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Note

Formation of a di-D-fructose dianhydride from levan by *Arthrobacter ureafaciens*

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It has been shown that three di-D-fructose dianhydrides are produced by acid hydrolysis of inulin[1–4]. Two of these, di-D-fructofuranose 1,2′:2,3′- and 1,2′:2,1′-dianhydrides, are produced by enzymic reaction on inulin[5,6]. In contrast to these (2→1)-linked di-D-fructose dianhydrides, di-D-fructofuranose 2,6′:6,2′-dianhydride (alliuminoside) has been isolated[7] from the bulbs of *Allium sewertzowi* Rgl. These findings led us to examine the microbial formation of di-D-fructose dianhydride from bacterial levan. The present paper reports the formation, by *Arthrobacter ureafaciens*, of a di-D-fructose dianhydride, probably an anomer of alliuminoside.

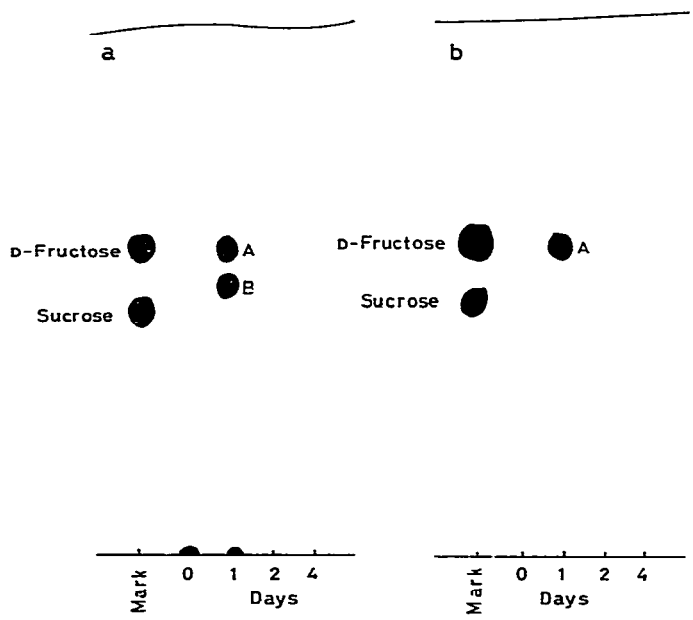


Fig. 1. Paper chromatograms showing formation of a nonreducing sugar in the culture medium containing levan as the sole carbon source: (a) Sprayed with anthrone–phosphoric acid reagent; and (b) sprayed with alkaline silver nitrate reagent.

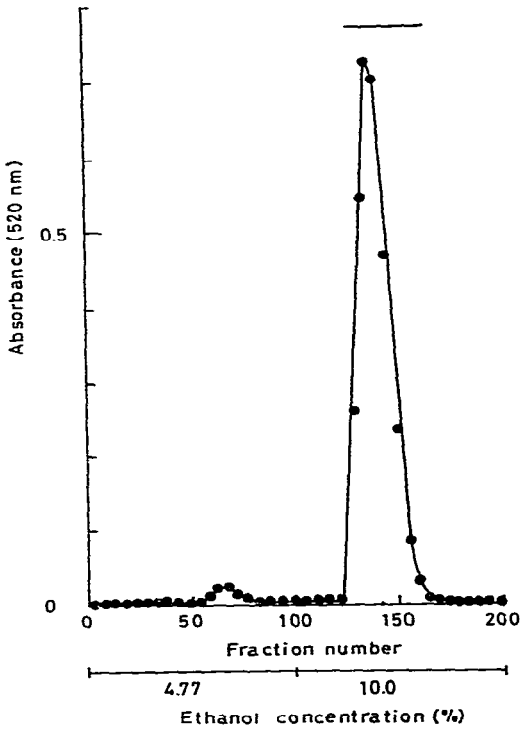


Fig. 2. Elution of the nonreducing sugar product from a carbon–Celite column.

