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Solphenazines A–F, Glycosylated Phenazines from *Streptomyces* sp. Strain DL-93

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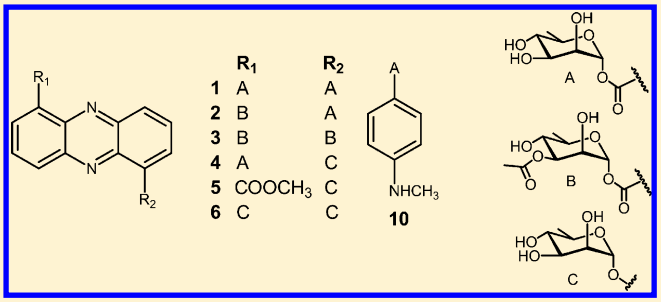
Supporting Information

ABSTRACT: During a survey of actinobacteria known to suppress the growth of *Streptomyces scabies* (the causative agent of potato scab disease) *in vivo*, six new rhamnosylated alkaloids, the solphenazines A–F (**1**–**6**), were isolated from a biological control strain of *Streptomyces* (DL-93). The known rhamnosyl analogue of paraben (**9**) was also isolated along with a new rhamnosylated derivative of *N*-methyl-*p*-aminobenzoic acid (**10**). None of the compounds exhibited any antibacterial or antifungal activity against a standard panel of microorganisms, but compounds **1**, **2**, and **6** displayed some cytotoxicity against HCT-116 cancer cells. Additional *in vitro* testing provided data suggesting that the cytotoxic activity is not due to DNA intercalation or topoisomerase inhibition.



The phenazines are a group of heterocyclic, nitrogen-containing compounds isolated primarily as natural products from *Pseudomonas* and *Streptomyces* bacteria as well as a single species of Archaea.[1] Natural and synthetic phenazines exhibit a wide range of biological activities including antibiotic, antiparasitic, cytotoxic, biofilm modulation, plant growth promotion, and virulence enhancement activities.[2,3] Simple substituted phenazines produced by pseudomonads play an important role in the suppression of many fungal phytopathogens, and several strains of phenazine-producing *Pseudomonas* are currently used as biological control agents in field applications.[4,5] More recently, phenazines have been implicated as redox-active compounds that may assist in the acquisition of biologically available iron in limiting environments.[6,7]

During a survey of actinobacteria known to suppress the growth of *Streptomyces scabies* (the causative agent of potato scab disease) *in vivo*, we identified a suite of novel glycosylated alkaloids, the solphenazines A–F (**1**–**6**) along with two known analogues, izuminosides C (**7**) and A (**8**), from a strain of *Streptomyces* (DL-93[8]). Compounds **1**–**4** and **6** are the first examples of diglycosylated phenazine natural products. The known rhamnosyl analogue of paraben (**9**) was also isolated along with a new rhamnosylated derivative of *N*-methyl-*p*-aminobenzoic acid (**10**). None of the compounds exhibited any inhibitory activity when tested against *S. scabies* and a panel of bacterial and fungal pathogens, suggesting that other antibacterial compounds are produced when grown in association with potatoes or *S. scabies* *in vivo*. However, several compounds (solphenazines A (**1**), B (**2**), and F (**6**)) did exhibit weak to moderate cytotoxicity against two mammalian cell lines. In order to explore the potential mechanism of cancer cell cytotoxicity, the active compounds were tested using *in vitro* topoisomerase and DNA intercalation assays but were inactive.

RESULTS AND DISCUSSION

NMR analysis of the ethyl acetate and butanol extracts from both the culture supernatant and mycelium suggested that they contained a suite of related aromatic compounds. Solphenazine A (**1**) was isolated as a yellow solid with a molecular formula of $C_{26}H_{28}N_2O_{12}$ established from a high-resolution ESIMS measurement of the $[M + H]^+$ peak at m/z 561.1715. Since the NMR data (Table 1) indicated the presence of only 13 carbons and 14 protons, it was apparent that **1** was a symmetrical dimer. Analysis of the aromatic region of the proton spectrum revealed the presence of an isolated ABC spin system with signals at δ 8.42 (d, J = 6.6 Hz), 8.03 (dd, J = 9.0, 6.6 Hz), and 8.64 (d, J = 9.0 Hz). The HMBC spectra indicated that two of these protons (δ 8.42 and 8.64) were coupled to the same quaternary carbon at δ 140.8, and the third proton was correlated to a different quaternary carbon at δ 143.0. The diagnostic UV absorbance peaks (λ_{max} = 240, 250, and 360 nm) combined with these NMR data and inclusion of two nitrogen atoms based on the molecular formula suggested that compound **1** was a disubstituted symmetrical phenazine.

