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Baraphenazines A−G, Divergent Fused Phenazine-Based Metabolites from a Himalayan *Streptomyces*

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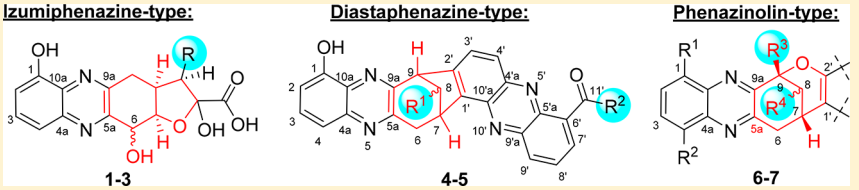
Supporting Information



ABSTRACT: The structures and bioactivities of three unprecedented fused 5-hydroxyquinoxaline/alpha-keto acid amino acid metabolites (baraphenazines A−C, **1**−**3**), two unique diastaphenazine-type metabolites (baraphenazines D and E, **4** and **5**) and two new phenazinolin-type (baraphenazines F and G, **6** and **7**) metabolites from the Himalayan isolate *Streptomyces* sp. PU-10A are reported. This study highlights the first reported bacterial strain capable of producing diastaphenazine-type, phenazinolin-type, and izumiphenazine A-type metabolites and presents a unique opportunity for the future biosynthetic interrogation of late-stage phenazine-based metabolite maturation.

Phenazine natural products are chorismate-derived dibenzo-annulated pyrazine-based secondary metabolites primarily produced by *Streptomyces* and *Pseudomonas*.[1−6] Phenazines are known to contribute to *Pseudomonas* virulence and to also display a broad range of biological activities (antihypertensive, antiparasitic, antimalarial, neuroprotective, radical scavenging, and/or antitumor),[1−3] primarily via the reduction of molecular oxygen to generate reactive oxygen species (ROS).[7] While over 150 naturally occurring phenazines have been reported,[1−3,8] only a small subset are composed of more than one phenazine-derived unit. These include six naturally occurring phenazine dimers (connected via a single covalent bond as exemplified by diphenazithinonin,[9] phenazostatins A−D,[10−12] and izumiphenazine B[13]) and 11 fused phenazine-based metabolites (in which the combination of two phenazine core units affords a new ring system as exemplified by esmeraldines A and B,[14] phenazinolins A−E,[15] izumiphenazine A,[13] and diastaphenazine,[16] Figure 1). Of the latter set, the recently reported diastaphenazine (isolated from an endophytic *Streptomyces* strain) stands out as the first example in which the new ring formed lacks a heteroatom.[16]

As part of an effort to characterize novel natural products from unusual terrestrial environments,[17−27] their unique molecular targets,[28] and/or corresponding biocatalysts,[29] herein we report the isolation and characterization of seven new fused phenazine-based metabolites (baraphenazines A−G; **1**−**7**, Figure 1), along with diastaphenazine and izumiphenazine A, from the Himalayan isolate *Streptomyces* sp. PU-10A. Of the new natural products disclosed herein, baraphenazines A−C (**1**−**3**) represent the first reported examples of fused 5-hydroxyquinoxaline/alpha-keto acid-based metabolites. In addition, baraphenazines D and E (**4** and **5**) embody two new diastaphenazine-type C−C-fused phenazine-based analogues, while baraphenazines F and G (**6** and **7**) exemplify two new phenazinolin-type C−O-fused compounds. This study highlights the first reported strain capable of producing the divergent phenazine ring-fused systems of diastaphenazine-type, izumiphenazine A-type, and phenoazinolin D/E-type

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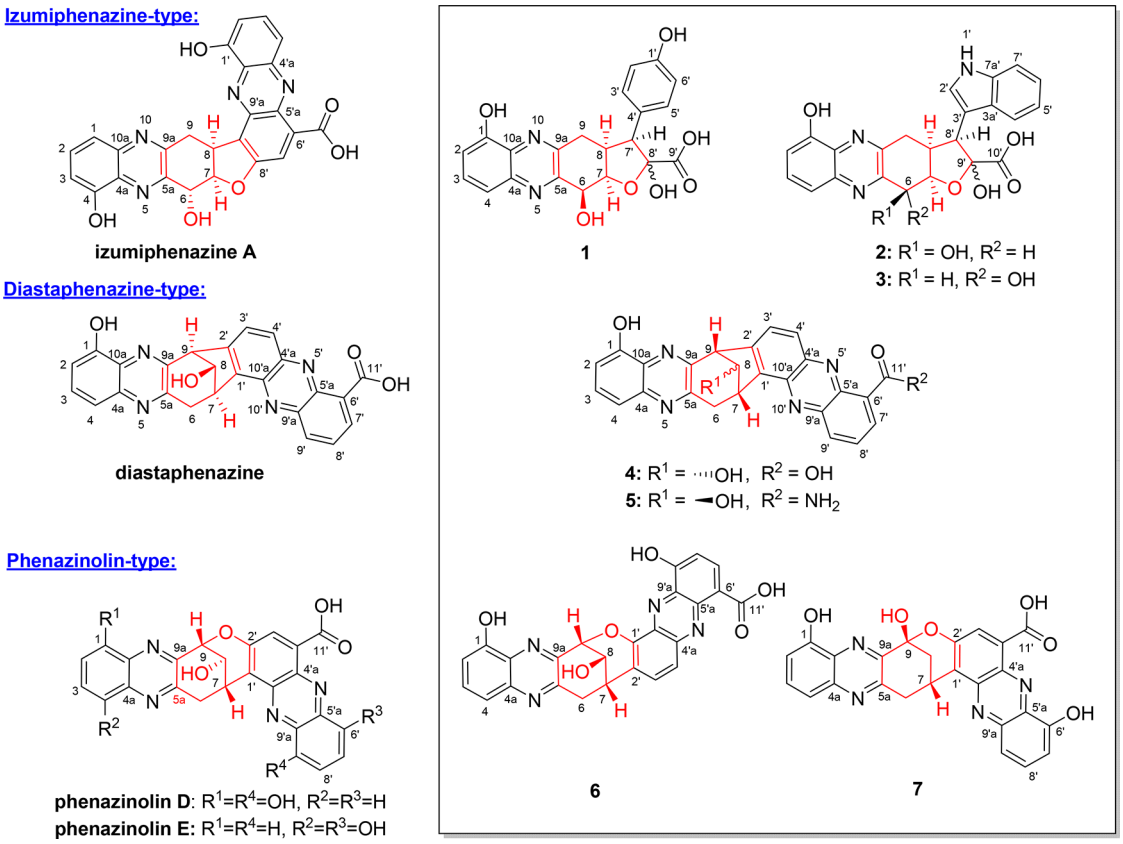

Figure 1. Structures of new compounds (**1**–**7**) isolated from *Streptomyces* sp. PU-10A and related phenazines.

