Chiral Analysis of Synthetic Peptides: High Performance Liquid Chromatography of Diastereoisomeric Carbamoyl Esters derived from N-Terminal Cleavage

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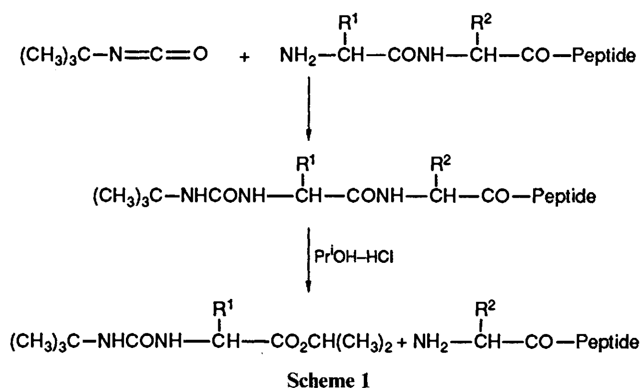
A cleavage routine for chiral analysis has revealed that the N-terminal residue in model dipeptides, after derivatisation with a chiral isocyanate reagent, can be cleaved off as the *N*-carbamoyl ester under relatively mild conditions ($MeOH/SOCl_2$/60 °C for 3 h) free from inherent problems of racemisation. The diastereoisomeric carbamoyl amino acid esters produced can be separated and identified by HPLC. The methodology has been optimised for a number of representative amino acids, including tyrosine, histidine, methionine and tryptophan which have side-functions capable of complicating the procedure.

The chemical synthesis of peptides and small proteins has evolved to become a routine and accessible goal in the hands of expert practitioners. There is a vast literature on peptide synthesis, as can be gleaned from recent reviews.[1] In the development of satisfactory syntheses, racemisation of the activated residue during the coupling stages has always been an undesirable complication in the making of biologically active peptides. Attempts over many years to eliminate racemisation are reflected by the number of racemisation tests[2] which have been developed to monitor the 'racemisation potential' of the chosen reaction, conditions and reagents. Consequently, in contemporary synthetic routines the stepwise addition of 'urethane'-protected amino acid residues, suitably activated by proven coupling agents, has served to reduce the chances of loss of chiral integrity.[3] However, certain residues, such as histidine[4] and cysteine[5] still demand caution, and fragment condensation using a chiral residue at the C-terminal position can still be susceptible to racemisation. Difficulties with the linking of the first amino acid residue to anchor links in solid phase peptide synthesis are still deemed to be vulnerable to racemisation.[6]

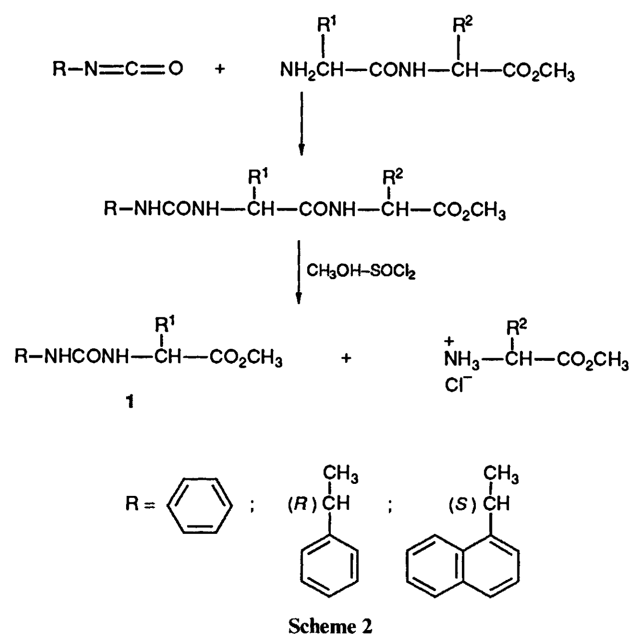
The utmost in chiral quality control would be a check on the chiral integrity of the added amino acid residue after it has undergone attachment to its neighbouring residue. It is to this end that we have undertaken an assessment of whether an N-terminal analysis technique based on the conventional 'Edman' sequencing technique could be developed. A procedure offering a variation on this philosophy has also been reported[7] recently, where the 'Edman' cleavage has been used to generate peptides whose N-terminal residues are then analysed *via* coupling with 5-fluoro-2,4-dinitro-L-phenylalanine. The latter is a modification of Marfey's reagent, which has also been used[8] as a precolumn derivatising agent for HPLC separation of diastereoisomeric amino acid derivatives. In previous publications we reported[9,10] that the use of chiral isothiocyanate reagents directly analogous to the Edman reagents[10] gave rise to thiazolinone intermediates that were inherently chirally unstable. Replacing the isothiocyanate by an isocyanate overcame this problem, but only limited success[9] was achieved in developing a satisfactory separation of diastereoisomeric hydantoin derivatives. The yields of hydantoin derivatives were also poor. In a development related to our procedure, König and co-workers[11] investigated the cleavage of *N*-alkylcarbamoyl derivatised peptides, to their corresponding N-terminal carbamoyl esters using propan-2-ol/hydrochloric acid for cleavage, as summarised in Scheme 1. The released carbamoyl amino acid esters were readily analysed and their chiral purity detected on chiral GC capillary columns. Up to 10



Scheme 1

residues could be sequenced, but some amino acid residues such as lysine, histidine, arginine and tryptophan failed to give sufficiently volatile derivatives.

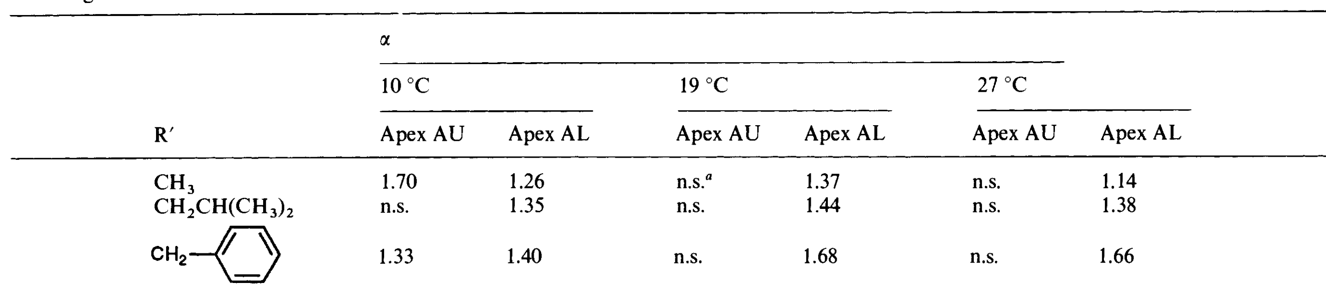
The thrust of our current approach is summarised in Scheme 2 and includes (*a*) an assessment of the separation of chiral



Scheme 2

isomers of carbamoyl amino acid esters on HPLC columns, (*b*) optimisation of the cleavage conditions and (*c*) the effect of reactive side chains on the analysis.

