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## Bacillamides from a Hypersaline Microbial Mat Bacterium

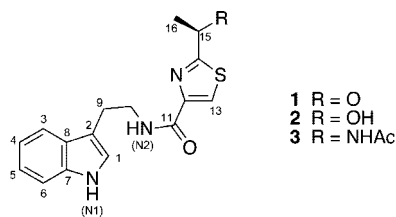
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Chemical studies of a *Bacillus endophyticus* isolated from a Bahamian hypersaline microbial mat led to the isolation of bacillamides B and C, new tryptamide thiazole metabolites. Bioassay-guided fractionation using a HPLC-UV-MS bioassay technique enabled the detection of these trace fermentation products, and their total structures were elucidated by combined spectroscopic techniques.

Microbial mats prevail in environments of extreme physical and chemical demands, and hypersaline mat communities can host bacterial assemblages with exceedingly high biodiversity.<sup>1</sup> Phyla are stratified by depth and employ mixed metabolic strategies to obtain nutrients cycled diurnally on the millimeter scale.<sup>2</sup> Gram-positive bacteria are indigenous to microbial mats,<sup>3–5</sup> but their secondary metabolites remain unexplored. In an effort to examine this chemistry, we cultivated 75 aerobic heterotrophic bacteria obtained from a tropical hypersaline pond. Over 70% of the isolates belonged to the genus *Bacillus*, and greater than 90% demonstrated antagonism toward other bacterial isolates from the same mat sample. Bioassay-guided investigation of isolate SP31, a *Bacillus endophyticus*, led to the isolation of the known algacide bacillamide A (**1**)<sup>6</sup> and two new analogues, bacillamides B (**2**) and C (**3**).



A 10 mm deep core sample was collected from a microbial mat on the eastern shore of Salt Pond, San Salvador Island, Bahamas. The core had five distinct laminations (1–3 mm), each distinguished by their color, which were (from upper to lower) green, pink, brown, white, and black. Strain SP31 was isolated from the black layer by cultivation on a high-salt marine medium and was subsequently identified as a *Bacillus endophyticus* using 16S rRNA gene sequence comparison. Using a bacterial antagonism assay,<sup>7</sup> SP31 demonstrated significant growth inhibition against 45 of the other 75 isolates from the core, and chemical extracts from small-scale Petri dish cultures reproduced the antibiotic activity. Large-scale cultivation was undertaken on 100 solid marine agar trays at 28 °C for 48 h. Ethyl acetate extracts of the mashed agar cultures were purified by solvent partitioning and LH20 column chromatography.

An HPLC-UV-MS bioassay revealed the presence of several metabolites with possible antibiotic activity in a late eluting LH20 fraction. Briefly, the sample was separated by analytical reversed-phase HPLC and the eluting flow was split between an electrospray mass spectrometer and a 96-well microtiter plate. The plate was concentrated *in vacuo* and subsequently inoculated with test strain SP02, a *Bacillus vietnamensis* isolated from the pink layer of the microbial mat. After incubation at 28 °C for 24 h, the retention

time of wells showing no bacterial growth was cross-referenced with corresponding UV and mass spectra. Distinct sets of sequential “kill wells” were observed. One series contained a metabolite with a UV absorbance at 280 nm and apparent molecular ions at 314.08 [M + H]<sup>+</sup> and 316.08 [M + H + 2]<sup>+</sup> in a 20:1 ratio, consistent with a compound containing a sulfur atom. A search of the microbial metabolite database AntiBase (Wiley-VCH) suggested that this compound might correspond to bacillamide A (**1**). A second series of kill wells appeared to be linked with a compound measuring a molecular ion [M + H]<sup>+</sup> at *m/z* 138.05, appropriate for the common bacterial metabolite anthranilic acid. Semipreparative purification of these compounds via HPLC and subsequent comparisons of <sup>1</sup>H NMR and mass spectral data with literature values confirmed the identification of the anticipated metabolites.

