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Isolation and Characterization of α,β-Unsaturated γ-Lactono-Hydrazides from *Streptomyces* sp.

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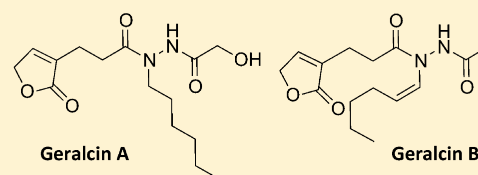
Supporting Information

ABSTRACT: Two novel α,β-unsaturated γ-lactono-hydrazides, geralcin A (**2**) and geralcin B (**3**), were isolated from *Streptomyces* sp. LMA-545. This unusual scaffold consists of the condensation of alkyl-hydrazide with an α,β-unsaturated γ-lactone, 3-(5-oxo-2*H*-furan-4-yl)propanoic acid (**1**), which was isolated from the same broth culture. Amberlite XAD-16 solid-phase extraction was used during the cultivation step, and the trapped compounds (**1**−**3**) were eluted from the resin with methanol. The structures were elucidated using ^{1}H, ^{13}C, and ^{15}N NMR spectroscopic analysis and high-resolution mass spectrometry. Geralcin B (**3**) was cytotoxic against MDA231 breast cancer cells with an IC_{50} of 5 μM.



Hydrazines and hydrazides are structural motifs often found in synthetic therapeutics that are in clinical use. An important example is iproniazid, a hydrazine monoamine oxidase inhibitor, used as an antidepressant.[1] In contrast, natural hydrazide compounds are notably scarce, and only four such structures have been reported to date. Hydrazidomycin A was isolated from the chloroform extract of *Streptomyces atratus*, together with hydrazidomycins B and C.[2] These compounds were also reported as elaiomycins B and C, respectively, isolated from the ethyl acetate extract of *Streptomyces* sp. BK190 mycelium.[3] The fourth compound reported was isolated from the seeds of *Centaurea montana* (Asteraceae) and is known as montamine.[4] The biological role of these metabolites is still unknown, and cytotoxic activities against tumor cell lines were reported. Montamine exhibited in vitro activity against a colon tumor cell line with an IC_{50} of 43.9 μM.[4] Hydrazidomycins A−C were screened against various cancer cell lines with an average IC_{50} of 0.37 μM.[2] These hydrazides were also reported as weak acetylcholinesterase and phosphodiesterase inhibitors.[5]

In this paper, we report the structural characterization of two novel alkylhydrazides produced by the bacterial strain *Streptomyces* sp. LMA-545. These compounds contain the α,β-unsaturated γ-lactone moiety 3-(5-oxo-2*H*-furan-4-yl)-propanoic acid (MH-031). MH-031 (**1**) was previously isolated from *Streptomyces* sp. A-5969 and showed hepatoprotective properties.[6] Compounds **1**−**3** were also evaluated for both antibiotic (*M. luteus*, *B. subtilis*, and *E. coli*) and cytotoxic (HCT116, MCF7, HT29, and MDA231) activities.

RESULTS AND DISCUSSION

The bacterial strain *Streptomyces* sp. LMA-545 was isolated from a soil sample collected in the Mare Longue forest, La Réunion Island (France), and was identified on the basis of analysis of 16S rRNA gene sequence. The compounds being studied were produced in a cultivation broth and recovered via in situ solid-phase extraction (SPE) using Amberlite XAD-16. Metabolic profiling of the crude extract by LC-MS with PDA and ELS detection revealed the presence of two unknown secondary metabolites (**2** and **3**). The structures were elucidated using both 1D and 2D ^{1}H and ^{13}C NMR spectroscopic analysis and high-resolution mass spectrometry. ^{1}H−^{15}N NMR experiments were required for full structural elucidation. The structures of compounds **1**−**3** are shown in Figure 1.

