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Structural Elucidation of Saponins

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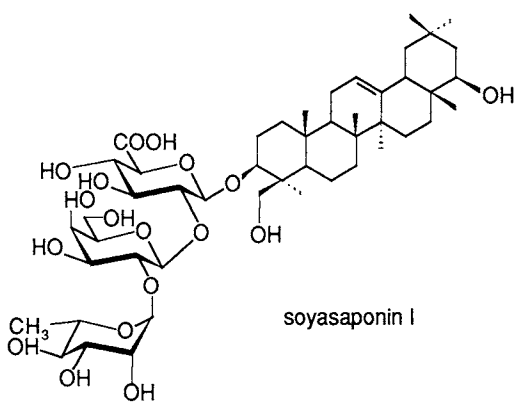
INTRODUCTION

SAPONINS ARE COMPLEX MOLECULES

Saponins constitute an important class of secondary metabolites from plant and animal domains (1-3). They display a broad spectrum of biological activities that have raised the interest of phytochemists for the past forty years. With molecular weights ranging from 600 to 2000 Daltons, their structures are complex. It was only in the seventies that, with the use of chemical degradation techniques, their precise structures were established (4-5). Since then, the field has benefitted from the recent progress in instrumentation. The purpose of this article is to describe the current techniques in use for the structural elucidation of saponins.

From a chemical standpoint, saponins are made of three entities: an aglycone (steroid or triterpene), sugars and sometimes acids. The determination of the structure therefore requires identification of the elements and sequencing. While the first problem is trivial, the second is generally more difficult to solve because of the large number of possible combinations. For example, let us consider the case of soyasaponin I (6), a saponin, which is made of soyasapogenol B, rhamnose, galactose and glucuronic acid. If there was only one branching point on each of the elements, there would be only 24 isomeric structures for soyasaponin I (4!). The larger number of iso



mers obtained with these four elements (35712) comes from the number of possible branching on each of them (3 for soyasapogenol B and rhamnose, 4 for glucuronic acid and galactose). The number which is obtained after this combinatory analysis must be further multiplied by 64 to account for α or β anomeric configurations and pyran or furan forms (table 1). Fortunately, all these possibilities are not met in nature (for example β-L-rhamnose) but this illustrates the difficulties of saponin sequencing.

TABLE 1.

Number of saponins made of soyasapogenol B, glucuronic acid, galactose and rhamnose.

Tridesmosides :			6 isomers
Bisdesmosides :			
22 disaccharides:	rha-gal-	(4 isomers)	
	rha-glcUA-	(4 isomers)	
	gal-glcUA-	(4 isomers)	x 3 branching points
	gal-rha-	(3 isomers)	x 2 possibilities for
	glcUA-rha-	(3 isomers)	the third sugar.
	glcUA-gal-	(4 isomers)	
		22 x 3 x 2 =	132 isomers
Monodesmosides :			
4 x rha-gal-	x 6 branching points		
4 x rha-glcUA-	x 6 branching points		
4 x gal-glcUA-	x 7 branching points		x 3 points of attachment
3 x gal-rha-	x 6 branching points		on aglycone
3 x glcUA-rha-	x 6 branching points		
4 x glcUA-gal-	x 7 branching points		
		140 x 3 =	420 isomers

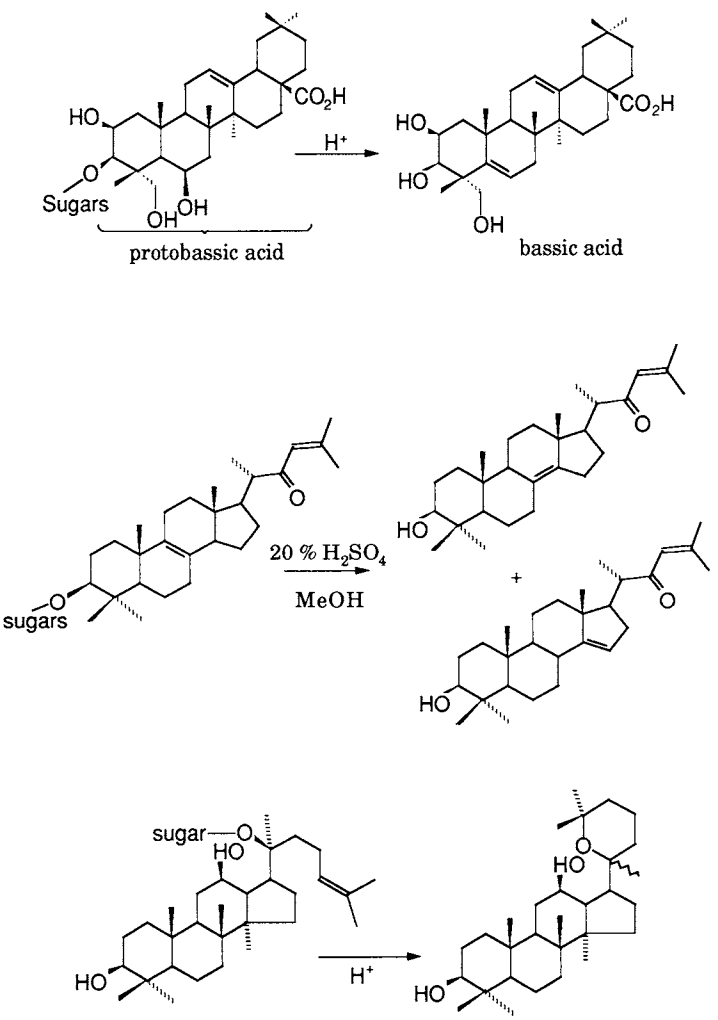
Total of isomers (including anomers, furanoses and pyranoses):

64 x (6 + 132 + 420) = 64 x 558 = 35712 isomers.

(this number must be multiplied by ten if a single acid substitutes the molecule)

THE IDENTIFICATION OF THE ELEMENTS OF A SAPONIN - ARTEFACT FORMATION

The elements of a saponin may be identified either on the intact saponin or after hydrolytic cleavage. It is always better to perform the identification on the intact saponin to eliminate the possibility of artefact formation during hydrolysis. This identification can be realized by mass spectrometry (MS) or nuclear magnetic resonance (NMR ^{1}H or ^{13}C). Artefacts are most often formed when the aglycone is sensitive to acid. The following equations give examples of such rearrangements (7-9).



Three examples of formation of artefacts after acid hydrolysis.

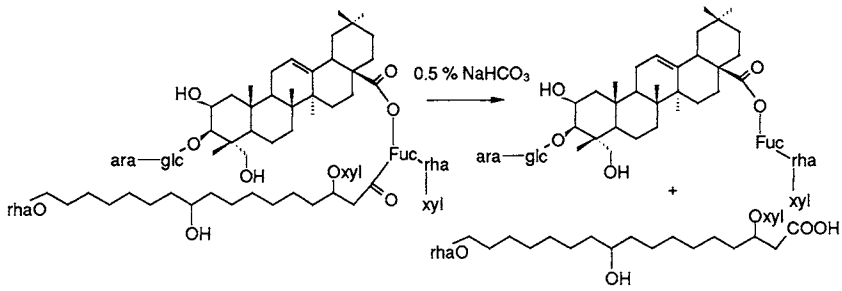
SUGARS PRESENT IN SAPONINS - IDENTIFICATION AND ABSOLUTE CONFIGURATION

The sugars present in saponins are common sugars such as D-glucose, D-galactose, L-rhamnose, D-fucose, D-xylose, L-arabinose, D-glucuronic acid and D-galacturonic acid. The enantiomers of these sugars are not found in plants a fact used as a clue in the determination of the configuration of the sugars. Less common sugars from saponins are for example : D-allomethylpyranose (10), D-ribose (11), D-apiose (12), D-quinovose (13), 2-acetamido-2-deoxy glucose (8, 14), 2-acetamido-2-deoxy galactose (15) and D-arabino-2-hexulopyranose (16),.

Sugars are obtained by hydrolysis of the saponins in acidic medium (1 or 2N HCl or H_2SO_4), in the free form if the solvent does not interfere (aqueous dioxane) or as methyl glycosides if the solvent contains methanol. Free sugars are separated from the aglycone by solvent extraction ($CHCl_3$) and the medium is neutralized with silver carbonate (with precipitation of AgCl) or basic resin (IRA 45, IRA OH^-). They are finally identified using reference samples by paper chromatography (17) or directly by HPLC (refractive index detection) (18). The methyl glycosides may also be analyzed by GC after silylation (O,N-bis trimethylsilyl trifluoroacetamide or hexamethyldisilazane and trimethylchlorosilane). Identification is based on retention times (8), FTIR (19) or mass spectrometry. The absolute configuration of the sugars is determined by running the same operations on a larger scale with a preparative separation stage. A variant is Nakanishi's method which consists in the parabromobenzylation of the methyl glycosides (*vide infra* for a complete discussion of the method) (15). Several analytical methods have been introduced to determine the absolute configuration of sugars on a microscale. The principle of these methods is based on the coupling of the sugar with an optically active reagent and the comparison of the conjugates with reference samples. A selection of the reagents comprises (S)-2-butanol (19), L-cysteine methyl ester (20) or L (-)-α-methylbenzylamine / $NaBH_3CN$ (21).

ACIDS PRESENT IN SAPONINS

The presence of acids in saponins further complicates the structural elucidation process and in most cases, one chooses to start the study with saponified compounds. It has been observed fortunately, that acyl groups on sugars are more labile than the hindered sugar chain esters at C-23, -24 or -28 and that they can be selectively cleaved with sodium bicarbonate (21):



Sodium bicarbonate hydrolysis of crocosmioside H.

Commonly encountered esters are angelates and tiglates, which are found, for example, in the saponins from horsechesnut *Aesculus glabrus* L. (22). Table 2 lists some of the acids present in saponins with their representative references.

TABLE 2 Examples of acids present in saponins.

tiglic acid	*Aesculus* saponins	22
angelic acid	*Dodonea* saponins	23
acetic acid	*Kalopanax* saponins	24
	ciwujianosides	25
malonic acid	ginsenosides	26
	malonylsaïkosaponins	27
3-hydroxybutyric acid	*Solidago* saponins	28
dicrotalic acid	lobatosides	29, 30
(hydroxymethylglutaric acid)	tubeimoside	31
	brownioside	32
gallic acid	*Castanopsis* saponins	33
digalloyl acid		33
phtalic acid	pseudoginsenoside	34
syringic acid	periandradulcins	35
p-coumaric acid	*Tragopogon* saponins	36
2, 9, 16-trihydroxyhexadecanoic acid	crocosmiosides	37
2, 6-dimethyl, 6-hydroxy-2-*trans*-	*Gleditsia* saponins	38
2, 7-octadienoic acid	*Entada* saponins	39

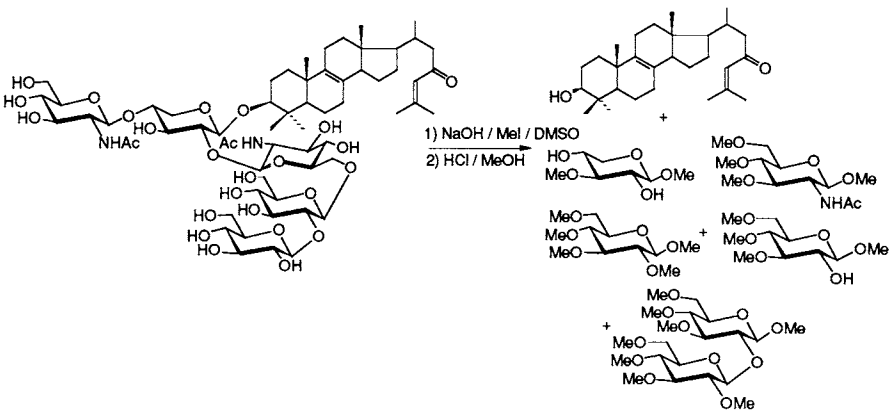
In most instances, the presence of esters is detected by comparison of the mass spectrum of the saponin with its desacetylated counterpart. This is also done with use of ^{1}H and ^{13}C NMR spectra. Some esters, particularly malonyl esters, are labile

and thus special care must be taken during the isolation process (26).

