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Inhibitors of human heart chymase based on a peptide library

(chymotrypsin/keto-amide/protease)

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ABSTRACT **We have synthesized two sets of noncleavable peptide-inhibitor libraries to map the S and S′ subsites of human heart chymase. Human heart chymase is a chymotrypsin-like enzyme that converts angiotensin I to angiotensin II. The first library consists of peptides with 3-fluorobenzylpyruvamides in the P_1 position. (Amino acid residues of substrates numbered P_1, P_2, etc., are toward the N-terminal direction, and P'_1, P'_2, etc., are toward the C-terminal direction from the scissile bond.) The P'_1 and P'_2 positions were varied to contain each one of the 20 naturally occurring amino acids and P'_3 was kept constant as an arginine. The second library consists of peptides with phenylalanine keto-amides at P_1, glycine in P'_1, and benzyloxycarbonyl (Z)-isoleucine in P_4. The P_2 and P_3 positions were varied to contain each of the naturally occurring amino acids, except for cysteine and methionine. The peptides of both libraries are attached to a solid support (pins). The peptides are evaluated by immersing the pins in a solution of the target enzyme and evaluating the amount of enzyme absorbed. The pins with the best inhibitors will absorb most enzyme. The libraries select the best and worst inhibitors within each group of peptides and provide an approximate ranking of the remaining peptides according to K_i. Through this library, we determined that Z-Ile-Glu-Pro-Phe-CO_2Me and (F)-Phe-CO-Glu-Asp-ArgOMe should be the best inhibitors of chymase in this collection of peptide inhibitors. We synthesized the peptides and found K_i values were 1 nM and 1 μM, respectively. The corresponding K_i values for chymotrypsin were 10 nM and 100 μM. The use of libraries of inhibitors has advantages over the classical method of synthesis of potential inhibitors in solution: the libraries are reusable, the same libraries can be used with a variety of different serine proteases, and the method allows the screening of hundreds of compounds in short periods of time.**

Inhibitors of proteolytic enzymes generally have the following structure: ---A_3-A_2-A_1-X-A'_1-A'_2-A'_3---, where A_1, A'_1, A_2, A'_2, etc., are amino acid residues that are required for optimal binding and specificity. X is an amino acid analog that interacts with the catalytic groups of the active site, as for example difluoro-ketones or keto-amides (1–12) and α-ketothiazole (13). How does one select the most effective combination of amino acid components of the inhibitor? If the kinetic parameters of the target enzyme are well known, V_{max}/K_m provides a useful guide for selection of amino acid components (14, 15). However, for a recently discovered enzyme, V_{max}/K_m data are not available, and another approach is needed. There are many reports of the syntheses of peptide libraries (16, 17), and many applications for these libraries have been reported, such as determination of substrate specificity (18), protease inhibitors (19, 20), ligand-binding activity (21, 22), mapping the S′ subsites of serine proteases (23), and optimization of enzyme substrates (24). We decided to explore the use of libraries consisting of peptides where the P_1 residue is substituted by an α-keto-amide. α-Keto-amides and esters are reversible inhibitors of serine proteases (9, 11, 25–27). Different amino acid combinations were added in either the P or the P′ positions (nomenclature of ref. 28). The peptides of the library are attached to pins (Fig. 1). The pins are immersed in a solution of the target enzyme contained in wells of microtiter plates. The amount of enzyme remaining in the wells (method II) and the amount absorbed on the pins (method I) are determined. The pins containing the best inhibitors will absorb the highest amount of enzyme and remove the greatest amount of enzyme from the wells. With these libraries, the best and the worst inhibitor(s) in the peptide collection can be identified, and an approximate rank order of inhibitors according to K_i values can be established. The libraries do not provide absolute values for K_i. Method II is the preferred method, since it is less subject to artifacts. Method I requires that the enzyme is released from the peptide. If k_{off} is slow, all of the enzyme will not be released and the efficacy of the inhibitor will be underestimated.

