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SYNTHESIS OF L-ASCORBATE 6-PHOSPHATE*

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ABSTRACT

L-Ascorbate 6-phosphate (**3**) was prepared in aqueous medium at pH 8.5–9.0 by reaction of 6-bromo-6-deoxy-L-ascorbic acid with hydrogen phosphate ion at 25°. It was not detected by liquid chromatography in fresh spinach leaves, nor in mouse brain or lungs at a concentration above 30 μg/100 g of wet tissue.

INTRODUCTION

Tolbert[1] has reported three major and six minor water-soluble metabolites of L-ascorbic acid in the urine of primates, including human. One of the major metabolites is 5-ketoascorbitol (D-*erythro*- or D-*threo*-3,5-hexodiulosono-1,4-lactone), while minor ones include the 2-sulfate ester[2] and the 2-methyl ether[3] of L-ascorbic acid. Perhaps others of those metabolites are phosphate esters, which are prevalent intermediates in the biological transformations of carbohydrates.

We prepared the 6-phosphate ester (**3**) of L-ascorbic acid, and assayed a plant leaf and two animal tissues for this derivative. No assays were attempted yet on urine. L-Ascorbate 2-phosphate, a well characterized ester[4–6] of L-ascorbic acid, has not been reported in nature.

RESULTS AND DISCUSSION

We attempted first to prepare L-ascorbate 6-phosphate (**3**) by displacement[7] of a 6-*O*-tosyl group from **5** by use of tetrabutylammonium phosphate in *N,N*-dimethylformamide. Under reflux, the reaction gave a complex mixture of products, as evidenced by t.l.c. No attempt was made to separate and identify these products. It is of interest that the preparation of **5** gave some difficulty. When L-ascorbyl 2-palmitate was treated with *p*-toluenesulfonyl chloride in pyridine alone, many

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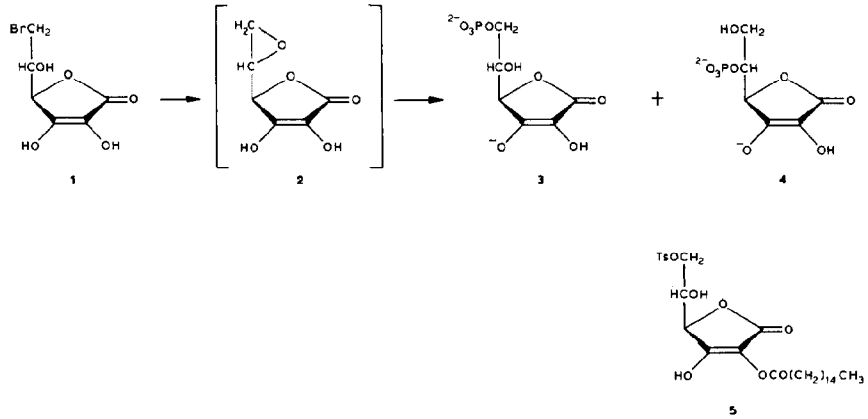
products were observed on t.l.c. However, *p*-toluenesulfonylation proceeded smoothly when ethyl ether was the solvent with traces of pyridine present.

The 6-phosphate ester **3** was obtained by ring-opening of the epoxide **2** intermediate by phosphate ion. The intermediate **2** was obtained from the 6-bromo derivative **1** under the conditions of Andrews[8]. In the presence of 1.8 equivalent of phosphate, the epoxide **2** reacted to give **3**, and presumably a small proportion of **4**. The pH of the aqueous reaction medium was critical to the formation of **3**. Using paper chromatography, we found that little or no **3** was formed at pH 3 and >12, whereas a good conversion was obtained at pH 8–9, in agreement with previous work[9].

