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Studies on *Medicago lupulina* saponins. 1. Isolation and identification of sapogenins from *M. lupulina* tops

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Abstract

Saponins from *M. lupulina* tops were investigated for the first time. Eight aglycones were found in the acid hydrolysates of saponins. All of the aglycones were isolated. On the basis of chromatography, mass spectrometry and infrared spectrometry the aglycones were identified as soyasapogenols B, C, D, E, F and medicagenic acid. Two new aglycones were also isolated and identified as pentacyclic triterpens of β-amyrin structure. Both possess a methyl ester group which is rarely present in nature.

Key words: Medicago lupulina, sapogenins, identification

INTRODUCTION

The isolation and chemical characterization of saponins from *M. lupulina* (black medic trefoil) seeds was reported several years ago (Jurzysta 1973a). It was shown that medicagenic acid glycosides and soyasapogenol glycosides were present in the seeds. The medicagenic acid glycosides are highly active biologically exhibiting fungistatic, hemolytic and antinutritional properties (Gestetner et al. 1971).

M. lupulina tops so far have not been investigated for saponin content, although *M. lupulina* hay is used as animal feed. Hence, the isolation of the aglycones (sapogenins) and the characterization of their composition in *M. lupulina* top saponins is the subject of our present paper.

MATERIAL AND METHODS

Plant material. Field grown *M. lupulina* cv. Renata was used. The tops were cut at the stage of flowering, oven dried at 60° C and ground.

Saponin isolation. One kilogram of ground *M. lupulina* tops was defatted with methylene chloride and saponins were isolated according to Wall et al. (1952).

Acid hydrolysis. 75 g of saponins were hydrolysed in 3 dm^3 of 2 N HCl methanol-water solution (1 : 1), for 0.5, 1, 6 and 12 h in a water bath under reflux. Aglycones were precipitated with water, filtered out; washed with water until washings were neutral and dried.

Fractionation of neutral and acid aglycones. 20 g of aglycones were dispersed in 1 dm^3 of 5% aqueous NaOH and extracted 3 times with chloroform in a separatory funnel. The chloroform phase (PhI) was washed with water until neutral and stored (neutral aglycones). The water phase was then acidified with HCl to pH 4.0 and extracted 3 times with ethyl acetate (PhII) the ethyl acetate phase was also neutralised and stored (acid aglycones).

Purification of aglycones. The two obtained extracts were subjected to column chromatography. The presence of aglycones in the eluates was analysed by TCL. PhI (chloroform extract) was layered on a preparative column — 12 × 6 cm Silica Gel 60, Merck. The filtrate containing impurities (TLC) was discarded. The column was then eluted with diethyl ether. The mixture of 7 neutral aglycones (named B, C, D, E, F, N and An) was found, in that eluate. PhII (ethyl acetate extract) was filtered through the gel column as above. The ethyl acetate filtrate was free of impurities and contained only one acid aglycone (named Ma). Ethyl acetate was evaporated and the precipitate crystallized from chloroform-methanol, yielding crystalline acid aglycone (Ma).

Isolation of neutral aglycones. 0.5 g of neutral aglycone precipitate was dissolved in chloroform and layered on a 50 × 3 cm gel column (Kiselgel 60, 70-230 mesh, Merck). The column was eluted with a gradient of 1-3% ethanol in benzene.

Separation of acetyl derivatives of aglycones. Acetyl derivatives of glycones were separated on a 32 × 2.5 cm Silica Gel column as above. Cyclohexane-acetone (95 : 5) was used for elution. The separated individual acetyl derivatives of the aglycones were then crystallized.

