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Mohangic Acids A–E, *p*-Aminoacetophenonic Acids from a Marine-Mudflat-Derived *Streptomyces* sp.

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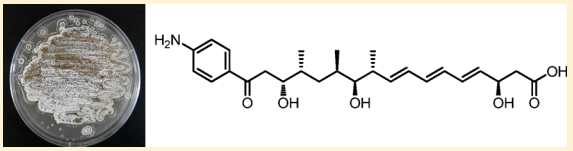
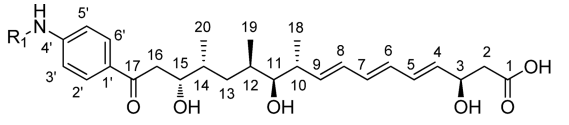
Supporting Information

ABSTRACT: Mohangic acids A–E (**1**–**5**) were isolated from a marine *Streptomyces* sp. collected from a mudflat in Buan, Republic of Korea. Comprehensive spectroscopic analysis revealed that the mohangic acids are new members of the *p*-aminoacetophenonic acid class. The relative and absolute configurations of the mohangic acids were determined by *J*-based configuration analysis and by the application of bidentate chiral NMR solvents followed by ^{13}C NMR analysis, chemical derivatization, and circular dichroism spectroscopy. Mohangic acid E (**5**), which is the first glycosylated compound in the *p*-aminoacetophenonic acid family, displayed significant quinone reductase induction activity.

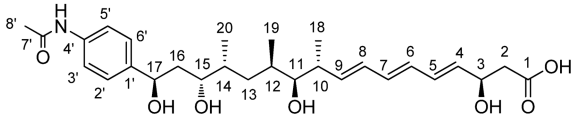


Over the past several decades, natural products derived from microbes have served as the most efficient natural source for drug discovery and development because of relatively convenient production of lead compounds by microbial cultivation.[1] Despite the previous success of microbial natural products, the constant clinical need for bioactive compounds with structural novelty has led natural product chemists to search for chemically prolific microorganisms such as actinomycetes that inhabit relatively uninvestigated environments.[2] Marine ecosystems, covering 70% of the surface of the earth and possessing great biological diversity, have been regarded as underinvestigated sources of microorganisms with biosynthetic machinery for bioactive secondary metabolites.[3]

As part of our efforts to discover new bioactive compounds from marine microorganisms, we isolated actinomycete strains from the intertidal mudflat in Buan, Republic of Korea, and studied their chemical profiles by LC/MS. Our chemical-analysis-based discovery strategy resulted in the efficient isolation of novel dilactone-tethered, pseudodimeric peptides that were identified as inhibitors of *Candida albicans* isocitrate lyase, the mohangamides,[4] and antibacterial cyclic depsipeptides with highly modified amino acid units, the hormaomycins,[5] from a *Streptomyces* strain (SNM55). Further chemical analysis of the actinomycete strains using LC/MS identified a *Streptomyces* strain (SNM31) that produces a major metabolite ($[M + Na]^+$ m/z at 482) bearing a triene moiety (UV λ_{max} 270 nm). Initial dereplication based on the UV and MS data indicated that this compound and its derivatives were previously unreported. A large-scale fermentation of the strain and purification of this series of compounds yielded five new *p*-aminoacetophenonic acid-derived metabolites, mohangic acids A–E (**1**–**5**). Here, we report the isolation, structure elucidation, and biological activities of mohangic acids A–E (**1**–**5**).



1 R_1 = H **2** R_1 = $^{7'}CH_3$ **3** R_1 = $^{7'}CO^{8'}CH_3$



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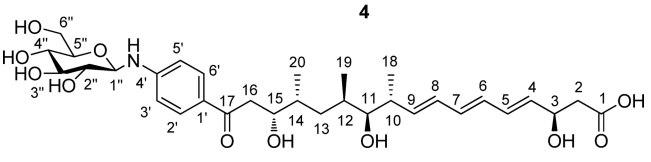

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RESULTS AND DISCUSSION

Mohangic acid A (**1**) was isolated as a yellow gum with a molecular formula of $C_{26}H_{37}NO_6$ according to ^1H and ^{13}C NMR and high-resolution FAB mass spectroscopy. The ^1H NMR (in CD_3OD) spectrum of **1** showed each of two overlapped aromatic protons at δ_H 7.77 and 6.64, six olefinic protons from 6.26 to 5.69 ppm, three carbinol protons at 4.48, 4.03, and 3.12 ppm, nine aliphatic protons between 2.97 and 1.22 ppm, and three methyl groups at 1.02, 0.90, and 0.87 ppm in the shielded region of the spectrum. The ^{13}C NMR and HSQC spectra of **1** displayed one ketone carbonyl at 200.3 ppm, one carboxylic acid carbon at 177.8 ppm, 12 sp^2 carbons

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Table 1. ^{1}H and ^{13}C NMR Data for Mohangic Acids A–E (1–5) in CD_3OD[a]

