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Synthesis of 3-Keto-levoglucosan Using Pyranose Oxidase and Its Spontaneous Decomposition via β-Elimination

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Abstract: 3-Keto-levoglucosan (3ketoLG) has been postulated to be the product of a reaction catalyzed by levoglucosan dehydrogenase (LGDH), a bacterial enzyme involved in the metabolism of levoglucosan (LG). To investigate the LG metabolic pathway catalyzed by LGDH, 3ketoLG is needed. However, 3ketoLG has not been successfully isolated from the LGDH reaction. This study investigated the ability of pyranose oxidase to convert LG into 3ketoLG by oxidizing the C3 hydroxyl group. During the oxidation of LG, 3ketoLG was spontaneously crystallized in the reaction mixture. Starting with 500 mM LG, the isolation yield of 3ketoLG was 80 %. Nuclear magnetic resonance analyses revealed that a part of 3ketoLG dimerized in aqueous solution, explaining its poor solubility. Even under normal conditions, 3ketoLG was unstable in aqueous solution, with a half-life of 16 h at pH 7.0 and 30 °C. The decomposition proceeded through β-elimination of the C–O bonds at both C1 and C5, as evidenced by decomposition products. This instability explains the difficulty in obtaining 3ketoLG via the LGDH reaction.

Key words: levoglucosan, 3-keto-levoglucosan, pyranose oxidase, β-elimination, levoglucosan dehydrogenase

INTRODUCTION

Levoglucosan (1,6-anhydro-β-D-glucose, LG, **1**; Fig. 1) is a compound generated through pyrolysis of cellulose or starch.[1)] Because LG is a naturally occurring product, microbial utilization system of LG has been investigated. Although LG can be chemically converted into D-glucose by acid hydrolysis, no enzyme that hydrolyzes LG has yet been found.[1)]

Early in the 1990s, researchers[2)3)4)5)] proposed two microbial pathways that would introduce LG into the glycolytic pathway. One is based on levoglucosan kinase (LGK), which catalyzes the synthesis of D-glucose 6-phosphate and ADP from LG, ATP, and H_2O.[2)3)] LGK has been isolated from several fungi and yeasts.[1)] The reaction involves the ring-opening of the β-1,6-linkage and phosphorylation at the C6 hydroxyl group. LGK specifically phosphorylates LG, but not D-glucose, indicating that the ring-opening is involved in the phosphorylation at C6. The structural analysis of LGK revealed its reaction mechanism, including the ring-opening and phosphorylation.[1)6)]

The other pathway involves levoglucosan dehydrogenase (LGDH), which catalyzes the synthesis of 3-keto-levoglucosan (1,6-anhydro-β-D-*ribo*-hexopyranos-3-ulose, 3ketoLG, **2**; Fig. 1) with the conversion of NAD^+ into NADH. Yasui *et al.*[4)] first reported that the bacterial strain I-552 converted LG into D-glucose in a pathway via the concerted actions of three or more enzymes, including an NAD^+-dependent dehydrogenase that oxidized LG. Nakahara *et al.*[5)] determined I-552 to be a strain of *Arthrobacter* sp. and characterized LGDH based on the absorption spectrum of the crude product under alkaline conditions and concluded that LGDH is involved in the synthesis of 3ketoLG. However, they did not successfully isolate the product. Because the equilibrium between a hydroxyl and a keto group, in reactions catalyzed by NAD^+-dependent dehydrogenases, highly favors reduction reactions,[7)] it is often difficult to efficiently synthesize keto products using NAD^+-dependent dehydrogenases. The scheme of the pathway after the LGDH-catalyzed reaction has not yet been identified due to the difficulty in the availability of 3ketoLG. Thus, easy accessibility to 3ketoLG is required to investigate the mechanism of this pathway.

The 3-keto-D-glucoside structure is known to undergo β-elimination under alkaline conditions.[8)] Such 3-keto structure is postulated as the reaction intermediate in the reac-

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Abbreviations: 3ketoLG, 3-keto-levoglucosan (1,6-anhydro-β-D-*ribo*-hexopyranos-3-ulose); 3ketoMeβGlc, methyl-3-keto-β-D-glucopyranoside (methyl-β-D-*ribo*-hexopyranosid-3-ulose); DMSO, dimethyl sulfoxide; DQF-COSY, double-quantum-filtered correlation spectroscopy; ESI-MS, electrospray ionization mass spectrometry; GH, glycoside hydrolase; HMBC, heteronuclear multiple-bond correlation; HPLC, high performance liquid chromatography; HSQC, heteronuclear single quantum coherence; LG, levoglucosan (1,6-anhydro-β-D-glucopyranose); LGDH, levoglucosan dehydrogenase; LGK, levoglucosan kinase; MeβGlc, methyl-β-D-glucopyranoside; MOPS, 3-morpholinopropanesulfonic acid; NMR, nuclear magnetic resonance; P2O, pyranose oxidase; TLC, thin-layer chromatography; TOPS, *N*-ethyl-*N*-(3-sulfopropyl)-3-methylaniline sodium salt monohydrate.

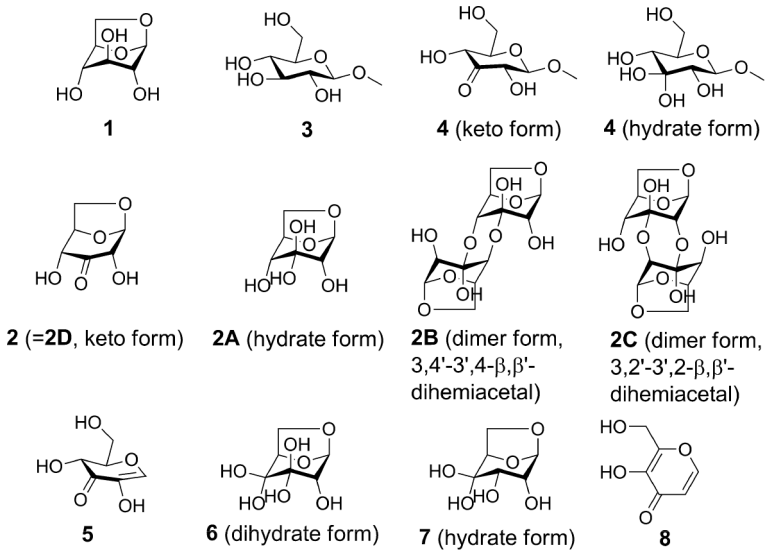

Fig. 1. Structural formulas of compounds mentioned in this study.
1, LG; **2**, 3ketoLG, **3**, MeβGlc; **4**, 3ketoMeβGlc; **5**, 1,5-anhydro-D-erythro-hex-1-en-3-ulose; **6**, 3,4-diketo-levoglucosan; **7**, 4-keto-1,6-anhydro-β-D-allopyranose; **8**, 1,5-anhydro-2-deoxy-hex-1,4-dien-3-ulose.

