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Small Hydroxyethylene-Based Peptidomimetics Inhibiting Both HIV-1 and *C. albicans* Aspartic Proteases

Alessandro Tossi,[a,b]* Fabio Benedetti,[b,c] Stefano Norbedo,[b,c] Damiano Skrbec,[a] Federico Berti[b,c] and Domenico Romeo[a,b]

[a]*Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Via Giorgieri 1, I-34127 Trieste, Italy*

[b]*Centre of Excellence on Biocrystallography, University of Trieste, Via Giorgieri 1, I-34127 Trieste, Italy*

[c]*Department of Chemical Sciences, University of Trieste, Via Giorgieri 1, I-34127 Trieste, Italy*

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Abstract—We have extended a highly flexible method for rapidly assembling aspartic protease inhibitors to produce symmetric and asymmetric monohydroxyethylene peptidomimetics. This method is based on the prior synthesis of the central non-cleavable peptide-bond isostere [NH_2–$P_1\psi P1'$–NH_2; ψ = hydroxyethylene isostere, HNCH(Bz)CHOHCH$_2$CH(Bz)NH], with the possibility of accurately controlling its stereochemistry (*S*,*S*,*S* or *S*,*R*,*S*), and subsequently adding appropriate flanking units, chosen from commercially available amino acids, aromatic carboxylic acids, or phenoxyacetic acid (Poa) derivatives. The method was used to make asymmetric inhibitors of general formula Kyn-Xaa-PheψPhe-dmPoa, (Kyn = kynurenic acid, Xaa = Val, Thr or D-thienylglycine, M_r = 716–754) and symmetric inhibitors of formula xPoa-PheψPhe-xPoa (xPoa = Poa or dimethyl-, hydroxy-, formyl- or acetyl-Poa, M_r = 553–609), with log$P_{o/w}$ values ranging from 4.1 to 7.6. Inhibition of HIV-PR did not depend on the stereochemistry of the hydroxyl group, while it depended markedly on the substituents present on the Poa residues, with dmPoa being preferred over Poa or its more hydrophilic derivatives. Conversely, inhibition of *Candida albicans* Sap2 was higher for the *S*,*S*,*S* epimers, and Poa or its hydrophilic derivatives were preferred over dmPoa.

Introduction

Aspartic proteases play significant roles in determining the pathogenicity of both viruses and fungi.[1–12] The design and synthesis of high affinity inhibitors of these enzymes can thus provide useful leads for the development of potential anti-infective drugs.[1–7,11,13] A large number of inhibitors have been characterised, but only a handful of those inhibiting the aspartic protease of HIV (HIV-PR), has reached clinical use.[4,6,7] For the aspartic proteases so far investigated, a high affinity of designed inhibitors has been achieved by maximizing the number of noncovalent contacts that they make with the enzyme binding site. To this effect a transition-state isostere, that is a functional group that can mimic the tetrahedral transition-state for amide bond hydrolysis, is incorporated into the designed inhibitors and appropriate flanking residues are attached to it.[1–3,5,7,8,13] Finally, to be exploited as potential drug leads, the protease inhibitors must possess a relatively low molecular weight and a hydrophilicity/hydrophobicity ratio suitable for a sufficient level of bioavailability.[4,5,7] In this context, we have been developing a flexible approach for the design and synthesis of such inhibitors.[14–22]

For HIV-PR, X-ray crystallography has shown that the two identical chains of this dimeric enzyme are related by a 2-fold rotation axis.[1,4,7] This has led to the design and synthesis of C_2-symmetric and nearly-symmetric compounds,[2,3,5,7,13,20] which in some cases have proved to be very efficient inhibitors and have been developed into drugs,[4,6] presently employed in the Highly Active Anti-Retroviral Therapy (HAART) of AIDS.[23–25] Much effort has been expended in reducing the size and complexity of these inhibitors while maintaining a high affinity, also towards mutant proteases which have developed resistance to the first generation of drugs.[7,26,27]

A variety of aspartic proteases are also produced and secreted by *Candida albicans*, a fungus responsible for

*Corresponding author. Tel.: +39-04-0558-3673; fax: +39-04-0558-3691; e-mail: tossi@bbcm.units.it

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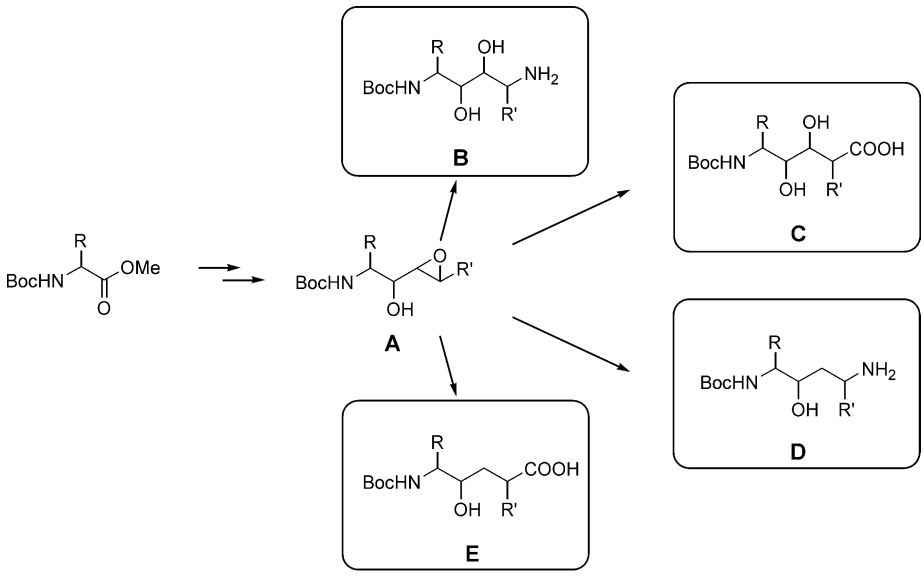


