Amino-acids and Peptides. Part XXIX.[1] The Use of *S*-Benzylthiomethyl-L-cysteine in Peptide Synthesis: Synthesis of Glutathione and Homoglutathione

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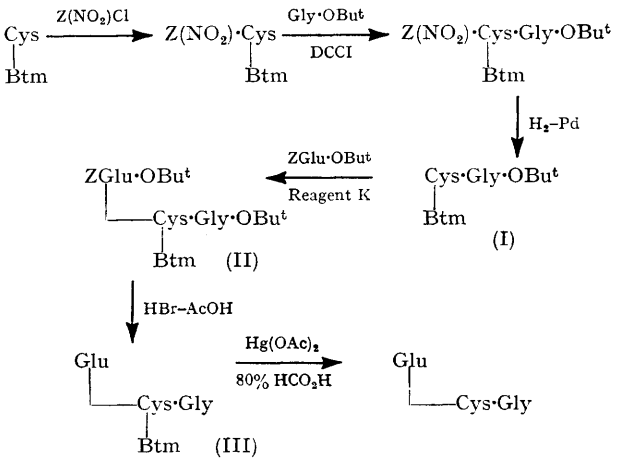
The usefulness of benzylthiomethyl as an *S*-protecting group has been further confirmed by the synthesis of glutathione and 'homoglutathione' (γ-L-glutamyl-L-cysteinyl-β-alanine). The benzylthiomethyl group was completely stable during the syntheses, and in the final stage was removed cleanly under the standard conditions to give quantitative or near-quantitative yields of glutathione and homoglutathione, which were analytically pure, and chromatographically pure except for traces of the corresponding disulphides, without the necessity for the usual purification by way of mercurous and cuprous salts.

THERE is now much evidence that the removal of the *S*-benzyl group from peptides containing *S*-benzylcysteine by means of sodium in liquid ammonia may be accompanied by side reactions, including fission of the peptide chain [2] and desulphuration.[3] In addition, the unambiguous synthesis of peptides containing more than one disulphide bridge requires a variety of *S*-protecting groups; for these reasons new and improved methods of thiol protection are still being sought.[4] We described earlier [5] the preparation of *S*-benzylthiomethyl-L-cysteine and its use in the synthesis of simple peptides, and we have continued our examination of this method of protection by the synthesis of glutathione and 'homoglutathione' (γ-L-glutamyl-L-cysteinyl-β-alanine).

Jeschkeit, Losse, and Knopf [6] list ten syntheses from which authentic glutathione has been isolated. To these must be added five subsequent syntheses, one using cystine [7] and the others using for *S*-protection benzyl,[8] benzyloxycarbonyl,[9] and *N*-ethylcarbamoyl [10] groups.

Berse, Boucher, and Piché [11] showed earlier that the *N*-protecting *p*-nitrobenzyloxycarbonyl group could be removed from di-*N*-*p*-nitrobenzyloxycarbonyl-L-cystine by catalytic hydrogenation; preliminary experiments [12] showed that this procedure is feasible also with derivatives of *S*-benzylthiomethyl-L-cysteine. We used this route (Scheme 1) for the preparation of *S*-benzylthiomethyl-L-cysteinylglycine t-butyl ester (I), but in the hydrogenation step coloured contaminants were difficult to remove, and the dipeptide ester could only be purified by way of its di-*p*-toluoyl-D-tartrate with loss of material. α-t-Butyl *N*-benzyloxycarbonyl-L-glutamate [13,14] (obtained crystalline) was condensed with the dipeptide ester (I) by means of 3-(2-ethyl-5-isoxazolio)-benzenesulphonate (Reagent K); [15] the protected tripeptide (II) was crystalline, but the yield was low. An alternative route (Scheme 2), using the racemisation-free coupling of acylpeptide piperidino-esters,[16] and similar to that used by Weygand and his co-workers [17] for the synthesis of many tripeptides, was more satisfactory. *S*-Benzylthiomethyl-L-cysteine was converted by means of phosgene into the (new) *N*-carboxy-anhydride (IV) this reacted with 1-hydroxypiperidine to give crystalline *S*-benzylthiomethyl-L-cysteine piperidino-ester hydrochloride (V), which was condensed with α-t-butyl *N*-benzyloxycarbonyl-L-glutamate by way of the carbonic



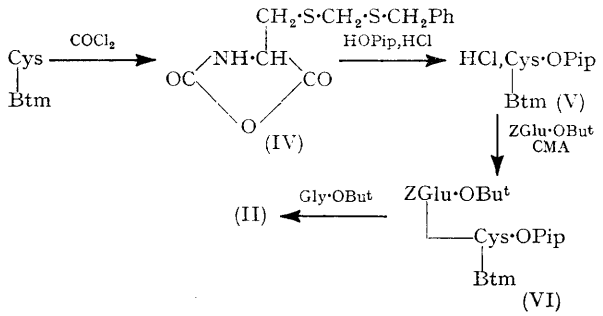
$Z(NO_2)$ = *p*-$NO_2{\cdot}C_6H_4{\cdot}CH_2{\cdot}O{\cdot}CO$; DCCI = $C_6H_{11}{\cdot}N{:}C{:}N{\cdot}C_6H_{11}$
Btm = $PhCH_2{\cdot}S{\cdot}CH_2$

SCHEME 1

Abbreviations follow the rules in 'Abbreviated Designation of Amino-Acid Derivatives and Polypeptides' (Information Bulletin No. 25, I.U.P.A.C.).

