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Antifungal Activity of Alkaloids from the Seeds of *Chimonanthus praecox*

by **Ji-Wen Zhang**[a]), **Jin-Ming Gao***[a])[b]), **Tian Xu**[a]), **Xing-Chang Zhang***[c]), **Ya-Tuan Ma**[a])[b]), **Suwatchai Jarussophon**[b])[d]), and **Yasuo Konishi**[b])

[a]) Research Centre for Natural Medicinal Chemistry, College of Science, Northwest A&F University, Yangling, Shaanxi 712100, P. R. China (phone: +86-29-87092335; fax: +86-29-87092335; e-mail: jinminggaocn@yahoo.com.cn)

[b]) Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, Que. H4P2R2, Canada

[c]) Institute of Soil & Water Conservation, Chinese Academy of Science, Yangling, Shaanxi 712100, P. R. China (e-mail: zhangxc@ms.iswc.ac.cn)

[d]) National Nanotechnology Center, National Science and Technology Development Agency, 30 Thailand Science Park, Paholyothin Rd., Klong Luang, Pathumthani 12120 Bangkok, Thailand

Two alkaloids, D-calycanthine (**1**) and L-folicanthine (**2**), were isolated from the active MeOH extract of the seeds of *Chimonanthus praecox* LINK. The structures of the two compounds were established by ^{1}H- and ^{13}C-NMR, and MS (FAB, ESI) analyses. In the *in vitro* tests, compounds **1** and **2** showed significant inhibitory activities against five plant pathogenic fungi *Exserohilum turcicum*, *Bipolaris maydis*, *Alternaria solani*, *Sclerotinia sderotiorum*, and *Fusarium oxysportium*, among which *B. maydis* was found to be the most susceptible to **1** with an EC_{50} value of 29.3 μg/ml, followed by *S. sderotiorum* to **2** with an EC_{50} value of 61.2 μg/ml. To our knowledge, this is the first report of isolation and LC/MS/MS identification as well as of antifungal properties of these alkaloids from the seeds of this plant.

Introduction. – Natural products have been a continuous source of new lead compounds, as well as chemical entities in the agrochemical and pharmaceutical industries [1–3]. Chemical crop protection plays a vital role in ensuring sufficient food supply to a growing world population. In the face of ever more stringent demands with regard to efficacy and environmental safety, the discovery of new agrochemicals has become a difficult and resource-intensive undertaking [4]. In addition, increasing incidence of resistance to commercial systemic fungicides has prompted synthetic chemists to search for natural products potentially useful as fungicidal lead compounds. On the other hand, many important crops are easily infected by certain phytopathogenic fungi that are difficult to control, leading to huge economic losses, and, as a result, development of bioactive compounds for control of those agricultural diseases is highly important.

The shrub *Chimonanthus praecox* LINK, a popular garden and ornamental plant, is a member of the family Calycanthaceae endemic to China [5]. The plant grown in the subtropical regions is mainly distributed in Southern China. Its roots and flowers are used as a single effective prescription in folk medicine for treatment of cold, sedative, antitussive, hypertension, as well as used in making perfume [5][6]. Previous

phytochemical studies revealed that plants of the genus *Chimonanthus* contain volatile oils [7][8], alkaloids, flavonoids, and coumarins, which were isolated from flowers, leaves, and roots [5][9]. The principal representative alkaloid of the family Calycanthaceae, calycanthine, has long been recognized as a very powerful convulsant [10–12]; furthermore, the potent antinociceptive activity of dimeric pyrrolidinoindolines, *e.g.*, chimonanthine alkaloids, that interact with opioid receptors has been reported [13–15]. However, very little is currently known about the antifungal activity of secondary metabolites produced by the seeds of the genus *Chimonanthus*.

Its seeds, commonly known as '*tubadou*', are very toxic and rich in fatty oil [5]. In our search for active substances of medicinal plants grown in Shaanxi Province, China, we have investigated the crude extract of the defatted seeds of *C. praecox* against phytopathogenic fungi. The antifungal activity-guided fractionation indicated that the activity was mainly associated with the polar alkaloids. Further purification of a MeOH extract from *C. praecox* seeds has afforded two dimeric alkaloids, one quinoline derivative, D-calycanthine (**1**), and one known pyrrolidinoindoline, L-folicanthine (**2**). Here, we report antifungal properties of the two major alkaloids **1** and **2**, which were isolated by silica-gel chromatography from the extracts and elucidated based on spectroscopic data. Furthermore, tandem-mass-spectrometric (MS^n) techniques as important tools are useful for distinguishing compounds [16]. We also report on the use of liquid chromatography/electrospray-ionization mass spectrometry (LC/ESI-MS) methods to confirm the structures of these alkaloids.

