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Synthesis of Novel Inhibitors of the HIV-1 Protease: Difunctional Enols of Simple N-Protected Amino Acids

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Abstract—A series of enol HIV-1 protease inhibitors which show competitive inhibition and the structure–activity relationship study which led to the design of these compounds are reported. By systematically modifying simple amino acids, Boc-Phe enol and Boc-Tyr enol derivatives yield nanomolar Ki_{app} values (Ki_{app} = 0.485 μM and Ki_{app} = 0.425 μM, respectively). These enols are of low molecular weight (< 500 g/mol) and of non-peptidic nature. The enols are synthesized in a one step chemical synthesis and modifications to increase their potency could easily be performed. Boc-Phe enol and Boc-Tyr enol showed low inhibitory effect on pepsin, Ki_{app}s of 23 and 149 μM, respectively, and Boc-Phe enol showed a Ki_{app} of 20 μM for cathepsin D. Neither of these two compounds inhibited renin (< 10% inhibition at 200 μM).

Introduction

The rapid spread of the human immunodeficiency virus (HIV), mainly HIV-1, the etiological agent of the Acquired Immunodeficiency Syndrome (AIDS) is urging scientists to develop new drugs that can interrupt the viral life cycle. Efficient vaccine development is still faced with the ongoing mutational events of HIV that enable it to evade the immune system. Efforts are now focused on the design of new drugs that will arrest the viral life cycle. Only a few drugs, such as AZT and ddI, have been approved so far. These nucleoside analogs are incorporated into the retroviral DNA by the reverse transcriptase resulting in chain termination.[3] Development of other effective compounds (nucleoside and non-nucleoside analogs) focusing on the reverse transcriptase inhibition are still underway. However, resistant strains appear within nine months of treatment and as a result these drugs are unable to stop the replication cycle.[4]

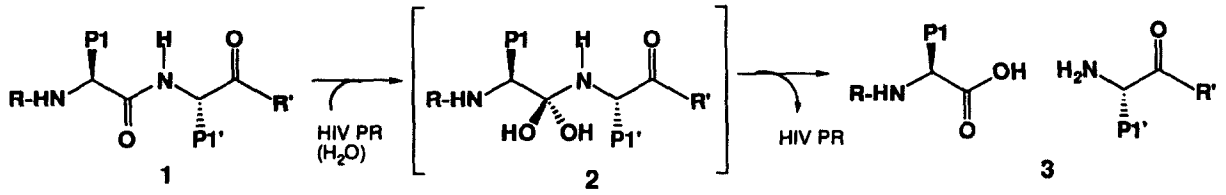
The HIV protease (HIV PR), which is indispensible for the specific cleavage of the polyproteins $pr55_{gag}$ and $pr160_{gagpol}$[5,6] has been identified as an excellent target in the search for an effective therapy against this illness. The specific cleavage of the two polyproteins results in the liberation of the structural proteins, p17 (matrix), p24 (capsid), p7 (nucleocapsid), p9-p6 (RNA stabilizers), and the viral enzymes, p20 (protease), p55–p61 (reverse transcriptase) and p31 (integrase). Mutations at specific amino acids (particularly Asp^{25}) generate a catalytically defective enzyme[7–9] which results in the production of noninfectious virions. Inhibition of the protease results in the same effect,[10–13] and consequently the spread of the viral infection should be slowed down.

Recently, it has been demonstrated that resistant strains of the virus appear very rapidly, characterized by mutations of specific amino acids of the protease.[14] Since no cross-resistance between the inhibitors of the protease has been reported, combinatorial therapies are now sought to prevent the occurrence of viral resistance. Most importantly, HIV PR inhibitors show synergy with AZT and ddI.[15]

Molecular modeling,[16] crystallographic data,[17–23] and molecular dynamic analysis[24] of the protease allow for the rational design of new drug candidates. Crystallographic data of enzyme–inhibitor complexes permit scientists to speculate on the enhancement of drug efficiency by the incorporation of substituents at some precise position on their lead compounds known to interact specifically with the protease.

Potent inhibitors of the HIV-1 PR have been synthesized, as analogs of the transition state illustrated in Scheme I. The transition state analogs include hydroxyethylene, hydroxyethylamine, dihydroxyethylene, difluoroketone, reduced peptide bond moieties,[25] and the list continues to increase. The enzyme interacts similarly with the backbone of these transition-state analogs, but side chain interactions of the protease–inhibitor complexes vary.[21–23] The hydroxyl group of these analogs replaces the hydrated amide intermediate of the substrate implicated in the proteolytic process and interacts directly with Asp^{25} and $Asp^{25'}$ (Figure 1).[26]

These transition state inhibitors can be separated into three distinct groups:[27] (1) proline and proline-like side chains at the P1' position, (2) inhibitors based on the study of renin inhibitors, and (3) C_2 symmetric inhibitors. The rapid appearance of highly potent inhibitors is mainly due to the study during the last decades of different enzymes, especially renin inhibitors as anti-hypertensive agents.



Scheme I.

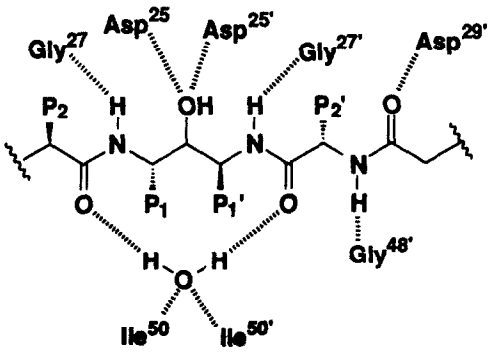
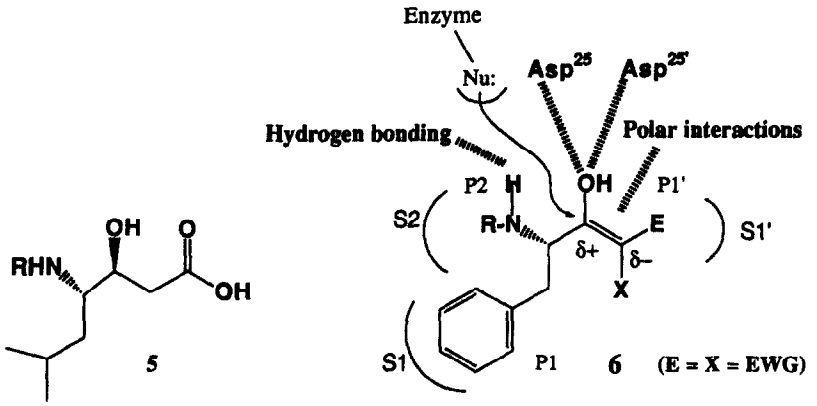


Figure 1. Interaction of transition-state inhibitor analogs at the active site of the HIV-1 PR. Interactions between the backbone of the inhibitors are mostly conserved, while side chain interactions are less conserved between inhibitors.

Our approach to the design of HIV-1 protease inhibitors is based on a novel isostere which relies on the chemistry of the enol functionality.[28] During the course of our study on serine protease inhibitors,[29] we became intrigued by the topographical similarities between statine **5** (Figure 2) and a prototypical enol **6**, and began to wonder about the effectiveness of these enols as inhibitors of aspartic proteases. Inhibitors like **7** which contain a hydrophobic group in the P1 and P1' positions are known to have high affinity for the protease. Also, compounds like **8** with a difluoroketone group, favoring the hydrated form, are known to be good inhibitors of HIV-1 PR.[30] In addition, the difluoro substitution polarizes the hydroxyls, which results in a strong interaction with Asp^{25} and $Asp^{25'}$ at the active site. As for the C_2 symmetric and the difluoroketone inhibitors, it can be hypothesized that an enol with a polarizable hydroxyl group may also interact with Asp^{25} and $Asp^{25'}$ at the active site. Furthermore, the enol can serve as an electrophilic center which may interact with nucleophilic groups of the amino acid side chains or water molecules at the active site.

