Influence of Stereochemistry on Activity and Binding Modes for C_2 Symmetry-Based Diol Inhibitors of HIV-1 Protease

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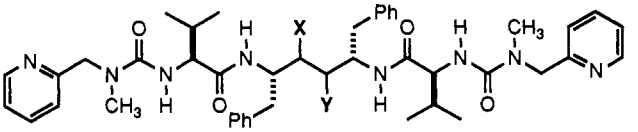
Abstract: The incorporation of C_2 symmetry has become a useful paradigm in the design of active site inhibitors for HIV-1 protease (HIV PR) and has led to the design of a series of highly potent, C_2 symmetry-based, diol-containing inhibitors of HIV PR, one of which, A-77003, has reached clinical trials. However, the stereochemistry of the diol core influences protease inhibition and antiviral activity in a manner that is not well understood. We analyzed the crystal structures of a diastereomeric series of C_2 symmetry-based diol inhibitors, along with a deshydroxy analogue, bound to HIV PR and found that the stereochemistry of the diol core influences the mode of binding to the active site aspartic acids. Diasteromers with similar binding affinity can bind in different, asymmetric and symmetric, modes, while those with different binding affinities can bind in a similar manner. The positional symmetry of an inhibitor with respect to the enzyme C_2 axis may be distinguished from its conformational symmetry. The structural differences between the inhibitor complexes were mainly confined to the central core portion of the diols, can be described by torsional differences about the central three bonds, and primarily affect interactions within the active site pocket formed by Asp 25/125 and Gly 27/127. Some flexibility in the enzyme backbone at Gly 127 was also apparent. Based on these results, we suggest that the binding mode for central hydroxy-bearing, C_2-symmetric inhibitors will be determined by how well the inhibitor can simultaneously optimize hydrogen bonding with the active site carboxylate groups and van der Waals contacts with the neighboring backbone atoms of the active site "ψ"-loops. A symmetric hydrogen-bonding arrangement with either one or two symmetrically positioned hydroxy groups appears to be preferred over less symmetric configurations.

Introduction

HIV-1 protease (HIV PR) has been the target of intensive efforts to design inhibitors which could serve as specific antiviral agents and arrest the progression of HIV infection in patients with AIDS.[1-4] Numerous laboratories have initiated X-ray crystallographic investigations of specific HIV PR/inhibitor complexes in an effort to use structure as an aid to inhibitor design.[5] Our initial attempt at the structure-based design of an HIV PR inhibitor utilized C_2 active site symmetry for the conceptualization of a C_2 symmetry-based diamino alcohol inhibitor core unit.[6,7] Key to this design strategy was the assumption that, upon binding, the inhibitor would adopt a position and orientation wherein the axes of symmetry of both inhibitor core and enzyme would coalign. This assumption was verified in the X-ray crystal structure of the symmetry-based inhibitor, A-74704, which bound to HIV PR in a highly symmetric conformation.[6]

Table 1. Chemical Structure and Biological Activity of C_2 Symmetry-Based Diol Analogues[a]



compd	X,Y	K_i, soln (pM)[b,c]	K_i, cryst (pM)[b,d]	EC_{50} (μM)[e]
A-77003 (I)	*R*-OH, *S*-OH	84	12	0.2
A-76889 (II)	*R*-OH, *R*-OH	1000	112	1.54
A-76928 (III)	*S*-OH, *S*-OH	77	11	0.17
A-78791 (IV)	*S*-OH,[f] H	35	4	0.16

[a] Synthesis of inhibitors for these studies was described in refs 8 and 9. [b] Protease activity was measured by continuous fluorometric assay[23] using the fluorogenic substrate DABCYL-GABA-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS (BACHEM, M-1865) or Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Lys(DABCYL)-Arg (kindly provided by Grant Krafft, Abbott Laboratories); data were analyzed using a mathematical model for tight-binding inhibitors[24] as described in ref 25. [c] Inhibition constants were determined at pH 4.7 in 0.125 M sodium acetate buffer containing 1.0 M NaCl as described previously.[10] [d] Inhibition constants were measured at pH 6.2 in 0.125 M MES buffer containing 1.2 M $(NH_4)_2SO_4$ as described previously.[25] [e] Concentration at which 50% inhibition of HIV replication was measured in MT4 cells using a cytopathic effect assay; data for compounds I–III from ref 10. [f] The priority rule for naming chiral carbons resulted in a different designation for this carbon. However, the absolute configuration of this hydroxy group is the same as that of the 1-*R*(OH) in the 1,2-hydroxy groups of the diols.

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Table 2. Summary of X-ray Data Collection and Refinement Statistics[a]

	compd			
	I (*R*,*S*)	II (*R*,*R*)	III (*S*,*S*)	IV (*S*,–)
resolution (Å)	7.0–1.8	7.0–1.8	7.0–1.8	7.0–2.0
reflections (total/unique)	52 676/17 998	82 259/17 974	85 425/16 239	29 397/10 031
R_{sym} (%)($\|F\| > 0.0$)[b]	6.52	7.36	8.36	8.92
R factor	0.181	0.186	0.177	0.158
no. of water molecules	83	72	80	84
rms bond (Å)	0.014	0.015	0.014	0.013
rms angle (deg)	2.61	2.84	2.63	2.48
mean *B* factor, protein (Å^2)	26	28	22	20
mean *B* factor, inhibitor (Å^2)	22	26	21	15

[a] Coordinates for the protein portion of 4HVP were used to provide starting phases for the structure determination and refinement of the complex with compound I. Protein coordinates from the complex with I were used to solve the structure of II–IV. Coordinates for all four data sets have been deposited with the Brookhaven Data Bank. [b] $R_{sym} = \sum|I - \langle I\rangle|/\sum I$, where I is the observed intensity and $\langle I\rangle$ is the average intensity obtained from multiple observations of symmmetry-related reflections.

