# 1,3-Oxazin-6-one Derivatives and Bohemamine-Type Pyrrolizidine Alkaloids from a Marine-Derived *Streptomyces spinoverrucosus*

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S *Supporting Information*

ABSTRACT: Two new 1,3-oxazin-6-one derivatives (**1** and **2**) and six new bohemamine-type pyrrolizidine alkaloids (**3−8**) were isolated from the marine-derived *Streptomyces spinoverrucosus* strain SNB-048. Their structures including the absolute configurations were fully elucidated on the basis of spectroscopic analysis, ECD spectra, quantum chemical calculations, and chemical methods. Compounds **1** and **2** possess a γ-lactam moiety and a 1,3-oxazin-6-one system.

■ RESULTS AND DISCUSSION
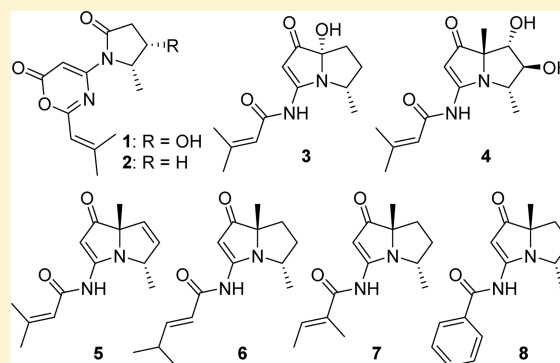
Pyrrolizidine alkaloids, a group of natural products produced by numerous plant families, are an interesting class of molecules due to their potent biological activities and diverse chemical structures.[1] More than 650 pyrrolizidine alkaloids have been isolated from over 6000 plants,[2] and many of them have been demonstrated to be hepatotoxic, genotoxic, and mutagenic.[3] Secondary metabolites from microbes possessing the pyrrolizidine nucleus, such as pyrrolams,[4] jenamidines,[5] and bohemamines,[6] have also been identified. These structures have attracted the attention of synthetic chemists,[7] especially bohemamines, which represented a rare pyrrolizidine subclass due to the methylation pattern and presence of the amide nitrogen on the pyrrolizidine skeleton. To date, only five bohemamine-type pyrrolizidine alkaloids, bohemamine (**12**), bohemamines B (**13**) and C (**14**), 5-chlorobohemamine C (**15**), and NP25302 (**9**), have been reported.[6]

As part of our studies to search for unusual structural features from marine-derived actinomycetes for further investigation, our attention was drawn to a series of metabolites contained in the extract of *Streptomyces spinoverrucosus* strain SNB-048. These compounds showed UV absorptions similar to those of bohemamines in an LC-UV-MS analysis.[6] Chemical investigation on this strain has resulted in the isolation of two novel bohemamine-type pyrrolizidine alkaloids, spithioneines A and B (**10** and **11**), which possess an unusual ergothioneine moiety.[8] Herein, we report another six new bohemamine analogues, bohemamines D−I (**3−8**), and two new 1,3-oxazin-6-one derivatives, spinoxazines A and B (**1** and **2**), together with a known bohemamine analogue, NP25302 (**9**). Structurally, compounds **1** and **2** possess a 1,3-oxazin-6-one system, which is rare in natural products.[21]

Spinoxazine A (**1**) was obtained as a white powder. The molecular formula was determined as $C_{13}H_{16}N_2O_4$ according to its HRESIMS peak at *m/z* 265.1183 [M + H]+. There were multiple IR stretches at 1740 cm−1 indicative of a lactam or ester moiety (five-membered-ring lactams absorb in the 1750−1700 cm−1 region; four-membered-ring lactams absorb at 1760−1730 cm−1; esters absorb at 1750−1735 cm−1; α,β-unsaturated esters appears at 1730−1715 cm−1). The 13C NMR spectrum showed 13 carbon signals that were classified by HSQC as three methyl carbons, one sp3 methylene carbon, four methine groups (two olefinic carbons, one carbon connected with oxygen, and one carbon connected with nitrogen), and five nonprotonated carbons (three carbonyls and two olefinic carbons) (Table 1). The 1H NMR spectrum showed five coupled signals at δ_H 1.23 (H-8, d, *J* = 6.5), 4.55 (H-4, qd, *J* = 6.7, 6.6), 4.43 (H-5, ddd, *J* = 8.8, 8.0, 6.7), 2.67 (H-6β, dd, *J* = 16.9, 8.0), and 2.63 (H-6α, dd, *J* = 16.9, 8.8). These data combined with COSY correlations of H-8/H-4/H-5/H-6 and the key HMBC correlations of H-8 to C-4/C-5 and H-4/H-5 to C-6/C-7 (Figure 1) constituted the 4-hydroxy-5-methyl-γ-lactam moiety. Two vinyl methyl groups at δ_H 2.25 (H-5′, d, *J* = 1.2 Hz) and 2.03 (H-4′, d, *J* = 1.2 Hz) and one olefinic proton signal at δ_H 5.98 (m), along with the HMBC correlations of H-2′ to C-4′/C-5′ and H-4′ to C-5′ (Figure 1), indicated the presence of an isobutenyl fragment. The isobutenyl fragment should be connected with C-1′ (δ_C 163.7), which was

