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Synthesis of α-Methyl- and β-Methyl-DL-Cystine

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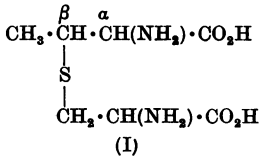
(*Received* 3 *July* 1957)

In view of the utilization of L-cystine as a precursor of the β-lactam ring of penicillin (Arnstein & Grant, 1954) it was of interest to study penicillin biosynthesis in the presence of compounds related to cystine. For this purpose, α-methyl- and β-methyl-cystine were required and the present paper deals with their synthesis. For some experiments, it was necessary to use isotopic labelling in order to detect any possible conversion of the substituted cystines into $C_{(5)}$- or $C_{(6)}$-substituted penicillins, which might be biologically inactive, and these two amino acids were therefore also labelled with ^{14}C, as described below. As will be shown in the following paper, no penicillin-like compounds were in fact formed, but α-methylcystine was found to inhibit penicillin production by washed mycelium of *Penicillium chrysogenum*.

β-Methylcysteine has recently been found to occur in the antibiotic subtilin as the *S*-(L-2-amino-2-carboxyethyl) derivative (I) (Alderton, 1953), but the configuration of the asymmetric β-carbon atom was not elucidated. The stereochemistry of β-methylcysteine is thus of some interest in connexion with the structure of this antibiotic.

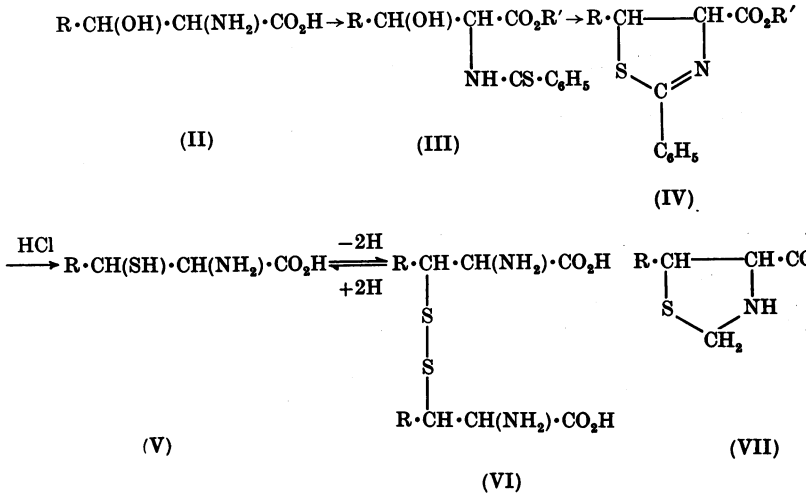


(I)

Synthesis of β-methyl-DL-cysteine and its disulphide from allo*threonine and threonine*

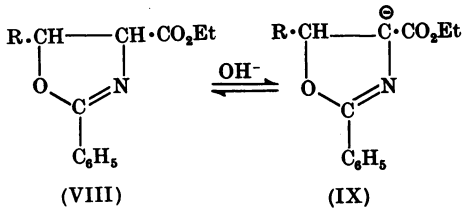
Of the four possible stereoisomers of β-methylcysteine (V, R = CH_3) which correspond in configuration to the L- and D-enantiomorphs of threonine and *allo*threonine, both pairs of DL-isomers (named isomers *A* and *B* of thiothreonine) have been synthesized by Carter, Stevens & Ney (1941), but their stereochemical relationship was not investigated. It was hoped to clarify this problem in the present work by applying the recent synthesis of cystine (VI, R = H) from serine (II, R = H) via the thiobenzoyl ester (III, R = H, R′ = C_2H_5) and thiazoline ester (IV, R = H, R′ = C_2H_5) derivatives (Crawhall & Elliott, 1951) to the preparation of β-methylcystine (VI, R = CH_3) from DL-threonine and DL-*allo*threonine (II, R = CH_3).

In view of the inversion at the β-carbon atom during ring closure to the thiazoline (IV, R = CH_3, R′ = C_2H_5), it was anticipated that this synthesis would yield the *erythro* and *threo* forms of DL-β-methylcysteine (V, R = CH_3) respectively. It was



found, however, that both threonine and *allo*-threonine unexpectedly gave rise to the same α-amino-β-mercaptobutyric acid (V), although, under identical conditions, a better yield was obtained from the latter. This amino acid (V) was identified as isomer *B* of thiothreonine (Carter *et al.* 1941) by mixed melting-point determinations and X-ray powder photographs of the thiazolidine (VII, R = CH_3) obtained by reaction with formaldehyde (Cook & Heilbron, 1949).

