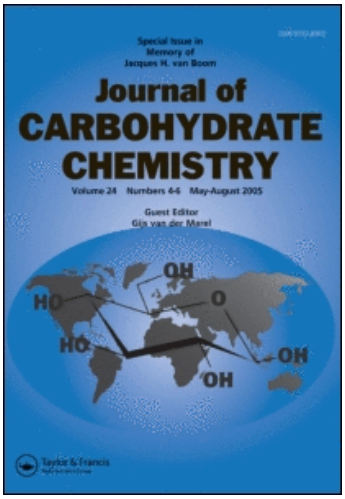

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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To cite this Article Seok, Yeong-Jae , Kim, Seong-Tae , Yang, Kap-Seok , Huh, Won-Ki and Kang, Sa-Ouk(1996) 'Characterization of Enediol-Containing Tautomers of L-Xylosone', Journal of Carbohydrate Chemistry, 15: 9, 1073 — 1083
To link to this Article: DOI: 10.1080/07328309608006498
URL: http://dx.doi.org/10.1080/07328309608006498

J. CARBOHYDRATE CHEMISTRY, 15(9), 1073-1083 (1996)

CHARACTERIZATION OF ENEDIOL-CONTAINING TAUTOMERS OF L-XYLOSONE

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Received January 15, 1996 - Final Form July 30, 1996

ABSTRACT

Two interconvertible enediol group-containing reductones were produced nonenzymatically from L-xylosone in citrate-phosphate buffer (pH 6.0). These compounds were oxidized by ascorbate oxidase (E.C. 1.10.3.3.) and had ability to reduce 2,6-dichloroindophenol. GLC-MS analysis of trimethylsilylated L-xylosone indicates that it exists in several tautomeric forms including the enediol group-containing reductones which are L-*glycero*-2-pentenopyranose and L-*glycero*-2-pentenofuranose. The production rates of both reductones from L-xylosone were proportional to pH within a pH range 4 to 7, and above that pH range further degradation of the reductones occurred.

INTRODUCTION

Aldos-2-uloses are generally important intermediates in oxidation and elimination reactions of free aldoses and ketoses.[1] Since these monosaccharides have both aldehyde and ketone groups with adjacent hydroxyl groups, they can exist in an equilibrium of open-chain and several ring-closed isomers under physiological conditions. L-Xylosone, the trivial name for L-*threo*-pentos-2-ulose, is a useful intermediate in the synthesis of L-ascorbic acid[2] and has been proposed as an intermediate in the oxidative degradation of L-

ascorbic acid in chemical and biological systems.[3,4] In an earlier study on the conformations of pentos-2-uloses including D-threo-pentos-2-ulose by means of ^{13}C NMR spectroscopy, the unhydrated aldopyranose, aldopyranose endocyclic hydrates, aldofuranose endocyclic hydrates and ketofuranose exocyclic hydrates were identified.[5] GLC-MS studies of per-*O*-trimethylsilylated aldos-2-uloses also revealed the presence of several isomeric forms.[6] The probable structures of the enediol compounds of aldos-2-uloses have been discussed in several reports.[7,8,9] The proposed structures of the enediol compounds have been inferred from the structures of aldos-2-uloses but no direct proof for the proposed structures of enediol compounds has been reported.

In the present paper, we report the formation and characterization of enediol group-containing tautomers of L-xylosone by means of HPLC and UV-visible absorption spectroscopy as well as their structural elucidation by means of GLC-MS methods.

RESULTS AND DISCUSSION

As shown in Fig. 1, a fresh solution of 10 mM L-xylosone (**1**) in citrate-phosphate buffer (0.1 M/0.2 M, pH 6.0) gave an absorption band at 227 nm with a weak shoulder at 263 nm, with the latter exhibiting time-dependent increase. This reaction mixture was analyzed by means of HPLC, as shown in Fig. 2. Two peaks [retention time (t_R) of about 3.6 and 4.1 min, respectively], which were monitored at 254 nm, appeared after 5 min and showed almost maximal intensities after 25 min. When this solution was treated with ascorbate oxidase, both peaks disappeared, as shown in Fig. 2d. Both compounds also reduced 2,6-dichloroindophenol, indicating that they contain enediol-groups.

