Specificity in the Binding of Inhibitors to the Active Site of Human/Primate Aspartic Proteinases: Analysis of P_2-P_1-P_1'-P_2' Variation[1]

Chetana M. Rao,[†] Paula E. Scarborough,[†] John Kay,[‡] Brian Batley,[§] Stephen Rapundalo,[§] Sylvester Klutchko,[§] Michael D. Taylor,[§] Elizabeth A. Lunney,[§] Christine C. Humblet,[§] and Ben M. Dunn*,[†]

Department of Biochemistry & Molecular Biology, University of Florida, Gainesville, Florida 32610-0245, Department of Biochemistry, University of Wales, College of Cardiff, Wales, United Kingdom, and Departments of Chemistry and Pharmacology, Parke-Davis Pharmaceutical Research, Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan 48106-1047

Received December 28, 1992[•]

To understand the differences in the binding specificities within the aspartic proteinase family of enzymes, we have carried out studies to determine the inhibition constants of a set of related compounds with various members of the human enzyme family. The inhibition constants (K_i values) were determined by competitive inhibition of the hydrolysis of chromogenic octapeptide substrates in the pH range of 3–5. For comparison, inhibition of monkey renin was studied by RIA at pH 6.0. All inhibitors were based on the general structure 4-(morpholinylsulfonyl)-L-Phe-P_2-(cyclohexyl)Alaψ[isostere]-P_1'-P_2'. The isosteric replacements of the scissile peptide bond included difluorohydroxyethylene, 1,2-diols, 1,3-diols, and difluoroketones. Side chain substituents in P_2 include hydrogen, allyl, ethylthio, (methoxycarbonyl)methyl, *N*-methylthiouridobutyl, imidazolylmethyl, and 4-amino-2-thiazolylmethyl. Our measurements have identified potent and selective inhibitors which are useful in evaluating the differences in the specificities among selected enzymes of this family.

Introduction

The aspartic proteinase family of human enzymes, which includes pepsin, gastricsin, cathepsin D, cathepsin E, and renin, is important in many biological and pathophysiological processes.[2-7] Pepsin and gastricsin are involved in the digestion of proteins in the stomach. The physiological roles of cathepsins D and E are not completely defined, despite their wide distribution in mammalian tissues.[8] Cathepsin D, a lysosomal enzyme, has been implicated in protein metabolism,[9] catabolism,[3] and antigen processing.[10] Cathepsin E has recently been shown to be involved in antigen processing and biosynthesis of peptide hormones.[11] Inhibitors that are known to discriminate between cathepsin D and cathepsin E include large naturally occurring proteins isolated from potato tubers that inhibit cathepsin D[12] or from the nematode *Ascaris* that inhibits cathepsin E.[13] However, these inhibitors have limited utility owing to their large size, restricting penetration into cells. Potent and selective inhibitors of cathepsins D and E that have improved permeability would prove valuable in exploring the specific functions of these intracellular enzymes. The renin–angiotensin system plays a vital role in the regulation of blood pressure and also in maintaining the sodium and volume homeostasis.[14] The aspartic proteinase renin catalyzes the conversion of antiogensinogen to angiotensin I, which is the precursor of angiotensin II, a major pressor substance.

The enzymes in this family share a strong sequence and both secondary and tertiary structural homology.[15] However, there are considerable differences in the substrate specificities,[16-21] presumably due to the amino acid side chains that make up the active site cleft, referred to as "secondary" interactions by Fruton.[22,23] The study of structurally diverse inhibitors has been essential for understanding the mode of binding to the active site leading to catalysis, as well as providing a basis for consideration of enzyme specificity.[24,25] This understanding has been enhanced by the solution of crystal structures of various aspartic proteinases complexed with transition-state analogue inhibitors at a high resolution.[26-29]

Herein, we report the results of our studies employing selected inhibitors as probes to develop structure–activity relationships for the human enzymes pepsin, gastricsin, cathepsin D, and cathepsin E and monkey renin. These results not only confirm the generally similar inhibitor specificity of aspartic proteinases but also illuminate some differences important for designing potent and selective inhibitors. Such results will be particularly useful when combined with the growing number of crystal structures of enzymes from this family.

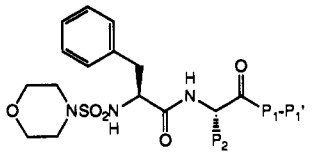
Results and Disussion

General Structure–Activity Relationships Based on Inhibitor Structures. A preliminary study evaluated selected aspartic proteinases for inhibition by several compounds containing transition-state isosteres, which are known to provide strong interaction with the catalytic site of aspartic proteinases. In this initial survey, we identified compound 7 (see Table I and Chart I) as a potent, broad-spectrum inhibitor exhibiting nanomolar inhibition of monkey renin and the human enzymes pepsin, gastricsin, cathepsin D, and cathepsin E. We then selected a structurally related group of compounds, listed in Table I, chosen to provide a series in which the P_2-P_2' region was varied. This series evaluated first the effect of changing the P_1-P_2' region by including several peptide bond isosteres: 2-amino-1-cyclohexyl-3,4- and 3,5-dihydroxy-6-methylheptane (3,4- and 3,5-ACDMH), 5(*R*)- and 5(*S*)-2-amino-1-cyclohexyl-3,5-dihydroxyheptane (*R*-ACDH, *S*-ACDH), 2-(2-amino-1-cyclohexyl-3,5-dihydroxypent-6-

[†] University of Florida.
[‡] University of Wales.
[§] Warner-Lambert Company.
[•] Abstract published in *Advance ACS Abstracts*, August 15, 1993.

0022-2623/93/1836-2614$04.00/0

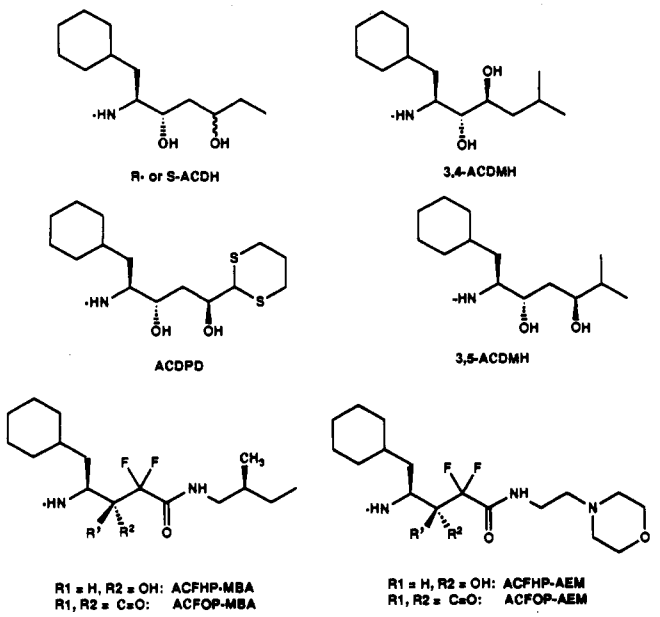
Table I. K_i Values from Inhibition Assays with Various Aspartic Proteinases[d]



compd	P_2	P_1–P_1' [a,b]	IC_{50} (nM): monkey[c] renin	K_i (nM): human pepsin	human gastricsin	human cathepsin D	human cathepsin E
1	allyl	ACFHP-AEM	0.7	1240	>4000	0.8	21
2	allyl	ACFHP-MBA	0.3	27	13	3.2	0.1
3	allyl	ACFOP-AEM	0.4	11	0.9	18	0.5
4	allyl	ACFOP-MBA	0.8	24	9	0.4	0.5
5	allyl	3,4-ACDMH	0.2	13	8	2.5	17
6	allyl	ACDPD	0.2	16	>4000	0.6	2.2
7	allyl	3,5-ACDMH	0.3	57	19	0.1	2.0
8	allyl	R-ACDH	0.2	77	>4000	5.4	11
9	allyl	S-ACDH	0.9	69	>4000	6.1	3.3
10	allylthio (*R*/*S*)	S-ACDH	13	516	>4000	15	2.9
11	CH_2CO_2Me	R-ACDH	0.6	185	>4000	13	44
12	$(CH_2)_4NHCSNHMe$	S-ACDH	>1	>10000	110	45	15
13	(*S*)-CH_2-(4-imidazole)	R-ACDH	1.1	>10000	>4000	>10000	850
14	(*S*)-CH_2-[4-(2-aminothiazole)]	R-ACDH	0.3	>10000	>4000	208	300
15	H	R-ACDH	44	>10000	>4000	>10000	>10000

