733. *Amino-acids and Peptides. Part XX.*[1] *S-Benzylthiomethyl-L-cysteine and its Use in the Synthesis of Peptides.*

By (Mrs.) P. J. E. Brownlee, (Mrs.) M. E. Cox, B. O. Handford, J. C. Marsden, and G. T. Young.

The preparation of *S*-benzylthiomethyl-L-cysteine and some simple derivatives is described. Contrary to another report, it is shown that *S*-benzylthiomethyl-L-cysteine is stable to hydrogen bromide in acetic acid under the conditions required for the removal of *N*-benzyloxycarbonyl groups. An improved procedure for the removal of the *S*-benzylthiomethyl group has been developed, and this new method of *S*-protection has been used in the synthesis of diglycyl-L-cystine and L-cystinyldiglycine in good yield. *S*-Phenylthiomethyl- and *S*-isobutoxymethyl-L-cysteine are also described.

Methods for the protection of the thiol group of cysteine during peptide synthesis have been kept under regular review,[2] and here it suffices to point out that the search for alternatives to the highly successful *S*-benzyl route [3] is prompted in part by evidence in certain cases of side-reactions caused by the sodium in liquid ammonia required for debenzylation,[4] and in part by the need for a variety of *S*-protection if the unambiguous synthesis of peptides containing more than one disulphide bridge is to be attempted. In an earlier Note [5] we reported a preliminary examination of the usefulness of the *S*-benzylthiomethyl group, and we give now a detailed account of our work.

$$RNH{\cdot}CH(CO{\cdot}R'){\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}S{\cdot}CH_2Ph$$

(I) R = H, R′ = OH (II) R = H, R′ = OMe (III) R = $PhCH_2{\cdot}OCO{\cdot}NH{\cdot}CH_2{\cdot}CO$, R′ = OMe
(IV) R = $PhCH_2{\cdot}OCO{\cdot}NH{\cdot}CH_2{\cdot}CO$, R′ = OH (V) R = $PhCH_2{\cdot}O{\cdot}CO$, R′ = $NH{\cdot}CH_2{\cdot}CO_2Et$
(VI) R = $PhCH_2{\cdot}OCO$, R′ = $NH{\cdot}CH_2{\cdot}CO_2H$

Initially we prepared *S*-benzylthiomethyl-L-cysteine (I) by the action of benzylthiomethyl chloride on L-cysteine hydrochloride suspended in methanol; this gave a mixture of the amino-acid with the amino-ester (II) hydrochloride, which could be converted entirely into the former by alkali or into the latter by thionyl chloride and methanol.[6] The heterogeneous reaction proved variable in its results, and it should be noted that the specific rotation reported originally for *S*-benzylthiomethyl-L-cysteine has to be corrected. As indicated in our Note, the best method of preparing this compound is by the reduction of L-cystine by sodium in liquid ammonia followed by the addition of benzylthiomethyl chloride, and provided that redistilled chloride is used and moisture is excluded a good yield of optically pure and beautifully crystalline amino-acid is obtained. Optical purity was established by conversion into the methyl ester hydrochloride, which absorbed an insignificant amount of oxygen in the presence of a D-amino-acid oxidase which caused considerable absorption by the racemic compound. The action of cold alkali on the ester regenerated amino-acid with the same specific rotation as the starting material. It is of course possible that the substrate in the enzymic reaction was the D-amino-acid formed by hydrolysis of the ester, but in either case this represents an addition to the known substrates of the enzyme. The *S*-benzylthiomethyl-L-cysteine methyl ester hydrochloride was prepared by the action of thionyl chloride and methanol on the amino-acid, and has

[1] Part XIX, *J.*, 1964, 3701.
[2] Young, Proc. Symp. on Methods of Peptide Synthesis, Prague, 1958 (*Coll. Czech. Chem. Comm.*, 1959, **24**, Special Issue, p. 39); Zervas, Photaki, Cosmatos, and Ghelis, in "Peptides: Proc. Fifth European Peptide Symp., Oxford, 1962," ed. G. T. Young, Pergamon Press, Oxford, 1963, p. 27.
[3] Wood and du Vigneaud, *J. Biol. Chem.*, 1939, **130**, 109.
[4] *E.g.*, Kappeler, in "Peptides: Proc. Fifth European Peptide Symp., Oxford, 1962," ed. G. T. Young, Pergamon Press, Oxford, 1963, p. 3; Guttmann, *loc. cit.*, p. 41.
[5] Pimlott and Young, *Proc. Chem. Soc.*, 1958, 257.
[6] Brenner and Huber, *Helv. Chim. Acta*, 1953, **36**, 1109.

since been prepared similarly by Hiskey and Tucker.[7] *N*-Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine was obtained in the usual fashion first as a syrup, but was later crystallised in this laboratory by Dr. M. Zaoral, and has been reported crystalline by Hiskey and Tucker.[7] For many purposes, the readily-crystallised dicyclohexylammonium salt is convenient and can be used in coupling reactions. The corresponding methyl ester was prepared by benzyloxycarbonylation of the methyl ester hydrochloride.

