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Design of Potent Competitive Inhibitors of Angiotensin-Converting Enzyme. Carboxyalkanoyl and Mercaptoalkanoyl Amino Acids†

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ABSTRACT: A hypothetical model of the active site of angiotensin-converting enzyme has been utilized to guide the design and synthesis of specific inhibitors. By analogy to bovine carboxypeptidase A, the active site of angiotensin-converting enzyme was proposed to contain three important groups that participate in binding of peptide substrates: a carboxyl-binding group, a group with affinity for the C-terminal peptide bond, and a tightly bound zinc ion that could coordinate with the carbonyl of the penultimate (scissile) peptide bond. According to the model, a succinyl amino acid could interact with each of these binding groups via its amino acid carboxyl, amide bond, and succinyl carboxyl, respectively, and thus act as a specific competitive inhibitor of the enzyme. Succinyl-L-proline was found to be such an inhibitor (I_{50} = 330 μM), and attempts to optimize its interaction with the active site of the enzyme as proposed in the model led to the synthesis of D-2-methylsuccinyl-L-proline (*R,S*) (K_i = 2.5 μM), and D-2-methylglutaryl-L-proline (*R,S*) (K_i = 0.8 μM). Replacement of the succinyl carboxyl group of these compounds by a sulfhydryl group led to a series of extremely potent competitive inhibitors of angiotensin-converting enzyme, including 3-mercaptopropanoyl-L-proline (SQ 13 863, K_i = 0.012 μM) and D-3-mercapto-2-methylpropanoyl-L-proline (*S,S*) (SQ 14 225, K_i = 0.0017 μM). These compounds are also potent orally active inhibitors of angiotensin-converting enzyme and have great potential as antihypertensive agents.

Angiotensin-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) is an exopeptidase that cleaves dipeptides from the carboxyl-terminal end of various peptide substrates (Piquilloud et al., 1970; Cushman and Cheung, 1971; Massay and Fessler, 1976; Angus et al., 1972; Yang et al., 1971; Elisseeva et al., 1971); like the similar carboxypeptidases, it is a zinc containing enzyme (Das and Soffer, 1975). Two reactions catalyzed by angiotensin-converting enzyme may play a role in blood pressure regulation: "conversion" of the inactive decapeptide angiotensin I to the potent vasopressor octapeptide angiotensin II (Skeggs et al., 1956) and inactivation of the vasodepressor nonapeptide bradykinin (Yang et al., 1971; Elisseeva et al., 1971; Dorer et al., 1974). The importance of one or both of these reactions in hypertensive disease has been greatly clarified in recent years by the development of a potent and specific inhibitor of angiotensin-converting enzyme, the nonapeptide SQ 20 881 (Ondetti et al., 1971, 1972c; Cushman and Cheung, 1972; Cheung and Cushman, 1973; Engel et al., 1972; Keim et al., 1972; Bianchi et al., 1973). SQ 20 881 lowers blood pressure in animal models of renovascular hypertension (Engel et al., 1973; Muirhead et al., 1974), in most patients with renovascular or malignant hypertension, and in many patients with essential hypertension (Gavras et al., 1974, 1975; Johnson et al., 1975; Case et al., 1976). In spite of these outstanding actions, however, the therapeutic utility of SQ 20 881 is limited by its lack of oral activity. Our understanding of the binding of peptide inhibitors to angiotensin-converting enzyme, based on extensive studies with SQ 20 881 and its analogues (Ondetti et al., 1972a,b; Pluščec et al., 1973; Cushman et al.,

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1973), and the general increase in understanding of the substrate specificity and other properties of angiotensin-converting enzyme have now enabled us to undertake the design of a new class of potent and specific inhibitors.

Since angiotensin-converting enzyme is a zinc-containing exopeptidase with many properties similar to those of the pancreatic carboxypeptidases, we felt that its mechanism of action and its active site might both be similar to those of bovine pancreatic carboxypeptidase A which are well understood (Quiocho and Lipscombe, 1971). Figure 1 presents our diagrammatic model of a hypothetical active site of angiotensin-converting enzyme based on the presumed similarity of this enzyme to carboxypeptidase A. A positively charged residue at the active site (analogous to Arg-145 of carboxypeptidase A) is postulated to bind with the negatively charged C-terminal carboxyl group of the peptide substrate. The enzyme-bound zinc ion of angiotensin-converting enzyme, which is expected to play a role in peptide bond cleavage, is shown as separated from the positively charged residue of the enzyme by the distance of a dipeptide residue, as opposed to the distance of a single amino acid residue in carboxypeptidase A. Angiotensin-converting enzyme, having no specificity for C-terminal hydrophobic amino acids, would not be expected to have at its active site a "hydrophobic pocket" such as that shown for carboxypeptidase A; but it may have some affinity for the side chains (R_1 and R_2) of the two terminal amino acid residues of a peptide substrate. We have assumed, in addition, that angiotensin-converting enzyme may bind, probably by hydrogen bonding, to the terminal, nonscissile, peptide bond of the substrate.