Chart 1
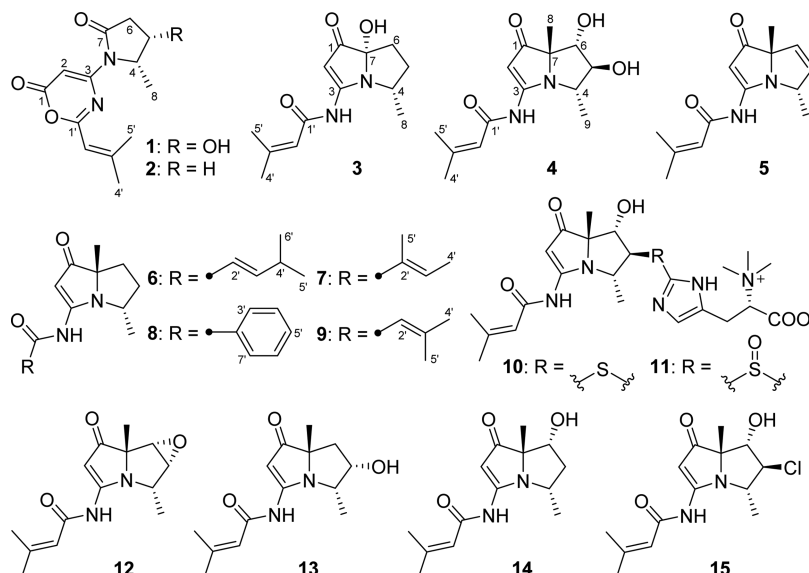
Table 1. ^{1}H (600 MHz) and ^{13}C (100 MHz) NMR Data for Compounds 1 and 2 in DMSO-d_6

	1		2	
no.	δ_C	δ_H, mult. (J in Hz)	δ_C	δ_H, mult. (J in Hz)
1	159.8, C		159.9, C	
2	89.0, CH	6.73, s	88.3, CH	6.79, s
3	155.3, C		155.5, C	
4	57.7, CH	4.55, qd (6.7, 6.6)	54.3, CH	4.65, qd (6.9, 6.6)
5	64.1, CH	4.43, ddd (8.8, 8.0, 6.7)	24.7, CH$_2$	2.21, m; 1.71, m
6	39.3, CH$_2$	2.67, dd (16.9, 8.0); 2.63, dd (16.9, 8.8)	31.4, CH$_2$	2.84, m; 2.45, m
7	173.4, C		176.0, C	
8	13.3, CH$_3$	1.23, d (6.5)	20.0, CH$_3$	1.31, d (6.4)
1'	163.7, C		163.7, C	
2'	116.2, CH	5.98, m	116.2, CH	5.98, m
3'	158.7, C		158.7, C	
4'	28.0, CH$_3$	2.03, d (1.2)	28.1, CH$_3$	2.03, s
5'	21.0, CH$_3$	2.25, d (1.2)	21.1, CH$_3$	2.25, s

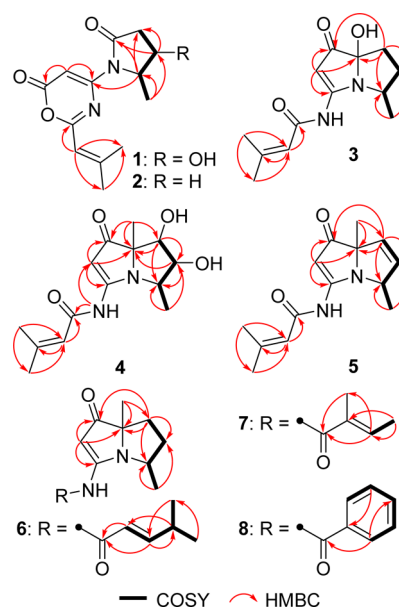**Figure 1.** Key correlations for the structural assignment of 1−8.

determined by the HMBC correlation of H-2' to C-1' (Figure 1). The remaining ^{1}H and ^{13}C NMR signals and the HMBC correlations of H-2 ($\delta_{H/C}$ 6.73/89.0) to C-1 (δ_C 159.8) and C-3 (δ_C 155.3) (Figure 1), along with the molecular formula, provided two possible structures that could not be easily distinguished: a 1,3-oxazin-6-one (1) or an unsaturated β-lactam unit (1a) (Figure 2). The proposed unsaturated β-lactam in 1a would be unprecedented in a natural product. Only one natural product possessing an unsaturated β-lactam moiety was reported,[9] but it was later revised to be a six-membered-ring metabolite.[10] Synthetic efforts to produce similar unsaturated β-lactams have been reported; however, literature reports suggest that such β-lactams rearrange to form the corresponding 1,3-oxazin-6-ones (Scheme 1).[11] In general, the ^{1}H and ^{13}C NMR data for the corresponding β-lactam and 1,3-oxazin-6-one structures for this natural product would be nearly identical. Predicted ^{13}C NMR shifts of both 1,3-oxazin-6-one (1) and β-lactam (1a), which were computed at the
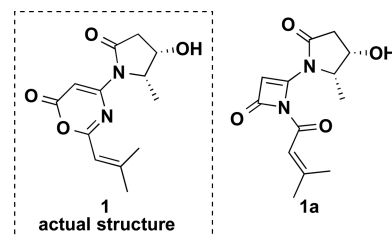

Figure 2. Two structures, 1 and 1a, in general agreement with the NMR data.

B3LYP/6-311++G(2d,p)//B3LYP/6-31G(d) level (full details on the calculation can be found in the Supporting Information),[12] strongly support that the chemical shift values of 1 are consistent with a 1,3-oxazin-6-one skeleton (Table S1), particularly the ^{13}C shifts of C-1, C-2, and C-3. The linkage

Scheme 1. Mechanism for the Conversion of *N*-Acyl-*β*-lactams into 1,3-Oxazin-6-ones
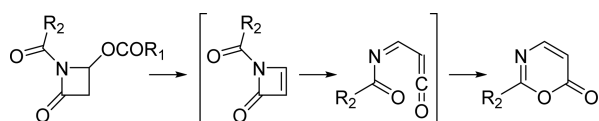
between the *γ*-lactam and the 1,3-oxazin-6-one could be confirmed by the HMBC correlation of H-4 to C-3.

