Polypeptides. Part III.[1] The Synthesis of the *C*-Terminal Tetrapeptide Sequence of Gastrin, its Optical Isomers, and Acylated Derivatives

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The synthesis is described, by various routes, of L-trytophyl-L-methionyl-L-aspartyl-L-phenylalanine amide, and various acylated derivatives of this tetrapeptide amide and of its D,D,D,D-, D,L,L,L-, L,D,L,L-, L,L,D,L-, L,L,L,D-L,D,D,D-, L,L,D,D-, D,L,D,D-, and D,D,L,L-isomers.

FOLLOWING the isolation, from hog antral mocosa, of the heptadecapeptide amides, gastrin I and II (which are believed to be related to the hormone which is released from the antral mucosa during digestion) by Gregory and Tracy,[2] the structures of these peptides were elucidated by Gregory, Hardy, Jones, Kenner, and Sheppard,[3] and their total synthesis was accomplished by Anderson, Barton, Gregory, Hardy, Kenner, MacLeod, Preston, Sheppard, and Morley.[4] In an associated study, Tracy and Gregory[5] described the physiological properties of a series of synthetic peptides structurally related to gastrin I and showed that of the 17 amino-acid residues of the molecule, only the *C*-terminal tetrapeptide amide sequence, Try·Met·Asp·Phe·NH_2 (which is found in both gastrins) was required for the whole range of physiological actions on gastric and pancreatic gland-cells and on the gastrointestinal musculature displayed by the natural hormones. Structure–function relationships in the *C*-terminal tetrapeptide amide sequence were subsequently described by Morley, Tracy, and Gregory.[6] The present Paper gives details of the synthesis of the tetrapeptide amide, and of various acylated derivatives of the tetrapeptide amide and its optical isomers. Subsequent Papers in this series will deal with the synthesis of analogues.

Various problems were presented in the synthesis of the tetrapeptide amide due to the simultaneous presence of tryptophyl, methionyl and aspartyl residues. The removal of protecting groups from methionine-containing peptides by hydrogenolysis (see, however, Medzihradszky and Medzihradszky[7]), strongly acidic reagents, or reducing agents is generally unsatisfactory,[8] so we chose the *t*-butoxycarbonyl- and *o*-nitrophenylsulphenyl- groups,[9] both of which may be removed under mild acidic conditions, for *N*-protection of this amino-acid. We also wished to avoid the saponification of aspartyl ester residues in the synthesis since this may be accompanied by transpeptidisation.[10]

We initially chose a stepwise procedure (Scheme 1) in which the aspartic β-carboxylic group was protected as a t-butyl ester and the methionine nitrogen was protected with the *o*-nitrophenylsulphenyl group to allow selective cleavage of the latter. β-t-Butyl-*N*-benzyloxycarbonyl-L-aspartate (I) and L-phenylalanine amide (II) were coupled by the mixed anhydride route or by means of *NN'*-dicyclohexylcarbodi-imide (DCCl), and the product (III) was hydrogenated, yielding (β-t-butyl)-L-aspartyl-L-phenylalanine amide (IV). This with *N*-(*o*-nitrophenylsulphenyl)-L-methionine (V) and DCCI gave the *o*-nitrophenylsulphenyl-tripeptide derivative (VI), from which the amino-protecting group was readily removed, without cleavage of the t-butyl ester group, by treatment at 0° with two equivalents of dry hydrogen chloride in chloroform. Further coupling of the resulting tripeptide derivative (VII) with *N*-t-butoxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (VIII) gave the

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[2] R. A. Gregory and H. J. Tracy, *Gut*, 1964, **5**, 103.
[3] H. Gregory, P. M. Hardy, D. S. Jones, G. W. Kenner, and R. C. Sheppard, *Nature*, 1964, **204**, 931.
[4] J. C. Anderson, M. A. Barton, R. A. Gregory, P. M. Hardy, G. W. Kenner, J. K. MacLeod, J. Preston, R. C. Sheppard, and J. S. Morley, *Nature*, 1964, **204**, 933.
[5] H. J. Tracy and R. A. Gregory, *Nature*, 1964, **204**, 935.
[6] J. S. Morley, H. J. Tracy, and R. A. Gregory, *Nature*, 1965, **207**, 1356.
[7] K. Medzihradszky and H. Medzihradszky, Proc. Seventh European Peptide Symp., Budapest, Sept. 1964, *Acta Chim. Acad. Sci. Hung.*, 1965, **44**, 15.
[8] See references cited by B. Iselin, *Helv. Chim. Acta*, 1961, **44**, 61.
[9] L. Zervas, D. Borovas, and E. Gazis, *J. Amer. Chem. Soc.*, 1963, **85**, 3660.
[10] See J. Rudinger, *Coll. Czech. Chem. Comm.*, 1959, **24**, 101.

N-t-butoxycarbonyl-tetrapeptide amide t-butyl ester (IX). Treatment of the latter with trifluoroacetic acid or hydrogen chloride–acetic acid gave salts of the well-characterised L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (X). It may be noted that whilst *N*-(*o*-nitrophenylsulphenyl)-L-tryptophan and the tripeptide derivative (VII) gave the *N*-(*o*-nitrophenylsulphenyl)-tetrapeptide amide t-butyl ester, selective cleavage of the nitrophenylsulphenyl group of the product was not achieved. Reasons for this failure have been previously commented upon.[11] However, the t-butyl ester of (X) was prepared [4] in moderate yield by selective cleavage of the t-butoxycarbonyl-tetrapeptide amide t-butyl ester (IX) (details will be published elsewhere).

Four other routes to the tetrapeptide amide (X) were subsequently developed. The preferred route (Scheme 2), which gave excellent yields of pure intermediates and product on the large scale, was again a stepwise procedure, but the use of a protecting group for the aspartyl β-carboxyl was avoided. This procedure, which has also recently been applied successfully in the synthesis of eledoisin,[12] was made possible by the use of active ester couplings in the presence of exactly one equivalent of a tertiary base (usually triethylamine). *N*-Benzyloxycarbonyl-(β-benzyl)-L-aspartyl-L-phenylalanine amide (XII) was prepared from α-2,4,5-trichlorophenyl β-benzyl *N*-benzyloxycarbonyl-L-aspartate (XI) and L phenylalanine amide (II) (other coupling procedures are also described in the Experimental section) and then hydrogenated to give L-aspartyl-L-phenylalanine amide (XIII). This coupled readily with *N*-t-butoxycarbonyl-L-methionine 2,4,5-trichlorophenyl ester (XIV) in aqueous dimethylformamide in the presence of exactly one equivalent of triethylamine (alternatively, salts of L-aspartyl-L-phenylalanine amide could be employed with 1·8—2 equivalents of triethylamine) to give, after acidification, *N*-t-butoxycarbonyl-L-methionyl-L-aspartyl-L-phenylalanine amide (XV), from which the *N*-protecting group was removed by treatment with cold 80% aqueous trifluoracetic acid or hydrogen chloride–acetic acid. The resulting salts of L-methionyl-L-aspartyl-L-phenylalanine amide (XVI) were coupled with *N*-t-butoxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (VII) in aqueous dimethylformamide in the presence of 1·8—2 equivalents of triethylamine and the butoxycarbonyl-tetrapeptide amide (XVII) was converted to the tetrapeptide amide (X).

A third route (Scheme 3) also employed a stepwise synthesis, but started from L-phenylalanine methyl ester (XVIII). By methods similar to these described in Scheme 2 for L-phenylalanine amide, this was converted, *via* the protected derivative (XIX), into L-aspartyl-L-phenylalanine methyl ester (XX). As expected, this gave mainly the piperazine (XXI) after treatment with methanolic ammonia, but condensation with *N*-t-butoxycarbonyl-L-methionine 2,4,5-trichlorophenyl ester (XIV) gave the butoxycarbonyl-tripeptide ester (XXI), which was smoothly converted into the corresponding amide (XXII). The synthesis then proceeded as in Scheme 2. Alternatively, treatment of the butoxycarbonyl-tripeptide ester (XXI) with hydrogen chloride in acetic acid gave the tripeptide ester (XXIII) as its hydrochloride, and this with *N*-t-butoxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (VIII) in dimethylformamide and triethylamine (2 mol.) gave the butoxycarbonyl-tetrapeptide ester (XXIV). Ammonolysis of this was again smooth, giving the butoxycarbonyl amide (XVII) and thence the unprotected amide (XVII).

The other two routes employed azide couplings of *N*-t-butoxycarbonyl-L-tryptophyl-L-methionyl azide (XXIX) with either L-aspartyl-L-phenylalanine amide (XIII) (Scheme 4) or methyl ester (XX) (Scheme 5). The latter route was initially developed by Professor G. W. Kenner and his co-workers at Liverpool University and will be described elsewhere. In Scheme 4, *N*-t-butoxycarbonyl-L-tryptophan (XXV) and L-methionine methyl ester (XXVI) gave, by two methods, *N*-t-butoxycarbonyl-L-tryptophyl-L-methionine methyl ester (XXVII) and thence the hydrazide (XXVIII). This was best converted into the azide (XXIX) by the Rudinger method [13] (other methods led to partial oxidation of the methionine sulphur). Coupling of the azide (XXIX) with the dipeptide amide (XIII) then gave the butoxycarbonyl-tetrapeptide amide (XVII). These two Schemes were frequently used in the preparation of analogues, but were inferior to Scheme 2 on the large scale.

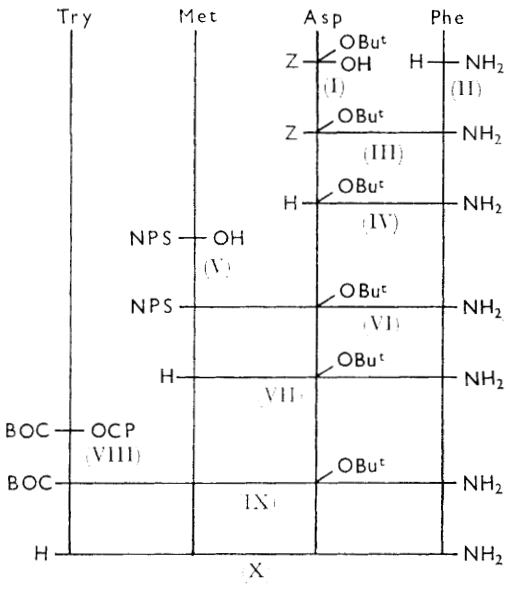
In the synthesis of optical isomers, D,D-, L,D-, and D,L-aspartylphenylalanine amide were first prepared by the method described for the L,L-isomer, using the appropriate L- or D-amino-acids. The D,D- or L,L-isomers were converted into the butoxycarbonyl derivatives of the D,D,D,D-, L,D,D,D-, D,L,D,D-, L,D,L,L-, D,L,L,L-, D,D,L,L-tetrapeptide amide using Scheme 2 and the appropriate L- or D-amino-acids. The L,L,D,L-, L,L,L,D-, and L,L,D,D-isomers were prepared by coupling *N*-t-butoxycarbonyl-L-tryptophyl-L-methionyl azide (XXIX) with L- or D-aspartyl-L- or D-phenylalanine amide (Scheme 4).

