Lorneic Acids, Trialkyl-Substituted Aromatic Acids from a Marine-Derived Actinomycete

Fumie Iwata,* Seizo Sato, Takako Mukai, Shoichi Yamada, Jiro Takeo, Akihisa Abe, Takaaki Okita, and Hiroyuki Kawahara

Central Research Laboratory, Nippon Suisan Kaisha, Ltd, 559-6 Kitano-machi Hachioji, Tokyo 192-0906, Japan

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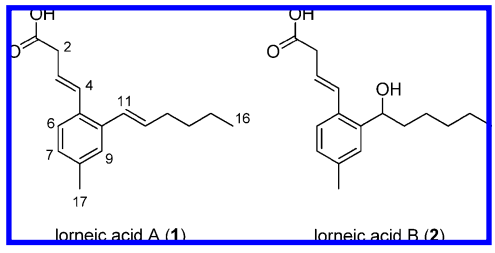
A marine-derived actinomyces strain (NPS554) isolated from a marine sediment sample collected from Miyazaki Harbor, Japan, at a depth of 38 m yielded two trialkyl-substituted aromatic acids, lorneic acid A (**1**) and lorneic acid B (**2**). The structures of the lorneic acids, which were elucidated by spectroscopic analysis, differed only in the side-chain, which contained either a conjugated double bond or a benzylic alcohol. Their structural differences affected inhibition activities against phosphodiesterase 5.

Actinomycetes from natural sources are widely recognized to produce secondary metabolites, including many antimicrobials such as streptomycin, erythromycin, and tetracycline, with original and ingenious structures and potent biological activities.[1] Therefore, actinomycetes are considered to be a potent resource for new lead compounds in drug development. Analysis of secondary metabolites produced by a *Streptomyces* strain (NPS554) with low sequence homology (16S rDNA 98.0%) resulted in the isolation of two trialkyl-substituted aromatic acids, lorneic acids A (**1**) and B (**2**). This report describes the isolation, structural elucidation, and biological activities of lorneic acids.

The strain NPS554 was cultured in an artificial seawater-based KG medium.[2] At the end of the culture period, the culture supernatant was partitioned between EtOAc and H_2O, and the H_2O phase was further extracted with *n*-BuOH. In addition, the mycelia were extracted with MeOH, and the MeOH extract was partitioned between EtOAc and H_2O. The combined crude extracts (EtOAc extract and *n*-BuOH extract) were subjected to C-18 reversed-phase flash chromatography and silica gel chromatography to yield lorneic acid A (**1**) and lorneic acid B (**2**).



Lorneic acid A (**1**) was obtained as a colorless oil. The molecular formula was established as $C_{17}H_{22}O_2$ by high-resolution electrospray-ionization time-of-flight mass spectrometry (HRESITOFMS: $[M - H]^-$ *m/z* 257.1505) in the negative mode. The IR spectrum exhibited an absorption band at 1636 cm^{-1}, indicative of a carboxyl functional group. The presence of a carboxylic acid functionality was confirmed by methylation of **1** with trimethylsilyldiazomethane[3] to yield the methyl ester **1a**, which produced a new ^1H NMR signal at δ 3.73. In addition, the signal correlated with the carbon signal at δ 174.1 in the HMBC experiment. The ^1H NMR spectrum in methanol-d_4 (Table 1) showed three aromatic methines, four olefinic methines, two allylic methylenes, two aliphatic methylenes, a singlet methyl, and a triplet methyl. Here, the two olefinic methines (H-4, δ 6.68; H-11, δ 6.68) overlapped in methanol-d_4. Thus, structure of lorneic acid A (**1**) was elucidated by an NMR spectrum in methanol-d_4 and chloroform-*d* (H-4, δ 6.75; H-11, δ 6.59). The ^{13}C NMR spectrum of **1**, including edited gs-HSQC experimental data,[4] showed one carboxylic acid carbon, six aromatic carbons including three quaternary carbons, four olefinic carbons, and six additional carbon signals between δ 14.3 and 43.9. $^1H-^1H$ COSY cross-peaks allowed assignment of the side-chain parts of the molecule (H-2 to H-4, H-11 to H-16) and the adjacent aromatic ring proton (H-6 to H-7). In the HMBC experiment (Figure 1), the H-12 signal correlated with C-10, and H-11 correlated with C-5, C-9, and C-10, suggesting a connection of C-10/C-11 and C-5/C-10/C-9. The H-4 signal correlated with both C-10 and C-6, and H-3 correlated with C-5, suggesting a connection of C-4/C-5/C-10/C-9 and C-4/C-5/C-6/C-7. The remaining structure was determined by the correlation from H-17 to C-7, C-8, and C-9, which established a 3,4-disubstituted toluene structure. The H-2 signal correlated with a carboxyl carbon (C-1), indicating an unsaturated carboxylic group. Finally, a large coupling constant ($J = 15.6$ Hz) was observed between H-3 and H-4 and between H-11 and H-12, suggesting that both C-3/C-4 and C-11/C-12 double bonds have the *E*-geometry.

