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Design and Synthesis of Unsymmetrical Peptidyl Urea Inhibitors of Aspartic Peptidases

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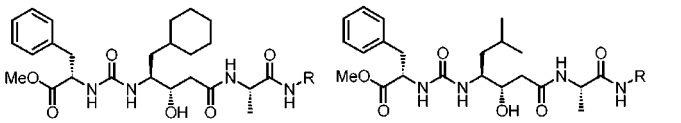
ABSTRACT



18; $K_i = 1 \pm 0.2$ nM (porcine pepsin) **20**; $K_i = 0.3 \pm 0.1$ nM (porcine pepsin)

The design, synthesis, and enzyme inhibition of a new class of aspartic peptidase inhibitors is described. Unsymmetrical ureas were designed from computer-generated structures. Using mechanism-based and substrate-based design techniques, potent pepsin inhibitors were developed and the binding mode was established. Two X-ray crystal structures of enzyme-bound inhibitors revealed a new binding mode that is closely related to the computer-generated binding mode.

The design and synthesis of peptidomimetics (highly modified peptides or small molecules that mimic the topography or action of peptides) is driven by the desire to obtain pharmaceutically useful agents.[1−3] The use of structure-generating programs can facilitate the discovery of peptidomimetics by suggesting novel structural scaffolds. Previous work has demonstrated that GrowMol,[4] a computational method for the de novo generation of novel structures complementary to the active site of a target enzyme, can accurately predict known metalloprotease[5] and aspartic peptidase inhibitors[6] and accurately predict the bound conformations of suggested inhibitors.[7] Here, we describe the use of GrowMol to create novel, unsymmetrical peptidyl ureas that inhibit aspartic peptidases.

Application of GrowMol to the 1.8 Å pepsin−Abbott renin inhibitor (A66702, Figure 1)[8] complex (inhibitor removed) generated approximately 25 000 unique, potential inhibitors. Growth was initiated at the critical hydroxyl group known

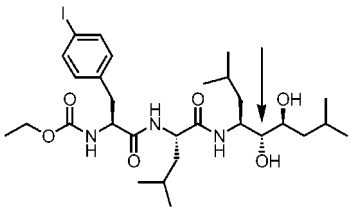


Figure 1. Abbott renin inhibitor A66702 with growth point indicated by the arrow.

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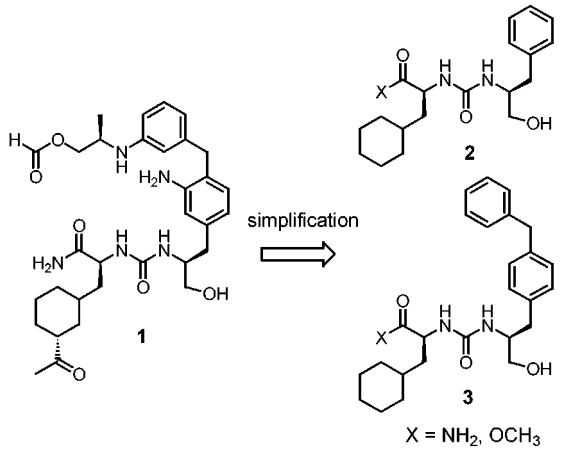
to mimic the tetrahedral intermediate for amide bond hydrolysis.[9] Inhibitor size was limited to 30 heavy atoms. Approximately 50 distinct structural classes were formed and examined. Several, highly diverse unsymmetrical peptidyl ureas were generated; most of these contained hydrophobic groups that spanned the S_1-S_2 subsites of pepsin.