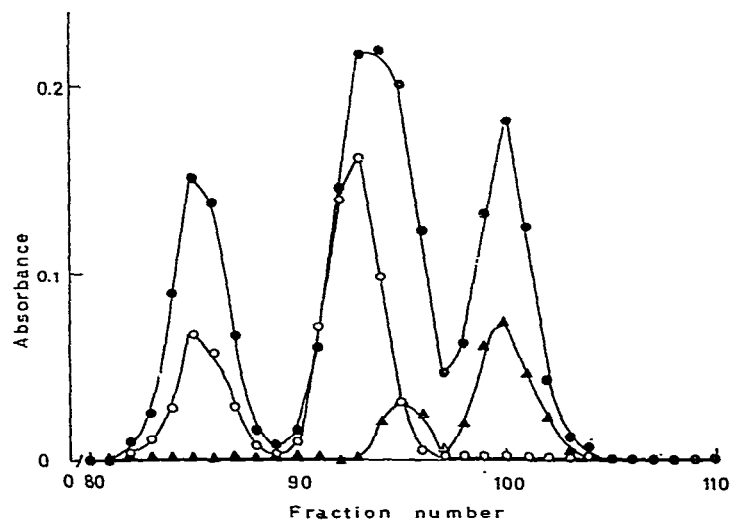


Fig. 3. Determination of degree of polymerization of the nonreducing sugar product by Bio-Gel P-2 gel-filtration: (▲) By Somogyi–Nelson method[10] (500 nm); (○) by resorcinol–hydrochloric acid method[11] (520 nm); and (●) by anthrone–sulfuric acid method[12] (620 nm).

One day after inoculation, paper chromatographic separation of the product of degradation, by *A. ureafaciens*, of a levan from *Bacillus mesentericus*, and spraying with the anthrone–phosphoric acid reagent[8] showed two spots (A and B, Fig. 1a). Spot B could not be detected by the alkaline silver nitrate reagent[9], suggesting it to be a nonreducing sugar (Fig. 1b). Neither spot could be detected after cultivation for more than two days.

The compound was isolated from the medium of a 12-h culture degraded by *A. ureafaciens*. After fractionation on a carbon–Celite column (Fig. 2) and treatment with ion-exchange resins, the product crystallized and was purified by repeated crystallizations. It was nonreducing toward the Somogyi–Nelson reagent[10], and nonfermentable by bakers' yeast. It was hydrolyzed completely by heating with 5mM sulfuric acid for 30 min at 100°. The resulting monosaccharide component was identified as D-fructose by t.l.c. and formation of the methylphenyl-osazone. Bio-Gel P-2 gel-filtration (Fig. 3) suggested it to be a dimer of D-fructose. Methylation, followed by hydrolysis with oxalic acid, gave, in 80% yield, 1,3,4-tri-*O*-methyl-D-fructose having a specific rotation close to that reported in the literature[13]. Periodate oxidation showed consumption of two moles of periodate per mole of the product, calculated as a di-D-fructose dianhydride.

The ^{13}C-n.m.r. spectrum for a solution in deuterium oxide (Fig. 4) shows six narrow signals at δ 103.5, 81.2, 77.4, 72.3, 60.7, and 59.8. C-2 (δ 103.5), C-1 and C-6 (60.7–59.8), and C-3, C-4, and C-5 (81.2–72.3) were assigned by comparison with the chemical shifts of related substances[14–17]. The present spectrum supports the deduction that the two D-fructose moieties are in the furanose form, that they occupy a symmetrical position relative to each other, and that both have the β-D configuration.