Scheme 1. Overview of dipeptide isosteres available from the common precursor **A**.

mucocutaneous and systemic infections, frequently encountered in immunodepressed patients such as those infected by HIV-1.[8,28] Although there are several members of this family, probably individually expressed at various stages of the infection process and playing different roles, Sap2 appears to be the major secretory aspartic protease in log-phase *Candida* cells.[29,30] Despite the difference in structure between the retroviral and the fungal aspartic proteases (Sap), which are monomeric proteins with two equivalent domains, several inhibitors of the HIV-1 protease are also active on Saps.[8,11,21,31–33] This may explain the beneficial effect on *Candida* infection observed in some AIDS patients undergoing HAART.[33]

Here, we report the results of the evaluation of the anti-aspartic protease activity of a series of diastereoisomeric peptidomimetics, containing a hydroxyethylene isostere as the central non-hydrolysable group, in either the *S*,*S*,*S* or *S*,*R*,*S* configuration, and rationally selected amino acids or organic acids as flanking units. These compounds were prepared by a simplified, modular synthetic approach that has been perfected in our laboratories, with the aim to maximally reduce both the size and complexity of inhibitors, while concomitantly achieving a potent inhibitory capacity. The aspartic proteases considered were a recombinant form of the HIV-1 enzyme and native Sap2, purified from a virulent strain of *C. albicans*.

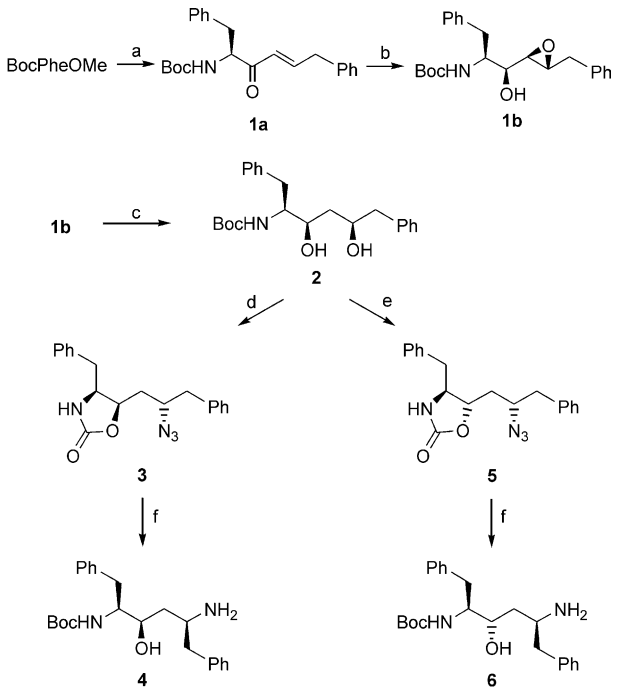
Results and Discussion

Chemistry

We have developed a rapid, efficient and very flexible method for assembling aspartic protease inhibitors.[16,18–22] This method is based on the prior synthesis of the desired type of central modules (a pseudo-dipeptide which contains the non-hydrolysable peptide bond isostere) (see Scheme 1), and subsequent assembly of inhibitors by adding appropriate flanking residues, using standard peptide synthesis methods. The flexibility of the method derives from the possibility of (i) obtaining from a common precursor **A** (Scheme 1) central modules with different types of non-hydrolysable amide-bond replacements (e.g., monohydroxyethylene **D**, **E** or dihydroxyethylene **B**, **C**); (ii) independently varying the type of side chain in positions P_1 and P_1' in these central modules; (iii) precisely controlling the stereochemistry of the central module; (iv) preparing modules with either two amino terminals (1,4-diamines **B**, **D**) or an amino and a carboxy terminal (δ-amino acids **C**, **E**)[19] and (v) selectively protecting one amino terminal, so as to permit assembly of either asymmetric or symmetric inhibitors on addition of appropriate flanking residues.[20,22] Another elegant method for the rapid synthesis of C_2-symmetric diaminodihydroxyethylene modules has been reported,[34] but it does not achieve the level of flexibility of our method. Furthermore, a key feature of our approach is the use of off-the-shelf components at all stages, which increases the rapidity with which novel inhibitor structures can be assembled.

We have previously integrated this synthetic methodology with a computational method that derives the structures of new potential HIV-PR inhibitors from a reference compound, whose inhibition constant and crystal structure in complex with HIV-PR are known.[14,20] Using molecular mechanics to compare complexation energies of these new structures relative to that of the original reference inhibitor[15] it is then possible to select the most promising ones for rapid assembly.

Recently, we have described several small diaminodihydroxyethylene type inhibitors obtained in this manner, which have IC_{50} values towards HIV-1 PR in the low nanomolar range.[18,20,21] These compounds confirmed the suitability of the phenylalanine side chain in both positions P_1 and P_1', whereas other tested possibilities



