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Chiral Analysis of the Reaction Stages in the Edman Method for sequencing Peptides [1]

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Chiral isothiocyanate reagents suitable for 'Edman sequencing' have been synthesised and used to assess the chiral features of individual stages in the Edman method. Using h.p.l.c. analysis of the diastereoisomeric thiohydantoins obtained, it has been deduced that the cyclisation and cleavage of thiazolinone step is the likely source of racemisation of the chiral centre derived from the *N*-terminal amino acid.

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Model racemisation tests [2] have made significant contributions to the understanding of the mechanism of racemisation during peptide synthesis, and have been a fundamental aid in the development of new protecting groups and racemisation-preventing additives in this field. Yet, however good the model is, it can only be a guide as to the eventual chiral purity of a synthetic peptide. There have been recent examples [3] where well recognised 'racemisation-free' conditions in model systems give rise to significant racemisation when used in the more realistic context of the synthesis of larger peptides. The ideal answer to the assessment of chiral purity would be the development of an analytical technique which could assess chiral purity in a post-synthetic context, *i.e.* on the exact peptide after the particular amino acid residue has been added. Traditionally the method most commonly used is the enzymic digestion of the final deprotected peptide. This technique suffers from the disadvantage of being unable to scrutinise each step just after a coupling has been made, and recent developments in the pharmaceutical context often involve substitution of unusual amino acid analogues with structure and stereochemistry not suited for enzyme digestion.

Some progress towards augmenting the methodology of chiral analysis has been made already by determining the optical purity of the derived amino acids using chromatography [4] on chiral stationary phases or using chiral derivatives, by hydrolysis with DCl and mass spectrometric analysis using selected ion monitoring,[5] or directly by h.p.l.c. of the intact peptide.[6] However, to our knowledge there is no record of a method which could systematically check on the chirality of the *N*-terminal residue immediately after it has been added to the sequence. An obvious advantage also would be to carry out the analysis with side-chain protecting groups intact. It is with this overall aim in mind that an investigation of the chiral stability of the various stages of the well known Edman peptide sequencing method [7] has been undertaken. The very extensive work [8] on the chromatographic analysis of thiohydantoins derived from the *N*-terminal analysis commended itself and the possibility of automation [9] was also attractive.

However, the development of a successful chiral analysis depends on the methodology itself being racemisation free. The principle underlying the current approach was to use a chiral isothiocyanate 'Edman' reagent to link to the *N*-terminal amino group of a peptide and then analyse the resulting thiohydantoins using h.p.l.c. Any racemisation of the *N*-terminal amino acid residue cleaved off would be detected as a diastereoisomeric mixture of thiohydantoins which could be separated on h.p.l.c. Scheme 1 shows the accepted stages [10] in the 'Edman' sequence of reactions so the incorporation of a chiral centre in R* of the reagent R*–N=C=S would provide the necessary criteria for separation of the final products as diastereoisomers.

Choice of chiral centre R* had to be made on the basis of its stability throughout the sequence of reactions and on the requirement for good chromatographic separation of the derived thiohydantoins. Four chiral isothiocyanates (**I**)—(**IV**) have been assessed.†

Isothiocyanate (**I**) was unsuccessful since its own chiral centre was subject to racemisation. Isothiocyanates (**II**) and (**III**) failed to provide satisfactory separations of the derived thiohydantoins at the h.p.l.c. analysis stage, and the Z-group in (**II**) was also susceptible to breakdown under extended contact with trifluoroacetic acid. Most of the results obtained in this work were therefore based on isothiocyanate (**IV**) which satisfied all the criteria mentioned above.

Isothiocyanate (**IV**) was synthesised from L-phenylalanine (Scheme 2). A check on the optical purity of the *p*-nitrophenylalanine produced *via* the Erlenmeyer and Lipp method [11] was carried out by converting the amino-acid into its methyl ester, and coupling this with benzoyl-L-alanine using DCCI–HOBt to give *N*-benzoyl-L-alanyl-L-*p*-nitrophenylalanine methyl ester. The n.m.r. spectrum of this derivative gave only one ester OCH_3 signal, at δ 3.73, confirming the existence of only one diastereoisomer [12]

In the application of reagent (**IV**) to the 'Edman' sequence of reactions it was considered prudent to try and achieve cleavage of the *N*-terminal group under the mildest conditions possible. Trial experiments revealed that coupling of the reagent (**IV**) in dioxane to a dipeptide could be carried out in aqueous pyridine–triethylamine (or $NaHCO_3$) at pH 9—10, and that cleavage of the resulting thiocarbamoyl derivative could be achieved using anhydrous trifluoroacetic acid for 1 h at room temperature under nitrogen. The thiohydantoins released in this manner were analysed successfully on a reversed-phase ODS–Hypersil column (25 × 0.43 cm) using acetonitrile as solvent.

