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Antimalarial Halorosellinic Acid from the Marine Fungus *Halorosellinia oceanica*

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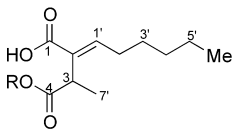
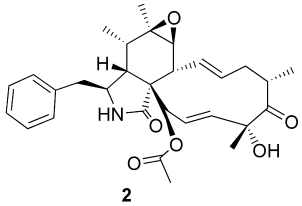
Abstract—Three known compounds, 2-hexylidene-3-methylsuccinic acid (**1**), cytochalasin Q (**2**), and 5-carboxymellein (**3**), together with two new derivatives, 2-hexylidene-3-methylsuccinic acid 4-methyl ester (**4**) and an ophiobolane sesterterpene named halorosellinic acid (**5**), were isolated from culture broth of the marine fungus *Halorosellinia oceanica* BCC 5149. Compounds **1**–**3** exhibited moderate cytotoxicity against KB and BC-1 cell lines with IC_{50} values of 1–13 μg/mL, while compounds **2**, **3**, **5**, and **6** showed antimalarial activity with respective IC_{50} values of 17, 4, 13, and 19 μg/mL. Halorosellinic acid (**5**) possessed only weak antimycobacterial activity with the minimum inhibitory concentration of 200 μg/mL.

Introduction

As part of our on-going research program on biologically active substances from Thai bioresources,[1] we have intensively screened biological activities of extracts from plants and microorganisms. Among these, a crude extract of the marine fungus *Halorosellinia oceanica* BCC 5149 exhibited cytotoxicity against KB cells with an IC_{50} of 6.5 μg/mL; this prompted us to investigate bioactive constituents of this fungus. We report herein the isolation, structure elucidation, and biological activities of secondary metabolites of *H. oceanica* BCC 5149, which include three known compounds, 2-hexylidene-3-methylsuccinic acid (**1**),[2] cytochalasin Q (**2**),[3] and 5-carboxymellein (**3**),[4] and two new metabolites, 2-hexylidene-3-methylsuccinic acid 4-methyl ester (**4**), and an ophiobolane sesterterpene named halorosellinic acid (**5**).



1, R = H
4, R = Me



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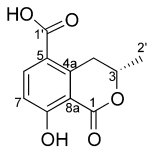

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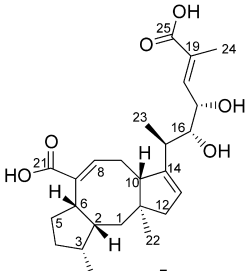

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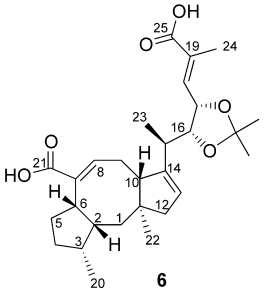

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Fungal Material, Extraction and Isolation

The marine fungus *H. oceanica* BCC 5149 was collected from Samutsongkram Province, Thailand, by Dr. A. Piluntanapark, identified by Professor E. B. G. Jones, and deposited at the BIOTEC Culture Collection, Bangkok, Thailand (registration no. BCC 5149). *H. oceanica* BCC 5149 was grown in a potato dextrose broth (DIFCO), and incubated for 5 days at 25 °C, then transferred into 250 mL of the same culture medium. The culture was subsequently incubated (at 25 °C) for 21 days, then harvested for further study. The culture (5 L) of *H. oceanica* BCC 5149 was filtered to separate cell and broth. The culture broth was extracted twice with an equal volume of EtOAc. EtOAc layers were combined and evaporated to dryness, yielding 2.3 g of a crude extract. The crude EtOAc extract was subsequently chromatographed on Sephadex LH-20 column and eluted with MeOH to provide three major fractions (fractions 1–3), which were further purified by either preparative HPLC or crystallization. Separation of fraction 1 by preparative HPLC (C_{18} reversed phase column, and MeCN/H_2O 30:70 as eluent) yielded 121 mg of 2-hexylidene-3-methylsuccinic acid (**1**). Fraction 2 was subjected to preparative HPLC (MeCN/H_2O 50:50) to furnish cytochalasin Q (**2**) (11 mg), 2-hexylidene-3-methylsuccinic acid 4-methyl ester (**4**) (7 mg), and halorosellinic acid (**5**) (19 mg). Crystallization of fraction 3 (MeOH) gave 6-hydroxy-3-methyl-3,4-dihydroisocoumarin-5-carboxylic acid (**3**) (9 mg).

