

# 11-Hydroxymonocerin from the Plant Endophytic Fungus *Exserohilum rostratum*

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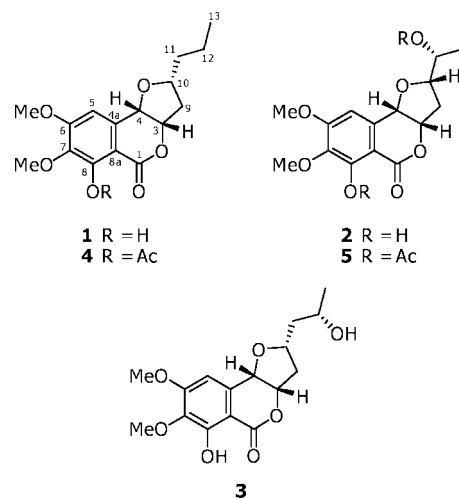
A new analogue of monocerin, 11-hydroxymonocerin (**2**), along with monocerin (**1**) and 12-hydroxymonocerin (**3**) were isolated from cultures of *Exserohilum rostratum*, a fungal strain endophytic in *Stemona* sp. The structure of **2** was determined by analysis of NMR and MS data and by comparison of spectroscopic data to those of **1**. Monocerin (**1**) and 11-hydroxymonocerin (**2**) displayed activity against *Plasmodium falciparum* (K1, multidrug-resistant strain) with IC<sub>50</sub> values of 0.68 and 7.70  $\mu$ M, respectively. None of the compounds were cytotoxic against any of the tumor cell lines tested.

Endophytic fungi have proven to be a rich source of bioactive secondary metabolites.<sup>1–5</sup> *Exserohilum rostratum* has been isolated from both marine and terrestrial sources and is known as a marine invertebrate pathogen<sup>6,7</sup> and a cause of foot and root diseases,<sup>8</sup> and its chemical study has led to the identification of phytotoxins useful as herbicides.<sup>9</sup> In the course of our search for biologically active metabolites from endophytic fungi from Thai medicinal plants, a subculture of an isolate of *E. rostratum*, obtained from leaves of a *Stemona* sp., was cultivated in yeast extract sucrose (YES) broth. An EtOAc extract of the culture showed significant antimalarial activity against *Plasmodium falciparum* (K1, multidrug-resistant strain). Herein, we report the isolation and structural elucidation of a new isocoumarin derivative, 11-hydroxymonocerin (**2**), together with known monocerin (**1**) and 12-hydroxymonocerin (**3**). The compounds were evaluated for antiplasmodial (potential antimalarial) and cytotoxic activities. This is a first report of the potential antimalarial activity of monocerin (**1**) and its analogues.

The culture broth of *E. rostratum* grown in YES broth for 3 weeks was extracted with EtOAc, and the crude extract was purified by column chromatography to yield compounds **1**–**3**.

Monocerin (**1**), the major metabolite, was characterized by analyses of its spectroscopic data and by comparison with data reported in the literature.<sup>10</sup> Monocerin has been isolated as an antifungal, insecticidal, and phytotoxic secondary metabolite from several fungal species including *Helminthosporium monoceras*,<sup>11</sup> *Exserohilum turcum*,<sup>12–14</sup> *Fusarium larvarum*,<sup>15,16</sup> and *Microdochium bolleyi*.<sup>10</sup>

Compound **2** was isolated as a white solid and was optically active. The molecular formula was determined to be C<sub>16</sub>H<sub>20</sub>H<sub>7</sub> by analysis of its HRESIMS (*m/z* 347.1128 [M + Na]<sup>+</sup>,  $\Delta$  –0.1 mmu), implying seven double-bond equivalents. The IR spectrum showed a strong broadened OH absorption band at 3431 cm<sup>–1</sup> and characteristic absorption bands for ester carbonyl (1665 cm<sup>–1</sup>) and aromatic ring (1521, 1455, and 1276 cm<sup>–1</sup>) groups. The <sup>1</sup>H NMR spectrum showed a chelated OH signal ( $\delta$ <sub>H</sub> 11.24, OH-8) and signals for an aromatic proton ( $\delta$ <sub>H</sub> 6.61), four oxygen-bearing methine groups ( $\delta$ <sub>H</sub> 5.10, 4.63, 4.02, and 3.53), and two OCH<sub>3</sub> groups ( $\delta$ <sub>H</sub>