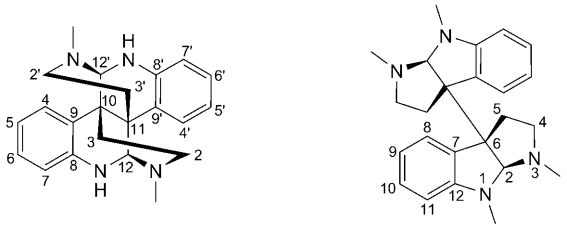

Fig. 1. *Structures of dimeric alkaloids* **1** *and* **2**

Results and Discussion. – Repeated column chromatography of the active MeOH extract of the seeds of *C. praecox* subjected to silica-gel column led to the isolation of two alkaloids, D-calycanthine (**1**) and L-folicanthine (**2**).

D-Calycanthine (**1**) was obtained as colorless crystals, m.p. 226–229°, which showed positive reaction toward the modified *Dragendorff* reagent. The FAB- and ESI-MS exhibited *pseudo*-molecular ions at *m/z* 347 ($[M+H]^+$) for a molecular mass of 346, and HR-ESI-MS (*m/z* 347.2240 ($[M+H]^+$)) delivered a molecular formula $C_{22}H_{26}N_4$. The ^{13}C-NMR spectrum of **1** showed only eleven C-atom signals (δ(C) 145.2, 126.6, 124.9, 124.3, 116.5, 112.1, 71.1, 46.5, 42.5, 35.9, 31.6), indicating that the alkaloid is a symmetrical dimer. The 'monomer' substructure would contain a MeN group with a signal at δ(C) 42.5, six C-atoms as a disubstituted aromatic ring, a quaternary C-atom appearing as a *singlet* at δ(C) 35.9, two CH_2 groups with signals at δ(C) 31.6 and 46.5,

and a CH group, probably between two N-atoms, with a signal at δ(C) 71.1. The ^{1}H-NMR spectrum indicated the presence of eight aromatic H-atoms with signals at δ(H) 6.30 (*d*, *J* = 7.8, 2 H), 6.59 (*t*, *J* = 7.4, 2 H), 6.86 (*t*, *J* = 7.6, 2 H), and 7.03 (*d*, *J* = 7.8, 2 H), supporting an *ortho*-disubstituted aromatic ring. The H-atom signals of the two CH_2 groups exhibited an *AA′XX′* pattern in the ^{1}H-NMR spectrum. The ^{1}H-NMR spectrum recorded in $CDCl_3$ exhibited the H-atom signals at δ(H) 1.31 (*dd*, *J* = 13, 2.6) and 3.16 (*ddd*, *J* = 13, 5.6) attributed to CH_2(3) and CH_2(3′), and the signals at δ(H) 2.66 (*dd*, *J* = 13, 5.4) and 2.28 (*ddd*, *J* = 13, 2.4) ascribed to CH_2(2) and CH_2(2′). Further, the ^{1}H-NMR spectrum showed a *singlet* at δ(H) 4.35 attributed to H−C(12) and H−C(12′). The identity was confirmed by comparison of NMR and MS data with those reported in the literature [17][18]. However, the optical rotation of **1** was $[\alpha]_D^{20} = +363$ (MeOH), which is opposite in sign to that of the frog L-calycanthine $[\alpha] = -570$ (MeOH) [17], and the plant L-calycanthine $[\alpha]^{20} = -688$ (EtOH) and $[\alpha] = -489$ (MeOH), which was previously derived from *Psychotria colorata* [19], and from *P. forsteriana* [20] and *P. glomerulata* [21]. Consequently, the plant-derived D-calycanthine reported in this work is the (+)-enantiomer of the known frog alkaloid calycanthine. In addition, calycanthine, isolated from *Calycanthus floridus*, had $[\alpha]_D = +684$ (MeOH) [22]. Compound **1**, therefore, was deduced as D-calycanthine (*Fig. 1*). To our knowledge, this is the first report of a D-configured alkaloid, *i.e.*, D-calycanthine (**1**), isolated from *C. praecox*.

