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WITHANOLIDE GLYCOSIDES FROM *DUNALIA AUSTRALIS**

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Key Word Index—*Dunalia australis*; Solanaceae; roots; withanolide glycosides; steroidal lactones; dunawithanines C, D, E and F.

Abstract—Four new withanolide glycosides, (20*R*,22*R*)-*O*-(3)-[β-D-xylopyranosyl(1→3),β-D-xylopyranosyl(1→4)]-β-D-glucopyranosyl-3β,20-dihydroxy-1α-acetoxy-witha-5,24-dienolide, (20*R*,22*R*)-*O*-(3)-[β-D-xylopyranosyl(1→3),β-D-glucopyranosyl(1→4)]-β-D-glucopyranosyl-3β,20-dihydroxy-1α-acetoxy-witha-5,24-dienolide, (20*R*,22*R*)-*O*-(3)-[β-D-glucopyranosyl(1→3),β-D-glucopyranosyl(1→4)]-β-D-glucopyranosyl-3β,20-dihydroxy-1α-acetoxy-witha-5,24-dienolide and (20*R*,22*R*)-*O*-(3)-[β-D-glucopyranosyl(1→3),β-D-glucopyranosyl(1→4)]-β-D-glucopyranosyl-3β,12β,20-trihydroxy-1α,acetoxy-witha-5,24-dienolide, named dunawithanines C, D, E and F, respectively, were isolated from *Dunalia australis*. Their structures were elucidated on the basis of spectral and chemical evidence, especially NMR data of the peracetates.

INTRODUCTION

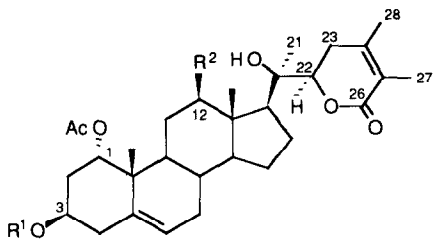
In earlier publications we reported on dunawithanines A and B from the sprouts of *Dunalia australis*, (Griseb.) Sleum. representing the first withanolide glycosides found in the plant kingdom [1, 2]. We now report on the isolation and structures of four further withanolide glycosides isolated from the roots of this plant.

RESULTS AND DISCUSSION

In a typical isolation, the dried and powdered roots of *Dunalia australis* were extracted with methanol and purified by repeated column chromatography on silica gel and finally HPLC on Lichrosorb RP18 (7 μm). In this way, we obtained in a low yield the following four new glycosides: dunawithanine C (**1a**), dunawithanine D (**2a**), dunawithanine E (**3a**) and dunawithanine F (**4a**), containing small amounts of impurities.

Acetylation of a mixture of **1a**–**4a** with acetic anhydride in pyridine gave after CC the peracetates **1b**, **2b**, **3b** and **4b** as suitable derivatives for a clear separation. Compounds **1b**–**4b** were structurally determined on the following spectral evidence. The IR spectra showed bands for α,β-unsaturated δ-lactone (1700–1710 cm^{-1}) and ester (1745 cm^{-1}) functions. All peracetates showed intense UV absorptions at 228–230 nm and ORD curves typical of a (22*R*)-configured withanolide with an α,β-unsaturated δ-lactone side chain moiety. The nature and connectivities of the sugar part in **1b**–**4b** were elucidated by detailed NMR studies.

Thus, the ^1H NMR spectra of compounds **1b**, **2b**, **3b** and **4b** (Table 1) indicated the presence of acetylated withanolide glycosides with dunawithagenine [2] and in the case of **4b** a 12β-hydroxylated dunawithagenine [3] as aglycone. All four compounds showed the following methyl signals of the unsaturated δ-lactone side chain: δ1.27 (H_3-21), 1.89 (H_3-27) and 1.94 (H_3-28) and a



	R^1	R^2
1a	Xyl(1→3)Xyl(1→4)Glc	H
2a	Glc(1→3)Xyl(1→4)Glc	H
3a	Glc(1→3)Glc(1→4)Glc	H
4a	Glc(1→3)Glc(1→4)Glc	OH

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Table 1. ^{1}H NMR spectral data of compounds **1a–4a** and **1b–4b**

