

## Notes

Metabolites of the “Smoke Tree”, *Dalea spinosa*, Potentiate Antibiotic Activity against Multidrug-Resistant *Staphylococcus aureus*Gil Belofsky,<sup>\*,†</sup> Roberto Carreno,<sup>†</sup> Kim Lewis,<sup>‡</sup> Anthony Ball,<sup>‡</sup> Gabriele Casadei,<sup>§,§</sup> and George P. Tegos<sup>⊥</sup>

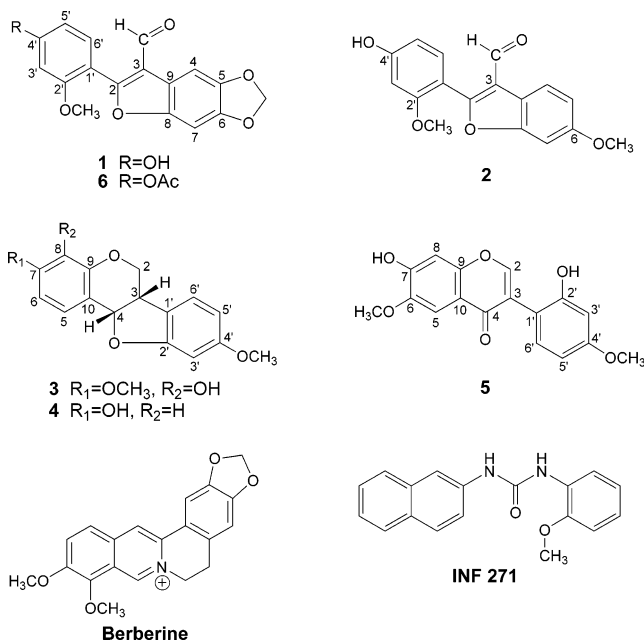
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Two new 2-arylbenzofuran aldehydes (**1** and **2**) and three known phenolic compounds (**3**–**5**) were isolated from organic extracts of *Dalea spinosa*. These compounds were evaluated for their intrinsic antimicrobial activity and their ability to perform as multidrug-resistance inhibitors by potentiating the activity of known antimicrobials against a variety of pathogenic microorganisms. Compound **1** and its acetate derivative **6** exhibited no direct antimicrobial activity but enhanced the effect of the weak plant antimicrobial berberine when tested against *Staphylococcus aureus*. Additional potentiation assays with *S. aureus* overexpression and knockout isogenic efflux mutants for the NorA pump were done in order to assess whether the potentiating effects were associated with inhibition of this known pump mechanism.

As one travels west through the south-central plains of the United States to Southern California, *Dalea* spp. generally change in morphology from herbs to woody shrubs to the largest member of the genus that grows up to eight meters in height, the “smoke tree”, *Dalea spinosa* A. Gray (syn: *Psorothamnus spinosus*) (Fabaceae). A methanolic extract of the lower bark of *D. spinosa* was subjected to successive fractionation by silica gel and Sephadex LH-20 chromatography. The resulting fractions were examined by <sup>1</sup>H NMR spectroscopy and TLC, to identify compounds with promising chemical functionalities and good chromatographic resolution. An aldehyde functionality, later observed to be visible in the HSQC NMR of the crude extract itself, was present in several fractions, which led us to focus on these materials, resulting in the isolation of spinosans A (**1**) and B (**2**). Spinosan A is a potent new potentiator of antibiotic activity against multidrug-resistant (MDR) *Staphylococcus aureus*. Further chemically guided fractionation led to the isolation of two known pterocarpanes (**3** and **4**) and a known isoflavone (**5**).

