Selectivity and Specificity in Substrate Binding to Proteases: Novel Hydrolytic Reactions Catalysed by α-Chymotrypsin Suspended in Organic Solvents with Low Water Content and Mediated by Ammonium Hydrogen Carbonate

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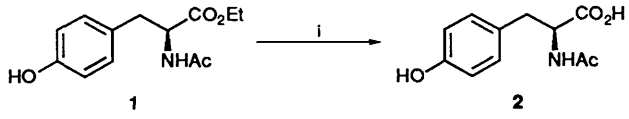
α-Chymotrypsin suspended in organic solvents with low water content catalysed hydrolytic reactions in the presence of ammonium hydrogen carbonate. Molecular modelling studies were carried out and structure–reactivity relationships were established by studying the hydrolysis of amino acid derivatives and analogues. The enzyme was found to be stereoselective with respect to the hydrolysis of L-amino acid derivatives, but no stereoselectivity was observed when α-hydroxy esters were used as substrates. A general procedure for the resolution of aromatic amino acid esters is given. The results are interpreted in terms of molecular modelling based on X-ray crystallographic data and literature data.

The primary role *in vivo* of chymotrypsin is to catalyse the hydrolysis of amide bonds of proteins adjacent to the carbonyl groups of aromatic L-amino acid residues such as those of phenylalanine, tyrosine and tryptophan. Most of the specificity data relevant to the use of chymotrypsin for resolution and in asymmetric synthesis have come from studies with ester and amide substrates.[1] Esters are hydrolysed much more rapidly than the corresponding amides. As a result, the structure–specificity relationships of the enzyme have been largely developed by using ester substrates in aqueous medium. On transition from an aqueous to an organic environment the enzyme usually exhibits altered properties.[2] The study of these provides valuable insights into the mechanistic and structural properties of the enzyme. The altered properties also make it possible to carry out new reactions which cannot be made to take place in water. Our previous studies dealing with the application of powdered α-chymotrypsin suspended in an organic solvent have already shown that the enzyme structure must have been modified, as it was possible, for example, to use D-amino acid nucleophiles in peptide synthesis.[3] However, the overall impression was that the enzyme seemed to be even more restricted in terms of specificity than when operating in a purely aqueous environment.

In order to establish structure–reactivity relationships, hydrolytic reactions provide a relatively simple experimental system because only part of the active site is involved. However, although chymotrypsin is able to hydrolyse selected substrates when suspended in dichloromethane containing 0.25% water,[4] the reactions are so slow, even with good substrates such as tyrosine derivatives, that this approach is impracticable for the screening of a large number of substrates.

This difficulty was overcome when a novel observation was made during the study of the potentially stereoselective enzymatic synthesis of primary amides. Primary amides can be synthesized easily according to a method described by Muramatsu[5] in which a coupling reagent is used in organic solvents containing ammonium hydrogen carbonate. Ammonium hydrogen carbonate acts as an efficient ammonia donor in organic solvents, and it was considered that it might therefore be used in an enzymatic reaction as a nucleophile, the coupling reagent being substituted by the enzyme (Scheme 1).

When the reaction depicted in Scheme 1 was carried out on the tyrosine derivative **1** using five equivalents of ammonium hydrogen carbonate, the isolated product was found to be not the desired amide Ac-L-TyrNH$_2$ but the acid, Ac-L-TyrOH **2**. This system for carrying out hydrolytic reactions in a medium of low water content proved to be a good vehicle for structure–activity studies. Active site mapping was possible and valuable information about the structure of the enzyme in the organic solvent could be obtained through screening simple substrates.



1 2

Scheme 1 *Reagents:* i, α-chymotrypsin, CH_2Cl_2–0.2% H_2O, NH_4HCO_3

Recent findings indicate that the essential water associated with enzymes can be substituted to some degree by other compounds.[6] When water is stripped from the enzyme by the solvent, it is suggested that areas of the protein which normally interact with water become exposed. This exposure is considered to have a detrimental effect on activity. If this is the case, then addition of compounds which can mimic the interaction of water with the protein (*e.g.* by forming hydrogen bonds) should to some degree restore the activity. Klibanov reported that the presence of 1% formamide increased the activity of alcohol dehydrogenase in butyl acetate containing 0.4% water.[6] This strategy was used for enzyme-catalysed peptide synthesis *via* segment condensation in the presence of water mimics.[7]

In an attempt to elucidate the nature of the ammonium hydrogen carbonate effect, several control experiments were carried out. Thus, no hydrolysis of Ac-L-TyrOEt **1** was observed in the absence of the added salt. Also, no reaction took place in the absence of the enzyme, or of the added water. In order to estimate the effect of the salt on the protein structure, experiments were carried out under anhydrous conditions. The model reaction was the synthesis of the dipeptide Ac-L-Tyr-L-PheNH$_2$ catalysed by chymotrypsin suspended in anhydrous dichloromethane containing three equivalents of ammonium hydrogen carbonate. After three days at room temperature, the desired dipeptide was isolated in 83% yield. The remaining starting material, Ac-L-TyrOEt, was recovered (84% conversion) together with traces of the hydrolysis product, Ac-L-TyrOH, which was detected by thin-layer chromatography. Although the reaction proceeded at a rate several orders of magnitude lower than the reaction with 0.25% water,[3] it proved nonetheless that peptide synthesis under anhydrous conditions was possible.

The effect was highly specific to ammonium hydrogen

Table 1 Effect of the nature of the salt on the hydrolysis of ethyl *N*-acetyl-L-tyrosinate by α-chymotrypsin suspended in dichloromethane

Salt	Yield (%)	Reaction time (h)
none	0	48
NH_4HCO_3	100	12
$NaHCO_3$	10	48
$(NH_4)_2SO_4$	10	48
$NH_4H_2PO_4$	10	48
formamide	10	48

Table 2 Hydrolysis of aromatic amino acid esters by a suspension of α-chymotrypsin in dichloromethane

Substrate	Reaction time (h)	Yield (%) Acid	Ester	ee (%) (unhydrolysed ester)
Ac-L-TyrOEt **1**	8	100 **2**	—	—
Ac-L-PheOEt **3**	12	100 **4**	—	—
Ac-L-TrpOEt **5**	21	45 **6**	—	—
Ac-L-PheOEt **7** + Ac-D-PheOEt **8**	18	50 **4**	50 **8**	100
L-TyrOEt **9**	12	100 **10**	—	—
L-TyrOMe **11**	15	100 **10**	—	—
DL-TyrOMe **12**	15	41 **10**	50 **13**	66[a]
L-PheOEt **14**	36	100 **15**	—	—
L-PheOMe **16**	48	100 **15**	—	—

[a] This value corresponds to an ee of 100% if hydrolysis had proceeded to 50%.

carbonate, as can be seen by comparison with the effect of other inorganic salts (Table 1). Two explanations can be proposed to explain the effect. Ammonium hydrogen carbonate shows a slight solubility in the organic phase.[5] If the theory of the stripping of water from the enzyme by the solvent is correct, dissolution of the salt in the organic phase could decrease the overall solubility of water in the organic solvent, thereby making it more available for hydrolysis. Furthermore, if the salt limits the solubility of water in the organic phase, the essential water will be conserved, and the enzyme will then exhibit some activity even under anhydrous conditions, as shown by its ability to catalyse the synthesis of dipeptides in anhydrous dichloromethane (see above). The second possibility could be a binding effect of the salt. By replacing water molecules on the protein molecule, the salt might help to keep the enzyme in its active form. However, the absence of an effect with formamide (Table 1) suggests that this could not be attributable solely to hydrogen bonding. A more specific effect might be envisaged if, by binding to the active site, the salt is able to stabilise the enzyme in its active conformation.

