The Journal of Antibiotics (2016) 69, 652–655

www.nature.com/ja

NOTE

New cytotoxic trichothecene macrolide epimers from endophytic *Myrothecium roridum* IFB-E012

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The Journal of Antibiotics (2016) **69,** 652–655; doi:10.1038/ja.2016.86; published online 13 July 2016

Since the mixture of verrucarin A and B was obtained as the antibiotic glutinosin, from *Myrothecium verrucaria* in 1946, and pure verrucarin A and B, and roridin A were isolated from *Myrothecium* sp. in 1962, over 100 macrocyclic trichothecenes have been isolated from the culture of various microorganisms such as *Myrothecium* sp., *Stachybotrys* sp., *Cylindrocarpon* sp., *Verticimonosporium* sp., and *Phomopsis* sp.[1–6] These fungal macrocyclic trichothecenes have complex structures and most of them have a 12,13-epoxide group in the sesquiterpenoid moiety.[2–4,6,7] Macrocyclic trichothecenes were classified as verrucarin, roridin, satratoxin, vertisporin and calcarisporin according to the variation of the macrolide ring. Macrocyclic trichothecenes exhibit significant bioactivity, especially anticancer activity, and this initiated further researches on their anticancer mechanism of action[6,8,9] and structure–activity relationships.[3,10]

In our continuous characterization of macrocyclic trichothecenes from the endophyte, *M. roridum* IFB-E012, present in the traditional Chinese medicinal plant, *Artemisia annua* (Asteraceae), a new cytotoxic trichothecene macrolide dihydromyrothecine C (**1**), was isolated as an epimeric mixture due to an unstable cyclic hemiacetal structure. In this paper, we report the structures and bioactivity of the new trichothecene macrolide epimers.

Dihydromyrothecine C (**1**) was isolated as white needles and its molecular formula was deduced as $C_{29}H_{38}O_{11}$ from the quasi-molecular ion at *m/z* 585.2311 (calcd for $C_{29}H_{38}O_{11}Na$ 585.2306) in the positive ESI-HR-MS, indicating 11 degrees of unsaturation. The ^{1}H- and ^{13}C-NMR spectra of **1** revealed that it was a 10,13-cyclotrichothecane-derived macrolide similar to myrothecines A–C,[4] but it had two more hydrogens than myrothecine C. Moreover, the one-dimensional NMR spectra showed that there were duplicated resonances of protons and carbons assigned to the macrocyclic residue (from C-1′ to C-14′) in a ratio of ~4:5. Attempting to separate this mixture was successful at low temperature (Supplementary Figure S9). However, when checked by HPLC immediately after separation without evaporation of the solvent, each separated peak showed exactly the same two peaks as before the separation. This clearly indicated that **1** existed in solution as a quickly equilibrating mixture of two stereoisomers (Figure 1). A close comparison of ^{1}H- and ^{13}C-NMR spectra with those of myrothecine C demonstrated that **1** was identical to myrothecine C[4] except for signals for an extra proton (**1a**: $\delta_{H\text{-}14'} = 5.65$ p.p.m. and **1b**: $\delta_{H\text{-}14'} = 5.03$ p.p.m.) and a fully substituted carbon (**1a**: $\delta_{C\text{-}14'} = 100.0$ p.p.m. and **1b**: $\delta_{C\text{-}14'} = 106.8$ p.p.m.) in place of the C-14′ ester carbonyl at δ 175.6 p.p.m. for myrothecine C, suggesting that **1** was a 14′-carbonyl reduction derivative of myrothecine C. This was confirmed by a set of 2D NMR spectra (HMQC, HMBC and ^{1}H–^{1}H COSY). The HMBC

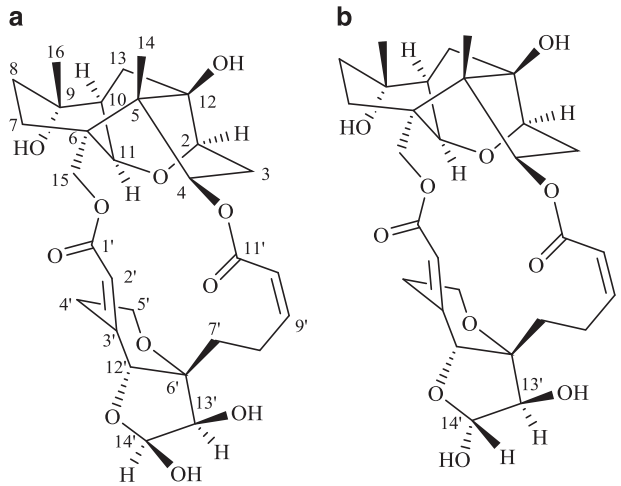

Figure 1 Structure of dihydromyrothecine C (**1**).

