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Note

L-(1-^{13}C)- and (2-^{13}C)ascorbic acid: synthesis and NMR characterization

Kenneth N. Drew [a], Timothy J. Church [a], Bidisa Basu [a], Tapani Vuorinen [b], Anthony S. Serianni [a,*]

[a] *Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556, USA*
[b] *Laboratory of Forest Products Chemistry, Helsinki University of Technology, FIN-02150 Espoo, Finland*

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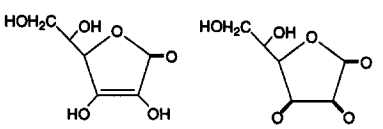
Apart from its well-known role in the prevention of scurvy, ascorbic acid (**1**, vitamin C) has been implicated in a number of diverse biological processes. For example, the monooxygenase, dopamine β-hydroxylase, which catalyzes the hydroxylation of 3,4-dihydroxyphenylethylamine (dopamine) to noradrenaline (norepinephrine), requires **1** for activity. Several dioxygenases also require **1** as a substrate or cofactor. Thus, **1** is required in the hydroxylation of peptidyl-L-prolyl to peptidyl-*trans*-hydroxy-L-prolyl by the enzyme, prolyl hydroxylase, during collagen biosynthesis. More recently, it has been proposed that **1** may mediate inter-cellular (inter-organ) energy transfer in living systems [1] (Scheme 1).



In this process, the glycolytic pathway of an erythrocyte generates **1** from dehydroascorbate (**2**) through the participation of NADH (generated via the reaction catalyzed by D-glyceraldehyde 3-phosphate dehydrogenase), and exports it. Compound **1** is subse-

* Corresponding author.

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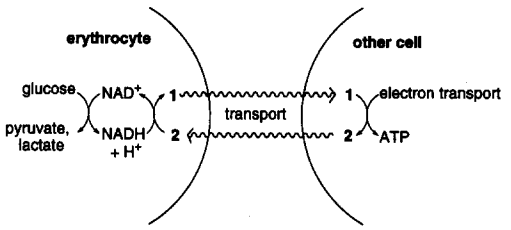
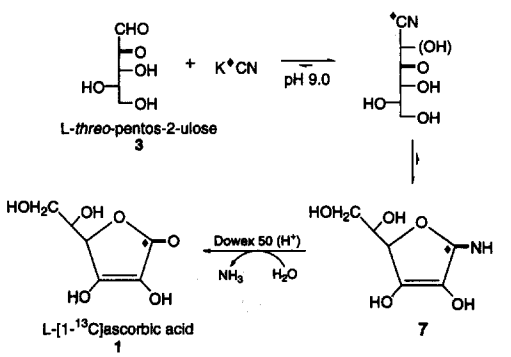


Scheme 1.

quently taken up by another cell, and its reducing equivalents utilized by the electron transport chain to generate energy in the form of ATP (Scheme 1). The redox properties of **1** may also be important in the diagnosis and treatment of cancer [2].

In vitro and in vivo investigations of the biological roles of vitamin C may benefit from the use of ^{13}C-labeled compounds, since the selectivity conferred to NMR investigations using labeled molecules makes it possible to monitor the fate of a specific metabolic precursor in an otherwise complex system. In anticipation of this application, we have refined a chemical route to the ^{13}C-labeling of **1** that may be adapted to prepare various ^{13}C isotopomers. In this report, we describe the preparation of L-(1-^{13}C)- and (2-^{13}C)ascorbic acid and discuss several ^{13}C–^{13}C and ^{13}C–^{1}H spin-coupling constants measured in these labeled forms.

A comprehensive review of chemical and enzymic routes to **1** has been published [3]. Based on considerations of ease of synthesis and adaptability to prepare various isotopomers in multigram quantities, a route involving the well-known condensation of KCN with L-*threo*-pentos-2-ulose (**3**) was adopted [4] (Scheme 2). Compound **3** was obtained from L-xylose (**4**) via oxidation with $Cu(OAc)_2$ in aqueous methanol as described previously [5]. This reaction gave a mixture of L-*threo*-pentos-2-ulose (**3**) and unreacted **4** (approximate 3:1 ratio, respectively, based on ^{13}C NMR spectroscopy), which was chromatographed on Dowex 50 × 8 (200–400 mesh) in the Ba^{2+} form [6]; **4** eluted prior to **3**. Thus, unreacted **4** can be recovered and reused, which is beneficial when **4** is ^{13}C-labeled.



