385. *Peptides. Part I. The Synthesis of Peptides through Anhydrides of Sulphuric Acid.*

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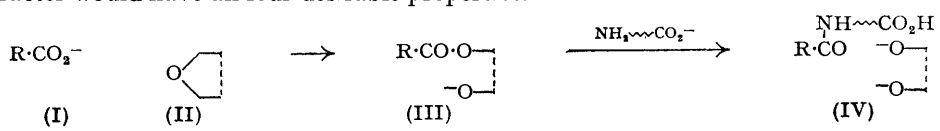
The possibility of peptide synthesis through water-soluble mixed anhydrides has been explored by studying the condensation of amines with toluene-*p*-sulphonyl-DL-alanine after treating its potassium salt with anhydrides of dibasic acids. Whereas *o*-sulphobenzoic and similar anhydrides are relatively ineffective in promoting condensation, the complex of sulphur trioxide with dimethylformamide gives high yields. A general procedure is described for lengthening the chain of a carbobenzyloxy-peptide by reaction of the salt of its sulphuric anhydride with the sodium salt of an amino-acid in aqueous solution. Among the examples given is the preparation of carbobenzyloxyglycyl-L-phenylalanylglycine from carbobenzyloxyglycyl-L-phenylalanine and glycine.

THE biological significance of peptides containing between five and thirty amino-acids has recently become increasingly apparent. This series of papers describes attempts to provide new methods for the synthesis and selective degradation of peptides of medium size to match those now available for their purification and determination of their amino-acid content.

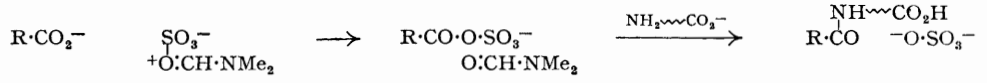
Four attributes seem desirable in a method for the synthesis of peptides containing more than some four amino-acids: (*a*) the avoidance of racemisation at the α-carbon atoms of the amino-acids, (*b*) the power to combine oligopeptides, (*c*) the use of polar and especially aqueous media for formation of the peptide link, and (*d*) the direct repeatable extension of a peptide chain in good yield without the isolation of intermediates. Of these, only (*a*) is a necessary property of useful synthetic methods, but the other three confer obvious advantages. Thus a scheme of synthesis in which amino-acids are added singly to the partly completed structure will be less flexible than one satisfying condition (*b*). The Bergmann–Curtius method has usually been chosen from those hitherto available (Fruton, *Adv. Protein Chem.*, 1949, **5**, 1; Wieland, *Angew. Chemie*, 1951, **63**, 7), for it satisfies conditions (*a*) and (*b*) and, to a certain extent, (*d*), in that the resultant carbobenzyloxy-ester may be converted *via* the hydrazide into a fresh acyl azide. The range of peptide syntheses would be usefully extended by a method of similar type, but one in which an anhydride was formed more directly from the free carboxyl group of a protected peptide and was coupled with the salt of a second peptide in an aqueous medium. A fully successful synthesis of this character would have all four desirable properties.



(I) (II) (III) (IV)

For initial study we selected the scheme of synthesis depicted above. This involves reaction between the anion (I) derived from a protected peptide and the cyclic anhydride

(II) of a dibasic acid, to give a mixed anhydride (III). The negative charge borne by (III) should confer water solubility on it and so facilitate its reaction with the salt of a second peptide leading to a larger protected peptide (IV), with which the whole process could be repeated. As the anhydrous medium necessary for the first step we chose dimethylformamide, since protected amino-acids and peptides dissolve freely in it and may then be directly converted into their anhydrous salts by neutralisation with methanolic alkali and fractional distillation of a portion of the solvent in a vacuum at about 50°. In this manner toluene-*p*-sulphonyl-DL-alanine was converted into its potassium salt and then condensed with morpholine by successive additions of one equivalent of *o*-sulphobenzoic anhydride and of excess morpholine to the dimethylformamide solution. The yield of morpholide was, however, only 59% and still lower when 3 : 5-dibromo-2-sulphobenzoic, β-sulphopropionic, or propane-1 : 3-disulphonic anhydride was used. The inefficiency of these syntheses might be attributed to retention of water or methanol by the salt solution or to incomplete reaction with the cyclic anhydride. But freshly fused potassium phenylacetate dissolved immediately when *o*-sulphobenzoic anhydride was added to its suspension in dimethylformamide, and only 50% of phenylacetanilide was isolated after addition of excess of aniline. We assume instead that the mixed anhydrides (III) either disproportionate very easily or, more probably, are attacked by the amino-group at both possible points (cf. Emery and Gold, *J.*, 1950, 1443). Better results might have been achieved by more careful selection of the cyclic anhydride and indeed, since we made these experiments, Wieland and Sehring (*Annalen*, 1950, **569**, 122) have described the synthesis of peptides through anhydrides of benzoic acid. It seemed, however, more profitable to examine mixed anhydrides with inorganic dibasic acids. Thus, Chantrenne (*Biochem. Biophys. Acta*, 1950, **4**, 484) had shown that the mixed anhydride of carbobenzyloxyglycine and phenyl dihydrogen phosphate acylates amino-acids and peptides in aqueous solution at pH 7·4. We found that the sodium salt of acetylsulphuric acid (van Peski, *Rec. Trav. chim.*, 1921, **40**, 103) likewise acetylated phenylalanine in 86% yield when the pH was kept at about 9 during the rapid reaction. With an aqueous solution of *cyclo*hexylamine the reaction was quantitative and even aniline gave 68% of acetanilide, although van Peski had claimed only poor yields for this reaction and the corresponding butyroylation (*ibid.*, p. 736). It was therefore an obvious step to attempt to prepare the analogous anhydride of toluene-*p*-sulphonyl-DL-alanine by substitution of sulphur trioxide for the cyclic anhydrides of the earlier experiments.



