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# Synthesis of Cystine-3,3′-$^{13}$C-3,3,3′,3′-$d_4$ for Use in Incorporation into Proteins

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Cystine labeled in the 3-position with both $^{13}$C and two deuteriums was prepared in 17% overall yield from Ba$^{13}CO_3$ by an aminomalonate condensation with $D_2CO$-$^{13}$C. The labeled formaldehyde precursor was prepared by catalytic oxidation of $^{13}CD_3OH$, which in turn was obtained by reduction of $^{13}CO_2$ with lithium aluminum deuteride. 

## INTRODUCTION

Specifically labeled cystine was desired for *in vivo* incorporation into the protein of *Escherichia coli* and other microorganisms so that enzymes containing such specifically labeled cysteine residues could be isolated. Similar incorporation of selectively $^{13}$C-enriched and $^{2}$H-labeled histidine has been reported and shown to facilitate NMR studies of macromolecules (*1*). These specific labeling techniques are of growing interest and utility as more genes encoding for proteins are cloned into microorganisms. In this paper, we describe a straightforward and reasonably large-scale preparation of cystine 3,3′-$^{13}$C-3,3,3′,3′-$d_4$ for such purposes.

## MATERIALS AND METHODS

$LiAlD_4$ was purchased from Merck, $BaCO_3$ (90% $^{13}$C) from Monsanto, and diethylaminomalonate hydrochloride from Aldrich; tetrahydrofurfuryl alcohol and dihydropyran (both practical grade) were obtained from Matheson, Coleman, and Bell. All other chemicals were reagent grade.

Melting and boiling points are uncorrected. The elemental analysis was performed by the Microanalytical Laboratory, University of California, Berkeley. PMR spectra were taken on a Varian T-60 spectrometer. CMR spectra (proton-

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decoupled) were obtained at 25 MHz with internal deuterium lock and quadrature phase detection on a Nicolet TT-23 spectrometer.

*Preparation of $^{13}CD_3OH$ (2)*

(*a*) *Preparation of tetrahydrofurfuryloxytetrahydropyran (TFTP) (3).* Tetrahydrofurfuryl alcohol (TFA) (569 g; 5.56 mol) was added dropwise with stirring to a solution of concentrated HCl (1 ml) in dihydropyran (499 g; 5.93 mol) at 0°C over 3 h. After stirring overnight at room temperature, the product was distilled under reduced pressure from several NaOH pellets. First fraction: 125 ml, bp 100–130°C at 15 mm Hg; product: bp 130°C at 15 mm Hg. The volume of the first fraction can be reduced by initial removal of unreacted starting materials by distillation using a column packed with glass beads. The last traces of TFA were removed from the product by treatment with either $LiAlH_4$ or Na followed by simple redistillation at the minimum feasible pressure into a cooled receiver. Removal of TFA or water can be monitored by observing the O–H stretching vibration in the IR at 3600 $cm^{-1}$.

(*b*) *Preparation of $LiAlD_4$ in TFTP.* Under nitrogen, $LiAlD_4$ (10.0 g; 0.238 mol) was ground in a mortar and added to 500 ml TFTP in a 1-liter three-neck flask, and the resulting suspension was stirred overnight at ~65°C. The solution was then filtered through a coarse sintered glass filter and standardized by iodometry (*4*).

(*c*) *Preparation of $^{13}CD_3OH$.* The reduction was performed on a vacuum manifold. Diagrams of the apparatus used may be found elsewhere (*2a, 5*). One flask contained the $LiAlD_4$/TFTP solution and a magnetic stirring bar. A second flask contained $Ba^{13}CO_3$ (23.6 g; 0.119 mol) and was fitted with an additional funnel which contained concentrated $H_2SO_4$ (80 ml; 36 N). The system was evacuated and flushed with nitrogen three times, evacuated to <50 $\mu$m pressure, and the $LiAlD_4$ solution was cooled to 0°C. $^{13}CO_2$ was generated over 40 min by dropwise addition of $H_2SO_4$ to the second flask while the $LiAlD_4$ solution was stirred continually. After further stirring for 3 h at room temperature, the reaction mixture was frozen using liquid nitrogen, the remainder of the system was heated with a heat gun, and the solution was then thawed.

The methanol complex was then destroyed by adding TFA (146 g, 1.43 mol) dropwise with stirring to the reaction vessel at 0°C over a period of 1.5 h. The methanol released was distilled from the vessel under reduced pressure and partial reflux into two traps cooled in liquid nitrogen. The product was distilled over a period of several hours at 70°C and <1 mm Hg. Any product in the secondary trap transferred to the primary, and the methanol was frozen and purified by sublimation under reduced pressure. The estimated overall yield from four batches of $Ba^{13}CO_3$ (0.476 mol) was about 74% (12.7 g; 0.352 mol). Purity, as determined by PMR, was at least 94%. The major impurity, TFA, will not interfere with the oxidation to formaldehyde.

