JOURNAL OF NATURAL PRODUCTS

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Chlorinated Polyketide Obtained from a *Daldinia* sp. Treated with the Epigenetic Modifier Suberoylanilide Hydroxamic Acid

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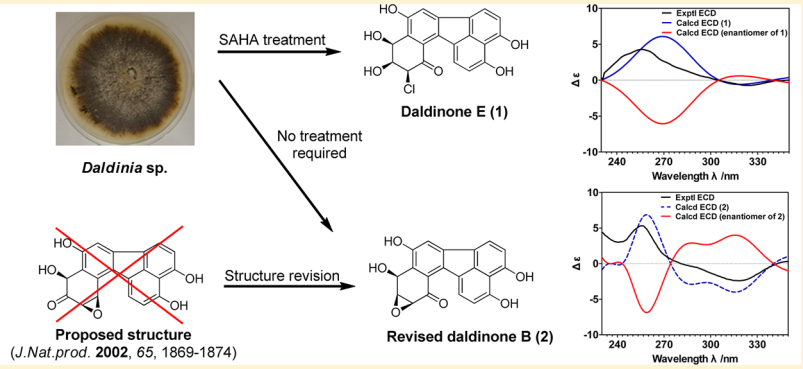
Supporting Information



ABSTRACT: A new chlorinated pentacyclic polyketide, daldinone E (**1**), was purified from a *Daldinia* sp. fungal isolate treated with the epigenetic modifier suberoylanilide hydroxamic acid (SAHA). A biosynthetically related epoxide-containing daldinone analogue, **2**, was also purified from the same fungus. The structures of both compounds were established by spectroscopic methods, and the absolute configurations were assigned by analysis of their NMR data (coupling constants and ROESY correlations) and DFT calculations of specific rotations and ECD spectra. During the course of these studies it was determined that metabolite **2** and the previously reported daldinone B shared the same spectroscopic data, leading to a revision of the reported structure. Both compounds **1** and **2** also exhibited DPPH radical scavenging activities with potency comparable to the positive control ascorbic acid.

Genome sequencing of filamentous fungi has revealed that many biosynthetic gene clusters involved in producing polyketide (PKS), nonribosomal peptide (NRPS), and hybrid PKS-NRPS (HPN) metabolites are transcriptionally suppressed under standard laboratory cultivation conditions.[1,2] Recently, epigenetic modifying agents, such as histone deacetylase (HDAC) and DNA methyltransferase inhibitors, have been introduced as important tools for expanding natural product discovery efforts via the transcriptional activation of silent biosynthetic genes.[3] The successful application of this method has been reported by our laboratory,[4−6] as well as other groups,[7−12] resulting in the generation of several structurally unique natural products.

Our group has recently implemented the use of a new solid-phase fermentation system that relies on culturing fungi on Cheerios breakfast cereal.[13,14] In light of this shift from liquid to solid-phase growth conditions, the question was asked whether chemical epigenetic modifiers could induce similar changes in the metabolomes of fungi prepared using this culture method. To test this, one hundred fungal isolates were grown on Cheerios supplemented with a 0.3% sucrose solution with and without the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA). The secondary metabolite profiles of the resulting EtOAc extracts were compared by LC-ESIMS. One of the test strains, a *Daldinia* sp., yielded an LC-ESIMS profile that contained a new peak bearing an isotope pattern characteristic for the presence of a single chlorine atom (Figure 1). Guided by the LC-ESIMS data, a new chlorinated aromatic polyketide, daldinone E (**1**), and the structurally related metabolite **2** were obtained. Whereas compound **2** appeared in both the SAHA-treated and control cultures, compound **1** was obtained exclusively from the SAHA-treated fungus.

