Glycolipids from Sponges. Part 16.[1] Discoside, a Rare *myo*-Inositol-Containing Glycolipid from the Caribbean Sponge *Discodermia dissoluta*

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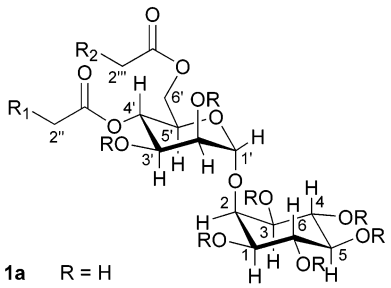
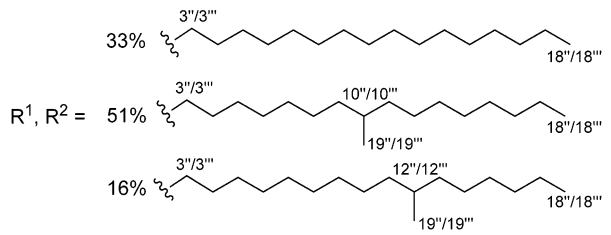
Discoside (**1a**), a glycolipid composed of 4,6-*O*-diacylated mannose attached to the 2-hydroxyl group of a *myo*-inositol unit, was isolated as a mixture of homologues from the marine sponge *Discodermia dissoluta*. The complete stereostructure of this new glycolipid was solved by interpretation of mass spectrometric and NMR data and CD analysis of degradation products.

Inositol-containing lipids are key membrane constituents and play important roles in essential metabolic processes in plants, animals, and some bacteria.[2] Phosphatidylinositols (PI), for example, are well known to be the anchor that links a variety of proteins to the external leaflet of the plasma membrane via a glycosyl bridge.

In the course of our ongoing efforts to study the glycolipids from marine sponges, we have found a new inositol-containing glycolipid (**1a**), which we have named discoside. Discoside is the first example of a 4,6-*O*-diacylated mannose attached to the 2-hydroxyl group of a *myo*-inositol unit [2-*O*-(4,6-di-*O*-acyl-α-D-mannopyranosyl)-*myo*-inositol]. The mannopyranosyl-*myo*-inositol partial structure is a well-known building block of phosphatidylinositol mannosides (PIMs) and of their multiglycosylated form, lipomannans (LMs), which are found in the cell walls of *Mycobacteria* and related species and exhibit a wide spectrum of immunoregulatory effects.[2,3]



1a R = H
1b R = Ac
1c R = CD_3CO



The only close analogue of **1a** is 1-*O*-pentadecanoyl-2-*O*-(6-*O*-heptadecanoyl-α-D-mannopyranosyl)-*myo*-inositol,[4] isolated in 1968 from various strains of *Propionibacterium*, where it constitutes up to 40% of the total lipid fraction. It has been suggested that this glycolipid is involved in the biosynthesis of PIMs. Since this first report, no other members of this class of glycolipids have been described.

Results and Discussion

The $CHCl_3$−MeOH-soluble extract of the marine sponge *D. dissoluta* (Schmidt 1880, Porifera, class Demospongiae, order Theonellidae, family Discodermiidae) collected around Berry Island (Bahamas) was partitioned between BuOH and H_2O. The organic fraction was successively purified by reversed-phase chromatography followed by direct-phase chromatography, yielding a crude fraction containing only glycolipids. This fraction was acetylated with Ac_2O in pyridine. This derivatization procedure is very useful in studying glycolipids. In fact, the nonpolar peracetylated glycolipid mixture is easy to purify on normal-phase HPLC. In addition, resonance assignment in the homonuclear NMR spectra of peracetylated glycolipids is made easier because of the better signal dispersion that allows also to discriminate between ether and ester oxymethine proton resonances on the basis of their different chemical shift range (δ 3.5−4.5 and 4.7−5.7, respectively).

The peracetylated glycolipid fraction was chromatographed by HPLC on a SiO_2 column using *n*-hexane−EtOAc (6:4). Discoside was isolated as its peracetyl derivative **1b**. Unfortunately, compound **1b** could not be deacetylated to give the natural discoside **1a** because of the presence of the ester-linked acyl chains. We tried to isolate discoside **1a** from the nonacetylated crude glycolipid fraction, but we did not succeed because of its low natural concentration and the difficulty of separating nonacetylated glycolipids.

