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NOTE

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Dinoxin B, a Withanolide from *Datura inoxia* Leaves with Specific Cytotoxic Activities

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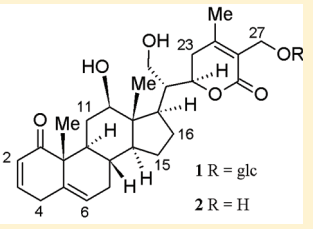
Supporting Information

ABSTRACT: A new withanolide, dinoxin B (12,21-dihydroxy-1-oxowitha-2,5,24-trienolide-27-*O*-β-D-glucopyranoside, **1**), was isolated from a methanol extract of *Datura inoxia* leaves, using bioassay-guided fractionation. The structure was determined by spectroscopic techniques, including ^{1}H, ^{13}C, and 2D NMR experiments as well as by HRMS. Extracts and the purified compound were tested for their antiproliferative activities toward a panel of human normal and cancer cell lines. Dinoxin B (**1**) and its aglycone (**2**) exhibited submicromolar IC_{50} values against multiple human cancer cell lines. Among the most sensitive were several breast cancer cell lines. Dinoxin B (**1**) was found only in *D. inoxia* and was not detected in *D. metel* or *D. stramonium*. The accumulation of this compound was limited largely to leaf tissue, with little to none detected in extracts from the flowers, fruits, roots, or stems of *D. inoxia*.



The genus *Datura* belongs to the family Solanaceae, commonly known as the nightshade family. This genus contains many species indigenous to the American Southwest including *Datura ferox*, *D. inoxia*, and *D. stramonium*. The genus originates from the Old World and has a worldwide geographical distribution.[1] Medicinally, *Datura* extracts have been used for their anesthetic, demulcent, expectorant, hypnotic, intoxicant, and sedative properties and are used traditionally to treat asthma, earache, headache, and tumors. The genus *Datura* has been an important commercial source of alkaloids, particularly for its content of tropane alkaloids such as hyoscyamine.[2]

Withanolides constitute a large family of plant steroids and steroid glycosides typified by a fused tetracyclic cholestane core and a side-chain unsaturated δ-lactone.[3] Within the genus *Datura*, withanolides have been isolated from several species, although Evans[2] suggests that not all species in this very large genus accumulate this class of secondary metabolite. To date, reports on this class have appeared for *D. fastuosa*,[4] *D. ferox*,[2,5] *D. inoxia*,[6,7] *D. metel*,[8,9] *D. quercifolia*,[8,9] and *D. stramonium*.[8,9] In all cases, the withanolides were obtained from aerial parts of the plant, usually the leaves but in one case the flowers.[4] There have been relatively few in vitro bioassays using withanolides from *Datura* spp. Kagale et al. demonstrated that extracts from *D. metel* leaves inhibited the growth of plant pathogens in vitro,[10] and the most abundant compound in this extract had a mass spectrum that matched the published report for the withanolide datruilin.[11] Pan et al. isolated 10 withanolides from *D. metel* flowers and demonstrated that four were cytotoxic at 10 μM or lower against three human cancer cell lines.[9] Withanolides, such as withaferin A, obtained from other plant species have been shown to induce apoptosis in the MCF-7 and MDA-MB-231 breast cancer cell lines.[12] Likewise, tubocapsenolide A induced apoptosis in the MDA-MB-231 cell line at low micromolar concentrations.[13]

In this study, we report the isolation and structure elucidation of a new withanolide (**1**) from a methanol extract of *D. inoxia* Mill. (*D. wrightii* Regel) leaves. The extract was fractionated by bioassay-guided separation using cytotoxicity toward the MCF-7 human breast cancer cell line to guide biological activity.

