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The Specificity of the S_1 and S_2 Subsites of Elastase

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Esters of tetrapeptides of the general formula ethoxycarbonyl-prolyl-alanyl-X-Y where either X or Y was an alanine residue were synthesised and their cleavage by elastase studied.

It was found that variation of the alcohol moiety between methyl, cyclohexyl and nitrophenyl residues had no effect on the catalytic rate constant for cleavage of ethoxycarbonyl-prolyl-dialanyl-alanine esters demonstrating that acylation is much faster than deacylation for this system and also that non-productive binding is not kinetically significant.

The effect of changing the amino acid residue in position X was small compared with that of change in position Y. The presence of valine and serine residues in position Y produced the highest specificity constant but the highest catalytic rate constant was found for a leucine residue in this position. The results are discussed in terms of the binding of the substrate to the enzyme.

Pancreatic elastase has been the subject of several studies using oligopeptide substrates to determine its specificity. The active site has been shown to have at least six subsites, each capable of binding one amino acid residue [1,2]. Four of these subsites lie on the acyl side of the cleaved bond and are labelled S_1, S_2, S_3 and S_4 in order from the cleavage site, the other two lie on the amide side of the cleaved bond and are labelled S_1' and S_2' [3]. The substrate residues which fit into these sites are correspondingly labelled P_4, P_3, P_2, P_1, P_1', P_2', where the bond between residues P_1 and P_1' is cleaved by the enzyme.

One problem in the study of elastase specificity arises from the possibility that a small peptide substrate can bind non-productively to the enzyme. It has been demonstrated [4] that this possibility can be minimised by using peptides which contain appropriately situated proline residues. Examination of the elastase-catalysed hydrolyses of a number of tetrapeptide amides showed that proline would bind in the S_4 and S_2 subsites but not in the S_3 subsite, in particular Ac-Pro-Ala-Ala-Ala-NH_2 was cleaved only at the amide bond.

The aim of the present study was to determine the specificity of the S_1 and S_2 sites of elastase towards the deacylation part of the reaction pathway using methyl ester substrates. Non-productive binding was to be minimised by utilising proline as the P_4 residue with respect to the ester bond and the final choice fell upon the series of peptides EtOC-Pro-Ala-X-Y-OMe where either X or Y was alanine. The ethoxycarbonyl group was chosen for its ease of introduction and water solubility.

MATERIALS AND METHODS

Enzyme and Substrates

Elastase was prepared from pancreatin by the method of Shotton [5] and showed a single band on electrophoresis. The concentration of the enzyme in a stock solution was determined in the pH-stat at pH 8 by rate assay using Ac-Ala_3-OMe as substrate. The preparation and properties of this substrate have been described previously [6].

The synthesis of other substrates is described in the miniprint section at the end of the paper.

Kinetic Measurements

The hydrolysis of the nitrophenyl ester was followed by spectrophotometry using the method previously described [6].

The other hydrolyses were followed using a Radiometer pH-stat apparatus consisting of a TTT2 pH meter, ABU12 automatic burette and REA300 recorder. All measurements were made in a reaction

Abbreviations. Ac-, acetyl; Boc-, *tert*-butoxycarbonyl; EtOC-, ethoxycarbonyl; -OMe, methoxyl; -OtBu, *tert*-butoxyl; Z-, benzyloxycarbonyl. The symbols for amino acids conform to those proposed by the IUPAC-IUB Commission on Biochemical Nomenclature [see *Eur. J. Biochem. 27*, 201 – 207 (1972)]. All amino acids, except glycine, had the L configuration.

Enzymes. Elastase (EC 3.4.21.11); chymotrypsin (EC 3.4.21.1).

Table 1. *Kinetic parameters for tetrapeptides*
Reactions were carried out in 1 mM Tris pH 8.00, 0.05 M KCl, at 25 °C. Solvent concentration was 2.5% (v/v)