tion mechanism of a 6-phospho-β-glucosidase, an NAD^+-dependent enzyme that belongs to the glycoside hydrolase (GH) family 4, in which the cleavage of glycosyl bond occurs in the 3-keto intermediate via β-elimination at C1.[9)10)] The same reaction mechanism via the 3-keto intermediate is also postulated in GH family 109 α-*N*-acetylgalactosaminidase.[11)]

Synthesis of 3ketoLG was first reported in the oxidation of LG catalyzed by platinum dioxide.[12)] The oxidation was specific at the C3 hydroxyl group of LG, but the reaction was not so efficient, as it required 1 g of the catalyst to obtain 400 mg of 3ketoLG from 2 g of LG. Recently, a palladium-based catalyst was developed to oxidize the C3 hydroxyl group of glycosides and 1,6-anhydrohexoses.[13)] However, this requires an equatorial hydroxyl group at C2 or C4 for efficient catalysis and is not capable of synthesizing 3ketoLG from LG, all the hydroxyl groups of which are axial due to its $^1C_4$ conformation.

Pyranose oxidase (EC 1.1.3.10, P2O) is a fungal enzyme that oxidizes D-glucose at the C2 hydroxyl group, with the consumption of O_2 to generate glucosone (2-keto-D-glucose, or D-*arabino*-hexos-2-ulose) and H_2O_2.[14)] P2O also oxidizes 2-deoxy-D-glucose (2-deoxy-D-*arabino*-hexose) and methyl-β-D-glucopyranoside (MeβGlc, **3**; Fig. 1) at their C3 hydroxyl groups to generate 3-keto-2-deoxy-D-glucose (2-deoxy-D-*erythro*-hexos-3-ulose) and 3-keto-methyl-β-D-glucopyranoside (methyl-β-D-*ribo*-hexopyranosid-3-ulose, 3ketoMeβGlc, **4**; Fig. 1), respectively, at much slower rates than it oxidizes D-glucose.[14)]

Because oxidation reactions catalyzed by oxidases that use O_2 as the oxidizing agent are irreversible, they are promising catalysts for the synthesis of oxidized products. Therefore, the author examined LG as a substrate of P2O and found that it gets specifically oxidized at the C3 hydroxyl group. In this report, the efficient synthesis of 3ketoLG using P2O is described. The author also found that 3ketoLG is labile in aqueous solution even under normal conditions. Some properties, especially on β-elimination, of 3ketoLG are also presented.

MATERIALS AND METHODS

Materials. LG (**1**; Fig. 1) was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan), P2O from *Coriolus* sp. (9.7 U/mg-solid according to the manufacture's measurement), MeβGlc (**3**; Fig. 1) from Sigma-Aldrich Corp. (St. Louis, USA). *N*-Ethyl-*N*-(3-sulfopropyl)-3-methylaniline sodium salt monohydrate (TOPS) was purchased from Dojindo Laboratories (Mashiki, Japan), horseradish peroxidase (Grade I, PEO-131) from Toyobo Co., Ltd (Osaka, Japan), and bovine liver catalase (10,000 U/mg-solid according to the manufacture's measurement) from Nacalai Tesque, Inc. (Kyoto, Japan). Other chemicals used were of reagent grade.

High Performance Liquid Chromatography (HPLC). HPLC analysis was performed using LC20A system (Shimadzu Corporation, Kyoto, Japan) equipped with a corona-charged aerosol detector (ESA Biosciences, Inc., Chelmsford, USA) and a Shodex SUGAR KS-801 column (8.0 mm φ × 300 mm, Showa Denko K.K., Tokyo, Japan), with H_2O as the solvent, at a flow rate of 1 mL/min at 60 °C or at a flow rate of 0.8 mL/min at 35 °C.

Thin layer chromatography (TLC). Analytical TLC was performed using a TLC plate (TLC Silica gel 60 F_{254}; 5 × 7.5 cm, Merck KGaA, Darmstadt, Germany) with acetonitrile:water (9:1 v/v) as the solvent phase. After development, the plate was dipped in a solution of sulfuric acid (5 %, v/v) in methanol and heated in an oven to detect the compounds.

General Methods. One-dimensional (^1H and ^{13}C) and two-dimensional [double-quantum-filtered correlation spectroscopy (DQF-COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC)] nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance 500 or Avance 800 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany)

in D_2O with 2-methyl-2-propanol as the internal standard (1.23 ppm for ^1H, 31.3 ppm for ^{13}C) or in dimethyl sulfoxide (DMSO)-d_6 referring solvent signals as the internal standard (2.49 ppm for ^1H, 39.5 ppm for ^{13}C) at 298 K. Signals were assigned using cross signals in the 2D spectra. Ring structures were confirmed with the cross signal on HMBC spectra. Electrospray ionization mass spectrometry (ESI-MS) was performed using a Velos Pro Dual-Pressure Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, USA).

