Synthesis and Properties of D-Erythroascorbic Acid and Its Vitamin C Activity in the Tobacco Hornworm (*Manduca sexta*)

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In a four-step synthesis of D-erythroascorbic acid (1), D-xylose was isomerized to a mixture of D-xylulose/D-xylose (8:2 w/w) using xylose isomerase in the presence of borate. After removal of borate, the syrupy mixture of sugars was acetonated to give mostly 2,3-*O*-isopropylidene-D-xylulose (5) plus some 1,2:3,5-di-*O*-isopropylidene-D-xylose. Compound 5, which was isolated in 70% crystalline yield by solvent extraction, was catalytically oxidized in aqueous solution at pH 8 to give 2,3-*O*-isopropylidene-β-D-*threo*-pentulosonic acid (6) in 85% yield. Finally, 6 was converted to 1 in methanolic hydrogen chloride in 64% yield. The overall isolated yield of 1 from D-xylose was 31%. L-Ascorbic acid (2) was slightly more readily oxidized than 1 at pH 4–8 by electrochemical oxidation at a glassy carbon electrode, as well as by O_2 in water. Compound 1 supported growth of *Manduca sexta* larvae but not ecdysis to pupae and adults.

INTRODUCTION

D-Erythroascorbic acid (1), which is the trivial name for D-*glycero*-2-pentenono-1,4-lactone, is closely related in structure to L-ascorbic acid (2) (Figure 1). D-Erythroascorbic acid occurs in yeasts and fungi which contain little or no L-ascorbic acid (Kim et al., 1993; Nick et al., 1986). Frank A. Loewus and his associates at Washington State University have postulated that 1 is catabolized to give oxalate originating from the C1 and C2 carbon atoms. Oxalate is known to accumulate in fungi, where it plays a role in pathogenesis. Evidence to support the hypothesis that 1 is the intermediary metabolite to oxalate was gained recently from an investigation on *Sclerotinia sclerotiorum*, a fungus that infects legumes, oil seeds, and leafy vegetables (Loewus, private communication).

The occurrence of 1 in yeast and fungi could also be important in the consideration of dietary sources of vitamin C for fin and shell fish (Masumoto et al., 1991), provided 1 has significant vitamin activity. If synthesized economically, D-erythroascorbic acid could replace certain food uses of the 6-carbon ascorbic acids (Bauernfeind, 1982).

The first synthesis of 1, which was reported by Yasuda (1969), began with the microbial fermentation (*Pseudomonas mildenbergii*) of D-xylose to give 88% D-*threo*-pentulosonic acid, which upon heating to 100 °C in concentrated hydrochloric acid under nitrogen produced 1 in 54% yield. In recent years, two chemical syntheses were reported (Liang et al., 1990; Gan and Seib, 1991). The starting material for both syntheses was D-glucose, and the overall yields were 11 and 20%. The objectives of this work were to develop an improved synthetic route to 1 and to determine the vitamin C activity of 1 using the tobacco hornworm, *Manduca sexta*, as the test animal (Kramer and Seib, 1982). The antiscorbutic activities of various compounds in the hornworm generally parallel those in the guinea pig, yet bioassay in *M. sexta* requires small quantities of a test substance.

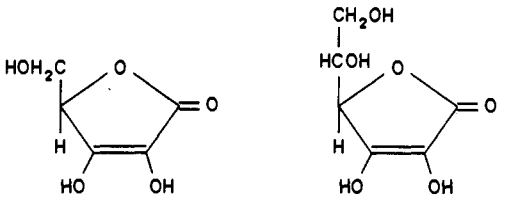

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Figure 1. D-Erythroascorbic acid (1) and L-ascorbic acid (2).

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of reagent grade unless otherwise stated. Norit (Fisher Scientific Co., Fair Lawn, NJ) was purified using the method of Mehltretter et al. (1951). D-Xylulose and 1,2-*O*-isopropylidene-D-xylofuranose were from Sigma Chemical Co., St. Louis, MO. Hydrogen hexachloroplatinate(IV) hydrate was from Aldrich Chemical Inc., Milwaukee, WI, and strongly acidic cation-exchange resin (Amberlite IR-120, H^+ form) from Mallinckrodt Co., Paris, KY. Ethyl acetate and acetone were dried over drierite and distilled. Immobilized xylose isomerase (Spezyme, IGI) was a gift from Genencor International Inc., Cedar Rapids, IA. *M. sexta* eggs were obtained from Dr. J. Buckner, Agriculture Research Service, U.S. Department of Agriculture, Fargo, ND, and the animals were reared according to procedures modified from those of Bell and Joachim (1976).

