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# Mutasynthesis of a Potent Anticancer Sibiromycin Analogue

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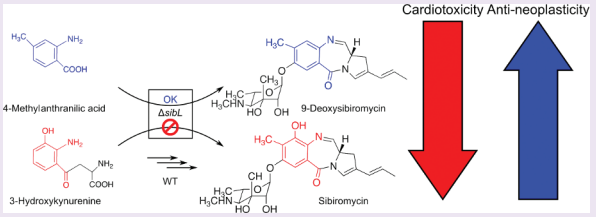
Supporting Information

**ABSTRACT:** Pursuit of the actinomycete pyrrolobenzodiazepine natural product sibiromycin as a chemotherapeutic agent has been limited by its cardiotoxicity. Among pyrrolobenzodiazepines, cardiotoxicity is associated with hydroxylation at position 9. Deletion of the methyltransferase gene *sibL* abolishes the production of sibiromycin. Supplementation of growth media with 4-methylanthranilic acid can substitute for its native 3-hydroxy congener. Cultures grown in this fashion yielded 9-deoxysibiromycin. In this study, we characterize the structure and biological activity of sibiromycin and 9-deoxysibiromycin methyl carbinolamines. Preliminary *in vitro* evidence suggests that 9-deoxysibiromycin exhibits reduced cardiotoxicity while gaining antitumor activity. These results strongly support further exploration of the production and evaluation of monomeric and dimeric glycosylated pyrrolobenzodiazepine analogues of sibiromycin.




The pyrrolobenzodiazepines (PBDs) represent a diverse family of natural products produced by diverse actinomyces including *Streptomyces*, *Micromonospora*, and *Streptosporangia*.[1] The PBDs consist of a tricyclic 6−7−5 system and exhibit antibacterial and anticancer activity by alkylating DNA. In this reaction the imine of the diazepine ring undergoes nucleophilic attack by an exocyclic guanine nitrogen upon binding in the minor groove of DNA. In solution, the imine form of the molecule is in dynamic equilibrium with the preferred carbinolamine form, but evidence[1] suggests that the imine form is the reactive species. The chirogenic five-membered ring imparts curvature to the molecule in such a way that the molecule nearly perfectly fits inside the minor groove.[2] DNA thus modified is believed to evade error-correction mechanisms, which typically sense errors by changes in DNA curvature.[3]

Although sibiromycin is one of the most potent anticancer PBDs, a major obstacle to animal testing is its cardiotoxicity attributable to the C-9 hydroxyl group (Figure 1).[1,4,5] Supplementation of coenzyme Q in cardiomyocyte cultures appears to mitigate this cardiotoxicity; possibly because C-9 hydroxyl containing PBDs disrupt the mitochondrial electron transport system.[5] Sibiromycin is one of only two known glycosylated PBDs (Figure 1), the other being sibanomicin,[6] and its remarkable potency is dependent on the presence of the cationic sugar sibirosamine.[7] Unfortunately, while some non-glycosylated PBDs are synthetically accessible,[1,8,9] yielding molecules such as SJG-136 (Figure 1) currently in clinical trials,[9] sibiromycin is synthetically intractable.[1] The producer strain for sibanomicin, which also features the sibirosamine group but not the C-9 hydroxyl group, is not available.

The PBD scaffold is assembled *via* a two-component nonribosomal peptide synthetase (NRPS) that merges an anthranilic acid derived from chorismate (as for tomaymycin[10]) or tryptophan (as for anthramycin and sibiromycin[11,12]) with a 3-substituted proline derived from tyrosine. Sibirosamine is derived from glucose-1-phosphate and added after completion of the PBD scaffold.[11] The convergent nature of PBD biosynthesis strongly suggests the use of mutasynthesis to first validate if the elimination of the C-9 hydroxyl group in sibiromycin mitigates cardiotoxicity while retaining its anticancer properties and later to derivatize this molecule. This strategy became possible when we identified the gene cluster responsible for sibiromycin biosynthesis.[11] Here we report the first preparation of a glycosylated PBD analogue, 9-deoxysibiromycin (Figure 1), and characterization of its DNA binding affinity, cardiotoxicity, and anticancer properties.

