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Molecular Basis of Absorption at 340 nm of 3-Ketoglucosides under Alkaline Conditions

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Abstract: Transient absorption at 340 nm under alkaline conditions has long been used to detect the presence of 3-keto-*O*-glycosides without understanding the molecular basis of the absorbance. The time course of $A_{340\ nm}$ for the alkaline treatment of 3-ketolevoglucosan, an intramolecular 3-keto-*O*-glycoside, was investigated to identify the three products generated through alkaline treatment. By comparing the spectra of these compounds under neutral and alkaline conditions, we identified 1,5-anhydro-D-*erythro*-hex-1-en-3-ulose (2-hydroxy-3-keto-D-glucal) as being the compound responsible for the absorption.

Key words: 3-ketoglucoside, alkaline treatment, 3-ketolevoglucosan, 2-hydroxy-3-ketoglucal, Absorption at 340 nm

INTRODUCTION

Recently, 3-keto-*O*-glycosides have attracted attention as intermediates of various metabolic systems. For instance, a multitude of microorganisms produce glucoside 3-dehydrogenase (EC 1.1.2.11) to oxidize various α- and β-glucosides at their C3 hydroxy group using cytochrome; the corresponding 3-ketoglucosides are produced extracellularly and the products are transported into their cells [1−5]. 3-Ketosucrose **1**, the oxidized product from sucrose, has been considered to be a building block for chemicals in organic synthesis [6,7]. *Escherichia coli* possesses a gene cluster consisting of those coding two NAD-dependent dehydrogenases oxidizing glucosides and gulosides at their C3 hydroxy groups, respectively, and a sugar isomerase interconverted the oxidized products [8,9]. Several bacteria metabolize 1,6-anhydro-β-D-glucopyranose (levoglucosan) **2** after oxidizing the C3 hydroxy group by NAD-dependent levoglucosan dehydrogenase (EC 1.1.1.425) to generate 3-ketolevoglucosan (1,6-anhydro-β-D-*ribo*-hexopyranos-3-ulose) **3** [10−12], followed by enzymatic intramolecular β-elimination [13].

The absorption at 340 nm under alkaline conditions was used for a long time to detect the presence of 3-keto-*O*-glycosides. This method was established by Fukui and Hayano in 1969 [14]. The researchers demonstrated rapid increases in absorption at 340 nm when such compounds were treated with an alkaline solution, followed by gradual decreases in absorbance. However, the molecular mechanisms underlying this absorption have not been elucidated.

Kitaoka produced **3** from **2** using pyranose oxidase (EC 1.1.3.10) and discovered that **3** decomposed spontaneously via β-elimination and had a half-life of 16 h at 30 ˚C and pH 7.0; 1,5-anhydro-D-*erythro*-hex-1-en-3-ulose (2-hydroxy-3-keto-D-glucal) **4** and 3-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one (ω-hydroxymaltol) **5** were generated [15]. We considered **3** to be a suitable model of a 3-ketoglucoside to study the molecular mechanism for the absorption at 340 nm under alkaline conditions. In this study, we identified a molecule that represents transient absorption.

MATERIALS AND METHODS

Chemicals and Enzymes. The carbohydrates used in this study (with their compound numbers) are shown in Fig. 1. Compound **3** was prepared by oxidizing **2** with pyranose oxidase, as described previously [15]. 3-Hydroxy-4*H*-pyran-4-one **6** and 5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one (kojic acid) **7** were purchased from Synthonix, Inc. (Wake Forest, NC, USA) and Fuji-Film Wako Pure Chemicals Co. (Osaka, Japan), respectively.

