Carbohydrate Research, 67 (1978) 127–138

CHEMICAL SYNTHESIS OF SEVERAL PHOSPHORIC ESTERS OF L-ASCORBIC ACID*

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(Received December 3rd, 1977, accepted for publication, January 30th, 1978)

ABSTRACT

The 5,6-isopropylidene acetal (**3**) of L-ascorbate 2-phosphate (**4**) was produced in almost quantitative yield by the action of phosphoryl chloride at 0–5° on 5,6-*O*-isopropylidene-L-ascorbate (**2**) in alkali (pH 12–13) containing a high concentration of pyridine After hydrolytic cleavage of the 5,6-acetal group and removal of inorganic phosphate and chloride, compound **4** was isolated, without chromatographic purification in 70% yield as its crystalline tricyclohexylammonium salt When no pyridine was present during the reaction of **2** with phosphoryl chloride in an alkaline solution, it was converted into a mixture of **3** and bis(5,6-*O*-isopropylidene-L-ascorbate) 2,2′-phosphate (**5**) Following removal of the 5,6-protecting groups of **5**, the phosphoric diester **6** was isolated in pure form in 32% yield by fractional recrystallization of its barium salt from cold water Reaction of **2** with phosphoryl chloride in acetone–pyridine gave, after removal of the protecting group, a mixture of four phosphoric esters that was resolved by column chromatography on an ion-exchange resin Two of the products were identical to **4** and **5**, and the third was tentatively identified as the 3-phosphate of L-ascorbic acid, the fourth was not identified The structures of the phosphoric esters were established by elemental analysis, u v and n m r spectroscopy (^{31}P and ^{1}H), and ionization constants

INTRODUCTION

Several phosphorylated derivatives of L-ascorbic acid are listed in the chronology presented in Table I We now report an improved method for chemically preparing, and readily isolating, the analytically pure tricyclohexylammonium salt of 2-*O*-phosphono-L-ascorbic acid (**4**) In our hands, the older methods of synthesis of **4** gave unreacted starting-material and a mixture of several phosphoric esters, from which compound **4** could be obtained in moderate to poor yield by column chromato-

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TABLE I

CHRONOLOGICAL SUMMARY OF PHOSPHORYLATED DERIVATIVES OF L-ASCORBIC ACID (1) OR 5,6-*O*-ISOPROPYLIDENE-L-ASCORBIC ACID (2)

Date	*References*	*Summary*
1961	1	Reacted **2** with phosphoryl chloride in acetone–pyridine; isolated crystalline tricyclohexylammonium salt of L-ascorbate 2-phosphate[a] (m.p. 169–170°); demonstrated 2-phosphate has vitamin C potency equivalent to that of L-ascorbic acid
1966	2	Reacted **2** with phenylphosphoric acid and *N*,*N'*-dicyclohexylcarbodiimide in pyridine; purified 2- and 3-(phenylphosphoric) esters of **1**
1969	3,4	Reacted **2** with phosphoryl chloride in acetone–pyridine; ion-exchange purification gave magnesium L-ascorbate 2-phosphate[a] and magnesium bis(L-ascorbate) 2,2'-phosphate[b]; two additional phosphorylated derivatives of **1** isolated
1969	5,6	Reacted an aqueous solution of **1** or **2** with phosphoryl chloride; high yields of magnesium L-ascorbate 2-phosphate[a] reported
1972	7	Reacted salts of **1** with phosphoryl chloride in acetone; structure of products not investigated
1973	8	Reacted **2** with morpholinophosphoryl dichloride in 1,4-dioxane–pyridine; product hydrolyzed in 0.5M hydrochloric acid; reported crystalline tricyclohexylammonium salt of L-ascorbate 2-phosphate[a] (m.p. 178–180°)

[a]Compound was originally assigned the 3-phosphate structure[1,3,8]. [b]Compound was originally assigned the structure of L-ascorbate 2-phosphate[4].

graphy on an ion-exchange resin. We also present data that strongly suggest that the L-ascorbic 3-phosphate reported by other workers is actually the 2-phosphate. Furthermore, we give a procedure for readily preparing the pure barium salt of bis(L-ascorbic acid) 2,2'-phosphate (**6**). Finally, we have apparently isolated the 3-phosphate of L-ascorbic acid in low yield by ion-exchange, column chromatography.

Compound **4**, like the naturally occurring 2-sulfuric ester[9,10], is oxidized by air in boiling water at approximately one-tenth the rate for L-ascorbate[11]. The two esters are equally stable at pH 13, but the 2-sulfate is hydrolyzed ten times as fast[11] as the 2-phosphate (**4**) in boiling acid (pH 1.0). Unlike the 2-sulfuric ester[12], the 2-phosphate retains its full antiscorbutic potency in the guinea pig[1,13] and, most probably, in man. The occurrence of **4** in Nature has not been demonstrated, possibly because of the wide-spread occurrence of phosphatases[14]. Compound **4** has been shown to be a powerful inhibitor of L-ascorbate 2-sulfate sulfohydrolase[14].

