PERGAMON

Phytochemistry 52 (1999) 453–457

PHYTOCHEMISTRY

Bioactive naphthoquinones from *Cordyceps unilateralis*

Prasat Kittakoop[a,*], Juntira Punya[a], Palangpon Kongsaeree[b], Yuwapin Lertwerawat[a,c], Amnuay Jintasirikul[a], Morakot Tanticharoen[a,c], Yodhathai Thebtaranonth[a,b]

[a]*National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 73/1, NSTDA building, Rama 6 Rd., Rajdhevee, Bangkok 10400, Thailand*

[b]*Department of Chemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

[c]*School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangmod, Bangkok 10140, Thailand*

Received 27 October 1998; received in revised form 10 March 1999

Abstract

Six bioactive naphthoquinone derivatives, erythrostominone, deoxyerythrostominone, 4-*O*-methyl erythrostominone, epierythrostominol, deoxyerythrostominol and 3,5,8-trihydroxy-6-methoxy-2-(5-oxohexa-1,3-dienyl)-1,4-naphthoquinone, were isolated from the insect pathogenic fungus *Cordyceps unilateralis* BCC1869. While the latter is synthetically known, both it and 4-*O*-methyl erythrostominone are products of fungus strain *C. unilateralis* BCC1869. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Cordyceps unilateralis*; Insect pathogenic fungi; Antimalarial; Cytotoxicity; Naphthoquinone; Erythrostominone; Epierythrostominol

1. Introduction

As part of the ongoing multidisciplinary research activity at the National Center for Genetic Engineering and Biotechnology (BIOTEC), insect pathogenic fungi have been routinely collected from various parts of Thailand. Fungi in our collection were grown under laboratory conditions and many strains were found in screening systems to produce secondary metabolites exhibiting various biological activities ranging from activity against malaria parasites, fungi, virus, mycobacteria and tumor cell lines. The strain of *Cordyceps unilateralis* BCC1869 is one of the fungi in our laboratory producing bioactive substances; hence activity-guided isolation and identification of its bioactive substances were undertaken and results are presented in this paper.

* Corresponding author. Tel.: +66-2-644-8103; fax: +66-2-644-8104.

E-mail address: prasat@biotec.or.th (P. Kittakoop)

2. Results and discussion

EtOAc extract of a culture broth of *C. unilateralis* BCC1869 yielded compounds **1**–**6** after purification with a gel filtration column chromatography and a MPLC using a C_{18} reversed phase column. The previously known red compounds **1**, **2**, **4** and **5** were identified, based upon their spectroscopic data, as erythrostominone, deoxyerythrostominone, epierythrostominol and deoxyerythrostominol, respectively. These compounds were previously reported to be antibacterial constituents in the fungus *Gnomonia erythrostoma* (Cross & Edinberry, 1970; Cross, Edinberry & Turner, 1972; Cross & Zammitt, 1973). Compound **3**, identified as 4-*O*-methyl erythrostominone and the quinone **6**, previously chemically synthesized from erythrostominone **1** (Cross & Edinberry, 1970; Cross et al., 1972) but never reported as a natural product, were also isolated and identified.

The ^{1}H NMR spectroscopic data of **1**, **2**, **4** and **5** were consistent with reported data (Cross & Edinberry, 1970; Cross et al., 1972; Cross & Zammitt,

0031-9422/99/$ - see front matter
PII: S0031-9422(99)00272-1

Table 1
^{13}C NMR spectral data for compounds **1**–**6**

C	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[b]
1	–	–	–	–	–	183.57
2	70.44	73.78	70.30	71.97	75.04	117.91
3	33.81	25.43	30.74	34.53	25.99	158.99
4	57.30	18.59	65.37	58.01	18.56	179.96
4a	122.18	122.23	120.23	122.41	122.29	111.68
5	174.93	175.22	174.18	175.95	175.78	161.94
5a	103.62	103.72	103.78	103.64	103.67	–
6	169.48	169.60	169.62	169.28	169.11	152.87
7	108.49	108.45	109.03	108.59	108.38	108.86
8	158.18	158.16	158.17	158.23	158.04	156.79
8a	–	–	–	–		103.89
9	162.59	162.54	163.88	162.26	162.01	–
9a	110.89	110.71	111.36	111.10	110.72	–
10	170.27	170.54	170.52	171.27	171.17	–
10a	153.86	153.62	154.25	154.27	153.73	–
1′	47.74	48.06	47.90	43.39	43.49	131.43
2′	204.96	205.22	205.00	64.25	64.22	133.70
3′	30.89	31.01	31.01	23.95	24.04	146.17
4′	–	–	–	–	–	131.28
5′	–	–	–	–	–	198.66
6′	–	–	–	–	–	27.43
4-OMe	–	–	57.15	–	–	–
6-OMe	–	–	–	–		57.23
8-OMe	56.73	56.64	56.66	56.72	56.61	–

[a] Recorded in $CDCl_3$.
[b] Recorded in DMSO-d_6.