H	1a*	1b†	2a*	2b†	3a*	3b‡	4a§	4b†
1β	5.03 *br s*	5.03 *br s*	5.06 *br s*	5.01 *s*	5.04 *br s*	4.16 *br s*	5.04 *br s*	4.99 *br s*
3α	3.88 *m*	3.74 *m*	overlapped	3.75 *m*	3.87 *m*	3.87 *m*	overlapped	3.74 *m*
6	5.54 *br d*	5.50 *br d*	5.52 *br d*	5.56 *br d*	5.53 *br d*	5.54 *br d*	5.54 *br d*	5.51 *br d*
12α							overlapped	3.39 *dd*
22	4.23 *dd*	4.21 *dd*	4.23 *dd*	4.22 *dd*	4.23 *dd*	4.20 *dd*	4.61 *dd*	4.78 *dd*
H_3-18	0.86 *s*	0.86 *s*	0.86 *s*	0.87 *s*	0.86 *s*	0.97 *s*	0.81 *s*	0.80 *s*
H_3-19	1.10 *s*	1.04 *s*	1.10 *s*	1.04 *s*	1.10 *s*	1.14 *s*	1.11 *s*	1.05 *s*
H_3-21	1.25 *s*	1.27 *s*	1.25 *s*	1.27 *s*	1.25 *s*	1.32 *s*	1.19 *s*	1.23 *s*
H_3-27	1.85 *s*	1.89 *s*	1.85 *s*	1.88 *s*	1.85 *s*	1.85 *s*	1.85 *s*	1.88 *s*
H_3-28	1.98 *s*	1.94 *s*	1.98 *s*	1.95 *s*	1.98 *s*	1.98 *s*	1.98 *s*	1.95 *s*
1α-OAc	2.02 *s*	overlapped	2.02 *s*	overlapped	2.02 *s*	overlapped	2.03 *s*	overlapped
	Glc	Glc	Glc	Glc	Glc	Glc	Glc	Glc
1	4.41	4.40	4.40	4.40	4.43	4.69	4.43	4.40
2		4.86		4.86		4.84		4.88
3		3.89		3.92		3.99		3.78
4		3.72		3.64		3.72		3.62
5		3.51		3.51		3.75		3.51
6	4.14	4.03/4.41		4.04/4.42		4.14/4.50		4.02/4.45
	Xyl	Xyl	Glc	Glc	Glc	Glc	Glc	Glc
1	4 52	4.61	4.44	4.56	4.61	4.85	4.62	4.46
2		4.90		4.98		5.07		4.99
3		5.14		5.15		5.28		5.18
4		4.99		5.14		5.23		5.16
5		3.43/4.29		3.68		3.96		3.66
6				4.20/4.57		4.18/4.65		4.18/4.59
	Xyl	Xyl	Xyl	Xyl	Glc	Glc	Glc	Glc
1		4.72	4.56	4.70		4.87		4.54
2		4.88		4.94		5.06		5.00
3		5.13		5.18		5.27		5.17
4		4.96		5.02		5.20		5.15
5		3.34/4.22		3.39/4.29		3.94		3.64
6						4.15/4.60		4.15/4.67

Typical J values (Hz)= Glc $J_{1,2}=8$; $J_{2,3}=9$; $J_{3,4}=9$; $J_{4,5}=9$; $J_{5,6a}=5$; $J_{5,6b}=3$; $J_{6a,6b}=-13$; Xyl $J_{1,2}=7$; $J_{2,3}=9$; $J_{3,4}=9$; $J_{4,5a}=8$; $J_{4,5b}=4$; $J_{5a,5b}=-12$; aglycone $J_{6,7a}=5$; $J_{6,7b}=<1$; $J_{11\alpha,12\alpha}=11$; $J_{11\beta,12\alpha}=4$; $J_{21a,22}=13$; $J_{21b,22}=4$.

*Solvent methanol-d_4, at 250 MHz.

†Solvent chloroform-d, at 300 MHz.

‡Solvent acetone-d_6, at 500 MHz.

§Solvent methanol-d_4, at 300 MHz.

methine signal in the low-field region at about 4.21 (H-22). The two methyl signals at δ0.86 and 1.04 were assigned to H_3-18 and H_3-19, respectively, of the steroidal skeleton. The broad doublet at δ5.50 was due to the olefinic proton H-6. The chemical shifts of the two methine signals at about δ3.8 (H-3α) and 5.0 (H-1β), respectively, indicated an acetyl group in the 1α-position and the sugar side chain bound to the C-3 oxygen at the β-face. Only in the case of compound **4b** was there an additional low-field methine signal at δ3.39, which was assigned to H-12α in the presence of a 12β-hydroxy group [3].