Table 1 Separation of $PhNHCONHCH(R')CO_2CH_3$ on chiral columns. Mobile phase 10% Pr^iOH–cyclohexane, flow rate 1 cm^3 min^{-1}, detector wavelength 258 nm.



R′	α 10 °C Apex AU	Apex AL	19 °C Apex AU	Apex AL	27 °C Apex AU	Apex AL
CH_3	1.70	1.26	n.s.[a]	1.37	n.s.	1.14
$CH_2CH(CH_3)_2$	n.s.	1.35	n.s.	1.44	n.s.	1.38
CH_2–Ph	1.33	1.40	n.s.	1.68	n.s.	1.66

[a] No separation.

Table 2 HPLC Analysis of (*R*)-α-methylbenzylcarbamoyl DL-amino acid methyl esters

Mobile phase	Residue	Retention time/min	Separation α	R_s
$MeOH–H_2O$ 60:40	L-Val/D-Val	10.85/11.49	1.07	0.82
	L-Trp/D-Trp	13.34/14.03	1.05	0.73
	L-Leu/D-Leu	14.42/16.30	1.15	1.07
	L-Phe/D-Phe	16.64/18.23	1.11	1.62
	L-His/D-His (N^{α},N^{Im}-disubstituted)	32.04/33.61	1.05	0.83
	L-Met/D-Met	11.29	1.00	0.00
	L-Lys/D-Lys (N^{α},N^{ϵ}-disubstituted)	30.76	1.00	0.00
$MeOH–H_2O$ 40:60	L-Ala/D-Ala	21.42/22.46	1.06	0.87
$MeCN–H_2O$ 40:60	D-Tyr/L-Tyr	8.82/9.25	1.06	0.57
$MeOH–H_2O$ 20:80	D-Ser/L-Ser	19.04/20.64	1.09	0.89

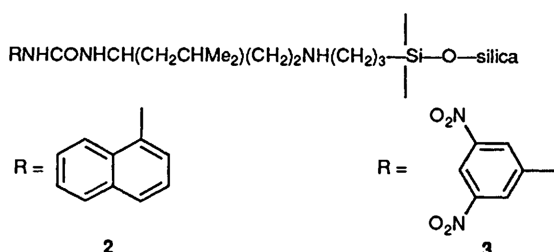
Results and Discussion

Separation of the Carbamoyl Amino Acid Isomers on HPLC Columns.—Key to the methodology is the need for an efficient means of assessing the chiral purity of the carbamoyl esters **1**. When the derivatising agent was phenyl isocyanate (*i.e.* R = Ph in Scheme 2), the derived carbamoyl esters were analysed on Pirkle-type chiral HPLC columns.[12] Separations achieved for L and D enantiomers have been expressed in Table 1 in terms of the selectivity factor α, and resolution R_s defined as

$$\alpha = \frac{T_1 - T_0}{T_2 - T_0}; \qquad R_s = 2 \times \frac{T_2 - T_1}{l_1 + l_2}$$

where T_1 and T_2 represent retention times (in min) of the enantiomeric carbamoyl esters and T_0 the retention time of the solvent, and l_1, l_2 are the widths (in min) of the first and second peaks eluted respectively.

The Apex AL column with chiral ligand **2** proved superior to the Apex AU column (chiral ligand **3**) in the three examples studied. But in general the peaks were extremely broad and



could never compare satisfactorily with the separation achieved by the combination of a chiral carbamoyl derivative [R = (*R*)-Ph-CH(CH_3)-NHCO or (*S*)-Naphthyl-CH(CH_3)-NHCO in Scheme 2] on a reversed-phase column.

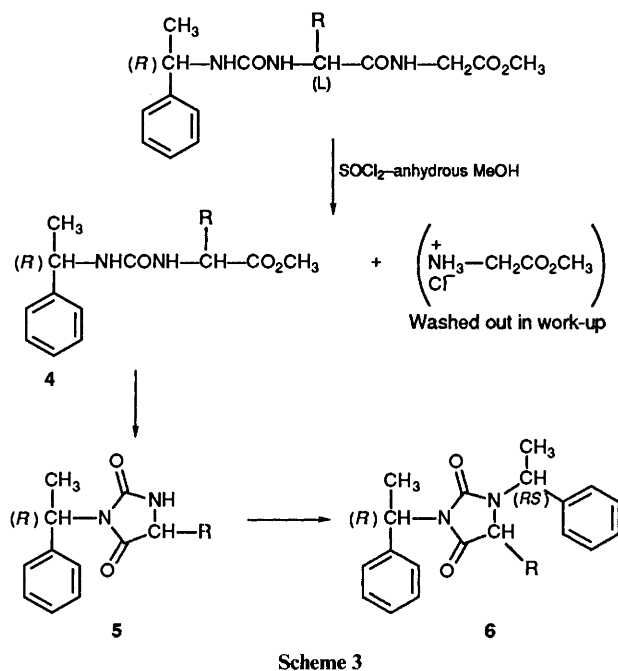
Tables 2 and 3 summarise the separations of the diastereoisomers on an ODS C_{18} column. All the examples studied, except lysine, could be separated, although methionine required the extra resolving power of the naphthylethyl derivative. Amino acid residues with polar side chains were eluted more rapidly than residues with more 'hydrophobic' side chains and consequently base-line resolution of diastereoisomers in the former cases was more difficult.

Optimisation of the Cleavage Conditions.—Preliminary studies had shown that HPLC traces derived from the cleavage of dipeptide esters could be explained by the reaction sequence summarised in Scheme 3. Optimisation amounted to maximising the yield of the carbamoyl ester **4** and minimising its further conversion to a hydantoin **5** and another compound giving a late peak on HPLC, which never interfered with the analysis, but nevertheless seemed always to be prominent at retention time R_t *ca.* 25 min. Hydantoin formation was favoured during hydrolysis of the carbamoyl peptide by dilute hydrochloric acid[9] and by elevated temperatures. Controlling the conditions to methanol–thionyl chloride at 60 °C for 3 h ensured complete cleavage of the carbamoyl dipeptide esters in all cases with only minimal conversion to hydantoin.

Since most runs were monitored utilising small quantities, it proved difficult to characterise the delayed peak at R_t *ca.* 25 min. However, a larger scale study on the (*R*)-(+)-α-methylbenzylhydantoin of glycine (**5**, R = H) showed that when this hydantoin was subjected to the optimised cleavage conditions

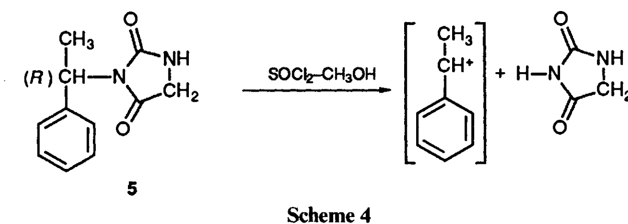
Table 3 HPLC Analysis of (*R*)-α-methylbenzylcarbamoyl DL-dipeptide methyl esters and (*S*)-1-(1-naphthyl)ethylcarbamoyl-methionine and -methionylglycine methyl esters

