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Use of N,S-Bis-*tert*-butoxycarbonyl-L-cysteine for Synthesis of Glutathione

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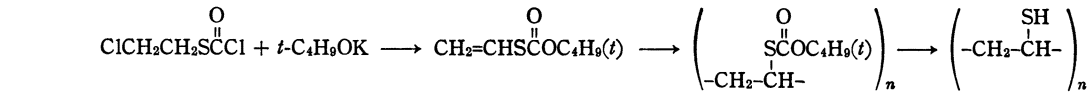
N,S-Bis-*tert*-butoxycarbonyl-L-cysteine (I), a novel protected cysteine derivative, is proposed for glutathione synthesis. I was obtained in one step reaction from L-cysteine and *tert*-butoxycarbonyl chloride. The reaction of *tert*-butoxycarbonyl azide with L-cysteine also gave I but in low yield. The protecting groups on the nitrogen and sulfur atoms of I are stable under the usual conditions used in peptide synthesis and can be removed selectively by mild acidic or alkaline conditions. These groups are also removed simultaniously by a strong acidic reagent such as hydrogen chloride of high concentration in acetic acid. Synthesis of glutathione was performed employing I as a new type of intermediate.

Since Harrington, *et al.* first synthesized glutathione in 1935,[2] numerous synthetic methods of this compound have been described in the course of progress in peptide chemistry. In these synthetic studies, various kinds of protecting groups have been developed for thiol function of the cysteine residue.

The S-benzyl group introduced by du Vigneaud, *et al.* in 1935[3] is still most commonly used because of its easy introduction to the SH group of cysteine and its stability under the usual reaction conditions employed in peptide synthesis. However, removal of this group requires rather drastic conditions such as use of sodium in liquid ammonia. Other forms employed for S-protection in glutathione synthesis are the cystine bond,[4] thiazolidine ring,[5] benzyloxycarbonyl,[6] trityl,[7] ethylcarbamoyl,[8] benzylthiomethyl,[9] and ethylthio[10] group.

During the course of our studies on syntheses of cysteine derivatives, N,S-bis-*tert*-butoxycarbonyl-L-cysteine (N,S-bis-Boc-L-cysteine) (I) which is obtained in one step from L-cysteine, was found to be useful as an intermediate for glutathione synthesis. The protecting groups on N and S of I are stable enough under the conditions of peptide synthesis and can be removed selectively under appropriate condition. A somewhat similar type of S-protection has been reported on the synthesis of S-containing polymers.[11] S-Vinyl-O-*tert*-butyl thiolcarbonate obtained by the reaction of potassium *tert*-butoxide with 2-chloroethylthiol carbonyl chloride was polymerized and then cleaved by dry hydrogen bromide in chloroform and tetrachloroethane to give polyvinylmercaptan.



1) Location: *2-2-50, Kawagishi, Toda-shi, Saitama.*
2) C.R. Harrington and T.H. Mead, *Biochem. J.*, **29**, 1602 (1935).
3) R.H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).
4) C. Berse, R. Boucher, and L. Piché, *Can. J. Chem.*, **37**, 1733 (1959).
5) F.E. King, J.W. Clark-Lewis, and R. Wade, *J. Chem. Soc.*, **1957**, 880.
6) M. Sokolovsky, M. Wilchek, and A. Patchornik, *J. Am. Chem. Soc.*, **86**, 1202 (1964).
7) G. Amiard, R. Heymès, and L. Velluz, *Bull. Soc. Chim. France*, **1956**, 698.
8) St. Guttmann, *Helv. Chim. Acta*, **49**, 83 (1966).
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10) N. Inukai, K. Nakano, and M. Murakami, *Bull. Soc. Chem. Japan*, **40**, 2913 (1967).
11) C.G. Overberger and W.H. Daly, *J. Am. Chem. Soc.*, **86**, 3402 (1964).

This type of S-protecting method has never been applied for peptide synthesis.

