SY FT spectrometer equipped with an Aspect 3000 computer. Acquisition parameters were as follows: spectral width, 1785 Hz (8.9 ppm); data size, 16K; recycle delay, 5 s; pulse width, 2 μs (40° tip angle); 128 transients. The ^1H decoupled spectra were examined with respect to a control spectrum in which the irradiated frequency corresponded to a blank region of the spectrum. The ^1H difference NOE spectra were recorded with a pulse program available in the Bruker library of programs. The spectra were recorded by sequential acquisition and storage of eight transients with irradiation at each of several frequencies. The list of frequencies included one corresponding to a blank spectral region, and the resulting spectrum was used as a control. A subsaturating irradiation pulse (0.8 s) was used before acquisition. The cycle was repeated ×32 to give a total of 256 transients. The stored free induction decays (FIDs) were processed with identical normalization and phase constants. The control spectrum was subtracted from each decoupled spectrum to give a difference spectrum.

Spectral regions of the 1D 500.133 MHz ^1H NMR spectrum of U50488 corresponding to the cyclohexyl ring were simulated with the LAOCOON-based PANIC software, available in the Bruker library of computer programs, using an Aspect 3000 computer. The computer program accepts a maximum of nine nuclear spins and requires the input of coupling constant and chemical shift data. Line width, spectral width, and data size are adjustable parameters. Chemical shifts and ^2J and ^3J coupling constant values estimated from analysis of the 1D and phase-sensitive 2D COSY spectra were used as starting points for an iterative simulation of subspectra.

The 2D ^1H-^1H NOE, COSY, and DQF COSY spectra were recorded on the Bruker WP-200 SY FT spectrometer. The pulse sequences for data acquisition and phase cycling routines were those available in the Bruker library of programs. Typical acquisition parameters for the experiments were as follows: 90° pulse width; 3.4 μs; initial t_1 value, 3 μs; 64 transients per t_1 value; sweep width in f_1 and f_2, 1638.2 Hz; recycle delay, 3.5 s. The 2D NOE spectra were recorded with a mixing time of 0.8 s with random variation for suppression of cross-peaks due to zero order scalar (J) coupling. The 2D DQF COSY spectra required a presaturation pulse. Data processing included application of a sine bell window function in f_1 and f_2, 2D Fourier transformation, and symmetrization of the data about the diagonal.

Materials. U50488H (the $CH_3SO_3^-$ salt) was obtained from the Drug Supply Program of the National Institute on Drug Abuse (Bethesda, MD). D_2O (99.5%) was obtained from the Aldrich Chemical Co. (Milwaukee, WI). U50488H was dissolved in D_2O, thoroughly degassed, and sealed in high-quality 5-mm NMR tubes (728-pp; Wilmad Glass Co., Buena, NJ). All spectra were recorded at 21.7 °C.

Acknowledgment. This work was supported by Grant DA 04762 from the National Institute on Drug Abuse.

Mapping the Binding Site of Tissue Kallikrein: Preparation and Testing of All Possible Substrate Analog Inhibitors Homologous with the Sequence of Kininogen between Ser^{386} and Gln^{392} †

Milind S. Deshpande‡ and James Burton*

Unit for Rational Drug Design, Department of Medicine, The University Hospital, Boston, Massachusetts 02118.
Received September 20, 1991

Programs aimed at converting peptide inhibitors of proteolytic enzymes into more traditional drug structures require an understanding of the role played by the individual amino acid residues in the inhibitor. To this end, all possible substrate analogues occurring within the sequence Ser^{386}-Pro-Phe-Arg-Ser-Val-Gln^{392} from bovine kininogen were synthesized and tested as inhibitors of tissue kallikrein (EC 3.4.21.35, β-PPK). Of the 21 sequences which can be formed from the heptapeptide, 11 have inhibitory constants which could be measured in the chromogenic assay employed in these studies. No dipeptide and only one tripeptide, Ac-Phe-Arg-Ser-NH_2 (K_i = 718 μM), measurably inhibits the enzyme. All longer peptides inhibit β-PPK. The heptapeptide Ac-Ser-Pro-Phe-Arg-Ser-Val-Gln-NH_2 is the most effective inhibitor in this series (K_i = 101 μM). Each amino acid residue in the sequence appears to alter binding in a relatively independent manner. The N-terminal seryl residue (P_4) and the prolyl residue (P_3) slightly improve the K_i of the various inhibitors. The phenylalanyl residue at P_2 appears to have a more pronounced effect on K_i. The arginyl residue at P_1 and the seryl residue at P_1' appear to be the most important residues in the inhibitory sequence. They contribute approximately one-third and one-fourth of the binding energy to the interaction between the substrate analogues and β-PPK, respectively. The valyl residue at P_2', and the C-terminal glutaminyl residue improve K_i of each of the peptides tested. Almost 80% of the binding energy of the substrate analogue inhibitors comes from the core sequence Phe-Arg-Ser which occurs between P_2 and P_1'. Molecular models developed from the Chen-Bode coordinates of the aprotinin-β-PPK complex have been used to interpret the results of these studies.

Introduction

Tissue kallikrein (EC 3.4.21.35) is a serine protease which releases kinins such as kallidin (lysylbradykinin) from low molecular weight kininogen.[1] Numerous functions have been assigned to kinins including regulation of blood flow in some organs,[2] the enhancement of pain,[3] and vascular leakage resulting from rhinitis.[4] Clinical evidence

† Abbreviations used: ACE, angiotensin I converting enzyme; BAEE, benzoyl-L-arginine ethyl ester; HUK, human urinary kallikrein (α-form); KKI, kallikrein inhibitor (Rational Drug Design Number); β-PPK, porcine pancreatic kallikrein (β-form); RUK, rat urinary kallikrein (α-form); S-2266, D-valylleucyl-arginyl-p-nitroanilide; PNA, 4-nitroaniline; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

* Unit for Rational Drug Design, E301, The University Hospital, 88 East Newton Street, Boston, MA 02118-2393.

‡ Current address: Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492.

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0022-2623/92/1835-3094$03.00/0

Table I. Physical Properties of Substrate Homologue Inhibitors

KKI no.	amino acid ratio						R_f (TLC)			Sp act., Ci/mol	HPLC[d] retention time, s
	P	F	R	S	V	Q	T2[a]	T3[b]	T4[c]		
1					1.19	1.02	0.2	0.5	0.2	0.027	600
2				0.95	0.99	1.06	0.2	0.48	0.2	0.018	620
3			1.00	1.01	0.97	1.05	0.2	0.48	0.2	0.018	680
4		0.94	1.01	0.94	0.98	1.04	0.2	0.46	0.2	0.017	820
5	1.02	1.00	0.98	0.97	1.01	1.01	0.2	0.46	0.2	0.18	900
6	0.99	1.00	0.98	1.98	0.97	0.96	0.2	0.46	0.2	0.17	870
71	1.00			0.95			0.2	0.48	0.2	0.018	1300[e]
72	1.06	0.95		1.01			0.2	0.46	0.2	0.018	1060[e]
24	1.08	0.90	1.03	0.98			0.2	0.45	0.17	0.145	880
25	1.02	0.94	1.00	2.02			0.2	0.45	0.17	0.011	820
66	1.05	1.01	1.07	1.87	0.99		0.2	0.48	0.18	0.004	995
21	1.07	0.93					0.2	0.45	0.18	0.019	1100[e]
20	1.00	0.97	1.02				0.2	0.45	0.18	0.005	870[e]
19	1.12	1.03	1.03	0.82			0.2	0.45	0.2	0.26	990[e]
9	1.12	0.98	0.86	1.00	1.01		0.2	0.45	0.2	0.018	950
22		0.95	1.01				0.2	0.45	0.18		740
23		0.80	0.83	1.36			0.2	0.45	0.18		985
69		1.11	0.99	1.04	0.98		0.2	0.43	0.19	0.018	790
26			1.01	1.00			0.2	0.47	0.19		640
70			0.96	1.00	1.01		0.01	0.40	0.10	0.018	660
65				1.01	1.00		0.01	0.40	0.10	0.019	620

