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Withanolides from *Physalis coztomatl*

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abstract

Six withanolides (1–6), as well as two known withanolides (physachenolide D 7 and withanoside VI 8), were isolated from the aerial parts of *Physalis coztomatl* (Solanaceae). Structural elucidations of 1–6 were achieved through 2D NMR and other spectroscopic techniques, while the structure of 1 was confirmed by X-ray crystallographic analysis. In addition, the stereochemical orientation of the 17-hydroxy group in withanolides was discussed in relation to ^{13}C NMR shifts of C-12, 13, 14 and 16. Such analysis established that coagulansin A contains a 17*α*-hydroxy moiety rather than the reported 17*b*-hydroxy functionality, and has been revised accordingly.

1. Introduction

The *Physalis* genus of Solanaceae continues to be a rich source of modified and highly-oxygenated C_{28} ergostane-type steroids with C-17 lactone/lactol side-chain substituents, collectively known as withanolides (Chen et al., 2011; Misico et al., 2011; Zhang et al., 2012a). Recently the isolation of a series of such compounds were reported, with structural variations in both the steroidal nucleus as well as the side-chain, from *Physalis hispida* (Cao et al., 2014) and *Physalis longifolia* (Zhang et al., 2011, 2012b). In continuing this research, *Physalis coztomatl* (Mociño & Sessé) Ex Dunal was cultivated and the aerial parts examined. Herein the isolation and structure elucidation of an array of new (1–6) and known (7 and 8) withanolides are reported, which are relatively more polar than those (physacoztolides A–E) previously reported in the same species (Pérez-Castorena et al., 2006).

2. Results and discussion

Compounds 1–8 were isolated from the *n*-butanol partition phase of the CH_2Cl_2-MeOH (1:1) extract of *P. coztomatl* (see Experimental). The structures of the two known withanolides (physachenolide D 7 and withanoside VI 8) were identified by comparing their NMR spectroscopic data with those of the published values (Maldonado et al., 2004; Matsuda et al., 2001). The molecular formula of 1 was determined to be $C_{32}H_{42}O_{10}$ by HRESIMS and NMR experiments, equating to twelve double-bond equivalents. The IR absorptions of 1 indicated the presence of double bond (1640 cm^{-1}), as well as hydroxy (3260 cm^{-1}) and ester (1740 cm^{-1}), groups. The ^1H NMR data of 1 (Table 1) displayed ten deshielded protons [δ_H 4.74 (1H, d, J = 11.6 Hz), 5.11 (1H, d, J = 11.6 Hz), 5.29 (1H, dd, J = 13.2, 2.9 Hz), 5.51 (1H, dd, J = 9.9, 8.0 Hz), 5.57 (1H, d, J = 6.0 Hz), 5.96 (1H, dd, J = 10.0, 2.0 Hz), 6.66 (1H, ddd, J = 10.0, 4.8, 2.4 Hz), 7.01 (1H, s), 7.21 (1H, s), and 9.76 (1H, s)], as well as six shielded CH_3 groups [δ_H 1.28 (3H, s), 1.76 (3H, s), 1.85 (3H, s), 2.02 (3H, s), 2.09 (3H, s), and 2.19 (3H, s)]. In addition to these CH_3 groups, the ^{13}C NMR (APT) and HSQC data established a further 26 carbon signals which were differentiated into seven CH_2 [including an oxygenated (δ_C 65.4)], seven CH [including three olefinic (δ_C 146.2, 128.4, and 126.3) and two oxygenated (δ_C 82.2 and 77.5)], as well as twelve C [including a keto (δ_C 204.4), three ester (δ_C 171.5, 171.2 and 167.2), three olefinic (δ_C 151.2, 135.6 and 122.1), and three oxygenated (δ_C 86.0, 80.3 and 79.7)] groups, which corresponded to $C_{32}H_{39}$ (Table 2). The remaining three hydrogen atoms were therefore assigned as OH groups, indicating the presence of a five-ringed structure.

The NMR spectroscopic data of 1 exhibited similarities to a major withanolide isolated in this study, namely the five-ringed physachenolide D (7) which was previously reported in *Physalis chenopodifolia* (Maldonado et al., 2004) and *P. coztomatl* (Pérez-Castorena et al., 2006) (Fig. 1). Compound 1 was found to contain

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three identical features also observed in 7: (1) a nine-carbon side-chain [$\delta_{C\text{-}20}$ 79.7 (C), $\delta_{C\text{-}21}$ 19.6 (CH_3), $\delta_{C\text{-}22}$ 82.2 (CH), $\delta_{C\text{-}23}$ 34.9 (CH_2), $\delta_{C\text{-}24}$ 151.2 (C), $\delta_{C\text{-}25}$ 122.1 (C), $\delta_{C\text{-}26}$ 167.2 (C), $\delta_{C\text{-}27}$ 13.1 (CH_3) and $\delta_{C\text{-}28}$ 20.8 (CH_3)] containing an**a**, b-unsaturated *d*-lactone [two vinylic CH_3 ($\delta_{H\text{-}27}$ 2.02, 3H, s; and $\delta_{H\text{-}28}$ 1.85, 3H, s), and an oxygenated CH ($\delta_{H\text{-}22}$ 5.29, 1H, dd, J = 13.2, 2.9 Hz)] unit; (2) a 1-oxo-2,5-diene [$\delta_{C\text{-}1}$ 204.4 (C), $\delta_{C\text{-}2}$ 128.4 (CH), $\delta_{C\text{-}3}$ 146.2 (CH), $\delta_{C\text{-}5}$ 135.6 (C), and $\delta_{C\text{-}6}$ 126.3 (CH)] functionality within the rings A and B of the steroid nucleus; and (3) oxygenation at C-14, 17, 18, and 20 [$\delta_{H\text{-}18}$ 4.74 (1H, d, J = 11.6 Hz), 5.11 (1H, d, J = 11.6 Hz); $\delta_{C\text{-}14}$ 80.3 (C), $\delta_{C\text{-}17}$ 86.0 (C), $\delta_{C\text{-}18}$ 65.4 (CH_2), $\delta_{C\text{-}20}$ 79.7 (C)].