The HRESIMS, <sup>13</sup>C NMR, and HSQC data for compound **1** indicated a molecular formula of C<sub>17</sub>H<sub>12</sub>O<sub>6</sub>. Distinctive signals were present in the <sup>1</sup>H and <sup>13</sup>C NMR spectra (Table 1) for an aldehyde group, a methylenedioxy functionality, and a methoxy group. Also present were five aromatic proton signals, three of which exhibited *ortho* and *meta* coupling (8.4 and 2.0 Hz, respectively), indicating their placement on one trisubstituted benzene ring. Assignment of the overall substitution pattern of this ring was accomplished in a straightforward manner from HSQC and HMBC correlations. The benzofuran ring system, with the methylenedioxy group placed at C-5,6, was established with the aid of key HMBC correlations from H-4 to C-3, C-5, and C-6 and from H-7 to C-5, C-6, C-8, and C-9, as well as by comparison of the <sup>1</sup>H and <sup>13</sup>C NMR spectra with known compounds.<sup>1–3</sup> Other key HMBC correlations, between H-6' and C-2 and from the formyl proton to C-2 and C-3, established



the connection of the trisubstituted benzene ring to C-2. The location of the methoxy group at C-2', rather than at C-4', was supported by the observation of only one NOESY correlation between the methoxy protons and H-3'. To further confirm the structure of **1**, the acetate derivative **6** was prepared. Complete NMR spectroscopic data for **6** were acquired (see Experimental Section) that agreed in all respects with the overall structure assignments for **1**. As observed for **1**, the methoxy group exhibited a NOESY correlation to H-3'. C-4' was shielded by 8.6 ppm upon acetylation of the attached oxygen and correlated to H-5' by HMBC. These data verified the relative positions of the hydroxy and methoxy groups in spinosan A (**1**).

The HRESIMS of **2** revealed a molecular formula of C<sub>17</sub>H<sub>14</sub>O<sub>5</sub>, requiring one less degree of unsaturation than **1**. The formyl group was present as in **1**, but the methylenedioxy group was replaced

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**Table 1.** NMR Data for Compounds **1** and **2** in Acetone-*d*<sub>6</sub>

| position                | <b>1</b>           |                                 | <b>2</b>     |                                 |
|-------------------------|--------------------|---------------------------------|--------------|---------------------------------|
|                         | $\delta_C^a$       | $\delta_H^b$ (mult.; $J_{HH}$ ) | $\delta_C^a$ | $\delta_H^b$ (mult.; $J_{HH}$ ) |
| 2                       | 163.6              |                                 | 163.6        |                                 |
| 3                       | 118.4              |                                 | 118.0        |                                 |
| 3-CHO                   | 187.8              | 9.98 (s)                        | 187.8        | 10.02 (s)                       |
| 4                       | 101.0              | 7.51 (s)                        | 123.0        | 8.01 (d; 8.6)                   |
| 5                       | <sup>c</sup> 147.8 |                                 | 114.0        | 7.00 (dd; 8.6, 2.2)             |
| 6                       | <sup>c</sup> 146.8 |                                 | 159.8        |                                 |
| 5,6-OCH <sub>2</sub> O- | 102.8              | 6.07 (s)                        |              |                                 |
| 6-OCH <sub>3</sub>      |                    |                                 | 56.2         | 3.88 (s)                        |
| 7                       | 94.2               | 7.14 (s)                        | 96.6         | 7.18 (d; 2.2)                   |
| 8                       | 150.4              |                                 | 156.3        |                                 |
| 9                       | 119.6              |                                 | 119.3        |                                 |
| 1'                      | 110.0              |                                 | 109.9        |                                 |
| 2'                      | 160.0              |                                 | 160.1        |                                 |
| 2'-OCH <sub>3</sub>     | 56.1               | 3.84 (s)                        | 56.2         | 3.86 (s)                        |
| 3'                      | 100.6              | 6.70 (d; 2.0)                   | 100.6        | 6.71 (d; 2.1)                   |
| 4'                      | 162.5              |                                 | 162.6        |                                 |
| 5'                      | 108.8              | 6.65 (dd; 8.4, 2.0)             | 108.9        | 6.66 (dd; 8.3, 2.1)             |
| 6'                      | 133.6              | 7.48 (d; 8.4)                   | 133.7        | 7.52 (d; 8.3)                   |

<sup>a</sup> 75 MHz. <sup>b</sup> 400 MHz. <sup>c</sup> Assignments may be interchanged.