[a] The stereochemistry of *R*- and *S*-ACDH was firmly established by spectroscopic analysis (NOE) of cyclic intermediates.[47] The stereochemical assignments of ACDPD and 3,5-ACDMH were made by analogy to ACDH based on spectroscopic and chromatographic properties. [b] Structures of the indicated fragments are illustrated in Chart I. [c] Cynomolgus monkey plasma renin. [d] The values reported have errors of ±15%, based on propagation of the errors of measurement.[43] Renin inhibition values are at pH 6.0. All other values were derived from studies with chromogenic substrates, as described in the Experimental Section.

Chart I. P_1-P_1' Isosteres



yl)-1,3-dithiane (ACDPD), 4-amino-5-cyclohexyl-2,2-difluoro-3-hydroxypentanamides (ACFHP), and 4-amino-5-cyclohexyl-2,2-difluoro-3-oxopentanamides (ACFOP). The P_4-P_2 region was initially maintained constant with substitutions [(morpholinylsulfonyl)-L-phenylalanyl-L-allylglycine, compounds 1–9] known to provide reasonable binding to the enzymes under study.[30,31] The P_2 substituent was then varied (compounds 10–15) to include hydrogen (glycine), allylthio, (methoxycarbonyl)methyl (aspartate), thiourea derivative of lysine, 4-imidazoylmethyl (histidine), and 4-(2-aminothiazolyl)methyl.

Variation of the P_1-P_1' portion of the inhibitor (1–9) was generally consistent with potent inhibition of most if not all of the enzymes tested, but with a range of measured K_i values. For example, compound 1 yielded subnanomolar K_i's against renin and cathepsin D, was about 20-fold weaker versus cathepsin E, and barely inhibited pepsin or gastricsin. A particularly good broad-spectrum aspartic proteinase inhibitor was 4, yielding K_i values in the low nanomolar range for all of the enzymes.

The effect of a basic group in the P_2' position, 2-morpholinylethylamide (AEM, in 1 and 3) vs the neutral 2-methylbutylamide (MBA, in 2 and 4), on relative inhibition of cathepsin D or E was dependent on whether an alcohol or ketone hydrate was present at P_1. With a basic moiety present, ketone hydrate (3) favored cathepsin E inhibition, while the alcohol 1 favored cathepsin D inhibition. The ketone hydrate 4 with the alkyl side chain inhibited all of the enzymes, while the alcohol 2 was the strongest cathepsin E inhibitor found. More generally, the oxidation of the alcohol to the ketone hydrate produced relatively small effects on inhibitory potency when P_2' contained MBA (compounds 2 and 4). However, when P_2' was AEM (compounds 1 and 3), there was a dramatic increase in potency for pepsin, gastricsin, and cathepsin E, while renin inhibition was unchanged and human cathepsin D was less inhibited by 3 relative to 1. These observations suggest an interdependency between the P_1 residue and the P_2' substituent, which could be due either to the juxtaposition of subsites S_1 and S_2' of the enzyme active site cleft (as a consequence of the extended binding mode) or to differences in the mode of binding between the alcohols and ketone hydrates that exist at the active sites of non-renin proteinases. Renin itself was uniformly insensitive to these changes.

The position of the second hydroxyl group in the 3,4 or 3,5 ACDMH dihydroxy derivatives (Chart I) produced little change in potency (5 vs 7), with the exception of cathepsin D and cathepsin E, where tighter binding was seen for compound 7. However, the deletion of a branch methyl at the 6-position eliminated inhibition of human gastricsin (8, 9 vs 5, 7). The stereochemistry of the ACDH

distal hydroxyl was not important for inhibition of the enzymes listed in the table.

Modification of the P_2 position was examined next. Addition of a heteroatom into the side chain (**10** vs **9**) reduced inhibitor potency for all enzymes except cathepsin E, although some of the reduction could be accounted for by the fact that **10** was a mixture of diastereomers. Addition of polar functionality (**11** vs **8** and **12** vs **9**) significantly reduced potency in most cases. One exception was human gastricsin, which seemed to tolerate the *N*-methylthiourea derivative of lysine at P_2. Installation of heteroaromatic side chains at P_2, particularly histidine, is widely known to produce selectivity for renin.[32] Evaluation of **13** and **14** confirmed that, while renin inhibition was maintained, substantial loss of potency was observed for all other enzymes. Finally, lack of substitution at P_2 (**15**), a modification studied rarely, was as good as histidine in terms of renin selectivity, which could be related to the more crowded renin active site.[33] However, a reduction in renin potency was noted.

Comparison of Results for Selected Human Enzymes. One of our main objectives in this study was to develop structure–activity relationships that could guide the design of more selective inhibitors for analysis of cellular processes. An additional concern in drug design is bioavailbility. Because the human enzymes pepsin, gastricsin, and cathepsin D are present in the body in relatively large amounts, binding of potential drugs may limit the concentration available to interact with the intended target, such as renin or HIV proteinase. Accordingly, it is valuable to compare the binding of the inhibitors listed in Table I from the separate perspective of the human enzymes.

One major revelation of the cathepsin D model[34] is that the S_2 region is extremely hydrophobic. Therefore, the anemic binding seen for compounds **13–15** to human cathepsin D could be explained on the basis of either the lack of a large hydrophobic side chain (**15**) or the presence of a hydrophilic one (**13** and **14**). The good inhibition seen for compound **12** versus human cathepsin D could be rationalized on the basis of the long, uncharged side chain at the P_2 finding an acceptable binding mode. The S_2 region of renin, being more crowded,[33] does not bind this derivative well into the active site.

The poor binding of **13** and **14** to human pepsin was more difficult to explain, since the pepsins have been shown to accept hydrophilic amino acids such as glutamic acid[37] or lysine[16] in the P_2 position of the substrate with excellent kinetic parameters. However, the work of Pohl and Dunn[16] on the closely related pig pepsin did establish that a positively charge side chain in the P_2 position was much less acceptable at lower pH. Since the values for human pepsin were obtained at pH 3.5, it seemed likely that the positive charge at P_2 of compounds **13** and **14** was not accommodated in the active site of pepsin at pH 3.5.

