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Research Article

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Synthesis and evaluation of 2-benzyl-3-hydroxybutanoic acid as inhibitor for carboxypeptidase A

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ABSTRACT

*2-Benzyl-3-hydroxybutanoic acid(**1**) was prepared starting with acetoacitic ester following benzylation, reduction using sodium dithionite and hydrolysis under alkaline condition. The inhibitory kinetic evaluation showed that **1**(K_i =107μM) is potent than 2-benzyl-3-hydroxypropanoic acid(K_i =610μM) as inhibitor for carboxypeptidase A, suggesting that introducing of a methyl group at the β-position of a inhibitor with weak zinc ligating moiety such as hydroxyl group to improve potency of inhibitor to CPA is possible.*

Key words: Carboxypeptidase A, Inhibitor, 2-Benzyl-3-hydroxybutanoic acid.

INTRODUCTION

Carboxypeptidase A(CPA, EC 3.4.17.1) is an exopeptidase which selectively cleaves off the C-terminal amino acid residue having a hydrophobic side chain from protein substrate[1]. It is one of much studied and well characterized zinc proteases known and whose X-ray crystal structure has been highly refined to 1.25 Å[2]. It has been used as a model enzyme for the development of enzyme inhibitor design strategies that can be effectively applied to other less well characterized zinc proteases with medical interest, such as angiotensin converting enzyme (ACE)[3-5], enkephalinase[6]and matrix metalloproteinases[7].

Asante-Appiah[8]reported the X-ray crystal structure of the complex of CPA with R- 2-ethyl-2-methylsuccinic acid, they found that the methyl group at the α-position of the inhibitor occupies a small hydrophobic cavity, which they called it a "methyl hole" and attributed the higher inhibition than 2-benylsuccinic acid for it. Subsequent studies[9-12]made by Kim and co-workers indicated it is hardly achievable to improve potency by the introduction of a methyl group at the α-position of known inhibitors especially with the high binding affinity zinc ligating moiety.Therefore, it was thought to be of interest to know the effect that the introduction of a methyl group at the β-position of a inhibitor with hydroxyl as the weak zinc ligating moiety. This paper reports synthesis of 2-benzyl-3-hydroxybutanoic acid (**1**) and its kinetic evaluation as inhibitor for CPA.

EXPERIMENTAL SECTION

Materials and instruments

The substrate *O*-(*trans*-*p*-chlorocinnamoyl)-*L*-β-phenyllactate (Cl-CPL) used in the kinetic study was prepared as described in the literature [13].Biochemical reagent CPA was purchased from Sigma Chemical Co.. All other chemicals and reagents were of analytical grade, and were used without further purification. UV absorbance measurement was detected by Perkin-Elmer HP 8453 UV-Vis spectrometer. Melting point was determined in open capillary tubes and was uncorrected. ^{1}H-NMR and ^{13}C NMR spectra were measured on a Bruker AM 300 (300MHz) NMR spectrometer. IR spectra was obtained with a FT-IR1730 spectro-photometer. Mass spectrometry was

recorded on a Hewlett Pachard 1100-HPLC/MSD instrument.

Preparation of 2-Benzyl-3-oxo-butyric acid ethyl ester (2):
To a suspension of 0.17 g sodium in 30 ml of absolue ethanol,0.91g ethyl acetoacetate was dropped in when the sodium disppeared.After refluxed for 0.5 hour, a solution of 0.974g benzyl chloride was dropped in. Then the mixture was refluxed for 11 hours. The pooled mixture was filtered to remove sodium chloride and the filtrate was concentrated on a rotary evaporator under reduced pressure. The resulting brown oil was purified by chromatography(PE:EA=10:1) to yield the product as a colourless oil(1.1 g, 72%) . ^{1}H-NMR: (300MHz,$CDCl_3$/TMS) δ(ppm): 7.31-7.18 (m,5H,-Ph), 4.19-4.12 (q,2H, J=7.2, -OCH_2-),3.82-3.77(t,1H,J=7.8,-CH-),3.18-3.14(d,2H,J=7.8, -CH_2Ph), 2.19 (s,3H,-$COCH_3$),1.23-1.16(t,3H,J=7.1,-CH_2CH_3).