by an *ortho*-coupled aromatic proton and a methoxy group. These data, along with HSQC and HMBC NMR correlations, and comparison with **1**, **6**, and known compounds<sup>1–3</sup> led us to elucidate the structure of spinosan B (**2**) shown. NOESY correlations from H<sub>3</sub>CO-2' to both H-3' and the formyl proton confirmed the relative positions of the hydroxy and methoxy groups in this portion of the molecule. The position of the methoxy at C-6 was further confirmed by NOESY correlations to both H-5 and H-7. To our knowledge, the basic structures of spinosans A and B are closely related to only two previously reported compounds, melimessanol C<sup>1</sup> and maginaldehyde.<sup>3</sup>

The structures of known compounds **3** and **4** were determined by NMR spectroscopic and mass spectrometric methods, and these data corresponded in all essential respects with those reported.<sup>4–11</sup> Complete NMR and other spectroscopic data for **3** are reported here for the first time. Compound **4** was also compared to an authentic sample, previously isolated in-house.<sup>8</sup>

Interpretation of NMR spectroscopic data and comparison of these data to known compounds<sup>12–14</sup> led us to elucidate the structure of compound **5** as 6,4'-dimethoxy-7,2'-dihydroxyisoflavone.<sup>15,16</sup> Key HMBC correlations that established regiochemistry were those from H-2 to C-1', from H-6' to C-3, and from H-5 to C-4. The relative positions of the two methoxy groups were established by HMBC correlations to their respective points of attachment, to which mutual correlations from aromatic ring protons were observed. The placement of the methoxy at C-6 was further supported by a NOESY correlation from this group to H-5. Extensive spectroscopic data for **5** (see Experimental Section) have not been previously reported.

Transmembrane efflux pump mechanisms are major components of resistance to many classes of antibiotics. Multidrug-resistance pumps (MDRs) expel a variety of structurally diverse compounds.<sup>17</sup> Various chemotypes have been shown to inhibit MDRs in microorganisms,<sup>18</sup> including compounds reported from another *Dalea* sp.<sup>8</sup> This effect enhances the utility of both conventional antibiotics and weak plant antimicrobials. Compounds **1–6** were evaluated in antimicrobial assays in order to determine both direct activity and MDR inhibitory activity against a panel of pathogenic microorganisms, including Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), and yeast (*Saccharomyces cerevisiae*, *Candida albicans*). Compounds **1–6** showed no direct antimicrobial activity (MIC > 140  $\mu$ M, data not shown) against these organisms. MDR inhibitory activities of **1–6** were evaluated by combining each of these compounds with a subinhibitory concentration of the relatively weak antimicrobial berberine. Compounds that inhibited

**Table 2.** Berberine Potentiation Assay: MICs ( $\mu$ M) of Berberine<sup>a</sup> against *S. aureus* Wild-Type (WT), NorA Knockout (NorA<sup>−</sup>), and Overexpression Mutant (NorA<sup>+</sup>), Alone and in the Presence of **1**, **4**, **5**, **6**, or INF 271 (at molar concentrations in the range 42–56  $\mu$ M)

|                                       | berberine (alone) | +1 | +4 | +5 | +6 | +INF 271 |
|---------------------------------------|-------------------|----|----|----|----|----------|
| <i>S. aureus</i> (WT)                 | 372               | 45 | 89 | 89 | 6  | 6        |
| <i>S. aureus</i> (NorA <sup>−</sup> ) | 89                | 6  | 21 | 45 | 45 | 3        |
| <i>S. aureus</i> (NorA <sup>+</sup> ) | >1488             | NP | NP | NP | 45 | 6        |

<sup>a</sup> Berberine test range in the potentiation study 1.5–89  $\mu$ M. NP = no potentiation at 89  $\mu$ M berberine.

cell growth under these conditions and having no activity when used alone were considered to exhibit MDR inhibitory activity. In preliminary assays, compounds **1**, **4**, **5**, and **6** showed potentiation activity against *S. aureus* when used in combination with berberine, while **2** and **3** did not. Accordingly, the active compounds were further analyzed in a more detailed potentiation assay, and the results are summarized in Table 2.