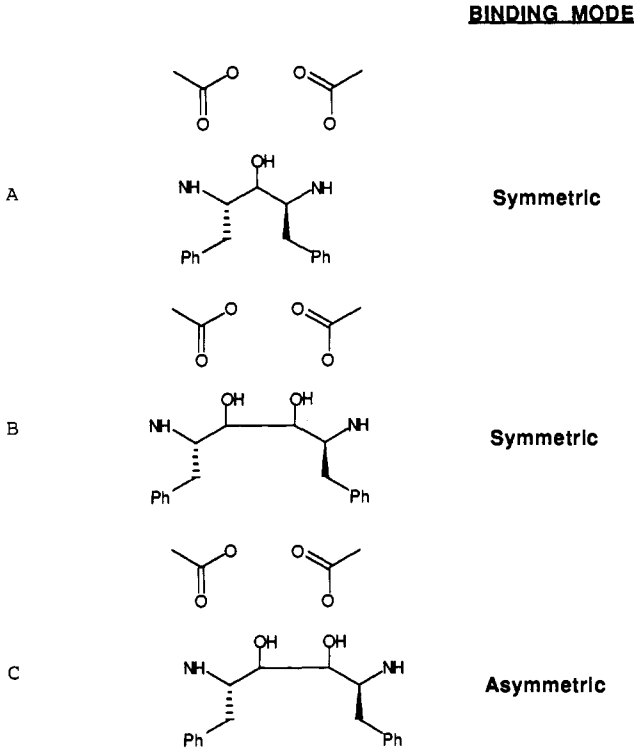

Figure 1. Symmetric vs asymmetric modes of inhibitor binding. (A, top) symmetric mode of binding exhibited by the pseudo-C_2-symmetric diamino alcohol, A-74704.[6] The C_2 axis of the enzyme relates the two active site carboxylate groups and nearly passes through the central carbon atom of A-74704 and within 0.2 Å of the hydroxy oxygen. (B and C, middle and bottom, respectively) possible modes of interaction for symmetry-based diols corresponding to type A and type B binding, respectively. Ph, phenyl.

This design strategy was extended to include a series of diamino diol core units, in which the C_2 axis bisected the bond connecting the two hydroxy-bearing carbon atoms.[7] Inhibitors derived from all three possible diol diastereomers, *R*,*R*, *S*,*S*, and *R*,*S* (or *S*,*R*), consistently showed greater potency than A-74704. However, the relative potencies of the diols differed for different diastereomers, and they did not exhibit a uniform dependence on the stereochemistry at the hydroxymethyl positions.[7,8] Moreover, inhibitors resulting from the removal of a single hydroxy group from the *R*,*R* diol showed an additional enhancement in activity.[9] Taken together, these results suggested that the mode of binding of different symmetric and pseudosymmetric core units might be dissimilar and substantially asymmetric. In an effort to define the structural basis for these observations, we determined the accurate K_i values and X-ray crystal structures of four related inhibitors derived from these core units (Table 1). The results demonstrate that C_2-symmetric inhibitors may bind in either symmetric or asymmetric modes with respect to the relative positioning of the C_2 axes of enzyme and inhibitor (Figure 1). The differences in binding appear to be determined by optimization of the binding of the diol core to a previously undefined active site pocket of the enzyme formed by the catalytic aspartic acids and their flanking glycine residues. Main-chain flexibility allows adjustment of the Gly 27 carbonyl group depending on the location of the second diol hydroxy group. The results suggest that, in addition to the well-recognized role of hydrogen bonding of the two aspartates, van der Waals interactions within the active site pocket play an important role in inhibitor binding.

The *R*,*S* diol inhibitor, A-77003, from this series possesses favorable pharmacokinetic and antiviral properties[10] and is in clinical trials. Recently, several A-77003-resistant HIV-1 strains have been isolated and found to contain single or double amino acid replacements in the protease sequence. The present crystal structure analysis of the A-77003/HIV PR complex provides a structural framework for the evaluation of these drug-resistant mutations which is described elsewhere.[11,12]

Results and Discussion

Structure and Activity of Inhibitors. The structures and biological activities of the inhibitors used in this study are shown in Table 1. The *R*,*S* and *S*,*S* diols were approximately equipotent, while the *R*,*R* diol was an order of magnitude less active in both the enzyme and antiviral assays. The deshydroxy analogue, IV, was the most potent protease inhibitor of this series. We measured K_i values under optimal solution conditions at pH 4.7, as previously determined for HIV PR activity,[13] and also under conditions similar to those used for crystallization at pH 6.2, in order to be certain that any structural differences that we observed were not due to differential solution effects on binding affinities. The K_i values of all four inhibitors were consistently 7–9 times lower under the crystallization conditions which employed 1.0–1.5 M $(NH_4)_2SO_4$. We found similar results with other types of inhibitors and also observed that 3.0 M NaCl substituted for 1.0 M $(NH_4)_2SO_4$ at pH 6.2.[14] These results extend the optimal pH

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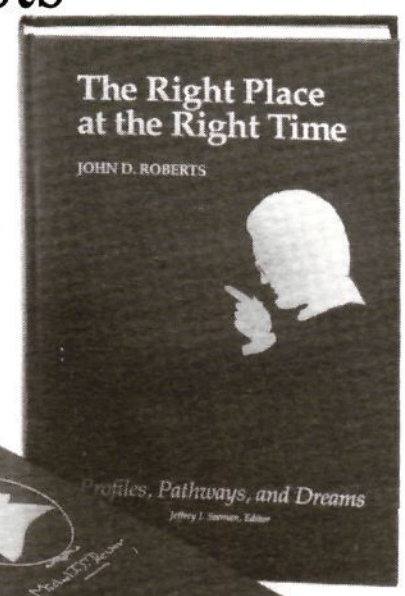

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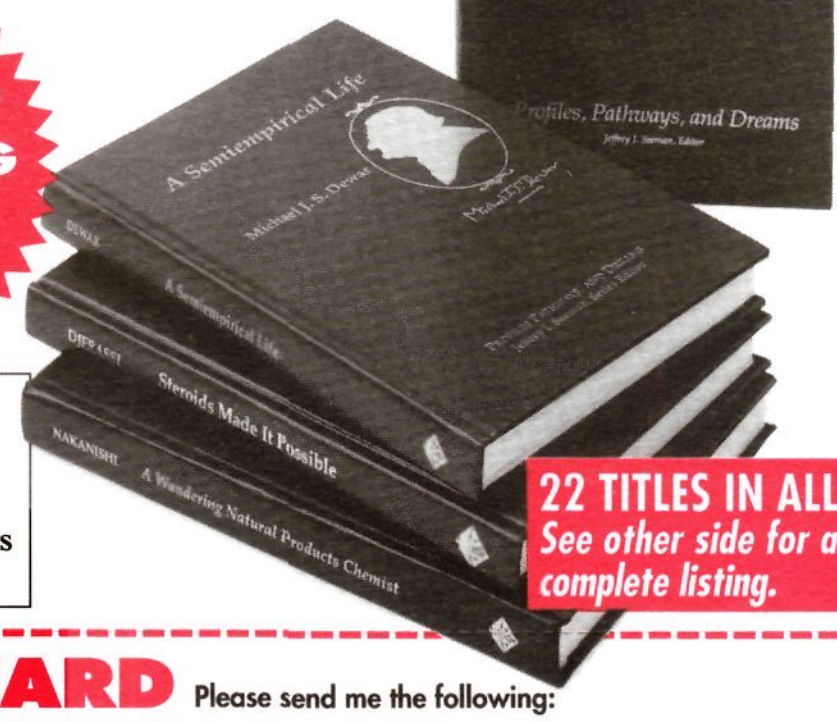