Measurement of P2O activity. The P2O reaction was monitored by the continuous measurement of H_2O_2 generated as follows: the P2O reaction was conducted in a reaction mixture (100 μL) containing TOPS (2 mM), 4-aminoantipyrin (2 mM), peroxidase (0.5 U/mL), and a substrate in 3-morpholinopropanesulfonic acid (MOPS)-Na buffer (25 mM; pH 7.0) in a well of a 384-well plate at 30 °C. The generation of H_2O_2 was continuously measured by monitoring A_λ at 550 nm. One unit of P2O activity was defined as the amount of enzyme that generated 1 μmol H_2O_2 under the designated conditions. Kinetic parameters were calculated by regressing the experimental data on the Michaelis–Menten equation using Grafit Ver. 7 (Erithacus Software Ltd., London, UK).

$$v = k[E]_0[S]/(K_m + [S])$$

Oxidation of LG. Preparative reactions by P2O were conducted in oxygen with a high relative gas–liquid surface area of the reaction mixture to prevent oxygen transfer at the gas–liquid surface from becoming the rate-limiting step. An aliquot of the reaction mixture (1 mL) containing LG (500 mM, 0.500 mmol), P2O (5 mg/mL), and catalase (0.05 mg/mL) in sodium phosphate buffer (50 mM; pH 6.0) was incubated at 10 °C under O_2 (normal pressure) in a cylindrical plastic tube (φ 3.4 cm). During the reaction, oxidized product precipitated as crystals. After a 30-h reaction, LG was depleted, and the reaction mixture was maintained at 0 °C for 16 h. The resultant crystals were collected by filtration and 63.6 mg of the compound was obtained after vacuum drying (0.398 mmol calculated as $C_6H_8O_5$, yield 80 %). The compound was identified as 3ketoLG (**2**; Fig. 1) by NMR analysis. ESI-MS: *m/z* 343.09 $[2M+Na]^+$ (calculated for $C_{12}H_{16}O_{10}Na^+$, 343.06), *m/z* 663.07 $[4M+Na]^+$ (calculated for $C_{24}H_{32}O_{20}Na^+$, 663.14).

Oxidation of MeβGlc. An aliquot of the reaction mixture (1 mL) containing MeβGlc (500 mM, 0.500 mmol), P2O (5 mg/mL), and catalase (0.01 mg/mL) in MOPS-Na buffer (50 mM, pH 7.0) was incubated at 30 °C under O_2 (normal pressure) in a cylindrical plastic tube (φ 2.4 cm) for 5 days. Based on the results of HPLC analysis, we found that approximately one-third of the starting material was oxidized. The reaction mixture was spotted on a preparative TLC plate (PLC Silica gel 60 F_{254}, 2-mm thickness, 20 × 20 cm, Merck), and the plate was developed with acetonitrile:water (85:15, v/v) solvent system. The silica gel from the plate was scratched off and the oxidized product was extracted using methanol. The extract was evaporated and 36.9 mg of the compound (0.192 mmol, yield 38 %) was isolated as syrup, which was identified as 3ketoMeβGlc (**4**; Fig. 1) by NMR analysis.

Stability of 3ketoLG. A 3ketoLG solution (10 mM) was made using one of the following buffers: sodium phosphate buffer (pH 8.0, 7.0, and 6.0; 50 mM each) and sodium acetate buffer (pH 5.0 and 4.0; 50 mM each). The solutions were incubated at various temperatures between 30 and 70 °C. For each measurement time, aliquots (10 μL) of the reaction mixtures were diluted with acetic acid solution (1 mM, 190 μL). The concentration of 3ketoLG was quantified using HPLC by injecting 50 μL of the diluted solution.

The rate constant (k) was determined by regressing the time-profile toward the following equation using Grafit Ver. 7.

$$[3ketoLG] = [3ketoLG]_0 \cdot e^{-kt}$$

The half-life ($t_{1/2}$) was calculated as $\ln 2/k$. The activation energy (E_a) at each pH was determined through linear regression of the Arrhenius equation.

$$\ln k = \ln A - E_a/RT \text{ (A: frequency factor)}$$

To determine its stability in acid, a 3ketoLG solution (10 mM) was prepared using HCl (1, 10, or 100 mM) and maintained at 100 °C for 1 h. After cooling, solutions were diluted with water (1:20 dilution) and the concentration of 3ketoLG was quantified using HPLC by injecting 50 μL of the diluted solution.

Isolation of the decomposed product from 3ketoLG. 3ketoLG (12 mg) was dissolved in 6 mL of sodium phosphate buffer (50 mM, pH 7.0), and the solution was incubated at 60 °C for 1 h. After cooling the sample on ice, ionic compounds were removed from the solution by electrodialysis using Microacylizer S1 with an AC-220-10 membrane cartridge (Astom Corp., Tokyo Japan) at 0 °C. The deionized solution was lyophilized and the resultant lyophilate (8.8 mg) was dissolved in H_2O (500 μL). An aliquot (50 μL) was injected into the HPLC (see HPLC section, without the detector) and the separation was performed at a flow rate of 0.8 mL/min and at 35 °C. Fractions eluted at 17.2–18.6 (Fraction 1) and 24.2–26.2 (Fraction 2) min were collected. The separation was repeated 10 times.

RESULTS

Specificity of P2O.

Specific activities of P2O on D-glucose, LG and MeβGlc (all at 10 mM) at 30 °C in MOPS-Na buffer (25 mM, pH 7.5) were 5.1 (100 %), 0.018 (0.45 %), 0.0033 (0.065 %) U/mg-solid, respectively. The enzyme did not oxidize either methyl-α-D-glucopyrnaoside or cellobiose. K_m value for D-glucose was 1.0 mM, whereas that of LG was at least >40 mM due to linear S–V relationship between 4 and 40 mM (data not shown).

Structural analysis of oxidized products from LG by P2O.