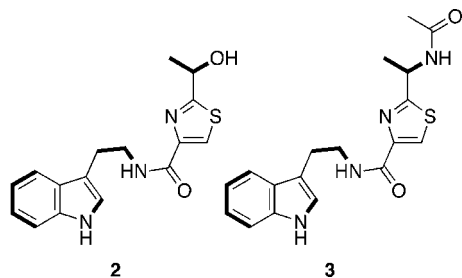
The third series of wells inhibiting microbial growth contained a metabolite (**2**) that appeared related to **1** on the basis of UV and MS data, but could not be similarly dereplicated. The compound was isolated by reversed-phase HPLC, and HRESIMS of the pure material gave a pseudomolecular ion [M + H]<sup>+</sup> at *m/z* 316.1134, appropriate for a molecular formula of C<sub>16</sub>H<sub>18</sub>N<sub>3</sub>O<sub>2</sub>S<sup>+</sup>. The two-unit difference in the molecular weight from **1** allowed for one additional degree of saturation. Analysis of the <sup>13</sup>C NMR spectrum provided chemical shifts of six methine and five quaternary aromatic resonances of the indole and thiazole rings, which closely matched that of **1**, and accounted for nine of the 10 degrees of unsaturation. The amide carbonyl resonance at δ160.5 of **2** was in accord with the C-11 assignment of **1**, accounting for the 10th degree of unsaturation. However, the C-15 carbonyl of the parent molecule (δ 191.4) was replaced by an oxygenated methine at δ 66.6. IR absorbances at 1652 and 3300 cm<sup>−1</sup> confirmed the presence of the C-11 amide carbonyl, while a broadened peak at 3410 cm<sup>−1</sup> was consistent with an additional alcohol functionality. Comparison of the <sup>1</sup>H NMR spectra further supported that compound **2** was closely related to **1**. Proton resonances for the tryptamine moiety were nearly identical for the two molecules. However, upfield chemical shifts were observed for the amide proton (δ 8.67 to δ 8.32) and H-13 of the thiazole (δ 8.66 to δ 8.09). The <sup>1</sup>H NMR spectrum for **2** also included an upfield shift of the H-16 methyl from δ 2.70 to δ 1.44, and this resonance now appeared as a doublet (*J* = 6.2 Hz). Furthermore, a new proton signal at δ 4.91 appeared as a doublet of quartets (*J* = 5.2, 6.2 Hz), and vicinal coupling from this resonance was observed to a doublet at δ 6.21 (*J* = 5.2 Hz). Examination of <sup>1</sup>H–<sup>1</sup>H COSY data verified a spin system between these three new resonances (Figure 1). Taken together, these data identified bacillamide B (**2**) as the C-15 alcohol analogue of bacillamide A.

Bacillamide C (**3**) was purified by reversed-phase HPLC from the same LH20 column fractions as **1** and **2**. HRESIMS analysis of **3** gave a pseudomolecular ion [M + H]<sup>+</sup> at *m/z* 357.1393, appropriate for the molecular formula of C<sub>18</sub>H<sub>21</sub>N<sub>4</sub>O<sub>2</sub>S<sup>+</sup>. Compared

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**Figure 1.**  $^1\text{H}$ – $^1\text{H}$  COSY correlations observed for bacillamides B (2) and C (3) (bold lines).

to that for 2, this formula required an additional degree of unsaturation and the installation of  $\text{C}_2\text{H}_3\text{N}$ . A broadened carbonyl absorbance at  $1700\text{ cm}^{-1}$  was observed in the IR spectrum. The  $^1\text{H}$  NMR spectral data for the tryptamide and thiazole portions of 3 were nearly identical to those of 2 (Table 1). The differences between these molecules resided in alteration of the substituent at C-14 on the thiazole ring. In comparison with the  $^1\text{H}$  NMR spectrum for 2, the H-16 methyl again appeared as a doublet ( $\delta$  1.46,  $J$  = 6.8 Hz), but the H-15 methine was now shifted 0.20 ppm downfield ( $\delta$  5.11,  $J$  = 6.8, 7.2 Hz). An additional NH resonance was observed at  $\delta$  8.66 ( $J$  = 7.2 Hz), and a COSY experiment confirmed a spin system comprising this proton with the H-15 methine and H-16 methyl (Figure 1). A new methyl singlet was observed at  $\delta$  1.86, consistent with the presence of an acetamide.  $^{13}\text{C}$  NMR data further supported this conclusion. A secondary amide carbonyl peak was observed at  $\delta$  169.1 (C-17), and the C-15 methine resonance was shifted upfield to  $\delta$  46.8. In sum, these data identified bacillamide C (3) as the C-15 acetamide analogue of 1.