By carrying out the sequence of reactions between reagent (**IV**) and in turn L-Leu-Gly, DL-Leu-Gly, L-Ala-Gly, DL-Ala-Gly, L-Val-Gly, and DL-Val-Gly, the resulting thiohydantoins from each cleavage were analysed by h.p.l.c. to give the results summarised in the Table. It can be deduced that there was significant racemisation of the L-amino acid residue in the three examples studied.

In an attempt to identify the exact location of racemisation, the sequence of reactions were examined stepwise. Proof that racemisation did not take place during step i (Scheme 1) came from n.m.r. studies on a thiocarbamoyl derivative derived from phenyl isothiocyanate and Ala-Gly. The n.m.r. spectrum of (**V**) in deuterioacetone was examined before and after addition of

† Abbreviations used: Z = benzyloxycarbonyl; Fmoc = fluorenylmethyloxycarbonyl; DCCI = *NN'*-dicyclohexylcarbodi-imide; HOBt = *N*-hydroxybenzotriazole.

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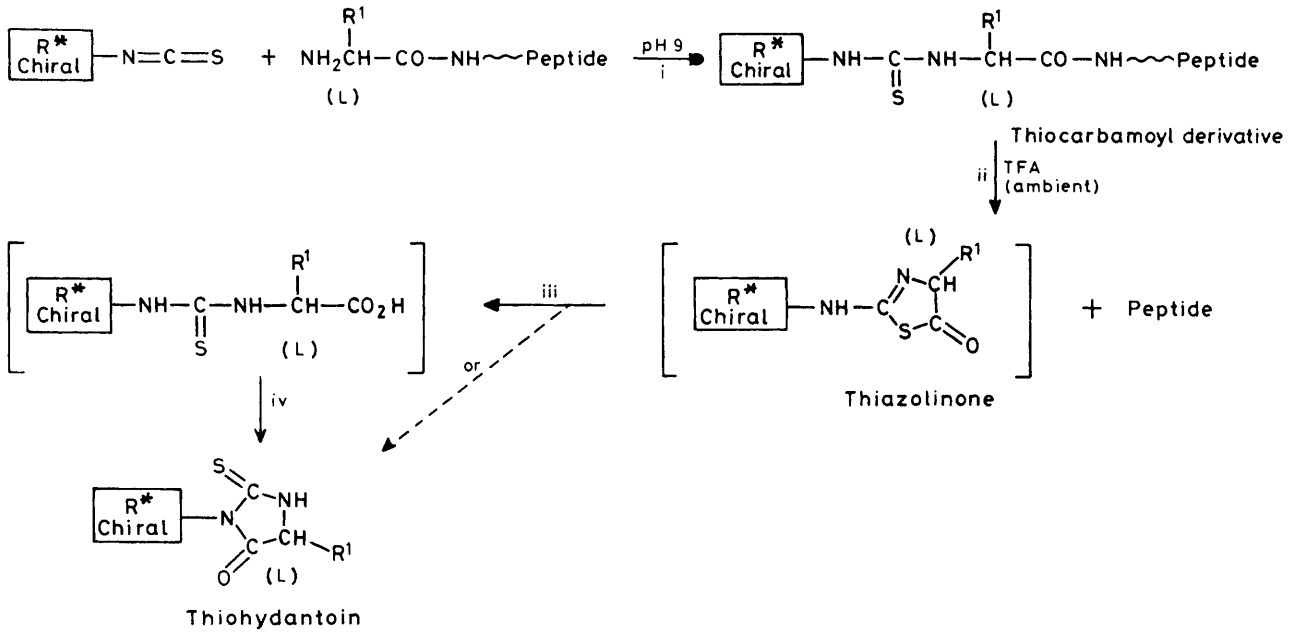
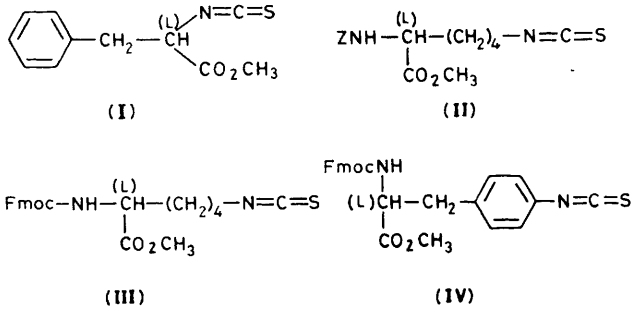


Scheme 1.



D_2O–pyridine or D_2O–triethylamine to the solution and checks were made for any evidence of H–D exchange at the α-C–H position. Even after leaving for 24 h no proton exchange was evident. The optical rotation of (**V**) with R = H or CH_3 also remained constant. It seems reasonable to deduce therefore that step i is racemisation free. When (**V**) was treated with deuteriated trifluoracetic acid (conditions for Edman cleavage) the α-C–H signal at δ 5.22 disappeared after 18 h and the δ 1.36 signal for the alanyl methyl became a singlet. This confirms further that loss of chirality must occur during cyclisation–cleavage.