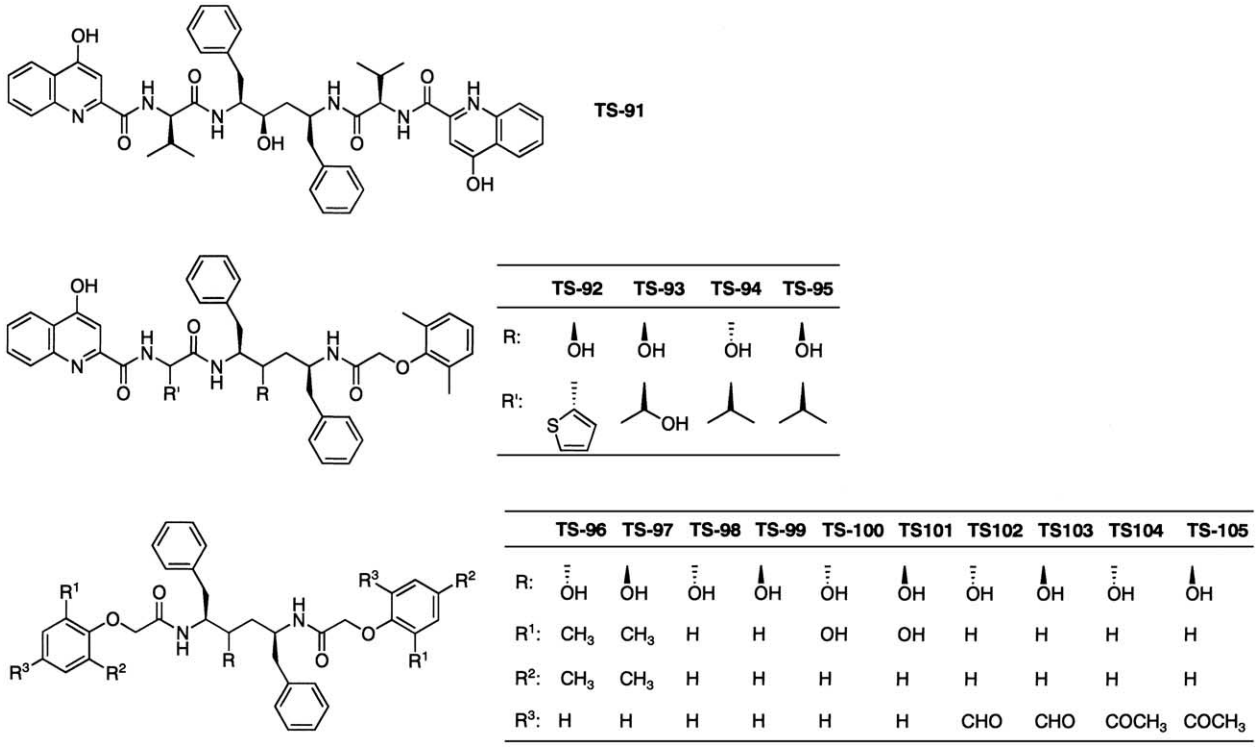
Scheme 2. Synthesis of the diamminoalcohols **4** and **6**, used as central modules in peptidomimetic aspartic protease inhibitors: (a) (i) $CH_3PO(OCH_3)_2$, *n*-BuLi, THF, 95%; (ii) BnCHO, K_2CO_3, EtOH, 50%; (b) (i) $NaBH_4$, MeOH, 0 °C, 75%; (ii) *m*CPBA, DCM, 70%; (c) RedAl, THF, 65%; (d) (i) NaH, THF, 80%; (ii) MsCl, Et_3N, DCM; (iii) NaN_3, DMSO, 18-crown-6, 80%; (e) (i) 2 equiv MsCl, DIPEA, 1,2-dichloroethane, 80 °C; (ii) NaN_3, DMSO, 18-crown-6, 50%; (f) (i) Boc_2O, NaH, THF; (ii) Cs_2CO_3, $MeOH/H_2O$; (iii) H_2, Pd/C, 80%.

(e.g., Ile and cyclohexylalanine) considerably reduced activity. This is in line with the substrate preference of the protease[2,4] and is also preferred in the clinically used inhibitors. We also found that small natural or non-proteinogenic amino acids, such as valine, threonine or D-thienylglycine (Dtg) were preferred in position P_2, and carboxylic acids based on the chromone core, such as kynurenic acid (Kyn), were suitable in position P_3. Such residues could also be placed in positions P_2' and P_3', respectively, so as to produce symmetric pseudo-hexapeptide inhibitors, or alternatively the small phenoxyacetic acid moiety (Poa) could be placed in P_2' to produce smaller and asymmetric, but highly efficient pentapeptide inhibitors.

We have now utilised this expertise for the assembly of inhibitors with the diamminomonohydroxyethylene type central module (Scheme 2). The synthesis of the monohydroxyethylene central cores **4** and **6**[22] exploits the same key intermediates we already used for dihydroxyethylene pseudodipeptides,[18,20] thus widening the scope of the strategy, while maintaining all the advantages described above. Briefly (Scheme 2), the methyl ester of *N*-Boc phenylalanine was converted into the corresponding phosphonoketone by reaction with lithiated methyl dimethylphosphonate; Horner–Emmons olefination with phenylacetaldehyde gave then the α,β-unsaturated ketone **1a** in 43% yield. The latter was then reduced with sodium borohydride to give a 3:1 mixture of diastereoisomeric alcohols; the *S,R* isomer was purified by column chromatography and crystallization and stereoselectively *syn*-epoxidized giving the epoxyalcohol **1b** in 23% overall yield from the starting aminoester.[16] Reductive ring opening of **1b** with Red-Al gave the diol **2** in 65% yield.[22] Selective protection of the first hydroxy group was achieved by cyclization of the *N*-Boc aminoalcohol to oxazolidinone under acyl transfer conditions (NaH/THF), while the second hydroxy group was mesylated and the mesylate was displaced by azide giving **3**. Finally, Boc was reinstalled on the amino group, the oxazolidinone was opened hydrolytically and the azide was hydrogenated to give the diaminoalcohol **4** in 51% yield from **2**. Alternatively, the pivotal intermediate **2** was mesylated giving a bis-mesylate that spontaneously cyclizes by a S_N2 mechanism; the second mesylate is then replaced by azide giving the oxazolidinone **5** in which the configuration at the ring *C(–O) is inverted with respect to **3**. The same sequence of reactions already described for **3** (step f, Scheme 2) finally gave the diaminoalcohol **6** in 32% yield from **2**. Thus, by choosing the appropriate conditions for the cyclisation of **2**, it was possible to control the configuration of the –OH group in the required isostere **4**/**6**.[22] While the synthetic effort required to obtain **4** or **6** is comparable, the latter was initially chosen for this study because this is the same isostere present in ritonavir.[5]

