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Lorneic Acid Analogues from an Endophytic Actinomycete

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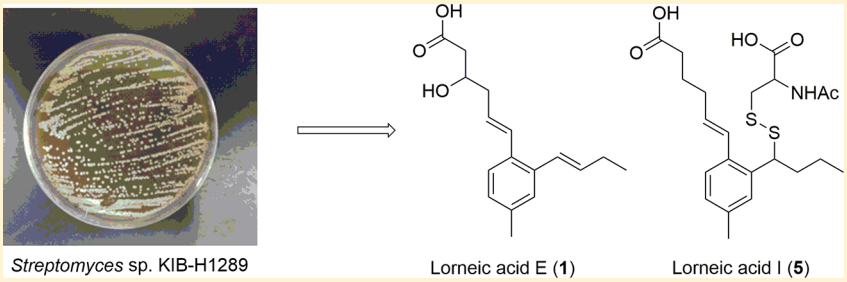
Supporting Information



ABSTRACT: Our natural products discovery program utilizes endophytic actinomycetes associated with plants and employs biological assays and HPLC-based metabolite profiles as the preliminary screen to identify strains of interest, followed by large-scale fermentation and isolation, leading to new and/or bioactive natural products. Six new trialkyl-substituted aromatic acids, namely, lorneic acids E–J (**1**–**6**), together with two known analogues (**7** and **8**), were isolated and identified from the culture extract of *Streptomyces* sp. KIB-H1289, an endophytic actinomycete obtained from the inner tissue of the bark of *Betula mandshurica* Nakai. The structures were characterized by interpretation of their spectroscopic data, mainly 1D and 2D NMR. Among them, compound **5** contains a unique disulfide bond that is presumably derived from *N*-acetylcysteine. All isolated metabolites were evaluated for their inhibitory activity on tyrosinase.

Micro-organisms that reside in the tissues of healthy living organisms are recognized as endophytes or endosymbionts. Special environments and selective pressures have led to endophytes that evolve into some new enzymes for adapting to their host; these micro-organisms possess and presumably benefit from these new enzymes, which thereby enables the biosynthesis of novel secondary metabolites.[1−4] Traditional Chinese medicine (TCM) plants have an extensive ethnobotanical history of use by local people. However, little is known about the endophytes associated with TCM plants for which there exists a tremendous cultural history in China.[5] This deficiency suggests that endophytes, particularly those living in TCM plants, are likely to produce new natural products.[6] Accordingly, we initiated a program designed to discover new or bioactive natural products from endophytic actinomycetes in TCM plants from unexplored and underexplored ecological niches for drug discovery.[7−9]

Betula mandshurica Nakai is widely distributed in China, and its bark has been used in traditional Chinese medicine for the treatment of pneumonia, chronic bronchitis, choloplania, and nephritis.[10] We recently isolated an endophytic actinomycete strain KIB-H1289 from the inner tissue of the bark of *B. mandshurica* Nakai collected from Yuanjiang county, People's Republic of China. Subsequent chemical screenings of its extract highlighted that this strain has a rich metabolite profile by high-performance liquid chromatography (HPLC) analysis. This strain was identified as *Streptomyces* sp. KIB-H1289 on the basis of the morphological characteristics and 16s rRNA gene sequence analysis (GeneBank no. KY465493) which shows 99.9% identity relative to strain *Streptomyces* sp. CC12J (GeneBank no: KM187147.1). In this context, we describe the compound isolation and structural elucidation of new lorneic acid[11−14] analogues **1**–**6**, as well as their inhibitory effects on tyrosinase.

RESULTS AND DISCUSSION

The fermentation broth of the strain *Streptomyces* sp. KIB-H1289 was centrifuged to obtain supernatant and a mycelial cake, which were extracted with acetone and ethyl acetate. Both extracts were combined and then applied on a repeated silica gel chromatograph column (CC), Sephadex LH-20 column, preparative HPLC, and semipreparative HPLC to yield six new lorneic acids E–J (**1**–**6**) as well as two known lorneic acids K (**7**)[11] and D (**8**) (Chart 1).[14]

Lorneic acid E (**1**) was obtained as colorless oil. Its molecular formula was deduced to be $C_{17}H_{22}O_3$ by HRESIMS. In the ^{1}H NMR data, a 1,2,4-trisubstituted benzene was observed on the basis of the ABX system for three aromatic protons (δ_H 7.28, d, *J*

