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Complete Assignment of the ^{1}H and ^{13}C NMR Spectra of Steroidal Sapogenins: Smilagenin and Sarsasapogenin†

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Ab initio **proton and ^{13}C NMR assignments for a stereoisomeric pair of 5β steroidal sapogenins, smilagenin (1) and sarsasapogenin (2), were accomplished using DEPT, COSY, TOCSY, HETCOR, HMQC, HMQC-TOCSY, HSQC-RELAY, HMBC and selective reverse INEPT techniques; the ^{13}C NMR assignments for 2 were confirmed using 2D INADEQUATE experiments. This work corrects earlier ^{13}C assignments for 1 and 2 proposed by Eggert and Djerassi.** © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Steroidal sapogenins, widely distributed secondary plant metabolites, are of importance as economically significant raw materials convertible into various hormonal drugs, in the natural defense of plants against fungal pathogens and/or predators, and by virtue of their medicinal value.[2] The spirostans represent the major subgroup of steroidal sapogenins. They include the perhydrocyclopentenophenanthrene nucleus, as present in androstane and cholestane, but contain in addition one five-membered ring (E) and one six-membered ring (F), both of which are heterocyclic and fixed in spiran fashion at C-22.

For about 100 years, smilagenin (**1**) has been isolated from plants such as *Smilax ornata* Hook and *Radix sarsaparilla* (Jamaica), and sarsasapogenin (**2**) has been extracted from *Smilax medica, officinalis* L. and *ornata* (Hooker), *Radix sarsaparilla* (Vera cruz), *Asparagus officinalis* and *Yucca filifera.* Originally identified as C_{26} compounds, they were revised to C_{27} in 1935,[3] and their side-chains were correctly identified by chemical analysis in 1939.[4] Smilagenin, (25α_f)-5β-spirostan-3β-ol (**1**), and sarsasapogenin, (25β_f)-5β-spirostan-3β-ol (**2**), are stereoisomers differing in configuration at C-25. Smilagenin is the 25*R* (25D) form and sarsasapogenin the 25*S* (25L) form.

^{13}C NMR assignments of some steroids, including androstane and cholestane, were given by Reich *et al.*[5] in 1969. Systematic investigations of the carbon spectra of steroids were undertaken by Eggert and Djerassi,[6] who studied the effects of keto groups in different positions and of different substitutions and skeletal stereochemistries of the steroidal sapogenins. In addition to proposing assignments for smilagenin and sarsasapogenin,[7] they reassigned androstane and cholestane, which are the parent hydrocarbons of the steroidal sapogenins.[6] The chemical shifts of C-1–C-10 and C-19 in the androstane and cholestane were shown to be similar, and the former assignments[5] for C-12 and C-16 were reversed.

Since the work of Eggert and Djerassi, full assignments of the ^{13}C spectra of steroidal sapogenins have been made routinely.[8] These assignments have been based largely on chemical shift arguments, although this can lead to problems in crowded regions of the spectrum. In contrast to ^{13}C NMR, ^{1}H spectra are sufficiently complicated by the smaller range of chemical shifts and the extensive interproton couplings that only a few signals, e.g. singlets for angular methyl groups (18, 19) and doublets for secondary methyl groups (21, 27), and signals appearing downfield (>3 ppm) are readily recognizable.[9] The rest of the ^{1}H NMR resonances are crowded into the region 1–2 ppm as a series of overlapping multiplets in the methylene envelope.

Complete ^{1}H assignments for a steroid of low degree of functionalization are difficult because more than 20 protons will typically have chemical shifts in this region. The first complete ^{1}H assignments for a steroidal molecule (1-dehydrotestosterone) were reported[10] in 1980, while only recently have full assignments for a steroidal sapogenin, hecogenin acetate [3β-acetoxy-(25*R*)-5α-spirostan-12-one][11] and subsequently for diosgenin [3β-hydroxy(25*R*)-spirost-5-ene][12] appeared. The

