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Latent Inhibitors Part 10. The Inhibition of Carboxypeptidase A by Tetrapeptide Analogues Based on 1-Aminocyclopropane Carboxylic Acid

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Abstract: In order to test the phenomenon of substrate activation of irreversible inhibition of carboxypeptidase A, extended inhibitors were designed. The synthesis of two N-protected tetrapeptide analogues containing C-terminal sulphones and 1-aminocyclopropane carboxylic acid and one similar N-unprotected tetrapeptide with C-terminal phenylalanine is described. The compounds were evaluated as inhibitors of carboxypeptidase A. The tetrapeptide sulphones exhibited time-dependent inhibition following the unusual 'substrate activated' pattern of related dipeptides but the phenylalanine containing dipeptide behaved as a mixed non-competitive inhibitor. A molecular modelling evaluation of the potential of aminocyclopropane carboxylic acid derivatives to act as irreversible inhibitors of peptidases was undertaken in an attempt to identify the properties of such compounds that lead to the unusual kinetic properties. A mechanism for the inhibition reactions of dipeptide and tetrapeptide analogues is proposed.

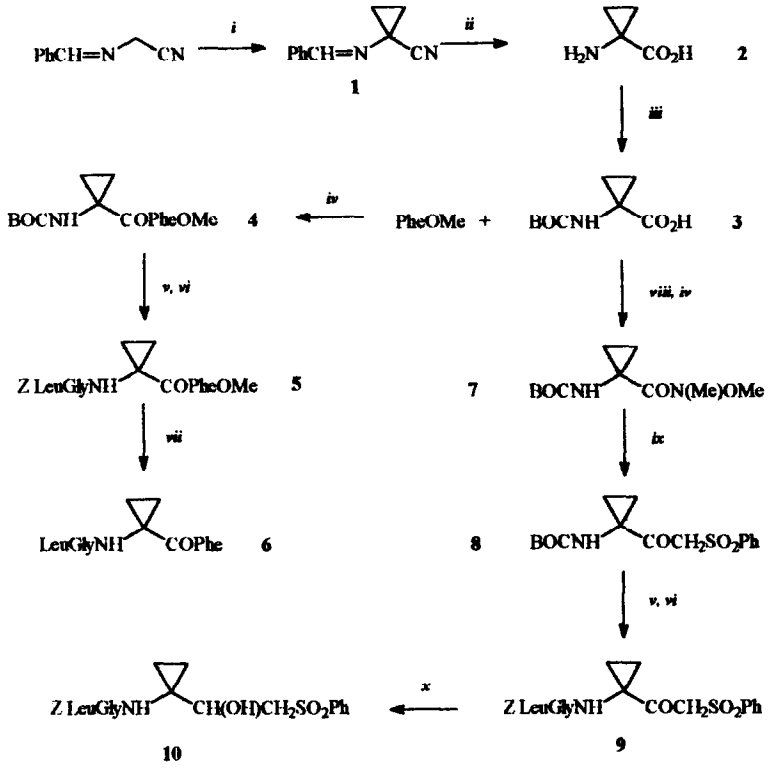
INTRODUCTION

Recently we reported that derivatives and analogues of peptides containing 1-aminocyclopropane carboxylic acid inhibited carboxypeptidase in a time-dependent, irreversible manner involving the unusual phenomenon of substrate activation[1]. In these reactions, inhibition was faster in the presence of the substrate than in its absence. This unusual behaviour was rationalised in terms of enzyme-substrate-inhibitor and enzyme-inhibitor-inhibitor complexes but the experimental data available did not lead to clearly defined kinetic constants for the inhibition reactions. Bearing in mind the multiple binding sites available to peptidases in general[2] and to carboxypeptidase in particular[3], we thought that a larger inhibitor that would occupy more binding sites than those previously studied, which were no longer than acyl dipeptides. We therefore selected some extended inhibitors of length equivalent to tetrapeptides for synthesis with the expectation that such

compounds might bind in an equivalent manner to the enzyme-substrate-inhibitor complex and lead to rapid inhibition in the absence of added substrate. A survey of the known test substrates and inhibitors of carboxypeptidase A showed that a wide range of residues could be tolerated[4]; moreover, some the activation or inhibition of hydrolysis catalysed by carboxypeptidase A can be observed with respect to distinct reactions such as amide and ester hydrolysis. For our experiments, the additional dipeptide selected was leucyl-glycine with the following features in mind: L-leucine has the potential to occupy a hydrophobic binding site and glycine poses the minimum steric barriers to rotation in the neighbourhood of the cyclopropane ring. In this way we hoped that the inhibitors would have increased binding together with sufficient rotational freedom to access conformations at the active site relevant to inhibition.

