MAGNETIC RESONANCE IN CHEMISTRY
Magn. Reson. Chem. **36**, S111–S117 (1998)

Isolation and identification by 2D NMR of two new complex saponins from *Michrosechium helleri*†

Ismael León,[1] Raul G. Enríquez,[2] Stewart McLean,[3] William F. Reynolds[3]* and Margaret Yu[3]

[1] Centro de Investigaciones en Quimica, Universidad Autonoma del Estado de Morelos, Avenida Universidad 1001, Colonia Champila 62210, Cuernavaca, Morelos, Mexico
[2] Instituto de Quimica, Universidad Nacional Autonoma de Mexico, Circuito Exterior, Ciudad Universitaria, Coyoacan, DF 45100, Mexico
[3] Department of Chemistry, University of Toronto, Toronto, Ontario, M5S 3H6, Canada

Received 29 December 1997; accepted 2 February 1998

ABSTRACT: Two complex saponins, amole F and G, were characterized and their spectra were assigned using only 1D and 2D ^{1}H and NMR methods. Amole F and G have seven and six monosaccharides, respectively, linked to the triterpene aglycone bayogenin. In addition to standard 2D methods, a series of TOCSY spectra with different mixing times and a high-resolution coupled HSQC spectrum were particularly useful for assigning the monosaccharide units. It is concluded that saponins of this complexity are approaching the limit of structural complexity that can be solved by NMR alone, although the limit might be pushed further by access to ultra-high field NMR spectrometers. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; ^{13}C NMR; complex saponins; coupled HSQC

INTRODUCTION

Michrosechium helleri (popularly known as amole) is a little studied plant which grows in Guatemala and the southern region of Mexico.[1] Conversations with the inhabitants of the region where it was harvested indicated that traditional uses of aqueous extracts of the rhizomes of this plant are as a laundry soap and as a lotion to prevent hair loss. The former use suggested the presence of saponins in the plant since many saponins have detergent properties.[2] However, no pharmacological or phytochemical investigations of this plant appeared to have been reported. Consequently, we decided to undertake such an investigation. Herein we report the isolation and the identification, mainly by 2D NMR, of two complex saponins, amole F and G, isolated from a methanolic extract of the roots of *M. helleri*.

RESULTS AND DISCUSSION

Since we were interested in subjecting the two isolated saponins, which were available in limited amounts, to subsequent pharmacological testing, we avoided classical degradative techniques and similar destructive methods which are commonly used in investigating complex saponins.[2] Instead, we used only NMR methods. ^{1}H, ^{13}C and DEPT-edited ^{13}C spectra of the two compounds suggested that each was a saponin. They appeared to share a common triterpene aglycone unit, with amole F having seven associated monosaccharide units whereas amole G had only six. Many of the ^{13}C peaks in the saccharide region also had identical chemical shifts (within 0.02 ppm) for the two compounds, suggesting close structural similarities for amole F and G.

Identification of the aglycone unit

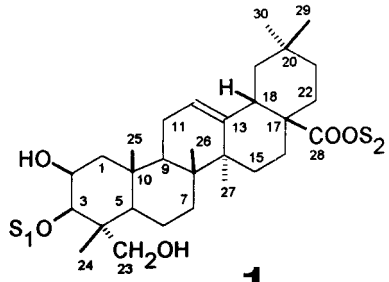
The structure and stereochemistry of the common aglycone unit was determined by a combination of COSY, ROESY, HSQC and HMBC spectra. ROESY spectra were used in place of NOESY since the molecules were found to have correlation times in solution such that NOE effects were near zero.[3] HSQC spectra were used in place of HMQC spectra to take advantage of the significantly better ^{13}C resolution (and sensitivity) of the former spectra.[4,5] Two sets of HMBC spectra were acquired with optimization of J_{CH} for 5 and 10 Hz.

