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21′-DI-DEHYDRO-DEACETYLLANATOSIDE C, A BIOTRANSFORMATION PRODUCT OF DEACETYLLANATOSIDE C FROM SENESCENT SHOOT CULTURES OF *DIGITALIS LANATA*

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Key Word Index—*Digitalis lanata*; Scrophulariaceae; cardiac glycosides; structural elucidation; 21′-di-dehydro-deacetyllanatoside C.

Abstract—Feeding deacetyllanatoside C to senescent shoot cultures of *Digitalis lanata* resulted in the formation of a new product, which was isolated by semi-preparative HPLC. The molecular structure was elucidated by means of HPLC-mass spectrometry and NMR as 21′-di-dehydro-deacetyllanatoside C. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Shoots of *Digitalis lanata* were grown axenically on modified MS nutrient medium according to Stuhlemmer *et al.* [1]. After addition of abscisic acid the cultivated shoots showed features of senescence (accumulation of polyphenols, fading of chlorophyll) [2]. Feeding of deacetyllanatoside C (**2**) to the senescent shoots resulted in the formation of a new product which was identified as 21′-di-dehydro-deacetyllanatoside C (**1**). This paper reports on the structural elucidation of this compound by HPLC–mass spectrometry and NMR.

RESULTS AND DISCUSSION

Shoot cultures of *D. lanata* were treated with abscisic acid for 10 days until the shoots appeared senescent. Compound **2** was then added and four days later the medium was extracted with a mixture of chloroform and isopropanol. The solvent was evaporated *in vacuo* to give a residue, which was purified by semi-preparative HPLC, yielding **1**.

Compound **1** showed a molecular peak at *m/z* 942 $[M+H]^+$ and fragment ions at *m/z* 652 $[M+H]^+$ (digoxigenin-bisdigitoxoside + H) and 522 $[M+H]^+$ (digoxigenin-monodigitoxoside + H) on HPLC–mass spectrometry. The molecular mass of the substrate (**2**) under the same conditions was 944 $[M+H]^+$, i.e. 2 units higher than the M_r of the product. The fragmentation pattern indicated that the loss of the two mass units took place at the final glucose or the third digitoxose of the sugar side chain because the fragment ions *m/z* 652 and 522 were present in the spectrum of **1** as well as in the spectrum of **2**.

All ^{13}C signals of the aglycone moiety were identical in **1** and **2**. The four methine signals at δ 106.98, 100.46, (2x C) and 96.85 indicated the presence of four sugar units. Starting from the signals of the corresponding anomeric protons one six-spin and three nine-spin systems were found based on the results of the double quantum filtered ^1H–^1H COSY spectrum.

One-bond and long-range ^1H–^{13}C shift correlation 2D experiments allowed the unambiguous assignment of the ^1H and ^{13}C sugar moiety signals even in the case of overlapping proton resonances. The three ^1H nine-spin systems were easily identified as belonging to three digitoxose units by the COSY correlation of the anomeric protons with two geminal protons in the high-field region and by the correlation between a methyl group with H-5 of the sugar moiety. The fourth sugar unit showed a ^1H six-spin system including a terminal CH_2OH group. Because there were six ^{13}C signals belonging to this sugar unit it had to be a hexose.

The chemical shift of one ^{13}C signal at δ 206.9 indicated the presence of a keto group for this sugar. The position of the carbon group was determined by

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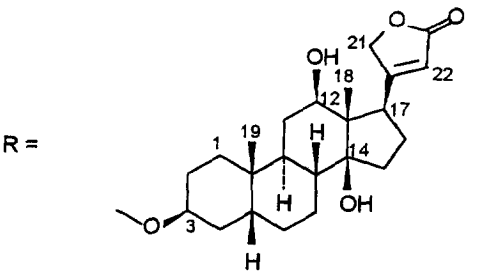