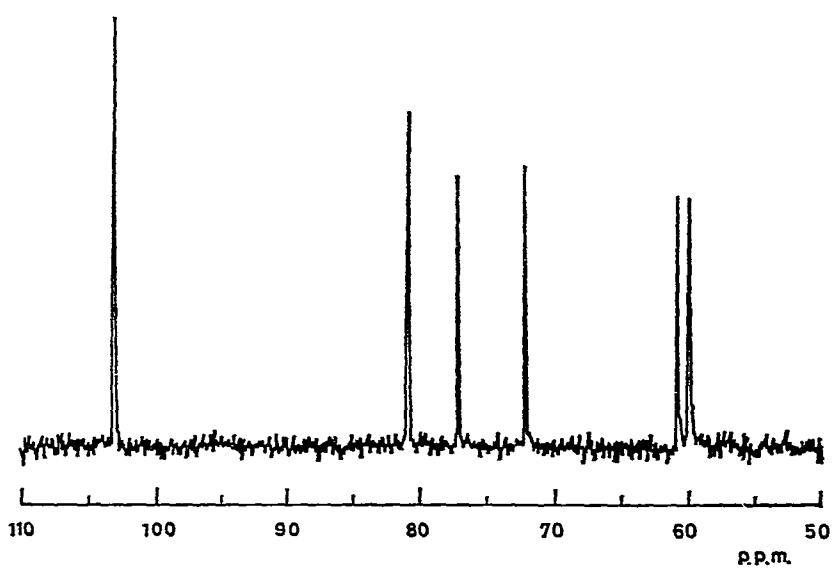


Fig. 4. Carbon-13 n.m.r. spectrum of di-fructose anhydride IV (1).

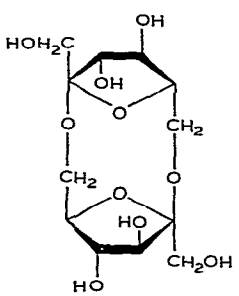
TABLE I

COMPARISON OF DI-D-FRUCTOSE ANHYDRIDE IV (1) WITH ALLIUMINOSIDE

Properties of	*Compound*	
	Di-D-fructose anhydride IV (1)	*Alliuminoside*[7]
Free sugar		
M.p.	177–178°	92–93°
$[\alpha]_D^{20}$	−32° (*c* 1.205)[a]	−23.8°
Hexa-*O*-acetyl derivative		
M.p.	142–143°	98–99°
$[\alpha]_D^{20}$	−54.9° (*c* 0.695)[b]	−29.3°
Hexa-*O*-methyl derivative		
$[\alpha]_D^{20}$	−56.6° (*c* 1.100)[b]	−39.7°

[a]Water solution. [b]Chloroform solution.

As mentioned earlier, the compound was estimated to be a dimer of D-fructose by Bio-Gel P-2 gel-filtration. Except for this finding, the results described earlier would not exclude the presence of 2,6-anhydro-β-D-fructofuranose. However, both melting point and specific rotation in water (lit.[18] m.p. 118–119°, $[\alpha]_D^{25}$ −107°) of the latter compound eliminate this possibility. Therefore, the present compound is di-β-D-fructofuranose 2,6′:6,2′-dianhydride (**1**). Alliuminoside[7] from the bulbs



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of *Allium sewertzowi* Rgl. has been reported as a di-D-fructose 2,6′:6,2′-dianhydride but having unknown anomeric configurations. Marked differences as to melting points of the free sugar and the hexa-*O*-acetyl derivative were observed between **1** and alliuminoside (Table I). Therefore, **1** is probably an anomer of alliuminoside, and we propose the name of "difructose anhydride IV" as a trivial name. Isolation of an enzyme responsible for the formation of **1** from levan is under study.

EXPERIMENTAL

General methods. — Melting points are uncorrected. The optical rotation was determined with a Jasco DIP 180 type polarimeter having a 1.0-dm light-path. The

^{13}C-n.m.r. spectrum for a solution in deuterium oxide (internal standard of tetramethylsilane; 0.2 mL of solution containing 50 mg of product in a 5-mm tube) was recorded with a Jeol FX 90-Q type spectrometer at room temperature; pulse angle, 45°; data point, 16 k; No. of pulses, 400; spectral width, 2.5 kHz; and digital resolution, 0.3 Hz. For moisture determination, samples were dehydrated *in vacuo* at 63° in the presence of phosphorus pentaoxide until a constant weight was obtained. Unless otherwise stated, all evaporations were performed under diminished pressure at 40°.

