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Anthraquinones from a Marine-Derived *Streptomyces spinoverrucosus*

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Abstract

Four new anthraquinone analogs including galvaquinones A-C (**1–3**) and an isolation artifact 5,8-dihydroxy-2,2,4-trimethyl-6-(3-methylbutyl)anthra[9,1-*de*][1,3]oxazin-7(2*H*)-one (**4**) were isolated from a marine-derived *Streptomyces spinoverrucosus* based on activity in an image-based assay to identify epigenetic modifying compounds. The structures of **1–4** were elucidated by comprehensive NMR and MS spectroscopic analysis. Galvaquinone B (**2**) was found to show epigenetic modulatory activity at 1.0 μM, and exhibited moderate cytotoxicity against non-small cell lung cancer (NSCLC) cell lines Calu-3 and H2887.

Actinomycetes derived from unique habitats are a rich source of bioactive natural products with bacterial isolates from the deep ocean[1] and symbiotic associations with insects providing unique molecules over the past few years.[2] The diverse chemistry from these studies has advanced a new frontier in the field of natural products. These new resources for natural products, coupled with advancements in phenotypic high-throughput screening approaches provide an excellent opportunity for the field to interrogate complex biological systems. For example, image-based screening [3] and cell reporter assays[4], can provide mechanistic insight into the biological role of a natural product in a primary assay. With these two concepts in mind, we have focused on building a collection of bacteria derived from the nutrient rich environments of mangrove swamps and estuaries along the Gulf of Mexico and the Bahamas. This collection of bacteria is composed of non-traditional sources of microbial natural products, such as α-proteobacteria, as well as a large number of actinomycetes.[5]

As part of our oncology screening program, we have screened a library of 1500 partially purified natural products fractions from these microorganisms for their ability to modify the epigenetic state of a cell.[6] Modification of the epigenetic state of a cancer cell, such as inhibition of histone deacetylases (HDACs), has been a validated therapeutic target with molecules in the clinic.[7] A number of natural products, including largazole are potent inhibitors of HDACs.[8] Other epigenetic targets, such as DNA methyltransferases (DNMTs) hold promise as cancer therapeutics.[9] The assay we have used for discovery of epigenetic modifiers, termed the Locus DeRepression assay (LDR), detects the derepression of a GFP reporter that is stably integrated in the mouse mammary carcinoma line C127.[10] In the vector, GFP transcription is controlled by a cytomegalovirus (CMV) promoter, which

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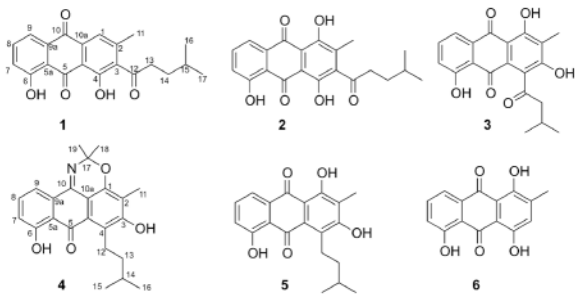
Supporting Information
NMR data tables and NMR spectra for compounds **1–4**. This material is available free of charge via the Internet at http://pubs.acs.org.

normally is strong and constitutively active. However, this cell line (LDR cell) was selected for lack of constitutive expression of the GFP reporter, presumably due to epigenetic silencing of the integration locus and/or methylation of the CMV promoter. GFP production can be induced by incubating the cells with HDAC inhibitors, such as TSA and butyrate or with DNMT inhibitors, such as 5-aza-deoxycytidine but not with general activators of transcriptional pathways, such as serum and steroids.[10b] There are other mechanisms, such as changes in protein phosphorylation state or additional post-translational modifications that might be observed with the LDR assay.[10a] The LDR assay has been previously used to screen large, pure compound libraries, but never as a primary screen for natural product discovery. We screened 1500 partially purified fractions at a concentration of 10μg/mL to obtain around 25 fractions that gave robust GFP expression. Herein we describe one of the GFP-inducing fractions, a non-polar fraction from *Streptomyces spinoverrucosus* strain SNB-032. Analysis of the active fraction by LC–UV–MS showed several peaks with the common UV profile indicative of anthraquinones. We report the bioassay-guided isolation of four new alkylated anthraquinone analogs, including galvaquinones A-C (**1–3**) and an isolation artifact 5,8-dihydroxy-2,2,4-trimethyl-6-(3-methylbutyl)anthra[9,1-*de*][1,3]oxazin-7(2*H*)-one (**4**), as well as the previously reported anthraquinones lupinacidin A (**5**) and islandicin (**6**). We have evaluated **1–6** for activity in the LDR assay and for cytotoxicity against a panel of non-small cell lung cancer (NSCLC) cell lines.