SYNTHESIS

The synthesis of the compounds evaluated is shown in scheme 1. Diphenylmethyleneaminoacetonitrile was cyclopropanated with 1,2-dibromoethane under phase transfer conditions to afford 1 (90%) which was hydrolysed with acid giving 1-aminocylopropane carboxylic acid 2 (100%). The aminoacid was BOC-protected using 2-t-butyloxycarbonyloximino-2-phenyl acetonintrile (BOC-ON) (3 66%)[5] and then coupled using standard carbodiimide conditions with L-phenylalanine methyl ester (58%); as found before[1], the yield in this reaction was never high. After purification by chromatography on silica, the BOC group was removed using 20% trifluoroacetic acid in dichloromethane to avoid hydrolysis of the methyl ester[6]. Coupling of this dipeptide 4 with benzyloxycarbonyl-L-leucylglycine was accomplished again in low yield to give the fully protected tetrapeptide 5 (30%) following the mixed anhydride procedure[7]. To obtain a suitable inhibitor, the methyl ester was hydrolysed with aqueous sodium hydroxide (1M); unexpectedly, this also led to cleavage of the benzyloxycarbonyl protecting group leading to 6. These cyclopropane containing tetrapeptides and analogues appear to favour hairpin conformations (see below). It is possible that the conformational limitations imposed by the cyclopropane ring might bring the carboxylate formed into close proximity with the urethane linkage and facilitate hydrolysis by a neighbouring group effect. The product was purified by ion exchange chromatography. To obtain the sulphones, BOC-protected aminocyclopropane carboxylic acid 3 was converted into the N,O-dimethylhydroxylamide 7 (70%) using carbodiimide coupling[8]. Addition of the lithium salt of phenylmethyl sulphone at -20° - -30°C in tetrahydrofuran solution to 7 afforded the ketosulphone 8 (66%)[9]. After deprotection with methanolic hydrogen chloride, 8 was coupled with Z-Leu-Gly by the mixed anhydride method (43%). The ketosulphone tetrapeptide analogue 9 was reduced with sodium borohydride in tetrahydrofuran to afford the third inhibitor, the hydroxysulphone 10 (51%).



Scheme 1 *Reagents*: i $BrCH_2CH_2Br$, NaOH, $PhCH_2NEt_3^+Cl^-$, toluene; ii ether, aq. HCl (1M); iii BOC-ON, Et_3N; iv DCC, CH_2Cl_2; v TFA, CH_2Cl_2; vi *i*-BuOCOCl, N-methylmorpholine, Z-LeuGly; vii aq. NaOH; viii MeONHMe.HCl, Et_3N; ix $PhSO_2Me$, LDA, THF; x $NaBH_4$, THF.

ENZYME ASSAYS

The procedures used to evaluate the inhibitors were identical to those described previously[1] in which the substrate N-benzoylhippuryl phenylalanine was incubated in the presence of enzyme in pH 7.5 TrisHCl (0.1M)and reactions were followed observing the change in absorbance at 254nm. The tetrapeptide **6** was shown to be a mixed non-competitive inhibitor with K_i = 0.065 mM as illustrated by the Dixon plot (figure 1) and no evidence of time-dependent inhibition whatsoever was found.

On the other hand, the N-benzyloxycarbonyl protected sulphones **9** and **10** behaved in the same manner as the dipeptide cyclopropane-containing inhibitors such as **11** - **13** described previously[1]. The comparison is shown in figure 2 which plots the fraction of hydrolysis of substrate completed in the

presence of inhibitors **9**, **10**, and **14**, (previous study[1]), against the concentration of inhibitor.

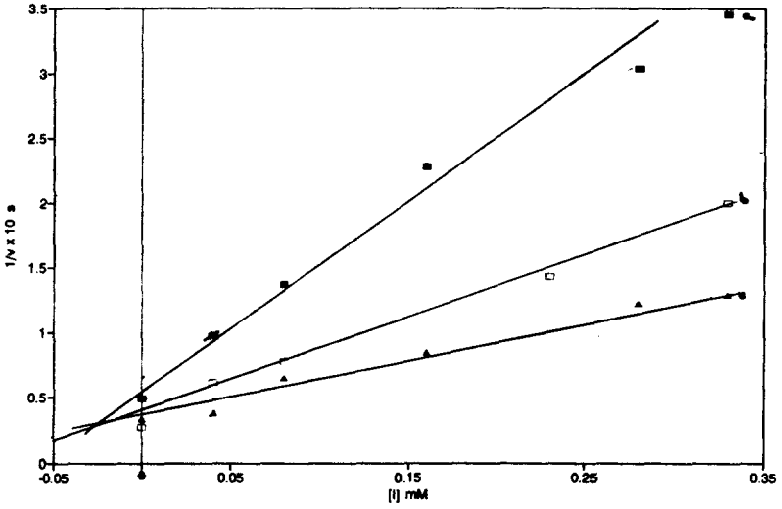


Figure 1 Dixon plot of data for the inhibition of carboxypeptidase A by **6**.

