Microthecaline A, a Quinoline Serrulatane Alkaloid from the Roots of the Australian Desert Plant *Eremophila microtheca*

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ABSTRACT

Chemical investigation of the roots of the Australian desert plant *Eremophila microtheca* yielded microthecaline A (**1**), a novel quinoline-serrulatane natural product. The structure of **1** was determined by spectroscopic analysis and the absolute configuration was assigned by ECD. Compound **1** exhibited moderate antimalarial activity against *Plasmodium falciparum* (3D7 strain) with an IC_{50} of 7.7 μM. Microthecaline A represents the first quinoline-serrulatane alkaloid to be isolated from Nature.

The Australian desert plant, *Eremophila microtheca* F. Muell. ex Benth. belongs to the family Scrophulariaceae, and is a member of a genus of approximately 215 species, the majority of which have not been chemically or pharmacologically investigated.[1] *Eremophila* species are currently known to produce a variety of structural classes such as terpenoid, flavonoid, sterol, lignan, and fatty acids.[2] While a number of different terpenoid subclasses have been reported from this genus, the major subclass is the serrulatane diterpenoids.[3-7] More than 100 serrulatanes have been reported from terrestrial plants and marine organisms[8] and approximately 20 serrulatane diterpenoids have been reported from the genus *Eremophila*.[9] Some of these *Eremophila* metabolites have been shown to exhibit antibacterial,[3,5-7] anti-inflammatory,[10] antimalarial[11] and cytotoxic effects.[12] *Eremophila microtheca* has been incompletely investigated with only one reported phytochemical study to date. The first chemical analyses of this plant focused on the aerial parts and yielded three new serrulatane derivatives, 3,7,8-trihydroxyserrulat-14-en-19-oic acid (**3**), 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid (**4**), and 3,19-diacetoxy-8-hydroxyserrulat-14-ene (**5**) (Figure 1), and the previously isolated natural products, verbascoside and jaceosidin.[4,13] Mild to moderate antibacterial activity was reported for several of these metabolites.[4]

As part of our search for structurally unique natural products from Australian endemic plants[4,14,15] the chemical investigation of the roots of *E. microtheca* was undertaken since LC-MS data analysis of the CH_2Cl_2 extract indicated potentially new alkaloidal chemistry. Subsequent large-scale extraction and isolation studies led to the identification of a novel quinoline serrulatane, microthecaline A (**1**) (Figure 1) in good yield, together with the known serrulatane natural product 3,7,8-trihydroxyserrulat-14-en-19-oic acid (**3**).

Herein, we report the purification and structure elucidation of the novel secondary metabolite **1** and its semi-synthetic derivative **2** along with the in vitro antimalarial profiling of these two compounds.

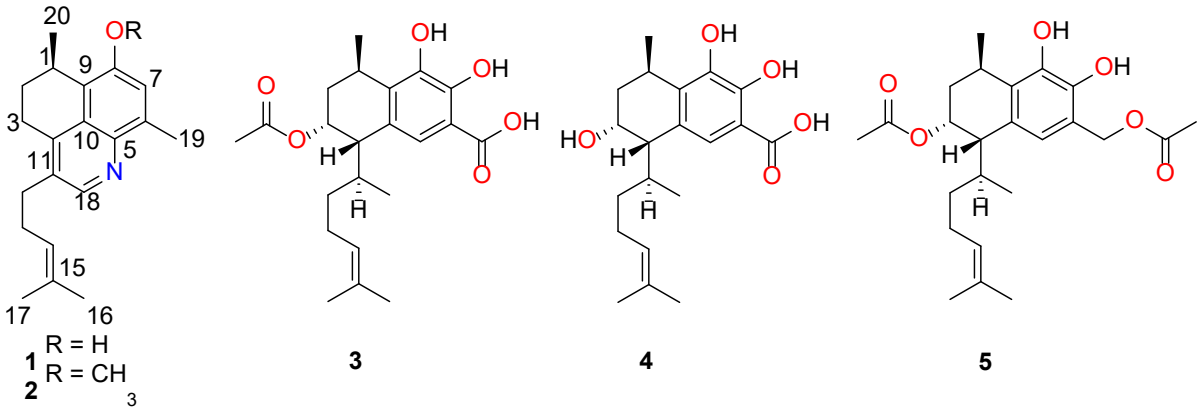

Figure 1. Chemical structures of microthecaline A (**1**), microthecaline A 8-methyl ether (**2**), and the previously isolated serrulatanes (**3**–**5**) from *Eremophila microtheca*.