NOE correlations between H-8 and H-5/H-6*β*, between H-4 and H-5, and between H-5 and H-6*α*/H-6*β* were observed in the 2D NOESY spectrum, suggesting the relative configuration of **1** as shown (Figure 3). NOE correlations combined with the
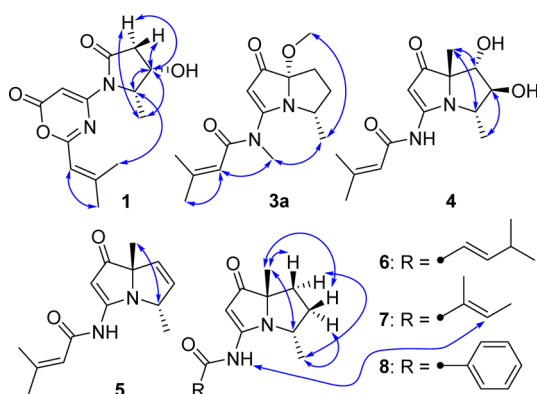

Figure 3. Selected NOESY correlations of **1**, **3a**, and **4**−**8**.

coupling constants between H-4 and H-5 (J = 6.7 Hz), between H-5 and H-6*α* (J = 8.8 Hz), and between H-5 and H-6*β* (J = 8.0 Hz) also indicated the conformation of **1** as shown in Figure 4. We utilized the empirical electronic circular dichrosim (ECD) rule of *γ*-lactams[13] to determine the absolute configuration of **1**. The ECD sign of *γ*-lactams can be determined by addition of two effects: configuration of C_α and ring chirality. If the *γ*-lactams have no lone pair electrons on the C_α substituent, the ECD signs are determined by the ring chirality (conformation A gives a negative sign, and B gives a positive sign) (Figure 4).[13] The conformation of (4*S*, 5*S*)-**1**, suggested by NOE correlations and chemical calculation (Supporting Information), is consistent with A, which was responsible for the negative Cotton effect ($\pi-\pi^*$ transition) in accordance with the measured negative ECD Cotton effect at λ_{max} 254 nm of **1** (Figure 4). As the 1,3-oxazin-6-one group connected with the nitrogen nearly lay on the same plane of the amide, it made a very small contribution to the lactam Cotton effect. Moreover, the predicted ECD spectrum was obtained by the TDDFT [B3LYP/6-31G(d)] method (Supporting Information),[14] which was subsequently compared with the experimental data. The measured ECD curve of **1** showed a Cotton effect at λ_{max} ($\Delta\varepsilon$) 254 (−4.7) nm, matching with the calculated ECD curve of (4*S*,5*S*)-**1** (Figure 5).

The molecular formula of spinoxazine B (**2**) was assigned as $C_{13}H_{16}N_2O_3$ based on the HRESIMS, which was only one oxygen less than that of **1**. Careful comparison of its ^{1}H and ^{13}C NMR spectra (Table 1) with those of **1** showed that methylene signals at $\delta_{C/H}$ 24.7/2.21 and 1.71 in **2** replaced the corresponding oxygenated methine signals at $\delta_{C/H}$ 64.1/4.43 in **1**. The COSY correlations of H-8/H-4/H-5/H-6 and the key HMBC correlations of H-8 to C-4/C-5 and H-4/H-5 to C-6/
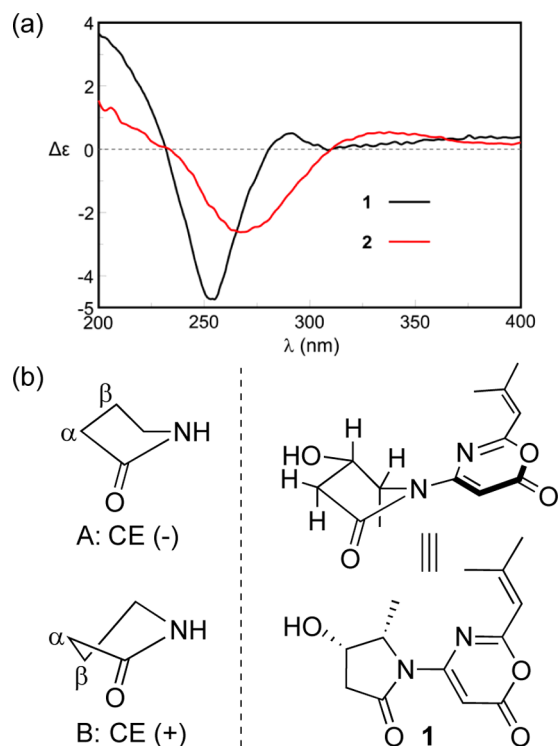

Figure 4. (a) ECD spectra of **1** and **2**. (b) Ring chirality and ECD sign of *γ*-lactams and the conformation of **1**.
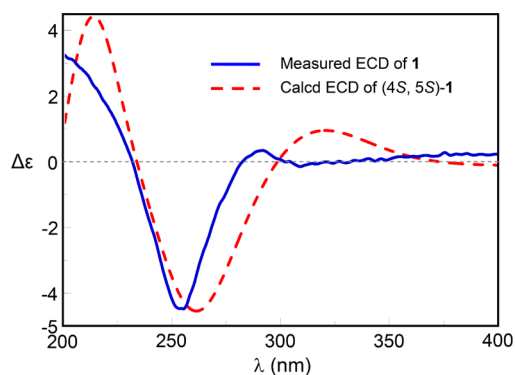

Figure 5. Measured and calculated ECD spectra for **1**.

C-7 also supported this change in **2** (Figure 1). The ECD Cotton effect at λ_{max} ($\Delta\varepsilon$) 267 (−2.6) was nearly identical to **1** (Figure 4), indicating the same absolute configuration.