L-Ascorbate 2-phosphate (**4**) has been used to prolong the storage life of whole blood[15]; in a related area, it might be used in organ culture[16]. In foods and feeds, dehydro-L-ascorbic acid, the first oxidation product of L-ascorbic acid, apparently reacts with amino acids and proteins to produce off-colors[17] and off-flavors[18]. Use of a more difficultly oxidizable form of vitamin C could alleviate these problems.

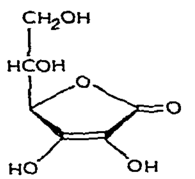
RESULTS AND DISCUSSION

Phosphoryl chloride, an inexpensive and fast-acting phosphorylating agent, is rarely used for synthesizing monophosphoric esters of carbohydrates, because of its multifunctional reactivity The reported 88–94% conversion of **2** into its 3-phosphate in an aqueous medium appeared to be an exception[5,6] We therefore repeated the reactions that reportedly gave these high yields (Examples 1 and 3 in ref 5, and Reaction No 13 in Table II of ref 6), but found that, instead, the reaction mixtures contained 20–36% of unreacted starting-material, 16–54% of **4**, and 16–38% of **6** Reaction No 13 in Table II of ref 6 gave, after mild deacetonation, the highest yield (54%) of **4** but the mixture also contained 20% of L-ascorbate and 16% of **6**

We re-investigated the use of phosphoryl chloride for phosphorylating L-ascorbic acid, and found that compound **2** is converted almost quantitatively into its 2-phosphate (**3**) under the following conditions (*1*) throughout the phosphorylation, the pH of the reaction mixture must be maintained at 12–13 by means of cesium potassium or rubidium hydroxide, (*2*) before the phosphoryl chloride is added, the mixture (pH 13 0) initially containing 0 4M 5,6-*O*-isopropylidene-L-ascorbate, must be made 2 2–3 0M in pyridine and (*3*) the mixture must be kept at 0–5° during slow addition of 1 4 equivalents of phosphoryl chloride Under those conditions, the phosphorylating mixture remains homogeneous despite the tendency for tertiary amines to separate into a second phase from an aqueous solution that is highly alkaline highly ionic, and at low temperature In fact, pyridine was the only tertiary amine found suitable among a number tested including triethylamine, 2,2′-bipyridine, 2-picoline, and 1,4-diazabicyclo[2 2 2]octane Phase separation of the tertiary amine also depended on the inorganic base used in the phosphorylation sodium hydroxide could not be used, because phase separation began after ~75% of the phosphoryl chloride had been added to the mixture

Inorganic bases of low solubility in water (barium, calcium, lithium, and magnesium hydroxides for example) could not be used in the phosphorylation, because they increase the volume too much during maintenance of the pH of the mixture The extra water competes with compound **2** for phosphoryl chloride resulting in unreacted starting-material in the reaction mixture It is essential that a constant high pH be maintained during the phosphorylation At pH values >8, only two products **3** and **5** were detected but if the phosphorylation was conducted under acidic conditions, more than two products were detected by chromatography

A series of phosphorylations was conducted at pH values between 8 and 13 in the absence of pyridine In each reaction, phosphoryl chloride (1 4 equiv) was treated at 0° with an alkaline solution of **2** (0 44M, after initial adjustment of the pH), and the pH of the mixture was maintained by periodically adding 10M aqueous potassium hydroxide As the pH was raised from 8 to 13, it was found that (*i*) the proportion of unreacted **2** (iodometric titration) decreased from 38 to 1% (*ii*) the proportion of 2-phosphate, as determined by high-performance, liquid chromatography (HPLC), changed very little (from 43 to 51%), and (*iii*) the proportion of **5** increased from 3 to 32% The barium salt of **6** was readily obtained in pure form, as it could be separated from the monoester **4** by fractional recrystallization from cold water



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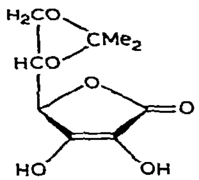

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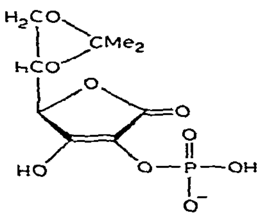

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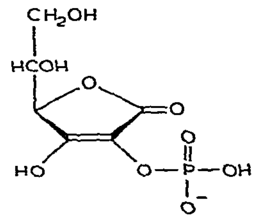

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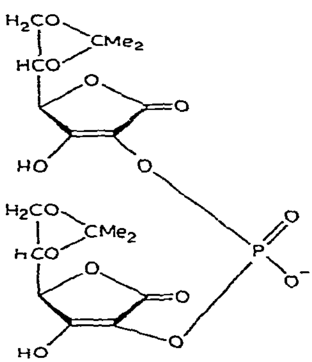

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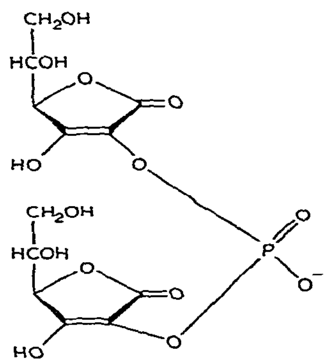