Tetrapeptide	k_{cat}	K_m	$10^{-3} \times k_{cat}/K_m$	Solvent
	s^{-1}	mM	$M^{-1} s^{-1}$	
EtOC-Pro-Ala-Gly-Ala-OMe	16.2	0.32	51	–
EtOC-Pro-Ala-Ala-Ala-OMe	94	0.079	1190	–
EtOC-Pro-Ala-Val-Ala-OMe	87	0.062	1400	acetonitrile
EtOC-Pro-Ala-Leu-Ala-OMe	21	0.013	1620	acetonitrile
EtOC-Pro-Ala-Ile-Ala-OMe	60	0.076	790	acetonitrile
EtOC-Pro-Ala-Thr-Ala-OMe	53	0.043	1230	–
EtOC-Pro-Ala-Lys-Ala-OMe · HCl	51	0.014	3640	–
EtOC-Pro-Ala-Phe-Ala-OMe	28	0.055	510	acetonitrile
EtOC-Pro-Ala-Tyr-Ala-OMe	19	0.009	2100	acetonitrile
EtOC-Pro-Ala-Trp-Ala-OMe	26	0.063	410	acetonitrile
EtOC-Pro-Ala-Pro-Ala-OMe	123	0.061	2020	–
EtOC-Pro-Ala-Glu-Ala-OMe	80	0.10	800	–
EtOC-Pro-Ala-Gln-Ala-OMe	41	0.015	2700	–
EtOC-Pro-Ala-Asp-Ala-OMe	34	0.048	710	–
EtOC-Pro-Ala-Asn-Ala-OMe	38	0.020	1900	–
EtOC-Pro-Ala-Ala-Gly-OMe	15.8	0.063	251	–
EtOC-Pro-Ala-Ala-Val-OMe	26	0.009	2890	ethanol
EtOC-Pro-Ala-Ala-Leu-OMe	250	0.33	760	acetonitrile
EtOC-Pro-Ala-Ala-Ile-OMe	7.0	0.17	41	acetonitrile
EtOC-Pro-Ala-Ala-Ser-OMe	116	0.030	3866	–
EtOC-Pro-Ala-Ala-Thr-OMe	43	0.077	560	acetonitrile
EtOC-Pro-Ala-Ala-Tyr-OMe	6.4	0.073	88	acetonitrile
EtOC-Pro-Ala-Ala-Trp-OMe	2.1	0.010	210	acetonitrile

vessel thermostatted at 25 °C which was continuously swept with a stream of nitrogen saturated with water vapour at 25 °C. The reactions were normally followed at pH 8.00 in 1 mM Tris (to stabilise the instrument) and 0.05 M potassium chloride.

A buffer solution containing the desired concentration of substrate was placed in the reaction vessel and allowed to equilibrate thermally before being titrated to pH 8.00 by the addition of sodium hydroxide solution (about 0.002 M). The amount of titrant added was noted in order to correct the initial concentration of substrate to the true value. A suitable volume of enzyme stock solution (which had been previously titrated to pH 8.00) was added and the reaction observed.

Plots which showed high curvature, usually those of lowest concentration, presented difficulties in determining the initial slopes by eye, particularly as pH-stats show a lag phase for the first 10–20 s after enzyme addition. The slopes in these cases were obtained from a backward extrapolation of later points by fitting them to a cubic equation using Chebyshev polynomials. The spontaneous hydrolysis rate of the substrate at pH 8.00 was determined and substracted from the observed initial slope; its value was always less than 50% of the total observed rate at any concentration. The values of K_m and k_{cat} for the substrate were determined from the initial rate measurements by the method of Wilkinson [7] using substrate concentrations between 0.2 K_m and 3 K_m. Enzyme concentrations were always in the range 0.1–0.01 μM.

RESULTS

The kinetic results obtained are given in Tables 1 and 2. The errors are ± 5% in k_{cat} and ± 10% in K_m except for those substrates with $K_m < 0.02$ mM where errors in K_m will be larger because 0.02 mM represents the lowest substrate concentration for which reproducible data could be obtained. The nitrophenyl ester, the results for which are given in Table 2, could not be studied at pH 8 because its spontaneous hydrolysis rate in several pH 8 buffers was always too large.

