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GLUTAMYL-L-CYSTEINYL-β-ALANINE

W. J. P. NEISH and A. RYLETT
Cancer Research Unit, The University, Western Bank, Sheffield 10, England

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Abstract—A synthesis of glutamyl-L-cysteinyl-β-alanine is described.

RECENTLY Carnegie[1] described the isolation of a new peptide L-γ-glutamyl-L-cysteinyl-β-alanine (homoglutathione) as its disulphide from etiolated seedings of *Phaseolus aureus*.

In attempts to identify an unknown glutathione-like peptide which occurred in extracts of livers of rats injected with the hepatocarcinogen, 3'-methyl-4-dimethylaminoazobenzene,[2] a number of possible isomers and homologues of gultathione were prepared including, at Dr. Carnegie's suggestion, glutamyl-L-cysteinyl-β-alanine. Although glutamyl-L-cysteinyl-β-alanine was not identical with the rat liver peptide it seems of interest to report our synthesis of this material.

EXPERIMENTAL

N-carbobenzoxy-S-benzylcysteinyl-β-alanine ethyl ester (I)

N-carbobenzoxy-S-benzylcysteine (3·45 g, m.p. 94–95°) was dissolved together with β-alanine ethyl ester hydrochloride (1·535 g, m.p. 69°) in dichloromethane (DCM, 25 ml) and the solution cooled in ice-water. After adding triethylamine (1·38 ml), a solution of dicyclohexylcarbodiimide (2·06 g) in DCM (5 ml) was added and the mixture kept for 48 hr at room temp. Dicyclohexylurea (1·3 g) was filtered off, the solvent removed *in vacuo* and the residue dissolved in ethyl acetate (50 ml). This solution was washed twice with 0·5 N HCl (15 ml), twice with 0·5 M $NaHCO_3$ (15 ml), 3 times with deionized water (15 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. The white residue was dissolved in ethyl acetate (30 ml) and treated gradually with pet. ether (b.p. 40–60°) until small white needles began to precipitate (2 g, m.p. 101·5–103·5°). After crystallization from absolute ethanol and drying in air, the dipeptide ester (I) was obtained as knots of fine white needles,* m.p. 104–105° (Found: C, 61·17, 61·24; H, 6·05, 5·94 $C_{23}H_{28}N_2O_5S$ requires: C, 62·15; H, 6·35%).

Decarbobenzoxylation of N-carbobenzoxy-S-benzylcysteinyl-β-alanine ethylester

The above dipeptide ester (I, 1 g) was decarbobenzoxylated in the usual way† with 45% (w/v) hydrobromic acid in acetic acid (2 ml). After stirring into anhydrous ether, the semi-solid peptide hydrobromide was washed repeatedly with ether. It yielded a white solid (434 mg) presumably the hydrobromide of S-benzylcysteinyl-β-alanine ethyl ester‡ (II) which was stored *in vacuo* over P_2O_5 and KOH.

N-carbobenzoxy-α-methyl-L-glutamyl-S-benzylcysteinyl-β-alanine ethyl ester (III)

N-carbobenzoxy-α-methyl-L-glutamic acid (328 mg) prepared by the method of Klieger and Gibian[3] was dissolved in DCM (10 ml) and a solution of II (434 mg) in DCM (3 ml) was added.

* Analysis suggests that ethanol may be present in the crystals. $C_{23}H_{28}N_2O_5S \cdot C_2H_5OH$ requires C, 61·19; H, 6·98. $C_{23}H_{28}N_2O_5S + \frac{1}{2} C_2H_5OH$ requires: C, 61·65; H, 6·68%).

† J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids* Vol. 2, p. 1245. J. Wiley, New York (1961).

‡ This product apparently absorbed one mole of water on exposure to air. (Found: C, 44·18; H, 6·36; $C_{15}H_{23}N_2SO_3Br$ requires C, 46·06; H, 5·92; $C_{15}H_{23}N_2SO_3Br + H_2O$ requires C, 44·02; H, 6·16%).

[1] P. R. Carnegie, *Arch. Biochem. Biophys.* **101**, 364 (1963).
[2] W. J. P. Neish and A. Rylett, Brit. *J. Cancer* **15**, 630 (1961).
[3] E. Klieger and H. Gibian, *Liebig's Ann.* **655**, 195 (1962).