Scheme 2.

The preparation of (2-^{13}C)**1** required L-(1-^{13}C)**4**, which was obtained via application of the cyanohydrin reduction reaction to L-threose (**5**) [7,8]. Compound **5** was prepared in good yield from 1,3-*O*-benzylidene-L-arabinitol (**6**) as described by Hudson and coworkers [9] with minor modification. Compound **6** was treated with sodium periodate as described by Perlin [10], yielding **5** after hydrolysis of the benzylidene group.

Earlier work [3,4] has shown that the addition of KCN to **3** yields **1** after acid hydrolysis. We observed considerable darkening (browning) of the reaction mixture when KCN was added directly to an aqueous solution of **3**, suggesting that degradation of **3** might be occurring under basic conditions, thus reducing the yield of **1**. In the present work, the addition of KCN was made under controlled pH (pH 8.5), analogous to the approach used for cyanohydrin reduction reactions [7,8]. With K^{13}CN as a reactant, the resulting (1-^{13}C)-labeled reaction intermediates were monitored via their time-lapse ^{13}C NMR spectra (Fig. 1). The data show an immediate production of several labeled signals between 118 and 122 ppm having chemical shifts characteristic of aldononitriles [11]. At least ten signals were observed in this region, indicating the production of several cyanohydrins which apparently arise from cyanide addition to acyclic and cyclic forms of **3** [5] (Fig. 1A). Enolization of the acyclic nitriles might also occur and thus contribute signals to this region. In contrast to the reaction of KCN with aldoses at pH 8.5–9.0 [11], ring-closure to the putative imido-1,4-lactone intermediate **7** (Scheme 2) was rapid and almost complete at pH 9.0, as indicated by the appearance of an intense labeled signal at 177.7 ppm after 10–20 min of reaction (Fig. 1B); a similar monitoring of the reaction of KCN with L-(1-^{13}C)*threo*-pentos-2-ulose by ^{13}C NMR revealed the C-2 signal of **7** at 116.3 ppm. At lower pH (pH 8), the conversion of nitrile to imidolactone appeared to yield less imidolactone (i.e., ring-closure appeared slower and less imidolactone formed). At pH 8–9, no darkening of the reaction mixture occurred.

As discussed earlier [12], products generated from the hydrolysis of imidolactones depend on solution pH, with low pH favoring lactone formation and high pH favoring amide formation. In the present case, the hydrolysis of **7** was conducted in the presence of Dowex 50 × 8 (200–400 mesh) ion-exchange resin in the H^+ form (Scheme 2). Since **7** is protonated in acidic solution, it binds to the resin early in the hydrolysis reaction; the product (1-^{13}C)**1** forms directly from (1-^{13}C)**7**, as indicated by the generation of a new signal at 174.8 ppm (Fig. 1C). The conversion of **7** to **1** was essentially complete after ~ 1.5 h; continued reflux beyond this point led to reduced yields of **1**. Other acid catalysts were examined (e.g., HCOOH and HCl), but better yields of **1** were obtained with the resin catalyst.

The ^{1}H-decoupled ^{13}C NMR spectrum (150 MHz) of L-(1-^{13}C)ascorbic acid contains an intense signal at 174.8 ppm corresponding to the enriched C-1. The C-2 signal at 119.2 ppm appears as a doublet, with $^1J_{C1,C2} = 85.0$ Hz. This latter coupling is considerably larger than $^1J_{C1,C2}$ in aldono-1,4-lactones (~ 56 Hz) [13] as expected, since $^1J_{CC}$ magnitude increases as percent *s*-character of the C–C bond increases [14]. The C-3 signal (157.2 ppm) shows a splitting of 19.7 Hz; by comparison, $J_{C1,C3}$ in aldono-1,4-lactones varies from 1.6–8.0 Hz [13]. While two coupling pathways affect $J_{C1,C3}$ in **1** (i.e., C-1–C-2–C-3 and C-1–O-4–C-4–C-3), the former is likely to be primarily responsible for the larger observed coupling in **1**, since the presence of

