CH_2Cl_2 and MeOH. The combined filtrate and washings were concentrated, and the residue was dissolved in 5% Na_2HPO_3 buffer (pH = 5). After 5 min, the aqueous solution was adjusted to pH = 9 with NH_4OH and extracted with ethyl acetate (3 × 50 mL). The extract was washed with brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was chromatographed over silica gel (5% MeOH/0.5% NH_4OH in CH_2Cl_2 followed by 10% MeOH/1% NH_4OH in CH_2Cl_2) to yield 25 mg (61%) of solid **12**: $[\alpha]_D$ -44.90° (*c* 1.6, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.92 (t, *J* = 7, 3 H), 1.12 (t, *J* = 7, 6 H), 1.20-1.26 (m, 6 H), 1.33 (d, *J* = 7, 3 H), 1.35 (s, 3 H), 1.39 (m, 1 H), 1.54-1.73 (m, 3 H), 1.93 (m, 2 H), 2.25 (d, *J* = 12, 1 H), 2.28 (s, 3 H), 2.38 (d, *J* = 15, 1 H), 2.44 (bs, 3 H), 2.72 (m, 1 H), 3.03 (t, *J* = 9, 1 H), 3.25 (dd, *J* = 9, *J* = 7, 1 H), 3.31 (s, 3 H), 3.48-3.56 (m, 2 H), 3.72-3.84 (AB, *J* = 12, 2 H), 3.82 (s, 1 H), 4.05 (dd, *J* = 9, *J* = 6, 1 H), 4.35 (m, 1 H), 4.44 (d, *J* = 7, 1 H), 4.88 (d, *J* = 4.5, 1 H), 4.97 (dd, *J* = 10, *J* = 3, 1 H); ^{13}C NMR ($CDCl_3/CD_3OD$) δ 9.43, 11.00, 14.48, 17.82, 20.98, 21.15, 22.09, 29.43, 33.10, 34.72, 40.10, 43.74, 43.79, 44.79, 49.43, 61.83, 64.45, 65.60, 68.85, 70.90, 72.49, 73.94, 75.43, 76.24, 77.21, 77.50, 77.63, 78.44, 79.39, 96.21, 102.93, 176.89; IR (KBr) 3600-3460, 2965, 2935, 1725, 1600, 1450, 1380, 1160, 1105, 1050, 1005 cm^{-1}; HRMS calcd for $C_{39}H_{75}N_2O_{13}$ (MH^+) 779.5269, found 779.5252.

(9*R*)-9-Deoxo-9-(*N*,*N*-dimethylamino)-21-hydroxyerythromycin B (13). Borane-THF (1 M BH_3 solution in THF) (2.9 mL, 2.86 mmol) was added to a stirring solution of **8** (255 mg, 0.286 mmol) in 6 mL THF at 0 °C. The solution was warmed to room temperature and allowed to stir for 18 h. The reaction was quenched by portionwise addition of 15 mL of water, and 10% KOH solution was added dropwise until the pH was 8. Hydrogen peroxide (30% w/v) (0.29 mL, 2.86 mmol) was added via syringe, and the solution was stirred for 2 h. The solution was further diluted with 20 mL of water and adjusted to pH = 9.5 by addition of NH_4OH. The solution was extracted with EtOAc (3 × 20 mL), and the EtOAc extract was washed with brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was redissolved in MeOH and heated to reflux for 20 h, and methanol was removed in vacuo. The residue was adhered onto silica gel and chromatographed over silica gel (5% MeOH/0.5% NH_4OH in CH_2Cl_2) to yield 160 mg of a mixture of two products, which was carried further. Palladium on carbon catalyst (10%) (50 mg) was added to a stirring solution of the mixture of products from above (160 mg) in MeOH (5 mL) under nitrogen. The flask was evacuated, and the mixture was hydrogenated at 1 atm for 30 min, filtered, and the residue was washed well with CH_2Cl_2 and MeOH. The combined filtrate and washings were concentrated, and the residue was chromatographed over silica gel (10% MeOH/1% NH_4OH in CH_2Cl_2 followed by 20% MeOH/2% NH_4OH in CH_2Cl_2) to give 80 mg of a mixture containing the desired intermediate. Formalin (37%) (162 μL, 2.17 mmol) was added to a stirring solution of the intermediate (80 mg, 0.108 mmol) in MeOH (5 mL) at room temperature. Palladium on carbon catalyst (10%) (50 mg) was added under nitrogen, and the flask was evacuated. The solution was hydrogenated at 1 atm for 5 h. The catalyst was filtered off and washed with CH_2Cl_2 and MeOH. The combined filtrate and washings were concentrated, and the residue was chromatographed over silica gel (7% MeOH/0.5% NH_4OH in CH_2Cl_2) to yield 55 mg (25% overall for three steps) of solid **13**: $[\alpha]_D$ -41.7° (*c* 0.9, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.94 (t, *J* = 7, 6 H), 1.13 (d, *J* = 7, 6 H), 1.23 (m, 9 H), 1.33 (d, *J* = 7, 3 H), 1.36 (s, 3 H), 1.50-1.72 (m, 9 H), 1.83 (m, 1 H), 1.90-2.00 (m, 2 H), 2.29 (s, 6 H), 2.32-2.50 (m, 6 H), 3.02 (t, *J* = 9, 1 H), 3.24 (dd, *J* = 12, *J* = 6, 1 H), 3.33 (s, 3 H), 3.42-3.55 (m, 2 H), 3.73 (dd, *J* = 12, *J* = 3, 1 H), 3.96 (dd, *J* = 14, *J* = 3, 1 H), 4.03 (dd, *J* = 10, *J* = 6, 1 H), 4.40 (d, *J* = 7, 1 H), 4.80 (d, *J* = 5, 1 H), 5.25 (m, 1 H); ^{13}C NMR ($CDCl_3/CD_3OD$) δ 10.00, 10.28, 14.00, 21.12, 21.40, 25.26, 29.16, 33.00, 34.98, 40.28, 44.07, 58.70, 64.98, 65.99, 69.16, 70.83, 70.57, 96.83, 103.93, 177.90; IR (KBr) 3600-3440, 2960, 2930, 2780, 1720, 1600, 1450, 1370, 1160, 1100, 1045, 1005 cm^{-1}; HRMS calcd for $C_{39}H_{74}N_2O_{12}$ (MH^+) 763.5320, found 763.5321.