To test whether the MDR inhibitory effects of the active compounds are exclusively related to the NorA pump of *S. aureus*, the isogenic pair of knockout and overexpression NorA mutants of *S. aureus*, containing none versus elevated levels of NorA, respectively, were used.<sup>19–21</sup> INF 271, a synthetic efflux pump inhibitor, was used as a positive control.<sup>22</sup>

Berberine exhibited MICs of 372 and 89  $\mu$ M against the wild-type *S. aureus* and the isogenic NorA mutant, respectively. As expected, the NorA-overexpressing *S. aureus* mutant was considerably less susceptible to berberine (MIC > 1488  $\mu$ M). Spinosan A (**1**, tested at 48  $\mu$ M) and its acetate (**6**, 42  $\mu$ M) decreased the MIC of berberine approximately 8-fold and 62-fold, respectively, against wild-type *S. aureus*. Compounds **4** and **5** (at 56 and 48  $\mu$ M, respectively) potentiated the activity of berberine to a lesser degree, each causing a 4-fold decrease in MIC. Compounds **1** (48  $\mu$ M) and **4** (56  $\mu$ M) potentiated berberine against the *S. aureus* knockout efflux mutant, causing 15- and 4-fold decreases in MIC, respectively, whereas the effects of **5** and **6** were less. Below ~40  $\mu$ M concentrations the inhibitory activity of all of the *D. spinosa* compounds decreased steadily. Moderate potentiation (berberine MIC = 89  $\mu$ M) was still observed, however, for **1** and **6** at concentrations of 6.3 and 5.6  $\mu$ M, respectively.

Compound **6** enhanced the antimicrobial effect of berberine against the NorA overexpression *S. aureus* mutant, while also causing a greater fold decrease in MIC in the wild-type strain compared to the knockout mutant, suggesting NorA-associated activity. Although the MICs of berberine against the *S. aureus* knockout efflux mutant changed when combined with **1**, **4**, and **5**, the magnitude of these changes relative to berberine alone were not sufficient to conclude NorA-associated activity. Additional efflux systems are present in *S. aureus* that have not yet been extensively studied or characterized,<sup>23</sup> and the inhibitory activities of these *D. spinosa* metabolites may be associated with more than one efflux system or with an unknown mechanism. None of the compounds showed potentiation against the other microorganisms tested in this study.

## Experimental Section

**General Experimental Procedures.** NMR spectra were acquired on a Varian UNITY INOVA 400 spectrometer, equipped with an inverse detection probe, and on a Varian Unity Plus 300 spectrometer. The NOESY NMR spectra of vanillin and *m*-anisaldehyde were obtained as controls for correlations between *ortho* aromatic substituents. Optical rotations were obtained on a JASCO model P-1010 polarimeter. IR spectra were recorded on a Nicolet Avatar 360 FT-IR spectrophotometer, and UV spectra were acquired on a Hewlett-Packard 8453 diode array spectrophotometer. EIMS were obtained on a Shimadzu GCMS-QP5000 equipped with a DI-50 direct sample inlet device. ESIMS/MS and HRESIMS were obtained in positive ion mode on a Micromass Q-TOF mass spectrometer.

**Plant Material.** Twigs, spines, stems, flower buds, and pieces of lower bark of *Dalea spinosa* A. Gray (Fabaceae) "smoke tree" were collected by one of the authors (Gil Belofsky) and Mr. Kavon Azadi on May 31, 2002, in Coachwhip Canyon, Anza-Borrego Desert, GPS position: N 33°17.217', W 116°09.064'. A voucher specimen (#11563) was authenticated by Dr. Paul Buck, Professor Emeritus, Department of Biological Science, The University of Tulsa, and deposited in the Barclay Herbarium at the same location. Plants were stored in a -20 °C freezer prior to extraction.

**Microbial Strains, Chemicals, and Susceptibility Testing.** *S. aureus* strains (the wild-type 8325-4, isogenic NorA knockout/K1758, and overexpression/K2361 mutants), *E. coli* (wild-type strain K12), and *P. aeruginosa* (PA14) were cultured in Mueller-Hinton (MH) broth. *E. faecalis* (V583) was cultured in brain heart infusion (BHI). *S. cerevisiae* (BY4742) and *C. albicans* (F5) cells were grown in yeast extract peptone and dextrose (YPD). INF 271 was kindly provided by Protez Pharmaceuticals, Malvern, PA. Berberine was purchased from Sigma Chemical Co. (St. Louis, MO). Cells ( $10^5$ /mL) were inoculated into MH, BHI, or YPD and dispensed at 0.2 mL/well in 96-well microtiter plates.

