# α-Bromo Ketone Substrate Analogues Are Powerful Reversible Inhibitors of Carboxypeptidase A

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ABSTRACT: The aldehyde (*RS*)-2-benzyl-4-oxobutanoic acid, which is 25% hydrated at pH 7.5, has recently been shown to be a strong reversible competitive inhibitor of carboxypeptidase A [$K_i$ = 0.48 nM; Galardy, R. E., & Kortylewicz, Z. P. (1984) *Biochemistry 23*, 2083–2087]. The ketone analogue of this aldehyde (*RS*)-2-benzyl-4-oxopentanoic acid (IV) is not detectably hydrated under the same conditions and is 1500-fold less potent ($K_i$ = 730 μM). The ketone homologue (*RS*)-2-benzyl-5-oxohexanoic acid (XIII) is also a weak inhibitor ($K_i$ = 1.3 mM). The α-monobrominated derivatives of these two ketones are, however, strong competitive inhibitors with $K_i$'s of 0.57 μM and 1.3 μM, respectively. Oximes derived from the aldehyde, the ketones IV and XIII, and a homologue of the aldehyde are weak inhibitors with $K_i$'s ranging from 480 to 7900 μM. The inhibition of carboxypeptidase A by the α-monobrominated ketones is reversible and independent of the time (up to 6 h) of incubation of enzyme and inhibitor together. Bromoacetone at a concentration of 30 mM does not inhibit carboxypeptidase A. Incubation of an equimolar mixture of 2-benzyl-4-bromo-5-oxohexanoic acid (XV) and enzyme for 1 h led to the recovery of 82% of XV, demonstrating that it is the major species reversibly bound during assay of inhibition. Taken together, these results indicate that tight binding of carbonyl inhibitors to carboxypeptidase A requires specific binding of inhibitor functional groups such as benzyl and an electrophilic carbonyl carbon such as that of an α-bromo ketone or aliphatic aldehyde. If the hydrate of XV (present at less than 5% as determined by $^{13}C$ NMR) is the species inhibiting the enzyme, its $K_i$ must be less than 65 nM.

Aldehyde and ketone substrate analogues have been shown to be strong reversible inhibitors of several zinc metalloproteases including leucine aminopeptidase (Birch et al., 1971; Andersson et al., 1982), *Aeromonas* aminopeptidase (Kettner et al., 1974), angiotensin converting enzyme (Almquist et al., 1980, 1982), and carboxypeptidase A (Galardy & Kortylewicz, 1984). In the case of leucine aminopeptidase, strong reversible inhibition by the aldehyde inhibitor has been attributed to its ability to react as an electrophile with an amino acid side chain nucleophile of the active site (Andersson et al., 1982). For *Aeromonas* aminopeptidase, the electrophilic halomethyl ketone inhibitors ($K_i$ = ~1 μM) were almost 2 orders of magnitude more potent than the parent methyl ketone (Kettner et al., 1979). Likewise, halomethyl ketones were somewhat more potent inhibitors of leucine aminopeptidase than the parent methyl ketone although all of these inhibitors had rather weak $K_i$'s (Fittkau et al., 1976). The ability of the aldehyde (*RS*)-2-benzyl-4-oxobutanoic acid to potently and reversibly inhibit carboxypeptidase A was attributed to the electrophilicity of the aldehydic carbon atom, which was shown to be 25% hydrated in $D_2O$ at pH 7.5 (Galardy & Kortylewicz, 1984). In the case of carboxypeptidase, the identity of the nucleophile postulated to be covalently but reversibly bonded to the aldehydic carbon atom was not established. It could be either an amino acid side chain of the enzyme or a water molecule.

Aldehyde and ketone substrate analogues are also strong reversible inhibitors of proteases containing active site hydroxyl (serine) and thiol (cysteine) nucleophiles (Thompson, 1974; Lewis & Wolfenden, 1977b). The degree of inhibition has been correlated with the electrophilicity of the carbonyl carbon atom (Lewis & Wolfenden, 1977a). For several enzymes, a tetrahedral complex between the active site nucleophile and the carbonyl carbon atom of the inhibitor has been observed by carbon-13 and fluorine-19 nuclear magnetic resonance spectroscopy (Rich et al., 1982; Malthouse et al., 1983; Gamcsik et al., 1983; Shah & Gorenstein, 1983).

We show here that the ketone (*RS*)-2-benzyl-4-oxopentanoic acid (IV) and its homologue (*RS*)-2-benzyl-5-oxohexanoic acid (XIII) are only weak inhibitors of carboxypeptidase A. However, the mixture of α-bromo ketones VII derived from IV and the α-bromo ketone XV derived from XIII are competitive and approximately 1000-fold more potent. The ketone IV, its α-bromo derivative XII, and their proposed mode of binding to carboxypeptidase A are shown in Figure 1. The bromo ketones are fully reversible inhibitors and do not exhibit any time-dependent inactivation of the enzyme. This result is in contrast to the time-dependent inactivation of carboxypeptidase A by the substrate analogue *N*-(bromoacetyl)-*N*-methyl-L-phenylalanine (Hass & Neurath, 1971a), an affinity label that reacts with glutamic acid-270 (Hass & Neurath, 1971b). The bromo ketone XV is recovered in 82% yield after 1 h of incubation with equimolar carboxypeptidase A under the assay conditions. A series of oximes related to the ketone IV were only weak inhibitors of the enzyme.