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The structure of the tricyclohexylammonium salt of **4** was established as follows Elemental analysis and enzymic hydrolysis (calf intestinal-mucosa alkaline phosphatase) showed the compound to be a monophosphoric ester[19] The ^{31}P-n m r spectrum of the compound gave only one signal, which, at pH $\sim$10, was a narrow doublet 3.6 p p m downfield from the reference signal of external, 50% aqueous phosphoric acid The small splitting indicated long-range coupling of the phosphorus to H-4 ($^5J_{P\text{-}H}$ $\sim$0 8 Hz)

The p m r spectrum of **4** was consistent with the structure assigned The chemical shifts and the major splittings of the signals of H-4, H-5, and H-6 of **4** were similar (see Table II) to those of 2-*O*-sulfo-L-ascorbic acid, a compound whose structure has been unequivocally determined[20] by X-ray crystallography The similarity of the chemical shifts of H-5 and H-6 to those of these protons in free L-ascorbic acid (**1**) shows that the phosphate group is situated on either C-2 or C-3. The ionization of the 3-hydroxyl group of **1** or its 2-sulfate shifts the signal of H-4 upfield by 0.5 p.p.m. (Δ H-4, see Table II). Ionization of OH-3 of compound **4** shifts the signal for H-4 by +0.4 p.p.m., and this strongly favors assigning of the 2-phosphate structure.

TABLE II

PROTON MAGNETIC RESONANCES OF L-ASCORBIC ACID AND SEVERAL OF ITS ESTERS

Derivative of L-*ascorbic acid*	*pH*	*Chemical shift*[a] (δ)			
		H-4	*Δ H-4*[b]	*H-5*	*H-6, H-6'*
Free acid	2	4.97	—	4.09	3.76
	7	4.50	+0.47	4.02	3.74
2-Sulfate	1	5.02	—	4.20	3.76
	7	4.57	+0.45	4.05	3.73
2-Phosphate	1	5.00	—	4.16	3.74
	7	4.60	+0.40	4.05	3.70
2,2'-Phosphoric diester	1	5.00	—	4.15	3.78
	12	4.50	+0.50	4.02	3.68
3-Phosphate[c]	8	4.92	—	4.22	3.77

[a]Determined in deuterium oxide, chemical shifts in p.p.m. from 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). [b]Δ H-4 is the upfield shift of H-4 on changing the pH of the medium. [c]Tentative, structural assignment.

TABLE III

ULTRAVIOLET-SPECTRAL PROPERTIES OF L-ASCORBIC ACID AND SEVERAL OF ITS DERIVATIVES[a]

Compound	*Acid (pH 2.0)*		*Neutral (pH 7.0)*		*Base (pH 10.0)*	
	λ_{max}	ε_{mM}	λ_{max}	ε_{mM}	λ_{max}	ε_{mM}
L-Ascorbic acid	243	10	265	16.5	—	—
3-*O*-Methyl-L-ascorbic acid	244	9	244	9	276	9
2-*O*-(Phenylphosphono)-L-ascorbic acid[b]	233	9.6	257	14.5	258	15
3-*O*-(Phenylphosphono)-L-ascorbic acid[b]	237	8	238	8	263	8
L-Ascorbate 2-sulfate	232	11	255	16.3	255	16.3
L-Ascorbate 2-phosphate	238	9	258	11.5	264	16.0
L-Ascorbate 2,2'-phosphate	236	17.3	258	21.6	259	30.2
L-Ascorbate 3-phosphate[c]	238	0.32[d]	250	0.370[d]	265[e]	0.300[d,e]

[a]All data in Table III except for those for the last four compounds, taken from Bond *et al.*[27] [b]5,6-Isopropylidene acetal. [c]Tentative, structural assignment. [d]Values of λ_{max} for the same solution at different pH values. [e]At pH 11.0.

U v -spectral data also supported the 2-phosphate structure assigned (see Table III) The absorption maxima and extinction coefficients of the 2-phosphate and 2-sulfate of L-ascorbic acid at pH 7 are practically identical to those of 5,6-*O*-isopropylidene-2-*O*-(phenylphosphono)-L-ascorbic acid, but differ from those of the corresponding 3-(phenylphosphonic) ester