Sulphur trioxide has commonly been brought into reaction with organic compounds which are labile to sulphuric acid in two forms, as its co-ordination complex with dioxan (Suter, Evans, and Kiefer, *J. Amer. Chem. Soc.*, 1938, **60**, 359) or pyridine (Baumgarten, *Ber.*, 1926, **59**, 1166). Neither of these complexes is suitable for preparation of mixed anhydrides of protected peptides. The former is an unstable solid used as a suspension in ethylene dichloride and therefore cannot be added in a measured quantity. The latter is an easily prepared stable solid but would probably yield an acylpyridinium salt rather than the desired acyl sulphate, if indeed it entered into reaction at all. As a base of intermediate strength, dimethylformamide, the solvent used by us, is more suitable for preliminary combination with the sulphur trioxide; its crystalline complex has been used for preparing sulphuric esters of leuco-dyes (Coffey, Driver, Fairweather, and Irving, B.P. 610,117, 642,206). A solution of this complex is easily prepared by distilling sulphur trioxide directly into dimethylformamide and may be stored with only minor decomposition for two months in the refrigerator. With the potassium salt of toluene-*p*-sulphonyl-DL-alanine, it rapidly gives a stable solution of potassium toluene-*p*-sulphonyl-DL-alanine sulphate (cf. p. 2073), which when run into excess of *cyclo*hexylamine in water afforded the *cyclo*hexylamide in 95% yield (5% of the original acid was recovered). The anhydride also reacted smoothly with an aqueous solution of glycine at pH 9 giving 80% of toluene-*p*-sulphonyl-DL-alanylglycine; and it gave 60% of the corresponding crystalline carbo-

benzyloxy-derivative. In more complicated cases the product could be profitably isolated by counter-current distribution between ethyl acetate and a phosphate buffer. The efficiency of separation is high in buffered systems (Craig, Golumbic, Mighton, and Titus, *J. Biol. Chem.*, 1945, **161**, 321) and adjustment of the pH gives the desired partition coefficient without change of the organic solvent. The precise yield of the synthesis is also calculable from the distribution curve.

We also studied the condensation of carbobenzyloxyglycyl-L-phenylalanine with glycine, in which the single centre of asymmetry would be subject to racemisation both directly in the mixed anhydride and through cyclisation of this to the oxazolone salt. As a preparatory experiment carbobenzyloxyglycyl-DL-phenylalanylglycine was synthesised in two steps, each of 70% yield, from carbobenzyloxyglycine, DL-phenylalanine, and glycine. The potassium salt of carbobenzyloxyglycine was sparingly soluble in dimethylformamide but dissolved on addition of the sulphur trioxide solution; so as to provide rapidity of manipulation it was replaced by the soluble trimethylphenylammonium salts. Carbobenzyloxyglycyl-L-phenylalanine (Hofmann and Bergmann, *ibid.*, 1940, **134**, 225) was converted into its sulphuric anhydride which was added to excess of aqueous sodium hydroxide; the recovered dipeptide derivative had been extensively racemised. The racemisation did not appear to have occurred in the dimethylformamide solution but rather through action of the aqueous alkali, for a second portion of the same anhydride solution kept for a longer time gave material of comparable optical activity. However, in the preparation of carbobenzyloxyglycyl-L-phenylalanylglycine the time allowed for anhydride formation was limited to one minute at 0°. This product was indistinguishable by melting point or optical rotation from a sample prepared by the customary azide procedure; further the recovered carbobenzyloxyglycyl-L-phenylalanine had not been appreciably racemised. In the preparation of carbobenzyloxy-L-phenylalanylglycine the yield was only 25%, owing to production of more water-soluble substances, which have not been fully investigated but appear to arise through loss of the benzyl radical from the carbobenzyloxy-group. Fruton and Bergmann (*ibid.*, 1942, **145**, 253) have remarked that the ethyl ester of this compound gives on treatment with ammonia 5-benzylhydantoin-3-acetamide and not the expected amide obtained in many analogous cases under the same conditions. In addition Wessely, Kemm, and Mayer (*Z. physiol. Chem.*, 1928, **180**, 64) have shown that ureido-compounds can arise in the alkaline hydrolysis of carbalkyloxy-dipeptides.

