HETEROCYCLES, Vol. 94, No. 9, 2017, pp. 1728 - 1735. © 2017 The Japan Institute of Heterocyclic Chemistry
Received, 31st March, 2017, Accepted, 23rd June, 2017, Published online, 6th July, 2017
DOI: 10.3987/COM-17-13704

A CYTOTOXIC PYRANONAPHTHOQUINONE FROM CULTURED LICHEN MYCOBIONTS OF *HAEMATOMMA* sp.

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Abstract – Spore-derived mycobionts of the lichen *Haematomma* sp. were cultivated on malt-yeast extract medium supplemented with 10% sucrose to produce a new pyranonaphthoquinone, **4**, as well as two known compounds (-)-usnic acid (**2**) and 5-deoxy-7-methylbostrycoidin (**3**). The structure of **4** was determined by spectroscopic methods. The inhibitory activities of **3**, **4**, and **6**–**8** against mammalian DNA polymerases (pols) and cytotoxic effects against a HeLa human cervical cancer cell line were also evaluated. Compound **4** showed the strongest mammalian pol inhibitory activity and cytotoxicity (ID_{50} = 6.7 μM) among the tested compounds.

Isolation and cultivation of mycobionts from the spores of lichen thalli could provide new sources of biologically active compounds. Lichens, which are symbiotic organisms of fungi (mycobiont) and algae (photobiont), are well known to produce characteristic secondary metabolites such as depsides, depsidones, dibenzofurans, and γ-lactones, so-called lichen substances. Some lichen substances have been found to exhibit a wide range of potentially useful biological activities.[1-5] Some of these secondary metabolites from lichen thalli are also produced by isolated cultured mycobionts of lichens without the participation of their photobionts.[6] Furthermore, cultured lichen mycobionts have the ability under osmotically stressed conditions to produce unusual substances that have never been detected in the lichenized state,[6-11] and some of the metabolites showed significant biological activities.[11,12] Based on our interests in the biological activities of the metabolites from cultured lichen mycobionts, we re-investigated the metabolites of *Haematomma* sp.

Many species of the lichen genus *Haematomma* produce red pigments, anthraquinones, such as haematommone (**1**) from *H. puniceum*.[13] The cultured mycobionts of *Haematomma* spp. could also produce haematommone (**1**) along with some other lichen substances.[14] On the other hand, a 2-aza-anthraquinone, 5-deoxy-7-methylbostrycoidin was isolated from our strain of cultured mycobionts of *Haematomma* sp.[15] We re-investigated the metabolites of the same strain of cultured mycobionts to isolate a new pyranonaphthoquinone. We report here the characterization of this novel compound and the biological activities of a 2-aza-anthraquinone, a pyranonaphthoquinone, and three metabolites isolated from the cultured mycobionts of *Trypethelium* sp.[10]

The previously established strain of mycobiont from a *Haematomma* sp. was cultured on conventional malt-yeast extract medium supplemented with 10% sucrose at 18 °C in the dark. After cultivation for 2 years, the colonies were harvested and extracted with Et_2O, with acetone and with MeOH. The extracts were separated by a combination of preparative TLC to afford three aromatic compounds **2**–**4**.

Compounds **2** and **3** were identified as (-)-usnic acid[16] and 5-deoxy-7-methylbostrycoidin,[15] respectively, by comparison of their spectroscopic data with those reported in the literature. (-)-Usnic acid is a common lichen substance isolated from several lichen genera containing *Haematomma*, but it was isolated for the first time from cultured mycobionts of *Haematomma* sp.

