The Journal of Antibiotics (2014), 1–5

www.nature.com/ja

ORIGINAL ARTICLE

Ascochlorin derivatives from the leafhopper pathogenic fungus *Microcera* sp. BCC 17074

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Two new ascochlorin derivatives, nectchlorins A (1) and B (2), together with eight known compounds (3–10), were isolated from cultures of the leafhopper pathogen *Microcera* sp. BCC 17074. The structures were elucidated on the basis of NMR spectroscopic and mass spectrometry data. The absolute configuration of 2 was determined by application of the modified Mosher's method. The absolute configuration of LL-Z 1272α epoxide (9), which is a plausible biosynthetic precursor of ascochlorins, was established by chemical correlations. Cytotoxic activities of these ascochlorin derivatives were evaluated.
The Journal of Antibiotics advance online publication, 2 July 2014; doi:10.1038/ja.2014.90

INTRODUCTION

Invertebrate pathogenic fungi have recently been proved to be unique and prolific sources of structurally diverse bioactive compounds.[1] Although a number of compounds with significant biological activities have been isolated from several genera such as *Cordyceps*, *Beauveria*, *Aschersonia*, *Isaria* and *Hirsutella*,[2] there are still many entomopathogenic genera/species in the order Hypocreales that remain chemically unexplored. As part of our research on bioactive secondary metabolites of invertebrate pathogenic fungi collected in Thailand, a strain of leafhopper pathogen, *Microcera* sp. BCC 17074 of the family Nectriaceae, has been chemically investigated. An extract from this strain exhibited cytotoxicity to KB (oral cavity cancer) cells with an MIC value of 24 μg ml^{-1} and its ^{1}H NMR spectrum suggested the presence of a known antibiotic ascochlorin[3] and several minor derivatives. To our knowledge, there has been no previous report on the bioactive secondary metabolites from this genus. Scale-up fermentation and chemical analysis led to the isolation and structure elucidation of two new ascochlorin-type metabolites, nectchlorins A (**1**) and B (**2**), along with eight known compounds **3–10** (Figure 1).

RESULTS AND DISCUSSION

Nectchlorin A (**1**) was obtained as a pale yellow solid, and the molecular formula was established as $C_{26}H_{35}ClO_6$, from the $[M+H]^+$ ion peak in the HRESIMS. The ^{1}H and ^{13}C NMR spectroscopic data showed resemblance to those of ascochlorin (**7**).[3] The ^{13}C NMR, DEPT135 and HMQC spectroscopic data revealed that it contained an aliphatic ketone (δ_C 213.5), a conjugated aldehyde (δ_C 193.5, δ_H 10.12), an ester carbonyl carbon (δ_C 173.7), seven sp^2 quaternary carbons, an sp^2 methine, an sp^3 quaternary carbon, three methines, five methylenes and six methyl groups (Table 1). In addition, the ^{1}H NMR spectrum showed resonances of two phenolic protons at δ_H 12.67 (s) and 6.40 (s). The 5-chloroorcylaldehyde unit was revealed by the HMBC correlations from a downfield phenolic proton at δ_H 12.67 (2-O*H*) to C-1, C-2 and C-3, from another phenolic proton at δ_H 6.40 (4-O*H*) to C-3, C-4 and C-5, from H_3-7 to C-1, C-5 and C-6 and from H-8 to C-1 and C-2. The planar structure of the sesquiterpene unit from C-9 to C-23, similar to ascochlorin, was elucidated by interpretation of COSY, HMQC and HMBC spectroscopic data. The linkage of the side chain to C-3 of 5-chloroorcylaldehyde was confirmed by the HMBC correlations from H_2-9 to C-2, C-3 and C-4 and the correlation from H-10 to C-3. The propionyloxy substituent at C-12 was demonstrated by the HMBC correlation from H-12 (δ_H 5.37) to the ester carbonyl carbon C-1′ (δ_C 173.7). The (*E*)-geometry of the trisubstituted olefin (C-10/C-11) was evident from the NOESY correlations H_2-9/H_3-23 and H-10/H-12. The relative configuration of the tetrasubstituted cyclohexanone was proved to be identical to that of ascochlorin (**7**) and other known analogs on the basis of the NOESY correlations H-15/H-19, H_β-16(axial)/H_3-20, H_3-20/H_3-21 and H_3-20/H_3-22.

