Note

L-Ascorbic acid 3-sulfate. Preparation and characterization

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INTRODUCTION

The function of L-ascorbic acid (**1**) in animals is still obscure and a possible relationship exists between **1** and biological sulfation. We have proposed, along with others, that this relationship may involve L-ascorbic acid 3-sulfate (**2**) as an intermediate in metabolic sulfation[1-3]. The recent isolation of **2** from brine-shrimp eggs in relatively large quantities also suggests a sulfate reservoir[4]. In a communication we reported the synthesis of radiolabeled **2** and its capability to sulfate an alcohol at elevated temperatures or under mild oxidizing conditions[2]. Subsequently, we have shown the *in vivo* sulfation of cholesterol by **2** in rats[5]. This manuscript reports in detail a synthesis and characterization of **2** in order that other investigators may more easily recognize this molecule in biological systems and may synthesize it for metabolic and medicinal investigations.

RESULTS AND DISCUSSION

L-Ascorbic acid 3-sulfate and 5,6-*O*-isopropylidene-L-ascorbic acid 3-sulfate (**3**) were synthesized by sulfation of 5,6-*O*-isopropylidene-L-ascorbic acid (**4**) with either pyridine–sulfur trioxide or with pyridine sulfate and acetic anhydride. The latter procedure was preferred because it was simpler, gave higher yields, and gave crystalline **2** more easily. This procedure is particularly desirable for the preparation of ^{35}S-**2** because pyridine ^{35}S-sulfate is less expensive, and easier to prepare, than pyridine–^{35}S-sulfur trioxide. A method also is given describing the synthesis, isolation, and purification of **3** (see Ref. 2). L-Ascorbic acid 3-sulfate and its isopropylidene acetal are relatively stable at room temperature in the absence of oxygen and can be stored in a vacuum desiccator for many months with no appreciable change. The *O*-isopropylidene group can be removed easily from **3** in acid (0.1M HCl, 5 min). Surprisingly, **2** possesses considerable acid stability and is only 20% hydrolyzed to sulfate in 6.5 h at pH 1. Although the sodium, potassium, and ammonium salts of **2** were prepared, the barium salt was found to be the easiest to recrystallize. When needed for biochemical studies, the barium salt of **2**, is easily converted into the

sodium or potassium salt by means of a cation-exchange column or by precipitation of the barium ion with sodium or potassium sulfate. The potassium salt of **2** gave the most satisfactory analytical data, presumably because the salt was not hydrated.

5,6-*O*-Isopropylidene-L-ascorbic acid possesses two hydroxyl groups (at C-2 and C-3) and theoretically both of these groups could be sulfated. The C-3 hydroxyl group is, however, the more acidic, and would be expected to be sulfated before the C-2 hydroxyl group. L-Ascorbic acid 3-sulfate absorbs strongly in the u.v., showing a maximum of 254 nm ($\varepsilon = 29{,}350$) at pH 7 and 233 nm ($\varepsilon = 21{,}900$) at pH 2 (Fig. 1). The u.v. absorption of **2** is essentially identical with that reported for L-ascorbic acid 3-phosphate[6], and to that of the isolated L-ascorbic acid 3-sulfate from brine-shrimp eggs[4]. The C-2 hydroxyl group of **2** is more acidic ($pK_2 = 2.77$, as determined by u.v.) than the C-3 hydroxyl group of **1** ($pK_1 = 4.17$).

The barium salt of **2** shows strong absorption in the i.r. (KBr disc) at 835 (C–O–S), 1045 (C–O), and 1250 (S=O) cm^{-1}, typical of sulfated carbohydrates[7,8] (Fig. 2). The C–O–S vibrational mode of 835 cm^{-1} is consistent for a planar, secondary, unhindered sulfate. The 60-MHz n.m.r. data for **2** are summarized in the

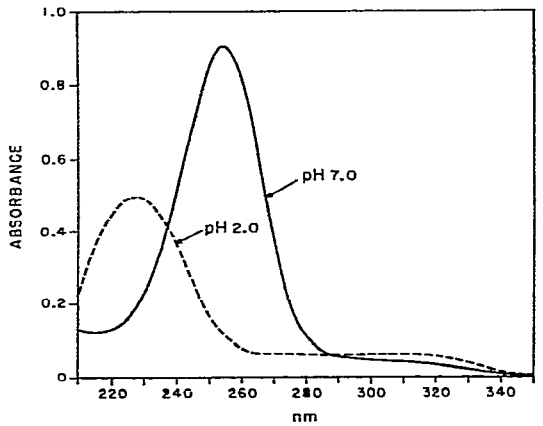

Fig. 1. U.v. spectra of L-ascorbic acid 3-sulfate at pH 2 and 7.