Table 1. ^{1}H NMR (400 MHz) Spectroscopic Data for Compounds 1–7 in DMSO-d_6 (δ in ppm, mult. J in Hz)

no.	1	no.	2	3	no.	4	5	6	7
2	7.13, d (7.7)	2	7.14, d (7.7)	7.08, d (7.5)	2	7.05, d (7.7)	7.04, d (7.8)	7.07, d (7.8)	7.12, d (7.8)
3	7.62, t (7.8, 7.9)	3	7.63, t (7.9, 8.1)	7.57, t (7.6, 7.9)	3	7.48, t (7.7, 8.0)	7.47, t (7.8, 8.2)	7.59, t (7.8, 8.2)	7.65, t (7.8, 8.4)
4	7.46, d (8.3)	4	7.46, d (8.3)	7.34, d (8.4)	4	7.24, d (8.0)	7.26, d (8.2)	7.35, d (8.2)	7.32,
6	4.92, d (4.0)	6	4.98, d (4.1)	5.09, br s	6α	3.14, d (18.0)	3.03, d (17.8)	3.34, d (18.0)	3.60, d (18.0)
7	4.59, dd (4.3, 8.7)	7	4.65, dd (4.1, 8.2)	4.54, t (5.7, 7.1)	6β	3.75, d (18.0)	3.67, dd (4.5, 17.8)	3.79	3.76, dd (5.0, 18.0)
8	3.29, m	8	3.38, m	3.18, m	7	4.46, br s	4.37, br s	3.82	4.91, br s
9α	2.78, dd (3.9, 14.8)	9α	2.90, m	2.40, m	8	4.95, br s	4.59, d (4.4)	4.79, br s	2.63, 2.86
9β	3.32, m	9β	3.39, m	3.24, m	9	4.58, br s	5.04, br s	5.74, br s	
2′	6.67, d (8.1)	1′	11.00, s	10.95, s	3′	8.08	7.96, d (8.8)	8.05, d (9.0)	7.91, s
3′	7.06, d (8.2)	2′	7.35, s	7.41, s	4′	8.22, d (8.9)	8.22, d (8.8)	7.88, d (9.0)	
5′	7.06, d (8.2)	4′	7.37, d (8.3)	7.56, d (7.6)	7′	8.52, d (6.6)	8.65, d (7.0)	8.59, d (8.2)	7.32
6′	6.67, d (8.1)	5′	6.89, t (7.2, 7.6)	6.98, t (7.5, 7.6)	8′	8.10	8.06, t (6.6, 8.4)	7.28, d (8.2)	7.84, t (7.7, 8.5)
7′	2.89, d (10.7)	6′	7.03, t (7.2, 7.5)	7.08, t (7.5, 7.7)	9′	8.58, d (8.9)	8.49, d (8.7)		7.74, d (8.6)
		7′	7.33, d (8.3)	7.39, d (8.4)	1-OH	10.25, br s	10.20, br s	10.57, s	10.20, s
		8′	3.30, d (10.6)	4.27, d (8.8)	8-OH	5.79, br s	6.19, s	6.16, s	
		1-OH	10.29, br s	10.11, s	9-OH				8.28, s
		6-OH		5.80, s	6′-OH				10.62, s
					9′-OH			11.99, s	

congeners and may implicate a broader biosynthetic relationship.

RESULTS AND DISCUSSION

Preliminary LC-MS metabolic profiling of purified actinomycete strains isolated from a soil sample collected in Northern Pakistan (Bara Gali) revealed *Streptomyces* sp. PU-10A as capable of novel secondary metabolite production (based on a comparison to the AntiBase 2017[8] database). Scale-up fermentation (10 L) of *Streptomyces* sp. PU-10A followed by extraction, fractionation, and standard chromatography (Supporting Information, Scheme S1) gave seven new

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Table 2. ^{13}C NMR (100 MHz) Spectroscopic Data for 1−7, Diastaphenazine, and Izumiphenazine in DMSO-d_6 (δ in ppm)

