Halorosellins A and B, unique isocoumarin glucosides from the marine fungus *Halorosellinia oceanica*

Maneekarn Chinworrungsee,[a] Prasat Kittakoop,*[a] Masahiko Isaka,[b] Ratchada Chanphen,[b] Morakot Tanticharoen[b] and Yodhathai Thebtaranonth[a,b]

[a] *Department of Chemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
[b] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Science Park, 113, Paholyothin Road, Klong 1, Klong Luang, Prathumthani 12120, Thailand. E-mail: prasat@biotec.or.th; Fax: +662-5646707; Tel: +662-5646700 ext. 3560*

Received (in Cambridge, UK) 12th August 2002, Accepted 4th September 2002
First published as an Advance Article on the web 15th October 2002

Structurally unique isocoumarin glucosides, named halorosellins A (**1**) and B (**2**), were isolated from the EtOAc extract of a broth of the marine fungus *Halorosellinia oceanica*. Other new minor metabolites including 4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**3**), 3-acetyl-7-hydroxy-5-methoxy-3,4-dimethyl-3*H*-isobenzofuran-1-one (**4**) and an ophiobolane sesterterpene, 17-dehydroxyhalorosellinic acid (**5**), were also isolated. Structures of these compounds were elucidated by analyses of spectroscopic data. Compound **4** exhibited mild antimycobacterial activity (MIC value of 200 μg mL^{-1}).

Introduction

As part of our continuing search for biologically active substances conducted at the National Center for Genetic Engineering and Biotechnology,[1–4] we have intensively screened biological activities of extracts from plants and microorganisms. Preliminary results showed that 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^{-1}, and this led us to isolate and identify biologically active principles from this fungus.[4] The marine fungus *Halorosellinia oceanica* is closely related to *Hypoxylon oceanicum*, from which several interesting bioactive compounds were isolated,[5,6] whilst the fungus *Halorosellinia oceanica* has rarely been chemically explored. We report herein our further study concerning the isolation and characterization of two new isocoumarin glucosides, named halorosellin A (**1**) and halorosellin B (**2**), together with other new metabolites, 4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**3**), 3-acetyl-7-hydroxy-5-methoxy-3,4-dimethyl-3*H*-isobenzofuran-1-one (**4**) and 17-dehydroxyhalorosellinic acid (**5**), from the marine fungus *H. oceanica* BCC 5149. Halorosellins A (**1**) and B (**2**) possess an isocoumarin aglycone, whose structure is uniquely decorated with a C-3 methylene group of an isochroman unit.

Results and discussion

A crude EtOAc extract of a culture broth (5 L) of *H. oceanica* BCC 5149 was sequentially chromatographed by sephadex LH-20 column and preparative HPLC (C_{18} reversed phase column), to yield halorosellin A (**1**) (1.87 mg), halorosellin B (**2**) (2.81 mg), compound **3** (2.17 mg), compound **4** (5.18 mg), and 17-dehydroxyhalorosellinic acid (**5**) (1.32 mg).