[substrate] = ■ 0.30, □ 0.67, ▲ 1.33 mM.

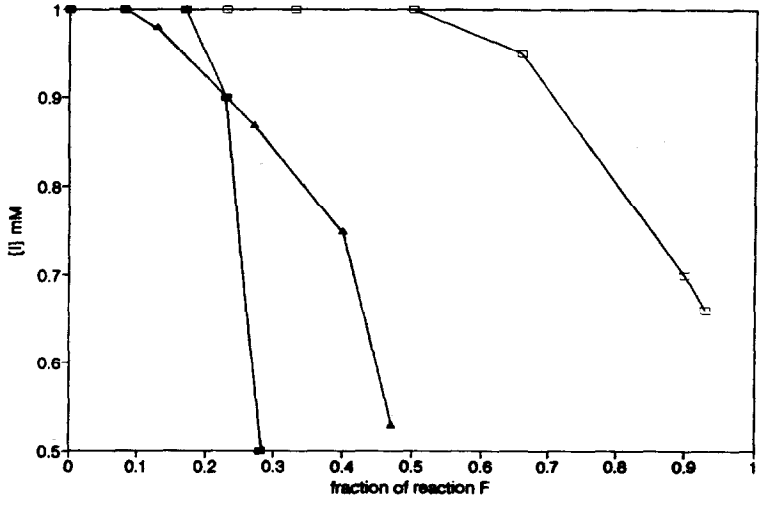


Figure 2 Inhibition of carboxypeptidase A by **9** (■), **10** (□), and **14** (▲). The fraction of reaction (F) complete under standard conditions (see experimental) is plotted against [I]

The fraction of reaction was determined directly from progress curves in the presence and absence of inhibitors. In the absence of inhibitors, reaction conditions were adjusted so that hydrolysis of the substrate, hippurylphenylalanine, was complete within 10-15 min leading to an absorbance increase of 1 - 2.

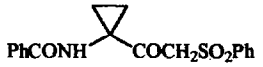
In the presence of inhibitor, the extent of the reaction was reduced both with respect ot the quantity of substrate hydrolysed (shown by a lower absorbance increase) and the duration of the hydrolysis reaction (shown by the quicker attainment of constant absorbance). The ratio of the absorbance in the presence and absence of inhibitor at the end of hydrolysis defines the fraction of reaction. Thus figure 2 shows that the fraction of reaction completed decreases sharply as inhibitor concentration is increased and that the benzamido-protected tetrapeptide analogues **9** and **10** behave in the same manner as the dipeptide analogues described in detail in our previous work[1]. Such plots are characteristic of time-dependent, irreversible inhibition in these reactions.

Thus the sulphones **9** and **10** surprisingly also showed substrate activation, an unusual feature for compounds of length comparable to tetrapeptides, which, as substrates normally exhibit Michaelis-Menten kinetics[10]. The different behaviour of the unprotected aminotetrapeptide **6** is not surprising since it is probable that the free amino group will remain in solvent outside the normal binding pocket of the enzyme. In contrast, the other inhibitors are polar but electrostatically neutral compounds.

11

12

13



14

15

16

INHIBITOR STRUCTURE

The interpretation of the new results described above and those reported previously[1] in terms of the structure of carboxypeptidase A is difficult because subsidiary binding sites have not been well characterised by X-ray crystallography of enzyme complexes with suitably large inhibitors[11,12]. Irreversible inhibition

requires activation of the cyclopropane group by the carbonyl group, a situation that would be expected to be sensitive to the conformation of the substituents around the cyclopropane ring[13]. Calculations therefore were carried out on inhibitors representative of the structural types included in this study and our previous work using the MM$^+$ force field[14].

Crystal structures have been determined for aminocyclopropane carboxylic acid homo-oligomers[15]. These compounds show torsion angles for C^b-C^a-C'-O in the same range as those calculated for the subjects of this paper as shown in figure 3. Other parameters of the cyclopropane ring are also similar, for example the torsion angle N-C^a-C^b-C^b likewise are similar (112°) indicating a skew conformation. These two angles define the relationship of the cyclopropane ring to its immediate meighbours in the peptide chain and are important in this problem. Calculated and measured bond lengths between 6 and 9 and the concensus bond lengths measured by crystallography were within 0.04A.