FULL STRUCTURAL DETERMINATION OF SAPONINS. THE CLASSICAL METHOD

IDENTIFICATION OF BRANCHING POINTS

Since information on branching is lost if complete hydrolysis is performed on a native saponin, the hydrolysis must be performed on a derivatized material. A general protocol proceeds as follows. The saponin is first permethylated according to Hakomori's method (NaH, DMSO, MeI) and cleaved with acid (TFA, 120°). The methylated sugars are reduced into alditols ($NaBH_4$), peracetylated and the mixture is analyzed by GC-MS (40). When a uronic acid is present, it is advised to reduce it into a bis-deuterated hexitol by treatment of the corresponding ester by means of $LiAlD_4$ before hydrolysis (41). Alternatively, the methylated sugars from the hydrolysis may be directly analyzed and identified by GLC (42) or HPLC (15).



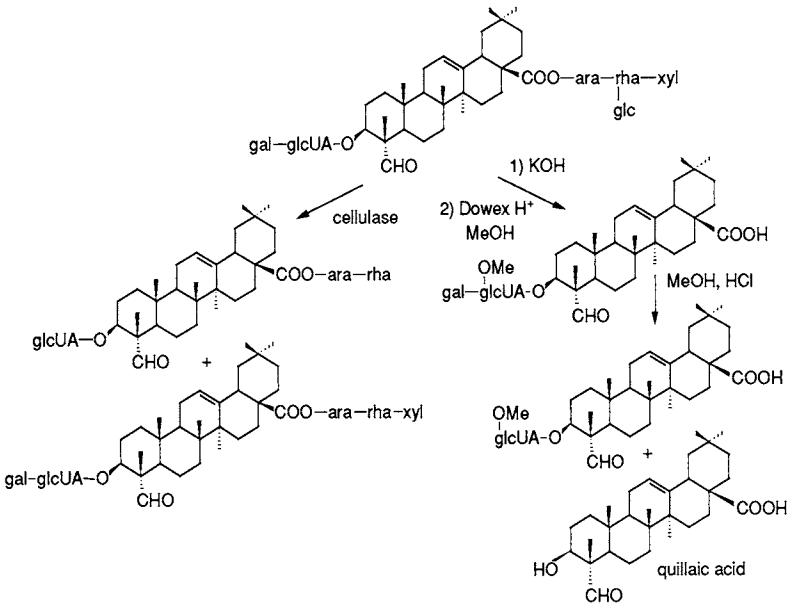
Derivatisation- hydrolysis of sarasinoside A_1 (15)

SEQUENCING OF THE SUGARS

In case where the chain of sugars has no more than two units, the permethylation-hydrolysis experiment suffices to identify the terminal sugar. In other cases, one must rely on incomplete hydrolysis experiments or on related series of compounds when they occur in the plant (43).

Even though purely degradative methods are no longer used, recent literature offers judicious examples of combination of degradations (18). The arsenal of degradative methods is impressive and contains tools such as microorganisms, isolated en-

zymes (18) and mineral salts (for example LiI for the cleavage of hindered esters) (21). Diazomethane itself, has been used as a cleavage reagent in quillaic acid derivatives. Participation of an aldehyde to the cleavage of a proximal ether bond explains this special reactivity (12).



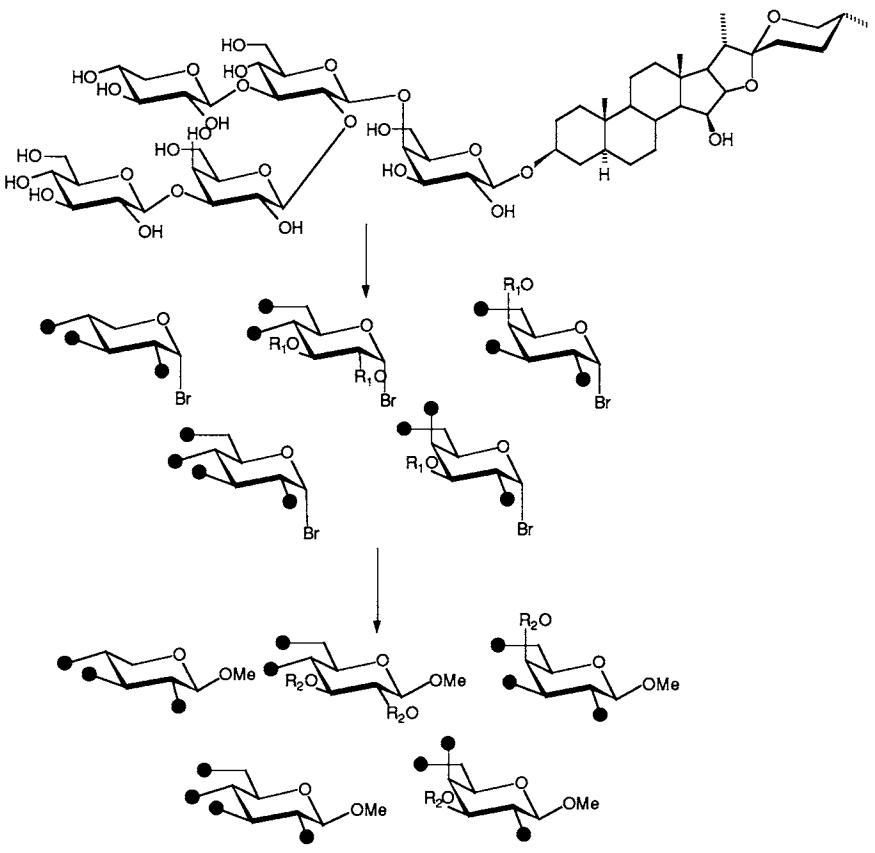
Combination of degradations of lucyoside P.

CIRCULAR DICHROISM - NAKANISHI' S METHOD

Nakanishi's method is based on the splitting of CD waves when two chromophores on a chiral molecule are close in space (the dibenzoate rule). Such is the case with dibromobenzoates of sugars which have a 1,2 or 1,3 relationship. In the first version of the method, saponins were permethylated, methanolyzed with acid and the liberated OH positions were *p*-bromobenzoylated (44). As terminal sugars are fully methylated and UV transparent, they need not be considered. Branched sugars (two substitutions at least) yield di-or tri-benzoates with exciton-split CD curves. The difference in $\Delta\varepsilon$ values of the two extrema of split CD curves is directly related to the respective positions of the benzoates (1, 2 eq-eq = 1, 2 eq-ax = 62 ; 1, 2 ax-ax = 6 ; 1, 3 eq-eq = 0 ; 1, 3 eq-ax = 16). The sensitivity of circular dichroism makes the method suitable for microassays. Typical analyses are performed on a 100 μg scale (nanomolar levels).

An improvement to the original method has recently been proposed (45). The saponin is not permethylated but is first parabromobenzoylated and cleaved with bro-

moacetylbromide. This operation transforms the sugars into 1-bromo-derivatives with bromoacetates on each of the linkage positions. The bromo sugars are converted into β-O-methylglycosides (Ag_2CO_3 / AgOTf) and the bromoacetates are exchanged for *p*-methoxy-cinnamates. After separation, one obtains derivatives of sugars bearing different combinations of benzoates and cinnamates. Their CD curves are characteristic of the sugar and of the substitution pattern.



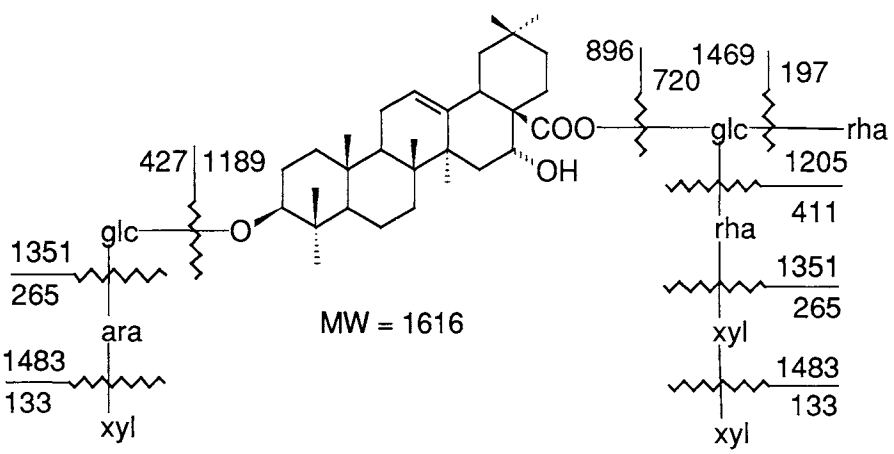
● = p-$BrC_6H_4CO_2$ R_1 = $BrCH_2CO$ R_2 = p-$MeOC_6H_4(CH)_2CO$

Preparation of derivatives suitable for CD from digitonin. (45)

MASS SPECTROMETRY AND SEQUENCING. RECENT ADVANCES

Saponins are too polar, too thermally unstable and not volatile enough to directly provide ions that are suitable for analysis. Until recently, derivatives (Me or TMS ethers) had to be prepared in order to obtain spectra. In the past ten years however, new techniques of soft ionization have emerged which have proved useful in the analysis of saponins. They are : Field Desorption (FD), Fast Atom Bombardment (FAB), Laser Field Desorption (LD) and Californium Plasma Desorption (CPD).

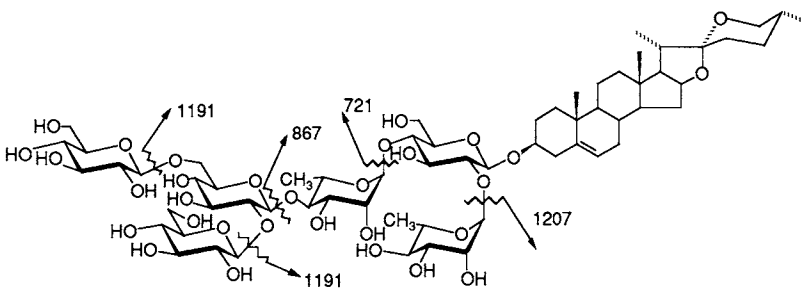
FDMS readily gives molecular ions and fragments with saponins (46, 47); in all cases cationized molecules are observed $(M+Na)^+$, $(M+K)^+$ and the spectra are often complicated by the presence of cationized fragment ions. For these reasons, it seems that FAB MS prevails over FDMS. Negative FAB MS gives $(M-H)^-$ ions of strong intensities and fragments corresponding to losses of the sugars. Positive FAB MS does not provide $(M+Na)^+$ or $(M+K)^+$ of strong intensity but the ionization may be increased by adding NaCl or KI to the glycerol matrix (48, 49).



Gleditsia japonica saponin. Calculated MS fragments (50)

1655	$[M+K]^+$	1243	$[M+Na-3x132]^+$
1639	$[M+Na]^+$	1229	$[M+Na-410]^+$
1523	$[M+K-132]^+$	1213	$[M+Na-426]^+$
1507	$[M+Na-132]^+$	936	$[M+K-719]^+$
1493	$[M+Na-146]^+$	920	$[M+Na-719]^+$
1391	$[M+K-264]^+$	839	$[M+Na+K]^{++}$
1374	$[M+Na-264]^+$	831	$[M+2Na]^{++}$

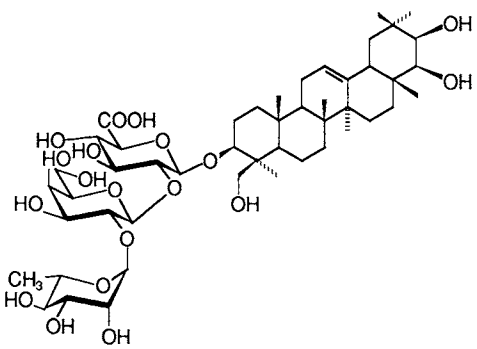
Gleditsia japonica saponin. Observed FD MS fragments (50)



Negative FAB fragmentation of *Alium vineale* saponin (19)

Laser desorption (time of flight or Fourier transform) also yields ions and fragments with saponins (15, 51, 52). Although promising the technique is not yet widely used (for cost reasons ?). Californium Plasma Desorption MS is the least expensive of the available MS techniques; it gives molecular ions of high intensity for non volatile, polar and fragile molecules of high molecular weight including saponins (53). While the spectra of underivatized saponins show little fragmentation (54), intense fragments for sequential losses of sugars are observed for peracetylated material (55). A disadvantage of CPDMS is the relatively low resolution of the peaks.

