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On the inhibition of HIV-1 protease by hydrazino-ureas displaying the N→C=O interaction

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ABSTRACT

To create novel HIV-1 protease (HIV PR) inhibitors, we have extended our investigations of the N→C=O interaction as a moiety that reproduces electrostatic properties of the transition state of peptidolysis. Consequently, we prepared a series of compounds with an unusual hydrazino-urea core. In polar protic media, these adopt solely a cyclic constitution displaying the interaction on one side of the molecule while offering a urea moiety on the opposite side meant to hydrogen-bond with the enzyme flaps. Each inhibitor candidate was obtained via a key series of three synthetic steps employing carbonyl-di-imidazole (CDI). It was thus possible to efficiently fuse two independent building blocks, a hydrazine and a protected aminoaldehyde in a convergent manner. NMR and UV analysis proved that all compounds, when dissolved in polar protic media, existed exclusively in the cyclic constitution exhibiting the N→C=O interaction. In total, five inhibitor candidates were tested with HIV PR for their potency. The one carrying the least bulk in peripheral substituents showed the highest activity. Its very low molecular weight (365 g/mol) holds great promise for future improvements in affinity without violating Lipinski's rule of remaining within the limit of 500 g/mol.

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1. Introduction

Moieties mimicking the transition-state of peptide hydrolysis have proved efficient in the design of inhibitors for aspartic proteases.[1–3] HIV-1 protease (HIV PR) belongs to this family of enzymes and remains to be one of the major targets in the search for drugs battling AIDS.[4,5] The development of novel agents against HIV is necessary because of the continued emergence of virus strains that resist current clinical drugs.[6,7] The established approach in designing inhibitors has been to incorporate a transition-state analog (i.e., *isostere*) into a peptidomimetic reproducing the linear shape of the peptide substrate. This strategy evolved from the discovery that certain naturally occurring peptides (e.g., pepstatine), containing a non-proteinogenic amino acid called statine, are generic aspartic protease inhibitors. The tetrahedral secondary alcohol moiety contained in statine is believed to act as a transition state mimic ever since it was shown that it interacts with the two aspartic acid residues of the catalytic center.[8,9] Many other transition-state mimicking moieties have been explored over the years, such as hydroxyethylamine, hydroxyethylene, reduced amide, α-perfluorinated ketones, α-ketocarboxamides, and phosphorus-based units.[10]

Alternatively, rigid heterocyclic cores have been used as templates for the development of potent agents that have become known as non-peptidic inhibitors. Two well-known representatives are the cyclic urea DMP 450 (**1**)[11] and tipranavir (**2**)[12,13] that is based on the 5,6-dihydro-4-hydroxy-2-pyrone core (Fig. 1). The rigid aspect of these cores can lead to higher affinity because a lower entropic penalty has to be paid upon binding.[11,14,15] It is also generally maintained that increased rigidity can result in better oral bioavailability.[16] Bioavailability also critically depends on molecular weight.[17] These points illustrate the need for finding as yet unexplored molecular motifs with superior affinity to their target site, allowing for reduction of overall inhibitor size. Another key feature of non-peptidic heterocycles **1** and **2** is the carbonyl function located opposite to the hydroxyl group(s). While the latter one(s) interact(s) with the catalytic aspartyl diad of HIV PR, the carbonyl undergoes hydrogen-bonding with two protein flaps of the enzyme. This results in the extrusion of a key enzyme bound water molecule that is habitually found in X-ray crystal structures of enzyme complexes with linear inhibitors and thus adds to the favorable entropic effect mentioned earlier.[11,18]

We have been interested[10] in an infrequently observed functional group for its capacity to mimic essential geometric and also electrostatic features of the transition states of peptide hydrolysis (see transition state structure TS (**8**)[19,20] and examples **4**,[21] **5**,[22] **6**,[23] **7a–c**[10] in Fig. 2). This molecular moiety results from the intramolecular interaction of a tertiary amine with a ketone or aldehyde

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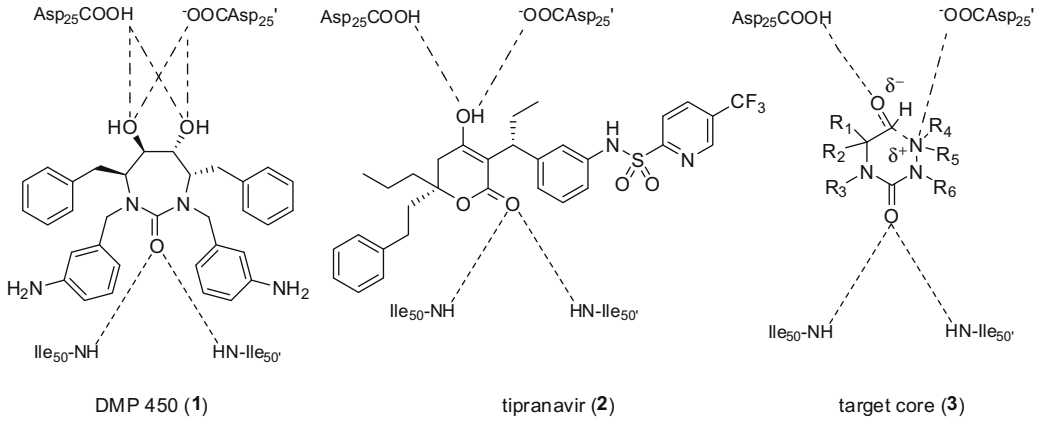


Figure 1. Hydrogen bonding between the catalytic diad (aspartic acid residues) as well as the enzyme flaps (isoleucines) with cyclic urea inhibitor DMP 450 (**1**), heterocyclic inhibitor Tipranavir (**2**), and hypothesized interactions with target structure **3**.

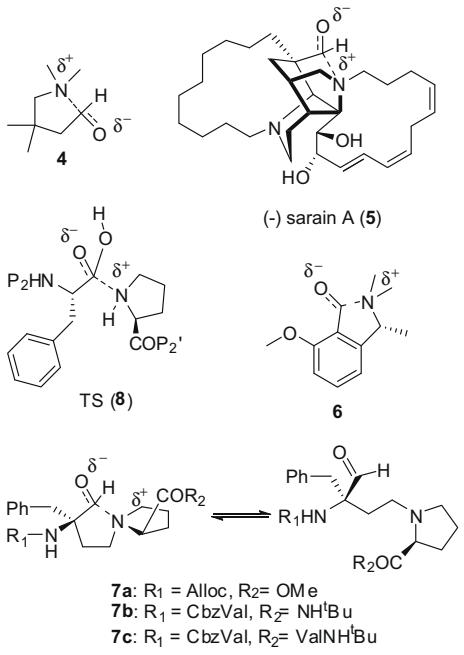


Figure 2. A selection of compounds (**4–7**), displaying the N→C═O interaction, and 2nd hydrolysis TS (**8**) of the preferred dipeptide sequence Phe-Pro of HIV PR.

function (N→C═O interaction), and has been observed initially in a rare class of alkaloids as a transannular contact across medium-sized rings.[24,25] The N→C═O interaction has been described as a through-space homoconjugation where electron density is shifted from the *n* nitrogen orbital to the carbonyl π^* orbital,[26] giving rise to an enhanced dipole moment[27] and a hypsochromic shift of the carbonyl UV absorption.[10,28] It has been shown that the interaction is favored in polar protic media, and when incorporated into conformationally restricted frameworks.[10,21,23,29–31] Recently, the N→C═O moiety has featured prominently in the total synthesis of the marine natural product (−) sarain A (**5**).[22] Its attractive features for this project are: (a) a carbon with a transient configuration between sp^2 and sp^3; (b) an N–C bond order between 0 and 1; (c) a C–O bond order between 1 and 2; and (d) partial charges on the oxygen and the nitrogen, resulting in an enhanced dipole moment.[27] This strongly polarized unit may thus form particularly strong hydrogen bonds and electrostatic interactions with the catalytic diad of the two aspartate residues.

