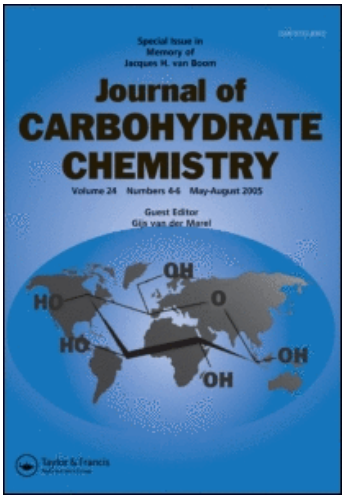

Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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To cite this Article Yang, Hanwoo and Seib, Paul A.(1992) '2-Phosphorylation of D-Erythorbic Acid', Journal of Carbohydrate Chemistry, 11: 4, 485 — 498
To link to this Article: DOI: 10.1080/07328309208017808
URL: http://dx.doi.org/10.1080/07328309208017808

J. CARBOHYDRATE CHEMISTRY, 11(4), 485-498 (1992)

2-PHOSPHORYLATION OF D-ERYTHORBIC ACID

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Received July 1, 1991 - Final Form February 11, 1992

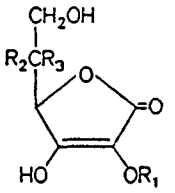
ABSTRACT

D-Erythorbate 2-phosphate (**3**) was prepared by phosphorylation of 5,6-*O*-isopropylidene-D-erythorbate (**5**) with phosphoryl chloride at high pH in the presence of pyridine. Following removal of the 5,6-acetal, a crude magnesium salt of **3** was isolated in 67-71% yield. The salt contained 74% of **3**, 9% of D-erythorbate 2-diphosphate (**7**), and 5% of *bis*-(D-erythorbyl) 2,2′-phosphate (**6**). Analytically pure **3** was obtained as its crystalline magnesium and tricyclohexylammonium salts in 26 and 47% yields, respectively, from **5**.

INTRODUCTION

D-Erythorbic acid (**1**) is the commercial name for D-*erythro*-2-hexenono-1,4-lactone, which is the C-5 epimer of L-ascorbic acid (**2**). Compound (**1**) has but 5% vitamin C activity, yet its strong reducing activity and low cost compared to **2** make it attractive for use in foods and industrial applications. The use of **1** in foods is regulated in the United States because **1** interferes[1] with absorption of **2** in the digestive tract.

The objective of this investigation was to chemically synthesize D-erythorbate 2-phosphate (**3**). This derivative is of interest since 2-phosphorylated derivatives of L-ascorbate have been found to inhibit warmed-over flavor[2] in cooked meats and browning[3] of fruits and vegetables.



1. R_1 = H, R_2 = OH, R_3 = H
2. R_1 = H, R_2 = H, R_3 = OH
3. R_1 = PO_3^-, R_2 = OH, R_3 = H
4. R_1 = PO_3^-, R_2 = H, R_3 = OH

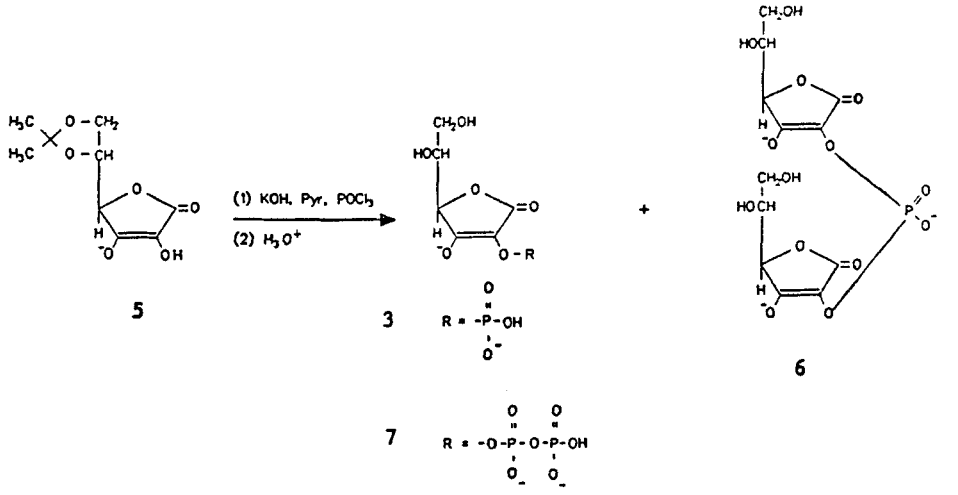
Compound (3) has been prepared[4] by the action of transphosphorylase on pyrophosphate in the presence of D-erythorbate. The transphosphorylase enzyme was produced by bacteria, and the frozen bacterial cells were used to produce both D-erythorbate 2-phosphate (3) and L-ascorbate 2-phosphate (4). The phosphate ester (4) has been prepared in a number of laboratories,[5-10] and the 2-triphosphate ester was recently reported.[11]