We propose that the reversible inhibition of carboxypeptidase A by the bromo ketones VII and XV is due to their enhanced electrophilicity compared to their parent ketones IV and XIII, respectively. The enhanced electrophilicity of the bromo ketones permits reaction with a nucleophile to form a tetrahedral complex that binds tightly but reversibly to the active site of the enzyme due to its resemblance to a tightly bound tetrahedral intermediate occurring during normal substrate hydrolysis. The nucleophile involved cannot be identified as yet but could be a water molecule or an enzyme-bound functional group normally involved in catalysis.

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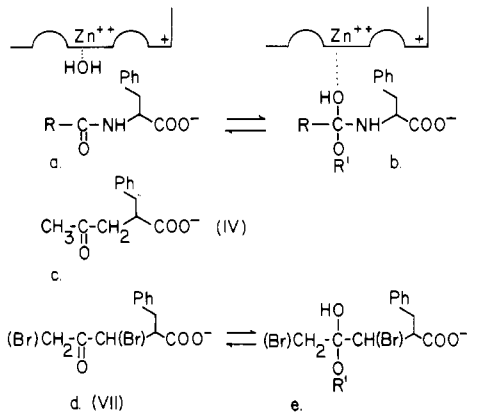

FIGURE 1: Model for the active site of carboxypeptidase A (Ondetti et al., 1979) showing proposed modes of binding for the following: (a) substrate bound as its Michaelis complex, (b) an intermediate resembling the transition state for substrate hydrolysis, where R′ is -H or an amino acid side chain from the enzyme such as that of glutamic acid-270, (c) ketone inhibitor IV bound as its Michaelis complex, (d) ketone inhibitor VII bound as its Michaelis complex, and (e) ketone inhibitor VII bound in a tetrahedral complex resembling (b).

These studies with carboxypeptidase A serve as a model for the inhibition of the dipeptidyl carboxypeptidase angiotensin converting enzyme by the ketone (5*S*)-5-benzamido-4-oxo-2-phenylhexanoic acid, which has been reported to be a more potent inhibitor in vitro than captopril (Almquist et al., 1980), a converting enzyme inhibitor presently being used clinically for the treatment of hypertension in humans.

## Experimental Procedures

Carboxypeptidase A (catalog no. C0386, 2× crystallized from bovine pancreas) was from Sigma Chemical Co., St. Louis, MO. *trans-p*-Chlorocinnamic acid from Aldrich Chemical Co., Milwaukee, WI, was recrystallized from chloroform–methanol (2:1) until its NMR spectrum was free of resonances from the cis isomer. L-β-Phenyllactic acid was from Aldrich. *O*-(*trans-p*-Chlorocinnamoyl)-L-β-phenyllactate was prepared according to Suh & Kaiser (1976); mp 125 °C (lit. mp 125–126.5 °C). The starting materials and solvents used in this work were reagent grade, 98% or higher purity, and were used without further purification or prepared by standard literature procedures. Thin-layer chromatography was performed on silica gel 60F-254 (EM Reagents), and compounds were visualized by UV light and iodine. Purification by column chromatography was performed on silica gel, 100–200-mesh size (EM Reagents). Infrared (IR)[1] measurements were done on a Perkin-Elmer 257 grating spectrophotometer, liquid samples were measured neat on NaCl plates, and solid samples were measured as KBr pellets. Proton nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-390 spectrometer. Carbon-13 NMR spectra of 150 mg of 2-benzyl-4-bromo-5-oxohexanoic acid (XV) at pH 7 in $D_2O$ and of a saturated solution of bromoacetone in $D_2O$ were recorded on a Varian XL-300 spectrometer in the Department of Chemistry at the University of Louisville, Louisville, KY. All chemical shifts (δ) are in parts per million (ppm) downfield from tetramethylsilane. The electron impact (EI) mass spectra (MS) at 70 eV were measured with an Hitachi 90E spectrometer and chemical ionization (CI) mass spectra on a Finnigan 3300 GC/MS at 150 eV with a solid probe. High-performance liquid chromatography was accomplished with a Varian 5000 chromatograph and a 20 cm × 4 mm Varian MCH-10 reversed-phase column or a Waters μBondapak $C_{18}$ column with detection at 205 nm at a flow rate of 1 mL·min$^{-1}$. The solvent systems are given with the retention times ($R_t$, in minutes after the solvent front) for individual compounds. Gas–liquid chromatography (GLC) was performed on a Hewlett-Packard F&M 5750 instrument with a 1.5 mm × 6 mm glass column on SE-30 Chromosorb. Melting points were taken on a hot stage and are corrected. The yields given were based on isolated products after purification. All compounds with one asymmetric carbon atom are racemic and are designated *RS* in Table I. All compounds with more than one asymmetric center are presumed to be mixtures of the maximum number of isomers.

