DIGITOXIGENIN-3β-O-[β-D-FUCOPYRANOSYL-4'-β-D-GLUCOPYRANOSIDE], THE MAIN CARDENOLIDE OF SOMATIC EMBRYOS OF *DIGITALIS LANATA*

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Abstract—The chemical structure of glucodigifucoside was established by ^{1}H NMR, ^{13}C NMR, and FABMS as digitoxigenin-3β-O-[β-D-fucopyranosido-4'-β-D-glucopyranoside]. Glucodigifucoside obtained from somatic embryos of *D. lanata* was shown to be identical with an authentic specimen from *D. lanata* leaves.

INTRODUCTION

Suitable cell strains of *Digitalis lanata* form large numbers of somatic embryos *in vitro* (for a summary of the literature see [1]). Embryogenesis was investigated most thoroughly with cell strain VII which is able to synthesize considerable quantities of cardenolides in the embryoid stage-II globules and late embryoid structures. Cardenolide accumulation depended upon the growth regulators and nutrients as well as on the regime of photoirradiation [2–4]. The cardenolides formed bind to antibodies against digitoxigenin (1) [2]. The main products on TLC and HPLC were identical with glucodigifucoside (digitoxigenin-3β-O-[β-D-fucopyranosido-4'-β-D-glucopyranoside]) (2), and odorobioside G (digitoxigenin-3β-O-[β-D-digitalopyranosideo-4'-β-D-glucopyranoside], (3) [5]). Compounds 2 and 3 have also been found in cultures of another embryogenic cell strain of *D. lanata* [6]. They are known constituents of *D. lanata* leaves first identified by Kaiser [7,8] through a comparison with authentic samples of 2 and 3 obtained from seeds of *D. purpurea* [9] and from cortex of *Nerium odorum* [10], respectively. In agreement with the proposed structures, 2 and 3 yielded 1, D-glucose, and D-fucose/D-digitalose on hydrolysis and identification by TLC comparison with authentic samples [5]. The suggested chemical structures of 2 and 3 have been derived from enzymatic hydrolysis and analysis of the products [9,10]. They have never been proven rigorously. The experiments described in this paper were conducted with the aim of verifying the structure of 2 isolated from leaves of *D. lanata* and to compare it with 2 obtained from the somatic *D. lanata* embryos, as well as to provide fundamental NMR data for this series of natural products.

RESULTS AND DISCUSSION

The negative ion FABMS of 2 showed prominent peaks corresponding to M$^+$, M$^+$ minus a hexose unit, and 1. In the positive mode FABMS spectrum ions were detected that were obviously derived from 1 and 2 as well as from the sugar moiety. These ion patterns agreed with those expected for 2 [11, 12] and were consistent with the structure of 2 proposed by Okano [9].

The combined ^{1}H and ^{13}C NMR spectral data sets provided unequivocal proof of the structure of 2. From these data it was possible to determine configurations for all of the ring fusions in the aglycone moiety, sites of oxygenations and their stereochemistries, the configuration at the site of butenolide attachment, and the identities and configurational assignments of the glycosyl residues.

Examination of the composite pulse proton decoupled ^{13}C NMR spectrum revealed lines for a total of 35 carbon atoms, the multiplicities of which could be determined from an edited DEPT analysis. For the 23 carbon atoms of the aglycone moiety, two quartets, ten triplets, six doublets and five singlets were identified. Carbon 13 chemical shifts were generally consistent with those of digitoxigenin (1), the suggested aglycone of glucodigifucoside (2) [13–15], as well as with glycosides of 1. The major differences in chemical shift were found for the resonances of C-3 (1, δ66.8, [14], 2, δ71.1), C-4 (1, δ33.5, [14], 2, δ30.9), and C-5 (1, δ35.9, 2, δ36.7). These comparisons also suggested a β-configuration for linkage of the first sugar residue with 1 [16]. Correlations of ^{13}C- and ^{1}H-chemical shifts were identified from the 2D-HMQC [17] experiment. For most of the protons of the aglycone unit only general region assignments were possible due to the complexity of the proton coupling patterns and the overlap in the upfield region of the proton spectrum (Table 1). Nevertheless, the correlations were adequate for determinations of the aglycone ring configurations.