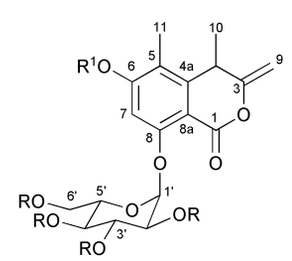
The ESI-TOF mass spectrum of halorosellin A (**1**) gave a molecular formula of **1** of $C_{19}H_{24}O_9$ [observed *m/z* 397.1500 $(M + H)^+$, Δ +0.2 millimass units (mmu)]. The ^1H NMR spectral data (acetone-d_6–D_2O 9 : 1) of halorosellin A (**1**) showed three methyl groups (at δ_H 1.31, 2.10 and 3.90), *exo*-methylene (at δ_H 4.58 and 4.60), an aromatic proton signal (at δ_H 7.09), and a number of protons attached to carbons bearing an oxygen atom (at δ_H 3.43–3.91). The ^1H and ^{13}C NMR spectra demonstrated the presence of a sugar unit in halorosellin A (**1**), showing the characteristics of an anomeric proton and carbon (at δ_H 5.48 and δ_C 100.7, respectively). Analyses of the ^{13}C and DEPT spectra of halorosellin A (**1**) revealed seven methine, two methylene, three methyl, and seven quaternary carbons. The HMQC spectral data of halorosellin A (**1**) assisted in the assignment of protons attached to their corresponding carbon (Table 1), while the ^1H–^1H COSY spectrum demonstrated the correlations from H-1′ through H-6′ and between H-4 and H-10. The HMBC spectrum of halorosellin A (**1**) showed correlations of H-9 to C-3 and C-4; H-10 to C-3 and C-4a; H-4 to C-4a, C-5, C-8a and C-10; H-11 to C-4a, C-5 and C-6; H-7 to C-5, C-6, C-8 and C-8a; and methoxy protons to C-6. Attachment of the unsaturated C-3 to an oxygen atom in **1** was evident from a downfield shift (at δ_C 157.9) of its ^{13}C resonance. Based upon these spectral data, the isocoumarin unit in halorosellin A (**1**) was readily established, and the presence of an *exo*-methylene moiety in the isocoumarin skeleton makes halorosellin A (**1**) structurally unique. Assignment of the relative stereochemistry of the sugar moiety in halorosellin A (**1**) was accomplished by analyses of coupling constants and NOESY spectrum. The *J* values of 3.6, 9.5, 9.3, and 9.4 Hz for $J_{H\text{-}1',H\text{-}2'}$, $J_{H\text{-}2',H\text{-}3'}$, $J_{H\text{-}3',H\text{-}4'}$, and $J_{H\text{-}4',H\text{-}5'}$ indicated equatorial, axial, axial, and axial orientations of H-1′, H-2′, H-3′, and H-4′, respectively; this spectral data suggested that the sugar unit in **1** is α-glucopyranose. Comparison of the ^{13}C NMR data of the sugar unit in **1** with those in the literature[7,8] conclusively confirmed the presence of α-glucopyranose in halorosellin A (**1**). The positive optical rotation ($[\alpha]_D^{30}$ +203.03, *c* 0.066 in EtOH) of **1**, together with the fact that D-glucopyranose was used as a carbon source in a culture medium for the fungus *H. oceanica* BCC 5149, implied that the sugar in **1** is more likely to be α-D-glucose. Acetylation of halorosellin A (**1**) with acetic anhydride in pyridine afforded a tetra-*O*-acetate derivative **1a** whose NMR data fully supported the identity of α-D-glucopyranose in **1**. On the basis of the described data, the chemical structure of halorosellin A (**1**) was therefore secured.

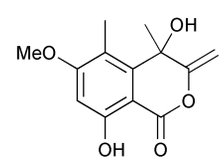
DOI: 10.1039/b207887m

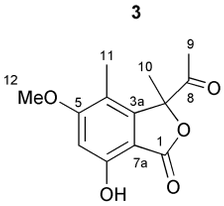
Table 1 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of halorosellins A (**1**) and B (**2**)

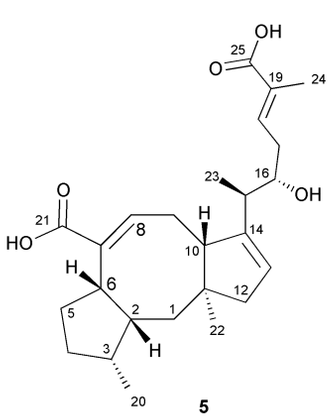
	Halorosellin A (**1**)[b]		Halorosellin B (**2**)[c]	
C	δ_C, multiplicity[a]	δ_H, multiplicity, *J* in Hz	δ_C, multiplicity[a]	δ_H, multiplicity, *J* in Hz
1	161.0, s	—	161.0, s	—
3	157.9, s	—	158.2, s	—
4	35.6, d	4.01, q, 7.0	35.1, d	4.10, q, 7.1
4a	144.8, s	—	143.4, s	—
5	116.9, s	—	117.0, s	—
6	164.1, s	—	163.1, s	—
7	100.8, d	7.09, s	101.5, d	6.73, s
8	160.2, s	—	163.0, s	—
8a	104.0, s	—	100.0, s	—
9	94.3, t	4.58, br s 4.60, br s	95.9, t	4.74, br s 4.75, br s
10	21.6, q	1.31, d, 7.1	22.3, q	1.38, d, 7.1
11	10.1, q	2.10, s	10.1, q	2.19, s
6-OMe	56.5, q	3.90, s	—	—
1′	100.7, d	5.48, d, 3.6	98.3, d	5.68, d, 3.5
2′	72.5, d	3.50, dd, 3.6, 9.5	72.8, d	3.63–3.74, m
3′	74.8, d	3.91, dd, 9.3, 9.3	74.8, d	3.92, dd, 9.0, 9.0
4′	70.6, d	3.43, dd, 9.4, 9.4	77.2, d	3.47–3.57, m
5′	74.5, d	3.83, m	74.7, d	3.47–3.57, m
6′	61.8, t	3.68, dd, 5.9, 11.9 3.81, m	62.1, t	3.63–3.74, m
6-OH	—	—	—	10.92, s