I was obtained in fairly good yield when L-cysteine was treated with *tert*-butoxycarbonyl chloride[12] in the presence of triethylamine in aqueous tetrahydrofuran. However, the reaction of L-cysteine with *tert*-butoxycarbonyl azide led to mainly the formation of N,N'-bis-Boc-L-cystine (II) and I was obtained only in extremely low yield. This result may be due to the oxidation of L-cysteine (or N-Boc-L-cysteine) by hydrogen azide, a mild oxidizing agent, which is liberated in the reaction. Weygand, *et al.*[13] have also reported that N,N'-bis-*p*-methoxybenzyloxycarbonyl-L-cystine was produced exclusively by the reaction of L-cysteine with *p*-methoxybenzyloxycarbazide.

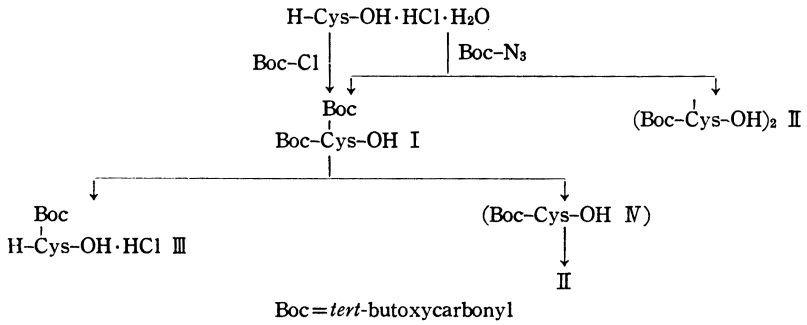

Chart 1

Selective cleavage of the N- or S-Boc group of I was attempted in both acidic and alkaline media. Treatment of I with 2.8 N dry hydrogen chloride in ethyl acetate gave S-Boc-L-cysteine hydrochloride (III) within a few minutes. On the other hand, the S-Boc group could be removed from I in 2.0 N sodium hydroxide solution at room temperature. N-Boc-L-cysteine (IV) thus obtained was an oil, which was characterized by converting it to II. However, this alkali treatment required rather long time for complete S-deprotection.

The compound I was then used for glutathione synthesis. The coupling reaction of I with glycine methyl ester took place by the dicyclohexylcarbodiimide (DCC) method (81% yield) or the mixed anhydride (MA) method (21% yield) giving N,S-bis-Boc-L-cysteinylglycine methyl ester (V). The N-Boc group of V was removed on treatment with 2.3 N dry hydrogen chloride in ethyl acetate to furnish S-Boc-L-cysteinylglycine methyl ester hydrochloride (VI) as a hydroscopic powder.

Table I. Cleavage of Boc Group from N,S-Bis-Boc-cysteinylglycine Methyl Ester (V)[a]

Reagent	Condition[b] (min)	Cleaved Boc	Presence of V
1.2N HCl/AcOH	10	N (and S)[c]	−
1.4N HCl/AcOH	90	N and S	−
3.1N HCl/Dioxane	30	N (and S)[c]	+
2.3N HCl/AcOEt	25	N (and S)[c]	−
100% HCOOH	30	N (and S)[c]	+
50% TFA[d]/CH_2Cl_2	30	N (and S)[c]	+
25% HBr/AcOH	10	N and S	−

a) examined by TLC, solvent systems: $CHCl_3$–AcOH–MeOH (95:3:10) and AcOEt detection: ninhydrin
b) at room temperature
c) slight cleavage of S-Boc group was also observed
d) trifluoroacetic acid

12) S. Sakakibara, I. Honda, K. Takada, M. Miyoshi, T. Ohnishi, and K. Okumura, *Bull. Chem. Soc. Japan*, **42**, 809 (1969).
13) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