Reactions were always followed in purely aqueous media where substrate solubility allowed, but in some cases acetonitrile and in one case ethanol had to be used as cosolvent to maintain solubility. We have investigated the effect of acetonitrile on the elastase-catalysed hydrolysis of Ac-Ala$_3$OMe and found that it behaves as a competitive inhibitor, $K_i \approx 0.5$ M (≈ 2.5% v/v in water). Hence substrates measured in this solvent will have a K_m twice the value in water alone, and a halved value for the specificity constant k_{cat}/K_m. The effect of ethanol as cosolvent will be to reduce the observed catalytic rate constant compared to that for water since ethanol will complete with water as a nucleophile for the acyl enzyme. If the par-

Table 2. *Effect of variation of alcohol moiety in esters of EtOC-Pro-Ala-Ala-Ala-OH*
Measurements were either by the pH-stat method (pH) or the spectrophotometric method (spectro.) with 2.5% acetonitrile present. The buffer was either 1 mM Tris + 0.05 M KCl (Tris/KCl), 0.05 M phosphate (phosphate) or 1 mM HCl + 0.05 M KCl (HCl/KCl)

Ester	Method	pH	k_{cat}	K_m	Buffer
			s^{-1}	mM	
Cyclohexyl	pH	8.00	89	0.095	Tris/KCl
Methyl	pH	8.00	94	0.17	Tris/KCl
4-Nitrophenyl	spectro.	6.10	59	0.12	phosphate
Methyl	pH	6.10	61	0.3	Tris/KCl
4-Nitropheynl	spectro.	4.50	4.2	0.16	HCl/KCl
Cyclohexyl	pH	4.50	4.2	0.21	HCl/KCl
Methyl	pH	4.50	4.0	0.14	HCl/KCl

tition factor is the same as that determined for methanol on chymotrypsin [8] then the effect will be to halve the observed k_{cat} value since at 2.5% ethanol the rate of conversion to acid and to ethyl ester will be approximately equal.

DISCUSSION

Multiple Binding of Substrates

One problem in the study of elastase specificity is that a particular oligopeptide can bind in more than one contiguous set of subsites on the enzyme. This has been observed using oligopeptide amides which exhibit cleavage by elastase at more than one site [2]; for example Ac-Ala_4-NH_2 was cleaved to give both Ac-Ala_4-OH and Ac-Ala_3-OH. However Ac-Ala_4-OMe was cleaved to give solely Ac-Ala_4-OH because esterolysis is much faster than the hydrolysis of peptide bonds with which it competes. Hence, for ester substrates, such alternative binding modes will be non-productive and will not give rise to detectable products.

If it is assumed that elastase follows the normal hydrolytic pathway for serine proteinases, and that there is a single non-productive complex ES* [Eqn (1)], it is

$$\begin{array}{ccccccc} E & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E \cdot S & \xrightarrow{k_2} & ES^1 & \xrightarrow{k_3} & E + P_2 \\ {\scriptstyle +N k_4}\uparrow\downarrow{\scriptstyle k_{-4}} & & & & {\scriptstyle +P_1} & & \\ E \cdot S^* & & & & & & \end{array}$$

possible to derive, making the usual assumptions and using steady-state kinetics (see Appendix) that the rate of reaction is given by Eqn (2):

$$v = \frac{k_2 [E]^\circ [S]}{K_s + [S]\left(1 + \dfrac{k_2}{k_3} + \dfrac{K_s}{K_N}\right)} \cdot \left(K_s = \frac{k_{-1} + k_2}{k_1}, K_N = \frac{k_{-4}}{k_4}\right). \quad (2)$$

This implies that the usual steady-state parameters, k_{cat} and K_m, are given by

$$k_{cat} = \frac{k_2}{1 + \dfrac{k_2}{k_3} + \dfrac{K_s}{K_N}}, \quad K_m = \frac{K_s}{1 + \dfrac{k_2}{k_3} + \dfrac{K_s}{K_N}}$$

so that $k_{cat}/K_m = k_2/K_s$, the specificity constant.

In the hydrolysis of ester substrates by serine proteinases it is usually found that deacylation is rate-determining; we shall demonstrate later that this is true for elastase. In this case $k_2 \gg k_3$ and, if we also assume that K_N is comparable with K_s, then $k_{cat} = K_m = k_3 K_s/k_2$. It is apparent therefore that non-productive binding could account for as much as 60% of the binding of the substrate to the enzyme and still show no detectable effect on the kinetics of ester hydrolysis providing $k_2/k_3 > 20$. However such non-productive binding would show a profound effect on the kinetics of amide hydrolysis where $k_2 \ll k_3$.

