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O-(HYDROXYACETYL)-L-β-PHENYLLACTIC ACID AS A NEW TYPE OF MECHANISM-BASED INACTIVATOR FOR CARBOXYPEPTIDASE A

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Abstract: *O*-(Hydroxyacetyl)-L-β-phenyllactic acid that was conceived as a mechanism-based inactivator for carboxypeptidase A from the X-ray crystal structure of the enzyme complexed with slowly hydrolyzed substrate, Gly-Tyr and a proposed mechanism for the catalytic action, indeed, inactivated the enzyme with the inactivation potency (k_{inact}/K_i) of 0.057 $M^{-1}s^{-1}$. © 1997 Elsevier Science Ltd.

Of various types of enzyme inhibitors, mechanism-based inactivators have received much attention because of their high selectivity towards target enzyme with long duration of inhibition.[1] We have been involved in the development of design protocols for such inactivators using well characterized proteases such as carboxypeptidase A and α-chymotrypsin as model target enzymes.[2] The design strategies developed with these enzymes are of considerable value in designing inactivators of other proteases of medicinal interest. In this communication, we wish to describe a new strategy which we have developed for the design of mechanism-based inactivator for carboxypeptidase A.

Carboxypeptidase A (CPA) which represents a larger number of zinc containing proteases cleaves C-terminal amino acid residue having a hydrophobic side chain.[3] At the active site of the enzyme there are present three major binding sites, *i.e.*, Arg-145, a hydrophobic pocket and a zinc ion, and a catalytic residue of Glu-270, functions of which may be summarized as the following: The guanidinium moiety of Arg-145 forms hydrogen bonds with the terminal carboxylate of substrate, and the hydrophobic pocket which is shaped in complementary to an aromatic ring accommodates the side chain aromatic ring of P_1' residue, and the zinc ion ligates to the scissile peptide carbonyl oxygen atom of substrate.[4] The carboxylate of Glu-270 in the catalytic site is intimately involved in the hydrolytic reaction either directly attacking the activated peptide bond to form an anhydride intermediate (anhydride pathway)[5] or activating zinc bound water molecule which in turn attacks the scissile peptide bond to generate a tetrahedral transition state (general base pathway).[6]

The X-ray crystal structure of CPA complexed with Gly-Tyr, a slowly hydrolyzed substrate for CPA revealed that its glycine portion chelates to the active site zinc ion in a bidentate fashion with both the amide carbonyl oxygen and the terminal amino nitrogen being involved in the ligation.[7] In this enzyme • substrate complex, the zinc-bound water molecule in the native enzyme is displaced by the amino group. It was occurred to us that *O*-(hydroxyacetyl)-L-β-phenyllactic acid (HPLA) would bind CPA in the same fashion as Gly-Tyr does by virtue of their mutual structural resemblance to form a complex in which the hydroxyl group coordinates to the zinc ion with displacement of the zinc coordinated water molecule. In this complex the carboxylate of Glu-270 becomes liable to attack the activated carbonyl carbon of the ligand to generate an acylenzyme in the form of an anhydride with expelling of β-phenyllactic acid. The anhydride intermediate[8] thus generated may undergo an intramolecular rearrangement initiated by the hydroxyl group present in the ligand, resulting in to modify the carboxylate of Glu-270 covalently in the form of an ester to cause impairment of the enzymic activity. The design rationale discussed above is illustrated in Figure 1.

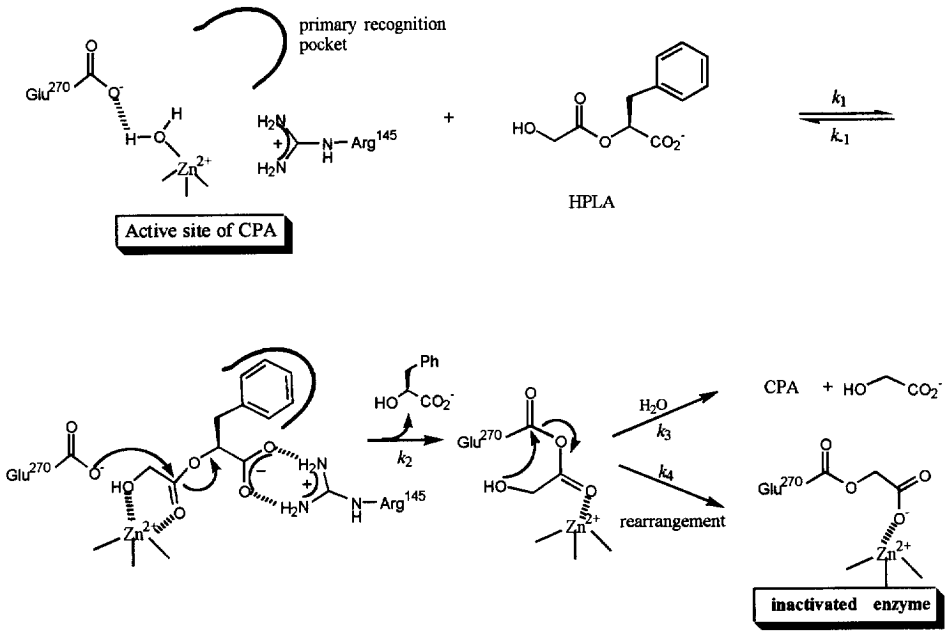
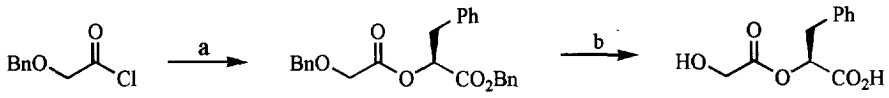