It seems likely that in the synthesis from threonine the intermediate *cis*-2-phenylthiazoline ethyl ester (IV, R = CH_3; R′ = C_2H_5) was converted into the *trans*-isomer. Such an isomerization is well known (see Elliott, 1953) for the corresponding oxazolines (VIII, where R may be methyl, phenyl,



pentadecyl or hydroxymethyl), but a strong base is required for this reaction. The ester anion (IX) is probably formed as an intermediate, whilst steric factors are believed to be responsible for the preferential formation of the *trans*-oxazoline from this intermediate (Elliott, 1949), and a similar mechanism may account for the conversion of *allo*-threonine and threonine into the same thiolamino acid.

The overall yield (20 %) of β-methylcystine from thiobenzoyl*allo*threonine ethyl ester is considerably lower than that obtained in the analogous synthesis of cystine (47 %; Crawhall & Elliott, 1951), probably due to formation of relatively large amounts of the oxazoline. Although the oxazoline was not isolated, its presence is indicated by the recovery of substantial quantities of α-amino-β-hydroxybutyric acid from the acid hydrolysis of the cyclization product. Moreover, cyclization of thiobenzoylhydroxyaspartic acid ester has been shown to result in the formation of the oxazoline as well as the thiazoline (Hauptmann & Berl, 1955). Substitution of the carbon atom carrying the hydroxyl group thus seems to favour cyclization to the oxazoline rather than to the thiazoline, at least when thionyl chloride is used. This is reminiscent of the reaction of amino alcohols with carbon disulphide and alcoholic alkali, where substitution of the carbinol carbon atom also results in oxazoline formation, as discussed by Crawhall & Elliott (1952).

The assumption that synthesis of α-amino-β-mercapto acids from the corresponding hydroxyamino acids via a thiazoline results only in inversion of configuration at the β-position, which has been made, for example, in a recent paper on the preparation of cystine-ββ′-dicarboxylic acid from *erythro*hydroxyaspartic acid (Hauptmann & Berl, 1955), is thus not valid. It is suggested that, in the present work, the *trans*-thiazoline was formed from both *allo*threonine and threonine; in the latter case, inversion of the β-configuration must have been followed by one at the α-position, probably by the mechanism discussed above. The formation of only one form of β-methylcystine would thus be accounted for and it is concluded from comparison of this compound with the *A* and *B* isomers of thiothreonine (Carter *et al.* 1941) that these are the *erythro*- and *threo*-isomers of β-methylcysteine

respectively. In the synthesis of cystine-ββ′-dicarboxylic acid mentioned above, both the *erythro* configuration of the starting material and saponification of the thiazoline ester with strong potassium hydroxide would favour formation of the *trans*-thiazoline carboxylic acid (see Elliott, 1953) and designation of the configuration of the product as *threo* (Hauptmann & Berl, 1955) is therefore not invalidated by the present work.

Synthesis of α-methyl-DL-cystine

Benzylthioacetone (cf. Wahl, 1922) was converted into the amino nitrile, which was hydrolysed without purification of the crude product, giving *S*-benzyl-α-methyl-DL-cysteine in 70% yield. After the present work was completed, Potts (1955) reported a synthesis of the latter, also from benzylthioacetone but via the hydantoin instead of the amino nitrile, the yield by this method being about 65%.

Reduction of the *S*-benzyl group by sodium in liquid ammonia (Wood & du Vigneaud, 1939) and oxidation of the product by air in the presence of a trace of ferric chloride at pH 8 gave α-methyl-DL-cystine. By using $K^{14}CN$ for synthesis of the amino nitrile, α-methyl-DL-[*carboxy*-^{14}C]cystine was prepared.

One noteworthy property of α-methylcysteine, and probably also of other α-substituted amino acids, is the very slow colour reaction with ninhydrin. Although the α-hydrogen atom of an amino acid is apparently not essential for reaction with ninhydrin (Spenser, Crawhall & Smyth, 1956), substitution of the α-position by a methyl group evidently has a profound effect on the rate of reaction.

EXPERIMENTAL

Radioactivity measurements. All samples were counted on 1 cm.^{2} polythene disks at infinite thickness, by using a thin-end-window Geiger-Müller counter (Popják, 1950). The specific radioactivities were obtained by direct comparison with a poly[^{14}C]methyl methacrylate standard containing 1 μc/g., which was obtained from the Radiochemical Centre, Amersham.