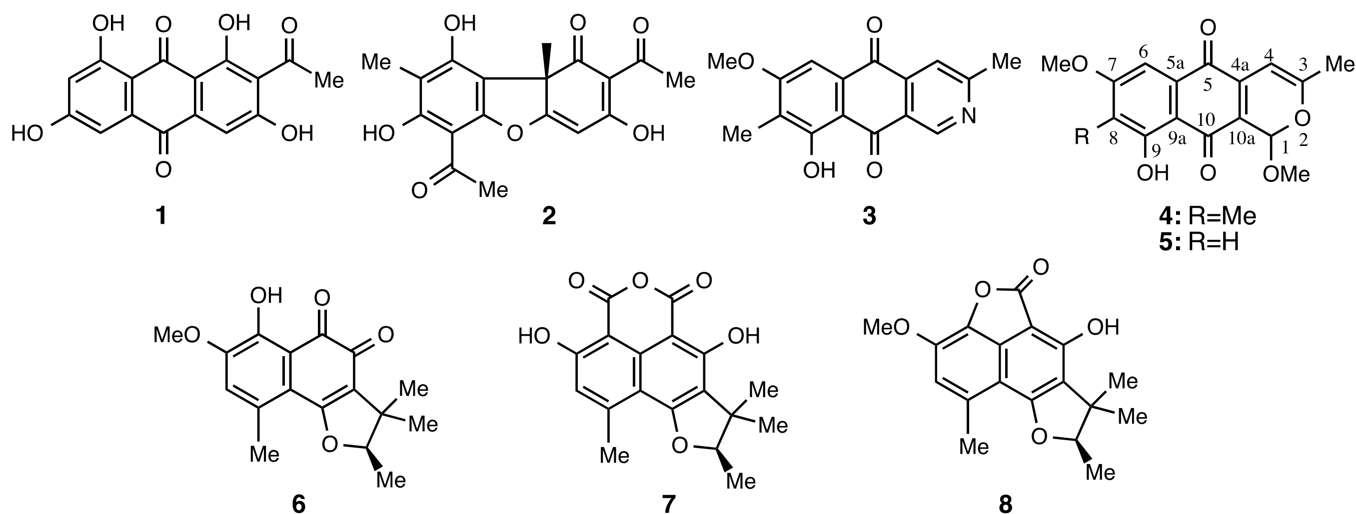

Figure 1. Structures of compounds **1**–**8**

New compound **4** was isolated as an orange solid. The HR-ESIMS of **4** established its molecular formula as $C_{17}H_{16}O_6$. The UV spectrum of **4** showed absorption at 222, 235sh, 259sh, 272, 365, and 484 nm. The ^{1}H-NMR spectrum of **4** showed signals for two methyls at δ 2.14 and 2.17 (each s), two methoxy groups at δ 3.95 and 3.60 (each s), three CH protons at δ 6.12, 6.23, and 7.16 (each s), and a hydrogen-bonded hydroxy group at δ 12.51. These spectroscopic features were similar to those of **3**, except for the presence of an additional methoxy group at δ 3.60 and differences in the chemical shifts of signals due to H-1, H-4,

H-6, and 3-Me. The ^{13}C-NMR spectrum of **4** demonstrated a methyl signal at δ_c 20.8, a methoxy signal at δ_c 55.8, two CH carbons at δ_c 94.6 and 95.2, and three quaternary carbons at δ_c 120.6, 137.0, and 162.1 in addition to ten carbon signals that were assignable to two carbonyls and a benzene ring substituted with a methyl, a methoxy and a hydroxy group as in **3** (Table 1).

Table 1. ^{1}H- and ^{13}C-NMR spectral data of **3**, **4** and **5** in $CDCl_3$

	3				**4**				**5**[a]			
position	δ_H		δ_C		δ_H		δ_C		δ_H		δ_C	
1	9.38	s	148.9	CH	6.23	s	95.2	CH	6.25	s	95.1	CH
3	----		165.7	C	----		162.1	C	----		162.3	C
4	7.84	s	118.4	CH	6.12	s	94.6	CH	6.14	s	94.6	CH
4a	----		138.5	C	----		137.0	C	----		137.1	C
5	----		182.3	C	----		182.5	C	----		182.4	C
5a	----		131.8	C	----		130.4	C	----		133.1	C
6	7.33	s	102.6	CH	7.16	s	102.3	CH	7.19	s	107.9	CH
7	----		163.7	C	----		162.6	C	----		165.4	C
8	----		121.9	C	----		121.1	C	6.64	s	106.7	CH
9	----		162.1	C	----		160.8	C	----		163.9	C
9a	----		110.5	C	----		110.0	C	----		109.8	C
10	----		186.6	C	----		186.6	C	----		186.1	C
10a	----		124.1	C	----		120.6	C	----		120.8	C
1-OMe	----		----		3.60	s	55.8	Me	3.61	s	55.8	Me
3-Me	2.77	s	25.3	Me	2.17	s	20.8	Me	2.29	s	20.8	Me
7-OMe	4.01	s	56.3	Me	3.95	s	56.1	Me	3.89	s	55.9	Me
8-Me	2.18	s	8.3	Me	2.14	s	8.2	Me	----	s	----	
9-OH	12.81	s	----		12.51	s	----		12.45	s	----	

[a]Data taken from ref. 17.

