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Two new flavones glycosides with antimicrobial activities from *Clerodendrum formicarum* Gürke (Lamiaceae)

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ABSTRACT

Clerodendrum formicarum Gürke from the Lamiaceae family is a Cameroonian medicinal plant. The crude methanol, methanol residual and ethyl acetate extracts of leaves have been phytochemically studied using chromatography column to afford four compounds; two new flavones glycoside: clerodendrononе 1a (**3**) and clerodendrononе 1b (**4**) along with two known compounds: 5,7-dihydroxy-4′-methoxyflavone (**1**) and 5-hydroxy-7,4′-dimethoxyflavone (**2**). Compound structures have been elucidated on the basis of their spectroscopy data and with literature information. The anti-microbial activities of extracts and three isolated compounds were performed. The antibacterial activity was evaluated against four gram positive, five gram negative and three fungus. Clerodendrononе 1b (**4**) showed good antibacterial activity against bacterial gram negative *Shigella flexineri* NR518 (MIC = 62.5 μg/ml) and moderate activity against *Staphylococcus aureus* NR46374 (MIC = 250 μ/ml). The ethyl acetate extract recorded good antibacterial activity against *Staphylococcus aureus* NR46003 (MIC = 125 μg/ml) and *Staphylococcus aureus* NR46374 (MIC = 125 μg/ml).

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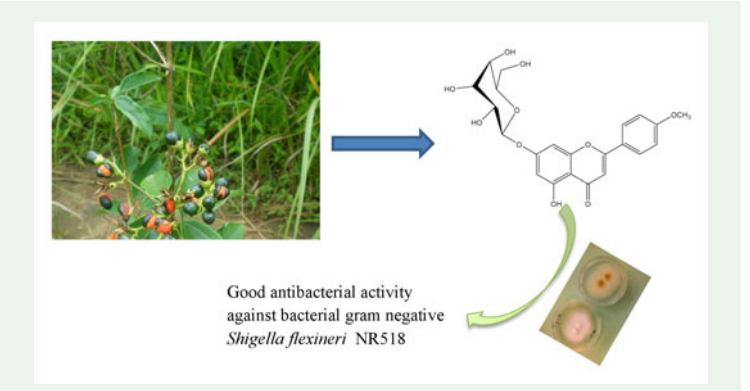

1. Introduction

Bacteria are ubiquitous and cause various types of human diseases including urinary tract infections (Bouza et al. 2001; Khan and Musharraf 2004), nosocomial bloodstream infections (Blot et al. 2005), wound infections (Wassilew 1989), brain abscess (Rau et al. 2002), pneumonia (Rubinstein et al. 2008), asthma (Chan-Yeung and Lam 1986), community acquired pneumonia (Tillotson and Lerner 1967) and skin infections (Delahaye et al. 2009). Some of the bacterial strains cause serious infections in human beings and in many cases death may result, for example, intestinal infection is the most common cause of diarrhea worldwide and is estimated to be responsible for the deaths of 3-4 million people each year (Anonymous 1996). Bacterial infections are common particularly in the tropics where Cameroon is located. They constitute a significant part of the disease burden and a large cause of mortality (Kumar and Clark 1990). Throughout the world, antibiotics are used to treat all microbial infections; however, bacteria become more and more resistant against antibiotics (Jacobs 1998). Due to those resistances there is a great interest in the search for new antimicrobial drugs. Furthermore, the increased use of antibiotics has created problem of dependence given. The fact that 80% of the worldwide populations use medicinal plants to treat various illnesses, plants can be a potential source of new antibacterial drugs.

The genus *Clerodendrum* (Lamiaceae) is very widely distributed in tropical and subtropical regions of the world. More than five hundred species of the genus are identified till now, which includes small trees, shrubs and herbs (Praveen et al. 2013). Plants of this genus have many uses in traditional medicine and showed several biological activities (Neeta and Tejas 2007). *Clerodendrum* genus is rich in various secondary metabolites such as steroids, terpenoids, flavonoids, alkaloids, ceramides (Yogesh et al. 2015; Neeta and Tejas 2007; Chaturvedi et al. 1983). *Clerodendrum formicarum* is a small tree; 2 to 4 m high with white flowers found in the western region of Cameroon where traditional healers used the leaves of this plant for the treatment of jaundice and infectious diseases (Maurice et al. 2016). The present work describes the isolation, structure elucidation, identification and antimicrobial activities of compounds and extracts obtained from the leaves of *Clerodendrum formicarum*. Previous work on this plant led to the