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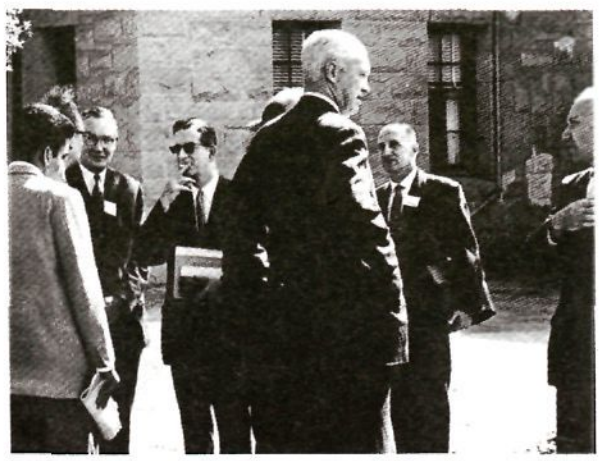

THE IUPAC MEETING, ZURICH 1955. L-R: Carl Djerassi, Michail Shemyakin (partially hidden), Derek Barton*, R.B. Woodward, Alexander Todd, Vladimir Prelog*, and Melvin Calvin*. The last five members of the group all became Nobel Prize winners. This assembly of remarkable scientists illustrates two phenomena found in the Profiles series: first, that genius attracts genius. Second, that despite the individuality, strength of personality, and competitiveness of each of these figures, they have forged close bonds and work together toward common goals. (*These men are included in the Profiles series.)*

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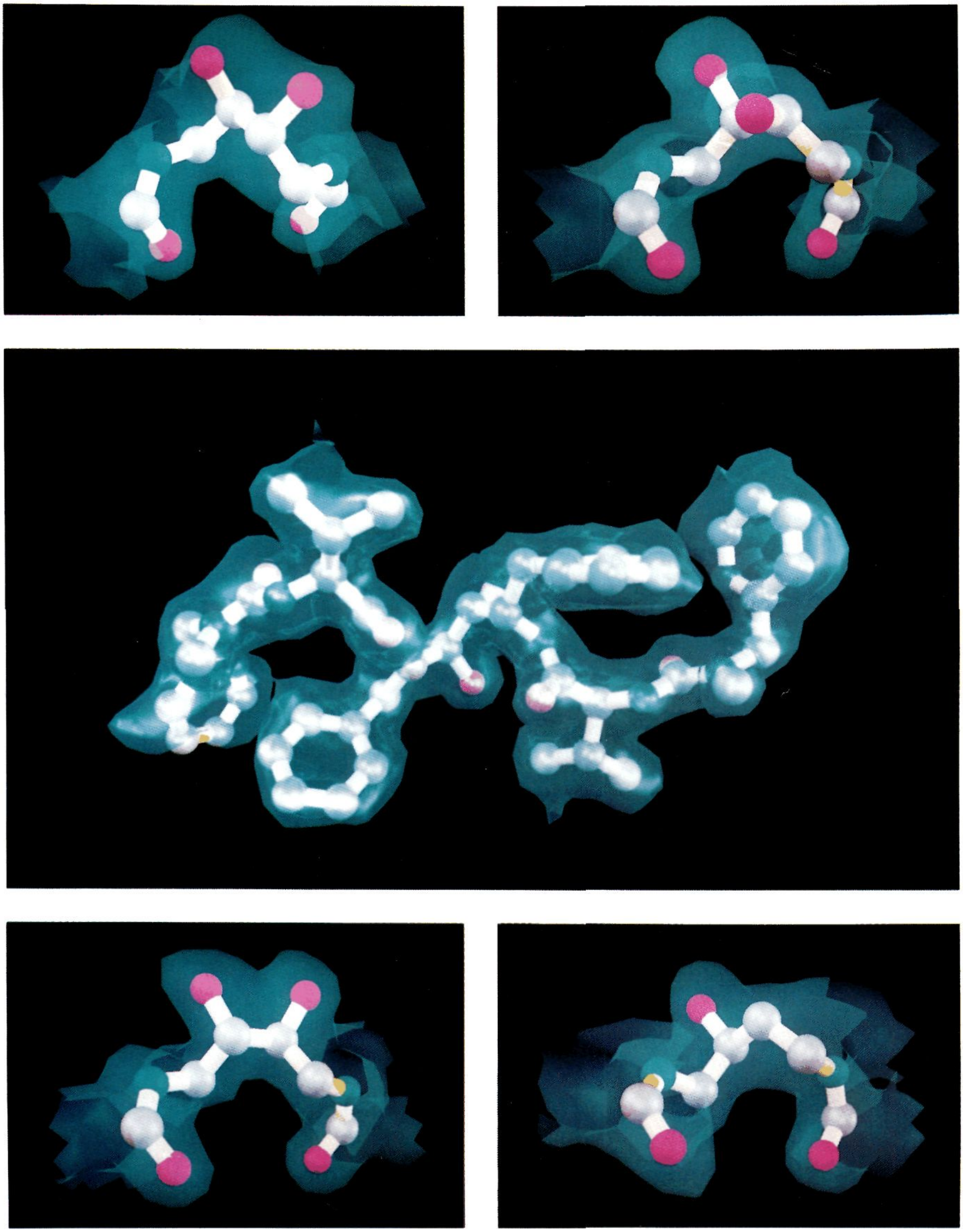

Figure 2. $F_o - F_c$ inhibitor–omit electron density maps for compounds **II** (A, top left), **I** (B, top right), **III** (C, bottom left), and **IV** (D, bottom right), showing the diol core portions only, and (E, middle) A-77003, compound **I**, in the complete $F_o - F_c$ inhibitor–omit map. Electron density maps were generated after removing the inhibitor from the structure of the complex, performing a round of refinement, and computing new $F_o - F_c$ maps using the phases of the enzyme only. All maps were contoured at 1.5σ.

range for this enzyme and demonstrate that the colligative properties of solution have a strong influence on HIV PR activity.

