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Peptides. Part XXVIII.[1] Synthesis of Ovine–Bovine Gastrin I

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The heptadecapeptide amide, pGlu-Gly-Pro-Trp-Val-Glu-Glu-Glu-Glu-Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2 (I), has been synthesised and shown to be identical with natural ovine–bovine gastrin I. Partial nitrosation of tryptophyl residues was detected during earlier attempts to synthesise (I) by routes involving azide couplings.

EARLIER papers in this series have described syntheses of the antral hormone gastrin from porcine [2] and human [3] species. More recently,[4] we and our colleagues have reported the isolation of gastrins from sheep and from cattle, and showed that the hormones from the two species were identical and consisted of sulphated and unsulphated forms of the heptadecapeptide amide (I). They are thus [Val^5, Ala^{10}]-analogues of the porcine and human hormones. We now give details of the synthesis of (I) which confirmed the amino-acid sequence assigned to the ovine and bovine gastrins.

pGlu-Gly-Pro-Trp-^{5}Val-Glu-Glu-Glu-Glu-^{10}Ala-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2 (I)

Several routes, based on condensation of peptide fragments comprising residues 1—5 and 6—17, or 1—6 and 7—17, and essentially analogous to those already described for the preparation of porcine gastrin were investigated.[2] In the present case, however, application of these and similar procedures yielded impure products.† The presence of the impurity was revealed only by enzymic degradation of the synthetic gastrins. During the structural studies,[4] it was found that natural ovine–bovine gastrin I was completely cleaved by subtilisin at the Trp-Val bond, but the synthetic products consistently yielded small amounts of a peptide fragment in which this bond was intact. Similar results were also obtained with thermolysin as the hydrolysing agent, and it seemed likely that this resistance to enzymic hydrolysis was due to some modification of the tryptophan residue during the synthesis. Since all these synthetic routes involved a final azide coupling of the *N*-terminal pentapeptide or hexapeptide, a small degree of nitrosation of the indole ring might have occurred during the formation of the peptide azide.[5]

This possibility was studied further with a simple model compound, acetyltryptophan methyl ester. Extended treatment of this ester under conditions otherwise similar to those used for the preparation of peptide azides readily gave a crystalline nitroso-derivative, $\lambda_{max.}$ 265 and 330 nm., almost identical to that ($\lambda_{max.}$ 264 and 331 nm.) reported [6] for 3-methyl-1-nitrosoindole, and formulation as an *N*-nitroso-derivative was also supported by the mass spectrum.‡ In contrast to the initial acetyltryptophan ester, the nitroso-derivative was completely inert to both subtilisin and chymotrypsin. These experiments thus supported the view that partial nitrosation of the tryptophan residue at position 4 in the peptide chain had occurred during the syntheses involving azide couplings. The resistance of *N*-nitroso-tryptophyl residues to enzymic hydrolysis may be a generally useful test for the detection of this side reaction, which in the past may have gone undetected in the synthesis of large peptides.

No improvement was obtained when azide formation

† We wish to thank Dr. J. Beacham, Dr. P. H. Bentley, and Dr. J. J. Mendive who carried out these experiments.

‡ The spectrum showed no parent ion but peaks at *m/e* 259 260, probably arising by (thermal) loss of nitric oxide with gain of a hydrogen atom from the spectrometer. A strong peak at *m/e* 30 (NO) was also present. Similar observations on other nitroso-compounds have been made in this laboratory [7] and elsewhere.[8]

[1] Part XXVII, K. L. Agarwal, G. W. Kenner, and R. C. Sheppard, *J. Chem. Soc.* (*C*), 1968, 1384.

[2] J. C. Anderson, G. W. Kenner, J. K. MacLeod, and R. C. Sheppard, *Tetrahedron*, 1966, Suppl. 8, 39.

[3] J. Beacham, P. H. Bentley, G. W. Kenner, J. K. MacLeod, J. J. Mendive, and R. C. Sheppard, *J. Chem. Soc.* (*C*), 1967, 2520.

[4] K. L. Agarwal, J. Beacham, P. H. Bentley, R. A. Gregory, G. W. Kenner, R. C. Sheppard, and Hilda, J. Tracy, *Nature*, 1968, **219**, 614.

[5] *Cf.* H. Zahn and D. Brandenburg, *Annalen*, 1966, **692**, 220.

[6] H. F. Hodson and G. F. Smith, *J. Chem. Soc.*, 1957, 3546.

[7] R. A. W. Johnstone and D. W. Payling, personal communication.