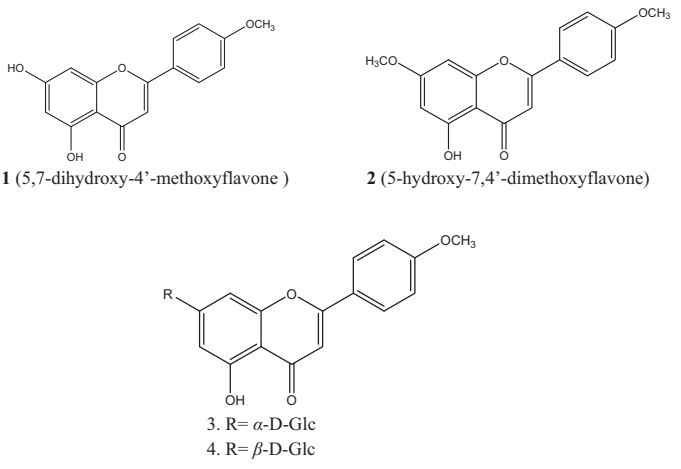

Figure 1. Chemical structures of compounds (1–4) isolated from *Clerodendrum formicarum*.

isolation of di and triterpenoids (Muhammad et al. 2010; Maurice et al. 2016), and of aromatic acids (Ali et al. 2010) with anti-proliferative activity (Maurice et al. 2016).

2. Results and discussion

2.1. Phytochemical results

The dried powder of the leaves of *Clerodendrum formicarum* was extracted with methanol. The dried crude methanol extract was partitioned with hexane to give hexane (50.75 g) and methanol residual (50.25 g) extracts after evaporation with rotavapor. The methanol residual extract was solubilised with ethyl acetate to give ethyl acetate (25.00 g) extract and methanol residual (25.25 g) extract. Repeated column chromatography of the ethyl acetate and residual methanol extracts resulted in the isolation of two new compounds named 5,7-dihydroxy-4′-β-D-glucopyranosylflavone (**4**, 15 mg), 5,7-dihydroxy-4′-α-D-glucopyranosylflavone (**3**, 21 mg) and two known compounds identified as 5,7-dihydroxy-4′-methoxy-flavone (**1**, 11 mg) and 5-hydroxy-7,4′-dimethoxyflavone (**2**, 6 mg) Figure 1.

Compound 3 was obtained as yellow amorphous powder. Its m.p. is 228.4 °C. The HR-ESI MS (Figure S2) of compound 3 led to the molecular formula $C_{22}H_{23}O_{10}$ this due to the presence of the Pseudo-molecular ion at m/z = 447.075, $[M+H]^+$ (calc for $C_{22}H_{22}O_{10}$). The UV spectrum (Figure S4) exhibited absorption maxima at 266 nm and 331 nm characteristic of band II and band I of flavones (Gaimei et al. 2009). The flavonic nature of compound 3 has been confirmed by the presence of a proton at 6.87 ppm (s, H-3) and carbon at 103.7 ppm corresponding respectively to proton H-3 and carbone C-3 of flavone (Gaimei et al. 2009).

The highly deshielded signal at 12.97 ppm suggested the evidence conjugation between the OH-5 and carbonyl moiety. The ^{1}H NMR spectrum (Figure S6) of compound 3 showed the presence of five aromatics protons at δ_H 6.96 ppm, 6.45ppm, 6.86ppm, 8.06 ppm and 7.13 ppm corresponding respectively to H-3, H-6, H-8, H-2′/6′ and H-3′/5′ and signal at 3.71 ppm corresponding to proton of the methoxyl group