Mass spectrometry is able to provide molecular ions and fragments corresponding to sequential losses of sugars. When the chains of sugars are linear and if the sugars have different molecular weights, the combined use of MS fragmentation and of methylated alditol analysis allows the full structural elucidation of saponins. This is the case for soyasaponin A_3 (from soyabean, *Glycine max*) where hydrolysis of the permethylate reveals the presence of soyasapogenol A, terminal rhamnose, 2-substituted galacturonic acid and 2-substituted galactose. Negative FAB MS shows sequential losses of deoxyhexose, hexose and hexuronic acid, thus allowing full structural elucidation (56).



Soyasaponin A3

Among further developments of MS in the field of saponins, it may be expected that LC/MS coupling will ease the tedious process of titration and analysis of saponins in mixtures. First results obtained with the Jeol FRIT-FAB system describe the analysis of saponins from *Panax* and *Bupleurum* (57).

^{13}C NMR AND STRUCTURES OF SUGAR CHAINS

The resolving power of ^{13}C NMR, which allows identification of individual resonances for each sugar carbon has rapidly contributed to the domain. The chemical shifts of carbon atoms in a chain of sugars may be recognized from the literature. The structures of the yunganosides, saponins isolated from a chinese liquorice and which possess the same chain as glycyrrhizin (rha-glcUA-glcUA), were, in this manner, determined by comparison of ^{13}C spectra (41).

^{13}C NMR is now used routinely to determine the substitution pattern of sugars in chains. Substitution of a sugar position by another sugar induces what is called glycosylation shifts (see table 3 for typical examples). The method seems perfectly reliable, its only limitations being the determination of ramification in chain and sequencing. In highly complex molecules however, it is worth wondering if in the absence of real correlations (*vide infra* for the use of 2D techniques), an assignment is unique or not. In this area of the field, it is expected that computers will help provide the operator with definitive or alternative assignments.

TABLE 3. Typical ^{13}C δ values for sugars (bold characters refer to substituted positions; starred values correspond to esters).

β-D-glucopyranose						ref.
C-1	C-2	C-3	C-4	C-5	C-6	
104.3	74.1	77.4	70.2	77.1	61.8	(60)
105.5	73.3	75.9	69.1	74.1	62	(60)
105.1	75.1	78.2	71.3	77.7	61.7	(58)
106.4	75.9	78	71.5	77.9	62.5	(29)
105.5	75.3	78.5	71.5	78.1	62.5	(62)
103.7	**79,8**	77.6	71.2	78.2	63.2	(59)
103.5	**83,5**	78	71.3	77.9	62.5	(29)
105.6	74	**84.8**	69.7	77.9	62.6	(62)
104.4	74.9	77.5	**78.4**	76.3	61.4	(64)
105.7	75.5	78.7	72.2	76.7	**69.7**	(21)
105.5	75.5	78.3	72.6	75.5	**68.8**	(59)
95.4*	73.5	78.3	70.1	76.6	**70.8**	(64)
105.8	75.5	75.5	72.2	76.7	**69.8**	(40)
105.1	**82**	76.2	**78.4**	77.4	63.8	(65)
105.1	**81.5**	**87.3**	**78.6**	77.6	62.8	(65)
β-D-galactopyranose						
106.4	74.5	74.9	70.1	77.1	62.1	(18)
103	72.5	74.3	69.2	75.4	60.7	(60)
106.3	74.4	74.9	69.8	77	61.7	(30)
101.7	**77.8**	73.6	71.1	76.9	61.6	(63)

β-D-glucuronic acid						ref.
C-1	C-2	C-3	C-4	C-5	C-6	
106.1	75.4	78.1	73.4	77.8	172.9	(16)
105.5	75.1	78.2	73.5	77.4	172.8	(16)
103.4	**83.5**	77.5	73	77.5	172.5	(18)
102.2	**81.3**	76.6	71.7	76.1	171	(60)
105.5	**78.7**	76.8	73.7	77.6	170.5	(63)
105.6	74.4	**85.1**	71.9	76.8	172.8	(16)
102.6	**77.6**	**85.2**	70.1	76	170.7	(60)
β-D-xylopyranose						
103.8	74.8	77.9	70.1	66		(60)
105.2	75.5	78.4	71.3	67.3		(18)
106.8	74.9	78.6	71.7	67.5		(18)
106.8	76	78.6	71	67.4		(58)
107.6	75.6	78.5	71.1	67.5		(43)
106	**79.5**	77.5	71.5	67		(43)
102.6	**79.4**	78.1	70.8	66.8		(63)
106.4	75.2	**83**	69.2	67.2		(62)
107	75.8	**82.9**	69.7	67.3		(43)
105.3	74.5	**85.8**	67.8	65.7		(60)
106.2	75	73.8	**76.7**	64.3		(43)
106.6	76	76.4	**76.5**	64.5		(40)
α-L-arabinopyranose						
105.1	72.6	74.4	69.2	66.5		(21)
105.6	71.9	73.4	68.3	65.9		(60)
106.5	73.8	74.3	69.2	67.2		(29)
101.3	**79.6**	72.1	66.9	63.3		(59)
94.3*	**74.9**	71.4	67.6	64.9		(29)
93.3*	**76.1**	70	66	62.9		(62)
93.8*	**75.7**	69.6	66	63.1		(18)
93.4*	**75.6**	70	65.9	62.8		(58)
105.6	72	73.1	**77.4**	65.4		(60)
104.1	**79.6**	73.2	**75.8**	64.3		(17)
α-L-rhamnopyranose						
101.6	72.3	72.6	73.8	70.6	18.7	(18)
101.9	72.3	72.5	74	69.9	18.6	(61)
102.3	72.4	72.1	73.5	69.4	18.1	(64)
101.5	71.8	**82.7**	72.9	69.6	18.5	(43)
101.5	71.7	**80.3**	73.6	71.7	18.5	(58)
101.5	71.5	**83.5**	72.7	70.1	18.4	(30)
101.2	72.1	72.6	**83.4**	68.3	18.6	(21)
101	72.7	72	**83.5**	68.6	18.5	(18)
99.9	70.8	71.2	**83**	67.1	17.3	(60)
102.2	72.6	72	**84.2**	67.9	18.5	(43)
101	71.6	**82.3**	**77.9**	71.2	18.5	(58)
101.1	71.7	**82.8**	**79**	68.9	18.8	(18)
100.8	69.8	**81.3**	**77.7**	67.8	17.7	(60)
β-D-fucopyranose						
93.7*	**73.1**	75.3	72	71.6	15.6	(60)
95*	**73.5**	76.9	73.4	72.7	17	(21)
95*	**72.5**	**85.6**	72.5	72.2	17	(40)
β-D-apiofuranose						
111.7	77.5	79.5	74.5	62.2		(58)
109.1	77.7	80.4	75.4	65.3		(21)
111.2	77.6	80.3	74.9	64.8		(62)
β-D-mannopyranose						
102.3	71.6	75.4	68.4	78.7	62.3	(16)

^{13}C RELAXATION TIMES AND SEQUENCING

It is well known that long relaxation times T_1 correspond to fast correlation times and this fact has been used to determine the position of a carbon in a chain. The more mobile sugars (end of chains) are therefore expected to have longer T_1 than those whose motions are restricted. This was first demonstrated with k-strophantoside (66) but only very few examples of sequence determination with this technique are found in the literature (67).

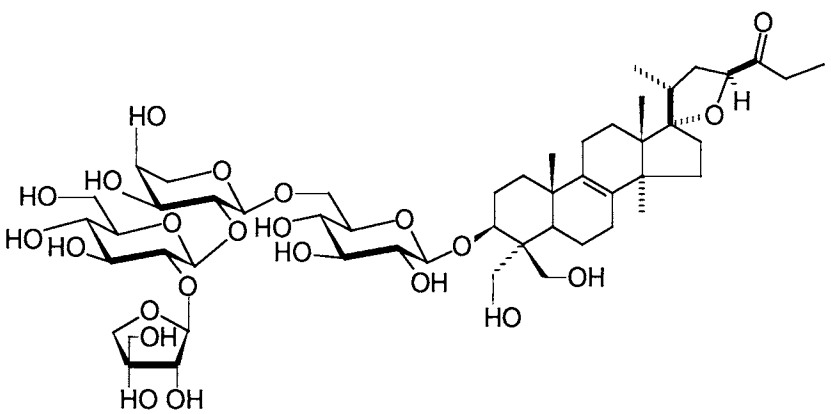
CONTRIBUTIONS OF ^1H NMR TO SAPONIN STRUCTURAL ELUCIDATION

An ^1H NMR spectrum of a saponin is characterized by a 3 to 5 ppm highly entangled area where most of the sugar proton resonances overlap. Until recently, these spectra were used to identify the aglycone by its methyl resonances (in analogy with the Zurcher steroid increments) or olefinic protons. The advent of high magnetic field and of 2D NMR has recently allowed the exploration of the sugar proton resonances, which had been hiding a wealth of information on sugar nature, configuration and linkage.

Basically, there are two approaches for the use of ^1H NMR spectroscopy of saponins which consist either in working on the underivatized (in deuterated MeOH, pyridine or DMSO) or on derivatized material (peracetates). Each of the methods has its advantage, and inconveniences, which will be discussed below.

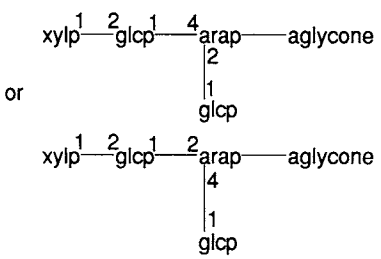
The first article in the domain was authored by Breitmaier in 1984 (59) and described a full strategy for the structural elucidation of saponins using 2D NMR techniques. The saponin chosen as an example was muscaroside C which contains glucose, arabinose and apiose in a 2:1:1 ratio (HPLC titration). The ^1H NMR spectrum was determined in pyridine d_5 at 500 MHz and assigned by means of a COSY-45. Despite the high field, only one of the glucose proton system could be directly assigned with COSY; the second glucose had H-2, H-3 and H-4 (δ 4.16, 4.14, 4.22) too close to give clear off diagonal signals and arabinose had H-2, H-3 and H-4 strongly coupled (δ 4.52, 4.55, 4.54). In fact, the COSY spectrum was mostly used as an aid to assign the ^{13}C spectrum by means of one-bond heteronuclear C-H correlations. Combination of ^1H-^1H and ^1H-^{13}C correlation provided a set of assignments for the ^{13}C resonances, which were then compared to literature reference resonances (methyl-β-D-glucopyranoside, methyl-α-L-arabinopyranoside and methyl-β-D-apiofuranoside). Deshielding of C-6 of one glucose (+ 6.3 ppm), of C-2 of the other glucose (+ 5 ppm) and

of C-2 of arabinose (+ 7.4 ppm) could tell that these positions were substituted but this was not sufficient to allow full sequencing of the saponin. Sequence was determinated by negative FAB MS which showed peaks at (M-H-pentose)$^-$, (M-H-pentose-hexose)$^-$, (M-H-pentose-hexose-pentose)$^-$ and (M-H-pentose-hexose-pentose-hexose)$^-$ at m/z 929, 767, 635, and 473. Apiose was determined to be the terminal sugar of the chain by its ^{13}C chemical shifts. The glucose substituted at position C-6 showed an nOe effect between its anomeric proton and H-3 of the aglycone. This allowed the structural determination of muscaroside C as :

