1259. *Amino-acids and Peptides. Part XXIII.*[1] *The Synthesis of Peptides Related to Arginine-vasopressin*

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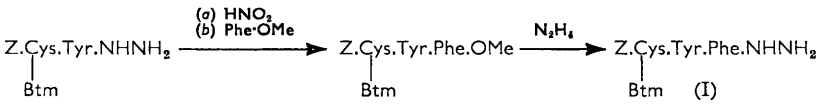
N-Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteinyl-L-tyrosyl-L-phenylalanylhydrazide (I) and *N*-benzyloxycarbonyl-L-asparaginyl-*S*-benzylthiomethyl-L-cysteinyl-L-prolyl-N^{ω}-nitro-L-arginylglycineamide (IX) have been synthesised, the latter by two routes. In the course of the preparation of N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycineamide, revised specific rotations for the corresponding acid and for the methyl and ethyl esters have been established; a by-product obtained during the amination of the methyl ester (V) has been shown to be the substituted hydantoin (III). This work confirms the stability of the *S*-benzylthiomethyl group during the normal operations of peptide synthesis.

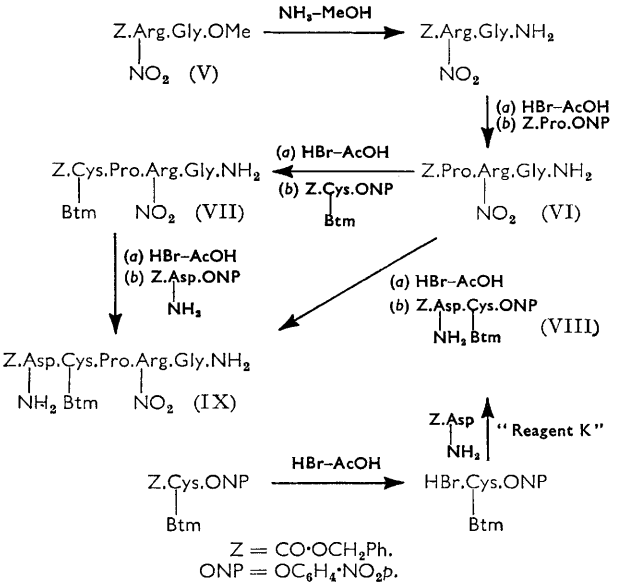
Part XX [2] described the use of the *S*-benzylthiomethyl protecting group in the synthesis of simple peptides of cysteine; this protection may be removed rapidly at room temperature by means of mercuric acetate in 80% formic acid, and is therefore available when the use of sodium in liquid ammonia (required for the removal of *S*-benzyl) is considered undesirable.[3] The usefulness of this procedure has now been investigated further in the synthesis of protected peptides related to arginine-vasopressin,[4] and this Paper describes the synthesis of the tripeptide *N*-benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteinyl-L-tyrosyl-L-phenylalanylhydrazide (I), and of the pentapeptide *N*-benzyloxycarbonyl-L-asparaginyl-*S*-benzylthiomethyl-L-cysteinyl-L-prolyl-N^{ω}-nitro-L-arginyl-glycineamide (IX). These syntheses are summarised in Schemes 1 and 2 respectively, using abbreviations [5] in the



Z = CO·OCH$_2$Ph; Btm = CH$_2$·S·CH$_2$Ph.

Scheme 1

[1] Part XXII, K. B. Walshaw and G. T. Young, *J.*, 1965, 786.
[2] P. J. E. Brownlee, M. E. Cox, B. O. Handford, J. C. Marsden, and G. T. Young, *J.*, 1964, 3832.
[3] *E.g.*, H. Kappeler, in "Peptides," Proc. Fifth European Peptide Symposium, Oxford, 1962, ed. G. T. Young, Pergamon Press, Oxford, 1963, p. 3; St. Guttmann, *ibid.*, p. 41.
[4] V. du Vigneaud, H. C. Lawler, and E. A. Popenoe, *J. Amer. Chem. Soc.*, 1953, **75**, 4880.
[5] Revised Tentative Rules for Abbreviations and Symbols for Chemical Names of Special Interest in Biological Chemistry (Information Bulletin No. 20, I.U.P.A.C.).

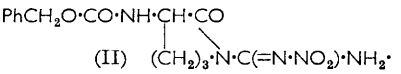


Z = CO·OCH$_2$Ph.
ONP = OC$_6$H$_4$·NO$_2$*p*.
" Reagent K " = 2-ethyl-5-*m*-sulphonatophenylisoxazolium.