The bromo ketone 2-benzyl-4-bromo-5-oxohexanoic acid (XV) was thoroughly characterized in terms of proving its structure and defining its reversible inhibition of carboxypeptidase A. The synthetic route to XV is outlined here. The detailed synthesis of XV and all other compounds is described in the supplementary material for this paper (see paragraph at end of paper regarding supplementary material). The first step in the synthesis of XV required conjugate Michael addition of methyl vinyl ketone to diethyl malonate in diethyl ether with potassium hydroxide as a base to give the keto diester diethyl 2-(3-oxobutyl)malonate (VIII). The ketone group of VIII was then protected by the formation of the 1,3-dioxolane, diethyl 2-[3,3-(ethylenedioxy)butyl]malonate (IX). The key intermediate, diethyl 2-benzyl-2-[3,3-(ethylenedioxy)butyl]malonate (X) was obtained by alkylation of the sodium salt of the monosubstituted diethyl malonate (IX) with benzyl bromide. High-vacuum distillation of the crude product gave analytically pure diethyl 2-benzyl-2-[3,3-(ethylenedioxy)butyl]malonate (X).

The next step was achieved by either of two routes: (a) hydrolysis of the diester X with aqueous potassium hydroxide followed by acidification of the salt without isolation of the diacid and heating at 100 °C or (b) isolation of the diacid XI from the hydrolysis reaction mixture and decarboxylation of this material by heating 20 °C above its melting point. Both routes gave a good yield of the monoacid 2-benzyl-5,5-(ethylenedioxy)hexanoic acid (XII). Cleavage of the 1,3-dioxolane in 60% aqueous acetic acid gave the δ-keto acid 2-benzyl-5-oxohexanoic acid (XIII). The final stage of the synthesis, bromination to give 2-benzyl-4-bromo-5-oxohexanoic acid (XV), was accomplished by reacting the keto acid XIII with bromine in $CCl_4$ at 0 °C. The bromo keto acid XV was obtained in the form of an oil, which upon standing at −80 °C gradually gave a small amount (120 mg) of crystalline XV. 2-Benzyl-4-bromo-5-oxohexanoic acid (XV) was characterized by melting point, chemical ionization mass spectrum, electron impact mass spectrum, proton NMR spectrum, carbon-13 NMR spectrum, and formation of a (2,4-dinitrophenyl)-hydrazone. All analytical data are given in the supplementary material for this paper.

*Kinetic Studies.* The hydrolysis of *O*-(*trans-p*-chlorocinnamoyl)-L-β-phenyllactate by carboxypeptidase A was measured spectrophotometrically at 25 °C as described by Suh & Kaiser (1976) in 50 mM Tris base–0.5 M sodium chloride adjusted to pH 7.5 with hydrochloric acid. The suspension of enzyme as received was diluted to give a stock solution with

[1] Abbreviations: GC/MS, gas chromatograph/mass spectrometer; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; $IC_{50}$, the concentration of inhibitor causing 50% inhibition of a given enzyme concentration at a given substrate concentration; IR, infrared; MS(CI), chemical ionization mass spectrometry; MS(EI), electron impact mass spectrometry; PPTS, pyridinium *p*-toluenesulfonate; SD, standard deviation; Tris, tris(hydroxymethyl)-aminomethane.

Table I: Inhibition of Carboxypeptidase A Catalyzed Hydrolysis of *O*-(*trans*-*p*-Chlorocinnamoyl)-L-β-phenyllactate by Ketones and Related Compounds at pH 7.5

| inhibitor | $K_i \pm$ SD (μM) | mode |
|---|---|---|
| (*RS*)-2-benzylsuccinate | 0.20 ± 0.1[a] | comp[e] |
| (*RS*)-2-benzyl-4-oxobutanoic acid | 0.48 ± 0.1[b] | comp |
| (*RS*)-2-benzyl-4-oxopentanoic acid (IV) | 770 ± 100[c] | comp or mixed |
| 2-benzyl-4-hydroxypentanoic acid (III) | 1100 ± 100 | comp |
| 2-benzyl-3-bromo-4-oxo-pentanoic acid and 2-benzyl-5-bromo-4-oxo-pentanoic acid (VII) | 0.57 ± 0.1 | comp |
| (*RS*)-2-benzyl-5-oxohexanoic acid (XIII) | 630 ± 100 | comp |
| 2-benzyl-5,6-dihydroxyhexanoic acid (XXI) | 180 ± 10 | comp |
| 2-benzyl-4-bromo-5-oxo-hexanoic acid (XV) | 1.3 ± 0.1 | comp |
| 2-oximino-3-phenylpropanoic acid (XVII) | 480 ± 20 | comp |
| (*RS*)-2-benzyl-3-oximino-4-oxo-pentanoic acid (VI) | 1200 ± 200 | comp |
| (*RS*)-2-benzyl-4-oximino-pentanoic acid (V) | 7900 ± 200 | mixed |
| (*RS*)-2-benzyl-5-oximino-hexanoic acid (XIV) | 1400 ± 200 | comp or mixed |
| bromoacetone | >100000[d] | |