Compounds **7a–c** are peptidomimetics incorporating the N→C═O interaction that mimic the Phe-Pro dipeptide sequence, one of the preferred cleavage sites of HIV PR. Molecules **7a–c** exist as an equilibrium of the open form, and a cyclized form that displays the N→C═O interaction. The highest degree of cyclization in methanol (70%) was achieved with **7a**. Mixtures including several constitutional isomers are however difficult to analyze. More importantly, the presence of an open form rules out any exploration as a drug candidate since aldehydes are justly regarded as too reactive towards nucleophiles in live organisms.

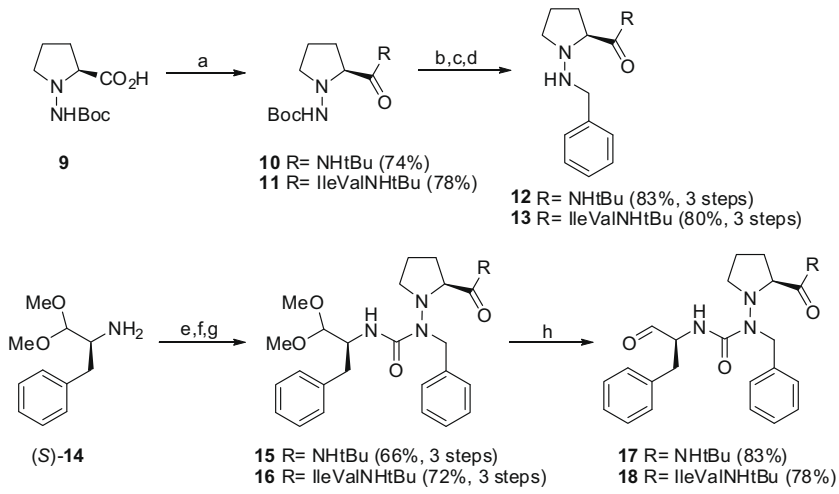
Recently, we have discovered a hydrazino-urea core unit of the generic structure **3** that exist to 100% in its cyclic, N→C═O interacted, form in polar protic media (Fig. 1).[32] In analogy to the potent heterocyclic inhibitor cores **1** and **2**, it also has a carbonyl group that might undergo hydrogen-bonding with the enzyme flaps. In this study, we present the synthesis of a number of structural variations of **3**, the analysis of their folding behavior, and the determination of their inhibitory potency with HIV PR.

2. Results and discussion

2.1. Chemical synthesis

Scheme 1 illustrates the convergent assembly of two examples of the generic structure in **3** that are derived from phenylalanine and proline. The synthesis of the hydrazine building block started with the amination of (*S*)-proline using freshly prepared *p*-cyanophenyl oxaziridine to furnish *N*-Boc protected hyrazino proline **9**.[33,34]

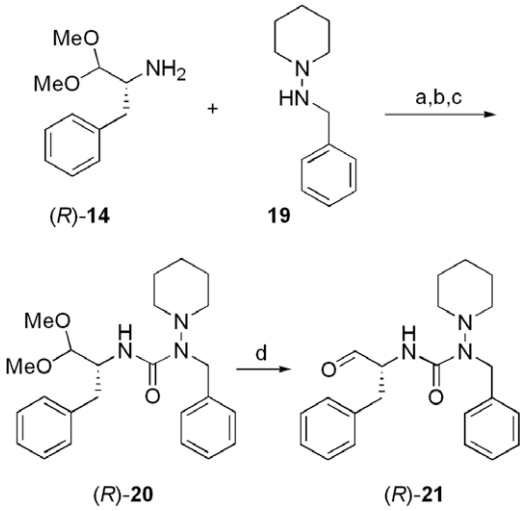
Hydrazinopeptides **10** and **11** were obtained by reacting compound **9** with H_2N-^{t}Bu, and H-Ile-Val-NH-^{t}Bu, respectively, using EDC and HOBt as coupling reagents.[35] The latter building block was initially prepared by peptide coupling of Cbz-Ile-OH and H-Val-NH-^{t}Bu, followed by Pd/C-catalyzed hydrogenolysis. A subsequent attempt to remove the Boc group from the hydrazine unit according to a reported procedure (HCl_{aq} in dioxane)[36] gave unsatisfactory results. These may be the consequence of reactions involving the highly nucleophilic hydrazine during water removal in the workup procedure at slightly elevated temperatures. On the other hand, exposure of **10** and **11** to neat TFA for two hours followed by solvent removal at room temperature, gave the desired hydrazine intermediate as the only product. This was immediately treated with benzaldehyde, followed by reduction of the resulting hydrazone with $NaCNBH_3$ and an excess of acetic acid to produce **12** and **13**, respectively. The overall yields of deprotection, hydrazone formation and reduction were 83% and 80%, respectively,



Scheme 1. Synthesis of target compounds **17** and **18**. Reagents and conditions: (a) RNH_2, EDC, HOBt, CH_2Cl_2, rt, 2 h; (b) TFA, rt, 2 h; (c) PhCHO, MeOH, rt, 12 h; (d) $NaCNBH_3$, HOAc, MeOH, rt, 2 h; (e) CDI, THF, rt, 15 min; (f) MeI, MeCN, rt, 12 h; (g) **12** (for **15**) or **13** (for **16**), NEt_3, CH_2Cl_2, rt, 12 h; (h) TMSCl, NaI, MeCN, rt, 1 h.

while purification by column chromatography was only necessary following the last step. The hydrazine building block can thus be prepared quite efficiently from commercially available (*S*)-proline. Carbonyl-di-imidazole (CDI) was used as coupling reagent to connect **12** and **13** with the readily available building block (*S*)-**14** to furnish unsymmetrical ureas **15** and **16**. Replacement of only one imidazole unit of CDI by (*S*)-**14** is achieved in only 15 min at room temperature. The resulting intermediate is much less reactive and does not react with **12** or **13**. By contrast, when the imidazole nitrogen is quaternized with iodomethane,[37] the corresponding, highly reactive imidazolium salt reacted smoothly with the hydrazine building blocks under mild conditions. The overall yields of CDI reaction with the amine part, activation and reaction with the hydrazine were 66% and 72%, respectively.

Deprotection of dimethyl acetals **15** and **16** with in situ generated TMSI gave target compounds **17** and **18**.[38] Scheme 2 shows the synthesis of a different example of the generic structure in **3** in which the stereocenter of the phenylalanine part is reversed. Readily available benzyl hydrazine **19**, and non natural amino acid derivative (*R*)-**14** were coupled with CDI as described above to produce unsymmetrical urea (*R*)-**20**. Subsequent deprotection gave target compound (*R*)-**21** in excellent yield.