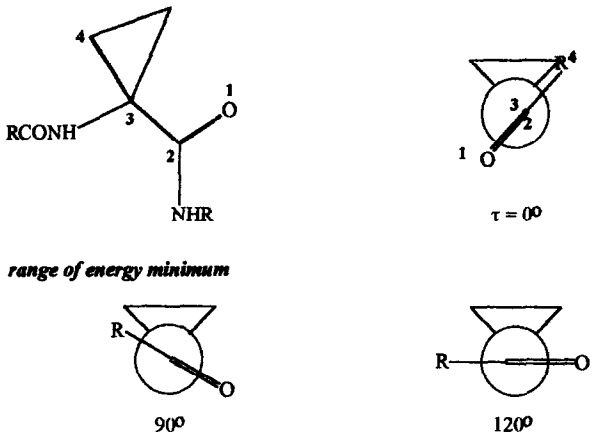


Figure 3 Torsion angle of α–carbonyl group and cyclopropane ring for minimum energy

Bond angles in and about the cyclopropane ring were generally within 1° of those measured. However, the calculations were unable to reproduce the full conformation of representative di- and tripeptides the structures of which were determined by X-ray crystallography[15]. On the other hand, results close to the cyclopropane ring were close enough to encourage an evaluation of the factors controlling the reactivity of the cyclopropane ring towards nucleophiles at the enzyme's active site.

Firstly, to investigate whether the cyclopropane ring imposed severe potential energy barriers to

rotation of either of the substituents, the total energies for the selected inhibitors (**11**, **13**, **14**) were computed as a function of the torsion angle cyclopropane C-C:C-C:C=O (figure 3). As would be expected, the three molecules showed qualitative similarities but quantitative differences, the major minimum being located between 90° and 120° with a significant barrier principally between -50° and -150° (figure 4 a, b, c). A previous theoretical study, supported by crystallographic data, of the available conformational space around the aminocyclopropane carboxylic acid group in small peptides showed similarly that a wide range of conformations was possible provided that no substituents were present on the cylopropane ring[16]. The inhibitors will be able to adopt conformations determined by the topography of the enzyme's active and binding sites and that the origin of the unusual inhibition mechanism observed with these compounds may be reasonably sought freely within these interactions.

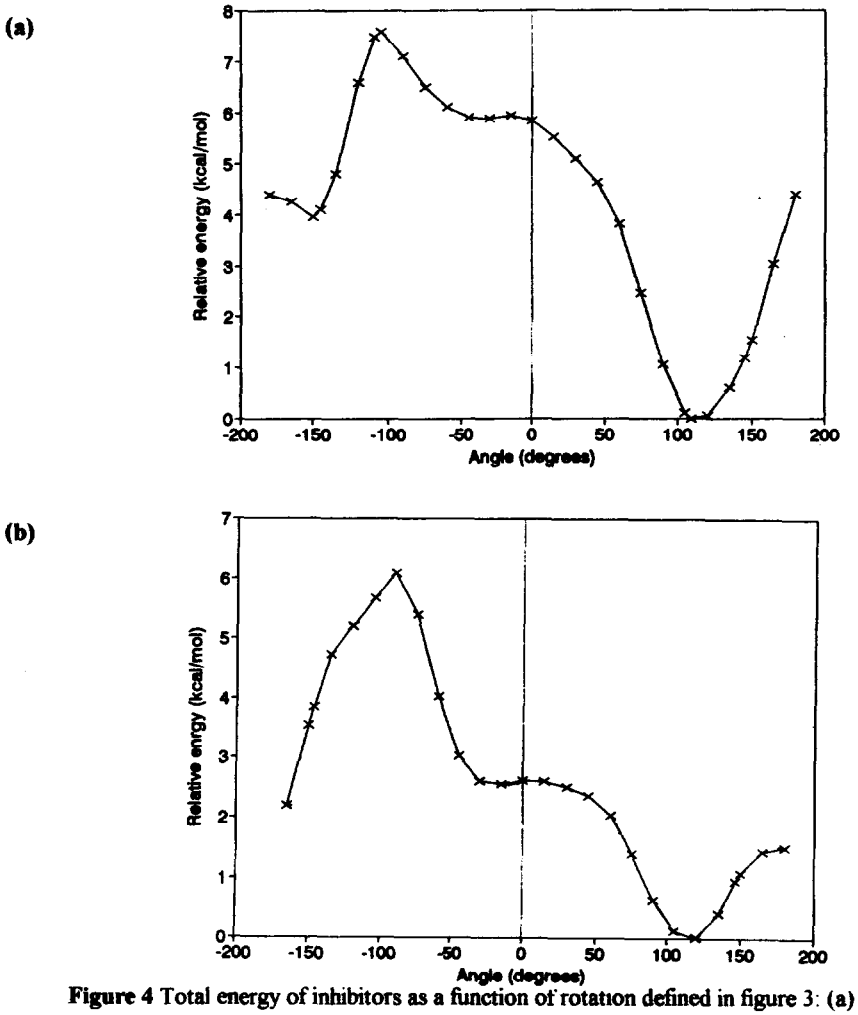


Figure 4 Total energy of inhibitors as a function of rotation defined in figure 3: (a) **11**, (b) **13**