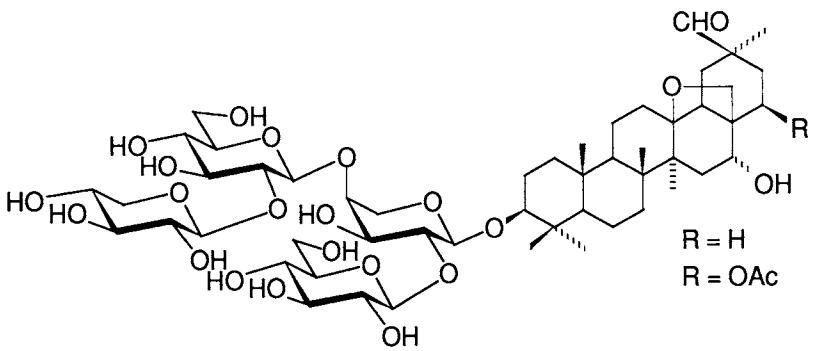


Muscaroside C

In 1986, three articles were published which presented important improvements of methodologies; two concerned the NMR of peracetates (68, 69) and one the NMR of a saponin in DMSO (17). The latter article dealt with the structures of saxifragifolins A and B from *Androsace saxifragifolia*. Combination of positive and negative FAB MS and of chemical degradation left two possibilities for the sugar chain of saxifragifolins A and B. They differ in the substitutions of an inner arabinose :



The point was settled by taking advantage of the special feature of NMR spectra run in DMSO that, as chemical exchanges are frozen, show individual lines for OH. OH protons are identified at low field and can be exchanged with D_2O; they show three bond couplings with CH or CH_2. The absence of couplings of CHO with OH means that the oxygen atom is not substituted by an hydrogen atom and COSY experiment verifies this. It was thus found that arabinose was linked through its 2- and 4-positions, that a glucopyranose was linked in turn through its 2- position and that xylopyranose and glucopyranose were terminal. A NOESY experiment, which showed transfers of magnetization from terminal glucose H-1 and arabinose H-2 and from the other glucose H-1 and arabinose H-4 allowed final proposals for the structure of saxifragifolins :



Saxifragifolins

The articles referenced (68) and (69) describe the advantages of running ^1H NMR spectra of peracetates :

- molecules are soluble in $CDCl_3$ and give spectra with better resolution
- sugar proton resonances are spread over 2.5 ppm
- protons α to acetates have chemical shifts larger than 4.5 ppm (except CH_2OAc which resonate between 4 and 4.5 ppm)
- protons α to ether linkages are shielded $\delta < 4$ ppm
- anomeric protons appear between 4.2 and 4.7 ppm except for those linked to acid functions.

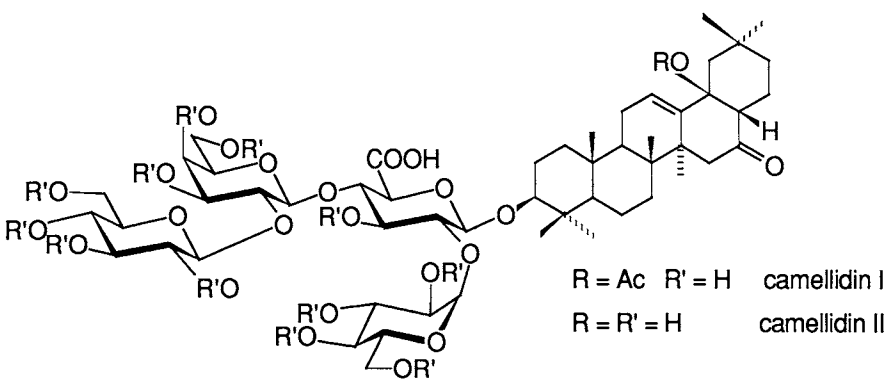
In general, assignments of ^1H NMR spectra of peracetates are easily performed with a COSY experiment; an example of such an analysis will be outlined below. Branching points, which correspond to the most shielded protons and interglycosidic

linkages are determined in two fashions :

- long range couplings
- Overhauser effects.

When the geometry is favourable (W or sickle paths), there exist ^4J between anomeric protons and protons situated on the other side of the glycosidic bond (69). These couplings do not split resonances because they are smaller than the proton natural lines ($J < 1$ Hz) but they are detectable in delayed COSY. In the delayed COSY experiments, a delay is introduced before and after the "read" pulse. To observe these long range effects, the delays must be of the order of 300 to 400 ms. Delayed COSY are complicated by a number of long range couplings (70) but it is unnecessary to examine the whole map to determine the sugar sequence : the information stands at the cross points of the rows corresponding to the anomeric protons and of the columns corresponding to the protons α to linkages (or vice-versa). With this technique, we were able to solve the structure of a four sugar saponin from *Medicago sativa* (69).

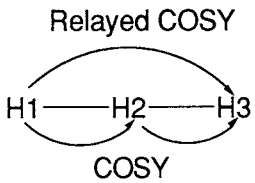
The second article on peracetyled saponins (68) describes the complete analysis of the ^1H NMR spectrum of a four sugar saponin using a COSY experiment. Little use was made in this article of ^1H chemical shifts to determine substitutions (except for H-2 of a galactose) which were established instead according to ^{13}C glycosylation shifts. Nuclear Overhauser effects were detected between H-3 of the aglycone and H-1 of the uronic acid, between H-4 of the uronic acid and H-1 of the 2- substituted galactose and between the anomeric proton of glucose and H-2 of this galactose. No nOe could substantiate the linking of the terminal galactose to C-2 of the uronic acid. The chemical shift of the anomeric proton of this galactose ($\delta = 5.49$ ppm) rather makes us think of an ester linked sugar (to the 6- position of galacturonic acid ?). In this case position 2 would have escaped acetylation for steric hindrance or for any other reason.



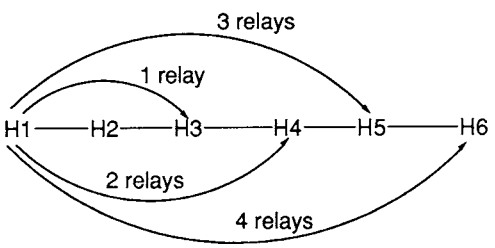
STRATEGY FOR ^{1}H NMR ASSIGNMENT OF SAPONINS. RELAYED COSY AND HOHAHA

The anomeric protons are most often doublets (or broad singlets for rhamnoses) readily detectable in the spectra of either native saponins (in CD_3OD, C_5D_5N or C_2D_6SO) or of peracetates. Starting from these protons, the H-2s are located by means of a COSY experiment, and so on (H-3, H-4..) until the terminal positions of the sugar. Unless the spin systems are especially well behaved, it is not possible to obtain an unambiguous assignment with this sole technique owing to crowding in the vicinity of the diagonal.

To circumvent the difficulty, one uses the relayed COSY experiment in which the coupling from H-2 to H-3 rebounds on H-1 through the H-1 to H-2 J relay. In this case there is a "relayed correlation" between H-1 and H-3 in the absence of genuine coupling between these protons.



The experiment may be extended to two, three, and more relays and if there is a continuous path of couplings, one can read H-4, H-5 and H-6 from H-1 in a single experiment. These experiments must be performed in sequence (in order of increasing complexity of maps). One must remember however that each of the relays brings an attenuation of the signal and more scans must be recorded for multi-relayed experiments.



To alleviate sentivity problems, Homo Hartmann-Hahn spectroscopy (HOHAHA or TOCSY, Total Correlation Spectroscopy) may be used to obtain correlations for all the protons of an isolated spin network (71). Glycosides are ideal systems for this experiment since they represent 5, 6 or 7 closed spin systems. This experiment was used *inter alia* in the structural elucidation of *Gypsophila* saponins (60). A variant of this experiment, which will surely become popular once NMR users are able to master it, is 1D-HOHAHA (71, 72) . Based on the spinlock principle and difference spectroscopy techniques, 1D-HOHAHA yields a subspectrum of complex spectra each time a proton may be selectively excited (shaped pulses or DANTE). The longer the spin-lock (usually up to 400 ms), the larger the spin system which is explored. Reference (58) presents 1D-HOHAHA spectra showing H-1, H-2 and H-3 (length of the spinlock is not precise). The 1D-HOHAHA signal may be utilised in other experiments such as 1D-HOHAHA-COSY in order to give selective COSY of selected sugars (73). Reference 74 presents impressive results based on selective experiments but with no experimental details unfortunately.

THE OVERHAUSER EFFECTS IN SAPONINS . SPECIFIC PROBLEMS

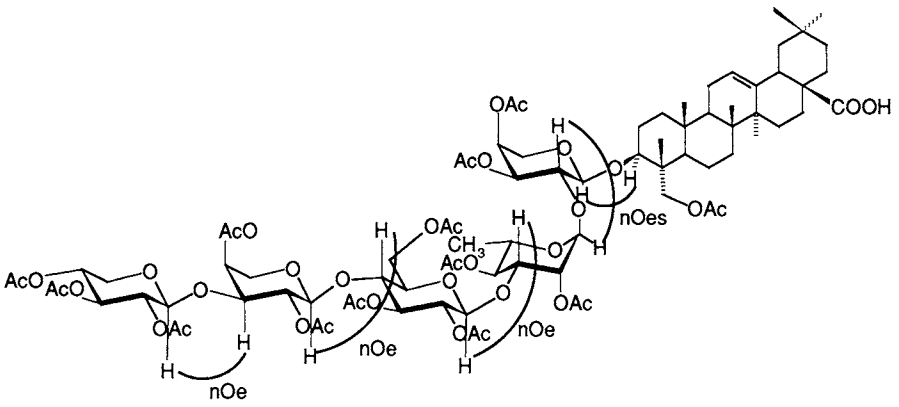
It is well known that nuclear Overhauser effects arise from dipole-dipole relaxation mechanisms depending on r^{-6} relationship (75).

There are three ways of observing nOes:

- saturate a frequency while measuring the integral of the spectrum. Changes are related to nOes.
- perform a subtraction between spectra or FIDs with the decoupler alternatively on and off (NOEDS)
- perform a 2D experiment called NOESY.

The first two means of observations are clean and devoid of artefacts (with the exception of Bloch-Seegert effects which are less important at higher magnetic fields). The third technique is sometimes more difficult to interpret, given that strong and, sharp lines often give rise to off-diagonal peaks at the point of encounter of their "ridges": weak nOe cross peaks are not easily distinguished from noise. As an example, reference 76 shows a series of COSY, relayed COSY and NOESY on a five sugar

saponin from *Blighia welwitschii.*



NOe and rOe correlations used for sequencing of a saponin from *Blighia welwitschii.*

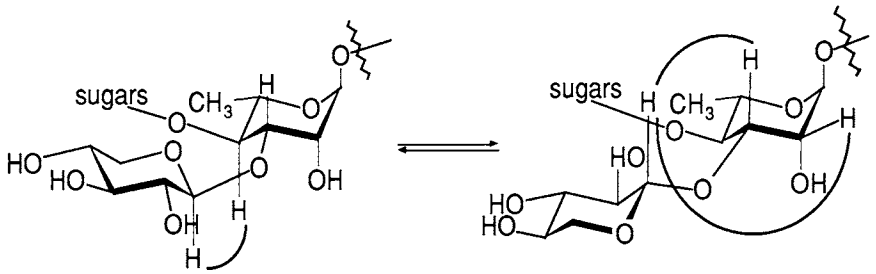
It is not so well known that nOes also depend on molecular mobility (correlation times τ_c) and observation frequency (ω) in a $1\text{-}\omega\tau_c$ fashion. This factor is almost null when one observes molecules of *ca* 1000 Daltons at 400 MHz. Saponins are thus not ideal molecules for the observation of strong nOes. NOes may be negative even though in some articles the nO**e** stand for **e**nhancement (29, 30)! The first negative nOes of saponins were recognized as such in a 1989 article by I. Kitagawa et al.(61).