After removal of insoluble magnesium phosphate, the magnesium salt of **3** was purified by fractional precipitation in 50% aqueous ethanol. Ion-exchange chromatography gave the sodium salt of **3** in 26% yield with a purity >95%, as evidenced by elemental analysis and iodometric titration. A chromatographic assay showed that the sodium salt **3** contained <1% of D-isoascorbate 5-phosphate (**4**), a result that was verified by the <1% D-isoascorbic acid observed in the product after phosphatase treatment. Compound **3** was isolated in reduced form as evidenced by its ^{1}H-n.m.r. spectrum and iodometric titration. Moreover, when **3** was treated with a 220 molar excess of dithiothreitol, a chromatographic assay of the product showed no increase in the concentration of **3**. The chemical shifts of the carbon atoms in the ^{13}C-n.m.r. spectrum of **3** were practically the same as those of L-ascorbic acid at pH 2 and 7, except for the signal of C-6 in **3**, which was shifted downfield, as expected[10], by 4.1–4.9 p.p.m., as compared to the signal of C-6 of L-ascorbic acid. The methylene protons at C-6 of **3** were also shifted downfield with reference to those of L-ascorbic acid. At pH 2 and pH 7, the λ_{max} values of 243 and 265 nm, respectively, verified the lack of esterification at O-2 or O-3 in compound **3**.



Scheme 1.

Fresh mouse lung, mouse brain, and spinach leaves were assayed for the presence of L-ascorbate 6-phosphate (**3**). No 6-phosphate ester was detected at a level >30 μg/100 g wet tissue, whereas L-ascorbic acid was found at a concentration of 29, 27, and 24 mg/100 g of wet lung, brain, and spinach, respectively. Rat lung and brain have been reported[11] to contain L-asorbic acid at a concentration of 20–40 and 35–50 mg/100 g, respectively, and spinach tissue[12] at a concentration of 15–120 mg/100 g. The phosphatase activity was not significant in the tissues tested. Known quantities of **3** were recovered quantitatively when added to the tissue.

EXPERIMENTAL

General methods. — N.m.r. spectra were recorded with a Bruker WM-400 instrument for solutions in D_2O and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal reference standard. Paper chromatography was performed on Whatman No. 1 paper in 15:4:1 (v/v/w) propanol–water–trichloroacetic acid. Components were detected with 0.7% $AgNO_3$ in acetone, followed by 0.05M NaOH in methanol. Cation exchange chromatography was done in a strongly acidic resin with u.v. monitoring of the eluent. Liquid chromatography under pressure used a reverse-phase column (Alltech C-18, 250 × 4.6 mm, 5 μm), maintained at 35°, and electrochemical detection (Model LC-4B, Bioanalytical System, West Lafayette, IN). The mobile phase was 1:19 (v/v) methanol–80mM acetate buffer (pH 4.5), containing 0.1mM disodium ethylenediaminetetraacetate and mM tetrabutylammonium phosphate, and the flow rate was 0.8 mL/min. Evaporations were done at reduced pressure <40°. Acid phosphatase was Type IV-S from Sigma Chemical Co., St. Louis, MO.

L-*Ascorbate 6-phosphate* (**3**). — The phosphate ester was prepared through an epoxide intermediate (**2**) starting from 6-bromo-6-deoxy-L-ascorbic acid[13] (**1**). The general procedure of Ballou and MacDonald[14] was modified as follows. To a solution of dipotassium hydrogen phosphate (12.6 g, 72 mmol) in water (40 mL) was added, with stirring under N_2 at 25°, **1** (9.6 g, 40 mmol). The pH of the mixture was adjusted to 8.5–9.0 with 10M aqueous KOH, and the pH maintained with an automatic pH controller. The formation of the intermediate epoxide **2** was complete in ~8 h, as indicated by alkali consumption. The reaction was allowed to proceed for a total of 24 h, after which the volume was completed to 100 mL, and an aliquot (1 mL) was titrated with 25mM I_2. The quantity of I_2 reduced indicated 83% retention of the reducing power of the starting material.

The mixture was passed through a column (750 mL) of strongly acidic, cation-exchange resin (H^+), and the column washed with water until the effluent had A_{243} <0.1. The pH of the washings was adjusted to 8.3 with MgO, and insoluble salts were removed by filtration. The filtrate (~2.5 L) was evaporated under reduced pressure to ~150 mL, and the concentrate kept overnight at 4° and filtered through a glass-fiber pad to remove a small amount of insoluble salts. Absolute ethanol (150 mL) was added with stirring, and the precipitate collected on Whatman No. 5 filter

paper. The solid product was dissolved in water (450 mL) and reprecipitated by addition of an equal volume of ethanol. The reprecipitation was repeated once.