Paper and thin-layer chromatographies. — For the paper-chromatographic monitoring of the levan degradation, an aliquot (2 mL) of the culture medium, withdrawn with a sterilized syringe, was centrifuged to remove bacterial cells, and desalted with a column of Amberlite IR-120 (H^+) and IR-45 (OH^-) ion-exchange resins. The sample was concentrated, dried in the presence of silica gel, and dissolved in distilled water (0.1 mL). Aliquots (10 μL) were spotted on Whatman No. 1 filter paper, and developed twice, ascending, with 6:4:3 (v/v) 1-butanol–pyridine–water[19].

T.l.c. monitoring of the acid hydrolysis of **1** was performed on Avicel SF plates (Funakoshi Chemical Co. Tokyo, Japan), developed twice with the same solvent system as just described (ascending). The plates were sprayed with the anthrone–phosphoric acid reagent[8].

The fractions obtained by silica gel column chromatography were monitored by t.l.c. on Silica gel 60 F_{254} plates (Merck), developed twice with the same solvent system, 1,3,4-tri-*O*-methyl-D-fructose being the reference. The spots were detected by spraying with the naphthoresorcinol–sulfuric acid reagent[20].

Microorganisms. — *Bacillus mesentericus* Trevisan (IFO 3034) was obtained from the Institute for Fermentation, Osaka. *Arthrobacter ureafaciens* used in this study was the strain previously described[21].

Preparation of levan. — Levan was prepared by cultivating, in a 500-mL volume Sakaguchi flask, the bacterium *B. mesentericus* in the medium (186 mL) described by Bell and Dedonder[22] with 0.2 g of yeast extract/L of medium at pH 5.8 by reciprocal shaking (110 strokes/min) for 10 days at 30°. Separation of levan from the medium was performed by the procedure A of alcoholic precipitation described by Avigad[23]. The levan was further purified by dialysis under toluene in a cellophane tube against tap water for 2 days, followed by treatment with Amberlite MB-1 resin. The purified levan was lyophilized to give 6–8 g/L of culture medium, $[\alpha]_D^{20}$ −47° (*c* 0.51, water); lit.[24,25] (with the same bacterial species) $[\alpha]_D^{18}$ −45.3°, and $[\alpha]_D^{20}$ −47° (water).

Anal. Calc. for $(C_6H_{10}O_5)_x$: C, 44.42; H, 6.20. Found: C, 44.59; H, 6.04.

1,3,4-Tri-O-methyl-D-fructose. — This compound, m.p. 71°, $[\alpha]_D^{20}$ −58° (*c* 0.505, water) was prepared as previously described[24] [lit.[24] m.p. 73°, lit.[13] $[\alpha]_D^{18}$ −57° (water)] from a tri-*O*-methyllevan preparation $[\alpha]_D^{20}$ −60.9° (*c* 0.855, chloroform), which had been prepared by application of the method of Hakomori[26] to levan, followed by dialysis in a cellophane tube against tap water.

Isolation of **1** *from the culture medium.* — The solid culture-medium for stock culture of *A. ureafaciens* had the same composition as described[21], except that in this