The further examples at present under study will evaluate our method, test more rigorously the retention of asymmetry (a small amount of racemisation would have escaped detection), and compare it with another, rather similar in concept, which has recently been described (Boissonas, *Helv. Chim. Acta*, 1951, **34**, 874; Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547; Wieland and Bernhard, *Annalen*, 1951, **572**, 190). The latter is simpler, but the intermediate mixed anhydrides with monoalkyl carbonates tend to disproportionate, and this is very unlikely with sulphuric anhydrides.

Experimental

M. p.s are corrected.

Toluene-p-*sulphonyl*-DL-*alanine Morpholide*.—Toluene-*p*-sulphonyl-DL-alanine (0·5 g.) and thionyl chloride (2 c.c.) were kept at 45—50° during 1 hour. After evaporation of the excess of chloride under reduced pressure, dry benzene (10 c.c.) was added and a portion removed under reduced pressure. Morpholine (0·5 c.c.) was added to the benzene solution, which was then diluted with chloroform before being washed with dilute acid, sodium hydrogen carbonate solution, and water. The *morpholide* obtained by evaporation of the solvent recrystallised from benzene in needles, m. p. 131·5° (Found, in material sublimed at 140°/10^{-4} mm.: C, 54·1; H, 6·2; N, 9·2. $C_{14}H_{20}O_4N_2S$ requires C, 53·8; H, 6·4; N, 9·0%).

Toluene-p-*sulphonyl*-DL-*alanine* cyclo*Hexylamide*.—Prepared in the same way as the morpholide, the cyclo*hexylamide* crystallised from benzene in needles, m. p. 144° (Found, in material sublimed at 140°/10^{-4} mm.: C, 59·2; H, 7·5; N, 8·4. $C_{16}H_{24}O_3N_2S$ requires C, 59·2; H, 7·5; N, 8·6%).

Identification of Acylamino-acids by Paper Chromatography.—The substances were applied in 0·3—1·0% solution to Whatman No. 1 paper. *n*-Butanol saturated with 2N-ammonia was used as the ascending solvent (Williams and Kirby, *Science*, 1948, **107**, 487). The paper was dried for

½ hour at 130° before being sprayed with an aqueous solution of sodium iodate (0·1%), sodium iodide (0·4%), and starch (0·5%), which revealed the location of the acids by blue spots (cf. Long, Quayle, and Stedman, *J.*, 1951, 2197). At 18° the following R_F values were obtained: toluene-*p*-sulphonyl derivatives of glycine (0·42), alanine (0·51), and alanylglycine (0·32); carbobenzyloxy-derivatives of glycine (0·45), alanine (0·52), phenylalanine (0·72), alanylglycine (0·41), glycylphenylalanine (0·65), and phenylalanylglycine (0·70).

Separation of Acylamino-acids by Countercurrent Distribution.—The solvent system was redistilled ethyl acetate and a mixture of M-aqueous KH_2PO_4 and K_2HPO_4, in proportions appropriate to the particular separation. Two all-glass apparatus of the type described by Craig and Post (*Analyt. Chem.*, 1949, **21**, 500), but with a tap at the end of each tube, were used, one with 12 tubes (each of 94 c.c.) for each phase and one with 23 tubes (each of 16 c.c.). At the end of distribution by the fundamental procedure phosphoric acid (1 c.c. for every 16 c.c. of buffer) was added to each tube, the whole was shaken without transfer and the aqueous phosphate layers were discarded. The substances were then recovered by evaporation in a vacuum, finally at 0·5 mm., in tared flasks. From the weight-distribution curve the amounts of product and recovered starting material in each tube and their partition coefficients were calculated (Williamson and Craig, *J. Biol. Chem.*, 1947, **168**, 687). The partition coefficients *K*, referred to later, are the ratios of the quantity in the ethyl acetate to that in the phosphate solution and the tubes are numbered from 0 at the "aqueous end," ethyl acetate being the moving phase and phosphate stationary.

