

Phytoconstituents from *Sidastrum micranthum* (A. St.-Hil.) Fryxell (Malvaceae) and antimicrobial activity of pheophytin a

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Sidastrum micranthum (A. St.-Hil.) Fryxell, a member of the Malvaceae family, is called *malva preta* in Brazil. As this species is commonly used to treat bronchitis, cough, and asthma, better knowledge of its chemical compounds is important. The phytochemical study of its hexane extract, using chromatographic techniques, led to isolation of six compounds: the triterpene isoarborinol, a mixture of sitosterol and stigmasterol, sitosterol-3-O- β -D-glucopyranoside, pheophytin a, and 13²-hydroxy-(13²-S)-pheophytin a. Structural identification of these compounds was carried out using spectroscopic methods such as IR and 1D and 2D NMR (HOMOCOSY, HMQC, HMBC, and NOESY). Compounds isolated from *S. micranthum* were screened for their *in vitro* antifungal and antibacterial activity against twenty fungal and bacterial standard strains. Pheophytin a exhibited antimicrobial action against all microorganisms tested.

Uniterms: Malvaceae/phytochemistry. *Sidastrum micranthum*/phytochemistry. *Sidastrum micranthum*/antimicrobial activity/*in vitro* study. Pheophytins. Medicinal plants.

Sidastrum micranthum (A. St.-Hil.) Fryxell, pertencente à família Malvaceae, é conhecida no Brasil como “malva preta”. A espécie é popularmente usada contra bronquite, tosse e asma, mostrando a relevância de conhecer melhor sua composição química. O estudo fitoquímico do extrato hexânico da espécie, utilizando técnicas cromatográficas, conduziu ao isolamento de seis compostos: o triterpeno isoarborinol, mistura de sitosterol e estigmasterol, sitosterol-3-O- β -D-glicopiranosídeo, feofitina a e de 13²-hidróxi-(13²-S)-feofitina a. A identificação estrutural destes compostos foi realizada com base em métodos espectroscópicos, tais como IV, RMN 1D e 2D (HOMOCOSY, HMQC, HMBC e NOESY). As substâncias isoladas de *Sidastrum micranthum* foram avaliadas quanto às suas atividades antimicrobianas *in vitro*, contra vinte cepas fúngicas e bacterianas. A feofitina a mostrou ação antimicrobiana contra todos os microrganismos testados.

Unitermos: Malvaceae/fitoquímica. *Sidastrum micranthum*/fitoquímica. *Sidastrum micranthum*/atividade antimicrobiana/estudo *in vitro*. Feofitinas. Plantas medicinais.

INTRODUCTION

Malvaceae is a widespread family comprising about 243 genera and 4225 species, distributed mainly in tropical areas (Costa *et al.*, 2007). Mainly because of their anti-oxidant and anti-inflammatory activities, the natural compounds isolated from Malvaceae species are used

worldwide to treat such diseases as asthma and gastritis, (Oliveira *et al.*, 2012; Teles *et al.*, 2014).

Sidastrum micranthum (A. St.-Hil.) Fryxell (Malvaceae), known as *malva preta*, is a small shrub commonly found in Cuba, Costa Rica, Venezuela, Guyana, and Brazil (Bovini, Carvalho-Okano, Vieira, 2001). The infusion prepared from its leaves has traditionally been used to treat bronchitis, cough, and asthma. The leaves are also used as cataplasms (poultices), with hot butter and olive oil, as a moisturizing agent (Agra *et al.*, 2007).

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Previous phytochemical studies of *Sidastrum* have reported the presence of steroids, triterpenes, and phenolic compounds (Cavalcante *et al.*, 2010; Teles *et al.*, 2015). We previously reported the isolation from *S. micranthum* of tiliroside, 4'-methoxy-5,7-dihydroxyflavone (acacetin), and 7,4'-di-*O*-methylisoscuteallarein, as well as other phenolic compounds (Gomes *et al.*, 2011).

The antimicrobial activity of Malvaceae species is well reported (Konaté *et al.*, 2012; Silva *et al.*, 2009). In recent decades, the emergence of microbial resistance to antibiotics has increased interest in exploring the potential of plant-derived antimicrobials (Silva *et al.*, 2010). To increase our knowledge of *S. micranthum* phytoconstituents, this species was submitted to a phytochemical investigation. In addition, the antimicrobial activity of the compounds isolated was evaluated.