	mohangic acid A (1)		mohangic acid B (2)		mohangic acid C (3)		mohangic acid D (4)		mohangic acid E (5)	
positon	δ_C, type	δ_H, mult (J in Hz)	δ_C, type	δ_H, mult (J in Hz)	δ_C, type	δ_H, mult (J in Hz)	δ_C, type	δ_H, mult (J in Hz)	δ_C, type	δ_H, mult (J in Hz)
1	177.8, C		178.0, C		176.5, C		179.0, C		178.5, C	
2	44.5, CH_2	2.41, d (6.5)	45.0, CH_2	2.41, d (6.5)	43.0, CH_2	2.40, d (6.5)	45.0, CH_2	2.37, d (6.5)	45.0, CH_2	2.40, d (6.5)
3	70.5, CH	4.48, dd (6.5, 6.5)	70.5, CH	4.49, dd (6.5, 6.5)	68.5, CH	4.47, dd (6.5, 6.5)	71.0, CH	4.46, dd (6.5, 6.5)	70.5, CH	4.47, dd (6.5, 6.5)
4	135.7, CH	5.69, dd (15.0, 6.5)	136.0, CH	5.69, dd (15.0, 6.5)	133.5, CH	5.67, dd (15.0, 6.5)	136.0, CH	5.66, dd (15.0, 6.5)	135.5, CH	5.71, dd (15.0, 6.5)
5	131.8, CH	6.26, dd (15.0, 10.5)	132.0, CH	6.27, dd (15.0, 10.5)	132.1, CH	6.25, dd (15.0, 10.5)	131.5, CH	6.26, dd (15.0, 10.5)	131.5, CH	6.27, dd (15.0, 10.5)
6	132.0, CH	6.12, m	131.5, CH	6.12, dd (15.0, 10.5)	129.5, CH	6.11, dd (15.0, 10.5)	132.5, CH	6.11, dd (15.0, 10.5)	132.0, CH	6.09, dd (15.0, 10.5)
7	134.7, CH	6.19, dd (15.0, 10.0)	135.0, CH	6.19, dd (15.0, 10.5)	133.0, CH	6.20, dd (15.0, 10.5)	134.5, CH	6.19, dd (15.0, 10.5)	134.5, CH	6.18, dd (15.0, 10.5)
8	132.2, CH	6.09, dd (15.0, 10.0)	132.5, CH	6.09, dd (15.0, 10.5)	130.0, CH	6.08, dd (15.0, 10.5)	132.0, CH	6.09, dd (15.0, 10.5)	131.8, CH	6.11, dd (15.0, 10.5)
9	138.4, CH	5.72, dd (15.0, 8.5)	138.5, CH	5.71, dd (15.0, 8.5)	137.0, CH	5.70, dd (15.0, 8.5)	138.5, CH	5.70, dd (15.0, 8.5)	138.0, CH	5.73, dd (15.0, 8.5)
10	41.6, CH	2.42, ddd (8.5, 6.5, 5.0)	41.5, CH	2.43, ddd (8.5, 6.5, 5.0)	39.5, CH	2.43, ddd (8.5, 6.5, 5.0)	42.0, CH	2.44, ddd (8.5, 6.5, 5.0)	41.5, CH	2.43, ddd (8.5, 6.5, 5.0)
11	80.8, CH	3.12, dd (9.0, 5.0)	79.8, CH	3.12, dd (9.0, 5.0)	79.0, CH	3.11, dd (9.0, 5.0)	81.0, CH	3.09, dd (9.0, 5.0)	80.5, CH	3.12, dd (9.0, 5.0)
12	34.5, CH	1.63, dddd (9.0, 8.5, 6.5, 3.5)	35.0, CH	1.62, dddd (9.0, 8.5, 6.5, 3.5)	34.5, CH	1.61, dddd (9.0, 8.5, 6.5, 3.5)	34.5, CH	1.58, dddd (9.0, 8.5, 6.5, 3.5)	34.5, CH	1.61, dddd (9.0, 8.5, 6.5, 3.5)
13a	37.0, CH_2	1.31, ddd (12.5, 8.5, 4.0)	37.0, CH_2	1.31, ddd (12.5, 8.5, 4.0)	36.9, CH_2	1.31, ddd (12.5, 8.5, 4.0)	37.0, CH_2	1.24, ddd (12.5, 8.5, 4.0)	37.5, CH_2	1.31, ddd (12.5, 8.5, 4.0)
13b		1.22, ddd (12.5, 8.5, 3.5)		1.21, ddd (12.5, 8.5, 3.5)		1.21, ddd (12.5, 8.5, 3.5)		1.21, ddd (12.5, 8.5, 3.5)		1.23, ddd (12.5, 8.5, 3.5)
14	37.4, CH	1.71, dddd (8.5, 8.0, 6.5, 4.0)	37.5, CH	1.71, dddd (8.5, 8.0, 6.5, 4.0)	37.5, CH	1.70, dddd (8.5, 8.0, 6.5, 4.0)	38.0, CH	1.63, dddd (8.5, 8.0, 6.5, 4.0)	37.5, CH	1.73, dddd (8.5, 8.0, 6.5, 4.0)
15	74.3, CH	4.03, ddd (9.0, 8.0, 4.0)	74.5, CH	4.04, ddd (8.5, 8.0, 4.0)	73.8, CH	4.05, ddd (9.0, 8.0, 4.0)	72.0, CH	3.75, ddd (9.0, 8.0, 3.0)	74.0, CH	4.05, ddd (9.0, 8.0, 4.0)
16a	42.4, CH_2	2.97, dd (15.5, 9.0)	42.5, CH_2	2.98, dd (15.5, 8.5)	43.2, CH_2	3.10, dd (15.5, 9.0)	43.2, CH_2	1.73, dd (12.0, 9.0, 3.0)	42.5, CH_2	3.03, dd (15.0, 9.0)
16b		2.95, dd (15.5, 4.0)		2.94, dd (15.5, 4.0)		2.96, dd (15.5, 4.0)		1.62, m		2.95, dd (15.0, 4.0)
17	200.3, C		200.5, C		200.0, C		71.5, CH	4.85, m	200.5, C	
18	18.4, CH_3	1.02, d (6.5)	18.7, CH_3	1.01, d (6.5)	17.0, CH_3	1.01, d (6.5)	19.0, CH_3	1.01, d (6.5)	18.5, CH_3	1.01, d (6.5)
19	14.2, CH_3	0.87, d (6.5)	14.4, CH_3	0.87, d (6.5)	13.0, CH_3	0.85, d (6.5)	14.5, CH_3	0.86, d (6.5)	14.5, CH_3	0.87, d (6.5)
20	15.5, CH_3	0.90, d (6.5)	15.5, CH_3	0.89, d (6.5)	14.0, CH_3	0.89, d (6.5)	15.4, CH_3	0.81, d (6.5)	15.5, CH_3	0.89, d (6.5)
1′	127.8, C		126.8, C		134.1, C		139.1, C		139.1, C	
2′	132.3, CH	7.77, d (8.0)	132.0, CH	7.80, d (8.0)	129.2, CH	7.94, d (8.0)	127.5, CH	7.28, d (8.0)	131.5, CH	7.84, d (8.0)
3′	114.6, CH	6.64, d (8.0)	112.2, CH	6.58, d (8.0)	118.5, CH	7.68, d (8.0)	121.5, CH	7.51, d (8.0)	114.0, CH	6.82, d (8.0)
4′	155.5, CH		155.0, C		144.8, C		143.5, C		153.6, C	
5′	114.6, CH	6.64, d (8.0)	112.2, CH	6.58, d (8.0)	118.5, CH	7.68, d (8.0)	121.5, CH	7.51, d (8.0)	114.0, CH	6.82, d (8.0)
6′	132.3, CH	7.77, d (8.0)	132.0, CH	7.80, d (8.0)	129.2, CH	7.94, d (8.0)	127.5, CH	7.28, d (8.0)	131.5, CH	7.84, d (8.0)
7′			30.4, CH_3	2.83, s	171.0, C		172.0, C			
8′					22.5, CH_3	2.13, s	24.0, CH_3	2.11, s		
1″									86.0, CH	4.63, d (9.0)
2″									71.5, CH	3.37, m
3″									79.4, CH	3.47, dd (9.0, 8.5)
4″									74.5, CH	3.35, m
5″									78.5, CH	3.41, ddd (9.0, 5.5, 2.5)
6″									63.0, CH_2	3.64, dd (12.0, 5.5)
										3.86, dd (12.0, 2.5)

[a] ^{1}H and ^{13}C NMR were recorded at 600 and 150 MHz, respectively.

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between 155.5 and 114.6 ppm, three oxygenated sp^3 carbons at 80.8, 74.3, and 70.5 ppm, six aliphatic sp^3 carbons between 44.5 and 34.5 ppm, and three methyl carbons at δ_C 18.4, 15.5, and 14.2. Because 12 olefinic carbon signals (six double bonds) and two carbonyl carbons accounted for eight of the nine degrees of unsaturation deduced from the molecular formula of **1**, mohangic acid A (**1**) must contain a ring structure.

