β-Lactam Derivatives as Inhibitors for Carboxypeptidase A. Enzyme Inhibitor Design, Part 17

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Received September 19, 1995

2-(Azetidin-2-one-1-yl)-3-phenylpropionic acid and 2-(azetidin-2-thione-1-yl)-3-phenylpropionic acid were designed as potential active site directed inactivators for carboxypeptidase A, but shown to be they are competitive reversible inhibitors for the enzyme. The observation was somewhat surprising, but is not unexpected considering the recent report of Page who questioned the validity of the generally believed notion that β-lactam ring is highly unstable.

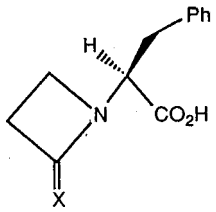
Introduction

β-Lactam is an enchanted ring which has received enormous attention last one-half a century as being an essential structural constituent of therapeutically invaluable antibiotics such as penicillins and cephalosporins. These antibiotics are known to manifest their antimicrobial activity *via* inhibition of the enzymic activity of D-alanyl-D-alanine transpeptidase which plays a critical role in the process of bacterial cell wall formation.[1,2] This enzyme inactivating property is attributed to the β-lactam ring present in the antibiotic molecules.[1,2] However, wide spread uses of these antibiotics have led to emergences of resistant strains. These resistant bacterial strains produce enzymes (β-lactamases) which catalyze the hydrolysis of β-lactam moiety in the antibiotics, thus rendering them to be ineffective as antibacterials.[3] Recently, β-lactam derivatives have also been studied as inhibitors of human leukocyte elastase whose aberrant activity is implicated to causes several pulmonary diseases such as emphysema and cystic fibrocis.[4]

Of these lactamases, Class B β-lactamases II from *Bacillus cereus* is a zinc containing enzyme. The studies by Little[6] *et al.* and Sutton and his associates[7] showed that the carboxylate of Glu-37 functions as the catalytic site of the enzyme. In these respects, the enzyme is very reminiscent to the well studied prototypic zinc containing enzyme, carboxypeptidase A (CPA).[8]

We have been actively involved in the development of novel designing methodology for enzyme inhibitors using CPA as a model target.[9] As an extension of these efforts we became interested in β-lactam derivatives as potential inhibitors for CPA. Such a study was thought to be important because it may provide valuable informations needed for successful designing of inhibitors which inactivates the β-lactamases. This report describes our rationale behind in designing β-lactam derivatives as inhibitors of CPA, and the synthesis and kinetic studies performed with these inhibitors.

The active site of CPA consists of a hydrophobic pocket which is responsible for the substrate specificity as being a primary substrate recognition site, Arg-145 whose protonated guanidinium ion forms a salt link with the carboxylate of the substrate, and Glu-270, the carboxylate of which is responsible for the catalytic activity by attacking the scissile carboxamide carbonyl carbon atom to form a high unstable anhydride intermediate. In addition, there is present a zinc ion which is coordinated to the backbone amino acid residues of His-69, Glu-72, and His-196. The presence of the zinc ion is essential for the catalytic activity of the enzyme, serving to activate the scissile peptide bond of the substrate.



1 : X = O

2 : X = S

On the basis of the topology of the active site and the catalytic mechanism of CPA, β-lactam derivatives **1** and **2** were designed as potential inhibitors of the enzyme. Upon binding the inhibitors to the enzyme, the aromatic moiety will be accommodated in the hydrophobic pocket and the carboxylate forms an ionic bond with the protonated guanidinium moiety of Arg-145. It is then expected that the carbonyl oxygen of the lactam would be positioned in the general area where the scissile peptide carbonyl of a substrate would position when it forms a complex with substrate.

