

## Lactone Derivatives Produced by a *Phaeoacremonium* sp., an Endophytic Fungus from *Senna spectabilis*

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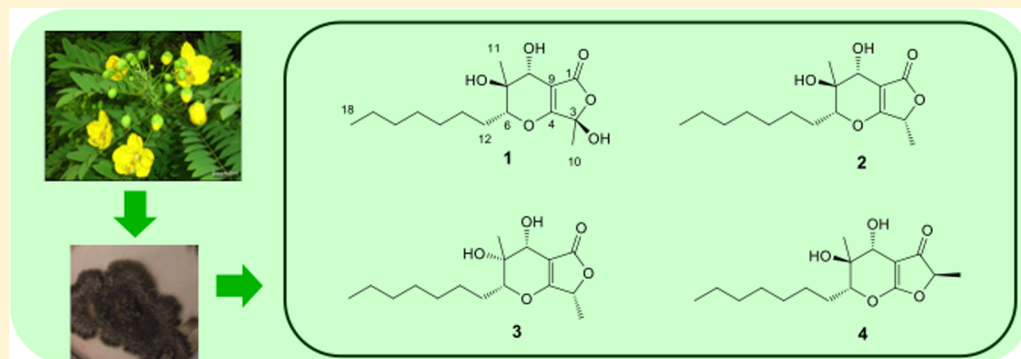
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### Supporting Information



**ABSTRACT:** Three new isoigalones, A, B, and C (1–3), along with aigalone (4), were isolated from the crude EtOAc extract of a *Phaeoacremonium* sp., an endophytic fungus obtained from the leaves of *Senna spectabilis*. The structures of these compounds were elucidated based on the analysis of spectroscopic data. Compounds 2 and 4 were active against the phytopathogenic fungi *Cladosporium cladosporioides* and *C. sphaerospermum*. This is the first report of metabolites produced by an *Phaeoacremonium* sp., associated with *S. spectabilis*.

Approximately one-quarter of all natural products have been isolated from fungi,<sup>1</sup> which are estimated to be 1.5–5.5 million species,<sup>2</sup> including mycoparasites, coprophiles, those from soil and freshwater, epiphytes, endophytes, and others. Endophytic fungi inhabit the interior of a plant during the fungus life cycle, without harming the plant.<sup>3</sup> A number of potentially bioactive and structurally diverse secondary metabolites have been isolated from endophytic fungi,<sup>4–6</sup> which are considered a promising source of novel products.<sup>6</sup>

In our continuing program studying metabolites from endophytic fungi,<sup>7–12</sup> we investigated endophytes associated with the plant *Senna spectabilis* (Leguminosae), selected because several species of *Senna* accumulate unusual phenolic

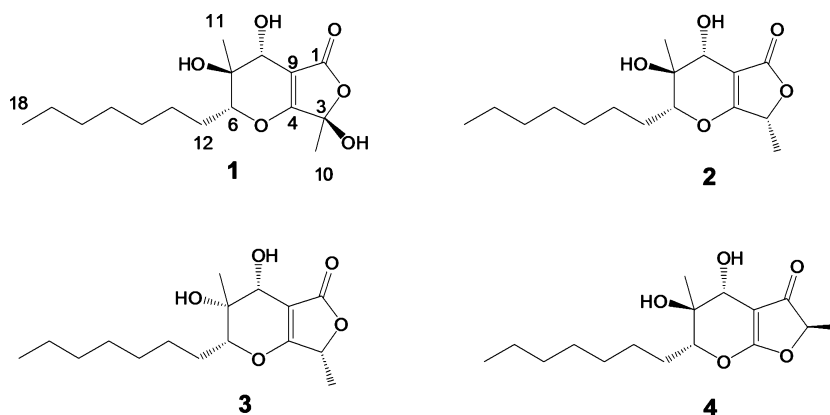
compounds.<sup>13</sup> Chapla et al.<sup>12</sup> described the isolation of bioactive phenolic compounds, including alternariol, 5'-epialtenuene, and cytosporone C, produced by the endophytic fungus *Phomopsis* sp., associated with *S. spectabilis*. These results, together with the hypothesis that endophytic fungi are capable of mimicking the chemistry of their hosts,<sup>13</sup> led us to investigate endophytic fungi associated with *S. spectabilis*.