ESIMS of **1b** showed the presence of three ion peaks [M + Na]$^+$ differing from each other by CH_2 (14 amu) at *m/z* 1219, 1205, and 1191, indicating that discoside is actually a mixture of homologues, which were not further separated. HREIMS of the most intense peak at *m/z* 1205.7159 supported the molecular formula $C_{63}H_{106}O_{20}$.

Inspection of the ^{1}H NMR spectrum of **1b** in $CDCl_3$ suggested that discoside is a glycolipid because of (i) a 6H methyl triplet at δ 0.88 attributable to two linear alkyl chains; (ii) a doublet at δ 0.83, which integrated for a nonintegral number of internal methyl branches and, therefore, indicated that the homologues have different branching; (iii) a broad band of resonances at δ 1.25 indicative of long aliphatic hydrocarbon chains; (iv) seven acetyl methyl resonances between δ 2.16 and 1.98; and (v) a cluster of carbinol proton resonances between δ 4.00 and 5.60.

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Table 1. ^{1}H and ^{13}C NMR Data of Discoside Peracetate **1b** (500 MHz)

	$CDCl_3$		C_6D_6	
pos.	δ_H (mult., J [Hz])[a]	δ_C (mult.)[b]	δ_H (mult., J [Hz])[c]	δ_C (mult.)[d]
1	5.00 (dd, 10.5, 2.3)	69.8 (CH)	4.88 (dd, 10.5, 2.4)	70.3 (CH)
2	4.29 (t, 2.3)	76.3 (CH)	4.17 (t, 2.4)	77.3 (CH)
3	5.08 (dd, 10.5, 2.3)	70.6 (CH)	5.01 (dd, 10.5, 2.4)	70.8 (CH)
4	5.53 (dd, 10.5, 9.8)	69.7 (CH)	5.91[f] (t, 10)	70.7 (CH)
5	5.19 (t, 9.8)	70.9 (CH)	5.33 (t, 9.7)	71.3 (CH)
6	5.49 (dd, 10.5, 9.8)	69.5 (CH)	5.91[f] (t, 10)	70.1 (CH)
1′	4.96 (d, 1.6)	99.6 (CH)	4.98 (d, 1.8)	100.0 (CH)
2′	5.37 (br s)	69.8 (CH)	5.66 (dd, 3.1, 1.8)	70.4 (CH)
3′	5.41[e]	68.8 (CH)	5.83 (dd, 10.1, 3.1)	69.3 (CH)
4′	5.42[e]	65.5 (CH)	5.92[f] (t, 10)	66.0 (CH)
5′	4.18 (m)	69.8 (CH)	4.56 (br d, 9.7)	70.7 (CH)
6′a	4.26 (dd, 12.2, 3.8)	61.9 (CH_2)	4.60 (dd, 12.1, 3.7)	62.1 (CH_2)
6′b	4.09 (dd, 12.2, 2.1)		4.48 (dd, 12.1, 1.5)	
1″		172.4 (C=O)		172.4 (C=O)
2″	2.31 (m)	34.1 (CH_2)	2.11 (m)	34.2 (CH_2)
3″	1.61 (m)	25.0 (CH_2)	1.53 (m)	25.2 (CH_2)
1‴		173.2 (C=O)		173.0 (C=O)
2‴	2.34 (m)	34.0 (CH_2)	2.37 (m)	34.4 (CH_2)
3‴	1.62 (m)	24.8 (CH_2)	1.72 (m)	25.2 (CH_2)
Ac's	2.14 (s), 2.09 (s), 2.07 (s), 2.03 (s), 2.02 (s), 2.01 (s), 1.99 (s)	20.7−20.4 (CH_3)	1.93 (s), 1.88 (s), 1.66 (12H, s), 1.58 (s)	20.4−19.9 (CH_3)
		169.8−169.2 (C=O)		169.6−168.9 (C=O)