The main observed differences corresponded to the signals of ring D, where the –C(15)H_2–C(16)H_2–fragment present in 7 was absent in 1. Instead, a ^{1}H–^{1}H COSY fragment of –CH(OR)–CH_2– [CH: δ_H 5.51 (1H, dd, J = 9.9, 8.0 Hz), δ_C 77.5; and CH_2: δ_H 3.08 (1H, dd, J = 11.2, 8.0 Hz), 2.86 (1H, dd, J = 11.2, 9.9 Hz), δ_C 43.4] was observed in 1. Based on HMBC correlations [CH (δ_H 5.51)/$OCOCH_3$ (δ_C 171.5); $OCOCH_3$ (δ_H 2.09, 3H, s)/$OCOCH_3$ (δ_C 171.5); 17-OH (δ_H 7.01, 1H, s)/CH_2 (δ_C 43.4, C-16)] and chemical shift values [CH_2 (δ_C 43.4, C-16)] this OR group was identified as an acetoxyl moiety, which suggested that compound 1 is 15-acetoxyphysachenolide D. Furthermore, the ROESY correlation between H-15 (δ_H 5.51, dd, J = 9.9, 8.0 Hz) and H-18 (δ_H 5.11, d, J = 11.6 Hz) revealed that the 15-acetoxy group was in an **a** orientation.

Finally, the structure of 1 was confirmed through a single-crystal X-ray diffraction experiment (Fig. 2), and its NMR spectra were assigned on the basis of 2D-NMR data from ^{1}H–^{1}H COSY, multiplicity edited-HSQC, HMBC and ROESY experiments (Tables 1 and 2). Even though oxygen was the heaviest element present in the crystal of compound 1, its absolute configuration was unambiguously determined using anomalous dispersion of the Cu X-rays while the Flack absolute structure parameter refined to a value of 0.03(3). Thus, withanolide 1 was determined as 15**a**-acetoxyphysachenolide D.

The molecular formula of compound 2 was determined to be $C_{30}H_{42}O_{10}S$ by HRESIMS and NMR experiments. The NMR spectroscopic data of 2 (Tables 1 and 2) were almost identical to those of 7, except for the ring A signals, where the olefinic protons of the conjugated 1-oxo-2-ene moiety present in 7 were absent in 2. Instead, the ^{13}C NMR (APT) and HSQC of 2 showed resonances for an isolated keto (δ_C 211.5), a CH_2 [δ_C 46.3; δ_H 3.23 (1H, dd, J = 12.7, 6.1 Hz) and 3.12 (1H, m)], and an oxymethine [δ_C 74.5 (CH); δ_H 5.06 (1H, brs)] group. These observations suggested that compound 2 was 2,3-dihydro-3-*O*-sulfonylphysachenolide D. This

Table 1
^{1}H NMR spectroscopic data [δ_H (J in Hz)] of withanolides 1–6 in C_5D_5N (400 MHz).

