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A new antifungal macrolide from *Streptomyces* sp. KIB-H869 and structure revision of halichomycin

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ABSTRACT

A new hygrolidin-type macrolide (**1**), together with one known derivative, oxohygrolidin (**2**), were isolated from the culture of *Streptomyces* sp. KIB-H869. The structure of the new compound was elucidated by means of extensive spectroscopic analysis. Moreover, by the biogenesis ratiocination and NMR data comparison, halichomycin and **2** were confirmed to have the same structure, which corrects the structural misrepresentation of halichomycin. The antifungal activities of the two compounds were evaluated.
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Bacteria of both terrestrial and marine origin have proven to be excellent sources of novel natural products[1] and recent advances in microbial genomics have unequivocally demonstrated that the biosynthetic potential of natural products in bacteria is much higher than previously appreciated.[2] Endophytes or endosymbiotic microorganisms that reside in the tissues of living plants, liverwort, and fungi, are prolific sources for novel natural products.[3] The most frequently isolated endophytes are fungi. As a result, most chemical studies of endophytic organisms have focused on natural products of fungal origin.[3a] Secondary metabolite production by the vast majority of endophytic actinomycetes has therefore not been extensively studied. Consequently, we have initiated a program to discover new natural products from endophytes (mainly actinomycetes) in traditional Chinese medicinal (TCM) plants and extremophiles from un- and underexplored ecological niches.[4]

Screening of extract libraries obtained from different endophytes and extremophiles against several fungal pathogens highlighted *Streptomyces* sp. KIB-H869, an endophyte isolated from the TCM plant *Dracaena cochinchinensis*, as a producer of antifungal toxins. Herein, we describe the isolation and structural elucidation of one new macrolide from this strain, as well as the structure revision of halichomycin by the biogenesis ratiocination and NMR data comparisons.

The fermentation broth (10 L) of the strain *Streptomyces* sp. KIB-H869 was centrifuged to obtain a mycelial cake, which was extracted with acetone. The extract was concentrated in vacuo and the residual aqueous concentrate was then extracted with EtOAc (500 mL × 3). The EtOAc layer (1.2 g) was then applied on repeated Sephadex LH-20 column, preparative HPLC and semipreparative HPLC to yield compounds **1** (2.0 mg) and **2** (42.0 mg).

Compound **1** was obtained as a white, amorphous powder. It gave a $[M+Na]^+$ ion at *m*/*z* 726.4188 in the positive ion HR-ESI-MS, consistent with a molecular formula of $C_{39}H_{61}NO_{10}$, requiring 10 sites of unsaturation. The ^1H NMR spectrum of **1** (Table 1) showed 11 methyl signals at δ_H 3.24, 3.10, 2.06, 1.98, 1.89, 1.07, 1.01, 0.97, 0.94, 0.93, and 0.89, of which two may be assigned for methoxy groups and three be assigned for olefinic methyl groups. Additionally, the signals of five vicinal olefinic protons [δ_H 7.04 (1H, d, *J* = 15.6 Hz), 6.71 (1H, d, *J* = 15.6 Hz), 6.58 (1H, dd, *J* = 15.0, 10.8 Hz), 5.77 (1H, d, *J* = 10.8 Hz), 5.12 (1H, dd, *J* = 15.0, 10.8 Hz)] and two oxygenated methylene groups [δ_H 5.28 (1H, dd, *J* = 11.3, 4.9 Hz) and 5.08 (1H, dd, *J* = 8.0, 0.7 Hz)] were also observed. The ^{13}C and DEPT spectra of **1** suggested 39 carbons, which were classified into 11 methyls, including two methoxy carbons, three

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Table 1
^{1}H and ^{13}C NMR data for compounds **1** and **2**[a] (δ in ppm)