General Methods. All evaporations were done under reduced pressure below 45 °C. Melting points (uncorrected) were determined on a Fisher-Johns apparatus and optical rotations on a Perkin-Elmer 241 polarimeter (Perkin-Elmer Co., Norwalk, CT). Elemental analyses were done by Huffman Laboratories, Inc., Wheatridge, CO. Thin-layer chromatography (TLC) was performed at 25 °C on aluminum sheets coated with silica gel 60 F_{254} (Alltech Associates Inc., Deerfield, IL). Components were detected by spraying with a mixture of 5% each of ammonium molybdate, sulfuric acid, and phosphoric acid in water or 50% aqueous sulfuric acid followed by charring on a hot plate. Column chromatography was carried out at 25 °C on silica gel (100–200 mesh, Sigma). Ultraviolet (UV) spectroscopy was done using a Varian spectrophotometer (Model DMS-80, Varian Associates, Walnut Creek, CA), and extinction coefficients were determined in the presence of 0.2% dithiothreitol (DTT). Ionization constants were determined by the changes in inflections of curves of λ_{max} *vs* pH (Albert and Serjeant, 1962). Infrared (IR) spectra were recorded on a Perkin-Elmer 1310 infrared spectrophotom-

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eter. Mass spectra (MS) were obtained on a HP 59827A mass spectrometer (Hewlett-Packard Co., Palo Alto, CA) with a HP 59980B particle beam LC/MS interface and a HP 1050 liquid chromatograph equipped with a HP 005 Hypersil column (5 μm, 10 × 2.1 mm). Both ^1H (400 MHz) and ^{13}C (100.6 MHz) nuclear magnetic resonance (NMR) spectra were recorded in aqueous solutions on a Bruker WM-400 instrument (USA Bruker Instruments Inc., Billerica, MA). Chemical shifts were reported in δ values from the internal reference signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) was done on a Dionex system (Dionex Co., Marlton, NJ) equipped with a CarboPac PA1 column (250 × 4 mm) and CarboPac PA guard column (25 × 3 mm) (Shi and Seib, 1992). The column was developed isocratically with 150 mM sodium hydroxide containing 25 mM sodium acetate at 1 mL/min and 25 °C. High-performance liquid chromatography with electrochemical detection (HPLC-EC) was carried out as described by Wang et al. (1988).

D-Xylulose (4). Three reaction mixtures were prepared without sodium borate decahydrate or with 1.0 or 10.2 g (2.68 and 26.8 mmol) plus D-xylose (3) (10 g, 67 mmol), immobilized xylose isomerase (1 g), and water (200 mL). In the absence of borate, the reaction mixture was adjusted to pH 7–8 by adding 0.5 mL of 1 M sodium hydroxide. In the presence of 1.0 or 10.2 g of borate, the mixtures gave pH values 8.2 and 9.2, respectively. Each reaction mixture was stirred at room temperature, and aliquots (1 mL) were removed at once and after 5, 20, 27, and 96 h; the conversion to 4 was determined either by the cysteine–carbazole test (Slein, 1955) or by HPAE-PAD.

To prepare quantities of 4, water (200 mL), sodium borate decahydrate (6.4 g), D-xylose (3, 10 g), and immobilized isomerase (1 g) were stirred together for 2 days at 25 °C and pH 8.8. Immobilized enzyme was removed by filtration, and borate was removed (Zill et al., 1953; Schlesinger et al., 1953) by passing the filtrate through a column of strongly acidic cation-exchange resin in the H^+ form and repeatedly adding and evaporating methanol (3 × 100 mL). The product was a syrup (10.9 g) containing an 82:18 (w/w) mixture of 4/3.

2,3-*O*-Isopropylidene-β-D-xylulose (5). The method of Tipson and Brady (1969) was followed with modification. The mixture (10.9 g) that contained 8.2 g of D-xylulose and 1.8 g of D-xylose was dried to constant weight (10.0 g) over phosphorus pentoxide in a vacuum desiccator. Anhydrous cupric sulfate (20 g), dried acetone (200 mL), and concentrated sulfuric acid (0.2 mL) were added, and the mixture was shaken vigorously at 25 °C. After ~2 days, no starting material (R_f <0.1) and no 1,2-*O*-isopropylidene-α-D-xylose (R_f 0.45) were detected by TLC (chloroform/methanol, 6:1 v/v); the major product in the reaction mixture was compound 5 (R_f 0.6), and the minor product was 1,2:3,5-di-*O*-isopropylidene-α-D-xylofuranose (R_f 0.8).