Determination of the configuration of the A/B ring junction followed readily from comparisons of ^{13}C NMR spectral data of 2 with a series of model compounds, [16, 18–21] and from applications of steric arguments. Compounds possessing *trans*-fused A/B junctions (e.g. uzarigenin (4), [22] and 5α-androstane-3β-ol (5), [13] exhibit chemical shifts for carbon atoms C-1 through C-6 that lie in a range between δ27 and 45 in methanol–water solutions, whereas these same carbon atoms resonate at

Table 1. ^{13}C and ^1H NMR assignments of 2*

C	^{13}C chemical shifts δ (ppm)† mult.‡		Correlated ^1H chemical shifts δ (ppm)† No. of H-atoms		Multi-plicity	J (Hz)
1	30.5	t	1.45	2 H		
2	27.9	t	1.75	2 H		
3	71.1	d	4.15	1 H		
4	30.9	t	1.45	2 H		
5	36.7	d	1.68	1 H		
6	27.0	t	1.59, 1.60	2 H		
7	22.0	t	not discernable			
8	42.4	d	1.15	1 H		
9	37.2	d	1.85	1 H		
10	35.8	s	----			
11	22.2	t	1.45	2 H		
12	40.8	t	1.46	2 H		
13	50.9	s	··			
14	86.9	s	----			
15	33.0	t	1.7, 2.2	2 H		
16	27.4	t	1.75, 1.85	2 H		
17	51.7	d	2.83	1 H		
18	16.3	q	0.85	3 H		
19	23.9	q	0.95	3 H		
20	178.4	s	----			
21a	75.8	t	4.95	1 H	dd	18.1, 2.6
21b			5.05	1 H	dd	18.1, 2.6
22	117.2	d	5.91	1 H	t	2.6
23	179.5	s	----			
1′	102.4	d	4.33	1 H	d	7.8
2′	72.5	d	3.52	1 H	dd	7.8, 9.2
3′	77.5	d	3.63	1 H	dd	9.2, 3.1
4′	81.8	d	3.91	1 H	dd	3.1, 1.5
5′	75.2	d	3.71	1 H	dq	6.3, 1.5
6′	17.1	q	1.28	3 H	d	6.3
1″	105.2	d	4.61	1 H	d	8.0
2″	71.2	d	3.35	1 H	dd	8.0, 8.6
3″	77.5	d	3.42	1 H	t	8.6, 8.6
4″	75.4	d	3.68	1 H	t	8.6, 8.6
5″	75.9	d	3.85	1 H	dt	8.6, 7.0
6″	62.2	t	3.35	2 H	m	7.0, ?

*The sample was dissolved in CD_3OD-D_2O 5:1.
†All values given relative to TMS.
‡Determined by DEPT experiments.

higher field ($\delta 22-\delta 36$) in A/B cis-fused systems such as 1 and in 2. These differences are due to steric compression shifts induced in the latter systems by forcing the ring system into the cis-configuration [19]. Particularly diagnostic is the chemical shift of C-7 (ca $\delta 22$ in cis-fused systems as well as in 2). Additionally, the A/B cis-configuration for 2 is indicated by the chemical shifts of methyl groups C-18 ($\delta 16.3$) and C-19 ($\delta 23.9$). In A/B trans- systems, such as 4 and 5, C-18 and C-19 resonate at relatively high field, with C-19 about 3–5 ppm upfield from C-18. In A/B cis-fused systems like 1, the C-19 resonance lies downfield from C-18. The C-18 resonance does not exhibit much change in position, but C-19 is deshielded by about 10 ppm.

The B/C trans-fusion is indicated [21] for 2 by the close correspondence of the ^{13}C-chemical shifts with those of compounds of known structural type, and by the observa-tion of a strong cross peak in the COSY spectrum due to a 1, 3-synaxial interaction between H-8 and H-11.