Pos.	1	2	3	4	5	6
2	5.96 dd (10.0, 2.0)	3.23 dd (12.7, 6.1 Hz), 3.12 m	2.95 dd (12.0, 5.0), 2.05 m	5.96 dd (10.0, 2.1)	5.99 dd (10.0, 2.0)	6.01 dd (10.0, 2.2)
3	6.66 ddd (10.0, 4.8, 2.4)	5.06 brs	1.50 br	6.67 ddd (10.0, 4.9, 2.4)	6.69 ddd (10.0, 4.9, 2.4)	6.70 ddd (10.0, 4.8, 2.4)
4	3.20 m, 2.73 m	3.06 m, 2.84 t (12.1)	1.34 dd (6.7, 5.2), 0.11 dd (5.2, 4.1)	3.20 d (21.9), 2.71 dd (21.9, 4.9)	3.24 m, 2.77 m	3.24 d (22.2), 2.71 m
6	5.57 d (6.0)	5.52 brs	3.87 s	5.57 d (5.3)	5.54 d (6.0)	5.56 d (6.0)
7	2.87 m, 2.14 m	2.58 dd (13.0, 11.2), 1.86 m	2.47 m, 2.25 m	2.63 m, 1.90 m	2.39 m, 1.84 m	2.39 m, 1.86 m
8	2.04 m	1.90 m	2.83 t (11.2)	1.88 m	2.01 m	1.95 m
9	2.94 m	2.69 m	2.30 m	2.93 m	2.81 m	2.78 m
11	2.70 m, 1.70 m	1.99 m, 1.58 q (12.8)	1.71 m, 1.58 m	2.66 m, 1.74 m	2.77 m, 1.81 m	2.68 m, 1.86 m
12	2.87 m, 2.14 m	2.69 m, 2.09 m	2.52 m, 2.10 m	2.86 m, 2.30 m	2.77 m, 2.41 m	2.84 m, 1.84 m
15	5.51 dd (9.9, 8.0)	1.79 m, 1.68 ddd (12.6, 12.3, 7.5)	2.00 m, 1.93 m	1.85 m, 1.73 m	2.06 m, 1.54 m	2.03 m, 1.75 m
16	3.08 dd (11.2, 8.0) 2.86 dd (11.2, 9.9)	3.06 m, 2.06 m	3.07 m, 2.04 m	3.08 ddd (14.2, 12.0, 8.5), 2.06 dd (14.2, 8.0)	2.77 m, 2.63 m	2.70 m, 2.66 m
18	5.11 d (11.6), 4.74 d (11.6)	4.99 d (11.5), 4.61 d (11.5)	5.16 d (11.1), 4.76 d (11.1)	4.99 d (11.3), 4.69 d (11.3)	4.99 d (11.2), 4.59 d (11.2)	1.49 s
19	1.28 s	1.28 s	1.44 s	1.21 s	1.27 s	1.30 s
21	1.76 s	1.79 s	1.78 s	1.76 s	1.75 s	1.57 s
22	5.29 dd (13.2, 2.9)	5.25 d (13.5)	5.26 dd (13.5, 2.2)	5.37 dd (13.2, 3.3)	5.03 dt (13.2, 2.1)	5.10 dt (12.7, 2.8)
23	3.15 m, 2.68 m	3.14 m, 2.65 m	3.11 m, 2.64 dd (13.5, 16.0)	3.20 d (21.3), 2.71 dd (21.3, 4.8)	3.29 m, 2.20 m	3.35 t (12.7), 2.31 m
27	2.02 s	2.00 s	2.01 s	2.13 s	5.03 d (10.8), 4.76 d (10.8)	5.03 d (10.8), 4.77 d (10.8)
28	1.85 s	1.82 s	1.86 s	4.76 d (12.7), 4.55 d (12.7)	1.96 s	1.96 s
15-OAc	2.19 s					
18-OAc	2.09 s	2.17 s	2.16 s	2.41 s	2.12 s	
1^0			4.82 d (7.7)	4.89 d (7.7)	4.97 d (7.7)	4.98 d (7.7)
2^0			3.99 m	4.02 t (7.7)	4.04 t (7.7)	4.04 t (7.7)
3^0			4.08 m	4.22 m	4.27 m	4.27 m
4^0			4.26 m	4.20 m	4.28 m	4.28 m
5^0			4.26 m	3.95 m	3.96 m	3.96 m
6^0			4.85 d (11.5), 4.34 dd (11.5, 6.3)	4.56 d (11.2), 4.35 m	4.57 d (11.2), 4.41 dd (11.2, 6.2)	4.57 d (11.1), 4.41 dd (11.1, 4.4)
1^{00}			5.19 d (7.8)			
2^{00}			4.08 m			
3^{00}			4.16 t (8.6)			
4^{00}			3.99 m			
5^{00}			3.97 m			
6^{00}			4.56 d (11.5), 4.41 dd (11.5, 4.5)			
14-OH	7.21 s	7.01 br	7.08 s	7.05 s	7.12 s	6.74 s
17-OH	7.01 s	6.47 s	6.48 s	6.32 s	6.89 s	6.76 s
20-OH	9.76 s	9.35 br	9.45 s	9.35 s	6.35 s	6.17 s

Table 2
^{13}C NMR spectroscopic data of withanolides 1–6 in C_5D_5N (125 MHz).