An excellent opportunity to test this purely hypothetical model of the active site of angiotensin-converting enzyme came from the observation of Byers and Wolfenden (1972, 1973) that D-2-benzylsuccinic acid was an unusually potent competitive inhibitor of carboxypeptidase A. They proposed that this compound served as a "biproduct analogue" that was bound to the active site of carboxypeptidase A in a manner that combined the modes of binding of the two products of the enzyme's action, i.e., the amino acid and the newly liberated carboxyl group. This latter group probably binds to the zinc ion of the enzyme (Figure 1). According to our hypothetical model, a succinyl amino acid might be expected to serve as a similar biproduct inhibitor of angiotensin-converting enzyme (Figure 1). In the present paper we will describe extensive structure–activity studies that originated from the assumptions summarized above. These studies have led to a confirmation of the general validity of the hypothetical model of the active site of angiotensin-converting enzyme and eventually to the development of an extremely potent class of inhibitors that have great potential as orally active antihypertensive drugs of novel mechanism.

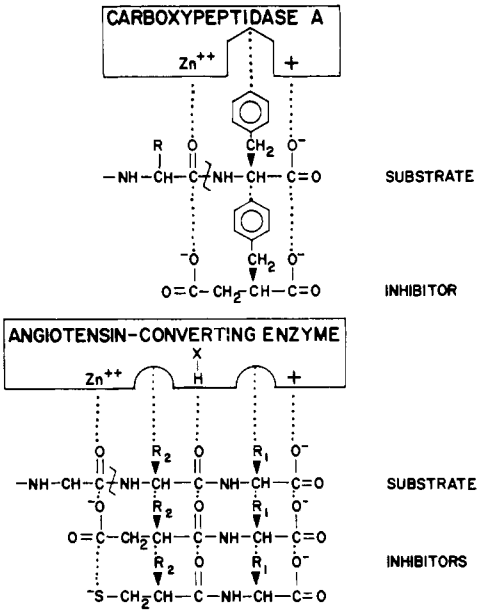

FIGURE 1: Diagrammatic model of the active site of carboxypeptidase A of bovine pancreas and the analogous hypothetical active site of angiotensin-converting enzyme. For each enzyme, the known or proposed binding of peptide substrates and competitive inhibitors is indicated. The hexagonal cleft in the model of the active site of carboxypeptidase A represents the hydrophobic pocket of this enzyme; the circular clefts shown for angiotensin-converting enzyme represents portions of the active site that interact with substituents R_1 and R_2 of substrates or competitive inhibitors by an undetermined mechanism; X–H represents a hydrogen bonding residue at the active site of angiotensin-converting enzyme.

Experimental Procedure

Enzymatic Studies. Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) was purchased from Bachem Fine Chemicals, Inc. Acetone powder of rabbit lung was obtained from Pel-Freez Biologicals, Inc. Homogeneous angiotensin-converting enzyme of rabbit lung was prepared from acetone powder of rabbit lung according to a previously published procedure (Cheung and Cushman, 1973). Carboxypeptidase A, trypsin, and chymotrypsin of bovine pancreas, leucine aminopeptidase of hog kidney, and carboxypeptidase B of hog pancreas were all obtained from Worthington Biochemical Corporation. Other chemicals were reagent grade and obtained from various commercial sources.

Spectrophotometric assay of the hydrolysis of Hip-His-Leu by angiotensin-converting enzyme was performed as described earlier (Cushman and Cheung, 1971). The fluorometric assay of His-Leu produced by the action of angiotensin-converting enzyme on Hip-His-Leu, and kinetic studies of inhibition of the enzyme employing this sensitive assay procedure were performed as described by Cheung and Cushman (1973).

Synthesis of Carboxyalkanoyl and Mercaptoalkanoyl Amino Acids. Two types of amino acid derivatives were synthesized to explore enzyme–inhibitor interactions: carboxyalkanoyl and mercaptoalkanoyl amino acids (Tables I–IV). The syntheses of some carboxyalkanoyl amino acids have been described in several isolated reports (e.g., Šormand Pravda, 1951; Berse et al., 1962; Bose and Strube, 1963; Flavin and Slaughter, 1965; Ogawa et al., 1973), but no comprehensive description of their preparation and properties is available. We have found that the method of choice for the synthesis of unsubstituted succinyl amino acids is the reaction with succinic anhydride in glacial acetic acid, as described by King et al. (1957) for maleyl amino acids. Hydrogenation of the corresponding maleyl amino acids, as described below for the case of L-serine, can also be used as an alternate route to succinyl amino acids. Heating of L-leucine with succinic anhydride in pyridine led to extensive racemization. However, this method was successfully used for the synthesis of glutaryl-L-proline. The oxalyl, malonyl, and adipoyl derivatives were synthesized via the monoester of the corresponding diacid.

In the case of substituted succinic acids the problem of the regiospecificity in the opening of the substituted succinic anhydride was a crucial one. Early attempts to obtain monobenzyl esters by reaction of methylsuccinic anhydride with benzyl alcohol led to mixtures of positional isomers. The high regiospecificity observed in the opening of the itaconic anhydride (Baker et al., 1952; Hancock and Linstead, 1953) allowed the preparation of pure intermediate monoesters of 2-methylsuccinic acid and the direct synthesis of the 3-methyl-

succinyl-L-proline by the direct reaction of proline with itaconic anhydride. The shielding effect observed on the chemical shift of the protons α to the free carboxyl group in going from the free acid to the salt (van Gorkom, 1966) was utilized to confirm the position of the methyl substituent in the methylsuccinyl derivatives.

The reaction of 2-methylglutaric anhydride with methanol and benzyl alcohol followed by fractional crystallization of the dicyclohexylammonium salts yielded the pure γ-methyl and γ-benzyl ester, respectively.