We have assembled a symmetric hexapeptide structure (TS-91) by adding the Kyn-Val dipeptide to both sides of module **6**, so as to ensure complete coverage of the enzyme binding site (Scheme 3). The pentapeptide inhibitors TS-92–TS-95 are instead based on results previously obtained with dihydroxyethylene inhibitors.[18,20] They have the general sequence Kyn-Xaa-**4**/**6**-dmPoa (Scheme 3), where Xaa is either Dtg, Thr or Val. In P_2', dimethylphenoxyacetic acid (dmPoa) was preferred to Poa, in analogy with the structure of the highly successful, new generation HIV-PR inhibitor lopinavir;[26,27] this choice was also supported by the results of a computational analysis.[35] In fact, compound TS-94, with the sequence Kyn-Val-**6**-dmPoa, differs from lopinavir only in position P_3 where Kyn replaces the cyclic urea residue. The synthesis of TS-94 is considerably simpler than that of lopinavir as the cyclic urea of the latter can not be introduced as such, but requires a specific, less direct approach.[27]

Compounds TS-96–TS-105 are symmetric tetramers with sequence xPoa-**4**/**6**-xPoa (Fig. 1), where xPoa represents the series Poa, dmPoa, 4-hydroxyphenoxyacetic acid (hPoa), 4-formylphenoxyacetic acid (fPoa), 4-acetylphenoxyacetic acid (aPoa). They are small and very facile to assemble. Our intention was to use this minimalist approach to (i) analyse the role of central module conformation when interaction of flanking residue with the enzyme binding-site are necessarily limited, and (ii) investigate the role of substituents on the Poa core in modulating interactions with the S_2 and S_2' enzyme binding subsites. Computational studies[35] had in fact indicated that the presence of H-bond donor/acceptor substituents on Poa could increase complexation with the HIV-PR binding site significantly, while also improving the hydrophobicity/solubility characteristics.



Scheme 3. Structures of peptidomimetic aspartic protease inhibitors reported in Table 1. The stereochemistry of the non-hydrolysable isostere hydroxyl group (R) is indicated.

The overall yield of synthesised peptidomimetics was relatively good, ranging from 10% to over 50% from modules **4** or **6**. The principal factors limiting yields are loss on extraction, which are significant at the limited synthetic scale (0.1 mmol) reported here, and formation of a side-product with flanking residues bound also to the core hydroxy group. For symmetric inhibitors synthesised in one-pot reactions, yields could be markedly increased by directly and inexpensively purifying the product from the reaction mixture with disposable RP-HPLC cartridges, thus avoiding extraction. Furthermore, the side-product (estimated at 10–40% of crude) can be efficiently reconverted to the desired product by treatment with a base, such as 80% aqueous hydrazine. For example, the yield of TS-96 increases from 34 to 44% after recovery. An alternative route, that of temporarily protecting the hydroxy with the trimethylsilyl group, was abandoned as it led to reduced yields due to precipitation under our reaction conditions.

Inhibition of HIV-1 aspartic protease

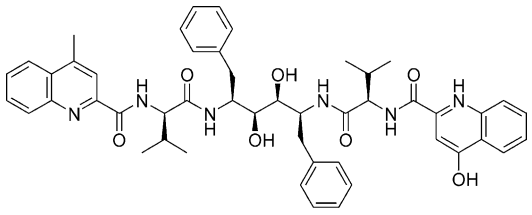
Determination of IC_{50} values on recombinant wild-type HIV-PR showed that the hexapeptide and pentapeptide compounds are all quite efficient in inhibiting the protease (Table 1). Values measured for three commercial inhibitors that present similar non-hydrolysable cores are also shown for comparison. Under our enzyme assay conditions, the hexapeptide shows a value comparable to that of the commercial inhibitors but is still quite large ($M_r = 825$). Comparison with the analogous diaminodihydroxy inhibitor TS-70[21] (Scheme 4) indicates that the monohydroxy isostere provides marginally better binding to HIV-PR. Replacing the Kyn-Val dipeptide on the P_2'/P_3' side of the central module with dmPoa (TS-92–TS-95) results in a slightly increased inhibitory activity, while molecular weight is reduced. Varying the amino acid in P_2 has little effect on IC_{50} (see TS-92–TS-94), but significantly modulates the solubility/hydrophobicity characteristics as indicated by the calculated $\log P_{o/w}$ values (Table 1). Furthermore, the conformation of the core hydroxy does not seem to be critical for inhibition as indicated by the identical activities of the epimeric compounds TS-94 and TS-95.

The negligible effect of the hydroxy group conformation for HIV-PR inhibition is confirmed in the tetrameric compounds TS-96 and TS-97, with dmPoa in P_2 and P_2'. These maintain a quite reasonable activity (≈25 nM) despite a considerably reduced size, confirming the effectiveness of dmPoa in interacting with the S_2 and S_2' binding subsites in the enzyme. That this interaction depends on the presence of the methyl groups in this residue is substantiated by the pair TS-98 and TS-99, with Poa in P_2 and P_2', for which the IC_{50} is shifted to the micromolar range. In this case, in the absence of strong interactions from the flanking residues with the S_2 and S_2' subsites of the enzyme, the compound with the *S*,*S*,*S* core configuration is the better inhibitor, but the effect is marginal. Furthermore, despite indications to the contrary provided by theoretical studies,[35] it appears that the presence of hydroxy, formyl or acetyl substituents on the aromatic ring, which might act as H-bonding donors or acceptors and considerably lower the inhibitors' $\log P_{o/w}$ value, does not improve the IC_{50} (see compounds TS-100–TS-105).