Bohemamine D (**3**) was isolated as a colorless oil. Its molecular formula was $C_{13}H_{18}N_2O_3$ based on the HRESIMS data. The UV spectrum showed similar absorptions to bohemamines at 252, 283, and 332 nm.[6] Comparison of its NMR spectra with those of known bohemamine analogues indicated that the ^{1}H and ^{13}C NMR data of **3** (Table 2) were similar to NP25302 (**9**).[6c] The signal of the C-7 methyl group in NP25302 was no longer present, and δ_C at C-7 was shifted deshielded to 95.3, leading to the assignment of a C-7 hydroxy group. The molecular formula and the key HMBC correlations of H-8 to C-4/C-5 and H-6 to C-4/C-5/C-7/C-1 (Figure 1) also confirmed the structure of **3**. In order to determine its relative configuration, a methylation reaction was carried out using iodomethane and sodium hydride to generate **3a** (Figure S2). In the NOESY spectrum of compound **3a**, the signal between H-8 and 7-OCH$_3$ could be observed, which indicated

Table 2. ^{1}H (600 MHz) and ^{13}C (100 MHz) NMR Data for Compounds 3−5 in DMSO-d_6

| no.	3		4		5	
	δ_C	δ_H, mult. (J in Hz)	δ_C	δ_H, mult. (J in Hz)	δ_C	δ_H, mult. (J in Hz)
1	198.9, C		201.5, C		203.2, C	
2	89.1, CH	5.42, s	93.5, CH	5.41, s	93.1, CH	5.58, s
3	168.4, C		166.7, C		168.0, C	
4	55.2, CH	3.71, qd (6.7, 3.7)	62.9, CH	3.76, qd (6.9, 3.3)	62.0, CH	4.40, ddd (6.7, 2.2, 1.4)
5	32.7, CH$_2$	1.91, m; 1.74, m	87.0, CH	3.92, brs	128.0, CH	5.74, dd (6.0, 2.2)
6	32.0, CH$_2$	1.82, m; 1.71, m	77.3, CH	3.70, brs	133.9, CH	5.67, dd (6.0, 1.4)
7	95.3, C		78.4, C		80.2, C	
8	22.0, CH$_3$	1.30, d (6.6)	23.2, CH$_3$	1.31, s	24.8, CH$_3$	1.22, s
9			16.4, CH$_3$	1.20, d (6.9)	17.9, CH$_3$	1.19, d (6.6)
1′	164.7, C		163.9, C		164.0, C	
2′	117.9, CH	6.13, m	117.7, CH	5.99, m	117.5, CH	5.98, m
3′	156.5, C		156.0, C		156.6, C	
4′	27.3, CH$_3$	1.90, d (1.2)	27.3, CH$_3$	1.89, d (1.3)	27.3, CH$_3$	1.90, d (1.2)
5′	20.1, CH$_3$	2.13, d (1.2)	20.0, CH$_3$	2.14, d (1.3)	20.0, CH$_3$	2.15, d (1.2)
NH		9.90, s		10.31, s		10.49, s
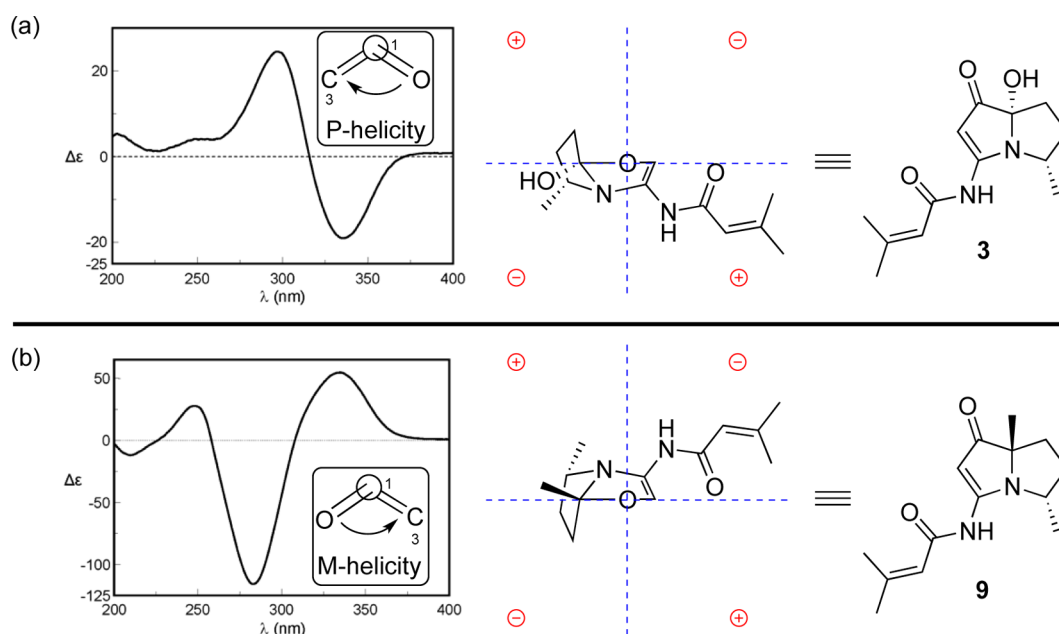
Figure 6. ECD spectra and the representation of the octant rule and helicity rule for the cyclopentenone moiety of **3** (a) and **9** (b).

the relative configuration of **3** as shown. The octant rule for cyclopentenone[8,15] was used to determine the absolute configuration (Figure 6). The conformation of (4S,7S)-**3** as shown in Figure 6 was placed in accordance with the octant rule model. The functional group at C-7 lying in the back lower left area was responsible for the negative Cotton effect (n−π*), which was consistent with the Cotton effect at 335 nm ($\Delta\varepsilon$ −19.0). Moreover, the ECD helicity rule of α,β-unsaturated ketones[16] was used to further confirm the absolute configuration of compound **3**. The P-helicity of the O=C−C$_\alpha$=C$_\beta$ torsion angle correlates the positive Cotton effect (π−π*) at 297 nm (Figure 6).

Bohemamine E (**4**) was also thought to be an analogue of bohemamines due to the similar UV absorptions at 252, 280, and 335 nm.[6] The molecular formula was determined as C$_{14}$H$_{20}$N$_2$O$_4$ by HRESIMS, with a H$_2$O unit more than bohemamine (**12**). The 1D NMR spectrum was similar to **12** except for the shifts of C-5 and C-6. So, we deduced that the epoxide ring in **12** was hydrolyzed to yield compound **4**. COSY

and HMBC data (Figure 1) confirmed this diol structure. NOE correlations between H-5 and H-9, between H-4 and H-8, and between H-6 and H-8 were observed in the 2D NOESY, suggesting the relative configuration of **4** as shown (Figure 3).

