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Reinvestigation of the Sapogenins and Prosapogenins from Alfalfa (*Medicago sativa*)

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The structures of the hydrolysis products of the alfalfa saponins have been reinvestigated as preliminary studies of the native saponins. Identified known products are the soyasapogenols A, B, C, and E, hederagenin, and medicagenic acid. Bayogenin and zanhic acid known from other sources have now been characterized in alfalfa. The elusive lucernic acid is presumed to be a lactone artefact derived from zanhic acid, i.e. 16-hydroxymedicagenic acid. Five prosapogenins containing medicagenic acid and zanhic acid (plus glucose, glucuronic acid, or sophorose) have been identified in the saponins hydrolysates; their structures have been established without degradations and mainly rest on NMR and MS measurements. An HPLC method is proposed for the determination of medicagenic acid content in alfalfa.

Alfalfa (lucerne, *Medicago sativa*, Papillionaceae) is one of the richest sources of vegetable proteins in temperate climates (Carlsson, 1983). The aerial parts of the plant are used as a forage or are industrially processed to yield leaf protein concentrates. These protein concentrates are well balanced in amino acids and are rich in vitamins, carotenoids, and xanthophylls (Gastineau and De Mathan, 1981). At the present time, they are one of the main pigmentation sources in poultry rations (Livingston et al., 1980). Their development however is somehow hampered by their high content in antifeeding substances, which, at high doses, bring loss of weight (Heywang and Bird, 1954) and a decrease in egg production (Anderson, 1957). Previous work has shown that these properties are linked to saponins and especially to a triterpene, medicagenic acid (1) (Gestetner et al., 1971), which was a subject of intense study in the 1950s. It has also been shown that these saponins strongly interact with cholesterol; they may show promise in the treatment of hypercholesteremia and atherosclerosis (Malinow et al., 1982). Despite all this work, little is known today about their structure–activity relationship (Morris et al., 1961; Gestetner, 1971; Massiot et al., 1986). As part of a cooperative program aiming at introducing new varieties of alfalfa devoid of these undesired properties, the structures of the saponins of all parts of the plant are being reinvestigated. This first article describes the extraction of the saponin mixture and their hydrolysis into triterpenes. The following paper in the series will report on the structures of the alfalfa root sa-

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ponin (Massiot et al., 1988).

RESULTS AND DISCUSSION

Saponin Extraction. Since the early work on alfalfa saponins, a large number of sophisticated extraction procedures have been published (Morris et al., 1961; Walter et al., 1954; Thompson et al., 1957; Van Atta et al., 1961; Jurzysta, 1973; Shany et al., 1970). They rely on the solubility of saponins in water and alcohols (methanol or butanol) and their insolubility in ether. Our extraction procedures incorporate a dialysis step to free saponins of small water-soluble molecules such as sugars. Some saponin preparations include precipitation of part of the saponin mixture with cholesterol (Walter et al., 1954); as we were interested in the entire saponin fraction, we have not used these methods.

Depending on the part of plant under study, slightly different extraction procedures are applied. Root saponins are first extracted in boiling methanol, defatted by means of several precipitations with ether, and dissolved in water. Final purification is achieved by dialysis against water with or without preliminary butanol extraction. In other material such as leaf, leaf protein concentrates, or seeds, saponins are first extracted with boiling water, then extracted with butanol, and finally submitted to ether precipitation and/or dialysis depending on the desired state of purity. In all cases, the final separation from water is accomplished by freeze-drying, which gives hygroscopic powders.

The average saponin content of the leaf protein concentrates is 2 g/kg; as previously shown, it varies according to the pH of precipitation of the proteins and is often higher than the saponin content of the whole leaf. The saponin content averages 1.3 g/kg in the seed and 30 g/kg in the root. These yields are highly dependent on the variety of alfalfa and on the time of its collection (Berrang et al., 1974; Hanson et al., 1973). The higher saponin content of the root is the main reason why they have been first studied.

Hydrolysis of the Saponins. Hydrolysis of the glycosidic bonds of saponins is often plagued with difficulties concerning yields, selectivity, and artefact formation (Kitagawa, 1981). The purposes of the hydrolyses performed in this study are to allow identification of all triterpenes present in the saponins, allow a reproducible estimation of these compounds, and provide products of partial hydrolysis (prosapogenins) that could be useful in structure elucidations.

In a first series of experiments, hydrolysis is performed in a two-phase system (1-butanol–water) at 100 °C in the presence of $HClO_4$, HBF_4, or CF_3CO_2H. More reproducible results are obtained when the reaction is performed in water–perchloric acid at 140 °C in sealed tubes. Under these conditions, precipitation of the genins from the solution limits the quantity of artefacts. The precipitate also contains prosapogenins, which must be accounted for in quantification experiments.

Alfalfa Sapogenins. Alfalfa triterpenoids have been intensively studied between 1950 and 1975, and several review articles on the topic are available (Hanson et al., 1973; Birk and Peri, 1980; Bondi et al., 1973). We felt however that some of the major compounds lacked adequate modern determination such as ^{13}C NMR, and since some compounds are still designated as unknown, a reinvestigation of these genins was needed.

Our isolation results are summarized in Table I.

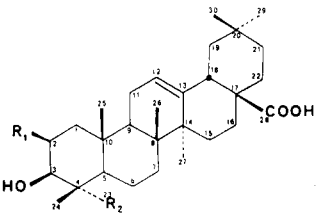
The most abundant alfalfa terpenoid is medicagenic acid (1), the structure of which was determined in 1957 (Djerassi et al., 1957). It is worth noting that this important compound has since only been isolated three times

Table I

genin[a]	no.	root	leaves	seeds
soyasapogenol C	5	×		×
soyasapogenol E	6	×		
soyasapogenol B	4		×	×
soyasapogenol A	3	×	×	
hederagenin	2	×	×	
bayogenin	7	×	×	
medicagenic acid	1	×	×	
lucernic acid	12		×	
zanhic acid	8		×	

[a] Increasing polarity order.

