SYNTHESIS OF [1,1'-$^{13}C_2$]-L-CYSTINE

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SUMMARY

An improved synthesis of [1,1'-$^{13}C_2$]-L-cystine which includes the optical resolution of the labeled N-acetyl-S-benzyl-DL-cysteine by *Aspergillus* acylase into S-benzyl-L-cysteine is described.

Key words : [1,1'-$^{13}C_2$]-L-cystine, S-benzyl[1-^{13}C]-L-cysteine
Aspergillus acylase

INTRODUCTION

The backbone carbonyl carbon NMR resonances of cysteinyl residues have recently been employed as sensitive probes to study the states of disulfide bonds in a protein having a molecular weight of 23kDa (1). As disulfide bonds have been known to stabilize the native conformation of proteins, intensive effort has been focused on the characterization of their structural roles (2-5). Such studies, however, have been hampered by the lack of the appropriate methods for this purpose, especially in the cases of larger proteins. A new approach to study the disulfide bonds in proteins using cysteinyl carbonyl carbon resonances seems therefore tc be a useful addendum (1), if one could obtain [1,1'-$^{13}C_2$]-L-cystine which is necessary to label proteins in their cysteinyl carbonyl carbons. Unfortunately, no convenient method has been published for synthesizing [1,1'-

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$^{13}C_2$]-L-cystine, which is not readily available commercially. Since microorganisms can utilize only L-cystine, which consists one quarter of racemic cystine, as the precursor for cysteinyl residues in proteins, versatile synthetic pathways of the optically active [1,1'-$^{13}C_2$]cystine should be established.

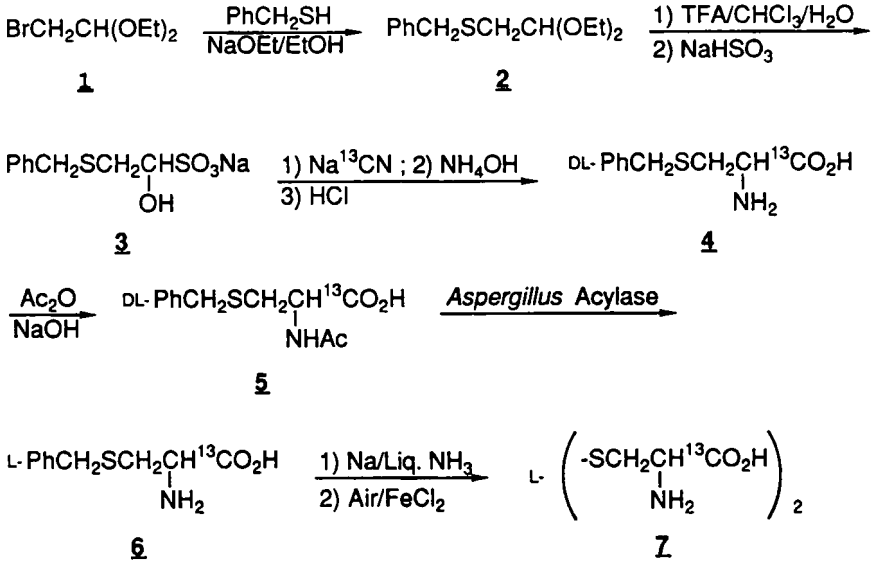
A variety of synthetic methods have been reported for L-cystine, although very few can be used for introducing the ^{13}C label at the carbonyl carbon. The methods from diethyl malonate derivatives (6-8) are not appropriate, since one of the two labeled carboxyl groups should be eliminated by decarboxylation. Methods that use labeled serine, β-chloroalanine, or acrylic acid derivatives (9-12) as starting materials suffer from the difficulties in obtaining the labeled compounds. On the other hand, the Strecker synthesis of cysteine from 2-benzylthio-acetaldehyde seems to be more facile, since one can introduce the ^{13}C label by using the labeled cyanide in the reaction step close to the end. Nadeau and Gaudry (13) reported a modified Strecker synthesis utilizing a hydantoin derivative. We found, however, that relatively poor yield of the hydantoin derivative made this process unsuitable for synthesizing the labeled cystine. Hruby *et al.* (14) have reported the synthesis of S-benzyl[1,1'-$^{13}C_2$]-DL-cystine from 2-benzylthioacetaldehyde by the method of Gawron and Glaid (15) who carried out the Strecker synthesis by a two-step reaction, namely a cyanohydrin synthesis followed by amination. This method seems to be appropriate for synthesis of [1,1'-$^{13}C_2$]-L-cystine, although the yield on their procedures should be improved. In this paper we describe a versatile method for synthesizing optically active [1,1'-$^{13}C_2$]-L-cystine in fairly good yield.

