1341. L-*Glutamyl-γ-aminobutyric Acid and Related Compounds*

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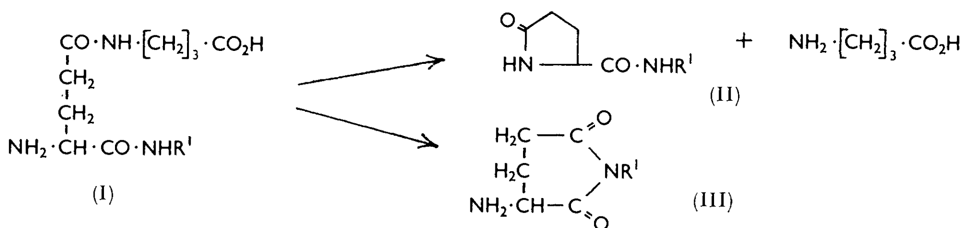
(α- and γ-L-Glutamyl)-γ-aminobutyric acid and various related compounds have been prepared for pharmacological evaluation. The decomposition of the γ-glutamyl isomers in aqueous solution has been studied. (γ-L-Glutamyl)-γ-aminobutyric acid is shown to be identical with a dipeptide previously isolated from seeds of *Lunaria annua* L.

This Paper is concerned with the synthesis and properties of various glutamyl derivatives of γ-aminobutyric acid, a compound which has been postulated to play an important role in central inhibitory processes in mammals.[1] Glutamyl derivatives were investigated because it was hoped that they, unlike γ-aminobutyric acid,[1] might cross the so-called blood–brain barrier and might, therefore, act as sources of γ-aminobutyric acid within the central nervous system. Compounds of type (I), for example, could release γ-aminobutyric acid by the formation of carboxypyrrolidone -(pyroglutamyl-, II) or imide (III) derivatives, or by the action of transpeptidases or peptidases. Although the compounds prepared were inactive in the biological systems investigated, these possible transformations are of chemical interest because of the wide occurrence of glutamyl and pyroglutamyl peptides. The α′- and γ′-isomers of glutamyl-γ-aminobutyric acid, which were also prepared, are of specific interest because unidentified isomers of this composition have been isolated from natural sources.

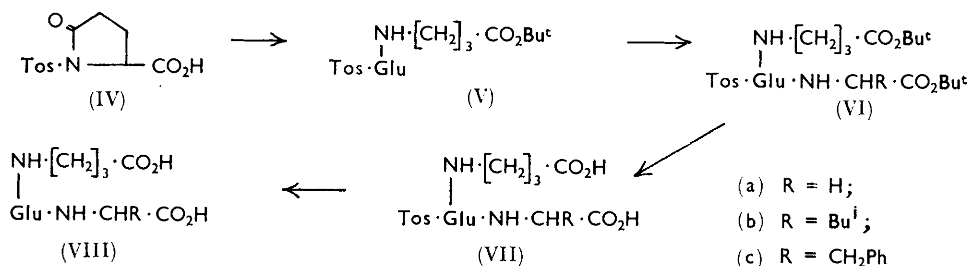


The approach which was adopted for the synthesis of compounds of type (I) is summarised below. It is based on the use of *N*-toluene-*p*-sulphonylpyrrolidonecarboxylic acid (IV) for the formation of the γ-glutamyl amide derivative (V).[2] The use of the toluene-*p*-sulphonyl protecting group seemed to offer good prospects of providing crystalline intermediates, whilst the formation of the γ-glutamyl bond at this stage provided a sizeable common intermediate. On the other hand, the presence of the toluene-*p*-sulphonyl group precluded hydrogenation as a cleaving reaction, which meant that the benzyl group could not be used to protect carboxyl functions selectively. Simple alkyl esters were avoided because of the dangers of transpeptidation[3,4] and racemisation[3] during saponification, but t-butyl esters, readily removed under acidic conditions,[5,6] proved ideal. Several routes were investigated for the synthesis of t-butyl γ-aminobutyrate; the most satisfactory, based on the general method of Roeske,[5] involved the reaction of benzyloxycarbonyl-γ-aminobutyric acid with isobutene, followed by catalytic hydrogenolysis to remove the benzyloxycarbonyl group. The ester was isolated as the phosphite.

Since the basicity of the amino-group in *N*-toluene-*p*-sulphonyl-α-amino-acids is not completely suppressed, the type of coupling reaction which may be used to form peptide

[1] For a review see K. A. C. Elliott, *Brit. Med. Bull.*, 1965, **21**, (1), 70.
[2] J. Rudinger, *Coll. Czech. Chem. Comm.*, 1954, **19**, 365, 375.
[3] E. Sondheimer and R. W. Holley, *J. Amer. Chem. Soc.*, 1954, **76**, 2467; 1957, **79**, 3767.
[4] A. R. Battersby and J. C. Robinson, *Chem. and Ind.*, 1954, 45; *J.*, 1955, 259; V. Bruckner, A. Kótai, and K. Kovács, *Acta Chim. Acad. Sci. Hung.*, 1959, **21**, 427.
[5] R. Roeske, *Chem. and Ind.*, 1959, 1121; *J. Org. Chem.*, 1963, **28**, 1251.
[6] G. W. Anderson and F. M. Callahan, *J. Amer. Chem. Soc.*, 1960, **82**, 3359.