Lorneic acid B (**2**) was obtained as a colorless oil, which had strong IR absorption bands at 3393 and 1671 cm^{-1}, indicative of hydroxy and carboxylic acid functional groups, respectively. The molecular formula was established as $C_{17}H_{24}O_3$ (HRESITOFMS: $[M - H]^-$ *m/z* 275.1655) in the negative mode. Treatment with trimethylsilyldiazomethane afforded the methyl ester **2a**, confirming the presence of a carboxylic acid functionality. The ^1H and ^{13}C NMR spectra differed from that of **1** at the quaternary aromatic carbon (C-10), methine carbon (C-11), and methylene carbons (C-12, C-13), indicating the presence of a benzylic hydroxy group at C-11 (δ_H 4.97, δ_C 70.9). This substitution was confirmed by $^1H-^1H$ COSY correlations between H-11 and H-12 along with the HMBC correlations from H-12 to C-10 and C-11 and from H-11 to C-5, C-9, C-12, and C-13.

The lorneic acids possess a common skeleton to the known lorneamides from a marine actinomycete (MSTMA190).[5] For the lorneic acids and the lorneamides, the side-chain substitution (carboxylic acid or carboxyamide functionality) is different.

The biological activity of lorneic acid A (**1**) is currently being examined in diverse bioassays. In initial screenings at 10 μM, lorneic acid A displayed significant activity in phosphodiesterase (PDE) inhibition experiments. It is interesting to note that lorneic acid A showed selectivity for PDEs (Figure 2.) Selective inhibition was further examined in detail with both lorneic acids A and B. As a consequence, PDE5 inhibitory activity of lorneic acid B (**2**) was weaker than that of **1** (estimated IC_{50} values: **1**, 12.6 μM; **2**, 87.1 μM). These results suggested that the conjugated double bond (C-11 to C-12) of the side-chain part of a molecule might be important for PDE5 inhibition.

* Corresponding author. Tel: +81-42-656-5192. Fax: +81-42-656-5188. E-mail: iwata@nissui.co.jp.

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Table 1. NMR Spectroscopic Data (400 MHz, CD_3OD) for Lorneic Acids A (**1**) and B (**2**)