Scheme 2

manner recommended in ref. 6; all are L-amino-acids. Certain of the stages have previously been reported briefly.[7]

The reactions outlined in Scheme 2 require some comment. Attempts to couple N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginine with glycineamide by the carbonic [8] or pivalic [9] mixed anhydride procedures or by means of phosphorus oxychloride [10] were unsuccessful and from the last experiment was obtained the lactam (II) [7] which has been encountered by

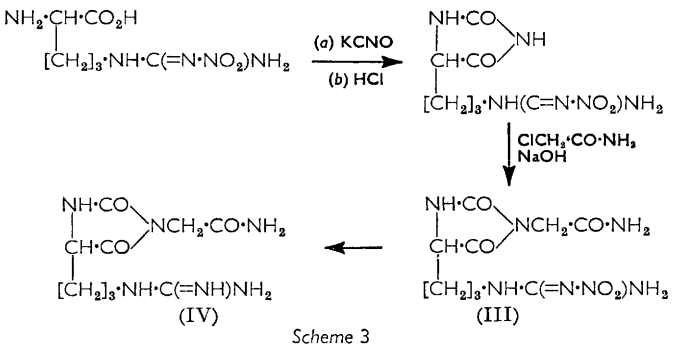


others [11] and which demonstrates the nucleophilicity still retained in the nitroguanidine residue. We therefore proceeded through the known methyl ester (V), but as in the case of the ethyl analogue [12] the specific rotation of this ester (prepared many times by a modification [12] of the carbonic mixed anhydride procedure used by Hofmann, Peckham, and Rheiner [13]) also was low, and hydrolysis of both esters gave N^{α}-benzyloxycarbonyl-N^{ω}-nitroargininylglycine of specific rotation below the literature values. However, hydrogenation of this material in 80% acetic acid gave a nearly quantitative yield of L-arginylglycine which without recrystallisation had a specific rotation close to the generally accepted value. [We have noted that the use of 80% acetic acid (cf. ref. 14) as solvent during this hydrogenation is much to be preferred to the use of methanol, which often gives product which is difficult to crystallise completely.] This crude product was hydrolysed by trypsin;

[6] R. Schwyzer, J. Rudinger, E. Wünsch, and G. T. Young, in " Peptides," Proc. Fifth European Peptide Symposium, Oxford 1962, ed. G. T. Young, Pergamon Press, Oxford, 1963, p. 261.
[7] M. E. Clubb, P. M. Scopes, and G. T. Young, *Chimia* (*Switz.*), 1960, **14**, 373.
[8] R. A. Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874; J. R. Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547; T. Wieland and H. Bernhard, *Annalen*, 1951, **572**, 190.
[9] M. Zaoral, *Coll. Czech. Chem. Comm.*, 1962, **27**, 1273.
[10] T. Wieland and B. Heinke, *Annalen*, 1956, **599**, 70.
[11] M. Bodanszky and J. T. Sheehan, *Chem. and Ind.*, 1960, 1268; R. Paul, G. W. Anderson, and F. M. Callahan, *J. Org. Chem.*, 1961, **26**, 3347.
[12] P. M. Scopes, K. B. Walshaw, M. Welford, and G. T. Young, *J.*, 1965, 782.
[13] K. Hofmann, W. D. Peckham, and A. Rheiner, *J. Amer. Chem. Soc.*, 1956, **78**, 238.
[14] L. Zervas, T. T. Otani, M. Winitz, and J. P. Greenstein, *J. Amer. Chem. Soc.*, 1959, **81**, 2878.

thin-layer chromatography detected no unchanged dipeptide under conditions which clearly detected 5% of D-isomer admixed with L-isomer. It appeared that our derivatives of nitro-L-arginylglycine must be substantially pure, and at our request Dr. K. Hofmann and Dr. E. Schröder kindly had the specific rotation of their samples of N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycine and of the methyl and ethyl esters redetermined; their revised values are close to those which we have found, and we are grateful for permission to quote these new figures in the Experimental section.

The amination of the methyl ester (V) required careful control as prolonged reaction gave the hydantoin (III), the identity of which was indicated by its infrared absorption,



Scheme 3

and proved by hydrogenation to the guanidino-derivative (IV) (isolated as the flavianate), and by the synthesis shown in Scheme **3**; the same product was obtained by the action of ammonia in methanol on the benzyloxycarbonyldipeptideamide, and such reactions have long been known.[15]

The protected tripeptide (VI) has been synthesised previously by another route, but no constants were reported;[16] from this point the synthesis of the pentapeptide (IX) proceeded by two routes: stepwise through the tetrapeptide (VII), and also by junction with the dipeptide unit (VIII). The latter was best prepared by the condensation of benzyloxycarbonyl-L-asparagine with *S*-benzylthiomethyl-L-cysteine *p*-nitrophenyl ester using 2-ethyl-5-*m*-sulphonatophenyl-isoxazolium,[17] which is especially useful in such a situation; t-butoxycarbonyl-L-asparaginyl-*S*-benzylthiomethyl-L-cysteine *p*-nitrophenyl ester has also been prepared analogously. Attempts to couple benzyloxycarbonyl-L-asparaginyl-L-glutamine with *S*-benzylthiomethyl-L-cysteine *p*-nitrophenyl ester in the same way failed because of the insolubility of its triethylamine salt in the preferred solvents, nitromethane and acetonitrile, and the use of dimethylformamide as solvent was unsatisfactory. The reaction of the *p*-nitrophenyl ester (VIII) with L-prolyl-N^{ω}-nitro-L-arginylglycineamide required more vigorous conditions than usual, but the protected pentapeptideamide (IX) had the same constants as that prepared by the stepwise route.