Paper chromatography. All R_F values refer to descending chromatograms on Whatman no. 1 paper. The solvent systems had the following composition (by vol.): *A*, phenol–water, 5:2 (with NH_3); *B*, pentan-1-ol–pyridine–water, 7:7:6; *C*, butanol–acetic acid–water, 63:10:27.

Synthesis of DL-[carboxy-^{14}C]allo*threonine*

[carboxy-^{14}C]*Butyric acid.* Propyl iodide (34 g., 0·2 mole, of material dried over $CaCl_2$ and freshly redistilled, b.p. 102–104°) was dissolved in ether (500 ml.) and added slowly to ether-washed Mg turnings (4·8 g.). When the initial reaction had subsided, the mixture was warmed on a water bath for 30 min. The yield of Grignard reagent, which was determined by adding a small portion of the above solution to excess of 0·1 N-HCl and back-titrating with 0·1 N-NaOH, was 90%. Carbonation of the Grignard reagent was carried out essentially as described by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949), by using $^{14}CO_2$ from $Ba^{14}CO_3$ (12 g., 0·15 mole; 1·5 mc). When absorption of CO_2 was complete, the product was poured on crushed ice (750 g.) and acidified with conc. H_2SO_4 (18 ml.) in water (150 ml.). The aqueous phase was saturated with NaCl and extracted with ether (3 × 200 ml.). After drying with Na_2SO_4, the ether was evaporated under reduced pressure. The residue was distilled at atmospheric pressure, giving 7 g. (53%, based on CO_2) of product, b.p. 160–162°.

iso*Butyl* α-*bromo*-[carboxy-^{14}C]*butyrate.* The labelled butyric acid was brominated in the presence of dry P (1 g.) by slow addition of dry Br_2 (8·5 ml.). The mixture was heated on the steam bath for 5 hr., kept overnight at room temp., and added dropwise to *iso*butanol (22·3 ml.) with stirring and cooling. The solution was boiled under reflux for 15 min., cooled and added to saturated $NaHCO_3$ (50 ml.) in a separating funnel. The bromo ester was separated and the aqueous solution extracted twice with ether, the ether extracts being mixed with the ester. The solution was dried ($CaCl_2$), the ether was evaporated and the residue distilled, giving 13·7 g. (78%) of *iso*butyl α-bromo-[*carboxy*-^{14}C]butyrate, b.p. 128–132° at 50 mm. Hg.

iso*Butyl* [carboxy-^{14}C]*crotonate.* The above bromo ester was added dropwise to boiling diethylaniline (20 g.) (Hunter & Popják, 1951), the last traces being added with a further 2 ml. of diethylaniline. The mixture was boiled under reflux for 23 hr., cooled, acidified with 6 N-HCl (9 ml.) and extracted twice with ether. The ether solution was dried (K_2CO_3 + $CaCl_2$) and the residue, after removal of ether, was distilled at 150 mm. Hg, giving 3·2 g. (37%) of product, b.p. 116–124°.

[carboxy-^{14}C]*Crotonic acid.* The above ester (3·2 g.) was refluxed with N-NaOH (35 ml.) for 2·5 hr. After cooling, the mixture was extracted twice with a little $CHCl_3$, acidified with 6 N-HCl (6 ml.) and extracted with ether. The ether solution was dried (Na_2SO_4) and the ether was evaporated in a stream of air. The residue was dried *in vacuo* over P_2O_5. The yield of crude [*carboxy*-^{14}C]crotonic acid, m.p. 76–78°, was 1·5 g. (78%), but on recrystallization from light petroleum (b.p. 60–80°; 5 ml.) only 0·88 g. was recovered. After successive additions of non-radioactive crotonic acid (0·8 g.; 1·0 g.; 2·0 g.), further crops of less active material (0·85 g., 1·3 g., and 1·7 g. respectively) were obtained, corresponding to a total recovery of 89% on crystallization.