The following three possibilities are anticipated when the potential inhibitors bind the enzyme: (i) The β-lactam derivatives simply behave as substrates with their lactam ring being cleaved. (ii) The anhydride intermediate that is generated by the nucleophilic attack of the catalytic carboxylate on the lactam ring may be reasonably stable to hydrolysis. In such a case, the compounds would behave as acylating agent, inactivating the enzyme transiently. This approach of inhibitor design has been extensively employed by Katzenellenbogen[10] and others[11] for designing inhibitors for serine protease. Lastly, the designed compounds may be reversible competitive inhibitors for the enzyme. This expectation is based on the recent reports of Page and his associates[12] who provided evidences against the generally believed notion that β-lactam ring is highly unstable due to its severe ring strain and hence disturbing resonance stabilization. These investigators asserted that β-lactams are rather stable entities which would not be hydrolyzed readily. In this regard, thio-

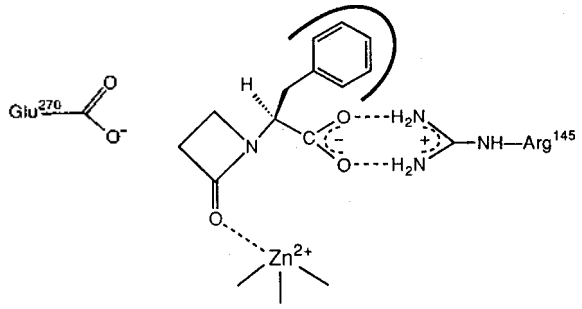
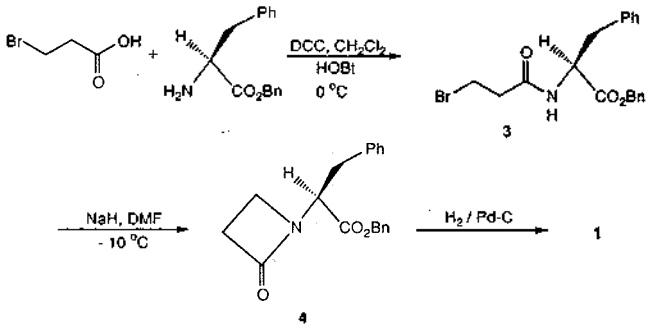
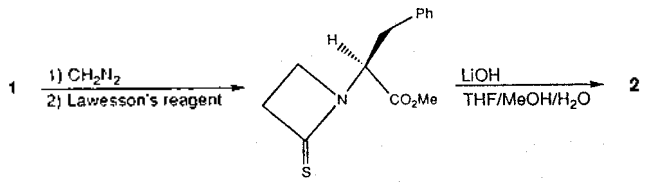


Figure 1. Schematic representation of the Michaelis complex formed by binding **1** to CPA.



Scheme 1.



Scheme 2.

lactam **2** was thought to be an interesting compound to be tested as a potential competitive inhibitor for the enzyme, since it has been known that thioamides are less reactive to hydrolyses compared with esters.[13]

Synthesis of Inhibitors and Enzyme Inhibitory Kinetics

The designed inhibitors were readily synthesized starting with L-phenylalanine benzyl ester as shown in Scheme 1. The cyclization of β-bromopropionamide **3** to form azetidinone **4** was effected under high dilution conditions following the general method developed by Wasserman and Hlasta,[14] giving the product in 53% yield. Debenzylation of **4** under catalytic hydrogenation conditions afforded **1** (Scheme 1). The conversion of **1** to thiolactam **2** was effected *via* its methyl ester, treating it with the Lawesson's reagent followed by hydrolysis of the ester moiety under alkaline conditions (Scheme 2).

As seen in Figure 2, the designed inhibitor (**1**) failed to exhibit a time-dependent loss of enzymic activity when the preincubated enzyme with the inhibitor was assayed, suggesting that the compound is not irreversible inhibitor for the