derivatives of these compounds is limited.[7,8] For example, with *N*-toluene-*p*-sulphonylglycine, only a low proportion of the desired amide is obtained under the standard conditions of mixed anhydride coupling.[7] However, pivaloyl mixed anhydrides of *N*-toluene-*p*-sulphonyl-α-amino-acids undergo the required coupling reaction in excellent yields [9] and this type of reaction proved satisfactory for the preparation of the diesters (VIa—c). Use of the *NN'*-carbonyldi-imidazole procedure,[10] which has not previously been employed with *N*-toluene-*p*-sulphonyl-α-amino-acids, was also satisfactory. Although yields of product were somewhat less, the isolation procedure proved easier than in the case of the pivalic acid mixed anhydride method, but attempts to extend the application of this coupling procedure to simple *N*-toluene-*p*-sulphonyl-α-amino-acids were less satisfactory. The coupling of the monoester (V) is not similarly favoured in the mixed anhydride procedure. When ethyl chloroformate was used in the preparation of the diester (VIb), the required product could only be isolated in 8% yield. A material which analysed as t-butyl *N*-ethoxycarbonylleucinate (11%) was also isolated from this reaction mixture, but it is not possible to say whether it was formed by nucleophilic attack at the " wrong " carbonyl or whether it resulted from a direct interaction between the amino-acid t-butyl ester and excess of chloroformate.



The t-butyl groups were cleaved from the diesters by the action of dry hydrogen chloride at room temperature to yield the diacids (VIIa—c). Subsequently, reduction by sodium in liquid ammonia was used to remove the toluene-*p*-sulphonyl group and the required tripeptides (VIIIa—c) were obtained chromatographically pure.

A similar two-stage removal of the protecting groups from the γ-glutamyl derivative (V) gave (γ-L-glutamyl)-γ-aminobutyric acid in good yield. This compound was also prepared by coupling benzyloxycarbonyl-α-benzyl-L-glutamate [11] with t-butyl γ-aminobutyrate by the *NN'*-carbonyldi-imidazole procedure, followed by acidolysis and hydrogenolysis to remove the protecting groups from the resulting γ-glutamyl derivative. t-Butyl (*N*-benzyloxycarbonyl-α-L-glutamyl)-γ-aminobutyrate was obtained from the reaction between phenyl *N*-benzyloxycarbonyl-α-L-glutamate [12] and t-butyl γ-aminobutyrate in solution in hot tetrahydrofuran. The t-butyl ester group was cleaved by the action of toluene-*p*-sulphonic acid and the benzyloxycarbonyl group was subsequently removed by hydrogenolysis to yield (α-L-glutamyl)-γ-aminobutyric acid.

The tripeptides (VIIIa—c) proved relatively stable in aqueous solution at room temperature, but, after several days, traces of free γ-aminobutyric acid were readily detected by thin-layer chromatography. At 100°, the decomposition was much more rapid. No appreciable differences in the rates of decomposition of (γ-glutamyl)-γ-aminobutyric acid and of the glycine (VIIIa), leucine (VIIIb), and phenylalanine tripeptides (VIIIc) were

[7] M. Zaoral and J. Rudinger, *Coll. Czech. Chem. Comm.*, 1961, **26**, 2316.
[8] C. Berse, T. Massiah, and L. Piché, *J. Org. Chem.*, 1961, **26**, 4514; *Canad. J. Chem.*, 1963, **41**, 2767.
[9] M. Zaoral, *Coll. Czech. Chem. Comm.*, 1962, **27**, 1273.
[10] R. Paul and G. W. Anderson, *J. Amer. Chem. Soc.*, 1960, **82**, 4596.
[11] R. A. Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874; J. R. Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547.
[12] E. Klieger and H. Gibian, *Annalen*, 1962, **655**, 195.

observed. (α-Glutamyl)-γ-aminobutyric acid gave very little or no γ-aminobutyric acid under these conditions.

The formation of pyrrolidone derivatives when γ-glutamyl peptides are heated in aqueous solution has been reported previously [13] and the absence of unidentified ninhydrin-positive spots on the above chromatograms suggests that this is the mode of decomposition involved here. Similar observations have been made with symmetrical diamide derivatives of glutamic acid; [14] asymmetrical diamides of type (I) have been prepared previously,[15] but stability studies were not reported. The participation of imide-formation in the decomposition of the tripeptides seems improbable since this route would give rise to new ninhydrin-positive materials. Furthermore, the resistance to saponification of the benzyl ester group in the supposedly favoured case of *N*-benzyloxycarbonyl-γ-benzyl-L-glutamyl-L-serineamide [16] makes a significant contribution from imide-formation in the decomposition of other αγ-glutamyl peptides seem unlikely. By the same token, the marked lability of the aspartic acid analogue of this benzyl ester [17] suggests that aspartyl homologues of type (I) should possess greater inherent instability than the glutamyl derivatives. The use of compounds of this type as potential drug-carriers is perhaps worthy of investigation.