Bioassay Procedures

Antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug resistant strain), which was cultured continuously according to the method of Trager and Jensen.[5] Quantitative assessment of antimalarial activity in vitro was determined by means of the microculture radioisotope technique based upon the method described by Desjardins et al.[6] The inhibitory concentration (IC_{50}) represents the concentration which causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. An IC_{50} value of 1 ng/mL was observed for the standard compound, artemisinin, in the same test system. The cytotoxicity of compounds **1**–**5** was determined, employing the colorimetric method as described by Skehan and co-workers.[7] The reference substance, ellipticine, exhibited activities toward BC-1 and KB cell lines, both with the IC_{50} of 0.3 μg/mL. The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[8] Standard drugs, isoniazid and kanamycin sulfate, the reference compounds for the antimycobacterial assay, showed the minimum inhibitory concentrations (MIC) of 0.040–0.090 and 2.0–5.0 μg/mL, respectively.

Structural Elucidation

Chemical structures of 2-hexylidene-3-methylsuccinic acid (**1**), cytochalasin Q (**2**) and 5-carboxymellein (**3**) were elucidated by analyses of ^{1}H, ^{13}C, DEPT, ^{1}H–^{1}H COSY, HMQC, and HMBC spectral data, and finally confirmed by literature data comparison.[2–4] Although 2-hexylidene-3-methylsuccinic acid (**1**) and 5-carboxymellein (**3**) are well known fungal metabolites, their ^{13}C NMR spectral data have not yet been recorded and assigned. The ^{13}C NMR spectrum (CD_3OD) of compound **1** revealed 11 carbon signals, 14.3 (C-6′), 16.7 (C-7′), 23.5 (C-5′), 29.2 (C-2′), 29.5 (C-3′), 32.6 (C-4′), 39.9 (C-3), 134.9 (C-2), 143.9 (C-1′), 171.6 (C-1), and 178.9 (C-4) (assigned by 2-D NMR techniques), while the ^{13}C NMR spectrum (DMSO-d_6) of 5-carboxymellein (**3**) exhibited 11 signals which were assigned by 2-D NMR experiments as follows: 20.4 (C-2′), 32.2 (C-4), 75.4 (C-3), 109.1 (C-5), 115.5 (C-7), 120.1 (C-8a), 138.4 (C-6), 143.4 (C-4a), 163.9 (C-8), 167.2 (C-1′), and 169.5 (C-1).

The molecular formula of compound **4**[9] was deduced from the ESITOF mass spectrum as $C_{12}H_{20}O_4$, showing an accurate mass at m/z 229.1423 [$(M+H)^+$, Δ −1.7 mmu]. The ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of **4** and those of 2-hexylidene-3-methylsuccinic acid (**1**) were almost superimposed, except for an additional methyl group in **4** (at δ_H 3.69 and δ_C 52.0). The ^{13}C NMR spectrum of compound **4** showed 12 signals, which could be classified, by the DEPT spectra, as two methine, four methylene, three methyl, and three quaternary carbons. The assignment of proton(s) attached to their corresponding carbons was readily accomplished by the HMQC technique. The ^{1}H–^{1}H COSY spectrum of **4** revealed the connectivity from H-1′ through H-6′, and between H-3 and H-7′. The HMBC spectral data of compound **4** demonstrated the correlation of H-1′ to C-1, H-2′ to C-2, H-7′ to C-2 and C-4, and methoxy protons to C-4. The IR spectrum of compound **4** showed absorption peaks at 1729 and 1713 cm^{-1}, confirming the presence of carbonyl functionality in **4**. On the basis of these spectral data, compound **4** was therefore 2-hexylidene-3-methylsuccinic acid 4-methyl ester (**4**). Assignments of protons and carbons in **4** are in Table 1.

