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ANTIMICROBIAL AND ANTILEISHMANIAL XANTHONES FROM THE STEM BARK OF *Allanblackia gabonensis*

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The phytochemical study of the stem bark of Allanblackia gabonensis *has resulted in the isolation and characterization of one new xanthone derivative, named allanxanthone D, together with ten known compounds, including six xanthone derivatives, allanxanthone A, 1,5-dihydroxyxanthone, 1,7-dihydroxyxanthone and 1,3,6,7-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone, forbexanthone, 6-deoxy-isojacareubin, one polyisoprenylated benzophenone, guttiferone F, one flavanol, epicathechin, two phytosterols,* β*-sitosterol, and campesterol. The structures of these compounds were established on the basis of one- and two-dimensional NMR homo- and heteronuclear evidence. These compounds were evaluated for their activity against Leishmania amazonensis* in vitro *and antimicrobial activities against a range of* Gram-*negative and* Gram-*positive bacteria.*

Key words: *Guttifereae*, *Allanblackia,* allanxanthone D, xanthones, antileishmanial and antimicrobial activities.

According to estimates of the World Health Organisation (WHO), 350 million individuals are living at risk with *Leishmania* parasite. Leishmaniasis incidence increases especially in Africa, Asia, and Latin America; and this illness become a more and more important cause of death. Antileishmanial drugs are few, expensive, and often toxic. Most of them (except miltefosine) are only usable by IV routes, and the resistance of the parasites is increasing. Thus, the search for new compounds as safe and efficient drugs for this disease becomes urgent. Natural substances could be an interesting source of such drugs, in particular natural products from plants as drug candidates and lead compounds against leishmaniasis and trypanosomiasis [1].

Plants of the genus *Allanblackia* are intriguing targets for phytochemical investigation not only because of their great structural variability but also because of the diverse biological activities of their secondary metabolites, among which are xanthones, benzophenones, biflavonoids, and triterpenoids [2–6]. *A. gabonensis,* locally named "agnoume," is a high sub-mountain dweller tree widely distributed in the Center Province of Cameroon [7, 8], where it is used as a medicinal plant against infections like dysentery, cold, and toothache [9, 10].

This paper described the isolation and structural elucidation of a new xanthone derivative named allanxanthone D (**1**), together with ten known compounds, allanxanthone A (**2**) [4], 1,5-dihydroxyxanthone (**3**) [11], 1,7-dihydroxyxanthone (**4**) [12] and 1,3,6,7-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone (**5**) [13], forbexanthone (**6**) [14], 6-deoxyisojacareubin (**7**) [15], one polyisoprenylated benzophenone, guttiferone F (**8**) [16], one flavanol, epicathechin (**9**) [17], two phytosterols, *β*-sitosterol (**10**), and campesterol (**11**). The antileishmanial activity against *Leishmania amazonensis* and antimicrobial activities against a range of *Gram*-negative and *Gram*-positive bacteria of some of the isolated compounds are also reported.

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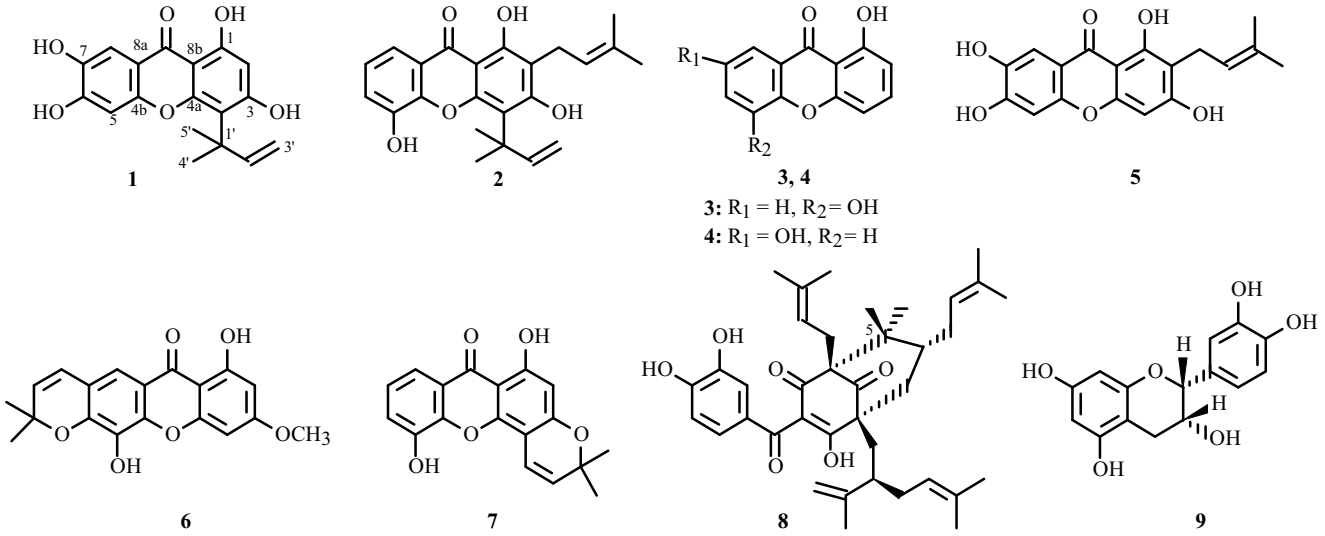