Inhibition of *C. albicans* Sap2

Inhibition by our compounds of another aspartic protease, Sap2, was also determined, using a protease purified from the H12 strain of *C. albicans*.[21] As the fungal Sap are monomeric proteins with two equivalent domains,[36] they are similar to the host aspartic proteases, so that they can be used as models to test the specificity of peptidomimetic inhibitors for the retroviral protease.

In effect, there does seem to be a considerable difference in the way the reported inhibitors interact with the fungal protease. The peptidomimetic TS-91, which extends from P_3 to P_3' and is a potent inhibitor of HIV-1 PR, also inhibits Sap2, albeit in the low micromolar range (IC_{50}, 12 μM). However, comparison of TS-91 with the dihydroxy analogue TS-70 (IC_{50}, 0.2 μM, Scheme 4),[21] indicates that in this case the dihydroxy isostere binds more strongly to the fungal protease than the monohydroxy isostere. Furthermore, regarding the stereochemistry, it is apparent that for this enzyme the *S*,*S*,*S*-configuration is preferred (see compounds TS-94–TS-105, Table 1). Finally, contrary to the viral protease, dmPoa is decidedly not suitable in P_2, as indicated by the high IC_{50} values of compounds TS-92–TS-97. Again, the methyl groups on the Poa ring seem to play a key role, that is however detrimental to binding for the fungal protease, so that Poa, as well as its derivatives with H-bond donor/acceptor groups, such as aPoa or fPoa, appear to be more suitable for positions P_2 and P_2'. Indeed, compound TS-98, with a M_r of only 553, has an IC_{50} value (4 μM), which is lower than that of the hexameric inhibitor TS-91, indinavir and nelfinavir, and is comparable to that of ritonavir.



TS-70

Scheme 4. Structure of the inhibitor TS-70, based on a dihydroxyethylene isostere in the central module P_1–P_1'.[19–21]

Molecular modelling

An explanation for the observed variations in the IC_{50} of selected peptidomimetics for Sap2 was obtained by molecular modelling. Docking of the *S*,*S*,*S* epimer TS-98 was carried out onto the structure of the inhibitor A-70450, bound to Sap2X.[36] This resulted in a good overlap with the central part of A-70450, which is in contact with the S_1 and S_1' enzyme subsites (Fig. 1A). After optimisation of the complex between TS-98 and the enzyme, a molecular dynamics run went to equilibration, indicating a good fit of TS-98 to the active site (Fig. 1B). In analogy with the reference inhibitor A-70450, the hydroxy group of TS-98 forms H-bonding interactions with catalytic Asp^{32} and Asp^{218}, the P_1' amide nitrogen forms H-bonding interactions with Asp^{218} and the Poa carboxy group in P_2 interacts with the main-chain nitrogen of Gly^{85} (Fig. 1B). This allows the inhibitor Phe side chain in P_1 to enter a hydrophobic pocket lined by Ile^{30} and Ile^{119}. The *S*,*R*,*S* epimer, TS-99, was more difficult to dock onto the reference inhibitor, and after optimization and molecular dynamics, it was apparent that two of the three sets of H-bonding

Table 1. Inhibition of HIV-1 and C. albicans aspartic protease by hydroxyethylamine-based peptidomimetics.[a]

	P_3-P_2-P_1-Ψ-P_1'-P_2'-P_3'	M_r (Da)	$\log P_{o/w}$[b]	IC_{50}	
				HIV-PR	Sap2 (μM)
TS-91	Kyn-Val-Phe-Ψ(*S*,*R*,*S*)[OH]-Phe-Val-Kyn	825	5.8	4 nM	12
TS-92	Kyn-Dtg-Phe-Ψ(*S*,*R*,*S*)[OH]-Phe-dmPoa	756	7.2	3 nM	80
TS-93	Kyn-Thr-Phe-Ψ(*S*,*R*,*S*)[OH]-Phe-dmPoa	718	4.3	4 nM	120
TS-94	Kyn-Val-Phe-Ψ(*S*,*S*,*S*)[OH]-Phe-dmPoa	716	5.2	3 nM	> 50
TS-95	Kyn-Val-Phe-Ψ(*S*,*R*,*S*)[OH]-Phe-dmPoa	716	5.2	3 nM	140
TS-96	dmPoa-Phe-Ψ(*S*,*S*,*S*)[OH]-Phe-dmPoa	609	7.6	28 nM	50
TS-97	dmPoa-Phe-Ψ(*S*,*R*,*S*)[OH]-Phe-dmPoa	609	7.6	25 nM	> 50
TS-98	Poa-Phe-Ψ(*S*,*S*,*S*)[OH]-Phe-Poa	553	5.6	5 μM	4
TS-99	Poa-Phe-Ψ(*S*,*R*,*S*)[OH]-Phe-Poa	553	5.6	17 μM	> 50
TS-100	hPoa-Phe-Ψ(*S*,*S*,*S*)[OH]-Phe-hPoa	584	4.1	7 μM	25
TS-101	hPoa-Phe-Ψ(*S*,*R*,*S*)[OH]-Phe-hPoa	584	4.1	9 μM	> 50
TS-102	aPoa-Phe-Ψ(*S*,*S*,*S*)[OH]-Phe-aPoa	636	5.0	19 μM	5
TS-103	aPoa-Phe-Ψ(*S*,*R*,*S*)[OH]-Phe-aPoa	636	5.0	23 μM	30
TS-104	fPoa-Phe-Ψ(*S*,*S*,*S*)[OH]-Phe-fPoa	608	5.0	68 μM	5
TS-105	fPoa-Phe-Ψ(*S*,*R*,*S*)[OH]-Phe-fPoa	608	5.0	80 μM	12
Ritonavir[c]		721	4.0	3 nM	1.2
Indinavir[c]		614	4.0	1.3 nM	50
Nelfinavir[c]		568	4.1	1.9 nM	234