The approach used to identify the aglycone unit and to assign its spectrum was similar to that which we had reported earlier for other triterpenes.[6–8] In particular, two and three-bond cross peaks from methyl protons in the HMBC spectra allow the determination and assignment of much of the molecular skeleton of the aglycone. Starting points were provided by a geminal methyl pair (subsequently assigned as C-29 and C-30) and a geminal CH_3/CH_2OH pair (subsequently assigned as C-24 and C-23). These results, in combination with the results of the COSY and HSQC spectra, allowed the identification of the aglycone as 2,3,23-trihydroxyolean-12-enoic

* *Correspondence to*: W. F. Reynolds, Department of Chemistry, University of Toronto, Toronto, Ontario M5S 3H6, Canada.
† Dedicated to Professor John D. Roberts on the occasion of his 80th birthday.
Contract/grant sponsor: CoNaCyT.
Contract/grant sponsor: DGAPA.
Contract/grant sponsor: NSERCC.

CCC 0749-1581/98/SI0111–07 $17.50

acid. The assignment of the stereochemistry at C-3 and confirmation that the third OH group was bonded to C-23 rather than C-24 was provided by the ROESY spectra. Key observations included ROESY cross peaks between H-3 and both H-1_{axial} and H-5, between H-5 and both H-23 protons and between H-24 and H-25 methyl peaks. Finally, the sterochemistry at H-2 was determined from high-resolution coupled HSQC spectra[5] (see below for further discussion). This showed only small (<5 Hz) and unresolved vicinal couplings of H-2 to H-3 and the two H-1 protons, indicating that it was α-equatorial. Other ROESY cross peaks confirmed the expected stereochemistry of the oleanene skeleton (**1**). Thus, the aglycone is 2β,3β,23-trihydroxyolean-12-en-28-oic acid, a known triterpenoic acid with the trivial name bayogenin.[9] The ^{1}H and ^{13}C chemical shifts for bayogenin in the two compounds were identical (within 0.02 ppm) and are listed in Table 1.



1

S_1 = G_1- (AMOLE G)

S_1 = G_2-(1→3)-G_1- (AMOLE F)

S_2 = R_2-(1→3)-X_1(1→3)-X_1-(1→4)-R_1-(1→2)-A-

G = β-D-Glucose
R = α-L-Rhamnose
X = β-D-Xylose
A = α-L-Arabinose

Structures of the polysaccharide portions of amole F and G

Inspection of the normal and DEPT-edited spectra of amole F and G suggested that the former contained two hexapyranose units, two 6-deoxy hexapyranose units and three pentapyranose units, whereas the latter had one less hexapyranose unit. However, identification of individual monosaccharide units proved to be a very challenging task. To aid in this assignment, several additional 2D spectra were obtained, starting with a series of five TOCSY spectra with mixing times ranging from 0.01 to 0.12 s. Inspection of cross-sections through anomeric protons (and also through CH_3 protons for the two 6-deoxy sugars) as a function of mixing time greatly facilitated the identification of sequences of protons in each monosaccharide unit. The HSQC spectra allowed the assignments of directly coupled ^{13}C–^{1}H pairs. However, this still left many ambiguities due to numbers of protons with very similar chemical shifts (particularly in the region δ3.15–3.40). Fortunately, many of these ambiguities could be resolved by observation of two- or three-bond connectivities in the HMBC spectra between clearly resolved and assignable protons and ambiguous carbons. However, this did not resolve all ambiguities, mainly because of the limited ^{13}C resolution in the HMBC spectrum (the presence of ^{1}H multiplet structure along f_1 in an HMBC spectrum limits resolution to >0.2 ppm, even if sufficient f_1 data

Table 1. ^{13}C and ^{1}H chemical shift for bayogenin aglycone in amole F[a] [in ppm relative to $(CH_3)_4Si$ in CD_3OD]

		δ_H[c]				δ_H	
Carbon[b]	δ_C	Axial	Equatorial	Carbon	δ_C	Axial	Equatorial
1	44.38	1.14	2.05	16	23.74	2.02	1.66
2	71.19	—	4.31	17	48.24	—	—
3	83.92	3.61	—	18	42.66	2.89	—
4	42.96	—	—	19	47.11	1.71	1.12
5	48.18	1.30	—	20	31.61	—	—
6	18.79	1.45	1.50	21	34.88	1.40	1.20
7	33.53	1.59	1.32	22	33.33	1.74	1.56
8	40.74	—	—	23	65.76	—	3.24, 3.61
9	49.31	1.56	—	24	14.81	0.94	—
10	37.54	—	—	25	17.60	1.28	—
11	24.70	1.99	1.92	26	18.04	0.78	—
12	123.94		5.30	27	26.39	1.16	—
13	144.99	—	—	28	177.74	—	—
14	43.15	—	—	29	24.03	0.92	—
15	28.88	1.65	1.14	30	33.53	—	0.89

