Design of Potent and Specific Inhibitors of Carboxypeptidases A and B†

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ABSTRACT: The combination in one molecule of functional groups that can interact specifically with different substrate binding areas at the active site of carboxypeptidases A and B has led to the development of potent and specific inhibitors of these enzymes. 2-Benzyl-3-mercaptopropanoic acid (SQ 14,603) has a K_i of 1.1×10^{-8} M vs. carboxypeptidase A and a K_i of 1.6×10^{-4} M vs. the B enzyme. 2-Mercaptomethyl-5-guanidinopentanoic acid (SQ 24,798) has a K_i of 4×10^{-10} M vs. carboxypeptidase B and a K_i of 1.2×10^{-5} M vs. carboxypeptidase A. It is proposed that the sulfhydryl groups of these inhibitors bind to the catalytically important zinc ions of these enzymes, and that, in conjunction with the benzyl and guanidinopropyl side chains, they are responsible for their specificity.

The concept that combination in one molecule of the modes of binding of two products of an enzymatic reaction could lead to the development of potent enzyme inhibitors ("bi-product analogues") was clearly demonstrated by Byers & Wolfenden (1973) for carboxypeptidase A with the development of the potent competitive inhibitor benzylsuccinic acid. The same approach has been recently extended to carboxypeptidase B by McKay & Plummer (1978). A more generalized version of this approach, namely, incorporation into one molecule of functional groups not necessarily present in reaction products but capable of interacting regio- and stereospecifically with the different binding areas of the active site of an enzyme, was used to develop potent and specific inhibitors of the angiotensin-converting enzyme, such as SQ 14,225 (Ondetti et al., 1977a,b). One of the most important interactions of SQ 14,225 [(D-3-mercapto-2-methylpropanoyl)-L-proline] with the angiotensin-converting enzyme is thought to be the binding of its sulfhydryl group with the catalytically important zinc ion of this enzyme (Cushman et al., 1977; Ondetti et al., 1977a,b). Since carboxypeptidases A and B are also zinc-containing peptidases, a similar interaction could be the basis for the development of potent inhibitors. However, in the design of these inhibitors careful attention should be given to other binding areas at the active site of these enzymes, e.g., the cationic center common to both, the hydrophobic pocket of carboxypeptidase A, and the anionic and partially hydrophobic pocket of carboxypeptidase B (Zisapel et al., 1973), in order to obtain the desired specificity. The inhibitors described in this paper combine functional groups capable of interacting specifically with these binding areas and show very high potency and remarkable specificity.

Experimental Procedure

Synthesis. All melting points were taken in open capillaries and are uncorrected. The NMR spectra (Varian T60) of all intermediates were in agreement with the proposed structures. Thin-layer chromatography was carried out on Merck silica gel plates.

DL-*2-Benzyl-3-mercaptopropanoic acid* (**1**, *SQ 14,603*).[1] Benzylacrylic acid (Mannich & Ritsert, 1924) (16.2 g) and thiolacetic acid (10.6 g) were stirred 1 h at room temperature and then heated 1 h on the steam bath. (NMR showed the absence of vinyl protons.) An aliquot of this preparation (4 g) was dissolved in an ice-cold mixture of 8 mL of water and 6 mL of concentrated ammonia. The reaction mixture was stored for 1 h at room temperature, chilled in an ice bath, acidified with concentrated HCl, extracted into $CHCl_3$, dried over $MgSO_4$, and concentrated to dryness in vacuo. The residue was dissolved in ether and dicyclohexylamine was added. The crystalline dicyclohexylammonium salt (5.6 g, mp 135–137 °C, sintering 129 °C) was distributed between ethyl acetate and 10% potassium bisulfate. The organic layer was dried ($MgSO_4$) and concentrated to dryness in vacuo to a clear oil: yield, 2.8 g (silica gel, benzene–acetic acid, 75:25; R_f 0.55). Anal. Calcd for $C_{10}H_{12}O_2S$: C, 61.21; H, 6.17; S, 16.34. Found: C, 61.04; H, 6.28; S, 16.17.