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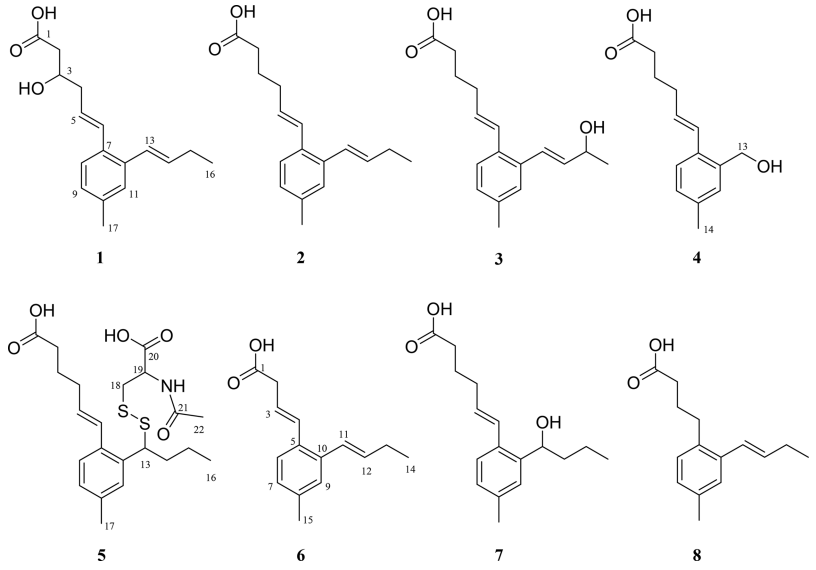
Chart 1



= 7.9 Hz, H-8; 6.96, brd, J = 7.9 Hz, H-9; 7.16, brs, H-11). In addition, the ^{1}H NMR data of **1** (Table 1) also revealed the presence of two methyl signals (δ_H 2.27, 3H, s, H_3-17; 1.09, 3H, t, J = 7.5 Hz, H_3-16) and two trans double bonds (δ_H 6.06, m, 2H; 6.69, brd, J = 15.6 Hz, H-6; 6.63, brd, J = 15.6 Hz, H-13). The ^{13}C NMR data (see Table 1) showed one carbonyl group (δ_C 176.2, C), 10 olefinic carbons at δ_C 127−138, including seven methines and three tertiary, one oxygenated methine (δ_C 69.4, CH), and five upfield shifted carbons (δ_C 42.8, 41.9, 27.4, 21.2, 14.2). In the HMBC data, the correlations of the methyl signal H_3-17 (δ_H 2.27, s) with three olefinic carbons [C-9 (δ_C 128.7, CH), C-10 (δ_C 137.8, C), and C-11 (δ_C 127.8, CH)] suggested that C-17 was connected to C-10 of the benzene ring. HMBC correlations of the double splitting olefinic proton H-6 with C-4 (δ_C 41.9, CH_2), C-8 (δ_C 127.3, CH), and C-12 (δ_C 137.0, C) as well as correlations of the benzene proton H-8 with C-6 (δ_C 131.7, CH), C-7 (δ_C 134.0, C), C-10 (δ_C 137.8, C), and C-12 (δ_C 137.0, C) indicated the benzene ring was substituted by C-5/C-6 trans double bonds at position C-7. In the same manner, the C-13/C-14 trans double bond was shown to be connected with C-12. In addition, HMBC correlations of H-5 with C-3, C-4, and C-7, of H-4 with C-2, C-3, C-5, and C-6, and of H-2 with C-1 and C-3 suggested the side chain (C-1/C-2/C-3/C-4/C-5/C-6) connected with C-7. The second side chain (C-13/C-14/C-15/C-16) was deduced from the HMBC correlations of H-16 with both C-15 and C-14 and of H-14 with C-12. Thus, the structure of compound **1** was determined as shown.

Lorneic acid F (**2**) was assigned the molecular formula $C_{17}H_{22}O_2$ as deduced from the positive HRESIMS. Comparison of the spectroscopic data of **2** with those of **1** revealed that they were quite similar except for C-3. The presence of a methylene (δ_C 24.4, CH_2) and the absence of an oxymethine carbon in the ^{13}C NMR data of **2** showed that a methylene carbon at the C-3 position was evident for **2** instead of a hydroxy group. This was confirmed by the cross-peaks of H-2 (δ_H 2.48, m) with C-1/C-3/C-4 and of H-3 (δ_H 1.89, m) and H-4 (δ_H 2.35, m) with C-5 (δ_C 130.5, CH) in the HMBC spectrum of **2**. Thus, the structure assigned to lorneic acid F (**2**) was as shown.