In general, biological activity is preserved or increased by acylation of the tryptophan α-nitrogen.[6] A variety of *N*-acylated derivatives were prepared by direct acylation of the all L-tetrapeptide amide (X) trifluoroacetate or hydrochloride (usually employing an active ester of the appropriate acid and two equivalents of triethylamine). Details of these are given in the Experimental section. The activity of one of the acylated derivatives, *N*-t-butoxycarbonyl-β-alanyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (I.C.I. 50,123) was outstanding (of the same order of activity of gastrin on a

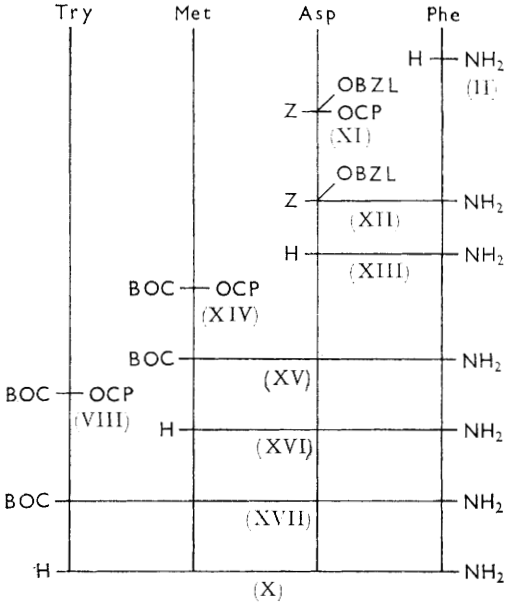
[11] J. C. Anderson, M. A. Barton, P. M. Hardy, G. W. Kenner, J. K. MacLeod, J. Preston, R. C. Sheppard, and J. S. Morley, *Acta Chim. Acad. Sci. Hung.*, 1965, **44**, 187.

[12] S. Bajusz, *Acta Chim. Acad. Sci. Hung.*, 1964, **42**, 383.

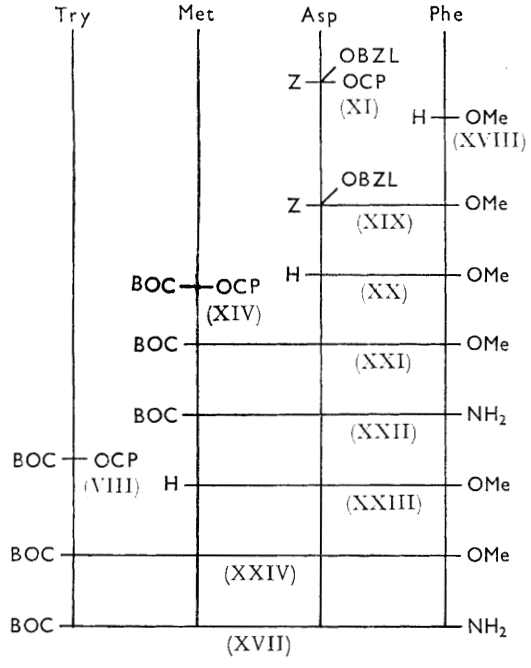
[13] J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, 1961, **26**, 2333.



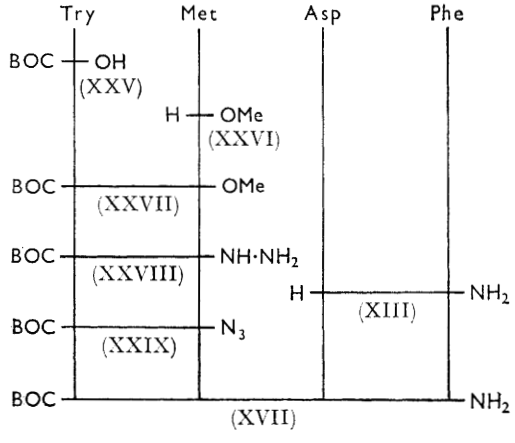
Scheme 1



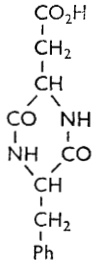
Scheme 2



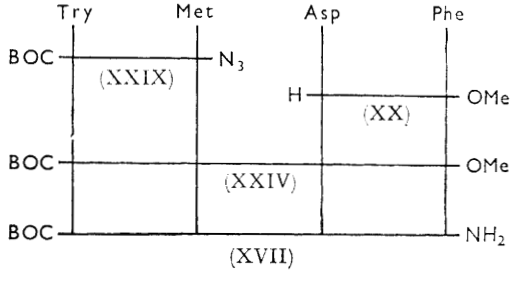
Scheme 3



Scheme 4



(XXI)



Scheme 5

BOC–β–Ala.Try.N$_3$ (XXX)

BOC–β–Ala.Try.Met·N$_3$ (XXXI)

(Z = benzyloxycarbonyl; BOC = t-Butoxycarbonyl; NPS = o-Nitrophenylsulphenyl; OBZL = Benzyl ester; OBu^t = t-Butyl ester; OCP = 2,4,5-Trichlorophenyl ester.)

simple weight-for-weight basis);[6,*] this derivative was also prepared by coupling *N*-t-butoxycarbonyl-β-alanyl-L-tryptophyl azide (XXX) with L-methionyl-L-aspartyl-L-phenylalanine amide (XVI), and by coupling *N*-t-butoxycarbonyl-β-alanyl-L-tryptophyl-L-methionyl azide (XXXI) with L-aspartyl-L-phenylalanine amide (XIII).

EXPERIMENTAL

Ascending, thin-layer chromatograms were run on Kieselgel-G using the solvent systems (R_{FA}–R_{FH}) described in Part II of this series.[1] Descending chromatograms were run on Whatman No. 3 paper with butan-1-ol–acetic acid–water (4 : 1 : 5 v/v) (R_{FM}) or butan-1-ol–acetic acid–water–pyridine (15 : 3 : 12 : 10) (R_{FN}). Thin-layer electrophoresis (400 v., 6—40 ma., 3—8 hr.) was carried out in the Desaga apparatus using silica gel HF plates, and high-voltage paper electrophoresis (5 kv., 70—80 ma., 15—50 min.) in the LoCarte apparatus using Whatman 3M paper: at pH 1·9 ($E_{1\cdot9}$) in pyridine–formic acid–water (5 : 15 : 80); at pH 6·5 ($E_{6\cdot5}$) in pyridine–formic acid–water (10 : 1 : 89); and at pH 8·0 ($E_{8\cdot0}$) in a mixture of 1% aqueous pyridine, 1% aqueous collidine, and sufficient acetic acid to give pH 8·0 . $E_{1\cdot9}$ (paper) = 0·62 × Phe–NH_2 indicates that on paper electrophoresis at pH 1·9 the substance migrates 0·62 times the distance that phenylalanine amide migrates. Spots were revealed by the methods previously described,[1] Ehrlich's reagent, and, in the case of thin-layer chromatography, by incorporating a fluorescent indicator in the thin-layer (Kieselgel GF 254, Merck). Acid, alkali, or enzymic hydrolysates of peptides or peptide derivatives were prepared using 6N-hydrochloric acid (110°/16 hr.), 5N-sodium hydroxide (110°/16 hr.), or leucine aminopeptidase [14] (LAP), and the amino-acid composition of the hydrolysates was determined with a Beckman–Spinco Amino Acid Analyser, model 120B. Optical rotations were determined with a Bendix NPL Automatic Polarimeter, model 143C, with Digital Converter, model 154C. Evaporations were carried out under reduced pressure, usually at a bath temperature of 40—50° in a rotary evaporator. M. p.s are uncorrected.

Preparation of Intermediates.—D- *and* L-*Phenylalanine amide* (II). Ethyl chloroformate (94 ml., 1 mole) was added dropwise over 15 min. at −10° to a stirred solution of *N*-benzyloxycarbonyl-L-phenylalanine (299 g., 1 mole) [15] and triethylamine (140 ml., 1 mole) in tetrahydrofuran (2 l.). The mixture was stirred at −15 to −10° for 20 min., then saturated below 0° with dry ammonia. The reaction mixture was kept at 20—22° overnight and then evaporated. The residue was stirred at 40—50° with water (1 l.) and the insoluble solid was collected, after cooling, washed with water, and dried at 40—60°. A solution of the resulting *N*-benzyloxycarbonyl-L-phenylalanine amide (249 g., 84%), m. p. 161—162°, in 80% acetic acid (2 l.) was hydrogenated over 5% palladised charcoal (50 g.) at room temperature and pressure for 6 hr. The mixture was filtered (kieselguhr) and evaporated, and the resulting residue was crystallised from ethyl acetate, yielding L-phenylalanine amide acetate (143·4 g., 64%), m. p. 116—117°, $[\alpha]_D^{22}$ +17·9° (*c* 2·0 in water). A solution of the acetate in water was shaken with DE-ACIDITE FF (OH form) (4 equiv.), then filtered and evaporated, giving L-phenylalanine amide (100% yield), m. p. 91—92°, R_{FB} 0·68, R_{FF} 0·34. In a similar manner, using *N*-benzyloxycarbonyl-D-phenylalanine, there was obtained *N*-benzyloxycarbonyl-D-phenylalanine amide, m. p. 162—163° (from ethyl acetate) (Found: C, 68·4; H, 6·0; N, 9·5. Calc. for $C_{17}H_{18}N_2O_3$: C, 68·4; H, 6·05; N, 9·4%), and D-phenylalanine amide acetate, m. p. 116—117°, $[\alpha]_D^{22}$ −18·0° (*c* 2·0 in water) (Found: C, 58·6; H, 7·4; N, 12·6. Calc. for $C_{11}H_{16}N_2O_3$: C, 59·0; H, 7·15; N, 12·5%).

β-*Benzyl N-benzyloxycarbonyl*-D- *and* -L-*aspartate*. *N*-Benzyloxycarbonyl-L-aspartic acid (267 g., 1 mole), *p*-toluene sulphonic acid hydrate (15 g.), benzyl alcohol (1·2 l.), and toluene (1·5 l.) were boiled under reflux for 1 hr., the water (37—39 ml.) formed during the reaction being removed continuously in a Dean and Stark assembly. The solvent and the excess benzyl alcohol were then removed rapidly at 0·4—10 mm., and the residual oil was added to light petroleum (b. p. 60—80°) (1·5 l.), yielding crystalline dibenzyl *N*-benzyloxycarbonyl-L-aspartate (428 g., 96%), m. p. 61—62°. A vigorously-stirred solution of the diester (89·4 g., 0·2 mole) in commercial acetone (2 l.) and ice–water (500 ml.) was treated dropwise over 45—60 min. at 20—22° with a solution of lithium hydroxide hydrate (91%) (9·20 g., 0·2 mole) in water (200 ml.). The acetone was then removed rapidly in a rotary evaporator and the resulting aqueous solution was extracted twice with ether, freed from ether, and acidified with concentrated hydrochloric acid (20 ml.) and ice. Light petroleum (b. p. 40—60°) (150 ml.) was added and the solid (46—48 g., 65—67%), m. p. 96—102°, was collected, washed with water, and dried *in vacuo*. The products from five such runs were combined and crystallised from benzene (450 ml.), yielding pure β-benzyl *N*-benzyloxycarbonyl-L-aspartate (193 g., 54%), m. p. 107—108°. In a similar manner, *N*-benzyloxycarbonyl-D-aspartic acid gave *dibenzyl N-benzyloxycarbonyl*-D-*aspartate*, m. p. 62—63° (from cyclohexane) (Found: C, 70·2; H, 5·6; N, 3·2. $C_{26}H_{25}NO_6$ requires C, 69·8; H, 5·6; N, 3·1%), and β-*benzyl N-benzyloxycarbonyl*-D-*aspartate*, m. p. 107—108° (from benzene) (Found: C, 63·6; H, 5·4; N, 4·0. $C_{19}H_{19}NO_6$ requires C, 63·8; H, 5·3; N, 3·9%).