[a] Galardy & Kortylewicz (1984); literature value, 1.1 μM (Byers & Wolfenden, 1973). [b] Galardy & Kortylewicz (1984); $K_i$ = 0.11 μM at pH 6.8 and 0.80 μM at pH 8.2. [c] $K_i$ = 260 μM at pH 6.8 and 1700 μM at 8.2. [d] No inhibition observed at 27 mM bromoacetone. [e] Comp = competitive.

a concentration of 60 nM in the assay buffer. The concentration of enzyme was determined by assay against hippuryl-L-phenylalanine as described by Polk & Schirmer (1963) using a specific activity of 37 units/mg. The enzyme concentration in the kinetic assay was usually 3 nM. Substrate concentrations were varied from 80 to 400 μM. The $K_m$ was found to be 170 ± 30 μM. Initial velocities were calculated from the linear initial slopes of the change in absorbance vs. reaction time curves where the amount of substrate consumed was always less than 10%. For each substrate and inhibitor at least four $K_i$'s were determined, one from a Lineweaver–Burk plot and one from a Dixon plot from each of two independent experiments done on different days. The four $K_i$'s were averaged, and a standard deviation (SD) was calculated.

*Recovery of Bromo Ketone XV from Its Complex with Carboxypeptidase A.* A concentrated solution of carboxypeptidase A (0.4 mM) equimolar in bromo ketone XV was incubated under the assay conditions, and XV was quantitatively determined by HPLC after recovery from the enzyme by acetone precipitation. A 1.8-mL aliquot (28.5 mg, 0.8 μmol) of the suspension of carboxypeptidase A as received from Sigma was diluted to 10 mL with water and centrifuged for 5 min. The supernatant was discarded, and 2 mL of the enzyme assay buffer was added to dissolve the pellet. To this solution was added 50 μL (0.25 mg, 0.8 μmol) of a solution of 5 mg·mL$^{-1}$ of 2-benzyl-4-bromo-5-oxohexanoic acid (XV) in enzyme assay buffer adjusted to pH 7.5, and the resulting solution was incubated at 25° C for 1 h. The enzyme was precipitated by the addition of 20 mL of acetone and centrifuged. The supernatant and three 2-mL acetone washes of the pellet were collected, combined, and evaporated at room temperature under vacuum. The concentrate was adjusted to a volume of 5 mL with the aqueous phase of the HPLC solvent system, 10 mM triethylamine adjusted to pH 6.0 with phosphoric acid. The bromo ketone was quantitated on the Waters reversed-phase column with isocratic elution by 11% acetonitrile–89% pH 6.0 buffer ($R_t$, 11.7 min).

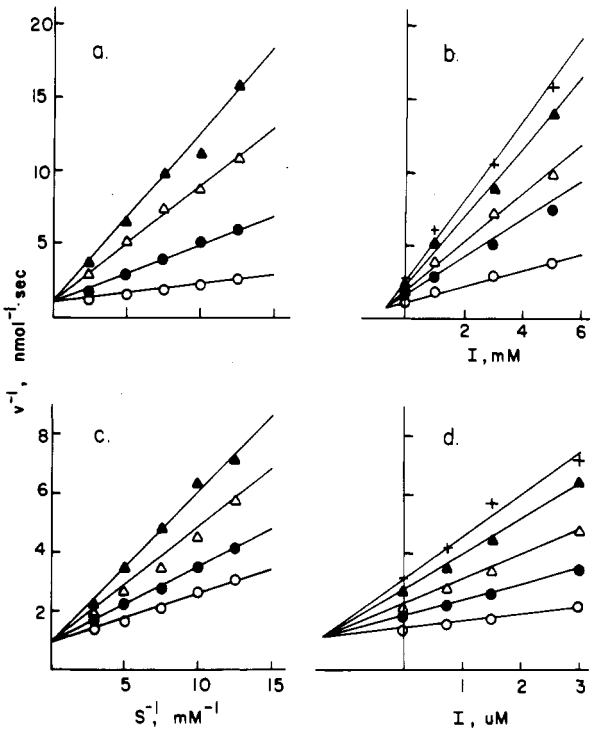


FIGURE 2: Lineweaver–Burk (a and c) and Dixon (b and d) plots for the inhibition of carboxypeptidase A catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate by (*RS*)-2-benzyl-5-oxohexanoic acid (XIII) (a and b) and 2-benzyl-4-bromo-5-oxohexanoic acid (XV) (c and d). Inhibitor concentrations are as follows: (a) 0 (○), 1 (●), 3 (△), and 5 mM (▲); (c) 0 (○), 0.75 (●), 1.5 (△), and 3 μM (▲). Substrate concentrations are as follows: (b and d) 400 (○), 200 (●), 133 (△), 100 (▲), and 80 μM (+).