The structure of compound **3** was elucidated by similar spectroscopic analysis, and it was identified as the known compound, chloronectrin.[4] Therefore, compounds **1** and **3** were assigned as propionate and acetate derivatives of the known co-metabolite **4**,[5] respectively. Nectchlorin A (**1**) is also closely related to cylindrols A_4 and A_5,[6,7] which are isovalerate and butanoate variants, respectively. Among these analogs, the 12*R* configuration was previously determined for **4** and cylindrol A_4.[6,8] Alkaline hydrolysis (1 M NaOH/dioxane) of chloronectrin (**3**) gave alcohol **4**, which confirmed the 12*R* configuration of **3**. Hydrolysis of a less reactive ester **1** did not take place under the same alkaline conditions. Hydrolysis of **1** under stronger basic conditions or acidic conditions

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Received 14 March 2014; revised 25 April 2014; accepted 1 June 2014

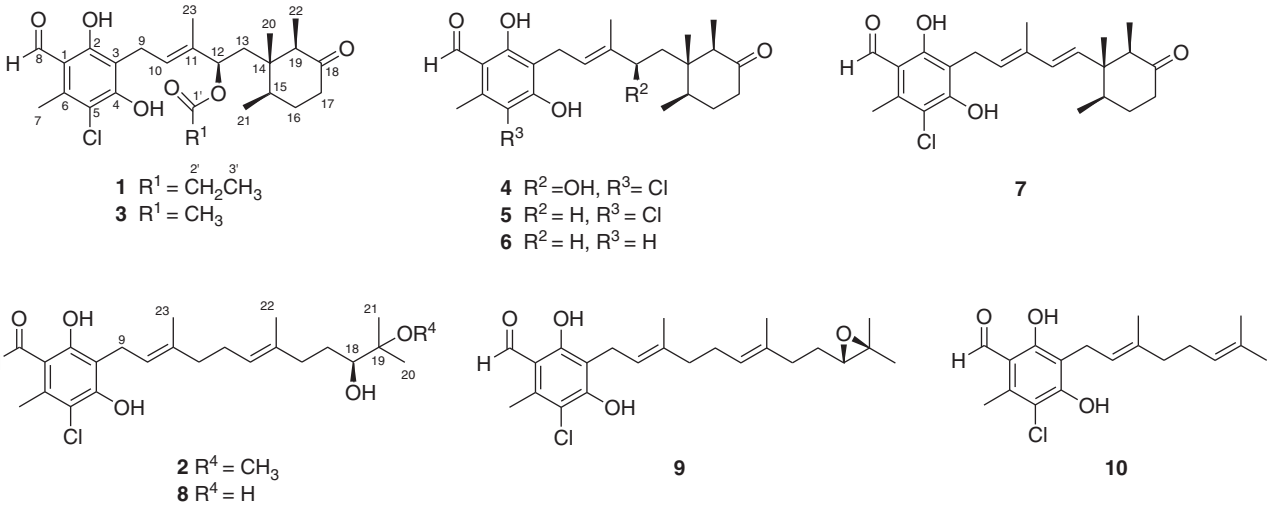


Figure 1 Structures of compounds **1**–**10**.