Communications to the Editor

Examination of HIV-1 Protease Secondary Structure Specificity Using Conformationally Constrained Inhibitors

A key step in the maturation of retroviruses, including the human immunodeficiency virus (HIV), is the post-translational cleavage of the *gag* and *pol* gene product polyprotein fusions into their constituent functional proteins, including the HIV protease (HIV-PR).[1-3] A protease-deficient mutant HIV-1 strain has been reported to produce noninfectious virions of immature morphology.[4,5] Thus, the HIV-1 protease is essential to viral replication[6-9] and represents an ideal target for antiviral therapy. The HIV-1 protease has been classified as an aspartic protease that functions as a homodimer based on its crystal structure,[10-12] its inhibition by pepstatin,[13] and the conservation of the characteristic Asp-Thr-Gly active-site sequence.[14,15]

The conformation of the polyprotein has been shown to be important in HIV-1 protease processing, since denaturation renders the substrate resistant to proteolytic

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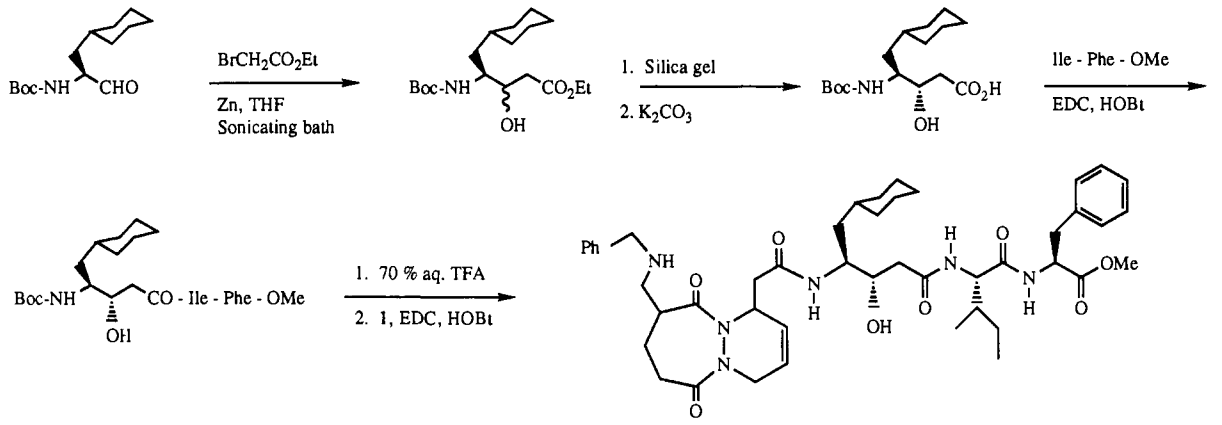
Scheme I