[a] Alkyl chain ^{1}H signals ($CDCl_3$): δ 1.25 (broad band, alkyl chain protons), 0.88 (t, J = 7.0, H_3-18), 0.83 (d, J = 6.4, H_3-18). [b] Alkyl chain ^{13}C signals ($CDCl_3$): 31.9 (CH_2, C-16), 22.7 (CH_2, C-17), 14.0 (CH_3, C-18); 10-Me branched chains: 37.2 (CH_2, C-9 and C-11), 32.8 (CH, C-10), 27.1 (CH_2, C-8 and C-11), 19.7 (CH_3, C-19). [c] Alkyl chain ^{1}H signals (C_6D_6): δ 1.32 (broad band, alkyl chain protons), 0.94 (t, J = 6.4, H_3-18), 0.91 (d, J = 6.8, H_3-18). [d] Alkyl chain ^{13}C signals ($CDCl_3$): 32.3 (CH_2, C-16), 23.0 (CH_2, C-17), 14.3 (CH_3, C-18); 10-Me branched chains: 37.6 (CH_2, C-9 and C-11), 33.3 (CH, C-10), 27.6 (CH_2, C-8 and C-11), 19.9 (CH_3, C-19). [e] Non-first-order multiplet. [f] Coupling constants were measured from a row of the HSQC spectrum.

As usual for glycolipid molecules, many resonances overlapped, preventing their analysis. To circumvent this problem, NMR spectra were recorded using two different solvents ($CDCl_3$ and C_6D_6).[5] From the ^{1}H NMR spectrum of **1b** in C_6D_6 two pairs of α-carbonyl methylene protons at δ 2.37 and 2.11, respectively, indicative of two fatty acid residues, were distinguished.

In the ^{13}C NMR spectrum in C_6D_6, the diagnostic value at δ 100.0, indicative of an anomeric carbon atom (C-1′), confirmed the presence of one sugar moiety. Building up the saccharide structure required a series of sequential steps: first, data obtained from a HSQC NMR experiment permitted assignment of the relevant anomeric proton at δ 4.97 (C_6D_6, H-1′). Then, the H-1′ resonance was the starting point to identify, in sequence, four methine protons at δ 5.66 (H-2′), 5.83 (H-3′), 5.92 (H-4′), and 4.57 (H-5′) and two methylene protons at δ 4.61 (H-6′a) and 4.48 (H-6′b), respectively, by means of COSY correlation peaks. These data are in accordance with a hexose structure. The relatively upfield chemical shift of H-5′ established that the hexose is in the pyranose form.

At this point, an accurate analysis of coupling constants was needed to identify the nature of the hexopyranose. The large (J = 10 Hz) coupling constant[6] between H-5′ and H-4′ indicated the *trans*-diaxial nature of these protons. H-3′ showed one large (J = 10.1 Hz) and one small (J = 3.1 Hz) coupling constant, showing H-3′ to be an axial proton flanked by the axial H-4′ and the equatorial H-2′ proton. These data revealed the *manno* configuration of the hexopyranose unit.

The equatorial position of H-2′ prevented the anomeric configuration of the mannopyranose from being determined on the basis of homonuclear coupling constant values. However, it is known that the $^1J_{CH}$ coupling constant of an axial anomeric proton is approximately 160 Hz, while that of an equatorial anomeric proton is ∼170 Hz.[7] The coupling constant between H-1′ and C-1′ (J = 174 Hz) in the HSQC clearly pointed to the equatorial orientation of H-1′ and, therefore, to the α anomeric configuration of the sugar unit.

Six oxymethine resonances in the mid-field region of the ^{1}H NMR spectrum remained to be interpreted. These resonances were part of the same spin system, as deduced from the COSY spectrum, and originated from the six cyclically arranged protons of an inositol. In fact, the proton at δ 4.29 ($CDCl_3$, H-2) was coupled with two protons at δ 5.05 (H-1) and 5.08 (H-3), which were in turn coupled with a signal at δ 5.49 (H-6) and a signal at δ 5.53 (H-4), respectively. Finally, both H-6 and H-4 were coupled with a signal resonating at δ 5.19 (H-5). Analysis of the coupling constants allowed establishment of the relative configuration of the inositol moiety. The coupling constants between H-1/H-6, H-3/H-4, H-4/H-5, and H-5/H-6 (see Table 1) indicated the *trans*-diaxial nature of these protons, while the small coupling constant (J = 2.3 Hz) of H-2 with H-3 and H-1, respectively, accounted for its equatorial orientation. The above data are consistent with a *myo*-inositol. The relatively upfield chemical shift of H-2 (δ 4.29) suggested that the *myo*-inositol is glycosylated at C-2, as confirmed by a cross-peak between H-1′ and C-2 and between H-2 and C-1′ in the HMBC experiments in both solvents.