1973), however, their ^{13}C and other 2D NMR data have not yet been recorded; ^{13}C NMR spectral data (with carbon assignments) of these compounds are in Table 1. The EIMS spectra of **1**, **2**, **4** and **5** exhibited molecular ion peaks at *m*/*z* 348, 332, 350 and 334, respectively. Based upon the published and additional spectroscopic data, quinones **1**, **2**, **4** and **5** were erythrostominone, deoxyerythrostominone, epierythrostominol and deoxyerythrostominol, respectively.

The ^{1}H NMR spectroscopic data of naphthoquinone **3** revealed two phenolic OH singlets at δ 12.70 and 13.42 ppm (D_2O exchangeable). Generally, the ^{1}H and ^{13}C NMR spectral data of **3** were similar to those of **1**, except that **3** had additional methyl group protons (at δ 3.54, *s*) as well as a carbon resonance at δ 57.15 ppm. The C-4 of **3** shifted downfield about 8 ppm relative to **1** (from 57.30 ppm of **1** to 65.35 ppm of **3**), suggesting a methoxyl group at C-4. DEPT and HMQC techniques were used to classify type of carbons and their connectivities with nearby protons. The ^{1}H-^{1}H COSY spectrum of **3** established the correlations of H-2 to H-3 and H-1′. The HMBC spectra of **3** revealed the correlations of methyl protons at H-3′ to the C-2′ carbonyl carbon; H-1′ to C-2′ and C-2; H-3 to C-2, C-4 and C-4a; H-4 to C-2, C-4a, C-10a and a carbonyl C-5; H-7 to C-5a, C-6, C-8 and C-9; phenolic OH at δ 12.70 to C-8, C-9 and C-9a; and phenolic OH at 13.42 to C-5a and C-7. The HMBC data of **3** also conclusively demonstrated the correlations of 4-OMe and 8-OMe protons to C-4 and C-8, respectively, hence confirming the presence of two methoxy groups at these two positions. The HR-EIMS spectrum of **3** showed a molecular ion at *m*/*z* 362.1010, suggesting a molecular formula of $C_{18}H_{18}O_8$. Based on these spectroscopic data, **3** was 4-*O*-methyl erythrostominone.

The H-4 of **1**–**5** had multiplicity of dd with small coupling constant (1.9–4.1 Hz), suggesting that H-4 had a rather small dihedral angle relative to H-3_{ax} and 3_{eq}, thus implying that H-4 was pseudo-equatorial. The $J_{\text{H-2,H-3}_{ax}}$ of ca. 12 Hz in compounds **1**, **3** and **4** indicated a pseudo-axial orientation of H-2. 2D NOESY data were used to establish the relative configuration of methylene protons at C-3 and C-4 in **2** and **5**. However, the relative configuration of the hydroxyl group on the propanol moiety attached to C-2′ of **4** and **5** could not conclusively be assigned based on available NMR spectroscopic data.

It is well known that the naphthoquinone with a naphthazarin nucleus can exist as an equilibrium mixture in which tautomer forms I and II are normally regarded to give major contributions in the equilibrium. The HMBC data demonstrated that the quinones **1**–**5**, in a solvent of $CDCl_3$, preferentially existed in form I; there were correlations of H-7 to the oxycarbons (C-6 and C-9), suggesting that H-7 was adjacent to both oxycarbons as expected for form I. In addition, no HMBC correlations of H-7 to the carbonyl carbons (C-5 and C-10), supporting the carbonyl carbons located in ring B of form I.

