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ALVELD-PRODUCING SAPONINS

I. CHEMICAL STUDIES

By

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ČEH, LOVRO and JENS GABRIEL HAUGE: *Alveld-producing saponins. I. Chemical studies.* Acta vet. scand. 1981, *22*, 391—402. — *Stabursvik* (1959) described the saponin fraction of Narthecium ossifragum as a sarsasapogenin glycoside with the structure arabinose-galactose-xylose-glucose-sarsasapogenin. In a renewed study of the phototoxic lamb disease alveld, in which this saponin has been implicated (*Ender* 1955), we have looked more closely at the saponin fraction. We find that there are two saponins, one major and one minor. Both have a branched trisaccharide on C-3 of the sapogenin. Galactose is directly attached to C-3 in both saponins. The major saponin has glucose and arabinose attached to galactose, the minor saponin has glucose and xylose. We suggest the names narthecin and xylosin for the spirostanol form of these two saponins. In fresh juice from leaves we find little narthecin, however. Most of the saponin is present in the furostanol form, with glucose on C-26. Enzymatic hydrolysis showed this glucose to be bound as a β-glucoside. From specific rotations in partial hydrolysates we conclude that the saccharide on C-3 is a β-D-glucoside, α-L-araboside, β-D-galactoside.

alveld; saponins.

During the digestion of chlorophyll in ruminants phylloerythrin is formed. Some of it is absorbed and excreted again with the bile. A group of phototoxic diseases in sheep is caused by reduced capacity for phylloerythrin excretion, leading to the appearance of phylloerythrin in the periferal circulation. *Ender* (1955) showed that this is the case also for alveld, a disease which each year affects a number of lambs, particularly in Southwestern Norway. It was known from studies by *Slagsvold* (unpublished) that the disease was related to ingestion of Narthecium ossifragum and that this plant contained considerable amounts of saponins. *Ender* (1955, 1960) showed that peroral administration of large amounts of crude saponins from N. ossi-

fragum would elicit the disease. He concluded that the saponins were the probable primary cause, but that further purification would be necessary in order to exclude a role for the contaminants remaining.

Staburšvik (1954, 1959) in a phytochemical study of N. ossifragum, isolated a saponin which he determined to be a sarsasapogenin glycoside with the suggested structure arabinose-galactose-xylose-glucose-sarsasapogenin. The xylose spot in paper chromatography was, however, much smaller than the other spots. He suggested that *Ender*'s preparation could be a mixture of partially hydrolyzed saponins, since such hydrolysis is known to take place in plant extracts on standing. The sarsasaponin isolated by *Staburšvik* was in the form later termed spirostanol (*Tschesche et al.* 1967). This form has sugar groups on C-3 only (Fig. 1a). Steroidal saponins may, however, as demonstrated by *Tschesche,* also have a sugar molecule at C-26, when the ring is open (furostanol form) (Fig. 1b). The second sugar group is easily cleaved off, by enzymes or acid, changing the water soluble

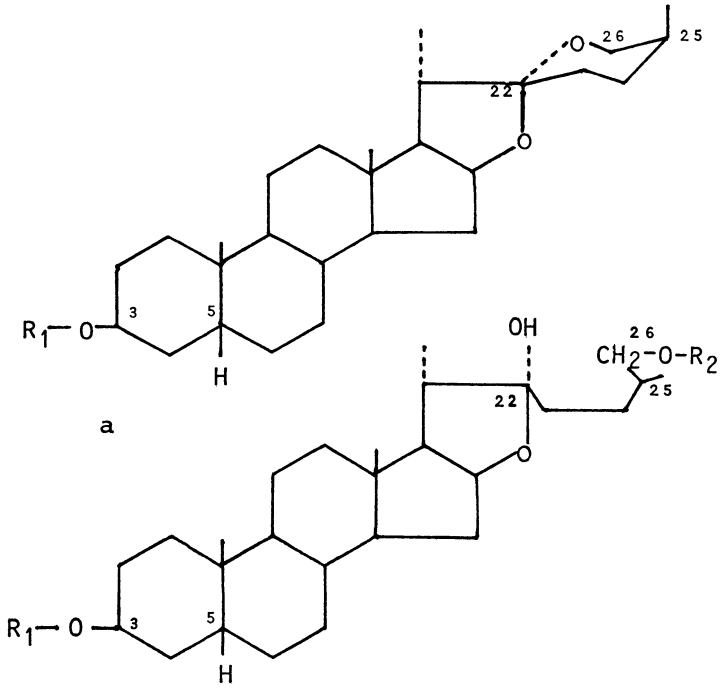

Figure 1. Sarsasaponin. a, spirostanol; b, furostanol.