α-2,4,5-*Trichlorophenyl* β-*benzyl N-benzyloxycarbonyl*-D- *and* -L-*aspartate* (XI). A solution of β-benzyl *N*-benzyloxycarbonyl-L-aspartate (178·5 g., 0·5 mole) and 2,4,5-trichlorophenol (108·6 g., 0·55 mole) in ethyl acetate (1250 ml.) was treated with *NN'*-dicyclohexylcarbodi-imide (103 g., 0·5 mole) at 0—5°, and the mixture was stirred at 0° for 4 hr. Acetic acid (5·7 ml.) was then added, and stirring was continued at 20—22° for 15 min. The filtered solution was evaporated and the residue was dissolved in warm ethanol (1 l.); colourless needles of the L-isomer (238 g., 88%), m. p. 93—94°, $[\alpha]_D^{22}$ −22·1° (*c* 1·0 in dimethylformamide) rapidly separated (lit.,[16] gives m. p. 92°). The D-*isomer*, prepared similarly, had m. p. 90—92°, $[\alpha]_D^{22}$ +22·5° (*c* 1·0 in dimethylformamide) (Found: C, 55·8; H, 3·3; N, 2·9. $C_{25}H_{20}NO_6Cl_3$ requires C, 55·8; H, 3·7; N, 2·6%).

N-t-Butoxycarbonyl-D-*tryptophan and* 2,4,5-*trichlorophenyl ester*. This *acid*, m. p. 143—144° (decomp.), $[\alpha]_D^{22}$ +18·6° (*c* 2·0 in acetic acid) (Found: C, 63·3; H, 6·6; N, 9·2.

* This and other active peptide derivatives are covered by pending Patent applications in the name of Imperial Chemical Industries Limited.

[14] K. Hofmann, H. Yajima, T.-Y. Liu, N. Yanaihara, C. Yanaihara, and J. L. Humes, *J. Amer. Chem. Soc.*, 1962, **84**, 4481.

[15] W. Grassmann and E. Wünsch, *Chem. Ber.*, 1958, **91**, 462.

[16] J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, 1963, **46**, 1609.

$C_{16}H_{20}N_2O_4$ requires C, 63·1; H, 6·6; N, 9·2%), and its *2,4,5-trichlorophenyl ester*, m. p. 132—133°, $[\alpha]_D^{22}$ +31·6° (*c* 1·0 in dimethylformamide) (Found: C, 54·4; H, 4·4; N, 5·9. $C_{22}H_{21}Cl_3N_2O_4$ requires C, 54·6; H, 4·4; N, 5·8%) were prepared by the methods described [1] for the L-isomers.

N-t-Butoxycarbonyl-D-methionine and 2,4,5-trichlorophenyl ester. This acid (an oil) and its 2,4,5-*trichlorophenyl ester*, m. p. 89—90° $[\alpha]_D^{22}$ +38·1° (*c* 2·0 in dimethylformamide) (Found: C, 45·1; H, 4·8; N, 3·3. $C_{16}H_{20}Cl_3NO_4S$ requires C, 44·8; H, 4·7; N, 3·3%), were prepared by the methods described [1] for the L-isomers.

Scheme 1.—*N-Benzyloxycarbonyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide* (III). Triethylamine (1·40 ml., 10 mmole) followed by ethyl chloroformate (0·94 ml., 10 mmole) were added at −10 to −5° to a stirred solution of β-t-butyl *N*-benzyloxycarbonyl-L-aspartate (3·23 g., 10 mmole) [17] in dry tetrahydrofuran (20 ml.). The mixture was stirred at −15 to −10° for 20 min.; a solution of L-phenylalanine amide acetate (2·24 g., 10 mmole) and triethylamine (1·40 ml.) in chloroform (30 ml.) was then added dropwise at −10 to −5°. The resulting mixture was stirred at 0—5° for 2 hr. and then at 20—22° for 16 hr. It was then evaporated, and the residue was shaken with ethyl acetate (250 ml.) and water (20 ml.). The ethyl acetate layer was separated and washed with 10% aqueous citric acid (20 ml.), water (20 ml.), N-aqueous potassium hydrogen carbonate (20 ml.), and water (3 × 20 ml.), then dried and evaporated to ~75 ml. The *protected dipeptide amide* (2·42 g., 51%), m. p. 157—158·5°, which separated, and a second crop (0·47 g., 10%), which was obtained by further evaporation of the mother-liquors, were collected and recrystallised from isopropanol or aqueous ethanol, giving fine needles, m. p. 158·5—159·5° (Found: C, 63·7; H, 6·8; N, 8·9. $C_{25}H_{31}N_3O_6$ requires C, 63·9; H, 6·7; N, 8·9%).

(β-t-Butyl)-L-aspartyl-L-phenylalanine amide (IV). A solution of the preceding protected derivative (2·348 g., 5 mmole) in pure methanol (75 ml.) was hydrogenated at room temperature and pressure for 5 hr. in the presence of 5% palladised charcoal (0·25 g.). The catalyst was removed (kieselguhr) and the solution was evaporated, yielding an oil which readily crystallised under ether. The *base* (1·481 g., 88%), m. p. 123—124° [unchanged after recrystallisation from benzene (25 ml.)] was collected and washed with ether (Found: C, 60·8; H, 7·6; N, 12·6. $C_{17}H_{25}N_3O_4$ requires C, 60·9; H, 7·5; N, 12·5%). In a subsequent experiment, the protected derivative (17·98 g., 38·3 mmole) was hydrogenated similarly, but 90% aqueous acetic acid (150 ml.) was used as the solvent. The acetate (14·0 g., 93%), which decomposed above 140°, was obtained after evaporation and drying of the resulting residue by azeotropic distillation with benzene. A suspension of this acetate (13·5 g.) in methylene chloride (500 ml.) was stirred at 0—5° for 15 min. with 50% aqueous potassium carbonate (15 ml.). Anhydrous magnesium sulphate was then added and the solution was filtered and evaporated. Recrystallisation of the resulting residue from benzene (60 ml.) gave the base (10·66 g., 93%), m. p. 123—124°.

N-(o-Nitrophenylsulphenyl)-L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide (VI). A solution of *N*-(*o*-nitrophenylsulphenyl)-L-methionine dicyclohexylammonium salt (6·78 g., 14 mmole) [9] in pure chloroform (350 ml.) was shaken at 0—5° with 0·5 N-sulphuric acid (56 ml.). The chloroform layer was quickly separated and washed with ice–water (2 × 70 ml.), then dried and evaporated to ~100 ml. (β-t-Butyl)-L-aspartyl-L-phenylalanine amide (4·70 g., 14 mmole) followed by *NN'*-dicyclohexylcarbodi-imide (3·17 g., 15·4 mmole) were added, and the resulting mixture was kept at 0—4° for 18 hr. Acetic acid (5 drops) was then added and the mixture was kept at 20—22° for a further 1 hr. The *NN'*-dicyclohexylurea (2·14 g.), m. p. 221—223°, was removed and the solution was evaporated. The resulting mixture was shaken with dry tetrahydrofuran (75 ml.) and the mixture was filtered. The filtrate was evaporated and the residue was triturated with ether, yielding the *nitrophenylsulphenyl-tripeptide derivative* (6·17 g., 71%), m. p. 165—167° (yellow needles, m. p. 168—169°, from ethanol) (Found: C, 54·4; H, 6·0; N, 11·0; S, 10·3. $C_{28}H_{37}N_5O_7S_2$ requires C, 54·3; H, 6·0; N, 11·3; S, 10·3%).

L-Methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide (VII). A solution of the preceding nitrophenylsulphenyl derivative (3·098 g., 5 mmole) in chloroform (50 ml.) was treated at 15° with 4·75N-hydrogen chloride in ether (2·10 ml., 10 mmole). The mixture was shaken at 15—20° for 5 min. then ether (100 ml.) was added and shaking was continued at 20—22° for 15 min. The solid was collected, washed with ether, and then dissolved in cold ethanol (20 ml.); colourless needles (1·606 g., 64%), m. p. 204—205° (effervescence) (unchanged after recrystallisation from ethanol–ether) of the *hydrochloride* began to separate within 2 min. and were collected after 30 min. (Found: C, 52·3; H, 7·3; N, 10·8. $C_{22}H_{34}N_4O_5S$·HCl requires C, 52·5; H, 7·0; N, 11·1%). A solution of the hydrochloride (7·33 g.) in hot water (100 ml.) was rapidly cooled and treated at 10° with 50% aqueous potassium carbonate (25 ml.). The *base* (6·39 g., 94%), m. p. 152—153° (unchanged after recrystallisation from ethyl acetate, ethanol, or water), R_{FA} 0·69, $E_{1\cdot9}$ (paper) = 0·51 × Phe–NH_2, was isolated by extraction with ethyl acetate (Found: C, 56·6; H, 7·5; N, 12·0. $C_{22}H_{34}N_4O_5S$ requires C, 56·6; H, 7·4; N, 12·0%).

N-t-Butoxycarbonyl-L-tryptophyl-L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide (IX). A solution of the preceding hydrochloride (0·251 g., 0·5 mmole), *N*-t-butoxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (0·241 g., 0·5 mmole), and triethylamine (0·07 ml., 0·5 mmole) in dimethylformamide (2 ml.) was kept at 20—22° for 18 hr. Ether (10 ml.) and water (10 ml.) were then added and the solid (0·317 g.), m. p. 171—174°, was collected and crystallised from ethanol, yielding the *tetrapeptide derivative*, m. p. 172—174° (Found: C, 60·9; H, 7·1; N, 11·0. $C_{38}H_{52}N_6O_8S$ requires C, 60·5; H, 6·95; N, 11·2%). The removal of the protecting groups of this derivative (IX ⟶ X) is described under Scheme 2.

N-(o-Nitrophenylsulphenyl)-L-tryptophyl-L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide. A solution of *N*-(*o*-nitrophenylsulphenyl)-L-tryptophan dicyclohexylammonium salt (0·538 g., 1 mmole) [9] in chloroform (50 ml.) was shaken at 20—22° for 30 min. with L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide hydrochloride (0·503 g., 1 mmole). *NN'*-Dicyclohexylcarbodi-imide (0·227 g., 1·1 mmole) was then added, and the resulting mixture was shaken at 20—22° for 18 hr. The solvent was removed and the residue was stirred at 40—50° for 10 min. with dry tetrahydrofuran. After cooling to 0°, the suspension was treated with 50% aqueous acetic acid (2 drops) and filtered after 20 min. The filtrate was evaporated and the resulting residue was triturated with ether,

[17] E. Schröder and E. Klieger, *Annalen*, 1964, **673**, 208.

yielding the *nitrophenylsulphenyl-tetrapeptide derivative* (0·781 g.), m. p. 170—172°, which separated from ethanol in yellow needles, m. p. 183—184° (Found: C, 57·8; H, 6·2; N, 12·0. $C_{39}H_{47}N_7O_8S_2$ requires C, 58·0; H, 5·9; N, 12·2%).