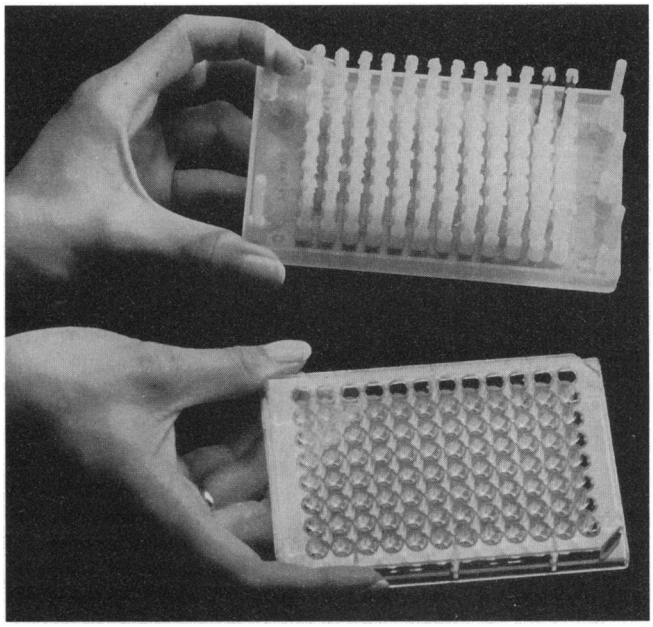

FIG. 1. Pins with peptides attached and microtiter plate.

The library described here differs from others in that the target enzyme is part of the detection and amplification system. This feature simplifies the use of the library since no HPLC or MS analysis is required. Peptide components of the library can be used many times. Also, to the best of our knowledge, no libraries have been prepared that contain pseudo amino acids.

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Abbreviation: Z, benzyloxycarbonyl.

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We chose human heart chymase as the target enzyme. This enzyme converts angiotensin I to angiotensin II and, thus, affects blood pressure (29–31). If inhibitors of chymase are to be used to control blood pressure, then these inhibitors must not inhibit enzymes of the digestive system (chymotrypsin, elastase, cathepsin D, etc.). The currently available data indicate that the substrate (inhibitor) specificities of chymase and chymotrypsin are similar (29). It was our primary goal to develop an inhibitor with the lowest possible K_i value for chymase. Additionally, we wanted to achieve maximal discrimination between chymotrypsin and chymase.

MATERIALS AND METHODS

Preparation of the Library of Inhibitors. The libraries were prepared by using standard solid-phase peptide methods. The inhibitors were prepared by using standard solution-phase peptide synthesis methods such as DCC and mixed anhydride couplings. Details of the syntheses of the libraries of inhibitors and the preparation of all new inhibitors and related compounds are available from the authors upon request.

Regeneration of the Library of Inhibitors. The pins are reusable after regeneration. Each block containing 96 pins was sonicated for two 1-h periods with 250 ml of 6 M urea containing 0.1% Triton X-100 and 10% (vol/vol) dimethylformamide and for three 15-min periods with 250 ml of an aqueous solution containing 0.1% Triton X-100 and 5% dimethylformamide. The pins were allowed to air dry at room temperature overnight.

Enzymes. α-Chymotrypsin from bovine pancreas was purchased from Sigma. Human heart chymase was a gift from Ahsan Husain (Cleveland Clinic Foundation) and was received as a 0.9 μM solution in 20 mM Tris·HCl, pH 8/0.5 M KCl/0.1% Triton X-100.

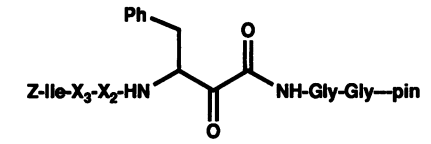
Evaluation of K_i Values. The K_i values were determined from the initial inhibited steady-state velocities in the presence of substrate. In the case of slow-binding kinetics, the K_i was determined by the procedure of ref. 32 or from the final steady-state velocities measured with inhibitor in the presence of substrate. Chymotrypsin assays contained 100 mM potassium phosphate (pH 7.2) and 2% (vol/vol) acetonitrile. The chymase assays contained 20 mM Tris·HCl (pH 8), 0.5 M KCl, 0.1% Triton X-100, and 2.5% acetonitrile. Assays were performed at 25°C. Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (where Suc is succinyl) was used as the substrate for both enzymes.