The 3-hydroxy-4-methylanthranilate unit of sibiromycin derives from kynurenine obtained by tryptophan degradation.[11] Recent biochemical studies have shown that 3-hydroxykynurenine is methylated by the SAM-dependent methyltransferase SibL, converted to 3-hydroxy-4-methylanthranilic acid by the PLP-dependent kynureninase SibQ and loaded on the NRPS SibE for formation of the PBD scaffold.[13] We have obtained a *Streptosporangium sibiricum* Δ*sibL* strain that, grown in unsupplemented media, does not produce sibiromycin (Figure 2). The inability of this strain to produce sibiromycin despite SibQ and SibE's recognition of 3-hydroxykynurenine and 3-hydroxyanthranilic acid as substrates,[13] respectively, is likely due to competitive depletion of 3-hydroxykynurenine by shunt pathways[11,14] combined with a decreased catalytic efficiency of SibQ for this substrate (Figure 1). Supplementation with 4-

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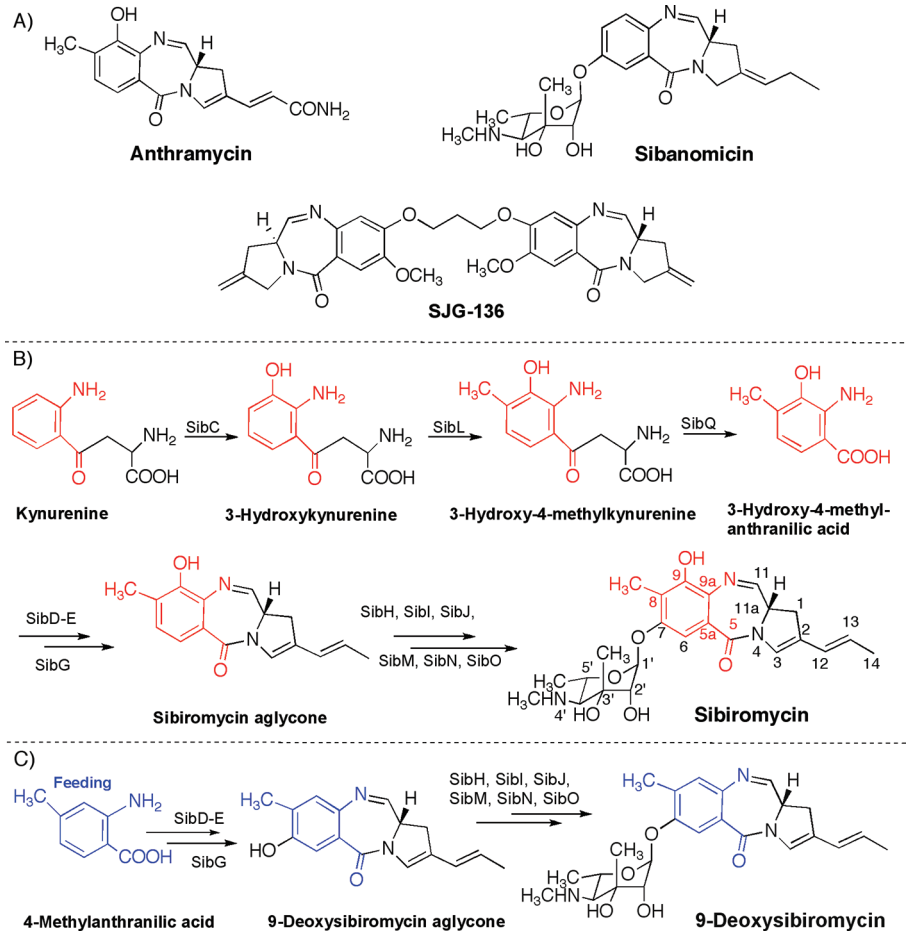