[1] Part XXVIII, J. H. Jones and G. T. Young, *J. Chem. Soc.* (*C*), 1968, 436.
[2] E.g., H. Kappeler in 'Peptides: Proc. 5th European Peptide Symposium, Oxford, 1962,' ed. G. T. Young, Pergamon, Oxford, 1963, p. 3; St. Guttmann, *ibid.*, p. 41; W. F. Berisek and R. D. Cole, *Biochem. Biophys. Res. Comm.*, 1965, **20**, 655.
[3] P. G. Katsoyannis, *Amer. J. Medicine*, 1966, **40**, 652.
[4] For a review, see E. Schröder and K. Lübke, 'The Peptides,' (trans. E. Gross), Vol. 1, Academic Press, New York, 1965.
[5] P. J. E. Brownlee, M. E. Cox, B. O. Handford, J. C. Marsden, and G. T. Young, *J. Chem. Soc.*, 1964, 3832.
[6] H. Jeschkeit, G. Losse, and D. Knopf, *Die Pharmazie*, 1963, **18**, 658.
[7] S. Goldschmidt, W. Lautenschlager, B. Kolb, and G. Zumach, *Chem. Ber.*, 1964, **97**, 2434.
[8] P. Baudet and I. Borecka, *Ann. Chim.* (*Italy*), 1963, **53**, 53.
[9] M. Sokolovsky, M. Wilchek, and A. Patchornik, *J. Amer. Chem. Soc.*, 1964, **86**, 1202; M. Fridkin, A. Patchornik, and E. Katchalski, *ibid.*, 1966, **88**, 3164.
[10] St. Guttmann, *Helv. Chim. Acta.*, 1966, **49**, 83.
[11] C. Berse, R. Boucher, and L. Piché, *J. Org. Chem.*, 1957, **22**, 805; *Canad. J. Chem.*, 1959, **37**, 1733.
[12] Unpublished work with D. M. Brunwin.
[13] E. Taschner, C. Wazielewski, T. Sokolowska, and J. F. Biernat, *Annalen*, 1961, **646**, 127.
[14] E. Klieger and H. Gibian, *Annalen*, 1962, **655**, 195.
[15] R. B. Woodward, R. A. Olofson, and H. Mayer, *J. Amer. Chem. Soc.*, 1961, **83**, 1010.
[16] B. O. Handford, J. H. Jones, G. T. Young, and T. F. N. Johnson, *J. Chem. Soc.*, 1965, 6814.
[17] F. Weygand, W. König, E. Nintz, D. Hoffmann, P. Huber, N. M. Khan, and W. Prinz, *Z. Naturforsch.*, 1966, **21***b*, 325.

mixed anhydride. In this way a racemisation-free coupling of an acyl dipeptide becomes possible;[16] condensation of the piperidino-ester (VI) with glycine t-butyl ester was slow but gave a good yield (58%



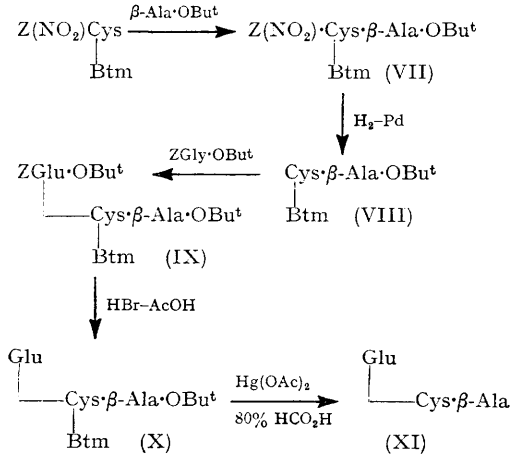
Pip = piperidino; CMA = carbonic mixed anhydride method

SCHEME 2

after recrystallisation) of pure protected glutathione (II). The benzyloxycarbonyl and t-butyl groups were removed smoothly under the standard conditions,[5] with hydrogen bromide in acetic acid (in the presence of methyl ethyl sulphide, to prevent *S*-benzylation in the cysteine side-chain). Paper chromatography detected only a trace of slow-running impurity in the crude product, thus confirming the stability of the benzylthiomethyl group during this step. *S*-Benzylthiomethylglutathione (III) was obtained analytically and chromatographically pure in 65% yield.

The benzylthiomethyl group was removed with mercuric acetate in 80% formic acid at room temperature for 30 min., under the conditions described earlier[5] to give glutathione. The whole product (89·5%) was analytically pure and chromatographically pure except for a faint spot believed to be due to the corresponding disulphide, normally found in solutions of such thiols; the usual purification by way of the mercurous and cuprous salts was not required. No unchanged *S*-benzylthiomethylglutathione, and no pyroglutamic acid (a common degradation product of glutamyl peptides) was detected.

Homoglutathione (XI) was isolated from Mung bean (*Phaseolus aureus*) seedlings by Carnegie,[18a] who elucidated the structure.[18b] A synthesis (using *S*-benzylcysteine) has been reported by Neish and Rylett,[19] but the tripeptide obtained by way of the cuprous thiolate was considered to contain some α-glutamyl isomer. Our synthesis (Scheme 3) is analogous to our first route to glutathione. β-Alanine t-butyl ester (a new compound) was prepared in the usual way [20] by way of its benzyloxycarbonyl derivative. Hydrogenation of the *p*-nitrobenzyloxycarbonyl derivative (VII) was again troublesome; *S*-benzylthiomethyl-L-cysteinyl-β-alanine t-butyl ester (VIII) was purified with difficulty by way of its di-*p*-toluoyl-D-tartrate. The removal of the benzyloxycarbonyl and t-butyl groups by the action of hydrogen bromide in acetic acid on the protected homoglutathione (IX) proceeded smoothly, with negligible decomposition of the benzylthiomethyl group. In this case again, the removal of the benzylthiomethyl group by the standard procedure was complete and quantitative; the freeze-dried product was analytically and



SCHEME 3

chromatographically pure (except for a very faint spot at the origin, corresponding in position and reactions to the disulphide).

Our experience in these syntheses confirms the value of the benzylthiomethyl *S*-protecting group; the intermediates were in general satisfactory to handle, and the removal was effected, without detectable side-reaction, in quantitative or near-quantitative yield.

EXPERIMENTAL

Melting points were determined with a Kofler hot-stage apparatus, and optical rotations with a Perkin-Elmer **141** automatic polarimeter (1 dm. cell). R_F Values refer, unless otherwise stated, to t.l.c. on Kieselgel G (unbaked), in the solvent denoted in brackets: (1) n-butanol–pyridine–water (2 : 1 : 2 v/v; upper layer after equilibration); (2) n-butanol–acetic acid–water (4 : 1 : 1; at equilibrium); (3) chloroform–methanol (95 : 5; R_F values sensitive to small changes in composition); (4) n-butanol–acetic acid–water (4 : 1 : 5; upper layer after equilibration); (5) n-butanol–acetic acid–water (70 : 10 : 20; at equilibrium); (6) phenol–water (4 : 1 *w/v*). Paper chromatograms were run on Whatman no. 1 paper. All thin-layer plates were developed with ninhydrin solution, then dried thoroughly, and exposed to chlorine for 15—20 sec.; they were then placed in a current of air for 1—3 min. and finally sprayed with a mixture (1 : 1 v/v) of 1% starch solution and 1% potassium iodide solution.[21] Paper chromatograms of thiols and disulphides were also developed with sodium nitroprusside and with sodium nitroprusside–sodium cyanide reagent.[22] Evaporation was performed with a rotary evaporator (usually at room temperature) and solutions in organic solvents were dried over magnesium sulphate.