Analysis of the COSY spectrum revealed one contiguous spin system formed by correlations of five oxygen-bearing methines and one doublet methyl signal at δ 1.38 indicating a deoxyhexapyranose. The small coupling between the anomeric proton δ 6.56 (s) and H-2′ at δ 4.10 (brs) suggested that H-1′ and H-2′ were in equatorial positions. Further analysis of the coupling constants for the remaining methines allowed assignment of

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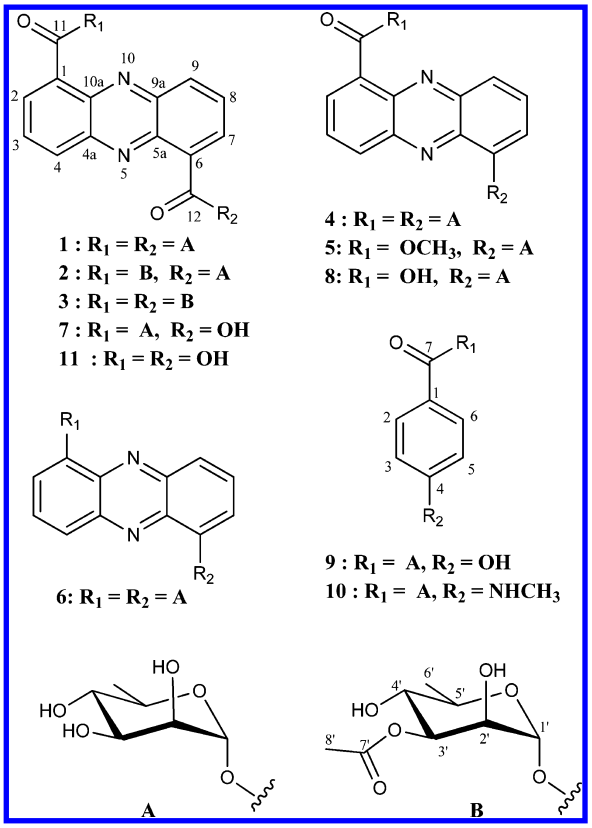
Table 1. ^{1}H and ^{13}C Data for Solphenazines A–C (1–3)

	δ_H and δ_C (ppm), mult. (J in Hz)					
	1		2		3	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	131.1		130.5		130.8	
2	133.9	8.42, d (6.6)	134.1	8.45, d (7.2)	134.2	8.45, d (7.2)
3	130.4	8.03, dd (9.0, 6.6)	130.1	8.03, dd (8.4, 7.2)	130.5	8.01, dd (8.9, 7.2)
4	134.4	8.64, d (9.0)	134.4	8.64, d (8.4)	134.6	8.71, d (8.9)
4a	143.0		142.9		142.8	
5a	140.8		140.5		140.6	
6	131.1		130.9		130.8	
7	133.9	8.42, 255	133.7	8.40, d (6.6)	134.2	8.45, d (7.2)
8	130.4	8.03, dd (9.0, 6.6)	129.9	7.99, dd (8.4, 6.6)	130.5	8.01, dd (8.9, 7.2)
9	134.4	8.64, d (9.0)	134.4	8.69, d (8.4)	134.6	8.71, d (8.9)
9a	143.0		142.9		142.8	
10a	140.8		140.5		140.6	
11	165.3		165.0		165.1	
12	165.3		165.3		165.1	
1′	95.8	6.56, s	95.0	6.33, s	95.1	6.35, s
2′	70.0	4.10, brs	71.5	4.24, brs	68.9	4.24, s
3′	71.2	4.00, dd (8.4, 1.8)	74.0	5.25, dd (9.6, 3.0)	73.9	5.27, dd (9.6, 3.3)
4′	72.4	3.58, t (9.6)	69.5	3.78, dd (10.2, 9.6)	69.4	3.79, t (9.6)
5′	71.8	4.18, m	71.6	4.31, m	71.2	4.31, m
6′	17.2	1.38, d (6.0)	17.0	1.39, d (6.0)	16.6	1.39 d (6.0)
7′			171.2		171.2	
8′			20.0	2.10, s	20.0	2.10, s
1″	95.8	6.56, s	95.6	6.35, s	95.1	6.35, s
2″	70.0	4.10, brs	71.5	4.09, brs	68.9	4.24, s
3″	71.2	4.00, dd (8.4, 1.8)	70.7	3.99. dd (9.6, 3.0)	73.9	5.27, dd (9.6, 3.3)
4″	72.4	3.58, t (9.6)	72.3	3.57, dd (9.6, 9.0)	69.4	3.79 t (9.6)
5″	71.8	4.18, m	71.5	4.18 m	71.2	4.31, m
6″	17.2	1.38, d (6.0)	17.5	1.32 d (6.0)	16.6	1.39, d (6.0)
7″					171.2	
8″					20.0	2.10, s

H-3′, H-4′, and H-5′ as axial protons (Table 1) and determination of the sugar unit as α-rhamnose. The stereochemistry of the rhamnose moieties was determined by cleavage of the sugars and subsequent analysis by CD spectroscopy. The Cotton effect in the CD spectrum of the sugar was negative and matched that of an authentic sample of L-rhamnose (Figure S67). This result was also supported by the optical rotation values of the hydrolyzed sugars compared to the standard ($[\alpha]^{24}_{D}$ −32 (*c* 0.025 in MeOH) and −16 (*c* 1 in MeOH), respectively).

