Antileishmanial Compounds from *Moringa oleifera* Lam.

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Z. Naturforsch. **69c**, 110 – 116 (2014) / DOI: 10.5560/ZNC.2013-0159
Received September 22 / November 8, 2013 / published online April 16, 2014

The antileishmanial activity of extracts and phytoconstituents of *Moringa oleifera* Lam. was investigated *in vitro* against promastigotes of *Leishmania donavani*. The 70 % ethanolic extract of roots and the methanolic extract of leaves showed moderate inhibitory activity with IC_{50} values of 83.0 μg/ml and 47.5 μg/ml, respectively. Antileishmanial activity of the methanolic extract of leaves increased upon fractionation, as its ethyl acetate fraction was found to be more active with an IC_{50} value of 27.5 μg/ml. The most active antileishmanial compound niazinin, a thiocarbamate glycoside isolated from this fraction, showed an IC_{50} value of 5.25 μM. Results presented in this study indicate that extracts from *M. oleifera* may be developed as an adjuvant therapy for the treatment of leishmaniasis.

Key words: Leishmaniasis, *Moringa oleifera*, Promastigotes, Thiocarbamate Glycosides

Introduction

Leishmaniasis is one of the most neglected tropical protozoal diseases. It has three main manifestations: cutaneous (CL), muco-cutaneous (MCL), and visceral (VL) leishmaniasis, with different spectra of symptoms ranging from skin and muco-cutaneous ulceration to systemic infection. According to World Health Organization (WHO, 2010) records, around 12 million people are infected with leishmaniasis and 350 million people are considered at risk of acquiring an infection. The disease is spreading at an alarming rate with estimated 2 million new cases occurring annually. In India, most of the leishmaniasis cases are of the VL type and are mainly being reported in the states of Bihar, Orissa, Jharkhand, West Bengal, Uttar Pradesh, and Assam. Antimonials remained the mainstay of treatment of leishmaniasis earlier, but are no longer useful for Indian VL due to the emergence of resistance. As a result, amphotericin B and its liposomal formulations are now preferred (Chappuis *et al*., 2007). However, treatment with the latter is expensive and remains unaffordable for the poor population. The use of other drugs such as pentamidine and miltefosine is also associated with side effects (Chappuis *et al*., 2007). Therefore, in order to find new drugs for the treatment of leishmaniasis, researchers have directed their attention to the use of natural products (Chan-Bacab and Pena-Rodriguez, 2001; Rocha *et al*., 2005); particularly, those from plants may be future treatment options for leishmaniasis.

As a part of our continuing efforts to find antileishmanial leads from medicinal plants, we have earlier reported antileishmanial activity of various extracts of *Piper cubeba*, *Piper retrofractum* (Bodiwala *et al*., 2007), *Alpinia galanga* (Kaur *et al*., 2010), and *Eucalyptus loxophleba* (Sidana *et al*., 2012). In this article, we report the evaluation of extracts of *Moringa oleifera* Lam. for their antileishmanial activity and the isolation of chemical compounds from active extracts. The active compounds of the most active extract were quantified by high performance thin-layer chromatography (HPTLC).

M. oleifera Lam. (syn. *M. pterygosperma* Gaertn.) is native to sub-Himalayan tracts of India, Pakistan, Bangladesh, and Afghanistan. It is now cultivated and

naturalized in tropical parts of Asia and Africa. It is commonly known as drumstick tree or horse radish tree, and is popular by the name of *Sahjana* in India. It is extensively used for its nutritional as well as medicinal benefits, and is one of the most studied and utilized members of the family Moringaceae. Various parts of this tree such as leaves, fruits, and pods are used as highly nutritive vegetables, particularly by poor populations of many parts of Asia and Africa where malnutrition is a major concern. Leaves are a good source of vitamins A and C, carotenes, calcium, and potassium. Seeds are useful as natural clarifiers for obtaining drinking water. Almost all parts of the tree are used for a variety of beneficial medicinal properties such as anti-inflammatory, antiurolithic, rubefacient, antirheumatic, antitumour, and antihypertensive. All such uses of *M. oleifera* have been reviewed (Anwar *et al*., 2007; Fahey, 2005). However, so far this highly useful tree has not been evaluated for its antileishmanial potential. Thiocarbamate, carbamate, and nitrile glycosides, which are important chemical constituents reported from *Moringa*, also have not been reported for their antileishmanial activity. To our knowledge, this is the first evaluation to date of the antileishmanial activity of extracts, fractions, and isolated phytoconstituents from *M. oleifera*.