Table 1 NMR spectroscopic data for 1 and 2 in $CDCl_3$

	1[a]			*2*[b]		
Position	δ_C, *mult.*	δ_H, *mult. (J in Hz)*	*HMBC*	δ_C, *mult.*	δ_H, *mult. (J in Hz)*	*HMBC*
1	113.5, qC			113.6, qC		
2	162.4, qC			162.1, qC		
2-O*H*		12.67, s	1, 2, 3		12.69, s	1, 2, 3
3	113.8, qC			114.5, qC		
4	156.3, qC			156.7, qC		
4-O*H*		6.40, s	3, 4, 5		6.79, s	3, 4, 5
5	113.3, qC			113.3, qC		
6	138.1, qC			137.6, qC		
7	14.7, CH_3	2.59, s	1, 5, 6	14.4, CH_3	2.60, s	1, 5, 6
8	193.5, CH	10.12, s	1, 2	193.2, CH	10.13, s	1, 2
9	21.8, CH_2	3.39-3.37, m	2, 3, 4, 10, 11	22.0, CH_2	3.39, d (7.0)	2, 3, 4, 10, 11
10	124.9, CH	5.56, br t (7.0)	3, 9, 12, 23	121.1, CH	5.20, br t (7.0)	3, 9, 12, 23
11	135.8, qC			136.6, qC		
12	75.9, CH	5.37, dd (7.4, 4.0)	10, 11, 13, 14, 23, 1′	39.6, CH_2	2.01, m	10, 11, 13, 14, 23
13	39.9, CH_2	1.81, m; 1.54, m	11, 12, 14, 15, 19, 20	26.3, CH_2	2.08, m	11, 12, 14, 15
14	44.3, qC			124.5, CH	5.12, br t (6.5)	12, 13, 16, 22
15	36.8, CH	1.91, m	20, 21	134.9, qC		
16	31.4, CH_2	1.78, m; 1.52, m	14, 15, 21	36.7, CH_2	2.24, m; 2.02, m	14, 15, 17, 18, 22
17	41.7, CH_2	2.24, m; 2.17, m	15, 16, 18, 19	29.5, CH_2	1.50, m; 1.39, m	15, 16, 18, 19
18	213.5, qC			76.3, CH	3.42, dd (10.2, 1.4)	16, 19, 21
19	50.6, CH	2.53, q (6.6)	14, 15, 17, 18, 20, 22	77.5, qC		
19-OCH_3				49.1, CH_3	3.22, s	19
20	15.6, CH_3	0.53, s	13, 14, 15, 19	20.7, CH_3	1.11, s	18, 19, 21
21	15.8, CH_3	0.95, d (6.7)	14, 15, 16	18.9, CH_3	1.09, s	18, 19, 20
22	8.2, CH_3	0.79, d (6.6)	14, 18, 19	16.0, CH_3	1.58, s	14, 15, 16
23	12.0, CH_3	1.80, br s	10, 11, 12	16.1, CH_3	1.78, s	10, 11, 12
1′	173.7, qC					
2′	28.2, CH_2	2.30-2.29, m	1′, 3′			
3′	9.3, CH_3	1.13, t (7.6)	1′, 2′			

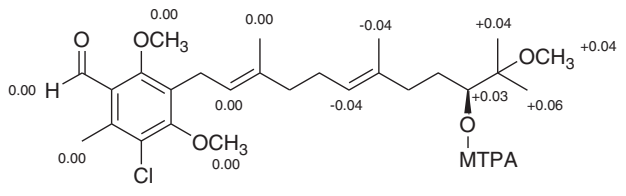
[a]400 MHz for ^1H and 100 MHz for ^{13}C.
[b]500 MHz for ^1H and 125 MHz for ^{13}C.

had been unsuccessful, giving polymeric products. However, close resemblance of the NMR spectroscopic data of **1** with those of **3**, and the co-production with **3** and **4** by the fungus BCC 17074 strongly suggested that **1** should have the same absolute configuration.