Preparation of Anhydrous Salts of Acylamino-acids.—The acylamino-acid, *e.g.*, toluene-*p*-sulphonyl-DL-alanine or carbobenzyloxyglycyl-L-phenylalanine (2—10 mmol.), was dissolved in dimethylformamide (30—50 c.c.) and neutralised with methanolic potassium methoxide. About one-half of the solvent was then removed by distillation at 50°/15 mm. through a 6" column packed with steel gauze (Dixon, *J. Soc. Chem. Ind.*, 1949, **68**, 88). During the latter part of this stripping process the column-head temperature was not affected by more than 0·1° on alteration of the reflux ratio from infinity to zero.

Alternatively, a solution of the trimethylphenylammonium salt was prepared in the same way after neutralisation with the filtrate from trimethylphenylammonium toluene-*p*-sulphonate (Rodionow, *Bull. Soc. chim.*, 1926, **39**, 305) and methanolic sodium methoxide.

*Condensation of Toluene-*p*-sulphonyl-DL-alanine with Morpholine by Means of Cyclic Anhydrides.*—The cyclic anhydride (3 mmol.) and, after 1 hour, morpholine (0·8 c.c.) were added to a dimethylformamide solution of the potassium salt (2 mmol.). After 3 hours further at 20° the solvent was evaporated under reduced pressure. The residue was dissolved in chloroform and washed with dilute acid, sodium hydrogen carbonate solution, and water. The morpholide, m. p. 129—130°, was obtained in yields of 59% from *o*-sulphobenzoic anhydride (Clarke and Dreger, *Org. Synth.*, 1929, **9**, 80), 45% from 3 : 5-dibromo-2-sulphobenzoic anhydride (Twiss and Ferniholt, *J. Amer. Chem. Soc.*, 1936, **58**, 1561), 27% from propane-1 : 3-disulphonic anhydride (McElvain, Jelinek, and Rorig, *ibid*, 1945, **67**, 1578), and 8% from β-sulphopropionic anhydride (Kharasch, Chao, and Brown, *ibid.*, 1940, **62**, 2393). Toluene-*p*-sulphonyl-DL-alanine, m. p. 139—140°, was recovered from the carbonate washings by acidification and four chloroform extractions in yields of 21, 28, 38, and 57% respectively.

Acetylation of Amines by Aqueous Solution of Sodium Acetyl Sulphate.—Sodium acetyl sulphate was prepared according to van Peski (*Rec. Trav. chim.*, 1921, **40**, 103) from sodium acetate, acetic anhydride, and 100% sulphuric acid; analysis by van Peski's method of alkali titrations showed it to contain about 25% of sodium hydrogen sulphate.

(a) *Aniline.* Sodium acetyl sulphate (0·96 g.) was stirred with aniline (3 c.c.) and ice-water (15 c.c.) during 2 hours. Acidification and chloroform extraction then yielded acetanilide (0·41 g., 68%).

(b) cyclo*Hexylamine.* The acetyl derivative was obtained similarly in quantitative yield (calc. on 75% purity of the sodium acetyl sulphate).

(c) DL-*Phenylalanine.* Sodium hydroxide (2N) was added dropwise during 15 minutes to a stirred solution of DL-phenylalanine (0·66 g.), sodium acetyl sulphate (1 g.), and phenolphthalein in N-sodium hydroxide (4 c.c.) and water (8 c.c.) at such a rate that the pink colour was maintained. After a further 15 minutes the solution was acidified with dilute sulphuric acid and extracted eight times with ethyl acetate, which then contained acetyl-DL-phneylalanine (0·81 g., 86%), m. p. 146—147°.

Dimethylformamide Solution of Sulphur Trioxide–Dimethylformamide Complex.—This was prepared by distilling sulphur trioxide directly on to the surface of dimethylformamide, which was stirred and cooled in an ice–salt bath. When crystals started to separate, the distillation

was stopped and sufficient dimethylformamide added to give a clear solution. This was standardised by titration with aqueous alkali and was about 1·3M.

The sulphur trioxide was prepared by passing sulphur dioxide and oxygen over platinised asbestos at 650° and was twice redistilled with rigorous exclusion of moisture. It was a colourless crystalline solid, which sublimed without melting. Sulphur trioxide prepared by fractional distillation of 63% oleum or from commercial sources contained appreciable amounts of moisture and gave a yield of no more than 86% in the preparation of toluene-*p*-sulphonyl-DL-alanine *cyclo*hexylamide.

