SECTION C
Organic Chemistry

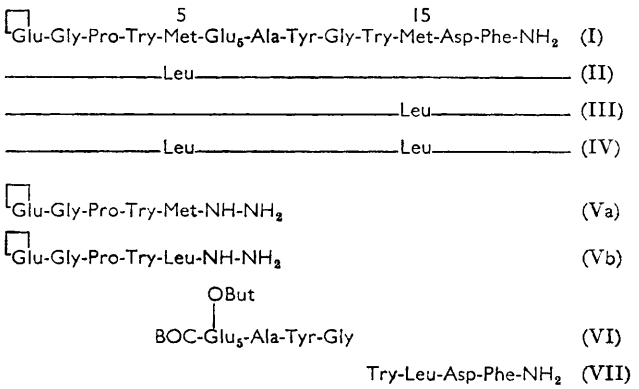
Peptides. Part XXVI.[1] Analogues of Gastrin Containing Leucine in Place of Methionine

By **G. W. Kenner,* J. J. Mendive,** and **R. C. Sheppard,** Robert Robinson Laboratories, University of Liverpool, Oxford Street, Liverpool

Syntheses of two heptadecapeptide analogues of the hormone gastrin, *viz.* [Leu^{15}] and [Leu^{5},Leu^{15}]-porcine gastrin I are described. Both compounds exhibit the full biological activity of the natural hormone.

PREVIOUS papers from this laboratory have discussed the degradative evidence leading to the heptadecapeptide amide structure (II) for human gastrin I.[1,2] At the outset of these investigations, the minute amounts of the natural substance isolated were sufficient only for amino-acid analysis. This indicated that the human hormone contained one residue less of methionine and one residue more of leucine than did its porcine counterpart (I), the structure of which we had already elucidated[3] and confirmed by synthesis.[4] A plausible hypothesis was therefore that human gastrin contained a leucine residue in place of either one of the two methionine residues of porcine gastrin. Both possible structures (II) and (III) were therefore immediately made the subject of synthetic studies. Meanwhile, isolation of further quantities of the natural hormone permitted more detailed structural investigation, and this provided strong evidence in favour of (II). The correctness of this amino-acid sequence was subsequently shown by synthesis and detailed comparison of the natural and synthetic products.[1,5] This paper describes the synthesis and biological activity of the isomeric peptide (III), and also of the congener (IV).

The interchange of methionine with other alkyl amino-acid residues in peptides has some general importance apart from the relationship between the various mammalian gastrins.[6] Methionine is one of the more difficult amino-acids to incorporate into synthetic peptides because of the facility with which side reactions occur at the sulphur atom. For example, the sensitivity of the sulphide linkage to conditions commonly used for the cleavage of benzyloxycarbonyl derivatives (catalytic hydrogenolysis, sodium in liquid ammonia, and hydrogen bromide in acetic acid) has largely precluded use of this otherwise valuable protecting group in the synthesis of methionyl peptides.[7] The sulphide grouping is also sensitive to adventitious oxidation. Insofar as the methionine sulphoxide analogues of ACTH and β-MSH,[8] and almost certainly of gastrin itself,[9,10] are, at most, only weakly biologically active, oxidation at methionine residues may be important in the *in vitro* and *in vivo* inactivation of these and other peptide hormones. Replacement of methionine in synthetic peptide hormones and their analogues is therefore of interest from the point of view of both ease of synthesis and chemical stability.



It has already been shown in the ACTH series that

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

[2] P. H. Bentley, G. W. Kenner, and R. C. Sheppard, *Nature*, 1966, **209**, 583.

[3] H. Gregory, P. M. Hardy, D. S. Jones, G. W. Kenner, and R. C. Sheppard, *Nature*, 1964, **204**, 931.

[4] (*a*) 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; (*b*) J. C. Anderson, G. W. Kenner, J. K. MacLeod, and R. C. Sheppard, *Tetrahedron*, 1966, Suppl. 8, 39; (*c*) J. C. Anderson, M. A. Barton, P. M. Hardy, G. W. Kenner, J. Preston, and R. C. Sheppard, *J. Chem. Soc.* (*C*), 1967, 108.

[5] J. Beacham, P. H. Bentley, R. A. Gregory, G. W. Kenner, J. K. MacLeod, and R. C. Sheppard, *Nature*, 1966, **209**, 584.

[6] J. Beacham, P. H. Bentley, G. W. Kenner, J. J. Mendive, and R. C. Sheppard, 'Proceedings Eighth European Peptide Symposium, Noordwijk, Holland, 1966,' North Holland Publishing Co., Amsterdam, 1967, p. 235.