RESULTS AND DISCUSSION

Synthesis. The method to prepare 3 (Scheme I) is identical to that[9] used to prepare L-ascorbate 2-phosphate (4). The starting compound, 5,6-*O*-isopropylidene-D-erythorbic acid (5), was prepared in 97% yield by reaction of 1 with 2,2-dimethoxypropane in 1,4-dioxane containing catalytic amounts of trifluoroacetic acid.[12] Poor yields of 5 were obtained when 1 was reacted[13] with acetone/HCl, which contrasts, for unexplained reasons, with the high yield of 5,6-*O*-isopropylidene-L-ascorbic acid obtained from 2.

A solution of the 5,6-acetal (5) in aqueous potassium hydroxide saturated with pyridine was reacted over a period of several hours with 1.5 equivalents of phosphorus oxychloride. The cold reaction mixture was maintained at pH

Scheme I.



12-13 by adding strong alkali. After the reaction mixture was passed through a strongly acidic ion-exchange resin in the hydrogen-ion form, reverse-phase HPLC with UV detection showed the presence of one major (R_T = 6.30 min), two minor (R_T = 7.56 and 8.51 min), and three trace components (R_T = 10.0-13.5 min). Iodometric titration of the acidic reaction mixture showed 2% unreacted **1**.

The organophosphates in the reaction mixture were purified using anion-exchange column chromatography. The order of elution from the column was the 2-monophosphate (**3**), 2-diphosphate (**7**), and 2,2′-phosphodiester (**6**). Our previous report[9] that *bis*-(L-ascorbyl) 2,2′-phosphate is eluted with 3M ammonium hydroxide rather than 0.6M HCO_3^- appears to be in error. The yields of **3**, **7**, and **6** from the column were 82, 4, and 3%, respectively, based on UV absorbance at pH 10 and 263 nm. The yields agreed with those found (85, 5, and 3%) by reverse-phase HPLC with UV detection.

D-Erythorbate 2-phosphate (**3**) magnesium salt was purified from the reaction mixture without anion-exchange

chromatography. The reaction mixture was passed through a bed of cation-exchange resin, and the effluent adjusted to pH 9 with magnesium oxide. After removal of insoluble magnesium phosphate, the filtrate was concentrated to ~ 1M in **3**. Adding several volumes of ethanol precipitated the organic salts, which were freed of magnesium chloride by washing with ethanol.

The crude magnesium salt of **3**, whose yield together with contaminating related phosphoesters was estimated at 67% from **5** by UV assay, was found to contain 19% water by Karl Fisher analysis. Its solids fraction was comprised of 74, 9, and 5% of phosphoesters (**3**), (**7**), and (**6**), respectively. The approximately 10% of solids unidentified in the crude magnesium salt may have been low levels of magnesium phosphate mixed with organic salts originating from alkaline decomposition of **5**. Analytically pure magnesium D-erythorbate 2-phosphate (**3**) was obtained in 26% yield from **5** by slow recrystallization of the crude salt from water at 25°C.

A pure tricyclohexylammonium salt of **3** was obtained (47% from **5**) after preparative anion-exchange chromatography of the crude magnesium salt. When the strongly basic anion-exchange resin (bicarbonate form) was developed with 0.4 M sodium bicarbonate, the contaminating inorganic phosphates eluted first and then **3**, which was converted to its crystalline tricyclohexylammonium salt.

Structure of Compound 3. The structure of the monophosphate ester (**3**) tricyclohexylammonium salt was determined by elemental analysis, UV spectroscopy, and NMR spectroscopy. The ^{31}P-NMR spectrum of **3** at pH 7.0 gave one signal, which was a singlet at 2.2 ppm downfield from the reference signal of external 85% aqueous phosphoric acid. The position of phosphate substitution was established by ^{31}P- ^{13}C coupling. The proton-decoupled ^{13}C-NMR spectrum of **3** showed that P was coupled to C-1, C-2, and C-3 but not to C-4, which indicated that the phosphate group was attached to C-2. Cabral and Haake[14] showed that the P atom in 2-

phosphoryl esters of 5,6-*O*-isopropylidene L-ascorbic acid is coupled to C-1, C-2, and C-3, whereas the P atom in 3-phosphoryl esters is coupled to C-2, C-3, and C-4.