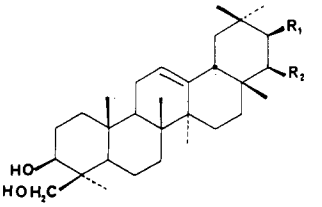
from other sources: as castanogenin in *Castanospermum australe* (Eade et al., 1963), in *Herniaria glabra* (Bukharov and Shcherbak, 1970), and in *Zanha golugensis* (Dimbi et al., 1984). Hederagenin (2), the major sapogenin of ivy, was identified in alfalfa long after its first isolation as sapogenin U (Shany et al., 1972). It has been identified in this study by direct comparison with a commercial sample. Soyasapogenols A (3), B (4), C (5), and E (6) are present in all parts of the plant and can be recognized by their spectral properties (MS, NMR). The structures of 3, 4, and 6 have recently been revised on the basis of an X-ray crystal structure determination (Kitagawa et al., 1982; Chiang and Chang, 1982).



R_1 = OH R_2 = COOH 1

R_1 = H R_2 = CH_2OH 2

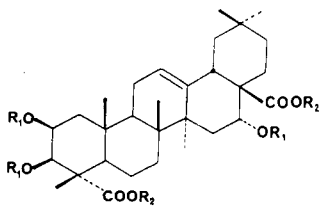
R_1 = OH R_2 = CH_2OH 7



R_1	R_2	
OH	OH	3
H	OH	4
>=<		5
H	>=O	6

Bayogenin (7) (Eade et al., 1963) possesses three alcohol functions and one acid function; it gives a triacetate (Ac_2O, DMAP) as well as a monomethyl ester (CH_2N_2). It has been found identical with an authentic sample prepared by diborane reduction of medicagenic acid. The reduction is not selective, and two triols are isolated; bayogenin displays retro-Diels–Alder MS fragments at m/z 203 and 248. Although isomeric with Hanson's unknown Y (Hanson et al., 1973), the mass spectra of the two compounds present dissimilarities and therefore ought to be different.

Compound 8 gives a triacetate 9 upon acetylation (Ac_2O, DMAP), a dimethyl ester 10 on methylation, and a dimethyl ester triacetate 11 after complete derivatization. Its full structure was determined as 2β,3β,16α-trihydroxyolean-12-ene-23,28-dioic acid by high-field ^1H NMR and by ^{13}C NMR as follows.



R_1 = R_2 = H 8

R_1 = $COCH_3$, R_2 = H 9

R_1 = H, R_2 = CH_3 10

R_1 = $COCH_3$, R_2 = CH_3 11

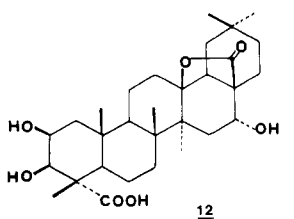


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Table II. ^{13}C NMR Data (δ) for 1a, 11, 21, and 22[a]

	1a	11	21	22
C-1	44.0	42.5	43.0	43.0
C-2	71.7	69.7	71.9	71.1
C-3	75.2	75.1	86.0	85.1
C-4	53.4	52.4	53.0	52.6
C-5	52.2	51.8	51.5	51.5
C-6	21.1	20.7	20.6	20.9
C-7	32.4	32.6	32.4	32.4
C-8	39.8	40.5	39.7	39.7
C-9	48.3	47.9	48.3	48.2
C-10	36.4	36.8	36.4	36.4
C-11	23.1	23.8	23.7	23.1
C-12	122.3	123.4	122.3	122.2
C-13	144.0	142.7	144.0	143.9
C-14	42.0	41.6	41.8	41.9
C-15	27.7	31.0	27.6	27.7
C-16	23.6	76.5	23.7	23.5
C-17	46.8	48.1	46.7	46.8
C-18	41.4	41.0	41.3	41.4
C-19	46.0	46.7	46.0	46.0
C-20	30.7	30.7	30.7	30.7
C-21	34.0	35.3	34.1	34.0
C-22	32.4	32.3	32.4	32.4
C-23	178.6	176.3	178.2	178.2
C-24	12.4	12.9	12.9	12.6
C-25	16.6	16.5	16.4	16.5
C-26	16.8	17.2	16.7	16.7
C-27	26.1	26.9	26.0	26.1
C-28	178.4	176.2	178.2	178.1
C-29	33.1	33.2	33.1	33.1
C-30	23.6	24.6	23.7	23.7
OCH_3	51.5	51.7	52.0	52.4
OCH_3	51.5	52.1	52.7	52.4

sugar carbon	21	22	sugar carbon	21	22
C-1′	101.5	101.6	C-1″		100.7
C-2′	69.5	71.6	C-2″		70.4
C-3′	72.7	74.9	C-3″		73.2
C-4′	68.7	68.8	C-4″		68.8
C-5′	72.3	71.6	C-5″		72.1
C-6′	62.0	62.3	C-6″		62.0

[a] Data on OAc signals (δ 20.6–21.9, 169.0–170.6) are not described.

The 401-MHz ^1H NMR spectrum of the derivative 11 ($[\alpha]_D = +10°$ (c 0.4, $CHCl_3$), amorphous) shows signals for the six angular methyl groups of the oleanane skeleton (δ 1.33, 1.19, 1.15, 0.92, 0.87, 0.67), for three acetates (δ 2.03, 2.01, 1.9), and for two methyl esters (δ 3.61, 3.58). A broad triplet at δ 5.40 indicates the presence of the trisubstituted Δ^{12}-olefin, and three multiplets at δ 5.63, 5.42, and 5.33 correspond to protons α to secondary acetates. The signals at δ 5.42 and 5.33 have the expected multiplicities for 2α H (dt, J = 3.5, 4.2 Hz) and 3α H (d, J = 4.2 Hz), respectively; their relationship is corroborated by a double-irradiation experiment. The third CHOAc proton appears as a triplet with a 3-Hz coupling constant (equatorial hydrogen). The mass spectrum of 11 is dominated by the usual retro-Diels–Alder fragmentation of Δ^{12} triterpenoids; the fragments corresponding to the D and E rings appear at m/z 201 (100%) and m/z 260 (60%), features indicative of the presence of a supplementary unsaturation in those rings. Although no peaks at m/z 320 can be detected, this unsaturation ought to correspond to the aforementioned acetate. The previous remark on the shape of the ^1H NMR signal for the proton vicinal to this ester fixes it at positions 15β, 16α, 21β, or 22α. This last ambiguity is cleared by ^{13}C NMR study of 11.