Pos.	1		2		3		4		5		6	
	d_C	Mult.	d_C	Mult.	d_C	Mult.	d_C	Mult.	d_C	Mult.	d_C	Mult.
1	204.4	C	211.5	C	218.3	C	204.5	C	204.4	C	204.5	C
2	128.4	CH	46.3	CH_2	40.4	CH_2	128.4	CH	128.4	CH	128.5	CH
3	146.2	CH	74.5	CH	16.9	CH	146.3	CH	146.4	CH	146.3	CH
4	34.0	CH_2	39.7	CH_2	19.4	CH_2	34.1	CH_2	34.1	CH_2	34.1	CH_2
5	135.6	C	135.7	C	35.0	C	136.1	C	136.3	C	136.3	C
6	126.3	CH	127.0	CH	79.6	CH	126.1	CH	125.4	CH	125.7	CH
7	26.7	CH_2	26.9	CH_2	31.2	CH_2	26.7	CH_2	25.9	CH_2	25.9	CH_2
8	38.6	CH	37.1	CH	34.7	CH	38.5	CH	37.9	CH	37.2	CH
9	37.2	CH	36.7	CH	40.9	CH	37.0	CH	37.2	CH	37.1	CH
10	51.5	C	53.7	C	53.4	C	51.7	C	51.7	C	51.8	C
11	24.1	CH_2	23.3	CH_2	23.4	CH_2	24.4	CH_2	23.6	CH_2	23.3	CH_2
12	26.8	CH_2	26.7	CH_2	26.7	CH_2	26.9	CH_2	23.8	CH_2	28.1	CH_2
13	58.3	C	58.5	C	59.1	C	58.4	C	55.2	C	52.5	C
14	80.3	C	81.5	C	82.3	C	81.7	C	85.7	C	86.7	C
15	77.5	CH	33.8	CH_2	34.0	CH_2	33.8	CH_2	33.8	CH_2	34.1	CH_2
16	43.4	CH_2	37.8	CH_2	37.8	CH_2	38.0	CH_2	34.3	CH_2	33.8	CH_2
17	86.0	C	89.0	C	89.1	C	89.1	C	88.7	C	88.9	C
18	65.4	CH_2	65.9	CH_2	65.7	CH_2	65.9	CH_2	64.4	CH_2	19.5	CH_3
19	19.3	CH_3	19.1	CH_3	15.2	CH_3	19.5	CH_3	19.5	CH_3	19.6	CH_3
20	79.7	C	79.5	C	79.5	C	79.7	C	78.6	C	78.3	C
21	19.6	CH_3	19.6	CH_3	19.5	CH_3	19.7	CH_3	19.5	CH_3	20.4	CH_3
22	82.2	CH	82.2	CH	82.3	CH	82.7	CH	81.8	CH	81.4	CH
23	34.9	CH_2	34.9	CH_2	35.1	CH_2	31.8	CH_2	35.0	CH_2	35.0	CH_2
24	151.2	C	151.4	C	151.4	C	149.5	C	158.9	C	158.8	C
25	122.1	C	122.1	C	122.1	C	125.3	C	123.6	C	123.6	C
26	167.2	C	167.4	C	167.4	C	167.3	C	166.4	C	166.4	C
27	13.1	CH_3	13.1	CH_3	13.1	CH_3	13.3	CH_3	63.8	CH_2	63.7	CH_2
28	20.8	CH_3	20.9	CH_3	20.9	CH_3	68.7	CH_2	21.1	CH_3	21.4	CH_3
15-OAc	171.2	C										
	21.8	CH_3										
18-OAc	171.5	C	171.4	C	171.1	C	172.0	C	171.7	C		
	21.9	CH_3	21.9	CH_3	21.9	CH_3	22.3	CH_3	21.9	CH_3		
1^0					102.3	CH	104.6	CH	105.3	CH	105.2	CH
2^0					75.3	CH	75.4	CH	75.6	CH	75.6	CH
3^0					77.9	CH	79.1	CH	79.0	CH	78.9	CH
4^0					72.1	CH	72.1	CH	72.1	CH	72.1	CH
5^0					78.9	CH	79.2	CH	79.1	CH	79.1	CH
6^0					70.8	CH_2	63.2	CH_2	63.2	CH_2	63.2	CH_2
1^{00}					106.2	CH						
2^{00}					75.8	CH						
3^{00}					79.6	CH						
4^{00}					72.4	CH						
5^{00}					79.0	CH						
6^{00}					63.3	CH_2						

was further supported by ^1H–^1H COSY fragment of –C(2)H_2–C(3)H–C(4)H_2–; the chemical shift values of H_2-2 (d_H 3.23 and 3.12) and H-3 (d_H 5.06); as well as the HMBC correlations of H_2-2, H-3/C-1 (d_C 211.5).

Comparing the NMR spectroscopic data of 2 with the published data of a 2,3-dihydro-3*b*-*O*-sulfate withanolide, cilistol y [3*b*-*O*-sulfonyl-1-oxo-24,25:22,26-diepoxy-3*b*,17*a*,26-trihydroxyergost-5-ene 26-*O*-*b*-D-glucopyranoside] established superimposable ring A signals (Zhu et al., 2001). These observations, in conjunction with the large proton-proton coupling constant (J = 12.1 Hz) present in 2[H-3/H-4*b* (d 2.84, t, J = 12.1 Hz)], affirmed that the 3-*O*-sulfonyl group in 2 was in a *b* orientation. Thus, withanolide 2 was determined as 2,3-dihydro-3*b*-*O*-sulfonylphysachenolide D.

The molecular formula of compound 3 was determined to be $C_{42}H_{62}O_{19}$ by HRESIMS and NMR experiments. Though the NMR spectroscopic data of 3 (Tables 1 and 2) were similar to 7 (presence of a nine-carbon side-chain containing an *a*,*b*-unsaturated *d*-lactone, as well as five CH_3groups), differences were observed corresponding to the signals of rings A and B. The NMR data of 3 displayed an additional 12 carbon resonances [in the 60–107 ppm range, which included two CH groups (d_C 106.2 and 102.3)], which in conjunction with the 18 proton signals (in the 3.8–5.5 ppm range), suggested that 3 was a withanolide saponin containing two sugar moieties. Furthermore, rather than a six-membered-keto ring, the aglycone of 3 presented NMR data indicative of a cyclopropane ring [low chemical shift (d_H 0.11); and a small geminal coupling constant (J = 5.2 Hz)] and a five-membered-keto ring [keto: (d_C 218.3); CH_2: d_C 16.9, d_H 1.34, 1H, dd (J = 6.7, 5.2 Hz), d_H 0.11, 1H, dd (J = 5.2, 4.1 Hz)].

Comparing NMR spectroscopic data of 3 with the published data of withawrightolide [(20*R*,22*R*,24*R*)-21,24-epoxy-3*a*,5*a*-cyclo-6*b*-hydroxy-1-oxowitha-25(27)-enolide] (Zhang et al., 2013) also established superimposable ring A signals, suggesting that a 1-oxo-3*a*,5*a*-cyclo-6*b*-hydroxy functionality was present in the aglycone of 3. This deduction was supported by the presence of the ^1H–^1H COSY fragment –C(2)H_2–C(3)H–C(4)H_2–; HMBC correlations of H_3-19 (d_H 1.44, 3H, s)/C-1 (d_C 218.3), C-5 (d_C 35.0), C-9 (d_C 40.9), and C-10 (d_C 53.4), as well as H_2-4 [d_H 1.34 dd (J = 6.7, 5.2 Hz), 0.11 dd (J = 5.2, 4.1 Hz)]/C-2 (d_C 40.4), C-3 (d_C 16.9), C-5 (d_C 35.0), C-6 (d_C 79.6) and C-10 (d_C 53.4); the small chemical shifts of C-3 (CH, d_C 16.9), C-4 (CH_2, d_C 19.4), and C-5 (C, d_C 35.0); the small coupling constant between H-6 (d_H 3.87, 1H, s) and H_2-7; as well as the H-4*a* [d_H 1.34]/H-6 ROESY correlation, observed in 3.