Compound **2** was obtained as colorless crystals, which showed positive reaction toward the modified *Dragendorff* reagent. The HR-ESI-MS provided a *pseudo*-molecular ion at *m*/*z* 375.2560 ($[M+H]^+$), corresponding to the molecular formula $C_{24}H_{30}N_4$. The ^{1}H- and ^{13}C-NMR data were in complete agreement with those reported in the literature for a known L-folicanthine [15][23][24]. This molecule was elucidated as L-folicanthine (*Fig. 1*) on the basis of their NMR and MS data by a comparison with the reported values.

In particular, LC/MS/MS methods were also used to identify the two major natural products **1** and **2**. On MS/MS analyses, the molecular ion of the D-calycanthine (**1**) at *m*/*z* 3047.2240 undergoes further fragmentations to form a set of characteristic ion peaks at *m*/*z* 316.1806, 304.1830, 290.1653, 285.1390, and 211.1226 (*Fig. 2*), whereas the molecular ion of L-folicanthine (**2**) at *m*/*z* 375.2566 provides only two ion peaks at *m*/*z* 344.2148 and 187.1218 (*Fig. 3*), suggesting that **2** is a highly symmetrical dimer. Accordingly, the first application of LC/MS/MS methods proved suitable to further elucidate the structures of these two metabolites present in the plant.

Compounds **1** and **2**, and the MeOH extract of *C. praecox* seeds were evaluated for their antifungal activities against five plant pathogenic fungi, *Exserohilum turcicum* and *Bipolaris maydis*, *Alternaria solani*, *Sclerotinia sderotiorum*, and *Fusarium oxysportium in vitro* using the protocol described in [25]. The inhibitory activities (the effective concentration for 50% growth inhibition, EC_{50} value) are collected in the *Table*. Bioassay results revealed that the MeOH extract inhibited the *in vitro* growth of five plant pathogenic fungi (*Table*) at a concentration of 250 μg/ml. Among these fungi, the extract was most sensitive to the first four fungi with more than 60% growth inhibition, indicating the presence of the inhibitory substances in the extract. The activity-guided fractionation of this extract afforded the two main antifungal compounds D-calycanthine (**1**) and L-folicanthine (**2**). Further bioassays for fungicidal activity of