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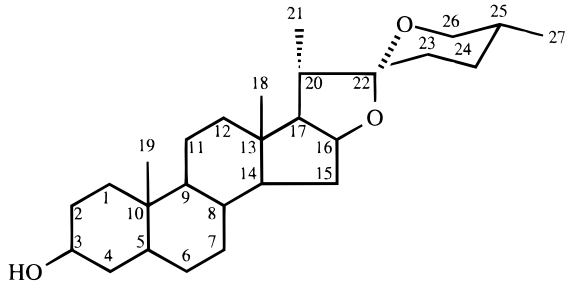

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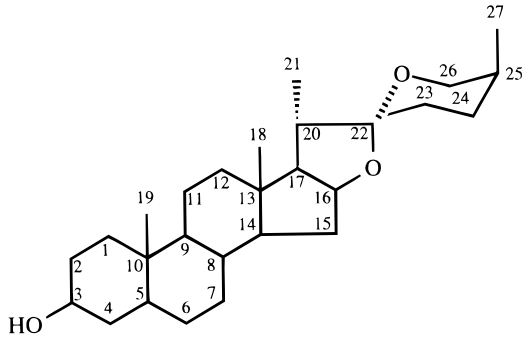

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absence of any unambiguous ^{1}H assignments for 5β-steroidal sapogenins,[1,13] and the need to test the limited assignments proposed hitherto,[6,7,14,15] prompted the application here of modern *ab initio* assignment techniques to **1** and **2**. The resulting proton assignments should serve as a basis for structural and spectral assignments of other members of the family of steroidal sapogenins and for derivatives of these compounds, and have also prompted a revision of the ^{13}C assignments in **1** and **2**.

EXPERIMENTAL

Sarsasapogenin (Sigma, Poole, UK) and smilagenin (ICN Biochemicals, Cleveland, OH, USA) were used without further purification; 'smilagenin' purchased from another supplier proved to be sarsasapogenin. The 1D and 2D NMR experiments were carried out on *ca.* 5% (w/v) solutions in $CDCl_3$ in 5 mm tubes at ambient temperature (20 °C) with an indirect detection probe on a Varian Unity 500 NMR spectrometer, except for the sarsasapogenin 2D INADEQUATE experiment, which used a near-saturated solution and was run on the same spectrometer in a 10 mm broadband probe. Chemical shifts were referenced to TMS. Standard methods were used throughout, except for the 2D INADEQUATE and selective reverse INEPT experiments.

The GROPE-16 compensated 2D INADEQUATE experiment[16] used a modified version of the procedure described by Levitt and Ernst[17] to integrate the GROPE-16 composite pulse[18] into the 2D INADEQUATE experiment. The pulse sequence used for this experiment is shown in (Fig. 1); under the experimental conditions used, this sequence gave up to twice the signal-to-noise ratio of the normal sequence for signals at the edges of the spectrum. The pulse sequence used for the selective reverse INEPT experiment was as described in Ref. 19, with selective excitation being accomplished using a DANTE sequence of 148 pulses of 0.55 μs spaced 250 μs apart.

RESULTS

The ^{1}H and ^{13}C NMR assignments for **1** and **2** are summarized in Tables 1 and 2, together with important homonuclear scalar couplings. The ^{13}C signal assignments rely on the initial delineation of the individual proton spin systems within each molecule using a variety of techniques, including double-quantum filtered COSY (DQF-COSY[20]), total correlation spectroscopy (TOCSY[21]), directly (HETCOR[22]) and indirectly (HMQC[23]) detected heteronuclear single-bond ^{1}H–^{13}C correlation and hybrid relayed methods such as HMQC-TOCSY[24] and HSQC-RELAY.[24] Assignments of quaternary carbon resonances (multiplicities were determined using DEPT[25] spectra) relied on the establishment of long-range heteronuclear connectivities using ^{1}H-detected heteronuclear multiple bond connectivity (HMBC[26]) experiments. To overcome problems with signal overlap and strong coupling in the proton spectrum, selective reverse INEPT (SRI) experiments were employed in some instances. For both **1** and **2**, the