The whole NMR spectra and assignment of each signal of the LG product recorded in D_2O are shown in Supplementary material Fig. S1 and Table 1, respectively. ^1H-NMR spectrum of the LG product suggested a mixture of four different forms evidenced by the four individual signals of the anomeric protons (Fig. 2, designated as **A**, **B**, **C**, and **D**). All four forms possessed 1,5-pyranose and 1,6-anhydro rings, determined by the existence of corresponding

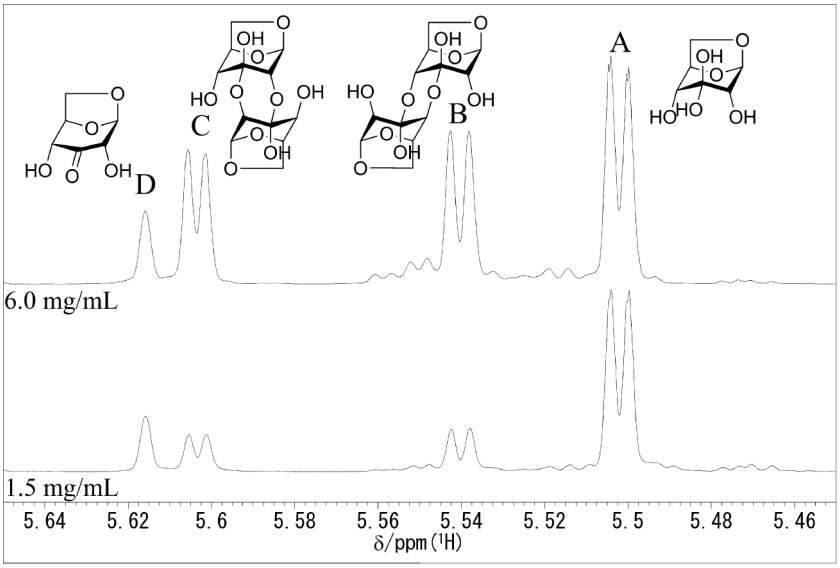

Fig. 2. ^{1}H-NMR spectra of the P2O product from LG (**1**) at the region of anomeric protons.
A, B, C, and D were identified to be isoforms of 3ketoLG (**2**) as illustrated.

HMBC cross peaks (Supplementary material Fig. S1_e-1). They did not possess hydrogen atoms at C3, as determined by HSQC spectrum (Table 2, Supplementary material Fig. S1_d), indicating that P2O oxidized C3 hydroxyl group to generate 3ketoLG (**2**; Fig. 1). Neither 2-keto nor 4-keto compound was detectable.

Signal strengths of **B** and **C** were similar at both concentrations (1.5 and 6.0 mg/mL) in D_2O, and ratios of signals **B** and **C** decreased with decrease in their concentration of the product (Fig. 2). These results suggested that **B** and **C** assumed dimer conformations existing as components of the equilibrium mixture of 3ketoLG. Based on the chemical shifts of C3, **A** and **D** were identified to be the hydrate and keto forms of **2**, respectively (**2A** and **2D**; Fig. 1). Those of **B** and **C** appeared at lower magnetic field than that of **A**, suggesting hemiacetal structures at C3 of **B** and **C**. According to the proton chemical shifts (Table 1) and the strength of HMBC cross peaks (Supplementary material Fig. S1_e-4), oxygen atoms at $C4^{B}$ and $C2^{C}$ assumed hemiacetal structure with C3 of other monomer units. No inter-ring HMBC cross signal was observed at $H4^{B}$-$C3^{C}$ or $H2^{C}$-$C3^{B}$ in the HMBC spectrum observing ^{13}C-NMR spectrum around 94.35 ppm with high resolution (Supplementary material Fig. S1_e-3), indicating that **B** and **C** are individual symmetric dimers. Finally, **B** and **C** were identified to be symmetric dimers, namely bis-(1,6-anhydro-β-D-*ribo*-hexopyranos-3-ulose)- 3,4′- 3′,4-β,β′-dihemiacetal and bis-(1,6-anhydro-β-D-*ribo*-hexopyranos-3-ulose)- 3,2′- 3′,2-β,β′-dihemiacetal, respectively [**2B** and **2C**; Fig. 1]. Such dihemiacetal dimeric structures in aqueous solution was observed with dihydroxyacetone,[15] the structure of which 3ketoLG possesses (between C2 and C4). The low solubility of 3ketoLG was probably due to the dimerization, which decreased the number of hydroxyl group to increase hydrophobicity. Only dimer forms **2B** and **2C** were observed in DMSO-d6 with different **B**/**C** ratio from that in D_2O (Supplementary material Fig. S2, Table 2).

Structural analysis of oxidized products from MeβGlc by P2O.

The oxidized product from MeβGlc also took two forms (Supplementary material Fig. S3 and Table 3), which were determined as the keto and hydrate forms of 3ketoMeβGlc (**4**; Fig. 1). No dimer was observed with 3ketoMeβGlc. The oxidation occurred only at the C3 hydroxyl group.

HPLC and TLC analyses of 3ketoLG and 3ketoMeβGlc.

The retention times of MeβGlc, 3ketoMeβGlc, LG, and 3ketoLG on HPLC analysis were 7.3, 8.5, 11.7, and 13.8 min, respectively, analyzed at 60 °C at the flow rate of 1 mL/min. On TLC, MeβGlc, 3ketoMeβGlc, and LG showed a single spot, whereas 3ketoLG yielded two spots (Fig. 3), suggesting that a portion of 3ketoLG may have decomposed before the development of the chromatogram. Sensitivities of 3-keto compounds were much less than those of the starting compounds. The Rf value of 3ketoMeβGlc was much larger than that of MeβGlc, whereas 3ketoLG migrated only a little more than LG. The difference is explainable with the ratio of the keto form, which is more hydrophobic than the hydrate form.

Stability of 3ketoLG.

When 3ketoLG and 3ketoMeβGlc were incubated in sodium phosphate buffer (50 mM, pH 8.0) for 60 min at 60 °C, 3ketoLG significantly decreased, whereas 3ketoMeβGlc remained intact (Fig. 4), suggesting that 3ketoLG was much more heat-labile than 3ketoMeβGlc.

The stability of 3ketoLG was examined under various conditions. In Table 4, we show that both high temperatures and high pH caused higher decomposition rate, suggesting that decomposition proceeded via β-elimination.[16] It should be noted that decomposition rate is considerably high under normal conditions such as pH 7.0 and 30 °C with the half-life of 16 h. On the other hand, 3ketoLG was rather stable under acidic conditions. During heat treatment at 100 °C for 1 h in 100 mM HCl, >90 % of the 3ketoLG remained.