**Figure 1.** (A) Structures of natural (anthramycin and sibanomycin) and synthetic (SJG-136) PBDs discussed. (B) Biosynthesis of sibiromycin. (C) Mutasynthesis of 9-deoxysibiromycin, a sibiromycin analogue. The analogue was obtained by feeding 4-methylanthranilic acid to an unproductive mutant strain of *S. sibiricum*.

methylanthranilic acid at 1 mM results in a peak that by reversed-phase high performance liquid chromatography exhibits a retention delay compared to sibiromycin and possesses a mass corresponding to 9-deoxysibiromycin (Figure 2). The production of the sibiromycin analogue in this mutant strain upon addition of 4-methylanthranilic acid shows that the broad substrate specificity of SibE[13] extends to this compound.

Sibiromycin and 9-deoxysibiromycin were isolated by extraction and subjected to silica gel and size exclusion chromatography. Per volume yields of ~2.5 mg $L^{-1}$ of sibiromycin and ~1.3 mg $L^{-1}$ of 9-deoxysibiromycin were obtained from wild-type and Δ*sibL* culture, respectively. Full confirmation of the identity of the PBD produced by the Δ*sibL* supplemented with 4-methylanthranilic acid as the 9-deoxy-sibiromycin was obtained by 1D and 2D NMR characterizations of the methyl carbinolamine adduct as present in $CD_3OD$ solvent (NMR spectra provided in the Supporting Information). Compared to the prior assignment of sibiromycin conducted in $d_6$-DMSO,[15] which left some resonances unresolved, our assignment completely accounts for resonances of all expected protons and carbon nuclei (Supplementary Table 2). We also report resonances for both diastereomers for 9-deoxy-sibiromycin and sibiromycin (Supplementary Tables 1and 2 and Figures 2−12) resulting from methanol attack on opposite faces of the diazepine ring. NMR analysis also indicated 9-deoxysibiromycin modestly prefers solvent attack in *cis* with respect to the pyrrole ring, whereas sibiromycin prefers *trans* attack.

Biological assays were performed to assess the relative activities of both sibiromycin and 9-deoxysibiromycin relative to a reference PBD, anthramycin. 9-Deoxysibiromycin was tested in the calf thymus DNA melting assay routinely used to evaluate the DNA binding affinity of a PBD compared to well-characterized PBDs.[1] 9-Deoxysibiromycin shows far stronger stabilization ($\Delta T_m$ = 22 °C) of the DNA against melting than either anthramycin ($\Delta T_m$ = 13 °C) or sibiromycin ($\Delta T_m$ = 16 °C). Thus, removal of the 9-hydroxy group has not adversely affected the DNA affinity but rather has increased it. In an initial evaluation on the cardiotoxicity properties, we tested sibiromycin and 9-deoxysibiromycin on the human cardiac cell line AC16.[16,17] This assay suggests that 9-deoxysibiromycin is approximately 5-fold less cardiotoxic than its parent compound (Supplementary Figure 1). Unfortunately, we were unable to obtain SJG-136 to establish a baseline acceptable cardiotoxicity for anticancer PBDs.

Sibiromycin and 9-deoxysibiromycin were submitted to the NCI cancer screen (http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html). *In vitro* antitumor activities against selected cell lines are detailed in Table 1 (for the full list of lines screened and activities see Supplementary Tables S3 and S4). The average concentration for 50% growth inhibition ($GI_{50}$) is 5-fold less for 9-deoxysibiromycin (24 nM) than for sibiromycin (117 nM). $GI_{50}$ values for 9-deoxysibiromycin vary


