Broadening of the substrate tolerance of α-chymotrypsin by using the carbamoylmethyl ester as an acyl donor in kinetically controlled peptide synthesis

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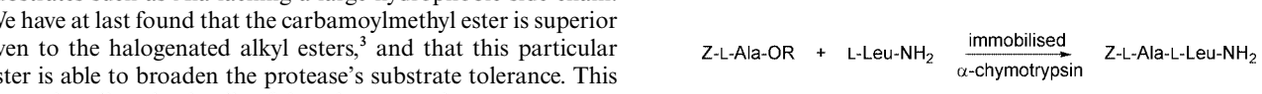
In the kinetically controlled approach of peptide synthesis mediated by α-chymotrypsin, the broadening of the protease's substrate tolerance is achieved by switching the acyl donor from the conventional methyl ester to the carbamoylmethyl ester. Thus, as a typical example, the extremely low coupling efficiency obtained by employing the methyl ester of an inherently poor amino acid substrate, Ala, is significantly improved by the use of this particular ester. Its ameliorating effect is observed also in the couplings of other amino acid residues such as Gly and Ser as carboxy components.

Introduction

Although enzymic methods for peptide synthesis[1] have several advantages over conventional chemical methodologies, a narrow substrate specificity is often regarded as a major drawback from a synthetic standpoint: only limited amino acid residues are acceptable as substrates. Accordingly, developing a means to broaden the applicability of proteases for peptide synthesis remains a challenging task. In the preceding paper,[2] we have reported that the coupling efficiency in the kinetically controlled peptide-bond formation catalysed by α-chymotrypsin is profoundly improved by the use of activated esters such as the 2,2,2-trifluoroethyl ester as an acyl donor instead of the conventional methyl ester in an organic solvent such as acetonitrile with low water content and that this approach is useful for the incorporation of non-protein amino acids such as halogenophenylalanines into peptides. The success of this approach prompted us to attempt to overcome the narrow substrate tolerance of the protease by modulating the ester moiety of an acyl donor. Thus, we have examined a series of activated esters for the couplings of α-chymotrypsin's inherently poor substrates such as Ala lacking a large hydrophobic side chain. We have at last found that the carbamoylmethyl ester is superior even to the halogenated alkyl esters,[3] and that this particular ester is able to broaden the protease's substrate tolerance. This paper describes the details of the relevant works.

Results and discussion

In the kinetically controlled peptide synthesis catalysed by a serine or cysteine protease[1b,e] (Scheme 1), an ester substrate is used as an acyl donor, which facilitates the formation of the acyl-enzyme intermediate. This intermediate is then deacylated either by a nucleophilic amine component to give a peptide product or by water to form the hydrolysis product of the donor ester. Such protease-catalysed peptide-bond formation can be carried out in organic solvents with only a small amount of water as well as in aqueous media.[4] In the former the water activity is reduced, which favours the synthetic reaction and virtually eliminates the secondary hydrolysis of the formed peptide. Along these lines, we have investigated the α-chymotrypsin-catalysed peptide synthesis using esters of *N*-protected amino acids (or peptides) as acyl donors in hydrophilic organic solvents containing only a few percent of water and we have found that such activated esters as the 2,2,2-trifluoroethyl ester are useful for the incorporation of non-protein amino acids such as halogenophenylalanines into peptides.[2] As an extension of this study, we have examined the couplings of α-chymotrypsin's inherently poor amino acid substrates, *e.g.*, Ala, by employing a series of its esters. Table 1 summarises the yields of the desired peptide and the hydrolysis product of the donor ester in the α-chymotrypsin-catalysed coupling of an ester of Z-L-Ala with L-Leu-NH_2 in acetonitrile containing 4% (v/v) Tris buffer (pH 7.8) after 48 h of incubation (Scheme 2),



Scheme 1 Kinetically controlled peptide synthesis: H-E, serine or cysteine protease; R^1CO_2R, carboxy component in the form of an ester; R^2NH_2, amino component.

Scheme 2

together with the relative initial rate (v_{rel}) of consumption of the substrate ester. The reaction conditions adopted here were the same as those determined as optimal ones in the previous investigation.[2] As was expected, the yield of the desired peptide, as well as that of the hydrolysed donor ester, was extremely low even after 48 h when the methyl ester (Entry 1) was used as an acyl donor. This was also the case with other alkyl esters carrying a longer chain (Entries 2, 3, 5–11). By contrast, when halogenated alkyl esters (Entries 12–15) were employed, the coupling efficiency was improved profoundly in accordance with our previous results.[2] As shown in Scheme 1, the acyl-enzyme intermediate is partitioned between aminolysis and hydrolysis, affording the peptide product and the hydrolysis product of the donor ester. Once the acyl-enzyme intermediate is formed, its competitive partitioning is supposed to be unaffected by the ester moiety of the acyl donor, according to

Table 1 α-Chymotrypsin-catalysed couplings of Z-L-Ala-OR with L-Leu-NH_2[a]

Entry	R	v_{rel}[b]	σ^*[c]	Yield (%)[d] Peptide	Yield (%)[d] Z-L-Ala-OH
1	CH_3	1[e]	0	6.7	1.0
2	CH_2CH_3	0.54	−0.100	4.2	0.7
3	$(CH_2)_2CH_3$	0.39	−0.115	2.5	0.5
4	$CH(CH_3)_2$			1.0	0
5	$(CH_2)_3CH_3$	0.53	−0.130	3.4	0.6
6	$CH_2CH(CH_3)_2$	0.76	−0.125	10.2	1.0
7	$(CH_2)_4CH_3$	0.34		3.0	0.4
8	$(CH_2)_5CH_3$	0.24		2.0	0.3
9	C_6H_{11}-*c*	0.41		3.4	0.6
10	$CH_2C_5H_9$-*c*	0.74		6.3	0.9
11	$CH_2C_6H_{11}$-*c*	0.68	−0.06	4.9	0.7
12	CH_2CF_3	17.5	+0.92	82.4	8.6[g]
13	$CH_2CF_2CF_3$	8.8		63.2	6.8[g]
14	CH_2CH_2Cl	3.4	+0.385	30.5	3.8[g]
15	CH_2CCl_3	14.1		87.8	6.9[g]
16	CH_2Ph	4.2	+0.215	28.6	4.2
17	$CH_2C_6H_4NO_2$-4	18.7		79.3	9.2
18	$CH_2C_6H_4CN$-4	11.4		72.7	9.6
19	$CH_2C_6H_4Cl$-4	5.3		33.6	4.0
20	$CH_2C_6H_4OMe$-4	2.8		19.2	2.2
21	$CH_2C_5H_4N$	29.1		89.9	10.0
22	$(CH_2)_2Ph$	0.61	+0.080	9.1	0.7
23	$(CH_2)_3Ph$	0.42	+0.02	6.8	0.5
24	CH_2CN	29.6	+1.30	88.3	6.4[g]
25	CH_2OCH_3	6.7	+0.64[f]	45.6	5.6[g]
26	CH_2COCH_3	6.9	+0.60	43.0	7.0[g]
27	CH_2COPh	0.51		7.5	4.1
28	CH_2CO_2Et	3.0		24.0	4.1
29	CH_2CONH_2	133		88.4	10.9[g]
30	$CH_2CONHCH_3$	110		89.1	10.8[g]
31	$CH_2CON(CH_3)_2$	4.8		34.4	5.2