[a] Chemical shifts for the same aglycone in amole G agreed to within 0.02 ppm with those listed here.
[b] Numbering as in **1**.
[c] Assignment of individual protons as axial or equatorial was determined from the width of multiplets in HSQC spectra and the presence or absence of 1–3 diaxial peaks in ROESY spectra.

points can be collected or linearly predicted). Fortunately, high-resolution coupled HSQC spectra,[5] which were obtained to aid in stereochemical assignments, allowed us to eliminate the remaining ambiguities. These spectra covered the saccharide region with an $f_2(^1H)$ spectral window of 1775 Hz and an $f_1(^{13}C)$ spectral window of 6200 Hz, 2048 data points with zero-filling to 8192 and 256 time increments with linear prediction to 2048 and, zero-filing to 4096. This provided excellent resolution along both axes. Parts of the spectrum for amole F is illustrated in Fig. 1. Two partially over lapping peaks are observed at δ78.22 and δ78.21 with similar ^1H chemical shifts. Cross-sections through the shoulders of the two peaks showed that the peak at δ78.22 was bonded to a proton at δ3.27 whereas the peak at δ78.21 was bonded to a proton at δ3.31. The former was a clean triplet, corresponding to two vicinal couplings of *ca.* 10 Hz, while the latter showed a similar triplet structure but with an additional coupling of *ca.* 4 Hz. The differences in coupling patterns (in conjunction with information from other spectra) aided the assignment of the former signal to C-3 of a xylose unit while the latter could be assigned to C-5 of a glucose unit. The same approach helped to resolve other ambiguous assignments.

However, the main advantage of the coupled HSQC spectrum was the useful stereochemical information which it supplied. This included not only the magnitudes of vicinal couplings but also the magnitudes of one-bond ^{13}C–^1H couplings for anomeric carbons. The latter information is useful because equatorial anomeric protons have $^1J(^{13}C,^1H) \approx 170$ Hz while the corresponding axial protons have $^1J(^{13}C,^1H) \sim 160$ Hz.[10] There was one additional advantage to the use of coupled HSQC spectra beyond those which we have described previously.[5] In some cases, protons on adjacent carbons have nearly coincident chemical shifts. The resultant strong coupling significantly complicates the determination of vicinal coupling constants from either 1D ^1H spectra or most 2D spectra. However, in a coupled HSQC spectrum, one is determining ^1H, ^1H coupling from a ^1H—^{13}C—^{12}C—^1H unit and the large one-bond ^{13}C, ^1H coupling effectively produces weak ^1H–^1H coupling (this corresponds to the long established technique of using ^{13}C satellite peaks in ^1H spectra to determine coupling between otherwise magnetically equivalent protons[11]). The information from the coupled HSQC spectrum allowed easy assignment of six of the seven monosaccharides in amole F as two β-glucose units, two α-rhamnose units and two β-xylose units. In the case of glucose and xylose units, this was based on the observation that each methine proton showed large vicinal couplings (*ca.* 10 Hz) to adjacent methine protons (and to one of the C-5 methylene protons of xylose). For rhamnose, the anomeric ^{13}C,^1H coupling was 171 Hz whereas H-1, H-2 and H-2, H-3 couplings were both small, requiring both H-1 and H-2 to be equatorial. Although the absolute stereochemistries of the individual monosaccharide units were not determined (since this would have required sample degradation), it is assumed that they are β-D-glucose, β-D-xylose and α-L-rhamnose (**2**), based on observations on other plant saponins.[2] Amole G differed in having only one β-glucose unit.

