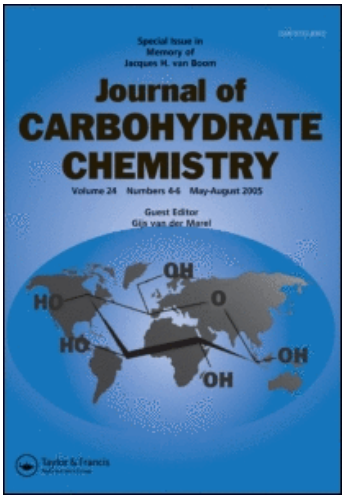

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CHARACTERIZATION OF QUINOXALINE DERIVATIVES OF DEHYDRO-D-ERYTHROASCORBIC ACID

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ABSTRACT

The quinoxaline derivatives formed between dehydro-D-erythroascorbic acid (**2**) and *o*-phenylenediamine (**3**) were separated by preparative HPLC and their structures were analyzed by HPLC-MS, UV-vis spectrophotometry and ^{1}H NMR spectroscopy. The reaction of **2** with an excess amount of **3** in 5% aq *m*-phosphoric acid gave three different products, depending on the concentration of **2**: below 0.1 mM of **2**, only 3-(D-*glycero*-1,2-dihydroxyethyl)quinoxaline-2-carboxylic γ-lactone (**4**) was produced, between 0.1 to 5 mM of **2**, another product, 2,2'-anhydro-[2-hydroxy-3-(D-*glycero*-2,3-dihydroxypropanal-1-yl)quinoxaline] (**5**) was formed as well as **4**, and over 10 mM of **2**, the third product, 2,1'-anhydro-[2-hydroxy-4-(D-*glycero*-1,2-dihydroxyethyl)-1,5-benzodiazepin-3-one] (**6**) was formed as well as **4** and **5**, with an overall production yield over 95%. Quinoxaline **6** was slowly converted to **4** via **5**. Based on these results, it was concluded that all three products retain the lactone ring moiety of **2**, and the most stable product is **4**. Compounds **5** and **6** were produced with higher concentration of **2**, but they were unstable and slowly converted to **4** in aqueous solution. A possible mechanism for this conversion was proposed.

INTRODUCTION

D-Erythroascorbic acid, which is the trivial name for D-*glycero*-2-pentenono-1,4-lactone, has been predicted as an intermediate produced during the oxidative degradation

of L-ascorbic acid (ASC) in chemical and biological systems.[1] D-Erythroascorbic acid (**1**) has been found recently as an analogue of ASC in *Candida* species, *Saccharomyces cerevisiae* and *Lypomyces starkeyi*.[2-4] On the other hand, it has been reported that L-erythroascorbic acid, the enantiomer of **1**, is produced during the catabolic pathway of ASC by purple sulfur bacteria,[5] and nonenzymatically by oxidative degradation of ASC via an α-ketoaldehyde, L-xylosone.[1] Studies of erythroascorbic acid in biological systems have focused on the occurrence or biosynthesis of erythroascorbic acid in relation to ASC. Its biological function could be explained in terms of a redox system between **1** and its oxidized form, **2**, like the redox system of ASC.[6] D-Erythroascorbic acid may be oxidized to **2** by ascorbate oxidase (E.C. 1.10.3.3.), which can catalyze the oxidation of enediol-containing compounds, such as ASC, erythorbic acid, and reductic acid.[1,7] However, the characteristics of **2** have not yet been reported. Although it is difficult to detect the native unstable form of **2** in aq solution, it can be monitored spectroscopically after quinoxaline derivatization with *o*-phenylenediamine (**3**), as was dehydro-L-ascorbic acid (DHA).[8-11] In the case of DHA, various quinoxaline derivatives are formed, depending on the reaction conditions and the ratio of the reactants. Recently, the condensation reactions of the 5-epimer of DHA with **3** have been also described.[12,13] In the present paper, the structures of quinoxaline derivatives prepared between **2** and **3**, are elucidated and a possible condensation pathway is proposed.