(β-*Benzyl*)-L-*aspartyl*-L-*phenylalanine amide*. β-Benzyl-*N*-benzyloxycarbonyl-L-aspartyl-L-phenylalanine amide (5·04 g., 10 mmole) in dry acetic acid (23 ml.) was treated at 15° with 45% hydrogen bromide in acetic acid (23 ml.). The solution was kept at 15—20° for 35 min., then diluted with ice-cold ether (700 ml.). The resulting gum was freed from the mother-liquors by decantation, washed four times with ether [the *hydrobromide hydrate* (51% yield), m. p. 185° (decomp.), could be obtained by recrystallisation of the resulting solid from methanol–ether and then from water (fine colourless needles) (Found: C, 51·6; H, 5·6; N, 9·1. $C_{20}H_{23}N_3O_4,HBr,H_2O$ requires C, 51·3; H, 5·4; N, 9·0%)] and dissolved in cold water (100 ml.). The solution was extracted with ether, then basified at 0° with 50% aqueous potassium carbonate (25 ml.) and extracted with ethyl acetate (3 × 60 ml.). The ethyl acetate extracts were washed with water, dried, and evaporated, yielding crystalline (β-*benzyl*)-L-*aspartyl*-L-*phenylalanine amide* (1·73 g., 47%), m. p. 99—101°, R_{FA} 0·73, which was collected with cyclohexane (Found: C, 65·1; H, 6·3; N, 11·3. $C_{20}H_{23}N_3O_4$ requires C, 65·0; H, 6·3; N, 11·4%).

N-(o-*Nitrophenylsulphenyl*)-L-*methionyl*-(β-*benzyl*)-L-*aspartyl*-L-*phenylalanine amide*. This (56% yield), m. p. 193—194·5° from methanol, was prepared in a manner similar to that described for the corresponding β-t-butyl ester but using (β-benzyl)-L-aspartyl-L-phenylalanine amide (Found: C, 56·9; H, 5·4; N, 10·7. $C_{31}H_{35}N_5O_7S_2$ requires C, 56·5; H, 5·2; N, 10·5%).

L-*Methionyl*-(β-*benzyl*)-L-*aspartyl*-L-*phenylalanine amide*. (*a*) The preceding nitrophenylsulphenyl derivative (0·251 g., 0·38 mmole) and 50% aqueous acetic acid (7 ml.) were stirred at 95° for 10 min. The bis(*o*-nitrophenyl) disulphide (28 mg., 50%), m. p. 192—194°, was removed by filtration, and the filtrate was evaporated. After basification of the residue with aqueous potassium carbonate, the resulting mixture was extracted with ethyl acetate. Evaporation of the dried extracts gave a solid (0·185 g.), m. p. 154—157°, which after recrystallisation from ethyl acetate, ethanol, and finally from ethyl acetate–light petroleum (b. p. 60—80°) yielded the pure *tripeptide amide ester* (93 mg., 48%), m. p. 174—175° (Found: C, 59·7; H, 6·9; N, 11·1. $C_{25}H_{32}N_4O_5S$ requires C, 59·95; H, 6·4; N, 11·2%).

(*b*) Cleavage of the nitrophenylsulphenyl derivative with 2 equiv. of dry hydrogen chloride in chloroform proceeded normally [Cf. procedure used for the corresponding β(t-butyl ester)] giving, after basification of the resulting hydrochloride, the tripeptide amide ester (90% yield), m. p. 174—175°.

Schemes 2 and 4, and Optical Isomers.—N-*Benzyloxycarbonyl*-(β-*benzyl*)-L-*aspartyl*-L-*phenylalanine amide* (XII). (*a*) A solution of L-phenylalanine amide acetate (112 g., 0·5 mole) in dimethylformamide (500 ml.) and triethylamine (67·2 ml., 0·48 mole) was treated dropwise with stirring over 20 min. at 5—10° with a solution of α-2,4,5-trichlorophenyl β-benzyl-*N*-benzyloxycarbonyl-L-aspartate (269 g., 0·5 mole) in dimethylformamide (250 ml.). The mixture was kept at 0—5° for 3 days then added to ice-water (2 l.) and cyclohexane (1 l.). The solid was collected, washed with water, and crystallised from 2-ethoxyethanol (1·5 l.), yielding the L,L-*isomer* (223 g., 89%), m. p. 170—171°, $[\alpha]_D^{22}$ −25·9° (*c* 1·0 in dimethylformamide) (Found: C, 66·6; H, 5·8; N, 8·2. $C_{28}H_{29}N_3O_6$ requires C, 66·8; H, 5·8; N, 8·35%).

(*b*) Triethylamine (1·40 ml., 10 mmole) followed by ethyl chloroformate (0·94 ml., 10 mmole) were added at −10° to a stirred solution of β-benzyl *N*-benzyloxycarbonyl-L-aspartate (3·57 g., 10 mmole) in dry tetrahydrofuran (20 ml.). The mixture was stirred at −15 to −10° for 20 min., then a solution of L-phenylalanine amide acetate (2·24 g., 10 mmole) and triethylamine (1·40 ml.) in chloroform (30 ml.) was added dropwise at −10 to −5°. The resulting mixture was stirred at 20—22° for 16 hr. and then evaporated. The solid residue was collected with water and crystallised from ethanol (60 ml.), yielding the L,L-isomer (2·54 g., 51%), m. p. 171—172°.

(*c*) A solution of β-benzyl *N*-benzyloxycarbonyl-L-aspartate (17·8 g., 50 mmole) and L-phenylalanine amide (8·20 g., 50 mmole) in dry tetrahydrofuran (100 ml.) was treated at 0° with *NN'*-dicyclohexylcarbodi-imide (10·3 g., 50 mmole) and the mixture was kept at 4° for 2 days. The solvent was removed and the resulting residue was crystallised twice from methanol (1·2 l.), yielding the L,L-isomer (18·0 g., 73%), m. p. 170·5—171°, containing traces of *NN'*-dicyclohexylurea.

N-*Benzyloxycarbonyl*-(β-*benzyl*)-L-*aspartyl*-D-*phenylalanine amide*. Obtained as described for the L,L-isomer [method (*a*)] using D-phenylalanine amide, the L,D-*isomer* separated from ethyl acetate in colourless needles (80% yield), m. p. 162—163°, $[\alpha]_D^{22}$ −0·9° (*c* 1·0 in dimethylformamide) (Found: C, 66·5; H, 5·9; N, 8·1%).

N-*Benzyloxycarbonyl*-(β-*benzyl*)-D-*aspartyl*-L-*phenylalanine amide*. Obtained as described for the L,L-isomer [method (*a*)] using α-2,4,5-trichlorophenyl β-benzyl *N*-benzyloxycarbonyl-D-aspartate, the D,L-*isomer*, m. p. 165—167°, $[\alpha]_D^{22}$ +0·3° (*c* 1·0 in dimethylformamide), was crystallised from ethanol (81% yield) (Found: C, 66·3; H, 5·3; N, 8·2%).

N-*Benzyloxycarbonyl*-(β-*benzyl*)-D-*aspartyl*-D-*phenylalanine amide*. Obtained as described for the L,L-isomer [method (*a*)] using α-2,4,5-trichlorophenyl β-benzyl D-aspartate and D-phenylalanine amide, the D,D-*isomer*, m. p. 169—170°, $[\alpha]_D^{22}$ +26·3° (*c* 1·0 in dimethylformamide) was crystallised from ethanol (74% yield) (Found: C, 66·9; H, 6·0; N, 8·3%).

L-*Aspartyl*-L-*phenylalanine amide* (XIII). A solution of *N*-benzyloxycarbonyl-(β-benzyl)-L-aspartyl-L-phenylalanine amide (222 g., 0·44 mole) in 95% aqueous acetic acid (3·5 l.) was hydrogenated over 6 hr. at room temperature and pressure in the presence of 5% palladised charcoal (45 g.). The filtered (kieselguhr) solution was evaporated, and the residue was dissolved in boiling water (500 ml.). Addition of acetone (2·5 l.) gave colourless needles which after drying for 24 hr. *in vacuo* at 50° gave the amide (94 g., 75%, m. p. 188—189° (decomp.), R_{FA} 0·38, R_{FC} 0·31, R_{FM} 0·36, $E_{1\cdot9}$ (paper) = 0·62 × Phe–NH_2, $[\alpha]_D^{22}$ +20·6° (*c* 1·0 in dimethylformamide containing 1 equiv. N-hydrochloric acid) (Found, immediately after drying: C, 55·7; H, 6·2; N, 15·0. $C_{13}H_{17}N_3O_4$ requires C, 55·8; H, 6·1; N, 15·1%). The hydrate was readily formed in moist air (Found: C, 52·9; H, 6·4; N, 13·8. $C_{13}H_{17}N_3O_4,H_2O$ requires C, 52·5; H, 6·45; N, 14·1%).

D-*Aspartyl*-L-*phenylalanine amide*. Prepared likewise in 64% yield, from *N*-benzyloxycarbonyl-(β-benzyl)-D-aspartyl-L-phenylalanine amide, the D,L-*isomer* separated from aqueous ethanol in colourless needles, m. p. 202—203°

(decomp.), $[\alpha]_D^{22}$ −9·6° (*c* 1·0 in N-hydrochloric acid) (Found: C, 55·7; H, 6·4; N, 15·0%).

L-*Aspartyl*-D-*phenylalanine amide*. Prepared likewise in 71% yield from *N*-benzyloxycarbonyl-(β-benzyl)-L-aspartyl-D-phenylalanine amide, the L,D-isomer separated from aqueous ethanol in colourless needles, m. p. 200—201° (decomp.), $[\alpha]_D^{22}$ +9·6° (*c* 1·0 in N-hydrochloric acid) (Found: C, 56·0; H, 6·3; N, 15·1%).

D-*Aspartyl*-D-*phenylalanine amide*. Prepared likewise in 84% yield from *N*-benzyloxycarbonyl-(β-benzyl)-D-aspartyl-D-phenylalanine amide, the D,D-isomer separated from aqueous acetone in colourless needles, m. p. 178—179° (decomp.), $[\alpha]_D^{22}$ −20·4° (*c* 1·0 in dimethylformamide containing 1 equiv. N-hydrochloric acid) (Found, after leaving a dried sample in moist air: C, 51·8; H, 6·6; N, 14·2%).

N-*t-Butoxycarbonyl*-L-*methionyl*-L-*aspartyl*-L-*phenylalanine amide* (XV). (*a*) L-Aspartyl-L-phenylalanine amide (55·8 g., 0·2 mole), *N*-t-butoxycarbonyl-L-methionine 2,4,5-trichlorophenyl ester (85·8 g., 0·2 mole),[1] dimethylformamide (700 ml.) and triethylamine (28 ml., 0·2 mole) were stirred at 0—5° for 18 hr., then the resulting solution was added to a stirred mixture of ice–water (3 l.), acetic acid (12 ml.), and cyclohexane (1 l.). The solid was collected, washed with water, then stirred with ethanol (1 l.) at 50—60° for 15 min. After cooling, the solid was collected and washed with ether, yielding *N*-t-butoxycarbonyl-L-methionyl-L-aspartyl-L-phenylalanine amide (83 g., 81%), m. p. 209—210° (decomp.) (unchanged after recrystallisation from 2-ethoxyethanol), R_{FA} 0·86, R_{FC} 0·82, $[\alpha]_D^{22}$ −39·3° (*c* 1·0 in dimethylformamide) (Found: C, 53·9; H, 6·6; N, 10·6. $C_{23}H_{34}N_4O_7S$ requires C, 54·1; H, 6·7; N, 11·0%).