RESULTS AND DISCUSSION

Marine bacterium SNB-032 was isolated from a sediment sample collected from Trinity Bay, Galveston, TX and isolated on a seawater based acidic Gauze medium. Analysis by 16S rRNA sequence revealed SNB-032 to have 99% identity to *Streptomyces spinoverrucosus*. A large-scale (20 L) shake fermentation was carried out to obtain sufficient material for full chemical and biological analysis of the new analogs. The excreted metabolites were collected using XAD-7 resin and the resulting extract purified by a combination of solvent/solvent extraction and normal phase flash chromatography to give fractions enriched in anthraquinone metabolites that continued to show activity in the LDR assay. Final purification by Sephadex LH-20 and gradient reversed-phase HPLC gave galvaquinones A (**1**, 0.70 mg), B (**2**, 0.75 mg), and C (**3**, 0.40 mg), and 5,8-dihydroxy-6-isopentyl-2,2,4-trimethylanthra[9,1-de][1,3]oxazin-7(2H)-one (**4**, 1.00 mg), as well as known compounds lupinacidin A (**5**, 2.00 mg)[11] and islandicin (**6**, 0.32 mg).[12]



Galvaquinone A (**1**) was isolated as a yellow powder. High-resolution ESIMS analysis gave an $[M + H]^+$ ion at 353.1382, consistent with a molecular formula of $C_{21}H_{20}O_5$, which indicated 12 degrees of unsaturation. The UV spectrum of **1** exhibited absorption bands at 437, 276, 254 and 218 nm, highly suggesting an anthraquinone chromophore.[11, 13] The ^1H NMR spectrum of **1** (Table 1) showed the presence of two exchangeable protons at δ_H 11.99 and 12.29; one singlet aromatic proton at δ_H 7.68 (s, H-1), three aromatic protons at δ_H 7.83 (d, J = 7.5 Hz, H-9), 7.69 (dd, J = 8.4, 7.5 Hz, H-8), and 7.31 (d, J = 8.4 Hz, H-7); as well as 14 aliphatic protons contributed by one methyl singlet at δ_H 2.36 (H-11); two methyl

doublets at δ_H 0.93 (H-16 and H-17), two methylenes at δ_H 2.87 and 1.63 (H-13 and H-14, respectively), and one methine at δ_H1.63 (H-15). In the ^{13}C NMR spectrum of **1** (Table 2), fourteen sp^2 carbon signals, including two oxygenated quaternary sp^2 carbon signals at δ_C 159.2 and 162.5, and two carbonyl carbon signals at δ_C 179.8 and 186.5 were observed, which highly suggested the presence of a dihydroxy-anthraquinone core. An additional carbonyl at δ_C 205.9 and six aliphatic carbons (δ_C 19.2, 21.4, 21.4, 27.0, 31.4, and 40.9) account for the remaining substituents on the anthraquinone ring.

The substituents and their location on the anthraquinone ring were established by analysis of the COSY and gHMBC spectra of **1** (Figure 1). COSY correlations between H-7/H-8 and H-8/H-9, combined with HMBC correlations from H-9 to C-7 (δ_C 124.8), C-5a (δ_C 115.9), and C-10 (δ_C 179.8), from H-8 to C-6 (δ_C 162.5), C-9a (δ_C 133.5), and C-9 (δ_C 120.2), and from H-7 to C-5a and C-9, as well as from the phenolic proton at δ_H 11.99 to C-5a, C-6, and C-7 established the 1,2,3-trisubstitued benzene ring with a phenol at C-6. The presence of a 4-methylpentyl side chain was suggested by COSY correlations (Figure 1) and supported by HMBC correlations from H-13 to C-12 (δ_C 205.9), C-14 (δ_C 31.4), and C-15 (δ_C 27.0), from H-16 to C-14, C-15, and C17 (δ_C 21.4). Additional HMBC correlations from a methyl singlet at δ_H 2.36 (H-11) to C-1 (δ_C 122.1), C-2 (δ_C 145.4), and C-3 (δ_C 136.5), and from H-1 to C-3, C-10, C-11(δ_C 19.2), and C-4a (δ_C 114.3) as well as from the exchangeable proton at δ_H 12.29 to C-3, C-4a, and C-4 (δ_C 159.2) established the location of a methyl at C-2 and a hydroxy group at C-4, which in turn indicated the 4-methylpentyl side chain was located at C-3 of the anthraquinone ring. As a result, the full structure of **1** was established and named as galvaquinone A.

Galvaquinone B (**2**) was isolated as a red powder. The molecular formula of **2** was determined to be $C_{21}H_{21}O_6$ on the basis of a HRESIMS data, one more oxygen than **1**. The ^1H and ^{13}C NMR spectra for **1** and **2** (Tables 1 and 2) showed very similar signals, with the exception that the H-1 singlet at δ_H 7.68 for **1** was replaced by a new exchangeable proton at δ_H 13.49 for **2**, and the carbon signal at δ_C 122.1 (C-1) for **1** was replaced by a carbon signal at δ_C 158.0 for **2**. These data indicated that the hydrogen at C-1 in **1** was substituted by a hydroxy group in **2**. This assignment was supported by HMBC correlations from H-11 (δ_H 2.25) to C-1 (δ_C 157.5), C-2 (δ_C 137.1), and C-3 (δ_C 141.1), and from the exchangeable proton at δ_H 13.49 (OH-1) to C-1, C-2, and C-10a (δ_C 111.7). The full structure of **2** was further confirmed by COSY and HMBC experiments (Supporting information, Table S2) and named as galvaquinone B.

