

A SYNTHESIS OF GLUTATHIONE

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The reduction of dicarbobenzoxycystinyldiglycine and of S-benzylcysteinylglycine to cysteinylglycine by metallic sodium in liquid ammonia was presented in the recent work on crystalline cystinyldiglycine by Loring and du Vigneaud (1). The effectiveness of these two reactions impressed us with their potentialities as possible key reactions for a new synthesis of glutathione; if N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine could be prepared, its reduction with sodium in liquid ammonia would be expected to yield glutathione.

This approach to glutathione would have a particular advantage in that the sulfhydryl group would be covered by a benzyl radical up to the final step and thus, during the course of the various reactions that might be employed up to this point, the likelihood of partial oxidation of the sulfhydryl group and its attendant difficulties would be eliminated. Even where the disulfide form might be used in any of the steps, the benzylthio ether linkage would still have the advantage of being more stable. The removal of the benzyl group would not involve an extra step, since it would be accomplished during the same reaction as that employed for the cleavage of the carbobenzoxo group.

We have undertaken the synthesis of glutathione along these lines and a method has been worked out which we feel offers excellent possibilities as a preparative procedure for obtaining this physiologically significant tripeptide. The yields throughout the synthesis have been encouragingly high and the reactions involved are such that one should be able to carry them out on a fairly large scale. The successful outcome of the synthesis has also

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afforded a confirmation of the synthetic proof of structure of glutathione, which was recently accomplished by Harington and Mead (2). The method of synthesis devised by these authors, although sufficing for structural proof, gave a very small yield.

In the present work, α -methyl-N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine methyl ester was prepared by the condensation of α -methyl-N-carbobenzoxyglutamyl chloride, which had been used by Harington and Mead (2), with S-benzylcysteinylglycine methyl ester in chloroform. 3 moles of the latter were employed in the reaction to 1 of the acid chloride. By using an excess of the basic ester the use of a foreign condensing agent was obviated. Furthermore, the use of an excess of the ester rather than an excess of the acid chloride seemed preferable to us, since the ester was very readily recoverable. After the condensation reaction was completed, the chloroform solution was extracted with dilute HCl. The excess S-benzylcysteinylglycine methyl ester was recovered from this extract and was used in subsequent reactions. The chloroform layer was evaporated and the condensation product so obtained was saponified. The resulting N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine was then dissolved in liquid ammonia and reduced by metallic sodium. After spontaneous evaporation of the ammonia, the residue was dissolved in sufficient 0.5 N H_2SO_4 to make the solution acid to litmus and the glutathione was precipitated as the mercury compound by the addition of mercuric sulfate. The mercury salt was decomposed with H_2S and the glutathione was reprecipitated with cuprous oxide. The cuprous glutathione showed the characteristic sheen mentioned by Hopkins (3). After the copper was removed with H_2S , the aqueous filtrate from the cuprous sulfide was evaporated to a small volume and the glutathione was crystallized in the usual manner.

The method of obtaining the free ester of S-benzylcysteinylglycine is worth calling attention to as it may find wider application. The free ester was prepared by the addition of an excess of diethylamine to a chloroform solution of the ester hydrochloride, followed by the addition of ether. The diethylamine hydrochloride was precipitated, leaving the free amino ester in solution. We had encountered much difficulty with diketopiperazine formation in attempts to make the free ester by other

procedures. In the diethylamine procedure, this tendency towards diketopiperazine formation was greatly minimized. The diethylamine, of course, can be readily recovered from the hydrochloride.

The glutathione obtained by the synthesis had the same crystalline form as that of an authentic sample isolated from natural sources. The compounds melted at the same temperature, namely 190° , and a mixture of the synthetic and naturally occurring compounds gave no depression of melting point. The compounds further gave the same specific rotation of $[\alpha]_D^{27} = -21.3^{\circ}$ for a 2 per cent solution in water.

EXPERIMENTAL

Preparation of N-Carbobenzoxy- γ -Glutamyl-S-Benzylcysteinylglycine—The S-benzylcysteinylglycine was prepared from dicarbobenzoxycystinylglycine by reduction in liquid ammonia with sodium and subsequent addition of benzyl chloride to the liquid ammonia solution of the reduced dipeptide, according to the directions of Loring and du Vigneaud (1). The ester hydrochloride was prepared by suspending 30 gm. of the S-benzylcysteinylglycine in 150 cc. of anhydrous methyl alcohol and saturating with dry HCl the mixture cooled in an ice-salt bath. As the HCl was being introduced the S-benzylcysteinylglycine readily passed into solution. The excess of HCl was removed with the aid of a water pump and the solution was concentrated *in vacuo* without application of heat. The syrupy residue was dissolved in 150 cc. of anhydrous methyl alcohol. The solution again was saturated at 0° with HCl and was concentrated as before. In order to remove excess HCl and methyl alcohol from the resulting residue, it was dissolved and reevaporated once with methyl alcohol and then three times with anhydrous chloroform.