Thin layer chromatography (TLC) of aglycones. Adsorbent — Silicagel (DC-Kieselgel 60, Merck) pre-coated plates. Solvent systems: S_1 petroleum ether-chloroform-acetic acid (7 : 2 : 1), S_2 benzene-ethanol (92 : 8), S_3 cyclohexane-acetone (90 : 10), S_4 hexane-benzene-acetone (50 : 45 : 5), S_5 hexane-diethyl ether (65 : 35). Detection reagents: dry developed plates were sprayed with the following reagents and heated at 100° C for 5 min.: R_1 — Liebermann-Burchard reagent (Van Atta and Gugg-

olz 1958), R_2 — mixture of phospho-molibdenic acid (25 g), Cs_2SO_4 (10 g), water (940 cm^3), concentrated H_2SO_4 (60 cm^3).

Preparation of methyl esters of aglycones. Aglycones dissolved in ether were treated with an etheral solution of CH_2N_2 until yellow colour was visible (Vogel 1964). Then ether was evaporated and the product crystallized from chloroform-methanol.

Preparation of acetyl derivatives of aglycones. All aglycones were treated overnight with a mixture of acetic anhydride, piridine (1 : 1) at room temperature, excluding Ma which was boiled for 3 h under reflux in the same solvent mixture. Acetylated aglycones were then crystallized as above.

Basic hydrolysis. Acetyl derivatives of aglycones were dissolved in 5% KOH in methanol and heated in a water bath for 3 h. Water was then added, the pH adjusted to 4.0 with HCl, methanol evaporated and the precipitate filtered. The obtained products were crystallized.

Melting point. Melting point (mp) was determined on a Büchi apparatus.

Spectral analyses. Infrared spectra (IR) were determined in KBr using a Perkin-Elmer 412 Spectrophotometer. Mass spectra (MS) were run on LKB 9000 Spectrophotometer (70 and 15 eV).

Standards. Standard samples of soyasapogenols and medicagenic acid were isolated and identified from soyabean and alfalfa as previously described (Jurzysta 1982).

Characterization of aglycones. Soyasapogenol B: needle crystals (chloroform-methanol), mp 257° (257-259°), IR identical with that of standard, MS (m/z) 458 (M^+), 440, 425, 409, 288, 234, 224, 219, 216, 106, 201, 175 same as of authentic soyasapogenol B.

Triacetate of soyasapogenol B: needle crystals chloroform-methanol), mp 178° (179-180°), IR identical with that of standard MS (m/z) 584 (M^+), 524, 509, 464, 307, 276, 247, 216, 201, 174, 132 same as of authentic 3 Ac soyasapogenol B.

Soyasapogenol C: needle crystals (benzene-chloroform), mp 239-244° (239-240°) IR indentical as standard MS (m/z) 440 (M^+), 425, 224, 216, 207, 201, 175, 134, 133, 132, 121, 95 same as authentic soyasapogenol C.

Diacetate of soyasapogenol C: needle crystals (chloroform-methanol), mp 200° (200°), IR identical as standard MS (m/z) 524 (M^+), 509, 464, 389, 307, 247, 216, 201, 173, 95 same as of authentic 2Ac soyasapogenol C.

Soyasapogenol D: plate crystals (chloroform-methanol), mp 295-297 (297-298°), IR identical as standard MS (m/z) 472 (M^+), 457, 440, 425, 248, 235, 224, 223, 216, 206, 203, 99 same as of authentic soyasapogenol D.

Diacetate of soyasapogenol D: plate crystals (chloroform-methanol) mp 189-190° (191°), IR identical as of standard MS (m/z) 556 (M^+), 541, 524, 307, 248, 235, 221, 216, 203, 99 same as of authentic 2 Ac soyasapogenol D.

Soyasapogenol F: needle crystals (chloroform-methanol), mp 314-316° (316-318°), IR identical as of standard, MS (m/z) 458 (M^+), 443, 440, 425, 422, 427, 399, 267, 234, 224, 221, 220, 216, 206, 205, 203, 201, 193, 189, 187, 175 same as of authentic soyasapogenol F.

