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NOTE

Complete ^{1}H and ^{13}C assignments of the *Digitalis lanata* Cardenolides, Glucodigifucoside and Glucogitoroside by 1D and 2D NMR

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The ^{1}H and ^{13}C NMR spectra of glucodigifucoside and glucogitoroside, cardenolides isolated from *Digitalis lanata* leaves, were completely assigned with the aid of 2D homonuclear (COSY) and heteronuclear (HETCOR and HMQC) correlation spectra. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

The cardenolides glucodigifucoside (Gdf) and glucogitoroside (Ggr) are steroidal compounds with a carbohydrate moiety in the 3β-position (Fig. 1). They occur exclusively in *Digitalis* species. High concentrations of these glycosides have been reported in the leaves of *D. lanata* cultivated in Brazil.[1] The 12β-hydroxylated cardenolides lanatoside C and digoxin are used in the treatment of congestive heart failure. Gdf shows a polarity close to those of these two cardenolides, suggesting pharmacological similarity. Nevertheless, the use of Gdf as a positive inotropic drug has never been reported.[2]

The complete ^{1}H NMR assignment of cardenolides, and of steroids in general, is made difficult even at high magnetic field by excessive signal overlap in the region between 1.0 and 2.5 ppm.[3] Although the ^{13}C NMR spectra of steroids have signals dispersed over a much larger chemical shift range, their assignment has previously required comparisons with appropriate model compounds.[4] However, two-dimensional spectroscopy now permits complete ^{1}H and ^{13}C assignments, without any need for model compounds.[5]

The chemical structure of Gdf was originally proposed on the basis of enzymatic hydrolysis and comparison of the aglycone unit and sugar moieties with authentic samples by thin-layer chromatography.[6] A partial ^{1}H NMR assignment of Gdf has been reported, in confirmation of its structure.[7] However, only general assignments were described for most of the hydrogens resonating below 2.5 ppm, because of the complexity of their couplings and severe overlapping.

^{1}H NMR spectral data for digitalis cardenolides are scarce, although complete ^{1}H assignments for digoxin[8] and digitoxin[9] have been made using 2D NMR. To date there has been no complete ^{1}H assignment for Ggr and Gdf, and also some of the ^{13}C assignments remain unproven. This paper reports the use of 1D and 2D NMR for the complete ^{1}H and ^{13}C assignment of Gdf and Ggr.

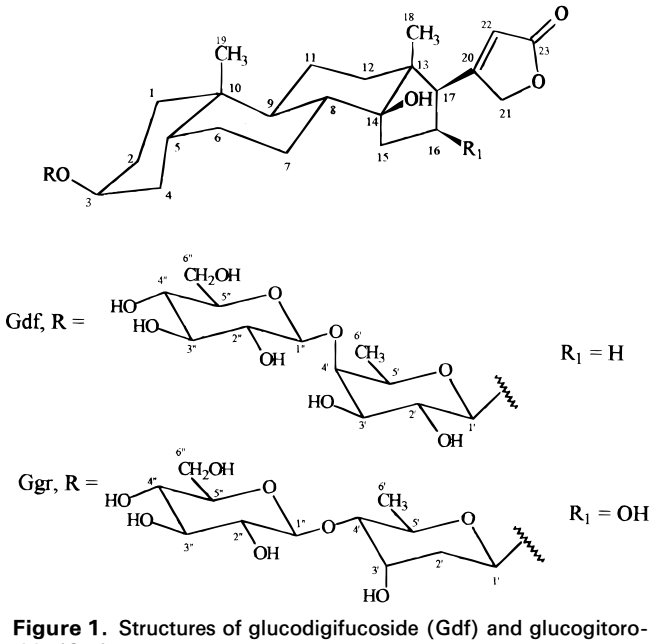

Figure 1. Structures of glucodigifucoside (Gdf) and glucogitoroside (Ggr).

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Table 1. Carbon-13 chemical shifts (ppm) for glucodigifucoside (Gdf) and glucogitoroside (Ggr)

	Chemical shift (ppm)				
Carbon	Gdf	Ggr	Carbon	Gdf	Ggr
1	30.1	30.0	19	23.1	23.3
2	27.0	26.5	20	177.6	172.7
3	75.0	67.5	21	74.4	76.9
4	30.2	30.4	22	116.8	119.6
5	35.3	36.9	23	176.3	176.3
6	26.5	26.8	1′	101.5	95.9
7	21.4	21.1	2′	74.1	38.1
8	41.7	41.9	3′	75.4	68.7
9	35.9	35.8	4′	85.9	83.2
10	36.5	35.2	5′	74.1	70.2
11	21.6	21.4	6′	17.2	17.6
12	40.0	40.0	1″	104.1	104.8
13	50.1	50.3	2″	74.1	74.1
14	85.5	84.7	3″	77.1	76.7
15	32.4	42.8	4″	70.5	72.1
16	26.8	73.5	5″	76.9	76.7
17	51.1	58.7	6″	61.5	61.3
18	15.4	16.1			