RESULTS AND DISCUSSION

Evaluation of Peptide Inhibitors. Two methods were used to evaluate the inhibitory efficacy of the peptide inhibitors. In method I, pins were immersed in a solution of the target enzyme contained in wells of microtiter plates. After the pins were removed, the amount of enzyme adsorbed by the pins was evaluated, and a score was assigned to the pins. Pins with the largest scores contained inhibitors with the lowest K_i values. In method II, pins were immersed in a solution of the target enzyme, and after 1 h the pins were removed, the amount of enzyme remaining in the wells was determined, and a score was assigned to each pin. The peptide inhibitors with lowest score will be the best inhibitors. Detailed description of score assignment and experimental conditions are in the tables.

The concentration of enzyme in the assay is important. If the concentration of enzyme used is too high, the percentage of enzyme removed from the wells, even by the best inhibitor, will be too small for a meaningful evaluation. On the other hand, if the concentration of enzyme used is too low, even poor inhibitors will adsorb too large a fraction of the enzyme. There is nonspecific absorption of enzyme to the pins. To correct for this a pin without peptide should be included.

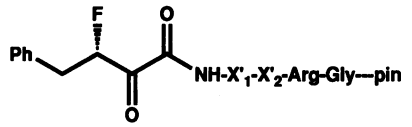
Identification of P Components with a Library of Peptidyl α-Keto-Amides. The peptide inhibitors of this library Z-Ile-X_3-X_2-Phe-CO-Gly-Gly-pin have the following structure:



X_2 and X_3 represent naturally occurring amino acids, except methionine and cysteine, and Z is benzyloxycarbonyl. A total of 324 (18 × 18) inhibitors were prepared. A complete set of data was obtained with this set of pins and chymotrypsin and chymase as target enzymes. The data obtained for chymase by method II are in Table 1. Examination of the data in Table 1 shows that the peptides with Pro-Glu and Gln-Ile as P_2-P_3 have the lowest score (0.07) of the 324 peptides in this group.

The peptides were also evaluated by method I. The score when P_2-P_3 is Pro-Glu is 0.19. Of the 324 combinations examined, 12 had higher scores. This ranking is lower than expected from Table 1. It is likely that a slow k_{off} is responsible for the lower than expected score. The best chymase inhibitor identified by method I is when P_2-P_3 is Asp-Glu and it has a score of 0.94. This peptide is also a good inhibitor of chymotrypsin. We felt that the peptide with P_2-P_3 as Pro-Glu will best meet our goals—i.e., minimal K_i value for chymase and, secondarily, maximum discrimination between chymase and chymotrypsin. We synthesized Z-Ile-Glu-Pro-Phe-CO_2Me and determined K_i values of 1 nM for chymase and 10 nM for chymotrypsin.

Identification of P′ Components of an Inhibitor of Chymase with a Library of Peptidyl-3-Fluorobenzylpyruvamides. The peptide inhibitors of this library (F)-Phe-CO-X_1'-X_2'-Arg-Gly-pin have the following structure:



X_1' and X_2' represent variation of the positions with each of the 20 naturally occurring amino acids. Thus, the library contained 400 inhibitors. The glycine residue was incorporated to minimize interference of the solid support with the binding of the enzyme to the inhibitor.

The data obtained for chymotrypsin and chymase are presented in Tables 2 and 3. Comparison of Tables 2 and 3 shows that the S′ sites of chymase are more selective than those of chymotrypsin. The scores for the S′ subsites of chymase are predominantly low (high K_i). In general the scores for chymotrypsin S′ subsites are higher (lower K_i) than scores for chymase. The data suggest that selectivity for chymase over chymotrypsin can be achieved through P′ components rather than P components.