Galvaquinone C (**3**) was isolated as yellow powder; its molecular formula was determined as $C_{20}H_{18}O_6$ by HRESIMS, one more oxygen and two fewer hydrogens than the known compound **5**. The ^1H and ^{13}C NMR spectra for **3** and **5** (Tables 1 and 2) showed very similar signals, with the exception that the H-12 multiplet at δ_H 3.21 for **5** was missing for **3**, and the carbon signal at δ_C 37.1 (C-13) for **5** was replaced by a carbon signal at δ_C 53.7 for **3**. These data indicated that the CH_2 at C-12 in **5** was substituted by a C=O in **3**. Due to limited material, we were not able to detect a signal for the ketone at C-12 in either the ^{13}C NMR or HMBC spectra. However, the COSY correlations between H-13 (δ_H 2.60) and H-14 (δ_H 2.18), and between H-14 and H-15/H-16 (δ_H 0.92), as well as the HMBC correlations from H-15 to C-13 (δ_C 53.7), C-14 (δ_C 26.7), and C-16 (δ_C 22.7) confirmed the presence of an isobutyl group. In addition, the ^{13}C chemical shift of C-13 at δ_C 53.7 highly suggested that the isobutyl group was directly connected to a ketone to form the isovaleryl group.[14] Other key HMBC correlations were from a methyl singlet at δ_H 2.24 (H-11) to C-1 (δ_C 163.8), C-2 (δ_C 120.9), and C-3 (δ_C 158.8) and from an exchangeable proton at δ_H 13.42 (OH-1) to C-1, C-2, and C-10a (δ_C 110.3). Finally, the full structure of **3** was established and named as galvaquinone C.

Compound **4** was isolated as yellow needles. High-resolution ESI MS analysis gave an $[M + H]^+$ at *m*/*z* 380.1873, consistent with a molecular formula of $C_{23}H_{25}NO_4$, which indicated 12 degrees of unsaturation. Comparison of the NMR data (Tables 1 and 2) for **4** with those for known compound lupinacidin A (**5**) showed a high degree of similarity in the aromatic rings and isopentyl side-chain. A few significant differences were observed, including the presence of two additional identical methyl singlets at δ_H 1.68/δ_C 27.9, and a quaternary carbon at δ_C 92.2 in **4**. Additionally, the C-10 carbonyl carbon signal at δ_C 186.9 in **5** was shifted upfield to δ_C 150.6 in **4**. Detailed analysis of COSY and gHMBC spectra (Supporting information, Table S4) confirmed the presence of an anthraquinone-type ring substituted by methyl (at C-2), isopentyl (at C-4) and hydroxy groups (at C-3 and C-6), as shown in substructure **a**. This substitution pattern is similar to the anthraquinone core of **5**. The assignment of C-5 was supported by its chemical shift at δ_C 191.2 and the presence of singlet phenolic proton H-6, which is strongly hydrogen-bonded to the carbonyl oxygen. Further HMBC correlations from the exchangeable proton at δ_H 5.31 to C-2, C-3, and C-4 (δ_C 124.1) indicated a phenol located at C-3. The presence of an isopentyl group was confirmed by COSY and HMBC correlations (Figure 2), while the location of the isopentyl side chain was assigned at C-4 by HMBC correlations from H-12 to C-3, C-4 and C-4a.

The remaining signals to be accounted for in **4** included two equivalent methyl groups (δ_H 1.68), a quaternary carbon at δ_C 92.2 and a sp^2 carbon at δ_C 150.0. The methyl singlets exhibited a strong HMBC correlation with the carbon at δ_C 92.2 (C-17) and a weak correlation with the carbon at δ_C 150.0 (C-10). This led us to propose substructure **b**, which contains an *N,O* acetonide moiety. The two substructures **a** and **b** account for $C_{22}H_{25}NO_4$ and 10 degrees of unsaturation. As a result, the remaining structure of **4** required two more rings. As there are only two exchangeable protons and four oxygen atoms in **4**, the oxygen at C-1 must be di-substituted and C-1 must be connected to C-17 by an oxygen atom. Moreover, the HMBC correlation from H-9 to C-10 and the chemical shift of C-10 at 150.0 ppm is suggestive of an iminoquinone. Thus, the full structure of **4** was established as 5,8-dihydroxy-2,2,4-trimethyl-6-(3-methylbutyl)anthra[9,1-*de*][1,3]oxazin-7(2*H*)-one. Compound **4** contains an *N,O*-acetonide, which is unusual in natural products.[15] As acetone was used in the extraction of *S. spinoverrucosus*, we surmised that the *N*, *O*-acetonide was an isolation artifact derived from an iminoquinone and acetone. To verify, LC-MS analysis of fermentation of *S. spinoverrucosus* extracted with methanol rather than acetone showed no peak corresponding to **4**, but a new peak with *m*/*z* 340 $[M + H]^+$ and 338 $[M - H]^-$ corresponding to the iminoquinone, confirming compound **4** is an isolation artifact. Despite repeated fermentation of *S. spinoverrucosus* we were unable to obtain sufficient material of the corresponding iminoquinone for full characterization. However, we speculate that **4** originates from known compound **5**, which can undergo conversion to the iminoquinone via the addition of ammonia (Figure 3). It should be noted that the conversion of anthraquinones into iminoquinones is readily accomplished synthetically via addition of ammonia in methanol.[16] However, as no exogenous source of ammonia is supplied to the fermentation or used in the extraction, it is likely that *Streptomyces spinoverrucosus* is the source of ammonia for production of **4**.