RESULTS AND DISCUSSION

Compound **1** was obtained as a yellow powder. It was assigned the molecular formula $C_{20}H_{13}ClO_6$ on the basis of its HRESIMS data (*m*/*z* 383.0330, $[M - H]^-$), which was indicative of 14 degrees of unsaturation. The ^1H NMR spectrum of **1** (Table 1) showed signals for two exchangeable protons (δ_H 5.60 and 5.91), five aromatic protons (δ_H 6.90, 6.92, 7.70, 7.96, and 8.78), and three protons (δ_H 4.33, 5.14, and 5.70) attached to carbons bonded to heteroatoms. The ^{13}C

Received: June 26, 2014
Published: October 22, 2014

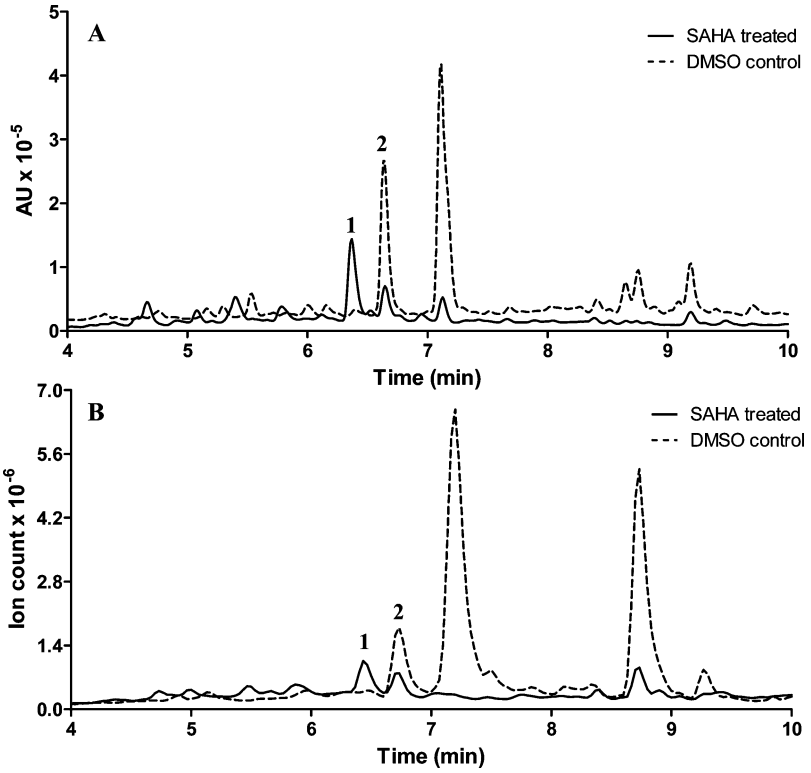
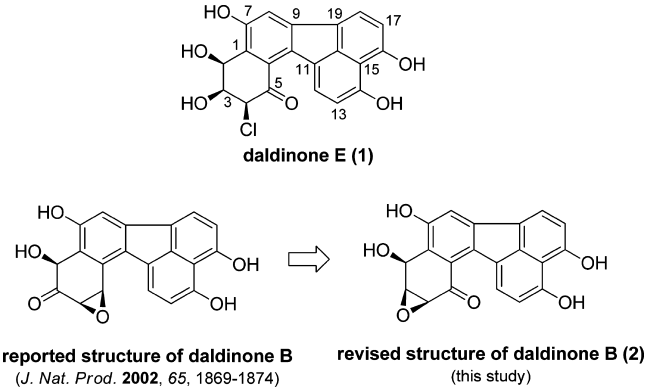


Figure 1. LC-ESIMS analysis of the EtOAc extracts for DMSO (dashed line) and SAHA (solid line) treated cultures of the *Daldinia* sp. (A) Extracted PDA chromatograms at 254 nm; (B) extracted ion chromatograms showing the MS range at m/z 300−400 in the negative ion mode.