We attempted to eliminate non-productive binding as far as possible in the present experiment by including a proline in the N-terminal position of the tetrapeptide chain. Since proline will not bind in the S_3 subsite [4], the only possible non-productive binding mode which allows the ethoxycarbonyl residue and the four amino-acid residues to be bound to an enzyme site is the one which places the proline in the S_2 subsite and so puts the four amino-acid residues in the S_2, S_1, S_1' and S_2' subsites.

The Rate-Determining Step in Elastase-Catalyzed Hydrolysis

It has been argued previously by two sets of workers [9, 10] that k_2 may well be effective in determining the rate of hydrolysis of ester substrates by elastase. These arguments were based on observations of hydrolyses of esters of N-protected amino acids. We have studied the hydrolysis of three esters of EtOC-Pro-$(Ala)_3$-OH at different pH values and find no effect of alcohol group on rate; these results are

recorded in Table 2. The identity of the results for 4-nitrophenyl and methyl esters at pH 6 is particularly indicative that k_2 is sufficiently large to have no effect on the observed k_{cat} since nitrophenyl esters would be expected to show a much higher acylation rate than methyl esters. We suspect that the previously observed results may have been due to large changes in the extent of non-productive binding.

The identity observed in the values of k_{cat} also indicates that, as suggested above, non-productive binding is not sufficiently large to compete kinetically with productive binding. The alcohol groups used are sufficiently different in structure to cause wide variation in the amount of non-productive binding.

Specificity of the S_1 Subsite

It can be seen from the rate constants given in Table 1 for the peptides EtOC-Pro-Ala-Ala-X-OMe that the peptide with $P_1(X)$ = serine has the highest specificity constant though this is approached by that for the peptide with P_1 = valine (and may be surpassed by it if the reduction in the observed k_{cat} due to the ethanol present is taken into account). Previous studies of elastase specificity have reported cleavage after one of two serine residues in the insulin A chain [11], and after one of the three serine residues in the ribonuclease S-peptide [12].

A skeletal model of elastase was built to the published coordinates [13] to which models of the substrates were fitted using the suggestions derived from crystallographic [14] and space-filling-model studies [15]. Fitting of a peptide with P_1 = serine into this model suggests that the binding may be aided by the formation of a hydrogen bond from the serine hydroxyl to the carbonyl group of Ser-214 of the enzyme. There is also in this region of the molecule a hole which, while not large enough to accomodate aromatic residues, could (and apparently does), accomodate aliphatic residues. The high k_{cat} value observed for the P_1 = leucine peptide suggests that binding of leucine in this area is possible though perhaps in a manner which leads to either a strained acyl-enzyme or a strained tetrahedral intermediate, so leading to a greatly enhanced deacylation rate. If strain is present then it might be expected that the rate of formation of acyl-enzyme would be slowed. In a study [16] of the hydrolysis of succinyl-tripeptide nitroanilides it was found that the k_{cat} values for peptides with P_1 = leucine (3.8 s^{-1}) and P_1 = valine (1.3 s^{-1}) were significantly slower than for P_1 = alanine (23.6 s^{-1}) which would support the hypothesis of some strain in the P_1 = leucyl enzyme.

The low K_m value for the P_1 = valine peptide suggests that this may be a case where non-productive binding is significant in causing a decrease in the observed K_m value, and so in the k_{cat} value; indeed it is possible that the true value of k_3 is similar to that found for P_1 = leucine. The need for a side chain of some sort on the P_1 residue is indicated by the low specificity for P_1 = glycine. The side chain must lock the acyl enzyme into a reactive conformation with respect to His-57 on the enzyme to allow hydrolysis and its absence results in a raising of the entropy of activation of the reaction and so reduces the rate.

The two peptides where P_1 is aromatic and that where P_1 = isoleucine were poor substrates and it is probable that they adopt a totally different conformation on the enzyme with the side chain pointing out into the solvent. The P_1 = tryptophanyl peptide behaves as a competitive inhibitor against Ac-$(Ala)_3$-OMe (K_i = 0.083 mM).

Specificity of the S_2 Subsite

As can be seen from the data in Table 1, this subsite shows little specificity supporting the hypothesis that the side chain on the P_2 residue points out into the solvent [14, 15].

The k_{cat} for the P_2 = prolyl peptide (132 s^{-1}) is somewhat higher than that for the P_2 = alanyl peptide (94 s^{-1}). Since we have already demonstrated that the rate constant for the peptide with P_2 = alanyl is unlikely to be affected by non-productive binding, this must be due to a conformational effect of the presence of the proline residue on the catalytic site. It is probable that the proline ring is pushed out of place relative to other residues by the side chain of Thr-41, and so distorts the position of the P_1 residue in a favourable sense for hydrolysis.

