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Difunctional Enols of N-Protected Amino Acids as Low Molecular Weight and Novel Inhibitors of HIV-1 Protease.

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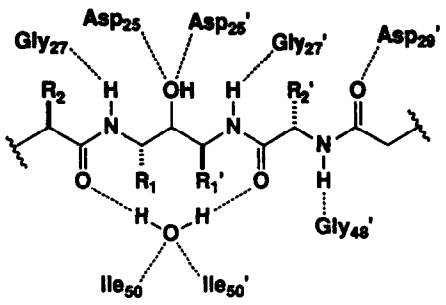
ABSTRACT: The synthesis of difunctional enols of simple amino acids and their novel inhibitory activity against human immunodeficiency virus type 1 protease (HIV-PR) are described. By modifying the substituents on these enols, we were able to achieve mid-nanomolar range in activity against HIV-1 protease which is, to our knowledge, the best reported activity for molecules that do not contain at least one peptide linkage.

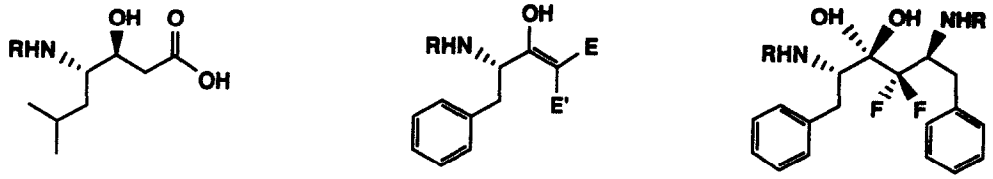
The catastrophic dimensions of the HIV-AIDS pandemic has spurred a worldwide quest for therapeutic agents that can interrupt the life cycle of HIV without harming the infected host.[1] There is much interest currently in combining antiviral therapy with drugs directed towards different targets in the viral life cycle.[2] One such possibility is the use of inhibitors of the virally encoded protease (PR) responsible for post-translational viral maturation. Although the HIV protease has been characterized extensively,[3] and its three-dimensional structure has been determined both alone and as a complex with a variety of inhibitors,[4] details as to the mode, time, and microenvironment of its action remain obscure. It is clear, however, that if the protease is catalytically defective or inhibited,[5] viral maturation of HIV is blocked and, consequently, the spread of infection is arrested.[6,7]

Figure 1



The most effective inhibitors of the HIV protease reported thus far are pseudopeptide/peptidomimetic compounds containing hydrophobic transition-state inserts in place of the natural substrate residues (Figure 1) in the R_1 and R_1' positions of the peptide.[8] Even though extensive research in the development of HIV-Pr inhibitors has focused in peptide and pseudopeptide modification, only haloperidol, cerulanin, and recently non-peptide carboxylates have been reported as non-peptide HIV-1 protease inhibitors.[9] Because peptidic inhibitors have shown short half-life *in vivo* and poor bioavailability, the search for non-peptidic inhibitors is crucial for development of potential drugs.

Figure 2



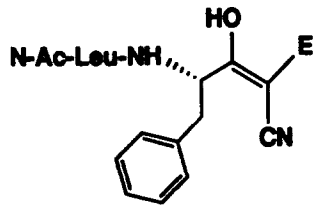
1 2 (E = E' = EWG) 3

Our approach is based on a novel isostere which relies on the chemical reactivity of the enol functionality. During the course of our studies on serine protease inhibitors,[10] we became intrigued by the topographical similarities between Boc-Statine **1** and prototypical enol **2** (Figure 2). In addition, analog **3** is known to efficiently inhibit HIV-1 protease.[8f] The presence of highly electronegative fluorine atoms adjacent to the ketone favor greatly the hydrated form which along with the proximal hydrophobic benzyl moieties enhance its ability as an inhibitor. We hypothesized that the highly polar enol group seemed interesting because the addition of two electron-withdrawing groups (E and E') provide a potential electrophilic center at the vinylic position for nucleophilic attack either by a water molecule or a nucleophile present at the active site of the protease. Also, certain substituents at the E and E' positions may favor enzyme-inhibitor interactions. For the above reasons, we undertook a research program aimed at establishing the effectiveness of these enols as inhibitors of HIV-1 protease.