The best way to avoid the $1\text{-}\omega\tau_c$ problem (and weak nOes) is to use a rotating frame experiment, for instance ROESY (Rotating frame Overhauser Effect Spectroscopy) also named CAMELSPIN by its inventors (77). The $\omega\tau_c$ dependence of rOes is complex and one may simply remember that rOes are always positive, never null. The ROESY sequence is similar to the sequence of HOHAHA; the main difference is the power of the spinlock which is generated by a long soft pulse rather than by a WALTZ sequence. In ROESY experiments, rOe cross peaks may be accompanied by Hartmann-Hahn correlations which are easily distinguished by their opposite sign (in phased experiments) (78).

There is no doubt that the ROESY experiment will become the best experiment to sequence chains of sugars by ^{1}H NMR. Many examples of this experiment are published in the recent literature, either 1D (58, 79) or 2D (62, 73, 76, 77, 80), even though reference 77 describes a ROESY on a triterpene !

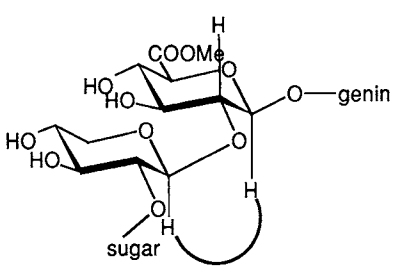
OVERHAUSER EFFECTS - SUGAR SEQUENCING AND CONFORMATIONS

When an Overhauser effect is detected between an anomeric proton and the proton of another sugar, a partial sequence is immediately deduced. With the spectra at hand, one sees that things are less simple and that generally two or three effects appear instead of a single one. For example in the xylose-arabinose part of acutoside H (79), there are ROEs between xylose H-1 and rhamnose H-2 , -3 and -4. It is possible to admit, in accordance with the authors, that one of the effects is larger than the other ones but this is not as obvious as stated. Molecular models of rhamnose show that H-2 and H-3 are on the β side of the mean plane while H-4 is on the α face. Why does a proton displays proximity effects with partners so far apart ? There is no answer with a single model and one has to admit that there exists at least two conformations around the glycosidic bonds (not to speak of freely rotating systems).



Rotating frame Overhauser Effects between H-1 of xylose and H-2, 3 and 4 of the neighbouring rhamnose ! (79)

Interestingly, these multiple nOes systems are only met with underivatized saponins. Peracetylated material where steric hindrance is more important do not show these phenomenoms. To further comment on nOes, there is at least one example of a molecule where the expected immediate effect (xyl H-1---> gluc H-2) does not appear and where instead one sees an effect with the second neighbour (xyl H-1---> gluc H-1).



Observation of a nOe between anomeric protons in wistariasaponin B1 (63)

SUGAR ANOMERIC CONFIGURATION

Sugar anomeric configurations were formerly derived from chiroptical properties such as the Klyne-Hudson rule. They are now determined by ^{1}H or ^{13}C NMR spectroscopy.

When pyranoses bear a substituent at position 5 (methyl or hydroxymethyl), they are locked in a single conformation with the C-5 substituent equatorial and the anomeric configuration can be determined from H1-H2 coupling constants. The pentoses (arabinose and xylose), which do not bear a C-5 substituent, do not behave so well and they may exist as interconvertible chairs in the $^{1}C_4$ and $^{4}C_1$ conformation depending on the substitution.

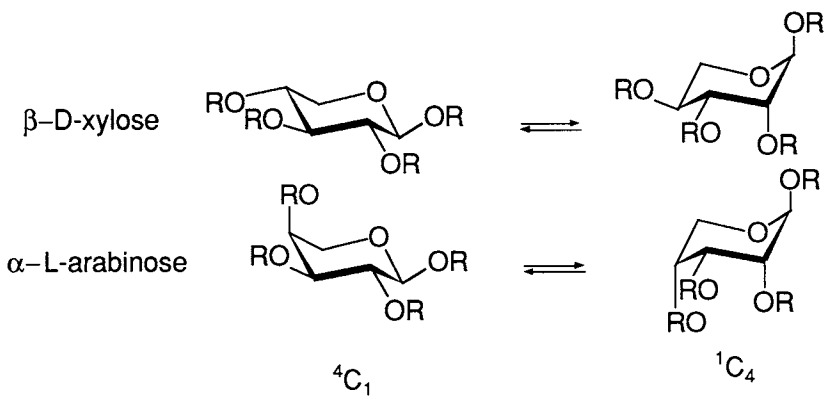


Table 4 lists some values found in the literature for J_{H1-H2}. These ^{3}J are Karplus dependent and they also vary according to the electronegative character of the oxygen substituents.

TABLE 4 . A selection of J_{H1-H2} found in recent literature. Variability may arise from $^{1}C_4$, $^{4}C_1$ equilibrium or strong couplings.

β-D-glucopyranose			
J	ref.	J	ref.
5.3	(19)	7.5	(29-32)
5.4	(19)	7.7	(17-31-32)
6	(19)	7.8	(17-19-21-32-37-59)
7	(12-19-29-43)	8	(18-28-29-30-36-37-60-31-83)
7.1	(65)	8.1	(65)
7.3	(19-59)	8.3	(15)

α-L-arabinopyranose

< 1	(29)
2.3	(81)
2.8	(81)
2.9	(81)
3	(18-29-30-79-81-83-85)
3.3	(81)
4	(29-30-85)
4.1	(59)
4.5	(81)
4.9	(81)
5	(84)
5.2	(81)
5.5	(17)
5.8	(81)
6	(25-30-85)
6.2	(81)
6.5	(81)
6.8	(37-81)
6.9	(31)
7	(28-79)
7.5	(28)
8	(28-30-36)
8.5	(36)

β-D-galactopyranose

7	(18-30)
7.5	(30)
8	(30-60-83)

β-D-glucuronic acid

7	(34)
7.5	(18)
8	(35-60)
8.5	(36)

β-D-xylopyranose

6	(13-34-61)
7	(13-18-21-35-37-85)
7.5	(43)
7.6	(17-31)
7.8	(21-37)
8	(12-60-62)
8.3	(15)
8.5	(36)

β-D-fucopyranose

7	(12-21-37-60)
8	(12)

α-L-rhamnopyranose

< 1	(13-18-28-43-85)
1	(12-35-60-61-79-85)
1.5	(21-37)
2	(12-60-79-85)

β-D-quinovose

3	(13)

β-D-apiofuranose

2	(59)
3	(21-37)
4	(60)

Before translating ^3J into configurations, one must make sure that what is measured is really coupling and not splitting. A β-D-glucose with $^3J_{H1\text{-}H2}$ = 5.4 Hz has been measured under conditions where δ_{H2} = 4.15 ppm and δ_{H3} = 4.12 ppm (at 400 MHz) (19); this is a case of second order effects even though in ABX system the apparent splitting of the X part equals the sum of $J_{AX} + J_{BX}$.

When the C(2)-O bond is axial (rhamnose and mannose for instance), it is not easy to distinguish $^3J_{H1ax\text{-}H2}$ from $^3J_{H1eq\text{-}H2}$ because the typical values of these couplings are similar and because of the antiparallelism of the C-O and C-H bonds in the α-L isomer decreases $J_{ax\text{-}eq}$ (Booth rule). As a consequence, L-rhamnose is char-

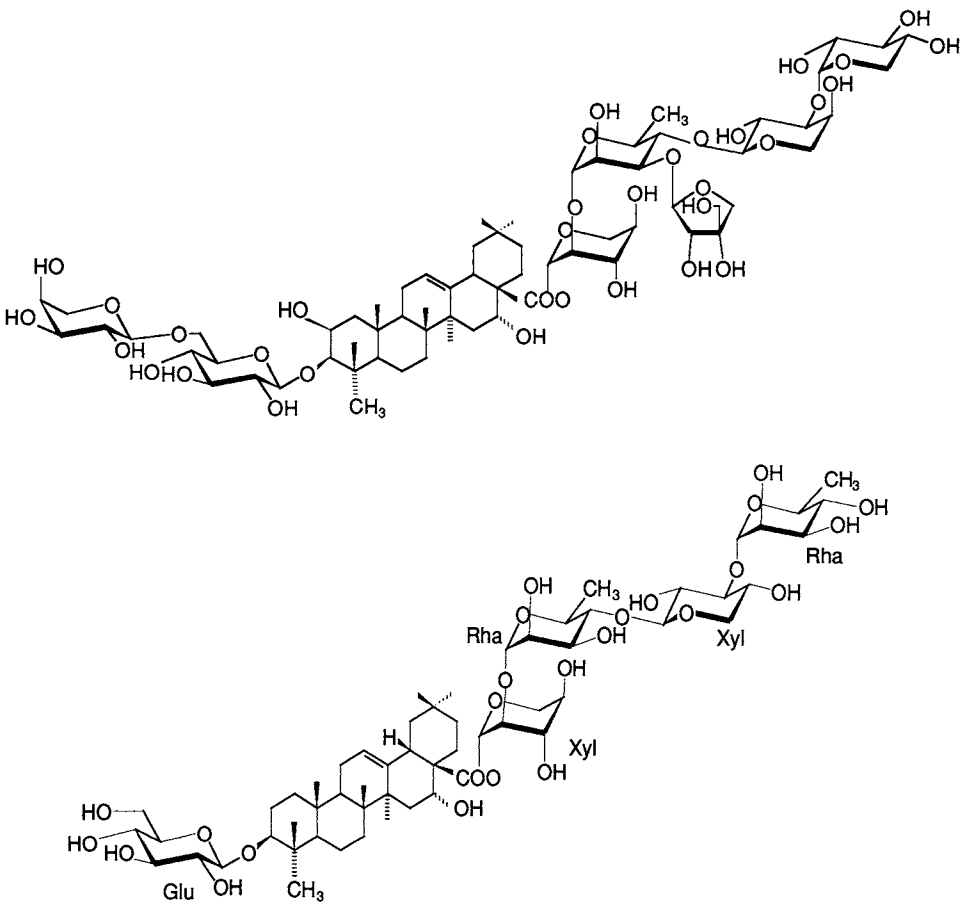
acterized as α with signal shapes ranging from singlets to doublets with J = 1.7 Hz (70). In those cases, one relies instead on ^{13}C chemical shifts and $^{1}J_{C-H}$ (76).

Xylose and arabinose must be analyzed more carefully. In principle, xylose should exist in the $^{4}C_{1}$ chair with all the substituents equatorial but there exist examples where the anomeric effect forces them all in axial positions. Arabinose should always be discussed in terms of chairs equilibrium and the first thorough examination of arabinose in saponins is due to Tori et al. (81). δ_C values range from 90.6 to 95.8 ppm, in esters J_{H1-H2} from 2.3 Hz to 5 Hz and $^{1}J_{CH}$ from 165 to 177 Hz. Of course these values depend on temperature and solvent.

TABLE 5. Typical $^{1}J_{C-H}$ values for some sugars in saponins.