furostanol into a water insoluble spirostanol. *Staburšvik* did not originally discuss the possible existence of furostanols in N. ossifragum, but has later reported (private communication) that these are present in vivo.

The aim of the present study was to elucidate further the composition of the saponin preparation extracted from N. ossifragum and the structure of the parent saponin(s). The biological activity of these saponins in lambs and rats will be described in a separate communication.

MATERIAL AND METHODS

Plant material

N. ossifragum leaves were picked by hand in the beginning of July on pastures in Bjerkreim, Rogaland, and forwarded to Oslo with railway on the same day. On arrival, the plant material was normally frozen. One half of the material was used for saponin production according to *Ender* (1955). This was kept frozen for several months. The other half, used for direct extraction with ethanol, was generally frozen before drying, but one year it was dried directly. The material picked in 1979 was, however, extracted without drying.

Chromatography

Column chromatography on silica gel (Merck 0.040—0.063 mm) for isolation of the total saponin fraction was carried out with chloroform-methanol-water (65:35:10). For separation of saponin components, a 65:25:10 mixture was used as the mobile phase.

Thin-layer chromatography (TLC) on ready made Merck silica gel G plates was carried out with chloroform-methanol-water (65:35:10) as the mobile phase. Plates were developed with a spray of 1 % vanillin in 50 % phosphoric acid (*Stahl* 1962). After heating for 10—20 min at 120°C, saponins produce red to violet spots. Already before heating the steroid containing spots are seen. Several other reagents tried were less specific. The agent does not distinguish between spirostanol and furostanol. Ehrlich's reagent (*Stahl*) could, however, be used to selectively develop furostanol spots (pink colour) (*Kiyosawa* 1968).

Paper chromatography for carbohydrate analysis was carried out with ethyl acetate-pyridin-water (3.6:1:1.15) and the sugars visualized with an anilin phosphate spray, followed by heating for 5 min at 100°C.

High pressure liquid chromatography (H P L C). Chromatograph ALC/GPC of Waters Associates was initially used with a 3.9 mm ID× 30 cm μ-porasil column for analytical and preparative saponin fractionation. Later the radial compression separation system (RCSS) with Radial-PAK B cartridges (straight phase silica, 5000 plates/10 cm) was used. The mobile phase was chloroform-methanol-water (70:30:3). Separation of some close fractions was better with a 75:25:3 mixture. Carbohydrates were analyzed with a 3.9 mm ID × 30 cm Waters carbohydrate analysis column, using acetonitrile-water (85:15). Samples were introduced with the U6K universal injector, and components detected with the analytical differential refractometer R-401.

Hemolytic index and physical determinations

Hemolytic index of saponin preparations was measured by the method of *Büchi & Dolder* (1950). Hemolytic index is here equal to the volume in ml of a 2 % dilution of cow blood which is completely hemolyzed in 24 h by 1 g of saponin. The method was standardized with a commercial saponin. The extremely low solubility of some preparations resulted in approximate values only.

Infra red spectrophotometry, mass spectrometry and determination of specific rotations were carried out in the Department of Chemistry, University of Oslo.