RESULTS AND DISCUSSION

Ascorbate oxidase catalyzes the oxidation of enediol-containing compounds like ASC,[1,6,14] and the oxidation product of ASC, DHA readily reacts with an excess of *o*-phenylenediamine (**3**) to give quinoxaline derivatives.[10] Likewise, a solution of **2**, prepared by the oxidation of **1** with ascorbate oxidase, reacted with **3** to produce quinoxaline derivatives. The reaction was complete after 40 min at 40 °C and more than 95% of the derivatives were extracted with ethyl acetate.

When the methanolic solution of the condensation products between **3** and the reaction product of **1** (>10 mM) by ascorbate oxidase was applied to HPLC-MS analysis, three peaks were found at scan numbers of 190, 358 and 456 using the total-ion-current

Table 1. HPLC-MS data of the three quinoxaline derivatives of **2**

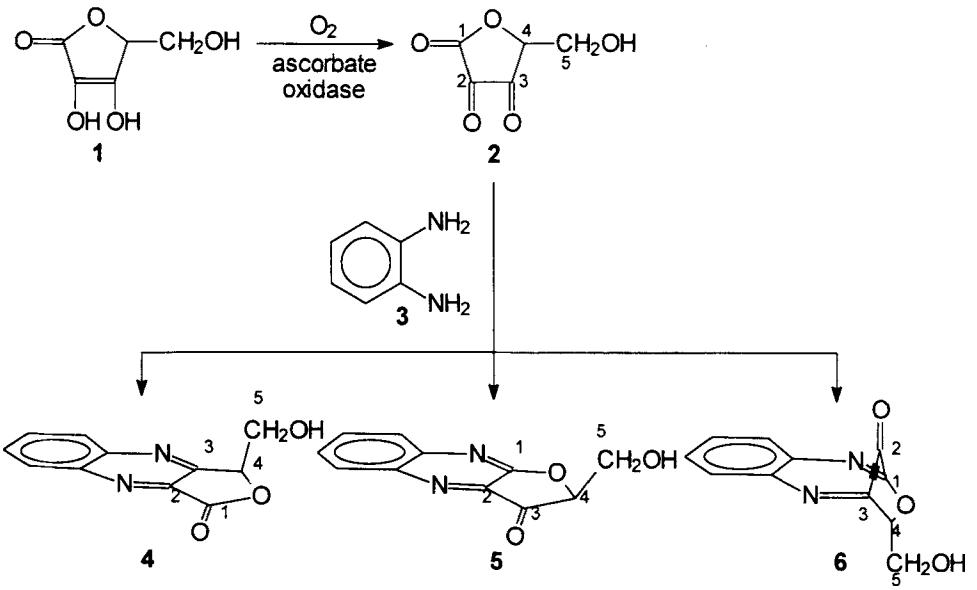
m/z	Assignments	Relative Abundance (%)		
		4	**5**	**6**
216	M^+	6.57	13.66	8.91
200	M-O	<1.00	1.27	3.39
198	M-H_2O	28.41	44.69	29.65
186	M-CHOH	100.00	100.00	100.00
170	186-O	6.03	7.69	7.22
158	186-CO	36.87	56.64	40.12
156	186-CH_2O	1.59	1.84	10.95
142	158-O	22.10	23.23	40.50
130	158-CO	35.35	50.00	51.16
129	158-CHO	78.79	81.42	69.77
115	142-HCN	4.39	4.98	7.66
103	130-HCN	17.17	25.66	25.39
102	129-HCN	18.81	18.47	23.64

detection mode. All three compounds showed the same fragmentation patterns with slight differences only in their relative peak intensities in MS (Table 1). Thus, the assignment of the structures of the three quinoxaline derivatives was made on the basis of relative abundance and the presence or absence of ion peaks of some fragments. In all mass spectra of the three peaks, the base peak and the molecular ion (M^+) peak are *m/z* 186 and 216, respectively. *m/z* 186 corresponds to the mass of the structure in which only the side chain is detached from the molecular ion. These results suggest that the reaction product of **1** by ascorbate oxidase is **2** and all three condensation products are monoquinoxaline derivatives retaining the lactone ring moiety. This result is different from that of previous work with the oxidized forms of 6-carbon reductones, in which the opening of the lactone ring was involved in the major pathway of the condensation process and only one type of derivative retaining the lactone moiety was reported as a minor product.[8-13]