Methyl N-(p-Methoxybenzyl)nipecotate Hydrochloride (**2a**). A mixture of 23 g (0.161 M) of methyl nipecotate, 24.3 g (0.176 M) of potassium carbonate, and 52 g (0.176 M) of *p*-methoxybenzyl trichloroacetate (Lee & Rapoport, 1975) in 800 mL of toluene was heated under reflux in a nitrogen atmosphere for 72 h. After cooling, the toluene was removed in vacuo. The residue was dissolved in chloroform and this solution was washed once with 400 mL of 10% potassium carbonate solution and then with 400 mL of 10% hydrochloric acid. The chloroform solution was dried and concentrated in vacuo to a viscous brown oil. Trituration of this with ethyl acetate gave 30.7 g (63.6%) of an off-white crystalline solid, which was recrystallized from ethyl acetate: yield, 26 g; mp 150–154 °C, sintering 148 °C. Anal. Calcd for $C_{15}H_{21}NO_3 \cdot HCl$: C, 60.10; H, 7.40; N, 4.67; Cl, 11.82. Found: C, 59.89; H, 7.23; N, 4.37; Cl, 11.69.

3-Methylene-2-piperidone (**2b**). A solution of **2a** (30.7 g, 0.102 M) and 8.4 g (0.210 M) of sodium hydroxide in 900 mL of methanol and 45 mL of water was stirred at room temperture for 17 h. The solution was taken to dryness in vacuo, and toluene was added to the residue twice and removed in vacuo. To the residue were added 1 L of acetic anhydride and 140 mL of triethylamine, and the mixture was heated under reflux for 4 h. After cooling the solvent was removed in vacuo. The residue was dissolved in chloroform, washed with water, dried, and concentrated in vacuo. The residual oil was chromatographed on 650 g of silica gel using 1:1 hexane–ethyl acetate as the eluant and yielded 16.9 g (72%)

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[1] The design of DL-2-benzyl-3-mercaptopropanoic acid as a potent inhibitor of carboxypeptidase A was reported in preliminary form in our paper on angiotensin-converting enzyme inhibitors (Cushman et al., 1977). We have since noted that reference to the inhibitory activity vs. carboxypeptidase A of a similar compound, 2-mercapto-3-phenylpropanoic acid, was made by Suh & Kaiser (1975), although no mention was made of the potency or the mechanism of its inhibitory action.

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of chromatographically pure (silica gel, EtOAc–hexane, 1:1; R_f 0.4) 1-(*p*-methoxybenzyl)-3-methylene-2-piperidone. A solution of this material (16.9 g, 73 mmol) and 21.3 g (177 mmol) of anisole in 400 mL of trifluoroacetic acid were refluxed under nitrogen for 48 h. The solution was taken to dryness in vacuo and the residue was chromatographed on 900 g of silica gel using ethyl acetate as the eluant to give 6.5 g (81%) of crystalline solid (silica gel, EtOAc; R_f 0.2), which was recrystallized from ether, mp 60–64 °C. Anal. Calcd for C_6H_9NO: C, 64.84; H, 8.16; N, 12.60. Found: C, 64.38; H, 7.97; N, 12.48.

2-Methylene-5-aminopentanoic Acid Hydrochloride (**2c**). A solution of 6.5 g (58.5 mmol) of **2b** in 350 mL of 6 N HCl was heated under reflux for 40 h. The cooled solution was extracted twice with chloroform and the aqueous layer was concentrated in vacuo. Toluene was added twice and removed in vacuo. Most of the residue was dissolved in hot isopropyl alcohol and filtered to remove insoluble material. The filtrate was concentrated in vacuo. Toluene was added three times to the residue and removed in vacuo to give 8.8 g (90%) of crystalline material, which was recrystallized from isopropyl alcohol, mp 138–144 °C, sintering 120 °C. Anal. Calcd for $C_6H_{11}NO_2$·HCl: C, 43.51; H, 7.30; N, 8.46; Cl, 21.40. Found: C, 43.33; H, 7.17; N, 8.23; Cl, 21.21.

