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A new fused tetracyclic heterocyclic antioxidant from *Serratia* sp. PAMC 25557

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ABSTRACT

A new fused tetracyclic heterocyclic compound, (4bR,10bR)-4b-hydroxy-10b,12-dihydrodibenzo[c,h][2,6]naphthyridine-5,11(4bH,6H)-dione (1), and a known compound, butyl 2-[(benzoyloxy)-methyl]benzoate, spatozoate 2, were isolated from the broth culture of *Serratia* sp. PAMC 25557. The structure of 1 was determined by analyzing spectroscopic data. Compound 1 did not exhibit antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, or *Candida albicans*. In addition, up to 100 μg/ml compound 1 did not show any toxicity against *Artemia salina* larvae. However, compound 1 showed DPPH free radical scavenging activity (IC_{50} = 16.7 ± 0.34 μg/ml). This was the first report of spatozoate isolation from bacterial sources.

1. Introduction

Several species of marine bacteria (Williams, 2008), including *Serratia* sp. (Dwivedi et al., 2008), produce biologically active secondary metabolites. We searched for new biologically active natural products produced by marine bacteria and selected *Serratia* sp. PAMC 25557, because preliminary data suggested that the crude extract had 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. In the present report, we describe a new tetracyclic heterocycle compound, (4bR,10bR)-4b-hydroxy-10b,12-dihydrodibenzo[c,h][2,6]naphthyridine-5,11(4bH,6H)-dione, and one known compound, spatozoate, which was originally isolated from the brown alga *Spatoglossum variabile* (Rahman et al., 1999). This is the first report of spatozoate isolation from bacterial sources.

2. Results and discussion

Compound **1** (2.5 mg) was isolated as a pale yellow solid from the ethyl acetate extract of the broth culture of *Serratia* sp. PAMC 25557. The specific rotation of **1** was $[\alpha]_D^{20}$ +3 (*c* 0.02, ethanol). The IR spectrum of compound **1** showed absorptions at 3333, 3252 (O–H, N–H), 1727, 1687 (C=O), 1621, 1469, 1341, and 1201 cm^{-1} (C=C, Ar). The HRESIMS of **1** showed the molecular ion $(M+Na)^+$ at *m/z* 303.0740, establishing the molecular formula of compound **1** as $C_{16}H_{12}N_2O_3$ with twelve degrees of unsaturation. The number of protons and carbons were supported by the respective NMR data of **1**. Eight aromatic protons, one hydroxyl, and two amide protons were observed. The NMR data of **1** are listed in Table 1.

The structure of **1** was further elucidated by analyzing data obtained from 2D NMR spectroscopy. The analysis of ^1H, ^1H correlation spectroscopy (COSY) data and splitting patterns of aromatic protons clearly indicated that two di-substituted benzene rings were included in the structure. Two carbonyl (C=O) carbons were resonated at 176.7 ppm and 173.9 ppm. Twelve sp2 carbons were observed, suggesting the presence of two benzene rings in the structure. Three exchangeable singlet protons appeared at 6.50 ppm (OH), 10.12 ppm (NH), and 10.27 ppm (NH). Two carbonyl carbons and two di-substituted benzene rings were considered to satisfy the twelve degrees of unsaturation in the molecule. We assigned two fused dihydroquinolinones in such a way that one benzene ring was attached with one heterocycle (Fig. 1).

Connections of these two aromatic rings with the remaining structural features were constructed by analyzing HMBC data (Table 1). Particularly, HMBC data from three exchangeable protons provided key correlations for the construction of the dihydrodibenzonaphthyridinedione skeleton. HMBC data for NH-12 with C-11, C-12a, and C-4a and consideration of the chemical

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; IR, ionizing radiation; ESIMS, electrospray ionization mass spectrometry; 1D, one-dimensional; 2D, two-dimensional; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; HMQC, heteronuclear multiple-quantum correlation; HMBC, heteronuclear multiple-bond correlation; COSY, correlation spectroscopy; NOE, nuclear Overhauser effect; MS, mass spectrometry; BHA, butylated hydroxyanisole; BST, brine shrimp lethality test.