Here, we describe the isolation, structure, and biological activity (antifungal and cytotoxic) of three new isoigalones, A,

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Chart 1

Table 1.  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data for Isoagialones 1–3, at 500 and 125 MHz

position	1		2		3	
	$\delta_{\text{C}}$ (mult.)	$\delta_{\text{H}}$ (mult., J in Hz)	$\delta_{\text{C}}$ (mult.)	$\delta_{\text{H}}$ (mult., J in Hz)	$\delta_{\text{C}}$ (mult.)	$\delta_{\text{H}}$ (mult., J in Hz)
1	168.8, C		171.1, C		171.1, C	
2						
3	99.9, C		72.5, CH	4.90, q (7.5)	72.7, CH	4.89, q (6.5)
4	172.3, C		175.8, C		176.0, C	
6	87.2, CH	4.10, br d (11.0)	87.2, CH	4.10, br d (12.5)	87.4, CH	4.08, br d (11.0)
7	70.7, C		70.7, C		70.6, C	
8	64.8, CH	3.80, s	64.5, CH	3.79, br d (6.0)	64.8, CH	3.88, s
9	101.2, C		100.4, C		100.5, C	
10	23.3, CH <sub>3</sub>	1.48, s	17.6, CH <sub>3</sub>	1.31, d (7.5)	17.3, CH <sub>3</sub>	1.32, d (6.5)
11	20.0, CH <sub>3</sub>	1.18, s	17.6, CH <sub>3</sub>	1.19, s	20.4, CH <sub>3</sub>	1.19, s
12	27.6, CH <sub>2</sub>	1.80, m	27.7, CH <sub>2</sub>	1.90, m	27.8, CH <sub>2</sub>	1.83, m
13	26.3, CH <sub>2</sub>	1.54, m	26.2, CH <sub>2</sub>	1.50, m	26.4, CH <sub>2</sub>	1.43, m
14	28.6, CH <sub>2</sub>	1.24, m	28.4, CH <sub>2</sub>	1.20, m	28.6, CH <sub>2</sub>	1.24, m
15	28.5, CH <sub>2</sub>	1.24, m	28.4, CH <sub>2</sub>	1.20, m	28.5, CH <sub>2</sub>	1.24, m
16	31.1, CH <sub>2</sub>	1.24, m	31.1, CH <sub>2</sub>	1.20, m	31.2, CH <sub>2</sub>	1.24, m
17	21.9, CH <sub>2</sub>	1.24, m	21.9, CH <sub>2</sub>	1.20, m	22.0, CH <sub>2</sub>	1.24, m
18	13.9, CH <sub>3</sub>	0.85, t (7.5)	13.8, CH <sub>3</sub>	0.84, t (7.5)	13.9, CH <sub>3</sub>	0.85, t (7.5)
7-OH				4.84, s		5.40, br s
8-OH				5.36, d (6.0)		4.80, s

B, and C (1–3), along with aigialone (4)<sup>14</sup> from a *Phaeoacremonium* sp. associated with *S. spectabilis*. These compounds are considered rare in nature due to a ketene acetal functional group.<sup>14</sup>

The chemical and biological evaluation of six endophytic axenic strains (CL-01–CL-06) isolated from leaves of *S. spectabilis* was conducted in order to prioritize these for additional studies. The crude extract of strain CL-06, classified as *Phaeoacremonium* sp., displayed antifungal activity against the phytopathogenic fungi *Cladosporium cladosporioides* and *C. sphaerospermum*; the endophytic fungus selected yielded three new isoagialones, A (1), B (2), and C (3), and the known aigialone (4).

Compound 1 was isolated as an optically active, white, amorphous powder. The molecular formula of 1 was established as C<sub>16</sub>H<sub>26</sub>O<sub>6</sub> by HRESIMS analysis. The UV spectrum of 1 showed an intense absorption at  $\lambda_{\text{max}}$  235 nm, while the IR spectrum presented bands at  $\nu_{\text{max}}$  3336 (OH), 1736 (C=O), and 1677 cm<sup>-1</sup> (enol).

