Diterpenes and Phenylpropanoids from *Clerodendrum splendens*

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Key words
- *Clerodendrum splendens*
- Verbenaceae
- clerodane diterpenes
- antiproliferative activity

Abstract

Four new clerodane diterpenes (**1**–**4**) and one new phenylpropanoid (**5**) have been isolated from *Clerodendrum splendens* aerial parts, together with nine known compounds. Their structures were determined by spectroscopic and spectrometric analysis and chemical methods. The absolute configuration of the 15,16-diol moiety in **4** was determined by Snatzke's method. Antiproliferative activity of diterpenes in HeLa cells was also evaluated. The IC_{50} values were 98 ± 11 μM for **3** and 101 ± 8 μM for **1**, respectively.

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Introduction

The genus *Clerodendrum* L., Verbenaceae family, is very widely distributed in tropical and subtropical regions of the world and comprises about 580 species of small trees, shrubs, and herbs [1]. The Latin name used in the beginning was "*Clerodendrum*", then giving place to the Greek form "*Clerodendron*"; in Greek "Klero" means chance and "Dendron" means tree. Later the Latinized name "*Clerodendrum*" was readopted and is now commonly used by taxonomists for the classification and description of the genus and species [2]. Many indigenous systems of medicines used various species of this genus as anti-inflammatory, antidiabetic, antimalarial, antiviral, antihypertensive, and hypolipidemic remedies. The tribal use of *C. inerme* as an antidote of poisoning from fish, crabs, and toads was also known [3]. In the last decade, this genus has attracted the attention of phytochemists due to the many monoterpenes, sesquiterpenes, diterpenes, triterpenes, and iridoids that have been isolated. Particularly, the *Clerodendrum* genus is one of the major sources of *neo*-clerodane diterpenoids, a large group of bioactive natural compounds isolated mainly from Asteraceae, Lamiaceae, and Verbenaceae [3]. *Clerodendrum splendens* G. Don is a climbing evergreen brush plant with attractive red flowers native to Sierra Leone (West Africa) and is used in traditional Nigerian medicine. Decoctions of the roots and leaves are used for the treatment of skin diseases, cancer, syphilis, gonorrhea, ulcers, and various inflammatory diseases [4]. Previous studies on this species reported the presence of flavonoids and *neo*-clerodane diterpenoids [4–7]. As a part of a program to search for bioactive diterpenes, the aerial parts of *C. splendens* were investigated. Four new clerodane diterpenes, 2*α*-acetoxy-3*β*-(2′,3′-diacetoxy-2′-methyl)-butanoyloxy-14-hydro-15-hydroxyclerodin (**1**), 3*β*,15-dihydroxy-14-hydro-clerodin (**2**), 2*α*,15-dihydroxy-3*β*-(2′-hydroxy-2′-methyl-3′-acetoxy)-butanoyloxy-6*α*,18-diacetoxy-4*α*,17-epoxy-clerodan-11,16-lactone (**3**), and 3*β*,14*S*,15-trihydroxy-6*α*,18-diacetoxy-4*α*,17-epoxy-clerodan-11,16-lactone (**4**), as well as one new phenylpropanoid *β*-(3,4-dihydroxyphenyl)ethyl-*O*-*β*-D-xylopyranosyl-(1 → 2)-*α*-L-rhamnopyranosyl-(1 → 3)-6-*O*-*t*-caffeoyl-*β*-D-glucopyranoside (**5**) (**Fig. 1**), along with nine known compounds, 14,15-dihydro-15-hydroxy-3-epicarioptin [8], phlinoside B [9], verbascoside [10], isoacteoside [11], hispidulin-7-*O*-*β*-D-glucopyranoside [12], hispidulin-7-*O*-neohesperoside [13], luteolin-7-*O*-neohesperoside [14], rosmarinic acid [15], and 2*β*-angeloyloxy-5*β*-hydroxy-7*α*,10*β*-methyl-eudesm-3-ene-1-one [16], were isolated. The antiproliferative activity of diterpenes against HeLa tumor cells was also investigated.