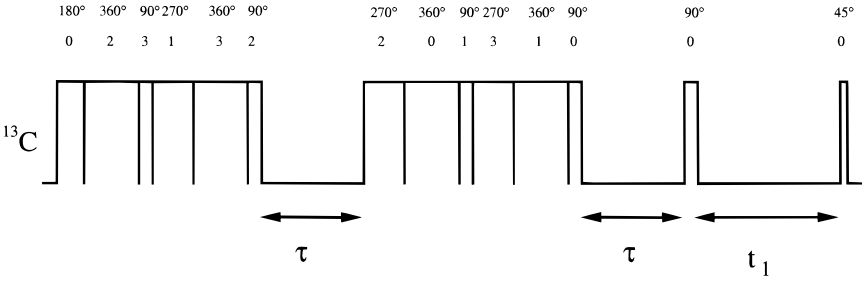

Figure 1. Pulse sequence used for the GROPE-16 compensated 2D INADEQUATE experiment; the flip angles of pulses are shown in degrees. Initial phase shifts of pulses (0, 1, 2, 3) are indicated in multiples of 90° (0°, 90°, 180°, 270°); subsequent phase cycling follows the normal pattern.[27] The read pulse should be increased from 45° to 90° if phase-sensitive data processing is to be used.

Table 1. ^{13}C and ^{1}H NMR assignments for smilagenin (1)

Position	^{13}C	^{1}H[a]
1	29.95	1.40, 1.53 (dt, J = 3.2, 10.1)
2	27.82	1.46, 1.58
3	67.12	4.11 (t, J = 2.8)
4	33.54	1.33, 1.98
5	36.58	1.72
6	26.56	1.16, 1.91 (t, J = 4.7)
7	26.54	1.07 (td, J = 3.8, 13.6), 1.46
8	35.28	1.58
9	39.85	1.33
10	35.28	—
11	20.90	1.25, 1.40
12	40.30	1.16, 1.72
13	40.70	—
14	56.48	1.16
15	31.80	1.25, 1.98
16	80.93	4.40 (ddd, J = 6.4, 7.8, 8.9)
17	62.27	1.77 (dd, J = 6.6, 8.8)
18	16.49	0.76
19	23.92	0.98
20	41.61	1.86 (qn, J = 6.8)
21	14.51	0.97 (d, J = 6.9)
22	109.26	—
23	31.39	1.58, 1.67 (d, J = 4.57)
24	28.80	1.46, 1.64
25	30.31	1.64
26	66.86	3.38 (t, J = 11.0), 3.48 (ddd, J = 1.9, 4.0, 11.0)
27	17.14	0.79 (d, J = 6.4)

[a] J in Hz.

Table 2. ^{13}C and ^{1}H NMR assignments for sarsasapogenin (2)

Position	^{13}C	^{1}H[a]	^{13}C–^{13}C[b]
1	29.95	1.39, 1.50	2, 10/8
2	27.79	1.40, 1.50	1, 3
3	67.12	4.11 (td, J = 2.5)	2, 4
4	33.51	1.32, 1.97	3, 5
5	36.51	1.72	4, 6, 8/10
6	26.56	1.15, 1.90 (dt, J = 2.3, 4.7)	5
7	26.54	1.04 (d, J = 4.6), 1.39	8/10
8	35.27	1.58 (td, J = 2.5, 11.7)	7, 9, 14
9	39.85	1.32	8/10, 11
10	35.27		1, 5, 9, 19
11	20.90	1.25, 1.39	9, 12
12	40.31	1.15, 1.72	11
13	40.67	—	14, 17, 18
14	56.47	1.15	8/10, 13, 15
15	31.74	1.25, 1.97	14, 16
16	81.02	4.40 (ddd, J = 6.6, 7.7, 8.8)	15, 17
17	62.09	1.75 (dd, J = 7.2, 8.8)	13, 16, 20
18	16.50	0.76	13
19	23.92	0.97	8/10
20	42.12	1.81 (qn, J = 6.9)	17, 21, 22
21	14.34	0.99 (d, J = 6.8)	20
22	109.74	—	20, 23
23	25.94	1.39, 1.87 (d, J = 5.2)	22
24	25.77	1.39, 2.02 (dt, J = 4.3, 13.3)	25
25	27.08	1.69	24, 26, 27
26	65.13	3.28 (dt, J = 1.8, 10.8), 3.95 (ddd, J = 0.5, 2.7, 10.8)	25
27	16.05	1.08 (d, J = 7.2)	25