Crude extracts were fractionated by a combination of chromatographic techniques from the methanol leaf extracts of *D. inoxia*. The fraction with the strongest cytotoxic activity against the MCF-7 cell line was then purified to homogeneity by preparative HPLC with multiwavelength PDA detection and characterized by high-resolution LC-MS. Infusion MS analysis on a Q-TOF mass spectrometer gave a large positive ion at m/z 655, which was determined to be $[M + Na]^+$, with major fragments at m/z 471 $[M - \text{glucose} + H]^+$ and 453 $[M - \text{glucose} - \text{water} + H]^+$. When the sample was analyzed by LC-MS, an ion at m/z 633 $[M + H]^+$ was associated with the single UV-absorbing peak. Using a calibrated infusion run on the Q-TOF mass spectrometer, the molecular formula of **1** was determined as

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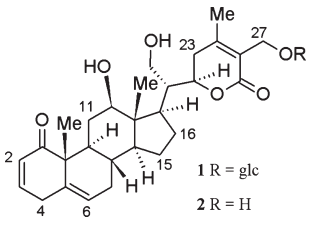
Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Dinoxin B (1) and Its Aglycone (2)

	compound **1**			compound **2**		
position	δ_C mult.	δ_H (J in Hz)	HMBC[a]	δ_C mult.	δ_H (J in Hz)	HMBC[a]
1	203.9, C			210.51		
2	127.3, CH	5.81, dd (9.9, 2.7)		126.86	5.67, m	1, 4
3	147.4, CH	6.94, ddd (9.9, 4.9, 2.3)	4, 10	130.06	6.07, d (8.4)	4
4	33.2, CH_2	3.30, 2.89, d (21.4) dd (21.4, 4.9)	1, 4, 5	39.61	3.31, 2.77, d (20), dd (20,5)	10, 1, 2, 3, 5
5	136.4, C			140.86		
6	124.6, CH	5.59, d (5.9)	4, 7, 8, 10	121.61	5.67, m	7, 10
7	30.5, CH_2	1.93, 1.49, dt (17.6, 4.9), m	5, 6, 8, 9	30.58	2.21, 1.6, m, m	5, 8, 9
8	32.3, CH	1.29, m	1, 9, 10, 13	30.74	1.51, m	[b]
9	42.0, CH	1.58, m	1, 7, 8, 10, 11, 19	39.79	1.91, m	[b]
10	50.2, C			51.47		
11	33.4, CH_2	2.26, 1.39, dt (13.0, 4.1), q (12.1)	8, 9, 10, 12, 13	33.91	2.05, 1.38, m, m	8, 9, 13, 12
12	77.8, CH	3.42, dd (11.5, 4.1)	11, 13, 18	76.10	3.69, m	18, 13
13	47.7, C			48.63		
14	54.2, CH	1.04, dd (11.1, 7.0)	8, 12, 13, 15, 18	54.20	1.22, m	[b]
15	24.0, CH_2	1.59, 1.19, m, m	8, 16	23.51	1.76, 1.38, m, m	[b]
16	26.9, CH_2	1.75, 1.50, m, m	13	26.65	1.85, 1.52, m, m	[b]
17	48.3, CH	1.73, m	13, 16, 20, 22	39.85	1.95, m	[b]
18	7.9, CH_3	0.71, s	12, 13, 14	7.68	0.75, s	12, 13, 14
19	18.9, CH_3	1.16, s	1, 5, 9, 10	20.23	1.35, s	1, 5, 10, 9
20	45.3, CH	1.84, m	17, 22	49.23	1.94, m	[b]
21	59.6, CH_2	3.74, 3.67, dd (11.5, 3.7), dd (11.4, 1.4)	17, 22	61.63	3.75, m	20, 17
22	77.7, CH	4.44, dt (12.8, 3.8)	20, 21, 24	75.77	4.61, m	17, 20
23	33.0, CH_2	2.76, 2.32, dd (18.3, 13.0), dd (18.3, 2.9)	22, 24, 25, 28	30.56	1.96	[b]
24	158.7, C			139.40		
25	122.4, C			129.20		
26	165.6, C			165.39		
27	62.5, CH_2	4.51, 4.24, d (10.9), d (10.9)	24, 25, 26, 1′	62.27	4.74, m	26
28	20.7, CH_3	2.04, s	23, 24, 25, 26, 27	25.88	1.45, s	23, 24, 25, 26
1′	103.2, CH	4.17, d (7.9)	27, 3′			
2′	73.8, CH	2.93, t (8.3)	1′, 3′			
3′	77.2, CH	3.13, t (8.6)				
4′	70.6, CH	3.04, m	5′			
5′	77.4, CH	3.08, m	6′			
6′	61.6, CH_2	3.67, 3.44, dd (11.5, 1.7), dd (11.9, 5.6)	4′, 5′			

[a] HMBC correlations, optimized for 10 Hz, are from proton(s) stated to the indicated carbon. [b] Correlations not assigned unambiguously due to multiplicity of the peaks in this region.