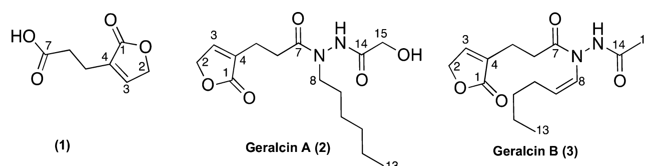


Figure 1. Structures of compounds **1**, **2**, and **3**.

3-(5-Oxo-2*H*-furan-4-yl)propanoic acid, or MH-031 (**1**), was obtained as colorless needles. High-resolution ion trap mass spectrometry indicated the molecular formula $C_7H_8O_4$. The NMR data (^{1}H and ^{13}C) were identical to those reported by Itoh et al.[6]

Compound **2** was obtained as a yellowish oil. The HRESIMS analysis gave the molecular formula $C_{15}H_{24}N_2O_5$. The NMR

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Table 1. NMR Spectroscopic Data for Compounds 2 and 3[a]

C	geralcin A (2)			geralcin B (3)		
	δ_C	δ_H (mult; J in Hz)	HMBC	δ_C	δ_H (mult ; J in Hz)	HMBC
1	174.4			174.8		
2	70.8	4.85, 2H, m[b]	C-1, 3, 4	71.5	4.74, 2H, dd (1.8, 2.9)	C-3, 4
3	147.0	7.48, 1H, m[b]	C-1, 2, 4, 5	147.5	7.24, 1H, m[b]	C-1, 2
4	132.3			132.7		
5	20.7	2.61, 2H, m[b]	C-3, 4, 6, 7	21.4	2.58, 2H, m[b]	C-1, 4, 6
6	30.5	2.47, 2H, dt (7.2, 1.6)	C- 4, 5, 7	31.9	2.52–2.62, 2H, m[b]	C-4, 5, 7
7	173.2			171.1		
8	47.4	3.58, 2H, m[b]	C-7, 9, N-2	125.4 (127.0)[d]	6.37, 1H, dd (9.0, 1.7) 6.27,[d] 1H, dd (9.0, 1.7)	C-9, N-1
9	27.2	1.52, 2H, qt (7.4)	C-8, 10, 11, N-1	122.0 (129.9)[d]	5.00, 1H, qd (9.0, 1.8) 5.35,[d] 1H, q (9.0)	C-8, 10, N-1
10	26.6	1.24–1.31, 2H, m[b]		27.5	2.03–2.10, 2H, qt (6.8)	C-8, 9, 11
11	31.8	1.24–1.31, 2H, m[b]		32.6	1.26–1.39, 2H, m[b]	C-12
12	22.7	1.24–1.31, 2H, m[b]		23.4	1.26–1.39, 2H, m[b]	C-11
13	13.9	0.86, 3H, t (6.9)	C-11, 12	14.7	0.88, 3H, m[b]	C-11, 12
14	171.8			168.7		
15	62.0	4.14, 2H, d (5.9)	C-14, H–O	20.8	2.02, 3H, s	C-14, N-2
H–N		10.44, 1H, s	C-7, 14, 15	H-N	8.01, 1H, s	C-14, N-1
H–O		5.79, 1H, t (5.9)	C-14, 15			
N-1	133.5[c], N			N-1	147.2,[c] N	
N-2	137.0,[c] NH		N-1	N-2	142.3,[c] NH	N-1

[a] ^{1}H chemical shifts were recorded at 600 MHz and ^{13}C chemical shifts at 150 MHz in DMF for compound **2** and CD_2Cl_2 for compound **3**. [b] Signals were not distinguishable. [c] ^{15}N chemical shift of the nitrogen atom δ_N in ppm. [d] NMR spectroscopic data recorded for the minor rotamer observed for compound **3**.