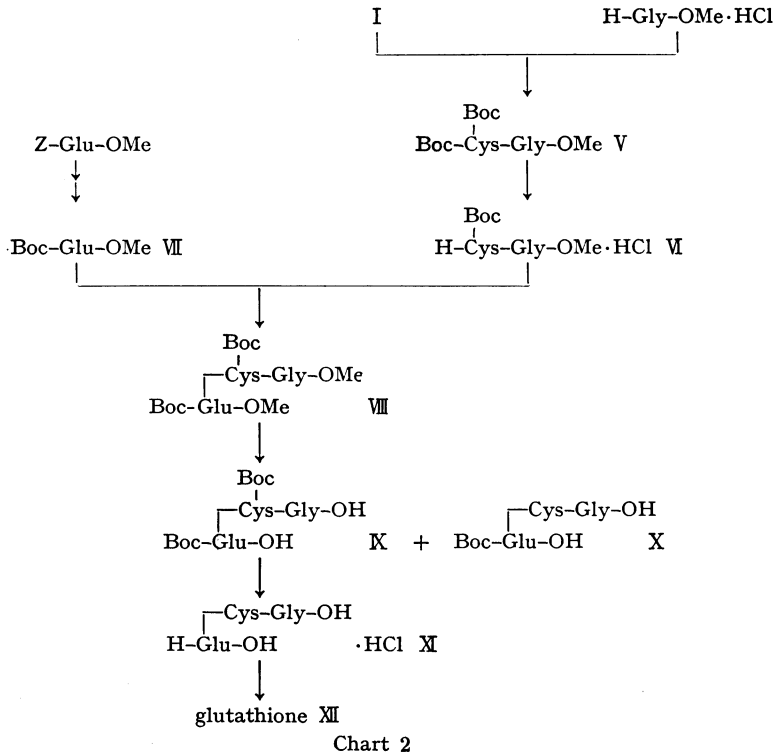

Chart 2

In addition, several other sets of conditions were tested for removal of the Boc groups from V and the reaction products were characterized by thin-layer chromatography (Table I). Dry hydrogen bromide or hydrogen chloride of high concentration in acetic acid cleaved both the N- and S-Boc groups, while short exposure to other milder reagents such as hydrogen chloride in ethyl acetate and trifluoroacetic acid in methylene chloride gave mostly the N-deprotected product with a trace of L-cysteinylglycine methyl ester.

Crude S-Boc-L-cysteinylglycine methyl ester hydrochloride thus obtained was then coupled with Boc-L-glutamic acid α-methyl ester, which had been liberated from its dicyclohexylamine (DCHA) salt, to give fully protected glutathione (VIII). Mild treatment of VIII with 0.33N sodium hydroxide in aqueous tetrahydrofuran at room temperature gave N,S-bis-Boc-glutathione (IX). The product was characterized as its dicyclohexylamine salt.

The Boc groups on IX were smoothly removed when IX was treated with 1.4 N hydrogen chloride in acetic acid to yield crude glutathione hydrochloride (XI) as a hygroscopic powder, which was then converted to the free peptide and purified by the method of du Vigneaud, *et al.*[14] The final product thus obtained was identical with an authentic sample in the respects of melting point, specific rotation, IR spectrum and paper chromatographic behavior.

I may have the following advantages as an intermediate for synthesis of glutathione or other L-cysteine derivatives:

1) Both NH_2 and SH groups of L-cysteine can be protected in one step.

2) The N-Boc and S-Boc groups are removed selectively or simultaniously under relatively mild conditions.

3) Both groups are stable enough under the reaction conditions for amide formation such as DCC and MA methods.

14) V. du Vigneaud and G.L. Miller, "Biochemical Preparations," Vol. 2, John Wiley and Sons, Inc., New York, 1952, p. 87.