All $^1J_{CH}$ correlations were fully assigned by analysis of HSQC spectroscopic data (Table 1). Interpretation of the COSY and HMBC spectra elucidated two partial structures (Figure 1).

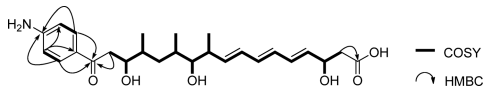

Figure 1. Key COSY and HMBC correlations of mohangic acid A (**1**).

First, the COSY correlations from H_2-2 (δ_H 2.41) to H-3 (δ_H 4.48) showed connectivity from C-2 (δ_C 44.5) to C-3 (δ_C 70.5). The oxygenated carbon C-3 was deduced to be directly connected to sp^2 carbon C-4 (δ_C 135.7) on the basis of the COSY correlation between H-3 and H-4 (δ_H 5.69). Further analysis of the COSY correlations of the six olefinic protons resulted in the construction of a triene moiety running from C-4 to C-9 (δ_C 138.4). The geometries of the double bonds in the triene structure were determined to be 4*E*, 6*E*, and 8*E* on the basis of the *trans* ^{1}H−^{1}H coupling constants (J = 15.0 Hz) observed in the olefinic proton peaks. The COSY correlation between H-9 (δ_H 5.72) and H-10 (δ_H 2.42) revealed that the triene moiety was connected to the C-10 aliphatic carbon (δ_C 41.6). In addition, COSY correlations from H-10 to H_2-16 (δ_H 2.97, 2.95) expanded the chain from C-10 to C-16 (δ_C 42.4). Three doublet methyl groups (C-18, C-19, and C-20; δ_C 18.4, 14.2, and 15.5) were located at C-10, C-12 (δ_C 34.5), and C-14 (δ_C 37.4), respectively, according to the H_3-18 (δ_H 1.02)/H-10, H_3-19 (δ_H 0.87)/H-12, and H_3-20 (δ_H 0.90)/H-14 (δ_H 1.71) homonuclear correlations. The $^2J_{CH}$ couplings from H_2-2 to C-1 (δ_C 177.8) and from H_2-16 to C-17 (δ_C 200.3) established the first partial structure, a chain composed of 20 carbons bearing three branch methyl groups.

The ^{1}H NMR spectroscopic data of **1** displayed a typical feature of *para*-substituted aromatic ring protons, H-2′/H-6′ (δ_H 7.77/δ_H 7.77) and H-3′/H-5′ (δ_H 6.64/δ_H 6.64). Strong COSY correlations between H-2′/H-6′ and H-3′/H-5′ and HMBC correlations from these protons indicated an aromatic ring. HMBC correlations from H-2′/H-6′ to C-17 indicated connectivity between the chain and the aromatic ring via the carbonyl carbon C-17. A primary amine group deduced from the molecular formula was assigned at C-4′ (δ_C 155.5), which was the last open position requiring a functional group based on the chemical shift of C-4′, thus completing the planar structure of **1**. The assigned structure was further confirmed by the analysis of 1D and 2D NMR data of **1** acquired in pyridine-d_5 (Table S1).

Mohangic acid B (**2**) was obtained as a yellow gum, and its molecular formula was deduced to be $C_{27}H_{39}NO_6$ on the basis of HRFABMS data combined with ^{1}H and ^{13}C NMR spectroscopic data (Table 1). Careful comparison of NMR spectroscopic data revealed that the ^{1}H NMR spectrum of **2** in CD_3OD was very similar to that of **1**. Careful analysis of the 1D and 2D NMR spectroscopic data of **2** revealed that mohangic acid B (**2**) differs from **1** by one *N*-methyl group (δ_H 2.83; δ_C 30.4). A $^3J_{CH}$ correlation from the *N*-methyl protons (δ_H 2.83) to C-4′ (δ_C 155.0) assigned the methyl group C-7′ to the amine at C-4′ in the *para*-substituted aromatic ring. Thus, the structure of mohangic acid B (**2**) was determined as an *N*-methyl analogue of **1**.

Mohangic acid C (**3**) was purified as a yellow gum determined to possess a molecular formula of $C_{28}H_{39}NO_7$ on the basis of HRFABMS data in combination with ^{1}H and ^{13}C NMR data (Table 1). The 1D and 2D NMR spectroscopic data of **3** displayed features analogous to those of **2** except for the absence of an *N*-methyl group (δ_H 2.83; δ_C 30.4) in **2** and the presence of a singlet methyl group (δ_H 2.13; δ_C 22.5) and an additional carbonyl carbon (δ_C 171.0) in **3**. Further analysis of 2D NMR spectra indicated the presence of an *N*-acetyl group, thus elucidating the structure of **3** as the *N*-acetylated congener of **1**.

Mohangic acid D (**4**) was isolated as a yellow gum. The molecular formula was assigned as $C_{28}H_{41}NO_7$ on the basis of HRFABMS and ^{1}H and ^{13}C spectroscopic data (Table 1). Although the NMR spectra of **4** were similar to those of **3**, an additional signal of a carbinol proton (δ_H 4.85) directly bound to an oxygenated carbon (δ_C 71.5) and the lack of the ketone carbonyl signal (δ_C 200.0) previously observed in the ^{13}C spectrum of **3** indicated a distinct difference between **3** and **4**. Analysis of 2D NMR spectra indicated that the C-17 ketone carbonyl in **3** was reduced to a hydroxy group in **4**, subsequently establishing the planar structure of **4**.

Mohangic acid E (**5**) was obtained as a yellow gum with a molecular formula of $C_{32}H_{47}NO_{11}$ on the basis of the HRFABMS and ^{1}H and ^{13}C spectroscopic data (Table 1). In contrast to the mohangic acids B−D (**2**−**4**), the molecular weight of **5** is 162 Da larger than that of **1**. In addition, mohangic acid E (**5**) is much more hydrophilic than the other congeners based on reversed-phase HPLC analysis. These observations led us to speculate that mohangic acid E (**5**) should be a glycosylated version of **1**. Careful analysis of the 1D and 2D NMR spectra revealed that mohangic acid E possesses one hexose. This hexose was determined to be a β-glucose by analysis of the coupling constants $^3J_{HH}$ and $^1J_{CH}$ (154 Hz)[6] and on the basis of ROESY NMR correlations among the axially oriented sugar protons. The HMBC correlation from the anomeric proton H-1″ (δ_H 4.63) to C-4′ (δ_C 153.6) bearing a nitrogen atom located the β-glucose moiety on the nitrogen attached to the aromatic ring. The ROESY correlation between H-1″ and H-3′/H-5′ (δ_H 6.82) also supported this assignment. To determine the absolute configuration of the glucose moiety in **5**, it was subjected to acid hydrolysis. The hydrolysate and authentic L- and D-glucose samples were respectively derivatized with hexamethyldisilazane (HMDS) and TMS-Cl.[7] GC/MS analysis of the derivatives clarified that the glucose in mohangic acid E (**5**) has a D-configuration because the derivative of the glucose in **5** exhibited a retention time consistent with that of an authentic D-glucose derivative.