The molecular formula of nectchlorin B (**2**) was determined by HRESIMS as $C_{24}H_{35}ClO_5$. The ^1H and ^{13}C NMR spectra suggested close structural resemblance to the known co-metabolite, chlorocylindrocarpol (**8**),[9] although **2** additionally bore a methoxy group

(δ_H 3.22; δ_C 49.1). Detailed interpretation of 2D NMR spectroscopic data revealed that the 5-chloroorcylaldehyde was identical to other ascochlorin derivatives. The sesquiterpene side chain structure was also deduced from 2D NMR (COSY, HMQC and HMBC) data. The location of the methoxy group was assigned by the HMBC correlation from OCH_3 to the quaternary carbon C-19 (δ_C 77.5). The neighboring secondary alcohol functionality was demonstrated by HMBC correlations from H_2-16, H_b-17 (δ_H 1.39), H_3-20 and H_3-21 to the oxymethine at δ_C 76.3 (C-18), and the correlations from H-18 to C-16, C-19 and C-21. The absolute configuration of this secondary alcohol carbon (C-18) was determined by application of the modified Mosher's method.[10] Compound **2** was methylated (MeI, K_2CO_3, 2-butanone) to give its 2,4-*O*-dimethyl derivative, which was then acylated with (*R*)- and (*S*)-MTPA-Cl in pyridine to obtain (*S*)- and (*R*)-MTPA ester derivatives **11a** and **11b**, respectively. The Δδ-values indicated the 18*S*-configuration (Figure 2), which is the same as **8**.[9] Although the absolute configuration of **8** was previously reported,[9] we confirmed it with our sample from the fungus *Microcera* sp. BCC 17074 using the same method as performed for **2**.

LL-Z 1272α epoxide (**9**) was previously isolated from a mutant of *Ascochyta viciae*,[11] but its absolute configuration of the epoxy methine carbon (C-18) remain unassigned. In earlier reports,[12–14] it has been proposed that farnesyl chain of LL-Z 1272α is epoxidized by a specific enzyme and then cyclized to a cyclohexanone ring to finally convert into ascochlorin (**7**). Since the cyclohexanone ring moiety of all known ascochlorin derivatives share the same absolute configuration, it is not unreasonable to assume an enantioselective enzymatic epoxidation of the achiral biosynthetic precursor, LL-Z 1272α, which should be the origin of the absolute configuration of ascochlorin. This hypothesis is consistent with the evidence that **2** and **8**, which are probably derived from **9**, shared the 18*S*-configuration. Nectchlorin B (**2**) could be an artifact from **9** during the isolation procedure using MeOH, especially silica gel column chromatography (CC), although we did not notice such an event. In this context, we examined conversion of **9** into **2** to determine the absolute configuration of **9**. Expected regioselective epoxide cleavage occurred when **9** was treated with *p*-TsOH in MeOH at room temperature for 16 h. The major reaction product (**2**) was purified and it was methylated and then acylated with (*R*)-MTPA-Cl by following the same procedures as described above. The ^{1}H NMR spectrum of the crude acylation product indicated the presence of a (*S*)-MTPA ester **11a** and the absence of *ent*-**11b**, which revealed the 18*S* absolute configuration of the product from acidic epoxide cleavage. Since the C-18–O bond of **9** was retained in the reaction, the absolute configuration of **9** was assigned to be 18*S*. Other ascochlorin-type metabolites isolated from BCC 17074 were identified as LL-Z 1272δ (**5**),[12] LL-Z 1272ε (**6**)[12] and colletochlorin B (**10**).[15]



11a (*S*)-MTPA ester
11b (*R*)-MTPA estert

Figure 2 Δδ-values (δS–δR) of the Mosher esters **11a** and **11b**.

Ascochlorin and its analogs have been known to exhibit broad range of biological activities including antiviral,[3,16] antifungal[7] and cytotoxic[16,17] activities. A variety of specific biological functions related to cancer chemotherapy have also been reported: inhibition of Ras farnesyl-protein transferase,[6] inhibition of mitochondrial cytochrome bc_1 complex,[18] inhibition of matrix metalloproteinase-9 expression[19] and activation of p53 in a manner distinct from DNA damaging agents.[20] New compounds **1** and **2**, their closely related analogs **3** and **8** and ascochlorin (**7**; for comparison) were tested for cytotoxic activities against cancer cell-lines, NCI-H187 (small-cell lung cancer), MCF-7 (breast cancer) and KB (oral cavity cancer), and nonmalignant Vero cells (African green monkey kidney fibroblasts) (Table 2). Cytotoxic activities of **1** and **2** were weaker when compared with ascochlorin (**7**).