Toluene-p-*sulphonyl*-DL-*alanine* cyclo*Hexylamide*.—A dimethylformamide solution (6·55 c.c. of 1·52M) of the sulphur trioxide–dimethylformamide complex (10 mmol.) was added to a dimethylformamide solution of the potassium salt of toluene-*p*-sulphonyl-DL-alanine (10 mmol.) at 20°. Aliquots (8 c.c. each) were withdrawn at intervals of 3, 10, 30, 55, and 130 minutes from this solution (total volume 52 c.c.) and pipetted into a solution of *cyclo*hexylamine (1 c.c.) in water (5 c.c.) at 0°. After 1 hour sodium hydrogen carbonate solution (5 c.c., saturated) was added to the mixture, which was then extracted six times with ethyl acetate (200 c.c. total). The ethyl acetate was washed with a little acid, dried ($CaSO_4$), and evaporated, finally in high vacuum, leaving the *cyclo*hexylamide as a syrup crystallising in rosettes of needles, m. p. 142—143° (0·476, 0·494, 0·484, 0·491, and 0·493 g. respectively; 0·499 g. corresponds to quantitative yield). The carbonate solutions from the first two experiments were acidified and extracted six times with ethyl acetate, which on evaporation left 0·016 g. and 0·021 g. respectively of syrup.

A similar experiment, in which an equimolar mixture of toluene-*p*-sulphonyl-DL-alanine and 4-methylmorpholine was used instead of the potassium salt, gave only 22% of *cyclo*hexylamide.

Toluene-p-*sulphonyl*-DL-*alanylglycine*.—To an ice-cooled solution of the mixed anhydride (2 mmol.), prepared as in the preceding experiment, was added a solution of glycine (0·225 g., 3 mmol.) and phenolphthalein in N-sodium hydroxide (3 c.c.) and water (8 c.c.) at 0°, followed by sufficient N-sodium hydroxide in portions to restore and maintain the pink colour. After 15 minutes the solution was neutralised with dilute sulphuric acid and thoroughly evaporated under reduced pressure. The residue was dissolved in ethyl acetate (20 c.c.) and 3N-sulphuric acid (10 c.c.), which was extracted five times more with ethyl acetate. The dried ethyl acetate extracts (200 c.c.) were evaporated to a syrup, which crystallised from hot water (5 c.c.) in colourless needles (3·48 g. 80%), m. p. 150—151° undepressed by an authentic specimen of the dipeptide derivative, m. p. 151° (Schönheimer, *Z. physiol. Chem.*, 1926, **154**, 203). Paper chromatography confirmed the identity of the preparations and showed the presence of both the dipeptide derivative (R_F 0·32) and toluene-*p*-sulphonyl-DL-alanine (R_F 0·51) in proportions of about 2 : 1 in the aqueous liquors.

Carbobenzyloxy-DL-*alanylglycine*.—This *compound*, prepared from carbobenzyloxy-DL-alanine (2 mmol.) as in the preceding experiment, had m. p. 130—131° (Found, in material dried at 20°: N, 9·9. $C_{13}H_{16}O_5N_2$ requires N, 10·0%) (0·34 g., 60%). A sample prepared by Dr. H. G. Khorana *via* the reaction between carbobenzyloxy-DL-alanyl chloride and glycine ethyl ester (cf. Hunt and du Vigneaud, *J. Biol. Chem.*, 1938, **124**, 699) had m. p. 128—129°. The liquors from its preparation through the sulphuric anhydride contained both the dipeptide derivative (R_F 0·41) and carbobenzyloxy-DL-alanine (R_F 0·52) in proportions of about 3 : 1.

Carbobenzyloxyglycyl-DL-*phenylalanine*.—A dimethylformamide solution (6·6 c.c.) of the sulphur trioxide–dimethylformamide complex (10 mmol.) was added to a suspension of the potassium salt of carbobenzyloxyglycine (10 mmol.) in dimethylformamide (30 c.c.). The mixture was kept at 20° with shaking for 5 minutes before being cooled in an ice–salt bath. A solution of DL-phenylalanine (1·98 g., 12 mmol.) and phenolphthalein in N-sodium hydroxide (12 c.c.) and water (10 c.c.) was then added in one portion to the stirred dimethylformamide solution and was followed rapidly by sufficient 0·5N-alkali to restore and maintain the pink colour. After 10 minutes the solution was neutralised with 3N-sulphuric acid and evaporated in a vacuum to a syrup, which was taken up in 3N-sulphuric acid (12 c.c.) and ethyl acetate (50 c.c.). The layers were separated and the water extracted four times more with ethyl acetate. Evaporation of the combined dried ethyl acetate extracts (350 c.c.) afforded a pale yellow oil (5·15 g.). An eleven-transfer distribution of this between ethyl acetate and M-phosphate buffer (7 mols. of KH_2PO_4 to 3 mols. of K_2HPO_4) separated the product (2·5 g., 7 mmol.; K 1·69) from carbobenzyloxyglycine (0·7 g., 3 mmol.; K 0·273). The material, m. p. 161° (2·35 g.), from tubes 5—10 was combined and recrystallised from ethyl acetate in colourless needles, m. p. 162° (Found, in material dried at 100°: C, 64·2; H, 5·7; N, 8·1. Calc. for $C_{19}H_{20}O_5N_2$: C, 64·0; H, 5·7; N, 7·9%). Neurath, Elkins, and Kaufmann (*J. Biol. Chem.*, 1947, **170**, 221) record m. p. 159·5—160·5° for carbobenzyloxyglycyl-DL-phenylalanine.