[a] Coupling conditions: Z-L-Ala-OR (0.05 mmol), L-Leu-NH_2·HCl (0.2 mmol), TEA (0.2 mmol), immobilised α-chymotrypsin on Celite (150 mg), acetonitrile (2 ml), 0.05 M Tris buffer (pH 7.8) (80 μl), 30 °C. [b] The initial rate of consumption of the substrate ester was determined through the periodical assay of the reaction mixture over 8 h (4 h and 1 h in the case of moderately fast reacting esters and fast reacting esters, respectively). [c] Ref. 11. [d] After 48 h of incubation. [e] 2.79×10^{-2} mM h^{-1} mg^{-1}. The value in Table 1 of ref. 3*b* was misprinted and should be corrected. [f] Ref. 12. [g] Corrected for non-enzymic hydrolysis (see Table 2).

the simplified reaction scheme. It may have some effect in the case when the leaving alkoxy group (OR) is not completely detached from the acyl-enzyme intermediate before the deacylation by nucleophiles occurs. In practice, the competing hydrolysis of acyl donors was little accelerated when the halogenated alkyl esters were used. The use of the 2,2,2-trifluoroethyl and 2,2,2-trichloroethyl esters (Entries 12 and 15, respectively) was especially effective.

With the benzyl ester[5] (Entry 16) the peptide yield was improved to a considerable extent, and its *p*-nitro or *p*-cyano derivative (Entries 17 and 18, respectively) had a further ameliorating effect, which was comparable to that shown by the above mentioned halogenated alkyl esters. The effect of substituents on the initial rate of consumption of the substrate ester resembled that on the rates of alkaline hydrolysis of substituted-benzyl acetates[6] or benzoates.[7] Thus, when the *p*-methoxybenzyl ester (Entry 20) was employed as an acyl donor, the peptide yield was lower than that obtained using the benzyl ester itself. It is interesting to note that the 4-pyridylmethyl ester (Entry 21) afforded a result similar to that given by the *p*-nitrobenzyl ester. The insertion of a methylene group between the phenyl ring and the ester oxygen atom resulted in a substantial decrease in the peptide yield (Entries 22 and 23), indicating the inductive (electron-withdrawing) effect of the phenyl ring. The importance of the electronic effect, over the steric effect which must be responsible mainly for the stability of the enzyme–substrate (ES) complex, was supported by the fact that the cyclohexylmethyl or cyclopentylmethyl ester (Entry 11 or 10, respectively), which had a steric demand more similar to that of the benzyl ester, exerted no marked influence compared with the other alkyl esters. Accordingly, substituted alkyl esters bearing electron-withdrawing groups other than halogens and phenyl rings were examined next. The cyanomethyl ester[8] (Entry 24) increased the peptide yield significantly. In this case, no peptide product was obtained non-enzymically, while spontaneous hydrolysis of the donor ester occurred to some extent (see Table 2).† The methoxymethyl and acetonyl esters (Entries 25 and 26, respectively) had a considerable ameliorating effect. Finally, it was gratifying to find that the carbamoylmethyl ester (Entry 29) was superior to the halogenated alkyl esters. The use of this ester as an enzyme substrate was reported some decades ago.[9] It was later examined as a donor ester in the α-chymotrypsin-catalysed coupling of Z- or Boc-Phe conducted in aqueous organic media.[10] The chief concern at that time was to take advantage of the better solubility of this ester in the aqueous phase for the coupling of the α-chymotrypsin's good amino acid substrates. The superiority of the carbamoylmethyl ester can be seen from its reaction profile as compared with those of the other esters, *e.g.*, the methyl and trifluoroethyl esters (Fig. 1): even after 2 h of incubation the peptide yield reached to 77% and the maximum yield (88%) was attained after *ca.* 6 h.

When the log v_{rel}-value for each ester substrate in Table 1 was plotted against the polar substituent constant, σ^*,[11] for R, which is a measure of its polar or inductive effect, a rough proportionality was found between them (Fig. 2): the higher

† Non-enzymic peptide synthesis was not detected when the other activated esters were employed, while a small amount of the donor ester was hydrolysed non-enzymically in some cases, as shown in Table 2. In Table 1, corrections were made for non-enzymic hydrolysis.

Table 2 Non-enzymic reactions of Z-L-Ala-OR with L-Leu-NH_2 [a]

R	Yield (%) [b]	
	Peptide	Z-L-Ala-OH
CH_2CF_3	0	1.9
$CH_2CF_2CF_3$	0	2.3
CH_2CH_2Cl	0	0.2
CH_2CCl_3	0	2.0
CH_2CN	0	5.3
CH_2OCH_3	0	2.9
CH_2COCH_3	0	1.6
CH_2CONH_2	0	0.7
$CH_2CONHCH_3$	0	0.1

[a] Reactions were conducted using Z-L-Ala-OR (0.05 mmol), L-Leu-NH_2·HCl (0.2 mmol), and TEA (0.2 mmol) in a solvent composed of acetonitrile (2 ml) and 0.05 M Tris buffer (pH 7.8) (80 μl) at 30 °C. [b] After 48 h.

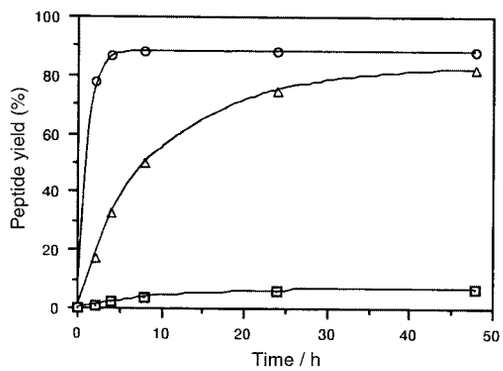


Fig. 1 Reaction profiles in the α-chymotrypsin-catalysed couplings of Z-L-Ala-OR with L-Leu-NH_2. Symbols: ○, R = CH_2CONH_2; △, R = CH_2CF_3; □, R = CH_3.