We describe also the preparation of benzyloxycarbonyl-L-asparaginyl-*S*-benzylthiomethyl-L-cysteine methyl ester (by the mixed pivalic anhydride procedure,[9] which again proved its value for coupling from asparagine), and of *S*-benzylthiomethyl-L-cysteinyl-L-proline methyl ester toluene-*p*-sulphonate. The work reported here confirms the stability of the *S*-benzylthiomethyl group during the normal operations of peptide synthesis.

Experimental

Melting points were taken on a Kofler hot-stage apparatus, infrared spectra were recorded on a Perkin-Elmer model 21 spectrophotometer, and optical rotations on an Ericsson automatic

[15] J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, 1942, **145**, 253; C. A. Dekker, S. P. Taylor, and J. S. Fruton, *ibid.*, 1949, **180**, 155.
[16] R. O. Studer and V. du Vigneaud, *J. Amer. Chem. Soc.*, 1960, **82**, 1499.
[17] R. B. Woodward, R. A. Olofson, and H. Mayer, *J. Amer. Chem. Soc.*, 1961, **83**, 1010.

polarimeter using a 20-mm. tube. Solutions in organic solvents were dried over magnesium sulphate; evaporation was normally by rotary evaporator. Paper chromatography was by ascending flow with Whatman No. 4 paper, using "BWA" (butan-1-ol–water–acetic acid; 62 : 26 : 12, freshly mixed). Samples for analysis were dried at 50°/0·3 mm. unless otherwise stated. *N*-Benzyloxycarbonylamino-acids and peptides were detected on chromatograms by ultraviolet irradiation followed by ninhydrin spray;[2] in some cases the purity of such compounds was examined by treating with 2N-hydrogen bromide in acetic acid at room temperature for 90 min., and precipitating with ether the hydrobromide so formed; this was chromatographed on paper as usual. The time required for the removal of the benzyloxycarbonyl group by hydrogen bromide in acetic acid was determined in every case by means of paper chromatography as described in Part XX.[2]

N-*Benzyloxycarbonyl*-S-*benzylthiomethyl*-L-*cysteinyl*-L-*tyrosyl*-L-*phenylalanine Methyl Ester.*—*N*-Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteinyl-L-tyrosylhydrazide[2] (2·84 g.) was dissolved in a mixture of dimethylformamide (DMF) (36 ml.) and hydrochloric acid (1·079N, 14 ml.). The solution was cooled to −10 to −15°, and a solution of sodium nitrite (0·345 g.) in water (6 ml.) was added. The solution was stirred for 5 min., and then a solution of L-phenylalanine methyl ester hydrochloride (1·08 g.) and triethylamine (2·025 ml.) in dimethylformamide (16 ml.) was added. After 1 hr. the cooling bath was removed. Next day, the solvents were removed below 50°, the residue was taken up in ethyl acetate and washed (water, dilute hydrochloric acid, water) and dried. Partial evaporation gave crystalline product (1·56 g., 43%) of m. p. 169—172°, which on recrystallisation from methanol–water (5 : 2) gave *peptide* of m. p. 172—173°, $[\alpha]_D^{19}$ −43° (*c* 1·0 in DMF) (Found: C, 62·7; H, 5·7; N, 5·5; S, 8·5. $C_{38}H_{41}N_3O_7S_2,H_2O$ requires C, 62·2; H, 5·9; N, 5·7; S, 8·7%); the product from the treatment of this compound with hydrogen bromide in acetic acid had R_F 0·88.

Preparation of the azide by the action of butyl nitrite and hydrogen chloride[18] in tetrahydrofuran, followed by coupling in ether–chloroform, gave a high yield (80%) of crude product which, however, in this case contained impurities which were difficult to remove; fractional crystallisation gave material of m. p. 171—172° in 23% yield.

N-*Benzyloxycarbonyl*-S-*benzylthiomethyl*-L-*cysteinyl*-L-*tyrosyl*-L-*phenylalanylhydrazide.*—The above methyl ester (0·715 g.) in methanol (70 ml.) was added dropwise to a solution of hydrazine hydrate (100%, 0·53 ml.) in methanol (30 ml.). After 1 week the crystals which had separated were removed; a little unchanged ester (detected by infrared absorption at 1750 $cm.^{-1}$) was extracted with boiling methanol leaving *hydrazide* (0·50 g., 69%) of m. p. 232·5—235°, $[\alpha]_D^{20}$ −44° (*c* 1·2 in DMF) (Found: C, 62·2; H, 5·9; N, 9·45; S, 9·3. $C_{37}H_{41}N_5O_6S_2$ requires C, 62·1; H, 5·8; N, 9·8; S, 8·95%); the product from the treatment of the compound with hydrogen bromide in acetic acid had R_F 0·91.