At 75 MHz all carbon resonances are resolved (Table II). The multiplicities are determined by a J-modulated spin echo experiment, which sorts out C/CH_2 on one hand and CH/CH_3 on the other (Le Cocq and Lallemand, 1981). An INEPT sequence then allows the distinction between C and CH_2 by suppressing the resonances due to carbon atoms not bound to protons (Doddrell and Pegg, 1980); CH and CH_3 are distinguished by their chemical shifts.

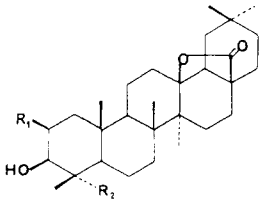
As a reference, the ^{13}C NMR spectrum of methyl medicagenate (**1a**) is assigned following Tori's characteristics for oleanane triterpenes (Tori et al., 1974). The agreement is good for all carbons of rings C–E, and carbons of rings A and B can be assigned provided attention is paid to deviations from additivity rules caused by *vic*-diols (Eggert et al., 1976; Van Antwerp et al., 1977). Hindrance brought about by the axially oriented 2β-alcohol produces the expected downfield shifts for C-24, C-25, and C-6 albeit in a diminished fashion due to possible flattening of ring A.

The assignments for the carbon atoms of rings A–C of **11** are easily deduced from the analysis of the spectrum of **1a**, the only point to be settled being the locus of the substitution on segments C-15 → C-16 or C-21 → C-22. The unassigned methylenes of **11** were examined. Four such carbons are found at δ 31.0, 32.3, 35.3, and 46.7 (**11**) in lieu of the five resonances at δ 23.6 (C-16), 27.7 (C-15), 32.4 (C-22), 34.0 (C-21), and 46.0 (C-19) (**1a**). Clearly, substitution by an OAc has occurred on the C-15 → C-16 branch. Choice of C-16 for the fixation of the OAc is permitted by examination of the ^{13}C NMR spectrum of a 16α-hydroxyolean-12-en-28-oic acid, quillaic acid (Tori et al., 1974). Comparison of the relevant carbons of **11** and of quillaic acid shows that **11** is the methyl ester of 2β,3β,16α-triacetoxyolean-12-ene-23,28-dioic acid. This assignment of course depends on previous structure determinations of 16-hydroxy triterpenoids (Kubota and Kitatani, 1968).

After this work was completed, we learned of the isolation and structure elucidation of compound **8** as zanhic acid in *Z. golugensis* (Dimbi et al., 1984). In this plant **8** is accompanied by lactone **12** whose properties closely match those of Livingston's lucernic acid (Livingston, 1959). HPLC comparison of alfalfa sapogenins samples and of a mixture of **8** and **12** shows that both compounds are present in alfalfa. We therefore deduce structure **12** for the long-sought-after lucernic acid.

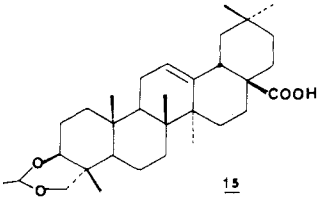
Hydrolysis Artefacts. The harshness of some hydrolytic conditions brings about formation of small quantities of artefacts.

One would expect 28 → 13 lactones formed by acid-catalyzed cyclization of the corresponding γ,δ-unsaturated acid. Thus, one finds lactones **13** and **14**, respectively, arising from hederagenin and medicagenic acid as well as compound **12** (lucernic acid), which may be considered as a hydrolysis artefact of zanhic acid.



$R_1 = H$, $R_2 = CH_2OH$ **13**

$R_1 = OH$, $R_2 = COOH$ **14**



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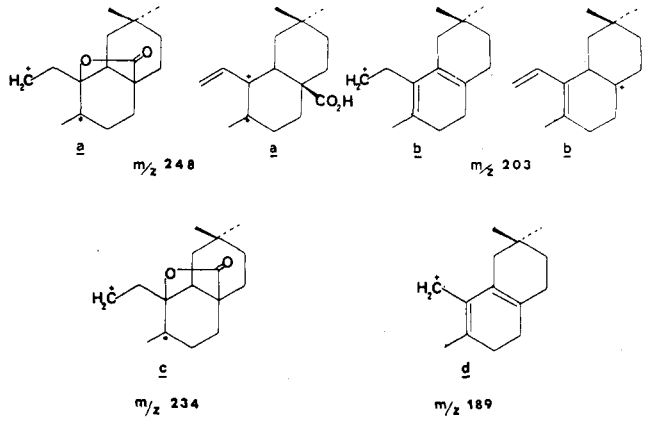
Identification of these compounds is based on their spectral properties: observation of a γ-lactone IR vibration band (1750 cm^{-1}) and absence of an olefinic proton in the ^1H NMR spectra. The mass spectra of the lactones are not basically different from the spectra of the corre-

Table III

	no.	root	leaves	protein concentrate
glucose(1→3)medicagenic acid	16	+	+	
sophorose(1→3)medicagenic acid	17	+		
glucuronic(1→3)medicagenic acid	18		+	+
glucose(1→3)zanhic acid	19		+	
glucuronic acid(1→3)zanhic acid	20		+	



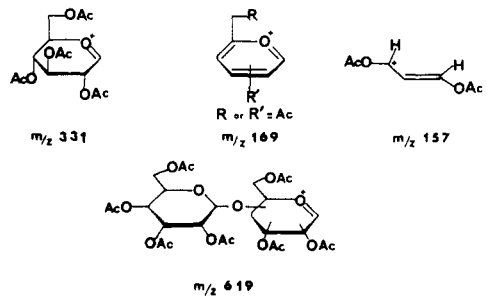
sponding acids: Fragmentation of ring C generates four ions **a–d**, the latter two being prominent in the lactones.