Analysis of NMR data of 1 (Table 1) indicated the presence of two olefinic carbons, three methyl groups, two O-substituted sp<sup>3</sup> carbons, two oxymethine groups, six methylene carbons, and one signal at  $\delta_{\text{C}}$  168.8 ppm in the <sup>13</sup>C NMR spectrum,

which suggested the presence of an ester group. The MS and NMR data indicated the presence of two cyclic systems in the molecule.

Analysis of the <sup>1</sup>H and <sup>13</sup>C NMR data (Table 1) indicated the presence of an *n*-heptyl moiety. A methylene group at  $\delta_{\text{H}}$  1.80 showed coupling with a methylene at  $\delta_{\text{H}}$  1.54, itself coupled to a methylene at  $\delta_{\text{H}}$  1.24 connected to the methyl group at  $\delta_{\text{H}}$  0.85. The *n*-heptyl group was shown to be connected to C-6 via a long-range correlation observed in the HMBC spectrum between H-6 ( $\delta_{\text{H}}$  4.10) and C-13 ( $\delta_{\text{C}}$  26.3). A vicinal <sup>1</sup>H–<sup>1</sup>H correlation between H-6 and H-12 ( $\delta_{\text{H}}$  1.80) observed in the COSY spectrum confirmed this connection. In the HMBC spectrum, H-6 showed correlations to both C-7 ( $\delta_{\text{C}}$  70.7) and C-4 ( $\delta_{\text{C}}$  172.3). The hydrogen H-8 ( $\delta_{\text{H}}$  3.80) showed long-range correlations to C-6 ( $\delta_{\text{C}}$  87.2), C-4 ( $\delta_{\text{C}}$  172.3), and C-1 ( $\delta_{\text{C}}$  168.8), while the methyl group C-11 showed correlations to C-6 and C-8. These data enabled us to establish the structure of the 3,4-dihydro-3-methyl-2-heptyl-2H-pyran-3,4-diol moiety in 1. Finally, correlations observed in the gHMBC spectrum between H<sub>3</sub>C-10 and C-3 and C-4, along with the UV absorption and the <sup>13</sup>C NMR chemical shifts of C-

1 ( $\delta_C$  168.8), C-4 ( $\delta_C$  172.3), and C-9 ( $\delta_C$  101.2), indicated the presence of a  $\beta$ -alkoxy- $\alpha,\beta$ -unsaturated lactone fragment in **1**.<sup>15</sup>

These data allowed us to establish the planar structure of **1**. The structure assignment, as well as its relative configuration, was finally confirmed by X-ray analysis (Figure 1).

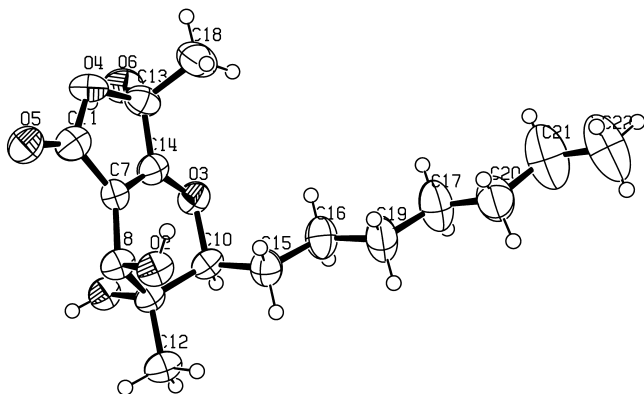


Figure 1. Thermal ellipsoid representation of **1**.