data for **2** are listed in Table 1. According to the molecular formula, five degrees of unsaturation should be present to account for the *α,β*-unsaturated lactone and two carbonyl groups. A characteristic IR band at 1741 cm^{-1} (O=C-NH) suggested that the two carbonyl groups are from amides. Moreover, the ^{1}H–^{15}N HMBC data revealed two nitrogen atoms at δ_N 133.5 (N-1, N) and 137.0 (N-2, NH). The correlations of H–N (δ_H 10.44, s, NH) to N-1 (δ_N 133.5, N) and H-8 (δ_H 3.58, m) to N-1 (δ_N 133.5, N) demonstrated that a N–N bond corresponding to a hydrazide group was present. The ^{13}C NMR revealed the presence of 15 carbon atoms, which included three carbonyl atoms at δ_C 174.4 (C-1), 173.2 (C-7), and 171.8 (C-14); two methine carbons at δ_C 147.0 (C-3) and 132.3 (C-4); nine methylene groups, which included two oxygen-bound methylenes at δ_C 70.8 (C-2) and 62.0 (C-15); and one methyl carbon at δ_C 13.9 (C-13). The $^nJ_{1H-13C}$ connectivities given by HSQC and HMBC NMR are listed in Table 1.

The key structural elements revealed by ^{1}H NMR were the singlet at δ_H 10.44 (H–N, s) associated with a nitrogen atom (^{15}N-HSQC in the Supporting Information) and a broad triplet at δ_H 5.79 (H–O, t, 5.9), linked to an oxygen atom and coupling with a methylene group at δ_H 4.14 (H-15, d, 5.9). The multiplet at δ_H 7.48 (H-3, m) was assigned to a double bond coupled to the methylene group at δ_H 4.85 (H-2, m). The ^{1}H–^{1}H COSY correlations indicated a pair of vicinal methylene groups at δ_H 2.61 (H-5, m) and 2.47 (H-6, td, 7.2, 1.6). A set of three methylene groups, with a broad signal between 1.24 and 1.31 ppm, was assigned to the aliphatic methylene chain (C-10 to C-12). This chain was connected to a pair of methylene groups at δ_H 1.52 (H-9, qt, 7.4) and 3.58 (H-8, m) and ended with a methyl group characterized by the triplet at δ_H 0.86 (H-13, t, 6.9). The ^{1}H–^{13}C HMBC connectivities of H-2 to, in particular, C-1/C-4 and H-3 to C-1/C-4/C-5 allowed for the construction of the *α,β*-unsaturated *γ*-lactone moiety (Figure 2a). The connectivities between H-5, H-6 and H-8, H-9 were

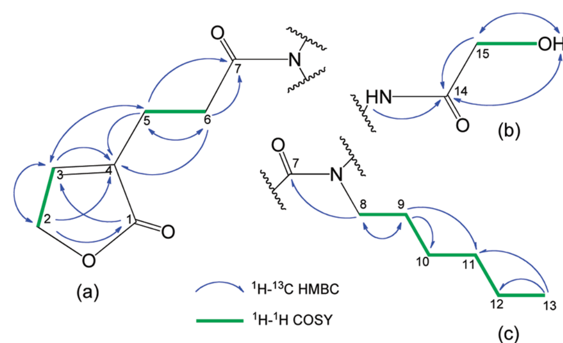

Figure 2. Substructures and 2D-NMR connectivities of compound **2**.

clearly deduced from NMR data recorded at different temperatures. Spectra recorded at room temperature gave broad signals for H-5, H-6 and H-8, H-9, whereas ^{1}H–^{13}C HMBC data obtained at high temperature (323 K) supported the connection of H-5 to C-4 and H-6 to C-7. The hydroxymethylene group at C-15 was linked to the carbonyl group at C-14. The ^{1}H–^{13}C HMBC data also accounted for the connectivities from H–N (δ_H 10.44, s, NH) and H–O (δ_H 5.79, t, OH) to C-14 (Figure 2b). Moreover, the aliphatic chain (C-8 to C-13, Figure 2c) was attached to C-7 of substructure 2a through the nitrogen (Figure 2). These results are in agreement with the bond between C-8 and C-7 deduced from the ^{1}H–^{13}C HMBC data. The chemical shift for C-8 (δ_C 47.4) confirmed the presence of a nitrogen atom in the local environment.