METHODS

General experimental procedures

Melting points were measured with an IA9100 digital melting point apparatus (Electrothermal, Essex, UK). Optical rotations were measured with a P-1030 digital polarimeter (JASCO, Tokyo, Japan). UV spectra were recorded on a Cintra 404 spectrophotometer (GBC Scientific Equipment, Braeside, VIC, Australia). Fourier transform infrared spectra were taken on an ALPHA spectrometer (Bruker, Bremen, Germany). NMR spectra were recorded on AV500D and DRX400 spectrometers (Bruker). ESI-time-of-flight mass spectra were measured with a micrOTOF mass spectrometer (Bruker).

Fungal material

The fungus used in this study was isolated from a leafhopper (Hemiptera) collected in Khao Sok National Park, Surat Thani Province, Thailand, by one of the authors (NLH-J). The fungus was deposited in the BIOTEC Culture Collection as BCC 17074. On the basis of the internal transcribed spacer sequence data (GenBank accession number, KF564779) and the results of the BLAST search, this strain was assigned to the genus *Microcera*, within the family Nectriaceae.

Fermentation, extraction and isolation

The fungus BCC 17074 was maintained on potato dextrose agar at 25 °C. The agar was cut into small plugs and inoculated into 3 × 250 ml Erlenmeyer flasks containing 25 ml of potato dextrose broth (potato starch, 4.0 g l^{-1}; dextrose, 20.0 g l^{-1}) and incubated at 25 °C for 6 days on a rotary shaker (200 r.p.m.). Each primary seed culture was transferred into a 1-l Erlenmeyer flask containing 250 ml of potato dextrose broth, and incubated at 25 °C for 5 days on a rotary shaker (200 r.p.m.). The seed cultures were combined and a 700-ml portion was transferred into a 10-l bioreactor containing 6.3 l of potato dextrose broth, and the final fermentation was carried out at 25 °C for 3 days under agitation at 120 r.p.m. and 0.3 vvm aeration rate, then for 2 days under agitation at 250 r.p.m. and 1 vvm aeration rate. The cultures were filtered to separate broth (filtrate) and mycelia (residue). The broth was extracted with

Table 2 Cytotoxic activities of 1–3, 7 and 8

	Cytotoxicity (IC_{50}, μg ml^{-1})			
Compound	*NCI-H187*	*MCF-7*	*KB*	*Vero*
Nectchlorin A (**1**)	>50	>50	17	35
Nectchlorin B (**2**)	40	>50	25	26
Chloronectrin (**3**)	46	39	5.9	21
Ascochlorin (**7**)	1.6	27	30	3.3
Chlorocylindrocarpol (**8**)	26	6.2	26	17
Doxorubicin[a]	0.10	8.6	0.46	—
Ellipticine[a]	1.2	—	0.55	0.36

[a]Standard compounds for cytotoxicity assays.