NMR spectrum (Table 1) exhibited 20 carbon resonances that were later assigned as eight methines and 12 quaternary carbons. An α,β-unsaturated cyclohexanone moiety was established based on ^{1}H−^{1}H COSY (OH-2 to H-2, H-2 to H-3, H-3 to OH-3, and H-3 to H-4) and ^{1}H−^{13}C HMBC (H-2 to C-6, H-3 to C-1 and C-5, and H-4 to C-3 and C-5) correlations (Figure 2). The remaining portion of the molecule consisting of $C_{16}H_8O_3$ was rationalized to comprise a fluoranthene system, which was fused to the α,β-unsaturated cyclohexanone moiety at C-1 and C-6. This was confirmed on the basis of ^{1}H−^{1}H COSY cross-peaks (H-12 to H-13 and H-17 to H-18) and ^{1}H−^{13}C HMBC correlations from H-8 to C-1, C-7, C-10, and C-19; from H-12 to C-10, C-14, and C-20; from H-13 to C-11 and C-15, from H-17 to C-15 and C-19; and from H-18 to C-9, C-16, and C-20 (Figure 2). Three hydroxy groups were rationalized to be bonded to C-7, C-14, and C-16 based on the chemical shifts of these quaternary carbons (δ_C 154.8, 155.5, and 156.4, Table 1). Thus, the planar structure of **1** was established. The relative configuration of **1** was assigned by analysis of ^{1}H−^{1}H coupling constants (Table 1) and NOE signals (Figure 2). The small $J_{2,3}$ (3.8 Hz) and $J_{3,4}$ (2.1 Hz) coupling constant values supported a 2,3-*cis*/3,4-*cis* relative configuration based on comparisons of these data with those for other similarly substituted cyclohexanones (Table S1).[15−29]

Table 1. ^{1}H and ^{13}C NMR Data for 1 and 2 (400 and 100 MHz, in DMSO-d_6, δ ppm)

	1		2	
no.	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	126.2		125.2	
2	66.5	5.14, br dd (3.8, 5.0)	60.3	5.56, br s
3	76.9	4.33, br dd (3.8, 5.5)	55.7	3.99, dd (2.6, 4.1)
4	66.6	5.70, d (2.1)	54.0	3.84, d (4.1)
5	191.2		197.2	
6	127.5		126.0	
7	154.8		153.9	
8	112.6	7.70, s	112.1	7.67, s
9	140.5		140.6	
10	127.9		127.3	
11	126.1		125.2	
12	129.2	8.78, d (8.0)	127.6	8.39, d (8.0)
13	109.9	6.90, d (8.0)	109.6	6.85, d (8.0)
14	155.5		156.1	
15	111.2		111.5	
16	156.4		157.1	
17	109.4	6.92, d (8.0)	109.5	6.89, d (8.0)
18	122.6	7.96, d (8.0)	122.8	7.94, d (8.0)
19	124.9		124.5	
20	135.2		135.2	
2-OH		5.60, d (5.0)		
3-OH		5.91, d (3.8)		

dx.doi.org/10.1021/np500522z | J. Nat. Prod. 2014, 77, 2454−2458

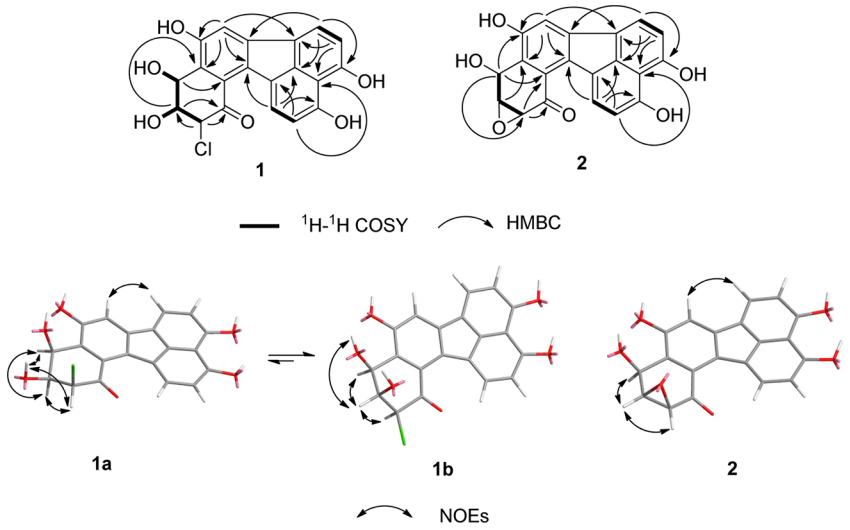


Figure 2. Key ^{1}H−^{1}H COSY, ^{1}H−^{13}C HMBC, and NOE correlations for **1** and **2**.