No.	**1**		**2**	
	δ_H (J, in Hz)	δ_C	δ_H (J, in Hz)	δ_C
1		173.0		172.4
2		123.1		123.0
3	7.27 s	148.2	7.25 s	147.8
4		134.9		134.5
5	5.96 d (8.6)	147.2	5.95 d (8.8)	146.7
6	2.56 m	38.6	2.53 m	38.6
7	3.25 m	81.4	3.26 m	81.2
8	1.98 m	40.9	1.85 m	41.1
9	2.03 m	42.7	2.02 m	42.6
10		144.8		144.3
11	5.77 d (10.8)	125.5	5.76 d (10.8)	125.4
12	6.58 dd (15.0, 10.8)	134.4	6.55 dd (14.9, 10.8)	134.1
13	5.12 dd (15.0, 8.0)	127.1	5.13 m	126.8
14	3.97 t (8.0)	85.3	3.93 t (7.8)	85.3
15	5.08 dd (8.0, 0.7)	77.5	5.10 m	76.7
16	1.98 m	40.9	1.99 m	40.5
17	3.49 d (10.3)	71.3	3.74 dd (8.7, 3.7)	73.7
18	2.13 m	39.7	3.06 m	47.6
19		104.8		204.9
20	1.98 m, 1.89 m	30.9	6.26 d (15.8)	129.4
21	5.28 dt (11.3, 4.9)	74.1	6.82 dd (15.8, 8.2)	151.2
22	2.04 m	35.9	2.37 m	44.2
23	3.57 m	74.1	3.37 m	76.8
24	1.61 m, 1.42 m	26.6	1.50 m, 1.32 m	28.5
25	0.97 t (7.4)	11.0	0.93 t (7.5)	10.7
26	2.06 s	14.2	2.03 s	14.1
27	1.98 s	15.5	1.96 s	15.4
28	1.07 d (7.0)	18.3	1.06 d (7.0)	18.3
29	0.93 d (4.9)	22.5	0.92 d (7.3)	22.6
30	1.89 s	20.2	1.85 s	19.9
31	3.24 s	56.1	3.21 s	56.0
32	0.94 d (5.4)	11.5	0.96 d (7.0)	11.3
33	1.01 d (7.0)	7.8	1.10 d (6.9)	9.8
34	0.89 d (7.2)	5.3	1.08 d (6.8)	15.4
35	3.10 s	47.2		
1′		166.2		
2′	6.71 d (15.6)	131.9		
3′	7.04 d (15.6)	137.7		
4′		168.4		

[a] ^{1}H NMR and ^{13}C NMR data were recorded in methanol-d_4 at 600 MHz and 150 MHz, respectively.

methylenes, 18 methines and seven quaternary carbons, containing three carbonyl carbons.

Comparison the NMR data of **1** with those of hygrolidin amide,[5] a 16-membered macrolide which was also isolated from *Streptomyces* in 1984, implied that **1** was a modified macrolide in this type. A methoxy group (δ_H 3.10, δ_C 47.2), which could coincidently balance the molecular weight variation (Δ 14) between **1** and hygrolidin amide, was clearly presented in the NMR spectra of **1**. The downfield shift of C-19 (Δ 5 ppm) in **1** and the long-range correlation from the oxymethyl (δ_H 3.10, Me-35) to the oxygen-bearing quaternary carbon (δ_C 104.8, C-19) disclosed that the methoxy group was attached to C-19 on the tetrahydropyran ring. As shown in Figure 2, the accurate assignments of all protons and carbons for compound **1** were preformed through the correlations in 2D-NMR spectra (^{1}H–^{1}H COSY, HSQC and HMBC, Fig. 2). The HMBC couplings Me-26/C-1, Me-26/C-3, H-3/C-4, Me-27/C-5, Me-28/C-7, Me-30/C-9, Me-30/C-11 and H-15/C-1, along with ^{1}H–^{1}H COSY correlations of H-5/H-6/H7/H-8/H-9 (Me-29) and H-11/H-12/H-13/H-14/H-15, revealed the 16-membered macrolide ring of **1**. Likewise, ^{1}H–^{1}H COSY correlations of H-15/H-16/H-17/H-18 and H-20/H-21/H-22/H-23/H-24/H-25, as well as the HMBC cross peaks Me-32/C-15, H-17/Me-19, H-23/C-19 and Me-34/C-23, demonstrated that the alkyl-substituted tetrahydropyran ring was linked to C-15 on the 16-membered core ring through a branched-chain containing three carbons. Besides, an amidated fumaric acid residue was supported to decorate the tetrahydropyran ring at C-21 by the HMBC spectrum, in which H-21 (δ_H 5.28) correlated to C-1′ (δ_C 166.2), H-3′ (δ_H 7.04) correlated to C-1′ (δ_C 166.2), and H-2′ (δ_H 6.71) correlated to C-4′ (δ_C 168.4), respectively.