In order to assess the potential inhibition of the HIV-1 protease by enols such as **2**, a screening of molecules with enol-like structures was performed to determine which functional groups would interact with the HIV-1 protease in a competitive way as observed by Dixon plot analysis.[11] The enols were prepared according to a general procedure : activation of a carboxylic acid (*N*-protected amino acid or dipeptide) by the use of 1,1'-carbonyldiimidazole at 0°C for 1 h, followed by addition of the carbanion at -78°C (generated by deprotonation of active methylene compound by sodium hydride at 0°C for 1 h) to give the

corresponding enols in high yields without any racemization.[10] All the difunctional enols exist in equilibrium between the E and Z isomers.[12]

Table 1

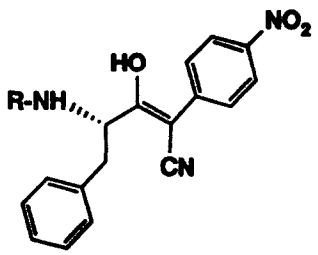


Compound No	E	Ki (μM)
4	CN	ni[b]
5	CO_2Me	209
6	Diethyl phosphonate	206
7	Phenylsulfonyl	152
8	Pyridyl	168
9	(4-NO_2)phenyl	9.5

a) All starting amino acids are of L configuration.
b) no inhibition observed at 400 μM.

A variety of enols **4-9** with different substituents at the E position were synthesized and tested (Table 1).[13] Amongst these was compound **9** (Ki_{app} = 9.5 μM) possessing the p-nitrophenyl moiety which showed significant activity as an inhibitor of HIV-1 PR. Next, we sought to modify this into a less peptidic and possibly more potent analog. First, the R_2 region was modified by changing the *N*-Ac-Leu moiety to a more simple protective group leading to compounds **10** (*N*-Cbz), **11** (*N*-Fmoc), **12** (*N*-Boc-Gly) and **13** (*N*-Boc). Compound **13** (*N*-Boc) proved to be the most active as seen in Table 2.

Table 2

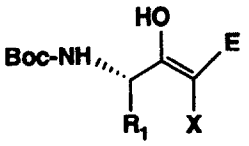


Compound No	R	Ki_{app} (μM)
9	N-Ac-Leu	9
10	Cbz	30
11	Fmoc	78
12	Boc-Gly	58[a]
13	Boc	2.3

a) IC_{50}

Our attention was then turned to the nature of the amino acid itself and we synthesized and evaluated compounds **14** to **23** (Table 3). There appears to be a strong preference for hydrophobicity in the amino acid portion (see compound: **14, 15, 19, 20** and **21**) and a significant difference in specificity between the L and D isomers (compound **13** and **21**). Changing the rather rigid enol system (compound **13**) to a more flexible one (compound **23**) had a profound effect on the activity. Removing the cyano group provided a compound that had a less polar hydroxyl group and a reduced contribution of the enol form,[14] and consequently little activity.

Table 3



Compound No	R_1[a]	E	X	Ki_{app} (μM)
14	ß-Naphtylalanine	p-nitrophenyl	CN	38
15	(4-NO_2)Phenylalanine	p-nitrophenyl	CN	60
16	Cyclohexylalanine	p-nitrophenyl	CN	15
17	Phenylglycine	p-nitrophenyl	CN	30
18	Proline	p-nitrophenyl	CN	181
19	Tyrosine(OBn)	p-nitrophenyl	CN	12
20	Tryptophan	p-nitrophenyl	CN	18
21	D-Phenylalanine	p-nitrophenyl	CN	54
22	Phenylalanine	benzoyl	CN	320
23	Phenylalanine	p-nitrophenyl	H	689

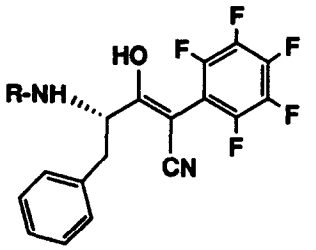
a) All starting amino acids are of L configuration unless otherwise indicated.