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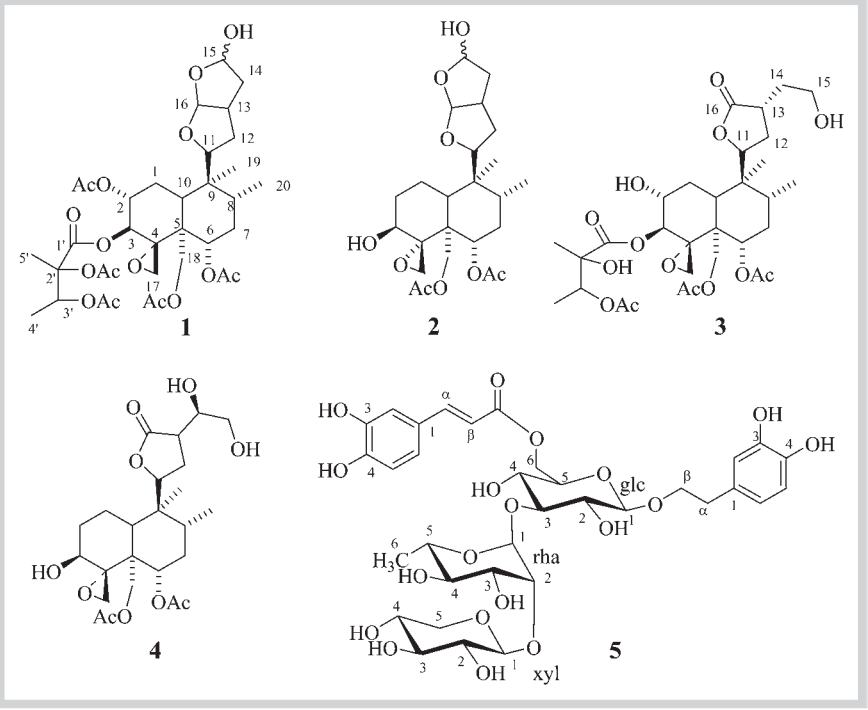

Fig. 1 Structures of compounds **1**–**5**.

Results and Discussion

Compound **1**'s molecular formula was determined as $C_{35}H_{50}O_{16}$ from its HR ESIMS (*m/z* 727.3204 [M + H]$^+$) and ^{13}C NMR data. The ESI MS spectrum of **1** showed a quasimolecular ion peak at *m/z* 749 [M + Na]$^+$ and peaks at *m/z* 689 [M + Na – 60]$^+$, 531 [M + Na – 218]$^+$, 471 [M + Na – 218–60]$^+$, and 411 [M + Na – 218–60–60]$^+$ due to the loss of two acetyl groups and an esterified acyl group. The ^{1}H and ^{13}C NMR spectra (**Table 1**) revealed the presence of five acetyl groups, a methine doublet (δ 1.20, d, J = 6.5 Hz) coupled with a methyne quartet (δ 5.10, q, J = 6.5 Hz), and a methyl singlet (δ 1.58) arisen from a 2,3-diacetoxy-2-methylbutanoyloxy moiety [17], together with the characteristic signals of a *neo*-clerodane diterpene having a 4α,17-oxirane bicycle (δ_H 2.63, 2.91 [H_2-17], δ_C 41.8 [C-17]; δ_C 64.0 [C-4]). Analysis of 2D NMR spectra showed that compound **1** possessed a hexahydrofurofuran ring moiety typical of 14-hydroclerodin [18]. In fact, protons H-11, H-15, and H-16 appeared as a pair of signals: H-11 δ 4.04 and 4.65 (0.5 H each); H-15 δ 5.33 and 5.45 (0.5 H each), and H-16 δ 5.61 and 5.65 (0.5 H each). The ^{13}C NMR spectrum of **1** was in complete agreement with the proposed structure and with the previously reported ^{13}C NMR data of other *neo*-clerodane diterpenes isolated from the genus *Clerodendrum* containing the same C-11/C-16 moiety [8,18]. Indeed, with the help of 1D TOCSY and DQF COSY spectra, it was possible to attribute the values of protons and carbons of both epimers. The elucidation of the whole skeleton was achieved on the basis of 1D TOCSY, DQF COSY, HSQC, and HMBC correlations, which also allowed the assignment of all the resonances in the ^{13}C NMR spectrum of the pertinent carbons. HMBC correlations from H-1 to C-2, C-5 and C-10, from H-3 to C-4 and C-17, from H-16 to C-11, C-13, and C-15, from H-12 to C-13, C-14, and C-16, and from H-18 to C-4, C-5, C-6, and C-10, confirmed the assignment of the *neo*-clerodane ring carbons. The peak correlating signals at δ 5.46 (H-3) and 169.2 ppm, δ 1.58 (Me-5′) and 169.2 and 83.5 ppm, and δ 4.90 (H-2) and 172.1 ppm showed that an acetoxy group and a 2,3-diacetoxy-2-methylbutanoyloxy moiety were attached at C-2 and C-3, respectively. Elucidation of the relative stereochemistry of **1** was mostly based on the close similarities of its NMR data to those of similar compounds [8,19]. Thus, compound **1** was assigned as a mixture of C-15 epimers of 2α-acetoxy-3β-(2′,3′-diacetoxy-2′-methyl)-butanoyloxy-14-hydro-15-hydroxyclerodin.

The molecular formula of compound **2** ($C_{24}H_{36}O_9$) was established by ^{13}C NMR and HR ESIMS spectrum (*m/z* 469.2500 for [M + H]$^+$). In the ESI MS spectrum, two main fragments at *m/z* 425 [M – H – 42]$^-$ and 383 [M – H – 42–42]$^-$ due to the loss of two C_2H_2O moieties were also observed. Its NMR spectral data (**Table 1**) suggested that the structure of **2** resembled that of **1** but differed in the A ring substitutions. The observed differences in the NMR spectra of **2** and **1** were consistent with the presence in **2** of a hydroxyl group at C-3 and no other substituents on ring A. Thus, the structure of **2** was deduced to be a mixture of C-15 epimers of 3β,15-dihydroxy-14-hydro-clerodin.