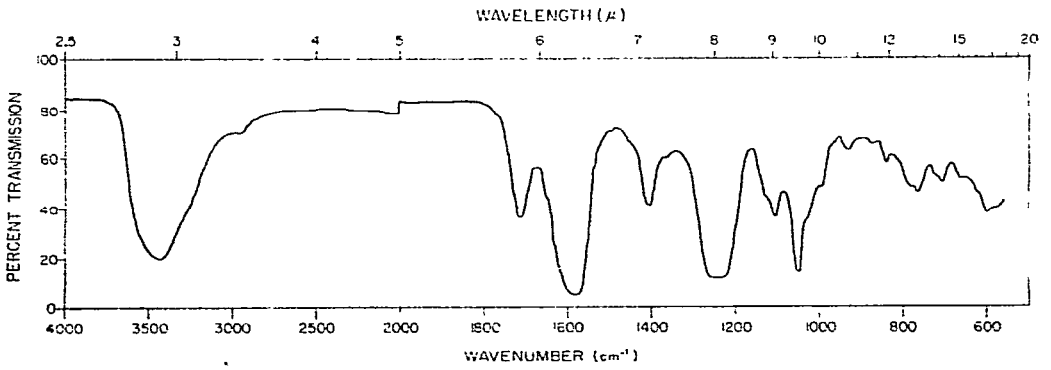

Fig. 2. I.r. spectrum of the barium salt of L-ascorbic acid 3-sulfate.

Experimental section. The presence of the sulfate ester group produces no significant change in the n.m.r. spectrum. The chromatographic properties of the potassium salts of **2**, **3** and related compounds are given in Table I. The sodium, potassium, and

TABLE I

THIN-LAYER AND PAPER CHROMATOGRAPHIC PROPERTIES OF **2**, **3**, AND RELATED COMPOUNDS

Compound	*R_F values[a] in solvent system given*						
	Thin-layer[b]			*Paper[c]*			
	A	*B*	*C*	*E*	*F*	*G*	*H*
SO_4^{2-}	0.0	0.0	0.0	0.0			
L-Ascorbic acid 3-sulfate	0.0	0.14	0.25 0.41[d]	0.22 0.28[d]	0.29 0.10[d]	0.22 0.0[d]	0.40 0.35[d]
L-Ascorbic acid	0.05	0.17	0.47				
5,6-*O*-Isopropylidene-L-ascorbic acid 3-sulfate	0.10	0.41	0.35	0.44	0.51		
5,6-*O*-Isopropylidene-L-ascorbic acid	0.55	0.69	0.88				
1-Octyl sulfate	0.62	0.75					
3-Cholesteryl sulfate	0.75	0.85					

[a]Date are presented for potassium salts, except where stated. Solvent systems are noted in the Experimental section. [b]On Supelcosil 12B. [c]On Whatman No. 4 paper. [d]Values for barium salt.

ammonium salts of **2** behave similarly, but, the barium salt of **2** possesses a considerably different chromatographic mobility than the monovalent cation salts. For example, by t.l.c. with solvent *C* the barium salt of **2** had R_F of 0.41 whereas the potassium salt has R_F value of only 0.25. In the phenol–water paper-chromatographic solvent system, **3** was relatively unstable and continually decomposed to give **2**.

TABLE II

REACTION OF VARIOUS COMPOUNDS WITH SPRAYS ON THIN-LAYER CHROMATOGRAPHY[a]

Sprays	*L-Ascorbic acid*	*5,6-O-Isopropylidene-L-ascorbic acid*	*L-Ascorbic acid 3-sulfate*	*5,6-O-Isopropylidene L-ascorbic acid 3-sulfate*
Silver nitrate (neutral)	+[b]	—	—	—
Silver nitrate (KOH)	+	+	+	+[c]
Iodine (vapor)	+[d]	+[d]	+	+[e]
$FeCl_3$	purple[f]	purple[f]	red[g]	red[g]
Phosphomolybdic acid	blue	blue	—	—
$HClO_4$ (char)[h]	+	+	+	+

[a]Data are presented for two adsorbents, Supelcosil 12B and Eastman CHROMAGRAM Sheet. + Indicates a positive reaction, — indicates a negative reaction. [b]Indicates immediate reaction, other compounds reacted eventually. [c]Did not stain on Supelcosil 12B. [d]Very intense. [e]Stains only weakly on Supelcosil 12B. [f]Light. [g]Intense. [h]Only for Supelcosil 12B, easily over-chars to give CO_2.