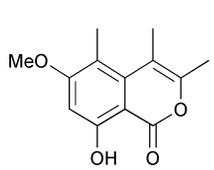
[a] Multiplicity was determined by analyses of DEPT spectra. [b] Acquired in acetone-d_6–D_2O (9 : 1). [c] Acquired in acetone-d_6.











The ^{1}H and ^{13}C NMR spectral data (acetone-d_6) of halorosellin B (**2**) were generally similar to those of halorosellin A (**1**), except that the methoxy signal (at δ_H 3.90, s; δ_C 56.5) in **1** was replaced by a hydroxy resonance (at δ_H 10.92, s) in **2**. A molecular formula of $C_{18}H_{22}O_9$ for halorosellin B (**2**) was deduced from the ESI-TOF mass spectrum [observed *m*/*z* 383.1355 $(M + H)^+$, Δ +1.3 mmu], which indicated that halorosellin B (**2**) was a desmethyl derivative of halorosellin A (**1**). In a similar fashion to that of **1**, protons and carbons in halorosellin B (**2**) were successfully assigned by analyses of the ^{1}H–^{1}H COSY and HMBC spectral data (Table 1). Important ^{1}H–^{13}C long ranged correlations (HMBC) of halorosellin B (**2**) are as follows: H-9 to C-3 and C-4; H-10 to C-3, C-4 and C-4a; H-4 to C-4a, C-5, C-8a, C-9 and C-10; H-11 to C-4a, C-5 and C-6; H-7 to C-5, C-6, C-8 and C-8a; and hydroxy proton to C-5, C-6 and C-7. The HMBC spectrum of halorosellin B (**2**) also showed the correlation of H-1′ to C-8, establishing the linkage between α-D-glucose and the isocoumarin unit.

The structurally unique isochroman unit in **1** and **2** was additionally confirmed by the presence of compounds **3** and **4** in the fungus extract. Chemical structures of the isomeric six and five membered ring lactones, **3** and **4** respectively, were closely related to an aglycone of **1** and **2**. The ^{1}H and ^{13}C NMR spectral data of **3** were similar to those of the aglycones in **1** and **2**, except that compound **3** possessed a singlet methyl (at δ_H 1.67) instead of a doublet methyl as in **1** and **2** (at δ_H 1.31 in **1** and 1.38 in **2**). In addition, a quartet methine signal (at δ_H 4.01 in **1** and 4.10 in **2**) was absent in compound **3**. The ^{13}C and DEPT spectra indicated that C-4 (at δ_C 72.0) in **3** was a quaternary carbon attached to an oxygen atom. The ESI-TOF mass spectrum established the molecular formula of **3** as $C_{13}H_{14}O_5$ [observed *m*/*z* 251.0923 $(M + H)^+$, Δ +0.4 mmu]. Protons and carbons in **3** were assigned by analyses of the ^{1}H–^{1}H COSY and HMBC spectral data. The HMBC spectra of **3** demonstrated long-range correlations of H-9 to C-3 and C-4; H-10 to C-3, C-4 and C-4a; H-11 to C-4a, C-5 and C-6; H-7 to C-5, C-6, C-8 and C-8a; and methoxy protons to C-6. Based on these spectral data, compound **3** was identified as 4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochroman-1-one.