Materials and Methods

General

All solvents used for extraction were of analytical grade. HPLC-grade methanol (JT Baker, Center Valley, PA, USA) was used for sample preparation and in high-performance liquid chromatography (HPLC) mobile phases. Precoated silica gel GF_{254} plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC) analysis. Silica gel (60 – 120 mesh; CDH Laboratory Reagents, New Delhi, India) was used for column chromatography (CC). TLC-grade silica gel G (SDFCL, Biosar, India) was used for vacuum liquid chromatography (VLC) of extracts. ^{1}H NMR and ^{13}C NMR spectra were recorded on 400- and 100-MHz spectrometers, respectively (Bruker, Fällanden, Switzerland). Deuterated chloroform ($CDCl_3$) and methanol (CD_3OD) (Sigma-Aldrich, St. Louis, MO, USA) were used for recording NMR spectra, and tetramethylsilane (TMS) was used as an internal standard. Mass spectra were acquired using a Bruker Apex 3 instrument. A Shimadzu HPLC system (Kyoto, Japan) was used for analytical and semi-preparative HPLC of the samples. Reverse-phase analytical columns C_8 and C_{18} (250 mm × 4.6 mm) Luna (Phenomenex, Torrance, CA, USA) were used for analytical separation of compounds. A semi-preparative column C_{18} (250 mm × 10 mm) (SPHER, Princeton, NJ, USA) was used for purification of compounds. RPMI-1640 HEPES modified medium, antibiotics, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich. Foetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). DMSO had no effect on the morphology and proliferation of the promastigotes.

Plant material and preparation of extracts

Leaves and roots of *M. oleifera* were collected from a tree grown in the Medicinal Plant Garden at the National Institute of Pharmaceutical Education and Research (NIPER), S. A. S. Nagar, Punjab, India (voucher specimen NIP-H-168). The plant material was authenticated by Dr. A. S. Sandhu, NIPER, S. A. S. Nagar, Punjab, India. Roots were dried in the shade and powdered, whereas fresh leaves were cut and used for the purpose of this study.

Extraction and isolation of compounds

Preparation of an aqueous ethanolic extract of roots was done by maceration with 70% ethanol for 7 d at room temperature. The extract was filtered and evaporated to dryness (25 g) under vacuum using a rotary evaporator (R-210; Büchi, Buchs, Switzerland) at 37 °C. The dried extract (25 g) was suspended in $MeOH/H_2O$ (9:1, v/v, 1.5 l) and partitioned sequentially with CH_2Cl_2, EtOAc, and *n*-BuOH (each 500 ml) to obtain 4 g MRC, 0.2 g MRE, and 0.5 g MRB fractions, respectively. Fraction MRC was subjected to CC on silica gel (60 – 120 mesh) using $CHCl_3$/MeOH (1:0 – 1:1) gradient elution, and five major subfractions, MRC1 – MRC5, were obtained. Subfraction MRC1 (0.2 g) was separated by CC on silica gel (60 – 120 mesh) using *n*-hexane/EtOAc (1:0 – 4:1) to obtain compound **1** (15 mg). Subfraction MRC2 (0.15 g) was chromatographed on a silica gel (60 – 120 mesh) column using *n*-hexane/EtOAc (1:0 – 7:3) to obtain compound **2** (7 mg). Subfraction MRC4 (0.4 g) was separated using semi-preparative HPLC under the following conditions: mobile phases, water (A) and MeOH (B); flow rate, 3 ml/min; solvent gradient, 45 – 70% B within 0 – 20 min to obtain compounds **3** (t_R 7.8 min, 10 mg) and **4** (t_R

15.2 min, 8 mg). Subfraction MRC5 (0.9 g) was separated on a silica gel (60 – 120 mesh) column using $CHCl_3$/MeOH (1:0 – 1:1) gradient elution to obtain compound **5** (30 mg).