Compound **1**, allanxanthone D, mp 238–240°C, was obtained as an orange powder and reacted positively with Gibbs and $FeCl_3$ reagent, indicating the presence of the phenolic group. The high-resolution ESI-TOF mass spectrum showed $[M+H]^+$ at *m/z* 329.0945, corresponding to the molecular formula $C_{18}H_{16}O_6$, indicating 11 degrees of unsaturation.

The UV spectrum of **1** displayed the maxima of absorption (MeOH) at 253 and 280 nm. The data obtained from the IR spectrum showed free hydroxyl (ν_{max} 3456 cm^{-1}), chelated hydroxyl (3290 cm^{-1}), conjugated carbonyl (1646 cm^{-1}), and aromatic ring (1620, 1585 cm^{-1}). All these absorptions were consistent with the presence of a xanthone skeleton tetraoxygenated [18].

The broad-band decoupled ^{13}C NMR spectrum of **1** showed 18 carbon signals which were attributed by APT, DEPT, and HSQC techniques to two methyls, one methylene, four methines, and 10 quaternary carbons, including a carbonyl (δ 181.2) The ^1H NMR spectrum (acetone) of compound **1** analyzed by ^1H–^1H COSY indicated three singlets of aromatic protons at δ 6.26; 6.91 and δ 7.51 whose positions remain to be established. The high deshielding of this last proton at δ 7.51 suggested that it is located in the paramagnetic anisotropy cone of carbonyl , i.e., at the C-8 position of the xanthone skeleton. Futhermore, the ^1H and ^{13}C NMR spectra also displayed an ABC system set of signals comprising a singlet of six protons at δ 1.67 (s)/ δ_C 29.5 and three doublet of doublets each of one proton at δ 4.91 (1H, dd, 10.5 Hz; 1.05 Hz)/δ_C 108.0 at δ 5.03 (1H, dd, 17.6; 1.05 Hz)/δ_C 108.0 and δ 6.35(1H, dd, 10.5 Hz; 17.6 Hz)/δ_C 151.5, suggesting the presence of a 1,1-dimethylallyl substituent. In addition, we observe a free hydroxyl signal at δ 8.10, a chelated hydroxyl signal at δ 13.47, and two chelated hydroxyl protons at δ 9.10; δ 9.16.