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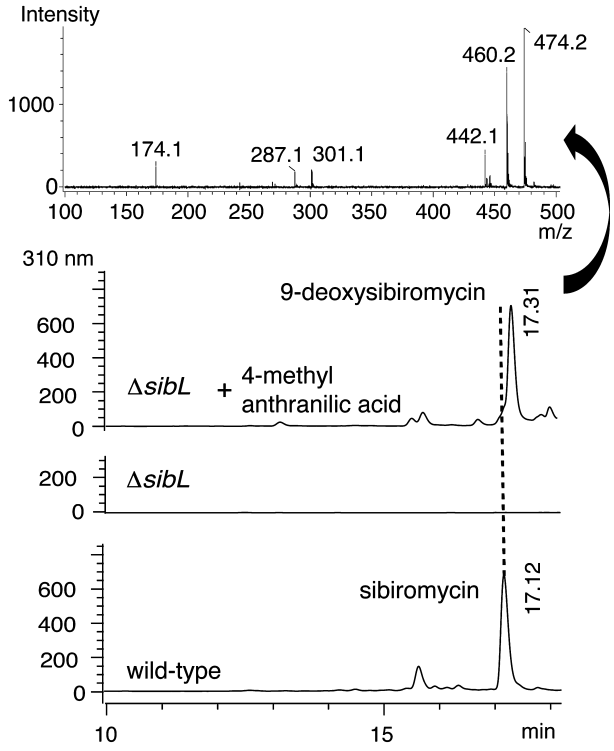


**Figure 2.** HPLC analysis of the secondary metabolites produced by wild-type and the Δ*sibL* mutant *S. sibiricum*. ESI-MS analysis of the 17.31 min peak shows mass peaks corresponding to the imine, carbinolamine, and carbinolamine methylether forms[11] of 9-deoxysibiromycin (442.1, 460.2, and 474.2 *m/z*, respectively) and of the sibirosamine and aglycone fragments (174.1, 287.1, and 301.1, respectively).

**Table 1. Selected $GI_{50}$ (nM) NCI 60 Cell Line Screen Results**

| cell line/type | sibiromycin | 9-deoxysibiromycin |
|---|---|---|
| SR/leukemia | 25.1 | 3.2 |
| NCI-H522/lung | 120.2 | 7.6 |
| OVCAR-8/ovarian | 195.0 | 17.8 |
| MCF7/breast | 131.8 | 8.5 |
| SK-MEL-5/melanoma | 154.9 | 14.1 |

from 3 to 239 nM. Whereas concentrations of sibiromycin higher than 1 μM are necessary to obtain 50% lethality in all cell lines ($LC_{50}$), concentrations as low as 58 and 25 nM of 9-deoxysibiromycin were sufficient for $LC_{50}$ and total growth inhibition (TGI) particularly in some melanoma and renal cancer cell lines. Overall, sibiromycin and 9-deoxysibiromycin display different cytotoxicity profiles based on the mean graph patterns. 9-Deoxysibiromycin is generally more potent than sibiromycin.

A COMPARE analysis[18] provided a comparison of the cytotoxicity profiles with other anticancer compounds tested in the NCI cancer screen. This indicated that sibiromycin and 9-deoxysibiromycin exhibit patterns of relative activity similar to those of DNA alkylating compounds as a class. However, sibiromycin and 9-deoxysibiromycin were each most similar to different compounds in the class and also distinct from SJG-136.[19] Although all three compounds are PBDs, the DNA sequence adjacent to the reactive guanine base interacts differently with the different substituents on the PBD scaffold. The PBD substitution also creates differences in the twist of the PBD scaffold and different modes of binding in the minor groove.[1] Combined, these differences in binding to DNA are likely the major factor contributing to the different cytotoxicity profiles that are observed in these three compounds. Other less studied properties such as cellular transport may also contribute to the different activities of these PBDs.