Roots (1.5 kg) were macerated with acetone (5 l) for 7 d at room temperature. The MRA extract was filtered and evaporated to dryness (23 g) under vacuum at 37 °C. The dried extract (23 g) was subjected to VLC on silica gel G using *n*-hexane/EtOAc (1:0 – 0:1) followed by EtOAc/MeOH (1:0 – 0:1) gradient elution to obtain seven subfractions, MRA1 – MRA7. Subfraction MRA2 (2 g) was further purified by CC using a $CHCl_3$/MeOH gradient to obtain compound **6** (20 mg). Subfraction MRA3 (4 g) was subjected to VLC using a $CHCl_3$/MeOH gradient, and compound **7** (150 mg) was obtained from it. Subfraction MRA7 (2 g) was further fractionated in seven subfractions, MRA7.1 – MRA7.7, by gel permeation chromatography on Sephadex LH-20 (Sigma-Aldrich) using MeOH as eluent. Sub-subfraction MRA7.3 was separated using semi-preparative HPLC under the following conditions: mobile phases, water (A) and MeOH (B); flow rate, 3 ml/min; solvent gradient, 25 – 60 % B within 0 – 25 min to obtain compounds **8** (t_R 10.1 min, 10 mg) and **9** (t_R 13.9 min, 12 mg). Sub-subfraction MRA7.4 was separated using semi-preparative HPLC under the following conditions: mobile phases, water (A) and MeOH (B); flow rate, 3 ml/min; solvent gradient, 10 – 55 % B within 0 – 20 min to obtain compound **10** (t_R 15.8 min, 15 mg). Sub-subfraction MRA7.5 was found to be the pure compound **11** (25 mg) on analysis by TLC and analytical HPLC.

Fresh, undried leaves (5 kg) were cut and macerated with methanol at room temperature for 7 d. The extract was dried under vacuum to 1.5 l of a thick syrup, which was diluted with an equal volume of water and sequentially partitioned with *n*-hexane, $CHCl_3$, and EtOAc to obtain 35 g MLH, 22 g MLC, and 20 g MLE fractions, respectively. Fraction MLE was subjected to VLC on silica gel G using *n*-hexane/EtOAc (1:0 – 0:1) followed by EtOAc/MeOH (1:0 – 0:1) gradient elution. Six subfractions, MLE1 – MLE6, were obtained. Subfraction MLE2 (400 mg) was separated using semi-preparative HPLC under the following conditions: mobile phases, water (A) and MeOH (B); flow rate, 3 ml/min; solvent gradient, 32 – 42 % B within 0 – 25 min, 42 – 54 % B within 26 – 29 min, 54 – 100 % B within 30 – 40 min to obtain compounds **3** (t_R 13.2 min, 10 mg), **12** (t_R 14.9 min, 7 mg), and **13** (t_R 31.6 min, 6 mg). Subfraction MLE3 (300 mg) was subjected to gel permeation chromatography on Sephadex LH-20 using MeOH as eluent to obtain compound **14** (30 mg).

Parasites and cell lines

Leishmania donovani wild-type (WT, MHOM/80/IN/Dd8) promastigotes were cultured at 24 °C in RPMI-1640, phenol red-free medium supplemented with 0.2 % sodium bicarbonate, 100 μg/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamycin, and 10 % heat-inactivated FBS, and the pH value of the medium was maintained at 7.2. The cells were washed in phosphate-buffered saline (PBS) (pH 7.4) at 6000 × *g* for 10 min at 24 °C. The number of cells was counted in a Neubauer haemocytometer (Sigma-Aldrich).