3.97 and 3.91). Analysis of <sup>13</sup>C NMR and HSQC experiments revealed the presence of a conjugated ester carbonyl ( $\delta$ <sub>C</sub> 167.6) strongly hydrogen-bonded with an OH, six aromatic carbons of which three were oxygenated ( $\delta$ <sub>C</sub> 158.7, 156.2, and 137.4), and one protonated ( $\delta$ <sub>C</sub> 104.6), four oxymethine ( $\delta$ <sub>C</sub> 81.8, 80.9, 75.0, and 74.6), two methylene ( $\delta$ <sub>C</sub> 35.9 and 25.9), and a methyl ( $\delta$ <sub>C</sub> 9.9) carbon. The <sup>1</sup>H and <sup>13</sup>C NMR spectra were very similar to those of monocerin (**1**), except for the marked differences in chemical shift values corresponding to position 11. In the <sup>1</sup>H NMR spectrum of **2**, the signal attributable to an oxygen-bearing methine at  $\delta$ <sub>H</sub> 3.53 replaced those corresponding to the methylene signal of **1** at  $\delta$ <sub>H</sub> 1.55 and 1.66.

Treatment of **2** with acetic anhydride resulted in formation of diacetate **5**, and the <sup>1</sup>H NMR spectrum of **5** revealed two acetate methyl singlets at  $\delta$ <sub>H</sub> 2.09 and 2.40, respectively. The shift in the signal corresponding to H-11 ( $\delta$ <sub>H</sub> 4.92) indicated that one OH group was attached to C-11 in **2**. The proposed structure was confirmed by a proton spin system from H-4 to H<sub>3</sub>-13, established by <sup>1</sup>H–<sup>1</sup>H correlation observed in the COSY spectrum and by HMBC correlations (Figure 1) of H-4 to  $\delta$ <sub>C</sub> 130.6 (C-4a), 101.9 (C-8a), 104.5 (CH-5), and 80.9 (CH-3) as well as H-3 to  $\delta$ <sub>C</sub> 38.9 (CH<sub>2</sub>-9). The full assignments and connectivity were determined by <sup>1</sup>H–<sup>1</sup>H COSY correlations as indicated by bold lines and HMBC correlations shown by arrows (Figure 1). By analysis of NOESY data, the compound exhibited NOEs between H-3 and H-4 and between

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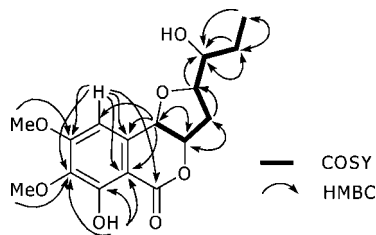


Figure 1. Key HMBC and COSY correlations for **2**.

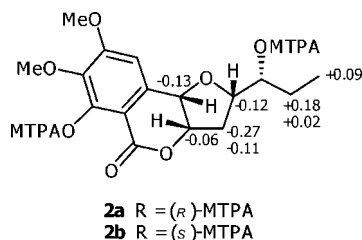


Figure 2.  $\Delta\delta$  values (in ppm) =  $\delta_S - \delta_R$  for (*S*)- and (*R*)-MTPA esters in **2a** and **2b**.

Table 1. In Vitro Antimalarial Activity of Compounds **1**, **2**, **4**, and **5** against *P. falciparum*

compound	IC <sub>50</sub> (μM)
<b>1</b>	0.68
<b>2</b>	7.70
<b>4</b>	0.82
<b>5</b>	9.10
dihydroartemisinin <sup>a</sup>	4 × 10 <sup>-3</sup>

<sup>a</sup> Standard antimalarial drug.

H-4 and H-10, indicating that these protons are all on the same face of the ring system.