APPENDIX

From Eqn (1) in the main text the following equations can be obtained, assuming $[S] \gg [E]^\circ$:

$$[E]^\circ = [E] + [E \cdot S] + [E \cdot S^*] + [ES^1] \quad (3)$$

$$\frac{d[E \cdot S^*]}{dt} = k_4 [E][S] - k_{-4} [E \cdot S^*] \quad (4)$$

$$\frac{d[E \cdot S]}{dt} = k_1 [E][S] - (k_{-1} + k_2)[E \cdot S] \quad (5)$$

$$\frac{d[ES^1]}{dt} = k_2 [E \cdot S] - k_3 [ES^1]\,. \quad (6)$$

Putting the left-hand sides of Eqns (4–6) equal to zero gives

$$[E \cdot S] = \frac{k_3}{k_2} [ES^1] \quad (7)$$

$$[E] = \frac{k_{-1} + k_2}{k_1} \cdot \frac{[E \cdot S]}{[S]} = \frac{K_s}{[S]} \cdot [E \cdot S]$$

$$= \frac{k_3}{k_2} \cdot \frac{K_s}{[S]} \cdot [ES^1].$$

where

$$K_s = \frac{k_{-1} + k_2}{k_1} \tag{8}$$

$$[E \cdot S^*] = \frac{k_4}{k_{-4}} [E][S] = \frac{1}{K_N} [E][S]$$

$$= \frac{k_3}{k_2} \cdot \frac{K_s}{K_N} [ES^1].$$

where

$$K_N = \frac{k_{-4}}{k_4} \tag{9}$$

Substituting into Eqn (3) gives

$$[E]^\circ = \left(\frac{k_3}{k_2} \cdot \frac{K_s}{[S]} + \frac{k_3}{k_2} + \frac{k_3}{k_2} \cdot \frac{K_s}{K_N} + 1 \right) [ES^1]$$

$$= \frac{k_3}{k_2} \left[\frac{K_s}{[S]} + \left(1 + \frac{k_2}{k_3} + \frac{K_s}{K_N} \right) \right] [ES^1].$$

But

$$v = k_3 [ES^1] = \frac{k_2 [E]^\circ [S]}{K_s + [S] \left(1 + \frac{k_2}{k_3} + \frac{K_s}{K_N} \right)}.$$

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Supplementary material to

The Specificity of the S_1 and S_2 Subsites of Elastase

by

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Synthesis of Substrates

N-Benzyloxycarbonyl protected methyl esters of dipeptides were prepared by coupling of a *N*-benzyloxycarbonyl amino acid with an amino acid methyl ester using dicyclohexylcarbodiimide in ethyl acetate solution. Where necessary amine side chains were protected by *tert*-butoxycarbonyl groups and carboxylic acid side chains as *tert*-butyl esters. These were removed after the synthesis of the tetrapeptides by treatment with trifluoroacetic acid at room temperature. The properties of the dipeptide derivatives synthesised in this manner are given in Miniprint Table 1. These and all other compounds synthesised in this work had satisfactory analyses.

Synthesis of EtOC-Pro-Ala-Ala-X-OMe

Preparation of Ethoxycarbonyl-L-Proline. L-Proline (11.5 g) and sodium hydrogen carbonate (21 g) were added to water (120 ml) and the suspension was stirred vigorously. Ethyl chloroformate (10 ml) was added in 3 portions over 30 min after which the stirring was continued for 1 h. Excess chloroformate was removed by extraction with ether and the aqueous layer acidified to pH 3. Extraction with ethyl acetate followed by drying and evaporation gave an oil which was recrystallised from diethyl ether/pentane to give *N*-ethoxycarbonyl-L-proline (13.0 g), m.p. 48-49 °C $[\alpha]_D^{20}$ - 54.1° (1%, $CHCl_3$).