[18] P. R. Carnegie, *Biochem. J.*, 1963, **89**, (*a*) p. 459, (*b*) p. 471.
[19] W. J. P. Neish and A. Rylett, *Tetrahedron*, 1963, **19**, 2031.
[20] G. W. Anderson and F. M. Callahan, *J. Amer. Chem. Soc.*, 1960, **82**, 3359.
[21] H. N. Rydon and P. G. W. Smith, *Nature*, 1952, **169**, 922.
[22] G. Toennies and J. J. Kolb, *Analyt. Chem.*, 1951, **23**, 823.

S-Benzylthiomethyl-N-p-nitrobenzyloxycarbonyl-L-cysteine.—*S*-Benzylthiomethyl-L-cysteine [5] (12·87 g., 0·05 mole) was dissolved in N-sodium hydroxide (125 ml.) and the solution was cooled in an ice-bath. A solution of *p*-nitrobenzyl chloroformate (10·78 g., 0·05 mole) in ether (250 ml.) and N-sodium hydroxide (125 ml.) were added simultaneously during 90 min., with vigorous stirring, which was continued for a further 45 min. with cooling for 1 hr. at room temperature. Water (400 ml.) was added, and the ether layer was separated; the aqueous layer was then washed with ether (250 ml.), cooled in an ice-bath, and acidified (Congo Red) with 6N-hydrochloric acid. The oil so obtained was extracted into ethyl acetate (4 × 300 ml.), and the solution was washed with aqueous sodium acetate (10%; 2 × 50 ml.) and brine (2 × 100 ml.) and dried. Evaporation left a plae yiellow oil which solidified after trituration with light petroleum. The crude product (20 g., 91%) was crystallised from warm ethyl acetate by the gradual addition of light petroleum, to give the *acid* (12·6 g., 58%), m.p. 87—89°, $[\alpha]_D^{20}$ −43·6° (*c* 0·954 in EtOAc), R_F (1) 0·80, R_F (2) 0·88 (Found: C, 51·7; H, 4·75; N, 6·35; S, 14·4. $C_{19}H_{20}N_2O_6S_2$ requires C, 52·3; H, 4·6; N, 6·4; S, 14·7%). The *cyclohexylammonium salt*, prepared in and recrystallised from ethyl acetate, had m.p. 117·5—118·5° (Found: C, 56·5; H, 6·4; N, 7·3; S, 11·85. $C_{25}H_{33}N_3O_6S_2$ requires C, 56·0; H, 6·2; N, 7·8; S, 12·0%).

S-Benzylthiomethyl-N-p-nitrobenzyloxycarbonyl-L-cysteinylglycine t-Butyl Ester.—A solution of glycine t-butyl ester (4·6 g., 0·035 mole) in ethyl acetate (20 ml.) was added to a stirred solution of *S*-benzylthiomethyl-*N*-*p*-nitrobenzyloxycarbonyl-L-cysteine (15·2 g., 0·035 mole) in a mixture of ethyl acetate (100 ml.) and dimethylformamide (10 ml.) at 0°. Dicyclohexylcarbodi-imide (8·6 g., 0·035 mole) in ethyl acetate (20 ml.) was added; after 3 hr. the temperature was allowed to rise. Next day acetic acid (5 ml.) was added, and after 30 min. the solution was filtered, the precipitate was washed with ethyl acetate, and the combined filtrates were evaporated. The yellow syrup was dissolved in ethyl acetate, and the solution was filtered and washed with N-hydrochloric acid (2 × 25 ml.), N-sodium hydrogen carbonate (4 × 40 ml.), water (2 × 40 ml.), and brine (3 × 40 ml.). Evaporation of the dried solution left a pale yellow gum which was crystallised from ether (80 ml.); the crystals were washed with light petroleum, to give crude product (15·2 g., 79·5%), m.p. 95—99°. Recrystallisation from ether gave the *protected dipeptide* (12·8 g., 67%), m.p. 100—101°. A sample crystallised again from ether had m.p. 98—99°, $[\alpha]_D^{20}$ −41·5° (*c* 1·1 in EtOAc), $[\alpha]_D^{20}$ −51·8° (*c* 0·99 in $HCO{\cdot}NMe_2$) (Found: C, 54·3; H, 5·9; N, 7·8; S, 12·0. $C_{25}H_{31}N_3O_7S_2$ requires C, 54·6; H, 5·7; N, 7·7; S, 11·7%).