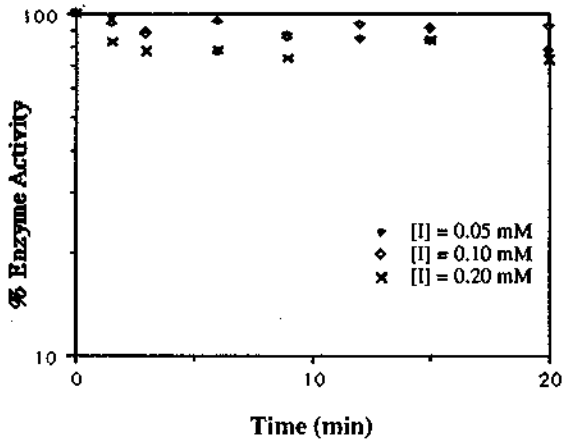


Figure 2. Time course for the inactivation of CPA at pH 7.5. CPA (2 μM) was incubated at 25 ℃ with varying amounts of **1**, and the remaining enzyme activity after given time intervals was determined by removing an aliquot of the incubation mix and addint it to the standard assay solution ([Hip-L-Phe]=500 μM, [CPA]=20 μM).

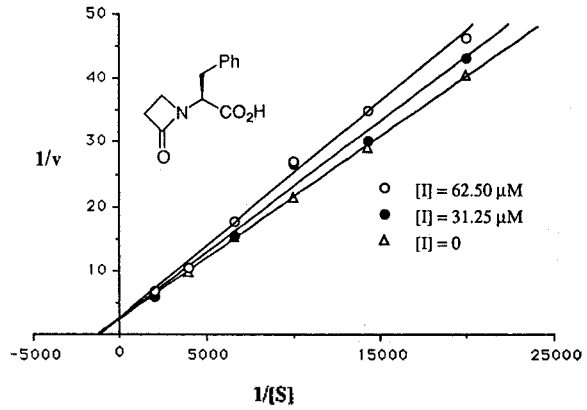


Figure 3. Lineweaver-Burk plot for the inhibition of CPA-catalyzed hydrolysis of hippuryl-L-phenylalanine by **1** at [CPA]=5.83 $\times 10^{-7}$ M.

enzyme.[15] However, the compound inhibited the enzymic activity competitively as seen from the Lineweaver-Burk plot (Figure 3). The inhibitory constant (K_i) for the compound was estimated from the Dixon plot[16] (Figure 4) to be 1.78×10^{-4} M. Similar inhibitory kinetic results were obtained with compound **2**, showing K_i value of 6.85×10^{-5} M (Figure 5). In comparison, the β-lactam ring in **1** was readily cleaved in a dilute alkaline aqueous medium at room temperature starting to show the formation of the product in about 10 min.

Results and Discussion

β-Lactam derivatives **1** and **2** were found to be only reversible competitive inhibitors having K_i values of 1.78×10^{-4} M and 6.85×10^{-5} M, respectively. This was somewhat surprising to us. However, we found that β-lactam ring is intact even after 2 days of incubation with the enzyme at room temperature, demonstrating that they are stable and thus

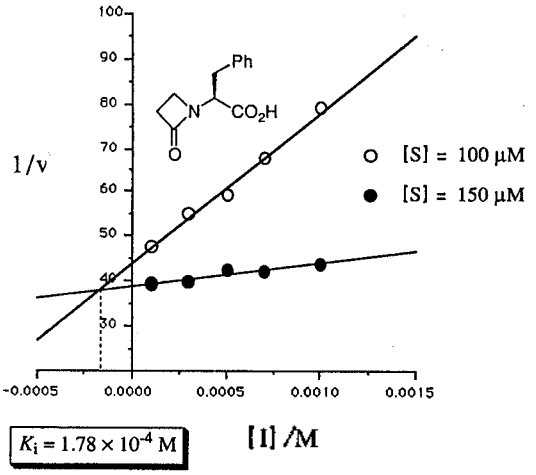


Figure 4. Dixon plot of data for the inhibition of CPA-catalyzed hydrolysis of hippuryl-L-phenylalanine by **1**.

