Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2967–2970

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

2-Benzyl-3,4-iminobutanoic Acid as Inhibitor of Carboxypeptidase A

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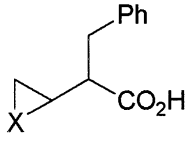
Received 13 June 2001; accepted 31 August 2001

Abstract—2-Benzyl-3,4-iminobutanoic acid (**3**) was evaluated as a novel class of inhibitor for carboxypeptidase A (CPA). All four stereoisomers of **3** are found to have competitive inhibitory activity for CPA, although their inhibitory potencies differ widely with (2*R*,3*R*)-**3** being most potent. The molecular modeling study for CPA(2*R*,3*R*)-**3** complex suggested that the lone pair electrons on the nitrogen of the aziridine ring in the inhibitor forms a coordinative bond with the active site zinc ion and the proton on the nitrogen is engaged in hydrogen bonding with one of the carboxylate oxygens of Glu-270.

Carboxypeptidase A (CPA) is one of the most extensively studied zinc-containing proteolytic enzyme and represents a large family of physiologically and pathologically important zinc proteases such as angiotensin converting enzyme and matrix metalloproteases.[1] CPA has been served as a model enzyme for developing design strategies of inhibitors that are potentially effective against zinc proteases of medicinal interest.[2] The most important residues at the active site are Glu-270 and Arg-145. The former is directly involved in the catalytic hydrolysis of substrate, and the latter forms hydrogen bonds with the C-terminal carboxylate of substrate. In addition, there is present a hydrophobic pocket, the primary function of which is to recognize substrate by accommodating the aromatic side chain in the P_1' residue of substrate. The catalytically essential zinc ion is held by His-69, Glu-72, His-196 and a molecule of water. The crystal structure of the enzyme revealed that the guanidinium moiety of Arg-145 and the carboxylate of Glu-270 are located near the surface of the enzyme molecule and the recognition pocket is invaginated deep into the core of the molecule. The zinc ion that is found also deep in the active site crevice is positioned in a transoid relationship to the hydrophobic pocket.[3]

We have reported that 2-benzyl-3,4-epoxybutanoic acid (**1**) is a highly efficient and fast acting irreversible inhibitor for CPA.[4] Upon binding the inactivator to CPA, its oxirane ring is activated by the zinc ion at the active site of the enzyme, and is subjected to a nucleophilic ring cleavage by the attack of the carboxylate of Glu-270 to result in covalent modification of the enzyme.[5] Its thiirane analogue (**2**) in which the oxirane ring of **1** is replaced with thiirane also showed potent inactivating property against CPA in comparable potency to **1**.[6] We have expanded the studies to include an aziridine analogue in which the oxirane in **1** is replaced with a three-membered nitrogen heterocycle, aziridine.



X = O : **1**
X = S : **2**
X = NH : **3**
X = CH_2: **4**

All of four possible stereoisomers of **3** were synthesized as described previously, starting with optically pure aspartic acid.[7] None of these compounds, however, exhibited time-dependent loss of CPA activity when they were assayed for the CPA, but instead acted as reversible competitive inhibitors of the enzyme. Their inhibitory constants (K_is) were determined from the respective Dixon[8] plot (Fig. 1) and are listed in Table 1. It is surprising to find that all four stereoisomers of **3** show inhibitory activity against CPA, which is a contrast to **1** that shows stereospecificity in inactivation of CPA. Thus, in the case of **1**, only two isomers having the (2*R*,3*S*)- and (2*S*,3*R*)-configurations are active as

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0960-894X/01/$ - see front matter
PII: S0960-894X(01)00592-3