The reaction mixture was filtered, and the filtrate was shaken with dry calcium hydroxide powder (12 g) for 1 h at 25 °C. The suspension was filtered and the filtrate added to a combination of barium carbonate (0.5 g) and magnesium perchlorate (2 g). After the mixture was shaken for 5 min, the salts were removed by filtration, and the filtrate was evaporated immediately to a syrup (14 g). The syrup was stirred with anhydrous ether (200 mL) for 10 min at 25 °C, and the insoluble material (0.4 g) was removed by filtration and discarded. The ether phase was extracted with water (5 × 20 mL) that contained traces of barium carbonate (0.2 g/100 mL). The combined aqueous washings, which contained the desired compound (5), were filtered to remove barium carbonate, and the solution was evaporated to a syrup (8.6 g). The water in the syrup was removed azeotropically using ethyl acetate (3 × 50 mL), and the dried syrup was held under reduced pressure (20–50 mmHg) for 2 days to afford a mass of colorless crystals. Recrystallization twice from ether–pentane gave 7.27 g (70%) of needles with mp 67–68 °C and $[\alpha]^{25}_D$ −9.0° (*c* 1.0, MeOH). Previously reported melting points were 74 (Tipson and Brady, 1956), 66–67 (Jarrell, 1978), and 67–68 °C (Hough and Theobald, 1962). Calcd for $C_8H_{14}O_5$: C, 50.52; H 7.36. Found: C, 50.29; H, 7.43.

2,3-*O*-Isopropylidene-β-D-*threo*-pentulosonic Acid (6). The isopropylidene derivative (5) (1.0 g, 5.26 mmol) was dissolved in water (30 mL) containing potassium bicarbonate (0.114 g) in a 100-mL, three-neck, round-bottom flask. A platinum on carbon catalyst (Mehltretter et al., 1951) (0.107 g) was introduced into the flask, and oxygen was bubbled through the reaction mixture with vigorous stirring at 50 °C. When the pH of the reaction mixture, which was monitored hourly, declined to below 7, potassium bicarbonate (0.114 g) was added. Generally, three additions of potassium bicarbonate (total 0.34 g) were required during the first 12 h. After 48 h, the reaction mixture was filtered through a glass-fiber filter pad, and the clear filtrate was evaporated to dryness. The crude product (1.5 g) was crystallized from methanol to give potassium 2,3-*O*-isopropylidene-β-D-*threo*-pentulosonate (87%) with melting point 263–265 °C. Prince and Reichstein (1937) reported mp 264–265 °C. The free acid (6) was generated from the potassium salt using a strongly acidic ion-exchange resin in the H^+ form. 2,3-*O*-Isopropylidene-β-D-*threo*-pentulosonic acid (6) was crystallized from ethyl acetate to give 85% of needles with mp 159–162 °C, $[\alpha]^{25}_D$ −18.04° (*c* 1.0, MeOH). Calcd for $C_8H_{12}O_6 \cdot {}^1/_2H_2O$: C, 45.07; H, 6.10. Found: C, 45.26; H, 5.89.

Methyl 2,3-*O*-Isopropylidene-β-D-*threo*-pentulosonate (7). Compound 6 (200 mg) was dissolved in 0.05 M hydrogen chloride in methanol at 25 °C. After 8 h, TLC (methanol/ethyl acetate/acetic acid, 1/4/0.05 v/v/v) showed that 6 (R_f 0.1) was converted to its methyl ester (7) (R_f 0.9). The mixture was evaporated to a solid, which was recrystallized from ethyl acetate: mp 64–66 °C; yield 180 mg (86%). Calcd for $C_9H_{14}O_6 \cdot {}^1/_2H_2O$: C, 47.57; H, 6.60. Found: C, 47.17; H, 6.46.

D-Erythroascorbic Acid (1). To determine optimum conditions to produce 1, 2,3-*O*-isopropylidene-β-D-*threo*-pentulosonic acid (6) (40 mg) was dissolved in dry methanol (5 mL) containing 0.05, 0.5, 1.0, or 2.5 M hydrogen chloride. The reaction mixtures were allowed to stand for up to 11 days either at 25 °C or at 25 and 4 °C in the case of 2.5 M HCl. Aliquots (0.5 mL) were withdrawn from a reaction mixture at intervals, and yields of 1 were measured with HPLC-EC. TLC using methanol/ethyl acetate/acetic acid (1/4/0.05 v/v/v) showed that at 25 °C compound 6 (R_f 0.1) was transformed to its methyl ester (7) (R_f 0.9) in less than 1 min, then more slowly to an unknown intermediate (R_f 0.5), and finally to 1 (R_f 0.3).

Preparative quantities of 1 were synthesized by adding 6 (1.0 g) to 2.5 M HCl in dry methanol (50 mL). The mixture was allowed to stand at room temperature for 4–5 days, at which time TLC showed no methyl ester (7) (R_f 0.9) and only traces of the unknown intermediate at R_f 0.5. The mixture was evaporated to a crystalline solid, which was recrystallized from hot acetonitrile. The needles (0.44 g, 64%) had mp 161–162 °C and $[\alpha]^{25}_D$ +12.5° (*c* 1.0, MeOH). Liang et al. (1990) and Gan and Seib (1991) reported mp 160–162 °C and $[\alpha]^{25}_D$ +12.5° (*c* 1.0, MeOH). Calcd for $C_5H_6O_5$: C, 41.10; H, 4.11. Found: C, 41.04; H, 4.29.