2-Acetylthiomethyl-5-(p-methoxybenzyloxycarbonyl)-aminopentanoic Acid (**2d**). To a solution of 8.8 g (53 mmol) of **2c** in 100 mL of water was added with stirring 6.36 g (159 mmol) of magnesium oxide, followed by a solution of 12.2 g (59 mmol) of *p*-methoxybenzyloxycarbonyl azide in 100 mL of dioxane, and the mixture was stirred at room temperature for 2 days. The mixture was filtered through Celite, and the filtrate was diluted with 200 mL of ethyl acetate. AG50WX2 Dowex 50 ion-exchange resin (200 mL wet volume, ~2 equiv) was added and the mixture was stirred at room temperature for 2 h. The resin was removed by filtration and washed with water. The layers in the filtrate were separated and the aqueous layer was extracted twice with ethyl acetate and the combined organic layers were dried and concentrated in vacuo to give 18.2 g. A solution of this material in 50 mL of thiolacetic acid was allowed to stand at room temperature for 48 h. The thiolacetic acid was removed in vacuo and three portions of benzene were added and removed in vacuo to remove excess thiolacetic acid. The remaining oil was dissolved in ether and treated with a slight excess of dicyclohexylamine. The crystalline material that precipitated was harvested and dried to give 60.8 g (62%) of the salt, mp 112–114 °C. Anal. Calcd for $C_{17}H_{23}NO_6S$·$C_{12}H_{23}N$: C, 63.24; H, 8.42; N, 5.09; S, 5.82. Found: C, 62.96; H, 8.34; N, 5.05; S, 5.62.

The salt was dissolved in chloroform and converted to the free acid by washing with 10% $KHSO_4$ solution. The chloroform solution was then washed with saturated NaCl solution and dried, and the solvent was removed in vacuo to give a quantitative yield of the free acid.

DL-*2-(Mercaptomethyl)guanidinopentanoic Acid* (**2**, *SQ 24,798*). To a stirred mixture of **2d** (9.65 g, 27 mmol) and 20 mL of anisole was added dropwise, over 15 minutes at 0–5 °C, 125 mL of trifluoroacetic acid. After the addition was complete, the solution was left at 0–5 °C for 1 h. The trifluoroacetic acid was removed in vacuo, the residue was taken up in water, this was thoroughly extracted with ether, and the aqueous layer was lyophilized to a pale yellow oil. The oil was taken up in water, applied to an AG50WX2 column (800 mL of resin), and eluted with water until the eluate was neutral. Elution with pH 6.5 buffer (200-mL fractions) afforded 3.7 g (66%) of 2-acetylthiomethyl-5-aminopentanoic acid. To a mixture of 3.7 g (18.0 mmol) of this material and 6.72 mL (48 mmol) of triethylamine in 40 mL of dry *N,N*-dimethylformamide was added 4.84 g (24 mmol) of guanyl-3,5-dimethylpyrazole nitrate, and the resulting mixture stirred at room temperature for 3 days. The clear yellow solution was taken to dryness in vacuo, the residue taken up in 10 mL of water and 10 mL of concentrated ammonium hydroxide, and the mixture stirred under nitrogen for 1 h at 0–5 °C. The solution was diluted with water and lyophilized. The residue was taken up in a small amount of pH 3.5 buffer, applied to an AG50WX2 column (200 mL of resin), and eluted with pyridine–acetate pH 3.5 buffer and then with a linear gradient of the same buffer (pH 3.5–5). Sakaguchi and nitroprusside fractions were lyophilized and the residue was triturated with water: yield, 0.115 g; mp 265–268 °C. Anal. Calcd for $C_7H_{15}N_3O_2S$: C, 40.98; H, 7.37; N, 20.48; S, 15.63. Found: C, 40.74; H, 7.32; N, 20.51; S, 15.41.

Enzymatic Studies. Hippuryl-L-phenylalanine (Hip-Phe), hippuryl-L-arginine (Hip-Arg), bovine pancreatic carboxypeptidase A (EC 3.4.12.2), and porcine pancreatic carboxypeptidase B (EC 3.4.12.3) were purchased from Worthington Biochemical Corp. Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) was obtained from Bachem Fine Chemicals, Inc., and acetone powder of rabbit lung from Pel-Freez Biologicals, Inc. Angiotensin-converting enzyme (EC 3.4.15.1) was purified from acetone powder of rabbit lung as described by Cheung & Cushman (1973).