no.	1	2	3	no.	4	5	6	7	diastaphenazine[a]	izumiphenazine[a]
1	153.6, C	153.6, C	153.2, C	1	153.4, C	153.3, C	153.6, C	153.3, C	153.3, C	118.4, CH
2	111.7, CH	111.7, CH	111.0, CH	2	111.9, CH	111.7, CH	111.8, CH	110.9, CH	111.7, CH	132.3, CH
3	130.4, CH	130.3, CH	130.0, CH	3	129.8, CH	129.5, CH	131.6, CH	132.1, CH	129.8, CH	111.8, CH
4	118.1, CH	118.1, CH	117.9, CH	4	117.7, CH	117.8, CH	117.8, CH	117.6, CH	117.7, CH	153.2, C
4a	142.4, C	142.4, C	141.9, C	4a	142.5, C	142.3, C	142.7, C	142.5, C	142.3, C	135.9, C
5a	151.1, C	151.2, C	150.8, C	5a	152.6, C	154.0, C	152.2, C	151.9, C	153.9, C	149.7, C
6	71.8, CH	71.8, CH	74.0, CH	6	35.5, CH_2	31.9, CH_2	35.5, CH_2	39.4, CH_2	31.9, CH_2	71.5, CH
7	80.5, CH	80.5, CH	84.7, CH	7	45.4, CH	40.2, CH	35.9, CH	27.0, CH	40.2, CH	88.7, CH
8	40.2, CH	40.9, CH	38.6, CH	8	80.6, CH	75.0, CH	62.9, CH	31.3, CH_2	75.0, CH	40.4, CH
9	32.5, CH_2	32.7, CH_2	32.0, CH_2	9	58.1, CH	55.0, CH	75.5, CH	97.2, C	54.9, C	33.3, CH_2
9a	153.9, C	154.1, C	154.6, C	9a	151.9, C	152.1, C	146.6, C	147.4, C	152.0, C	153.6, C
10a	131.4, C	131.4, C	130.6, C	10a	130.6, C	131.1, C	132.6, C	130.6, C	131.5, C	139.6, C
1′	156.4, C			1′	140.5, C	140.2, C	146.6, C	122.2, C	140.4, C	153.5, C
2′	114.7, CH	124.2, CH	124.6, CH	2′	148.0, C	146.8, C	123.1, C	152.7, C	147.7, C	111.7, CH
3′	130.3, CH	109.0, C	109.5, C	3′	129.3, CH	128.1, CH	135.8, CH	127.9, CH	128.6, CH	130.6, CH
3a′		127.7, C	127.7, C	4′	128.9, CH	129.6, CH	119.8, CH	127.6, CH	128.8, CH	118.0, CH
4′	125.8, C	118.7, CH	118.1, CH	4′a	140.8, C	141.3, C	139.7, C	136.9, C	140.7, C	142.3, C
5′	130.3, CH	118.2, CH	118.4, CH	5′a	139.3, C	139.9, C	140.5, C	135.1, C	139.3, C	137.3, C
6′	114.7, CH	120.6, CH	120.9, CH	6′	128.4, C	130.9, C	114.1, C	153.2, C	129.8, C	129.9, C
7′	56.0, CH	111.2, CH	111.2, CH	7′	133.8, CH	133.9, CH	138.9, CH	111.5, CH	133.2, CH	121.1, CH
7a′		135.9, C	135.5, C	8′	130.6, CH	130.5, CH	110.3, CH	132.4, CH	130.7, CH	158.7, C
8′	101.7, C	48.9, CH	45.2, CH	9′	133.6, CH	133.1, CH	159.3, C	118.0, CH	133.0, CH	124.9, C
9′	170.4, C	101.4, C	104.9, C	9′a	142.5, C	142.7, C	134.2, C	139.3, C	142.4, C	138.7, C
10′		170.8, C	169.6, C	10′a	140.5, C	139.1, C	134.2, C	139.8, C	139.6, C	131.3, C
				11′	166.3, C	165.6, C	165.5, C	165.4, C	166.7, C	165.8, C

[a]The atom numbers correspond to the atom numbers in compound 1−7 for comparison.

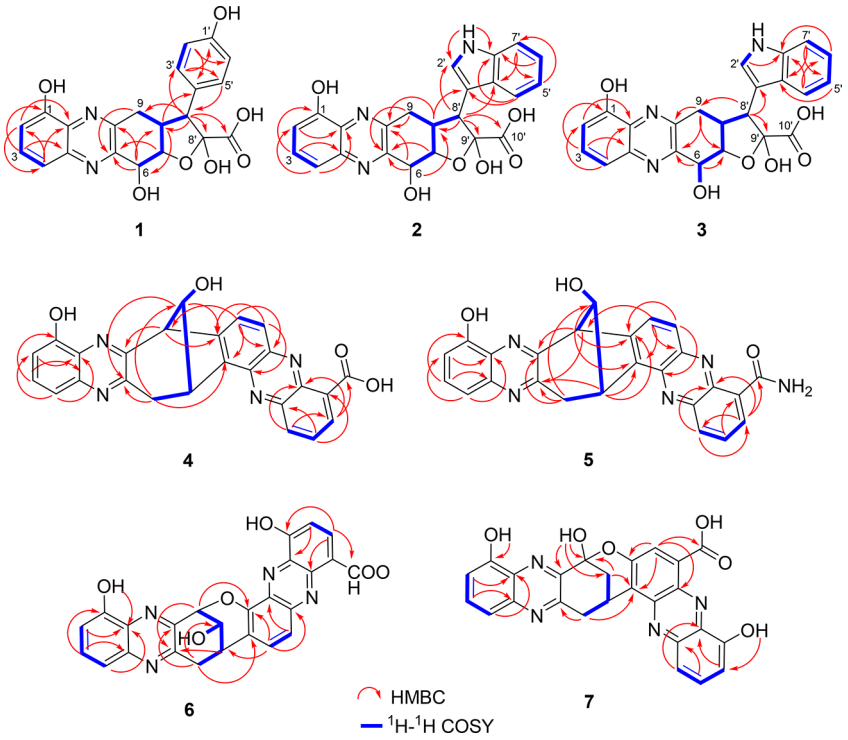

Figure 2. ^{1}H−^{1}H COSY (bold lines) and key HMBC (→) correlations for 1−7.

compounds [baraphenazines A (1, yield: 1.02 mg/L), B (2, yield: 1.15 mg/L), C (3, yield: 0.48 mg/L), D (5, yield: 0.94 mg/L), E (4, yield: 0.75 mg/L), F (6, yield: 0.31 mg/L), and G (7, yield: 0.40 mg/L)] and two previously reported metabolites [diastaphenazine (yield: 1.02 mg/L) and izumiphenazine A (yield: 1.30 mg/L)] (Figure 1).

