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Spirobacillenes A and B, Unusual Spiro-cyclopentenones from *Lysinibacillus fusiformis* KMC003

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ABSTRACT

Two previously unreported spiro-cyclopentenones, spirobacillenes A (1) and B (2), were isolated from the 24 h broth culture of *Lysinibacillus fusiformis* KMC003 derived from acidic coal-mine drainage. The structures of 1 and 2 were elucidated by analyses of the NMR, HRFABMS, single-crystal X-ray diffraction crystallography, and circular dichroism (CD) spectral data. Compound 1 possessed moderate inhibitory activity against the production of nitric oxide (NO) and reactive oxygen species (ROS).

Microbial metabolites have provided a variety of bioactive chemical structures in drug discovery programs, and the chemical diversity from microbial resources is continuously increasing.[1,2] Multidirectional approaches, such as exploitation of new species, have been developed for discovering novel chemical scaffolds from microorganisms. One effective approach for new secondary metabolites is to study microorganisms derived from unexplored extreme environments (e.g., acidic mine drainage), where microorganisms can create a unique offensive and defensive biochemical metabolism under ecological pressure.[3] Recently, diverse microbes derived from acidic mine environments, which can grow and produce new secondary metabolites under laboratory conditions, have been described in the literature.[4] The most prominent example is a *Penicillium* fungal strain isolated from the Berkeley Pit, which is an acid mine waste lake that is extremely contaminated by heavy metals and sulfuric acid. Structurally remarkable bioactive compounds were isolated from the *Penicillium* sp.[5]

Recently, we isolated a bacterial strain, *Lysinibacillus fusiformis* KMC003,[6] from acidic coal mine drainage that

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(6) The KMC-003 strain was classified as a *Lysinibacillus* sp. Gram-positive bacterium based on a 16S rRNA sequence analysis. The strain shares 99.9% sequence identity with *Lysinibacillus fusiformis*.

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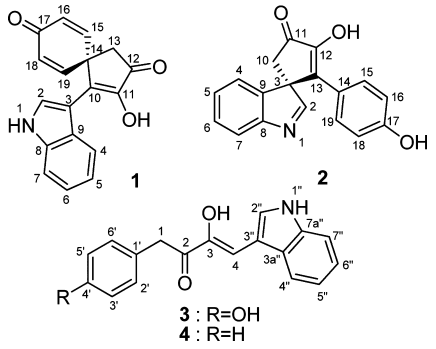
was highly contaminated by iron-rich heavy-metal ions and sulfuric acid (pH 3.0). *Lysinibacillus fusiformis*, a Gram-positive bacterium, was reclassified via reinspection of rRNA group 2 of the *Bacillus fusiformis* and was found to have a distinctive cell-wall peptidoglycan composition.[7] They were isolated from heavy-metal-contaminated wastewater and from the livers of the puffer fish.[8,9] Previous studies of *L. fusiformis* reported their ability to produce tetrodotoxin and convert oleic acid to 10-hydroxystearic acid by oleate hydratase as well as their resistance to multiple metals.[8−10] However, no bioactive secondary metabolites have been reported from the genus *Lysinibacillus*. Therefore, we investigated the production of secondary metabolites from the bacterial strain *L. fusiformis* KMC003 because the *Bacillus* species are known for their ability to produce structurally diverse bioactive molecules, such as polyene, macrolide, and especially peptide antibiotics.[11]



A chemical analysis of the secondary metabolites produced by *L. fusiformis* KMC003 revealed the presence of six major metabolites in the 24 h broth culture. Interestingly, these metabolites disappeared after 48 h of culturing. To identify their chemical structures, the 24 h liquid culture of *L. fusiformis* KMC003 was extracted with ethyl acetate. The ethyl acetate extracts were subsequently subjected to reversed-phase HPLC separation to afford two novel compounds, spirobacillenes A (**1**) and B (**2**) along with two new natural compounds [(*Z*)-3-hydroxy-4-(3-indolyl)-1-hydroxyphenyl-2-butenone (**3**) and (*Z*)-3-hydroxy-4-(3-indolyl)-1-phenyl-2-butenone (**4**)] and two known indole derivatives [soraphinol A (**5**)[12] and kurasoin B (**6**)[13]]. Spirobacillenes A (**1**) and B (**2**) featured a unique indole and indolenine moiety that contained spiro-cyclopentenones, respectively. Herein, we report the isolation, structure elucidation, and possible biosynthetic pathway of **1** and **2**.