***Preparation of recombinant LgdB1 (3-ketolevoglucosan intramolecular lyase, EC 5.5.1.X) from* Bacillus smithii.** The expression vector pET-28-*lgdB1* was constructed using the SLiCE method [16] with the primers listed in Table 1 amplifying the insert and vector; pGEX-6P-1-*lgdB1* [13] and pET-28(a) were used as templates. The *lgdB1* gene was inserted into pET-28 between the NdeI and XhoI sites;

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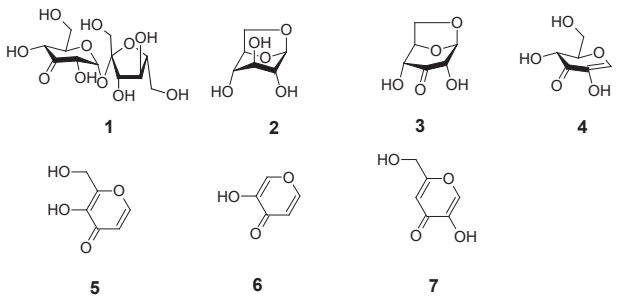

Fig. 1. List of the compounds appearing in this study.

Systematic names are provided with their trivial names within parentheses. **1**, β-D-fructofuranosyl-α-D-*arabino*-hexopyranosid-3-ulose (3-ketosucrose); **2**, 1,6-anhydro-β-D-glucopyranose (levoglucosan); **3**, 1,6-anhydro-β-D-*ribo*-hexopyranos-3-ulose (3-ketolevoglucosan); **4**, 1,5-anhydro-D-*erythro*-hex-1-en-3-ulose (2-hydroxy-3-keto-D-glucal); **5**, 3-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one (ω-hydroxymaltol); **6**, 3-hydroxy-4*H*-pyran-4-one; **7**, 5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one (kojic acid).

Table 1. Primers used to construct pET-28-*lgdB1*.

Name	Sequence
Insert-Forward	CCGCGCGGCAGC*CATAT*GAAATTCGGTTAT
Insert-Reverse	GGTGGTGGTG*CTCGATC*AAACTTGATTCGT
Vector-Forward	ACGAATCAAGTTTG*ATCGAG*CACCACCACC
Vector-Reverse	ATAACCGAATTT*CATATG*GCTGCCGCGCGG

Underlined, sequences based on the insert; italicized, NdeI site (CATATG) and the mutated XhoI site (originally CTCGAG).

however, one base pair at the XhoI site was mutated because of seamless cloning by SLiCE.

LgdB1 [13] with an additional N-terminal His_6 sequence and a thrombin site was prepared using *E. coli* BL21 (DE3) cells harboring the plasmid and purified using the standard procedure [11]. The concentration of the protein was determined by the absorption coefficient (ε) at 280 nm (34,840 $M^{-1}cm^{-1}$) and the molecular weight (32,785) was calculated based on the amino-acid sequence [17].

Preparation of compound 4. Compound **4** was prepared from **3** using LgdB1 [13]. A 125-mL reaction mixture containing 2.0 g of **3** (not completely dissolved at the start of the reaction due to its low solubility) and 0.36 mg LgdB1 in 10 mM Tris-HCl buffer (pH 7.5) was rotary-shaken (150 rpm) at 30 °C for 18 h. After the reaction, His_6-tagged LgdB1 was removed by adding 1 mL of Nuvia IMAC Ni-charged Resin (Bio-Rad Laboratories, Inc. Hercules, CA, USA) followed by filtration. The mixture was deionized by electrodialysis using a Microacylyzer S1 with an AC220-10 membrane cassette (Astom Corporation, Tokyo, Japan), concentrated to approximately 20 mL using a rotary evaporator, and lyophilized to obtain 1.89 g of **4** (yield 94 %).