(c)

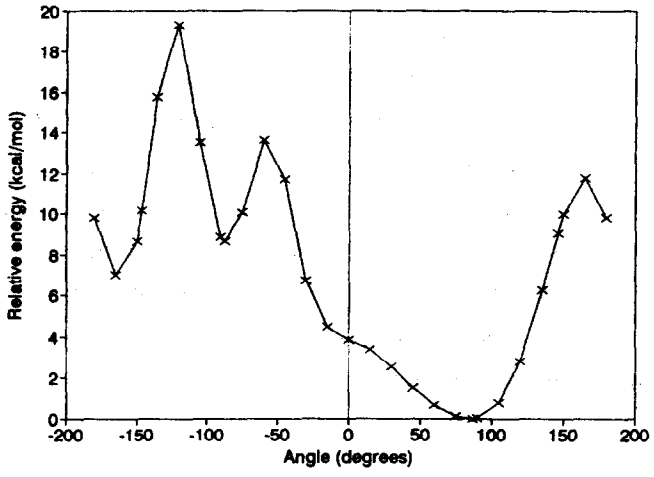


Figure 4 (cont.) Total energy of inhibitor **14** as a function of the rotation defined in figure 3.

A MODEL FOR SUBSTRATE ACTIVATED INHIBITION

Taking the evidence presented in this paper together with that described previously[1], it is possible to explain the behaviour of carboxypeptidase in terms of two interdependent binding sites, one of which is catalytically active. The relevant facts derived from our previous study[1] are: 1. The extent of the inhibition reaction at high concentration of inhibitor depends upon the square of the concentration of the inhibitor. 2. The rate of inhibition is only significant in the presence of substrate. 3. Nmr experiments suggest that inhibition takes place by forming a carbon-oxygen bond, such as between Glu-270 and the inhibitor molecule. Consistently, from the current work we can add: 4. Inhibition takes place equally with tetrapeptides 5. There appear to be no conformational impediments to inhibition around the cyclopropane ring. A model that takes account of all of these facts is shown in figure 5.

On binding a molecule of substrate, the enzyme undergoes a conformational change leading to the formation of a binding site suitable for occupation by a cyclopropane-containing inhibitor. Hydrolysis of the substrate and dissociation of the products (single amino acids) then takes place with the inhibitor still bound. A further conformational change permits the inhibitor to bind now at the active site and inhibition takes place by covalent modification. This sequence of events accounts for inhibition through an EI_2 complex. If the order of events is changed and the substrate binds firstly to the second binding site thereby promoting a conformational change in the active site so that the inhibitor now binds, inhibition through an ESI complex can be understood.

In the kinetic equations, the rates of conformational change would probably be significant and this additional factor accounts for the difficulty in extracting kinetic constants (k_i and K_i) from a model including

only association/dissociation equilibria and rate constants for chemical changes in substrates or inhibitors. It would be satisfying to correlate these suggested events with the crystal structure of carboxypeptidase A through a computer model. We have already developed such a model for the dipeptide inhibitors **11** and **12**[13,1]; the model is consistent with nucleophilic inhibition via a glutamate residue but it cannot reasonably be pressed further to deal with this correlation. Such an experiment would require a treatment of the conformational mobility of substantial regions of the enzyme, well beyond what can reasonably be derived from a crystal structure of the carboxypeptidase-glycyltyrosine complex.[17]. There may even be interactions remote from the active site that could account for the observed behaviour of our inhibitors.