Finally, the ^{1}H–^{15}N HMBC experiments confirmed the position of the two nitrogen atoms and the connectivities

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between the three substructures, particularly parts 2a and 2b (Figure 2).

The overall structural assignments led to compound **2**, shown in Figure 3. This compound consists of a novel natural scaffold that connects an alkylhydrazide to an α,β-unsaturated γ-lactone.

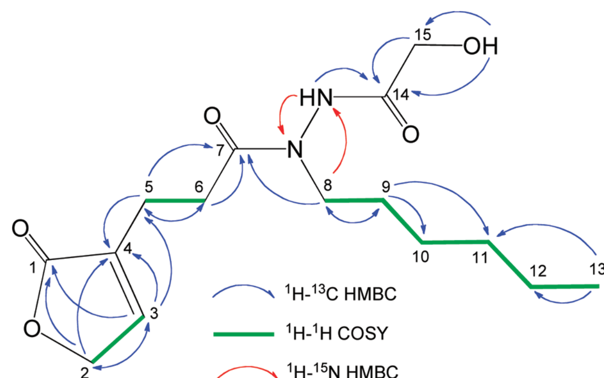

Figure 3. Structure and selective 2D-NMR connectivities of compound **2**.

Compound **3** was obtained as a yellowish oil. The HRESIMS analysis provided the molecular formula $C_{15}H_{22}N_2O_4$. The NMR data for **3** (Table 1) showed a number of similarities to those for **2**. The molecular weight of **3** had a difference of 18 Da compared to **2**. Six degrees of unsaturation were deduced from the molecular formula and accounted for by an α,β-unsaturated lactone, two carbonyl groups (δ_C 168.7 and 171.1), and two olefinic carbons (δ_C 122.0 and 125.4). A characteristic IR band at 1743 cm^{-1} (O=C-NH) suggested that the two carbonyl groups should be amide type, as found for compound **2**. Moreover, the $^1H-^{15}N$ HMBC data, recorded in CH_2Cl_2 at room temperature, revealed two nitrogen atoms at δ_N 147.2 (N-1, N) and 142.3 (N-2, NH). The correlations of H−N (δ_H 8.01, s, NH) to N-1 (δ_N 147.2, N) and H-8 (δ_H 6.37, dd, 9.0, 1.7) to N-1 (δ_N 147.2, N) demonstrated that a N−N bond corresponding to a hydrazide group was also present.

The ^{13}C NMR spectrum of **3** revealed 15 carbon atoms including three carbonyl groups at δ_C 174.8 (C-1), 171.1 (C-7), and 168.7 (C-14); two methine carbons at δ_C 147.5 (C-3) and 132.7 (C-4); six methylene groups, which included one oxygen-bound methylene at δ_C 71.5 (C-2); and two methyl carbons at δ_C 14.7 (C-13) and 20.8 (C-15). The complete carbon assignment was deduced from the $^nJ_{1H-13C}$ connectivities obtained from the HSQC-NMR and the HMBC-NMR spectra (Table 1).