Compound **2** was isolated as an optically active, white, amorphous powder, with the formula  $C_{16}H_{26}O_5$  based on analysis by HRESIMS and  $^1H$  and  $^{13}C$  NMR data (Table 1). The  $^1H$  and  $^{13}C$  NMR spectra (Table 1) of **2** were very similar to that of isoagialone A (**1**), differing in the chemical shift of C-3, which changed from  $\delta_C$  99.9 to  $\delta_C$  72.5, as well as the coupling pattern of  $H_3C-10$ , which appeared as a singlet in **1** ( $\delta_H$  1.48) and as a doublet in **2** ( $\delta_H$  1.32, 7.5 Hz). Long-range correlations observed in the HMBC data between  $H_3C-10$  ( $\delta_H$  1.31) and C-4 ( $\delta_C$  175.8), as well as from H-3 ( $\delta_H$  4.90) to C-9 ( $\delta_C$  100.4) and C-1 ( $\delta_C$  171.1), confirmed the absence of the hydroxy group in C-3. The loss of 16 mass units in **2** relative to compound **1**, along with the analysis of the NMR data of **2**, clearly indicated the reduction of C-3 in **2**. The relative configuration of isoagialone B (**2**) was established by analysis of NOESY 1D data, which showed an NOE between  $H_3C-11$  and  $H_3C-10$ , indicating a pseudoaxial position for  $H_3C-11$  in a *cis* relationship to  $CH_3-10$ . A dipolar NOE coupling was observed between H-6 and H-8, positioning them in a 1,3 pseudodiaxial relationship. Hence the relative stereochemistry of isoagialone B (**2**) was assigned as shown in Figure 2.

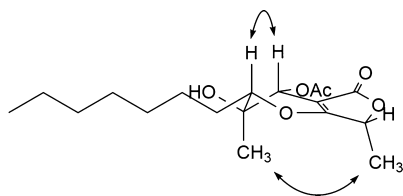


Figure 2. Dipolar couplings (NOEs) observed for isoagialone B (**2**).

Compound **3** was also isolated as an optically active, white, amorphous powder. Its molecular formula was established as  $C_{16}H_{26}O_5$  by HRESIMS analysis, indicating that **3** is isomeric to **2**. Indeed, analysis of the NMR data indicated that compound **3** had the same planar structure as **2**. Considering that the only difference observed between **3** and **2** was the C-11 chemical shift ( $\delta_C$  20.4 in **3**,  $\delta_C$  17.6 in **2**), we suggest that **3** is epimeric at C-7 relative to compound **2**.

Compound **4** was identified as the known metabolite aigialone by comparing its spectroscopic data with literature data.<sup>14</sup> Aigialone was first described from the fungus *Aigialus parvus* and has not been reported from any other natural source.

Compounds **1–4** were evaluated against *Cladosporium cladosporioides* and *C. sphaerospermum* using direct bioautography.<sup>16</sup> The results showed that **2** and **4** exhibited antifungal activity, with a detection limit of 5  $\mu g$ , for both fungi, while compound **3** displayed weak activity (detection limit >5  $\mu g$ ), with a detection limit of 25  $\mu g$ . Nystatin was used as a positive control, showing a detection limit of 1  $\mu g$ .

The cytotoxicity of compounds **1–4** against a human cervical tumor cell line (HeLa) was tested using the MTT assay (Mosmann, 1983);<sup>18</sup> compound **4** exhibited an  $IC_{50}$  of 50  $\mu mol L^{-1}$ , compound **2** presented an  $IC_{50}$  value of 100  $\mu mol L^{-1}$ , and the other compounds were inactive ( $IC_{50} > 100 \mu mol L^{-1}$ ). Camptothecin was used as positive control and presented an  $IC_{50}$  of 0.12  $\mu mol L^{-1}$ .

In conclusion, we have isolated three new polyketides, isoagialones A, B, and C, from *Phaeoacremonium* sp. To the best of our knowledge, only one natural metabolite presenting the furo[3,4-*b*]pyran ring system, named massarilactone B, has been previously reported, in this case from the freshwater aquatic fungus *Massarina tunicata*.<sup>15</sup> Compounds **2–4** are active against the phytopathogenic fungi *C. cladosporioides* and *C. sphaerospermum*, which suggests that these metabolites may exert a defensive role in *S. spectabilis* host species against microbial pathogens.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** Optical rotations were measured in MeOH using a PerkinElmer polarimeter with a sodium lamp operating at 598 nm and 25 °C. UV spectra were recorded in MeOH or  $CHCl_3$  on a Shimadzu UV-2401 PC spectrophotometer. IR spectra were run on a Nicolet Impact-400 spectrophotometer.  $^1H$  (500 MHz) and  $^{13}C$  (125 MHz) NMR spectra were recorded on a Varian DRX-500 spectrometer, using tetramethylsilane as internal standard. HRESIMS data were obtained on a Q-TOF mass spectrometer from Bruker maXis, with an electrospray ionization (ESI) interface (Bruker, Fremont, CA, USA).