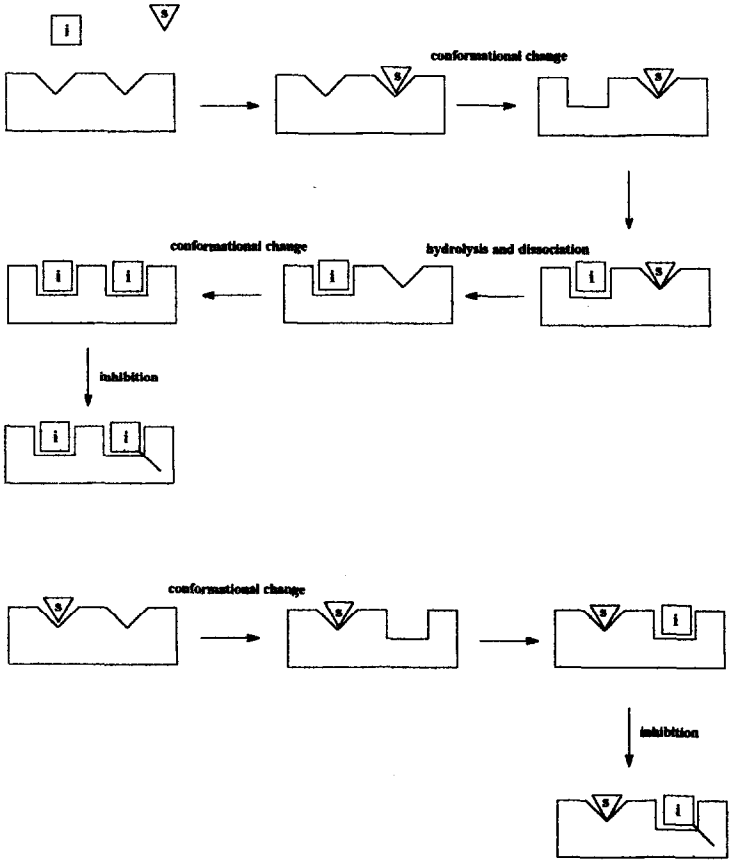


Figure 5. A two-site mechanism that accounts for substrate-activated inhibition.

We therefore believe that the above multiconformational state model together with the molecular model previously described[13,1] is the best currently available explanation of substrate activated inhibition of

carboxypeptidase A by cyclopropane-containing peptide analogues.

EXPERIMENTAL

1-(t-Butyloxycarbonyl)amino cyclopropane-1-carboxylic acid (**3**). 1-Aminocyclopropane-1-carboxylic acid hydrochloride (1.37 g; 10 mmol), BOC-ON (11.2 g; 44 mmol), triethylamine (5.5 ml; 40 mmol), dioxane (10 ml) and water (10 ml) were all stirred at room temperature overnight. Ethyl acetate (100 ml) and water (100 ml) were added and the aqueous layer collected and acidified with ice-cold 1N HCl. Extraction with ethyl acetate, drying (Na_2SO_4) and removal of solvent yielded a white solid (1.3 g; 66%), m.p. 175-178°C; δ_H (90 MHz; CD_3OD) 1.1-1.6 (13H, m, 3xCH_3 plus cyclopropyl); ν_{max}(nujol) 3250 (NH), 1700, 1650 (C=O); Found C:53.5, H:7.5, N:7.4, $C_9H_{15}NO_4$ requires C:53.7, H:7.5, N:7.0%.

1-(t-Butyloxycarbonyl)aminocyclopropane-1-carbonyl-phenylalanine methyl ester (**4**). The BOC protected cyclopropyl amino acid (**3**) (0.48 g; 2.4 mmol), dicyclohexylcarbodiimide (0.54 g; 2.6 mmol), phenylalanine methyl ester (0.51 g; 2.4 mmol), triethylamine (0.34 ml; 2.4 mmol), acetonitrile (10 ml) and dichloromethane (10 ml) were all stirred together at room temperature overnight. The white solid formed was filtered off and the solvent removed before adding ethyl acetate (20 ml). Again the solid was filtered off and the filtrate reduced to dryness to leave a three component, white solid as shown by tlc. Silica gel chromatography (hexane:ethyl acetate, (60:40) gave the required amide as a white solid (0.52 g; 60%); m.p. 109-110°C; δ_H(90 MHz; $CDCl_3$), 1.10-1.60 (13H, m, 3xCH_3 plus cyclopropyl), 3.15 (2H, d, CHCH_2), 3.70 (3H, s, CO_2CH_3), 4.91 (1H, CHCH_2), 5.10 (1H, s, NH), 7.25 (5H, m, Ph); Found C:63.2, H:7.3, N:7.7, $C_{19}H_{26}N_2O_5$ requires C:63.0, H:7.2, N:7.7%.

1-[(Benzyloxycarbonyl-L-leucylglycyl)amino]cyclopropane-1-carbonylphenylalanyl methyl ester (**5**). The BOC protected dipeptide (**4**) (1.0 g; 2.8 mmol) was stirred in 20% trifluoroacetic acid in dichloromethane (20 ml) for two hours in an ice bath. The product was extracted in water and removal of the solvent gave the product (0.85 g; 82%) as a thick green oil. Z-LeuGly (0.81 g, 2.5 mmol) in THF (13 ml, dry) was chilled to -5°C in an ice/salt bath before adding N-methylmorpholine (0.28 ml; 2.5 mmol) followed by isobutylchlorocarbonate (0.34 mml; 2.5 mmol). After a couple of minutes a solution of the dipeptide acid (0.94 g; 2.5 mmol) and N-methylmorpholine (0.28 ml; 2.5 mmol) in DMF (5 ml:dry) was added. The solution was allowed to warm to room temperature and then stirred for five hours. The solvents were then removed *in vacuo* and the crude product extracted into ethyl acetate from water. Silica gel chromatography (ethyl acetate:hexane, 80:20) gave the product **5** as white crystals (0.42 g; 30%); δ_H (250 MHz; $CDCl_3$) 0.93 and 1.50-1.61 (13H, m, 2xCH_3, CHCH_2, cyclopropyl), 3.01 (2H, m, $PhCH_2O$), 3.58 (3H, s, CO_2CH_3), 3.60-4.10