Alkaline treatment of compounds 3 and 4. An aliquot (50 μL) of 1.25 mM **3** (dissolved in 1 mM HCl) or 0.325 mM **4** (dissolved in water) was mixed with the same volume of 0.2 M NaOH in a well of a 384 microplate (light path = 0.861 cm) at 30 °C. The absorbance at 340 nm ($A_{340\,nm}$) was continuously measured every minute after mixing, using a temperature-controlled microplate reader (Multiskan GO, ThermoFisher Scientific, Waltham, MA). The first-order kinetic constants (k) were calculated by regressing the experimental data in Equation (1) using Grafit Ver. 7 (Erithacus Software Ltd., London, UK).

$$y = A_0 \cdot \exp(-k \cdot t) \quad (1)$$

Substances generated via the alkaline treatment. Compound **3** or **4** was dissolved in 0.1 M NaOH at 30 °C to a concentration of 10 mg/mL. Aliquots (0.5 mL) were collected periodically and neutralized by mixing with 0.5 mL of 0.2 M NaH_2PO_4, followed by evaporation to dryness using a centrifugal evaporator (CVE-3100; Eyela, Tokyo, Japan) equipped with an oil vacuum pump. Each residue was dissolved in 0.7 mL of D_2O and subjected to nuclear magnetic resonance (NMR) analysis.

General Methods. The absorbance spectrum of each compound was measured between 200 and 400 nm in water or NaOH (0.1 M) using a spectrophotometer (UV1800, Shimadzu, Kyoto, Japan). ^{1}H-NMR spectra were recorded at room temperature (20−25 °C) using a Bruker Avance 400 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) in D_2O with 2-methyl-2-propanol as the internal standard (1.23 ppm).

RESULTS

Alkaline treatment of compounds 3 and 4.

The time course of $A_{340\,nm}$ of **3** dissolved in 0.1 M NaOH was monitored at 30 °C. $A_{340\,nm}$ increased during the early stages and reached a maximum after 7 min with ε of 1.06×10^3 $M^{-1}cm^{-1}$ and then decreased gradually (Fig. 2). $A_{340\,nm}$ exceeded half the maximum within 2 min, suggesting that the half-life of **3** under the alkaline conditions was less than 2 min, much shorter than that at pH 7.0 (16 h) [15]. The reaction products were analyzed by ^{1}H-NMR (Fig. 3). After 5 min, the signals corresponding to **4** (7.53 ppm (s), 4.53 ppm (d, J 13.4 Hz)) and **5** (8.07 ppm (d, J 5.5 Hz), 6.54 ppm (d, J 5.5 Hz)), both of which were detected in the decomposition under neutral conditions [15], were detected, indicating that **4** and **5** were the main components. The following signals (8.10 ppm (d, J 0.7 Hz), 8.06 ppm (dd, J 0.7, 5.5 Hz), 6.56 ppm (d, J 5.5 Hz)) are identical with those of authentic **6**, which was not detected under neutral conditions [15]. The ratio of **4**:**5**:**6** at 5 min was 1.00:0.47:0.36 based on the integrations of the signals at 7.53, 6.54, and 6.56 ppm, respectively. After 1 h, the signals corresponding to **3** were not detectable. The signals of **4** decreased as the reaction proceeded, whereas those of the 4*H*-pyrones (**5** and **6**)

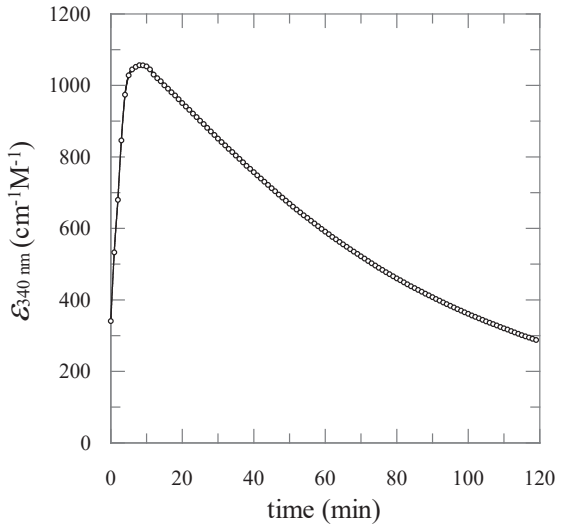

Fig. 2. Time course of the absorption coefficient of **3** at 340 nm in alkaline solution at 30 °C.