Cyclic Voltammetry of D-Erythroascorbic Acid (1) and L-Ascorbic Acid (2). Cyclic voltammetric studies of D-erythroascorbic acid (1) and L-ascorbic acid (2) were performed using a Bioanalytical Systems 100 B/W electrochemical analyzer. Voltammograms on 1.0 mM solutions of 1 and 2 in 0.5 M H_2SO_4 (pH 0.3), 0.1 M acetate buffer (pH 4.0), 0.1 M phosphate buffer (pH 6.2), and 0.1 M tris(hydroxymethyl)aminomethane (pH 8.0) were recorded using a glassy carbon working electrode. The oxidation peak potentials and peak currents were determined at a scan rate of 10 mV/s, and potentials are reported *vs* a Ag/AgCl (0.1 M KCl) reference electrode. Peak heights were measured at scan rates of 10–200 mV/s, and plots of peak height *vs* the square root of scan rate were constructed.

Stabilities of D-Erythroascorbic Acid (1) and L-Ascorbic Acid (2) in Aqueous Solution. Solutions (4 mM, 150 mL) of 1 and 2 were prepared in 0.1 M sodium phosphate buffers at pH values 4, 6, and 8 in open-topped Erlenmeyer flasks (250 mL). The flasks were placed in constant temperature baths at 25, 35, and 45 °C and stirred rapidly; aliquots (3 mL) were removed at various times. The decline in UV absorbance at 258 (at pH 4) or 264 nm (at pH 6–8) was determined to follow losses of 1 and 2.

Bioassay of D-Erythroascorbic Acid for Vitamin C Activity Using *M. sexta*. Bioassays for vitamin C activity were done using *M. sexta* as the test animal (Shao, 1992; Kramer et al., 1978). Larvae were fed a gelled diet containing 0.5 mM 1 (EAA) or 2 (AsA) in formula diet 2 described by Shao (1992). None of the components found in the diet contained 2 above the

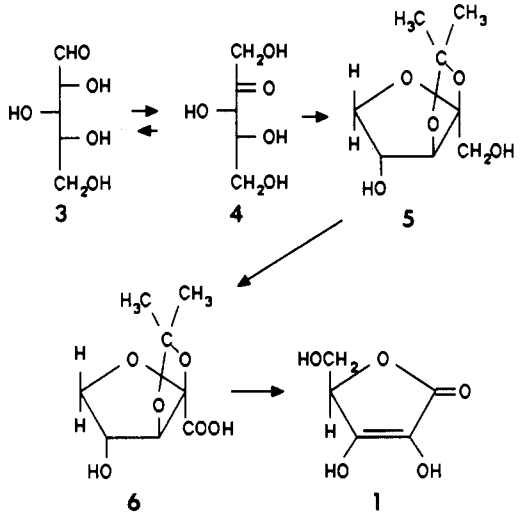

Figure 2. Scheme to prepare D-erythroascorbic acid (1) from D-xylose (3).

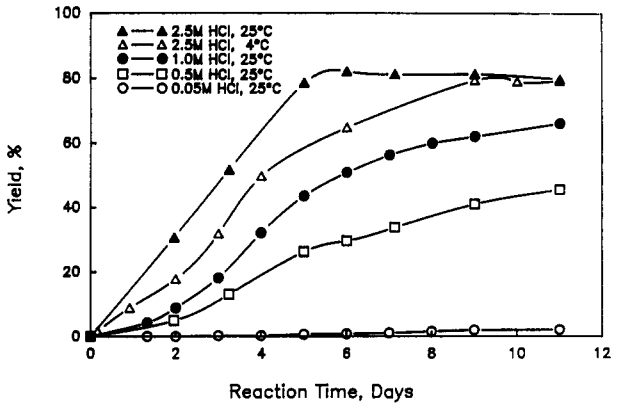

Figure 3. Conversion of 2,3-*O*-isopropylidene-β-D-*threo*-pentulosonic acid (6) to D-erythroascorbic acid (1) in methanolic hydrogen chloride determined by HPLC-EC.

detection limit of the HPLC-EC assay (~3.5 ppm). After eggs were hatched, two groups of 20 neonate larvae each were fed with diets containing 1 and 2. Another 92 neonate larvae were initially fed the blank diet (no added vitamin C). On the third day of feeding, 20 larvae from the blank diet were placed on the diet containing 1 (group EAA-R3), and another 20 larvae were placed on the diet containing 2 (AsA-R3). On the sixth day of feeding, 16 larvae from the blank diet were placed on the diet containing 1 (EAA-R6) and another 16 larvae on the diet containing 2 (AsA-R6). The remaining 20 larvae continued to be fed with the blank diet. Bioassay data were analyzed using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS, 1985).

RESULTS AND DISCUSSION

Synthesis of D-Erythroascorbic Acid (1). The four-step scheme to prepare D-erythroascorbic acid (1) starting from D-xylose (3) is similar to the one used commercially to synthesize L-ascorbic acid (2) from L-sorbose (Crawford and Crawford, 1980).