Mobile phase	Residues	Retention time/min	Separation α	Separation R_s
	(*R*)-α-methylbenzylcarbamoyl derivatives of:			
$MeOH-H_2O$ 60:40	-L-Val-Gly-OMe	7.61		
	-L-Trp-Gly-OMe	12.25		
	-L-Leu-Gly-OMe	12.20	1.08	0.77
	-D-Leu-Gly-OMe	12.98		
	-L-Phe-Gly-OMe	13.04	1.00	0.00
	-D-Phe-Gly-OMe	13.04		
	-L-His-Gly-OMe ($N^{\alpha,Im}$-disubstituted)	25.63		
	-L-Met-Gly-OMe	8.82		
$MeOH-H_2O$ 40:60	-L-Ala-Gly-OMe	17.61	1.06	0.75
	-D-Ala-Gly-OMe	18.56		
$MeCN-H_2O$ 40:60	-L-Tyr-Gly-OMe (N^{α},*O*-disubstituted)	14.16		
$MeOH-H_2O$ 60:40	L-Ser-Gly-OMe (N^{α},*O*-disubstituted)	15.52		
$MeCN-H_2O$ 40:60	L-Ser-Gly-OMe (N^{α},*O*-disubstituted)	20.53		
	(*S*)-1-(1-naphthyl)ethylcarbamoyl derivatives of:			
$MeOH-H_2O$ 60:40	-D-Met-OMe/L-Met-OMe	25.03/28.18	1.13	1.96
$MeOH-H_2O$ 60:40	-DMet-Gly-OMe	19.86	1.07	0.76
	-LMet-Gly-OMe	21.13		
$MeOH-H_2O$ 70:30	-DMet-Gly-OMe	10.47	1.09	0.98
	-LMet-Gly-OMe	11.30		



Scheme 3

($MeOH/SOCl_2$/60 °C/3 h) a peak at R_t = 28 min was obtained, which could be attributed to the di-*N*-substituted hydantoin **6**, R = H. A plausible summary of events is given in Scheme 4, and was supported by an NMR analysis, which showed that in the 3–4 ppm region of the spectrum, signals reminiscent of a diastereoisomeric mixture were present, explained by the racemisation of the $Ph\overset{+}{C}HCH_3$ centre before it re-attacks the hydantoin ring. This, at present, is our most plausible explanation for the later HPLC peak, which would be expected to be slightly different for each amino acid.



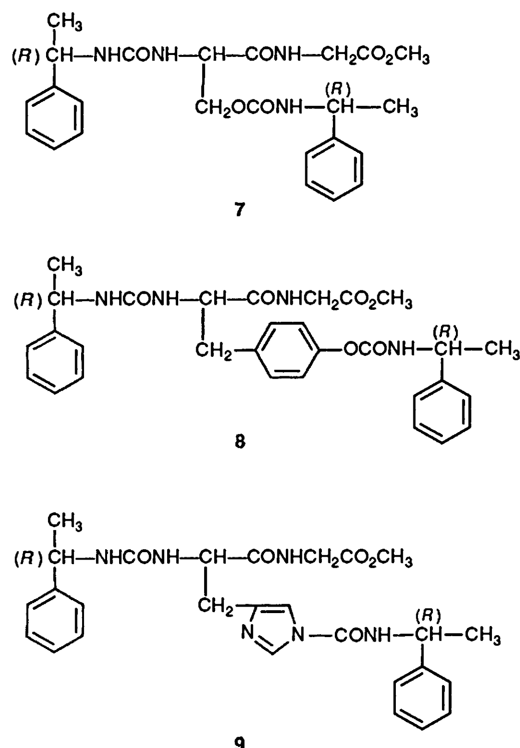
Scheme 4

The Effect of Reactive Side-chains on the Analysis.—The carbamoyl dipeptide esters without a 'reactive' side chain could be readily synthesised in high purity, as shown by NMR and sharp single peaks on HPLC, by reacting the corresponding dipeptide methyl esters with one mole equivalent of (*R*)-α-methylbenzyl isocyanate. The optimised conditions of cleavage gave the derived (*R*)-α-methylbenzylcarbamoyl amino acid methyl esters in good yield for the leucyl, alanyl and phenylalanyl residues. Within the 3 h reaction time the valyl residue was a little more sluggish, giving a 90% cleavage yield. The tryptophyl residue could be analysed successfully on the basis of only a 20% yield of the carbamoyl tryptophyl ester being present (owing to acid breakdown).

For the methionyl residue, both α-methylbenzyl- and 1-(1-naphthyl)ethylcarbamoylmethionylglycine methyl esters were prepared, as cleavage of the latter gave a superior derivative for chiral analysis on the HPLC columns. More side reactions occurred in this case, attributed to oxidation and alkylation of the side-chain thioether group. These did not hamper the chiral analysis, provided efficient washing of the column was carried out after each run.

It was proposed that under analytical conditions, the side chains of tyrosine, serine and histidine might react with two

moles of the derivatising agent, (*R*)-(+)-methylbenzyl isocyanate. With two mole equivalents of the isocyanate, the carbamoyl dipeptide esters **7–9** were readily synthesised. The possible



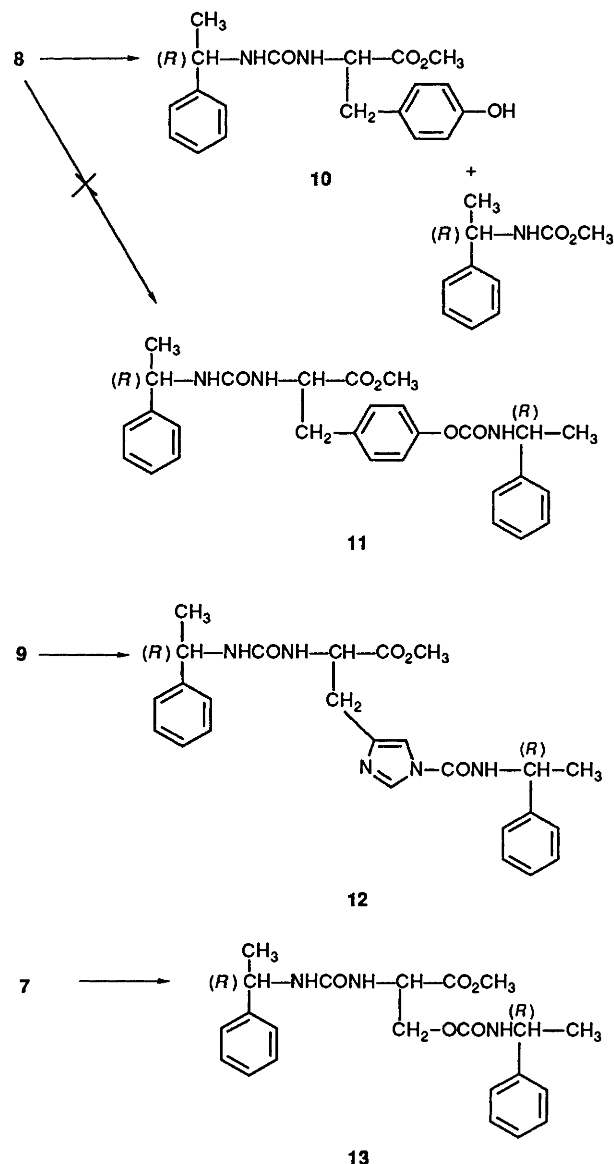
cleavage pathways are outlined in Scheme 5. For tyrosine the chiral analysis could be based on the HPLC of **10**, since the urethane side-chain was cleaved in the methanolic conditions to yield **10** and not **11**.