Experimental[15]

N,S-Bis-*tert*-butoxycarbonylcysteine I)——a) *tert*-Butoxycarbonyl Chloride Method: To a solution of cysteine hydrochloride monohydrate (17.56 g; 0.1 mole) in distilled water (100 ml) was added a solution of Et_3N (40.4 g; 0.4 mole) in abs. THF (100 ml) with stirring at 0° in an amtsphere of nitrogen. To this solution was added *tert*-butoxycarbonyl chloride[12] in absolute ether (prepared from *tert*-butyl alcohol (44.5 g) and phosgene (71 g)) with vigorous stirring during 1.25 hr at 0—5° and the mixture was stirred at 0° for 1.5 hr during which time Et_3N (7 g) was added in small portions to keep the solution alkaline, and the mixture was stirred for 2 hr at room temperature. The organic phase was separated and extracted twice with 10% aqueous Et_3N. The combined aqueous layer and extracts were acidified with citric acid, saturated with NaCl and extracted with ether. The extracts were washed with 0.5 M citric acid and water, dried over Na_2SO_4 and evaporated to leave crystals of mp 119—122° (21.67 g; 67.5%). Recrystallization from ether–*n*-hexane afforded 17.60 g (54.8%) of I, mp 127°, $[\alpha]_D^{21}$ −26.7° (c=1.0, EtOH). NMR ($CDCl_3$) τ: 8.58 (N-Boc), 8.53 (S-Boc) (total 18H), 7.1—6.8 (2H, multiplet, SCH_2), 5.45 (1H), 4.55 (1H), −1.25 (1H). IR ν_{max}^{Nujol} cm^{-1}: 1720 (COOH), 1695 (N-Boc), 1660 (S-Boc). *Anal.* Calcd. for $C_{13}H_{23}O_6NS$: C, 48.58; H, 7.21; N, 4.36; S, 9.98. Found: C, 48.67; H, 7.04; N, 4.48; S, 9.82.

b) *tert*-Butoxycarbonyl Azibe Method: *tert*-Butyl carbazate (19.82 g; 0.15 mole) was treated with $NaNO_2$ in aquous AcOH in the usual manner to give 1.988 g; (92.5%) of crude *tert*-butoxycarbonyl azide as an oil.

To a solution of cysteine hydrochloride monohydrate (8.78 g; 0.05 mole) and Et_3N (22.81 g; 0.125 mole) in 50% (v/v) aqueous dioxane (60 ml) was added the above *tert*-butoxycarbonyl azide (16.16 g; 0.125 mole) at room temperature, and the mixture was stirred for 23 hr at 35° under an atmosphere of nitrogen. After neutralization with citric acid the solution was concentrated *in vacuo* to remove dioxane, and pH of the remaining solution was adjusted to 3 with addition of solid citric acid. The mixture was extracted with ether, and the extracts were washed with water, dried and evaporated to leave a crystalline residue (9.2 g; mp 117—120°). This was recrystallized four times from AcOEt–petroleum ether to yield 4.7 g (37%) of prisms, mp 147—148°, $[\alpha]_D^{22}$ −141° (c=2.5, MeOH), which was identified with N,N′-bis-*tert*-butoxycarbonylcystine (II) (mp 145—146°, $[\alpha]_D^{19}$ −139° (c=2.5, MeOH)).[16] The mother liquor was concentrated *in vacuo* and recrystallized three times from ether–*n*-hexane to yield 1.2 g (7.5%) of I, mp 127°.

S-*tert*-Butoxycarbonylcysteine Hydrochloride (III)——To 330 mg of I (1.0 mmole) was added 2.8 N HCl/AcOEt (1 ml) and the solution was allowed to stand at room temperature for 10 min. To the reaction mixture was added ether (3 ml) and the resulting crystalline material was collected by filtration and washed well with ether. The crude III (220 mg; 83.5%) of mp 184—185° (decomp.) was obtained. After recrystallization from MeOH–AcOEt–*n*-hexane the melting point was raised to 191—192° (decomp.). $[\alpha]_D^{24}$ −28.5° (c=0.8, MeOH). *Anal.* Calcd. for $C_8H_{16}O_4NSCl$: C, 37.27; H, 6.26; N, 5.44. Found: C, 37.48; H, 6.29; N, 5.98. IR ν_{max}^{Nujol} cm^{-1}: 1715 (COOH), 1660 (S-Boc), 1590, 1500 (NH_3^+).