RESULTS AND DISCUSSION

The synthetic pathway for [1,1'-$^{13}C_2$]-L-cystine (7) is shown in the Scheme. 2-Bromoacetaldehyde diethylacetal (1) was

converted to the 2-benzylthioacetaldehyde bisulfite adduct (3) (13, 16), which was used for further reactions. The yield of the amination reaction of the cyanohydrin intermediate (see the Scheme) was improved by lowering the reaction temperatures, namely S-benzyl[1-^{13}C]-DL-cysteine was obtained in 55% yield at room temperature which compared favorably with 37% yield at 100 °C. S-Benzyl[1-^{13}C]-DL-cysteine (4) was prepared from the bisulfite adduct (3) without isolating the cyanohydrin and aminonitrile intermediates and then acetylated with acetic anhydride to give N-acetyl-S-benzyl[1-^{13}C]-DL-cysteine (5).

SCHEME



A crucial step in this synthesis was to hydrolyze N-acetyl-S-benzyl[1-^{13}C]-DL-cysteine (5) with *Aspergillus* acylase to S-benzyl[1-^{13}C]-L-cystein (6). We found that hog kidney acylase I, which has been used frequently in amino acid syntheses, was not capable of hydrolizing the N-acetyl intermediate efficiently. We found that this microbial acylase showed a much higher hydrolytic activity toward N-acetyl-S-benzylcysteine and gave S-benzyl-L-cysteine quantitatively. The remaining D-isomer in the reaction mixture was racemized (18) and subjected also to optical

resolution. The combined S-benzyl[1-^{13}C]-L-cysteine (6) was oxidized to [1,1'-$^{13}C_2$]-L-cystine (7) by the method of Wood and Vigneaud (18). The [1,1'-$^{13}C_2$]-L-cystine prepared was obtained in an overall yield of 35%, calculated from Na^{13}CN. The labeled cystine prepared by the present method could be incorporated into a protein very efficiently (1). This improved synthesis of [1,1'-$^{13}C_2$]-L-cystine (7) may be recommended to non-organic chemists, since every step could be carried out quite reproducibly.

EXPERIMENTAL

Spectral measurements

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer (300 MHz for ^{1}H and 75.4MHz for ^{13}C). Optical rotations were measured with a DIP-370 polarimeter (Japan Spectroscopic Co.,Ltd.)

2-Benzylthioacetaldehyde diethylacetal (2)

The method of Nadeau and Gaudry was used (13): to 200 mL of 1 M sodium ethoxide in ethanol, benzylmercaptane (Aldrich; 25 g, 0.2 mol) and 2-bromoacetaldehyde diethylacetal (1) (Aldrich; 40 g, 0.2 mol) was added at room temperature with stirring. After refluxing for 2 hours, the reaction mixture was poured into 500ml of cold water and the product was extracted with ether. The ethereal extract, dried over magnesium sulfate, afforded by removing the solvent 2-benzylthioacetaldehyde diethylacetal (2), which was distilled at 161-162°C / 17 mmHg (lit.(13), 178°C 25mmHg). Yield: 41 g (85%); ^{1}H NMR δ ($CDCl_3$): 1.21 (6H, t, CH_3), 2.58 (2H, d, SCH_2CH), 3.57 (4H, m, OCH_2CH_3), 3.79 (2H, s, $PhCH_2$), 4.54 (1H, t, SCH_2CH), 7.31 (5H, s, Ph).

2-Benzylthioacetaldehyde bisulfite adduct (3)

To a mixture of trifluoroacetic acid (10 ml), chloroform (20 ml) and water (10 ml), kept at 0 °C, benzylthioacetaldehyde

diethylacetal (2) (6.0 g, 25 mmol) was slowly added with vigorous stirring. The mixture was kept at 0 °C for 1.5 hour. The organic layer was separated, washed with water, and the solvent was evaporated in vacuo. Sodium bisulfite (2.6 g, 25 mmol) and a few ml of water were added to the residual syrup with vigorous stirring. After the exothermic reaction ceased, the mixture was allowed to stand at room temperature. The cooled reaction mixture was diluted with 100 ml of water, extracted with ether, and the aqueous layer was evaporated under reduced pressure until precipitates came out. Several volumes of ethanol were added to the suspension, which was allowed to stand overnight at room temperature. The crystals were collected by suction and dried at 100 °C to a white powder. Yield: 4.5 g (75%); ^{1}H NMR δ (D_2O): 2.74, 3.04 (2H, m, CH_2CH), 3.88 (2H, s, $PhCH_2$), 4.36 (1H, m, CH), 7.24 (5H, s, Ph).