$C_{34}H_{48}O_{11}$, with a formula weight of 632.74 (calcd for $C_{34}H_{48}O_{11}Na$, 655.3088).



The diagnostic mass and NMR spectra suggested a steroidal lactone glucoside (Table 1: the ^{1}H, ^{13}C, DEPT, HSQC, HMBC, and NOSEY NMR spectra are provided as Supporting Information; Figures S1−S11). The ^{13}C and HSQC spectra showed the presence of a ketone, a carboxyl moiety, and six olefinic carbons, three of which are protonated. The HMBC and COSY spectra showed all the double-bonded carbons to be isolated from each other. There were three methyls found on unprotonated carbons.

Beginning with the ketone at position 1, it was possible to follow the connectivity of the ABCD rings with the HMBC and COSY spectra and assign the basic steroidal structure. The COSY and HMBC spectra both showed the C-17 to C-20 connection as the branch point for the rest of the molecule. The COSY spectrum then followed from H-20 to H-22 to H-23, after which the HMBC spectrum was used to assign the rest of the lactone ring. The HMBC spectrum also showed the connection between methylene C-27 and C-1′ of the glucose anomeric carbon. There were strong NOESY cross-peaks from H-1′ to H-3′ and H-1′ to H-5′, implying that H-1′, H-3′, and H-5′ are on the same side of the glucose ring. This requires that the glucose have the β-O configuration. Strong NOESY cross-peaks from H-12 to H-9, H-11a, H-14, and H-17 and weak or absent cross-peaks from H-12 to H-8, H-11b, H-18, and H-19 were used to determine the stereochemistry for most of

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the molecule. H-9, H-12, H-14, and H-17 were shown to be on one side of the molecule, and H-8, H-11B, H-18, and H-19 were on the other. Strong NOESY cross-peaks from H-18 to H-8, H-11b, and H-19 and a lack of cross-peaks from H-18 to H-9, H-11a, H-12, and H-14 agreed with the configuration suggested above. The cross-peak from H-18 to H-20 did not imply any stereochemistry due to the free rotation around the C-17−C-20 bond, as a strong NOESY signal would be present for any configuration at C-13, C-17, or C-20. Related 12-hydroxy compounds showing both *R* and *S* stereochemistry are found in *Datura* species at C-12.[14]

The configurations at carbons 8, 9, 10, 12, 13, 14, and 17 are as shown in the structure. The stereochemistry at C-20 was difficult to establish from the NOESY spectrum due to the free rotation of the C-17−C-20 and C-20−C-22 bonds. However, it was determined as *R* on the basis of a biogenetic argument because all reported withanolides unsubstituted at C-20 have the same configuration.[9] The stereochemistry at C-22 was determined to be *R*, as the literature indicates that an α-oriented H at C-22 gives rise to $J_{22,23}$ values of between 0.5−7 and 9−13.8 Hz, whereas the β-oriented form exhibits values between 2.5−7 and 2−5 Hz. The observed coupling constants for the proton at C-22 in **1** (J = 12.8, 3.8 Hz) fell under the former limits.[15,16] Withametelinol,[6] withametelin, and other related compounds were also shown to have C-22 *R* stereochemistry on the basis of a positive Cotton effect around 250 nm.[17]

Accordingly, **1** was assigned the structure 12,21-dihydroxy-1-oxowitha-2,5,24-trienolide-27-*O*-β-D-glucopyranoside. It can be alternatively named ergosta-2,5,24-trien-26-oic acid, 27-(β-D-glucopyranosyloxy)-12,21,22-trihydroxy-1-oxo, δ-lactone. Compound **1** can also be classified as a sitoindoside, which is a withanolide containing a glucose group at the C-27 position.[18]

The ^{1}H and ^{13}C NMR spectra of compound **2** contained shifts corresponding to the aglycone of compound **1** (Table 1: the ^{1}H, ^{13}C, HMBC, COSY, and TOCSY NMR spectra are provided as Supporting Information; Figures S13−S17). The NMR spectrum was similar to that of **1**, without the presence of the glucose moiety, but also suggests that it is a compound that is in equilibrium with an inseparable isomer.