ROESY spectrum and coupling constants were used to establish the relative stereochemistry of **1**. Although we have not yet determined its absolute stereochemistry, we envision **1** to possess the same stereochemistry from C-1 to C-18 as the known compound **2** on the basis of its biosynthetic origin and similar NMR data. Thus, as shown in Figure 2, the rest of chiral centers was assigned by the ROESY correlations observed for H-23/Me-35 and H-21/H-23, which displayed that OMe-19, H-23, and H-21 were α-oriented. Hence, the structure of compound **1** was established as shown in Figure 1.

Compound **2** was obtained as a white amorphous powder. It was found to possess the molecular formula $C_{34}H_{54}O_7$ from the HR-ESI-MS data (m/z 597.5591 $[M+Na]^+$, calcd 597.5591), indicating 8 degrees of unsaturation. Acquisition and analysis of NMR data for **2** (Table 1) and comparison to data acquired for the known macrolide suggested that compound **2** might be a 16-membered macrolactone possessing the same structure as that of oxohygrolidin.[6] Interestingly, we found that the NMR data of **2** were coincident with another known natural product halichomycin produced by a strain of *Streptomyces hygroscopicus*, which was obtained from the gastrointestinal tract of a marine fish.[7] This compound is structurally different from **2** and possesses an unprecedented tricyclic hemimacrolactam scaffold. Both **1** and oxohygrolidin belongs to the bafilomycin family, which has a common 16-membered lactone ring with two conjugated diene moieties at the same positions and the same side chains at C-6, C-7, C-8, and C-14.[8] This family contains more than 20 members and has been proved to be biosynthesized by type I polyketide synthase (PKS).[9] The structure diversity of type I PKS comes from the different starter and extender units as well as the cyclization pattern. The proposed wrong structure of halichomycin contains a very unique side chain at C-8 position, indicating a strange extender unit, which has never been reported and present in any other natural products. Kobayashi and Ishibashi also commented that the biosynthetic provenance of halichomycin appeared to be strange.[10] Above information implicated the possible wrong structure assignment of halichomycin.

To clarify our deduction, we measured the detailed 2D NMR data to confirm the structure of **2**. As was the case with compound **1**, the HMBC and ^{1}H–^{1}H COSY correlations of **2** supported a same 16-membered macrolide core ring (Fig. 3). Two spin systems out of the core ring shown in its ^{1}H–^{1}H COSY spectrum, H-21/H-22/H-23/H-24/H-25 and Me-32/H-16/H-17/H-18/Me-33, together with the HMBC correlations from H-20 (δ_H 6.26) and H-18 (δ_H 3.06) to C-19 (δ_C 204.9), and from H-16 (δ_H 1.99) to C-15 (δ_C 76.7), indicated that a side chain containing 13 carbons was attached to the core ring at C-15 via a carbon–carbon bond. Thus, as shown in Figure 1, the structure of compound **2** was determined to be the same with the known 16-membered macrolide oxohygrolidin.[6] The reported NMR data of halichomycin was recorded in $CDCl_3$, while NMR data of our isolate **2** was measured in CD_3OD. This led to the minor mismatch for the NMR data between **2** and halichomycin. Then, ^{1}H and ^{13}C NMR data of **2** were acquired in chloroform, which was identical with reported NMR data of halichomycin (Supplementary Material, Table S1). In the course of thoroughgoing NMR data examination and assignment for **2**, we firstly noticed that the totally overlapped ^{13}C NMR signals for C-32 and C-33 at δ_C 10.4 ppm in $CDCl_3$ were very misleading for the carbon number assignment of halichomycin. Therefore, we conclude that **2** and halichomycin should be the same compound and the structure of halichomycin should be revised to **2** as shown in Figure 1.