The absolute configuration of **2** was assigned by application of the modified Mosher method.<sup>17,18</sup> Treatment of **2** with (*S*)- and (*R*)-MTPA Cl afforded the (*R*)-MTPA ester (**2a**) and (*S*)-MTPA ester (**2b**), respectively. The difference in chemical shift values ( $\Delta\delta = \delta_S - \delta_R$ ) for the diastereomeric esters **2b** and **2a** was calculated in order to assign the absolute configuration at C-11. Calculation for all relevant signals suggested the *R* absolute configuration at C-11, as shown in Figure 2. Therefore, compound **2** was determined to be 11(*R*)-hydroxymonocerin.

Compound **3** had the same molecular formula as **2**, C<sub>16</sub>H<sub>20</sub>O<sub>7</sub>. Comparison of the optical rotation and its NMR spectroscopic data with those in the literature<sup>10</sup> indicated that compound **3** is 12(*R*)-hydroxymonocerin.

Monocerin (**1**), 11-hydroxymonocerin (**2**), and their acetylated products **4** and **5** were evaluated for antiplasmodial activity against the multidrug-resistant K1 strain of *Plasmodium falciparum*. Monocerin exhibited antiplasmodial activity (IC<sub>50</sub> value of 0.68 μM). Activity of the 11-hydroxy analogue (**2**) was 10-fold lower, indicating that an additional OH group in the *n*-propyl chain reduces the activity (Table 1). Conversion of the OH groups to acetyl esters (**4** and **5**) did not show any significant effect on their activity. The compounds were also tested for cytotoxicity against five human tumor cell lines: BT474, CHAGO, Hep-G2, KATO-3, and SW-620. None were cytotoxic at a concentration of 20 μg/mL.

## Experimental Section

**General Experimental Procedures.** Optical rotations were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp at wavelength 589 nm, and UV data were recorded on a Varian 50Probe UV/vis spectrophotometer. Melting points were measured using a Fisher-Johns melting point apparatus. IR spectra were recorded on a Perkin-Elmer model 1760X Fourier transform infrared spectrophotometer. HRESIMS spectra were obtained using a Micromass LCT mass spectrometer. The

NMR spectra were recorded on a Varian YH400 spectrometer at 400 MHz for <sup>1</sup>H NMR and at 100 MHz NMR for <sup>13</sup>C NMR using TMS (tetramethylsilane) as internal standard.

**Isolation of Endophytic Fungi.** Healthy leaves and roots of a *Stemona* sp. were collected from Amphur Bangban, Ayutthaya Province, Thailand, in June 2007. Plant samples were washed in tap water and air-dried. The cleaned leaf and root fragments were surface-sterilized as described by Schulz and co-workers<sup>19</sup> with some modifications. Plant fragments were sequentially immersed in 70% EtOH for 1 min, 6% NaOCl solution for 5 min, and sterile distilled H<sub>2</sub>O for 1 min (two times). Then, the surface-sterilized fragments were cut into small pieces (ca. 5 mm in length) using a sterile blade and placed on sterile water agar plates for further incubation at 30 °C. The hyphal tip of the endophytic fungus growing out from the plant tissue was cut by a sterile pipet and transferred onto a potato dextrose agar (PDA) plate. After incubation at 30 °C for 7–14 days, culture purity was determined from colony morphology.

**Identification of Endophyte.** The fungal endophyte isolate (stem3) was identified on the basis of both morphology of the fungus grown on banana leaf agar at 25 °C and analysis of the DNA sequences of the ITS region of the rRNA gene. The fungus grew on PDA as brown filamentous colonies, and they developed characteristic brown spores. A GenBank search for sequences similar to their ITS region revealed *Exserohilum rostratum* in the family Pleosporaceae as the closest matches, with sequence identity of 99%. The culture of isolate stem3 (accession number EU571210) has been deposited at the Department of Microbiology, Mahidol University, Thailand.