The known compounds were identified as lupinacidin A (**5**)[11] and islandicin (**6**)[12], respectively, based on NMR and mass spectra and comparison with the reported data.

Compounds **1–6** were evaluated for their activities as potential epigenetic modulators in the LDR assay at an initial concentration of 10 μM. Only **2** exhibited significant activity at 10 μM and subsequently displayed activity at 1.0 μM (Figure 4) as seen by expression of GFP. Compound **1** demonstrated weak activity at 10 μM (data not shown). We measured the fluorescence spectra of compounds **1** and **2** (Supporting Information, Fig. S24), to confirm

the fluorescence measured in the LDR assay was generated through the expression of GFP, not by fluorescence of the compounds.

Compounds **1–6** were also examined for their cytotoxic activities against three non-small cell lung cancer (NSCLC) cell lines (Calu-3, H2887). Compound **2** showed cytotoxicity against the H2887 cell line with an IC_{50} of 5.0 μM, while it was slightly less active against Calu-3 with an IC_{50} of 12.2 μM. Known compound **5** exhibited cytotoxicity against Calu-3 and H2887 cell lines with IC_{50} values of 8.8 μM and 3.1 μM, respectively. None of the remaining compounds had IC_{50} values < 50 μM against the cell lines tested.

Anthraquinones are well known for their anti-inflammatory, antioxidant, antimicrobial, antitumor, and antiviral properties.[12] The close analogs of galvaquinones, lupinadicins A-C were found to show significant inhibitory effects on the invasion of murine colon 26-L5 carcinoma cells with IC_{50} of 0.21 μM, 0.92 μM, and 0.054 μM, respectively.[11] This is the first report on the epigenetic modulator property of anthraquinones. As the LDR assay detects the transcriptional derepression of a silenced reporter in cells, this assay is not biased toward a particular enzyme or activity, thereby permitting the detection of new and diverse epigenetic targets. However, this also provides a challenge to move towards a specific mechanism of action. We are further exploring the mechanism(s) by which galvaquinone B changes the epigenetic state of cells.

EXPERIMENTAL SECTION

General Procedures

UV spectra were recorded on a Shimadzu UV-1601 UV-vis spectrophotometer. ^{1}H and 2D NMR spectroscopic data were recorded at 600 MHz in $CDCl_3$ or DMSO-d_6 solution on a Varian System spectrometer, and chemical shifts were referenced to the corresponding solvent residual signal. ^{13}C NMR spectra were acquired at 100 MHz on a Varian System spectrometer. High resolution ESI-TOF mass spectra were provided by The Scripps Research Institute, La Jolla, CA. Low-resolution LC/ESI-MS data were measured using an Agilent 1200 series LC/MS system with a reversed- phase C_{18} column (Phenomenex Luna, 150 mm × 4.6 mm, 5 μm) at a flow rate of 0.7 mL/min. Preparative HPLC was performed on an Agilent 1200 series instrument with a DAD detector, using a Phenyl-Hexyl column (Phenomenex Luna, 250 × 10.0 mm, 5 μm). Sephadex LH-20 (GE Healthcare, Sweden) and ODS (50 mm, Merck) were used for column chromatography.

Collection and Phylogenetic Analysis of Strain SNB-032

The marine-derived bacterium, strain SNB-032, was isolated from a sediment sample collected from Trinity Bay, Galveston, TX (29° 36′36″ N, W 94° 46′30″ W). Bacterial spores were collected via stepwise centrifugation as follows: 2 g of sediment was dried over 24 h in an incubator at 35 °C and the resulting sediment added to 10 mL seawater (sH_2O) containing 0.05% Tween 20. After vigorous vortexing for 10 min, the sediment was centrifuged at 2500 rpm for 5 min (4 °C). The supernatant was removed and transferred into a new tube and centrifuged at 18,000 rpm for 25 min (4 °C) and the resulting spore pellet collected. The resuspended spore pellet (4 mL sH_2O) was plated on an acidified Gauze medium, giving rise to individual colonies of SNB-032 after two weeks. Analysis of the 16S rRNA sequence of SNB-032 revealed 99% identity to *Streptomyces spinoverrucosus*. The sequence is deposited in GenBank under accession #JX511995.

Cultivation and Extraction

Bacterium SNB-032 was cultured in 20 2.8 L Fernbach flasks each containing 1 L of a seawater based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40mg

$Fe_2(SO_4)_3.4H_2O$, 100 mg KBr) and shaken at 200 rpm at 27 °C. After seven days of cultivation, sterilized XAD-7-HP resin (20 g/L) was added to adsorb the organic products, and the culture and resin were shaken at 200 rpm for 2 h. The resin was filtered through cheesecloth, washed with deionized water, and eluted with acetone. The acetone soluble fraction was dried *in vacuo* to yield 6.0 g of extract.