Compound **3** ($C_{31}H_{46}O_{14}$) showed a quasimolecular ion peak at *m/z* 643.2899 [M + H]$^+$ in the positive HR ESIMS. The prominent fragment ion observed in the ESI MS spectrum at *m/z* 486 [M + Na – 176]$^+$ suggested the presence of a 2′-hydroxy-3′-acetoxy-2′-methylbutanoyl moiety in **3**. Analysis of the ^{1}H and ^{13}C NMR spectra (**Table 1**) of **3** clearly indicated that it also belonged to the *neo*-clerodane diterpenoid class. The ^{1}H NMR spectrum showed signals due to one tertiary methyl (δ 1.02, 3H, s), one secondary methyl (δ 0.93, 3H, J = 6.0 Hz), three acetate residues (δ 1.91. 1.99, and 2.11, each 3H, s), a methyl doublet (δ 1.27, d, J = 6.5 Hz) coupled with a methine quartet (δ 5.12, q, J = 6.5 Hz), and a methyl singlet (δ 1.29, s) arising from the 2′-hydroxy-3′-acetoxy-2′-methylbutanoyloxy moiety at the C-3β position. It also showed the two AB quartets, typical of a primary carbinol methylene group at δ 4.51 and 4.65 (1H, J = 12.0 Hz, H-18a and H-18b), an epoxide methylene group at δ 2.64 and 2.90 (1H, J = 4.0 Hz, H-17a and H-17b), a doublet of doublets at δ 4.82 (1H, J = 11.0, 4.5 Hz, H-6) due to a C-6 proton, a multiplet at δ 3.64 (1H, m, H-2), and a doublet at δ 5.15 (1H, J = 10.0 Hz, H-3) due to a C-3 pro-

Table 1 ^{1}H and ^{13}C NMR data of compounds **1–4** (CD_3OD, 600 MHz, *J* in Hz).

Position	1		2		3		4	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1a	2.75 m	28.0	2.28 m	21.1	2.19 m	32.0	1.85 m	22.7
1b	1.85 m	–	1.75 m	–	1.83 m	–	–	–
2a	4.90[a]	71.6	1.82 m	31.3	3.64 m	71.0	2.12 br dd (11.5, 4.5)	34.3
2b	–	–	1.30 m	–	–	–	1.40 m	–
3	5.46 d (10.5)	71.2	3.98 dd (11.5, 5.0)	65.4	5.15 d (10.0)	74.4	4.02 dd (11.5, 5.5)	66.3
4	–	64.0	–	68.0	–	64.0	–	68.4
5	–	47.0	–	49.4	–	47.2	–	47.9
6	4.87 dd (11.0, 4.5)	72.0	4.80 dd (11.0, 4.5)	71.7	4.82 dd (11.0, 4.5)	72.2	4.84 dd (11.0, 5.0)	73.0
7a	1.92 m	32.4	1.70 m	33.0	1.70 m	33.7	1.75 m	34.3
7b	1.60 m	–	1.42 m	–	1.50 m	–	1.50 m	–
8	1.55 m	37.0	1.52 m	35.8	1.67 m	35.6	1.65 m	35.5
9	–	41.6	–	42.0	–	42.5	–	42.8
10	1.92 dd (10.0, 3.0)	43.0	1.78 dd (10.0, 2.5)	47.6	1.95 dd (9.5, 3.0)	43.1	1.92 dd (10.0, 3.9)	48.0
11	4.65 dd (11.0, 6.2)	84.0	4.63 dd (11.0, 6.2)	83.6	4.70 t (9.0)	89.9	4.72 t (9.0)	88.5
	4.04 dd (12.0, 5.0)	85.1	3.99 dd (12.0, 5.0)	83.7				
12a	1.70 m	33.7	2.06 m	32.8	2.37 br dd (13.0, 9.0)	28.4	2.31 br dd (13.0, 9.0)	23.0
12b	1.48 m	–	1.61 m	–	2.06 m	–	2.22 m	–
13	3.11 m	41.0	2.97 m	40.2	2.86 m	39.9	2.90 m	45.0
14a	2.26 m	40.6	2.07 m	39.7	1.95 m	34.3	4.06 m	72.6
14b	1.78 m	–	1.78 m	–	1.80 m	–	–	–
15a	5.45 br d (5.5)	99.3	5.54 br d (4.6)	98.5	3.72 m	60.0	3.60 dd (12.0, 8.0)	64.9
	5.33 br d (3.5)	99.6	5.45 br d (5.0)	97.9				
15b	–	–	–	–	3.67 m	–	3.49 dd (12.0, 7.0)	–
16	5.65 d (5.5)	109.5	5.71 d (4.7)	106.8	–	181.0	–	181.0
	5.61 d (5.5)	108.2	5.67 d (6.2)	108.7				
17a	2.91 d (4.0)	41.8	2.84 d (3.5)	41.6	2.90 d (4.0)	43.2	2.90 d (4.5)	43.2
17b	2.63 d (4.0)	–	2.72 d (3.5)	–	2.64 d (4.0)	–	2.75 d (4.5)	–
18a	4.82 d (12.3)	62.7	4.91 d (12.5)	61.6	4.65 d (12.0)	62.5	4.94 d (12.0)	62.8
18b	4.52 d (12.3)	–	4.33 d (12.5)	–	4.51 d (12.0)	–	4.36 d (12.0)	–
19	0.94 s	15.0	0.95 s	13.0	1.02 s	13.8	1.03 s	14.0
20	0.96 d (6.0)	15.2	0.92 d (6.0)	15.9	0.93 d (6.0)	16.2	0.96 d (6.5)	16.4
$OCO\underline{CH_3}$	2.10 s	21.3	2.10 s	19.7	2.11 s	21.3	2.12 s	21.2
	2.10 s	21.3	1.93 s	20.2	1.99 s	21.2	1.96 s	21.4
	1.97 s	20.3						
$O\underline{CO}CH_3$	–	171.8	–	172.8	–	172.6	–	172.7
		172.1		171.2		171.5		171.4
		170.0						
1′	–	169.2	–		–	172.0	–	
2′	–	83.5	–		–	77.0	–	
3′	5.10 q (6.5)	73.0	–		5.12 q (6.5)	75.0	–	
4′	1.20 d (6.5)	15.0	–		1.27 d (6.5)	13.6	–	
5′	1.58 s	15.2	–		1.29 s	22.0	–	
$CO\underline{CH_3}$	2.10 s	21.3	–		1.91 s	21.2	–	
	1.92 s	21.2						
$\underline{CO}CH_3$	–	171.9	–		–	171.7	–	
		170.5						

Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments. [a] Overlapped signal

ton. 1D TOCSY and DQF COSY measurements showing couplings between H-11, H_2-12, H-13, H_2-14, and H_2-15 established the spin system C-11–C-15, indicating the presence of a *β*-substituent carrying a five membered lactone ring. The linkage between C-14 and the butanolide lactone ring was deduced by HMBC correlations; the signal at *δ* 4.70 (1H, t, *J* = 9.0 Hz, H-11) correlated to C-8, C-9, C-10, and C-13; *δ* 2.86 (1H, m, H-13) to C-12, C-15, and C-16; *δ* 3.72 (1H, m, H-15a) to C-14 and C-15. Signals attributable to the three acetate groups appeared at *δ* 171.5, 171.7, 172.6, and at *δ* 21.2 (2 CH_3) and 21.3, while the signals due to carbons bearing the acetate groups appeared at *δ* 62.5 (C-18), 72.2 (C-6), and 75.0 (C-3′). The elucidation of the whole skeleton from the above subunits was achieved on the basis of 1D TOCSY, DQF COSY, HSQC, and HMBC correlations, which also allowed the assignment of all the resonances in the ^{13}C NMR spectrum. The relative stereochemistry of rings A and B was established by comparison of NMR data to those of similar compounds [8, 19]; the relative stereochemistry at C-11 and C-13 was indicated by a 2D ROESY experiment showing ROE cross-peaks between Me-20 and H-12a and H-14a and between H-11 and Me-19 and Me-20. Consequently, **3** was characterized as 2*α*,15-dihydroxy-3*β*-(2′-hydroxy-2′-methyl-3′-acetoxy)-butanoyloxy-6*α*,18-diacetoxy-4*α*,17-epoxy-clerodan-11,16-lactone.

Table 2 ^{1}H and ^{13}C-NMR data of compound **5** (CD_3OD, 600 MHz, *J* in Hz).

Position	5	
	δ_H	δ_C
Aglycone 1	–	130.5
2	6.64 d (2.0)	115.9
3	–	146.2
4	–	145.0
5	6.70 d (8.0)	116.8
6	6.56 dd (8.0, 2.0)	121.2
α	3.76 m	36.3
	3.96 m	–
β	2.78 m	72.1
Glc 1	4.36 d (7.8)	104.1
2	3.55 dd (9.0, 7.8)	74.8
3	3.50 t (9.0)	83.8
4	3.45 t (9.0)	70.5
5	3.28 m	75.0
6a	4.52 dd (12.0, 3.0)	64.4
6b	4.36 dd (12.0, 4.5)	–
Rha 1	5.45 d (1.8)	101.6
2	3.99 dd (3.0, 1.8)	82.3
3	3.77 dd (9.0, 3.0)	72.1
4	3.38 t (9.0)	74.0
5	3.98 m	69.7
6	1.24 d (6.5)	17.7
Xyl 1	4.34 d (7.5)	107.3
2	3.38 dd (9.0, 7.5)	74.5
3	3.90 t (9.0)	77.8
4	3.47 m	70.5
5a	3.86 dd (12.0, 3.0)	67.0
5b	3.22 dd (12.0, 4.5)	–
Caffeic acid 1	–	127.8
2	7.05 d (2.0)	115.0
3	–	149.7
4	–	145.6
5	6.80 d (8.0)	116.4
6	6.90 dd (8.0, 2.0)	123.2
α	6.30 d (16.0)	114.7
β	7.58 d (16.0)	147.5
COO	–	169.0

Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments

The HR ESIMS of **4** showed a quasimolecular ion $[M + H]^+$ at *m/z* 485.2423, consistent with a molecular formula of $C_{24}H_{36}O_{10}$. In the ESI MS spectrum, two fragments at *m/z* 447 $[M + Na - 60]^+$ and 387 $[M + Na - 60–60]^+$ indicated the loss of two acetoxy residues. Its NMR spectral data (**Table 1**) suggested that the structure of **4** resembled that of **3** but differed in the shifts of A ring signals and shifts of H-13/C-13, H-14/C-14, H-15/C-15 (2.90/45.0 in **4** vs. 2.86/39.9 in **3**, 4.06/72.6 in **4** vs. 1.95, 1.80/34.3 in **3**, 3.49, 3.60/64.9 in **4** vs. 3.67, 3.72/60.0 in **3**, respectively). In particular ^{1}H and ^{13}C NMR signals due to the A ring atoms were different. The NMR spectra of **4** contained one less hydroxyl group and acyl moiety signals. The 1D TOCSY and DQF COSY showed the presence of a hydroxyl group at C-3. Another difference was the presence in **4** of a 14,15-diol moiety. The absolute configuration of the 14,15-diol moiety of **4** was determined by the circular dichroism (CD) induced after *in situ* complexation with dimolybdenum tetracetate in DMSO solution [20]. According to a rule proposed by Snatzke [21], the sign of the diagnostic band at about 305 nm correlated with the absolute configuration of the chiral centers in the 1,2-diol moiety. In particular an *S*-monosubstituted glycol gives rise to a positive Cotton effect at 305 nm, while a negative sign leads to the assignment of *R*-configuration. Thus, the 305 nm sign observed in the CD spectrum of **4** allowed us to assign the *S*-configuration to C-14. Therefore, compound **4** was assigned the structure of 3*β*,14*S*,15-trihydroxy-6*α*,18-diacetoxy-4*α*,17-epoxy-clerodan-11,16-lactone.

The absolute stereochemistry of compounds **1**–**4** is assumed, while the side chain stereochemistry of **1** and **3** was not determined.

Compound **5**'s molecular formula was determined as $C_{34}H_{44}O_{19}$ by HR ESI mass spectrometry (*m/z* 757.2637 $[M + H]^+$). Its ESI MS spectrum showed a quasimolecular ion peak at *m/z* 779 $[M + Na]^+$. Two main fragments at *m/z* 647 $[M + Na - 132]^+$ and 501 $[M + Na - 132–146]^+$, due to the subsequent loss of one pentose and one deoxyhexose moiety, were also observed. The ^{13}C NMR spectrum (**Table 2**) of **5** displayed 34 signals, of which 17 were assigned to the aglycone moiety; the remaining 17 signals corresponded to two hexoses and one pentose sugar residues. The ^{1}H NMR spectrum (**Table 2**) of **5** displayed proton signals characteristic of a *trans*-caffeoyl group [three aromatic protons resonating at *δ* 6.80 (1H, d, *J* = 8.0 Hz), 6.90 (1H, dd, *J* = 8.0, 2.0 Hz), 7.05 (1H, d, *J* = 2.0 Hz) as an ABX system and two *trans* olefinic protons as an AB system at *δ* 6.30, 7.58 (1H, d, *J* = 16.0 Hz)] and a 3,4-dihydroxyphenylethanol moiety [three aromatic protons at *δ* 6.56 (1H, dd, *J* = 8.0, 2.0 Hz), 6.64 (1H, d, *J* = 2.0 Hz), 6.70 (1H, d, *J* = 8.0 Hz) as an ABX system and an A_2B_2 system assigned to a hydroxyethyl group at *δ* 2.78 (2H, m), 3.76 (1H, m), 3.96 (1H, m)] [22]. Additionally, three signals assignable to anomeric protons indicated the presence of three sugar moieties: a doublet at *δ* 4.36 (1H, *J* = 7.8 Hz, H-1 of glucose), a broad singlet at *δ* 5.45 (1H, *J* = 1.8 Hz, H-1 of rhamnose), and one doublet at *δ* 4.34 (1H, *J* = 7.5 Hz, H-1 of xylose), consistent, with the help of the ^{13}C NMR spectrum, with the following C-1 configuration: *β* for glucose, *α* for rhamnose, and *β* for xylose. These findings matched those in the HSQC/^{13}C NMR spectra, where three corresponding anomeric carbons resonated at *δ* 104.1, 101.6, and 107.3, respectively. Other remaining ^{1}H and ^{13}C NMR signals were assigned with the aid of 2D NMR spectra. The downfield shift of C-3′ (83.8 ppm) of glucose indicated that this position was a glycosylation site. This finding was further confirmed by an HMBC experiment, where a cross-peak between H-1_{rha} (*δ* 5.45) and C-3_{glc} (83.8 ppm) was observed. The carbon resonances assigned to the *β*-xylose unit suggested its terminal position. A further site of connectivity was proved to be C-2 of rhamnose, on the basis of ^{1}H and ^{13}C NMR spectra, as well as HMBC correlations between H-1_{xyl} (*δ* 4.34) and C-2_{rha} (82.3 ppm). The acylation site was on C-6 of glucose as evidenced by the strong deshielding of H-$6a_{glc}$ and H-$6b_{glc}$ at *δ* 4.52 and 4.36 and the cross-peak between H-$6a_{glc}$ and H-$6b_{glc}$ and COO at 169.0 ppm. The configuration of the sugar units was assigned after hydrolysis of **5** with 1 N HCl and GC analysis of trimethylsilylated sugar through a chiral column. On the basis of this data, compound **5** was determined to be the new phenylpropanoid glycoside *β*-(3,4-dihydroxyphenyl) ethyl-*O*-*β*-D-xylopyranosyl-(1 → 2)-*α*-L-rhamnopyranosyl-(1 → 3)-6-*O*-*t*-caffeoyl-*β*-D-glucopyranoside.