Several enzymes have been reported which might effect the release or transfer of 4-aminobutyric acid from the tripeptides (VIIIa—c). One of these, γ-glutamyl lactamase, was isolated from rabbit liver by the method of Cliffe and Waley,[18] and incubated with the γ-aminobutyric acid peptides under appropriate conditions. In each case, free γ-aminobutyrate could be detected on chromatograms, but other ninhydrin-positive species were present and reactions other than simple pyrrolidone formation were clearly taking place.

Glutamyl-γ-aminobutyric acid has been isolated from the seeds of *Lunaria annua* L.[19] and from chloramphenicol-resistant strains of *E. coli*.[20] Chromatographic comparisons with our synthetic materials indicated that the seed peptide is (γ-glutamyl)-γ-aminobutyric acid and this has since been confirmed by Larsen,[21] who has synthesised the γ-glutamyl derivative independently by another route. The two isomers are also clearly distinguished by their infrared spectra, which provide confirmation of the structure of the natural peptide. On the other hand, neither isomer behaves on chromatograms exactly like the compound isolated from *E. coli* and it is suggested tentatively that the natural material in this case might be a simple derivative of glutamyl-γ-aminobutyric acid.

EXPERIMENTAL

N-Benzyloxycarbonyl-γ-aminobutyric Acid.—4-Aminobutyric acid (25·75 g., 0·25 mole) was dissolved in a mixture of acetone (128 ml.) and aqueous sodium hydroxide (154 ml.; 1·67N). The mixture was stirred vigorously at 0—5° whilst a solution of benzyloxycarbonyl chloride (65·6 g., 0·38 mole) in acetone (128 ml.) was added over 5 hr. Aqueous sodium hydroxide (240 ml.; 2N) was added simultaneously at a suitable rate to maintain the pH at 10·5. The reaction mixture was finally stirred at room temperature overnight. Evaporation of the acetone, followed by acidification of the aqueous phase, resulted in the formation of a crystalline precipitate. This was filtered off, dried, and recrystallised from ethyl acetate to give the *benzyloxycarbonylamino-acid* (47·3 g., 80%), m. p. 64—67°. A small sample recrystallised from water had m. p. 65·5—67° (Found: C, 61·0; H, 6·5; N, 6·0. $C_{12}H_{15}O_4N$ requires C, 60·75; H, 6·4; N, 5·9%).

[13] W. J. Le Quesne and G. T. Young, *J.*, 1952, 594.
[14] T. Shiba and T. Kaneko, *Bull. Chem. Soc. Japan*, 1960, **33**, 1721.
[15] H. Sachs and E. Brand, *J. Amer. Chem. Soc.*, 1954, **76**, 1811.
[16] A. P. Fosker, R. W. Hanson, and H. D. Law, *Chem. and Ind.*, 1963, 569.
[17] S. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, M. Sela, and Y. Shalitin, *J. Amer. Chem. Soc.*, 1962, **84**, 2421.
[18] E. E. Cliffe and S. G. Waley, *Biochem. J.*, 1961, **79**, 118.
[19] P. O. Larsen, *Acta Chem. Scand.*, 1962, **16**, 1511.
[20] F. Šorm and J. Černá, *Coll. Czech. Chem. Comm.*, 1960, **25**, 565.
[21] P. O. Larsen, *Acta Chem. Scand.*, 1965, **19**, 1071.

t-Butyl N-*Benzyloxycarbonyl-γ-aminobutyrate*.—(*a*) *N*-Benzyloxycarbonyl-γ-aminobutyric acid (21 g., 0·89 mole) was dissolved in methylene chloride (150 ml.) containing concentrated sulphuric acid (1 ml.) and the solution was saturated with isobutene. After 3 days at room temperature, the reaction mixture was poured into saturated aqueous sodium carbonate and the organic phase was separated off, washed, and dried. Evaporation of the methylene chloride yielded the required *t-butyl ester* as a pale yellow oil (24·3 g., 93%) (Found: C, 65·7; H, 7·9; N, 4·9. $C_{16}H_{23}O_4N$ requires C, 65·5; H, 7·9; N, 4·8%).

(*b*) Isobutene (300 ml.) was added to a solution of *N*-benzyloxycarbonyl-γ-aminobutyric acid (35 g., 0·15 mole) in dioxan (300 ml.) containing concentrated sulphuric acid (30 ml.) and the mixture was shaken overnight at room temperature in a glass-lined steel reaction vessel. The reaction mixture was afterwards poured into excess of saturated aqueous sodium carbonate and the product was extracted into ether. Evaporation of the washed and dried ethereal fraction yielded a colourless oil (36 g., 83%) indistinguishable by infrared spectra and thin-layer chromatography (t.l.c.), from the analytical sample.

t-Butyl γ-Aminobutyrate.—(*a*) The benzyloxycarbonylamino-acid t-butyl ester (27·5 g., 0·094 mole) was dissolved in ethanol (200 ml.) and hydrogenolysed (26 hr.) over 10% palladised charcoal (4 g.). After the removal of the catalyst, the filtrate was concentrated to 70 ml. and a solution of phosphorous acid (7·7 g.) in dry ether (100 ml.) was slowly added to the concentrate. The *t-butyl ester phosphite* rapidly crystallised (21·2 g., 93%), m. p. 163—166° [overall yield, from γ-aminobutyric acid, 69·5%]. A sample recrystallised from methanol had m. p. 166·5—168·5° (Found: C, 40·05; H, 8·5; N, 5·73. $C_8H_{20}NO_5P$ requires C, 39·8; H, 8·4; N, 5·8%).