S-Benzylthiomethyl-L-cysteinylglycine t-Butyl Ester Di-p-Toluoyl-D-tartrate.— *S*-Benzylthiomethyl-*N*-*p*-nitrobenzyloxycarbonyl-L-cysteinylglycine t-butyl ester (7·6 g., 0·0138 mole) in methanol (200 ml.) and acetic acid (20 ml.) was hydrogenated overnight at atmospheric pressure in the presence of palladium–charcoal (5%; 4·0 g.). The dark yellow mixture was then filtered through Celite and washed with methanol through a charcoal-Celite bed (2:1; 30 g.). The clear filtrate and washings were evaporated, and the residue was dissolved in ethyl acetate (150 ml.) and the solution was washed with N-sodium hydrogen carbonate (6 × 30 ml.) and brine (3 × 30 ml.) and then dried. It was then evaporated and the residual gum (3·7 g., 72%, after 3 hr. at 0·1 mm./20°) was dissolved in ether (20 ml.). The solution was filtered, and di(*p*-toluoyl)-D-tartaric acid (3·865 g., 0·01 mole) in ether (20 ml.) was added. The white gelatinous precipitate was filtered off and twice reprecipitated from methanol with ether and light petroleum, to give the *salt* as a fine white powder (5·28 g., 50·5% overall), m.p. 170—170·5°, $[\alpha]_D^{20}$ −76·8° (*c* 0·95 in MeOH), R_F (1) 0·85, R_F (4) 0·72 (Found: C, 58·9; H, 6·1; N, 4·0; S, 8·65. $C_{37}H_{44}N_2O_{11}S_2$ requires C, 58·7; H, 5·9; N, 3·7; S, 8·5%). T.l.c. of the solutions after hydrogenation always showed a spot which corresponded to the *p*-nitrobenzyloxycarbonyl derivative and which could not be removed completely by increasing the hydrogenation time, by the addition of more catalyst, or by hydrogenation at 5 atm. pressure. No *p*-toluidine (which gives a strong purple colouration with ninhydrin) could be detected in the products. The free amino-ester was liberated from the salt (1·133 g., 1·15 mmole) dissolved in ethanol–water (75%; 200 ml.) by shaking with Amberlite IRA-400 (OH^-) resin (10 ml.). After 45 min. the resin was filtered off and the filtrate was evaporated; the residue was taken up in ether (10 ml.) and the solution was filtered and evaporated to leave the ester as a syrup (0·55 g., 99%), $[\alpha]_D^{20}$ −20·7° (*c* 2·63 in EtOAc), R_F (MeOH) 0·75, R_F (3) 0·73. It was used immediately.

α-t-Butyl Benzyloxycarbonyl-L-glutamate.—α-t-Butyl γ-methyl benzyloxycarbonyl-L-glutamate was prepared by the reaction of isobutene with γ-methyl benzyloxycarbonyl-L-glutamate [23] in dichloromethane in the presence of sulphuric acid, according to the method of Anderson and Callahan; [20] the product (78%) was an oil, R_F (1) 0·91, R_F (2) 0·85. It has previously been prepared by use of t-butyl acetate.[13] This product (3·5 g., 0·01 mole) was saponified as described in ref. 13 to give an oil (2·9 g., 85%) which after reprecipitation from ether by light petroleum crystallised overnight in the refrigerator. The crude product had R_F (MeOH) 0·6 (main spot), with a streak from the origin to 0·3, apparently due to benzyloxycarbonylglutamic acid; since the starting material had been washed thoroughly with sodium hydrogen carbonate, it seems likely that this arose either from saponification of some t-butyl ester,[24] or from the presence of the isobutyl ester (which would be saponified by alkali). The impurity was not eliminated by repeated recrystallisation from ether–light petroleum, but was removed as follows. Crude product (1·69 g., 5·0 mmoles) in ether (10 ml.) was extracted with 0·01M-sodium carbonate (5 × 5 ml.); t.l.c. showed that the impurity was no longer present after the fourth wash. The ether layer was then washed with water, dried, and evaporated. The residual oil was recrystallised from ether–light petroleum to give fine needles of the *ester* (1·33 g., 79%), m.p. 82—84°, $[\alpha]_D^{20}$ −10·6° (*c* 2·0 in EtOAc), $[\alpha]_D^{20}$ −26·2° (*c* 1·2 in MeOH), $[\alpha]_D^{20}$ +5·6° (*c* 1·76 in $CHCl_3$) (Found: C, 60·8; H, 6·6; N, 4·4. $C_{17}H_{23}NO_6$ requires C, 60·5; H, 6·8; N, 4·2%). The overall yield of pure ester was 53—55%, in several preparations. This ester has also been crystallised in Professor Kenner's laboratory.[25]

S-Benzylthiomethyl-N-carboxy-L-cysteine Anhydride (IV).—Phosgene was passed through a suspension of *S*-benzylthiomethyl-L-cysteine [5] (12·87 g., 0·05 mole) in dry dioxan (250 ml.) at 40—50° for 45 min. A clear solution was obtained after 5—10 min. Excess of phosgene was removed

[23] W. E. Hauby, S. G. Waley, and J. Watson, *J. Chem. Soc.*, 1950, 3239.

[24] *Cf.* S. Bajusz, T. Lázár, and Z. Paulay, *Acta Chim. Acad. Sci. Hung.*, 1964, **41**, 329.

[25] J. J. Mendive, Ph.D. Thesis, Liverpool, 1967.

by passing carbon dioxide through the solution for 90 min. at room temperature; the solution was then filtered and evaporated. The residual oil crystallised after trituration with light petroleum, and the solid (from ethyl acetate–light petroleum) gave needles (11·1 g., 78·5%), m.p. 92—94°. Further recrystallisation gave the *anhydride* (9·5 g., 67%), m.p. 96—97°, $[\alpha]_D^{20}$ −107·4° (*c* 1·0 in $HCO{\cdot}NMe_2$) (Found: C, 51·05; H, 4·6; N, 4·8. $C_{12}H_{13}NO_3S_2$ requires C, 50·9; H, 4·6; N, 4·9%). The compound is unstable and was always recrystallised immediately before use.

S-*Benzylthiomethyl*-L-*cysteine Piperidino-ester Hydrochloride*.[17,26]—1-Hydroxypiperidine hydrochloride was prepared by the addition of hydrogen chloride in ether (3·3N; 1 equiv.) to a solution of 1-hydroxypiperidine in ether; it had m.p. 142—146° (softening at 122—123°) (from chloroform–ether) (lit.,[27] 141°). Freshly recrystallised material (1·375 g., 0·01 mole) dissolved in chloroform (20 ml.) was added to a stirred suspension of freshly recrystallised *S*-benzylthiomethyl-*N*-carboxy-L-cysteine anhydride (2·834 g., 0·01 mole) in chloroform (20 ml.) at room temperature, with exclusion of moisture and light. After 75 min., the mixture was placed in the refrigerator, and after 6 hr. the crystals were collected and washed with a little cold chloroform and ether. The crude product (2·4 g., 64%) was dissolved in cold ethanol (200 ml.) and the solution was filtered and concentrated at room temperature until crystallisation began, when an excess of ether was added. The *piperidino-ester hydrochloride* (2·24 g., 59·5%) had $[\alpha]_D^{20}$ −33·4° (*c* 1·0 in MeOH), R_F (MeOH) 0·70, R_F ($CHCl_3$) 0·30 (faint ninhydrin-positive spots at the origin in each case) (Found: C, 50·85; H, 7·0; N, 7·6; S, 17·15. $C_{16}H_{25}ClN_2O_2S_2$ requires C, 51·0; H, 6·7; N, 7·4; S, 17·0%). The yields varied considerably, and it was found to be important to use freshly recrystallised reactants in exact proportions, and purified chloroform [washed with water, dried ($CaCl_2$) and distilled]. These ester hydrochlorides do not have definite melting points.[17]

