Maklamicin, an Antibacterial Polyketide from an Endophytic *Micromonospora* sp.

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ABSTRACT: A new spirotetronate-class polyketide, maklamicin (1), was isolated from the culture extract of an endophytic actinomycete of the genus *Micromonospora*. The structure and relative configuration of 1 were elucidated by interpretation of NMR and other spectroscopic data, and the absolute configuration was determined using the modified Mosher method. Maklamicin (1) showed strong to modest antimicrobial activity against Gram-positive bacteria.

RESULTS AND DISCUSSION

Actinomycetes are Gram-positive bacteria, which have served as a significant source for drug discovery for more than five decades and have yielded more than 10,000 bioactive compounds. Of these, approximately 75% are produced by *Streptomyces* species, the dominant genus in terrestrial environments such as soil and leaf litter, while 25% are the products of non-*Streptomyces* species traditionally termed “rare actinomycetes.”1 Within this group, *Micromonospora* is the most prolific in producing metabolites, accounting for more than 700 compounds to date including pharmaceutically important chemical classes such as calicheamicin and gentamicin.1 Wide distribution of this genus in various aquatic and terrestrial environments has been demonstrated, but its association with plants is not fully recognized.2 Recently, members of this genus were recovered from nitrogen-fixing root nodules of diverse legumes in significant numbers.3 In addition, some of these endophytic actinomycetes were suggested to have nitrogen fixation ability, and they were speculated to promote the growth of host plants.3d,4 Although evidence is still lacking, their phytoprotective properties through antibiotic production is also possible.5 Intrigued by their potential as a source of medicinally useful molecules, we examined the metabolites of *Micromonospora lupini* isolated from a root nodule of the legume *Lupinus angustifolius* and found new anthraquinones possessing anti-invasive activity.6 As part of our continuing chemical investigation on plant-associated actinomycetes,7 we now report on the isolation of a new tetronate-class polyketide, maklamicin (1), from the culture extract of *Micromonospora* sp. GMKU326 isolated from the root of a leguminous plant, Maklam pheuak (*Abrus pulchellus* Wall. Ex Thwaites subsp. *pulchellus*), collected in Thailand.

Received: October 11, 2010  
Published: March 09, 2011

attributable to three oxygen-bearing quaternary sp² carbons, seven sp² carbons (five are proton-bearing), three quaternary sp³ carbons (one is oxygen-bearing), seven sp³ methylenes (one is oxygen-bearing), seven sp¹ methines (one is oxygen-bearing), and five methyl groups. These data accounted for all of the observed NMR resonances except for three exchangeable protons. The 13C NMR spectrum of 1 showed distinctive resonances of sp² quaternary carbons at δ 204.3, 201.9, 167.0, and 107.2, characteristic of tetrionic acid carbons, although its UV absorption maxima λmax at 246 and 291 nm were obviously different from those of the known compounds. Interpretation of HMBC NMR data allowed the assignment of the remaining 1H and long-range 1H/13C coupling constants. An NOE between H-5 and H-6 and between H-16 and H-17b and between H-14 and H-15 was observed in the NOESY spectrum.
and determined on the basis of a small coupling constant between H-22\(\beta\) and H-21 (Figure 5). These data allowed assignment of the absolute configuration at C-31 (Figure 3a). Within this side chain, small \(J_{\text{CH}}\) coupling constants for C-32/H-30a and C-32/H-30b indicated a gauche relationship of H-32 methyl to H-30a and H-30b, and a large \(J_{\text{CH}}\) value for C-31/H-30a and a small \(J_{\text{CH}}\) value for C-31/H-30b indicated an anti relationship of the 31-OH group to H-30b, thereby establishing the configuration at C-31 (Figure 3b).\(^{10}\) Finally, the configuration at the spirocarbon C-23 was determined on the basis of a small coupling constant between H-22\(\beta\) and C-23 (\(J_{\text{CH}} < 3\) Hz) and a large coupling constant between H-22\(\alpha\) and C-23 (\(J_{\text{CH}} = 4.8\) Hz) that allowed an anti orientation of H-22\(\beta\) to the oxygen atom at C-23 for the C-22–C-23 bond (Figure 4).