Once the gross structure was defined, the location of the two acyl chains was established on the basis of HMBC data. In fact, the carbonyl carbon atom at δ 172.4 ($CDCl_3$, C-1″) of one fatty acyl chain, identified from its HMBC correlation peak with the α-methylene protons at δ 2.31 (H-2″), was also coupled with the mannose proton H-4′ (δ 5.42). Likewise, the carbonyl carbon atom of the second fatty acyl chain (δ 173.2, C-1‴) was coupled with the α-methylene protons at δ 2.33 (H-2‴) as well as with H-6′a (δ 4.26).

Because the chemical shifts of H-4′ and H-3′ were nearly coincident in the spectra recorded in $CDCl_3$, these data did not positively prove that the fatty acyl chain was linked to O-4′ and not to O-3′. Therefore, the HMBC experiment in C_6D_6 was also analyzed. The long-range coupling between

the carbon at δ 172.3 (C-1″) and the proton at δ 5.92 (H-4′) demonstrated that one fatty acyl group was indeed linked to O-4′.

The nature and the relative amounts of the fatty acyl chains of **1b** were identified using chemical degradation and GC-MS. Acid-catalyzed methanolysis of **1b** liberated the fatty acid methyl esters, methyl mannoside, and *myo*-inositol. The reaction mixture was partitioned between $CHCl_3$ and H_2O, giving an organic layer containing a mixture of fatty acid methyl esters and an aqueous layer containing methyl mannoside and *myo*-inositol.

The mixture of fatty acid methyl esters was subjected to GC-MS analysis, and three different compounds were identified on the basis of their GC retention times and mass fragmentation: methyl octadecanoate (33%), methyl 10-methyloctadecanoate (51%), methyl 12-methyloctadecanoate (16%). The unbranched compound showed GC retention time and mass spectra that matched those of an authentic sample. As for methyl 12-methyloctadecanoate ($M^+ = m/z$ 312), methyl branch positions were indicated by the mass spectrometric fragmentation pattern:[8] two relatively intense peaks separated by 28 amu at *m/z* 199 ($C_{12}H_{23}O_2$) and 227 ($C_{14}H_{27}O_2$), originating from α-cleavages with respect to the tertiary carbon atoms carrying the methyl branch, and two peaks at *m/z* 195 ($C_{14}H_{27}O_2$ − MeOH) and 177 ($C_{14}H_{27}O_2$ − MeOH − H_2O). Analogously, the methyl branching of methyl 10-methyloctadecanoate ($M^+ = m/z$ 312) was indicated by the peaks at *m/z* 171 ($C_{10}H_{19}O_2$), 199 ($C_{12}H_{23}O_2$), 167 ($C_{12}H_{23}O_2$ − MeOH), and 149 ($C_{12}H_{23}O_2$ − MeOH − H_2O).

After structure **1b** was fully established, it remained to verify whether any of the acetyl groups in **1b** were already present in the native glycolipid. This was accomplished by subjecting a small portion of the crude glycolipid fraction to acetylation with trideuteroacetic anhydride instead of acetic anhydride.[9] The ^{1}H NMR spectrum of the derivative **1c** was identical to that of **1b** except for the absence of the seven acetyl methyl singlets, showing that none of the acetyl groups in **1b** were present in the natural product **1a**. In addition, in the ESIMS spectrum of the pertrideuteroacetate derivative **1c**, the three $[M + Na]^+$ pseudomolecular ion peaks were present at *m/z* 1212, 1226, and 1240, 21 amu higher than the corresponding ions of **1b**.