α-*Bromo*-β-*hydroxy*[carboxy-^{14}C]*butyric acid.* The labelled crotonic acid (4·6 g.) obtained above was dissolved in 160 ml. of water and gaseous Br_2, generated from Br_2 (9 g.) by a stream of N_2, was passed through the solution for 5·5 hr. (cf. Carter & Zirkle, 1949). The solution was concentrated *in vacuo* (bath temp. 40–50°) to 20 ml. and extracted with benzene (5 × 40 ml.) to remove αβ-dibromobutyric acid, m.p. 88° (Found: Br, 65·3, Calc. for $C_4H_6O_2Br_2$: Br, 65·0%). After evaporation of the aqueous layer, the residue failed to crystallize and was therefore dissolved in water (30 ml.) and again extracted with benzene (5 × 30 ml.). Evaporation of the aqueous solution to dryness (bath temp. <50°) yielded crude α-bromo-β-hydroxybutyric acid, m.p. 70–75°. After recrystallization from benzene by addition of light petroleum (b.p. 60–80°), α-bromo-β-hydroxy[*carboxy*-^{14}C]butyric acid (3·1 g., 32%; m.p. 84–86°; mixed m.p. with αβ-dibromobutyric acid, 63–66°) was obtained.

DL-[carboxy-^{14}C]allo*Threonine*. The above α-bromo-β-hydroxy[*carboxy*-^{14}C]butyric acid (3 g.) was mixed with carrier (5 g.) and aminated at room temp. with a mixture of $(NH_4)_2CO_3$ (24 g.), conc. aq. NH_3 soln. (80 ml.) and water (32 ml.). After 24 hr. the mixture was heated at 60–65° for 6 hr. and then evaporated to dryness. The residue was dissolved in hot water (25 ml.) and boiling ethanol (250 ml.) was added. After standing overnight at 4°, 4·8 g. of product was obtained. Recrystallization from hot water (15 ml.) by adding boiling ethanol (150 ml.) gave 4·5 g. (87%) of DL-[*carboxy*-^{14}C]*allo*threonine, m.p. 241–242°, specific radioactivity 11·2 μc/g. The overall chemical and radiochemical yields from $Ba^{14}CO_3$ were 3·0 and 3·4% respectively.

Conversion of allo*threonine into β-methylcysteine (thiothreonine, isomer* B*) and its disulphide*

DL-[*carboxy*-^{14}C]*allo*Threonine (2·1 g., 1·34 μc/m-mole, 23·3 μc) was suspended in dry ethanol (50 ml.). Dry HCl gas was passed through for 15 min., when the ethanol was boiling. After cooling in ice, the solution was saturated with HCl and left at room temp. overnight. The ethanol was removed *in vacuo*, the residue was dried in a desiccator and seeded. The crystalline product was washed with dry ether; yield 3·2 g. (99%). The ester hydrochloride was dissolved in pyridine (50 ml.) and triethylamine (3·6 g.) and (thiobenzoylthio)acetic acid (4·2 g., prepared as described by Crawhall & Elliott, 1951) were added. After 24 hr., the reaction mixture was added to water (500 ml.) and extracted with ether (150 ml., 75 ml. and 75 ml.). The ethereal extracts were combined, washed with water, 2N-HCl (7 × 6 ml.), saturated $NaHCO_3$ and water (10 ml.). The ether layer was separated, dried (Na_2SO_4) and the ether was evaporated. The residue was dried in a desiccator overnight and crystallized from ether–light petroleum (b.p. 40–60°). The yield of *N*-thiobenzoyl*allo*threonine ethyl ester, m.p. 80–82°, was 3·1 g. (66%). The solid was finely powdered and added to redistilled $SOCl_2$ (5·8 ml.). After 15 min., the $SOCl_2$ was evaporated *in vacuo* at <30° and the residue was triturated with ether, when it crystallized. The crystalline solid was washed with ether, dissolved in $CHCl_3$ and treated with charcoal. After filtering, the $CHCl_3$ was evaporated, giving a cryst. product (3·5 g.), which was hydrolysed by boiling under reflux with 3N-HCl (120 ml.) for 18 hr. Benzoic acid was removed by extraction with ether and the aqueous solution was concentrated to dryness by evaporation *in vacuo*. Paper chromatography (solvent *B*) showed the presence of approximately equal quantities of β-methylcysteine and *allo*threonine. The crude product was therefore chromatographed on a column (approx. 2·5 cm. diam.) containing 250 g. of Zeo-Karb 225 resin (Permutit Co. Ltd., London) in the H^+ form, 1·5N-HCl being used as eluent. Fractions of about 10 ml. each were collected; fractions no. 50–70 contained *allo*threonine together with small amounts of an unidentified ninhydrin-positive impurity (detected by paper chromatography with solvent system *B*), whilst β-methylcysteine was eluted later (fractions no. 110–200). Evaporation of the appropriate fractions yielded *allo*threonine hydrochloride (0·52 g.) and β-methylcysteine hydrochloride (0·64 g., 33%) respectively. The radioactivity of this material, measured after combustion to CO_2 and conversion into $BaCO_3$, was found to be 8·1 μc/g. (1·38 μc/m-mole), which is in good agreement with that of the starting material. The overall chemical and radiochemical yields from *allo*threonine were 19·8 and 22·3% respectively.