Isolation

The extract (6.0 g) was partitioned between *n*-hexane and 90% aqueous MeOH, and the MeOH (aq) fraction was diluted to 60% MeOH (aq) and extracted with $CHCl_2$. The bioactive *n*-hexane fraction (1.0 g) which contained anthraquinones was fractionated by flash column chromatography on silica gel (50 g), eluting with a step gradient of CH_2Cl_2 and MeOH (100:0–50:50), and 11 fractions (Fr.1–Fr.11) were collected. The active fractions (Fr.1 and Fr.2) were further purified to give pure compounds. Fraction 1 was purified by reversed-phase HPLC (Phenomenex Luna, Phenyl-Hexyl, 250 × 10.0 mm, 2.5 mL/min, 5 mm, UV = 210 nm) using a gradient solvent system from 80% to 99% CH_3CN (0.1% formic acid) over 30 min to afford lupinacidin A (**5**, 2.0 mg, t_R = 13.4 min) and galvaquinone B (**2**, 0.75 mg, t_R = 16.3 min). Fraction 2 was purified by Sephadex LH-20 eluted with CH_2Cl_2/Hexane (4:1) to give 5 sub-fractions (Fr.2-1–Fr.2-5). Sub-fraction Fr.2-1 was further purified by Sephadex LH-20 eluted with MeOH to give 4 fractions (Fr.2-1-1–Fr.2-1-4). Fraction Fr.2-1-2 was purified by reversed phase HPLC (Phenomenex Luna, Phenyl-Hexyl, 250 × 10.0 mm, 2.5 mL/min, 5 mm, UV = 210 nm) using a gradient solvent system from 70% to 90% CH_3CN (0.1% formic acid) over 30 min to afford galvaquinone A (**1**, 0.7 mg, t_R = 20.6 min). Fraction Fr.2-1-4 was purified with the same HPLC method to afford galvaquinone C (**3**, 0.4 mg, t_R = 13.3 min) and islandicin (**6**, 0.3 mg, t_R = 16.3 min). Fraction Fr.2-4 was further purified by Sephadex LH-20 eluted with MeOH to give compound **4** (1.0 mg).

Galvaquinone A (**1**, 0.70 mg) yellow powder; UV (MeOH) λ_{max} (log ε) 218 (4.3), 254 (3.9), 276 (4.3), 437 (3.8) nm; ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Tables 1 and 2. ESI-MS *m*/*z* 351.2 $[M - H]^-$. HRESIMS *m*/*z* 353.1382 $[M + H]^+$ (calcd for $C_{21}H_{21}O_5$, 353.1389).

Galvaquinone B (**2**, 0.75 mg) red powder; UV (MeOH) λ_{max} (log ε) 233 (4.3), 254 (3.6), 293 (3.3), 492 (3.6) nm; ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Tables 1 and 2. ESI-MS *m*/*z* 367.2 $[M - H]^-$. HRESIMS *m*/*z* 369.1333 $[M + H]^+$ (calcd for $C_{21}H_{21}O_6$, 369.1338).

Galvaquinone C (**3**, 0.40 mg) yellow powder; UV (MeOH) λ_{max} (log ε) 218 (4.1), 254 (3.9), 276 (4.1), 437 (3.9) nm; ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Tables 1 and 2. ESI-MS *m*/*z* 353.1 $[M - H]^-$. HRESIMS *m*/*z* 355.1185 $[M + H]^+$ (calcd for $C_{20}H_{19}O_6$, 355.1176); *m*/*z* 353.1038 $[M - H]^-$ (calcd for $C_{20}H_{17}O_6$, 353.1031).

5,8-dihydroxy-2,2,4-trimethyl-6-(3-methylbutyl)anthra[9,1-*de*][1,3]oxazin-7(2*H*)-one (**4**, 1.00 mg) yellow needles; UV (MeOH) λ_{max} (log ε) 241 (4.3), 287 (3.9), 401(3.7) nm; ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Tables 1 and 2. ESI-MS *m*/*z* 378.2 $[M - H]^-$, 380.1 $[M + H]^+$. HRESIMS *m*/*z* 380.1873 $[M + H]^+$ (calcd for $C_{23}H_{26}NO_4$, 380.1861).

Epigenetic Assay

Compounds **1–6** were tested for their activity as epigenetic modulators using the Locus DeRepression assay.[10] LDR cells were plated on four-well chambered glass slides (Lab-

TekII, Nunc) at a density of 15,000 cells per well, and cultured in complete media at 37 °C, 5% CO_2 and 95% air overnight. DMSO (0.5%) was used for the negative control and 25 mM sodium butyrate was used as the positive control. Twenty-four hours after seeding the cells into assay wells, the cells were treated with compounds **1–6** (10 μM and 1.0 μM final for each compound). After a 24 hour treatment with the chemicals, the assay wells were subjected to fixation with 4% formaldehyde for 10 min. After fixation, the assay plates were washed with 500 μL of PBS before being imaged on a Nikon Eclipse TE2000-U fluorescence microscope equipped with a CCD Roper camera, at 20 ×, 4 sites per well, 20 ms exposure for GFP. As for bioassay of the fractions, a same process was used and the concentration of each fraction was 10μg/mL.