A further attribute of the system is illustrated by the results shown in Table 2 which demonstrate that the system can be used for the resolution of racemates of amino acid derivatives. Tyrosine esters appear to be the best substrates for hydrolysis, far better than the phenylalanine and tryptophan equivalents. Molecular modelling studies were used to gain an insight into the origins of these differences. Chymotrypsin has been exceptionally well studied by X-ray crystallography. High resolution structures of α-chymotrypsin,[8] γ-chymotrypsin[9] and the ovomucoid-α-chymotrypsin complex[10] are available. In the following discussion, favourable binding of the oxyanion intermediate is assumed to be the key factor in determining overall rates of the reaction catalysed by chymotrypsin. It should be noted that in all of the molecular modelling studies described below, the peptide backbone of the protein has been treated as a rigid entity. Relaxation of side-chain conformations was also disallowed, with one exception (Trp-215).

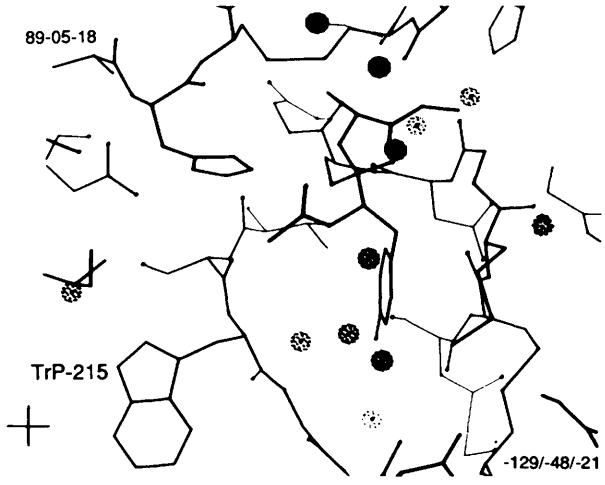


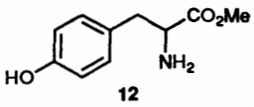
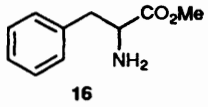
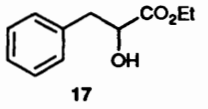
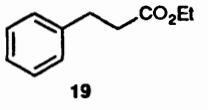
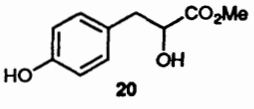
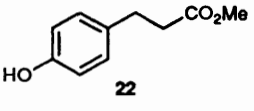
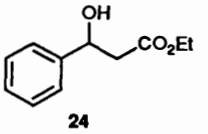
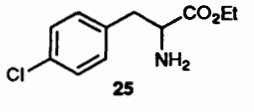
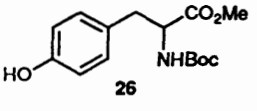
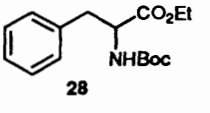
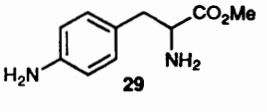
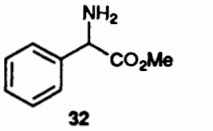
Fig. 1 Covalently bound tetrahedral intermediate Ac-L-Tyr-L-AlaOMe with inclusion of water molecules

Fig. 1 shows the covalently bound tetrahedral intermediate from Ac-L-Tyr-L-AlaNH$_2$ with inclusion of the water molecules present in the free enzyme. The oxyanion replaces one water molecule. The tyrosine side-chain of the substrate must replace one water molecule in the specificity pocket, whereas the other four conserved water molecules in the specificity pocket may simply rearrange themselves so as to maintain the hydrogen bonding network. The very slow hydrolysis of the tryptophan ester **5** (Table 2) cannot be readily explained. On bonding, the indole side-chain will displace the same number of water molecules as the tyrosine and phenylalanine derivatives. In water, the indolyl residue of tryptophan affords a near perfect fit to the specificity pocket (Ac-L-TrpOMe: K_M = 0.095 mmol dm^{-3} and $k_{cat}/K_M = 2.9 \times 10^5$ mol^{-1} dm^3 s^{-1}). For *N*-acetyl-L-tryptophanamide, *N*-acetyl-L-tyrosinamide and *N*-acetyl-L-phenylalaninamide respectively, k_{cat}/K_M(rel) = 5.5:3.5:1.[11] To explain the low reactivity of tryptophan esters, one could postulate that the three dimensional structure of the protein might have been altered by the new organic environment. If such modifications occurred near the active site, the binding of the bulkier tryptophan derivative would be more affected than the monocyclic side-chains of tyrosine and phenylalanine. Without knowing the actual structure of the enzyme in an organic solvent, no conclusive answer can be provided. However, if any conformational changes do occur, they will have to affect the binding segment which is constituted by the hydrophobic cavity with the Met-192 residue as the lid.

As indicated in Table 2, there is no need for a protecting group on the α-amino moiety of the substrate, as observed for aqueous systems.[12] Unprotected amino acid esters were readily hydrolysed, again with a marked preference for tyrosine derivatives (Tables 2, 3).

The ester functionality of the substrate in chymotrypsin-catalysed hydrolyses exhibits a limited influence on the reaction rates. Ethyl esters appear to be better than their methyl counterparts. The difference between methyl and ethyl esters becomes very noticeable for the phenylalanine derivatives. As shown in Table 2, resolution of racemic amino acid esters was possible. The L-enantiomer of the substrate **7** (Table 2) was readily hydrolysed but not the D-enantiomer **8**. The lower rates of hydrolysis of the racemic substrates relative to those of the pure enantiomers are attributable to the competitive inhibition of the reaction by the D-enantiomers.[1] Interpretation of the stereoselectivity of the enzyme towards L-enantiomers was assisted by molecular modelling studies, which indicated that binding of the D-ester induces a significant torsional strain

Table 3 Hydrolysis of aromatic amino acid analogues by a suspension of α-chymotrypsin in dichloromethane [40 mmol dm^{-3} substrate, 16 μmol dm^{-3} enzyme, 5 molar equivalents of salt, 0.2% (v/v) water]

	Config.	Hydrolysis	*t*/h	Products
 **12**	DL	+	15	L-acid **10** D-ester **13**
 **16**	L	+	48	L-acid **15**
 **17**	L	+(slow)	96	L-acid **18**
 **19**	—	—	72	non substrate
 **20**	DL	+	60	DL-acid **21**
 **22**	—	+	18	acid **23** (24% yield)
 **24**	DL	—	48	non substrate
 **25**	DL	—	48	non substrate
 **26**	L	+(slow)	30	acid **27** (35% yield)
 **28**	L	—	48	non substrate
 **29**	DL	+	26	L-acid **30** D-ester **31**
 **32**	DL	—	48	non substrate

amounting to some 8 kcal mol^{-1} * in the case of D-tyrosine derivatives. This is sufficient in itself to explain the stereoselectivity of the enzyme. However, additionally, in the D-enantiomer, no possibility of hydrogen bonding exists between the amino group and the peptide backbone-CO of Ser-214 and a steric conflict will result (see below).