We were also interested in the relative inhibition of human cathepsin D versus human cathepsin E. These two enzymes not only have distinct amino acid sequences but also are targeted to different cellular compartments. Cathepsin D is targeted to the lysosomal compartment as a consequence of the presence of mannose 6-phosphate, which leads to binding to a lysosomal receptor.[38]

Human cathepsin E is definitely not lysosomal, but appears to be associated with the vesicular network of the cell cytoplasm.[39] It may play a role in the activation of

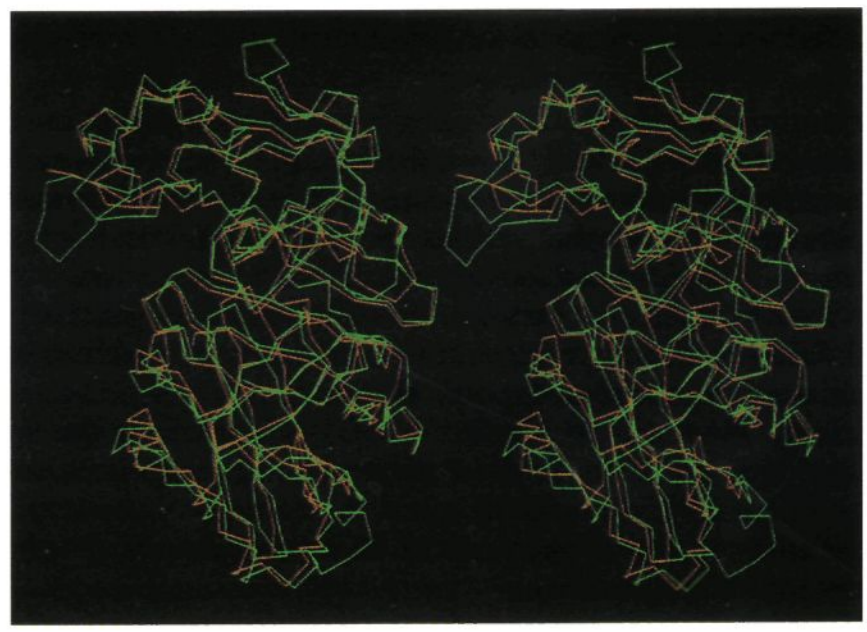


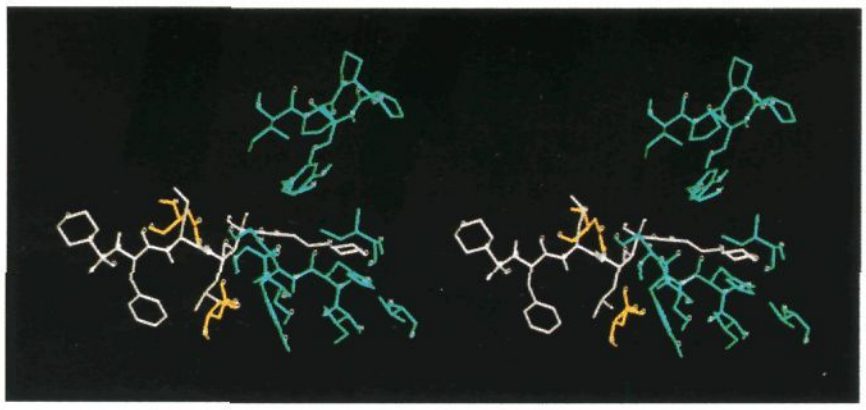
Figure 1. Stereoview of the overlap of the α carbon tracings for the cathepsin D model[34] (blue) and the mouse renin crystal structure[33] (yellow).

hormones such as endothelin and in antigen processing events.[11] Defining strong and selective inhibitors for both the cathepsins would contribute to the on-going studies of these processes.

Compounds **1–3** and **7** display the largest differences in binding to the two human cathepsins, with **1** and **7** binding more tightly to cathepsin D whereas **2** and **3** bind strongly to cathepsin E. An exact rationale for the observed differences must await the determination at high resolution of the human cathepsin D (Baldwin and Erickson, personal communication) and E structures.

It can be seen in Table I that all the compounds examined in this study are strong inhibitors of monkey renin. In sharp contrast, only five of the 15 compounds, **2–5** and **7**, show strong binding to human gastricsin. Because the structure of gastricsin has not been defined by crystallographic analysis at this point, it is difficult to rationalize the distinction seen here; however, it is anticipated that gastricsin will exhibit some unique features at the active site. We have previously reported[37] that gastricsin also exhibits weak interaction with peptide substrates that demonstrate excellent binding to pig pepsin and other members of the aspartic proteinase family. Due to the large amount of gastricsin in human stomach tissue, further study of the unusual properties of this enzyme are needed.

Molecular Modeling. To better understand the binding of the compounds reported in Table I and, specifically, the selectivity of **1** for cathepsin D and renin, we have examined, by molecular modeling, the active site regions of several of the enzyme under study. In particular, selected residues in the S_1', S_2', and S_3' subsites of cathepsin D were identified by studying a rule-based model of the enzyme[34] (Figure 1) and, for comparison, by referring to a description of these sites in the human and mouse renin crystal structures.[33] The variable C-terminal segments of the inhibitors in Table I could potentially interact with the S_1', S_2', and S_3' enzyme regions (Figure 2A). The amino acid sequences found in these binding sites were compared with the analogous residues in the other proteins listed in Figure 2B. [The sequence for monkey renin has not been reported. However, since it has been shown that the binding affinities for human renin parallel those determined for the monkey enzyme,[31] the sequence for human renin was considered in this evaluation.] Despite the overall high percentage of amino acid identity observed



Human Enzyme Residue	34	35	73	74	75	76	128	130	189	291	292	293	294	295
Cathepsin D	Gly.	Ser	Ile	His	Tyr	Gly	Ile	Val	Tyr	Ile	Pro	Pro	Pro	Ser
Cathepsin E	Gly.	Ser	Ile	Gln	Tyr	Gly	Leu	Val	Tyr	Ile	His	Pro	Pro	Ala
Renin	Gly.	Ser	Leu	Arg	Tyr	Ser	Gln	Ile	Val	Ile	Pro	Pro	Pro	Thr
Pepsin	Gly.	Ser	Ile	Thr	Tyr	Gly	Ile	Ser	Tyr	Leu	Pro	Thr	Glu	Ser
Gastricsin	Gly.	Ser	Leu	Gln	Tyr	Gly	Leu	Val	Tyr	Leu	Ser	Ser	Gln[]	Pro
													[Asn	Gly Gln]

Figure 2. (A, top) Stereoview of 1 (white) bound in the cathepsin D model. The residues in the "prime" binding sites of the enzyme are colored blue, while Asp32 and Asp 215 are in yellow. (B, bottom) The sequence alignment of residues in the "prime" binding sites for five aspartic proteinases. Above are shown 1 and the approximate locations of the cathepsin D residues (indicated by residue number) around the inhibitor as shown in A.

in comparing active sites at the C-terminal end of a bound inhibitor (the "prime" side of the cleft[1]), three out of four residues in cathepsin D that differ from cathepsin E are most closely related to the human renin sequence (Figure 2B). Two of these differing residues, Pro292 and Ser295, are situated in a rigid proline loop segment recently described for human and mouse renins.[33]

A further distinguishing feature is located at residue 74 in the "flap", a β-hairpin loop contributed by the N-terminal domain that partially occludes the active site cleft.[15] In cathepsin D, this is a His (Figure 2B) and the analogous residue in human renin is Arg. By contrast, in cathepsin E and gastricsin, Gln is found while human pepsin has a Thr. Thus, for both cathepsin D and renin, a basic residue occupies this site that is able to interact with parts of a bound inhibitor contributed by the "prime" or C-terminal half, while neutral, polar amino acids are present in the other enzymes.