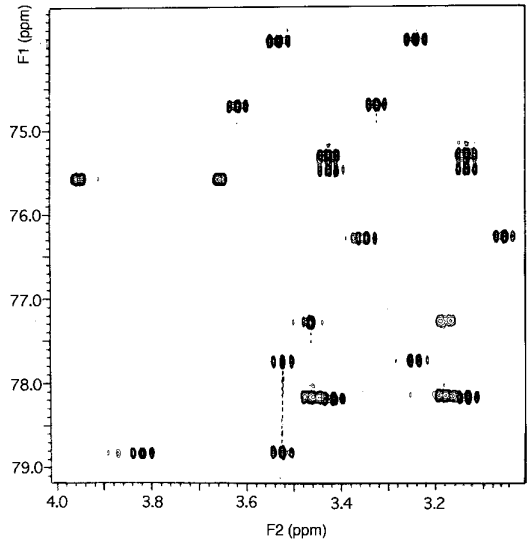
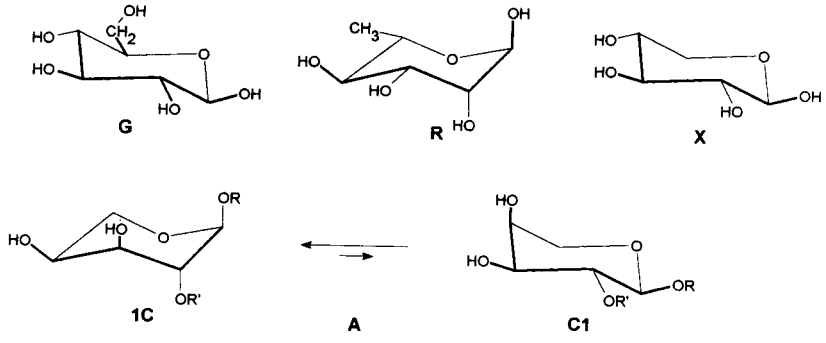


Figure 1. Expansion illustrating one region of the coupled HSQC spectrum of amole F. Both positive and negative contours are plotted to allow clearer observation of multiplet patterns.

The remaining pentapyranose unit differed from the other monosaccharides in showing only one large vicinal coupling constant, between H-4 and one of the H-5 protons. Cross-sections through individual carbon peaks in the coupled HSQC spectrum are illustrated in Fig. 2. The anomeric ^{13}C,^1H coupling constant is 172 Hz, suggesting an equatorial orientation for the anomeric hydrogen. This is supported by the observation that H-1 shows stronger cross peaks to C-3 and C-5 than to C-2 in the HMBC spectrum optimized for $J(^{13}C,^1H) = 10$ Hz, suggesting that H-1 is *anti* to C-3 and C-5. Vicinal coupling constants for protons H-1 through H-4 are all *ca.* 4 Hz while there is a coupling of *ca.* 10 Hz between H-4 and one of the H-5 protons. These data are entirely consistent with α-arabinose, assumed to be the L-isomer based on observations on related plant saponins.[2] However, the coupling constants indicate that α-arabinose is in its normally less stable[12] 1C chair conformation (the 1C chair has three of four OH or OR groups axial compared with only one of four for the alternate C1 chairs; **2**). A possible reason for this observation is provided below. Complete ^1H and ^{13}C chemical shift assignments for the various monosaccharide units are given in Table 2.

The final information required is the linkage sites between monosaccharides and to the aglycone unit. This was easily established from the HMBC spectra. In each case of an interglycosic link, three-bond connectivity peaks were observed between one anomeric hydrogen and a particular carbon on the second monosaccharide unit, along with a cross peak between the corresponding attached proton on the second monosaccharide unit with the anomeric carbon of the first unit.



R = Aglycone

R' = Tetrasaccharide

1C, C1 = Chair conformations of A

2

Similarly, cross peaks between H-1 of one glucose and C-3 of the aglycone (and H-3 of the aglycone with C-1 of glucose) established one point of attachment to the aglycone while a clear cross peak between H-1 of arabinose and the ester carbon (C-28) of the aglycone established the second point of attachment. The interglycosidic links are shown in **1** along with points of attachment to the aglycone. Hence amole F has the structure 3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl]-28-*O*-{α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-[β-D-xylopyranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl-(1 → }
-