The molecular formula of halorosellinic acid (**5**),[10] $C_{25}H_{36}O_6$, was obtained from the ESITOF mass spectrum, showing an accurate mass of m/z 455.2418 [$(M+Na)^+$, Δ +0.8 mmu]. The ^{1}H NMR spectrum (acetone-d_6/D_2O 9:1) of halorosellinic acid (**5**) revealed four methyl groups (two doublets and two singlets), three olefinic protons (at δ_H 5.38, 6.27, and 6.81), two methine protons on carbons bearing oxygen atom (at δ_H 3.83 and 4.48), and a number of methine and methylene protons at δ_H 1.2–3.3. This evidence, together with its ^{13}C NMR spectrum, which showed 25 signals, suggested that halorosellinic acid (**5**) possessed a sesterterpenoid skeleton. Analyses of ^{1}H–^{1}H COSY spectrum of halorosellinic acid (**5**) led to the assignments of the partial structures from H-1 through H-6, from H-8 through H-13, and from H-15 through H-18, and also demonstrated the correlation between H-3 and H-20, and H-15 and H-23. The HMBC spectral data of halorosellinic acid (**5**) demonstrated the correlations of H-2 to C-7, C-11 and C-20; H-4 to C-20; H-5 to C-7; H-8 to C-10 and C-21; H-12 to C-14; H-16 to C-14, C-18 and C-23; H-17

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of 2-hexylidene-3-methylsuccinic acid 4-methyl ester (**4**) ($CDCl_3$) and halorosellinic acid (**5**) (acetone-d_6:D_2O/9:1)

Compound **4**			Halorosellinic acid (**5**)		
C	δ_C, multiplicity[a]	δ_H, multiplicity	C	δ_C, multiplicity[a]	δ_H, multiplicity
1	171.3, s	—	1	35.4, t	1.38 (H-1α); 1.50 (H-1β)
2	131.4, s	—	2	43.7, d	2.44, m
3	37.4, d	3.62, q, 7.0	3	35.9, d	2.04, m
4	174.1, s	—	4	31.6, t	1.45 (H-4α); 1.64 (H-4β)
1′	146.9, d	7.10, t, 7.7	5	26.3, t	1.70 (H-5β); 2.00 (H-5α)
2′	28.7, t	2.22, m	6	38.4, d	3.30, m
3′	28.1, t	1.50, quin, 6.8	7	136.2, s	—
4′	31.5, t	1.33, m	8	131.6, d	6.27, br d, 7.8
5′	22.4, t	1.33, m	9	25.2, t	1.94 (H-9α); 2.53 (H-9β)
6′	13.9, q	0.92, t, 6.7	10	46.3, d	3.30, m
7′	15.7, q	1.38, d, 7.2	11	43.2, s	—
O*CH*$_3$	52.0, q	3.69, s	12	43.6, t	1.76, br d, 15.3 (H-12α); 2.18, br d, 15.3 (H-12β)
			13	118.8, d	5.38, br s
			14	146.1, s	—
			15	32.1, d	2.24, dq, 7.3, 6.7
			16	73.3, d	3.83, dd, 4.3, 7.5
			17	66.2, d	4.48, dd, 4.4, 9.2
			18	137.3, d	6.81, d, 8.9
			19	128.1, s	—
			20	12.1, q	0.85, d, 6.4
			21	169.2, s	—
			22	22.0, q	0.86, s
			23	13.4, q	1.06, d, 6.8
			24	10.5, q	1.82, s
			25	167.4, s	—

[a]Multiplicity was determined by analyses of the DEPT spectra.

to C-19; H-18 to C-19 and C-25; H-24 to C-18, C-19 and C-25. The information from the ^{1}H–^{1}H COSY and HMBC spectral data conclusively revealed an ophiobolane sesterterpenoid skeleton in **5**. The IR spectrum of **5** demonstrated an absorption peak at 1689 cm^{-1}, suggesting the existence of a conjugated carbonyl of a carboxylic acid. Based upon these spectral data, the chemical structure of halorosellinic acid (**5**) was secured. Detailed assignments of protons and carbons of halorosellinic acid (**5**) are shown in Table 1.