The filter cake was dissolved in water (100 mL) containing a strongly acidic, cation-exchange resin (~140 mL, Na^+), and the mixture passed through a column (750 mL) of strongly acidic, cation-exchange resin (Na^+). After the column had been washed with water, the total eluent (~2 L) was evaporated to a small volume (100 mL), followed by repeated addition of absolute ethanol and evaporation. The sodium salt of L-ascorbate 6-phospate (**3**) was a slightly yellow solid (3.5 g, 26%) having $[\alpha]_D^{25}$ +29° (*c* 2, 3% HPO_3). Upon drying *in vacuo* at 60°, the product lost 5.3% moisture. L.c. assay of the solid product showed one major component with R_T 11.7 min, one minor component with R_T 14.2 min (intensity <1% of the major product), and a trace of L-ascorbic acid with R_T 6.1 min. The sodium salt of **3** (152 mg) consumed 16.1 mL of 25mM iodine (90% theory); λ_{max}^{pH2} 243 (ε_{mM} 9.6), $\lambda_{max}^{pH\ 7,10}$ 265 nm; ^{1}H-n.m.r. (D_2O, pH 2.0): δ 5.04 (1 H, $J_{4,5}$ 1.93 Hz, H-4), 4.26–4.22 (1 H, H-5), and 4.05–4.02 (2 H, H-6a,6b); (pH 7.0), 4.57 (1 H, H-4), 4.17–4.11 (1 H, H-5), and 3.96–3.88 (2 H, H-6a,6b); ^{13}C-n.m.r. (D_2O, pH 2.0): δ 175.6 (C-1), 157.7 (C-3), 120.5 (C-2), 78.5 (C-4), 70.3 (C-5), and 68.0 (C-6); (pH 7.0), 179.9 (C-1), 178.3 (C-3), 115.6 (C-2), 80.9 (C-4), 71.4 (C-5), and 67.7 (C-6).

Anal. Calc. for $C_6H_6Na_3O_9P \cdot H_2O$: C, 21.18; H, 2.35; Na, 20.29; P, 9.18. Found: C, 21.89; H, 2.44; Na, 19.10; P, 9.23.

Treatment of L-*ascorbate 6-phosphate* (**3**) *with phosphatase and 1,4-dithiothreitol.* — Acid phosphatase (10 mg) was added to a solution of the sodium salt of **3** (50 μg) in 0.08M acetate buffer (10 mL, pH 4.5) containing 1.3mM 1,4-dithiothreitol (DTT). The mixture was incubated for 10 min at 35°. L.c. assay showed initially two peaks at R_T 11.7 min (L-ascorbate 6-phosphate) and 14.2 min (DTT), and without added DTT, a small peak at R_T 14.2 min, which was presumed to be D-isoascorbate 5-phosphate. After a 10-min incubation, three peaks were observed at R_T 6.1 min (L-ascorbate), 6.7 min (D-isoascorbate), and 14.2 min (DTT).

To determine the dehydro content in the sodium salt of **3**, the sodium salt (10 mg) was added to either 4% aqueous HPO_3 with 65mM DTT or 4% aqueous HPO_3 alone (100 mL each). After 15 min of stirring at 25°, l.c. showed that the detection response of **3** in the first solution was 98% of that in the second.

L.c. assay for L-*ascorbate 6-phosphate and* L-*ascorbic acid.* — Fresh leaves (15 g) of spinach (*Spinacia oleracea*) were ground with cold 6% aqueous HPO_3 (15.0 mL) in a mortar with pestle. The ground material was washed into a centrifuge tube by additional 6% HPO_3 (10.0 mL). After centrifugation at 1065*g* for 15 min, cold, degassed 0.05M $HClO_4$ was added to an aliquot (10 mL) to a volume of 25 mL. The mixture was centrifuged (8160*g*) for 3 min, a portion of the supernatant was forced through a 0.45-μm syringe filter, and the clear filtrate (20 μL) was injected into the chromatograph. Standard curves for L-ascorbic acid and **3** were prepared without the tissue present. When added to tissue (42 mg/100 g wet weight) **3** was recovered quantitatively.

Fresh lung (0.40 g) and brain (0.86 g) tissue from mice was assayed in the same manner, except that the tissue was ground in 6% HPO_3 (5 mL) with a Tekmar

Tissumizer (Tekmar Co., Cincinnati, OH). The clarified extract (5 mL) was diluted to 10.0 mL with 0.05M $HClO_4$ prior to injection into the chromatograph.