Table 2. Chemical shifts (ppm) and coupling constants (Hz) for the aglycone unit of glucodigifucoside (Gdf) and glucogitoroside (Ggr)

	Gdf		Ggr	
Hydrogen	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)
1α	1.50		1.50	
1β	1.50		1.50	
2α	1.61	$J_{2\alpha,3}=4.0$	1.66	
2β	1.68		1.66	
3	4.03		4.34	$J_{3,4\beta}=2.9$
4α	1.57		1.52	
4β	1.83		1.87	
5	1.73		1.73	
6α	1.89		1.72	
6β	1.28		1.22	
7α	1.27	$J_{7\alpha,\beta}=-18.0$	1.22	
7β	1.79		1.46	$J_{7\beta,8}=3.5$
8	1.68	$J_{8,9}=11.6$	1.59	$J_{8,9}=8.7$
9	1.82	$J_{9,11\beta}=3.3$	1.65	
11α	1.43		1.86	
11β	1.27		1.29	
12α	1.52		1.42	
12β	1.52		1.55	
15α	2.20		2.63	$J_{15\alpha,\beta}=-13.7$
15β	1.76		1.83	$J_{15\alpha,16}=8.0$
16α	1.89	$J_{16\alpha,17}=7$	4.67	$J_{15\beta,16}=2$
16β	2.20	$J_{16\beta,17}=10$	—	$J_{16,17}=7.6$
17	2.85		3.14	
18	0.90		0.94	
19	0.99		0.95	
21a	4.94	$J_{21\alpha,\beta}=-18.4$	4.93	$J_{21\alpha,\beta}=-18.0$
21b	5.03	$J_{21,22}=1.7$	5.15	
22	5.92	$J_{21,22}=1.7$	5.95	$J_{21,22}=2.6$

RESULTS AND DISCUSSION

^{1}H and ^{13}C assignments for Gdf

The $^{13}C\{^{1}H\}$ NMR spectrum of Gdf showed a total of 35 carbon atoms. Their multiplicities were determined using DEPT. The ^{13}C resonances (Table 1) were com-