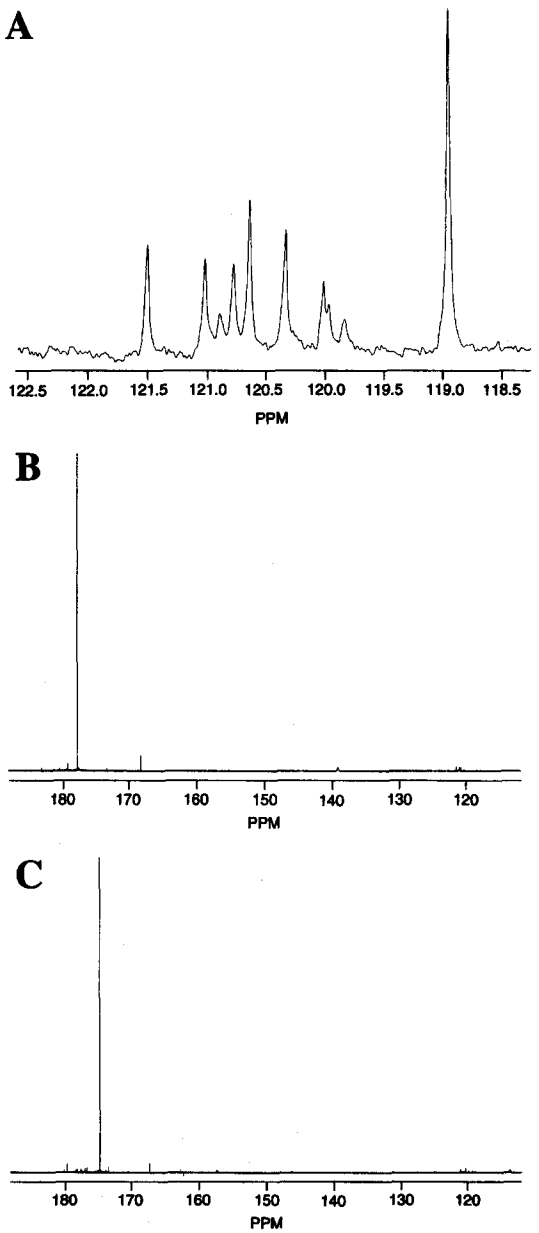


Fig. 1. (A) ^{1}H-Decoupled ^{13}C NMR spectrum (150 MHz) of the reaction mixture after addition of K^{13}CN to L-*threo*-pentos-2-ulose (**3**) (pH 8.5, 10 min), showing the production of multiple (1-^{13}C)cyanohydrins. (B) ^{1}H-Decoupled ^{13}C NMR spectrum (150 MHz) of the same reaction as in (A) conducted at pH 9.0 (20 min of reaction), where ring-closure of the cyanohydrins is favored, generating the putative (1-^{13}C)imido-1,4-lactone **7**. (C) ^{1}H-Decoupled ^{13}C NMR spectrum (150 MHz) of the reaction mixture in (B) after treatment with Dowex 50 (H^+) resin (reflux, 1.5 h), showing the conversion of (1-^{13}C)**7** to (1-^{13}C)**1**.

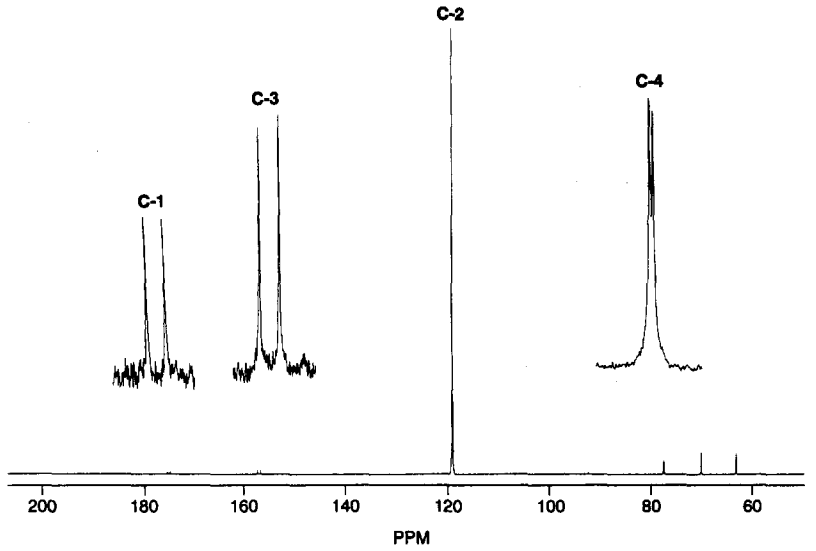


Fig. 2. ^{1}H-decoupled ^{13}C NMR spectrum (150 MHz) of purified (2-^{13}C)**1**, showing ^{13}C–^{13}C spin-couplings between the enriched C-2 and C-1, C-3 and C-4 (see text).

unsaturation along a C–C–C coupling pathway should enhance coupling substantially. No ^{13}C–^{13}C coupling is observed between C-1 and C-4, C-5 and C-6.