(3H, m, CHCHO plus CH_2CO), 5.10 (2H, m, $PhCH_2O$), 7.10-7.41 (10H, m, 2xPh).

1-[(L-leucylglycyl)amino]cyclopropane-1-carbonylphenylalanine carboxylic acid (**6**). The diprotected tetrapeptide **5** (300 mg; 0.53 mmol) was stirred in 1N NaOH (1.06 ml; 1.06 mmol) in acetone (2 ml) for one hour at room temperature. The acetone was removed and the aqueous layer acidified to pH=1 with 2N HCl. The product was then extracted with ethyl acetate, the solution dried (Na_2SO_4) and evaporated to leave a white solid (203 mg) m.p. 118-120°C. d_H (250 MHz; MeOD) 0.93 and 1.40-1.74 (13H, m, $2xCH_3$, $CHCH_2$, cyclopropyl), 3.13 (2H, m, CH_2Ph), 3.70 (2H, s, $NHC\underline{H}_2CO$), 4.31 (1H, m, NCHCO), 4.58 (1H, m, NCHCO), 7.25 (5H, s, Ph). A sample was further purified by dissolving this material (50 mg) in ethyl acetate and passing ammonia through the solution which caused a precipitate to form. This was extracted in water, the water evaporated to leave a white solid (48 mg); m.p. 135-138°C, Found m/z 400.2066; $C_{21}H_{28}N_4O_4$ (M^+-H_2O) requires 400.2111. HPLC (ODS:Reverse phase) water:CH_3CN, 60:40, showed one peak at 195s, uv detection at 254 nm.

1-[(t-Butyloxycarbonyl)amino]cyclopropane-1-carbonyl-N,O-dimethylhydroxylamide (**7**). To a slurry of the BOC protected acid (**3**) (0.48 g, 2.4 mmol) in acetonitrile (10 ml) was added dicyclohexylcarbodiimide (0.54 g; 2.6 mmol), N,O-dimethylhydroxylamine hydrochloride (0.23 g; 2.4 mmol) and finally, triethylamine (0.34 ml; 2.4 mmol) in acetonitrile (5 ml) and dichloromethane (10 ml). The solution was stirred at room temperature for three days. The white precipitate of dicyclohexylurea was filtered off and the solvents removed under reduced pressure. The product was obtained by silica gel chromatography (hexane:ethyl acetate, 80:20) as white crystals (0.43 g, 73%), m.p. 72-73°C; δ_H (250 MHz; $CDCl_3$) 1.10-1.60 (13H, m, $3xCH_3$ plus cyclopropyl), 3.2 (3H, s, Me), 3.8 (3H, s, OMe), 5.5 (1H, s, NH); Found C:56.7, H:7.9, N:11.0, $C_{12}H_{20}N_2O_4$ requires C:56.2, H:8.3, N:10.9%. ν_{max}(nujol) 3380 (N-H), 2980 (C-H), 1700 (C=O) cm^{-1}.

1-t-Butyloxycarbonylamino-1-(2-phenylsulfone-1-oxoethyl)cyclopropane (**8**). Diisopropylamine (0.41 g; 1.84 mmol) was added to THF (10 ml) at 0°C, then n-butyllithium (1.8 ml; 2.5 M; 4.42 mmol), added dropwise at -5°C. Methylphenylsulfone (0.63 g; 4.04 mmol) in THF (3 ml) was added dropwise to give a creamy/yellow precipitate. The amide (**7**) (0.45 g; 1.84 mmol) in THF (6 ml) was then added, again at -5°C, at which point the solution became clear. After 30 minutes the solvents were removed under reduced pressure and the crude product extracted into ether from water and the ether removed under reduced pressure. The product was purified by silica gel chromatography (dichloromethane:hexane, 65:35) to yield a white solid. (0.41 g; 66%); m.p. 166-168°C; δ_H (90 MHz; $CDCl_3/CD_3OD$) 1.20-1.70 (13H, m, $3xCH_3$ plus cyclopropyl), 3.50 (2H, s, CH_2), 7.61-8.02 (5H, m, Ph); Found C:56.2, H:6.1, N:4.0, S:9.8, $C_{16}H_{21}N_1O_5S$ requires C:56.6, H:6.2, N:4.1, S:9.5%.