The ^{1}H NMR spectrum of the naphthoquinone **6**, recorded in DMSO-d_6, showed four olefinic protons along the *trans*-butadiene moiety at δ 7.62 (1H, dd, J = 15.5 and 15.4 Hz, H-2′), 7.42 (1H, dd, J = 15.5 and 15.4 Hz, H-3′), 7.24 (1H, d, J = 15.5 Hz, H-1′) and 6.21 (1H, d, J = 15.5 Hz, H-4′). In addition, two methyl singlets at δ 3.93 and 2.26, two phenolic OHs at δ 13.71 (s) and 12.26 (s) (D_2O exchangeable) and one aromatic proton at δ 6.83 (s), were visible in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum of **6** revealed 17 carbons (Table 1) and DEPT and HMQC techniques were employed for the classification of the type of carbons and C–H connectivities, respectively. The ^{1}H–^{1}H COSY spectrum showed correlations of H-1′ to H-2′, H-2′ to H-3′ and H-3′ to H-4′. The HMBC spectrum of **6** demonstrated the correlations of methyl protons at H-6′ to the carbonyl C-5′; H-4′ to C-5′ and C-6′; H-3′ to C-5′; H-2′ to C-4′ and C-2; H-1′ to C-1 and C-3; H-7 to C-6, C-8 and C-8a; 6-OMe protons to C-6 and phenolic OH at δ 13.71 to C-7, C-8 and C-8a. The HR-EIMS spectrum of **6** revealed a molecular ion peak at *m*/*z* 330.0743, representing a molecular formula of $C_{17}H_{14}O_7$. Based upon these spectral data, compound **6** was therefore 3,5,8-trihydroxy-6-methoxy-2-(5-oxohexa-1,3-dienyl)-1,4-

naphthoquinone. The naphthoquinone (**6**) is known and was chemically prepared by the cleavage of ring C of erythrostominone (**1**) in a hot ethanolic solution (Cross et al., 1972). The presence of **6** in a broth of the culture has now been confirmed using HPLC. When a filtered culture broth was subjected to HPLC, a peak having the same retention time (2.9 min) as that of the standard **6** was detected. The presence of **6** in a culture broth clearly indicated that naphthoquinone **6** was a naturally occurring naphthoquinone, produced by the fungus *C. unilateralis* BCC1869 and not an artifact.

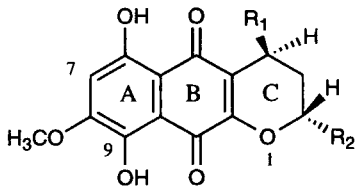
While naphthoquinones **1**–**5** showed both antimalarial activity (against the protozoa *Plasmodium falciparum*) and cytotoxicity (against BC, KB and Vero cell lines), napthoquinone **6** was active against the malarial parasites but not against the above cell lines (Table 2).

3. Experimental

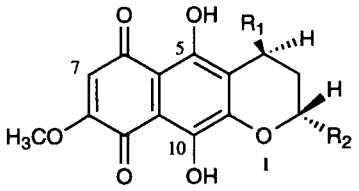
^{1}H, ^{13}C, DEPT (Doddrell, Pegg & Bendall, 1982), ^{1}H–^{1}H COSY (Derome & Williamson, 1990), HMQC (Bax, Griffey & Hawkins, 1983) (optimized for $^{1}J_{HC} = 145$ Hz) and HMBC (Bax & Summers, 1986) (optimized for $^{n}J_{HC} = 8.0$ Hz) spectra were recorded on a Bruker DRX 400, operating at 400.1 MHz for proton and 100.6 MHz for carbon. EIMS (70 eV) spectra were obtained from a Micromass Platform II mass spectrometer.

3.1. Insect pathogenic fungi

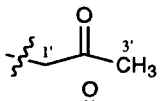
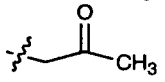
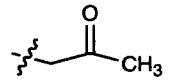
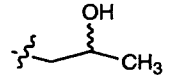
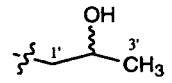
C. unilateralis BCC1869 was collected from the Khao Luang National Park, Thailand and identified by Dr. Nigel Leslie Hywel-Jones of the Mycology Research Unit, National Center for Genetic

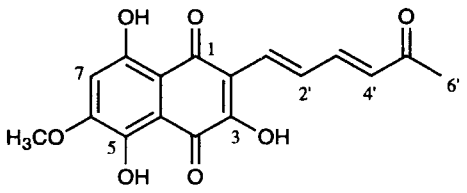


I



II

	R_1	R_2
1	OH	
2	H	
3	OCH_3	
4	OH	
5	H	



6

Table 2
Bioactivities of compounds **1**–**6**

Compound	Antimalaria activity (EC_{50}, μg/mL)	Cytotoxicity (EC_{50}, μg/mL)		
		BC cell line	KB cell line	Vero cell line
1	4.0	9.7	23.0	15.0
2	7.5	6.0	12.4	ca. 30
3	10.1	ca. 5	24.0	ca. 10
4	7.0	4.2	7.2	7.5
5	8.5	ca. 10	20.0	10.0
6	2.5	Inactive at 50 μg/mL	Inactive at 50 μg/mL	Inactive at 50 μg/mL
Ellipticine	–	0.3	0.3	1.0

Engineering and Biotechnology (BIOTEC). The culture was deposited at the BIOTEC Culture Collection.