Symmetric vs Asymmetric Binding Modes. Unlike the diamino alcohol, A-74704, which is pseudo-C_2 symmetric,[6] the *R*,*R* (**II**) and *S*,*S* (**III**) diamino diols can, in principle, exhibit an infinite number of exact, C_2-symmetric conformations owing to the fact that the C_2 axis is orthogonal to and bisects the central C–C bond of the diols. Thus, one can envision that these compounds might

bind to HIV PR with exact or nearly exact C_2 symmetry with respect to the structures of the inhibitor, enzyme, or both (Figure 1). Fully symmetric binding would result in each diol OH group making preferential interactions with one of the two active site carboxylate groups. On the other hand, if interaction of the two aspartates with a single central hydroxy group is energetically favored, then the diols should bind asymmetrically, in which one of the two hydroxy groups would occupy the position close to that observed for A-74704 (Figure 1A). This asymmetric mode of binding has been observed in a complex with an asymmetric peptidomimetic diol-based inhibitor.[15]

The overall conformation of the inhibitor is, in principle, independent of the mode of diol core binding. We designate the following categories of binding to distinguish different combinations of positional and conformational symmetry: type A, a symmetric inhibitor backbone conformation bound symmetrically in the active site, in which the C_2 axes of inhibitor and enzyme coalign (Figure 1B); type B, a symmetric inhibitor conformation bound asymmetrically, in which the C_2 axes are non-collinear (Figure 1C); and type C, an asymmetric inhibitor conformation. In the case of type B binding, the C_2 axes of the inhibitor and enzyme would be displaced, in apparent contradiction to the original design principles for symmetric inhibitors.[6,7] Asymmetric peptidomimetic inhibitors follow type C binding by definition.

Structures of Diol Complexes: General Features. To address the above issues, the compounds shown in Table 1 were cocrystallized with HIV PR, and the structures of the complexes were determined by X-ray crystallography. Data collection, processing, and refinement statistics for the four complexes are shown in Table 2. All inhibitors were built initially without their central hydroxy groups. After a single cycle of SA–refinement, the resulting $F_o - F_c$ maps clearly indicated the absolute configurations for the diol OH groups (Figure 2A–D), which agreed in every case with the chemical assignments. No evidence for disorder arising from two different inhibitor orientations was observed for any of the complexes. The overall quality of the structure solutions for the inhibitor complexes is exemplified by the $F_o - F_c$ inhibitor–omit electron density map for I (Figure 2E).

It may be noted that the mean temperature factors for the four inhibitors (Table 2) rank in the same order as the inhibitor K_i values (Table 1), suggesting that the *B* factor may be an indicator of relative inhibitor potency. This correlation is consistent with thermodynamic principles that relate an increase in energetic stability of a system to a decrease in the number of highly populated states. However, we regard the current observation as fortuitous pending a more careful assessment of the systematic errors that may be hidden in the *B* factor values.

The four inhibitor structures were similar overall (Figure 3A), and their interactions with HIV PR exhibited features common to most other peptidomimetic inhibitors.[5] These features include a tetrahedrally-coordinated water molecule that bridges the carbonyl oxygens of the inhibitor with the backbone amides of the flap (Figure 3B), a conserved hydrogen-bonding arrangement between the backbone of the inhibitor and the backbone and side chains of the enzyme, and placement of the P3–P3′ side-chain substituents of the inhibitors into the respective S3–S3′ binding pockets of the enzyme.[16] However, the mode of interaction of the central diol core portion of the inhibitors is specific to each compound.

A novel binding feature shared by this series of inhibitor–enzyme complexes is the stacked arrangement of the P3/P3′ pyridyl groups on the inhibitor and Arg 8/108 guanidinium side chains of the enzyme (Figure 3C). The planes of the guanidinium and pyridyl groups are parallel, with an interplanar distance ranging from 3.3 to 4.0 Å for all eight examples. This interaction is partially stabilized by an amino-aromatic, induced dipole electrostatic interaction and can be classified as a weakly polar contact.[17] This arrangement is stabilized further by the adjacent P1/P1′ phenyl ring that interacts in an orthogonal fashion with the pyridyl ring and also by the carboxylate group of Asp 129 that forms a salt bridge with Arg 8 at the dimer interface. The importance of the Arg 8–P3 pyridyl interaction in these complexes is underscored by the tendency of HIV to generate R8Q protease mutants in the presence of A-77003.[11,12] Other mutations conferring resistance to A-77003 have been identified at positions 32 and 82, and affect primarily inhibitor side-chain interactions in the S2 and S1 subsites, respectively.[12]

Asymmetric Binding Mode for the *R,S* Diol: Importance of the Second Hydroxy Group. Examination of the 1.8-Å crystal structure of A-77003 (I) indicated that this pseudo-C_2-symmetric *R,S* diol interacts with the active site region of HIV PR in an asymmetric mode (Figure 4A). The *R*-OH group is centrally located near the approximate plane formed by the carboxylate oxygens of Asp 25 and 125. The O···O distances between the *R*-OH and the four carboxyl oxygens are 2.7, 2.7, 3.0, and 3.0 Å. The *S*-OH group, in contrast, can form only a single hydrogen bond with Asp 125. Superposition analysis of the complex of I with that of A-74704 indicates that the *R*-OH oxygen atom lies within 0.2 Å of the central hydroxy group in the latter. These results suggest that the shared binding of a single hydroxy group by the two aspartates is preferred for the *R,S* analogue and that the *S*-OH, which points away from the active site pocket, appears to play a minor role in binding. This hypothesis is supported biochemically by the fact that the deshydroxy diol, IV, which lacks the second (*S*) hydroxy group, was a more potent inhibitor than the *R,S* compound (Table 1).

The 2.0-Å crystal structure of the complex with IV was solved to evaluate the structural influence of removal of the *S*-OH group on binding. The bound conformations of I and IV are virtually indistinguishable (Figures 3A and 4A), and the two inhibitor structures superimpose to within 0.2 Å root mean square (rms) for 54 identical, non-hydrogen atom pairs. The absence of detectable structural differences between the two complexes indicates that the difference in binding energy between I and IV is most likely not due to differences in enzyme–inhibitor interactions. Preliminary calculations indicate that the presence of the *S*-OH group on I results in both a higher desolvation energy and a greater entropy loss on binding relative to the deshydroxy analogue.[18] These effects, together with the limited hydrogen-bonding compensation provided by the *S*-OH–Asp 125 interaction, are most likely responsible for the decreased potency of the *R,S* compound.

Asymmetric vs Symmetric Binding Modes: *R,R* and *S,S* Diols. The relatively high potencies of the three diastereomeric diols sharply contrast with the structure–activity relationships of asymmetric peptidomimetic inhibitors, which exhibit potent activity for only one hydroxy stereoisomer. The *S,S* diol differs from I only in the stereochemistry of the hydroxy group that interacts strongly with Asp 25 and Asp 125. Thus, we anticipated that the potency of III might arise from adoption of a binding mode dissimilar to that of I. On the other hand, the *R,R* diol, which differs from I in the stereochemistry of the hydroxy group that interacts weakly with the aspartates, might be expected to bind similarly to the R,S compound.