The molecular formula of bohemamine F (**5**) was determined to be C$_{14}$H$_{18}$N$_2$O$_2$ from the protonated molecule peak at m/z 247.1441 [M + H]$^+$ in the positive-ion HRESIMS spectrum, which was two hydrogen atoms less than that of NP25302 (**9**). Comparison of its ^{1}H and ^{13}C NMR spectra (Table 2) with those of **9** revealed that the signals of two methylene groups (C-5 and C-6) were replaced by two coupled olefinic methine signals (C-5: $\delta_{C/H}$ 128.0/5.74, dd, J = 6.0, 2.2; C-6: $\delta_{C/H}$ 133.9/5.67, dd, J = 6.0, 1.4). This structure can be confirmed by COSY correlations of H-9/H-4/H-5/H-6 and the key HMBC correlations from H-9 to C-4/C-5, H-6 to C-4/C-5/C-7/C-1, and H-8 to C-6/C-7. The relative configurations of C-4 and C-7 could be determined by the NOE correlation between H-8 and H-4.

Table 3. ^{1}H (600 MHz) and ^{13}C (100 MHz) NMR Data for Compounds 6–8 in DMSO-d_6

	6		7		8	
no.	δ_C	δ_H, mult. (J in Hz)	δ_C	δ_H, mult. (J in Hz)	δ_C	δ_H, mult. (J in Hz)
1	204.0, C		203.8, C		203.9, C	
2	93.5, CH	5.54, s	93.3, CH	5.46, s	93.9, CH	5.60, s
3	166.3, C		166.9, C		167.1, C	
4	54.2, CH	3.97, qd (6.6, 6.6)	54.3, CH	4.03, qd (6.6, 6.6)	54.4, CH	4.10, qd (6.6, 6.6)
5	34.5, CH$_2$	2.39, m; 1.74, m	34.4, CH$_2$	2.37, m; 1.71, m	34.5, CH$_2$	2.41, m; 1.75, m
6	27.6, CH$_2$	1.67, dd (12.5, 6.2);	27.7, CH$_2$	1.67, dd (12.4, 6.3);	27.8, CH$_2$	1.71, dd (12.3, 6.2);
		1.52, dd (12.1, 6.2)		1.53, dd (12.2, 6.5)		1.56, dd (12.0, 6.9)
7	73.1, C		73.3, C		73.4, C	
8	24.7, CH$_3$	1.15, s	24.6, CH$_3$	1.16, s	24.6, CH$_3$	1.20, s
9	16.7, CH$_3$	1.00, d (6.4)	16.8, CH$_3$	1.01, d (6.5)	16.9, CH$_3$	1.09, d (6.4)
1'	163.7, C		167.5, C		165.8, C	
2'	120.3, CH	6.19, dd (15.5, 1.5)	131.5, C		133.4, C	
3'	154.3, CH	6.91, dd (15.5, 6.3)	133.9, CH	6.50, qd (6.8, 1.4)	128.2, CH	7.91, d (7.5)
4'	30.3, CH	2.50, m	14.1, CH$_3$	1.79, d (6.8)	128.5, CH	7.55, t (7.6)
5'	21.1, CH$_3$	1.04, d (6.8)	12.3, CH$_3$	1.80, s	132.5, CH	7.64, t (7.4)
6'	21.1, CH$_3$	1.04, d (6.8)		10.28, s	128.5, CH	7.55, t (7.6)
7'					128.2, CH	7.91, d (7.5)
NH		10.49, s				10.92, s

The molecular formulas of bohemamines G–I (6–8) were determined as $C_{15}H_{22}N_2O_2$, $C_{14}H_{20}N_2O_2$, and $C_{16}H_{18}N_2O_2$ based on their HRESIMS spectra at m/z 263.1754 [M + H]$^+$, 249.1596 [M + H]$^+$, and 271.1443 [M + H]$^+$, respectively. Examination of their ^{1}H and ^{13}C NMR spectra (Table 3) showed that they had the same pyrrolizidine skeleton as 9, with variation only in the amide moiety. For compound 6, the COSY correlations of H-2'/H-3'/H-4' and H-5'/H-4'/H-6' and the key HMBC correlations from H-2' to C-1', H-3' to C-1'/C-4', H-4' to C-6', H-5' to C-6', and H-6' to C-3' suggested the presence of a 4-methylpent-2-enamide side chain (Figure 1). The large coupling constant between H-2' and H-3' (J = 15.5 Hz) indicated the E- configuration. Compound 7 was determined to be an isomer of 9 (Table 3), where the amide moiety is a 2-methylbut-2-enamide group. This structure was supported by the COSY correlation of H-3'/H-4' and the HMBC correlations from H-3' to C-1'/C-5', H-4' to C-2'/C-3', and H-5' to C-1' (Figure 1). The E-configuration for this side chain was determined by the NOE correlation between H-3' and 3-NH (Figure 3). The NMR data were also consistent with those reported in the literature.[7b] In the 1D NMR spectrum of compound 8 (Table 3), the signals of a monosubstituted phenyl group were observed, which were further supported by 2D correlations (Figure 1). The NOE correlations between H-9 and H-5'β/H-6'β and between H-8' and H-4'/H-5'α/H-6'α could be observed in the 2D NOESY spectra of compounds 6–8, which revealed that they had the same relative configuration as shown (Figure 3).

The absolute configuration of compound 9 has been determined as (4S,7S) by total synthesis,[5c] which can be confirmed with the octant rule for cyclopentenone (Figure 6).[8,15] The ECD helicity rule of α,β-unsaturated ketones can also be used to confirm this conclusion. M-Helicity of the O═C–C$_\alpha$═C$_\beta$ torsion angle of compound 9 was consistent with the negative Cotton effect ($\pi–\pi^*$) at 283 nm (Figure 6), which suggested the absolute configuration of 9 as (4S,7S). The ECD spectra of compounds 4–8 showed the same Cotton effects as those of 9 (Figure 7), indicating the same core configurations.