The absolute configurations of bacillamides B and C were investigated by comparison of their circular dichroism (CD) spectra to that reported for the closely related bacterial metabolite *N*-3'- $\beta$ -indolyethyl-2- $\alpha$ -aminoethylthiazole-4-carboxamide. This alkaloid from a thermophilic actinomycete was assigned the *S*-configuration at C-15 by comparison of CD spectra of its *N*-salicylidene derivative to model  $\alpha$ -arylalkylamines.<sup>8,9</sup> It produces a negative first Cotton effect at 253 nm, accompanied by a positive second effect at 223 nm. In contrast, both 2 and 3 produce positive first Cotton effects at 252 nm and negative second effects at 210 nm. Bacillamides B and C have thus inverse spectra in comparison with the previously

assigned molecule and are therefore assigned the *R*-configuration at C-15. Attempts to prepare the Mosher's ester derivatives of 2 were unsuccessful.

Bacillamide A was first isolated from a marine *Bacillus* sp. collected during termination of a toxic algal bloom. It showed antibiosis against dinoflagellates and raphidophytes, but lacked activity against other algae, bacteria, fungi, and yeast. Due to a lack of sufficient material and the appropriate test organisms, bacillamides B and C were not tested for algicidal activity. In a broth dilution assay,<sup>10</sup> none of the purified bacillamides demonstrated antibiotic activity against target isolates of hypersaline pond *Bacillus* sp. at concentrations less than  $500\text{ }\mu\text{M}$ . Hence, these compounds do not appear to be responsible for the antibiosis originally observed in our small-scale screening assays. Anthranilic acid is produced in abundance by this isolate and may account for the initially observed activities. Further tests demonstrated that the bacillamides do not inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) and were inactive in an assay that measures the reduction of  $\alpha$ -toxin secretion by a biofilm-forming strain of MRSA<sup>11</sup> (concentrations tested  $<150\text{ }\mu\text{M}$ ).

The tryptamide thiazole motif observed in the bacillamides frequently occurs as a building block in potentially bioactive cyclic peptides. Examples include the antibiotic zekovamycin produced by a *Streptomyces* sp.,<sup>12</sup> the protein synthesis inhibitors A-21459 A and B from an *Actinoplanes* sp.,<sup>13</sup> and the immunosuppressive argyris from the myxobacterium *Archangium gephyra*.<sup>14</sup> Bacillamides A–C are the first reported secondary metabolites from the newly described species *Bacillus endophyticus*.<sup>15</sup> Further investigation is currently underway to identify antibiotics produced by other *Bacillus* isolates from the Salt Pond hypersaline mat ecosystem.

## Experimental Section

**General Experimental Procedures.** Optical rotations were measured on an Autopol III automatic polarimeter (Rudolf Research), circular dichroism (CD) measurements were taken on a J-810 spectropolarimeter (JASCO), and UV spectra were recorded on a DU 800 spectrophotometer (Beckmann-Coulter). IR spectra were obtained on a Nexus 470 FT-IR (Thermo-Nicolet) equipped with a Continuum microscope. NMR spectra were recorded on a Bruker Biospin spectrometer (400 MHz for  $^1\text{H}$ , 100 MHz for  $^{13}\text{C}$ ) and were referenced to residual solvent signals with resonances  $\delta_{\text{H/C}}$  2.50/39.5 ( $\text{DMSO}-d_6$ ). High-resolution ESI mass spectrometry was accomplished in 1:1  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (+0.1% formic acid) on a Micromass Q-Tof Ultima

**Table 1.** NMR Data for Bacillamides B (2) and C (3) in  $\text{DMSO}-d_6^a$

position	2			3		
	$\delta_{\text{C}}$	$\delta_{\text{H}}$	COSY	$\delta_{\text{C}}$	$\delta_{\text{H}}$	COSY
1	122.6	7.14 (d, 1.0)	NH-1	122.6	7.14 (d, 1.0)	NH-1
2	111.7			111.7		
3	118.2	7.57 (d, 7.8)	4	118.2	7.57 (d, 7.8)	4
4	118.2	6.93 (dd, 7.6, 7.8)	3,5	118.2	6.94 (dd, 7.6, 7.8)	3,5
5	120.9	7.02 (dd, 7.6, 7.8)	4,6	121	7.03 (dd, 7.6, 7.8)	4,6
6	111.3	7.29 (d, 7.8)	5	111.4	7.29 (d, 7.8)	5
7	136.2			136		
8	127.2			127.2		
9	25.3	2.89 (t, 7.2)	10	25.4	2.90 (t, 7.4)	10
10	40.4	3.50 (dt, 6.2, 7.2)	9, NH-2	40.4	3.51 (dt, 6.6, 7.4)	9, NH-2
11	160.5			160.4		
12	149.9			149.8		
13	123	8.09 (s)		123.2	8.08 (s)	
14	178.6			176.5		
15	66.6	4.91 (dq, 5.2, 6.2)	16, OH	46.8	5.11 (dq, 6.8, 7.2)	16, NH-3
16	24.1	1.44 (d, 6.2)	15	20.5	1.46 (d, 6.8)	15
17				169.1		
18				22.5	1.86 (s)	
OH		6.21 (d, 5.2)	15			
NH-1		10.78 (d, 1.0)	1		10.79 (d, 1.0)	1
NH-2		8.32 (t, 6.2)	10		8.35 (t, 6.6)	10
NH-3					8.66 (d, 7.2)	15