Scheme 2. Synthesis of target compound (*R*)-**21**. Reagents and conditions: (a) (*R*)-**14**, CDI, THF, rt, 15 min; (b) MeI, MeCN, rt, 12 h; (c) **19**, NEt_3, CH_2Cl_2, rt, 12 h, 73% (three steps); (d) TMSCl, NaI, MeCN, rt, 1 h, 93%.

2.2. Configurational and constitutional analysis

Prior to in vitro tests with HIV PR, it was important to explore the tendency of the new compounds to exist in their cyclized form. NMR and UV spectroscopy are an appropriate means to prove the existence of the N→C=O interaction.[10,21,23,29–31] The proton and carbon NMR spectra in chloroform of compounds **17**, **18** and (*R*)-**21** all exhibit only one set of signals including one resonance corresponding to the aldehyde group. By contrast, in methanol all signals corresponding to the aldehyde forms are no longer present. In effect, the ^{13}C NMR spectra now show two independent signal sets of equal intensity, including two characteristic resonances (101.4 and 100.0 ppm in **17**, Fig. 3), right in the middle of the region between the olefinic/aromatic and the aliphatic region. These signals may be ascribed to the newly formed pseudo-tetrahedral methine carbon, adopting a hybridization state between those of sp^2 and sp^3. The former prochiral aldehyde group has thus been turned into a new stereogenic unit resulting in the formation of two diastereomers without any particular stereoselectivity; similar observations were made in a previous study.[23] The characteristics of the carbon spectra are reflected by those of the proton ones, in that the signals of the aldehyde protons have disappeared, while new resonances were observed in the shift interval between 4.61 and 4.46 ppm that can be attributed to the methine protons of the folded forms. These combined observations agree with literature data, according to which the N→C=O interaction is favored in polar protic media.[10,21,23,30,31]

In our previous work, compounds **7a–c** adopted only the linear aldehydic form in chloroform, while in methanol they existed as a mixture of varying proportions between the open and the cyclized forms.[10] In fact, the cyclization tendency of **7a–c** decreased with the increase in length of the peptide side chain, shifting from 70% cyclic **7a** to 55% cyclic **7b** and 45% cyclic **7c**. This was explained with the hydrophobic nature of the peripheral chains that may fold back onto each other in order to minimize exposure to the polar solvent, resulting in the rupture of the N→C=O interaction. In the present work, cyclization is complete for all compounds. This demonstrates the pronounced preorganizing effect of the planar urea moiety, and thus corroborates our molecular design.

It has been previously demonstrated that UV spectroscopy is an independent means to detect the N→C=O interaction.[10,28,32]

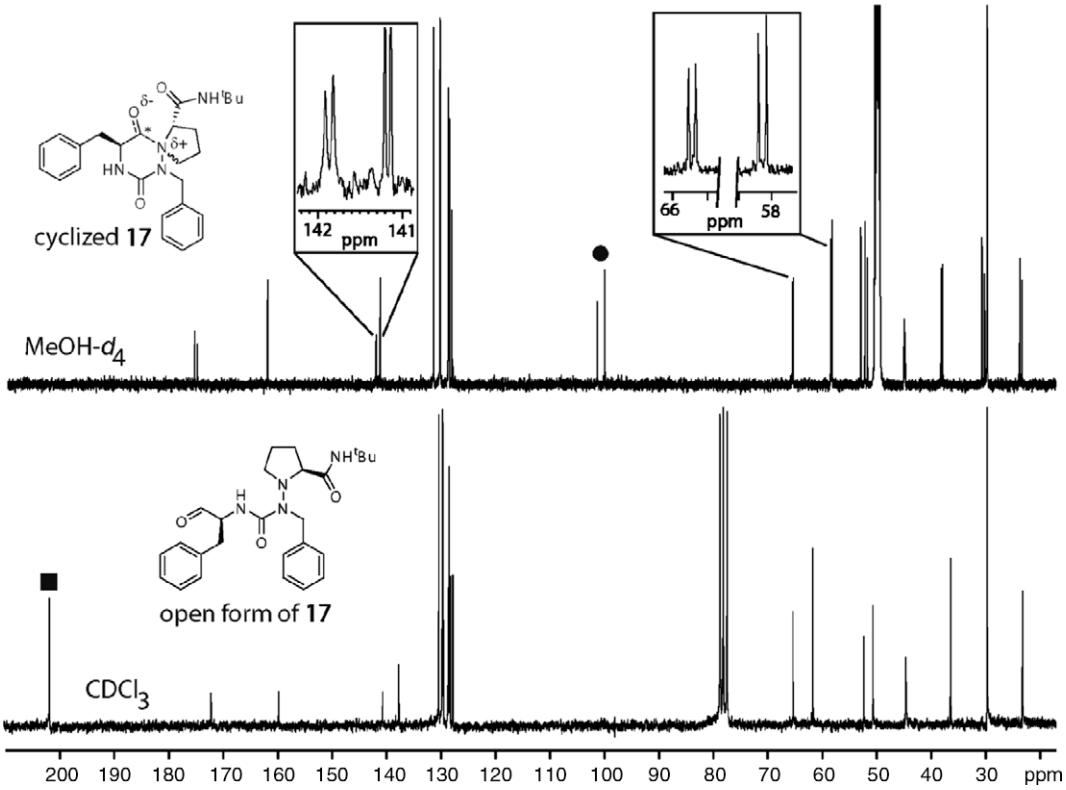


Figure 3. ^{13}C NMR spectra (127 MHz) of **17** in $CDCl_3$ (■ signal of the open-form aldehydic carbon) and MeOH-d_4 (● signals of the cyclized form methine carbons). Expanded regions illustrate the generation of two diastereomers with no particular selectivity.

The N→C═O group is characterized by an absorption that is much more intense and appears at a shorter wavelength than that caused by the aldehyde moiety. Figure 4 shows the UV spectra of compounds **17**, **18**, and (*R*)-**21** in chloroform and in methanol. In chloroform, all spectra have their maximum at 241 nm, corresponding to the absorption of the aldehyde function. Their extinction coefficients were calculated to be $\varepsilon = 674$, 731, and 871 L mol^{-1} cm^{-1}, being in the typical range for aldehydes. By contrast, in methanol this band is shifted to 208 nm and has become much more intense. The corresponding extinction coefficients were $\varepsilon = 20{,}350$, 18,201, and 21,200 L mol^{-1} cm^{-1} for compounds **17**, **18** and (*R*)-**21**, respectively. This absorption behavior of the N→C═O interaction is now well established in the literature and perfectly correlates with the NMR data.[10,28,32]

2.3. In vitro Inhibition of HIV PR

The capacity of compounds **17**, **18** and (*R*)-**21** and of previously synthesized ureas (*S*)-**21** and **22** (see Table 1),[32] to inhibit HIV PR was tested in a well-established continuous FRET (fluorescence resonance energy transfer) assay.[39,40] Pepstatine was tested simultaneously as standard. Initially, we determined the kinetic parameters of our recombinant source of HIV PR with a commercial FRET