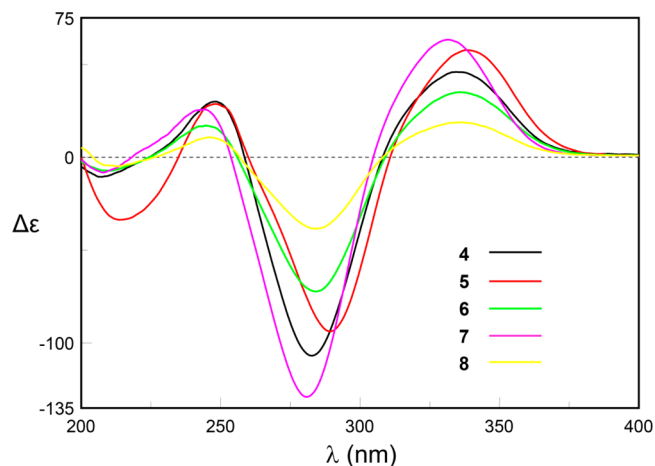A plausible biogenetic pathway for spinoxazines A and B (1 and 2) and bohemamine D (3) is postulated (Scheme 2). L-
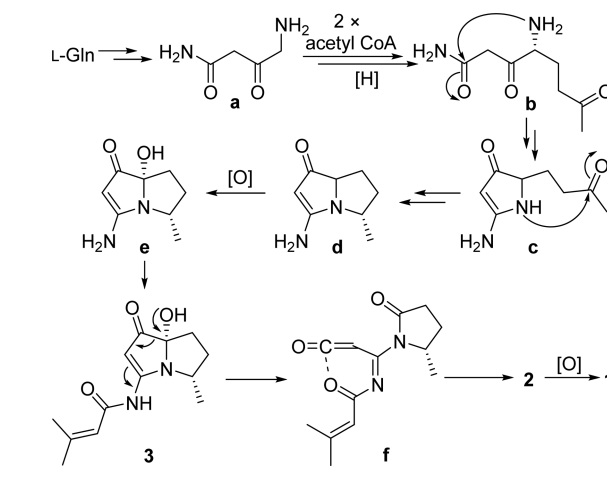

Figure 7. ECD spectra of 4–8.

Scheme 2. Plausible Biogenetic Pathway of Compounds 1–3

Glutamine undergoes decarboxylation and oxidation to generate the primary amine intermediate a, followed by condensation with two acetyl-CoA units and reduction to

yield intermediate **b**.[17] Dual nucleophilic addition of the amine to the amide and methyl ketone carbonyls, respectively, followed by elimination and reduction would provide the pyrrolizidine core **d**. Oxidation of the bridgehead carbon would result in the hemiaminal intermediate **e**, which undergoes N-acylation to yield compound **3**.[8,18] Dehydrogenation of the pyrrolizidine core would give the ring cleavage intermediate **f**,[19] which could undergo a pericyclic reaction to form the 1,3-oxazin-6-one compound spinoxazine B (**2**). Further oxidation would generate spinoxazine A (**1**).

Compounds **1−8** did not show cytotoxicity to non-small-cell lung cancer cell lines HCC366, A549, HCC44, and HCC1171. In addition, they showed no antibacterial activities against *Pseudomonas aeruginosa* and *Bacillus subtilis*.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded with an AUTOPOL AP IV-6W polarimeter equipped with a halogen lamp (589 nm). UV spectra were recorded on a Shimadzu UV-1601 UV−vis spectrophotometer. ECD spectra were measured on a JASCO J-815 spectrometer. IR spectra were obtained on a PerkinElmer Spectrum 1000 FT-IR spectrometer. ^{1}H and 2D NMR spectroscopic data were recorded at 600 MHz in DMSO-d_6 or CDCl$_3$ solution on a Varian System spectrometer, and chemical shifts were referenced to the corresponding residual solvent signal ($\delta_{H/C}$ 2.50/39.52 for DMSO-d_6 and $\delta_{H/C}$ 7.26/77.16 for CDCl$_3$). ^{13}C NMR spectra were acquired at 100 MHz on a Varian System spectrometer. High-resolution ESI-TOF mass spectra were provided by The Scripps Research Institute (La Jolla, CA, USA). Low-resolution LC/ESIMS data were measured using an Agilent 1200 series LC/MS system with a reversed-phase C$_{18}$ column (Phenomenex Luna, 150 mm × 4.6 mm, 5 μm) at a flow rate of 0.7 mL/min. Preparative HPLC was performed on an Agilent 1200 series instrument with a DAD detector, using a reversed-phase C$_{18}$ column (Phenomenex Luna, 250 × 10.0 mm, 5 μm), a phenyl-hexyl column (Phenomenex Luna, 250 × 10.0 mm, 5 μm), or a CN column (Phenomenex Luna, 250 × 10.0 mm, 5 μm). Sephadex LH-20 (GE Healthcare) and ODS (50 mm, Merck) were used for column chromatography. Artificial seawater was used in microbial fermentations as described in a previous reference.[20]

Collection and Phylogenetic Analysis of Strain SNB-048. *Streptomyces* sp. strain SNB-048 was isolated from a sand sample collected from a Bahamian tidal flat. The sediment was desiccated and stamped onto agar plates using gauze 1 acidic media (10 g starch, 1 g NaNO$_3$, 0.5 g K$_2$HPO$_4$, 0.5 g MgSO$_4$, 0.5 g NaCl, 0.01 g FeSO$_4$, 1 L seawater, 15 g agar, pH adjusted to 5.3 with phosphate buffer). Bacterial colonies were selected and streaked to purity using the same agar media. Analysis of the strain by 16S rRNA revealed 99.9% identity to *Streptomyces spinoverrucosus*. The sequence is deposited in GenBank under accession no. KR091963.

Cultivation and Extraction of SNB-048. Bacterium SNB-048 was cultured in 20 × 2.8 L Fernbach flasks each containing 1 L of a seawater-based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g CaCO$_3$, 40 mg Fe$_2$(SO$_4$)$_3$·4H$_2$O, 100 mg KBr) and shaken at 200 rpm at 27 °C. After 7 days of cultivation, sterilized XAD-7-HP resin (20 g/L) was added to adsorb the organic products, and the culture and resin were shaken at 200 rpm for 2 h. The resin was filtered through cheesecloth, washed with deionized H$_2$O, and eluted with acetone. The acetone-soluble fraction was dried *in vacuo* to yield 1.2 g of extract. In order to get more material, another cultivation of 10 L was done under the same condition. From this cultivation, 460 mg of extract was obtained.