The C/D ring junction of 2 is assigned as cis, with the butenolide ring attached at the C-17β position. In sup-port of this configuration, protons attached to methyl group H-18 ($\delta 0.85$) give rise to strong synaxial J-correla-tions with proton H-11 ($\delta 1.45$), and proton H-8 ($\delta 1.15$), as well as to H-17α ($\delta 2.83$). Moreover, this configuration is supported by the ^{13}C NMR data, based on comparisons of ^{13}C- chemical shifts of 2 with those of 17β-H-digitoxi-genin, 6 [23]. The upfield steric compression shift of the C-18 resonance from $\delta 19.2$ in 6 to $\delta 16.1$ in 2 is diagnostic for this arrangement. The overall ring configuration of 2 may be checked by application of the well-known addit-ivity rules for steroids [24]. Comparisons of the experimentally determined proton chemical shifts for methyl groups C-18 and C-19 with shifts calculated from

the reference compound 5β, 14β-androstane (7) correspond with a high degree of precision to the *cis, trans, cis* (5β, 14β, 17α-H)-configuration.

The configuration at C-3, the site of glycosyl attachment, is easily identified as 3β-O based on the chemical shift of the H-3 proton (δ4.08) when compared to a number of reference steroids [25]. The equatorial proton H-3 in 2 is deshielded by the sigma electrons of the adjacent carbon–carbon bonds compared to the corresponding axial proton of 3α-O steroids, which resonates at *ca* δ3.1.

The ¹³C- resonances for the sugar moiety of 2 indicated that with the exception of one, each carbon atom was bonded to at least one oxygen atom. The chemical shifts of the proposed D-fucopyranosyl moiety showed strong similarities with the signals of the D-digitalo (3-O-methyl-D-fuco) pyranosyl moieties of digoxigenin digitaloside (8) [26], diginatigenin digitaloside (9) [26], and gitoxigenin digitaloside (10) [26]. The smaller chemical shift difference at C-2′ (8, 71.2; 9, 71.0; 10, 71.3 [14]; 2, 72.5) compared with C-4′ (8, 68.8; 9, 68.6; 10, 68.9 [14]; 2, 81.8) is in agreement with the results of ¹H NMR studies which indicated that the terminal D-glucose moiety is linked to C-4′ of the D-fucose residue. (At least part of the chemical shift difference at C-2′ is probably brought about by the different solvents used. Compounds 8, 9, and 10 were measured in CD₃OD/CDCl₃ [27]. Compound 2 was examined in CD₃OD/D₂O, *cf.* Table 1 due to poor solubility in the former solvent. The ¹³C NMR signals of the D-glucopyranosyl moiety resembled those of terminal D-glucopyranosyl residue in lanatoside E [11] as well as in several cardenolides from *Adonis vernalis* [27].

Proton connectivity patterns were traced for each of the sugar units with data from the COSY-90 experiment. These data were used to identify the chemical shift values and coupling information for all of the protons in the disaccharide moiety. Proton chemical shift values, magnitudes of the coupling constants, and line multiplicities were consistent with β-D-fucopyranosido-[1,4]-β-D-glucopyranoside. The 2D-NOESY experiment revealed strong cross-relaxation correlations between transglycosidic protons H-3α (δ4.08)-H-1′ (δ4.33), (consistent with

the β-D fucose linkage at the 3β-O position of the aglycone), *trans*-glycosidic protons H-4′$_{ax}$ (δ3.91)-H-1″$_{ax}$ (δ4.61), (consistent with the β [1,4] linkage between the fucose and glucose residues), as well as between H-1″$_{ax}$ (δ4.61)-H-5′$_{ax}$ (δ3.71).

In summary, this paper provides NMR data that allows the structural formula of 2 to be unequivocally derived (Fig. 1).