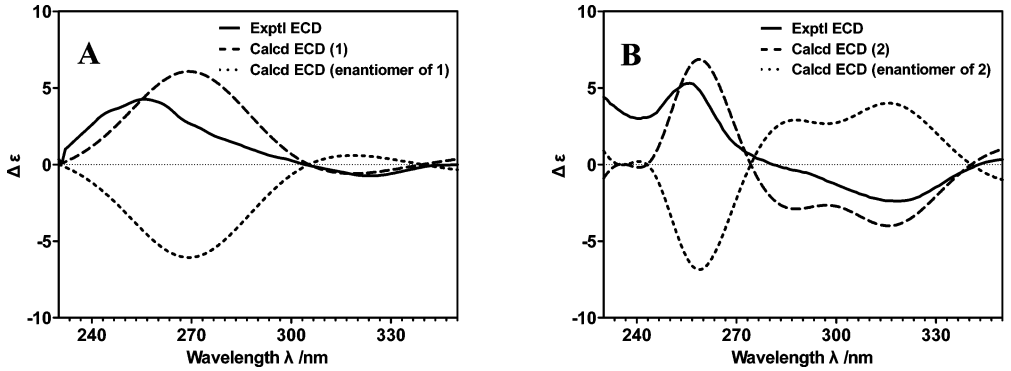


Figure 3. Comparison of the experimental and calculated ECD spectra of **1** (A, calculated at the B3LYP/6-31+G** level in the gas phase) and **2** (B, calculated at the B3LYP/6-31+G** level in EtOH).

The ROESY correlations between H-2 and OH-3, H-4 and OH-3, and H-3 and OH-2, as well as the NOE enhancement of H-2 and H-4 upon irradiation of H-3, supported the presence of two interchangeable half-chair conformations, **1a** and **1b**, as shown in Figure 3. In order to determine the absolute configuration of **1**, a lowest energy conformation search was carried out in Spartan'10[30] using the proposed configuration 2*S*,3*S*,4*S*. Geometry, frequency, and specific rotation calculations for 21 low-energy conformers were carried out at the DFT level (B3LYP functional/6-31+G** basis set) in the gas phase with Gaussian 09.[31] The Gaussian calculations resulted in four conformers with energies within 1.2 kcal/mol of the lowest energy conformer. The UV and CD spectra of the low-energy conformers, which were calculated at the TD-DFT level (B3LYP functional/6-31+G** basis set), were averaged using SpecDis 1.60.[32] The calculated specific rotation value for **1** ($[\alpha]_D$ 57) yielded a good match with the experimental datum ($[\alpha]^{24}_D$ 60). Furthermore, Cotton effects observed at ∼252 nm (positive) and ∼320 nm (negative) in the experimental ECD spectrum of **1** matched well with the predicted spectral data (Figure 3A). Thus, the absolute configuration of **1** was assigned as 2*S*,3*S*,4*S*.

A molecular formula consisting of $C_{20}H_{12}O_6$ was established for **2** based on HRESIMS data (*m*/*z* 347.0563, $[M - H]^-$). The physicochemical properties and ^{1}H and ^{13}C NMR data (Table 1) of **2** were similar to those of **1**, indicating both metabolites shared the same pentacyclic skeleton. This was later confirmed by analysis of the 2D NMR data (^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC, Figure 2). Considering the absence of the chlorine atom in **2**, the existence of an additional degree of unsaturation, and the upfield shifts of H-3 (δ_H 4.33 for **1** and δ_H 3.99 for **2**) and H-4 (δ_H 5.70 for **1** and δ_H 3.84 for **2**) and C-3 (δ_C 76.9 for **1** and δ_C 55.7 for **2**) and C-4 (δ_C 66.6 for **1** and δ_C 54.0 for **2**) (Table 1), a C-3−C-4 epoxide was proposed for **2**. The presence of the epoxide was confirmed by analysis of the relevant ^{1}H−^{1}H COSY and ^{1}H−^{13}C HMBC correlations illustrated in Figure 2. The relative configuration of **2** was established as 2,3-*cis*/3,4-*cis* by comparing the $J_{2,3}$ (2.6 Hz) and $J_{3,4}$ (4.1 Hz) coupling values with coupling constants for other cyclohexanone oxides (Table S1).[15−29] This conclusion was also supported by ROESY correlations between H-2 and H-3 and between H-3 and H-4 (Figure 2). DFT calculations (ECD and specific rotation) were performed to determine the absolute configuration of **2**. The calculated specific rotation value ($[\alpha]^{24}_D$ −195) and ECD spectrum (Figure 3B) of 2*S*,3*S*,4*S*-**2** matched well with experimental data ($[\alpha]^{24}_D$ −216, refer to Figure 3B for the ECD spectrum). Thus, the planar structure and absolute configuration of the new metabolite were established as illustrated for **2**.