The ionization constants of compound **4** are more in accord with the 2- than with the 3-phosphate structure By use of u v data[21] and ^{31}P-n m r data[22 23], it was found that, at 25°, **4** has $pK_1 < 1$, pK_2 3 3 and pK_3 6 8, from u v data and potentiometric titrations, Nomura and co-workers[24] found pK_1 0 01, pK_2 3 27, and pK_3 6 70 at 28° The first ionization-constant (pK_1 0 01) can undoubtedly be assigned to the ionization of the phosphate group even though it is 1–2 pK units lower than that found for simple monoalkyl phosphates[25] The pK_2 and pK_3 constants of 3 3 and 6 8 were assigned, respectively, to the 3-hydroxyl group and to the second hydroxyl group of the phosphate group The 2- and 3-hydroxyl groups of free L-ascorbic acid have pK_a values of 11 79 and 4 25 respectively[26] It is unlikely that the ionization constant of the 2-hydroxyl group would be lowered by 3 orders of magnitude (from pK_a 11 8 to 6 8) upon esterification of the 3-hydroxyl group Assignment of pK_2 3 3 to the 3-hydroxyl group in compound **4** agrees closely with ionization data for the known 2-sulfate of L-ascorbic acid In the latter, 2-sulfation decreases[27 28] the ionization constant of the 3-hydroxyl group from pK_a 4 25 to 2 75

The structure of barium bis(L-ascorbic acid) 2 2′-phosphate (**6**) was deduced from the following evidence Elemental analysis indicated that the compound is a phosphoric diester as expected[19], it is stable in the presence of alkaline phosphatase The ^{31}P-n m r spectrum of compound **6** showed only one resonance signal a narrow triplet due to long-range coupling of the phosphorus to H-4 and H-4′ ($^5J_{P,H} = {}^5J_{P,H}$ = ~0 8 Hz)

The p m r spectrum of **6** gave three signals similar to those of **4** (see Table II) The H-4 (H-4′) signal of compound **6** shifted 0 5 p p m upfield when OH-3 (OH-3′) of the phosphoric diester ionized The appearance of only one ^{31}P signal and only three proton signals indicated that the phosphorus atom in the phosphoric diester **6** is symmetrically substituted The 2 2′-linkage is strongly suggested by the p m r data The u v -spectral data (see Table III) and the ionization constants (pK_1 2 3, pK_2 5 8 and pK_3 8 0) of the compound support the structure assigned

In their original work, Nomura and his colleagues[3 4] treated compound **2** with phosphoryl chloride in pyridine–acetone, and reported the isolation of four phosphates of L-ascorbic acid by use of column chromatography on a strongly basic, anion-exchange resin We repeated those experiments, and found that ion-exchange chromatography does, indeed, separate four components from the phosphorylation reaction-mixture Unlike their results, however, we found that our reaction mixture also contained unreacted L-ascorbic acid, which was eluted much more rapidly than the four other components In addition, we consider that incorrect structures were originally assigned to three of the more slowly eluting compounds In the original papers[3 4], fraction I (designated compound II), fraction III (compound IV), and fraction IV (compound V) were assigned, respectively, to the structures L-ascorbate 3-phosphate, bis(L-ascorbate) 3,3'-phosphate, and L-ascorbate 2-phosphate We found, instead, that fractions I and IV are L-ascorbate 2-phosphate (4) and bis(L-ascorbate) 2,2'-phosphate (6), respectively, and that fraction III is probably L-ascorbate 3-phosphate We did not investigate fraction II, which was originally assigned the structure L-ascorbate 3-diphosphate

We have evidence that fraction III (designation of Nomura and co-workers[4]) from the column is L-ascorbate 3-phosphate We separated fraction III only once, and have not completed its characterization In paper chromatography, fraction III gave a single spot that co-migrated with L-ascorbate 2-phosphate But fraction III gave a deep-purple color with ferric chloride spray, instead of the brick-red color produced by the 2-phosphate and the 2,2'-phosphoric diester The different colors of the ferric ion complexes indicate that the two compounds have different enolic hydroxyl groups[29]

The ^{31}P-n m r spectrum of fraction III showed the presence of only one phosphorus atom at δ -1 75 (pH 9 3) with a major splitting of $\sim$2 Hz The ^{31}P signal became a very sharp doublet (δ -1 20) at pH 12, with $^4J_{P,H}$ $\sim$2 7 Hz

The ^{1}H-n m r spectrum of the tricyclohexylammonium salt of fraction III was also in accord (Table II) with the 3-phosphate structure The chemical shifts of H-5 and H-6 indicated that the phosphate group was on C-2 or C-3 as, otherwise the signal of H-5 or H-6 would have been shifted downfield from those observed for L-ascorbate The chemical shift of H-4 at pH 8 indicates that OH-3 in fraction III did not undergo ionization at pH 8 0 When we isolated fraction III several years ago, we were unaware of the significant shift of H-4 when the 3-hydroxyl group of L-ascorbic acid ionizes Unfortunately, we did not record the spectrum of fraction III at pH 1 0 We should point out that our p m r data agree well with those originally reported[4] for fraction III (compound IV)

The u v data in Table III also support the 3-phosphate structure for fraction III The absorbance of the compound, like that of 5 6-*O*-isopropylidene-3-*O*-(phenylphosphono)-L-ascorbic acid did not change much in magnitude on passing from pH 2 to pH 10 At pH 7 0, L-ascorbate 3-phosphate however had λ_{max} 250 nm, whereas the 3-(phenylphosphate) derivative has λ_{max} 238 nm Apparently, the second ionization of the monosubstituted phosphate group in L-ascorbate 3-phosphate is responsible for that difference