N-*Benzyloxycarbonyl*-α-*t-butyl*-γ-L-*glutamyl*-S-*benzylthiomethyl*-L-*cysteine Piperidino-ester* (VI).—A solution of ethyl chloroformate (0·434 g., 4·0 mmoles) in tetrahydrofuran (5 ml.) was added dropwise during 1·5 min. to a stirred solution of α-t-butyl *N*-benzyloxycarbonyl-L-glutamate (1·350 g., 4·0 mmoles) and triethylamine (0·405 g., 4·0 mmoles) in tetrahydrofuran (24 ml.) at −15°. After 10 min., *S*-benzylthiomethyl-L-cysteine piperidino-ester hydrochloride (1·508 g., 4·0 mmoles) was added, followed by a solution of triethylamine (0·405 g.) in tetrahydrofuran (10 ml.), added during 10 min. The mixture was allowed to reach room temperature, and next day it was filtered. The filtrate was evaporated, and ethyl acetate was twice added and then evaporated from the residue; this was dissolved in ethyl acetate and washed with 0·2N-hydrochloric acid (2 × 10 ml.), water, N-sodium hydrogen carbonate (2 × 10 ml.), water, and brine, and dried. Evaporation and trituration of the residue with ether gave a solid (1·6 g., 61%), m.p. 86—91°; after two recrystallisations from di-isopropyl ether the m.p. was 89—92°, $[\alpha]_D^{20}$ −46·5°, $[\alpha]_{365}^{20}$ −149·7° (*c* 0·47 in MeOH); this material was used for the next stage, but traces of two contaminants were shown on t.l.c. plates: R_F (3) 0·95, with very faint spots at 0·0 and 0·55. A sample (100 mg.) was therefore purified on a t.l.c. plate (1 mm. layer, 1 m. long; silica gel HF 254) in solvent (3). The main band was extracted into ethyl acetate (3 × 100 ml.) and the residue after evaporation of the solvent gave the *ester* as needles (87 mg.), m.p. 87—89° (from di-isopropyl ether); a very faint spot on the starting line [solvent (3)] was presumably due to decomposition on the plate; $[\alpha]_D^{20}$ −47·1°, $[\alpha]_{365}^{20}$ −149·7° (*c* 0·46 in MeOH) (Found: C, 59·9; H, 6·9; N, 6·6; S, 10·2. $C_{33}H_{45}N_3O_7S_2$ requires C, 60·1; H, 6·9; N, 6·4; S, 9·7%).

N-*Benzyloxycarbonyl*-γ-L-*glutamyl*-S-*benzylthiomethyl*-L-*cysteinylglycine Di-t-butyl Ester* (II).—(*a*) A solution of triethylamine (0·25 ml., 1·79 mmoles) in acetonitrile (10 ml.) was added to a stirred solution of α-t-butyl *N*-benzyloxycarbonyl-L-glutamate (0·604 g., 1·79 mmoles) in acetonitrile (10 ml.) at 0°. 3-(2-Ethyl-5-isoxazolio)benzenesulphonate[15] (Reagent K; 0·454 g., 1·79 mmoles) was added, and a clear solution resulted after 1 hr. An ice-cold solution of *S*-benzylthiomethyl-L-cysteinylglycine t-butyl ester (0·664 g., 1·79 mmoles) in acetonitrile (10 ml.) was added and the mixture was stirred for 1 hr. at 0° and overnight at room temperature. Evaporation left a foam which was distributed between ethyl acetate and water; the ethyl acetate solution was washed with water, 2N-hydrochloric acid (2 × 10 ml.), water, N-sodium hydrogen carbonate (3 × 10 ml.), water, and brine, and dried. Evaporation left a gum which solidified after trituration with ether; the solid was collected and washed with ether to give a product (0·36 g., 29%) which gave the *protected tripeptide ester* as needles (0·30 g., 24%), m.p. 128—130° (from di-isopropyl ether) $[\alpha]_D^{20}$ −54·7°, $[\alpha]_{365}^{20}$ −179° (*c* 0·47 in MeOH), $[\alpha]_D^{20}$ −45·8°, $[\alpha]_{365}^{20}$ −147° (*c* 0·56 in $HCO{\cdot}NMe_2$), R_F (3) 0·6 (Found: C, 59·4; H, 6·8; N, 6·35; S, 9·3. $C_{34}H_{47}N_3O_8S_2$ requires C, 59·2; H, 6·9; N, 6·1; S, 9·3%).

(*b*) A solution of glacial acetic acid (0·120 g., 2·0 mmoles) in chloroform (0·5 ml.) was added to a mixture of α-t-butyl-*N*-benzyloxycarbonyl-γ-L-glutamyl-*S*-benzylthiomethyl-L-cysteine piperidino-ester (0·660 g., 1·0 mmole) and glycine t-butyl ester (0·263 g., 2·0 mmoles). The viscous solution was stirred at room temperature; after 3 hr. more chloroform (2·0 ml.) was added to allow stirring to continue. After 6 days the solvent was evaporated and the residue was dissolved in chloroform and washed with 0·1N-hydrochloric acid, water, N-sodium hydrogen carbonate, and water. The dried solution was evaporated to leave a solid which was reprecipitated from chloroform with light petroleum. The powder (0·61 g., 88%) gave the *protected tripeptide ester* (0·40 g., 58%), m.p. 127—129° (from di-isopropyl ether), $[\alpha]_D^{20}$ −54·4°, $[\alpha]_{365}^{20}$ −177° (*c* 0·49 in MeOH), $[\alpha]_D^{20}$ −45·2°, $[\alpha]_{365}^{20}$ −146° (*c* 0·53 in $HCO{\cdot}NMe_2$), R_F (3) 0·6 (Found: C, 59·2; H, 6·8; N, 6·4; S, 9·4%). Product (0·20 g.) recovered from the mother-liquor of the recrystallisations was found suitable for the preparation of *S*-benzylthiomethylglutathione.