Past investigations have shown that the transformation of D-xylose (3) to D-xylulose (4) can be achieved by chemical isomerization in hot pyridine (Tipson and Brady, 1969; Ramchander and Feather, 1977; Morgenlie, 1982) or in hot alkali with borate (Mendicino, 1960). Alternatively, 4 has been prepared by enzyme-catalyzed isomerization using xylose isomerase (Hochster and Watson, 1953; Mitsuhashi and Lampen, 1953; Slein, 1955). Base-catalyzed isomerization yielded, at most, 42% of 4 in hot alkali/borate (Mendicino, 1960), whereas isomerization catalyzed by xylose isomerase in the presence of borate gave 81.5% of 4 (Hochster and Watson, 1953). In this work, a commercial immobilized enzyme converted D-xylose (5–10 wt %) in 27 mM borate at pH 9.2 into 84% of 4 after 30 h at 25 °C. In 2.7 mM borate at pH 8.2, the yield of 4 was 70% after 96 h at 25 °C. But in the absence of borate, the yield of 4 reached only 21% after 24 h and then remained constant. The level of 4 in reaction mixtures was determined either by the cysteine carbazole test or by HPLC-EC; both assays gave almost identical results.

In the second step of the synthesis (Figure 2), D-xylulose (4) was acetonated to its 2,3-acetal (5). The method of Tipson and Brady (1969) was followed, except that the starting material was an 8:2 (w/w) mixture of D-xylulose/D-xylose, rather than pure D-xylulose. In spite of the impure starting material, the acetal (5) was readily isolated in crystalline form in 70% yield. Starting with the mixture of 3 and 4 avoided column chromatographic purification of 4, as was done by previous authors (Tipson and Brady, 1969; Ramchander and Feather, 1977).

In the next step of the synthesis (Figure 1), the free primary alcohol group on 5 was oxidized to a carboxyl group. Oxidation of 5 with alkaline potassium permanganate gave only 12% 2,3-*O*-isopropylidene-β-D-*threo*-pentulosonic acid (6), in agreement with the 14% yield previously reported by Prince and Reichstein (1937). The selectivity of the oxidation reaction was much improved by catalytic O_2 oxidation of 5 using platinum/carbon in a slightly alkaline medium (Trenner, 1947; Mehltretter et al., 1951; Marsh, 1951; Overend et al., 1954). Catalytic oxidation of 5 yielded 85% 6. Occasionally, though, the catalyst had reduced activity and yields were low. Trenner (1947) reported the catalytic O_2 oxidation of 2,3-*O*-isopropylidene-L-sorbofuranose to 2,3-*O*-isopropylidene-L-*xylo*-hexasaccharic acid in 85% yield.

The 2,3-*O*-isopropylidene group on 6 showed unusual stability toward aqueous acid, probably because of the electron-withdrawing effect of the neighboring carboxyl group. Because the acetal group in 6 is stable to acid, it was possible to crystallize 6, in spite of its containing a free carboxylic acid group.

In the final step of the scheme (Figure 2), compound 6 was converted to the desired D-erythroascorbic acid (1) by acid-catalyzed methanolysis. Exposure of 6 to 2.5 M hydrogen chloride in methanol for 5 days at 25 °C gave 82% 1 as determined by HPLC-EC (Figure 3). From the same reaction, the isolated yield of crystals of 1 was 64%. Conversion of 6 to 1 was decreased when 6 was held in 0.5 or 1.0 M methanolic hydrogen chloride at 25 °C for 11 days (Figure 3).

The first step in the conversion of 6 to 1 is rapid esterification of the carboxyl group on 6 to produce its methyl ester (7), followed by slow methanolysis of the isopropylidene group. The 4-OH then probably attacks the methyl ester to form a lactone ring, and the 2-keto-1,4-lactone tautomerizes to 1. The methyl ester intermediate (7) in fact, was isolated in 85% yield when 6 was reacted in 0.05 M methanolic hydrogen chloride, and the ester (7) was shown by thin-layer chromatography to be an intermediate in the conversion of 6 to 1. Moreover, 7 was converted to 84% 1 after 5 days in 2.5 M hydrogen chloride in methanol as determined by HPLC-EC.

The structures of the intermediates (5–7) and the desired product (1) were verified by ^1H and ^{13}C NMR spectra (Tables I and II). The mass spectrum (electron impact) of 1 also agreed with its assigned structure (Shao, 1992). Compound 1, as well as 2, gave weak molecular ions but much more intense M + 1 ions, apparently due to protonation of the molecular ion.