We determined the ionization constants for fraction III by using two different methods (^{31}P-chemical shifts and u v data) We found pK_1 3 2, pK_2 7 0, and pK_3 10 5 The last constant, which we have assigned to the ionization of OH-2, is much higher than any of the ionization constants found for L-ascorbate 2-phosphate The 2-hydroxyl group of L-ascorbic acid has pK 11 79 and the lowering of that ionization constant to pK 10 5 is consistent with 3-*O*-phosphorylation

Finally, Nomura and his colleagues[4] reported that fraction III (their compound IV, to which they assigned the phosphoric diester structure, and to which we assign the 3-phosphate structure) is readily hydrolyzed in hot alkali At the same time, they reported that fraction IV (compound V, to which they assigned the 2-phosphate structure, and to which we assign the 2,2′-phosphoric diester structure) is stable to hot alkali They also reported that passage of fraction III together with M hydrochloric acid through a strongly basic, anion-exchange resin (chloride form) at 25° produced a mixture of L-ascorbic acid and L-ascorbate 3-phosphate Finally, they reported that fraction III was unstable and that it disappeared if the phosphorylation reaction-mixture was not separated rapidly by ion-exchange chromatography using 0 7M sodium hydrogencarbonate

Those properties of fractions III and IV are more readily explained if fraction III (compound IV) is assigned the structure L-ascorbate 3-phosphate, and fraction IV (compound V), the structure bis(L-ascorbate) 2,2′-phosphate L-Ascorbate 3-phosphate is a mixed anhydride of an inorganic and an organic acid, it would be expected to be hydrolyzed much faster in hot alkali than the 2,2′-phosphoric diester of L-ascorbic acid Converting fraction III (our asssigned L-ascorbate 3-phosphate) by M hydrochloric acid at 25° into a mixture of L-ascorbic acid and L-ascorbate 2-phosphate (our assignment to fraction I) may be explained by acid-catalyzed hydrolysis of the 3-phosphate and concurrent migration[30] of the phosphate group to afford the more stable 2-phosphate (4) We have found that L-ascorbate 2-phosphate is very stable at pH 0 5–1 0 at 25° The variable proportions of L-ascorbate 3-phosphate reported[4] in the products of phosphorylation of L-ascorbic acid in acetone are understandable, because of base- and acid-catalyzed hydrolysis, and phosphate migration of the 3-phosphate

EXPERIMENTAL

General — High-performance liquid chromatography (h p l c) was conducted at 25° in a Waters Model ALC/G PC 201 instrument (Waters Associates, Inc , Milford, MA) The stainless-steel column (1 22 m × 3 17 mm) was packed with a pellicular, strongly basic, anion-exchange resin (Bondapak AX/Corasil, 37–50 μm, Waters Assoc , Inc) Samples (10 μL) were injected through a stop-flow injector, the column was developed with 0 1M potassium dihydrogenphosphate (pH 4 4), and the effluent was monitored at 254 nm The relative mobilities (R_{Asc}) of the materials examined were as follows **1**, 1 0, **2**, 1 3, dipotassium L-ascorbate 2-sulfate, 1 8, tricyclohexylammonium L-ascorbate 2-phosphate, 2 8, barium bis(L-ascorbate) 2,2′-phosphate, 2 3, **3**, 3 4, and **5**, 6 0 Paper chromatography was performed on Whatman No 1 paper using 15 4 1 (v/v/w) 1-propanol–water–trichloroacetic acid as the developing solvent Components were detected with either 1% ferric chloride in 95% ethanol[29] or acid-molybdate spray[31]

Ionization constants were determined from u v [21] or ^{31}P-n m r data[22,23] N m r spectra were recorded with a Varian XL-100 spectrometer Proton chemical shifts are reported in δ values from the internal reference signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate For ^{31}P-n m r spectra, a 50% aqueous solution of phosphoric acid was used as the external reference material to determine δ values

5,6-O-Isopropylidene-L-ascorbic acid (**2**) — The procedure is a modification of that of Jackson and Jones[32] To a slurry of L-ascorbic acid (**1**, 300 g) in acetone (1 350 L) was added acetyl chloride (37 5 mL), and the mixture was stirred vigorously for 2 h at 25–30°, by then, crystalline **2** had separated The crystals were collected by filtration, washed with cold, 4 7 (v/v) acetone–hexane, and dried in a vacuum desiccator over potassium hydroxide pellets, needle-shaped crystals, yield 348 g (97%), m p 217–223° Thin-layer chromatographic analysis of the crystals on plates coated with silica gel, with 24 1 (v/v) ethyl ether–acetic acid as the developing solvent, showed only one component (R_F 0 7) Compound **2** reduced the same amount of iodine as an equivalent weight[33] of **1**