The CH_2Cl_2 extract of the roots of *E. microtheca* was subjected to C_{18} HPLC that yielded microthecaline A (**1**, 47.7 mg, 0.076% dry wt), which was isolated as an optically active brown gum. The (+)-LRESIMS of microthecaline A (**1**) displayed a peak for a protonated molecule at *m/z* 296 $[M + H]^+$, indicating the presence of an odd number of nitrogen(s) in the molecule. Following analysis of NMR (Table 1) and (+)-HRESIMS data a molecular formula of $C_{20}H_{25}NO$ was assigned to **1**, requiring nine double bond equivalents. The ^{1}H NMR spectrum of microthecaline A (**1**) displayed four methyl groups (δ_H 1.27, 1.45, 1.67, and 2.74), four methylenes (δ_H 2.01/2.16, 2.44, 3.02, and 3.25/3.38), one methine (δ_H 3.65), one olefinic proton (δ_H 5.23), and two aromatic singlets (δ_H 7.49, and 8.57). The ^{13}C NMR spectrum together with the HSQC spectrum of **1** displayed 20 carbon signals, consisting of four methyls (δ_C 17.1, 17.6, 18.7, and 25.8), four methylenes (δ_C 24.0, 28.2, 29.0, and 30.8), one sp^3 methine (δ_C 27.1), three sp^2 methines (δ_C 123.4, 127.1, and 140.9), and eight sp^2 non-protonated carbons (δ_C 123.1, 128.6, 129.2, 131.4,

133.5, 134.9, 154.7, and 156.4). These NMR data accounted for six out of the nine degrees of unsaturation indicating the presence of a three-ring system.

The NMR data of compound **1** compared to previously isolated compounds, in particular 3,7,8-trihydroxyserrulat-14-en-19-oic acid (**4**) from *E. microtheca*,[4] and the analysis of 2D NMR data (Figure 2, COSY, HMBC, and ROESY), indicated that **1** also possessed a serrulatane carbon skeleton. The aromatic proton at δ_H 7.49 showed HMBC correlations to C-5, C-8, and C-9 which allowed it to be assigned to H-7. Furthermore, the HMBC correlation from H-7 to the methyl carbon resonating at δ_C 17.1 (C-19), the three-bond HMBC correlations from H-19 (δ_H 2.74) to C-5 (δ_C 131.4) and C-7 (δ_C 127.1), and a two-bond HMBC correlation to C-6 (δ_C 129.2) allowed the methyl group to be placed on C-6. HMBC correlations from H-1 to carbons resonating at δ_C 123.1 (C-9), 128.6 (C-10), H-2 to δ_C 123.1 (C-9) and H-3 displayed a HMBC correlation to δ_C 128.6 (C-10) and indicated H-1, H-2, and H-3 to be adjacent to a penta-substituted aromatic ring system. The low-field methine proton at δ_H 8.57 was assigned to H-18 due to the inductive effect of the N atom and the HMBC correlations to non-protonated carbons C-4 (δ_C 156.5), C-5 (δ_C 131.4), and C-11 (δ_C 133.5) resulted in the formation of a tricyclic system with an embedded quinoline moiety, accounting for all the double bond equivalents.

Table 1. NMR Data for Microthecaline A (**1**)[a]