(table S2). Apigenin skeleton was occurred from a hydroxyl δ 12.95 ppm (s, OH-5), two doublets at δ_H 6.21 (d, $J = 5.0$ Hz, H-6) and δ 6.51 (d, $J = 5.0$ Hz, H-8) on the B-ring; the presence of the A_2B_2 aromatic system was indicated by the chemical shift at δ_H 8. 04 ppm (d, $J = 5.0$Hz, H-2′, H-6′) and 7.13 ppm (d, $J = 5.0$ Hz, H-3′, H-5′). On the other hand AB-system was observed on ring A because of the presence of two protons signals at 6, 21 (1H, d, $J = 5.0$Hz) and 6.51 ppm (1H, d, $J = 5.0$ Hz). The occurrence of one olefinic proton δ_H 6.87 ppm (s, H-3) on a flavone C-ring (Hong-yun et al. 2016). The ^{13}C NMR spectrum (Figure S7, table S1) compound 3 revealed the presence of one primary carbon; δ_C 55.5 (C-1″) attributable to the methoxy carbon, ten tertiary carbons and of eight quaternary carbons. However, the presence of δ_C (157.3, 98.8, 164.2 and 94.0) for A-ring, δ_C (163.3, 103.7, 182.7, 161.4 and 103.7) for C-ring and δ_C (128.8, 122. 8, 114.6 and 162.3) are observed and attributable according the HSQC spectrum and located in these rings. The presence on its ^{13}C NMR spectrum of a signals at δ 100. 4 ppm related according HSQC spectrum to a proton at 5.07 ppm suggested the presence of a sugar moiety. This hypothesis is confirmed by the presence of other signal 77.1, 76.4, 73.1, 69.5 and 60.6 corresponding to four methines and one methylene respectively. Correlation observed on its HMBC spectrum (Figure S8) between H-1″ and C-7 and between O**C**$\underline{\mathbf{H}}_3$ and C-4′ allowed us to say that sugar moiety is in position 7 while the methoxyl group is in position 4′ (Teng et al. 1999; Gorin and Can 1975). The *alpha* or *beta* nature of sugar was deduced from the coupling constancy value of the anomeric proton. Indeed, according to the literature when this value is from ^{3}J (H-1, H-2) = 10.0 Hz (>5 Hz) then the sugar is *beta* whereas when it is ≤ 5.0 Hz the sugar is a*lpha*. The coupling constancy of anomeric proton of compound 3 is 5.0 Hz that means that the sugar is the *alpha* one (Rongwei et al. 2002). All those information's and those found in literature allowed us to say that compound 3 is 5,7-dihydroxy-4′-α-D-glucopyranosylflavone, a new flavone to which trivial name clerodendronone 1a has been given.

Compound 4 was also obtained as a yellow amorphous powder. Its UV spectrum exhibited the maxima absorption 206 nm and 331 nm characteritic of those of flavone skeleton. The LC-MS, ESI-MS spectrum (Figure S4) showed pseudo-molecular ion at *m/z* 447.1288 $[M + H]^+$ corresponding to $C_{22}H_{23}O_{10}$. The ^{13}C NMR and ^{1}H spectra (Figure S10 and S11) were similar to those of compound 3 but the coupling constancy value 10.1Hz of the anomeric proton suggest that in compound 4 we have a β-D-glycoside. The structure of compound (4) was determined as dihydroxy-4′-β-D-glucopyranosylflavone, a new flavone trivially named clerodendronone 1b.

2.2. Antimicrobial assay

The antibacterial activity was evaluated by determining the Minimum inhibitory concentration (MIC) of extracts (crude methanol, ethyl acetate extract, hexane extracts), compounds:5,7-dihydroxy -4′-α-D-glucopyranosylflavone (**3**), 5,7-dihydroxy-4′-β-D-glucopyranosylflavoneand (**4**) 5,7-dihydroxy-4′-methoxy-flavone (**1**) against multi-resistant bacteria (table S3) according the method of the Clinical Laboratory Standard Institute (CLSI 2008b) M7-A9 microdilution. The result obtained showed that the plant extracts displayed distinct antibacterial activities against the tested bacterial strains, with MICs

ranging from 125 μg/ml to >500 for extracts and 62.5 to >500 μg/ml for the compounds. The ethyl acetate extract was the most active followed by the crude methanol extract, residual methanol extract and hexane extract. Hence the moderate and lowest MIC value (250 μg/ml) and (62.5 μg/ml) corresponding to the moderate and highest activities were obtained with the 5,7-dihydroxy -4′-β-D-glucopyranosylflavone against bacteria gram positive *Staphylococcus aureus* NR46374 and bacteria gram negative *Shigella flexineri* NR518 respectively. The activities of extracts and products were lower than those obtained with the reference antibiotics (amoxicillin and fluconazole).