	lorneic acid A (**1**)			lorneic acid B (**2**)		
no.	δ_C[a] mult.[b]	δ_H (J in Hz)	HMBC	δ_C[a] mult.[b]	δ_H (J in Hz)	HMBC
1	180.2, qC			179.8, qC		
2	43.9, CH_2	3.12, d (7.2)	1, 3, 4	43.4, CH_2	3.13, d (7.2)	1, 3
3	128.3, CH	6.26, dt (15.6, 7.2)	2, 5	128.3, CH	6.24, dt (15.6, 7.2)	1, 2, 5
4	129.9, CH	6.68,[c] d (15.6)	2, 6, 10	129.4, CH	6.73, d (15.6)	2, 5, 6
5	134.2, qC			134.0, qC		
6	127.3, CH	7.34, d (8.0)	4, 8, 10	127.2, CH	7.36, d (8.0)	4, 8, 10
7	128.7, CH	6.97, d (8.0)	5, 17	128.7, CH	7.01, d (8.0)	5, 9, 17
8	137.6, qC			137.7, qC		
9	127.7, CH	7.17, s	5, 7, 17	127.0, CH	7.26, s	5, 7, 17
10	137.0, qC			143.2, qC		
11	129.2, CH	6.68,[d] d (15.6)	5, 9, 10, 13	70.9, CH	4.97, t (6.4)	5, 9, 10, 12, 13
12	133.7, CH	6.05, dt (15.6, 6.8)	10, 13	39.7, CH_2	1.68, dt (6.8, 6.4)	10, 11, 13, 14
13	34.1, CH_2	2.26, dt (6.8, 6.8)	11, 12, 14, 15	26.7, CH_2	1.39, m	
14	32.9, CH_2	1.50, tt (7.2, 6.8)	12, 13, 15, 16	32.9, CH_2	1.32, m	
15	23.4, CH_2	1.44, tq (7.2, 7.2)	14, 16	23.7, CH_2	1.33, m	13, 14, 16
16	14.3, CH_3	0.98, t (7.2)	14, 15	14.4, CH_3	0.90, t (6.8)	14, 15
17	21.2, CH_3	2.30, s	7, 8, 9	21.4, CH_3	2.33, s	7, 8, 9

[a] 100 MHz. [b] Assignments by edited gs-HSQC experiments. [c] δ_H 6.75 ($CDCl_3$). [d] δ_H 6.59 ($CDCl_3$).

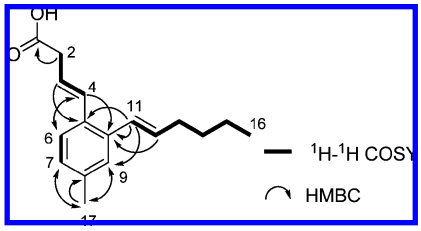

Figure 1. Key $^1H-^1H$ COSY and HMBC correlations of lorneic acid A (**1**).

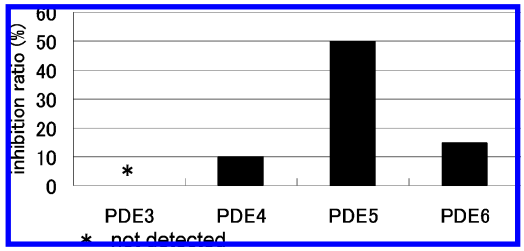

Figure 2. Phosphodiesterase inhibitory activities of lorneic acid A (**1**) at 10 μM.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were measured with a JASCO V-560 UV/vis spectrophotometer. IR spectra were obtained on a JASCO FTIR VALOR-III spectrophotometer. NMR spectra were recorded on a Bruker AVANCE DPX 400 spectrometer with methanol-d_4 and chloroform-d, the chemical shift of which was used as internal standard. High-resolution ESITOFMS spectra were measured on a Waters LCT-Premier XE using an electrospray ionization, time-of-flight mass analyzer.

Isolation of the NPS 554 Strain and Cultivation. *Streptomyces* strain NPS554 was isolated on a modified HV agar[6] (1.0 g of humic acid, 0.5 g of Na_2HPO_4, 1.71 g of KCl, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.02 g of $CaCO_3$, 0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxine-HC1, inositol, Ca-pantothenate, and *p*-aminobenzoic acid, 0.25 mg of biotin, 50 mg of cycloheximide, 18 g of agar, 18 g of Daigo's artificial seawater, and 1 L of distilled water) from marine sediment collected at a depth of 38 m near Miyazaki Harbor, Japan. The 16S rDNA sequence of the NPS554 strain (1503 base pairs) was deposited in the DDBJ Genbank (DDBJ accession number AB515328). This strain shared 98.0% 16S rDNA sequence identity with *Streptomyces* sp. CNQ-233_SD01_MAR2. The strain was cultured in 50 × 100 mL volumes of KG medium (25 g of glucose, 6 g of soytone, 2 g of yeast extract, 4 g of $CaCO_3$, 18 g of Daigo's artificial seawater, 800 μL of 2 M NaOH, and 1 L of distilled water; pH was adjusted to 6.95 before autoclaving) while shaking at 200 rpm at 28 °C for 6 days.