Mohangic acids A−E (**1**−**5**) have several stereogenic centers in their chains. To determine their configurations, we performed comprehensive spectroscopic analyses and various chemical derivatizations. We first conducted homodecoupling spectroscopic experiments of mohangic acid A (**1**) to extract exact $^3J_{HH}$ values for H-10/H-11, H-11/H-12, H-12/H_2-13, H_2-13/H-14, and H-14/H-15 couplings; the ^{1}H−^{1}H coupling constants for these protons were not measurable in the ordinary ^{1}H NMR spectra because of their complicated multiplicities. After ^{1}H−^{1}H coupling constants were clearly measured, long-range heteronuclear coupling constants were

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acquired by a HETLOC (hetero half-filtered TOCSY) NMR experiment.[8] *J*-based configuration analysis was performed using the obtained $^3J_{HH}$, $^3J_{CH}$, and $^2J_{CH}$ values and ROESY NMR correlations (Figure 2).[9] On the basis of the *J*-based configuration analysis, the most appropriate rotamers from C-10 to C-15 were selected.

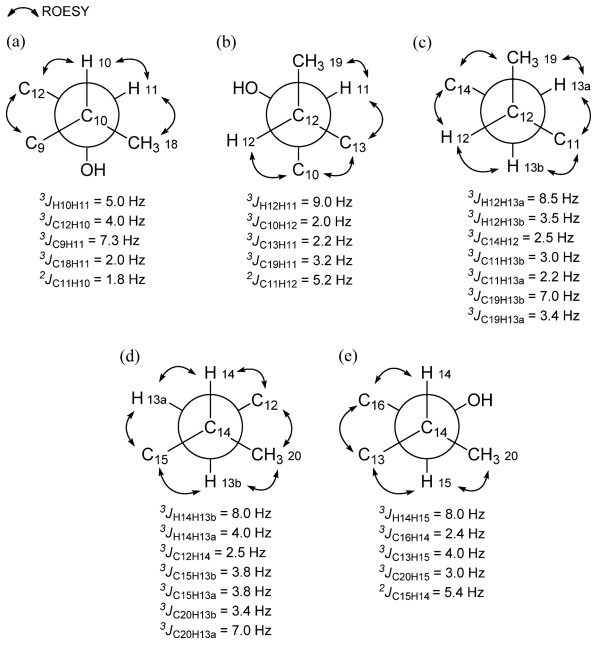

Figure 2. *J*-based configuration analysis of mohangic acid A (**1**) at (a) C-10 and C-11, (b) C-11 and C-12, (c) C-12 and C-13, (d) C-13 and C-14, and (e) C-14 and C-15.

The relationship between C-9 and H-11 was deduced as *anti* because of the large corresponding $^3J_{C9H11}$ value (7.3 Hz). The $^3J_{H10H11}$ (5.0 Hz), $^3J_{C12H10}$ (4.0 Hz), and $^3J_{C18H11}$ (2.0 Hz) values indicated *gauche* relationships between H-10/H-11, C-12/H-10, and C-18/H-11. The $^2J_{C11H10}$ value (1.8 Hz) indicated an *anti* relationship between the hydroxy group at C-11 and H-10, and ROESY correlations between the H-10/H-11, H-10/H-12, and H-11/H_3-18 supported the rotamer depicted in Figure 2a. An *anti* relationship between H-11 and H-12 was established on the basis of the large $^3J_{H11H12}$ value (9.0 Hz). The $^3J_{C10H12}$ (2.0 Hz), $^3J_{C13H11}$ (2.2 Hz), and $^3J_{C19H11}$ (3.2 Hz) completely assigned the relationships of C-10/H-12, C-13/H-11, and C-19/H-11 as *gauche*. Moreover, the large $^2J_{C11H12}$ (5.2 Hz) value and observed ROESY correlations supported the rotamer shown in Figure 2b. The methylene group at C-13 is not a stereogenic center. However, the two protons bound to this carbon are distinguishable in the ^{1}H NMR spectrum, thus allowing for consecutive *J*-based analysis to relate configurations from C-12 to C-14 through C-13. The $^3J_{H12H13a}$ (8.5 Hz) and $^3J_{C19H13b}$ (7.0 Hz) values implied *anti* relationships of H-12/H-13a and C-19/H-13b. *Gauche* relationships of C-14/H-12, C-11/H-13b, C-11/H-13a, and C-19/H-13a were deduced on the basis of the small $^3J_{CH}$ values (2.2−3.4 Hz). A strong ROESY correlation between H_3-19 and H-13a supported a *gauche* relationship between C-19 and H-13a, clarifying the proper rotamer depicted in Figure 2c. This rotamer was consistent with the observed ROESY correlations around C-12 and C-13. The $^3J_{H13bH14}$ (8.0 Hz) and $^3J_{C20H13a}$ (7.0 Hz) values indicated *anti* relationships of H-13b/H-14 and C-20/H-13a. The other coupling constants supported the rotamer depicted in Figure 2d. The H_3-20/H-13b ROESY correlation, along with the other observed ROESY correlations, further supported this rotamer structure. An *anti* relationship between H-14 and H-15 was indicated by the $^3J_{H14H15}$ (8.0 Hz) value. The C-16/H-14, C-13/H-15, and C-20/H-15 relationships were deduced as *gauche* on the basis of the $^3J_{C16H14}$ (2.4 Hz), $^3J_{C13H15}$ (4.0 Hz), and $^3J_{C20H15}$ (3.0 Hz) values. The large $^2J_{C15H14}$ value (5.4 Hz) and the ROESY NMR signals between H-13 and H-16 indicated that the rotamer in Figure 2e was the most appropriate structure. Overall, detailed *J*-based configuration analysis revealed that the relative configurations of the stereogenic centers in the chain moiety are 10*R**, 11*S**, 12*R**, 14*R**, and 15*R**.

