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DESIGN, SYNTHESIS, AND ACTIVITY OF CONFORMATIONALLY-CONSTRAINED MACROCYCLIC PEPTIDE-BASED INHIBITORS OF HIV PROTEASE

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Abstract: Conformationally-constrained macrocyclic peptide-based hydroxyethylamines, with 17- to 19-membered ring systems, have been designed and synthesized as HIV protease inhibitors. Structure-activity relationships were consistent with molecular modeling studies, and certain cyclic inhibitors were developed with HIV protease IC_{50} values of ~1 nM, and antiviral activities (HIV-1/RF infected MT-2 cells) of EC_{50} 4-8 nM.

Introduction. The aspartic proteinase encoded by the Human Immunodeficiency Virus (HIV), essential for the processing of the *gag* and *gag-pol* polyprotein gene products, has been identified as a viable chemotherapeutic target for the Acquired Immune Deficiency Syndrome (AIDS)[1]. As a result, this proteinase has been the subject of intensive research, and a great number of inhibitors have been developed for this enzyme[2,3]. Several transition-state analog inhibitors[2,3], including reduced amides, statines, hydroxyethylenes, dihydroxyethylenes, hydroxyethylamines, and hydroxyamides (norstatines)[4] have been reported, with many examples expressing low nanomolar or subnanomolar HIV protease inhibitory activity. Of particular note, potent C2-symmetric and pseudosymmetric inhibitors, including linear peptide-based molecules[5] and non-peptide cyclic ureas[6], have been developed through the aid of molecular modeling studies of HIV-protease/inhibitor complex X-ray structures.

The incorporation of conformationally-constrained macrocyclic ring structures in peptide-based enzyme inhibitors has the potential to provide a number of advantages relative to the acyclic analogs. These include increased binding affinity resulting from an entropic advantage, enhanced stabilization toward proteolytic enzymes, and specificity for the target enzyme. Several examples of macrocyclic peptide-based inhibitors of the aspartic proteinase renin have been reported[7]. In efforts to develop novel and potent HIV protease inhibitors with such favorable properties, we have therefore designed and synthesized macrocyclic peptide-based inhibitors, as analogs of the linear hydroxyethylamine inhibitors reported by Rich and coworkers[8] and by the Roche group[9].

Results and Discussion. Examination[10] of the X-ray crystal structure of the heptapeptide-derived hydroxyethylamine inhibitor 'JG-365' (Ac-Ser-Leu-Asn-PheΨ[CH(OH)CH_2N]Pro-Ile-Val-OMe) complexed to

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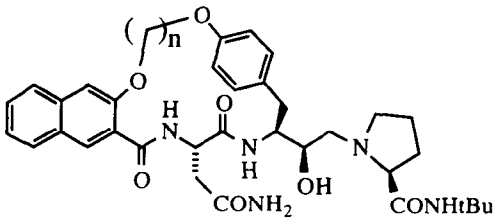
HIV protease[8b], as well as other HIV protease inhibitor complexes[2,3], revealed that a cleft extended from the S_1 binding subsite (terminology of Schechter and Berger[11]) to the S_3 subsite. It appeared that this cleft would allow for the binding of an aliphatic chain linking the phenyl ring of the inhibitor P_1 residue to the side-chain of the P_3 residue. Therefore, molecular modeling studies[10] were carried out, in which the conformation of JG-365 ('extracted' from the enzyme-inhibitor complex X-ray structure[8b]) was used as a template for the construction of various potential cyclic analogs incorporating an *N*-benzoyl group at P_3 and a P_3-to-P_1 aliphatic ether linkage. Energy minimization of these structures was followed by their comparison (RMS fit) to the X-ray structure of JG-365[8b], for an evaluation of the potential for these cyclic analogs to adapt to the putative bioactive conformation of JG-365. These modeling studies generated structures **1a-c** as promising initial targets, with the 17- and 18-membered ring systems (**1a,b**; n = 2, 3) predicted to be approximately equal in HIV inhibitory potency, and the 19-membered ring compound (**1c**, n = 4) expected to be somewhat less effective.