Coupling of the succinic or glutaric acid monoesters with the amino acid esters was carried out by the carbodiimide procedure to avoid the possibility of isomerization observed with more drastic activation procedures (Chase and Hey, 1952). Formation of acylurea by-products was observed in most of the cases but they could be easily separated by silica gel chromatography or by an acid–base wash after cleavage of the *tert*-butyl esters. The succinyl derivatives were found to be very acid sensitive, particularly in the case of proline. Acid hydrolysis of 2-methylsuccinyl-L-proline proceeds several times faster than that of 2-methylglutaryl-L-proline. These findings are in agreement with the known acid lability of aspartyl peptide bonds and particularly Asp-Pro bonds (Piszkiewicz et al., 1970). Aqueous solutions of 2-methylsuccinyl-L-proline (pH 3) hydrolyze spontaneously at room temperature with liberation of proline.

The synthesis of mercaptoalkanoyl amino acids has been the subject of several literature reports (Mita et al., 1966, 1975; Vasilevskii et al., 1970). However, these studies have been mostly directed toward α-mercaptoalkanoyl derivatives. The methods for the synthesis of these compounds are adaptations of the classical Fisher method for synthesis of peptides, e.g., acylation of amino acids with haloalkanoyl chlorides, followed by nucleophilic displacement. For the synthesis of mercaptoalkanoyl amino acids the nucleophile of choice has been thiobenzoic acid. We found this procedure very suitable for the synthesis of 3-mercaptopropanoyl amino acids of varied functionality. The most suitable intermediates for the synthesis of the substituted mercaptopropanoyl amino acids were the corresponding 3-acetylthiopropionic acids obtained by addition of thiolacetic acid to the substituted acrylic acid (Holmberg and Schjänberg, 1940). The separation of the two diastereomeric proline derivatives could be achieved by a short fractional crystallization of the dicyclohexylammonium salts. The reasonable ease with which the diastereomeric proline derivatives could be separated in these two series (carboxy- and mercaptoalkanoyl) is in agreement with the observations of Halpern and Westley (1965) that proline derivatives could be useful resolving agents for amino acids.

The isolation of the mono- or dicarboxylic acids was carried out whenever possible by extraction with organic solvents. In those cases in which water solubility was very high a sulfonic acid resin [AG-50W-X2 (Bio-Rad Laboratories)] was used; the free acids were eluted with water and the amino or guanidino acids with 1 M pyridinium acetate buffer (pH 6.5). Dicyclohexylammonium salts (DCHA)[1] were prepared in organic solvents by addition of dicyclohexylamine until pH 8–9 (moist pH paper). The dicyclohexylammonium salts were converted to the free acids by distribution between ethyl acetate and 10% aqueous potassium bisulfate, or by passage through a column of AG-50W-X2 in water. Sodium or potassium salts were prepared in ether or ethyl acetate with sodium or potassium 2-ethylhexanoate. Homogeneity of intermediates and final products was determined by paper electrophoresis and thin-layer chromatography. The latter was carried out on Merck silica gel plates developed with one of the following solvent systems: (1) 1-butanol–pyridine–acetic acid–water (30:20:6:24); (2) 1-butanol–acetic acid–water (4:1:1); (3) chloroform–methanol–ammonia (60:45:20); (4) chloroform–methanol–38% acetic acid (6:4:2); (5) benzene–acetic acid (7:3); (6) benzene–acetic acid (7:1). Paper electrophoresis was carried out on a Camag high-voltage electrophoresis apparatus at 50 V/cm with buffer pH 6.5 (1.2 M pyridinium acetate) or pH 1.9 [formic acid–acetic acid–water (2.6:12:100)]. The following reagents were used for detection (Block et al., 1958): aniline–xylose (carboxylic acids), sodium nitroprusside (thiols), Sakaguchi (arginine), and iodine vapor. Nuclear magnetic resonance (NMR) spectra were obtained for all intermediates and final products in a Varian T-60 spectrometer and were in agreement with the proposed structures. Rotations were measured with a Perkin-Elmer automatic polarimeter Model 141. Melting points were determined in open capillary tubes and are not corrected.

The synthesis of the most active of all the derivatives discussed in this paper, SQ 14 225, is described in full detail below. The description of the syntheses of all other carboxy and mercaptoalkanoyl amino acids is available as supplementary material (see paragraph at end of paper).

1-(D-3-Acetylthio-2-methylpropanoyl)-L-proline (*S,S*). To a solution of proline *tert*-butyl ester (20.5 g) and dicyclohexylcarbodiimide (24.7 g) in dichloromethane (180 mL) chilled in an ice bath, 3-acetylthio-2-methylpropionic acid (19.4 g) (Fredga and Mårtensson, 1942) is added and the mixture is stirred at room temperature for 16 h. The dicyclohexylurea is removed by filtration and the filtrate is washed neutral, dried ($MgSO_4$), and concentrated to dryness in vacuo. The crude *tert*-butyl ester (41.8 g) is dissolved in a mixture of trifluoroacetic acid (500 mL) and anisole (240 mL) and the solution is stored at room temperature for 1 h. The solvent is removed in vacuo and the residue is distributed between saturated sodium bicarbonate (300 mL) and ethyl acetate (600 mL). The aqueous layer is acidified and extracted with ethyl acetate (2 × 300 mL). The organic layers are dried ($MgSO_4$) and concentrated to dryness in vacuo. The residue is converted to the DCHA salt in acetonitrile. The crude salt (mp 173–178 °C) is boiled with acetonitrile, chilled, filtered, and recrystallized from 2-propanol: yield, 13.3 g; mp 188–190 °C (sintering 173 °C); $[\alpha]^{22}_D$ −67.0° (*c* 1.4, EtOH).