The ^{1}H-decoupled ^{13}C NMR spectrum of L-(2-^{13}C)ascorbic acid (Fig. 2) contains an intense signal at 119.2 ppm corresponding to the enriched C-2 carbon. The signal at 174.8 ppm (C-1) shows a splitting of 85.0 Hz ($^{1}J_{C1,C2}$), in agreement with results obtained from L-(1-^{13}C)**1** (see above). A large splitting is also observed in the signal at 157.2 ppm, which can be unequivocally assigned to C-3; $^{1}J_{C2,C3} = 91.7$ Hz, which is comparable in magnitude to $^{1}J_{C1,C2}$, since both couplings involve two sp^2-hybridized carbons. The signal at 77.6 ppm appears as a doublet, indicating that C-4 is coupled to C-2 (8.5 Hz; sign unknown). This latter coupling can arise from two pathways (C-2–C-3–C-4 and C-2–C-1–O-4–C-4), but the relative contributions to the observed coupling cannot be assessed at present, although the former is expected to make the major contribution. The remaining carbons (C-5, 70.3 ppm; C-6, 63.5 ppm) are not coupled to C-2.

Two intra-ring J_{CH} values ($^{3}J_{C1,H4}$ and $^{3}J_{C2,H4}$) are accessible with the use of L-(1-^{13}C)- and (2-^{13}C)ascorbic acid in $^{2}H_2O$ solvent. These couplings were measured directly from 1D ^{1}H NMR spectra ($^{3}J_{C1,H4} = 1.9 \pm 0.1$; $^{3}J_{C2,H4} = 1.9 \pm 0.1$ Hz) and are similar to those measured previously by Paukstelis et al. [15] via ^{1}H-coupled ^{13}C NMR spectra. In D-aldono-1,4-lactones [13], $^{3}J_{C1,H4}$ is either very small or zero (^{3}E forms) or relatively large (3.7–4.6 Hz; E_3 forms), reflecting the different C-1–O-4–C-4–H-4 dihedral angles ($\sim 90°$ and $> 135°$, respectively) in these two envelope forms. $^{3}J_{C1,H4}$ in **1** corresponds to a relatively fixed C-1–O-4–C-4–H-4 dihedral angle of $\sim 120°$ imposed by the C-2–C-3 double bond, and thus assumes the intermediate value of 1.9 Hz.

This report describes the synthesis of **1** singly substituted with ^{13}C at C-1 or C-2. The method can be extended to prepare other ^{13}C isotopomers of **1** using suitable ^{13}C substituted forms of **4**. L-(1-^{13}C)Xylose was prepared in this work by reaction of L-threose with $K^{13}CN$. This reaction also yields L-(1-^{13}C)lyxose (**8**) as a byproduct. Molybdate-catalyzed epimerization [16] of **8** yields L-(2-^{13}C)xylose, which can be used to prepare **1** singly substituted with ^{13}C at C-3. L-(3-^{13}C)Xylose can be prepared from L-(2-^{13}C)threose (**9**), and **9** is obtained by molybdate-catalyzed epimerization of L-(1-^{13}C)erythrose. The latter labeled tetrose is obtained from L-glyceraldehyde [17] and $K^{13}CN$ via cyanohydrin reduction [18]. L-(4-^{13}C)Xylose (**10**) and L-(5-^{13}C)xylose (**11**) can be obtained from L-(2-^{13}C)arabinitol (**12**) and L-(1-^{13}C)arabinitol (**13**), respectively. These labeled pentitols are readily prepared from L-(1-^{13}C)arabinose and L-(1-^{13}C)ribose [8]; the former is reduced with $NaBH_4$ to give **13**, whereas the latter is epimerized to give L-(2-^{13}C)arabinose, which is reduced to yield **12**. Alternatively, **11** can be obtained from L-(6-^{13}C)glucose as described previously [19]. Suitable modifications can be envisioned to prepare multiply-labeled forms of **1**. Thus, for example, **13** can be used to prepare **11**, and **11** can be used in reactions described herein to prepare L-(1,6-$^{13}C_2$)**1**.

