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ENDOTHIOPEPTIDE INHIBITORS OF HIV-1 PROTEASE

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Abstract: Endothiopeptide inhibitors of HIV-1 protease were synthesized by chemical and enzymatic methods to individually replace each backbone amide bond in **1** with a thioamide-linkage. Interestingly, agent **7**, which contains a thioamide-linkage between the P_2' and P_3' positions of **1**, was the most potent, competitive inhibitor of HIV-1 protease with a K_i of 3.4 μM.

Introduction

The use of peptidomimetics to replace proteolyzable amide linkages has been a successful strategy in the design of novel inhibitors of enzymes and receptors.[1] Thioamides, for example, have been used as amide bond surrogates in a number of systems, including cyclosporin,[2] thyrotropin-releasing hormone,[3] cholecystokinin dipeptoids,[4] leu-enkephalin,[5] and cyclic enkephalin analogues,[6] pentagastrin,[7] and inhibitors of carboxypeptidase A,[8] papain,[9] triosephosphate isomerase,[10] astin B,[11] peptidyl-prolyl isomerase,[12] and angiotensin-converting enzyme.[13] The thioamide replacement in these agents has led to a number of interesting findings, such as, increased proteolytic stability,[8,13] alternate conformations in cyclic peptides,[6,10] restriction in the allowable φ, Ψ angles in the vicinity of the thioamide linkage,[14] and higher barriers of rotation about the C-N bond.[15]

Numerous examples of amide bond surrogates and transition state analogues have been implemented for inhibitors of the aspartyl protease of HIV-1.[16] To date, a thioamide replacement strategy has not been applied to inhibition of any of the aspartyl proteases. In this paper, we describe the synthesis of inhibitors of HIV-1 protease utilizing a sequential thioamide-replacement approach within a peptide substrate of the protease, and disclose a unique pattern of inhibition obtained with these agents, along with an unusual lack of cleavage by HIV-1 protease.

Results and Discussion

HIV-1 protease has been shown to cleave peptides at Phe-Phe sequences,[17] and a hydroxyethylene isostere mimetic **2** of peptide **1**, a substrate for HIV-1 protease, has potent inhibitory properties against HIV-1 protease with a K_i of 0.4 nM.[17] We choose to study the inhibitory effect of a thioamide bond in place of the scissile bond in **1**(compound **5**). While developing synthetic procedures for the preparation of **5**, we also prepared analogs of **1**, which contained thioamide substitutions at each of the individual amide bonds of **1** (compounds **3-4** and **6-7**).

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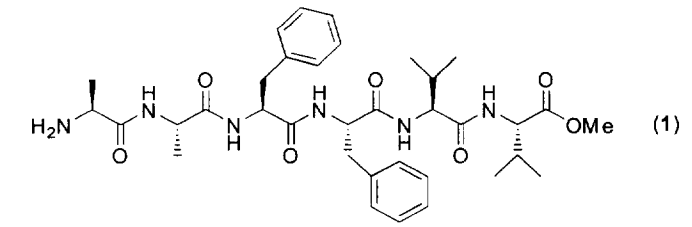
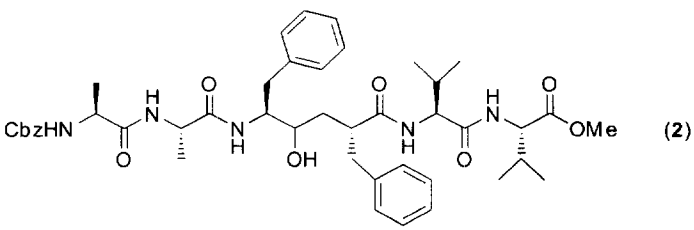


(1)