Structure Elucidation. Compound 1 was isolated as a green, amorphous powder, and its molecular formula was established by (+)-HR-ESIMS as $C_{21}H_{18}N_2O_7$, indicating 14 degrees of unsaturation. The analysis of the ^{1}H/^{13}C and HSQC NMR data suggested the presence of one methylene, 11 methine (seven aromatic), one hemiketal, seven sp^2

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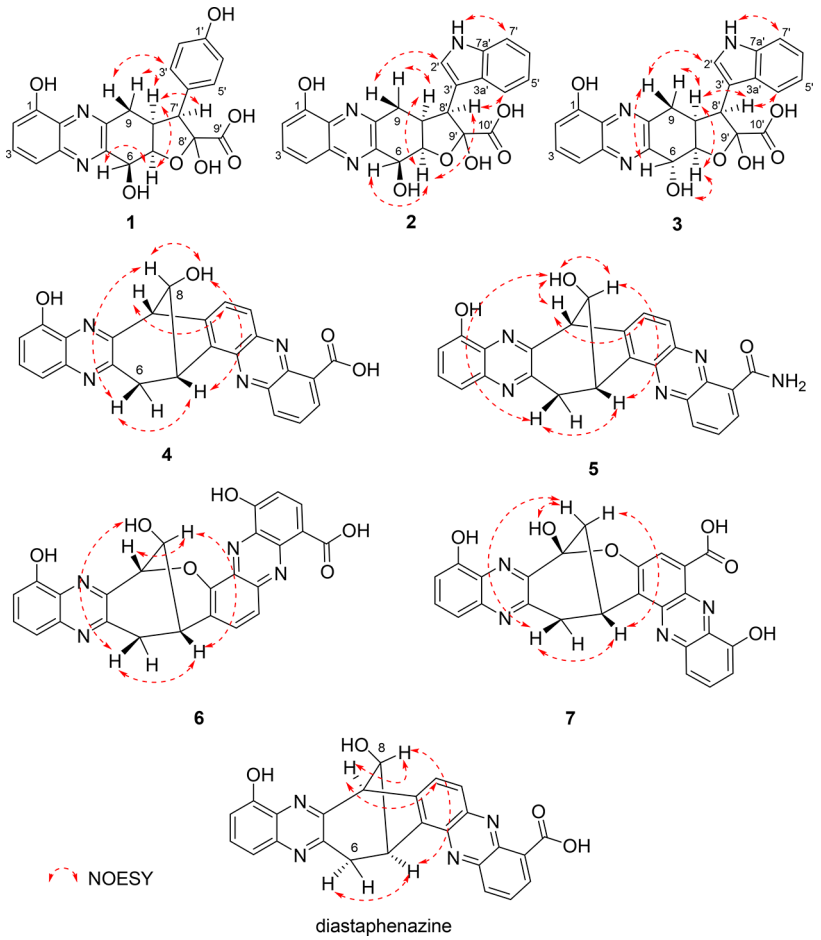
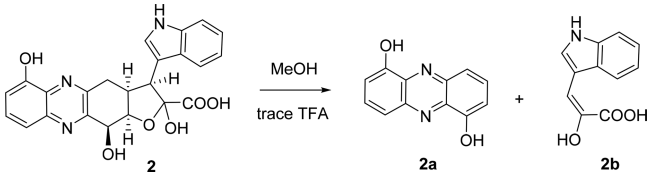


Figure 3. Key NOESY correlations for **1**−**7**.

nonprotonated carbons, and one carboxylic acid (Tables 1 and 2). Analysis of the COSY spectrum revealed the presence of three $^{1}H-^{1}H$ spin systems, including a 1,2,3-trisubstituted benzene ring (CH-2/CH-3/CH-4), a 1,4-disubstituted benzene ring (CH-2′/CH-3′ and CH-4′/CH-5′) and the connectivity of CH-6/CH-7/CH-8/CH-7′/CH_2-9. Key HMBC correlations (Figure 2) established **1** to comprise two key substructures: a 7,8-disubstituted tetrahydrophenazine-1,6-diol (supported by HMBC correlations from H-2 to C-4 and C-10a, from H-3 to C-1 and C-4a, and from H-4 to C-2 and C-10a) and an 1′,8′,8′-trihydroxy-benzenepropanoic acid (based on HMBC correlations from H-7′ to C-8′, C-9′, and C-3′; from H-3′ to C-7′ and C-1′; and from H-2′ to C-4′ and C-6′). The crucial HMBC correlations from H-8 (δ_H 3.29) to C-4′ (δ_C 125.8) and from H-7′ (δ_H 2.89, d) to C-8 (δ_C 40.2) and C-9 (δ_C 32.5) served as a basis for substructure connectivity to establish the preliminary **1** scaffold. The relative configuration of **1** was established by NOESY, which highlighted H-6, H-7, H-8, and H-9α to adopt the same facial orientation (Figure 3). Consistent with the putative **1** 8′-hemiketal, methylation of **1** in the presence of methyl iodide and silver oxide[30] yielded two *per*-methyl-**1** C-8′ diastereomers (compounds **1a** and **1b**, Scheme 1). Thus, the structure of **1** was established as a new natural product, and **1** was named baraphenazine A to reflect the producing strain's point of origin.

Compounds **2** and **3** share a common molecular formula ($C_{23}H_{19}N_3O_6$) and also display similar UV and NMR signatures to that of **1** (Tables 1 and 2) with apparent

Scheme 1. Decomposition of 2



structural divergence deriving from C-7′ substitution (Figure 1). Substitution of the **1** C-7′ phenol side chain with an indole in both **2** and **3** was established by the presence of characteristic indole ^{1}H NMR signals [e.g., for **2**: δ_H 7.35 (s, H-2′), 7.33 (d, H-7′), 7.03 (t, H-6′), 6.89 (t, H-5′), and 7.37 (d, H-4′)]; corresponding ^{13}C NMR signals [e.g., for **2**: δ_C 124.2 (C-2′), 109.0 (C-3′), 127.7 (C-3a′), 118.7 (C-4′), 118.2 (C-5′), 120.6 (C-6′), 111.2 (C-7′), 135.9 (C-7a′)]; and key HMBC [e.g., for **2**: from H-8 (δ_H 3.38, m) to C-3′ (δ_C 109.5) and from H-8′ (δ_H 3.30, s) to C-2′ (δ_C 124.2) and C-3a′ (δ_C 127.7)]; and NOESY correlations (Tables 1 and 2, Figures 1−3). NOESY revealed **2** and **3** to solely differ in C6-OH orientation (Figure 3) and further established **2** and **3** as unprecedented fused 5-hydroxyquinoxaline/indolylpyruvic acid-based metabolites. Compounds **1**−**3** decomposed in MeOH, and, as exemplified by **2**, the decomposition products were identified as 1,6-dihydroxyphenazine and the enol form of indole-3-pyruvic acid (Schemes 1 and S2). As new natural products, **2** and **3** were designated as baraphenazines B and C.