EXPERIMENTAL

Isolation of 2 from D. lanata embryos. D. lanata cell strain VII was cultivated as described [3]. Lyophilized, powdered somatic embryos (250 g) were refluxed with 2 l EtOH (20%) for 15 min. The extract was treated with 400 ml Pb(OAc)₂ soln (15%). After centrifugation and addition of 600 ml Na₂HPO₄ soln it was again centrifuged. The supernatant was extracted × 6 with 200 ml CHCl₃-*i*-PrOH (3:2) and the extract concd under red. pres. The residue was mixed with silica gel 60 (E. Merck, Darmstadt; particle size 0.063–0.200 mm) and chromatographed on a silica gel column (3 × 20 cm). After treatment of the column with C₆H₆ (1 l) and EtOAc (1 l), the cardenolides were eluted with EtOH (96%, 250 ml), and the cardenolide-containing extract was concd under red. pres. Compound 2 was obtained by HPLC on Spherisorb ODS 5 (Pye Unicam), MeOH-H₂O (63:37), UV 225 nm, retention factor $k' = 5.0$. The sample was crystallized from Me₂CO-Et₂O to give white needles which were readily soluble in H₂O and EtOH. The total amount of 2 isolated was 4 mg.

FABMS was carried out using a VG-7070 E-HF instrument with glycerol as the matrix and a 6 keV accelerated Xe neutral atom beam. The ion source accelerating potential was 8 kV. The results were calculated after subtraction of ions derived from the matrix, e.g. [2 (glycerol) + Na⁺]⁺ at *m/z* 207, [2 (glycerol) + H]⁺ at *m/z* 185, [glycerol + Na⁺]⁺ at *m/z* 115, and [2 (glycerol) − H]⁻ at *m/z* 183 in the positive and negative mode, respectively. FABMS *m/z* (rel. int.) Negative mode: 681(100) [2−H]⁻; 519(19), [2 − hexose]⁻; 373(11), [1]⁻; 319(14), [1 − 3 H₂O]⁻. Positive mode: 721 (27), [2 + K]⁺; 705 (100), [2 + Na]⁺; 375 (36), [1 + 2H]⁺; 357(38), [1 + 2H − H₂O]⁺; 339 (34), [1 + 2H − 2 H₂O]⁺; 309 (38), [hexose + methylpentose]⁺.

D-Glucopyranosyl moiety   D-Fucopyranosyl moiety   Digitoxigenin (1) moiety
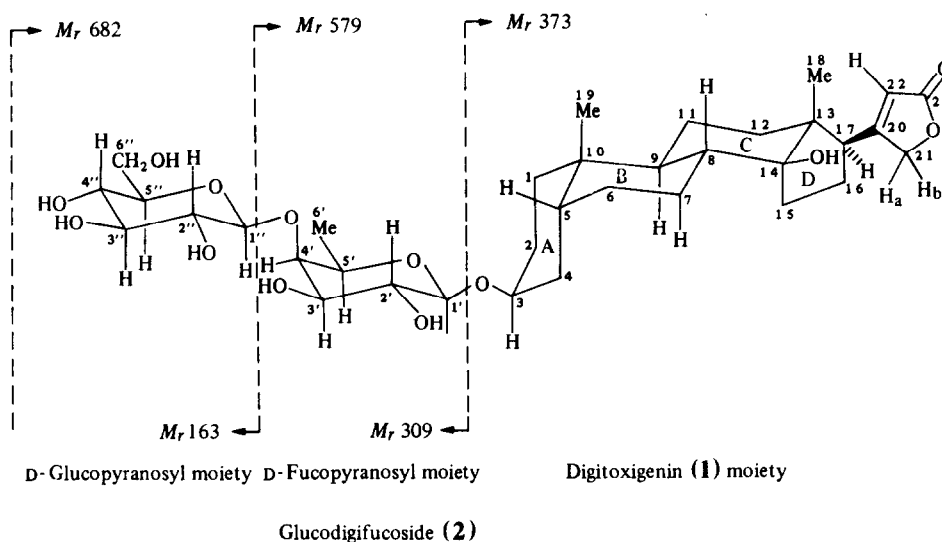
Glucodigifucoside (2)

Fig. 1.