It remained to establish, at this stage of the discussion, not only the position of the 1,1-dimethylprop-2-enyl group, but also those of the three other groups of hydroxyls on the xanthone skeleton. Thus, the fact that in the HMBC spectrum, the chelated hydroxyl proton at δ 13.41 displayed cross-peaks with the carbons at δ_C 99.6 (C-2), 156.8 (C-4a), and 163.9 (C-1) in addition to cross-peaks between the proton at δ 6.26 (H-2) and carbons δ 162.2 (C-3), 163.9 (C-1), suggested that this aromatic proton is localized at the C-2 position. In the same way, correlations observed between the proton at δ 6.26 with carbons δ 162.2 (C-3), 111.7 (C-4) and those observed between the protons of the *gem*-methyl group at δ 1.67 with carbons δ 41.8 (C-1′), 111.7 (C-4), indicated that the *α,α*-dimethylallyl group is located at the C-4 position. Moreover, the fact that the H-8 proton appears as a singlet indicates that it is in the *para* position compared to the other aromatic proton at δ 6.91, i.e., in the C-5 position. This assumption is confirmed by the analysis of the NOESY spectrum, on which we correlated peaks between this aromatic proton at δ 6.91 and one of the hydroxyls slightly chelated at δ 9.10 as well as the interactions with the proton in the *peri* position, i.e., C-8 and the other hydroxyl slightly chelated at δ 9.16. On the basis of the above analysis, the structure of allanxanthone D **1** was assigned to be 1,3,6,7-tetrahydroxy-4-(1,1-dimethylprop-2-enyl)xanthone.

The antileishmanial activity of compounds **1**, **4**, **5**, and **9** (IC_{50} μg/mL: 13.9, N.a., 13.3, N.a releafevily) may be related to some of their structural features. Xanthones have been shown to be more active than flavonoids. Among xanthones, tetraoxygenated prenylated xanthones were more active than dioxygenated simple xanthones, and their activities may be attributed to the presence of the prenyl group at position 2 or 4.

TABLE 1. Minimum Inhibition Concentration[a] (μg/mL and μM) of Compounds **1**, **2**, **5** and Reference Antibiotics

Microorganisms	Tested samples			
	1	**2**	**5**	RA[b]
		Gram-negative bacteria		
Citrobacter freundii	2.44 (7.44)	4.88 (12.84)	39.06 (119.1)	4.88 (9.0)
Enterobacter aerogens	39.06 (119.1)	39.06 (102.79)	>78.12 (>238.17)	9.76 (18)
Enterobacter cloacae	2.44 (7.44)	>78.12 (>205.58)	9.76 (29.77)	4.88 (9.0)
Escherichia coli	2.44 (7.44)	>78.12 (>205.58)	19.53 (59.54)	1.22 (2.25)
Klebsiella pneumoniae	2.44 (7.44)	2.44 (6.42)	4.88 (14.89)	2.44 (4.5)
Morganella morganii	39.06 (119.1)	>78.12 (>205.58)	19.53 (59.54)	2.44 (4.5)
Proteus mirabilis	4.88 (14.88)	>78.12 (>205.58)	19.53 (59.54)	2.44 (4.5)
Proteus vulgaris	1.22 (3.72)	4.88 (12.84)	>78.12 (>238.17)	1.22 (2.25)
Pseudomonas aeruginosa	1.22 (3.72)	>78.12 (>205.58)	4.88 (14.89)	4.88 (9.0)
Shigella dysenteriae	4.88 (14.88)	>78.12 (>205.58)	39.06 (119.1)	2.44 (4.5)
Shigella flexneri	19.53 (59.54)	39.06 (102.79)	78.12 (238.17)	2.44 (4.5)
Salmonella typhi	2.44 (7.44)	19.53 (51.37)	39.06 (119.1)	2.44 (4.5)
		Gram-positive bacteria		
Streptococcus faecalis	1.22 (3.72)	39.06 (102.79)	9.76 (29.77)	4.88 (9.0)
Staphylococcus aureus	1.22 (3.72)	9.76 (25.68)	2.44 (7.44)	4.88 (9.0)
Bacillus cereus	1.22 (3.72)	>78.12 (>205.58)	>78.12 (>238.17)	2.44 (4.5)
Bacillus megaterium	2.44 (7.44)	39.06 (102.79)	2.44 (7.44)	4.88 (9.0)
Bacillus stearothermophilus	2.44 (7.44)	4.88 (12.84)	19.53 (59.54)	4.88 (9.0)
Bacillus thurengiensis	4.88 (14.88)	39.06 (102.79)	>78.12 (>238.17)	9.76 (18)
Bacillus subtilis	2.44 (7.44)	>78.12 (>205.58)	9.76 (29.77)	2.44 (4.5)
		Fungi		
Candida albicans	19.53 (59.54)	78.12 (>205.58)	>78.12 (>238.17)	4.88 (5.21)
Candida gabrata	1.22 (3.72)	2.44 (6.42)	2.44 (7.44)	4.88 (5.21)
Candida krusei	19.53 (59.54)	39.06 (102.79)	>78.12 (>238.17)	9.76 (10.42)
Absidia sp.	1.22 (3.72)	>78.12 (>205.58)	>78.12 (>238.17)	1.22 (2.11)