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Received 27 February 2016; revised 13 June 2016; accepted 15 June 2016; published online 13 July 2016

spectrum of **1** showed correlations of $\delta_{H\text{-}14'}$ (5.65 for **1a** and 5.03 for **1b**) with $\delta_{C\text{-}12'}$ (84.0 for **1a** and 85.9 for **1b**) and $\delta_{C\text{-}13'}$ (73.9 for **1a** and 79.1 for **1b**), suggesting the formation of a hemiacetal between C-12′ and C-14′. The mixture was determined as the result of the epimerization of this hemiacetal group by coupling constants, COSY and NOESY spectra. The α-orientation of 14′-OH in **1b** was evident by NOE correlations between $\delta_{H\text{-}14'}$ 5.03 (**1b**) and $\delta_{H\text{-}12'}$ 3.95 (**1b**). In addition, the α-orientation of H-13′ was suggested, as both H-13′ (**1b**) and H-14′ (**1b**) are singlets and only a weak NOE correlation between them was observed. The strong COSY and NOE correlations between $\delta_{H\text{-}14'}$ 5.65 (**1a**) and $\delta_{H\text{-}13'}$ 3.66 (**1a**), along with $^3J_{H\text{-}13',\,14'} =$ 3.8 Hz, confirmed that the H-13′ is α-oriented. To confirm this result, a molecular modeling was conducted using Gaussian 09 at b3pw91/6-311g(d,p) level. The optimized conformations were shown in Figure 2. The calculated distances between H-14′/H-12′ (3.5 Å in **1b**), H-14′/H-13′ (2.4 Å in **1a** and 2.7 Å in **1b**), and dihedral angles of $H_{14'}$–$C_{14'}$–$C_{13'}$–$H_{13'}$ (−24.9° in **1a** and 91.0° in **1b**) are consistent with the proposed configuration. Thus, the structure of dihydromyrothecine C was determined as a mixture of the C-14′ stereoisomers, as depicted in Figure 1. The absolute configuration of myrothecines A and C was previously determined by X-ray diffraction and Mosher's method.[4] Dihydromyrothecine C is postulated to follow the same absolute configuration of myrothecines A and C because they are isolated from the culture of the same fungal strain. As methanol was used in the extraction and separation procedure, the stability of **1** in methanol was test by measuring ^{1}H NMR at 5 min, 4 h and 18 h. No change was observed indicating that **1** was stable in methanol.