We report the first production of a glycosylated PBD, 9-deoxysibiromycin, and the biological characterization of this compound and its parent compound, sibiromycin. 9-Deoxysibiromycin showed diminished cardiotoxicity but increased potency against cancer cell lines relative to sibiromycin. Variations in relative potency of these compounds toward the cell lines in the panel are likely to be due to different DNA selectivity. The improved overall activity can be rationalized by the stronger binding toward DNA as reflected by the increase in $\Delta T_m$. This rationale is consistent with the results observed for SJG-136.[20] This is mechanistically distinct from the electron transport disruption caused by the 9-hydroxyl group present in the sibiromycin parent compound. We speculate that an evolutionary preference for two moderate antibiotic activities may be preferable to a single potent activity in the host's environmental context to provide broad-spectrum activity or to hinder the development of resistance to the compound. To the best of our knowledge this is the first reported instance of a targeted mutasynthesis performed specifically to remove an undesirable side effect from a natural product compound.

## METHODS

**Bacterial Strains and Plasmids.** *Escherichia coli ET12567*[21] and plasmids pIJ790 and pIJ773 were a generous gift from Dr. B. Gust (University of Tübingen). Growth and maintenance of *S. sibiricum* (*DSM 44039*) was performed as previously described.[11]

***sibL* Inactivation by Gene Replacement.** Single gene inactivation experiments were carried out in *E. coli* using the protocol based on REDIRECT technology[22] and as previously described.[11] Insertional inactivation of the target gene was confirmed as previously described.[11]

**Batch Production and Purification of Sibiromycin and 9-Deoxysibiromycin.** Twenty 500 mL Erlenmeyer flasks with metal springs and filled with 100 mL of sibiromycin media [30 g $L^{-1}$ of corn starch (Spectrum), 15 g $L^{-1}$ of Bacto soytone (BD), 50 mM $CaCO_3$ (Fisher), and 70 mM NaCl (Fisher)] were inoculated with a 1 mL culture plug of wild-type or Δ*sibL S. sibiricum*. Cultures of Δ*sibL S. sibiricum* were supplemented upon inoculation with 15 mg of 2-amino 4-methylbenzoic acid (Maybridge) dissolved in 0.1 mL of DMSO (Fisher) for a final concentration of 1 mM. Cultures were grown for ~36 h at 30 °C and harvested by centrifugation. The mycelial pellet was removed, and the media was filtered. The filtered media (~1.5 L) was washed 5 times in 200 mL of 5% isopropanol/95% pentanes. The aqueous layer was partitioned into 250 mL portions and extracted 3 times in 250 mL of dichloromethane. The organic and emulsion layers were pooled and frozen overnight to further separate the aqueous components. Frozen extract was cold-filtered at 4 °C and pooled; the dichloromethane was then removed by rotary evaporation at 10 °C. The remaining, dried crude extract was resuspended in 10 mL of methanol and stored at −20 °C. PBDs exist in aqueous solution in equilibrium between the imine and carbinolamine forms.[1] Because neither form is ideal for long-term storage, PBDs are usually stored in methanol as the carbinolamine methyl ether.

Silica gel (Fisher) chromatography was performed by dry-packing the column (4.5 cm diameter, ~20 cm vertical length; liquid column volume ~200 mL) under positive pressure into 100% chloroform, followed by equilibration by gravity drip (1 cv) and under positive pressure (1.5 cv) of 5% methanol/95% chloroform. The stored crude extract in methanol was dried under rotary evaporation at 10 °C, reconstituted in a minimal volume of 5% methanol/95% chloroform,


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and loaded on the silica gel column. The column was washed with 300 mL of 5% methanol/95% chloroform. Sibiromycin or 9-deoxysibiromycin was eluted with 20% methanol/80% chloroform. Collected fractions were analyzed by spot TLC of 10 $\mu$L. UV-active fractions were analyzed by HPLC; fractions assessed to be of high yield and purity were pooled and dried under rotary evaporation at 10 °C, resuspended in 10 mL of methanol, and stored at −20 °C.