Because mohangic acid D (**4**) bears an additional stereogenic center at C-17, further chemical derivatization and NMR spectroscopic analysis were required to establish its configuration. We first hydrogenated mohangic acid D (**4**) with 10% Pd/C under 1 atm of H_2 at room temperature to increase its stability for further derivatization. The hydrogenated product (**6**) was then derivatized with 2,2-dimethoxy propane to furnish an acetonide derivative (**7**) for the 1,3-diol moiety at C-15 and C-17. The ^{1}H and HSQC NMR spectra of the acetonide derivative (**7**) showed that the ^{1}H and ^{13}C chemical shifts of two acetonide methyl groups are almost identical (δ_C 24.6−δ_H 1.39; δ_C 24.6−δ_H 1.39), indicating an *anti* relationship of the 1,3-hydroxy groups (Figure 3).[10]

For the assignment of the absolute configurations of the asymmetric carbons at C-3, C-10, C-11, C-14, and C-15 in **1**, we initially attempted MTPA derivatization on the secondary alcohol groups in mohangic acid A to apply the modified Mosher's method.[11] However, mohangic acid A (**1**) was degraded as soon as *S*- or *R*-MTPA-Cl reagent was added to **1**. We therefore attempted to stabilize **1** by hydrogenation of the triene; however, the hydrogenated product was also degraded during MTPA derivatization. Therefore, we used an alternative method developed by Kishi and co-workers that involves bidentate chiral NMR solvents, (*R*,*R*)- and (*S*,*S*)-bis-α-methylbenzylamine-*p*-Me (BMBA).[12] The NH groups in the bidentate chiral NMR solvents build N···H−O hydrogen bonds through interactions with isolated alcohols, resulting in the discrimination of the chemical shifts of the carbons adjacent to

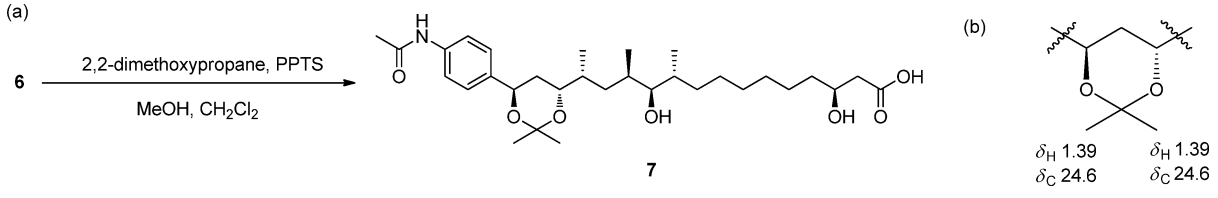

Figure 3. (a) Acetonide formation of hydrogenation product **6** of mohangic acid D (**4**). (b) ^{1}H and ^{13}C NMR chemical shifts of acetonide derivative **7** indicating an *anti* relationship of 1,3-diol.

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the secondary hydroxy groups. The analysis of these ^{13}C chemical shift behaviors in (*R*,*R*)- and (*S*,*S*)-bidentate chiral NMR solvents by calculation of $\Delta\delta_{(R,R)-(S,S)}$ values can establish the relative and absolute configurations of secondary alcohols.[12] Because the bidentate chiral solvents were not commercially available, we synthesized the solvents (*R*,*R*)- and (*S*,*S*)-bis-α-methylbenzylamine-*p*-Me.[12] The ^{13}C NMR spectra of 15 mg of mohangic acid A (**1**) in a 5:2 mixture of (*R*,*R*)-BMBA and pyridine-d_5 and in a 5:2 mixture of (*S*,*S*)-BMBA and pyridine-d_5 were recorded (Figures S38 and S39, respectively). Careful comparison of the ^{13}C NMR spectra in the bidentate solvents enabled clear detection of shifts of the ^{13}C signals associated with the carbons flanking C-3, C-11, and C-15 each with a secondary hydroxy group (Figures 4 and S40). By calculating

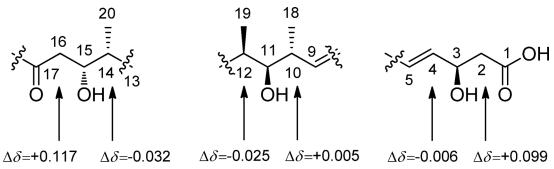

Figure 4. Chemical shift differences [$\Delta\delta = \delta(R,R) - \delta(S,S)$] observed for the indicated carbons in a 5:2 mixture of (*R*,*R*)- and (*S*,*S*)-BMBA and pyridine-d_5.

$\Delta\delta_{(R,R)-(S,S)}$ values at C-4/C-2, C-12/C-10, and C-14/C-16, we determined the absolute configurations of the stereogenic centers of C-3, C-11, and C-15 as 3*R*, 11*S*, and 15*R*. Subsequently, we assigned the absolute configurations of the other stereogenic centers in mohangic acid (**1**) as 10*R*, 12*R*, and 14*R* on the basis of the relative configuration determined *vide supra*.

Mohangic acids B–E (**2**–**5**) were proposed to possess configurations identical to those of **1** at the common stereogenic centers because of the strong similarity among the NMR data for their chains and because of their common biosynthetic origin. In addition, circular dichroism (ECD) spectra of mohangic acids A–E displayed almost identical features, with a strong Cotton effect at 218 nm, clearly supporting their identical absolute configurations of 3*R*, 10*R*, 11*S*, 12*R*, 14*R*, and 15*R* (Figure 5). The additional stereogenic center at C-17 of mohangic acid D was also deduced as 17*R* on

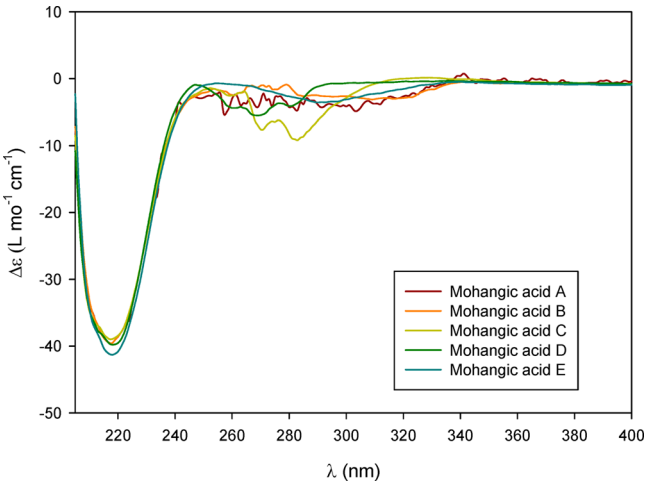

Figure 5. ECD spectra of mohangic acids A–E (**1**–**5**).

the basis of the relative configuration established by acetonide derivatization, as previously mentioned.

The biological activities of mohangic acids A–E (**1**–**5**) were evaluated in several ways. In a cytotoxicity assay against human cancer cell lines such as A549 (lung cancer), HCT116 (colon cancer), SNU638 (gastric cancer), K562 (leukemia), SK-HEP1 (liver cancer), and MDA-MB231 (breast cancer), mohangic acids A–E did not display significant cytotoxic activities (IC_{50} > 10 μM). The mohangic acids (**1**–**5**) were also evaluated for growth inhibition activity against pathogenic bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Kocuria rhizophila*, *Salmonella enterica*, *Proteus hauseri*, and *Escherichia coli*) and fungi (*Candida albicans* and *Aspergillus fumigatus*). However, no antimicrobial effects were observed (MIC > 128 μM).