A comparison of the spectroscopic data (^{1}H and ^{13}C NMR data in CD_3OD, UV−vis, and ECD) for **2** with those reported for daldinone B[33] (Figures S15 and S16) revealed that the two

dx.doi.org/10.1021/np500522z | J. Nat. Prod. 2014, 77, 2454−2458

compounds were identical; however, our structure determination efforts had guided us to a plausible alternative structure solution. Probing the two possible structures further, a DFT calculation was performed to generate a UV−vis spectrum for daldinone B. This provided a blue-shifted maximum absorption at 305 nm that was markedly different from experimental data (exptl 350 nm) (Figure S23). Curiously, a large positive specific rotation value ($[\alpha]_D$ +300.9) was reported for daldinone B in contrast to the large negative value we observed for **2** ($[\alpha]_D$ −216). This is in spite of the nearly superimposable ECD data for daldinone B and **2**. Unfortunately, we were not able to resolve the source of this discrepancy through communications with Dr. Y. Asakawa (corresponding author of ref 33). Thus, the structure of daldinone B has been revised to that illustrated for **2**.

With both **1** and **2** in hand, we questioned whether one of these metabolites might be an artifact generated during the purification process. Although the formation of chlorhydrins from epoxides is well known, these reactions generally provide products bearing *trans* configurations. To confirm this, a sample of **2** was treated with pyridine hydrochloride in ethanol, which yielded the expected *trans-epi*-4-chloro analogue of **1** (Figure S18).[14] In addition, base treatment of **1** was not successful in generating **2**. Thus, it appears that these two metabolites might be generated through enzymatic processes.

The antibacterial, cell cytotoxicity, and DPPH radical scavenging activities of **1** and **2** were also tested in our lab using previously described methods.[14,34,35] Both **1** and **2** displayed DPPH radical scavenging activities with IC_{50} values of 3.6 and 3.1 μM, respectively. Ascorbic acid was used as a positive control, giving an IC_{50} value of 3.2 μM. Neither compound showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* or *Klebsiella pneumoniae*, nor did they exhibit cytotoxic effects in a human pancreatic carcinoma (MIA PaCa-2) cell line.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter. UV data were measured on a Hewlett-Packard 8452A diode array spectrophotometer. IR spectra were obtained on a Bruker Vector 22 FT-IR spectrometer. NMR data were obtained on a Varian VNMR spectrometer (400 MHz for ^{1}H and 2D NMR, 100 MHz for ^{13}C) with a broad band resonance probe at 25 ± 0.5 °C. Electrospray-ionization mass spectrometry data were collected on an Agilent 6538 high-mass-resolution QTOF mass spectrometer. HPLC separations were performed on a Shimadzu system using an SCL-10A VP system controller. All solvents were of ACS grade or better.

Fungal Strain and Fermentation. A large library of commercially available dried herbs was procured and tested for culturable fungi. The fungal isolate described here was isolated from dried and finely powdered star apple (*Chrysophyllum cainito*) leaves. It was the lone fungus isolated from this herb when spread onto Czapek plates; no fungi were obtained on soil extract agar plates. The strain was identified as a *Daldinia* sp. based on its ribosomal internal transcribed spacer (ITS) and the 5.8S rRNA gene (ITS1-5.8S-ITS2) sequence data (GenBank accession no. KM000127).

Fermentations were performed by inoculating fungal mycelia and spores into 24 1 L Erlenmeyer flasks each containing Cheerios (80 mL by volume) with 48 mL of sterile sucrose solution (3 g/L) and 800 μM SAHA. The flasks were incubated for 30 days at room temperature under ambient lighting.