The histidyl residue could be analysed as the dicarbamoylhistidyl methyl ester **12**, giving unambiguous evidence that there was no racemisation during the cleavage of this highly susceptible residue. For the seryl residue the carbamoyl-derivatised side-chain remained intact (as **13**) for the analysis, in contrast to the tyrosine case. Unfortunately, the diastereoisomeric disubstituted seryl methyl ester could not be resolved on the HPLC columns under all conditions tried, and needs further optimisation.

In the majority of the cases studied here, there is no doubt that the *N*-carbamoyl amino acid methyl esters lend themselves to a sensitive method for chiral analysis. When side reactions do occur in the series studied, they do not interfere in the region of the HPLC trace where the esters are eluted. No racemisation of the N-terminal residues could be detected in any of the samples studied.

Experimental

^{1}H NMR spectra were recorded at 250 MHz on a Bruker WM250 spectrometer using Me_4Si as internal standard. Peak multiplicities are reported as s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet. Mass spectra were determined using a VG Analytical VG 12-250 instrument for low resolution EI and CI measurements. Accurate masses were measured on a VG Analytical ZAB-E instrument at the SERC Mass Spectrometer Centre, Swansea. C, H and N microanalyses were carried out at the University of Wales College, Cardiff. Optical rotations were measured on a Perkin-Elmer 141 automatic polarimeter using the Na D line (589 nm) or the Hg line (546 nm) and are in 10^{-1} deg cm^2 g^{-1}. M.p.s were measured on a Gallenkamp hot-stage apparatus and are uncorrected.



Scheme 5

HPLC measurements were carried out using LDC/Milton Roy instrumentation. Columns had dimensions of 25 × 0.43 cm and the flow rate was 1 cm^3 min^{-1} at pressures of 3000–4000 p.s.i. The chiral columns (Apex AL and Apex AU) were purchased or loaned from Jones Chromatography Ltd. TLC was carried out on Merck silica gel $60F_{254}$ or on Fluka alumina UV_{254} plates. Either ethyl acetate–chloroform or ethyl acetate–light petroleum were used for development of the chromatograms. For flash chromatography, alumina (Phase Sep, 100 > mesh) and 15% ethyl acetate in light petroleum (30–40 °C) were used.

(*R*)-(+)-α-Methylbenzyl isocyanate (99%) and (*S*)-(+)-1-(naphthyl)ethyl isocyanate (99%) were used as purchased from Aldrich Chemicals.

Synthesis of Phenylcarbamoyl Amino Acid Methyl Esters.— Each amino acid methyl ester hydrochloride[13] (1 mmol) was suspended in anhydrous dichloromethane (20 cm^3). Triethylamine (1.1 mmol) and phenyl isocyanate (1.1 mmol) were added at 0 °C under stirring. After 15 min the mixture was left at room temperature overnight, under stirring. The reaction mixture was then washed with 1 mol dm^{-3} HCl (10 cm^3), saturated aqueous $NaHCO_3$ (10 cm^3) and water (2 × 10 cm^3). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to

dryness to produce a white powder, recrystallised from dichloromethane–light petroleum to yield each of the following compounds.

Phenylcarbamoyl-L-*alanine methyl ester*. M.p. 122–123 °C (65% yield); $[\alpha]_D^{20}$ −1.8° (*c* 0.1 in MeOH) (Found: C, 59.6; H, 6.3; N, 12.5. $C_{11}H_{14}N_2O_3$ requires C, 59.5; H, 6.3; N, 12.6%); $\delta_H(CDCl_3)$ 1.38–1.41 (3 H, d, CH_3), 3.72 (3 H, s, OCH_3), 4.52–4.60 (1 H, q, C*H*), 7.00–7.06 (2 H, m, 2 × N*H*) and 7.21–7.32 (5 H, m, C_6H_5); *m/z* $(M + H)^+$, 223.

Phenylcarbamoyl-D-*alanine methyl ester*. M.p. 122–125 °C (70% yield); $[\alpha]_D^{20}$ +2.5° (*c* 0.1 in MeOH); spectral data as above.

Phenylcarbamoyl-L-*leucine methyl ester*. M.p. 106 °C (75% yield); $[\alpha]_D^{20}$ −23.2° (*c* 0.1 in MeOH); $\delta_H(CDCl_3)$ 0.92–0.97 (6 H, 2 × d, 2 × CH_3), 1.50–1.69 [3 H, m, $CH_2CH(CH_3)_2$ overlapping with $CH_2CH(CH_3)_2$], 3.73 (3 H, s, OCH_3), 4.56–4.62 (1 H, q, C*H*), 7.03–7.06 (2 H, m, 2 × N*H*) and 7.22–7.31 (5 H, m, C_6H_5); *m/z* $(M + H)^+$, 265.

Phenylcarbamoyl-D-*leucine methyl ester*. M.p. 107 °C (72% yield); $[\alpha]_D^{20}$ +22.3° (*c* 0.1 in MeOH); spectral data as above.

Phenylcarbamoyl-L-*phenylalanine methyl ester*. M.p. 112–114 °C (43% yield); $[\alpha]_D^{20}$ −19.6° (*c* 0.1 in MeOH) (Found: C, 68.4; H, 6.0; N, 9.4. $C_{17}H_{18}N_2O_3$ requires C, 68.5; H, 6.0; N, 9.4%); $\delta_H(CDCl_3)$ 2.95–3.15 (2 H, q, $CHCH_2$-Ph), 3.69 (3 H, s, OCH_3), 4.79–4.84 (1 H, q, C*H*), 5.6–6.0 (2 H, v br, 2 × N*H*) and 6.99–7.33 (10 H, m, 2 × C_6H_5); *m/z* $(M + H)^+$, 299.

Phenylcarbamoyl-D-*phenylalanine methyl ester*. M.p. 113–114 °C (70% yield); $[\alpha]_D^{20}$ +18.9° (*c* 0.1 in MeOH); other spectral data as above.

Synthesis of (R)-α-*Methylbenzylcarbamoyl Amino Acid Methyl Esters*.—Starting with the appropriate amino acid methyl ester hydrochloride[13] (1.0 mmol), suspended in anhydrous dichloromethane, followed by addition of triethylamine (1.1 mmol) and (*R*)-(+)-α-methylbenzyl isocyanate (1.1 mmol) under stirring at room temperature, the following were prepared using the same work up as for the phenylcarbamoyl analogues.