The connection of the sugar to the phenazine core via an ester bond was established at the C-1 position by the HMBC correlations between H-2 (δ 8.42) and an ester carbonyl (C-11) signal at δ 165.3. The remaining rhamnose ester could be connected at either the C-6 or C-9 position. The cleavage of the ester-linked sugars yielded a dicarboxylic acid phenazine, which matched the published spectroscopic properties of the 1,6-substituted derivative **11**.[8−10] Since some phenazine



substitutions have been incorrectly assigned in the literature due to reliance on similar hydrolysis derivatives,[9] we performed a ^{15}N HMBC experiment with an asymmetrical dirhamnosyl derivative (**4**, see below), which corroborated the 1,6 substitution and was assumed for all of the analogues (**1**–**6**) in this series. Solphenazine A (**1**) was therefore identified as 1,6-dicarbo-α-L-rhamnosyloxyphenazine.

The molecular formula of solphenazine B (**2**) was established as $C_{28}H_{30}N_2O_{13}$ on the basis of the HRESI peak at *m*/*z* 603.1817 ($[M + H]^+$). Analysis of the NMR data clearly indicated that **2** was closely related to solphenazine A (**1**), but was asymmetrical due to the presence of six aromatic protons, 10 protons attached to oxygenated carbons, two methyl doublets, and a methyl singlet. The additional C_2H_2O in the molecular formula and methyl singlet in the proton NMR spectrum suggested the presence of an acetate group. This was confirmed by HMBC correlations between the methyl protons (δ 2.10) and the downfield shifted C-3′ sugar proton (δ 5.25) with the same ester carbonyl signal at δ 171.2, placing the acetate at the C-3′ position on one of the rhamnosyl sugars to give 1-(3-*O*-acetylcarbo-α-L-rhamnosyloxy)-6-carbo-α-L-rhamnosyloxyphenazine.

Solphenazine C (**3**) was found to have a molecular weight of 644 by low-resolution mass spectrometric analysis, 42 mass units higher than solphenazine B (**2**), suggesting an additional acetate unit. The carbon and proton NMR data indicated that the structure was symmetrical with only a single set of three coupled aromatic signals, five methines, and two methyl proton signals. The additional acetate ester was therefore assigned to the symmetrical C-3″ position on the second rhamnosyl to give 1,6-di(3-*O*-acetylcarbo-α-L-dirhamnosyloxy)phenazine (**3**). The diacetate derivative was unstable and readily degraded into the monoacetate analogue solphenazine B (**2**) within 12 h.

Solphenazine D (**4**) was isolated as a yellow solid with a molecular formula of $C_{25}H_{28}N_2O_{11}$ established from the high-resolution

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Table 2. ^{1}H and ^{13}C Data for Solphenazines D−F (4−6)

	δ_H and δ_C (ppm), mult.(J in Hz)					
	4		5		6	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	132.5		131.1		154.2	
2	134.0	8.36, d (7.2)	132.1	8.46 dd (8.6)	113.5	7.52, d (6.0)
3	129.5	7.99, dd (8.4, 7.2)	128.8	7.96, dd (8.6, 7.9)	131.5	7.83, dd (9.0, 6.0)
4	133.2	8.51, d (8.4)	132.9	8.28, dd (6.6, 1.3)	122.0	7.86, d (9.0)
4a	141.8		141.4		143.8	
5a	137.0		136.1		136.8	
6	152.0		151,6		154.2	
7	114.5	7.62, d (7.8)	113.5	7.98, d (6.6)	113.5	7.52, d (6.0)
8	134.0	7.89, dd (8.4, 7.8)	130.8	7.88, dd (6.6, 5.6)	131.5	7.83, dd (9.0, 6.0)
9	124.2	8.12, d (8.4)	123.8	7.61, d (5.6)	122.0	7.86, d (9.0)
9a	144.6		144.2		143.8	
10a	141.5		140.5		136.8	
11	165.8		167.3			
1′	95.6	6.33, s			99.7	5.85, s
2′	70.8	4.10, brs			70.7	4.40, brs
3′	71.2	3.96, dd (9.6, 3.0)			71.2	4.30, dd (9.6, 3.0)
4′	72.3	3.57, t (9.6)			72.4	3.57, t (9.6)
5′	72.5	4.15, m			70.0	3.92, m
6′	17.5	1.36, d (6.0)			16.6	1.22, d (6.0)
1″	101.5	5.84, s	99.8	5.82, s	99.7	5.85, s
2″	70.0	4.36, brs	70.6	4.37, d (3.0)	70.7	4.40, brs
3″	71.5	4.22, dd (9.6, 3.6)	71.0	4.21, dd (9.2, 3.0)	71.2	4.30, dd (9.6, 3.0)
4″	72.5	3.55, t (9.6)	72.4	3.54, dd (9.9, 9.2)	72.4	3.57, t (9.6)
5″	71.8	3.80, m	69.8	3.80, m	70.0	3.92, m
6″	17.0	1.24, d (6.0)	16.6	1.24, d (6.0)	16.6	1.22, d (6.0)
O-Me			51.6	4.08, s		