(*b*) Crude *N*-t-butoxycarbonyl-L-methionyl-L-aspartyl-L-phenylalanine methyl ester (5·26 g., 10 mmole) (see Scheme 3) and dry methanol saturated with ammonia (50 ml.) were stirred at 0° for 18 hr. The mixture was then evaporated, and the residue was collected with ether and dissolved in warm methanol (30 ml.). After cooling, the solution was acidified to pH 3 with aqueous citric acid and diluted with ice–water (100 ml.), yielding the amide (2·21 g., 43%) identical with the sample prepared in (*a*).

N-*t-Butoxycarbonyl*-D-*methionyl*-L-*aspartyl*-L-*phenylalanine amide*. L-Aspartyl-L-phenylalanine amide (1·4 g., 5 mmole), *N*-t-butoxycarbonyl-D-methionine 2,4,5-trichlorophenyl ester (2·15 g., 5 mmole), dimethylformamide (30 ml.), water (1 ml.), and triethylamine (0·7 ml.) were stirred at 0° for 16 hr. After evaporation, the residue was treated with water (50 ml.) and the pH was adjusted to 2 with 10% aqueous citric acid. The solid was collected, washed well with ether, and crystallised from aqueous ethanol, yielding the D,L,L-*isomer* (1·9 g., 75%), m. p. 189—190° (decomp.), $[\alpha]_D^{22}$ −43·7° (*c* 1·0 in dimethylformamide) (Found: C, 54·1; H, 7·0; N, 10·9%).

N-*t-Butoxycarbonyl*-L-*methionyl*-D-*aspartyl*-D-*phenylalanine amide*. Prepared similarly to the D,L,L-isomer using D-aspartyl-D-phenylalanine amide and *N*-t-butoxycarbonyl-L-methionine 2,4,5-trichlorophenyl ester, the L,D,D-*isomer* (72% yield), m. p. 188—189° (decomp.), $[\alpha]_D^{22}$ +44·0° (*c* 1·0 in dimethylformamide), was crystallised from methanol–ethyl acetate (Found: C, 54·3; H, 6·4; N, 10·6%).

N-*t-Butoxycarbonyl*-D-*methionyl*-D-*aspartyl*-D-*phenylalanine amide*. Prepared similarly to the D,L,L-isomer using D-aspartyl-D-phenylalanine amide and *N*-t-butoxycarbonyl-D-methionine 2,4,5-trichlorophenyl ester, the D,D,D-*isomer* (74% yield), m. p. 204—205° (decomp.), $[\alpha]_D^{22}$ +39·0° (*c* 1·0 in dimethylformamide), was crystallised from ethanol (Found: C, 54·3; H, 6·8; N, 11·1%).

L-*Methionyl*-L-*aspartyl*-L-*phenylalanine amide*. (*a*) Finely powdered *N*-t-butoxycarbonyl-L-methionyl-L-aspartyl-L-phenylalanine amide (78·9 g., 0·155 mole) was added over 15 min. to vigorously stirred 1·75 N-hydrogen chloride in acetic acid (400 ml.) at 10—15°. Stirring was continued at 10—15° for 3 hr., then the solution was evaporated. The residue was collected and washed with ether and then crystallised from methanol (400 ml.)–ethyl acetate (1·5 l.) yielding colourless needles of the *tripeptide hydrochloride hemihydrate* (60·4 g., 87%), m. p. 185° (decomp.), R_{FA} 0·53, R_{FC} 0·38, R_{FM} 0·49, $E_{1\cdot9}$ (paper) = 0·57 × Phe–NH_2, $[\alpha]_D^{22}$ −12·7° (*c* 1·0 in dimethylformamide) (Found: C, 47·4; H, 6·3; N, 12·2. $C_{18}H_{26}N_4O_5S$,HCl, 0·5 H_2O requires C, 47·4; H, 6·2; N, 12·3%).

(*b*) A solution of *N*-t-butoxycarbonyl-L-methionyl-L-aspartyl-L-phenylalanine amide (0·511 g., 1 mmole) in trifluoracetic acid (2 ml.) was kept at 20—22° for 1 hr. Addition of dry ether (20 ml.) gave the tripeptide trifluoracetate (0·496 g.), which was collected and washed well with ether. A solution of the trifluoracetate or the hydrochloride, prepared as described in (*a*), (1 mmole) in water (3 ml.) was shaken with DE-ACIDITE G (acetate form); the filtered solution was evaporated and the residue was dried at 50—60° for 24 hr. *in vacuo*, yielding the *tripeptide* (100% yield) (colourless needles from dimethylformamide or aqueous methanol), m. p. 223—225° (decomp.) (Found: C, 52·3; H, 6·5; N, 13·4. $C_{18}H_{26}N_4O_5S$ requires C, 52·6; H, 6·4; N, 13·7%).

D-*Methionyl*-L-*aspartyl*-L-*phenylalanine amide hydrochloride monohydrate*. The *N*-t-butoxycarbonyl derivative (0·51 g., 1 mmole), acetic acid (3 ml.), and 3N-hydrogen chloride in ethyl acetate (5 ml.) were stirred at 20—25° for 25 min. The D,L,L-*hydrochloride monohydrate* (0·45 g., 97%), m. p. 189—192° (decomp.), (192—194° after several reprecipitations with di-isopropyl ether from methanolic solution), $[\alpha]_D^{22}$ −82·3° (*c* 1·0 in dimethylformamide) (Found: C, 46·6; H, 6·3; N, 12·3. $C_{18}H_{26}N_4O_5S$,HCl,H_2O requires C, 46·5; H, 6·2; N, 12·1%).

L-*Methionyl*-D-*aspartyl*-D-*phenylalanine amide hydrochloride hemihydrate*. Prepared from the *N*-t-butoxycarbonyl derivatives similarly to the D,L,L-isomer (100% yield), the L,D,D-*hydrochloride hemihydrate* had m. p. 197—198° (decomp.), $[\alpha]_D^{22}$ +82·0° (*c* 1·0 in dimethylformamide), after precipitation with ethyl acetate from methanolic solution (Found: C, 47·4; H, 6·2; N, 12·2. $C_{18}H_{26}N_4O_5S$,HCl, 0·5 H_2O requires C, 47·4; H, 6·15; N, 12·3%).

D-*Methionyl*-D-*aspartyl*-D-*phenylalanine amide hydrochloride hemihydrate*. Prepared from the *N*-t-butoxycarbonyl derivatives similarly to the D,L,L-isomer (100% yield), the D,D,D-*hydrochloride hemihydrate* had m. p. 182° (decomp.), $[\alpha]_D^{22}$ +12·4° (*c* 1·0 in dimethylformamide) (Found: C, 47·3; H, 6·2; N, 12·0%).

N-*t-Butoxycarbonyl*-L-*tryptophyl*-L-*methionine methyl ester* (XXVII). (*a*) *N*-t-Butoxycarbonyl-L-tryptophan (60·8 g., 0·2 mole),[1] L-methionine methyl ester hydrochloride (40 g., 0·2 mole),[18] and triethylamine (28 ml., 0·2 mole) were dissolved in AnalaR chloroform (400 ml.), and the solution was treated at 0° with *NN'*-dicyclohexylcarbodi-imide (41·2 g., 0·2 mole). The mixture was kept at 4° for 16 hr., then filtered from *NN'*-dicyclohexylurea and washed with

[18] M. Brenner and R. W. Pfister, *Helv. Chim. Acta*, 1951, **31**, 2085.

water (100 ml.), 10% aqueous citric acid (100 ml.), saturated aqueous sodium chloride (100 ml.), N-potassium hydrogen carbonate (100 ml.), and saturated aqueous sodium chloride (3 × 100 ml.) (backwashing with chloroform). The residue, obtained after drying and evaporating the chloroform solution, and backwashings, was dissolved in warm di-isopropyl ether (200 ml.) (peroxide-free) and the solution was cooled slowly, yielding colourless needles of the *dipeptide ester derivative* (80·1 g., 89%), m. p. 98—99° (Found: C, 58·9; H, 6·9. $C_{22}H_{31}N_3O_5S$ requires C, 58·8; H, 7·0%).

(*b*) A solution of *N*-t-butoxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (4·83 g., 10 mmole),[1] L-methionine methyl ester hydrochloride (2·0 g., 10 mmole), and triethylamine (1·40 ml., 10 mmole) in dimethylformamide (25 ml.) was kept at 4° for 36 hr. The product, isolated by evaporating the solution and extracting the resulting residue with ethyl acetate, was a mixture of the dipeptide ester derivative and 2,4,5-trichlorophenol. The mixture was not separated, as the phenol did not interfere in the preparation of the hydrazide (see below).

N-t-Butoxycarbonyl-L-tryptophyl-L-methionine hydrazide (XXVIII). 100% Hydrazine hydrate (11 ml., 0·22 mole) was added at 0—5° to a solution of the preceeding ester (44·9 g., 0·1 mole) [or a mixture of the ester and 2,4,5-trichlorophenol, obtained as described in (*b*) above] in methanol (100 ml.), and the mixture was left at 20—22° for 16 hr. The *hydrazide* was collected with ether and recrystallised from methanol (370 ml.) or ethyl acetate (600 ml.), yielding long, colourless needles, m. p. 177—178° (effervescence) (Found: C, 56·2; H, 7·0; N, 15·4. $C_{21}H_{31}N_5O_4S$ requires C, 56·1; H, 6·9; N, 15·6%).

N-t-Butoxycarbonyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (XVII). (*a*) Water (100 ml.), followed by triethylamine (14 ml. 0·1 mole), were added at 5—10° to a solution of L-methionyl-L-aspartyl-L-phenylalanine amide hydrochloride (22·4 g., 0·05 mole) and *N*-t-butoxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (26·6 g., 0·055 mole) in dimethylformamide (500 ml.), and the mixture was stirred at 0—5° for 2 days. Water (50 ml.) was then added and stirring was continued at 20—22° for a further 2 days. Addition of the resulting solution to ice–water (2 l.) and acetic acid (10 ml.) gave a solid, which was collected, washed with water, and then stirred for 5 min. with boiling ethanol (750 ml.). The *N-t-butoxycarbonyl-tetrapeptide amide* (28·9 g., 85%), m. p. 209—210° (decomp.) was collected after cooling, and washed well with ether (colourless needles from aqueous 2-ethoxyethanol), R_{FA} 0·81, R_{FC} 0·44, $[\alpha]_D^{22}$ −35·7° (*c* 1·0 in dimethylformamide) (Found: C, 58·6; H, 6·4; N, 12·0. $C_{34}H_{44}N_6O_8S$ requires C, 58·6; H, 6·3; N, 12·05%).

(*b*) 1·92N-Hydrogen chloride in dry tetrahydrofuran (0·52 ml., 1 mmole) was added to a vigorously stirred suspension of *N*-t-butoxycarbonyl-L-tryptophyl-L-methionine hydrazide (0·225 g., 0·5 mmole) in dry tetrahydrofuran (0·5 ml.) at −20°. As soon as all the solid had dissolved (1—2 min.), n-butyl nitrite (0·06 ml., 0·525 mmole) was added, and stirring was continued at −25 to −20° for 6 min. The resulting azide solution was then added to a solution of L-aspartyl-L-phenylalanine amide (0·14 g., 0·5 mmole) in dimethylformamide (2 ml.) and triethylamine (0·21 ml., 1·5 mmole). The mixture was kept at 0° for 2 days, then diluted with ice–water (10 ml.). The suspension was acidified to pH 2 with citric acid, and the solid was collected, washed with water and ether, and crystallised from ethanol (20 ml.), affording the *N*-t-butoxycarbonyl-tetrapeptide amide (0·206 g., 59%), identical with the sample prepared in (*a*).