[a] J in Hz.
[b] Carbon–carbon correlations observed in GROPE-16 compensated 2D INADEQUATE experiments. A solidus (/) indicates that the identification of a cross peak is ambiguous because of the similarity of the chemical shifts concerned.

carbon assignments, with the exception of those for carbons bearing oxy substituents, were completed without the need to invoke chemical shift arguments. An independent check on the ^{13}C assignments for **2** was provided by a 2D GROPE-16 compensated INADEQUATE experiment, which allowed the identification of almost all the one-bond ^{13}C–^{13}C couplings in **2**.

Interpretation and assignment of spectra

The ^{1}H NMR spectrum of **1** in deuterochloroform is complex, with considerable overlap in the region 1–2 ppm and few recognizable resonances: singlets at δ 0.76 and 0.98 and doublets at δ 0.79 and 0.97 for four methyl groups, a triplet at δ 3.38 coupled (J = 11.0 Hz) to a multiplet at δ 3.48, a triplet (J = 2.8 Hz) at δ 4.11 and a quartet at δ 4.40. The proton-decoupled ^{13}C NMR spectrum shows 26 peaks, with a double intensity signal at δ 35.28; the DEPT subspectra identify four methyl, eleven methylene, nine methine and three quaternary carbons. The assignment of the quaternary carbon at δ 109.26 to C-22 is straightforward as it is the only carbon attached to two oxygen atoms; the methylene at δ 66.86 can similarly be assigned to C-26. The methine carbons at δ 80.93 and 67.12 must correspond to C-3/C-16. Of the four methyl carbons, those at δ 23.92 and 16.49 show one-bond couplings to singlet methyl proton signals and those at 17.14 and 14.51 to doublet signals, indicating that the former pair corresponds to C-18/C-19 and the latter to C-21/C-27.

Combining information from the DQF-COSY and HMQC experiments, the methyl proton doublet at δ 0.79 is coupled to a proton signal at δ 1.64, as are the signals of H_2-26 at δ 3.48 and 3.38, so the signal at δ 1.64 (δ_C 30.31) must be that of methine 25, and that at δ 0.79 is methyl 27. The other doublet methyl proton resonance, at δ 0.97 (J = 6.9 Hz), must be C-21, and shows a vicinal connectivity to a resonance at δ 1.86 (δ_C 41.61) which must be C-20. This also leads to the assignment of the oxy-substituted methine resonance at δ 80.93 to C-16, from its TOCSY correlation between δ 4.40 and 1.86 (H-20), and thus the remaining oxymethine signal at δ 67.12 corresponds to C-3. The latter assignment is supported by the DQF-COSY correlations from δ 4.11 to methylene resonances at δ 1.98 and 1.58, which would identify these resonances as belonging to C-2/C-4. The resonance at δ 4.40 (H-16) shows a ^{1}H–^{1}H correlation to δ 1.77 and δ 1.98 in the DQF-COSY spectrum, and their $^{1}J_{CH}$ correlations to methine (δ 62.27) and methylene (δ 31.80) carbons lead to their assignment as C-17 and C-15, respectively.

Further assignments of the proton and carbon resonances were carried out using long-range ^{1}H–^{13}C correlation (Table 3), exploiting the much higher resolution

Table 3. Heteronuclear correlations observed[a] in HMBC, HMQC-TOCSY and HSQC-RELAY experiments for smilagenin (1)