The remarkable differences between the NMR spectra of **3** and **4**, as well as their molecular formulas, implied that compound **4** possessed a 2-methoxy-2*H*-pyran ring instead of pyridine core in **3**. This proposed structure was deduced from correlations in the HSQC, HMBC, and NOESY spectra (Figure 2). The proton singlet at δ_H 6.12 (H-4), which was correlated with a carbon signal at δ_C 94.6 in the HSQC spectrum, showed HMBC correlations with an oxygenated sp^2 carbon at δ_C 162.1 (C-3) and a carbonyl at δ_C 182.5 (C-5), and a NOESY cross-peak with a methyl signal at δ_H 2.17. Another proton singlet at δ_H 6.23 (H-1) showed a HSQC correlation with an acetalic carbon signal at δ_C 95.2, an HMBC correlation with δ_C 186.6 (C-10), and a NOESY interaction with a *O*-methyl group, which showed an HMBC

correlation to the carbon signal at δ_C 95.2 in turn. Further support was obtained from the comparison of ^{1}H- and ^{13}C-NMR spectroscopic data of **4** with those of ascomycone A (**5**).[17] Thus, the new compound **4** was characterized as 9-hydroxy-1,7-dimethoxy-3,8-dimethyl-1*H*-benzo[*g*]isochromene-5,10-dione and designated as 8-methylascomycone A.

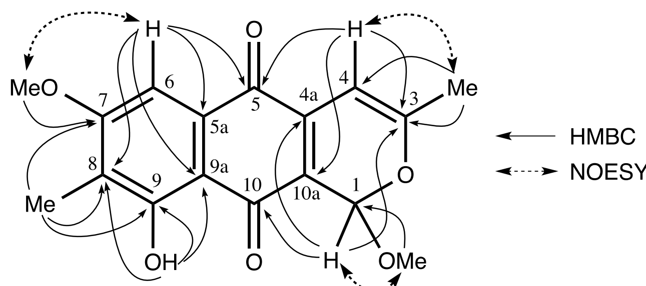

Figure 2. HMBC and NOESY correlations of compound **4**

Quinones constitute an important group of natural products with wide-ranging biological activities. For instance, a pyranonaphthoquinone thysanone[18] exhibited activity against human rhinovirus 3C-protease. The binding ability to DNA of the 2-aza-anthraquinone-type cytotoxic compound TPL-1[19] and induction of DNA topoisomerase II-mediated DNA cleavage by a 1,2-naphthoquinone, β-lapachone[20] were also reported. These findings prompted us to investigate the DNA polymerase (pol) inhibitory activities of the isolated 2-aza-anthraquinone **3** and pyranonaphthoquinone **4** as well as a 1,2-naphthoquinone **6** and its

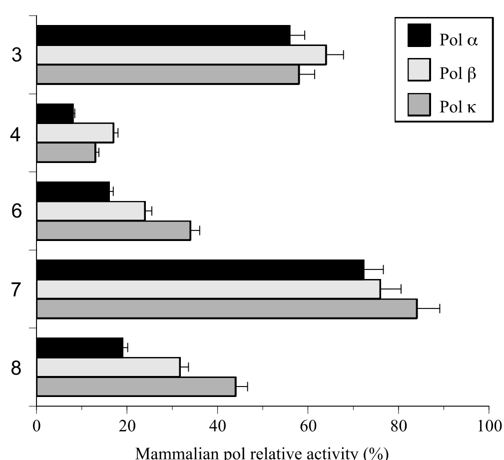

Figure 3. Effect of compounds **3**, **4**, **6**–**8** (100 μM each) on the activities of mammalian pols. The enzymes (0.05 unit each) were immune affinity-purified calf thymus pol α (black bars), rat recombinant pol β (light-gray bars) and human pol κ (dark-gray bars). Pol activity in the absence of the compound was taken as 100%. Data are shown as mean ± S.D. of 3 independent experiments.