Preparation of EtOC-Pro-Ala-OH. Ethoxycarbonyl proline (7.48 g) and isobutylchloroformate (5.25 ml) were dissolved in dichloromethane (60 ml) and the solution cooled to -5 °C. Triethylamine (5.54 ml) was added dropwise with stirring while the temperature was maintained at -5 °C. The mixture was stirred for 15 min and a precooled solution of L-alanine benzyl ester (obtained by treating the toluenesulphonate (14.05 g) with potassium carbonate solution and extracting with dichloromethane) was added. After stirring for 20 min at -5 °C, 2h at room temperature, the solution was washed successively with 1 M hydrochloric acid and saturated sodium hydrogen carbonate solution and dried over sodium sulphate. Evaporation of the solvent gave an oil which was crystallised from ether/pentane to give EtOC-Pro-Ala-OBz (10.2 g) m.p. 77-78 °C, $[\alpha]_D^{20}$ -78.7° (1%, chloroform).

The benzyl ester (10.23 g) was dissolved in methanol (80 ml) and 5% palladium in charcoal (0.1 g) added. Hydrogenation for 6h at atmospheric pressure resulted in uptake of the theoretical amount of hydrogen. The solution was filtered and the methanol evaporated to give a white solid which was recrystallised from ether/hexane to give EtOC-Pro-Ala-OH (7.0 g) m.p. 151-152 °C, $[\alpha]_D^{20}$ -88.1° (1%, chloroform).

Preparation of EtOC-Pro-Ala-Ala-OH. EtOC-Pro-Ala-OH (1.29 g), L-alanine benzyl ester p-toluenesulphonate (1.76 g), N-hydroxysuccinimide (0.7 g) and *N*-methylmorpholine (0.56 ml) were dissolved in tetrahydrofuran (15 ml) and the solution cooled to 2 °C. Dicyclohexylcarbodiimide (1.04 g) was added to the stirred solution which was allowed to warm to room temperature and stirred for 16 h. Ethyl acetate (50 ml) was added to the mixture which was filtered to remove dicyclohexylurea, and then treated as above to give an oil which crystallised very slowly from 2-propanol/ether to give EtOC-Pro-Ala-Ala-OBz (1.20 g) m.p. 140-141 °C, $[\alpha]_D^{20}$ -79.2° (1%, chloroform). Hydrogenolysis of this ester (0.84 g) as above, followed by recrystallisation of the product from ether gave EtOC-Pro-Ala-Ala-OH (0.61 g) m.p. 175-176 °C, $[\alpha]_D^{20}$ - 58.9° (1% tetrahydrofuran).

Preparation of EtOC-Pro-Ala-Ala-X-OMe. The preparation of EtOC-Pro-Ala-Ala-OMe is given as an example. EtOC-Pro-Ala-Ala-OH (0.33 g), glycine methyl ester hydrochloride (0.126 g) and *N*-methylmorpholine (0.111 ml) were dissolved in N,N-dimethylformamide (10 ml). N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.25 g) was added and the solution left at room temperature for 16 hrs. The whole was evaporated to dryness on a rotary evaporator and the residue taken up in dichloromethane. This solution was washed once with 50% saturated sodium chloride solution, to remove amine hydrochloride, and then dried over sodium sulphate. Evaporation of the solvent and crystallisation from chloroform/petrol gave EtOC-Pro-Ala-Ala-Gly-OMe (0.24 g).

Preparation of EtOC-Pro-Ala-X-Ala-OMe

The preparation of EtOC-Pro-Ala-Gly-Ala-OMe is given as an example.

Preparation of EtOC-Pro-Ala-OSu. EtOC-Pro-Ala-OH (5.16 g) and *N*-hydroxy succinimide (2.31 g) were dissolved in a 1:1 mixture of dioxan and ethyl acetate (50 ml). The solution was cooled to 2 °C and dicyclohexylcarbodiimide (4.12 g) added. The mixture was stirred at 0 °C for 1h and at room temperature for 1h and the solvent evaporated. The residue was taken up in ethyl acetate and washed as described for the benzyl esters above. The product was obtained on evaporation of the solvent as an oil which crystallised from 2-propanol to give EtOC-Pro-Ala-OSu (6.1 g), m.p. 98-100 °C, $[\alpha]_D^{20}$ + 111° (1%, ethanol).

