Ubiquinone Q_9 from a marine isolate of an actinobacterium *Nocardia* sp.

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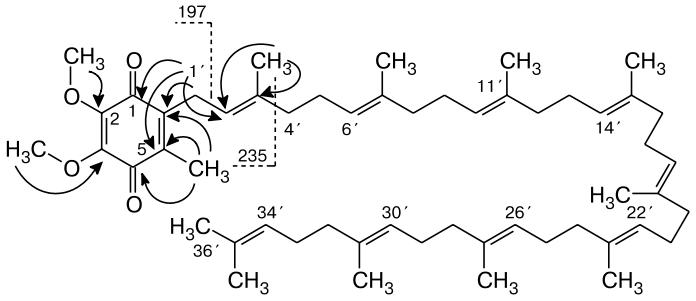
The major component of a nonpolar fraction of an extract of an actinobacterium *Nocardia* sp. KMM 3749 isolated from an unidentified marine ascidian was shown, using NMR spectroscopy and mass spectrometry, to be 2,3-dimethoxy-5-methyl-6-polyprenyl-1,4-benzoquinone (ubiquinone Q_9).

Key words: marine actinobacterium *Nocardia* sp., ubiquinone Q_9.

Recently, marine microorganisms as producers of biologically active compounds have attracted attention of researchers working in various fields.[1—3] When performing screening for biologically active compounds in ethanol—chloroform extracts of marine actinobacteria, we found that the strain KMM 3749, attributed to the *Nocardia* genus judging by some phenotypic signs, synthesized cytotoxic compounds. They inhibited the development of fertilized eggs of sea urchin *Strongylocentrotus intermedius*, the minimum inhibiting concentration (MIC) being 40 μg mL^{-1} , and caused hemolysis of mouse erythrocytes (MIC = 30—50 μg mL^{-1}). Chromatography of the actinobacterium extract on silica gel in gradient systems with increasing polarity (see Experimental) yielded seven fractions. The activity assays showed the benzene-eluted nonpolar fraction to be responsible for the cytotoxicity. This fraction did not exhibit antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus vulgaris*, *Bacillus subtilis*, *Enterococcus faeces*, and *Candida albicans* as test cultures. This fraction was repeatedly purified by adsorption chromatography on silica gel in a 15 : 1 hexane : ethyl acetate system and by HPLC. This gave chromatographically pure compound **1**. The UV spectrum of compound **1** showed an absorption maximum at 276 nm, which is typical of benzoquinones.[4] Analysis of the ^1H and ^{13}C NMR spectra of **1** (DEPT and HMQC experiments) (Table 1) showed the presence of eleven methyl groups at double bonds (δ_C 25.7, 17.8, 16.4, and 16.0 (7C)), one of them occurring in the benzoquinone ring (δ_C 11.9). Other signals identified in the spectra corresponded to seventeen methylene groups, nine methine groups, and fifteen carbon atoms bearing no protons, four of which were bound to oxygen-containing functional groups (two oxo and two methoxy groups) (see Table 1). These data and comparison of the NMR spectra of compound **1** with those for ubiquinones Q_7 [5] and Q_8 [6] confirmed that **1** contains a benzoquinone ring and a polyprenyl chain. The electron impact mass spectrum (EI MS) of compound **1** exhibited fragment peaks with m/z 235 and 197, typical of ubiquinone fragmentation.[4] The molecular mass of **1** (794 Da) found from MALDI mass-spectrometric study indicated that **1** contains nine isoprene units. The positions of substituents in the benzoquinone ring were determined in HMBC and ^1H-^1H COSY experiments. The HMBC spectrum manifested correlations between the



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Note. The arrows show the correlations observed in the HMBC spectrum.

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Table 1. Data from the ^1H and ^{13}C NMR spectra of compound **1** ($CDCl_3$, δ, *J*/Hz)

Atom[a]	δ_C[b]	δ_H
C(1)	184.8 s [c]	
C(2)	144.3 s [d]	
C(3)	144.4 s [d]	
C(4)	183.9 s [c]	
C(5)	138.9 s	
C(6)	141.7 s	
C(1′)	25.3 t	3.18 (d, 2 H, *J* = 7)
C(2′)	118.9 d	4.93 (m, 1 H)
C(3′)	137.7 s	
C(4′), C(8′), C(12′), C(16′), C(20′), C(24′), C(28′), C(32′),	39.8 (t, 8 C)	1.98 (m, 8 CH_2)
C(5′), C(9′), C(3′), C(17′), C(21′), C(25′), C(29′), C(33′)	26.7 (t, 7 C); 26.5 t	2.06 (m, 8 CH_2)
C(6′), C(10′) C(14′), C(18′), C(22′), C(26′), C(30′), C(34′)	124.4, 124.3 (both d, 5 C); 124.2 d; 123.9 d	5.11 (m, 8 H)
C(7′), C(11′), C(15′), C(19′), C(23′), C(27′), C(31′)	135.3, 135.0, 134.9 (all s, 5 C)	
C(35′)	131.3 s	
C(36′)	17.8 q	1.60 (s, 3 H)
CH_3 (C(35′))	25.7 q	1.68 (br.d, 3 H, *J* = 1.1)
CH_3 (C(7′)—C(31′))	16.0 (q, 7 C)	1.60 (s, 7 CH_3)
CH_3 (C(3′))	16.4 q	1.74 (br.d, 3 H, *J* = 1.1)
CH_3 (C(5))	11.9 q	2.01 (s, 3 H)
CH_3O (C(2), C(3))	61.9 (q, 2 C)	4.00 (s, 3 H, 3.98 (s, 3 H)