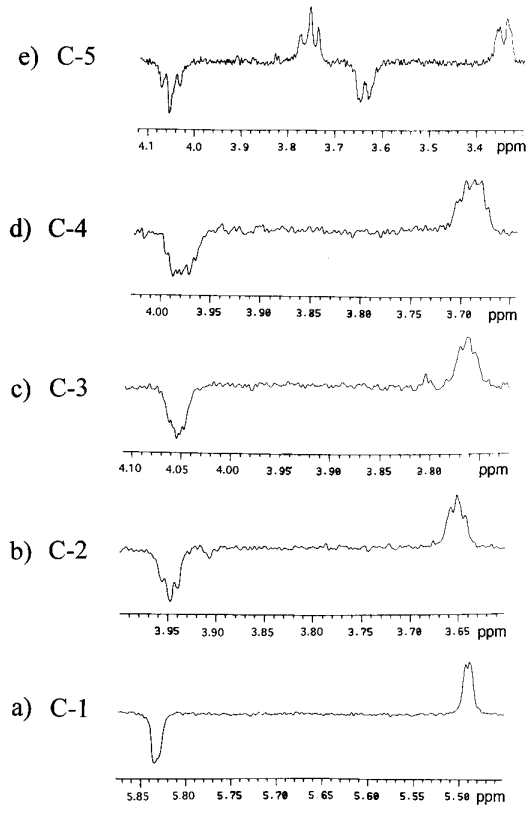


Figure 2. Cross-sections through individual carbons of arabinose from the coupled HSQC spectrum of amole F.

bayogenin. Amole G differs in having only one glucose ring attached to C-3 of the aglycone.

The α-L-arabinose unit has the aglycone unit *O*-linked to C-1 and a branched tetrasaccharide unit *O*-linked to C-2. Adoption of the 1C chair form (**2**) puts these two bulky groups in an *anti* arrangement, minimizing steric hindrance. Hence it appears that it is this need to minimize repulsion between attached groups which requires the α-arabinose unit to adopt the normally less stable 1C chair.

Finally, the ROESY spectra show a number of significant interglycosidic NOE peaks within the pentasaccharide unit ester linked to C-28, in addition to those between the pairs of protons at either end of each interglycosidic link. Clearly assignable peaks are listed in Table 3. The presence of these additional peaks suggests that there are strongly preferred conformations within the pentasaccharide unit, along with restricted rotation about interglycosidic bonds. The presence of these peaks has potential value in determining the preferred conformation within the pentasaccharide unit. At the same time, they make the ROESY spectra less useful than the HMBC spectra for determining specific sites of interglycosidic links, since the HMBC cross peaks are specific to these sites while the ROESY cross peaks are not.

Comparisons with structures of related saponins

Saponins based on a hydroxylated olean-12-en-28-oic acid aglycone unit are the most common of all plant saponins.[2] Of these, 3-hydroxyolean-12-en-28-oic acid and 3β,23-dihydroxy-olean-12-en-oic acid are the most common aglycones. Relatively fewer saponins are based on the 2β,3β,23-trihydroxy compound (bayogenin) found in this investigation.[2] A search of the literature revealed no saponins with an identical structure to either amole F or G. However, a series of even more complex saponins with a bayogenin aglycone unit have been isolated and characterized.[13] Three of these saponins, isolated from *Solidago canadensis*, have a core

Table 2. ^{13}C and ^{1}H chemical shifts for monosaccharide units in amole F and G [in ppm relative to $(CH_3)_4Si$ in CD_3OD]

Monosaccharide[a]	Carbon	δ_C [b]	S_H [b]	$[^1J(^{13}C,^1H)]$[c]	Monosaccharide	Carbon	δ_C	δ_H	$[^1J(^{13}C,^1H)]$
A	1	93.80	5.66	[172]	X_2	1	106.05	4.54	[161]
	2	75.61	3.79			2	75.32	3.26	
	3	70.50	3.90			3	78.22	3.26	
	4	66.77	3.82			4	71.04	3.47	
	5	63.31	3.49(e), 3.89(a)			5	67.04	3.22(a), 3.85(e)	
R_1	1	101.00	5.00	[171]	G_1	1	105.12	4.48	[159]
	2	72.22	4.03			2	74.72	3.46	
	3	82.16	3.87			3	87.95	3.53	
	4	78.83	3.67			4	69.50	3.48	
	5	69.03	3.71			5	77.33	3.30	
	6	18.36	1.25			6	62.14	3.71, 3.80	
R_2	1	102.66	5.14	[171]	G_2	1	105.24	4.55	[161]
	2	72.27	3.93			2	75.49	3.27	
	3	72.16	3.97			3	77.79	3.36	
	4	73.97	3.38			4	71.54	3.26	
	5	69.96	4.00			5	78.21	3.31	
	6	17.90	1.23			6	62.62	3.62, 3.87	
X_1	1	104.80	4.70	[163]	G_1G[d]	1	105.48	4.43	[160]
	2	76.28	3.19			2	75.39	3.27	
	3	84.39	3.38			3	78.21	3.36	
	4	70.21	3.49			4	71.09	3.36	
	5	66.95	3.16(a), 3.82(e)			5	77.73	3.27	
						6	62.28	3.70, 3.80	

