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

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Abstract

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 dendritic 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, antiinflammatory, antispasmodic, and general tonic effects.² The alcoholic and/or the aqueous extracts of the stem of *T. cordifolia* have been evaluated using several different biological models, and were found to possess immunomodulatory, endocrine, hypolipidemic, antiinfective, 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

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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>.

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 diterpene derivatives, of which **1-3** (Fig. 1) are new compounds, with **1** being an unprecedented 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 room temperature (3×2 L). The methanol solution was concentrated in vacuo (30 g) and partitioned to give hexane-soluble (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 columbin⁸ (**4**, 15 mg). Fr. 4 was purified on an open RP-18 column with $\text{MeOH-H}_2\text{O}$ (60:40 to 80:20) as solvent system to yield tinosporaside^{4b} (**5**, 27 mg). Fr. 5 was chromatographed using RP-18 silica gel with a $\text{MeOH-H}_2\text{O}$ gradient to furnish eight subfractions (Fr.501-Fr.508). Palmatoside C⁹ (**6**, 10 mg) and palmatoside D⁹ (**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 semi-preparative RP-18 column with acetonitrile- H_2O (20:80) and $\text{MeOH-H}_2\text{O}$ (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.

Cordifolide A (**1**) was obtained as a pale yellow powder, and was recrystallized in a solvent mixture of CD_3OD and CH_3OH , to afford colorless prisms.¹⁰ The molecular formula of compound **1** was established as $\text{C}_{28}\text{H}_{38}\text{O}_{12}\text{S}$ based on the accurate sodiated molecular ion peak at m/z 621.1996 $[\text{M}+\text{Na}]^+$ (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 ^1H 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 ^1H 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 ^1H NMR spectrum of **1** showed signals for two tertiary methyls at δ_{H} 1.22 (3H, s, H-19) and 1.17 (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 compound **1** is a clerodane diterpene derivative.^{4,9,10,11}

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 ($-\text{CH}_2-\text{CH}_2-\text{S}$) and 70.4 ($-\text{CH}_2-\text{CH}_2-\text{S}$).

The corresponding protons of these two methylenes appeared at δ_{H} 2.90 and 2.80 (each 1H, m, -CH₂-CH₂-S), and δ_{H} 3.99 and 3.75 (each 1H, m, -CH₂-CH-S) in the ¹H 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 compound **1**, with a hydroxy group located on the α -position of the carbonyl functionality, which was supported by HMBC correlations between H₃-19 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, H₃-20 with C-8, C-9 and C-11, as well as H-12 and H-16 with C-13, C-14 and C-15, respectively. The proposed aglycone moiety of compound **1**, based on the above analysis, is similar to that of borapetol A, a known non-sulfur-containing clerodane 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 ¹³C NMR spectrum are designated as C-19 and C-20, respectively, which corrected the possible reverse 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/H₃-19, H-6/H-1 β , H-8 and H-7 β , H₃-20/H-11 β and H-8, and H-12/H₃-19 and H-11 α , which is consistent with those of known *cis*-clerodane diterpenes (Fig 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 position of the hydroxy group on C-4, as well as the relative configurations of the other chiral centers, were established conclusively by X-ray crystallographic analysis, as shown in Figure 2.¹³ Thus, the structure of **1** was determined as represented, and the trivial 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 natural occurring sulfur-containing clerodane diterpene.

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, C₂₆H₃₂O₁₃. In the H- and 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 around 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 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 ¹³C 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 compounds **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-11 α and H-10, which demonstrated the α orientation of H-12 in **2** and **3**, the same as that of compound **1**.

The NOE correlations of H-14 and H-16, two protons on the furan ring in compounds **2** and **3**, were quite different, however. For compound **2**, besides strong correlations observed between H-16 with 11 α and H-11 β , 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-11 α and H-11 β . Based on these key NOE observations, it could be deduced that in compound **2**, the OH group at C-16 is on the same side of the molecule as H-11, while in compound **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 compounds **2** and **3**, as shown in Figure 3. Furthermore, when taking the presumed structural differences 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 compounds **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 co-stimulatory 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 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 A (**1**) and columbin (**4**) were found to inhibit LPS-induced upregulation of these co-stimulators, with the suppressing effect on CD40 being more significant than 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. However, they may also play a detrimental role in autoimmune diseases where the inflammatory response is exacerbated.¹⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- (10). Cordifolide A (**1**): colorless prisms; mp 185–188 °C (MeOH/MeOH-*d*₄); [α]_D²⁰ +22 (*c* 0.1, MeOH); UV (MeOH) $\gamma_{\max}$ (log ϵ) 278 (3.48) nm; IR (KBr) $\nu_{\max}$ 3418, 1774, 1748, 1645, 1447, 1240, 1073, 1020 cm⁻¹; ¹H and ¹³C NMR data: see Tables 1 and 2; HRESIMS *m/z* 621.1996 [M+Na]⁺ (calcd for 621.1982).
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- (14). Mixture of cordifolides B (**2**) and C (**3**): white, amorphous powder; UV (MeOH) $\gamma_{\max}$ (log ϵ) 278 (3.48) nm; IR (KBr) $\nu_{\max}$ 3388, 1758, 1678, 1444, 1296, 1133, 1075 cm⁻¹; ¹H and ¹³C NMR data: see Tables 1 and 2; HRESIMS *m/z* 575.1736 [M+Na]⁺ (calcd for 575.1741).
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Figure 1.
Structures of compounds **1-3**

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

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

Table 1¹H NMR (400 MHz, MeOH-*d*₄, *J* in Hz) Spectroscopic Data for **1-3**

position	1	2	3
1 α	1.72, m	5.32, d (4.8)	5.29, d (4.8)
1 β	1.62, m		
2	2.04 ^a	6.63 ^a	6.63 ^a
3	3.17 ^a	6.83 ^a	6.83 ^a
6 α		1.94 ^a	1.94 ^a
6 β	4.80, dd (12.0, 4.0)	1.50, m	1.50, m
7 α	2.39 ^a	2.54 ^a	2.54 ^a
7 β	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
11 α	2.36 ^a	2.40, dd (14.4, 4.4)	2.59 ^a
11 β	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)

^aOverlapping signals

Table 2¹³C 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