Figure 1. Schematic illustration of the ratonale used for designing HPLA as a mechanism-based inactivator for CPA

The potential inhibitor was synthesized by allowing benzyloxyacetyl chloride to react with L-β-phenyllactic acid benzyl ester in the presence of pyridine in THF followed by hydrogenolysis to remove benzyl moieties (Scheme 1).[9]



Scheme 1. *Reagents and conditions*: (a) L-*β*-phenyllactic acid benzyl ester, pyridine, THF; (b) H_2, Pd/C (1 atm), MeOH, r.t.

Enzymic assay and inhibition kinetics were carried out at 25 ℃ in Tris buffer (0.05 M) of pH 7.5 using *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactic acid (Cl-CPL) by monitoring the absorbance change at 320 nm. The time course of inactivation of CPA by HPLA was examined and semilogarithmic plot of the percent activity remaining *vs* time at different concentrations of HPLA gave straight lines (Figure 2), suggesting that the inhibition occurs in an irreversible fashion. The rate of inactivation was reduced in the presence of 2-benzylsuccinic acid,[10] a well known potent competitive inhibitor for CPA, which suggests that HPLA binds the active site of CPA as 2-benzlylsuccinic acid does. Kinetic parameters for the irreversible inhibition, K_I and k_{inact} were calculated from Figure 3, in which pseudo-first-order rate constants obtained from Figure 2 were plotted against concentrations of the inhibitor according to the method of Kitz and Wilson,[11] to provide 3.5 mM and 0.012 min^{-1}, respectively. The involvement of the hydroxyl group of the bound HPLA in the irreversible inhibition of the enzyme was supported by the observation that the use of *O*-(benzyloxyacetyl)-L-β-phenyllactic acid in lieu of HPLA in the kinetic experiment fails to inactivate the enzyme. The inactivated enzyme failed to regain its enzymic activity upon dialysis, confirming the irreversible nature of the inhibition. The partition ratio of the inhibition, which is a measure of the efficiency of the inhibitor was determined by the titration method[1] to be 1246. The high partition ratio exhibited by HPLA suggests that the turn over process competes favorably with the inactivation, *i.e.*, there appears to occur extensive hydrolytic cleavage of the anhydride intermediate by water with regeneration of the enzyme (Figure 1). The reversible inhibitory constant (K_i) which reflects the affinity of HPLA for the enzyme was calculated from the Dixon plot (Figure 4),[12] a semireciprocal plot of the initial velocity (v) of the enzymic action in the presence of HPLA *vs* $[HPLA]_o$ to give 0.4 mM. The kinetic analysis and experimental observations presented above suggest strongly that HPLA is indeed a mechanism-based inactivator for CPA, verifying the design paradigm.

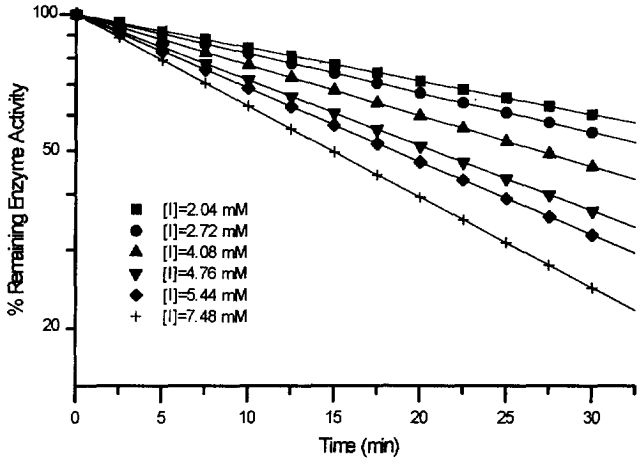


Figure 2. Loss of enzymic activity of CPA as a function of time during incubation with HPLA of different concentrations.