The DCHA salt was distributed between ethyl acetate and 5% potassium bisulfate. The organic layer was dried ($MgSO_4$) and concentrated to dryness. The residue was crystallized from ether–hexane: yield, 6.5 g; mp 83–85 °C (sintering 78 °C); $[\alpha]^{22}_D$ −164.4° (*c* 2.4, EtOH); R_f[5] 0.28. Anal. Calcd for $C_{11}H_{17}NO_4S$: C, 50.96; H, 6.61; N, 5.40; S, 12.38. Found: C, 50.96; H, 6.65; N, 5.35; S, 12.52.

From the acetonitrile mother liquors of the DCHA salt of the D,L isomer the DCHA of the L,L isomer can be isolated by digestion with ether: mp 136–137 °C (sintering 132 °C). The free acid is obtained as described above: mp 94–95 °C (sintering 92 °C) (ethyl acetate–hexane); $[\alpha]^{22}_D$ +17.8° (*c* 1.3, EtOH).

1-(D-3-Mercapto-2-methylpropanoyl)-L-proline (*S,S*) (**32**). A solution of 1-(D-3-acetylthio-2-methylpropanoyl)-L-proline (5.5 g) in 5.5 N methanolic ammonia (50 mL) was stored at room temperature under argon for 2 h. The solvent was removed in vacuo and the residue was applied to a column of AG-50W-X2 and eluted with water. The free acid was converted to a DCHA salt in acetonitrile: yield 6.2 g; mp 188–189 °C (sintering 175 °C). The DCHA was distributed between ethyl acetate and 5% aqueous potassium bisulfate. The

[1] Abbreviation used is: DCHA, dicyclohexylammonium.

TABLE I: Inhibition of Angiotensin-Converting Enzyme of Rabbit Lung by Succinyl and 3-Mercaptopropanoyl Derivatives of Amino Acids.

		I_{50} (μM)[a]	
		a	b
No.	Amino acid (AA)	$HOOCCH_2CH_2CO$-AA	$HSCH_2CH_2CO$-AA
1	L-Pro	330	0.20
2	L-Arg	470	0.65
3	L-Phe	550	0.43
4	L-Leu	610	1.6
5	L-Met	750	
6	L-Ile	1040	
7	L-Val	1100	
8	L-Ala	1340	0.85
9	Gly	1990	2.8[b]
10	L-Ser	2440	
11	L-His	>3000	
12	D-Phe	>3000	
13	L-Lys		2.4
14	L-Asp		68
15	β-Ala		490
16	D-Pro		1800

[a] Concentration inhibiting 50% of the activity of angiotensin-converting enzyme at pH 8.3 in 100 mM potassium phosphate buffer containing 300 mM NaCl with the substrate, Hip-His-Leu, at a concentration of 5 mM. [b] The commercially available isomer DL-2-mercaptopropanoylglycine was found to have an I_{50} value of 1.7 μM.

organic layer was dried ($MgSO_4$) and concentrated to dryness. The residue was crystallized from ethyl acetate–hexane: yield, 2.6 g; mp 87–88 °C (resolidifies and melts 104–105 °C); $[\alpha]^{22}_D$ −131.0° (*c* 1.7, EtOH); R_f[4] 0.85, R_f[5] 0.26. Anal. Calcd for $C_9H_{15}NO_3S$; C, 49.78; H, 6.96; N, 6.45; S, 14.77. Found: C, 49.62; H, 6.83; N, 6.74; S, 14.58.

By a similar procedure the 1-(L-3-mercapto-2-methylpropanoyl)-L-proline (*R,S*) (**34**) can be obtained from the corresponding acetylthio derivative: mp 104–105 °C (ethyl acetate–hexane); $[\alpha]^{22}_D$ −41.1° (*c* 2.3, EtOH).

A sample of 1-(D-3-mercapto-2-methylpropanoyl)-L-proline (217 mg) was refluxed with 1 N hydrochloric acid (15 mL) for 30 h, chilled to room temperature, and extracted with chloroform. The solvent was removed in vacuo, the residue was dissolved in water, and 0.5 M iodine in ethanol was added until persistent yellow color, while maintaining the pH at 6–7. After acidification the aqueous solution is extracted with ethyl acetate. The organic layer is concentrated to dryness and the residue was crystallized from water to give 56 mg of (*S,S*)-3,3′-dithiobis-2-methylpropionic acid: mp 120–121 °C; $[\alpha]^{22}_D$ −223° (*c* 1, 1 N NH_4OH) [lit. (Ställberg, 1957), $[\alpha]^{25}_D$ −219.7°]. When a similar procedure was applied to 434 mg of 1-(L-3-mercapto-2-methylpropanoyl)-L-proline 1.2 mg of (*R,R*)-3,3′-dithiobis-2-methylpropionic acid [mp 119–120 °C; $[\alpha]^{22}_D$ +219° (*c* 1.1, 1 N NH_4OH)] was obtained.