MATERIAL AND METHODS

General procedures

Chromatographic columns were packed with silica gel 60 (ASTM, 230-400 mesh, Merck). Thin-layer chromatography (TLC) was performed on PF₂₅₄ plates, and the spots were visualized under ultraviolet light (244 and 366 nm) and by exposure to iodine vapor. Isolated compounds were identified by infrared (IR; Perkin-Elmer, FT-IR-1750, and Shimadzu, IR prestige 21) and extensive one- and two-dimensional nuclear magnetic resonance (NMR) analysis (¹H 200 MHz and ¹³C 50 MHz, Varian-Mercury; or ¹H 500 MHz and ¹³C 125 MHz, Bruker-AC) using deuterated solvents.

Plant material

The aerial parts of *S. micranthum* were collected in Monteiro City, Paraíba/Brazil, in June 2006. A voucher specimen (JPB 6865) was authenticated and deposited at the Professor Lauro Pires Xavier Herbarium, Federal University of Paraíba (CCEN/JPB/UFPB).

Extraction and isolation of compounds

Plant material was dehydrated in an oven at 40 °C for 72 hours and then ground with a mechanical mill, yielding 6 kg of powder, which was macerated with 95% ethanol at room temperature. This process was repeated to maximize the extraction. The ethanolic extract was concentrated using a rotary evaporator, yielding 200 g of crude ethanol extract. This material was solubilized in ethanol:water (8:2) and submitted to liquid-liquid extraction with

hexane, chloroform, ethyl acetate, and *n*-butanol, affording 46 g of hexane extract, 7 g of chloroform extract, 6 g of ethyl acetate extract, and 8 g of *n*-butanol extract.

Hexane extract (10 g) was chromatographed on silica gel (column A) eluted with hexane, ethyl acetate, and methanol. From this process, 139 fractions were obtained and combined by TLC. Compound **1** was the pure white powder (15 mg) from fractions 29-34. Recrystallization of fractions 39-64 with chloroform yielded 79 mg of colorless crystals, later identified as a mixture of compounds **2** and **3**. Fractions 65-90 (400 mg) were chromatographed on a silica gel column under medium-pressure liquid chromatography (MPLC-Model BÜCHI 688), using hexane, ethyl acetate, and methanol and yielding 90 fractions. The pure fractions 60-64 (40 mg) and 71-83 (30 mg), both amorphous dark green solids, were named compounds **4** and **5**, respectively. The combined fractions 95-129, from column A, yielded a white solid precipitated (48 mg) that was separated and identified as the compound **6** (Figure 1).

To identify the compounds isolated, the fractions were analyzed by IR, ¹H and ¹³C NMR, and two-dimensional techniques (HMQC, HMBC, COSY, and NOESY).

β -Isoarborinol (**1**). ¹H NMR (δ , CDCl₃, 500 MHz): 5.20 (d, *J* = 6.5 Hz, H-11), 3.19 (dd, *J* = 4.5 and 11.25 Hz, H-3), 1.01 (s, H-25), 0.96 (s, H-23), 0.88 (d, H-30), 0.80 (s, H-26), 0.79 (s, H-24), 0.79 (d, H-29), 0.75 (s, H-27), 0.74 (s, H-28). ¹³C NMR (δ , CDCl₃, 125 MHz): 148.89 (C-9), 114.35 (C-11), 78.97 (C-3), 59.68 (C-21), 52.38 (C-5), 52.09 (C-18), 42.87 (C-17), 41.01 (C-8), 39.66 (C-10), 39.08 (C-4), 38.21 (C-14), 36.81 (C-13), 36.10 (C-1), 36.04 (C-12), 35.96 (C-16), 30.76 (C-22), 29.67 (C-15), 28.23 (C-23), 28.21 (C-20), 27.85 (C-2), 26.70 (C-7), 22.97 (C-29), 22.14 (C-25 and C-30), 21.45 (C-6), 20.18 (C-19), 17.02 (C-26), 15.30 (C-24 and C-27), 13.99 (C-28). The ¹H- and ¹³C-NMR data are consistent with literature (Farruque *et al.*, 2003).

