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Note

New structure for the O-polysaccharide of *Providencia alcalifaciens* O27 and revised structure for the O-polysaccharide of *Providencia stuartii* O43

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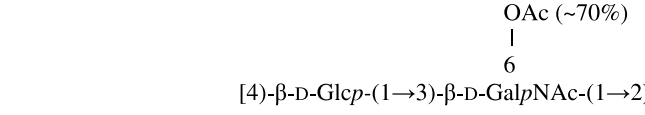
Abstract—The O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide from *Providencia alcalifaciens* O27 and studied by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, ROESY, H-detected $^{1}H,^{13}C$ HSQC, and HMBC experiments. It was found that the polysaccharide is built up of linear partially O-acetylated tetrasaccharide repeating units and has the following structure:



where Qui4NFo stands for 4-formamido-4,6-dideoxyglucose (4-formamido-4-deoxyquinovose).

The O-polysaccharide structure of *Providencia stuartii* O43 established earlier was revised with respect to the configuration of the constituent 4-amino-4,6-dideoxyhexose (from Rha4N to Qui4N).

Keywords: *Providencia alcalifaciens*; *Providencia stuartii*; O-Antigen; Lipopolysaccharide; Polysaccharide structure

Gram-negative bacteria of the genus *Providencia* are divided into six species, including *P. alcalifaciens*, *P. rustigianii*, *P. stuartii*, *P. heimbachae*, *P. rettgerii*, and *P. vermicola*.[1,2] They are facultative pathogens that under favorable conditions cause enteric diseases, as well as wound and urinary-tract infections.[2] These infections are frequently persistent, difficult to treat and may even result in fatal bacteremia. The serological classification scheme of three *Providencia* species, *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii*, used in serotyping of clinical isolates, is based on the lipopolysaccharide (LPS, O-antigen, endotoxin) and flagella (H-antigens) and includes 63 serogroups.[3] Immunochemical studies of *Providencia* O-antigens aim at the creation of the molecular basis for the serological classification and cross-reactivity of *Providencia* strains and related bacteria, including *Proteus*. At present, 25 *Providencia* O-polysaccharide structures have been established (Refs. 4–14 and references cited in Refs. 4 and 7). In this paper, we report on a new structure of the O-polysaccharide of *P. alcalifaciens* O27 and a revised structure of the O-polysaccharide of *P. stuartii* O43 studied by us earlier.[8]

Mild acid degradation of the LPS of *P. alcalifaciens* O27 gave a high-molecular-mass polysaccharide eluted from Sephadex G-50 immediately after the void volume.

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Sugar analysis using GLC–MS of the alditol acetates derived after hydrolysis with CF_3CO_2H showed the presence of glucose (Glc), 2-amino-2-deoxygalactose (GalN), and 4-amino-4,6-dideoxyglucose (Qui4N) in ratios ~1:0.6:0.8. GLC–MS of the acetylated methyl glycosides demonstrated the presence of glucuronic acid (GlcA). The D configuration of all monosaccharides was determined by GLC of the acetylated (*S*)-2-octyl glycosides and confirmed by NMR spectroscopy (see below).

Linkage analysis by GLC–MS of the partially methylated alditol acetates derived from the methylated polysaccharide revealed 4-substituted Glc, 3-substituted GalN, and 2-substituted Qui4N. In addition to these monosaccharides, similar analysis after carboxyl-reduction of the methylated polysaccharide showed the presence of 4,6-disubstituted Glc, which was evidently derived from 4-substituted GlcA.