^{13}C and ^{1}H NMR data (Table) were also consistent with the assigned structure of D-erythorbate 2-monophosphate for compound (**3**). The resonance signals of C-5 and C-6 and of H-5 and H-6 were similar to those resonances in D-erythorbate (**1**), indicating no esterification at O-5 and O-6.

The ^{13}C-chemical shift of C-3 in compound (**3**) was observed at 173.7 ppm at pH 7, showing that the 3-OH was ionized and, therefore, unsubstituted. The 3-OH of 3-methyl L-ascorbic acid, which is not ionized at pH 7, has[15] its C-3 signal at 155 ppm.

The wavelength of maximum absorption in the UV and the molar absorptivity of D-erythorbate 2-phosphate varied with pH. The inflection points in the absorbance (λ max) curve *versus* pH occurred at pH 3.5 and 7.5, which were attributed, respectively, to the pK of the 3-OH and to pK_2 of the phosphate group. The pK_1 of the phosphate group was below pH 2, most likely at a value[6] near 0.01.

The UV absorptivity of compound (**3**) increased when its 3-OH ionized. That increase in absorptivity confirms 2-substitution, because absorptivity decreases[16] when the 2-OH ionizes in a 3-derivative of **2**.

Phosphodiester of D-Erythorbate. When D-erythorbate was reacted with phosphoryl chloride (1.5 equivalents) at pH 13 in barium hydroxide in the absence of pyridine, barium *bis*-(D-erythorbyl) 2,2′-phosphate (**6**) was crystallized directly from the reaction mixture in 42% yield from **1**, although the crystals were contaminated with approximately one-third by weight of barium chloride. Ion-exchange chromatography gave pure **6** in 22% yield as a trihydrate. Pyridine seems to be required in the phosphorylation of D-erythorbate to inhibit formation of the phosphodiester (**6**). Formation of a phosphodiester was also observed[9] during phosphorylation of L-ascorbate with phosphoryl chloride in alkali without pyridine.

Table. Chemical Shifts of L-Ascorbate, D-Erythorbate and Their 2-Monophosphate Esters[a] in D_2O at pH 6.5-7.5.

Compound	C-1	C-2	C-3	C-4	C-5	C-6	P-2	H-4	H-5	H-6 H-6′
L-ascorbate (**2**)	178.0	114.1	176.2	79.2	70.6	63.6	-	4.50	4.02[b]	3.74[b]
2-monophosphate (**4**)	177.4	113.2	177.0	78.7	70.1	62.9	3.6[c]	4.60	4.05	3.70
D-erythorbate (**1**)	179.9	115.6	177.9	82.8	74.0	63.4	-	4.61	4.08	3.61
2-monophosphate (**3**)	177.2	114.8	173.7	81.1	73.5	63.0	2.2[d]	4.70	4.08	3.67

[a]Data on **2** and **4** from reference 11. Chemical shifts of ^{13}C and ^{1}H with reference to $(CH_3)_3Si(CH_2)_3SO_3Na$ or $(CH_3)_4$ Si, and pH recorded directly from pH meter.

[b]Reported as center of multiplet. Signal of H-4 was a doublet.

[c]Downfield from external 50% aqueous H_3PO_4.

[d]Downfield from external 85% aqueous H_3PO_4.

The ^{13}P-NMR spectrum of the phosphodiester (**6**) showed a singlet, which was 4.24 ppm upfield from the reference signal of 85% aqueous phosphoric acid. It is known[17] that phosphodiesters give ^{31}P-signals upfield from 85% H_3PO_4, whereas phosphomonoesters give signals downfield. The signals of C-5 and C-6 and of H-5 and H-6 in (**6**) were similar to those resonances in D-erythorbate, indicating no esterification at O-5 and O-6. The appearance of one ^{31}P and three ^{1}H signals indicated that the phosphorus atom in the phosphodiester (**6**) was symmetrically substituted. Furthermore, the proton-decoupled ^{13}C NMR showed that the phosphorus in compound (**6**) was coupled to C-1, C-2, and C-3 but not to C-4. Those results show that compound (**6**) is *bis*(D-erythorbyl) 2,2′phosphate.