Preparations

Saponin mixtures. Saponin prepared according to *Ender* (1955) and further purified by crystallization from aqueous ethanol (*Videm et al.* in prep.) was obtained from I. W. Dishington. This is referred to as saponin preparation A. Its hemolytic index was ca 180,000. An alternative method, method B, was worked out starting with a 4 h extraction of dry plant powder with boiling 85 % ethanol. Ethanol was removed from the extract in vacuo and chlorophyll extracted with petrol ether. To the aqueous suspension was added 5 % NaCl, and saponin

extracted with water-saturated n-butanol (3 extractions). Butanol was evaporated and the dry material dissolved in methanol. A crude saponin product was produced by dripping the methanol solution into excess acetone. The crude saponin was dissolved in boiling 40 % ethanol. On cooling, crystalline saponin was obtained. This was further purified by repeated crystallizations from 60—70 % ethanol and, if necessary, chromatography on silica gel columns. This saponin is referred to as saponin B. Its hemolytic index was ca 200,000. The crystals of this preparation melted fairly sharply at 235°C. Mass spectrometry gave 578 as the highest molecular weight, corresponding to sarsasapogenin with one hexose. Infra red analysis (KBr) gave strong absorption in the region 900—1100 nm with peaks at 895 and 915 nm.

Carbohydrates and sarsasapogenin

10—30 mg saponin preparation or isolated component was suspended in a mixture of 3 ml dioxan and 9 ml 2 N H_2SO_4 and heated on a boiling water bath with reflux condenser for 4½ h. After cooling and dilution with water, $BaCO_3$ was added to neutralize the acid. The $BaSO_4$ precipitate was removed by centrifugation, and the supernatant filtered and evaporated to dryness. Carbohydrates were dissolved in acetonitrile-water 1:1, in preparation for chromatography on paper and HPLC.

The sapogenin was dissolved in benzene when TLC-chromatography was to follow (running phase benzene-acetone, 85:15). For further characterization it was purified by recrystallization from acetone. This gave a product of melting point 194—197°C. Commercial sarsasapogenin (Upjohn) melted at 195—198°C. Mixed melting point was 195—198°C. The highest molecular weight in the mass spectrometer was 416, the theoretically expected value. $(\alpha_D)^{20}$ was —78.5 (c = 1, chloroform).

RESULTS

Composition of saponin preparations

TLC-chromatography of a type A saponin preparation reveals the presence of 8 components with a steroid nucleus (Fig. 2b). With B-type preparations components corresponding Nos. 6, 7 and 8 only are found (Fig. 2a). This finding was expected, since the saponins are considerably more exposed to enzymatic hydrolysis in method A.

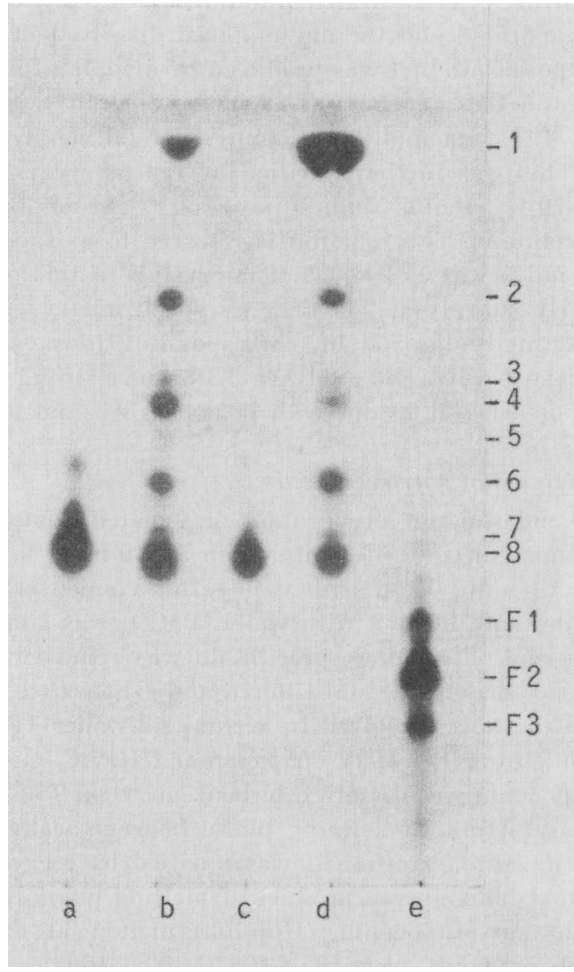

Figure 2. Thin-layer chromatography of saponin preparations. a, saponin B; b, saponin A; c, saponin B further purified; d, saponins as in c, subjected to partial hydrolysis; e, a furostanol preparation.