Compound **4** also exhibited the same molecular formula, $C_{13}H_{14}O_5$, as that of **3** (observed *m*/*z* 251.0928 $(M + H)^+$, Δ +0.9 mmu). The ^{1}H NMR spectrum of **4** showed four methyl groups at δ_H 1.69, 1.97, 1.97 and 3.88, and an aromatic proton at δ_H 6.60. Protons and carbons in **4** were readily assigned by analyses of the HMBC spectrum, from which the following correlations were observed: H-6 to C-4, C-5, C-7 and C-7a; H-11 to C-3a, C-4 and C-5; OMe to C-5; Me-9 to C-8; and Me-10 to C-8, C-3 and C-3a. On the basis of these spectral data, compound **4** was identified as 3-acetyl-7-hydroxy-5-methoxy-3,4-dimethyl-3*H*-isobenzofuran-1-one.

Compounds **1**–**4** possess the same aromatic part but different lactone units; it is most likely that their biosynthetic pathways are closely related. However, based upon the spectroscopic data available, the absolute stereochemistry of compounds **1**–**4**

could not be determined due to the limited amount of these minor metabolites (major metabolites being halorosellinic acid, 2-hexylidene-3-methylsuccinic acid, 5-carboxymellein and cytochalasins).[4] The isocoumarins halorosellins A (**1**) and B (**2**) might be biosynthesized from **I** *via* the intermediate **II**,[9–11] *e.g.* a naturally occurring isocoumarin, sclerin A.[10] Dehydration followed by glycosylation of a sclerin A-like intermediate (**II**) gives rise to the formation of halorosellins A (**1**) and B (**2**) (Fig. 1). The presence of a C-3 methylene group on the isochroman unit in **1**–**3** is exceptionally unique; this structural feature has not yet been isolated from a natural environment. Surprisingly, the more stable isomer of the aglycone part, *e.g.* compound **6**, was not obtained from this isolation.

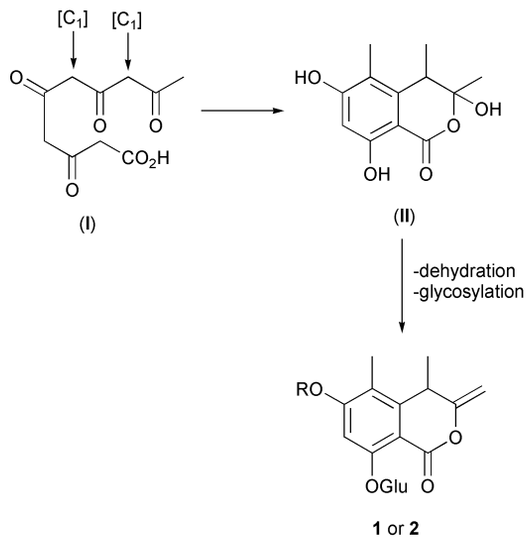


Fig. 1 Possible biosynthetic pathway of halorosellins A (**1**) and B (**2**).

17-Dehydroxyhalorosellinic acid (**5**) was also isolated from the culture broth of the *H. oceanica* BCC 5149, and identified by analyses of spectral data as well as comparison spectral data with those of halorosellinic acid.[4] The ^{1}H NMR spectrum ($CDCl_3$) of **5** was similar to those of halorosellinic acid,[4] except that a hydroxy signal (H-17) of halorosellinic acid was replaced by a methylene resonance in **5**. The molecular formula of compound **5**, $C_{25}H_{36}O_5$, was obtained from the ESITOF mass spectrum (negative ion), showing an accurate mass of *m*/*z* 415.2479 [(M − H)$^-$, Δ −0.6 mmu]. These spectral data indicated that compound **5** was a dehydroxy derivative of halorosellinic acid, and identified as 17-dehydroxyhalorosellinic acid. Analyses of ^{1}H–^{1}H COSY and NOESY spectra of **5**, in combination with spectral data correlation of **5** to those of halorosellinic acid,[4] led to the assignment of protons in **5**, however, the ^{13}C NMR spectrum of **5** could not be recorded due to the limited amount of the isolated substance.

Unfortunately, compounds **1**, **2**, **3** and **5** showed no biological activities (antimalaria, antimycobacterium, antivirus and cytotoxicity against BC-1 and KB cells), while **4** exhibited only mild antimycobacterial activity with the minimum inhibitory concentration (MIC) value of 200 μg mL^{-1}.