Further analysis of the impact of the basic residues, in contrast to a polar group, at position 74 was carried out using the Grin/Grid software.[35] This program evaluates the interaction energies between selected probe groups (that is, water and a methyl group) and a target molecule. The energy contour was then created to localize the favorable interaction sites. The energies were calculated between an oxygen probe, to mimic the oxygen in a morpholine group, and the "flap" region of cathepsin D. The "flap" sequence was replaced with the cathepsin E sequence, and the calculation was repeated. These calculations resulted in the contours illustrated in Figure 3. The arrows indicate a large favorable interaction site for cathepsin D in the "prime" area of the enzyme active site

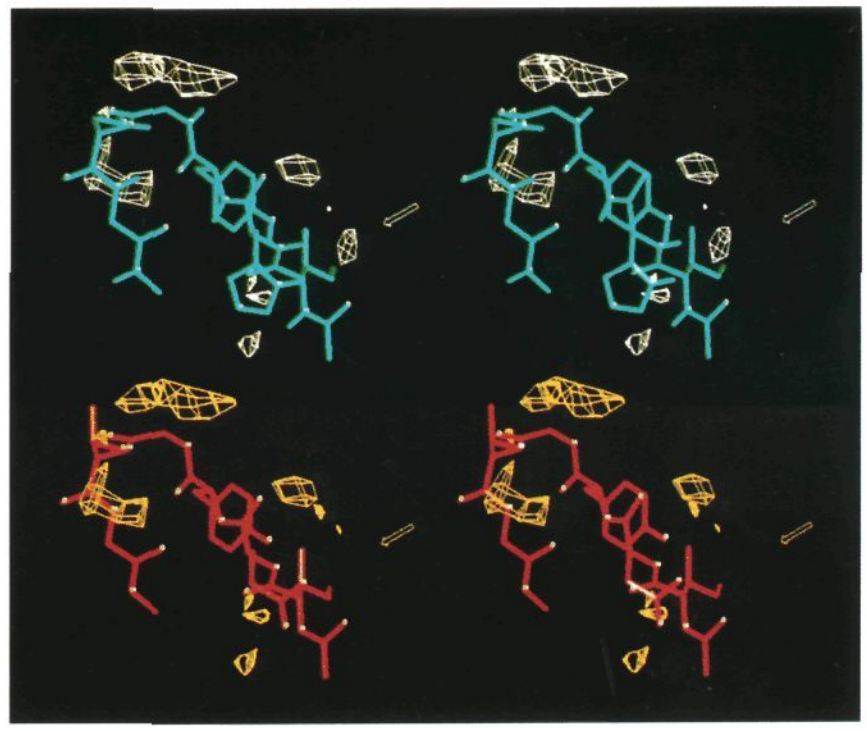


Figure 3. Stereoviews of the energy plots generated by the Grin/Grid program for the cathepsin D model "flap" region (blue) and the "flap" segment substituted with the residue sequence for cathepsin E (red). The contours represent a −2 kcal/mol level, using an ether oxygen probe. The arrows indicate the favorable interaction site in cathepsin D that is not found in cathepsin E.

that does not exist with cathepsin E. This may, in part, explain the selectivity for 1 observed for cathepsin D, and also renin, with a basic Arg residue in position 74.

In the rigid loop segment, the analogous residues to Pro292 and Ser295 in cathepsin D are Pro and Thr, respectively, in renin. In cathepsin E these sites are identified as His and Ala (Figure 2B). In extending the analysis to the other enzymes, it was apparent that although all proteins have at least one proline in this loop region, only human renin and cathepsin D contain three sequential prolines. Cathepsin E was the only enzyme in the series with two proline residues in the loop, and although it was over 20-fold less selective for 1 than renin and cathepsin D, cathepsin E was at least 60-fold more selective for this analogy relative to gastricsin and human pepsin. The prolines generate a more rigid loop in cathepsin D, human renin, and, to a lesser extent, cathepsin E relative to the analogous sites in the other enzymes. Furthermore, the proline amide bonds can exist in the cis configuration, which would significantly impact the secondary structure at this site and in turn the interaction with the inhibitor. In the crystal structures of the human and mouse renins, the third proline amide bond of the proline tripeptide segment was indeed found to exist in a cis orientation.[33]

The crystal structures of a difluorostatone- and a difluorostatine-containing peptide complexed with penicillopepsin provide new insight regarding the binding of the difluoro analogs.[36] Upon superimposting of the two enzyme crystal structures, the regions of the inhibitors that differed in a significant manner were the $StaF_2NHCH_3$ and the $StoF_2NHCH_3$ segments. The presence of the (*R*)-3-hydroxyl group caused the entire $StoF_2$ residue to be positioned further away from the catalytic Asps than the $StaF_2$. These different binding modes, along with the homology between renin and human cathepsin D in the "prime" binding regions described above, could explain the selectivity of the alcohol with a AEM at P_2' for monkey renin and human cathepsin D. Although it is yet unclear how these features impact one another, this analysis pinpoints key sites to be explored in designing

inhibitors with selectivity among the aspartic proteinases, in particular between cathepsin D and cathepsin E. Further evidence of the impact of the "prime" sites on binding affinities was the slightly increased selectivity for cathepsin D versus cathepsin E observed in going from a 3,4- to 3,5-diol substitution at the P_1-P_1' site (**5** vs **7**) and the drop in binding potency for gastricsin upon the addition of a bulky 1,3-dithiane group (**6** vs **7**). In the latter case, this phenomenon could be explained by the extended proline loop region present in gastricsin, restricting the size of the binding pocket in the "prime" region (Figure 2). However, this rationale does not hold for the loss in gastricsin binding observed for **8** and **9** containing *R*- or *S*-ACDH relative to **7** having a 3,5-ADCMH group.

The data presented in Table I and the preceding discussion underscore the distinctions that are readily apparent in the active site cleft of each of these structurally and mechanistically related enzymes. These data also provide leads for the further analyses of each member of the aspartic proteinase family with the aim of exploiting the structural distinctions within the enzyme active sites for the purpose of targeted inhibitor design.

Experimental Section

Molecular Modeling. The molecular modeling analysis was carried out using the Sybyl Software package[40] on a Silicon Graphics 4D/35 computer. Analog 1 was docked in the cathepsin D model following the conserved binding mode and interactions observed in aspartic proteinase crystal structures.[27] Energy optimizations of 1 in the enzyme cleft (composed of residues within an approximate 8-Å sphere around the ligand) were carried out using Maxmin. During the minimization, the enzyme residues were aggregated as were three atoms of the P_1 moiety found at the catalytic site. The aspartic proteinase sequences were aligned according to Blundell et al.[41] Grin/Grid probe calculations were run on the cathepsin D loop sequence Ile73-Gly78 capped with the CCO of Asp72 and the NC of Ser79. The sequence was then replaced with that of cathepsin E using the biopolymer-change command with Sybyl, and the Grin/Grid process was repeated. The energy plots shown in Figure 3 were contoured at −2 kcal/mol levels for the two loop regions.

Substrates. Series of substrates were synthesized by Ruth Davenport of the Protein Chemistry Core Facility (University of Florida) using the solid-phase method on an Applied Biosystems Model 430A Synthesizer. The substrates used in the inhibition assays were based on the parent peptide Lys-Pro-Ala-Lys-Phe*Nph-Arg-Leu.

Stock peptide solutions were made in distilled water and were quantified by amino acid analyses on a Beckman 6300 amino acid analyzer. The purity of the peptides (≥90%) was verified by HPLC analyses.

Enzymes. Human pepsin was supplied by Dr. A. P. Ryle of the University of Edinburgh Medical School, Edinburgh, Scotland, U.K. Prof. Jordan Tang of the Oklahoma Medical Research Foundation provided the human gastricsin. Human cathepsin D was prepared from human spleen as described by Afting and Becker.[42] Human cathepsin E was generously given by Dr. I. M. Samloff of the Veteran's Administration Medical Center, Sepulveda, CA. Monkey plasma was used as the source of primate renin activity.