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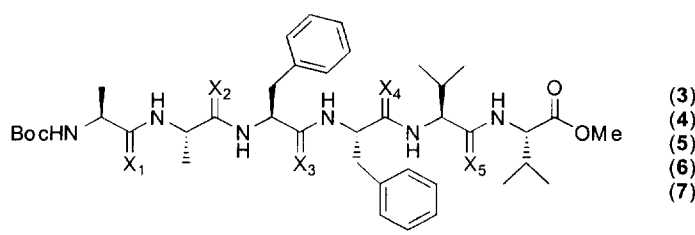
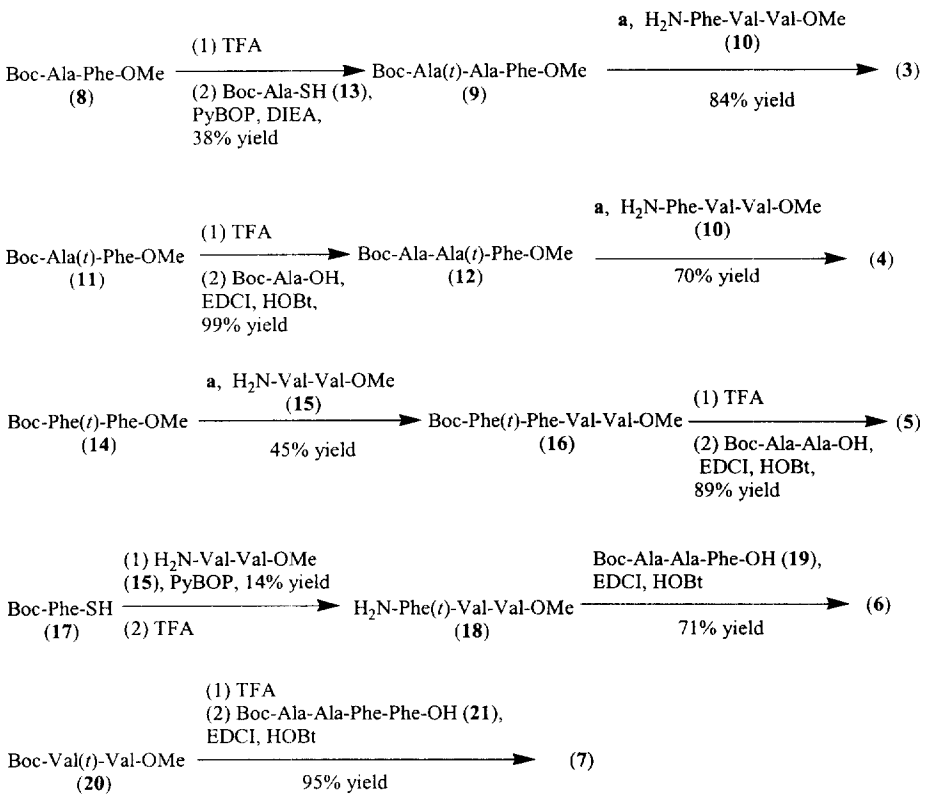


(3) X_1 = S, X_{2-5} = O
(4) X_2 = S, X_1, X_{3-5} = O
(5) X_3 = S, X_{1-2}, X_{4-5} = O
(6) X_4 = S, X_{1-3}, X_5 = O
(7) X_5 = S, X_{1-4} = O

Synthesis of Agents 3-7

Many methods have been developed to synthesize peptides containing thioamide linkages (endothiopeptides), including direct Thionation with Lawesson's reagent[18] and thioacylation with alkyl dithioesters,[19] thiobenimidazolones,[20] thioacylbenztriazoles,[21] and thioacids with reagents such as PyBOP or PyNOP.[22] The use of enzymatic coupling of endothiopeptide methyl esters with peptide amino-termini has also been reported.[23] Our general strategy for the synthesis of **3-7** involved first incorporating the thioamide bond into the dipeptide starting materials **8**, **13**, and **15** using Lawesson's reagent to give **11**, **14**, and **20** (Scheme 1).[18] The synthesis of the endothiotripeptide starting materials (**9**, **12**, and **18**) was accomplished either by addition of a Boc-protected amino acid to the amino-terminal end of an endothiodipeptide methyl ester **11** with EDCI, or by coupling Boc-protected amino thio-acids (**13** and **17**) to the amino-terminal end of dipeptide methyl esters **8** or **15** with PyBOP.[22] The synthesis of endothiotetrapeptide **16** and endothiohexapeptides **3** and **4** was carried out using enzymatic (chymotrypsin) coupling procedures when the specificity for coupling was appropriate, whereas endothiohexapeptides **5-7** were prepared by chemical coupling (EDCI) methods when enzymatic coupling was not feasible (Scheme 1).