A portion of the amino acid hydrochloride was converted into the free amino acid by adsorption on Zeo-Karb 215 resin in the H^+ form (about 10 mg. of resin/mg. of amino acid) and elution with aq. 0·2N-NH_3 soln. This procedure also resulted in oxidation of the sulphydryl group to the disulphide. The eluate was evaporated to dryness *in vacuo* and the residue crystallized from water by adding ethanol, giving *β-methylcystine*, m.p. 193–194° (Found: C, 35·3; H, 6·6; N, 9·7; S, 24·9. $C_8H_{16}O_4N_2S_2$ requires C, 35·8; H, 6·0; N, 10·45; S, 23·9%). The R_F values were 0·58 in solvent *A*, 0·03 in solvent *B* and 0·06 in solvent *C*.

In another experiment, non-radioactive β-methylcystine (1·5 g.) was dissolved in conc. HCl (5 ml.) and reduced with Sn (1 g.). After dilution with approx. 50 ml. of water, the Sn was precipitated with H_2S, and the solution filtered and evaporated to dryness. The amino acid hydrochloride was converted into the free amino acid by treatment with Zeo-Karb 215 resin as described previously. Owing to the rapid oxidation of sulphydryl groups by air at an alkaline pH, this procedure resulted in the re-oxidation of β-methylcysteine to β-methylcystine (Found: C, 35·8; H, 6·2; N, 9·7; S, 24·7%). Several further attempts to obtain more satisfactory analyses for nitrogen and sulphur were unsuccessful, although no impurities could be detected by paper chromatography (solvent systems *A*, *B* and *C*).

Conversion of β-methylcysteine into 4*-carboxy-5-methylthiazolidine* (cf. Cook & Heilbron, 1949). Non-radioactive β-methylcysteine hydrochloride (0·59 g.), which had been prepared from non-radioactive *allo*threonine and chromatographed as described above, but with Dowex 50 ion-exchange resin (Microchemical Specialities Co., Berkeley, California), was dissolved in water (1·1 ml.), formalin (0·5 ml.) was added and the solution was kept at room temp. for about 18 hr. Pyridine (0·5 ml.) and ethanol (1 ml.) were added and the solution was cooled to 4°, when a crystalline product, m.p. 204°, separated. After recrystallization from water by addition of ethanol, pure 4-carboxy-5-methylthiazolidine (0·1 g., m.p. 209–210°) was obtained (Found: C, 40·9; H, 6·5; N, 8·9; S, 21·95. Calc. for $C_5H_9O_2NS$: C, 40·8; H, 6·1; N, 9·5; S, 21·8%). A simultaneous mixed m.p. determination with 4-carboxy-5-methylthiazolidine prepared from the *B* isomer of thiothreonine (cf. Carter *et al.* 1941) showed no depression, and the mixed m.p. with the corresponding derivative of thiothreonine, isomer *A*, was 192°.

Conversion of threonine into β-methylcysteine

DL-Threonine (10 g.) was suspended in dry ethanol (200 ml.) and dry HCl was passed in until the solution boiled. After cooling to 0°, the solution was saturated with HCl and kept at room temp. overnight. The ethanol was evaporated *in vacuo* and the residue was freed from excess of HCl in a vacuum desiccator over NaOH; the yield of crude DL-threonine ethyl ester hydrochloride was 16·1 g. The ester was dissolved in pyridine (200 ml.), redistilled triethylamine (17 g.) and (thiobenzoylthio)acetic acid (20 g.) were added and the mixture was allowed to stand at room temp. overnight. The reaction mixture was poured into water (2 l.) and extracted with ether (500 ml., 250 ml., 250 ml.) and the ether extracts were washed with water

(3 × 25 ml.), 2N-HCl until the aqueous phase was acid, saturated $NaHCO_3$ (25 ml.) and water (25 ml.). The ether solution was then dried (Na_2SO_4) and evaporated. The residue was crystallized from ether by adding light petroleum (b.p. 40–60°), giving *N-thiobenzoyl-DL-threonine ethyl ester*, m.p. 83–85° (12·8 g., 58%). After two further recrystallizations the m.p. was 84–85° (Found: C, 58·2, H, 6·6; N, 5·2; $C_{13}H_{17}O_3NS$ requires C, 58·4; H, 6·4; N, 5·2%).