There are three carbonyl groups in **2** and two *ortho*-amino groups in **3**. Therefore, the reaction can yield three different quinoxalines. Thus three peaks in the HPLC-MS analysis can be referred to as isomers, each with a different condensation site. It is

thought that the order of the electrophilicities of the three carbonyl carbons in **2** is C-2 > C-1 > C-3. Thus, the relatively abundant product might be made from condensation between **3** and C-1 or C-2 carbonyl groups in **2**. The steric consideration also supports this possibility because the side group in **2** is farthest from the ring system in this configuration. When an oxygen atom is lost from the molecular ion, the peak at *m/z* 200 (M^+ - 16) is produced. The production of the peak at *m/z* 200 was pronounced in the scan number 190 product in comparison to the others, which suggests that the scan number 190 has the structure in which the oxygen in the remaining keto group is more easily lost by electron bombardment than is the oxygen in the other products. Thus, it is possible that the scan number 190 product results from condensation between **3** and C-1 or C-3 carbonyl groups of **2** and is 2,1'-anhydro-[2-hydroxy-4-(D-*glycero*-1,2-dihydroxyethyl)-1,5-benzodiazepin-3-one] (**6**). In the MS of scan number 358, the intensity of *m/z* 158 was the highest of all three peaks. These results suggest that the compound of scan number 358 has a structure in which the lactone ring moiety of **2** is relatively easily eliminated and, especially, that the carbonyl group is most easily lost from the ring of *m/z* 186 on electron bombardment. Considering the fact that the ratio of *m/z* 142 to *m/z* 158 in scan number 456 (0.6) is much higher than that of scan number 358 (0.4), the oxygen in scan number 456 might be linked directly to the carbonyl group. From these considerations, it is concluded that the compounds of scan numbers 358 and 456 in the HPLC-MS analysis can be referred to 2,2'-anhydro-[2-hydroxy-3-(D-*glycero*-2,3-dihydroxypropanal-1-yl)quinoxaline] (**5**) and 3-(D-*glycero*-1,2-dihydroxy-ethyl) quinoxaline-2-carboxylic γ-lactone (**4**), respectively (Scheme 1).

To confirm this conclusion, the three quinoxaline derivatives were purified by means of preparative HPLC. The methanolic solution of quinoxaline derivatives gave only three peaks in 14.20, 19.05 and 22.08 min, respectively, when recorded at 320 nm (Fig. 1). These corresponded to the peaks of scan numbers 190, 358 and 456, respectively, in HPLC-MS analysis. These compounds had typical UV-vis spectra of quinoxaline derivatives in distilled water and their λ_{max}'s for n-π^* transitions were 321.2, 323.5, and 328.7 nm, respectively (Fig. 2). Absorption spectra of retention time (t_R) 19.05 (**5**) and 22.08 (**4**) min were very similar to each other and to that of DHA. This result



Scheme 1

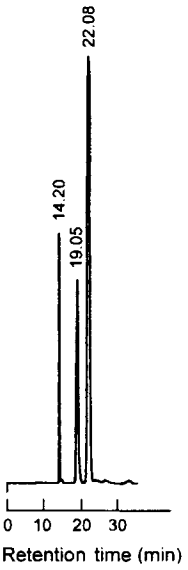


Figure 1. High-performance liquid chromatogram of quinoxaline derivatives of **2**.