EXPERIMENTAL

General Methods. All evaporations were done under reduced pressure below 40 °C. Elemental analyses were done by Huffman Laboratories, Inc., Wheatridge, CO. Thin-layer chromatography was performed on plates coated with silica gel G. The compounds were located by spraying with 50% aqueous sulfuric acid followed by charring on a hot plate.

Ultraviolet (UV) spectroscopy was done using a Model DMS-80 Varian spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on aqueous solutions with a Bruker WM-400 instrument. The pH values of solutions that had been previously exchanged with D_2O were adjusted prior to measurement. The pH reported was that read from the pH meter. Proton chemical shifts are reported in δ-values from the internal reference signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). For ^{31}P NMR spectra, an 85% aqueous solution of phosphoric acid was used as external reference.

High performance liquid chromatography (HPLC) was carried out with a Knauer pump fitted with a column heater, a Rheodyne loop injector, an integrating recorder, and an

UV detector. For HPLC-UV analysis, samples (20 μL) were injected onto a reverse-phase column (C-18, Alltech), and components were eluted with a 68:32 (v/v) mixture of 0.08M acetate buffer (pH 4.7) and methanol containing 1.0 mM tetrabutylammonium phosphate and 0.2 mM EDTA. The column was maintained at 45 °C, the flow rate at 1.0 mL/min, and the detector at 250 nm. The retention times for D-erythorbate 2-monophosphate, (**3**) D-erythorbate 2-diphosphate (**7**), and *bis*(D-erythorbyl) 2,2′-phosphate (**6**) were approximately 6.37, 7.52, and 8.34 min, respectively. Standard curves were derived using the pure tricyclohexylammonium salt of **3** and the barium salt of **6**. The standard curve of **3** was also used to quantitate **7**.

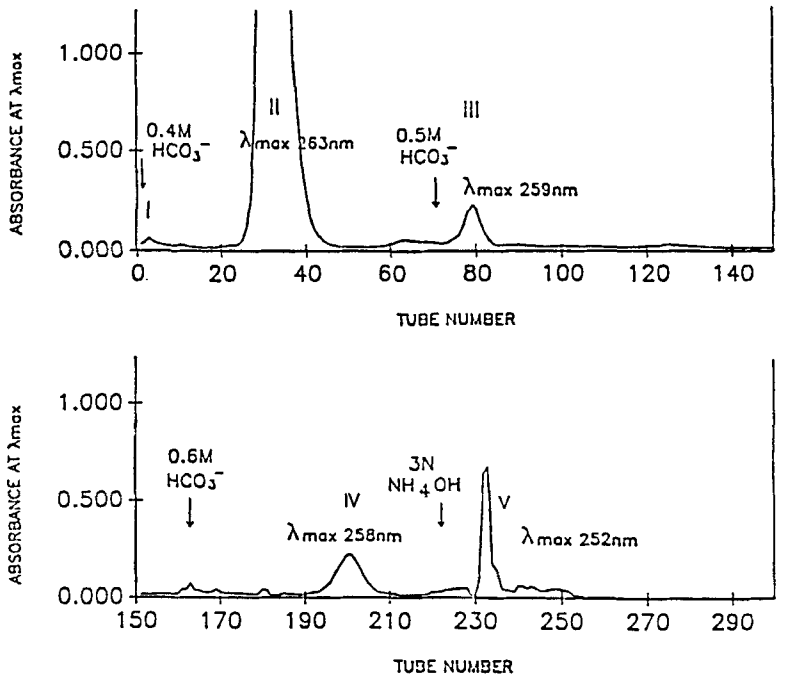
Preparation of 5,6-*O*-Isopropylidene-D-Erythorbic Acid (5). The method used was that reported by Tolbert and Ward.[12] D-Erythorbic acid (50 g) gave **5** in 97% yield with mp 217-220 °C (dec.). Thin layer chromatography using 65/25/4 (v/v/v) chloroform-methanol-water as developing solvent showed one component (Rf=0.77) in the crystalline solid.