After incubation of the solution, as exhibited in Figs. 1 and 2, at 25 °C for 40 min, that solution was acidified to pH 3.0 with *o*-phosphoric acid upon which the peak of t_R 3.6 disappeared and the peak of t_R 4.1 increased by the corresponding amount (Fig. 3a). The peak of t_R 3.6 reappeared by raising the pH to 6.0 (Fig. 3e). When the reaction mixture exhibiting Fig. 3a was incubated at 25 °C, the peak of t_R 4.1 decreased and almost disappeared after 20 h (Figs. 3b and 3c). When the pH of the solution of Fig. 3c was raised to 6.0 and that solution was incubated at 25 °C for 10 min, two peaks reappeared (Fig. 3d). From these results, it was deduced that the two compounds of Fig. 2 were interconvertible,

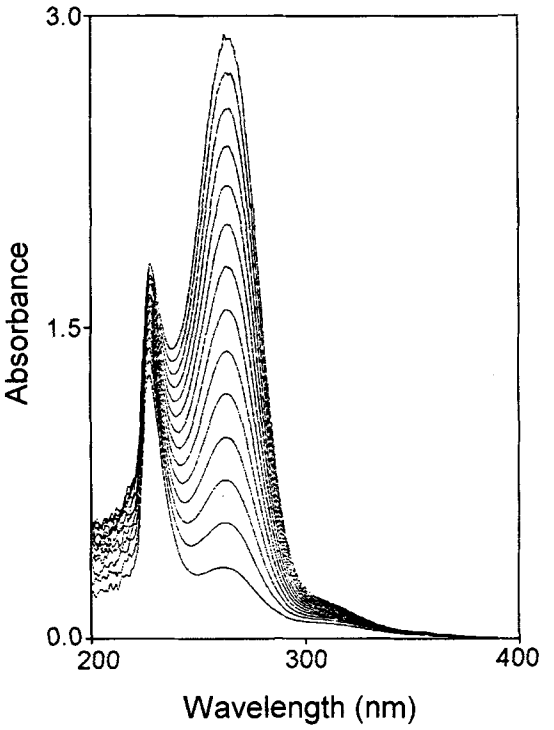

Figure 1. Time-dependent changes of absorption spectra of **1** in citrate-phosphate buffer (0.1 M/0.2 M, pH 6.0). Freshly prepared **1** was dissolved in the buffer to a final concentration of 10 mM and its absorbance was scanned from 200 nm to 400 nm at 25 °C every minute.

depending on pH. The chromatograms in Figs. 2 and 3 suggested that the compounds with reducing ability formed from **1** may be a mixture of tautomers containing enediol-groups. The isolated enediol compounds of t_R 3.6 and 4.1 changed to mixtures of tautomers in a couple of hours, which made it impossible to analyze the structures of the isolated compounds by NMR spectroscopy or mass spectrometry. TLC (1-propanol:acetic acid:water = 8:1:3) showed that the same enediol compounds as those formed in citrate-phosphate buffer were also formed in pyridine (data not shown). Thus, the formation of enediol compounds was monitored in pyridine and the compounds were analyzed by GLC-MS.

Fig. 4 shows the GLC-chromatogram recorded in the relative total-ion-current mode with a scan time of 2 s. Five mass spectra shown in Table 1 correspond to the peaks at scan

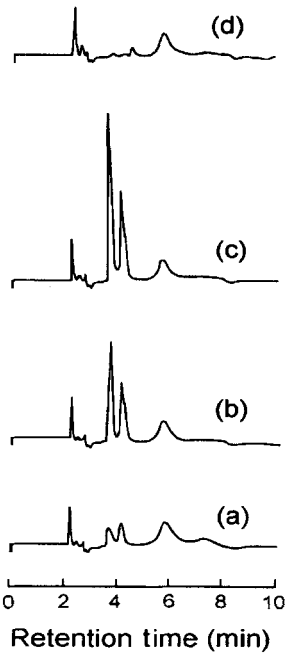

Figure 2. Time-dependent changes of HPLC-chromatograms of **1** dissolved in citrate-phosphate buffer (0.1 M/0.2 M, pH 6.0). (a), (b) and (c) show the chromatograms of reaction mixture incubated at 25 °C for 5, 15 and 25 min, respectively. (d) shows the chromatogram of reaction mixture incubated for 25 min and treated with ascorbate oxidase at 25 °C. Chromatograms were recorded at 254 nm.

numbers 422, 468, 478, 526 and 563 in the chromatogram, respectively. All spectra were very uniform in pattern with only slight differences. The assignment of structures (Fig. 5) was made on the basis of fragmentation patterns observed.