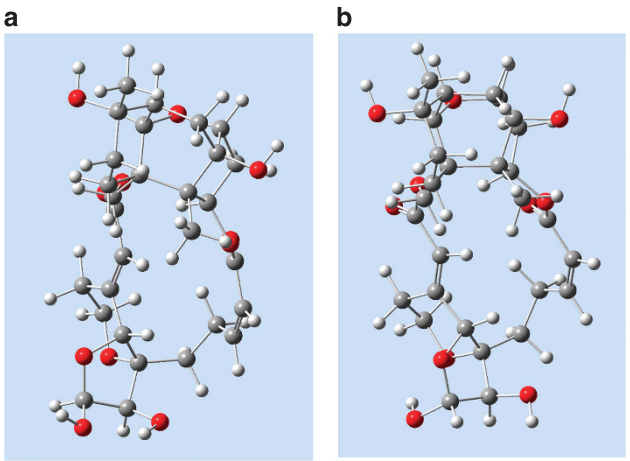
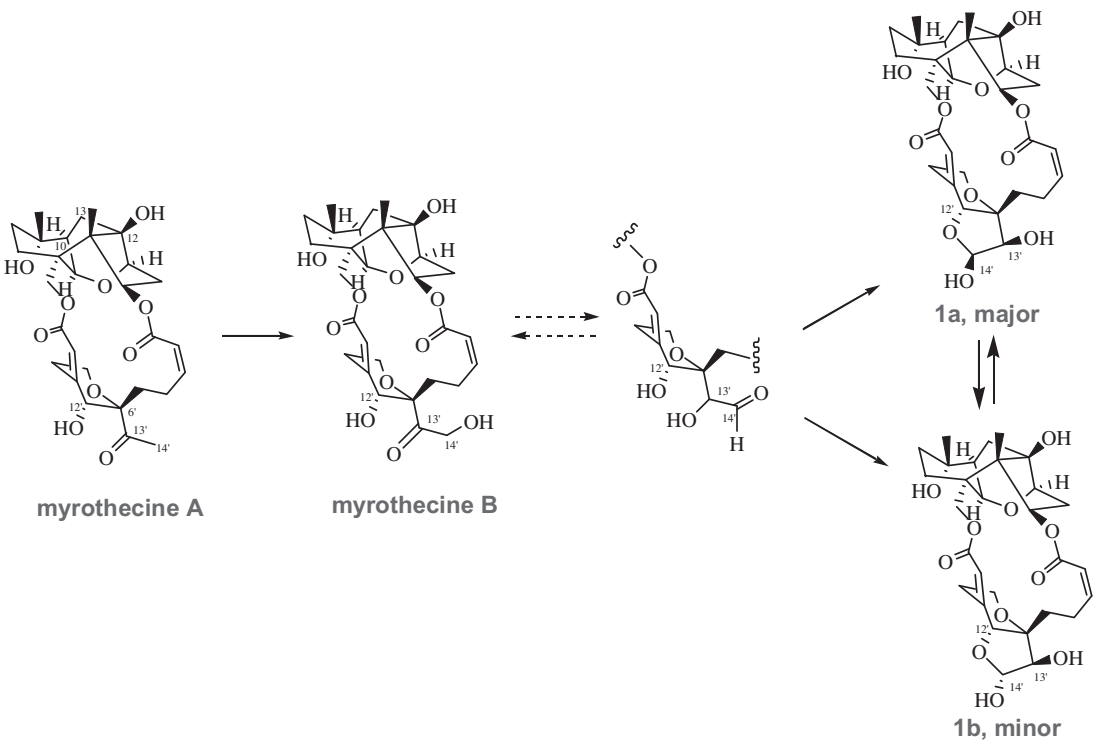


Figure 2 Optimized conformations of dihydromyrothecine C (**1**) at b3pw91/6-311g(d,p) level.

Biosynthetically, it could be hypothesized that hydroxylation of myrothecine A yielded myrothecine B, and tautomerization of myrothecine B followed by acetal formation provided dihydromyrothecine C (**1**) as an equilibrium mixture (Scheme 1). The minimized energies of the conformations of the two epimers of dihydromyrothecine C, 14′*S*- and 14′*R*-isomer, were calculated at b3pw91/6-311g (d,p) level and the conformers are shown in Figure 2. The optimized energies of 14′*S*-isomer and 14′*R*-isomer were −1955.12187 and −1955.12125 a.u. (1 a.u. = 2625.505 kJ mol^{-1}), respectively. The calculation result indicated that 14′*S*-isomer was more stable than 14′*R*-isomer, which was in accordance with the phenomenon that 14′*S*-isomer was the major conformer in solution as determined by the ^{1}H NMR spectrum of **1**. The equilibrium mixture in solution caused by epimerization of cyclic hemiacetals has been reported.[11–13]

The *in vitro* cytotoxicity of **1** against the human nasopharyngeal carcinoma cell line KB was evaluated by the 3-(4,5-dimethylthiazol-2-



Scheme 1 Proposed biosynthesis of dihydromyrothecine C (**1**) . A full color version of this scheme is available at *The Journal of Antibiotics* journal online.

Table 1 ^{1}H-NMR data for 1 and myrothecine C in acetone-d_6 recorded at 500 MHz (δ in p.p.m. and J in Hz)