[8] J. Collin, *Bull. Soc. roy. Sci. Liège*, 1954, **22**, 201.

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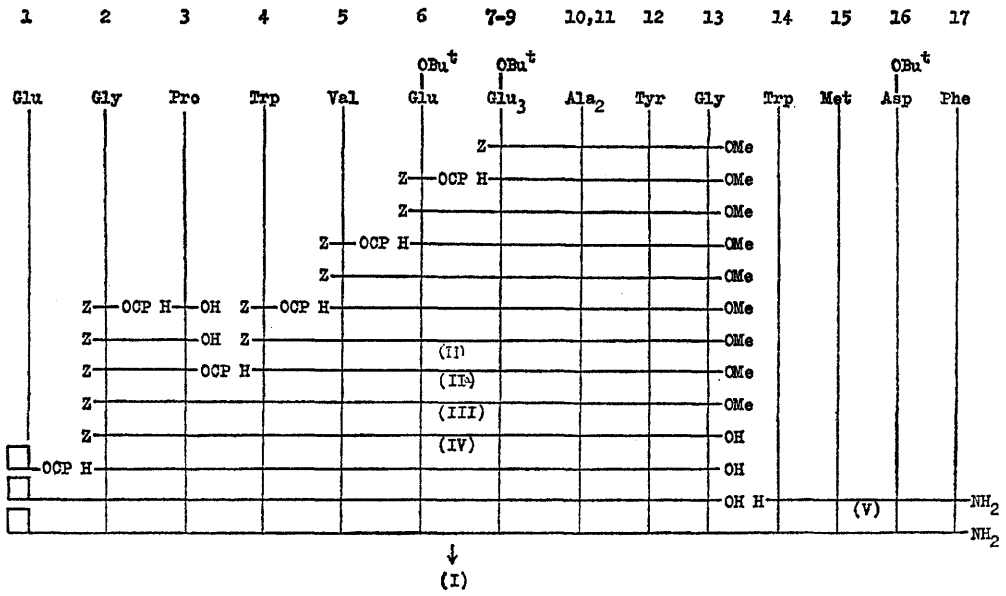
was effected under other conditions (*e.g.* by the anhydrous procedure of Honzl and Rudinger [9]), and the problem was therefore avoided completely in redesigned syntheses, as indicated in the Schemes. The absence of methionine from the *N*-terminal region of (I) allows more extensive use of stepwise procedures than was possible in the case of porcine gastrin, and in fact obviates the need for azide couplings completely. Indeed, routes similar to those employed here have been used [10] in a synthesis of human gastrin, which also lacks methionine in the *N*-terminal region. For those cases where methionine is present in this part of the molecule, we have recently developed a new, largely stepwise route, which has been applied to the synthesis of canine gastrin [11] and may be generally applicable in the gastrin series.

ing only 60% reaction. The cause of this difficulty was not obvious, but in any case it was avoided completely by the simultaneous addition of residues 2 and 3 as a dipeptide unit. Neither the benzyloxycarbonyl nor the t-butoxycarbonyl derivative of glycylproline trichlorophenyl ester could be obtained crystalline, but both oily active esters afforded the desired dodecapeptide derivatives (III) and (VI) when treated with the hydrogenated decapeptide (IIa).

The synthesis of (I) was completed in two ways. The terminal methyl ester groups in (III) and (VI) were selectively saponified in the presence of the side-chain t-butyl esters without difficulty. The benzyloxycarbonyl group in (IV) (Scheme 1) was cleaved by hydrogenolysis and the *N*-terminal pyroglutamyl residue was



SCHEME 1

The synthesis of the decapeptide derivative (II) (Scheme 1) was straightforward. We have recently described [1] an improved procedure for the preparation of the tripeptide ester, Ala-Tyr-Gly-OMe, and seven residues were added to this peptide by use of benzyloxycarbonylamino-acid trichlorophenyl esters exclusively. In continuation of our previous practice, t-butyl esters were used for the protection of acidic side chains, and intermediate benzyloxycarbonyl derivatives were cleaved by hydrogenolysis. In initial experiments residue 3 (proline) was then added by the *p*-nitrophenyl ester method and the *N*-protecting group was removed completely without difficulty. However, residue 2 (glycine) could not then be efficiently incorporated by use of either the benzyloxycarbonyl or t-butoxycarbonyl derivative of glycine trichlorophenyl ester; at best the product contained only 1·6 residues of glycine, indicat-

added, again by the active ester method. Condensation of the resulting tridecapeptide with the *C*-terminal tetrapeptide amide (V)[10] *via* the intermediate pivalic anhydride then yielded the fully protected gastrin from which all the protecting groups were cleaved by trifluoroacetic acid. Alternatively (Scheme 2), the t-butoxycarbonyl derivative (VII) was combined with the tetrapeptide amide (V), all the protecting groups were stripped from the product with trifluoroacetic acid, and the terminal pyroglutamyl residue was added in the final step. In both cases the synthetic gastrins were purified by gel filtration and were homogeneous by the usual criteria of paper electrophoresis, t.l.c., and amino-acid analysis.

