Latent Inhibitors. Part 9. Substrate Activated Time-dependent Inhibition of Carboxypeptidase A by Aminocyclopropanecarboxylic Acid Derivatives and Analogues

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A series of 1-aminocyclopropanecarboxylic acid derivatives and analogues have been synthesised as potential inhibitors of carboxypeptidase A. Whereas simple cyclopropylcarboxamido derivatives of Gly, Phe and Pro showed no indications of time-dependent, irreversible inhibition, benzamido-1-aminocyclopropane carboxamido-Phe and Pro were characterised as latent inhibitors. The former was also a substrate for carboxypeptidase A but the latter was purely an inhibitor. This behaviour suggested that cyclopropylketones should also be inhibitors of carboxypeptidase A; this suggestion was confirmed experimentally. Kinetic experiments showed surprisingly that the rate of inhibition is increased in the presence of substrate, hippurylphenylalanine. Related secondary alcohols also acted as time-dependent inhibitors. The results are evaluated in the context of current views on the mechanism of action of carboxypeptidase A.

The cyclopropane group has many attractions for the design of latent inhibitors of enzymes. Its smallness makes it possible to incorporate the ring into molecules that are close substrate analogues affording compounds that are usually able to bind to the target enzyme. The multifaceted reactivity of the cyclopropane ring also offers opportunities for activation through both radical and electrophilic mechanisms.[1] We have exploited the latter in the design of inhibitors of NAD-dependent enzymes,[2] of dihydrofolate reductase,[3] and of dihydroorotate dehydrogenase.[4] In this paper we describe the inhibition of the metalloexopeptidase, carboxypeptidase A.[5]

Our studies on horse liver alcohol dehydrogenase (HLADH) had shown that a Lewis acid catalyst (Zn^{2+}) at an enzyme's active site was a powerful force for activating cyclopropyl methanols and cyclopropyl aldehydes and ketones to nucleophilic attack at the cyclopropane ring even in the absence of a redox reaction.[6] Carboxypeptidase A also contains a zinc ion that acts as a Lewis acid[7] and an obvious development was to prepare a series of cyclopropane-containing amino acid derivatives for investigation as inhibitors of carboxypeptidase A. Fig. 1 summarises the design principle. The structures of the compounds studied in this paper are shown in Table 1 together with the relevant kinetic data (see below).

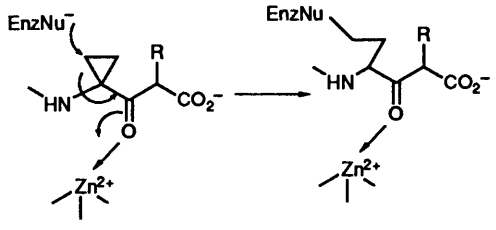

Fig. 1 The design strategy for cyclopropane-containing inhibitors of metallopeptidases

Results

Synthesis.—The simple acyl amino acid derivatives **1–3** were prepared with cyclopropylcarbonyl chloride in the presence of pyridine. 1-Benzamidocyclopropanecarboxylic acid was prepared by alkylation of benzylideneglycine ethyl ester[3] and transposition of the protecting groups, and by phase transfer catalysed diacylation of the dibenzylimine of aminoacetonitrile.[8] Peptide coupling at room temperature with this acyl amino acid was effected with dicyclohexylcarbodiimide or with mixed anhydrides (Scheme 1); the reactions were slow and yields moderate with 2-phenylspiro[cyclopropane-4-oxazol]-5-one being a significant by-product. Better yields of peptides were obtained using the oxazolone as the acylating agent. The oxazolone was also used in the preparation of sulfones (**9**, **10**) from the corresponding carbanions. The benzamidocyclopropylketones, **11**, **12**, were prepared from the corresponding *N*-methyl-*O*-methylhydroxamide **13**.[9] Reduction of the ketones with sodium borohydride led to the secondary alcohols, **14**, **15**. The reactions used were unremarkable except for the fact that no method of peptide coupling attempted gave a high yield in contrast to the experience of others in coupling reactions of ring-substituted aminocyclopropanecarboxylates.[10]

Enzyme Inhibition.—The effect of the potential inhibitors on the hydrolysis of hippurylphenylalanine by carboxypeptidase A was determined by standard spectrophotometric assay at 254 nm.[11] In some preliminary experiments, *O-trans*-3-furylacryloylphenyllactic acid (FAPLA),[12] which responds at a higher wavelength (320 nm) beyond the absorption range of the inhibitors, was used. Reactions were followed by methods routinely used to characterise latent inhibitors.[6,13] Kinetic data are shown in Table 1. Time-dependent inhibition was not observed with compounds **1–3** that lack a benzamido substituent or with the glycine-containing peptide **6**; all other compounds showed time-dependent inhibition. Observed rate constants (k_{obs}) were determined from the progress curves for hydrolysis of substrate (hippurylphenylalanine) or by incubation of the enzyme with the inhibitor alone as indicated in the notes to Table 1. From the values of k_{obs} at several concentrations, the apparent kinetic parameters k_i and K_i were calculated from plots of eqn. (1). Typical progress curves are shown in Fig. 2 for peptides **7** and **8**. The significance of the above parameters will be discussed below.

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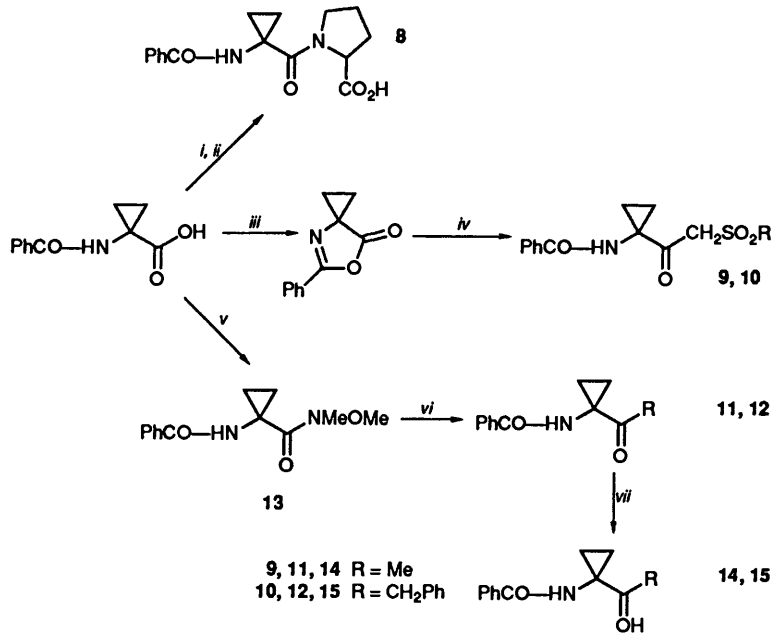
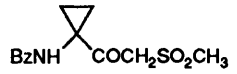
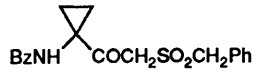
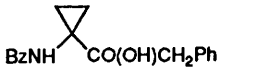