(R)-α-*Methylbenzylcarbamoyl*-L-*alanine methyl ester*. M.p. 101–102 °C (65% yield); $[\alpha]_D^{20}$ +3.8° (*c* 0.1 in MeOH), $[\alpha]_{546}^{20}$ +40.0° (*c* 0.1 in CH_2Cl_2); $\delta_H(CDCl_3)$ 1.25–1.28 [3 H, d, $CHCH_3$(Ala)], 1.37–1.39 (3 H, d, $PhCHCH_3$), 3.65 (3 H, s, OCH_3), 4.35–4.44 (1 H, q, NHC*H*CO), 4.78–4.86 (1 H, q, PhC*H*CH_3), 5.67 (2 H, br, 2 × N*H*) and 7.17–7.30 (5 H, m, C_6H_5); *m/z* $(M + H)^+$, 251.

(R)-α-*Methylbenzylcarbamoyl*-D-*alanine methyl ester*. M.p. 118–120 °C (63% yield); other spectral data as above.

(R)-α-*methylbenzylcarbamoyl*-L-*leucine methyl ester*. M.p. 92–93 °C (75% yield); $[\alpha]_D^{20}$ +4.1° (*c* 0.1 in MeOH), $[\alpha]_{546}^{20}$ +26.6° (*c* 0.1 in CH_2Cl_2); $\delta_H(CDCl_3)$ 0.80–0.85 [6 H, 2 d, $CH(CH_3)_2$], 1.25–1.52 [6 H, m, overlapping $CH_2CH(CH_3)_2$, $CH_2CH(CH_3)_2$ and $PhCHCH_3$], 3.64 (3 H, s, OCH_3), 4.43 (1 H, t, NHC*H*CH_2), 4.79 (1 H, q, PhC*H*CH_3), 5.35 (1 H, br, N*H*), 5.62 (1 H, br, N*H*) and 7.17–7.30 (5 H, m, C_6H_5); *m/z* $(M + H)^+$, 293.

(R)-α-*Methylbenzylcarbamoyl*-D-*leucine methyl ester*. M.p. 125–126 °C (61% yield); other spectral data as above.

(R)-α-*Methylbenzylcarbamoyl*-DL-*phenylalanine methyl ester*. M.p. 88–93 °C (70% yield); $\delta_H(CDCl_3)$ 1.35–1.39 (3 H, q, $PhCHCH_3$), 2.96–2.98 (2 H, d, CH_2Ph), 3.61–3.63 (3 H, 2 s, OCH_3), 4.69–4.71 (2 H, q, overlapping PhC*H*CH_3 and NHC*H*CO), 6.93 (2 H, br s, 2 × N*H*) and 6.93–7.32 (10 H, m, 2 × C_6H_5); *m/z* $(M + H)^+$, 327.

(R)-α-*Methylbenzylcarbamoyl*-L-*valine methyl ester*. M.p. 82–84 °C (67% yield); $[\alpha]_D^{20}$ +20.2° (*c* 0.1 in MeOH), $[\alpha]_{546}^{20}$ +13.4° (*c* 0.1 in CH_2Cl_2); $\delta_H(CDCl_3)$ 0.76–0.88 [6 H, 2 d, $CH(CH_3)_2$], 1.98–2.06 [1 H, q, $CH(CH_3)_2$], 3.63 (3 H, s, OCH_3), 4.35–4.37 [1 H, d, $CHCH(CH_3)_2$], 4.79–4.87 (1 H, q, PhC*H*CH_3), 5.8–6.0 (2 H, br, 2 × N*H*) and 7.19–7.30 (5 H, m, C_6H_5); *m/z* $(M + H)^+$, 279.

(R)-α-*Methylbenzylcarbamoyl*-D-*valine methyl ester*. M.p. 89–90 °C (69% yield); other spectral data as above.

(R)-α-*Methylbenzylcarbamoyl*-DL-*tryptophan methyl ester*. M.p. 142–143 °C (70% yield); $\delta_H(CDCl_3)$ 1.25–1.31 (3 H, t, $PhCHCH_3$), 3.16–3.18 (2 H, t, $CHCH_2$), 3.55 (3 H, s, OCH_3), 4.68–4.76 (2 H, m, overlapping PhC*H*CH_3 and NHC*H*-CO_2CH_3), 5.0–5.2 (2 H, br, 2 × N*H*), 6.68–7.29 (9 H, m, aromatic protons), 7.45–7.48 (1 H, d, 2-position in indole nucleus) and 8.55–8.65 (1 H, d, 1-NH of indole nucleus); Found: $(M + H)^+$, 366.1818. Calc. for $C_{21}H_{24}N_3O_3$: *M* + 1, 366.1739.

(R)-α-*Methylbenzylcarbamoyl*-L-*serine methyl ester*. M.p. 123–124 °C (60% yield); $[\alpha]_D^{20}$ +21.0° (*c* 0.1 in MeOH), $[\alpha]_{546}^{20}$ +49.6° (*c* 0.1 in CH_2Cl_2); $\delta_H(CDCl_3)$ 1.38–1.40 (3 H, d, $PhCHCH_3$), 3.64 (3 H, s, OCH_3), 3.72–3.80 (3 H, m, overlapping CH_2OH and CH_2OH), 4.43 (1 H, s, NHC*H*CO), 4.80–4.82 (1 H, q, PhC*H*CH_3), 5.5–6.2 (2 H, br, 2 × N*H*) and 7.2–7.29 (5 H, m, C_6H_5); Found: M^+, 266.1267. Calc. for $C_{13}H_{18}N_2O_4$: *M*, 266.1267.

(R)-α-*Methylbenzylcarbamoyl*-D-*serine methyl ester*. M.p. 113–116 °C (58% yield); other spectral data as above.

(R)-α-*Methylbenzylcarbamoyl*-L-*tyrosine methyl ester*. M.p. 143–144 °C (76% yield); $[\alpha]_D^{20}$ 0° (*c* 0.1 in MeOH), $[\alpha]_{546}^{20}$ +51.6° (*c* 0.1 in CH_2Cl_2); $\delta_H(CDCl_3)$ 1.32–1.35 (3 H, d, PhCH-CH_3), 2.86–2.87 (2 H, d, $CH_2C_6H_4OH$), 3.60 (3 H, s, OCH_3), 4.65–4.68 (2 H, m, overlapping PhC*H*CH_3 and NHC*H*CO), 5.34 (1 H, br, N*H*), 5.56 (1 H, br, N*H*), 6.59–6.74 (4 H, dd, C_6H_4OH) and 7.17–7.30 (5 H, m, C_6H_5); Found: $(M + H)^+$, 343.1568. Calc. for $C_{19}H_{23}N_2O_4$: *M* + 1, 343.1579.

(R)-α-*Methylbenzylcarbamoyl*-D-*tyrosine methyl ester*. M.p. 157–159 °C (59% yield); $[\alpha]_D^{20}$ 0° (*c* 0.1 in MeOH), $[\alpha]_{546}^{20}$ −29.9° (*c* 0.1 in CH_2Cl_2); other spectral data as above.