(*c*) *N*-t-Butoxycarbonyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine methyl ester (0·712 g., 1 mmole) (see Scheme 3) and dry methanol saturated with ammonia (10 ml.) were stirred at 0° for 18 hr. The mixture was then evaporated, and the residue was dissolved in warm 50% aqueous methanol (20 ml.). After cooling, the solution was acidified to pH 3 with aqueous citric acid, yielding the amide (0·621 g., 89%), identical with the sample prepared in (*a*).

N-t-Butoxycarbonyl-D-tryptophyl-D-methionyl-D-aspartyl-D-phenylalanine amide. Obtained as described for the L,L,L,L-isomer [method (*a*)] using D-reactants, the D,D,D,D-*isomer* (60% yield), m. p. 210—202° (decomp.), $[\alpha]_D^{22}$ +36·2° (*c* 1·0 in dimethylformamide), was recovered from aqueous ethanol (Found: C, 57·8; H, 6·4; N, 12·0%).

N-t-Butoxycarbonyl-D-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide. Obtained as described for the L,L,L,L-isomer [method (*a*)] using *N*-t-butoxycarbonyl-D-tryptophan 2,4,5-trichlorophenyl ester, the D,L,L,L-*isomer* (59% yield) had m. p. 198—200° (decomp.), R_{FB} 0·68, $[\alpha]_D^{22}$ −23·8° (*c* 1·0 in dimethylformamide), after recrystallisation from methanol–ethyl acetate–light petroleum (b. p. 60—80°).

N-t-Butoxycarbonyl-L-tryptophyl-D-methionyl-D-aspartyl-D-phenylalanine amide. Obtained as described for the L,L,L-isomer [method (*a*)] using D-methionyl-D-aspartyl-D-phenylalanine amide hydrochloride, the L,D,D,D-*isomer* (57% yield) had m. p. 201—202° (decomp.) $[\alpha]_D^{22}$ +24·9° (*c* 1·0 in dimethylformamide), after recrystallisation from aqueous methanol (Found: C, 58·7; H, 6·7; N, 11·9%).

N-t-Butoxycarbonyl-L-tryptophyl-D-methionyl-L-aspartyl-L-phenylalanine amide. Obtained as described for the L,L,L,L-isomer [method (*a*)] using D-methionyl-L-aspartyl-L-phenylalanine amide hydrochloride hydrate, the L,D,L,L-*isomer* (74% yield) had m. p. 207—208° (decomp.), $[\alpha]_D^{22}$ −52·0° (*c* 1·0 in dimethylformamide), after recrystallisation from aqueous ethanol (Found: C, 58·2; H, 6·4; N, 11·8%).

N-t-Butoxycarbonyl-D-tryptophyl-D-methionyl-L-aspartyl-L-phenylalanine amide. Obtained as described for the L,L,L,L-isomer [method (*a*)] using D-methionyl-L-aspartyl-L-phenylalanine amide hydrochloride hydrate and *N*-t-butoxycarbonyl-D-tryptophan 2,4,5-trichlorophenyl ester, the D,D,L,L-*isomer* (52% yield) had m. p. 208—209° (decomp.), $[\alpha]_D^{22}$ −33·7° (*c* 1·0 in dimethylformamide), after recrystallisation from aqueous methanol (Found: C, 58·3; H, 6·0; N, 11·9%).

N-t-Butoxycarbonyl-D-tryptophyl-L-methionyl-D-aspartyl-D-phenylalanine amide. Obtained as described for the L,L,L,L-isomer [method (*a*)] using L-methionyl-D-aspartyl-D-phenylalanine amide hydrochloride hemihydrate and *N*-t-Butoxycarbonyl-D-tryptophan 2,4,5-trichlorophenyl ester, the D,L,D,D-*isomer* (53% yield) had m. p. 206—207° (decomp.), $[\alpha]_D^{22}$ +50·9° (*c* 1·0 in dimethylformamide), after recrystallisation from methanol–ethyl acetate (Found: C, 58·4; H, 6·5; N, 12·0%).

N-t-Butoxycarbonyl-L-tryptophyl-L-methionyl-D-aspartyl-L-phenylalanine amide. Obtained as described for the L,L,L,L-isomer [method (*b*)] using D-aspartyl-L-phenylalanine amide, the L,L,D,L-*isomer* (34% yield) had m. p. 150—151° (decomp.), $[\alpha]_D^{22}$ −0·4° (*c* 1·0 in dimethylformamide), after recrystallisation from methanol–ethyl acetate–

light petroleum (b. p. 60—80°) (Found: C, 58·3; H, 6·4; N, 11·9%).

N-*t*-*Butoxycarbonyl*-L-*tryptophyl*-L-*methionyl*-L-*aspartyl*-D-*phenylalanine amide*. Obtained as described for the L,L,L,L-isomer [method (*b*)] using L-aspartyl-D-phenylalanine amide, the L,L,L,D-*isomer* (55% yield) had m. p. 209—210° (decomp.), $[\alpha]_D^{22}$ −19·7° (*c* 1·0 in dimethylformamide), after recrystallisation from methanol–ethyl acetate–light petroleum (b. p. 60—80°) (Found: C, 58·2; H, 6·2; N, 11·8%).

N-*t*-*Butoxycarbonyl*-L-*tryptophyl*-L-*methionyl*-D-*aspartyl*-D-*phenylalanine amide*. Obtained as described for the L,L,L,L-isomer [method (*b*)] using D-aspartyl-D-phenylalanine amide, the L,L,D,D-*isomer* (52% yield) had m. p. 196—197° (decomp.), $[\alpha]_D^{22}$ +34·9° (*c* 1·0 in dimethylformamide), after recrystallisation from methanol–ethyl acetate (Found: C, 58·2; H, 6·5; N, 11·9%).

L-*Tryptophyl*-L-*methionyl*-L-*aspartyl*-L-*phenylalanine amide* (X). (*a*) *N*-t-Butoxycarbonyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (6·97 g., 10 mmole) and 80% aqueous trifluoracetic acid (30 ml.) were stirred under nitrogen at 20—22° for 2 hr. Ether (200 ml.) was then added and the solid was collected, washed exhaustively with ether and dissolved in cold methanol (50 ml.); addition of dry ether (200 ml.) gave colourless needles of the *tetrapeptide amide trifluoracetate* (6·96 g., 98%), m. p. 197° (decomp.), R_{FA} 0·63, R_{FB} 0·94, R_{FC} 0·37, $E_{1\cdot9}$ (paper) = 0·40 × Phe–NH_2, amino-acid ratios in acid hydrolysate, asp 1·00 : met 0·98 : phe 1·00 : try 0·61, amino-acids in L.A.P. digest of 1 μmole, asp 0·98, met 1·01, phe 0·98, try 0·98 μmole, $[\alpha]_D^{22}$ −26·5° (*c* 1·0 in dimethylformamide) (Found: C, 52·1; H, 5·4; N, 11·7. $C_{29}H_{36}N_6O_6S,C_2HO_2F_3$ requires C, 52·4; H, 5·25; N, 11·8%).

(*b*) A solution of *N*-t-butoxycarbonyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (6·97 g., 10 mmole) in boiling acetic acid (50 ml.) was rapidly cooled to 50° and then added in one portion, with stirring, to 1·75N-hydrogen chloride in acetic acid (60 ml., ~0·1 mole). The temperature of the mixture was rapidly brought to 20°, by vigorous cooling, and stirring was continued at 20—22° for 2 hr. Dry ether (500 ml.) precipitated the *tetrapeptide hydrochloride* (6·065 g., 96%), m. p. 215—216° (decomp.), $[\alpha]_D^{22}$ −31·7° (*c* 1·0 in dimethylformamide) (Found: Cl, 5·4. $C_{29}H_{36}N_6O_6S,HCl$ requires Cl, 5·6%), which separated from hot water (100 ml.) in gelatinous needles. The *free tetrapeptide* (0·552 g.), m. p. 251—252° (decomp.) was precipitated when a solution of the trifluoracetate (prepared as described above) or hydrochloride (1 mmole) in water (25 ml.) and N-sodium hydroxide (2·2 ml.) was acidified with acetic acid (1 ml.); it was collected and washed by centrifugation (Found: C, 57·0; H, 6·2; N, 13·8. $C_{29}H_{36}O_6N_6S,H_2O$ requires C, 56·6; H, 6·2; N, 13·7%).

(*c*) *N*-t-Butoxycarbonyl-L-tryptophyl-L-methionyl-(β-t-butyl)-L-aspartyl-L-phenylalanine amide (8 g.) and 80% aqueous trifluoracetic acid (32 ml.) were stirred under nitrogen at 20—22° for 3 hr. The product (100% yield) obtained as described in (*a*) was identical with the trifluoracetate described in (*a*).

Scheme 3.—N-*Benzyloxycarbonyl*-(β-*benzyl*)-L-*aspartyl*-L-*phenylalanine methyl ester* (XIX). A solution of L-phenylalanine methyl ester hydrochloride (21·6 g., 0·1 mole),[19] and α-2,4,5-trichlorophenyl β-benzyl *N*-benzyloxycarbonyl-L-aspartate (53·7 g., 0·1 mole) in dimethylformamide (100 ml.) was treated at 0° with triethylamine (14·0 ml., 0·1 mole). The resulting mixture was stirred at 0—4° for 3 days, and then added to a mixture of ice–water (200 ml.) and ether (100 ml.), yielding the *protected dipeptide ester* (49·9 g., 96%), m. p. 116—117° (unchanged after crystallisation from methanol), $[\alpha]_D^{22}$ −15·3° (*c* 1·0 in dimethylformamide), which was collected and washed with water and ether (Found: C, 67·2; H, 5·8; N, 5·4. $C_{29}H_{30}N_2O_7$ requires C, 67·1; H, 5·8; N, 5·4%).

L-*Aspartyl*-L-*phenylalanine methyl ester* (XX). A solution of the preceding protected derivative (49 g.) in acetic acid (500 ml.) and water (25 ml.) was hydrogenated over 5 hr. at room temperature and pressure in the presence of 5% palladised charcoal (5 g.). The filtered (kieselguhr) solution was evaporated, and the residue was dried, first by azeotropic distillation with benzene, and then for 48 hr. at 60° *in vacuo*, yielding the *dipeptide ester* (22·9 g., 87%), m. p. 246—247° (colourless needles from water), R_{FA} 0·61, $[\alpha]_D^{22}$ −2·3° (*c* 1·0 in N-hydrochloric acid) (Found: C, 57·1; H, 6·3; N, 9·4. $C_{14}H_{18}N_2O_5$ requires C, 57·2; H, 6·2; N, 9·5%).