The different pyranoid or furanoid forms may be distinguished on the basis of the ratio of mass number (*m/z*) 217 over *m/z* 204. This ratio is larger in the furanoid form than in the pyranoid form.[6] The mass number of the base peak was equal to 217, in the mass spectra of scan numbers 422, 468 and 478, whereas it was equal to 204 in that of scan numbers 526 and 563 (Table 1). Therefore, the compounds of scan numbers 422, 468 and 478 are assigned to be furanoid forms, and those of scan numbers 526 and 563 are assigned to be pyranoid forms. On the other hand, the *m/z* value of 205 implies fragmentation of a trimethylsilylated open-chain sugar, but none was found in any of the reported mass spectra.

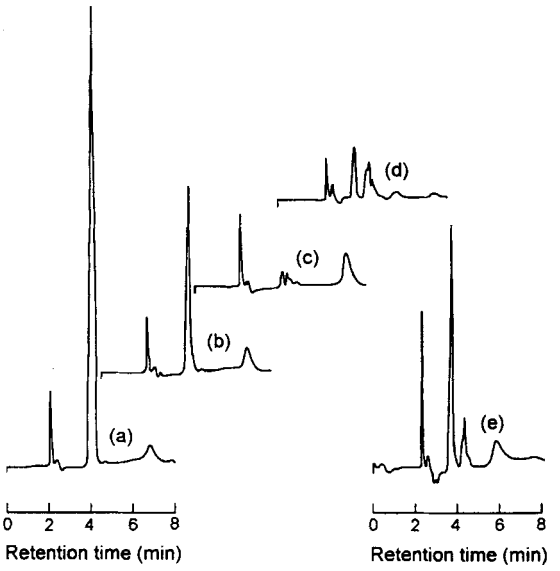

Figure 3. Changes of liquid chromatograms of **1** at various conditions. After 10 mM of **1** solution in citrate-phosphate buffer (0.1 M/0.2 M, pH 6.0) was incubated for 40 min at 25 °C, this solution was acidified to pH 3.0 with *o*-phosphoric acid, and then applied to HPLC. (a), (b) and (c) are the chromatograms of the solution after incubation at 25 °C for 0, 2 and 20 h, respectively. (d) is the chromatogram of solution (c) incubated for 10 min at 25 °C after pH was raised to 6.0 again. (e) is the chromatogram of the solution (a) neutralized to pH 6.0 without delay. Chromatograms were recorded at 254 nm.

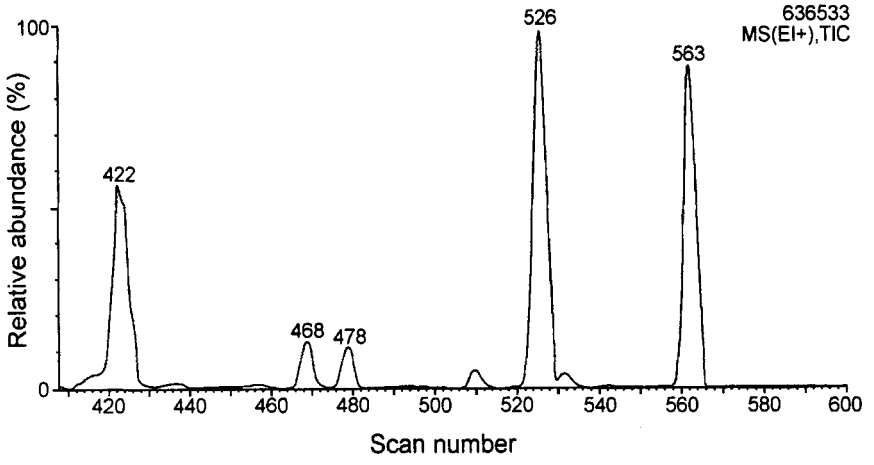

Figure 4. GLC-chromatogram of trimethylsilyl derivatives of **1**. Detection was carried out with mass spectrometry and the chromatogram was recorded in the relative total-ion-current mode with a scan time of 2 s.