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The determined molecular formula of **4** ($C_{25}H_{16}N_4O_4$) and 2D NMR (Figures 2 and 3) revealed **4** to share a planar structure identical to the previously reported diastaphenazine,[16] also a confirmed metabolite of *Streptomyces* sp. PU-10A. NOESY of **4** and diastaphenazine (Supporting Information, Figures S29 and S74) revealed these compounds to stereochemically differ at C7, C8, and C9 with the observed H-6/H-8 NOE in **4** (Figure 3) and lack thereof in diastaphenazine, consistent with a **4**/diastaphenazine C8 enantiomeric relationship. Consistent with this, the determined optical rotation of **4** ($[\alpha]^{25}_D$ +15.1) differed from that of diastaphenazine (determined $[\alpha]^{25}_D$ −278.0; literature $[\alpha]^{20}_D$ −1248).[16] NMR also highlighted a close structural relationship between **4** and **5**, with divergence at C-6′ (C-6′ carboxylic acid in **4** versus a C-6′ carboxamide in **5**). The relative configuration of **5** was also determined by NOESY (Figure 3). In a similar manner, HRESIMS and 1D/2D NMR (Tables 1 and 2, Figure 2) revealed baraphenazines F and G (**6** and **7**) as two new phenazinolin-type[15] C−O-fused phenazine dimers, where the unique dimeric orientation of **6** is unprecedented compared to previously reported phenazine-based metabolites (Figure 1).

All isolated compounds were evaluated in standard antibacterial, antifungal, and cancer cell line cytotoxicity assays (Supporting Information, Table S1). Only **5** displayed appreciable activity at the concentrations tested [≤50 μM in cytotoxicity assays and ≤120 μM (49.2−56.2 μg/mL) in antibacterial/fungal assays]. Specifically, the similar cancer cell line cytotoxicity [human non-small-cell lung A549, IC_{50} 2.4 μM; human prostate PC3, IC_{50} 4.7 μM] and antimycobacterial [*M. aurum* MIC 30 μM (13.1 μg/mL) and anti-Gram-(+) (*S. aureus*, MIC 4 μM (1.7 μg/mL); *M. luteus*, MIC 7.5 μM (3.3 μg/mL); *B. subtilis*, MIC 7.5 μM (3.3 μg/mL)] potencies of **5** are consistent with those previously reported for the structurally related diastaphenazine. This bioactivity data may implicate a general toxicity-based mechanism of action and the impact of the **5** C-11′-amide on cellular uptake (in contrast to the corresponding free acid on **1**−**4**, **6**, and **7**).[16] Compounds **1**−**7** also lacked activity and/or toxicity at the concentrations tested (≤10 μM) in our recently developed axolotl embryo tail regeneration assay.[25,31]

Discussion. Given the role of phenazines in *Pseudomonas* virulence and quorum-sensing, the biosynthesis of the phenazine core scaffold (phenazine-1-carboxylic acid, phenazine-1,6-dicarboxylic acid, and phenazine) has been well studied.[1−6,32−36] However, the genes/enzymes that contribute to species-specific structural/functional divergence of phenazines remain poorly understood. Previous studies highlighted *Streptomyces* sp. IFM 11204, *Streptomyces diastaticus* subsp. *ardesiacus*, and *Streptomyces diastaticus* YIM DT26 as capable of producing izumiphenazines, diastaphenazine, and phenazinolins, respectively. In contrast, the current study establishes *Streptomyces* sp. PU-10A as the first reported strain to produce diastaphenazine-type (baraphenazines D and E; **4** and **5**, respectively), phenazinolin-type (baraphenazines F and G; **6** and **7**, respectively), and izumiphenazine A-type (baraphenazines A−C; **1**−**3**, respectively) metabolites. Among the diverse set of novel phenazine metabolites identified in this study, baraphenazines A−C (**1**−**3**) stand out as the first reported examples of fused 5-hydroxyquinoxaline/alpha-keto acid-based phenazines. Cumulatively, this study implicates *Streptomyces* sp. PU-10A as a new genomic and metabolic enabling resource for future studies to elucidate the key late-stage biosynthetic transformations/enzymes that contribute to the structural divergence of major phenazine classes.[1−6,32−36]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was recorded on a Jasco DIP-370 digital polarimeter (Jasco, Easton, MD, USA). UV spectra were recorded on an Ultrospec 8000 spectrometer (GE, Pittsburgh, PA, USA). All NMR data were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C with Varian Inova NMR spectrometers (Agilent, Santa Clara, CA, USA). HPLC-MS was conducted with an Agilent 6120 Quadrupole MSD mass spectrometer (Agilent Technologies) equipped with an Agilent 1200 Series Quaternary LC system and an Eclipse XDB-C_{18} column (150 × 4.6 mm, 5 μm). HRESIMS spectra were recorded on an AB SCIEX Triple TOF 5600 System (AB Sciex, Framingham, MA, USA). HPLC analyses were performed on an Agilent 1260 system equipped with a photodiode array (PDA) detector and a Phenomenex C_{18} column (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA). Semipreparative HPLC separation was performed on a Varian Prostar 210 HPLC system equipped with a PDA detector using a Supelco DiscoveryBio wide-pore C_{18} column (250 × 21.2 mm, 10 μm; flow rate, 8 mL/min; Sigma-Aldrich, St. Louis, MO, USA). Size exclusion chromatography was performed on Sephadex LH-20 (25−100 μm; GE Healthcare, Piscataway, NJ). Amberlite XAD16N resin (20−60 mesh) was purchased from Sigma-Aldrich. TLC silica gel plates (60 F_{254}) were purchased from EMD Chemicals Inc. (Darmstadt, Germany). *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella enterica*, *Mycobacterium aurum*, and *Saccharomyces cerevisiae* strains and PC3 and A549 cells were obtained from ATCC (Manassas, VA, USA); *Micrococcus luteus* and *Escherichia coli* were obtained from NRRL (Peoria, IL, USA). All solvents used were of ACS grade and purchased from the Pharmco-AAPER (Brookfield, CT, USA). All other reagents used were reagent grade and purchased from Sigma-Aldrich.