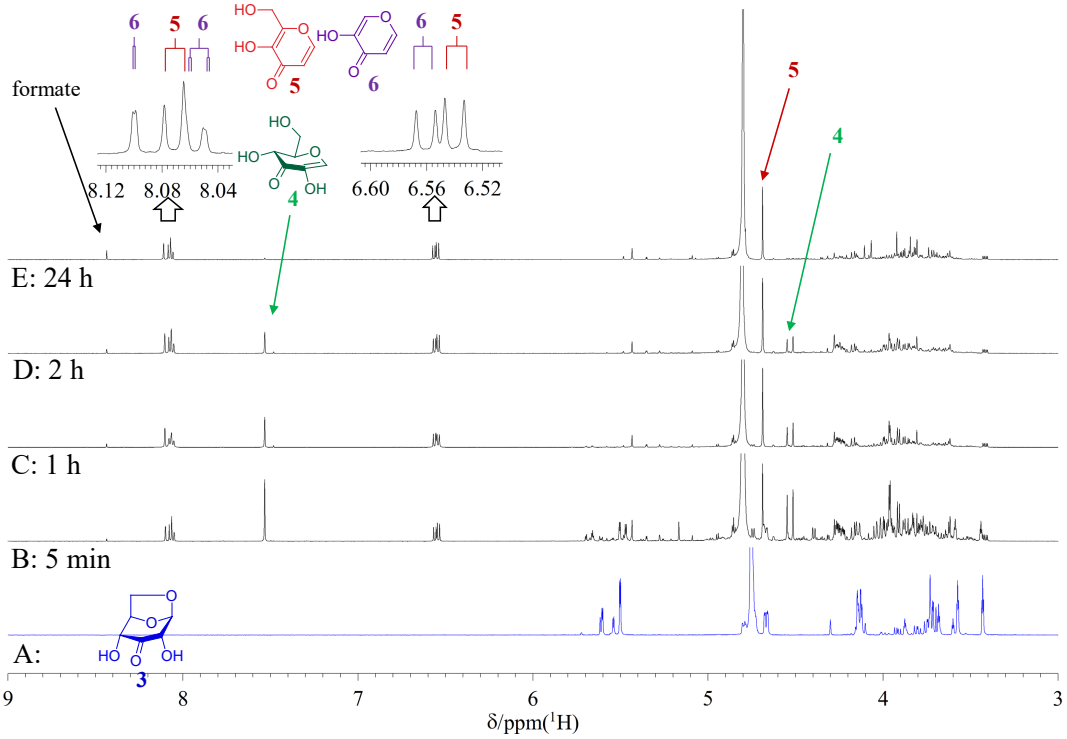

Fig. 3. ^{1}H-NMR analyses of the products generated in alkaline solution of **3**.
A, compound **3** in D_2O; B-E, alkaline treatment of **3** for 5 min, 1 h, 2 h, and 24 h, respectively. The specific signals for compounds **4**, **5**, **6**, and formate are indicated by arrows. The assignments of ^{1}H-NMR signals of compounds **3**, **4**, **5**, and **6** are shown in Table S1 (see J. Appl. Glycosci. Web site).

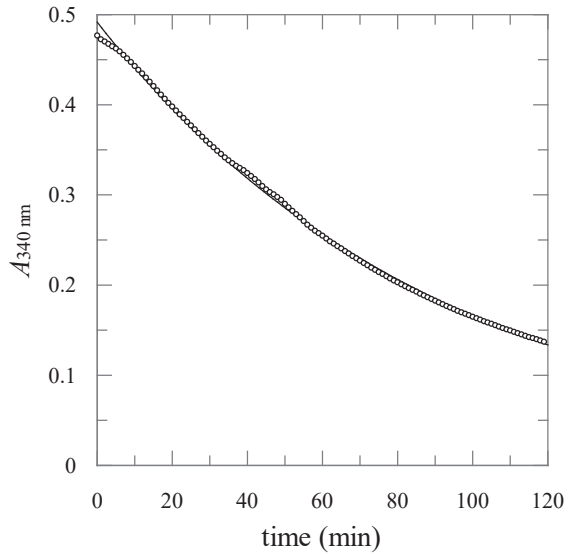

Fig. 4. Time course of $A_{340\ nm}$ of **4** in alkaline solution at 30 °C.
The line is a theoretical one based on Equation (1). The first-order kinetic constant (k) was calculated to be 1.09 (±0.01) × 10^{-2} min^{-1}.

remained unchanged. After 24 h, the signals of **4** disappeared. Notably, the singlet signal at 8.44 ppm corresponding to a salt of formic acid, increased as the intensity of the signals associated with **4** decreased.