Preparation of 2-Benzyl-3-hydroxbutyric acid ethyl ester (3):
In a three necked 250 ml round bottom flask a solution of 2-benzyl-3-oxo-butyric acid ethyl ester (0.93g, 0.0042mol) in dioxane (37.5ml) was added to water (37.5ml) containing sodium bicarbonate (2.325g, 0.028mol) under nitrogen. Sodium dithionite (1.0575g,0.006mol) was added all at once and the reaction mixture was stirred at 85℃ for 3hr. during which time additional sodium dithionate was added in three portions of 3g each. The reaction mixture was cooled to room temperature and clod water was added untill solution became clear. The product was extracted with ether (2×25 ml) and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure and the residue chromatographed over silica gel to furnish pure product (0.52g , 56%). ^{1}H-NMR: (300MHz,$CDCl_3$/TMS) δ(ppm): 7.31-7.18 (m,5H,-Ph), 4.13- 4.01 (m,3H, -CH_2O-,-CHOH), 3.00-2.98(m, 2H,-CH_2Ph), 2.79-2.67 (m,1H,-CH-), 1.28-1.25(m,3H,-$CHCH_3$), 1.16-1.08(m,3H,-CH_2CH_3). ^{13}C-NMR (75 MHz,$CDCl_3$/TMS) δ(ppm): 174.6, 139.1, 129.0,128.4,126.5 68.12, 60.59, 54.40,35.61, 21.87,14.16.

2-Benzyl-3-hydroxybutanoic acid (1):
To a stirred solution of 3 (0.5g, 0.0022mol) in absolute eathnol (20 ml) was added potassium hydroxide (0.25g, 0.0044mol) over 24 hr. at room temperature, then evaporated under reduced pressure to remove the eathnol. The resultant residue was added 30 ml water and extracted with EtOAc(20ml×3) to remove unhydrosis 3. Aqueous layer acidified with 3 N HCl solution to pH 2-3 and then extracted with EtOAc(40ml×3).The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to give a solid residue. (0.383g, 87%). MP 126-127℃(Literature[14]:128℃).IR (KBr cm^{-1}):3237,3029-2928,1704,1496,1453. ^{1}H-NMR: (300MHz,$CDCl_3$/ TMS) δ(ppm): 7.31-7.19(m,5H,-Ph), 7.31-7.18 (s, -COOH), 4.08- 3.90 (m,1H, -CHOH), 3.07-2.95(m, 2H, -CH_2Ph), 2.90 -2.72 (m,1H, -CH-), 1.30-1.28 (m,3H, -CH_3). ^{13}C-NMR (75MHz, $CDCl_3$/TMS) δ(ppm): 179.36,138.86,128.93,126.63,68.01,54.13,35.09,21.56. MS:193[M-1].

Determination of K_i value:
All solutions for deternination of K_i value were prepared by dissolving in double distilled and deionized water. The concentration of CPA stock solutions which by dissolving CPA in 0.05 mol•L^{-1} Tris/0.5 mol•L^{-1} NaCl (pH 7.5) was mensurated from the absorbance at 278 nm (ε_{278} = 64, 200 L•g^{-1}•cm^{-1}). CPA stock solutions were added to a solution containing Cl-CPL (final concentrations: 50 and 100 μM) and inhibitor (five different final concentrations in the range of 0.5-2 K_i) in 0.05M Tris/0.5M NaCl, pH 7.5 buffer (1-mL curette). Absorption at 320 nm was measured promptly. The final concentration of CPA was 20 nM. The K_i values were estimated according to the method of Dixon[15] and the experimental operation[16-17]. The correlation coefficients for the Dixon plots were above 0.990.

RESULTS AND DISCUSSION

The compound **1** was prepared starting with acetoacitic ester following benzylation as described method in the literature[18], reduction using sodium dithionite according to the literature method[19]and hydrolysis under alkaline condition as shown in Figure 1.

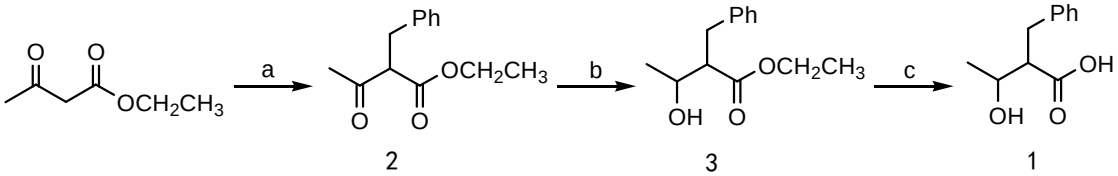
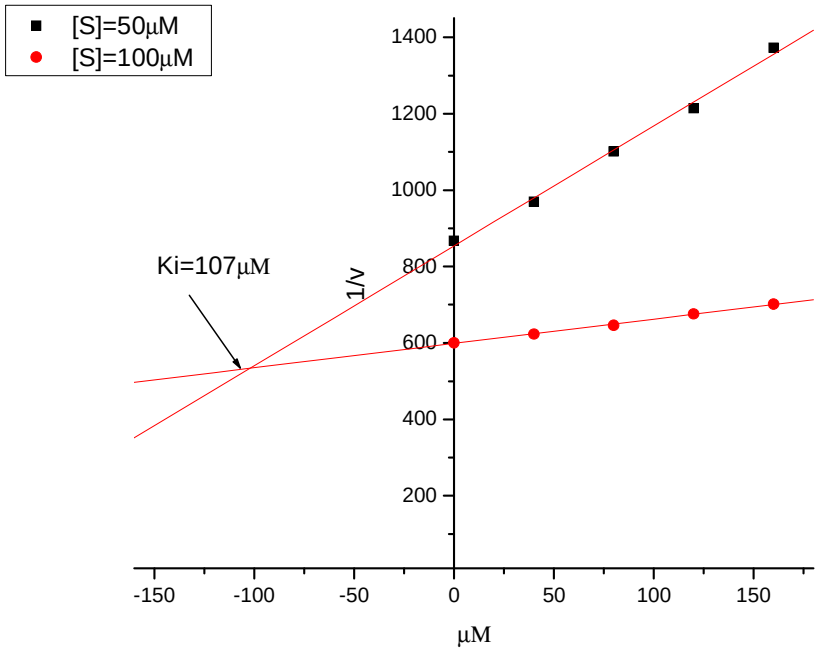