position	δ_C, mult.	δ_H (J in Hz)	COSY	HMBC	ROESY
1	27.1, CH	3.65, m	2, 20	2, 3, 8, 9, 10, 20	
2α	28.2, CH_2	2.01, dddd (13.6, 13.6, 4.8,	1, 2β, 3β	1, 3, 20	20
2β		4.8)			
		2.16, dddd (13.6, 4.8, 2.4, 2.4)	2 α, 3α	1, 3, 4, 9, 20	
3α	24.0, CH_2	3.38, ddd (18.6, 4.8, 2.4)	3β	1, 4, 10, 11	12
3β		3.25, ddd (18.6, 13.6, 2.4)	2α, 2β, 3α	2, 4, 10, 11	20
4	156.4, C				
5	131.4, C				
6	129.2, C				
7	127.1, CH	7.49, s	19	5, 8, 9, 19	19
8	154.7, C				
9	123.1, C				
10	128.6, C				
11	133.5, C				
12	30.8, CH_2	3.02, t (7.5)	13	4, 11, 13, 14, 18	14, 18
13	29.0, CH_2	2.44, m	12, 14	11, 12, 14, 15	14, 18
14	123.4, CH	5.23, t (5.7)	13, 16, 17	13, 16, 17	12, 13, 16
15	134.9, C				
16	25.8, CH_3	1.67, s	14	13, 14, 15, 17	14
17	17.6 CH_3	1.45, s	14	13, 14, 15, 16	13
18	140.9, CH	8.57, s		4, 5, 11, 12	12, 13
19	17.1, CH_3	2.74, s	7	5, 6, 7	7
20	18.7, CH_3	1.27, d (7.0)	1	1, 2, 9	2β, 3β

[a]Recorded in CD_3OD at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR) at 30 °C.

The NMR data of compounds possessing the quinoline moiety, such as 4,8-dimethyl-6-*O*-(2′,4′-di-*O*-methyl-β-D-xylopyranosyl)-hydroxyquinoline and 4,8-dimethyl-6-hydroxyquinoline isolated from the marine cyanobacterium *Lyngbya majuscula*[16] and 6-hydroquinoline-8-carboxylic acid isolated from the fungus *Cortinarius subtortus*,[17] showed a high degree of similarity with the quinoline moiety of **1**. Finally, a bathochromic shift was noted in the UV spectrum upon addition of base to a solution of **1** indicating the presence of a free phenolic moiety.[18] Furthermore, this hydroxy group was attached to the quaternary aromatic carbon C-8 (δ_C 154.7) on the basis of the deshielded ^{13}C NMR

resonance. ROESY correlations (Figure 2) between H-20, H-2β, and H-3β showed that these protons are cofacial.

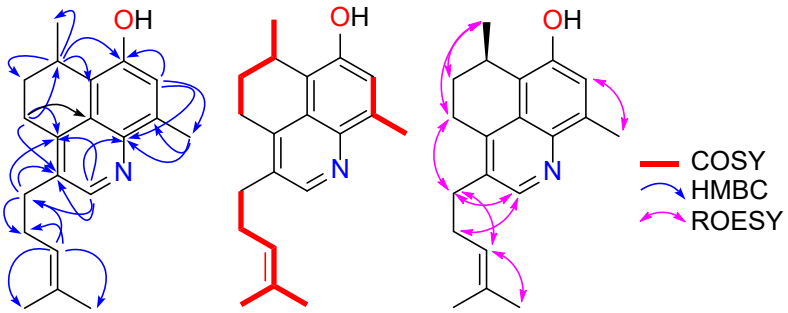

Figure 2. Key COSY, HMBC, and ROESY correlations for **1**

Moreover, the ROESY correlations from H-18 to H-12 and H-13 and importantly from H-3α to H-12 established the position of the nitrogen atom adjacent to C-18 and also the placement of the side chain at C-11 further confirming the structure. Based on these data, the planar structure of microthecaline A was assigned to **1**. Attempts to crystallize **1** for X-ray diffraction analysis proved unsuccessful. Methylation of **1** was also conducted to obtain another analogue for crystallization studies. The methylated derivative of **1** was generated using TMS-diazomethane in CH_2Cl_2/MeOH (1:1) and C_{18} HPLC (MeOH/H_2O/0.1% TFA) afforded microthecaline A 8-methyl ether (**2**, 2.2 mg, 20%). Compound **2** was also characterized using NMR, MS, and UV experiments. To date, neither crystals of **1** nor **2** have been successfully obtained. Thus, to determine the absolute configuration of microthecaline A (**1**), ECD calculations were employed. The experimental ECD spectrum (Figure 3) of **1** showed a negative Cotton effect at 339 nm ($\Delta\varepsilon$ −12.1) and positive Cotton effects at 247 ($\Delta\varepsilon$ +8.8), and 207 nm ($\Delta\varepsilon$ +7.4).