(*b*) A mixture of γ-aminobutyric acid (0·198 g., 1·9 mmoles), t-butyl acetate (25 g.), and perchloric acid (0·23 ml. of a 60% aqueous solution) was shaken in a stoppered flask at room temperature for 7 days. The mixture was cooled to 0°, extracted rapidly with dilute hydrochloric acid and the aqueous extract was immediately basified by the addition of solid sodium hydrogen carbonate followed by a few drops of aqueous sodium hydroxide (final pH −9). Excess of sodium hydrogen carbonate was filtered off and the filtrate was extracted with ether (20 ml.). Addition of phosphorous acid (0·16 g.) to the ethereal solution caused the t-butyl ester phosphite to crystallise (0·95 g., 21%), m. p. 166—168°.

(*c*) A mixture of γ-aminobutyric acid (15 g., 0·146 mole), dioxan (150 ml.), isobutene (150 ml.), and concentrated sulphuric acid (15 ml.) was shaken in a glass-lined steel vessel for 18 hr. The mixture was afterwards poured into aqueous sodium hydroxide and the t-butyl ester was extracted into ether. Addition of phosphorous acid to the dried ethereal solution resulted in the crystallisation of the t-butyl ester phosphite (7 g., 20%), m. p. 170—171°.

For conversion into the free base, the phosphite salt of the t-butyl ester was dissolved in a small volume of water and the solution was basified by the addition of aqueous sodium hydroxide. The mixture was immediately extracted with ether. Evaporation of the washed and dried ethereal solution yielded the free base as a colourless oil (~90%).

t-Butyl (N-*Toluene*-p-*sulphonyl-γ*-L-*glutamyl*)-*γ-aminobutyrate*.—A solution of t-butyl γ-aminobutyrate (9·0 g., 0·056 mole) in dioxan (75 ml.) was added over 2 hr. to a vigorously stirred, boiling solution of *N*-toluene-*p*-sulphonyl-2-carboxypyrrolidone (16 g., 0·056 mole, prepared from L-glutamic acid) in dioxan (100 ml.) containing triethylamine (7·85 ml., 0·056 mole). After the addition, refluxing was continued for 3 hr. before the solvent was evaporated under reduced pressure. The resulting oil solidified upon trituration in the cold under hydrochloric acid (21 ml. of 2·7N). Recrystallisation from acetone–water gave the required *γ-glutamyl derivative* (17·7 g., 77·5%), m. p. 140—141°, $[\alpha]_D^{18}$ +28·6° (*c* 1·01 in MeOH) (Found: C, 54·3; H, 7·0; N, 6·42. $C_{20}H_{30}N_2O_7S$ requires C, 54·3; H, 6·8; N, 6·3%).

t-Butyl N^{α}-*Toluene*-p-*sulphonyl*-N^{γ}-(*3-t-butoxycarbonylpropyl*)-L-*glutaminylglycinate*.—(*a*) A solution of pivaloyl chloride (1·2 g., 0·01 mole) in dry tetrahydrofuran (10 ml.) was added at −10° to a vigorously stirred solution of the above γ-glutamyl derivative (4·42 g., 0·01 mole) in tetrahydrofuran (40 ml.) containing triethylamine (1·39 ml., 0·01 mole). The reaction mixture was allowed to warm slowly to 0° (2 hr.) and was then recooled to −10° prior to the addition of a solution of t-butyl glycinate (1·31 g., 0·01 mole) in tetrahydrofuran (10 ml.) containing another equivalent of triethylamine. After a further hour at −10°, the reaction mixture was stirred at room temperature overnight. Triethylammonium hydrochloride was filtered off and washed with a small volume of tetrahydrofuran, and the combined filtrate and washings were evaporated under reduced pressure. The residual solid was dissolved in ethyl acetate and the solution was washed with saturated aqueous sodium hydrogen carbonate and water. Evaporation of the

solvent left a white solid which was recrystallised by the addition of water to a methanolic solution to give the required *diester* (2·7 g., 50%), m. p. 137—138°, $[\alpha]_D^{19}$ +7·25° (*c* 0·9 in MeOH) (Found: C, 56·3; H, 7·3; N, 7·7. $C_{26}H_{41}N_3O_8S$ requires C, 56·2; H, 7·4; N, 7·6%).

(*b*) t-Butyl (toluene-*p*-sulphonyl-γ-L-glutamyl)-γ-aminobutyrate (4·9 g., 11 mmoles) was dissolved in dry tetrahydrofuran (100 ml.) and *NN'*-carbonyldi-imidazole (1·92 g. of 90%, 10·7 mmoles) was added to the stirred solution at −10°. The temperature of the mixture was allowed to rise to 0° over 1 hr. and was kept at 0° for a further 30 min. Afterwards, the reaction mixture, which during this time had become almost solid, was cooled once more to −10° and a solution of t-butyl glycinate (1·4 g., 10·7 mmoles) in tetrahydrofuran (10 ml.) was added to it. The reaction mixture was stirred at 0° for several hours and then at room temperature overnight. Evaporation of the solvent under reduced pressure gave an oil which was dissolved in ethyl acetate. The resulting solution was washed (thrice with 10% acetic acid, thrice with 5% aqueous sodium hydrogen carbonate, twice with water), dried, and evaporated to give a white solid (3·6 g.), m. p. 80—110°. Recrystallisation of this material from ethyl acetate–light petroleum gave the required diester (2·26 g., 40%), m. p. 132° (identical with authentic sample by infrared spectroscopy and t.l.c.).