Isolation of *Streptomyces* sp. PU-10A and Taxonomical Identification. A soil sample containing PU-10A was collected from the Himalayan mountain range (Bara Gali, Pakistan; 34°6′0″ N, 73°21′0″ E; altitude 7710 ft). *Streptomyces* sp. PU-10A was isolated following previously reported methods.[18,37] A full colony isolated on M2 medium (glucose, 4.0 g; yeast extract, 4.0 g; malt extract, 10.0 g; $CaCO_3$, 2.0 g; agar, 18.0 g) was used to inoculate M2 broth. After 3 days of incubation at 28 °C the cell pellet was collected and genomic DNA was prepared using UltraClean microbial DNA isolation kit (Mo Bio Laboratories, CA, USA). The partial 16S rRNA gene fragment was amplified using universal primers (27F, 5′-AGAGTTTGATCMTGGCTCAG-3′; 1492R, 5′-GGTTACCTTGTTACGACTT-3′)[38] and Advantage GC 2 polymerase (Clontech, Mountain View, CA, USA). QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) was used to gel-purify the amplified product, which was cloned into pGEM T-easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. The corresponding ligated plasmid construct was transformed into NEB 5-alpha Competent *E. coli* (New England Biolabs, Ipswich, MA, USA) following manufacturer's recommendations. The plasmids were purified using QIAprep Spin Miniprep plasmid purification kit (Qiagen) and sequenced using plasmid forward and reverse primers. Three different clones were sequenced for verification. The insert 16S rRNA sequence (1370 bp) displayed 99% identity (BLAST search) to the 16S rRNA gene sequence of *Streptomyces diastaticus* subsp. *ardesiacus* strain NBRC 15402 and has been deposited in the NCBI nucleotide database with the accession number KY484991.

Fermentation, Extraction, Isolation, and Purification. The terrestrial *Streptomyces* sp. PU-10A was cultivated on M2 agar plates at 28 °C for 7 days. To prepare the seed culture, small pieces of the agar with the fully grown strain were used to inoculate three 250 mL Erlenmeyer flasks, each containing 50 mL of medium A (glucose, 10.0 g; soluble starch, 20.0 g; yeast extract, 5.0 g; peptone, 5.0 g; NaCl, 4.0 g; $CaCO_3$, 2 g; K_2HPO_4, 0.5 g; and $MgSO_4{\cdot}7H_2O$, 0.5 g, dissolved in 1 L of H_2O, pH 7.0; sterilized by autoclaving for 30 min at 121 °C) and grown at 28 °C with shaking (210 rpm) for 3 days. An aliquot of

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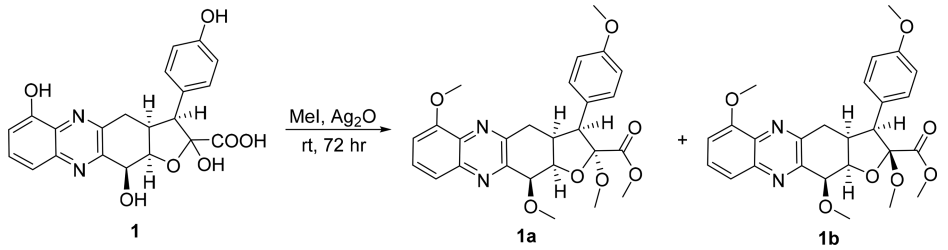
Scheme 2. Methylation of 1



1 1a 1b

seed culture (1 mL) was subsequently used to inoculate 100 250 mL baffled flasks, each containing 100 mL of medium A. The fermentation (10 L total) was continued at 28 °C with shaking (210 rpm) for 8 days. After 8 days, the culture broth was combined and centrifuged at 3000*g* for 30 min (4 °C). The biomass (mycelium) was extracted with MeOH (3 × 800 mL), and the corresponding organics were evaporated *in vacuo* at 40 °C to yield 28.0 g of mycelium crude extract. The supernatant was mixed with 3% (w/v) XAD-16 resin and stirred overnight, followed by filtration. The resin was washed with H_2O (3 × 500 mL) and then extracted with MeOH until the eluent was colorless. The MeOH extract was subsequently evaporated to afford 6.2 g of XAD crude extract. Both extracts (obtained from the biomass and supernatant) revealed a similar metabolite profile based on HPLC and TLC analyses and were therefore combined (34.2 g).

As highlighted in Scheme S1, the combined crude extract was subjected to HP-20SS resin column chromatography (8 × 40 cm, 800 g) eluted with a gradient of aqueous CH_3CN (10−100%) to yield 10 fractions, A−J. Fraction C (1.2 g) was subjected to a Sephadex LH-20 column (4 × 100 cm, 2 mL/min, MeOH) to obtain three subfractions, C1−C3. Subfraction C2 (0.3 g) was further purified by semipreparative HPLC (25−45% aqueous CH_3CN over 30 min) to yield compound **1** (10.2 mg, retention time: 13.4 min) as a green, amorphous powder. Fraction D (1.7 g) was initially purified by a Sephadex LH-20 column (4 × 100 cm, 2 mL/min, MeOH) to obtain four subfractions, D1−D4. Subfraction D2 (0.4 g) was further purified by semipreparative HPLC (30−35% aqueous CH_3CN over 25 min) to yield compound **4** (9.4 mg, retention time: 17.2 min) as yellow, amorphous powders. Subfraction D3 (0.5 g) was further purified by semipreparative HPLC (30−35% aqueous CH_3CN over 22 min) to yield compounds **2** (11.5 mg, retention time: 15.7 min), **3** (4.8 mg, retention time: 17.0 min), and diastaphenazine (10.2 mg, retention time: 14.5 min) as green, amorphous powders. Fraction g (2.2 g) was purified by a Sephadex LH-20 column (4 × 100 cm, 2 mL/min, MeOH) to obtain three subfractions, G1−G3. Subfraction G2 (0.4 g) was further purified by semipreparative HPLC (30−55% aqueous CH_3CN over 30 min) to yield compounds **6** (3.1 mg, retention time: 19.0 min), **7** (4.0 mg, retention time: 20.6 min), and izumiphenazine A (13.0 mg, retention time: 19.9 min) as yellow, amorphous powders. Fraction I (2.3 g) was subjected to a Sephadex LH-20 column (4 × 100 cm, 2 mL/min, MeOH) to obtain five subfractions, I1−I5. Subfraction I3 (0.4 g) was further purified by semipreparative HPLC (20−45% aqueous CH_3CN over 30 min) to yield compound **5** (7.5 mg, retention time: 15.1 min) as a yellow, amorphous powder.