[a] Atom numbering corresponds to the IUPAC-IUB rules (see *Biochim. Biophys. Acta*, 1965, **107**, 5).
[b] The signal multiplicity was determined in the DEPT and HMQC experiments.
[c,d] The assignment of the signal is ambiguous.

methoxy-group protons and the C(2) and C(3) atoms and between the protons of the methyl group at the C(5) atom and the C(5), C(6), and C(4) atoms, and also coupling of protons at C(1′) with the C(6), C(5), C(2′), and C(1) atoms.

The COSY spectrum exhibited cross-peaks between the protons at C(1′) and the methyl-group protons at C(5) and C(3′). Thus, we identified the major component of the nonpolar fraction of an extract of the actinobacterium *Nocardia* sp as ubiquinone Q_9. This component accounts for 7% of the total chloroform—ethanol extract from bacterial cells.

1,4-Benzoquinones are widely distributed in nature and are mainly isolated from fungi and various tissues of higher plants.[7] They have also been found in the marine bacteria of *Alteromonas*, *Marinomonas*, *Deleya*, *Shewanella*, and *Pseudomonas* genera and used in taxonomic studies. It was shown that alteromonads and marinomonads predominantly contain ubiquinone Q_8. In the bacteria *Deleya* and *Pseudomonas*, ubiquinone Q_9 was detected, and bacteria of the *Shewanella* genus produce mixtures of ubiquinones, menaquinones, and methylmenaquinones.[8] This is the first isolation of ubiquinone Q_9 from actinobacteria. The composition of the ubiquinone fraction from *Nocardia* sp. does not virtually change when the actinobacterium is cultivated on different media.

Experimental

The actinobacterium *Nocardia* sp. KMM 3749 was isolated from an unidentified marine ascidium (23-128) (an expedition of the research ship "Academician Oparin", Simushir Island, Kuril Islands, August 1999).

^1H and ^{13}C NMR spectra were recorded on Bruker DPX-300 (300 and 75.4 MHz) and Bruker DRX-500 (500 and 125 MHz) instruments in $CDCl_3$ using Me_4Si as the internal standard. COSY, HMQC, and HMBC experiments were performed on a Bruker DRX-500 spectrometer using standard Bruker programs. UV spectrum was measured on a Specord M-40 spectrophotometer (Carl Zeiss, Jena, Germany). Electron impact mass spectrum was run on a LKB 9000s mass spectrometer (70 eV), and MALDI mass spectrometry was done on a Bruker Biflex III spectrometer (N_2 laser, 337 nm, accelerating voltage 20 kV). 2,5-Dihydroxybenzoic acid was used as the matrix.

Isolation of compound 1. The fermentation of actinobacteria was carried out in 20 L of a medium containing (g L^{-1}): peptone (5); glucose (1); yeast extract (2); potassium monophosphate (2); sea water (500 mL), and distilled water (500 mL). The fermentation time was 5 days and the temperature was 20—22 °C. The cells were collected by centrifugation and washed twice with distilled water. The biomass was extracted three times with a 2 : 1 chloroform : ethanol mixture. The extract was concentrated to give 200 mg of a dry residue.

The residue was dissolved in 70 mL of a 2 : 1 chloroform : ethanol mixture. The insoluble precipitate was separated and the supernatant was concentrated to dryness. The dry residue (53 mg) was dissolved in chloroform (0.7 mL) and separated on a column with 20 g of silica gel (KSK, Russia). The compounds were eluted with benzene (fraction *1*, 21 mg), benzene : ethyl acetate (5 : 1), benzene : ethyl acetate (1 : 1), ethyl acetate, ethyl acetate : ethanol (9 : 1), ethyl acetate : ethanol (5 : 1), ethyl acetate : ethanol (3 : 1), ethyl acetate : ethanol (1 : 1), and ethanol.

Fraction *1* was rechromatographed on silica gel in a 15 : 1 hexane : ethyl acetate system, 5-mL fractions being collected. According to TLC, compound **1** was contained in fractions *12* and *13* (13 mg). This was subjected to HPLC on a column (250×4 mm) with Silasorb 600 (10 μ) in a 20 : 1 hexane : ethyl

acetate system using a Beckman Altex chromatograph with a Differential Refractometer RIDK-102 detector an an elution rate of 0.7 mL min^{-1}. The retention time of ubiquinone Q_9 (9.75 mg) was 7 min. Three minor components were eluted with retention times of 2.5, 4.2, and 9 min.

Determination of cytotoxic activity using mouse erythrocytes. A suspension of mouse erythrocytes (OD_{720} = 0.75) in a physiological saline was prepared. Saline (50 μL), the erythrocyte suspension (50 μL), and 50, 25, or 10-μL aliquots of the tested solution (concentration 1 mg mL^{-1}) were placed into wells of a plate for enzyme immunoassay. The solution was incubated at 37 °C. Hemolysis was observed directly within 1—120 min.

Determination of cytotoxic activity using fertilized eggs of sea urchin *Strongylocentrotus intermedius* was carried out by a previously described procedure.[9]

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