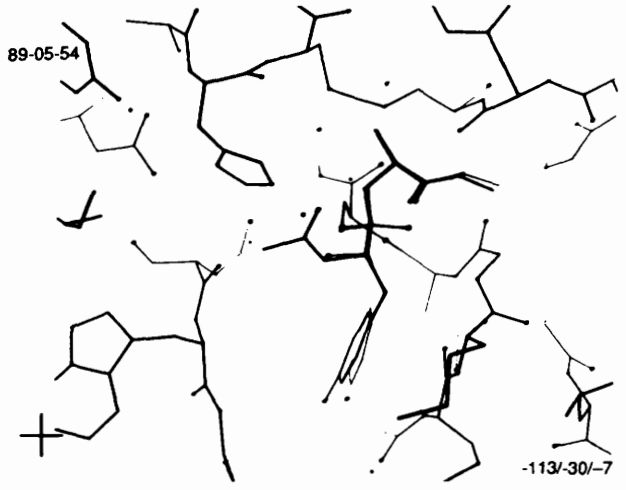


Fig. 2 Superposition of covalently docked Ac-L-Tyr-L-AlaNHMe and L-phenyllactyl-L-AlaNHMe

In order to draw structure–reactivity relationships, the hydrolysis of aromatic amino acid analogues bearing new functionalities at the α- and β-positions and in the aromatic ring was investigated (Table 3). It was noted above that the hydroxy group of the tyrosine derivative can be part of a network of hydrogen bonds created by the water molecules in the specificity pocket (Fig. 1). When the aromatic ring was substituted at the *para*-position by a chloro group, no hydrolysis could be observed. The *p*-chloro derivative **25** (Table 3) goes deeper into the specificity pocket than phenylalanine derivatives (*cf.* Table 2). Consequently, the network of water molecules at the bottom of the pocket is heavily disturbed. Although this explanation for the lack of reacitivity of the *p*-chloro derivative **25** seems reasonable on thermodynamic grounds, the additional possibility of a modification of the three dimensional structure of the enzyme cannot be ruled out. In water, substitution at the *para*-position is usually not detrimental.[1]

As shown in Table 3, substitution at the *para*-position by an amino group as in substrate **29** immediately restored activity. The amino group could again participate in hydrogen bonding with the water molecules in the specificity pocket, and therefore proved to be a much better substrate than the phenylalanine equivalent. The reaction is stereoselective and the optically pure L-enantiomer of the product acid could be obtained.

By replacing the α-amino group by an hydroxy group, the substrate reactivity was diminished to a great extent as shown for ethyl L-phenyllactate **17**. With the tyrosine analogue **20**, low substrate reactivity and loss of stereoselectivity was observed. No resolution was possible and the racemic DL-acid was obtained. In water, the hydrolysis of phenyllactate derivatives is stereoselective, the L-enantiomer being hydrolysed more rapidly than its D-counterpart.[13]

When the reaction is carried out in organic solvents, one can clearly see that the enzyme has lost its stereoselectivity for α-hydroxy esters. Insight into this problem was provided by molecular modelling. In Fig. 2 is shown the superposition of covalently docked Ac-L-Tyr-L-AlaNMe and L-Phelac-L-AlaNMe. From the molecular modelling experiments, one can see that not much difference in the conformations of the bound substrates is to be expected. The lactate OH sits at the position of the *N*-terminal acylamino group of Ac-L-Tyr-L-AlaNMe.

* 1 cal = 4.184 J.

Table 4 Hydrolysis catalysed by α-chymotrypsin of *p*-nitrophenyl esters of *N*-acetylphenylalanine and *O*-acetylphenyllactate[18]

Acyl group	k_{da}[a]/s^{-1}	K_M/mol dm^{-3}
N-Acetyl-L-phenylalanyl	94.8	$4.8.10^{-6}$
N-Acetyl-D-phenylalanyl	0.0148	
O-Acetyl-L-phenyllactyl	9.2	$6.1.10^{-6}$
O-Acetyl-D-phenyllactyl	1.03	

[a] k_{da} = rate constant for deacylation of the acyl–enzyme intermediate.

Although Fig. 2 provides an explanation for the stereoselectivity observed in aqueous medium, it does not explain the loss of stereoselectivity for α-hydroxy esters when the reaction is carried out in an organic solvent. It has been proposed that the orientation of the L-enantiomer of amino acid derivatives at the active site is determined by the hydrogen bond between the amino group and the peptide backbone-CO of Ser-214 (see below).[14] No evidence for the formation of such a hydrogen bond was revealed in our modelling studies. However, it was emphasised above that the protein was treated as a rigid entity; it is therefore possible that a conformational change might bring the backbone carbonyl group of Ser-214 within hydrogen bonding range. (Ser-214 lies below the tetrahedral carbon atom of the oxyanion in the views of Figs. 1–3). In view of the clear evidence for pH-dependent conformational change in α-chymotrypsin (admirably summarised by Laidler and Bunting),[15] the suggestion is reasonable that an altered conformation, induced by the organic environment, might make possible the formation of a hydrogen bond, as noted above. If this hydrogen bond were lost, then no stereoselectivity would be expected. In an aqueous environment, this hydrogen bond is established by the α-amino- and α-hydroxy groups. To explain the loss of stereoselectivity for α-hydroxy esters in the organic medium, one can suggest that the capacity for forming a strong hydrogen bond has been lost in the case of the α-hydroxy derivative, but conserved for the amino acid derivative.

In general, N–H • • • O=C hydrogen bonds are longer than O–H • • • O=C hydrogen bonds.[16] It is therefore possible that if the protein structure became more rigid in the organic environment,[2] only the amino group would be able to establish a strong hydrogen bond with the backbone-CO of Ser-214. The rigidity of the protein would prevent an α-hydroxy group from approaching sufficiently closely. Whether or not this hypothesis might explain the low reactivity of phenyllactate esters must await further experimental investigation.

If the α-functionality is replaced by a hydrogen atom, the direct effect of the binding of the aromatic substituent can be measured. Once again, a striking difference is observed between the tyrosyl **22** and the phenylalanyl **19** analogues (Table 3). The ester **22** is hydrolysed to the acid in 24% yield over 18 h. However, this rate is low compared to the natural tyrosine derivative **12**. For the phenylalanine analogue **19**, the rate is so low that no reaction could be observed. These results again emphasize the importance of the putative hydrogen bond between the α-functionality and the carbonyl oxygen atom of Ser-214 in the binding process. The binding role of Ser-214 would be more effective for groups such as the amino group which, for geometrical reasons, can form strong hydrogen bonds, as noted above.

These results should be compared with results for aqueous systems. It is well established that if the amide group in *N*-acetylphenylalanine esters is replaced by an hydroxy group as in phenyllactate esters[17,18] there is a marked decrease in discrimination between L- and D-isomers. It has also been clearly demonstrated that the presence of an α-amido hydrogen atom influences the catalytic steps but has no effect on substrate binding. The markedly different influences of an α-hydrogen bonding group on the catalytic step and on binding is particularly evident in the data of Ingles and Knowles, reproduced in Table 4.[18] The magnitude of both the rate constant for deacylation and the discrimination between stereoisomers is greatly diminished in the phenyllactate isomers compared with their *N*-acetylphenylalanine counterparts. The effect of replacing the acetamido group by an hydroxy group in the α-chymotrypsin–ammonium hydrogen carbonate system is similar, at least qualitatively, to that observed in aqueous systems. [Compare the results given in Table 4 with data for compounds **7**, **8** and **12** (Table 2) and **20** (Table 3).] As noted above, our modelling experiments revealed no evidence for hydrogen bonding of such an α-amido group to Ser-214 in oxyanion intermediates. Such a hydrogen bond was also absent in a complex between α-chymotrypsin and the *pseudo*-substrate *N*-formyltryptophan.[19] However, all of the evidence summarised above suggests that a conformational change occurs during catalysis that brings together the carbonyl oxygen atom of Ser-214 and the α-substituent in residues at the S_1 subsite with the L- (but not the D-) configuration. This question is returned to below after discussions of interactions at the S_1–S_3 subsites.