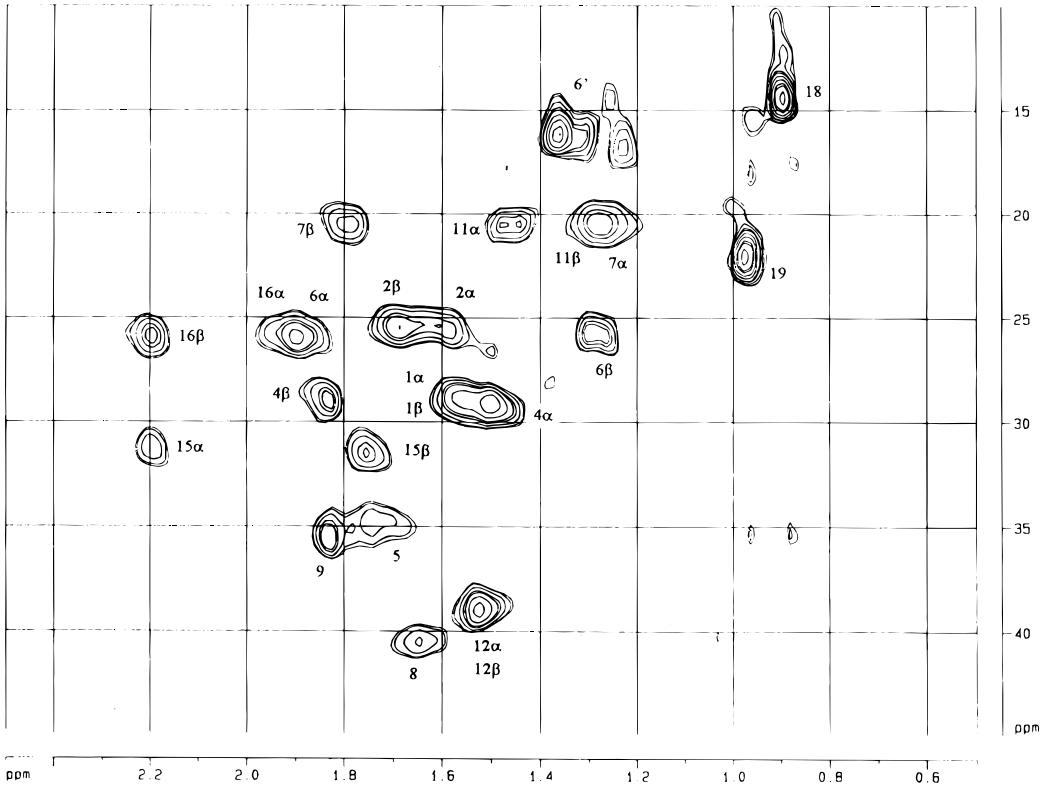

Figure 2. Expanded region of an HMQC spectrum of glucodigifucoside, obtained in CD_3OD solution.

Table 3. Chemical shifts (ppm) and coupling constants (Hz) for the carbohydrate hydrogens of glucodigifucoside (Gdf) and glucogitoroside (Ggr)

	Gdf				Ggr		
H	δ (ppm)	J (Hz)	Multiplicity	H	δ (ppm)	J (Hz)	Multiplicity
1′	4.33	$J_{1',2'}=8.0$	d	1′	4.93	$J_{1',2'\beta}=9.5$; $J_{1',2'\alpha}=2.4$	dd
2′	3.27	$J_{2',3'}=9.0$	dd	2′α	1.96	$J_{2\alpha,\beta'}=-14.2$; $J_{2'\alpha,3'}=3.0$	td
3′	3.47	$J_{3',4'}=4.2$	dd	2′β	1.75	$J_{2'\beta,3'}=3.0$	td
4′	3.19	$J_{4',5'}=1.5$	dd	3′	4.04	$J_{3',4'}=3.0$	dd
5′	3.52	$J_{5',6'}=6.2$	dq	4′	3.28	$J_{4',5'}=9.6$	dd
6′	1.37		d	5′	3.89	$J_{5',6'}=6.4$	dd
1″	4.38	$J_{1'',2''}=8.0$	d	6′	1.31		d
2″	3.26	$J_{2'',3''}=8.6$	dd	1″	4.37	$J_{1'',2''}=7.6$	d
3″	3.38	$J_{3'',4''}=9.0$	t	2″	3.27	$J_{2'',3''}=9.0$	dd
4″	3.32	$J_{4'',5''}=9.0$	t	3″	3.37		
5″	3.37	$J_{5'',6''a}=6.2$	dt	4″	3.38		
6″a	3.70	$J_{6''a,b}=-11.8$	dd	5″	3.37	$J_{5'',6''a}=4.8$	
6″b	3.90	$J_{6''b,5''}=2.2$	dd	6″a	3.72	$J_{6''a,b}=-11.7$	dd
				6″b	3.84	$J_{6''b,5''}=2.6$	dd

pletely assigned as described below and the chemical shifts compared with the earlier data for this cardenolide[7] and for its aglycone unit.[8,9] The small differences probably arise from variations in solvent and from the reassignment of peaks in crowded regions of the spectra.

Independent assignments were carried out by combining data from ^1H–^1H (COSY) and ^1H–^{13}C (HMQC) spectra (Fig. 2). The latter were valuable in identifying methylene pairs and for ^{13}C assignments. As expected, the 1D ^1H NMR spectrum of Gdf was very complex, and the only resonances readily assignable below δ 2.5 were those of the two methyl groups (C-18 at 0.90 ppm and C-19 at 0.99 ppm). Their chemical shifts were similar to those in other cardenolides .[7–9] Above 2.5 ppm it was easy to assign H-22 (5.92 ppm), H-21 (4.94 and 5.03 ppm), H-3 (4.03 ppm) and H-17 (2.85 ppm). These assignments (Table 2) were confirmed by the 2D spectra. The chemical shifts were similar to those reported previously for Gdf[7] and for digitoxigenin.[8,9] These literature data were adequate to distinguish C-10 from C-13, as their ^{13}C shifts differ by 13.6 ppm.