Experimental

General

^{1}H, ^{13}C, DEPTs, ^{1}H–^{1}H COSY, NOESY, HMQC, and HMBC experiments were carried out on a Bruker DRX 400 NMR spectrometer, operating at 400 MHz for protons and 100 MHz for carbons. ESI-TOF mass spectra were obtained from a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of the accurate mass. Optical rotations were measured on JASCO DIP 370 polarimeter, while UV spectra were recorded on a Cary 1E UV–VIS spectrophotometer. Optical rotations are given in 10^{-1} deg cm^2 g^{-1}.

Fungal material

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). The fungus was grown in a potato dextrose broth, 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, and harvested for further study.

Extraction and isolation

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, and the EtOAc layers were combined and evaporated to dryness. The crude EtOAc extract (2.3 g) was subsequently chromatographed on Sephadex LH-20 column, and eluted with MeOH to provide three major fractions (Fr. 1–3). Fraction 2 was repeatedly purified on Sephadex LH-20 column, using MeOH as eluent, to give five major fractions (Fr. 2.1–2.5), which were further purified by preparative HPLC. Separation of fraction 2.2 by preparative HPLC (C_{18} reversed phase column, and MeCN–H_2O 40 : 60 as eluent) yielded 1.32 mg of 17-dehydroxyhalorosellinic acid (**5**). Fraction 2.5 was purified by preparative HPLC (MeCN–H_2O 30 : 70 as eluent) to furnish halorosellin B (**2**) (2.81 mg). Fraction 3 was rechromatographed on a Sephadex LH-20 column (MeOH as eluent) to give four major fractions (Fr. 3.1–3.4), which were further purified by preparative HPLC. Fraction 3.2 was subjected to preparative HPLC (MeCN–H_2O 40 : 60) to afford halorosellin A (**1**) (1.87 mg), while fraction 3.4 was subsequently purified by preparative HPLC (MeCN–H_2O 30 : 70), followed by semi-preparative HPLC with a solvent system of MeCN–H_2O (70 : 30), yielding compound **3** (2.17 mg) and compound **4** (5.18 mg).

Halorosellin A (1). Amorphous solid; $[\alpha]_D^{30}$ +203.03 (*c* 0.066, EtOH); UV (EtOH) λ_{max} 222, 268 and 302 nm; ESITOF MS *m*/*z* 397.1500 (M + H)$^+$, calcd. for $[C_{19}H_{24}O_9 + H]^+$ 397.1498; ^{1}H and ^{13}C NMR see Table 1.

Halorosellin A acetate (1a). Amorphous solid; ESITOF MS *m*/*z* 565.1940 [M + H]$^+$, calcd. for $[C_{27}H_{32}O_{13} + H]^+$ 565.1921; ^{1}H NMR ($CDCl_3$) δ_H 6.59 (1H, s H-7), 5.94 (1H, d, *J* = 3.7 Hz, H-1′), 5.94 (1H, t, *J* = 9.5 Hz, H-3′), 5.22 (1H, t, *J* = 9.7 Hz, H-4′), 5.07 (1H, dd, *J* = 3.5, 10.1 Hz, H-2′), 4.70 (1H, H-9a), 4.49 (1H, H-9b), 4.49 (1H, H-5′), 4.27 (1H, H-6′a), 4.09 (1H, dd, *J* = 3.8, 12 Hz, H-6′b), 3.88 (3H, s, OMe), 3.80 (1H, q, *J* = 7.2 Hz, H-4), 2.15 (3H, s, H-11), 2.05–2.09 (12H, OAc), and 1.41 (3H, d, *J* = 7.1 Hz, H-10).

Halorosellin B (2). Amorphous solid; $[\alpha]_D^{30}$ +240.57 (*c* 0.140, EtOH); UV (EtOH) λ_{max} 218, 267 and 315 nm; ESITOF MS *m*/*z* 383.1355 [M + H]$^+$, calcd. for $[C_{18}H_{22}O_9 + H]^+$ 383.1342; ^{1}H and ^{13}C NMR see Table 1.