[a]Three letter codes are used to identify aminoacids in P_1/P_1', P_2/P_2'. Other abbreviations: Kyn = kynurenic acid; Dtg = D-α-(2-thienyl)glycine; Poa = phenoxyacetic acid; dmPoa = 2,6-dimethylphenoxyacetic acid; hPoa = 2-hydroxyphenoxyacetic acid; aPoa = 4-acetlyphenoxyacetic acid; fPoa = 4-formylphenoxyacetic acid. See also Scheme 3.
[b]Calculated using the cLogP programme (v 4.01, www.daylight.com).
[c]Ritonavir, indinavir and nelfinavir were extracted from drug formulations and purified by RP-HPLC.

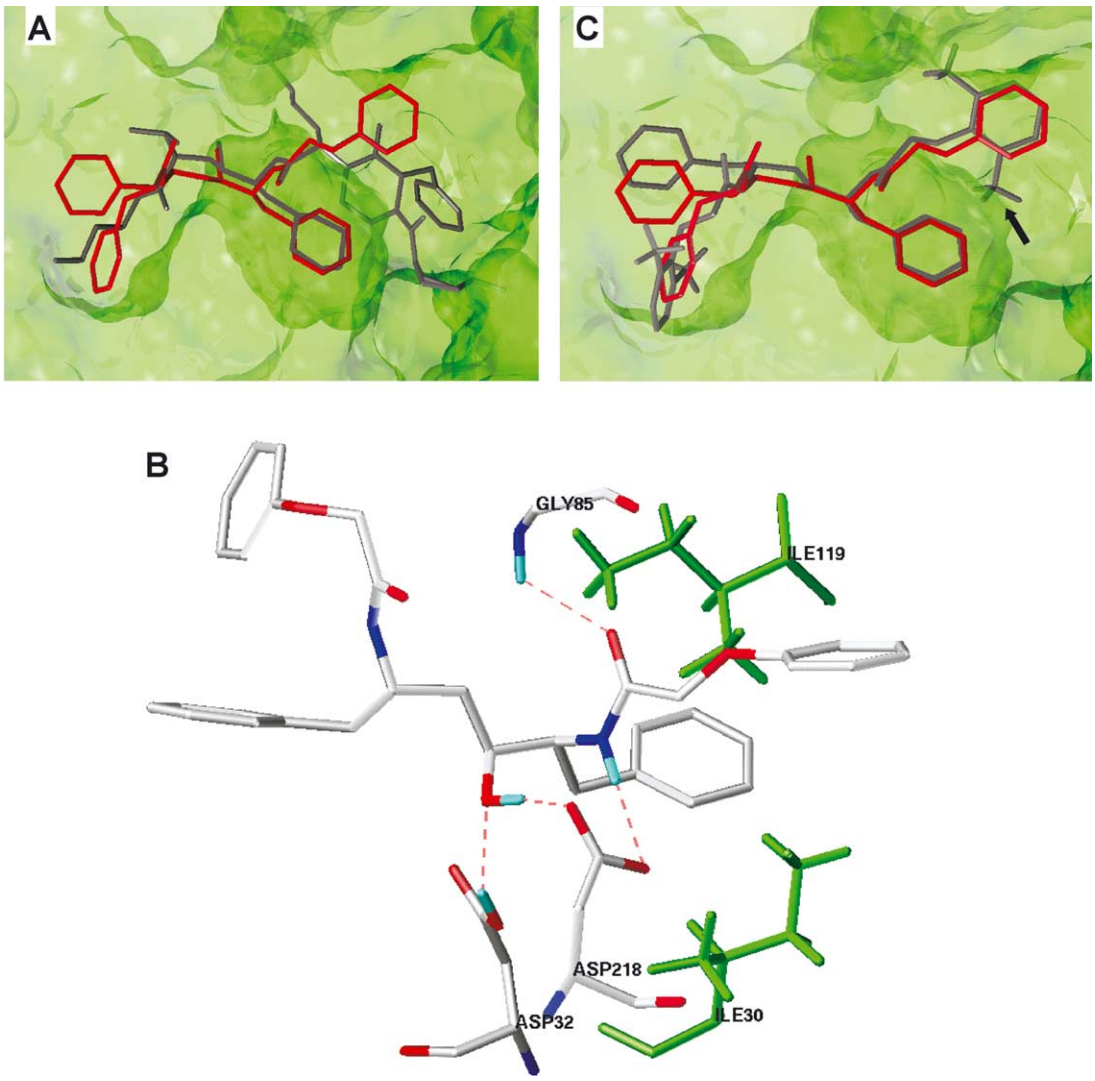


Figure 1. Model structure of TS-98 bound to the active site of *C. albicans* Sap2. The enzyme structure was based on that of PDB entry 1zap. (A) TS-98 was first docked onto the original inhibitor so as to give a maximum overlap. A-70450 is in grey and TS-98 in red. (B) The original inhibitor was then removed from the active site and the model structure of Sap2 bound to TS-98 minimized and subjected to a molecular dynamics run. H-bonding interactions of TS-98 with enzyme residues, and Ile residues lining a hydrophobic pocket for the Poa group are shown. (C) TS-98 was then replaced by TS-96 and the process repeated; TS-98 is in red, TS-96 is in grey. The region where a methyl group from the P_2' dmPoa residue in TS-96 sterically clashes with the active site is indicated by an arrow.

interactions, namely that between the Poa carbonyl and Gly^{85} nitrogen and that between the P_1' amide nitrogen and Asp^{218} (Fig. 1B), were broken; at the same time neither of the inhibitor Phe side chains could fit into the S_1 hydrophobic pocket. The loss of these interactions lead to a calculated complexation energy for the *S*,*R*,*S* epimer less favourable by 6.8 kcal/mol, consistent with a lower affinity of this configuration for the binding site. This appears to be a general feature as a lower affinity of the *S*,*R*,*S* epimer was also observed for the other tetrameric inhibitors (Table 1).