Table 1. Assignment of NMR signals of the oxidized product from LG (**1**) in D_2O.

Position	^{13}C-NMR	^{1}H-NMR		
	δ (ppm)	δ (ppm)	Pattern	*J* (Hz)
A [40 % (6 mg/mL), 62 % (1.5 mg/mL)]				
1	102.6	5.50	dd	0.3, 2.2 ($J_{1,2}$)
2	73.3	3.43	ddd	0.4($J_{2,5}$), 1.8($J_{2,4}$), 2.1($J_{1,2}$)
3	92.2			
4	74.2	3.57	ddd	0.2, 1.9($J_{2,4}$), 2.0($J_{4,5}$)
5	77.4	4.67	dddd	0.4($J_{2,5}$), 1.1($J_{5,6}$), 2.3($J_{4,5}$), 5.9($J_{5,6'}$)
6	66.7	4.14	ddd	0.2, 1.1($J_{5,6}$), 7.6($J_{6,6'}$)
6′		3.71	dd	6.0($J_{5,6}$), 7.6($J_{6,6'}$)
B [27 % (6 mg/mL), 14 % (1.5 mg/mL)]				
1	102.2	5.54	d	2.2($J_{1,2}$)
2	72.3	3.60	t-like	1.9($J_{1,2}$, $J_{2,4}$)
3	94.3			
4	69.5	3.90	dd	1.9($J_{2,4}$), 2.1($J_{4,5}$)
5	75.1	4.79	m	
6	66.8	4.11	dd	0.9($J_{5,6}$), 7.9($J_{6,6'}$)
6′		3.80	dd	5.9($J_{5,6'}$), 7.9($J_{6,6'}$)
C [24 % (6 mg/mL), 13 % (1.5 mg/mL)]				
1	100.6	5.60	d	2.1($J_{1,2}$)
2	68.6	3.68	t-like	1.9($J_{1,2}$, $J_{2,4}$)
3	94.4			
4	73.0	3.73	t-like	1.9($J_{2,4}$, $J_{4,5}$)
5	76.9	4.73	m	
6	66.0	4.13	dd	1.1($J_{5,6}$), 8.0($J_{6,6'}$)
6′		3.75	dd	6.1($J_{5,6'}$), 7.8($J_{6,6'}$)
D [9 % (6 mg/mL), 11 % (1.5 mg/mL)]				
1	105.9	5.62	s	
2	77.3	4.14	d	
3	208.9			
4	77.6	4.30	t-like	0.8($J_{2,4}$, $J_{4,5}$)
5	80.9	4.80	m	
6	69.4	4.15	dd	1.0($J_{5,6}$), 8.3($J_{6,6'}$)
6′		3.92	dd	5.4($J_{5,6'}$), 8.1($J_{6,6'}$)

Structures of A, B, C, and D are indicated in Fig. 2.

No significant decrease of 3ketoLG was observed during the same treatment in 1 mM HCl. Under such acidic conditions, minimal degradation observed occurred through acid hydrolysis, not β-elimination.

Analysis of the products from 3ketoLG.

After the heat treatment, several new peaks appeared on the HPLC chromatogram (Fig. 5). The two major peaks (retention time 17.9 and 25.2 min) were fractionated as described in Materials and Methods. The two fractions (Fractions 1 and 2) were individually lyophilized to obtain 2.0 and 1.1 mg of residues, respectively. NMR analyses revealed that more than two compounds were present in each fraction.

Fraction 1 (retention time 17.9 min in Fig. 5) contained two compounds (Table 5, Supplementary material Fig. S4). The major compound was identified as 1,5-anhydro-D-*erythro*-hex-1-en-3-ulose (**5**; Fig. 1). The other compound was identified as the dihydrate form of 3,4-diketo-levoglucosan (1,6-anhydro-β-D-*erythro*-hexopyranos-3,4-diulose, **6**; Fig. 1).

Table 2. Assignment of NMR signals of the oxidized product from LG (**1**) in DMSO-d6.

Position	^{13}C-NMR	^{1}H-NMR		
	δ (ppm)	δ (ppm)	Pattern	*J* (Hz)
B [40 %]				
1	101.1	5.25	d	2.2($J_{1,2}$)
2	70.6	3.34	m	
3	92.3			
4	67.5	3.59	t-like	1.9($J_{2,4}$, $J_{4,5}$)
5	72.8	4.51	ddd	1.0($J_{5,6}$), 2.1($J_{4,5}$), 5.9($J_{6,6'}$)
6	64.5	3.95	dd	1.0($J_{5,6}$), 7.1($J_{6,6'}$)
6′		3.52	dd	6.0($J_{5,6'}$), 6.9($J_{6,6'}$)
2-OH		4.67	d	5.1($J_{2,OH}$)
3-OH		6.47	s	
C [60 %]				
1	98.8	5.33	d	2.1($J_{1,2}$)
2	66.8	3.48	t-like	1.9($J_{1,2}$, $J_{2,4}$)
3	92.3			
4	71.2	3.40	m	
5	75.3	4.45	ddd	1.0($J_{5,6}$), 2.4($J_{4,5}$), 5.9($J_{6,6'}$)
6	63.9	3.92	dd	1.0($J_{5,6}$), 7.0($J_{6,6'}$)
6′		3.47	dd	6.0($J_{5,6'}$), 7.1($J_{6,6'}$)
3-OH		6.53	s	
4-OH		4.67	d	5.1($J_{4,OH}$)

Structures of B and C are indicated in Fig. 2.

Table 3. Assignment of NMR signals of the oxidized product from MeβGlc (**3**) in D_2O.