Carbobenzyloxyglycyl-L-phenylalanine.—This was prepared in the same way as the DL-compound and crystallised from ethyl acetate–ether in colourless needles, m. p. 127°, $[\alpha]_D^{18}$ +41·5° (±1°) (*c*, 2 in ethanol) (Found, in material dried at 65°: N, 7·9. Calc. for $C_{19}H_{20}O_5N_2$: N, 7·9%). Hofmann and Bergmann (*ibid.*, 1940, **134**, 225) give m. p. 125—126°, $[\alpha]_D^{24}$ +38·5° (*c*, 5 in ethanol) for carbobenzyloxyglycyl-L-phenylalanine.

The dipeptide derivative (1 mmol.) was converted through its potassium salt into the mixed sulphuric anhydride, as in the above preparations. About one-half of the anhydride solution was poured directly into ice-cold 0·2N-sodium hydroxide (10 c.c.) and the dipeptide derivative recovered by acidification, evaporation, and ethyl acetate extraction from dilute sulphuric acid. It crystallised from ether in needles, m. p. 159—160°, $[\alpha]_D^{17}$ +9° (Found, in material dried at 65°: N, 8·2%) and the material in the mother-liquors had $[\alpha]_D^{16}$ +14°. The second half of the anhydride solution was kept at 20° during 15 minutes before being treated in the same way; in this case the recovered materials had $[\alpha]_D^{15}$ +19°.

Carbobenzyloxyglycyl-DL-phenylalanylglycine.—A dimethylformamide solution (0·72 c.c.) of the sulphur trioxide–dimethylformamide complex (1 mmol.) was added to an ice-cooled solution of the trimethylphenylammonium salt (1 mmol.) of carbobenzyloxyglycyl-DL-phenylalanine in dimethylformamide (10 c.c.). After 1 minute this mixture was treated successively with a solution of glycine (0·15 g., 2 mmol.) and phenolphthalein in N-sodium hydroxide (2 c.c.) and water (5 c.c.) and with sufficient 0·5N-alkali to restore and maintain the pink colour. After 10 minutes the solution was neutralised with dilute sulphuric acid, evaporated, and separated between ethyl acetate (20 c.c.) and N-sulphuric acid (5 c.c.). The aqueous layer was extracted four times more with ethyl acetate, and the combined extracts (120 c.c.) were evaporated to an oil (0·59 g.). An eleven-transfer distribution between ethyl acetate and M-phosphate buffer (8 mols. of KH_2PO_4 to 2 mols. of K_2HPO_4) showed the presence of *carbobenzyloxyglycyl-DL-phenylalanylglycine* (0·322 g., 78%; *K* 0·835), but separation was relatively poor. The material from tubes 0—6 was combined (0·283 g.) and twice crystallised from ethyl acetate in colourless plates, m. p. 141—142° (Found, in material dried at 80°: C, 61·4; H, 5·6; N, 10·5. $C_{21}H_{23}O_6N_3$ requires C, 61·0; H, 5·6; N, 10·2%).

Carbobenzyloxyglycyl-L-phenylalanylglycine.—Carbobenzyloxyglycyl-L-phenylalanine was condensed with glycine as in the preceding experiment. The crude product was separated by a twenty-two transfer distribution between ethyl acetate and M-phosphate buffer (7·5 mols. of KH_2PO_4 to 2·5 mols. of K_2HPO_4) into *carbobenzyloxyglycyl-L-phenylalanylglycine* (*K* 0·69) and carbobenzyloxyglycyl-L-phenylalanine (*K* 2·54). The yield of tripeptide derivative was only 52% in this experiment, doubtless owing to the use of an old sample of the sulphur trioxide–dimethylformamide complex. The combined material from tubes 5—10 had $[\alpha]_D^{15}$ −13·3° (±2°) (*c*, 1·2 in ethanol) and crystallised from ethyl acetate–ether, m. p. 155·5—157·5°, $[\alpha]_D^{16}$ −15·2° (±1·5°) (*c*, 1·1 in ethanol) (Found, in material dried at 55°: C, 61·1; H, 5·7; N, 10·5. $C_{21}H_{23}O_6N_3$ requires C, 61·0; H, 5·6; N, 10·2%). The dipeptide derivative recovered from tubes 17—19 had $[\alpha]_D^{15}$ +38·8° (±2°) (*c*, 1 in ethanol).