The NMR spectroscopic data of 3 exhibited similarities to a major isolate in this study, namely, withanoside VI (8) (Matsuda

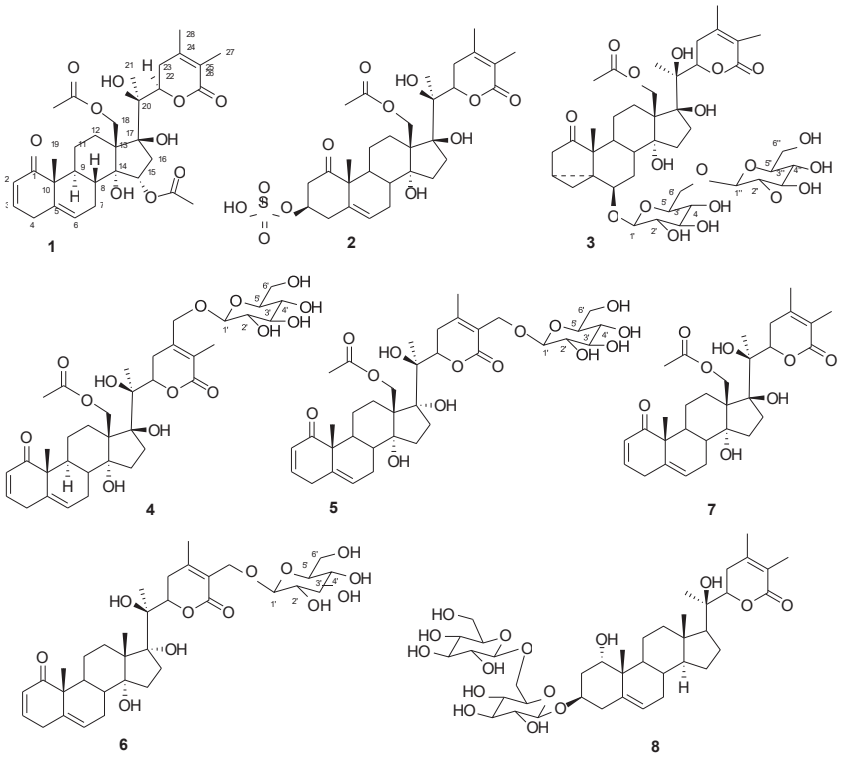

Fig. 1. Withanolides 1–8 isolated from *Physalis coztomatl*.

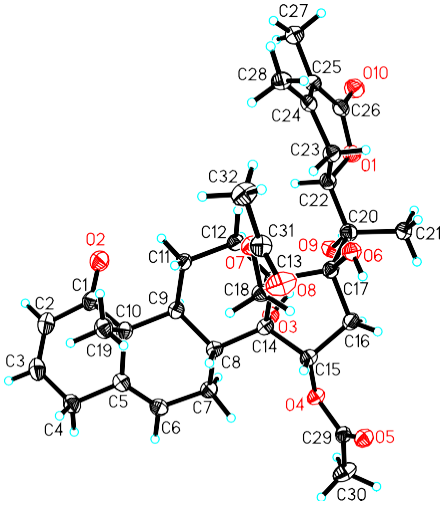

Fig. 2. X-ray ORTEP drawing of 15*α*-acetoxyphysachenolide D (1).

et al., 2001), a withanolide saponin with a 3-*O*-[*b*-D-glucopyranosyl-(1?6)-*b*-D-glucopyranosyl] moiety. Comparisons suggested that both withanolides contained identical -*O*-[*b*-D-glucopyranosyl-(1?6)-*b*-D-glucopyranosyl] units. The observed chemical shift values of C-6 (d_C 79.6, CH; d_H 3.87, 1H, s), and HMBC correlations of H-6/C-1^0 (d_C 102.3, CH) and H-1^0 (d_H 4.82, 1H, d, J = 7.7 Hz)/C-6 established that the sugar moiety in 3 was attached at C-6. Thus, compound 3 was determined as 6*b*-*O*-[*b*-D-glucopyranosyl-(1?6)-*b*-D-glucopyranosyl]-(20*S*,22*R*)-14*α*,17*b*,20-trihydroxy-18-acetoxy-3*α*,5*α*-cyclo-1-oxowitha-24-enolide, and subsequently named physacoztolide F.

The molecular formula of compound 4 was determined to be $C_{36}H_{50}O_{14}$ by HRESIMS and NMR experiments. The similarities between the NMR spectroscopic data of 4 (Tables 1 and 2) and the published data of 28-hydroxyphysachenolide D (Maldonado et al., 2012), suggested that 4 contained a nine-carbon side-chain with an *α*,*b*-unsaturated *d*-lactone, as well as four CH_3 groups. However, the presence of six additional oxygenated carbons [five CH (d_C 104.6, 79.2, 79.1, 75.4, 72.1) and a CH_2 (d_C 63.2)] and seven protons in the range of 4–5 ppm suggested the presence of a pyranose form *b*-glucose moiety in 4. This *b* configuration was further supported by the observed large coupling constant of the anomeric proton H-1^0 (d_H 4.82, d, J = 7.7 Hz). Finally, the presence of high frequency shifts [H_2-28 (d_H 4.55 and 4.76) and C-28 (d_C 68.7 CH_2)] and the HMBC correlations [H-1^0/C-28 and H_2-28/C-1^0 (d_C 104.6 CH)] indicated that this *b*-glucose moiety was attached to C-28 in 4. Thus, the structure of 4 was resolved as 28-*O*-*b*-D-glucopyranosyl-physachenolide D.