Spirobacillene A (**1**)[14] was isolated as a yellow chunk crystal. The molecular formula was determined to be $C_{18}H_{13}NO_3$ ($[M+H]^+$ at m/z 292.0977) based on a HRFABMS measurement, which indicated that **1** contained 13 degrees of unsaturation. The IR spectrum exhibited absorption bands that corresponded to hydroxyl (3228 cm^{-1}) and carbonyl (1693 cm^{-1}) functional groups. The ^1H NMR spectrum of **1** recorded in acetonitrile-d_3 displayed characteristics of a NH signal [δ_H 9.63 (1H, br s)], three olefinic methine proton signals [δ_H 7.46 (1H, s, overlap), 7.06 (2H, d, J = 10.0 Hz), 6.33 (2H, d, J = 10.0 Hz)], four aromatic proton signals [δ_H 7.98 (1H, d, J = 8.0 Hz), 7.46 (1H, m, overlap), 7.23 (1H, ddd, J = 8.0, 7.0, 1.2 Hz), 7.16 (1H, ddd, J = 8.0, 7.0, 1.2 Hz)], and a methylene group [δ_H 2.76 (2H, s)]. The ^{13}C NMR spectrum indicated resonances for 16 carbons attributable to two α,β-unsaturated ketone carbons (δ_C 197.6, 185.0), a methylene carbon (δ_C 42.1), three olefinic methine carbons (δ_C 154.2, 129.1, 125.3), four aromatic methines (δ_C 122.7, 122.5, 120.1, and 111.7), five aromatic (or sp^2) quaternary carbons (δ_C 148.2, 137.0, 136.3, 126.0, and 109.2), and a quaternary carbon (δ_C 47.0). The HSQC spectrum of **1** enabled the assignment of all of the protons to the directly bonded carbons. All of the above data suggested the presence of both an indole moiety and a cross-conjugated dienone group. The substructures were assigned by analyses of the $^1H-^1H$ COSY and $^1H-^{13}C$ HMBC spectral data. The $^1H-^1H$ COSY spectrum indicated connectivity between NH (δ_H 9.63) and H-2 (δ_H 7.46). The sequential COSY correlations from H-4 (δ_H 7.98) to H-7 (δ_H 7.46) and the HMBC correlations from H-5 (δ_H 7.16) to C-9 (δ_C 126.0), H-6 (δ_H 7.23) to C-8 (δ_C 136.3), and H-2 (δ_H 7.46) to C-3 (δ_C 109.2) and C-9 (δ_C 126.0) established the presence of an indole structure. In addition, the COSY correlations of H-15/H-19 (δ_H 7.06) and H-16/H-18 (δ_H 6.33) and the observed HMBC correlations from both H-15/H-19 and H-16/H-18 to C-17 (δ_C 185.0) and C-14 (δ_C 47.0) established the presence of a 2,5-cyclohexadienone. This six-membered ring contains a C-14 spiro-carbon center connected to a cyclopentenone through C-14, which was assigned based on HMBC correlations from H-15/H-19 (δ_H 7.06) to C-13 (δ_C 42.1) and C-10 (δ_C 137.0). The additional HMBC correlations from H-13 (δ_H 2.76) to C-10 (δ_C 137.0), C-11 (δ_C 148.2), C-12 (δ_C 197.6), C-14 (δ_C 47.0), and C-15/C19 (δ_C 154.2) led to the construction of a spiro[4.5]decane skeleton of **1**.[15,18] 2D NOESY experiments also supported the assignment of this spiro-[4.5]decane structure based on an NOE correlation of H-13

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(14) Spirobacillene A (**1**): yellow chunk crystal; mp 217−220 °C; $[\alpha]_D^{25}$ −4.4 (*c* 0.10, CH_3CN); UV (CH_3CN) λ_{max} (log ε) 342 (3.73), 224 (4.07), 193 (4.06) nm; IR (film) ν_{max} 3228, 1693, 1657, 1619, 1394, 1221, 858, 661 cm^{-1}; ^1H and ^{13}C NMR spectra, see Table S2 in the SI; HRFABMS$[M+H]^+$ m/z 292.0977 (calcd for $C_{18}H_{14}NO_3$, 292.0974).

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and H-15/H-19. The structure of **1** has been unambiguously confirmed by single-crystal X-ray diffraction analysis as 3-hydroxy-4-(1*H*-indolyl)-spiro[4,5]deca-3,6,9-trienone (Figure 1).

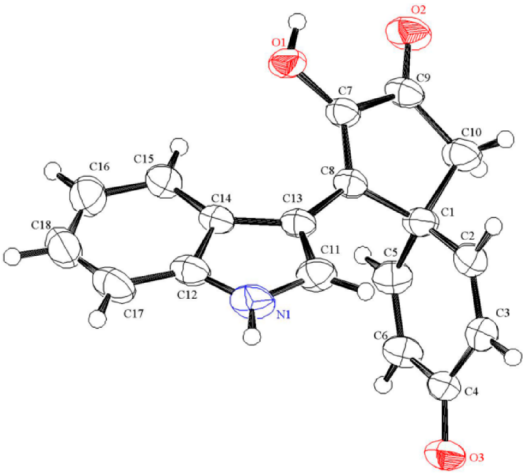

Figure 1. X-ray ORTEP drawing of **1**.