Scheme 1 *Reagents:* i, Bu^{t}COCl, *N*-Me-morpholine, ProOMe; ii, aq. KOH; iii, Ac_2O; iv, Bu-Li, CH_3SO_2R; v, $(COCl)_2$, MeNHOMe; vi, $PhCH_2MgBr$ or MeMgI, THF; vii, $NaBH_4$

Table 1 Inhibitors of carboxypeptidase A[e]

Compound	K_i/mmol dm^{-3}	$k_1/10^{-3}$ s^{-1}	
BzGlyPhe (substrate)	0.68 (K_m)	—	—
1 ▽ Gly	0.83[a]	—	—
2 ▽ Phe	0.44[a]	—	—
3 ▽ Pro	0.84[a]	—	—
6 BzCypGly	0.26[a]	—	—
7 BzCypPhe	0.20[a], 0.86[b]	5.6[c]	0.37[d]
8 BzCypPro	0.25[a], 0.55[b]	6.9[c]	—
9 	0.46[b]	12.5[c]	—
10 	0.59[b]	33.0[c]	—
11 BzCypMe	0.49[b]	4.9[c]	0.02[d]
12 BzCypCH$_2$Ph	0.12[b]	7.5[c]	0.03[d]
13 BzCypN(Me)OMe	0.12[b]	8.5[c]	—
14 	0.13[b]	5.9[c]	0.02[d]
15 	0.13[b]	—	0.10[d]

[a] Determined in competition with FAPLA. [b] Determined in competition with hippurylphenylalanine. [c] Determined by progress curve analysis of reaction in presence of substrate (0.7 mmol dm^{-3}). [d] Determined by incubation in presence of inhibitor and sampling at time intervals up to 2 h. [e] Values *b* and *c* were estimated from the values of k_{obs} at a series of inhibitor concentrations (0–2 mmol dm^{-3}) using the methods described previously.[13]

$$(1/k_{obs}) = (1/k_i) + (K_i/k_i)(1/[I]) \quad (1)$$

The reversibility of inhibition was investigated. A sample of carboxypeptidase A inhibited by peptide **8** recovered activity neither on standing (48 h) nor on gel filtration through Sephadex G-25 (Table 2). The ketone **11** also caused irreversible inhibition as shown by gel filtration.

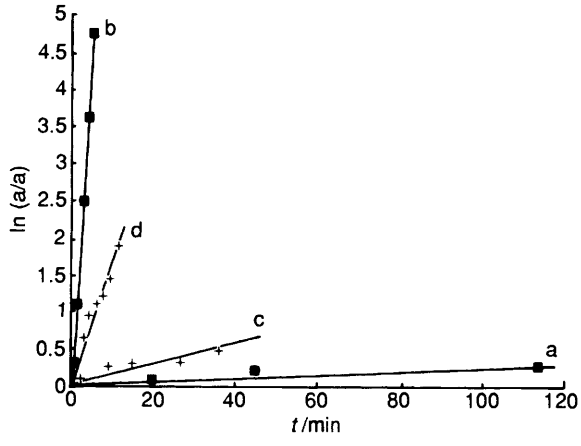


Fig. 2 Time courses for the inhibition of carboxypeptidase A by representative cyclopropane-containing inhibitors. (a) ■ [**12**] = 1.03 mmol dm^{-3} without substrate, (b) ■ [**12**] = 0.33 mmol dm^{-3} [substrate] = 1.33 mmol dm^{-3}, (c) +[**7**] = 0.92 mmol dm^{-3}, (d) +[**7**] = 0.77 mmol dm^{-3} [substrate] = 1.0 mmol dm^{-3}.

Table 2 Tests for reversibility of inhibition

Inhibitor		Sample and activity (%)		
8	Before	E 100	E + I	29.5
	After	E 106	E + I	28.0
11	Before	E 100	E + I	28.6
	After	E 102	E + I	21.4

HLADH had shown the ability to catalyse hydrolytic cleavage of a cyclopropane ring in our earlier studies.[6] Evidence for a similar reaction catalysed by carboxypeptidase A was sought by incubating alcohol **14** (1 mmol) with a large quantity of enzyme (10 mg) as if an enzyme-catalysed preparative reaction was being carried out. Extraction of the low molecular weight organic product and characterisation by NMR spectroscopy and HPLC revealed that starting material alone was recovered. Although this non-natural hydrolysis reaction

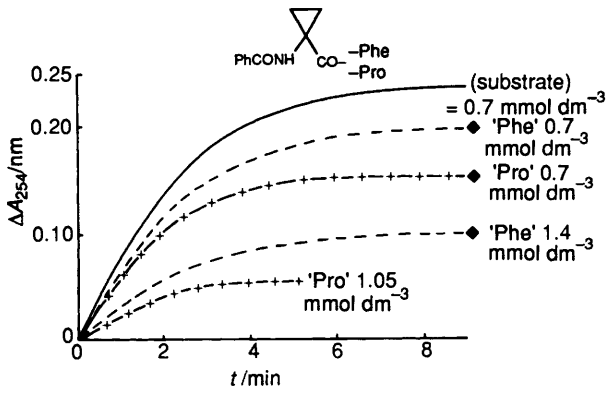


Fig. 3 Progress curves for the inhibition of carboxypeptidase A by the dipeptides **7** and **8** in the presence of substrate showing the plateaux from which the fraction of reaction parameter was measured

was not observed, the phenylalanine dipeptide **7** was found to be a substrate for carboxypeptidase A by the standard spectrophotometric assay; the rate of inhibition was 2.3 times that of hydrolysis. On the other hand the proline dipeptide **8** was not a substrate. No other compound was found to be a substrate by HPLC and spectrophotometric analysis.

The cyclopropylcarboxy amino acids **1–3**, although they bound measurably to the enzyme, showed no evidence for time-dependence. This result was not surprising because the substrate preference for acyl dipeptides, especially those of phenylalanine, is well established for carboxypeptidase A.[7] Indeed, the acyl dipeptides **6–8** all bound more strongly to the enzyme, as expected from the cyclopropyl inhibitor design strategy. Time-dependent irreversible inhibition was demonstrated for the direct substrate analogue containing phenylalanine **7** and the molecule was also a substrate. It was surprising that the proline dipeptide **8** was such a good inhibitor because prolyl dipeptides are not notably good substrates for carboxypeptidase A; in the latter respect, however, the behaviour of dipeptide **8** is consistent with precedent.