The absolute configuration of the mannopyranose unit was established as D using a circular dichroism (CD) method. The aqueous layer from the methanolysis, containing methyl mannosides and the *meso myo*-inositol, was perbenzoylated with benzoyl chloride in pyridine, and methyl α-mannopyranoside perbenzoate was isolated by normal-phase HPLC and identified from its ^{1}H NMR spectrum. The CD spectrum of methyl α-mannopyranoside perbenzoate obtained from discoside matched that of an authentic sample of methyl α-D-mannopyranoside perbenzoate.

In summary, we reported the isolation and the complete stereostructure determination of discoside, a unique glycolipid containing a *myo*-inositol mannoside. Such compounds have never been reported from marine sponges, since the only close analogue of discoside reported was isolated from various strains of *Propionibacterium*. Recent studies suggested that secondary metabolites may be produced by symbiotic microorganisms rather than by the sponges to which they have been attributed.[10] It is worth noting that the most important secondary metabolite isolated form *D. dissoluta*, the antitumor polyketide discodermolide,[11] is thought to be produced by bacterial symbionts.[12] Because of the strong similarity between discoside and the *myo*-inositol-containing glycolipid of bacterial origin, it is reasonable to suppose that also discoside could be produced by symbionts associated with the sponge.

Experimental Section

General Experimental Procedures. High-resolution ESIMS spectra were obtained on a Micromass QTOF Micro mass spectrometer, dissolving the sample in MeCN−H_2O (1:1) with 0.1% TFA. ESIMS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. The spectra were recorded by infusion into the ESI source using MeOH as the solvent. Optical rotations were measured at 589 nm on a Perkin-Elmer 192 polarimeter using a 10 cm microcell. CD spectra were recorded on a Jasco J-710 spectrophotometer using a 1 cm cell. ^{1}H and ^{13}C NMR spectra were determined on a Varian Unity Inova spectrometer at 500.13 and 125.77 MHz, respectively; chemical shifts were referenced to the residual solvent signal ($CDCl_3$: $\delta_H = 7.26$, $\delta_C = 77.0$; C_6D_6: $\delta_H = 7.15$, $\delta_C = 128.0$). Homonuclear ^{1}H connectivities were determined by COSY experiments. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with a mixing time of 500 ms. Coupling constants of overlapping signals in the ^{1}H spectrum were measured from the rows of the HSQC. The reverse multiple-quantum heteronuclear correlation (HSQC) spectra were recorded optimized for an average $^1J_{CH}$ of 140 Hz. The gradient-enhanced multiple-bond heteronuclear correlation (HMBC) experiment was optimized for a $^3J_{CH}$ of 8 Hz. GC-MS spectra were recorded on a Hewlett-Packard 5890 gas chromatograph with a mass selective detector MSD HP 5970 MS, a split/splitness injector, and a fused-silica column, 25 m × 0.20 mm HP-5 (cross-linked 25% Ph Me silicone, 0.33 mm film thickness); the temperature of the column was varied, after a delay of 3 min from the injection, from 150 to 280 °C with a slope of 10 °C min^{-1}; quantitative determination was based on the area of the GC peaks. High-performance liquid chromatographies (HPLC) were achieved on a Varian Prostar 210 apparatus equipped with an Varian 350 refractive index detector or a Varian 325 UV detector.

Collection. Specimens of *D. dissoluta* (Porifera, class Demospongiae, order Theonellidae, family Discodermiidae) were collected by scuba (depth 23 m) during the third "Pawlik Expedition" (July 2000) along the coast of Little San Salvador (Bahamas) and identified by Prof. M. Pansini, University of Genova. A reference sample (#28-00) has been deposited at the Istituto di Zoologia, Genova, Italy. The samples were stored at −20 °C until extraction.

Extraction. The frozen sponge sample of *D. dissoluta* #28-00 (36 g of dry weight after extraction) was blended in MeOH, then in sequence extracted with MeOH, MeOH−$CHCl_3$ (2:1), MeOH−$CHCl_3$ (1:2), and $CHCl_3$.