**Fermentation, Extraction, and Isolation.** The endophytic fungus *Exserohilum rostratum* was cultured in 1000 mL Erlenmeyer flasks (×25) containing 200 mL of yeast extract sucrose (YES) broth at 30 °C for 21 days under static conditions. The fungal cells were separated from the broth by filtration, and a culture broth was subsequently extracted with EtOAc (equal volume × 3), yielding 4.10 g of crude extract. The extract was subjected to SiO<sub>2</sub> column chromatography (CC) eluted with hexane–EtOAc and MeOH–CH<sub>2</sub>Cl<sub>2</sub> mixtures of increasing polarity to afford eight fractions (I–VIII). Fraction II was rechromatographed over silica gel eluted with EtOAc–hexane (2:3) to afford monocerin (**1**, 764.2 mg). Fraction IV was further subjected to flash CC on silica gel (benzene–EtOAc, 1:1) to yield compound **2** (165.4 mg). Fraction V was rechromatographed by flash CC on SiO<sub>2</sub> eluted with benzene–EtOAc (3:2), followed by preparative TLC (MeOH–CH<sub>2</sub>Cl<sub>2</sub>, 1:19) to give compound **3** (3.6 mg).

**Monocerin (1):** colorless oil; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 11.25 (1H, s, 8-OH), 6.57 (1H, s, H-5), 5.03 (1H, brs, H-3), 4.52 (1H, brs, H-4), 4.08 (1H, m, H-10), 3.92 (3H, s, 6-OCH<sub>3</sub>), 3.86 (3H, s, 7-OCH<sub>3</sub>), 2.58 (1H, m, H-9β), 2.12 (1H, dd, *J* = 14.4, 5.2 Hz, H-9α), 1.66 (1H, m, H-11a), 1.55 (1H, m, H-11b), 1.38 (2H, m, H<sub>2</sub>-12), 0.88 (3H, t, *J* = 7.0 Hz, H<sub>3</sub>-13); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 167.8 (C, C-1), 158.7 (C, C-6), 156.2 (C, C-8), 137.2 (C, C-7), 131.2 (C, C-4a), 104.9 (CH, C-5), 101.9 (C, C-8a), 81.3 (CH, C-3), 78.7 (CH, C-10), 74.7 (CH, C-4), 60.7 (CH<sub>3</sub>, 7-OCH<sub>3</sub>), 56.2 (CH<sub>3</sub>, 6-OCH<sub>3</sub>), 39.0 (CH<sub>2</sub>, C-9), 38.0 (CH<sub>2</sub>, C-11), 19.1 (CH<sub>2</sub>, C-12), 13.9 (CH<sub>3</sub>, C-13); HRESIMS *m/z* 331.1151 [M + Na]<sup>+</sup> (calcd for C<sub>16</sub>H<sub>20</sub>O<sub>6</sub> Na, 331.1158).

**11-Hydroxymonocerin (2):** white solid; mp 118–121 °C; [α]<sub>D</sub><sup>20</sup> +50.0 (c 0.1, in EtOH); UV (EtOAc) λ<sub>max</sub> (log ε) 308 (3.78), 273 (4.23) nm; IR (KBr) ν<sub>max</sub> 3431, 2930, 1665, 1455, 1276, 1117 cm<sup>-1</sup>; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 11.24 (1H, s, 8-OH), 6.61 (1H, s, H-5), 5.10 (1H, brs, H-3), 4.63 (1H, brs, H-4), 4.02 (1H, m, H-10), 3.97 (3H, s, 6-OCH<sub>3</sub>), 3.91 (3H, s, 7-OCH<sub>3</sub>), 3.53 (1H, brs, H-11), 2.57 (1H, m, H-9β), 2.27 (1H, dd, *J* = 14.4, 5.0 Hz, H-9α), 1.53 (1H, m, H-12a), 1.45 (1H, m, H-12b), 1.02 (3H, t, *J* = 6.8 Hz, H<sub>3</sub>-13); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 167.6 (C, C-1), 158.7 (C, C-6), 156.2 (C, C-8), 137.4 (C, C-7), 130.6 (C, C-4a), 104.6 (CH, C-5), 101.9 (C, C-8a), 81.8 (CH, C-10), 80.9 (CH, C-3), 75.0 (CH, C-11), 74.6 (CH, C-4), 60.8 (CH<sub>3</sub>, 7-OCH<sub>3</sub>), 56.3 (CH<sub>3</sub>, 6-OCH<sub>3</sub>), 35.9 (CH<sub>2</sub>, C-9), 25.9 (CH<sub>2</sub>, C-12), 9.9 (CH<sub>3</sub>, C-13); HRESIMS *m/z* 347.1106 [M + Na]<sup>+</sup> (calcd for C<sub>16</sub>H<sub>20</sub>O<sub>7</sub> Na, 347.1107).