Carbobenzyloxyglycyl-L-phenylalanine Ethyl Ester.—Sodium carbonate (0·742 g., 7 mmol.) was added to a solution of L-phenylalanine ethyl ester hydrochloride (1·607 g., 7 mmol.; m. p. 148—150°, $[\alpha]_D^{19}$ −7·33°±0·25° [*c*, 3·9 in water]) in ice-cold water (6 c.c.) which was then extracted thrice with cold ether. The combined extracts (15 c.c.) were dried (Na_2SO_4) during 5 minutes and then mixed with triethylamine (1·1 c.c.) before being added to an ice-cooled solution of the sulphuric anhydride prepared in the usual way from the trimethylphenylammonium salt of carbobenzyloxyglycine (1·045 g., 5 mmol.) and a solution of the sulphur trioxide–dimethylformamide complex in dimethylformamide (5·2 c.c., equivalent to 11·6 c.c. of N-sodium hydroxide, but containing only 5 mmol. of sulphur trioxide). After 5 minutes the solution was neutralised with dilute sulphuric acid and evaporated, finally at 2 mm., to an oil, which was partitioned between ethyl acetate (50 c.c.) and 4N-sulphuric acid (20 c.c.). The aqueous layer was five times re-extracted with ethyl acetate, and the combined ethyl acetate solutions (300 c.c.) were washed thrice with a saturated sodium hydrogen carbonate solution (75 c.c. total), from which carbobenzyloxyglycine (0·191 g., 0·915 mmol.) was recovered by acidification and ethyl acetate extraction. The main ethyl acetate solution contained the oily product (1·825 g.); Hofmann and Bergmann (*loc. cit.*) likewise report carbobenzyloxyglycyl-L-phenylalanine ethyl ester as an oil.

Carbobenzyloxyglycyl-L-phenylalanine Hydrazide.—Hydrazine hydrate (0·5 c.c. of 100%) was added to a solution of the ethyl ester (1·39 g.), prepared in the preceding experiment, in ethanol (6 c.c.). The mixture was kept at 18° for 15 hours and then between 50° and 60° for 5 hours. Crystalline material (0·68 g.) separated after dilution with ethanol (3 c.c.), filtration, and addition

of ether (100 c.c.), and a further quantity was obtained from the liquors by a repetition of the hydrazine treatment. The two crops were recrystallised from ethanol {0·93 g., 70%; m. p. 142—144°; $[\alpha]_D^{16\cdot5}$ +6·9° (±2°) (*c*, 2 in 0·5N-hydrochloric acid)} and then twice more, to give the pure *hydrazide*, m. p. 143—144·5° (Found, in material dried at 50°: C, 61·5; H, 5·4; N, 15·4. $C_{19}H_{22}O_4N_4$ requires C, 61·6; H, 6·0; N, 15·1%).

Carbobenzyloxyglycyl-L-phenylalanylglycine by the Azide Method.—Sodium nitrite (0·16 g.) in water (3 c.c.) was added during 15 minutes to a stirred ice-cooled solution of carbobenzyloxyglycyl-L-phenylalanine hydrazide (0·65 g.) in acetic acid (0·7 c.c.) and 0·5N-hydrochloric acid (7 c.c.). The gum which separated was extracted into ice-cold ether (16 c.c.) and washed at 0° with water, twice with saturated sodium hydrogen carbonate solution, and again with water. The ethereal solution was dried (Na_2SO_4) at 0° and added during 20 minutes to glycine ethyl ester (0·26 c.c.) in dry ether (5 c.c.). The mixture was kept at 0° during 20 hours and then for 4 hours at room temperature. Ethyl acetate was added to give a homogeneous solution, which was washed twice with N-sulphuric acid, twice with sodium hydrogen carbonate solution and once with water. Carbobenzyloxyglycyl-L-phenylalanylglycine ethyl ester remained on evaporation, finally at 0·01 mm., as a pale yellow glass (0·492 g., 63%), which was dissolved in ethanol (4 c.c.) and saponified with N-sodium hydroxide (1·4 c.c., 1·25 equivalents) during 45 minutes. After neutralisation with dilute acid and evaporation of the solvent the product was obtained by three extractions with sodium hydrogen carbonate solution (60 c.c.) from ethyl acetate (30 c.c.), followed by washing with ethyl acetate, acidification with dilute acid, and extraction by ethyl acetate (4 × 30 c.c.). The colourless glass (0·433 g., 60%) was twice crystallised from ethyl acetate by addition of ether, yielding carbobenzyloxyglycyl-L-phenylalanylglycine (0·236 g.) of constant m. p. 155·5—157·5°, $[\alpha]_D^{16}$ −15·7° (±1·5°) (mean of five determinations at *c*, 1·2 in ethanol). The m. p. was unchanged by admixture of the sample, m. p. 155·5—157·5°, prepared through the sulphuric anhydride, but was depressed to 140·5—141·5° by only a small quantity of the DL-compound, m. p. 141—142°.