Washing of B-crystals with chloroform-benzene-methanol 80:10:10 selectively removed component 6 (Fig. 2c). Such a preparation was used to study the result of partial acid hydrolysis. When the preparation was dissolved in 75 % ethanol, 0.5 mol/l HCl and heated on a boiling water bath, hydrolysis products gradually appeared, corresponding to components 1—6 (Fig. 2d). This confirms our view that these components in the

saponin preparations are hydrolytic degradation products of the saponin(s) found in the plant extract.

Identification of the components

In order to identify the substances found in preparation A, it was necessary to isolate each of them in a pure state. Complete separation of all components except the pairs 8—7, 6—5, and 4—3 was achieved on long silica gel columns. Separation of the latter pairs was possible on HPLC, using the Radial PAK B cartridges.

Each of the 8 substances was submitted to acid hydrolysis. Substance 1 did not undergo any change, and on TLC moved with the Rf-value of authentic sarsasapogenin.. The sapogenins extracted from the other 7 hydrolysates all moved as sarsasapogenin. When recrystallized from acetone, the sapogenin obtained on hydrolysis of a saponin B preparation gave a melting point, a specific rotation and a molecular weight in mass spectrometry as expected for sarsasapogenin.

Analysis of the sugar mixture obtained from saponin A and B on complete acid hydrolysis by paper chromatography and HPLC showed arabinose, glucose, galactose and a small amount of xylose. This confirms the report by *Staburšvik* (1959). Analysis of the hydrolysate of the separated components demonstrate, however, that these sugars do not belong to the same saponin, but to one major and one minor sarsasapogenintrisaccharide (Table 1). For each of the substances 3—8 the HPLC-tracing indicated an equimolar amount of the sugars found. This is shown for the parent saponin in Fig. 3.

Table 1. Composition of a saponin A preparation.

Component	Rf	Abundance	Sugars found	Substance	$[\alpha]_D^{20}$
1	0.93	+++	none	sarsasapogenin	—78.5
2	0.74	+++	gal.	monogalactoside	—47.9
3	0.63	+	gal., xyl.	desglucoxylosin	
4	0.61	+++	gal., ara.	desgluconarthecin	—32.3
5	0.56	+	gal., glu.	desxyloxylosin	
6	0.51	+++	gal., glu.	desarabonarthecin	—45.3
7	0.44	+	gal., glu., xyl.	xylosin	
8	0.41	+++++	gal., glu., ara.	narthecin	—29.5

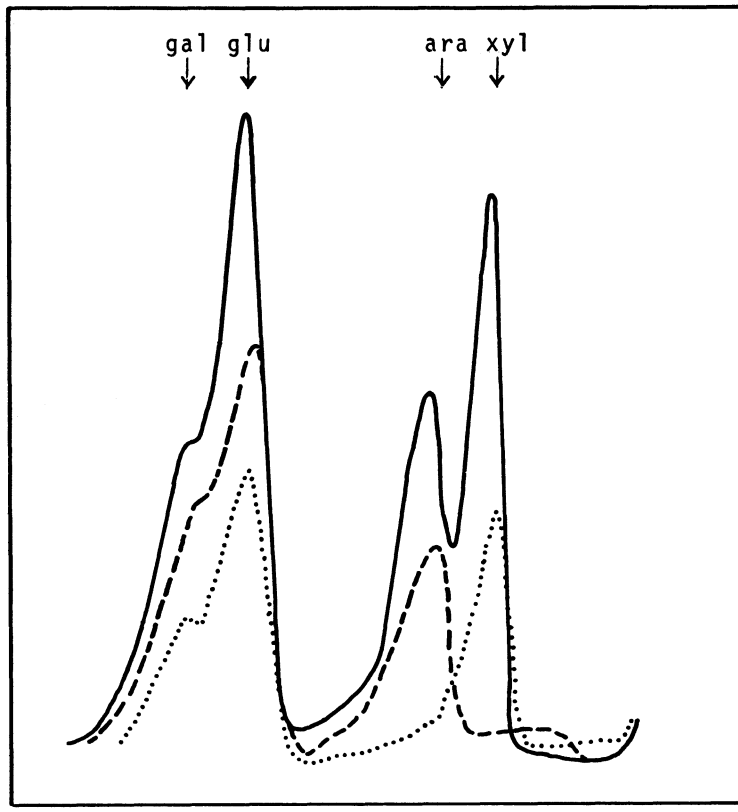

Figure 3. Sugars of narthecin and xylosin analyzed on HPLC. ——— standards (equimolar mixture), - - - - - narthecin, ... xylosin.