In vitro promastigote assay

In vitro leishmanicidal activity of the compounds was investigated against *L. donovani* promastigotes using the MTT (thiazolyl blue tetrazolium bromide) assay (Mosmann, 1983). Cells (2×10^5/200 μl per well) were seeded onto a 96-well plate and incubated at 24 °C for 48 h. The compounds were dissolved in 20 % DMSO, and the final concentration was maintained at 1 % (v/v). After 48 h, different dilutions (10 – 100 μg/ml) of each compound were prepared and added to the cells in triplicate which were further incubated at 24 °C for 48 h. MTT was added to a final concentration of 400 μg/ml, and the plates were further incubated at 37 °C for 4 h. The cells were centrifuged at 3000 × *g* for 10 min and the supernatant was removed. The resultant purple formazan formed was dissolved in 100 μl DMSO, and the absorbance was read at 540 nm on a Tecan microplate reader (Model Infinite M200, Männedorf; Switzerland). The IC_{50} values of the treated leishmanial cells were calculated relative to the untreated control cells, and the results were expressed as the concentration of the compounds inhibiting 50 % of the parasite growth. Miltefosine was used as the standard antileishmanial drug. Three independent experiments were performed in triplicate for each compound. IC_{50} values are given as mean ± SD of three independent determinations.

Cytotoxicity

Human leukaemia monocytes (THP-1) were grown at 37 °C with 5 % CO_2 in RPMI-1640, phenol red-free medium (supplementation of antibiotics and FBS was

same as for promastigote medium). Cells were harvested in the exponential growth phase by centrifugation at 3000 × *g* for 5 min and counted in a haemocytometer. The cells were then exposed to different dilutions of the compounds and kept for incubation at 37 °C for 48 h. The MTT assay was performed as described earlier. The cytotoxicity of the compounds (CC_{50}) was expressed as 50% reduction in the viability of the cells after treatment with compounds in comparison to the untreated cells. Experiments were performed in triplicate for each compound.

Results and Discussion

There are very few reports on phytochemical investigations of roots of *M. oleifera* (Sashidhara *et al.*, 2009) as compared to leaves (Faizi *et al.*, 1994, 1995; Manguro and Lemmen, 2007). For evaluation of the antileishmanial activity, we prepared acetone and 70% ethanolic extracts, respectively, of roots. The acetone extract contained non-polar to medium polar compounds, while the ethanolic extract contained polar compounds; therefore, the entire spectrum of chemical classes present in roots was covered by these two extracts. For preparation of extracts from leaves, a sequential extraction method was used. First, leaves were defatted by *n*-hexane, followed by extraction with chloroform and ethyl acetate. In several earlier reports, isolation of bioactive compounds was achieved from the ethyl acetate fraction of leaves (Faizi *et al.*, 1994, 1995).

Five compounds, **1**–**5**, were isolated from the 70% ethanolic extract of roots. These were identified as benzyl isothiocyanate (**1**), *O*-ethyl benzyl thiocarbamate (**2**) (Murakami *et al.*, 1998), 4-(α-L-rhamnopyranosyloxy) benzylnitrile (niazirin) (**3**) (Faizi *et al.*, 1994), *N*-ethoxythiocarbonyl-4-(α-rhamnopyranosyloxy) benzylamine (niazimicin) (**4**) (Leuck and Kunz, 1998), and benzylamine (**5**). Six compounds, **6**–**11**, were isolated from the acetone extract of roots which were identified as *p*-hydroxybenzoic acid (**6**), gallic acid (**7**), benzyl (6-*O*-