Previous results also indicated that the overall structure of the S_1-subsite might have been modified. The evidence for this was that Boc-derivatives and dipeptides are not substrates for peptide synthesis catalysed by chymotrypsin suspended in organic solvents.[3] To explore further the apparent difference in reactivity between phenylalanine and tyrosine derivatives, the hydrolyses of Boc-L-TyrOMe **26** and Boc-L-PheOEt **28** were studied (Table 3). Despite the indication in molecular modelling experiments of a perfect interaction with the specificity pocket, Boc-L-TyrOMe **26** was only very slowly hydrolysed, whereas for the phenylalanine derivative **28**, no reaction was observed. Fig. 3 shows the hydrophobic interactions (dotted lines) between the covalently docked Boc-L-Tyr-L-AlaGlyNMe and chymotrypsin. The Boc-unit occupies the space between His-57 and Trp-215. Crystallographic studies of native chymotrypsin show that the side chain of Trp-215 is kept in place only by hydrophobic interactions in a thermodynamically unfavourable conformation. If molecules of the organic solvent were able to diffuse into the crystal lattice, it would be expected that hydrophobic clusters would be the first to be disturbed. The free energy value for the transfer of the side chain of tryptophan from water to ethanol at 25 °C is by far the lowest amongst the amino acids.[20] This suggests the possibility that hydrophobic solvent molecules might interact with the side-chain of Trp-215, causing it to swing free from its association with underlying hydrophobic side-chains and partially to obstruct the S_1-subsite. Fig. 3 shows Trp-215 in the altered conformation that may arise in this way. (Compare with Figs. 1 and 2.) A large number of compounds have been shown to act as competitive inhibitors of α-chymotrypsin[1] including benzene, toluene[21] (K_i = 25, 13 mmol dm^{-3} respectively) and dioxane,[22] indicating that molecules of such solvents can penetrate into the interior of the protein to bind at the active site. Further, Steitz *et al.*[19] found a dioxane molecule on X-ray diffraction analysis of a crystal of α-chymotrypsin that had been formed in a dioxane-containing medium. The dioxane was bound in the hydrophobic pocket, disturbing the Met-192 residue at the entrance to the pocket.

As shown in Fig. 3, the Boc-protecting group does not interact strongly with the Trp-215 side-chain. However, only a very slightly unfavourable interaction would be responsible for the absence of reactivity. The apparent modification of the S_1-subsite is further emphasized by the result obtained with the ester **24** (Table 3). The resolution of the hydroxy ester **24** was not possible and no hydrolysis was observed. In water, separating the chiral centre bearing the hydroxy group from

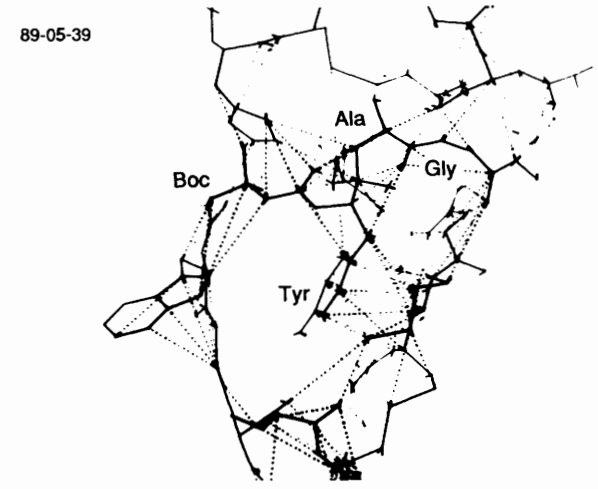


Fig. 3 Display of hydrophobic interactions between the covalently docked Boc-L-Tyr-L-Ala-GlyNHMe and chymotrypsin

Table 5 Hydrolysis of dipeptide derivatives by a suspension of α-chymotrypsin in dichloromethane

Dipeptide	Hydrolysis	Reaction time (h)	Yield (%)[a]
Z-L-Leu-L-PheOEt **33**	—	24	0
Z-L-Leu-L-LeuOMe **34**	—	24	0
Z-L-Leu-L-TyrOEt **35**	+(**36**)	24	35
Z-L-Ala-L-TyrOEt **37**	+(**38**)	24	100
Z-D-Ala-L-TyrOEt **39**	+(**40**)	24	20

[a] Determined by ^{1}H NMR spectroscopy.

the ester causes a reversal in stereoselectivity.[14] This type of inversion of steroselectivity was readily explained in terms of the active site model proposed by Cohen.[23]

The phenylglycine derivative **32** was not a substrate under these conditions despite the fact that acetyl-DL-phenylglycine methyl ester is readily hydrolysed in water, yielding the L-acid whereas the D-enantiomer is unaffected.[1] Our results indicate that at the β-position, no substituent other than hydrogen can be accommodated in the hydrophobic pocket, once again indicating a modification of the protein structure at the S_1-subsite. Optimum binding appears to occur when a two-carbon unit separates the aromatic ring from the ester carbonyl group.[1]

The result of the hydrolysis of Boc-L-TyrOMe **26** led us to consider closely the structure of the S_2- and S_3-subsites. If the S_2-subsite is not totally obstructed (as indicated by the peptide synthesis experiments,[3] and if one provides a perfect interaction with the specificity pocket (tyrosine derivatives), one might be able to get some reactivity towards dipeptide derivatives. The results are summarized in Table 5, which shows that this prediction was confirmed. (The two dipeptides Z-D-Ala-L-TyrOH **40** and Z-L-Leu-L-TyrOH **36** could not be isolated owing to the low chemical yields in which they were formed.) Again, the favourable interaction of the tyrosyl side-chain in the specificity pocket appears to be the factor that determines whether or not hydrolysis occurs.

Segal and co-workers[24] studied, by X-ray crystallography, complexes of α-chymotrypsin with a covalently-bound inhibitory species obtained by incubating the enzyme with chloromethyl ketone analogues of peptide substrates. In these, the carboxy-terminal group was replaced by $COCH_2Cl$, which reacted to form a covalent bond to the imidazole ring of His-57. It was pointed out that the hydroxy group of the catalytic Ser-195 was positioned with respect to the carbonyl group of what

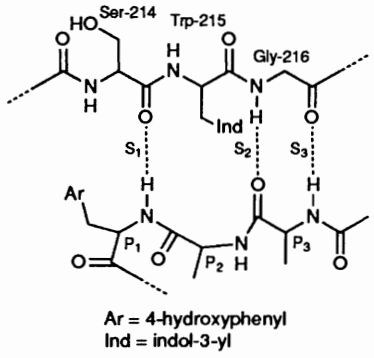


Fig. 4 Schematic diagram of the subsite binding of the covalently bound inhibitor from *N*-acetyl-L-Ala-L-Ala-L-TyrCOCH$_2$ ··· according to Segal.[24] The hydrogen bonds involved in the antiparallel β-pleated sheet configuration with the residues 214–216 of the enzyme are shown (dotted lines).

was originally the chloromethyl ketone in the manner expected for incipient attack on the scissile amide or ester bond of a substrate molecule. The inhibitor–enzyme complex can thus be taken as a reasonable representation of the conformation of a bound substrate just prior to the formation of the oxyanion intermediate. In the inhibitor–enzyme complex, it was found that an antiparallel β-pleated sheet-type interaction existed between the amino acyl chain and an extended peptide chain at the enzyme surface adjacent to the primary specificity site shown schematically in Fig. 4. The amino acid residues involved in the interaction are Ser-214, Trp-215 and Gly-216. Specifically, a hydrogen bond was seen to be formed between the P_1-residue and the carbonyl group of Ser-214. As indicated by the results shown in Table 5, dipeptides lacking a strong interaction with the specificity pocket, **33** and **34**, were not hydrolysed by chymotrypsin. However, when tyrosine constituted the *C*-terminal residue, hydrolysis was observed. When L-alanine occupied the P_2-position, complete hydrolysis of the dipeptide **37** was observed. However, with the bulkier L-leucine, **35**, a slow hydrolysis took place which was fractionally faster than for the dipeptide **39** where D-alanine occupied the P_2-position.