Diacetate of soyasapogenol F: needle crystals (chloroform-methanol), mp 206-207° (208-210°), IR identical as of standard, MS (m/z) 584 (M^+), 524, 522, 509, 464, 391, 389, 307, 276, 263, 248, 247, 221, 216, 204, 203, 187, same as of authentic 2 Ac soyasapogenol F.

Medicagenic acid: needle crystals (dioxane-water) mp 354-355° (350-351°), IR identical as of standard, MS (m/z) 502 (M^+), 456, 248, 233, 221, 203, 189, 133 same as of authentic medicagenic acid.

Diacetate of medicagenic acid: small needle crystals (chloroform-methanol), mp 210-215° (209-210°), IR identical as of standard, MS (m/z) 586 (M^+), 526, 466, 248, 233, 203, 189, 133 same as of authentic 2Ac medicagenic acid.

Dimethyl ester of medicagenic acid: plate crystals (chloroform-methanol), mp 216-219° (218-220°).

Aglycon An: white needle crystals (chloroform-methanol), mp 286-288°, IR V_{max} (cm^{-1}) 1730 (COO), 3200-3400 (OH), (Fig. 4) MS (m/z) 502 (12%) M^+, 443 (23) M-COOMe, 420 (13), 278 (37) DE_1, 265 (46), 260 (13) DE_1-H_2O, 247 (35) DE_1-MeOH, 224, (32) AB_1, 219 (36) DE_1-COOMe, 206 (51) AB_2, 187 (80) DE_1-COOMe+MeOH, 175 (77) AB_3, 135 (35), 121 (55), 109 (57), 105 (56), 91 (50), 81 (60), 79 (42), 69 (42), 55 (79), 43 (67), 41 (100), 27 (44).

Triacetate of aglycon An: plate crystals (chloroform-methanol), mp 200-201°, IR V_{max} (cm^{-1}) 1730 (COO), lack of 3200-3400 (OH). MS (m/z) 628 (1.2) M^+, 568 (8) M-HOAc, 508 (1.5) M-2x HOAc, 448 (1.2) M-3x HOAc, 320 (100) DE_1, 308 (12) AB_1, 260 (53) DE_2, 248 (18) AB_1-HOAc, 228 (8), 200 (28), 188 (11), 175 (2), AB_3, 172 (7), 120 (5), 106 (5), 95 (8).

An_2 derivative: white needle crystals (chloroform-methanol), mp 315-319°, IR V_{max} (m^{-1}) 1730 (COO), 3200-3400 (OH), MS (m/z) 488 (4) M^+, 470 (4) M-H_2O, 452 (3) M-2H_2O, 264 (100) DE_1, 246 (28) DE_1-H_2O, 224 (27) AB_1, 219 (10) DE_1-COOH, 217 (58), 206 (23), AB_2, 175 (32) AB_3, 147 (8), 135 (9), 122 (7), 112 (4).

Aglycon N: white needles (chloroform-methanol), mp 268-269°, IR V_{max} cm^{-1} 1710 (C =O), 1730 (COO), 3200-3400 (OH) MS (m/z) 500 (3) M^+, 482 (2) M-H_2O, 44 (3) M-COO Me, 276 (85), DE_1, 261 (8) DE_1-CH_3, 247 (17), 224 (25) AB_1, 206 (30) AB_2, 175 (71) AB_3, 173 (25), 133 (45), 121 (27), 119 (45), 114 (100), 55 (54).

Diacetate of aglicon N: white needles (chloroform-methanol), mp 241°, IR V_{max} (cm^{-1}) 1710 (C = O), 1730 (COO), lack of 3200-3400 (OH). MS (m/z) 584 (3) M^+, 524 (3) M-HOAc, 464 (2) M-2x HOAc, 327 (2) AB_1,

276 (77) DE_1, 261 (5) DE_1-CH_3, 248 (14) AB_2, 188 (32), 187 (23), 175 (11) AB_3, 114 (74), 43 (100), CH_3-$C^+ = 0$.