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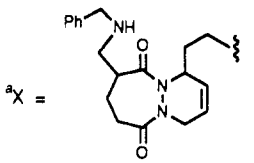
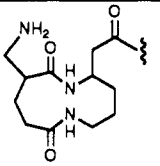
cleavage.[16] Eight sites within the HIV-1 *gag–pol* polyprotein are known cleavage sites for the HIV-PR.[17,18] These sites exhibit very limited primary sequence identity. An extended β-strand-like conformation for the cleavage site is suggested by the crystal structure of the HIV-1 protease complexed with substrate-based inhibitors[19–23] and by recent work on HIV-1 and HIV-2 protease cleavage of calcium-free calmodulin.[24] The cleavage sites are also generally characterized by short hydrophobic peptides flanked by relatively polar sequences. These features are thought to be prominent on the surface of the precursor molecules. The inability of HIV-PR to cleave denatured substrates in vitro is consistent with the hypothesis that secondary structure is a critical element in proteolytic processing.[16] Extensive conformational analysis of other peptide and protein systems has also shown that cleavage sites are often located adjacent to turns.[25] Thus, in this report, we outline an approach to examine the importance of secondary structure in proteolytic processing[26] by utilizing our previously reported β-turn mimetic systems, 1 and 2.[27]

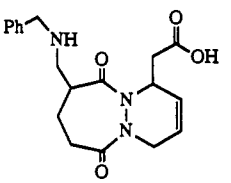
Table I. Inhibition of HIV Protease Activity

inhibitor	IC_{50} (M)[c]
X-ACHPA-Ile-Phe-OMe,[a] **3**	3.6×10^{-4}
X-Sta-Ile-Phe-OMe, **4**	1.4×10^{-4}
X-Sta-Ile-Phe-NH_2, **5**	2.1×10^{-5}
X-AHPPA-Ile-Phe-OMe, **6**	3.5×10^{-4}
Y-ACHPA-Ile-Phe-NH_2,[b] **7**	2.6×10^{-8}

[a]X = 
[b]Y = 

AHPPA = (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid. [c] IC_{50}'s were estimated from plots of percent inhibition vs inhibitor concentration.

Recent work by others has shown that peptides in which the scissile amide bond is replaced by a transition-state analogue provide a useful starting point in the design of tight-binding inhibitors of the HIV protease.[28–37] The



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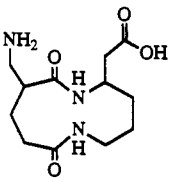

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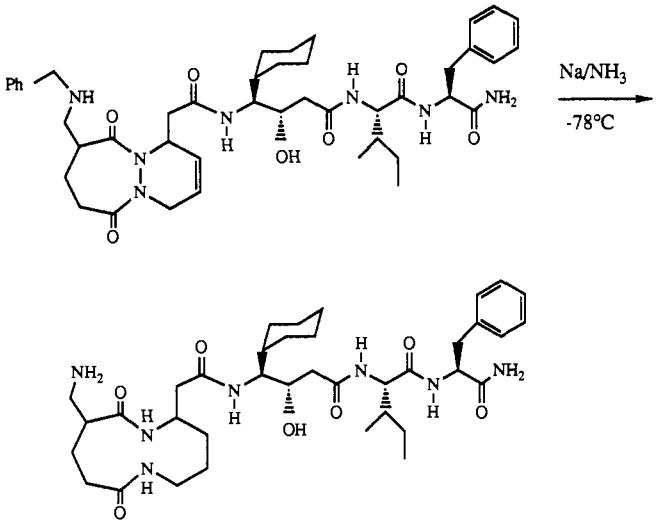
Scheme II



7

design of inhibitors for the closely related aspartic protease, renin, has also shown that replacement of the P_4-P_2 residues with various turn mimetics or cyclic moieties provides good retention of activity, as compared to unconstrained, linear inhibitors.[38-41] Thus, when coupled to a peptide that contains a statine type hydroxymethylene unit, the turn mimetics 1 and 2 should impart inhibitory potency toward aspartic proteinases.

Preliminary in vitro evaluation demonstrated that our designed inhibitors exhibit moderate to good inhibitory activity with IC_{50}'s $\sim 10^{-4}$–10^{-8} M, consistent with the use of statine-like transition-state analogues, and suggest that the optimum inhibitor will have an extended structure with a length complementary to that of the HIV-PR active site.