N^{α}-*Benzyloxycarbonyl*-N^{ω}-*nitro*-L-*arginylglycine Methyl Ester.*—This was prepared by the modification of the method of Hofmann, Peckham, and Rheiner[13] described in Part XXI,[12] except that the time for coupling was 45 min.; after evaporation of the dimethylformamide the residue was taken up in ethyl acetate, and the solution was then washed and dried. The product crystallised after partial evaporation of the solvent; yield 65%, of ester of m. p. 75—77°; $[\alpha]_D^{21}$ −10·4° (*c* 1·0 in MeOH) {lit.,[13] m. p. 70—73°, $[\alpha]_D^{29}$ −13·8° (*c* 3·6 in MeOH)}; many repetitions by different workers gave similar product (Found after drying at 20°/0·1 mm.: C, 48·3; H, 5·9; N, 20·15. Calc. for $C_{17}H_{24}N_6O_7$: C, 48·1; H, 5·7; N, 19·8%).

Evidence on the Optical Purity of N^{α}-*Benzyloxycarbonyl*-N^{ω}-*nitro*-L-*arginylglycine and its Methyl and Ethyl Esters.*—In Part XXI[12] specific rotations markedly lower than those given in the earlier literature[13,19] were reported for N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycine and its ethyl ester. The specific rotation of the methyl ester (above) is also below the literature value; hydrolysis to the acid by means of 1 equivalent of N-sodium hydroxide with tetrahydrofuran as solvent gave product with the same specific rotation as that of the acid obtained by hydrolysis of our ethyl ester.[12] We believe this N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycine to be optically pure because hydrogenation gave a nearly quantitative yield of L-arginylglycine diacetate having the generally accepted specific rotation and being hydrolysed by trypsin, as described:

(*a*) *Hydrogenation to* L-*arginylglycine.* N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycine

[18] J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, 1961, **26**, 2333.
[19] H. Gibian and E. Schröder, *Annalen*, 1961, **642**, 145.

{1·67 g., $[\alpha]_D^{21}$ −10·2° (*c* 1·0 in MeOH)} was dissolved in 80% acetic acid (25 ml.) and palladium-on-charcoal (10%, 1·5 g.) was added. Hydrogenation was continued for 24 hr. when the catalyst was filtered off and washed with water. The combined filtrates were evaporated to dryness; a little ethanol was added and then evaporated, and this was done three times in all. After the final evaporation the whole product soldified (1·32 g., 94%) and had $[\alpha]_D^{23}$ +39·1° (*c* 1·0 in water) (Found: C,41·3; H, 7·4; N, 20·15. Calc. for $C_8H_{17}N_5O_3,C_4H_8O_4$: C, 41·0; H, 7·2; N, 19·9%) {lit., $[\alpha]_D^{28}$ +38·9° (*c* 5·75 in H_2O); [13] $[\alpha]_D^{24}$ +37·6° (*c* 1 in H_2O); [14] $[\alpha]_D^{22}$ +47·1° (*c* 0·89 in H_2O) [20]}.

(*b*) *Hydrolysis of the* L-*arginylglycine by trypsin.* The arginylglycine diacetate so obtained (25 mg.) was dissolved in 0·033M phosphate buffer of pH 7·2 (1 ml.); a solution of trypsin (B.D.H, crystalline, 2 mg.) in the same buffer (2 ml.) was added, and the solution was kept at 37°. The progress of the hydrolysis was followed by means of thin-layer chromatography (Kieselgel-G) using a mixture of phenol saturated with water (94 vol.) and ammonium hydroxide (*d* 0·880, 1 vol.); in this system arginylglycine had R_F 0·41; arginine, R_F 0·31; glycine, R_F 0·21. After 10 days, chromatography of a sample of the hydrolysate showed no ninhydrin-positive spot of R_F 0·41, whereas samples taken from parallel experiments using DL-arginylglycine diacetate, and a mixture of the L-peptide with 10% of DL-peptide, after 10 days gave easily observed spots in this position. We conclude that our L-arginylglycine diacetate contained less (and, by estimate, considerably less) than 5% of D-isomer.

We believe therefore that our derivatives of nitro-L-arginylglycine are substantially pure, and we give below the most recent values for their specific rotations (the solvent in each case is methanol): N^{α}-*Benzyloxycarbonyl*-N^{ω}-*nitro*-L-*arginylglycine:* $[\alpha]_D^{21}$ −10·2° (*c* 1·0) (this Series, Part XXI) [12]; Dr. E. Schröder (personal communication, quoted in Part XXI [12]): $[\alpha]_D^{23}$ −10·2° (*c* 1); Dr. K. Hofmann (personal communication): $[\alpha]_D^{26}$ −8·7° (*c* 1·15). *Methyl ester:* $[\alpha]_D^{21}$ −10·4° (*c* 1·0) (this Paper); Dr. K. Hofmann (personal communication): $[\alpha]_D^{26}$ −8·7° (*c* 1·15). *Ethyl ester:* $[\alpha]_D^{22}$ −10·3° (*c* 2·1) (Part XXI [12]); Dr. E. Schröder (personal communication quoted in Part XXI [12]): $[\alpha]_D^{20}$ −11·8 and −11·5° ±0·5° (*c* 1).