[a] Minimum Inhibition Concentration: lowest concentration at which there was 100% growth inhibition of the tested pathogens in μM (in parenthesis); No effect of the DMSO used as dilution solvent was observed on the tested microbial strains.
[b] RA: Reference antibiotics (gentamycin for bacteria, nystatin for *Candida species*, and amphotericin B for *Absidia* sp.); The others compounds were in small quantities for the tests.

The results of the antimicrobial assay demonstrated that compounds **1**, **2**, **5** exhibited a wide spectrum of antimicrobial activity with important inhibition either on *Gram*-positive or *Gram*-negative bacteria, yeasts, and mycelial fungus. The MIC values range from 1.22–39.06 μg/mL (3.72–119.1 μM) on *Gram*-negative bacteria, 1.22–4.88 μg/mL (3.72–14.88 μM) on *Gram*-positive bacteria, 1.22–19.53 (3.72–59.54 μM/mL) on *Candida* species, while the MIC obtained on the mycelial fungi was 1.22 μg/mL (3.72 μM/mL) (Table 1). The activity of compound **1** is greater than that of reference antibiotics on all *Gram*-positive and some of the tested *Gram*-negative bacteria as well as against *Candida krusei*. Xanthones are known to complex irreversibly with nucleophilic amino acids in proteins, often leading to the inactivation of proteins and loss of function [19]. This appeared to be the possible mechanism by which compounds **1**, **2**, and **5** exhibited their antimicrobial effects.

EXPERIMENTAL

The IR spectra were recorded using an infrared spectrophotometer in Fourier transform of Nicolet 400 type on KBr pellet. UV spectra were recorded in MeOH solution on a Kontron-Uvikon 932 spectrophotometer. The mass spectra were

obtained in the positive mode on spectrometers of APCI QSTSAR^{TM} type equipped with an analyzer in time of flight and using an electrospray like the ionization technique. The NMR experiments were carried out in various deuterated solvents (MeOH, acetone, pyridine, DMSO, $CDCl_3$) on a Brucker AC300 spectrometer with fourrer transform for the experiments 1D (300.13 MHz for ^1H and 75.47 MHz for the ^{13}C) and on a Brucker 400 spectrometer with fourier transform for the experiments 2D (400.13 MHz for the ^1H and 100.71 for the ^{13}C). The chemical shifts of the ^1H are expressed in ppm and the coupling constants J in Hz. The direct spectra of correlation ^1H–^{13}C, HSQC were carried out to 400.13 MHz in two dimensions (proton dimension) and to 100 in carbon dimension. HMBC experiments were recorded to 400.13 MHz at an average frequency of 4401 MHz. NOESY spectra were recorded with a time of mixing of 500 ms. The column chromatographies were carried out on variable diameters of columns by using like stationary phase silica gel with granulometry, 60 Merck between 70–230 mesh and 230–400 mesh. The flash chromatographies were done on a short column (12×10 cm). The preparative and analytical thin layer chromatographies were carried out on aluminum plates covered with G.60 silica of GF_{254} (Merck, Darmstadt, Germany) type. The spots in CCM are visualized in UV light or by pulverization using sulfuric acid (H_2SO_4) or revealed with iodine. The various mass obtained were measured on an electronic balance of type "Sartorius". The various fractions were concentrated on an evaporator of the type Heidolph VV 200.

Plant Material. The stem bark of *Allanblackia gabonensis* was collected with the Kala mount located in the district of Mbankomo, Mefou Afamba division, Center province of Cameroon in October 2003 by M. Nana, botanist at the National Herbarium, Yaounde, where a voucher specimen was deposited under reference number 23255/HNC.