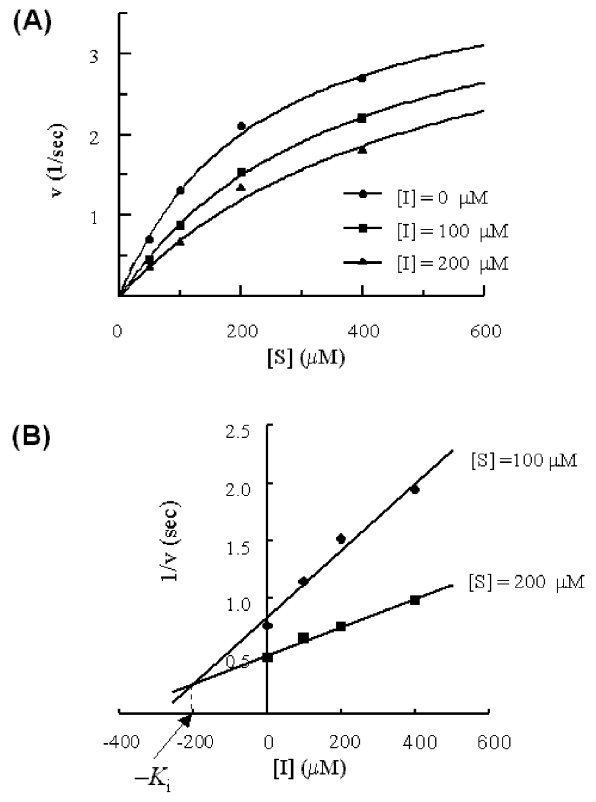


Figure 1. (a) Progress curves for CPA catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-3-phenyllactate (ClCPL) at 25 °C in the presence of different concentration of (2*R*,3*S*)-**3** in Tris buffer of pH 7.5 containing NaCl (0.5 M). The enzyme activity was measured by monitoring hydrolysis of ClCPL at 320 nm. (b) The Dixon plot for the inhibition of CPA by (2*R*,3*S*)-**3**.

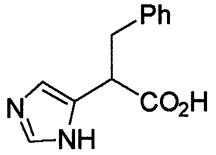
Table 1. Kinetic parameters for the inhibition of CPA

Compd	Inhibition K_i[a] (μM)
(2*R*,3*S*)-**3**	230±27
(2*S*,3*R*)-**3**	510±48
(2*R*,3*R*)-**3**	35±4
(2*S*,3*S*)-**3**	120±15
rac-**4**	792±58
(*R*)-**4**	803±62
(*S*)-**4**	646±45
rac-**5**	0.8[b]

[a]Values are means of three experiments, and standard deviations are derived from Grafit® program.
[b]Ref 18.

irreversible inhibitors of CPA.[4,5] Furthermore, while the most active stereoisomer of **1** bears the (2*S*,3*R*)-configuration,[4] in the case of the present aziridine bearing inhibitor, (2*R*,3*R*)-**3** is shown to be most potent with the K_i value of 35±4 μM. The failure of **3** to irreversibly inactivate CPA may be envisioned on the basis of the insensitivity of the nitrogen heterocycle towards nucleophile.[9] Being uniquely different from other three-membered heterocycles such oxirane and thiirane, aziridine ring is known to be relatively stable in spite of its inherent high ring strain energy[10] and the nucleophilic cleavage of aziridine requires activation of the ring by protonation and/or a stronger nucleophile such as mercapto group.[11] It seems that the activation of aziridine ring through interaction with metallic Lewis acid appears problematic[12] by virtue of the increased *s*-character of the lone pair electrons of the aziridine nitrogen.[13,14] It appears therefore that the aziridine moiety of the CPA-bound **3** may not be sufficiently activated for the ring cleavage to take place by the attack of relatively weak nucleophile of the carboxylate of Glu-270.

It was thought to be of interest to compare the binding affinity of **3** with that of **4** in which the aziridine moiety in **3** is replaced with a cyclopropane ring. Compound **4** was readily prepared from 2-benzyl-3-butenoic acid methyl ester by the modified Simmons–Smith method[15] followed by saponification.[16] Optically active 2-benzyl-3-butenoic acid methyl ester was obtained by the enzymic resolution using α-chymotrypsin as reported in the literature.[17] As expected, the binding affinity of **4** toward CPA was diminished significantly compared with that of **3**, suggesting that the aziridine nitrogen forms a coordinative bond with the active site zinc ion. However, the increase of the K_i value of 1.3–3.5-fold[18] by the replacement corresponds to 0.15–0.74 kcal mol^{-1}, which indicates that the interactions between the unshared electrons on the nitrogen of the aziridine ring and the metal ion are considerably weak. This is consistent with the reduced basicity reported for the aziridine nitrogen as described above.[14] The K_i value of 230 μM for (2*R*,3*S*)-**3** corresponds to 288-fold decrease in binding affinity compared with that of 2-(4-imidazolyl)hydrocinnamic acid (**5**), a CPA inhibitor reported in the literature.[19] The latter is the compound in which the aziridine in **3** is replaced with an imidazole ring that is thought to ligate to the active site zinc ion much more effectively.[19] The stronger zinc binding property shown by the imidazole ring in **5** may be attributed to the lone pair electrons on the second nitrogen atom in the ring, which are not involved in the aromatic π-electron system and thus can participate in a coordinative bond formation with the zinc ion.[20]