Besides these compounds, a minor product is found and assigned the structure of hederagenin acetal (**15**). The most noticeable characteristic of **15** is the presence in its ^1H NMR spectrum of a three-proton doublet at δ 1.32 (J = 5 Hz), attributed to the acetal methyl group. Reaction of hederagenin with acetaldehyde under acid catalyst gave **15** as final structural proof for this compound.

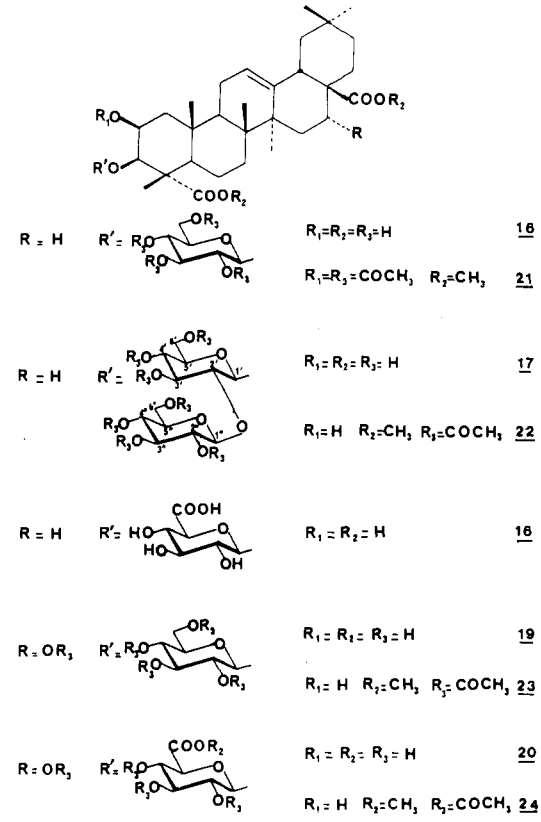
Alfalfa Prosapogenins. Among the polar products of the hydrolysis experiments, several glycotriterpenes have been isolated. Their occurrence is summarized in Table III.

All these very polar compounds have been characterized as their peracetylated derivatives; to increase their solubility, they all have also been methylated (CH_2N_2). If glucuronic acid is present, better yields are obtained when methylation is performed prior to acetylation.

Compound **16** is the glucoside of medicagenic acid, previously isolated as a saponin (Morris et al., 1961) and also synthesized from medicagenic acid and acetobromoglucose under Koenigs–Knorr conditions (Morris and Tankersley, 1963). This compound gives a dimethyl ester and a pentaacetate **21** that does not show a molecular ion in its mass spectrum recorded under electron impact. The sugar is identified as glucose by the observation on the MS of ions at m/z 331, 169, and 157 (Budzikiewicz et al., 1964) and by ^1H NMR spectroscopy. Thus, at 400 MHz the seven protons of the sugar residue give rise to well-separated resonances on which coupling constants can be measured. The observation of large vicinal couplings between H-1, H-2, H-3, H-4, and H-5 is in favor of a sugar possessing all its substituents in equatorial positions, i.e. glucose. The anomeric proton appears as a doublet (δ 4.47, J = 8 Hz) suggesting a β configuration for the glycosidic bond. The genin protons H-2 and H-3 are found at δ 5.47 and 4.18, respectively; this indicates that position 2 is acylated and that the sugar is bound to the C-3 oxygen of the genin (the acidic functions are free since they can be methylated).



The second highly polar compound of the root saponin hydrolysis is a triterpenoid disaccharide sophorose(1→3)medicagenic acid **17**. Upon acetylation it gives an heptaacetate, and on diazomethane treatment, a dimethyl ester. The mass spectrum of the acetylated and methylated derivative **22** shows as most intense peaks ions at m/z 331 (hexose, peracetylated) and 262 (rings D and E of the triterpene); another important peak is found at m/z 619, corresponding to a peracetylated disaccharide composed of hexoses. Under electron impact, no molecular ion is observable and the peak of highest mass (m/z 1088) is obtained after a loss of acetic acid.



The ^1H NMR spectrum of **22** displays the expected signals for a diester of medicagenic acid (see the Experimental Section). The protons bound to C-2 and C-3 identified by double-irradiation experiments resonate at δ 4.2 and 4.1; they are surrounded by 14 sugar protons found between δ 3.5 and 5.1. The analysis of the interproton connectivities is based on double-resonance experiments starting at the anomeric protons (doublets at δ 4.36 and 4.58). As above, the measured coupling constants are those of two glucoses: One is a terminal glucose as shown by its deshielded proton, and the other is substituted at the 2′-position (H-2′ found at δ 3.9 instead of δ 4.83 for an acylated glucose). Both sugars are in the pyranose form, and the glycosidic bonds are β. The disaccharide (glucose(1→2)glucose = sophorose) is placed on the O-3 of the genin; position 2, which is severely hindered, is not acetylated under normal conditions. The alternative situation (sugars on position 2) would give a

less congested position for the free alcohol and therefore would allow its acylation.

The same conclusions regarding the structures of **21** and **22** are reached by using ^{13}C NMR spectroscopy (Table II). All the carbon resonances of medicagenic acid methyl ester are found at values similar to those found in the free triterpene, except the resonance of C-3, which is 10 ppm downfield as a consequence of the sugar substitution. The chemical shifts of the peracetylated glucose and sophorose closely fit literature values (Seo et al., 1978; Gagnaire et al., 1976).

The third derivative of medicagenic acid (**18**) is found in the hydrolysates of the leaves and in the saponin of the leaf protein concentrates. Medicagenic acid is identified by ^1H NMR spectroscopy (six methyl singlets; two coupled HCO: H-2 and H-3; an olefinic proton). Upon methylation, a triester is obtained, suggesting that the sugar is an uronic acid; acetylation gives a triacetate. The interproton couplings are detected in a COSY experiment that shows a five-spin system for the sugar residue. An important peak at m/z 317 is indicative of an uronic acid methyl ester, which is identified as a glucuronic acid by its large interproton coupling constants. As with all other sugar derivatives described here, the sugar is in a β-pyranosyl form and is attached to the 3-position of the genin; position 2 is not acylated.