The data presented below confirm this suggestion. The data in Tables 2 and 3 show that the peptide with P_1'-P_2' as Glu-Asp has a low score with chymotrypsin (0.01) and a high score with chymase (0.57). This peptide should have a low K_i for the inhibition of chymase and a relatively high K_i for chymotrypsin. We synthesized (F)-Phe-CO-Glu-Asp-ArgOMe and found K_i values of 1 μM for chymase and 100 μM for chymotrypsin. This is a 10-fold larger discrimination than was obtained with the inhibitor based on P interactions. Apparently, it is not possible to discriminate between inhibitors with $K_i < 1$ μM by method I with keto-amide peptides.

Interaction Between the P and the P′ Binding Sites. We have, at this point, identified a peptide inhibitor that primarily

Table 1. Scores for inhibitors of chymase P_2 and P_3 varied (method II)

	G	A	V	L	I	P	F	Y	W	S	T	N	Q	H	K	R	D	E	
G	0.14	0.12	0.14	0.15	0.15	0.14	0.14	0.17	0.15	0.17	0.17	0.15	0.15	0.15	0.15	0.13	0.13	0.13	G
A	0.16	0.12	0.14	0.16	0.15	0.14	0.16	0.15	0.15	0.13	0.14	0.16	0.15	0.15	0.16	0.09	0.15	0.14	A
V	0.16	0.16	0.20	0.19	0.20	0.17	0.17	0.21	0.21	0.19	0.19	0.20	0.16	0.19	0.19	0.20	0.17	0.12	V
L	0.16	0.15	0.15	0.15	0.16	0.12	0.15	0.16	0.16	0.14	0.15	0.14	0.13	0.17	0.10	0.13	0.14	0.10	L
I	0.16	0.15	0.17	0.17	0.18	0.10	0.18	0.14	0.16	0.12	0.15	0.10	0.07	0.16	0.14	0.16	0.17	0.09	I
P	0.16	0.16	0.20	0.15	0.15	0.12	0.16	0.20	0.21	0.22	0.20	0.15	0.12	0.17	0.15	0.18	0.14	0.14	P
F	0.16	0.16	0.17	0.18	0.14	0.15	0.16	0.15	0.17	0.15	0.17	0.15	0.15	0.14	0.13	0.15	0.15	0.17	F
Y	0.19	0.20	0.18	0.19	0.19	0.20	0.19	0.15	0.17	0.19	0.19	0.19	0.19	0.17	0.18	0.18	0.18	0.18	Y
W	0.19	0.18	0.19	0.18	0.18	0.17	0.18	0.20	0.20	0.19	0.19	0.19	0.16	0.19	0.18	0.20	0.17	0.17	W
S	0.18	0.17	0.17	0.17	0.18	0.13	0.19	0.19	0.19	0.16	0.17	0.17	0.16	0.17	0.14	0.18	0.13	0.13	S
T	0.17	0.16	0.15	0.12	0.17	0.14	0.22	0.19	0.18	0.12	0.13	0.16	0.14	0.18	0.14	0.16	0.17	0.10	T
N	0.14	0.15	0.13	0.16	0.15	0.15	0.14	0.14	0.14	0.16	0.15	0.17	0.15	0.19	0.17	0.17	0.19	0.17	N
Q	0.19	0.19	0.17	0.16	0.16	0.13	0.19	0.18	0.19	0.16	0.15	0.16	0.16	0.14	0.15	0.15	0.14	0.13	Q
H	0.15	0.16	0.15	0.16	0.16	0.17	0.16	0.13	0.14	0.15	0.16	0.17	0.17	0.16	0.17	0.17	0.17	0.15	H
K	0.15	0.15	0.15	0.16	0.16	0.14	0.10	0.10	0.15	0.14	0.10	0.16	0.15	0.15	0.13	0.15	0.16	0.15	K
R	0.19	0.21	0.18	0.22	0.23	0.21	0.22	0.19	0.18	0.16	0.17	0.22	0.22	0.23	0.20	0.15	0.22	0.20	R
D	0.15	0.14	0.18	0.18	0.18	0.17	0.15	0.17	0.18	0.18	0.18	0.18	0.19	0.11	0.17	0.19	0.12	0.14	D
E	0.14	0.09	0.12	0.11	0.12	0.07	0.11	0.15	0.12	0.08	0.15	0.14	0.11	0.14	0.15	0.13	0.14	0.10	E