Results and Discussion

Carboxyalkanoyl Amino Acids. The first succinyl amino acid synthesized to test the validity of the hypothetical model of the active site of angiotensin-converting enzyme depicted in Figure 1 was succinyl-L-proline. Proline was chosen as the amino acid moiety because of its presence as the carboxyl-terminal amino acid residue in SQ 20 881 and other potent and specific inhibitors of angiotensin-converting enzyme found in snake venoms (Ondetti et al., 1971; Ferreira et al., 1970; Kimura et al., 1972). As shown in Table I, succinyl-L-proline (**1a**) has an I_{50} value of 3.3×10^{-4} M for inhibition of angiotensin-converting enzyme of rabbit lung. Thus, it is only about 1/500 as potent as the snake venom peptide SQ 20 881 ($I_{50} = 5.6 \times 10^{-7}$ M; Cheung and Cushman, 1973). Although it is only a moderately potent inhibitor, succinyl-L-proline appears to be reasonably specific for inhibition of angiotensin-converting enzyme as judged by its effects on various agonists contracting strips of guinea pig ileum in vitro (Ondetti et al., 1977; Rubin et al., submitted for publication); it inhibits the contractile response of this smooth muscle to angiotensin I and augments the contractile response to bradykinin (two activities shared by all inhibitors of angiotensin-converting enzyme), but has no effect on the contractile responses elicited by other agonists such as angiotensin II and acetylcholine.

TABLE II: Effect of Acyl Chain Length on Inhibition of Angiotensin-Converting Enzyme by Carboxyalkanoyl and Mercaptoalkanoyl Derivatives of Proline.

No.		I_{50} (μM)[a]
	R = carboxyalkanoyl	
17	HOOCCO	4800
18	$HOOCCH_2CO$	2600
1a	$HOOCCH_2CH_2CO$	330
19	$HOOCCH_2CH_2CH_2CO$	70
20	$HOOCCH_2CH_2CH_2CH_2CO$	>4000
	R = mercaptoalkanoyl	
21	$HSCH_2CO$	1.1
1b	$HSCH_2CH_2CO$	0.20
22	$HSCH_2CH_2CH_2CO$	9.7

[a] See footnote *a* of Table I.

As shown in Table I, column A, we have tested succinyl derivatives of 11 other amino acids. None of these compounds was more inhibitory than succinyl-L-proline, and several were at least 3 to 10 times less active. The succinyl derivatives of L-histidine (**11a**) and D-phenylalanine (**12a**) did not inhibit angiotensin-converting enzyme of rabbit lung at the highest concentrations tested. Because of these results, most structure–activity studies with the carboxyalkanoyl amino acids were carried out with derivatives of L-proline.

If succinyl-L-proline binds to the active site of angiotensin-converting enzyme in the manner visualized in the model of Figure 1, there should be an optimal length for the carboxyalkanoyl group that would allow for the most favorable alignment of its carboxyl function with the zinc ion at the active site of the enzyme without interfering with the binding of the amide carbonyl and the proline carboxyl group at their respective binding sites. This is confirmed by the results shown in Table II. Glutaryl-L-proline (**19**, $I_{50} = 7 \times 10^{-5}$ M) and succinyl-L-proline (**1a**, $I_{50} = 3.3 \times 10^{-4}$ M) are the most potent of the carboxyalkanoyl derivatives of L-proline; the homologous derivatives containing longer or shorter carboxyalkanoyl chains are considerably less potent as inhibitors of angiotensin-converting enzyme. If the optimal chain length for a carboxyalkanoyl function were intermediate between those of the succinyl and glutaryl groups, it could explain why the greatest inhibition was obtained with the more flexible glutaryl derivative, binding in a folded conformation.

In the hypothetical model of Figure 1, the acyl (carboxyalkanoyl) group of the carboxyalkanoyl amino acids not only binds to the zinc ion of the enzyme via its free carboxyl group, but it also occupies the same position at the hypothetical active site of angiotensin-converting enzyme as the penultimate amino acid residue of a peptide substrate for the enzyme. Since it is known from structure–activity studies with substrates

TABLE III: Effect of Methyl Substitution on Inhibition of Angiotensin-Converting Enzyme by Carboxyalkanoyl and Mercaptoalkanoyl Derivatives of Proline.

No.	R—N—COOH	I_{50} (μM)[a]
	R = methylsuccinyl	
23	HOOCCH$_2$CHCO (CH$_3$)	22
24	HOOCCHCH$_2$CO (CH$_3$)	610
25	HOOCCH$_2$CHCO (CH$_3$)	1480
26	HOOCCHCH$_2$CO (CH$_3$)	2600
	R = methylglutaryl	
27	HOOCCH$_2$CH$_2$CHCO (CH$_3$)	4.9
28	HOOCCH$_2$CHCH$_2$CO (CH$_3$)	1200[b]
29	HOOCCHCH$_2$CH$_2$CO (CH$_3$)	260
30	HOOCCH$_2$CH$_2$CHCO (CH$_3$)	950
	R = methyl-2-mercaptoacetyl	
31	HSCHCO (CH$_3$)	1.1
	R = methyl-3-mercaptopropanoyl	
32	HSCH$_2$CHCO (CH$_3$)	0.023
33	HSCHCH$_2$CO (CH$_3$)	1.1
34	HSCH$_2$CHCO (CH$_3$)	2.4

[a] See legend to Table I. [b] The absolute configuration of this methyl group is not known. The value in the table is that for isomer a; the I_{50} value for isomer b is 1600 μM.