Based on HRESIMS and NMR experiments, compound 5 was determined to be an isomer of 28-*O*-*b*-D-glucopyranosylphysachenolide D (4) and assigned a molecular formula of $C_{36}H_{50}O_{14}$. Though the NMR spectroscopic data of 5 and 4 were similar to each other (identical functional groups and multiplicities for all carbons present), differences were observed corresponding to the position of the pyranose form *b*-glucose moiety as well as the stereochemistry of the C-17 hydroxy group (Tables 1 and 2).

HMBC correlations [H_2-27 (d_H 5.03, d, J = 10.8 Hz; d_H4.76, d, J = 10.8 Hz)/C-1^0 (d_C105.3, CH); H-1^0 (d_H 5.00, d, J = 7.7 Hz)/C-27 present in 5] and ^{13}C NMR shift differences between 4 [d_C 13.3 (C-27,CH_3); d_C 68.7 (C-28,CH_2)] and 5[d_C 63.8 (C-27,CH_2); d_C21.1 (C-28,CH_3)] suggested that the sugar moiety was attached to C-27 in 5 rather than at C-28 as observed in 4. This deduction was supported by comparing the NMR signals of 5 against the published data of a 27-*O*-*b*-D-glucopyranyl withanolide, namely sitoindoside IX (Ghosal et al., 1988; Zhang et al., 2011), revealing superimposable side-chain lactone signals.

Though 2D NMR spectroscopic data indicated that 4 and 5 contained an identical planar aglycone structure, noticeable ^{13}C NMR shift differences were observed corresponding to the rings C and

D. The chemical shifts difference of C-12 and C-14 (d_C 26.9 and 81.7 in 4; d_C 23.8 and85.7 in 5, respectively) induced by the *c*-effect of the hydroxy group at C-17 (where a 17*a*-OH shields C-12 and a 17*b*-OH shields C-14); as well as the chemical shifts of C-13 and C-16 (d_C 58.4 and 38.0 in 4; d_C 55.2 and 34.3 in 5, respectively) induced by the *c*-effect of the hydroxy group at C-20 (where a 20-OH shields C-14 and C-16 in 17*a*-OH withanolides), implied that a 17*a*-OH moiety was present in 5 (Gottlieb and Kirson, 1981; Huang et al., 2009; Kirson and Gottlieb, 1980) instead of a 17*b*-OH moiety in 4. This deduction was further supported by comparing the NMR signals of 5 against the published data of withanolides containing 14*a*,17*a*,20-trihydroxy functionalities (Gottlieb and Kirson, 1981; Abdeljebbar et al., 2007). Thus, compound 5 (physacoztolide G) was determined as 27-*O*-*b*-D-glucopyranosyl-(20*S*,22*R*)-14*a*,17*a*,20-trihydroxy-18-acetoxy-1-oxowitha-2,5,24-trienolide.

Compound 6 was assigned a molecular formula of $C_{34}H_{48}O_{12}$ by HRESIMS and NMR experiments. Though the NMR spectroscopic data of 5 and 6 were similar (Tables 1 and 2), differences were observed corresponding to the C-18 substituent, where the acetoxy methylene [C-18, d_C 64.4; d_H 4.99 (d, J = 11.2 Hz), 4.59 (d, J = 11.2 Hz); –OAc group, d_C 171.7, 21.1; d_H 2.12 (s)] moiety observed in 5, was absent in 6. Instead, a methyl d_C 19.6 and d_H1.49 (3H, s) group was observed in 6, implying that it is the 18-deacetoxy derivative of 5. This observation was supported by HMBC correlations (H_3-18/C-12, 13, 14, 17), and by the high-frequency shift of C-12 methylene [d_C 28.1 in 6 (with a C-18 methyl group) and d_C 23.8 in 5 (with a 18-acetoxy methylene group) due to the *c*-effect of the –OAc group at C-18]. In addition, comparing the superimposable aglycone NMR signals of 6 against the published data of a 14*a*,17*a*,20-trihydroxy withanolide, namely withacoagulin D (Huang et al., 2009), further substantiated this hypothesis. Thus, compound 6 (physacoztolide H) was determined as 27-*O*-*b*-D-glucopyranosyl-(20*S*,22*R*)-14*a*,17*a*,20-trihydroxy-1-oxowitha-2,5,24-trienolide.

Withanolides 1–7 are highly oxygenated steroids with oxygenation at C-14, 17 and 20. In addition, 1–4 present additional oxygenation at C-18, whereas both C-15 and C-18 are oxygenated in withanolide 1. Furthermore, withanolides 1–4 and 7 have a 17*b* hydroxy group, whereas 5 and 6 contain a 17*a* hydroxy moiety. It has been reported in the literature that the stereochemistry of the 17-hydroxy substituent can be determined by ^{1}H NMR spectroscopy by comparing the H_3-19 and H_3-21 chemical shifts difference of a withanolide measured in C_5D_5N against in $CDCl_3$ (Bessalle and Lavie, 1992; Kirson and Gottlieb, 1980). It is, however, far more convenient to simply compare the ^{13}C NMR shifts of C-12, 13, 14, and 16; where an observed 3–5 ppm difference can distinguish between a pair of 17-*epi* isomers, and determine the precise orientation of the C-17 hydroxy moiety of each compound.