It has been stated by Katsoyannis [8] that exposure of *S*-benzylthiomethylcysteine-containing peptides to hydrogen bromide in acetic acid should be avoided, and he reported the presence of four ninhydrin-positive components when *S*-benzylthiomethyl-L-cysteine was left for 45 minutes in 2N-hydrogen bromide in acetic acid at room temperature. This is not our experience. Paper chromatography of the product after this treatment shows a very faint additional ninhydrin-positive spot, but even after 1 hour's contact with the reagent, chromatographically, analytically, and optically pure amino-acid can be recovered in 94% yield. After 15 hours' treatment the additional spot is stronger in intensity, but the recovery of pure material is still 83%. It is to be noted that Katsoyannis prepared *S*-benzylthiomethyl-L-cysteine from cysteine hydrochloride in methanol, which gives material needing careful purification. It is not unexpected that the action of hydrogen bromide on *N*-benzyloxycarbonyl-*S*-benzylthiomethylcysteine should give rise to side-products, presumably analogous to those encountered with derivatives of methionine. We have for some time been using the modified procedure devised by Guttmann and Boissonnas [9] for such cases, carrying out the reaction in the presence of ethyl methyl sulphide. In this way, benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine can be converted in 92% yield into chromatographically, analytically, and optically pure *S*-benzylthiomethyl-L-cysteine. As in other cases, it is wise to determine by means of paper chromatography the minimum time required for the debenzyloxycarbonylation, and for sulphur-containing compounds we use the procedure developed with Handford and Welford;[10] ultraviolet radiation cleaves the benzyloxycarbonyl derivative [11] on the chromatogram, which is then sprayed with ninhydrin to reveal the amino-compound so formed. We have used hydrogen bromide in acetic acid for the debenzyloxycarbonylation of a considerable number of peptides containing *S*-benzylthiomethylcysteine residues, and, provided that the reaction is not unduly prolonged, no difficulty has been encountered.

The removal of the *S*-benzylthiomethyl group has given rise to a more real difficulty, and we have spent much time in attempting to improve this procedure. In our preliminary Note [5] we referred to the use of warm aqueous mercuric chloride with warm N-hydrochloric acid, but with these reagents the removal is only partial and the yield of L-cystine is low (*ca.* 25%); results are variable because an insoluble addition compound may be precipitated by the mercuric chloride before fission has occurred, in which case the reaction proceeds no further. It seems likely that this is the reason for the lack of reaction found in another case by Hiskey and Tucker.[7] * Further, an important by-product is thiazolidine-4-carboxylic acid, readily detected by paper chromatography with phenol–water as solvent. After a lengthy investigation of a variety of conditions we now use mercuric acetate in 80% formic acid; in this solvent removal is rapid, being complete in 5—20 minutes at room temperature. The addition of ethanedithiol at this stage reduces very considerably the formation of thiazolidine-4-carboxylic acid, and optically and

* On page 4791 of this Paper it is stated that the *S*-benzylthiomethyl group "is reported to be removed from *N*-carbobenzoxy-*S*-benzylthiomethylcysteine with aqueous mercuric chloride and from *S*-benzylthiomethylcysteine with warm N-hydrochloric acid." It is not clear what report is referred to; our preliminary Note does not make either of these statements.

[7] Hiskey and Tucker, *J. Amer. Chem. Soc.*, 1962, **84**, 4789.
[8] Katsoyannis, *J. Amer. Chem. Soc.*, 1961, **83**, 4053.
[9] Guttmann and Boissonnas, *Helv. Chim. Acta*, 1958, **41**, 1852.
[10] Handford, Welford, and Young, in "Peptides: Proc. Fifth European Peptide Symp., Oxford, 1962," ed. G. T. Young, Pergamon Press, Oxford, 1963, p. 56.
[11] Barltrop and Schofield, *Tetrahedron Letters*, 1962, **16**, 697.

chromatographically pure L-cystine can then be obtained in 80% yield from *S*-benzylthiomethyl-L-cysteine. We determine the minimum time required for the reaction with mercuric acetate in each case by means of paper chromatography, again using the ultraviolet radiation–ninhydrin technique for the detection of benzyloxycarbonyl compounds.

Benzyloxycarbonylglycine was condensed with *S*-benzylthiomethyl-L-cysteine methyl ester by means of dicyclohexylcarbodi-imide to give crystalline benzyloxycarbonylglycyl-*S*-benzylthiomethyl-L-cysteine methyl ester (III) (84% yield), which was hydrolysed by alkali to the acid (IV). Hiskey and Tucker [7] report the ester (III) as an oil, but for the acid (IV) they found m. p. 127—128°; we have prepared it on several occasions and find m. p. 108·5—110°, although in early experiments we obtained crystals which melted first at 64° and again at 107—108°; recrystallisation gave product melting only at 108—110°. Apparently the compound is polymorphic. Removal of the *S*-protection in the above fashion followed by aerial oxidation gave crystalline di(benzyloxycarbonylglycyl)-L-cystine in 78% yield, and this with hydrogen bromide in acetic acid gave diglycyl-L-cystine (89% yield). Alternatively, the action of hydrogen bromide in acetic acid (in the presence of ethyl methyl sulphide) on the acid (IV) gave crystalline glycyl-*S*-benzylthiomethyl-L-cysteine (81% yield), which with mercuric acetate in 80% formic acid (and ethanedithiol) followed by aerial oxidation gave diglycyl-L-cystine in 60% yield.

Analogously, benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine was condensed with glycine ethyl ester; alkaline hydrolysis of the peptide ester (V) gave the acid (VI), which by the standard removal procedure gave crystalline di(benzyloxycarbonyl)-L-cystinyldiglycine (in 79% yield), which with hydrogen bromide in acetic acid gave L-cystinyldiglycine (79% yield). Alternatively, the acid (VI) with hydrogen bromide in acetic acid gave *S*-benzylthiomethyl-L-cysteinylglycine (71%), which on removal of the *S*-protection gave L-cystinyldiglycine in 57% yield.

Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteinyl-L-tyrosine methyl ester was prepared by the dicyclohexylcarbodi-imide method; its conversion into the hydrazide in high yield shows the stability of the *S*-benzylthiomethyl grouping towards hydrazine under normal conditions.

We have prepared, through the appropriate alkyl chlorides, *S*-phenylthiomethyl- and *S*-isobutoxymethyl-L-cysteine. The first closely resembles the *S*-benzylthiomethyl derivative and was cleaved by mercuric acetate in 80% formic acid (with ethanedithiol) and oxidised to L-cystine, in 74% yield; thiazolidine-4-carboxylic acid was detected in the filtrate. The second analogue, being a monothioacetal, is more readily decomposed by acid, but is fairly stable to 2N-hydrochloric acid at room temperature.