(R)-α-*Methylbenzylcarbamoyl*-L-*methionine methyl ester*. M.p. 103–104 °C (73% yield); $[\alpha]_D^{20}$ +4.6° (*c* 0.1 in MeOH), $[\alpha]_{546}^{20}$ +38.4° (*c* 0.1 in CH_2Cl_2); $\delta_H(CDCl_3)$ 1.42–1.45 (3 H, d, $PhCHCH_3$), 2.0 (3 H, s, SCH_3), 1.78–2.16 (2 H, m, $CH_2CH_2SCH_3$), 2.27–2.33 (2 H, t, $CH_2CH_2SCH_3$), 3.67 (3 H, s, OCH_3), 4.50–4.55 (1 H, q, NHC*H*CO), 4.75–4.78 (1 H, q, PhC*H*CH_3), 5.35 (2 H, br, 2 × N*H*) and 7.21–7.32 (5 H, m, C_6H_5); Found $(M + H)^+$, 311.1429. Calc. for $C_{15}H_{23}N_2O_3S$: *M* + 1, 311.1351.

(R)-α-*Methylbenzylcarbamoyl*-DL-*methionine methyl ester*. M.p. 101–104 °C (75% yield); other spectral data as above.

For amino acids with reactive side chains, the reaction conditions and the work up techniques were as previously described but two mole equivalents of (*R*)-(+)-α-methylbenzyl isocyanate were used per mole of amino acid methyl ester. Derivatives produced in this way were as follows.

$N,^{\alpha}N^{\varepsilon}$-*Bis*[(R)-α-*Methylbenzylcarbamoyl*]-L-*lysine methyl ester*. M.p. 142–144 °C (60% yield); $\delta_H([^2H_6]DMSO)$ 1.16–1.49 (4 H, m, $CH_2CH_2CH_2CH_2NH$), 1.33–1.49 (6 H, d, 2 × $PhCHCH_3$), 1.49–1.65 (2 H, m, $CH_2CH_2CH_2$), 2.50–2.98 (2 H, m, CH_2NH), 3.62 (3 H, s, OCH_3), 4.09–4.17 (1 H, q, NHC*H*CO), 4.71–4.77 (2 H, m, 2 × $PhCHCH_3$), 5.79–5.83 (1 H, t, N*H*), 6.21–6.30 (2 H, 2 d, 2 × N*H*), 6.47–6.51 (1 H, d, N*H*) and 7.20–7.29 (10 H, m, 2 × C_6H_5); Found: $(M + H)^+$, 455.2658. Calc. for $C_{25}H_{35}N_4O$: *M* + 1, 455.2580.

$N,^{\alpha}N^{\varepsilon}$-*Bis*[(R)-α-*Methylbenzylcarbamoyl*]-DL-*lysine methyl ester*. M.p. 119–121 °C (70% yield); other spectral details as above.

$N,^{\alpha}N^{im}$-*Bis*[(R)-α-*Methylbenzylcarbamoyl*]-L-*histidine methyl ester*. M.p. 62–64 °C (72% yield); $[\alpha]_D^{20}$ +2.5° (*c* 0.1 in MeOH), $[\alpha]_{546}^{20}$ +2.7° (*c* 0.1 in CH_2Cl_2); $\delta_H(CDCl_3)$ 1.29–1.37 (3 H, d, $PhCHCH_3$), 1.53–1.58 (3 H, d, $PhCHCH_3$), 2.86–2.88 (2 H, d, $CHCH_2$), 3.59 (3 H, s, OCH_3), 4.57–4.63 (1 H, q, NHC*H*CO), 4.65–4.76 (1 H, q, PhC*H*CH_3), 5.05–5.13 (1 H, q, PhC*H*CH_3), 5.85–5.92 (1 H, m, N*H*), 5.99–6.02 (1 H, d, N*H*), 7.09–7.41 (11 H, d, overlapping 2 × C_6H_5 and imidazole C*H*),

6.3–7.66 (1 H, d, imidazole C*H*), 8.05–8.08 (1 H, d, N*H*); *m*/*z* $(M + H)^+$, 464.

Synthesis of (R)-α-*Methylbenzylcarbamoyl*-L-*leucylglycine Ethyl Ester*.—Much of the fundamental work on optimisation was carried out on this compound and it was therefore synthesised especially for this purpose.

L-Leucylglycine ethyl ester hydrochloride[13] (0.32 mmol) in anhydrous dichloromethane (10 cm^3), was treated with triethylamine (0.4 mmol) and (*R*)-(+)-α-methylbenzyl isocyanate (0.4 mmol) under stirring at room temperature. The reaction mixture was left overnight, then washed with 1 mol dm^{-3} HCl (10 cm^3), saturated aqueous $NaHCO_3$ (10 cm^3) and water (2 × 10 cm^3). After drying, the organic layer on evaporation yielded white crystals (66% yield). Recrystallisation from dichloromethane–light petroleum gave (R)-α-*methylbenzylcarbamoyl*-L-*leucylglycine ethyl ester*, m.p. 177 °C [Found: C, 62.6; H, 8.1; N, 11.7, $C_{19}H_{29}N_3O_4$ requires C, 62.7; H, 8.0; N, 11.6%); $\delta_H(CDCl_3)$ 0.87–0.95 [6 H, 2 d, $CH_2CH(CH_3)_2$], 1.19–1.24 (3 H, t, OCH_2CH_3), 1.37–1.39 (3 H, d, $PhCHCH_3$), 1.42–1.63 [3 H, m, $CH_2CH(CH_3)_2$ overlapping with $CH_2CH(CH_3)_2$], 3.56–3.88 (2 H, m, $NHCH_2CO$), 4.06–4.15 (2 H, q, OCH_2CH_3), 4.40 (1 H, t, NHC*H*CO), 4.77–4.79 (1 H, q, PhC*H*CH_3), 5.93 (2 H, m, 2 × N*H*), 7.12–7.27 (5 H, m, C_6H_5) and 7.57 (1 H, m, N*H*); *m*/*z* $(M + H)^+$, 364.

General Synthesis of the (R)-α-*Methylbenzylcarbamoyldipeptide Methyl Esters*.—These were synthesised as for the dipeptide ethyl ester derivative above, but using the dipeptide methyl ester hydrochlorides[13] as starting material. The following esters were prepared.

(R)-α-*Methylbenzylcarbamoyl*-L-*alanylglycine methyl ester*. M.p. 212–213 °C (65% yield); $\delta_H(CDCl_3)$ 1.26–1.28 (3 H, d, $CHCH_3$), 1.37–1.39 (3 H, d, $PhCHCH_3$), 3.69 (3 H, s, OCH_3), 3.89–3.92 (2 H, d, $NHCH_2CO$), 4.32–4.34 (1 H, q, NHC*H*CO), 4.82–4.85 (1 H, q, PhC*H*CH_3), 7.19–7.20 (5 H, m, C_6H_5) and 7.98 (1 H, t, N*H*).

(R)-α-*Methylbenzylcarbamoyl*-DL-*alanylglycine methyl ester*. M.p. 201–203 °C (70% yield); other spectral data similar to above.