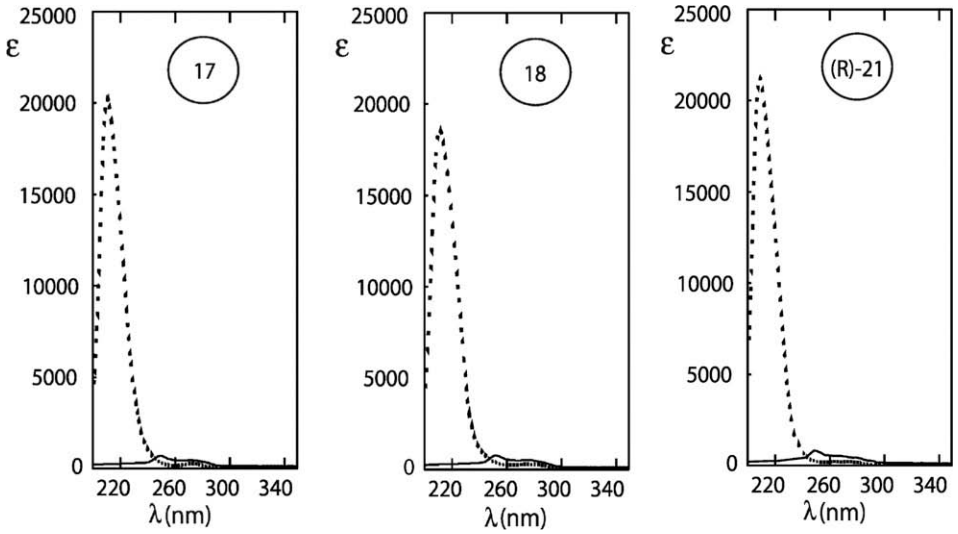


Figure 4. UV spectra of compounds **17**, **18** and (*R*)-**21** in MeOH (dotted line) and in $CHCl_3$ (continuous line).

substrate in order to evaluate our assay performance in comparison to the literature data. The obtained mean values of K_M = 25 μM, and V_{max} = 272 nM/min are in the range of those from previous reports.[10,39] The potency of the inhibitors was first estimated by determining their IC_{50} values by fitting the experimental data to $v/v_0 = 1/(1 + [I]/IC_{50})$ (Table 1). The exact K_i constants were derived from the same set of experiments.[41] For comparison, this table also includes the inhibition data for compounds **7a**–**c**.[10] The IC_{50} values of all hydrazino-ureas tested were slightly higher than the K_i values, which is in accord with the general relation for competitive inhibitors ($IC_{50} = K_i(1 + [S]/K_M)$ when the substrate concentrations are low compared to the K_M value, as they were in this study.[42] The K_i value obtained for pepstatine serves as a positive control and closely corresponds to the literature values that range from 0.4 to 250 nM depending on the experimental conditions used.[39] Matayoshi and Krafft obtained a K_i value of 17 nM with a similar FRET substrate.[39] We have earlier reported an inhibition constant of K_i = 36 nM using the same substrate and conditions as in the present work.[10] In general, the compounds of the hydrazino-urea series have better inhibitory potency than the first-generation inhibitors **7a**–**c**. Among the newly synthesized molecules, the small (*S*)-**21** and **17** were the best compounds having about one order of magnitude higher affinity than **18**, **22** and (*R*)-**21**. They were also approximately three times more effective than the best compound of the earlier series (**7c**). This increase in performance has to be appreciated in the light of the considerable reduction in size (46% drop) when switching from **7c** to (*S*)-**21** (see Table 1).

As a structurally novel entity the hydrazino urea motif had to be examined also in regard to its mode of inhibition. The curve patterns of the graphical representation in Figure 5, obtained from the experimental data for compound (*S*)-**21**, shows that this molecule is a competitive inhibitor.[41] Corresponding representations were obtained for the other four derivatives. The (*R*)-enantiomer of **21** had the lowest inhibitory effect of the new compounds with a K_i about 20-fold higher than that of (*S*)-**21**. Clearly an (*S*)-configured phenylalanine-derived motif is much more tolerated by HIV PR. In this context, it is interesting to note that subtle configurational changes such as a single stereocenter inversion can have a huge impact on affinity. In an extreme case a K_i difference of approximately seven orders of magnitude was reported.[3,43,44]

Interestingly, the new hydrazino-urea series exhibited an inhibitory tendency opposite to our previous series: while inhibitor candidates **7a**–**c** showed increasing potency (of more than one order of magnitude) with growing length of the peripheral chains, our current molecule candidate (*S*)-**21**, being the smallest, is the best performer in the series. While **17** is only marginally less effective, compounds **18** and **22** exhibit a quick drop in affinity. We are tempted to speculate that the hydrazino-urea core has appreciable complementarity with the active site of HIV PR. Since (*S*)-**21** sports a molecular weight of only 365, there is considerable room for improvement in affinity and ADME properties while plainly remaining within the Lipinski limit of 500 g/mol.[17] On the other hand, extending the peptide chain beyond the proline-carbonyl results in lower potency (**22** vs **17**). However, further expansion of this peripheral chain (**18** vs **22**) recovers some affinity, maybe because of the increase in surface area available for hydrophobic interactions.

3. Conclusion

This study broadens our exploration of the N→C=O motif as a potential isostere that reproduces key stereoelectronic properties of the transition states of peptide hydrolysis. In contrast to our first-generation inhibitors, only one constitutional component is present in all molecule candidates in a protic medium provided by methanol. In no instance have we noticed an impairment to the mutual approach of the two interacting functional groups for steric reasons. Generally, the new compounds exhibit higher inhibitory potency than those from our earlier work (**7a**–**c**), conceivably caused by the combined effects of simultaneous satisfaction of the di-Asp motif and the binding partners on the protein flaps; this remains of course to be confirmed by further SAR studies concerning all points of variation on the core and by a complementary X-ray structure analysis of the corresponding enzyme–inhibitor complex. It should be stressed that the demonstrated absence of any aldehydic component constitutes a major stride towards the consideration of the N→C=O motif as a future pharmacophore. The most potent inhibitor in this study is so small that great improvements in affinity and ADME properties may be obtained by introduction of additional molecular bulk without violating Lipinski's rule of remaining within the limit of 500 g/mol. We feel thus encouraged to initiate a hit-to-lead transformation[17] by a combined computational and combinatorial tactic.