Purification. The extract (1.2 g) of the first cultivation was fractionated by flash column chromatography on ODS (50 μm, 30 g), eluting with a step gradient of MeOH and H$_2$O (10:90−100:0), and 12 fractions (Fr1.1−Fr1.12) were collected. Fractions 7 and 8 (403.7 mg) were combined and separated into six fractions (Fr1.7.1−Fr1.7.6) on Sephadex LH-20, eluting with MeOH. Fr1.7.3 (151.2 mg) was further separated into 16 fractions (Fr1.7.3.1−Fr1.7.3.16) by flash

column chromatography on ODS (50 μm, 30 g), eluting with a step gradient of MeOH and H$_2$O (10:90−100:0). Fr1.7.3.6 (6.3 mg) was purified by HPLC on a phenyl-hexyl column (Phenomenex Luna, 250 × 10.0 mm, 5 μm, 2.5 mL/min) using a gradient solvent system from 20% to 100% CH$_3$CN (0.1% formic acid) over 20 min to yield compound **4** (1.4 mg, t_R = 9.5 min). Fr.7.3.8 (17.5 mg) was purified by HPLC on a phenyl-hexyl column (Phenomenex Luna, 250 × 10.0 mm, 5 μm, 2.5 mL/min) using a gradient solvent system from 20% to 85% CH$_3$CN (0.1% formic acid) over 20 min to afford compound **3** (1.9 mg, t_R = 11.1 min). Fr1.7.3.9 (8.1 mg) was also separated by HPLC on a phenyl-hexyl column (Phenomenex Luna, 250 × 10.0 mm, 5 μm, 2.5 mL/min) using a gradient solvent system from 20% to 85% CH$_3$CN (0.1% formic acid) over 20 min to afford compound **7** (0.9 mg, t_R = 12.5 min). Fr1.7.3.10 (31.7 mg) was separated by a reversed-phase HPLC (Phenomenex Luna, C$_{18}$, 250 × 10.0 mm, 5 μm, 2.5 mL/min) using a gradient solvent system from 20% to 80% CH$_3$CN (0.1% formic acid) over 20 min to yield compound **9** (3.8 mg, t_R = 13.4 min) and Fr1.7.3.10.4 (12.6 mg, t_R = 14.3 min). Fr1.7.3.10.4 (12.6 mg) was further purified by HPLC on a CN column (Phenomenex Luna, 250 × 10.0 mm, 5 μm, 2.5 mL/min) using a gradient solvent system from 20% to 100% CH$_3$CN (0.1% formic acid) over 20 min to afford compounds **5** (3.3 mg, t_R = 11.5 min) and **8** (2.3 mg, t_R = 12.0 min). Fr1.7.3.11 (9.1 mg) was purified by HPLC on a phenyl-hexyl column (Phenomenex Luna, 250 × 10.0 mm, 5 μm, 2.5 mL/min) using a gradient solvent system from 20% to 100% CH$_3$CN (0.1% formic acid) over 20 min to afford compound **6** (1.8 mg, t_R = 13.6 min). Fr1.7.5 (21.7 mg) was purified by reversed-phase HPLC (Phenomenex Luna, C$_{18}$, 250 × 10.0 mm, 5 μm, 2.5 mL/min) using a gradient solvent system from 20% to 90% CH$_3$CN (0.1% formic acid) over 15 min to afford Fr1.7.5.1 (1.2 mg, t_R = 12.2 min). Fr1.7.5.1 (1.2 mg) was further purified by HPLC on a phenyl-hexyl column (Phenomenex Luna, 250 × 10.0 mm, 5 μm, 2.5 mL/min) using a gradient solvent system from 20% to 100% CH$_3$CN (0.1% formic acid) over 20 min to yield compound **1** (0.8 mg, t_R = 13.4 min). The extract (460 mg) of the second cultivation was fractionated by flash column chromatography on ODS (50 μm, 30 g), eluting with a step gradient of MeOH and H$_2$O (10:90−100:0), and eight fractions (Fr2.1−Fr2.8) were collected. Fr2.4 (21.1 mg) separated into nine fractions (Fr2.4.1−Fr2.4.9) on Sephadex LH-20, eluting with MeOH. Fr2.4.6 (3.5 mg) was purified by reversed-phase HPLC (Phenomenex Luna, C$_{18}$, 250 × 10.0 mm, 5 μm, 2.5 mL/min) using a gradient solvent system from 30% to 100% CH$_3$CN (0.1% formic acid) over 20 min to yield compound **2** (0.6 mg, t_R = 15.7 min).

Spinoxazine A (1): white powder, $[\alpha]^{23}_D$ +17 (c 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 254 (4.04), 318 (3.79) nm; ECD (c = 0.87 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 291 (+0.5), 254 (−4.7) nm; IR (NaCl disk) ν_{max} 3398, 2982, 2925, 1740, 1594, 1541, 1394, 1272, 1258, 1208, 1187, 1102, 1071, 830 cm^{-1}; ^{1}H and ^{13}C NMR, Table 1; HRESIMS m/z 265.1183 [M + H]$^{+}$ (calcd for C$_{13}$H$_{17}$N$_2$O$_4$, 265.1183).

Spinoxazine B (2): white powder, $[\alpha]^{23}_D$ +6 (c 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 255 (4.10), 320 (3.82) nm; ECD (c = 1.33 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 333 (+0.5), 267 (−2.6) nm; IR (NaCl disk) ν_{max} 2918, 2850, 1739, 1734, 1593, 1540, 1387, 1254, 1184, 830 cm^{-1}; ^{1}H and ^{13}C NMR, Table 1; HRESIMS m/z 249.1235 [M + H]$^{+}$ (calcd for C$_{13}$H$_{17}$N$_2$O$_3$, 249.1234).

