Bioorganic & Medicinal Chemistry Letters, Vol.1, No.10, pp. 557-562, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

SUBSTRATE ACTIVATED TIME DEPENDENT INHIBITION OF CARBOXYPEPTIDASE A BY AMINOCYCLOPROPANE CARBOXYLIC ACID DERIVATIVES AND ANALOGUES

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(*Received* 20 *August* 1991)

Abstract: A series of aminocyclopropane carboxylic acid derivatives and analogues that are time dependent inhibitors of carboxypeptidase A has been synthesised. Kinetic experiments show surprisingly that the rate of inhibition is increased in the presence of substrate. A related secondary alcohol also acts as a time dependent inhibitor of carboxypeptidase A and this result is evaluated in the context of current views on the mechanism of action of carboxypeptidase A.

As part of our studies on the use of cyclopropane-containing compounds as enzyme inhibitors, we showed that dipeptides containing aminocyclopropane carboxylic acid could act as time dependent irreversible inhibitors of carboxypeptidase A (CPA)[1]. These studies have been extended to include peptide analogues and two unusual features have emerged. Firstly, kinetic studies show that the rate of inhibition is increased in the presence of substrate, the converse of the normal expectation for time dependent inhibition; an analysis of the data suggests that inhibition takes place principally through an EIS complex. Secondly we find that a secondary alcohol is equally effective as related carbonyl compounds in the inhibition reaction, a result that has significance with respect to the chemistry of the inhibition reaction and the mechanism of action of carboxypeptidase A.

The original design of the inhibitors was based upon the concept that the electrophilicity of the cyclopropane ring would be enhanced by coordination of the scissile carbonyl group to the zinc ion at the active site (figure 1). In order to develop the concept further towards compounds that might have interest

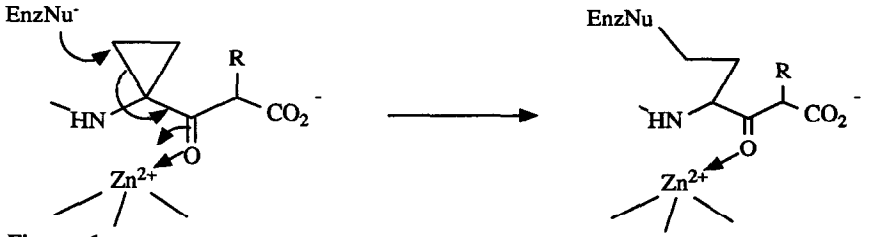
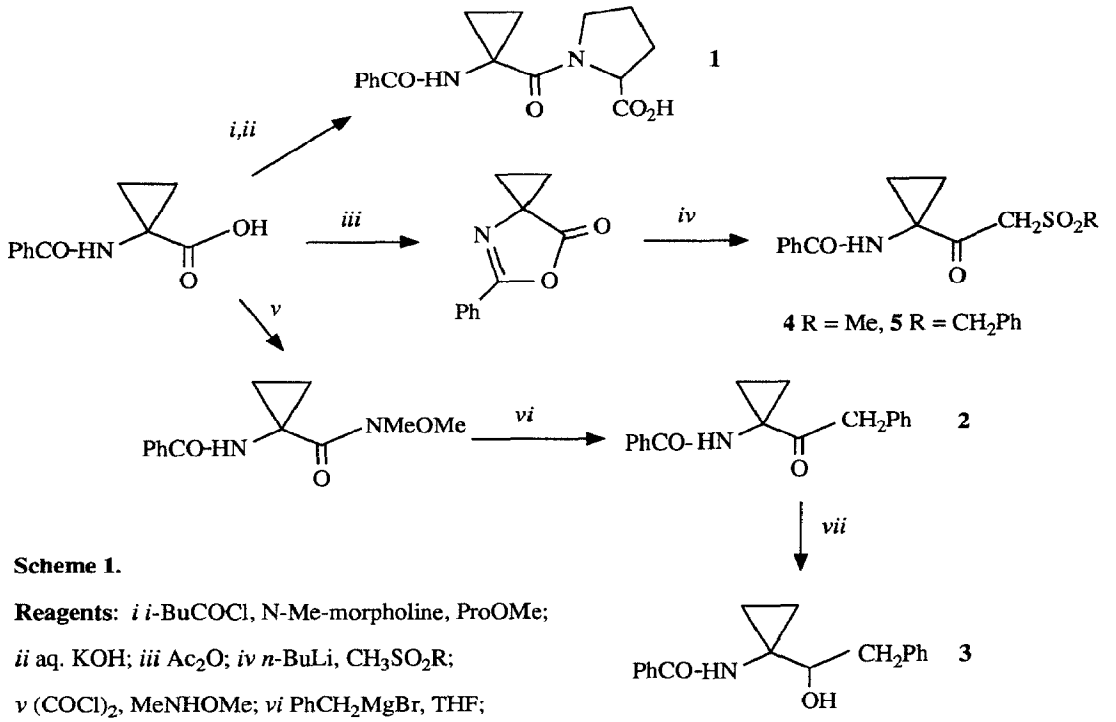


Figure 1

in medicinal chemical applications, we examined a series of non-peptide analogues of the original

inhibitors, some of which were available as a result of synthetic work directed towards inhibitors of dihydrofolate reductase[2]. The compounds were prepared as shown in scheme 1.