The synthetic gastrin was fully active in stimulating the flow of gastric juice in the conscious dog, and was

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

[10] J. S. Morley, *J. Chem. Soc.* (*C*), 1967, 2410.

[11] K. L. Agarwal, G. W. Kenner, and R. C. Sheppard, *Experientia*, in press.

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indistinguishable on paper electrophoresis and t.l.c. from natural bovine gastrin I and from a desulphated sample of ovine gastrin II. Convincing evidence for the identity of the natural and synthetic products was furnished by electrophoretic and t.l.c. comparison of enzymic digests prepared by use of subtilisin (cf. ref. 3) and thermolysin. Cleavage at the tryptophyl-valine bond by both enzymes was complete, and no differences could be detected in the patterns of products obtained from the natural and the synthetic gastrins.

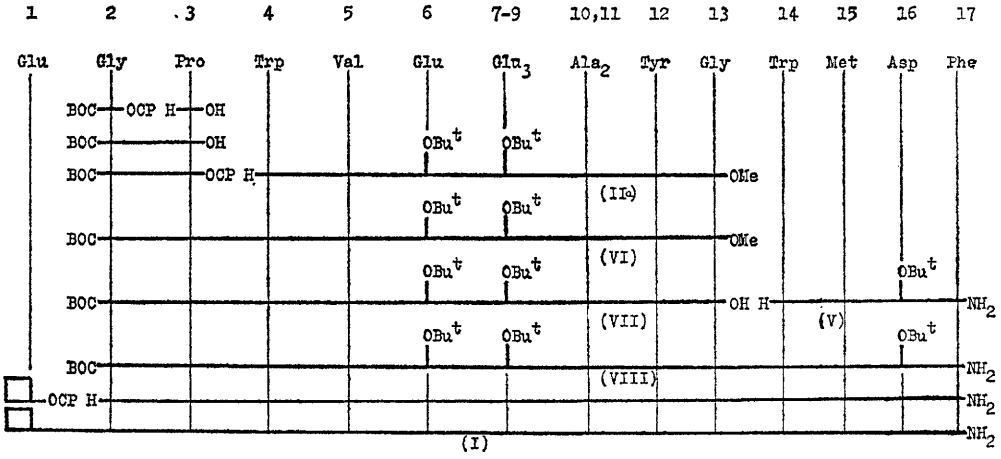
EXPERIMENTAL

The general instructions given in Parts XXV [3] and XXVII [1] apply. The additional solvent systems for t.l.c. were TLC-13, chloroform–methanol (20 : 1); TLC-14, chloroform; and TLC-15, chloroform–methanol (10 : 1).

N(α)-*Acetyl-1-nitroso-L-tryptophan Methyl Ester*.—*N*-Acetyl-L-tryptophan methyl ester (130 mg., 0·5 mmole) was dissolved in dimethylformamide (2·0 ml.) and treated with 2N-hydrochloric acid (0·5 ml., 1·0 mmole) at −5° followed by N-sodium nitrite (1·0 ml., 1·0 mmole). The mixture was kept for 12 hr. at 0°, then another portion of 2N-hydrochloric acid (0·5 ml., 1·0 mmole) and N-sodium nitrite (1·0 ml., 1·0 mmole) was added. After 12 hr. at 0° the solution was evaporated under reduced pressure and the residue was dissolved in ethyl acetate (10 ml.), washed with water (2 × 25 ml.), and dried ($MgSO_4$). The *nitroso-derivative* (130 mg.) had m.p. 135—137° (from ethyl acetate–petroleum) (Found: C, 58·0, H, 5·0; N, 14·3. $C_{14}H_{15}O_4N_3$ requires C, 58·1; H, 5·2; N, 14·5%).