Position	^{13}C-NMR	^{1}H-NMR		
	δ (ppm)	δ (ppm)	Pattern	*J* (Hz)
Keto form [65%]				
1	106.1	4.49	d	8.1 ($J_{1,2}$)
2	78.3	4.26	dd	1.7 ($J_{2,4}$), 8.1 ($J_{1,2}$)
3	208.1			
4	73.8	4.36	dd	1.7 ($J_{2,4}$), 10.4 ($J_{4,5}$)
5	77.6	3.54	ddd	2.2 ($J_{5,6}$), 5.1 ($J_{5,6'}$), 10.3 ($J_{4,5}$)
6	62.7	3.99	dd	2.2 ($J_{5,6}$), 12.6 ($J_{6,6'}$)
6′		3.84	dd	5.1 ($J_{5,6'}$), 12.6 ($J_{6,6'}$)
Me	59.1	3.61	s (3H)	
Hydrate form [35%]				
1	104.1	4.43	d	8.1 ($J_{1,2}$)
2	75.6	3.31	d	8.1 ($J_{1,2}$)
3	96.0			
4	72.1	3.45	d	10.0 ($J_{4,5}$)
5	76.7	3.57	ddd	2.3 ($J_{5,6}$), 6.0 ($J_{5,6'}$), 10.1 ($J_{4,5}$)
6	62.4	3.89	dd	2.3 ($J_{5,6}$), 12.3 ($J_{6,6'}$)
6′		3.70	dd	6.0 ($J_{5,6}$), 12.3 ($J_{6,6'}$)
Me	58.8	3.55	s (3H)	

Fraction 2 (retention time 25.2 min in Fig. 5) contained several compounds (Table 6, Supplementary material Fig. S5). The major compound was identified as the hydrate form of 4-keto-1,6-anhydro-β-D-allopyranose (1,6-anhydro-β-D-*ribo*-hexopyranos-4-ulose, **7**; Fig. 1), generated through the isomerization of 3ketoLG via the 3,4-enol intermediate. The moderate $J_{2,3}$ value (4.9 Hz) suggested the axial proton at C3 in the $^{1}C_4$ conformation, supporting the

Table 4. Rate constant and half-life for β-elimination of 3ketoLG (**2**).

	k (h^{-1}) [$t_{1/2}$ (h)]					E_a (kJ/mol)
	30 ºC	40 ºC	50 ºC	60 ºC	70 ºC	
pH 4.0					0.062±0.002 [11]	
pH 5.0					0.20±0.01 [3.5]	
pH 6.0		0.028±0.003 [25]	0.085±0.005 [8.2]	0.32±0.04 [2.1]	1.0±0.1 [0.67]	109
pH 7.0	0.043±0.003 [16]	0.13±0.01 [5.3]	0.33±0.02 [2.1]	1.7±0.1 [0.42]	4.4±0.1 [0.16]	102
pH 8.0	0.083±0.005 [8.4]	0.21±0.02 [3.3]	0.57±0.03 [1.2]	3.0±0.1 [0.23]	9.1±0.4 [0.076]	104

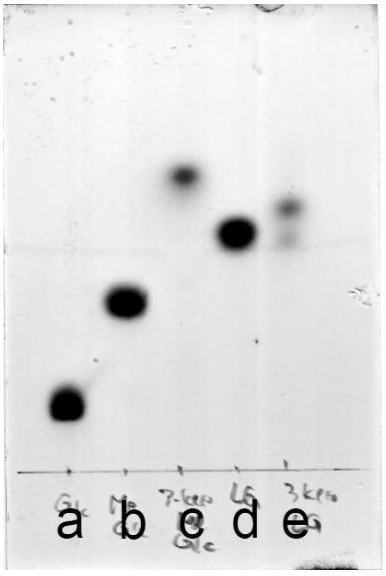

Fig. 3. TLC analysis of the oxidized products.
An aliquot (1 μL) of each sample (50 mM) was spotted on the TLC plate. Lane a, D-glucose; lane b, MeβGlc (**3**); lane c, 3ketoMeβGlc (**4**); lane d, LG (**1**); lane e, 3ketoLG (**2**).

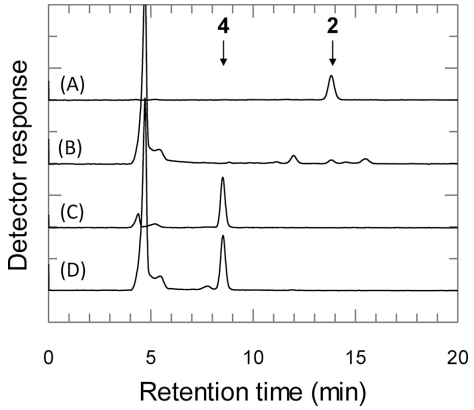

Fig. 4. HPLC chromatogram of the heat treatment of 3ketoLG (**2**) and 3ketoMeβGlc (**4**).
The conditions of separation were a flow rate of 1 mL/min at 60 °C. The positions of compounds **2** and **4** on the chromatogram are indicated by arrows. (A) 3ketoLG before heat treatment; (B) 3ketoLG after heat treatment; (C) 3ketoMeβGlc before heat treatment; (D) 3ketoMeβGlc after heat treatment.

allo structure, not *gluco*. The second major compound was 1,5-anhydro-2-deoxy-hex-1,4-dien-3-ulose (**8**: Fig. 1). The structures of the other minor compounds could not be confidently determined, but one was probably the keto form of **7**.

The 3ketoLG solution in D_2O (6 mg/mL, pH unadjusted)

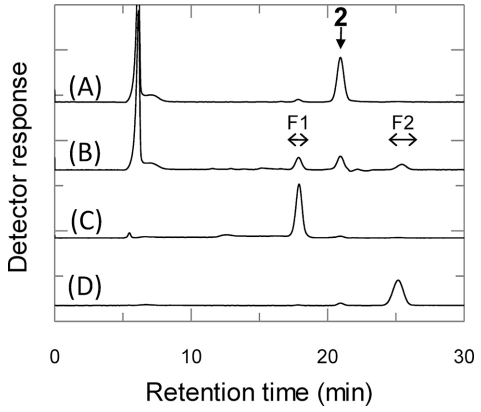

Fig. 5. HPLC chromatogram of the separated products from 3ketoLG (**2**).
The conditions of separation include a flow rate of 0.8 mL/min at 35 °C. The position of compound **2** on the chromatogram is indicated by an arrow. The ranges of Fractions 1 and 2 are indicated by double-headed arrows. (A) 3ketoLG before heat treatment; (B) 3ketoLG after heat treatment; (C) Fraction 1; (D) Fraction 2.