Extraction and Isolation. Ground, air-dried, stem bark of *Allanblackia gabonensis* was extracted with CH_2Cl_2/MeOH at room temperature. The extracts, concentrated under reduced pressure and submitted to flash chromatography, afforded four series, A (17 g), B (8 g), C (34 g), and D (42 g). By repeated column chromatography on silica gel of series A, we obtained a novel derivative compound, allanxanthone D (**1**), together with ten known compounds, including four xanthone derivatives allanxanthone A (**2**), 1,5-dihydroxyxanthone (**3**), 1,7-dihydroxyxanthone (**4**) and 1,3,6,7-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone (**5**), forbexanthone (**6**), 6-deoxyisojacareubin (**7**) one polyisoprenylated benzophenone, guttiferone F (**8**), one flavanol, epicathechin (**9**), two phytosterols, β-sitosterol (**10**), and campesterol (**11**).

From series A eluted with a mixture of hexane ethyl acetate 150 fractions were collected. Fractions 1–5, rechromatographed on column silica gel, gave 0.03 g of yellow greenish guttiferone F **8**. Fractions 13–18 left at the room temperature precipitated allanxanthone A (**2**) (1.02 g) in the form of soluble yellow powder, which dissolved in acetone. From fractions 20–26 was precipitated, at ambient temperature, in the form of a white spangle soluble in acetone, a mixture of β-sitosterol (**10**) and campesterol (**11**). From fractions 29–35 left at room temperature was precipitated 1,5-dihydroxyxanthone (**3**, 0.096 g), in the form of a yellow powder soluble in acetone; also, from fractions 40–50 left at room temperature was precipitated 1,7-dihydroxyxanthone (**4**, 0.06 g) in the form of a yellow powder soluble in acetone. Fraction 139, allanxanthone D (**1**, 1.05 g) precipitated at room temperature in the form of an orange powder soluble in acetone. From fractions 117–120 left at ambient temperature precipitated epicathechin (**9**, 0.075 g) as shining crystals soluble in MeOH. From fractions 142–145 left at room temperature precipitated a yellow powder soluble in acetone and corresponding to 1,3,6,7-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone (**5**, 0.75g). Series B was column chromatographed over silica gel (230–400 mesh). Left at room temperature, from fractions 41–64, on the one hand, and fractions 65–80, on the other hand, was precipitated, in the form of a mixture, a soluble yellow powder in acetone. Purification of this mixture was made possible by means of preparative chromatography using for the elution a mixture of hexane and ethyl acetate (4:1). With three successives migrations, the most polar bands are recovered. The extraction and evaporation allowed us to obtain two pure amorphous compounds, forbexanthone (**6**, 0.078 g) and 6-deoxyisojacareubin (**7**, 0.03 g).

Series C and D contained very polar compounds difficult to separate.

Allanxanthone D (1) (1,3,6,7-tetrahydroxy-4-(1,1-dimethylprop-2-enyl)xanthone). Orange powder; + HRESI-TOF-MS *m/z* 329.0945 ($C_{18}H_{16}O_6$). IR (KBr, cm^{-1}): 3456, 3290, 1646, 1620, 1585, 1470, 1421, 1243, 1140, 1116. UV (λ_{max}, nm): 248, 253, 311, 342; (+NaOH): 261, 292, 352; (+$AlCl_3$): 260, 336, 397; (+NaOAc): 291, 344. For ^1H NMR 13.41 (1H, s, OH-1); 9.16 (1H, br.s, OH-7); 9.10 (1H, br.s, OH-6); 8.10 (1H, br.s, OH-3); 7.51 (1H, s, H-8); 6.91 (1H, s, H-5); 6.35 (1H, dd, 2.7; 17.6 Hz, H-2′); 6.26 (1H, s, H-2); 5.03 (1H, dd, 1.05; 10.5 Hz, H-3′); 4.91 (1H, dd, 1.05; 17.6 Hz, H-3′); 1.67 (3H, s, H-4′, H-5′) and ^{13}C NMR, 181.0 (C-9); 163.9 (C-1); 162.2 (C-3); 156.8 (C-4a); 154.2 (C-6); 152.3 (C-4b); 144.0 (C-7); 133.4 (C-3′); 123.5 (C-2′); 113.3 (C-5); 111.7 (C-4); 108.8 (C-8); 103.7 (C-8b); 99.6 (C-2); 41.8 (C-1′); 29.5 (C-4′, C-5′).