N,N′-Bis-*tert*-butoxycarbonylcystine (II)——A solution of I (330 mg; 1.0 mmole) in 2 N NaOH (8 ml) was allowed to stand for 14 hr at room temperature and acidified by addition of 2 N HCl. To this solution were added AcOEt and a solution of iodine in MeOH until colour of iodine appeared. The solution was separated and extracted with AcOEt. The extracts were washed with saturated NaCl solution and water, dried and evaporated to give II, 145 mg (65.5%), mp 143—145°, $[\alpha]_D^{23}$ −122° (c=2.5, MeOH).

N,S-Bis-*tert*-butoxycarbonylcysteinylglycine Methyl Ester (V)——a) DCC Method: To a solution of 3.21 g of I (10 mmoles) in 30 ml of $CHCl_3$ was added 2.28 g of DCC (11 mmoles) in 10 ml of $CHCl_3$ during 4 min with stirring at 3—4°. After 5 min a solution of glycine methyl ester hydrochloride (1.26 g; 10 mmoles) and Et_3N (1.02 g; 10 mmoles) in $CHCl_3$ (25 ml) was added thereto during 15 min at 0—4° and stirring was continued for 5 hr below 10°. Dicyclohexylurea formed was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was dissolved in AcOEt and washed successively with water, 4% $NaHCO_3$, saturated NaCl, 0.5 M citric acid and saturated NaCl and dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue thus obtained was crystallized from isopropyl ether–petr. ether; 2.59 g (66%), mp 95—98°. And mp 100.5—101° after recrystallization from ether–petr. ether, $[\alpha]_D^{29}$ −25.5° (c=1.0, EtOH). *Anal.* Calcd. for $C_{16}H_{28}O_7N_2S$: C, 48.96; H, 7.19; N, 7.14; S, 8.16. Found: C, 49.36; H, 7.38; N, 6.85; S, 7.96.

b) MA Method: To a solution of 3.21 g (10 mmoles) of I in THF (30 ml) was added 1.01 g (10 mmoles) of Et_3N at −16°, and then a solution of 1.09 g (10 mmoles) of ethyl chloroformate in THF (5 ml) was added thereto. After 5 min, a solution of 1.26 g (10 mmoles) of glycine methyl ester hydrochloride and 1.01 g of Et_3N in THF (25 ml) and $CHCl_3$ (10 ml) was added at −15°. The solution was stirred for 2 hr at the same temperature and for 30 min at room temperature and the solvent was evaporated *in vacuo*. The residue was worked up in the same way as described in a). The organic extract thus obtained was evaporated *in vacuo* to give a crude crystalline residue, 3.9 g (99%), mp 71—80°. After recrystallization from

15) All the melting points are uncorrected. Thin-layer chromatography (TLC) was carried out on Silica Gel G (E. Merck Co.). Configuration of amino acids described in this experimental part is L-form.
16) I. Photaki, *J. Am. Chem. Soc.*, **88**, 2292 (1966).

ether–isopropyl ether, the melting point was raised to 99—100.5°, 0.82 g (21%), $[\alpha]_D^{25}$ −26.1° (c=1.0, EtOH). This product was identical with the sample prepared by the DCC method described above.

***tert*-Butoxycarbonylglutamic Acid α-Methyl Ester (VII) Dicyclohexylamine Salt**——Benzyloxycarbonylglutamic acid α-methyl ester (17.72 g; 0.06 mole) was treated with 25% HBr/AcOH (40 ml), under cooling in an ice–bath, and then the solution was kept at room remperature for 1 hr. After evaporation of the solution *in vacuo*, the residue was washed once with petr. ether and four times with ether. After drying over KOH–P_2O_5, oily glutamic acid α-methyl ester hydrobromide was obtained in a nearly quantitative yield.

To a solution of this oily product and *tert*-butoxycarbonyl azide (17.2 g; 0.12 mole) in 50% (v/v) aqueous dioxane (140 ml) was added Et_3N (24.3 g; 0.24 mole), and the solution was stirred at 35° for 22 hr. After cooling and neutralization with citric acid the solution was concentrated *in vacuo*, and then pH of the remaining solution was adjusted to 3 with addition of citric acid. The resulting mixture was extracted with AcOEt and the extract was washed with water, dried and evaporated to leave 1.05 g (67%) of an oily product (VII).