Comparison of model structures for TS-98 and TS-96 bound to the enzyme indicated that the methyl groups on the aromatic ring in the latter severely interfere with binding to the Sap2 active site. In correspondence with P_2, steric interactions are relieved by a rotation of the dmPoa moiety and its extension outwards from the binding site, while P_2' is constrained at the cost of bumping of one methyl group in particular with enzyme residues Thr^{221}, Thr^{222}, Ile^{223} and Tyr^{225} (Fig. 1C). These interactions, together with the enhanced exposition to solvent of the aromatic ring in P_2, and a severe strain induced in the whole protein structure, causes a loss of calculated complexation energy of more than 40 kcal/mol with respect to TS-98. The binding site of Sap2 is narrower than that of HIV-PR, and the S_2,S_2' subsites impose more stringent requirements on residues that can be accepted. In this respect, the S_2 sub-site in the retroviral protease appears to be quite tolerant, and potent inhibitors result from the incorporation in P_2 of different residues such as dmPoa, Thr, Val or Dtg (Table 1).

Conclusions

We have extended a highly flexible method for rapidly assembling diverse aspartic protease inhibitors, which could be generally advantageous for rapidly generating

Table 2. Yields, MS and HPLC data

Compd	Yield (%)	ES-MS (*m*/*z*)	HPLC (min)
TS-91	10	825.7	30.5
TS-92	10	757.4	39.0
TS-93	10	718.7	29.6
TS-94	15	717.4	34.0
TS-95	15	717.4	36.5
TS-96	34	610.4	41.8
TS-97	25	610.3	42.8
TS-98	54	553.2	34.6
TS-99	45	553.2	34.3
TS-100	38	585.4	25.2
TS-101	30	585.3	28.4
TS-102	21	637.2	27.7
TS-103	20	637.2	27.6
TS-104	43	608.9	28.1
TS-105	37	609.3	27.6

lead compounds active against aspartic proteases of pathogens, including retroviral and secreted fungal proteases or the digestive proteases of the *Plasmodium falciparum*.[37] It may also be useful in probing for solutions to the problem of resistance in HIV-PR mutants. In this respect, it has been shown that mutations that lead to resistance often affect the hydrophobic enzyme residues interacting with the inhibitor residues in the P_3/P_3' positions[7] and that molecules without a P_3 group or with one of reduced size exhibit a strong inhibition against both wild-type and drug resistant mutant proteases. Our tetrameric inhibitors based on the dmPoa moiety thus appear to be excellent candidates for further development.

Experimental

General

Fmoc-protected amino acids, benzotriazol-1-yl-oxy-*tris*(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were from ABI (Columbia, MD, USA). *N*,*N*-Diisopropylethylamine (DIPEA), dimethylsulfoxide, dimethylformamide (DMF) hydroxybenzotriazole (HOBT), phenoxyacetic acid (Poa), 2-hydroxyphenoxyacetic acid (hPoa), 4-formylphenoxyacetic acid (fPoa), 4-acetylphenoxyacetic acid (aPoa) and kynurenic acid (Kyn) were from Fluka Chemie (Buchs, CH), D-α-(2-thienyl)glycine (Dtg), Fmoc-*N*-hydroxysuccinimide ester (Fmoc-OSu), *N*-(*tert*-butoxycarbonyl)-L-phenylalanine methyl ester, and all other reagents used in the synthesis of **4** and **6** (see caption to Scheme 2) were from Sigma-Aldrich (St Louis, MO, USA). All other reagents were technical or analytical grade as appropriate. Syntheses were monitored using ES–MS spectrometry (API-*I* Sciex) which was also used to determine the correct structure and purity of each final product in conjunction with analytical RP-HPLC (Water Symmetry C18, 3.5 μm, 4.6×50 mm) carried out on a Gilson instrument, using a 20–60% gradient of CH_3CN in 0.05% aqueous TFA in 30 min, and a flow of 0.8 mL/min. Semi preparative purification (up to 4 mg per elution) was carried out either on a Waters Radial-PAK C18, 8×10 mm cartridge using a gradient of 30–80% of CH_3CN in 0.05% aqueous TFA in 50 min, and a flow of 1.5 mL/min, after extraction, (TS-91–TS-95), or by directly injecting the reaction mixture into a Waters Sep-Pak Plus disposable C18 cartridge, using a gradient of 20–70% of CH_3CN in 0.05% aqueous TFA in 100 min, and a flow of 0.8 mL/min, (TS-96–TS-105). A Gilson UV/VIS 155 detector set at the wavelength of 214 and 254 nm was used in all the analyses.

Synthesis of diaminoalcohol isosteres

Diaminoalcohols **4** and **6** were synthesized as described.[22] Reagents and conditions are summarized in Scheme 2.