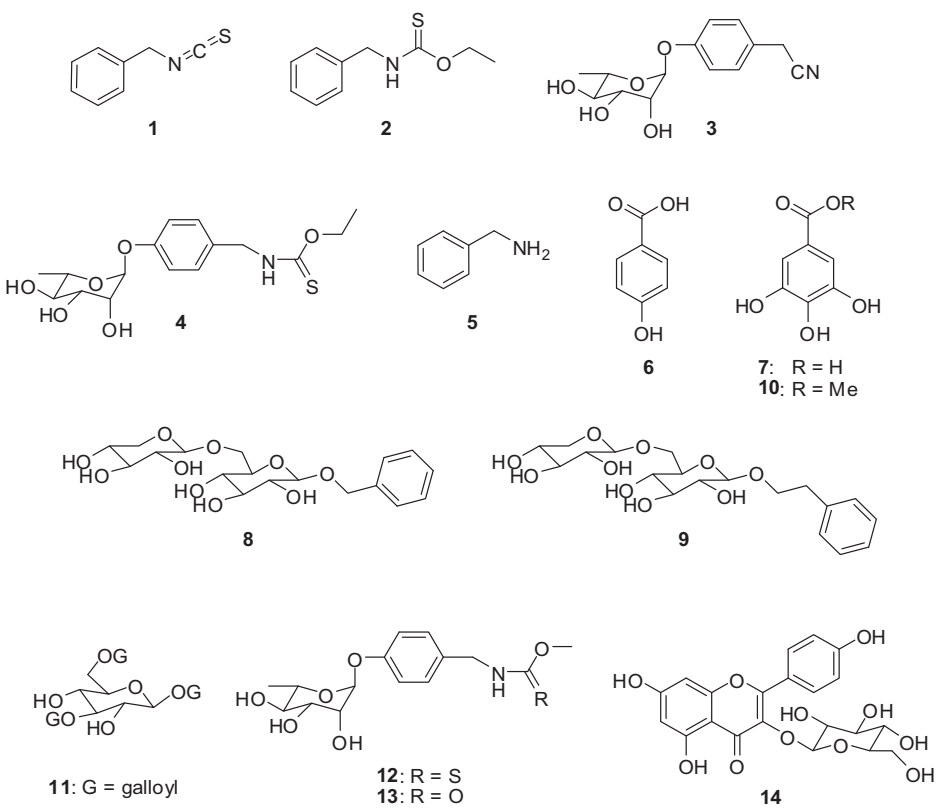

Fig. 1. Chemical structures of compounds isolated from *M. oleifera*.

β-D-xylopyranosyl)-β-D-glucopyranoside (**8**) (Otsuka *et al*., 1990), phenethyl (6-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside (**9**) (Otsuka *et al.*, 1990), methyl gallate (**10**), and 1,3,6-tri-*O*-galloyl-β-D-glucose (**11**) (Nonaka *et al*., 1981). Four compounds, **3** and **12**–**14**, were isolated form leaves and identified as niazirin (**3**), *N*-methoxythiocarbonyl-4-(α-L-rhamnopyranosyloxy) benzylamine (niazinin) (**12**) (Leuck and Kunz, 1998), *N*-methoxycarbonyl-4-(α-rhamnopyranosyloxy) benzylamine (**13**) (Sahakitpichan *et al*., 2011), and kaempferol-3-*O*-β-D-glucopyranoside (**14**). The structures of the compounds (Fig. 1) were confirmed by comparison of the ^{1}H, ^{13}C NMR, and MS data with those reported in the literature. Compounds **2**, **4**, and **12** were thiocarbamates, whereas **13** was a carbamate-type compound. These compounds were structurally similar by possessing a benzylamine nucleus. Phenolic compounds isolated from roots included the low-molecular weight compounds **6**, **7**, and **10**, as well as the high-molecular weight trigalloylated glucose **11**.

All extracts, fractions, and compounds were tested for their activity against *L. donovani* promastigotes (Table I). The 70% ethanolic extract of roots exhibited moderate inhibitory activity against promastigotes with an IC_{50} value of 83.0 μg/ml. The thiocarbamate glycoside niazimicin (**4**) isolated from this extract also showed moderate antileishmanial activity with an IC_{50} value of 68.5 μM. Thiocarbamate and carbamate glycosides with similar structures have been reported mainly from the leaves of *Moringa*, therefore extracts of leaves were also prepared and tested for their activity. The methanolic extract of leaves had an IC_{50} value of 47.5 μg/ml which increased on fractionation, as the ethyl acetate fraction was found to be more active with an IC_{50} value of 27.5 μg/ml. Chromatographic separation of this fraction led to the thiocarbamate glycoside niazinin (**12**) and the carbamate glycoside *N*-methoxycarbonyl-4-(α-rhamnopyranosyloxy) benzylamine (**13**) with IC_{50} values of 5.25 and 37.25 μM, respectively. It may be suggested that the antileishmanial activity of the extracts is due to the presence of thiocarbamate and carbamate glycosides. It was observed that substitution of the ethyl thiocarbamate aglycone of **4** with an aglycone one carbon atom shorter resulted in the methyl thiocarbamate aglycone in **12** and to a significant increase in activity. Substitution of the thiocarbamate unit in **12** with the carbamate unit in **13** decreased the antileishmanial activity.