N^{α}-*Benzyloxycarbonyl*-N^{ω}-*nitro*-L-*arginylglycineamide.*—N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycine methyl ester (5·1 g.) was dissolved in dry methanol (90 ml.) at 0° and ammonia was passed in for 2 hr. After one further hour at 0° and 2 hr. at room temperature, the solvent was removed rapidly below 40° and the residue was recrystallised from methanol, giving the *amide* (3·8 g., 70%) of m. p. 105—107°, $[\alpha]_D^{20}$ −3·6° (*c* 3·5 in MeOH), R_F 0·72 (BWA) (Found after drying at 20°/0·1 mm.: C, 46·8; H, 5·5; N, 23·75. $C_{16}H_{23}N_7O_6$ requires C, 46·9; H, 5·7; N, 24·0%). When the residue was recrystallised from methanol–propan-2-ol (1 : 1), the amide was obtained as a *hydrate*, m. p. 149—151°, $[\alpha]_D^{25}$ −3·3° (*c* 1·0 in MeOH) (Found after drying at 20°/0·1 mm.: C, 44·9; H, 6·1; N, 22·6. $C_{16}H_{23}N_7O_6,H_2O$ requires C, 45·0; H, 5·85; N, 22·95%). Recrystallisation of this material from methanol gave amide of m. p. 105—107°. When this reaction with ammonia was prolonged, *e.g.*, for 24 hr. at room temperature, or when the ester was treated with liquid ammonia in the presence of ethylene glycol for 4 days, the main product was 5-(3-nitroguanidinopropyl)hydantoin-3-acetamide (III) (m. p. 220—222°, unchanged on admixture with authentic material), the identity of which was proved by reduction and by synthesis (see below). The same compound was obtained in 66% yield by the action of ammonia in methanol on N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycineamide for 36 hr. The best conditions (described above) for the conversion of the ester into amide were found by following the course of the reaction by paper chromatography of samples which had been evaporated to dryness and treated with hydrogen bromide in acetic acid. In some preparations the crude product contained some substituted hydantoin, detected by infrared absorption at 1782 $cm.^{-1}$ (in liq. paraffin), but recrystallisation gave pure amide.

Hydrogenation of By-product from Prolonged Amination of N^{α}-*Benzyloxycarbonyl*-N^{ω}-*nitro*-L-*arginylglycine Methyl Ester.*—The by-product (0·1 g.) was hydrogenated in dilute acetic acid in the presence of palladium-on-charcoal (10%; 0·05 g.). The catalyst was filtered off, the filtrate was concentrated to 4 ml., and a solution of flavianic acid (0·2 g.) in water (1 ml.) was added. The crystalline product was recrystallised twice by dissolving in warm 2N-ammonium hydroxide and adding an equivalent volume of 2N-sulphuric acid, giving 5-(3-*guanidinopropyl*)-*hydantoin*-3-*acetamide monoflavianate monohydrate*, m. p. 190—191° (Found: C, 39·2; H, 4·0; N, 19·3; S, 5·5. $C_{19}H_{22}N_8O_{11}S,H_2O$ requires C, 38·9; H, 4·1; N, 19·1; S, 5·4%).

5-(3-*Nitroguanidinopropyl*)*hydantoin.*—Nitro-L-arginine (2·0 g.) was suspended in boiling

[20] C. Berse and L. Piche, *J. Org. Chem.*, 1956, **21**, 808.

water (10 ml.) and potassium cyanate (0·9 g.) was added. After 5 min. the solution was clear, 20% hydrochloric acid (10 ml.) was added, and the solution was boiled for 30 min. The product which separated on cooling was filtered off and recrystallised from 50% aqueous acetic acid, giving the substituted *hydantoin* of m. p. 254—255°, $\nu_{max.}$ 1777 cm.$^{-1}$ (in liq. paraffin) (Found: C, 34·6; H, 5·0; N, 34·5. $C_7H_{12}N_6O_4$ requires C, 34·4; H, 4·9; N, 34·4%).

5-(3-*Nitroguanidinopropyl*)*hydantoin*-3-*acetamide*.—5-(3-Nitroguanidinopropyl)hydantoin (0·98 g.) was dissolved in a hot solution of sodium hydroxide (0·16 g.) in water (10 ml.). Chloroacetamide (0·75 g.) was added, and the solution was boiled for 30 min. After 1 hr. at 0°, the solid product was filtered off (0·75 g., 63%) and recrystallised from boiling water, giving substituted *hydantoin* of m. p. 219—221°, $\nu_{max.}$ 1782 cm.$^{-1}$ (in liq. paraffin) (Found: C, 35·8; H, 5·2; N, 32·4. $C_9H_{15}N_7O_8$ requires C, 35·9; H, 5·0; N, 32·6%).