Assays. The hydrolyses of the substrates were monitored at 37 °C, in a 0.1 M sodium formate buffer by averaging the decrease in absorbance over a range of 234–324 nm using a Hewlett-Packard diode-array spectrophotometer. The pH for the assays were as follows: human pepsin, pH = 3.5; human gastricsin, pH = 3.1; human cathepsin D, pH = 3.7; human cathepsin E, pH = 4.5. The K_m and V_{max} values were measured using the initial rate at six different substrate concentrations. For human pepsin a K_m of 95 μM and a V_{max} of 380 × 10^{-6} AU/s at an enzyme concentration of 2.3 nM were obtained. For human gastricsin the corresponding values were 300 μM and 145 × 10^{-6} AU/s at an enzyme concentration of 14 nM. For human cathepsin D, the values were 60 μM and 100 × 10^{-6} AU/s at an enzyme concentration of 3 nM, while for human cathepsin E, the values were 165 μM and 100 × 10^{-6} AU/s at an enzyme concentration of 8 nM. The cleavage of the peptide was monitored on a HPLC column followed by amino acid analysis of the respective peaks to ensure that site of cleavage was at the Phe*Nph bond. Inhibition of monkey renin at pH 6.0 was carried out as described.[31]

Active Concentration. The active concentrations of human pepsin, human cathepsin D, human cathepsin E, and human gastricsin were determined by titration against an Upjohn Pharmaceutical Co. inhibitor, U85548E, kindly provided by Dr. Tomi Sawyer.

Inhibition Constants. The K_i values were determined by quantitating the competitive inhibition of the hydrolyses of the chromogenic substrates. The peptide used to assay human pepsin and human cathepsin D was Lys-Pro-Ile-Glu-Phe*Nph-Arg-Leu, whereas Lys-Pro-Ala-Ala-Phe*Nph-Arg-Leu and Pro-Pro-Thr-Ile-Phe*Nph-Arg-Leu were used to assay human gastricsin and human cathepsin E, respectively. K_i values were determined with an error range of ±15%, as determined by propagation of errors using procedures outlined by Meyer.[43]

Inhibitor Synthesis. Melting points were determined with a Thomas-Hoover capillary melting point apparatus, which was calibrated against known standards. NMR spectra were run on a Varian XL 200, Bruker AM 250, or IBM WP 100SY spectrometer using Me_4Si as an internal standard. Mass spectra were run on a Finnigan TSQ-70 or a VG Analytical 7070E/HF spectrometer. Elemental analyses of target compounds check to within 0.4% of the calculated values except where indicated otherwise. The preparation of **1**–**4**[31] and **14**[44] have been reported previously.

[1*S*-(1*R,2*R**,3*S**)]-[*N*-(4-Morpholinylsulfonyl)-L-phenylalanyl]-*N*-[1-(cyclohexylmethyl)-2,3-dihydroxy-5-methylhexyl]-4,5-didehydro-L-norvalinamide (5).** This compound was prepared from (morpholinylsulfonyl)-L-phenylalanine (SMO-Phe)[45] and *N*-[1-(cyclohexylmethyl)-2,3-dihydroxy-5-methylhexyl]-4,5-didehydro-L-norvalinamide (α-allylglycinyl-ACDMH) by the procedure described for **6**: NMR ($CDCl_3$) δ 0.090 (t, 6H), 1.05–1.50 (m, 6H), 1.50–1.80 (m, 8H), 1.80–2.05 (m, 1H), 2.50–2.85 (m, 4H), 2.85–3.05 (m, 3H), 3.05–3.65 (m, 8H), 3.95 (q, 1H), 4.20–4.45 (m, 2H), 4.55 (q, 1H), 4.90 (d, 1H), 5.10–5.25 (m, 2H), 5.60–5.85 (m, 1H), 6.70 (d, 1H), 6.90 (d, 1H), 7.20–7.45 (m6, H); MS (FAB) *m/z* 637.2 (M + 1). Anal. Calcd for $C_{32}H_{52}N_4O_7S \cdot 0.13CHCl_3$: C, H, N.

[1*S*-(1*R,2*R**,4*S**)]-[*N*-(4-Morpholinylsulfonyl)-L-phenylalanyl]-*N*-[1-(cyclohexylmethyl)-4-(1,3-dithian-2-yl)-2,4-dihydroxybutyl]-4,5-didehydro-L-norvalinamide (6).** A solution of 0.100 g (0.24 mmol) of *N*-[1-(cyclohexylmethyl)-4-(1,3-dithian-2-yl)-2,4-dihydroxybutyl]-4,5-didehydro-L-norvalinamide (allylglycinyl-ACDPD[46]) in 5 mL of CH_2Cl_2 was treated successively with 0.075 g (0.24 mmol) of (morpholinylsulfonyl)-L-phenylalanine (SMO-Phe),[45] 0.036 g (0.24 mmol) of 1-hydroxybenzotriazole hydrate (HOBT), and 0.050 g (0.24 mmol) of 1,3-dicyclohexylcarbodiimide (DCC). The resulting solution was allowed to stand at room temperature overnight. The precipitate was filtered and the filtrate was washed successively with 5 mL of saturated $NaHCO_3$, 5 mL of water, and 5 mL of 10% citric acid solution. Chromatography on silica gel eluting with $CHCl_3$ to $CHCl_3$/EtOAc (50/50) gave a single isomer as an amorphous solid: wt 0.140 g (82%); NMR ($CDCl_3$) δ 0.70–1.05 (m, 2H), 1.05–1.55 (m, 7H), 1.55–2.15 (m, 9H), 2.50–3.00 (m, 11H), 3.15 (q, 1H), 3.30–3.50 (m, 4H), 3.70–3.90 (m, 2H), 3.90–4.15 (m, 4H), 4.45 (q, 1H), 5.10–5.25 (m, 2H), 5.70–5.90 (m, 1H), 6.85 (d, 1H), 7.20 (d, 1H), 7.25–7.45 (m, 6H), 7.60 (d, 1H); MS (FAB) *m/z* 714 (M + 1). Anal. Calcd for $C_{33}H_{52}N_4O_7S_3$: C, H, N.

[1*S*-(1*R,2*R**,4*S**)]-[*N*-(4-Morpholinylsulfonyl)-L-phenylalanyl]-*N*-[1-(cyclohexylmethyl)-2,4-dihydroxy-5-methylhexyl]-4,5-didehydro-L-norvalinamide (7).** This compound was prepared by a DCC coupling of SMO-Phe and allylglycinyl-3,5-ACDMH[46] by a procedure similar to that for compound **6**: 42% yield of a single isomer; NMR ($CDCl_3$) δ 0.75–0.95 (m, 8H), 1.05–1.80 (m, 14H), 2.50–2.75 (m, 4H), 2.75–3.00 (m, 4H), 3.20–3.40 (m, 2H), 3.40–3.65 (m, 5H), 3.80 (br. s, 1H), 3.90–4.10 (m, 2H), 4.55 (q, 1H), 5.10–5.25 (m, 3H), 5.60–5.80 (m, 1H), 6.75 (d, 1H), 6.95 (d, 1H), 7.20–7.40 (m, 5H); MS (EI) *m/z* 637 (M). Anal. Calcd for $C_{32}H_{52}N_4O_7S$: C, H, N.

[1*S*-(1*R,2*R**,4*S**)]-[*N*-(4-Morpholinylsulfonyl)-L-phenylalanyl]-*N*-[1-(cyclohexylmethyl)-2,4-dihydroxyhexyl]-4,5-didehydro-L-norvalinamide (8).** This compound was prepared by a coupling of SMO-Phe and allylglycinyl-*R*-ACDH[46,47] by the procedure for 6: NMR ($CDCl_3$) δ 0.70–1.00 (m, 5H), 1.00–1.20 (m, 5H), 1.40–1.85 (m, 10H), 2.50–2.90 (m, 5H), 2.90–3.00 (m, 2H), 3.15–3.35 (m, 1H), 3.40–3.60 (m, 4H), 3.70–3.90 (m, 2H), 3.90–4.10 (m, 2H), 4.45–4.55 (q, 1H), 4.90 (d, 1H), 5.05–5.25 (m, 2H), 5.60–5.80 (m, 1H), 6.65 (d, 1H), 6.90 (d, 1H), 7.20–7.45 (m, 7H); MS (FAB) *m/z* 623 (m). Anal. Calcd for $C_{31}H_{50}N_4O_7S$: C, H, N.