In accordance with the antiparallel β-pleated sheet configuration proposed by Segal,[24] acyl–enzyme intermediates derived from such tyrosine derivatives will have a hydrogen bond between the NH-group of tyrosine and the CO-group of Ser-214. There are no hydrogen bonds between the P_2-residue and Trp-215, but there are two possible hydrogen-bonded interactions between the P_3-residue and Gly-216 (Fig. 4). The experimentally demonstrated formation of a hydrogen bond between the P_1-residue and Ser-214 in a complex that corresponds to a slightly altered conformation of a normal substrate lends credence to the view expressed above that formation of such a hydrogen bond occurs during the catalytic process.

From X-ray structures and molecular modelling experiments, the S_2-subsite is seen to be formed by a groove constituted by the side-chain of Trp-215 on one side, and Asp-102/His-57 on the other. The driving force for the binding at the S_2-subsite is provided by hydrophobic interactions with the hydrophobic cluster on which Trp-215 is lying. Therefore, one might predict that hydrophobic residues at the S_2-subsite should be good substrates as indeed is found for peptide hydrolysis in water. (The preference for binding to the P_2-subsite for a series of Gly-X-Tyr tripeptides was found to be X = Val > Leu > Ala > Gly).[25] However, the results shown in Table 5 indicate that L-alanine is preferred to L-leucine at the P_2-position. This result is an indication that the leucine side-chain is in conflict with part of the protein structure, and as indicated previously, the

Trp-215 indole side-chain would seem to be the most likely source of steric hindrance. If the Trp-215 side-chain were to adopt the new altered conformation (Fig. 3), it would interact more strongly with the leucine derivative than with the L-alanine one. If D-alanine is present at the P_2-position, the antiparallel β-pleated sheet-type structure puts the side-chain of D-alanine in direct conflict with the β-methylene group of Trp-215, whatever the conformation of the latter residue.[24] The experimental results (Table 5) are therefore rather well explained by this interpretation, based on molecular modelling and X-ray structure data.

In conclusion, hydrolytic reactions catalysed by chymotrypsin suspended in dichloromethane in the presence of ammonium hydrogen carbonate provide a useful system for the study of structure–activity relationships. By direct comparison with the binding of the substrates in aqueous medium through molecular modelling, valuable insights into the three dimensional structure of the enzyme in the organic medium can be gained. The structure of the active site of the enzyme seems to have been modified by conformational changes during the transition from an aqueous to an organic environment. The enzyme exhibits a significant preference for tyrosyl residues at the P_1-position. We have suggested that this is associated with replacement of a conserved water molecule in the hydrophobic pocket by the phenolic hydroxy group of the tyrosine side-chain, a compensating replacement not possible with phenylalanine or tryptophan derivatives. A similar compensation was observed with a *p*-aminophenylalanine ester (**29**, Table 3) the oxyanion from which also displaces a water molecule. These observations are interesting in the light of observed correspondences between water clusters and bound substrates in the active sites, for example, of *Streptomyces griseus* proteinase A[26] and proteinase K.[27] Interactions lost by displacing water molecules are restored through corresponding interactions involving polar groups in the substrates. In the latter case, the oxyanion is stabilised by interactions with Ser-224 and Asn-161, which, in the native enzyme, bind a water molecule.

Although hydrolysis was stereoselective for amino acid derivatives, no stereoselectivity was observed for α-hydroxy esters. This may be attributable to loss of a hydrogen bond to Ser-214. We have suggested that conformational changes might have occurred leading to a new structure for the active site. More drastic changes might have resulted in complete inactivation. This may be the case with trypsin, a protease very closely related to chymotrypsin, with which we could never demonstrate activity when it was used as a suspension in an organic solvent. However, if these changes involve only restricted parts of the active site, this could result in a new range of properties, explaining, for example, with chymotrypsin, why D-amino acid derivatives can be used as nucleophiles in synthesis[3] and why no stereoselectivity was found for α-hydroxy esters.

Hydrolytic reactions in organic solvents appeared as a method of choice for the study of the properties of an enzyme in an organic environment. The amount of water is controlled and can be adjusted to the optimal level. The procedure allows easy recovery of the products, and can be used for hydrolysing substrates exhibiting very low water solubility, thus avoiding the use of cosolvents that may be difficult to remove, or inimical to the action of the enzyme.

By providing a simple experimental system, hydrolytic reactions in organic solvents can be used to screen the activity of enzymes in organic solvents. It is also possible that kinetic parameters in such systems might be obtained.

Experimental

Crystalline bovine pancreatic α-chymotrypsin (EC 3.4.2.1) (type II) was purchased from Sigma as a lyophilized powder with specific activity of 51 units mg^{-1} protein. The enzyme was used without further purification. Ammonium hydrogen carbonate was purchased in the highest purity available. The salt was extensively washed with anhydrous solvents (THF, CH_2Cl_2) to remove any contaminants prior to drying under high vacuum. Dichloromethane was obtained through continuous distillation over calcium hydride. Thin-layer chromatography (TLC) was performed on Merck Kieselgel F254 pre-coated aluminium plates using dichloromethane–methanol (0–20%, v/v) or ethyl acetate–pyridine–water–acetic acid (6:2:1:0.6, v/v) as the mobile phase. Products were visualised by UV fluorescence and spraying with a solution of ninhydrin in butanol. NMR spectra were determined at 220 MHz using a Perkin-Elmer R34 spectrometer or at 400 MHz using a Bruker WH400 spectrometer. Mass spectra were determined using a Kratos MS80 spectrometer. $[\alpha]_D$ Values are given in units of 10^{-1} deg cm^2 g^{-1}.

Assays.—The standard reaction mixture in dichloromethane consisted of the substrate (40 mmol dm^{-3}), enzyme (16 μmol dm^{-3}), and 5 moles of ammonium hydrogen carbonate per mole of ester substrate. The reaction was started by the addition of 0.2% (v/v) water. The reaction was followed by TLC (dichloromethane–methanol 0–10%). At a given time, the reaction mixture was filtered, the solvents were evaporated, and the remaining residue was dissolved in the appropriate NMR solvent (D_2O–[$^2H_6$]acetone, $CDCl_3$–trifluoroacetic acid). In most cases, the unchanged ester was isolated (*via* extraction or chromatography on silica gel), analysed by ^1H NMR spectroscopy (220 MHz), and its optical rotation and melting point were compared with commercially available samples. Unprotected amino acids were identified by TLC [ethyl acetate–pyridine–water–acetic acid (6:2:1:0.6, v/v)] by comparison with authentic samples.