Benzyloxycarbonyl-L-alanyl-L-alanyl-L-tyrosylglycine Methyl Ester.—Benzyloxycarbonyl-L-alanyl-L-tyrosylglycine methyl ester [1] (4·57 g., 10 mmoles) was hydrogenated in 80% acetic acid over palladium–charcoal (5%; 1·2 g.) at room temp. until carbon dioxide evolution ceased. After filtration and evaporation, the residue was dissolved in dimethylformamide (20 ml.) and treated with benzyloxycarbonyl-L-alanine 2,4,5-trichlorophenyl ester (4·5 g., 11 mmoles) and triethylamine (1·0 ml., 7 mmoles). After 18 hr. at 37°, the solution was evaporated, the residue was dissolved in ethyl acetate (100 ml.), and 3-dimethylaminopropylamine (1·0 ml.) was added. The *tetrapeptide derivative* crystallised from this solution and was collected, washed with ether, and recrystallised from ethyl acetate; yield 4·8 g. (90%), m.p. 212—213°, $[\alpha]_D^{23°}$ −22·9° (*c* 0·63 in dimethylformamide), TLC-1 R_F 0·43, TLC-13 R_F 0·56, $Ala_{2·00}$ $Gly_{1·00}$ $Tyr_{0·83}$ (Found: C, 58·8; H, 6·0; N, 10·5. $C_{26}H_{32}N_4O_8$ requires C, 59·1; H, 6·1; N, 10·6%).

Benzyloxycarbonyl-γ-t-butyl-L-glutamyl-L-alanyl-L-alanyl-L-tyrosylglycine Methyl Ester.—The foregoing tetrapeptide derivative (5·28 g., 10 mmoles) was hydrogenated in acetic acid (80%; 50 ml.) over 5% palladium–charcoal (1·1 g.) at room temp. The resulting tetrapeptide acetate was coupled with *N*-benzyloxycarbonyl-α-(2,4,5-trichlorophenyl)-γ-t-butyl-L-glutamate (5·72 g., 11 mmoles) in dimethylformamide (20 ml.) in the presence of triethylamine (1·0 ml.) and the product was treated with 3-dimethyl-



SCHEME 2

aminopropylamine (1·0 ml.) as described before. The crude product yielded the *pentapeptide derivative* (6·6 g., 91%), m.p. 207—208° (from ethyl acetate), $[\alpha]_D^{23}$ −19·8° (*c* 1·0 in dimethylformamide), TLC-1 R_F 0·40, TLC-13 R_F 0·53, $Glu_{1·01}$ $Ala_{2·00}$ $Gly_{1·00}$ $Tyr_{0·94}$ (Found: C, 58·8; H, 6·8; N, 9·5. $C_{35}H_{47}N_5O_{11}$ requires C, 58·9; H, 6·6; N, 9·8%).

Benzyloxycarbonyl-γ-t-butyl-L-glutamyl-γ-t-butyl-L-glutamyl-L-alanyl-L-alanyl-L-tyrosylglycine Methyl Ester.—The preceding pentapeptide (6·5 g., 9·1 mmoles) was hydrogenated in acetic acid (80%; 100 ml.) over palladium–charcoal (5%; 2·0 g.). The residue was coupled with *N*-benzyloxycarbonyl-α-2,4,5-trichlorophenyl-γ-t-butyl-L-glutamate (5·1 g., 10 mmoles) in dimethylformamide (20 ml.) in presence of triethylamine (0·9 ml.). The product was isolated as before and gave the *hexapeptide derivative* (8·3 g., 93%), m.p. 203—204° (from methanol–ethyl acetate, $[\alpha]_D^{23}$ −24·1° (*c* 1·2 in dimethylformamide), TLC-1 R_F 0·36, TLC-13 R_F 0·49, $Glu_{2·02}$ $Ala_{2·00}$ $Gly_{1·01}$ $Tyr_{0·91}$ (Found: C, 58·6; H, 6·7; N, 9·4. $C_{44}H_{62}N_6O_{14}$ requires C, 58·8; H, 7·0; N, 9·4%).

Benzyloxycarbonyl-γ-t-butyl-L-glutamyl-γ-t-butyl-L-glutamyl-γ-t-butyl-L-glutamyl-L-alanyl-L-alanyl-L-tyrosylglycine Methyl Ester.—The foregoing hexapeptide derivative (7·7 g., 8·55 mmoles) was hydrogenated in 80% acetic acid (50 ml.) for 3 hr. over palladium–charcoal (10%, 1·2 g.). The residue was dissolved in dimethylformamide (20 ml.) and triethylamine (0·76 ml., 5·5 mmoles) and *N*-benzyloxycarbonyl-α-(2,4,5-trichlorophenyl)-γ-t-butyl-L-glutamate