There is already mechanistic evidence [10] to suggest that the rearrangement of the thiazolinone to thiohydantoin (steps iii—iv in Scheme 1) occurs *via* the carboxylic acid. It was therefore possible to check the final step [step (iv) Scheme 1] and also the chiral stability of the final thiohydantoin by preparing thiocarbamoyl derivatives of amino acids directly and converting them into thiohydantoins. This stage was checked by reaction between chiral isothiocyanate (**IV**) and the amino acids leucine and alanine in both their L- and DL-forms (the latter to check the optimisation of the h.p.l.c. separation). Coupling between isothiocyanate and amino acid was carried out in aqueous pyridine or sodium hydrogencarbonate solution at pH 9 and cyclisation to thiohydantoin was effected with anhydrous trifluoroacetic acid at room temperature for 1 h. The L-forms of both amino acids yielded thiohydantoins which showed only single peaks on h.p.l.c. thus confirming the stability of the chiral

Table.

Diastereoisomeric hydantoins from reagent (**IV**) [in (*S*) form] + dipeptides below	Retention times (R_t) of diastereoisomeric peaks from ODS–Hypersil column (25 × 0.4 cm)–CH_3CN solvent (0.5 ml min^{-1})	
	R_{t1}[L-(*S*)]/min (%)	R_{t2}[D-(*S*)]/min (%)
L-Leu-Gly	6.0 (60)	6.2 (40)
DL-Leu-Gly	6.0 (52)	6.2 (48)
L-Ala-Gly	5.5 (66)	5.7 (34)
DL-Ala-Gly	5.5 (54)	5.7 (46)
L-Val-Gly	5.6 (65)	5.8 (35)
DL-Val-Gly	5.5 (55)	5.8 (45)

centre under the conditions of direct cyclisation of the acid (step iv, Scheme 1). This is contrary to the report of Edman [13] and others [14] but the conditions of cyclisation currently reported are milder. Chiral stability over this stage was also confirmed when the same amino acids were treated with phenyl isothiocyanate and converted into thiohydantoins. Optical rotation measurements and n.m.r. studies (deuterium exchange) confirmed that on extended contact with trifluoroacetic acid no chiral loss occurred. However, by chance it was discovered that if any of the thiohydantoins were purified off thick-layer silica plates or chromatographed on silica gel, then the products produced in this way had been significantly racemised. Addition of an organic base (triethylamine) and D_2O to a solution of the phenylthiohydantoins also catalysed rapid exchange of the α-protons. It is clear therefore that strict adherence to the trifluoroacetic acid conditions is necessary and that the most plausible point for racemisation in the complete sequence must take place at the thiazolinone stage, and not at the final stage.

This racemisation at the thiazolinone stage is most likely to occur due to the ready 'aromatisation' of the thiazolinone with consequent loss of chirality [see resonance forms (**VI**)]. To proceed therefore towards a successful method of post-synthetic chiral analysis, it is clear that the initial and final stages of the 'Edman' sequence of reactions, together with analysis by h.p.l.c., would be acceptable. So the way ahead must

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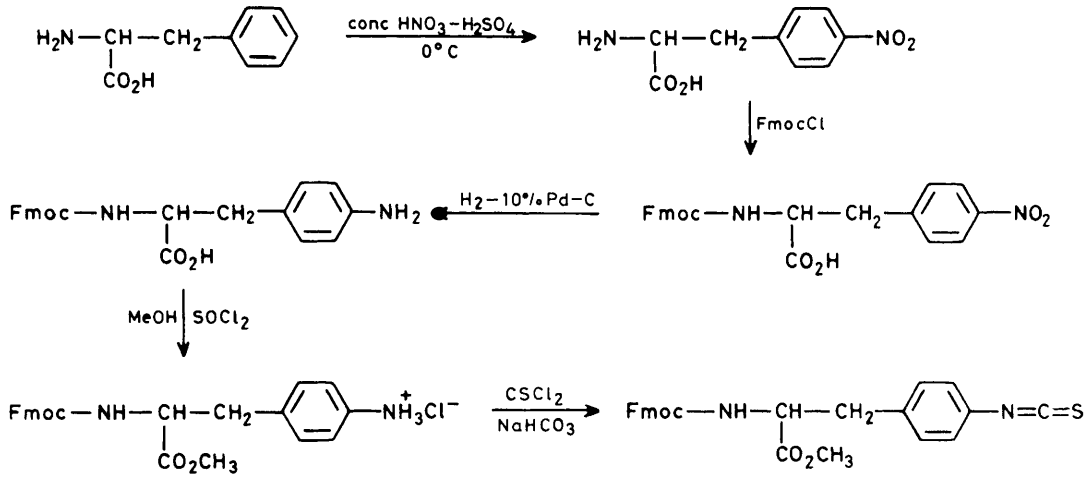
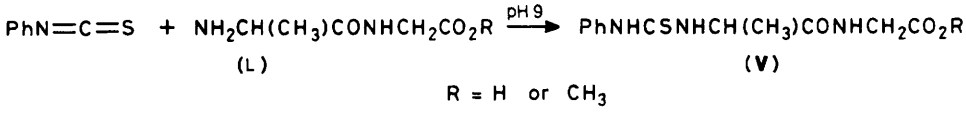
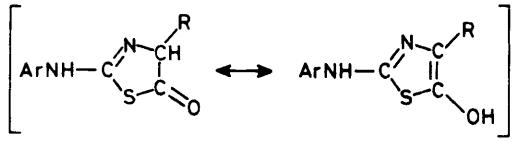


Scheme 2.