General Procedure for the Hydrolysis of Ester Substrates by Chymotrypsin Suspended in Dichloromethane in the Presence of Ammonium Hydrogen Carbonate. Resolution of Ethyl N-*Acetyl*-DL-*phenylalaninate* **7**.—To a solution of ethyl *N*-acetyl-DL-phenylalaninate **7** (1 mmol) in dichloromethane (25 cm^3) were added chymotrypsin (25 mg) and ammonium hydrogen carbonate (395 mg, 5 mmol). The resulting suspension was vigorously stirred while water (50 mm^3, 0.2% v/v) was added. After completion of the reaction as monitored by TLC (dichloromethane–10% methanol), the mixture was filtered. The precipitate was dissolved in methanol and crystallisation was induced by the addition of diethyl ether. *N*-Acetyl-L-phenylalanine **4** (0.38 mmol) was obtained as a white powder [m.p. 168–169 °C; $[\alpha]_D^{25}$ +38.0 (*c* 1, MeOH)] (lit.,[28] m.p. 172 °C). The specific rotation agrees with that quoted by a commercial supplier {Aldrich Chemical Co. $[[\alpha]_D^{22}$ +40 (*c* 1, MeOH)]}. The filtrate which contained ethyl *N*-acetyl-D-phenylalaninate **8** and the remaining *N*-acetyl-L-phenylalanine **4**, was washed with aqueous sodium carbonate (0.5 mol dm^{-3}; 5 cm^3) and water (5 cm^3). After drying ($MgSO_4$) and evaporation of the solvent, the pure ethyl *N*-acetyl-D-phenylalaninate **8** (0.5 mmol) was recrystallised from ethyl acetate–light petroleum (b.p. 40–60 °C), m.p. 92–94 °C, $[\alpha]_D^{25}$ −87.7 (*c* 1, $CHCl_3$) (lit.,[29] m.p. 68 °C).

Ethyl L-3-*Phenyllactate* **17**.—Acetyl chloride (5 cm^3, 70.3 mmol) was added to anhydrous ethanol (50 cm^3) cooled in an ice–water bath. L-Phenyllactic acid (1 g, 6 mmol) was added and the remaining solution was boiled under reflux overnight. The solvent was evaporated under reduced pressure and ethyl acetate (25 cm^3) was added. The organic phase was washed with aqueous sodium carbonate (0.5 mol dm^{-3}, 5 cm^3) and water

(5 cm^3). After drying ($MgSO_4$), the solvent was evaporated under reduced pressure to give an oil which solidified on standing (1.095 g, 94%), m.p. 26–28 °C (Found: C, 68.0; H, 7.15. $C_{11}H_{14}O_3$ requires C, 68.0; H, 7.3%); $[\alpha]_D^{25}$ −19.3 (*c* 0.75, $CHCl_3$) [lit.,[30] $[\alpha]_D^{22}$ −54.9 (*c* 3.5, $CHCl_3$)]; δ_H(220 MHz; $CDCl_3$) 1.3 (3 H, t, *J* 7, CH_3), 2.85 (1 H, d, *J* 5, OH), 3.05 (1 H, q, ^2J 14.7, ^3J 3.20, CH_2), 3.20 (1 H, q, ^2J 14.7, ^3J 4.9, CH_2), 4.3 (2 H, q, *J* 7, OCH_2), 4.55 [1 H, q, *J* 7.3, 4.9, C*H*(OAc)] and 7.4 (5 H, m, Ar-H).

Methyl 3-(p-*Hydroxyphenyl*)-DL-*lactate* **20**.—3-(*p*-Hydroxyphenyl)-2-oxopropionic acid (2 g, 12 mmol) was added to a concentrated solution of hydrochloric acid in anhydrous methanol (25 cm^3). The mixture was boiled under reflux for 2 h. The solvent was evaporated and anhydrous dichloromethane (20 cm^3) was added. The pale yellow precipitate was filtered off and the solvent was removed from the filtrate under reduced pressure. The bright orange residue was dissolved in anhydrous THF (18 cm^3) and the mixture was cooled to 0 °C. Sodium borohydride (450 mg, 12 mmol) was added and the stirring was continued for 2 h. The reaction was quenched by the addition of aqueous hydrochloric acid (0.5 mol dm^{-3}) and the solvent was evaporated under reduced pressure. The ester **20** was purified by flash column chromatography (CH_2Cl_2–MeOH 5%) (800 mg, 34%), R_F 0.35 (CH_2Cl_2–MeOH 95:5, v/v); ν_{max}/cm^{-1} 3400–3300, 1880, 1730, 1600, 1500 and 820; δ_H(220 MHz; CD_3OD) 3.45 (1 H, q, ^2J 12.2, ^3J 9.8, CH_2), 3.75 (1 H, q, *J* 12.2, CH_2), 4.05 (3 H, s, CH_3), 4.85 [1 H, t, *J* 2.4, 9.8, C*H*(OH)], 7.45 (2 H, d, Ar*H*) and 7.85 (2 H, d, Ar*H*); *m/z* 196 (M^+); *m/z* (FAB) 197 (MH^+, 20%), 137 (74, M − CO_2Me), 107 (72), 91 (20) and 77 (10).

Methyl 3-(p-*Hydroxyphenyl*)*propionate* **22**.—Acetyl chloride (5 cm^3, 70.3 mmol) was added to anhydrous methanol (50 cm^3) cooled in an ice–water bath. 3-(4-Hydroxyphenyl)propionic acid (1 g, 6 mmol) was added and the solution was boiled under reflux overnight. The solvent was then evaporated under reduced pressure and ethyl acetate (25 cm^3) was added. The organic phase was washed with aqueous sodium carbonate (0.5 mol dm^{-3}, 5 cm^3) and water (5 cm^3). After drying ($MgSO_4$), the solvent was evaporated under reduced pressure. The oily residue was purified by flash column chromatography (CH_2Cl_2–MeOH 2.5%) to give the ester **22** (962 mg, 89%), m.p. 24–26 °C; R_F 0.83 (CH_2Cl_2–MeOH 90:10, v/v) (Found: M^+, 180.078. $C_{10}H_{12}O_3$ requires *M*, 180.079); δ_H(220 MHz; $CDCl_3$) 2.65 (2 H, t, *J* 7.3, 2-H), 2.9 (2 H, t, *J* 7.3, 3-H), 3.7 (3 H, s, Me), 6.8 (2 H, d, *J* 9.8, ArH) and 7.1 (2 H, d, *J* 9.8, ArH); *m/z* 180 (M^+).

Ethyl 3-*Hydroxy*-3-*phenyl*-DL-*propionate* **24**.—Sodium borohydride (76 mg, 2 mmol) was added to a solution of ethyl 3-oxo-3-phenylpropionate (192 mg, 1 mmol) in ethanol (3 cm^3). The solution was stirred at 0 °C for 1 h. The reaction was quenched by the addition of hydrochloric acid (0.5 mol dm^{-3}) and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2–MeOH 97.5:2.5, v/v) to give the ester **24** (155 mg, 80%),[31] R_F 0.63 (CH_2Cl_2–MeOH, 96:4, v/v) (Found: M^+, 194.094. $C_{11}H_{14}O_3$ requires *M*, 194.094); ν_{max}/cm^{-1} 3440, 1705, 1600, 1490 and 755; δ_H(220 MHz; $CDCl_3$) 1.25 (3 H, t, *J* 6, Me), 2.75 (2 H, m, CH_2), 3.55 (1 H, d, *J* 4, OH), 4.2 (2 H, q, *J* 6, OCH_2), 5.15 [1 H, m, C*H*(OH)] and 7.4 (5 H, m, ArH); *m/z* 194 (M^+).