The ^{13}C NMR spectrum of the polysaccharide showed structural heterogeneity, which, most likely, was caused by nonstoichiometric O-acetylation. The ^{13}C JMOD NMR spectrum of the O-deacetylated polysaccharide (OPS) (Fig. 1) showed a regular structure. It contained signals for four sugar residues, including those for four anomeric carbons at δ 99.0, 101.5, 104.8, and 105.7, 14 sugar-ring carbons in the region δ 67–83, one C–CH_3 group at δ 18.0 (minor *E* isomer) and 18.1 (major *Z* isomer), two C–CH_2OH groups at δ 61.9 and 62.6, one C–CO_2H group at δ 176.5, and two nitrogen-bearing carbons at δ 52.8 and 57.0. The spectrum also contained signals for one *N*-acetyl group at δ 176.7 (CO) and δ 23.8 (CH_3) as well as one *N*-formyl group at δ 166.1 and 169.0 (major *Z* and minor *E* isomer, respectively). There was only one signal within the region δ 82–88, at δ 82.5 later assigned to C-2 of Qui4N; hence, all sugar residues in the repeating unit are pyranosidic.[15] The ^1H spectrum of the OPS contained signals for four anomeric protons at δ 4.52, 4.67, 5.32, and 5.69, one C–CH_3 group at δ 1.13 (major) and 1.18 (minor), one *N*-acetyl group at δ 2.01, one *N*-formyl group at 8.18 (major *Z* isomer) and 7.99 (minor *E* isomer) (compare published data[10]), and other protons in the region δ 3.33–4.18.

The ^1H and ^{13}C NMR spectra of the OPS were assigned using $^1H,^1H$ COSY, TOCSY, ROESY, $^1H,^{13}C$ HSQC (Fig. 2), $^1H,^{13}C$ HSQC–TOCSY, and $^1H,^{13}C$ HMBC (Fig. 3) experiments (Table 1). The spin system of Qui4N was distinguished by correlations of each of H-1 and H-6 with all other protons of this residue in the TOCSY spectrum, and the H-2 to H-5 signals were assigned using the COSY spectrum. The ^{13}C NMR signals were assigned by H/C correlations in the HSQC spectrum, H-6/C-4 and H-6/C-5 correlations in the HMBC spectrum, and H-6/C-2 and H-6/C-3 correlations in the HSQC–TOCSY spectrum. A difficulty in the assignment of the C-2 and C-3 signals owing to an overlap of the H-2 and H-3 signals at δ 3.71 was overcome by taking into account the methylation analysis data: the lower field resonance at δ 82.5 was assigned to C-2 of the 2-substituted Qui*p*4N and, accordingly, the other signal at δ 70.6 to C-3. A cross-peak at δ 8.18/57.0 between the *N*-formyl group and C-4 of Qui4N in the HMBC spectrum demonstrated formylation at N-4. A small $J_{1,2}$ coupling constant <3 Hz and chemical shifts of H-1 (δ 5.69) and C-5 (δ 68.3, compare published data[16]) indicated that Qui*p*4NFo is α-linked.

The assignment of the ^1H NMR signals for another monosaccharide was performed using the COSY spectrum, which demonstrated correlations between all neighboring protons from H-1 to H-5, and were confirmed by the TOCSY experiment. A triplet form of the H-3 signal at δ 3.96 ($J_{2,3} \approx J_{3,4} \sim 9$ Hz) revealed the *gluco*-configuration of this residue. A doublet splitting of the H-5 signal and a H-5/C-6 cross-peak at δ 4.07/176.5 in the HMBC spectrum indicated that this residue is GlcA. A small $J_{1,2}$ coupling constant <3 Hz and chemical shift of the H-1 (δ 5.32) showed its α configuration.

The ^{13}C NMR signals of Glc*p* were distinguished by the HSQC–TOCSY experiment showing correlations