[a] T2 = 2-butanol–3% NH_3, 150:66. [b] T3 = 1-butanol–pyridine–acetic acid–water, 75:50:15:60. [c] T4 = 1-butanol–water–acetic acid, 143:42:14. [d] HPLC: solvent A, 0.2% CF_3COOH; solvent B, CH_3CN–0.2% CF_3COOH; gradient (linear), 0–100% solvent B over 20 min. [e] HPLC: 0.2% CF_3COOH (isocratic).

also indicates that decreases in mean arterial pressure in human essential hypertension, caused by administration of ACE inhibitors, are better correlated with increases in circulating levels of kinins than with decreases in levels of circulating angiotensin II.[5]

Additional biologic roles have been suggested for tissue kallikrein. These include processing proenzymes and prohormones such as prorenin,[6] proinsulin,[7] atriopeptigen,[8] and tissue plasminogen activator.[9]

Understanding the importance of tissue kallikrein in various biologic processes may depend on development of *specific* tissue kallikrein inhibitors which can be used in vivo. The kinin receptor blockers[10,11] have been extremely valuable for the study of the biological effects of kinins. However, these compounds do not identify the source of kinins, making it difficult to know whether tissue (EC 3.4.21.35) or plasma (EC 3.4.21.34) kallikrein is responsible for a specific physiologic effect. The receptor blockers also function as partial agonists[10] in some systems, complicating the analysis of results from in vivo tests.

Protease inhibitors such as aprotinin[12] and its fragments,[13] benzamidine,[14] aromatic diamidines,[15] and pep-

Table II. Inhibitory Constants and ΔG for Substrate Analogue Inhibitors

KKI no.	sequence	K_i, μM	ΔG, cal/mol
1	Ac-Val-Gln-NH_2	NI[a]	
2	Ac-Ser-Val-Gln-NH_2	NI[b]	
3	Ac-Arg-Ser-Val-Gln-NH_2	950	−4287
4	Ac-Phe-Arg-Ser-Val-Gln-NH_2	230	−5161
5	Ac-Pro-Phe-Arg-Ser-Val-Gln-NH_2	167	−5358
6	Ac-Ser-Pro-Phe-Arg-Ser-Val-Gln-NH_2	101	−5668
9	Ac-Pro-Phe-Arg-Ser-Val-NH_2	266	−5071
19	Ac-Pro-Phe-Arg-Ser-NH_2	578	−4593
20	Ac-Pro-Phe-Arg-NH_2	NI[c]	
21	Ac-Pro-Phe-NH_2	NI[d]	
22	Ac-Phe-Arg-NH_2	NI[d]	
23	Ac-Phe-Arg-Ser-NH_2	718	−4459
24	Ac-Ser-Pro-Phe-Arg-NH_2	2292	−3744
25	Ac-Ser-Pro-Phe-Arg-Ser-NH_2	223	−5180
26	Ac-Arg-Ser-NH_2	NI[c]	
65	Ac-Ser-Val-NH_2	NI[c]	
66	Ac-Ser-Pro-Phe-Arg-Ser-Val-NH_2	135	−5489
69	Ac-Phe-Arg-Ser-Val-NH_2	479	−4860
70	Ac-Arg-Ser-Val-NH_2	NI[c]	
71	Ac-Ser-Pro-NH_2	NI[c]	
72	Ac-Ser-Pro-Phe-NH_2	NI[c]	

[a] NI: No inhibition (<5%) at 28 mM concentration of inhibitor. [b] NI: No inhibition at 20 mM concentration of inhibitor. [c] NI: No inhibition at 0.5 mM concentration of inhibitor. [d] NI: No inhibition at 1 mM concentration of inhibitor.

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Table III. Calculation of $\Delta\Delta G$ for Amino Acid Residues in the Substrate Analogue Inhibitors

site	residue	KKI no.	ΔG, cal/mol	KKI no.	ΔG, cal/mol	$\Delta\Delta G$, cal/mol
P_4	$+Ser^{386}$	5	-5358	6	-5668	-310
		9	-5071	66	-5489	-418
		19	-4593	25	-5180	-587
		20	-	24	-3744	-
		21	-	72	-	-
					average	-438
P_3	$+Pro^{387}$	4	-5161	5	-5358	-197
		69	-4860	9	-5071	-211
		23	-4459	19	-4593	-134
		22	-	20	-	-
					average	-181
P_2	$+Phe^{388}$	3	-4287	4	-5161	-847
		70	-	69	-4860	-
		26	-	23	-4459	-
		71	-	72	-	-
P_1	$+Arg^{389}$	2	-	3	-4287	-
		65	-	70	-	-
		72	-	24	-3744	-
		21	-	20	-	-
P_1'	$+Ser^{390}$	1	-	2	-	-
		24	3744	25	-5180	-1436
		20	-	19	-4593	-
		22	-	23	-4459	-
P_2'	$+Val^{391}$	25	-5180	66	-5489	-309
		19	-4593	9	-5071	-478
		23	-4459	69	-4860	-401
		26	-	70	-	-
					average	-396
P_3'	$+Gln^{392}$	66	-5489	6	-5668	-179
		9	-5071	5	-5358	-287
		69	-4860	4	-5161	-301
		70	-	3	-4287	-
		65	-	2	-	-
					average	-256

tides containing a chloromethyl ketone group[16] do not appear to be specific for tissue kallikrein. Tissue kallikrein is inhibited by peptides which are homologous with the amino acid sequence of the substrate around the cleavage site.[17,18] Importantly, the substrate analogue inhibitors appear to be reasonably specific for tissue kallikrein.[19] Development of these compounds may thus yield the specific inhibitors sought for study of the in vivo function of the enzyme.

To better understand how the various amino acid residues in the substrate analogues interact with tissue kallikrein, all possible peptides which can be formed from residues comprising the P_4–P_3' sequence were synthesized and tested as inhibitors. Data from these studies are evaluated to show the contributions of the various amino acid residues to the interaction between the inhibitors and β-PPK.

Results

There are 21 synthetic peptides which can be formed from the heptapeptide sequence that occurs between Ser^{386} and Gln^{392} of bovine kininogen.[20] These were synthesized by solid-phase techniques,[21,22] purified to homogeneity by gel filtration and HPLC, and characterized by amino acid composition, TLC, HPLC, and specific activity (Table I).