t-Butyl N^{α}-*Toluene*-p-*sulphonyl*-N^{γ}-(3-*t-butoxycarbonylpropyl*)-L-*glutaminyl*-L-*leucinate*.—(*a*) The crude product, prepared by the pivaloyl chloride method described, was recrystallised from warm methanol by the addition of water to give the required *diester* (70%), m. p. 130—133°, $[\alpha]_D^{19}$ +4·8° (*c* 1 in MeOH) (Found: C, 58·9; H, 7·9; N, 6·9. $C_{30}H_{49}N_3O_8S$ requires C, 58·9; H, 8·1; N, 6·9%).

(*b*) By the *NN'*-carbonyldi-imidazole method described above, a crude product (8·4 g., 76%), m. p. 114—118°, was obtained. Recrystallisation from ethyl acetate–light petroleum gave the required dipeptide derivative (5·65 g., 51%), m. p. 129° (identical with authentic sample by infrared spectroscopy and t.l.c.). A second crop (1·76 g., 16%), which was not investigated further, had m. p. 152—158°.

(*c*) When ethyl chloroformate was used instead of pivaloyl chloride in the mixed anhydride procedure and the quantity of triethylamine adjusted accordingly, 8% of the required tripeptide derivative was obtained by precipitation from ethyl acetate–light petroleum. Evaporation of the petroleum phase gave an oily residue which was distilled in a bulb tube to yield a clear liquid [b. p. 100—117° (air-bath)/0·17 mm.] which analysed as *t-butyl* N-*ethoxycarbonylleucinate* (11% based on t-butyl leucinate) (Found: C, 60·2; H, 9·7; N, 5·5. $C_{13}H_{25}NO_4$ requires C, 60·2; H, 9·7; N, 5·4%).

t-Butyl N^{α}-*Toluene*-p-*sulphonyl*-N^{γ}-(3-*t-butoxycarbonylpropyl*)-L-*glutaminyl*-L-*phenylalaninate*.—(*a*) The crude product obtained by the pivaloyl chloride method described above was recrystallised from warm aqueous methanol to give the required *diester* (75%), m. p. 108—109°, $[\alpha]_D^{19}$ +18·4° (*c* 1 in MeOH) (Found: C, 61·1; H, 7·5; N, 6·6. $C_{33}H_{47}N_3O_8S$ requires C, 61·4; H, 7·3; N, 6·5%).

(*b*) The main product obtained by the *NN'*-carbonyldi-imidazole procedure was the required diester (70%), m. p. 98—100° (identical with authentic sample by infrared spectroscopy and t.l.c.).

(N^{α}-*Toluene*-p-*sulphonyl*-γ-L-*glutamyl*)-γ-*aminobutyric Acid*.—Dry hydrogen chloride was bubbled through a stirred mixture of the t-butyl ester (4 g., 9 mmoles) in dry benzene (300 ml.) for ½ hr. at room temperature and the reaction mixture was left to stand at room temperature for a further 2 hr. Evaporation of the benzene under reduced pressure gave a white residue which was dissolved in aqueous sodium hydrogen carbonate. The filtered solution was acidified with concentrated hydrochloric acid and the precipitate which formed was filtered off and dried in air to give the required *diacid* (2·83 g., 81%), m. p. 153—154°, $[\alpha]_D^{19}$ +6·07° (*c* 1·58 in 5% aqueous $NaHCO_3$) (Found: C, 49·5; H, 5·9; N, 7·2. $C_{16}H_{22}N_2O_7S$ requires C, 49·7; H, 5·7; N, 7·25%).

N^{α}-*Toluene*-p-*sulphonyl*-N^{γ}-(3-*carboxypropyl*)-L-*glutaminylglycine*.—The di-t-butyl ester (1·9 g., 3·4 mmoles) of this compound was treated with dry hydrogen chloride as described above. After the benzene had been removed, the residue was dissolved in aqueous sodium hydrogen carbonate and the filtered solution acidified. The product was extracted into ethyl acetate and the dried (Na_2SO_4) solution yielded by evaporation a white solid. Recrystallisation of this material from ethanol–light petroleum gave the required *diacid* (1·13 g., 75%), m. p. 171·5—172·5°, $[\alpha]_D^{16\cdot5}$ −20·1° (*c* 0·51 in 5% aqueous $NaHCO_3$) (Found: C, 48·6; H, 5·9; N, 9·25. $C_{18}H_{25}N_3O_8S$ requires C, 48·8; H, 5·7; N, 9·5%).