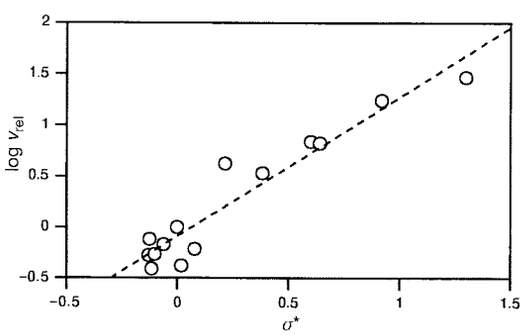


Fig. 2 Plot of log v_{rel} against σ^* of R for the α-chymotrypsin-catalysed coupling of Z-L-Ala-OR with L-Leu-NH_2 (see Table 1).

the electron-withdrawing ability of the R group, the higher the v_{rel}-value. The log v_{rel}-value corresponds to the relative activation free energy which is dependent both on the stability of the ES complex and on the rate of acyl-enzyme formation. The correlation between log v_{rel} and σ^* is indicative of the predominance of the effect of the R group on the acyl-enzyme formation over the binding of the substrate ester onto the enzyme. In the case of the carbamoylmethyl ester, however, its effect on the binding stage must also be relatively important, because the electron-withdrawing ability of this group should not be markedly large compared with other groups, *e.g.*, the acetonyl group ($\sigma^* = +0.60$), though its σ^*-value is not reported in the literature.‡ Carbamoylmethyl esters whose amide hydrogens were substituted by methyl group(s) were examined next (Entries 30 and 31 in Table 1). The monomethylated ester gave almost the same result as the parent ester, while with the dimethylated ester the peptide yield was diminished to a great extent. This result implies the necessity of at least one amide proton of the carbamoylmethyl ester for stabilisation through hydrogen bonding of the ES-complex.

‡ The σ^*-value for the carbamoylmethyl group is estimated to be +0.59 according to the following equation,[13] $pK_a(RCO_2H) = -1.700\sigma^*(R) + 4.644$, by employing the reported K_a-value for propanedioic acid monoamide (2.284×10^{-4} at 25 °C).[14]

Table 3 α-Chymotrypsin-catalysed couplings of Z-Xaa-OR with L-Leu-NH_2 [a]

Xaa	R	Yield (%) [b]	
		Peptide	Z-Xaa-OH
Gly	CH_3	0.4	0.6
	CH_2CF_3	74.3	9.1 [c]
	CH_2Ph	3.9	1.3
	$CH_2C_6H_4NO_2$-4	23.4	3.3
	CH_2CONH_2	90.9	6.0 [c]
L-Ser	CH_3	8.7	1.2
	CH_2CF_3	87.9	0.5 [c]
	CH_2Ph	69.7	4.6
	$CH_2C_6H_4NO_2$-4	92.4	4.6
	CH_2CONH_2	91.3	6.3 [c]
L-Val	CH_3	0	0.3
	CH_2CF_3	0.9	0.8
	CH_2CONH_2	13.6	6.8
D-Ala	CH_3	0	0.8
	CH_2CONH_2	40.6	7.0
L-Pro	CH_2CF_3	0.6	0.5
	CH_2CONH_2	0.6	0.3
Aib	CH_2CONH_2	6.4	1.4

[a] The coupling conditions were the same as described in Table 1, using 0.05 mmol of acyl donor. [b] After 48 h. [c] Corrected for non-enzymic hydrolysis. The yields (%) of Z-Xaa-OH formed after 48 h of incubation under the same conditions as described in Table 2 were as follows: Z-Gly-OCH_2CF_3, 1.8; Z-Gly-OCH_2CONH_2, 2.1; Z-L-Ser-OCH_2CF_3, 10.2; Z-L-Ser-OCH_2CONH_2, 2.2. The corresponding values in Table 2 of ref. 3*b* were uncorrected ones.

The ameliorating effect of the carbamoylmethyl ester was observed also in the couplings of other amino acid residues as carboxy components. As shown in Table 3, Gly behaved as a poorer amino acid substrate for α-chymotrypsin than did L-Ala, the yield of the desired peptide being negligible even after 48 h of incubation when the methyl ester was used as the acyl donor. In this case also, a marked enhancement of peptide yield by the use of the trifluoroethyl ester and the superiority of the carbamoylmethyl ester over other activated esters were demonstrated. The same trend was observed also among the methyl, trifluoroethyl and carbamoylmethyl esters of L-Ser. But a feature observed only in this particular case was that the *p*-nitrobenzyl ester was as good a donor ester as the carbamoylmethyl ester. The methyl ester of Z-L-Val failed in the production of the desired peptide, probably due to the steric hindrance caused by the β-branching in the side chain, while the carbamoylmethyl ester managed to give the peptide product, albeit in low yield. This was also the case with another bulky amino acid, α-aminoisobutyric acid (Aib, 2-amino-2-methylpropanoic acid),[15] the peptide yield being even lower. Furthermore, the use of the carbamoylmethyl ester allowed even the coupling of a D-amino acid as the carboxy component.[16] Thus, the Z-D-Ala ester gave the desired D-L-peptide in moderate yield, although the v_{rel}-value was less than one sixth of that for the L-counterpart. On the other hand, it proved to be quite difficult to obtain the peptide of L-Pro even by the use of the carbamoylmethyl ester.

In summary, α-chymotrypsin's narrow substrate specificity was broadened by the use of the carbamoylmethyl ester as the acyl donor in the kinetically controlled approach of peptide synthesis mediated by this protease.