β-Porcine pancreatic kallikrein (β-PPK) hydrolyses the chromogenic substrate S-2266 (D-Val-Leu-Arg-pNA)[17,23] with a concomitant increase in absorbance at 406 nm. Addition of the inhibitors to the reaction mixture slows the rate of hydrolysis (v) of the chromogenic substrate. Analysis of the rates of hydrolysis in the presence of the inhibitors listed here show kinetics characteristic of competitive inhibition.[24] K_i values were obtained from the kinetic data by the method of Dixon[24] (Table II and supplementary material).

HPLC of the assay mixtures shows that the inhibitors are stable under the conditions employed for K_i determination. No significant hydrolysis of the inhibitor is observed when the solution used to determine K_i is subjected to HPLC. An automated assay in which free α-

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amino groups that would arise from cleavage of the acetylated substrate analogue inhibitors is quantitated by reaction with TNBS has also been used to confirm the lack of significant hydrolysis under assay conditions (Patel, P. H. and Burton, J., manuscript in preparation).

K_i and Length of the Inhibitors. None of the dipeptides reported here measurably inhibits β-PPK in the assay employed (Table II). One tripeptide, **KKI-23** (Ac-Phe-Arg-Ser-NH_2, 718 μM), inhibits β-PPK.

All tetrapeptides and longer peptides inhibit β-PPK.

Role of Individual Residues in Binding. Each residue in the substrate analogue appears to alter binding of the inhibitors to the enzyme in an independent and relatively consistent manner.[25] The effect of addition of the seryl residue at P_4 (serine) on K_i may potentially be evaluated in five cases (Table III). Comparison of the K_i value for **KKI-5** (Ac-Pro-Phe-Arg-Ser-Val-Gln-NH_2, 167 μM) with that of **KKI-6** (Ac-Ser-Pro-Phe-Arg-Ser-Val-Gln-NH_2, 101 μM) indicates that addition of the seryl residue to the substrate analog sequence causes a measurable, though not marked, improvement in K_i. In two other cases a similar effect on K_i is also noted. These are **KKI-9** (266 μM) and **KKI-66** (135 μM), and **KKI-19** (578 μM) and **KKI-25** (223 μM). In two remaining cases (**KKI-21** and **KKI-72**, and **KKI-20** and **KKI-24**), any change in affinity caused by addition of the seryl residue is undefined because at least one member of the pair does not have a measurable K_i.

Addition of the P_3 residue (proline) to the substrate analogues appears to have less effect on K_i than does addition of the seryl residue at P_4 (Table III). Changes in K_i may potentially be measured in four cases. Three of these are defined and show a slight improvement in K_i. One case is undefined. The effect of the addition of the P_2 residue (phenylalanine) on K_i can potentially be evaluated in four cases. In three of these, at least one of the numbers of each pair has an undefined K_i. In the one case which is defined, **KKI-4** vs **KKI-3**, K_i is reduced approximately 4-fold by addition of the phenylalanyl residue.

No inhibitor which lacks the arginyl residue measurably inhibits tissue kallikrein. Thus, while there are four potential ways to judge alterations in binding caused by addition of the arginyl residue, none yield useful information (Table III).

The effect of addition of the seryl residue at P_1' to the inhibitor sequence can potentially be evaluated in four cases. In only one of these (**KKI-25** vs **KKI-24**) are K_i values for both members defined. The effect of addition of the seryl residue at this position is marked. The K_i value of the compound containing the seryl residue at P_1' is about $^1/_{10}$ of that of the homologous inhibitor lacking this residue. This strong effect may be contrasted with the relatively weak effect caused by addition of the amino acid residue (serine) at P_4.

Addition of the P_2' residue (valine) slightly improves the affinity of the inhibitors for β-PPK. Comparison of K_i values for **KKI-25** and **KKI-66**, **KKI-19** and **KKI-9**, **KKI-23** and **KKI-69**, and **KKI-26** and **KKI-70** (Table III) indicates that addition of the valyl residue tends to increase binding somewhat.

Addition of glutamine at P_3' improves binding of the inhibitors by a smaller amount than observed with the valyl residue at P_2'. The effect of this addition could be quantitated in two cases. Comparison of **KKI-25** and **KKI-6** (200 vs 120 μM) and **KKI-19** and **KKI-1** (700 vs 380 μM) indicates that affinity is almost doubled by addition of the glutaminyl residue at P_3'.

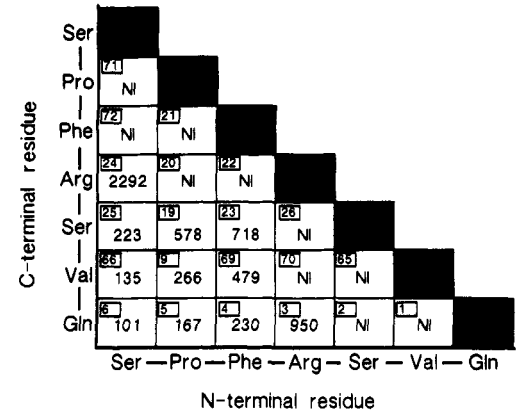

Figure 1. Substrate analogues arranged in a checkerboard. The N-terminal residue of the inhibitors is on the ordinate, and the C-terminal residue on the abscissa. The number in the inset squares corresponds to the peptide number (see Table I). The K_i value (μM) for each peptide is shown in the larger box (see Table II).

Discussion

Conversion of a peptide inhibitor into a more traditional drug structure typically involves both size reduction and the elimination of moieties which may be labile under biologic conditions. However, simplification of a peptide must be done with care to avoid eliminating groups which are important for binding to the target structure. In order to rationally eliminate nonessential residues in the peptide, quantitation of the interaction between the various residues and the target is necessary.

A systematic approach, in which all possible homologous peptides existing between any two residues in a peptide sequence are prepared and tested, is presented here. The number of nontrivial (>1 residue) substrate analogs is $\sum n - n$, where n is the number of amino acid residues in the synthetic sequence. For the kininogen sequence[20] occurring between Ser^{386} and Gln^{392}, $n = 7$ and the number of analogues is 21.

In order to simplify evaluation of possible relationships between various substrate analogues, sequence and biologic data are organized as shown in Figure 1. Each square represents *one* peptide. Both the inhibitory constant (K_i) and the identifying number of the peptide (Table II) are shown in each square. The N-terminal amino acid residue of the peptide is listed on the ordinate and the C-terminal residue on the abscissa of Figure 1. **KKI-9**, for example occurs in the second column in the fifth row of Figure 1. The N-terminal residue of this pentapeptide is proline (abscissa) and the C-terminal residue is valine (ordinate). K_i for this peptide is 266 μM. Blacked out squares are single amino acids (peptides having the same residue as both the N- and C-terminus).

Three types of information may be gained from results arranged as shown in Figure 1: (A) Identification of the shortest sequence having the desired inhibitory properties, (B) The effect of N-terminal extension on various properties, and (C) The effect of C-terminal extension on various properties.