Pheophytin a (**4**). ¹H NMR (δ , CDCl₃, 500 MHz): 9.51 (s, H-10), 9.35 (s, H-5), 8.60 (s, H-20), 7.95 (dd, *J* = 17.85 and 11.48 Hz, H-3¹), 6.30 (s, H-13²), 6.27 (*trans*, d, *J* = 17.95 Hz, H-3²) and 6.18 (*cis*, d, *J* = 11.10 Hz, H-3²), 4.34 (m, H-18), 4.15 (m, H-17), 3.91 (s, H-13⁴), 3.69 (s, H-12¹), 3.63 (m, H-8¹), 3.39 (s, H-2¹), 3.19 (s, H-7¹), 1.84 (d, H-18¹), 1.66 (m, H-8²). ¹³C NMR (δ , CDCl₃, 125 MHz): 189.81 (C-13¹), 173.18 (C-17³), 172.63 (C-19), 169.77 (C-13³), 161.19 (C-16), 155.55 (C-6), 150.92 (C-9), 149.59 (C-14), 145.25 (C-8), 142.34 (C-1), 138.14 (C-11), 136.82 (C-3), 136.51 (C-4), 136.14 (C-7), 131.1 (C-2), 129.19 (C-

3¹), 129.14 (C-13), 129.03 (C-12), 123.11 (C-3²), 105.10 (C-15), 104.59 (C-10), 97.66 (C-5), 93.72 (C-20), 64.9 (C-13²), 53.07 (C-13⁴), 51.42 (C-17), 50.36 (C-18), 31.42 (C-17²), 29.89 (C-17¹), 23.28 (C-18¹), 19.60 (C-8¹), 17.52 (C-8²), 12.32 (C-12¹), 12.26 (C-2¹), 11.35 (C-7¹). The ¹H- and ¹³C-NMR data are consistent with the literature (Chaves *et al.*, 2013).

13²-Hydroxy-(13²-*S*)-pheophytin a (5). ¹H NMR (δ , CDCl₃, 500 MHz): 9.46 (s, H-10), 9.58 (s, H-5), 8.60 (s, H-20), 8.01 (dd, J = 18.0 Hz, 11.5 Hz, H-3¹), 6.28 (*trans*) (d, J = 18.0 Hz, H-3²) and 6.17 (*cis*) (d, J = 11.5 Hz, H-3²), 4.55 (m, H-18), 4.15 (m, H-17), 3.88 (s, H-13⁴), 3.60 (s, H-12¹), 3.71 (s, H-8¹), 3.46 (s, H-2¹), 3.22 (s, H-7¹), 1.65 (d, H-18¹), 1.68 (t, H-8²). ¹³C NMR (δ , CDCl₃, 125 MHz): 192.01 (C-13¹), 172.78 (C-17³), 172.43 (C-19), 173.56 (C-13³), 162.51 (C-16), 155.35 (C-6), 151.06 (C-9), 149.88 (C-14), 145.21 (C-8), 142.04 (C-1), 137.85 (C-11), 137.0 (C-3), 136.32 (C-4), 136.25 (C-7), 131.74 (C-2), 129.1 (C-3¹), 129.70 (C-13), 129.40 (C-12), 122.82 (C-3²), 107.71 (C-15), 104.24 (C-10), 97.92 (C-5), 93.62 (C-20), 89.0 (C-13²), 51.9 (C-13⁴), 51.76 (C-17), 50.35 (C-18), 31.65 (C-17²), 29.40 (C-17¹), 23.65 (C-18¹), 19.70 (C-8¹), 17.40 (C-8²), 12.26 (C-12¹), 12.07 (C-2¹), 11.25 (C-7¹). The ¹H and ¹³C NMR data are consistent with the literature (Teles *et al.*, 2014).