Table II summarizes the reaction of **1**, **2**, **3**, and **4**, with various spray reagents. The t.l.c. adsorbent influenced the sensitivity of the sprays. Iodine vapor is more sensitive with Eastman CHROMAGRAM Sheets than with Supelcosil 12B. Silver nitrate spray (basic) did not stain **3** when it was chromatographed on Supelco 12B but it did stain **3** when it was chromatographed on Eastman CHROMAGRAM Sheets. Iodine vapor and phosphomolybdic acid spray are very sensitive for **1** and **4**, and these compounds can be detected in trace amounts. The ferric chloride spray (1% in methanol) gave a purple color with **1** and **4**, and an intense brick-red color with **2** and **3**. This red color can be used as a sensitive qualitative test for **2** in biological tissues; however, this spray is not necessarily specific, it is indicative of 3-substituted L-ascorbic acids and not of 2-substituted L-ascorbic acids[9,10]. Aqueous ferric chloride spray does not form the brick-red color with **2** or **3** because the ferric ion does not form a chelate with 3-substituted L-ascorbic acids in aqueous solutions.

EXPERIMENTAL

Materials. — All solvents were redistilled. The purity of all reagents was determined by t.l.c. The ^{35}S-chlorosulfonic acid and the $^{35}SO_4^{2-}$ were obtained from New England Nuclear Corporation, 1-octadecanol from Supelco, Inc., and other reagents from Eastman Organic Chemicals.

Instruments of analysis. — U.v. spectra were recorded with a Cary Model 14 spectrophotometer, and i.r. spectra with a Perkin–Elmer Model 421 recording infrared spectrophotometer, with potassium bromide discs. N.m.r. spectra were recorded with a Varian A-60 n.m.r. spectrometer.

Chromatography. — T.l.c. was used to follow all reactions and procedures. Three adsorbents were used: Supelcosil 12B (Supelco, Inc.), Eastman CHROMAGRAM Sheet (Distillation Products Industries), and ChromAR Sheet 500 (Mallinckrodt). Solvents used for development were: *A*, chloroform–methanol–water (65:25:4, v/v/v); *B*, chloroform–methanol–water–pyridine (65:50:8:0.5, by vol.); *C*, chloroform–methanol–water–acetic acid (65:50:15:1, by vol.); and *D*, ethyl ether–benzene–ethanol–acetic acid (40:50:2:0.2, by vol.). Paper chromatography was also employed, utilizing *E*, phenol–water (100:40, w/w); *F*, butyl alcohol–propionic acid–water (10:5:7, v/v/v); *G*, butyl alcohol–pyridine–water (6:4:3, v/v/v); and *H*, butyl alcohol–acetic acid–water (5:2:3, v/v/v).

Compounds were visualized with various sprays. Exposure to iodine vapors and a 20% perchloric acid spray were used as general detection methods. Three sprays, specific for reducing compounds, were used: phosphomolybdic acid; 1.0% silver nitrate in ethanol; and 1.0% silver nitrate followed by 0.1M potassium hydroxide. The phosphomolybdic acid method of detecting reducing compounds was modified from that reported[11] (Spray No. 120b), by subsequently spraying with NH_4OH in methanol, which removed the yellow background. A ferric chloride spray (1% in methanol) was used to detect **2** and **3**. Radioautography was also employed to locate ^{35}S-labeled compounds on chromatograms.

Preparation of 5,6-O-isopropylidene-L-ascorbic acid (**4**). — This procedure is a variation of that reported by Salomon[12]. Finely powdered **1** (20.0 g) was added to 200 ml of anhydrous acetone and the mixture was placed in an ice bath. Anhydrous hydrogen chloride was rapidly bubbled into this cooled, magnetically stirred, mixture for 0.5 h. Subsequently, 160 ml of hexane was added, the mixture was swirled and allowed to settle. The solvent was decanted and the solid residue was repeatedly washed with 200 ml of 4:7 acetone–hexane until all of the hydrogen chloride had been removed, giving 18 g (75%) of **4**.

Anal. Calc. for $C_6H_{12}O_6$: C, 50.0, H, 5.56. Found: C, 50.15; H, 5.64.