Glycosides **19** and **20** have been isolated after hydrolysis of the leaf saponins and separated as the permethyl esters and peracetates, **23** and **24**. Their separation was difficult and has been achieved by preparative TLC after two plate developments. Compounds **19** and **20** share a common genin, namely zanhic acid (**8**), as shown by typical MS fragments (m/z 201, 260) and by the observation of a deshielded H-16 in the ^1H NMR of derivatives **23** and **24**. Arguments similar to those developed in the structure elucidations of **16** and **18** allowed determination of the sugars as glucose and glucuronic acid (see the Experimental Section for details).

The isolation of **19** and **20** is proof of the existence of zanhic rather than lucernic acid in alfalfa.

The formation of large quantities of these prosapogenins (see the Experimental Section) with medicagenic or zanhic acid is probably a consequence of the encumbrance around the most inner glycosidic bond of the saponin. Attack of the reagents is impeded on one side by the two C-4 substituents (methyl and acid) and on the other by the axial C-2 hydroxyl group. This steric hindrance is also demonstrated by the difficult acylation of the genin OH-2.

HPLC Determination of Medicagenic Acid. In order to monitor a genetic selection or to compare samples of alfalfa, it is important to know their genin profile. So far, several analytical methods have been proposed, based on TLC (Jurzysta et al., 1988). or GLC (Brawn et al., 1981; Rao and Bories, 1987). Other methods rely on biological tests such as hemolysis or fungi growth inhibition. As an alternative to these techniques, we have chosen HPLC to rapidly quantify the medicagenic acid content of alfalfa. Samples are prepared as above and dissolved in methanol (10 mg/5 mL of MeOH, 20-μL injections); chromatographic conditions are similar to those used in the titration of the soyabean triterpenoids (Lin et al., 1981). Columns are 15 cm long and are filled with C_{18} ODS reverse phase; the solvent is a mixture of MeOH (75%), H_2O (25%), and 88% formic acid (0.05%). Detection is performed at 210 nm (double-bond absorption). With a 2 mL/min flow rate, the following retention times (min) are obtained: zanhic acid, 1.63; medicagenic acid, 3.9; bayogenin, 4.2; hederagenin, 7.47. The prosapogenins are rapidly eluted with the solvent front. This method has been applied to more than 50 varieties of leaves of alfalfa; in all cases zanhic and medicagenic acids are present in important quantities. Other genins are often found as minor compounds, and the ratios of the different genins greatly vary from species to species.

The accuracy of the titration depends on the quality of the saponin hydrolysis technique. As pointed out above, hydrolysis artefacts and partial hydrolysis are the two major problems. In order to ensure the reproducibility and the self-consistency of the titration, mild hydrolysis conditions and a measurement of medicagenic acid as well as of some of its glycoside precursors are proposed.

CONCLUSION

The structures of the previously isolated alfalfa sapogenin have been verified, and two new substances have been added to the list of the alfalfa triterpenoids: bayogenin and zahnic acid. This latter compound is very important since it is probably related via a lactone to the elusive lucernic acid, a major constituent of some cultivars. The structures of five prosapogenins have been established; four of them are novel compounds. From their structures, it appears that the alfalfa triterpenes are often substituted by a glucose or glucuronic acid at position 3; this remark has been verified in the native saponins whose structures have been determined. The sole prosapogenin containing two sugars (sophorose(1→3)medicagenic acid) has also been found as a subunit of two alfalfa saponins (Lavaud, 1986). The 1 → 2 linkage between the two sugars has been established by a nondegradative technique based on the observation of NMR signals; this approach for the determination of complex chains of sugars shows great promise in the field of polyglycoside chemistry (Massiot et al., 1986; Nishino et al., 1986; Waltho et al., 1986; Lanzetta et al., 1986).

EXPERIMENTAL SECTION

General Procedures. Melting points are uncorrected. Optical rotations were measured in a 1-dm cell on a Perkin-Elmer 241 automatic polarimeter. IR spectra were recorded on a Beckman Acculab 2 spectrometer. ^1H NMR were measured at 401 MHz on a prototype at the Institut d'Electronique Fondamentale (Orsay) or at 300 MHz on a AC Bruker spectrometer. ^{13}C NMR were obtained at 15 MHz on a Bruker WP 60 instrument, at 50 MHz on a Bruker WM 200 instrument, or at 75 MHz on a Bruker AM 300 spectrometer. Mass spectra were run on a Jeol D 300 spectrometer. TLC were run on Whatman or Merck plates; solvents of elution were mixtures of MeOH and $CHCl_3$ (from 2 to 8%).

Root saponins were extracted from the Lutece cultivar of *M. sativa*, grown at the Lusignan INRA center. Seeds were of the Europe cultivar. Leaves and protein concentrates of saponins were obtained from France-Luzerne and corresponded to a mixture of Europe (ca. 80%) and Resis (ca. 20%) cultivars.

Saponin Extraction. Dried, powdered root (2 kg) was boiled in 15 L of MeOH–H_2O (4:1) for 4 h. After cooling and filtration, the solvent was removed in vacuo and the residue was suspended in 1.75 L of MeOH at 60 °C. After filtration, the saponins were precipitated from the solution by means of 8.75 L of ether. The precipitate was filtered and dissolved in 1.3 L of water. The solution was then dialyzed against pure water for 4 days after which time the content of the tube was freeze-dried. The solid residue was dissolved in methanol and decolorized with carbon black. After filtration, the solution was diluted with five volumes of ether. The white saponin precipitate was filtered and

dried in vacuo over P_2O_5; 44 g, yield 22 g/kg.

Sapogenin Preparation and Isolation. The saponin mixture (3.6 g) was dissolved in 100 mL of 4% aqueous $HClO_4$ and heated at 140 °C in a thick-wall sealed tube for 2 h. After cooling, the sapogenin precipitate was filtered, rinsed with water, and dried in vacuo over P_2O_5. It was thus obtained 1.41 g of sapogenins, which were purified by column chromatography on silica gel. Elution pressure was 10 bar; solvent was $CHCl_3$ and a gradient of $CHCl_3$ in MeOH. Fractions (20 mL) were checked by TLC.