γ-L-*Glutamyl*-S-*benzylthiomethyl*-L-*cysteinylglycine* ('S-*Benzylthiomethyl-glutathione*').—*N*-Benzyloxycarbonyl-γ-L-glutamyl-*S*-benzylthiomethyl-L-cysteinylglycine di-t-butyl ester (0·345 g., 0·5 mmole) and methyl ethyl sulphide (0·91 ml., 10 mmoles) were dissolved in glacial acetic acid (1·5 ml.), and hydrogen bromide in acetic acid (6·5N; 0·8 ml., 5 mmoles) was added. After 1 hr. at room temperature, ether (100 ml.) was added; the sticky solid so obtained was triturated three times with ether and then dried *in vacuo* over sodium hydroxide. The hydrobromide was dissolved in 20% acetic acid (20 ml.; if the solution is too concentrated, the product will be precipitated on the resin),

[26] S. Bittner, Y. Knobler, and M. Frankel, *Tetrahedron Letters*, 1965, 95.

[27] W. Wernick and P. Wolffenstein, *Ber.*, 1898, **31**, 1553; M. Auerbach and R. Wolffenstein, *ibid.*, 1899, **32**, 2507.

passed down a column (9 × 1·5 cm.) of Dowex-3 resin (acetate form), and eluted with 20% acetic acid. The first 50 ml. of eluate contained the product, and the solution was evaporated at 0·5 mm./30—35°. The residue was triturated with acetone and dried to give the crude product (0·180 g., 81%). It was dissolved in water (100 ml.) at 90—100° and the solution was filtered, and then concentrated at 4 mm./40—50° to 6 ml. and rapidly cooled. (This procedure was used to avoid decomposition during the time required for dissolution in a small volume of water). The crystalline product was filtered off and washed with water and acetone to give S-*benzylthiomethylglutathione* (0·144 g., 65%), m.p. 202—207·5°, $[\alpha]_D^{20}$ −23·1°, $[\alpha]_{365}^{20}$ −78·4° (*c* 0·50 in 0·025M-Na_2CO_3), paper chromatography R_F (1) 0·43, R_F (5) 0·52, and R_F (6) 0·62, t.l.c. R_F (1) 0·5, R_F (4) 0·27 (the plates showed no other chlorine-positive spots) (Found: C, 48·5; H, 5·4; N, 9·5; S, 14·8. $C_{18}H_{25}N_3O_6S_2$ requires C, 48·7; H, 5·7; N, 9·5; S, 14·5%). A second experiment, with 0·166 moles of protected glutathione, gave a 69% yield of recrystallised product.

γ-L-*Glutamyl*-L-*cysteinylglycine* (*Glutathione*).—As far as practicable, all operations after the removal of the *S*-benzylthiomethyl group were carried out in nitrogen purified by passage through Fieser's solution;[28] distilled water was boiled out, and ethanol was of spectroscopic grade, freed from oxygen by the passage of nitrogen. A solution of γ-L-glutamyl-*S*-benzylthiomethyl-L-cysteinylglycine (44 mg., 0·10 mmole) in formic acid (98—100%; 1 ml.) was added to a stirred partial suspension of powdered mercuric acetate (0·128 g., 0·40 mmole) in water (0·5 ml.); the remainder of the solution was rinsed in with formic acid (2 × 0·5 ml.). After 30 min. at room temperature, ethanedithiol (0·086 ml., 1·0 mmole) was added, to give a cream-coloured gelatinous precipitate which turned grey after 2—3 min. After 15 min., hydrogen sulphide was passed in for 2 hr., with stirring; the precipitate was centrifuged off and washed with 80% formic acid (4 × 3 ml.). The solution and washings were evaporated at room temperature under high vacuum to leave a clear thick gum containing particles of mercuric sulphide; it was dissolved in water (2 ml.), hydrogen sulphide was passed through for 15 min., and the mixture was filtered through a pad of Celite (1 cm. deep) over two Whatman no. 42 filter papers, on a sintered glass filter (porosity 4). The filter bed was washed with water (5—10 ml.) and the filtrate and washings were evaporated. The clear thick syrup remaining was triturated three times with ethanol (the ethanol was evaporated from the residue each time) to leave a white amorphous solid. This was washed twice with ethanol (separation by centrifuge) and dried for 15 hr. at 0·5 mm. over silica gel at room temperature, to give glutathione (0·030 g., 89·5% calc. as dihydrate), $[\alpha]_D^{20}$ −24·3° (*c* 0·90 in H_2O; calc. for anhydrous glutathione), paper chromatography R_F (5) 0·16 (with a faint spot at the starting line, positive to ninhydrin, and to nitroprusside only after cyanide), R_F (6) 0·42 (commercial glutathione gave a main spot in the same position as the synthetic preparation, in each solvent, but there was much streaking) (Found: C, 34·7; H, 5·7; N, 11·9. Calc. for $C_{10}H_{17}N_3O_6S,2H_2O$: C, 35·0; H, 6·2; N, 12·2%) {lit.,[29] $[\alpha]_D^{27}$ −21·3° (*c* 2 in H_2O)}.

In a second preparation (on the same scale) the syrup was scratched under ethanol to give an amorphous solid which was centrifuged off immediately and washed with ethanol three times (centrifuge); the partly crystalline solid was dried for 22 hr. at room temperature and 0·5 mm. (P_2O_5), and finally for 17 hr. at 50°. The product (18·7 mg.; some was lost in the first ethanol washings) had the correct analysis for the monohydrate (Found: C, 36·4; H, 5·6; N, 13·0. Calc. for $C_{10}H_{17}N_3O_6S,H_2O$: C, 36·9; H, 5·9; N, 12·9%) and had $[\alpha]_D^{20}$ −22·0° (*c* 0·136 in H_2O; calc. for anhydrous glutathione). In neither preparation was any unchanged *S*-benzylthiomethylglutathione detected; no pyroglutamic acid was detected by t.l.c. by use of solvents (4) and (5), in which the product had R_F 0·04 and 0·10 respectively.