Synthesis of peptidomimetics

Peptidomimetics were assembled in solution from the central modules **4** and **6**, on a 0.1 mmol scale, using standard peptide synthesis protocols as described previously.[20] Amino acidic or carboxylic acid residues, or pre-synthesised dipeptides were added to the central module (2 equiv per free amine group on modules **4** or **6)**, in the presence of equimolar amounts of TBTU and HOBT and a 4-fold excess of DIPEA, in DMF. Synthetic intermediates, as well as the hexameric and pentameric final products TS-91–TS-95, were extracted from 5% $NaHSO_4$ with ethyl acetate (EtOAc), washed with brine, dried over $MgSO_4$, evaporated under vacuum and recrystallised from EtOAc/hexane prior to semipreparative RP-HPLC purification, using Waters Delta-Pak C18 cartridges. The tetrameric products TS-96–TS-105 were instead purified without extraction, by directly loading the reaction mixture onto disposable Waters Sep-Pak cartridges adapted for use on HPLC equipment. All products were confirmed to have the correct structure and to be pure by analytical RP-HPLC/mass spectrometry. Yields from modules **4** and **6**, mass spectral data and HPLC details are reported in Table 2.

Synthesis of symmetric products

TS-91 and TS-96–TS-105 were prepared in one-pot reactions by adding 4 equiv each of the pre-synthesized dipeptide Kyn-Val-OH and TBTU or the appropriate Poa derivatives to the central modules **4** or **6**, previously deprotected from the Boc group by treatment with 95% TFA.

Synthesis of asymmetric products

TS-94 and TS-95 were synthesised by addition of the dipeptide Kyn-Val-OH to the unprimed side, followed by Boc deprotection and addition of dmPoa to the primed side. The dipeptide was prepared by reacting Kyn with H-Val-O*t*Bu and then removing the protecting group with 95% TFA. When using the dipeptide for couplings, to reduce racemization, PyBop and collidine were used in preference to TBTU and DIPEA.

Synthesis of TS-92 and TS-93

These compounds were obtained by adding Fmoc-Thr(*t*Bu)-OH or Fmoc-Dtg-OH (2 equiv) to the unprimed side, extracting and deprotecting from Boc with 95% TFA, adding dmPoa (2 equiv) to the primed side, extracting and deprotecting from Fmoc with 95% piperidine in DMF and then adding Kyn (2 equiv) to the unprimed side. Fmoc-Dtg-OH, used in compound TS-92, was previously prepared by treating Dtg with 0.8 equiv Fmoc-*O*-succinammide and extracting as described above.

Enzyme assays

Recombinant HIV-1 protease was purchased from Bioczech (Prague, Czech Republic) and Sap2 was purified from *C. albicans* H12, as reported.[21] The fluorogenic substrates Abz-Thr-Ile-Nle-Phe(NO_2)-Gln-Arg-NH_2 (HIV-protease) and Arg-Glu-[5-(2-aminoethylamino)-1-naphthalenesulfonate]-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys-[4′-dimethylaminoazobenzene-4-carboxylate]-Arg (*C. albicans* protease) were from Bachem (Bubendorf, CH) and Molecular Probes Europe (Leiden, NL), respectively. The extent of inhibition of the activity of the aspartic protease of HIV-1 and *C. albicans* (Sap2) was evaluated as previously reported for compounds based on a dihydroxyethylene module[20] and for some of the inhibitors described here.[21] In particular, assays of HIV-PR activity were performed at 25 °C in 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 mM MES–NaOH buffer, pH 5.5, and 30 μM fluorogenic substrate Abz-Thr-Ile-Nle-Phe(NO_2)-Gln-Arg-NH_2. Titration of HIV-PR was carried out with two reference inhibitors, ritonavir and indinavir, using the equation $IC_{50} = [E]/2 + K_i\ (1 + [S]/K_M)$,[38] where K_i are the published inhibition constants for the reference inhibitors[7,39,40] and K_M was 25 μM, as determined under our conditions. The active enzyme concentration in the assay, [E], was estimated to be 3.6 ± 2.3 nM.

Molecular modelling

Calculations were carried out on Silicon Graphics Octane 1 workstations. Sybyl 6.8 (Tripos Inc., 1699 South Hanley Road, St. Louis, MO 63144, USA; www.tripos.com) was used for all the molecular mechanics and molecular dynamics simulations. The initial coordinates for the Sap2 structure correspond to those of the complex of A-70450 with *C. albicans* Sap2X,[36] and were obtained from the Protein Data Bank, Brookhaven National Laboratory (PDB entry 1zap). After adding hydrogens and removing crystallographic water molecules, the starting model of the A-70450–Sap2X complex was obtained by the multistage minimization procedure described by Levit and Lifson[41] and previously used also by other authors on Sap2X.[42] The Cornell's version of the AMBER force-field,[43] as implemented in Sybyl 6.8, was used in all energy minimisations and dynamics runs. The starting structure, as well as all other structures of ligands and enzyme complexes, was minimized until a convergence criterion of 0.001 kcal/mol/Å was achieved for all energy gradients. The conjugate gradient minimisation algorithm was always used after running 20 initial steps of Simplex linear minimization. All the calculations were carried out in a continuum dielectric of relative permittivity $\varepsilon = 4rij$.[44]

The structures of the two *S*,*S*,*S* and *S*,*R*,*S* epimers TS-98 and TS-99, and the structure of the dimethyl-Poa derivative TS-96 were built with standard bond lengths and angles, minimized and submitted to an extensive conformational search driven by a genetic algorithm operating on all the rotatable bonds.[45] The energies of the absolute conformational minima thus obtained were taken as reference energies of the free ligands.

The ligands were then manually docked onto the structure of the A-70450/Sap2X complex so as to achieve maximum overlap at the level of the fundamental hydroxy group and of the core hydrogen-bonded and hydrophobic groups. After removal of the reference inhibitor, the new model structures were first optimised by running an energy minimisation on the full structure to a 0.05 kcal/mol/Å energy gradient. The models were then subjected to a molecular dynamics run in the NTV ensemble, at 300 K for 500 ps. The complexes resulted stable at the dynamic simulation, and equilibration was always achieved within 300 ps. The lowest potential energy equilibrium geometries were finally re-optimised to a final gradient of 0.001 kcal/mol/Å.

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