Scheme 1.

Reagents: *i* *i*-BuCOCl, N-Me-morpholine, ProOMe; *ii* aq. KOH; *iii* Ac_2O; *iv* *n*-BuLi, CH_3SO_2R; *v* $(COCl)_2$, MeNHOMe; *vi* $PhCH_2MgBr$, THF; *viii* $NaBH_4$.

The normal assay for time dependent inhibition involves the incubation of the inhibitor together with the enzyme and the removal of samples at suitable time intervals for assay for residual enzyme activity. When this assay was applied to the inhibitors **1** - **5** ([inhibitor] 10^{-3}-10^{-4}M, [CPA] 10^{-7}M), the loss of enzyme activity occured very slowly. However when the inhibitors were incubated in the presence of both enzyme and substrate ([hippurylphenylalanine] 10^{-4}M), inhibition occurred rapidly over a few minutes. Typical time courses for the reaction monitoring the formation of product at 254nm are shown in figure 2: the curves show that the extent of hydrolysis of the substrate decreases as the concentration of the inhibitor increases. Thus the presence of substrate enhances the rate of inhibition of the CPA. Similar curves were obtained for all five inhibitors.

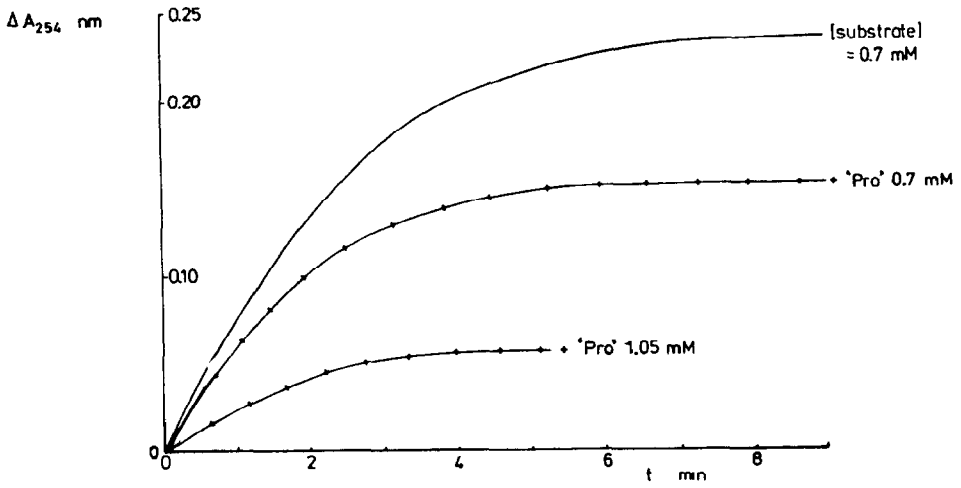


Figure 2. Progress curves for the hydrolysis of hippuryl phenylalanine by CPA in the presence and absence of inhibitor **1**.

In order to characterise kinetically the substrate activation phenomenon, the initial rates of reaction were measured in the presence of inhibitor and substrate. Whilst at zero inhibitor concentration, the value for K_m (0.68mM) derived from Lineweaver-Burk plots was close to that reported by Ondetti (0.72mM)[3], the data obtained in the presence of inhibitor did not lead to linear plots and K_i values could not be obtained. Similar problems have been encountered previously with CPA for N-substituted dipeptide and ester analogue substrates and these problems have been attributed to activation and inhibition by substrates and products[4,5]. However, such substrate *activation* of time dependent inhibition is essentially antithetic to the normal understanding of the mechanisms of time dependent irreversible inhibition and its origin merited further investigation.

