ORGANIC LETTERS

2012
Vol. 14, No. 8
2118–2121

Cordifolide A, a Sulfur-Containing Clerodane Diterpene Glycoside from *Tinospora cordifolia*

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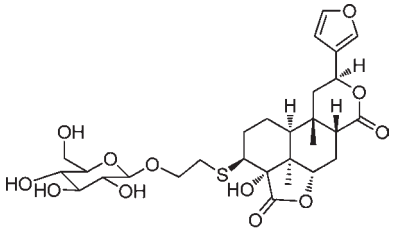
Received March 14, 2012

ABSTRACT



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Cordifolide A (1), a novel unprecedented sulfur-containing clerodane diterpene glycoside, together with other two new diterpene glycosides, cordifolides B (2) and C (3), and four known analogues, was isolated from a methanol-soluble extract of the stems of *Tinospora cordifolia*. The structures of the new compounds were determined on the basis of spectroscopic data interpretation, with that of cordifolide A (1) confirmed by a single-crystal X-ray crystallographic analysis. All isolates were evaluated for their in vitro immunomodulatory activity using mouse bone marrow-derived dentritic cells (BMDCs).

Tinospora cordifolia Miers (Menispermaceae), also known as "Guduchi", is a woody climbing shrub distributed throughout tropical and subtropical areas of India, mainland China, Myanmar, and Sri Lanka.[1] This plant is widely used as a folk medicine in India and the People's Republic of China for its medicinal properties, inclusive of antiallergic, antiarthritic, antidiabetic, anti-inflammatory, antispasmodic, and general tonic effects.[2] The alcoholic and/or the aqueous extracts of the stems of *T. cordifolia* have been evaluated using several different biological models and were found to possess immunomodulatory, endocrine, hypolipidemic, anti-infective, antipyretic, anti-inflammatory, and antioxidant activities.[2] Previous phytochemical studies on this medicinal plant have led to the isolation of alkaloids,[3] clerodane diterpenoids,[4] phenolic derivatives,[5] sesquiterpenes,[6] and sterols.[7]

As part of an ongoing investigation on the discovery of naturally occurring immunomodulatory agents from plants, a $CHCl_3$-soluble extract of the stems of *T. cordifolia* was subjected to investigation and yielded seven clerodane

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10.1021/ol300657h
Published on Web 04/12/2012

diterpene derivatives, of which **1**–**3** (Figure 1) are new compounds, with **1** being an unprecendented sulfur-bearing diterpene glycoside. Herein, we present the isolation and structure elucidation of compounds **1**–**3**, and the biological evaluation of all compounds isolated.

The dried and powdered stems of *T. cordifolia* were obtained from India, in August 2010. The plant material (480 g) was extracted with methanol overnight at rt (3 × 2 L). The methanol solution was concentrated in vacuo (30 g) and partitioned to give hexane- (4.5 g) and $CHCl_3$-soluble extracts (11 g). Fractionation of the $CHCl_3$-soluble partition by column chromatography on silica gel with gradient elution, using CH_2Cl_2/acetone mixtures of increasing polarity, afforded seven subfractions (Fr.1–7). Fr.1 was chromatographed over silica gel with a hexane/acetone gradient to afford columbin[8] (**4**, 15 mg). Fr.4 was purified on an open RP-18 column with MeOH/H_2O (60:40 to 80:20) as the solvent system to yield tinosporaside[4b] (**5**, 27 mg). Fr.5 was chromatographed using RP-18 silica gel with a MeOH/H_2O gradient to furnish eight subfractions (Fr.501–Fr.508). Palmatoside C[9] (**6**, 10 mg) and D[9] (**7**, 25 mg) were crystallized from Fr.505 and Fr.506, respectively. Fr.501 and Fr.502 were subjected to purification by HPLC, using a semipreparative RP-18 column with acetonitrile/H_2O (20:80) and MeOH/H_2O (30:70) as solvent systems, to afford an epimeric mixture of cordifolides B and C (**2**/**3**, 4.0 mg) and cordifolide A (**1**, 3.2 mg), respectively.

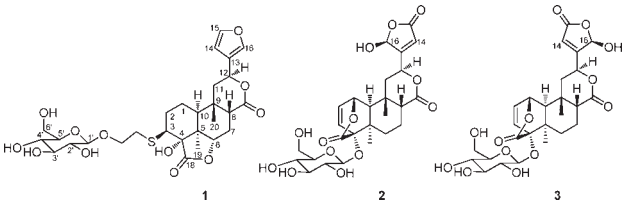

Figure 1. Structures of compounds **1**–**3**.