Identification of the sugars and their branching points was done by combined use of 2D COSY [4] and 2D ROESY [5] NMR techniques. Starting from the three anomeric proton signals of compound **1b** at δ4.40, 4.61 and 4.72 in the COSY spectrum, one seven-spin system due to glucose and two six-spin systems corresponding to two xylose residues were found. Observation of H-3 of glucose at δ3.89 and H-4 at 3.72 indicated that these positions were not acetylated and were, therefore, considered to be the branching points of the sugar chain. From their chemical shifts the two xylose sugars were recognized to be fully acetylated, hence they have to be terminal. The coupling constants $J_{1,2}$ of the anomeric protons showed the sugars to have the β-D conformation.

The assumption of a [β-D-triacetyl-xylopyranosyl-(1→4),β-D-triacetyl-xylopyranosyl(1→3)]-β-D-diacetyl-glucopyranosyl sugar chain was confirmed by a 2D NMR ROESY spectrum. Cross peaks between Xyl-1 and Glc-4 and Xyl-1′ and Glc-3, respectively, verified the spatial proximity of these protons. Furthermore, a ROESY cross peak between Glc-1 and H-3α of the aglycone, indicated the sugar chain to be placed at C-3 of dunawithagenine.

In a similar manner, the type of sugars and their points of attachment were determined by 2D COSY and

ROESY NMR spectra for compounds **2b**, **3b** and **4b**. Compound **2b** was identified as (20*R*,22*R*)-*O*-(3)-[β-D-triacetyl-xylopyranosyl(1→3),β-D-tetracetyl-glucopyranosyl(1→4)]-β-D-diacetyl-glucopyranosyl-3β,20-dihydroxy-1α-acetoxywitha-5,24-dienolide, compound **3b** as (20*R*,22*R*)-*O*-(3)-[β-D-tetraacetyl-glucopyranosyl(1→3), β-D-tetraacetyl- glucopyranosyl(1→4)] β-D-diacetyl-glucopyranosyl-3β,20-dihydroxy-1α-acetoxy-witha-5,24-dienolide and compound **4b** as (20*R*,22*R*)-*O*-(3)-[β-D-tetraacetyl-glucopyranosyl(1→3),β-D-tetraacetyl-glucopyranosyl(1→4)]-β-D-diacetyl-glucopyranosyl-3β,12β,20-trihydroxy-1α-acetoxy-witha-5,24-dienolide.

The COSY spectrum of compound **2b** showed the presence of two glucoses and one xylose with $J_{1,2}$ values of 7.6, 7.6 and 7.0 Hz, respectively, which revealed β-D conformations. The appearance of H-3 and H-4 signals of one glucose at δ3.92 and 3.64, respectively, showed that these positions were not acetylated, therefore indicating the branching points. The other glucose and the xylose were fully acetylated and had to be terminal. In the ROESY spectrum of **2b** beside cross peaks due to 1–3 diaxially oriented sugar protons two structurally important correlations were found. One of these correlations related Glc-1 to H-3α of the dunawithagenine, the other between Xyl-1 and Glc-3 showed the sequencing of the sugar chain to be Glc′(1→4), Xyl(1→3)-Glc. The ROESY cross peak between Glc-1′ and Glc-4 was overlapped by the cross peak Glc-1′/Glc-5′. Nevertheless, with only one of the two expected cross peaks it was possible to assign the sugar chain structure.

The COSY NMR spectra of **3b** and **4b** showed them to have the same sugar chain. The COSY experiment yielded two terminal glucoses and one glucose with H-3 and H-4 signals located at higher field than δ4.1, indicating these positions to be the branching points. The observation of ROESY cross peaks between Glc-1′/Glc-4 and Glc-2″/Glc-3 confirmed the structure of the sugar chain to be Glc′(1→4), Glc″(1→3)-Glc.

In addition, by use of the COSY technique it was also possible to elucidate the structure of the sugar chain of dunawithanine B [2] which remained open until now. Following the COSY correlations of the peracetylated compound two seven spin systems were found corresponding to the two glucose units. Due to the chemical shifts one of them had to be fully acetylated and was therefore terminal. In the case of the second glucose the H-2 signal appeared at high field at δ3.74. Thus, the structure of dunawithanine B was determined as (20*R*,22*R*)-*O*-(3)-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-3β,20-dihdyroxy-1α-acetoxy-witha-5,24-dienolide.