[a] Labeling of monosaccharides as illustrated in **1**. A = α-L-arabinose; G = β-D-glucose; R = α-L-rhamnose; X = β-D-xylose.
[b] Chemical shifts were determined for amole F, unless indicated otherwise. The corresponding ^1H and ^{13}C chemical shifts for all A, R and X monosaccharides in amole G agreed to within 0.02 ppm with those for amole F.
[c] $^1J(^{13}C,^1H)$ for anomeric carbon (in Hz), determined from a coupled HSQC spectrum.
[d] Data for single β-D-glucose unit in amole G.

Table 3. Assignable interglycosidic NOE peaks from ROESY spectrum of amole F

A(1)–R_1(5)[a]	R_1(4)–X_2(1)
A(2)–R_1(1)	R_2(1)–X_1(2)
R_1(3)–X_1(1)	R_2(1)–X_1(3)
R_1(4)–X_1(1)	R_2(5)–X_1(3)
R_1(5)–X_1(1)	G_2(1)–G_1(3)
R_1(2)–X_2(1)	G_2(1)–G_1(4)
R_1(3)–X_2(1)	

[a] A letter with or without a subscript corresponds to monosaccharide defined in **1**. Numbers in parentheses corresponds to the proton within that monosaccharide unit for which an NOE peak is observed.

skeleton identical with that of amole F but with one or two additional attached monosaccharide units. The first two compounds have either an α-L-rhamnose unit of a β-D-apiose unit linked to C-3 of the arabinose unit while the third has the same additional α-rhamnose unit plus a β-D-galactose unit linked to C-2 of the terminal rhamnose unit. All three were characterized with the aid of chemical degradation and derivatization, in addition to spectroscopic methods.[13a] NMR assignments were provided for only the first two compounds.[13b] These are generally in good agreement with our results with two main exceptions. First, the chemical shifts for the arabinose units were significantly different from those which we have obtained. This is undoubtedly related to the presence of the additional group linked to C-3 of arabinose. With this third attached group, there is a major steric hindrance in either chair form of α-arabinose. In fact, it was concluded that their arabinose units existed in strongly distorted chair conformations.[13b] Second, there was a much larger number of uncertain assignments in the earlier investigation, e.g. 13 in the compound with the one additional α-rhamnose unit.[13b] This is undoubtedly partly due to the increased spectral complexity but also to the additional spectral information which was available to us, particularly from the coupled HSQC spectra. For example, in the earlier work, the carbon at δ78.21 was tentatively assigned to C-5 of the internal glucose unit and that at δ77.33 was assigned to C-5 of the external glucose. The main problem in making this assignment was obviously the nearly equal chemical shifts of the two attached protons (see Table 2). Our investigation reverses these assignments. As discussed above, the coupled HSQC spectrum shows that the proton attached to the carbon at δ78.21 shows a large (*ca.* 10 Hz) and a small (*ca.* 4 Hz) coupling to the two H-6 protons in addition to a diaxial coupling (*ca.* 10 Hz) to H-4. By contrast, the proton attached to the carbon at δ77.33 shows only one large coupling (to H-4) and two smaller unresolved couplings (see Fig. 1). Cross-sections through the two C-6 carbons shows that one of the protons bonded to the carbon at δ62.62 shows a large vicinal coupling while both protons attached to the carbon at δ62.14 show small vicinal couplings. Thus the carbon at δ77.33 is bonded to the carbon at δ62.14 rather than the carbon at δ62.62, as previously assigned. Cross-sections through anomeric protons in a TOCSY spectrum with a 0.12 s mixing time allowed completion of the glucose assignments by demonstrating that the methylene protons at δ3.71 and δ3.80 attached to the carbon at δ62.14 were part of the same coupled spin system as the anomeric hydrogen at δ4.56 while the methylene protons at δ3.62 and δ3.87 belonged to the same spin system as the δ4.48 anomeric proton.