(R)-α-*Methylbenzylcarbamoyl*-L-*leucylglycine methyl ester*. M.p. 151–153 °C (75% yield); $\delta_H(CDCl_3)$ 0.85–0.93 [6 H, 2 d, $CH(CH_3)_2$], 1.32–1.35 (3 H, d, $PhCHCH_3$), 1.48–1.64 [3 H, m, overlapping $CH_2CH(CH_3)_2$ and $CH_2CH(CH_3)_2$], 3.58 (3 H, s, OCH_3), 3.36–3.78 (2 H, m, $NHCH_2CO$), 4.50–4.52 (1 H, q, PhC*H*CH_3), 4.74–4.80 (1 H, t, NHC*H*CO), 6.09–6.11 (1 H, d, N*H*), 6.23 (1 H, br, N*H*), 7.09–7.27 (5 H, m, C_6H_5) and 8.08–8.13 (1 H, t, N*H*); *m*/*z* $(M + H)^+$, 350.

(R)-α-*Methylbenzylcarbamoyl*-DL-*leucylglycine methyl ester*. M.p. 157–159 °C (73% yield); other spectral data similar to above.

(R)-α-*Methylbenzylcarbamoyl*-L-*phenylalanylglycine methyl ester*. M.p. 217–218 °C (67% yield); $\delta_H(CDCl_3)$ 1.25–1.38 (5 H, m, $PhCHCH_3$ overlapping with CH_2Ph), 3.61 (3 H, s, OCH_3), 3.65–3.70 (2 H, m, $NHCH_2CO$), 4.69–4.73 (2 H, m, overlapping PhC*H*CH_3 and NHC*H*CO), 5.60–6.20 (2 H, br, 2 × N*H*), 7.10–7.25 (10 H, m, 2 × C_6H_5) and 7.46 (1 H, s, N*H*); *m*/*z* $(M + H)^+$, 384.

(R)-α-*Methylbenzylcarbamoyl*-D-*phenylalanylglycine methyl ester*. M.p. 207–209 °C (58% yield); other spectral data as above.

(R)-α-*Methylbenzylcarbamoyl*-L-*valylglycine methyl ester*. M.p. 224–225 °C (65% yield); $\delta_H(CDCl_3)$ 0.88–0.96 [6 H, 2 d, $CH(CH_3)_2$], 1.38–1.41 (3 H, d, $PhCHCH_3$), 2.10–2.12 [1 H, q, $CH(CH_3)_2$], 3.62 (3 H, s, OCH_3), 3.86–3.88 (2 H, d, NH-CH_2CO), 4.18–4.23 (1 H, q, PhC*H*CH_3), 4.81–4.87 (1 H, t, NHC*H*CO), 6.08–6.11 (1 H, d, N*H*), 6.42–6.46 (1 H, d, N*H*), 7.17–7.30 (5 H, m, C_6H_5) and 7.83 (1 H, m, N*H*); *m*/*z* $(M + H)^+$, 336.

(R)-α-*Methylbenzylcarbamoyl*-L-*tryptophylglycine methyl ester*. M.p. 120–122 °C (70% yield); $\delta_H(CDCl_3)$ 1.30–1.32 (3 H, d, $PhCHCH_3$), 3.11–3.13 (2 H, d, $CHCH_2$), 3.55–3.58 (5 H, m, overlapping OCH_3 and $NHCH_2CO$), 4.60 (1 H, t, NHC*H*CO), 4.74–4.76 (1 H, q, PhC*H*CH_3), 5.8–6.0 (2 H, br, 2 × N*H*), 6.87 (1 H, s, N*H*), 7.00–7.29 (9 H, m, aromatic protons), 7.48–7.51 (1 H, d, 2-H indole nucleus) and 8.56 (1 H, s, 1-H indole nucleus); *m*/*z* $(M + H)^+$, 423.

(R)-α-*Methylbenzylcarbamoyl*-L-*methionylglycine methyl ester*. M.p. 174–176 °C (72% yield); $\delta_H(CDCl_3)$ 1.36–1.39 (3 H, d, $PhCHCH_3$), 1.88–2.02 (2 H, m, $CH_2CH_2SCH_3$), 2.06 (3 H, s, SCH_3), 2.45–2.51 (2 H, t, $CH_2CH_2SCH_3$), 3.64 (3 H, s, OCH_3), 3.54–3.89 (2 H, 2 d, $NHCH_2CO$), 4.57–4.62 (1 H, t, NHC*H*CO), 4.73–4.78 (1 H, q, PhC*H*CH_3), 7.16–7.27 (5 H, m, C_6H_5) and 7.79 (1 H, s, N*H*); *m*/*z* $(M + H)^+$, 368.

N^α,N^{im}-*Bis*[(R)-α-*Methylbenzylcarbamoyl*]-L-*histidylglycine methyl ester*. This compound was prepared using the standard methodology but more than two mole equivalents of the isocyanate reagent were used to give white crystals, m.p. 157–158 °C (63% yield); $\delta_H([^2H_6]DMSO)$ 1.25–1.28 (3 H, d, $PhCHCH_3$), 1.49–1.52 (3 H, d, $PhCHCH_3$), 2.50 (2 H, s, $CHCH_2$), 3.58 (3 H, s, OCH_3), 3.78–3.81 (2 H, m, $NHCH_2CO$), 4.41–4.46 (1 H, t, C*H*CH_2), 4.67–4.73 (1 H, q, PhC*H*CH_3), 4.99–5.05 (1 H, q, PhC*H*CH_3), 6.01–6.10 (1 H, d, N*H*), 6.61–6.64 (1 H, d, N*H*), 7.18–7.41 (10 H, m, 2 × C_6H_5), 7.45 (1 H, s, imidazole C*H*), 8.21 (1 H, s, imidazole C*H*), 8.23–8.33 (1 H, t, N*H*) and 8.71–8.74 (1 H, d, N*H*).

N^α,O-*Bis*[(R)-α-*Methylbenzylcarbamoyl*]-L-*tyrosylglycine methyl ester*. This compound was prepared using the standard method but more than two mole equivalents of the isocyanate reagent were used to give white crystals, m.p. 129–132 °C (51% yield); $\delta_H(CDCl_3)$ 1.23–1.30 (3 H, d, $PhCHCH_3$), 1.26–1.33 (3 H, d, $PhCHCH_3$), 2.59–2.91 (2 H, m, $CHCH_2$), 3.62 (3 H, s, OCH_3), 3.54–3.91 (2 H, m, $NHCH_2CO$), 4.13–4.48 (1 H, m, NHC*H*CO), 4.50–4.84 (2 H, m, 2 × PhC*H*CH_3), 5.96–6.14 (2 H, m, 2 × N*H*), 6.40–6.50 (2 H, d, 2 × N*H*), 6.61–7.04 (4 H, dd, $CH_2C_6H_4O$) and 7.10–7.46 (10 H, m, 2 × C_6H_5).

N^α,O-*Bis*[(R)-α-*Methylbenzylcarbamoyl*]-L-*serylglycine methyl ester*. This compound was also synthesised using excess isocyanate reagent to give an uncrystallisable oil (yield 63%); $\delta_H(CDCl_3)$ 1.43–1.46 (3 H, d, $PhCHCH_3$), 1.56–1.59 (Ph-$CHCH_3$), 3.62–3.66 (5 H, m, overlapping $NHCH_2CO$ and OCH_3), 4.03–4.07 (1 H, q, NHC*H*CO), 4.69–4.80 (2 H, q, 2 × PhC*H*CH_3), 4.82 (2 H, m, 2 × N*H*), 5.16 (2 H, m, 2 × N*H*) and 7.13–7.39 (10 H, m, 2 × C_6H_5).