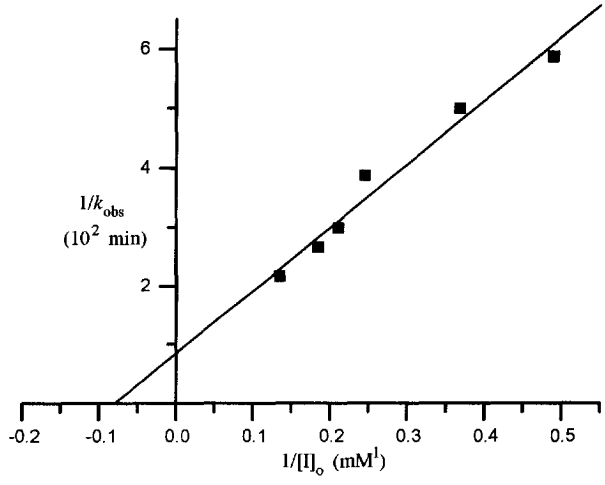


Figure 3. Double reciprocal plot of k_{obs} *vs* $[HPLA]_o$. Values of k_{inact} and K_I were calculated from the y intercept and the slope, respectively.

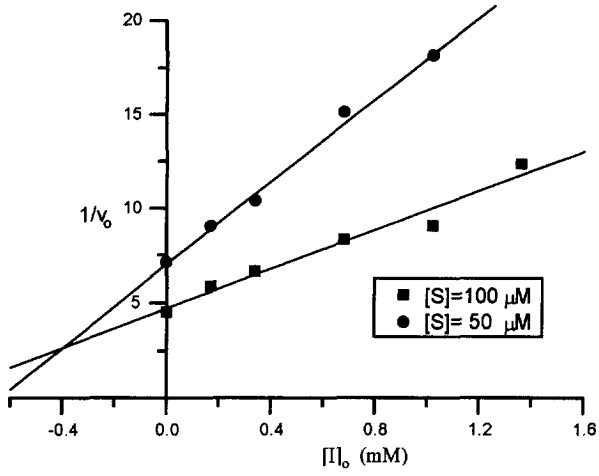


Figure 4. The Dixon plot for the reversible inhibition of CPA by HPLA.

HPLA is a novel type of mechanism-based irreversible inhibitor for CPA, which was designed rationally on the basis of the X-ray crystal structure of CPA complexed with slowly hydrolyzed substrate, Gly-Tyr and a proposed reaction mechanism of the catalytic action. The design protocol may be of value in designing inactivators of other zinc proteases.

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8. The formation of the anhydride intermediate in the interaction of HPLA with the enzyme is expected from the anhydride mechanism but may also be envisioned from the general base pathway under the conditions of no catalytic water being present. In fact, it has been generally believed that the hydrolysis of ester susbstrate goes through anhydride intermediate: (a) Breslow, R.; Wernick, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *74*, 1303 - 1307. (b) Makinen, M. W.; Kuo, L. C.; Dymowski, J. D.; Jaffer, S. *J. Biol. Chem.* **1979**, *254*, 356 - 366. (c) Breslow, R.; Schepartz, A. *Chem. Lett.* **1987**, 1 - 4. (d) Britt, B. M.; Peticolas, W. L. *J. Am. Chem. Soc.* **1992**, *114*, 5295 - 5303.

9. Experimental Procedure for the preparation of HPLA: To a solution of L-β-phenyllactic acid benzyl ester (0.5 g, 1.95 mmol) and pyridine (0.46 g, 6 mmol) in THF (10 ml) was added benzyloxyacetyl chloride (0.36 g, 2 mmol) dropwise at 0 ℃ and the mixture was stirred at room temperature for 8 h. The reaction mixture was diluted with ethyl acetate (30 ml), washed with 3 N HCl, saturated $NaHCO_3$ solution, and brine, then dried ($MgSO_4$) and evaporated *in vacuo*. The crude product was purified by column chromatography to give *O*-(benzyloxyacetyl)-L-β-phenyllactic acid benzyl ester as a colorless oil (0.64 g, 81%). The product (0.6 g) was dissolved in MeOH (5 ml) and was hydrogenated (1 atm) in the presence of 10% Pd-C (100 mg) at room temperature for 6 h. The mixture was filtered through a celite pad and washed with methanol. The filtrate was concentrated *in vacuo* to give HPLA as a colorless oil in a quantitative yield. $[\alpha]_D = -23.0$ ° (c 1, $CHCl_3$); IR (neat) 3000-3500 (br), 1750, 1720 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.10 - 3.32 (m, 2H), 4.16 (d, J = 18 Hz, 2H), 5.38 (m, 1H, α-H), 7.21 - 7.34 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 37.4, 60.8, 73.5, 127.8, 129.1, 129.6, 135.6, 167.3, 173.3; EI HRMS Calcd for $C_{11}H_{12}O_5$: 224.0685, Found: 224.0685.

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