*PMR spectrum of $^{13}CD_3OH$ (neat, ext. TMS).* $\delta$4.53 (s, OH).

*Preparation of $D_2{}^{13}CO$.* $D_2{}^{13}CO$ was prepared from $^{13}CD_3OH$ by the oxidative method of Arnstein (*6*) using a molybdenum oxide/ferric oxide catalyst. The yield (72%) based on labeled methanol was determined either by formation of the dime-

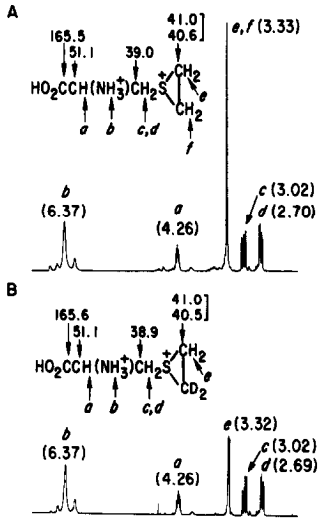



FIG. 2. $^1$H NMR spectra of thiiranium ions derived from (A) HEC and (B) HEC-$d_2$ in trifluoromethanesulfonic acid : sulfur dioxide at 22°C. The $^{13}$C chemical shifts are given with the structural formulae.

chemical shifts reported for thiiranium ions with a methyl substituent on the ring (*12*) when the effect of a β-methyl group is taken into account.

Dissolution of HEC-1,1-$d_2$ in trifluoromethanesulfonic acid : sulfur dioxide as above results in a similar $^1$H NMR spectrum (Fig. 2B), except that the signal ascribed to the ring protons appears as two singlets at 3.29 and 3.31 ppm and is reduced in area by one-half. The $^{13}$C NMR spectrum is also similar to that observed for the product obtained from HEC, except that the signals assigned to the ring carbons are of lesser intensity.

Other reasonable reaction pathways for the parent compound in strong acid include dehydration to the olefin, which would rapidly polymerize in super acid (the $^1$H NMR spectra showed evidence for a small amount of polymeric material) and formation of a protonated sulfide. The $^1$H NMR of the latter would be distinctive, showing additional couplings in the resonances from the protons adjacent to the sulfur (*13*), and no evidence for such a species was obtained.

### *Chlorination of HEBS-1,1-$d_2$*

HEBS-1,1-$d_2$ was prepared to determine if an organosoluble β-hydroxyethylsulfide could be converted to the corresponding chloride without randomization of the deuterium label. Reaction of HEBS-1,1-$d_2$ with a 10% molar excess of thionyl chloride in $CDCl_3$ at room temperature produces a 50 : 50 mixture of chlorides CEBS-1,1-$d_2$ and CEBS-2,2-$d_2$. The reaction is complete in less than 5 min and no NMR signals attributable to the intermediate chlorosulfite ester are observed. Identical results are obtained when the reaction is conducted in the presence of a 10% molar excess of pyridine-$d_5$. This extremely rapid reaction suggests

accomplished by treatment of a 0.1–0.2 M solution in 1 N HCl with decolorizing carbon and reprecipitation as described above.

*Anal.* Calcd for $^{12}C_4{}^{13}C_2H_8D_4N_2O_4S_2$: C, 30.08; H, 6.50; N, 11.38. Found: C, 29.86; H, 6.64; N, 11.20.

*PMR spectrum of cystine-3,3'-$^{13}$C-3,3,3',3'-$d_4$ (0.5 M in 1.2 N DCl, ext. TMS).* $\delta$4.7 (d, = CH–, $J_{^{13}C\text{-}C\text{-}H}$ = 5 Hz).

*CMR spectrum of cystine-3,3'-$^{13}$C-3,3,3',3'-$d_4$.* As expected, the labeled carbon (in 1.2 N DCl) appears as a quintet [(dioxane) $\delta$ ppm = 30.78] due to coupling with the two deuteriums ($J_{^{13}C\text{-}D}$ = 22 Hz). The corresponding $\delta$ value for this carbon in non-deuterated cystine is 30.33.