Methyl N-tert-*Butoxycarbonyl*-L-*tyrosinate* **26**.—To a solution of methyl L-tyrosinate (1.96 g, 10 mmol) in dioxane (20 cm^3) cooled in an ice–water bath, was added di-*tert*-butyl dicarbonate (2.4 g, 11 mmol) and stirring was continued for 3 h. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (20 cm^3). The organic phase was washed with water (5 cm^3), aqueous hydrochloric acid (0.5 mol dm^{-3}, 5 cm^3), dried ($MgSO_4$) and evaporated under reduced pressure to give the ester **26** (2.63 g, 89%), m.p. 102–104 °C [ethyl acetate–light petroleum (b.p. 40–60 °C)] (lit.,[32] m.p. 102–104 °C) (Found: C, 60.6; H, 7.2; N, 4.5. Calc. for $C_{15}H_{21}NO_5$: C, 61.0; H, 7.2; N, 4.75%); $[\alpha]_D^{25}$ +5.6 (*c* 1, MeOH); δ_H(220 MHz; CD_3OD) 1.6 (9 H, s, Me_3C), 3.1 (2 H, m, CH_2), 3.9 (3 H, s, OMe), 4.5 (1 H, t, H-2), 6.95 (2 H, d, *J* 7, ArH) and 7.25 (2 H, d, *J* 7, H-ArH).

Ethyl N-tert-*Butoxycarbonyl*-L-*phenylalaninate* **28**.—To a solution of ethyl L-phenylalaninate hydrochloride (2.165 g, 10 mmol) in dry dichloromethane (20 cm^3) was added triethylamine (1.011 g, 10 mmol) and di-*tert*-butyl dicarbonate (2.4 g, 11 mmol). The solution was stirred at room temperature overnight, and was then washed with water (5 cm^3) and aqueous hydrochloric acid (0.5 mol dm^{-3}; 5 cm^3). The organic phase was dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2) to give the ester **28** (2.064 g, 74%), m.p. 25–27 °C (Found: C, 65.7; H, 8.0; N, 4.65. $C_{16}H_{23}NO_4$ requires C, 65.5; H, 7.9; N, 4.8%); R_F 0.33 (CH_2Cl_2); $[\alpha]_D^{25}$ +40.2 (*c* 1.3, $CHCl_3$); δ_H(220 MHz; $CDCl_3$) 1.2 (3 H, t, *J* 5, Me), 1.4 (9 H, s, Me_3C), 3.1 (2 H, m, CH_2), 4.15 (2 H, q, *J* 5, OCH_2), 4.55 (1 H, m, C*H*CO_2Et), 5.15 (1 H, br, NH) and 7.3 (5 H, m, ArH).

Methyl p-*Amino*-DL-*phenylalaninate Dihydrochloride* **29**.—Acetyl chloride (5 cm^3, 70.3 mmol) was added to anhydrous methanol (50 cm^3) cooled in an ice–water bath. 4-Amino-L-phenylalanine hydrate (1 g, 5.5 mmol) was then added and the solution was boiled under reflux overnight. The solvent was evaporated under reduced pressure and the dried residue was dissolved in the minimum amount of methanol and precipitated by the addition of diethyl ether to give the methyl ester **29** as the dihydrochloride (823 mg, 56%), m.p. 208–210 °C (Found: C, 43.45; H, 6.15; Cl, 17.45; N, 10.15. $C_{10}H_{16}Cl_2N_2O_2$ requires C, 44.95; H, 6.05; Cl, 17.6; N, 10.5%); δ_H(220 MHz; D_2O) 3.2 (2 H, m, CH_2), 3.7 (3 H, s, Me), 4.35 (1 H, t, C*H*CO_2Me) and 7.35 (4 H, s, ArH).

Methyl DL-*Phenylglycinate Hydrochloride* **32**.—Acetyl chloride (5 cm^3, 70.3 mmol) was added to anhydrous methanol (50 cm^3) cooled in an ice–water bath. DL-Phenylglycine (5 g, 33 mmol) was added and the remaining solution was boiled under reflux overnight. The solvent was evaporated under reduced pressure. The residue was recrystallised (methanol–diethyl ether) to give the salt of ester **32** (5.89 g, 89%), m.p. 205–207 °C (Found: C, 53.5; H, 6.1; N, 7.05. $C_9H_{12}ClNO_2$ requires C, 53.6; H, 6.0; N, 6.95%); R_F 0.38 (CH_2Cl_2–MeOH 90:10, v/v); δ_H(220 MHz; D_2O) 3.8 (3 H, s, Me), 5.3 (1 H, s, C*H*CO_2Me) and 7.55 (5 H, m, ArH).

General Procedure for the Coupling with eedq.—The coupling reagent eedq (ethyl 1,2-dihydro-2-ethoxyquinoline-1-carboxylate) (2.47 g, 10 mmol) was added to a solution of the *N*-protected amino acid (10 mmol) and the amino acid ester (10 mmol). If the hydrochloride was to be used, it was first neutralized by the addition of one equivalent of triethylamine in dry dichloromethane (30 cm^3) and the mixture was stirred at room temperature for 24 h. The solution was extracted with hydrochloric acid (1 mol dm^{-3}), sodium hydrogen carbonate (0.5 mol dm^{-3}) and water. The organic phase was dried ($MgSO_4$) and evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography.

Ethyl N-*benzyloxycarbonyl*-L-*leucyl*-L-*phenylalaninate* **33**.[33] (1.23 g, 92%), m.p. 76–78 °C (ethyl acetate–diethyl ether) (Found: C, 68.0; H, 7.25; N, 6.8. $C_{25}H_{32}N_2O_5$ requires C, 68.15;

Table 6 Characteristics of some of the amino acid products

Derivative	M.p./°C	$[\alpha]_D^{25}$
Ac-L-TyrOH **2**	150–152	+62.0 (*c* 1, MeOH) [lit.,[37] m.p. 148 °C; $[\alpha]_D^{25}$ ***41*** (*c* 0.4, H_2O)]
Ac-L-TrpOH **6**	200 (decomp.)	+17.8 (*c* 1.2, MeOH) (lit.,[39] m.p. 189–190 °C)
D-TyrOMe **13**	133–135	−22.9 (*c* 2, MeOH) [lit.,[36] m.p. 136–137 °C; $[\alpha]_D^{25}$ − 19.8 (*c* 2.4, pyridine)]
Boc-L-TyrOH **27**	136–138	+4.2 (*c* 1.5, MeOH) [lit.,[36] m.p. 136–138 °C; $[\alpha]_D^{25}$ +3.9 (*c* 2, AcOH)]
D-*p*-$NH_2C_6H_4CH_2CH(NH_2)CO_2H$ **30**	204–206	−16.0 (*c* 1, MeOH)

H, 7.3; N, 6.4%); $[\alpha]_D^{25}$ −25.0 (*c* 0.5, MeOH); δ_H(220 MHz; $CDCl_3$) 0.95 (6 H, d, *J* 5, Leu-Me), 1.25 (3 H, t, *J* 7.3, *Me*CH_2O), 1.4–1.8 (3 H, m, $CHCH_2$), 3.15 (2 H, m, PhCH_2), 4.2 (2 H, q, *J* 7.3, OCH_2), 4.85 [1 H, m, α-C*H*(Leu)], 5.15 (2 H, s, OCH_2Ar), 5.25 [1 H, m, α-C*H*(Phe)], 6.55 (1 H, br, NH) and 7.1–7.4 (11 H, m, ArH and NH).