Analytical HPLC was performed on a Varian Pro Star 230 System with a UV-vis/DAD detector, using a Phenomenex  $C_{18}$  column (250 mm  $\times$  4.6 mm, 5  $\mu m$ ). Column chromatography (CC) was performed over reversed-phase silica gel 230–400 mesh (Merck). TLC was performed using Merck silica gel 60 (>230 mesh) and precoated silica gel 60 PF<sub>254</sub>. Spots on TLC plates were visualized under UV light and by spraying with anisaldehyde- $H_2SO_4$  reagent followed by heating at 120 °C. Preparative HPLC was performed on a Varian Prep-Star 400 system, using a Phenomenex  $C_{18}$  (250 mm  $\times$  21.2 mm, 10  $\mu m$ ) preparative column.

**Plant Material.** Leaves of *Senna spectabilis* were collected in the Araraquara Cerrado area, in June 2001, Araraquara, Sao Paulo state, Brazil. A voucher specimen number (SILVA N<sup>o</sup>-193) has been deposited in the herbarium of the Botanic Garden of São Paulo, Brazil.

**Isolation of the Endophytic Fungus.** The fungal strains were isolated from healthy leaves of *S. spectabilis* following a published protocol.<sup>17</sup> Six strains were obtained. Strains were coded as CL-01 to CL-06 and preserved in sterile water. The fungal strain CL-06 was identified as *Phaeoacremonium* sp. by Prof. Dr. Ludwig H. Pfening and Camila P. Nicolli, Federal University of Lavras, based on morphological markers and ITS sequences. The sequence reported has been deposited in the GenBank database (accession no. KY486755). The fungal strain was also deposited at Coleção Micológica de Lavras under the number CML 139, at the Phytopathology Department, Federal University of Lavras, Brazil.

**Growth of *Phaeoacremonium* sp. and Preparation of the EtOAc Extract.** The strain CL-06, identified as *Phaeoacremonium* sp., was inoculated in a Petri dish containing potato dextrose agar and incubated for 7 days. After this period, preinoculi were incubated in potato dextrose broth (5.6 L, total volume) and incubated at 160 rpm, under controlled temperature (25 °C) for 28 days. After growth, the broth was separated from the mycelium by filtration and extracted with EtOAc three times (50% of the broth volume each). The organic layer was evaporated to dryness, to give 866.0 mg of crude extract.

**Antifungal Assay.** The assay for antifungal potential (bioautography) of the crude AcOEt extract and compounds 1–4 was carried out with the phytopathogenic fungi *C. sphaerospermum* (Perzig) SPC 491 and *C. cladosporioides* (Fresen) of Vries SPC 140, using a previously described method.<sup>16</sup> Both strains are maintained at the Institute of Botany, São Paulo, Brazil. The assays were performed at concentrations of 5, 10, 20, 50, and 100  $\mu\text{g L}^{-1}$ , using nystatin as positive control.

**Cytotoxicity Bioassay.** The human cervical cancer cell line assay was performed as previously described.<sup>18</sup> Camptothecin was used as positive control.

**Extraction and Isolation.** The EtOAc extract (866.0 mg) was fractionated by CC using reversed-phase silica gel and a gradient of MeOH in H<sub>2</sub>O as eluent, to give six fractions of 200 mL each: MeOH 15% (FrCL-06-1, 108.0 mg), MeOH 25% (FrCL-06-2, 83.0 mg), MeOH 35% (FrCL-06-3, 19.4 mg), MeOH 50% (FrCL-06-4, 26.0 mg), MeOH 100% (FrCL-06-5, 553.0 mg), and CH<sub>2</sub>Cl<sub>2</sub> (FrCL-06-6, 63.0 mg). Fraction FrCL-06-5 (553.0 mg) was further purified using reversed-phase preparative HPLC (H<sub>2</sub>O/MeCN (65:35), 15.0 mL min<sup>-1</sup>), yielding the isoaiqualones 1 ( $t_R$  = 43 min; 30.0 mg), 2 ( $t_R$  = 52 min; 20.0 mg), and 3 ( $t_R$  = 65 min; 12.0 mg) and aigialone 4 ( $t_R$  = 69 min; 180.0 mg).