Because dipeptide **8** failed to be hydrolysed, it could be considered to be ketone-like with respect to carboxypeptidase A and not a typical peptide. This result suggested some extensions to the potential range of inhibitors. Ketones are known to be competitive inhibitors of carboxypeptidase A[14] and since the ketoamides **11** and **12** were available from other work, they were tested as inhibitors of carboxypeptidase A. The aqueous insolubility of the benzylketone **12** necessitated the use of methanol as a cosolvent in the enzyme assays. Both ketones bound to carboxypeptidase similarly strongly to the dipeptides but were weaker latent inhibitors, reacting 10 times more slowly than the dipeptides when inhibition was carried out in the absence of substrate. The measurable but weak activity of these ketones suggested a further stage in this extension of the application of activated cyclopropanes to metallopeptidase inhibition.

Recalling the reactivity of cyclopropane methanols with HLADH[6] and the fact that transition state analogues are potent inhibitors of carboxypeptidase A[14,15] the secondary alcohols **14** and **15** were prepared. These compounds contain tetrahedral carbon at the position equivalent to the scissile peptide bond and therefore have some characteristics of transition state analogues. Although the methyl derivative **14** showed little improvement on the ketones, the benzyl derivative **15** inhibited carboxypeptidase A five times more rapidly than **14** and only four times slower than the dipeptides **7** and **8** in the absence of substrate. This secondary alcohol contains a benzyl group that could be recognised by the primary binding site of the enzyme and together with the geometrical features appropriate to a transition state may account for the improvement in activity with respect to ketones **11** and **12**.

The best inhibitors in this extended series are, however, the ketosulfones **9** and **10**. These compounds were designed to include a non-ionic polar group to act as a substitute for the C-terminal carboxylate. The effectiveness of this substitution on the rate of inhibition in the presence of substrate is substantial. Finally, an amide with enhanced donor and co-ordination properties, **13**, was tested. Improved binding was not observed but inhibition was rapid in the presence of substrate.

Discussion

The data shown in Table 1 refer to rate constants obtained in two ways that differ in the presence and absence of substrate. Inhibition is clearly more rapid in the presence of substrate (Fig. 2) and this observation would normally be taken to argue against a latent or suicide inhibition mechanism. The inhibition of dehydrogenases by cyclopropane-containing compounds,[2,3,6,13] was characterised by normal substrate protection. However, peptidases, unlike dehydrogenases, contain multiple binding subsites and, with small substrates, substrate activation towards hydrolysis by additional small peptides has been observed.[17] Synergism has also been observed recently in the binding of inhibitors to a metalloaminopeptidase.[18] Further, there are evolutionary grounds for believing that peptidases possess additional binding sites that may interact in kinetically significant ways.[19] Carboxypeptidase A also has been shown to exhibit substrate activation with *N*-substituted dipeptide and ester analogue substrates.[20,21] The results obtained are therefore not inconsistent with latent inhibition although a more complex kinetic and molecular mechanism than that usually encountered would be implied.

Analysis of the progress curves showed that the rate of loss of enzyme activity was first-order in enzyme (Fig. 2). Although rate constants (k_{obs} for the inhibition reaction) could be derived from these data, the most precise empirical measure relating 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, Table 3) could be calculated simply from the flat plateaux of the progress curves (Fig. 3). The fraction of reaction was used as a measure from which to derive a model for the inhibition reactions studied.

The key observations were: (a) progress curves for the loss of catalytic activity were first-order in enzyme, (b) inhibition in the absence of substrate was very slow, (c) initial rates of hydrolysis of substrate were only slightly affected by added inhibitor at concentrations of less than 1 mmol dm^{-3}, and (d) the behaviour of all of the inhibitors was qualitatively the same. In order to provide some insight into the origin of these phenomena, fraction of reaction completed data (f) at fixed substrate concentration (Table 3) were subjected to a regression analysis (Table 4) which showed that the extent of inhibition was well represented by a quadratic equation in which the inhibitor concentration was the variable, eqn. (2). The coefficients

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

calculated imply that inhibition takes place in ternary complexes; where the coefficient b is large, an EIS complex is indicated and where coefficient c is large, an EI_2 complex may be involved. This formulation is consistent with the unusual pattern of inhibition shown by these cyclopropane-containing peptides and peptide analogues. The stoichiometry implied by the first-order loss of enzyme activity and the regression analysis led to a reaction scheme constructed to model the observed

Table 3 Fraction of hydrolysis (f) of hippurylphenylalanine completed in the presence of inhibitors

		[S]/mmol dm^{-3}				
I	[I]/mmol dm^{-3}	1.33	1	0.67	0.33	0.19
8	0	1	1	1	1	1
	0.33	0.615	0.778	0.894	0.949	0.947
	0.47		0.651	0.847		0.842
	0.67	0.196	0.373	0.623	0.845	0.842
	0.8	0.108	0.23	0.447		
	1	0	0.063	0.176	0.385	
9	0	1	1	1		
	0.33	0.715	0.886	0.942		
	0.47	0.611	0.813	0.919		
	0.67	0.382	0.65	0.759		
	0.8	0.271	0.52	0.713		
	1	0.139	0.321	0.54		
10	0	1	1	1		
	0.13	0.858	0.92	0.977		
	0.27	0.723	0.864	0.874		
	0.33	0.574				
	0.4	0.453	0.64	0.747		
	0.47	0.236	0.424	0.529		
12	0	1	1	1		
	0.33	0.641	0.782	0.901		
	0.47	0.437	0.672	0.827		
	0.67	0.19	0.437	0.63		
	0.8	0.099				
	1	0.07	0.084	0.173		
	1.2	0	0	0.025		
15	0	1	1	1		
	0.2		0.932	0.987		
	0.33	0.622	0.821	0.853		
	0.47	0.415	0.581	0.8		
	0.57			0.72		
	0.67	0.207	0.427			

Table 4 Empirical correlation of inhibitor action

Inhibitor	[S]/mmol dm^{-3}	a	b	c	r^2
8	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
9	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
10	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
12	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
15	1.33	1.00	−1.17	0.009	0.986
	1.00	1.02	−6.4	−0.68	0.967
	0.67	1.01	−0.16	−0.62	0.956

behaviour (Fig. 4).[22] This model readily accounts qualitatively for the results and was tested quantitatively as follows.