Position	**1a** (major, 14′S-isomer)	**1b** (minor, 14′R-isomer)	Myrothecine C
2	3.88 (d, 3.9)	3.88 (d, 3.9)	3.89 (d, 3.8)
3α	2.12 (m)	2.12 (m)	2.15 (m)
3β	1.83 (m)	1.83 (m)	1.86 (m)
4	5.12 (dd, 8.2, 3.1)	5.13 (dd, 8.2, 3.1)	5.23 (dd, 8.0, 2.9)
7α	1.58 (m)	1.58 (m)	1.58 (m)
7β	2.15 (m)	2.15 (m)	2.11 (m)
8α	1.35 (br dd, 14.3, 5.2)	1.35 (br dd, 14.3, 5.2)	1.36 (dd, 14.3, 5.2)
8β	1.76 (m)	1.76 (m)	1.82 (m)
10	2.08 (m)	2.08 (m)	2.16 (m)
11	3.56 (d, 3.3)	3.56 (d, 3.3)	3.59 (d, 3.6)
13α	1.85 (m)	1.85 (m)	1.91 (m)
13β	1.55 (dd, 13.8, 5.4)	1.55 (dd, 13.8, 5.4)	1.56 (m)
14	1.14 (s)	1.14 (s)	1.16 (s)
15a	4.45 (d, 11.6)	4.46 (d, 11.6)	4.50 (d, 11.5)
15b	3.58 (d, 11.6)	3.60 (d, 11.6)	3.64 (d, 11.5)
16	1.17 (s)	1.17 (s)	1.19 (s)
2′	5.94 (s)	5.99 (s)	6.26 (s)
4′α	2.53 (m)	2.70 (m)	2.48 (td, 12.8, 5.9)
4′β	3.40 (br d, 12.5)	3.45 (br d, 12.5)	3.55 (br d, 13.0)
5′α	3.83 (m)	3.95 (m)	3.88 (m)
5′β	4.09 (m)	4.14 (m)	4.26 (td, 12.8, 2.2)
7′a	1.75 (m)	1.75 (m)	1.88 (m)
7′b	1.70 (m)	1.70 (m)	1.82 (m)
8′a	2.83 (m)	2.83 (m)	2.89(m)
8′b	1.92 (m)	1.92 (m)	1.95 (m)
9′	6.51 (m)	6.51 (m)	6.55 (m)
10′	5.82 (d, 11.5)	5.82 (d, 11.6)	5.86 (dd, 11.5, 2.1)
12′	4.01 (s)	3.95 (s)	4.61 (s)
13′	3.66 (d, 3.8)	3.73 (s)	3.79 (s)
14′	5.65 (d, 3.6)	5.03 (s)	

Table 2 ^{13}C-NMR data for 1 and myrothecine C in acetone-d_6 recorded at 500 MHz (δ in p.p.m. and J in Hz)

Position	**1a** (major, 14′S-isomer)	**1b** (minor, 14′R-isomer)	Myrothecine C
2	81.3	81.3	84.4
3	41.0	41.0	40.9
4	79.1	79.1	79.1
5	51.5	51.5	51.6
6	44.2	44.2	44.3
7	28.6	28.6	27.6
8	31.4	31.4	31.6
9	72.7	72.7	72.6
10	44.6	44.6	44.8
11	69.8	69.8	69.8
12	77.1	77.1	77.2
13	29.0	29.0	30.4
14	10.6	10.6	10.6
15	73.7	73.7	73.9
16	27.5	27.5	25.8
1′	166.2	166.2	165.7
2′	117.1	117.5	120.2
3′	154.6	154.3	149.5
4′	26.4	26.4	24.4
5′	65.0	65.4	65.0
6′	88.3	87.5	83.8
7′	25.1	24.6	28.7
8′	21.6	21.7	21.5
9′	148.0	148.0	147.9
10′	121.8	121.8	122.1
11′	166.9	166.9	166.8
12′	84.0	85.9	81.3
13′	73.9	79.1	73.0
14′	100.0	106.8	175.6

yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Compound **1** showed moderate cytotoxicity against KB cells with an half maximal inhibitory concentration (IC_{50}) value of 44.48 μM, whereas the control, 5-fluorouracil (5-FU) had an IC_{50} value of 14 μM.

Many researchers have shown that minor structural variation of macrocyclic trichothecenes significantly affected their anticancer activity. 10,13-Cyclotrichothecane macrolides mainly found in symbiotic fungi were proved to be less cytotoxic.[3,4,6] Change of the 2′,3′-double bond to 2′-OH could effectively increase the cytotoxicity, whereas breakage of the macrocyclic ring greatly reduces the activity.[3] Macrocyclic trichothecenes bearing the tetrahydropyranyl ring within the macrolide chain are considerably more potent than other roridins and verrucarins.[14] Reduction of the 7′,8′,9′,10′-diene system diminished cytotoxicity greatly, especially reduction of the 7′,8′-double bond.[14] The stereochemistry at C6′–C13′ also has a role in affecting both selectivity and potency.[3] Therefore, seeking new macrocyclic trichothecenes from fungal metabolites for extensive structure–activity relationship investigation can help to develop new trichothecene macrolide-type anticancer drugs in the future.