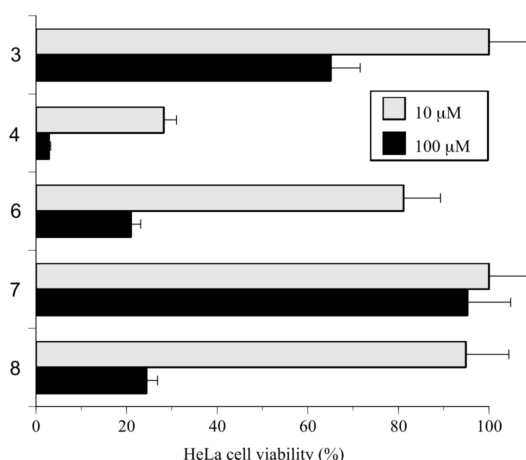

Figure 4. Effect of compounds **3**, **4**, **6**–**8** on the proliferation of human cancer cells. HeLa cells were incubated with 10 and 100 μM (gray bars and black bars, respectively) of each compound for 24 h, and the rate of cultured cell growth inhibition was determined by MTT assay. Cell growth inhibition of the cells in the absence of the compound was taken as 100%. Data are shown as mean ± S.D. of 5 independent experiments.

related substances **7** and **8**, which have been previously isolated from cultured mycobionts of *Trypethelium* sp.[10]

Mammalian DNA pols are essential for DNA replication, repair, and cell division.[21] Selective inhibitors of mammalian pols have been considered a group of potentially useful chemotherapy agents for anticancer, antiviral, and anti-inflammatory treatments.[22] The inhibitory activities against three mammalian pols, namely, calf pol α, rat pol β, and human pol κ, by 100 μM of compounds **3**, **4**, and **6**–**8**, were evaluated (Figure 3). Compounds **3** and **7** showed weak activities against three pols, whereas the activities of pyranonaphthoquinone **4** and phenolic compounds **6** and **8** were stronger than those of **3** and **7**. These compounds were also assayed for their cytotoxic effects against the HeLa human cervical cancer cultured cell line (Figure 4). Compound **4** was the strongest suppressor of HeLa cell growth among the tested compounds, with an IC_{50} of 6.7 μM. The much stronger activity of **4** compared with **3** indicated that the 2*H*-pyran ring with 2-methoxy group is crucial for the cytotoxic activity against HeLa cells.

EXPERIMENTAL

General procedures. The UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and the IR spectra on a Shimadzu FTIR-8200 infrared spectrophotometer. Optical rotation was measured on a Jasco DIP-370 digital polarimeter. HR-ESIMS were obtained with a Thermo Scientific Q Exactive. The NMR experiments were performed with Varian Gemini-300, Varian NMR System-500 and Varian UNITY INOVA (500 MHz) spectrometers with TMS as an internal standard. TLC was performed on precoated Kieselgel $60F_{254}$ plates (Merck) and spots were visualized using UV light.

Plant material. Cultivation of spore-derived mycobionts of *Haematomma* sp. (registration No. NH9830572) was already described in ref. 15. The strain of mycobiont was cultivated in 80 test tubes containing modified MY10 medium (malt extract 10 g, yeast extract 4 g, sucrose 100 g, agar 15 g, H_2O 1 L, pH 7.0) at 18 °C in the dark. After cultivation for 2 years, the colonies were harvested.

Isolation of compounds. The harvested colonies (dry weight 12.7 g) were extracted continuously with Et_2O, with acetone and then with MeOH. The Et_2O, acetone, and MeOH extracts were concentrated to give residues R-1 (358 mg), R-2 (275 mg), and R-3 (6.44 g), respectively. Residue R-3 was suspended in H_2O and partitioned with *n*-BuOH. Concentration of the *n*-BuOH extracts gave a residue R-4 (1.54 g). Residues R-1, R-2 and R-4 were repeatedly subjected to preparative TLC ($CHCl_3$-MeOH 49:1, 99:1, benzene-AcOEt-EtOH 8:2:1, toluene-acetone 17:3, toluene-AcOH 9:1), giving rise to **2** (R-1: 6.0 mg, R-2: 4.5 mg), **3** (R-1: 18.5 mg, R-2: 15.3 mg, R-3: 22.2 mg), and **4** (R-1: 88.5 mg, R-2: 45.6 mg, R-3: 12.0 mg).