Chemistry

The bicyclic heterocycle 1 was prepared according to the procedure of Kahn and Bertenshaw.[42] The statine acid related analogues were then prepared by Reformatsky reaction of the appropriate *N*-Boc protected (*S*)-amino aldehydes with the zinc enolate of ethyl bromoacetate. The desired major *S,S* diastereomer (~4:1) was separated via flash chromatography and isolated in 45–60% yield. Saponification and coupling to the dipeptide ester provided the P_1-P_3' section of the inhibitors, as shown in Scheme I. Amino terminal deprotection and coupling to 1 provided inhibitor 3. The congeners 4, 5, and 6 were prepared by analogous routes and assayed via the procedure of Heimbach et al.[43] without modification. Their in vitro inhibitory activity against HIV protease is shown in Table I.

Molecular dynamics analysis, discussed below, suggested that the *N*-benzyl substituent was outside of the HIV protease binding pocket, thus we also investigated the effect of removing the *N*-benzyl substituent. This was readily accomplished via sodium ammonia reduction of the C-terminal amide of inhibitor 3, which also cleaved the N–N bond and reduced the double bond in the ring, to provide inhibitor 7 as shown in Scheme II. Gratifyingly, inhibitor 7 is the most potent of our inhibitors, with an IC_{50} ~26 nM.

Molecular Modeling

To improve inhibitor activity, we utilized computer-assisted molecular modeling to evaluate the detailed interactions between our inhibitors and HIV-1 PR. The statine based inhibitor 4, which incorporates the bicyclic turn template 1, was built using the DISCOVER program (Biosym Technologies, Inc.) with *S* stereochemistry at the statine hydroxyl carbon. Electrostatic charges at the ring and statine were determined by MNDO molecular orbital calculations. The lowest energy conformation of the bicyclic ring structure was obtained from a Monte Carlo conformational search by BATCHMIN with the MM2/MACROMODEL force field.[44] The crystal structure of the HIV-1 protease dimer complexed with the hexapeptide inhibitor MVT101[19] was used as the starting point to study the nonbonded interactions between the protease and our inhibitors. The torsional angles in statine, isoleucine, and phenylalanine were set equal to those in the MVT101 inhibitor. The five torsional angles around the bicyclic ring were determined from the total energy by rotating the torsional angles 360° with an increment of 10° using the DISCOVER program. All water molecules found in the X-ray structure were included. The minimized inhibitor was placed in the cleft of the HIV-1 protease dimer. The new complex structure was then energy-minimized by 50 steps of steepest descent and 400 steps of conjugate gradient minimization. The overall hydrogen-bond interactions were found to be very similar to those in the MVT101 complex except for one hydrogen bond from the carbonyl oxygen of Gly-48 at the flap that is absent in the new complex. One additional hydrogen bond was found between the carbonyl oxygen in Asp-29 and the benzylamine hydrogen in the side chain of the bicyclic ring.

Nonbonded interactions between inhibitor 4 and the protease were estimated from the van der Waals and Coulomb energies with a cut-off distance of 20 Å and a dielectric constant of 3.5. The total nonbonded energy between 4 and the HIV-PR was 5.5 kcal/mol lower than