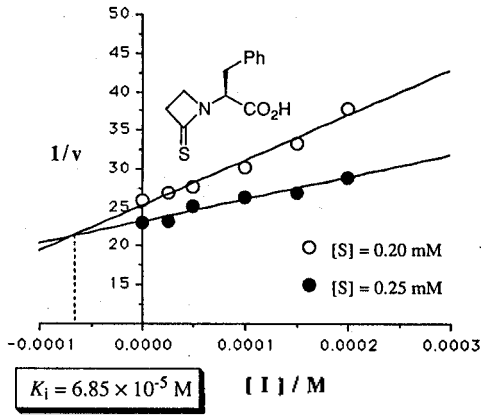


Figure 5. Dixon plot for the inhibition of CPA-catalyzed hydrolysis of hippuryl-L-phenylalanine by 2 at $[CPA]=1.2\times10^{-6}$ M.

successfully compete for the active site with the substrate. Recently, Page and his associate[12] have raised a question on the generally accepted rationalization that the enzyme inhibitory activity rendered by β-lactam antibiotics is due to the high chemical reactivity of the β-lactam moiety arising from its high degree of ring strain and the reduced resonance stabilization. Very recently, Mustafi and Makinen[17] offered an explanation for the less than expected stability of the ring on the basis of stereoelectronic and steric effects: In the ring cleavage reaction, the sterically preferred mode of nucleophilic attack is not allowed stereoelectronically, while the stereoelectronically favored attack is sterically hindered.

The higher affinity of **1** toward the enzyme compared with the substrate, hippuryl-L-phenylalanine ($K_m=6.80\times10^{-4}$ M)[18] may be envisaged from the molecular geometry of the β-lactam ring. X-ray crystallographic studies of azetidin-2-ones showed them to have an essentially planar ring configuration with the nitrogen atom slightly out of the mean plane of its substituents.[19] Standard bond distances and bond angles[20] of a typical peptide are shown in Figure 6 along with those of β-lactam.[20] It is seen from Figure 6 that the N-CO bond

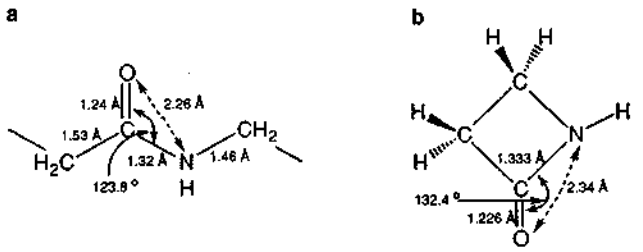


Figure 6. Bond distances and bond angles of peptide (a) and β-lactam (b).

distance in β-lactams is somewhat longer than that of a normal amide of 1.32 Å, while the C=O distance is shorter relative to that of a typical amide. Accordingly, the distance between the nitrogen and the carbonyl oxygen in the lactam is significantly greater than that in the peptide, and thus the carbonyl oxygen of the azetidinone would be rested somewhat closer to the active site zinc than a normal substrate would upon forming a complex with CPA, resulting in to have a tighter binding.

The further improved binding of thiolactam **2** to the enzyme appears to be mainly due to the larger size and higher polarizability of sulfur atom in the thiolactam compared with oxygen in **1**. The bond length (1.7 Å) of thiocarbonyl is known to be considerably longer than that (1.2 Å) of carbonyl.[13] Furthermore, it has been recently reported by Abbound *et al.*[21] that thiocarbonyl derivatives are in general more basic than their carbonyl counterparts, which may certainly also contribute to the improved binding of **2** compared with **1**. Our result, however, is not consistent with that of Bartlett *et al.*[13] who reported that the replacement of the scissile amide carbonyl of Cbz-Gly-Phe with a thiocarbonyl moiety does not appreciably affect the enzyme binding property.

Presently, we are not certain whether the failure of the enzyme to hydrolyze the lactam ring of **1** is due to the inherent property of the ring or there are involved other factors such as that the ethylene linkage of the β–lactam ring impedes the attack of the catalytic carboxylate of the enzyme at the electrophilic center of **1** which is bound to the enzyme at the active site.

Conclusion

We have designed and synthesized **1** and **2** as inhibitors of carboxypeptidase A and evaluated their fate upon binding to its active site. In kinetic assays, both were found to be reversible competitive inhibitors for the enzyme. The β-lactam moiety in them was not hydrolyzed by the enzyme even after incubating the mixture for 2 days. The results indicate that the azetidin-2-one ring in **1** is fairly stable at least at the active site of the enzyme, which is contrary to the generally believed notion that β-lactams would undergo facile ring opening reaction due to the high ring strain and prohibited resonance stabilization.