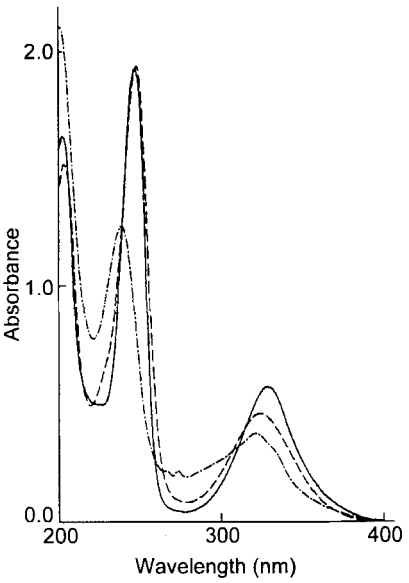


Figure 2. UV-scanning spectra of quinoxaline derivatives of **2** in distilled water. Solid, dashed and chain line represent absorption spectra of the peaks at t_R 22.08, 19.05 and 14.20 min in **Figure 1**, respectively.

indicates that the compounds have common ring structures, which is consistent with the results from the HPLC-MS experiments. However, the spectrum of t_R 14.20 (**6**) is slightly blue-shifted, especially the absorption peak of π-π^* transition (238.5 nm), when compared with the others (246.5 and 248 nm for **4** and **5**, respectively). This shift may originate from the greater ring strain in the structure of **6** than the other compounds.

The condensation reaction gave different products according to the concentration of **2**. When the concentration of **2** was lower than 0.1 mM, the reaction produced only one product, **4**, at t_R 22.08 min in HPLC. However, when **2** of a concentration between 0.1 and 5 mM reacted with an excess of **3**, two peaks at t_R 19.05 and 22.08 min were recorded. At a concentration range between 10 and 50 mM of **2**, all three products appeared on the HPLC chromatogram. The yields of three condensation products **4**, **5** and **6** were 72, 22, and 6% of the total amount of quinoxalines, respectively.

Moreover, purified **6** was found to be gradually converted to **4** and **5**, and purified **5** to **4** in aq solution, which was corroborated by HPLC analysis. These facts were also

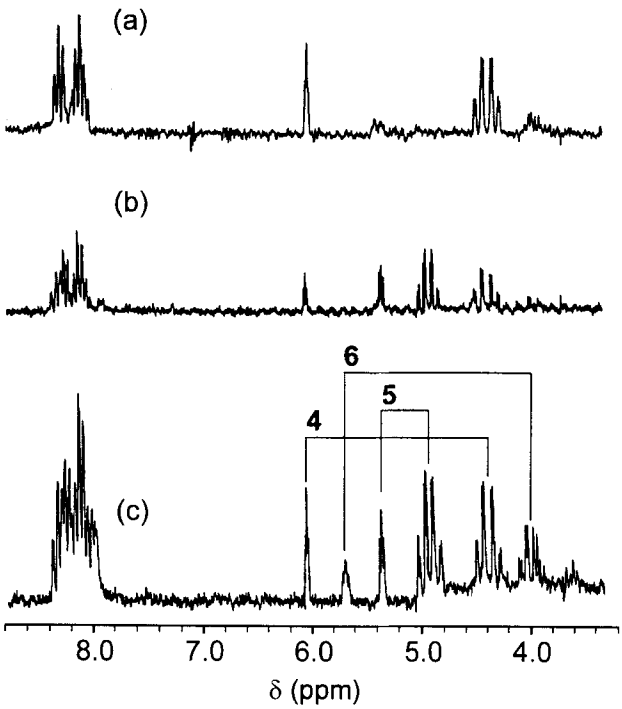
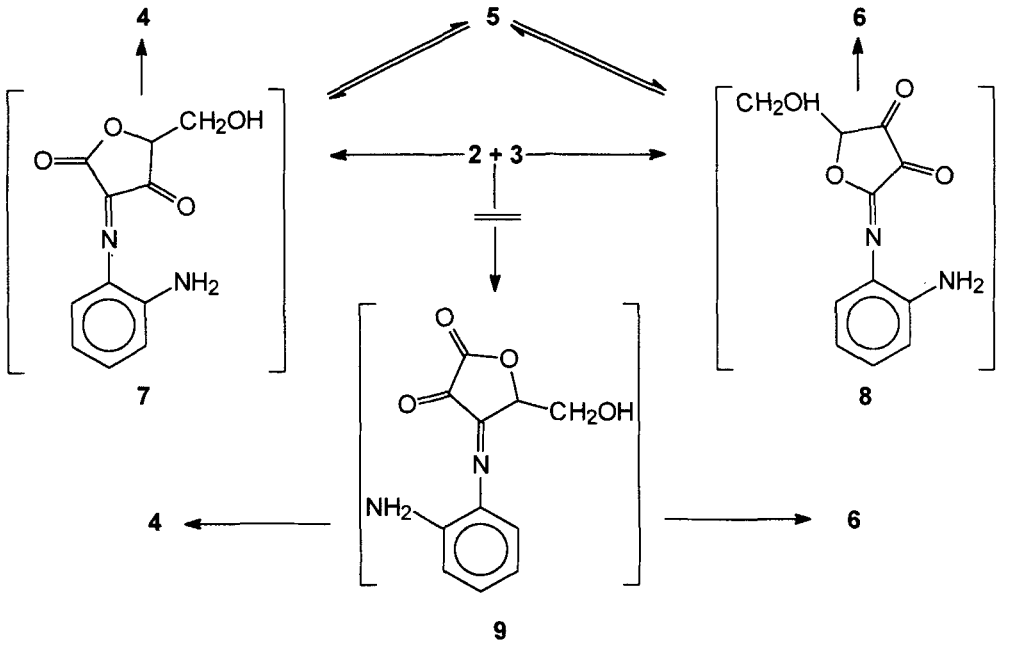