Growth inhibition was determined by serial 2-fold dilution of test compounds, starting at 50  $\mu$ g/mL (42–50  $\mu$ M), combined with 30  $\mu$ g/mL (89  $\mu$ M) of berberine against Gram-positive bacteria, 10  $\mu$ g/mL (13.6  $\mu$ M) erythromycin for Gram-negative bacteria, and 1  $\mu$ g/mL (3.3  $\mu$ M) fluconazole for yeast. An MDR inhibitor was defined as a compound that completely prevented cell growth in the presence of subinhibitory concentrations of an antibiotic during an 18-h incubation at 37 °C for *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* and a 24-h incubation at 30 °C for *S. cerevisiae* and *C. albicans*. All tests were done in triplicate by following National Center for Clinical Laboratory Standards (NCCLS) recommendations.<sup>24</sup> Growth was assayed with a microtiter plate reader (Spectramax PLUS384, Molecular Devices) by absorption at 600 nm.

**Berberine Potentiation Assay.** A checkerboard assay was conducted to specify the degree of potentiation of berberine by **1**, **4**, **5**, and **6** and to determine the specificity of these compounds for the NorA efflux pump. Serial 2-fold dilutions of berberine and a test compound were mixed in each well of a 96-well microtiter plate so that each row (and column) contained a fixed amount of one agent and increasing amounts of the second agent. The resulting plate presents a pattern in which every well contains a unique combination of concentrations between the two molecules. The concentrations of berberine (row) ranged from 30 to 0.5  $\mu$ g/mL (89–1.5  $\mu$ M), while plant compound (column) concentrations ranged from 15 to 0.015  $\mu$ g/mL (corresponding to molar concentrations in the ranges 56–42  $\mu$ M to 0.06–0.04  $\mu$ M). Each plate also contained a row and column in which a serial dilution of each agent was present alone. The >89  $\mu$ M (>30  $\mu$ g/mL) MIC values for berberine alone, against *S. aureus* wild-type and NorA overexpression strains (Table 2), were determined in a separate assay. Cells were added to each well at a final concentration of  $5 \times 10^6$  CFU/mL, and plates incubated at 37 °C for 24 h. Growth was assayed by absorption at 600 nm with a microtiter plate reader (Spectramax PLUS384, Molecular Devices). An OD less than 0.04 was considered to reveal no bacterial growth.

**Extraction and Isolation.** Finely divided *D. spinosa* bark (220 g) was extracted with MeOH (1.5 L, 24 h) to provide, after evaporation, 4.1 g of crude extract. This material was preadsorbed in a CH<sub>2</sub>Cl<sub>2</sub>–MeOH solution onto ~10 g of silica gel, the solvent removed under vacuum, and the resulting powder subjected to vacuum liquid chromatography (VLC) over a prepacked column bed, 10 cm (i.d.)  $\times$  3.5 cm (h), of TLC-grade silica gel (Selecto Scientific). The column was eluted using a stepwise gradient of solvents (500 mL each), beginning with hexane and continuing with mixtures of EtOAc in hexane (20%, 40%, 60%, 80%, 100%), followed by mixtures of MeOH in CH<sub>2</sub>Cl<sub>2</sub>, up to 30%. The five fractions that eluted with 20–100% EtOAc were combined on the basis of TLC analysis (EM Science, silica gel 60, F<sub>254</sub>, with vanillin/concentrated H<sub>2</sub>SO<sub>4</sub> spray reagent, 1% w/v), and the solvents were evaporated. The residue (1.5 g) was further fractionated by Sephadex LH-20 (Sigma) column chromatography (2.5 cm  $\times$  58 cm) eluting with 1 L of 3:1:1 hexane–toluene–MeOH at a flow rate of 0.5 mL/min, collecting ~5 mL fractions. Fractions of similar composition as determined by TLC were pooled, resulting in 15 fractions. Fraction 11 (81 mg) from this column was further purified over silica gel (2.5 cm  $\times$  9 cm, Davison Chemical 100–200 mesh) by gravity elution using a step-gradient of 100% CH<sub>2</sub>Cl<sub>2</sub>, followed by