***R,R* Diol.** The X-ray crystal structure of the complex of II indicated that the *R,R* diol core bound in an asymmetric mode in which the two hydroxy groups were shared unequally by both aspartates (Figure 4A). Superposition analysis indicated that the conformations of I and II were similar overall (Figure 3A). The two inhibitor structures superposed to within 0.37 Å rms for

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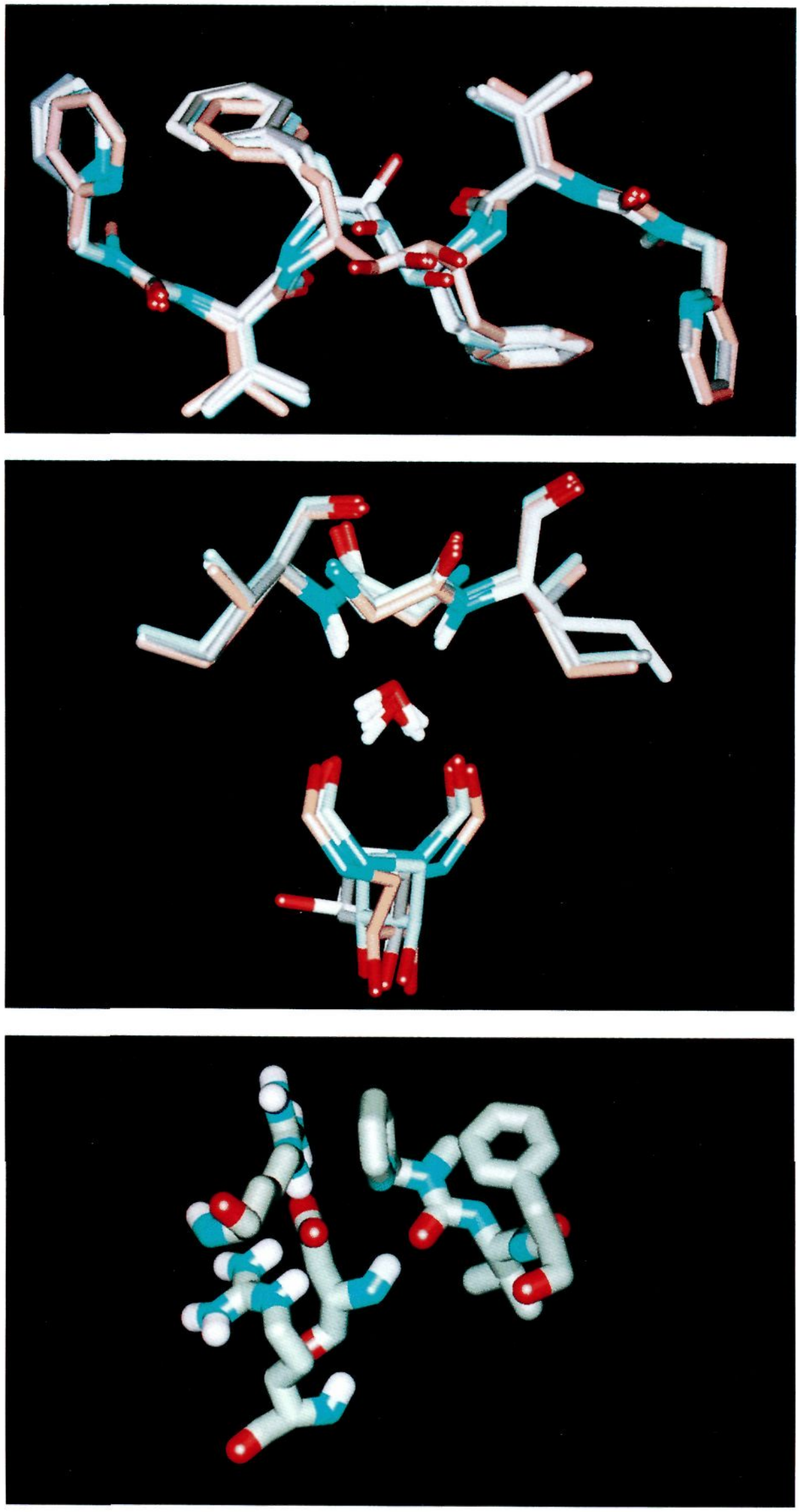
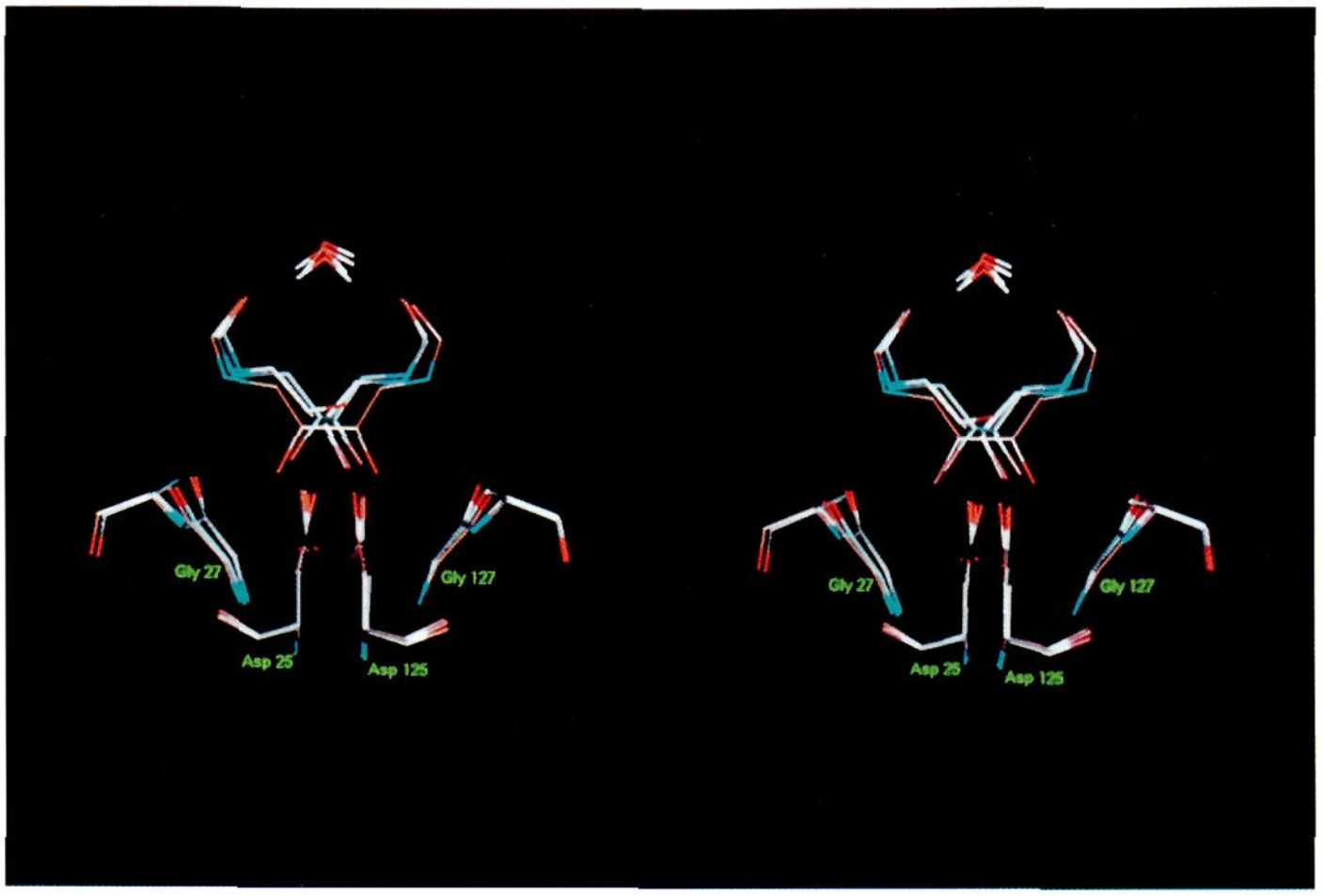