case inulin was replaced by levan (10 g), and 0.2 g of yeast extract was added per L of medium. The cultivation to isolate **1** was performed at 30°, in 150 mL of liquid medium in a 500-mL volume Sakaguchi flask, by reciprocal shaking at 110 strokes/min. The 12-h culture media were combined (about 750 mL) and centrifuged for 15 min at 0° and 10 000*g* to remove the bacterial cells. The clear solution was heated for 20 min at 80°, concentrated to ~100 mL, and stored under toluene in a refrigerator for several days. The sediment produced was removed by centrifugation. The supernatant contained sugars at the concentration of about 80 mg/mL as D-fructose by the resorcinol–hydrochloric acid method[11]. One half of the volume of the solution was fractionated on an active carbon–Celite column (4.7 × 31.8 cm) composed of Tokusei Shirasagi carbon (60 g, Takeda Pharma. Indust. Co., Osaka, Japan) and Celite No. 535 (120 g, Johns-Manville Co., U.S.A.), prewashed as reported earlier[21]. After being washed with distilled water to remove any monosaccharide, the column was eluted with 4.77 and 10% ethanol solutions, successively, at a flow rate of 5 mL/min, and 20-mL fractions were collected, which were monitored for ketohexose[11]. The fractions eluted with 10% ethanol (indicated by the bar in Fig. 2) were pooled, and concentrated to about 5 mL. The sediment produced was removed by centrifugation, and the clear solution was desalted with Amberlite MB-1, IR 120 (H^+), and IR 45 (OH^-) ion-exchange resins, and then further concentrated to a thick syrup. The product, difructose anhydride IV (**1**), crystallized from 95% ethanol in the cold (yield ~3.5%, based on the levan). Recrystallization was repeated until a constant melting point was obtained.

Methylation and acid hydrolysis of the methylated product. — Compound **1** (30 mg) was methylated as previously described[21] (yield 27.3 mg).

Anal. Calc. for $C_{18}H_{32}O_{10}$ (hexa-*O*-methyl derivative): C, 52.93; H, 7.90. Found: C, 52.88; H, 8.07.

A portion of the methylated derivative (39 mg) was hydrolyzed with oxalic acid in methanol and subsequently in water as described previously[27], except that the periods of hydrolysis were 6 and 2 h, respectively. After neutralization with calcium carbonate and extraction with chloroform as described previously[24], the extract was evaporated at 30° under diminished pressure to a syrup $[\alpha]_D^{20}$ −57.9° (*c* 1.04, water). The amount of methylated D-fructose derivative in the syrup was estimated by the phenol–sulfuric acid method[13] using D-fructose as the standard.

Hexa-O-acetyl derivative. — Compound **1** (24 mg) was acetylated[6] with acetic anhydride (1 mL) and pyridine (1 mL) for 24 h at 0°. The product was crystallized by evaporation from ethanol and recrystallized from ethyl ether (yield 31 mg).

Anal. Calc. for $C_{24}H_{32}O_{16}$: C, 49.89; H, 5.60. Found: C, 49.24; H, 5.68.

Periodate oxidation. — Portions of **1** (13.2 and 11.8 mg; 40.7 and 36 μmol, respectively, as di-D-fructose dianhydride) were oxidized, each in 10 mL of 20mM sodium metaperiodate for 24 and 48 h, respectively, at 25°, and a periodate consumption of 81.4 and 71.4 μmol, respectively, was determined as previously described[28].

Preparation of the osazone from the acid hydrolyzate of **1**. — Compound **1** (20 mg) was completely hydrolyzed by heating for 30 min at 100° with 5mM sulfuric

acid (5 mL). The mixture was made neutral with 20mM sodium hydroxide, desalted with Amberlite MB-1 resin, and concentrated. A small portion of the resulting syrup was used for t.l.c., and the rest was used for the preparation, by the method previously described[29], of methylphenylosazone, m.p. 149°, lit.[29] 153°; mixed m.p. with authentic osazone (m.p. 150°) 149–150°.

Bio-Gel P-2 gel-filtration. — Bio-Gel P-2 gel-filtration was performed as previously described[30] by applying a 2.8-mL mixture containing 800 μg each of **1**, D-glucose, maltose, and raffinose on a column (2.5 × 100 cm) of Bio-Gel P-2 (Pharmacia, Uppsala, Sweden, 40–120 μm). The column was eluted with water at a flow rate of 12 mL/h, and 4-mL fractions were collected.

Silica gel column chromatography. — The acid hydrolyzate of methylated **1** (39 mg) was dissolved in a small volume of chloroform and fractionated on a column (3.3 × 30 cm) of Wakogel C-200 (Wako Pure Chemical Co., Osaka, Japan) by elution with the upper layer of 200:47:15 (v/v) benzene–ethanol–water at a flow rate of 2.2 mL/h and collecting 20-mL fractions.

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