Cordifolide A (**1**) was obtained as a pale yellow powder and recrystallized in a CD_3OD/CH_3OH solvent mixture to afford colorless prisms.[10] The molecular formula of **1** was established as $C_{28}H_{38}O_{12}S$ based on the accurate sodiated molecular ion peak at *m*/*z* 621.1996 $[M+Na]^+$ (calcd 621.1982) in the HRESIMS. A monosaccharide unit was recognized from the signals observed at δ_H 4.31 (1H, d, J = 7.6 Hz, H-1′; the anomeric proton) and oxygenated protons distributed in the region of δ 3.1–4.0 ppm in the ^{1}H NMR spectrum, as well as the corresponding chemical shifts observed at δ_C 104.6 (CH, C-1′), 75.1 (CH, C-2′), 78.0 (CH, C-3′), 71.5 (CH, C-4′), 77.9 (CH, C-5′), and 62.7 (CH_2, C-6′) in the ^{13}C NMR spectrum. These data were quite comparable with those of the glucosyl residue present in several known clerodane glycosides isolated from the genus *Tinospora*.[11] The β-configuration of the glycosidic linkage was elucidated from the coupling constant (J = 7.6 Hz) of the anomeric proton. Besides the signals of the sugar unit, a β-substituted furan ring was present, as evidenced by the signals of three olefinic protons at δ_H 6.54 (1H, brs, H-14), 7.63 (1H, brs, H-15), and 7.52 (1H, brs, H-16) in the ^{1}H NMR spectrum, which were consistent with the ^{13}C NMR signals of two double bonds at δ_C 126.0 (C, C-13), 109.6 (CH, C-14), 141.6 (CH, C-15), and 145.1 (CH, C-16). Two lactone rings were also present, based on two oxygenated methine protons at δ_H 5.89 (dd, J = 12.8, 4.8 Hz, H-12) and 4.80 (1H, dd, J = 12.8, 4.0 Hz, H-6), in combination with resonances for two carbonyl groups at δ_C 179.7 (C-18) and 175.6 (C-17), and two oxygen-bearing methines at δ_C 72.2 (C-12) and 76.8 (C-6) in the ^{13}C NMR spectrum. In addition to the signals attributed to the furan ring and two lactones, the ^{1}H NMR spectrum of **1** showed signals for two tertiary methyls at δ_H 1.22 (3H, s, H-19) and 1.12 (3H, s, H-20), as well as a number of protons of alkyl methylenes and methines that appeared in the high-field region from 1.4 to 2.9 ppm. In the ^{13}C NMR spectrum, two quaternary carbon signals at δ_C 48.3 and 36.1, and two tertiary carbon signals at δ_C 48.3 and 48.7, were assigned to the ring junction carbon atoms of C-5, C-9, C-8, and C-10, respectively. These characteristic NMR data suggested that **1** is a clerodane diterpene derivative.[4,9–11]

In the ^{13}C NMR spectrum of **1**, besides all signals assigned to the diterpene skeleton, two extra methylene carbon signals occurred at δ_C 34.8 (-CH_2-$\underline{CH_2}$-S) and 70.4 (-$\underline{CH_2}$-CH_2-S). The corresponding protons of these two methylenes appeared at δ_H 2.90 and 2.80 (each 1H, m, -CH_2-$C\underline{H_2}$-S), and δ_H 3.99 and 3.75 (each 1H, m, -$C\underline{H_2}$-CH_2-S) in the ^{1}H NMR spectrum and showed strong COSY correlations to each other. Key HMBC correlations from the anomeric proton of the glucose moiety to δ_C 70.4, and the methylene protons at δ_H 2.90 and 2.80 to δ_C 48.8 (C-3) were observed. Thus, it could be deduced that the glucose unit in **1** is connected with the aglycone through an ethanethiolate functionality. For the aglycone unit, instead of forming a δ-lactone ring between C-1 and C-18, as in most of the other clerodane diterpenes isolated in the current study, a γ-lactone ring occurred between C-18 and C-6 in **1**, with a hydroxy group located on the α-position of the carbonyl functionality, which was supported by HMBC correlations between H_3-19 with C-4 and C-6. The presence of both a six-membered lactone ring and a β-substituted furan ring was confirmed by HMBC correlations between H-8 with C-9 and C-17, H_3-20 with C-8, C-9, and C-11, and H-12 and H-16 with C-13, C-14, and C-15, respectively. The proposed aglycone moiety of **1**, based on the above analysis, is similar to that of borapetol A, a known nonsulfur-containing clerodane

(8) Geraldo Pacheco, A.; Machado de Oliveira, P.; Pilo-Veloso, D.; de Carvalho, A. F. *Molecules* **2009**, *14*, 1245–1262.