To establish the absolute configuration of microthecaline A, the ECD curve of the simplified and truncated chemical structures of **1**, models A and B (Figure 3) were calculated using time-dependent density functional theory (TDDFT) with Gaussian 09.[19]

Due to the insignificant contribution to the ECD spectrum by the aliphatic chain in **1**, models A (*R*) and B (*S*) were considered appropriate for theoretical ECD calculations.[20] The OPLS_2005 conformational search of **1** followed by DFT optimization at the B3LYP/6-31G(d, p) level afforded four different conformers (see Supporting Information). Theoretical ECD spectra of the major conformer with a 89% Boltzmann population was calculated with the B3LYP/6-31G(d, p) basis set. Subsequent comparison of the ECD spectra of models A (*R*) and B (*S*) (Figure 3) with the experimental ECD spectrum of **1** revealed good agreement with the ECD data of model A (*R*). Thus, the absolute configuration of **1** and **2** were determined to be 1*R*.

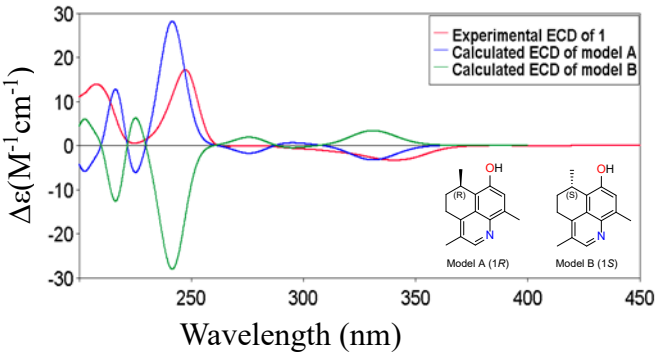

Figure 3. Experimental ECD spectrum of **1** in MeOH and the calculated ECD spectra of the model structures A and B.

A proposed biosynthetic pathway of **1** is shown in Figure 4. It is believed that the biosynthesis of the serrulatane quinoline takes place in two major stages. Geranylgeranyl pyrophosphate (GGPP) loses the pyrophosphate group, which initiates the cyclization cascade to form the diterpene cyclase[21] product (vi). This species then undergoes aromatization to form the intermediate (vii), an enantiomer of a brown algal natural product, erogorgiaene.[22,23] Following oxidation of erogorgiaene, the plant natural product, 5,8-dihydroxyserrulat-14-en-18-al[24] (viii) is formed. The second stage of the proposed biosynthesis is not well understood, however it may be postulated that the introduction of

the nitrogen atom (via a nitrogen source such as L-serine[25,26]) results in the ring closure that leads to a quinoline moiety, and thus the formation of microthecaline A (**1**).