Figure 3. Various views of inhibitor structures. (A, top) Overlay of compounds **I**–**IV** after superposing the protease active site regions for the four complexes. (B, middle) Overlay of the inhibitor (lower)/water (middle)/flap (Ile 50/150, upper) interactions for the four complexes. (C, bottom) Stacking interaction between the P3 pyridyl (middle), Arg 8 guanidinium (left), and P1 phenyl (right) groups for the complex with **I**. Heteratoms in A and B are color-coded by atom type. Carbon atoms are color-coded by inhibitor: **I**, white; **II**, light blue; **III**, light brown; **IV**, light gray. In C, carbon atoms are green.

all atoms. Most of the deviations betweeen the two structures could be ascribed to the inner three torsion angles in the diol cores, which differed by an average of 7.0° (Figure 4B), and resulted in a slight positional displacement of the diol bond position in **II** relative to **I**. The more central *R*-OH group in **II** makes only three potential hydrogen bonds within 3.1 Å with the carboxylate



R,S	87	-179	177	-123	105	-178	-126	69	70	48	-120	-171	87	-106	171	180	106
S,-	84	-176	174	-123	109	-175	-132	62	69	53	-122	-171	92	-108	172	185	113
S,S	87	-178	177	-104	86	-186	-108	161	-79	164	-109	-185	90	-110	174	177	102
R,R	90	-179	175	-117	94	-180	-124	64	62	56	-122	-179	94	-115	172	185	108

Figure 4. (A, top) Close-up stereoview of the interactions of the diol cores with the active site pocket. Color scheme as in Figure 3A. (B, bottom) Torsion angles for the different inhibitor–enzyme complexes. The backbone of the *S,S* diol is illustrated for reference purposes. The torsion angles for the different inhibitors (rows) are listed under the corresponding bonds (columns).

oxygens. The more distal *R*-OH group can form two hydrogen bonds with Asp 125 and is 3.6–4.2 Å from Asp 25.

The R,R diol was a 10-fold less potent inhibitor than the R,S compound despite the similar number of potential hydrogen bonds made by the two diols with the active site (Table 1). However, comparison of the active site vicinities of the two structures suggests a reason for the weaker activity of **II**. If we assume that **I** binds in a preferred mode based on comparing the structures of **I** and **IV**, then it can easily be shown that the second *R*-OH of **II** would make highly repulsive van der Waals contacts with Gly 127 (O–O and O–C distances = 2.5 and 3.0 Å, respectively) if **II** were to bind in precisely the same conformation as that observed for **I** and **IV**. To avoid this situation, the backbone dihedral angles of both Gly 127 and the *R,R* diol core must be altered such that the second *R*-OH group makes only weakly repulsive contacts with the carbonyl carbon atom of Gly 127 (O–C distance = 3.3 Å). *Examination of the structure reveals* that the main chain at Gly 127 is displaced relative to **I** and **IV** (Figure 4A). We presume that the slightly repulsive contact, along with the energetic price of altering the Gly 127 conformation, results in a weaker binding affinity for **II** and may explain the origin of the torsion angle differences for the two inhibitor cores. The different hydroxy–carboxylate interactions in the complexes may also contribute to the affinity differences.

The hydrogen bond interactions between the active site aspartates and the *R*-OH observed with the *R,S* and *R,R* diols evidently favors an asymmetric mode of diol interaction for these compounds. The importance of optimizing hydrogen bond sharing

with both carboxylates is underscored by the fact that the deshydroxy analogue, IV, bound isostructurally to I. Recent structural studies have shown that the two hydroxy groups of an *R,R* diol for both an asymmetric[15] and a C_2-symmetric[19,20] inhibitor also interact unequally where one of the two hydroxy groups is found more centrally positioned in a manner analogous to that of the (*S*)-hydroxyethylene-based inhibitors.

S,S Diol. Examination of the structure of III bound to HIV PR revealed that the *S,S* diol bound in a novel, symmetric mode (Figure 4A), in which each OH group makes nearly equivalent interactions with a single aspartate as envisioned in our initial proposal (Figure 1B). Each *S*-OH oxygen is 2.7 Å from the nearest oxygen atom on the adjacent carboxylate group and can make three additional hydrogen bonds within 3.1–3.3 Å with the remaining oxygens for a total of eight potential O—H···O contacts. The C_2 axis of the enzyme passes within 0.1 Å of the midpoint of the diol bond, in accord with the original design principle that the inhibitor and enzyme symmetry axes should coalign. The backbone structures of I and III differ by 0.6 Å rms overall. However, most of the structural differences are confined to the diol core portion (Figures 3A and 4A). Outside this region, the P3, P2, P2′, and P3′ portions of the *S,S* inhibitor align remarkably well onto those of I and II despite the large shift in the location of the central diol bond and the different disposition of the two OH groups in III. As in the *R,R* diol, the binding differences of the *S,S* core can be largely accounted for by dihedral flexibility involving rotations about only the inner three bonds (Figure 4B), which differ by up to 149°, relative to **1**, with an average deviation of 119°. By contrast, the torsion angles for the 10 freely rotatable bonds outside the central core region differ by an average of only 6°. The *S,S* diol structure demonstrates that markedly different central active site interactions can occur while still preserving essential hydrogen-bonding interactions with the flaps and the buried water molecule, as well as the interactions of the P2/P2′ and P3/P3′ groups with the respective S2/S2′ and S3/S3′ subsites.