1a, n = 2

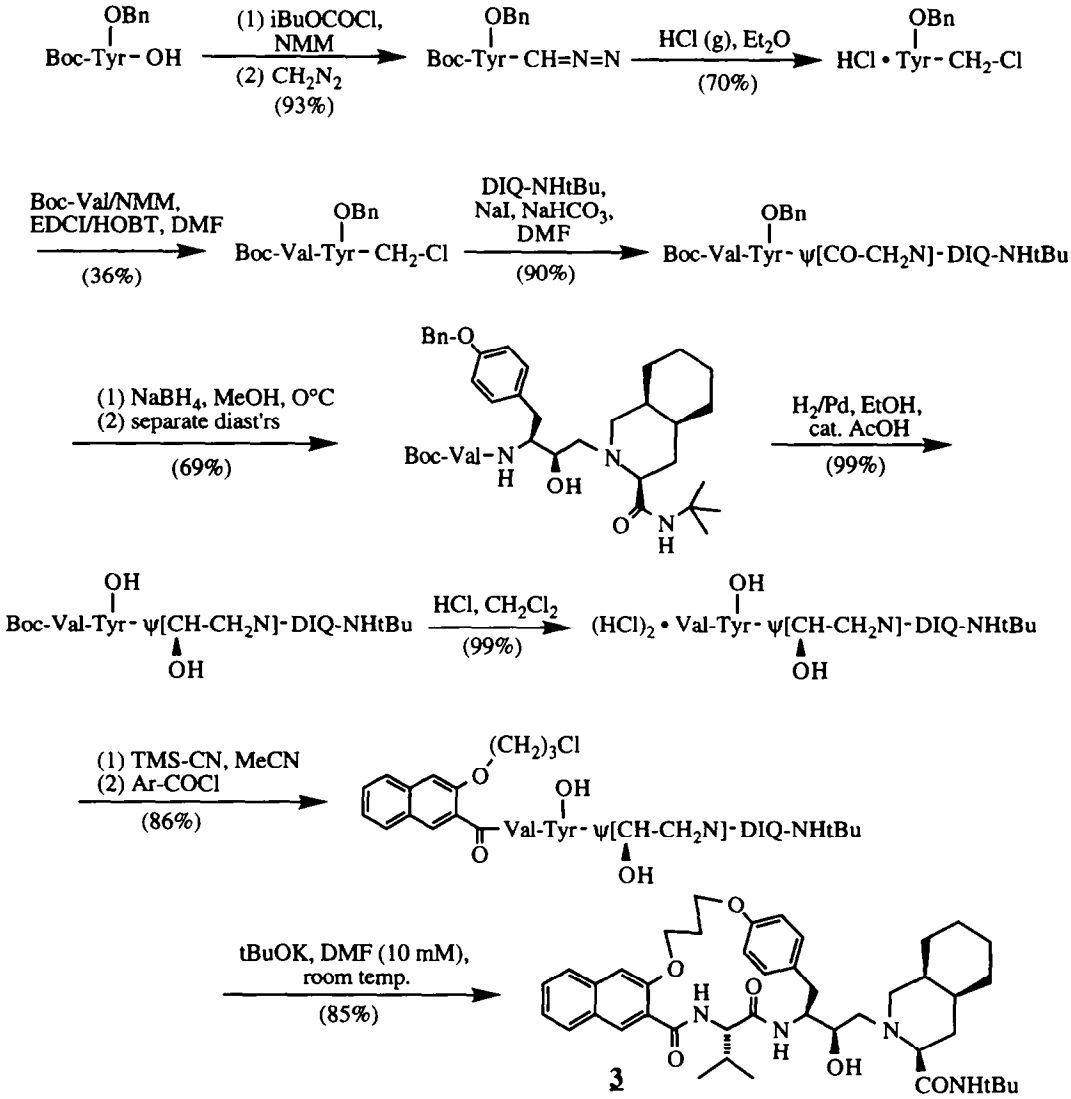
1b, n = 3

1c, n = 4

Our most efficient synthetic route to compounds such as **1** is outlined in the Scheme, as described for cyclic inhibitor **3**. This sequence involves the preparation of the P_2-P_1 chloromethyl ketone, based on methods described by Rich et al.[8], followed by incorporation of the P_1' group (such as (4a*S*, 8a*S*)-decahydro-3(*S*)-isoquinolinecarbonyl *tert*-butyl amide (DIQ–NHtBu)[9,12]). The resulting aminomethyl ketone was then reduced with sodium borohydride in methanol to provide the corresponding hydroxyethylamine, typically as a 3:1 mixture of *R* and *S* diastereomers. Consistent with previous studies of the effect of hydroxyl group configuration in peptidyl hydroxyethylamines on HIV protease inhibition[8c,9b], the (*R*)-hydroxyethylamine (shown for **1** and **3**) was the more potent HIV protease inhibitor of the two diastereomers, in all of our examples[13]. Standard deprotection steps were then followed by a trimethylsilylcyanide-mediated[14] neutralization and *N*-acylation step, to afford the acylic penultimate species. Macrocyclization was then achieved very efficiently by the action of a slight excess of *tert*-butoxide in DMF solution (10 mM) at room temperature[15]. For this nine step sequence, this represents an overall yield of 10.4% (not including the preparation of DIQ-NHtBu and Ar-COCl). For various cases of the macrocyclization reaction, the 17-membered ring system was obtained in 10-25% yields, while the 18- and 19-membered ring systems were generally obtained in 35-85% yields. These cyclization yields are quite respectable, if not remarkable, in comparison with related examples[7].