1. Experimental

Materials.—Ion-exchange resins were purchased from Sigma Chemical Company. L-Xylose was purchased from Pfanstiehl Laboratories and Sigma Chemical Company. Potassium (^{13}C)cyanide ($K^{13}CN$, 99 atom%) and deuterium oxide (^2H_2O, 99.8 atom%) were purchased from Cambridge Isotope Laboratories. Thin-layer chromatography (TLC) plates (Silica Gel GF, 0.25 mm) were obtained from Analtech.

General procedures.—L-Threose (**5**) was prepared from 1,3-*O*-benzylidene-L-arabinitol as described by Perlin [10]. L-(1-^{13}C)Xylose was prepared by cyanohydrin reduction [7,8] using $K^{13}CN$ and **5** as reactants. The reaction yielded L-(1-^{13}C)lyxose and L-(1-^{13}C)xylose, which were separated by chromatography on Dowex 50 × 8 (200–400 mesh) ion-exchange resin in the Ca^{2+} form [20]. In a typical chromatographic run, the column (12 cm i.d. × 85 cm) was loaded with 42 mmol of the pentose mixture and eluted with water at a flow rate of 4 mL per min, and 20 mL fractions were collected. The *xylo* isomer (17 mmol) eluted first (fractions 51–125), and the *lyxo* isomer second (25 mmol) (fractions 145–235).

Instrumentation.—^1H-decoupled ^{13}C NMR spectra were obtained on a Varian Unity-*Plus* 300 MHz FTNMR spectrometer (75.36 MHz for ^{13}C), and a Varian Unity*Plus* 600 FTNMR spectrometer (150.84 MHz for ^{13}C), both operating at ambient temperature. Samples were analyzed in 3 mm (Nalorac microprobe) or 5 mm NMR tubes. ^{13}C NMR spectra of ^{13}C-labeled **1** in ^2H_2O were referenced to the C-1 chemical shift of α-D-(1-^{13}C)mannopyranose (95.52 ppm). ^{13}C chemical shifts are accurate to ±0.1 ppm, and ^{13}C–^{13}C couplings are accurate to ±0.1 Hz. ^1H NMR spectra (600 MHz) in ^2H_2O were obtained on a Varian Unity*Plus* 600 FTNMR operating at ambient temperature. Samples for time-lapse ^{13}C NMR determinations were prepared by adding deuterium oxide (0.1 mL) to 0.6 mL of the reaction mixture.

1,3-O-Benzylidene-L-arabinitol **6** *[9].*—L-Arabinitol (100 g, 0.66 mol) was placed in a 500-mL, large-mouth Erlenmeyer flask, and benzaldehyde (85 mL, 0.82 mol) was

added. A glass tube (~ 20 cm in length) was inserted into the flask, and dry HCl was slowly bubbled through the mixture for ~ 15 min at room temperature, with occasional stirring with the glass tube. The viscous reaction mixture was allowed to stand at room temperature for 18 h, during which time it turned into a solid crystalline mass. The mass was broken up and placed in an evacuated desiccator containing KOH and H_2SO_4 for 24 h. The mass was triturated with diethyl ether (large mortar and pestle), neutralized with M NH_4OH (~ 150 mL), and filtered and washed with H_2O until the pH of the filtrate was neutral. The product was washed with diethyl ether and recrystallized from 2-propanol containing 0.5% v/v NH_4OH to give 175 g of wet **6**. Drying afforded colorless **6**: 145 g (91%); mp 129–130 °C, ref. [9] 151–152 °C; TLC $R_f = 0.39$, (3:3:0.5 EtOAc–CH_2Cl_2–MeOH); ^{13}C NMR (acetone-d_6): 139.9, 129.3, 128.6, 127.1, 101.7, 80.1, 73.5, 70.8, 63.9, 63.7.