4,8-Dihydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochroman-1-one (3). Colorless needles; $[\alpha]_D^{29}$ +92.63 (*c* 0.048, EtOH); UV (EtOH) λ_{max} 218, 261 and 306 nm; ESITOF MS *m*/*z* 251.0923 [M + H]$^+$, calcd. for $[C_{13}H_{14}O_5 + H]^+$ 251.0919; ^{1}H NMR (acetone-d_6) δ_H 6.53 (1H, s, H-7), 5.12 (1H, br s, H-9a), 4.88 (1H, br s, H-9b), 3.94 (3H, s, OMe), 2.40 (3H, s, H-11), and 1.67 (3H, s, H-10); ^{13}C NMR δ_C 166.0 (s, C-6), 164.0 (s, C-8), 162.1 (s, C-3), 161.0 (s, C-1), 144.0 (s, C-4a), 117.0

(s, C-5), 100.3 (s, C-8a), 98.7 (d, C-7), 95.1 (t, C-9), 72.0 (s, C-4), 56.6 (q, OMe), 20.1 (q, C-10), and 10.1 (q, C-11).

3-Acetyl-7-hydroxy-5-methoxy-3,4-dimethyl-3*H*-isobenzofuran-1-one (4). Colorless needles; $[\alpha]_D^{29}$ +200.00 (*c* 0.050, EtOH); UV (EtOH) λ_{max} 216, 260 and 303 nm; ESITOF MS *m*/*z* 251.0928 $[M + H]^+$, calcd. for $[C_{13}H_{14}O_5 + H]^+$ 251.0919; ^{1}H NMR (acetone-d_6) δ_H 6.60 (1H, s, H-6), 3.88 (3H, s, OMe), 1.97 (3H, s, H-9), 1.97 (3H, s, H-11), and 1.69 (3H, s, H-10); ^{13}C NMR δ_C 203.4 (s, C-8), 166.0 (s, C-5), 164.0 (s, C-1), 160.0 (s, C-7), 147.8 (s, C-3a), 113.0 (s, C-4), 103.5 (s, C-7a), 100.8 (d, C-6), 90.3 (s, C-3), 56.6 (q, OMe), 23.6 (q, C-9), 20.2 (q, C-10) and 9.9 (q, C-11).

17-Dehydroxyhalorosellinic acid (5). Colorless needles; $[\alpha]_D^{28}$ +42.42 (*c* 0.066, MeOH); UV (MeOH) λ_{max} 210 nm; ESITOF MS *m*/*z* 415.2479 $[M - H]^-$, calcd. for $[C_{25}H_{36}O_5 - H]^-$ 415.2485; ^{1}H NMR ($CDCl_3$) δ_H 7.00 (1H, t, J = 6.3 Hz, H-18), 6.56 (1H, br d, J = 7.42 Hz, H-8), 5.37 (1H, br s, H-13), 3.80 (1H, H-16), 3.32 (1H, H-10), 3.32 (1H, H-6), 2.55 (1H, H-9β), 2.48 (2H, H-17), 2.45 (1H, H-2), 2.26 (1H, H-12β), 2.23 (1H, H-15), 2.11 (1H, H-3), 2.11 (1H, H-5α), 2.11 (1H, H-9α), 1.88 (3H, s, H-24), 1.87 (1H, H-12α), 1.85 (1H, H-5β), 1.68 (1H, H-4β), 1.57 (1H, H-1β), 1.54 (1H, H-4α), 1.39 (1H, H-1α), 1.10 (3H, d, J = 6.8 Hz, H-23), 0.90 (3H, d, J = 6.4 Hz, H-20), and 0.90 (3H, s, H-22).

Bioassays

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.[12] 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.*.[13] 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^{-1} 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 coworkers.[14] The reference substance, ellipticine, exhibited activities toward BC-1 and KB cell lines (both with the IC_{50} of 0.3 μg mL^{-1}). The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[15] 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^{-1}, respectively.

Acknowledgements

We are indebted to the Biodiversity Research and Training Program (BRT) for financial support. We are grateful to the Fermentation Technology Laboratory for mass cultivation of the fungus. Y. T. thanks BIOTEC for the Senior Research Fellowship Award. M. C. acknowledges the Thailand Graduate Institute of Science and Technology (TGIST) for the student's grant.

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