In the continuation of new antimicrobial drug discovery, extracts (crude methanol, residual methanol, ethyl acetate and hexane extracts) of leaves of *Clerodendrum formicarum* and three isolated compounds were investigated for their antimicrobial potential. The antibacterial activity of the plants extracts is attributed to the presence of various bioactive compounds such as phenolic, tannins and flavonoids (Ouattara et al. 2012). Several flavonoids isolated from Cameroonian medicinal plants have been reported for their antimicrobial activities (Kuete 2010). The results of the antibacterial assays justify the use of the plants in the traditional medicine. It's the first reports about the antibacterial activity test of *Clerodendrum formicarum*. Many species from the genus *Clerodendrum* were documented in the literature and have showed antimicrobial potential (Pallab et al. 2014). The results of our investigation showed that the most active compound is 5,7-dihydroxy-4′-β-D-glucopyranosylflavone while its isomer showed lower activity. This observation indicates the influence of the sugar configuration in the biological activity of compounds.

3. Experimental

3.1. General experimental procedures

High resolution mass spectra were obtained with a LC-MS-QTOF Spectrometer (Bruker, Germany) equipped with a HESI source. The spectrometer was operated in positive mode (Hmass range: 100–1500, with a scan rate of 1.00 Hz) with automatic gain control to provide high-accuracy mass measurements within 0.40 ppm deviation using Na Formate as calibrant.

The NMR data were recorded with a Bruker spectrometer with tetramethylsilane (TMS) as standard ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz).

Column chromatography (CC) was performed with silica gel 60 (Merck, 70-230 mesh particle size) as adsorbent and thin layer chromatography (TLC) on pre-coated silica gel on aluminum sheets F-254 Merck (20x20 cm). The melting points of the products were recorded in an open capillary using Stuart melting point apparatus.

3.2. Plant material

The leaves of *C. formicarum* were collected from Mont Elumdem Nkolbisson in the Centre Region of Cameroon in November 2013 (dried season). The plant was localized at the geographical coordinates of 3°49′0″ North, 11°25′60″ East and 1.010 m of altitude with a 'QK72' UTM position and a Joint Operation Graphic of NA32-04. The voucher specimen was identified and authenticated by Mr. Nana Victor a botanist of the

national herbarium of Yaounde Cameroon this by comparison with a known specimen under voucher number No 10842 SRF/Cam.

3.3 Extraction and isolation

The leaves collected were air-dried at room temperature (26-27 °C) during six days and powdered. The air-dried powder of *C. formicarum* (1.8 kg) was macerated in the pure methanol (15 l) during 24 h at room temperature. Removal of the solvent from the extract under reduced pressure at 55 °C of a dark green residue. The dark green methanolic extract (475 g) corresponding to 36.53% in terms of extraction yield was partitioned for the first time between methanol and hexane. Dried methanol phase was extracted with ethyl acetate to obtain methanol residual extract and ethyl acetate extract.

The ethyl acetate extract (15 g) was separated using column chromatography on silica gel and eluted with gradient of hexane, ethyl acetate and methanol solvent system at Hex-EA (60/40) and Hex/AE (60/40) two yellow amorphous powder was obtained (compound 1 and 2). The methanol residual extract (15 g) was submitted to the same process and afforded two compounds: 3 and 4.

3.4. General information's of compounds

Compound 1 (5,7-dihydroxy-4′-methoxyflavone)**:** Yellow amorphous powder, ^{1}H NMR (δ, DMSO-d6, 500 MHz): 8.04 (dd, $J = 7.0$ Hz and $J = 2.0$ Hz, H-2′/6′), 7.12 (dd, $J = 7.0$ Hz and $J = 2.0$ Hz, H-3′/5′), 6.84 (s, H-3), 6.53 (s, H-6), 3.88 (s, OCH_3-7), 3.85 (s, OCH_3-4′). ^{13}C NMR (125 MHz): 182.4 (C-4), 163.5 (C-2), 162.4 (C-4′), 154.3 (C-7), 153.0 (C-5), 144.4 (C-9), 128.5 (C-2′/6′), 126.2 (C-9), 123.0 (C-1′), 114.5 (C-3′/5′), 103.9 (C-10), 103.0 (C-3), 95.7 (C-6), 56.3 (OCH_3-7), 55.6 (OCH_3-4′).