MeOH in CH<sub>2</sub>Cl<sub>2</sub> (0.5%, 1%, 1.5%, 2%, 3%, 5%, 8%, 10%, 30%; 200 mL fractions). Fraction 3 (35 mg) from this column was then purified using a second silica gel gravity elution column (2.5 cm  $\times$  7.5 cm) with a step gradient of 100% hexane, followed by EtOAc in hexane (10%, 15%, 20%, 24%, 28%, 32%, 36%, 40%, 50%, 75%, 100%; 100 mL fractions). Fractions 6 and 7 from this column were combined and evaporated (20 mg), and MeOH was added to precipitate pure spinosan A (**1**, 12 mg). An additional 5 mg of **1** was later isolated from other fractions using similar procedures.

Fraction 9 (86 mg) from the Sephadex column was further purified using column chromatographic procedures nearly identical to that described above, resulting in a 23 mg fraction that, following addition of MeOH, precipitated pure spinosan B (**2**, 13 mg).

Fractions 8 (62 mg) and 10 (56 mg) from the Sephadex column were purified over silica gel using a sequence similar to that described above (step gradients of MeOH in CH<sub>2</sub>Cl<sub>2</sub>, followed by EtOAc in hexane), resulting in, respectively, compound **3** (9 mg) and, with one additional EtOAc–hexane silica gel column procedure, compound **4** (8 mg).

Fraction 12 (86 mg) from the Sephadex column was subjected to three successive silica gel chromatography procedures (step gradients in MeOH–CH<sub>2</sub>Cl<sub>2</sub>, EtOAc–hexane, and MeOH–CH<sub>2</sub>Cl<sub>2</sub>) to provide a 22 mg fraction of interest (primarily a purple spot with vanillin/H<sub>2</sub>SO<sub>4</sub> spray reagent on TLC,  $R_f = 0.6$  in 9:1 CH<sub>2</sub>Cl<sub>2</sub>–MeOH). This fraction was purified over silica gel (2.5 cm  $\times$  7 cm; Davison 100–200 mesh) using a linear gradient of MeOH (0 to 2%) in CH<sub>2</sub>Cl<sub>2</sub>, at a flow rate of ~20 mL/min, to afford compound **5** (6 mg).

**Spinosan A (1):** pale yellow solid; UV(MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 211 (4.32), 248 (4.03), 296 (3.72), 347 (3.88) nm; IR  $\nu_{\max}$  (CHCl<sub>3</sub>) 3300 (br OH), 3019, 1664, 1616, 1458, 1310, 1125, 1034 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; HMBC correlations (acetone-*d*<sub>6</sub>) CHO  $\rightarrow$  C-2, 3, 9; H-4  $\rightarrow$  C-3, 5, 6, 7\*, 8; OCH<sub>2</sub>O  $\rightarrow$  C-5, 6; H-7  $\rightarrow$  C-4\*, 5, 6, 8, 9; H<sub>3</sub>CO  $\rightarrow$  C-2'; H-3'  $\rightarrow$  C-1', 2', 4', 5'; H-5'  $\rightarrow$  C-1', 2', 3', 4', 6'; H-6'  $\rightarrow$  C-2, 2', 3', 4' (\*indicates weak four-bond correlation); HRESIMS found  $m/z$  313.0670 (M + H)<sup>+</sup>, calcd for C<sub>17</sub>H<sub>13</sub>O<sub>6</sub> 313.0712.

**Spinosan B (2):** pale yellow solid; UV(MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 207 (4.42), 244 (4.28), 279 (3.80), 343 (3.98) nm; IR  $\nu_{\max}$  (film on NaCl) 3323 (br OH), 2905, 1654, 1618, 1589, 1498, 1315, 1266, 1202, 1143, 1062 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; HMBC correlations (acetone-*d*<sub>6</sub>) CHO  $\rightarrow$  C-2, 3; H-4  $\rightarrow$  C-3, 5, 6, 7\*, 8; H-5  $\rightarrow$  C-6, 7, 9; H<sub>3</sub>CO-6  $\rightarrow$  C-6; H-7  $\rightarrow$  C-5, 6, 8, 9; H<sub>3</sub>CO-2'  $\rightarrow$  C-2'; H-3'  $\rightarrow$  C-1', 2', 4', 5'; H-5'  $\rightarrow$  C-1', 2', 3', 4'; H-6'  $\rightarrow$  C-2', 3', 4' (\*indicates weak four-bond correlation); HRESIMS found  $m/z$  299.0894 (M + H)<sup>+</sup>, calcd for C<sub>17</sub>H<sub>15</sub>O<sub>5</sub> 299.0919.

