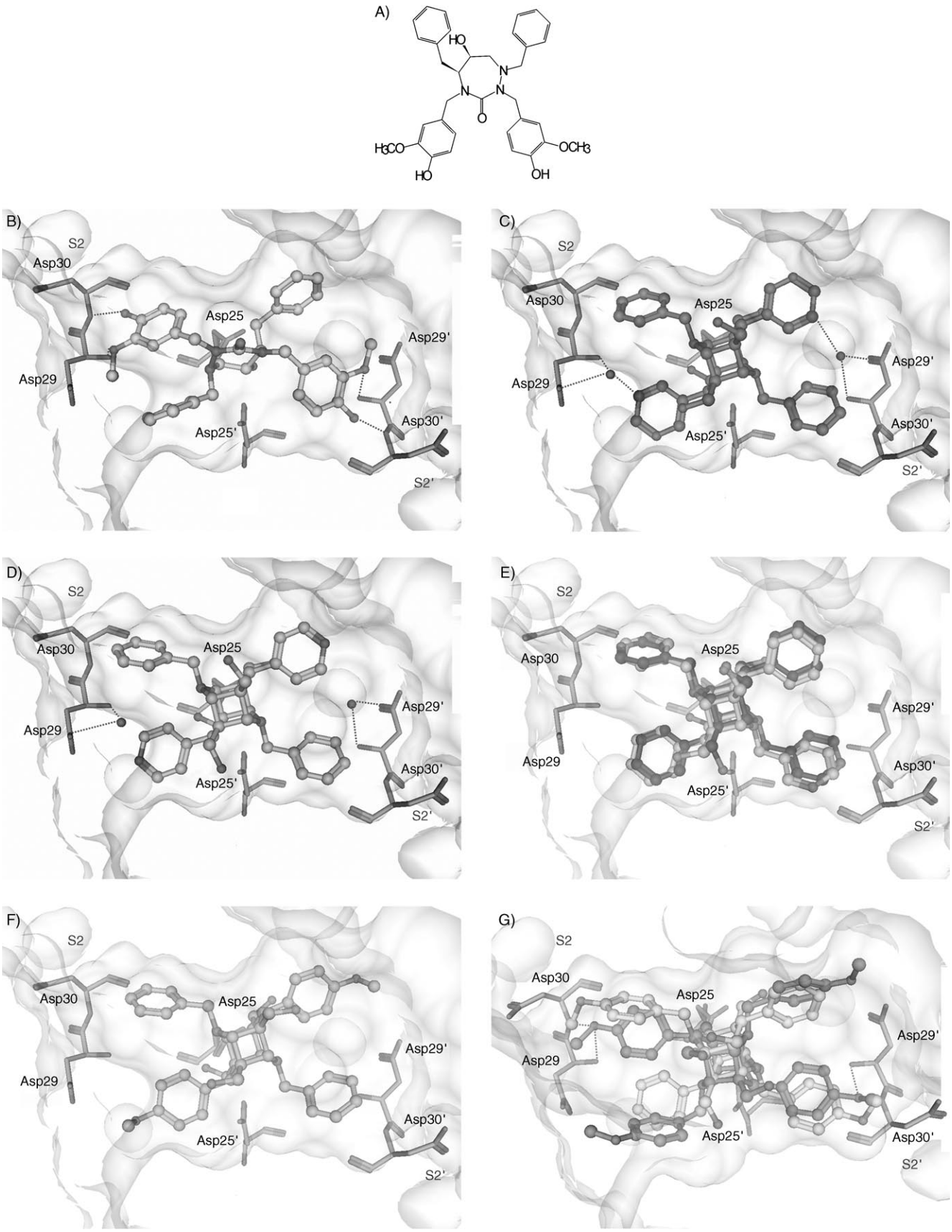

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The introduction of the methoxy function into the *N*-benzyl substituent in compound **5f** was comparably more favorable with a K_i value of 5.7 μM,[9] so that it may be concluded that the methoxy benzyl substituted residue R^2 binds to the S2/S2′ subsite of the enzyme similiar to the cyclic urea compounds. This was again confirmed by docking of the compound to the enzyme's subsites (Figure 1G). The binding of the *N*-methoxybenzyl substituent to the S2/S2′ subsite may also be preferred because of the larger space demand of this substituent compared to the methoxyphenyl substituent.

We then introduced the methoxy function into the para position of both the phenyl and the benzylic functions in compound **5g**. Surprisingly, this compound exhibits the almost highest affinity constant in the nanomolar range. It may be concluded that the completely substituted derivative with the highest degree of symmetry is favorable with respect to a totally symmetric binding to the active site cavity of HIV protease. This contradicts many previous observations which suggested that the the binding mode of nonsymmetrical inhibitors were much more advantagous with respect to their higher affinity for the active site cavity of HIV protease.[9,10] Docking studies show that the totally symmetric substitution patterns in derivative **5g** lead to a most favorable binding of the compound with possible hydrogen bonding of the methoxy functions to the NH functions of the amino acids Asp29/29′ (distance 3.1–3.2 Å) and Asp30/30′ (distance 3.4–3.5 Å) within the S2/S2′ subsite (Figure 1G).