In order to develop small and non-peptidic inhibitors, a combination of properties has been taken into consideration. First, the introduction of the polarized hydroxyl (enol function **6**, Figure 2) which is similar to



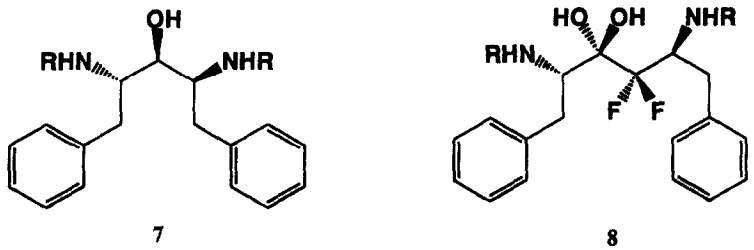


Figure 2. Structural comparison between inhibitors. Transition-state analog inhibitors are based on statine (**5**) and all comprise one or many hydroxyls (**7**). The highly polarized hydroxyl of the difluoroketone (**8**) suggests that the polarized enol may interact strongly with the enzyme (**6**).

the tetrahedral intermediate, should ensure the appropriate orientation and emplacement of the molecule at the active site. Second, a judicious choice of electron-withdrawing substituents adjacent to the enol function provide adequate polarization of the hydroxyl such that it interacts with the amino acids responsible for substrate cleavage (Asp^{25} and $Asp^{25'}$). Finally, the nature of the different substituents E, X and the amino acid side chain should also possess good complementarity with the S1 and S1' enzyme subsites and strong affinity through hydrophobic interactions, hydrogen bonds, etc.

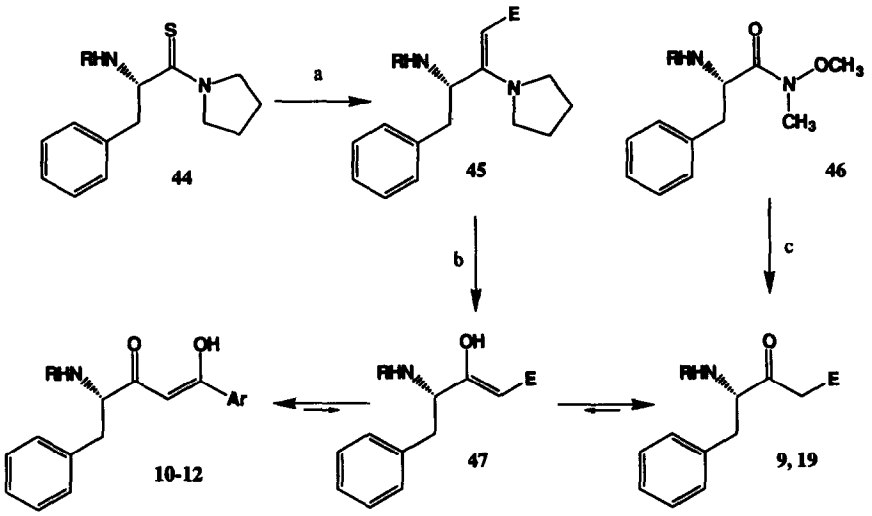
These characteristics should counterbalance the small size of the analogs when compared to the polypeptidic analogs having additional binding sites at other protease subsites. The proposed enols may provide the strong interactions sought for allowing small molecules to be highly potent inhibitors. Herein, we describe novel enol inhibitors of HIV-1 PR which may give way to the design of potent inhibitors without having the peptidic bond limitations encountered thus far.

Chemistry

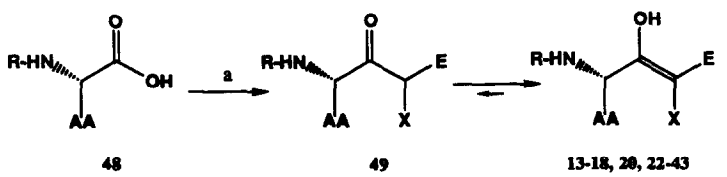
Compounds **9–43**[31](Tables 1–3) were prepared according to Schemes 2–4 by application of our own methodology.[32–34] The monofunctionalized compounds **9–12** were synthesized (Scheme II) from the corresponding thioamide (**44**) and functionalized bromomethylene reagent in the presence of sodium iodide. The Eschenmoser reaction proceeds through a sulfide contraction of the thioiminium intermediate with diisopropylethylamine, followed by the extrusion of sulfur on the episulfide intermediate with triethylphosphite. The resulting enamine (**45**) is hydrolysed under mild acid conditions (hydrochloric acid, 0.4 N).[32] The yields varied between **47** and 66 %. The benzyl compound **19** (E = C_6H_5) was obtained in 67 % yield by treatment of Boc-Phe amide **46** (prepared in 88 % from commercial Boc-Phe-OH according to Weinreb's procedure:[35,36] HN(Me)OMe, $ClCO_2Me$, Et_3N, CH_2Cl_2) with phenylmagnesium bromide (2 eq.) in THF.

We observed exclusively the ketone form by ^1H NMR spectra (typical AB pattern around 3.5 ppm for 2H) for the monofunctionalized compounds (**9, 19**) with the exception of compounds **10–12** where the arylketone moiety exists in the enol form (singlet signal around 6.0 ppm for one vinyl proton). The enols of the aryl diketones exist as tautomers as indicated in Scheme II (structures **10–12** and **47**), where **10–12**, as represented, is the predominant form due to resonance of the double bond (extended conjugation) with the aromatic ring. In **47**, delocalization (crossed conjugation) is less efficient.[37] No racemization was detected by NMR and HPLC with these reaction conditions.

The difunctionalized enols **13–18, 20** and **22–43** were obtained according to Scheme III.[33] The carboxylic acid (**48**: N-protected amino acid or dipeptide) was activated with 1,1'-carbonyldiimidazole, and in parallel a carbanion was generated by the addition of the functionalized methylene to a suspension of sodium hydride in THF. The imidazolide solution was then cooled to -78 °C, and the carbanion solution was added dropwise. After work-up, the purification of the product was achieved by flash chromatography. The yields varied between 10 and 95 % depending on the carbanion. We observed only the enol form with these difunctionalized compounds by ^1H NMR, existing as a mixture of *E* and *Z* isomers in rapid equilibrium. This method proceeded without racemization.



Scheme II. a) $BrCH_2E$, NaI, CH_3CN; $P(OEt)_3$, $N(iPr)_2$ Et; b) HCl 0.4N, MeOH; c) Mg, BrBn, THF; Boc–Phe amide, THF.



Scheme III. a) CDI, THF; NaH, X-CH_2-E, THF.

The difunctionalized enamine **21** was prepared (Scheme IV) from the corresponding thioamide (**44**) and methyl trifluoromethanesulfonate giving the methylthioiminium intermediate which was treated with the *p*-nitrophenylacetonitrile carbanion (generated from sodium hydride and *p*-nitrophenylacetonitrile in dimethoxyethane).[34] The enamine **21** was obtained in 75 % yield and exists as a mixture of *E* and *Z* isomers as determined by ^{1}H NMR studies at low temperatures. No racemization occurs under the reaction conditions.

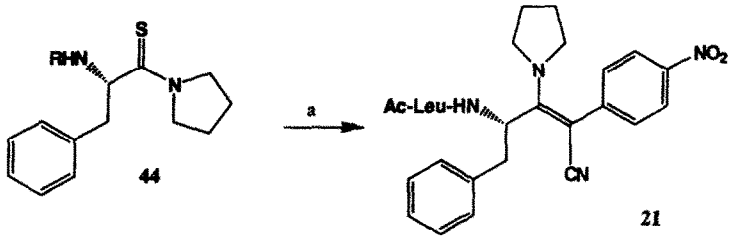
Enzyme assays

The HIV-1 protease assays, based on HPLC hydrolysate and peptide substrate quantification, were described earlier.[6] Each experiment was at least duplicated and the error on *Ki* measurements is 15–25 %. Cathepsin D, pepsin and renin were also assayed according to published methods.[38–40]

Results and Discussion

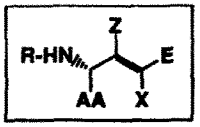
The HIV-1 PR inhibitors designed thus far have been subject to problems inherent to peptide-based drugs: rapid biliary excretion, low solubility, poor bioavailability, and rapid degradation in the blood.[41,42] The search for non-peptidic inhibitors is crucial for development of potential drugs. To date, only haloperidol,[43] cerulanin,[44] and recently non-peptidic carboxylates[45] have been reported as non-peptidic HIV-1 PR inhibitors with weak anti-enzymatic activity. In order to assess the potential HIV-1 inhibition of enols, a screening of molecules **9–21** with enol and ketone structures, synthesized in the course of our study on serine protease inhibitors, was performed to evaluate the type (hydrophobic, non-hydrophobic) and nature (electronic) of the substituents required around the enol moiety to interact with the HIV-1 protease in either a competitive, non-competitive or uncompetitive way. These results are shown in Table 1.