Synthesis of L-ascorbate 2-phosphate (**4**) — In a 250-mL beaker fitted with a pH electrode, a magnetic stirring-bar, and a nitrogen-inlet tube were placed pyridine (310 mmol, 25 mL) and distilled water (100 mL) that had been prepurged with nitrogen To this mixture was added **2** (12 3 g, 56 9 mmol), and the pH was adjusted to 13 by adding 10M aqueous potassium hydroxide (~12 mL) (sodium hydroxide should not be used) The mixture was cooled to −5° in a salt–ice bath At this point, the concentrations of pyridine and **2** in the mixture were ~2 3M and 0 44M, respectively Phosphoryl chloride (7 3 mL, 79 8 mmol) was slowly added (~3 mL/h) to the mixture by use of a small buret or a syringe-pump (Model No 975, Howard Apparatus Co , Inc , Melis, MA) fitted with Teflon tubing (0 25 mm i d) The temperature of the mixture was maintained at −5 to +5°, and the pH at 13 by periodic addition of 10M aqueous potassium hydroxide The total volume of 10M potassium hydroxide added during the reaction was ~46 mL When addition of phosphoryl chloride was complete, the volume of the mixture was made to 250 mL with water, and an aliquot (10 0 mL) of the mixture was titrated[33] with 0 1M iodine The iodine titer indicated that the mixture contained 2 2% of unreacted **2**

The yields of phosphorylated components in the reaction mixture were determined by h p l c , both before and after hydrolytic removal of the isopropylidene group It was advantageous to examine the reaction mixture by h p l c before removal of the isopropylidene group, as the resolution of components in the reaction mixture was improved An aliquot (1 0 mL) of the total reaction-mixture was concentrated to ~0 5 mL under diminished pressure below 50°, to remove pyridine The volume of the residual, alkaline solution was made to 100 mL, and an aliquot (4 0 mL) of the solution was combined with 1 0 mL of a solution of analytically pure tricyclohexylammonium L-ascorbate 2-phosphate (50 mg in 100 mL of water) in a 10-mL volumetric flask After making to volume (10 0 mL) with water, triplicate 10 0-μL aliquots of the latter solution were injected into the chromatograph The amounts of **3** (R_{Asc} 3 4) and **5** (R_{Asc} 6 0) were determined from the areas of their peaks relative to that of the tricyclohexylammonium salt of **4** (R_{Asc} 2 8) We calculated that 97% of **2** had been converted into **3**, only a trace of **5** was detected

The high conversion of **3** into **4** was confirmed by h p l c assay after hydrolytic removal of the isopropylidene group An aliquot (1 0 mL) of the reaction mixture previously made to 250 mL was placed on a column of strongly acidic, cation-exchange resin (15 mL) in the hydrogen form The column was washed with water, and the effluent made to volume (250 mL) Triplicate aliquots (10 0 μL) of the latter solution were injected into the chromatograph Using response areas and a standard curve obtained with the tricyclohexylammonium salt of **4**, it was determined that 96 2% of the starting material had been converted into **4** Again, the chromatogram showed that the reaction mixture contained only a trace of **6**, R_{Asc} 2 3, and that the isopropylidene group had been completely removed by passing the reaction mixture through the strongly acidic, cation-exchange resin

Isolation and purification of the tricyclohexylammonium salt of **4** — The rest (248 mL) of the reaction mixture analyzed by h p l c 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 2 L The absorbance at 264 nm of the column effluent at pH 10 showed a 93 4% recovery of the theoretical amount of L-ascorbate 2-phosphate The column effluent, pH 1 0, was kept for 2 h at 25°, and then magnesium oxide (~ 11 g) was added to pH 9 0 The mixture was kept overnight at 5°, and filtered Recovery of L-ascorbate 2-phosphate in the filtrate was 91 7% according to u v analysis The filtrate was concentrated under diminished pressure to 50 mL, and the concentrate was added to absolute ethanol (300 mL) The magnesium L-ascorbate 2-phosphate precipitated was collected by centrifugation, and washed free of magnesium chloride with 95% ethanol (2 × 100 mL) The magnesium salt of **6** is more soluble in ethanol than the magnesium salt of **4** The sedimented material was dried under vacuum, to give 19 5 g (86% yield by u v) of compound **4** as a white powder