On the basis of literature evidence on related compounds [6], the isolated clerodane diterpenes were tested for antiproliferative activity, exposing HeLa cells to 50 µM of each chemical for 24 h and 15 µM of phenethylisothiocyanate (PEITC) as control. Only compounds **3** and **1** displayed any cell growth inhibition activity. Further experiments were performed to study the activity of **1**

and **3** by using different doses (range 20–100 µM). The IC_{50} values calculated from cell viability dose-response curves obtained by incubating HeLa cells for 72 h were 98 ± 11 µM for **3** and 101 ± 8 µM for **1**.

Materials and Methods

General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin-Elmer-Lambda spectrophotometer. CD spectra were measured on a JASCO J-810 spectropolarimeter with a 0.1-cm cell in DMSO at room temperature under the following conditions: speed 50 nm/min, time constant 1 s, bandwidth 2.0 nm. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. 2D NMR spectra were acquired in CD_3OD in the phase-sensitive mode with the transmitter set at the solvent resonance and TPPI (time proportional phase increment) was used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for DQF-COSY, TOCSY, HSQC, HMBC, and NOESY experiments. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using UXNMR software. ESIMS were obtained using a Finnigan LC-Q Advantage Termoquest spectrometer, equipped with Xcalibur software. HRESIMS were acquired in the positive ion mode on a Q-TOF premier spectrometer (Waters-Milford). TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck); compounds were detected by spraying with $Ce(SO_4)_2/H_2SO_4$ (Sigma Aldrich) and NTS (Naturstoffe reagent)-PEG (Poliethylene glycol 4000) solutions. Column chromatography was performed over Sephadex LH-20 (Pharmacia); reversed-phase (RP) HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID10A refractive index detector and a Shimadzu injector, using a C_{18} µ-Bondapak column (30 cm × 7.8 mm) and a mobile phase consisting of MeOH-H_2O mixtures at a flow rate of 2 mL/min. GC analyses were performed using a Dani GC 1000 instrument. Dimolybdenum tetracetate was purchased from Sigma Aldrich.

Plant material

Aerial parts of *C. splendens* G. Don were collected in El Zoharia Research Garden of Cairo, Egypt, in March 2010 and identified by Dr. Mamdouh Shokry (El Zoharia Research Garden, Cairo, Egypt). The voucher specimen (No. 7191 *C. splendens* G. Don/1) was deposited at the Herbarium Hortii Botanici Pisani, Flora Aegyptiaca, Pisa, Italy.