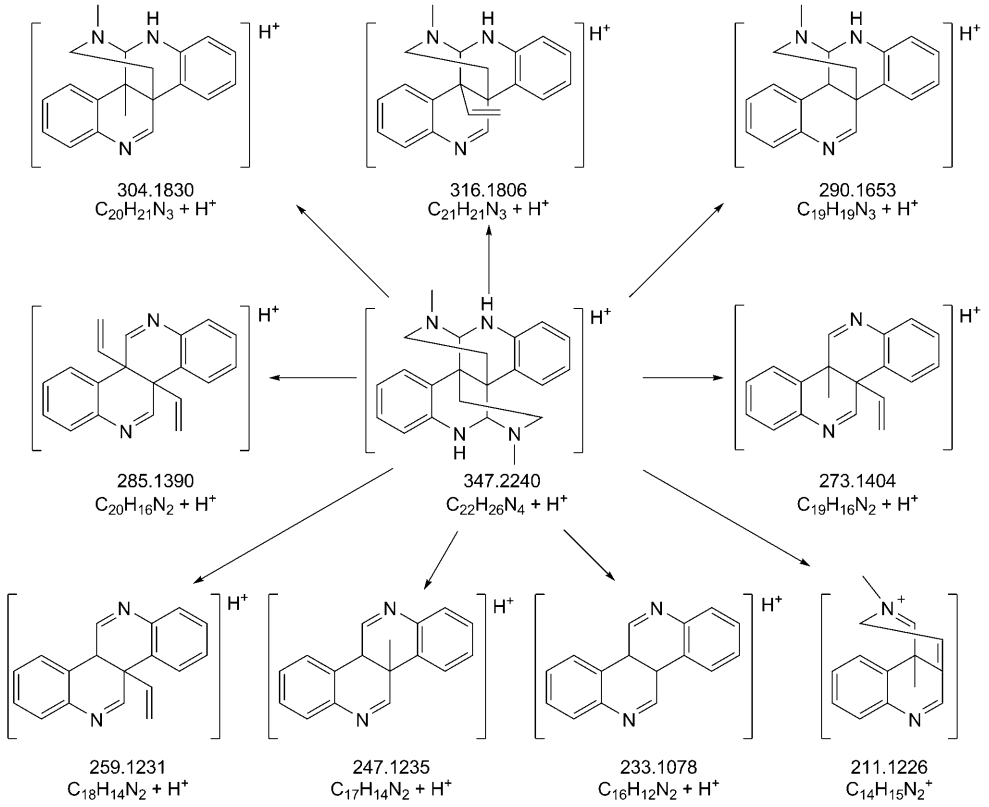

Fig. 2. *Observed MS/MS fragmentation pathways of compound* **1** (values in *m*/*z*)

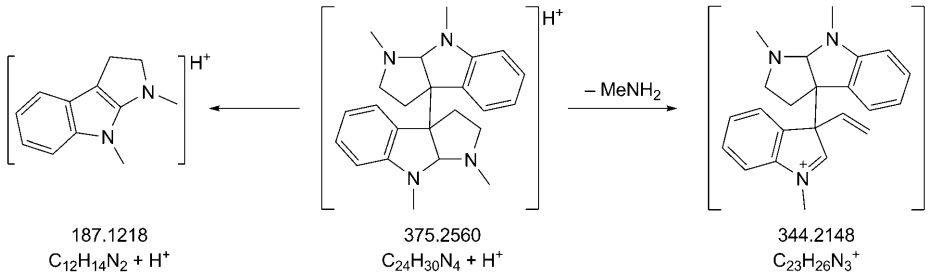

Fig. 3. *Observed MS/MS fragmentation pathways of compound* **2** (values in *m*/*z*)

the compounds indicated that compound **1**, at a concentration of 250 μg/ml, was effective in reducing *E. turcicum* and *B. maydis*, with more than *ca.* 76 and 81% growth inhibition, and with EC_{50} values (*Table*) of 103.1 and 29.3 μg/ml, respectively, whereas **1** effected *ca.* 42–27% inhibition of *A. solani* and *F. oxysportium* at the same concentration (*Table*). These results suggest that compound **1** may have potential as

fungicidal agent in controlling both pathogenic fungi *E. turcicum* and *B. maydis*. Compounds **2** showed a marked inhibition of *Bipolaris maydis*, *Sclerotinia sderotiorum*, and *Alternaria solani* at a concentration of 250 μg/ml (*Table*) with inhibition rates of 78.6, 82.6, and 71.3%, and with EC_{50} values (*Table*) of 79.6, 61.2, and 125.7 μg/ml, respectively. All samples tested exhibited a weak inhibitory effect on *Fusarium oxysportium*. However, all test samples failed to show any insecticidal activity to *Mythimna separate*.

Table. *Inhibitory Effects of MeOH Extract, and Compounds* **1** *and* **2** *on Phytopathogenic fungi* in vitro (mean ± SD, $n = 3$)[a])

Sample	*E. t.*	*B. m.*	*S. s.*	*A. s.*	*F. o.*
Growth inhibition [%] at 250 μg/ml					
MeOH Extract	62.3	77.9	75.5	62.3	43.8
1	76.9	81.1	inactive	42.7	27.9
2	47.3	78.6	82.6	71.3	33.4
EC_{50} Values [μg/ml]					
1	103.1 ± 2.4	29.3 ± 0.8	inactive	328.3 ± 3.8	> 500
2	261.3 ± 3.6	79.6 ± 1.1	61.2 ± 1.2	125.7 ± 1.9	> 500

[a]) *E. t.: Exserohilum turcicum*; *B. m.: Bipolaris maydis*; *S. s.: Sclerotinia sderotiorum*; *A. s.: Alternaria solani*; *F. o.: Fusarium oxysportium.*

Both calycanthine- and pyrrolidinoindoline-type alkaloids have been known to be widespread bioactive compounds that have been isolated from plants belonging to the genera of Calycanthaceae, Idiospermaceae, and Rubiaceae [18][19][24][26]. Within the large reservoir of natural fungicides that exist in plants and microorganisms, it is reasonable that examples exist that would serve as safe and effective alternatives to synthetic fungicides. Such compounds could be used directly or could act as templates for synthetic analogs. Some antibiotics are effective as fungicides against a number of plant pathogens. However, there is considerable resistance to the use of antibiotics in agriculture. It has been argued that such use will risk the development of resistance in animal pathogens to the antibiotic and thereby diminish its usefulness in animal-disease therapy.