N^{α}-*Benzyloxycarbonyl*-L-*prolyl*-N^{ω}-*nitro*-L-*arginylglycineamide*.—N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginylglycineamide (2·1 g.) was dissolved in acetic acid (6 ml.) and treated with hydrogen bromide in acetic acid (7·3N, 4 ml.) and left at room temperature for 1 hr. The dihydrobromide of N^{ω}-nitro-L-arginylglycineamide was precipitated with dry ether, quickly filtered and washed with dry ether. The dihydrobromide was taken up in the minimum quantity of dimethylformamide, and dry triethylamine (1·38 ml.) was added, followed by benzyloxycarbonyl-L-proline *p*-nitrophenyl ester [21] (1·85 g.). After 1 hr. triethylamine (0·69 ml.) was added, and the pH was maintained at 7 by addition of triethylamine as necessary. After 24 hours' stirring the ninhydrin reaction was negative; the dimethylformamide was removed under vacuum below 40° and the residual gum was triturated with dry ether and then ethyl acetate. The hygroscopic solid was dissolved in pyridine, the insoluble triethylamine hydrobromide was filtered off, and the filtrate was diluted with ethyl acetate. The precipitate was collected and washed with chloroform giving product (1·92 g., 71%) of m. p. 94—97°. After recrystallisation from propan-2-ol the protected tripeptideamide monohydrate had m. p. 102—104°, $[\alpha]_D^{19}$ −44·4° (*c* 1·0 in MeOH), R_F 0·72 (BWA) (Found: C, 48·2; H, 6·7; N, 21·2. Calc. for $C_{21}H_{30}N_8O_7,H_2O$: C, 48·1; H, 6·15; N, 21·4%). Studer and du Vigneaud [16] record no m. p. or specific rotation, but characterised their product by conversion into L-prolyl-L-arginylglycineamide diflavianate.

N-*Benzyloxycarbonyl*-S-*benzylthiomethyl*-L-*cysteine* p-*Nitrophenyl Ester*.—To *N*-benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine [2] (3·91 g.) and *p*-nitrophenol (1·67 g.) in ethyl acetate at 0° was added dicyclohexylcarbodi-imide (2·06 g.), and the solution was stirred at 0° for 1 hr. and then at 25° for 2 hr. After the addition of a few drops of acetic acid, the solution was filtered, the filtrate was evaporated to dryness and the residue was washed with ether and recrystallised from ethanol, giving 4·29 g. (84%) of product of m. p. 106—107·5°. Recrystallisation from ethanol gave material of m. p. 107·5—108°, $[\alpha]_D^{22}$ −44° (*c* 1·8 in DMF) (Found C, 58·8; H, 4·8; N, 5·6; S,12·35. Calc. for $C_{25}H_{24}N_2O_6S_2$: C, 58·6; H, 4·7; N, 5·5; S, 12·5%). Hiskey and Tucker [22] obtained a 46% yield in this preparation, and give m. p. 105·5—106° but do not report the optical rotation.

S-*Benzylthiomethyl*-L-*cysteine* p-*Nitrophenyl Ester Hydrobromide*.—*N*-Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine *p*-nitrophenyl ester (5·12 g.) was dissolved in warm acetic acid (9 ml.) and diethyl phosphite (6·9 g.); hydrogen bromide in acetic acid (3·7N, 11 ml.) was added and the mixture was heated at 70° for 2 min. The crystalline hydrobromide was precipitated by ether and washed repeatedly with ether (yield, 3·77 g., 81%); recrystallisation from ethanol–ether gave *hydrobromide* of m. p. 141—143°, $[\alpha]_D^{23}$ −13° (*c* 1·6 in DMF) (Found: C, 44·5; H, 4·2; N, 6·1; S, 14·1. $C_{17}H_{19}BrN_2O_4S_2$ requires C, 44·4; H, 4·2; N, 6·1; S, 14·0%).

N-*Benzyloxycarbonyl*-L-*asparaginyl*-S-*benzylthiomethyl*-L-*cysteine* p-*Nitrophenyl Ester*.—2-Ethyl-5-*m*-sulphonatophenylisoxazolium [7] (0·506 g.) was stirred in nitromethane (7 ml.) at 15—20° and a solution of benzyloxycarbonyl-L-asparagine (0·532 g.) and triethylamine (0·27 ml.) in nitromethane (10 ml.) was added. After 10 min., *S*-benzylthiomethyl-L-cysteine *p*-nitrophenyl ester hydrobromide (0·918 g.) and triethylamine (0·27 ml.) were added, and the solution was stirred overnight at 25°. The solvent was evaporated and the solid residue was washed (0·5N-sodium hydrogen carbonate, N-hydrochloric acid, water) and dried in a vacuum desiccator (0·90 g., 72%). It was washed with ethanol and then ether, and recrystallised from aqueous acetic acid, giving *protected dipeptide ester* of m. p. 171—172·5°, $[\alpha]_D^{20}$ −51° (*c* 1·4 in

[21] M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, 1959, **81**, 5688.
[22] R. G. Hiskey and W. P. Tucker, *J. Amer. Chem. Soc.*, 1962, **84**, 4789.