General Method of Acetylation. Starting material (10 mg) was suspended in CH_2Cl_2 (5 mL) and the resultant mixture stirred overnight with 20 mg of 4-(dimethylamino)pyridine (DMAP) and 100 μL of acetic anhydride. In case of larger quantities of starting material, pyridine is added to the mixture (1 mL/g). The organic layer was washed with 10% aqueous $CuSO_4$ and then with saturated $NaHCO_3$ and pure water. The organic solution was dried over Na_2SO_4; after filtration solvent was removed in vacuo.

General Method of Methylation of Acids. Starting material (100 mg) was dissolved in 5 mL of MeOH and the resultant mixture stirred at 0 °C with an excess of an ethereal solution of diazomethane. The reaction was followed by TLC; after disappearance of starting material, excess diazomethane was destroyed by 1 drop of AcOH and the methylated product was obtained after removal of the solvent.

Diborane Reduction of Medicagenic Acid (1). Compound 1 (20 mg, 0.04 mmol) was suspended into 3 mL of BH_3–THF solution. After the mixture was stirred for 10 h, excess diborane was destroyed by 1 drop of AcOH. Dilution with water and usual treatment yielded 22 mg of an oil, showing three spots on TLC. The compound of medium polarity was purified by preparative TLC and identified with natural bayogenin (7) (MS, ^1H NMR).

Zanhic acid (8): mp 265 °C (MeOH); ^1H NMR (400 MHz, $CDCl_3$–CD_3OD) δ 0.68 (s, 3 H), 0.88 (s, 3 H), 1.16 (s, 3 H), 1.23 (s, 3 H), 1.24 (s, 3 H), 3.9 (d, J = 4 Hz, H-3), 4.03 (m, H-16), 4.33 (m, H-2), 5.3 (m, H-12).

Acetylation of 8. A solution of 8 (8 mg) was acetylated according to the general procedure. The resulting oily residue (10 mg) was purified by TLC. Triacetate **9** was thus obtained: 6 mg, yield 65%; IR ($CHCl_3$) ν_{max} (cm^{-1}) 3450, 1740, 1720 (sh), 1240; ^1H NMR (400 MHz, $CDCl_3$) δ 0.79 (s, 3 H), 0.9 (s, 3 H), 0.95 (s, 3 H), 1.2 (s, 3 H), 1.21 (s, 3 H), 1.42 (s, 3 H), 1.95 (s, 3 H), 2.04 (s, 3 H), 2.08 (s, 3 H), 3.09 (dd, J = 11, 4 Hz, H-18), 5.32 (d, J = 4 Hz, H-3), 5.45 (m, 2 H, H-12 + H-2), 5.56 (br t, J = 4 Hz, H-16).

Esterification of 8 with diazomethane (10): ^1H NMR (400 MHz, $CDCl_3$) δ 0.71 (s, 3 H), 0.88 (s, 3 H), 0.95 (s, 3 H), 1.23 (s, 3 H), 1.32 (s, 3 H), 1.33 (s, 3 H), 3.01 (dd, J = 11, 4 Hz, H-18), 3.58 (s, 3 H), 3.71 (s, 3 H), 4.0 (m, H-2), 4.16 (m, H-16), 4.5 (d, J = 4 Hz, H-3), 5.38 (m, H-12).

Preparation of 11. Triacetate **9** (6 mg) was treated with CH_2N_2 as above, and **11** was obtained in virtually quantitative yield: $[\alpha]_D$ +10° ($CHCl_3$, c 0.4); MS, m/z (rel intens) 672 (M^+) (0.5), 613 (27), 612 (40), 580 (15), 553 (27), 552 (22), 493 (10), 433 (8), 260 (60), 247 (18), 245 (19), 203 (35), 202 (28), 201 (100), 187 (24), 173 (20), 145 (14), 133 (25), 131 (31); IR ($CHCl_3$) ν_{max} (cm^{-1}) 2920, 2850, 1740, 1730, 1440, 1420, 1365, 1240, 1220, 1100, 1050, 1015 cm^{-1}; ^1H and ^{13}C NMR, see text.

Hederagenin lactone (13): mp 298 °C (acetone–ether); $[\alpha]_D$ +14° ($CHCl_3$, c 0.4); IR (KBr) ν_{max} (cm^{-1}) 3300, 1750; MS, m/z (rel intens) 472 (M^+) (6), 454 (7), 436, 424, 410, 395, 248, (100), 236, 235, 234 (69), 223, 203 (46), 189 (81); ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (s, 6 H, 2 Me), 0.93 (s, Me), 1.00 (s, Me), 1.07 (s, Me), 1.17 (s, Me), 3.43 (d, J = 11 Hz, H-23), 3.65 (t, J = 8 Hz, H-3), 3.75 (br d, J = 11 Hz, H-23).

Medicagenic acid lactone (14): characterization as diacetate methyl ester; $[\alpha]_D$ +16° ($CHCl_3$, c 0.17); IR, ν_{max} (cm^{-1}) 1740, 1240; MS, m/z (rel intens) 600 (M^+) (0.6), 568, 554 (6), 480, 452, 435, 421, 332, 305, 248 (29), 235 (63), 234 (46), 203 (77), 189 (100); ^1H NMR (400 MHz, $CDCl_3$) δ 0.90 (s, Me), 1.03 (s, Me), 1.07 (s, Me), 1.19 (s, Me), 1.21 (s, Me), 1.42 (s, Me), 1.98 (s, 3 H, OCOMe), 2.08 (s, 3 H, OCOMe), 3.70 (s, 3 H, COOMe), 5.33 (d, J = 4 Hz, H-3), 5.47 (m, H-2).

Hederagenin ketal (15): $[\alpha]_D$ +46° ($CHCl_3$, c 0.13); IR, ν_{max} (cm^{-1}) 1775, 1700, 1460, 1385; ^1H NMR (400 MHz, $CDCl_3$) δ 0.82 (s, Me), 0.90 (s, Me), 0.92 (s, 6 H, 2 Me), 1.07 (s, Me), 1.13 (s, Me), 1.35 (d, J = 5 Hz, 3 H), 2.82 (dd, J = 12, 4 Hz, H-18), 3.23 (br d, J = 11 Hz, H-23), 3.50 (m, H-3), 3.75 (d, J = 11 Hz, H-23), 4.73 (d, J = 5 Hz, 1 H), 5.28 (br t, H-12).