The computer-generated ureas were considered attractive synthetic targets for several reasons. Although natural peptidase inhibitors (e.g., antipain, chymostatin, and elastinal) are known that contain urea bonds in place of amide bonds,[10] these occur between the P_3 and P_4 residues, and not between P_1 and P_2 residues. Ureas can form stronger hydrogen bonds than amides,[11] but they also place an additional heteroatom within the peptidyl chain. Finally, the stereochemistry predicted for the computer-generated P_2-P_3 replacement (L,L) differed from that expected if the unit functioned as a retro-inverso unit (D,L).[12] Consequently, we developed synthetic routes to simplified versions of these molecules to determine whether the urea analogues would inhibit aspartic peptidases and, if so, how they would bind.

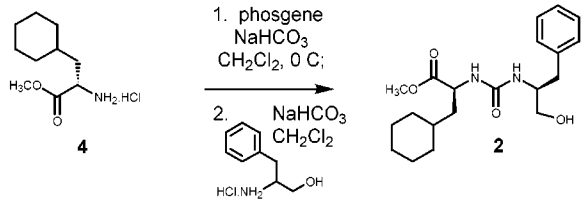
Examination of many GrowMol-generated ureas, e.g., **1**, in the enzyme active site showed that some functionality could be removed. The simplified unsymmetrical dipeptidyl ureas **2** and **3** were selected as the first synthetic targets (Scheme 1).

Scheme 1. Simplification of GrowMol-Generated Urea **1**



All diastereomers of the P_1-P_2 template **2** were synthesized according to the general procedure shown in Scheme 2; see Supporting Information, inhibitors were characterized by NMR, MS, and HPLC and shown to be greater than 95% pure). The benzylphenylalaninol ureas **3** and **15** were

Scheme 2. Synthesis of Unsymmetrical Urea Target **2**



synthesized via an iodinated phenylalanine derivative (see Scheme 3, in Supporting Information).

During structure generation, growth was directed into the S_2-S_3 subsites of the enzyme so that no P′ substitutents (to the right of the alcohol TS mimic) were formed. To increase inhibitor interactions to the S′ subsites, a statine unit[13] was added and used to extend the inhibitors at the C-terminus (see Table 1). These additions enhance inhibitor potency in analogues related to pepstatin and were expected to enhance inhibitor potency if the ureas were binding to the aspartic protease in a similar fashion.[14]

Enzyme Inhibition. Inhibition of porcine pepsin and *Rhizopus chinensis* pepsin was determined by established methods (Table 1).[15,16] Compounds **2** and **3** are the first aspartic peptidase inhibitors to contain a urea between P_1 and P_2. The 4-benzyl functionality in **3** increased inhibition, but only by a factor of 2. However, since all four urea diastereomers, **2** and **8**−**10**, and all four diastereomers of the Boc-Cha-Phe-ol control dipeptides (not shown) inhibit pepsin, we believe that these inhibitors bind to the enzyme in multiple binding modes.[17] X-ray crystallography is needed to determine the binding mode of these small inhibitors.

The statine-derived urea inhibitors **11**−**17** were designed to mimic the structure of known pepstatin analogues. The effect of structure in the P′ sites on binding for the ureas was similar to that of known pepstatin inhibitors,[18] which suggested they both bind in a similar fashion. The statine-derived urea **11** is an order of magnitude more potent than the simple alcohol template **2**. Addition of the P_2' and P_3' binding elements increased binding of the urea inhibitors **16**−**21** 1000-fold. Urea−statine analogue **20** was the best pepsin inhibitor (K_i = 0.3 nM). As predicted by GrowMol, the *S* stereochemistry was preferred in P_2 (**16** vs **17** and **20** vs **21**). These are the tightest-binding pepsin inhibitors that lack a P_3 component.