RESULTS

ISOLATION OF AGLYCONES

Crude saponins were isolated from *M. lupulina* tops with a yield of 9%. The saponins were subjected to acid hydrolysis for 0.5, 1, 6 and 12 hours. In acid hydrolysates, 8 aglycones were found and named B, C, D, E, F, N, An and Ma. The relative quantities of the aglycones varied with the time of hydrolysis.

The yield of aglycon B decreased with the time of hydrolysis and other aglycone yields increased. For this reason, 0.5 h hydrolysis was employed to obtain B and 12 h hydrolysis to obtain the other aglycones.

Aglycones were then fractionated to neutral and acidic ones taking advantage of their different solubility in acidic and basic water solutions. In the acid fraction only one aglycone, Ma, was found. After purification on the silica gel column, Ma was isolated in the crystalline form (1.2 g).

The neutral fraction contained 7 aglycones. These aglycones were purified by filtration on silica gel and separated using column chromatography. By column chromatography, 70 mg of C, 20 mg of B, 60 mg of D, 50 mg of F, 50 mg of N, were isolated.

One chromatographically homogenous fraction of E and a fraction of unseparated F and An were also obtained. Since no resolution of N and An aglycones was obtained with further column separation, the mixture of aglycones F and An was subjected to acetylation and acetyl (Ac) derivatives chromatographed on a silica gel column. Separation of AcF and AcAn was obtained. AcF and AcAn were subjected to basic hydrolysis yelding F and An. After crystallization, 60 mg of F and 30 mg of An were obtained.

Summing up, the following quantities of individual aglycones were isolated: Ma — 1200 mg, B — 20 mg, C — 70 mg, D — 60 mg, F — 110 mg, N — 50 mg and An — 30 mg.

IDENTIFICATION OF AGLYCONES

Aglycones were chromatographed on silica gel plates using S_1 and S_2 solvent systems. Acetyl derivatives of aglycones were developed in the S_3, S_4, and S_5 solvent systems. The standard soyasapogenols and their acetyl derivatives were also chromatographed (Fig. 1).

