Inhibition of carboxypeptidase A by (*S*)-2-mercapto-3-phenylpropanoic acid

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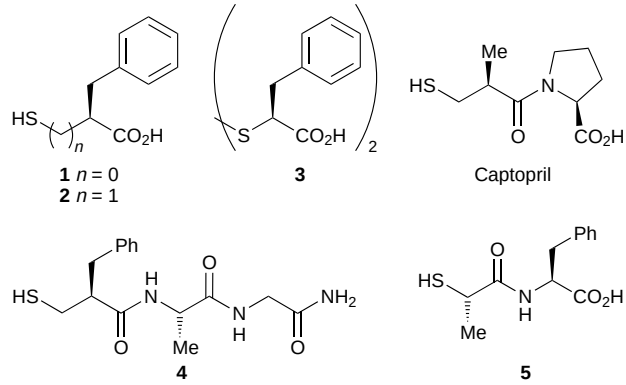
(*S*)-2-Mercapto-3-phenylpropanoic acid is an effective competitive inhibitor of carboxypeptidase A, comparable in potency to (*S*)-3-mercapto-2-benzylpropanoic acid.

Divalent metal ion-dependent proteolytic enzymes are involved in a wide variety of physiologically important processes and play a role in a number of disease states. It is widely recognized that selective inhibition of such enzymes is a viable strategy for therapeutic intervention. Notable successes have been reported in the development of very effective antihypertensive agents which function through inhibition of zinc-dependent angiotensin converting enzyme (ACE).[1] Intensive research efforts are underway aimed at developing related specific inhibitors for other zinc-dependent proteases such as neutral endopeptidase 24.11 and the matrix metalloproteases.[2,3] The ability to carry out rational design of inhibitors for such enzymes has been built upon detailed observations made in the development of specific inhibitors for the better characterized and understood zinc-dependent proteases such as carboxypeptidase A (CPA) and thermolysin.[4] Thus CPA has served as a valuable model system for the elucidation of guiding principles for the design of inhibition strategies for other less well understood but therapeutically important zinc-dependent proteases.

The now classical studies of the inhibition of CPA by benzylsuccinic acid[5] and by mercaptocarboxylic acid derivatives[6–8] played an important role in the definition of design criteria for the extremely successful commercial antihypertensive agents enaloprilat[9] and captopril.[10] The observation of the relative potencies of (±)-2-mercapto-3-phenylpropanoic acid **1** (K_i = 1.2 μM)[7] and its homologue (*S*)-2-benzyl-3-mercaptopropanoic acid **2** (K_i = 7.8 nM)[8] as competitive inhibitors of CPA has led to the conclusion that the insertion of a methylene group between the metal binding mercapto group and the α-carbon of an α-mercapto carboxylic acid derivative results in an optimal interaction between the active site and the mercapto, carboxylate and hydrophobic side chains of the inhibitor. The fact that inclusion of a similar methylene spacer between the mercapto group and the remainder of the structure of the ACE inhibitor captopril results in very high inhibitory potency (K_i = 1.7 nM)[10] reinforced the belief that a spacing of approximately 4.1 Å between the sulfur atom and the carbonyl group carbon represents the ideal spatial relationship.

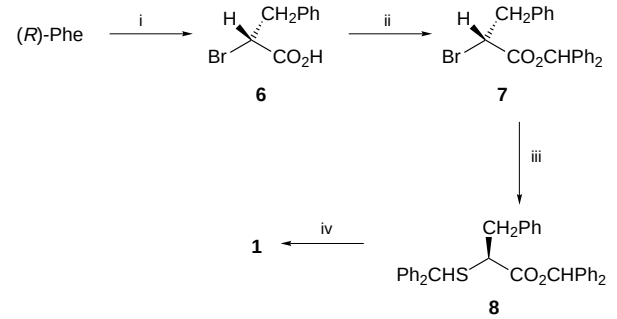
In the course of studies of the hydrolysis of thioesters by CPA we have prepared optically active 2-mercapto-3-phenylpropanoic acid **1** and have examined the kinetics of inhibition of CPA by this thiol. We report herein our observation that **1** is a much more effective inhibitor of the peptidase activity of CPA than previously believed, rivaling the potency of 3-mercapto-2-benzylpropanoic acid **2**, and point out the possible significance of this observation with respect to the rational design of specific zinc-binding inhibitors for metalloproteases.

The thiol **1** was synthesized *via* the route shown in Scheme 1. The known bromoacid **5**, prepared with retention of configuration by diazotization of (*R*)-phenylalanine in the presence of bromide, was converted to the benzhydryl ester **6** which was subjected to an S_N2 displacement with 1,1-diphenylmethanethiol as the nucleophile. Deprotection using trifluoroacetic acid–phenol yielded the free thioacid **1** (89% ee as measured by ^{1}H NMR analysis of the Mosher's thioester[11] form of **1**.



Progress curves† for the CPA catalyzed hydrolysis of hippuryl-L-phenylalanine (HP)[12] in the presence of **1** were found to be biphasic with an initial low rate observed at short reaction times and a higher reaction rate at longer reaction times. That this phenomenon was associated with air oxidation of the thiol to the corresponding disulfide **3** was revealed by the fact that preincubation of solutions of **1** in air until no thiol could be detected using DTNB[13] assays prior to use in inhibition studies abolished the biphasic nature of the progress curves. The slopes of such progress curves were equal to those observed for the second phase of the biphasic curves observed in experiments employing solutions with equal concentrations of **1** which had not been preincubated in air. The inhibition by the disulfide **3**, which was isolated from such solutions and fully characterized, was found to be of the same order of magnitude as reported previously for the thiol **1** [K_i = 3.6 ± 0.5 μM (competitive) for **3** *vs.* reported apparent K_i of 1.2 μM (ref. 7) for racemic **1**].