The molecular formula of lorneic acid G (**3**) was determined as $C_{17}H_{22}O_3$ from its HRESIMS. The ^{1}H and ^{13}C NMR data of **3** were similar to those of **2**, except for the absence of a methylene carbon. A hydroxy group at the C-15 position was evident for **3** on the basis of the observation of a double splitting methyl signal (δ_H 1.32, d, J = 6.4 Hz, H-15) and a methine signal (δ_C 69.5, CH, C-15) in the ^{1}H and ^{13}C NMR data, instead of the triple splitting signal (δ_H 1.17, t, J = 7.5 Hz) in the spectrum of **2**. The HMBC correlations of H_3-16 with C-15/C-14 (δ_C 136.7, CH) and of H-13 (δ_H 6.82, d, J = 15.6 Hz)/H-14 (δ_H 6.07, m) with C-15 allowed a determination of lorneic acid G (**3**) as shown.

Lorneic acid H (**4**) was isolated as colorless oil. The molecular formula was determined to be $C_{14}H_{18}O_3$. Comparison of the ^{1}H and ^{13}C NMR data of **4** with that of **2** and **3** revealed many similarities except for the absence of the signals for one of the trans double bonds and ethyl moiety connected to C-12 of the benzene ring. Observation of the presence of an additional oxygenated methylene (δ_H 4.62, s; δ_C 63.2, CH_2) in the ^{1}H, ^{13}C, and HSQC NMR spectra of **4** permitted a hydroxymethyl group to be placed at the C-12 position. This was confirmed by the HMBC correlations of H-13 with C-7, C-11, and C-12 in **4**. Accordingly, compound **4** was structurally determined as shown.

Lorneic acid I (**5**) was obtained as colorless oil. Its molecular formula was determined as $C_{22}H_{31}NO_5S_2$ according to HRESIMS. The ^{13}C and HSQC spectra discriminated 22 carbon atoms into three carbonyl groups, eight olefinic carbons including five protonated, six methylenes, two sp^3 methine carbons (δ_C 51.3 and 53.5), along with three methyl groups (Table 1). In the ^{1}H−^{1}H COSY and HSQC spectra of **5**, the presence of the partial structures −$CH_2CH_2CH_2CHCH$− (C-2 to C-6) and −$CHCH_2CH_2CH_3$− (C-13 to C-16) was inferred. Through analysis of the HMBC data (Figure 1), these two partial structures were connected to C-7 and C-12 positions of the benzene ring, respectively, on the basis of the observation of the cross-peaks of H-5 (δ_H 6.00, m) with C-7 (δ_C 136.4, C), of H-6 (δ_H 6.81, brd, J = 15.6 Hz) with C-7 (δ_C 136.4, C), C-8 (δ_C 128.1, CH), and C-12 (δ_C 138.2, C), of H-14 (δ_H 1.90 and 2.08, m) with C-12 (δ_C 138.2, C), and of H-13 (δ_H 4.33, m) with C-7(δ_C 136.4, C), C-11 (δ_C 128.7, CH), and C-12 (δ_C 138.2, C). Furthermore, the HMBC correlations of H_3-17 (δ_H 2.30, s) with C-9 (δ_C 129.3, CH), C-10 (δ_C 138.1, C), and C-11 (δ_C 128.7, CH) and of H_2-2 (δ_H 2.36, m) and H_2-3 (δ_H 1.79, m) with C-1 (δ_C 177.7, C) of **1** allowed the determinations of C-2 being connected with the C-1 carbonyl carbon, and the methyl group C-17 was placed at C-10.

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J. Nat. Prod. 2017, 80, 2615−2619