<sup>a</sup> Measured at 400 MHz ( $^1\text{H}$ ) and 100 MHz ( $^{13}\text{C}$ ).

instrument at the University of Illinois Urbana-Champaign Noyes Laboratory. LC/MS experiments were conducted on an Agilent 1100 analytical HPLC with the flow split between an Applied Biosystems Mariner mass spectrometer and a Gilson FC203B fraction collector. Preparatory HPLC was performed using a Waters 600 pump equipped with a Waters 2487 dual  $\lambda$  absorbance detector.

**Isolation and Fermentation.** The *Bacillus endophyticus* strain SP31 was isolated from a 1 cm deep core taken from a microbial mat collected on the eastern shore of Salt Pond, San Salvador Island, Bahamas (24°05' N, 74°30' W). Colored layers were thin sectioned with a sterile razor blade; a fresh blade was used at each interface. Each layer was placed into a sterile microcentrifuge tube containing autoclaved 0.2  $\mu$ m filtered seawater (salinity adjusted to 72 ppt), and the sample was vortexed to disrupt the matrix and dislodge bacteria. Aliquots were spread on ZoBell 2216 Marine agar plates (1 g of yeast extract, 5 g of peptone, and 15 g of agar per 72 g/L sea salt water (Instant Ocean)). Isolates were restreaked three times to ensure a single genotype and then stored at -80 °C in 20% glycerol stocks. Large-scale cultivation of SP31 was performed on solid marine agar in 100 aluminum trays (20 × 27 × 4 cm). Each tray received 300 mL of YP agar medium (1 g of yeast extract, 5 g of peptone, 10 g of agar per L seawater) and was inoculated with 2 mL of a 10 mL culture grown overnight in YP broth (155 rpm, 28 °C).

Phylogenetic identification of SP31 was accomplished using 16S rRNA gene sequence comparison. Total DNA was extracted from a 1.4 mL aliquot of an overnight culture using DNeasy tissue kit (Qiagen, Valencia, CA) followed by the recommended proteinase K lysis step. A 5  $\mu$ L portion of genomic DNA template (39 ng/ $\mu$ L) was added to a 25  $\mu$ L PCR reaction mixture containing Taq-PCR-Mastermix (Qiagen), 0.1  $\mu$ M of each B27F forward and B1392R reverse primer,<sup>16</sup> and molecular biology grade water. The amplification reaction occurred in an Eppendorf Mastercycler ep thermal cycler. The PCR product was purified using a QIAquick PCR purification kit (Qiagen), quantified using a D-1000 spectrophotometer (NanoDrop Technologies), and sequenced using an Applied Biosystems 3130 × 1 sequencer (URI Genomics and Sequencing Center, Kingston, RI). A BLAST search (NCBI, 3–21–07) revealed that the amplified 16S rRNA sequence (1308 bases, deposited with GenBank as Accession No. EF514218) matched that of *Bacillus endophyticus* (99% similarity).