L-Ascorbic acid 3-sulfate (**2**). — Two methods were employed for the preparation of **2**: A, sulfation of **4** with pyridine–sulfur trioxide and B, sulfation of **4** with pyridine sulfate and acetic anhydride[13]. In all cases ^{35}S-labeled reactants were used.

Method A. To 10 g of **4** dissolved in 70 ml of stirred, anhydrous *N,N*-dimethylformamide was added 10 g of pyridine–sulfur trioxide prepared from chlorosulfonic acid[14]. The reaction mixture turned golden brown after stirring for 20 h at room temperature. The solvent was removed under vacuum and 100 ml of water was added to the residue. Sulfuric acid was added to adjust the pH to 1. The acidic solution was stirred for 0.5 h to ensure complete removal of the *O*-isopropylidene group. Potassium hydroxide was then added to bring the pH to 6.5. The solvent was removed under vacuum. The residue was extracted twice with 150 ml of 85% methanol. The methanol extracts were combined and concentrated by flash evaporation at 35° to a viscous liquid. The addition of anhydrous methanol produced a brownish-white precipitate. The mixture was filtered and the precipitate washed 4 times with abs. methanol to give 2.5 g (15%) of chromatographically homogeneous, dipotassium **2**.

Anal. Calc. for $C_6H_6K_2O_9S \cdot CH_3OH$: C, 23.08, H, 2.75; K, 21.43; S, 8.79. Found: C, 22.87; H, 2.53; K, 22.29; S, 8.72.

The dipotassium salt of **2** was converted into the barium salt by the use of an ion-exchange column. Dipotassium **2** was dissolved in water and passed through a column of Dowex 50-WX8 (Ba^{2+} form). The barium salt was obtained upon evaporation of the solution or it was precipitated from a concentrated aqueous solution with abs. methanol.

Anal. Calc. for $C_6H_6BaO_9S \cdot CH_3OH \cdot H_2O$: C, 19.04; H, 2.72; Ba, 31.07; S, 7.26. Found: C, 19.03; H, 2.40; Ba, 31.23; S, 7.28.

Method B. To 12.5 g (49 mmole) of pyridine sulfate dissolved in 50 ml of anhydrous pyridine was added 5.0 ml (53 mmole) of acetic anhydride with stirring. After 45 min the solution became clear and 4.59 g (22 mmole) of **4** dissolved in a minimal amount of pyridine was added. The reaction was allowed to proceed at 25° for 15 h in the dark with stirring. The solution was adjusted to pH 7.5 with potassium hydroxide (20%) and the solvent removed under vacuum. The residue was dissolved in 40 ml of water and the pH was adjusted to 1.0 with sulfuric acid. After 30 min the solution was neutralized with potassium hydroxide. The solvent was removed under vacuum and the residue was washed with 200 ml of 85% methanol. The isolation of **2** from the methanolic solution was essentially the same as in Method A, producing

1.62 g (22%) of dipotassium **2**; n.m.r. data (60 MHz, at pH 7 in deuterium oxide, 4,4-dimethyl-4-silapentane-1-sulfonate as internal standard) δ 4.58 (doublet, $J_{4,5}$ 2.0 Hz, H-4), 4.08 (multiplet, H-5), 3.74 (multiplet, H-6); corresponding data for L-ascorbic acid δ 4.57 ($J_{4,5}$ 1.6Hz, H-4), 4.13 (H-5), 3.77 (H-6).

Preparation of 5,6-O-isopropylidene-L-ascorbic acid 3-sulfate (**3**). — This alternative method for the synthesis of **3** describes the actual isolation and purification of **3**. Pyridine–^{35}S-sulfur trioxide (0.535 mmol) and 0.39 mmole of **4** were suspended in 0.3 ml of *N,N*-dimethylformamide in a 12 ml centrifuge tube. After 1 h with stirring at 25° the reactants were heated for 10 min to 37°. The solution was cooled to 25° and 0.7 ml of 0.77M potassium hydroxide (0.535 mmole) was added (pH 6.0). The solvent was removed under vacuum and the crude **3** was dissolved in 1 ml of water and washed three times with 5 ml of ethyl ether. The ether removed most of the unreacted **4**. The aqueous solution was evaporated under vacuum. The residue, a glossy amorphous solid, was resuspended in 2 ml of acetone. A portion of the amorphous solid did not redissolve. This insoluble residue was potassium sulfate and it was removed from the acetone solution by centrifugation. Removal of the acetone under vacuum gave 0.3 mmole of **3**, which was stored at −40°, and could be recovered unchanged by redissolving in acetone.

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