A comparison of compound **3** with compound **2** indicated two notable differences, involving the C-8, C-9, and C-15 carbons. The ^{13}C NMR spectrum showed that two of the methylene carbons (δ_C 27.2 and 47.4) were replaced by the two olefinic signals at δ_C 122.0 (δ_C 129.9) and 125.4 (δ_C 127.0). The HMBC spectra indicated the connection of H-8 to C-9 and H-9 to C-10 and placed the double bond between C-8 and C-9. The two methine proton signals at δ_H 6.37 (6.27) (H-8, dd, 9.0, 1.7) and 5.00 (5.35) (H-9, dq, 9.0, 1.8) corroborated this observation. Duplication of the ^1H and ^{13}C NMR signals for the double bond was observed for compound **3** only. This phenomenon was due to the equilibrium between amide rotamers:[7] doubling of some signals present in the ^{13}C spectrum obtained at room temperature was typical of a conformational equilibrium that was slow compared with the NMR time-scale. The presence of two rotamers was confirmed by equilibrium spots present in the $^1H-^1H$ NOESY spectrum (Supporting Information): these are EXSY correlations. The two forms were found to have a 70/30 ratio at room temperature, favoring the amide form, drawn as compound **3**. The existence of a predominant form could be due to stabilization by hydrogen bonding between H−N (δ_H 8.01) and the oxygen atom in the carbonyl group at C-7. No duplication of the ^1H and ^{13}C NMR signals due to such an equilibrium between potential rotamers was recorded for compound **2**. Possible supplementary stabilization by hydrogen bonding between the hydrogen H−O (δ_H 5.79) and the oxygen atom in the carbonyl group at C-7 could inhibit interconversions for the amide moiety, resulting in one unique rotamer. All the spectra for compound **2** were recorded in DMF-d_7, which might stabilize compound **2**, in particular the amide moiety, thus producing a unique rotamer.

The coupling constant of 9.0 Hz between H-8 and H-9 of compound **3** indicated a cis configuration for the C-8/C-9 double bond. The ^{13}C NMR spectrum also revealed the absence of a hydroxy-methylene signal and the presence of a methyl group at δ_C 20.8. The correlation of H-15 to C-14 located this methyl group at the α-position of the C-14 carbonyl group. The structure of **3** is presented in Figure 4.

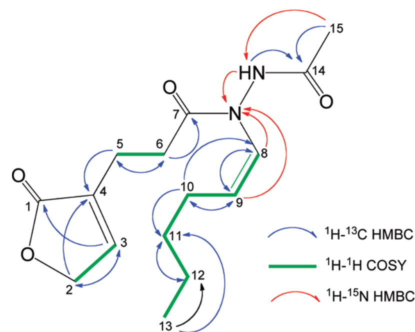

Figure 4. Structure and selective 2D-NMR connectivities of compound **3**.

Thus, compounds **2** and **3** represent a new natural scaffold combining alkylhydrazide and α,β-unsaturated γ-lactone moieties. Preliminary antibiotic and cytotoxic studies were conducted as previously reported.[8] None of the compounds demonstrated antibacterial activity. In vitro tumor-cell growth inhibition was tested using cell lines HCT116, MCF7, HT29, and MDA231. Geralcin B (**3**) inhibited the growth of MDA231 breast cancer cells with an IC_{50} of 5 μM (Supporting Information).

Natural γ-butyrolactone derivatives are notably common in natural products.[9] A significant γ-butyrolactone found in certain strains of *Streptomyces* is A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone), which triggers secondary metabolism and morphogenesis in *Streptomyces*.[9,10] The biosynthesis of A-factor involves the ketoacyl transfer from an 8-methyl-3-oxononanoyl-acyl carrier protein to the hydroxyl group of dihydroxyacetone phosphate, which was catalyzed by the enzyme AfsA.[9] This enzyme resembles fatty acid synthase in mammals.[10] A-factor was obtained via the intramolecular aldol condensation of the AfsA reaction product and the subsequent reduction of the α,β-unsaturated lactone.[9] Geralcins A and B may be derived from the condensation of their corresponding

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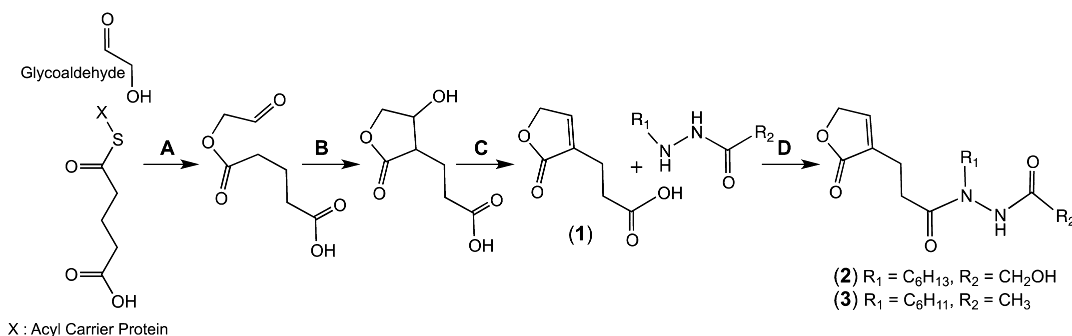