(+)-**Melilotocarpin A (3):** yellow oil; [ $\alpha$ ]<sub>D</sub> +21.0 (*c* 0.20, CHCl<sub>3</sub>); UV(MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 208 (4.67), 228sh (4.11), 283 (3.75) nm; IR  $\nu_{\max}$  (CHCl<sub>3</sub>) 3528 (br OH), 3020, 2938, 1623, 1605, 1497, 1477, 1278, 1147, 1090 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  7.19 (1H, d,  $J = 8.4$ , H-5), 7.15 (1H, d,  $J = 8.8$ , H-6'), 6.71 (1H, d,  $J = 8.4$ , H-6), 6.47 (1H, d,  $J = 2.2$ , H-3'), 6.47 (1H, dd,  $J = 8.8$ , 2.2, H-5'), 5.51 (1H, d,  $J = 6.5$ , H-4), 4.36 (1H, dd,  $J = 10.5$ , 4.8, H-2 $\beta$ ), 3.92 (3H, s, H<sub>3</sub>CO-7), 3.78 (3H, s, H<sub>3</sub>CO-4'), 3.64 (1H, d,  $J = 10.5$ , H-2 $\alpha$ ), 3.57 (1H, dd,  $J = 6.5$ , 4.8, H-3); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  161.4 (C-4'), 160.9 (C-2'), 149.9 (C-8), 148.7 (C-9), 135.1 (C-7), 126.3 (C-5), 125.0 (C-6'), 119.1 (C-1'), 113.4 (C-10), 109.2 (C-6), 106.7 (C-3'), 97.1 (C-5'), 78.7 (C-4), 66.9 (C-2), 61.4 (OCH<sub>3</sub>-7), 55.7 (OCH<sub>3</sub>-4'), 39.6 (C-3); HMBC correlations (CDCl<sub>3</sub>) H<sub>2</sub>-2  $\rightarrow$  C-3, 4, 9, 1'; H-3  $\rightarrow$  C-2, 1', 2'; H-4  $\rightarrow$  C-2, 3, 5, 9, 10; H-5  $\rightarrow$  C-4, 7, 9; H-6  $\rightarrow$  C-7, 8, 10; H<sub>3</sub>CO-7  $\rightarrow$  C-7; H-3'  $\rightarrow$  C-1', 2', 5'; H<sub>3</sub>CO-4'  $\rightarrow$  C-4'; H-5'  $\rightarrow$  C-1', 3'; H-6'  $\rightarrow$  C-3, 2', 4', 5'; HRESIMS found  $m/z$  301.1053 (M + H)<sup>+</sup>, calcd for C<sub>17</sub>H<sub>17</sub>O<sub>5</sub> 301.1076.

(+)-**Medicarpin (4):** yellow oil; [ $\alpha$ ]<sub>D</sub> +16.7 (*c* 0.20, CHCl<sub>3</sub>); UV, <sup>1</sup>H NMR, and <sup>13</sup>C NMR spectra were consistent with an authentic sample;<sup>8</sup> the structure of **4** was also confirmed by H<sup>13</sup>C and HMBC NMR spectroscopy; EIMS  $m/z$  271 (M + H)<sup>+</sup>, rel int 100, 256 (36), 242 (47), 213 (7), 185 (5), 175 (8), 162 (33), 148 (29), 135 (24).