ESI/APCI MS peak at m/z 533.1756 $[M + H]^+$. Analysis of the NMR data indicated the presence of two rhamnosyl sugars and a disubstituted asymmetrical phenazine. However, the formula contained one less CO group than solphenazine A (**1**), and the HMBC spectrum included only one downfield carbonyl peak, confirming the presence of a single rhamnosyl ester at the C-1 position. Compared to the ester-linked rhamnosyl moieties in compounds **1**−**3**, the anomeric proton on the second rhamnosyl of **4** was shifted upfield (δ 5.84) and correlated to a downfield shifted sp^2 quaternary carbon (C-6, δ 152.0). The second rhamnosyl sugar was therefore connected by an ether bond at the C-6 position. The ^{15}N HMBC spectrum (Figure S24) of this compound showed single three-bond correlations of H-4 to a nitrogen at δ_N 258.0 (N-5) and H-9 to the second nitrogen at δ_N 261.5 (N-10), confirming the position of the ester (C-1) and ether (C-6) moieties on the phenazine core unit to give 1-(carbo-α-L-rhamnosyloxy)-6-α-L-rhamnosyloxyphenazine.

Solphenazine E (**5**) had a molecular formula of $C_{20}H_{20}N_2O_7$ based on the measurement of the HRESI peak at m/z 401.1355 $[M + H]^+$. The proton NMR spectrum was almost identical to that of **4**. Instead of the rhamnosyl at C-1, a methoxy group at δ 4.08 was assigned by HMBC correlations of a carbonyl to the methoxy and H-2 (δ 8.46). Further analysis of the HMBC, HMQC, and COSY data indicated that the remainder of the structure was identical to solphenazine D (**4**) to give 1-carbomethoxy-6-α-L-rhamnosyloxyphenazine.

The molecular formula of solphenazine F (**6**) was $C_{24}H_{28}N_2O_{10}$ established from a high-resolution ESIMS measurement of the $[M + H]^+$ peak at m/z 505.1806. Analysis of the NMR data indicated that **6** was a symmetrical disubstituted phenazine containing two rhamnose moieties. The lack of carbonyl signals in the carbon spectrum and HMBC correlations between the anomeric sugar protons (H-1′ and H-1″) and sp^2 quaternary carbons on the phenazine core (C-1 and C-6) confirmed the identity of solphenazine F (**6**) as 1,6-di-α-L-rhamnosyloxyphenazine.

The known monoglycosylated analogues izuminosides C (**7**) and A (**8**) were identified by comparison of the mass spectrometric and NMR data to published values.[10,11]

Compounds **9** and **10** were isolated from HPLC fractions adjacent to the phenazine compounds but were clearly not members of the phenazine class on the basis of the UV and NMR data. Comparison of the mass spectra, proton NMR shifts, and optical rotation data allowed the identification of compound **9** as 4-hydroxybenzoyl-α-L-rhamnopyranoside, previously identified through feeding 4-hydroxybenzoic acid or 4-hydroxyphenylglycine to cultures of *S. griseoviridis* (strain Tü 3634).[12,13] Compound **10** was a closely related analogue with a molecular formula of $C_{14}H_{19}NO_6$ based on the measurement of a HRESIMS peak at m/z 298.1276 $(M + H)^+$. Analysis of the NMR data revealed the same α-rhamnose connected to a para-substituted aromatic ring via an ester bond, and the optical rotation ($[\alpha]^{24}_D = -56$ (c 0.025 MeOH)) confirmed the L configuration of the sugar. The additional NCH_3 and loss of one oxygen in the molecular formula suggested that the ring was substituted by an amino methyl moiety. This was confirmed by analysis of the HMBC data, which showed correlations from the methyl proton (δ 2.83, s) to an aromatic carbon (C-4, δ 155.2). Compound **10** is therefore 4-methylaminobenzoyl-α-L-rhamnopyranoside, a new member of the acyl rhamnoside class of natural products.