(-)-Usnic acid (2): Pale yellow crystalline solid. Mp 179–180 °C; $[\alpha]_D^{24}$ –377 (*c* 0.25, $CHCl_3$);

HR-ESIMS *m/z*: Calcd for $C_{18}H_{15}O_7$ $[M-H]^-$: 343.0818. Found: 343.0824.

5-Deoxy-7-methylbostrycoidin (3): Orange crystalline solid. Mp 221 °C; UV (EtOH) λ_{max} nm (log ε): 241 (4.32), 283 (4.30), 329sh (3.66), 412 (3.80). IR (KBr) $\nu_{max}cm^{-1}$: 1673, 1634, 1591. HR-ESIMS *m/z*: Calcd for $C_{16}H_{14}NO_4$ $[M+H]^+$: 284.0923. Found: 284.0918.

8-Methylascomycone A (4): Orange solid. UV (EtOH) λ_{max} nm (log ε): 222 (4.35), 235sh (4.28), 259sh (4.21), 272 (4.22), 365 (3.85), 484 (3.75). IR (KBr) $\nu_{max}cm^{-1}$: 1669, 1650, 1562. HR-ESIMS *m/z*: Calcd for $C_{17}H_{15}O_6$ $[M-H]^-$: 315.0870. Found: 315.0879.

DNA pol Assay. DNA pol α was purified from the thymus by immuno-affinity column chromatography as described previously.[23] Recombinant rat DNA pol β was purified from *Escherichia coli* JMpβ5, as described previously.[24] A truncated form of pol κ (residues 1–560) with 6 × His-tags attached at the C-terminus was overproduced in *E. coli* and purified as described previously.[25]

The standard reaction mixture for pol α (24 μL final volume) contained 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM $MgCl_2$, 5 μM poly(dA)/oligo$(dT)_{18}$ (= 2/1), 10 μM $[^3H]$dTTP (100 cpm/pmol), 15% (v/v) glycerol, and 8 μL of an enzyme inhibitor solution. The standard reaction mixture for pol β was the same, except that it also contained 150 mM KCl. The reaction mixture for pol κ was the same as for pol α. The test compounds were dissolved in distilled DMSO at various concentrations and sonicated for 30 s. Aliquots of 4 μL sonicated samples were mixed with 16 μL of each enzyme (final amount 0.05 units) in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol, and 0.1 mM EDTA, and kept at 0 °C for 10 min. These inhibitor-enzyme mixtures (8 μL) were added to 16 μL of each of the enzyme standard reaction mixtures and incubation was carried out at 37 °C for 60 min. Activity without the inhibitor was considered 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol dTTP into synthetic DNA template-primers in 60 min at 37 °C in the normal reaction conditions for each enzyme.

Human cancer cell culture and measurement of the cell viability. A human cervical cancer cultured cell line, HeLa, was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and 1.6 mg/mL $NaHCO_3$ at 37 °C in a humid atmosphere of 5% CO_2/95% air. For the cell viability assay, cells were seeded at 1×10^3 cells/well in a 96-well microplate with various concentrations of the test compounds and incubated for 24 h. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) solution was added to a final concentration of 0.6 mg/mL in purified water for 2 h, after which time the medium was discarded and the cells were lysed in DMSO. A_{540} was then measured using a Molecular Devices Vmax-K microplate reader.

ACKNOWLEDGEMENTS

The excellent technical assistance of Miss A. Sumada is acknowledged with appreciation. We thank Dr. M. Tode (Kobe Pharmaceutical University) for providing ^{1}H- and ^{13}C-NMR spectra, and to Dr. A. Takeuchi (Kobe Pharmaceutical University) for mass spectral measurements. This research was supported financially in part by Kobe Pharmaceutical University Collaboration Fund and Grant-in-Aid for Scientific Research (C) (No. 26460137) from the Japan Society for the Promotion of Science (JSPS).

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