Latent infections are especially difficult to control in harvested commodities, because the pathogen resides in an inactive state within the host tissue. Nonsystemic, synthetic fungicides and biological control agents are ineffective in controlling such infections. Natural plant-derived fungicides should provide a wide variety of compounds as alternatives to synthetic fungicides, both as fumigants and as contact pesticides. They may also prove valuable as 'lead structures' for the development of synthetic compounds [4]. It behoves us to explore more intensely this rich source of fungicides.

In the current study, we demonstrated for the first time that the two substances **1** and **2** isolated from *C. praecox* might serve as the main components responsible for pronounced *in vitro* antifungal properties of this plant extract against *E. turcicum*, *B. maydis*, and *S. sderotiorum*, which also show certain synergistic effects. Additionally, the occurrence of the antifungal substances in *C. praecox* may indicate the ecological

impact of these compounds in the plant, which produce defense effects against pathogens to survive in the ecosystem. Further trials will be carried out in the near future in order to test the effectiveness of these molecules also under field conditions, and to better understand the mechanism of action involved in pathogen inhibition. D-Calycanthine and L-folicanthine could be considered as potential candidates for the development as new fungicides.

Experimental Part

General. Column chromatography (CC): over silica gel (SiO_2; 200–300 mesh; *Qingdao Marine Chemical Ltd.*, Qingdao, P. R. China). TLC: Plates precoated with silica gel F_{254} (*Qingdao Marine Chemical Ltd.*); detection by UV light and by spraying with modified *Dragendorff* reagent. M.p.: *X-4* apparatus; uncorrected. Optical rotations: *Perkin-Elmer 241MC* automatic polarimeter. ^{1}H- and ^{13}C-NMR spectra: *Bruker AM 200* instrument (D-Rheinstetten); Me_4Si as an internal standard; coupling constants *J* in Hz. FAB-MS: *VGZAB-HS* mass spectrometer. Liquid chromatography (LC)/ESI-MS/MS: *Micromass Waters Q-TOF Ultima Global* mass spectrometer; in *m*/*z*.

Plant Materials. Seeds of *C. praecox* were collected from campus of Northwest A&F University, Yangling, Shaanxi, China. The voucher specimen was deposited with the College of Sciences, Northwest A&F University.

Isolation of the Antifungal Constituents. Dried powder of seeds of *C. praecox* (671g) was extracted three times with petroleum ether (b.p. 30–60°) at r.t., and the combined org. phase was concentrated under reduced pressure to give an oil (111 g). The residue was extracted with MeOH (3 × 3 l) at r.t., and the combined org. layer was evaporated *in vacuo* to provide 61 g of extract. The extract (2 g) was subjected to flash CC (SiO_2; gradient of $CHCl_3$/MeOH 9 : 1, 8 : 1, 7 : 1, 6 : 1, 5 : 1, 4 : 1, 3 : 1). The same fractions were combined according to TLC analysis to yield compounds **1** (183 mg) and **2** (56 mg).