EXPERIMENTAL PROCEDURE

General

Melting point was measured on an XT-4 apparatus and was uncorrected. The IR spectrum was determined in a KBr disk on a Nexus 870 FT-IR spectrometer (Thermo Nicolet Corporation, Madison, WI, USA). NMR spectra were acquired on a Bruker DRX-500 spectrometer (Bruker Corporation, Karlsruhe, Germany) using solvent signals as internal standards. ESI-HR-MS was taken on a Mariner Mass 5304 instrument (Applied Biosystems, Lincoln, CA, USA). Silica gel (200–300 mesh) for column chromatography and silica GF_{254} for TLC were produced by Qingdao Marine Chemical Company, Qingdao, China. Octadecylsilyl silica gel was from Nacalai Tesque, Kyoto, Japan. HPLC analysis was performed on an Agilent 1260 (Agilent Technologies Inc., Santa Clara, CA, USA) using a Sinochrom ODS-AP column (5 μm, 250 × 4.6 mm) from Dalian Elite Analytical Instruments Co., Ltd (Dalina, China). The enzyme-linked immunosorbent assay plate reader was from Sunrise, Tecan Company, Switzerland. Fetal bovine serum was produced by Hangzhou Sijiqing Co (Hangzhou, China). RPMI-1640 and MTT were purchased from Gibco Co. (New York, NY, USA). 5-FU was provided by the Medical College of Nantong University, China. The human nasopharyngeal carcinoma cell line KB was supplied by Nanjing University, China.

Microorganism

Strain IFB-E012 was isolated from surface-sterilized stems of apparently healthy *A. annua* collected in May 2003 from the coast of the Yangtze River (Nanjing, China). It was identified as *M. roridum* by comparing the morphological character and 18 S ribosomal DNA sequence with those of standard records;[4] the sequences of the strain have been deposited in GenBank (DQ102373). *M. roridum* IFB-E012

is kept at the Institute of Functional Biomolecules, Nanjing University, China.

Extraction and isolation

M. roridum IFB-E012 was cultured in liquid medium and the culture filtrate was extracted exhaustively with ethyl acetate.[4] Evaporation of the solvent from the extract *in vacuo* yielded a black residue (35 g). The extract was dissolved in methanol and H_2O in a ratio of 85 to 15 (v/v) and then kept at − 4 °C overnight. After removing of the waxy substances by filtration and evaporation of the filtrate, a residue (27 g) was obtained. This residue was then chromatographed over a silica gel column eluted successively with $CHCl_3$/MeOH gradient (100:0 → 0:100, v/v) to provide six fractions (Fr.-1 ~ Fr.-6). Fr.-4 (3.0 g) was subjected to further column chromatography fractionation over silica gel with $CHCl_3$/MeOH gradient (100:0 → 100:32, v/v). Then, purification of Fr-4-2 (0.9 g) on ODS silica gel column chromatography eluting with H_2O/MeOH (100:0 → 60:40) gave 10 mg of **1**.

1 (dihydromyrothecine C): white needle crystals (acetone); melting point 208–210 °C; IR (KBr) ν_{max} 3494, 3330, 2963, 1713, 1686, 1253, 1226, 1188, 1155, 1097, 1058, 1020 and 1008 cm^{-1}; ^1H- and ^{13}C-NMR data were given in Tables 1 and 2; (+)-ESI-HR-MS *m*/*z* 585.2311 $[M+Na]^+$ (calcd for $C_{29}H_{38}O_{11}$ 585.2306).

Theoretical calculations

All calculations were performed using the Gaussian 09 suite of programs.[15] For two isomers of dihydromyrothecine C, full geometry optimizations, vibrational frequency calculation as well as energy calculation were performed at the b3pw91/6-311 g(d,p) level of theory.[16,17]

Cytotoxicity assay

In vitro cytotoxicity of **1** was evaluated using the MTT method as described previously.[18]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China (nos 21372191 and 30701045). This article is dedicated to the fond memory of the late Professor Lester Mitscher, a great scholar, teacher and Emeritus Editor of this Journal.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)