Although *Streptomyces* sp. DL-93 inhibits the *S. scabies* potato pathogen *in vivo*,[8] crude extracts of the strain grown in liquid culture were not inhibitory, and none of the pure compounds reduced the growth of *S. scabies* in a disk diffusion assay (25 μg/disk). The compounds also did not inhibit any members of a microbial pathogen panel in broth dilution assays (MIC > 25 μg/mL, see Experimental Section for test strains). These results are in contrast to the potent antimicrobial activities of phenazines produced by biological control strains of *Pseudomonas* used in agricultural systems for pathogen suppression.[14]

When tested against mammalian cell lines, solphenazine A (**1**) was moderately cytotoxic against HCT-116 cancer cells (EC_{50} = 18 μM) and less active against Vero cells (EC_{50} = 84 μM). Solphenazines B (**2**) and F (**6**) had lower activity against HCT-116 cells (EC_{50} = 52 and 45 μM, respectively) and Vero cells (inhibited growth by 30−40% at 150 μM), while compounds **4**, **5**, **7**, **8**, and **11** were inactive. The planar structures, activities of published analogues, and selective activity toward cancer cells suggested that the active compounds might interact with DNA or cell replication enzymes such as topoisomerases.[15] Additional *in vitro* assays showed that **1** does not intercalate DNA and does not inhibit Topo I catalytic activity or act as a Topo I or Topo II poison (Figures S68 and S69).[16,17] Solphenazine A (**1**) weakly inhibited the catalytic

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activity of eukaryotic Topo II (EC_{50} = 253 ± 41 μM, etoposide EC_{50} = 116 ± 9 μM, Figure S70) but was inactive toward prokaryotic type II topoisomerase (*E. coli* Topo IV, Figure S71). Compounds **2**, **4**, **6**, **7**, and **11** were inactive in the Topo II decatenation assay (EC_{50} > 250 μM). Collectively, the results of the *in vitro* experiments suggest that the moderate cytotoxic activities exhibited by solphenazine A (**1**) are not due to DNA intercalation or interactions with topoisomerases.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Analytical Autopol III polarimeter. IR spectra were obtained using a JASCO 4100 FT-IR spectrophotometer. Mass analyses were performed using an Agilent TOF II TOF/MS spectrometer with a dual ESI and APCI source. A JASCO 200 system was used to record the CD spectra. Standard 1D and 2D NMR spectra were recorded on a Varian 600 MHz spectrometer in CD_3OD. Proton and carbon chemical shifts are reported in ppm and referenced with the ^1H and ^{13}C signals of residual methanol (δ 3.31 and 49.9 ppm, respectively). The ^{15}N HMBC data were obtained using a Bruker 700 MHz NMR equipped with a 5 mm TXI cryoprobe and Z gradient. Flash chromatography separations were performed using a Teledyne ISCO Combiflash Rf system. TLC separations were performed using Whatman silica gel 60 F_{254} aluminum-backed TLC plates. Sephadex LH-20 (GE Healthcare) and silica gel 60 (230−400 mesh, Merck) were used as the stationary phases for column chromatography. HPLC separations were performed with an Agilent 1200 instrument.

Microorganisms and Susceptibility Testing. *Streptomyces* sp. DL-93 (Genbank AY277380) was a gift from Professor Linda Kinkel and originally isolated from the surface of a potato tuber grown in soil from a scab-conducive agricultural plot in Grand Rapids, Minnesota.[8] The isolate was identified as a species of *Streptomyces* based on morphological characteristics and analysis of 16s rRNA sequence data suggesting that it is most closely related to *S. nojiriensis* LMG 20094 (NR_042303.1) and *S. spororaveus* strain LMG 20313 (NR_042306.1) (99% similarity out of 1399 base pairs). A seed culture (50 mL) of the strain was grown for 3 days in ISP2 medium and inoculated into 500 mL of the same medium in (2) 2 L flasks fitted with steel springs and cultured for 7 days with shaking at 30 °C.

Microbial susceptibility testing was performed using a standard microbroth dilution assay[18] with the following strains purchased from the American Type Culture Collection (ATCC): Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, vancomycin-resistant *Enterococcus faecalis* (VRE) ATCC 51299, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumonia* ATCC 13883, *Cryptococcus neoformans* ATCC 66031, and *Candida albicans* ATCC 10231.

Compounds were also tested against two strains of *Streptomyces scabies* (NRRL B 2795 and LK 82) using a double-layer agar method.[8] Briefly, *S. scabies* strains grown in liquid ISP2 for 5 days were diluted in molten ISP2 agar 1:10. The test compounds (25 μg) dissolved in 20 μL of methanol were added to sterile paper discs, and the discs were placed on Petri dishes over a thin layer of agar. Then 10 mL of the *S. scabies* agar suspension was poured over the discs and allowed to solidify. The plates were incubated for 2 days at 30 °C, and the diameters of the zones of inhibition were measured.