DMF); R_F 0·60 (after treatment with hydrogen bromide in acetic acid) (Found: C, 55·4; H, 5·05; N, 8·9; S, 10·7. $C_{29}H_{30}N_4O_8S_2$ requires C, 55·6; H, 4·8; N, 8·9; S, 10·2%).

N-Benzyloxycarbonyl-S-benzylthiomethyl-L-cysteinyl-L-prolyl-N$^{\omega}$-nitro-L-arginylglycineamide.—To *N*-benzyloxycarbonyl-L-prolyl-*N*$^{\omega}$-nitro-L-arginylglycineamide (1·94 g.) in glacial acetic acid (6 ml.) was added hydrogen bromide in acetic acid (7·3N; 4 ml.). After 1 hr. at room temperature, the hydrobromide of the tripeptideamide was precipitated by dry ether, quickly filtered off, washed with dry ether, and immediately dissolved in the minimum volume of dimethylformamide containing triethylamine (1·1 ml.); more triethylamine (0·5 ml.) was added to bring to pH 7. *N*-Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine *p*-nitrophenyl ester (1·8 g.) was added, and the solution was stirred at room temperature; the pH was maintained at 7 by the addition of a few drops of triethylamine as required. After 24 hr., the solution gave a negative ninhydrin reaction, and it was then poured on to crushed ice, and the gummy product was separated by decantation and taken up in chloroform. The solution was washed (N-sodium hydrogen carbonate, water, N-hydrochloric acid, water) and dried (Na_2SO_4). The filtered solution was diluted with di-isopropyl ether, giving protected tetrapeptideamide (0·92 g., 34% overall) which was purified by precipitation from the same solvents, giving *amide* of m. p. 92—94°, $[\alpha]_D^{22}$ −43·3° (*c* 0·8 in DMF) (Found after drying at 20° and 0·1 mm.: C, 51·1; H, 6·0; N, 17·2; S, 8·6. $C_{32}H_{43}N_9O_8S_2$ requires C, 51·5; H, 5·8; N, 16·9; S, 8·6%).

Benzyloxycarbonyl-L-asparaginyl-S-benzylthiomethyl-L-cysteinyl-L-prolyl-N$^{\omega}$-nitro-L-arginylglycineamide.—Route A. To benzyloxycarbonyl-L-prolyl-*N*$^{\omega}$-nitro-L-arginylglycineamide (1·01 g.) in acetic acid (2·25 ml.) was added hydrogen bromide in acetic acid (4·5N, 1·8 ml.) and the solution was heated at 60° until carbon dioxide evolution ceased (2—3 min.). The hydrobromide was precipitated and washed repeatedly with ether, and precipitated from methanol by ether (yield, 80%); R_F 0·29. It was dissolved in water, and Amberlite IRA-400 (HCO_3^-) resin was added in portions until bromide had been removed. The resin was filtered off and the filtrate was freeze-dried, giving the free tripeptideamide (80%) as a white powder of R_F 0·29 (single spot).

Benzyloxycarbonyl-L-asparaginyl-*S*-benzylthiomethyl-L-cysteine *p*-nitrophenyl ester (0·266 g.) was dissolved in the minimum volume of dimethylformamide, L-prolyl-*N*$^{\omega}$-nitroarginylglycineamide (0·156 g.) was added, and the solution was heated at 70° for 6 hr. The solvent was removed; the residue was extracted with ether to remove *p*-nitrophenol and was taken up in warm methanol; the solution was filtered and addition of ether to the filtrate precipitated the protected pentapeptideamide (0·25 g., 69%). Reprecipitation from methanol by water gave *amide hydrate* of m. p. 189—191°, $[\alpha]_D^{22}$ −51° (*c* 1·0 in DMF), R_F 0·80, (Found after drying at 20°/0·1 mm.: C, 49·45; H, 5·9; N, 17·0; S, 7·7. $C_{36}H_{49}N_{11}O_{10}S_2,H_2O$ requires C, 49·2; H, 5·8; N, 17·55; S, 7·3%).