Figure 3. ^{1}H NMR spectra of quinoxaline derivatives of **2**. (a), (b) and (c) are the spectra of **4**, **5** and **6**, respectively, incubated in D_2O at room temperature for 20 h.

confirmed by a ^{1}H NMR experiment. When the 20 h-incubated solutions of the isolated **4**, **5** and **6** in D_2O were subjected to NMR spectroscopic analysis, the proton signals for **4** and **5** appeared among the signal of **6**, and the signal for **4** appeared among the signal of **5** (Fig. 3).

The protons bonded to C-4 and C-5 of **4** showed their ^{1}H NMR signals at the slightly downfield [δ 4.30 (dd, 1H, H-5; $J_{4,5}$=3.42 Hz), 4.44 (dd, 1H, H-5'; $J_{4,5'}$=2.44 Hz) and 6.04 (dd, 1H, H-4)] to those of **6** [δ 3.91 (dd, 1H, H-5; $J_{4,5}$=6.83 Hz), 4.05 (dd, 1H, H-5'; $J_{4,5'}$=3.91 Hz) and 5.67 (dd, 1H, H-4)], but very different from those of **5** [δ 4.84 (dd, 1H, H-5; $J_{4,5}$=4.39 Hz), 4.98 (dd, 1H, H-5';$J_{4,5'}$=2.93 Hz) and 5.34 (dd, 1H, H-4)]. Thus, the electronic environment of the protons in **4** and **6** must be similar to each other, which was also proved in the MS experiment. Ring current due to the quinoxaline moiety may give the strongest deshielding effect on the proton in the lactone moiety of **4**. The lactone



Scheme 2

ring proton (H-4) in **5** is most shielded because it is farthest from the quinoxaline ring. Thus, the lactone proton in **5** appears at more upfield chemical shift (5.34 ppm) when compared with other compounds (5.67 and 6.04 ppm for **6** and **4**, respectively). Aromatic protons in all three products appeared as two multiplets near 8.2 ppm. These NMR spectra show good agreement with the structures assigned from HPLC-MS analysis.

The spontaneous conversions of **6** to **5** and/or **4** and **5** to **4** in aq solutions suggest that there must be a few common intermediates between **4** and **5**, between **5** and **6**, or between **4** and **6**. It is probable that the process occurs mainly through the intermediates **7** and **8** (Scheme 2). The carbonyl group of C-2 in **2** must be the most reactive of the three isomers. At a lower concentration of **2**, the first amino group of **3** may react with the C-2 to produce **7**, followed by cyclization with either C-3 or C-1 to produce **4** or **5**, respectively. At a higher concentration of **2**, an amino group of **3** may react with C-1 to produce **8**, at a lower extent than with C-2, followed by the cyclization with either C-2 or C-3 to produce **5** or **6**, respectively. In the present case, C-3 was found to be much less

reactive than C-1. The process rarely seems to occur through **9**. With the concentration of **2** below 5 mM, virtually no **6** was produced. This is partially explained by the steric hindrance of the bulky -CH_2OH group.