(9) Yonemitsu, M.; Fukuda, N.; Kimura, T.; Komori, T. *Liebigs Ann. Chem.* **1987**, *1987*, 193–197.

(10) Cordifolide A (**1**): colorless prisms; mp 185–188 °C (MeOH/MeOH-d_4); $[\alpha]^{20}_D$ +22 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 278 (3.48) nm; IR (KBr) ν_{max} 3418, 1774, 1748, 1645, 1447, 1240, 1073, 1020 cm^{-1}; ^{1}H and ^{13}C NMR data: see Tables 1 and 2; HRESIMS *m*/*z* 621.1996 $[M+Na]^+$ (calcd for 621.1982).

(11) (a) Martin, T. S.; Ohtani, K.; Kasai, R.; Yamasaki, K. *Phytochemistry* **1995**, *40*, 1729–1736. (b) Martin, T. S.; Ohtani, K.; Kasai, R.; Yamasaki, K. *Phytochemistry* **1996**, *42*, 153–158.

first isolated from *Tinospora tuberculata*.[12] It should be indicated that, according to the HSQC and HMBC analysis, two methyl groups at δ_C 18.5 and 33.3 in the ^{13}C NMR spectrum are designated as C-19 and C-20, respectively, which corrects the presumed reversed assignments reported.

Attempts to generate the aglycone of **1** by acid hydrolysis were unsuccessful, due to the paucity of sample obtained. The relative configuration of **1** was established from the NOE effects of H-10/H_3-19, H-6/H-1β, H-8 and H-7β, H_3-20/H-11β and H-8, and H-12/H_3-19 and H-11α, which is consistent with those of known *cis*-clerodane diterpenes (Figure 1). The configuration of the glucose was presumed as D based on biogenetic considerations. No informative cross peaks resulted from a NOESY experiment to assign the relative configuration of C-3 and C-4. Finally, the presence of a sulfur atom, the β-orientation of the glucose-ethanethiolate functionality substituted on C-3, the α-orientation of the hydroxy group on C-4, and the relative configurations of the other chiral centers were established conclusively by X-ray crystallographic analysis (Figure 2).[13] Thus, the structure of **1** was determined as represented, and the trival name, cordifolide A, was given to this compound. Sulfur-containing secondary metabolites are rare in the plant kingdom. To the best of our knowledge, this is the first report of the isolation and identification of a naturally occurring clerodane diterpene containing a sulfur atom in the aglycone.

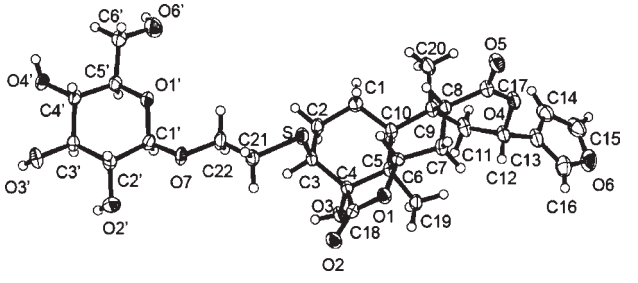

Figure 2. Single-crystal X-ray structure of **1** (drawn with 50% probability displacement ellipsoids; hydrogen atoms are drawn with an artificial radius).

Cordifolides B (**2**) and C (**3**) were isolated as a white powder in the form of a mixture that could not be further separated by any chromatographic methods used in the present study.[14] In the HRESIMS, only one sodiated molecular ion $[M+Na]^+$ was observed at m/z 575.1736 (calcd 575.1741), corresponding to the elemental formula, $C_{26}H_{32}O_{13}$. In the ^1H and ^{13}C NMR spectra of this mixture, the resonances appeared as pairs or were overlapped. The ratio of the amounts of **2** and **3** was estimated to be ~3:2 based on the integration of certain clearly discernible paired protons in the ^1H NMR spectrum, which allowed for the unambiguous assignments of the signals for each compound. Analysis of the NMR spectra revealed that the structures of **2** and **3** are somewhat comparable with that of palmatoside C (**6**),[9] a known diterpene isolated in the current investigation, except for the furan ring located on C-12.