The ester hydrochloride was dissolved in 60 cc. of purified chloroform. The solution was cooled in a solid CO_2 -trichloroethylene bath and to it were added 23 cc. of diethylamine. The solution was allowed to warm to about 0° , and 250 cc. of anhydrous ether were added with shaking. The crystalline diethylamine hydrochloride was filtered and was washed several times with ether. The clear, pale yellow filtrate was concentrated at a temperature below 0° . In order to remove excess diethyl-

amine from the syrupy residue, it was twice taken up in cold chloroform and was concentrated after each addition. The final product was dissolved in 75 cc. of chloroform and was stored temporarily in a solid CO_2 -trichloroethylene bath while the acid chloride with which it was to be condensed was being prepared.

The α -methyl-N-carbobenzoxyglutamate was prepared according to the directions of Harington and Mead (2) who utilized the observation of Melville (4) that treatment of carbobenzoxyglutamic anhydride with sodium ethylate yielded preponderantly the α ester and that the γ -chloride of the latter could then be prepared. 11 gm. of α -methyl-N-carbobenzoxyglutamate were dissolved in 35 cc. of anhydrous ether. The solution was cooled in an ice-salt bath and 10 gm. of finely pulverized PCl_5 were added in one portion. After the mixture was shaken for 20 minutes, the supernatant solution was decanted from a small amount of unchanged PCl_5 and was concentrated *in vacuo* at 0°C . When the solution began to acquire a syrupy consistency, the distillation was stopped. The acid chloride was precipitated by adding 50 cc. of ice-cold anhydrous petroleum ether (boiling point, 70°). After the mixture was thoroughly shaken to dissolve the POCl_3 , the petroleum ether layer was decanted. The acid chloride again was washed by shaking vigorously with 50 cc. of cold petroleum ether. The process was repeated a third time. The acid chloride was next dissolved in 50 cc. of chloroform which had been precooled in a solid CO_2 bath and the solution was used immediately for the next reaction. Since the compound has been found to decompose very rapidly, too much emphasis cannot be placed on the speed with which the latter steps should be carried out.

The chloroform solution of the acid chloride was poured immediately into the cold solution of the methyl ester of S-benzylcysteinylglycine which had just been prepared as indicated above. The mixture was allowed to stand at room temperature for 3 hours. The clear, yellow solution was extracted ten times with 25 cc. portions of 0.1 N HCl, then four times with water. The chloroform layer was dried over anhydrous sodium sulfate and was evaporated *in vacuo* to a dry solid residue. The crude condensation product was removed from the flask with the aid of a little ethyl acetate and precipitation of the material which had

dissolved in the ethyl acetate was accomplished by the addition of several volumes of petroleum ether. The material was filtered, washed with petroleum ether, and was used for the saponification.

The excess ester hydrochloride used in the condensation reaction was recovered to the extent of 88 per cent. The pH of the combined acid and water extracts was adjusted to approximately 4.0 and the solution was concentrated to a syrup. The residue was taken up in alcohol and the solution was evaporated. The compound was dissolved in anhydrous chloroform, was filtered to remove the NaCl, and the filtrate was concentrated. The residue was dissolved again in chloroform and was reevaporated. After this treatment the material was suitable for use again in another condensation reaction.

For saponification of the condensation product, the method used by Harrington and Mead for the saponification of α -methyl-N-carbobenzoxy- γ -glutamylcysteinylglycine ethyl ester (2) was found to be applicable. 20 gm. of the crude α -methyl-N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine methyl ester were dissolved in 200 cc. of dioxane. Sufficient alkali was added to make the solution alkaline to litmus. After this point was reached, there was gradually added to the solution a 20 per cent excess of the theoretical amount of N NaOH required to saponify the ester. The reaction mixture was allowed to stand at room temperature for 1 hour, at the end of which time it was neutralized with an amount of N HCl equivalent to the NaOH added. The solution was decolorized by allowing it to stand with carbex E for 2 hours at room temperature. The filtrate from the carbex E was concentrated *in vacuo* without application of heat. The semisolid residue was taken up in ethyl acetate. The acidic product was extracted from the ethyl acetate solution with saturated NaHCO₃ solution. After the NaHCO₃ extract was washed with ether, it was carefully acidified. At a pH of about 5.0 a small amount of amorphous impurity separated. This was filtered and the filtrate was further acidified. The compound came out of solution as an oil which hardened on standing. The solidified material was broken up, filtered, washed with water, and dried. 14 gm. of the crude material suitable for the reduction step were obtained. Attempts at crystallization have so

far proved unsuccessful. The compound, after solution in absolute alcohol and precipitation with water, was analyzed for sulfur and nitrogen. The values of 6.32 per cent for sulfur and 8.03 per cent for nitrogen were obtained in comparison with the theoretical values of 6.00 and 7.91 respectively based on the empirical formula of $C_{25}H_{29}O_8N_3S$.