Baraphenazine A (1): green, amorphous powder; $[\alpha]^{25}_D$ −66.7 (*c* 0.45, DMSO); UV (MeOH) λ_{max} (log ε) 228 (8.69), 274 (0.64) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 411.0 $[M + H]^+$; (−)-ESIMS *m/z* 408.9 $[M - H]^-$; (+)-HRESIMS *m/z* 411.1190 $[M + H]^+$ (calcd for $C_{21}H_{19}N_2O_7$, 411.1192).

Baraphenazine B (2): green, amorphous powder; $[\alpha]^{25}_D$ −144.9 (*c* 0.44, DMSO); UV (MeOH) λ_{max} (log ε) 228 (8.72), 260 (1.46), 280 (0.23) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 434.0 $[M + H]^+$; (−)-ESIMS *m/z* 431.9 $[M - H]^-$; (+)-HRESIMS *m/z* 434.1358 $[M + H]^+$ (calcd for $C_{23}H_{20}N_3O_6$, 434.1352).

Baraphenazine C (3): green, amorphous powder; $[\alpha]^{25}_D$ +35.0 (*c* 0.13, DMSO); UV (MeOH) λ_{max} (log ε) 227 (8.50), 263 (0.83), 281 (0.96) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 434.0 $[M + H]^+$; (−)-ESIMS *m/z* 431.8 $[M - H]^-$; (+)-HRESIMS *m/z* 434.1341 $[M + H]^+$ (calcd for $C_{23}H_{20}N_3O_6$, 434.1352).

Baraphenazine D (4): yellow, amorphous powder; $[\alpha]^{25}_D$ +15.1 (*c* 0.57, DMSO); UV (MeOH) λ_{max} (log ε) 226 (9.13), 262 (2.96), 376 (0.76) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 437.0 $[M + H]^+$; HRESIMS *m/z* 437.1252 $[M + H]^+$ (calcd for $C_{25}H_{17}N_4O_4$, 437.1250).

Baraphenazine E (5): yellow, amorphous powder; $[\alpha]^{25}_D$ +38.0 (*c* 0.51, DMSO); UV (MeOH) λ_{max} (log ε) 228 (8.94), 260 (0.71), 372 (0.76) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 436.0 $[M + H]^+$; (−)-ESIMS *m/z* 433.8 $[M - H]^-$; (+)-HRESIMS *m/z* 436.1407 $[M + H]^+$ (calcd for $C_{25}H_{18}N_5O_3$, 436.1410).

Baraphenazine F (6): yellow, amorphous powder; $[\alpha]^{25}_D$ +117.2 (*c* 0.24, DMSO); UV (MeOH) λ_{max} (log ε) 227 (9.09), 260 (1.80), 280 (0.21) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 469.0 $[M + H]^+$; HRESIMS *m/z* 469.1176 $[M + H]^+$ (calcd for $C_{25}H_{17}N_4O_6$, 469.1148).

Baraphenazine G (7): yellow, amorphous powder; $[\alpha]^{25}_D$ −36.3 (*c* 0.18, DMSO); UV (MeOH) λ_{max} (log ε) 227 (8.18), 272 (0.34) nm; ^{13}C and ^1H NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 469.0 $[M + H]^+$; HRESIMS *m/z* 469.1176 $[M + H]^+$ (calcd for $C_{25}H_{17}N_4O_6$, 469.1148).

Diastaphenazine: yellow, amorphous powder; $[\alpha]^{25}_D$ −278.0 (*c* 0.65, DMSO) [lit., $[\alpha]^{20}_D$ −1248 (*c* 0.11, DMSO)];[16] ^{13}C NMR data, see Table 2; (+)-ESIMS *m/z* 437.0 $[M + H]^+$; HRESIMS *m/z* 437.1249 $[M + H]^+$ (calcd for $C_{25}H_{17}N_4O_6$, 437.1250).

Izumiphenazine A: yellow, amorphous powder; $[\alpha]^{25}_D$ −360.3 (*c* 0.28, DMSO) [lit., $[\alpha]^{20}_D$ −480 (*c* 0.14, MeOH)];[13] ^{13}C NMR data, see Table 2; (+)-ESIMS *m/z* 469.0 $[M + H]^+$; HRESIMS *m/z* 469.1127 $[M + H]^+$ (calcd for $C_{25}H_{17}N_4O_6$, 469.1148).

Methylation of Compound 1.[30] CH_3I (0.1 mL) and Ag_2O (23.17 mg, 0.1 mmol) were added to **1** (4.1 mg, 0.01 mmol) dissolved in *tert*-butyl ether (0.1 mL), and the mixture was stirred at room temperature for 72 h. The reaction was subsequently evaporated to dryness under vacuum, dissolved in 0.5 mL of MeOH, and purified via semipreparative HPLC (35−70% aqueous CH_3CN over 30 min) to yield **1a** (1.9 mg, 47.5% yield) and **1b** (1.5 mg, 37.5% yield) (Scheme 2).

O-Permethyl-(8′S)-baraphenazine A (1a): green, amorphous powder; ^{13}C and ^1H NMR data, see Table 3; (+)-ESIMS *m/z* 481.1 $[M + H]^+$; (+)-HRESIMS *m/z* 481.2004 $[M + H]^+$ (calcd for $C_{26}H_{29}N_2O_7$, 481.1975); *m/z* 503.1823 $[M + Na]^+$ (calcd for $C_{26}H_{28}N_2O_7Na$, 503.1794).