S-Benzyl[1-^{13}C]-DL-cysteine (4)

To a solution of benzylthioacetaldehyde bisulfite adduct (3) (5.4 g, 20 mmol) in 20 ml of water, $Na^{13}CN$ (90% ^{13}C; CEA) (1.0 g, 20 mmol) in 5 ml of water was added dropwise over 20 minutes. After the mixture was stirred for 2 hours, it was extracted with ether. The ethereal solution was evaporated to dryness. Ethanol (28 ml) and 28% NH_4OH (28 ml) were added to a pale yellow residue and stirred for 12 hours at room temperature. After the solution was concentrated to about 30ml in vacuo, an equal volume of brine was added and the oily product was extracted with ether. The ether was evaporated and 30 ml of 35% aq. HCl was added to the residual oil. The resulting lumpy solid was crushed well with a glass rod at 0 °C, and dry hydrogen chloride was introduced for 20 minutes to the mixture at 0 °C. The mixture was then kept at this temperature for 24 hours and then refluxed for 2 hours. After treatment with active charcoal, the solution was brought to pH 6 by adding 28% NH_4OH; the solution was then chilled for

several hours. S-benzyl[1-^{13}C]-DL-cysteine (4) was collected by suction, dissolved in 1N NH_4OH, and decolorized with charcoal again. The solution was adjusted to pH 6 with 1N HCl and stored overnight at 4 °C. The crystals were collected by suction and washed repeatedly with a small amount of cold water, ethanol, and ether. Yield: 2.3 g (55% from Na^{13}CN); ^{1}H NMR δ (0.5N NaOD): 2.51, 2.59 (2H, m, C$\underline{H}_2$CH), 3.19 (1H, m, C$\underline{H}$), 3.79 (2H, s, PhC$\underline{H}_2$), 7.21 (5H, s, Ph). ^{13}C NMR δ (0.5N NaOD): 182.30 ($\underline{C}$=O).

N-Acetyl-S-benzyl[1-^{13}C]-DL-cysteine (5)

To a solution of S-benzyl[1-^{13}C]-DL-cysteine (4) (2.1 g, 10 mmol) and NaOH (1.0 g) in 3.5 ml of water, acetic anhydride (1.4 g) was added dropwise over 30 minutes at 37-40 °C. After the solution was kept at the temperature for 30 minutes, it was brought to pH 2 and stored at 4 °C overnight. The white precipitate was collected by suction and washed with a small amount of cold 0.01N HCl and ether. Yield: 2.3 g (91%); ^{1}H NMR δ (0.5N NaOD): 2.00 (3H, s, COC$\underline{H}_3$), 2.76, 2.82 (2H, m, C$\underline{H}_2$CH), 3.80 (2H, s, PhC$\underline{H}_2$); 4.32 (1H, m, $CH_2C\underline{H}$), 7.40 (5H, s, $\underline{Ph}$). ^{13}C NMR δ (0.5N NaOD): 178.22 ($\underline{C}$=O).

S-Benzyl[1-^{13}C]-L-cysteine (6)

N-Acetyl-S-benzyl[1-^{13}C]-DL-cysteine (5) (2.5 g, 10 mmol) and $CoCl_2.6H_2O$ (10 mg) was dissolved in 40 ml 1N NaOH, and the solution was brought to pH 8.0 with 1N HCl. A crude powder of *Aspergillus* acylase (80 mg) (Amano Seiyaku) was added to the solution which was incubated for 1 day at 37 °C with occasional gentle shaking. The mixture was then adjusted to pH 6 with 1N HCl and stored at 4 °C overnight. The crystals were collected by suction and washed repeatedly with cold water, ethanol, and ether. N-Acetyl-S-benzyl[1-^{13}C]-D-cysteine was recovered from the mother liquid and aqueous washings and was racemized as described below to N-acetyl-S-benzyl[1-^{13}C]-DL-cysteine. Yield: 1.0 g (95%

recovery of L-form). [α]D= +19.4 (c=1, 1N NaOH, 22 °C)(Lit.(17), +25.5, c=1, 1N NaOH, 25 °C). ^{1}H NMR and ^{13}C NMR spectra were identical with those of DL-form (4).