(Yang et al., 1971; Elisseeva et al., 1971) and with competitive peptide inhibitors (Cushman et al., 1973) of angiotensin-converting enzyme that the nature of the penultimate amino acid residue can influence binding to the enzyme, it is reasonable that substituents in the 2 position of the acyl group of carboxyalkanoyl amino acids (R_2 in Figure 1) might also influence binding to the enzyme.

As shown in Table III (cf. Table II), 2-methyl substituents with a D configuration (above the plane of the paper) enhance by about 15-fold the inhibitory potencies of succinyl (**23**) or glutaryl (**27**) derivatives of L-proline. In this class of compounds, the D configuration is isosteric with the L configuration of the side chain of an amino acid (Byers and Wolfenden, 1972). The L-2 methyl-substituted succinyl and glutaryl derivatives of proline (**25** and **30**) are both about 100 times less inhibitory than the corresponding D enantiomers (Table III), and D or L methyl substituents in the 3 position of succinyl-L-proline (**24, 26**) or in the 3 or 4 positions of glutaryl-L-proline (**28, 29**) either have no effect or weaken the inhibitory potency of these compounds. The remarkable stereochemical and positional specificity of these substitutions in regard to their influence on the inhibitory potency strongly supports the mode of binding proposed for these inhibitors.

D-2-Methylsuccinyl-L-proline (SQ 13 297, **23**) was found to inhibit vasopressor responses to angiotensin I and augment vasodepressor responses to bradykinin when administered orally to rats (Ondetti et al., 1977; Rubin et al., submitted for publication), but in either the guinea pig ileum test system in vitro or in studies in vivo this compound was still only 1/300 as potent as SQ 20 881 as an inhibitor of angiotensin-converting enzyme; D-2-methylglutaryl-L-proline (SQ 14 102, **27**) is about 4 times more potent than the succinyl derivative.

Mercaptoalkanoyl Amino Acids. A large number of structural modifications were undertaken in the hope of producing analogues of the carboxyalkanoyl amino acids with greater affinity for the active site of angiotensin-converting enzyme as it is conceptualized in the model of Figure 1. The most important alteration attempted proved to be the replacement of the succinyl carboxyl group of succinyl-L-proline by other chemical groups having greater affinity for the enzyme-bound zinc ion. Replacement of the carboxyl group of succinyl-L-proline by a sulfhydryl function yielded a strikingly potent inhibitor (**1b**, Table I) that was 1000 times more potent than succinyl-L-proline, with an I_{50} value of 2×10^{-7} M. This compound, 3-mercaptopropanoyl-L-proline, designated SQ 13 863, was 10 to 20 times more potent than SQ 20 881 as an inhibitor of angiotensin-converting enzyme of rabbit lung. It was approximately equipotent with SQ 20 881 as an inhibitor of the contractile response of guinea pig ileum to angiotensin I, or as an inhibitor of the vasopressor activity of angiotensin I in rats when oral doses were compared with parenteral doses of SQ 20 881 (Ondetti et al., 1977; Rubin et al., submitted for publication). The remarkably high affinity of this mercaptoalkanoyl derivative for the enzyme is strong although indirect evidence that the mercapto function binds to the zinc ion of the enzyme as proposed in the model, since sulfur is a better ligand for zinc than oxygen. The high inhibitory activity of such mercaptoalkanoyl amino acids made it possible to study in a more quantitative fashion the enzyme–inhibitor interactions previously described, e.g., influence of the amino acid moiety, amide functionality, and substitution and length of the acyl chain.

As in the case of the succinyl derivative, the 3-mercaptopropanoyl derivative of L-proline (**1b**) is 2 to 10 times more potent that the corresponding derivatives of most other L-amino acids (Table I); it is about 300 times more potent than the 3-mercaptopropanoyl derivative of aspartic acid (**14b**), and over 2000 times more potent that those of β-alanine (**15b**) or D-proline (**16b**). These results are quite consistent with the known substrate specificity of angiotensin-converting enzyme and provide supportive evidence for the binding of these inhibitors at the active site. Among the peptides that are poor substrates for angiotensin-converting enzyme are those that contain a carboxyl-terminal D-amino acid (Oparil et al., 1973), those with a carboxyl-terminal dicarboxylic amino acid (Elisseeva et al., 1971), and those that lack two true peptide bonds at the carboxyl-terminal end (Yang et al., 1971). We have also observed similar structure–activity correlations with analogues of the peptide competitive inhibitors of angiotensin-converting enzyme from snake venom (Cushman et al., 1973). The superiority of L-proline as the amino acid moiety is probably due to the rigid ring structure of this amino acid that may lock the

TABLE IV: Requirement of Free Carboxyl and Sulfhydryl Groups and Amide Bond for Inhibition of Angiotensin-Converting Enzyme by Mercaptoalkanoyl Amine Acids.

No.	Structure	I_{50} (μM)[a]
1b	$HSCH_2CH_2CO$—N(pyrrolidine)—COOH	0.20
35	$HSCH_2CH_2CO$—N(pyrrolidine)—$COOC(CH_3)_3$	39
36	$HSCH_2CH_2CO$—N(pyrrolidine)—$COOCH_2CH_3$	17
37	$CH_3SCH_2CH_2CO$—N(pyrrolidine)—COOH	4300
9b	$HSCH_2CH_2CONHCH_2COOH$	2.8
38	$HSCH_2CONHCH_2CH_2COOH$	460
39	$HSCH_2CH_2CH_2CH_2CH_2COOH$	1100

[a] See footnote *a* of Table I.