Assay conditions were as follows: The pins were immersed for 1 h in microtiter plates containing 0.1 nM chymase in 100 mM Hepes/2.5% acetonitrile/0.1% Triton X-100, pH 7.5. The pins were removed, and the substrate (Suc-Ala-Ala-Pro-Phe-pNA, where Suc is succinyl and pNA is *p*-nitroanilide; final concentration, 0.75 mM) was added to the microtiter plates. The reaction proceeded for 4 h; hydrolysis was monitored at 410 nm. The final A_{410} value is referred to as the score. Rows represent P_3 positions. Columns represent P_2 positions.

interacts with S subsites and a peptide inhibitor that utilizes S′ sites (Table 4, compounds **1** and **2**). An inhibitor was synthesized that contained amino acids of both peptides (compound **3**). If the interaction at the active site of the precursor peptides (compounds **1** and **2**) is precise, then K_i value of the hybrid peptide (compound **3**) should be considerably greater than the product of the K_i values for each component (33). The result obtained for chymase and chymotrypsin is shown in Table 4. For chymotrypsin, the K_i value of the hybrid molecule (**3**) is 4 $\times$ 10^3 larger than the K_i value for the inhibitor (**2**) and somewhat smaller than K_i for compound **1**. For chymase, the K_i for compound **3** is 10^2-fold larger than the K_i for compound **2**. The discrimination (K_i chymase/K_i chymotrypsin) is 400. The failure to obtain a K_i for the inhibitor (compound **3**) that

Table 2. Scores for inhibitors of chymase P_1' and P_2' varied (method I)

	G	A	V	L	I	P	F	Y	W	S	T	N	Q	H	K	R	D	E	C	M	
G	0.01	0.01	0.02	0.01	0.01	0.00	0.03	0.00	0.00	0.02	0.01	0.00	0.00	0.00	0.00	0.01	0.03	0.01	0.00	0.00	G
A	0.05	0.05	0.00	0.08	0.07	0.01	0.01	0.00	0.01	0.02	0.01	0.07	0.04	0.03	0.05	0.06	0.22	0.14	0.02	0.04	A
V	0.06	0.02	0.00	0.03	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.03	0.06	0.04	0.03	0.04	0.08	0.05	0.03	0.04	V
L	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.04	0.01	0.00	0.01	L
I	0.06	0.05	0.01	0.02	0.02	0.01	0.01	0.02	0.02	0.03	0.02	0.03	0.06	0.02	0.02	0.07	0.10	0.06	0.01	0.02	I
P	0.02	0.03	0.03	0.03	0.02	0.01	0.02	0.01	0.01	0.08	0.02	0.04	0.03	0.05	0.03	0.05	0.27	0.02	0.02	0.04	P
F	0.04	0.03	0.01	0.07	0.05	0.01	0.02	0.01	0.01	0.04	0.03	0.04	0.04	0.04	0.03	0.05	0.28	0.10	0.03	0.03	F
Y	0.06	0.04	0.01	0.02	0.03	0.02	0.01	0.00	0.00	0.04	0.02	0.03	0.04	0.04	0.02	0.01	0.01	0.20	0.04	0.01	Y
W	0.01	0.02	0.01	0.02	0.05	0.01	0.0	0.02	0.01	0.03	0.02	0.02	0.02	0.01	0.01	0.02	0.17	0.06	0.02	0.02	W
S	0.05	0.06	0.02	0.01	0.04	0.01	0.03	0.01	0.01	0.15	0.04	0.09	0.01	0.03	0.02	0.11	0.27	0.27	0.03	0.08	S
T	0.10	0.02	0.03	0.04	0.02	0.01	0.01	0.02	0.02	0.08	0.03	0.02	0.03	0.03	0.02	0.05	0.14	0.06	0.01	0.02	T
N	0.01	0.00	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.02	0.00	0.00	0.00	0.00	N
Q	0.05	0.01	0.00	0.04	0.02	0.00	0.01	0.01	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.06	0.06	0.03	0.01	0.01	Q
H	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.00	0.03	0.01	0.10	0.02	0.00	0.00	H
K	0.02	0.01	0.03	0.01	0.02	0.00	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.03	0.01	0.02	K
R	0.01	0.01	0.00	0.01	0.01	0.00	0.01	0.00	0.01	0.01	0.00	0.02	0.01	0.01	0.00	0.01	0.03	0.01	0.03	0.01	R
D	0.08	0.05	0.02	0.12	0.05	0.01	0.02	0.07	0.07	0.07	0.03	0.09	0.04	0.10	0.05	0.10	0.55	0.44	0.08	0.04	D
E	0.08	0.04	0.01	0.13	0.08	0.02	0.03	0.02	0.03	0.04	0.02	0.10	0.04	0.07	0.06	0.10	0.57	0.34	0.06	0.08	E
C	0.01	0.00	0.01	0.03	0.01	0.00	0.00	0.01	0.00	0.02	0.01	0.01	0.01	0.00	0.01	0.02	0.14	0.07	0.00	0.00	C
M	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	M