N^{α}-*Toluene*-p-*sulphonyl*-N^{γ}-(3-*carboxypropyl*)-L-*glutaminyl*-L-*leucine*.—The di-t-butyl ester (3·37 g., 5·5 mmoles) was treated with dry hydrogen chloride as described above. Acidification of the sodium hydrogen carbonate solution resulted in the precipitation of a white solid which was filtered off and air-dried. Reprecipitation from sodium hydrogen carbonate solution by the addition of acid gave the required *diacid* (2·33 g., 81%), m. p. 209—212°, $[\alpha]_D^{17}$ $-2·2°$ (*c* 1·87 in 5% aqueous $NaHCO_3$) (Found: C, 53·1; H, 6·7; N, 8·2. $C_{22}H_{33}N_3O_8S$ requires C, 52·9; H, 6·7; N, 8·4%).

N^{α}-*Toluene*-p-*sulphonyl*-N^{γ}-(3-*carboxypropyl*)-L-*glutaminyl*-L-*phenylalanine*.—The di-t-butyl ester (4·1 g., 6·4 mmoles) was treated with dry hydrogen chloride as described above. An oil precipitated when the sodium hydrogen carbonate solution was acidified but it solidified on rubbing. Reprecipitation from sodium hydrogen carbonate solution gave the required *diacid* (2·92 g., 90%), m. p. 132—135°, $[\alpha]_D^{18}$ $+8·54°$ (*c* 2·68 in 5% aqueous $NaHCO_3$) (Found: C, 55·6; H, 6·0; N, 7·7. $C_{25}H_{31}N_3O_8S,0·5H_2O$ requires C, 55·3; H, 5·9; N, 7·7%).

(γ-L-*Glutamyl*)-γ-*aminobutyric Acid*.—(*a*) (N^{α}-Toluene-*p*-sulphonyl-γ-L-glutamyl)-γ-aminobutyric acid (2·04 g., 5·3 mmoles) was dissolved in liquid ammonia and small pieces of sodium were added to the stirred solution until a permanent blue colour resulted. The blue colour was finally discharged by the addition of a few drops of acetic acid and the ammonia was allowed to evaporate. Subsequently, the residue was dissolved in water and treated with Amberlite IRC 50 and barium acetate in the manner described by Rudinger.[2] Concentration of the final aqueous solution to a very small volume, followed by the addition of ethanol, resulted in the deposition of the required γ-*glutamyl derivative* (0·9 g., 73%), m. p. 177—179°. A sample recrystallised from aqueous ethanol had m. p. 190—191°, $[\alpha]_D^{17}$ $+7·3°$ (*c* 0·9 in H_2O), $\nu_{max.}$ 1640s, 1690m cm.$^{-1}$ (Found: C, 46·65; H, 7·12; N, 11·9. $C_9H_{16}N_2O_5$ requires C, 46·5; H, 6·9; N, 12·1%).

(*b*) Benzyl N^{α}-benzoxycarbonyl-N^{γ}-(3-carboxypropyl)-L-glutaminate (1 g., 2·19m moles, see below) was hydrogenolysed in aqueous methanol over 10% palladised charcoal (0·1 g.). The catalyst was filtered off and the filtrate was evaporated under reduced pressure, leaving a solid residue. Recrystallisation from aqueous ethanol gave the required γ-glutamyl derivative (0·2 g., 40%), m. p. 190—192° (identical by infrared spectroscopy and t.l.c. with the above material).

N^{γ}-(3-*Carboxypropyl*)-L-*glutaminyl*-L-*glycine*.—The N^{α}-toluene-*p*-sulphonyl derivative (1·13 g., 2·55 mmoles) was reduced by the sodium–liquid ammonia reagent and the product was isolated by the Amberlite IRC 50–barium acetate procedure described above. Recrystallisation from aqueous methanol gave the required *peptide* (0·36 g., 49%), m. p 192—193° (Found: C, 46·05; H, 6·7. $C_{11}H_{19}N_3O_6$ requires C, 45·7; H, 6·6%).

N^{γ}-(3-*Carboxypropyl*)-L-*glutaminyl*-L-*phenylalanine*.—The N^{α}-toluene-*p*-sulphonyl derivative (1·74 g., 3·26 mmoles) was reduced by the sodium–liquid ammonia reagent and the product was isolated by the Amberlite IRC 50–barium acetate procedure described above. Recrystallisation of the crude product from ethanol–water gave the required *peptide* (50%), m. p. 197—199° (Found: C, 56·7; H, 6·75; N, 11·0. $C_{18}H_{25}N_3O_6$ requires C, 57·0; H, 6·6; N, 11·1%).

N^{γ}-(3-*Carboxypropyl*)-L-*glutaminyl*-L-*leucine*.—The N^{α}-toluene-*p*-sulphonyl derivative of this compound (2·6 g., 5·2 mmoles) was reduced by the sodium–liquid ammonia reagent and the product isolated by the Amberlite IRC 50–barium acetate procedure described above. Recrystallisation of the crude product from aqueous ethanol gave the required *peptide* (0·75 g., 42%), m. p. 189—191°. A sample recrystallised several times from aqueous ethanol had m. p. 194—194·5°, $[\alpha]_D^{18}$ $+11·15°$ (*c* 0·5 in H_2O) (Found: C, 49·5; H, 7·7; N, 11·15. $C_{15}H_{27}O_6N_3,H_2O$ requires C, 49·6; H, 8·0; N, 11·55%).