Table 4 Elemental analyses of new compounds

Compound	Molecular formula	C (%) Found (required)	H (%) Found (required)
Z-L-Ala-OCH_2CH_3	$C_{13}H_{17}NO_4$	62.21 (62.14)	6.87 (6.82)
Z-L-Ala-$O(CH_2)_2CH_3$	$C_{14}H_{19}NO_4$	63.11 (63.38)	7.28 (7.21)
Z-L-Ala-$OCH(CH_3)_2$	$C_{14}H_{19}NO_4$	63.39 (63.38)	7.23 (7.21)
Z-L-Ala-$O(CH_2)_3CH_3$	$C_{15}H_{21}NO_4$	64.22 (64.50)	7.60 (7.58)
Z-L-Ala-$OCH_2CH(CH_3)_2$	$C_{15}H_{21}NO_4$	64.33 (64.50)	7.62 (7.58)
Z-L-Ala-$O(CH_2)_4CH_3$	$C_{16}H_{23}NO_4$	65.33 (65.51)	7.98 (7.90)
Z-L-Ala-$O(CH_2)_5CH_3$	$C_{17}H_{25}NO_4$	66.29 (66.43)	8.23 (8.20)
Z-L-Ala-OC_6H_{11}-*c*	$C_{17}H_{23}NO_4$	66.61 (66.86)	7.61 (7.59)
Z-L-Ala-$OCH_2C_5H_9$-*c*	$C_{17}H_{23}NO_4$	66.71 (66.86)	7.75 (7.59)
Z-L-Ala-$OCH_2C_6H_{11}$-*c*	$C_{18}H_{25}NO_4$	67.23 (67.69)	7.82 (7.89)
Z-L-Ala-OCH_2CF_3	$C_{13}H_{14}F_3NO_4$	51.46 (51.15)	4.63 (4.62)
Z-L-Ala-$OCH_2CF_2CF_3$	$C_{14}H_{14}F_5NO_4$	47.51 (47.33)	3.92 (3.97)
Z-L-Ala-OCH_2CH_2Cl	$C_{13}H_{16}ClNO_4$	54.70 (54.65)	5.63 (5.64)
Z-L-Ala-OCH_2CCl_3	$C_{13}H_{14}Cl_3NO_4$	44.30 (44.03)	4.06 (3.98)
Z-L-Ala-OCH_2Ph	$C_{18}H_{19}NO_4$	68.81 (69.00)	6.19 (6.11)
Z-L-Ala-$OCH_2C_6H_4NO_2$-4	$C_{18}H_{18}N_2O_6$	60.41 (60.33)	5.03 (5.06)
Z-L-Ala-$OCH_2C_6H_4CN$-4	$C_{19}H_{18}N_2O_4$	67.14 (67.45)	5.24 (5.36)
Z-L-Ala-$OCH_2C_6H_4Cl$-4	$C_{18}H_{18}ClNO_4$	62.17 (62.16)	5.19 (5.22)
Z-L-Ala-$OCH_2C_6H_4OMe$-4	$C_{19}H_{21}NO_5$	66.29 (66.46)	6.14 (6.16)
Z-L-Ala-$O(CH_2)_2Ph$	$C_{19}H_{21}NO_4$	69.66 (69.71)	6.48 (6.47)
Z-L-Ala-$O(CH_2)_3Ph$	$C_{20}H_{23}NO_4$	70.25 (70.36)	6.78 (6.79)
Z-L-Ala-OCH_2CN	$C_{13}H_{14}N_2O_4$	59.41 (59.54)	5.54 (5.38)
Z-L-Ala-OCH_2OCH_3	$C_{13}H_{17}NO_5$	58.21 (58.42)	6.47 (6.41)
Z-L-Ala-OCH_2COCH_3	$C_{14}H_{17}NO_5$	60.24 (60.21)	6.19 (6.13)
Z-L-Ala-OCH_2CO_2Et	$C_{15}H_{19}NO_6$	58.05 (58.25)	6.16 (6.19)
Z-L-Ala-$OCH_2CONHCH_3$	$C_{14}H_{18}N_2O_5$	57.03 (57.14)	6.24 (6.16)
Z-L-Ala-$OCH_2CON(CH_3)_2$	$C_{15}H_{20}N_2O_5$	58.08 (58.43)	6.64 (6.54)
Z-L-Ser-OCH_2CONH_2	$C_{13}H_{16}N_2O_6$	52.64 (52.70)	5.54 (5.44)
Z-L-Val-OCH_2CONH_2	$C_{15}H_{20}N_2O_5$	58.10 (58.43)	6.73 (6.54)
Z-L-Pro-OCH_2CONH_2	$C_{15}H_{18}N_2O_5$	58.63 (58.82)	5.91 (5.92)
Z-Aib-OCH_2CONH_2	$C_{14}H_{18}N_2O_5$	56.87 (57.14)	6.02 (6.16)
Z-D-Ala-L-Leu-NH_2	$C_{17}H_{25}N_3O_4$	61.08 (60.88)	7.36 (7.51)
Z-Gly-L-Leu-NH_2	$C_{16}H_{23}N_3O_4$	59.72 (59.80)	7.17 (7.21)
Z-L-Ser-L-Leu-NH_2	$C_{17}H_{25}N_3O_5 \cdot 1/2H_2O$	56.61 (56.65)	7.18 (7.27)
Z-L-Val-L-Leu-NH_2	$C_{19}H_{29}N_3O_4$	62.69 (62.79)	8.07 (8.04)
Z-L-Pro-L-Leu-NH_2	$C_{19}H_{27}N_3O_4$	62.84 (63.14)	7.53 (7.53)
Z-Aib-L-Leu-NH_2	$C_{18}H_{27}N_3O_4$	61.61 (61.87)	8.01 (7.79)

Experimental

All solvents were distilled, and dried over molecular sieves prior to use. L-Leu-NH_2·HCl was purchased from Kokusan Chemical Works (Japan). α-Chymotrypsin (type II, ex bovine pancreas) was purchased from Sigma and had a specific activity of 48 units per mg solid with *N*-Bz-Tyr ethyl ester. It was immobilised on Celite using a pH 7.8 buffer as described before.[2] IR spectra were recorded on a Nicolet N-750B FT-IR spectrometer using attenuated total reflectance (ATR). ^{1}H NMR spectra (300 MHz) were recorded with a Varian Unity 300 spectrometer using $CDCl_3$ as solvent with TMS as internal standard unless otherwise stated. Mps were determined on a Yamato MP-21 apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 digital polarimeter. $[a]_D$-Values are given in units of 10^{-1} deg cm^2 g^{-1}. Elemental analyses of new compounds are compiled in Table 4. Petroleum spirit refers to the fraction with distillation range 30–70 °C.

Preparation of *N*-Z-amino acid esters

The physical and ^{1}H NMR data of the esters of Z-L-Ala prepared as described below are compiled in Table 5, and those of the carbamoylmethyl esters of other *N*-Z-amino acids are in Table 6.

(i) The methyl esters were prepared through treatment of the corresponding *N*-Z-amino acids with an ethereal solution of diazomethane in a nearly quantitative yield. Z-Gly-OMe: oil; Z-L-Ser-OMe: oil; $[a]_D^{25}$ −14.0 (*c* 1.0, MeOH) {lit.,[17] mp 45–46 °C; $[a]_D^{22}$ −12.5 (*c* 1, MeOH)}; Z-L-Val-OMe: oil; $[a]_D^{25}$ −19.4 (*c* 1.0, MeOH) {lit.,[18] mp 54–55 °C; $[a]_D^{20}$ −18.9 (*c* 1, MeOH)}; Z-D-Ala-OMe: mp 47–48.5 °C; $[a]_D^{25}$ +33.8 (*c* 1.0, MeOH).

(ii) The following esters were prepared through the reaction of an *N*-Z-amino acid (2.2 mmol) with the corresponding alcohol (2.5 mmol for the group A esters or 2.1 mmol for the group B esters) in the presence of DMAP (135 mg, 1.1 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) (460 mg, 2.4 mmol) in DCM (8 ml) according to the procedure of Dhaon *et al.*:[19] (A) ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, cyclohexyl, cyclopentylmethyl, cyclohexylmethyl, 2,2,2-trifluoroethyl, 2,2,3,3,3-pentafluoropropyl, 2-chloroethyl, or 2,2,2-trichloroethyl ester; (B) *n*-pentyl, *n*-hexyl, benzyl, 4-chlorobenzyl, 4-methoxybenzyl, 2-phenylethyl, 3-phenylpropyl, or ethoxycarbonylmethyl ester.

Z-Gly-OCH_2CF_3: mp 73–73.5 °C (Found: C, 49.56; H, 4.10; N, 4.87. $C_{12}H_{12}F_3NO_4$ requires C, 49.49; H, 4.15; N, 4.81%); Z-Gly-OCH_2Ph: mp 71–72 °C (Found: C, 68.26; H, 5.84; N, 4.70. $C_{17}H_{17}NO_4$ requires C, 68.22; H, 5.72; N, 4.68%); Z-L-Ser-OCH_2CF_3: mp 73–77 °C; $[a]_D^{25}$ −14.2 (*c* 1.0, MeOH) (Found: C, 48.87; H, 4.36; N, 4.48. $C_{13}H_{14}F_3NO_5$ requires C, 48.60; H, 4.39; N, 4.36%); Z-L-Val-OCH_2CF_3: mp 58 °C; $[a]_D^{25}$ −17.8 (*c* 1.0, MeOH) (Found: C, 53.97; H, 5.42; N, 4.24. $C_{15}H_{18}F_3NO_4$ requires C, 54.05; H, 5.44; N, 4.20%); Z-L-Pro-OCH_2CF_3: oil; $[a]_D^{25}$ −48.8 (*c* 1.0, MeOH) (Found: C, 54.17; H, 4.87; N, 4.23. $C_{15}H_{16}F_3NO_4$ requires C, 54.38; H, 4.87; N, 4.23%).

