A New Synthesis of Glutathione *via* the Thiazoline Peptide

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A convenient synthesis of glutathione (GSH) by the use of minimal protecting groups was investigated. *N*-Formyl-L-2-amino-4-cyanobutyric acid ethyl ester was condensed with ethyl L-cysteinylglycinate to give (4*R*)-2-[(3*S*)-3-ethoxycarbonyl-3-(formylamino)propyl]-4-(ethoxycarbonylmethylcarbamoyl)-2-thiazoline. This compound was saponified in aqueous acetone at −15——20 °C and subsequently treated with dilute H_2SO_4 (pH 4) to yield formylglutathione, whose formyl group was then hydrolyzed with 0.5 M (1 M=1 mol dm^{-3}) H_2SO_4 to give free GSH. For purification, this was changed to a copper thiolate, which was then decomposed with H_2S to afford pure GSH.

Glutathione (GSH) is a tripeptide with the sequence γ-L-Glu–L-Cys–Gly that occurs in many cells in a variety of species. It is currently used as a medicine for some diseases in several countries. GSH has thus been produced on a large-scale by means of both fermentation and chemical synthesis.

Although GSH is a small-sized peptide, a large-scale synthesis of it seems to be difficult because it has a free SH group that is susceptible to oxidation, and a γ-linkage in the molecule. A variety of methods[1] for synthesizing GSH have been reported so far, but only a few of them are applicable to a large-scale synthesis of GSH; in most of these reported syntheses, all the functional groups of the amino acids have been protected, and expensive benzyloxycarbonyl and benzyl groups have often been employed for protecting the amino and the mercapto groups, respectively. Moreover, in most of these methods, drastic conditions such as sodium in liquid ammonia have been used for removal of the protecting groups.

In order to find a more convenient synthetic method for GSH, we have investigated a new route which uses minimal protection. The distinctive feature of the present method is to form a tripeptide with a thiazoline ring as an intermediate peptide (**6**), which is subsequently hydrolyzed to give GSH. We concentrated our efforts on forming the thiazoline ring and opening it at the C–S bond to form a peptide linkage and a free SH group.

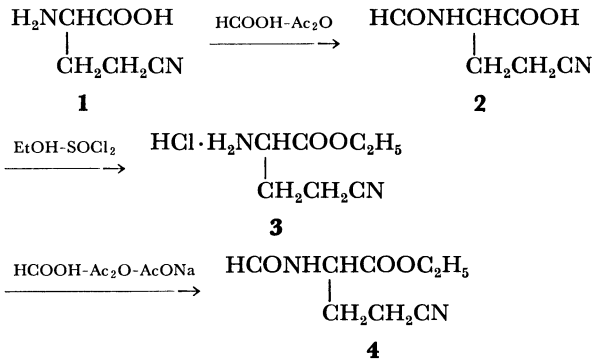
L-2-Amino-4-cyanobutyric acid (**1**),[2] which was prepared by dehydration of *Z*-L-Gln with *N*,*N*′-dicyclohexylcarbodiimide followed by hydrogenolysis, was employed as a starting material instead of L-Glu.

A partner peptide, L-Cys-Gly-OEt (**5**), was prepared by hydrolysis of (4*R*)-(3-formyl-2,2-dimethyl-4-ethoxycarbonylmethylcarbamoyl)thiazoline, which was prepared according to the method described in the synthesis of the corresponding methyl ester.[3]