Benzyl N^{α}-*Benzyloxycarbonyl*-N^{γ}-(3-*t-butoxycarbonylpropyl*)-L-*glutaminate*.—t-Butyl γ-aminobutyrate was coupled in the usual way with benzyl *N*-benzyloxycarbonyl-α-L-glutamate by the *NN'*-carbonyldi-imidazole procedure. After the evaporation of the solvent the residual oil was dissolved in ethyl acetate and the washed solution was dried (Na_2SO_4). The ethyl acetate was removed under reduced pressure to give an oil which solidified on rubbing. Recrystallisation from acetone–water gave the required γ-*glutamyl derivative* (1·06 g., 51%), m. p. 73—74°, $[\alpha]_D^{17}$ $-14·1°$ (*c* 0·67 in MeOH) (Found: C, 65·4; H, 7·2; N, 5·8. $C_{28}H_{36}N_2O_7$ requires C, 65·6; H, 7·1; N, 5·5%).

Benzyl N^{α}-*Benzyloxycarbonyl*-N^{γ}-(3-*carboxypropyl*)-L-*glutaminate*.—The above t-butyl ester (4·7 g., 9·2 mmoles) was treated with dry hydrogen chloride in the usual manner. Recrystallisation of the crude product from aqueous methanol gave the required *monoester* (1·4 g., 35%),

m. p. 115—117° (Found: C, 63·1; H, 6·2; N, 6·4. $C_{24}H_{28}N_2O_7$ requires C, 63·1; H, 6·2; N, 6·1%).

t-Butyl (N-*Benzyloxycarbonyl-α-L-glutamyl)-γ-aminobutyrate.*—A solution of phenyl *N*-benzyloxycarbonyl-α-L-glutamate (3·57 g., 0·01 mole) and t-butyl γ-aminobutyrate (1·7 g., 0·011 mole) in tetrahydrofuran was heated at 45° for 2 days. Evaporation of the solvent resulted in the formation of an oil which was dissolved in ethyl acetate. The resulting solution was washed quickly at 0° with dilute hydrochloric acid and water, dried (Na_2SO_4), and evaporated to yield the required *α-glutamyl derivative* (2·7 g., 61%), m. p. 80°. A sample recrystallised twice from ethyl acetate–light petroleum had m. p. 85—87°, $[\alpha]_D^{18}$ −9·6° (*c* 0·67 in methanol) (Found C, 60·0; H, 7·2; N, 6·7. $C_{21}H_{30}O_7N_2$ requires C, 59·7; H, 7·2; N, 6·6%).

(N-*Benzyloxycarbonyl-α-L-glutamyl)-γ-aminobutyric Acid.*—A solution of the t-butyl ester (1 g., 2·4 mmoles) and toluene-*p*-sulphonic acid (0·15 g.) in benzene (15 ml.) was refluxed for 1 hr. and after it had cooled to room temperature the resultant emulsion was extracted with aqueous sodium hydrogen carbonate (50 ml. of 5%). The aqueous layer was acidified and the product was extracted into ethyl acetate. Evaporation of the dried (Na_2SO_4) organic phase gave an oil which solidified on trituration under light petroleum. Recrystallisation from ethyl acetate–light petroleum gave the required *α-glutamyl derivative* (0·48 g., 53%), m. p. 62—65° (foams) (Found: C, 55·8; H, 6·2; N, 7·8. $C_{17}H_{22}N_2O_7$ requires C, 55·7; H, 6·05; N, 7·65%).

(*α-L-Glutamyl)-γ-aminobutyric Acid.*—(*N*-Benzyloxycarbonyl-α-L-glutamyl)γ-aminobutyric acid (0·48 g., 1·3 mmoles) in solution in aqueous methanol (20 ml., 70%) was hydrogenolysed over palladised charcoal (0·08 g. of 10%) for 1 hr. at room temperature. Filtration of the resulting solution and evaporation of the filtrate gave an oil which solidified on trituration under ether. The solid was dissolved in a small volume of water and absolute ethanol was added to the solution. After several days at 0° a crystalline precipitate had formed. Recrystallisation, in the same manner, gave the *dipeptide monohydrate* (0·13 g., 40%), m. p. 122—125°, $[\alpha]_D^{17}$ +33·6° (*c* 1·02 in H_2O), $\nu_{max.}$ 1650m, 1675s cm.$^{-1}$ (Found: C, 43·3; H, 7·3; N, 11·5. $C_9H_{16}N_2O_5,H_2O$ requires C, 43·2; H, 7·3; N, 11·2%).

Methyl N-*Toluene*-p-*sulphonyl-γ-L-glutamylglycinate.*—This compound was prepared by the reaction of methyl glycinate with *N*-toluene-*p*-sulphonylpyrrolid-5-one-2-carboxylic acid in boiling dioxan solution in a method similar to that employed by Rudinger [2] for the synthesis of the corresponding ethyl ester. The crude product was reprecipitated from aqueous sodium hydrogen carbonate by the addition of dilute hydrochloric acid and was finally recrystallised from aqueous methanol to give the *dipeptide methyl ester derivative* (70%), m. p. 184—186° (Found: C, 48·5; H, 5·5; N, 7·5. $C_{15}H_{20}N_2O_7S$ requires C, 48·4; H, 5·4; N, 7·5%).