Extraction and isolation

Dried powdered aerial parts of *C. splendens* (775 g) were successively and separately extracted for 48 h with *n*-hexane, $CHCl_3$, $CHCl_3$–MeOH (9 : 1), and MeOH, by exhaustive maceration (2 L), to give 15.6, 13.5, 21.7, 26.8 g of the respective residues. The MeOH extract was partitioned between *n*-BuOH and H_2O, to afford an *n*-BuOH residue (5.7 g). The *n*-BuOH fraction was submitted to a Sephadex LH-20 column (5 × 70 cm, flow rate 1.5 mL/min) using MeOH as the eluent, yielding 133 fractions of 15 mL that were grouped by TLC into twelve major fractions (A–N). Fraction B (1 g, 810–1170 mL) was partitioned between *n*-BuOH and H_2O, to afford a *n*-BuOH residue (780 mg) that was purified by RP-HPLC with MeOH–H_2O (2 : 3) as the eluent to give pure phlinoside B (9 mg, t_R = 13 min), compound **5**, (1.1 mg, t_R = 20 min), and hispidulin-7-*O*-neohesperoside (1 mg, t_R = 38 min). Fraction E (248 mg, 1350–1410 mL), fraction G (77 mg, 1545–1620 mL), and fraction I (115 mg, 1725–1830 mL) were directly subjected to RP-HPLC with MeOH–H_2O (2 : 3) as the eluent to give pure verbascoside (2.4 mg, t_R = 13 min), compound **5** (5.6 mg, t_R = 20 min), hispidulin-7-*O*-neohesperoside (7.4 mg, t_R = 38 min) from fraction E, isoacteoside (5.1 mg, t_R = 20 min) from fraction G, and luteolin-7-*O*-neohesperoside (2.2 mg, t_R = 29 min) and hispidulin-7-*O*-*β*-D-glucopyranoside (2 mg, t_R = 45 min) from fraction I. Fraction N (120 mg, 1950–1995 mL) was purified by RP-HPLC with MeOH–H_2O (35 : 65) as the eluent to give pure rosmarinic acid (6.6 mg, t_R = 35 min). Part of the $CHCl_3$–MeOH extract (10 g) was chromatographed over Sephadex LH-20 column (5 × 70 cm, flow rate 2.0 mL/min) using MeOH as the eluent and collecting 164 fractions of 20 mL that were grouped by TLC into twelve major fractions (A–N). Fraction F (189 mg, 1220–1480 mL) was purified by RP-HPLC with MeOH–H_2O (35 : 65) as the eluent to yield phlinoside B (11.9 mg, t_R = 22 min). Fraction H (70 mg, 2040–2200 mL) was also purified by RP-HPLC with MeOH–H_2O (2 : 3) as the eluent to yield hispidulin-7-*O*-*β*-D-glucopyranoside (8.6 mg, t_R = 38 min). Part of the $CHCl_3$ extract (10 g) was chromatographed over silica gel column (9 × 20 cm) eluting with $CHCl_3$ followed by increasing concentrations of MeOH in $CHCl_3$ (between 1% and 50%, $CHCl_3$ 7.5 L, $CHCl_3$-MeOH 99 : 1 3 L, $CHCl_3$-MeOH 98 : 2 4 L, $CHCl_3$-MeOH 97 : 3 1.5 L, $CHCl_3$-MeOH 95 : 5 3.5 L, $CHCl_3$-MeOH 9 : 1 1 L, $CHCl_3$-MeOH 1 : 1 1 L). Fractions of 75 mL were collected and grouped by TLC into 18 fractions (A–T). Fraction H–L (1.6 g, 5.5–7.5 L) was purified by RP-HPLC with MeOH–H_2O (7 : 3) as the eluent to yield 2*β*-angeloyloxy-5*β*-hydroxy-7*α*,10*β*-methyl-eudesm-3-ene-1-one (1.5 mg, t_R = 19 min). Fraction O (500 mg, 12.4–13 L) was purified by RP-HPLC with MeOH–H_2O (3 : 2) as the eluent to yield 14,15-dihydro-15-hydroxy-3-epicarioptin (5.5 mg, t_R = 11 min) and compound **1** (5.4 mg, t_R = 22 min). Fraction Q (292 mg, 17.3–17.8 L) was purified by RP-HPLC with MeOH–H_2O (2 : 3) as the eluent to yield compound **2** (1.8 mg, t_R = 27 min). Fractions R (294 mg, 19.7–20.2 L) and S (270 mg, 20.3–20.4 L) were purified by RP-HPLC with MeOH–H_2O (45 : 55) as the eluent to yield compound **3** (1.8 mg, t_R = 20 min) from fraction R and compound **4** (1.3 mg, t_R = 12 min) from fraction S. All the compounds met the criteria of ≥ 95% purity, as inferred by HPLC and NMR analyses.

2α-acetoxy-3β-(2′,3′-diacetoxy-2′-methyl)-butanoyloxy-14-hydro-15-hydroxyclerodin (**1**): colorless amorphous powder; $[\alpha]_D^{25}$ − 9.4 (*c* 0.45, MeOH); ^1H and ^{13}C NMR data, see **Table 1**; ESI MS *m/z* 749 $[M + Na]^+$, 689 $[M + Na - 60]^+$, 531 $[M + Na - 218]^+$, 489 $[M + Na - 218–42]^+$, 471 $[M + Na - 218–60]^+$, 411 $[M + Na - 218–60–60]^+$; HR ESIMS $[M + H]^+$ 727.3204 (calcd. for $C_{35}H_{51}O_{16}$ 727.3177).

3β,15-dihydroxy-14-hydro-clerodin (**2**): colorless amorphous powder; $[\alpha]_D^{25}$ +5.7 (*c* 0.15, MeOH); ^1H and ^{13}C NMR data, see **Table 1**; ESI MS *m/z* 491 $[M + Na]^+$, 431 $[M + Na - 60]^+$, 371 $[M + Na - 60–60]^+$, 467 $[M - H]^-$, 425 $[M - H - 42]^-$, 383 $[M - H - 42–42]^-$; HR ESIMS *m/z* 469.2500 $[M + H]^+$ (calcd. for $C_{24}H_{37}O_9$ 469.2438).