3.2. Cultivation of fungi

The culture of *C. unilateralis* BCC1869 was maintained (at 22°C) on potato dextose agar slants (DIFCO). Primary inoculum in a potato dextose broth (DIFCO) was incubated for 4 days (at 22°C), then transferred into 250 mL of the same culture medium. The culture was subsequently incubated (at 22°C) for 38 days, then harvested for further study.

3.3. Extraction and isolation

A culture (10 L) of *C. unilateralis* was filtered to separate cell and broth and the broth was subsequently extracted twice, each with an equal volume (5 L) of EtOAc. A crude EtOAc extract was partially purified with gel filtration column chromatography on Sephadex LH-20 to give 160 mg of a pure naphthoquinone **6** as well as a reddish fraction, which was further purified by MPLC using a C_{18} reversed phase column (MeOH:H_2O (70:30, v/v) as an eluent) to yield red compounds **1** (190 mg), **2** (55 mg), **3** (7 mg), **4** (40 mg) and **5** (38 mg).

3.4. Analysis of 6 in the C. unilateralis culture broth by HPLC

A broth of *C. unilateralis* was filtered in order to separate fungus cells from the medium. The filtrate was subsequently filtered through a 0.25 μm membrane and directly subjected (20 μL) to HPLC (a WATERS 600 pump connected with a WATERS 996 photodiode array detector). A reversed phase C_{18} (WATERS RCM) column and an amino column (300 NH_2 LiChrospher, E. Merck) were employed. MeCN:H_2O (70:30, v/v) was used as the mobile phase for the reversed phase column, whilst MeCN:H_2O (80:20, v/v) was employed for the amino column. Under these HPLC conditions, compound **6** was detected both in the reversed phase system (retention time of 2.9 min) and in the amino column (retention time of 8.3 min). Co-injection of the culture broth with the standard **6** further confirmed the result.

3.5. Erythrostominone (1)

EIMS *m/z* (rel. int.): 348 (15) $[M]^+$, 290 (23), 274 (20), 272 (26), 95 (33), 85 (36), 83 (43), 77 (34), 69 (100); ^{13}C NMR (100 MHz; $CDCl_3$): Table 1.

3.6. Deoxyerythrostominone (2)

EIMS *m/z* (rel. int.): 332 (60) $[M]^+$, 290 (15), 275 (34), 274 (100), 261 (22), 250 (25), 249 (32), 221 (22), 219 (30), 69 (28); ^{13}C NMR (100 MHz; $CDCl_3$): Table 1.

3.7. 4-O-methyl erythrostominone (3)

Red solid; mp 149–151°; $[\alpha]_D^{28}$ +232° (acetone; *c* 0.1); UV λ_{max}^{MeOH} nm (log ε): 231 (4.65), 278 (3.96), 310 (3.90), 478 (3.77), 507 (3.81), 546 (3.62); IR ν_{max}^{KBr} cm^{-1}: 3497 (OH), 1716 (C=O), 1603, 1457, 1278, 1137, 1082, 731; EIMS *m/z* (rel. int.): 362 (14) $[M]^+$, 332 (15), 274 (25), 272 (100), 85 (28), 83 (31), 69 (30); HR-EIMS *m/z*: 362.1010 ($C_{18}H_{18}O_8$ $[M]^+$, requires 362.1002); ^1H NMR (400 MHz; $CDCl_3$): 13.42 (1H, *s*, phenolic OH), 12.70 (1H, *s*, phenolic OH), 6.42 (1H, *s*, H-7), 4.82 (1H, dddd, J = 12.3, 6.3, 2.5 and 2.1 Hz, H-2), 4.57 (1H, dd, J = 2.5 and 2.5 Hz, H-4), 3.94 (3H, *s*, 8-OMe), 3.54 (3H, s, 4-OMe), 3.15 (1H, dd, J = 16.8 and 6.3 Hz, H-$1_a'$), 2.75 (1H, dd, J = 16.8 and 6.3 Hz, H-$1_b'$), 2.38 (1H, ddd, J = 14.4, 2.5 and 2.1 Hz, H-3_{eq}), 2.31 (3H, *s*, H-3′), 1.58 (1H, ddd, J = 14.4, 12.3 and 2.9 Hz, H-3_{ax}); ^{13}C NMR (100 MHz; $CDCl_3$): Table 1.

3.8. Epierythrostominol (**4**)

EIMS *m/z* (rel. int.): 350 (11) $[M]^+$, 236 (22), 95 (43), 85 (45), 83 (45), 77 (42), 69 (100); ^{13}C NMR (100 MHz; $CDCl_3$): Table 1.