Table 5. Assignment of NMR signals of Fraction 1 in D_2O.

Position	^{13}C-NMR	^{1}H-NMR		
	δ (ppm)	δ (ppm)	Pattern	J (Hz)
1,5-anhydro-D-*erythro*-hex-1-en-3-ulose (**5**) [83%]				
1	152.7	7.53	s	
2	134.7			
3	194.2			
4	69.1	4.52	d	13.4 ($J_{4,5}$)
5	84.4	4.26	dddd	0.5, 2.3 ($J_{5,6}$), 4.5 ($J_{5,6'}$), 13.4 ($J_{4,5}$)
6	61.7	3.98	dd	2.3 ($J_{5,6}$), 12.9 ($J_{6,6'}$)
6′		3.89	dd	4.5 ($J_{5,6'}$), 12.9 ($J_{6,6'}$)
1,6-anhydro-β-D-*erythro*-hexopyranos-3,4-diulose (**6**) [17%]				
1	103.1	5.48	d	2.1 ($J_{1,2}$)
2	74	3.56	d	2.1 ($J_{1,2}$)
3	93.6			
4	93.9			
5	78.5	4.36	dd	0.9 ($J_{5,6}$), 5.6 ($J_{5,6'}$)
6	66.2	4.22	dd	0.9 ($J_{5,6}$), 7.8 ($J_{6,6'}$)
6′		3.69	dd	5.8 ($J_{5,6'}$), 7.9 ($J_{6,6'}$)

used for NMR spectroscopy was kept at 37 °C for 48 h. After the heat treatment, compounds **5, 7,** and **8** were observed in the ^{1}H-NMR spectrum (Fig. 6), suggesting that decomposition occurred in the absence of salts.

DISCUSSION

β-Elimination often occurs at a C–O bond that is two car-

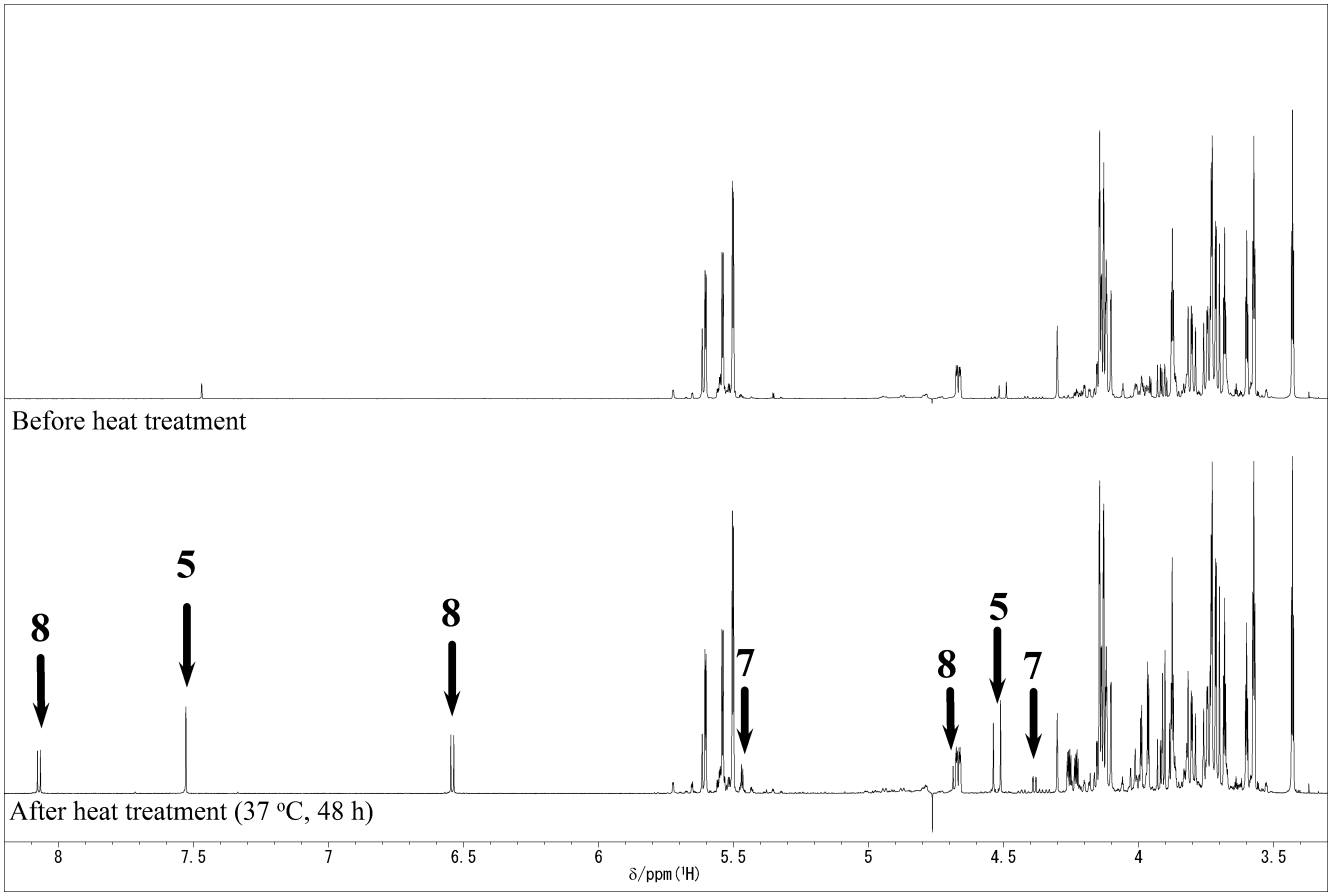

Fig. 6. ^{1}H-NMR spectra of 3ketoLG after heat treatment.
Upper, before heat treatment; lower, after heat treatment. Isolated signals for compounds **5**, **7**, and **8** are indicated with arrows.