Carbon No.	HMBC	HMQC-TOCSY	HSQC-RELAY
1	3, 4α, 19	3, 2α, 1α, 1β	2α, 1α, 1β
2		3, 2α, 1α, 1β, 2β	2α, 1α, 1β
3		3, 4β, 2β	3, 4β, 2α, 4α, 2β
4	2β/7α	3, 4α, 5, 4β	4α, 5, 4β
5	3, 1α, 7α, 19	5, 4β, 4α	5, 4β, 6α, 4α
6 + 7	14/12β/6β	14/12β/6β, 8, 7α, 6α, 7β, 12α/5, 9/4β	6β, 7α, 6α, 7β, 8
8 + 10	14/12β, 9/4β, 15β/11β, 1α/7α, 1β/11α, 19	14/12β/6β	14/12β/6β, 9, 8
9	12α/5	14/12β/6β, 12α/5, 1β, 11α	9/4β, 8, 15β/11β, 1β, 11α
11		14/12β/6β, 12α/5, 15β/11β, 1β/11α, 9/4β	
12	17	14/12β/6β, 12α/5, 9/4β, 15β/11β, 1β/11α	
13	16, 14/12β, 20, 12α, 15α		
14	12α/5, 15β/11β	15α	
15	14/12β/6β	16, 14/12β/6β, 4α/15α, 15β/11β, 17	16, 14/12β/6β, 4α/15α, 15β/11β, 17
16	12α, 15β, 21	16, 21, 17, 15α/4α, 15β/11β	16, 17, 4α/15α, 15β/11β
17	20, 18, 21	16, 17, 21	16, 17, 20
18	17, 14/12β	18	
19		19	
20	17, 21	17, 20, 21	
21	17, 20, 7β	17, 20, 21	
22	20, 25/24α, 21, 26α	16, 17, 21, 15α, 15β	
23	20	23β, 23α, 25/24α, 24β, 27	23β, 23α, 25/24α, 24β
24	26α	23β, 25/24α, 24β, 27, 23α	23β, 23α, 24α, 24β
25		23β, 25/24α, 24β, 23α, 27	25/24α
26	27	26α, 26β, 25/24α, 27	26α, 26β, 25/24α
27	26α, 26β, 25/24α, 27	26α, 26β, 25/24α	

[a] A solidus (/) indicates that the identification of a cross peak is ambiguous because of the similarity of the chemical shifts concerned.

of the ^{13}C spectrum as compared with the proton spectrum. The HMBC connectivities for the angular methyl at δ 16.49, δ_C 16.49 → H-17 and C-17 → δ_H 0.76, led to its assignment to C-18, leaving the methyl group at δ 23.92 as C-19. The remaining quaternary carbon signals at δ 40.70 and 35.28 were assigned to C-13 and C-10, respectively, by virtue of the HMBC signal δ 40.70 → H-20. The HMBC cross peaks δ 56. 48 → H-20 and δ 36.58 → H-3 led to their assignments to methines C-14 and C-5, respectively. Signals which were ambiguous in the DQF-COSY spectrum because of their similar shifts, but are well separated in the structure of **1**, could now be assigned. For example, the DQF-COSY cross peak between δ 1.98 and δ 1.72 could now be attributed to coupling between H-4 or H-15 and H-5 or H-12; since the only pair of signals close enough in the structure to show sufficient coupling are H-4 and H-15, this sufficed to identify the methylene signal at δ 33.54 as C-4. At this point, only three methine carbons (C-8, C-9 and C-14) and four pairs of adjacent methylene carbons (C-1/C-2, C-6/C-7, C-11/C-12 and C-23/C-24) remained to be assigned.

The use of HMQC-TOCSY spectra with a range of mixing times, allowing discrimination between short- and long-range relayed correlations, enabled some further progress to be made in assigning the methylene resonances. Correlations from δ_C 27.82 to H-3 (δ 4.11) and H-1 (δ 1.40) identified this carbon as C-2 and hence that at δ_C 29.95 as C-1, and the correlation from δ_C 56.48 to H-15 (δ 1.98) identified methine 14. The HMQC-TOCSY and DQF-COSY correlations together showed a chain of proton–proton and proton–carbon connectivities linking C-6, C-7, C-8 and C-9, completing the assignment of the methine resonances. The HMQC-TOCSY signals from δ_C 40.30 to ^1H signals at δ 1.33 and δ 1.25 raised two possibilities: if this carbon were C-7, it would identify those at δ_C 39.85 and 20.90 as C-8 and C-6; if it were C-11 or C-12, δ_C 39.85 and 20.90 would be C-9 and C-12 or C-11. As with HMQC, the resolution of the carbon domain in HMQC-TOCSY was insufficient to separate the two signals around δ 26.5 (26.56 and 26.54 ppm), but these two methylenes were shown by DQF-COSY to be adjacent, and hence either C-6/C-7 or C-11/C-12. At this stage only two further pieces of information were needed to complete the assignment, making the use of selective excitation techniques more efficient than 2D NMR.