D-*Calycanthine* (**1**). Colorless crystals. M.p. 226–229°. $[\alpha]_D^{20} = +363$ ($c = 0.74$, MeOH) ([22a]: $[\alpha]_D = +684$ (MeOH)). ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.03 (*d*, *J* = 7.8, H–C(7), H–C(7′)); 6.86 (*t*, *J* = 7.6, H–C(6), H–C(6′)); 6.59 (*t*, *J* = 7.4, H–C(5), H–C(5′)); 6.30 (*d*, *J* = 7.8, H–C(4), H–C(4′)); 4.60 (br. *s*, 2 NH); 4.35 (*s*, H–C(12), H–C(12′)); 3.16 (*ddd*, *J* = 13, 5.6, H_α–C(3), H_α–C(3′)); 2.66 (*dd*, *J* = 13, 5.4, H_α–C(2), H_α–C(2′)); 2.44 (*s*, 2 MeN); 2.28 (*ddd*, *J* = 13, 2.4, H_β–C(2), H_β–C(2′)); 1.31 (*dd*, *J* = 13, 2.6, H_β–C(3), H_β–C(3′)). ^{13}C-NMR ($CDCl_3$, 50 MHz): 145.2 (*s*, C(8), C(8′)); 126.6 (*s*, C(9), C(9′)); 124.9 (*d*, C(6), C(6′)); 124.3 (*d*, C(4), C(4′)); 116.5 (*s*, C(5), C(5′)); 112.1 (*d*, C(7), C(7′)); 71.1 (*d*, C(12), C(12′)); 46.5 (*t*, C(2), C(2′)); 42.5 (*t*, MeN(1), MeN(1′)); 35.9 (*s*, C(10), C(11)); 31.6 (*t*, C(3), C(3′)). FAB-MS (pos.): 347 ($[M+H]^+$), 316 ($[M-2\,Me]^+$), 91 ($[C_7H_7]^+$). HR-ESI-MS (pos.): 347.2240 ($[M+H]^+$).

L-*Folicanthine* (= *Methylchimonanthine*; **2**). Colorless crystals. M.p. 118–120°. $[\alpha]_D^{20} = -331$ ($c = 1.2$, EtOH). ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.96–2.46 (*m*, CH_2(5), CH_2(5′)); 2.53–2.63 (*m*, CH_2(4), CH_2(4′)); 2.45 (*s*, MeN(3), MeN(3′)); 2.99 (*s*, MeN(1), MeN(1′)); 4.38 (*s*, H–C(2), H–C(2′)); 6.25 (*d*, *J* = 8.0, H–C(11), H–C(11′)); 6.48 (*t*, *J* = 8.0, H–C(9), H–C(9′)); 6.94–7.12 (*m*, H–C(8), H–C(8′), H–C(10), H–C(10′)). ^{13}C-NMR ($CDCl_3$, 50 MHz): 153.7 (*s*, C(12), C(12′)); 133.6 (*s*, C(7), C(7′)); 128.8 (*d*, C(10), C(10′)); 124.4 (*d*, C(8), C(8′)); 117.4 (*d*, C(9), C(9′)); 106.6 (*d*, C(11), C(11′)); 92.7 (*d*, C(2), C(2′)); 63.4 (*s*, C(6), C(6′)); 53.4 (*t*, C(4), C(4′)); 38.7 (*q*, MeN(3), MeN(3′)); 36.3 (*q*, MeN(1), MeN(1′)); 36.1 (*t*, C(5), C(5′)). HR-ESI-MS (pos.): 375.2566 ($[M+H]^+$). These data were similar to literature values [23][24].

Antifungal Bioassay. The tested pathogenic fungi, *Alternaria solani*, *Fusarium oxysportium*, *Sclerotinia sderotiorum*, *Exserohilum turcicum*, and *Bipolaris maydis*, were provided by Institute of Pesticides, Northwest A&F University. All samples dissolved in MeOH were tested for antifungal activity *in vitro* by mycelial growth inhibitory rate method, a *Poison Food Technique* [25]. Potato dextrose agar (PDA) medium was used as the medium for all test fungi.

The media (100 ml) incorporating test samples were inoculated at the center of agar discs of the test fungi (4 mm diameter). Three replicate plates for each fungus were incubated at 26 (±2)° for all test fungi. Control plates containing media mixed with MeOH (1 ml) were included. After incubation for 72–

96 h, the mycelial growth of fungi (mm) in both treated (T) and control (C) *Petri* dishes was measured diametrically in three different directions (decussation method) until the fungal growth in the control dishes was almost complete. The percentage of growth inhibition (*I*) was calculated using the following formula:

$$I\,[\%] = [(C - T)/C] \times 100$$

Insecticidal Bioassay. Third instar larva of *Mythimna separate* was used as test insect for evaluation of insecticidal activity. Contact toxicity assay was performed by capillary quantitative drop method and by the leaf disk assay [27].

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