## RESULTS

*Inhibition of Carboxypeptidase A by Ketone Substrate Analogues.* The $K_i$'s for inhibition of carboxypeptidase A by the ketone substrate analogues and related compounds are given in Table I. The $K_i$ of (*RS*)-2-benzylsuccinate is given for comparison. Figure 2 shows one set of Lineweaver–Burk and Dixon plots of the inhibition by each of the ketones (*RS*)-2-benzyl-5-oxohexanoic acid (XIII) and 2-benzyl-4-bromo-5-oxohexanoic acid (XV). The results in Table I demonstrate that the two bromo ketones are much more potent competitive inhibitors than their less electrophilic parent ketones. 2-Benzyl-4-hydroxypentanoic acid (III), the alcohol derived from IV, is also a weak inhibitor in spite of the tetrahedral configuration about the alcoholic carbon proposed to be positioned near the zinc atom at the active site of the enzyme (see Figure 1). These results are in agreement with the weak inhibition of carboxypeptidase A by 2-benzyl-4-hydroxybutanoic acid compared to that by its parent aldehyde 2-benzyl-4-oxobutanoic acid (Galardy & Kortylewicz, 1984). Lack of strong inhibition by the diol XXI demonstrates that bidentate chelation of zinc by two hydroxyls either does not occur or does not result in tight binding.

Table I also gives $K_i$'s for a series of oximes related to the parent ketone IV. These oximes are proposed to fit the active site of carboxypeptidase A as shown in Figure 3 for 2-benzyl-3-oximino-4-oxopentanoic acid (VI). This oxime was designed to be a by-product analogue similar to 2-benzylsuccinic acid (Byers & Wolfenden, 1973). However, VI, with the oxime oxygen atom in the "correct" position for coordination to the active site zinc atom, is a weak inhibitor. Oximes

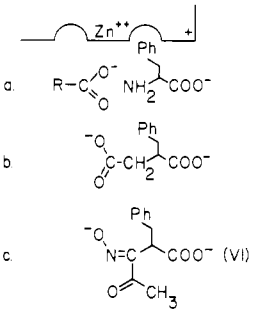


FIGURE 3: Proposed mode of interaction of 2-benzyl-3-oximino-4-oxopentanoic acid (VI) with carboxypeptidase A: (a) binding of the two products of substrate hydrolysis, (b) binding of the by-product inhibitor 2-benzylsuccinate, and (c) the proposed binding of oxime VI.

XVII, V, and XIV are also weak inhibitors. The weak inhibition by XVII, V, and XIV may be due to the high $pK_a$ of simple oximes (the $pK_a$ of acetoxime is 12.4; Fasman, 1976) or the lack of tetrahedral hybridization at the nitrogen atom attached to the oxime oxygen atom. Although oxime VI probably has a $pK_a$ near 8 (the $pK_a$ of phenylglyoxaldoxime is 8.3; Fasman, 1976), it is still a weak inhibitor. The weak inhibition of carboxypeptidase by the oximes is surprising considering the strong inhibition of other metalloproteases by hydroxamates, which, like oximes, contain an ionizable –NOH group (Nishino & Powers, 1979; Petrillo & Ondetti, 1982). This could be due to the bidentate, five-membered ring chelation of zinc possible in the hydroxamates, which is absent in the oximes.

The $K_i$'s of the ketone IV and the aldehyde 2-benzyl-4-oxobutanoic acid were determined at pH 6.8 and 8.2 in addition to pH 7.5. The same buffer composition was used at each pH with care being taken not to exceed the buffering capacity at pH 6.8 and 8.2. The pH dependence of these $K_i$'s showed the same trend as that of the by-product inhibitor 2-benzylsuccinate (Byers & Wolfenden, 1973) and the transition-state analogue *N*-[[[(benzyloxycarbonyl)amino]-methyl]hydroxyphosphinyl]-L-phenylalanine (Jacobsen & Bartlett, 1981), increasing with pH (see Table I).

*Stability of the Bromo Ketones at pH 7.5.* 2-Benzyl-4-bromo-5-oxohexanoic acid (XV) was shown to be stable for at least 24 h under the conditions of assay of carboxypeptidase A by HPLC analysis using the Varian column. A solution of 0.9 mM XV in 50 mM Tris buffer adjusted to pH 7.5 with hydrochloric acid, 0.5 M sodium chloride, was incubated at 25 °C. Aliquots were chromatographed in the solvent system 20% acetonitrile–80% 10 mM triethylamine adjusted to pH 7.0 with phosphoric acid [$R_t$(XV), 4 min] and 40% acetonitrile–60% 10 mM triethylamine adjusted to pH 3.0 with phosphoric acid [$R_t$(XV), 5 min]. There was no change in either the area of the peak or its $R_t$ in either solvent system over 24 h at pH 7.5 compared to the area and $R_t$ of a reference solution of XV in acetonitrile, which was also unchanged. Ten milligrams of XV was incubated in the pH 7.5 buffer overnight at 25 °C, the buffer was acidified, and XV was extracted into ether and dried under high vacuum to give XV with mass spectrum unchanged from starting material: MS(CI) 299 (M − 1), 297 (M − 3). The mixture of bromo ketones 2-benzyl-3-bromo-4-oxopentanoic acid and 2-benzyl-5-bromo-4-oxopentanoic acid (VII) likewise was stable at pH 7.5 and 25 °C for at least 24 h.