Fluorescence Measurement

The fluorescence spectra of compounds **1–2** were measured separately at a concentration of 10 μM in PBS scanning from 480–550 nm with excitation wavelengths of 475 nm and 395 nm using PerkinElmer LS55 Fluorescence spectrometer.

Cytotoxity Assays

Cell lines were cultured in 10 cm dishes (Corning, Inc.) in NSCLC cell-culture medium: RPMI/L-glutamine medium (Invitrogen, Inc.), 1000 U/ml penicillin (Invitrogen, Inc.), 1 mg/mL streptomycin (Invitrogen, Inc.), and 5% fetal bovine serum (Atlanta Biologicals, Inc.). Cell lines were grown in a humidified environment in the presence of 5% CO_2 at 37 °C. For cell viability assays, Calu-3 and H2887 cells (60 mL) were plated individually at a density of 750 and 500 cells/well, respectively, in 384-well microtiter assay plates (Bio-one; Greiner, Inc.). After incubating the assay plates overnight under the growth conditions described above, purified compounds were dissolved and diluted in DMSO and subsequently added to each plate with final compound concentrations ranging from 50 μM to 1 nM and a final DMSO concentration of 0.5%. After an incubation of 96 h under growth conditions, Cell Titer Glo reagent (Promega, Inc.) was added to each well (10 mL of a 1:2 dilution in NSCLC culture medium) and mixed. Plates were incubated for 10 min at room temperature and luminescence was determined for each well using an Envision multi-modal plate reader (Perkin- Elmer, Inc.). Relative luminescence units were normalized to the untreated control wells (cells plus DMSO only).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. For a review on marine-derived actinomycetes see: Fenical W, Jensen PR. Nat Chem Biol. 2006; 2:666–673. [PubMed: 17108984]
2. (a) Crawford JM, Clardy J. Chem Commun. 2011; 47:7559–7566.(b) Oh DC, Poulsen M, Currie CR, Clardy J. Org Lett. 2011; 13:752–755. [PubMed: 21247188] (c) Oh DC, Poulsen M, Currie CR, Clardy J. Nat Chem Biol. 2009; 5:391–393. [PubMed: 19330011]
3. (a) Ohnuki S, Oka S, Nogami S, Ohya Y. PLoS ONE. 2010; 5:e10177. [PubMed: 20418956] (b) Yarrow JC, Totsukawa G, Charras GT, Mitchison TJ. Chem Biol. 2005; 12:385–395. [PubMed:

15797222] (c) Peach KC, Bray WM, Shikuma NJ, Gassner NC, Lokey RS, Yildiz FH, Linington RG. Mol Biosyst. 2011:1176–1184. [PubMed: 21246108]