Methyl N-*benzyloxycarbonyl*-L-*leucyl*-L-*leucinate* **34**.[34] (3.54 g, 84%), m.p. 86–88 °C [ethyl acetate–light petroleum (b.p. 40–60 °C)] (Found: C, 64.1; H, 8.2; N, 7.2. $C_{21}H_{32}N_2O_5$ requires C, 64.25; H, 8.2; N, 7.15%); $[\alpha]_D^{25}$ −24.7 (*c* 1, $CHCl_3$); δ_H(220 MHz; $CDCl_3$) 0.95 (12 H, m, 2 × Me_2C), 1.65 (6 H, m, 2 × $CHCH_2$), 3.8 (3 H, s, OMe), 4.3 (1 H, m, C*H*CO_2Me), 4.7 (1 H, m, C*H*CONH), 5.2 (2 H, s, OCH_2Ar), 5.5 (1 H, d, NH), 6.7 (1 H, d, NH) and 7.5 (5 H, s, ArH).

Ethyl N-*benzyloxycarbonyl*-L-*leucyl*-L-*tyrosinate* **35**. (0.897 g, 75%); *m*/*z* 456 (M^+) (Found: M^+, 456.3201. $C_{25}H_{32}N_2O_6$ requires *M*, 456.226); $[\alpha]_D^{25}$ +20.0 (*c* 1.2, $CHCl_3$); δ_H(220 MHz; $CDCl_3$) 0.9 (6 H, m, Me_2CH), 1.2 (3 H, t, *J* 5, Me), 1.45–1.75 (3 H, m, $CHCH_2$), 3.0 (2 H, m, CH_2-Tyr), 4.15 (2 H, q, *J* 5, OCH_2), 4.35 (1 H, m, C*H*CON), 4.8 (1 H, m, C*H*CO_2Et), 5.08 (2 H, q, OCH_2Ar), 6.05 (1 H, d, *J* 5, NH), 6.8 and 7.0 (4 H, each d, *J* 7, TyrArH) and 7.35 (6 H, s, ArH, NH).

Ethyl N-*benzyloxycarbonyl*-L-*alanyl*-L-*tyrosinate* **37**.[35] (1.23 g, 88%), m.p. 133–135 °C [ethyl acetate–light petroleum (b.p. 40–60 °C)] (Found: C, 63.1; H, 6.3; N, 6.55. $C_{22}H_{26}N_2O_6$ requires C, 63.7; H, 6.3; N, 6.77%); $[\alpha]_D^{25}$ +29.2 (*c* 0.9, $CHCl_3$); δ_H(220 MHz; $CDCl_3$) 1.3 (3 H, t, *J* 5, Me), 1.35 (3 H, d, *Me*CH), 3.05 (2 H, m, ArCH_2), 4.25 (2 H, q, *J* 5, OCH_2), 4.3 (1 H, m, CHCON), 4.85 (1 H, m, C*H*CO_2Et), 5.15 (2 H, q, OCH_2Ar), 5.65 (1 H, d, *J* 5, NH), 6.8 (3 H, m, TyrArH, NH), 7.0 (2 H, d, *J* 7, ArH) and 7.45 (5 H, s, Ph).

Ethyl N-*benzyloxycarbonyl*-D-*alanyl*-L-*tyrosinate* **39**. (534 mg, 93%) (Found: C, 63.7; H, 6.35; N, 6.55. $C_{22}H_{26}N_2O_6$ requires C, 63.7; H, 6.3; N, 6.77%); $[\alpha]_D^{25}$ +44.0 (*c* 1, $CHCl_3$); δ_H(220 MHz; $CDCl_3$) 1.35 (6 H, m, *Me*CHN, *Me*CH_2O), 3.1 (2 H, m, ArCH_2), 4.3 (2 H, q, OCH_2), 4.35 (1 H, m, CHCON), 4.9 (1 H, m, C*H*CO_2Et), 5.2 (2 H, q, *J* 12.2, OCH_2-Ar), 5.8 (1 H, d, *J* 5, NH), 6.85 (2 H, d, *J* 7, TyrArH), 7.05 (3 H, m, TyrArH, NH), 7.5 (5 H, s, Ph) and 7.65 (1 H, br s, OH).

L-3-*Phenyllactic acid* **18**. M.p. 115–117 °C (lit.,[36] m.p. 122–124 °C); $[\alpha]_D^{25}$ −20.8 (*c* 2, H_2O) (Found: C, 65.0; H, 6.10. Calc. for $C_9H_{10}O_3$: C, 65.05; H, 6.10%); δ_H(220 MHz; D_2O) 2.9 (1 H, m, C*H*H), 3.1 (1 H, m, CH*H*), 4.4 [1 H, m, C*H*(OH)] and 7.5 (5 H, m, Ph).

3-(p-*Hydroxyphenyl*)-DL-*lactic acid* **21**. M.p. 133–135 °C (lit.,[37] 172 °C) (Found: C, 59.35; H, 5.5. Calc. for $C_9H_{10}O_4$: C, 59.6; H, 5.8%); R_F 0.44 (CH_2Cl_2–MeOH 80:20, v/v); δ_H(220 MHz; D_2O) 2.75 (1 H, q, ^2J 15, C*H*H), 2.95 (1 H, q, ^2J 15, CH*H*), 4.15 [1 H, m, C*H*(OH)], 6.8 (2 H, d, *J* 9, ArH) and 7.15 (2 H, d, *J* 9, ArH).

3-(p-*Hydroxyphenyl*)*propionic acid* **23**. M.p. 125–127 °C (lit.,[37] 130 °C) (Found: C, 65.05; H, 6.1. Calc. for $C_9H_{10}O_3$: C, 65.05; H, 6.05%); δ_H(220 MHz; D_2O) 3.45 (2 H, t, *J* 7, ArCH_2), 3.7 (2 H, t, *J* 7, CH_2CO_2H), 7.7 (2 H, d, *J* 7, ArH) and 8.0 (2 H, d, *J* 7, ArH).

N-*Benzyloxycarbonyl*-L-*alanyl*-L-*tyrosine* **38**.[38] M.p. 145–147 °C; $[\alpha]_D^{25}$ +14.0 (*c* 0.5, MeOH); R_F 0.28 (CH_2Cl_2–MeOH 80:20, v/v) (Found: M^+, 386.743. $C_{20}H_{22}N_2O_6$ requires *M*, 386.147); δ_H(220 MHz; [$^2H_6$]acetone) 1.4 (3 H, d, *J* 7.3, Me), 3.0 (1 H, q, *J* 14.6, ArC*H*H), 3.1 (1 H, q, ^2J 14.6, ArCH*H*), 4.4 (1 H, m, CHCON), 4.8 (1 H, q, C*H*CO_2H), 5.2 (2 H, q, ^2J 12, $ArCH_2O$), 6.9 (3 H, m, ArH, NH), 7.2 (2 H, d, *J* 7.3, ArH) and 7.5 (7 H, m, Ph, OH, NH); *m*/*z* 386 (M^+) *m*/*z* (FAB) 387 [$(M + H)^+$, 5%], 343 (4), 233 (2), 136 (23), 107 (18) and 91 (100).

Further data on some of the amino acid products of hydrolysis are given in Table 6.

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