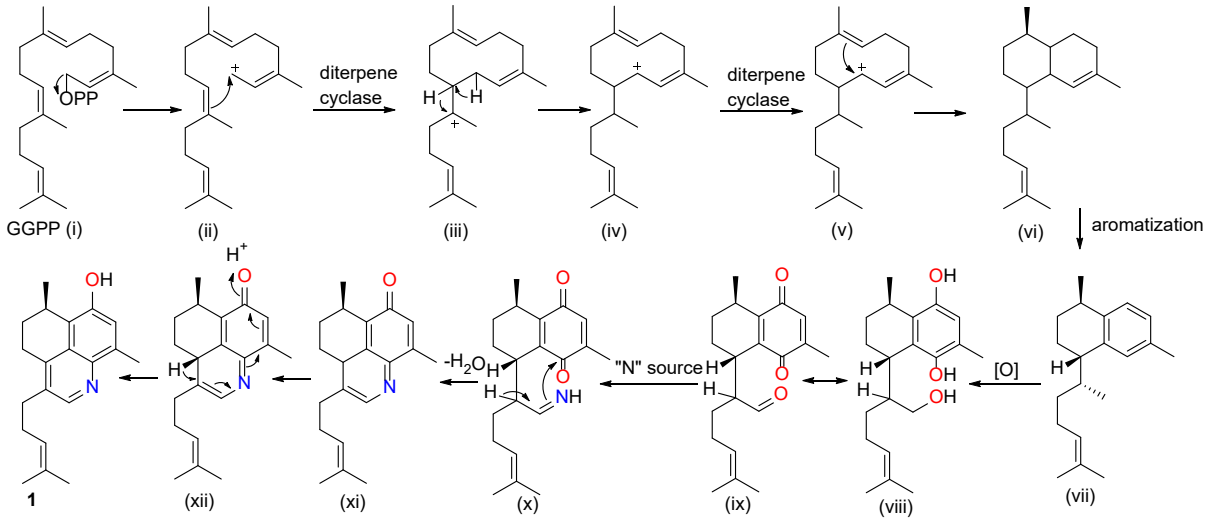

Figure 4. Proposed biosynthetic pathway for **1.**

Due to an interest in identifying potential antimalarial compounds from natural sources,[11] and our recent report of antiplasmodial activity for several serrulatane NPs and semi-synthetic derivatives,[27] it was decided to evaluate **1** and **2** for their activity against 3D7 (chloroquine-sensitive) and Dd2 (chloroquine-resistant) *Plasmodium falciparum* parasites.[28] Preliminary cytotoxicity data were also acquired for **1** and **2** using a human embryonic kidney cell (HEK293) (Table 2). The in vitro antimalarial image-based and cytotoxicity assays were performed using previously reported methods (see Supporting Information).[28] Microthecaline A (**1**) exhibited moderate activity against 3D7 and low activity against Dd2 *P. falciparum* strain with IC_{50} values of 7.7 and 17.2 µM, respectively. The semi-synthetic derivative **2** was inactive against both parasitic strains at 10 µM, indicating the importance of the C-8 phenolic hydroxyl group for activity against malaria parasites. Microthecaline A (**1**) represents the first example of a natural product possessing the novel quinoline-serrulatane carbon skeleton. Furthermore, since this is first

alkaloidal identification from an *Eremophila* species, this genus warrants further chemical investigation.

Table 2. Biological Data for Microthecaline A (**1**) and Microthecaline A 8-Methyl Ether (**2**)[28]

compound	3D7[a]	Dd2[b]	HEK293[c]	SI	SI
	IC_{50} ± SD (μM)	IC_{50} ± SD (μM)		(HEK293/3D7)	(HEK293/Dd2)
1	7.7 ± 1.2	17.2 ± 0.4	62	5.2	2.3
2	NA	NA	NT		
chloroquine	0.0053 ± 0.0007	0.082 ± 0.035	NA	7512	486
artesunate	0.0009 ± 0.0001	0.0010 ± 0.0000	91% (20 μM)	11641	10030
puromycin	0.053 ± 0.004	0.044 ± 0.003	0.46 ± 0.09	9	10.5

[a]3D7 = *Plasmodium falciparum* (chloroquine sensitive) strain, [b]Dd2 = *P. falciparum* (chloroquine resistant) strain, [c]HEK293 = human embryonic kidney cell line, average percent inhibition measured at 80 μM. SD = standard deviation. NA = not active at 10 μM. NT = not tested. SI = selectivity index = IC_{50} HEK293/IC_{50} malarial strains.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter. UV spectra were acquired on a JASCO V-650 UV/vis spectrophotometer. ECD spectra were recorded on a JASCO J-715 spectropolarimeter. FTIR data were recorded on a Universal Attenuated Total Reflectance Two (UATR) on a PerkinElmer spectrophotometer. NMR spectra were acquired on a Varian 600 MHz Unity INOVA spectrometer or on a Bruker Avance HDX 800 MHz spectrometer equipped with a cryoprobe in CD_3OD (δ_H 3.31 and δ_C 49.0). A Waters ZQ mass spectrometer was used to record LRESIMS while the HRESIMS were acquired on a 12 T SolariX XR FT-ICR-MS instrument. A Waters 600 pump, 996 PDA detector and a Gilson 715 liquid handler were used for HPLC purification. Alltech C_{18} bonded silica gel (35-75 μm, 150 Å) was used for pre-adsorption work and was packed into a Grace stainless steel guard cartridge (10 × 30 mm). A ThermoElectron C_{18} Betasil 5 μm 143 Å (21.2 × 150 mm) column was used for HPLC. All solvents used were of HPLC grade (Honeywell Burdick & Jackson), and the H_2O was filtered using a Sartorius Arium® pro VF Ultrapure Water System.