The abundance of withanolides in the various organs of *D. inoxia* was investigated by monitoring methanol extracts by LC-MS and for the presence of the indicator m/z 655 ion (Figure S18, Supporting Information). Extracts from leaves, stems, roots, and flowers were prepared using the ASE extraction protocol up through the methanol extraction. At least four other withanolides were detected, but the most abundant form was the second peak, hence the name dinoxin B for compound **1**. The most abundant source of **1** was the leaves; this organ accumulated 10-fold greater concentrations of this compound than found in the flowers or leaves from immature plants. Neither of the other organs, the stems or roots, had any appreciable concentrations of **1**. Commercial seed sources of *D. inoxia*, *D. metel,* and *D. stramonium* were germinated and cultivated in the greenhouse. Leaves were collected and extracted. Again, LC-MS analyses were conducted to screen for the indicator m/z 655 ion. Only leaf extracts from *D. inoxia* contained **1**; it was not detected in extracts from leaves of *D. metel* or *D. stramonium*.

The estrogen receptor-positive (ER^{+}) breast cancer cell line MCF-7[19] was used throughout the extraction process in the bioassay-guided fractionation of the *Datura* extracts. The characteristic mass spectrum of the active HPLC fraction against MCF-7 was determined, and this chemical signature was used to purify **1**. The specificity of **1** was investigated by bioassays against a number of normal (n = 3) and cancer cell lines (n = 21); these IC_{50} values are presented in Table 2. Compound **1** exhibited submicromolar activity against several cancer cell lines with breast cancer cell lines being consistently among the most sensitive. Compound **1** is unusual in that it has submicromolar cytotoxicity levels as a glucosylated form of a withanolide. All other withanolide glycosides have less cytotoxicity relative to their aglycone forms.[15,20,21] To determine if the presence of the glucosyl group at C-27 on **1** influenced the biological activity, **2** was generated and used in bioassays. The IC_{50} values for **2** against the T47D, MDA-MB468, and MCF-7 cell lines were 0.99 ± 0.09, 0.75 ± 0.05, and 0.87 ± 0.22, respectively. In each case, the most potent cytotoxic compound was **1**; compound **2** was slightly less active (Table 2). This is in direct contrast to the bioactivities of other withanolides; the published IC_{50} values for withaferin A and its glycoside form against MCF-7 cells are 0.6 and 7.9 μM, respectively.[20] In the case of withaferin A, unlike compound **1**, the presence of a glucose group on the withanolide reduces the bioactivity 10-fold.

Table 2. Cytotoxicity in Human Cancer Cell Lines of Dinoxin B (1)[a]

type	cell line	IC_{50} (μM)	type	cell line	IC_{50} (μM)
breast	MDA-MB453	3.0 ± 0.05	liver	HEPG2	1.5 ± 0.04
	MDA-MB231	1.0 ± 0.07		HUH7	1.5 ± 0.02
	MCF7	0.61 ± 0.05		HEP3B	0.67 ± 0.29
	MDA-MB468	0.58 ± 0.07	normal	IEC6	3.3 ± 0.05
	HS578T	0.44 ± 0.38		NHF177	1.23 ± 0.06
	T47D	0.22 ± 0.06		HUVEC	0.74 ± 0.09
colon	Colo205	2.2 ± 0.12	melanoma	SK-MEL28	2.3 ± 0.05
	HCT116	1.0 ± 0.07		SK-MEL5	1.4 ± 0.03
	SW48	0.58 ± 0.08		UACC62	0.50 ± 0.03
	RKO	0.36 ± 0.02		SK-MEL2	0.36 ± 0.05
lung	A549	3.4 ± 0.02	ovarian	OVCAR3	3.2 ± 0.06
	A427	0.87 ± 0.03			

[a] Normal and cancer cell lines, organized by organ source, were treated with varying doses of **1** to determine the IC_{50} using the CellTiter-Glo luminescent cell viability assay.