$A_{340\ nm}$ of **4** in 0.1 M NaOH decreased with a first-order rate constant of 1.09 (±0.01) × 10^{-2} min^{-1} at 30 °C (Fig. 4). Twenty-four hours after dissolution, the signal associated with **4** disappeared but the signals from 4*H*-pyrones (**5** and **6**) were not detected (Fig. 5), indicating that the signals observed in the alkaline reaction mixture from **3** were not generated via **4**.

Spectrum of each compound.

The spectra of **4** in H_2O and 0.1 M NaOH, as well as those of **6** and **7** (an isomer of **5**, which was not commercially available), are shown in Fig. 6. Compound **4** displayed maximum absorbances in water and 0.1 M NaOH at 294 nm (ε = 5.2 × 10^3 $M^{-1}cm^{-1}$) and 340 nm (ε = 4.0 × 10^3 $M^{-1}cm^{-1}$), respectively. The values for the derivatives of 4*H*-pyrones were almost identical. Compounds **6** and **7** exhibited maximum absorbances in water at 270 nm (ε = 7.8 × 10^3 and 7.5 × 10^3 $M^{-1}cm^{-1}$, respectively). The maximum absorbances in 0.1 M NaOH occurred at 312 nm (ε = 5.6 × 10^3 and 5.8 × 10^3 $M^{-1}cm^{-1}$, respectively). The ε values of compounds **6** and **7** at 340 nm in 0.1 M NaOH were 1.2×10^3 and 1.7×10^3 $M^{-1}cm^{-1}$, respectively, suggesting that **4** contributed mainly to the absorption at 340 nm.

DISCUSSION

We established that the compound responsible for a transient increase in $A_{340\ nm}$ in an alkaline solution of 3-ketoglucosides was **4**. The compound was already found in an alkaline solution of **1** as the major product [6], but the spectral data were not available. Though 1,5-anhydro-D-*erythro*-hexo-2,3-diulose **8**, the keto-enol tautomerized isomer of **4**, was reported as the alkaline degradation product of 3-ketoglucoside in the 1950s [18,19]; we hypothesize that this was due to the misidentification of **4** for the following reasons. Compound **8** was not found in the alkaline solutions of **1** [6] and **3** [15], and the absorption at 340 nm was not predictable because of the lack of a resonance structure. Furthermore, **8** is stable as a dihydrate and is produced from 1,5-anhydro-D-fructose **9** via oxidation by pyranose oxidase [20], suggesting that keto-enol tautomerization between **4**