1-[(Benzyloxycarbonylleucylglycyl)amino]-1-(2-phenylsulfone-1-oxoethyl)cyclopropane (**9**). The protected peptide (**8**) (2.4 g; 7.1 mmol) was dissolved in the minimum volume of methanol:ethyl acetate, (1:1 v/v) and cooled in an ice bath. The solution was stirred while conc. HCl was added in 1 ml portions until no starting material was left. The solvents were then removed and the product extracted into water from ethyl acetate. Evaporation of the water gave a white solid (1.0 g; 51%); m.p. 107-110°C; δ_H (250 MHz; CD_3OD) 1.55 (2H, t, 2xH on ring), 1.95 (2H, t, 2xH on ring), 4.90 (2H, s, CH_2SO_2), 7.65-7.95 (5H, m, Ph).

Benzyloxycarbonylleucylglycine (0.34 g; 0.91 mmol) in THF (5 ml:dry) was cooled in an ice/salt bath before adding N-methylmorpholine (0.10 ml; 0.91 mmol) followed by isobutylchlorocarbonate (0.12 ml; 0.91 mmol). The mixture was stirred for five minutes before adding a solution of the peptide sulfone (**8**) deprotected as above (0.25 g; 0.9 mmol), N-methylmorpholine (0.10 ml; 0.91 mmol) in DMF (4 ml:dry). The solution was stirred for six hours at room temperature, and the solvents then removed under reduced pressure. Silica gel chromatography (ethyl acetate:hexane, 80:20) gave the product (0.21 g; 43%) as white crystals, m.p. 139-141°C; δ_H (250 MHz; $CDCl_3$) 0.9 (6H, m, 2xCH_3), 1.25 and 1.60 (4H, m, cyclopropyl), 1.60 3H, m, C<u>H</u>C<u>H</u>$_2$), 3.7-4.1 (3H, m, NHC<u>H</u>CO plus NHC<u>H</u>$_2$CO), 4.47 (2H, s, CH_2S), 5.14 (2H, m, CH_2Ph), 7.33-7.90 (10H, m, 2xPh); Found C:60.0, H:6.4, N:7.7, S:5.8, $C_{27}H_{33}N_3O_7S$ requires C:59.6, H:6.1, N:7.7, S:5.9%.

1-[(Benzyloxycarbonyl)amino]-1-(2-phenylsulfone-1-hydroxyethyl) cyclopropane (**10**). The ketone (**9**) (171 mg; 0.31 mmol) was dissolved in THF (6 ml) and sodium borohydride (12 mg; 0.32 mmol) added slowly. Ater two hours the THF was removed, the product taken up in ethyl acetate and washed with water. The solution was dried (Na_2SO_4) and the solvent removed under reduced pressure to leave the product (170 mg; 99%); m.p. 59-61°C; δ_H (250 MHz; $CDCl_3$), 0.91 (6H, m, 2xCH_3), 1.20-1.69 (7H, m, $CHCH_2$ + cyclopropyl), 3.41-4.12 (6H, m, C<u>H</u>OH,CH_2S, NHC<u>H</u>CO, NHC<u>H</u>$_2$), 5.11 (2H, m, CH_2Ph), 7.34-7.95 (10H, m, 2xPh); Found C:59.0, H:6.8, N:7.3, S:5.4, $C_{27}H_{35}N_3O_7S$ requires C:59.4, H:6.5, N:7.7, S:5.8%.

Enzyme Assays. The enzyme assays were carried out by following changes in U.V. absorption using a Phillips Pye Unicam SP800 ultraviolet spectrometer at 254 nm and at high substrate concentrations reverse cell mode was used. Carboxypeptidase A was Bovine Pancreas, (Sigma Chemical Company). The substrate (Sigma) was hippuryl-L-phenylalanine. Stock solutions were made up for enzyme, substrate and inhibitors. The enzyme solution was made up in phosphate buffer (Na_2HPO_4, NaCl 5 mM at pH 7.0) and the reactions carried out in Tris/HCl buffer (Tris(hydroxymethyl)aminomethane) (0.1M Tris, 1M NaCl at pH 7.5). The concentrations were as follows:

5.5×10^{-6}M enzyme (11×10^{-3} ml enzyme made up to 1 ml);

50 mM substrate (in 0.01M NaOH);

20 mM inhibitor, dissolved in DMSO.

Molecular modelling was carried out using the Hyperchem suite of programmes based upon the references cited.

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