The absolute stereochemistry of I was determined by applying the modified Mosher method\(^{11}\) to the secondary hydroxyl group at C-31. Prior to the esterification, the enolic hydroxy group at C-24 was protected as a methyl ether by treating I with TMSC\(\text{HCH}_2\)Cl/MerOH. The methylated derivative 2 was then treated with \((S)\)- and \((R)\)-MTPA chloride, yielding the bis-\((R)\)- and \((S)\)-MTPA esters (3 and 4), respectively. In the \(^1\)H NMR spectra of 3 and 4, positive \(\Delta\delta_{S,2-R}\) values were observed for the protons for H-32, while negative \(\Delta\delta_{S,2-R}\) values were observed for H-30, H-21, H-22, H-29, H-19, H-18, and H-17 (Figure 5). These data allowed assignment of the absolute configuration of C-31 as R. The absolute configurations of the polycyclic stereogenic centers of I were identical with those of the previously reported spirotetrone antibiotics.\(^{8,9}\)

Maklamicin (1) is a new member of spirotetrone acids comprising a trans-decalin unit and a tetronic acid moiety spiro-linked with a cyclohexene ring. Although over 50 related spirotetrones have been reported from actinomycetes, two structural features differentiate this new compound from others.\(^{12}\) First, the presence of a stereogenic center (C-31) on the substituent of the cyclohexene unit has not been reported.

**EXPERIMENTAL SECTION**

**General Experimental Procedures.** Optical rotations were measured using a JASCO DIP-3000 polarimeter. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. IR spectra were measured on a Perkin-Elmer Spectrum 100. NMR spectra were obtained on a Bruker AVANCE 400 or a Bruker AVANCE 500 spectrometer and referenced to residual solvent signals (\(\delta_H = 7.26, \delta_C = 77.0\)). The mass spectra were measured using a JASCO DIP-3000 polarimeter. UV spectra were recorded on a JASCO UV-2500 spectrophotometer.

**Microorganism.** Strain GMKU326 was isolated from a root of Maklam phueak (Abrus pulchellus Wall. Ex Thwaites subsp. pulchellus) collected at Eastern Botanical Garden (Kha Hin Son), Chachoengsao Province, Thailand, according to the reported protocol.\(^{13}\) The strain was identified as a member of the genus Micromonospora on the basis of...
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99.3% 16S rRNA gene sequence identity (1368 nucleotides; GenBank accession number GU45831) with the Micromonospora aurantia strain (accession number AB159779).

**Fermentation.** Strain GMKU326 cultured on a slant agar medium consisting of soluble starch 0.5%, glucose 0.5%, agar 1.5% was inoculated into 100 mL of the V-22 seed medium consisting of soluble starch 1%, glucose 0.5%, and agar 1.5%. The mycelium was harvested after 5 days of cultivation. Evaporation of the mycelium gave 10 g of extract from 5 L of medium.

**Extraction.** At the end of the fermentation period, 100 mL of 1-butanol was added to each flask, and they were allowed to shake for one hour. The mixture was centrifuged at 6000 rpm for 10 min, and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the solvent gave 7.5 g of extract from 5 L of medium.

**Antimicrobial assays.** Antimicrobial assays were carried out using Escherichia coli NIH-JC2, Micrococcus luteus ATCC9343, Bacillus subtilis PC1219, Bacillus cereus NBRC15305, Staphylococcus aureus IFO12732, Enterococcus faecalis NBRC100480, and Candida albicans IFO1594. Mueller Hinton broth (DIFCO Laboratories) was used for bacteria, and Yeast Nitrogen Base (DIFCO Laboratories) supplemented with 2% glucose was used for C. albicans. Test microorganisms were inoculated into a 32 mL test tube containing 8 mL of the liquid medium. After incubation on a reciprocal shaker for 20 h at 30 °C, the cultures were centrifuged (3000 rpm, 5 min), and the cell suspension (1 x 10^9 cells/mL) was prepared in saline. Then, the liquid medium (1.5 mL, the cell suspension (15 mL), and the sample solution in each well, and the plates were placed in the incubator 37 °C, the absorbance at 650 nm was measured using a microplate reader. MIC values of the standard antibiotic tetracycline hydrochloride (Sigma-Aldrich Co.) against the above-described bacteria E. coli, M. luteus, B. subtilis, B. cereus, S. aureus, and E. faecalis were 0.8, 0.1, 0.05, 1.5, 0.05, and 0.2 μg/mL, respectively.