4. (a) Sista P, Edmiston S, Darges JW, Robinson S, David J, Burns DJ. Mol Cell Biochem. 1994; 141:129–134. [PubMed: 7891670] (b) Cherry JJ, Evans MC, Ni J, Cuny GD, Glicksman MA, Elliot J, Androphy EJ. J Biomol Screen. 2012; 17:481–495. [PubMed: 22233647]
5. (a) Hu Y, MacMillan JB. Org Lett. 2011; 13:6580–6583. [PubMed: 22106936] (b) Hu Y, Legako AG, Espindola AP, MacMillan JB. J Org Chem. 2012; 77:3401–3407. [PubMed: 22384985] (c) Hu Y, Espindola AP, Stewart NA, Wei S, Posner BA, MacMillan JB. Bioorg Med Chem. 2011; 19:5183–5189. [PubMed: 21807523] (d) Pan E, Jamison M, Yousufuddin M, MacMillan JB. Org Lett. 2012; 14:2390–2393. [PubMed: 22515470]
6. The natural product fraction library was created from crude bacterial extracts of 5 L fermentations of bacterial strains. Fractions were generated using reversed phase C_{18} chromatography on an ISCO medium pressure automatic purification system (gradient from 90:10 H_2O:CH_3CN to 0:100 H_2O:CH_3CN over 25 minutes) to generate 10–20 fractions.
7. (a) Grant S, Easley C, Kirkpatrick P. Nat Rev Drug Discov. 2007; 6:21–22. [PubMed: 17269160] (b) Shah MH, Binkley P, Chan K, Xiao J, Arbogast D, Collamore M, Farra Y, Young D, Grever M. Clin Cancer Res. 2006; 12:3997–4003. [PubMed: 16818698]
8. Taori K, Paul VJ, Luesch H. J Am Chem Soc. 2008; 130:1806–1807. [PubMed: 18205365]
9. (a) Ganesan A, Nolan L, Crabb SJ, Packham G. Curr Cancer Drug Targets. 2009; 9:963–981. [PubMed: 20025605] (b) Ghoshal K, Bai S. Drugs Today. 2007; 43:395–422. [PubMed: 17612710] (c) George SM. Nat Biotechnol. 2010; 28:1259–1266. [PubMed: 21139608]
10. (a) Johnson RL, Huang W, Jadhav A, Austin CP, Inglese J, Martinez ED. Anal Biochem. 2008; 375:237–248. [PubMed: 18211814] (b) Best AM, Chang J, Dull AB, Beutler JA, Martinez ED. J Biomed Biotech. 2011:Article ID 868095.
11. (a) Igarashi Y, Trujillo ME, Martinez-Molina E, Yanase S, Miyanaga S, Obata T, Sakurai H, Saiki I, Fujita T, Furumai T. Bioorg Med Chem Lett. 2007; 17:3702–3705. [PubMed: 17475486] (b) Igarashi Y, Yanase S, Sugimoto K, Enomoto M, Miyanaga S, Trujillo ME, Saiki I, Kuwahara S. J Nat Prod. 2011; 74:862–865. [PubMed: 21226490]
12. Tietze LF, Gericke KM, Schuberth I. Eur J Org Chem. 2007:4563–4577.
13. Abdelfattah M. Nat Prod Res. 2009; 23:212–218. [PubMed: 19235021]
14. (a) Wu XF, Wang YD, Yu SS, Jiang N, Ma J, Tan RX, Hu YC, Qu J. Tetrahedron. 2011; 67:8155–8159.(b) Lopez-Perez JL, Olmedo QA, Olmo ED, Vasquez Y, Solis PN, Gupta MP, Feliciano AS. J Nat Prod. 2005; 68:369–373. [PubMed: 15787438] (c) Verotta L, Lovaglio E, Vidari G, Finzi PV, Neri MG, Raimondi A, Parapini S, Taramelli D, Riva A, Bombardelli E. Phytochemistry. 2004; 65:2867–2879. [PubMed: 15501254] (d) Takasaki M, Konoshima T, Kozuka M, Haruna M, Ito K, Shingu T. Chem Pharm Bull. 1994; 42:2591–2597.(e) Helesbeux J-J, Duval O, Dartiguelongue C, Séraphin D, Oger J-M, Pascal Richomme P. Tetrahedron. 2004; 60:2293–2300.
15. Yan YX, Chen JC, Sun Y, Wang YY, Su J, Li Y, Qiu M-H. Chem Biodivers. 2010; 7:1822–1827. [PubMed: 20658671]
16. Farina F, Molina MT, Noheda P, Paredes MC. Tetrahedron. 1992; 48:8437–8450.

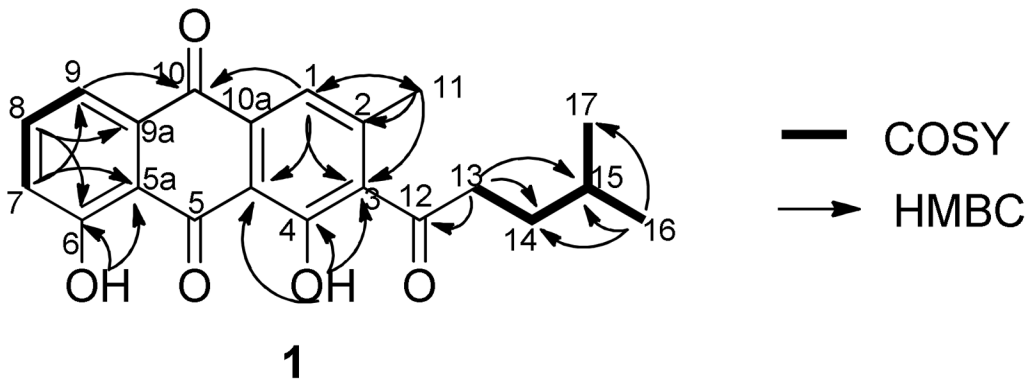

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Figure 1.
COSY and key HMBC correlations for **1**.

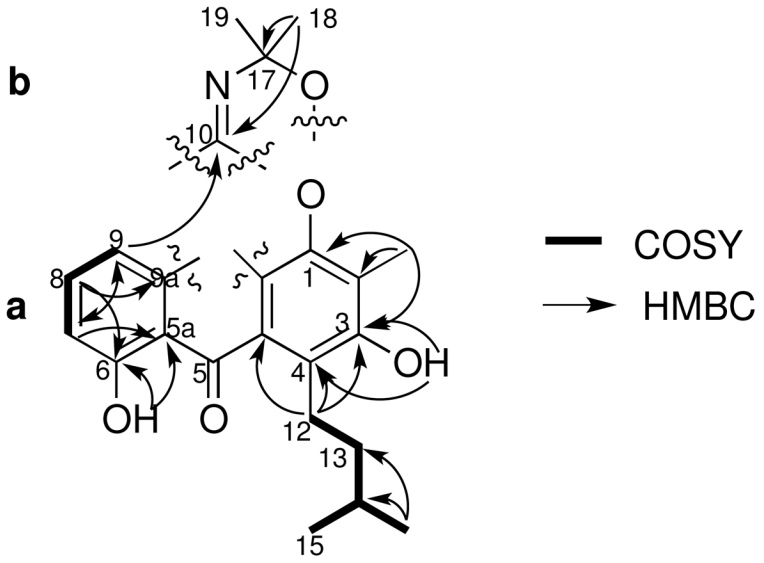

Figure 2.
Substructures of **4** from COSY and HMBC correlations.