**Extraction and Purification.** Whole agar cultures were mashed and extracted with EtOAc. Contents were filtered through cheesecloth, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo* to yield 1.53 g. The material was solvent-partitioned between H<sub>2</sub>O and EtOAc, and the latter fraction was concentrated and further partitioned between MeOH and isooctane. The MeOH fraction (988 mg) was separated by Sephadex LH20 (Fluka) column chromatography (100% MeOH, 4 × 86 cm resin bed, 10–12 mL fractions). Weak antibiotic activity against hypersaline pond isolate SP02, a *Bacillus vietnamensis*, was observed among LH20 fractions 71–105 (IC<sub>50</sub> 125–250  $\mu$ g/mL in a broth dilution assay). The pooled material from these fractions (98 mg) was purified by reversed-phase HPLC (Phenomenex Gemini C-18 column, 5  $\mu$ m 110 Å, 250 × 10 mm). A method using 10 mL/min of 0–70% CH<sub>3</sub>CN + 0.1% trifluoroacetic acid in H<sub>2</sub>O over 16 min yielded anthranilic acid (25 mg, *t*<sub>R</sub> 8.5 min) and the pure metabolites **1** (0.4 mg, *t*<sub>R</sub> = 13.75 min), **2** (1.0 mg, *t*<sub>R</sub> = 11.5 min), and **3** (0.8 mg, *t*<sub>R</sub> = 11.25 min).

**HPLC-UV-MS Antibacterial Bioassay.** A 375  $\mu$ g aliquot of pooled LH20 fractions 91–95 was separated by analytical HPLC using a linear gradient of 0–100% CH<sub>3</sub>CN + 0.2% formic acid in H<sub>2</sub>O over 20 min at 1 mL/min (Phenomenex Gemini C-18 column, 5  $\mu$ m 110 Å, 100 × 3 mm). After elution through the diode array detector, 50  $\mu$ L/min of the flow was split to an ESI mass spectrometer operating in the positive ion mode (nozzle potential 70 V, nozzle temperature 140 °C). The remainder of the flow was collected every 0.2 min into separate wells of a 96-well microtiter plate, which was subsequently dried in a vacuum centrifuge (Thermo Savant SPD1010 SpeedVac). To each well was then added 80  $\mu$ L culture of test strain SP02 in YP broth (optical

absorbance at 650 nm = 0.004) and shaken at 28 °C for 24 h. Growth of the test strain was assessed by measuring optical absorbance at 650 nm on a Molecular Devices Spectramax M2 spectrometer with Softmax Pro 4.6 software. The wells corresponding to the retention times (*t*<sub>R</sub>) of anthranilic acid (*t*<sub>R</sub> 6.8–7.2 min), **1** (*t*<sub>R</sub> 12.6–13 min), and **2** (*t*<sub>R</sub> 11.2 min) showed significant growth inhibition compared to media-spiked control wells. A “kill well” was defined as optical absorbance <0.130 after 24 h. Control wells typically grew to an optical absorbance of 0.450 under the identical conditions.

**Bacillamide B:** light brown, amorphous solid; [ $\alpha$ ]<sub>D</sub><sup>20</sup> +7.4 (c 0.095, MeOH); CD (EtOH)  $\lambda$  ( $\Delta\epsilon$ ) 210 (−0.38), 252 (+0.26), 296 (−0.03); IR (NaCl)  $\nu_{\max}$  3410 (br), 3300, 2927, 1652, 1546, 1493, 1457, 1190, 1109, 1021, 1010 cm<sup>−1</sup>; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 223 (4.22), 271 (3.52), 280 (3.52), 289 (3.43) nm; HRESIMS [M + H]<sup>+</sup> 316.1134 (316.1120 calcd for C<sub>16</sub>H<sub>18</sub>N<sub>3</sub>O<sub>2</sub>S<sup>+</sup>,  $\Delta$  = 4.4 ppm).

**Bacillamide C:** light brown, amorphous solid; [ $\alpha$ ]<sub>D</sub><sup>24</sup> −15.2 (c 0.082, MeOH); CD (EtOH)  $\lambda$  ( $\Delta\epsilon$ ) 206 (−0.22), 256 (+0.13), 300 (−0.02); IR (NaCl)  $\nu_{\max}$  3279 (br), 2924, 2246, 1700, 1656, 1546, 1489, 1454, 1372, 1255, 1021, 1010, 911 cm<sup>−1</sup>; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 223 (4.05), 280 (3.50), 288 (3.41), 340 (2.71) nm; HRESIMS [M + H]<sup>+</sup> 357.1393 (357.1385 calcd for C<sub>18</sub>H<sub>21</sub>N<sub>4</sub>O<sub>2</sub>S<sup>+</sup>,  $\Delta$  = 2.2 ppm).

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**Supporting Information Available:** <sup>1</sup>H and <sup>1</sup>H–<sup>1</sup>H COSY NMR spectra for bacillamides A–C (**1–3**) as well as CD spectra for bacillamides B (**2**) and C (**3**). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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