The *S,S* diol is equipotent to I despite the fact that the *R*-OH group, shown to be critical for binding to I, II, and IV is replaced by an *S*-OH group. The conformational readjustment of the inner three core torsion angles relative to II results in a novel, symmetric set of interactions with both aspartates. In the *S,S* complex, the individual interactions of each *S*-OH group are presumed to be weaker in strength than those of a single *R*-OH group located near the enzyme 2-fold axis (as in I, II, and IV), since the symmetric binding mode presents a suboptimal configuration for individual hydroxy group sharing with the two aspartates; in I, three of four *R*-OH hydrogen bond distances are significantly shorter than the hydrogen bonds observed with the *S,S* hydroxy groups. As in II, the presence of the second *S*-OH in III requires a small adjustment of the enzyme backbone at Gly 127 to avoid a repulsive contact with the carbonyl oxygen atom. However, unlike the case with II, this rearrangement, combined with the large torsional difference of the inhibitor backbone, results in a closer approach, by about 0.5 Å, of the diol core of III to the center of the active site region of the protease (Figure 4A). This leads, in turn, to a tighter fit of III with the enzyme in this region and presumably results in improved van der Waals interactions. This central region of the active site that interacts differently with the different diol cores can be described as a shallow binding pocket, formed by the carboxylate groups of Asp 25 and Asp 125 and by the carbonyl groups of Gly 27 and Gly 127 (Figure 4A). The recognition of an active site pocket in HIV PR extends the classical subsite description of aspartic proteases.

Conformational Symmetry and Binding. Analysis of the *R,R*, *R,S*, *S,*– and *S,S* inhibitor conformations indicated that each possessed nearly exact C_2 symmetry despite the structural differences of their core portions. Equivalent atoms from the two halves of each inhibitor molecule superpose to within 0.2–0.5 Å by a rotation axis of approximately 179°. The *S,S* diol binding falls into type A, as defined above, in which the inhibitor conformation is symmetric and the C_2 axes of inhibitor and enzyme approximately coalign. The *R,S* diol and the *S,*– analogue can be classified as type B binders since the inhibitor and enzyme C_2 axes are displaced by 0.8 Å, or half a bond length, relative to each other. This is more than the 0.2-Å shift found in the case of the pseudo-C_2-symmetric diamino alcohol, A-74704, and extends the limits on the initial design constraint that the two-fold symmetry axes of the inhibitor and enzyme should superpose.[6] The *R,R* diol should perhaps also be classified as a type B binder. However, steric constraints force a conformational adjustment of the diol core, with the result that it is more symmetrically bound than I. Modeling analysis revealed that if the *R,R* diol were to bind in a symmetric mode with a conformation similar to that of the *S,S* diol, the alternate stereochemistry would not permit the close approach of the *R,R* hydroxy groups to the binding pocket that is observed with the *S,S* diol. Thus, while it appears that symmetric conformations are energetically stable for symmetric and pseudosymmetric inhibitors, the binding mode, or positional symmetry, exhibited by a C_2 symmetry-based inhibitor may be independent of its conformational symmetry and is strongly influenced by the optimum hydrogen-bonding arrangement that can be achieved. Moreover, there is no correlation between either positional symmetry or conformational symmetry of an inhibitor and its binding affinity.

Besides the issues of inhibitor conformation and diol interactions, we can ask how symmetric are the complexes overall for the different diols. We addressed this question by rotating the entire complex using the enzyme dyad and comparing the rotated complex with the original structure. This procedure resulted in a mismatch of the P1 and P1′ groups for the *R,S* diol owing to the positional displacemet of enzyme and inhibitor two-fold axes (Figure 5A). However, atoms beyond P1 and P1′ were still approximately related by the enzyme two-fold axis except for some slight shifts of the P3/P3′ aromatic rings. In contrast to the *R,S* diol, the *S,S* and *R,R* inhibitors superpose nearly exactly onto themselves when transformed using the symmetry operation of the enzyme. The high degree of structural correspondence occurs not just at P2,P2′ and beyond but also at P1,P1′ and at the diol core atoms as well (Figure 5B and C). This is a consequence of the approximate collinearity of symmetry axes in those complexes. In all four inhibitors, the hydrogen-bonding atoms beyond P1,P1′ maintain equivalent hydrogen bonds in both halves of the inhibitor. Atoms at P1,P1′, together with the diol atoms, adjust to optimize the interactions of the diol cores with both aspartates within the constraints of the residues forming the active site pocket. When the diols are able to be contained in a symmetric fashion within the active site pocket of the enzyme, the entire inhibitor displays the approximate symmetry of the enzyme. Otherwise, the atoms at P1,P1′ adjust their positions in an asymmetric way to accomodate the diols within the pocket and thus leave the rest of the atoms to obey the approximate symmetry of the enzyme. The latter situation leads to the observed asymmetric binding mode. The enzymes structures of all four inhibitor complexes are highly symmetric; Cα atoms of the two chains of the enzyme superpose to within 0.4 Å with a rotation ranging between 178.3° and 178.6°.

The conformational adjustments of both II and III resulted in a slight shift of the P1′ benzyl groups within the S1′ pockets, with a concomitant shift in the positions of the P3′ pyridyl rings (Figure 3A). Since the latter movement is an inplane shift, the Arg 8

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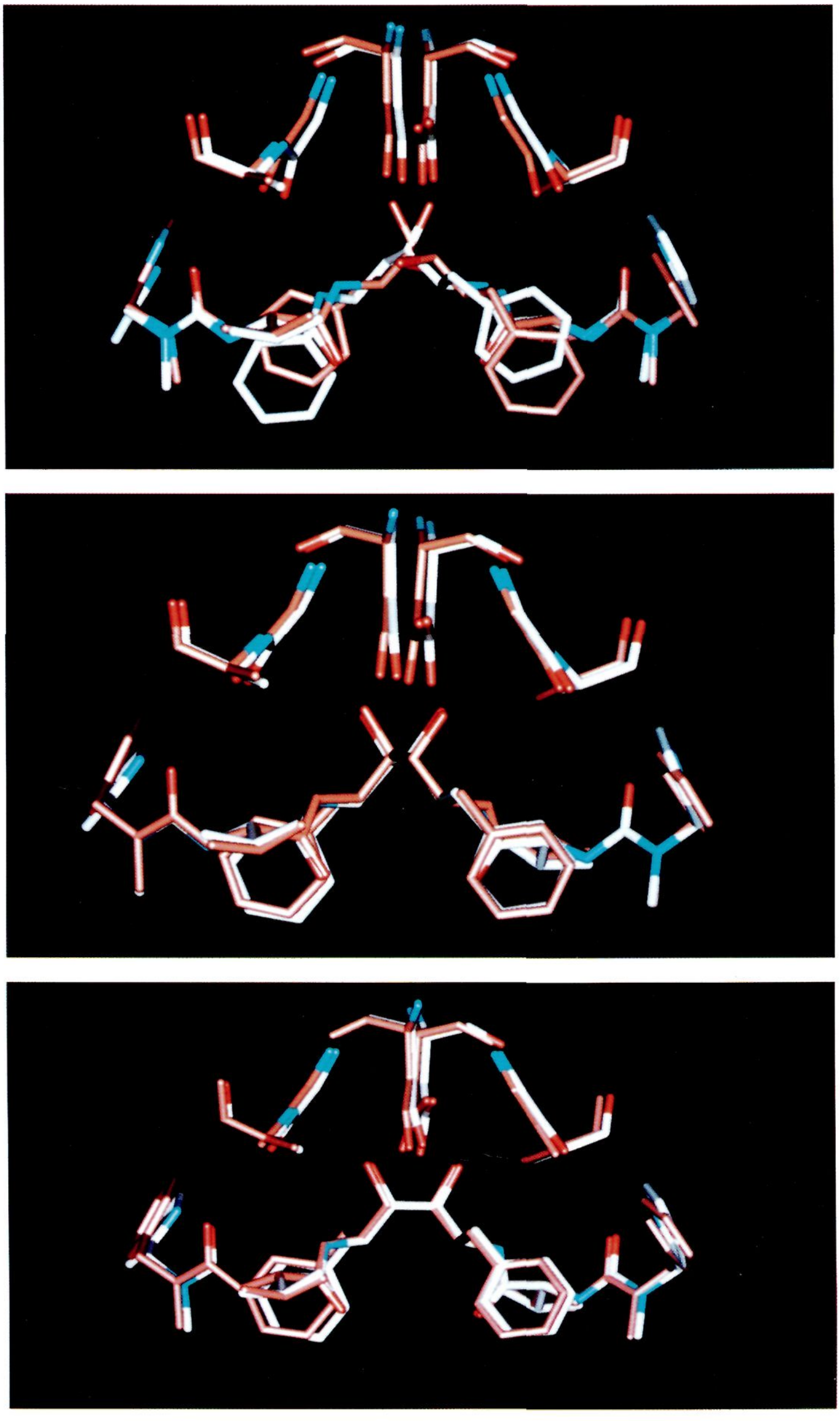