(iii) The following esters were prepared *via* reaction of the Ce salt of an *N*-Z-amino acid with the corresponding chloride:[20] the carbamoylmethyl, cyanomethyl, methoxymethyl, acetonyl, benzoylmethyl, *N*-methylcarbamoylmethyl, or *N*,*N*-dimethyl-

Table 5 Physical and ^{1}H NMR data of Z-L-Ala-OR

R	Mp (T/°C)[a]	$[a]_D^{25}$ (10^{-1} deg cm^2 g^{-1})[b]	IR (ATR) (ν_{max}/cm^{-1})[c]	^{1}H NMR δ_H ($CDCl_3$)
CH_3[d]	46–47.5 (A)	−33.8	3339, 1752, 1691	1.41 (3H, d, *J* 6.9), 3.75 (3H, s), 4.39 (1H, quint, *J* 6.9), 5.11 (2H, s), 5.24–5.40 (1H, br d), 7.26–7.40 (5H, m)
CH_2CH_3	oil	−31.1	3334, 1720	1.27 (3H, t, *J* 7.2), 1.41 (3H, d, *J* 6.9), 4.20 (2H, q, *J* 7.2), 4.36 (1H, quint, *J* 6.9), 5.11 (2H, s), 5.26–5.44 (1H, br), 7.25–7.40 (5H, m)
$(CH_2)_2CH_3$	oil	−32.0		0.94 (3H, t, *J* 7.5), 1.42 (3H, d, *J* 7.2), 1.57–1.74 (2H, m), 4.10 (2H, t, *J* 6.5), 4.39 (1H, quint, *J* 7.2), 5.11 (2H, s), 5.28–5.42 (1H, br d), 7.26–7.41 (5H, m)
$CH(CH_3)_2$	oil	−28.4	3347, 1720	1.24 (3H, d, *J* 6.1), 1.26 (3H, d, *J* 6.1), 1.40 (3H, d, *J* 6.9), 4.33 (1H, quint, *J* 6.9), 4.97–5.05 (1H, sept, *J* 6.1), 5.11 (2H, s), 5.28–5.42 (1H, br d), 7.26–7.41 (5H, m)
$(CH_2)_3CH_3$	oil	−29.0	3349, 1721	0.93 (3H, t, *J* 7.5), 1.27–1.43 (2H, m), 1.41 (3H, d, *J* 7.2), 1.54–1.69 (2H, m), 4.14 (2H, t, *J* 6.6), 4.38 (1H, quint, *J* 7.2), 5.11 (2H, s), 5.26–5.41 (1H, br d), 7.27–7.42 (5H, m)
$CH_2CH(CH_3)_2$	oil	−31.3	3349, 1722	0.93 (6H, d, *J* 6.6), 1.43 (3H, d, *J* 7.2), 1.86–2.02 (1H, m), 3.82–4.00 (2H, m), 4.40 (1H, quint, *J* 7.2), 5.11 (2H, s), 5.28–5.42 (1H, br d), 7.26–7.40 (5H, m)
$(CH_2)_4CH_3$	oil	−29.1	3342, 1723	0.90 (3H, t, *J* 6.9), 1.23–1.37 (4H, m), 1.41 (3H, d, *J* 7.2), 1.59–1.69 (2H, m), 4.13 (2H, t, *J* 6.8), 4.38 (1H, quint, *J* 7.2), 5.11 (2H, s), 5.27–5.41 (1H, br d), 7.26–7.40 (5H, m)
$(CH_2)_5CH_3$	oil	−27.6	3355, 1723	0.89 (3H, t, *J* 6.9), 1.19–1.40 (6H, m), 1.42 (3H, d, *J* 7.2), 1.57–1.68 (2H, m), 4.13 (2H, t, *J* 6.5), 4.38 (1H, quint, *J* 7.2), 5.11 (2H, s), 5.28–5.40 (1H, br d), 7.26–7.38 (5H, m)
C_6H_{11}-*c*	57.5–58.5 (B)	−31.1	3363, 1751, 1694	1.17–1.90 (10H, m), 1.41 (3H, d, *J* 7.2), 4.35 (1H, quint, *J* 7.2), 4.74–4.86 (1H, m), 5.11 (2H, s), 5.28–5.41 (1H, br d), 7.26–7.40 (5H, m)
$CH_2C_5H_9$-*c*	oil	−30.5	3335, 1722	1.10–1.82 (8H, m), 1.42 (3H, d, *J* 7.2), 2.11–2.37 (1H, m), 3.93–4.10 (2H, m), 4.38 (1H, quint, *J* 7.2), 5.11 (2H, s), 5.28–5.42 (1H, br d), 7.27–7.40 (5H, m)
$CH_2C_6H_{11}$-*c*	oil	−25.7	3357, 1735, 1709	0.84–1.82 (11H, m), 1.42 (3H, d, *J* 7.2), 3.85–4.30 (2H, m), 4.39 (1H, quint, *J* 7.2), 5.11 (2H, s), 5.37 (1H, br d, *J* 6.9), 7.26–7.42 (5H, m)
CH_2CF_3	59 (B)	−28.6	3331, 1773, 1683	1.46 (3H, d, *J* 7.2), 4.34–4.54 (2H, m), 4.54–4.70 (1H, m), 5.12 (2H, AB q, *J* 13), 5.16–5.29 (1H, br), 7.26–7.41 (5H, m)
$CH_2CF_2CF_3$	56–56.5 (A)	−27.6	3351, 1756, 1687	1.45 (3H, d, *J* 7.5), 4.40–4.57 (2H, m), 4.72 (1H, AB q, *J* 13), 5.12 (2H, AB q, *J* 12), 5.18–5.29 (1H, br d), 7.27–7.41 (5H, m)
CH_2CH_2Cl	53–54 (B)	−30.3	3330, 1752, 1691	1.44 (3H, d, *J* 7.2), 3.67 (2H, t, *J* 5.6), 4.23–4.51 (3H, m), 5.11 (2H, s), 5.21–5.36 (1H, br), 7.25–7.45 (5H, m)
CH_2CCl_3	oil	−31.7	3327, 1763, 1705	1.51 (3H, d, *J* 7.2), 4.54 (1H, quint, *J* 7.2), 4.65 and 4.93 (2H, AB q, *J* 12), 5.12 (2H, AB q, *J* 12), 5.23–5.36 (1H, br d), 7.27–7.40 (5H, m)
CH_2Ph	38–39 (B)	−31.4	3338, 1747, 1688	1.42 (3H, d, *J* 7.2), 4.44 (1H, quint, *J* 7.2), 5.11 (2H, s), 5.17 (2H, AB q, *J* 12), 5.29–5.40 (1H, br d), 7.24–7.43 (10H, m)
$CH_2C_6H_4NO_2$-4	100.5–101 (C)	−17.2	3324, 1744, 1688	1.46 (3H, d, *J* 7.2), 4.47 (1H, quint, *J* 7.2), 5.12 (2H, s), 5.15–5.34 (1H, br), 5.27 (2H, AB q, *J* 9.3), 7.24–7.40 (5H, m), 7.55 (2H, d, *J* 8.4), 8.22 (2H, d, *J* 8.4)