Because the mohangic acids exhibited no cytotoxicity, their biological activities were tested with respect to cancer chemoprevention. Cancer chemoprevention is considered to play an important role in decreasing the risk of cancer development, involving prevention, delay, or reversal of the process of carcinogenesis.[13] In particular, one important cancer prevention strategy is to enhance the deactivation of radicals and electrophiles via phase II enzymes.[14] Quinone reductase (QR), one of the representative phase II detoxification enzymes, is known to function as a cancer chemopreventative.[15] A QR induction assay was conducted for evaluating the bioactivities of **1**–**5** in the cultured Hepa-1c1c7 murine hepatoma cell line. In this assay, 0.1% dimethyl sulfoxide (DMSO) was used as a control and β-naphthoflavone (2 μM) was used as a positive control. As shown in Figure 6, mohangic

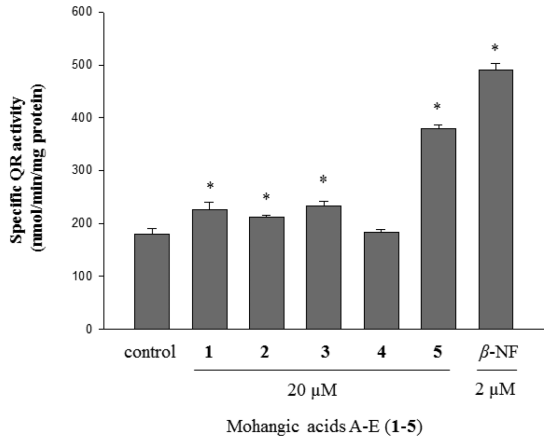

Figure 6. Effect of **1**–**5** on the induction of quinone reductase in murine Hepa1c1c7 cells. Cells were grown for 24 h, then exposed to **1**–**5** for 24 h. Quinone reductase activities were measured in cell lysates by reduction of a tetrazolium dye and expressed as nmol/min/mg protein. Values represent the mean ± SD of four determinations. *P < 0.001 indicates statistically significant differences from the control group.

acid E (**5**) at a concentration of 20 μM displayed a significant 2.1-fold increase in QR induction activity compared with the control. However, mohangic acids A–D (**1**–**4**) exhibited no remarkable activities. Mohangic acid E (**5**) induced QR activity in a concentration-dependent manner without cytotoxicity and with QR activity enhancements of 1.3-, 1.5-, 1.8-, 2.1-, and 2.2-fold at 2, 5, 10, 20, and 30 μM of **5**, respectively (Figure 7). These results suggest that the glucose moiety in **5** is particularly important for the QR activity in this class of compounds.

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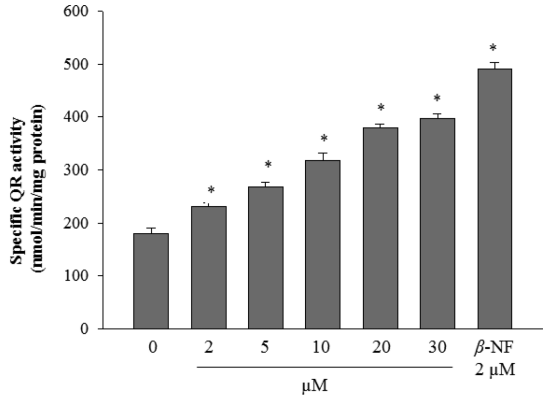

Figure 7. Effect of **5** on the induction of quinone reductase in murine Hepa1c1c7 cells. Cells were grown for 24 h, then exposed to **5** for 24 h. Quinone reductase activities were measured in cell lysates by reduction of a tetrazolium dye and expressed as nmol/min/mg protein. Values represent the mean ± SD of four determinations. *P < 0.001 indicates statistically significant differences from the control group.

Mohangic acids A–E commonly bear a *para*-substituted aromatic ring with an amine and an alkyl chain. The structural feature of the mohangic acids is related to (2*E*)-11-(4′-aminophenyl)-5,9-dihydroxy-4,6,8-trimethyl-11-oxoundec-2-enoic acid[16] and (5*E*)-7-(4′-aminophenyl)-2,4-dimethyl-7-oxohept-5-enoic acid,[17] both of which were independently isolated from mangrove-derived *Streptomyces griseus* strains. However, these known compounds have different chain lengths and do not incorporate the triene moiety in the mohangic acids. In addition, modification at the amine group of **2**–**5** by substitution with a methyl, acetyl, or glycosyl group has not previously been reported in this class. An antifungal metabolite candicidin D, discovered from *S. griseus*, incorporates an aminoacetophenonic acid fragment identical to the gross structure of mohangic acid A without C-1, C-2, and C-3.[18] However, the overall structure of candicidin D is a heptaene macrolide different from mohangic acids A–E.

Even though relative configurations of some aminoacetophenonic acids including candicidin D were proposed based on NMR analysis, their absolute configurations have not been determined.[16−18] It is possibly a consequence of the structural instability of the aminoacetophenonic group during chemical derivatizations. Our extensive spectroscopic analysis using bidentate chiral NMR solvents enabled us to determine the absolute configurations of the mohangic acids by avoiding chemical derivatizations. The biological activities of the compounds belonging to the *p*-aminoacetophenonic acids have not been reported previously.[16,17] Our report of QR activity induced by treatment of mohangic acid E (**5**), which is a glycosylated version of *p*-aminoacetophenonic acid, represents the first biological evaluation of this class of compounds and reveals the biological importance of glycosylation at the amino group in **5**. The discovery of the mohangic acids, along with the results of our previous studies,[4,5,19] highlights that microorganisms inhabiting relatively uninvestigated marine environments, such as an intertidal mudflat, could be a promising source of biologically active new secondary metabolites.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded using a JASCO P-200 polarimeter with a 1 cm cell. UV spectra were obtained on a PerkinElmer Lambda 35 UV/vis spectrophotometer. ECD spectra were acquired on an Applied Photopysics Chirascan-Plus circular dichroism spectrometer. IR spectra were obtained using a Thermo Nicolet iS10 detector. ^{1}H, ^{13}C, and 2D NMR spectra were recorded on Bruker Avance 600 MHz spectrometers at the National Center for Inter-University Research Facilities (NCIRF) at Seoul National University and on a Bruker Avance II 900 MHz NMR spectrometer at the Korea Basic Science Institute at Ochang. NMR spectra were referenced to the residual protonated solvent signals for CD_3OD (δ_H/δ_C, 3.30/49.0) or pyridine-d_5 (δ_H/δ_C, 8.74/150.4). Electrospray ionization (ESI) low-resolution LC/MS data were acquired on an Agilent Technologies 6130 quadrupole mass spectrometer coupled with an Agilent Technologies 1200-series HPLC. High-resolution fast atom bombardment (HR-FAB) mass spectra were obtained using a Jeol JMS-600W high-resolution mass spectrometer at NCIRF. GC analysis was performed on an Agilent 6850-series gas chromatograph using an HP-5 column (cross-linked 5% PHM/Siloxan, Macherey and Nagel) and a flame ionization detector. Instant Ocean sea-salt mix was used to prepare seawater-based media.