To overcome the problem of signal overlap and strong coupling in the two methylene pairs, a selective reverse INEPT experiment with homonuclear decoupling[19] was used. This restricts observation to a particular proton or group of protons by one-bond

polarization transfer from a selectively excited ^{13}C resonance, and then establishes connectivity with other protons by classical double resonance. The presence of the one-bond ^{13}C–proton coupling lifts degeneracies in the proton spectrum, allowing individual multiplets to be identified. For the C-11/C-12 assignment, the ^{13}C signals at δ 20.90 and δ 40.30 were excited selectively. For excitation at δ_C 20.90, irradiation at the shift of H-9 collapsed the proton multiplet structure of the resolved high field ^{13}C satellite, establishing the assignment of C-11 through the coupling H-9–H-11. Similarly, polarization transfer from δ_C 28.80 and irradiation at H-25 showed the existence of the coupling H-25–H-24, allowing the assignment of C-24. With C-12 and C-23 following by elimination, this completed the *ab initio* proton and ^{13}C assignments for smilagenin (**1**), which are shown in Table 1; they are similar to the assignments of Eggert and Djerassi except that C-9 and C-12 are interchanged.

Sarsasapogenin was assigned in a similar manner by the combined application of homonuclear (DQF-COSY and TOCSY) and heteronuclear (HMQC, HMBC, HMQC-TOCSY and HSQC-RELAY) experiments. As a check on the carbon assignments, a 2D GROPE-16 compensated INADEQUATE experiment was carried out. The use of GROPE-16 compensation for off-resonance effects allowed very uniform excitation and detection of the double quantum coherence with normal radiofrequency power levels. The results of the 2D INADEQUATE experiment are shown in (Fig. 2). Since the INADEQUATE results confirmed all of the assignments with the exception of three strongly coupled pairs (C-12/C-13, C-6/C-7 and C-23/C-24), the argument for the assignments of sarsasapogenin, which follows the same pattern as that for smilagenin, is not given here. The *ab initio* assignments for sarsasapogenin are summarized in Table 2, again largely in agreement with Refs 6 and 7 but with the positions of C-9 and C-12 reversed, and with changes to the assignments of C-23, C-24 and C-25.

DISCUSSION

The two sets of carbon assignments for **1** and **2** are consistent with each other to within 0.1 ppm for rings A, B, C, D and E, with the exception of C-17 and C-20, which show slightly larger deviations. As expected, the major differences in the ^{13}C spectra are for ring F resonances; all the carbon resonances except C-22 occur at higher field in **2** than in **1** (shift differences $\delta_2 - \delta_1$: C-22 0.48, C-23–5.45, C-24 – 3.03, C-25 – 3.23, C-26 – 1.73 and C-27 – 1.09 ppm). The effect on the proton chemical shifts of the change in configuration at C-25 between **1** and **2** is to shift 27-CH_3 0.29 ppm downfield and to increase the dispersion of the neighbouring geminal protons three- to fourfold (shift separations for methylenes 24 and 26: 0.63 and 0.67 ppm in **2** and 0.18 and