Size exclusion chromatography was performed by gravity-packing 40 g of LH-20 resin (GE Healthcare) in 200 mL of methanol into a 1.5 m column, inner diameter 1.5 cm, followed by elution and pressure equilibration at a flow rate of 1 mL $min^{-1}$. Silica purified material was subjected to rotary evaporation at 10 °C (∼50% yield) and resuspended in a total of 1 mL of methanol and loaded onto the column, three times, to minimize volume and maximize yield. Collected fractions were analyzed and pooled as previously described. The purified sibiromycin or 9-deoxysibiromycin was resuspended in 10 mL of methanol and stored at −20 °C. Production of sibiromycin and 9-deoxysibiromycin were performed in 2 L batches as described, netting ∼30 mg of sibiromycin from a total 12 L of wild-type culture and ∼40 mg of 9-deoxysibiromycin from a total 16 L of Δ*sibL* culture.

**HPLC-ESI.** Production of sibiromycin or sibiromycin analogue was tested by HPLC-ESI on a Zorbax Eclipse XDB-C8 column (4.6 mm × 150 mm, Agilent) pre-equilibrated in 90% solvent A (0.1% (v/v) TFA in $H_2O$) and 10% solvent B (methanol) at a flow rate of 1 mL $min^{-1}$. A linear gradient method (hold for 2 min solvent A at 90%, 10−70% solvent B for 18 min) was applied to the column. The various compounds were detected at 230 and 310 nm using an Agilent 1100 HPLC system (Agilent) and by ESI-MS in the positive ion mode using a JEOL AccuTOF-CS mass spectrometer. Depending on the conditions of the analytical technique used, one or more forms were detected. Under the ESI-MS conditions three forms are detected for sibiromycin $[M + H]^+$ at $m/z$ = 458.2 for the imine form, $m/z$ = 476.3 for the carbinolamine form, and $m/z$ = for the 490.3 for the carbinolamine methyl ether form consistent with the molecular formulas $C_{24}H_{31}N_3O_6$ (calculated 457.2), $C_{24}H_{33}N_3O_7$ (calculated 475.2), and $C_{25}H_{35}N_3O_7$ (calculated 489.3), respectively.[11] Similarly, for 9-deoxysibiromycin three forms are detected $[M + H]^+$ at $m/z$ = 442.1 for the imine form, $m/z$ = 460.2 for the carbinolamine form, and $m/z$ = 474.2 for the carbinolamine methyl ether form consistent with the molecular formulas $C_{24}H_{31}N_3O_5$ (calculated 441.2), $C_{24}H_{33}N_3O_6$ (calculated 459.2), and $C_{25}H_{35}N_3O_6$ (calculated 473.2), respectively. Fragmentation at the anomeric bond is observed with the appearance of peaks at 287.1 and 301.1 for the aglycone fragment in addition to 174.1 $m/z$ assigned to the sibirosamine fragment. Similar fragmentation has been observed for sibiromycin.[11]

**NMR Spectroscopy.** Samples of sibiromycin and 9-deoxysibiromycin were prepared in 1 mL of $CD_3OD$ to a final concentration of approximately 10 and 5 mM, respectively. $^1H$ and $^{13}C$ NMR spectra (Supporting Information) were acquired on a Bruker DRX-400 at 300 K, and 2D NMR spectra ($^1H-^1H$ COSY, $^{13}C-^1H$ HSQC, and $^{13}C-^1H$ HMBC spectra; Supporting Information) were acquired on a Bruker AVIII-600 at 300 K. Standard pulse sequences were used and spectra chemical shifts were referenced against solvent $CHD_2OD$ or $^{13}CD_3OD$ peaks. Simulations of certain peak sets for sibiromycin were performed by fitting the frequency-domain data to a sum of Gaussians separated by fixed intervals whose integration correspond to the appropriate binomial coefficients corresponding to the splitting conditions. This simulation was performed using Mathematica's Nonlinearregress function with the Levenburg-Marquadt algorithm and used to extract chemical shift and *J*-coupling values for the following resonances: 1$\beta$ (both diastereomers), 5′ (both diastereomers), 11a (minor diastereomer), and 13 (both diastereomers) (Supplementary Figure S3).