EtOAc (3 × 6.5 l) and concentrated under reduced pressure to obtain a brown gum (extract A, 371 mg). The wet mycelia were macerated in MeOH (1.1 l, room temperature, 2 days) and filtered. Hexane (1.0 l) and H_2O (300 ml) were added to the MeOH solution and the layers were separated. The hexane (upper) layer was concentrated under reduced pressure to give a pale brown viscous oil (extract B, 380 mg). The H_2O/MeOH (bottom) layer was partially concentrated by evaporation, and the residue was extracted with EtOAc (3 × 700 ml). The combined EtOAc layer was concentrated under reduced pressure to obtain a brown gum (extract C, 1.23 g). Extract C was subjected to CC on silica gel (3.0 × 16 cm, MeOH/CH_2Cl_2, step gradient elution from 0:100 to 5:97) to afford six pooled fractions, fraction C-1–C-6. Fraction C-1 (626 mg) was further fractionated by preparative HPLC using a reversed phase column HPLC (SunFire Prep C_{18} OBD, 19 × 250 mm, 10 μm, Waters Corporation, Milford, MA, USA; mobile phase MeCN/H_2O, 70:30, flow rate 15 ml min^{-1}) to furnish **7** (282 mg), **5** (145 mg), **9** (16.6 mg) and **2** (6.4 mg). Fraction C-4 (178 mg) was also purified by preparative HPLC (gradient elution with MeCN/H_2O from 30:70 to 80:20 over 30 min) to afford **4** (10.4 mg), **8** (16.1 mg), **6** (12.9 mg), **3** (23.1 mg) and **1** (13.1 mg). Extract B was also fractionated by CC on silica gel and preparative HPLC to furnish **5** (62.5 mg), **10** (7.3 mg), **7** (78.5 mg), **9** (31.8 mg), **8** (7.5 mg), **3** (7.0 mg) and **1** (5.3 mg). No unique metabolite was isolated by chromatographic fractionation of extract A.

*Nectchlorin A (**1**):* pale yellow solid; mp 134–135 °C; $[\alpha]^{26}_D + 25$ (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 229 (4.33), 292 (4.17), 345 (4.02) nm; IR (ATR) ν_{max} 1728 sh, 1710, 1629, 1421, 1249, 1188, 755 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 1; HR-MS (ESI-time-of-flight, positive) *m/z* 479.2184 $[M+H]^+$ (calculated for $C_{26}H_{36}ClO_6$, 479.2195).

*Nectchlorin B (**2**):* colorless oil; $[\alpha]^{26}_D$–12 (*c* 0.32, MeOH); UV (MeOH) λ_{max} (log ε) 228 (4.58), 294 (4.43), 345 (4.14) nm; IR (ATR) λ_{max} 1629, 1420, 1284, 1249, 1079, 751 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 1; HR-MS (ESI-time-of-flight, positive) *m/z* 461.2064 $[M+Na]^+$ (calculated for $C_{24}H_{35}ClNaO_5$, 461.2065).

Alkaline hydrolysis of chloronectrin (3)

To a solution of **3** (1.0 mg) in dioxane (0.5 ml) was added 1-M aqueous NaOH (0.1 ml) and the mixture was stirred at room temperature for 2 h. The mixture was neutralized to pH 3 by addition of 0.1-M HCl and partially concentrated by evaporation. The residual aqueous solution was extracted twice with EtOAc, and the combined organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by CC on silica gel (0–4 % acetone/CH_2Cl_2) to furnish a pale yellow gum (0.7 mg), whose ^1H NMR ($CDCl_3$) spectroscopic and HR-MS data were identical to those of **4**.

Synthesis of the 2,4-*O*-dimethyl derivative of 2 and application of the modified Mosher's method