Microbial Strains. A total of 23 microbial cultures showing resistance to the commonly used antibiotics and belonging to seven *Gram*-positive bacterial species (*Bacillus cereus* LMP0404G, *Bacillus megaterium* LMP0204G, *Bacillus*

stearothermophilus LMP0104G, *Bacillus thurengiensis* LMP9901E, *Bacillus subtilis* LMP0304G, *Staphylococcus aureus* LMP0206U, *Streptococcus faecalis* LMP0207U), 12 *Gram*-negative bacteria (*Escherichia coli* LMP0101U, *Shigella dysenteriae* LMP0208U, *Proteus vulgaris* LMP0103U, *Proteus mirabilis* LMP0504G, *Shigella flexneri* LMP0313U, *Klebsiella pneumoniae* LMP0210U, *Pseudomonas aeruginosa* LMP0102U, *Salmonella typhi* LMP0209U, *Morganella morganii* LMP0904G, *Enterobacter aerogens* LMP 1004G, *Citrobacter freundii* LMP0904G, *Enterobacter cloacae* LMP1104G), three yeasts from Candida species (*Candida albicans* LMP0204U, *Candida krusei* LMP0311U, and *Candida gabrata* LMP0416U), and one mycelial fungus (*Absidia* sp LMP9903) were used in this study. Three of the four *Bacillus* species were provided by the ''L'Institut Appert de Paris,'' while *Bacillus cereus* was provided by the A.F.R.C Reading Laboratory of Great Britain. The other strains were clinically isolated from patients in the Centre Pasteur de Yaounde-Cameroon (health institution). They were then maintained on agar slants at 4°C in the Laboratory of Applied Microbiology and Molecular Pharmacology (Faculty of Science, University of Yaounde I), where the antimicrobial tests were performed. The strains were activated at 37°C for 24 h on nutrient agar (NA) (bacteria) or Sabouraud glucose agar (fungi). The nutrient broth (NB) was used to determine the minimal inhibition concentration (MIC) of compounds **1**, **2**, and **5** against the tested pathogens.

Antimicrobial Assays. MICs of compounds **1**, **2**, and **5** were evaluated against the pathogens. The inocula of microorganisms were prepared from 12 h broth culture and the suspensions were adjusted to 0.5 McFarland turbidity. The compounds were first dissolved in dimethyl sulfoxide (DMSO) to the highest dilution (78.12 µg/mL), and serial twofold dilutions were made in a concentration ranging from 0.31 to 78.12 µg/mL in the 96-well microplate containing NB. MIC values of the tested compounds against pathogens were determined based on the microdilution method, as the lowest concentration at which there was 100% growth inhibition of the tested pathogens [20]. Gentamycin (bacteria) and Nystatin (yeasts) and Amphotericin B (*Absidia* sp) diluted in water were also used as reference antibiotics. The final concentration of DMSO in the well was less than 1% [preliminary analyses with 1% (v/v) DMSO do not affect the growth of the test organisms].

Antileishmanial Assays. The antileishmanial activity of the molecules was evaluated against axenic amastigote of *Leishmania amazonensis* clone 1 (MHOM/BR/76/LTB-012) by a colorimetric method based on reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The culture of axenic amastigote of *Leishmania amazonensis* was maintained at 32±1°C by subpassages using MAA medium (medium for axenically grown amastigote), supplemented with 20% heat-inactivated fetal calf serum [21]. To estimate the 50% inhibitory concentrations (IC_{50}), parasites from the late log phase of growth (axenic amastigote) were seeded in 96-well flat-bottom microplates in a volume of 100 µL [22]. All tests were performed in triplicate. After 72 h of incubation at 32°C, 10 µL of MTT (10 µg/mL) was added to each well and the plates were incubated for 3½ h more. Enzyme reaction was stopped by addition of 100 µL of 50% isopropanol-10% SDS. The plates were shaked at room temperature for an additional 30 min. The optical density at 600 nm was read using a 96-well scanner. IC_{50} were determined graphically. Amphotericin B was used as inhibition control. Each experiment was performed three times.

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