K_i and Length of the Inhibitors. Figure 2 shows how data in Figure 1 may be arranged to identify all sequences containing a fixed number of amino acid residues. Inhibitors which lie on a line parallel to the hypotenuse of the triangle have the same number of amino acid residues. All dipeptides, for example, lie on a line between proline on the ordinate and valine on the abscissa. Tripeptides

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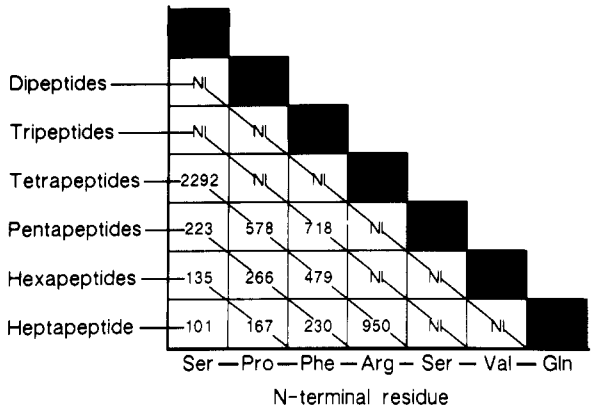

Figure 2. Substrate analogue inhibitors arranged as shown in Figure 1. Dipeptides lie on a line adjacent to and parallel with the hypotenuse, etc. (see the text).

lie on a line between phenylalanine and serine, etc.

Examination of Figure 2 shows that the shortest sequence which inhibits tissue kallikrein in the system employed is the tripeptide **KKI-23**. Previous research by Okunishi et al.[17] indicates that the shortest substrate analog to inhibit β-PPK is the tetrapeptide **KKI-3** (Ac-Arg-Ser-Val-Gln-NH_2). The evaluation of length vs K_i presented here has identified a tripeptide inhibitor not found in the original search. The shortest kallikrein inhibitor is the tripeptide Ac-Phe-Arg-Ser-NH_2 (MW 449, K_i = 718 μM) rather than the tetrapeptide Ac-Arg-Ser-Val-Gln-NH_2 (MW 529, K_i = 950 μM) reported earlier.[17]

Fielder[26] evaluated a series of kininogen analogues as substrates of β-PPK at pH 9.0 to show that deletion of the N-terminal six residues from the sequence Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Val-Gln had little effect on k_{cat}/K_m. Removal of the seventh residue, proline, then decreased k_{cat}/K_m 75-fold. Replacement of the prolyl residue at P_3 with an acetyl group restored k_{cat}/K_m to about one-fifth of the value observed for the longer peptides. The side chains of residues on the N-terminal side of P_2 thus do not markedly influence k_{cat}/K_m.[26]

Results obtained in this study parallel Fiedler's findings and indicate that the side chains of residues at P_3 and P_4 do not have much effect on K_i.

On the C-terminal side of the cleavage site, Fiedler[26] reported that the glutaminyl residue at P_3' can be replaced with an amide group to increase k_{cat}/K_m by 1 order of magnitude. Elimination of the valyl residue from the sequence Ac-Phe-Arg-Ser-Val-NH_2 then decreases k_{cat}/K_m back to values observed for the longer substrates.

The observation (Table II) that taken together the Val-Gln residues of the kininogen analogues have a limited effect on K_i is in agreement with the lack of marked change in k_{cat}/K_m reported by Fiedler.[26] K_i values for Ac-Phe-Arg-Ser-Val-Gln-NH_2 (**KKI-4**, 230 μM) and Ac-Phe-Arg-Ser-NH_2 (**KKI-23**, 718 μM) show a decrease in affinity for β-PPK that mirrors changes in k_{cat}/K_m for Ac-Phe-Arg-Ser-Val-Gln (22 mM^{-1} s^{-1}) and Ac-Phe-Arg-Ser-NH_2 (10 mM^{-1} s^{-1}). Fiedler reported, however, that replacement of the C-terminal glutaminyl residue with an amide moiety to yield Ac-Phe-Arg-Ser-Val-NH_2 increased k_{cat}/K_m (290 mM^{-1} s^{-1}) 1 order of magnitude. Elimination of the valyl residue then reduces this value to 10 mM^{-1} s^{-1}.

Data from the study reported here indicates that elimination of the glutaminyl residue always increase K_i. One possible explanation for this difference might be that the kininogen analogues listed by Fiedler which have a glutamine residue at P_3' *all* have substantially lower values for k_{cat}/K_m than would be observed with analogues lacking this residue. Thus, the 1 order of magnitude increase in k_{cat}/K_m seen on replacement of the glutamine residue with an amide group could be due to the elimination of interactions which slow cleavage of the Arg-Ser peptide bond rather than interactions which speed hydrolysis.

Chen and Bode[27] report that the α-carboxamide group of Ile[18] which occurs at the P_3' of the aprotinin–β-PPK complex is H-bonded to another segment of the inhibitor. The second segment does not exist in the kininogen analogues and these intrachain interactions are not possible. Model building using the Chen–Bode coordinates indicates that the side chain carboxamide group of glutamine may be involved in interactions which could distort the Arg-Ser cleavage site of the substrate analogues, lowering k_{cat}/K_m. Elimination of this interaction by removal of the C-terminal glutaminyl residue then allows the Arg-Ser peptide bond to be cleaved more efficiently. With the series presented here, elimination of the interaction between the glutamine residue and β-PPK is observed as weaker binding (increased K_i).

Kettner et al.[16] determined K_i for a series of peptidyl chloromethyl ketones based on the sequence of kininogen with human urinary kallikrein (HUK) at pH 7.0. K_i increases, intermittently, with peptide chain length until addition of the phenylalanyl residue at P_5, when a sharp drop in K_i signals tighter binding to the enzyme. This is interpreted to mean that the S_2 and S_5 subsites are of major importance in enzyme–substrate interactions. Filling the S_3 and S_4 subsites seems to decrease affinity of the chloromethyl ketones for the enzyme.

There are some differences between research reported by Kettner et al.[16] and that shown in Table II. Results listed in Table II support Kettner et al.'s[16] observation that the P_4 side chain is relatively unimportant in binding to the enzyme. It should also be noted that Chen and Bode[27] did not observe any interactions between the P_4 residue of aprotinin (Gly^{12}) and β-PPK that would alter K_i. The phenylalanyl residue at P_2 substantially improves K_i in the one case reported here (Table III) in a manner similar to that found by Kettner and co-workers. The differences between the two studies occur at the P_3 position where Kettner et al.[16] found that the prolyl residue at P_3 increased K_i. Although the improvement in binding found in the study reported here is small, the opposite results were observed by Kettner et al. Several explanations can be invoked to explain this discrepancy.

Research from this laboratory indicates that the volume of the P_3 side chain can have a marked effect on both K_i and K_m (Raju, B. and Burton, J., manuscript in preparation). Thus whether an inhibitor is bound in the ground state or transition state may affect the role played by residues remote from the cleavage site. Similar results have been reported for elastase.[28]

(26) Fiedler, F. Effects of secondary interactions on the kinetics of peptide and peptide ester hydrolysis by tissue kallikrein and trypsin. *Eur. J. Biochem.* **1987**, *163*, 303–312.

(27) Chen, Z.; Bode, W. Refined 2.5 Å X-ray crystal structure of the complex formed by porcine kallikrein A and the bovine pancreatic trypsin inhibitor. Crystallization, Patterson search, structure determination, refinement, structure and comparison with its components and with the bovine trypsin-pancreated trypsin inhibitor complex. *J. Mol. Biol.* **1983**, *164*, 283–311.