If, as the experimental results show, inhibition through a binary EI complex is negligible, then the fraction of reaction observed during inhibition in the presence of inhibitor and substrate together is given by

$$f = \frac{V(\mathrm{P})}{V(\mathrm{E\text{–}I}) + V(\mathrm{P})} \qquad (3)$$

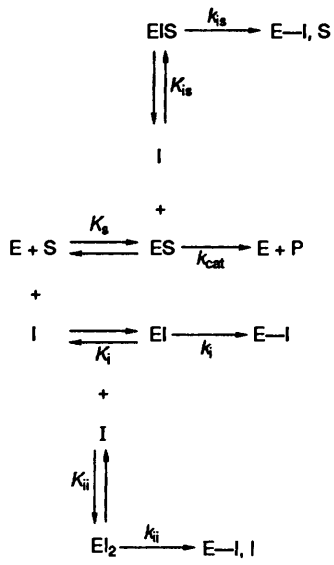


Fig. 4 Hypothetical reaction scheme to model the properties of inhibition of carboxypeptidase A by the cyclopropane-containing compounds

where $V(\mathrm{P})$ represents the rate of formation of product and $V(\mathrm{E\text{–}I})$ the rate of formation of covalently inhibited enzyme. Substituting for these rates using the concentrations and constants implied by the model shown in Fig. 4, we obtain eqn. (4). This representation assumes that the rate and equilibrium

$$\frac{1}{f} = \frac{k_{is}[\mathrm{I}]}{K_{is}k_{cat}} + \frac{k_{ii}K_s[\mathrm{I}]^2}{K_{ii}k_{cat}[\mathrm{S}]} \qquad (4)$$

constants for inhibition do not vary with inhibitor and substrate concentrations. Since f depends upon [I], eqn. (5) holds.

$$\frac{\mathrm{d}\frac{1}{f}}{\mathrm{d}[\mathrm{I}]} = \frac{k_{is}}{K_{is}k_{cat}} + \frac{2k_{ii}K_s[\mathrm{I}]}{K_{ii}k_{cat}[\mathrm{S}]} \qquad (5)$$

Hence, if the slopes of a plot of $1/f$ *versus* [I] are measured and replotted *versus* [I], a straight line should result. This was not found to be the case from which it can be concluded that either the model (Fig. 4) is wrong, or the 'constants' symbolised by k_{ii} *etc.* are also functions of the conditions of inhibition. This situation may well follow the well known fact that carboxypeptidase A has multiple binding sites[23] and that only peptide substrates larger than tetrapeptides exhibit Michaelis Menten kinetics. In conclusion, kinetic studies indicate an unusual kinetic mechanism but do not lead to any conclusions concerning the molecular mechanism of inhibition. An approach to understanding this was made by structural and molecular orbital considerations.

Molecular Mechanism of Inhibition.—For the inhibition described above to occur it is necessary that there be a nucleophile in proximity to the cyclopropane ring apex. A testable hypothesis for the mechanism of the reactions can be derived by considering two factors, firstly the stereoelectronic requirements for opening of the cyclopropane ring attached to a polarised carbonyl group, and secondly the interaction of the inhibitor at the active site of the enzyme allowing for the stereoelectronic requirements.

It would be expected that the preferred orientation of a carbonyl substituent on a cyclopropane ring would be in a bisected conformation in which the antibonding acceptor orbital of the carbonyl group can interact strongly with the HOMO of the cyclopropane.[24,25] Nucleophilic attack of a cyclopropane ring has been shown to occur preferentially with inversion of configuration at the site of attack[26] and this result

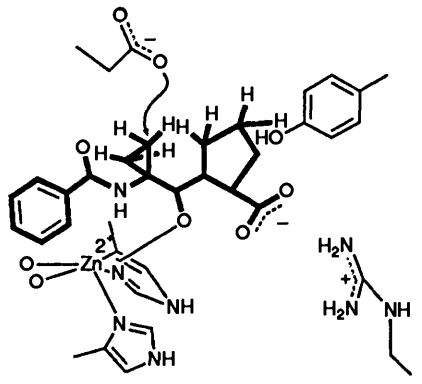


Fig. 5 A possible molecular explanation of the inhibition reactions as suggested by NMR experiments and theoretical arguments

can be rationalised by an interaction of the donor orbital of the nucleophile with the LUMO of the cyclopropane.[27] The preferred direction of attack is then seen to be at 150° to the cyclopropane ring in the plane of the ring carbon.

In our original rationalisation of these results,[5] we considered that the carbonyl group corresponding to the scissile peptide bond co-ordinated directly to the zinc ion at the active site with Glu-270 acting as the nucleophile. Whilst this hypothesis may be correct, we cannot be certain at this stage in view of the current formulation of the mechanism of action of carboxypeptidase A. Nevertheless, the substrate, hippurylphenylalanine, and the two peptides **7** and **8** make up an interesting series of reactivity. Their behaviour can be evaluated by the accessibility of water to attack the carbonyl group; this can be investigated for each case by examining the solvent accessible surface.[27] For the substrate and the phenylalanine derivative, it appeared that a water molecule could readily approach the carbonyl group leading to hydrolysis. In contrast, the five-membered ring of proline butts closely up to the cyclopropane ring in a probable conformation for binding and inhibition and may thereby prevent the access of water (Fig. 5). This hypothesis accounts for the ability of **8** to act as an inhibitor but not as a substrate. It also provided the first suggestion of the possibility that ketones might also act as enzyme-activated inhibitors.

Some evidence in support of this mechanism was obtained using $[^2H]_4$-labelled **8** in the cyclopropane ring; the deuteriated compound was prepared as described for **8** from $[^2H]_4$-1,2-dibromoethane. The free inhibitor shows a broad triplet (δ 0.9, 1.3, 1.8) for the cyclopropane deuterons. When carboxypeptidase A was inactivated using the deuteriated inhibitor, a new signal was observed in the ^{2}H-NMR spectrum at δ 3.85 as a sharp peak on the shoulder of the broad ^{2}H signal of the bound inhibitor centred at δ 1.3. While not being conclusive, this result is consistent with the formation of an ester during inhibition. Interestingly, Mobashery[28] has rationalised his results in which cyanoacetyl and chloropropanoyl amides of phenylalanine act as inhibitors of carboxypeptidase A also through the formation of an ester with Glu-270. His compounds bound more tightly to carboxypeptidase A than ours ($K_i \sim 10^{-6}$ mol dm^{-3} cf 10^{-4} mol dm^{-3}) which may reflect the conformational restrictions imposed in our compounds by the cyclopropane ring.

The results described in this paper show that cyclopropane-containing peptides and analogues act as inhibitors of metallopeptidases thereby extending the range of application of electrophilic activation of cyclopropanes in enzyme inhibition. Whatever detailed mechanism is supported by further research, the results also suggest important conceptual developments for drug design using latent inhibitors. A close substrate analogue such as is generated by cyclopropane substitutions will not necessarily be the most desirable compound for an inhibitor. Although it will have selectivity with respect to activation by the target enzyme, it may nevertheless interact with other related biological systems. The inherent flexibility for structural modification to optimise the structure is also restricted in substrate analogues. We have shown here, however, that more distant relatives to the substrate analogue **7**, namely the ketones, the corresponding secondary alcohols, and especially the ketosulfones are also enzyme inhibitors presumably activated by Lewis acid catalysis at the active site.

Experimental

NMR spectra were obtained on a Perkin-Elmer R32 spectrometer (90 MHz) or a Bruker WM. 250 spectrometer (250 MHz). *J* values are given in Hz. Melting points are uncorrected. Enzyme kinetic studies were performed on a Philips PU 8800 spectrophotometer equipped with a thermostatted cell block at 20 °C.