O-Permethyl-(8′R)-baraphenazine A (1b): green, amorphous powder; ^{13}C and ^1H NMR data, see Table 3; (+)-ESIMS *m/z* 481.1 $[M + H]^+$; (+)-HRESIMS *m/z* 481.2002 $[M + H]^+$ (calcd for $C_{26}H_{29}N_2O_7$, 481.1975); *m/z* 503.1825 $[M + Na]^+$ (calcd for $C_{26}H_{28}N_2O_7Na$, 503.1794).

Decomposition of 2. Compound **2** (5.0 mg) was dissolved in 0.5 mL of MeOH with a trace of trifluoroacetic acid at room temperature for 48 h, and the decomposition products were purified via semipreparative HPLC (35−45% aqueous CH_3CN over 25 min) to yield two compounds [1,6-dihydroxyphenazine (**2a**, 2.2 mg, 44.0%

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Table 3. ^{1}H and ^{13}C NMR (400 and 100 MHz) Spectroscopic Data for Compounds 1a and 1b in DMSO-d_6 (δ in ppm)

	compound 1a		compound 1b	
no.	δ_C, type	δ_H, mult. (J in Hz)	δ_C, type	δ_H, mult. (J in Hz)
1	155.1, C		155.2, C	
2	108.3, CH	7.26, d (7.8)	108.5, CH	7.29, d (7.9)
3	130.1, CH	7.74, t (7.8, 8.6)	130.6, CH	7.77, t (7.7, 8.4)
4	119.7, CH	7.58, d (8.4)	119.8, CH	7.60, d (8.5)
4a	142.6, C		142.6, C	
5a	149.3, C		149.0, C	
6	81.3, CH	4.66, d (3.7)	80.4, CH	4.65, br s
7	81.7, CH	5.14, dd (3.3, 8.2)	78.7, CH	4.82, d (8.8)
8	41.7, CH	3.23, m	40.4, CH	3.44, m
9α	34.0, CH_2	3.06, m	31.2, CH_2	2.68, d (14.6)
9β		3.40, m		3.37, m
9a	153.9, C		153.5, C	
10a	132.5, C		132.7, C	
1′	158.3, C		158.6, C	
2′	113.5, CH	6.85, d (8.2)	113.3, CH	6.85, d (6.9)
3′	129.1, CH	7.08, d (8.4)	130.6, CH	7.09, d (7.2)
4′	129.5, C		126.0, C	
5′	129.1, CH	7.08, d (8.4)	130.6, CH	7.09, d (7.2)
6′	113.5, CH	6.85, d (8.2)	113.3, CH	6.85, d (6.9)
7′	59.6, CH	3.05, d (5.1)	57.0, CH	2.49, d
8′	109.8, C		104.6, C	
9′	167.3, C		167.4, C	
1-OCH_3	55.9, CH_3	4.02, s	55.9, CH_3	4.02, s
6-OCH_3	57.2, CH_3	3.37, s	56.7, CH_3	3.26, s
1′-OCH_3	54.9, CH_3	3.72, s	54.9, CH_3	3.74, s
8′-OCH_3	50.8, CH_3	2.60, s	50.8, CH_3	3.22, s
9′-OCH_3	51.5, CH_3	3.23, s	52.0, CH_3	3.38, s

yield) and the enol form of indole-3-pyruvic acid (**2b**, 1.7 mg, 34.0% yield)].

*1,6-Dihydroxyphenazine (**2a**):* green, amorphous powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.20 (2H), 7.70 (2H), 7.83 (2H),10.35 (2H, br s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 109.1 (C-2,7), 118.6 (C-4,9), 132.60 (C-3,8), 132.67 (C-5a, 10a), 143.7 (C-4a, 9a), 153.2 (C-1,6); (+)-ESIMS m/z 213.1 $[M + H]^+$; (−)-ESIMS m/z 210.9 $[M - H]^-$.

*Indole-3-pyruvic acid: enol form (**2b**):* yellow, amorphous powder; ^{1}H NMR (400 MHz, CD_3OD) δ 6.90 (1H, s), 7.04−7.13 (2H, m), 7.35 (1H, d, J = 8.1 Hz), 7.66 (1H, d, J = 7.6 Hz), 7.90 (2H, br s); ^{13}C NMR (100 MHz, CD_3OD) δ 104.1, 110.8, 117.5, 119.1, 121.4, 126.8, 127.1, 135.9, 140.1, 160.6, 169.7; (+)-ESIMS m/z 213.1 $[M + H]^+$; (−)-ESIMS m/z 210.9 $[M - H]^-$.

Antibacterial, Antifungal, Cell Line Cytotoxicity, and Axolotl Embryo Tail Regeneration Assays. Antibacterial (*Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* NRRL B-287, *Bacillus subtilis* ATCC 6633, *Mycobacterium Aurum* ATCC 23366, *Escherichia coli* NRRL B-3708, *Salmonella enterica* ATCC 10708), antifungal (*Saccharomyces cerevisiae* ATCC 204508), and cell line cytotoxicity [A549 (lung) and PC3 (prostate) human cancer cell lines] and axolotl embryo tail regeneration assays were accomplished in triplicate following our previously reported protocols.[17−27,39,40] Antibacterial/antifungal MIC values were obtained after 16−48 h of incubation. Kanamycin and ampicillin (*S. aureus*, *M. luteus*, *B. subtilis*, *M. aurum*, *S. enterica*, and *E. coli*), amphotericin B (*S. cerevisiae*), and actinomycin D (A549 and PC3) were used as positive controls. The Hsp90 inhibitor geldanamycin was used as a positive control for the axolotl embryo tail regeneration assays.[25,31]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.9b00289.

Workup isolation scheme; 1D/2D NMR and HRMS spectra of isolated compounds (PDF)

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Notes

The authors declare the following competing financial interest(s): J.S.T. is a co-founder of Centrose (Madison, WI, USA).

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