Relative stereochemistry of halorosellinic acid (**5**) was successfully assigned by analysis of the NOESY spectral data. An intense cross peak between H-2 and H-6 was observed on the NOESY spectrum, revealing a *cis*-orientation between H-2 and H-6. The following correlations were also observed from the NOESY spectrum: H-2 to H-1β, H-5β and H-12β; H-12β to H-1β, H-2 and H-10; H-10 to H-23 methyl protons, indicating that H-2, H-10 and H-23 methyl group were on the same plane. An intense cross peak observed between H-2 and H-3, but none between H-2 and H-20 methyl protons (α position), implied that H-2 and H-3 were on the same plane of the molecule, and therefore an α orientation of C-20 methyl group. Also, a cross peak between H-12α and H-22 methyl protons (but no correlation between H-10 and H-22) was observed, which indicated a *trans*-relationship between H-10 and C-22 methyl group. The NOESY spectrum of **5** revealed the correlation of H-24 methyl and H-17, but no correlation between H-24 methyl and an olefinic proton (H-18), an indication for an *E* geometry of the double bond at C-18 and C-19. The relative stereochemistry at C-16 and C-17 of halorosellinic acid (**5**) was conclusively assigned by the formation of its acetonide derivative (**6**).[11] The $J_{H\text{-}16,H\text{-}17}$ value of 6.0 Hz indicated a *cis* fused acetonide of **6**. The $J_{H\text{-}15,H\text{-}16}$ value of 10.0 Hz suggested a β orientation of H-16, and this was also confirmed by the NOESY spectrum of **6** (an intense cross peak observed from H-16 to H-23 methyl, but none to H-15). The NOESY spectrum of **6** also revealed the close proximity between H-8 and H-22 methyl, and between H-15 and H-18. Molecular model inspection revealed that ring B of **6** adopted a boat conformation, which is similar to that of ophiobolin K,[12] and the model also clarified the proximity of H-8 and H-22 methyl. Possible conformation in solution of **6**, as well as NOE correlations, is depicted in Figure 1.

Ophiobolane sesterterpenes are reported to be metabolites of fungi and insect wax,[12,13] and are not commonly

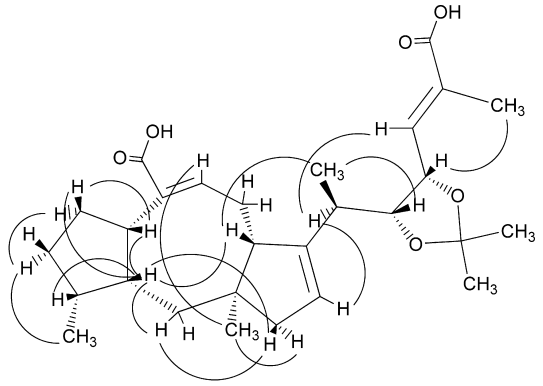

Figure 1. Conformation in solution of an acetonide derivative (**6**) with NOE correlations observed from the NOESY spectrum.

Table 2. Biological activities of compounds **1**–**6**

Compound	Cytotoxicity (IC_{50}, μg/mL)		Antimalaria	Antimycobacteria
	KB[a]	BC-1[b]	(IC_{50}, μg/mL)	(MIC, μg/mL)
1	13	5	Inactive[c]	Inactive[d]
2	3	1	17	Inactive[d]
3	3	3	4	Inactive[d]
4	Inactive[c]	Inactive[c]	Inactive[c]	Inactive[d]
5	Inactive[c]	Inactive[c]	13	200
6	Inactive[c]	Inactive[c]	19	200

[a]Human epidermoid carcinoma in the mouth.
[b]Human breast cancer cells.
[c]Inactive at 20 μg/mL.
[d]Inactive at 200 μg/mL.

found in nature. While the fusion of rings B and C of ophiobolanes always adopts a *trans*-configuration, that of rings A and B can be either *cis* or *trans*. Biological activities of ophiobolanes, including nematocidal, antimicrobial, antimalarial, and cytotoxic activities, have previously been reported.[12,13]