Glucoside of medicagenic acid (21): acetylated and methylated derivative; IR, ν_{max} (cm^{-1}) 1760, 1750, 1720, 1230; MS, m/z (rel intens) 860 $(M - 42)^+$ (0.3), 842 (0.3), 810, 772, 512 (2.9), 454, 453, 452, 393, 331 (27), 262 (36), 203 (35), 169 (35), 153; ^1H NMR (400 MHz, $CDCl_3$) δ 0.73 (s, Me), 0.92 (s, Me), 0.94 (s, Me), 1.09 (s, Me), 1.11 (s, Me), 1.27 (s, Me), 2.00 (s, 3 H, AcO), 2.03 (s, 3 H, AcO), 2.07 (s, 3 H, AcO), 2.17 (s, 3 H, AcO), 2.33 (s, 3 H, AcO), 2.87 (dd, J = 14, 5 Hz, H-18), 3.62 (s, 3 H, CO_2Me), 3.67 (s, 3 H, CO_2Me), 4.10 (dd, J = 12.5, 2.5 Hz, H-6′), 4.18 (d, J = 4 Hz, H-3), 4.47 (d, J = 8 Hz, H-1′), 4.88 (dd, J = 10, 8 Hz, H-2′), 5.00 (t, J = 10 Hz, H-4′), 5.13 (t, J = 10 Hz, H-3′), 5.27 (br t, H-12), 5.47 (dt, J = 7, 4 Hz, H-2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.9 (q, C-24), 16.4 (q, C-25), 16.7 (q, C-26), 20.6 (t, C-6), 20.6 (5 q, AcO), 23.7 (q, C-30), 23.7 (t, C-16), 23.7 (t, C-11), 26.0 (q, C-27), 27.6 (t, C-15), 30.7 (s, C-20), 32.4 (t, C-22), 32.4 (t, C-7), 33.1 (q, C-29), 34.1 (t, C-21), 36.4 (s, C-10), 39.7 (s, C-8), 41.3 (d, C-18), 41.8 (t, C-14), 43.0 (t, C-1), 46.0 (t, C-19), 46.7 (s, C-17), 48.3 (d, C-9), 51.5 (d, C-5), 52.0 (q, CO_2Me), 52.7 (q, CO_2Me), 53.0 (s, C-4), 62.0 (t, C-6′), 68.7 (d, C-4′), 69.6 (d, C-2′), 71.9 (d, C-2), 71.9 (d, C-5′), 72.7 (d, C-3′), 86.0 (d, C-3), 101.5 (d, C-1′), 122.3 (d, C-12), 144.0 (s, C-13), 169.3–169.4–170.2–170.6 (4 s, 5 OAc), 178.2 (2 s, C-23, C-28).

Sophorose(1→3)medicagenic acid (22): acetylated and methylated; $[\alpha]_D$ +30.7° ($CHCl_3$, c 0.78); IR, ν_{max} (cm^{-1}) 3560, 1530, 1370, 1250–1200; MS, m/z (rel intens) 1088 $(M - 60)^+$ (0.2), 861, 858, 801, 740, 726, 619 (6), 527, 512 (7.5), 494 (3), 453 (3.5), 331 (100), 303, 262 (5); ^1H NMR (400 MHz, $CDCl_3$) δ 0.65 (s, Me), 0.83 (s, Me), 0.87 (s, Me), 1.05 (s, Me), 1.20 (s, Me), 1.33 (s, Me), 1.93 (s, 9 H, AcO), 1.97 (s, 3 H, AcO), 2.01 (s, 3 H, AcO), 2.05 (s, 3 H, AcO), 2.07 (s, 3 H, AcO), 2.80 (dd, J = 14, 4 Hz, H-18), 3.57 (s, 3 H, COOMe), 3.70 (s, 3 H, COOMe), 3.58 (m, 2 H, H-5′, H-5″), 3.90 (m, H-2′), 4.00 (dd, J = 13, 2.5 Hz, H-6″), 4.10 (d, J = 4 Hz, H-3), 4.11 (dd, J = 13, 2.5 Hz, H-6′), 4.16 (dd, J = 13, 4 Hz, H-6′), 4.23 (dd, J = 13, 5 Hz, H-6″), 4.20 (m, H-2), 4.36 (d, J = 8 Hz, H-1′), 4.58 (d, J = 8 Hz, H-1″), 4.83 (t, J = 9.5 Hz, H-2″), 4.88 (t, J = 9.5 Hz, H-4″), 5.00 (t, J = 9.5 Hz, H-3″), 5.08 (t, J = 9.5 Hz, H-4′), 5.10 (t, J = 9.5 Hz, H-3′), 5.23 (br t, H-12); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.6 (q, C-24), 16.5 (q, C-25), 16.7 (q, C-26), 20.6–20.7–20.9 (br q, OAc), 20.9 (t, C-6), 23.1 (t, C-11), 23.5 (t, C-16), 23.7 (q, C-30), 26.1 (q, C-27), 27.7 (t, C-15), 30.7 (s, C-20), 32.4 (t, C-22), 32.4 (t, C-7), 33.1 (q, C-29), 34.0 (q, C-27), 36.4 (s, C-10), 39.7 (s, C-8), 41.4 (d, C-18), 41.9 (s, C-14), 43.0 (t, C-1), 46.0 (t, C-19), 46.8 (s, C-17), 48.2 (d, C-9), 51.5 (d, C-5), 52.4 (2 52.6 2 CO_2Me), 52.6 (s, C-4), 62.0 (t, C-6′), 62.3 (t, C-6″), 68.8–70.4–71.6–72.1–76.7–73.2–74.9 (d, C-2′, C-3′, C-4′, C-5′, C-2″, C-3″, C-4″,

C-5″), 71.1 (d, C-2), 85.1 (d, C-3), 100.7 and 101.6 (2 d, C-1′, C-1″), 122.2 (d, C-12), 143.9 (s, C-13), 169.2–169.4–170.0–170.3–170.4–170.6 (7 s, OAc), 178.1 and 178.2 (2 s, CO_2Me).