Ethyl N-*cyclopropylcarbonylglycinate.* To cyclopropanecarbonyl chloride (0.5 g) in diethyl ether was slowly added ethyl glycinate (1.0 g) and the mixture stirred at 0 °C for 1 h. The crude solid was recrystallised from ethanol to yield 0.71 g (83%) of the amide m.p. 76 °C (Found: C, 55.8; H, 7.8; N, 8.3. $C_8H_{13}O_3$ requires C, 56.1; H, 7.6; N, 8.2%); ν_{max}(Nujol)/cm^{-1} 3300, 1630; δ_H(90 MHz, $CDCl_3$) 6.8–6.25 (s, 1 H), 4.32–4.12 (q, 2 H), 4.15–4.10 (d, 2 H, 1.6–1.40 (m, 1 H), 1.4–1.20 (t, 3 H), 1.05–0.80 (m, m, 4 H).

Methyl N-*cyclopropylcarbonylphenylalaninate.* To cyclopropanecarbonyl chloride (0.5 g) in diethyl ether was slowly added methyl phenylalaninate (1.15 g). The mixture was stirred and gently refluxed for 3 h, cooled, and the ether removed under reduced pressure to leave a gummy solid. Recrystallisation from methanol/water yielded 0.7 g (57%) of the amide m.p. 87–89 °C (Found: C, 67.6; H, 6.8; N, 5.6. $C_{14}H_{17}NO_3$ requires C, 68.0; H, 6.9; N, 5.7%); δ_H(90 MHz, $CDCl_3$) 7.25 (m, 5 H), 4.6–4.40 (t, 1 H), 3.6 (s, 3 H), 2.0–2.9 (d, 2 H), 1.7–1.5 (s, 1 H), 0.7–0.6 (m, m, 4 H).

Methyl N-*cyclopropylcarbonylprolinate.* To cyclopropanecarbonyl chloride (0.5 g) and pyridine (1 cm^3) in ether (20 cm^3) was slowly added with stirring methyl prolinate (0.62 g). Upon complete addition the reaction mixture was stirred for a further 2 h at room temperature. The crude product was purified by column chromatography (silica gel 60–20 μm mesh, 2% methanol in dichloromethane as eluent) yielding 61% of the amide as a pale yellow viscous oil (Found: C, 61.2; H, 7.6; N, 6.7. $C_{10}H_{15}NO_3$ requires C, 60.9; H, 7.6; N, 7.1%); ν_{max}/(liquid film)/cm^{-1} 3460, 1745, 1640; δ_H(90 MHz, $CDCl_3$), 4.70–4.4 (m, 1 H), 3.86–3.70 (s, t, 3 H, 2 H), 2.3–1.90 (m, 4 H), 1.80–1.60 (m, 1 H), 1.05–0.65 (m, 4 H).

N-*Cyclopropylcarbonylglycine* (**1**). Ethyl *N*-cyclopropylcarbonylglycinate (0.5 g) was hydrolysed using sodium hydroxide (5.26 cm^3 of 0.5 mol dm^{-3}) solution yielding 70% of the required acyl amino acid m.p. 135–138 °C from methanol (Found: C, 50.5; H, 6.3; N, 9.6. $C_6H_9NO_3$ requires C, 50.3; H, 6.3; N, 9.8%); ν_{max}(Nujol)/cm^{-1} 3400, 3320, 1710, 1640; δ_H(250 MHz, [$^2H_6$]DMSO) 8.45–8.3 (t, 1 H; *J* 8.52), 3.79–3.73 (d, 2 H), 1.70–1.63 (m, 1 H), 0.70–0.63 (s, 4 H).

N-*Cyclopropylcarbonylphenylalanine* (**2**). Methyl *N*-cyclopropylcarbonylphenylalanate (0.5 g) was hydrolysed using sodium hydroxide solution (4.05 cm^3 of 0.5 mol dm^{-3}). Recrystallisation from methanol yielded 0.32 g (71%) of the required acyl amino acid m.p. 123.5–126 °C (Found: C, 67.0; H, 6.3; N, 6.2. $C_{12}H_{15}NO_3$ requires C, 67.0; H, 6.4; N, 6.0%); ν_{max}(Nujol)/cm^{-1} 3400, 3310, 1705, 1645, 1600; δ_H(250 MHz, [$^2H_6$]DMSO) 8.43–8.34 (d, 1 H), 7.25 (s, 5 H), 4.5–4.3 (dd, 1 H), 3.1–2.96 (d, 1 H), 1.68–1.55 (m, 1 H), 0.63, 0.56 (s, 4 H); *J* (–CH_2–CH_2Ph) 12.8, *J* (–CH–NH–) 7.67.

N-*Cyclopropylcarbonylproline* (**3**). Methyl *N*-cyclopropylcarbonylprolinate (0.5 g) was hydrolysed using sodium hydroxide solution (5.10 cm^3 of 0.5 mol dm^{-3}). Recrystallisation from methanol yielded 45% of the required acyl amino acid m.p. 131–133 °C (Found: C, 58.4; H, 7.4; N, 7.5. $C_9H_{13}NO_3$ requires C, 58.7; H, 7.6; N, 7.6%); ν_{max}(Nujol)/cm^{-1} 3350, 1700, 1640; δ_H(90 MHz, [$^2H_6$]DMSO) 4.60 (m, 1 H, *J* 4.7), 3.80 (t, 2 H), 2.35–2.0 (m, 5 H), 1.81–1.62 (m, 1 H), 1.06–0.70 (m, 4 H).

Methyl N-*benzoyl*-1-*aminocyclopropane*-1-*carbonylphenylalaninate*. Methyl phenylalaninate hydrochloride (2.64 g, 2 mol eq.) was suspended in dry dichloromethane (15 cm^3) and acid ammonia gas bubbled through for 15 min. The insoluble ammonium chloride was filtered off and the residue evaporated to dryness under reduced pressure. The residue was dissolved in dichloromethane (15 cm^3) together with *N*-benzoylamino-1-aminocyclopropanecarboxylic acid (1.0 g) and dicyclohexylcarbodiimide (1.0 g). The reaction mixture was stirred at room temperature for 20 h, the precipitated dicyclohexyl urea filtered off and the solution evaporated to dryness under reduced pressure. The residue was dissolved in diethyl ether and the ether solution washed with aqueous hydrocyloric acid (1 mol dm^{-3}), water, and saturated aqueous sodium carbonate solution. The solution was dried (Na_2SO_4) and evaporated to dryness yielding a white solid. Recrystallisation from acetone–light petroleum (b.p. 60–80 °C) yielded 1.05 g (60%) of the dipeptide m.p. 110–112 °C (Found: C, 68.7; H, 5.6; N, 7.3. $C_{21}H_{22}N_2O_4$ requires C, 68.8; H, 6.0; N, 7.6%); ν_{max}(Nujol)/cm^{-1} 3260, 1745–55, 1670, 1640, 1600; δ_H(250 MHz, $CDCl_3$) 7.8–7.4 (m, 5 H), 7.1 (s, 5 H), 4.86 (t, 1 H), 3.68 (s, 3 H), 3.11 (d, 2 H), 2.15 (s, 1 H), 1.4–1.06 (m, 4 H).