Spirobacillene B (**2**)[16] was obtained as an optically active pale-yellow oil ($[\alpha]_D^{25}$ −6.5) that possessed the same molecular formula, $C_{18}H_{13}NO_3$, as **1** based on the positive-ion HRFABMS (obsd $[M+H]^+$ at m/z 292.0978, calcd, 292.0974). The strong UV absorptions with λ_{max} values of 219 and 322 nm suggested the presence of an indole or phenyl conjugated α,β-unsaturated ketone chromophore in the structure of **2**.[17] The critical differences between the ^{1}H NMR signals of **1** and **2** were the absence of a NH (δ_H 9.63) signal and the coupling constants (9.0 Hz) for H-15/H-19 (δ_H 7.10) and H-16/H-18 (δ_H 6.65) in the ^{1}H NMR spectrum of **2**, which suggested that **2** contained a hydroxyphenyl group instead of the cross-conjugated dienone group in **1**.[18] A hydroxylated aromatic carbon signal (δ_C 158.0) and the chemical resonances of C-15/C-19 (δ_C 129.4) and C-16/C-18 (δ_C 115.4) in the ^{13}C NMR spectrum supported this result. The ^{13}C NMR spectral data of **2** also exhibited a nitrogen-bearing sp^2 carbon (or ester/acid carbonyl carbon) (δ_C 177.2),[19]suggesting the presence of an indolenine core. The gross structure of **2** was constructed by analysis of the detailed ^{1}H−^{1}H COSY and HMBC spectral data (Figure 2). The COSY correlations from H-15/H-19 (δ_H 7.10) and H-16/H-18 (δ_H 6.65) and the HMBC correlations from H-16/H-18 (δ_H 6.65) to C-14 (δ_C 124.6) and H-15/H-19 (δ_H 7.10) to C-17 (δ_C 158.0) led to the identification of a 1,4-disubstituted phenyl group. The linkage of the indolenine core and 1,4-disubstituted phenyl group was achieved based on the HMBC correlations from the methylene proton H-10 (δ_H 2.92, 2.55) to C-2 (δ_C 177.2), C-3 (δ_C 61.4), C-9 (δ_C 142.4), C-11 (δ_C 199.0), and C-13 (δ_C 135.8) and on the correlations from H-15/H-19 (δ_H 7.10) to C-13 (δ_C135.8). Interestingly, these HMBC correlations indicated the presence of an α-hydroxy β-phenyl substituted cyclopentenone that was connected to the indolenine structure via spiro-carbon C-3 (δ_C 61.4).[19,20] The 2D NOESY experiments supported the existence of this spiro structure by NOE correlations between H-4 and H-10β (or H-10α), H-2 and H-10α (or H-10β), and H-2 and H-15/H-19. Therefore, the planar structure of **2** was assigned as 3-hydroxy-2-(4-hydroxyphenyl)-spiro(cyclopentene-1,3′-indole)-4-one.

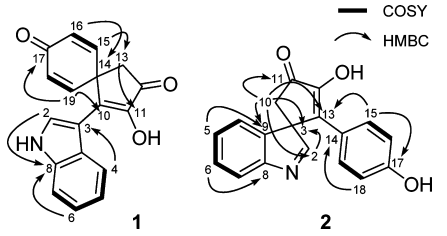

Figure 2. Key COSY and HMBC correlations of **1** and **2**.

The absolute configuration at spiro carbon C-3 of **2** was determined by application of the exciton-coupled circular dichroism method (Figure 3). The CD spectrum of **2**, which was acquired in acetonitrile, showed distinguishable absorption bands at 220 and 320 nm, similar to the UV spectrum. The Cotton effect was observed as a negative band at 223 nm and a positive band at 234 nm and is associated with the exciton coupling of the phenyl and indole chromophores.[21,22] In addition, the CD spectrum exhibited a negative Cotton effect at 313 nm and a positive band at 324 nm, which could have originated from the exciton coupling between the phenyl conjugated cyclopentanone and indole chromophores. The completely split circular dichroism curve of **2** suggested that the helicity of the phenyl and indole chromophores was positive, which indicated that the absolute configuration of spiro-carbon C-3 was *S*.[22,23] Therefore, the structure of **2** was

(16) Spirobacillene B (**2**): pale-yellow oil; $[\alpha]_D^{25}$ −6.5 (*c* 0.05, CH_3CN); CD (CH_3CN) λ_{max} ($\Delta\varepsilon$) 324 (+4.06), 313 (−1.66), 234 (+4.04), 223 (−9.88) nm; UV (CH_3CN) λ_{max} (log ε) 322 (3.90), 219 (4.08), 194 (4.19) nm; IR (film) ν_{max} 3361, 2359, 1698, 1607, 1384, 835, 759 cm^{-1}; ^{1}H and ^{13}C NMR spectra, see Table S2 in the Supporting Information; HRFABMS$[M+H]^+$ m/z 292.0978 (calcd for $C_{18}H_{14}NO_3$, 292.0974).