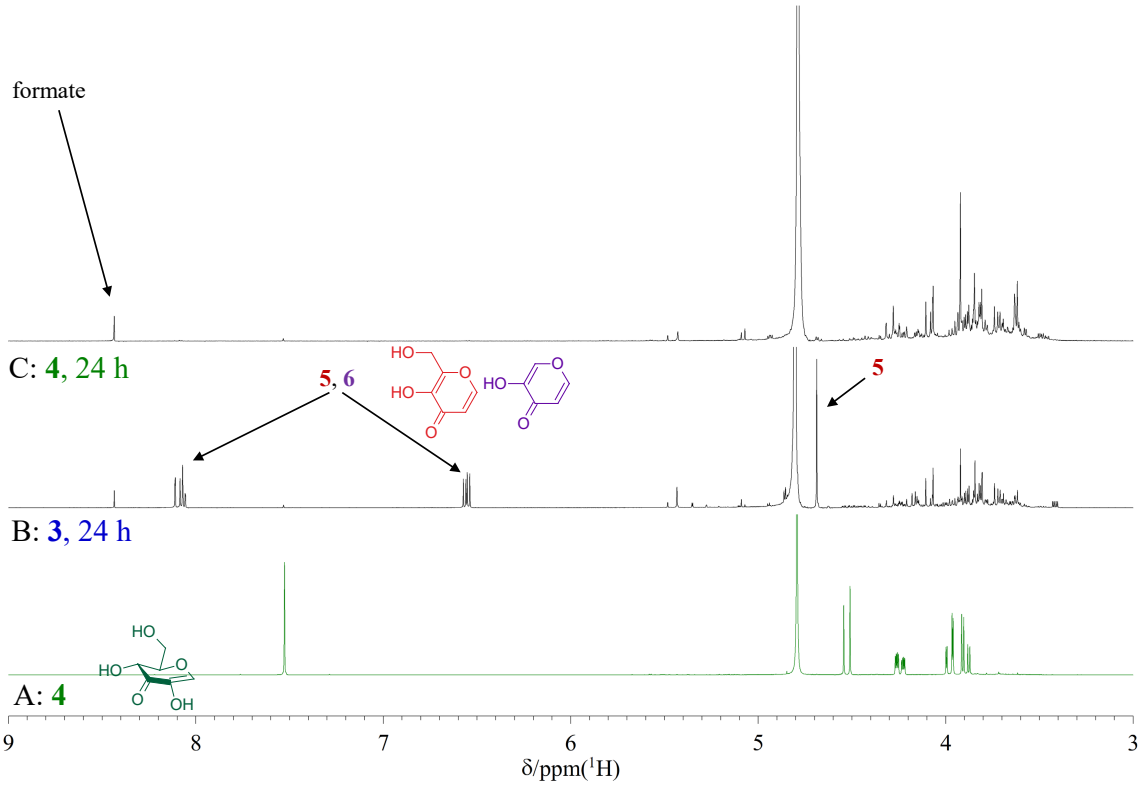

Fig. 5. ^{1}H-NMR analyses of the products generated in alkaline solution of **3** and **4**.
A, compound **4** in D_2O; B, alkaline treatment of **3** for 24 h; C, alkaline treatment of **4** for 24 h. The specific signals for compounds **5**, **6**, and formate are indicated by arrows. The assignments of ^{1}H-NMR signals of compounds **3**, **4**, **5**, and **6** are shown in Table S1 (see J. Appl. Glycosci. Web site).

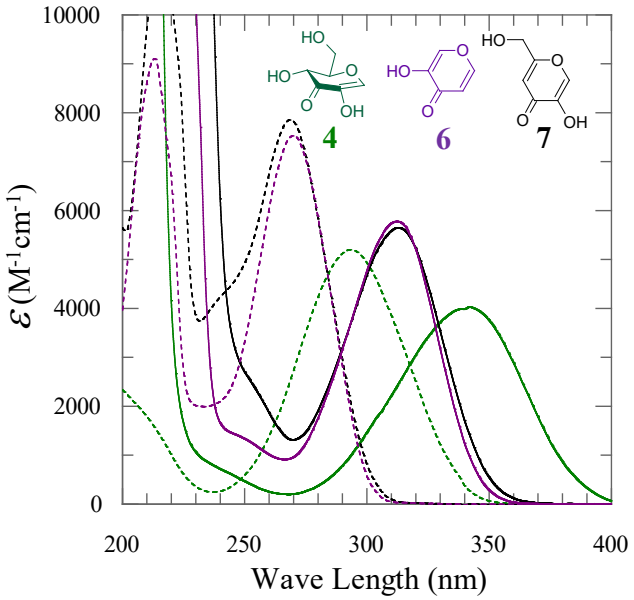

Fig. 6. Spectra of compounds **4**, **6**, and **7**.
The green, violet, and black lines represent the spectra of **4**, **6**, and **7**, respectively. The broken and solid lines indicate the spectra in H_2O and 0.1 M NaOH, respectively.