Antimicrobial activity assay

Test-microorganisms

Among the strains (bacteria and yeast) selected for evaluation of the antimicrobial activity of the isolated compounds, six were obtained from the Mycology Laboratory of the Pharmaceutical Sciences Department collection, Universidade Federal da Paraiba (LM-UFPB): *Salmonella flexineri* (LM 412), *Candida albicans* (LM 142V), *Candida tropicalis* (LM 028), *Candida krusei* (LM 12), *Candida guilliermondii* (LM 2101), and *Candida guilliermondii* (LM 011). Twelve standard strains are from the American Type Culture Collection (ATCC): *Staphylococcus aureus* (ATCC 6538), *S. aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 25853), *P. aeruginosa* (ATCC 9027), *Escherichia coli* (classic C), *E. coli* (ATCC 18739), *E. coli* (ATCC 8733), *C. albicans* (ATCC 90028), *C. albicans* (ATCC 76615), *Candida tropicalis* (ATCC 13803), and *Candida krusei* (ATCC 6258). One strain was obtained from the Institute of Biomedical Sciences, Universidade de São Paulo (ICB-USP/SP): *C. albicans* (ICB 12).

Determination of minimum inhibitory concentration (MIC)

Chloramphenicol and ketoconazole (Sigma-Aldrich) were used as reference antibacterial and antimycotic controls. Antifungal and antibacterial activities were determined using microbroth dilution assays in 96-well microplates, in duplicate.

Stock bacterial strains were maintained in Muller Hinton agar (MHA), and the yeast, on Sabouraud dextrose agar (SDA), under refrigeration (8°C). Microorganism inocula were prepared and standardized in sterilized saline (0.85%) containing Tween 80 (1%). Turbidity was adjusted to a 0.5 McFarland tube to obtain an inoculum of 10⁶ colony-forming units (CFU)/mL. Minimum inhibitory concentrations (MICs) were determined with the microdilution method using 96-well plates (INLAB) (Hadacek, Greger, 2000).

To each well was added 100 μ L of double-concentrated liquid medium CSD or HCM. Ten microliters of each compound was then added to the wells of the first row of the plate, and by serial dilution, concentrations from 300 to 9 μ g/mL were obtained. Later, 10 μ L of the microorganism inoculum was added to the wells. The plate was incubated at 35 °C for 24 h. Afterward, 20 μ L of resazurin sodium 0.01% (w/v) (Sigma-Aldrich) was added. Resazurin is a colorimetric indicator of oxidation-reduction for bacteria. As an indicator for yeast, 20 μ L of 1% triphenyltetrazolium chloride (TTC) (Sigma-Aldrich) was used. The assay was incubated at 35 °C. Results were read by viewing the color change from blue to pink for bacteria, and from colorless to pink for yeast, indicating growth of the microorganism.

The MIC of the active compound was defined as the lowest concentration able to inhibit growth (Deswal, Chand, 1997). Compounds were considered either active or nonactive according to the following criteria: Compounds with MICs between 50 and 500 μ g/mL were classified as having strong/great antimicrobial activity; those with MICs from 500 to 1500 μ g/mL had moderate activity; and those with MICs higher than 1500 μ g/mL were considered to have weak antimicrobial activity (Houghton *et al.*, 2007; Sartoratto *et al.*, 2004).

RESULTS AND DISCUSSION

Structural elucidation of the isolated compounds

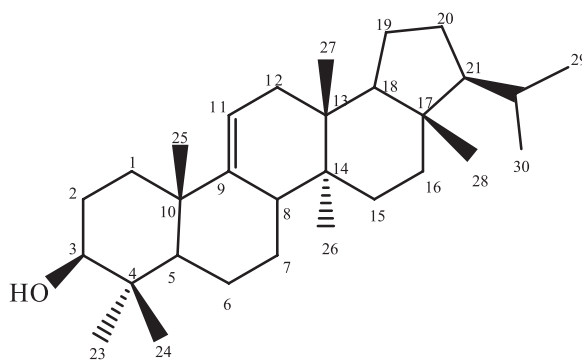
The structural assignments of compounds **2**, **3**, and **6** isolated from *S. micranthum* (Figure 1) were made based on spectral analysis and are in good agreement with those reported in the literature. Thus, their structures were

identified as sitosterol (**2**), stigmasterol (**3**), and sitosterol 3-*O*- β -D-glucopyranoside (**6**) (Kojima et al., 1990; Kongduang et al., 2008; Rashed *et al.*, 2014). Steroids are widespread in plants and play an important role as components of vegetable cell walls and membranes. They have been previously reported in many Malvaceae species, such as *Sidastrum paniculatum*, *Wissadula periplocifolia*, *Sida rhombifolia*, *Sida galheirensis*, *Bakeridesia pickelli*, and *Herissantia crispa* (Teles *et al.*, 2015; Teles *et al.*, 2014; Chaves *et al.*, 2013; Silva *et al.*, 2006; Costa *et al.*, 2007; Costa *et al.*, 2009).