(VI)

be towards criteria which would reduce the pseudo-aromatic character of (**VI**). This is currently underway by utilising a chiral isocyanate in place of the isothiocyanate, thus affording the oxy-analogue of (**VI**) which has reduced 'aromatic' character.

Experimental

^{1}H N.m.r. spectra were determined at 100 MHz on a Varian HA-100 spectrometer with tetramethylsilane as the internal reference. Optical rotations were measured on a Perkin-Elmer 141 automatic polarimeter (10 cm cells) using the sodium D line at 589 nm. I.r. spectra were determined using KBr discs or sodium chloride plates on a Pye–Unicam SP 1050 spectrophotometer. C, H, and N Microanalyses were carried out using a Carlo Erba 1106 instrument, while h.p.l.c. measurements were carried out on an LDC SpectroMonitor III with a model 2601 gradient master using 25 × 0.43 cm ODS–Hypersil columns with a variety of solvents, but the best separation of thiohydantoin diastereoisomers was accomplished using CH_3CN at a flow rate of 0.5 ml min^{-1} with initial pressure of 800—830 lb in^{-2}. Purifications of products were carried out either on columns of Kieselgel 60 (230—400 mesh) or on preparative plates of Kieselgel G. Purity was routinely checked using Kieselgel G t.l.c. plates in (i) $CHCl_3$ or (ii) 1—10% MeOH—$CHCl_3$ or (iii) n-butanol–glacial acetic acid–water (4:1:1) as solvents. I_2 vapour or u.v. light on GF_{254} Kieselgel plates were used for detection of spots. M.p.s were determined on a Kofler block and are uncorrected.

Synthesis of N-*Fluorenylmethoxycarbonyl*-L-p-*isothiocyanophenylalanine Methyl Ester.*—L-p-*Nitrophenylalanine. Method of Erlenmeyer and Lipp.*[11] A mixture of concentrated nitric acid (28.5 g, 20 cm^3) and concentrated sulphuric acid (37 g, 20 cm^3) was cooled to 0 °C. To this mixture with stirring was added L-phenylalanine (32 g) in portions over 15 min controlling the temperature to < 5 °C. The mixture was stirred for a further 1 h at 0 °C and 30 min at room temperature when it was poured with stirring into cold water (500 cm^3). Dilute ammonium hydroxide was added until pH 8 was reached, which caused precipitation. Recrystallisation of the solid from water gave L-*p*-nitrophenylalanine (15 g, 75% yield), m.p. 239—241 °C (decomp. > 230 °C) (lit.,[11] 238—240 °C); $[\alpha]_D^{27}$ +9.4° (*c*, 1 in 1M HCl) (lit.,[11] +9.8°). Optical purity of this acid was also confirmed by its conversion into the methyl ester *via* the thionyl chloride[15] method, and coupling this ester hydrochloride (260 mg) with benzoyl-L-alanine[16] (193 mg) in the presence of DCCI (206 mg), HOBt (135 mg), and triethylamine (101 mg) in tetrahydrofuran (7 cm^3) at −5 °C. On work-up the coupling yielded *benzoyl*-L-*alanyl*-L-p-*nitrophenylalanine methyl ester* (356 mg, 86% yield), m.p. 181—182 °C (Found: C, 57.85; H, 5.8; N, 9.8. $C_{20}H_{23}N_3O_7$ requires C, 57.55; H, 5.6; N, 10.1%), $[\alpha]_D^{26}$ +15.2° (*c*, 1.38 in $CHCl_3$); δ ($CDCl_3$); (prior to purification to include all possible diastereoisomers) 1.45 (3 H, d, $CHCH_3$), 3.37 (2 H, m, $CHCH_2$), 3.73 (3 H, s OCH_3), 4.84 (2 H, m, α-CH Ala and Phe), 6.97 (1 H, br, CONH), and 7.22—7.99 (9 H, m, ArH and $ArNO_2$). A single ester peak at δ 3.73 confirms the presence of only one diastereoisomer, *i.e.* the L-L form.