Scheme 1. Syntheses of endothiopeptides (**3-7**).



a. Chymotrypsin, DMF/Phosphate buffer, pH 9, 15 min[24]

Using a variety of chemical and enzymatic procedures agents **3-7** were prepared in high purity after reverse phase HPLC purification, and then characterized by ^{1}H & ^{13}C NMR, HRMS and amino acid analysis.[25] The reaction of the dipeptides with Lawesson's reagent proceeded in moderate to high yield (54% and 90%), whereas thioacylation of peptide amines with the corresponding amino thioacid and PyBOP generally provided low yields of the thioamide products (14% and 38%) due to competing activation at sulfur instead of oxygen. Other reagents have been developed that avoid this difficulty,[19-21] but in our case enough compound was obtained to continue the synthesis, and the amide and thioamide products were easily separated and identified. Both chemical and enzymatic coupling of the peptide fragments proceeded in high yield (71-99%, and 45-84%, respectively), but the enzymatic coupling allowed the use of endothiodipeptide esters as coupling agents; active esters of endothiodipeptides generally being prone to cyclization and racemization.[26] Moreover, the enzyme catalyzed reactions were extremely fast with typical reaction times of approximately 5 min, as compared to the reported reaction times of 24 h.[23]

HIV-1 Protease Inhibition and Proteolysis with 3-7

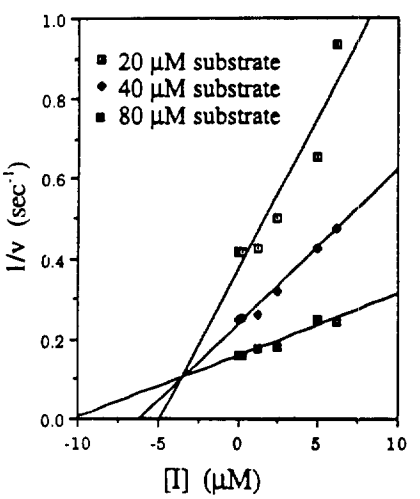
HIV-1 protease inhibition was evaluated using a fluorogenic substrate assay developed by Toth and Marshall.[27] The effect of 30 min pre-incubation of agents **3-7** with HIV-1 protease upon the hydrolysis of substrate[27] was evaluated by monitoring the increase in fluorescence at 430 nm with respect to time (Table 1). Agent **5**, which contains the thioamide replacement at the scissile bond, was reasonably good inhibitor of HIV-1 protease with an IC_{50} value of 18 μM. Surprisingly, agent **7**, which contains an amide linkage at the scissile bond and a thioamide linkage between Val-Val, was the best inhibitor of this series with an IC_{50} value of 4.5 μM, whereas substitution of a thioamide between the Ala-Ala (**3**), Ala-Phe (**4**) or Phe-Val (**6**) positions produced much weaker inhibitors with IC_{50} values of 160 μM, 51 μM, and >200 μM, respectively. K_i value was obtained for the best inhibitor, **7**, to confirm competitive inhibition. Agent **7** inhibited HIV-1 protease with a K_i of 3.4 μM, and was indeed a competitive inhibitor (Figure 1).

Table 1. Inhibition of HIV-1 Protease

Compound	IC_{50} (μM)
Boc-A(t)AFFVV-OMe (**3**)	160
Boc-AA(t)FFVV-OMe (**4**)	51
Boc-AAF(t)FVV-OMe (**5**)	18
Boc-AAFF(t)VV-OMe (**6**)	>200
Boc-AAFFV(t)V-OMe (**7**)	4.5

Enzyme Assay: 50 μL of 100 nM protease solution in a buffer containing 20 mM phosphate, 20% glycerol, 1 mM DTT, 1 mM EDTA and 0.1% CHAPS at pH 5.5 was incubated with 10 mL of the inhibitor solution in DMSO (14% final) for 30 min. This was followed by the addition of the incubated solution to 40 μL of 163 μM substrate at room temperature. The change in fluorescence 430 nm (λ_{ex} = 355 nm) was monitored over a period of 300 seconds. A control experiment with no inhibitor was also assayed in a similar manner.

Figure 1. K_i Determination for agent **7**



Compounds 1 and **3-7** were assayed for proteolytic cleavage by HIV-1 protease. Compound **1** was a substrate for the protease and was completely cleaved at a concentration of 270 μM in 30 minutes as monitored by reverse phase HPLC. Interestingly, compounds **3-7** were not substrates for the protease; no detectable proteolysis was observed after 12 h using identical conditions as were used with **1**, even though the scissile bond in these agents is in the proteolyzable amide form. This interesting result seems to indicate a different binding mode for agents containing thioamide replacements in the protease binding pocket, which may remove the scissile bond from its necessary proximity to Asp 25 and 25'. The differing hydrogen bond donor and acceptor properties of the thioamide moiety in comparison to the amide linkage, in addition the larger van der Waals radius of the sulfur and

the longer C=S bond may account for the different binding mode of the peptides in the HIV-1 protease binding pocket in the region of the Val-Val linkage. How this information is relayed to alter the conformation or position of the Phe-Phe scissile bond at the active site still remains to be discerned.