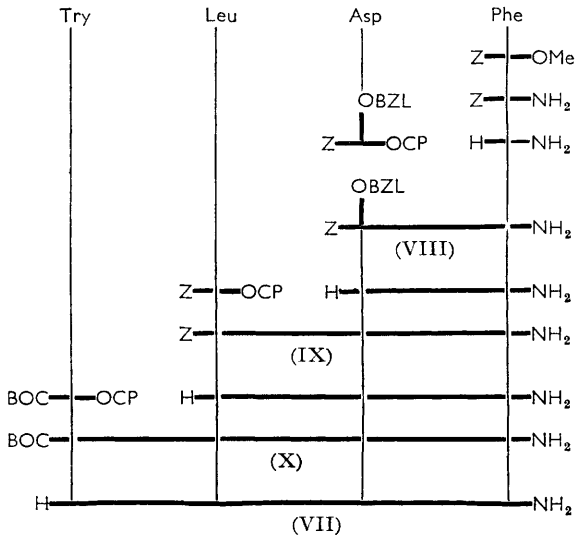
[7] E. Schröder and K. Lübke, 'The Peptides,' Academic Press, New York, 1965, vol. 1, p. 241, but see also K. Medzihradszky and H. Medzihradszky-Schweiger, Proc. Seventh European Peptide Symp., Budapest, 1964, *Acta Chimica Acad. Sci. Hung.*, 1965, **44**, 15; 1966, **50**, 339.

[8] K. Hofmann, J. Rosenthaler, R. D. Wells, and H. Yajima, *J. Amer. Chem. Soc.*, 1964, **86**, 4991, and references there cited.

[9] J. S. Morley, H. J. Tracy, and R. A. Gregory, *Nature*, 1965, **207**, 1356.

[10] J. S. Morley, 'Proceedings Eighth European Peptide Symposium, Noordwijk, Holland, 1966,' North Holland Publishing Co., Amsterdam, 1967, p. 226.

methionin may be replaced by the approximately isosteric amino-acid norleucine to give highly active analogues.[11] The *C*-terminal tetrapeptide sequence of gastrin also retains its ability to stimulate the secretion of gastric acid when methionine is replaced by norleucine.[9,10] The synthetic gastrin analogue (III) ([Leu^{15}]-porcine gastrin I) proved to be equiactive with porcine gastrin in stimultating gastric secretion in the conscious dog.* We have also synthesised the analogue (IV) ([Leu^{5},Leu^{15}]-porcine gastrin I, [Leu^{15}]-human gastrin I) in which both methionine residues have been replaced by leucine, and which is, as expected, also biologically fully active in the conscious dog.* The leucine-containing *C*-terminal tetrapeptide derivative is also highly active in the anaesthetised rat.[10] Because of the high biological activity of the leucine analogues of gastrin and of their relative ease of synthesis, our current work in this series employs [Leu^{15}]-derivatives exclusively.[12]



SCHEME

The synthesis of (III) and (IV) was achieved by the union of peptide fragments (V), (VI), and (VII), comprising respectively residues 1—5, 6—13, and 14—17 of the final gastrin analogues. The two *N*-terminal pentapeptide hydrazides (Va) and (Vb) had been prepared earlier during our syntheses of porcine [4] and human [1] gastrin, as had the central octapeptide derivative (VI). The new *C*-terminal tetrapeptide amide (VII) was prepared (Scheme) *via* its t-butoxycarbonyl derivative by a simple stepwise procedure using activated ester coupling methods exclusively. It was found more convenient to prepare the starting phenylalanine amide by ammonolysis of benzyloxycarbonylphenylalanine methyl ester than by the described [13] mixed-anhydride procedure. Hydrogenolysis of the benzyloxycarbonyl derivative and coupling of the resulting phenylalanine amide with the α-2,4,5-trichlorophenyl ester of β-benzyl benzyloxycarbonylaspartate gave the known dipeptide derivative (VIII),[13] and further hydrogenolysis and active ester coupling gave successively the benzyloxycarbonyl tripeptide (IX) and t-butoxycarbonyl tetrapeptide (X) amides. The choice of t-butoxycarbonyltryptophan 2,4,5-trichlorophenyl ester for this last coupling was made initially purely because of the availability of this reagent from other studies. Subsequent experience [12] in related cases, however, has shown that the t-butoxycarbonyl derivative may sometimes be superior to the benzyloxycarbonyl derivative in terms of ease of removal of the *N*-protecting group in compounds of this type. The benzyloxycarbonyl tetrapeptide amide analogous to (X) has been prepared elsewhere by a different route.[14]