Experimental

Melting points were determined on a Kofler hot-stage apparatus. The solvents for paper chromatography were: "BWA": butan-1-ol–water–acetic acid (62 : 26 : 12 by volume); "PW": phenol saturated with water; ascending flow, with Whatman No. 4 paper, unless otherwise stated. Solutions in organic solvents were dried with $MgSO_4$. Evaporation was nsually by rotary evaporator.

Benzylthiomethyl Chloride.—The method of Wood and du Vigneaud [3] was used. It is important that the polyoxymethylene should be freshly prepared; paraformaldehyde gave poor yields.[12] For large batches (using 350 g. of benzyl mercaptan) two hydrogen chloride generators were used and saturation took 7 hr. Each piece of apparatus was plunged into alkaline permanganate solution immediately after use, and with care much of the unpleasantness of this preparation can be avoided. Filtration from the calcium chloride was effected through a No. 2 sintered glass filter covered by a Whatman No. 54 filter paper and Celite filter-aid. Product distilling at 78°/0·2 mm., and 98—99°/2·5 mm., with n_D^{25} 1·571—1·578, has given

[12] Böhme, Fischer, and Franck, *Annalen*, 1949, **563**, 54.

S-benzylthiomethyl-L-cysteine of satisfactory purity; high-boiling fractions have given amino-acid of low optical rotation. The specified fractions represent a yield of *ca.* 65% on the benzyl mercaptan. Unchanged benzyl mercaptan distils first, and the end fractions may contain formaldehyde dibenzyl mercaptal. *S*-Benzylthiomethyl-L-cysteine of low specific rotation gives in many cases methy ester hydrochloride of good rotation.

S-*Benzylthiomethyl*-L-*cysteine*.—(a) *In liquid ammonia.* Ammonia was distilled into a 3-necked flask, which was cooled to −70° and provided with a stirrer and a protecting tube containing sodium hydroxide. When 100 ml. had been condensed, the cooling bath was removed and L-cystine (4·80 g.) was dissolved with stirring, during which time the ammonia reached boiling point. Small, clean chips of sodium were added until a permanent blue colour was obtained. The colour was discharged with a few crystals of dried ammonium chloride, and benzylthiomethyl chloride (6·90 g.; 5·9 ml.) was added in one portion. The ammonia was evaporated rapidly and completely on a water-pump, and the residue was washed twice with ether by decantation. Addition of ice-cold N-hydrochloric acid (to pH 6) gave a bulky precipitate, which was filtered off and washed with water, ethanol, and finally ether. The crude product (9·36 g., 91%) had m. p. 193—194° (decomp.) and was recrystallised by suspension in hot aqueous methanol (1 : 1, by vol.) and addition of concentrated hydrochloric acid until a clear hot solution was obtained.

S-*Benzylthiomethyl*-L-*cysteine* crystallised as shining plates, which were washed with water, ethanol, and ether; it had m. p. 193° (decomp.), $[\alpha]_D^{23}$ −49° (less 1° per degree rise in temperature) (*c* 2·5 in MeOH–6N-HCl, 9 : 1 by volume), $[\alpha]_D^{22}$ −24·5° (*c* 1·0 in 3N-HCl); R_F 0·77 (BWA), 0·92 (PW) (Found: C, 51·3; H, 5·9; N, 5·4; S, 25·1. $C_{11}H_{15}NO_2S_2$ requires C, 51·3; H, 5·9; N, 5·4; S, 24·9%). Katsoyannis [9] gives m. p. 200° and Hiskey and Tucker [7] m. p. 193—194°, but no other data for this compound. The specific rotation recorded in our preliminary Note [5] is erroneous; this material was made by method (b) below and apparently contained highly lævorotatory impurity.

(b) *In methanol.* Dry L-cysteine hydrochloride (1·58 g.) and benzylthiomethyl chloride (1·90 g.) were heated at 100° for 15 min. with exclusion of moisture. Dry methanol (0·7 ml.) was added, and the temperature was kept at 70° for a further 30 min. The crude mixture of amino-acid and amino-ester was then either converted completely into the methyl ester hydrochloride as described below, or hydrolysed to yield the amino-acid as follows. After the reaction mixture had been cooled to room temperature, dioxan (12·5 ml.) was added, followed by a solution of sodium hydroxide (2·0 g.) in water (12·5 ml.). After 30 min. at room temperature, the solution was adjusted to pH 4, and the precipitate was filtered off and purified as under (a) above. The product (1·60 g., 62%) had m. p. 193° (decomp.), $[\alpha]_D^{23}$ −31° (*c* 0·6 in 3N-HCl). This procedure was used in our initial experiments, but method (a) is preferred.

S-*Benzylthiomethyl*-DL-*cysteine*.—This was prepared from DL-cystine as described for the L-isomer, method (a); the *amino-acid* had m. p. 195—198° (decomp.) (Found: C, 51·3; H, 6·0; N, 5·6; S, 25·2%).

S-*Benzylthiomethyl*-L-*cysteine Methyl Ester Hydrochloride*.—Dry methanol (12 ml.) was cooled to −60°, and thionyl chloride (1·7 ml.; purified by distillation from quinoline and then from linseed oil) was added carefully; [6] the mixture was added to *S*-benzylthiomethyl-L-cysteine (5·14 g.) in a vessel cooled to −60°. The cooling bath was then removed, and finally the solution was boiled for 2 hr. under reflux with protection against moisture. After cooling, dry acetone (30 ml.) and dry ether (50 ml.) were added successively, giving shining needles of S-*benzylthiomethyl*-L-*cysteine methyl ester hydrochloride* (6·0 g., 96%). Recrystallisation from a mixture of methanol, acetone, and ether (17 ml. of each) containing a little hydrogen chloride gave needles, m. p. 150—151° (decomp.), $[\alpha]_D^{22}$ −50° (less 1° per degree rise in temperature) (*c* 1·0 in dry MeOH), $[\alpha]_D^{21}$ −25° (*c* 1·0 in dimethylformamide), R_F 0·81 (BWA) (Found: C, 47·1; H, 5·7; Cl, 11·8; N, 4·5; S, 20·6. $C_{12}H_{18}NO_2S_2Cl$ requires C, 46·8; H, 5·9; Cl, 11·5; N, 4·6; S, 20·8%). Hiskey and Tucker [7] give m. p. 152·5—154° but no other data.