This was converted to dicyclohexylamine salt in ether and was washed with ether. This was recrystallized from $CHCl_3$–ether to give 14.0 g (overall yield 50%) of the salt, mp 160—163°, and mp 165—166.5° after repeated recrystallizations, $[\alpha]_D^{28}$ −12.0° (c=1.0, MeOH) (lit.[17] mp 167—168°, $[\alpha]_D^{25}$ −13.0° (c=1.0, MeOH)).

This salt was treated with 0.5 M citric acid to give oily VII in 85—90% yield.

S-*tert*-Butoxycarbonylcysteinylglycine Methyl Ester Hydrochloride (VI)——V (3.92 g; 10 mmoles) was treated with 2.3 N dry HCl/AcOEt (28 ml) under cooling in an ice–bath. After the solution was kept at room temperature for 25 min, the solvent was removed *in vacuo*. AcOEt was added to the residue and evaporated. This operation was repeated three times and the residue was dried over KOH and P_2O_5 to give 3.10 g (94.5%) of very hygroscopic powder. This was used for the next step without further purification.

***tert*-Butoxycarbonyl-α-methyl-γ-glutamyl-S-*tert*-butoxycarbonylcysteinylglycine Methyl Ester (VIII)**——a) MA Method: To a solution of VII (2.46 g; 9.4 mmoles) and N-methylmorpholine (0.95 g; 9.4 mmoles) in abs. THF (40 ml) was added a solution of ethyl chloroformate (1.02 g; 9.4 mmoles) in abs. THF (15 ml) over a period of 5 min with stirring at −25°. After 5 min, VI (3.10 g; 9.45 mmoles) and N-methylmorpholine (0.95 g) in abs. THF (40 ml) were added during 25 min below −25°, and then the mixture was stirred for 2 hr. The solution was filtered and the filtrate was concentrated *in vacuo* below 35°. The residue was dissolved in AcOEt, and the solution was washed with 0.5 M citric acid, NaCl solution, 4% $NaHCO_3$, and NaCl solution and finally dried over Na_2SO_4. The solvent was evaporated *in vacuo*, and the residue was crystallized from petr. ether and washed with ether; mp 82—86°; 3.36 g (66.6%). Recrystallization from AcOEt–petr. ether afforded 2.48 g of VIII, mp 88.5—90°. The ethereal washings and the mother liquor of recrystallization were combined and evaporated to leave an oil (1.8 g), which was purified by column-chromatography (silicic acid, solvent: AcOEt–ether 1:2 v/v) and the resulting crystalline material was recrystallized from AcOEt–petr. ether to yield 0.7 g of the second crop of VIII, mp 88—90°; total 3.18 g (63%). Further recrystallization gave an analytically pure sample (mp 92—94°, $[\alpha]_D^{25}$ −37.2° (c=1.0, EtOH)). *Anal.* Calcd. for $C_{22}H_{37}O_{10}N_3S \cdot 1/2CH_3COOC_2H_3$: C, 49.73; H, 7.13; N, 7.25; S, 5.53. Found: C, 50.12; H, 7.19; N, 7.48; S, 5.80.

b) DCC Method: To a solution of VII (230 mg; 0.88 mmole) in CH_2Cl_2 (5 ml) were added DCC (182 mg; 0.88 mmole) at 0—5° during 5 min and, after 5 min, a solution of VI, prepared from 397 mg (1.0 mmole) of V and N-methylmorpholine (105 mg; 1.0 mmole) in DMF and CH_2Cl_2 under the same condition. The solution was stirred for 1 hr at 5° and for 2 hr at approximately 10° and allowed to stand in a refrigerator overnight. The solution was filtered and the filtrate was concentrated *in vacuo*. The residue obtained was dissolved in AcOEt and the insoluble material was filtered off. This procedure was repeated three times. The AcOEt solution was washed with 0.5 M citric acid, water, 4% $NaHCO_3$ and water, dried and evaporated. The remaining crystals (370 mg) were recrystallized from AcOEt–petr. ether to give 180 mg (34%) of VIII, mp 86—89°, and mp 92—94° on further recrystallization. This was identified with the product obtained by the MA method a) by mixted melting point test and comparison of their IR spectra.