The free tetrapeptide amide (VII) obtained by the action of trifluoroacetic acid or anhydrous hydrogen chloride on the t-butoxycarbonyl derivative (X) was coupled with the central octapeptide derivative (VI) by the mixed-anhydride procedure. A smooth coupling was achieved when the time of anhydride formation between pivaloyl chloride and the triethylammonium salt of (VI) was limited to 3 min. at −15°,[15] and after cleavage of all protecting groups, the product was purified by gel filtration on G-25 Sephadex. The yield of purified dodecapeptide amide was 43%. For the synthesis of (III), this dodecapeptide amide was condensed with an excess of the diazotised pentapeptide hydrazide (Va), and the product was purified on Sephadex. The gastrin analogue (III) was thus obtained in 74% yield and with excellent amino-acid ratios; the pure analogue (IV) was similarly obtained in 92% yield from the pentapeptide hydrazide (Vb). These procedures were essentially those developed for the synthesis of the natural gastrins,[1,4] but in both the present cases less difficulty was encountered in the purification of the products than had been observed previously. Almost certainly this is due to the absence of methionine in (IV) and the introduction of the single residue of methionine in (III) at the last stage of the synthesis. The synthesis of human gastrin I recently described by Morley [10,16] also introduced the methionine residue at a late stage.

EXPERIMENTAL

The general instructions given in Part XXV [1] apply.

Benzyloxycarbonyl-L-phenylalanine Amide.—Benzyloxycarbonyl chloride (95%; 4·2 ml., 23·1 mmole) was added dropwise during 30 min. to a stirred and cooled (0°) solution of L-phenylalanine methyl ester hydrochloride (4·97 g., 23·1 mmole) and sodium carbonate (4·90 g., 46·2 mmole) in water (250 ml.). After a further 4 hr. the solution was extracted with ether and the ethereal solution was washed

* We are grateful to Professor R. A. Gregory and Dr. H. J. Tracy, Department of Physiology, Liverpool University, for these determinations.

[11] R. A. Boissonnas, St. Guttmann, and J. Pless, *Experientia*, 1966, **22**, 526.

[12] K. L. Agarwal, G. W. Kenner, and R. C. Sheppard, to be published.

[13] J. M. Davey, A. H. Laird, and J. S. Morley, *J. Chem. Soc.* (*C*), 1966, 555.

[14] J. S. Morley, to be published.

[15] Cf. R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, 1966, **49**, 134.

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

with N-hydrochloric acid, N-aqueous sodium hydrogen carbonate, and brine, before being dried ($MgSO_4$) and evaporated. The residual oily benzyloxycarbonyl derivative was added to a saturated solution of ammonia in methanol and set aside overnight. Evaporation of the solvent and crystallisation of the residue from ethyl acetate yielded benzyloxycarbonyl-L-phenylalanine amide (6·0 g., 88% overall), m. p. 164—165°, $[\alpha]_D^{18}$ −6·8° (*c* 1 in MeOH) (lit.,[13] m. p. 161—162°).

*Benzyloxycarbonyl-*L*-leucine 2,4,5-Trichlorophenyl Ester.*—Dicyclohexylcarbodi-imide (7·05 g., 34·2 mmole) was added to a cooled (−15°) solution of benzyloxycarbonyl-L-leucine (9·1 g., 34·2 mmole) and 2,4,5-trichlorophenol (6·85 g., 34·2 mmole) in methylene dichloride (20 ml.). The solution was kept for 2 hr. at −15° and 2 hr. at room temp. before being filtered and the filtrate evaporated. The initial preparation was purified by chromatography on silica, but subsequent preparation crystallised directly from light petroleum giving the *active ester* (12·1 g., 75%), m. p. 62—64°, $[\alpha]_D^{23}$ −53·3° (*c* 1·12 in ethyl acetate) (Found: C, 54·1; H, 4·55; N, 3·2. $C_{20}H_{20}Cl_3NO_4$ requires C, 54·0; H, 4·5; N, 3·1%).