A small sample of the above product was hydrolysed to the amino-acid as follows. The ester hydrochloride (0·31 g.) was dissolved in dioxan (2·5 ml.) and N-sodium hydroxide (2·5 ml.) and left at room temperature for 30 min. The pH was adjusted to 6·0 and the precipitate was filtered off and washed thoroughly with water, ethanol, and ether, and dried. The product (0·23 g., 90%) had $[\alpha]_D^{21}$ −48·5° (*c* 2·3 in MeOH–6N-HCl, 9 : 1 by volume).

S-*Benzylthiomethyl*-DL-*cysteine Methyl Ester Hydrochloride*.—This was prepared analogously to the L-isomer, and after recrystallisation from methanol (containing a little hydrogen chloride)

and ether the *amino-ester hydrochloride* had m. p. 129° (Found: C, 46·6; H, 5·7; N, 4·5; S, 21·2%).

Estimation of the Optical Purity of S-*Benzylthiomethyl-*L-*cysteine Methyl Ester Hydrochloride by means of a* D-*Amino-acid Oxidase.*—We are grateful to Dr. H. Blaschko, of the Department of Pharmacology, for examining a sample of *S*-benzylthiomethyl-L-cysteine methyl ester hydrochloride (the free amino-acid was too insloluble for this purpose). The enzyme preparation was a dialysed extract of an acetone-dried powder of pig's kidney and was used in a sodium pyrophosphate buffer of pH 8·0. This solution (1·8 ml.) was allowed to react in a conical Warburg flask at 37·5° with (*a*) 0·2 ml. of 0·1-molar-D-phenylalanine, (*b*) 0·2 ml. of 0·1 molar-*S*-benzylthiomethyl-L-cysteine methyl ester hydrochloride, $[\alpha]_D^{18}$ −50·5° (*c* 3 in MeOH), and (*c*) 0·2 ml. of 0·1 molar-*S*-benzylthiomethyl-DL-cysteine methyl ester hydrochloride. After 30 min., the volumes of oxygen absorbed were (*a*) 221, (*b*) 1, (*c*) 115 µl., in excess of the blank experiment.

N-*Benzyloxycarbonyl*-S-*benzylthiomethyl*-L-*cysteine.*—To *S*-Benzylthiomethyl-L-cysteine (2·57 g.) in 18 ml. of N-sodium hydroxide were added simultaneously benzyl chloroformate (1·7 ml.) and N-sodium hydroxide (4 ml.) with stirring at 0°, during 20—30 min., pH being maintained at *ca.* 10·5. Stirring was continued until a drop of the solution, acidified with acetic acid, gave a weak ninhydrin test. Water was then added and the solution was extracted with ether; the aqueous layer was made acid to Congo Red and the oil liberated was extracted into ether, which was dried and evaporated. Addition of light petroleum (b. p. 40—60°) caused crystallisation; recrystallisation from carbon tetrachloride–light petroleum gave *acid* (74% yield), m. p. 67·5—68°, $[\alpha]_D^{22}$ −42° (*c* 3·8 in EtOH) (Found: C, 58·5; H, 5·8; N, 3·5; S, 16·3. $C_{19}H_{21}NO_4S_2$ requires C, 58·3; H, 5·4; N, 3·6; S, 16·4%). This compound, which was crystallised by Dr. M. Zaoral in this laboratory, has been reported by Hiskey and Tucker [7] who give m. p. 68—70°.

In our initial work, the compound was isolated as the readily crystalline *dicyclohexylammonium salt*, which is obtained analytically pure by the addition of redistilled dicyclohexylamine in ether to a dried ether solution of the crude product from the above preparation (77% yield); it has m. p. 121—122°, $[\alpha]_D^{23}$ +8° (*c* 1·0 in $CHCl_3$) (Found: C, 64·8; H, 7·8; N, 4·9; S, 11·4. $C_{31}H_{44}N_2O_4S_2$ requires C, 65·0; H, 7·7; N, 4·9; S, 11·2%).

N-*Benzyloxycarbonyl*-S-*benzylthiomethyl*-L-*cysteine Methyl Ester.*—A solution of *S*-benzylthiomethyl-L-cysteine methyl ester hydrochloride (1·0 g.) and sodium hydrogen carbonate (0·56 g.) in water (10 ml.) was covered with a layer of ethyl acetate (10 ml.) and cooled to 0°. The mixture was stirred vigorously while benzyl chloroformate (0·5 ml.) was added dropwise; the pH was kept at 8·5 by occasional addition of N-sodium hydroxide. After 1½ hr. the ethyl acetate layer was separated, a drop of pyridine was added, and the solution was washed with water and brine and dried (Na_2SO_4). To remove a little *S*-benzylthiomethylcysteine, a few drops of dicyclohexylamine were added, the solution was cooled to 0°, and the fine precipitate was then filtered off. Evaporation of the filtrate gave a syrup, which was taken up in ether; addition of light petroleum (b. p. 40—60°) gave crystalline *ester* (1·0 g., 75%). Recrystallisation twice from methanol containing a little water gave product, m. p. 70—70·5°, $[\alpha]_D^{24}$ −34° (*c* 2·0 in EtOAc) (Found: C, 59·1; H, 5·6; N, 3·6; S, 16·2. $C_{20}H_{23}NO_4S_2$ requires C, 59·3; H, 5·7; N, 3·5; S, 15·8%).