Methyl N-*benzoyl*-1-*aminocyclopropane*-1-*carbonylprolinate*. This was prepared in 51% yield from 2 equivalents of amino acid ester. The product had m.p. 157–160 °C (Found: C, 64.8; H, 6.5; N, 8.9. $C_{17}H_{20}N_2O_4$ requires C, 64.6; H, 6.3; N, 8.9%); ν_{max}(Nujol)/cm^{-1} 3320, 3280, 1735, 1670, 1615; δ_H(90 MHz, $CDCl_3$) 7.85–7.3 (m, 5 H), 4.55 (s, 1 H), 3.90–3.85 (d, 2 H), 3.6 (s, 3 H), 2.1–1.80 (m, 4 H), 1.6–1.10 (m, 4 H).

Ethyl N-*benzoyl*-1-*aminocyclopropane*-1-*carbonylglycinate*. This was prepared from 2 equivalents of the amino acid ester. Recrystallisation from acetone/diethyl ether yielded 54% of the dipeptide m.p. 131–134 °C (Found: C, 62.4; H, 6.6; N, 9.8. $C_{15}H_{18}N_2O_4$ requires C, 62.1; H, 6.2; N, 9.7%); ν_{max}(Nujol)/cm^{-1} 3340, 3250, 1720, 1650, 1630, 1600; δ_H(90 MHz, $CDCl_3$) 7.85–7.40 (m, 5 H), 4.0–4.25 (q, 2 H), 3.95 (s, 2 H), 1.80–1.60 (m, 2 H), 1.20 (t, 3 H), 1.30–1.10 (m, 2 H).

N-*Benzoyl*-1-*aminopropane*-1-*carbonylphenylalanine*. (**7**). The hyrolysis of the corresponding methyl ester above (0.5 g) was achieved using 0.5 mol dm^{-3} sodium hydroxide (2.73 cm^3). The pH of the solution was maintained at 12.0 throughout using a pH stat meter. Acidification with concentrated hydrochloric acid and recrystallisation from methanol yielded the required dipeptide (0.3 g, 60%) m.p. 101–103 °C (Found: C, 64.8; H, 5.8; N, 7.6. $C_{20}H_{20}N_2O_4 \cdot H_2O$ requires C, 64.9; H, 5.9; N, 7.6%); ν_{max}(Nujol)/cm^{-1} 3300, 1700, 1650, 1600; δ_H(250 MHz [$^2H_6$]DMSO) 8.98 (s, 1 H), 7.94–7.47 (m, 5 H), 7.13 (s, 5 H), 4.6–4.3 (t, 1 H), 3.02–2.95 (d, 2 H), 1.47–0.87 (m, 4 H). *J* (–CH–CH_2Ph) 12.7 *J* (–CH–NH–) 9.0.

N-*Benzoyl*-1-*aminocyclopropane*-1-*carbonylproline* (**8**). Using the above method the corresponding methyl ester (0.5 g) was hydrolysed using sodium hydroxide solution (3.17 ml 0.5 mol dm^{-3}). Recrystallisation from methanol yielded the required dipeptide (0.36 g, 75%) m.p. 187–189 °C (Found: C, 63.4; H, 6.0; N, 9.2. $C_{16}H_{18}N_2O_4$ requires C, 63.6; H, 6.0; N, 9.3%); ν_{max}(Nujol)/cm^{-1} 3300, 1695, 1640, 1625, 1600; δ_H(360 MHz [$^2H_6$]DMSO) 9.05 (s, 1 H), 7.92–7.4 (m, 5 H), 4.21–4.17 (m, 1 H; *J* 4.5), 3.88–3.60 (t, 2 H), 2.12–0.85 (m, t, m, m, 2 H, 2 H, 4 H).

N-*Benzoyl*-1-*aminocyclopropane*-1-*carbonylglycinate* (**6**). Hydrolysis of the corresponding ester using the above conditions afforded the dipeptide which was recrystallised from methanol (52%, m.p. 210–212 °C) (Found: C, 59.5; H, 4.5; N, 10.6. $C_{13}H_{14}N_2O_4$ requires C, 59.5; H, 5.3; N, 10.7%). ν_{max}(Nujol)/cm^{-1} 3420, 3320, 1720, 1650–25, 1600. δ_H(360 MHz, [$^2H_6$]DMSO) 8.99 (s, 1 H), 8.10–7.38 (m, 5 H), 3.75–3.68 (d, 2 H), 3.49–3.20 (d, 1 H, *J* 5.8), 1.42–0.94 (m, 4 H).

2-*Phenylspiro*[*cyclopropane*-4-*oxazol*]-5-*one*. (a) 1-Benzamidocyclopropanecarboxylic acid (1 g, 4.9 mmol) was heated on a steam bath with acetic anhydride (13 cm^3) for 30 min. The reaction mixture was then poured into ice/water and left to stand for 20 min with occasional stirring. The resultant precipitate was removed by filtration, air dried and recrystallised from ethanol. This gave the required oxazolone (0.57 g, 63%) as a pale yellow solid; m.p. 145–147 °C. (b) Freshly distilled benzoyl chloride (1.03 cm^3, 2.3 mol equivalent) was added dropwise to a stirred solution of 1-aminocyclopropanecarboxylic acid (0.4 g, 4.0 mmol) in pyridine (8 cm^3, distilled over KOH) at 0 °C. This was allowed to warm up to 25 °C and left to stir for 24 h. The reaction was followed by TLC [stationary phase, silica gel; eluent, chloroform:ethanol, 12:1; R_f (product) = 0.78]. The pyridine was then removed under reduced pressure and the product extracted with chloroform, washed with 1 mol dm^{-3} HCl, 1 mol dm^{-3} NaOH and water. The organic layer was dried ($MgSO_4$), filtered and evaporated. The resultant orange solid was recrystallised from ethanol and further purified by column chromatography (silica gel, 400 mesh; eluent: dichloromethane; R_f: impurity = 0.64 and product = 0.43). The required oxazolone was obtained as a pale yellow solid; m.p. 144–145 °C, in 49% yield. ν_{max}(KCl disc)/cm^{-1} 2920, 1795, 1630, 1600; δ_H(250 MHz, $CDCl_3$) 8.05–7.16 (m, 2 H), 7.62–7.45 (m, 3 H), 1.96–1.76 (m, 4 H) (Found: C, 70.2; H, 4.8; N, 7.4. $C_{11}H_9N$ requires C, 70.6; H, 4.8; N, 7.5%).