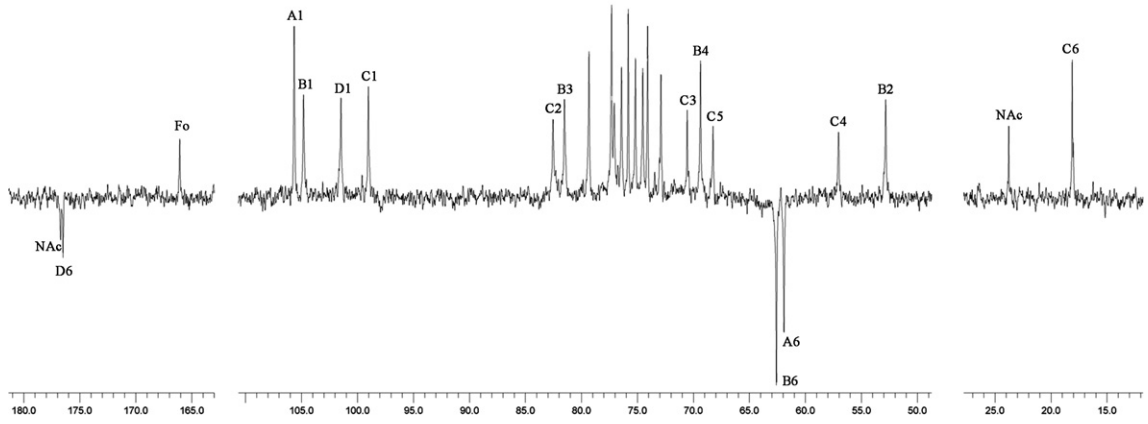

Figure 1. JMOD spectrum of the O-deacetylated polysaccharide from *P. alcalifaciens* O27. Arabic numerals refer to carbons in sugar residues denoted by capital letters as shown in Table 1.

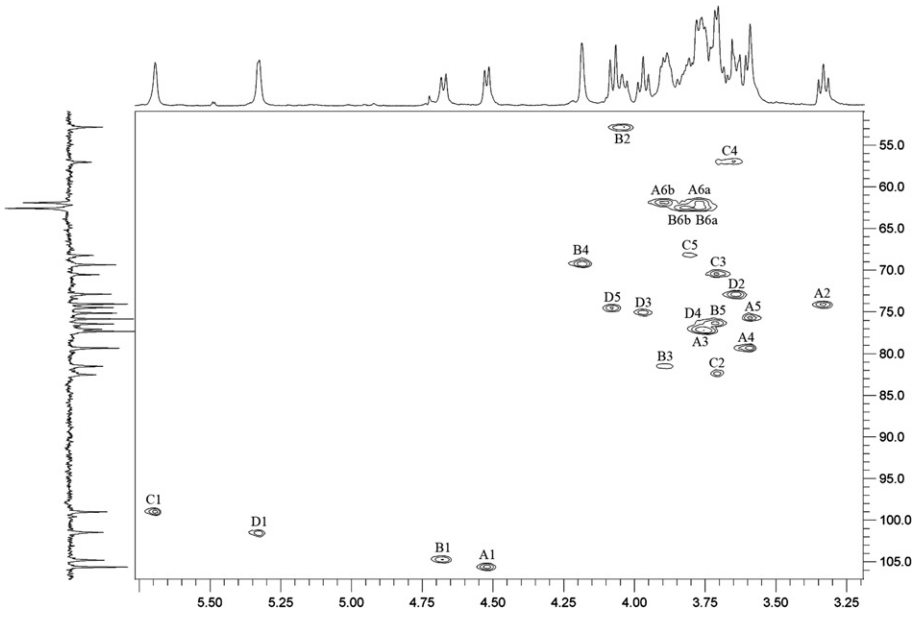

Figure 2. Part of a $^{1}H,^{13}C$ HSQC spectrum of the O-deacetylated polysaccharide from *P. alcalifaciens* O27. The corresponding parts of the ^{1}H and JMOD ^{13}C NMR spectra are shown along the horizontal and vertical axes, respectively. Arabic numerals refer to H/C pairs in sugar residues denoted by capital letters as shown in Table 1.