**Acetylation of 1 and 2.** Acetic anhydride (0.3 mL) and DMAP (catalytic amount) were added to a solution of monocerin (**1**) (10 mg) in pyridine, and the mixture was left stirring at room temperature for 5 h. After removing the solvent under reduced pressure, acetate **4** was purified by CC on silica gel with EtOAc–hexane (3:1); 95% yield (10.8 mg); colorless oil; [α]<sub>D</sub><sup>20</sup> −3.0 (c 0.1, in EtOH); <sup>1</sup>H NMR of **4** (400 MHz, CDCl<sub>3</sub>) δ 6.89 (1H, s, H-5), 4.96 (1H, brs, H-3), 4.54 (1H, d, *J* = 3.2 Hz, H-4), 4.11 (1H, m, H-10), 3.94 (3H, s, 6-OCH<sub>3</sub>), 3.82

(3H, s, 7-*OCH*<sub>3</sub>), 2.51 (1H, m, H-9 $\beta$ ), 2.39 (3H, s, 8-*OCOCH*<sub>3</sub>), 2.10 (1H, dd, *J* = 14.4, 5.6 Hz, H-9 $\alpha$ ), 1.65 (1H, m, H-11a), 1.53 (1H, m, H-11b), 1.34 (2H, brm, H<sub>2</sub>-12), 0.88 (3H, t, *J* = 7.2 Hz, H<sub>3</sub>-13); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  168.8 (C, 8-*OCOCH*<sub>3</sub>), 159.5 (C, C-1), 157.5 (C, C-6), 145.5 (C, C-8), 142.6 (C, C-7), 132.2 (C, C-4a), 109.8 (C, C-8a), 109.5 (CH, C-5), 79.4 (CH, C-3), 78.4 (CH, C-10), 74.0 (CH, C-4), 60.7 (CH<sub>3</sub>, 7-*OCH*<sub>3</sub>), 55.8 (CH<sub>3</sub>, 6-*OCH*<sub>3</sub>), 38.5 (CH<sub>2</sub>, C-9), 37.6 (CH<sub>2</sub>, C-11), 20.5 (CH<sub>3</sub>, 8-*OCOCH*<sub>3</sub>), 18.6 (CH<sub>2</sub>, C-12), 13.5 (CH<sub>3</sub>, C-13); HRESIMS *m/z* 373.1260 [M + Na]<sup>+</sup> (calcd for C<sub>18</sub>H<sub>22</sub>O<sub>7</sub> Na, 373.1263).