A β-substituted 2-oxy-5-hydroxyfuran ring was proposed in **2**/**3** from the resonances of an olefinic proton at δ_H 6.14/6.20 (1H, brs, H-14) and a hemiacetal proton at 6.22/6.25 (1H, s, H-16) in the ^1H NMR spectrum, as well as corresponding carbon signals of a double bond at δ_C 168.3/167.7 (C-13) and 118.6/119.6 (C-14), a carbonyl group at δ_C 172.0/172.1 (C-15), and a hemiacetal methine carbon at δ_C 98.9/99.4 (CH, C-16) in the ^{13}C NMR spectrum. This deduction was confirmed by HMBC correlations from H-14 to C-12, C-15, and C-16. In the ^1H NMR spectrum, the major differences between **2** and **3** were focused on the protons on the furan ring and the adjacent protons including H-12 and H-11, which implied that **2** and **3** are *epi*-isomers of either the chiral carbon C-12 or C-16. In the NOESY spectrum, the H-12 signal of both compounds was observed to show correlations with H-11α and H-10, which demonstrated the α-orientation of H-12 in **2** and **3**, the same as that of **1**.

The NOE correlations of H-14 and H-16, two protons on the furan ring in **2** and **3**, were quite different, however. For **2**, besides strong correlations observed between H-16 with 11α and H-11β, a NOE correlation could be recognized between H-12 with H-14. In turn, for compound **3**, H-16 was observed to exhibit only a strong correlation with H-12, and H-14 was found to correlate with both H-11α and H-11β. Based on these key NOE observations, it could be deduced that in **2** the OH group at C-16 is on the same side of the molecule as H-11, while in **3** the double bond, C-13(14), is closer to H-11. The above analysis implied that the furan ring located on C-12 is rotated nearly 180° from the C-12−C-13 bond to result in different orientations in **2** and **3** (Figure 3). Furthermore, when the presumed structural differences are taken into consideration, the relative configuration of the hydroxy group on C-16 in **2** and **3** was elucidated as β, based on a NOESY analysis in each case. Thus, the structures of **2** and **3** (cordifolides B and C) were elucidated as shown in Figure 1.

The immunomodulatory properties of all compounds (**1**−**7**) were evaluated by measuring their ability to modulate the surface expression of costimulatory molecules, inclusive of CD40, CD80, and CD86, on bone marrow-derived dendritic cells (BMDCs) (Supporting Information).[15,16] When the lipopolysaccharide (LPS)-stimulated BMDCs were treated

(12) Fukuda, N.; Yonemitsu, M.; Kimura, T.; Hachiyama, S.; Miyahara, K.; Kawasaki, T. *Chem. Pharm. Bull.* **1985**, *33*, 4438–4444.

(13) Crystallographic data for **1**: $C_{28}H_{38}O_{12}S \cdot CH_3OH$, MW = 630.69, Mo K$\alpha$ radiation, colorless rectangular block, size 0.23 × 0.27 × 0.50 mm^3, monoclinic, $P2_1$, a = 10.0464(1), b = 7.8254(1), c = 19.3562(3) Å, β = 102.457(1)°, V = 1485.90(3) Å^3, T = 180 K, Z = 2, μ = 0.177 mm^{-1}, 32 214 collected reflections, 6788 independent reflections (*Rint* = 0.034), *R*1 (all data) = 0.046, *wR*2 (all data) = 0.089, Flack parameter = 0.02(6). Crystallographic data of **1** have been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 870595). Copies of these data can be obtained free of charge via http://www.ccdc.cam.ac.uk/cgi-bin/catreq.cgi.

(14) Mixture of cordifolides B (**2**) and C (**3**): white, amorphous powder; UV (MeOH) λ_{max} (log ε) 278 (3.48) nm; IR (KBr) ν_{max} 3388, 1758, 1678, 1444, 1296, 1133, 1075 cm^{-1}; ^1H and ^{13}C NMR data: see Tables 1 and 2; HRESIMS m/z 575.1736 $[M+Na]^+$ (calcd for 575.1741).

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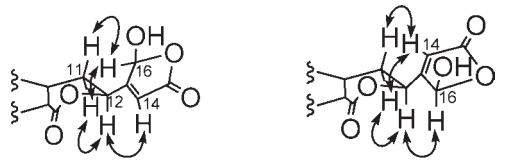
(16) Terrazas, C. A.; Gómez-García, L.; Terrazas, L. I. *Int. J. Parasitol.* **2010**, *40*, 1051–1062.