The above ester (8·3 g.) was dissolved in $CHCl_3$ (200 ml.), the solution was cooled in ice and PCl_5 (15 g.) was added in small portions with shaking (see Hauptmann & Berl, 1955). After 30 min. at 0° and 30 min. at room temp., the $CHCl_3$ solution was extracted with sat. $NaHCO_3$, dried (Na_2SO_4) and evaporated. The crude thiazoline was hydrolysed and the product chromatographed on Zeo-Karb 225 resin, essentially as described above for the separation of β-methylcysteine from *allo*threonine. β-Methylcysteine hydrochloride was obtained by evaporation of the appropriate fractions from the column; the yield was 1·07 g. (20%).

Cyclization of thiobenzoylthreonine ethyl ester (8·2 g.) with $SOCl_2$ under the same conditions as described for thiobenzoyl*allo*threonine ethyl ester, followed by hydrolysis and chromatography on Zeo-Karb 225 resin as above, also gave β-methylcysteine hydrochloride, but in lower yield (0·42 g., 8%).

Reaction with formaldehyde. A portion of the above product (0·52 g.) in water (0·97 ml.) was mixed with formalin (0·44 ml.) and kept overnight at room temp. Pyridine (0·44 ml.) and ethanol (0·88 ml.) were added and the solution was cooled, when 4-carboxy-5-methylthiazolidine (0·11 g.) crystallized. After recrystallization from water by addition of ethanol, 80 mg. of pure product, m.p. 206°, was obtained (Found: C, 40·4; H, 6·1; N, 9·4. Calc. for $C_5H_9O_2NS$: C, 40·8; H, 6·1; N, 9·5%). There was no depression of m.p. when this material was mixed with the thiazolidine from β-methylcysteine prepared from *allo*threonine or with the thiazolidine from thiothreonine, isomer *B*, but a mixture with the thiazolidine from thiothreonine, isomer *A*, had m.p. 194°.

Preparation of DL-[carboxy-^{14}C]*thiothreonine, isomer* A

[carboxy-^{14}C]*Hippuric acid.* Redistilled benzoyl chloride (6·3 ml., 0·066 mole) and 1·92N-NaOH (28·6 ml., 0·055 mole) were added over 20 min. with stirring to [*carboxy*-^{14}C]glycine (4·1 g., approx. 200 μc; 0·055 mole) in N-NaOH (55 ml.), the rate of addition of the solution of NaOH being about four times that of the benzoyl chloride. After a further 30 min. the mixture was acidified to Congo red, cooled and the product was filtered off, washed with water and dried; yield, 9 g. (92%); specific radioactivity, 20·6 μc/g.

4-Ethylidene-2-phenyl-[5-^{14}C]*oxazolone.* The radioactive hippuric acid (9 g., 0·05 mole), acetic anhydride (16 ml., 0·15 mole) and basic lead acetate (7·6 g.) (Finar & Libman, 1949) were added to redistilled acetaldehyde (4·4 g., 0·1 mole), the mixture was shaken at 23° for 18 hr., poured into water (150 ml.) and extracted with benzene (2 × 50 ml.). The benzene layer was washed once with water (10 ml.) and evaporated, giving 7·2 g. (77%) of crude product, m.p. 82–85°. After recrystallization from ethanol by adding water, 5·5 g. (58%), m.p. 87–90°, was obtained (specific radioactivity, 17·4 μc/g.).

2-Benzamido-3-benzylthio-[carboxy-^{14}C]*butyric acid.* Addition of phenylmethanethiol to the above oxazolone, hydrolysis and benzoylation of the product was carried out as described by Carter *et al.* (1941). The yield of mixed *A* and *B* isomers was 6·6 g. (69%), having specific radioactivity 9·94 μc/g. The *A* isomer was separated from this mixture via the phenylethylamine salt (Carter *et al.* 1941), yield, 1·72 g. (26%), m.p. 151–152°; specific radioactivity, 10·2 μc/g.