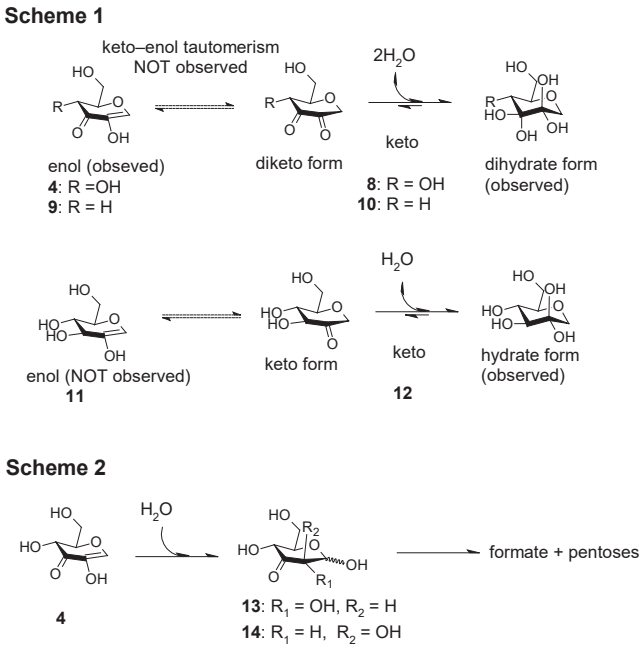

Fig. 7. Schemes of the reactions.
Scheme **1**, keto-enol tautomerism of compounds with 2-hydroxyglucal structure; Scheme **2**, alkaline treatment of **4**. Systematic names of the compounds are provided with their trivial names in parentheses. **8**, 1,5-anhydro-D-*erythro*-hex-2,3-diulose (3-keto-1,5-anhydro-D-fructose); **9**, 1,5-anhydro-4-deoxy-D-*glycero*-hex-1-en-3-ulose (ascopyron P), **10,** 1,5-anhydro-4-deoxy-D-*glycero*-hex-2,3-diulose (ascopyronT);**11**,1,5-anhydro-D-*ribo*-hex-1-enitol(2-hydroxy-D-glucal); **12**, 1,5-anhydro-D-*ribo*-hex-2-ulose (1,5-anhydro-D-fructose); **13,** D-*ribo*-hexos-3-ulose (3-keto-D-glucose); **14**, D-*arabino*-hexos-3-ulose (3-keto-D-mannose).

and **8** does not occur spontaneously (Fig. 7, Scheme 1). Keto-enol tautomerization between ascopyron P **9** and ascopyron T (dihydrate form) **10**, which are 4-deoxy derivatives of **4** and **8**, also does not occur spontaneously [21,22]. Notably, the 3-keto structure is necessary to stabilize the 2-hydroxyglucal-like structures of **4** and **9** because 2-hydroxy-D-glucal **11** is unstable and has never been isolated, whereas 1,5-anhydro-D-furctose **12**, the keto tautomer of **11**, is commercially produced from starch using α-1,4-glucan lyase (EC 4.2.2.13) [23].

It is assumed that 3-ketoglucosides in alkaline solution

undergo a fast β-elimination at the β-glucosidic C-O bond with the proton at C2 to form **4**; **4** is gradually hydrated to generate 3-keto-D-glucose **13** and 3-keto-D-mannose **14**, which have been reported as being unstable compounds that decompose into formic acid and pentoses [8] (Fig. 7, Scheme 2). The hydration is akin to a reverse reaction of β-elimination and can occur in the presence of abundant water molecules. The detection of formic acid during the alkaline treatments of **3** and **4** supported this hypothesis. We concluded that **4** exhibited absorption at 340 nm when 3-ketoglucosides were treated in an alkaline solution.

CONFLICTS OF INTERESTS

The authors declare no conflicts of interest associated with this manuscript.

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