Carboxypeptidase A activity was assayed by spectrophotometic measurement of its hydrolysis of Hip-Phe (Folk & Schirmer, 1963), at pH 7.5 in the presence of 0.5 M NaCl. Carboxypeptidase B was similarly assayed with Hip-Arg as substrate (Folk et al., 1960) at pH 7.65 in the presence of 0.1 M NaCl. Angiotensin-converting enzyme was assayed by spectrophotometric or spectrofluorometric determination of the rate of hydrolysis of Hip-His-Leu (Cushman & Cheung, 1971; Cheung & Cushman, 1973). K_i values for tight-binding inhibitors of carboxypeptidase A (SQ 14,603, **1**), carboxypeptidase B (SQ 24,798, **2**), or angiotensin-converting enzyme (SQ 14,225, Cushman et al., 1977) were derived from kinetic data plotted according to Lineweaver & Burk (1934) and Dixon (1953) using the following low enzyme concentrations to avoid mutual depletion of enzyme and inhibitor: carboxypeptidase A, 1.4×10^{-9} M; carboxypeptidase B, 2.8×10^{-10} M; angiotensin-converting enzyme, 4.0×10^{-10} M. At these low concentrations of enzme, it was necessary, for assay of carboxypeptidases A and B, to record absorbance at 254 nm for 20 min to 1 h to obtain linear changes of 0.005–0.05, using the most sensitive scale on the Gilford Model 2400 recording spectrophotometer; the sensitive fluorometric assay of angiotensin-converting enzyme was employed for such kinetic studies at low enzyme concentration. No significant losses of activity were observed when such low concentrations of these enzymes were incubated under these conditions for a 1–2 h period, as judged by the linear rates of substrate hydrolysis. Other K_i values for determination of the specificity of each of the three inhibitors were estimated according to Cheng & Prusoff (1973) using I_{50} values at a single substrate concentration and the experimentally determined K_m values for Hip-Phe, Hip-Arg, and Hip-His-Leu obtained under our assay conditions.

Results

As shown in Table I DL-2-benzyl-3-mercaptopropanoic acid (SQ 14,603) is a potent inhibitor of carboxypeptidase A (K_i = 1.1×10^{-8} M) with 15 000-fold lower affinity for carboxypeptidase B. DL-2-Mercaptomethyl-5-guanidinopentanoic

Table I: Specificity of Tight-Binding Competitive Inhibitors of Carboxypeptidases A and B and Angiotensin-Converting Enzyme

inhibitor	K_i (nM)[a] carboxypeptidase A	carboxypeptidase B	angiotensin-converting enzyme
$HSCH_2CH(CH_2C_6H_5)CO_2H$ (SQ 14,603)	11	163 000	58 000
$HSCH_2CH(CH_2CH_2CH_2NHC(=NH)NH_2)CO_2H$ (SQ 24,798)	11 600	0.42	288 000
$HSCH_2CH(CH_3)CON$(C$_5$H$_6$)CO_2H (SQ 14,225)	623 000	>250 000	1.7

[a] K_i values for each of the three inhibitors vs. the enzymes for which they were designed were calculated from kinetic data plotted according to Lineweaver & Burk (1934) and Dixon (1953), as shown in Figures 1-3. Other K_i values are estimated from I_{50} values according to Cheng & Prusoff (1973), using the following substrate concentrations and experimentally determined K_m values: [Hip-Arg] = 1 mM, K_m = 0.33 mM; [Hip-Phe] = 1 mM, K_m = 0.71 mM; [Hip-His-Leu] = 5 mM, K_m = 1.2 mM.

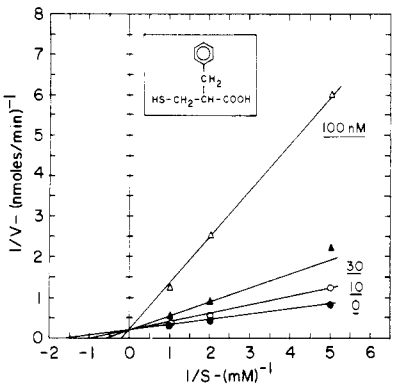

FIGURE 1: Double-reciprocal plot of the effect of substrate (Hip-Phe) on inhibition of carboxypeptidase A by SQ 14,603. At the low enzyme concentration employed (1.4×10^{-9} M), it was necessary to measure changes in absorption at 254 nm for 20 min to 1 h in order to measure the linear rate of hydrolysis of Hip-Phe.