Even though most of the compounds listed in Table 1 showed weak inhibitory potential, some general conclusions can be drawn. Compound **9** with one non-hydrophobic electron-withdrawing group and compounds **10–12** and **19** with one hydrophobic group at the E position exist in the ketone form (in reference to the carbonyl adjacent to the C_α of the phenylalanine moiety) and gave inactive to mid-micromolar Ki_{app}s (> 2000, 823, 446, 336 and 689 μM, respectively). In general, monofunctionalized analogs exist in the ketone form. For the aryl β-diketones (e.g. **10**), the predominant species has the carbonyl next to the aromatic group existing in the enol form, while the carbonyl adjacent to the phenylalanine C_α remains in the ketone form. Since the enolic hydroxyl group is thought to mimic the substrate tetrahedral intermediate and thus would interact strongly with the



Scheme IV. a) $CF_3SO_2OCH_3$, DME; NaH, $(4\text{-}NO_2)C_6H_4\text{-}CH_2CN$, DME.

Table 1.



Compound No[31]	R	AA[a]	E	X	Z	Ki_{app} (μM)
9	NAc Leu	Benzyl	CN	H	OH	>2000
10	NAc Leu	Benzyl	2-Naphthoyl	H	OH	823
11	NAc Leu	Benzyl	Benzoyl	H	OH	446
12	NAc Leu	Benzyl	$(4\text{-}NO_2)$benzoyl	H	OH	338
13	NAc Leu	Benzyl	N-Piperidinocarbonyl	CN	OH	286
14	NAc Leu	Benzyl	CN	CN	OH	>2000
15	NAc Leu	Benzyl	Phenyl sulfonyl	CN	OH	152
16	NAc Leu	Benzyl	Diethyl phosphonate	CN	OH	206
17	NAc Leu	Benzyl	Methoxycarbonyl	CN	OH	209
18	NAc Leu	Benzyl	2-Pyridyl	CN	OH	168
19	Boc	Benzyl	phenyl	H	OH	689
20	NAc Leu	Benzyl	$(4\text{-}NO_2)$phenyl	CN	OH	9.5
21	NAc Leu	Benzyl	$(4\text{-}NO_2)$phenyl	CN	Pyrrolidyl	>2000

[a]All starting amino acids are of L configuration: Leu and Phe.

enzyme at the active site, it was hypothesized that the ketone form had low affinity with the enzyme. Thus, compounds with an electron-withdrawing group at both X and E positions should exhibit enhanced inhibition. This hypothesis is partly verified with compounds **13** and **15–18** which showed lower $K_{i_{app}}$s (286, 152, 206, 209 and 168 μM, respectively). The relatively small difference in their $K_{i_{app}}$s suggests poor complementarity with the enzyme at the S1' subsite. This hypothesis is reinforced when comparing **14** with **20**. Compound **14**, with two small and non-hydrophobic groups, did not inhibit the HIV-1 protease, while compound **20** with two electron-withdrawing groups, one being an electron-deficient hydrophobic aromatic ring, generated a $K_{i_{app}}$ of 9.5 μM.

Structure–activity relationship studies of the compounds listed in Table 1 led to an understanding of the specific requirements surrounding the enol moiety as shown in Figure 3; typical structures are shown at the left in Figure 3 with $K_{i_{app}}$s for different substituents at the R position and the presence of an electron-withdrawing substituent on the aromatic ring at the E position for comparison. Strong electron-withdrawing groups such as cyano and *p*-nitrophenyl are needed at the X and E positions, respectively, in order to favor the enol form. Replacement of the aromatic ring by cyano (**20** compared to **14**) led to a complete loss of inhibitory potential, indicating a requirement for hydrophobic ring substituents at the E position in order to achieve good complementarity with the groups at the S1' subsite of the active center. Substitution of an electron-deficient aromatic ring (nitrophenyl) at the E position by an unsubstituted phenyl (**26** compared to **37**) reduced the inhibitory potential by a factor of 50. Replacement of one electron-withdrawing group (cyano to hydrogen, compound **19**), gave a compound with a ketone function, resulting in a loss of inhibitory potential. It was also noticed that changing the rather rigid conjugated aromatic ring (**26** or **37**) to a more flexible one (**35**, insertion of a carbonyl group, Figure 3) at the E position had a profound effect on the activity, reducing inhibition by more than 100-fold as compared to **26**. Enolization is still taking place, however the location of the enol function is different in order to allow a more favorable extended conjugated system. It is believed that this difference in the enol position orients the phenyl group (E substituent) differently resulting in less favorable interactions with the protease. Finally, replacing the hydroxyl group by a pyrrolidyl (**20** compared to **21**) led to a complete loss of inhibitory potential, suggesting no favorable interaction of the pyrrolidyl compound in the active site of the HIV-1 PR. This may be caused by the increase of steric hindrance from the large pyrrolidyl group compared to the hydroxyl, but the enol function is also expected to be of crucial importance as it may insert into the active site in a manner very similar to the tetrahedral transition-state intermediate and consequently interact strongly with Asp^{25} and $Asp^{25'}$ of the protease.

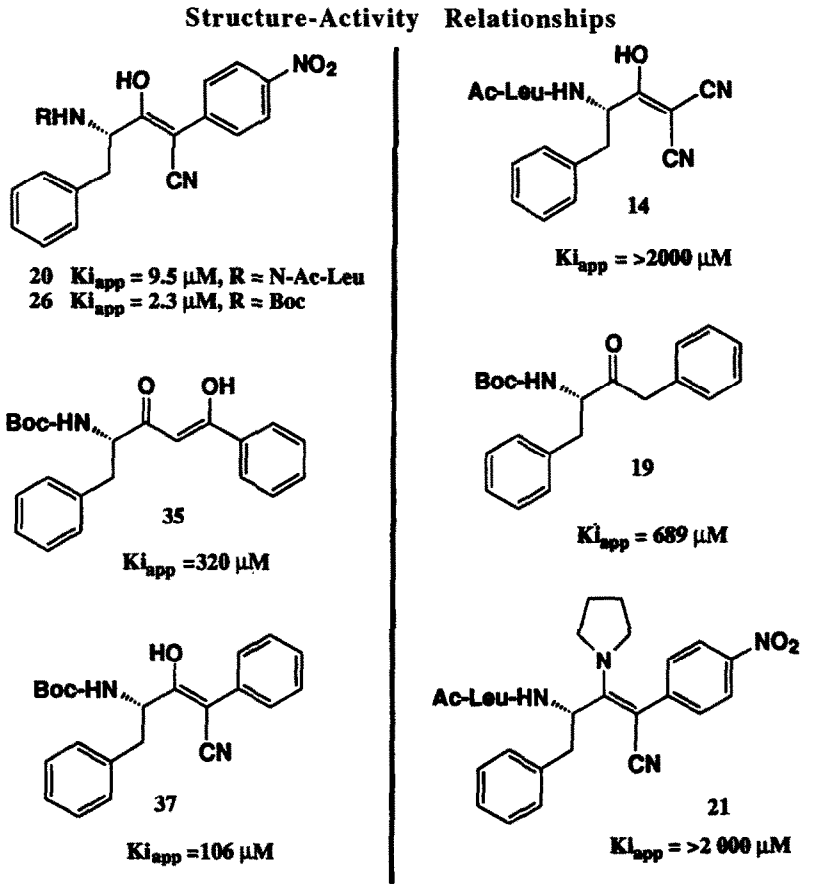


Figure 3. Structure–activity relationships. The requirement for specific substituents at the E position, (**14**, **21** and **19**), at the X position (**19**), and for the hydroxyl (**21**) is shown and compared with similar structures which generate lower $K_{i_{app}}$ (**20**, **26** and **37**). Also, the inhibitory potential increases as the EWG potential increases (**19** < **37** < **26**).