Glucuronide of medicagenic acid (18); description of the triacetate trimethyl ester of 18: MS, *m/z* (rel intens) 317 (5), 262 (60), 203 (100); ^1H NMR (300 MHz, $CDCl_3$) δ 0.7 (s, 3 H), 0.88 (s, 3 H), 0.9 (s, 3 H), 1.1 (s, 3 H), 1.19 (s, 3 H), 1.3 (s, 3 H), 2 (s, 6 H), 2.05 (s, 3 H), 2.09 (s, 3 H), 2.87 (dd, *J* = 14, 5 Hz, H-18), 3.58 (s, 3 H), 3.65 (s, 3 H), 3.7 (s, 3 H), 4.02 (d, *J* = 8 Hz, H-5′), 4.08 (d, *J* = 4 Hz, H-3), 4.25 (m, H-2), 4.58 (d, *J* = 7 Hz, H-1′), 5 (dd, *J* = 7, 8 Hz, H-2′), 5.17 (t, *J* = 8 Hz, H-4′), 5.21 (t, *J* = 8 Hz, H-3′), 5.27 (m, H-12).

Glucoside of zanhic acid (19); description of the pentaacetate dimethyl ester (23): MS, *m/z* (rel intens) 858 $(M - 60)^+$ (1), 798 (0.2), 331 (60), 260 (70), 201 (80), 169 (100); ^1H NMR (300 MHz, $CDCl_3$) δ 0.71 (s, 3 H), 0.9 (s, 3 H), 0.97 (s, 3 H), 1.2 (s, 3 H), 1.25 (s, 3 H), 1.3 (s, 3 H), 2–2.2 (5 s, 15 H), 3.08 (dd, *J* = 14, 5 Hz, H-18), 3.62 (s, 3 H), 3.71 (s, 3 H), 3.72 (m, H-5′), 4.08 (d, *J* = 4 Hz, H-3), 4.1 (dd, *J* = 11, 3 Hz, H-6′), 4.18 (dd, *J* = 11, 5 Hz, H-6′), 4.25 (m, H-2), 4.52 (d, *J* = 8 Hz, H-1′), 4.98 (t, *J* = 8 Hz, H-2′), 5.01 (t, *J* = 8 Hz, H-4′), 5.18 (t, *J* = 8 Hz, H-3′), 5.43 (m, H-12), 5.64 (m, H-16).

Glucuronide of zanhic acid (20); description of the tetraacetate trimethyl ester (24): MS, *m/z* (rel intens) 844 $(M - 60)^+$ (1), 785 (2), 317 (10), 260 (80), 201 (100), 155 (40); ^1H NMR (300 MHz, $CDCl_3$) δ 0.71 (s, 3 H), 0.9 (s, 3 H), 0.97 (s, 3 H), 1.2 (s, 3 H), 1.25 (s, 3 H), 1.3 (s, 3 H), 2.02 (s, 3 H), 2.05 (s, 3 H), 2.1 (s, 3 H), 2.18 (s, 3 H), 3.1 (dd, *J* = 14, 5 Hz, H-18), 3.62 (s, 3 H), 3.7 (s, 3 H), 3.74 (s, 3 H), 4.03 (d, *J* = 8 Hz, H-5′), 4.1 (d, *J* = 4 Hz, H-3), 4.25 (m, H-2), 4.58 (d, *J* = 8 Hz, H-1′), 5 (t, *J* = 8 Hz, H-2′), 5.18 (t, *J* = 8 Hz, H-4′), 5.24 (t, *J* = 8 Hz, H-3′), 5.43 (m, H-12), 5.64 (m, H-16).

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Structural Characterization of Nitrosylhemochromogen of Cooked Cured Meat: Implications in the Meat-Curing Reaction

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Cured meat pigment from cooked corned beef was isolated and completely characterized by IR and visible spectroscopies and shown to be identical with synthetic material that was further identified by fast atom bombardment mass spectrometry as the mononitrosyl species nitrosyliron(II) protoporphyrin. The ^{1}H NMR spectrum of this paramagnetic complex is reported for the first time. It was shown that this pigment can be formed from chloroiron(III) protoporphyrin by autoreduction with imidazole and nitric oxide. The same compound is formed by reacting nitric oxide gas with metmyoglobin followed by protein denaturation. These results lead to the proposal of a new mechanism for the meat-curing process involving (1) oxidation of myoglobin to metmyoglobin by nitrite, which is reduced to nitric oxide, (2) formation of the unobserved intermediate nitrosylmetmyoglobin, (3) rapid autoreduction to a nitrosylmyoglobin radical cation, (4) further reduction to nitrosylmyoglobin, and (5) formation of nitrosylhemochromogen (nitrosyliron(II) protoporphyrin) and incorporation of a second mole of nitrite into the denatured protein on heating.

The structure of the nitrosylhemochromogen pigment of cooked cured meat has long been a subject of dispute. This structure has been suggested as being either a five-coordinate mononitrosyliron(II) protoporphyrin complex or a six-coordinate dinitrosyliron(II) protoporphyrin complex.

Hornsey (1956) demonstrated that the characteristic red pigment of cooked cured meat could be extracted completely by an 80% acetone–water mixture. Electronic adsorbance and reflectance data of the pigments of cooked cured ham, heat-denatured nitric oxide hemoglobin, and the acetone extracts of these compounds were identical with each other and indicative of a low-spin ferrous coordination complex (Tarladgis, 1962). Attempts by Tarladgis (1962) to obtain an electron spin resonance spectrum of this pigment were unsuccessful under the conditions used. The absence of an ESR signal was assumed to indicate a diamagnetic six-coordinate dinitrosyl heme complex 1 as the pigment of cooked cured meats. It was proposed that this pigment was formed on heating the uncooked cured meat pigment, nitric oxide myoglobin, resulting in denaturation of the protein and displacement of the globin by nitric oxide, which occupied both axial coordination positions of the iron.

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