$CH_2C_6H_4CN$-4	91.5–92.5 (D)	−20.0	3333, 1743, 1685	1.44 (3 H, d, *J* 7.2), 4.46 (1H, quint, *J* 7.2), 5.11 (2H, s), 5.22 (2H, AB q, *J* 15), 5.24–5.34 (1H, br d), 7.23–7.40 (5H, m), 7.43 and 7.62 (4H, AB q, *J* 8.0)
$CH_2C_6H_4Cl$-4	88.5–89.5 (A)	−25.4	3300, 1736, 1689	1.41 (3H, d, *J* 7.2), 4.43 (1H, quint, *J* 7.2), 5.10 (2H, s), 5.13 (2H, AB q, *J* 12), 5.21–5.35 (1H, br d), 7.15–7.40 (9H, m)
$CH_2C_6H_4OMe$-4	61 (D)	−29.8	3336, 1735, 1686	1.40 (3H, d, *J* 7.2), 3.81 (3H, s), 4.40 (1H, quint, *J* 7.2), 5.10 (4H, s), 5.27–5.37 (1H, br d), 6.88 and 7.28 (4H, AB q, *J* 8.4), 7.30–7.40 (5H, m)
$CH_2C_5H_4N$[e]	113–114 (E)	−18.8 (DMF)	3215, 1747, 1709	1.47 (3H, d, *J* 7.2), 4.49 (1H, quint, *J* 7.2), 5.12 (2H, s), 5.19 (2H, AB q, *J* 13), 5.32–5.45 (1H, br d), 7.23 (2H, d, *J* 5.4), 7.27–7.40 (5H, m), 8.60 (2H, d, *J* 5.4)
$(CH_2)_2Ph$	oil	−32.6	3307, 1734, 1682	1.34 (3H, d, *J* 7.2), 2.95 (2H, t, *J* 6.9), 4.27–4.43 (3H, m), 5.10 (2H, s), 5.24–5.36 (1H, br d), 7.16–7.41 (10H, m)
$(CH_2)_3Ph$	49–50 (D)	−24.7	3304, 1738, 1686	1.42 (3H, d, *J* 7.7), 1.95–2.04 (2H, m), 2.68 (2H, t, *J* 12), 4.16 (2H, t, *J* 6.6), 4.39 (1H, quint, *J* 7.7), 5.11 (2H, s), 5.24–5.37 (1H, br d), 7.11–7.40 (10H, m)
CH_2CN	oil	−45.3	3329, 1762, 1705	1.46 (3H, d, *J* 7.2), 4.46 (1H, quint, *J* 7.2), 4.76 (2H, AB q, *J* 17), 5.11 (2H, AB q, *J* 14), 5.21–5.34 (1H, br d), 7.26–7.42 (5H, m)
CH_2OCH_3	oil	−36.6	3352, 1801, 1713	1.45 (3H, d, *J* 6.9), 3.46 (3H, s), 4.42 (1H, quint, *J* 6.9), 5.11 (2H, AB q, *J* 12), 5.25 and 5.33 (2H, AB q, *J* 6.0), 5.28–5.40 (1H, br), 7.27–7.40 (5H, m)
CH_2COCH_3	68–68.5 (D)	−43.0	3333, 1756, 1725, 1692	1.51 (3H, d, *J* 7.2), 2.16 (3H, s), 4.50 (1H, quint, *J* 7.2), 4.64 and 4.78 (2H, AB q, *J* 17), 5.11 (2H, AB q, *J* 13), 5.23–5.38 (1H, br d), 7.27–7.41 (5H, m)
CH_2COPh[f]	156–157 (E)	−24.8 ($CHCl_3$)	3367, 1754, 1687	1.58 (3H, d, *J* 7.2), 4.58 (1H, quint, *J* 7.2), 5.12 (2H, AB q, *J* 12), 5.29 and 5.51 (2H, AB q, *J* 17), 5.34 (1H, br d, *J* 8.1), 7.26–7.40 (5H, m), 7.44–7.97 (5H, m)
CH_2CO_2Et	50–52.5 (A)	−46.6	3329, 1761, 1744, 1686	1.28 (3H, t, *J* 7.2), 1.50 (3H, d, *J* 7.2), 4.22 (2H, q, *J* 7.2), 4.50 (1H, quint, *J* 7.2), 4.56 and 4.76 (1H, AB q, *J* 16), 5.12 (2H, AB q, *J* 13), 5.23–5.38 (1H, br d), 7.26–7.41 (5H, m)
CH_2CONH_2[g]	71.5–72.5 (F)	−16.3 (DMF)	3421, 3328, 1747, 1711, 1686	1.46 (3H, d, *J* 7.2), 4.33 (1H, quint, *J* 7.2), 4.63 (2H, AB q, *J* 16), 5.10 (2H, AB q, *J* 11), 5.44 (1H, br), 5.73 (1H, br), 6.78 (1H, br), 7.28–7.41 (5H, m)
$CH_2CONHCH_3$	98.5–99 (E)	−21.8		1.46 (3H, d, *J* 7.2), 2.78 (3H, d, *J* 4.8), 4.31 (1H, quint, *J* 7.2), 4.64 (2H, AB q, *J* 16), 5.12 (2H, AB q, *J* 12), 5.31–5.42 (1H, br d), 6.80 (1H, br), 7.28–7.41 (5H, m)
$CH_2CON(CH_3)_2$	oil	−40.7	3308, 1753, 1716, 1663	1.53 (3H, d, *J* 7.2), 2.95 (6H, s), 4.51 (1H, quint, *J* 7.2), 4.67 and 4.87 (2H, AB q, *J* 14), 5.11 (2H, AB q, *J* 11), 5.44 (1H, br d), 7.27–7.39 (5H, m)

[a] Recrystallisation solvent: A, cyclohexane; B, CCl_4–petroleum spirit; C, benzene–petroleum spirit; D, CCl_4; E, EtOAc; F, EtOAc–petroleum spirit. [b] *c* 1.0 in MeOH unless otherwise noted. [c] Wavenumbers of only the peaks ascribed to the N–H and C=O stretching vibrations are quoted. [d] Lit.,[19] mp 45–46 °C; $[a]_D^{22}$ −33.9 (*c* 2, MeOH). [e] 4-Picolyl ester. Lit.,[24] mp 111–112.5 °C; $[a]_D^{20}$ −20.2 (*c* 1.0, DMF). [f] Lit.,[25] mp 154–155 °C; $[a]_D^{18}$ −26.5 (*c* 2.0, $CHCl_3$). [g] Lit.,[26] mp 74–75 °C; $[a]_D^{24}$ −16.8 (*c* 2.0, DMF).