N-*t*-*Butoxycarbonyl*-L-*methionyl*-L-*aspartyl*-L-*phenylalanine methyl ester* (XXI). A solution of L-aspartyl-L-phenylalanine methyl ester (2·94 g., 10 mmole), *N*-t-butoxycarbonyl-L-methionine 2,4,5-trichlorophenyl ester (4·29 g., 10 mmole),[1] and triethylamine (1·4 ml., 10 mmole) in dimethylformamide (50 ml.) and water (10 ml.), was kept at 4° for 3 days. The resulting solution was acidified at 0° to pH 4 with aqueous citric acid, and then added immediately to ice–water (100 ml.). The mixture was extracted with ethyl acetate (3 × 50 ml.) and the extracts were washed with 5% aqueous citric acid and water (4 times), dried, and evaporated. The residue was freed from 2,4,5-trichlorophenol by digestion with boiling cyclohexane (5 × 50 ml.), leaving the crude tripeptide ester derivative (6·0 g.) as a solid, which was used without further purification. The ammonolysis of this ester is described under *N*-t-butoxycarbonyl-L-methionyl-L-aspartyl-L-phenylalanine amide in Scheme 2.

N-*t*-*Butoxycarbonyl*-L-*tryptophyl*-L-*methionyl*-L-*aspartyl*-L-*phenylalanine methyl ester* (XXIV). A solution of the preceding crude tripeptide ester derivative (1·05 g., 2 mmole) in 1·75 N-hydrogen chloride in acetic acid (6 ml.) was kept at 10—15° for 2 hr., and then evaporated. The residue was dissolved in dimethylformamide (10 ml.), *N*-t-butoxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (1·06 g., 2·2 mmole)[1] followed by triethylamine (0·56 ml., 4 mmole) were added at 0°, and the resulting solution was kept at 4° for 3 days. After the addition of ice–water (50 ml.), the reaction mixture was acidified to pH 3 with aqueous citric acid and extracted with ethyl acetate. The extracts were washed with 5% aqueous citric acid and water, dried, and evaporated, yielding a gum which slowly solidified under water. The solid was collected, washed with water, dried, and then digested with boiling benzene (3 × 6 ml.). The insoluble solid was crystallised from isopropanol (6 ml.), yielding colourless needles of the *tetrapeptide ester derivative* (0·71 g., 50%), m. p. 168—170°, R_{FA} 0·82, R_{FC} 0·56, $[\alpha]_D^{22}$ −24·2° (*c* 1·0 in dimethylformamide) (Found: C, 58·6; H, 6·7; N, 9·7. $C_{35}H_{45}N_5O_9S$ requires C, 59·0; H, 6·5; N, 9·8%). The ammonolysis of this ester is described under *N*-t-butoxycarbonyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide in Scheme 2.

3-L-*Benzyl*-6-L-(*carboxymethyl*)-2,5-*dioxopiperazine* (XXI).

[19] R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud, and J.-P. Waller, *Helv. Chim. Acta*, 1956, **39**, 1421.

A solution of L-aspartyl-L-phenylalanine methyl ester (2·94 g., 10 mmole) in dry methanol saturated with ammonia (50 ml.) was kept at 0° for 16 hr., and then evaporated. The residue was dissolved in water (100 ml.) at 50°, and the solution was filtered and acidified with acetic acid, yielding the *piperazine* (1·39 g., 53%), which separated from acetic acid (20 ml.) in gelatinous needles, m. p. 256—258° (Found: C, 59·4; H, 5·3; N, 10·5. $C_{13}H_{14}N_2O_4$ requires C, 59·5; H, 5·4; N, 10·7%).

Preparation of Acylated Derivatives.—*N-Benzyloxycarbonyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide.* Water (30 ml.), followed by triethylamine (2·80 ml., 20 mmole), were added at 5—10° to a solution of L-methionyl-L-aspartyl-L-phenylalanine amide hydrochloride (4·47 g., 10 mmole) and *N*-benzyloxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (5·7 g., 11 mmole) in dimethylformamide (100 ml.). The mixture was stirred at 0—5° for 3 days and then at 20—22° for 1 day, and then added to a stirred mixture of ice–water (500 ml.), N-hydrochloric acid (22 ml.), and ethyl acetate (100 ml.). The *N-benzyloxycarbonyl-tetrapeptide amide* (6·789 g., 93%), m. p. 237—238° (decomp.), R_{FA} 0·86, R_{FB} 0·80, R_{FC} 0·47, R_{FD} 0·62, $[\alpha]_D^{22}$ −41·0° (*c* 1·0 in dimethylformamide) was collected and washed well with water followed by ether. For recrystallisation, the product was dissolved rapidly in boiling 2-ethoxyethanol (700 ml.). The solution was immediately cooled to 50° and treated with water (700 ml.), yielding colourless needles (6·174 g.) of unchanged m. p. (Found: C, 60·6; H, 5·8; N, 11·4. $C_{37}H_{42}N_6O_8S$ requires C, 60·7; H, 5·8; N, 11·5%).

Preparation of other N-acylated derivatives of L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide. A solution of the tetrapeptide trifluoracetate (0·178 g.) or hydrochloride (0·158 g.) (0·25 mmole) in warm (40—60°) dimethylformamide (2 ml.) was cooled to 0° and treated with triethylamine (0·07 ml., 0·5 mmole) followed by the appropriate active ester (0·3 mmole) (2,4,5-trichlorophenyl esters were used in all cases except the first four cited, when *p*-nitrophenyl esters were used). The solution was kept at 0—2° for 1—4 days (until there was no colour reaction on testing a sample with ninhydrin reagent), then the product was isolated by an appropriate procedure. In the case of acylated derivatives containing a t-butoxycarbonyl group, the reaction solution was usually added to a mixture of ice–water (25 ml.), hydrochloric acid (0·25 mmole), acetic acid (0·5 mmole), and cyclohexane, ether, or ethyl acetate (15 ml.). The product was then collected, washed four times with water, four times with ether and dried *in vacuo* at 40°. The procedure in other cases was usually similar except that hydrochloric acid (0·5 mmole) was used and the acetic acid omitted. When salts separated from the reaction mixture, these were first brought into solution by adding water (2 ml.) and warming to 60°; the warm solution was then worked up as described. Occasionally, after dilution with ice–water, the reaction mixture was extracted with ethyl acetate and the product precipitated from the aqueous phase with citric or hydrochloric acid. The products were invariably pure as judged by t.l.c. in two-solvent systems, but were crystallised from ethanol, 2-ethoxyethanol, acetic acid, or mixtures of these solvents with water. The following *N*-acyl derivatives of L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide were prepared [details in parenthesis refer to % yield, m. p. (decomp.), and R_F]: *N*-acetyl- (37, 245—246°); *N*-benzoyl- (29, 228—230°); *N*-DL-2-ethylhexanoylglycyl- (73, 229—230°); *N*-benzyloxycarbonyl-L-prolyl- (55, 238—240°); *N*-pivaloyl-β-alanyl- (66, 212—214°, R_{FA} 0·75, R_{FC} 0·43); *N*-benzoyl-β-alanyl- (75, 210—212°, R_{FC} 0·57); *N*-acetyl-β-alanyl- (58, 241—243°, R_{FA} 0·59, R_{FC} 0·32); *N*-benzyloxycarbonyl-β-alanyl- (84, 230—232°, R_{FA} 0·81, R_{FC} 0·40); *N*-t-butoxycarbonyl-L-alanyl-L-phenylalanyl-L-isoleucylglycyl- (76, 248—250°, R_{FA} 0·79, R_{FC} 0·46); *N*-t-butoxycarbonyl-L-alanyl- (84, 223—225°, R_{FA} 0·80, R_{FC} 0·47); *N-p*-(trifluoracetylamino)benzoyl- (76, 227—229°); N^{α}-t-butoxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl- (68, 207—208°); N^{α}-benzyloxycarbonyl-N^{ϵ}-t-butoxycarbonyl-L-lysyl- (87, 209—210°); *N*-hippuryl- (76, 237—238°); *L*-pyroglutamyl- (61, 232°); N^{α}-benzyloxycarbonyl-N^{ϵ}-(4-chloro-6-dimethylaminotriazin-2-yl)-L-lysyl- (80, 233°); *N*-benzyloxycarbonyl-L-phenylalanyl- (94, 241—243°); *N*-acetyl-γ-aminobutyryl- (51, 232°, R_{FC} 0·46); *N*-benzyloxycarbonyl-L-asparaginyl- (73, 231—232°); *N*-t-butoxycarbonyl-*S*-benzyl-L-homocysteinyl- (45, 215—216°); N^{α}-benzyloxycarbonyl-N^{ϵ}-butoxycarbonyl-L-lysylglycyl- (68, 200—201°); *N*-benzyloxycarbonyl-L-valyl- (87, 246—247°); *N*-t-butoxycarbonyl-*N*-methyl-L-alanyl- (68, 180—185°).

Hydrolysis of the appropriate *N*-t-butoxycarbonyl derivative (0·1 g.) with 80% aqueous trifluoracetic acid (1 ml.) (1 hr. at 20—22°) gave quantitative yields of the trifluoracetates of the following *N*-acyl derivatives of L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide: N^{α}-benzyloxycarbonyl-L-lysyl- m. p. 197—200° (decomp.), R_{FA} 0·80; N^{ϵ}-benzyloxycarbonyl-L-lysyl-, R_{FA} 0·67; *S*-benzyl-L-homocysteinyl-, R_{FA} 0·77; N^{α}-benzyloxycarbonyl-L-lysylglycyl-, R_{FA} 0·67.

N-Carbamoyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (Dr. H. Gregory). L-Tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide trifluoracetate (177 mg., 0·25 mmole) in water (1 ml.) and dimethylformamide (1 ml.) was treated with *N*-methylmorpholine (0·3 ml.). The mixture was stirred for 30 min., then acetic acid was added to bring the solution to pH 8. Potassium cyanate (108 mg., 1·3 mmole) in water (0·5 ml.) was added and the solution kept at 18—22° for 24 hr. Water (10 ml.) was added, then 2N-hydrochloric acid to adjust the solution to pH 2. After 2 hr., the precipitate was collected and recrystallised from aqueous dimethylformamide, yielding the *carbamoyl tetrapeptide* (132 mg., 82%), m. p. 205—206° (decomp.), R_{FC} 0·15 (Found: C, 55·8; H, 6·2; N, 15·1; $C_{30}H_{37}N_7O_7S,0·5H_2O$ requires C, 55·6; H, 5·9; N, 15·1%).

Various Routes to I.C.I. 50,123.—L-Tryptophyl-L-methionine methyl ester hydrochloride.* A solution of *N*-t-butoxycarbonyl-L-tryptophyl-L-methionine methyl ester (8·99 g., 20 mmole) in warm ethyl acetate (20 ml.) was cooled to 20° and treated with 3·45 N-hydrogen chloride in ethyl acetate (50 ml.). The solution was kept at 20—22° for 2 hr., dry ether (500 ml.) was added, and the solid (7·74 g., 100%), m. p. 95—105° (decomp.), R_{FA} 0·66, R_{FC} 0·83, was collected and washed with dry ether. A solution of this hydrochloride (3·86 g., 10 mmole) in water (5 ml.) was cooled to 0° and then shaken with ethyl acetate (7 ml.) and 50% aqueous potassium carbonate (2 ml.). The ethyl acetate phase was separated and combined with two further extracts of the aqueous phase with ethyl acetate. The combined extracts (30 ml.) were dried ($MgSO_4$) and evaporated, giving the corresponding base as a brown oil. This was dissolved in dimethylformamide to give 6·0 ml. of solution, and the resulting solution was used immediately

* Patent pending.

in the preparation of *N*-t-butoxycarbonyl-β-alanyl-L-tryptophyl-L-methionine methyl ester [described in (*b*) below].