The scheme reported here to synthesize 1 is an improvement over other chemical methods (Liang et al., 1990;

Table I. ^1H NMR Chemical Shifts[a] of Intermediate Compounds, D-Erythroascorbic Acid (1), and L-Ascorbic Acid (2) in D_2O at pH 7.0–7.5

compd	H-1 H-1′	H-3	H-4	H-5 H-5′	methyls of isopropylidene
5	3.73d	4.52s	4.34d	3.98d	1.40s
	3.80d			4.22dd	1.51s
6		4.69s	4.30d	4.04d	1.42s
				4.20dd	1.51s
7[b]		4.91s	4.46d	4.16d	1.42s
				4.33dd	1.53s
1[c]			4.89dd	3.89dd	
				4.03dd	
2[d]			4.97d	4.09ddd	

[a] Chemical shifts in ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Multiplicity of peak indicated by s, singlet; d, doublet; and dd, doublet at doublets. [b] The proton on the methyl ester group was at 3.96 ppm. [c] Data in agreement with those previously reported (Liang et al., 1990). [d] Chemical shift of H-6 and H-6′ was 3.76 ppm, which was the center of the recorded multiplet. Data taken from Lee et al. (1978).

Table II. ^{13}C NMR Chemical Shifts[a] of Intermediate Compounds, D-Erythroascorbic Acid (1), and L-Ascorbic Acid (2) in D_2O at pH 7.0–7.5

compd	C-1	C-2	C-3	C-4	C-5	isopropylidene methine	isopropylidene methyl
5	64.8	115.6	87.0	76.1	76.8	117.2	28.3
							29.0
6	176.0	114.6	89.4	77.0	76.9	116.3	27.8
							28.7
7[b]	176.0	112.6	90.0	77.6	76.4	117.8	27.4
							28.5
1[c]	180.0	115.7	178.0	82.4	63.5		
2[d]	178.0	114.1	176.2	79.2	70.6		

[a] Chemical shifts in ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). [b] Methyl group on ester at 56.4 ppm. [c] Data in agreement with those previously reported (Liang et al., 1990). [d] The signal for C-6 on 2 was at 63.6 ppm. Data from Lee et al. (1978).

Gan and Seib, 1991). The scheme gave the highest overall yield of 31% starting from D-xylose, compared to 10–20% starting from D-glucose. However, it should be noted that Yasuda (1969) patented a fermentation/chemical method to convert D-xylose to 1 in 48% yield. Our new scheme requires no chromatography to purify intermediate reactants and no high-temperature/pressure conditions, which were necessary in the past (Liang et al., 1990; Gan and Seib, 1991).

Because D-glucose can be converted (Sowden, 1951) (60% yield) to D-xylose, which is the starting material used here, the present scheme provides an alternate route to isotopically labeling 1 starting from D-glucose. In the new scheme, the 6-methylol group of D-glucose is lost, whereas in the former schemes (Liang et al., 1990; Gan and Seib, 1991) the 1-formyl group of D-glucose is lost during the synthesis of 1. Isotopically labeled 1 would be useful in metabolic investigations.

Properties of D-Erythroascorbic Acid. Compound 1 was found to have $pK_1 = 4.0$ and $pK_2 = 11.6$. Those values are similar to $pK_1 = 4.2$ and $pK_2 = 11.8$ of 2 (Crawford and Crawford, 1980). Both 1 and 2 showed identical UV properties at acidic or neutral pH (Table III).

The infrared spectrum of D-erythroascorbic acid (1) showed a strong broad band at 1660 cm^{-1} and a strong sharp band at 1740 cm^{-1}, which were assigned to C=C and C=O (lactone) stretching, respectively. The corresponding bands in 2 were reported at 1680 and 1745 cm^{-1}, respectively (Weigl, 1952; Lohmann et al., 1984). Interestingly, the OH stretch region of 1 showed a single but broad and strong band centered at 3300 cm^{-1}. On the other hand, four bands were well resolved in the OH stretch region of 2, which have been assigned to its 6-OH (3520 cm^{-1}), 3-OH (3400 cm^{-1}), 5-OH (3200 cm^{-1}), and 2-OH (3202 cm^{-1}) (Lohmann et al., 1984). It is not clear why the OH bands in 1 were not resolved, whereas those in 2 were. L-Ascorbic acid (2) contains four molecules in its unit cell, and the interatomic distances and angles are almost identical in each molecule. Thus, the hydroxyls in crystalline 2 repeat regularly throughout the structure (Hvoslef, 1968). The crystal structure of 1 has not yet been determined.

Table III. UV Spectral Properties of D-Erythroascorbic Acid (1) and L-Ascorbic Acid (2) in Aqueous Media

	pH 2		pH 7		pH 10	
compd	λ_{max}	ϵ_{mM}	λ_{max}	ϵ_{mM}	λ_{max}	ϵ_{mM}
1	243	10	265	16.5	265	
2	243	10	265	16.5	265	

Table IV. Cyclic Voltammetry of D-Erythroascorbic Acid (1) and L-Ascorbic Acid (2) Using a Glassy Carbon Electrode and a Scan Rate of 10 mV/s

	peak potential, V		peak current, μA	
pH	1	2	1	2
0.3[a]	0.48	0.60	8.1	6.5
4.0	0.39	0.25	10.3	9.2
6.2	0.41	0.25	7.2	7.4
8.0	0.45	0.20	6.5	7.5

[a] The solution 1.0 N H_2SO_4 was reported to have pH of 0.3 (Robinson, 1985). The other pH values were measured with a pH meter.