Racemization of N-acetyl-S-benzyl[1-^{13}C]-D-cysteine

The mother liquor and aqueous washings of the above reaction were combined and acidified to pH 2 with 35% HCl. The suspension was stored overnight at 4 °C. The precipitated N-acetyl-S-benzyl[1-^{13}C]-D-cysteine was collected by filtration and washed with a small amount of cold 0.01N HCl. The racemization was performed by using the modified method of Wood and Vigneaud (18): the solution of N-acetyl-S-benzyl[1-^{13}C]-D-cysteine (1.0 g, 4 mmol) in 2 ml of 2.5N NaOH was heated on a water bath at 60-65 °C. To this solution, acetic anhydride (0.64 g, 6.4 mmol) was added dropwise over 1 hour. The solution was kept at this temperature for 30 minutes and was allowed to cool. The solution was acidified to pH 2 with 35% HCl and was stored at 4 °C overnight. The precipitate was filtered and washed with 0.01N HCl. Yield: 1.0 g (99%).

[1,1'-$^{13}C_2$]-L-cystine (7)

S-Benzyl[1-^{13}C]-L-cysteine (6) was converted to [1,1'-$^{13}C_2$]-L-cystine (7) according to the method of Wood and Vigneaud (18): 1.0g of S-benzyl[1-^{13}C]-L-cysteine (4.7 mmol) was treated with sodium in dry liquid ammonia, and the deprotected L-cysteine was oxidized to L-cystine by air with $FeCl_2$ as a catalyst. Yield: 0.42 g (75%); [α]D=-210.2 (c=1, 1N HCl, 22 °C)(lit.(19), -223.4, c=1, 1N HCl, 20°C). ^{1}H NMR δ (0.5N NaOD):2.91, 3.11(2H,m, C$\underline{H}_2$), 3.58 (1H, m, C$\underline{H}$). ^{13}C NMR δ (0.5N NaOD) 182.10 ($\underline{C}$=O).

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REFERENCES

(1) Uchida K., Miyake Y. and Kainosho M., - J. Biomol. NMR, 1, 000 (1991).

(2) Creighton T. E. - Methods Enzymol., 131, 83 (1986)

(3) Wagner G., Kalb A. J. and Wuethrich K. - Eur. J. Biochem., 95, 249(1979)

(4) Goto Y. and Hamaguchi K. - J. Mol. Biol., 146, 321(1981)

(5) Thornton J. M. - J. Mol. Biol., 151, 261(1981)

(6) Wood J. L. and du Vigneaud V. - J. Biol. Chem. 131 : 267 (1939)

(7) Crawhall J. C. and Elliott D. F. - J. Chem. Soc. : 2071 (1951)

(8) Arnstein H. R. V. and Crawhall J. C. - Biochem. J. 67 : 180 (1957)

(9) Farlow M. W. - J. Biol. Chem. 176 : 71 (1948)

(10) Fry E. M. - J. Org. Chem. 15 : 438 (1950)

(11) Rambacher P. - Chem. Ber. 101 : 3433 (1968)

(12) Schoberl A., Rimpler M. and Magosch K. H. - Chem. Ber. 102 : 1767 (1969)

(13) Nadeau P. G. and Gaudry R. - Can. J. Research 27 : 421 (1949)

(14) Hruby V. J., Viswanatha V. and Yang Y. C. S. - J. Label. Compounds Radiopharmaceuticals 17 : 801 (1979)

(15) Gawron O. and Glaid III A. J. - J. Am. Chem. Soc. 71 : 3232 (1949)

(16) Ellison R. A., Lukenbach E. R. and C. Chiu - Tetrahedron Lett. : 499 (1975)

(17) Brinbaum S. M. and Greenstein J. P. - Arch. Biochem. Biophys. 39 : 108 (1952)

(18) Wood J. L. and du Vigneaud V. - J. Biol. Chem. 130 : 109 (1939)

(19) Loring H. S. and du Vigneaud V. - J. Biol. Chem. 107 : 267 (1934)