MTT Cell Viability (Cytotoxicity). Cytotoxicity assays were performed with Vero green monkey kidney cells (ATCC CCL-81) and human colon tumor cells HCT 116 (ATCC CCL-247). Cells were grown in MEM (Vero) and RPMI (HCT-116) media supplemented with 10% fetal bovine serum, 1% penicillin−streptomycin (5 mg/mL), and 1% Glutamax. Cells were seeded at 3.0×10^4 cells per well in a 96-well microtiter plate (Corning) and allowed to adhere overnight. Medium was carefully aspirated and replaced with 195 μL of fresh medium, and compound solutions in DMSO (5 μL) were added to give a final concentration of 150−0.1 μM. Plates were incubated for 72 h at 37 °C and 4.5% CO_2 in a humidified chamber, and cell viability was measured using the MTT assay method.[19,20]

DNA Unwinding Assay. A DNA unwinding assay was used to measure intercalation into DNA as a function of a change in DNA topology.[21] A reduction in relaxed plasmid substrate and increase in supercoiled DNA indicates increased levels of intercalation. Reaction mixtures (20 μL) contained 50 mM Tris-HCl (pH 7.5 at 23 °C), 1 mM $MgCl_2$, 0.5 mM DTT, 50 μg/mL BSA, 0.3 μg of relaxed pBR322, 1 unit of calf thymus Topo I (Invitrogen, Carlsbad, CA, USA), and test compounds or ethidium bromide (control) in DMSO (1 μL). Reaction mixtures were incubated at 37 °C for 15 min and then terminated by addition of SDS to 1% and further incubated at 37 °C for 10 min. EDTA and proteinase K were added to a final concentration of 40 mM and 100 μg/mL, respectively, and the mixtures were then incubated 37 °C for 15 min. DNA products were purified by extraction with phenol−chloroform−isoamyl alcohol (25:24:1, v/v) and analyzed by electrophoresis through vertical 1.2% agarose gels at 2 V/cm for 15 h in TAE buffer. Gels were stained with ethidium bromide prior to being photographed and quantified using an Eagle Eye II system (Stratagene, Santa Clara, CA, USA).

Relaxation Assay. The effect of the compounds on the relaxation activity of calf thymus Topo I was assessed using negatively supercoiled plasmid DNA as the substrate.[17] A reduction in relaxed DNA and increase in supercoiled plasmid substrate indicates inhibition of topoisomerase relaxation activity. Reactions mixtures (20 μL) contained 50 mM Tris-HCl (pH 7.5 at 23 °C), 1 mM $MgCl_2$, 0.5 mM DTT, 50 μg/mL BSA, 0.3 μg of pBR322, 0.5 unit of Topo I, and test compounds or camptothecin (control). Reaction mixtures were incubated at 37 °C for 15 min, and reactions were terminated by chilling on ice and addition of a quarter volume of a loading dye mixture (15% glycerol, 2% sarkosyl, 0.05% xylene·cyanol, 0.05% bromophenol blue, and 50 mM EDTA). Reaction products were analyzed by agarose gel electrophoresis as described for the DNA unwinding assay. All experiments were performed in duplicate.

Decatenation Assays. The effect of the compounds on the catalytic activity of the α form of human Topo II was assessed by a decatenation assay using kDNA as the substrate.[16,22] The reduction of decatenated DNA indicates inhibition of toposiomerase decatenation activity. Topo II and kDNA were purchased from Topogen (Fort Orange, FL, USA). Reaction mixtures (20 μL) contained 50 mM Tris-HCl (pH 8.0 at 23 °C), 10 mM $MgCl_2$, 200 mM potassium glutamate, 10 mM DTT, 50 μg/mL BSA, 1 mM ATP, 0.3 μg of kDNA, 5 μg/mL tRNA, 2 units of Topo II, and the indicated concentrations of the compounds or etoposide (control). Reactions were incubated at 37 °C for 15 min and were terminated by addition of EDTA to 25 mM and further incubation at 37 °C for 5 min. Reaction products were analyzed by agarose gel electrophoresis as described for the unwinding assay. All experiments were performed in duplicate.

DNA Cleavage Assays. The ability of the compounds to poison topoisomerases was determined using DNA cleavage assays.[17,21] Poisoning of Topo I and Topo II results in the generation of nicked and linear DNA, respectively, in these assays. For Topo I, reaction mixtures (20 μL) were as described for the DNA unwinding assay except that 2 units of Topo I were used. Topo II DNA cleavage reaction mixtures (20 μL) were as described for the decatenation assays except that potassium glutamate was omitted and 5 units of human Topo II was used. Camptothecin and etoposide were used as the controls for Topo I and Topo II, respectively.

For both assays, reaction mixtures were incubated for 10 min at 37 °C, then SDS was added to 1%, and reactions were continued at 37 °C for 10 min. After addition of EDTA and proteinase K to a final concentration of 40 mM and 100 μg/mL, respectively, the reaction mixtures were further incubated either at 37 °C for 15 min (for Topo I) or at 50 °C for 1 h (for Topo II). The DNA products were purified by extraction with phenol−chloroform−isoamyl alcohol (25:24:1, v/v) and analyzed by electrophoresis through vertical 1.2% agarose gels at 2 V/cm for 12 h in TAE buffer. Ethidium bromide was present in both agarose gels and the TAE buffer at a concentration of 0.5 μg/mL. Gels were destained in water and then photographed and quantified using an Eagle Eye II system. The poisoning effect was determined by

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comparing the intensity of either nicked or linear DNA in the presence and absence of compound. All experiments were performed at least in duplicate.