The *N*-protected dipeptide ester was converted to γ-L-glutamylglycine by saponification, followed by sodium–liquid ammonia reduction, as described by Rudinger.[2]

Stability of Glutamyl-γ-aminobutyric Acid Derivatives in Aqueous Solution.—The peptides (10 mg.) were dissolved in water (0·5 ml.) and the resulting solutions were examined by thin-layer chromatography after various periods at room temperature and at 100°. Each of the γ-glutamyl derivatives gave solutions containing γ-aminobutyric acid. This component was detectable after 20 min. heating; after 24 hr. at 100° the decomposition was generally complete. At room temperature, the γ-glutamyl peptides were relatively stable but after 1 week, γ-aminobutyric acid was clearly detectable. Slight traces of unknown ninhydrin-positive components were sometimes detected on these chromatograms but the amounts present were not considered significant. [α-L-Glutamyl)-γ-aminobutyric acid showed no decomposition after 1 hr. at 100°.

Details of the chromatography were the same as those reported for the enzymic studies.

Enzymic Digestion of (γ-L-Glutamyl)-γ-aminobutyric Acid Derivatives.—γ-Glutamyl lactamase was prepared as an acetone-dried powder from the liver of freshly-killed rabbit by the procedure of Cliffe and Waley.[18] To confirm its identity, the enzyme was extracted from the powder by a phosphate-salt buffer (pH 7·4), as described by these authors, and incubated at 37° with γ-L-glutamylglycine. The course of the digestion was followed by thin-layer chromatography on Kieselgel G using phenol–water as solvent. γ-L-Glutamylglycine, glycine, and pyrrolidonecarboxylic acid were clearly separated in this solvent system. Pyrrolidonecarboxylic acid was visualised under u.v. light after the plate had been left overnight at room temperature and heated to 105° for 10 min. immediately prior to viewing. Glycine and γ-L-glutamylglycine were detected by ninhydrin. γ-L-Glutamylglycine was readily digested by the enzyme preparation, glycine and pyrrolidonecarboxylic acid being the only apparent products.

Digestions of the (γ-L-glutamyl)-γ-aminobutyric acid derivatives were carried out under identical conditions (0·03 mmole of derivative, 0·17 ml. buffer, 0·05 ml. enzyme preparation, 37°, 4 hr.), but whereas the γ-L-glutamylglycine digestion remained clear, the 4-aminobutyrate digestions quickly solidified. Thin-layer chromatography in the phenol system revealed that 4-aminobutyric acid was liberated in each case, but in lesser amounts than expected. In addition, at least one other ninhydrin-positive substance, as well as the starting material, was present in each digest of the 4-aminobutyrate compounds. This was particularly noticeable with the leucine derivative.

The compositions of the mixtures after 4 hr. digestion are summarised below [substance, R_F, (relative intensity of ninhydrin colour)].

(*a*) γ-L-*Glutamylglycine.* Starting material, 0·09, (+++); glycine, 0·185, (+++); pyrrolidonecarboxylic acid, 0·25.

(*b*) (γ-L-*Glutamyl*)-γ-*aminobutyric acid.* Starting material, 0·15, (+++); γ-aminobutyric acid, 0·28, (++); other, 0·39 (+).

(*c*) N^{γ}-(3-*Carboxypropyl*)-L-*glutaminylglycine.* Starting material, 0·13, (+++); γ-aminobutyric acid, 0·27, (+); other, 0·37, (+).

(*d*) N^{γ}-(3-*Carboxypropyl*)-L-*glutaminyl*-L-*leucine.* Starting material, 0·21, (+++); γ-aminobutyric acid, 0·24, (+); other, 0·40, (+++).

(*e*) N^{γ}-(3-*Carboxypropyl*)-L-*glutaminyl*-L-*phenylalanine.* Starting material, 0·22, (+++); γ-aminobutyric acid, 0·23, (+); other, 0·46, (++).

Chromatographic Behaviour of (α- and γ-L-*Glutamyl*)-γ-*Aminobutyric Acid on Whatman No.* 1 *Filter Paper* (*Descending Technique*).—In the system butanol–acetic acid–water (12 : 3 : 5) (γ-L-glutamyl)-γ-aminobutyric acid (R_F 0·37) ran slightly ahead of threonine (R_F 0·29) and less than tyrosine (R_F 0·47). This is similar to the behaviour of the peptide isolated from seeds of *Lunaria annua* L.[19] (α-L-Glutamyl)-γ-aminobutyric acid (R_F 0·45) is almost indistinguishable from tyrosine in this system. In phenol–water–ammonia (120 : 30 : 1) both isomers, like the natural peptide,[19] behave like threonine (R_F 0·56).

By single development in the system butanol–acetic acid–water (11 : 1 : 3), (α-L-glutamyl)-γ-aminobutyric acid (R_F 0·24) and (γ-L-glutamyl)-γ-aminobutyric acid (R_F 0·17) ran ahead of glutamic acid (R_F 0·12). By repeated development, the peptide from *E. coli* behaves like glutamic acid in this system.[20] In phenol–water, (α- and γ-L-glutamyl)-γ-aminobutyric acid were indistinguishable (R_F 0·65) and ran ahead of threonine (R_F 0·48). The peptide from *E. coli* behaves " approximately like threonine " in this system.[20]

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