TABLE V: Structure and K_i Values of Potent Competitive Inhibitors of Angiotensin-Converting Enzyme.

Compound	Structure	K_i (μM)[a]
SQ 14225 (32)	$HSCH_2CH(CH_3)CO$—N(pyrrolidine)—COOH	0.0017
SQ 13863 (1b)	$HSCH_2CH_2CO$—N(pyrrolidine)—COOH	0.012
SQ 20881	<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	0.10
SQ 14102 (27)	$HOOCCH_2CH_2CH(CH_3)CO$—N(pyrrolidine)—COOH	0.80
SQ 13297 (23)	$HOOCCH_2CH(CH_3)CO$—N(pyrrolidine)—COOH	2.5

[a] Kinetic studies were carried out in 0.1 M potassium phosphate, 0.3 M NaCl, pH 8.3, with Hip-His-Leu (5 mM) as substrate at 37 °C.

carboxyl group into a conformation favorable for interacting with the positively charged residue at the active site of the enzyme.

The optimal acyl chain length for mercaptoalkanoyl derivatives of proline is that of 3-mercaptopropanoyl-L-proline (**1b**, Table II). The inhibitory activity of this compound vs. angiotensin-converting enzyme of rabbit lung is five times greater than that of the shorter analogue 2-mercaptoacetyl-L-proline (**21**), and 50 times greater than that of the longer derivative 4-mercaptobutanoyl-L-proline (**22**).

The D-3-mercapto-2-methylpropanoyl-L-proline (**32**, Table III) was 10 times more active as an inhibitor of angiotensin-converting enzyme than the unsubstituted analogue (**1b**, Table I). The 3-methyl analogue **33** and the L-2-methyl analogue **34**, however, were 5 and 10 times less potent, respectively, than the unsubstituted 3-mercaptopropanoyl-L-proline. These results are quite similar to those obtained with substituted carboxyalkanoyl derivatives of proline.

The D-3-mercapto-2-methylpropanoyl-L-proline (**32**, Table III), designated SQ 14 225, is the most potent inhibitor of angiotensin-converting enzyme that we have developed, with an I_{50} value of 2.3×10^{-8} M. Even though the added hydrophobic interactions of the 2-methyl group might contribute to the increased binding of this derivative, it is more likely that the main contribution in this respect is the restriction in conformation introduced with this stereospecific substitution, which would diminish the entropy "expenditure" of the enzyme required to restrict the mobility of the acyl moiety.

Analogues of 3-mercaptopropanoyl-L-proline with substituted carboxyl or sulfhydryl groups (Table IV) are much less active than the parent compound, emphasizing the importance of these terminal groups for binding to the active site of the enzyme. The *tert*-butyl (**35**) or ethyl (**36**) esters of 3-mercaptopropanoyl-L-proline are at least 100 to 200 times less inhibitory than the unesterified compound; the methyl sulfide (**37**) is 20 000 times less inhibitory than the parent compound. *S*-Acetyl derivatives of mercaptoalkanoyl amino acids (not shown in Table IV) retained 3–5% of the inhibitory potency of the unesterified compounds, but it is difficult to rule out the generation from these thioesters of a small amount of unesterified mercaptoalkanoyl amino acids under our assay conditions. The importance of the amide bond in the binding of the inhibitor to the enzyme was explored with the synthesis of suitably modified derivatives of 3-mercaptopropanoylglycine (Table IV). Derivatives that lack the amide carbonyl (**39**), or in which the position of this residue is shifted (**38**), are significantly less active as inhibitors than the parent compound.

Enzymatic Studies. Inhibition of angiotensin-converting enzyme by carboxyalkanoyl or mercaptoalkanoyl amino acids was not progressive with time, and was reversed by dilution or dialysis of the enzyme–inhibitor mixture. The most potent of these compounds (Table V) have been shown to be fully competitive inhibitors from enzyme kinetic data plotted according to the methods of Lineweaver and Burk or according to the method of Dixon (Webb, 1963, p 149). The structures and inhibitor constants of these compounds are shown in Table V in comparison with another competitive inhibitor, the venom peptide SQ 20 881. The fully competitive nature of the inhibition with respect to substrate is quite consistent with the binding of these inhibitors to the active site of angiotensin-converting enzyme.

D-3-Mercapto-2-methylpropanoyl-L-proline (SQ 14 225, Table V) has a K_i value of 1.7×10^{-9} M, and must, therefore, classify as one of the more potent competitive enzyme inhibitors. In kinetic studies with an inhibitor of such great potency, Michaelis–Menten kinetics become inapplicable when enzyme concentrations are employed that approach the K_i value of the inhibitor because of "mutual depletion" of both free enzyme and free inhibitor (Webb, 1963, p 66). For the enzyme kinetic studies summarized in Table V, we employed homogeneous angiotensin-converting enzyme (Cheung and Cushman, 1973) at a concentration of 4×10^{-10} M, as calculated from the molecular weight of 129 000 (Das and Soffer, 1975). At this enzyme concentration, 5 times lower than the K_i value of SQ 14 225, and with substrate concentrations varying from 2.5×10^{-3} to 2.5×10^{-4} M, less than 5% error is expected in determination of enzyme inhibition due to deviation from Michaelis–Menten kinetics (Webb, 1963, p 66). The I_{50} value for SQ 14 225 (substrate = 5×10^{-3} M) was evaluated over a wide range of enzyme concentrations, and was found to be invariable at enzyme concentrations lower than 2×10^{-9} M, but to increase linearly between 2×10^{-9} and 3×10^{-8} M enzyme.