Deacetylation of the peracetates **1b**–**4b** under mild conditions ($NaHCO_3$ in methanol; 2 hr at 20°) led to the withanolide glycosides **1a**–**4a** which were identical with the compounds isolated directly from plant material. The withanolide glycosides were obtained as amorphous powders or gums with the following yields: dunawithanine C (**1a**): 0.07%, dunawithanine D (**2a**): 0.07%, dunawithanine E (**3a**): 0.08% and dunawithanine F (**4a**): 0.21%. The IR spectra of all four compounds showed the presence of a hydroxy group at 3400 cm^{-1}, an ester function at 1730–1720 cm^{-1} and an α,β-unsaturated δ-lactone at 1710–1700 cm^{-1}. The ^1H NMR spectra (Table 1) showed five methyl singlets for the 18-, 19-, 21-, 27- and 28-methyl groups and an acetyl singlet at δ2.02 which was due to the native 1α-acetyl group. In the high field region, signals overlapped strongly and only that of the anomeric proton of the glucose attached to the withanolide and the olefinic proton H-6 were separated from the bulk of the resonances.

Acid hydrolysis of compound **1a** (1 M HCl in methanol) gave xylose and glucose, identified by TLC, PC and HPLC as well as the aglycone 1α-acetyl-dunawithagenine besides dunawithagenine [2, 6]. Similar acid hydrolysis of **2a** gave the same aglycones and sugars as in the case of **1a**. Acidic hydrolysis of **3a** yielded dunawithagenine, 1α-acetyl-dunawithagenine and glucose. Acid hydrolysis of **4a** gave glucose, 12β-hydroxy-dunawithagenine and 1α-acetyl-12β-hydroxy-dunawithagenine [3].

EXPERIMENTAL

^1H NMR spectra were recorded at 250, 300 and 500 MHz, respectively. Mps: corr. CC silica gel Merck 60 (0.04–0.063 μm) unless otherwise stated.

Isolation. Dried and powdered roots (380 g) of *Dunalia australis* (grown in a greenhouse) were extracted exhaustively with MeOH (3 l). The MeOH soln was concd under red. pres. and the residue (15 g) was chromatographed over silica gel (600 g). Elution with $CHCl_3$ gave 477 mg (0.13%) sitosterol (identical to an authentic specimen). Elution with $CHCl_3$–MeOH (19:1) yielded 380 mg (0.1%) sitosterol glucoside (identical to an authentic specimen). Elution with $CHCl_3$–MeOH–H_2O (100:16:1.6) gave a mixture of four polar glycosides **1a**, **2a**, **3a** and **4a** (6.975 g). Acetylation of this mixture (8 hr at 20°) with Ac_2O (70 ml) and pyridine (70 ml) gave after concn under red. pres. a mixture of the crude peracetates **1b**, **2b**, **3b** and **4b** (7.5 g). CC over silica gel (375 g) afforded the following pure peracetates.

Dunawithanine C peracetate (**1b**). Elution with $CHCl_3$–EtOAc (9:1) gave 1.027 g **1b**: mp 123–126° (petrol–$CHCl_3$), $[\alpha]_D^{22}$ −21.5° ($CHCl_3$; *c* 0.2), IR ν_{max} cm^{-1}: 1700 (α,β-unsaturated δ-lactone), 1745 (ester), 3550 (OH). UV λ_{max} nm (log ε): 229 (6300), ORD: $[\Theta]_{270}$ +1705°, $[\Theta]_{253}$ 0°, $[\Theta]_{240}$ −3510° (a = +52, *c* 0.5 in $CHCl_3$).

Dunawithanine C (**1a**). Compound **1b** (103 mg) in MeOH (20 ml) was stirred with $NaHCO_3$ (80 mg) for 2 hr at room temp. Neutralization ($MeCO_2H$), evapn and TLC purification of the residue yielded 27 mg (0.07%) **1a** and 15 mg (0.04%) 1α-deacetyldunawithanine C. **1a**: amorphous, $[\alpha]_D^{25}$ +15.2° (MeOH; *c* 0.1). UV λ_{max} nm (log ε): 230 (1200). IR ν_{max} cm^{-1}: 1710 (α,β-unsaturated δ-lactone), 1730 (ester), 3400 (OH).

Acid hydrolysis of compound **1a**. Compound **1a** (20 mg) was refluxed for 4 hr with 1 M methanolic HCl (5 ml). The soln was concd under red. pres. and diluted with 5 ml H_2O. It was extracted with EtOAc and the organic solvent gave dunawithagenine and 1α-acetyl-dunawithagenine [2, 6]. The aq. phase was concd, xylose and glucose were identified by PC [Schleicher & Schuell 2043b, *n*-BuOH–HOAc–H_2O (4:1:5) and H_2O-satd C_6H_5OH, detection with aniline-phthalic acid].