2-Phosphorylation of 5,6-*O*-Isopropylidene-D-Erythrobic acid. The acetal (**5**) (12.3 g, 56.9 mmol) was dissolved in aqueous potassium hydroxide (pH 13) that had been saturated with pyridine, and phosphoryl chloride (7.3 mL, 79.8 mmol) was added according to the conditions described by Lee et al.[9] The reaction mixture was made to volume (250 mL) with water to give Solution A. An aliquot (2 mL) of Solution A was passed through a strongly acidic cation exchange resin (30 mL) in the hydrogen-ion form, which had been previously washed with generous amounts of water to remove all UV-absorbing materials. The ion-exchange column was washed with water (150 mL), and the effluent made to volume (250 mL), to give Solution B. An aliquot (2 mL) of Solution B was made to 100 mL volume with carbonate buffer, pH 10, and the absorbance of the solution was measured at 263nm. The percentage of 2-phosphorylation of D-erythorbate was esti-

mated, assuming that molar extinction coefficients of compounds (**3**), (**7**), and (**6**) are equal to $\epsilon = 16.0 \times 10^3$, which was found[9,18] for **L**-ascorbate 2-phosphate (**4**), its 2-diphosphate, and its phosphodiester at pH 10 and 263nm. The absorbance of the solution was 0.55, which corresponded to 93% of phosphorylation at the 2-OH of (**5**). A second aliquot of Solution B was injected into the liquid chromatograph. The major peak (R_T 6.30 min) co-eluted with pure **3**, and was present in 85% yield from **5**. The minor components with R_T 7.56 min and 8.51 min, were the 2-diphosphate (**7**) and phosphodiester (**6**), respectively, which were formed in 5 and 3% yields. Three trace components with R_T = 10.0-13.5 min were also resolved in the chromatogram.

Another aliquot (5.0 mL) of Solution A was titrated[19] immediately with 0.05M iodine. The iodine titer indicated that the mixture contained 2% unreacted **1**.

Separation of Components in Phosphorylation Reaction Mixture. An aliquot (20 mL) of Solution A, which was 0.23 M in **D**-erythorbate equivalents, was brought to pH 8 by stirring with a small quantity (~ 20 mL) of strongly acidic cation-exchange resin in hydrogen ion form. An aliquot (0.2 mL) of the phosphorylation product was placed on a column (21 x 1.7 cm) of strongly basic ion exchange resin in the bicarbonate form (AG-1 x 8, 200-400 mesh, BioRad Laboratories). The column was developed at a flow rate of 1.0 mL/min, using sequential elution with 0.4, 0.5, and 0.6 M ammonium bicarbonate and finally with 3 M ammonium hydroxide. Fractions (10 mL) were collected, and eluted components were located by diluting fractions with 0.1 M sodium carbonate buffer (pH 10) and determining UV absorbance at λ max. The components were identified by their UV properties at different pHs, and their yields were estimated after pooling fractions and using a molar absorptivity ϵmM=16 at λ max 263 nm and pH 10.



Fraction I was **D**-erythorbate (**1**), Fraction II **D**-erythorbate 2-phosphate (**3**), Fraction III **D**-erythorbate 2-diphosphate (**7**), Fraction IV *bis*-(**D**-erythorbyl) 2,2'phosphate (**6**), and Fraction V higher phosphate esters as evidenced by the trace components with R_T > 10 min observed by HPLC.

Isolation and Purification of the Magnesium and Tricyclohexylammonium Salts of D-Erythorbate 2-Phosphate (3). The remainder (223 mL) of Solution A was passed through a column (600 mL) of strongly acidic, cation-exchange resin (H^+). The column was washed until the total volume of the effluent was exactly 2 L. The absorbance at 263 nm of the column effluent at pH 10 (ϵ = 16,000 L/mol-cm) showed a 88.9% recovery of **D**-erythorbate 2-phosphate (**3**) and related derivatives. The column effluent, which was at pH 1.0, was allowed to stand for 2 h at 25 °C and solid magnesium oxide (12 g) was added with stirring to reach pH 9.0. The mixture was kept overnight at 5 °C and filtered. Recovery of **D**-erythorbate 2-phosphate (**3**) in the

filtrate was 86.2% by UV analysis. The filtrate was concentrated under diminished pressure to 1.1 M in D-erythorbate, and the dilute syrup was added to absolute ethanol (200 mL), which precipitated magnesium D-erythorbate 2-phosphate (**3**). The precipitate was collected by centrifugation, washed free of magnesium chloride with 95% ethanol (3 x 100 mL), and dried under vacuum to give 16.6g of compound **3** as a white powder. UV assay at 263 nm and pH 10 showed that the yield of **3** from **5** was 67%. HPLC-UV showed the white powder contained 74% of magnesium D-erythorbate 2-phosphate, while Karl Fisher assay gave 19.2% water. Those data indicate a 71% yield of **3** from **5**. The magnesium salt (4 g) was recrystallized twice from water (10% solution) at 25 °C to produce 1.1 g (26% from **5**) analytically pure magnesium salt with mp 265 °C (dec). The pure salt showed one peak in a reverse-phase HPLC chromatogram. ^{1}H-NMR (D_2O, pH 7.5) δ 4.64 (1H, d, H-4); 4.11 (1H, m, H-5); and 3.73 (2H, m, H-6,6′).