The COSY spectrum was then analysed, starting from the connectivities of the above resonances. Thus, H-17 showed cross peaks to signals at 1.89 and 2.20 ppm, i.e. H-16α and H-16β, respectively. These were

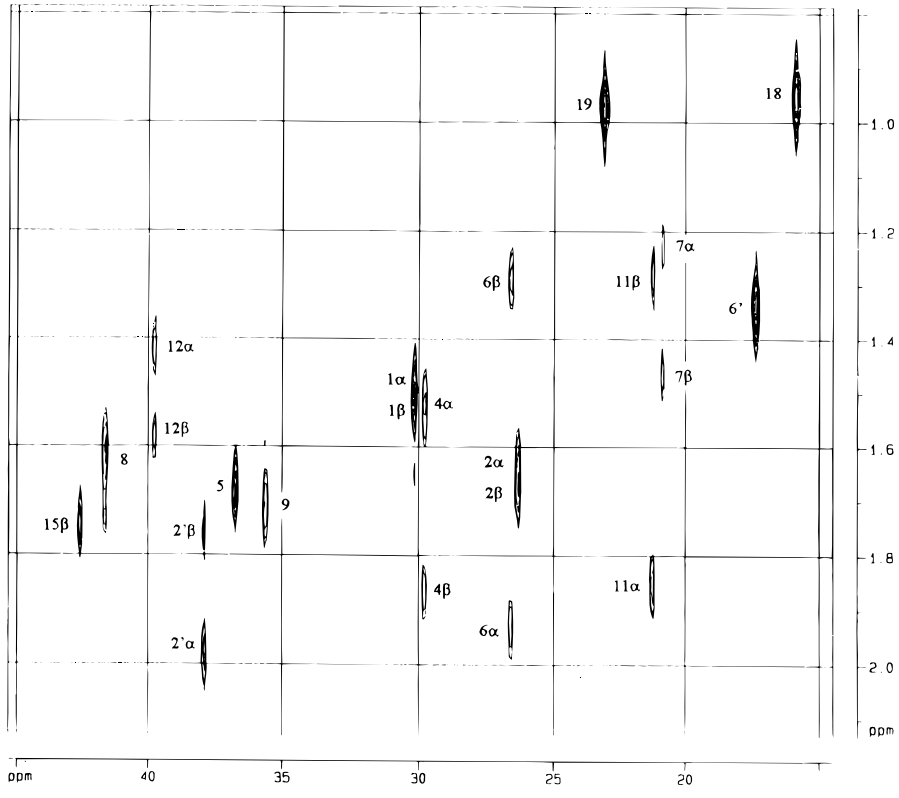

Figure 3. Expanded region of a HETCOR spectrum of glucogitoroside, obtained in CD_3OD solution.

also correlated in the HMQC spectrum with the ^{13}C resonance at 26.8 ppm. Couplings were used to distinguish α from β, together with modelling where necessary. Similarly, the H-16 signals correlated with resonances at 2.20 and 1.76 ppm, assigned to H-15α and H-15β, and each of these linked with the ^{13}C peak at 32.4 ppm. Similar assignments were made for the A ring, starting from the glycosidic bond to C-3, and from the partially resolved 2D coupling patterns in the other two rings. Careful inspection of the 1D spectra then also permitted the measurement of further interproton coupling constants. The 11.6 Hz coupling between H-8 and H-9 confirmed the *trans* fusion of the B and C rings.

The corresponding assignment of the carbohydrate portion of Gdf was more straightforward and is reported in Table 3. Our shifts agree only approximately with reported data for Gdf[7] and for the terminal glucose residue in lanatoside C.[10]

^{1}H and ^{13}C assignments in Ggr

The $^{13}C\{^{1}H\}$ NMR spectrum of Ggr was unambiguously assignable (Table 1) using the above Gdf data, together with DEPT and HETCOR ^{1}H–^{13}C shift correlation data. The HETCOR method was chosen for its high resolution in the ^{13}C dimension, as adequate sample (20 mg) was available to overcome any loss of signal-to-noise ratio. The ^{13}C chemical shifts for its steroid moiety are largely in agreement with previous assignments.[11] C-16 in Ggr must be assigned to the resonance at 72.1 ppm, since it bears a β-hydroxyl group. This group also deshields the neighbouring carbons, relative to Gdf. The C-15 resonance moves from 32.4 to 42.8 ppm and C-17 from 51.1 to 58.7 ppm. In contrast, C-3 moves from 75.0 to 67.5 ppm, presumably because of a conformational change at the glycosidic bond, induced by the removal of O-2′. Also, C-20 becomes shielded by a further 4.9 ppm, because it is now *γ-gauche* to O-16. The anomeric carbon in the digitoxose residue was assigned by its ^{1}H shift and couplings to the peak at 95.9 ppm, and similarly the methylene signal at 38.1 ppm to the C-2′ resonance. The shifts agreed with those for other cardenolides possessing this sugar residue.[8–10,12] The ^{1}H shifts were then assigned via HETCOR (see Fig. 3) and some interproton coupling constants were calculated from careful inspection of the 1D spectrum. The ^{1}H chemical shifts of the digitoxose ring obtained in this work were also fairly close to those reported for this sugar residue in digoxin,[8] digitoxin[9] and lanatoside C.[10]

In summary, the present work provides, for the first time, the complete ^{1}H and ^{13}C assignments for Ggr and also confirms the earlier partial assignments for Gdf.