Several of the pepstatin-like ureas were also assayed for inhibition of the related aspartic peptidase *R. chinensis* pepsin (Table 1). In each case, the inhibitors had less affinity for Rhizopus pepsin than porcine pepsin (4−95-fold decreased

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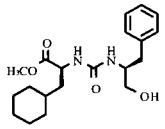
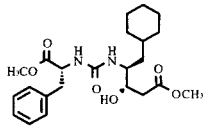
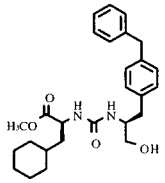
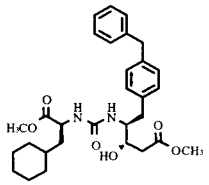
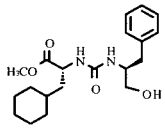
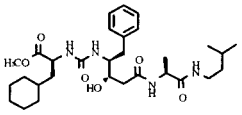
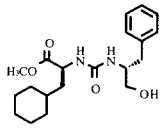
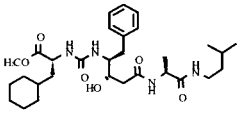
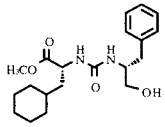
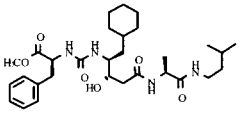
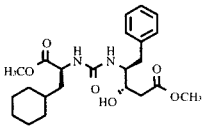
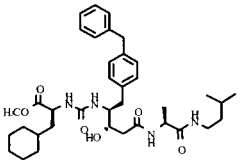
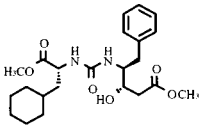
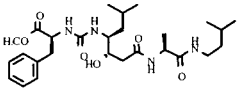
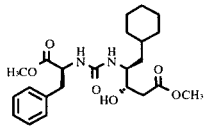
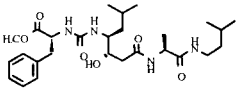
Table 1. Porcine Pepsin Inhibition of Unsymmetrical Ureas

cpd. #	structure	K_i (μM)	cpd. #	structure	K_i (μM)
2		77 ± 9	**14**		44 ± 4
3		35 ± 10	**15**		13 ± 2
8		133 ± 2	**16**		0.016 ± 0.001 (1.5 ± 0.1)*
9		19 ± 4	**17**		0.13 ± 0.02
10		11 ± 2	**18**		0.001± 0.0002 (0.033 ± 0.003)*
11		8 ± 1	**19**		0.035 ± 0.005
12		26 ± 2	**20**		0.0003 ± 0.0001 (0.006 ± 0.0004)*
13		2 ± 0.2	**21**		0.46 ± 0.06 (1.9 + 0.3)*

**Rhizopus chinensis* pepsin inhibition data (μM).

inhibition). Similar trends in inhibition were observed for both enzymes; the *S* stereochemistry is preferred in the P_2 region (**16** vs **17**) and the isobutyl side chain is preferred in P_1 (**20** vs **16** and **18**).

Crystallographic Evaluation. Although the potency of the statine-derived and pepstatin-derived urea inhibitors is consistent with the predicted active site binding, only a biophysical method such as X-ray crystallography can unequivocally establish how these compounds bind. Cocrystallization of these urea inhibitors with porcine pepsin has not been achieved; however, cocrystallization of ureas **16** and **20** bound in *R. chinensis* pepsin has been successful. The structure of urea **16** in the Rhizopus pepsin active site (Figure 2 resolved to 1.8 Å) is consistent with the computer prediction. The cyclohexyl binds in S_2, the phenyl in S_1, and the alanine in S_2'. The inhibitor hydroxyl group is hydrogen bonded to the two catalytic aspartic acid residues (Asp32 2.64 Å, 3.20 Å and Asp215 2.78 Å, 2.98 Å (not shown)). Several hydrogen bonds typical of pepstatin-derived inhibitors are present: P_1 NH and Gly220 (3.3 Å); P_2 carbonyl and Gly78 (3.0 Å), Asp79 (3.5 Å); P_1' carbonyl and Gly78 (2.7 Å); P_2' NH and Gly36 (2.3 Å). However, subtle variations in binding mode also are clearly evident. A new hydrogen bond is formed between the urea P_2 NH and the Thr221 side chain hydroxyl, and there is a nonideal interaction between the P_1 NH and the Gly220 carbonyl. The