Compound 2 (5-hydroxy-7,4′-dimethoxyflavone)**:** Yellow amorphous powder, m.p. 259.6 °C HRESI-MS (+) m/z 285, 0756 ($[M + H]^+$ calcd for $C_{16}H_{12}O_5$, 285). UV (MeOH): $\lambda_{(Max)}$ nm log (ε) 210, 266, 331. ^{1}H NMR (δ, 500 MHz): 8.06 (d, $J = 5.0$ Hz, H-2′/6′), 7.12 (d, $J = 5.0$ Hz, H-3′/5′), 6.87 (s, H-3), 6.21 (s, H-6), 10.50 (OH-7), 12.95 (OH-5), 3.97 (s, OCH_3-4′). ^{13}C NMR (δ, 125 MHz): 181.7 (C-4), 163.2 (C-2), 103.7 (C-3), 161.4 (C-5), 98.8 (C-6), 164.5 (C-7), 94.0 (C-8), 157.3 (C-9), 103.5 (C-10), 122.8 (C-1′), 128.3 (C-2′/5′), 114.5 (C-3′/6′), 162.3 (C-4′), 55.8 (OCH_3-4′).

Compound 3 (clerodendronone 1a): Yellow amorphous powder, m.p.228.4 °C HRESI-MS (+) m/z 447, 1282 ($[M + H]^+$ calcd for $C_{22}H_{23}O_{10}$, 4417,) UV (MeOH): $\lambda_{(Max)}$ nm log (ε) 206, 266, 331. ^{1}H NMR (δ, 500 MHz): 8.06 (d, $J = 5.0$ Hz, H-2′/5′), 7.13 (d, $J = 5.0$ Hz, H-3′/6′), 6.96 (s, H-3), 6.45 (s, H-6), 6.86 (s, H-8), 12.92 (OH-5), 5.07 (d, $J = 5.0$ Hz, H-1″), 3.26 (s, H-2″), 3.46 (H-3″), 3.17 (s, H-4″), 3.29 (s, H-6″a), 3.71 (s, H-6″b). ^{13}C NMR (δ, 125 MHz): 182.5 (C-4), 164.3 (C-2), 103. 7 (C-3), 161.5 (C-5), 100.0 (C-6), 164.3 (C-7), 96.4 (C-8), 157.4 (C-9), 104.2 (C-10), 123.1 (C-1′), 128.9 (C-2′/5′), 115.1 (C-3′/6′), 162.9 (C-4′), 56.0 (OCH_3-4′), 100.3 (C-1″), 77.6 (C-2″), 76.9 (C-3″), 73.5 (C-4″), 70.0 (C-5″), 61.0 (C-6″).

Compound 4 (clerodendronone 1b): Yellow amorphous powder, m.p.221.4 °C HRESI-MS (+) m/z 447.1288 ($[M + H]^+$ calcd for $C_{22}H_{23}O_{10}$, 4417) UV (MeOH): $\lambda_{(Max)}$

nm log (ε) 202, 266, 331. ^{1}H NMR (δ, 500 MHz): 8.06 (d, $J = 5.0$ Hz, H-2′/6′), 7.14 (d, $J = 5.0$ Hz, H-3′/5′), 6.96 (s, H-3), 12.92 (OH-5), 6.46 (s, H-6), 6.86 (s, H-8), 12.92 (OH-5), 3.86 (OCH_3-4′), 5.06 (d, $J = 10,1$ Hz, H-1″), 3.27 (s, H-2″), 3.44 (s, H-3″), 3.17 (s, H-4″), 3.30 (s, H-5″), 3.70 (s, H-6″a), 3.48 (s, H-6″b). ^{13}C NMR (δ, 125 MHz): 163.8 (C-2), 103.7 (C-3), 182.0 (C-4), 161.1 (C-5), 99.5 (C-6), 163.0 (C-7), 94.9 (C-8), 156.9 (C-9), 106.3 (C-10), 122.6 (C-1′), 128.4 (C-2′/5′), 114.6 (C-3′/6′), 162.4 (C-4′), 55.5 (OCH_3-4′), 99.9 (C-1″), 77.1 (C-2″), 76.4 (C-3″), 73.0 (C-4″), 69.5 (C-5″), 60.6 (C-6″).