Fmoc-L-p-*nitrophenylalanine.* 9-Fluorenylmethoxycarbonyl chloride (5.59 g, 10 mmol) in dioxane (20 cm^3) was added with stirring at 0 °C to L-*p*-nitrophenylalanine (2.1 g, 10 mmol) in 10% sodium carbonate solution. After 2 h at room temperature the mixture was poured into water (400 cm^3) and washed (2 × 50 cm^3) with ethyl acetate. The aqueous layer was cooled and acidified with concentrated hydrochloric acid until acid to Congo Red paper. The solid precipitate was dissolved in ethyl acetate (90 cm^3), washed with water (3 × 30 cm^3), and dried to yield a solid, crystallised from dichloromethane–ether as N-*Fmoc*-L-p-*nitrophenylalanine* (3.65 g, 86% yield), m.p. 233—234 °C (Found: C, 66.6; H, 52.5; N, 6.4. $C_{24}H_{20}N_2O_6$ requires C, 66.7; H, 4.9; N. 6.5%); $[\alpha]_D^{25}$ +15.2° (*c*, 1.7 in dioxane); δ ([$^2H_6$]DMSO) 2.99—3.55 (2 H, m, $PhCH_2CH$), 4.03—4.51 (4 H, m, $CHCH_2O$, α-C-H), 7.17—7.71 [8 H, m, ArH (Fmoc)], 7.75 (1 H, br, NH), and 7.86 and 8.15 (4 H, dd, C_6H_4).

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N-*Fmoc*-L-p-*aminophenylalanine*. A solution of Fmoc-L-*p*-nitrophenylalanine (2.16 g, 5 mmol) in dioxane (25 cm^3) was hydrogenated at atmospheric pressure over 10% Pd–C (0.5 g). Uptake of hydrogen was complete in 1 h, and the solution after removing the catalyst was evaporated to dryness and the residual solid crystallised from methanol–ether to give pale yellow crystals of *Fmoc*-L-p-*aminophenylalanine* (1.79 g, 90% yield), m.p. 218—219 °C (Found: C, 71.3; H, 5.6; N, 7.1. $C_{24}H_{22}N_2O_4$ requires C, 71.6; H, 5.5; N, 7.0%); $[\alpha]_D$ +23.1 (*c*, 1.2 in dioxane) δ([$^2H_6$]DMSO) 2.77—3.05 (2 H, m, PhCH_2CH), 3.97—4.41 (4 H, m CHCH_2O, CHCH$_2$Ph), 6.51, 6.95 (4 H, dd, C_6H_4), and 7.11—7.99 [9 H, m, ArH (Fmoc) and NH].

N-*Fmoc*-L-p-*isothiocyanophenylalanine methyl ester* (**IV**). The Fmoc-L-*p*-aminophenylalanine was esterified using methanol–thionyl chloride[15] and the resulting methyl ester hydrochloride (2.26 g, 5 mmol) in water (15 cm^3) with stirring was added to a mixture of thiophosgene (0.42 cm^3, 5.5 mmol), 1M-sodium hydrogencarbonate (3 cm^3, 17 mmol), and chloroform (25 cm^3). The reaction mixture was stirred at room temperature for 2 h, the aqueous layer removed, and the organic extract was washed with 1M-$NaHCO_3$, water, and dried ($MgSO_4$). On evaporation of the solvent, the residual solid recrystallised from ether–light petroleum to give *Fmoc*-L-p-*isothiocyanophenylalanine methyl ester* (**IV**) (1.93 g, 79%), m.p. 143—144 °C (Found: C, 67.9; H, 5.0; N, 6.2. $C_{26}H_{22}N_2O_4S$ requires C, 68.3; H, 4.85; N, 6.1%); $[\alpha]_D^{27}$ +46.7° (*c*, 1.07 in $CHCl_3$); $\nu_{max.}$ (KBr) 3 340 (NH), 2 100—2 000 br (N=C=S), 1 750 (ester CO), and 1 710 cm^{-1} (urethane CO); δ ($CDCl_3$) 2.82—3.26 (2 H, m, PhCH_2), 3.72 (3 H, s, OCH_3), 4.22 (1 H, m, α-CH), 4.36—4.72 (3 H, m, $CHCH_2OCO$), 5.32 (1 H, br, NH), and 6.98 (4 H, s, C_6H_4).

Coupling of Isothiocyanate (**IV**) *with Model Dipeptides to form Thiocarbamoyl Derivatives and their Conversion into Thiohydantoins. (Standard Procedure represented by Method used for* DL-(*Leucylglycine*).—DL-Leucylglycine (1 mmol) in aqueous pyridine (4 cm^3) was treated with triethylamine until pH reached 9—10. The solution was stirred for 15 min by bubbling a gentle stream of nitrogen through it. To this solution was added isothiocyanate (**IV**) (1 mmol) in freshly distilled (peroxide free) dioxane (5 cm^3). The reaction mixture was stirred for 2 h at 40 °C in the dark and under nitrogen. The pH was maintained at 9—10 by periodic addition of triethylamine. The solvent was removed *in vacuo* at < 40 °C and the solid residue was washed with benzene (3 × 25 cm^3). The solid in water (50 cm^3) was acidified to pH 5 using 5% citric acid solution and extracted into ethyl acetate, washed with water (3 × 50 cm^3), and dried ($MgSO_4$). Further purification of residue after evaporation of ethyl acetate was carried out using a silica gel column, initial elution with $CHCl_3$–MeOH (95:5) to remove impurity followed by elution of product with methanol to yield N-*Fmoc*-L-*Phe*-(p-*thiocarbamoyl*-DL-*Leu-Gly*)*OMe* as light yellow crystals (69%), m.p. 155—156 °C (from ethanol–ether) (Found: C, 62.9; N, 5.8; N, 8.4. $C_{34}H_{38}N_4O_7S$ requires C, 63.1; H, 5.9; N, 8.7%); $\nu_{max.}$ (KBr) 1 760—1 710 (ester and urethane CO) and 1 640 cm^{-1} (amide CO); δ ([$^2H_6$]acetone) 0.7—1.18 [6 H, m, CH(CH_3)$_2$], 1.42—1.90 [3 H, m, CH_2CH(CH$_3$)$_2$], 2.8—3.2 (2 H, m, CH_2Ph), 3.58 (3 H, s, OCH_3), 3.94—4.64 [5 H, m, CHCH_2O, α-CHs Phe and Leu), 5.20 (2 H, br, urethane NH), 6.70 (1 H, br, peptide NH), 6.90—7.88 [12 H, m, C_6H_4NH, ArH (Fmoc)], and 9.12 (1 H, br, C_6H_4NH).