4. Experimental

4.1. Materials and general methods

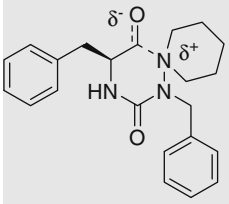
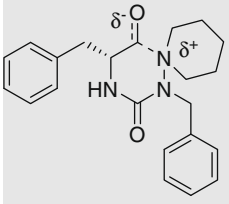
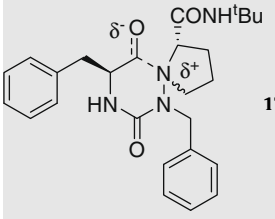
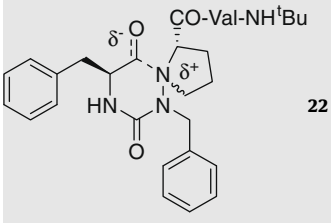
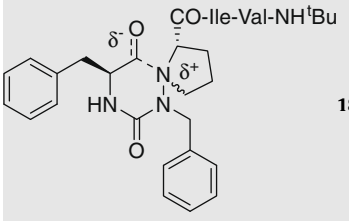
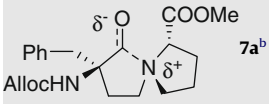
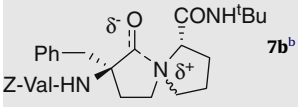
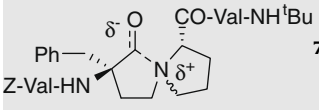
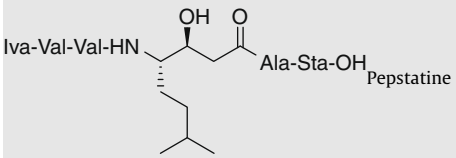
Reagents and solvents were purchased from Aldrich, Acros and Alfa Aesar. THF was distilled from Na/benzophenone, and dichloromethane was dried using $CaCl_2$ prior to distillation. Triethylamine was distilled from CaH_2. Compounds **9**, (*R*)-**14,** (*S*)-**14,** and **19** were prepared according to literature protocols.[32,33] All reactions were performed in anhydrous solvents under argon atmosphere in dried glassware. Column chromatography was performed using Merck silica gel Si 60 (40–63 μm). ^1H and ^{13}C NMR spectra were recorded on a Varian Unity 500 spectrometer (499.83 MHz, 126.7 MHz, respectively), or a Bruker DPX 200 instrument (200.13 MHz, 50.13 MHz, respectively). Chemical shifts (δ) are reported in ppm (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad) and referenced from tetramethyl silane or from solvent references.[45] NMR coupling constants (J) are reported in hertz. HRMS, MS, and elemental analyses were carried out by the Service Central d'Analyse du CNRS, Solaize, France. UV spectra were recorded on a JASCO V550 UV–vis spectrophotometer using 10 mm path length cells. Optical rotations were measured on a JASCO P1010 polarimeter with a 100 mm path length cell thermostated at 25 °C. Melting point (mp) determinations were carried out on a Büchi melting point B-540 apparatus and are uncorrected.

4.2. Synthetic protococls

4.2.1. (*S*)-(2-*tert*-Butylcarbamoyl-pyrrolidin-1-yl)-carbamic acid *tert*-butyl ester (10)

Compound **9** (320 mg, 1.04 mmol) was dissolved in CH_2Cl_2 (10 mL) and EDC (280 mg, 1.46 mmol), then HOBt (1.56 mL of a 1 N solution in *N*-methylmorpholine, 1.56 mmol) were added. After 5 min freshly distilled $^tBuNH_2$ (330 μL, 3.12 mmol) was added, and the reaction was stirred overnight. $NaHCO_3$ (satd aqueous solution, 10 mL) was added in order to quench the reaction and the mixture was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and the solvent was evaporated under vacuum. Purification by flash chromatography (33% EtOAc/CH_2Cl_2) gave **10** as a white solid (219 mg, 74%). Mp 207 °C; $[\alpha]_D^{25}$ −43.1 (*c* 1.02, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.87 (br s, 1H), 5.53 (br s, 1H), 3.40–3.35 (m, 1H), 3.14 (dd, J = 10.1, 4.6 Hz, 1H), 2.65–2.59 (m, 1H), 2.27–2.19 (m, 1H), 1.90–1.78 (m, 3H), 1.45 (s, 9H), 1.35 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.1, 155.7, 80.1, 69.8, 56.2, 50.3, 28.8, 28.5, 28.2, 21.9; HRMS (ESI) calcd for $C_{14}H_{28}N_3O_3$ $[M+H]^+$ 286.2132, found 286.2138.

Table 1
Inhibition data of hydrazino-ureas and related inhibitors towards HIV PR

Compound	IC_{50} (μM)	K_i (μM)[a]			MW (g/mol)
		Lineweaver and Burk	Hanes	Dixon	
 (*S*)-**21**	37.2	26.2	26.2	34.8	365
 (*R*)-**21**	543	461.0	461.0	453.9	365
 **17**	44.5	34.3	34.3	43.9	450
 **22**	341	292.0	292.0	286.2	549
 **18**	169	148.6	148.6	137.8	662
 **7a**[b]	>1000	–	–	–	388
 **7b**[b]	545	574	618	577	578
 **7c**[b]	204	99	96	96	677
 Pepstatine	0.041	0.038	0.038	0.050	686

[a] K_i values were obtained by use of three different methods.
[b] Data are from Gautier et al.[10]

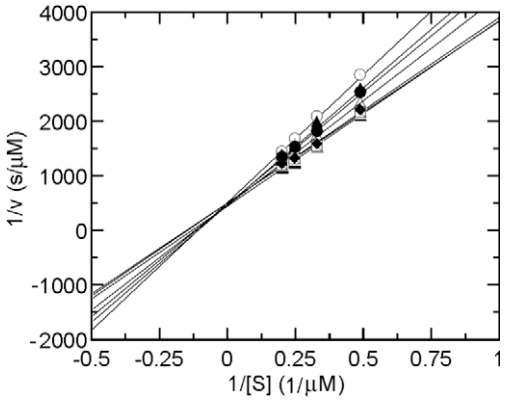


Figure 5. Inhibition of HIV PR by (*S*)-**21** at pH 4.7 and 37 °C: diagram according to the method of Lineweaver and Burk[41] with inhibitor concentrations (from bottom to top) [*I*] = 0 μM, 5 μM, 10 μM, 22 μM, 52 μM, 74 μM, 104 μM.

4.2.2. (*S,S,S*)-{2-[1-(1-*tert*-Butylcarbamoyl-2-methyl-propylcarbamoyl)-2-methyl-butylcarbamoyl]-pyrrolidin-1-yl}-carbamic acid *tert*-butyl ester (11)

Cbz-Ile-Val-NH-^{t}Bu: Cbz-Ile-OH (2.23 g, 8.43 mmol) was dissolved in CH_2Cl_2 (60 mL), and EDC (2.27 g, 11.77 mmol), then HOBt (12.65 mL of a 1 N solution in *N*-methylmorpholine, 12.65 mmol) were added. After 5 min, a suspension of HValNH^{t}Bu·HCl (1.75 g, 8.43 mmol) and triethylamine (1.17 mL, 8.43 mmol) in CH_2Cl_2 (20 mL) was added and the reaction was stirred overnight. $NaHCO_3$ (satd aqueous solution, 80 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic extracts were dried over Na_2SO_4 and the solvent was evaporated under vacuum. After purification by flash chromatography (50% EtOAc/cyclohexane), Cbz-Ile-Val-NH-^{t}Bu was obtained as a white solid (3.35 g, 95%). Mp 175 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33–7.29 (m, 5H), 6.68 (br d, J = 8.5 Hz, 1H), 5.89 (br s, 1H), 5.54 (br d, J = 8.5 Hz, 1H), 5.08 (s, 2H), 4.14–4.03 (m, 2H), 2.12–2.03 (m, 1H), 1.85–1.77 (m, 1H), 1.52–1.44 (m, 1H), 1.32 (s, 9H), 1.15–1.07 (m, 1H), 0.91–0.87 (m, 12H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.9, 170.3, 156.4, 136.5, 128.3, 127.9, 127.8, 66.6, 59.6, 59.5, 51.2, 37.6, 30.4, 28.5, 24.9, 19.1, 18.5, 15.3, 11.2; MS (ESI) *m/z* [M$^+$+H] 420 (100).