We have given the main spirostanol saponin of N. ossifragum the name narthecin, in conformance with the naming of several other saponins in the literature. The minor saponin is provisionally named xylosin. Narthecin gives during preparation method A rise to the major disaccharide components 6 and 4, xylosin to the minor disaccharides 5 and 3. Sarsasapogenin and its monogalactoside are formed from both. The finding in each series of 2 different disaccharides shows that the structure must be branched. The existence of 2 glucose- and galactose-containing disaccharides with different Rf-values shows, in addition, that glucose must be bound differently in narthecin and xylosin. Schematically these structures thus must be as shown in Fig. 4. Narthecin crystals showed a melting point of 270—273°C (decomp) and $(\alpha_D)^{20}$ —29.5° (c = 1, pyridin).

Figure 4. Structure of narthecin and xylosin.

Furostanol saponins in N. ossifragum

We noticed that the yield of crystallizable spirostanol saponin with procedure B was greatly reduced for a batch of N. ossifragum which was not frozen before drying. In the early stages of purification large amounts of material with a steroid nucleus was seen which moved with lower Rf-values than narthecin. This material followed the spirostanol as far as the acetone powder stage, but remained in ethanol solution when the spirostanol saponins crystallized. On TLC, the spots stained with Ehrlich's reagent. These properties were as expected for the furostanol form of a saponin. When frozen N. ossifragum was rapidly thawed and pressed while cold, and the juice analysed on TLC, the plate sprayed with Ehrlich's reagent showed 3 strong pink spots. Another plate sprayed with vanillin-phosphoric acid showed 3 green violet spots with the same Rf-values as the first plate. Spots corresponding to narthecin and its degradation products were, however, absent. The juice thus contained furostanol only.

In the press juice, hydrolysis of the furostanol was relatively slow, 50 % remaining after 1 week at room temperature.

Boiling the press cake with 80 % methanol resulted in extraction of material which on TLC revealed some narthecin, xylosin and desarabonarthecin, but the narthecin spot was still weak compared to the furostanol spots. It thus appears that in N. ossifragum leaves in vivo the saponins are almost exclusively in the furostanol form.

Enzymatic conversion of furostanol to spirostanol

A yellowish brown powder containing approximately 75 % furostanol saponin was prepared from acetone powder by chromatography on a silica gel column (chloroform-methanol-water, 65:35:10). The preparation produced 3 separate spots on TLC (Fig. 2e, Rf 0.33, 0.27, 0.20) and 3 clearly separated fractions

in HPLC. Two of these may be the 22-hydroxy-furostanol and 22-O-methyl-furostanol, respectively, since the C-22 hemiketal group may change to a methylketal in methanolic solution. The third component could be the Δ^{22}-furostanol which is formed in the presence of silica by release of water or methanol from the former (*Tschesche* 1967).

To 250 mg of the furostanol preparation in 20 ml phosphate buffer, pH 5.0, was added 200 units of β-glucosidase (Sigma). After 1 h at 37 °C the solution was turbid from precipitation of spirostanols. After 3 h ca ⅓ of the original furostanol had reacted. TLC showed concomitant appearance of narthecin and some xylosin and desarabonarthecin. In a separate preparation narthecin was carefully purified from the digest in a final yield of 0.52 g/g furostanol. Digestion with the same amount of α-glucosidase had no effect.

The supernatant from the enzyme digestion was analysed for carbohydrates with paper chromatography and HPLC. Both methods showed that it was glucose which was liberated in the process of transforming the furostanol to spirostanol.