[1*S*-(1*R,2*R**,4*R**)]-[*N*-(4-Morpholinylsulfonyl)-L-phenylalanyl]-*N*-[1-(cyclohexylmethyl)-2,4-dihydroxyhexyl]-4,5-didehydro-L-norvalinamide (9).** This compound was prepared by a coupling of SMO-Phe and allylglycinyl-*S*-ACDH[46,47] by the procedure for 6. The pure product was obtained by recrystallization from ethyl acetate/pentane: 42% yield; mp 160–162 °C; NMR ($CDCl_3$) δ 0.80–1.05 (m, 5H), 1.05–1.32 (m, 4H), 1.35–1.85 (m, 11H), 2.45–2.75 (m, 4H), 2.75–2.95 (m, 3H), 3.20 (q, 1H), 3.40–3.55 (m, 4H), 3.70–4.05 (m, 4H), 4.70 (q, 1H), 5.15 (d, 1H), 5.50 (d, 1H), 5.65–5.85 (m, 1H), 6.88 (d, 1H), 7.05 (d, 1H), 7.20–7.40 (m, 7H); MS (FAB) *m/z* 624 (M + 1). Anal. Calcd for $C_{31}H_{50}N_4O_7S$: C, H, N.

[1*S*-[1*R[*RS*)*R**)],2*R**,4*R**]]-*N*-[2-[[1-(Cyclohexylmethyl)-2,4-dihydroxyhexyl]amino]-2-oxo-1-(2-propenylthio)ethyl-α-[(4-morpholinylsulfonyl)amino]benzenepropanamide (10).** This compound was prepared by coupling SMO-Phe-(allylthio)-glycine[45] and *S*-ACDH[47] by the procedure for compound 6: NMR ($CDCl_3$) δ 0.75–1.05 (m, 4H), 1.05–1.90 (m, 14H), 2.60–3.30 (m, 10H), 3.30–3.65 (m, 6H), 3.65–4.10 (m, 4H), 5.05–5.50 (m, 2H), 5.50–5.70 (m, 1H), 5.70–6.00 (m, 1H), 6.85 (q, 1H), 7.15–7.50 (m, 7H); MS (FAB) *m/z* 655.3 (M); HPLC, 90%. Anal. Calcd for $C_{31}H_{50}N_4O_7S_2$: C, 60.11; H, 8.09; N, 9.00. Found: C, 56.34; H, 7.31; N, 8.06.

Methyl [1*S*-(1*R,2*R**,4*S**)]-[*N*-(4-Morpholinylsulfonyl)-L-phenylalanyl]-*N*-[1-(cyclohexylmethyl)-2,4-dihydroxyhexyl]-L-α-aspartamide (11).** This compound was prepared by coupling SMO-Phe and Asp(OME)-*R*-ACDH by the procedure for compound 6: 45% yield; NMR ($CDCl_3$ + DMSO-d_6) δ 0.50–0.80 (m, 4H), 0.80–1.50 (m, 14H), 1.50–1.65 (m, 2H), 2.45–2.65 (m, 4H), 2.70–2.95 (m, 2H), 3.05–3.25 (m, 4H), 3.30–3.60 (m, 7H), 3.60–3.80 (m, 2H), 4.50–4.65 (m, 1H), 6.75 (d, 1H), 6.95–7.15 (m, 6H), 7.20–7.30 (m, 2H), 7.82 (d, 1H); MS (FAB) *m/z* 655 (M). Anal. Calcd for $C_{31}H_{50}N_4O_9S$: C, H, N.

The Asp(OMe)-*R*-ACDH intermediate in the above preparation of 11 was synthesized by coupling Boc-Asp(β-OMe) (Sigma) and *R*-ACDH by the procedure for compound 6: TLC (EtOAc) one spot, R_f 0.6 (ninhydrin); MS (EI) 459 (M + 1). Deblocking with MeOH/CH_2Cl_2/HCl gave Asp(OMe)-*R*-ACDH, which was used directly in the above preparation of compound 11.

[1*S*-[1*R[*R**(*R**)],2*R**,4*S**]]-[*N*-(4-Morpholinylsulfonyl)-L-phenylalanyl]-*N*-[1-(cyclohexylmethyl)-2,4-dihydroxyhexyl]-*N*^{6}-[(methylamino)thioxomethyl]-L-lysinamide (12).** This compound was prepared by coupling SMO-Phe-Lys[C(S)-NHMe][46] and *S*-ACDH by the procedure for compound 6 except that DMF was used as a solvent. The product was purified by silica gel chromatography eluting with 2–10% MeOH/$CHCl_3$: 59% yield; NMR (DMSO-d_6) δ 0.65–1.00 (m, 4H), 1.00–1.90 (m, 20H), 2.55–3.00 (m, 8H), 3.20–3.55 (m, 10H), 3.55–3.70 (m, 1H), 3.80–4.10 (m, 2H), 4.30 (q, 1H), 4.45 (d, 1H), 4.65 (d, 1H), 7.15–7.50 (m, 8H), 7.75 (d, 1H), 8.30 (d, 1H); MS (FAB) *m/z* 727 (M). Anal. Calcd for $C_{34}H_{58}N_6O_7S_2$: C, H, N, S.

[1*S*-(1*R,2*R**,4*S**)]-[*N*-(4-Morpholinylsulfonyl)-L-phenylalanyl]-*N*-[1-(cyclohexylmethyl)-2,4-dihydroxyhexyl]-L-histidinamide (13).** This compound was prepared by coupling SMO-Phe and (Trt)His-*R*-ACDH[46] by the procedure for compound 6. This intermediate [MS (FAB) 905.3 (M + 1)] was deprotected by treating it with 20% aqueous acetic acid at 95 °C for ca. 5 min. Ph_3COH was precipitated by the addition of 2 volumes of water and filtered. The filtrate was concentrated at reduced pressure and the residue was extracted into CH_2Cl_2. The extract was washed with saturated $NaHCO_3$, dried ($NaSO_4$), and concentrated to give pure product as a solid foam: NMR ($CDCl_3$) δ 0.65–0.95 (m, 5H), 0.95–1.80 (m, 18H), 2.45–2.65 (m, 2H), 2.65–2.90 (m, 3H), 3.00–3.55 (m, 7H), 3.70–4.05 (m, 4H), 4.70 (s, 1H), 6.85 (s, 1H), 7.00 (d, 1H), 7.30 (m, 6H), 7.55 (s, 1H), 8.45 (br s, 1H); MS (FAB) *m/z* 663.4 (M + 1). Anal. Calcd for $C_{32}H_{50}N_6O_7S \cdot 0.5CH_2Cl_2$: C, H, N.

[1*S*-(1*R,2*R**,4*S**)]-[*N*-(4-Morpholinylsulfonyl)-L-phenylalanyl]-*N*-[1-(cyclohexylmethyl)-2,4-dihydroxyhexyl]glycinamide (15).** This compound was prepared by coupling SMO-Phe-Gly[46] and *R*-ACDH by the procedure of compound 6; 47% yield; NMR ($CDCl_3$) δ 0.75–1.05 (m, 4H), 1.05–2.00 (m, 14H), 2.55–2.75 (m, 2H), 2.75–2.95 (m, 3H), 3.20 (q, 1H), 3.30–3.55 (m, 4H), 3.65–3.90 (m, 3H), 3.95–4.15 (m, 4H), 5.90 (m, 1H), 6.80 (d, 1H), 7.20–7.40 (m, 7H), 7.60 (m, 1H); MS (FAB) *m/z* 583 (M). Anal. Calcd for $C_{28}H_{46}N_4O_7S$: C, H, N.