Consideration of the limitations of NMR for determining structures of saponins and complex polysaccharides

The discussion immediately above concerning one specific assignment problem clearly illustrates the difficulties in using NMR spectroscopic methods to determine the structure and assign the spectrum of a compound of this level of complexity.[14] In certain important ways, the investigation of a complex polysaccharide is more difficult than the investigation of a protein of higher molecular weight. First, almost all of the ^{1}H and ^{13}C chemical shifts occur in much narrower spectral windows for a polysaccharide than for a protein. Second, the individual peptide fragments are bonded in a regular and predictable fashion in a protein whereas a hexapyranose unit can be linked to an adjacent monosaccharide through one of C-1, C-2, C-3, C-4 or C-6. Finally, polysaccharides have only two spin-$\frac{1}{2}$ nuclei compared with three for a doubly labelled protein, limiting the possibilities of spreading frequency information along three or four axes. The possibility of using 3D methods for polysaccharide structure elucidation has been discussed.[15] However, it would be difficult to obtain adequate resolution along the two time-incremented axes, even with the aid of linear prediction,[16] in any reasonable time. This problem is further aggravated by the necessity of working at natural ^{13}C abundance. These problems balance against the larger number of assignments for a protein to create a problem of comparable difficulty.

Based on this investigation, we doubt if it will be generally feasible to use NMR methods alone to characterize a polysaccharide much more complex than amole F, at least while working at magnetic field strengths commonly available in chemistry laboratories. If one has sufficient sample to allow degradative methods, combined with chemical derivatization to identify individual monosaccharides, the degree of complexity of problems which can be tackled successfully can be at least modestly increased.[13] However, if one wishes to determine structures of more complex polysaccharides by spectroscopy alone, the only viable solution appears to be access to higher field spectrometers. Since these molecules are still of low enough molecular weight to produce sharp ^{1}H and ^{13}C peaks, the 60% increase in spectral dispersion on going from 500 to 800 MHz would be of great value in resolving spectral ambiguities.

EXPERIMENTAL

Isolation of amole F and G

Michrosechium helleri was collected in May 1993 in Santiago Tlazoyoaltepec, Municipio Elta, Oaxaca, Mexico. A voucher specimen is deposited at the IMSS herbarium in Mexico City. The rhizomes were cut into slices, air dried and extracted successively with hexane, CH_2Cl_2 and CH_3OH. The methanolic extract was injected into an HPLC system (Varian 1090) using CH_3OH–H_2O (3:1) with a flow-rate of 1 ml min^{-1} at 31 °C, on a C_{18} reversed phase column of 300 mm × 4 mm i.d. Peaks were detected with a UV detector (Varian 5090) at 220 nm. Several peaks were observed in the chromatogram, of which two were purified by repeated HPLC injections, yielding 8 mg of amole F and 10 mg of amole G.

NMR methods

All spectra were collected on a Varian Unity 500 spectrometer, equipped with an inverse detection 5 mm probe (90° ^1H pulse width 9.5 μs, ^{13}C 90° decoupler pulse width 9.4 μs). Spectra were collected for samples in CD_3OD at a probe temperature of 25 °C. Peak positions of the residual CD_2H peak (δ3.30) and the $^{13}CD_3$ peak (δ49.02) were measured relative to internal $(CH_4)_3Si$ on the solutions used in the experiments. In subsequent, experiments, most measurements were then made relative to the CD_2H and $^{13}CD_3$ peaks to allow narrowing of spectral windows. Most experiments were run using standard Varian software, except for the coupled HSQC spectra, which were obtained with minor modification of the standard HSQC pulse sequence.[5] ^1H and ^{13}C spectra were obtained with spectral windows of 2900 and 23 000 Hz, respectively, 32 768 points (zero-filled to 65 536) and 60° pulses. HMBC spectra were obtained using the same ^1H and ^{13}C spectral windows as listed above, 1024 data points (zero filled to 2048), 256 time increments (linear predicted to 2048 with zero-filling to 4096), a 0.6 s relaxation delay and 80 transients per increment.