Extraction and Isolation of Lorneic Acids. The culture was centrifuged (2000*g*, 10 min), and the upper layer was partitioned with EtOAc and *n*-BuOH. The mycelia were extracted with MeOH, the solvent was removed, and the residue was partitioned between H_2O and EtOAc. Removal of the solvents (EtOAc and *n*-BuOH) provided 3.5 g of dry extract per 5 L of culture solution. The extract (3.5 g) was subjected to C-18 reversed-phase column chromatography purification eluting with H_2O/MeOH (3:1), H_2O/MeOH (1:1), H_2O/MeOH (1:3), and MeOH, successively. The MeOH eluting fraction was fractionated by silica gel column chromatography eluting with hexane to EtOAc. The hexane/EtOAc (1:2) fraction was further subjected to preparative TLC purification with $CHCl_3$/MeOH (3:1) to obtain pure lorneic acid A (**1**, 3.4 mg) [R_f value 0.51; solvent $CHCl_3$/MeOH (3:1)]. The H_2O/MeOH (1:3) fraction was purified by silica gel column chromatography eluting with $CHCl_3$ to MeOH. Final purification of the $CHCl_3$/MeOH (9:1) fraction by preparative TLC with the eluent $CHCl_3$/MeOH (9:1) afforded pure lorneic acid B (**2**, 3.1 mg) [R_f value 0.41; solvent $CHCl_3$/MeOH (9:1)].

Lorneic acid A (1): colorless oil; UV (MeOH) λ_{max} (log ϵ) 240 nm (4.59), 265 nm (4.48); IR (KBr) 2954, 2927, 2856, 1636, 1577, 1457, 1428, 1396, 1372, 1247, 1192, 1099, 965, 934, 826 cm^{-1}; NMR data, see Table 1 (CD_3OD); ^1H NMR (400 MHz, $CDCl_3$) δ 7.30 (1H, d, J = 8.0 Hz, H-6), 7.18 (1H, s, H-9), 6.99 (1H, d, J = 8.0 Hz, H-7), 6.75 (1H, d, J = 15.6 Hz, H-4), 6.59 (1H, d, J = 15.6 Hz, H-11), 6.09 (1H, dt, J = 15.6, 6.8 Hz, H-3), 6.04 (1H, dt, J = 15.6, 6.8 Hz, H-12), 3.31 (2H, d, J = 6.8 Hz, H-2), 2.31 (3H, s, H-17), 2.23 (2H, q, J = 6.8 Hz, H-13), 1.46 (2H, m, H-14), 1.38 (2H, m, H-15), 0.93 (3H, t, J = 7.2 Hz, H-16); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.7 (C-1), 137.3 (C-8), 136.1 (C-10), 133.7 (C-12), 131.9 (C-4), 131.7 (C-5), 127.8 (C-7), 127.4 (C-11), 127.0 (C-9), 126.4 (C-6), 121.8 (C-3), 38.3 (C-2), 33.0 (C-13), 31.6 (C-14), 22.3 (C-15), 21.2 (C-17), 13.9 (C-16); HRESITOFMS $[M - H]^-$ m/z 257.1505, calcd for $C_{17}H_{22}O_2$, 257.1540.

Lorneic acid B (2): colorless oil, $[\alpha]_D$ −22 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 252 nm (4.00); IR (KBr) 3393, 2956, 2930, 2859, 1718, 1671, 1577, 1457, 1389, 1255, 1163, 1057, 969 cm^{-1}; HRESITOFMS: $[M - H]^-$ m/z 275.1655, calcd for $C_{17}H_{24}O_3$, 275.1645.