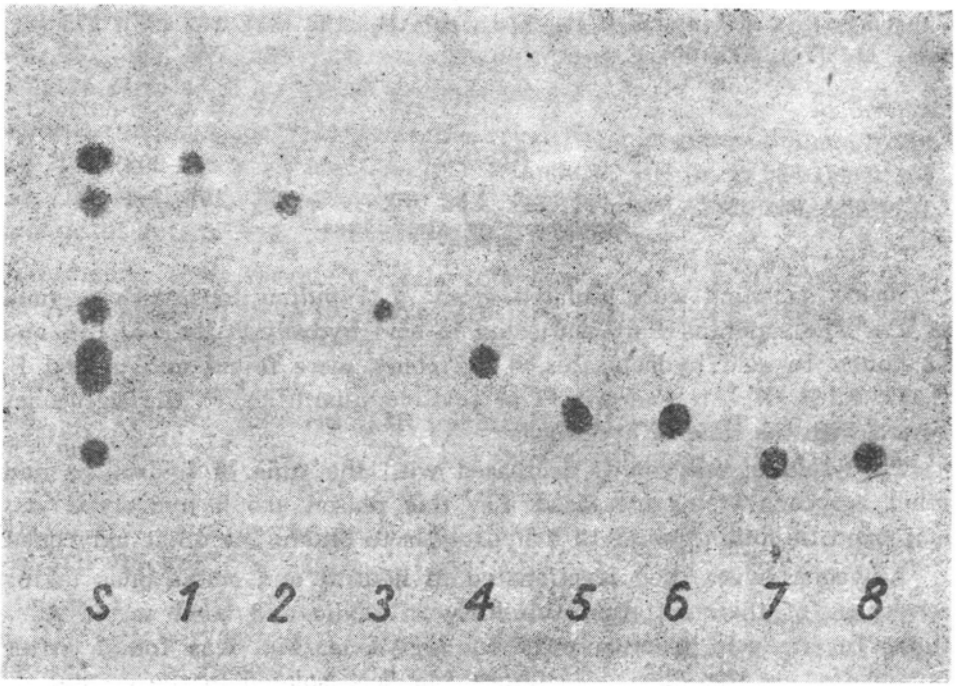

Fig. 1. TL chromatogram of *M. lupulina* sapogenins. S — standard soyasapogenols (from top to bottom): C, D, E, B, F and medicagenic acid. 1-8 aglycones isolated from *M. lupulina* tops: 1-5 soyasapogenols C, D, E, B, F; 6 — aglicone An; 7 — aglicone N; 8 — medicagenic acid. Chromatogram developed in S_1 detected with R_2 reagent

On the basis of TLC, co-chromatography, IR and MS analyses it was found that aglycones B, C, D, E, F and Ma were identical with soyasapogenols B, C, D, E, F and medicagenic acid. But aglycones N and An were not identical with any of the standards.

MS of aglycon An is characteristic of β-amyrine (B u d z i k i e w i c z et al. 1963). Molecular weight (MW) determined as M^+ was 502 (MW calc. for $C_{31}H_{50}O_5 = 502$). MW of acetylated aglycone An was 628 suggesting the presence of 3 acetate (Ac) groups: $502 + 3 \times 42$ (Ac) $= 628$ (Fig. 2). MS confirm that suggestion showing 3 peaks (m/z) 568, 508, 448. Because only hydroxyl groups were acetylated, aglycon An possessed 3 OH groups.

Aglycone An subjected to basic hydrolysis gave An_2 derivative of MW 448. Demethylation of aglycone An to An_2 was confirmed with MW changes 502-14 $(CH_2) = 488$. An_2 derivative after methylation gave aglycon An (the same TLC, IR, mp), which indicated that An carried a COOMe function. The presence of a COO group was also confirmed by IR (Fig. 4).

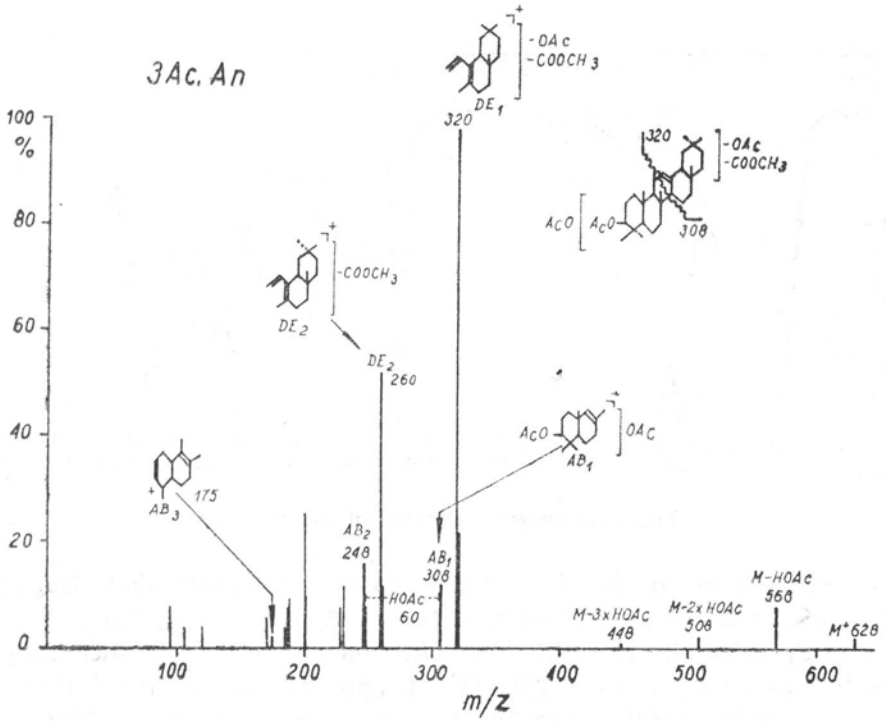

Fig. 2. Mass spectrum of triacetate of aglycone An (3AcAn)