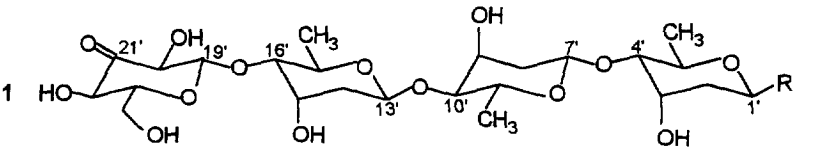

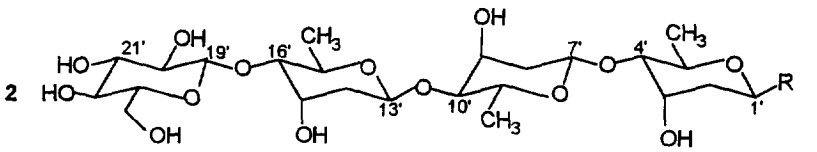

Table 1. ^{13}C NMR chemical shifts of 21′-di-dehydro-deacetyllanatoside C in methanol-d_4

Aglycone C	[4]		Sugar	C	
1	31.0	30.97*	β-D-Dx†	1′	96.85
2	27.4	27.45*		2′	38.87[a]
3	74.4	74.34*		3′	68.38
4	31.4	31.39*		4′	84.31
5	37.9	37.91		5′	69.37
6	27.8	27.75*		6′	18.55
7	22.7	22.77*	β-D-Dx	7′	100.46
8	42.2	42.14*		8′	38.69[a]
9	33.5	33.55		9′	68.21
10	36.2	36.15		10′	83.51[b]
11	30.8	30.81*		11′	69.43
12	75.3	75.56		12′	18.55
13	57.3	57.21	β-D-Dx	13′	100.46
14	86.3	86.72		14′	38.46[a]
15	33.6	33.51*		15′	68.21
16	28.3	28.34		16′	83.72[b]
17	47.1	46.99		17′	69.43
18	9.8	9.91		18′	18.55
19	24.2	24.24	β-D-Gl	19′	106.98
20	178.4	178.40		20′	78.22
21	75.4	75.38		21′	206.92
22	117.7	117.64		22′	73.31
23	177.3	177.46		23′	77.93
				24′	62.13

* Assignments were performed according to ref. [4].

† Sugar bound to the aglycone.

[a,b] Assignemnts may be interchanged.

Table 2. ^{1}H NMR chemical shifts of 21′-di-dehydro-deacetyllanatoside C in methanol-d_4 (multiplicity in parenthesis)

H	Aglycone	Sugar	H	
1 A/B	*1.46/1.81*	β-D-Dx*	1′	4.89
2 A/B	*ca* 1.5–*ca* 1.7		2′ A/B	*1.70/1.93*
3	4,0		3′	4.34
4 A/B	*ca* 1.4–*ca* 1.5		4′	3.35
5	1.65		5′	3.93
6 A/B	*1.27/1.88*		Me-6′	1.292 (*d*)
7 A/B	*1.26/1.74*	β-D-Dx	7′	4.90[a]
8	*1.6*		8′ A	1.99[b]
9	1.74		8′ B	1.75[c]
11 A/B	*1.27/1.59*		9′	4.23
12	3.38		10′	3.23
15 A/B	*1.73/1.97*		11′	3.81
16 A/B	*1.94/2.14*		Me-12′	1.200 (*d*)
17	3.32	β-D-Dx	13′	4.94[a]
Me-18	0.783 (*s*)		14′ A	2.02[b]
Me-19	0.949 (*s*)		14′ B	1.78[c]
21 A/B	*4.90/4.97* (*dd*)		15′	4.23
22	5.901 (*br*)*s*		16′	3.23
			17′	3.81
			Me-18′	1.198 (*d*)
		β-D-Gl	19′	4.490 (*d*)
			20′	4.162 (*dd*)
			21′	4.17
			22′	4.25
			23′	3.33
			24′ A	3.90
			24′ B	3.80

J (Hz): H-21 A/H-21 B = 18.3; Me-6′/H-5′ = 6.1; Me-12′/H-11′ = 6.4; Me-18′/H-17′ = 6.1; H-19′/H-20′ = 7.8; H-20′/H-22′ = 1.2.

Values in italics are chemical shifts of cross peaks (DQFCOSY, HMQC).

* Sugar bound to the aglycone.