Dunawithanine D peracetate (**2b**). Elution with $CHCl_3$–EtOAc (4:1) yielded 63 mg **2b**: mp 118–120° (petrol–$CHCl_3$), $[\alpha]_D^{22}$ −9.5° ($CHCl_3$; *c* 0.3). IR ν_{max} cm^{-1}: 1710 (α,β-unsaturated δ-lactone), 1745 (ester), 3600 (OH). UV λ_{max} nm (log ε): 230 (5400), ORD: $[\Theta]_{270}$ +1082°, $[\Theta]_{253}$ 0°, $[\Theta]_{244}$ −2803° (a = +39, *c* 1.0 in $CHCl_3$).

Dunawithanine D (**2a**). Deacetylation of **2b** (50 mg) (analogous to **1b**→**1a**) and purification by TLC led to 27 mg **2a**: amorphous, $[\alpha]_D^{24}$ +5.7° (MeOH; *c* 0.2). IR ν_{max} cm^{-1}: 1710 (α,β-unsaturated δ-lactone), 1720 (ester), 3400 (OH). UV λ_{max} nm (log ε): 230 (4200).

Acid hydrolysis of compound **2a**. Compound **2a** (13 mg) was refluxed for 4 hr with 1 M methanolic HCl (3 ml). Analogous work-up gave dunawithagenine, 1α-acetyl-dunawithagenine, glucose and xylose.

Dunawithanine E peracetate (**3b**). Elution with $CHCl_3$–EtOAc (7:3) gave 965.4 mg **3b**: mp 117–123° (petrol–$CHCl_3$), $[\alpha]_D^{22}$ −2.1° ($CHCl_3$; *c* 0.2). IR ν_{max} cm^{-1}: 1710 (α,β-unsaturated δ-lactone), 1745 (ester), 3600 (OH). UV λ_{max} nm (log ε): 230 (16 300), ORD: $[\Theta]_{270}$ +3018°, $[\Theta]_{260}$ 0°, $[\Theta]_{226}$ −11469° (a = +145, *c* 0.4 in $CHCl_3$).

Dunawithanine E (**3a**). Deactylation of **3b** (100 mg) (analogous to **1b**→**1a**) yielded after TLC purification 32 mg (0.08% yield) **3a** and 17 mg 1α-deacetyl-dunawithanine E. **3a**: amorphous, $[\alpha]_D^{24}$ +12.3° (MeOH; *c* 0.8). IR ν_{max} cm^{-1}: 1700 (α,β-unsaturated δ-lactone), 1730 (ester), 3400 (OH). UV λ_{max} nm (log ε): 230 (1100).

Acid hydrolysis of compound **3a**. Glycoside **3a** (20 mg) on hydrolysis with 1 M methanolic HCl (5 ml) under reflux for 4 hr followed by usual work-up gave dunawithagenine, 1α-acetyl-dunawithagenine [2, 6] and only glucose.

Dunawithanine F peracetate (**4b**). Elution with $CHCl_3$–EtOAc (3:2) gave 1.257 g 12β-acetyl-dunawithanine F peracetate. Further elution with $CHCl_3$–EtOAc (1:1) gave 424 mg **4b**: mp 120–124° (petrol–Me_2CO), $[\alpha]_D^{26}$ −23.4° ($CHCl_3$; *c* 0.2). IR ν_{max} cm^{-1}: 1710 (α,β-unsaturated δ-lactone), 1745 (ester), 3400 (OH). UV λ_{max} nm (log ε): 230 (11600), ORD: $[\Theta]_{272}$ +4930°, $[\Theta]_{260}$ 0°, $[\Theta]_{226}$ −10563° (a = +155, *c* 0.4 in $CHCl_3$).

Dunawithanine F (**4a**). Deacetylation of **4b** (100 mg) (analogous to **1b**→**1a**) gave after CC 50 mg (0.21% yield) **4a**: amorphous, $[\alpha]_D^{25}$ −18° (MeOH; *c* 0.5). IR ν_{max} cm^{-1}: 1700 (α,β-unsaturated δ-lactone), 1720 (ester), 3400 (OH). UV λ_{max} nm (log ε): 228 (7800), ORD: $[\Theta]_{270}$ +1021°, $[\Theta]_{260}$ 0°; $[\Theta]_{230}$ −4086° (a = +51.1, *c* 0.3 in MeOH).

Acid hydrolysis of compound **4a**. Compound **4a** (20 mg) was refluxed for 4 hr with 1 M methanolic HCl (5 ml). Analogous work-up yielded glucose (detected by PC) and 12β-hydroxy-dunawithagenine [3].

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