Experimental

Melting points were determined on Thomas-Hoover capillary melting point apparatus and were uncorrected. ^{1}H NMR

spectra were recorded on Bruker 300 MHz FT-NMR spectrometer in deuteriochloroform and chemical shifts are expressed in ppm relative to tetramethylsilane. IR spectra were recorded on BOMEM FT-IR M100-C15 spectrometer. Low resolution mass spectra were obtained with KRATOS MS25 RFA instrument. High resolution mass spectrum and elemental analysis were performed by Korea Basic Science Center. Enzyme assays were monitored using Hewlett Packward 8452 A Diode Array spectrophotometer fitted with a cell-temperature controller. The spectrometer was interfaced to a microcomputer (HP 89500 UV/VIS ChemStation). All compounds described in this report showed a single spot on TLC plates. Carboxypeptidase A was obtained from Sigma Chemical Co. and used without purification. Buffer solution (0.05 M Tris buffer) containing NaCl (0.5 M) was adjusted to pH 7.5.

S-N-(β-Bromopropanoyl)-phenylalanine benzyl ester

To a chilled mixture of 3-bromopropanoic acid (0.153 g, 1 mmol), *S*-phenylalanine benzyl ester hydrochloride (0.29 g, 1 mmol), *N*-ethylmorpholine (0.13 g, 1 mmol) in methylenechloride (100 mL) was added under stirring 1-hydroxybenzotriazole hydrate (0.07 mg, 1 mmol) and dicyclohexylcarbodiimide (0.1 g, 1 mmol). The resulting mixture was allowed to stir at room temperature for 5 hr, then was chilled in ice. Dicyclohexylurea that precipitated was removed by filtration, and the filter residue was evaporated under reduced pressure to give an oil which was purified by silaca gel column chromatography, giving 0.2 g (48% yield) of the product. Mp 83.5-85 ℃; ^{1}H NMR δ 7.36-6.98 (m, 10H), 5.96 (d, 1H), 5.14 (dd, 2H), 4.94 (m, 1H), 3.57 (m, 2H), 3.13 (m, 2H), 2.74 (m, 2H); IR (KBr), 3300 (NH), 1725 (C=O ester), 1640 (C=O amide), 1534 cm^{-1} (NH bending); $[\alpha]^{24.5}=-9.1°$ (c=1.0, EtOH).

S-2-(Azetidin-2-one-1-yl)-3-phenylpropanoic acid benzyl ester

S-N-(β-Bromopropanoyl)phenylalanine benzyl ester (5.36 g, 13.7 mmol) was dissolved in dried DMF (150 mL) and was chilled at −10 ℃. To this solution was added slowly under stirring a solution of sodium hydride (0.6 g of NaH dispersed in oil, 13.7 mmol) dissolved in dried DMF using a cannula. The progress of reaction was traced with a TLC. When the reaction was completed, the mixture was treated with aqueous ammonium chloride solution, and then was added ethyl acetate (200 mL). The resulting mixture was washed with water, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The residue was purified by silaca gel column chromatography, giving 2.25 g (53% yield) of the product in the form of an oil. ^{1}H NMR δ 7.11-7.32 (m, 10H), 5.10 (s, 2H), 4.67 (dd, 1H), 3.29 (dt, 1H), 3.19 (dd, 1H), 3.13 (dt, 1H), 3.00 (dd, 1H) 2.80 (m, 1H), 2.72 (m, 1H); IR (KBr) 3055, 2985, 1753 (C=O β-lactam), 1740 cm^{-1} (C=O).