Enzyme inhibition studies were carried out with protease from the BRU (IIIB) strain of HIV-1 virus, as described previously[4a]. These measurements revealed IC_{50} values for **1a**: 450 nM; **1b**: 400 nM; **1c**: 900 nM; i.e., in the same order of magnitude as those measured[4a,8c,9a,9b] for related acyclic analogs having a carbobenzyloxy group at the P_3 position (e.g., Z-Asn-PheΨ[CH(OH)CH_2N]Pro-NHtBu, IC_{50} 210 nM[9a]). In sharp contrast to the 1.4- to 9-fold difference in potency observed for the *R* and *S* diastereomers (epimeric at –CHOH–) of these acyclic analogs, the (*S*)-diastereomer of **1b** was found to have an IC_{50} value of 90 μM,

Scheme

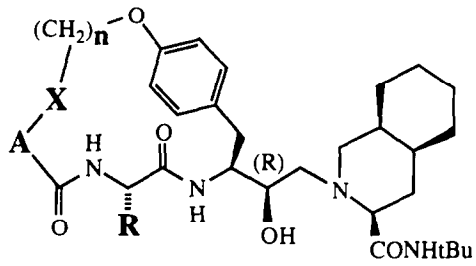


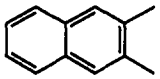
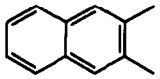
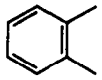
3

i.e., 200-fold less potent than (*R*)-**1b**. This suggests that the constraints imposed upon binding of the macrocycle in (*R*)-**1b** to the S_1-to-S_3 subsites of HIV protease do not allow for variance in the chirality of the –CHOH– moiety, to the extent that this change in chirality is tolerated for the more flexible acyclic analogs. In accordance with the investigations by the Roche group[9], incorporation of the DIQ residue at P_1' provided inhibitors with markedly enhanced HIV protease inhibitory activity (see Table). Consistent with our molecular modeling design studies (*vide supra*), the 17- and 18-membered ring inhibitors (**2a**, **2b**) were found to be approximately equal in potency, and somewhat more effective than the 19-membered ring analog (**2c**). This trend was also observed in the EC_{50} values for anti-HIV activity in a cell assay, using MT-2 cells infected with virus

strain HTLV-1 RF, and measuring for the level of p24 core antigen[16]. Incorporation of valine in place of asparagine at the P_2 position afforded compound **3**, with comparable activity to **2b** in both the protease and antiviral assays.

Table



A	X	R	cpd	n	HIV Protease IC_{50} (nM)	HIV Cell Assay (RF/MT-2) EC_{50} (nM)
	O	CH_2CONH_2	**2a**	2	13.	18.
			2b	3	10.	14.
			2c	4	88.	315.
	O	$CH(CH_3)_2$	**3**	3	5.5	17.
	O	$CH(CH_3)_2$	**4**	3	8.0	20.
	CH_2	$CH(CH_3)_2$	**5a**	2	7.3	36.
			5b	3	9.0	31.
			5c	4	25.	216.
	CH_2	$CH(CH_3)_2$	**6a**	2	1.5	8.0
			6b	3	1.0	4.0
			6c	4	3.4	26.
Ro31-8959: QC-Asn-PheΨ[CH(OH)CH_2N]DIQ-NHtBu					0.2	5.0
Npth-Val-PheΨ[CH(OH)CH_2N]DIQ-NHtBu					0.9	8.0
Ac-Val-PheΨ[CH(OH)CH_2N]DIQ-NHtBu					3.0	29.
nC_6H_{13}CO-Val-PheΨ[CH(OH)CH_2N]DIQ-NHtBu					6.0	378.