Decoupled HSQC spectra were obtained with the same ^1H spectral window and data points as above, a ^{13}C spectral window of 17 000 Hz, again with 256 increments linear predicted to 2048, a relaxation delay of 2.0 s (to minimize decoupler heating), a BIRD nulling delay of 0.3 s and 16 transients per increment. Many of the acquisition parameters for the coupled HSQC spectra are described in the text. In this case a BIRD null delay of 0.85 s was used (chosen to minimize OH and CD_2H peaks) along with a relaxation delay of 0.5 s, with 16 transients per increment. All of the above 2D spectra were acquired and processed in the phase-sensitive mode, except for the HMBC spectra, which were processed in the mixed mode (phase sensitive along f_1 and absolute value along f_2), as recommended by the original authors.[17] Gaussian weighting was used in all cases except for the absolute value HMBC axis, where a 1/3 shifted sine-bell was used.

COSY spectra were acquired in absolute value mode with 2900 Hz spectral windows along both axes, 1024 data points and time increments (both zero-filled to 2048), 16 transients per increment and a 0.6 s relaxation delay. Pseudo-echo processing was used along both axes, followed by triangular folding. TOCSY and ROESY spectra were both measured in the phase-sensitive mode, using the same spectral windows and data points as for COSY, 16 transients per increment, 256 increments (linear predicted to 1024 with zero-filling to 2048) and relaxation delays of 1.5 s. Isotropic mixing times for TOCSY spectra ranged from 0.01 to 0.12 s and the ROESY spin-lock duration was 0.15 s. Gaussian weighting was used for both spectra.

Acknowledgements

Financial support from CoNaCyT (Mexico), DGAPA (Mexico) and NSERCC (Canada) is acknowledged by I.L., R.G.E. and W.F.R., respectively.

REFERENCES

1. J. V. A. Dieterle, *Fieldiana: Botany* **24**, Part XI, 358 (1976).
2. K. Hostettmann and A. Marston, *Saponins*. Cambridge University Press, Cambridge (1995).
3. D. Neuhaus and M. P. Williamson, *The Nuclear Overhauser Effect in Structural and Conformational Analysis*. VCH, New York (1989).
4. A. Bax, M. Ikura, L. E. Kay, D. A. Torchia and R. Tschudin, *J. Magn. Reson.* **86**, 304 (1990).
5. W. F. Reynolds, S. McLean, L.-L. Tay, M. Yu, R. G. Enriquez, D. M. Estwick and K. O. Pascoe, *Magn. Reson. Chem.* **35**, 455 (1997).
6. W. F. Reynolds, S. McLean, J. Poplawski, R. G. Enriquez, L. I. Escobar and I. Leon, *Tetrahedron* **42**, 3419 (1986).
7. S. McLean, M. Perpick-Dumont, W. F. Reynolds, H. Jacobs and S. S. Lachmansing, *Can. J. Chem.* **65**, 2519 (1987).
8. S. McLean, W. F. Reynolds, J.-P. Yang, H. Jacobs and L. L. Jean-Pierre, *Magn. Reson. Chem.* **32**, 422 (1992).
9. R. A. Eade, J. J. H. Simes and B. Stevenson, *Aust. J. Chem.* **16**, 900 (1963).
10. A. S. Perlin and B. Casu, *Tetrahedron Lett.* 2921 (1969).
11. A. D. Cohen, N. Sheppard and J. J. Turner, *Proc. Chem. Soc.* 188 (1958).
12. P. L. Durette and D. Horton, *Adv. Carbohydr. Chem. Biochem.* **26**, 49 (1971).
13. (a) K. Hiller, G. Baden, G. Reznicek, J. Jurenitsch and W. Kulbelka, *Pharmazie* **46**, 405 (1991); (b) G. Reznicek, J. Jurenitsch, M. Plasun, S. Korhammer, E. Haslinger, K. Hiller and W. Kublka, *Phytochemistry* **30**, 1629 (1991).
14. P. K. Agrawal, D. C. Jain and A. K. Pathak, *Magn. Reson. Chem.* **33**, 923.
15. S. T. Thiilborg, C. Cornett and E. Lemmich, in *Saponins Used in Traditional and Modern Medicine*, edited by G. Waller and K. Yamasaki, p. 151. Plenum Press, New York (1996).
16. W. F. Reynolds, M. Yu, R. G. Enriquez and I. Leon, *Magn. Reson. Chem.* **35**, 505 (1997).
17. A. Bax and M. F. Summer, *J. Am. Chem. Soc.* **108**, 2093 (1986).