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Table 1
NMR[a] data for compound **1**.

	δc	C-type	δH (mult. J, Hz.)	HMBC (1H-C)	COSY
1	123.44	CH	6.20 (d, 7.4 Hz)	2,12a	6.66, 7.13
2	120.79	CH	6.66 (t, 7.5 Hz)	1,3,4,4a$^{\Delta}$,12a	6.20, 6.75, 7.13
3	129.36	CH	7.13, (t, 7.7 Hz)	1,2,4,4a,12a$^{\Delta}$	6.66, 6.75
4	109.38	CH	6.75 (d, 7.7 Hz)	1$^{\Delta}$,2,3,4a,12a	6.66, 7.13
4a	128.11	Cq	–	–	–
4b	75.40	Cq	–	–	–
5	173.91	C=O	–	–	–
6		NH	10.12, (s)	5, 4b,6a,10a,10b$^{\Delta}$	
6a	143.12	Cq	–	–	–
7	126.18	CH	7.46, (d, 7.5 Hz)	6a,9	6.99
8	120.87	CH	6.99 (t, 7.5 Hz)	6a,7,9,10,10a$^{\Delta}$	7.25, 7.46, 6.75
9	128.20	CH	7.25, (t, 7.7 Hz)	6a$^{\Delta}$,7,8,10,10a	6.76, 6.99
10	108.83	CH	6.76 (d, 7.7 Hz)	6a, 8,10a	7.25, 6.99
10a	125.61	Cq	–	–	–
10b	51.23	CH	4.0, (s)	4a,4b,6a,10,10a,11	
11	176.74	C=O	–	–	–
12		NH	10.27, (s)	4a,4b$^{\Delta}$,11,12a,	
12a	142.51	Cq	–	–	–
13		OH	6.50, (s)	4a,4b,5,10b	

[a] NMR data were recorded in DMSO-d6, ^{1}H NMR was recorded in 300 MHz, ^{13}C NMR were recorded in 125 MHz, $^{\Delta}$4-bond HMBC correlation

shift for C-12a allowed linkage of C-12a and C-11 via a nitrogen atom. The signal corresponding to the proton of the hydroxyl group at C-4b showed heteronuclear correlations with C-4a, C-4b, C-5, and C-10. Based on these correlations, the quaternary carbon C-4b was connected to C-4a, C-5, and C-10. Next, heteronuclear correlations of 6-NH with C-4b, C-5, C-6a, and C-10a placed this NH group between C-5 and C-6a. Finally, HMBC data for H-10b with C-10, C-10a, and C-11 allowed completion of the planar structure for **1** as shown. NOE correlation of H-10b with OH-4b suggested that these protons are located on the same side of the ring system. Therefore, the ring junction at C-4b and C-10b appeared to be *cis*.

Compound **2** was isolated as an oily compound (1.5 mg). Analysis of the ^{1}H NMR data of **2** indicated the presence of nine aromatic protons, one methyl, and four methylene protons. Signals in the aromatic region indicated the presence of two benzene rings. One benzene ring was disubstituted, and the other was mono-substituted. High-resolution (HR)-ESIMS established the molecular formula of **2** as $C_{19}H_{20}O_4$ with *m/z* 335.1254 $[M+Na]^+$ and ten degrees of unsaturation. Twelve sp2 carbons, two carbonyl carbons (C=O), four methylene carbons, and one methyl carbon were observed. Mass spectrometry (MS) and NMR data were comparable with those of spatozoate, which was originally isolated from the brown alga *Spatoglossum variable* (Rahman et al., 1999).