*Reversibility of the Inhibition of Carboxypeptidase A by Bromo Ketones VII and XV.* The bromo ketones VII and XV were tested for progressive, time-dependent inhibition of carboxypeptidase A by assaying a mixture of enzyme and inhibitor as a function of time of incubation of the mixture. The reversibility of inhibition was determined by diluting an enzyme–inhibitor mixture exhibiting about 50% inhibition into buffer to a concentration expected to give less than 10% inhibition. For 2-benzyl-4-bromo-5-oxohexanoic acid (XV), incubation of enzyme (3.2 nM) and inhibitor (5 μM, 48% inhibition) for 5.5 h led to a decrease in initial velocity to 83% of that present at zero time at this inhibitor concentration at 80 μM substrate. Incubation of enzyme alone at 3.2 nM gave a decrease to 85% of the initial velocity at zero time. Incubation of enzyme (100 nM) and XV (9 μM, 44% inhibition compared to uninhibited enzyme) for 1 h followed by 30-fold dilution gave 96% recovery of enzyme activity compared to dilution of the uninhibited control. Therefore, the inhibition of carboxypeptidase A by the bromo ketone XV is neither time-dependent nor irreversible.

Similar results were obtained with the mixture of bromo ketones VII. Incubation of enzyme (3.2 nM) and VII (2 μM, 68% inhibition) for 5 h led to a decrease to 84% of the initial velocity at zero time at 80 μM substrate. Incubation of enzyme (320 nM) and VII (27 μM, 93% inhibition compared to an uninhibited control) for 1 h followed by 100-fold dilution gave 97% recovery of activity compared to dilution of the uninhibited control.

*Recovery of Bromo Ketone XV from Its Complex with Carboxypeptidase A.* A concentrated solution of carboxypeptidase A (0.4 mM) equimolar in 2-benzyl-4-bromo-5-oxohexanoic acid (XV) was incubated for 1 h at 25 °C in assay buffer, and the recovery of XV was determined in order to prove that the enzyme does not significantly convert XV to another product. At these concentrations it can be calculated from the $K_i$ that over 95% of XV is bound to the enzyme. This enhances the probability of detecting conversion of a significant amount of XV to another product by a factor of about 3000 compared to the usual kinetic assay conditions. For example, 5 μM XV gives 48% inhibition of a 3.2 nM solution of enzyme in the presence of 80 μM substrate, and the enzyme–inhibitor complex concentration is therefore about 1.6 nM and contains only about 1.6 nM × (5 μM)$^{-1}$ or 0.03% of the total inhibitor concentration. The turnover of a small percentage of inhibitor to another compound by the enzyme would be difficult to detect in the presence of such a large excess of intact inhibitor. In the equimolar mixture of enzyme and inhibitor at high concentration, turnover, if it occurs, should be easily detectable. The high concentration of XV, a potential alkylating agent, was not expected to nonspecifically inactivate the enzyme by alkylation since 30 mM bromoacetone, which must be more reactive because of its smaller size, did not detectably inhibit carboxypeptidase A.

Under the conditions of high equimolar concentrations (0.4 mM) of enzyme and XV, 82 ± 4% of XV was recovered as determined by HPLC assay. In a control experiment without enzyme 100 ± 2% of (XV) was recovered. The loss of about 18% of XV with the enzyme could be due to turnover of 18% of XV to another product, to occlusion of XV in the precipitated enzyme (no precipitate occurred in the control), or to nonspecific alkylation of the protein by XV. If nonspecific alkylation occurred, only about one in six carboxypeptidase molecules would each carry a single molecule of XV. If turnover of XV occurred to the extent of 18%, then turnover of XV during the kinetic assays where $K_i$ was measured must have been 3000-fold less. If turnover occurred during the kinetic assays to produce an inhibitor that accounted for all of the observed inhibition of carboxypeptidase A, then this inhibitor would have to have a $K_i$ of about 1.3 × 3000$^{-1}$ × 0.18

or 80 pM. Turnover to a more potent inhibitor seems to be an improbable explanation for the inhibition of the enzyme by the bromo ketone XV.