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determined to be (*S*)-3-hydroxy-2-(4-hydroxyphenyl)-spiro(cyclopentene-1,3′-indole)-4-one.

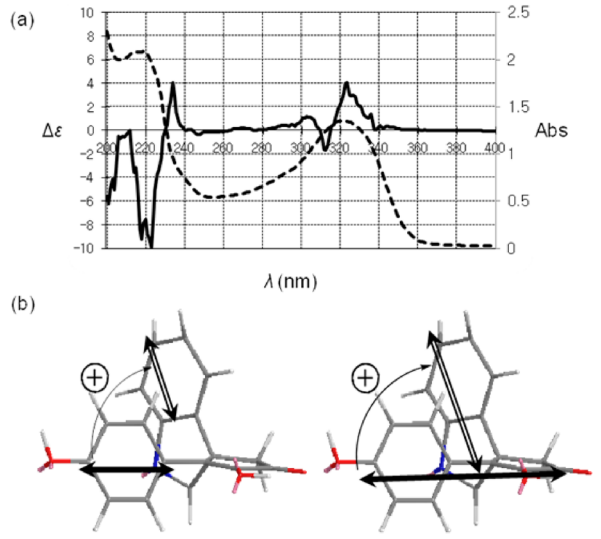

Figure 3. (a) CD (—) and UV (--) spectra of **2**. (b) Sign of exciton chirality of **2**.

To the best of our knowledge, spirobacillenes are a new type of naturally occurring indole alkaloid. The structure of **1** contains a single C−C bond between a spiro-[4.5]decane moiety and an adjacent indole ring, which is a novel skeleton for a natural product. The only report of spiro[4.5]decane natural products having originated from a microorganism involved the isolation of a bicarbonyl structure, spirodionic acid.[15] In addition, although the carbon backbone of **2** has been previously reported as an intermediate in the synthesis of carbazole alkaloids,[24] the fact that a highly functionalized spiro-cyclopentenone that contains this carbon backbone has a natural origin is intriguing. The proposed biosynthetic pathway of spirobacillenes is shown in Scheme S1 (see Supporting Information (SI)). A time-course analysis for the metabolite profiles of *L. fusiformis* KMC003 resulted in the optimized production of **3** in an 18 h broth culture of *L. fusiformis* KMC003, which nearly disappeared after 24 h of culturing (see Figure S3, SI). Notably, the production of **1** and **2** was maximized after the disappearance of **3**, which suggested that **3** may be a key precursor for the biosynthesis of **1** and **2**. Therefore, we presume that **3** could be the biosynthetic origin of the spirobacillenes. The biosynthesis of spirobacillenes most likely arises from subsequent decarboxylation and oxidation of **3a**, which was proposed for the biosynthesis of the cyanobacterial metabolite, scytonemin.[25] The production of **3** could then undergo a keto−enol tautomerism and cyclization derived from the intramolecular reaction to afford the cyclopentenone ring and spiroaminal. The unique spiro[4.5]decane system in **1** may be generated from the divergent oxidative coupling of the carbanion from precursor **3**. Compounds **1**−**3** were tested for their antimicrobial effects against 11 pathogenic strains. **1** and **2** exhibited weak activity with an MIC value greater than 50 μg/mL, whereas **3** exhibited moderate antimicrobial activity against three strains, including *Micrococcus luteus*, *Enterococcus hirae*, and *Staphylococcus aureus* [MIC of **3**: 3.13, 3.13, 12.5 μg/mL, respectively] (see Table S1). In addition, **1** and **2** were tested for inhibition against NO and ROS production in the LPS-induced RAW 264.7 macrophage cell line. The cytotoxic effect of **1** and **2** in RAW264.7 cells was evaluated by a CCK-8 assay. Compounds **1** and **2** did not affect cell viability for 48 h at concentrations up to 50 μM. For following experiments, 2.5−50 μM concentrations of **1** and **2** were used. Compound **1** exhibited a weak inhibitory effect against the production of NO and ROS with IC_{50} values of 39 and 43 μM, respectively, whereas **2** was found to be inactive.

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Supporting Information Available. Experimental procedure, HPLC trace, NMR spectral data of **1**−**4**, X-ray crystallographic data, and possible biosynthetic pathway of **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.