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In an effort to explore the origin of the potency differences shown between the four stereoisomers of **3** in the inhibition of CPA, we have performed modeling study, generating energy-minimized CPA·inhibitor complexes.[21] Figure 2 shows binding modes of the four stereoisomers of **3** to the CPA obtained by the computer modeling. It may be concluded from the study that the aziridine nitrogen of (2*R*,3*S*)- and (2*S*,3*R*)-**3** would form coordinative bond with the zinc ion at the active site albeit weak. The aziridine ring of (2*R*,3*R*)-**3** also forms a coordinative bond to the zinc ion but in this case there may form an additional bonding, that is, the amino proton is engaged in hydrogen bonding with one of the carboxylate oxygens of Glu-270. The much higher inhibitory potency shown by this isomer may then be accounted for. The binding mode of (2*S*,3*S*)-**3** is different

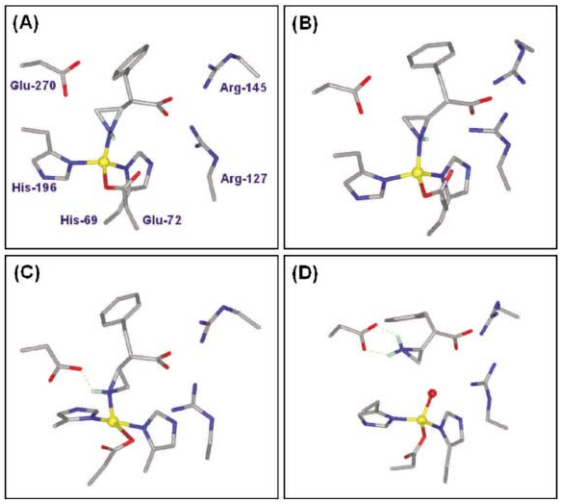

Figure 2. Energy minimized CPA complexes formed with each of four stereoisomers of **3**. The yellow sphere represents the zinc ion that is held by His-69, Glu-72, and His-196. In the case of CPA complex with (2*S*,3*S*)-**3**, the fourth ligand for the zinc ion is a water molecule represented by a red sphere. (A), CPA·(2*R*,3*S*)-**3**; (B), CPA·(2*S*,3*R*)-**3**; (C), CPA·(2*R*,3*R*)-**3**; (D), CPA·(2*S*,3*S*)-**3**.

from the other three inhibitors: in the CPA complex formed with (2*S*,3*S*)-**3** the aziridine nitrogen does not appear to form a coordinative bond to the zinc ion, but instead hydrogen bonded to Glu-270 in the form of ammonium ion and the water molecule present in the native enzyme remains being coordinated to the zinc ion.

In summary, contrary to inhibitors **1** and **2**, which inactivate CPA in an irreversible manner to modify covalently the catalytic carboxylate of Glu-270, their aziridine analogue **3** exhibits reversible inhibitory activity against CPA. Furthermore, while in the case of inhibitors **1** and **2**, the stereoisomers having (2*S*,3*R*)- and (2*R*,3*S*)-configuration showed the inactivating property, all four stereoisomers of **3** were active with (2*R*,3*R*)-**3** being most potent. The lack of irreversible inactivating property shown by **3** may be due to the unique property of the aziridine ring, that is, much reduced basicity and reasonable stability toward nucleophilic ring cleavage.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation and by the Brain 21 Project.