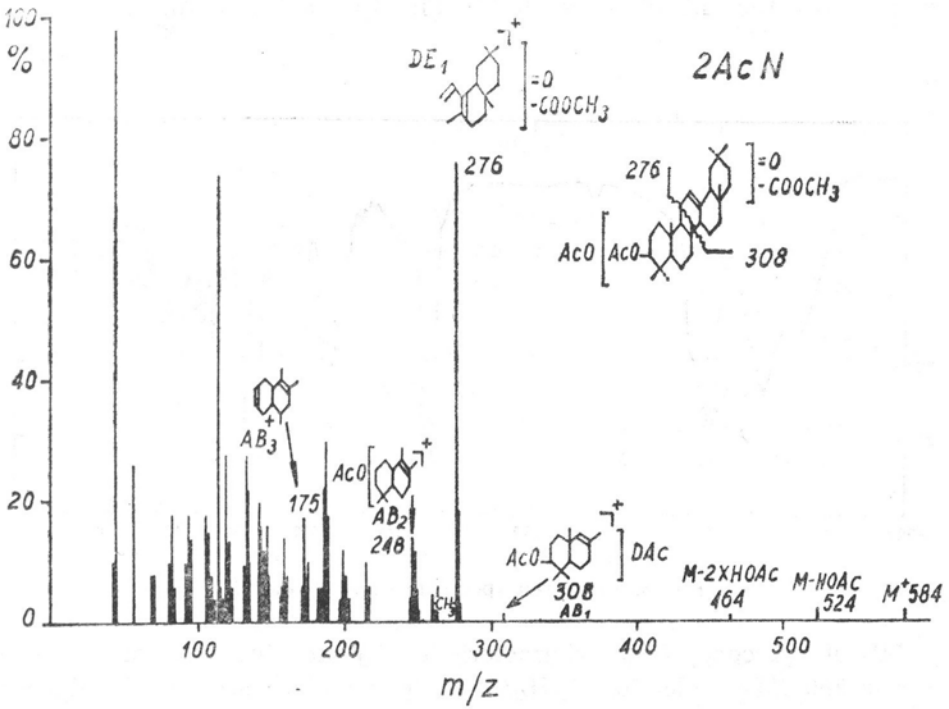

Fig. 3. Mass spectrum of diacetate of aglycone N (2AcN)