Figure 5. Structural correspondence of enzyme and inhibitor symmetry. The enzyme dyad was computed by superposition analysis of the 99 Cα atom for subunits 1 and 2. The entire enzyme–inhibitor complex was then rotated using this dyad. The before (unrotated) and after (rotated) structures are superposed for comparison. (A, top) *R*,*S* diol; (B, middle) *R*,*R* diol; (C, bottom) *S*,*S* diol. Structures with different orientations have different colors for their carbon atoms.

guanidinium group remains in place and the P1 benzyl–Arg 8–P3 pyridyl interaction is preserved. These results are consistent with the observation that compounds II and III are cross-resistant to an R8Q mutant of HIV PR that was initially selected on the basis of resistance to A-77003.[11]

The aromatic ring shifts observed in the diols occur on the P1′ side only; the P halves of the inhibitor superpose more closely (Figure 3A). This may reflect an inherent asymmetry in the kinetics or thermodynamics of binding in which, for example, the P1–P3 groups on the inhibitors form preferentials subsite interactions. A similar observation was noted for the asymmetric peptidomimetic inhibitor complexes of HIV PR, in which the N-terminal subsite interactions are more highly conserved than are those with the C-terminal halves of the inhibitors.[26] That study also noted that the C_2 symmetry-based inhibitor, A-74704, aligns best with the N-terminal portion of substrate-based peptidomimetic inhibitors. This result also holds true for I–IV.

Summary and Conclusions

The binding of symmetric and pseudosymmetric inhibitors is governed by a combination of factors which, if well understood, may lead to certain predictions for compound design. Our initial design assumption required an inhibitor that can adopt an intrinsically stable C_2-symmetric conformation and simultaneously make complementary interactions with the enzyme in order to be energetically favorable.[6] However, the current results relax the initial constraint of collinearity of enzyme and inhibitor two-fold axes and reveal that interactions within the central active site region, which itself forms a shallow subsite binding pocket, may be optimized independently of those outside this region. The main binding differences between the diasteromers are localized to the central diol core; the conformations and interactions of all the inhibitors are highly preserved outside this region. Thus, the different binding potencies for the diastereomers result from differences in the abilities of these analogues to simultaneously optimize the interactions within the active site and P1/P1′ pockets on one hand, while on the other hand preserving the critical hydrogen-bonding interactions with the flap, accommodating the buried water in the optimal location, and making favorable contacts in the outer subsites. The interplay of the outer subsite contacts with the core binding can be illustrated by the fact that replacement of the P3/P3′ pyridyl–urea groups with Cbz moieties resulted in a flattening out of the stereochemical dependence on binding potency.[17] Intrinsic solvation free energy differences for the binding of the different inhibitors may also play a role in determining binding potency but were not explicitly considered in this study. With flexible inhibitors, it has been difficult to make accurate model structures and to predict relative binding affinities. This study provides a valuable combination of comparative affinity and structural data for ongoing modeling and computational chemistry studies aimed at improving our ability to predict *a priori* the binding and relative affinity of newly designed inhibitors.

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Experimental Section

Crystallization. Crystals of HIV PR/inhibitor complexes were grown by hanging drop vapor diffusion. HIV PR, at 8 mg/mL, in 50 mM sodium acetate buffer, pH 4.5, containing 10 mM dithiothreitol, was mixed with inhibitor in a 1:5 molar ratio at a DMSO concentration of 13%. Crystallization well buffer consisted of 37% saturated ammonium sulfate, 63 mM sodium citrate, and 126 mM sodium phosphate, pH 6.2. Drops were prepared by mixing 2 μL of protein and 2 μL of well buffer, with subsequent addition of 10 μL of 1.0 M β-mercaptoethanol, 100 μL of DMSO, and 40 μL of 2-propanol to the 1.0-mL well solution. Drops were sometimes seeded with microcrystals prior to sealing. Crystals grew over a period of one to a few weeks at room temperature.

Structure Determination of HIV PR/Inhibitor Complexes. Crystals of HIV PR/inhibitor complexes were grown by cocrystallization in the space group $P2_12_12_1$ that was first described for MVT-101.[21] Crystals diffracted to between 1.6- and 2.0-Å resolution. Crystal structures were solved by molecular replacement using X-ray diffraction data collected on either a Siemen's Multiwire or an R-axis image plate area detector. Cu Kα X-rays were produced using a Rigaku Ru-200H rotating anode X-ray generator. In each case, a single SA–refinement cycle with the enzyme only as a starting model was performed using the program XPLOR.[22] Inhibitors were fitted into the resulting $F_o - F_c$ electron density map, and the complex was refined with several additional cycles of SA–refinement followed by individual atom *B* factor refinement. Water molecules were added at each step in the refinement using $F_o - F_c$ and $2F_o - F_c$ maps, and chemical environment as a guide.

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Supplementary Material Available: Overlay of inhibitor structures I and IV after aligning on the enzyme structures only (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.