Table 1. Mass spectra (20 eV) of the *O*-trimethylsilyl derivatives of **1**

m/z	Scan number				
	422	468	478	526	563
436				1.43	
364		0.57			
349	1.00	0.43		0.71	
347				1.43	
335	1.29				
333		0.57	0.86		
320	1.29				
305	1.14	0.43	1.43	1.43	1.49
259	1.57	0.86	1.00	1.43	2.14
246	1.14				
244		5.29			
217	100.00	100.00	100.00	39.29	41.43
204	1.43	5.29	2.71	100.00	100.00
191	9.00	8.00	9.00	34.29	35.71
147	16.43	6.00	1.14	5.43	4.29
103	4.29	4.14	3.57	1.86	2.57
73	10.71	10.86	2.57	7.86	6.43

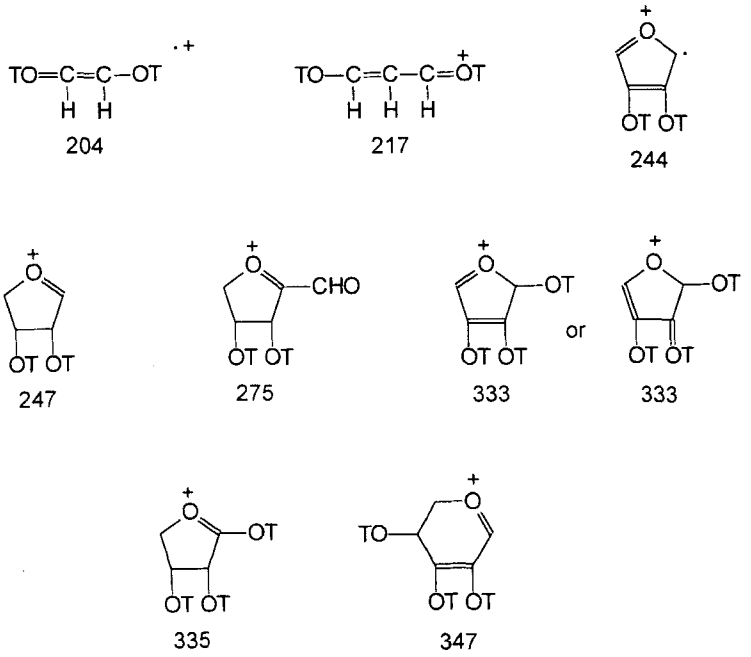

Figure 5. The possible structures of the characteristic fragment ion peaks derived from *O*-trimethylsilyl derivatives of **1**.

At the lower mass range of all spectra, ions at *m/z* 73 (TMS radical ion), 103 (CH_2=O^+TMS), and 147 [TMS-O^+=Si(CH_3)$_2$] were recorded. These peaks were previously shown to be characteristics of *O*-trimethylsilylated derivatives of other compounds. The ion peaks at *m/z* 191 and 305 which are observed in all spectra, represent TMSO-CH=O^+TMS and TMSO^+=CH-C(OTMS)=CH-OTMS, respectively. The most favored cleavage is a rupture of the bond between the side chain and the ring. It is thus to be expected that great differences will exist for the TMS-derivatives of different ring structures with different side chains. The ion peaks at *m/z* 246[364(M^+)–CHO–OTMS], 320 (335–CH_3), and 335 (364–CHO) were found only in the spectrum of scan number 422, and they were assigned to be specific for the ketofuranoid structure. The ion peak at *m/z* 244 was observed only with the spectrum of scan number 468, and this peak indicates the presence of the enediol group within the furanoid structure. The ion peak at *m/z* 333 is formed when a hydroxymethyl group is split off from the mother ion. Thus this peak was relatively abundant in the furanoid structure containing the side chain hydroxymethyl group and found in the spectra of scan numbers 468 and 478. The mass spectra of *O*-trimethylsilylated sugars generally show very weak peaks for the molecular ions. However, several fragment ions can be used to determine molecular weight. More intense peaks are generally given by the ion M–15 (M–CH_3·), M–73 (M–TMS), M–89 (M–TMSO), and M–90 (M–TMSOH), etc. than by the molecular ion.[10] All the mass spectra in Table 1 show excellent agreements with these facts.

The peaks at *m/z* 259, 347, 349, 364, and 436 were used in determining the molecular weights of the peaks in the GLC-chromatogram shown in Fig. 4. The peak at *m/z* 259 corresponds to the sequential loss of a methyl TMSOH and TMS (when M is 436) radicals and is observed in all spectra. The peak at *m/z* 347 corresponds to the loss of a TMSO radical from the molecule containing an enediol group within the ring and is found only in the spectrum of scan number 526. The peak at *m/z* 349 corresponds to the loss of a methyl radical from the molecule containing an aldehyde group within the ring (scan numbers 422, 468 and 526 in Table 1). The peaks at *m/z* 364 and 436 correspond to the molecular weights of the isomers containing the carbonyl group and enediol group within the ring, respectively.