Lorneic Acid A Methyl Ester (1a). To a solution of lorneic acid A (**1**) (1.0 mg, 3.9 μmol) in MeOH (0.5 mL) was added trimethylsilyldiazomethane (0.12 mL, 0.23 mmol, 2.0 M in hexane solution) at room temperature. After stirring at room temperature for 20 min, the reaction mixture was concentrated under reduced pressure to give the methyl ester **1a** as a colorless oil: ^1H NMR (400 MHz, CD_3OD) δ 7.30 (1H, d, J = 8.0 Hz, H-6), 7.20 (1H, s, H-9), 7.00 (1H, d, J = 8.0 Hz, H-7), 6.76 (1H, d, J = 15.6 Hz, H-4), 6.64 (1H, d, J = 15.6 Hz, H-11), 6.10 (1H, dt, J = 15.6, 7.2 Hz, H-3), 6.06 (1H, dt, J = 15.6, 7.2 Hz, H-12), 3.73 (3H, s, OMe), 3.29 (2H, dd, J = 7.2, 1.6 Hz, H-2), 2.32 (3H, s, H-17), 2.26 (2H, dt, J = 7.2, 7.2 Hz, H-13), 1.50 (2H, m, H-14), 1.42 (2H, m, H-15), 0.98 (3H, t, J = 7.2 Hz, H-16); ^{13}C NMR (100 MHz, CD_3OD) δ 174.1 (C-1), 138.3 (C-8), 137.3 (C-10), 134.3 (C-12), 133.3 (C-5), 132.6 (C-4), 129.0 (C-11), 128.8 (C-7), 128.0 (C-9), 127.4 (C-

6), 124.0 (C-3), 52.4 (OMe), 39.0 (C-2), 34.1 (C-13), 32.8 (C-14), 23.4 (C-15), 21.2 (C-17), 14.3 (C-16); HRESITOFMS $[M - H]^+$ *m/z* 273.1829, calcd for $C_{18}H_{25}O_2$, 273.1855.

Lorneic Acid B Methyl Ester (2a). Lorneic acid B (**2**) (1.0 mg) was converted to the corresponding methyl ester **2a** by the same experimental procedure as described for methyl ester **1a**: colorless oil; ^{1}H NMR (400 MHz, CD_3OD) δ 7.32 (1H, d, $J = 8.0$ Hz, H-6), 7.28 (1H, s, H-9), 7.04 (1H, d, $J = 8.0$ Hz, H-7), 6.84 (1H, d, $J = 16.0$ Hz, H-4), 6.09 (1H, dt, $J = 16.0$, 4.0 Hz, H-3), 4.91 (1H, dd, $J = 6.0$, 4.8 Hz, H-11), 3.73 (3H, s, OMe), 3.29 (2H, dd, $J = 6.8$, 1.6 Hz, H-2), 2.34 (3H, s, H-17), 1.68 (2H, m, H-12) 1.48−1.25 (6H, m, H-13, H-14, and H-15), 0.90 (3H, t, $J = 6.8$ Hz, H-16); ^{13}C NMR (100 MHz, CD_3OD) δ 174.0 (C-1), 143.4 (C-10), 138.4 (C-8), 133.4 (C-5), 132.2 (C-4), 128.8 (C-7), 127.3 (C-6), 127.3 (C-9), 124.2 (C-3), 71.3 (C-11), 49.9 (OMe), 39.7 (C-12), 39.7 (C-2), 32.9 (C-14), 26.7 (C-13), 23.7 (C-15), 21.4 (C-17), 14.4 (C-16); HRESITOFMS $[M - H]^+$ *m/z* 291.1952, calcd for $C_{18}H_{27}O_3$, 291.1960.

PDE5 Assay. Human platelet phosphodiesterase (PDE5) is used. Test compound and vehicle were preincubated with 35 μg/mL enzyme in Tris-HCl buffer (pH 7.5) for 15 min at 25 °C. The reaction was initiated by the addition of 1 μM cGMP and 0.01 μM [^{3}H]cGMP for another 20 min incubation period and terminated at 100 °C. The resulting [^{3}H]GMP is converted to [^{3}H]guanosine by the addition of snake venom nucleotidase and separated by AG1X2 resin. An aliquot was removed and counted to determine the amount of [^{3}H]guanosine formed. All test compounds were assayed in duplicate.

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Supporting Information Available: ^{1}H and ^{13}C NMR and UV spectra of **1** and **2** and ^{1}H and ^{13}C NMR spectra of **1a** and **2a** are available free of charge via the Internet at http://pubs.acs.org.

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