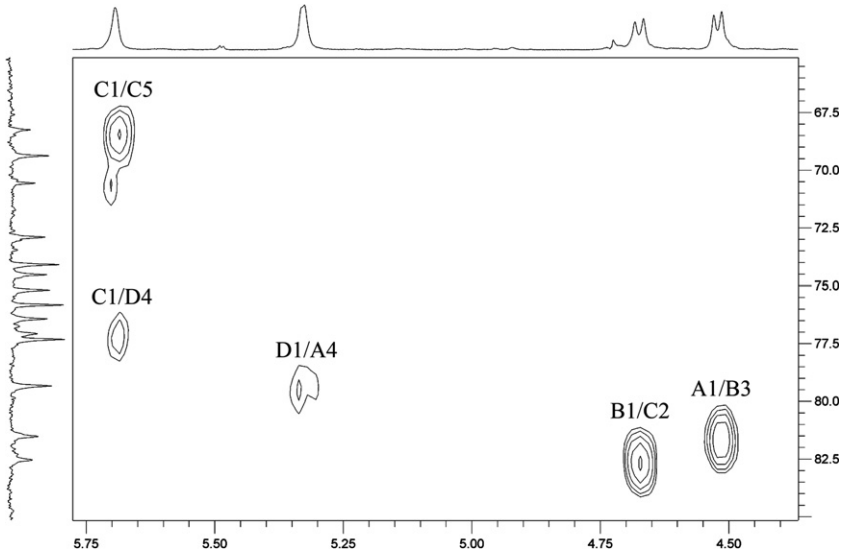

Figure 3. Part of a $^{1}H,^{13}C$ HMBC spectrum of the O-deacetylated polysaccharide from *P. alcalifaciens* O27 showing correlations for anomeric protons. The corresponding parts of the ^{1}H and JMOD ^{13}C NMR spectra are shown along the horizontal and vertical axes, respectively. Arabic numerals refer to atoms in sugar residues denoted by capital letters as shown in Table 1.

of H-1 with all carbons of this residue, none of which bears nitrogen. The corresponding proton signals were found using the HSQC spectrum and assigned by the COSY spectrum. The H-1 to H-4 signals of the fourth spin system, belonging evidently to GalNAc, were assigned using the COSY spectrum, and then the C-1 to C-4 signals were found by the HSQC spectrum, the remaining C-5 and C-6 signals were assigned by their chemical shifts of δ 76.4 and δ 62.6, respectively. The $J_{1,2}$ values of 7.8 and 8.8 Hz as well as H-1 chemical shifts δ 4.52 and 4.67, respectively, demonstrated the β configuration of Glc*p* and Gal*p*NAc.

Significant downfield displacements of the signals for α-Glc*p*A C-4, β-Gal*p*N C-3, and β-Glc*p* C-4 (Table 1), as compared with their positions of the corresponding nonsubstituted monosaccharides,[15] defined the modes of substitutions of these residues. The HMBC spectrum demonstrated the following inter-residue cross-peaks: Glc H-1,GalNAc C-3 at δ 4.52/81.5; GalN H-1,Qui4NFo C-2 at δ 4.67/82.5; Qui4NFo H-1,GlcA C-4 at δ

Table 1. ^{1}H and ^{13}C NMR data (δ, ppm) for the O-deacetylated polysaccharide from *P. alcalifaciens* O27

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→4)-β-D-Glc*p*-(1→	**A**	4.52	3.33	3.75	3.60	3.58	3.76	3.89
→3)-β-D-Gal*p*NAc-(1→	**B**	4.67	4.05	3.89	4.18	3.72	3.76	3.80
→2)-α-D-Qui*p*4NFo-(1→	**C**	5.69	3.71	3.71	3.67	3.80	1.13	
→4)-α-D-Glc*p*A-(1→	**D**	5.32	3.64	3.96	3.74	4.07		
		C-1	C-2	C-3	C-4	C-5	C-6	
→4)-β-D-Glc*p*-(1→	**A**	105.7	74.1	77.3	79.3	75.8	61.9	
→3)-β-D-Gal*p*NAc-(1→	**B**	104.8	52.8	81.5	69.4	76.4	62.6	
→2)-α-D-Qui*p*4NFo-(1→	**C**	99.0	82.5	70.6	57.0	68.3	18.1	
→4)-α-D-Glc*p*A-(1→	**D**	101.5	72.9	75.2	77.1	74.5	176.5	

The chemical shifts for the *N*-acetyl group are δ_H 2.01, δ_C 23.8 (CH_3) and 176.7 (CO); for the *Z*- and *E*-forms of the *N*-formyl group δ_H 8.18 and 7.99, δ_C 166.1 and 169.0, respectively. The chemical shifts for the *O*-acetyl group in the initial O-polysaccharide are δ_H 2.12; δ_C 21.9 (CH_3) and 175.2 (CO).