All compounds have (*R*)-chirality at –CHOH–; QC = quinolin-2-carbonyl; Npth = 2-naphthoyl

In order to characterize the solution conformation of one cyclic inhibitor (**1b**), nuclear Overhauser enhancement (NOE) data was obtained at both 300 and 500 MHz, in ROESY spectra (dmso-d_6). Proton-proton distance constraints derived from these data were incorporated in a distance geometry computation[10], to generate a family of related low energy conformations which were consistent with these constraints. In 'docking' studies[10] with the HIV protease X-ray structure[8b], this NMR/NOE-derived conformation was found to be a reasonable complement to the active site structure. In addition, it appeared that the P_3 naphthoyl group was oriented out of the active site into the solvent shell, without having any substantial interaction with the enzyme surface. This suggested that one or both of the aromatic rings at P_3 might be eliminated without a significant loss in binding affinity.

Accordingly, the *N*-benzoyl derivative **4** and α,β-unsaturated amides **5a-c** were synthesized by related methods[17]. Consistent with the docking studies, 18-membered ring inhibitors **3**, **4**, and **5b** were approximately equally potent HIV protease inhibitors. Unlike various acyclic HIV protease inhibitors described in the literature, an aromatic group fused at the P_3 position of these conformationally constrained inhibitors is neither required for effective activity, nor does it provide any enhancement in activity. Hydrogenation of inhibitors **5a-c** provided compounds **6a-c**, which were our most potent HIV protease inhibitors and antiviral agents in this series. Indeed, although **6b** (IC_{50} 1.0 nM) is five-fold less effective than the lead Roche inhibitor[9] **Ro31-8959** (see Table), it is completely equivalent in the anti-HIV cell assay (**6b**: EC_{50} 4 nM, EC_{90} 10 nM; **Ro31-8959**: EC_{50} 5 nM, EC_{90} 9 nM (our data)), and is more potent than closely-related acyclic analogs (see Table). Furthermore, we find that the ratio of inhibitory activities in the enzyme versus cell assays (e.g., IC_{50}/EC_{50} or IC_{50}/EC_{90}) for our cyclic peptide inhibitors is, in general, superior to the corresponding ratio calculated for any type of acyclic peptide-based HIV protease inhibitor reported to date. This may suggest that our cyclic peptide inhibitors have improved cell permeability and/or resistance to cellular enzymes, relative to acyclic peptide-based inhibitors.

In summary, we have designed and synthesized a series of macrocyclic peptide-based hydroxyethylamines as HIV protease inhibitors, with 17- to 19-membered ring systems. Low nanomolar IC_{50} values were obtained for several of these inhibitors, and one example had HIV antiviral activity equaling that of the lead Roche inhibitor Ro31-8959. We believe this represents a novel class of potent, conformationally-constrained macrocyclic peptide-based inhibitors designed for HIV protease.

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10. All molecular modeling studies were carried out with Sybyl software ver. 5.32-6.0 (Tripos Associates, St. Louis, MO) on a Silicon Graphics Personal Iris 4D35 workstation. Energy minimizations of inhibitor structures were performed in the absence of the protein, *in vacuo*, using Gasteiger-Marsali charges and the Tripos forcefield. The global energy minimum conformations of the candidate macrocycle structures were not pursued.
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13. Diastereomeric hydroxyethylamines were separated and purified by silica gel column chromatography. Assignments of configuration for our hydroxyethylamines are based on relative polarities, as per assignments given to the Roche hydroxyethylamine inhibitors[9c].
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15. All final products gave appropriate 300 MHz ^{1}H NMR, FAB-MS, and HPLC criteria. Bn = benzyl; NMM = *N*-methyl-morpholine; EDCI = *N*-ethyl-*N'*-dimethylaminopropyl-carbodiimide hydrochloride; HOBT = 1-hydroxybenzotriazole.
16. MT-2 Cells, a continuous human T-cell line, were infected with HTLV-1/RF for 1.5 h at 37°C, then washed to remove unabsorbed virus. The infected cells (and control uninfected cells) were then treated with inhibitor, incubated for 72 h, then the p24 core antigen level was determined by ELISA using the DuPont p24 Core Antigen test kit.
17. The (*Z*)-isomers of $Cl(CH_2)_nCH{=}CHCOOH$ were obtained from propargyl alcohol by the following sequence: 1.(a) nBuLi; (b) $Cl(CH_2)_nBr$; 2. Jones [O]; 3. CH_2N_2; 4. H_2/Pd-$BaSO_4$; 5. NaOH, H_2O-dioxane.

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