Route B. To a solution of benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteinyl-L-prolyl-*N*$^{\omega}$-nitro-L-arginylglycineamide (0·2 g.) and ethyl methyl sulphide (0·24 ml.) in acetic acid (0·8 ml.) was added hydrogen bromide in acetic acid (7·3N, 0·28 ml.). After 30 min. at room temperature, the peptideamide hydrobromide was precipitated with dry ether, filtered quickly, and washed with dry ether. It was immediately dissolved in the minimum volume of dimethylformamide, and triethylamine (0·1 ml.) and benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester [21] (0·09 g.) were added. The solution was stirred at room temperature; after 1 hr. a second addition of benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester (0·09 g.) was made; the pH was maintained at 7 by the addition of a few drops of triethylamine as required. After 24 hr. the solution gave a negative ninhydrin reaction, and the mixture was poured on to crushed ice; the precipitate was collected and drained, and washed successively with hot chloroform and ethyl acetate, giving product (0·092 g., 32% overall) of m. p. 188—190°, $[\alpha]_D^{22}$ −51·9° (*c* 1·0 in DMF).

t-Butoxycarbonyl-L-asparaginyl-S-benzylthiomethyl-L-cysteine p-Nitrophenyl Ester.—2-Ethyl-5-*m*-sulphonatophenylisoxazolium [17] (0·506 g.) was suspended in nitromethane (7 ml.) at 18° and a solution of t-butoxycarbonyl-L-asparagine [23] (0·464 g.) and triethylamine (0·27 ml.) in nitromethane (10 ml.) was added with stirring. After 10 min. the solution cleared, and *S*-benzylthiomethyl-L-cysteine *p*-nitrophenyl ester hydrobromide (0·918 g.) and triethylamine (0·27 ml.) were then added. By next day a gelatinous precipitate had separated; the solvent was evaporated and the residue was washed (0·5N-sodium hydrogen carbonate, ice-cold N-hydrochloric acid, water) and dried in a vacuum desiccator, giving the crude ester (0·70 g., 60%)

[23] E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, 1963, **46**, 1637.

as a pale yellow solid which was recrystallised with difficulty (gels were readily formed) from dry acetone to give colourless needles of *ester*, m. p. 175—176°, $[\alpha]_D^{25}$ −50·5° (*c* 1·0 in DMF) (Found: C, 52·8; H, 5·6; N, 9·7; S, 11·0. $C_{26}H_{32}N_4O_8S_2$ requires C, 52·7; H, 5·4; N, 9·5; S, 10·8%).

Benzyloxycarbonyl-L-*asparaginyl*-S-*benzylthiomethyl*-L-*cysteine Methyl Ester*.—Benzyloxycarbonyl-L-asparagine (1·3 g.) and triethylamine (0·7 ml.) were dissolved in dimethylformamide and the solution was cooled to −10°. Pivaloyl chloride (0·6 ml.) was added, followed after 5 min. by a solution of *S*-benzylthiomethyl-L-cysteine methyl ester hydrochloride [2] (1·54 g.) and triethylamine (0·7 ml.) in dimethylformamide (10 ml.). The mixture was shaken and a further 0·7 ml. of triethylamine was added. The cooling bath was removed, and after 48 hr. water (125 ml.) was added. The solid was collected, washed, and dried (1·62 g., 62%). Recrystallisation from methanol–water gave *ester* of m. p. 149°, $[\alpha]_D^{22}$ −31° (*c* 2·0 in DMF) (Found: C, 55·3; H, 5·4; N, 8·0; S, 12·3. $C_{24}H_{29}N_3O_6S_2$ requires C, 55·5; H, 5·6; N, 8·1; S, 12·3%).

S-*Benzylthiomethyl*-L-*cysteinyl*-L-*proline Methyl Ester Toluene*-p-*sulphonate*.—Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine dicyclohexylammonium salt [2] (5·7 g.), L-proline methyl ester toluene-*p*-sulphonate [24] (3·03 g.) and dicyclohexylcarbodi-imide (2·25 g.) were dissolved in dichloromethane (25 ml.) with stirring. After 3 hr. the solution was filtered, the filtrate was evaporated to dryness, and the residue was extracted into ethyl acetate. The solution was washed, dried, and evaporated. The residual syrup (4·6 g., 93%) was dissolved in acetic acid (16 ml.) and hydrogen bromide in acetic acid (5N, 4·1 ml.) was added. After 1 hr., ether was added, the hygroscopic hydrobromide (80%) was collected and dissolved in chloroform containing one equivalent of triethylamine. The solvent was removed and the residue was extracted into ethyl acetate; to this solution was added an excess of anhydrous toluene-*p*-sulphonic acid. Addition of ether precipitated the *dipeptide ester toluene*-p-*sulphonate* (75% yield from the hydrobromide), which was recrystallised from a mixture of methanol and di-isopropyl ether, when it had m. p. 123—124°, $[\alpha]_D^{22}$ −87° (*c* 1·9 in MeOH), R_F 0·82 (Found: C, 53·1; H, 5·9; N, 5·3; S, 18·3. $C_{24}H_{32}N_2O_6S_2$ requires C, 53·3; H, 6·0; N, 5·2; S, 17·8%).

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[24] J. M. Theobald, M. W. Williams, and G. T. Young, *J.*, 1963, 1927.