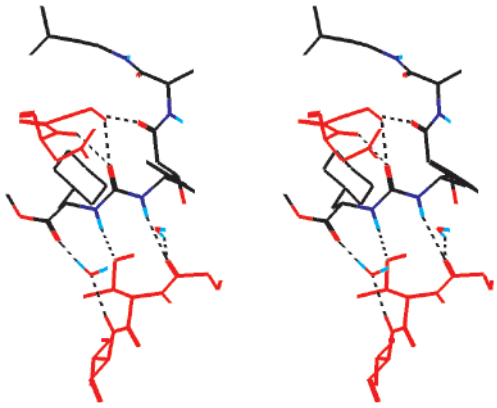


Figure 2. Crossed stereo representation of urea portion of **16** bound to *R. chinensis* pepsin.

latter hydrogen bond is conserved among peptide-derived aspartic peptidase inhibitors and is believed to be necessary for tight-binding inhibition. Although the P_1 NH in the urea **16**-Rhizopus crystal structure is within hydrogen bonding distance of Gly220, the directionality of the hydrogen bond is not ideal. A bound water molecule is quite close to Gly220 carbonyl (2.5 Å), and this water may mediate a better hydrogen bond to the P_1 NH (3.4 Å). This arrangement may be necessary since Gly220 is adjacent to Thr221, which forms the new hydrogen bond to the P_2 NH. Data from a 1.8 Å structure of urea **20** in the Rhizopus pepsin active site show a similar binding pattern.

These interactions are remarkably consistent with the major features of the binding mode predicted by structure generation in the active site of pepsin. However, hydrogen bonds between the methyl ester and the Thr222 backbone NH mediated by a water molecule (ester to water 3.5 Å, water to Thr222 3.0 Å) were not predicted.

Rhizopus pepsin and porcine pepsin are not identical, and although the initial GrowMol calculations were done on porcine pepsin, the X-ray structures were obtained on the *R. chinensis* pepsin−inhibitor complexes. Two important active site residues are different in these related enzymes: pepsin Thr77 corresponds to Rhizopus Asp77, and Ile30 in pepsin corresponds to Asp30 in Rhizopus pepsin. Both mutations could contribute to the decreased potency of the inhibitors against Rhizopus pepsin.

When we carried out GrowMol structure generation in the active site of *R. chinensis* pepsin by the described methods, we obtained a direct analogue of urea **20**. Figure 3 shows

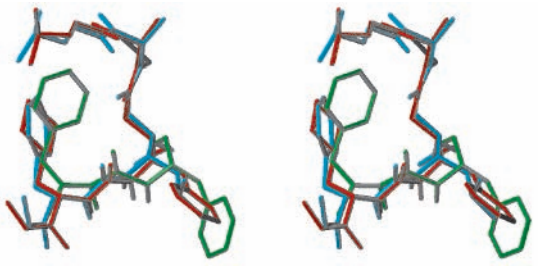


Figure 3. Crossed stereo projections of enzyme-bound conformations of inhibitors **16** (red) and **20** (blue) in the active site of *R. chinensis* aspartic protease I, superimposed on inhibitor designed by hybridizing GrowMol-generated partial structures (green).

crossed stereo projections of the two X-ray structures (**16** and **20**) in comparison with the GrowMol-generated structure. The agreement between prediction and experimental is quite good.

This work demonstrates that GrowMol predicts novel structural features that interact with the target enzymes essentially as predicted. When coupled with synthetic medicinal chemistry, GrowMol can be a very powerful tool for stimulating the design of new inhibitors.

Acknowledgment. We thank the NIH (GM50113) for financial support and Dr. George Flentke for providing *R. chinensis* pepsin and for assistance with the biological assays.

Supporting Information Available: Detailed synthetic procedures and spectral data; Scheme 3. This material is available free of charge via the Internet at http://pubs.acs.org.

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