The supernatant liquor from the centrifugation was combined with the ethanol washings, and the resulting solution which contained 6% (u v analysis) of **4** was evaporated to dryness under vacuum The solid residue was dissolved in water (50 mL), and barium hydroxide (2 g) was added to precipitate the barium salt of **4** which is insoluble at neutral or alkaline pH, but dissolves at pH ⩽4 The precipitate was collected by centrifugation, and the supernatant liquor was discarded (u v loss 0 24%) The precipitated barium salt of **4** was combined with the solid magnesium salt of **4**, and the mixture was placed on top of a column (600 mL) of strongly acidic, cation-exchange resin (H^+) The pH of the effluent (200 mL) which contained 82% of the theoretical amount of **4** (as determined by u v spectroscopy), was adjusted to 9 with cyclohexylamine, and the solution was evaporated under vacuum to a thick syrup Absolute ethanol was added, and the solution was cooled overnight, to give 18 9 g of tricyclohexylammonium L-ascorbate 2-phosphate as flat prisms, m p 178–182° (dec), $[\alpha]_D^{25}$ +30° (*c* 1 0, water) A second crop (3 3 g) of crystals brought the total yield of material to 22 2 g (70%) [The mother liquors retained 12% (u v) of L-ascorbate 2-phosphate Paper chromatography showed that the crystalline material contained traces of inorganic phosphate (R_{Asc} 1 2) along with only one other component (R_{Asc} 0 6) The minor traces of inorganic phosphate could not be completely eliminated from the product by repeated recrystallization from 95% ethanol] U v data $\lambda_{max}^{pH\ 2\ 0}$ 238 nm, ε_{mM} 9, $\lambda_{max}^{pH\ 7\ 0}$ 258, ε_{mM} 11 5, $\lambda_{max}^{pH\ 10\ 0}$ 264 nm, ε_{mM} 16, p m r data (pH 7) δ 3.70 (H-6), 4.05 (H-5), and 4.60 (H-4). Nomura and his colleagues[3] reported the following properties for the compound to which they assigned the structure of magnesium L-ascorbate 3-phosphate pentahydrate: $[\alpha]_D^{22}$ +42° (*c* 1.0, H_2O), u.v. data: $\lambda_{max}^{pH\ 1.0}$ 237 nm, ε_{mM} 9.8, $\lambda_{max}^{pH\ 13}$ 261 nm, ε_{mM} 16.1; p.m.r. data (D_2O): δ 3.83 (H-6), 4.16 (H-5), and 4.64 (H-4). Cutolo and Larizza[1] reported that the tricyclohexylammonium salt of **4** had $[\alpha]_D^{20}$ +44° (*c* 1.6, H_2O); $\lambda_{max}^{pH\ 1}$ 237, ε_{mM} 9.77, $\lambda_{max}^{pH\ 6.5}$ 258, ε_{mM} 11.8, and $\lambda_{max}^{pH\ 13}$ 265, ε_{mM} 15.1.

Anal. Calc. for $C_{24}H_{49}N_3O_9P$: C, 52.06; H, 8.74; N, 7.59; P, 5.34. Found: C, 51.95; H, 8.81; N, 7.39; P, 5.34.

The ionization constants of L-ascorbate 2-phosphate were determined from plots[21] of λ_{max} *versus* pH: pK_1 < 1.0, pK_2 3.25, and pK_3 7.85; lit.[24] pK_1 0.01, pK_2 3.27, and pK_3 6.70.

Calcium L-ascorbate 2-phosphate. — A solution of tricyclohexylammonium L-ascorbate 2-phosphate (m.p. 180–182°) (1.00 g) in water (~10 mL) was passed through a column (12 mL) of a strongly acidic, cation-exchange resin (H^+). The column was washed with water (2 vol.) and the effluents were combined, adjusted to pH 9.0 by addition of calcium hydroxide, and filtered. Evaporation of the filtrate gave 0.574 g of calcium L-ascorbate 2-phosphate, m.p. 220–240° (dec.); recovery (based on u.v. absorption) 82.9%.

Barium salt of bis(L-ascorbic acid) 2,2'-phosphate (**6**). — The phosphoric diester **6** was prepared by starting with either **2** or **1**, the latter providing the easier procedure. To a de-aerated solution of L-ascorbic acid (30.0 g, 170 mmol) in water (300 mL) was added solid barium hydroxide at 50°, with mechanical stirring, until the pH of the solution reached 10.5. Phosphoryl chloride (23.4 mL, 39.1 g, 255 mmol) was added dropwise, and the pH of the mixture was kept at 9.5–10.5 by periodic addition of solid barium hydroxide (total, 190 g). The reaction was complete in 100 min; the precipitated barium phosphate and barium L-ascorbate 2-phosphate were then removed by rapidly filtering the mixture. The clear filtrate was kept overnight at 5° to induce crystallization of the salt of phosphoric diester **6**. The crystals were collected by filtration and dried over phosphorus pentaoxide, yield 15.2 g (28.9%). Recrystallization from cold water gave analytically pure barium salt of **6**, m.p. 250° (dec.); $[\alpha]_D^{25}$ +65° (*c* 1.0 of sodium salt, H_2O); u.v. data: $\lambda_{max}^{pH\ 1.0}$ 235 nm, ε_{mM} 18.2; $\lambda_{max}^{pH\ 7.0}$ 258, ε_{mM} 25.6; $\lambda_{max}^{pH\ 11-13}$ 258, ε_{mM} 30.6; ionization constants, pK_1 2.3, pK_2 5.8, and pK_3 8.0; p.m.r. data at pD 11.0: δ 3.70 (H-6), 4.01 (H-5), and 4.50 (H-4). [Nomura *et al.*[4] reported the following properties for their component IV (compound V) to which they erroneously assigned the structure of magnesium L-ascorbate 2-phosphate: u.v. data: $\lambda_{max}^{pH\ 1.0}$ 235 nm, ε_{mM} 8.7, and $\lambda_{max}^{pH\ 13.0}$ 259, ε_{mM} 14; p.m.r. data (read from Fig. 4 in ref. 4): δ 3.76 (H-6), 4.10 (H-5), and 4.63 (H-4).]