Anal. Calcd for $C_6H_6O_9PMg_{3/2}$ 3 H_2O: C, 20.96; H, 3.49; P, 9.02. Found: C, 20.0; H, 3.69; P 8.28.

The crude magnesium salt of **3** (6.03 g) was dissolved in water, and the slurry was added to the top of a column (5 x 45 cm) of a strongly basic ion-exchange resin (600 g, BioRad AG-1, X 8) in bicarbonate form. The column had been previously prepared by washing the resin (chloride form) with water (200 mL) followed by 0.4 M sodium bicarbonate. Elution with 0.4 M $NaHCO_3$ (2.2 liters at 1.8 mL/min) gave pure D-erythorbate 2-phosphate (**3**) as determined by HPLC/UV. The fractions (10 mL each) containing **3** and sodium bicarbonate were combined and passed through a column of strongly acidic cation exchange resin (H-form, 600 mL). The column effluent was adjusted to pH 9 with cyclohexylamine, and the solution was concentrated to a thick syrup, which, upon addition of absolute ethanol and cooling, deposited tricyclohexylammonium D-erythorbate 2-phosphate as white crystals (4.7 g, 47% from **5**). Recrystallization from 95% ethanol produced fine needles with mp 142-144 °C. NMR data on the salt are given in the Table.

Anal. Calcd for C_{24} H_{48} N_3 O_9 P: C, 52.8; H, 8.68; N, 7.60; P, 5.61. Found: C, 51.87; H, 8.70; N, 7.37; P, 5.57.

Barium Salt of Bis(D-Erythorbyl) 2,2′-Phosphate (6). To a deaerated solution of D-erythorbic acid (30.0 g; 170 mmol) in water (300 mL) was added solid barium hydroxide at 50 °C with mechanical stirring until the pH of the solution reached 10.5. Phosphoryl chloride (39.1 g, 255 mmol) was added dropwise, and the pH of the mixture was kept at pH 9.5-10.5 by periodic addition of solid barium hydroxide (total, 190 g). The reaction was complete in 100 min, and the precipitated barium phosphate was removed by rapidly filtering the mixture at 50 °C. The clear filtrate was kept overnight at 5 °C to induce crystallization of the barium salt of the phosphodiester (**6**). The crystals were collected by filtration and dried; yield 24 g (42%). Elemental analysis indicated that the crystals of **6** were contaminated with barium chloride. The crude barium salt (0.3g) was placed atop a column (1.7 x 20 cm) of strongly basic anion-exchange resin in the bicarbonate form, and fractions (15 mL) were collected and monitored at 263 nm. The column was developed with 0.4M ammonium bicarbonate (300 mL), which eluted compound **3**, and then with 0.6 M ammonium bicarbonate (660 mL), which eluted the phosphodiester **6**. The fractions containing **6** were combined, and the solution was concentrated to remove ammonium bicarbonate. The ammonium salt of **6** was converted to its free acid form using a strongly acidic cation exchange resin (H^+-form) and then to its barium salt by neutralizing with barium hydroxide. The barium salt of **6** was crystallized from water. Yield 0.16 g (22% based on **1**): mp 250-5 °C (dec); ^{13}C-NMR (D_2O, pH 7.5) δ 179.7 (C-1), 177.8 (C-3), 113.5 (C-2), 82.2 (C-4), 73.5 (C-5), 63.5 (C-5), 63.5 (C-6). ^{1}H NMR (D_2O, pH 7.5) δ 4.64 (1H, d, H-4); 4.11 (1H, m, H-5); and 3,67 (2H, m, H-6,6′).

Anal. Calcd for $C_{12}H_{12}0P$ $Ba_{3/2}$ $3H_20$ C, 21.48%; H, 2.68; P, 4.62; Ba, 30.65. Found. C, 21.69; H, 2.56; P, 4.58; Ba, 31.88%.

ACKNOWLEDGEMENT

The authors thank Ming-Long Liao for discussions and Yun-Teh Liang for purification of barium *bis*-(D-erythorbyl) 2,2'phosphate.

Contribution No. 91-558-J from the Kansas Agriculture Experiment Station.

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