H-Ile-Val-NH-^{t}Bu: 10% Pd–C (840 mg) was added to a solution of Cbz-Ile-Val-NH-^{t}Bu (3.32 g, 7.92 mmol) in MeOH (100 mL). The solution was purged with hydrogen and stirred under a hydrogen atmosphere (1 bar) for 1.5 h. The catalyst was filtered off and the filtrate was evaporated to dryness. The residue was redissolved in CH_2Cl_2 and dried over Na_2SO_4. The solvent was removed under vacuum to give pure dipeptide H-Ile-Val-NH-^{t}Bu as a white solid (2.26 g, 100%); Mp 118 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87 (br d, J = 8.6 Hz, 1H), 5.91 (br s, 1H), 4.00 (dd, J = 8.6, 7.4 Hz, 1H), 3.37 (d, J = 3.9 Hz, 1H), 2.10–1.81 (m, 3H), 1.33 (s, 9H), 1.20–1.09 (m, 1H), 0.95–0.90 (m, 12H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.3, 170.5, 59.8, 58.8, 51.1, 37.9, 30.8, 28.6, 23.7, 19.3, 18.4, 16.0, 11.7; MS (ESI) *m/z* [M$^+$+H] 286 (100).

As described in the synthesis of **10**, usage of **9** (1.94 g, 8.43 mmol), EDC (2.26 g, 11.77 mmol), HOBt (12.65 mL of a 1 N solution in *N*-methylmorpholine, 12.65 mmol) and H-Ile-Val-NH-^{t}Bu (2.40 g, 8.43 mmol) in CH_2Cl_2 (80 mL) gave **11** as colorless oil (3.27 g, 78%) after purification by flash chromatography (50% CH_2Cl_2/EtOAc). $[\alpha]_D^{25}$ −38.4 (*c* 1.09, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.42 (br s, 1H), 6.68 (br d, J = 8.5 Hz, 1H), 6.04 (br s, 1H), 5.94 (br s, 1H), 4.20 (dd, J = 7.9, 6.9 Hz, 1H), 3.99 (dd, J = 8.5, 6.9 Hz, 1H), 3.50 (dd, J = 10.0, 4.8 Hz, 1H), 3.41–3.37 (m, 1H), 2.84–2.78 (m, 1H), 2.21–1.86 (m, 6H), 1.47–1.27 (m, 2H), 1.44 (s, 9H), 1.31 (s, 9H), 0.93–0.83 (m, 12H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.2, 171.3, 170.1, 155.7, 80.8, 68.3, 59.4, 58.5, 56.0, 51.1, 35.9, 29.5, 29.4, 28.6, 28.4, 24.8, 23.1, 19.4, 17.9, 15.7, 11.2; HRMS (ESI) calcd for $C_{25}H_{48}N_5O_5$ [M+H]$^+$ 498.3658, found 498.3659.

4.2.3. (*S*)-1-Benzylamino-pyrrolidine-2-carboxylic acid *tert*-butylamide (12)

Compound **10** (770 mg, 2.70 mmol) was dissolved in TFA (60 mL) and stirred for 2 h at rt. TFA was evaporated and the residue was dried under vacuum. The yellow oil was redissolved in anhydrous MeOH (20 mL), then benzaldehyde (329 mg, 3.11 mmol) was added, and the reaction was stirred overnight. Acetic acid (4 mL) and $NaCNBH_3$ (850 mg, 13.50 mmol) were added and stirring was continued for 2 h. Most part of the solvent was removed under vacuum, then a pH value around 8 was adjusted by adding $NaHCO_3$ (satd aq solution, 10 mL) and NaOH (aq solution), and the mixture was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and the solvent was evaporated under vacuum. After purification by flash chromatography (5% MeOH/CH_2Cl_2), **12** was obtained as a yellow oil (620 mg, 83%). $[\alpha]_D^{25}$ −45.5 (*c* 1.02, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (br s, 1H), 7.40–7.31 (m, 5H), 6.96 (br s, 1H), 4.04 (d, J = 16.1 Hz, 1H), 3.97 (d, J = 16.1 Hz, 1H), 3.60–3.55 (m, 1H), 3.17 (dd, J = 9.6, 5.5 Hz, 1H), 2.47–2.43 (m, 1H), 2.21–2.17 (m, 1H), 1.87–1.77 (m, 3H), 1.25 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.2, 138.6, 128.7, 128.3, 127.1, 70.3, 56.5, 54.1, 49.9, 28.6, 28.4, 22.3; HRMS (ESI) calcd for $C_{16}H_{26}N_3O$ [M+H]$^+$ 276.2077, found 276.2086.

4.2.4. (*S,S,S*)-1-Benzylamino-pyrrolidine-2-carboxylic acid [1-(1-*tert*-butylcarbamoyl-2-methyl-propylcarbamoyl)-2-methyl-butyl]-amide (13)

As described in the preparation of **12**, compound **11** (2.30 g, 4.62 mmol) was dissolved in TFA (100 mL) and stirred for 2 h at rt. After solvent evaporation, the dried residue was reacted with benzaldehyde (564 mg, 5.32 mmol) in methanol (35 mL). Subsequent treatment with $NaCNBH_3$ (1.46 g, 23.10 mmol) and acetic acid (7 mL) gave **13** as a colorless oil (1.80 g, 80%) after purification by flash chromatography (5% MeOH/CH_2Cl_2). $[\alpha]_D^{25}$ −39.6 (*c* 1.07, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (br s, 1H), 7.38–7.26 (m, 6H), 6.69 (br d, J = 8.6, 1H), 5.91 (br s, 1H), 4.21 (dd, J = 7.9, 6.9 Hz, 1H), 4.03–3.98 (m, 3H), 3.53–3.49 (m, 1H), 3.25 (dd, J = 9.4, 5.6 Hz, 1H), 2.32–2.15 (m, 4H), 1.91–1.76 (m, 3H), 1.45–1.28 (m, 1H), 1.32 (s, 9H), 1.01–0.86 (m, 13H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.1, 171.3, 170.0, 138.1, 128.6, 128.2, 127.2, 69.5, 59.2, 57.5, 56.0, 54.6, 51.1, 36.7, 29.9, 28.5, 27.9, 24.7, 22.2, 19.1, 17.9, 15.5, 11.1; HRMS (ESI) calcd for $C_{27}H_{46}N_5O_3$ [M+H]$^+$ 488.3603, found 488.3608.

4.2.5. (*S,S*)-1-[1-Benzyl-3-(1-benzyl-2,2-dimethoxy-ethyl)-ureido]-pyrrolidine-2-carboxylic acid *tert*-butylamide (15)