Figure 5. Hypothetic biosynthetic pathway of compounds **1**, **2**, and **3**. (A) Condensation of glucoaldehyde to glutaric acid catalyzed by an AfsA-like protein as reported in ref 9. (B) Aldol condensation. (C) Dehydration catalyzed by a dehydratase. (D) Condensation as reported in ref 11.

hydrazines with the carboxyl group of **1** (Figure 5). The enzymatic catalysis of such a reaction has been reported in the literature and involves hydrolytic enzymes, such as lipases or amidases.[11]

EXPERIMENTAL SECTION

General Experimental Procedures. The IR spectra were obtained using a Perkin-Elmer Spectrum 100 model instrument. NMR experiments were performed using a Bruker Avance 600 MHz spectrometer equipped with a microprobe head (1.7 TXI, Bruker) for compounds **2** and **3** and a Bruker Avance 500 MHz spectrometer for compound **1**. The spectra for compound **2** were acquired in DMF-d_7 (δ_H 2.75, 2.92, 8.03 ppm; δ_C 29.74, 34.89, 163.15 ppm) at 253 K, room temperature, and 323 K to observe all of the correlations present. The spectra for compound **3** were obtained in methylene chloride-d_2 (δ_H 5.32; δ_C 54.0 ppm) at room temperature. The $^1H-^{15}N$ HMBC for compound **3** was performed in DMF-d_7 (δ_N 105.0 ppm) at 253 K.

LC-MS experiments were performed using a Waters-Micro-massZQ2000 simple-stage quadrupole mass spectrometer equipped with an ESI (electrospray ionization) interface coupled to an Alliance Waters 2695 HPLC instrument with PDA and ELS detection.

HRESIMS was conducted using a Waters-Micromass mass spectrometer equipped with an ESI-TOF (electrospray-time-of-flight).

Biological Materials. *Streptomyces* sp. LMA-545 was isolated from a soil sample collected in La Réunion Island and grown on a PDB agar (potato dextrose broth, DIFCO) at 30 °C. The microorganism was examined for chemotaxonomic and morphological properties known to be useful in the systematics of *Streptomyces.* A phylogenetic analysis was performed using a fragment of the 16S rRNA gene amplified from the genomic DNA of *Streptomyces* sp. LMA-545. The 16S rRNA gene amplification and sequencing were performed, and the resulting material was compared to the corresponding sequences in the related *Streptomyces* using the NCBI/BLAST Web site (GenBank): *Streptomyces* sp. LMA-545. The primers used for PCR amplification were 16 S F 27: AGA GTT TGA TC(AC) TGG CTC AG (T_m: 56.3 °C) and 16 S R 1492: TAC GG(CT) TAC CTT GTT ACG ACT T (T_m: 57.5 °C). The GenBank accession number for *Streptomyces* sp. LMA-545 sequence is BankIt-1535712 8404357.seq JX025158.

Fermentation. Batch fermentation of *Streptomyces* sp. LMA-545 was conducted in a 15 L fermentor (Chemap 20 L unit) in a PDB medium over 5 days at 30 °C with an aeration rate of 16 volumes of air per volume per minute and 200 rpm agitation. The initial pH of 7.2 was progressively decreased to 4.3. Amberlite XAD-16 (30 g/L) was added prior to sterilization to allow the in situ trapping of the microbial metabolites.