**Calf-Thymus DNA Melting Point Assay.** Calf-thymus DNA (CT-DNA) type I, highly polymerized (Sigma-Aldrich D1501) was reconstituted in 10 mM sodium phosphate, 1 mM EDTA, 0.02% (w/v) $NaN_3$, pH 7.0, at RT for 2 h and aliquoted to 100 $\mu$M ($OD_{260}$ = 0.6). Samples were prepared to a final concentration ratio of DNA:PBD = 1:20. DNA was subjected to thermal denaturation from 25 to 94 °C at a melting rate of 1 °C per minute. Two different melting curves were collected at time zero and after 18 h incubation at 37 °C. Experiments were performed in triplicate. The melting curve for CT-DNA treated with 9-deoxysibiromycin did not show a wide post-transition region necessary for thermodynamic modeling, so the melting temperature $T_m$ for all samples was estimated by calculating the discrete derivative of the melting curve using $\Delta OD_{260}/\Delta T$ for each pair of successive data points on the melting curve. $T_m$ was assigned to the maximum of this derivative. Data processing for this step was performed in Mathematica.

**Cardiomyocyte-Based Assay.** The human cardiac cell line AC16[17] was maintained in DMEM:F12 (1:1) supplemented with 10% FBS, 1 mM HEPES buffer, 2 mM L-glutamine, 100 units $mL^{-1}$ penicillin, and 100 $\mu$g $mL^{-1}$ streptomycin. Two- to four-day-old cultures (∼70% confluent) were used for the experiments. The human cardiac AC16 cells were seeded in black wall, clear bottom, tissue culture treated 96-well plates (Costar) at a density of 250 cells per well, in Opti-MEM supplemented with 5% FBS, 1 mM HEPES buffer, 2 mM L-glutamine, 100 units $mL^{-1}$ penicillin, 100 $\mu$g $mL^{-1}$ streptomycin, and 0.05 mg $mL^{-1}$ $CaCl_2$ and incubated overnight at 37 °C. Serial dilutions of sibiromycin and 9-deoxysibiromycin (stocks 20 mM in EtOH) were prepared in Hank's Balanced Salt Solution (HBSS) at concentrations ranging from 20 $\mu$M to 0.2 nM and then diluted 1:1 with Opti-MEM supplemented with 0.8 mg $mL^{-1}$ BSA, 2 mM HEPES buffer, 4 mM L-glutamine, 200 units $mL^{-1}$ penicillin, 200 $\mu$g $mL^{-1}$ streptomycin, and 0.1 mg $mL^{-1}$ $CaCl_2$. The medium from the cells was removed and replaced immediately by the diluted test compounds or similarly diluted HBSS (vehicle control) and incubated 24 and 48 h at 37 °C after which cell viability was evaluated by resazurin reduction assay.[17] Briefly, 10 $\mu$L per well of resazurin (500 $\mu$M, PBS) was added to each well and incubated for 2−3 h at 37 °C. Viable cells reduce resazurin to the highly fluorescent resorufin dye, which is quantitated in a multiwell plate fluororeader (Ex/Em 530/590 nm, Tecan Safire2). Cell viability was calculated as percentage of fluorescence relative to cells treated with vehicle only (100% viability) after subtraction of blank fluorescence (wells without cells). All experimental conditions were performed in triplicate. Averages and SEM corresponding to 2 independently performed experiments were calculated. To obtain experimental $EC_{50}$ values, the data were analyzed by nonlinear regression using a sigmoidal dose response curve model with variable slope (GraphPrism). The model $R^2$ values were >0.98 in all cases (Supporting Figure 1).

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

PBDs, pyrrolobenzodiazepines; NRPS, nonribosomal peptide synthetase

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