Having established the necessity for a specific amino acid (L-Phe) and one protective group (*N*-Boc), the requirements for the enol group were then investigated. After carefully inspecting the data in Table 1 we were struck by the electron deficient nature of the p-nitrophenyl group and began searching for a substituent that would increase this trend. Therefore, the p-nitrophenyl group was replaced by a pentafluorophenyl moiety to obtain compounds **24** (*R* = *N*-Ac-Leu, Ki_{app} = 7 μM) and **25** (R = *N*-Boc, Ki_{app} = 0.48 μM) (Figure 3). We were pleased to find that the pentafluorophenyl series was 4-5 times more active than the corresponding p-nitrophenyl series.

We have shown that difunctional enols derived from simple amino acids are a potent, novel class of HIV-1 protease inhibitors. The structure activity relationship study shows that: 1) the *N*-Boc group generated, to date, the best inhibition at the R_2 position, 2) a benzyl group at the R_1 position gave the best interaction, and 3) an electron-poor hydrophobic moiety is needed for the R_1' position. By modifying the electron-withdrawing nature of the substituents on

these enols we were able to achieve mid-nanomolar range in activity against HIV-1 protease which is, to our knowledge, the best reported activity for molecules that do not contain at least one peptide linkage.

Figure 3



24, R = *N*-Ac-Leu, K*i app* = 7 μM
25, R = *N*-Boc, K*iapp* = 0.48 μM

These enolic inhibitors may steer us in a different direction in the development of a new class of AIDS drugs. Studies aimed at increasing their activity and determining their mode of action are currently underway. Full experimental details[15] and the complete enzymatic studies using these analogs will be reported elsewhere.

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[11] HIV-1 substrate: the substrate, based on the cleavage site of the p17-p24 *gag* residues, Val-Ser-Gln-Asn-Tyr*-Pro-Ile-Vla-Gln-OH (Km = 1.80 mM), was synthesized by solid-phase method using Applied BioSystem (ABS) synthesizer, Model 430A. Purification of the newly synthesized nonapeptide was performed on an ABS type HPLC, model 1400A, on a water-acetonitrile with 0.1% TFA gradient. Verification of the amino acid content of the peptide was performed using the Picotag derivative method of Waters. Assay & kinetics: a stock inhibitor solution was prepared in 5% DMSO. 2 µl of the stock solutions of inhibitor were added at different concentrations 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 at pH 5.5, containing EDTA 5 mM, BSA 0.1%, 1 M NaCl. The HIV-1 protease is added (2 µl) and the reaction mixture incubated for 30 min at 37°C. The reaction is quenched by the addition of 158 µl TFA 5%, and kinetic parameters determined on a Waters HPLC using reverse phase chromatography with a 100 X 4.6 mm, C_{18} column (purchased from ABS) at 210 nm. The inhibition parameters were determined using the Dixon plot for competitive inhibition. All the enols reported in this paper showed competitive inhibition of the HIV-1 protease.

[12] The energy barriers for rotation around the carbon-carbon double bond are low for non-symmetrical ß,ß'-difunctionalized enol groups (see reference 10). We observed for compound **13** only one set of signals in NMR spectra for both *E* and *Z* isomers at room temperature that correspond to rapid equilibrium, and two sets of signals in acetone-d_6 when cooled to -55°C which correspond to the E and Z isomers.

[13] As N-protected Valine was found to be optimal at the R_2 position for inhibitors of HIV-1 Pr (see, Griffiths, J. T.; Phylip, L. H.; Konvalenka, J.; Strop, P.; Gustchina, A.; Wlodawer, A.; Davenport, R. J.; Briggs, R.; Dunn, B. M.; Kay, J. *Biochemistry* **1992**, 31, 5193-5200) we decided to test a similar hydrophobic residue at the R_2 position, namely a Leucine analog with a phenylalanine residue at the R_1 position in our initial screening.

[14] Having two electron-withdrawing groups adjacent to the ketone favor completely the enol form (see ref. 10), compound **23** with one electron-withdrawing group showed by NMR greater than 90% of the ketone form in $CDCl_3$.

[15] All compounds were characterized by NMR, IR and high resolution MS.