Isolation and Purification. The supernatant was separated from the mycelium by centrifugation and extracted consecutively with EtOAc and n-BuOH. Methanol and ethyl acetate extracts of the mycelium were combined, concentrated and further partitioned with water and EtOAc. The aqueous fraction was then further partitioned with n-BuOH. The BuOH extracts were combined and fractionated using a Fisher Prepsep C_{18} column (1 g) with a step gradient elution from water to methanol. The compounds eluted with 70% aqueous MeOH were separated using flash chromatography (12 g Gold silica column, CH_2Cl_2 to 50% MeOH gradient), yielding compound **4** (3.9 mg) during the 25% MeOH−CH_2Cl_2 elution. The subsequent fraction during the 25% MeOH−CH_2Cl_2 elution was further purified using HPLC (Altima HP C18, 5 μm, 7 × 250 mm, 20−55% aqueous CH_3CN) to give compounds **1** (1.5 mg) and **6** (0.9 mg). All of the phenazine compounds were easily identified in HPLC separations by characteristic UV absorbances in the 250−260 and 350 nm peak areas.

The EtOAc extracts from the mycelium and supernatant were combined, partitioned with 90% aqueous MeOH, and fractionated by vacuum silica chromatography (step gradient of CH_2Cl_2−MeOH). The compounds eluting in 20% MeOH−CH_2Cl_2 were separated using flash chromatography (12 g Gold silica column, CH_2Cl_2/50% MeOH) to yield additional compound **1** (8.1 mg) in the 12% MeOH−CH_2Cl_2 elution. The compounds eluting in 10% MeOH−CH_2Cl_2 were purified by HPLC (Altima HP C18, 5 μm, 7 × 250 mm, 20−55% aqueous CH_3CN) to give compounds **3** (0.9 mg), **5** (1.5 mg), and **9** (2.1 mg). Two fractions containing mixtures of phenazines were rechromatographed by HPLC (Altima HP C18, 5 μm, 7 × 250 mm, 5−40% aqueous CH_3CN) to give compounds **7** (0.7 mg), **8** (0.8 mg), and **10** (0.7 mg). The 40% MeOH−CH_2Cl_2 fraction from the flash column was further purified with an additional flash column separation (4 g Gold silica column, CH_2Cl_2−50% MeOH). The compounds eluting in 100% CH_2Cl_2 were purified by a final Sephadex LH-20 column (MeOH) to afford compound **2** (2.0 mg).

The trivial name solphenazine was given due to the isolation of the producing strain from potatoes of the genus *Solanum*.

Solphenazine A (1): yellow solid; $[\alpha]^{24}_D$ −16 (*c* 0.025 in MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.26), 240 (4.40), 250 (4.39), 360 (4.09); IR (film) ν_{max} 3500 − 3100 (broad), 2900, 1720 (sh), 1600, 1400, 1300, 1200, 1100 cm^{-1}; ^1H NMR (600 MHz) and ^{13}C NMR (151 MHz), see Table 1; ESIMS *m/z* 561 $[M + H]^+$; HRESI-APCIMS *m/z* 561.1722 $[M + H]^+$ (calcd for $C_{26}H_{29}N_2O_{12}$, 561.1715).

Phenazine Hydrolysis. Three equivalents of TEA and 10 equivalents of LiBr were added to a solution of solphenazine A (**1**) (3 mg in 500 μL of acetonitrile containing 2% of water) and stirred at room temperature overnight.[23] The reaction mixture was concentrated *in vacuo* and extracted with EtOAc and H_2O. The aqueous fraction was partitioned with n-BuOH to give pure L-rhamnose (1.2 mg). The EtOAc fraction was submitted to semipreparative HPLC to afford 1,6-dicarboxylic phenazine, **11** (0.8 mg).

Solphenazine B (2): yellow solid; $[\alpha]^{24}_D$ −32 (*c* 0.025 in MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.15), 250 (4.37), 360 (3.80); IR (film) ν_{max} 3500 − 3100 (broad), 2920, 1718, 1508, 1270, 1131, 1059 cm^{-1}; ^1H NMR (600 MHz) and ^{13}C NMR (151 MHz), see Table 1; ESIMS *m/z* 603 $[M + H]^+$; HRESIMS *m/z* 603.1817 $[M + H]^+$ (calcd for $C_{28}H_{31}N_2O_{13}$, 603.1821).

Solphenazine C (3): yellow solid; ^1H NMR (600 MHz) and ^{13}C NMR (151 MHz), see Table 1; ESIMS 643 $[M - H]^-$.