Conclusion

Endothiopeptide inhibitors of HIV-1 protease have been successfully synthesized using a wide variety of chemical and enzymatic techniques, with this work being the first application of Kessler's enzymatic coupling strategy[23] to the synthesis of endothiopeptide inhibitors. The agent **5** with the thioamide-linkage at the scissile bond of **1** was a modest inhibitor of HIV-1 protease. Interestingly, peptidomimetic **7**, which contains a thioamide-linkage away from the scissile bond between the P_2' and P_3' positions of **1**, was a competitive inhibitor of HIV-1 protease, and was four fold more effective as compared to agent **5**. More interestingly, however, agent **7** was not a substrate for HIV-1 protease although it still contains an amide-linkage at the scissile bond. This is the first report of a thioamide replacement outside of the scissile bond position which produces an enzyme inhibitor, and also the first time that such a replacement has inhibited proteolysis at the scissile bond, facts which will be useful in future inhibitor designs. These experiments serve to underscore the fact that there is still much to learn about the hydrogen bonding and conformational properties of thioamide-containing molecules if they are to be utilized in inhibitor design strategies.

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24. **General Procedure for Enzymatic Synthesis of Thioamide-containing Peptides.** The N-terminal peptide methyl ester (1 equiv), and the C-terminal peptide (2-4 equiv), were added to a solution containing DMF (50% v/v) and phosphate buffer (0.1 M, pH 9; final pH of the mixture was adjusted to 9 with 1 N NaOH if necessary). Chymotrypsin was added and the reaction was stirred at rt for 15 min.. The reaction was worked up by extraction with CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$, 10% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to yield the desired product. Further purification was performed if necessary.
25. ^{1}H NMR (300 MHz, $CDCl_3$) & HRMS calculated for $C_{40}H_{58}N_6O_8S$ 783.4115: (**3**) δ 9.25 (br, 1H), 7.70 (br, 1H), 7.30-6.90 (m, 14H), 5.40-5.05 (br, 3H), 4.88-4.50 (br, 3H), 3.78 (s, 3H), 2.92 (m, 4H), 2.20 (m, 1H), 1.99 (m, 1H), 1.43 (s, 9H), 1.40 (m, 6H), 0.96 (m, 12H), HRMS obtained 783.4066; (**4**) δ 8.62 (br, 1H), 7.25-7.06 (m, 11H), 7.01 (br, 1H), 6.85 (br, 1H), 6.79 (br, 1H), 6.67 (br, 1H), 5.19 (br, 1H), 4.93 (br, 1H), 4.79 (br, 1H), 4.66 (br, 1H), 4.51 (m, 1H), 4.28 (m, 1H), 3.73(s, 3H), 3.37 (m, 1H), 3.21 (m, 1H), 3.01 (m, 2H), 2.34 (m, 1H), 2.20 (m, 1H), 1.38 (s, 9H), 1.35-1.25 (m, 6H), 0.97-0.91 (m, 12H), HRMS obtained 783.4122; (**5**) δ 8.20-7.50 (br, 1H), 7.25-6.90 (m, 14H), 6.63 (br, 1H), 5.33 (m, 1H), 5.00 (m, 1H), 4.60-4.10 (m, 4H), 3.75 (s, 3H), 3.30-2.90 (m, 4H), 2.25-2.00 (m, 2H), 1.40 (s, 9H), 1.35-1.20 (m, 6H), 1.00-0.80 (m, 12H), HRMS obtained 783.4094; (**6**) δ 9.62 (br, 1H), 7.90 (br, 1H), 7.52 (br, 1H), 7.25 (m, 11H), 6.70 (m, 2H), 5.75 (br, 1H), 5.29 (m, 1H), 4.80 (m, 2H), 4.58 (m, 1H), 4.37 (m, 1H), 3.74 (s, 3H), 3.20-2.85 (m, 4H), 2.35 (m, 1H), 2.20 (m, 1H), 1.46 (s, 9H), 1.30 (m, 6H), 0.95 (m, 12H), HRMS obtained 783.4106; (**7**) δ 8.50 (br, 1H), 7.25-6.65 (m, 15 H), 5.25-4.50 (m-br, 5H), 4.30 (m, 1H), 3.78 (s, 3H), 3.05 (m-br, 2H), 2.81 (m-br, 2H), 2.37 (m-br, 2H), 1.45 (s, 9H), 1.40-1.15 (m, 6H), 1.00 (m, 12H), HRMS obtained 783.4104.
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