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(5·0 g., 9·7 mmoles) were added at 0°. The mixture was kept at 37° for 24 hr., then worked up by the usual procedure. Crystallisation from methanol–ethyl acetate gave the *heptapeptide derivative* (6·7 g., 79%), m.p. 220—223°, $[\alpha]_D^{25}$ −53·0° (*c* 2·5 in dimethylformamide), TLC-4 R_F 0·78, TLC-13 R_F 0·61, $Glu_{3\cdot09}$ $Ala_{1\cdot96}$ $Gly_{1\cdot00}$ $Tyr_{0\cdot94}$ (Found: C, 58·4; H, 7·3; N, 8·95. $C_{53}H_{77}O_{17}N_7$ requires C, 58·7; H, 7·1; N, 9·05%).

*Benzyloxycarbonyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycine Methyl Ester*.—A solution of the preceding heptapeptide derivative (1·08 g., 1 mmole) in 80% acetic acid (30 ml.) was hydrogenated over 10% palladium–charcoal (0·2 g.). The product was coupled with *N*-benzyloxycarbonyl-α-(2,4,5-trichlorophenyl)-γ-t-butyl-L-glutamate (572 mg., 1·1 mmole) in dimethylformamide (10 ml.). The usual work up and precipitation from methanol with water gave the *octapeptide derivative* (1·1 g., 85%), m.p. 238—241° (decomp.), $[\alpha]_D^{23}$ −24·12° (*c* 1·1 in dimethylformamide), TLC-4 R_F 0·74, TLC-13 R_F 0·64, $Glu_{4\cdot0}$ $Gly_{1\cdot0}$ $Ala_{2\cdot06}$ $Tyr_{0\cdot93}$ (Found: C, 57·8; H, 6·9; N, 8·9. $C_{62}H_{92}O_{20}N_8,H_2O$ requires C, 57·85; H, 7·3; N, 8·7%).

*Benzyloxycarbonyl-*L*-valyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycine Methyl Ester*.—The foregoing octapeptide derivative (3·12 g., 2·5 mmoles) was hydrogenolysed in 90% acetic acid over palladium–charcoal (0·6 g.). The product was coupled with *N*-benzyloxycarbonyl-L-valine 2,4,5-trichlorophenyl ester (1·075 g., 2·5 mmoles) in dimethylformamide solution (10 ml.). Isolation in the usual manner gave the *nonapeptide derivative* (1·0 g., 83%), m.p. 270—272° (decomp.) (from methanol), $[\alpha]_D^{23}$ −20·34° (*c* 1·0 in dimethylformamide), TLC-4 R_F 0·81, TLC-13 R_F 0·72, $Glu_{4\cdot02}$ $Gly_{1\cdot00}$ $Ala_{2\cdot09}$ $Val_{0\cdot87}$ $Tyr_{0\cdot96}$ (Found: C, 58·25; H, 7·6; N, 9·35. $C_{67}H_{101}O_{21}N_9,H_2O$ requires C, 58·05; H, 7·4; N, 9·1%).

*Benzyloxycarbonyl-*L*-tryptophyl-*L*-valyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycine Methyl Ester* (II).—The preceding nonapeptide derivative (3·24 g., 2·4 mmoles) was hydrogenated in 90% acetic acid (100 ml.) over 5% palladium–charcoal (600 mg.) for 6 hr. The acetic acid was evaporated off below 40° and the residue was coupled with *N*-benzyloxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (1·224 g.) in dimethylformamide (20 ml.). Crystallisation from methanol gave the *decapeptide derivative* (3·4 g., 95%), m.p. 256—258° (decomp.), $[\alpha]_D^{23}$ −20·11° (*c* 1·11 in dimethylformamide), TLC-13 R_F 0·51, TLC-7 R_F 0·71, $Glu_{4\cdot06}$ $Ala_{2\cdot09}$ $Gly_{0\cdot94}$ $Tyr_{1\cdot00}$ $Val_{0\cdot81}$ (Found: C, 59·0; H, 7·1; N, 9·9. $C_{78}H_{111}O_{22}N_{11},2H_2O$ requires C, 58·9; H, 7·2; N, 9·7%).