On the basis of these results, it is concluded that as expected, **1** exists in several isomeric forms. The compounds of scan numbers 422, 468, 478, 526, and 563 in Table 1

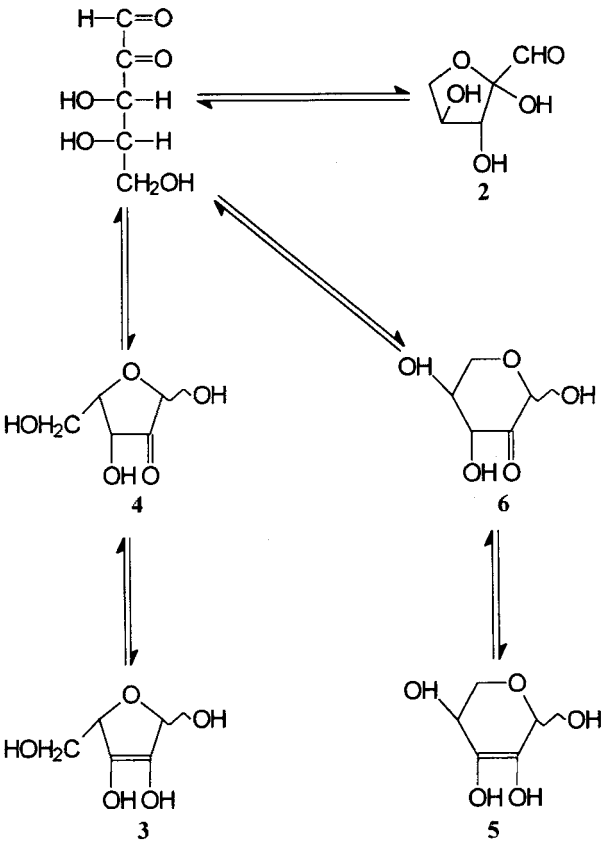

Figure 6. Proposed scheme for the tautomeric interconversions of **1**.

were assigned to *O*-trimethylsilylated ethers of L-*threo*-pentos-2-ulo-2,5-furanose (**2**), L-*glycero*-2-pentenofuranose (**3**), L-*threo*-pentos-2-ulo-1,4-furanose (**4**), L-*glycero*-2-pentenopyranose (**5**), and L-*threo*-pentos-2-ulo-1,5-pyranose (**6**), respectively, but an open-chain form was not detected. Compounds **2**, **4** and **6** analyzed by GLC-MS have similar structures to those of D-erythropentos-2-ulose and D-threopentos-2-ulose, which were proposed by means of ^{13}C NMR spectroscopy.[5] However, it seems that two peaks at scan numbers 526 and 468 in the GLC-MS analysis correspond to tautomers as their *O*-trimethylsilylated derivatives, with the structures **3** and **5** containing enediol groups. These tautomers have not been previously reported.

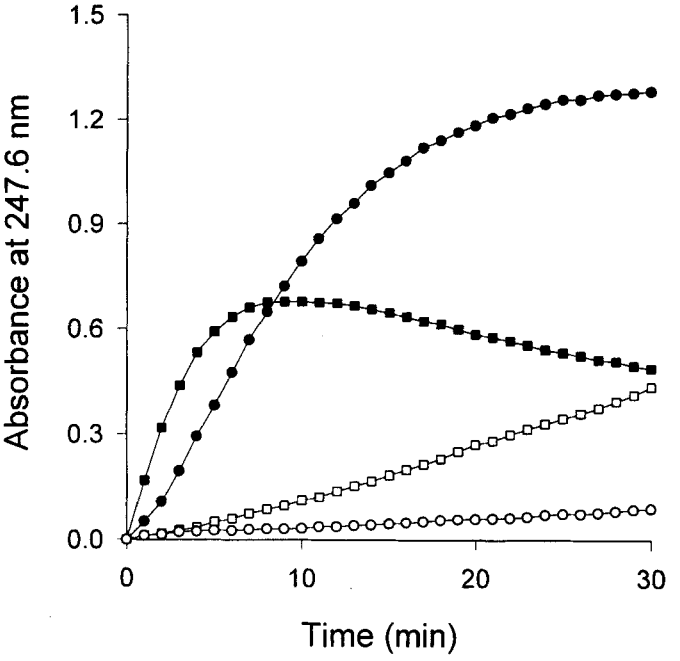

Figure 7. Production of enediol compounds from **1** in citrate-phosphate (0.1 M/0.2 M) buffer at various pH's: ○, pH 4.0; , pH 5.0; ●, pH 6.0; ■, pH 7.0.