N,S-Bis-*tert*-butoxycarbonylglutathione (IX)——To a stirred solution of VIII (2.42 g; 4.5 mmoles) in abs. MeOH (53 ml) was added 1.67 N NaOH (13.3 ml; 22.5 mmoles) with cooling in an ice–bath under an atmosphere of nitrogen. The solution was allowed to stand for 30 min at room temperature. The solution was neutralized with 10% H_2SO_4 and then extracted with ether. From the extracts there was obtained 1.70 g (74.5%) of crude IX as a viscous oil. This oil was used directly for the next step. Didicyclohexylamine salt: mp 142—144° (EtOH–ether). *Anal.* Calcd. for $C_{44}H_{79}O_{10}N_5S$: C, 60.73; H, 9.15; N, 8.05; S, 3.68. Found: C, 59.78; H, 9.11; N, 7.92; S, 3.51. The acidic aquous layer was saturated with NaCl and extracted with AcOEt. The extracts were evaporated to give an oil (0.45 g) which was found to be a mixture of two components, IX and S-*tert*-butoxycarbonylglutathione (X) (the ratio being about 1:1 as measured by NMR). This oil was also used for the next step.

17) E. Schröder and E. Klieger, *Ann.*, **673**, 196 (1964).

Glutathione Hydrochloride (XI)——To a solution of the above IX (1.63 g) in AcOH (8 ml) was added 2.1 N HCl/AcOH (16 ml), the solution was kept for 1 hr at room temperature and evaporated *in vacuo*. The residue was washed twice with ether–petr. ether (1:1 v/v) and dried *in vacuo* over NaOH and P_2O_5 to give a very hygroscopic powder (1.2 g, quantatative). IR spectrum of this product was practically identical with that of authentic XI.

Glutathione (XII)——A 340 mg portion of XI was dissolved in distilled water and filtered. The solution was adjusted to pH 2.8—2.9 with addition of Et_3N, concentrated *in vacuo* below 40° and diluted with EtOH (10 ml) and a solid thus precipitated was collected by decantation, washed with cold EtOH and dried over P_2O_5 to give crude glutathione, 200 mg, mp 140—150°. The combined mother liquor and washings were diluted with ether and a separated solid was treated again as described above to give another crop of glutathione (27 mg, mp 140—150°; total yield 74.7%). To a 215 mg (0.63 mmole) portion of this product in 1 N H_2SO_4 (8 ml) was added freshly prepared Cu_2O (43.6 mg; 0.31 mmole) at 40—50°. After cooling, the resulting precipitate was collected, washed repeatedly with oxygen-free water until the washing had become free of sulfate ion, each time the precipitate being collected by centrifugation, and dried to give cuprous glutathione (215 mg, 83%). This was treated with H_2S according to the method of du Vigneaud, *et al.*[14] to give pure glutathione (78 mg, 45%), mp 187—188° (decomp.), $[\alpha]_D^{30}$ −19.8° (c=2.0, water). *Anal.* Calcd. for $C_{10}H_{17}O_6N_3S$: C, 39.08; H, 5.58; N, 13.67; S, 10.43. Found: C, 38.97; H, 5.73; N, 13.10; S, 10.00. This was identified with authentic glutathione by comparison of their IR spectra. In paper chromatography (Toyo Roshi; No. 51, ninhydrin), this showed *Rf* 0.35 in *n*-BuOH–AcOH–water (2:1:1 v/v) and *Rf* 0.13 in *n*-BuOH–AcOH–pyridine–water (5:1:3:4 v/v); these *Rf* values also gave good agreement with those of glutathione.

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