We reported interesting observations: The inhibitor binding towards HIV protease subsites is determined not in the first place by the possibility of hydrogen bonding of functional groups but by additional relevant steric effects. This could be demonstrated by our series of compounds in which we consequently placed hydrogen bond acceptor/donator functions in substituents which could bind to both the S1/S1′and the S2/S2′ subsite. As our results were supported by docking studies this method is a valuable tool to further substitute our compounds and thus increase activity. The highest activity of the totally symmetric structure could be explained by the docking studies whereas structural features did not suggest the observed improved activity.

Experimental Section

Target compound synthesis: Selected monomer **3a** was given by a cyclocondensation reaction of pyridine 3-carbaldehyde (1.07 g, 10 mmol), ethyl propiolate (1.96 g, 20 mmol), and benzyl amine (1.05 g, 10 mmol) in freshly distilled acetic acid (1 mL).[4] Hydrolysis work up procedure of the cyclocondensation product **3a** which has been given after stirring in a water bath for 1 h followed extraction with chloroform, drying over sodium sulfate and, finally, removal of the organic layer. Spectral data of selected compound **3a**: ^{1}H NMR ($CDCl_3$): $\delta = 1.16$ (t, $J = 7.2$ Hz, 6H; $COOCH_2CH_3$), 4.04 (q, $J = 7.2$ Hz, 4H; $COOCH_2CH_3$), 4.60 (s, 2H; NCH_2), 5.03 (s, 1H; 4-H), 7.26–7.29 (m, 4H; 2-, 6-H of Ph, 2-, 6-H), 7.37–7.45 (m, 4H; 5-H of pyr, 3-, 4-, 5-H of Ph), 8.00 (s br, 1H; 6-H of pyr), 8.41 (d, $J = 5.3$ Hz, 1H; 4-H of pyr), 8.56 (s, 1H; 2-H of pyr); IR (ATR): $\nu = 1696$ (CO), 1582 (N-C=C-CO); MS (ESI) *m/z* (%): 415 (100) $[M+H]^+$. Alkylation of the corresponding N-unsubstituted monomer (0.93 g, 3.1 mmol) with picolyl bromide hydrobromide (0.56 g, 9.3 mmol) in dry dimethylpropylene urea (DMPU) after N-hydrogen abstraction using a sevenfold excess of sodium hydride (0.52 g, 21.7 mmol) yielded compound **3d** after work up procedure with ice water hydrolysis, filtration of the precipitating oil, and purifying by column chromatography over silica gel using an eluent mixture of chloroform/acetic acid ethyl ester/methanol (75:25:5).

Selected monomer derivative **3a** (0.50 g, 1.2 mmol) has been dissolved in dry THF (30 mL) in a quarz flask. Irradiation was carried out under nitrogen atmosphere using Ultra Vitalux lamps^R at wavelengths $\lambda > 270$ nm with an irradiation distance of 60 cm.[4] During irradiation, cage dimer **4a** crystallized from the solution and irradiation was continued until no more starting compound could be detected by TLC. During evaporation of the solvent under atmosphere pressure the isolated yields of the dimer **4a** were collected and recrystallized from alcohol. Spectral data of selected compound **4a**: ^{1}H NMR ($CDCl_3$): $\delta = 1.01$ (t, $J = 7.2$ Hz, 12H; $COOCH_2CH_3$), 4.00 (q, $J = 7.2$ Hz, 8H; $COOCH_2CH_3$), 4.27 (s, 2H; 6-, 12-H), 4.28 (s, 4H; 2-, 4-, 8-, 10-H), 4.45 (s, 4H; NCH_2), 6.97 (dd, $J = 8.1$, 5.0 Hz, 2H; 5-H of pyr), 7.27–7.32 (m, 10H; ArH of Ph), 7.78 (d, $J = 8.1$ Hz, 2H; 6-H of pyr), 8.36 (d, $J = 5.0$ Hz, 2H; 4-H of pyr), 8.42 (s, 2H; 2-H of pyr); IR (ATR): $\nu = 1721$ (CO); MS(ESI) *m/z* (%): 785 (100) $[M+H]^+$.