Typical coupling reaction using a mixed anhydride. Proline methyl ester hydrochloride (0.83 g, 5 mmol) was dissolved in DMF (10 cm^3) with warming. Triethylamine (0.70 cm^3, 5 mmol) was added and this was allowed to stir while the anhydride was prepared. *N*-Benzoyl-1-aminocyclopropanecarboxylic acid (1.0 g, 5 mmol) and THF (20 cm^3) were cooled in an acetone/dry ice bath and neutralised with *N*-methylmorpholine (0.55 cm^3). Isobutylchloroformate (0.66 cm^3, 5 mmol) was then added to the solution. This was allowed to react for 1 h after which time the filtered proline methyl ester solution was added in small aliquots. The reaction mixture was allowed to warm up to 25 °C and stirred overnight. The reaction was then checked by infrared spectroscopy and as no anhydride bands (1825 ± 25 and 1765 ± 25 cm^{-1}) were observed, the reaction was worked up. The *N*-methylmorpholine hydrochloride was removed by filtration, washed with a little THF and the filtrate reduced to dryness. The residue was extracted with chloroform (50 cm^3) and washed with 5% citric acid (20 cm^3), 5% sodium hydrogencarbonate (twice) and water. The aqueous layer was backwashed with chloroform and the combined organic layer was dried ($MgSO_4$), filtered and evaporated. The product was then recrystallised from acetone/light petroleum (b.p. 60–80 °C), yielding 0.54 g, (34%); m.p. 155–157 °C.

Typical coupling reaction using oxazolone. (a) 2-Phenylspiro[cyclopropane-4-oxazol]-5-one (1 g, 5.3 mmol), proline methyl ester hydrochloride (1.7 g, 10.0 mmol) and triethylamine (1.4 cm^3, 10.0 mmol) in acetonitrile (35 cm^3) were refluxed for 2 h, the reaction being followed by TLC (eluent, chloroform:ethanol, 12:1; R_f starting material = 0.6, product = 0.43). The solvent was removed and the residue was extracted with chloroform (40 cm^3), washed with 1 mol dm^{-3} HCl (twice), 5% sodium hydrogencarbonate (twice) and water. The aqueous layer was back extracted with chloroform and the combined organic layer was dried ($MgSO_4$), filtered and evaporated to dryness. Recrystallisation was from ethanol. This gave a yield of 1.06 g (62%) of a white crystalline solid; m.p. 158–160 °C.

1-Benzamidocyclopropane-1-carbonyl-N,O-dimethylhydroxylamide, (**13**). The above oxazolone (0.51 g, 2.7 mmol) *N,O*-dimethylhydroxylamine hydrochloride (0.31 g, 3.2 mmol) distilled pyridine (0.26 cm^3, 3.2 mmol) and dichloromethane (dried and distilled, 15 cm^3) were stirred at room temperature for six days. The reaction mixture was then washed with brine (twice) and the organic layer was dried, filtered and evaporated. The product recrystallised from ethyl acetate to yield 0.49 g (72%) of a white crystalline solid; m.p. 161–162 °C. (Found: C, 62.9; H, 6.5; N, 11.4. $C_{13}H_{16}N_2O_3$ requires C, 62.9; H, 6.5; N, 11.3%.) ν_{max}(Nujol)/cm^{-1} 3320, 2960–2850, 1650, 1625, 1600, 1525; δ_H(250 MHz) 7.90–7.72 (d, 2 H), 7.56–7.38 (m, 3 H), 7.02 (s, 1 H), 3.65 (s, 3 H), 3.19 (s, 3 H), 2.65–2.55 (m, 2 H), 1.24–1.10 (m, 2 H).

1-Acetyl-1-benzamidocyclopropane (**11**). Iodomethane (3.76 cm^3, 60 mmol) was added gradually to a suspension of magnesium turnings (1.44 g, 60 mmol) in dry tetrahydrofuran (40 cm^3). A solution of the methoxymethylhydroxylamide (**13**, 1.49 g, 6 mmol) in warm tetrahydrofuran (30 cm^3) was added over 5–10 min during which time a vigorous reaction ensured. Reaction was allowed to continue for 2 h and the reaction mixture than added to crushed ice mixed with dilute hydrochloric acid. The product was extracted with dichloromethane, the organic solution dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The required ketone was recrystallised from carbon tetrachloride to give pale yellow crystals (0.61 g) m.p. 144–147 °C (Found: C, 71.1; H, 6.5; N, 6.9. $C_{12}H_{13}NO_2$ requires C, 70.94; H, 6.45; N, 6.89%). δ_H(250 MHz) 7.82–7.79 (d, 2 H), 7.56–7.38 (m, 3 H), 7.40 (bd s, 1 H), 2.22 (s, 3 H), 1.71–1.58 (m, 2 H), 1.29–1.23 (m, 2 H).

1-Benzamido-1-(2-phenyl-1-oxoethyl)cyclopropane (**12**). Benzyl bromide (23.7 cm^3, 0.20 mol) in THF dried and distilled (400 cm^3) was added dropwise over a period of 20 min, to a mixture of magnesium turnings (0.05 g, 0.20 mol) and iodine in sodium-dried diethyl ether (200 cm^3) and was allowed to stir for a further 40 min refluxing gently. A solution of the methoxymethylhydroxylamide **13** (4.6 g, 0.02 mol) in THF (100 cm^3) was added dropwise over 30 min and the reaction was left to stir for 3 h at room temperature. The solution was poured onto crushed ice/1 mol dm^{-3} HCl (150 cm^3) and extracted with dichloromethane (thrice) and the organic layer was washed with water. This was dried, filtered and evaporated. Prior to recrystallisation, the residue was washed with light petroleum (b.p. 30–40 °C) successively to remove any bibenzyl impurity. Recrystallisation was from CH_2Cl_2–light petroleum (b.p. 60–80 °C) initially followed by ethanol and then water to give the required ketone (2.5 g) as a white crystalline solid; m.p. 125–127 °C (Found: C, 77.4; H, 6.1; N, 5.1. $C_{18}H_{17}NO_2$ requires C, 77.4; H, 6.1; N, 5.0%). ν_{max}(KCl disc)/cm^{-1} 3240, 3050, 3030, 2920, 1690, 1635, 1600, 1525; δ_H(250 MHz, $CDCl_3$) 7.76–7.68 (m, 2 H), 7.60–7.40 (m, 3 H), 7.34–7.20 (m, 3 H), 7.15–7.08 (m, 2 H, 6.63 (s, 1 H), 3.95 (s, 2 H), 1.80–1.70 (m, 2 H), 1.62 (s, 1 H), 1.30–1.20 (m, 2 H).