The Stability of S-*Benzylthiomethyl*-L-*cysteine in* 2N-*Hydrogen Bromide in Acetic Acid.*—The amino-acid (200 mg.) was dissolved in 2N-hydrogen bromide in acetic acid (10 ml.) at 20°. After 45 min., the solution was concentrated in a rotary evaporator (0·1 mm. pressure, below 20°). Precipitation of the product was completed by the addition of dry ether; paper chromatography (descending flow, in Partridge's solvent,[13] n-butanol–acetic acid–water, 40 : 10 : 50 by volume, Whatman No. 1 paper; in this system *S*-benzylthiomethyl-L-cysteine has R_F 0·85) showed a very faint additional spot of R_F 0·09. A similar experiment, continued for 15 hr., gave a stronger spot of R_F 0·09 and a very faint spot of R_F 0·13. In quantitative experiments the solution was evaporated to dryness after 1 hr. at 21°, dry ether was added and the product was collected on a sintered-glass filter, washed with ether, and sucked dry. The residue was washed thoroughly with small volumes of water (5 ml. in all), sucked dry, washed finally with ether, and dried to constant weight. 0·321 g. of *S*-benzylthiomethyl-L-cysteine gave 0·304 g. (95%) and 0·298 g. (93%), respectively, of recovered starting material, chromatographically pure (BWA); $[\alpha]_D^{23\cdot5}$ −48° (*c* 1·0 in MeOH–6N-HCl, 9 : 1 by volume) (Found: C, 51·2; H, 6·0;

[13] Partridge, *Biochem. J.*, 1948, **42**, 238.

N, 5·5; S, 24·7%). After 15 hr., the recovery of chromatographically pure material was 0·270 g. (84%) and 0·263 g. (82%), respectively.

General Procedure for Removing Benzyloxycarbonyl Groups from S-*Benzylthiomethylcysteine Derivatives*.—Sufficient hydrogen bromide in acetic acid (*ca.* 4·5N; standardised by titration with silver nitrate) to provide 0·004 mole of hydrogen bromide for each benzyloxycarbonyl group was added to a magnetically-stirred suspension of the benzyloxycarbonyl derivative (0·001 mole) in ethyl methyl sulphide (0·91 ml.) and acetic acid; the amount of acetic acid (AnalaR in each case) was such that the hydrogen bromide was 2N in the reaction mixture. After the required time at room temperature the solution was taken to dryness in a rotary evaporator (0·1 mm. pressure; bath temperature below 20°). The residue was taken up in water (10 ml.) and the aqueous solution was extracted with ethyl acetate (3 × 5 ml.). The concentration of acetic acid in the aqueous layer was brought to *ca.* 25%, and the solution was passed through a column of Dowex-3 acetate (equivalent to 0·008 mole of hydrogen bromide, and previously equilibrated with 25% acetic acid). The eluate was taken to dryness as before, and the product was treated as described in each case.

The time required for debenzyloxycarbonylation was determined for each compound by means of paper chromatography; unchanged material was detected on the paper by the procedure of Handford, Welford, and Young;[10] at various time intervals an aliquot was removed and added to a test tube containing an excess of Dowex-3 acetate covered with BWA. An aliquot part of this solution was spotted on paper and chromatographed using BWA as solvent. The paper was then dried and supported in a concave fashion 9 cm. from a Hanovia 501/1 120 w. ultraviolet lamp. Exposure for 90 min. was usually satisfactory, and the paper was then sprayed with ninhydrin solution; over-exposure gives much background colour. An identical chromatogram was sprayed with ninhydrin but without irradiation, to confirm that no spot appeared in the same position as the benzyloxycarbonyl derivative.

Removal of the N-*Benzyloxycarbonyl Group from* N-*Benzyloxycarbonyl*-S-*benzylthiomethyl*-L-*cysteine*. *N*-Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine (0·391 g.) was treated with hydrogen bromide in acetic acid for 30 min. according to the general procedure, except that the residue after the evaporation was collected and washed as in the quantitative experiments on stability described above; the use of the Dowex-3 acetate column was unnecessary in this case. The *S*-benzylthiomethyl-L-cysteine (0·236 g., 92%) was chromatographically pure (BWA) and had $[\alpha]_D^{23}$ −48·5° (*c* 1·0 in MeOH–6N-HCl, 9 : 1 by volume) (Found: C, 51·1; H, 6·2; N, 5·4; S, 24·7%).

General Procedure for the Removal of the S-*Benzylthiomethyl Group*.—The *S*-benzylthiomethyl derivative (0·001 mole) was dissolved in formic acid (98—100%; 20 ml.) and added to a partial suspension of powdered mercuric acetate (1·28 g., 0·004 mole) in water (5 ml.) with swirling, giving a clear solution. After the required time at room temperature, the solution was stirred (magnetically) and ethanedithiol (0·84 ml., 0·01 mole) was added. After 15 min., hydrogen sulphide was passed in, and the precipitated mercuric sulphide was filtered off and washed with small volumes of 80% formic acid. The filtrate and washings were taken to dryness below 20°. The residue was treated as described in each case. The time required for the reaction with mercuric acetate was determined for each compound by means of paper chromatography using BWA for compounds with a free amino-group and n-butanol saturated with 2N-ammonium hydroxide for *N*-benzyloxycarbonyl derivatives; the latter were detected by the irradiation procedure.

Conversion of S-*Benzylthiomethyl*-L-*cysteine into* L-*Cystine* (*with* J. Hollowood).—*S*-Benzylthiomethyl-L-cysteine (0·321 g.) was treated by the above general procedure, except that in this case the mercuric acetate was partially dissolved in water (4 ml.), and formic acid (98—100%; 16 ml.) was added to give a solution which was then added to a solution of the *S*-benzylthiomethyl derivative in 80% formic acid (5 ml.); the cleavage stage required 5 min. (Solutions of mercuric acetate in formic acid must not be heated). The residue from freeze-drying was taken up in water, the solution was extracted with ethyl acetate, the aqueous layer was separated, and sodium hydroxide was added to give pH 8. Air was passed in until the solution gave a negative nitroprusside test; the pH was brought to 6 and the L-cystine {0·12 g., 80%, $[\alpha]_D^{21}$ −224° (*c* 1·0 in N-HCl), chromatographically pure (BWA)} was collected from the cooled solution.