Carbobenzyloxy-L-phenylalanylglycine.—A solution of the trimethylphenylammonium salt (10 mmol.) of carbobenzyloxy-L-phenylalanine in dimethylformamide (15 c.c.) was cooled to 0° and treated first with a dimethylformamide solution (7·6 c.c.) of the sulphur trioxide–dimethylformamide complex (10 mmol.) and then after 1 minute with a solution of glycine (1·13 g., 15 mmol.) and phenolphthalein in N-sodium hydroxide (15 c.c.) and water (6 c.c.) followed by sufficient 0·5N-alkali to restore and maintain the pink colour. By neutralisation, evaporation, and ethyl acetate extraction from acid as in previous experiments an oil (4·50 g.) was obtained, which was distributed in eleven transfers between ethyl acetate and M-phosphate buffer (5 mols. of KH_2PO_4 to 5 mols. of K_2HPO_4). Tube 0 contained more material (0·712 g.) than any other and the weight distribution curve had maxima at tubes 5 (0·231 g.) and 10 (0·327 g.) and minima at tubes 3 (0·142 g.) and 8 (0·167 g.). This is consistent with the presence of four substances: recovered carbobenzyloxy-L-phenylalanine (*K* 6·10; 0·731 g., 2·44 mmol.), the desired product (*K* 0·937; 0·887 g., 2·48 mmol.), and two substances "X" (*K* 0·081; 0·814 g.) and "Y" (*K* 0·00; 0·364 g.). Carbobenzyloxy-L-phenylalanylglycine (0·663 g.) was obtained by recrystallisation of the solid from tubes 4—7 from ethyl acetate–ether and had m. p. 154°, $[\alpha]_D^{17}$ −8° (±1·5°) (*c*, 1·5 in acetic acid) (Found, in material dried at 65°: N, 7·9. Calc. for $C_{19}H_{20}O_5N_2$: N, 7·9%). Behrens, Doherty, and Bergmann (*ibid.*, 1940, **136**, 61) give m. p. 151—152°, $[\alpha]_D^{26}$ −9·6° (*c*, 5 in acetic acid), for this substance. The material in tube 0 did not dissolve completely in benzene; "Y" (0·317 g.; m. p. 70—80°) separated from it on dissolution in dioxan (7 c.c.) and addition of benzene (15 c.c.) and was recrystallised twice in the same way, having m. p. 73—89° (Found: C, 55·5; H, 6·6; N, 8·3%). Its ultra-violet spectrum showed normal benzene absorption with maxima at 233, 258, and 264 mμ ($E_{1\,cm.}^{1\%}$ 3·96, 5·02, and 3·82 respectively), similar too, but less than half as intense as, that of carbobenzyloxy-L-phenylalanylglycine (maxima at 253, 259, and 264 mμ; $E_{1\,cm.}^{1\%}$ 8·71, 10·95, and 8·71 respectively). A second sample of "Y" was obtained from the liquors of the two dioxan–benzene recrystallisations by crystallisation from ethyl acetate–carbon tetrachloride, then having m. p. 82—86° (Found, in material dried at 60°: C, 53·8; H, 5·7; N, 10·1. $C_{12}H_{14}O_5N_2$ requires C, 54·1; H, 5·3; N, 10·5%). The substances were chromatographed on Whatman No. 1 paper in *tert.*-butanol (80 parts by volume), water (16 parts), ammonia (4 parts; *d*, 0·88) and detected as the ammonium salts by the blue colour with ninhydrin (Long, Quayle, and Stedman, *loc. cit.*). Carbobenzyloxy-L-phenylalanine had R_F 0·78, carbobenzyloxy-L-phenylalanylglycine R_F 0·67, and "Y" R_F 0·56. The material in tube 1, which should have been almost pure "X," was freely soluble in benzene and gave a single spot R_F 0·61. The first liquors from crystallisation of "Y" gave a streak between the limits R_F 0·53 and 0·65. Hydrolysis of both "X" and "Y"

by 9N-hydrochloric acid during 15 hours at 110° liberated approximately equivalent amounts of phenylalanine and glycine, detected by paper chromatography.

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