Figure 1. synthesis route of 2-benzyl-3-hydroxybutanoic acid

Reagents and conditions: (a) Na, EtOH, $PhCH_2Cl$, reflux; (b) $Na_2S_2O_4$, $NaHCO_3$, H_2O/dioxane, 85-90 ℃; (c) KOH, EtOH.

The compound **1** was assayed for its inhibitory activity for CPA at 25℃ in Tris buffer (0.05M) of pH 7.5 using Cl-CPL as substrate. It is found to be competitive inhibitor for CPA as shown by the Dixon plot (Figure 2).



Figue 2. The Dioxn plot for the inhibition of CPA with 2-benzyl-3-hydroxybutanoic acid

Inhibitory constants (K_i values) of **1** and its analogs known are collected in Table 1. It can be seen from Table 1 that inhibitory constant of **4** is less potent than **5**. The structure of **4** is regarded as **5** introduced a methyl group at γ position. The reduced potency may be due to incorrect positioning of the hydroxy of **4** near the zinc atom at the active site of the enzyme for steric hindrance generated by methyl group. Correspondingly inhibitory constant of **7** should be more potent than **1**.But it can be seen from Table 1 that inhibitory constant of **7** is less potent than **1**.The more inhibitory constant may be due to methyl group introduced at the β-position next to the terminal carboxylate in the case of **1** for CPA at the correct position.

Table 1. K_i values for CPA inhibition

Inhibitors	K_i (μM)	Reference
2-benzyl-3-hydroxybutanoic acid (**1**)	107	
2-benzyl-4-hydroxypentanoic acid (**4**)	1100	[20]
2-benzyl-4-hydroxybutanoic acid (**5**)	540	[21]
2-benzylbutanoic acid (**6**)	6300	[21]
2-benzyl-3-hydroxypropanoic acid (**7**)	610	[22]

One of the inhibitory constant of **1**,**4**,**5** and **7** is more potent than 6, indicates the hydroxyl group of **1**,**4**,**5** and **7** may coordinate to the Zinc(Ⅱ) ion at the active site of CPA. The binding mode of **1** to CPA is schematically shown in Figure 3.

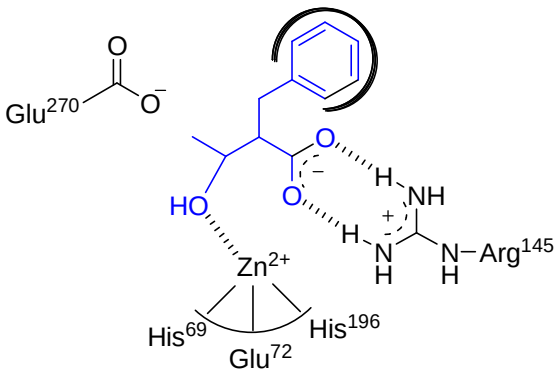


Figure 3. Postulated binding mode of 2-benzyl-3-hydroxybutanoic acid to carboxypeptidae A

The carboxylate of **1** is thought to form bifurcated hydrogen bonds with the guanidinium moiety of Arg-145,the

phenyl ring is fitted in the hydrophobic pocket at the active site of CPA and the hydroxyl group coordinates to the active site zinc ion.

CONCLUSION

In conclusion, we have shown that 2-benzyl-3-hydroxybutanoic acid has more potency to CPA than its analogs and it is likely to improve potency of inhibitor to CPA by introducing of a methyl group at the β-position of a inhibitor with weak zinc ligating moiety such as hydroxyl group. Consequently it may result in development of more potent inhibitor for zinc proteases.

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