N-t-Butoxycarbonyl-β-alanyl-L-tryptophyl-L-methionine methyl ester. (*a*) A solution of *N*-t-butoxycarbonyl-β-alanine trichlorophenyl ester (0·184 g., 0·5 mmole) [1] in dimethylformamide (2 ml.) was treated at 0° with L-tryptophyl-L-methionine methyl ester hydrochloride (0·192 g., 0·5 mmole) and triethylamine (0·07 ml.). The resulting solution was kept at 0° for 48 hr. and then added to a mixture of ice (10 g.) and acetic acid (0·006 ml.). The mixture was extracted with ethyl acetate (3 × 10 ml.) and the ethyl acetate extracts were washed with 5% aqueous citric acid (10 ml.), water (5 ml.), N-aqueous sodium hydrogen carbonate (3 ml.), and water (3 × 3 ml.), then dried ($MgSO_4$), and evaporated. The residue (0·224 g., 86%) was collected with light petroleum (b. p. 60—80°) (5 ml.) and crystallised from a mixture of ethyl acetate (5 ml.) and cyclohexane (10 ml.), giving the *tripeptide ester derivative* (0·194 g., 75%), m. p. 137—139° (138—139° after further recrystallisation from ethyl acetate), R_{FA} 0·96, R_{FC} 0·88, R_{FF} 0·71 (Found: C, 57·5; H, 7·0; N, 10·6. $C_{25}H_{36}N_4O_6S$ requires C, 57·7; H, 7·0; N, 10·8%).

(*b*) *N*-t-Butoxycarbonyl-β-alanine 2,4,5-trichlorophenyl ester (1·84 g., 5 mmole) [1] in dimethylformamide (4 ml.) was added at 0° to a solution of L-tryptophyl-L-methionine methyl ester (6 mmole) in dimethylformamide (3·6 ml.). Acetic acid (0·03 ml., 0·6 mmole) was added and the mixture was kept at 0° for 48 hr. The product (2·23 g., 86%), m. p. 132—134° [1·97 g. (76%), m. p. 138—139° after recrystallisation from ethyl acetate], was isolated as described in (*a*) above.

N-t-Butoxycarbonyl-β-alanyl-L-tryptophyl-L-methionine hydrazide. A solution of the above ester (1·972 g., 3·79 mmole) in methanol (25 ml.) was treated with 100% hydrazine hydrate (0·405 ml., 8·34 mmole). The mixture was kept at 20—22° for 18 hr., and the hydrazide (0·842 g., 43%), m. p. 218—219° (decomp.) (unchanged after crystallisation from ethanol) was collected (Found: C, 55·0; H, 7·1; N, 16·0. $C_{24}H_{36}N_6O_5S$ requires C, 55·4; H, 7·0; N, 16·1%). A second crop (0·232 g., 12%), m. p. 216—217° (decomp.) was obtained by evaporation of the mother-liquors to 10 ml.

N-t-Butoxycarbonyl-β-alanyl-L-tryptophan methyl ester. A solution of L-tryptophan methyl ester hydrochloride (6·43 g., 25·25 mmole) [20] in water (40 ml.) was treated at 0—5° with 50% aqueous potassium carbonate (10 ml.). The oil was extracted into ethyl acetate (3 × 20 ml.) and the extracts were dried ($MgSO_4$) and treated with *N*-t-butoxycarbonyl-β-alanine (3·78 g., 20 mmole). A salt separated; the solvent was therefore removed *in vacuo* at 20—25° and the residue dissolved in acetone (100 ml.). The solution was treated at 0° with *NN'*-dicyclohexylcarbodi-imide (4·12 g., 20 mmole) and stirred at 0° for 48 hr. Acetic acid (5 drops) was added and the mixture was kept at 20—25° for 20 min., and then filtered. The residue and the solid obtained by evaporating the filtrate were combined and digested with boiling ethyl acetate. The suspension was quickly cooled to 30° and filtered from *NN'*-dicyclohexylurea. The filtrate was washed with 0·5N-hydrochloric acid, water, N-sodium hydrogen carbonate solution, and water, then dried ($MgSO_4$) and evaporated. The residue (6·07 g., 77%), m. p. 155—157°, was collected and washed with ether, and crystallised from ethyl acetate, yielding the *ester*, m. p. 157—158°, R_{FA} 0·87, R_{FC} 0·82 (Found: C, 62·3; H, 7·0; N, 10·5. $C_{20}H_{27}N_3O_5$ requires C, 61·6; H, 7·0; N, 10·8%).

N-t-Butoxycarbonyl-β-alanyl-L-tryptophan hydrazide. A solution of the preceding methyl ester (3·89 g., 10 mmole) in warm methanol (50 ml.) was cooled to 20° and treated with 100% hydrazine hydrate (1·07 ml., 22 mmole). The solution was kept at 20—22° for 48 hr. and the solid (2·1 g., 54%) and a second crop (1·36 g., 35%), obtained by evaporation of the mother-liquors, were collected. The two crops, m. p. 186—188° (decomp.) were combined and crystallised from ethanol, yielding the *hydrazide*, m. p. 198—199° (decomp.), R_{FF} 0·28 (Found: C, 58·5; H, 6·9; N, 17·7. $C_{19}H_{27}N_5O_4$ requires C, 58·6; H, 7·0; N, 18·0%).

N-t-Butoxycarbonyl-β-alanyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (I.C.I. 50,123). (*a*) A solution of L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide trifluoracetate (3·55 g., 5 mmole) and *N*-t-butoxycarbonyl-β-alanine 2,4,5-trichlorophenyl ester (2·20 g., 6 mmole) [1] in dimethylformamide (30 ml.) was treated at 0° with triethylamine (1·40 ml., 10 mmole). The mixture was stirred at 4° for 36 hr. and then at 20—23° for 24 hr., and then added to a mixture of ice–water (100 ml.), N-hydrochloric acid (5 ml.), acetic acid (0·6 ml.), and ethyl acetate (25 ml.). The solid was collected, washed five times with water and five times with ether, and dried at 40° *in vacuo*, yielding the pure *pentapeptide derivative* (3·785 g., 98%), m. p. 229—230° (decomp.) (variable with rate of heating), R_{FA} 0·83, R_{FB} 0·74, R_{FC} 0·54, R_{FD} 0·82, $E_{1\cdot9}$ (thin-layer) = 0, $E_{1\cdot9,\ 6\cdot5,\ or\ 8\cdot0}$ (paper) = 0, $[\alpha]_D^{22}$ −28·8° ± 0·5° (*c* 1·0 in dimethylformamide), amino-acids in acid hydrolysate of 1 μmole, β-ala 0·97, asp 1·00, met 0·99, phe 1·00, try 0·60 μmole, and in alkali hydrolysate of 1 μmole, β-ala 0·65, asp 0·97, met 1·00, phe 1·03, try 0·97 μmole (Found: C, 57·4; H, 6·4; N, 12·4. $C_{37}H_{49}N_7O_9S$ requires C, 57·9; H, 6·4; N, 12·8%). A solution of the derivative in water containing two equiv. of ammonium hydroxide had $\lambda_{min.}$ 246·5 mμ (ε 2080); λ_{inf} 272—275 (5040); $\lambda_{max.}$ 280 (5340) and 289 (4590). For recrystallisation, this product was treated with boiling 2-ethoxyethanol (100 ml.) and dissolved rapidly by vigorous stirring at the boil (1—2 min.). The hot solution was filtered and the filtrate and washings (total 150 ml.) were cooled to 80° and treated with distilled water (100 ml.). Colourless needles (3·306 g., 86%), which were indistinguishable from the product prior to crystallisation in m. p., chromatographic behaviour, and optical rotation, were obtained on cooling.

(*b*) *N*-t-Butoxycarbonyl-β-alanyl-L-tryptophyl-L-methionine hydrazide (0·521 g., 1 mmole) was added at −20° to a solution of 0·91N-hydrogen chloride in tetrahydrofuran (2·2 ml., 2 mmole). The mixture was stirred for 2 min., then n-butyl nitrite (0·12 ml., 1·05 mmole) was added dropwise, and the mixture was further stirred at −20 to −25° for 6 min. Half the resulting solution was then added at −10° to a solution of L-aspartyl-L-phenylalanine amide (0·153 g., 0·55 mmole) in a mixture of dimethylformamide (2 ml.), water (0·1 ml.), and triethylamine (0·294 ml., 2·1 mmole). Water (0·1 ml.) was added and the solution was stirred at 0° for 72 hr. The resulting mixture was acidified to pH 3·5 with 5% aqueous citric acid then added to ice–water (20 g.). The solid (0·280 g., 73%), m. p. 210—211° (decomp.), was collected and washed with water (3 × 10 ml.), and dissolved in boiling 2-ethoxyethanol (7 ml.); the solution was rapidly cooled to 80° when water

[20] R. A. Boissonnas, St. Guttmann, R. L. Huguenin, R.-A. Jaquenoud, and Ed. Sandrin, *Helv. Chim. Acta*, 1958, **41**, 1867.

(7 ml.) was added, giving crystals of the pentapeptide derivative (0·120 g., 31%), m. p. 226—227° (decomp.) on cooling. The pure compound, m. p. 229—230° (decomp.), was obtained after a further recrystallisation from aqueous 2-ethoxyethanol and was identical with the sample prepared as described in (*a*) above.

(*c*) *N*-t-Butoxycarbonyl-β-alanyl-L-tryptophan hydrazide (0·389 g., 1 mmole) was added at −20° to a stirred solution of 0·91N-hydrogen chloride in tetrahydrofuran (2·2 ml., 2 mmole). The solution was stirred at −20° for 2 min., then freshly distilled n-butyl nitrite (0·12 ml., 1·06 mmole) was added and the mixture was further stirred at −20 to −25° for 6 min. The resulting solution was added at 0° to a solution of L-methionyl-L-aspartyl-L-phenylalanine amide hydrochloride (0·447 g., 1 mmole) in a mixture of dimethylformamide (5 ml.), water (2·5 ml.), and triethylamine (0·56 ml., 4 mmole). The mixture was stirred at 0° for 1 hr., then dimethylformamide (5 ml.) and water (7·5 ml.) were added. The resulting mixture was stirred at 0° for 96 hr., then acidified to pH 3·5 with 5% aqueous citric acid, and added to a mixture of ice–water (50 g.) and ethyl acetate (50 ml.). The solid was collected and crystallised from aqueous 2-ethoxyethanol, yielding the pure pentapeptide derivative (0·162 g., 21%) identical with the sample prepared as described in (*a*) above.

β-Alanyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide hydrochloride. This *hydrochloride* (90% yield), m. p. 208°, R_{FA} 0·47, $E_{1\cdot9}$ (paper) = 0·33 × his, 0·32 × β-ala, 0·43 × phe, $E_{1\cdot9}$ (thin-layer) = 0·72 × ala, 0·64 × phe, $E_{6\cdot5}$ (thin-layer) = 0·38 × ala, $E_{8\cdot0}$ (thin-layer) = 0·48 × his, 0·50 × β-ala, was obtained by cleavage of the preceding t-butoxycarbonyl-derivative (0·154 g., 0·2 mmole) in acetic acid (1 ml.) with 3·8 N-hydrogen chloride in ethyl acetate (0·27 ml., 1 mmole) (45 min. at 18—20°) (Found: C, 54·5; H, 6·1; Cl, 5·0. $C_{32}H_{41}N_7O_7S,HCl$ requires C, 54·6; H, 6·0; Cl, 5·05%).

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