acid (SQ 24,798) is an extremely potent inhibitor of carboxypeptidase B ($K_i = 4.2 \times 10^{-10}$ M) with a 25 000-fold lower affinity for carboxypeptidase A. Lineweaver–Burk plots (Figures 1 and 2) for these inhibitors and Dixon plots (not shown) indicate purely competitive inhibition. Linear rates of substrate hydrolysis were obtained at all inhibitor concentrations after steady-state equilibrium was obtained, indicating that the inhibition was not of a progressive or irreversible nature. Both compounds have practically no inhibitory activity vs. angiotensin-converting enzyme, whereas D-3-mercapto-2-methylpropanoyl-L-proline (SQ 14,225), a potent competitive inhibitor of this enzyme (Table I), shows no significant inhibition of carboxypeptidase A or carboxypeptidase B.

Discussion

Benzylsuccinic acid is a potent inhibitor of carboxypeptidase A (K_i = 0.4–1.1 × 10^{-6} M) (Byers & Wolfenden, 1973; McKay & Plummer, 1978), but it is also highly inhibitory when assayed against carboxypeptidase B (K_i = 1.2–8 × 10^{-6} M) (Zisapel & Skolovsky, 1974; McKay & Plummer, 1978). The lack of specificity of this inhibitor is not surprising since

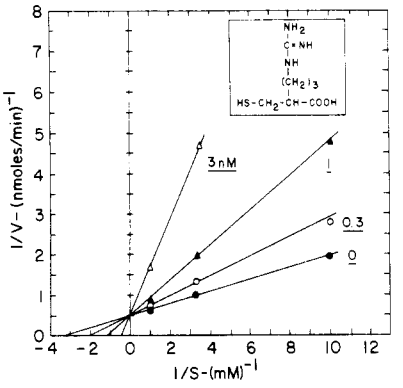

FIGURE 2: Double-reciprocal plot of the effect of substrate (Hip-Arg) on inhibition of carboxypeptidase B by SQ 24,798. At the low enzyme concentration employed (2.8×10^{-10} M), it was necessary to measure changes in absorption at 254 nm for 20 min to 1 h to measure the linear rate of hydrolysis of Hip-Arg.

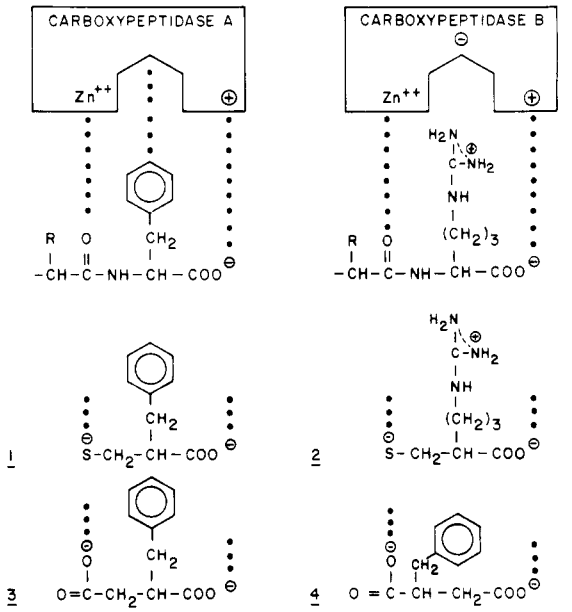

FIGURE 3: Schematic representation of binding of substrates and proposed mode of binding of inhibitors to the active sites of carboxypeptidase A and B. **1**, SQ 14,603; **2**, SQ 24,798; **3**, benzylsuccinic acid; **4**, benzylsuccinic acid shown binding to carboxypeptidase B in the reverse direction with a partial interaction of its aromatic ring with the anionic–hydrophobic pocket.