Selected cage dimer **4a** (0.05 g, 0.07 mmol) was dissolved in dry THF (20 mL) under warming. After stirring at RT for 1 h the solution was cooled to −8 °C and a twofold molar excess of lithium aluminum hydride (1 M solution in THF) was added dropwise. After additional stirring at the low temperature the solution was diluted with ice water at 0 °C and extracted with chloroform for several times. The organic layer was then dried over sodium sulfate and filtered. After evaporation to dryness the oily residue was dissolved in chloroform. Diethylether and petrol ether (bp 30–50 °C) were added dropwise until dimming. The resulting precipitating crystalline alcoholic compound **5a** was recrystallized from methanol. Spectral data of compound **5a**: ^{1}H NMR ([D_6]DMSO): $\delta = 2.95$ (s, 4H; 2-, 4-, 8-, 10-H), 3.00 (ABX, $J = 10.2$, 4.3 Hz, 4H; CH_BOH), 3.18 (ABX, $J = 10.2$, 4.3 Hz, 4H; CH_AOH), 3.71 (s, 2H; 6-, 12-H), 4.13 (s, 4H; NCH_2), 4.52 (t, $J = 4.3$ Hz, 4H; OH), 6.94 (dd, $J = 8.2$, 6.5 Hz, 1H; 5-H of pyr), 7.12 (d, $J = 6.5$ Hz, 1H; 6-H of pyr), 7.25 (m, 3H; 5-H of pyr, 4-H of Ph), 7.36 (m, 8H; 2-, 3-, 5-, 6-H of Ph), 7.68 (d, $J = 6.5$ Hz, 1H; 6-H of pyr), 8.06 (d, $J = 8.2$ Hz, 1H; 4-H of pyr), 8.25 (d, $J = 8.2$ Hz, 1H; 4-H of pyr), 8.30, 8.43 (2 x s, 2H; 2-H of pyr); IR (ATR): $\nu = 3250$ (OH); MS(ESI) *m/z* (%): 617 (100) $[M+H]^+$.

Figure 1. A) GOLD docking solutions for A-98881 and the inhibitors under study. Only the important amino acid residues Asp25/25′, Asp29/29′, and Asp30/30′ are displayed. Nitrogen atoms are colored dark gray, whereas oxygen atoms are colored gray. Hydrogen bonds between the inhibitors and the enzyme are shown as red dashed lines. B) A-98881 shows hydrogen bonds to the backbone NH functions of Asp29/29′ and Asp30/30′. C) Favorable docking solutions for **5a** were obtained when considering two conserved water molecules (indicated as balls). The meta pyridyl nitrogen makes hydrogen bonds to the water molecules. D) Docking solution for **5b** where no hydrogen bonds to the water molecules are observed. E) Comparison of the docking solutions for **5c** (light gray carbon atoms) and **5d** (dark gray carbon atoms), where the picolyl residues are located in the S1/S1′ subsite. F) Docking solution for **5e**. The methoxyphenyl substituents are located in the S1/S1′ subsites where no hydrogen bonding possibilities exist. G) Comparison of the docking solutions for **5f** (white carbon atoms) and **5g** (light gray carbon atoms). Only in case of **5g** the 4-methoxy groups are located close to the backbone NH functions of Asp29/Asp29′ and Asp30/Asp30′.

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HIV protease affinity: K_i values have been determined by the use of the IC_{50} values of competitive HIV protease inhibition in a fluorescence assay with a fluorogenic HIV protease substrate hexapeptide, Abz (2-aminobenzoic acid)-Thr-Ile-Nle-Ph(p-NO_2)-Gln-Arg-NH_2,[11] and the measurement of increased fluorescence upon cleavage at 410 nm. Assays were carried out in buffer solutions at pH 6.25 after preincubation of protease and varying inhibitor concentrations.

Docking study: All calculations were performed on a Pentium IV 1.8 GHz based Linux cluster. The molecular structures of the inhibitors were generated using the MOE modeling package (Chemical Computing Group).[12] The structures were energy minimized using the implemented MMFF94 s force field and the conjugate gradient method, until the default derivative convergence criterion of 0.01 kcal mol^{-1} $Å^{-1}$ was met. The PDB entry 1PRO was chosen as a starting point for the docking studies since the co-crystallized cyclic urea inhibitor A-98881 in this X-ray crystal structure most closely resembled the compounds under investigation. In advance of docking the novel inhibitors into the binding pocket it was first tested whether the docking program GOLD 3.2[13] is able to reproduce the experimentally observed binding mode of A-98881. The inhibitor structures were docked within a sphere (radius 15 Å) around amino acid Asp25. All torsion angles in each compound were allowed to rotate freely. Goldscore was chosen as the fitness function. For each molecule, 30 docking runs were performed. The top-ranked poses for each ligand were retained and analyzed together with the HIV-1 protein structure within MOE (Chemical Computing Group). The top ranked docking solution showed an rmsd value of 0.35 (heavy atoms), which indicated an excellent reproduction of the binding mode in the crystal structure (data not shown). This result gave confidence for using GOLD for the docking of the novel HIV protease inhibitors. Compounds **5a**–**5i** were subsequently docked to the active site of HIV-1 protease as described above. The resulting GOLD solutions were clustered on the basis of the heavy atom rmsd values (1 Å). The top-ranked poses for each ligand were retained and analyzed using the MOE software.

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Keywords: binding region · ligand docking · medicinal chemistry · structure–activity relationships · symmetric structure

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