Although the carboxyalkanoyl and mercaptoalkanoyl derivatives of L-proline shown in Table V were developed initially

by analogy to an inhibitor of carboxypeptidase A,[2] they have been designed for multifunctional interaction with a different active site of rather precise geometry, and should, therefore, be quite specific for inhibition of angiotensin-converting enzyme. The compounds shown in Table V are competitive inhibitors of angiotensin-converting enzyme with inhibitor constants ranging from 10^{-6} to 10^{-9} M. However, none of these compounds at 10^{-3} M inhibited by more than 50% the activities of carboxypeptidase A, trypsin, or chymotrypsin of bovine pancreas, or that of carboxypeptidase B of hog pancreas. Leucine aminopeptidase of hog kidney was not inhibited by the carboxyalkanoyl amino acids SQ 13 297 or SQ 14 102 at 10^{-3} M, but was inhibited 50% by the mercaptoalkanoyl amino acids SQ 13 863 and SQ 14 225 at 3.9×10^{-5} and 5.4×10^{-6} M, respectively, concentrations that are 200 times those required for equivalent inhibition of angiotensin-converting enzyme by these compounds.

The studies described above exemplify the great heuristic value of an active-site model in the design of inhibitors, even when such a model is a hypothetical one. Only when suitable information on substrate specificity and mechanism of action of an enzyme is available, can one make a reasonable working hypothesis with regard to complementary functionality needed in an inhibitor. If several complementary groups can be combined in one molecule, a very strong but still reversible inhibitor can be obtained, as we have demonstrated in the case of angiotensin-converting enzyme. Since this enzyme plays an important role in the pathogenesis of hypertension, a potent specific and orally active inhibitor such as SQ 14 225 should have great therapeutic value.

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Supplementary Material Available

Description of syntheses of other carboxy and mercaptoalkanoyl amino acids (17 pages). Ordering information is given on any current masthead page.

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Angiotensin I Converting Enzyme from Human Plasma†

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ABSTRACT: The angiotensin I converting enzyme was purified 101 000-fold to homogeneity from human plasma by a combination of chromatographic and electrophoretic techniques. The enzyme is similar to other angiotensin I converting enzymes. It is an acidic glycoprotein consisting of a single polypeptide chain of molecular weight 140 000 with an isoelectric point of 4.6. The enzyme requires chloride ion for activity and is inhibited by ethylenediaminetetraacetic acid, angiotensin II, bradykinin, bradykinin potentiating factor nonapeptide, and 3-mercapto-2-D-methylpropanoyl-L-proline (SQ-14,225). The purified preparation cleaves bradykinin as well as angiotensin I and hippuryl-L-histidyl-L-leucine. Its specific activity with angiotensin I is 2.4 units per mg and with hippuryl-L-histidyl-L-leucine is 31.4 units per mg.

The renin–angiotensin system has been implicated as a major system in the pathogenesis of hypertension (Skeegs et al., 1976) and hypertensive vascular damage (Giese, 1973) in man. A component of this system, the angiotensin I converting enzyme, converts the relatively inactive decapeptide angiotensin I to the potent pressor octapeptide angiotensin II. Detailed physical properties of this enzyme from human tissue have not been available because of the difficulty of purifying the enzyme. One problem has been the lack of a suitable supply of human tissue. Another has been that the most readily available human tissue, outdated human plasma, contains only a small quantity of enzyme. To use human plasma as the starting material, a high-resolution protocol had to be developed to enable purification of the enzyme in a reasonable number of steps.

In this paper, we describe a method for purifying the enzyme 101 000-fold to homogeneity from human plasma. This protocol should be readily adaptable to purifying the enzyme from any animal tissue. Some properties of the human enzyme are described. A purified enzyme from a human source will allow immunologic studies on human material not otherwise possible due to species specificity of angiotensin I converting enzyme (Conroy et al., 1976; Polsky-Cynkin and Fanburg, 1977).

Materials and Methods

Materials. Outdated human plasma was obtained from the Blood Bank of the New England Medical Center Hospital. Hip-His-Leu[1] was custom synthesized by Vega-Fox Biochemicals. Angiotensin I was from Beckman Instruments, Inc.; BPP_{9a} and 3-mercapto-2-D-methylpropanoyl-L-proline (SQ-14,225) were gifts from Dr. D. W. Cushman, Squibb Institute for Medical Research. Bradykinin was from Schwarz/Mann. Whatman DE 42 microgranular DEAE-cellulose was from Reeve-Angel. Bio-Gel HTP hydroxylapatite was from Bio-Rad Labs. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc. All other chemicals were reagent grade.

Angiotensin I Converting Enzyme Activity. The assay used for monitoring column and electrophoretic procedures was

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[1] Abbreviations used are: Hip-His-Leu, hippuryl-L-histidyl-L-leucine; BPP_{9a}, bradykinin potentiating factor nonapeptide; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; DEAE, diethylaminoethyl; TLC, thin-layer chromatography; Bis, *N,N'*-methylenebis(acrylamide); Tris, tris(hydroxymethyl)aminomethane.