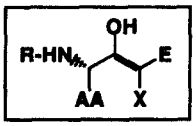
Knowing the requirement for an electron-withdrawing aromatic group at the E position, another electron-withdrawing group at the X position and the hydroxyl group at the Z position, we then turned our attention to the R position. The *N*-Ac-Leu moiety at that position was found to be a requirement from previous studies on serine protease, and better complementarity was sought with the HIV-1 PR. The P2 region was modified by changing the *N*-Ac-Leu moiety to a more simple protective group. The results are shown in Table 2.

Compounds **22** and **23**, Cbz and Fmoc, respectively, were evaluated. It has been described previously that the P2 position is preferentially occupied by a small hydrophobic substituent.[46] Hence, Cbz and Fmoc which are larger in size than *N*-Ac-Leu gave a higher Ki_{app} for the enol. Compound **24** with *N*-acetyl, a small substituent, did not inhibit the protease. The *N*-Boc compound **26**, an intermediate-sized substituent at the P2 position, generated the best inhibition with a Ki_{app} of 2.3 μM. Valine has shown good complementarity at the P2 position of many inhibitors of the HIV-1 protease,[47] and the structure of the Boc group is similar to the side chain of valine. Compound **25** (Boc-Val-Phe enol) showed poor inhibitory potential, suggesting poor complementarity of the Boc group with the enzyme S3 subsite. Charged amino acids or substituents are preferred at the substrate P3 position in order to interact with Arg^8 in the protease active site. So far the results suggest that the *N*-Boc group goes into the S2 subsite of the protease as observed recently for other inhibitors,[48] while the E substituent might fit into S1'.

As we were also interested in modifications at the AA position, we undertook the synthesis of a series of enols starting with different amino acids. Modifications at the AA position were made keeping the *N*-Boc at the P2 position. First, compounds with hydrophobic moieties such as **27**, **28**, **32** and **33** were synthesized, but such modifications did not improve the Ki_{app}. Compound **28** which presents an electron-deficient aromatic ring at the P1 position increases the Ki_{app} to 60 μM. Because tyrosine at the P1 position of many inhibitors showed better results than phenylalanine,[47] it can be postulated that electron-rich aromatic rings are preferred rather than electron-poor substituents. This hypothesis is partly verified with the lower Ki_{app} generated with compounds **32** and **33**. On the other hand, the 2-naphthylmethyl and phenyl side chain analogs **27** and **30** are in between compounds **28** and **33** with Ki_{app}s of 38 and 30 μM, respectively. The relatively big difference observed in Ki_{app} for compounds **26** and **30** may also indicate that these interactions occur specifically in the hydrophobic subsite (S1). The 10-fold increase in Ki_{app} with an isopropyl group as in analog **36**, which may not fit as well as benzyl or other substituted benzyl groups in the enzyme S1 pocket, further suggests that specific interactions are occurring at the hydrophobic subsite(s).

Replacement of the phenyl group, as in analog **26**, by a saturated ring (cyclohexyl compound **29**, Ki_{app} = 15 μM) did not improve the inhibitory potential, but still indicates good affinity with the enzyme. In fact, inhibition is similar to the one observed with Trp or O-protected Tyr. The cyclohexylmethyl residue at the P1 position has been reported to interact specifically at the S1 subsite of the enzyme with inhibitors based on renin-like substrates.[49–51]

Table 2.



Compound No	R	AA[a]	E	X	Ki_{app} (μM)
22	Cbz	Benzyl	(4-NO_2)phenyl	CN	30
23	Fmoc	Benzyl	(4-NO_2)phenyl	CN	78
24	Ac	Benzyl	(4-NO_2)phenyl	CN	ni[b]
25	BocVal	Benzyl	(4-NO_2)phenyl	CN	ni[b]
26	Boc	Benzyl	(4-NO_2)phenyl	CN	2.3
27	Boc	2-Naphthylmethyl	(4-NO_2)phenyl	CN	38
28	Boc	(4-NO_2)Benzyl	(4-NO_2)phenyl	CN	60
29	Boc	Cyclohexylmethyl	(4-NO_2)phenyl	CN	15
30	Boc	Phenyl	(4-NO_2)phenyl	CN	30
31	Boc	-C_3H_6-[c]	(4-NO_2)phenyl	CN	181
32	Boc	4-O-(2,6-Dichlorobenzyl)-Benzyl	(4-NO_2)phenyl	CN	12
33	Boc	3-Indolylmethyl	(4-NO_2)phenyl	CN	18
34	Boc	Benzyl[d]	(4-NO_2)phenyl	CN	54
35	Boc	Benzyl	benzoyl	CN	320
36	Boc	Isopropyl	(4-NO_2)phenyl	CN	275

[a]All starting amino acids are of L configuration, except for **34** starting with D-Phe.
[b]ni = no substancial inhibition at 400 μM.
[c]N and C_α are part of pyrrolidine derived from the amino acid L-proline.
[d]Attached to chiral center (C_α) with R configuration.

Proline (compound **31**) showed poor inhibition, and this result can be explained by the fact that the modified peptidic bond is at the C-terminal position of proline compared to other proline-like inhibitors where the modification (e.g. hydroxyethylene isostere) generally takes place adjacent to the N-terminal position.[52–54] In this way proline should fit into the S1' protease subsite.

The enzyme showed different inhibition values for D and L-phenylalanine (**34** and **26** respectively), demonstrating specificity of the enzyme for L-Phe and also suggesting competitive inhibition. All the results so far place the proposed enol analogs in the C_2 symmetric inhibitor group with a hydrophobic group in P1 and P1' positions, and have established the necessity for a specific amino acid (L-Phe) and a P2 ligand (*N*-Boc).

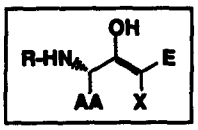
After carefully inspecting the data in Table 1, we were struck by the electron-deficient nature of the *p*-nitrophenyl group needed at the E position and began searching for a functionality that would increase this trend. It was thought that by increasing the electron-withdrawing nature of the ring, the inhibitory potential would also increase. This new series of modifications is shown in Table 3 with compounds **37–40**.

The hypothesis that a highly polarized hydroxyl group, generated by the electron-withdrawing strength of the E and X groups would enhance the inhibitory potential is confirmed. The inhibition improves as the number of fluorine atoms increases on the phenyl ring (**37**, **38** and **40** show Ki_{app}s of 106, 40 and 0.480 μM, respectively). The trifluoromethyl group, a weak electron-withdrawing substituent of the phenyl (**39**), slightly enhanced the inhibitory potential when compared to the unsubstituted phenyl analog **37**. Some electron-deficient aromatic rings have been incorporated into the P1' position of hydroxyethylene isostere inhibitors without improvement of inhibitory potential.[55] These results seem in contrast to our observations, however the role of the electron-withdrawing substituent at the E position might be more implicated in the polarization of the adjacent hydroxyl than strictly involving the binding affinities toward the S1' subsite. It is known that the keto–enol equilibrium is displaced to the enol form as the electron-withdrawing strength increases.[56] Since the enol hydroxyl mimics the substrate tetrahedral transition-state intermediate, the stronger the electron-withdrawing groups are, the stronger the interactions between the highly polarized hydroxyl of the enol with Asp^{25} and $Asp^{25'}$ at the active site should be. Secondary binding interactions at the S1' subsite may act differently and this may explain why divergent results are obtained with inhibitors harboring the enol isostere. There are, however, differences in protease affinity between the pentafluorophenyl and the 4-nitrophenyl groups in the E enol position as can be seen from the comparison of analog **26** with **40** and **20** with **41**. Although both rings exert similar inductive effects on the enol double bond, the nitro substituent presents a dipole that may contribute to less effective hydrophobic interactions in the S1' subsite when compared to the symmetrical pentafluorophenyl electron-withdrawing group.