(28) Thompson, R. C. Binding of peptides to elastase: Implications for the mechanism of substrate hydrolysis. *Biochemistry* **1974**, *13*, 5495–5501.

(29) Eisenberg, D.; Crothers, D. Physical Chemistry with Applications to the Life Sciences; Benjamin/Cummings: Menlo Park, CA, 1979; p 149.

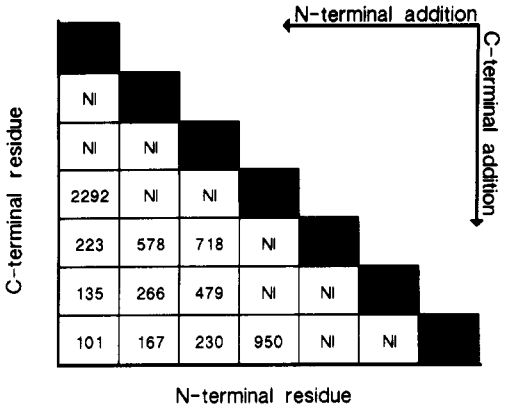

Figure 3. Substrate analogue inhibitors arranged as shown in Figure 1. Comparison of adjacent squares in a vertical column shows the effect of addition of a residue to the C-terminus. Comparison of adjacent squares in a horizontal row shows the effect of addition on the N-terminus (see the text).

Second, work reported here was performed with the two-chain β-PPK, which lacks amino acid residues between Ser^{80} and Ala^{89}. Research from Kettner and co-workers employed single-chain α-HUK. Chen and Bode[27] have called attention to the fact that residues missing from β-PPK form part of the P_3 subsite. The β-forms of kallikrein may thus have a larger S_3 subsite than do the corresponding α-forms of the enzyme. The smaller S_3 subsite in the enzyme used by Kettner and co-workers could interact with the prolyl residue to prevent binding.

A third explanation could be species specificity. Kettner et al.[16] report that the binding patterns for rat urinary kallikrein (RUK) are somewhat different than that from HUK. The differences in K_i could be due to the fact that the tissue kallikreins used in this and the previous studies were isolated from the pig and rat, respectively.

Role of Individual Residues in Binding. The approach presented here[32] makes it possible to evaluate the effect of addition of a residue on K_i in $n - 3$ cases for internal residues and $n - 2$ cases for amino acid residues occurring at either terminus. Thus, addition of the N-terminal serine and C-terminal glutamine residues on K_i may potentially be evaluated in $n - 2$ or 5 cases. Changes in K_i caused by addition of other residues may potentially be compared in $n - 3$ or 4 cases (Figure 3).

It is well-known that removal or addition of an amino acid residue may change the conformation of a peptide. One would not expect a priori that addition of a particular residue would alter the values for K_i of a series of substrate analogues to the same degree, unless the various amino acids in the peptide chain functioned in a relatively independent manner. In the series presented here, this appears to be the case. For the most part, similar changes in K_i are observed on addition of a particular residue to various core peptides (Table III).

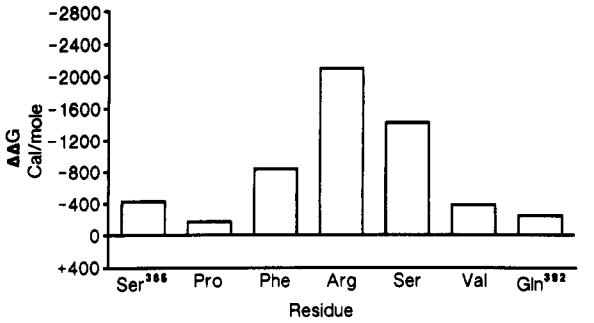

Figure 4. Average value for $\Delta\Delta G$ for each residue in the substrate analogue inhibitors.

Changes in the free energy of binding caused by addition of a single amino acid residue, $\Delta\Delta G$, may also be evaluated in quantitative terms by converting K_i values to ΔG ($\Delta G = -RT \ln K_i$[29]) and then determining the effect of each residue on ΔG (Table III). The average value of $\Delta\Delta G$ associated with each residue is plotted in Figure 4.

The values of $\Delta\Delta G$ obtained for addition of each amino acid residue appear to be reasonably consistent. The relatively similar changes in K_i caused by addition of a residue to a series of peptides, e.g. **KKI-9** and **KKI-5**, and **KKI-66** and **KKI-6**, indicate that, for tissue kallikrein, inhibitors gain in affinity by filling discrete binding sites rather than by altering conformation of the inhibitors to open up new binding modes.

$\Delta\Delta G$ values for the arginyl residues at P_i cannot be calculated directly as analogues lacking this residue do not inhibit β-PPK in the assay employed here. Some idea of the importance of this residue can be obtained by comparing ΔG for the heptapeptide **KKI-6** (−5668 cal mol^{-1}) with the sum of the individual residue shown in Table III. These calculations [−5668 − (−438 − 181 − 847 − 1436 − 396 − 256)] give a value of −2114 cal/mol for the $\Delta\Delta G$ of the arginyl residue. The percentage contribution of each residue to the value of ΔG for **KKI-6** may then be calculated: Ser, 8%; Pro, 3%; Phe, 15%; Arg, 37%; Ser, 25%; Val, 7%; Gln, 5%. Almost 80% of the total binding energy comes from the core sequence Phe-Arg-Ser. Summing the individual values of ΔG for each of the core residues (Table III) indicates that the tripeptide should have a ΔG of −4392 cal mol^{-1} (K_i = 800 μM), which compares well with the experimental value determined for **KKI-23** (−4459 cal mol^{-1}; K_i = 718 μM).

Detailed physical studies are, of course, needed to provide a molecular explanation for the values of $\Delta\Delta G$. Since these studies have not been performed with any kininogen analogue, molecular interpretations cannot be made with great confidence. An attempt to relate the average values of $\Delta\Delta G$ calculated for the various residues can be made from a model for the interaction between the kininogen substrate analogues and β-PPK which is based on structures proposed by Chen and Bode[27] for the aprotinin–PPK complex. The approach used to develop the model is given in the Experimental Section.

At S_4 Chen and Bode proposed that the naturally occurring glycyl residue of aprotinin does not interact with β-PPK. In the model used in which Gly^{12} is replaced with a seryl residue which does not appear to have any possible new interactions with β-PPK that could enhance binding. The value for $\Delta\Delta G$ at P_4 (−438 cal mol^{-1}) is consistent with this lack of major new interactions between the enzyme and inhibitor.

Both kininogen and aprotinin have a prolyl residue at P_3. At this position Chen and Bode propose that the O

(30) Taylor, R.; Kennard, O.; Verichel, W. Geometry of the N–H···O=C bond. 1. Lone-pair directionality. *J. Am. Chem. Soc.* **1983**, *105*, 5761–5766.

(31) Jencks, W. P. Catalysis in Chemistry and Enzymology; Dover Publications: New York, 1987; pp 400–403.