Measurement of the true inhibitory potency of the thiol **1** required experimental conditions which inhibited the air oxidation process. This was eventually achieved by working with an argon atmosphere or, much more conveniently, by adding glutathione as an antioxidant. It was found that concentrations of glutathione as high as 0.1 mM could be tolerated without any observable influence on the rate of CPA



Scheme 1 *Reagents and conditions*: i, NaBr, $NaNO_2$, H_2SO_4; ii, Ph_2CN_2, ether; iii, Ph_2CHSH, acetone; iv, CF_3CO_2H, CH_2Cl_2, PhOH

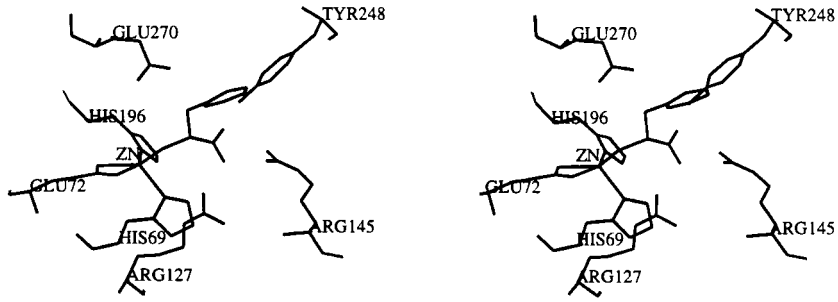


Fig. 1 Stereoview of the predicted binding mode of **1** to the active site of CPA

catalyzed hydrolysis of HP. Inhibition of the CPA catalyzed hydrolysis of HP by **1** in the presence of glutathione was found to fit a competitive inhibition model as indicated in the Lineweaver–Burk plots.

Estimation of the potency of inhibition by **1** was complicated by the fact that the affinity of **1** for CPA was sufficiently high that inhibitor and enzyme depletion phenomena are significant and render invalid the normal assumptions associated with kinetic models of reversible inhibition. As a result, the graphical method of Dixon[14] which avoids such assumptions was employed to determine K_i for inhibition of CPA catalyzed hydrolysis of HP to be 12.6 ± 1.0 nM. It thus appears likely that, in the earlier studies which concluded that the apparent K_i for inhibition of peptidase activity by (±)-**1** was 1.2 μM (ref. 7), the inhibition observed resulted largely from the presence of the disulfide **3** rather than the thiol **1**.

The recognition that **1** (K_i = 12.6 nM) is comparable in potency to **2** (K_i = 7.8 nM)[8] raises interesting questions concerning the dimensions of the pharmacophore for selective zinc-binding inhibitors for Zn^{2+}-dependent proteases. It has been assumed previously that the apparent substantially higher inhibitory potency of **2** arose from the ability of the benzyl side chain of **2** to bind in the hydrophobic $S_{1'}$ pocket in the active site with the thiol sulfur atom interacting favorably with the Zn^{2+} ion and the carboxylate group interacting with Arg-145. Docking of a model of **2** (not shown) in the active site of CPA‡ as defined by X-ray crystallographic coordinates suggests that such a hypothesis is reasonable and consistent with the active site interactions observed between the related thiol-type inhibitor **4** and the zinc-dependent endopeptidase thermolysin.[15] The apparent lower potency of the thiol inhibitor **1** has been assumed to result from an inability of the hydrophobic side chain, the thiol and the carboxylate group to simultaneously interact with the potential binding sites which are accessible to the same groups in the homologue **2**. A molecular modeling analysis of **1** docked in the active site of CPA confirms that the benzyl group in **1** has a diminished interaction with the hydrophobic pocket in the $S_{1'}$ subsite when the sulfhydryl sulfur atom is liganded to zinc and also reveals an interesting favorable interaction between the carboxylate of **1** and Arg-127 which might offset the loss of binding energy associated with the diminished hydrophobic interactions (Fig. 1). This model, although speculative, does suggest strategies for modification of the structure of 2-mercaptocarboxylic acid inhibitors to enhance inhibitor potency by increasing the interaction with the hydrophobic pocket which are now being pursued in this laboratory.

Although our observations clearly indicate the need for consideration of the stability of thiol type inhibitors in evaluation of inhibitory potency against zinc dependent proteases, it should be emphasized that some thiols are much better behaved than **1** and do not require the use of anaerobic conditions or radical scavengers in inhibition studies. For example the rates of air oxidation of the 3-mercaptocarboxylic acid **2** or the 2-mercaptocarboxamide **5**, which is a potent inhibitor of angiotensin converting enzyme,[16] are substantially lower than that observed for **1** such that no special precautions were found to be necessary in kinetic investigations with these compounds. Studies aimed at developing a mechanistic rationale for the susceptibility of 2-mercaptocarboxylates towards air oxidation are in progress.

Footnotes and References

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† CPA activity assays were conducted in 25 mM tris-HCl, 0.5 M NaCl, pH 7.5 containing 3.5% ethanol (v/v) at 25 °C using hippuryl-L-phenylalanine as substrate. Enzyme concentrations ranged from 113 to 126 nM, as determined using absorption at 280 nm (ε_{280} = 6.42 × 10^4 M^{-1} cm^{-1}).[7]

‡ Molecular modeling studies were conducted using the software package SYBYL 6.2 (Tripos Associates Inc.) with crystal coordinates for carboxypeptidase A bound to [(−)-2-carboxy-3-phenylpropyl]methylsulfodiimine[17] (pdb1cps) obtained from the Brookhaven Protein Data Bank.

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Received in Corvallis, OR, USA, 4th August 1997; 7/05741E