[carboxy-^{14}C]*Thiothreonine* (A *isomer*). The foregoing product (1·72 g., 17·5 μc) was mixed with non-radioactive 2-benzamido-3-benzylthiobutyric acid (10·7 g.) and hydrolysed by boiling with formic acid (163 ml.)–conc. HCl (188 ml.)–water (213 ml.) for 3 hr. [*carboxy*-^{14}C]*S*-Benzylthiothreonine *A* was isolated essentially as described by Carter *et al.* (1941), the crude product being recrystallized by dissolving it in 2N-HCl (30 ml.) and neutralizing with 15N-NaOH (4 ml.), followed by solution in aq. 6N-NH_3 soln. (40 ml.) and evaporation of the NH_3 in a desiccator containing HCl; yield, 5 g. (11·7 μc, 67%), m.p. 198°; specific radioactivity, 2·34 μc/g. Reduction of the *S*-benzyl group with Na and isolation of thiothreonine hydrochloride (2 g.) were carried out as described by Carter *et al.* (1941). The hydrochloride was converted into the free amino acid by dissolving it in ethanol and adding pyridine, giving 0·85 g. (28%; specific radioactivity, 3·1 μc/g.) of [*carboxy*-^{14}C]thiothreonine *A*. The R_F values of the disulphide, obtained by oxidation at an alkaline pH, were identical with those of the B form in all three solvents.

Preparation of α-*methyl-*DL-*cystine*

Benzylthioacetone. Chloroacetone (20·9 g.) was added with stirring to the sodium derivative of phenylmethanethiol (25·9) in ethanol (160 ml.). After 3 hr., the ethanol was evaporated, the residue was extracted with ether and a saturated aqueous solution of $NaHSO_3$ was added to the ether layer with shaking. The bisulphite compound was filtered off, washed with ethanol and ether and dried. It was decomposed by warming with 2N-HCl and the ketone was extracted with ether. After evaporation of the ether the residue was distilled at 0·03 mm. Hg, giving benzylthioacetone (23·7 g., 58%), b.p. 108–110° (Wahl, 1922, gives b.p. as 155–156°/17 mm. Hg.). The 2:4-dinitrophenylhydrazone derivative was prepared by adding the ketone (100 mg.) to 2:4-dinitrophenylhydrazine (150 mg.), dissolved in conc. H_2SO_4 (0·3 ml.) and ethanol (3 ml.). After dilution with water (3 ml.), the precipitate (0·14 g., 70%) was filtered off, dried and recrystallized from ethanol. *Benzylthioacetone 2:4-dinitrophenylhydrazone* had m.p. 132° (Found: C, 53·1; H, 4·5; N, 15·8; $C_{16}H_{16}O_4N_4S$ requires C, 53·3; H, 4·5; N, 15·6%).

S-Benzyl-α-methylcysteine. Benzylthioacetone (17·8 g.) was added with stirring during 0·5 hr. to a mixture of NH_4Cl (11·5 g.), NaCN (10·4 g.), conc. aq. NH_3 soln. (70 ml.) and ethanol saturated with gaseous NH_3 (40 ml.). After shaking overnight at room temp., the amino nitrile was extracted with ether. The ether was evaporated and the oily residue hydrolysed by refluxing with conc. HCl (50 ml.) for 2·5 hr. The solution was evaporated to dryness, the residue was dissolved in water and the pH was adjusted to 6, when *S*-benzyl-α-methylcysteine crystallized; yield 15·6 g. (70%), m.p. 244° (decomp.). This material gave a blue colour with ninhydrin only after 5–10 min. at 100° in 0·1M phosphate buffer, pH 6·9 (Found: C, 58·4; H, 6·6;

N, 6·3; S, 13·8. Calc. for $C_{11}H_{15}O_3NS$: C, 58·7; H, 6·7; N, 6·2; S, 14·2%). The R_F values were 0·91 in solvent *A*, 0·54 in solvent *B* and 0·70 in solvent *C*.

S-Benzyl-N-formyl-α-methylcysteine. *S*-Benzyl-α-methylcysteine (5 g.) was formylated by the procedure of Wood & du Vigneaud (1939), giving 2·8 g. of crude product. After two recrystallizations from 30% ethanol, pure *S-benzyl-N-formyl-α-methylcysteine*, m.p. 154°, was obtained (Found: C, 57·2; H, 5·9. $C_{12}H_{15}O_3NS$ requires C, 56·9; H, 6·0%).