Biological Activities of Compounds 1–5

Cytotoxic, antimalarial, and antimycobacterial activities of the metabolites **1**–**5** isolated from the marine fungus, *H. oceanica* BCC 5149, are shown in Table 2. Compounds **1**–**3** exhibited moderate cytotoxicity against KB and BC-1 cell lines with the IC_{50} values of 1–13 μg/mL, while compounds **4** and **5** were inactive at 20 μg/mL. Substances **2**, **3**, **5**, and **6** showed moderate antimalarial activity with respective IC_{50} values of 17, 4, 13, and 19 μg/mL, whilst halorosellinic acid (**5**) and its acetonide derivative (**6**) possessed only weak antimycobacterial activity at the MIC of 200 μg/mL. Halorosellinic acid (**5**) and its acetonide derivative (**6**) showed similar trends of biological activities, thus, the hydroxyl groups at positions 16 and 17 are not essential for those activities.

Acknowledgements

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References and Notes

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9. 2-Hexylidene-3-methylsuccinic acid 4-methyl ester (**4**). Colorless oil; $[\alpha]_D^{29}$ −15.77° (*c* 0.35, MeOH); UV (MeOH) λ_{max} 212 nm; IR (neat) ν_{max} 3416, 2930, 2859, 1729, 1713, 1695, 1453, 1432, 1414, 1295, 1223, 1158, 1058, 1098, 789 cm^{-1}; ESITOF MS *m*/*z* 229.1423 $(M+H)^+$, calcd for $C_{12}H_{21}O_4$ 229.1440; ^1H and ^{13}C NMR, see Table 1.
10. Halorosellinic acid (**5**). Colorless needles; $[\alpha]_D^{29}$ +20.67° (*c* 0.59, MeOH); UV (MeOH) λ_{max} 207 nm; IR (KBr) ν_{max} 3401, 2957, 2931, 1689, 1456, 1383, 1260, 1227, 1065, 1015, 983, 927, 808, 772 cm^{-1}; ESITOF MS *m*/*z* 455.2418 $(M+Na)^+$, calcd for $C_{25}H_{36}O_6Na$ 455.2410; ^1H and ^{13}C NMR, see Table 1.
11. The reaction mixture comprising of halorosellinic acid (**5**) (4 mg), 2,2-dimethoxypropane (0.6 mL) and *p*-toluenesulfonic acid (0.5 mg) was stirring and left standing at room temperature for 2 h. EtOAc (8 mL) was added to the mixture, which was subsequently washed with H_2O (5×8 mL). The organic layer was dried, yielding ca. 4 mg of an acetonide derivative

(**6**). ESITOF MS *m*/*z* 495.2737 $(M+Na)^+$, calcd for $C_{28}H_{40}O_6Na$; ^1H NMR (acetone-d_6) δ 6.69 (1H, d, $J=10.3$ Hz, H-18), 6.38 (1H, br d, $J=8.1$ Hz, H-8), 5.38 (1H, br s, H-13), 5.02 (1H, dd, $J=10.2$ and 6.0 Hz, H-17), 4.45 (1H, dd, $J=10.0$ and 6.0 Hz, H-16), 3.35 (2H, H-6 and H-10), 2.54 (1H, H-9β), 2.46 (1H, H-2), 2.30 (1H, H-15), 2.18 (1H, H-12β), 2.10 (1H, H-3), 2.07 (1H, H-5α), 1.99 (1H, H-9α), 1.82 (3H, s, H-24), 1.77 (1H, H-12α), 1.75 (1H, H-5β), 1.68 (1H, H-4β), 1.50 (1H, H-4α), 1.49 (1H, H-1β), 1.40 (3H, s, acetonide-Me), 1.37 (3H, s, acetonide-Me), 1.35 (1H, H-1α), 1.15 (3H, d, $J=6.4$ Hz, H-23), 0.88 (3H, d, $J=6.7$ Hz, H-20), and 0.72 (3H, s, H-22).

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