N-*Benzyloxycarbonyl*-β-*alanine t-Butyl Ester*.—*N*-Benzyloxycarbonyl-β-alanine[30] (39 g., 0·175 mole) was suspended in dichloromethane (400 ml.) in a screw-topped pressure bottle, and concentrated sulphuric acid (2 ml.) and then isobutene (200 ml.) were added. The mixture was stirred magnetically at room temperature. After 3 days the solution was clear; after 14 days it was concentrated to 200 ml. and washed with 0·2N-hydrochloric acid (2 × 100 ml.), N-sodium hydrogen carbonate (2 × 100 ml.), and water. The dried solution was evaporated, and the residual yellow oil was dissolved in an equal volume of ether. A large excess of light petroleum was added, and after storage overnight in a refrigerator a first crop of crystals (19·5 g.), m.p. 42—44°, was obtained. Concentration of the mother-liquors and the addition of light petroleum gave more product (in all, 36·4 g., 75%). A sample of *ester* had m.p. 41—43° (from ether–light petroleum) (Found: C, 64·8; H, 7·5; N, 5·0. $C_{15}H_{21}NO_4$ requires C, 64·5; H, 7·6; N, 5·0%).

β-*Alanine t-Butyl Ester*.—*N*-Benzyloxycarbonyl-β-alanine t-butyl ester (30·7 g., 0·11 mole) in methanol (250 ml.) was hydrogenated at atmospheric pressure in the presence of palladium–charcoal (10%; 4 g. initially, and a further 1 g. after 3 hr.). The carbon dioxide liberated during the reaction was absorbed on filter paper impregnated with concentrated potassium hydroxide solution. After 4·5 hr. the uptake of hydrogen had ceased; the mixture was filtered through Celite, and the filtrate was concentrated to 75 ml. A solution of phosphorous acid (9·02 g., 0·11 mole) was added and a portion of the precipitate (21 g., 84%) was twice recrystallised from methanol–di-isopropyl ether to give the *phosphite* of the amino-ester, m.p. 137—140° (Found: C, 37·1; H, 8·0; N, 6·0. $C_7H_{18}NO_5P$ requires C, 37·0; H, 8·0; N, 6·2%). The main product (19 g.) was dissolved in water (250 ml.) and the solution was made alkaline with sodium hydroxide (7 g.). The oil which separated was extracted into ether (2 × 100 ml.) and dried. Evaporation left a yellow oil (8 g., 66%) which was distilled to give the *ester*, b.p. 46—48°/1·5 mm., n_D^{19} 1·4290 (Found: C, 57·95; H, 10·7; N, 9·65. $C_7H_{15}NO_2$ requires C, 57·9; H, 10·4; N, 9·65%).

S-*Benzylthiomethyl*-N-p-*nitrobenzyloxycarbonyl*-L-*cysteinyl*-β-*alanine t-Butyl Ester* (VII).—β-Alanine t-butyl ester (3·63 g., 0·025 mole) in ethyl acetate (25 ml.) was added to a solution of *S*-benzylthiomethyl-*N*-*p*-nitrobenzyloxycarbonyl-L-cysteine (10·91 g., 0·025 mole) in ethyl acetate (150 ml.) at 0°. Dicyclohexylcarbodi-imide (5·60 g., 0·027 mole) in ethyl acetate (25 ml.) was added. After 15 min., the reaction mixture had become nearly solid; more ethyl acetate (50 ml.) was added, and the temperature was allowed to rise. Yet more ethyl acetate (25 ml.) had to be added to facilitate stirring. (In other experiments, a small amount

[28] L. F. Fieser, *J. Amer. Chem. Soc.*, 1924, **46**, 2639.
[29] V. du Vigneaud and G. L. Miller, *Biochem. Prep.*, 1952, **2**, 87.
[30] R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, 1935, **108**, 753.

of dimethylformamide was added to keep the reactants in solution). Next day, acetic acid (10 drops) was added, and the chilled solution was filtered. The precipitate was washed with ethyl acetate, and the combined filtrates were evaporated; the residue was taken up in ethyl acetate and the solution was filtered and then washed with 0·2N-hydrochloric acid (2 × 50 ml.), water (2 × 25 ml.) N-sodium hydrogen carbonate (2 × 50 ml.), water, and brine. The dried solution was evaporated and the residue was taken up in acetone (100 ml.). After 3 days the solution was filtered and evaporated to leave an oil which was dissolved in hot di-isopropyl ether (100 ml.) with the aid of the minimum amount of acetone. Cooling gave a gum which solidified in the refrigerator after trituration with light petroleum. The product could not be crystallised, but was reprecipitated as above and from ether–light petroleum to give the *ester* as a pale yellow amorphous solid (9·0 g., 64%), m.p. 50° (with previous softening), $[\alpha]_D^{20}$ −27·4° (*c* 1·47 in EtOAc), R_F (Et_2O) 0·83, with a faint spot at 0·96 (Found: C, 55·6; H, 6·0; N, 7·7. $C_{26}H_{33}N_3O_7S_2$ requires C, 55·5; H, 5·9; N, 7·4%).

S-*Benzylthiomethyl*-L-*cysteinyl*-β-*alanine t-Butyl Ester Di*-p-*toluoyl*-D-*tartrate*.— *S*-Benzylthiomethyl-*N*-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-β-alanine t-butyl ester (0·564 g., 1 mmole) was hydrogenated in methanol as described for the glycine analogue. Evaporation of the eluate (500 ml.) from the charcoal–Celite column left a gum (0·20 g., 52%) which was dissolved in ether (20 ml.); the solution was filtered, and di-*p*-toluoyl-D-tartaric acid (0·201 g.) in ether (20 ml.) was added. The gel was filtered off, washed with ether, and dried (0·22 g., 28·5% overall); reprecipitation from methanol with ether and light petroleum gave the *salt* as a fine powder (0·185 g., 24% overall), m.p. 176—177°, $[\alpha]_D^{20}$ −70·3° (*c* 1·37 in MeOH), R_F (1) 0·84, with a very faint ninhydrin-negative chlorine-positive spot at 0·3, R_F (2) 0·72, with a very faint ninhydrin-negative chlorine-positive spot at 0·4 (Found: C, 59·2; H, 6·2; N, 3·75; S, 8·15. $C_{38}H_{46}N_2O_{11}S_2$ requires C, 59·1; H, 6·0; N, 3·6; S, 8·3%). The free dipeptide ester was liberated from the salt as described for the glycine analogue (98·5% yield) to give the ester, $[\alpha]_D^{20}$ −20·4° (*c* 2·15 in EtOAc), R_F (1) 0·8, R_F (2) 0·7. It was used immediately.