1-Benzamido-1-(2-methylsulfonyl-1-oxoethyl)cyclopropane (**9**). Butyllithium (1.75 cm^3, 4.4 mmol) was added dropwise to diisopropylamine (0.66 cm^3, 4.7 mmol) in THF (10 cm^3) under an atmosphere of nitrogen at −75 °C and allowed to stir for 15 min. A solution of methyl sulfone (0.33 g, 3.5 mmol) in dry THF (20 cm^3) was then slowly added to the reaction mixture and left stirring for a further 30 min. This was followed by oxazolone **7** (0.6 g, 3.2 mmol) dissolved in THF (20 cm^3) which was added dropwise and allowed to react for 2 h. The reaction mixture was then quenched by the addition of aqueous acetic acid (30 cm^3, 5%) at −78 °C and brought up to room temperature. Water was added to the reaction and then extracted with diethyl ether (twice). The ether layer was washed with water followed by saturated sodium hydrogencarbonate and the resultant white solid was filtered and recrystallised from ethanol to give the required ketosulfone (0.33 g, 36%); m.p. 219–220 °C; (Found: C, 55.3; H, 5.1; N, 5.0; S, 11.3. $C_{13}H_{15}O_4NS$ requires C, 55.5; H, 5.4; N, 5.0; S, 11.4%). ν_{max}(KCl disc)/cm^{-1} 3320, 3090, 3030 2960, 2910, 1705, 1645, 1605, 1525, 1310, 1125; δ_H(250 MHz, [$^2H_6$]DMSO) 9.24 (s, 1 H), 7.94–7.88 (m, 2 H), 7.62–7.45 (s, 2 H), 3.07 (s, 3 H), 1.64–1.56 (m, 2 H), 1.35–1.28 (m, 2 H).

1-Benzamido-1-(2-phenylsulfonyl-1-oxoethyl)cyclopropane (**10**). This compound was prepared similarly to the methyl analogue **9** using phenylmethylsulfone. The required ketosulfone was obtained in 32% yield as a white solid; m.p. 181–184 °C; (Found: C, 62.7; H, 5.0; N, 3.9; S, 9.5. $C_{18}H_{17}NO_4S$ requires C, 63.0; H, 5.0; N, 4.1; S, 9.3%) ν_{max}(KCl disc)/cm^{-1} 3370, 3030, 2970, 1695, 1645, 1605, 1515, 1330, 1155; δ_H(90 MHz, [$^2H_6$]DMSO) 9.15 (s, 1 H, 8.08–7.80 (m, 4 H), 7.80–7.45 (m, 6 H), 4.79 (s, 2 H), 1.70–1.40 (m, 2 H), 1.40–1.11 (m, 2 H).

1-Benzamido-1-(1-hydroxymethyl)cyclopropane (**14**). (Experiment carried out by Mr. E. Lucas). To a solution of sodium borohydride (0.058 g) in ethanol (50 cm^3) solid ketone (**11**, 0.5 g, 1.8 mmol) was added. Reaction was allowed to continue for 1 h and was then quenched by the addition of a little acetic acid. The solution was evaporated to dryness under reduced pressure and the residue dissolved in water. The product was extracted with dichloromethane (4 × 40 cm^3), the organic extracts dried (Na_2SO_4) and evaporated under reduced pressure to give an oil which readily crystallised. Recrystallisation from carbon tetrachloride gave the required alcohol (0.45 g, 60%), m.p. 115–117 °C. (Found: C, 70.1; H, 7.35; N, 6.72. $C_{12}H_{16}NO_2$ requires C, 70.2; H, 7.32; N, 6.82%). ν_{max}(KCl)/cm^{-1} 3220, 1630, 1600. δ_H(250 MHz, $CDCl_3$) 7.4–7.9 (m, 5 H); 6.78 (s, 1 H); 4.75 (d, 1 H); 3.41 (m, 1 H); 1.25 (d, 3 H); 1.21–0.8 (m, 4 H).

Similarly prepared was *1-benzamido-1-(1-hydroxy-2-phenylethyl)cyclopropane* (**15**) in 35% yield, m.p. 141–142 °C (white needles from carbon tetrachloride) (Found: C, 76.8; H, 6.75; N, 4.9. $C_{18}H_{19}NO_2$ requires C, 76.9; H, 6.76; N, 4.98%). ν_{max}(KCl)/cm^{-1} 3345, 3045, 1630, 1600. δ_H(250 MHz, $CDCl_3$) 7.69 (2 H, d), 7.45 (3 H, m), 7.24 (5 H, m) 6.43 (1 H, s), 4.77 (1 H, d, *J* 5.0), 3.40 (1 H, dd, *J* 5.0 and 5.0), 2.94 (2 H, m), 1.14 (1 H, m), 1.08 (1 H, m), 0.91 (1 H, m), 0.77 (1 H, m).

Enzyme Assays.—The enzyme assays monitored by changes in the UV absorption were performed using a Phillips Pye Unicam SP800 ultraviolet spectrophotometer, using cuvettes of 1 cm light path. The reactions were all monitored at 254 nm and using reverse cell mode. The enzyme used in all experiments was carboxypeptidase A from bovine pancreas, as supplied by Sigma Chemical Company unless otherwise stated. The substrate was hippuryl-L-phenylalanine (obtained from Sigma, H6875).

The enzyme solution was made up in phosphate buffer (Na_2HPO_4, NaCl 5 mmol dm^{-3} at pH 7.0) and the reactions were carried out in Tris/HCl buffer (Tris[hydroxymethyl]-aminomethane) (0.1 mol dm^{-3} Tris, 1 mol dm^{-3} NaCl at pH 7.5). The concentration of the stock solutions were as follows: (a) 5.5×10^{-6} mol dm^{-3} carboxypeptidase (11×10^{-3} cm^3 CPA made up to 1 cm^3). (b) 50 mmol dm^{-3} hippurylphenylalanine (in 0.1 mol dm^{-3} NaOH). (c) 30 mol dm^{-3} inhibitor, dissolved in the following: peptides and acylaminoacids, 0.1 mol dm^{-3} NaOH; alkyl ketones, ethanol and buffer (3:2); secondary alcohols, ethanol; sulfones, dimethyl sulfoxide. Appropriate dilutions with buffer afforded the concentrations quoted in the tables.

NMR Experiments.—Carboxypeptidase A (55 mg, suspension in buffer containing toluene) was dissolved in 0.1 mol dm^{-3} Tris, 1 mol dm^{-3} NaCl, pH 7.5 (2.0 cm^3) and the solution dialysed against distilled water at 4 °C overnight. The suspension of enzyme was centrifuged down at 4000 rpm and 4 °C for 0.5 h. The pellet was resuspended in deuterium oxide (0.5 cm^3) and

centrifuged; this process was repeated. To obtain the ^{2}H-NMR spectrum, the enzyme (55 mg, 1.6×10^{-6} mol) was dissolved in deuterium oxide (0.5 cm^3) together with [$^2H_4$]-**7** (1 mg, 3.2×10^{-6} mol) and allowed to react for 18 h. The ^{2}H-NMR spectrum was obtained using 2500 scans.

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