Preparation of EtOC-Pro-Ala-Gly-Ala-OMe. Z-Gly-Ala-OMe (0.3 g) was hydrogenolysed over 5% paddadium on charcoal in methanol (20 ml) in the presence of one equivalent of acetic acid. The catalyst was removed by filtration and EtOC-Pro-Ala-OSu (0.178 g) and *N*-methylmorpholine (0.056 ml) added to the solution which was kept at room temperature for 24 hr. The methanol was evaporated and the residue worked up as described for EtOC-Pro-Ala-Ala-Gly-OMe giving the product as an oil which crystallised from ethyl acetate to give EtOC-Pro-Ala-Gly-Ala-OMe (0.130 g).

The properties of all the tetrapeptide methyl esters are given in Miniprint Table 2.

Miniprint Table 1. Properties of dipeptides.

Peptide	Observed value for			Literature value for			Reference [a]
	Melting Point	$[\alpha]_D^{20}$		Melting Point	$[\alpha]_D^{20}$		
	°C	°	(%,solvent)	°C	°	(%,solvent)	
Z-Gly-Ala-OMe	90-91	-8.5	(1,ethyl acetate)	62-64	-8.1	(1,ethyl acetate)	1
Z-Ala-Ala-OMe	105-106	-15.0	(1,chloroform)	105-107	-16	(1,chloroform)	2
Z-Val-Ala-OMe	156-157	-49.4	(1,methanol)	162-163	-49.4	(1,methanol)	3
Z-Leu-Ala-OMe	96-97	-43.1	(1,ethanol)	95-96	-38.0	(1,ethanol)	4
Z-Ile-Ala-OMe	164-166	-46.1	(4,methanol)	171-175	-44	(4,methanol)	5
Z-Thr-Ala-OMe	128-129	-33.8	(1,methanol)	126-128	-33.6	(1,methanol)	6
Z-(ε-Boc)Lys-Ala-OMe	118-119	- 9.3	(1,chloroform)	-	-	-	-
Z-Phe-Ala-OMe	111-112 [b]			132-133	-20.4	(1,dimethylformamide)	7
	131-133 [b]	-20.9	(1,dimethylformamide)				
Z-Tyr-Ala-OMe	136-137 [b]			135-136	-18.5	(1,methanol)	8
	157-159 [b]	-18.7	(1,methanol)	157-159		-	9
Z-Trp-Ala-OMe	67-68 [b]						
	127-128	- 7.8	(1,chloroform)	131-133		-	9
Z-Pro-Ala-OMe	76-78	-76.7	(1,ethanol)	79-80	-74	(2,ethanol)	10
Z-Glu(OtBu)-Ala-OMe	100-102	-13.4	(1,dimethylformamide)	102	-15.5	(1,dimethylformamide)	11
Z-Gln-Ala-OMe	210-211	-10.8	(1,dimethylformamide)	210-211	-12.2	(0.4,dimethyl-formamide)	12
Z-Asp(OtBu)-Ala-OMe	88-89	+26.5	(1,chloroform)	-		-	-
Z-Asn-Ala-OMe	205-206	-7.7	(3,dimethylformamide)	200-201	- 7.0	(3,dimethylformamide)	13

[a] The absence of a reference indicates a new compound.

[b] Each of these compounds melted initially over the lower temperature range but resolidified on maintaining the temperature just above the melting point and melted finally over the higher range.

Miniprint Table 2. Properties of tetrapeptides.

All concentrations for rotations were 1% w/v.