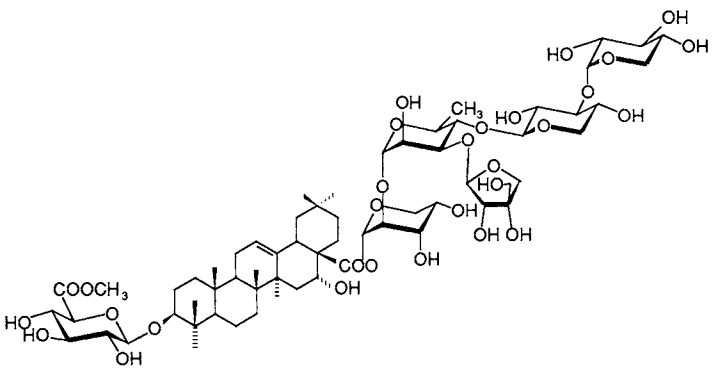
β-D-glucopyranose		α-L-arabinopyranose	
157.5	(19)	162	(30)
159.4	(19)	169	(30-81-85)
161.8	(19)	173	(81-83)
β-D-xylopyranose		**α-L-rhamnopyranose**	
161	(15)	168	(21-82)
170	(82)	172	(13)
		174.9	(19)

The interested reader is referred to a series of recent articles of Okabe *et al.* (82-84) where the configurations of xylose and arabinose are examined in detail. In the xylose ester of *Aster* saponin A (82) J_{H1-H2} is measured equal to 4 Hz and $^{1}J_{C1-H1}$ = 170 Hz suggesting a $^{4}C_{1}$ conformation with α-configuration. These values may also fit a β-linkage in the $^{1}C_{4}$ conformer. The only way to decide which configuration is correct, is to analyze the full spin system; this is done by 1D-HOHAHA. The vicinal coupling constants are found equal to 2 Hz (H2-H3), 5 Hz (H3-H4) and 5 and 4 Hz (H4-H5). These values support the β-configuration in the $^{1}C_{4}$ conformation (with probably a slight contribution of the $^{4}C_{1}$ conformation to account for the somehow large J values). The same analysis and deduction were done on xylose in chrysanthellin (73) and the spin-spin analysis was supported by 1D-HOHAHA and ROESY.

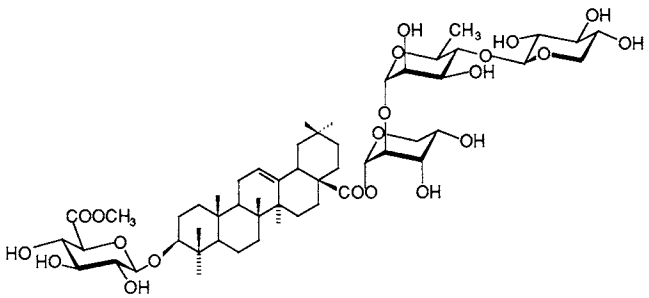


β-D-Xyloses in $^1C_4$ conformation in astersaponin A and chrysanthellin B.

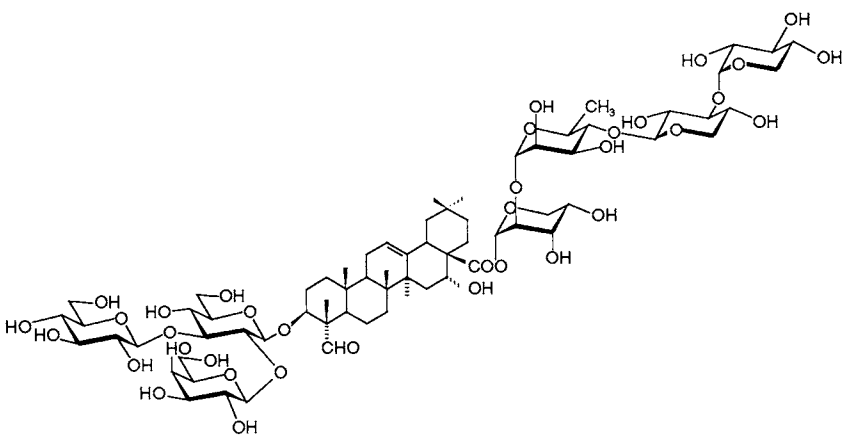
Aster saponin H (84), the scaberosides (85) and the dubiosides (83) are examples of arabinoside esters with L-arabinose in a α-configuration and $^1C_4$ conformation. It is worth noting that in all of these examples, arabinose and xylose are linked to hindered C-28 acids and that they are substituted by one or several sugars at C-2. The steric congestion introduced by the equatorial chains at positions -1 and -2 of the sugar are relieved by a full inversion of the chair and a predominant all *trans* configuration.



Aster saponin H_d



scaberoside B_5



dubioside F

α-L-Arabinoses in $^1C_4$ conformations

CONTRIBUTIONS FROM C-H 2D NMR EXPERIMENTS

The domain is concerned with mainly two varieties of CH 2D experiments : the one-bond correlation and the long range (two- or three-bond) correlation. These experiments were formerly run in the so-called normal mode (^{13}C acquisition with ^{1}H modulation) and were named CH COSY (XH CORR or HETCOR) and COLOC (long range). These acronyms are now being replaced with HMQC (Heteronuclear Multiple Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation) and they are run in the reverse mode (^{1}H acquisition with ^{13}C modulation). The differences between normal and reverse experiments are sensitivity (a factor of 5) and resolution (in the dimension of acquisition).

HMQC (and CH COSY) have been used to assign ^{1}H and ^{13}C spectra or at least to assign one spectrum when the other is known. Two types of determinations have been made with these experiments : the count and assignment of ^{1}H and ^{13}C anomeric signals and therefore the count of the number of sugars. This contribution may seem trivial but in some cases overlap in ^{1}H or ^{13}C NMR spectra makes the determination hazardous. In one of the first examples of the structural determination of a saponin with 2D NMR, CH COSY was used to assign sugar carbon resonances and hence, to deduce branching points with use of glycosylation shifts (59).

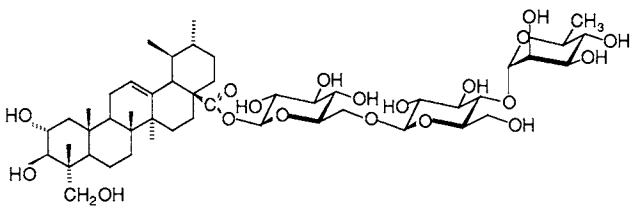
The main use of HMBC (^{2}J and ^{3}J CH) is in the determination of glycoside linkages and it is one of the rare experiments which allow passing through osidic bonds. For ether bound sugars, there are two CH correlations going from one sugar to the other; both are Karplus dependent and usually one of them at least, yields a correlation. It must not be forgotten that in the tuning of HMBC (and HMQC) experiments, there is one delay which must be introduced in the pulse sequence and which is J dependant ($0.25\ J^{-1}$ or $0.5\ J^{-1}$). The adjustment of this delay is crucial for some correlations, such as the COOCH (values must be adjusted stepwise from 70 to 200 ms).

When the assignment of a CH direct correlation is ambiguous owing to proton overlap, the correlation may be relayed to another proton which couples to the first. Reference 60 reports the use of such relayed CH experiments in the assignment of the spectra of 7 sugar saponins.

Modern variants of these experiments include CH - HOHAHA where the information is relayed to the entire proton system via a spin lock (86) and which constitutes a multiple check of ^{13}C and ^{1}H resonances. The resolution problem in 2D NMR may be alleviated through phased experiments - this must always be considered before running them.

CONTRIBUTIONS FROM X-RAY CRYSTALLOGRAPHY AND MOLECULAR MODELING

Saponins are usually isolated as white solids with high melting points but it is uncommon to obtain high quality monocrystals suitable for X - ray analysis. To the best of our knowledge, there is only one three sugar saponin whose structure has been determined by crystallography : asiaticoside (64) and a mono glycoside (87). It would be interesting to obtain more saponin atom coordinates to verify if solid state conformation of chains of sugars are similar to the ones deduced from nOe data.



Asiaticoside

Molecular modeling is another means of determination of the φ and ϕ angles linking two sugars. Some efforts are being made in view of obtaining data on sugar chain conformation and the method is starting to be applied to the saponin field (64, 88). This work is related to the important field of molecular recognition.

THE PROBLEM OF ESTER LOCALIZATION

Amongst saponins, those with ester functions are the source of redoubtable structural problems. They can be solved chemically, i.e. by derivatization followed by hydrolysis and identification of the fragments. Reduction reactions may be used to complement hydrolysis reactions in the fragmentation of saponins. A reagent of choice is $LiAlH_4$ which reduces esters into diols; prior protection of alcohol functions as ethers is necessary to avoid solubility problems (5). The chemoselective reduction of acids in the presence of esters by diborane is an excellent method of distinguishing these two functions in multifunctional compounds (55).

The effects of esterification on chemical shifts are well known and have been used to localize esters. It is worth noting, given the fact that α effects (+ 2 ppm) and β effects (- 2 ppm) have a small amplitude in a congested area of the spectra, that early examples of ester sequencing by NMR relied on ^{13}C chemical shifts. Although popular, the method is relatively inaccurate as witnessed by references 24 and 25 in which acylations of position C-6 of glucose induce α and β shifts equal to 1.9 and 3.1 ppm

(24), 3.3 and 2.3 ppm (25). ^{1}H acylation shifts on the other hand have a magnitude in the 1 ppm range which displaces protons in relatively unencombered region of the spectra (4.5 - 5 ppm). Pattern recognition suffices then to determine points of acylation of a sugar residue (21).

Complications arise when two esters of different acids are present or when acylation occurs on the aglycone. One must then rely on partial hydrolysis. The tactics are examplified by the *Entada* saponins which contain C_2 and C_{10} acids (89). The acetate was selectively removed by 0.025 % K_2CO_3 while both acids were removed by 1 % KOH. Comparison of ^{13}C NMR spectra of the parent compound and of derivatives allowed determination of the points of acylation. The dicrotalic (3-hydroxy 3-methyl glutaric acid) esters of the tubeistemosides and related compounds (29-32) provide more complicated examples where the double anchoring of the diacid transforms a prochiral carbon atom into a center of chirality.

The only general solution to the problem of ester localisation is the recently introduced 2D NMR experiment HMBC. The nature of the CO_2 groups prevents observation of interproton couplings beyond the C - O bond. The problem is made even more difficult by the fact that acid functions on triterpenes are planted on quaternary carbon atoms. Three bond C - H couplings can be observed through ester bonds and this is becoming the technique of choice to determine esters (60, 80, 90).

CONCLUSION

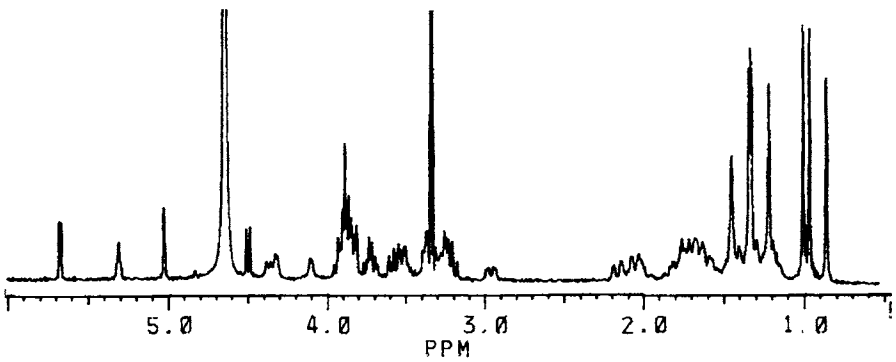
The recent advances in MS and NMR instrumentation provide chemists with tools which enable them to rapidly solve the structure of complex saponins. This will hopefully open new explorations into the vast structure-activity relationship domain. To those who would be tempted to use their instrumental skills to illuminate the field, it is reminded, as a last remark, that the separation of saponins remains a major and primordial task which brings little glory to the actors.

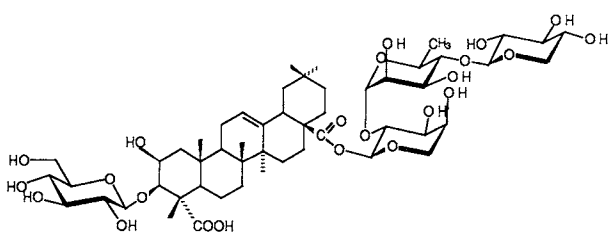
APPENDIX

In order to illustrate some of the points raised above, the following pages display typical examples of some important NMR experiments presently in use to solve saponin structures. They all concern a saponin from *Medicago sativa*, isolation of which is reported in reference 55.