EXPERIMENTAL SECTION

General Experimental Procedures. UV/vis spectra were recorded on a Beckman-Coulter DU-640 UV/vis spectrophotometer. IR spectra were recorded on a Biorad FTS-6000 spectrometer from KBr pellets. NMR spectra were collected in d_6-DMSO at 27 °C with a Bruker Avance 500 using a 5 mm BBO probe. The data were processed with Bruker Topspin 1.3 and the chemical shift predictions made using ACD/ChemSketch (Toronto, Ontario, Canada) version 12.01. Chemical shifts are reported as parts per million from TMS based on the lock solvent. NMR spetra were also collected at 25 °C in $CDCl_3$ on a Varian Unity Inova 500 MHz NMR spectrometer equipped with a Varian triple resonance/pulse field gradient probe. HRMS was performed on an Applied Biosystems/MDS Sciex Q-Star Elite Q-TOF mass spectrometer with a Turboionspray electrospray source, with an Agilent 1100 series HPLC system (G1379A degasser, G1357A binary capillary pump, G1389A autosampler, G1315B photodiode array detector, and G1316A column oven) all running under Applied Biosystems Analyst 2.0 (build 1446) LC-MS software. The MS was calibrated at least once daily with a standard calibration mixture recommended by Applied Biosystems, and the signal detection was optimized as needed. The data were acquired in the positive TOF MS mode. The MS parameters were as follows: accu-

mulation time 1 s, mass range 200 to 1000 Da, source gas 1−50 units, source gas 2−35 units, curtain gas 25 units, ion spray voltage 4500, source heater 400 °C, declustering potential 80, focusing potential 265, declustering potential 2−15, ion release delay 6, ion release width 5. For HRMS determination the samples were mixed with a standard calibrant and infused directly into the MS. For LC-MS analysis, the column used was an Inertsil ODS-3 reversed-phase C_{18} column (3 μm, 150 × 3 mm, with a Metaguard column, from Varian). The initial conditions were 20% methanol and 0.2% acetic acid in water, at a flow rate of 0.25 mL per min. The effluent was monitored at 280 nm using the PDA detector. After a delay of 2 min, the column was developed to 100% methanol with a linear gradient over 60 min.

Plant Material. *Datura inoxia* Mill. (*Datura wrightii* Regel) and *Datura stramonium* L. samples were collected on the New Mexico State University (NMSU) campus in Las Cruces, Doña Ana county, in southern New Mexico; *D. inoxia*, *D. metel* L., and *D. stramonium* plants were also grown from seed (Horizon Herbs, Willliams OR) in a greenhouse on the NMSU campus. A voucher specimen of the *D. inoxia* collected from the NMSU unmanaged campus area on October 2004 was submitted to the Range Science Herbarium at NMSU [Holguin 1 (NMCR)]. Plant identification for field-collected plants was confirmed by K. Allred, range plant taxonomist at NMSU. Re-collections of *D. inoxia* from this same population on the NMSU campus have been carried out annually in the fall months of 2006, 2008, and 2009.

Extraction and Isolation. A 1 kg amount of dried, pulverized *D. inoxia* leaves was extracted in 10 g aliquots in an ASE 350 liquid extraction apparatus. A 5 mL layer of sand was placed in each 35 mL cell, followed by a mixture of the crushed leaves and sufficient sand to fill the cell (ca. 15 g). The samples were extracted three times with hexane (80 °C, 8 min static extraction, 50% rinse volume, 60 s purge), followed by three extractions with chloroform (same conditions, but 100 s purge). Finally, the sample was extracted four times with methanol (same conditions as for chloroform extractions). The methanol extracts contained the active principle and were diluted with an equal volume of water, then centrifuged to remove the insoluble precipitate. The supernatant was extracted with half a volume of hexane and pooled. The methanol phase was loaded onto a 20 mL (5 g) LC-18 column (Supelco) in 250 mL aliquots. After washing the column extensively with 30% (v/v) methanol, the concentrated sample was eluted with 100% methanol. Four volumes of anhydrous ethyl ether were slowly added to the extract (with continuous stirring) at 0 °C. The sample was centrifuged for 15 min (10000*g*, 4 °C) to remove the insoluble precipitate. The supernatant was collected, and the ether was removed under a stream of nitrogen at ~50 °C. Semipreparative HPLC was then performed using a Waters HPLC system: 10 × 100 mm Waters Atlantis Prep C_{18} column (5 μm), using a 6 mL/min linear gradient of 20% to 60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Following 1 min of 20% acetonitrile, the gradient slope was 5% per min. The column was prerun at 54 °C with 100% acetonitrile and equilibrated with 20% acetonitrile prior to each injection. Absorbance was monitored at 271 nm using a Waters 960 photodiode array with the major peak collected at approximately 7.35 min. This material was diluted with an equal part of water and concentrated on an LC-18 column as before. Approximately 50 mg of purified material was obtained.