Analysis of the progress curves showed that the rate of loss of enzyme activity was first order in enzyme. Although rate constants ($k_{obs}$for the inhibition reaction) could be derived from these data, the most precise empirical measure related to the extent of inhibition was the fraction of the substrate hydrolysis reaction completed by the time the enzyme sample in an incubation was fully inhibited at a fixed concentration of substrate. This fraction (f) could be calculated simply from the flat plateaux of the

progress curves. The fraction of reaction was used as a measure from which to derive a model for the inhibition reactions studied. In summary, the key observations are: a) inhibition in the absence of substrate was very slow, b) initial rates of substrate hydrolysis were only slightly affected by added inhibitor except at the highest inhibitor concentrations, c) the rate of inhibition was greatly accelerated in the presence of substrate, d) the progress curves were first order in enzyme, e) the behaviour of all compounds **1** - **5** was essentially the same. From these observations, the following conclusions can be drawn: 1) rapid inhibition occurs on formation of an EIS complex (from a,c, and d), 2) direct binding of the inhibitor to the enzyme is weak (from b), 3) the activation of the inhibition reaction does not require the presence of a C-terminal carboxylate group.

To obtain some insight into the complexes involved in the inhibition reaction at lower substrate concentrations, the data was analysed by regression analysis[6]. The equation fitted was of the form

$$f[S] = a + b[I] + c[I]^2 \quad (1)$$

where a, b, and c are constants and the other symbols have the usual meanings. Good fits to equation 1 were found for all inhibitors with R^2 greater than 0.98 in all but one case (Table 1). The magnitudes of the coefficients b and c indicates the relative contributions of complexes involving respectively one and two molecules of inhibitor in the inhibition reaction. When the substrate concentration is low, the large, negative coefficients imply that a complex involving two molecules of inhibitor is important in the inhibition reaction, a fact not included in the simple model. In one case, the phenyl sulphone (**5**), the I^2 term dominates at all substrate concentrations indicating that this molecule has a greater affinity for the enzyme than the other inhibitors. Since the fit to the quadratic equation 1 is so good, it is probable that there are three kinetically significant reactions leading to inhibition by these cyclopropane containing compounds, the predominant mechanism depending upon the substrate/inhibitor ratio.

Table 1. Empirical correlation of inhibitor action

Inhibitor	[S] mM	a	b	c	r^2
1	1.33	1.01	-1.47	0.44	0.996
	1.00	1.02	-0.73	-0.26	0.990
	0.67	1.00	0.003	-0.84	0.985
2	1.33	1.03	-1.53	0.44	0.984
	1.00	1.00	-0.57	-0.36	0.990
	0.67	1.00	0.002	-0.84	0.983

3	1.33	1.00	-1.17	0.009	0.986
	1.00	1.02	-0.46	-0.68	0.967
	0.67	1.01	-0.16	-0.62	0.956
4	1.33	1.01	-0.92	0.004	0.992
	1.00	1.00	-0.19	-0.55	0.999
	0.67	1.00	-0.002	-0.44	0.989
5	1.33	0.99	-0.43	-0.239	0.993
	1.00	0.98	0.18	-0.284	0.986
	0.67	1.00	0.25	-0.264	0.998

The molecular explanation of these phenomena cannot be explicitly stated but it is well known that CPA has multiple binding sites[7] and that only with peptide substrates longer than tetrapeptides is Michaelis-Menten kinetics obeyed. It is probable that the differing kinetic properties observed with these inhibitors relate to conformational changes as well as to the occupancy of different binding sites on the enzyme. However at this stage in the investigation, it is not possible to give a clearer view.

Finally, the relationship between the structure of the inhibitors and their kinetic behaviour is notable. We have already commented upon the lack of a requirement for a C-terminal carboxyl group. In this context it is interesting that the phenyl sulphone (**5**) appears to interact most strongly with the CPA; this inhibitor is structurally similar to the normally preferred C-terminal amino acid, phenylalanine, in which the sulphone mimics the carboxylate and the phenyl rings occupy the same binding pocket. Perhaps the most significant observation is that the secondary alcohol (**3**) was a time dependent inhibitor and was equally effective as the corresponding ketone (**2**). The ketone would be expected to have electrophilic activity according to the initial design hypothesis[1] but for the alcohol to react in the same way, an unusual carbon-oxygen cleavage reaction would be required. Such a reaction is not unprecedented in a zinc-containing enzyme[8] but we have no direct evidence as was available in that case. The tetrahedral structure of (**2**) is also noteworthy in that it mimics the currently held view of an intermediate in the hydrolysis of peptides by CPA[9]. In this sense, (**2**) could be considered to be an analogue of statine, an aminoacid that is found in potent inhibitors of aspartate peptidases[10]. Although we have demonstrated that inhibition by the dipeptide (**1**) is irrveresible[1], the remaining compounds in this series have not been examined in detail for this property. It remains a possibility, therefore, that the time dependence reported in this paper is due to slow conformational changes in the structure of the enzyme on binding the inhibitors to form the EIS complex. We are currently synthesising extended analogues of these compounds to investigate in greater detail the binding of inhibitors and the molecular mechanism of inhibition.

Acknowledgements

We thank SERC for a research studentship (A.K.) and the Wolfson Foundation for financial support.

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