After addition of triethylamine (0·154 ml), a solution of dicyclohexylcarbodiimide (229 mg) in DCM (2 ml) was added and the mixture kept for 48 hr at room temp. Dicyclohexylurea (166 mg) was filtered off, DCM removed *in vacuo*, the residue dissolved in ethyl acetate and the solution washed with HCl, $NaHCO_3$ and water as previously described. The tripeptide ester (III) was crystallized from ethyl acetate–pet ether, yield 423 mg, m.p. 135–138° (decomp.). Recrystallization from absolute ethanol gave a white product (365 mg) which shrank slightly at 135° and melted at 140–142° with a white turbidity developing in the melt.* (Found: C, 59·16; H, 6·22. $C_{29}H_{37}N_3SO_8$ requires: C, 59·28; H, 6·35%).

Saponification. The tripeptide ester (III, 340 mg) was dissolved in methanol (8 ml), 2 N NaOH (0·63 ml, 10% excess) was added dropwise and the solution kept at room temp overnight.† After removal of methanol *in vacuo*, the residue was dissolved in water and filtered. Acidification with 6 N HCl gave an oil (IV) which failed to crystallize. It was washed repeatedly with water and dried *in vacuo* over P_2O_5.

Removal of carbobenzoxy and benzyl groups from IV

The product (IV) was dissolved in liquid ammonia (25 ml) and pieces of sodium (cleaned with n-butanol) were added until the blue colour persisted for 3 min. Ammonium sulphate was added to discharge the blue colour, and the ammonia removed. The residue was dissolved in 10% acetic acid (12 ml) and filtered. A saturated solution of mercuric acetate in 10% acetic acid was added until no more precipitate was produced. The mixture was diluted with an equal volume of water and kept at 4° overnight. The precipitate, collected on the centrifuge, was washed repeatedly with deionized water and decomposed with H_2S. After centrifugation, the colourless supernatant, freed from H_2S in a stream of nitrogen, was freeze-dried over P_2O_5.

The resulting tripeptide (glutamyl-L-cysteinyl-β-alanine) formed white crystals, m.p. began at 78°, complete at 94–95°. It gave a strong positive nitroprusside reaction and an orange-red colour when boiled with 0·2% ninhydrin in n-butanol. On adding a drop of water to the butanol solution, the colour changed to purple which was fairly persistent. Similar reactions are given by glutathione. The compound was colourless in the solid form, in solution in water (acidic) and in phosphate buffer (pH 7·2).

The thiol peptide was not stable in air at room temp. After several days it failed to yield a cuprous mercaptide and analysis revealed that it may have been transformed into the sulphonic acid. (Found C, 35·30; H, 5·21. $C_{11}H_{19}N_3O_9S$ requires: C, 35·76; H, 5·18%).

The freshly prepared thiol gave a stable cuprous mercaptide by the method of Waelsch and Rittenberg.[4] (Found: C, 32·86; H, 5·01. $C_{11}H_{18}N_3O_6SCu$ requires: C, 34·40; H, 4·73. $C_{11}H_{18}N_3O_6SCu + H_2O$ requires: C, 32·87; H, 5·02%).

It is concluded that the cuprous mercaptide of homoglutathione (homoGSH) contains 1 mole of water. Waelsch and Rittenberg[4] stated that the cuprous mercaptide of glutathione (GSH) contains rather more than 1 mole water (in our experience,[5] 1·5 mole water).

The N-ethylmaleimide (NEM) derivatives of homoglutathione has R_f values 0·81, 0·22 on Whatman No. 1 paper as compared with values of 0·64, 0·18 for the NEM derivative of GSH in phenol and butanol-methylethylketone–dicyclohexylamine solvents.[6] HomoGSH (7·1 cm) and GSH (7·3 cm) have about the same mobility (anodewards) in electrophoresis on Whatman No. 1 paper (350 volts, pH 8·6 barbitone–sodium barbitione buffer, 3 hr). The NEM derivatives of homoGSH and GSH have each a mobility of 4·5 cm (anodewards) under our electrophoretic conditions.

According to Dr. P. R. Carnegie, the product derived from our preparations of homoGSH cuprous mercaptide is not pure γ-glutamylcysteinyl-β-alanine but contains some α-glutamyl isomer.

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* In a second preparation, m.p. 135–139°.

† In a second preparation, the saponification time was reduced to 3 hr with some improvement in yield.

[4] H. Waelsch and D. Rittenberg, *J. biol. Chem.* **139**, 761 (1941).

[5] W. J. P. Neish and A. Rylett, *Biochem. Pharmacol.* In press, **12**, 893 (1963).

[6] C. H. Bowden. *Clin. Chim. Acta.* **4**, 539 (1959).