**6,4'-Dimethoxy-7,2'-dihydroxyisoflavone (5):** pale yellow solid; mp 198–200 °C (lit. 195–198 °C);<sup>16</sup> UV(MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 204 (4.46), 216 (4.38), 256 (4.15), 288 (3.97), 320 (3.92), 359 (3.46) nm; IR  $\nu_{\max}$  (film on NaCl) 3393 (br OH), 1616, 1576, 1506, 1480, 1437, 1285, 1201, 1160 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  8.08 (1H, s, H-2), 7.69 (1H, s, H-5), 7.09 (1H, d,  $J = 8.6$ , H-6'), 7.07 (1H, s, H-8), 6.67 (1H, d,  $J = 2.6$ , H-3'), 6.56 (1H, dd,  $J = 8.6$ , 2.6, H-5'), 4.06 (3H, s, OCH<sub>3</sub>-6), 3.84 (3H, s, OCH<sub>3</sub>-4'); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  178.6 (C=O), 162.2 (C-4'), 158.2 (C-2'), 154.7 (C-2), 152.9 (C-9), 152.5 (C-7), 146.2 (C-6), 130.5 (C-6'), 124.4 (C-3), 116.9 (C-10), 113.3 (C-1'), 107.9 (C-5'),

104.64 (C-5), 104.55 (C-3'), 102.6 (C-8), 56.9 (OCH<sub>3</sub>-6), 55.6 (OCH<sub>3</sub>-4'); HMBC correlations (CDCl<sub>3</sub>) H-2 → C-3, 4, 9, 1'; H-5 → C-4, 6, 7, 8\*, 9, 10; H<sub>3</sub>CO-6 → C-6; H-8 → C-4\*, 6, 7, 9, 10; H-3' → C-1', 2', 4', 5'; H<sub>3</sub>CO-4' → C-4'; H-5' → C-1', 3', 4'; H-6' → C-3, 2', 3', 4'; \*indicates weak four-bond correlation; EIMS *m/z* 314 (M<sup>+</sup>, rel int 40), 297 (11), 282 (2), 271 (2), 175 (3), 166 (13), 157 (9), 148 (100), 133 (16).

**Spinosan A Acetate (6).** To 9 mg of **1** were added 2 mL of triethylamine and 1 mL of Ac<sub>2</sub>O. The reaction mixture was stirred at RT for 1.5 h and evaporated under dry N<sub>2</sub>. To the residue was added 3 mL of H<sub>2</sub>O, which was then extracted twice in succession with 3 mL of EtOAc. The combined EtOAc extracts were evaporated under N<sub>2</sub> to afford 9 mg of the acetate, **6**: pale yellow solid; mp 175–179 °C; UV(MeOH) λ<sub>max</sub> (log ε) 209 (4.44), 244 (4.11), 289 (3.86), 341 (3.96) nm; IR ν<sub>max</sub> (CHCl<sub>3</sub>) 3018, 1766, 1670, 1614, 1591, 1500, 1462, 1277, 1203, 1190, 1156, 1134 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 10.1 (1H, s, CHO), 7.65 (1H, s, H-4), 7.61 (1H, d, *J* = 8.4, H-6'), 7.02 (1H, s, H-7), 6.89 (1H, dd; *J* = 8.4, 2.1, H-5'), 6.84 (1H, d, *J* = 2.1, H-3'), 6.04 (2H, s, OCH<sub>2</sub>O), 3.86 (3H, s, OCH<sub>3</sub>), 2.36 (3H, s, OCOCH<sub>3</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 187.7 (CHO), 169.2 (OCOCH<sub>3</sub>), 160.9 (C-2), 158.2 (C-2'), 153.9 (C-4'), 150.2 (C-8), 147.2 (C-5), 146.1 (C-6), 132.4 (C-6'), 118.9 (C-3), 118.8 (C-9), 115.8 (C-1'), 114.4 (C-5'), 106.0 (C-3'), 101.8 (OCH<sub>2</sub>O), 101.2 (C-4), 93.5 (C-7), 56.2 (OCH<sub>3</sub>), 21.4 (OCOCH<sub>3</sub>); HMBC correlations (CDCl<sub>3</sub>) CHO → C-2, H-4 → C-3, 6, 8; OCH<sub>2</sub>O → C-5, 6; H-7 → C-5, 8, 9; H<sub>3</sub>CO → C-2'; H-3' → C-1', 2', 4', 5'; H-5' → C-1', 3', 4'; H-6' → C-2, 2', 4'; EIMS *m/z* 354 (M<sup>+</sup>, rel int 41), 312 (74), 296 (12), 281 (49), 269 (10), 253 (3), 241 (9), 211 (4), 151 (6).

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