3.5. Antimicrobial activities assessment

3.5.1. Microorganisms

The following bacterial strains were used for the screening: *Staphylococcus* ATCC43300, *Klebsiella* NR41916, *Shigella flexineri* NR518, *Klebsiella pneumonia* ATCC13883, *Pneudmonas enteric* NR13555, *Staphylococcus aureus* NR46003, *Staphylococcus aureus* NR46374, *Candida krusei* ATCCL26, *Candida parapsilosis* ATCC22019. Isolates were obtained from the Yaoundé Central Hospital, Cameroon and the reference strains from BEI resources and the American Type Culture Collection. The microorganisms were maintained on agar slope at 4 °C and sub-cultured for 24 h and 48 h before use.

3.5.2. Preparation of stock solutions of fungal extracts and reference drugs

The stock solutions of crude extracts were prepared at 1 mg/ml using 10% dimethyl sulfoxide (DMSO). Fluconazole and amoxicillin (Sigma Aldrich) were used as reference drugs respectively for fungal and bacteria. The stock solutions were filter-sterilized with a 0.20 μm syringe filter and stored at −20 °C until use.

3.5.3. Antimicrobial assay

For the estimation of the antimicrobial activities of extracts and isolated compounds, a broth dilution method was employed for minimum inhibitory concentration (MIC) determination following the Clinical and Laboratory Standards Institute (CLSI) guidelines M27-A3 for yeast and M7-A9 for bacteria (CLSI 2008a). Each extract/compound was first of all tested in duplicate at 500 μg/ml and only extracts showing inhibition were subsequently considered for MIC determination.

More specifically, 50 μl of Sabouraud dextrose broth (SDB) or Mueller Hinton broth (MHB) were introduced in a 96-well microplate respectively for fungal and bacteria. 50 μl of extract/compound concentrated at 1 mg/mL were added to wells of the first line. A serial twofold dilution was made by transferring 50 μL of the mixture of the first wells to the next one up to the last, final concentrations varying from 500 to 31.25 μg/ml. Then, 50 μL of an inoculum of 1×10^5 cells/ml for yeast and 1×10^6 cells/mL for bacteria were introduced in all the wells except those of the sterility control. Fluconazole and Amoxicillin were used as positive control respectively for fungal and bacteria. Each plate also contained a positive control (Fluconazole or Amoxicillin), a negative control and a blank. Plates were incubated during 24 and 48 hours for bacterial and fungal respectively. The lowest concentration of extract/compound that inhibited the visible growth of a microorganism was defined as minimum inhibitory concentration (MIC). Cut-off points for significant activity of extracts were as follow:

very good (MIC $<$ 62.5 μg/ml), good (62.5 $<$ MIC $\leq$ 125 μg/ml), moderate (250 $<$ MIC $\leq$ 500 μg/ml) or weak (MIC $>$ 500 μg/ml).

4. Conclusion

The present work concerns phytochemical and antimicrobial activity of *Clerodendrum formicarum*. Column chromatography of residual methanol and ethyl acetate extracts afforded four compounds: 5,7-dihydroxy-4′-α-D-glucopyranosylflavone (clerodendrononе 1a); 5,7-dihydroxy-4′-β-D–glucopyranosylflavone (clerodendrononе 1b), 5,7-dihydroxy-4′-methoxy-flavone and 5-hydroxy-7,4′-dimethoxyflavone. Clerodendrononе 1b isolated from residual methanol extract was exhibited antibacterial activity against *Staphylococcus aureus* NR46374 bacteria gram positive and bacteria gram negative *Shigella flexineri* NR518 respectively. Taking into account the polar nature of the secondary metabolites present in this plant which makes the isolation difficult, we plan in the future to isolate compounds of the other parts of this plant and to evaluate their biological potentialities.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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