The electrochemical properties of 1 were compared to those of 2 at several pH values using cyclic voltammetry with a glassy carbon electrode. Anodic peak potentials were measured using a scan rate of 10 mV/s. The cyclic voltammographic data (Table IV) of 1 and 2 indicate that at pH values of 4.0, 6.2, and 8.0 compound 2 was easier to oxidize than 1 by 0.15 V (±0.01 V). Surprisingly, at pH 0.3, 1 was easier to oxidize than 2. Examination of Table IV reveals that the oxidation of 2 at pH 0.3 was shifted anodically by over 0.3 V compared to higher pH data, whereas the potential of 1 was relatively unaffected by decreasing pH to a value of 0.3. The higher peak potential of 2 in 1 N sulfuric acid may be due to a large overpotential, i.e., slow electron transfer from L-ascorbic acid to the carbon electrode. The oxidation potentials varied little between pH 4 and 8 for compounds 1 and 2.

The peak oxidation currents (Table IV) for 1 and 2 at a given pH were similar and ranged between 6.5 and 10.30 μA. Because it is established that 2 undergoes a two-electron transfer during oxidation (Weis, 1975; Liao and Seib, 1988; Sharma and Kumbhat, 1990), it can be concluded that 1 also undergoes two-electron oxidation.

Oxidations of 1 and 2 at all pH values were chemically irreversible, which indicated very rapid reaction of the first oxidation product (the dehydro derivatives of 1 or 2), probably to give 2,3-diketo-D-xylonic acid (D-*glycero*-penta-2,3-diulosonate) or 2,3-diketo-L-gulonic acid (D-*threo*-hexa-2,3-diulosonate). Even at a scan rate of 5000 mV/s, no dehydro product was observed from 1 and 2. However, oxidation of 2 in DMF was reversible (Sawyer et al., 1982). During the electrochemical oxidation of compound 1 or 2, a linear relationship was observed between peak current and the square root of the scan rate over the range of scan rates from 10 to 200 mV/s (data not shown). Those results indicated a diffusion-controlled oxidation of 1 and 2 at the electrode.

In aqueous solutions at three temperatures and three pH values, compound 1 was slightly more stable to O_2

Table V. Half-Lives of D-Erythroascorbic Acid (1) and L-Ascorbic Acid (2) in Phosphate Buffer (0.1 M) under Aerobic Conditions at 25, 35, and 45 °C

pH	temp, °C	half-life ($t_{1/2}$), h		
		1	2	$t_{1/2}$ of 1/$t_{1/2}$ of 2
4	25	55.01	44.45	1.24
	35	18.13	11.0	1.65
	45	4.08	3.36	1.22
6	25	19.08	13.15	1.45
	35	11.14	5.89	1.89
	45	4.03	2.02	2.00
8	25	59.47	49.84	1.19
	35	20.37	14.43	1.41
	45	6.89	5.15	1.34

oxidation than 2 (Table V). The magnitude of the ratio of the half-lives of 1 to 2 ranged between 1.2 and 2.0. These results for 2 agreed with previous data, which showed that 2 was more stable at pH 4 and 8 than at pH 6 (Liao and Seib, 1990). Compound 1 behaved similarly. Oxidation of 2 is known to proceed through the L-ascorbate monoanion, and at pH 4, ionization is partially suppressed. The oxidation reaction is also known to be mediated by copper and ferric ions, which are probably more highly chelated by the phosphate ions in the buffer at pH 8 than at pH 4 or 6.

Effect of D-Erythroascorbic Acid on Growth and Development of *M. sexta*. Larvae fed a diet devoid of 2 for 2 days and then rescued on the third day with the 0.5 mM AsA diet (group AsA-R3) grew to the same size as control animals (group AsA), although their development was delayed by several days (Figure 4). Larvae rescued with the diet containing 0.5 mM 1 (group EAA-R3) or fed the EAA diet from the beginning of the experiment (group EAA) were somewhat smaller than those fed the AsA diet. Insects fed the blank diet (group no-AsA) grew most slowly to 13 days, and those rescued after 6 days on either the AsA or EAA diet also grew slowly relative to AsA and AsA-R3 groups (Figure 4).