3.9. Deoxyerythrostominol (**5**)

EIMS *m/z* (rel. int.): 334 (100) $[M]^+$, 275 (51), 261 (38), 249 (40), 248 (45), 221 (35), 219 (70), 95 (28), 85 (80), 77 (31), 69 (60); ^{13}C NMR (100 MHz; $CDCl_3$): Table 1.

3.10. 3,5,8-Trihydroxy-6-methoxy-2-(5-oxohexa-1,3-dienyl)-1,4-naphthoquinone (**6**)

Red needles from acetic acid, mp 234.0–237.9° (lit.[2] 238–242°); UV λ_{max}^{MeOH} nm (log ε): 236 (3.97), 280 (3.94), 331 (4.05), 402 (4.12); IR ν_{max}^{KBr} cm^{-1}: 3498 (OH), 1716 (C=O), 1667, 1583, 1477, 1289, 1220, 855, 811, 736; EIMS *m/z* (rel. int.): 330 (100) $[M]^+$, 315 (32), 287 (86), 273 (35), 245 (60), 244 (52), 121 (25); HR-EIMS *m/z*: 330.0743 ($C_{17}H_{14}O_7$ $[M]^+$, requires 330.0739); ^1H NMR (400 MHz; DMSO-d_6): 13.71 (1H, s, phenolic OH), 12.26 (1H, s, phenolic OH), 7.62 (1H, dd, J = 15.5 and 15.4 Hz, H-2′), 7.42 (1H, dd, J = 15.5 and 15.4 Hz, H-3′), 7.24 (1H, d, J = 15.5 Hz, H-1′), 6.83 (1H, s, H-7), 6.21 (1H, d, J = 15.5 Hz, H-4′), 3.93 (3H, s, 6-OMe), 2.26 (3H, s, H-6′); ^{13}C NMR (100 MHz; DMSO-d_6): Table 1.

3.11. Bioassays

Antimalarial assay was performed according to the method of Trager and Jensen (1976) using continuous cultures (in vitro) of asexual erythrocytic stages of *Plasmodium falciparum* (K1, multidrug resistant strain). Quantitative assessment of antimalarial activity in vitro was determined by means of the microculture radioisotope technique based upon the method described by Desjardins, Canfield, Haynes & Chulay (1979). Effective concentration (EC_{50}) represents the concentration which causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^3H]-hypoxanthine by *P. falciparum*. An EC_{50} value of 0.16 μg/mL (3.1 μM) was observed for the standard sample, chloroquine diphosphate, in the same test system. Cytotoxicity was conducted on a breast cancer cell line (BC), a human epidermoid carcinoma in the mouth (KB) and Vero cell line, using the method as previously described by Skehan et al. (1990).

Acknowledgements

We are grateful to the Biodiversity Research and Training Program and the BIOTEC/NSTDA, for financial support. One of us (Y.T.) thanks the National Science and Technology Development Agency (NSTDA) for the Senior Research Fellowship Award. We thank the BIOTEC fermentation technology laboratory and bioassay research facility for mass cultivation of the fungus and bioactivity test, respectively. We thank Dr. Ryohei Yamaoka, Kyoto Institute of Technology, Japan, for HR-EIMS data.

References

Bax, A., Griffey, R. H., & Hawkins, B. L. (1983). *Journal of Magnetic Resonance*, *55*, 301.

Bax, A., & Summers, M. F. (1986). *Journal of the American Chemical Society*, *108*, 2093.

Cross, B. E., & Edinberry, M. N. (1970). *Journal of the Chemical Society (D)*, *4*, 209.

Cross, B. E., Edinberry, M. N., & Turner, W. E. (1972). *Journal of the Chemical Society, Perkin Transactions 1*, *3*, 380.

Cross, B. E., & Zammitt, L. J. (1973). *Journal of the Chemical Society, Perkin Transactions 1*, *23*, 2975.

Derome, A. E., & Williamson, M. P. (1990). *Journal of Magnetic Resonanace*, *88*, 177.

Desjardins, R. E., Canfield, C. J., Haynes, J. D., & Chulay, J. D. (1979). *Antimicrobial Agents and Chemotherapy*, *16*, 710.

Doddrell, D. M., Pegg, D. T., & Bendall, M. R. (1982). *Journal of Magnetic Resonance*, *48*, 323.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., & Boyd, M. R. (1990). *Journal of the National Cancer Institute*, *82*, 1107.

Trager, W., & Jensen, J. B. (1976). *Science*, *193*, 673.