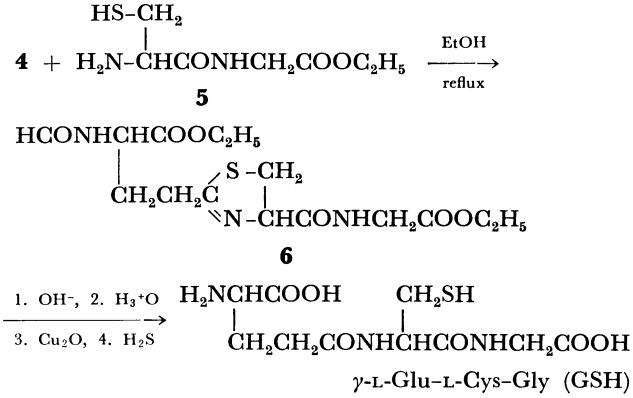
Esterification of **1** by a conventional method was always accompanied with a considerable amount of Glu formed by hydrolysis of the nitrile group. The amino acid (**1**) was successfully esterified with EtOH–$SOCl_2$ after blocking the amino group by a formyl group to give ethyl L-2-amino-4-cyanobutyrate hydrochloride (**3**). This compound was then treated with HCOOH–Ac_2O–AcONa for formylation because the formyl group of **2** was cleaved during the esterification (Scheme 1). An attempt to convert the nitrile group into an imidic ester failed. Treatment of *N*-formyl-L-2-amino-4-cyanobutyric acid ethyl ester (**4**) with EtOH–HCl was accompanied with a considerable amount of by-products, such as Glu and Glu ester, by concomitant cleavage of the formyl group and hydrolysis of the nitrile group. Therefore, we decided to form the thiazoline ring by direct condensation of **4** with L-Cys–Gly–OEt (**5**). Compound **4** was condensed with **5** in ethanol under reflux and in an atmosphere of nitrogen to give (4*R*)-2-[(3*S*)-ethoxycarbonyl-3-(formylamino)propyl]-4-(ethoxycarbonylmethylcarbamoyl)-2-thiazoline (**6**) in 62.5% yield (Scheme 2).

Care must be taken in treating **6** with alkali, since it is known that a thiazoline ring is susceptible to racemization.[4] No appreciable racemization occurred when **6** was treated with alkali at a low temperature of −15——20 °C.

Careful saponification of the ester group of **6** at −15——20 °C in aqueous acetone and subsequent mild



Scheme 1.



Scheme 2.

acid hydrolysis of the thiazoline ring gave *N*-formylglutathione. This was passed through a column of Diaion SK1B[5] (H^+ form) in order to remove the sodium ion which was found to cause the free SH group to change into an –S–S– bond at the final stage. Removal of the formyl group with dilute sulfuric acid gave free GSH, which was changed to a copper thiolate. Subsequent decomposition of the thiolate with hydrogen sulfide afforded pure GSH (Scheme 2).

This method shows considerable promise for large-scale syntheses of GSH.

Experimental

Melting points were measured in glass capillaries and are uncorrected. The UV-spectrum was recorded on a Shimadzu MPS-5000 spectrometer. The optical rotations were measured on a Perkin-Elmer 141 polarimeter.

*N-Formyl-L-2-amino-4-cyanobutyric Acid Ethyl Ester (**4**).* To an ice-cooled mixture of formic acid (55.2 g, 1.2 mol) and acetic anhydride (45.9 g, 0.60 mol) was added **1** (38.4 g, 0.30 mol) with stirring. After stirring for 3 h in an ice-bath, ether (500 ml) was added to the reaction mixture, and crystals (**2**) thus formed were collected by filtration; yield, 42.9 g (91.7%); mp 127—128 °C. To a suspension of **2** (42.1 g, 0.27 mol) in anhydrous ethanol (300 ml) was added dropwise thionyl chloride (21 ml) at −5——10 °C. After the addition was complete, the mixture was permitted to warm to room temperature. After the mixture was stirred at 40 °C for 40 min, the solvent was evaporated *in vacuo* at a bath temperature of 30 °C. The residue was dissolved in ethanol (140 ml) and filtered, and ether (300 ml) was added to the filtrate, which was then kept in a refrigerator overnight. Crystals (**3**) thus formed were collected by filtration and recrystallized from ethanol–ether; yield, 39.5 g (76.1%); mp 143—144 °C. To a mixture of formic acid (46.0 g, 1.0 mol) and sodium acetate (16.4 g, 0.2 mol) was added acetic anhydride (30.6 g, 0.3 mol) with cooling in an ice-bath. After stirring for 10 min, **3** (38.5 g, 0.2 mol) was added to the mixture, which was stirred with cooling in an ice-bath for 3 h. Ice water (30.6 g) was then added to the reaction mixture. After stirring at room temperature for 30 min, the reaction mixture was concentrated *in vacuo* to give an oily residue, which was dissolved in water (100 ml), adjusted to pH 6.0 with sodium carbonate, and extracted twice with 150-ml portions of chloroform. The chloroform layer was washed with water (100 ml), dried on anhydrous sodium sulfate, and evaporated *in vacuo* to give the desired compound (**4**) as an oil; yield, 30.4 g (82.6%).