Such a pattern was observed when comparing the ^{13}C NMR chemical shifts of the 17-*epi* isomeric aglycones of 4 (C-12, 13, 14, 16: d_C 26.9, 58.4, 81.7, 38.0) and 5 (C-12, 13, 14, 16: d_C23.8, 55.2, 85.7, 34.3), where the 17-OH and 20-OH groups induce the *c*-effect (Table 2). Comparing these values it becomes apparent that shielding of C-12, C-13 and C-16 (but not C-14) is observed when a 17*a*-OH is present. Conversely, the presence of a 17*b*-OH substituent results in the shielding of C-14, whereas C-12, C-13 and C-16 remain unaffected. Analogous shift differences are consistent with the published data of other 17-*epi* isomers such as withanolide E (17*b*-OH functionality) and *iso*-withanolide E (17*a*-OH functionality) (Gottlieb and Kirson, 1981); as well as withanolide F (17*b*-OH functionality) and withanolide J (17*a*-OH functionality) (Abdeljebbar et al., 2007).

Utilizing this method to examine the published data of withanolides isolated from *Withania coagulans* reveals that coagulansin A (Jahan et al., 2010) contains a 17*a*-hydroxy moiety rather than the reported 17*b*-hydroxy functionality. This revised structure is identical to that of another *W. coagulans* withanolide, namely withacoagulin D (Huang et al., 2009). Not only are the reported ^{13}C NMR spectroscopic data sets of these two withanolides superimposable, but they are very similar to reported data of other 17*a*-OH withanolides rather than 17*b*-OH withanolides. For instance, the C-12 to C-16 ^{13}C NMR data of coagulin A (d_C 27.0, 51.0, 84.0, 32.0, 33.0) (Jahan et al., 2010) are similar to those of the 17*a*-OH, yet quite different to those of 17*b*-OH withanolides, such as withanolide F (d_C 30.1, 53.7, 81.4, 31.9, 35.7) (Abdeljebbar et al., 2007).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a Rudolph RS Autopol IV automatic polarimeter. IR data were obtained with a Thermo Nicolet Avatar 380 FT-IR spectrometer. NMR spectra were recorded with a Bruker AV-400 spectrometer equipped with a X-channel observed quadruple nuclei probe or an AVIII-500 instrument with a cryogenically-cooled carbon observe probe for ^{1}H, APT ^{13}C, COSY, HSQC, HMBC, and ROESY. Chemical shift values are given in *d* (ppm) using the peak signals of the solvent C_5D_5N (d_H 8.74 and d_C 150.3) as references and coupling constants were reported in Hz. HRESIMS data were collected with a LCT Premier time of flight mass spectrometer (Waters Corp., Milford, MA). Column chromatography (CC) was performed on CombiFlash columns (Teledyne Isco, Lincoln, NE). Normal-phase silica gel G TLC plates (w/UV 254) and reversed-phase C_{18} TLC plates (w/UV 254) (Sorbent Technologies, Atlanta, GA) were used for fractionation and compound detection. The spots were visualized using UV light at 254 nm and spraying with 10% EtOH-sulfuric acid reagent. Semi-preparative HPLC was performed on an Agilent 1200 unit equipped with a DAD detector, utilizing a Lichrospher RP-18 column (250 × 10 mm, 5 μm).

3.2. Plant material

Seeds of *P. coztomatl* were planted in flats in the greenhouse at the University of Kansas in January 2011. Seedlings were transplanted to research beds of the Native Medicinal Plant Research Program garden at the University of Kansas (latitude: 30.0094°; longitude: 95.20645°, Douglas County, Kansas, USA) in April. Fresh aerial parts of *P. coztomatl* were harvested at about 8 months of age on September 27, 2011. The species was identified by plant taxonomist Dr. Kelly Kindscher at the Kansas Biological Survey, University of Kansas. A voucher specimen (Kelly Kindscher 4073) is deposited in the R.L. McGregor Herbarium of the University of Kansas.

3.3. Extraction and isolation

The collected biomass was air dried indoors, ground to a coarse powder (2.8 kg) and stored in an air-tight dark container until processing time. It was extracted three times with CH_2Cl_2-MeOH (50:50, 12.0 L) at room temperature. After removing the solvents *in vacuo*, the extract (220 g) was suspended in H_2O (1.0 L), followed by successive partitions with *n*-hexane, ethyl acetate and *n*-butanol (3 × 1.0 L).

The resulting *n*-butanol fraction (55 g) obtained was subjected to MCI CHP20P gel CC (2.0 Kg) eluted with a mixture of H_2O-MeOH (100:0, 80:20, 60:40, 40:60, 85:15, 0:100), in order of increasing concentrations of MeOH. The 85% MeOH fraction (10.8 g) was subjected to silica gel CC, eluted with CH_2Cl_2-CH_3COCH_3 with increasing amounts of CH_3COCH_3 to afford compounds 1

(12 mg) and 7 (500 mg). The 60% MeOH fraction (4.2 g) was subjected to silica gel CC, eluted with CH_2Cl_2-MeOH-H_2O (7:1:0.1) with increasing amounts of MeOH-H_2O (10:1); followed by reversed-phase C18 Si gel CC (200 g, particle size 40–63 μm), eluted by MeOH-H_2O (40:60, 50:50, 60:40, 65:35). The resulting fractions were subjected to semi-preparative HPLC, with a CH_3CN-H_2O (18:82; 26:74; 28:72; 30:70) mobile phase, to afford compounds 2 (70 mg), 3 (57 mg), 4 (92 mg), 5 (42 mg), 6 (66 mg), and 8 (1.3 g).