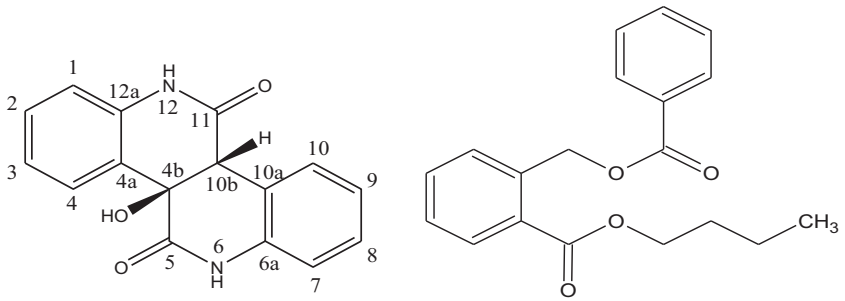
Compound **1** showed moderate antioxidant activity in terms of DPPH free radical scavenging activity with an IC_{50} of 16.7 ± 0.34 μg/ml. In comparison, the IC_{50} for butylated hydroxyanisole (BHA), which is a commercial synthetic standard compound, was 4.98 ± 0.9 μg/ml. Compound **1** did not show antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, or *Candida albicans* at concentrations as high as 100 μg/disk. In addition, compound **1** did not show any toxic effects against *Artemia salina* larvae at concentrations up to 100 μg/ml.

3. Materials and methods

Optical rotation was measured with a polarimeter (Autopol III, Rudolph, USA). Ionizing radiation (IR) spectra were recorded with FT-IR Nicolet 6700 (Thermo, USA). Electrospray ionization mass spectrometry (ESIMS) data were obtained with a Mariner ESI-MS instrument (Perseptive Biosystem, USA). One-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectra were recorded in dimethyl sulfoxide (DMSO)-d_6 with a JEOL JNM ECP-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Chemical shifts were recorded relative to tetramethylsilane ($\delta_H/\delta_{C=O}$). Heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple-bond correlation (HMBC) experiments were optimized for $^{1}J_{CH}$ = 140 Hz and $^{n}J_{CH}$ = 8 Hz, respectively. Flash-column chromatography was performed with silica gel (MERCK, Germany, particle size 60–100 μm). Size-exclusion chromatography was performed with Sephadex LH-20 (Sigma).

3.1. Serratia *sp. PAMC 25557*

The bacterial species included in this study was isolated from the Antarctic sea near the Korean Antarctic Research Station site on



1 **2**

Fig. 1. Structures of compounds **1** and **2**.

King George Island (61°50′–62°15′S and 57°30′–59°01′W), Antarctica. The bacterial species was identified by analyzing 16S rDNA sequencing data obtained by Dr. Soon Gyu Hong and was deposited in the KOPRI culture collection with the name PAMC 25557. The strain was assigned to the genus *Serratia* and the family Enterobacteriaceae.

3.2. Fermentation, extraction, and purification of compounds

Approximately 10 L of *Serratia* sp. PAMC 25557 broth culture was grown for eight days in M2 broth in sea water (10 g/L maltose, 4 g/L yeast extract, 4 g/L glucose, pH 7.8) at 27 °C. The culture broth was filtered through a celite filter. The aqueous fractions were extracted with an Amberlite XAD-16 (Sigma) column (open type), and the adsorbed organic extract was eluted with methanol (10 L). The methanol extract was concentrated with a vacuum at 40 °C, and the water residue was re-extracted with ethyl acetate. The celite filter bed was extracted with acetone. After the acetone evaporated, the aqueous residue was extracted with ethyl acetate. All of the ethyl acetate extracts were combined and dried, yielding a 2.0-g product, which was purified by silica gel column chromatography, Sephadex LH-20, and preparative thin layer chromatography.

3.3. Biological activity

Antibacterial activity was tested against human pathogenic strains, *S. aureus*, *E. coli*, and *C. albicans*. Antimicrobial activity was evaluated with a paper disk assay as previously described (Paudel et al., 2010). The antioxidant activity of compound **1** was evaluated by measuring the DPPH free radical scavenging capacity as described previously (Blois, 1958; Paudel et al., 2011). The toxicity of compound **1** was determined by the brine shrimp lethality test (BST) as described previously (Meyer et al., 1982) with slight modification. (A detailed description is provided in the supplementary section).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2013.07.006.

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