Bohemamine D (3): colorless oil, $[\alpha]^{23}_D$ −34 (c 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 252 (4.05), 283 (3.79), 332 (3.69) nm; ECD (c = 0.40 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 336 (−19.0), 297 (+24.5) nm; IR (NaCl disk) ν_{max} 3430, 2100, 1645, 1506, 1394, 1206, 1135, 1058, 793, 733 cm^{-1}; ^{1}H and ^{13}C NMR, Table 2; HRESIMS m/z 251.1391 [M + H]$^{+}$ (calcd for C$_{13}$H$_{19}$N$_2$O$_3$, 251.1390).

Bohemamine E (4): colorless oil, $[\alpha]^{23}_D$ −7 (c 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 252 (4.01), 280 (3.78), 335 (3.70) nm; ECD (c = 0.25 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 335 (+45.8), 282 (−106.8), 248 (+29.9), 207 (−10.5) nm; IR (NaCl disk) ν_{max} 3422, 2092, 1638, 1201, 1130, 734 cm^{-1}; ^{1}H and ^{13}C NMR, Table 2; HRESIMS m/z 281.1496 [M + H]$^{+}$ (calcd for C$_{14}$H$_{21}$N$_2$O$_4$, 281.1496).

Bohemamine F (5): colorless oil, $[\alpha]^{23}_D$ +422 (c 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 246 (4.02), 288 (3.77), 338 (3.66) nm; ECD (c = 0.41 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 339 (+57.6), 290 (−93.7), 248

(+28.7), 207 (−26.2) nm; IR (NaCl disk) ν_{max}: 3432, 2101, 1643, 1506, 1361, 1228, 1134, 1099, 756 cm^{-1}; ^{1}H and ^{13}C NMR, Table 2; HRESIMS m/z 247.1441 [M + H]$^{+}$ (calcd for $C_{14}H_{19}N_2O_2$, 247.1441).

Bohemamine G (6): colorless oil, $[\alpha]^{23}_D$ +17 (c 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 245 (4.09), 284 (3.81), 337 (3.71) nm; ECD (c = 0.38 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 335 (+34.9), 284 (−72.3), 244 (+16.8), 209 (−7.1) nm; IR (NaCl disk) ν_{max} 3428, 2100, 1504, 1132, 677 cm^{-1}; ^{1}H and ^{13}C NMR, Table 3; HRESIMS m/z 263.1754 [M + H]$^{+}$ (calcd for $C_{15}H_{23}N_2O_2$, 263.1754).

Bohemamine H (7): colorless oil, $[\alpha]^{23}_D$ +37 (c 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 244 (4.09), 284 (3.81), 337 (3.71) nm; ECD (c = 0.40 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 331 (+63.2), 281 (−129.0), 244 (+25.7), 207 (−8.2) nm; IR (NaCl disk) ν_{max} 3429, 2099, 1638, 1506, 1260, 1174, 1121, 734 cm^{-1}; ^{1}H and ^{13}C NMR, Table 3; HRESIMS m/z 249.1596 [M + H]$^{+}$ (calcd for $C_{14}H_{21}N_2O_2$, 249.1598).

Bohemamine I (8): colorless oil, $[\alpha]^{23}_D$ +39 (c 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 248 (4.02), 282 (3.78), 336 (3.69) nm; ECD (c = 0.37 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 335 (+18.7), 284 (−38.4), 246 (+10.7), 214 (−4.9) nm; IR (NaCl disk) ν_{max} 3431, 2100, 1645, 1506, 1258, 1137, 794, 723, 704 cm^{-1}; ^{1}H and ^{13}C NMR, Table 3; HRESIMS m/z 271.1443 [M + H]$^{+}$ (calcd for $C_{16}H_{19}N_2O_2$, 271.1441).

NP25302 (9): $[\alpha]^{23}_D$ +104 (c 0.10, MeOH), literature[6c] $[\alpha]^{24}_D$ +116 (c 0.11, MeOH); ECD (c = 0.40 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 335 (+54.3), 283 (−115.9), 248 (+27.4), 210 (−12.2) nm.

Methylation of 3. To a solution of **3** (1.5 mg) in DMF (anhydrous, 0.5 mL) was added 2.0 mg of NaH. After allowing it to stir at 0 °C for 0.5 h, 20 μL of CH$_3$I was added into the reaction mixture. It was stirred at room temperature for another 0.5 h. Then, a saturated solution of NH$_4$Cl (2.0 mL) was added to quench the reaction. The product was extracted with EtOAc (3 × 3.0 mL) and purified by reversed-phase HPLC (Phenomenex Luna, C$_{18}$, 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system from 20% to 100% CH$_3$CN (0.1% formic acid) over 20 min to afford compound **3a** (0.5 mg, t_R = 14.4 min). Compound **3a**: white powder; ^{1}H NMR (600 MHz, CDCl$_3$) δ 5.99 (m, 1H, H-2′), 5.05 (s, 1H, H-2), 3.62 (m, 1H, H-4), 3.29 (s, 3H, 3-NCH$_3$), 3.27 (s, 3H, 7-OCH$_3$), 2.17 (s, 3H, H-5′), 2.17 (m, 1H, H-5), 2.09 (m, 1H, H-6), 2.00 (m, 1H, H-5), 1.90 (s, 3H, H-4′), 1.88 (m, 1H, H-6), 1.28 (d, J = 6.8, 3H, H-8); ^{13}C and 2D NMR, Figure S2; HRESIMS m/z 279.1704 [M + H]$^{+}$ (calcd for $C_{15}H_{23}N_2O_3$, 279.1703).

ASSOCIATED CONTENT

⬡ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnat-prod.5b00604.

^{13}C and 2D NMR data of **3a**, calculated ^{13}C NMR shifts for compound **1**, computation methods of NMR and ECD, HRESIMS and NMR spectra for compounds **1–8** and **3a**, a description of the bioassay protocols used (PDF)

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Notes

The authors declare no competing financial interest.

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DEDICATION

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