*L-Cys-Gly-OEt (**5**).* (4*R*)-(3-Formyl-2,2-dimethyl-4-ethoxycarbonylmethylcarbamoyl)thiazolidine (mp 70—72 °C) prepared according to the method described for the corresponding methyl ester[3] was hydrolyzed in ethanolic hydrochloric acid to give L-Cys–Gly–OEt·HCl (83.5%; mp 135—137 °C), which was neutralized with Et_3N in ethyl acetate. The solvent was evaporated *in vacuo* to give **5** as an oil.

*(4R)-2-[(3S)-3-Ethoxycarbonyl-3-(formylamino)propyl-4-(ethoxycarbonylmethylcarbamoyl)-2-thiazoline (**6**).* A mixture of **4** (25.0 g, 0.136 mol) and **5** (33.6 g, 0.163 mol) in anhydrous ethanol (50 ml) was heated under reflux with stirring in an atmosphere of nitrogen for 16 h. After the reaction was complete, insoluble materials were filtered off, and the filtrate was concentrated *in vacuo*. The residue was taken up in chloroform (300 ml) and washed with a 20% aqueous citric acid solution (50 ml), and then twice with 100-ml portions of water. The chloroform layer was dried on anhydrous sodium sulfate and then evaporated *in vacuo* to give an oily residue, which was crystallized from ethyl acetate–petroleum ether to give **6** as crystals; yield, 31.7 g (62.5% based on **4**); mp 78—80 °C; UV (35% HCl): λ_{max} 268 nm (ε 4900); $[\alpha]_D^{18}$ +13.2° (*c* 1.0, ethanol). Found: C, 48.05; H, 6.19; N, 11.08; S, 8.69%. Calcd for $C_{15}H_{23}O_6N_3S$: C, 48.24; H, 6.21; N, 11.25; S, 8.59%.

Glutathione (GSH). To a stirred solution of **6** (70.0 g, 0.187 mol) in a mixture of acetone (617 ml) and O_2-free water (94 ml) was added dropwise 1 M (1 M=1 mol dm^{-3}) NaOH (374 ml) at −15——20 °C. After stirring at the same temperature for 1 h, the reaction mixture was acidified with 0.5 M H_2SO_4 (380 ml) to pH 4. In order to open the thiazoline ring, the mixture was stirred at room temperature for 30 min, and then the acetone was evaporated *in vacuo* from the mixture. The resulting solution, which contained formylglutathione, was passed through a column of Diaion SK1B (H^+ form) and the column was then washed with O_2-free water. The eluate and washings were combined and concentrated *in vacuo* to a small volume (336 ml), and then 5 M H_2SO_4 (37 ml) was added to the concentrate, which was stirred at 45—47 °C for 6 h in order to remove the formyl group. Copper(I) oxide (13.37 g, 0.0935 mol) was added to the reaction mixture, which was then stirred at the same temperature for 15 min. After storage in a refrigerator overnight, the copper thiolate thus formed was collected by filtration and washed thoroughly with water. The thiolate was suspended in water (500 ml) and H_2S gas was bubbled into the suspension. The copper sulfide thus formed was filtered off and the filtrate was concentrated *in vacuo* to give a syrupy residue, which was dissolved in O_2-free water (96 ml), and then ethanol (480 ml) was added to the solution at 40 °C. After standing at the same temperature for 15 min, a yellow oil was removed by decantation. Crystals of GSH (1.0 g) were added as seed to the solution, which was kept at room temperature for 2 d and then placed in a refrigerator overnight. Crude crystals along with a syrupy material were collected by filtration; yield, 20.0 g (34.7%; one gram (seed) is subtracted from the yield). The crystals (20.0 g) were dissolved in O_2-free water (60 ml) and 2-propanol (80 ml) was added at 40 °C. Crystals of GSH (1.0 g) were added as seed to the mixture, which was stirred at 40 °C for 2 h and kept at room temperature for 20 h, then kept in a refrigerator overnight. The crystals thus formed were collected by filtration; yield, 16.6 g (28.8%; one gram (seed) is subtracted from the yield). Mp 184—185 °C (dec); lit,[1] 186—190 °C (dec); $[\alpha]_D^{23}$ −21.9° (*c* 2.74, H_2O), lit,[1] $[\alpha]_D^{27}$ −21° (*c* 2.74, H_2O); Found: C, 38.88; H, 5.52; N, 13.75; S, 9.86%. Calcd for $C_{10}H_{17}O_6N_3S$: C, 39.08; H, 5.58; N, 13.67; S, 10.43%.

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