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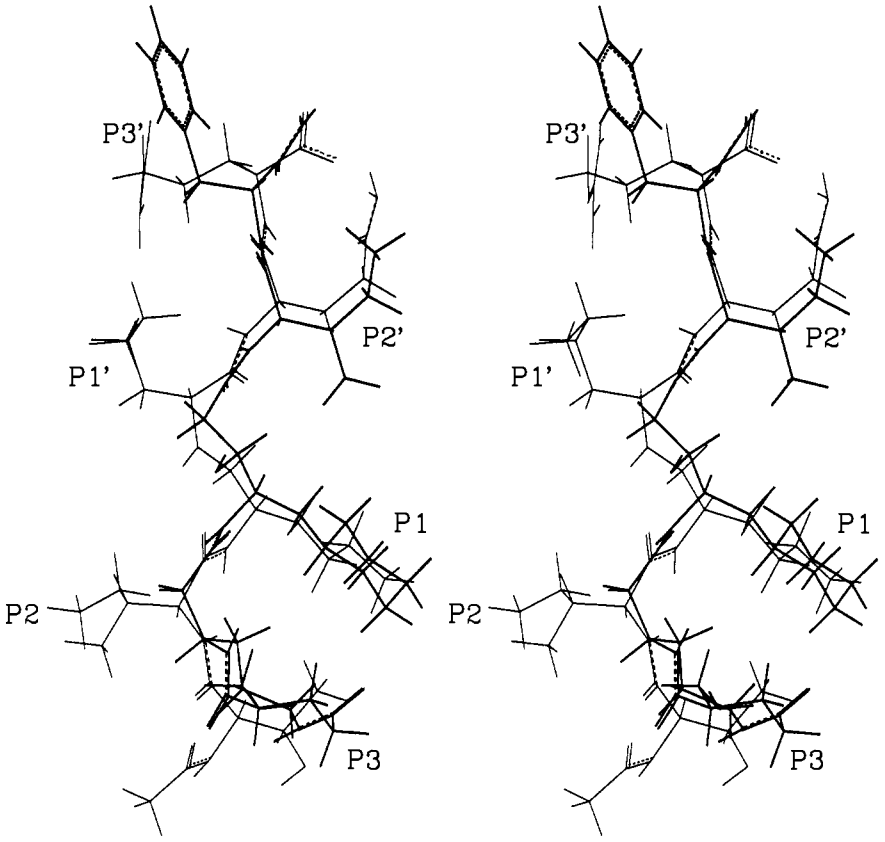

Figure 1. Stereo overlay of the minimum energy conformation of **7** (heavy line) with that of MVT101 (light line), from the respective inhibitor/HIV-PR complexes. All hydrogens are shown.

the nonbonded energy between the MVT101 and the protease. The nonbonded interaction energy between the two monomers of protease dimer was 2 kcal/mol less than the value found in the original HIV-PR/MVT101 complex.

The minimized complex structure was then used for a molecular dynamics simulation at 300 K for 25 ps in an effort to find a better fit between the inhibitor and the protease. The MD search found improved conformations until the total energy became stable after 20 ps. Analysis of the MD trajectory shows that the inhibitor is tightly packed in the protease cleft, and only slight movement, which is in accord with protease cleft movement, was observed in the backbone structure including the bicyclic ring. The low energy bound conformation of our inhibitor is very similar to that of MVT101, with a root-mean-square value of 0.57 Å for the four α-carbon positions. The side chains at the P_1, P_2', and P_3' positions occupy the hydrophobic pockets in the same manner, and the bicyclic ring occupies the S_3 pocket. Interestingly, the phenyl ring of the *N*-benzyl substituent of the turn mimetic shows dramatic movement due to the fact that it is protruding from the protease cleft and suggests that this ring is not contributing to the binding stabilization. Other work has also suggested that solvent exposure of the isovaleryl group in isovaleryl pepstatin results in a K_i about 2 orders of magnitude lower than that of acetyl pepstatin.[20] Based on this analysis, inhibitor **7** was synthesized, as described above.

Molecular dynamics simulation was then used to compare the binding mode of **7** with that of **4**. After 25 ps of molecular dynamics simulation to allow for conformational relaxation of the inhibitor, followed by energy minimization, the 11-membered ring structure of inhibitor **7** was found to fit in the S_3 pocket nicely, in a manner similar to that of the MVT101 complex, as shown in Figure 1, where the minimum energy bound conformation of **7** is superimposed with the MVT101. The statine and other inhibitor residues of **7** exhibit exactly the same hydrogen-bonding pattern (shown in the dashed lines in Figure 2) and very similar conformations as found in the crystal structure of HIV-PR complexed with acetylpepstatin.[20] However, the ring portion of **7** exhibits a hydrogen bond between a ring NH and the Gly-27 carbonyl that is not possible for the bicyclic ring of **4**. Other work has shown that a single hydrogen bond can contribute up to ~3–4 kcal/mol to inhibitor binding affinity,[45] thus the increased hydrogen-bonding potential of the monocyclic ring of **7** may contribute significantly to the increased activity of **7** over that of **4**. Model binding energy calculations also indicate that removal of the phenyl group and opening of the bicyclic ring both contribute to binding stabilization and that the 11-member ring plays a major role in binding. Details of the calculations will be reported elsewhere.

Conclusions

HIV-1 PR exhibits substrate specificity that cannot be predicted with respect to simple analysis of potential peptide or protein primary sequences.[46] Thus the design of an HIV proteinase inhibitor presents an additional challenge to try to incorporate secondary structure specificity.[47] Additional criteria that should be included in

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(47) For an earlier attempt to include secondary structure specific elements into renin inhibitors, see: Boger, J. in *Third SCI-RSC Medicinal Chemistry Symposium Royal Society of Chemistry*; Burlington House: London, 1986; pp 271–292.