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14. The p*K*a value of the aziridine in **3** was found to be 7.7 which is substantially lower than the p*K*a value of 9.6 determined for the corresponding non-cyclic amine in 2-benzyl-3-methylaminopropanoic acid.
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16. Preparation of (*R*)-2-cyclopropyl-3-phenylpropanoic acid ((*R*)-**4**). A solution of trifluoroacetic acid (310 μL, 4 mmol) in CH_2Cl_2 (2 mL) was added slowly under stirring to the ice chilled solution of Et_2Zn (4.2 mL of 1.0 M solution in hexanes, 4.2 mmol) in CH_2Cl_2 (4 mL) under N_2 and the stirring was continued for 30 min. To the resulting mixture was added a solution of CH_2I_2 (320 μL, 4 mmol) in CH_2Cl_2 (2 mL) and the stirring was continued for 30 min. A solution of (*R*)-2-benzyl-3-butenoic acid methyl ester[17] (380 mg, 2 mmol) in CH_2Cl_2 (2 mL) was added to the mixture. The reaction mixture was stirred for 3 h at room temperature, then treated with saturated aq NH_4Cl solution. The product was extracted with diethyl ether. The organic layer was washed successively with saturated aq $NaHCO_3$ solution, water, and brine, then dried over $MgSO_4$, and concentrated. Purification of the crude product thus obtained by column chromatography (hexane/ethyl acetate = 15:1) yielded (*R*)-2-cyclopropyl-3-phenylpropanoic acid methyl ester (345 mg, 85%): $[\alpha]_D = -10.3\ ^\circ$ ($c = 1.2$, $CHCl_3$); IR (KBr) 1738 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.15–7.29 (m, 5H), 3.62 (s, 3H), 3.06 (dd, $J = 13.5$, 9.1 Hz, 1H), 2.94 (dd, $J = 13.5$, 5.9 Hz, 1H), 1.90 (m, 1H), 1.00 (m, 1H), 0.51 (m, 2H), 0.30 (m, 1H), 0.06 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.8, 129.3, 128.7, 126.6, 126.3, 53.2, 51.8, 39.0, 14.2, 5.10, 3.94; MS *m*/*z* (FAB) 205.02 (M + 1). A mixture of (*R*)-2-cyclopropyl-3-phenylpropanoic acid methyl ester (120 mg, 0.6 mmol), THF (3 mL), MeOH (1 mL), H_2O (1 mL), and 1 M LiOH (0.6 mL) was stirred overnight at room temperature, acidified with 1 N HCl solution, and extracted with diethyl ether. The combined extracts were washed with brine, dried over $MgSO_4$, and concentrated. The crude product thus obtained was purified by column chromatography (hexane/ethyl acetate = 2:1) to yield (*R*)-**4** as an oil (101 mg, 90%): $[\alpha]_D = -13.1\ ^\circ$ ($c = 0.7$, $CHCl_3$); IR (KBr) 3063 (br), 1717 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.15–7.29 (m, 5H), 3.09 (dd, $J = 13.7$, 8.6 Hz, 1H), 2.95 (dd, $J = 13.7$, 6.0

Hz, 1H), 1.90 (m, 1H), 1.00 (m, 1H), 0.53 (m, 2H), 0.35 (m, 1H), 0.07 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 129.4, 128.7, 126.7, 52.8, 38.7, 14.1, 5.3, 4.0; MS *m*/*z* (FAB) 190.93 (M + 1). (*S*)-2-Cyclopropyl-3-phenylpropanoic acid ((*S*)-**4**) was prepared in a similar fashion starting with (*S*)-2-benzyl-3-butenoic acid methyl ester[17] in an overall yield of 78%: $[\alpha]_D = +12.6°$ ($c = 0.8$, $CHCl_3$).

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18. In the calculation of the relative binding affinities, the K_i value of (*R*)-**4** was compared with that of (2*R*,3*S*)-**3** because the modeling study (Fig. 2) showed that the nitrogen atom of (2*R*,3*S*)-**3** ligates the zinc ion, whereas the nitrogen of (2*R*,3*R*)-**3** is involved in hydrogen bonding with the carboxylate of the Glu-270 in addition to the coordination to the metal ion. On the basis of the similar reasoning, the K_i value of (*S*)-**4** was compared with that of (2*S*,3*R*)-**3**.

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21. The initial geometries of the complexes were generated by superimposing **3** on the X-ray crystal structure[5] of the ring cleaved **1** to form a covalent complex with CPA followed by removal of **1** from the structure. The active sites of the CPA structures were fractioned to include the residues laid less than 5 Å away from **3**. Residues of Glu-270, Arg-145, Arg-127, Tyr-248, Glu-72, His-69, His-196, and the zinc ion are present in the fraction. The chain ends were terminated by the addition of an acetyl or *N*-methyl group. The geometry optimization and atomic charge calculations were performed by the PM3 method of the MOPAC program package with 0.05 kcal/(mol Å) rms gradient, where the convergence limit for SCF procedure was 10^{-3} eV/atom. A Silicon Graphics Indigo 2 workstation was used. In the case of **3** having *threo* configuration, the initial complexes were energy-minimized first with Affinity/Insight II (ver. 98, MSI, Inc., San Diego, CA), then full optimization with the PM3 method. For CPA·(2*S*,3*S*)-**3** complex, since the aziridine nitrogen was separated from the zinc ion by more than 5 Å, the zinc bound water molecule was introduced in the form of hydroxide and the aziridine nitrogen is protonated. In the other three CPA·**3** complexes, the zinc bound water molecule appears to be displaced by the aziridine ring. There have been numerous experimental observations and molecular dynamic calculations which suggest that in the presence of an inhibitor at the active site of CPA the zinc bound water molecule becomes mobile and is easily displaced.[22]

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