Table 6 Physical and ^{1}H NMR data of Z-Xaa-OCH_2CONH_2

Xaa	Mp (T/°C)[a]	$[\alpha]_D^{25}$ (10^{-1} deg cm^2 g^{-1})[b]	IR (ATR) (ν_{max}/cm^{-1})[c]	^{1}H NMR
Gly[d]	104–105.5		3446, 3328, 1770, 1745, 1687	δ_H (DMSO-d_6) 3.89 (2H, d, J 6.0), 4.45 (2H, s), 5.04 (2H, s), 7.22–7.37 (6H, m), 7.44 (1H, br), 7.75 (1H, t, J 6.0)
L-Ser	122.5–124	−16.6	3308, 1766, 1684	δ_H (DMSO-d_6) 3.61–3.79 (2H, m), 4.24–4.31 (1H, m), 4.47 (2H, AB q, J 15), 5.04 (2H, s), 5.14 (1H, t, J 5.7), 7.24–7.46 (7H, m), 7.68 (1H, d, J 7.8)
L-Val	89–89.5	−17.5	3445, 3317, 1750, 1707, 1666	δ_H ($CDCl_3$) 0.98 (3H, d, J 6.9), 1.02 (3H, d, J 6.9), 2.05–2.24 (1H, m), 4.16 (1H, dd, J 7.1 and 6.3), 4.55 and 4.73 (2H, AB q, J 16), 5.10 (2H, s), 5.36 (1H, br d, J 7.1), 5.73 (1H, br), 6.70 (1H, br), 7.28–7.42 (5H, m)
L-Pro	oil	−53.8	3339, 1754, 1678	δ_H ($CDCl_3$) 1.87–2.36 (4H, m), 3.50–3.69 (2H, m), 4.37–4.47 (1H, m), 4.65 (2H, AB q, J 16), 5.14 (2H, s), 5.49 (1H, br), 7.21 (1H, br), 7.28–7.43 (5H, m)
Aib	153.5–154.5		3373, 3284, 1745, 1675	δ_H (DMSO-d_6) 1.39 (6H, s), 4.40 (2H, s), 5.01 (2H, s), 7.18 (1H, br), 7.30–7.40 (6H, m), 7.94 (1H, br)

[a] From EtOAc. [b] c 1.0 in MeOH. [c] Wavenumbers of only the peaks ascribed to the N–H and C=O stretching vibrations are quoted. [d] Lit.,[26] mp 105–108 °C.

carbamoylmethyl ester. The preparation of the carbamoylmethyl ester of Z-L-Ala is described as a typical example. To a solution of Z-L-Ala (447 mg, 2 mmol) in MeOH (4 ml) was added a solution of Cs_2CO_3 (326 mg, 1 mmol) in water (1.5 ml), and the mixture was evaporated under reduced pressure. After repeated evaporation to dryness with toluene, the residue was stored over P_4O_{10} in a vacuum desiccator. The Ce salt of Z-L-Ala thus obtained was mixed with 2-chloroacetamide (187 mg, 2 mmol) in DMF (8 ml) and the mixture was stirred at 60 °C overnight. The mixture was distributed between EtOAc (40 ml) and water (10 ml), and the aqueous phase was extracted further with EtOAc (2 × 10 ml), and the combined organic extracts were washed successively with 1 M aq. $NaHCO_3$ and water and dried over Na_2SO_4. Evaporation of the solvent afforded white crystals, which were recrystallised from EtOAc–petroleum spirit; yield 485 mg (87%).

The benzyl ester of Z-L-Ser was also prepared by the same route: mp 83–83.5 °C; $[\alpha]_D^{25}$ +3.8 (c 1.0, $CHCl_3$) {lit.,[21] mp 83–84 °C; $[\alpha]_D^{20}$ +5.1 (c 2.95, $CHCl_3$)}.

(iv) The 4-nitrobenzyl and 4-cyanobenzyl esters were prepared *via* reaction of the triethylamine (TEA) salt of an *N*-Z-amino acid with the corresponding bromide.[22] The preparation of the 4-nitrobenzyl ester of Z-L-Ala is described as a typical example. A mixture of Z-L-Ala (447 mg, 2 mmol), 4-nitrobenzyl bromide (432 mg, 2 mmol), and TEA (202 mg, 2 mmol) in EtOAc (7 ml) was refluxed for 8.5 h. The white precipitate was filtered off and the filtrate was washed successively with 2 M HCl, water, 1 M aq. $NaHCO_3$ and brine, and dried over Na_2SO_4. Evaporation of the solvent *in vacuo* afforded white crystals, which were recrystallised from benzene–petroleum spirit; yield 464 mg (65%).

Z-Gly-$OCH_2C_6H_4(4NO_2)$: mp 111 °C (lit.,[23] 107–109.5 °C); Z-L-Ser-$OCH_2C_6H_4(4NO_2)$: mp 117.5–118 °C; $[\alpha]_D^{25}$ −10.8 (c 1.0, MeOH) {lit.,[21] mp 116–117 °C; $[\alpha]_D^{20}$ −11.0 (c 1, MeOH)}.

(v) The 4-picolyl ester[24] of Z-L-Ala was prepared by stirring a mixture of Z-L-Ala (447 mg, 2 mmol), 4-picolyl chloride hydrochloride (392 mg, 2 mmol), and 1,1,3,3-tetramethylguanidine (461 mg, 4 mmol) in DMF (7 ml) at 90 °C for 9 h. Similar work-up as above yielded pale yellow crystals, which were recrystallised from EtOAc; yield 392 mg (62%).

Preparation of *N*-substituted 2-chloroacetamides

***N*-Methyl-2-chloroacetamide.** Methylamine hydrochloride (14.9 g, 0.22 mol) was dissolved in 24% (w/v) aq. NaOH (100 ml) and 1,2-dichloroethane (100 ml) was added. To the stirred mixture was added a solution of chloroacetyl chloride (24.9 g, 0.22 mol) in 1,2-dichloroethane (25 ml) below −5 °C over a period of 40 min. After stirring for 30 min, the organic layer was separated, washed successively with 0.5 M HCl and water, and dried over Na_2SO_4. Evaporation of the solvent *in vacuo* afforded white crystals, which were recrystallised from benzene–petroleum spirit; yield 4.9 g (21%); mp 37–38.5 °C (lit.,[27] 45–46 °C) (Found: C, 33.77; H, 5.50; N, 13.36. C_3H_6ClNO requires C, 33.51; H, 5.62; N, 13.62%); δ_H ($CDCl_3$) 2.90 (3H, d, J 5.1 Hz), 4.06 (2H, s), 6.70 (1H, br).