*Hydration of Bromo Ketone XV in $D_2O$ at pH 7.* A carbon-13 NMR spectrum of XV in $D_2O$ at pH 7 gave the following resonances [ppm downfield from tetramethylsilane (height in mm, assignment)]: 25.1 (10, $CH_3$); 33.9, 37.2, 38.0, and 38.3 (44, 22, 26, and 40, $CH_2$ and $CH_2Ph$); 45.8, 46.4, 49.2, 74.4, and 74.6 (17, 33, 18, 15, and 30, COCH and CHBr); 125.1, 126.2, 128.3, 138.9, and 139.4 (76, 197, 184, 211, and 10, Ph); 182.0, 182.2, and 182.6 (4, 3, and 4, COOH); 214.0 (3, CO). No resonances were observed in the region from 74.6 to 125.1 ppm at a 10-fold expansion of the amplitude of this spectrum. This is the region expected for the hydrate of this ketone by comparison with chemical shifts of the *gem*-diol carbon of the following hydrated ketones: bromoacetone, 94.3 ppm; acetone, 94.6 ppm (Grobelny and Galardy, unpublished results); 7-amino-2-(carbobenzoxyamido)-1-chloro-2-heptane, 95.4 ppm (Malthouse et al., 1983). On the basis of the ratio of the signal height at 74.6 ppm to the noise in the 74.6–125.1 ppm region, bromo ketone XV must be less than 5% hydrated in $D_2O$ at pH 7. This is not inconsistent with the 7% hydration of bromoacetone reported by Lewis & Wolfenden (1977a).

## Discussion

The strong inhibition of carboxypeptidase A by the $\alpha$-bromo ketones VII and XV compared to their respective parent ketones IV and XIII must be due to the increased electrophilicity of the bromo ketone carbonyl carbon atom. We propose that the bromo ketones are able to add a nucleophile to this position to produce a tetrahedrally hybridized adduct that mimics a tetrahedral intermediate that occurs during normal substrate hydrolysis and is strongly bound by the enzyme. Thus the bromoketones VII and XV are commonly called transition-state analogoues, as described by Wolfenden (1969), or analogues of labile intermediates that are not transition states (Anderson et al., 1984). The fact that electrophilic $\alpha$-bromo ketones appear to be transition-state analogues for carboxypeptidase is in agreement with our finding that the aldehyde 2-benzyl-4-oxobutanoic acid is also a transition-state analogue for this enzyme (Galardy & Kortylewicz, 1984). The electrophilicity of this aldehyde is apparent from its 25% extent of hydration at pH 7.5.

The hydration of bromo ketone XV at pH 7.5 could not be conclusively detected by proton NMR and was found to be undetectable ($\leq$5% hydrate) by carbon-13 NMR at very high signal to noise ratios. This is consistent with the low extent of hydration of bromoacetone (7%) reported by Lewis & Wolfenden (1977a) although the increased electrophilicity of bromoacetone compared to acetone is reflected by the large increase in hydration of the former (7.0%) compared to the latter (0.2%). We conclude that the increased electrophilicity of the bromo ketone XV compared to that of ketone XIII is responsible for its decreased $K_i$ even though its hydration was not detectable by NMR. If the hydrate of XV is the species inhibiting the enzyme, then its $K_i$ must be less than 1.3 $\mu$M $\times$ (5% hydrate) = 65 nM. The fact that 2-benzyl-4-hydroxypentanoic acid (III) is no more potent an inhibitor than its parent ketone IV demonstrates that tetrahedral geometry at carbon 4 is not sufficient for tight binding. Tetrahedral geometry and two heteroatoms attached to this carbon, one of which being contributed by water or an enzyme-bound nucleophile, appear to be required, as found for the aldehyde 2-benzyl-4-oxobutanoic acid (Galardy & Kortylewicz, 1984). X-ray crystallography of the complex between this aldehyde and carboxypeptidase A demonstrates that it is indeed the hydrate that is bound (personal communication, D. W. Christianson and W. N. Lipscomb, Harvard University).

The reversibility and time independence of the inhibition by the bromo ketones are consistent with their being transition-state analogues rather than affinity labels. It is not clear why the $\alpha$-bromo amide *N*-(bromoacetyl)-*N*-methyl-L-phenylalanine is an irreversible affinity label (Hass & Neurath, 1971a,b) while the bromo ketones are not. The conclusion that the bromo ketones are transition-state analogues requires that an adduct of the $\alpha$-bromo ketone is the species bound at the active site of the enzyme. 2-Benzyl-4-bromo-5-oxohexanoic acid (XV) is a relatively labile compound that could hydrolyze in solution to the 4-hydroxy ketone, cyclize to a $\gamma$-lactone with loss of bromine, or cyclize to a $\delta$-lactone hemiacetal. Indeed, at high pH XV loses bromine accompanied by cyclization, and during derivatization with (2,4-dinitrophenyl)hydrazine the bromine is replaced by hydroxyl to give XVI. This lability required the demonstration that the bromo ketone XV was stable during the enzyme assay at pH 7.5. If it were not stable, then the species that strongly inhibits the enzyme was less likely to be a reversible adduct of XV. The identity of the crystalline bromo ketone XV was established by proton and carbon-13 NMR, chemical ionization and electron impact mass spectroscopy, and conversion to a (dinitrophenyl)-hydrazone derivative XVI). The stability of XV at pH 7.5 at 25 °C for 24 h in buffer alone and in the presence of an equimolar amount of enzyme for 1 h was proven by HPLC analysis and reisolation of starting XV. Therefore, no other species is likely to be responsible for the strong reversible inhibition occurring with XV. The mixture of bromo ketones VII was less extensively characterized because it was not a single structural isomer. The equal inhibitory potency of the $\alpha$-bromo-4-oxo compound VII and the $\alpha$-bromo-5-oxo compound XV was unexpected since the aldehyde 2-benzyl-5-oxopentanoic acid is bound 48-fold more weakly than 2-benzyl-4-oxobutanoic acid (Galardy & Kortylewicz, 1984).