Bacterial Isolation. A mud sample was collected from the Mohang mudflat in Buan, Republic of Korea. The sample was dried at room temperature (rt) for 3 h, and the dry sediment (1 g) was diluted in 4 mL of sterilized artificial seawater. The mixture was spread onto actinomycete isolation agar, A4 medium (1 L seawater, 18 g agar, 100 mg/L cycloheximide), A5 medium (750 mL seawater, 250 mL distilled water, 18 g agar, 100 mg/L cycloheximide), and chitin-based agar (1 L seawater, 18 g agar, 5 g chitin powder, 100 mg/L cycloheximide) by stamping, spreading, or both. The single-strain SNM31 was isolated on A5 medium. The strain SNM31 (GenBank accession no. KT921418) was phylogenetically identified as a *Streptomyces* sp. on the basis of the 16S rDNA sequence analysis (most closely related to *Streptomyces griseus*, 99% identity).

Cultivation and Extraction of the Bacterial Strain. The strain SNM31 was cultivated in 50 mL of YEME medium (4 g yeast extract, 10 g malt extract, and 4 g glucose in 1 L artificial seawater) in a 125 mL Erlenmeyer flask. After cultivation for 3 days on a rotary shaker at 160 rpm at 30 °C, 10 mL of the culture was inoculated directly to 1 L of YEME liquid medium in 2.8 L Erlenmeyer flasks (12 each × 1 L, total volume 12 L). After the bacteria were incubated for 4 days, the 12 L culture of the SNM31 strain was extracted with 18 L of EtOAc. The EtOAc layer was separated, and anhydrous sodium sulfate was added to remove residual water. The extraction procedure was repeated four times (total culture volume: 48 L). The extract in EtOAc was concentrated *in vacuo* to yield 4 g of dried material in total.

Isolation of Mohangic Acids (1–5). The organic extract of SNM31 was filtered with a syringe filter and injected directly onto a semipreparative reversed-phase HPLC column (Kromasil C_{18} (2): 250 × 10 mm, 5 μm) with a gradient solvent system (30% MeOH/H_2O to 80% MeOH/H_2O over 60 min, UV 280 nm detection, flow rate: 2 mL/min). Five major peaks at retention times of 44, 45, 48, 50, and 54 min were observed. Each compound was further purified under gradient solvent conditions (10% CH_3CN/H_2O to 100% CH_3CN over 40 min, UV 280 nm detection, flow rate: 2 mL/min) using a reversed-phase C_{18} HPLC column (Kromasil C_{18} (2): 250 × 10 mm, 5 μm). Mohangic acids A–E (**1**–**5**) (72, 12, 15, 13, and 8 mg) were obtained in pure form at retention times of 24.0, 22.5, 23.5, 25, and 18.5 min, respectively.

*Mohangic acid A (**1**):* yellow gum; $[\alpha]^{25}_D$ −6.9 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 270 (4.46) nm, 325 (4.15) nm; ECD (*c* 4.3 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 218 (−39.5), 257 (−5.5) nm; IR (neat) ν_{max} 3392, 2958, 1672, 1588, 1541 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HRFABMS *m*/*z* 482.2517 [M + Na]$^+$ (calcd for $C_{26}H_{37}NO_6Na$, 482.2519).

*Mohangic acid B (**2**):* yellow gum; $[\alpha]^{25}_D$ −16.7 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 270 (4.57) nm, 325 (4.26) nm; ECD (*c* 4.2

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× 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 218 (−39.5) nm; IR (neat) ν_{max} 3410, 2964, 1602, 1546, 1395 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRFABMS *m/z* 496.2681 $[M + Na]^+$ (calcd for $C_{27}H_{39}NO_6Na$, 496.2675).

*Mohangic acid C (**3**):* yellow gum; $[\alpha]^{25}_D$ −7.1 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 270 (4.63) nm; 325 (4.32) nm; ECD (*c* 4.0 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 218 (−39.1), 269 (−7.5), 275 (−6.2), 282 (−9.2) nm; IR (neat) ν_{max} 3402, 2967, 1675, 1593, 1536 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRFABMS *m/z* 524.2629 $[M + Na]^+$ (calcd for $C_{28}H_{39}NO_7Na$, 524.2624).

*Mohangic acid D (**4**):* yellow gum; $[\alpha]^{25}_D$ −11.7 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 270 (4.46) nm; ECD (*c* 4.0 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 217 (−39.9), 259 (−4.4), 268 (−5.5), 280 (3.7) nm; IR (neat) ν_{max} 3452, 2958, 1737, 1598, 1366 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRFABMS *m/z* 526.2784 $[M + Na]^+$ (calcd for $C_{28}H_{41}NO_7Na$, 526.2781).

*Mohangic acid E (**5**):* yellow gum; $[\alpha]^{25}_D$ −25.7 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 270 (4.19) nm, 325 (3.91) nm; ECD (*c* 3.2 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 218 (−41.3), 292 (−3.5) nm; IR (neat) ν_{max} 3368, 2968, 1737, 1599, 1373 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRFABMS *m/z* 644.3052 $[M + Na]^+$ (calcd for $C_{32}H_{47}NO_{11}Na$, 644.3047).

Hydrogenation of Mohangic Acid D. Mohangic acid D (**4**) (4 mg) was lyophilized for 24 h and subsequently suspended, along with a 10% Pd/C catalyst, in absolute EtOH (1.5 mL). The mixture solution of **4** was stirred for 2 h under 1 atm of H_2 at rt. The reaction mixture in EtOH was filtered using a PTFE syringe filter and evaporated *in vacuo* to yield the hydrogenation product (**6**) of **4**. The product was isolated in pure form by reversed-phase HPLC (Phenomenex Luna 5 μm C_{18} (2) 250 × 10.0 mm, flow rate 2 mL/min, UV 254 nm detection) using a gradient solvent system (10% to 100% CH_3CN/H_2O over 40 min). The hydrogenated product (**6**) was obtained at a retention time of 27 min (3 mg, yield: 75%) under HPLC conditions. The molecular formula of product **6** was confirmed as $C_{28}H_{47}NO_7$ on the basis of ESIMS data ($[M + H]^+$ *m/z* at 510).

*Hydrogenation product **6**:* ^1H NMR (600 MHz, CD_3OD) δ_H 7.49 (d, *J* = 8.0 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 4.58 (m, 1H), 3.95 (m, 1H), 3.75 (m, 1H), 3.04 (dd, *J* = 7.5 Hz, 4.5 Hz, 1H), 2.42 (dd, *J* = 15.5 Hz, 5.5 Hz, 1H), 2.34 (dd, *J* = 15.5 Hz, 5.5 Hz, 1H), 2.10 (s, 3H), 1.75−1.72 (m, 2H), 1.66−1.62 (m, 3H), 1.56 (m, 1H), 1.47−1.42 (m, 4H), 1.38−1.30 (m, 5H), 1.24−1.20 (m, 2H), 1.12−1.05 (m, 2H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.84 (d, *J* = 6.5 Hz, 3H), 0.82 (d, *J* = 6.5 Hz, 3H).