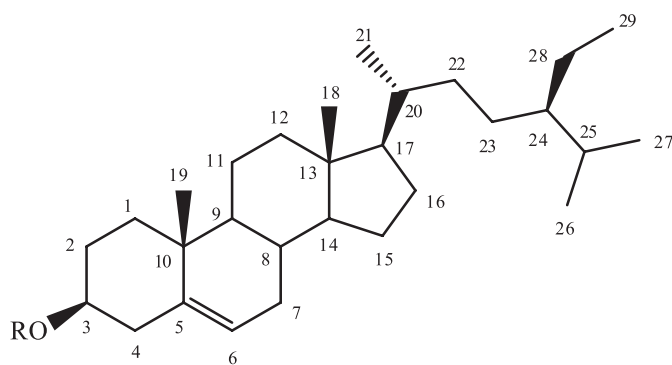
Compound 1, on ^1H NMR, exhibited a triterpene profile, characterized by six singlet methyls, two doublet

methyls, one oxymethinic proton (δ_{H} 3.19), and one olefinic proton (δ_{H} 5.20). The presence of 30 signals on ^{13}C NMR and the correlations observed on HMQC, HMBC, COSY, and NOESY spectra led to identification of compound 1 as β -isoarborinol, a hopane triterpene previously isolated from *Melicope indica* Wt. (Rutaceae) (Farruque *et al.*, 2003). Triterpenes have been reported in Malvaceae species, and hopane triterpenes have recently been reported in *Wissadula periplocifolia* and *Sidastrum paniculatum* (Teles *et al.*, 2014; Teles *et al.*, 2015).

Compounds **4** and **5** appeared similar (dark green amorphous solids) and had similar IR bands. On the basis of ^1H and ^{13}C NMR spectra, we confirmed that compounds



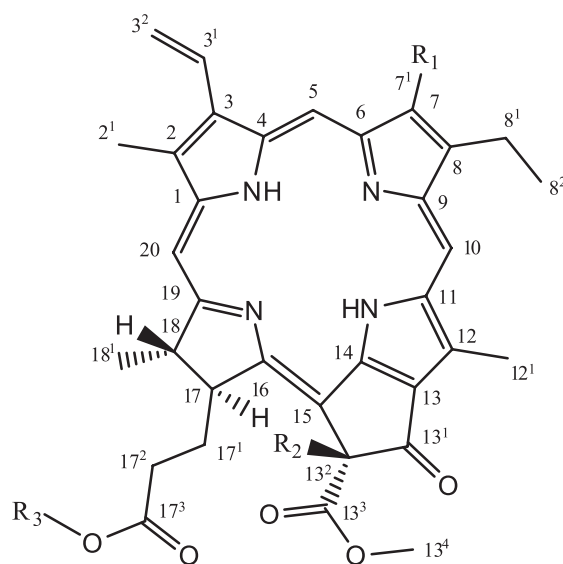
1: β -isoarborinol



2: R= H

3: R= H; $\Delta^{22,23}$

6: R= β -D-Glucopyranosyl



4: R₁= CH₃, R₂=H, R₃= phytol

5: R₁= CH₃, R₂=OH, R₃= phytol

FIGURE 1 - Chemical constituents isolated from *Sidastrum micranthum*.

4 and 5 are structurally related, both being formed by porphyrin rings. Extensive analyses of spectral data and comparisons with the literature led to identification of compounds 4 and 5 as pheophytin a and 13²-hydroxy-(13²-S)-pheophytin a, respectively, previously isolated from the Malvaceae species *W. periplocifolia*, *S. rhombifolia*, and *S. galheirensis* (Teles *et al.*, 2014; Chaves *et al.*, 2013; Silva *et al.*, 2006).

Antibacterial and antifungal activities

The isolated compounds sitosterol/stigmasterol (2/3), pheophytin a (4), acacetin (7), and 7,4'-di-*O*-methylisoescutelarein (8) were assessed against bacteria and yeast strains. Isolation of the flavones 7 and 8 has previously been reported (Gomes *et al.*, 2011).