N-*Benzyloxycarbonylglycyl*-S-*benzylthiomethyl*-L-*cysteine Methyl Ester*.—To a solution of *S*-benzylthiomethyl-L-cysteine methyl ester hydrochloride (6·16 g.) and 1-methylpiperidine

(2·41 ml.) in acetonitrile (50 ml.) was added benzyloxycarbonylglycine (4·18 g.) in acetonitrile (50 ml.) and then dicyclohexylcarbodi-imide (4·13 g.). After stirring for 4 hr., a few drops of acetic acid were added, the solution was filtered and then evaporated to dryness; the residue was taken up in ethyl acetate, filtered, washed, and dried in the usual way. Evaporation gave an oil which was crystallised from warm ether, giving needles (7·80 g., 84%). Recrystallisation from ether gave protected *dipeptide*, m. p. 59—61°, $[\alpha]_D^{23}$ −21° (*c* 1·0 in ethyl acetate), $[\alpha]_D^{25}$ −29° (*c* 1·0 in dimethylformamide) (Found: C, 57·1; H, 5·5; N, 6·0; S, 13·7. $C_{22}H_{26}N_2O_5S_2$ requires C, 57·1; H, 5·7; N, 6·1; S, 13·9%). Hiskey and Tucker [7] report this compound as an oil.

N-*Benzyloxycarbonylglycyl*-S-*benzylthiomethyl*-L-*cysteine*.—The above methyl ester (9·25 g.) was hydrolysed in dioxan (100 ml.) and N-sodium hydroxide (22 ml.) for 1 hr. at room temperature. Ether (100 ml.), water (78 ml.), and N-hydrochloric acid (22 ml.) were added, the ether layer was separated, and the aqueous layer extracted again with ether (2 × 100 ml.). The ether extracts were washed with brine until neutral and dried. Dicyclohexylamine (3·99 g.) in ether (20 ml.) was added and the precipitate was collected and recrystallised from chloroform–light petroleum (b. p. 40—60°), giving needles of the *dicyclohexylammonium salt* (10·63 g., 84%), m. p. 125—127°, $[\alpha]_D^{23}$ +22° (*c* 1·0 in chloroform) (Found: C, 62·7; H, 7·4; N, 6·6; S, 10·1. $C_{33}H_{47}N_3O_5S_2$ requires C, 62·9; H, 7·5; N, 6·7; S, 10·2%).

The free acid was obtained by shaking the salt (12·60 g.) with ethyl acetate (100 ml.) and N-sulphuric acid (100 ml.) until dissolution was complete. The layers were separated, the aqueous layer was extracted again with ethyl acetate (2 × 100 ml.), and the combined extracts were washed with brine and dried. The solution was concentrated to *ca.* 50 ml., then warmed to 50° and di-isopropyl ether was added to cloudiness which was cleared by warming. The *acid* (7·84 g., 87%) separated as rosettes, m. p. 107—108°, raised by recrystallisation to m. p. 108·5—110°, $[\alpha]_D^{22}$ −25° (*c* 1·0 in ethyl acetate), $[\alpha]_D^{22}$ −29° (*c* 1·0 in acetone), $[\alpha]_D^{23}$ −32° (*c* 1·0 in dimethylformamide) (Found: C, 56·1; H, 5·3; N, 6·5; S, 14·6. $C_{21}H_{24}N_2O_5S_2$ requires C, 56·2; H, 5·4; N, 6·3; S, 14·3%). In early experiments we obtained material which melted first at 64°, solidified on the block and melted finally at 107—108°; recrystallisation gave product melting only at 108—110°. Hiskey and Tucker [7] give m. p. 127—128° for product recrystallised from acetone–ether; from this solvent our material had m. p. 107—108°.

Di(*benzyloxycarbonylglycyl*)-L-*cystine*.—*N*-Benzyloxycarbonylglycyl-*S*-benzylthiomethyl-L-cysteine (0·449 g.) was cleaved by mercuric acetate (reaction time, 15 min.) according to the general procedure. The residue from the final evaporation was taken up in half-saturated sodium hydrogen carbonate (10 ml.), and the solution was extracted with ethyl acetate (3 × 5 ml.). The aqueous layer was aerated until the nitroprusside test was negative (*ca.* 3 hr.), when the solution was made acid to Congo Red with 4N-hydrochloric acid (with cooling); the product was extracted into ethyl acetate, and the solution was dried and evaporated to dryness (below 20°). The residue was swirled with AnalaR acetone (10 ml.) until all had dissolved (no heat), the flask was stoppered and set aside at room temperature. After 24 hr. the crystals were collected and washed with acetone; concentration of the mother-liquors gave a further crop; total yield of di(benzyloxycarbonylglycyl)-L-cystine, 0·241 g. (78%), m. p. 136—138°, $[\alpha]_D^{21}$ −90° (*c* 1·0 in dimethylformamide) (Found: C, 50·2; H, 4·9; N, 9·3; S, 10·4. Calc. for $C_{26}H_{30}N_4O_{10}S_2$: C, 50·2; H, 4·8; N, 9·0; S, 10·3%). Greenstein [14] gives m. p. 142°. Omission of the ethanedithiol from the removal procedure gave a 43% yield of low m. p. material.

Glycyl-S-*benzylthiomethyl*-L-*cysteine*.—*N*-Benzyloxycarbonylglycyl-*S*-benzylthiomethyl-L-cysteine (0·449 g.) was debenzyloxycarbonylated by the general procedure for 20 min. The final residue after evaporation was swirled with boiling ethanol (10 ml.) and after cooling the solid was collected, washed with ethanol, and recrystallised from hot water (10 ml.), giving the *dipeptide* as colourless plates (0·255 g., 81%), m. p. 192—194°, $[\alpha]_D^{22}$ −23° (*c* 1·0 in N-HCl) (Found: C, 49·8; H, 6·0; N, 9·2; S, 20·2. $C_{13}H_{18}N_2O_3S_2$ requires C, 49·7; H, 5·8; N, 8·9; S, 20·4%).