After the body-wetting stage, the percentages of live larvae in the various groups were EAA, 85%; EAA-R3, 85%; AsA, 95%; AsA-R3, 90%; and EAA-R6, AsA-R6, and blank, 0%. The slopes of the growth curves shown in Figure 4 were similar for the EAA and EAA-R3 groups and the AsA and AsA-R3 groups. However, the percentages of animals that transformed into pupae and adults were less than 5% for EAA and EAA-R3 groups (Table VI). Moreover, half of the pupae that did develop were deformed. Thus, EAA supported larval growth of *M. sexta* but not larval–pupal ecdysis or pupal–adult ecdysis. It appears ascorbate-type compounds may have both larval growth-supporting activity and insect-transformation activity, i.e., larva to pupa to adult. In the 6-carbon analogs of L-ascorbic acid (Kramer and Seib, 1982), D-ascorbic, D-isoascorbic, and L-isoascorbic acids were found to have 40, 10, and 0%, respectively, of activity relative to L-ascorbic acid, on the basis of ecdysis of pupae to adult. We estimate that D-erythroascorbic acid exhibited about 90% of the activity required for larval growth but less than 5% for the transformation of larvae to pupae relative to L-ascorbic acid.

In all tests, larvae appeared to be normal until the second instar (day 3–4), perhaps because they contained a pool of L-ascorbic acid in their eggs obtained maternally. In larvae on diets lacking vitamin C activity in the present study, pathological consequences occurred at the third instar (day 5). The larvae began to shrivel and had a pale yellow-green color. They had difficulty in molting and exhibited mouth parts and tarsi that were abnormally dark.

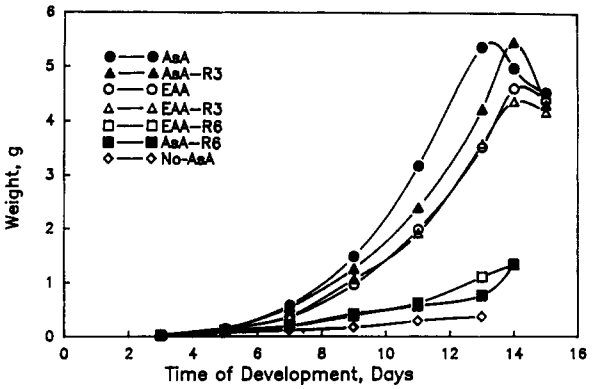

Figure 4. Growth of *M. sexta* fed a diet containing 0.5 mM L-ascorbic acid (AsA), 0.5 mM D-erythroascorbic acid (EAA), blank diet for days 1–2 followed by rescue with 0.5 mM AsA (AsA-R3) and EAA (EAA-R3), blank diet for days 1–5 followed by 0.5 mM AsA (AsA-R6) and EAA (EAA-R6), or blank diet (no-AsA).

Table VI. Proportion of Insect Larvae (*M. sexta*) Transformed to Pupae and/or Adults on Diets Containing 0.5 mM D-Erythroascorbic Acid or L-Ascorbic Acid

insect group	no. of larvae	mean weight before pupation,[a] g	no. of pupae (%)	no. of adults (%)
AsA	20	2.57 a	19 (95)	17 (85)
AsA-R3[b]	20	2.31 b	18 (90)	11 (55)
AsA-R6[b]	16	0.46 de	0 (0)	0 (0)
EAA	20	2.01 c	5 (25)	0 (0)
EAA-R3[b]	20	1.90 c	5 (25)	1 (5)
EAA-R6[b]	16	0.51 d	0 (0)	0 (0)
blank	20	0.19 e	0 (0)	0 (0)

[a] Different letters in a column show statistically significant differences in larval weight at $P < 0.05$. [b] R3 and R6 indicate larvae fed blank diet from day 1 to day 2 and from day 1 to day 5, respectively, and then transferred to diet containing AsA or EAA.

Finally, the insects became moribund. These observations confirm previous reports about the pathology of L-ascorbate-deficient insects (Kramer et al., 1978; Kramer and Seib, 1982; Navon et al., 1985). Compounds 1 and 2 did not rescue larvae fed a diet lacking vitamin C for 5 days (Figure 4) because symptoms in pathological abnormalities had already occurred at that time.

It was previously reported (Dadd, 1985) that vitamin C deficiency in insects frequently involves abnormalities at ecdysis, indicating faulty control over phenol oxidase-dependent processes in cuticle tanning. L-Ascorbic acid, as a reducing agent, can inhibit the catechol oxidase activity of tyrosinase and favor instead its tyrosine hydroxylase activity (Hopkins and Kramer, 1992). The absence of L-ascorbic acid apparently permits premature oxidation of metabolites, which causes an abnormally dark cuticle appearance.

Since EAA is almost as readily oxidized as AsA in an aqueous system, it should have reducing power similar to that of AsA. A possible cause for the low degree of pupation (~5%) brought about by EAA may be the result of a more rapid metabolism relative to L-ascorbic acid, its more rapid excretion, or its lower level of storage in tissues where cuticle development occurs.

Conclusions. D-Erythroascorbic acid can be synthesized in four steps from D-xylose in 31% yield. The intermediates in the scheme are readily isolated in crystalline form without the use of chromatographic methods. D-Erythroascorbic acid is slightly more stable than L-ascorbic acid to oxidation. Bioassay for vitamin C activity in *M. sexta* showed D-erythroascorbic acid supported well the growth of the hornworm but did not facilitate larval–pupal transformation.

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