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of 1−6

	1		2[a]		3		4		5		6	
no.	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	176.2, C		180.1, C		178.3, C		177.8, C		177.7, C		176.3, C	
2	42.8, CH_2	2.52 m, 2.41m	33.4, CH_2	2.48 m	34.9, CH_2	2.33 m	34.4, CH_2	2.34 m	34.4, CH_2	2.36 m	39.5, CH_2	3.21 m
3	69.4, CH	4.12 m	24.4, CH_2	1.89 m	26.0, CH_2	1.78 m	25.8, CH_2	1.78 m	25.8, CH_2	1.79 m	124.8, CH	6.11 m
4	41.9, CH_2	2.41 m	32.5, CH_2	2.35 m	33.6, CH_2	2.27 m	33.7, CH_2	2.27 m	33.5, CH_2	2.30 m	132.0, CH	6.73 brd (15.6)
5	128.3, CH	6.06 m	130.5, CH	6.03 m	132.4, CH	6.01 m	132.0, CH	6.06 m	133.4, CH	6.00 m	133.5, C	
6	131.7, CH	6.69 brd (15.6)	128.9, CH	6.71 brd (15.6)	129.6, CH	6.66 brd (15.6)	128.8, CH	6.68 brd (15.6)	129.6, CH	6.81 brd (15.6)	127.3, CH	7.28 d (7.9)
7	134.0, C		132.7, C		134.6, C		135.0, C		136.4, C		128.8, CH	6.98 brd (7.9)
8	127.3, CH	7.28 d (7.9)	126.3, CH	7.33 d (7.9)	127.4, CH	7.27 d (7.9)	126.8, CH	7.34 d (7.9)	128.1, CH	7.27 d (7.9)	138.2, C	
9	128.7, CH	6.96 brd (7.9)	127.8, CH	7.05 brd (7.9)	129.3, CH	6.99 brd (7.9)	129.4, CH	7.03 brd (7.9)	129.3, CH	7.01 brd (7.9)	127.9, CH	7.18 brs
10	137.8, C		136.7, C		137.7, C		137.7, C		138.1, C		137.2, C	
11	127.8, CH	7.16 brs	126.9, CH	7.26 brs	127.9, CH	7.21 brs	129.9, CH	7.15 brs	128.7, CH	7.14 brs	128.0, CH	6.63 brd (15.6)
12	137.0, C		135.7, C		136.0, C		138.5, C		138.2, C		135.6, CH	6.09 m
13	128.2, CH	6.63 brd (15.6)	126.7, CH	6.67 brd (15.6)	128.1, CH	6.82 brd (15.6)	63.2, CH_2	4.62 s	51.3, CH	4.33 m	27.3, CH_2	2.24 m
14	135.4, CH	6.06 m	134.7, CH	6.16 m	136.7, CH	6.07 m	21.1, CH_3	2.28 s	37.8, CH_2	1.90 m, 2.08 m	14.0, CH_3	1.09 t (7.5)
15	27.4, CH_2	2.23 m	26.4, CH_2	2.31 m	69.5, CH	4.42 m			21.6, CH_2	1.26 m	21.1, CH_3	2.28 s
16	14.2, CH_3	1.09 t (7.5)	13.8, CH_3	1.17 t (7.5)	23.7, CH_3	1.32 d (6.4)			14.1, CH_3	0.88 t (7.5)		
17	21.2, CH_3	2.27 s	21.2, CH_3	2.38 s	21.0, CH_3	2.29 s			21.2, CH_3	2.30 s		
18									41.4, CH_2	2.69 m, 2.82 m		
19									53.5, CH	4.57 m		
20									174.1, C			
21									173.1, C			
22									22.5, CH_3	1.97 s		

[a]Recorded in $CDCl_3$, others in CD_3OD.

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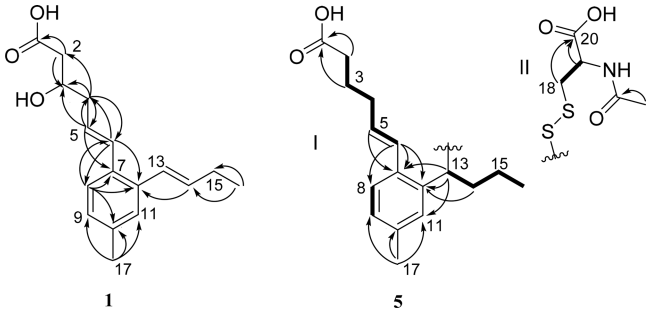

Figure 1. Key HMBC (arrows) and $^{1}H-^{1}H$ COSY (bold lines) correlations of **1** and **5**.