(a) (b)

Figure 3. Selected key NOESY correlations of compounds **2** and **3**.

Table 1. ^{1}H NMR (400 MHz, MeOH-d_4, J in Hz) Spectroscopic Data for **1**–**3**

position	1	2	3
1α	1.72, m	5.32, d (4.8)	5.29, d (4.8)
1β	1.62, m		
2	2.04[a]	6.63[a]	6.63[a]
3	3.17[a]	6.83[a]	6.83[a]
6α		1.94[a]	1.94[a]
6β	4.80, dd (12.0, 4.0)	1.50, m	1.50, m
7α	2.39[a]	2.54[a]	2.54[a]
7β	2.24, m	2.10, m	2.10, m
8	2.72, dd (12.8, 5.6)	2.57[a]	2.57[a]
10	1.91, dd (14.0, 5.6)	1.75, brs	1.74, brs
11α	2.36[a]	2.40, dd (14.4, 4.4)	2.59[a]
11β	1.99, m	1.91[a]	1.91[a]
12	5.89, dd (12.8, 4.8)	5.55, dd (12.4, 4.0)	5.47, brd (12.4)
14	6.54, brs	6.14, brs	6.20, brs
15	7.63, brs		
16	7.52, brs	6.22, s	6.25, s
19	1.22	1.05	1.06
20	1.12	1.22	1.24
-CH_2-CH_2-S	2.90, m		
	2.80, m		
-CH_2-CH_2–S	3.99, m		
	3.75, m		
1′	4.31, d (7.6)	4.72[a]	4.72[a]
2′	3.17[a]	3.34[a]	3.34[a]
3′	3.31[a]	3.30[a]	3.30[a]
4′	3.30[a]	3.34[a]	3.34[a]
5′	3.31[a]	3.34[a]	3.34[a]
6a′	3.90[a]	3.80 brd (11.5)	3.80 brd (11.5)
6b′	3.69[a]	3.68 dd (11.5, 4.8)	3.68 dd (11.5, 4.8)

[a] Overlapping signals.

with compounds **2**/**3**, **5**, and **7**, a significant upregulation of the surface expression of CD80 and CD86 in each case was observed compared to LPS alone. In contrast, cordifolide

Table 2. ^{13}C NMR (100 MHz, MeOH-d_4) Spectroscopic Data for **1**–**3**

position	1	2	3
1	21.0	74.6	74.7
2	29.5	131.6	131.6
3	48.9	132.7	132.6
4	83.9	87.4	87.4
5	48.3	40.0	40.0
6	76.8	27.0	27.0
7	26.3	18.6	18.5
8	48.3	45.3	45.3
9	36.1	36.3	36.1
10	48.8	47.3	47.5
11	45.2	39.9	39.4
12	72.2	73.9	73.2
13	126.0	168.3	167.7
14	109.6	118.6	119.6
15	141.6	172.0	172.1
16	145.1	98.9	99.4
17	175.6	175.4	175.4
18	179.7	175.1	175.1
19	18.5	24.7	24.7
20	33.3	28.0	28.1
-CH_2-CH_2-S	34.8		
-CH_2-CH_2–S	70.4		
1′	104.6	101.5	101.5
2′	75.1	75.2	75.2
3′	78.0	78.2	78.2
4′	71.5	71.2	71.2
5′	77.9	78.0	78.0
6′	62.7	62.5	62.5

A (**1**) and columbin (**4**) were found to inhibit LPS-induced upregulation of these costimulators, with the suppressing effect on CD40 being more significant than that for CD80 and CD86.

The results demonstrate that the clerodane diterpenes isolated in the present study can modulate the immune response in vitro by promoting the maturation of BMDCs and regulating the expression of LPS-induced costimulatory molecules, which play an important role in immunity against pathogens.

Acknowledgment. This study was supported by Grant RC4 AI092624, awarded to A.R.S. and A.D.K. by NIAID, NIH. We thank Mr. John Fowble, College of Pharmacy, and Dr. Chun-hua Yuan, Campus Chemical Instrumentation Center (CCIC), The Ohio State University (OSU), for facilitating the acquisition of NMR spectra. We are also grateful to Mr. M. E. Apsega and Ms. J. C. Hach of CCIC, OSU, for the mass spectrometric data.

Supporting Information Available. The CIF document of X-ray analysis of **1**, 1D and 2D NMR spectra of **1** and **2**/**3**, a summary of the biological evaluation data of all the isolated compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.