Plant Material. The roots of *E. microtheca* were harvested in April of 2013 (Brisbane, Australia). A voucher sample (RAD039) has been archived at the Griffith Institute for Drug Discovery, Australia.

Extraction and Isolation. The roots of *E. microtheca* (63 g) were extracted with CH_2Cl_2 (3 × 1 L) and the resulting extract was dried to yield a brown gum (1.9 g). This extract was pre-adsorbed to C_{18}-bonded silica gel, packed into a guard cartridge and subjected to semi-preparative HPLC purification. Isocratic conditions of 85% $MeOH_{(aq)}$ (0.1% TFA) were held initially for 10 min, followed by a linear gradient to 100% MeOH (0.1% TFA) over 40 min, and was maintained at 100% MeOH (0.1% TFA) for the last 10 min, at a flow rate of 9 mL/min. Fractions 39–41 contained pure microthecaline A (**1**, 47.7 mg, 0.076% dry wt).

Microthecaline A (**1**): stable brown gum; $[\alpha]_D^{25}$ –18 (*c* 0.1, MeOH); UV (*c* 0.25 MeOH) λ_{max} (log ε) 213 (4.35), 247 (4.47), 298 (3.58), 339 (3.53) nm; ECD (*c* 0.25 MeOH) λ_{ext} 207 nm (Δε +7.4), 247 (Δε +8.8), 339 nm (Δε −12.1); IR (UATR) ν_{max} 3230, 2921, 1616, 1669, 1178, 1129, 718; ^1H and ^{13}C NMR data, see Table 1; (+)-LRESIMS *m/z* 296 (100) $[M + H]^+$; HRESIMS *m/z* 296.2013 $[M + H]^+$ (calcd for $C_{20}H_{26}NO$, 296.2009).

Methylation of Microthecaline A. Compound **1** (10.0 mg, 0.0339 mmol) was methylated using a previously reported method.[13] The product was purified by C_{18} HPLC using the isocratic system of 55% $MeOH_{(aq)}$ (0.1% TFA) for 10 min, followed by an increase to 100% MeOH (0.1% TFA) over 40 min, and was maintained at 100% MeOH (0.1% TFA) for the last 10 min, all at a flow rate of 9 mL/min. Microthecaline A 8-methyl ether (**2**, 2.2 mg, 20%) eluted between 34–35 min.

Microthecaline A 8-Methyl Ether (**2**): stable brown gum; $[\alpha]_D^{24}$ –211 (*c* 0.052, MeOH); UV (*c* 0.25 MeOH) λ_{max} (log ε) 246 (4.27), 265 (3.87), 330 (3.46) nm; ECD (*c*

0.25 MeOH) λ_{ext} 209 nm (Δε +7.8), 247 (Δε +9.3), 341 nm (Δε −12.8); ^{1}H and ^{13}C NMR data, see Table 1 (Supporting Information); (+)-LRESIMS *m/z* 310 (100) $[M + H]^+$; HRESIMS *m/z* 310.2164 $[M + H]^+$ (calcd for $C_{21}H_{28}NO$, 310.2165).

Computational Analysis. The theoretical calculations of the simplified models (A and B) of compound **1** was performed using Gaussian 09.[19] Conformational analysis was performed using MacroModel in Schrödinger 2015-1. All the conformational searching was carried out using the OPLS_2005 force field with a 21 kJ/mol energy window. The OPLS_2005 conformers were optimized at the B3LYP/6–31G(d, p) level and the lowest relative energy conformer was obtained from the Boltzmann distribution calculated from Schrödinger software. The ECD calculation was carried out using TDDFT at the B3LYP/6–31G(d, p) level in MeOH with a polarizable continuum model (PCM) model. The calculated ECD spectrum was generated using SpecDis 1.64.[29] The software SDAR v3.2 was used to process the raw ECD data.[30]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Copies of ^{1}H, ^{13}C, COSY, HSQC, HMBC, and ROESY NMR spectra of compounds **1** and **2**.

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Notes

The authors declare no competing financial interest.

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