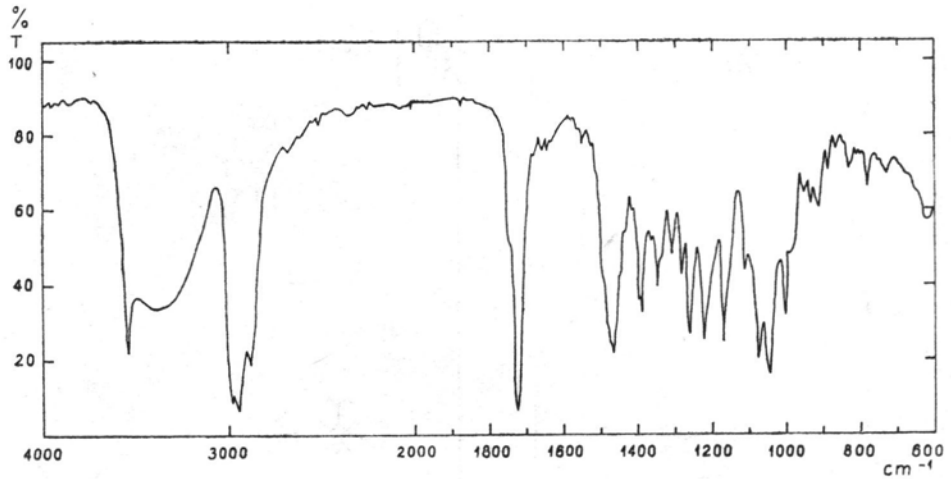

Fig. 4. Infra red spectrum of aglycone An

Mass spectrum of An showed the typical retro-Diels-Alder fragmentation of the C ring, resulting in a fragment AB_1 — m/z 224 and fragment DE_1 m/z 278. The MS fragmentation of Ac An revealed AB_1 of m/z 308 and DE_1 of m/z 320. This fragmentation confirmed that An carried two hydroxyl groups in the A/B ring and one hydroxy and one COOMe function in the D/E ring. On the basis of the data obtained we proposed the formula presented in Fig. 6 for aglycon An.

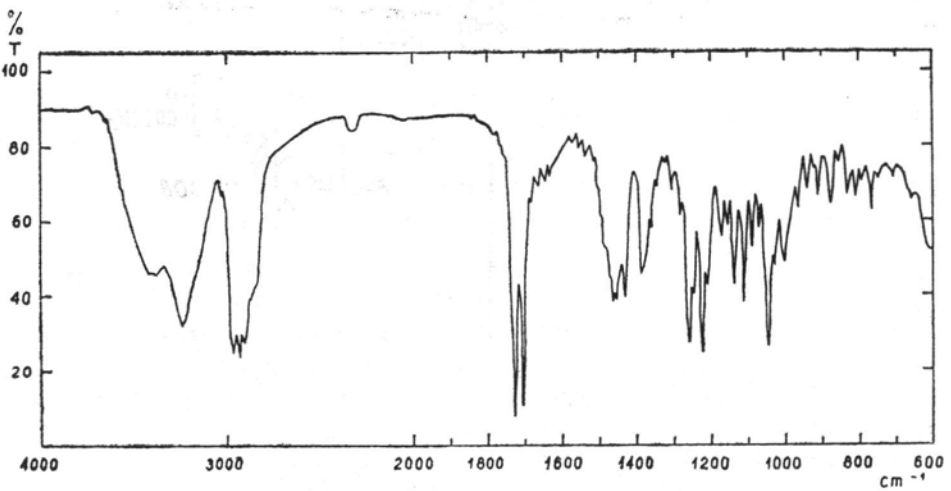

Fig. 5. Infra red spectrum of aglycone N

MS of aglycone N was characteristic of β-amyrine. MW of aglycone N was 500 (MW calc. for $C_{31}H_{48}O_5$ = 500) and of acetylated aglycone N was 584 suggesting the presence of 2 Ac groups in AcN and 2 OH

groups in aglycone N: 500 + 2 × 42 (Ac) = 584 (Fig. 3). Aglycone N subjected to acid hydrolysis yielded N_2 derivative. Further methylation of N_2 derivative gave again aglycone N (the same TLC, mp) which indicated the presence of a COOMe group in N. The presence of C = O and COO groups was also confirmed by IR (Fig. 5).

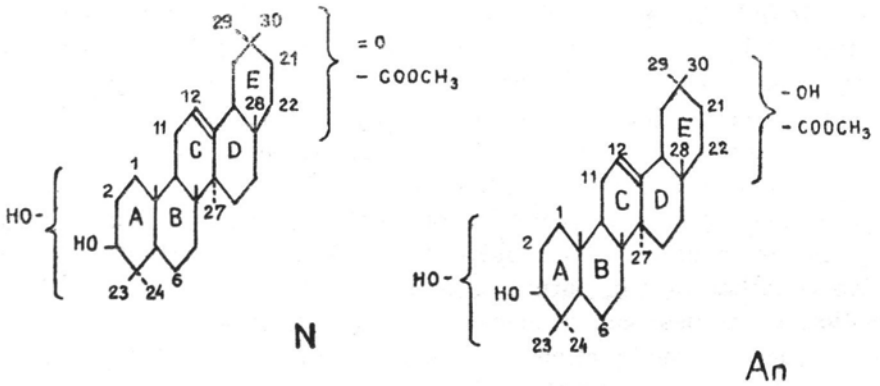

Fig. 6. The proposed formulas of aglycones An and N, respectively

From the retro-Diels-Alder fragmentation of N it was found that the A/B ring carried two hydroxyl groups (AB_1 — m/z 244) and the D/E ring carried one hydroxyl and one COOMe group (DE_1 = m/z 276). One the basis of the data obtained we proposed for aglycone N the formula presented in Fig. 6.