EXPERIMENTAL

Preparation of plant material

The 12-month-old leaves of *Digitalis lanata* were collected from an experimental cultivar at Itatiaia, Brazil. The dense rosette leaves (1 kg) were dried at 40 °C for 48 h and then percolated with 70% MeOH (3 × 3 l) at room temperature for 72 h. The extract was evaporated to dryness under reduced pressure to give a dark-green residue (400 g).

Isolation of the cardenolides

A portion of the crude aqueous methanolic extract (50 g) was dissolved in 30% MeOH (1500 ml). Pigments were removed by shaking with polyvinylpyrrolidone (150 g), following adsorption on Amberlite XAD-7 (150 g). The resin was washed with H_2O (3 × 900 ml) and 30% MeOH (3 × 900 ml). Cardenolides were recovered by elution with 70% MeOH (3 × 900 ml). This extract was partitioned with $CHCl_3$–Pr^iOH (3:2) (3 × 90 ml). The residue (1.3 g) was dissolved in dimethyl sulphoxide (4 ml) and submitted to flash chromatography in a reversed-phase silica column (Merck LiChroPrep RP-8, 40–63 μm, pressure 1.2 bar) (460 × 25 mm i.d.). The cardenolides were eluted with MeOH–H_2O (50:50) and 50 ml fractions were collected. The composition of the individual fractions was monitored by HPLC. Fractions 11–13 were combined and concentrated, at 40 °C under reduced pressure, to give 82 mg of residue. The high-performance liquid chromatographic (HPLC) profile of these combined fractions indicated Ggr as the major component. HPLC analysis of the combined fractions 15–18, after concentration to 116 mg of residue, indicated they were mainly composed of Gdf. Final purification of cardenolides included preparative chromatography on silica gel plates (0.2 mm, 20 × 20 cm; Merck, Darmstadt, Germany), employing $CHCl_3$–MeOH–H_2O (80:18:2) as eluent. Gdf (26 mg) and Ggr (24 mg) were obtained with purity > 90%, according to HPLC analysis.

HPLC analysis

Analyses were carried out with a Hewlett–Packard (Waldbronn, Germany) Model 1090 apparatus Series II with a diode-array detector. An ODS C_{18} column (100 × 2.1 mm i.d., Hewlett–Packard) was used at 40 °C, with flow-rate 0.2 ml min^{-1} and observation at 220 nm. Gradient elution with H_2O (A) and 84% CH_3CN (B) was performed as follows: 0–5 min, 85% A–15% B; 5–10 min, 80% A–20% B; 10–12 min, 80% A–20% B; 12–25 min, 73% A–27% B; 25–35 min, 55% A–45% B; 35–50 min, 40% A–60% B; 50–52 min, 5% A–95% B.

Identification of the isolated cardenolides

The isolated cardenolides were identified by their UV spectra using a diode-array detector and by comparison of their retention times with those of standard cardenolides by HPLC analysis.

Chemicals

Acetonitrile (LiChrosolv) was obtained from Merck. Water was purified using a Milli-Q 50 purification

system (Millipore, Eschborn, Germany). Standards of glucodigifucoside (Gdf) and glucogitoroside (Ggr), were obtained from Boehringer Mannheim (Mannheim, Germany).

Spectroscopy

NMR spectra were obtained at the Departamento de Química, ICEX, UFMG, on a Bruker Avance spectrometer at 100.6 MHz (^{13}C) and 400 MHz (^{1}H); 20 mg of sample were dissolved in CD_3OD at 25 °C with tetramethylsilane (TMS) as internal reference for both nuclei. COSY spectra were obtained with 512 time points and spectral widths of 4.6 ppm. Both dimensions used sine-bell windows and were zero-filled to 2K points before symmetrization. HETCOR spectra used (^{1}H) 32 time points, filled to 512 points after a sine-bell window, 5.7 ppm width and (^{13}C) 4K points, 1 Hz exponential window, 170 ppm width, 1 s relaxation delay.[13] HMQC used (^{1}H) 1K time and data points with a sine-bell window, 14 ppm width, and (^{13}C) 128 points filled to 512 after a squared sine-bell window, 175 ppm width, 1.2 s relaxation delay.[14] The refocusing delay in each case was 0.003 46 s, i.e. set for a 125 Hz coupling.

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