The above thiocarbamoyl derivative (0.5 mmol) in anhydrous trifluoroacetic acid (2 cm^3) under nitrogen was shaken for 30 min at room temperature and the excess of trifluoroacetic acid then removed *in vacuo*. The residue was dissolved in ethyl acetate, washed with water (3 × 50 cm^3), and dried ($MgSO_4$) to give DL-5-(2-*methylpropyl*)-3-{4-[(2S)-*Fmocamino*-2-*methoxycarbonylethyl*]*phenyl*}-2-*thiohydantoin* (68%), m.p. 180—187 °C (from dichloromethane–ether) (Found: 67.4; H, 6.6; N, 7.2. $C_{32}H_{33}N_3O_5S$ requires C, 67.2; H, 5.8; N, 7.35%); $\nu_{max.}$ 1 750 (ester CO) and 1 710—1 640 cm^{-1} (broad CO, urethane and thiohydantoin); δ ([$^2H_6$]acetone) 0.93 [6 H, d, CH(CH_3)$_2$], 1.53—2.07 [3 H, m, CH_2CH(CH$_3$)$_2$], 3.00—3.30 (2 H, m, CH_2C$_6$H$_4$), 3.64 (3 H, s OCH_3), 4.09—4.57 (5 H, m, CHCH_2O, CHCH$_2$CH, and CHCH$_2$Ph), 7.82 (1 H, br, urethane NH), 7.14—7.78 (12 H, m, ArH and C_6H_4), and 9.16 (1 H, br, thiohydantoin NH). H.p.l.c. results are recorded in the Table.

The above methodology was repeated for the L-Leu-Gly analogue giving physical parameters for the thiocarbamoyl derivative and thiohydantoin as follows: N-*Fmoc*-L-*Phe*-(p-*thiocarbamoyl*-L-*Leu-Gly*)*OMe*, m.p. 156—157 °C (from ethanol–ether); 5-(2-*methylpropyl*)-3-{4-[(2S)-*Fmocamino*-2-*methoxycarbonylethyl*]*phenyl*}-2-*thiohydantoin* (70%) m.p. 185—187 °C (from dichloromethane–ether) (h.p.l.c. results indicated presence of both L-L and L-D diastereoisomers).

Similarly the following data were obtained for thiohydantoin derivatives derived from DL-Ala-Gly, L-Ala-Gly; DL-Val-Gly and L-Val-Gly. H.p.l.c. data appear in the Table and show that products from the L-dipeptides were always contaminated with partially racemised forms. DL-5-*Methyl*-3-{4-[(2S)-*Fmocamino*-2-*methoxycarbonylethyl*]*phenyl*}-2-*thiohydantoin* (67%) had m.p. 134—144 °C (Found: C, 65.3; H, 4.9; N, 8.4. $C_{29}H_{27}N_3O_5S$ requires C, 65.7; H, 5.1; N, 8.0%); δ ([$^2H_6$]DMSO) 2.40 (3 H, d, CHCH_3), 2.90—3.14 (2 H, m, CHCH_2Ph), 3.31 (3 H, s, OCH_3), 4.10—4.50 [5 H, m, (CHCH_2O), (CHCH$_2$Ph), and (CHCH$_3$)], 7.16 (1 H, br, urethane NH), and 7.20—8.00 (13 H, m, ArH, C_6H_4, and thiohydantoin NH). DL-5-*Isopropyl*-3-{4-[(2S)-*Fmocamino*-2-*methoxycarbonylethyl*]*phenyl*}-2-*thiohydantoin* (71%) had m.p. 131—134 °C (from ether–light petroleum) (Found: C, 66.7; H, 6.0; N, 7.2. $C_{31}H_{31}N_3O_5S$ requires C, 66.75; H, 5.6; N, 7.5%); δ ([$^2H_6$]acetone) 0.89 [6 H, 2d (overlapping), CH(CH_3)$_2$], 2.26 [1 H, m, CH(CH$_3$)$_2$], 2.94—3.18 (2 H, m, CHCH_2Ph), 3.58 (3 H, s, OCH_3), 4.10—4.55 [5 H, m, CHCH_2O, CHCH$_2$Ph, CHCH(CH_3)$_2$], 7.83 (1 H, br, urethane NH), 7.19—7.80 (12 H, m, ArH and C_6H_4), and 0.95 (1 H, br, thiohydantoin NH).