Solphenazine D (4): yellow solid; $[\alpha]^{24}_D$ −24 (*c* 0.025 in MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.28), 260 (4.32), 360 (3.65); IR (film) ν_{max} 3500 − 3100 (broad), 2900, 2059 (broad), 1718, 1561, 1407, 1283, 1252, 1088, 1058 (sh) cm^{-1}; ^1H NMR (600 MHz) and ^{13}C NMR (151 MHz), see Table 2; ESI-APCIMS *m/z* 533 $[M + H]^+$; HRESI-APCIMS *m/z* 533.1756 $[M + H]^+$ (calcd for $C_{25}H_{29}N_2O_{11}$ 533.1766).

Solphenazine E (5): yellow solid; $[\alpha]^{24}_D$ −16 (*c* 0.025 in MeOH); UV (MeOH) λ_{max} (log ε) 200 (3.99), 210 (3.99), 250 (4.11), 260 (4.11), 360 (3.53); IR (film) ν_{max} 3500−3100 (broad), 2900, 1718, 1561, 1407, 1252, 1088, 1058 (sh) cm^{-1}; ^1H NMR (600 MHz) and ^{13}C NMR (151 MHz), see Table 2; ESIMS *m/z* 401, $[M + H]^+$; HRESIMS *m/z* 401.1355 $[M + H]^+$ (calcd for $C_{20}H_{21}N_2O_7$ 401.1343).

Solphenazine F (6): yellow solid; $[\alpha]^{24}_D$ −16 (*c* 0.025 in MeOH); UV (MeOH) λ_{max} (log ε) 200 (3.93), 210 (3.88), 260 (4.07), 360 (3.15); IR (film) ν_{max} 3500−3100 (broad), 1653, 1235 (sh), 1089 (sh) cm^{-1}; ^1H NMR (600 MHz) and ^{13}C NMR (151 MHz), see Table 2; APCIMS *m/z* 503, $[M - H]^+$; ESIMS *m/z* 505, $[M + H]^+$; HRESIMS *m/z* 505.1806 $[M + H]^+$ (calcd for $C_{24}H_{29}N_2O_{10}$ 505.1817).

4-Hydroxybenzoyl-α-L-rhamnopyranoside (9): yellowish-brown solid; $[\alpha]^{24}_D$ −40 (*c* 0.03 in MeOH); ^1H NMR (600 MHz) and ^{13}C NMR (151 MHz), see Table S10.

4-Methylaminobenzoyl-α-L-rhamnopyranoside (10): pale yellow solid; $[\alpha]^{24}_D$ −56 (*c* 0.025 in MeOH); UV (MeOH) λ_{max} (log ε) 200 (3.98), 250 (3.72), 310 (4.14); IR (film) ν_{max} 3500−3100 (broad), 2934, 1698, 1604 (sh), 1269, 1056 cm^{-1}; ^1H NMR (600 MHz) δ 7.80 (d, *J* = 8.8 Hz, H-2 and H-6), 6.58 (d, *J* = 8.8 Hz, H-3 and H-6), 6.11 (s, H-1′), 3.91 (dd, *J* = 3.3 and 1.1 Hz, H-2′), 3.83 (dd, *J* = 9.5 and 3.3 Hz, H-3′), 3.75 (m, H-5′), 3.49 (t, *J* = 9.5 Hz, H-4′), 2.83 (s, N-CH_3), 1.28 (d, *J* = 6.2 Hz, H-6′); ^{13}C NMR (151 MHz) δ 167.4 (C-7), 155.2 (C-4), 132.6 (C-2 and C-6), 116.8 (C-1), 111.8 (C-3 and C-5), 95.0 (C-1′), δ 73.4 (C-4′), 72.3 (C-3′), 72.2 (C-5′), 71.4 (C-2′), 29.6 (N-CH_3), 17.9 (C-6′); ESIMS *m/z* 298 $[M + H]^+$; HRESIMS *m/z* 298.1276 $[M + H]^+$ (calcd for $C_{14}H_{20}NO_6$ 298.1285).

Phenazine-1,6-dicarboxylic acid (11): greenish-yellow solid; ^1H NMR (CF_3COOD, 600 MHz) δ 9.30 (d, *J* = 7.3 Hz, H-2 and H-7), 8.99 (d, *J* = 9.2 Hz, H-4 and H-8), 8.51 (dd, *J* = 9.2 and 7.3 Hz, H-3 and H-9); ^{13}C NMR (CF_3COOD, 151 MHz) δ 168.5 (C-11 and C-12), 144.2 (C-2 and C-7), 139.8 (C-4a and C-9a), 138.0 (C-3 and C-8), 133.8 (C-4 and C-9), 123.8 (C-1 and C-6); α-rhamnose $[\alpha]^{24}_D$ −32 (*c* 0.025 in MeOH).

■ ASSOCIATED CONTENT

Supporting Information

^1H, ^{13}C, and 2D NMR and high-resolution mass spectra of compounds **1**−**11**, CD spectrum of hydrolyzed rhamnose from **1**, ^{15}N HMBC spectrum of **4**, table of ^1H and ^{13}C NMR data for compounds **9** and **10**, and DNA intercalation and topoisomerase inhibition data. 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.

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