5.69/77.1; and GlcA H-1,Glc C-4 at δ 5.32/79.3. These correlations confirmed the substitution pattern and revealed the monosaccharide sequence in the repeating unit of the OPS.

Analysis of the glycosylation effects on the ^{13}C NMR chemical shifts showed that Glc, GalN, and Qui4N have the same absolute configuration. Thus, the α-glycosylation effect of $+\delta$ 9.1 on GalNAc C-3 demonstrated that both residues in the β-Glc*p*-(1→3)-β-Gal*p*NAc fragment have the same absolute configurations.[17] The α-glycosylation effect value on C-2 α-Qui*p*4NFo (as compared with δ 73.2 in unsubstituted α-Qui*p*4NFo[16]) was $+\delta$ 9.3, and, hence, both residues in the β-Gal*p*NAc-(1→2)-α-Qui*p*4NFo disaccharide have the same absolute configuration too.[17]

The O-acetylation site in the initial O-polysaccharide was found using HSQC, HMQC–TOCSY, and COSY spectra. The HSQC spectrum, as compared to that of the OPS, demonstrated strong partial displacement from δ 3.80/62.6 to δ 4.30/65.1 of the β-GalNAc H-6/C-6 cross-peak caused by a deshielding effect of the *O*-acetyl group. The β-GalNAc H-5/C-5 cross-peak, found by the C-6/H-5 correlation in the HMQC–TOCSY spectrum, shifted correspondingly from δ 76.4/3.72 to 73.7/3.92, whereas displacements of the other signals were insignificant. The acetylation at O-6 was further confirmed by correlations in the HMBC spectrum of the carboxyl carbon and methyl protons of the *O*-acetyl group with H-6 and C-6 of β-GalNAc at δ 175.2/4.30 (strong) and 2.12/65.1 (weak), respectively. A comparison of integral intensities of proton signals in the O-acetylated and non-acetylated units (e.g., those of α-Glc*p*A H-1 and β-Gal*p*NAc6Ac H-6) showed that ~70% of the repeating units were O-acetylated.

Therefore, the O-polysaccharide of *P. alcalifaciens* O27 has the structure **1** shown in Chart 1.

A distinguished feature of the O27 polysaccharide is the presence of a derivative of D-Qui4N, an uncommon component of bacterial polysaccharides. Remarkably, when occurring, D-Qui4N often bears an uncommon *N*-acyl substituent (see Bacterial Carbohydrate Structure Database at http://www.glyco.ac.ru/bcsdb/). For instance, in various *Providencia* O-polysaccharides Qui4N is N-acylated with such rarely occurring groups as D-aspartyl (in serogroup O33[18]), L-aspartyl (O4[19]), L-Ala-L-Ala dipeptide (O35[20]), or formyl (O30[10] and O27 (this work)).

Recently, the O-polysaccharide of *P. stuartii* O43 has been reported to contain 4-acetamido-4,6-dideoxy-D-mannose (4-acetamido-4-deoxy-D-rhamnose, D-Rha4NAc).[8] Reinvestigation in this work of the composition of the O43 polysaccharide by GLC of alditol acetates resulted in revision of Rha4NAc to Qui4NAc. Analysis of the ^{1}H and ^{13}C NMR spectroscopy data of the polysaccharide confirmed this finding and showed that all other structure details are as reported.[8] The revised structure

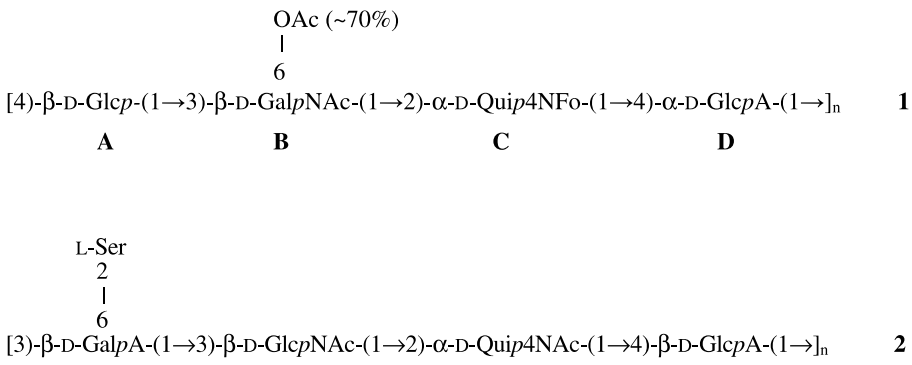

Chart 1. Structures of the O-polysaccharides of *Providencia alcalifaciens* O27 (**1**) and *Providencia stuartii* O43 (**2**).

of the O-polysaccharide of *P. stuartii* O43 (**2**) is shown in Chart 1.