Thus, an electron-withdrawing aromatic group is required at the E position, and the pentafluorophenyl group appears to be the most potent moiety at this position as can be seen from the results presented in Table 3. Compound **40** with one pentafluorophenyl ring revealed mid-nanomolar Ki_{app} (0.485 μM) and has potential in the development of non-peptidic HIV-1 PR inhibitors. Slight modifications to the aromatic side chain of **40** (AA position) such as introducing a hydroxyl substituent leading to compound **43** does not significantly affect inhibition. The Ki_{app}s are roughly the same for **40** and **43** (0.485 and 0.425 μM, respectively), however when a larger methoxy group is introduced at this same position, inhibition of the resulting compound **42** is less efficient (Ki_{app} =13 μM). Tyrosine (**43**) interacted strongly with the enzyme with an IC_{50} of 3.5 μM, while phenylalanine (**40**) gave an IC_{50} of 19 μM.

Inhibitors aimed at disrupting the activity of the HIV-1 PR would have to be highly specific to avoid side effects. The compounds described in this paper, particularly **40** and **43**, set up the basis for the development of a new series of inhibitors. These two enol compounds have been tested to determine their affinity towards other aspartic acid proteases: renin, pepsin and cathepsin D. Ki_{app}s of 23 and

Table 3.



Compound No	R	AA[a]	E	X	Ki_{app} (μM)
37	Boc	Benzyl	Phenyl	CN	106
38	Boc	Benzyl	3,5-Difluorophenyl	CN	40
39	Boc	Benzyl	4-(Trifluoromethyl)phenyl	CN	62
40	Boc	Benzyl	Pentafluorophenyl	CN	0.485
41	N-Ac Leu	Benzyl	Pentafluorophenyl	CN	7.0
42	Boc	4-Methoxybenzyl	Pentafluorophenyl	CN	13
43	Boc	4-Hydroxybenzyl	Pentafluorophenyl	CN	0.425

[a]All starting amino acids are of L configuration.

149 μM have been determined for **40** and **43**, respectively, with pepsin, indicating decreased specificity factors of 47- and 349-fold, respectively. Also, a Ki_{app} of 20 μM has been determined using cathepsin D for **40**, indicating a decreased specificity factor of 41-fold relative to HIV-1 PR. When these two inhibitors were tested with renin, less than 10 % inhibition was observed at 200 μM. These results may be explained by the fact that such inhibitors are not very similar to renin substrates, while phenylalanine and tyrosine are found at the P1 position of many pepsin and cathepsin D substrates.

Conclusion

A new series of compounds that potently inhibit the HIV-1 PR has been described. Starting from compounds that generated little inhibition, and by analysing the data carefully, it was possible to improve the efficiency of these inhibitors. By systematically modifying key positions and applying structure–activity-relationship studies, Ki_{app}s in the mid-nanomolar range have been obtained. The following general conclusions may be drawn with regard to the proposed function of the enol moiety:

1) At the R position, the Boc group generated the best interaction with the enzyme.
2) At the enol AA position (P1), electron deficient aromatic rings decreased inhibition.
3) At the E position, electron-withdrawing aromatic rings which favor the enol form and contribute to the hydroxyl polarization increased inhibition.
4) At the X position, cyano is thought to favor the enol form and is small enough to fit into the active site.

In Table 3, where inhibitors with sub-micromolar Ki_{app}s are shown, these general rules apply and are confirmed. Phenylalanine and tyrosine residues at the AA position demonstrated potent inhibition, with a Ki_{app}s of 485 and 425 nM, respectively. Improvement of the inhibition potencies is now underway since this class of inhibitors are not limited by long chemical synthesis.

Thus, we have demonstrated a novel class of HIV-1 protease inhibitors by using structure–activity relationship. These non-peptidic inhibitors show good promise as a new class of potent AIDS drugs.

Experimental Section

Chemistry

Proton NMR spectra were recorded on a Bruker WH-400 (400 MHz) spectrometer with DMSO-d_6 or $CDCL_3$ as solvents and Me_4Si as an internal standard. NMR abbreviations are as follows: s = singlet, d = doublet, dd = doublet of doublet, m = multiplet, t = triplet. IR spectra were obtained on a BOMEM Michelson 100 FT IR with KBr pellets or NaCl pastels. Mass spectra were taken on a Kratos MS-50 TCTA or VG AUTOSPEC Q mass spectrometer. Fast-atom bombardment (FAB) mass spectra were obtained using thio-glycerol matrix. Optical rotatory measurements were made on a Perkin Elmer 783 polarimeter. All the melting points were measured on a Büchi 530 apparatus and are not corrected. TLC (silica gel 60 F_{254}, Merck, Darmstadt) was used to monitor reactions and to visualize product homogeneity. Flash column chromatography was conducted on silica gel, 40–63 μm.

It should be noted that the conditions of the chemical synthesis are not optimized which may explain the low yields obtained with some compounds reported in this article.

The detailed procedures (Schemes II and IV) and physical data of the compounds **9–21**, **24**, **26**, **35** and **41** are available and can be requested from the authors.[31]

General procedure for the preparation of N*-protected amino acid difunctionalized enols.* The following procedure for the preparation of (*E*,*Z*)-(4*S*)-4-benzyloxycarbonylamino-3-hydroxy-2-(4-nitrophenyl)-5-phenyl-2-pentenenitrile (**22**) is representative. To a solution of *N*-benzyloxycarbonyl-L-phenylalanine in dry tetrahydrofuran (440 mg, 1.47 mmol) at 0 °C was added 1,1'-carbonyldiimidazole (300 mg, 1.1 eq.), and the solution was kept at 0 °C for 1 h. In parallel, a solution of 4-nitrophenylacetonitrile in dry tetrahydrofuran (262 mg, 1.62 mmol) at 0 °C was added dropwise to a solution of sodium hydride in dry tetrahydrofuran (50 % oil dispersion, 78 mg, 1.62 mmol), and the solution was kept at 0 °C for 30 min. The activated acid solution was then cooled at -78 °C prior to the dropwise addition of the carbanion. The resulting solution was kept at -78 °C for 1 h, and at room temperature overnight. Citric acid 5 % was then added and the mixture evaporated *invacuo* to remove the tetrahydrofuran. The aqueous fraction was extracted with ethyl acetate and the organic layer washed with sodium bicarbonate 5 % and brine. The solution was dried with magnesium sulfate, filtered and evaporated. The compound was purified by flash chromatography (15 % methanol, 85 % ethyl acetate), to give 87 mg of pure material (13 %): $[\alpha]_D$ = -196 ° (*c* = 0.64, MeOH); mp 123 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.90 (dd, J_{vic} = 6.4 Hz, J_{gem} = 13.2 Hz, 1H), 3.04 (m, 1H), 4.79 (m, 1H), 4.93 (d, J_{gem} = 13.9 Hz, 1H), 4.99 (d, J_{gem} = 13.9 Hz, 1H) 6.45 (s, 1H), 7.06–8.00 (m, 14H); IR (KBr) 729, 818, 857, 1049, 1110, 1187, 1455, 1720, 2170, 2343, 2366, 2961; HR MS (FAB) m/z 466.1350 $(M + Na)^+$, calcd for $C_{25}H_{21}N_3O_5Na$ 466.1379.

The following compounds were prepared from the indicated starting materials using the general procedure described above.

(*E*,*Z*) - (4*S*) -4- (9 - Fluorenylmethyloxycarbonylamino) -3-hydroxy-2-(4-nitrophenyl)-5-phenyl-2-pentenenitrile (**23**) was prepared from *N*-(9-fluorenylmethyloxycarbonyl)-L-phenylalanine and 4-nitrophenylacetonitrile, and isolated as described above (16 %): $[\alpha]_D$ = -198 ° (*c* = 0.71, MeOH); mp 167 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.87 (dd, J_{vic} = 9.5 Hz, J_{gem} = 13.6 Hz, 1H), 3.10 (dd, J_{vic} = 4.0 Hz, J_{gem} = 13.6 Hz, 1H), 4.00–4.23 (m, 4H), 7.16–7.88 (m, 18 H); IR (KBr) 621, 700, 782, 820, 935, 1653,

1793, 1846, 2169, 2344; HRMS (FAB) m/e 554.1753 (M + Na)$^+$, calcd for $C_{32}H_{25}N_3O_5Na$ 554.1692.