*Benzyloxycarbonyl-*L*-prolyl-*L*-tryptophyl-*L*-valyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycine Methyl Ester*.—A solution of the preceding decapeptide derivative (II) (931 mg., 0·6 mmole) was hydrogenolysed in 90% glacial acetic acid (25 ml.) over palladium–charcoal (200 mg.). The product was coupled with benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (242 mg., 10% excess) in dimethylformamide in the presence of triethylamine (0·084 ml.). Precipitation from methanol with water gave the *undecapeptide derivative* (750 mg., 76%), m.p. 246—249° (decomp.), $[\alpha]_D^{23}$ −19·32° (*c* 1·12 in dimethylformamide), TLC-4 R_F 0·62, TLC-13 R_F 0·42, $Glu_{4\cdot00}$ $Pro_{0\cdot95}$ $Gly_{1\cdot00}$ $Ala_{2\cdot10}$ $Val_{0\cdot86}$ $Tyr_{1\cdot02}$.

*t-Butoxycarbonylglycyl-*L*-proline*.—L-Proline (2·308 g., 20 mmoles) was dissolved in water (5 ml.) and dimethylformamide (20 ml.) and triethylamine (2·8 ml., 20 mmoles) were added. The solution was cooled to 0°, t-butoxycarbonylglycine 2,4,5-trichlorophenyl ester (7·08 g., 20 mmoles) was added, and the mixture was kept at 37—40° for 24 hr. The residue after evaporation was treated with 10% aqueous citric acid (200 ml.) and extracted with ethyl acetate (4 × 100 ml.), and the combined extracts were washed with citric acid and water (2 × 100 ml.), dried ($MgSO_4$), and evaporated. Crystallisation from ethyl acetate gave the dipeptide derivative (5·0 g., 91%), $[\alpha]_D^{25}$ −70·1° (*c* 2·1 in dimethylformamide), m.p. 143—144° (lit.,[12] m.p. 142—144°).

*t-Butoxycarbonylglycyl-*L*-proline 2,4,5-Trichlorophenyl Ester*.—A solution of benzyloxycarbonylglycyl-L-proline (2·72 g., 10 mmoles) and 2,4,5-trichlorophenol (1·96 g., 10 mmoles) in tetrahydrofuran (25 ml.) was treated with dicyclohexylcarbodi-imide (2·06 g., 10 mmole) at 0°. Dicyclohexylurea was filtered off and washed with ethyl acetate (10 ml.) and the combined filtrate and washings were evaporated to dryness. The residual oil could not be crystallised even after chromatography on silica gel; TLC-14 R_F 0·38, TLC-15 R_F 0·93.

*t-Butoxycarbonylglycyl-*L*-prolyl-*L*-tryptophyl-*L*-valyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycyl Methyl Ester* (VI). The foregoing decapeptide derivative (II) (1·086 g., 0·7 mmole) was hydrogenated in 90% acetic acid (40 ml.) over 5% palladium–charcoal (250 mg.). The product was dissolved in dimethylformamide (15 ml.) and treated with triethylamine (0·66 ml.) followed by t-butoxycarbonylglycyl-L-proline 2,4,5-trichlorophenyl ester (452 mg., 1 mmole) at 37° for 48 hr. The *dodecapeptide derivative* was obtained by precipitation from methanol with water (800 mg.). It had m.p. 230—234° (from acetic acid), $[\alpha]_D^{23}$ −19·6 (*c* 1·01 in dimethylformamide), TLC-13 R_F 0·14, TLC-4 R_F 0·49, $Glu_{4\cdot03}$ $Pro_{0\cdot99}$ $Gly_{1\cdot88}$ $Ala_{2\cdot00}$ $Val_{0\cdot94}$ $Tyr_{1\cdot00}$.

*t-Butoxycarbonylglycyl-*L*-prolyl-*L*-tryptophyl-*L*-valyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycine* (VII).—The above dodecapeptide derivative (668 mg., 0·4 mmole) was dissolved in warm dioxan (50 ml.) and treated with N-sodium hydroxide (2·0 ml.) at 20° for 40 min. The mixture was then poured into ice-cold citric acid solution (20%; 200 ml.) and the precipitate was collected by centrifugation and washed well with water (50 ml.), cold methanol (5·0 ml.), and ether (3 × 50 ml.). The dodecapeptide acid (600 mg.) was obtained by precipitation from pyridine with ether; TLC-9 R_F 0·66; TLC-7 R_F 0·48, TLC-13 R_F 0·75.

N-*Benzyloxycarbonylglycyl-*L*-proline 2,4,5-Trichlorophenyl Ester*.—To a solution of benzyloxycarbonylglycyl-L-proline (3·06 g., 10 mmoles) and 2,4,5-trichlorophenol (1·95 g., 10 mmoles) in tetrahydrofuran (50 ml.) was added dicyclohexylcarbodi-imide (2·06 g., 10 mmoles) at 0° with stirring. The solution was stirred at room temp. for 12 hr. then filtered and evaporated. The residue was dissolved in ethyl acetate (100 ml.) and washed with 10% sodium hydrogen carbonate (2 × 50 ml.), 10% hydrochloric acid (2 × 25 ml.), and water (2 × 100 ml.). The residual oil obtained by evaporation could not be crystallised; TLC-14 R_F 0·31, TLC-15 R_F 0·88.