In a similar fashion, compound **2** (10 mg), acetic anhydride (0.3 mL), and DMAP (catalytic amount) in pyridine (1 mL) were allowed to react at room temperature for 5 h, and the reaction mixture was processed as described above for **4** to afford diacetate **5** (12.1 mg, 96% yield): [ $\alpha$ ]<sub>D</sub><sup>20</sup> +24.0 (c 0.1, in EtOH); <sup>1</sup>H NMR of **5** (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.90 (1H, s, H-5), 4.98 (1H, brs, H-3), 4.92 (1H, m, H-11), 4.59 (1H, d, *J* = 3.6 Hz, H-4), 4.26 (1H, m, H-10), 3.97 (3H, s, 6-*OCH*<sub>3</sub>), 3.84 (3H, s, 7-*OCH*<sub>3</sub>), 2.48 (1H, m, H-9 $\beta$ ), 2.40 (3H, s, 11-*OCOCH*<sub>3</sub>), 2.21 (1H, dd, *J* = 14.4, 6.0 Hz, H-9 $\alpha$ ), 2.09 (3H, s, 8-*OCOCH*<sub>3</sub>), 1.62 (2H, m, H<sub>2</sub>-12), 0.87 (3H, t, *J* = 7.6 Hz, H<sub>3</sub>-13); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  171.5 (C, 11-*OCOCH*<sub>3</sub>), 169.5 (C, 8-*OCOCH*<sub>3</sub>), 160.0 (C, C-1), 158.0 (C, C-6), 146.0 (C, C-8), 143.2 (C, C-7), 132.2 (C, C-4a), 110.4 (C, C-8a), 110.0 (CH, C-5), 78.9 (CH, C-3), 78.7 (CH, C-10), 74.7 (CH, C-11), 74.4 (CH, C-4), 61.2 (CH<sub>3</sub>, 7-*OCH*<sub>3</sub>), 56.3 (CH<sub>3</sub>, 6-*OCH*<sub>3</sub>), 35.6 (CH<sub>2</sub>, C-9), 23.7 (CH<sub>2</sub>, C-12), 21.0 (CH<sub>3</sub>, 8-*OCOCH*<sub>3</sub>), 21.0 (CH<sub>3</sub>, 11-*OCOCH*<sub>3</sub>), 9.9 (CH<sub>3</sub>, C-13); HRESIMS *m/z* 431.1321 [M + Na]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>24</sub>O<sub>9</sub> Na, 431.1318).

**Preparation of (R)-MTPA Ester (2a) and (S)-MTPA Ester (2b).** A reaction mixture of **2** (2 mg), (S)- or (R)-MTPA Cl (20  $\mu$ L), and DMAP (catalytic amount) in pyridine (0.25 mL) was stirred at room temperature overnight. After removing the solvent under reduced pressure, the (R)- and (S)-MTPA esters (**2a** and **2b**) were purified by mini-column chromatography on silica gel with EtOAc (1:4).

**Compound 2a:** <sup>1</sup>H NMR of 2*R*-MTPA ester (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.57 (1H, s, H-5), 5.25 (1H, m, H-11), 5.03, (1H, m, H-3), 4.37 (1H, d, *J* = 3.6 Hz, H-4), 4.24 (1H, m, H-10), 3.98 (3H, s, 6-*OCH*<sub>3</sub>), 3.91 (3H, s, 7-*OCH*<sub>3</sub>), 2.56 (1H, m, H-9 $\beta$ ), 2.15 (1H, dd, *J* = 14.0, 6.2 Hz, H-9 $\alpha$ ), 2.10 (1H, m, H-12a), 1.74 (1H, m, H-12b), 1.17 (3H, t, *J* = 7.2 Hz, H<sub>3</sub>-13).

**Compound 2b:** <sup>1</sup>H NMR of 2*S*-MTPA ester (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.58 (1H, s, H-5), 5.23 (1H, m, H-11), 4.97, (1H, m, H-3), 4.50 (1H, d, *J* = 3.2 Hz, H-4), 4.12 (1H, m, H-10), 3.96 (3H, s, 6-*OCH*<sub>3</sub>), 3.94 (3H, s, 7-*OCH*<sub>3</sub>), 2.29 (1H, m, H-9 $\beta$ ), 2.12 (1H, m, H-12a), 2.04 (1H, dd, *J* = 14.2, 5.8 Hz, H-9 $\alpha$ ), 1.92 (1H, m, H-12b), 1.26 (3H, t, *J* = 7.0 Hz, H<sub>3</sub>-13).

**Biological Assays.** Antimalarial activity in vitro was determined by means of the microculture radioisotope technique based on the method described by Desjardins.<sup>20</sup> The parasite *P. falciparum* (K1, multidrug-resistant strain) was cultured continuously according to the method of Trager and Jensen.<sup>21</sup> An IC<sub>50</sub> value of  $4.0 \times 10^{-3}$   $\mu$ M (*n* = 3) was observed for the positive control, dihydroartemisinin. Cytotoxicity was assessed against human cell cultures, BT474 (breast carcinoma),

CHAGO (lung carcinoma), Hep-G2 (hepatocarcinoma), KATO-3 (gastric carcinoma), and SW-620 (colon carcinoma), using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.<sup>22</sup>

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