2-O-Palmitoyl-6-O-p-toluenesulfonyl-L-ascorbic acid (**5**). — 2-*O*-Palmitoyl-L-ascorbic acid[15] (0.3 g, 0.95 mmol) was dissolved in ethyl ether (30 mL) and pyridine (0.3 mL, 3.8 mmol), and the mixture was stirred at 5° while a solution of *p*-toluenesulfonyl chloride (0.15 g, 0.78 mmol) in ethyl ether (5 mL) was added dropwise. The mixture was stirred at room temperature until conversion of the starting material (R_F 0.3) to a new compound (R_F 0.9) was complete, as determined by t.l.c. in 4:1 (v/v) chloroform–methanol. After removal of ethyl ether by evaporation, tetrahydrofuran (30 mL) and ice (0.5 g) were added, and the mixture was stirred for 3 h. Ether (100 mL) was added, and the pyridine was removed from the ether solution by repeatedly washing with aqueous $CuSO_4$, followed by water. The organic layer was dried (Na_2SO_4) and filtered, and the filtrate evaporated to a syrup that was subjected to silica gel (200–400 mesh) column chromatography in 15:1 (v/v) dichloromethane–2-propanol. The fractions containing the major product were collected and, upon evaporation of the solvents, a crystalline solid (0.27 g, 60%) was obtained, m.p. 72–74°.

Anal. Calc. for $C_{29}H_{44}O_9S$: C, 61.27; H, 7.75; S, 5.63. Found: C, 61.20; H, 7.91; S, 5.93.

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REFERENCES

1 B. M. TOLBERT, *Int. J. Vitam. Nutr. Res.*, Suppl. 27 (1985) 121–138.
2 E. M. BAKER, D. C. HAMMER, S. C. MARCH, B. M. TOLBERT, AND J. E. CANHAM, *Science*, 173 (1971) 826–827.
3 P. W. LU, D. W. LILLARD, JR., P. A. SEIB, K. J. KRAMER, AND Y. T. LIANG, *J. Agric. Food Chem.*, 32 (1984) 21-28.
4 C. H. LEE, P. A. SEIB, Y. T. LIANG, R. C. HOSENEY, AND C. W. DEYOE, *Carbohydr. Res.*, 67 (1978) 127–138.
5 J. JERNOW, J. BLOUNT, E. OLIVETO, A. PERROTTA, P. ROSEN, AND V. TOOME, *Tetrahedron*, 35 (1979) 1483–1486.
6 H. NOMURA, H. NAKAMACHI, AND Y. WADA, *Chem. Pharm. Bull.*, 30 (1982) 1024–1029.
7 A. K. CHATTERJEE, AND D. L. MACDONALD, *J. Org. Chem.*, 33 (1968) 1584–1585.
8 G. C. ANDREWS, *Carbohydr. Res.*, 134 (1984) 321–326.
9 G. P. LAMPSON, AND H. A. LARDY, *J. Biol. Chem.*, 181 (1949) 693–696.
10 J. V. PAUKSTELIS, D. D. MUELLER, P. A. SEIB, AND D. W. LILLARD, JR., *Adv. Chem. Ser.*, 200 (1982) 125–151.
11 D. HORNIG, *Ann. N. Y. Acad. Sci.*, 258 (1975) 103–118.
12 S. W. SOUCI, W. FACHMANN, AND H. KRAUT, *Food Composition and Nutrition, Tables 1981/82*, 2nd Edn., Wissenschaftliche Verlagsgesellschaft, Stuttgart, 1981, pp. 868–869.
13 K. BOCK, I. LUNDT, AND C. PEDERSEN, *Carbohydr. Res.*, 68 (1979) 313–319.
14 C. E. BALLOU, AND D. L. MACDONALD, *Methods Carbohydr. Chem.*, 2 (1963) 289–293.
15 R. C. COUSINS, P. A. SEIB, R. C. HOSENEY, C. W. DEYOE, Y. T. LIANG, AND D. W. LILLARD, JR., *J. Am. Oil Chem. Soc.*, 54 (1977) 308–312.