Though the acetone extract of roots did not show any significant inhibition of promastigotes, two

Table I. *In vitro* antileishmanial and cytotoxic activities.

Extract/Fraction/Compound	IC_{50}^{a}	CC_{50}
Roots (ethanolic extract)	83.0 ± 5.0	> 100
Roots (acetone extract)	> 100	> 100
Leaves (methanolic extract)	47.5 ± 2.5	> 100
Leaves (*n*-hexane fraction)	–[b]	–
Leaves (chloroform fraction)	–[b]	–
Leaves (ethyl acetate fraction)	27.5 ± 2.5	78
Benzyl isothiocyanate (**1**)	10 ± 2.83	30
O-Ethyl benzyl thiocarbamate (**2**)	17 ± 4.24	53.9
4-(α-L-Rhamnopyranosyloxy) benzylnitrile (niazirin) (**3**)	31.5 ± 0.71	28
N-Ethoxythiocarbonyl-4-(α-rhamnopyranosyloxy) benzylamine (niazimicin) (**4**)	68.5 ± 1.5	71.5
Benzylamine (**5**)	21.5 ± 14.85	> 100
p-Hydroxybenzoic acid (**6**)	16.5 ± 0.71	> 100
Gallic acid (**7**)	36 ± 2.83	> 100
Benzyl (6-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside (**8**)	10.0 ± 1.0	60.5
Phenethyl (6-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside (**9**)	24.25 ± 3.25	12
Methyl gallate (**10**)	> 100	> 100
1,3,6-Tri-*O*-galloyl-β-D-glucose (**11**)	8.5 ± 0.71	83.4
N-Methoxythiocarbonyl-4-(α-L-rhamnopyranosyloxy) benzylamine (niazinin) (**12**)	5.25 ± 0.25	31.6
N-Methoxycarbonyl-4-(α-rhamnopyranosyloxy) benzylamine (**13**)	37.25 ± 5.25	31.6
Kaempferol-3-*O*-β-D-glucopyranoside (**14**)	39.0 ± 1.0	76.2
Miltefosine[c]	12.5	32

[a]Extracts/fractions in μg/ml and compounds in μM.
[b]Could not be tested, not soluble.
[c]Positive standard.

compounds isolated from this extract, namely benzyl (6-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside (**8**) and phenethyl (6-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside (**9**), showed promising activity with IC_{50} values of 10.0 and 24.25 μM, respectively.

The cytotoxicity of the extracts, fractions, and compounds was also assessed (Table I). Results of this assay indicated lack of selective toxicity by most of the compounds; however, the most active compound, niazinin (**12**), was cytotoxic at substantially higher concentration (CC_{50} 31.6 μM) than its antileishmanial concentration (IC_{50} 5.25 μM). Similarly, benzyl (6-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside (**8**) and 1,3,6-tri-*O*-galloyl-β-D-glucose (**11**) showed higher CC_{50} values compared to their IC_{50} values.

The ethyl acetate fraction of leaves was found to be the most active; therefore, compounds **3** and **12**–**14** were quantified in this fraction by HPTLC using precoated silica gel GF_{254} HPTLC plates as stationary phase and toluene/EtOAc/formic acid (1.5:7.5:1, v/v) as mobile phase. The ethyl acetate extract of *M. oleifera* leaves was found to contain (2.81 ± 0.42) % (w/w) **3**, (0.98 ± 0.03) % **13**, (1.73 ± 0.16) % **12**, and (11.62 ± 0.22 %) **14** as determined by this method.

Conclusion

Our study is the first report on the antileishmanial activity of extracts and phytoconstituents of *M. oleifera*. Leishmaniasis is mainly a disease of poor African and Asian countries, where *Moringa* is widely used for its medicinal properties and as a food item. Wide use of *Moringa* as a vegetable is an evidence for its safety and non-toxicity. Therefore, *Moringa*-based herbal preparations may be used in the future as an adjuvant therapy for the treatment of leishmaniasis.

Acknowledgement

This work was supported by Department of Science and Technology, Government of India, New Delhi (Grant No. SR/SO/HS-03/2005).

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