Preparation of Acetonide Derivative of 6 (7). The hydrogenation product of **4** (**6**) (3 mg) was prepared in a 40 mL vial and dried under high vacuum for 24 h. After adding pyridinium *p*-toluenesulfonate (1 mg), the mixture was dissolved in anhydrous MeOH (200 μL) and anhydrous CH_2Cl_2 (2 mL) at rt. Then, 2,2-dimethoxypropane (4 mL) was added to the reaction vial. The solution was stirred at rt under argon for 48 h. To quench the reaction, a saturated $NaHCO_3$ aqueous solution was added. The reaction mixture was fractionated by flash column reversed-phase chromatography using a gradient solvent system of $MeOH/H_2O$ (20%, 40%, 60%, 80%, and 100%). The acetonide derivative (**7**) eluted in the 80% and 100% $MeOH/H_2O$ fractions. The final product (**7**) was purified by reversed-phase HPLC (Phenomenex Luna 5 μm C_{18} (2) 250 × 10.0 mm, flow rate 2 mL/min, UV 254 nm detection) with a gradient solvent system (10% to 100% $MeOH/H_2O$) for 40 min. The acetonide product (**7**) eluted at a retention time of 35 min (2.5 mg, yield: 83%) under the HPLC conditions. The molecular formula of **7** was confirmed as $C_{31}H_{51}NO_7$ by ESIMS analysis ($[M + H]^+$ *m/z* at 550).

*Acetonide product (**7**):* ^1H NMR (600 MHz, CD_3OD) δ_H 7.52 (d, *J* = 8.0 Hz, 2H), 7.32 (d, *J* = 8.0 Hz, 2H), 3.95 (dd, *J* = 10.0 Hz, 6.0 Hz, 1H), 3.92 (m, 1H), 3.74 (m, 1H), 3.06 (dd, *J* = 7.0 Hz, 4.0 Hz, 1H), 2.38 (dd, *J* = 15.0 Hz, 5.0 Hz, 1H), 2.30 (dd, *J* = 15.0 Hz, 8.5 Hz, 1H), 2.12 (s, 3H), 1.98 (m, 1H), 1.87 (m, 1H), 1.77 (m, 1H), 1.69−1.67 (m, 2H), 1.57−1.55 (m, 2H), 1.47−1.42 (m, 4H), 1.39 (s, 6H), 1.38−1.30 (m, 5H), 1.22 (m, 1H), 1.12−1.06 (m, 2H), 0.89 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.84 (d, *J* = 6.5 Hz, 3H).

Analysis of the Absolute Configuration of the Glucose in Mohangic Acid E (5). Mohangic acid E (3 mg) was dissolved in 3 N HCl (0.5 mL) and stirred at 80 °C for 2 h. After the solution cooled to rt, the HCl was evaporated *in vacuo* to yield the hydrolysate. HMDS and TMSCl (50 μL, v/v = 2:1) were added to the hydrolysate with pyridine (0.5 mL). After being stirred at 60 °C for 30 min, the mixture was dried *in vacuo* and separated using H_2O and CH_2Cl_2 (1 mL, v/v = 1:1). The CH_2Cl_2 layer was injected into a GC equipped with an HP5 column (0.32 mm × 30 m). The temperature of the injector and the detector in the GC was maintained at 200 °C. During analysis, the temperature of the GC column was controlled (60 °C for 3 min, 60−200 °C at 4 °C/min, and 200 °C for 3 min). The glucose derivative from the hydrolysate of **5** was detected at a retention time of 32.65 min. The authentic D-glucose and L-glucose samples were treated and analyzed using the same procedures. The derivatives of D-glucose and L-glucose were detected at 32.67 and 32.85 min, respectively. Co-injection of the silylated derivative of the hydrolysate and authentic D-glucose gave a single peak at 32.64 min, thereby determining the absolute configuration of the glucose in **5** as the D-form.

Synthesis of Bidentate Chiral NMR Solvents. *N*-Bromosuccinimide (2.2 mmol) was added to a mixture of 2-methyl-1,3-propanediol (1.0 mmol) and triphenylphosphine (2.2 mmol) in CH_2Cl_2 (10.0 mL) at 0 °C. The reaction mixture was stirred at rt for 30 min. The solvent was removed, and the dibromopropane was purified by column chromatography (silica gel, 40−63 μm, pentane). With the acquired dibromopropane, bidentate chiral solvents (*R,R*)- and (*S,S*)-bis-α-methylbenzylamine-*p*-Me were synthesized according to previously reported procedures.[11,20]

Quinone Reductase Assay. QR activities were determined spectrophotometrically according to a modified microtiter method.[21] Hepa-1c1c7 cells were plated at a density of 20 000 cells/mL and cultured for 24 h in a humidified incubator containing 5% CO_2 at 37 °C. The cells were then exposed to mohangic acids (**1**−**5**) and incubated for an additional 24 h. The media was subsequently decanted, and the cells in each well were lysed by incubation at 37 °C for 10 min with 250 μL of a solution containing 10 mM Tris-HCl pH 8.0, 140 mM NaCl, 15 mM $MgCl_2$, and 0.5% NP-40 (IGEPAL CA-630) (Sigma-Aldrich). The plates were then agitated on an orbital shaker for an additional 10 min at 25 °C, after which 1 mL of the complete reaction mixture containing 12.5 mM Tris-HCl pH 7.4, 0.67 mg/mL bovine serum albumin, 0.01% Tween-20, 50 μM flavin adenine dinucleotide, 1 mM glucose-6-phosphate, 2 U/mL glucose-6-phosphate dehydrogenase, 30 μM NADP, 50 μg/mL 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 50 μM menadione was added. A blue color developed, and the reaction was stopped after 10 min. The rate of the NADPH-dependent, menadiol-mediated reduction of MTT was measured at 610 nm. Protein was detected by crystal violet staining of an identical set of test plates. The specific activity (SA) of quinone reductase (nmol/min/mg of protein) was calculated from the equation

$$SA = [MTT_{abs}/CV_{abs}] \times 3345$$

where MTT_{abs} is the change in the absorbance of MTT per min, CV_{abs} is the absorbance of crystal violet, and 3345 is the ratio of the proportionality constant determined for crystal violet and the extinction coefficient of MTT and has units of nmol/mg.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00956.

1D and 2D NMR spectra for **1**−**5**, homodecoupling NMR experiment data of **1**, ^1H NMR spectrum of **6**, ^1H and HSQC NMR spectra of **7**, ^{13}C NMR spectra for **1** in a 5:2 mixture of (*R,R*)-, (*S,S*)-BMBA and pyridine-d_5, and detailed quinone reductase assay (PDF)

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Notes

The authors declare no competing financial interest.

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