(*E,Z*)-(4*S*)-4-Acetylamino-3-hydroxy-2-(4-nitrophenyl)-5-phenyl-2-pentenenitrile (**24**) was prepared from *N*-acetyl-L-phenylalanine and 4-nitrophenylacetonitrile, and isolated as described above (29 %): $[\alpha]_D$ = +59 ° (*c* = 1.07, MeOH); mp 202 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.70 (s, 3H), 2.68 (dd, J_{vic} = 9.8 Hz, J_{gem} = 13.3 Hz, 1H), 3.03 (dd, J_{vic} = 3.9 Hz, J_{gem} = 13.3 Hz, 1H), 5.06 (ddd, J_{vic} = 9.8, 3.9 and 8.8 Hz, 1H), 7.11–7.26 (m, 5 H), 7.81 (d, *J* = 8.8 Hz, 1H), 7.96 (m, 4 H); IR (KBr) 699, 756, 852, 1042, 1418, 1718, 1794, 2173, 2344, 3401; HRMS (EI) m/z 351.1219 (M)$^+$, calcd for $C_{19}H_{17}N_3O_4$ 351.1215.

(*E,Z*)-(4*S*)-4-[*N*-(*N*-*tert*-Butoxycarbonyl-L-valyl)-amino]-3-hydroxy-2-(4-nitrophenyl)-5-phenyl-2-pentenenitrile (**25**) was prepared from *N*-*tert*-butoxycarbonyl-L-valyl-L-phenylalanine and 4-nitrophenylacetonitrile and isolated as described above (< 10 %) : $[\alpha]_D$ = -16 ° (*c* = 0.20, MeOH); mp 182 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.67 (2d, *J* = 6.8 Hz, 6H), 1.38 (s, 9H), 1.82 (m, 1H), 2.71 (dd, J_{vic} = 9.2 Hz, J_{gem} = 13.5 Hz, 1H), 3.03 (dd, J_{vic} = 3.8 Hz, J_{gem} = 13.5 Hz, 1H), 3.68 (dd, J_{vic} = 7.8 Hz and 9.6 Hz, 1H), 5.11 (ddd, J_{vic} = 3.8, 9.2 and 8.7 Hz, 1H), 6.76 (d, *J* = 9.6 Hz, 1H), 7.10–7.20 (m, 5H), 7.60 (d, *J* = 8.7 Hz, 1H), 7.70–7.98 (m, 4H); IR (KBr) 699, 757, 1044, 1648, 1655, 1793, 2171, 2345, 2971, 3412, 3567; HRMS (FAB) m/z 531.2248 (M + Na)$^+$, calcd for $C_{27}H_{32}N_4O_6Na$ 531.2219.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(4-nitrophenyl)-5-(2-naphthyl)-2-pentenenitrile (**27**) was prepared from *N*-*tert*-butoxycarbonyl-L-naphthylalanine and 4-nitrophenylacetonitrile, and isolated as described above (49 %): $[\alpha]_D$ = +3 ° (*c* = 1.12, MeOH); mp 191 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.25 (s, 9H), 2.87 (dd, J_{vic} = 9.9 Hz, J_{gem} = 13.6 Hz, 1H), 3.19 (dd, J_{vic} = 2.9 Hz, J_{gem} = 13.6 Hz, 1H), 4.86 (ddd, J_{vic} = 2.9, 9.9 and 8.6 Hz, 1H), 6.37 (d, *J* = 8.6 Hz, 1H), 7.20–7.97 (m, 11H); IR (KBr) 620, 730, 818, 1793, 1846, 1926, 2174, 2344, 2373, 3400; HRMS (FAB) m/z 482.1658 (M + Na)$^+$, calcd for $C_{26}H_{25}N_3O_5Na$ 482.1692.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2,5-bis-(4-nitrophenyl)-2-pentenenitrile (**28**) was prepared from *N*-*tert*-butoxycarbonyl-L-4-nitrophenylalanine and 4-nitrophenylacetonitrile, and isolated as described above (51 %): $[\alpha]_D$ = +113 ° (*c* = 0.39, MeOH); mp 210 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.27 (s, 9H), 2.84 (dd, J_{vic} = 9.7 Hz, J_{gem} = 13.5 Hz, 1H), 3.14 (dd, J_{vic} = 4.4 Hz, J_{gem} = 13.5 Hz, 1H), 4.75 (ddd, J_{vic} = 4.4, 9.7 and 8.5 Hz, 1H), 6.49 (d, *J* = 8.5 Hz, 1H), 7.52 (d, *J* = 8.1 Hz, 2H), 7.90–7.97 (m, 4H), 8.14 (d, *J* = 8.1 Hz, 2H); IR (KBr) 685, 757, 786, 820, 1017, 1048, 1637, 1654, 1792, 1830, 1846, 1870, 1939, 2170, 2344, 2374, 3412; HRMS (FAB) m/z 477.1386 (M + Na)$^+$, calcd for $C_{22}H_{22}N_4O_7Na$ 477.1386.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(4-nitrophenyl)-5-cyclohexyl-2-pentenenitrile (**29**) was prepared from *N*-*tert*-butoxycarbonyl-L-cyclohexylalanine and 4-nitrophenylacetonitrile, and isolated as described above (57 %): $[\alpha]_D$ = +3 ° (*c* = 0.88, MeOH); mp 182 °C red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.81 (m, 12H), 1.36 (s, 9H), 1.92 (m, 1H), 4.67 (m, 1H, ..11 (d, *J* = 8.4 Hz, 1H), 7.91–7.99 (m, 4H); IR (KBr) ..70, 714, 756, 1017, 1046, 1654, 1870, 1935, 2174, 2344, 2372; HRMS (FAB) m/z 438.2031 (M + Na)$^+$, calcd for $C_{22}H_{29}N_3O_5Na$ 438.2005.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(4-nitrophenyl)-4-phenyl-2-butenenitrile (**30**) was prepared from *N*-*tert*-butoxycarbonyl-L-phenylglycine and 4-nitrophenylacetonitrile, and isolated as described above (18 %): $[\alpha]_D$ = -294 ° (*c* = 0.66, MeOH); mp 183 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.36 (s, 9H), 5.66 (d, *J* = 7.9 Hz, 1H), 6.64 (d, *J* = 7.9 Hz, 1H), 7.19–7.43 (m, 5H), 7.94 (s, 4H); IR (KBr) 699, 727, 755, 935, 1049, 1653, 1717, 2172, 2344, 2371, 2881, 2969, 3403; HRMS (FAB) m/z 418.1350 (M + Na)$^+$, calcd for $C_{21}H_{21}N_3O_5Na$ 418.1379.