Glycolipid Isolation Procedure. The MeOH extracts were partitioned between BuOH and H_2O. The BuOH phase was concentrated in vacuo and combined with the $CHCl_3$ extract. The organic phases (35.97 g) were subjected to chromatography on a column packed with RP-18 by elution with a gradient of H_2O−MeOH to $CHCl_3$. The fraction eluted with $CHCl_3$ (7.5 g) was further chromatographed on a SiO_2-packed column eluted with a gradient of *n*-hexane−EtOAc (9:1) to MeOH. The fraction eluted with EtOAc−MeOH (7:3, 345 mg) was composed only of glycolipids. This fraction was acetylated with Ac_2O in pyridine and chromatographed by HPLC on a SiO_2 column using *n*-hexane−EtOAc (6:4) to afford 7.5 mg of peracetylated discoside (**1b**).

Discoside Peracetate (1b): colorless oil, $[\alpha]_D^{25}$ +12° ($CHCl_3$, *c* 0.5); ^{1}H and ^{13}C NMR, Table 1; ESIMS *m/z* 1219, 1205, 1191, $[M + Na]^+$ series; HRESIMS *m/z* 1205.7159 (calcd for $C_{63}H_{106}NaO_{20}$, 1205.7170).

Fatty Acyl Chains Determination. Compound **1b** (1 mg) was subjected to acidic methanolysis with 1 M HCl−MeOH. The reaction mixture was kept for 12 h at 80 °C in a sealed

tube, then dried under nitrogen and partitioned between $CHCl_3$ and H_2O−MeOH (8:2) to give an aqueous layer containing the methylmannoside and the inositol (fraction A) and an organic layer containing the fatty acyl methyl esters (fraction B). After removal of the solvent, fraction B was analyzed by GC-MS and its components were identified as methyl octadecanoate (t_R = 22.88 min, 33%), methyl 10-methyloctadecanoate (t_R = 23.66 min, 51%), and methyl 12-methyloctadecanoate (t_R = 23.71 min, 16%).

Methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranoside. D-Mannose (2.0 mg) was subjected to acidic methanolysis, and the resulting methyl glycosides were benzoylated with benzoyl chloride (20 μL) in pyridine (200 μL) at 25 °C for 16 h. The reaction was then quenched with MeOH and after 30 min was dried under nitrogen. Methyl benzoate was removed by keeping the residue under vacuum for 24 h with an oil pump. The residue was purified by HPLC (column: Luna SiO_2, 5 μm, 4 × 250 mm, eluent: *n*-hexane−*i*-PrOH, 99:1, flow: 1 mL/min), affording methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranoside (t_R = 9.5 min): ^{1}H NMR ($CDCl_3$) δ 8.10 (2H, d, *J* = 7.8 Hz, benzoyl *ortho* protons), 8.06 (2H, d, *J* = 7.8 Hz, benzoyl *ortho* protons), 7.95 (2H, d, *J* = 7.8 Hz, benzoyl *ortho* protons), 7.84 (2H, d, *J* = 7.8 Hz, benzoyl *ortho* protons), 7.61−7.23 (12H, overlapping signals, benzoyl protons), 6.11 (1H, t, *J* = 10.1 Hz, H-4), 5.91 (1H, dd, *J* = 10.1 and 3.3 Hz, H-3), 5.70 (1H, dd, *J* = 3.3 and 1.7 Hz, H-2), 5.01 (1H, d, *J* = 1.7 Hz, H-1), 4.71 (1H, dd, *J* = 12.1 and 2.6 Hz, H-6a), 4.50 (1H, dd, *J* = 12.1 and 4.5 Hz, H-6b), 4.42 (1H, ddd, *J* = 10.1, 4.5, and 2.6 Hz, H-5), 3.55 (3H, s, OMe); CD (MeCN) λ_{max} = 240 nm (Δε = −31), 223 nm (Δε = +15).

Absolute Configuration Determination. Fraction A from methanolysis of compound **1b** was benzoylated as described above and purified by HPLC as above. The chromatogram contained a peak (t_R = 9.5 min), which was identified as methyl α-D-mannopyranoside by a comparison of its retention times and its ^{1}H NMR and CD spectra with those of the synthetic glycosides prepared from D-mannose.

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Supporting Information Available: 1D and 2D NMR spectra of discoside peracetate (**2b**) in $CDCl_3$ and C_6D_6; ^{1}H NMR and CD spectra of methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-mannoside. This material is available free of charge via the Internet at http://pubs.acs.org.

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