NMR spectra were measured as methanol-d_4–D_2O 5:1 solns at 313 K (300.13 MHz ^1H and 75.46 MHz ^{13}C). Proton signals were assigned by 1-D proton spectroscopy, 2-D proton homonuclear correlated spectroscopy (COSY) [28], 2-D proton *J*-resolved spectroscopy [29], and phase-sensitive homonuclear correlated nuclear Overhauser (NOESY) [30] experiments. ^{13}C signals were examined by 1-D composite pulse proton-decoupled ^{13}C analysis, and multiplicities were determined by an edited DEPT (Distortionless Enhancement by Polarization Transfer) experiment. $^{13}C/^1H$ heteronuclear chemical shift correlations were determined by an Inverse HMQC (heteronuclear multiple quantum coherence) proton-detected [17] experiment. Assignments of NMR spectral data are as follows: ^{13}C NMR (75.46 MHz, methanol-d_4–D_2O 5:1): δ30.5 (*t*, C-1), 27.9 (*t*, C-2), 71.1 (*d*, C-3), 30.9 (*t*, C-4), 36.7 (*d*, C-5), 27.0 (*t*, C-6), 22.0 (*t*, C-7), 42.4 (*d*, C-8), 37.2 (*d*, C-9), 35.8 (*s*, C-10), 22.2 (*t*, C-11), 40.8 (*t*, C-12), 50.9 (*s*, C-13), 86.9 (*s*, C-14), 33.0 (*t*, C-15), 27.4 (*t*, C-16), 51.7 (*d*, C-17), 16.3 (*q*, C-18), 23.9 (*q*, C-19), 178.4 (*s*, C-20), 75.8 (*t*, C-21), 117.2 (*d*, C-22), 179.5 (*s*, C-23), 102.4 (*d*, C-1'), 72.5 (*d*, C-2'), 77.5 (*d*, C-3'), 81.8 (*d*, C-4'), 75.2 (*d*, C-5'), 17.1 (*q*, C-6'), 105.2 (*d*, C-1''), 71.2 (*d*, C-2''), 77.5 (*d*, C-3''), 75.4 (*d*, C-4''), 75.9 (*d*, C-5''), 62.2 (*t*, C-6''). ^1H NMR (300.13 MHz, methanol-d_4–D_2O 5:1) fucose unit, δ 4.33 (1H, *d*, $J_{1',2'}$ = 7.8 Hz, H-1'), 3.52 (1H, *dd*, $J_{1',2'}$ = 7.8 Hz, $J_{2',3'}$ = 9.2 Hz, H-2'), 3.63 (1H, *dd*, $J_{2',3'}$ = 9.2 Hz, $J_{3',4'}$ = 3.1 Hz, H-3'), 3.91 (1H, *dd*, $J_{3',4'}$ = 3.1 Hz, $J_{4',5'}$ = 1.5 Hz, H-4'), 3.71 (1H, *dq*, $J_{4',5'}$ = 1.5 Hz, $J_{5',6'}$ = 6.3 Hz, H-5'), 1.28 (3H, *d*, $J_{5',6'}$ = 6.3 Hz, H-6'); glucose unit, δ4.61 (1H, *d*, $J_{1'',2''}$ = 8.0 Hz, H-1''), 3.35 (1H, *dd*, $J_{1'',2''}$ = 8.0 Hz, $J_{2'',3''}$ = 8.6 Hz, H-2''), 3.42 (1H, *t*, $J_{2'',3''}$ = 8.6 Hz, $J_{3'',4''}$ ≡ 8.6 Hz, H-3''), 3.68 (1H, *t*, $J_{3'',4''}$ = 8.6 Hz, $J_{4'',5''}$ = 8.6 Hz, H-4''), 3.85 (1H, *dt*, $J_{4'',5''}$ = 8.6 Hz, $J_{5'',6''}$ = 7.0 Hz, H-5''), 3.35 (2H, *m*, $J_{5'',6''}$ = 7.0 Hz, H-6''); aglycone unit, 0.85 (3H, *s*, H-18), 0.95 (3H, *s*, H-19), 4.08 (1H, *br m*, H-3α), 4.95 (1H, *dd*, J_{gem} = 18.1 Hz, J_{all} = 2.6 Hz, H-21a), 5.05 (1H, *dd*, J_{gem} = 18.1 Hz, J_{all} = 2.6 Hz, H-21b), 5.91 (1H, *t*, *J* = 2.6 Hz, H-22).

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