(*E,Z*)-(2'*S*)-3-[2'-(1'-*tert*-Butoxycarbonyl)-pyrrolidinyl]-3-hydroxy-2-(4-nitrophenyl)-2-propenenitrile (**31**) was prepared from *N*-*tert*-butoxycarbonyl-L-proline and 4-nitrophenylacetonitrile, and isolated as described above (36 %): $[\alpha]_D$ = -80 ° (*c* = 1.07, MeOH); mp 187 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.23 and 1.38 (2s, 9H; 2:1), 1.65–1.83 (m, 4H), 2.08–2.18 (m, 2H), 4.70 (m, 1H), 7.91–7.96 (m, 4H); IR (KBr) 696, 714, 743, 852, 1367, 1559, 1654, 1726, 2183, 2343, 2877, 3445; HRMS (FAB) m/z 382.1360 (M + Na)$^+$, calcd for $C_{12}H_{21}N_3O_5Na$ 382.1379.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-5-[4-(2,6-dichlorobenzyloxy)-phenyl]-2-(4-nitrophenyl)-2-pentenenitrile (**32**) was prepared from *N*-*tert*-butoxycarbonyl-L-(*O*-2,6-dichlorobenzyl)-tyrosine and 4-nitrophenylacetonitrile, and isolated as described above (31 %): $[\alpha]_D$ = +23 ° (*c* = 0.48, MeOH); mp 169 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.30 (s, 9H), 2.67 (dd, J_{vic} = 10.9 Hz, J_{gem} = 13.2 Hz, 1H), 2.96 (dd, J_{vic} = 2.6 Hz, J_{gem} = 13.2 Hz, 1H), 4.69 (ddd, J_{vic} = 2.6, 10.9 and 8.6 Hz, 1H), 5.18 (s, 2H), 6.33 (d, *J* = 8.6 Hz, 1H), 6.93 (d, *J* = 8.4 Hz, 2H), 7.19 (d, *J* = 8.4 Hz, 2H), 7.46 (t, *J* = 8.4 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 2H), 7.96 (s, 4H); IR (KBr) 685, 744, 855, 1017, 1048, 1367, 1654, 1721, 1792, 1831, 1846, 2170, 2344, 2373, 3406; HRMS (FAB) m/z 584.1296 (M + Na)$^+$, calcd for $C_{29}H_{28}N_3O_6Cl_2Na$ 584.1355.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(4-nitrophenyl)-5-(3-indolyl)-2-pentenenitrile (**33**) was prepared from *N*-*tert*-butoxycarbonyl-L-tryptophan and 4-nitrophenylacetonitrile, and isolated as described above (21 %): $[\alpha]_D$ = +126 ° (*c* = 0.26, MeOH); mp 151 °C, red powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.33 (s, 9H), 2.99 (dd, J_{vic} = 7.6 Hz, J_{gem} = 13.4 Hz, 1H), 3.14 (dd, J_{vic} = 4.2 Hz, J_{gem} = 13.4 Hz, 1H), 3.96 (m, 1H), 6.45 (m, 1H), 6.90–7.96 (m, 9H), 10.72 (m, 1H); IR (KBr) 747, 852, 1110, 1292, 1499, 1594, 1686, 2170, 2976, 3396;

HRMS (FAB) m/z 471.1591 $(M + Na)^+$, calcd for $C_{24}H_{24}N_4O_5Na$ 471.1644.

(*E,Z*)-(4*R*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(4-nitrophenyl)-5-phenyl-2-pentenenitrile (**34**) was prepared from *N*-*tert*-butoxycarbonyl-D-phenylalanine and 4-nitrophenylacetonitrile, and isolated as described above (32 %): $[\alpha]_D$ = -75 ° (*c* = 0.24, MeOH); mp 169 °C, red powder; ^1H NMR (400 MHz, DMSO-d_6) δ 1.29 (s, 9H), 2.70 (m, 1H), 2.99 (m, 1H), 4.73 (m, 1H), 6.37 (d, *J* = 7.4 Hz, 1H), 7.15–7.26 (m, 5H), 7.97–8.00 (m, 4H); IR (KBr) 699, 744, 856, 1049, 1417, 1654, 1720, 2175, 2344, 2881, 2972, 3404; HRMS (FAB) m/z 432.1556 $(M + Na)^+$, calcd for $C_{22}H_{23}N_3O_5Na$ 432.1535.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(4-nitrophenyl)-5-(2-propyl)-2-pentenenitrile (**36**) was prepared from *N*-*tert*-butoxycarbonyl-L-valine and 4-nitrophenylacetonitrile, and isolated as described above (< 10 %): $[\alpha]_D$ = -65 ° (*c* = 0.32, MeOH); mp 193 °C, red powder; ^1H NMR (400 MHz, DMSO-d_6) δ 0.74 and 0.88 (2 d, *J* = 6.7 Hz, 6H), 1.37 (s, 9H), 2.02 (m, 1H), 4.49 (m, 1H), 5.86 (d, *J* = 8.3 Hz, 1H), 7.92–7.98 (m, 4H); IR (KBr), 685, 758, 857, 1017, 1044, 1417, 1654, 1720, 1793, 2173, 2878, 2930, 3404; HRMS (FAB) m/z 362.1751 $(M + H)^+$, calcd for $C_{18}H_{24}N_3O_5$ 362.1716.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2,5-diphenyl-2-pentenenitrile (**37**) was prepared from *N*-*tert*-butoxycarbonyl-L-phenylalanine and phenylacetonitrile as the methylene, and isolated as described above except that sodium hydride has been replaced by sodium bis(trimethylsilyl) amide (< 10 %): $[\alpha]_D$ = +52 ° (*c* = 0.83, MeOH); mp 59 °C, pale yellow powder; ^1H NMR (400 MHz, DMSO-d_6) δ 1.30 (s, 9H), 2.86 (dd, J_{vic} = 7.8 Hz, J_{gem} = 13.4 Hz, 1H), 3.00 (dd, J_{vic} = 5.3 Hz, J_{gem} = 13.4 Hz, 1H), 4.80 (ddd, J_{vic} = 5.3, 7.8 and 7.6 Hz, 1H), 6.36 (d, *J* = 7.6 Hz, 1H), 7.18–7.72 (m, 10H); IR (KBr) 699, 849, 966, 1159, 1265, 1518, 1608, 1695, 2178, 2979, 3369; HRMS (FAB) m/z 387.1685 $(M + Na)^+$, calcd for $C_{22}H_{24}N_2O_3Na$ 387.1685.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(3,5-difluorophenyl)-5-phenyl-2-pentenenitrile (**38**) was prepared from *N*-*tert*-butoxycarbonyl-L-phenylalanine and 2,4-difluorophenylacetonitrile as the methylene. The product was isolated using the same procedure as described for compound **37** (< 10 %): $[\alpha]_D$ = +48 ° (*c* = 0.65, MeOH); mp 69 °C, white powder; ^1H NMR (400 MHz, DMSO-d_6) δ 1.31 (s, 9H), 2.79 (dd, J_{vic} = 10.6 Hz, J_{gem} = 13.3 Hz, 1H), 3.00 (dd, J_{vic} = 4.4 Hz, J_{gem} = 13.3 Hz, 1H), 4.74 (ddd, J_{vic} = 4.4, 10.6 and 7.4 Hz, 1H), 6.20 (d, *J* = 7.4 Hz, 1H), 6.86–7.99 (m, 8H); IR (KBr) 699, 1159, 1265, 1367, 1518, 1695, 2178, 2979, 3369; HRMS (FAB) m/z 423.1496 $(M + Na)^+$, calcd for $C_{22}H_{22}N_2O_3F_2Na$ 423.1496.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(4-trifluoromethylphenyl)-5-phenyl-2-pentenenitrile (**39**) was prepared from *N*-*tert*-butoxycarbonyl-L-phenylalanine and 4-trifluoromethylphenylacetonitrile as the methylene, and was isolated as described above (< 10 %): $[\alpha]_D$ = +5 ° (*c* = 0.65, MeOH); mp 140 °C, white powder; ^1H NMR (400 MHz, DMSO-d_6) δ 1.29 (s, 9H), 2.75 (dd, J_{vic} = 10.4 Hz, J_{gem} = 13.3 Hz, 1H), 3.01 (dd, J_{vic} = 3.9 Hz, J_{gem} = 13.3 Hz, 1H), 4.07 (ddd, *J* = 3.9 Hz, 10.4 Hz and 8.3 Hz, 1H), 6.27 (d, *J* = 8.3 Hz, 1H), 7.19–7.29 (m, 5H), 7.42 (d, *J* = 7.8 Hz, 2H), 8.02 (d, *J* = 7.8 Hz, 2 H); IR (KBr) 700, 844, 1017, 1067, 1457, 1614, 1735, 2177, 2982, 3396; HRMS (FAB) m/z 433.1696 $(M + H)^+$, calcd for $C_{23}H_{24}N_2O_3F_3$ 433.1739.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(2,3,4-5,6-pentafluorophenyl)-5-phenyl-2-pentenenitrile (**40**) was prepared from *N*-*tert*-butoxycarbonyl-L-phenylalanine and 2,3,4,5,6-pentafluorophenylacetonitrile as the methylene, and was isolated as described above (84 %): $[\alpha]_D$ = +51 ° (*c* = 0.92, MeOH); mp 90 °C, gray powder; ^1H NMR (400 MHz, DMSO-d_6) δ 1.30 (s, 9H), 2.78 (m, 1H), 3.02 (dd, J_{vic} = 3.9 Hz, J_{gem} = 13.1 Hz, 1H), 4.68 (m, 1H), 6.01 (d, *J* = 8.6 Hz, 1H), 7.04–7.69 (m, 5H); IR (KBr) 700, 755, 1053, 1164, 1255, 1457, 1519, 1676, 2190, 2981, 3380; HRMS (FAB) m/z 455.1414 $(M + H)^+$, calcd for $C_{22}H_{20}N_2O_3F_5$ 455.1394.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-5-(4-methoxyphenyl)-2-(2,3,4,5,6-pentafluorophenyl)-2-pentenenitrile (**42**) was prepared from *tert*-butoxycarbonyl-L-(*O*-methyl)tyrosine and 2,3,4,5,6-pentafluorophenylacetonitrile as the methylene, and was isolated as described above (73 %): $[\alpha]_D$ = +44 ° (*c* = 0.10, MeOH); mp 177 °C, white powder; ^1H NMR (400 MHz, DMSO-d_6) δ 1.30 (s, 9H), 2.70 (dd, J_{vic} = 8.1Hz, J_{gem} = 13.6 Hz, 1H), 2.96 (dd, J_{vic} = 2.7 Hz, J_{gem} = 13.6 Hz, 1H), 3.70 (s, 3H), 4.61 (ddd, *J* = 2.7, 8.1 and 8.4 Hz, 1H), 6.02 (d, *J* = 8.4 Hz, 1H), 6.52–7.16 (m, 4H); IR (KBr) 998, 1167, 1249, 1514, 1648, 2188, 2958, 3421; HRMS (FAB) m/z 485.1480 $(M + H)^+$, calcd for $C_{23}H_{22}N_2O_4F_5$ 485.1500.