carboxypeptidase B, in addition to its affinity toward substrates with terminal basic amino acids, has also been shown to possess intrinsic activity against substrates with nonbasic hydrophobic amino acids. However, it has been noted that the modes of binding of these two types of substrates with carboxypeptidase B are not the same (Zisapel et al., 1973). Since benzylsuccinic acid has two carboxyl groups, one could also postulate different modes of binding of this inhibitor to the active sites of carboxypeptidases A and B, one with the carboxyl α to the benzyl moiety binding to the cationic center of the enzyme, and the other with this carboxyl binding to the zinc atom. The first mode of binding would be naturally preferred in the interaction with carboxypeptidase A (Figure 3) because it allows for an optimal interaction between the phenyl ring and the hydrophobic pocket. The second might be operative in the binding of this inhibitor to carboxypeptidase B (Figure 3). In this latter case the benzyl moiety might bind to another hydrophobic binding site (Akanuma et al., 1971), or it might penetrate only partially into the specific binding site of this enzyme thereby avoiding the unfavorable interaction with the negatively charged side chain of the Asp^{255} residue present in this partially

hydrophobic pocket (Zisapel et al., 1973; Schmid & Herriott, 1976). By replacing the β-carboxyl of benzylsuccinic acid with a sulfhydryl group, which has a strong affinity for zinc, but not for the cationic center of the enzyme, we have eliminated the possibility for a nonspecific mode of binding for this inhibitor. The 14 000-fold difference in the binding of 2-benzyl-3-mercaptopropanoic acid (SQ 14,603) to carboxypeptidase A and B (Table I) is a clear indication of the specificity of its interaction with these enzymes. The proposed binding of the sulfhydryl group to the zinc ion of the enzyme, as postulated in Figure 3, is supported by the evidence that, in bidentate zinc ligands, the replacement of oxygen by sulfur increases the stability constant 100 000-fold (Valee & Coleman, 1964). Even though sulfhydryl groups are not commonly found as zinc ligands in metalloenzymes, they do occur as such at the active site of horse liver alcohol dehydrogenase (Eklund et al., 1974).

To design a potent and specific inhibitor of carboxypeptidase B, we have retained the mercaptopropanoic acid moiety to assure the specificity of the interactions with the zinc atom and the cationic center of the enzyme (Figure 3) and have added the guanidinopropyl side chain characteristic of the arginine substrates of this enzyme. The compound thus obtained, SQ 24,798, has a very low enzyme–inhibitor dissociation constant (4×10^{-10} M), and a very high specificity for carboxypeptidase B. Since carboxypeptidase A does not bind to any significant extent to basic substrates, the bi-product analogues recently described by McKay & Plummer (1978), e.g., guanidinoethylmercaptosuccinic acid (GEMSA) and aminopropylmercaptosuccinic acid (APMSA), are also very specific inhibitors of carboxypeptidase B, with fairly low dissociation contants ($K_i = 1 \times 10^{-6}$ and 5×10^{-6} M). The 2500-fold increase in potency observed with the mercapto derivatives supports the assumption that the β-carboxyl group of GEMSA and the sulfhydryl group of SQ 24,798 are interacting directly with the zinc atom of the enzyme.

If one considers that the distances between the zinc atom and the carboxyl-binding site in carboxypeptidases A and B are quite different from that expected to exist on angiotensin-converting enzyme (Cushman et al., 1977), it is not surprising that the two mercaptopropanoic acid derivatives described in this paper show no significant inhibitory activity against the latter enzyme, and that the specific inhibitor of angiotensin-converting enzyme (SQ 14,225) is not an inhibitor of the pancreatic carboxypeptidases.

In our studies with angiotensin-converting enzyme, we had proposed a hypothetical model of the active site of this enzyme based on the similarities with carboxypeptidase A (Ondetti et al., 1977a,b). The development, based on this model, of the potent and specific inhibitor SQ 14,225 provided indirect but strong evidence that this proposal was substantially correct (Cushman et al., 1977). This proposal has now been further corroborated by the observation that a similar reasoning can lead to very potent and specific inhibitors of carboxypeptidase A and B, for which the model of the active site is based on direct X-ray crystallographic evidence.

The mercaptopropanoic acid derivatives described in this paper should be considerably more useful than the corresponding succinic acid derivatives in exploring the architecture of the active site of these carboxypeptidases, not only because of their greater potency, but also because their specificity excludes alternate modes of binding.

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