*α-Methyl-*DL*-cystine.* *S*-Benzyl-α-methylcysteine (5 g.) was dissolved in liquid NH_3 (200 ml.) and Na (approx. 1 g., 2 equiv.) was added slowly with stirring until a permanent blue colour appeared. The colour was just discharged with NH_4Cl and more NH_4Cl (2·4 g., 2 equiv.) was added. Ammonia was evaporated, the residue was extracted thoroughly with ether, dissolved in water and oxidized at pH 8, after addition of a trace of $FeCl_3$, by passing a stream of air overnight. The pH was adjusted to 6 with HCl and the solution was evaporated to a small volume, when *α-methyl-*DL*-cystine*, m.p. 260° (decomp.), (1·49 g., 50%) crystallized. After recrystallization from water–ethanol the product had m.p. 260° (decomp.) (Found: C, 35·7; H, 6·0; N, 10·4; S, 22·9. $C_8H_{16}O_4N_2S_2$ requires; C, 35·8; H, 6·0; N, 10·4; S, 23·85%). From the mother liquors of the first crystallization a second crop (0·3 g.) was obtained by precipitation as the Hg complex (Neuberg & Kerb, 1912), regeneration to the amino acid hydrochloride with H_2S, conversion into the free amino acid by addition of pyridine and recrystallization from water–ethanol (Found: C, 36·2; H, 6·1; N, 10·6; S, 23·3.) The R_F values were 0·62 in solvent *A*, 0·03 in solvent *B* and 0·06 in solvent *C*.

*α-Methyl-*DL*-[carboxy-*^{14}*C]cystine.* In the synthesis of the labelled amino acid from $K^{14}CN$, obtained from the Radiochemical Centre, Amersham, an attempt was made to avoid the use of excess of labelled cyanide by adding $K^{14}CN$ (2·3 g., 35 μc) to a mixture of benzylthioacetone (6·5 g.), NH_4Cl (4·3 g.), conc. aq. NH_3 soln. (26 ml.) and saturated ethanolic NH_3 soln. (14·6 ml.). After 18 hr. at room temperature with shaking, excess of non-isotopic KCN (2·9 g.) was added and the reaction mixture was shaken at room temperature for a further 24 hr., when the product was isolated as described previously. The chemical yield of *S*-benzyl-α-methyl-DL-[*carboxy-*^{14}C]cysteine (3·3 g., 40·5%; m.p. 243°) was, however, somewhat less than in the trial experiment with unlabelled material, and the recovery of ^{14}C was very poor (3·0 μc, 9%). The *S*-benzyl-α-methyl-DL-[*carboxy-*^{14}C]cysteine (1·7 g., 1·5 μc) was treated with Na in liquid NH_3 and α-methyl-DL-[*carboxy-*^{14}C]cystine was isolated as before. After recrystallization from water–ethanol, 0·2 g. (22%) of the labelled amino acid (specific radioactivity, 1·4 μc/g.) was obtained. On paper chromatography this product and unlabelled α-methyl-DL-cystine had identical R_F values in solvent systems *A*, *B* and *C*.

SUMMARY

1. A synthesis of β-methyl-DL-cystine from DL-*allo*threonine is described. Cyclization of *N*-thiobenzoyl*allo*threonine ethyl ester with thionyl chloride gave 4-carboxy-5-methyl-2-phenylthiazoline, which was hydrolysed by acid to β-methyl-DL-cysteine (thiothreonine, α-amino-β-mercaptobutyric acid). This amino acid was found to be identical with isomer *B* of thiothreonine, previously synthesized by Carter *et al.* (1941).

2. By a similar series of reactions DL-threonine was also converted into isomer *B* of thiothreonine.

3. The significance of formation of the same isomer of thiothreonine from both threonine and *allo*threonine is discussed. It is suggested that isomers *A* and *B* of thiothreonine have the *erythro* and *threo* configuration respectively.

4. The synthesis of α-methyl-DL-cystine is reported.

5. The preparation of DL-[*carboxy-*^{14}C]*allo*threonine, β-methyl-DL-[*carboxy-*^{14}C]cystine and α-methyl-DL-[*carboxy-*^{14}C]cystine is also described.

I should like to thank Professor V. du Vigneaud, Department of Biochemistry, Cornell University Medical College, New York, for the samples of 4-carboxy-5-methylthiazolidine from isomers *A* and *B* of thiothreonine, which were used as reference compounds in the present work. I am also very grateful to the following members of the staff of the National Institute for Medical Research: Mrs O. Kennard for the X-ray powder photographs; Dr D. F. Elliott for helpful advice and discussion on the synthesis of β-methylcystine; Mr E. J. Toms and Mr E. Stevens for technical assistance.

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