Acknowledgment. The authors wish to acknowledge the contributions of A. Doherty, J. Hamby, I. Sircar, B. Steinbaugh, and H. Hamilton, for the preparation of some of the compounds used in this study, and M. Reily, for assistance in assigning the stereochemistry of ACDH. We also thank Ruth Davenport (Protein Chemistry Core Facility, University of Florida) for the synthesis of substrate peptides. We also acknowledge the gift of enzymes from several colleagues. This work was supported by NIH Grant DK18865.

References

(1) Designation of amino acid residues of a substrate, P_n·····P_2-P_1*P_1'-P_2'·····P_n', where * indicates the scissile peptide bond, and the corresponding regions of the enzyme which interact with those substrate residues, S_n·····S_2-S_1*S_1'-S_2'·····S_n', is according to Schechter and Berger (Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157). The extended active site cleft is then divided into two regions, one that corresponds to the "non-prime" or N-terminal half and one that corresponds to the "prime" or C-terminal half of a substrate or inhibitor.

(2) Kay, J.; Jupp, R. A.; Norey, C. G.; Richards, A. D.; Reid, W. A.; Taggart, R. T.; Samloff, I. M.; Dunn, B. M. Aspartic Proteinases and Inhibitors for Their Control in Health and Disease. In *Proteinases: Potential Role In Health & Disease*; Horl, W. H., Heidland, A., Eds.; Plenum Press: New York, 1988; pp 1–11.

(3) Kay, J.; Jupp, R. A.; Richards, A. D.; Thomas, D. J.; Yamamoto, K.; Reid, W. A.; Dunn, B. M.; Samloff, I. M. The Aspartic Proteinases, Cathepsin E and Cathepsin D in Relation to Protein Degradation within Cells. In *Intracellular Protein Catabolism*; Katunuma, N., Kominami, E., Eds.; Springer-Verlag: 1989; pp 155–162.

(4) Kay, J. Aspartic Proteinases and Their Inhibitors. In *Aspartic Proteinases and Their Inhibitors*; Kostka, V., Ed.; Walter de Gryuter & Co.: Berlin, New York, 1985; pp 1–14.

(5) Tanaka, Y.; Mine, K.; Nakai, Y.; Mishima, N.; Nakagawa, T. Serum Pepsinogen I Concentrations in Peptic Ulcer patients in Relation to Ulcer Location and Stage. *Gut* **1991**, *32*, 849–852.

(6) Pillai, S.; Zull, J. E. Production of Biologically Active Fragments of Parathyroid Hormone by Isolated Kupffer Cells. *J. Biol. Chem.* **1988**, *261*, 14919–14923.

(7) Rochefort, H. Cathepsin D in Breast Cancer. *Breast Cancer Res. Treat.* **1990**, *16*, 1–13.

(8) Sakai, H.; Saku, T.; Kato, Y.; Yamamoto, K. Quantification and Immunohistochemical Localization of Cathepsins E and D in Rat Tissues and Blood Cells. *Biochem. Biophys. Acta* **1989**, *991*, 367–375.

(9) Helseth, D. L. Jr.; Veis, A. Cathepsin D Mediated Processing of Procollagen: Lysosomal Involvement in Secretory Processing of Procollagen. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3302–3306.

(10) van Noort, J. M.; van der Drift, A. C. M. The Selectivity of Cathepsin D Suggests an Involvement of the Enzyme in the Generation of T-cell Epitopes. *J. Biol. Chem.* **1989**, *264*, 14159–14164.

(11) Lees, W. E.; Kalinka, S.; Meech, J.; Capper, S. J.; Cook, N. D.; Kay, J. Generation of Human Endothelin by Cathepsin E. *FEBS Lett.* **1990**, *273*, 99–102.

(12) Ritonja, A.; Krizaj, I.; Mesko, P.; Kopitar, M.; Lucovnik, P.; Strukelj, B.; Pungercar, J.; Buttle, D. J.; Barrett, A. J.; Turk, V. The Amino Acid Sequence from a Novel Inhibitor of Cathepsin D from Potato. *FEBS Lett.* **1990**, *267*, 13–15.

(13) Martzen, M. R.; McMullen, B. A.; Smith, N. E.; Fujikawa, K.; Peansky, R. J. Primary Structure of the Major Pepsin Inhibitor from the Intestinal Parasitic Nematode Ascaris suum. *Biochemistry* **1990**, *29*, 7366–7372.

(14) Greenlee, W. J. Renin Inhibitors. *Med. Res. Rev.* **1990**, *10*, 173–236.

(15) Davies, D. R. The Structure and Function of Aspartic Proteinases. *Annu. Rev. Biophys. Biophys. Chem.* **1990**, *19*, 189–215.

(16) Pohl, J.; Dunn, B. M. Secondary Enzyme-Substrate Interactions: Kinetic Evidence for Ionic Interactions Between Substrate Side Chains and the Pepsin Active Site. *Biochemistry* **1988**, *27*, 4827–4834.

(17) Rao, C.; Scarborough, P. E.; Lowther, W. T.; Kay, J.; Batley, B.; Rapundalo, S.; Klutchko, S.; Taylor, M. D.; Dunn, B. M. Structure-Function Database for Active Site Binding to the Aspartic Proteinases. In *Structure and Function of the Aspartic Proteinases*; Dunn, B. M., Ed.; Plenum Press: New York, 1991; pp 143–147.

(18) Lowther, W. T.; Chen, Z.; Lin, X.; Tang, J.; Dunn, B. M. Substrate Specificity Study of Recombinant Rhizopus chinensis Aspartic Proteinase. In *Structure and Function of the Aspartic Proteinases*; Dunn, B. M., Ed.; Plenum Press: New York, 1991; pp 275–279.

(19) Scarborough, P. E.; Richo, G. R.; Kay, J.; Conner, G. E.; Dunn, B. M. Comparison of Kinetic Properties of Native and Recombinant Human Cathepsin D. In *Structure and Function of the Aspartic Proteinases*; Dunn, B. M., Ed.; Plenum Press: New York, 1991; pp 343–347.

(20) Dunn, B. M.; Valler, M. J.; Rolph, C. E.; Foundling, S. I.; Jimenez, M.; Kay, J. The pH Dependence of the Hydrolysis of Chromogenic Substrates of the Type, Lys-Pro-Xaa-Yaa-Phe-(NO_2)Phe-Arg-Leu, by Selected Aspartic Proteinases: Evidence for Specific Interactions in Subsites S_3 and S_2. *Biochem. Biophys. Acta* **1987**, *913*, 122–130.

(21) Kay, J.; Dunn, B. M. Substrate Specificity and Inhibitors of Aspartic Proteinases. *Scand J. Clin. Lab. Invest.* **1992**, *52*, 1–8.

(22) Fruton, J. S. The Specificity and Mechanism of Pepsin Action. *Adv. Enzymol.* **1970**, *33*, 401–443.

(23) Voynick, I. M.; Fruton, J. S. The Comparative Specificity of Acid Proteinases. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 257–259.

(24) Wolfenden, R. Transition State Analog Inhibitors and Enzyme Catalysis. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271–306.

(25) Veerapandian, B.; Cooper, J. B.; Sali, A.; Blundell, T. L.; Rosati, R. L.; Dominy, B. W.; Darmin, D. B.; Hoover, D. J. Direct Observation by X-ray Analysis of the Tetrahedral "Intermediate" of Aspartic Proteinases. *Protein Sci.* **1992**, *1*, 322–328.