DISCUSSION

The saponins in *M. lupulina* tops have not been investigated so far, hence our first attempt was to analyse their sapogenin composition. The chemical composition of the saponins will be the subject of our next paper.

In the acid hydrolysate of isolated saponins, 8 aglycones were found. All aglycones except one were isolated in crystalline form. On the basis of MS, IR and mp analyses using standards, the aglycones were identified as soyasapogenols B, C, D, F and medicagenic acid. Soyasapogenol E was identified only with TLC. Two aglycones (N, An) were isolated for the first time.

Soyasapogenols were also found in the saponins isolated from the tops of soybean, alfalfa and clover (Gestetner et al. 1966, 1970, Walter et al. 1955). Medicagenic acid was found to be the predominant sapogenin in roots and tops of alfalfa (Gestetner et al. 1970), although it is absent in the seeds (Jurzysta 1973b). Unlike the seeds of alfalfa the seeds of *M. lupulina* were found to contain medicagenic

acid (Jurzysta 1973a). Now it was found that medicagenic acid was the major component of saponins from the tops of *M. lupulina*.

The presence of medicagenic acid in the tops of *M. lupulina* is important because of its high biological and antinutritional activities (Roshef et al. 1976). The biological activity of *M. lupulina* saponins will be the subject of our next paper.

We found that the relative quantity of soyasapogenols varied with the time of hydrolysis. The yield of soyasapogenol B decreased and that of soyasapogenols C, D, F increased with the time of hydrolysis. These results agreed with the previous findings of Jurzysta (1978) that soyasapogenols C, D and F are artefacts produced by the destruction of soyasapogenol B during acid hydrolysis.

Two new isolated aglycones were identified as pentacyclic triterpens of β-amyrin structure. Both aglycones possess the methyl ester group which is rather rare in nature (Connolly and Overton 1972). The question is if these aglycons are natural compounds or whether they are products of methylation developed during the time of acid hydrolysis in a solution containing methanol.

The structural formulas for the two new aglycones N and An are proposed (Fig. 6). Although the exact position of some functional groups in both aglycones was not diffined, on the basis of the fragmentation pattern of new aglycones we supposed that COOMe functions in An and N are bound to C 17 because the M — 59 peak exceeded the M^+ peak. If the COOMe group was in C 30, the M^+ peak would exceed the M — 59 peak (Budzikiewicz et al. 1963). Likewise from other characteristic fragment patterns we proposed that OH groups were attached to C 23 in An and N. The third hydroxyl group was in the C 21/C 22 position in An and keto group was attached to C 21/C 22 in N.

The purification and chemical characterization of saponins from the tops of *M. lupulina* are being investigated and will be the subject of our next paper.

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Badania nad saponinami Medicago lupulina. 1. Izolacja i identyfikacja sapogenin z części nadziemnych M. lupulina

Streszczenie

Po raz pierwszy badano skład aglikonowy saponin z części nadziemnych lucerny chmielowej. W kwaśnych hydrolizatach saponin stwierdzono obecność 8 aglikonów. Aglikony wyizolowano i zidentyfikowano na podstawie chromatografii i analiz spektralnych (IR, MS) jako sojasapogenole B, C, D, E, F i kwas medikagenowy. Wśród wyizolowanych aglikonów znaleziono dwa nowe, które zidentyfikowano jako trójterpeny pięciocykliczne o strukturze ß-amyryny.