As a result, the partial structure I (Figure 1) was given. The remaining ^{1}H and ^{13}C NMR data were suggestive of a *N*-acetylmercaptocysteine residue II (Figure 1), which was confirmed by COSY and HMBC correlations (Figure 1) and by comparison of the NMR data with *N*-acetylmercaptocysteine and other known derivatives.[15,16] We next sought to determine the connectivity of residues I and II. Detailed comparison of NMR shifts demonstrated that the C-1 carbonyl carbon in **5** was almost identical to that in compounds **2**–**4**, thus excluding the possibility that residue II was attached to C-1 through a thiol ester bond. Therefore, carbon C-13 in partial structure I has to be connected with the *N*-acetylmercaptocysteine moiety via a C–S bond, which was also supported by the ^{1}H and ^{13}C NMR chemical shift of C-13 methine.[15,16] This complete structure elucidation of compound **5** showed a novel lorneic acid analogue featuring a disulfide bond at C-13 (Figure 1). This rare disulfide moiety is presumably derived from *N*-acetylcysteine via a PLP-dependent C–S lyase catalyzing C–S bond cleavage[17,18] to generate the active *N*-acetylmercaptocysteine, which can easily react with isolated lorneic acid F (**2**) to generate lorneic acid I (**5**) (Figure S1).

Lorneic acid J (**6**) was obtained as colorless oil. Its molecular formula was determined from HRESIMS. During comparison of the spectroscopic data of **6** with that of **2** (Table 1), great similarities were found, except that two methylenes in **2** were missing in **6**. On the basis of the HMBC correlations of H-2 (δ_H 3.21, m) with C-1 (δ_C 176.3, C), C-3 (δ_C 124.8, CH), and C-4 (δ_C 132.0, CH), of H-3 (δ_H 6.11, m) with C-5 (δ_C 133.5, C), and of H-4 (δ_H 6.73, brd, 15.6) with C-5 (δ_C 133.5, C), C-6 (δ_C 127.3, CH), and C-10 (δ_C 137.2, C), the structure of compound **6** was deduced as shown.

All the isolates were evaluated for inhibitory activity on tyrosinase. In the inhibitory activity test for tyrosinase, which is mainly involved in melanin biosynthesis and also important in cosmetic applications for skin-whitening effects,[19,20] lorneic acid K (**7**) showed moderate inhibitory effects with an inhibition ratio of 27.0% at the concentration of 0.18 mM. Compounds **2** and **5** also showed weak inhibition with values of 12.9 and 18.9% at the concentration of 0.19 and 0.11 mM, respectively. Kojic acid served as a positive control with the inhibition value of 70.1% at the concentration of 0.07 mM.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P1020 digital polarimeter. NMR spectra were recorded in $CDCl_3$ or CD_3OD using a Bruker AV600 MHz spectrometer with TMS as an internal standard. ESIMS spectra were recorded using a Waters Xevo TQ-S ultra-high-pressure liquid chromatography triple quadrupole mass spectrometer. HRESIMS data were obtained using an Agilent 1290 UPLC/6540 Q-TOF mass instrument. Silica gel (200–300 mesh and 300–400 mesh, Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (Pharmacia Biotech Ltd., Sweden) were used for the chromatograph column. Semi-preparative HPLC was conducted on a HITACHI Chromaster system equipped with a DAD detector, a YMC-Triart C_{18} column (250 × 10 mm i.d., 5 μm), and a flow rate of 3.0 mL/min.

Isolation of Strain KIB-H1289 and the Cultivation. The strain designated KIB-H1289 was isolated from the bark of *B. mandshurica* Nakai, which was collected in Yuanjiang county, Yunnan Province, China, in 2015. It was identified as *Streptomyces* sp. on the basis of the morphological characteristics and 16s rRNA gene sequence analysis (GeneBank No. KY465493). This strain was grown on ISP2 agar plates (glucose 4 g, malt extract 10 g, yeast extract 4 g, and agar 20 g in 1 L of water, pH 7.2) for 3 days at 30 °C. Then, it was inoculated into 250 mL baffle Erlenmeyer flasks containing 50 mL of sterile seed medium (Tryptone soy broth, 30g/L) and cultivated for 2 days at 30 °C on a rotary shaker (250 rpm). After that, aliquots (12.5 mL) of the culture were transferred into 1000 mL baffled Erlenmeyer flasks filled with 250 mL of a production medium consisting of 1% soluble starch, 0.5% tryptone, 1% glucose, 1% glycerol, 0.5% yeast extract, and 0.3% $CaCO_3$ (pH 7.0) and cultured on a rotary shaker (220 rpm) at 30 °C for 8 days.

Extraction and Isolation of Lorneic Acids. The fermentation broth (20 L) was centrifuged (4000 rpm, 15 min), and the supernatant was extracted with EtOAc (10 L × 3). The EtOAc extract was subsequently evaporated in vacuo to afford 3.0 g of oily crude extract. The mycelia were extracted with acetone (1 L × 2) and then concentrated in vacuo to remove the acetone to yield the aqueous concentrate. This aqueous concentrate was finally extracted with EtOAc (1 L × 3) to give 1.0 g of oily crude extract after removing the EtOAc. Both extracts revealed an identical set of metabolites based on HPLC and TLC analyses, and therefore, they were combined for further purification.