Peptide	Recrystallisation Solvent	Melting Point	$[\alpha]_D^{20}$	
		°C	°	(solvent)
EtOC-Pro-Ala-Gly-Ala-OMe	Ethyl acetate	204-206	-68.1	(water)
EtOC-Pro-Ala-Ala-Ala-OMe	Ethyl acetate	229-230	-103	(water)
EtOC-Pro-Ala-Val-Ala-OMe	Ethyl acetate/ether	229-230	-100	(chloroform)
EtOC-Pro-Ala-Leu-Ala-OMe	Ethyl acetate	242-243	-79.5	(acetonitrile)
EtOC-Pro-Ala-Ile-Ala-OMe	Ethyl acetate	228-229	-94.1	(chloroform)
EtOC-Pro-Ala-Thr-Ala-OMe	Ethyl acetate/ethanol	237-238	-71.1	(water)
EtOC-Pro-Ala-(ε-Boc)Lys-Ala-OMe	Ethyl acetate	188-189	-64.2	(chloroform)
EtOC-Pro-Ala-Lys-Ala-OMe.HCl	Ethanol/ether	189-191	-77.3	(water)
EtOC-Pro-Ala-Phe-Ala-OMe	Ethyl acetate/ether	159-161	-75.8	(acetonitrile)
EtOC-Pro-Ala-Tyr-Ala-OMe	Ethyl acetate/ethanol	232-234	-95.2	(acetonitrile)
EtOC-Pro-Ala-Trp-Ala-OMe	Ethyl acetate/ether	124-125	-65.1	(acetonitrile)
EtOC-Pro-Ala-Pro-Ala-OMe	Chloroform/petrol	128-130	-147	(chloroform)
EtOC-Pro-Ala-Glu(OtBu)-Ala-OMe	Chloroform/petrol	170-172	-47.4	(dimethylformamide)
EtOC-Pro-Ala-Glu-Ala-OMe	Ether	195-196	-79.5	(water)
EtOC-Pro-Ala-Gln-Ala-OMe	Ethyl acetate	263-265	-89.0	(water)
EtOC-Pro-Ala-Asp(OtBu)-Ala-OMe	Chloroform/petrol	158-159	-64.4	(chloroform)
EtOC-Pro-Ala-Asp-Ala-OMe	Chloroform/petrol	179-180	-77.6	(water)
EtOC-Pro-Ala-Asn-Ala-OMe	Ethyl acetate/ethanol	222-224	-59.4	(water)
EtOC-Pro-Ala-Ala-Gly-OMe	Chloroform/petrol	201-202	-86.1	(water)
EtOC-Pro-Ala-Ala-Val-OMe	Ethyl acetate	205-208	-96.2	(ethanol)
EtOC-Pro-Ala-Ala-Leu-OMe	Ethyl acetate/ether	176-177	-99.0	(ethanol)
EtOC-Pro-Ala-Ala-Ile-OMe	Ethyl acetate	198-200	-66.3	(acetonitrile)
EtOC-Pro-Ala-Ala-Ser-OMe	Ethanol/ether	251-255	-98.0	(water)
EtOC-Pro-Ala-Ala-Thr-OMe	Ethyl acetate/ethanol	261-263	-101	(acetonitrile)
EtOC-Pro-Ala-Ala-Tyr-OMe	Ethyl acetate	226-229	-31.1	(acetonitrile)
EtOC-Pro-Ala-Ala-Trp-OMe	Ethyl acetate/ethanol	228-229	-29.5	(acetonitrile)

Preparation of EtOC-Pro-Ala-Ala-Ala-OH cyclohexyl ester

Alanine cyclohexyl ester hydrochloride Alanine (3.56 g) and cyclohexanol (4.16 ml) were mixed, saturated with dry hydrogen chloride and the mixture kept at room temperature for 48 h. Addition of diethyl ether precipitated the ester hydrochloride as an oil.

Alanine cyclohexyl ester The alanine ester hydrochloride (1 g) was suspended in ethyl acetate and extracted with saturated potassium carbonate solution. The organic layer was dried over ethyl acetate and evaporated to give alanine cyclohexyl ester as an oil.

EtOC-Pro-Ala-Ala-Ala-OH, cyclohexyl ester Alanine cyclohexyl ester (0.425 g) and EtOC-Pro-Ala-Ala-OH (0.82 g) were dissolved in dichloromethane (15 ml) and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.62 g) added. The mixture was kept overnight at room temperature and the product obtained as an oil which crystallised from diethyl ether to give EtOC-Pro-Ala-Ala-Ala-OC$_6$H$_{11}$ (0.52 g) m.p. 175-176 °C, $[\alpha]_D^{20}$ -83.6° (1%, ethanol).

Preparation of EtOC-Pro-Ala-Ala-Ala-ONp Alanine nitrophenyl ester hydrobromide 14 (0.291 g) and EtOC-Pro-Ala-Ala-OH (0.335 g) were dissolved in tetrahydrofuran at 0 °C. N-Methyl morpholine (0.111 ml) and dicyclohexylcarbodiimide (0.210 g) were added and the mixture kept overnight at room temperature. The product was obtained by the usual procedure as an oil which crystallised from 2-propanol/diethyl ether to give EtOC-Pro-Ala-Ala-Ala-ONp (0.21 g) m.p. 162-164 °, $[\alpha]_D^{20}$ -77.5°.

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