The results obtained indicate that the mixture of phytosterols 2/3 and the flavones acacetin (7) and 7,4'-di-

O-methylisoescutelarein (8) did not have an inhibitory effect on the test strains. In contrast, pheophytin a (4) exhibited strong antimicrobial activity against all test strains (Table I).

Two yeast (*C. albicans* ATCC – 90028 and *C. albicans* ATCC – 76615) were sensitive to pheophytin a at 38 µg/mL and were the most sensitive test strains. At 75 µg/mL, pheophytin a was able to inhibit growth of 85% of all strains, indicating strong antimicrobial activity for this compound, according to the criteria reported by Sartoratto *et al.* (2004) and Houghton *et al.* (2007).

Pheophytins are formed by the degradation of chlorophyll by Mg-dechelatase and chlorophyllase enzymes and have been previously reported to have anti-leishmanial and cytotoxic activity (Hörtensteiner *et al.*, 1998; Cheng *et al.*, 2001; Sakata *et al.*, 1990). The results obtained are in agreement with the antimicrobial effect of some chlorophyll derivatives, including

TABLE I - MIC values (µg/mL) of compounds isolated from *Sidastrum micranthum*

Microorganisms	2/3	4	7	8	Control		
					Tween 80	Microorganisms	Antimicrobial*
<i>S. aureus</i> ATCC - 6538	+	75	+	+	+	+	-
<i>S. aureus</i> ATCC - 25923	+	75	+	+	+	+	+
<i>S. epidermidis</i> ATCC - 12228	+	75	+	+	+	+	-
<i>B. subtilis</i> ATCC - 6633	+	75	+	+	+	+	-
<i>P. aeruginosa</i> ATCC – 25853	+	150	+	+	+	+	+
<i>P. aeruginosa</i> ATCC – 9027	+	150	+	+	+	+	+
<i>E. coli</i> (classical C)	+	75	+	+	+	+	-
<i>E. coli</i> ATCC – 18739	+	75	+	+	+	+	+
<i>E. coli</i> ATCC – 8733	+	75	+	+	+	+	+
<i>S. flexineri</i> LM – 412	+	75	+	+	+	+	-
<i>C. albicans</i> ATCC – 90028	+	38	+	+	+	+	-
<i>C. albicans</i> ATCC – 76615	+	38	+	+	+	+	-
<i>C. albicans</i> LM – 142 V	+	75	+	+	+	+	-
<i>C. albicans</i> ICB – 12	+	150	+	+	+	+	-
<i>C. tropicalis</i> ATCC – 13803	+	75	+	+	+	+	+
<i>C. tropicalis</i> LM – 028	+	75	+	+	+	+	-
<i>C. krusei</i> ATCC – 6258	+	75	+	+	+	+	+
<i>C. krusei</i> LM – 12	+	75	+	+	+	+	-
<i>C. guilliermondii</i> LM – 2101	+	75	+	+	+	+	-
<i>C. guilliermondii</i> LM – 011	+	75	+	+	+	+	+

+ = Presence of microbial growth (compound not active), - = Absence of microbial growth

pheophorbide, as determined by Gerola *et al.* (2011). Thus, the antimicrobial activity shown by these compounds may be evidence of their additional role protecting plants from microorganisms in their environment.

CONCLUSION

This phytochemical study of *Sidastrum micranthum* hexane extract resulted in the isolation of six compounds: β -isoarborinol (**1**), a mixture of sitosterol (**2**) and stigmaterol (**3**), pheophytin a (**4**), 13²-hydroxy-(13²-S)-pheophytin a (**5**) and sitosterol-3-*O*- β -D-glucopyranoside (**6**). Compounds **1**, **4**, and **5** are reported here for the first time in the *Sidastrum* genus. In addition, pheophytin a exhibited significant antimicrobial activity against all bacteria and yeasts tested. According to the criteria reported by Sartoratto *et al.* (2004) and Houghton *et al.* (2007), pheophytin a is considered to possess strong antimicrobial activity against *C. albicans* (ATCC 90028) and *C. albicans* (ATCC 76615).

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