(*E,Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-5-(4-hydroxyphenyl)-2-(2,3,4,5,6-pentafluorophenyl)-2-pentenenitrile (**43**) was prepared from *tert*-butoxycarbonyl-L-tyrosine 2,3,4,5,6-pentafluorophenylacetonitrile and 1,1-carbonyldiimidazole (2 eq.) and isolated as described above (85 %): $[\alpha]_D$ = +49 ° (*c* = 0.36, MeOH); mp 217 °C, white powder; ^1H NMR (400 MHz, DMSO-d_6) (2 isomers 5:1) δ 1.33 and 1.28 (2s, 9H), 2.74 (dd, J_{vic} = 8.5 Hz, J_{gem} = 13.1 Hz, 1H), 2.85 (m, 1H), 4.68 (m, 1H), 6.33 and 6.12 (2m, 1H), 6.63 and 7.10 (2d, *J* = 7.8 Hz and 8.2 Hz, respectively, 2 H), 7.01 and 7.26 (2d, *J* = 7.8 Hz and 8.2 Hz, respectively, 2H), 9.11 (s, 1H); IR (KBr) 827, 894, 1055, 1127, 1250, 1374, 1615, 1655, 2184, 2932, 3403; HRMS (EI) m/z 426.1377 $(M - CO_2)^+$, calcd for $C_{21}H_{19}N_2O_2F_5$ 426.1367.

Biology

Inhibition of HIV-1 protease. The substrate, H_2N–Val–Ser–Gln–Asn–Tyr*–Pro–Ile–Val–Gln–OH, based on the cleavage site of the p17–p24 gag residues, was synthesized by solid-phase method using Fmoc-amino acid, DCC and HOBT chemistry on an 430A Applied Biosystem (ABI)

synthesizer. Purification was performed on an ABI HPLC apparatus using a water–acetonitrile gradient with 0.1 % TFA. Verification of the amino acid content of the peptide was realized using the Waters Picotag phenyl isothiocyanate derivative method.

Assay and kinetics: stock inhibitor solutions were prepared in 5 % DMSO. The inhibitor solution (2 μL) was added to the substrate solution at various concentrations for a final volume of 40 μL. The substrate was previously dissolved in sodium acetate buffer 100 mM, pH 5.5, EDTA 5 mM, BSA 0.1 %, NaCl 1 M. The HIV-1 protease was added (3–6 nM) (generously provided by Dr P. L. Darke from Merck Sharp & Dohme) and the reaction mixture incubated for 30 min at 37 °C. The reaction was quenched by the addition of 158 μL TFA 5 %, and the kinetic parameters were determined by reverse phase chromatography with an ABI aquapore OD-300, 7 μm, 100 x 4.6 mm, reverse phase column (flow rate: 2.0 mL/min, solvent A: 100 % H_2O, 0.1 % TFA, solvent B: 60 % CH_3CN, 40 % H_2O, 0.1 % TFA). Gradient: 0 min, 100 % A, 2 min, 100 % A, 10 min, 70 % A, 11 min, 100 % B, 16 min, 100 % B. The substrate and hydrolysates were monitored using a UV detector (λ = 210 nm), and the inhibition parameters determined using the Dixon plot for competitive inhibition.

Inhibition of other aspartic acid proteases. Pepsin and cathepsin D were purchased from Sigma. For pepsin and cathepsin D, inhibitory potential of compounds **40** and **43** were assessed using the HPLC method described above. The choice of the substrate, H_2N–Phe–Gly–Phe–His–Phe(NO_2)–Phe–Ala–Phe–OH, was based on a previously reported pepsin substrate,[37] except that the C-terminal carboxylic acid was used rather than the methyl ester. This substrate analog generated a K_m of 0.08–0.10 mM compared to 0.04 mM reported for the OMe substrate. Different assay conditions and/or the small modification on the substrate may account for the K_m difference. Since the sequence His–Phe(NO_2)–Phe–Ala is also known for cathepsin D affinity, the same substrate was used for both enzymatic assays. We determined a K_m of 0.14 mM for cathepsin D with this substrate. The following assay conditions were used for pepsin and cathepsin D: 0.04 M formate buffer, pH 4.0, 10 mM EDTA, 37 °C and 0.01 M formate buffer, pH 4.0, 37 °C, respectively. For both enzymes, the reactions were initiated by the addition of the enzyme to the substrate solution with and without inhibitors. After 30–60 min, the reactions were stopped by the addition of 1 % aqueous TFA and proteolytic cleavages were quantified after separation of the hydrolysates using the same HPLC conditions as for the HIV-1 protease assays. The inhibition parameters for both enzymes were determined using the Dixon plot for competitive inhibition and the Cha equation[57] using the IC_{50} values.

Renin assays are based on previously reported methodology.[40] Rat plasma (250 μL, with essentially no renin activity) was used as the source of angiotensinogen, the renin substrate. In brief, plasma was incubated in presence of 0.125 mU of porcine kidney renin with 250 μL of maleate buffer 0.2 M, pH 6.5, 0.25 % 8-hydroxyquinoline and with increasing concentrations of inhibitors (0–200 μM). The tubes were incubated at 4 °C (controls) or 37 °C for 2 h. One hundred μL was then removed in duplicate to measure by radioimmunoassay the amount of angiotensin I liberated by renin. To the samples were added angiotensin I [I^{125}] (6000 cpm/mL), angiotensin I buffer (potassium phosphate 0.1 M, pH 7.4, 0.25 % BSA, 0.02 % sodium azide and 0.3 mM EDTA) and angiotensin I antibody. Following overnight incubation at 4 °C bound angiotensin I was separated from free by centrifugation after addition of 1 mL of charcoal–dextran. Supernatants were collected for monitoring radioactivity and results were calculated by comparison with a standard curve of angiotensin I (78–9999 pg/mL) and expressed as ng angiotensin I liberated per mL per hour.

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Supplementary Material Available

Detailed procedures for Schemes II and IV and the physical data for compounds **1–12**, **17**, **26** and **32** are available and can be requested from the authors.

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