S-2-(Azetidin-2-one-1-yl)-3-phenylpropanoic acid (1)

S-2-(Azetidin-2-one-1-yl)-3-phenylpropanoic acid benzyl ester (2.20 g, 7.1 mmol) was dissolved in a mixture (100 mL) of ethyl acetate and methanol (1 : 1), and the resulting reaction mixture was hydrogenated in the presence of a catalytic amount of Pd/C (10%). After about 1 hr, the reaction mixture was filtered to remove the catalyst, and the filtrate was evaporated under reduced pressure to give a white solid (1.56 g, quantitive yield) which was recrystallized from methylenechloride and hexane. Mp 165-166 ℃; ^{1}H NMR δ 7.33-7.19 (m, 5H), 6.88 (s, 1H), 4.62 (dd, 1H), 3.31 (dt, 1H), 3.23 (dd, 1H), 3.15 (dt, 1H), 3.04 (dd, 1H), 2.84 (m, 1H), 2.75 (m, 1H); IR (KBr) 3446, 2900-2570, 1756 (C=O β-lactam), 1725 cm^{-1} (CO carboxylic acid); MS (FAB) m/z 242, 220 (M^++H), 178, 132, 105; $[\alpha]=-9.06°$ (c=1.0, EtOH); Anal. Calcd for $C_{12}H_{13}NO_3$: C, 65.74; H, 5.98; N, 6.39. Found: C, 65.43; H, 6.15; N, 6.24.

S-2-(Azetidin-2-thione-1-yl)-3-phenylpropanoic acid (2)

S-(2-Azetidin-2-one-1-yl)-3-phenylpropanoic acid (**1**) was converted into its methyl ester by a standard method using diazomethane in a quantitative yield. The oily methyl ester (0.34 g, 1.46 mmol) thus obtained was dissolved in THF (50 mL), and to this solution was added Lawesson's reagent (0.3 g, 0.73 mmol). The resulting mixture was heated under reflux for 20 hr, then the solvent was removed under reduced pressure to give an oil (*S*-2-(azetidin-2-thione-1-yl)-3-phenylpropanoic acid methyl ester) which was purified by silica gel column chromatography. ^{1}H NMR δ 7.25-7.14 (m, 5H), 5.16 (dd, 1H), 3.94 (m, 1H), 3.69 (s, 3H), 3.61 (m, 1H), 3.28 (dd, 1H), 3.08 (dd, 1H), 2.91 (m, 1H), 2.81 (m, 1H); IR (KBr window) 3028, 2950, 1742 (C=O ester), 1597 cm^{-1} (C=S); MS (EI) m/z 249 (M^+), 190, 161, 131, 91, 76, 71; $[\alpha]=+18.4°$ (c=1.0, EtOH). The methyl ester (169 mg, 0.68 mmol) was dissolved in a mixture (17 mL) of THF and methanol (3 : 1), and was chilled in ice. To this solution was added under stirring aqueous LiOH solution (0.1 M) (17 mL) and the stirring was continued for about 10 min, after which the reaction mixture was acidified with hydrochloric acid (3 N). The product was extracted with ethyl acetate, and the ethyl acetate solution was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to give an oil which was purified by silica gel column chromatography. overall yield, 80%; ^{1}H NMR δ 7.37-7.24 (m, 5H), 5.28 (dd, 1H), 3.99 (m, 1H), 3.65 (m, 1H), 3.41 (dd, 1H), 3.20 (dd, 1H), 2.99 (m, 1H), 2.89 (m, 1H); IR (KBr window) 3200-2800, 1710 (C=O carboxylic acid), 1590 cm^{-1} (C=S); MS (EI) m/e: 235 (M^+), 191, 147, 129, 104, 97, 91, 77, 71; HRMS (EI$^+$) m/z: 235.0668 (M^+, calcd 235.0667); $[\alpha]=+26.7°$ (c=1.0, EtOH).

Enzyme Activity Assay. The enzymic hydrolysis of Hip-L-Phe (assay substrate) in pH 7.5 Tris buffer solution at 25 ℃ was monitored by measuring the UV absorption at 254 nm during the first 30 sec after the initiation of the enzymic action of CPA. The initial rates were determined in the absence and pressure of different concentrations of inhibitor. K_i values were calculated from kinetic plot obtained by the method of Dixon.

Acknowledgment. The authors express their thanks to the Korea Science and Engineering Foundation for the financial support of this study.

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