The dicarbonyl sugar **1** has a potentially free aldehyde and a ketone group. Thus it can exist at equilibrium with the enediol form in aqueous solution, especially at non-acidic pH, and the resulting enediol will be an active reducing agent. The production rate of enediol compounds from 1 mM of **1** was determined by increase in UV absorbance at isosbestic wavelength (247.6 nm) at several pH values at 25 °C (Fig. 7). The enediol compounds were scarcely formed below pH 4.0. However, as pH was raised, the formation rate increased. But, above pH 7.0, the enediol-containing tautomer degraded further. This result corresponds to that from HPLC analysis, in which the tautomeric transformation does not occur at lower pH values.

EXPERIMENTAL

Chemicals. Starting **1** was prepared by the method proposed by Salomon et al.[12] L-Xylose (4 g) was dissolved in 96% aq methanol (250 mL) and heated under reflux in a

round-bottomed flask. Cupric acetate monohydrate (17 g) was added and the mixture was heated for an additional 15 min, cooled and filtered through 0.45 μm glass filter to remove the precipitate of cuprous oxide. The excess cupric acetate was removed by passage of the methanolic solution through a 1.5 × 20 cm column of Dowex 50X 8-100 (Sigma, U.S.A.). The resin had been previously converted to the hydrogen form by washing with 2 N hydrochloric acid, followed by water and thorough washing with methanol. After passing through the column, the solution was concentrated in vacuo to a syrupy substance. This syrup was dissolved in methanol (10 mL), applied onto a 1.5 × 20 cm column of Dowex 1X 8-100 and eluted with methanol (300 mL). The resin had been previously converted to the formate form by washing with 2 N formic acid, followed by water and thorough washing with methanol. Each fraction was subjected to a spot test with 2,4-dinitrophenylhydrazine saturated in 2 N HCl, and the fractions giving immediate precipitate were pooled and concentrated in vacuo to a final concentration of 2 M, and diluted with the same volume of ethanol and stored by -20 °C. The concentration of **1** was determined according to the semicarbazide method proposed by Alexander and Boyer.[13]

L-Ascorbic acid was purchased from Merck (Germany), 3,4-*O*-benzylidene-D-ribono-1,5-lactone, anhydrous pyridine, and 1-(trimethylsilyl)imidazole from Aldrich (U.S.A.), ascorbate oxidase from Boehringer Mannheim (Germany), and 2,6-dichloroindophenol and trifluoroacetic acid from Sigma (U.S.A.). D-Erythroascorbic acid (EASC) was prepared from 3,4-*O*-benzylidene-D-ribono-1,5-lactone according to the method proposed by Gan and Seib.[11] Other reagents used were of the highest quality commercially available.

High-performance liquid chromatography (HPLC) A Waters Associates Liquid Chromatography System (Waters, U.S.A.) equipped with Model 510 pump, U6K injector, Model 440 absorbance detector and Model 741 data module was used for analytical purpose. 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 for preparative purpose. Waters μBondapak C18 column (30 cm × 3.9 mm) was employed as stationary phase for analytical chromatography at a flow rate of 1 mL/min and DELTA PAK C18 column (30 cm × 19 mm) for preparation using 0.1% aq trifluoroacetic acid as eluent. Chromatograms were recorded at 254 nm.

GLC-MS spectrometry of trimethylsilyl ethers of L-xylosone. To prepare fully trimethylsilylated ethers, 50 mmol of **1** was dissolved in 80 μL of dry pyridine. To this solution, 20 μL of 1-(trimethylsilyl)imidazole was added. The reaction mixture was shaken at least for 30 min at 40 °C, and 1 μL of the sample was applied to the GLC-column.

For the GLC-MS spectrometric analyses, a Hewlett-Packard 5890A gas chromatography equipped with a fused-silica capillary column (30 m × 0.25 mm) coated with a 0.25 mm layer of SPB-1 (Supelco, U.S.A.) was used in combination with a VG-Quattro mass spectrometer (U.K.), operating in EI ionization mode (20 eV). Helium was used as a carrier gas, at a flow rate of the 1 mL/min. The samples were introduced into the column at 150 °C and the elution was programmed linearly, after a 2-min delay, to 250 °C at 5 °C/min. All spectra were acquired in the mass range 50-500 in atomic mass units, with a scan time of 2 s.

ACKNOWLEDGEMENT

This work was supported by a reseach grant for SRC (Research Center for Molecular Microbiology, Seoul National University) from the Korea Science and Engineering Foundation (KOSEF).

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