[a,b,c] Assignments may be interchanged.

considering the ^{1}H–^{1}H COSY correlations and the ^{1}H–^{1}H coupling constants. The anomeric proton H-19′ (δ 4.490, *d*, *J* = 7.8 Hz) showed a correlation with a double doublet (δ 4.162, *dd*, *J* = 7.8 and 1.2 Hz), which therefore had to be H-20′; H-20′ gave a further correlation with a signal at δ 4.25. Unfortunately, this signal was partly overlapped with the signals of H-9′ and H-15′. Thus, the coupling constants could not be extracted from the routine ^{1}H NMR spectrum. However, even though the ^{1}H signal of H-23′ was heavily overlapped with H-12, H-4′ and the solvent signal, in the 1D proton spectrum the ^{1}H chemical shift of H-23′ were known by its COSY correlations with H-24A′ and H-24B′.

By using homodecoupling with irradiation at H-23′, the coupling constant of H-23′ with the signal at δ 4.25 could be estimated by the shift of the most low-field multiplet line to be >7 Hz and hence to correspond to a vicinal coupling whereas the coupling to H-14′ (1.2 Hz) had to be a long-range coupling. Therefore, the ^{1}H signal at δ 4.25 was assigned to H-22′, and the ^{13}C carbonyl signal at δ 206.9 was assigned to C-21′.

Finally, ^{1}H–^{13}C long-range (HMBC) shift correlations were used to establish the connection of the sugar components and the aglycone. HMBC correlation peaks were detected between H-1′ and C-3, H-7′ and C-4′, H-13′ and C-10′ and H-19′ and C-16′, respectively. Therefore, the structure of **1** was established as 21′-di-dehydro-deacetyllanatoside C. The ^{1}H and ^{13}C NMR data are summarized in Tables 1 and 2.

EXPERIMENTAL

General. 1D and 2D NMR spectra of **1** were recorded in MeOH-d_4 at 301 K on a Varian Unity 500 NMR spectrometer (^{1}H: 500 MHz; ^{13}C: 125.7 MHz). Samples were fractionated with a Hewlett Packard HPLC System 1050. Mass spectra were obtained on a Hewlett Packard HP 5989A mass spectrometer 5989A equipped with a HP electrospray source 59987A (HPLC-MS). Semi-prep. HPLC was performed on a Beckman HPLC System Gold (pump 125, DAD detector 168).

Plant material. Shoot-forming cultures were

initiated from axillary buds of individual plants of *D. lanata* EHRH. cv. Boehringer Mannheim GmbH in May 1989 and established as continuous cultures in liquid medium by Stuhlemmer *et al.* [1]. The cultures were propagated in half-strength MS medium [1, 3] (except for NH_4NO_3 and KH_2PO_4, which were full-strength) supplemented with 0.6 μM IAA, 4.5 μM BA and 0.1 μM glucose. The pH was adjusted to 6.0 before autoclaving. The shoots were subcultured weekly: 15 g wet wt of tissue were transferred into 300 ml Erlenmeyer flasks containing 50 ml medium. The flasks were kept on a gyratory shaker (80 rev min^{-1}, 24°) under continuous white light (fluorescent tubes, 10 W m^{-2}).

On the 1st day after transfer into fresh liquid medium, abscisic acid (0.1 mM) was added to the liquid medium. Compound **2** (0.6 mM) was added 10 days later, when the shoots appeared senescent. The largest amount of **1** (90%) was detected in the medium whereas the shoots contained less of the product. Therefore, only the medium was used for isolation of **1**.

Extraction and isolation. The medium was extracted (× 3) with 20 ml $CHCl_3$–*iso*-PrOH (3:2). The organic phases were combined and dried over Na_2SO_4. The solvent was evapd under red. press. and the residue dissolved in 2 ml MeOH. After centrifugation at 14000 *g* the clear supernatant was used directly for HPLC analysis and semi-prep. HPLC.

Compound **1** was isolated by semi-prep. HPLC (Beckman, System Gold) on a combination of two spherisorb-ODS 2–5 μm columns (precolumn 8 mm i.d. × 4 cm; main column 8 mm i.d. × 25 cm). Flow rate: 3 ml min^{-1}. Samples were eluted with a step gradient of aq. 84% MeCN (solvent B) and double-distilled H_2O (solvent A): start (20% B); 5 min (32% B); 15 min (35% B); 25 min (38% B), 30 min (92% B); 32 min (20% B). Cardenolides were detected at 220 nm.

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