(32) One reviewer pointed out that S-2266 has been reported to bind to a second site in tissue kallikrein and display cleavage kinetics with a two-site model (Oliveira, L.; Araujo-Viel, M. S.; Juliano, L.; Prado, E. S. *Biochemistry* **1987**, *26*, 5032). Use of the model and the equations proposed by these authors to analyze data gathered in these experiments reported here would lead to curved Dixon plots which were not observed in the present study. It appears that the enzyme kinetics obtained for studies reported here are consistent with the standard Michaelis–Menten kinetics reported by other authors for cleavage of S-2266 by β-PPK (see the above reference for a list).

of Pro^{13} in aprotinin H-bonds to the NH of Gly^{216} in the enzyme. In the model developed here, the O of Pro in **KKI-5** and the NH of Gly^{216} are 1.74 Å apart and the N–H–O angle is 175.4°, close to optimal for H-bond formation.[30] As discussed previously, this interaction would exist where the prolyl residue is replaced by an acetyl group whose oxygen can also accept this H-bond. Loss of the prolyl ring should not drastically alter the affinity of the inhibitor for the enzyme. The low value for $\Delta\Delta G$ (–181 cal mol^{-1}) is consistent with this lack of interaction.

At the S_2 subsite the substrate analogues have a phenylalanyl residue while aprotinin has a cysteinyl residue that is part of a disulfide bridge that cross-links the chain between positions 14 and 35. Chen and Bode hypothesize that the phenylalanyl residue of kininogen fits into a wedge-shaped cavity formed between Trp^{215} and Tyr^{99} of β-PPK. The $\Delta\Delta G$ values (–847 cal mol^{-1}) for this residue are consistent with a moderate apolar interaction at this position. The value is substantially less than the 5 kcal mol^{-1} observed[31] for desolvation of an aromatic moiety. Whether the lower value is due to incomplete desolvation of the P_2 residue or to a poor fit of P_2 cannot be determined.

Available evidence indicates that the arginyl residue in the substrate analogue sequences fills S_1 rather than S_2' as it does in aprotinin. For example, several of the substrate analogues are cleaved at the Arg-Ser peptide bond by β-PPK.[19] N^{ϵ} of Lys^{15} of aprotinin is thought to form a salt bridge with Asp^{189} of the enzyme. Hydrogen bonds between the ammonium group of the aprotinin lysine and Thr^{190} and Ser^{226} of β-PPK also seem possible.[27] H-bonds and/or a salt bridge could explain the relativley high value for $\Delta\Delta G$ calculated when arginine replaces lysine (–2114 cal mol^{-1}).

At S_1' the relatively large contribution of the seryl residue to binding of the substrate analogues may also be explained by the model developed from the Chen–Bode coordinates. These authors proposed that the side chain of the P_1' residue in aprotinin (alanine) fits into a narrow channel formed between His^{57} and Cys^{42} of β-PPK. Replacement of Ala^{16} by a seryl residue indicates that the H-atom of the side chain hydroxyl group is well placed to interact with either the O of the side chain amide of Gln^{41} or the O of His^{57}.

At S_2' no obvious explanation for the increase in $\Delta\Delta G$ is observed in the model. In the aprotinin–β-PPK complex S_2 is occupied by Arg^{27}. Chen and Bode suggested that energy gained by the binding of the arginyl residue permits the anomalous binding of kininogen, so that the cleavage which releases lysylbradykinin occurs between the Met-Lys bond rather than, as expected, the Lys-Arg bond. Model building indicates that there is adequate room for the valyl residue in the S_2' pocket. In addition, the N*H* of the valyl residue should be able to maintain the H-bond with O of Gln^{41} seen when the arginyl residue is in place.

At S_3' the Ile^{18} residue which occurs at P_3' of aprotinin forms two intramolecular hydrogen bonds in the Chen–Bode model. As discussed previously, these H-bonds are not possible with the kininogen substrate analogues which are missing the critical residues. Both the relatively large value for $\Delta\Delta G$ observed in these studies and the change in k_{cat}/K_m reported by Fiedler indicate that some type of interaction between the enzyme and the inhibitor does occur at this location.

Experimental Section

Enzyme Inhibition. Porcine pancreatic kallikrein (β-kallikrein, lot 26F-0197, 50 units mg^{-1} protein; 1 unit hydrolyses 1.0 μM BAEE to *N*-α-benzoyl-L-arginine min^{-1} at pH 8.7 at 25 °C) was purchased from Sigma (St. Louis, MO). The rate of hydrolysis of BAEE was unaffected in the presence of lima bean trypsin inhibitor, indicating that the enzyme preparation contained little, if any, active trypsin.

K_i was determined from the capacity of the substrate analogues to inhibit β-PPK. Automated assay were performed on a Biomek 1000 laboratory workstation (Beckman Instruments). These involved inhibition of the cleavage of the chromogenic substrate S-2266 (S-2266, D-Val-Leu-Arg-pNA). Assays were performed in 0.12 M Tris·HCl buffer (pH 8.0). Concentration of the chromogenic substrate was either 30 or 60 μM. Full details of the assay are provided as supplementary material.

Stability of the inhibitors was evaluated by taking samples of the reaction mixture after 5 min. These were subjected to reversed-phase HPLC under conditions used for purification of the peptide. None of the inhibitors were significantly hydrolyzed under the conditions used to determine K_i.

Model Building. Molecular modeling was done on a Silicon Graphics IRIS 4D-70 workstation using Quanta 3.0 (Polygen, Waltham, MA) software for creation, manipulation, and visualization of molecular structures as previously reported.[13] The coordinates for aprotinin–β-PPK complex were obtained from Brookhaven Protein Data Bank (Brookhaven, NY) and read into Quanta. The coordinates for polar hydrogen atoms were then created using the HBUILD subroutine. The resulting structure was refined by using 250 steps of steepest descents energy minimization. Residues 12–18 of aprotinin were cut from the rest of the aprotinin molecule by deleting the bonds between Thr^{11}-Gly^{12}, Ile^{18}-Ile^{19}, and the disulfide bond between Cys^{14}-Cys^{38}. The aprotinin segment devoid of residues 12–18 was deleted to yield the new structure which displayed β-PPK in contact with aprotinin residues 12–18 only. These residues were then replaced by residues Ser, Pro, Phe, Arg, Ser, Val, and Gln, respectively, to mutate the aprotinin segment into that of the substrate analogues. After each replacement, a relatively unhindered conformation for the side chain atoms of each residue was obtained by using the SPIN subroutine followed by energy minimization for 250 steps of steepest descents and 250 steps of the adapted-basis Newton–Raphson method. The structure in which all residues had been altered was finally subjected to an additional 250 steps of steepest descents and 250 steps of the adapted-basis Newton–Raphson method to yield the model used in these studies.