*Benzyloxycarbonyl-*L*-leucyl-*L*-aspartyl-*L*-phenylalanine Amide* (IX).—The foregoing active ester (1·34 g., 2·9 mmole), aspartylphenylalanine amide [13] (0·80 g., 2·9 mmole), and triethylamine (0·31 ml., 2·9 mmole) were dissolved in dimethylformamide (3 ml.) and the solution was kept at 36·5° for 16 hr. The solution was added to a mixture of hydrochloric acid (0·03N; 100 ml.) and ether (100 ml.), and the precipitated solid was collected and washed with ether. This product was added to warm ethanol, the suspension was cooled to 0° and filtered to yield the *tripeptide derivative* (1·16 g., 76%), m. p. 224—226°, $[\alpha]_D^{23}$ −49·4° (*c* 0·53 in dimethylformamide) (Found: C, 61·5; H, 6·5; N, 10·55. $C_{27}H_{34}N_4O_7$ requires C, 61·6; H, 6·5; N, 10·6%).

*t-Butoxycarbonyl-*L*-tryptophyl-*L*-leucyl-*L*-aspartyl-*L*-phenylalanine Amide* (X).—The foregoing benzyloxycarbonyl derivative (1·06 g., 1·9 mmole) was hydrogenated in 80% aqueous acetic acid solution (40 ml.) for 2½ hr. at room temp. Evaporation of the filtered solution and trituration of the residue with ether gave the tripeptide amide (0·76 g.) which was dissolved in dimethylformamide (5 ml.) together with triethylamine (0·21 ml., 1·9 mmole) and t-butoxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester (0·70 g., 1·9 mmole). The solution was kept for 16 hr. at 37° and then added to a mixture of hydrochloric acid (0·019N; 100 ml.) and ether (100 ml.). The precipitated solid was collected and washed with ether. This product was added to warm ethanol, the suspension was cooled to 0° and filtered to yield the *tetrapeptide derivative* (1·07 g., 81%), m. p. 217° (decomp.), $[\alpha]_D^{22}$ −46·5° (*c* 0·35 in dimethylformamide) (Found: C, 61·7; H, 6·7; N, 12·2. $C_{35}H_{46}N_6O_8$ requires C, 61·9; H, 6·8; N, 12·4%).

*L-Tryptophyl-*L*-leucyl-*L*-aspartyl-*L*-phenylalanine Amide Trifluoroacetate and Hydrochloride.*—The foregoing t-butoxycarbonyl derivative (0·618 g., 0·91 mmole) was dissolved in 98% aqueous trifluoroacetic acid (10 ml.) and the solution was kept for 1 hr. at room temp. under nitrogen. The solution was added to ether (60 ml.), cooled to 0°, and the precipitated solid was collected and washed with ether. Crystallisation of the product from methanolic ether gave the *trifluoroacetate salt* (0·60 g., 95%), m. p. 200—202° $[\alpha]_D^{22}$ −21·4° (*c* 0·579 in methanol), TLC-9, R_F 0·7, TLC-6, R_F 0·9, TLC-7, R_F 0·4 (Found: C, 54·1; H, 5·8; N, 11·9. $C_{32}H_{39}F_3N_6O_7,H_2O$ requires C, 54·1; H, 5·8; N, 11·8%). Similarly, cleavage of the t-butoxycarbonyl derivative (0·92 g., 1·36 mmole) with 3N-hydrogen chloride in acetic acid (25 ml.) for 1 hr. at room temp. under nitrogen and crystallisation of the product from methanol–ether gave the *hydrochloride* (0·71 g., 85%), m. p. 201—203°, $[\alpha]_D^{22}$ −30·4° (*c* 0·56 in methanol), $Leu_{0·97}Asp_{1·00}Phe_{1·00}$ (Found: C, 57·1; H, 6·6. $C_{30}H_{39}ClN_6O_5,H_2O$ requires C, 56·9; H, 6·5%).