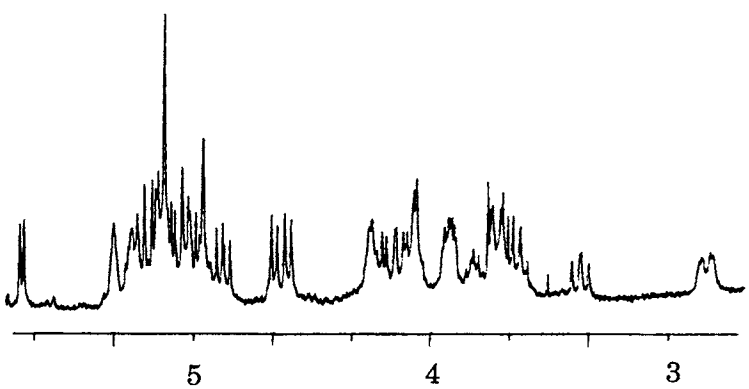
1D ^{1}H and ^{13}C NMR

A. ^{1}H NMR of a non derivatized saponin in CD_3OD (recorded at 300 MHz). At high field, one distinguishes the methyl resonances. The large peak at 4.7 ppm is OH (from solvent and saponin). The 5.3 ppm signal is from the triterpene double bond; other signals in this area are anomeric protons from arabinose (5.7), rhamnose (5), xylose (4.5) and glucose (4.4 ppm).

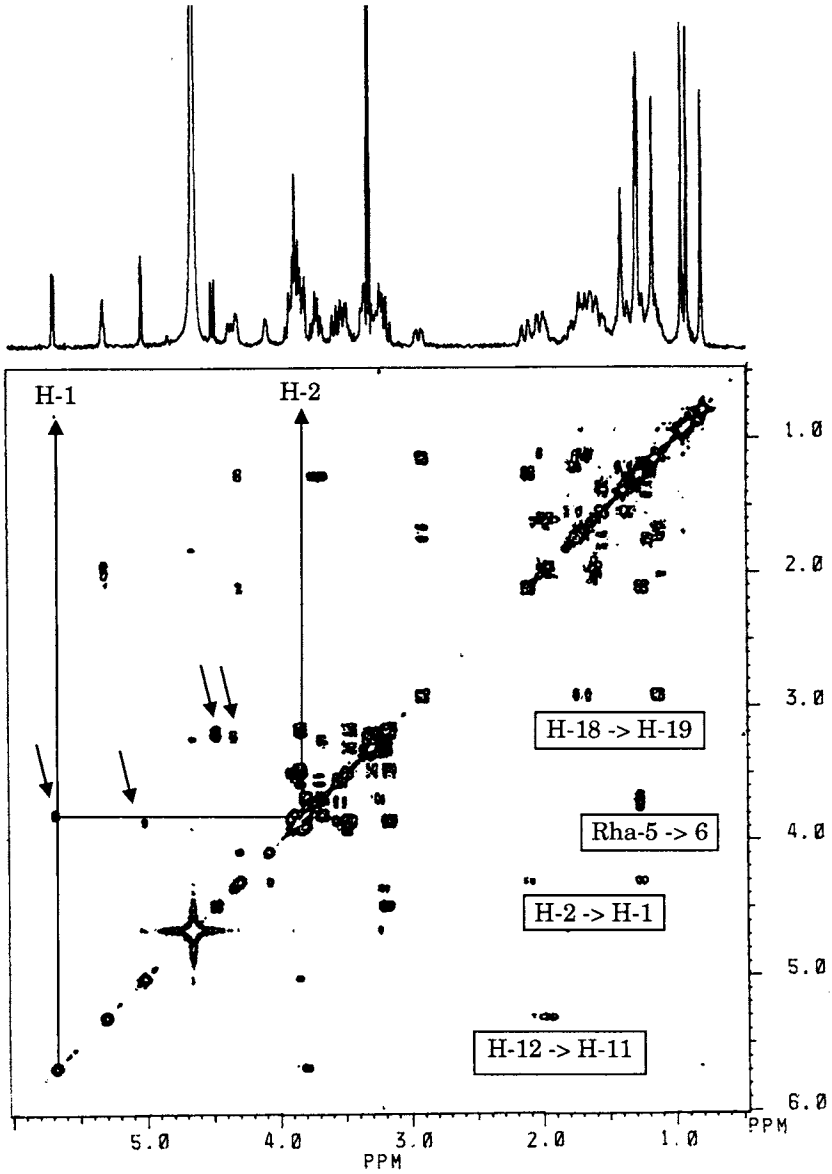




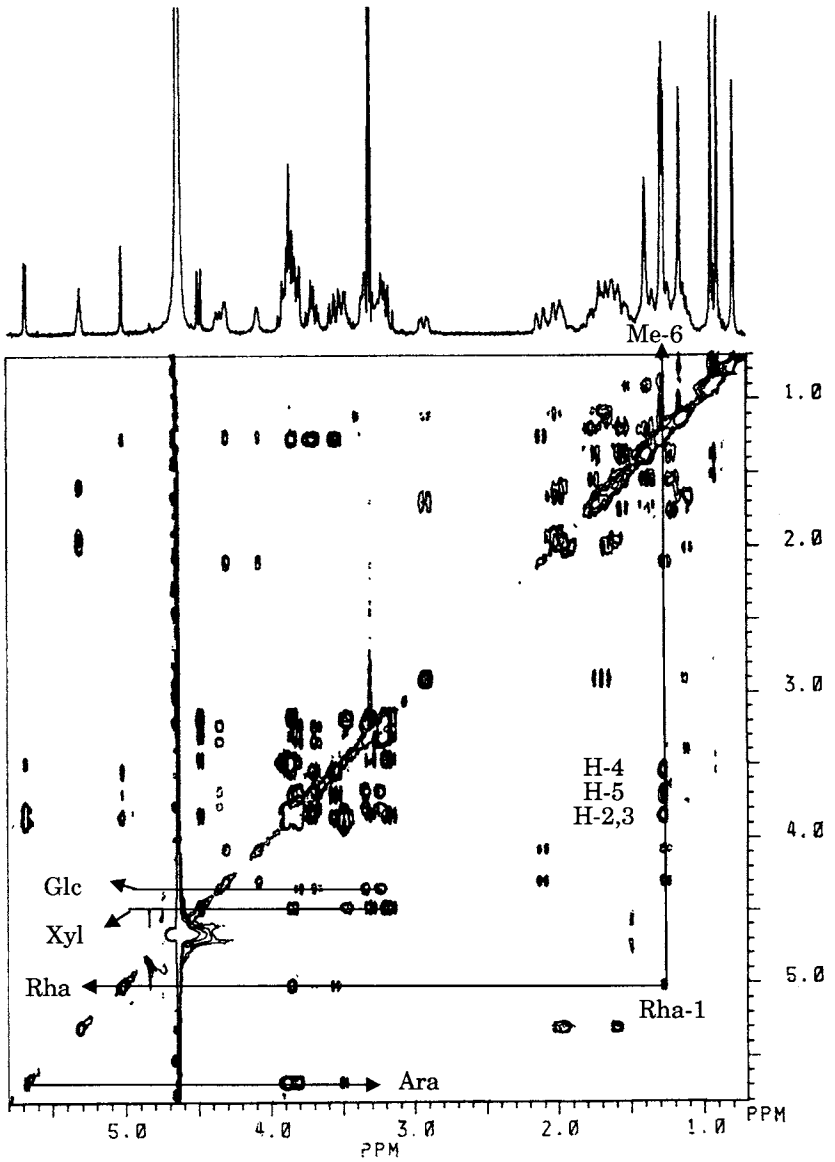
B. The ^{1}H NMR of the same derivatized saponin in $CDCl_3$. The part of the spectrum corresponding to the sugar resonances is only shown. The rhamnose H-1 resonance is now buried among other resonances (5ppm) but it can be detected with a COSY experiment.



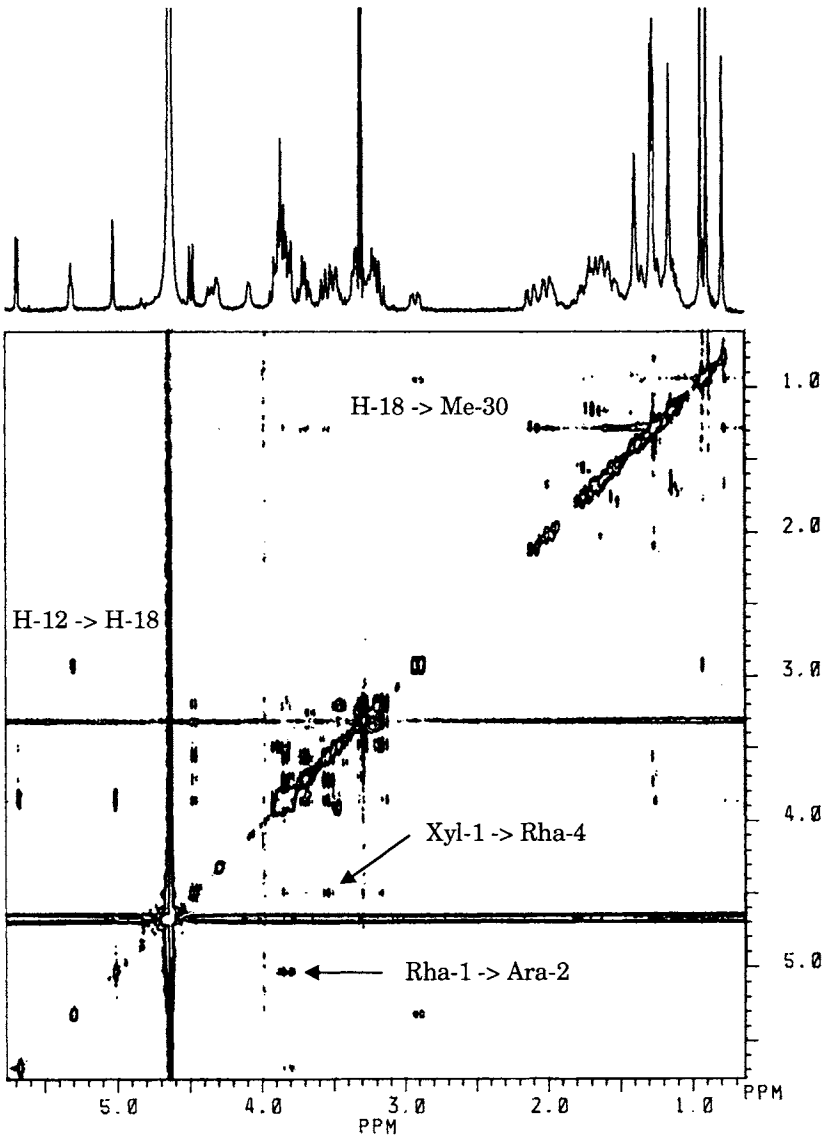
C. COSY-45 experiment performed on the underivatized saponin (256 experiments of 1K). Arrows point H-1 to H-2 correlations in sugars. Note that the experiment is symmetrized (same information on each side of the diagonal).