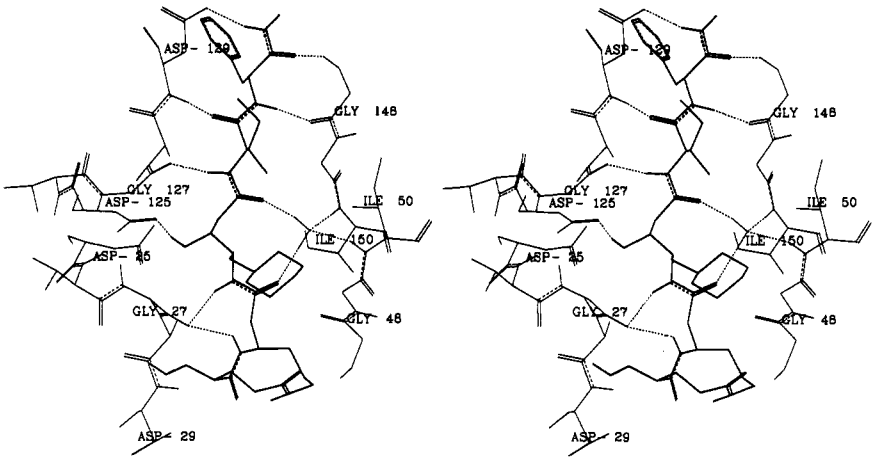

Figure 2. Stereoview of the hydrogen bonding pattern found for inhibitor **7** following molecular dynamics. This pattern is identical to that found for the HIV-1/acetylpepstatin complex.[20] For clarity, only polar hydrogens are shown.

the design are sufficient lipophilicity to allow for cell membrane penetration and resistance to proteolytic degradation.

We have designed a series of novel inhibitors that exhibit moderate to good potency against HIV-1 protease. We have incorporated a β-turn peptide mimetic unit which extends from the P_3-P_2 sites of the inhibitor. Despite the incorporation of less than optimal P_1-P_1' isosteres, which are known to play a significant role in binding affinity, and the known preference for branched residues in P_2, **7** displays 26 nM inhibition in vitro. This suggests that the turn mimetic framework may serve as an excellent foundation for the development of yet more potent HIV-1 PR inhibitors. There are several potential functions that the 11-membered ring may serve. The natural substrates of HIV-1 PR may have a propensity for a chain reversal adjacent to the cleavage site. Alternatively it may play a similar role to large heterocyclic species that can occupy the expanded S_3 site of HIV-1 PR.[29] Finally, it should be noted that recent work on the design of inhibitors for the closely related aspartic protease, renin, has also shown that replacement of the P_4-P_2 residues with various cyclic moieties provides good retention of activity, as compared to unconstrained, linear inhibitors.[38-41] Further investigations with extended substrates incorporating secondary structure mimetics will be required to evaluate their importance in HIV-1 PR specificity.

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† Department of Chemistry.
‡ Department of Medicinal Chemistry and Pharmacognosy.

Michael Kahn,*,† Hiroshi Nakanishi†,‡
R. Alan Chrusciel,† Dennis Fitzpatrick†
Michael E. Johnson*,‡
Department of Chemistry and
Department of Medicinal Chemistry and Pharmacognosy
University of Illinois at Chicago
P.O. Box 6998
Chicago, Illinois 60680
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Delineating the Pharmacophoric Elements of Huperzine A: Importance of the Unsaturated Three-Carbon Bridge to Its AChE Inhibitory Activity

While the acetylcholinesterase (AChE) inhibitor tetrahydroaminoacridine (THA) has been used in a number of clinical trials in the United States for the treatment of Alzheimer's disease (AD), results have been modest, and the studies have been hampered by its toxicity.[1] Indeed, in two primate model studies conducted by Iversen et al., THA was shown not to have a superior profile to physostigmine as a cognitive enhancer in primates.[2] Although physostigmine is also undergoing therapeutic trials, its usefulness appears to be limited by its short duration of action.[3] To the extent that AChE inhibitors can serve as useful adjuncts in the treatment of AD, two relatively new lycopodium alkaloids, huperzine A and B isolated from *Huperzia serrata* (Thunb.) Trev., a Chinese folk medicine, appear superior to THA and physostigmine.[4]

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