(26) Abad-Zapatero, C.; Rydel, T. J.; Neidhart, D. J.; Luly, J.; Erickson, J. W. Inhibitor Binding Induces Structural Changes in Porcine Pepsin. In *Structure and Function of the Aspartic Proteinases*; Dunn, B. M.; Ed.; Plenum Press: New York, 1991; pp 9–21.

(27) Fraser, M. E.; Strynadka, N. C. J.; Bartlett, P. A.; Harrison, J. E.; James, M. N. G. Crystallographic Analyses of Transision-State Mimics Bound to Pencilliopepsin-Phosphorus-Containing Peptide Analogues. *Biochemistry* **1992**, *31*, 5201–5214.

(28) Blundell, T. L.; Cooper, J. B.; Foundling, S. I.; Jones, D. M.; Atrash, B.; Szelke, M. On the Rational Design of Renin Inhibitors: X-ray Studies of Aspartic Proteinases Complexed with Transition State Analogues. *Biochemistry* **1987**, *26*, 5585–5589.

(29) Sawyer, T. K.; Hester, J. B.; Schostarez, H. J.; Thaisrivongs, S.; Bundy, G. L.; Liu, L.; Bradford, V. S.; DeVaux, A. E.; Staples, D. J.; Maggiora, L. L.; TenBrink, R. E.; Kinner, J. H.; Smith, C. W.; Pals, D. T.; Couch, S. J.; Hinzmann, J. S.; Poorman, R. A.; Einspahr, H. M.; Finzel, B. C.; Watenpaugh, K. D.; Mao, B.; Epps, D. E.; Kezdy, F. J.; Heinrikson, R. L. Exploiting the Molecular Template of Angiotensinogen in the Discovery and Design of Peptidyl and Pseudopeptidyl and Peptidomimetic Inhibitors of Human Renin: A Structure-Activity Perspective. In *Structure and Function of the Aspartic Proteinases*; Dunn, B. M., Ed.; Plenum Press: New York, 1991; pp 325–334.

(30) Hodges, J. C.; Connolly, C. J.; Kornberg, B. E.; Repine, J. T.; Klinkefus, B. A.; Batley, B. L.; Ryan, M. J. American Chemical Society 199th National Meeting, 1990, Boston, MEDI 129. Batley, B. L. unpublished results.

(31) Doherty, A. M.; Sircar, I.; Kornberg, B. E.; Quin, J., III; Winter, R. T.; Kaltenbronn, J. S.; Taylor, M. D.; Batley, B. L.; Rapundalo, S. R.; Ryan, M. J.; Painchaud, C. A. Design and Synthesis of Potent, Selective, and Orally Active Fluorine Containing Renin Inhibitors. *J. Med. Chem.* **1992**, *35*, 2–14.

(32) Jupp, R. A.; Dunn, B. M.; Jacobs, J. W.; Vlasuk, G.; Arcuri, K. E.; Veber, D. F.; Perlow, D. S.; Payne, L. S.; Boger, J.; Greenlee, J. W.; De Laszlo, S.; Chakravarty, P. K.; Ten Broeke, J.; Hangauer, D. G.; Ondeyka, D.; Kay, J. The Selectivity of Statine-based Inhibitors Against Various Human Aspartic Proteinases. *Biochem. J.* **1990**, *265*, 871–878.

(33) Dhanaraj, V.; Dealwis, C. G.; Frazao, C.; Badasso, M.; Sibanda, B. L.; Tickle, I. J.; Cooper, J. B.; Driessen, H. P. C.; Newman, M.; Aguilar, C.; Wood, S. P.; Blundell, T. L.; Hobart, P. M.; Geoghegan, K. F.; Ammirati, M. J.; Danley, D. E.; O'Conner, B. A.; Hoover, D. J. X-ray Analyses of Peptide-Inhibitor Complexes Define the Structural Basis of Specificity for Human and Mouse Renins. *Nature* **1992**, *357*, 466–472.

(34) Scarborough, P. E.; Guruprasad, K.; Topham, C.; Richo, G. R.; Conner, G. E.; Blundell, T. L.; Dunn, B. M. Exploration of Subsite Binding Specificity of Human Cathepsin D Through Kinetics and Rule-based Molecular Modelling. *Protein Sci.* **1993**, *2*, 264–276.

(35) Grin/Grid Software Program, Molecular Discovery Ltd., West Way House, Elms Parade, Oxford, OX2 9LL, England.

(36) James, M. N. G.; Sielecki, A.; Hayakawa, K.; Gelb, M. H. Crystallographic Analysis of Transition State Mimics Bound to Penicillopepsin: Difluorostatine- and Difluorostatone-Containing Peptides. *Biochem. J.* **1992**, *31*, 3872–3886.

(37) Dunn, B. M.; Jimenez, M.; Parten, B. F.; Valler, M. J.; Rolph, C. E.; Kay, J. A Systematic Series of Synthetic Chromophoric Substrates for Aspartic Proteinases. *Biochem. J.* **1986**, *237*, 899–906.

(38) Takahashi, T.; Schmidt, P. G.; Tang, J. Oligosaccharide Units of Lysosomal Cathepsin D from Porcine Spleen: Amino Acid Sequence and Carbohydrate Structure of the Glycopeptides. *J. Biol. Chem.* **1983**, *258*, 2819–2830.

(39) Sakai, H.; Saku, T.; Kato, Y.; Yamamoto, K. Quantitation and Immunohistochemical Localization of Cathepsin E and D in Rat Tissue and Blood Cells. *Biochim. Biophys. Acta* **1989**, *991*, 367–375.

(40) Sybyl Software Package, Tripos Associates Inc., A subsidiary of Evans and Sutherland, 1699 S. Hanley Road, Suite 303, St. Louis, MO 63144.

(41) Blundell, T. L.; Jenkins, J. A.; Sewell, B. T.; Pearl, L. H.; Cooper, J. B.; Tickle, I. J.; Veerapandian, B.; Wood, S. P., X-ray Analyses of Aspartic Proteinases, *J. Mol. Biol.* **1990**, *211*, 919–941.

(42) Afting, E. G.; Becker, M. L. Two-Step Affinity-Chromatographic Purification of Cathepsin D from Pig Myometrium with High Yield. *Biochem. J.* **1981**, *197*, 519–522.

(43) Meyer, S. L. *Data Analysis for Scientists and Engineers*; John Wiley and Sons, Inc.: New York, 1975.

(44) Patt, W. C.; Hamilton, H. W.; Taylor, M. D.; Ryan, M. J.; Taylor, D. G., Jr.; Connolly, C. J. C.; Doherty, A. M.; Klutchko, S. R.; Sircar, I.; Steinbaugh, B. A.; Batley, B. L.; Painchaud, C. A.; Rapundalo, S. T.; Michniewicz, B. M.; Olson, S. C. Structure-Activity Relationships of a Series of 2-Amino-4-thiazole-Containing Renin Inhibitors, *J. Med. Chem.* **1992**, *35*, 2562–2572.

(45) Repine, T. J.; Katlenbronn, J. S.; Doherty, A. M.; Hamby, J. M.; Himmelsbach, R. J.; Kornberg, B. E.; Taylor, M. D.; Lunney, E. A.; Humblet, C.; Rapundalo, S. R.; Batley, B. L.; Ryan, M. J.; Painchaud, C. A. Renin Inhibitors Containing α-Heteroatom Amino Acids as P_2 Residues, *J. Med. Chem.* **1992**, *35*, 1032–1042.

(46) Hamby, J. M.; Hodges, J. C.; Klutchko, S. R. 1990, WO Patent Application 90/07, 521.

(47) Klutchko, S.; Hamby, J. M.; Reily, M.; Taylor, M. D.; Hodges, J. C. Synthesis of Amino 1,3-diols. Ring Opening of N-acyl Activated Lactams with Carbon Nucleophiles. *Synth. Commun.* In press.