*Penta-(*L*-glutamyl)-*L*-alanyl-*L*-tyrosylglycyl-*L*-tryptophyl-*L*-leucyl-*L*-aspartyl-*L*-phenylalanine Amide.*—A solution of t-butoxycarbonyl-penta-(γ-t-butyl-L-glutamyl)-L-alanyl-L-tyrosylglycine [1,4b] (383 mg., 0·29 mmole) in dimethylformamide (4 ml.) was dried by concentration under reduced pressure to *ca.* 2 ml. To this cooled (−15°) solution was added triethylamine (0·04 ml., 0·29 mmole) and pivaloyl chloride (0·036 ml., 0·30 mmole), and after 3 min. at −15°, a solution of L-tryptophyl-L-leucyl-L-aspartyl-L-phenylalanine amide hydrochloride (177 mg., 0·29 mole) in dimethylformamide (2 ml.) (dried by vacuum concentration as above), and triethylamine (0·12 ml., 0·87 mmole). The solution was stirred for 1 hr. at 0° and then for 4 hr. at room temp. before being evaporated. The residue was dissolved in 98% aqueous trifluoroacetic acid (10 ml.) and kept for 1 hr. at room temp. in a nitrogen atmosphere. The trifluoroacetic acid was evaporated and the residue was dissolved in water (5 ml.) made just alkaline with ammonia, and applied to a column (90 cm. × 2·7 cm. diam.) of G-25 Sephadex. The column was eluted with 0·4% aqueous ammonium hydrogen carbonate, fractions of 1·7-ml. being collected every 15 min. and their optical density at 280 mμ determined. Fractions 158—200 containing the major peak were combined and evaporated, and the residue re-chromatographed on the same column. Fractions 154—190 were combined and evaporated to yield the *dodecapeptide amide ammonium salt* (195 mg., 43%), TLC-6, R_F 0·7, $Asp_{0·95}Glu_{4·6}Gly_{1·00}Leu_{1·05}Tyr_{1·00}Phe_{1·05}$.

*L-Pyroglutamylglycyl-*L*-prolyl-*L*-tryptophyl-*L*-leucyl-(penta-*L*-glutamyl)-*L*-alanyl-*L*-tyrosylglycyl-*L*-tryptophyl-*L*-leucyl-*L*-aspartyl-*L*-phenylalanine Amide* (IV).—The foregoing dodecapeptide amide ammonium salt (12·2 mg., 7·5 μmole) was dissolved in water, excess of triethylamine was added and the solution was evaporated. This procedure was repeated three times in all and the resulting triethylammonium salt was dissolved in dimethylformamide (0·5 ml.). To this solution at −5° was added a solution of L-pyroglutamylglycyl-L-prolyl-L-tryptophyl-L-leucine azide, prepared by treatment of the corresponding hydrazide [1] (6·9 mg., 11·5 μmole) dissolved in dimethylformamide (1 ml.) at 0° with N-hydrochloric acid (0·0345 ml.) and aqueous sodium nitrite (0·4N; 0·032 ml.) for 10 min., and then with 10% triethylamine in dimethylformamide (0·044 ml.). The mixture was kept at 0° for 16 hr. when a second equal portion of pentapeptide azide was added. After a further 16 hr. at 0°, the solution was evaporated and the residue was chromatographed on G-25 Sephadex as in the foregoing experiment. Fractions 145—165 containing the major peak were combined and evaporated to yield the *heptadecapeptide amide ammonium salt* (6·9 μmole, 92%), $E^{6·5}_{Glu}$ 0·70, $Asp_{0·96}Glu_{5·88}Pro_{1·05}Gly_{2·05}Ala_{1·00}Leu_{1·94}Tyr_{0·96}Phe_{0·96}$.

*L-Pyroglutamylglycyl-*L*-prolyl-*L*-tryptophyl-*L*-methionyl-(penta-*L*-glutamyl)-*L*-alanyl-*L*-tyrosyl-*L*-glycyl-*L*-tryptophyl-*L*-leucyl-*L*-aspartyl-*L*-phenylalanine Amide* (III).—L-Pyroglutamylglycyl-L-prolyl-L-tryptophyl-L-methionine hydrazide [4c] (9·2 mg., 16 μmole) dissolved in dimethylformamide (0·5 ml.) at −5° was treated with N-hydrochloric acid

(0·048 ml.) and and 0·4N-sodium nitrite (0·041 ml.) and after 10 min. at −5° with 10% triethylamine in dimethylformamide (0·067 ml.). This solution was added to the dodecapeptide triethylammonium salt (14 mg., 8·2 μmole), dissolved in dimethylformamide (0·5 ml.) and after 16 hr. at 0° a second equal portion of pentapeptide azide was added. After a further 16 hr. at 0°, the product was isolated as in the foregoing experiment, to yield the *heptadecapeptide ammonium salt* (13·4 mg., 74%), $E^{6\cdot5}_{Glu}$ 0·70, $Asp_{1\cdot01}Glu_{6\cdot1}Pro_{1\cdot02}Gly_{1\cdot98}Ala_{1\cdot08}Met_{0\cdot95}Leu_{0\cdot97}Tyr_{1\cdot00}Phe_{0\cdot95}$.

We are grateful to Imperial Chemical Industries Limited, Pharmaceuticals Division, for generous financial support.

[7/1304 *Received, October 10th,* 1967]