On the basis of the α -methyl-N-carbobenzoxyglutamate used to prepare the acid chloride employed in the condensation, the yield of 14 gm. of saponified product represents 72 per cent of the theoretical yield. On the basis of the S-benzylcysteinylglycine used, allowing for the amount of excess ester actually recovered from the condensation reaction, the yield was 57 per cent. This represents the over-all yield through the esterification of S-benzylcysteinylglycine, freeing of the ester, the condensation with the acid chloride, and the saponification. It might also be mentioned that the S-benzylcysteinylglycine was obtained in a 50 per cent yield based on the original amount of cystine started with and the α -methyl-N-carbobenzoxyglutamate was obtained in a 63 per cent yield based on the glutamic acid used.

Reduction of N-Carbobenzoxy- γ -Glutamyl-S-Benzylcysteinylglycine—7 gm. of crude N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine were dissolved in 100 cc. of dry liquid ammonia in a 3-neck 200 cc. flask equipped with a mercury seal stirrer and a soda-lime tube. 1.5 gm. of sodium were added gradually in small pieces and the reaction mixture was stirred at a moderate rate. 4.3 gm. of ammonium sulfate, which were equivalent to the amount of sodium used, were added. After the solution was stirred for about 15 minutes longer, the ammonia was allowed to evaporate spontaneously. The last portions of ammonia were removed in a vacuum desiccator over H_2SO_4 . Another 7 gm. of the saponified condensation product were reduced with sodium in a manner similar to that described above and the combined solid residues from the two reactions were dissolved in 250 cc. of cold 0.5 N H_2SO_4 . The solution which resulted was acid to litmus. The mercaptide was first precipitated with a modified Hopkins' mercuric sulfate reagent (5). The mercury salt was washed with water several times at the centrifuge. Then it was suspended in about 50 cc. of water and decomposed with H_2S . The mercuric sulfide was separated by centrifugation and was washed

several times with water. The washings were combined with the main solution. After the removal of excess H_2S by bubbling hydrogen through the solution, an equal volume of $\text{N H}_2\text{SO}_4$ was added. The solution was heated to 40° and was treated with cuprous oxide according to Hopkins' procedure (3). A crystalline precipitate possessing the sheen characteristic of cuprous glutathione separated. The cuprous salt was washed with oxygen-free water until it was free of H_2SO_4 . It was then suspended in about 50 cc. of water and decomposed with H_2S . The cuprous sulfide was filtered and the excess H_2S was removed from the filtrate by passing a stream of hydrogen through it. The solution then was evaporated rapidly in a vacuum desiccator over P_2O_5 . The glassy residue was dissolved in sufficient water to give a heavy syrup. After a small amount of alcohol was mixed with the syrup, the solution was seeded and was placed in a desiccator over solid NaOH and P_2O_5 . The desiccator was filled with hydrogen and was placed in the refrigerator. Crystallization was allowed to proceed for 3 days, at the end of which time the material was taken up with the aid of a little 75 per cent alcohol and filtered. The product was washed first with 75 per cent alcohol and finally with 95 per cent alcohol and then dried. 1.3 gm. of beautifully crystalline product were obtained. The filtrate and washings were evaporated and the residue was dissolved in 0.5 $\text{N H}_2\text{SO}_4$ and treated with cuprous oxide as above. After decomposition of the cuprous salt another crop of the crystals, amounting to 0.9 gm., was obtained. The total yield of 2.2 gm. represented 27 per cent of the theoretical yield based on the amount of saponified condensation product used in the reduction.

The crystalline product melted at 190° and, when mixed with an authentic sample of glutathione isolated from natural sources, no depression of the melting point resulted. The specific rotation of the synthetic material was identical with that of the naturally occurring material, namely $[\alpha]_D^{27} = -21.3^\circ$ for a 2 per cent solution in water. The crystalline form of the two products was also identical. The synthetic material yielded on analysis 13.54 per cent nitrogen and 10.66 per cent sulfur. These figures are in close agreement with the theoretical values of 13.68 per cent nitrogen and 10.44 per cent sulfur for glutathione.

SUMMARY

A synthesis of glutathione has been presented. S-Benzylcysteinylglycine methyl ester was condensed with the acid chloride of α -methyl-N-carbobenzoxyglutamate. After saponification of the condensation product, the N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine so obtained was reduced with sodium in liquid ammonia. The resulting glutathione was isolated through the mercury and copper salts and finally as the free crystalline tripeptide.

The yields obtained in the various steps and the reagents and procedures involved in the reactions are of such a nature that the synthesis should prove to be a feasible preparative method for obtaining this physiologically important compound.

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