Extraction and Isolation. The fungal cultures were extracted three times with equal volumes of ethyl acetate. The solvent was pooled and evaporated *in vacuo* to generate the crude extract (20.8 g). The extract was separated into six fractions by silica vacuum column chromatography (eluted with gradients of hexanes−EtOAc and CH_2Cl_2−MeOH). Fraction 4 was separated into nine subfractions by Sephadex LH20 column chromatography (eluted with 1:1 CH_2Cl_2−MeOH), and subfraction 4-5 was then subjected to preparative-HPLC (Gemini 5 μm C_{18} column, 110 Å, 250 × 21.2 mm, 55% MeOH in 0.1% formic acid, flow rate 10 mL/min) to yield compounds **1** (7.2 mg, 0.0034% yield) and **2** (12.1 mg, 0.0058% yield). Subsequent repurification steps were carried out several times with both compounds owing to their decomposition into multiple trace products upon purification.

*Daldinone E (**1**):* yellow solid; $[\alpha]^{24}_D$ 60 (*c* 0.04, EtOH); UV (EtOH) λ_{max} (log ε) 216 (4.25), 248 (4.20), 350 (3.97); IR (film) ν_{max} 3378, 2920, 2848, 1611, 1420, 1234, 1180, 1109, 1064, 1019 cm^{-1}; CD (EtOH) λ_{max} ($\Delta\varepsilon$) 256 (4.2), 322 (−0.7), 391 (0.8); HRESIMS *m*/*z* 383.0330, $[M - H]^-$ (calcd for $C_{20}H_{12}ClO_6$, 383.0328).

*Daldinone B (**2**):* yellow solid; $[\alpha]^{24}_D$ −216 (*c* 0.05, EtOH); UV (EtOH) λ_{max} (log ε) 218 (4.25), 248 (4.19), 350 (3.93); IR (film) ν_{max} 3389, 2922, 2850, 1606, 1443, 1424, 1409, 1238, 1189, 1117, 1045, 1018 cm^{-1}; CD (EtOH) λ_{max} ($\Delta\varepsilon$) 256 (3.5), 318 (−1.6), 379 (−0.7); HRESIMS *m*/*z* 347.0563, $[M - H]^-$ (calcd for $C_{20}H_{11}O_6$, 347.0561).

Computational Details. Conformational analyses were carried out using Spartan'10.[30] Geometry, frequency, specific rotation, and ECD calculations were applied at the DFT level with Gaussian 09.[31] SpecDis 1.60[32] was used to average single ECD or UV−vis spectra after Boltzmann statistical weighting.

Bioactivity Assays. The antimicrobial assays against methicillin-resistant *Staphylococcus aureus* and *Klebsiella pneumoniae* were performed as previously described.[34] The cytotoxic effects in a human pancreatic carcinoma (MIA PaCa-2) cell line were assessed using the MTT assay.[35] For the DPPH radical scavenging assay,[14] samples (10 mM in DMSO) and ascorbic acid (10 mM in DMSO, positive control) were dissolved in 2-propanol, and the solution (50 μL) or the vehicle (50 μL of 2-propanol, negative control) was dispensed into wells of a 96-well microtiter tray. An aliquot of 150 μL of the DPPH solution in 2-propanol (40 uM) was added to each well. The mixture was shaken and left to stand for 30 min. After the reaction, the absorbance of each well was measured at 520 nm using a microplate reader (Infinite M200, Tecan Group Ltd.), and the percent inhibition calculated by comparisons to control wells. All the samples were tested in triplicate. The reported IC_{50} values denote the concentration of sample required to scavenge 50% of the DPPH free radicals.

ASSOCIATED CONTENT

Supporting Information

Tables of selected spectroscopic data for cyclohexenones and cyclohexenone epoxides, NMR (^{1}H and ^{13}C NMR, HSQC, HMBC, COSY, and ROESY) data for compounds **1** and **2**, and LC-ESIMS analysis of compound **2** and its chlorinated product. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Institutes of Health (1R01GM092219 and 1R01AI085161). The LC-ESIMS instrument used for this project was provided in part by a Challenge Grant from the Office of the Vice President for Research, University of Oklahoma, Norman Campus, and an award through the

Shimadzu Equipment Grant Program. The authors appreciate the help of Y. Asakawa for sharing original NMR spectral data for daldinone B.

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