3.3.1. 15α-Acetoxyphysachenolide D (1)

Colorless needles; $[\alpha]_D^{25}$ -47.5 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 226 (2.57) nm; IR (neat) ν_{max} 3260 (broad), 2833, 2833, 1740, 1640, 1100, 1015 cm^{-1}; HRESIMS *m/z* 609.2670 $[M+Na]^+$(calcd for $C_{32}H_{42}O_{10}Na$, 609.2676, Δ = 1.0 ppm), 1195.5483 [2M+Na] (calcd for $C_{64}H_{84}O_{20}Na$, 1195.5454); For ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

3.3.2. Single-crystal X-ray structure determination of 15α-acetoxyphysachenolide D (1)

Crystal analysis was performed with a colorless cubic crystal (dimensions 0.51 × 0.07 × 0.03 mm^3) obtained from CH_3COCH_3-CH_3CN (1:1) using Cu Kα radiation (λ = 1.54178 Å)on a Bruker APEX2 diffractometer equipped with a Bruker MicroStar microfocus rotating anode X-ray source and Helios multilayer optics. Crystal data for 1: $C_{32}H_{42}O_{13}$-$3H_2O$ (formula weight 640.70), Orthorhombic, space group $P2_12_12_1$, T = 100(2) K, crystal cell parameters *a* = 10.8337(13) Å, *b* = 15.0357(18) Å, *c* = 19.525(2) Å, *V* = 3180.5(7) $Å^3$, D_c = 1.338 Mg/m^3, *Z* = 4, *F*(000) = 1376, absorption coefficient μ = 0.863 mm^{-1}. A total of 18,789 reflections were collected in the range 3.71 < θ < 68.09°, with 5564 independent reflections [$R_{(int)}$ = 0.0256], completeness to θ = 66° was 99.4%. Multi-scan absorption correction applied; full-matrix least-squares refinement on F^2, the number of data/restraints/parameters were 5564/0/579; goodness-of-fit on F^2 = 1.049; final *R* indices [$I > 2\sigma(I)$], R_1 = 0.0276, wR_2 = 0.0726; *R* indices (all data), R_1 = 0.0282, wR_2 = 0.0732; largest difference peak and hole, 0.177 and -0.168 e/$Å^{-3}$.

3.3.3. 2,3-Dihydro-3b-O-sulfonylphysachenolide D (2)

Solid; $[\alpha]_D^{25}$ −66.4 (*c* 0.63, MeOH); UV (MeOH) λ_{max} (log ε) 225 (1.97) nm; IR (neat) ν_{max} 3346 (broad), 2948, 2832, 1701, 1392, 1233, 1140, 1020 cm^{-1}; HRESIMS *m/z* 649.2314 $[M+Na]^+$(calcd for $C_{30}H_{42}O_{12}SNa$, 649.2295, Δ = 2.9 ppm); For ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

3.3.4. Physacoztolide F (3)

Solid; $[\alpha]_D^{25}$ −30.1 (*c* 1.82, MeOH); UV (MeOH) λ_{max} (log ε) 220 (1.80) nm; IR (neat) ν_{max} 3347 (broad), 2942, 2832, 1700, 1685, 1395, 1023 cm^{-1}; HRESIMS *m/z* 893.3796 $[M+Na]^+$(calcd for $C_{42}H_{62}O_{19}Na$, 893.3783, Δ = 1.5 ppm); For ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

3.3.5. 28-O-b-D-Glucopyranosylphysachenolide D (4)

Solid; $[\alpha]_D^{25}$ 21.4 (*c* 0.36, MeOH); UV (MeOH) λ_{max} (log ε) 226 (2.28) nm; IR (neat) ν_{max} 3312 (broad), 2944, 2832, 1702, 1685, 1450, 1021 cm^{-1}; HRESIMS *m/z* 729.3121 $[M+Na]^+$(calcd for $C_{36}H_{50}O_{14}Na$, 729.3098, Δ = 3.2 ppm); For ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

3.3.6. Physacoztolide G (5)

Solid; $[\alpha]_D^{25}$ 48.1 (*c* 0.69, MeOH); UV (MeOH) λ_{max} (log ε) 222 (2.30) nm; IR (neat) ν_{max} 3340 (broad), 2944, 2831, 1700, 1662, 1405, 1022 cm^{-1}; HRESIMS *m/z* 729.3101 $[M+Na]^+$(calcd for $C_{36}H_{50}O_{14}Na$, 729.3098, Δ = 0.4 ppm); For ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

3.3.7. Physacoztolide H (6)

Solid; $[\alpha]_D^{25}$ 12.8 (*c* 040, MeOH); UV (MeOH) λ_{max} (log ε) 222 (2.37) nm; IR (neat) ν_{max} 3344 (broad), 2942, 2830, 1700, 1680, 1662, 1397, 1021 cm^{-1}; HRESIMS *m/z* 671.3035 $[M+Na]^+$(calcd for $C_{34}H_{48}O_{12}Na$, 671.3044, Δ = 1.3 ppm); For ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

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Appendix A. Supplementary data

^1H, ^{13}C(APT), and 2D NMR spectra of withanolides 1–6 are available. Crystallographic data for the structure of 1 as reported in this paper were deposited with the Cambridge Crystallographic Data Centre with the CCDC number 1020813. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk). Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.10.012.

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