Anal. Calc. for $C_{12}H_{12}Ba_{1.5}O_{14}P$: C, 23.35; H, 1.96; P, 5.02. Found: C, 23.46; H, 2.04; P, 4.70.

ACKNOWLEDGMENT

The authors thank Dr Joseph Paukstelis for recording and interpreting the ^{31}P-n m r. spectra

REFERENCES

1 E CUTOLO AND A LARIZZA *Gazz Chim Ital*, 91 (1961) 964–972
2 V M CLARK, J W B HERSHEY, AND D W HUTCHINSON, *Experientia*, 22 (1966) 425
3 H NOMURA, T ISHIGURO, AND S MORIMOTO, *Chem Pharm Bull*, 17 (1969) 381–386
4 H NOMURA, T ISHIGURO, AND S MORIMOTO, *Chem Pharm Bull* 17 (1969) 387–393
5 H NOMURA, T ISHIGURO, AND K MAEDA, Ger Pat 1,805,958 (1969), *Chem Abstr* 71 (1969) 70 897*n*
6 H NOMURA, M SHIMOMURA AND S MORIMOTO *Chem Pharm Bull*, 17 (1971) 1433–1437
7 D F HINKLEY, U S Pat 3,671,549 (1972), *Chem Abstr*, 71 (1969) 91,827*e*
8 S ISHII, Jpn Pat 73 15, 605 (1973), *Chem Abstr*, 79 (1973) 66,743*g*
9 C G MEAD AND F S FINAMORE, *Biochemistry*, 8 (1969) 2652–2655
10 B M TOLBERT, M DOWNING, R W CARLSON, M K KNIGHT, AND E M BAKER, *Ann N Y Acad Sci*, 258 (1975) 48–69
11 C H LEE *Synthesis and Characterization of* L-*Ascorbate Phosphates and Their Stabilities in Model Systems*, Ph D Dissertation Kansas State University, 1976
12 W KUENZIG, R AVENIA AND J J KAMM *J Nutrition* 104 (1974) 952–956
13 Y IMAI, T USUI H MATSUZAKI, H YOKOTANI H MIMA, AND Y ARAMAKI, *Jpn J Pharmacol*, 17 (1967) 317–324
14 R W CARLSON, M DOWNING, P A SEIB, AND B M TOLBERT, *Biochem Biophys Res Commun* 68 (1976) 949–953
15 T A BENSINGER AND T F ZUCK *Transfusion* 16 (518) (1976) 109–110
16 J FENG, A H MELCHER, D M BRUNETTE, AND H K MOE *In Vitro* 13 (2) (1977) 91–99
17 J R BRIDGES AND D M MORRIS, *J Insect Physiol*, 23 (1977) 497–501
18 E ABDERHALDEN, *Fermentforschung* 15 (1938) 522–525
19 T W REID AND I B WILSON *Enzymes*, 3rd edn, 4 (1971) 417–443
20 B M MCCLELLAND *Acta Crystallogr*, 30 (1974) 178–186
21 A ALBERT AND E P SERJEANT *Ionization Constants of Acids and Bases, A Laboratory Manual*, Wiley, New York, 1962 pp 69—91
22 M M CRUTCHFIELD, C F CALLIS, R R IRANI, AND G C ROTH *Inorg Chem* 1 (1962) 813–817
23 A COSTELLO, T GLONEK, AND T C MYERS, *Carbohydr Res*, 46 (1976) 159–171
24 N NOMURA M KUWAYAMA, T ISHIGURO AND S MORIMOTO, *Chem Pharm Bull*, 19 (1971) 341–354
25 J R WAZER *Phosphorus and its Compounds*, Vol 1, Interscience, New York 1958, p 538
26 G W HAY, B A LEWIS, AND F SMITH, in W H SEBRELL, JR, AND R S HARRIS (Eds), *The Vitamins*, 2nd edn, Vol 1 Academic Press, New York, 1967, p 308
27 A D BOND, B W MCCLELLAND, J R EINSTEIN, AND F J FINAMORE, *Arch Biochem Biophys* 153 (1972) 207–214
28 C G MEAD AND F J FINAMORE, *Biochemistry*, 8 (1969) 2652–2655
29 C S VESTLING AND M C REBSTOCK, *J Biol Chem* 161 (1945) 285–291, F ARNDT, L LOEWE, AND E AYCA, *Chem Ber*, 85 (1952) 1150–1160
30 D L MACDONALD, in W PIGMAN AND D HORTON (Eds), *The Carbohydrates*, Vol IA, Academic Press, New York, 1972, pp 256–257
31 C S HANES AND F A ISHERWOOD, *Nature*, 164 (1949) 1107–1112
32 K G A JACKSON AND J K N JONES, *Can J Chem* 47 (1969) 2498
33 *Food Chemicals Codex*, 2nd edn, National Academy of Sciences, Washington, D C, 1972, pp 66–67