CDI (390 mg, 2.40 mmol) was suspended in anhydrous THF (3 mL), and a solution of amine (*S*)-**14** (425 mg, 2.18 mmol) in THF (2 mL) was added dropwise at rt. The reaction was stirred for 15 min, then the solvent was evaporated. The residue was redissolved in CH_2Cl_2 (10 mL) and washed with water (2 × 5 mL). The organic phase was dried over Na_2SO_4 and the solvent was evaporated under vacuum. The residue was dissolved in anhydrous acetonitril (4 mL), and iodomethane (527 μL, 8.45 mmol) was added. The reaction was stirred overnight at rt, then the solvent was evaporated and the yellow oil was dried under vacuum. The residue was redissolved in anhydrous CH_2Cl_2 (22 mL), and hydrazine **12** (600 mg, 2.18 mmol) and triethylamine (300 μL, 2.18 mmol) were added. After stirring overnight at rt, the reaction was quenched by adding $NaHCO_3$ (satd aq solution, 10 mL) and the mixture was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and the solvent was removed under vacuum. Compound **15** was obtained as a white

solid (714 mg, 66%) after purification by flash chromatography (33% EtOAc/CH_2Cl_2). Mp 112 °C; $[\alpha]_D^{25}$ −66.7 (*c* 1.05, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.33–7.10 (m, 10H), 6.55 (br s, 1H), 5.35 (br s, 1H), 4.76 (d, *J* = 16.2 Hz, 1H), 4.40–4.29 (m, 1H), 4.26–4.10 (m, 2H), 3.46 (s, 1H), 3.44 (s, 1H), 3.24–3.12 (m, 1H), 3.06 (dd, *J* = 14.1, 4.4 Hz, 1H), 2.75 (dd, *J* = 14.1, 10.1 Hz, 1H), 2.64–2.55 (m, 1H), 2.52–2.41 (m, 1H), 1.95–1.66 (m, 4H), 1.23 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.9, 158.5, 139.6, 138.7, 129.1, 128.3, 128.0, 126.9, 126.7, 125.8, 105.8, 63.7, 55.8, 55.3, 52.7, 50.7, 49.2, 43.2, 34.6, 28.3, 28.1, 21.5; HRMS (ESI) calcd for $C_{28}H_{41}N_4O_4$ $[M+H]^+$ 497.3130, found 497.3133.

4.2.6. (*S,S,S,S*)-1-[1-Benzyl-3-(1-benzyl-2,2-dimethoxy-ethyl)-ureido]-pyrrolidine-2-carboxylic acid [1-(1-*tert*-butylcarbamoyl-2-methyl-propylcarbamoyl)-2-methyl-butyl]-amide (16)

As in the preparation of **15**, compound (*S*)-**14** (642 mg, 3.29 mmol) was initially reacted with CDI (588 mg, 3.62 mmol) in THF (7.5 mL). The resulting residue was treated with iodomethane (795 μL, 12.77 mmol) in acetonitril (6 mL), followed by reaction with hydrazine **13** (1.60 g, 3.29 mmol) and triethylamine (460 μL, 3.29 mmol) in CH_2Cl_2 (35 mL) to give **16** as a white solid (1.68 g, 72%) after purification by flash chromatography (75% EtOAc/cyclohexane). Mp 156 °C; $[\alpha]_D^{25}$ −59.5 (*c* 1.06, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.25–7.11 (m, 10H), 6.81 (br s, 1H), 6.52 (br s, 1H), 6.27 (br s, 1H), 5.77 (br s, 1H), 4.68 (d, *J* = 16.2, 1H), 4.36–4.28 (m, 1H), 4.23–4.14 (m, 3H), 3.96 (dd, *J* = 8.8, 7.1 Hz, 1H), 3.44 (s, 1H), 3.42 (s, 1H), 3.38–3.32 (m, 1H), 3.06 (dd, *J* = 14.0, 4.3 Hz, 1H), 2.74 (dd, *J* = 14.0, 10.3 Hz, 1H), 2.69–2.61 (m, 1H), 2.58–2.50 (m, 1H), 2.08–1.89 (m, 2H), 1.82–1.59 (m, 4H), 1.46–1.37 (m, 1H), 1.30 (s, 9H), 1.07–0.98 (m, 1H), 0.91–0.79 (m, 12H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.8, 171.0, 169.9, 158.5, 139.4, 138.9, 129.2, 128.3, 128.0, 126.8, 126.7, 125.7, 105.2, 63.4, 59.1, 57.7, 56.2, 55.0, 52.6, 51.1, 50.1, 44.4, 37.1, 34.7, 30.4, 29.3, 28.4, 24.8, 22.5, 19.0, 18.2, 15.3, 11.2; HRMS (ESI) calcd for $C_{39}H_{61}N_6O_6$ $[M+H]^+$ 709.4656, found 709.4662.

4.2.7. (*S,S*)-1-[1-Benzyl-3-(1-benzyl-2-oxo-ethyl)-ureido]-pyrrolidine-2-carboxylic acid *tert*-butylamide (17)

To a solution of compound **15** (64 mg, 0.13 mmol) in anhydrous acetonitrile (3 mL) were added NaI (49 mg, 0.33 mmol) and TMSCl (42 μL, 0.33 mmol). The reaction was stirred at rt for 1 h, and was then quenched by adding $NaHCO_3$ (satd aq solution, 5 mL). The mixture was extracted with CH_2Cl_2 (3 × 10 mL) and the combined organic extracts were washed with $Na_2S_2O_3$ (satd aq solution, 10 mL), and then with water (10 mL). The organic phase was dried over Na_2SO_4 and the solvent was evaporated under vacuum. After purification by flash chromatography (25% EtOAc/CH_2Cl_2), **17** was obtained as colorless oil (49 mg, 83%). $[\alpha]_D^{25}$ −63.4 (*c* 1.05, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 9.67 (s, 1H), 7.33–7.20 (m, 11H), 5.17 (br s, 1H), 4.90 (d, *J* = 16.1, 1H), 4.49–4.43 (m, 1H), 4.28 (d, *J* = 16.1, 1H), 3.26–3.14 (m, 2H), 2.96 (dd, *J* = 14.0, 8.9 Hz, 1H), 2.83–2.76 (m, 1H), 2.71–2.63 (m, 1H), 1.97–1.87 (m, 1H), 1.85–1.64 (m, 3H), 1.23 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 201.0, 171.2, 158.8, 139.7, 136.7, 129.4, 128.7, 128.5, 127.4, 127.2, 126.8, 64.2, 60.6, 51.2, 49.5, 43.4, 35.2, 28.7, 28.5, 21.9; ^1H NMR (500 MHz, MeOH-d_4), (1:1 mixture of two diasteromers) δ 7.35–7.19 (m, 16H), 7.15 (d, *J* = 7.5, 2H), 7.08 (d, *J* = 7.5, 2H), 4.58–4.46 (m, 6H), 4.08–4.02 (m, 2H), 3.50–3.46 (m, 2H), 3.10 (dd, *J* = 13.8, 4.4 Hz, 1H), 3.02 (dd, *J* = 13.6, 5.7 Hz, 1H), 2.85–2.65 (m, 6H), 2.08–1.98 (m, 2H), 1.92–1.69 (m, 6H), 1.33 (s, 9H), 1.31 (s, 9H); ^{13}C NMR (127 MHz, MeOH-d_4) δ 175.2, 174.7, 161.7, 141.9, 141.8, 141.2, 141.1, 131.43, 131.40, 130.31, 130.25, 130.17, 130.15, 128.63, 128.61, 128.53, 128.47, 128.07, 128.05, 101.4, 100.0, 65.5, 65.3, 58.3, 58.1, 52.93, 52.90, 52.2, 51.7, 44.9, 44.8, 38.1, 37.9, 30.6, 30.2, 29.8, 29.7, 23.71, 23.3; HRMS (ESI) calcd for $C_{26}H_{35}N_4O_3$ $[M+H]^+$ 451.2711, found 451.2711. Anal. Calcd for $C_{26}H_{34}N_4O_3$: C, 69.31; H, 7.61; N, 12.43. Found: C, 69.23; H, 7.64; N 12.41.