Synthesis of (S)-1-(1-*Naphthyl*)*ethylcarbamoyl Amino Acid and Dipeptide Methyl Esters*.—The appropriate methyl ester hydrochloride (1 mmol) was suspended in anhydrous dichloromethane, then triethylamine (1.1 mmol) and (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate (1.2 mmol) were added under stirring at room temperature. The reaction mixture was left overnight, then washed in turn with 1 mol dm^{-3} HCl (10 cm^3), saturated aqueous $NaHCO_3$ (10 cm^3) and water (2 × 10 cm^3). Work up involved the same procedure as described before to yield a white solid which was recrystallised from dichloromethane–light petroleum. Compounds prepared were as follows.

(S)-1-(1-*Naphthyl*)*ethylcarbamoyl*-DL-*methionine methyl ester*. M.p. 108–110 °C (75% yield); $\delta_H(CDCl_3)$ 1.47–1.58 (3 H, 2 d, $C_{10}H_7CHCH_3$), 1.57–1.59 (3 H, 2 s, SCH_3), 1.79–2.06 (2 H, m, $CH_2CH_2SCH_3$), 2.10–2.50 (2 H, br, CH_2SCH_3), 3.60–3.64 (3 H, d, OCH_3), 4.51–4.56 (1 H, m, NHC*H*CO), 5.55–5.65 (1 H, m, $C_{10}H_7CHCH_3$) and 7.20–8.12 (7 H, m, $C_{10}H_7$). Similar diastereoisomeric effects were observed in $[^2H_6]$DMSO with the 2 NH signals appearing at 6.45 and 6.65 ppm. Found: M^+, 360.1508. Calc. for $C_{19}H_{24}N_2O_3S$: *M*, 360.1508.

(S)-1-(1-*Naphthyl*)*ethylcarbamoyl*-DL-*lysine methyl ester*. M.p. 226–230 °C (64% yield); $\delta_H([^2H_6]DMSO)$ 1.43–1.45

(6 H, d, 2 × CHCH_3), 1.11–1.87 [8 H, m, CH(CH_2)$_4$NH], 3.38 (3 H, s, OCH_3), 4.05–4.09 (1 H, m, NHC*H*CO), 5.53–5.59 (2 H, q, 2 × $C_{10}H_7$C*H*CH_3), 6.43–6.46 (2 H, d, 2 × N*H*) and 7.46–8.16 (14 H, m, 2 × $C_{10}H_7$).

(S)-1-(1-*Naphthyl)ethylcarbamoyl*-L-*methionylglycine methyl ester*. M.p. 205–207 °C (61% yield); δ_H($CDCl_3$) 1.43–1.45 (3 H, d, CHCH_3), 1.47 (3 H, s, SCH_3), 1.62–2.54 (4 H, br, overlapping CH_2CH_2SCH_3 and C$H_2$$CH_2SCH_3$), 3.71 (3 H, s, OC$H_3$), 3.92–4.05 (3 H, m, overlapping NHC*H*CO and NHCH_2CO), 5.30–5.61 (1 H, q, $C_{10}H_7$C*H*CH_3) and 7.15–8.10 (7 H, m, $C_{10}H_7$).

(S)-1-(1-*Naphthyl)ethylcarbamoyl*-D-*methionylglycine methyl ester*. M.p. 210–212 °C (57% yield); other spectral data as above.

Synthesis of (R)-α-*Methylbenzylhydantoins of some* DL *Amino Acids*.—The corresponding (*R*)-α-methylbenzylcarbamoyl-DL-amino acid methyl ester (1 mmol) was dissolved in purified dioxane (5 cm^3) and concentrated HCl (5 cm^3) was added under stirring. The solution was heated to 80 °C for 3 h. Evaporation of the reaction mixture to dryness produced an oil in each case, which failed to crystallise. The compounds gave the following spectral data.

(R)-α-*Methylbenzylhydantoin of* DL-*leucine*. δ_H($CDCl_3$) 0.91–0.98 [6 H, d, CH(CH_3)$_2$], 1.25–1.45 [1 H, m, CH_2C*H*(CH_3)$_2$], 1.64–1.77 [2 H, m, CH_2CH(CH_3)$_2$], 1.81–1.84 (3 H, d, Ph-CHCH_3), 3.91–3.97 (1 H, m, NHC*H*CO), 5.29–5.32 (1 H, q, PhC*H*CH_3), 7.00 (1 H, s, N*H*) and 7.26–7.45 (5 H, m, C_6H_5). HPLC: R_t = 18.21 min (MeOH–H_2O, 60:40).

(R)-α-*Methylbenzylhydantoin of* DL-*alanine*. δ_H($CDCl_3$) 1.35–1.40 (3 H, d, PhCHCH_3), 1.78–1.85 (3 H, d, CHCH_3), 3.94–4.00 (1 H, q, NHC*H*CO), 5.26–5.36 (1 H, q, PhC*H*CH_3), 6.45 (1 H, s, N*H*) and 7.23–7.46 (5 H, m, C_6H_5). HPLC: R_t = 6.33 min (MeOH–H_2O, 60:40).

(R)-α-*Methylbenzylhydantoin of* DL-*phenylalanine*. δ_H($CDCl_3$) 1.67–1.70 (3 H, d, PhCHCH_3), 3.62–3.76 (2 H, t, CHCH_2Ph), 4.14–4.20 (1 H, t, NHC*H*CO), 5.18–5.23 (1 H, q, PhC*H*CH_3), 6.15–6.19 (1 H, d, N*H*) and 7.09–7.34 (10 H, m, 2 × C_6H_5). HPLC: R_t = 17.80, 18.20 min (MeOH–H_2O, 60:40).

Optimised Cleavage Reaction and Anlaysis of Hydrolysates.—The dried (*R*)-α-methylbenzylcarbamoyldipeptide methyl ester (5 mg) or (*S*)-1-(1-naphthyl)ethylcarbamoyl-DL-methionylglycine methyl ester (7 mg) was suspended in anhydrous MeOH (10 cm^3) and thionyl chloride (1 cm^3) was added to the stirred, cooled mixture. After 15 min the mixture was heated for 3 h at 60 °C. Removal of the solvent under reduced pressure produced a brown residue which was dissolved in dichloromethane, washed with 1 mol dm^{-3} HCl (10 cm^3), saturated aqueous $NaHCO_3$ (10 cm^3) and water (2 × 10 cm^3). The organic layer was dried ($MgSO_4$), filtered and evaporated to dryness to yield a brown residue. This was dissolved in anhydrous MeOH for all the analyses. HPLC conditions:—Mobile phases: Mixtures of (*a*) MeOH–H_2O or (*b*) MeCN–H_2O + 0.1% TFA. Flow rate 1 cm^3 min^{-1}. Detector wavelength 258 nm. Normal temperature of analysis, 20 °C.

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