Pure L-(*S*) forms of the above thiohydantoins could be obtained by direct coupling to the chiral amino acids followed by cyclisation with trifluoroacetic acid as typified by the following standard method for leucine.

L-Leucine (1 mmol) was dissolved in a mixture of pyridine (2 cm^3) and water (2 cm^3). The pH was adjusted to 9 by addition of 1M-$NaHCO_3$, and the solution was flushed with a stream of nitrogen for 15 min, when isothiocyanate (**IV**) (1 mmol) in dioxane (5 cm^3) was added with stirring. Small portions of 1M-$NaHCO_3$ were added during the reaction to keep the pH at 9. After stirring at room temperature for 1 h, the solvent was removed *in vacuo* at ambient temperature. To the residue, water (25 cm^3) was added, and acidified to pH 5 using 5% citric acid and extracted with ethyl acetate. Purification on a silica gel column [eluting with $CHCl_3$–MeOH (95:5)] gave yellow crystals (63%) of N-*Fmoc*-L-*Phe*-(p-*thiocarbamoyl*-L-*Leu*)*OMe*, m.p. 153—154 °C (from dichloromethane–ether) (Found: C, 62.6; H, 6.1; N, 6.4. $C_{32}H_{35}N_3O_6S{\cdot}1H_2O$ requires C, 63.1; H, 6.1; N, 6.9%); δ ([$^2H_6$]DMSO) 0.93 [6 H 2d (overlapping) CH(CH_3)$_2$], 1.17—1.93 [3 H, m, CH_2CH(CH$_3$)$_2$], 2.81—3.11 (2 H, m, CH_2Ph), 3.61 (3 H, s, OCH_3), 3.87—4.45 [4 H, m, CHCH_2O and α-H Leu), 4.93 (1 H, m, α-C-H Phe), 7.03 (1 H, br, urethane NH), and 7.13—7.97 (12 H, m, ArH, C_6H_4). The above thiocarbamoyl derivative (0.5 mmol) was treated with anhydrous trifluoroacetic acid (2 cm^3) and shaken at ambient temperature under a stream of nitrogen for 1 h. Extraction and isolation using the same procedure as described for thiohydantoins generated from dipeptides gave L-5-*isopropyl*-3-{4-[(2S)-*Fmocamino*-2-*methoxycarbonylethyl*]*phenyl*}-2-*thiohydantoin* (90%), m.p. 180—181 °C (from dichloromethane–

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ether), $[\alpha]_D^{27}$ −10.2° (*c*, 0.9 in MeOH). Physical and spectral data were identical with those of a sample of thiohydantoin from DL-dipeptide except that h.p.l.c. on an ODS column with CH_3CN as solvent at 0.5 ml min^{-1} gave only one peak, at R_t 6.0 min.

Similarly the chirally pure form of the thiohydantoin from L-alanine had the following data, with all other data identical with its racemised counterpart reported earlier.

L-5-*Methyl*-3-{4-[(2S)-*Fmocamino*-2-*methoxycarbonylethyl*]*phenyl*}*thiohydantoin* (88%) had m.p. 134—137 °C, $[\alpha]_D^{28}$ −8.0° (*c*, 1.2 in MeOH), R_t 5.5 min under the conditions above for h.p.l.c.

Chiral Stability Experiments.—These were carried out using n.m.r. and optical rotation techniques on products obtained by coupling peptides and amino acids with phenyl isothiocyanate yielding the following compounds by standard methods described above. The compounds produced were: phenylthiocarbamoyl-L-leucine, m.p. 84—85 °C (from ether), $[\alpha]_D^{27}$ + 2.1° (*c*, 1.2 in EtOH); δ ([$^2H_6$]acetone) 0.85 [6 H, 2d (overlapping), $CH(CH_3)_2$], 1.61—1.93 [3 H, m, $CH_2CH(CH_3)_2$], 5.15 (1 H, m, α-CH), 6.25 (1 H, br, CSNH), 6.93—7.69 (5 H, m, ArH), and 9.57 (1 H, br, PhNH). On addition of [$^2H_1$]TFA there was no evidence of α-C-H exchange nor was there any alteration in the $CHCH_2$ (side-chain signal) although evidence was obtained of thiohydantoin formation.