Peptide Synthesis. *tert*-Butyloxycarbonyl amino acids were purchased from Peninsula Laboratories (San Mateo, CA). Side-chain-protecting groups are as follows: glutamine, xanthyl; serine, benzyl; and arginine, tosyl. Tritated Boc-Pro and Boc-Val were prepared by reaction of labeled amino acid (Du Pont, Boston, MA), diluted approximately 0.1 Ci mol^{-1} with unlabeled amino acid (Eastman Kodak, Rochester, NY), with di-*tert*-butyl dicarbonate[33] (Tridom, Hauppage, NY). Both CF_3COOH and DCC were obtained from Aldrich (Milwaukee, WI). CH_2Cl_2 (Dow, Midland, MI) was redistilled before use. The support for solid-phase synthesis was *p*-methylbenzylhydrylamine resin containing 0.47 mmol of NH_2 g^{-1} (USB, Cleveland, OH). Dioxane (Fisher, Medford, MA) was redistilled from Na; 6 N HCl–dioxane was prepared by bubbling electronic-grade HCl gas (Matheson, Gloucester, MA) through dioxane cooled in an ice bath. The concentrated solution was then diluted to 6 N and stored in a closed container. Other reagents were of analytical grade.

Synthetic reactions were performed in 60-mL polypropylene syringes fitted with a frit (70-μm porosity, Bolab, Derry, NH) by using apparatus and techniques previously described.[22] Completeness of coupling was judged by the ninhydrin test[34] both by observing the bulk reaction and by viewing the beads on filter paper at 40× under a dissecting microscope.[22]

Peptides were hydrolyzed at 105 °C for 24 h under vacuum in 6 N HCl. Amino acid analyses were performed with a Beckman D-6000 analyzer.

UV spectra were obtained on an HP8590A spectrophotometer equipped with an HP7445A plotter (Hewlett-Packard, Palo Alto,

(33) Bodanszky, M.; Bodanszky, A. *The Practice of Peptide Synthesis*; Springer-Verlag: New York, 1984; p 20.
(34) Sarin, V. K.; Kent, S. B.; Tam, J. P.; Merrifield, R. B. Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.* **1981**, *117*, 147–157.

CA). HPLC purifications were done on a Beckman ODS column (1 × 25 cm) with a gradient of 0–100% CH_3CN–0.2% CF_3COOH in H_2O–0.2% CF_3COOH over 20 min.

Data from the analysis of the synthetic peptides is summarized in Table I.

Acetyl[^{3}H]prolylphenylalaninamide, KKI-21. *p*-Methylbenzhydrylamine resin (3.0 g) containing 0.47 mmol of NH_2 was placed in a synthesis syringe,[22] thoroughly washed with CH_2Cl_2, and neutralized with 5% (v/v) Et_3N–CH_2Cl_2. After washing, a 5-fold excess of Boc-Phe and DCC were used to quantitatively couple the C-terminal amino acid to the polymer over a period of 1 h. The aminoacyl polymer was deprotected, neutralized, and coupled with Boc[^{3}H]Pro. After addition of the prolyl residue, the polymer was deprotected, neutralized, and acetylated with a 5-fold excess of Et_3N–Ac_2O in CH_2Cl_2. The peptidyl polymer was then washed well with EtOH and dried to constant weight over P_2O_5. The dipeptidyl resin (3.0 g) was treated with HF–anisole (9:1) for 1 h at 0 °C. After evaporation at high vacuum, the cleaved resin was transferred to a coarse Hirsch funnel with cold AcOEt and sequentially extracted with 100 mL of 1, 5, 10, and 25% (v/v) AcOH solution. Most of the radioactivity (90%) was found in the 1% extract which was lyophilized to yield the crude peptide (20 mg). This was purified by gel filtration on Sephadex G-15 (1 × 110 cm) in 5% AcOH solution with a flow rate of 15 mL h^{-1}. Fractions from the column were counted for radioactivity, and the absorbance (258 nm) was measured. Fractions (77–82 mL) which had a constant ratio of absorbance and cpm mL^{-1} were then pooled and lyophilized to yield 14 mg (75%) of homogeneous **KKI-21**.

Acetyl[^{3}H]valylglutaminamide, KKI-1. The C-terminal glutaminyl residue of **KKI-1** was quantitatively coupled to 3.0 g 4-methylbenzhydrylamine resin using the nitrophenyl ester in DMF. After addition of Boc[^{3}H]valine, deprotection, neutralization, and acetylation, 3.0 g of peptidyl resin was cleaved with HF–anisole 9:1 for 1 h at 0 °C. Radioactivity (90%) was found in the 1% extract, which was lyophilized to yield 17 mg of peptide. This material was dissolved in 1% acetic acid solution and gel filtered on Sephadex G-15. Material eluting between 85 and 89 mL had a constant ratio of absorbance (220 nm) and radioactivity (cpm mL^{-1}) and was pooled and lyophilized to yield 12 mg of homogeneous **KKI-1** (71%).

Acetylseryl[^{3}H]valylglutaminamide, KKI-2. Peptide **KKI-2** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded 25 mg of material which was gel filtered on Sephadex G-15. Fractions eluting between 75 and 79 mL were lyophilized to yield 21 mg of homogeneous **KKI-2** (84%).

Acetylarginylseryl[^{3}H]valylglutaminamide, KKI-3. Peptide **KKI-3** was synthesized on 1.5 g of 4-methylbenzhydrylamine resin using standard procedures. HF clevage, extraction, and lyophilization of the 1% acetic acid extract yielded a white powder (99%) which was dissolved in 5% acetic acid solution and gel filtered on Sephadex G-15 (2.5 × 110 cm) in the same solvent. Fractions eluting between 294 and 333 mL were lyophilized to yield 135 mg of homogeneous **KKI-3** (51%).

Acetylphenylalanylarginylseryl[^{3}H]valylglutaminamide, KKI-4. Peptide **KKI-4** was synthesized on 1.50 g of 4-methylbenzhydrylamine resin using standard procedures. After HF cleavage and extraction, 90% of the radioactivity was found in the 1% acetic acid extract and 10% in the AcOEt extract. The latter was evaporated and the residue combined with 1% extract which was lyophilized to yield a white powder (81%). This was gel filtered on Sephadex G-15 (1.1 × 110 cm) in 5% acetic acid solution. Fractions eluting between 48 and 58 mL were lyophilized to yield 169 mg of homogeneous **KKI-4** (60%).

Acetylprolylphenylalanylarginylseryl[^{3}H]valylglutaminamide, KKI-5. Peptide **KKI-5** was synthesized on 1.4 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (91%) which was gel filtered on Sephadex G-15 (1.1 × 110 cm) in 5% acetic acid solution. Fractions eluting between 49 and 58 mL were lyophilized to yield 155 mg of homogeneous **KKI-5** (56%).

Acetylserylprolylphenylalanylarginylseryl[^{3}H]valylglutaminamide, KKI-6. Peptide **KKI-6** was synthesized on 1.50 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (94%) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 43 and 55 mL were lyophilized to yield 344 mg of homogeneous **KKI-6** (88%).

Acetylprolylphenylalanylarginylseryl[^{3}H]valinamide, KKI-9. Peptide **KKI-9** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (80%) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 44 and 50 mL were lyophilized to yield 72 mg of homogeneous **KKI-9** (90%).

Acetyl[^{3}H]prolylphenylalanylarginylserinamide, KKI-19. Peptide **KKI-19** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (35 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 64 and 68 mL were lyophilized to yield 28 mg of homogeneous **KKI-19** (80%).

Acetyl[^{3}H]prolylphenylalanylargininamide, KKI-20. Peptide **KKI-20** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (34 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 48 and 52 mL were lyophilized to yield 29 mg of homogeneous **KKI-20** (85%).