Diglycyl-L-*cystine*.—(a) *From di*(*benzyloxycarbonylglycyl*)-L-*cystine*. The dibenzyloxycarbonyl derivative (0·311 g., 0·0005 mole) was treated with hydrogen bromide in acetic acid by the general procedure but using 4 times the stated volumes of ethyl methyl sulphide, acetic acid, and hydrogen bromide in acetic acid; the peptide derivative did not dissolve but coagulated, and the mixture was shaken until the sticky solid was replaced by needles of the

[14] Greenstein, *J. Biol. Chem.*, 1939, **128**, 241.

dihydrobromide. After a further 5 min. the product was isolated in the usual way, but using 5% acetic acid for the chromatography on Dowex-3 acetate. The residue left after evaporation of the eluate was triturated with ethanol, giving diglycyl-L-cystine (0·158 g., 89%), $[\alpha]_D^{22}$ −115° (*c* 1·0 in N-HCl); Greenstein [14] gives $[\alpha]_D^{24}$ −108° (*c* 0·75 in N-HCl).

(b) *From glycyl-S-benzylthiomethyl-L-cysteine.*—The *S*-benzylthiomethyl derivative (0·314 g.) was cleaved by mercuric acetate (reaction time, 20 min.) according to the general procedure. The residue after the final evaporation was taken up in water (10 ml.) and extracted with ethyl acetate (3 × 5 ml.); the aqueous layer was brought to pH *ca.* 8·5 by the addition of pyridine (1 ml.) and 2,4,6-collidine (1 ml.) and the solution was aerated until the nitroprusside test was negative (up to 24 hr.), and evaporated to dryness (below 20°). The residue was triturated with ethanol and then dissolved in aqueous dimethyl sulphoxide; slow removal of water in a rotary evaporator (0·1 mm. pressure) gave peptide (0·106 g., 60%), $[\alpha]_D^{22}$ −108° (*c* 1·0 in N-HCl).

N-Benzyloxycarbonyl-S-benzylthiomethyl-L-cysteinylglycine Ethyl Ester.—*N*-Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine dicyclohexylammonium salt (11·46 g.) was stirred vigorously with a suspension of finely powdered glycine ethyl ester hydrochloride (2·79 g.) in dichloromethane (100 ml.). When the formation of fluffy needles of dicyclohexylammonium chloride was complete, dicyclohexylcarbodi-imide (4·13 g.) in dichloromethane (20 ml.) was added. The usual procedure gave a solution of the coupling product in ethyl acetate, which was evaporated to *ca.* 50 ml. and warmed to 50°; di-isopropyl ether was then added to cloudiness which was cleared by warming. White needles separated (7·92 g., 83%) and recrystallisation from the same solvents gave *protected dipeptide*, m. p. 103—104°, $[\alpha]_D^{21}$ −45° (*c* 1·0 in ethyl acetate), $[\alpha]_D^{23}$ −55° (*c* 1·0 in dimethylformamide) (Found: C, 57·6; H, 5·9; N, 5·8; S, 13·7. $C_{23}H_{28}N_2O_5S_2$ requires C, 57·9; H, 5·9; N, 5·9; S, 13·5%).

N-Benzyloxycarbonyl-S-benzylthiomethyl-L-cysteinylglycine.—The above ethyl ester was hydrolysed as described above for the glycylcysteine analogue, and again the product was isolated as the *dicyclohexylammonium salt*, which was recrystallised from ethyl acetate, giving white needles (8·58 g., 68%), m. p. 127—129°, $[\alpha]_D^{25}$ −37·5° (*c* 1·0 in methanol), $[\alpha]_D^{24}$ −40° (*c* 1·0 in dimethylformamide) (Found: C, 62·8; H, 7·6; N, 6·7; S, 10·3. $C_{33}H_{47}N_3O_5S_2$ requires C, 62·9; H, 7·5; N, 6·7; S, 10·2%). The *acid* was liberated from the salt as described above for the analogue, except that the final solution in ethyl acetate was evaporated to dryness, and the remaining solid was crystallised from aqueous dimethylformamide, giving needles (7·62 g., 85%), m. p. 88·5—90°, $[\alpha]_D^{22}$ −52° (*c* 1·0 in dimethylformamide) (Found: C, 56·3; H, 5·2; N, 6·6; S, 14·4. $C_{21}H_{24}N_2O_5S_2$ requires C, 56·2; H, 5·4; N, 6·3; S, 14·3%).

Dibenzyloxycarbonyl-L-cystinyldiglycine.—*N*-Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteinylglycine (0·449 g.) was cleaved by mercuric acetate (reaction time, 15 min.) according to the general procedure. The product was isolated as described for the glycylcysteine analogue, except that crystallisation began when the volume of the final solution in ethyl acetate had been reduced to 10 ml.; a further quantity was obtained by concentration of the mother-liquor. The crystals were washed with ethyl acetate, giving di(benzyloxycarbonyl)-L-cystinyldiglycine (0·246 g., 79%), m. p. 181—183°, $[\alpha]_D^{23}$ −153° (*c* 1·0 in dimethylformamide), $[\alpha]_D^{25}$ −132° (*c* 1·0 in methanol); lit., m. p. 161—163°,[15] 182—183°,[16] 176—177°; $[\alpha]_D^{24}$ −129° (in methanol) [17] (Found: C, 49·9; H, 4·9; N, 9·0; S, 10·5. Calc. for $C_{26}H_{30}N_4O_{10}S_2$: C, 50·2; H, 4·8; N, 9·0; S, 10·3%).

S-Benzylthiomethyl-L-cysteinylglycine.—*N*-Benzyloxycarbonyl-*S*-benzylthiomethyl-L-cystinylglycine (0·449 g.) was debenzyloxycarbonylated by the general procedure (20 min.). The final residue was dissolved rapidly in hot ethanol (10 ml.), from which on cooling the *dipeptide* separated as very small white needles; recrystallisation from ethanol–water (3 : 7 by volume; 10—15 ml.) gave needles (0·222 g., 71%), m. p. 197—199°, $[\alpha]_D^{22}$ +34·5° (*c* 1·0 in N-HCl) (Found: C, 49·7; H, 6·0; N, 8·9; S, 20·6. $C_{13}H_{18}N_2O_3S_2$ requires C, 49·7; H, 5·8; N, 8·9; S, 20·4%).