L-5-(2-*Methylpropyl*)-3-*phenyl*-2-*thiohydantoin* was obtained as crystals, m.p. 175—176 °C (from ethyl acetate) (Found: C, 62.7; H, 6.6; N, 10.9. $C_{13}H_{16}N_2OS$ requires C, 62.9; H, 6.45; N, 11.3%); $[\alpha]_D^{27}$ −50° (*c*, 1 in MeOH); δ ([$^2H_6$]DMSO) 0.93 [6 H, 2d (overlapping), $CH(CH_3)_2$], 1.51—2.05 (3 H, m, CH_2CH), 4.43 (1 H, t, α-H on thiohydantoin ring), 7.13—7.47 (5 H, m, ArH), and 7.53 [1 H, br, NH (overlapping with ArH)]; $\nu_{max.}$ 1 770 cm^{-1} (thiohydantoin CO). When D_2O–TFA was added no change occurred in the n.m.r. spectrum (other than N-H exchange). When this optically active leucine thiohydantoin was chromatographed on silica gel with $CHCl_3$ as eluant, the final product from the plate showed zero optical rotation and m.p. 177—179 °C (from ether).

Phenylthiocarbamoyl-L-*alanine* was obtained as crystals, m.p. 193—195 °C (from ether); $[\alpha]_D^{27}$ +23.4° (*c*, 1.3 in MeOH); δ ([$^2H_6$]acetone) 1.68 [3 H, d (overlapping), $CHCH_3$], 4.74 (1 H, m, α-H), 7.16—7.68 (5 H, m, ArH), 7.70 (1 H, br, CSNH), and 9.00 (1 H, br, PhNH). [$^2H_1$]TFA was added to the n.m.r. tube and after 24 h there was no obvious exchange of α-H (signal converted to quartet because of N-H exchange) and the alanyl methyl signal at δ 1.68 became a symmetrical doublet (due to cyclisation to the thiohydantoin).

L-5-*Methyl*-3-*phenyl*-2-*thiohydantoin* gave crystals, m.p. 181—182 °C (from ether) (Found: C, 57.8; H, 5.0; N, 13.6. $C_{10}H_{10}N_2OS$ requires C, 58.25; H, 4.9; N, 13.6%); $[\alpha]_D^{27}$ −31° (*c*, 1.14 in MeOH); δ ([$^2H_6$]acetone) 1.41 (3 H, d, $CHCH_3$), 4.41 (1 H, q, $CHCH_3$), 7.21—7.55 (5 H, m, ArH), and 9.14 (1 H, br, NH). When this thiohydantoin was purified by chromatography on silica gel ($CHCl_3$ as eluant), the product was optically inactive and had m.p. 187—190 °C (from ether).

Phenylthiocarbamoyl-L-*alanylglycine methyl ester* was prepared by reacting phenyl isothiocyanate with L-alanylglycine methyl ester obtained by hydrogenolysis of Z-L-Ala-GlyOMe.[17] The product was obtained in 80% yield, m.p. 150 °C (from dichloromethane–light petroleum) (Found: C, 52.8; H, 6.0; N, 13.9. $C_{13}H_{17}N_3O_3S$ requires C, 52.8; H, 5.83; N, 24.2%); $[\alpha]_D^{28}$ −6.6° (*c*, 1 in EtOH); δ ([$^2H_6$]acetone) 1.36 (3 H, d, $CHCH_3$), 3.64 (3 H, s OCH_3), 4.00 (2 H, d, $NHCH_2$), 5.22 (1 H, m, α-CH Ala), 7.28 (1 H, br, peptide NH), 7.28—7.58 (5 H, m, ArH), 7.82 (1 H, br, CSNH), and 8.88 (1 H, br, PhNH). On addition of D_2O–Et_3N or D_2O–pyridine there was no evidence of α-proton exchange, nor any change in the alanyl CH_3 doublet. After 24 h the original spectrum was obtained.

On addition of [$^2H_1$]TFA the signal at δ 5.22 (α-H) disappeared after 18 h and the doublet at δ 1.36 (CH_3) was reduced to a singlet which is proof of thiohydantoin formation and proton exchange.

5-Methyl-3-phenyl-2-thiohydantoin was obtained from the above peptide ester after treatment with anhydrous trifluoroacetic acid, m.p. 187—190 °C (lit.,[13] 184—185 °C for the thiohydantoin from DL-alanine; $[\alpha]_D^{26}$ 0° (*c*, 2 in EtOH). N.m.r. data were as given for the compound prepared directly from the amino acid (see above). On addition of D_2O–Et_3N to the n.m.r. sample after 2 h the quartet at δ 4.41 (α-CH) disappeared and the doublet at 1.41 (CH_3) was reduced to a singlet, thus confirming the chiral instability of the thiohydantoin to basic conditions. On the other hand, when the original solution of the thiohydantoin was treated with D_2O–TFA there was no evidence of α-H exchange, nor was there any change in the methyl doublet signal even on leaving the sample for 24 h in contact with D_2O–TFA at 40 °C.

Similar results were obtained when n.m.r. proton exchange studies were carried out on phenylthiocarbamoyl-Leu-Gly[18] and the derived leucine thiohydantoin[13] after acid cleavage.

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