4.2.8. (*S,S,S,S*)-1-[1-Benzyl-3-(1-benzyl-2-oxo-ethyl)-ureido]-pyrrolidine-2-carboxylic acid [1-(1-*tert*-butylcarbamoyl-2-methyl-propylcarbamoyl)-2-methyl-butyl]-amide (18)

As described in the preparation of **17**, acetal **16** (99 mg, 0.14 mmol) was treated with NaI (53 mg, 0.35 mmol) and TMSCl (45 μL, 0.35 mmol) in anhydrous acetonitrile (3 mL). After purification by flash chromatography (75% EtOAc/cyclohexane), **18** was obtained as a colorless oil (72 mg, 78%). $[\alpha]_D^{25}$ −53.7 (*c* 1.03, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 9.62 (s, 1H), 7.32–7.13 (m, 11H), 6.50 (br s, 1H), 6.38 (br s, 1H), 5.76 (br s, 1H), 4.71 (d, *J* = 16.2 Hz, 1H), 4.52–4.45 (m, 1H), 4.33 (d, *J* = 16.2 Hz, 1H), 4.27 (dd, *J* = 8.8, 7.1 Hz, 1H), 3.93 (dd, *J* = 8.8, 7.1 Hz, 1H), 3.50–3.41 (m, 1H), 3.22 (dd, *J* = 14.0, 5.6 Hz, 1H), 2.96 (dd, *J* = 14.0, 9.0 Hz, 1H), 2.82–2.73 (m, 1H), 2.69–2.60 (m, 1H), 2.02–1.93 (m, 2H), 1.78–1.65 (m, 4H), 1.30 (s, 9H), 1.27–1.20 (m, 1H), 1.09–0.98 (m, 1H), 0.92–0.79 (m, 12 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 201.7, 172.6, 170.8, 169.7, 158.6, 139.1, 136.9, 129.3, 128.6, 128.4, 127.2, 127.1, 126.7, 63.7, 60.4, 59.4, 57.4, 51.5, 50.0, 44.1, 37.6, 35.1, 30.7, 29.5, 28.5, 24.8, 22.4, 19.0, 18.3, 15.3, 11.2; ^1H NMR (500 MHz, MeOH-d_4), (1:1 mixture of two diasteromers) δ 7.35–7.18 (m, 16H), 7.11 (d, *J* = 7.4 Hz, 2H), 7.06 (d, *J* = 7.5 Hz, 2H), 4.58–4.46 (m, 6H), 4.25–4.19 (m, 2H), 4.09–3.98 (m, 4H), 3.69–3.65 (m, 2H), 3.11 (dd, *J* = 13.8, 4.3 Hz, 1H), 3.02 (dd, *J* = 13.7, 5.7 Hz, 1H), 2.86–2.64 (m, 6H), 2.17–1.96 (m, 4H), 1.88–1.69 (m, 8H), 1.56–1.45 (m, 2H), 1.36 (s, 9H), 1.34 (s, 9H), 1.20–1.07 (m, 2H), 0.99–0.90 (m, 24H); ^{13}C NMR (127 MHz, MeOH-d_4) δ 175.7, 175.6, 174.3, 174.2, 173.28, 173.25, 161.5, 142.0, 141.7, 141.1, 141.0, 131.5, 131.4, 130.3, 130.2, 130.2, 130.1, 128.6, 128.5, 128.09, 128.05, 127.87, 127.85, 101.5, 100.0, 65.0, 64.5, 61.6, 61.4, 60.5, 60.1, 58.2, 58.0, 53.1, 53.0, 52.5, 52.1, 45.3, 45.0, 38.8, 38.6, 38.0, 37.8, 32.7, 32.0, 30.8, 30.4, 29.6, 27.1, 27.0, 24.0, 23.5, 20.5, 19.9, 19.8, 16.8, 16.7, 12.3, 12.2; HRMS (ESI) calcd for $C_{37}H_{54}N_6O_5Na$ $[M+Na]^+$ 685.4056, found 685.4059. Anal. Calcd for $C_{37}H_{54}N_6O_5$: C, 67.04; H, 8.21; N, 12.68. Found: C, 66.94; H, 8.23; N 12.64.

4.2.9. (*R*)-1-Benzyl-3-(1-benzyl-2,2-dimethoxy-ethyl)-1-piperidin-1-yl-urea ((*R*)-20)

According to the preparation of **15**, (*R*)-**14** (300 mg, 1.54 mmol) was first reacted with CDI (274 mg, 1.69 mmol) in THF (3.5 mL). The resulting residue was treated with iodomethane (372 μL, 5.98 mmol) in acetonitril (3 mL), followed by reaction with hydrazine **19** (293 mg, 1.54 mmol) and triethylamine (214 μL, 1.54 mmol) in CH_2Cl_2 (15 mL) to give (*R*)-**20** as a colorless oil (461 mg, 73%) after purification by flash chromatography (33% EtOAc/cyclohexane). $[\alpha]_D^{25}$ +28.7 (*c* 1.09, $CHCl_3$); HRMS, ^1H and ^{13}C NMR spectra were identical to those reported for the (*S*) enantiomer.[32]

4.2.10. (*R*)-1-Benzyl-3-(1-benzyl-2-oxo-ethyl)-1-piperidin-1-yl-urea ((*R*)-21)

As described in the preparation of **17**, (*R*)-**20** (193 mg, 0.47 mmol) was treated with NaI (178 mg, 1.18 mmol) and TMSCl (151 μL, 1.18 mmol) in anhydrous acetonitrile (10 mL). After purification by flash chromatography (50% EtOAc/cyclohexane), (*R*)-**21** was obtained as a colorless oil (160 mg, 93%). $[\alpha]_D^{25}$ + 30.6 (*c* 1.09, $CHCl_3$); Anal. Calcd for $C_{22}H_{27}N_3O_2$: C, 72.30; H, 7.45; N, 11.50. Found: C, 72.21; H, 7.47; N 11.48; HRMS, ^1H and ^{13}C NMR spectra were identical to those reported for the (*S*) enantiomer.[32]

4.3. Enzyme assays

HIV PR was purchased from Bachem, and HIV PR substrate 1 (Arg-Glu(EDANS)SerGlnAsnTyr-ProIleValGlnLys-(DABCYL)Arg) was

obtained from Sigma–Aldrich. Enzymatic activity and inhibition were determined by a fluorogenic assay using a microplate spectrofluorimeter (Spectramax Gemini XS, Molecular Devices) and black 384-well plates (NUNCLONE, Nunc Inc.). Hydrolysis of the fluorogenic substrate was investigated at pH 4.7, 37 °C in 0.1 M acetate, 1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mg/mL bovine serum albumin. The entire time course was recorded by monitoring the fluorescence at 490 nm with an excitation wavelength set to 340 nm. Each initial rate was the average value of five independently performed reactions.

K_M determination was performed using a range of fluorogenic substrate concentrations from 0.5 to 10 μM and an HIV PR concentration of 4 nM. Final solutions contained 10% DMSO.

For determination of IC_{50} and K_i values, inhibitors were preincubated with HIV PR for 30 min. IC_{50} determinations were performed using 10% DMSO solutions containing a final of 5 nM HIV PR and 4 μM fluorogenic substrate. Initial rates were determined for at least five inhibitor concentrations. K_i determinations were performed using 10% DMSO solutions containing a final of 5 nM HIV PR. Initial rates were determined for at least five inhibitor concentrations and four fluorogenic substrate concentrations (2, 3, 4, and 5 μM).

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