In summary, the electrophilic bromo ketones VII and XV are powerful inhibitors of carboxypeptidase A compared to their less electrophilic parent ketones. They appear to be transition-state analogues for this enzyme as proposed for the aldehyde 2-benzyl-4-oxobutanoic acid (Galardy & Kortylewicz, 1984). These results serve as a model for the inhibition of the dipeptidyl carboxypeptidase angiotensin converting enzyme by [(5*S*)-5-benzamido-4-oxo-6-phenylhexanoyl]-L-proline, with a $K_i$ of 0.6 nM [Grobelny and Galardy, unpublished results; reported $K_i$ of 100 nM (Almquist et al., 1980); under conditions of potential enzyme-inhibitor depletion, $K_i$ = 1 nM (Almquist et al., 1982)]. This ketone may be a transition-state analogue for converting enzyme.

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## Supplementary Material Available

Description of the preparation of inhibitors not given here (12 pages). Ordering information is given on any current masthead page.

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# Inhibition of Carboxypeptidase A by Ketones and Alcohols That Are Isosteric with Peptide Substrates

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ABSTRACT: The $K_i$'s of three peptide ketone and three peptide alcohol inhibitors of carboxypeptidase A are compared with $K_i$'s of their respective isosteric peptide substrates, $N^{\alpha}$-benzoyl-L-phenylalanine, $N^{\alpha}$-benzoylglycyl-L-phenylalanine, and $N^{\alpha}$-carbobenzoxyglycylglycyl-L-phenylalanine. For the isosteric ketone analogues of these substrates, the respective $K_i$'s are as follows: (2*RS*)-2-benzyl-4-(3-methoxyphenyl)-4-oxobutanoic acid, 180 ± 40 μM; (2*RS*)-5-benzamido-2-benzyl-4-oxopentanoic acid (V), 48 ± 7 μM; (2*RS*)-2-benzyl-5-(carbobenzoxyglycinamido)-4-oxopentanoic acid (IX), 9 ± 0.1 μM. For the alcohols derived by reduction of each of these ketones, $K_i$'s are as follows: (2*RS*,4*RS*)-2-benzyl-4-(3-methoxyphenyl)-4-hydroxybutanoic acid, 190 ± 10 μM; (2*RS*,4*RS*)-5-benzamido-2-benzyl-4-hydroxybutanoic acid (IV), 160 ± 62 μM; (2*RS*,4*RS*)-2-benzyl-5-(carbobenzoxyglycinamido)-4-hydroxypentanoic acid (XI), 600 ± 100 μM. $K_i$ values for the competitive peptide ketone inhibitors decrease with increasing peptide chain length. This is consistent with the possibility of increased binding interaction between inhibitor and enzyme by simple occupation of additional binding subsites by adding more amino acid residues to the inhibitor. In contrast, the $K_i$ values of the alcohols (competitive or mixed inhibition) increased or remain essentially unchanged with increasing chain length. Increasing the chain length of ketone inhibitor V to give IX decreases $K_i$ by one-fifth. The $K_i$ of ketone IX is also less than $^1/_{30}$th the $K_i$ of its isosteric peptide and almost $^1/_{70}$th that of its isosteric alcohol, XI. Taken together, these results suggest that binding of ketones V and IX to the enzyme may not be via a simple Michaelis-type complex but requires an additional interaction with the enzyme.

Carboxypeptidase A (EC 3.4.12.2) is a zinc metalloprotease that cleaves a single amino acid residue from the carboxy terminus of peptides. Aldehyde and ketone substrate analogues and isosteres strongly inhibit several zinc metalloproteases: angiotensin-converting enzyme (Almquist et al., 1980, 1982; Meyer et al., 1981; Gordon et al., 1984; Natarajan et al., 1984), leucine aminopeptidase (Andersson et al., 1982), and carboxypeptidase A [Galardy & Kortylewicz (1984, 1985) and references cited therein]. Carbonyl compounds have been proposed to be transition-state analogues for proteases due to their ability to add a nucleophile to form a tetrahedral species

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