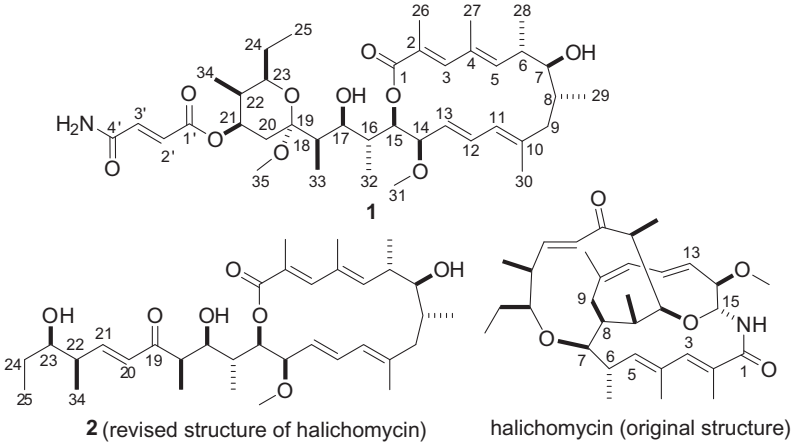

Figure 1. The structures of **1**, **2**, and halichomycin.

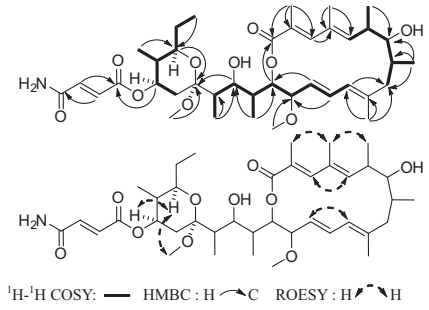

Figure 2. Key ^{1}H–^{1}H COSY, HMBC, and ROESY correlations of **1**.

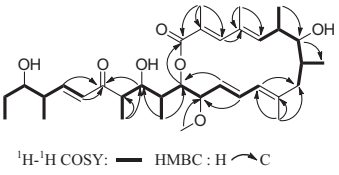

Figure 3. Key HMBC and ^{1}H–^{1}H COSY correlations of **2**.

Table 2
Antifungal activities of compounds **1** and **2**[a]

Test fungi	1	2	Carbendazim[b]
Botrytis cinerea	12.05	0	23.02
Gibberella saubinetti	15.02	0	20.03
F. oxysporum	5.06	0	33.00
F. oxysporum f. sp. *Vasinfectum*	5.08	0	30.53
Fusarium solani	15.02	4.06	24.09

[a] The antifungal activities were evaluated in inhibition zones (mm) at 9.0 μg/disk.
[b] Carbendazim was used as the positive control.

The inhibitory activities of the two isolates were tested in vitro against five plant pathogenic fungi (*Botrytis cinerea*, *Gibberella saubinetti*, *F. oxysporum*, *F. oxysporum* f. sp. *Vasinfectum*, *Fusarium solani*) by the filter paper method (Supporting Information, antifungal bioassay).[11] The results indicated that compound **1** showed broad antifungal activities (Table 2).

In conclusion, we isolated a new hygrolidin-type macrolide (**1**), together with a known derivative, oxohygrolidin (**2**), from the culture of *Streptomyces* sp. KIB-H869. From the biosynthetic consideration and detailed structure elucidation for compound **2**, we strongly predicated that the reported structure of halichomycin had not been correctly assigned, which were further verified by observing the identical NMR data to compound **2** we isolated. Thus, the structure revision was proposed for halichomycin. Nearly over the past twenty years, the structure of halichomycin reported previously has been a hot issue in the area of total synthesis,[12–16] because of its potent cytotoxicity and its extraordinary molecular architecture. To some extent, structure revision of halichomycin will provide a scientific basis for the relevant researches on its total synthesis and biosynthesis.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.02.061.

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