The crude extract (4.0 g) was subjected to silica gel CC using a successive elution of petroleum ether/EtOAc (1:0, 10:1, 5:1, 1:1 and 1:0, v/v) and EtOAc/CH_3OH (1:1, v/v), yielding six fractions (A–F). Main composition was distributed in eluate of petroleum ether/EtOAc (10:1, 5:1, 1:1) by HPLC analysis. Compound **5** (4.3 mg) was obtained from the fraction F (EtOAc/CH_3OH 1:1) (0.4 g) by semipreparative HPLC. Fraction B (petroleum ether/EtOAc 10:1) (0.7 g) was sequentially subjected to Sephadex LH-20 CC ($CHCl_3$/CH_3OH 1:1) and semipreparative HPLC in sequence to afford compounds **2** (6.1 mg) and **8** (2.0 mg). Fractions C (petroleum ether/EtOAc 5:1) and D (petroleum ether/EtOAc 1:1) were combined and subjected to Sephadex LH-20 CC (CH_3OH) to generate seven subfractions, which were further purified by semipreparative HPLC to yield compounds **1** (2.3 mg), **3** (4.1 mg), **4** (2.5 mg), **6** (4.0 mg), and **7** (8.5 mg).

*Lorneic Acid E (**1**):* colorless oil; $[\alpha]_D^{23.0}$ −3.8 (*c* 0.26, CH_3OH); for ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 297 $[M + Na]^+$; HRESIMS *m*/*z* 297.1462 $[M + Na]^+$ (calcd for $C_{17}H_{22}O_3Na$ 297.1461).

*Lorneic Acid F (**2**):* colorless oil; for ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 259 $[M + H]^+$; HRESIMS *m*/*z* 259.1694 $[M + H]^+$ (calcd for $C_{17}H_{23}O_2$ 259.1693).

*Lorneic Acid G (**3**):* colorless oil; $[\alpha]_D^{23.0}$ +0.99 (*c* 0.37, CH_3OH); for ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 297 $[M + Na]^+$; HRESIMS *m*/*z* 297.1459 $[M + Na]^+$ (calcd for $C_{17}H_{22}O_3Na$ 297.1461).

*Lorneic Acid H (**4**):* colorless oil; for ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 257 $[M + Na]^+$; HRESIMS *m*/*z* 257.1148 $[M + Na]^+$ (calcd for $C_{14}H_{18}O_3Na$ 257.1148).

*Lorneic Acid I (**5**):* colorless oil; $[\alpha]_D^{23.0}$ −49.2 (*c* 0.44, CH_3OH); for ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 476 $[M + Na]^+$; HRESIMS *m*/*z* 476.1534 $[M + Na]^+$ (calcd for $C_{22}H_{31}NO_5S_2Na$ 476.1536).

*Lorneic Acid J (**6**):* colorless oil; for ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 253 $[M + Na]^+$; HRESIMS *m*/*z* 231.1375 $[M + H]^+$ (calcd for $C_{15}H_{19}O_2$ 231.1380).

Tyrosinase Inhibitory Activity. Mushroom tyrosinase was purchased from Sigma Chemical (St. Louis, MO, USA). The concentration of test material (50 μg/mL) was dissolved in phosphate-buffered saline. Tyrosinase inhibitory activity was performed

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according to the reported protocol.[21,22] The incubation mixture containing L-DOPA (1.25 mM) was used as a substrate; the test compound solution was prepared, and kojic acid served as a positive control (inhibition value of 70.1% at the concentration of 0.07 mM). The reaction was initiated by the addition of tyrosinase (25.0 units/mL) at room temperature for 5 min. The amount of dopachrome in the reaction mixture was measured after incubation. The inhibitory activity of the sample was determined based on the optical density at 490 nm using a spectrophotometer.

$$\text{inhibition}\ (\%) = (A - B)/A \times 100$$

where A and B indicate the absorbance of vehicle- and test-compound-treated groups, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00056.

Spectroscopic data of **7** and **8**, proposed biosynthetic pathway of lorneic acid I (**5**), and HRESIMS and NMR spectra of compounds **1**−**8** (PDF)

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Notes

The authors declare no competing financial interest.

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