L-threo-*Pentos-2-ulose* **3** *[5].*—L-Xylose (**4**) (2.55 g, 17 mmol) was dissolved in 96% aqueous methanol (150 mL) and heated under reflux (oil bath, 80 °C) in a three-neck round-bottom flask. Cupric acetate monohydrate (10.2 g, 51.3 mmol) was added, and the mixture was heated for an additional 10 min. The resulting orange–brown inorganic precipitate was removed by centrifugation, and the blue filtrate (pH ~ 4.6) was transferred to a 1-L beaker containing a stir bar. To the solution was added H^+ ion-exchange resin (HCR-W2, 20–50 mesh) until the pH was lowered to ~ 2.9 and the blue color disappeared. The resin was removed by filtration and washed with water (~ 1.5 L) until the washings gave a negative phenol–sulfuric acid test [21]. The filtrate and washings were concentrated in vacuo to a small volume (~ 25 mL); ^{13}C NMR spectroscopy [5] showed the solution contained ~ 75% **3** and ~ 25% unreacted **4**, based on a comparison of anomeric carbon peak intensities. The reaction mixture was applied to a column (4.8 cm i.d. × 124 cm) of Dowex 50 × 8 (200–400 mesh) cation-exchange resin in the Ba^{2+} form [6] and eluted with distilled water (1.6 mL/min, 10 mL/fraction). Xylose (**4**) eluted at ~ 1.1–1.6 L and **3** eluted at 1.7–2.0 L; some overlap of the two compounds occurred between 1.6–1.7 L. Compound **3** was characterized by ^1H and ^{13}C NMR spectroscopy as described previously [5].

L-(1-^{13}C)*threo*-Pentos-2-ulose was prepared as described above using L-(1-^{13}C)xylose instead of unlabeled **4** [5] (see General procedures).

L-*(1-^{13}C)Ascorbic acid* **1**.—$K^{13}CN$ (1.38 g, 20.9 mmol) was added to a three-neck 500 mL round-bottom flask containing H_2O (20 mL), and the pH was adjusted to ~ 9.0 with HOAc (M; ~ 14 mL). L-*threo*-Pentos-2-ulose **3** (3.0 g syrup; 2.4 g as determined by iodometric titration [22]; 16.2 mmol) in H_2O (80 mL) was added dropwise over a 10 min period and allowed to stir for an additional 10 min while maintaining the pH between 9.0 and 9.1 with additions of M HOAc. During this time, the reaction mixture turned a pale yellow color. Dowex HCR-W2 resin (H^+ form; washed with H_2O and vacuum filtered to dryness; ~ 100 mL) was added with a minimal amount (~ 5 mL) of H_2O. The reaction vessel was placed in a pre-heated oil bath at 115 °C, and the mixture was allowed to reflux under an N_2 atmosphere. After 1.5 h, the reaction appeared complete as determined by ^{13}C NMR spectroscopy. The reaction mixture was filtered, the resin washed with H_2O (4 × 50 mL), and the filtrate and washings were combined and concentrated at 30 °C in vacuo to give a brown solid (~ 2.2 g; 77% crude yield). The crude **1** was dissolved in H_2O (~ 25 mL), and the solution was applied to a column

(3 cm i.d. × 23 cm) of Dowex 1 × 2 (200–400 mesh) ion-exchange resin in the formate form, which was eluted with formic acid (M) at a flow rate of 1.5 mL/min. Fractions (10 mL) were collected and assayed with phenol–H_2SO_4 [21]. Compound **1** eluted after ~ 300 mL of formic acid had passed through the column. The fractions were pooled and concentrated at 30 °C in vacuo. Absolute EtOH was added to the resulting syrup, and the solution was concentrated at 30 °C in vacuo. Evaporation from EtOH was repeated twice more. Fine white crystals appeared upon concentration. Absolute EtOH was added (~ 10 mL), and the flask was allowed to sit overnight at −5 °C. The crystals were filtered and washed with cold absolute EtOH to give 0.55 g of **1**. The mother liquor afforded an additional 0.5 g of **1**. Total isolated yield of white crystalline **1**: 1.05 g (37%). mp 185–186 °C; ref. [23] 190 °C; FABMS (glycerol matrix): m/z $[M + H]^+$ 178. Anal. Calcd for $C_5{}^{13}C_1H_8O_6$: C, 41.25; H, 4.55. Found: C, 40.77; H, 4.79 (authentic unlabeled **1**: Anal. Calcd $C_6H_8O_6$: C, 40.92; H, 4.58. Found: C, 40.45; H, 4.70).

L-(2-^{13}C)*Ascorbic acid* **1**.—L-(2-^{13}C)Ascorbic acid **1** was prepared as described above using KCN and L-(1-^{13}C)*threo*-pentos-2-ulose as the reactants; mp 183–184 °C; ref. [23] 190 °C; FABMS (glycerol matrix): m/z $[M + H]^+$ 178; Anal. Calcd for $C_5{}^{13}C_1H_8O_6$: C, 41.25; H, 4.55. Found: C, 40.82; H, 4.72.

Acknowledgements

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