Table 6. Assignment of NMR signals of Fraction 2 in D_2O.

Position	^{13}C-NMR	^{1}H-NMR		
	δ (ppm)	δ (ppm)	Pattern	*J* (Hz)
1,6-anhydro-β-D-*ribo*-hexopyrnos-4-ulose (**7**) [77 %]				
1	102.8	5.47	d	2.3 ($J_{1,2}$)
2	71.2	3.83	dd	2.4 ($J_{1,2}$), 4.9 ($J_{2,3}$)
3	69.2	3.79	d	4.9 ($J_{2,3}$)
4	94.7			
5	79.2	4.39	dd	0.7 ($J_{5,6}$), 5.4 ($J_{5,6'}$)
6	66.3	4.02	dd	0.9 ($J_{5,6}$), 8.4 ($J_{6,6'}$)
6′		3.75	dd	5.4 ($J_{5,6'}$), 8.4 ($J_{6,6'}$)
1,5-anhydro-2-deoxy-hex-1,4-dien-3-ulose (**8**) [23 %]				
1	158.3	8.07	d	5.5 ($J_{1,2}$)
2	115.3	6.54	d	5.5 ($J_{1,2}$)
3	178.1			
4	144.5			
5	153.7			
6	57.6	4.69	s (2H)	

bons away from a carbonyl residue. As shown in Fig. 7, generations of compounds **5** and **8** are explainable by β-elimination at C1 and C5, respectively, both of which are two carbons away from the C3 carbonyl residue.

In β-elimination at C1, the elimination occurred only at the C–O bond that forms the 1,6-linkage, not on the bond that forms the 1,5-linkage. The C–O bond was cleaved with the elimination of the hydrogen atom at C2, resulting in compound **5** having a pyranose structure with a double bond between C1 and C2. This compound was reported as the product obtained through the decomposition of 3^{G}-keto-sucrose via β-elimination under alkaline conditions.[8] Because both 3ketoLG and 3^{G}-keto-sucrose are glycosides of 3-keto-D-glucopyranose, generation of the same compound is explainable with β-elimination.

In β-elimination at C5, bond cleavage occurs at the C–O bond with the elimination of the hydrogen atom at C4, resulting in a double bond between C4 and C5. The resulting unstable hemiacetal (7-membered ring) is converted into a linear aldehyde and then rearranged to form compound **8,** which has a pyranose structure with another double bond between C1and C2, as the stable product. Compound **8** is achiral and has other names—ω-hydroxymaltol and 3-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one (nomenclature based on pyran). The generation of compound **8** was observed during the storage of starch after irradiation.[17]

The stability of 3ketoLG in aqueous solution is considerably low. Similar level of instability caused by β-elimination was found with 1,5-anhydro-4-*O*-β-D-glucopyranosyl-D-fructose, whose half-life is 17 h at 30 °C and pH 7.0.[18] The instability of 3ketoLG explains several questions raised in the previous study.[4)5)] Yasui *et al.*[4] found a compound having *m*/*z* of 286, which was formed during acetylation of oxidized LG, synthesized with the help of LGDH, and postulated as a triacetate form of 3ketoLG. However, such structure is not readily explainable with the structure

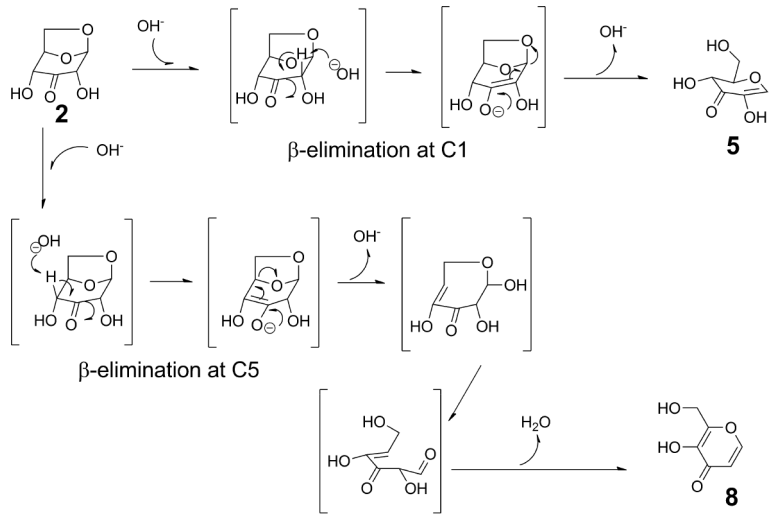

Fig. 7. Scheme of the β-elimination of 3ketoLG (**2**) at C1 and C5.
Compounds shown in the brackets are intermediates and were not detected.

of 3ketoLG because it has only two hydroxyl groups that can be acetylated (see **2** keto form; Fig. 1). Their observation is explainable with the triacetate of compound **5**, which possesses three hydroxyl groups and may be formed during both the LGDH reaction and acetylation of 3ketoLG under alkaline conditions. The instability also explains the difficulty to obtain 3ketoLG by the action of LGDH. Nakahara *et al.*[5] reported the synthesis of 3ketoLG by the action of LGDH using cytochrome P450nor to regenerate NAD^+, but could not successfully isolate the compound. Judging from the reaction conditions described (pH 7.5, 30 °C, 24 h),[5] a significant part of the product had already been decomposed during the reaction, as the half-life of 3ketoLG at pH 7.0 and 8.0 are 16 and 8.4 h, respectively, at 30 °C, which is shorter than the reaction time.

In this study, the author demonstrates that P2O can oxidize LG at its C3 hydroxyl group to generate 3ketoLG. The fact that 3ketoLG can be readily crystallized in the reaction mixture, due to its low solubility, suggests a simple isolation process. The improved availability of 3ketoLG makes it possible for us to revisit the LG-metabolizing pathway through LGDH.

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