Assay conditions were as follows: The pins were immersed for 1.5 h in microtiter plates containing 1.8 nM chymase in 20 mM Tris·HCl/0.5 M KCl/2.5% acetonitrile/0.1% Triton X-100, pH 8. The pins were removed and washed for two 15-sec periods with the same buffer. The pins were immersed for 30 min in a 0.17 mM substrate solution. The reaction proceeded for 5 h; hydrolysis was monitored at 410 nm. The final A_{410} value is referred to as the score. Rows represent P_1' positions. Columns represent residues in the P_2' positions.

Table 3. Scores for inhibitors of chymotrypsin P_1' and P_2' varied (method I)

	G	A	V	L	I	P	F	Y	W	S	T	N	Q	H	K	R	D	E	C	M	
G	0.10	0.17	0.09	0.20	0.27	0.19	0.13	0.01	0.08	0.24	0.17	0.08	0.06	0.03	0.05	0.19	0.02	0.02	0.03	0.04	G
A	0.08	0.18	0.06	0.26	0.44	0.04	0.10	0.08	0.04	0.12	0.07	0.13	0.07	0.07	0.14	0.15	0.03	0.01	0.04	0.07	A
V	0.28	0.79	0.39	0.44	0.61	0.31	0.12	0.05	0.14	0.07	0.55	0.68	0.34	0.35	0.43	0.43	0.14	0.29	0.05	0.55	V
L	0.22	0.20	0.57	0.21	0.54	0.50	0.03	0.00	0.12	0.74	0.68	0.43	0.36	0.14	0.20	0.52	0.06	0.36	0.03	0.27	L
I	0.29	0.54	0.08	0.26	0.53	0.28	0.15	0.07	0.08	0.33	0.14	0.73	0.47	0.19	0.27	0.51	0.11	0.34	0.08	0.47	I
P	0.04	0.10	0.04	0.02	0.09	0.09	0.33	0.00	0.04	0.16	0.05	0.05	0.03	0.07	0.03	0.14	0.00	0.00	0.00	0.05	P
F	0.13	0.45	0.30	0.35	0.63	0.48	0.09	0.02	0.14	0.30	0.35	0.24	0.25	0.16	0.21	0.09	0.07	0.20	0.45	0.15	F
Y	0.20	0.43	0.37	0.26	0.40	0.44	0.09	0.08	0.18	0.42	0.40	0.29	0.22	0.22	0.20	0.26	0.03	0.45	0.07	0.27	Y
W	0.16	0.24	0.20	0.29	0.31	0.16	0.21	0.08	0.13	0.34	0.30	0.20	0.18	0.12	0.17	0.32	0.11	0.08	0.10	0.16	W
S	0.13	0.53	0.22	0.35	0.34	0.29	0.19	0.05	0.09	0.71	0.05	0.30	0.29	0.13	0.12	0.64	0.10	0.10	0.20	0.16	S
T	0.28	0.51	0.65	0.34	0.68	0.38	0.07	0.05	0.25	0.75	0.74	0.24	0.18	0.68	0.19	0.52	0.07	0.10	0.12	0.12	T
N	0.11	0.03	0.33	0.45	0.42	0.15	0.19	0.01	0.08	0.48	0.47	0.19	0.22	0.10	0.11	0.26	0.04	0.03	0.05	0.19	N
Q	0.14	0.57	0.39	0.35	0.40	0.21	0.16	0.06	0.12	0.63	0.58	0.27	0.18	0.10	0.16	0.34	0.04	0.04	0.04	0.21	Q
H	0.08	0.06	0.09	0.16	0.26	0.03	0.13	0.03	0.07	0.08	0.08	0.07	0.00	0.07	0.06	0.06	0.06	0.02	0.02	0.03	H
K	0.08	0.24	0.04	0.18	0.24	0.16	0.04	0.03	0.04	0.34	0.20	0.10	0.10	0.08	0.06	0.22	0.03	0.03	0.03	0.10	K