**Isoaiqualone A (1):** white, amorphous powder;  $[\alpha]_D^{25}$  -36.0 (c 0.38, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 235 (3.65) nm; IR (KBr)  $\nu_{\text{max}}$  3336, 1736, and 1677 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR see Table 1; HRESIMS  $m/z$  315.1788 [M + H]<sup>+</sup> (calcd for C<sub>17</sub>H<sub>26</sub>O<sub>6</sub>, 315.1808).

**Isoaiqualone B (2):** white, amorphous powder;  $[\alpha]_D^{25}$  -141.5 (c 0.27, CHCl<sub>3</sub>); IR (KBr)  $\nu_{\text{max}}$  3336, 1736, and 1677 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR see Table 1; HRESIMS  $m/z$  299.1853 [M + H]<sup>+</sup> (calcd for C<sub>16</sub>H<sub>25</sub>O<sub>5</sub>, 299.1858).

**Isoaiqualone C (3):** white, amorphous powder;  $[\alpha]_D^{25}$  +39.1 (c 0.14, CHCl<sub>3</sub>); IR (KBr)  $\nu_{\text{max}}$  3336, 1736, and 1677 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR see Table 1; HRESIMS  $m/z$  321.1677 [M + Na]<sup>+</sup> (calcd for C<sub>16</sub>H<sub>26</sub>O<sub>5</sub>Na, 321.1678).

**X-ray Structure Determination of Isoaiqualone 1.** The X-ray crystallographic data were measured on an Enraf-Nonius KAPPA CCD diffractometer (95 mm CCD camera on a  $\kappa$ -goniostat) with a CCD area detector at 293(2) K using graphite-monochromated Mo K $\alpha$  radiation ( $\lambda$  = 0.710 73 Å). The structure was solved by direct methods and refined by full-matrix least-squares on  $F^2$  using the program SHELXS-97.<sup>19</sup> The crystal data are summarized as follows: empirical formula C<sub>16</sub>H<sub>24</sub>O<sub>6</sub>; formula weight 312.35 amu; monoclinic, space group P2<sub>1</sub>, Z = 2, a = 5.433(1) Å, b = 8.456(1) Å, c = 18.775(3) Å,  $\beta$  = 94.864(7)°, V = 859.4(2) Å<sup>3</sup>; D<sub>calcd</sub> = 1.207 mg/m<sup>3</sup>; F(000) = 336,  $\mu$  = 0.092 mm<sup>-1</sup>; 6387 collected reflections (3.25° ≤  $\theta$  ≤ 27.36°), -6 ≤ h ≤ 6, -10 ≤ k ≤ 9, -21 ≤ l ≤ 23; 2830 independent reflections (R<sub>int</sub> = 0.0739); goodness-of-fit on  $F^2$  is 1.099, final R indices for I > 2 $\sigma$ (I), R<sub>1</sub> = 0.0739, wR<sub>2</sub> = 0.1698, R indices for all data R<sub>1</sub> = 0.1345, wR<sub>2</sub> = 0.2022; refining 224 parameters and no restraints; the largest difference peak and hole was 0.262 and -0.267 e Å<sup>-3</sup>; completeness to  $\theta$  (27.36°). Crystallographic data are deposited with number 763495 at the Cambridge Crystallographic Data Centre, Cambridge, UK.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00828.

Selected <sup>1</sup>H and <sup>13</sup>C NMR, <sup>1</sup>H–<sup>1</sup>H COSY, HSQC, HMBC, and HRMS spectra of compounds 1–3 (PDF)

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### Notes

The authors declare no competing financial interest.

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