For structural analysis, an additional purification using a Shimadzu (Columbia, MD) preparative HPLC system was used with dual 8A pumps, SIL 10vp autoinjector, SPD M10Avp photodiode array detector, and SCL 10Avp system controller, all operating under the Shimadzu Class VP operating system. Sample aliquots (1 mL) in methanol were injected on a Phenomenex (Torrance, CA) Luna C18(2) semipreparative reversed-phase column (5 μm, 100 A, 250 × 25 mm). The column was pre-equilibrated with 1% acetic acid, 20% methanol, and 79% water at a flow rate of 10 mL per min, and the effluent was monitored at 280 nm. The column was developed to 100% methanol over 45 min. The procedure was repeated to obtain sufficient purified material. Pooled material was allowed to evaporate to remove organic solvent and then freeze-dried to recover **1**.

Dinoxin B (1):. Light brown, amorphous solid; $[\alpha]^{25}_D$ −36.5 (c 5, MeOH); UV (MeOH) λ_{max} (log ε) 230, 314 (3.60) nm; IR (KBr, disk) λ_{max} 3409, 2910, 1683, 1664(sh), 1395, 1077, 1050(sh), 798; HREIMS positive ion *m*/*z* 655.3146 $[M + Na]^+$ (calcd for $C_{34}H_{48}O_{11}Na$, 655.3088); with major fragments *m*/*z* 471.2830 $[M - \text{glucose} + H]^+$ (calcd for $C_{28}H_{39}O_6$ 471.2741) and 453.2686 $[M - \text{glucose} - \text{water} + H]^+$ (calcd for $C_{28}H_{37}O_5$ 453.2635); ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) spectroscopic data, see Table 1.

Hydrolysis of Dinoxin B (1). Dinoxin B (**1**, 10 mg) was dissolved in 1 mL of 10% aqueous HCl−dioxane (1:1). The solution was heated at 85−90 °C for 5 h in an oil bath. The mixture was neutralized ($NaHCO_3$) and extracted with ethyl acetate (2 × 1 mL), dried (Na_2SO_4), and evaporated to afford a brown residue. The residue was purified using flash chromatography with 5% MeOH−CH_2Cl_2 to afford the aglycone **2** as a colorless oil (3 mg, 40% yield): $[\alpha]^{25}_D$ −15.2 (*c* 1, MeOH); HRMS positive ion *m*/*z* 470.2884 $[M + H]^+$ (calcd for $C_{28}H_{38}O_6$ 470.2741); ^{1}H NMR and ^{13}C NMR ($CDCl_3$, 500 MHz) spectroscopic data, see Table 1.

Mammalian Cancer Cell Assays. Cell viability evaluation was carried out using an ATP-sensitive CellTiterGlo luminescent assay. Cells, typically 5000−15 000 per well, were plated in 96-well microtiter plates. Cells were allowed to adhere using a standard growth culture medium and conditions as recommended by the ATCC. After 24 h, the medium was replaced and test compounds were added with a final DMSO concentration of <0.1%. Cells were grown in the presence of test compounds for 48 h, at which time medium was replaced with drug-free medium. Cells were allowed to recover for 24 h. The CellTiter Glo reagent was added directly to the medium, and luminescence was measured using a Packard TopCount microtiter plate luminometer. Cytotoxicity, relative to vehicle-treated controls, as determined using this method, was highly consistent with the ^{3}H-thymidine incorporation assay.[22]

■ ASSOCIATED CONTENT

Supporting Information. The ^{1}H, ^{13}C, DEPT, NOESY, HMBC, HSQC NMR spectra and IR spectrum for dinoxin B (**1**) and the ^{1}H, ^{13}C, COSY, HMBC, and TOCSY NMR spectra for the aglycone **2** are available free of charge via the Internet at http://pubs.acs.org.

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products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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