Cytotoxic assay was carried out using HeLa human cervical cancer cells and MCF7 human breast cancer cells. Cancer cells were suspended in RPMI medium containing 10% FBS (Sigma-Aldrich Co.) and 2 mM L-glutamine and seeded into the wells of a 96-well culture plate (1 x 10^4 cells/50 μL/well). Then, test compounds at various concentrations in RPMI medium (0.8-92.2 μM/L) were added to the wells. After incubation for 48 h in a humidified 5% CO2 incubator at 37 °C, MTT (0.25 mg, Sigma-Aldrich Co.) in PBS (50 μL) was added to each well, and the plates were placed in the incubator 37 °C for 4 h. Medium in the wells was removed by suction, and DMSO (100 μL) was added to each well. After 10 min, the absorbance at 570 nm was read by a

**NMR data, see Table 1; HRESITOFMS [M + Na]^+ 547.3034 (calc for C_{53}H_{50}F_{6}O_{10}Na 993.3983).**

**Bi(3)-MTPA Ester of 2 (3).** In the same manner as described for 2 (5.0 mg, 0.0093 mmol) was reacted with (R)-MTPA chloride to give 4 (2.6 mg, 33.2 min, 29% yield): colorless, amorphous solid; H NMR (500 MHz, CDCl3) δ 2.01 (3H, s, H-28), 1.73 (1H, d, J = 15.0 Hz, H-22β), 1.76 (1H, m, H-30a), 1.82 (1H, d, J = 14.5 Hz, H-17α), 1.85 (1H, m, H-32), 2.07 (1H, m, H-30b), 2.08 (1H, d, J = 15.0, 6.5 Hz, H-22α), 2.24 (1H, m, H-21), 2.35 (1H, dd, J = 12.5, 11.5 Hz, H-17β), 4.67 (1H, d, J = 12.5 Hz, H-29α), 4.75 (1H, d, J = 12.5 Hz, H-29β), 5.19 (1H, dd, J = 9.9, 6.0, 2.1 Hz, H-31), 5.33 (1H, s, H-19); HRESITOFMS m/z 993.3989 [M + Na]^+ (calc for C_{53}H_{50}F_{6}O_{10}Na 993.3983).**

**Bi(5)-MTPA Ester of 2 (4).** To a solution of 1 (5.0 mg, 0.0095 mmol) in CHCl3/MeOH (0.25 mL each) was added a solution of TMSCHN3 in Et2O (2.0 M, 0.25 mL, 0.50 mmol) at room temperature. After stirring for 15 min, the reaction mixture was concentrated to dryness. The residue was purified on a silica gel column (hexane/EtOAc, 20:1–1:1) to give 2 (3.2 mg, 62% yield) as a colorless, amorphous solid: [α]_D^25 −110 (c 0.50, CHCl); UV (MeOH) λ max (log ε) 246 (4.06), 291 (3.90) nm; [α]_D^25 −104 (c 1.01, H2O); 13C-NMR data, see Table 1; HRESITOFMS [M + Na]^+ 547.3034 (calc for C_{53}H_{50}F_{6}O_{10}Na 993.3983).**

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**Bi(5)-MTPA Ester of 2 (4).** To a solution of 1 (5.0 mg, 0.0095 mmol) in CHCl3/MeOH (0.25 mL each) was added a solution of TMSCHN3 in Et2O (2.0 M, 0.25 mL, 0.50 mmol) at room temperature. After stirring for 15 min, the reaction mixture was concentrated to dryness. The residue was purified on a silica gel column (hexane/EtOAc, 20:1–1:1) to give 2 (3.2 mg, 62% yield) as a colorless, amorphous solid: [α]_D^25 −110 (c 0.50, CHCl); UV (MeOH) λ max (log ε) 246 (4.06), 291 (3.90) nm; [α]_D^25 −104 (c 1.01, H2O); 13C-NMR data, see Table 1; HRESITOFMS [M + Na]^+ 547.3034 (calc for C_{53}H_{50}F_{6}O_{10}Na 993.3983).**
microplate reader. Staurosporin (Wako Pure Chemical Industries, Ltd.) was used as a positive control. Its IC_{50} values against HeLa and MCF-7 cell lines were 4 pM and 50 nM, respectively.

**ASSOCIATED CONTENT**

5 Supporting Information. 1D and 2D NMR spectra of maklamicin (1) and ^1H NMR spectra of 2–4. This material is available free of charge via the Internet at http://pubs.acs.org.

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**ACKNOWLEDGMENT**

We acknowledge Dr. T. Okuda and Ms. Y. Sudoh at Tama-gawa University and Dr. F. Fujimori at Tokyo Kasei University for assistance with the antimicrobial assay and cytotoxicity assay, respectively. C.I. was awarded a Ph.D. scholarship from the Thailand Research Fund, CHE, and Ministry of Education, Culture, Sports, Science and Technology, Japan.

**REFERENCES**