L-*Cystinyldiglycine.*—(a) *From dibenzyloxycarbonyl-L-cystinyldiglycine.* The dibenzyloxycarbonyl derivative (0·311 g.) was treated with hydrogen bromide in acetic acid by the procedure described for the glycylcysteine analogue, giving L-cystinyldiglycine (0·140 g., 79%), $[\alpha]_D^{20\cdot5}$ −89° (*c* 1·0 in N-HCl); lit.,[16] $[\alpha]_D^{27}$ −86·0° (*c* 1 in N-HCl).

(b) *From S-benzylthiomethyl-L-cysteinylglycine.* The *S*-benzylthiomethyl derivative

[15] White, *J. Biol. Chem.*, 1934, **106**, 141.
[16] Loring and du Vigneaud, *J. Biol. Chem.*, 1935, **111**, 385.
[17] King and Suydam, *J. Amer. Chem. Soc.*, 1952, **74**, 5499.

(0·314 g.) was cleaved by mercuric acetate (reaction time, 20 min.) according to the general procedure. The L-cystinyldiglycine was isolated as described for diglycyl-L-cystine, except that precipitation was from aqueous dimethylformamide, giving peptide (0·100 g., 57%), $[\alpha]_D^{21}$ −87° (*c* 1·0 in N-HCl).

N-*Benzyloxycarbonyl*-S-*benzylthiomethyl*-L-*cysteinyl*-L-*tyrosine Methyl Ester*.—A solution of *N*-benzyloxycarbonyl-*S*-benzylthiomethyl-L-cysteine dicyclohexylammonium salt (1·14 g.) in dry dichloromethane (2 ml.) was added to a fine suspension of L-tyrosine methyl ester hydrochloride [6] (0·46 g.) in dichloromethane (20 ml.). The mixture was stirred at room temperature and dicyclohexylcarbodi-imide (0·45 g.) was added. After 4 hr. a drop of acetic acid was added and the solid dicyclohexylurea was filtered off and washed thoroughly with ethyl acetate. The filtrate and washings were evaporated to dryness, and the residue taken up in ethyl acetate and washed and dried as usual. Evaporation left a syrup, which was dissolved in cold methanol; careful addition of water yielded soft needles of the *ester* (0·80 g., 71%), m. p. 110—112°. Two recrystallisations from methanol–water raised the m. p. to 112—114°, $[\alpha]_D^{18}$ −37° (*c* 2·6 in methanol). Before analysis the specimen was dried for 20 hr. at 78°/0·03 mm. (Found: C, 61·3; H, 5·5; N, 5·2; S, 11·1. $C_{29}H_{32}N_2O_6S_2$ requires C, 61·4; H, 5·7; N, 4·9; S, 11·3%).

N-*Benzyloxycarbonyl*-S-*benzylthiomethyl*-L-*cysteinyl*-L-*tyrosylhydrazide*.—The corresponding methyl ester (2·85 g.) was dissolved in methanol (free from carbonyl compounds; 50 ml.) and hydrazine hydrate (100%; 0·77 ml.) was added dropwise. Next day the solid obtained on cooling to 0° was collected, and concentration of the filtrate gave more crude product (98% in all). Recrystallisation from n-propanol (free from carbonyl compounds) gave needles of *hydrazide* (2·12 g., 75%), m. p. 192·5—194·5°, $[\alpha]_D^{23}$ −46° (*c* 1·8 in dimethylformamide) (Found: C, 59·1; H, 5·9; N, 9·9; S, 11·6. $C_{28}H_{32}N_4O_5S_2$ requires C, 59·1; H, 5·7; N, 9·85; S, 11·3%).

S-*Phenylthiomethyl*-L-*cysteine* (*with* R. G. Nichols *and* J. Hollowood).—The use of phenylthiomethyl chloride [12] in the normal procedure [method (a)] gave the *amino-acid* in 88% yield. It was recrystallised from hot dilute hydrochloric acid giving needles, m. p. 187—188° (decomp.), $[\alpha]_D^{18}$ −48° (*c* 1·0 in MeOH–6N-HCl, 9 : 1 by volume), R_F 0·66 (BWA) (Found: C, 49·8; H, 5·6; N, 5·8; S, 26·4. $C_{10}H_{13}NO_2S_2$ requires C, 49·7; H, 5·4; S, 26·3%).

Conversion of S-*Phenylthiomethyl*-L-*cysteine into* L-*Cystine* (*with* J. Hollowood).—The procedure described above for *S*-benzylthiomethyl-L-cysteine (using mercuric acetate in 80% formic acid, with addition of ethanedithiol) gave L-cystine in 74% yield. Chromatography (PW) of the filtrate showed the presence of thiazolidine-4-carboxylic acid, R_F 0·75.

S-*Isobutoxymethyl*-L-*cysteine* (*with* R. Purkayastha).—The use of isobutoxymethyl chloride [18] in the normal procedure [method (a)] gave the *amino-acid* in 82% yield. The product was recrystallised from 0·1N-hydrochloric acid, which yielded lustrous plates, m. p. 210—214° (decomp.), $[\alpha]_D^{24}$ +12° (*c* 1·0 in 0·1N-HCl), R_F 0·70 (BWA), 0·86 (PW) (Found: C, 46·3; H, 8·6; N, 7·0; S, 15·6. $C_8H_{17}NO_3S$ requires C, 46·35; H, 8·3; N, 6·8; S, 15·5%).

The compound remained chromatographically homogeneous after treatment at room temperature with 2N-hydrochloric acid or 50% acetic acid for 4 hr., but showed decomposition after 2 hr. in 2N-sodium hydroxide, and decomposed rapidly in 2N-hydrogen bromide in acetic acid.

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[18] Hill and Keach, *J. Amer. Chem. Soc.*, 1926, **48**, 257.