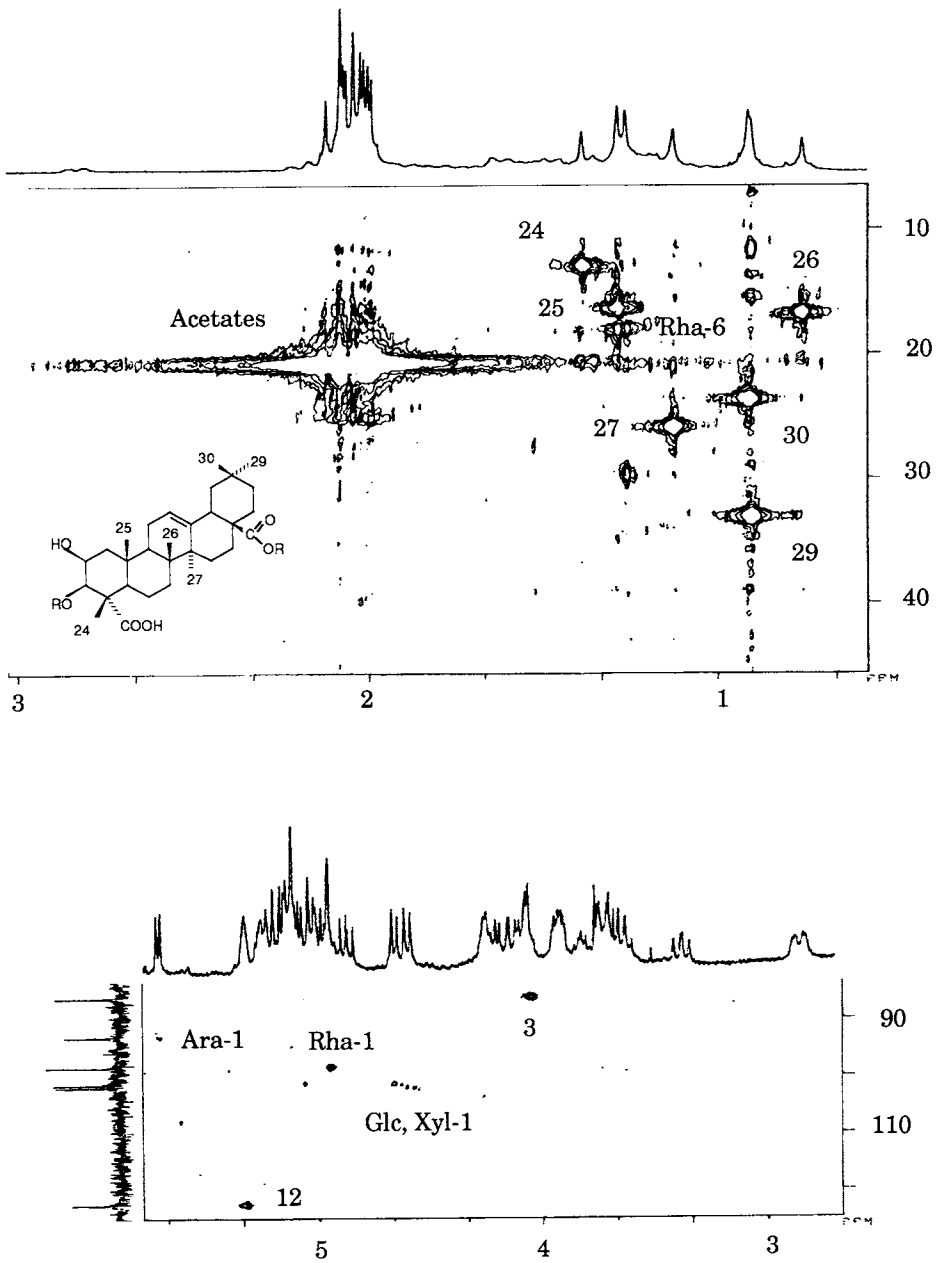
D. HOHAHA experiment performed on the underivatized saponin (256 experiments of 2K, spin lock : 250 ms MLEV16 sequence; reverse mode; experiment is phased). Horizontal lines cross the correlation peaks of each sugar. On the column corresponding to rhamnose H-6 are indicated the correlations observed between Me-6, H-1, 2 and 3 (superimposed), H-4 and H-5.



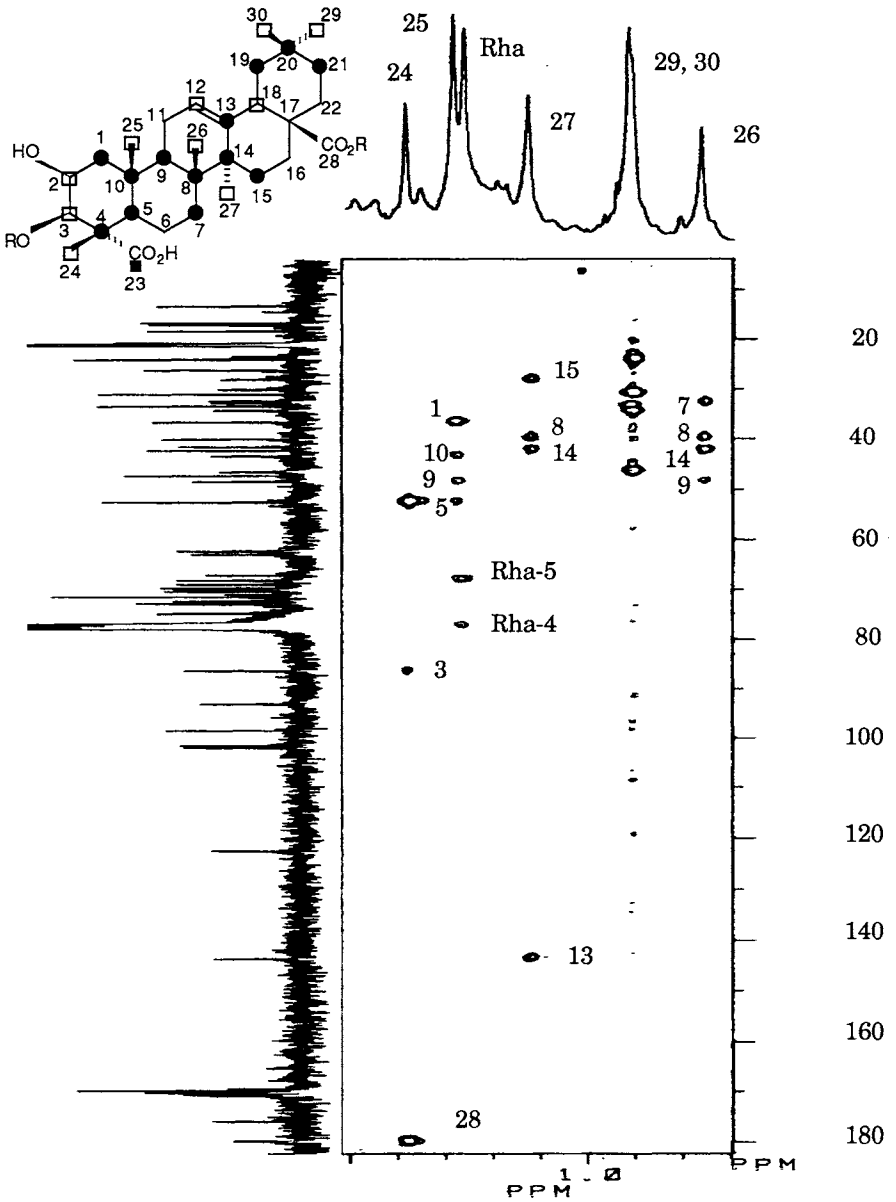
E. ROESY experiment performed on the underivatized saponin (256 experiments of 2K, spin lock : 200 ms single long pulse generated through the decoupling channel; normal mode with phase coherence between transmitter and decoupler; experiment is phased). Heavy lines correspond to solvent peaks. Roes have phase opposite to the phase of diagonal peaks (here positive and negative levels are plotted). Arrows point correlations which were useful for sequencing. The H-12 to H-18 correlation is indicative of the 18 β-H configuration.



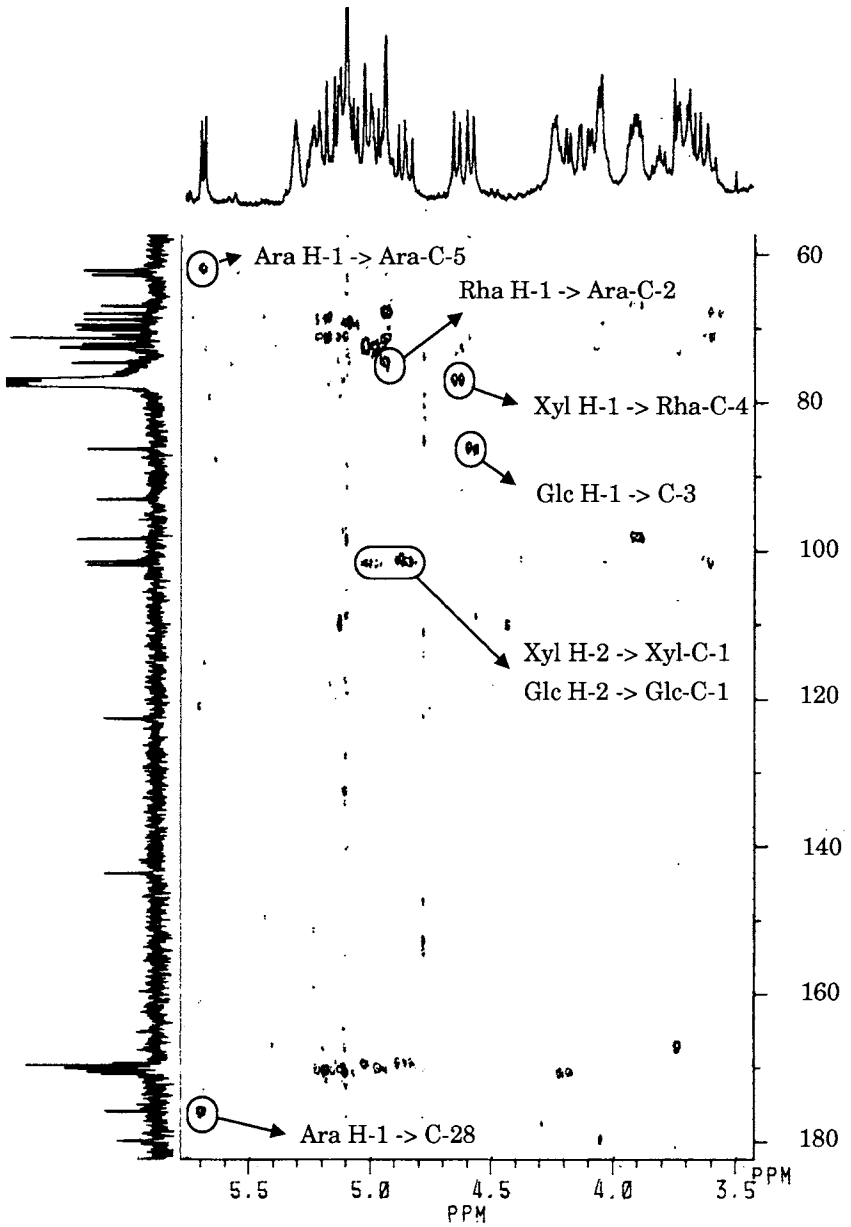
F. HMQC experiment performed on the derivatized saponin (256 experiments of 2K, reverse mode with decoupling in the carbon dimension; experiment is not phased). The part of the map corresponding to the high field resonances (methyls) is shown on top. At the bottom is the part of the map corresponding to anomeric resonances (plus H,C-3 and -12 of the triterpene).



G. HMBC experiment performed on the derivatized saponin (256 experiments of 2K, reverse mode without decoupling in the carbon dimension; experiment is not phased). Part of the map corresponding to the high field resonances (methyls) is shown below. The observed correlations allow assignment of most of the carbons of the triterpene (medicagenic acid). Carbon assigned by means of HMQC appear as squares on the formula and carbon assigned by means of HMBC appear as circles.



H. HMBC experiment performed on the derivatized saponin (256 experiments of 2K, reverse mode without decoupling in the carbon dimension; experiment is not phased). Part of the map corresponding to the low field resonances (sugars) is shown below. The observed correlations allow sequencing of the chains of sugars.



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