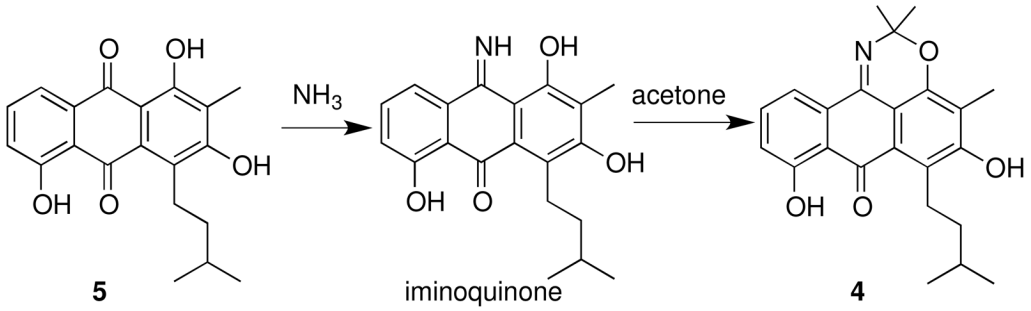

Figure 3.
Proposed conversion of **5** to **4**.

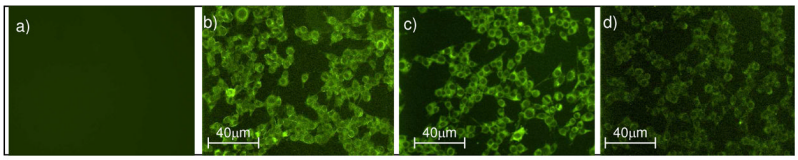

Figure 4.

The locus derepression assay. a) cells treated with DMSO. b) cells treated with 5-azacytidine (positive control). c) cells treated with galvaquinone B (**2**) at 10 μM. d) cells treated with galvaquinone B (**2**) at 1.0 μM. Cells imaged for GFP expression.

Table 1

^{1}H NMR (600 MHz) data of compounds **1–5** in $CDCl_3$ (δ_H, mult., J in Hz)

no.	1	2	3	4	5
1	7.68, s				
7	7.31, dd (8.4)	7.32, dd(8.5, 1.3)	7.30, d (8.2)	7.10, dd (8.3, 1.1)	7.26, d (8.2)
8	7.69, dd (8.4, 7.5)	7.72, dd (8.5, 7.6)	7.69, dd (8.2, 7.4)	7.53, dd (8.3, 7.7)	7.62, dd (8.2, 7.5)
9	7.83, d (7.5)	7.90, dd (7.6, 1.3)	7.84, d (7.4)	7.85, dd (7.7, 1.1)	7.79, d (7.5)
11	2.36, s	2.25, s	2.24, s	2.18, s	2.27, s
12				3.18, m	3.21, m (6.6)
13	2.87, t (7.1)	2.85, m	2.60, m	1.45, m	1.46, m(6.6)
14	1.63, m	1.63, m	2.18, m (6.7)	1.78, m	1.80, m (6.6)
15	1.63, m	1.63, m	0.92, d (6.7)	1.03, d (6.7)	1.04, d (6.6)
16	0.93, d (6.2)	0.93, d (6.2)	0.92, d (6.7)	1.03, d (6.7)	1.04, d (6.6)
17	0.93, d (6.2)	0.93, d (6.2)			
18/19				1.68, s	
1-OH		13.49, s	13.42, s		14.18, s
3-OH				5.31, s	
4-OH	12.29, s	12.50, s			
6-OH	11.99, s	12.14, s	12.05, s	13.06, s	12.96, s

Table 2

^{13}C NMR (100 MHz) data of compounds **1–5** in $CDCl_3$

no.	1	2	3[a]	4	5
1	122.1	157.5	163.8	150.6	159.6
2	145.4	137.1	120.9	117.8	117.5
3	136.5	141.1	158.8	156.6	159.1
4	159.2	153.8	*b*	124.1	130.4
4a	114.3	116.2	*b*	124.3	127.3
5	186.5	190.7	*b*	191.2	190.2
5a	115.9	116.2	115.8	117.3	117.1
6	162.5	162.7	162.4	162.4	162.5
7	124.8	124.8	124.7	120.4	124.3
8	137.3	137.1	137.3	135.5	136.0
9	120.2	119.7	119.6	115.1	118.3
9a	133.5	133.4	133.0	134.6	133.0
10	179.8	186.5	187.4	150.0	186.9
10a	122.2	111.7	110.3	108.4	109.0
11	19.2	13.2	8.8	8.4	8.4
12	205.9	204.9	*b*	24.2	24.8
13	40.9	42.4	53.7	38.4	37.7
14	31.4	31.9	26.7	28.8	28.9
15	27.0	27.6	22.7	22.5	22.5
16	21.4	22.4	22.7	22.5	22.5
17	21.4	22.4		92.2	
18/19				27.9	

*a)*Derived from HSQC and HMBC experiments.

*b)*Signal missing due to limited material.