A mixture of compound **2** (1.5 mg), MeI (20 μl) and K_2CO_3 (20 mg) in 2-butanone (0.2 ml) was stirred at room temperature for 15 h. The mixture was diluted with EtOAc and washed with H_2O, and the organic layer was concentrated *in vacuo* to afford the 2,4-*O*-dimethyl derivative (1.6 mg, pale yellow gum): ^1H NMR (400 MHz, $CDCl_3$) δ 10.41 (1H, s, H-8), 5.16 (1H, t, $J = 6.3$ Hz, H-10), 5.13 (1H, t, $J = 7.0$ Hz, H-14), 3.87 (3H, s, 2-OCH_3 or 4-OCH_3), 3.82 (3H, s, 4-OCH_3 or 2-OCH_3), 3.40 (2H, d, $J = 6.3$ Hz, H-9), 3.39 (1H, m, H-18), 3.21 (3H, s, 19-OCH_3), 2.63 (3H, s, H-7), 2.24 (1H, m, H_a-16), 2.07 (2H, m, H-13), 2.02 (2H, m, H-12), 2.01 (1H, m, H_b-16), 1.79 (3H, s, H-23), 1.58 (3H, s, H-22), 1.49 (1H, m, H_a-17), 1.35 (1H, m, H_b-17), 1.11 (3H, s, H-20), 1.08 (3H, s, H-21). A small portion (0.5 mg) of this reaction product was treated with (−)-(*R*)-MTPA-Cl (10 μl) in pyridine (0.2 ml) at room temperature for 15 h. The mixture was diluted with EtOAc and washed with H_2O and 1-M $NaHCO_3$, and the organic layer was concentrated *in vacuo*. The residue was purified by preparative HPLC (MeCN/H_2O) to afford a (*S*)-MTPA ester derivative **11a** (0.2 mg). Similarly, (*R*)-MTPA ester derivative **11b** was prepared using (+)-(*S*)-MTPA-Cl. It should be noted that the definition of *R* and *S* at C-2 of MTPA switches by esterification of MTPA-Cl, due to the priority order of -COCl > -CF_3 > -COOR.

*(S)-MTPA ester **11a**:* colorless gum; ^1H NMR (400 MHz, $CDCl_3$) partial assignments, δ 10.41 (1H, s, H-8), 5.17 (1H, tm, H-18), 5.16 (1H, m, H-10), 5.02 (1H, m, H-14), 3.86 (3H, s, 2-OCH_3 or 4-OCH_3), 3.81 (3H, s, 4-OCH_3 or 2-OCH_3), 3.57 (3H, s, CH_3 of MTPA), 3.40 (2H, d, $J = 6.2$ Hz, H-9), 3.19 (3H, s, 19-OCH_3), 2.63 (3H, s, H-7), 1.79 (3H, s, H-23), 1.50 (3H, s, H-22), 1.13 (3H, s, H-20), 1.11 (3H, s, H-21).

*(R)-MTPA ester **11b**:* colorless gum; ^1H NMR (400 MHz, $CDCl_3$) partial assignments, δ 10.41 (1H, s, H-8), 5.16 (1H, tm, H-10), 5.14 (1H, m, H-18), 5.06 (1H, m, H-14), 3.86 (3H, s, 2-OCH_3 or 4-OCH_3), 3.81 (3H, s, 4-OCH_3 or 2-OCH_3), 3.55 (3H, s, CH_3 of MTPA), 3.40 (2H, d, $J = 6.1$ Hz, H-9), 3.15 (3H, s, 19-OCH_3), 2.63 (3H, s, H-7), 1.79 (3H, s, H-23), 1.54 (3H, s, H-22), 1.09 (3H, s, H-20), 1.05 (3H, s, H-21).

Transformation of 9 into 2

To a solution of **9** (5.0 mg) in MeOH (0.5 ml) was added *p*-TsOH · H_2O (25 mg) and the mixture was stirred at room temperature for 16 h. The reaction was terminated by addition of 1-M $NaHCO_3$, and the mixture was partially concentrated by evaporation. The residual aqueous solution was extracted with EtOAc and the organic phase was concentrated under reduced pressure to obtain a yellow gum, which was purified by CC on silica gel (0–3 % acetone/CH_2Cl_2) to afford **8** (1.6 mg). The ^1H NMR spectroscopic and ESI-MS data of this reaction product were consistent with those of the natural product **8**.

Biological assays

Cytotoxic activities against KB cells (oral cavity cancer), MCF-7 cells (breast cancer) and NCI-H187 cells (small-cell lung cancer) were evaluated using the resazurin microplate assay.[21] Cytotoxicity to Vero cells (African green monkey kidney fibroblasts) were performed using the green fluorescent protein microplate assay.[22]

ACKNOWLEDGEMENTS

We gratefully acknowledge Financial support from the National Center for Genetic Engineering and Biotechnology (BIOTEC). We also thank Ms Donnaya Thanakitpipattana for assistance in identification of the fungus.

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