2α,15-dihydroxy-3β-(2′-hydroxy-2′-methyl-3′-acetoxy)-butanoyloxy-6α,18-diacetoxy-4α,17-epoxy-clerodan-11,16-lactone (**3**): colorless amorphous powder; $[\alpha]_D^{25}$ − 5.7 (*c* 0.11, MeOH); ^1H and ^{13}C NMR data, see **Table 1**; ESI MS *m/z* 665 $[M + Na]^+$, 605 $[M + Na - 60]^+$, 486 $[M + Na - 176]^+$; HR ESIMS *m/z* $[M + H]^+$ 643.2899 (calcd. for $C_{31}H_{47}O_{14}$ 643.2966).

3β,14S,15-trihydroxy-6α,18-diacetoxy-4α,17-epoxy-clerodan-11,16-lactone (**4**): colorless amorphous powder; $[\alpha]_D^{25}$ +2.6 (*c*

0.13, MeOH); ^{1}H and ^{13}C NMR data, see ◉ **Table 1**; ESI MS *m/z* 507 $[M + Na]^+$, 447 $[M + Na - 60]^+$, 387 $[M + Na - 60–60]^+$; HR ESIMS *m/z* 485.2423 $[M + H]^+$ (calcd. for $C_{24}H_{37}O_{10}$ 485.2387).

β-(3,4-dihydroxyphenyl)ethyl-O-β-D-xylopyranosyl-(1 → 2)-α-L-rhamnopyranosyl-(1 → 3)-6-O-t-caffeoyl-β-D-glucopyranoside (**5**): brown amorphous powder; $[\alpha]_D^{25}$ − 10.7 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 220 sh (3.88), 288 sh (3.95), 330 (4.09) nm; ^{1}H and ^{13}C NMR data, see ◉ **Table 2**; ESI MS *m/z* 755 $[M - H]^-$,623 $[M - H - 132]^-$, 593 $[M - H - 162]^-$, 779 $[M + Na]^+$, 647 $[M + Na - 132]^+$, 501 $[M + Na - 132–146]^+$; HR ESIMS *m/z* 757.2637 $[M + H]^+$ (calcd. for $C_{34}H_{45}O_{19}$ 757.2555).

Acid hydrolysis of compound 5

A solution of compound **5** (2.0 mg) in HCl 1 N (1 mL) was stirred at 80 °C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between water and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a L-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of L-rhamnose, D-xylose, and D-glucose (Sigma Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

Determination of absolute configuration of the 15,16-diol moiety in compound 4 using Snatzke's method

According to the published procedure [20, 23], about 1 : 1 diol-to-molybdenum mixtures were prepared using 0.5 mg/mL of compound **4**. Soon after mixing, the first CD spectrum was recorded and its evolution monitored until stationary (30–60 min). The sign of the diagnostic band at 305 nm is correlated to the absolute configuration of the 15,16-diol moiety.

Cells cultures and chemicals

HeLa cells (human epitheloid cervix carcinoma) were from American Type Culture Collection (ATTC). Cells, growth in DMEM supplemented with 10% (v/v) FBS, 100 mg/L streptomycin, and 100 IU/mL penicillin were maintained at 37 °C in a humidified atmosphere with 5% CO_2. To ensure logarithmic growth, cells were subcultured every three days. Under given experimental conditions, control cells were able to double their number within 24 h. Fetal bovine serum (FBS) was from GIBCO; Dulbecco's modified Eagle's medium (DMEM) and antibiotics were from Lonza BioWhittaker. PEITC was obtained from Sigma (W4401404). All the other reagents were from Sigma Aldrich.

Cell viability evaluation

Stock solutions (50 mM) of purified compounds in DMSO were stored in the dark at 4 °C. Working solutions were prepared in culture medium immediately prior to use. HeLa, being cells growing as monolayer, were plated one day before the beginning of treatment at a density of 1.2×10^5/mL. After established incubation time with different concentrations of each chemical, cell viability was determined by the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) assay [24]. Data were subtracted of the corresponding appropriate blank. The number of viable cells in treated samples was calculated as percentage of control samples containing equal amounts of vehicle (DMSO). To exclude any interference of test compounds with the tetrazolium salt-based assay, cell growth inhibition was randomly verified also by cytometric count (trypan blue exclusion test). PEITC was used as a positive control and showed an IC_{50} of 15 µM.

Statistical analysis

Data reported are the mean values ± SD of at least three experiments performed in duplicate.

Supporting information

NMR spectra of compounds **1**–**5** are available as Supporting Information. This material is available online at http://www.thieme-connect.de/ejournals/toc/plantamedica.

Conflict of Interest

The authors declare no conflict of interest.

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