R	0.18	0.59	0.27	0.51	0.53	0.29	0.10	0.04	0.13	0.50	0.41	0.49	0.25	0.02	0.33	0.29	0.13	0.15	0.09	0.60	R
D	0.06	0.15	0.08	0.22	0.19	0.05	0.26	0.21	0.07	0.10	0.07	0.10	0.05	0.11	0.04	0.22	0.01	0.03	0.05	0.04	D
E	0.02	0.06	0.00	0.12	0.19	0.01	0.22	0.11	0.07	0.03	0.02	0.04	0.01	0.11	0.09	0.36	0.01	0.02	0.03	0.02	E
C	0.03	0.09	0.03	0.06	0.12	0.04	0.05	0.01	0.04	0.05	0.04	0.05	0.04	0.02	0.04	0.06	0.02	0.00	0.02	0.00	C
M	0.06	0.40	0.18	0.24	0.27	0.25	0.35	0.01	0.06	0.39	0.09	0.11	0.09	0.04	0.06	0.18	0.00	0.02	0.01	0.10	M

Assay conditions were as follows: Same as Table 2, except that the pins were immersed for 30 min and the chymotrypsin concentration was 2.9 μM and 10% acetonitrile and no Triton X-100 was added. The pins were washed for two 1-min periods. Rows represent residues in the P_1' positions. Columns represent residues in the P_2' positions.

is less than the product of the K_i for the component peptide is most likely due to imperfect fit to the subsites. The keto-amide inhibitor contains one more carbon than the analogous amino acid. It is possible that the active site can accommodate an inhibitor better that consists primarily of either P or P′ components than one that contains both P and P′ components.

Library Reliability. We synthesized several peptide inhibitors to determine whether the ranking of solution K_i values is in agreement with the ranking predicted from the library. For chymase, the score for the peptide with P_3-P_2 as Glu-Pro is 0.07 (Table 1) and K_i is 1 nM. Peptides with higher scores should be worse inhibitors. The peptides with P_3-P_2 as Pro-Val, Phe-Gly, and Ser-Ile that had scores 0.20, 0.16, and 0.18, respectively, were synthesized. The K_i values are 30 μM, 4 μM, and 0.01 μM, respectively. This result is in agreement with our prediction. For chymotrypsin, the score of peptide with P_3-P_2 as Glu-Pro is 0.08 and K_i is 10 nM. We synthesized peptides with P_3-P_2 as Phe-Gly, Val-Ala, and Pro-Ala that had scores of 0.20, 0.13, and 0.18, respectively. Based on these scores, we expected K_i values of >10 nM for these peptides. The K_i values for the peptides with P_3-P_2 as Phe-Gly and Pro-Ala were 800 nM and 8 μM, respectively, as expected. For the peptide with P_3-P_2 as Val-Ala, K_i was 10 nM, which is unexpectedly low.