The orientation of the glycosidic bond at C-26 is evident from the above. The orientations of the other glycosidic linkages can be inferred from the specific rotations given in Table 1, when these are compared with the known molar rotations of α- and β-methylglycosides for the 3 sugars. We conclude that the saccharide in C-3 is a β-D-glucoside, α-L-araboside, β-D-galactoside. The main saponin of N. ossifragum, which we call narthecioside, thus has the structure shown in Fig. 5. The corresponding aglycone is (25S)-5β-furostan-3β, 22α, 26-triol (*Tschesche et al.* 1967).

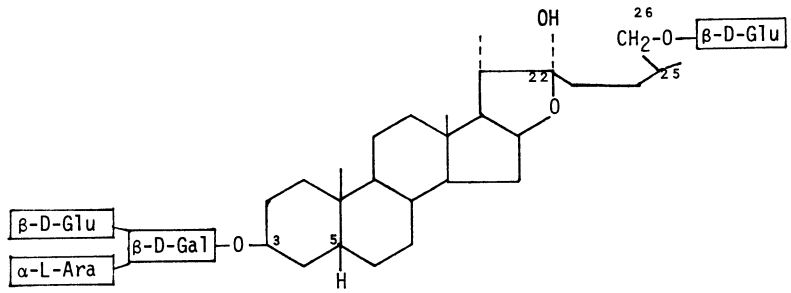

Figure 5. Structure of narthecioside.

DISCUSSION

The present work has confirmed the finding by *Stabursvik* of a sarsasapogenin glycoside in extracts of N. ossifragum. His tentative assignment of the carbohydrate structure to be arabinose-galactose-xylose-glucose has, however, not been confirmed. The small amount of xylose found is explained by its occurrence in a minor saponin, containing xylose instead of arabinose. From the number of hydrolysis products, their sugar composition, and the equimolar ratios observed, it is evident that both the major saponin, narthecin and the minor saponin, xylosin, have branched trisaccharides with galactose directly linked to the sapogenin. It appears, however, that glucose is bound to galactose in different positions in narthecin and xylosin. The elucidation of the exact mode of binding of the sugars will require further studies involving methylation of the saponins.

The spirostanols narthecin and xylosin were found in extracts in yields which varied with the nature of the extracts and the mode of pretreatment of the plant material. The reason for this variation is now clear. Narthecin and xylosin are the products of hydrolysis of the corresponding furostanol, which is the form in which almost all of these saponins exist in the plant. This seems to be the case for other saponins as well (*Tschesche* 1967).

Cases of alveld are produced relatively rapidly when lambs are placed on pastures rich in N. ossifragum, while large amounts of spirostanol saponins given by stomach tube are required to elicit, and sometimes fail to elicit, the disease. It is tempting to speculate that this could be related to the furostanol nature of the saponins in fresh N. ossifragum.

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SAMMENDRAG

Alveldproduserende saponiner.

Stabursvik (1959) bestemte saponinfraksjonen fra Narthecium ossifragum til å være et sarsasapogeninglykosid med struktur arabinose-galaktose-xylose-glukose-sarsasapogenin. I en fornyet studie av den fototoksiske lammesykdommen alveld, der dette saponin synes a spille en sentral rolle (*Ender* 1955) har vi undersøkt saponinfraksjonen nøyere. Vi finner at der er to saponiner, et hovedsaponin og et bisaponin. Begge har et forgrenet trisakkarid på sapogeninets C-3. Galaktose er direkte bundet til C-3 i begge saponiner. Hovedsaponinet har glukose og arabinose bundet til galaktose, bisaponinet har glukose og xylose. Vi foreslår navnene narthecin og xylosin for spirostanolformen av disse to saponiner. I frisk saft fra bladene finnes det imidlertid lite narthecin. Mesteparten av saponinet er til stede i furostanolformen, med et molekyl glukose bundet i C-26. Enzymatisk hydrolyse viste at denne glukose er bundet som β-glukosid. Fra spesifikke rotasjoner i partielle hydrolyseprodukter slutter vi at sakkaridet på C-3 er et β-D-glukosid, α-L-arabosid, β-D-galaktosid.

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