[12] J. C. Anderson, M. A. Barton, P. M. Hardy, G. W. Kenner, J. Preston, and R. C. Sheppard, *J. Chem. Soc.* (*C*), 1967, 108.

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N-*Benzyloxycarbonyl-glycyl-*L*-prolyl-*L*-tryptophyl-*L*-valyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycine Methyl Ester* (III).—The decapeptide derivative (II) (931 mg., 0·6 mmole) was hydrogenated in 90% glacial acetic acid (25 ml.) over 5% palladium–charcoal (200 mg.). The residue was coupled with benzyloxycarbonylglycyl-L-proline 2,4,5-trichlorophenyl ester (311 mg., 10% excess) in dimethylformamide (10 ml.) in the presence of triethylamine (0·084 ml.). The *dodecapeptide derivative* was obtained by isolation in the usual manner and precipitation from methanol with water (600 mg.), m.p. 245—248° (decomp.), $[\alpha]_D^{23}$ −17·97° (*c* 1·0 in dimethylformamide), TLC-13 R_F 0·28, TLC-4 R_F 0·57, $Glu_{4\cdot20}$ $Pro_{0\cdot97}$ $Gly_{1\cdot87}$ $Ala_{2\cdot00}$ $Val_{0\cdot96}$ $Tyr_{1\cdot03}$.

N-*Benzyloxycarbonylglycyl-*L*-prolyl-*L*-tryptophyl-*L*-valyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycine* (IV).—The foregoing dodecapeptide derivative (857 mg., 0·5 mmole) was treated with N-sodium hydroxide (2·2 ml.) in dioxan (50 ml.) at room temp. for 40 min. The mixture was poured into an ice-cold solution of citric acid (20%; 250 ml.) and the precipitate was collected and washed with water (50 ml.), cold methanol (2 × 5·0 ml.), and ether (3 × 50 ml.). The dodecapeptide acid was obtained by precipitation from pyridine with ether (660 mg.), TLC-9 R_F 0·54, TLC-7 R_F 0·46, TLC-13 R_F 0·72.

L-*Pyroglutamylglycyl-*L*-prolyl-*L*-tryptophyl-*L*-leucyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycine.*— The foregoing dodecapeptide derivative (400 mg., 0·230 mmole) was hydrogenated in 90% acetic acid (50 ml.) over palladium–charcoal (5%; 100 mg.). The product was dissolved in hexamethylphosphoramide (25 ml.), and 2,4,5-trichlorophenyl L-pyroglutamate (308 mg.) and triethylamine (0·034 ml.) were added. The mixture was kept for 6 days at 37°. The product was precipitated by the addition of citric acid solution (20%; 200 ml.), filtered, and washed with N-hydrochloric acid (25 ml.), methanol (2 × 5 ml.), and ether (2 × 50 ml.). Crystallisation of the crude product from ethanol (50 ml.) gave the *tridecapeptide derivative* (200 mg., 50%), TLC-3 R_F 0·42, TLC-4 R_F 0·29, $Glu_{4\cdot80}$ $Pro_{0\cdot86}$ $Gly_{1\cdot90}$ $Ala_{2\cdot14}$ $Val_{0\cdot91}$ $Tyr_{1\cdot0}$.