The interaction of peptides with the structure Xaa-Ala-Ala-Ala, where Xaa is an amino acid other than alanine, with the S′ subsite of chymotrypsin has been evaluated (23) by comparing the ratio of acyl group transfer from acyl chymotrypsin to the peptide and to water. The peptide with Xaa as Arg had the highest nucleophilicity. We expected that peptide inhibitors with P_1'-P_2' as Arg-Ala should have a lower K_i value than those with any other amino acid in P_1' and should also have a high score (method I). In Table 3, the score of the 20 peptides with P_2' as Ala ranges from 0.79 when P_1' is Val to 0.06 when P_1' is Glu or His. The second highest score is 0.59 when P_1' is Arg. Peptides with low nucleophilicity were Glu and Asp (23). The scores in Table 3 are low, 0.06 and 0.15, respectively, which is in agreement with previous results.

Table 4. Interaction between P and P′ binding sites

Compound	K_i, μM	
	Chymotrypsin	Chymase
1. (F)-Phe-CO-Glu-Asp-Arg-OMe	100	1
2. Z-Ile-Glu-Pro-Phe-CO_2Me	0.01	0.001
3. Z-Ile-Glu-Pro-Phe-CO-Glu-Asp-Arg-OMe	40	0.1

General Observations. Combinations of amino acids that result in the lowest K_i and highest K_i values occur infrequently. The majority of peptides have modest K_i values. For instance, in Table 1, three peptides have a low rating from 0.07 to 0.08 (low K_i) and nine peptides have the two highest ratings 0.22 and 0.23. Intermediate scores of 0.16–0.18 were assigned to 112 peptides. The reason for the low probability of obtaining high-K_i inhibitors is the presence of the keto-amide at P_1. The keto-amide is an inhibitor and its inhibitory capability must be overcome to obtain a high-K_i inhibitor.

It is of interest to consider whether the efficacy of the amino acid component of an inhibitor is influenced by neighboring amino acids. The scores within each row or each column are generally very similar. For instance, in Table 1, the average score is 0.12 ± 0.02 for row E, 0.20 ± 0.02 for row D, and 0.14 ± 0.03 for column P. This indicates that each amino acid component of the inhibitor makes an intrinsic contribution to K_i; i.e., this contribution is not strongly influenced by adjacent amino acids. There are, however, some exceptions. In Table 1, row E, the score for P_2-P_3 as Pro-Glu is 0.07. This can be compared to the average score of 0.14 ± 0.03 for all peptides with Pro in P_2 and 0.12 ± 0.02 for Glu in P_3. The low score for Pro-Glu could be due to an exceptionally precise fit of Pro and Glu to the active site, the result of the chelation effect (33), or the interaction of Pro and Glu with each other to form a structure that interacts well with the active site.

These results show that, in selecting combinations of amino acids for a peptide ligand, the action of groups of amino acids

must be considered, rather than individual amino acids. The standard approach in designing a peptide ligand, consisting of n amino acids, is to keep $n - 1$ amino acids constant and vary one amino acid at a time. This approach can easily miss good ligands. All possible combinations of amino acids must be evaluated to ensure optimal amino acid composition.

Note. In addition to Tables 1–3, we generated three other tables. These tables, which are entitled Scores of inhibitors of chymase P_2 and P_3 varied (method I), Scores of inhibitors of chymotrypsin P_2 and P_3 varied (method II), and Scores of inhibitors of chymotrypsin P_2 and P_3 varied (method I), could not be included because of space limitations but are available from the corresponding author upon request.

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