1. Experimental

1.1. Bacterial strain and isolation of the LPS

P. alcalifaciens O27:H17, strain 5824/50 obtained from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest) was cultivated under aerobic conditions in tryptic soy broth supplemented with 0.6% yeast extract. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water, and lyophilized. The LPS was isolated by phenol–water extraction[21] followed by dialysis of the extract without layer separation and centrifugation of the retentate to remove insoluble contaminations. The crude LPS preparation was purified by treatment of the supernatant with cold aq 50% CCl_3CO_2H to precipitate proteins and nucleic acids followed by centrifugation; the supernatant was dialyzed and freeze-dried to give the purified LPS in a yield of 5.8% of dry bacteria weight.

1.2. Isolation and O-deacetylation of LPS

A LPS sample (200 mg) was heated with 2% AcOH for 3 h at 100 °C and the carbohydrate-containing supernatant was fractionated on a column (60 × 2.5 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer, pH 4.5. The yield of the O-polysaccharide was 24% of the LPS weight.

O-Deacetylation of the polysaccharide was performed with aq 12% ammonia (20 °C, 16 h) and the OPS was isolated by GPC on Sephadex G-50 as above.

1.3. Sugar analyses

The OPS was subjected to methanolysis (0.5 M HCl/MeOH, 85 °C, 1 h) followed by acetylation (Ac_2O–pyridine, 100 °C, 1 h). The resultant acetylated methyl glycosides were analyzed by GLC–MS on a ThermoQuest Finnigan model Trace series GC 2000 instrument equipped with an EC-1 column (0.32 mm × 30 m) using a temperature gradient of 10 °C min^{-1} from 150 to 250 °C.

The OPS was hydrolyzed with 2 M CF_3CO_2H for 2 h at 120 °C. Alditol acetates were prepared by reduction with an excess of $NaBH_4$ (20 °C, 2 h) followed by acetylation and analysis by GLC on a Hewlett-Packard HP 5890 chromatograph equipped with an Ultra-2 column (0.2 mm × 25 m) using a temperature gradient of 7 °C min^{-1} from 160 to 290 °C.

For determination of the absolute configuration of the monosaccharides, the OPS was hydrolyzed with 1 M CF_3CO_2H (100 °C, 1 h), N-acetylated (60 μL Ac_2O in 400 μL aq satd $NaHCO_3$, 0 °C, 1 h), hydrolyzed with 10 M HCl (80 °C, 30 min), N-acetylated as above, subjected to (+)-2-octanolysis[22] (100 μL (+)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h), acetylated and studied by GLC as in analysis of the alditol acetates.

1.4. Methylation analysis

Methylation of the OPS was performed according to the Hakomori procedure.[23] The methylated polymer was recovered using a Sep-Pak cartridge, and a portion was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were derived by hydrolysis with 2 M CF_3CO_2H (2 h, 120 °C), converted into the alditol acetates and analyzed by GLC–MS as above.

1.5. NMR spectroscopy

Samples were freeze-dried twice from a ^2H_2O soln and dissolved in 99.96% ^2H_2O with internal TSP (δ_H 0) and external acetone (δ_C 31.45) as references. ^1H and ^{13}C NMR spectra were recorded at 30 °C using a Bruker DRX-500 NMR spectrometer and XWINNMR Bruker software on SGI Indy/Irix 5.3 workstation. Mixing time of 300 ms and spin-lock time of 30 ms were used in ROESY and TOCSY experiments, respectively. Other NMR experimental parameters were essentially as described.[24]

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