Acetylphenylalanylargininamide, KKI-22. Peptide **KKI-22** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (18 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 55 and 61 mL were lyophilized to yield 14 mg of homogeneous **KKI-22** (77%).

Acetylphenylarginylserinamide, KKI-23. Peptide **KKI-23** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (28 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 58 and 64 mL were lyophilized to yield 21 mg of homogeneous **KKI-23** (75%).

Acetylseryl[^{3}H]prolylphenylalanylargininamide, KKI-24. Peptide **KKI-24** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (200 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 59 and 63 mL were lyophilized to yield 170 mg of homogeneous **KKI-24** (85%).

Acetylseryl[^{3}H]prolylphenylalanylarginylserinamide, KKI-25. Peptide **KKI-25** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (60 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 59 and 63 mL were lyophilized to yield 50 mg of homogeneous **KKI-25** (83%).

Acetylarginylserinamide, KKI-26. Peptide **KKI-26** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF clevage, extraction, and lyophilization of the 1% extract yielded a white powder (18 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 78 and 82 mL were lyophilized to yield 12 mg of homogeneous **KKI-26** (66%).

Acetylseryl[^{3}H]valinamide, KKI-65. Peptide **KKI-65** was synthesized on 3.0 g 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and concentration of the 1% AcOH extract yielded a white solid (15 mg) which was purified to homogeneity by HPLC.

Acetylserylprolylphenylalanylarginylseryl[^{3}H]valinamide, KKI-66. Peptide **KKI-66** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and lyophilization of the 1% extract yielded a white powder (56 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 62 and 68 mL were lyophilized to yield 48 mg of homogeneous **KKI-66** (86%).

Acetylphenylalanylarginylseryl[^{3}H]valinamide, KKI-69. Peptide **KKI-69** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage,

extraction, and lyophilization of the 1% extract yielded a white powder (62 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 53 and 58 mL were lyophilized to yield 54 mg of homogeneous **KKI-69** (87%).

Acetylarginylseryl[^{3}H]valinamide, KKI-70. Peptide **KKI-70** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and concentration of the 1% extract yielded a white solid (21 mg) which was purified to homogeneity by HPLC.

Acetylseryl[^{3}H]prolylphenylalaninamide, KKI-72. Peptide **KKI-71** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and concentration of the EtOAc extract yielded a white solid (20 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 79 and 83 mL were lyophilized to yield 15 mg of homogeneous **KKI-71** (75%).

Acetylseryl[^{3}H]prolinamide, KKI-72. Peptide **KKI-72** was synthesized on 3.0 g of 4-methylbenzhydrylamine resin using standard procedures. HF cleavage, extraction, and concentration of the EtOAc extract yielded a white solid (15 mg) which was gel filtered on Sephadex G-15 (1.1 × 110 cm). Fractions eluting between 93 and 97 mL were lyophilized to yield 8 mg of homogeneous **KKI-72** (53%).

Acknowledgment. This research was supported by the NIH Grant HL-38212. Amino acid analyses were performed by Mr. George Crombie, Department of Biochemistry, Boston University School of Medicine, Boston, MA. We are grateful to Mr. O. David Carretero, currently of Hypertension Research, Henry Ford Hospital, Detroit, MI, for the synthesis of **KKI-19**, **KKI-26**, and **KKI-20**; Dr. H. Okunishi, Department of Pharmacology, Kyoto Medical College, Kyoto, Japan, for the synthesis of **KKI-1-6**; and Dr. C. Nakaie, Department of Biophysics, Escola Paulista de Medicina, São Paulo, Brazil, for the synthesis of **KKI-9.**

Supplementary Material Available: A description of the computer programs and the use of BIOMEK 1000 for K_i determinations (11 pages). Ordering information is given on any current masthead page.

Synthesis and Pharmacological Evaluation of Enantiomerically Pure 4-Deoxy-4-fluoromuscarines

Pierfrancesco Bravo,*[†] Giuseppe Resnati,*[‡] Piero Angeli,*[§] Massimo Frigerio,[†] Fiorenza Viani,[‡] Alberto Arnone,[‡] Gabriella Marucci,[§] and Franco Cantalamessa[∥]

Dipartimento di Chimica del Politecnico, C.N.R.-Centro Studio Sostanze Organiche Naturali, Piazza Leonardo da Vinci 32, I-20133 Milano, Italy, Dipartimento di Scienze Chimiche, Via S. Agostino 1, 62032 Camerino (MC), Italy, and Instituto di Farmacologia e Farmacognosia, Via Scalzino, 62032 Camerino (MC), Italy. *Received January 13, 1992*

Four isomers of [(4-fluoro-5-methyl-tetrahydrofuran-2-yl)methyl]trimethylammonium iodide (4-deoxy-4-fluoromuscarines) were prepared in enantiomerically and diastereomerically pure form from (*S*)-(−)-methyl 4-methylphenyl sulfoxide, ethyl fluoroacetate, and allyl bromide. Their absolute configurations were assigned by ^{1}H NMR analyses. The four optically pure compounds were tested in vitro on guinea pig and their muscarinic potency was evaluated at M_3 (ileum and bladder) and M_2 (heart) muscarinic receptor subtypes. Compound **1a**, the most potent isomer of the series, was also tested in vivo on pithed rat and its muscarinic activity at the M_1 receptor subtype was compared with that of muscarine. Moreover, affinity and relative efficacy were calculated in vitro for this compound at M_2 (heart force and rate) and M_3 (ileum and bladder) receptors in order to investigate muscarinic receptor heterogeneity. The 4-deoxy-4-fluoromuscarines display a similar trend of potency as the corresponding muscarines and compound **1a** shows differences in the affinity constants among the studied tissues. Replacement of a hydroxyl group for a fluorine atom in the 4 position of muscarine produces 1 order of magnitude increase in affinity for cardiac M_2 muscarinic receptors controlling rate, while the affinity at cardiac M_2 muscarinic receptors controlling force is unchanged, opening the possibility of a further classification of cardiac muscarinic receptors.

Fluorine-substituted analogues of naturally occurring and biologically active organic compounds have become the focus of increasing interest for the purpose of creating new drugs.[1a,b] Moreover, there are a number of new fluorinated products which are useful probes for studying biochemical processes and there may also be a future for some of them in clinical diagnostics.[1c-f] Introduction of fluorine atoms allows preparation of modified structures which differ only slightly in their steric hinderance from the corresponding naturally occurring molecules. As a result, several of these fluorinated analogs retain the biological activity of the parent compounds.[1g,h]

Considerable attention has been recently devoted to investigations on muscarinic receptors.[2] Many of these efforts have involved exploration of the drug-receptor interactions for novel structures in order to better distinguish the different muscarinic receptor subtypes and their presence and role in different tissues.[3] Some of us have been already involved in those studies[4] and have recently developed new synthetic methods for the construction of fluoroorganic molecules in their optically pure forms.[5] We expanded our efforts by synthesizing and testing a number

[†] Dipartimento di Chimica del Politecnico.
[‡] C.N.R.-Centro Studio Sostanze Organiche Naturali.
[§] Dipartimento di Scienze Chimiche.
[∥] Instituto di Farmacologia e Farmacognosia.

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