The concentration-dependent product formation may be due to the kinetics of the condensation reaction. Under the reaction conditions employed, the nucleophilic attack of an amino group of **3** to carbonyl carbon of **2** seems to be the rate-limiting step of the condensation reaction. The destruction of the lactone moiety is not expected as an intermediary step through the whole condensation process, which is different from the results obtained by the reaction between **3** and 6-carbon reductones.[10,12,13]

In neutral and alkaline solutions, DHA is known to be very rapidly delactonized to 2,3-diketogulonic acid, which is subsequently decarboxylated to pentosone or pentonic acids[1,16-18] and thus, its 5-epimer and **2** will follow a similar pathway as DHA in neutral pH's. The oxidized form of 6-carbon reductones exists in bicyclic form in aq solution,[19,20] whereas that of a 5-carbon reductone cannot exist in bicyclic and the three adjacent carbonyl groups are exposed to the attack by two amino groups of **3**. Although there are great structural differences between the oxidized form of 6-carbon reductones and **2**, it is thought that the condensation process of **2** with **3** is not very different from those of DHA and its 5-epimer.

EXPERIMENTAL

General methods. HPLC-MS analyses were performed using ODS hypersil column (10 cm × 4.6 mm i.d.) (Hewlett Packard, U.S.A.) coupled to VG LINC Particle Beam interface (VG Instruments, U.K.). MS measurements were made in the EI ionization mode (15 eV) on a VG Quattro system (VG Instruments, U.K.). All spectra were acquired in the mass range 50-300 amu, with a scan time of 0.8 s. 25% methanol in water was used as eluent at a flow rate of 0.5 mL/min. For the purification of condensation products, a Waters Delta Prep 4000 Preparative Chromatography System (Waters, U.S.A.) equipped with Waters 4000 system controller, Waters 484 tunable absorbance detector, and Waters 746 data module was used. DELTA PAK C18 column (30 cm × 7.8 mm i.d.) was employed as stationary phase and 25% methanol in Milli-Q

water was used as mobile phase at a flow rate of 1.5 mL/min. UV-vis spectra were obtained with a Shimadzu model UV-265 spectrophotometer (Shimadzu, Japan). ^{1}H NMR spectra were recorded at room temperature with a VXR-200S FT-NMR spectrometer (Varian, U.S.A.) using 3-(trimethylsilyl)-1-propane sulfonic acid as an internal reference, and chemical shifts are reported on δ-scale. Excess water signals were suppressed by homogated decoupling.

Reaction of dehydro-D-erythroascorbic acid (2) with an excess of *o*-phenylenediamine (3). D-Erythroascorbic acid (**1**) was prepared from 3,4-*O*-benzylidene-D-ribono-δ-lactone according to the method proposed by Gan and Seib.[15] The recrystallization of **1** from hot acetonitrile gave colorless needles.

A 0.1 M citrate-phosphate buffer (0.1 M/0.2 M, pH 5.6) solution of **1** (40 mg/mL) was oxidized by O_2/ascorbate oxidase (Boehringer Mannheim, Germany) at 25 °C until absorbance at 265 nm disappeared. This solution of **2** was immediately diluted to various concentrations (20 μM - 100 mM) with distilled water and mixed with the same volume of 10% *m*-phosphoric acid solution containing 200 mM of **3**. This reaction mixture was incubated at 40 °C for 60 min and extracted five times with the same volume of ethyl acetate. The ethyl acetate layer was collected, dried in vacuo, and redissolved in the initial reaction volume of methanol. This methanolic solution was applied to direct HPLC-MS analysis or preparative HPLC. Each peak in preparative HPLC was collected, freeze-dried and stored at -70 °C.

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