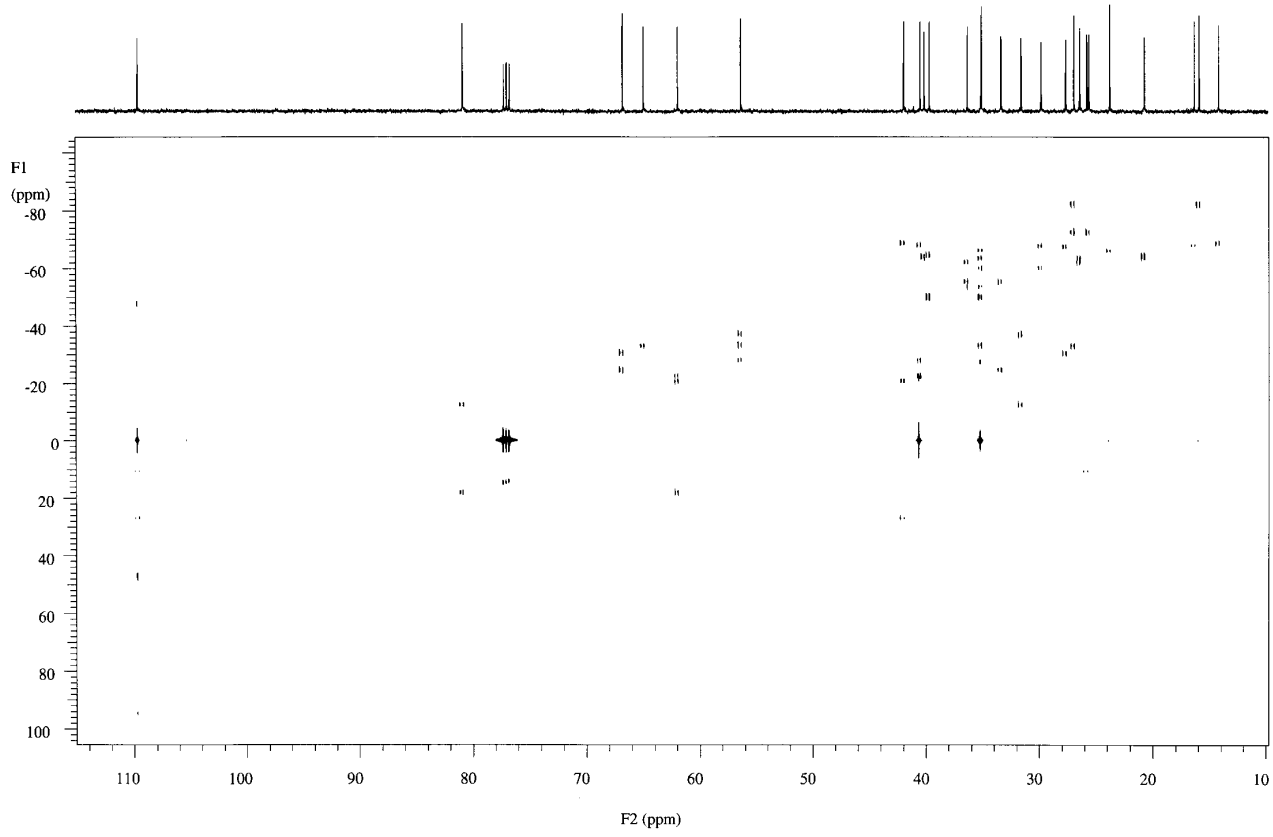

Figure 2. Absolute value GROPE-16 compensated 2D INADEQUATE spectrum of **2**. Axial artefacts are visible along the axis $f_1 = 0$ for signals with long spin–lattice relaxation times. 128 transients were acquired for each of 256 t_1 increments of 4096 complex data points, in a total time of 20 h, using a 90° pulse width of 22 μs and spectral widths of 13 240 and 26 480 Hz in f_2 and f_1, respectively.

0.10 ppm in **1**). A comparison of the observed NMR data for **1** and **2** with NMR data reported[12] for diosgenin [(25α_f)-spirost-5-ene-3β-ol] suggests that the presence of the Δ^5-olefinic bond does not have any strong effect on the chemical shifts of the ring F resonances.

The concerted use of multiple pulse 1D and 2D NMR techniques allows a much higher standard of accuracy in assignment than methods relying on chemical shift comparisons, and the availability of proton chemical shift assignments for steroidal sapogenins offers detailed structural and stereochemical information. The observation that the published ^{13}C assignments of C-9 and C-12 in **1** and **2** should be reversed suggests the possibility that similar changes may be necessary in the assignments of other members of this family.

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