Isolation. The XAD-16 resin was separated from the broth culture via filtration and washed with water before being eluted with MeOH (500 mL). The eluate was concentrated to dryness in vacuo (5.4 g) and extracted with MeOH. The crude extract (4.7 g) was subjected to flash chromatography on a Combiflash Companion using a Redisep 80 g silica column, with a heptane−ethyl acetate mixture serving as the eluent. The fractions containing compounds **1**−**3** were separated as pure compounds by preparative RP-HPLC (Sunfire Prep C_{18} 5 μm, 10 × 250 mm) eluted using a linear H_2O-CH_3CN gradient supplemented with 0.1% formic acid (100−0 to 0−100). After concentrating in vacuo, compound **1** (118 mg) was obtained as translucent white needles, while compounds **2** (7 mg) and **3** (5 mg) were obtained as yellowish oils.

MH-031 (1): white needles (Hept/AcOEt); UV λ_{max} 225 nm; IR ν_{max} 3184, 1744, 1710, 1652, 1431, 1406, 1355, 1209, 1056 cm^{-1}; ^{1}H NMR (CD$_3$OD 500 MHz) 7.23 (1H, td, J = 1.7 Hz), 4.80 (2H, dd, J = 1.7, 1.7 Hz), 2.69 (2H, m), 2.66 (2H, m); ^{13}C NMR (CD$_3$OD, 125 MHz) 176.6 (qC), 176.1 (qC), 148.4 (qC), 133.2 (CH), 72.2 (CH$_2$), 32.6 (CH$_2$), 21.9 (CH$_2$); HRESIMS m/z [M − H]$^-$ 155.0346 (calcd for $C_7H_7O_4$, 155.0344).

Geralcin A (2): yellowish oil; UV λ_{max} 236 nm; IR ν_{max} 3430, 3275, 2959, 2929, 2860, 1741, 1647, 1425, 1080, 1050 cm^{-1}; see Table 1 for complete NMR data; HRESIMS m/z [M + Na]$^+$ 335.1575 (calcd for $C_{15}H_{24}N_2O_5Na$, 335.1583).

Geralcin B (3): yellowish oil; UV λ_{max} 246 nm; IR ν_{max} 3267, 2961, 2931, 2869, 1743, 1660, 1449, 1375, 1083, 1050 cm^{-1}; For complete NMR data see Table 1; HRESIMS m/z [M + H]$^+$ 295.1612 (calcd for $C_{15}H_{23}N_2O_4$, 295.1658)

Antibacterial and Antitumor Cell Assays. The antibacterial activity was measured using the disk inhibition zone method against *Bacillus subtilis* ATCC.6633, *Micrococcus luteus* ATCC.10240, and *Escherichia coli* ATCC.25922. Inhibition was compared for 10 μg of gentamicin and 30 μg of chloramphenicol.

Cytotoxicity Assays. A tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT]-based colorimetric assay was used to measure the inhibition on the proliferation of the colonic epithelial cancer cell line HCT116, the hormone-responsive breast cancer cell line MCF7, the colon adenocarcinoma cell line HT29, and the breast cancer cell line MDA231, as previously reported.[12] All of the test compounds were formulated in DMSO and added to the cells such that the final DMSO concentration ranged from 1% to 3%. Cells were grown in D-MEM medium supplemented with 10% fetal calf serum (Invitrogen), in the presence of penicillin, streptomycin, and fungizone, and plated in 96-well microplates. After 24 h of growth, cells were treated with target compounds from 100 μM to 10 nM. After 72 h, MTS reagent (Promega) was added, and the absorbance was monitored (490 nm) to measure the inhibition of cell proliferation compared to untreated cells. IC$_{50}$ determination experiments were performed in separate duplicate experiments.

ASSOCIATED CONTENT

Supporting Information

The experimental section, physicochemical properties, 1D and 2D NMR spectroscopic data, IR data, and high-resolution Orbitrap-ESIMS are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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