## RESULTS AND DISCUSSION

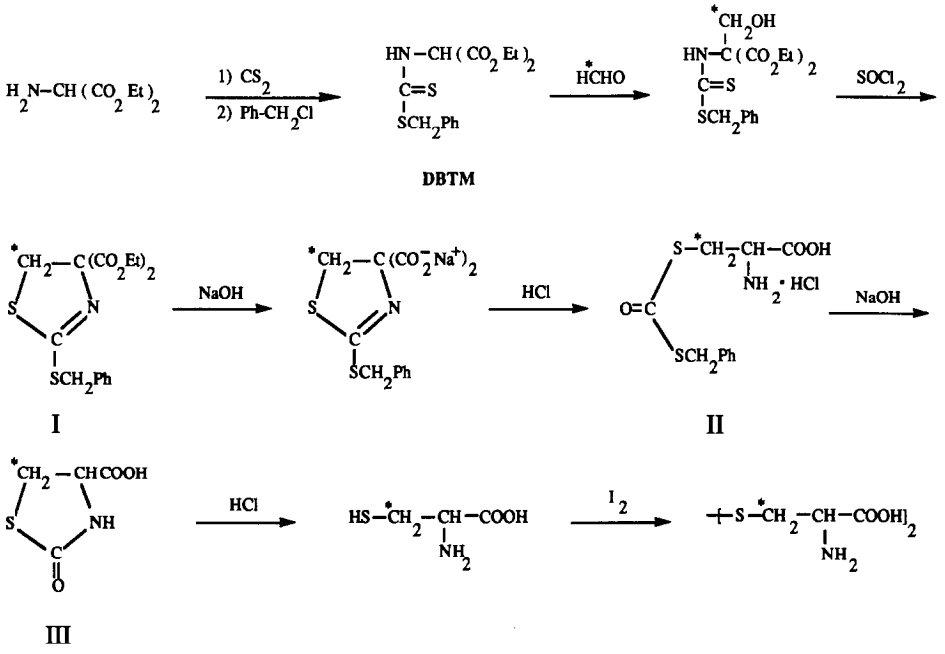
Most of the methods reported in the literature for synthesizing cysteine can be placed in one of three main categories: those including an acrylate intermediate, those utilizing malonate condensation, or those involving the conversion of serine to cysteine. Syntheses via an acrylate usually proceed through the addition of a mercaptan across the double bond:

$$R'\text{—}SH + H_2C{=}\overset{\displaystyle R}{\overset{|}{C}}\text{—}COOH \rightarrow R'\text{—}S\text{—}CH_2\text{—}\overset{\displaystyle R}{\overset{|}{C}}H\text{—}COOH \qquad (1)$$

A major obstacle to our employing a synthesis of this type, however, is presented by the difficulty of achieving specific labeling of the 3-carbon of the acrylate molecule. Based on reported yields, the simplest and best method of producing the desired labeled compound should be that of Rambacher (*10*) for converting serine to cysteine (via the azlactone) with thiolacetic acid in acetic anhydride and pyridine. Serine can easily be made in 70% yield from formaldehyde (*11*), and Rambacher claims a 72–78% conversion of serine to cystine. The overall yield from formaldehyde could therefore be greater than 50%, better than any other total synthesis reported in the literature. However, in our hands the Rambacher method was found to give variable yields far below that reported.

Cystine-3,3'-$^{13}$C was ultimately synthesized using adaptations of the method of Crawhall and Elliott (*9a*) and Arnstein and Crawhill (*9b*) starting from formaldehyde and diethyl[(benzylthio)thiocarbonylamino]malonate (Scheme 1). Although the yield is somewhat lower (48% based on formaldehyde) than that reported for the alternate route, this procedure is facile and reliable. It involves the condensation of formaldehyde with DBTM and cyclization with thionyl chloride to give the thiazoline (**I**). Saponification, decarboxylation, and hydrolysis afford *S*-[(benzylthio)carbonyl] cysteine (**II**), which is cyclized to 2-ketothiazolidine-4-carboxylic acid (**III**) and finally hydrolyzed in acid to cysteine.

The final problem in the synthesis was finding an efficient means of obtaining the appropriately labeled formaldehyde from $Ba^{13}CO_3$. Here again a variety of methods are described in the literature. Of those attempted, oxidation of methanol




SCHEME 1.

with a molybdenum oxide/ferric oxide catalyst (*6*) gave the highest yields in our hands. An additional advantage of this method is that it directly provides the Formalin solution required for the reaction with DBTM.

Labeled methanol was prepared by reduction of $^{13}CO_2$ (*2*), generated from $Ba^{13}CO_3$, with lithium aluminum deuteride in a high boiling ether, tetrahydrofurfuryloxytetrahydropyran.

Such labeled cystine may be readily converted either to the corresponding labeled cysteine by reduction with tin in 3 N HCl (*12*) or to the correspondingly labeled cysteic acid by performic acid oxidation (*5*). Once obtained, specifically labeled cysteine can be incorporated into bacterial proteins as described by Beilan *et al.* (*13*). Such specifically labeled proteins can be useful in both structural and mechanistic studies on proteins using $^{13}C$ NMR spectroscopy (*1*). Examples of $^{13}C$ NMR spectra taken at 25 MHz after the labeled cysteine was incorporated into *Escherichia coli* tryptophan synthase $\alpha$-subunit may be seen elsewhere (*5*).

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