N-*Benzyloxycarbonyl*-γ-L-*glutamyl*-S-*benzylthiomethyl*-L-*cysteinyl*-β-*alanine Di-t-butyl Ester* (IX).—α-t-Butyl *N*-benzyloxycarbonyl-L-glutamate (0·49 g., 1·45 mmoles) was added to a stirred solution of *S*-benzylthiomethyl-L-cysteinyl-β-alanine t-butyl ester (0·56 g., 1·45 mmole, liberated from its salt immediately prior to use) in acetonitrile (10 ml.). The solution was cooled in an ice-bath and dicyclohexylcarbodi-imide (0·34 g., 1·6 mmoles) in acetonitrile (5 ml.) was added. After 1 hr., the temperature was allowed to rise, and next day the solvent was evaporated. The residue was taken up in ethyl acetate and the solution was filtered and then washed with 0·2N-hydrochloric acid (2 × 10 ml.), water, N-sodium hydrogen carbonate (3 × 10 ml.), water, and brine. The dried solution was evaporated. The residue was taken up in ethyl acetate, the solution was filtered, and the product was precipitated with ether and light petroleum to give an amorphous solid (0·77 g., 75%), m.p. 109—113° (with earlier softening). It could not be crystallised, but was reprecipitated from di-isopropyl ether to give the *protected tripeptide ester* (0·70 g., 68·5%), m.p. 108—111°, $[\alpha]_D^{20}$ −35·8° (*c* 1·04 in EtOAc) and −41·8° (*c* 1·04 in MeOH), R_F (1) 0·88, R_F (2) 0·95, R_F (3) 0·95 (trace at 0·81) (Found: C, 59·55; H, 7·0; N, 6·25; S, 9·0. $C_{35}H_{49}N_3O_8S_2$ requires C, 59·75; H, 7·0; N, 6·0; S, 9·1%).

γ-L-*Glutamyl*-S-*benzylthiomethyl*-L-*cysteinyl*-β-*alanine* ('S-*Benzylthiomethylhomoglutathione*') (X).—This was prepared from *N*-benzyloxycarbonyl-γ-L-glutamyl-*S*-benzylthiomethyl-L-cysteinyl-β-alanine di-t-butyl ester (0·352 g., 0·5 mmole) as described for the glycine analogue. Acetate was exchanged for bromide on a Dowex-3 (acetate) column in 25% acetic acid; the product was contained in the first 75 ml. of eluate, which was evaporated. The residue was triturated with acetone to give the crude product (0·21 g., 93%), purified as for the glycine analogue to give S-*benzylthiomethylhomoglutathione* (0·175 g., 76·5%), m.p. 180—185° (softening at 171°) (when preheated to 170°), $[\alpha]_D^{20}$ −30·5°, $[\alpha]_{365}^{20}$ −105·2° (*c* 1·00 in 95% AcOH), $[\alpha]_D^{20}$ −17·1°, $[\alpha]_{365}^{20}$ −55·0° (*c* 0·53 in 0·025M-Na_2CO_3), paper chromatography R_F (1) 0·40, R_F (5) 0·48, and R_F (6) 0·73, t.l.c. R_F (1) 0·50 and R_F (4) 0·31 (the plates showed no other chlorine-positive spot) (Found: C, 49·5; H, 6·05; N, 8·95; S, 14·0. $C_{19}H_{27}O_3O_6S_2$ requires C, 49·9; H, 5·95; N, 9·2; S, 14·0%).

γ-L-*Glutamyl*-L-*cysteinyl*-β-*alanine* (*Homoglutathione*) (XI).—The benzylthiomethyl group was removed from *S*-benzylthiomethylhomoglutathione (0·092 g., 0·2 mmole) as described for the preparation of glutathione. The clear thick syrup so obtained was dissolved in water (4 ml.), the solution was filtered, and the filtrate was freeze-dried. The fluffy amorphous solid was then dried (P_2O_5) at 0·5 mm. at room temperature for 96 hr. The hygroscopic solid (65·9 mg., quantitative for the hemihydrate) had $[\alpha]_D^{20}$ −16·1° (calc. for the anhydrous compound, *c* 1·09 in H_2O) (Found: C, 39·5; H, 5·9; N, 12·8. Calc. for $C_{11}H_{19}N_3O_6S,\frac{1}{2}H_2O$: C, 40·0; H, 6·1; N, 12·7%), paper chromatography R_F (4) 0·10 (with a very faint spot at the origin positive to ninhydrin, and to nitroprusside only after cyanide), R_F (5) 0·19 and 0·03 (very faint), and R_F (6) 0·66 (faint tail). No unchanged benzylthiomethyl derivative, and only a trace of the disulphide, could be detected: {lit.,[18b] $[\alpha]_D^{20}$ −16·4° (*c* 1·3 in H_2O) for product estimated to contain *ca.* 10% of the disulphide and a trace of glutamic acid}.

In a second experiment (0·1 mmole scale), the syrup first obtained was triturated four times with ethanol (the ethanol was evaporated from the syrup each time) to leave an amorphous solid. This was immediately transferred to a desiccator and dried for 34 hr. at 0·5 mm. (P_2O_5). The partly crystalline solid (36 mg., quantitative) had the correct analysis for the sesquihydrate (Found: C, 37·8; H, 5·9. Calc. for $C_{11}H_{19}N_3O_6S,1{\cdot}5H_2O$: C, 37·9; H, 6·35%). After a further 20 hr. drying, the hemi-hydrate was obtained (Found: C, 39·75; H, 6·1; N, 13·15%). The behaviour of this product during paper chromatography was identical with that of the product from the first experiment; again no unchanged benzylthiomethyl derivative was detected.

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