N-*t-Butoxycarbonylglycyl-*L*-prolyl-*L*-tryptophyl-*L*-valyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycyl-*L*-trytophyl-*L*-methionyl-β-t-butyl-*L*-aspartyl-*L*-phenylalanine Amide* (VIII).—A solution of t-butoxycarbonyltetra-(γ-t-butyl-L-glutamyl)-L-alanyl-L-alanyl-L-tyrosylglycine (VII) (334 mg., 0·2 mmole) in dimethylformamide (20 ml.) was dried by concentration *in vacuo* to *ca.* 10 ml. To this solution was added at −20° triethylamine (0·028 ml., 0·2 mmole) and pivaloyl chloride (0·028 ml., 0·23 mmole). The solution was stirred for 3 min. at −30° and a solution of L-tryptophyl-L-methionyl-β-t-butyl-L-aspartyl-L-phenylalanine amide (V) (261·2 mg., 0·4 mmole) in dimethylformamide (15 ml.), dried by vacuum distillation to *ca.* 5·0 ml., was added. The solution was stirred for 1 hr. at −20° and then overnight at room temp. The mixture was cooled to 0°, treated with aqueous potassium carbonate (25%; 1·0 ml.), and then immediately poured into ice-cold water (100 ml.). The white precipitate was collected, washed with N-hydrochloric acid (2 × 50 ml.), ethanol (2 × 5 ml.), and ether (2 × 50 ml.), and crystallised from glacial acetic acid to give the *hexadecapeptide derivative* (250 mg.), TLC-6 R_F 0·59, TLC-9 R_F 0·33, $Asp_{0\cdot95}$ $Glu_{4\cdot20}$ $Pro_{0\cdot85}$ $Gly_{1\cdot80}$ $Ala_{2\cdot10}$ $Val_{0\cdot95}$ $Met_{0\cdot90}$ $Tyr_{1\cdot05}$ $Phe_{1\cdot00}$.

L-*Pyroglutamylglycyl-*L*-prolyl-*L*-tryptophyl-*L*-valyl-*L*-glutamyl-*L*-glutamyl-*L*-glutamyl-*L*-glutamyl-*L*-alanyl-*L*-alanyl-*L*-tyrosylglycyl-*L*-tryptophyl-*L*-methionyl-*L*-aspartyl-*L*-phenylalanine Amide (Ovine–Bovine Gastrin I).*—(*a*) Solutions of the foregoing tridecapeptide derivative (168·3 mg., 0·1 mmole) and of the tetrapeptide amide (V) (130 mg., 0·2 mmole) in dimethylformamide were dried by vacuum concentration to 2·5 ml. and 1·5 ml. respectively. To the tridecapeptide solution at −20°, triethylamine (0·015 ml., 0·11 mmole) and pivaloyl chloride (0·0135 ml., 0·11 mmole) were added with stirring. After 3·5 min., the dried solution of the tetrapeptide amide was added and the temperature was kept at −20° for 30 min. The mixture was kept for 18 hr. at room temp., treated at 0° with 25% aqueous potassium carbonate (1·0 ml.) for 2 min., and then poured into ice-cold water (100 ml.). The precipitate was collected and washed with 0·2N-hydrochloric acid (2 × 25 ml.), water (2 × 50 ml.), ethanol (2 × 50 ml.), and finally ether (2 × 100 ml.). The dried crude material was then dissolved in trifluoroacetic acid (98%, 5 ml.) under nitrogen and kept at room temp. for 1 hr. The residue after evaporation was dissolved in 5% ammonium hydroxide (2·5 ml.) and chromatographed on G-25 Sephadex with 0·4% ammonium hydrogen carbonate as eluant. Fractions (2 ml.; nos. 168—210) corresponding to the major peak were combined and evaporated to yield the *heptadecapeptide amide ammonium salt* (66 mg.) $E_{glu}^{6\cdot5}$ 0·62, TLC-6 R_F 0·55, TLC-9 R_F 0·31, $Asp_{1\cdot02}$ $Glu_{5\cdot09}$ $Pro_{0\cdot94}$ $Gly_{1\cdot90}$ $Ala_{2\cdot00}$ $Val_{0\cdot98}$ $Met_{1\cdot00}$ $Tyr_{1\cdot02}$ $Phe_{1\cdot00}$ $Trp_{1\cdot97}$.

(*b*) The hexadecapeptide amide (VIII) (50 mg.) was treated with trifluoroacetic acid (98%, 4·0 ml.) under nitrogen for 1 hr. at room temp. The solution was then poured into ether (100 ml.) and the precipitate (45 mg.) was collected and washed well with ether. This product (25 mg.) was converted into its triethylammonium salt by solution in 1% aqueous triethylamine and evaporation to dryness. The residue was dissolved in aqueous dimethylformamide (90%, 2 ml.) and treated at intervals with L-pyroglutamic acid 2,4,5-trichlorophenyl ester (5 × 30 mg.) until the mixture gave no positive reaction with ninhydrin (8 days at 37°). The product was chromatographed on G-25 Sephadex as before; evaporation of fractions 179—220 gave the pure ammonium salt (15 mg.), $Asp_{1\cdot0}$ $Glu_{5\cdot1}$ $Pro_{0\cdot96}$ $Gly_{1\cdot86}$ $Ala_{2\cdot00}$ $Val_{0\cdot95}$ $Met_{1\cdot00}$ $Tyr_{1\cdot00}$ $Phe_{1\cdot00}$.

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