***N*,*N*-Dimethyl-2-chloroacetamide.** This compound was prepared from dimethylamine hydrochloride and chloroacetyl chloride in the same manner as above. An oil obtained after washings was distilled under reduced pressure; yield 33%; bp 100 °C at 14 mmHg (lit.,[27] 98.5–99.5 °C at 11 mmHg) (Found: C, 39.71; H, 6.75; N, 11.46. C_4H_8ClNO requires C, 39.52; H, 6.63; N, 11.52%); δ_H ($CDCl_3$) 2.99 (3H, s), 3.06 (3H, s), 4.09 (2H, s).

Preparation of authentic dipeptides

The authentic samples of *N*-protected dipeptide amides, Z-Xaa-L-Leu-NH_2, were prepared as illustrated below for the preparation of Z-L-Ala-L-Leu-NH_2. To a stirred solution of Z-L-Ala (447 mg, 2.0 mmol), L-Leu-NH_2·HCl (333 mg, 2.0 mmol), TEA (203 mg, 2.0 mmol) and HOBT (270 mg, 2.0 mmol) in DMF (8 ml) was added EDC·HCl (384 mg, 2.0 mmol) under ice-cooling. After stirring at this temperature for 2 h and then at ambient temperature overnight, the reaction mixture was diluted with EtOAc, washed successively with 1 M HCl, water, 1 M aq. $NaHCO_3$ and brine, and dried over Na_2SO_4. Evaporation of the solvent *in vacuo* afforded white crystals, which were recrystallised from aq. EtOH; yield 532 mg (94%). Physical and ^{1}H NMR data of Z-Xaa-L-Leu-NH_2 thus prepared are compiled in Table 7.

HPLC analyses

The liquid chromatograph employed was a Shimadzu (Japan) LC-10AS instrument, equipped with a Rheodyne 7725i sample injector and a Shimadzu SPD-10A variable-wavelength UV monitor. A Shimadzu C-R6A data processor was used for data acquisition and processing. The amounts of the donor ester, peptide and hydrolysis product of the donor ester were determined by HPLC analysis on an ODS column under the following conditions: column, Cosmosil 5C_{18} (4.6 mm id × 150 mm, Nacalai Tesque, Japan); mobile phase, 30–55% aq. MeOH containing H_3PO_4 (0.01 M); flow rate, 1.0 ml min^{-1}; column temperature, 30 °C; detection, UV at 254 nm.

Peptide synthesis mediated by immobilised α-chymotrypsin in acetonitrile with low water content

The preparation of Z-L-Ala-L-Leu-NH_2 is described as a typical example. A mixture of Z-L-Ala-OCH_2CONH_2 (14 mg, 0.05 mmol), L-Leu-NH_2·HCl (33 mg, 0.2 mmol), TEA (28 μl, 0.2 mmol) and the immobilised enzyme on Celite (150 mg,

Table 7 Physical and ^{1}H NMR data of Z-Xaa-L-Leu-NH_2

Xaa	Mp (*T*/°C)[a]	$[\alpha]_D^{25}$ (10^{-1} deg cm^2 g^{-1})[b]	^{1}H NMR δ_H (DMSO-d_6)
L-Ala[c]	191–191.5	−18.3 (DMF)[d]	0.82 (3H, d, *J* 6.5), 0.85 (3H, d, *J* 6.5), 1.17 (3H, d, *J* 7.2), 1.40–1.46 (2H, t-like), 1.48–1.65 (1H, m), 3.93–4.11 (1H, quint-like), 4.16–4.24 (1H, q-like), 5.00 (2H, s), 6.97 (1H, s), 7.28 (1H, s), 7.19–7.40 (5H, m), 7.46 (1H, br d, *J* 8.4), 7.77 (1H, br d, *J* 8.4)
D-Ala	189.5–191	−2.8	0.81 (3H, d, *J* 6.0), 0.85 (3H, d, *J* 6.0), 1.18 (3H, d, *J* 6.9), 1.25–1.61 (3H, m), 3.99–4.10 (1H, quint-like), 4.14–4.23 (1H, m), 5.00 (2H, s), 7.04 (1H, s), 7.25 (1H, s), 7.21–7.39 (5H, m), 7.47 (1H, d, *J* 7.2), 8.01 (1H, d, *J* 8.4)
Gly	124–126	−19.1	0.82 (3H, d, *J* 6.5), 0.86 (3H, d, *J* 6.5), 1.36–1.64 (3H, m), 3.55–3.72 (2H, m), 4.16–4.28 (1H, q-like), 5.02 (2H, s), 7.00 (1H, s), 7.24–7.40 (6H, m), 7.44 (1H, t, *J* 5.7), 7.88 (1H, d, *J* 8.1)
L-Ser	184–184.5	−19.6	0.82 (3H, d, *J* 6.3), 0.86 (3H, d, *J* 6.3), 1.40–1.50 (2H, m), 1.50–1.67 (1H, m), 3.56 (2H, t, *J* 5.9), 4.04–4.11 (1H, q-like), 4.16–4.24 (1H, m), 5.02 (2H, s), 5.04 (1H, t, *J* 5.4), 7.04 (1H, s), 7.27–7.40 (7H, m), 7.96 (1H, d, *J* 8.7)
L-Val	260–261	−43.7	0.80–0.88 (12H, m), 1.32–1.48 (2H, m), 1.48–1.66 (1H, m), 1.87–2.04 (1H, m), 3.84 (1H, dd, *J* 8.7 and 6.9), 4.21–4.29 (1H, m), 5.02 (2H, s), 6.96 (1H, s), 7.30–7.40 (7H, m), 7.80 (1H, d, *J* 8.4)
L-Pro	189–194	−78.2	0.67–0.95 (6H, m), 1.29–1.64 (3H, m), 1.71–1.89 (3H, m), 2.01–2.21 (3H, m), 3.27–3.52 (2H, m), 4.13–4.32 (2H, m), 4.78–5.13 (2H, m), 6.94 and 6.99 (1H, s + s), 7.16–7.41 (6H, m), 7.95 and 7.98 (1H, d + d, *J* 7.5)
Aib	137.5–139	−28.4	0.79 (3H, d, *J* 6.0), 0.84 (3H, d, *J* 6.0), 1.31 (6H, s), 1.44–1.60 (3H, m), 4.08–4.18 (1H, m), 4.99 (2H, AB q, *J* 13), 7.00 (1H, s), 7.11 (1H, s), 7.26–7.38 (5H, m), 7.57 (1H, s), 7.65 (1H, br d, *J* 8.4)

[a] From aq. EtOH. [b] *c* 1.0 in MeOH unless otherwise noted. [c] Lit.,[10] mp 187–189 °C; $[\alpha]_D^{22}$ −17.1 (*c* 1, DMF). [d] −43.7 in MeOH.

corresponding to 4.7 mg of α-chymotrypsin) was incubated with shaking (180 strokes min^{-1}) in a solvent composed of acetonitrile (2 ml) and 0.05 M Tris buffer (pH 7.8) (83 μl) at 30 °C. An aliquot (10 μl) of the reaction mixture was withdrawn periodically, diluted with AcOH (100 μl) and injected onto the HPLC column.

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