36. Saponins with Molluscicidal Properties from *Lonicera nigra* L.

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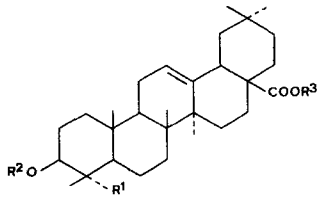
Summary

Eight triterpenoid saponins (**1–8**) have been isolated from the berries of *Lonicera nigra* L. *(Caprifoliaceae)* by combination of droplet counter-current chromatography and column chromatography on silica. The structures have been established by ^{1}H-NMR., ^{13}C-NMR. spectroscopy, FD.-MS., FAB.-MS. and D./CI.-MS., and on the basis of acidic and basic hydrolyses. The monodesmosidic saponins **1–5** possess strong molluscicidal activity against the schistosomiasis-transmitting snail *Biomphalaria glabrata,* whereas the bidesmosidic saponins **6–8** are inactive.

Introduction. – Although chemotherapy is one of the most valuable methods for the fight against schistosomiasis, there is still a need for more selective and efficient molluscicides. The use of plants with molluscicidal properties is a simple, inexpensive and appropriate technology for the control of snail vector [1]. Since the discovery of potent saponins in the fruits of *Phytolacca dodecandra* L'HERIT *(Phytolaccaceae)* [2] [3], naturally occurring molluscicides are receiving considerable attention.

In connection with our systematic screening studies on compounds with molluscicidal activity from medicinal plants [4], we reported recently about some structure/activity-relationship considerations which should facilitate the selection of active plant sources [5]. Among the selected plants, there is *Lonicera nigra* L. *(Caprifoliaceae).* The methanolic extract of the berries exhibits strong molluscicidal activity against *Biomphalaria glabrata* snails. We now report on the isolation and structure elucidation of the active saponin fraction.

Results. – The fresh berries (500 g) were extracted with MeOH. The crude extract (15 g) was chromatographed on a silica gel column with $CHCl_3$/MeOH/H_2O 75 : 23 : 2; during elution, the amount of MeOH was gradually increased. Three saponin fractions were collected. The biological active molluscicidal fraction I (0.9 g) was submitted to repeated droplet counter-current chromatography (DCCC.) [6] with $CHCl_3$/MeOH/H_2O 25 : 46 : 29 in the descending mode. The pure saponins **1–5** (70, 10, 50, 15 and 10 mg, respectively) were obtained.



Compound	R^1	R^2	R^3
1	CH_2OH	Arabinosyl	H
2	CH_3	Glucosyl-(1→2)-arabinosyl	H
3	CH_2OH	Glucosyl-(1→2)-arabinosyl	H
4	CH_3	Glucuronyl	H
5	CH_2OH	Glucuronyl	H
6	CH_2OH	Arabinosyl	Gentiobiosyl
7	CH_3	Glucosyl-(1→2)-arabinosyl	Gentiobiosyl
8	CH_2OH	Glucosyl-(1→2)-arabinosyl	Gentiobiosyl
9	CH_2OH	H	H
10	CH_3	H	H

Arabinosyl: α-L-arabinopyranosyl
Glucosyl: β-D-Glucopyranosyl
Glucuronyl: β-D-Glucuronopyranosyl
Gentiobiosyl: β-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl

The more polar saponins **6–8** (without molluscicidal activity) have been isolated from fractions II (1.3 g) and III (1.2 g). Fraction II was submitted to DCCC. with $CHCl_3$/MeOH/H_2O 25:45:30 in the descending mode. From the remaining stationary phase, compounds **6** and **7** were recovered. Saponin **6** was further purified on silica gel with $CHCl_3$/MeOH/H_2O 60:32:8 (lower phase). Finally, 70 mg of pure **6** were obtained. Saponin **7** was purified by DCCC. with BuOH/acetone/H_2O 35:10:55 in the descending mode, followed by gel filtration on *Sephadex LH-20* with MeOH, which afforded 10 mg of pure **7**. Fraction III (1.2 g) was chromatographed on silica gel with $CHCl_3$/MeOH/H_2O 25:45:30 (lower phase) and yielded a mixture of **7** and **8**. Further purification on *Sephadex LH-20* with MeOH, followed by silica gel column chromatography with the lower layer of $CHCl_3$/MeOH/H_2O 65:28:7 afforded 15 mg of **7** and 25 mg of **8**.

Acidic hydrolysis of compounds **1**, **3**, **5**, **6** and **8** afforded the same aglycone (mol.-wt. = 472, $C_{30}H_{48}O_4$) identified as hederagenin (**9**) from MS. data of its methyl ester (Mol.-wt. = 486) [7] and by ^{1}H- and ^{13}C-NMR. spectroscopy [8], whereas the aglycones of **2**, **4** and **7** were identified as oleanolic acid (**10**) by comparison with an authentic sample. The sugars obtained from the saponin hydrolysates were arabinose in **1**, arabinose and glucose in **2**, **3** and **6–8** and glucuronic acid in **4** and **5**. Basic hydrolysis of **6** yielded **1**, of **7** yielded **2**, of **8** afforded **3**, whereas **1–5** were not affected. In all the three cases, the sugar moiety was identified as gentiobiose (β-D-glucopyranosyl-(1→6)-β-D-glucopyranose) by comparison with an authentic sample (TLC. and HPLC.). Thus, compounds **1–5** are monodesmosidic saponins and **6–8** bidesmosidic saponins with the gentiobiosyl attached through an ester bond at C(28) of the aglycone.

Compound **1** was identical in all respects with hederagenin-3-*O*-*α*-L-arabinopyranoside [9], previously isolated in our laboratory [4]. The molecular weight and the sugar sequence of saponins **2–8** were established by the combination of different MS. methods without derivatization of the glycosides. The desorption/chemical-ionization MS. (D./CI.-MS.) [10] of **3** (reactant gas NH_3) shows signals at *m/z* 784 and 767 corresponding to the quasi-molecular ions $[M+NH_4]^+$ and $[M+H]^+$, respectively. Signals at *m/z* 622 ($[(M+NH_4)-162]^+$) and 605 ($[(M+H)-162]^+$) correspond to the fragments produced by the loss of a glucosyl unit and, thus, indicate that glucose is the terminal sugar. Signals at *m/z* 490 ($[(M+NH_4)-294]^+$) and 473 ($[(M+H)-294]^+$) are due to the loss of a glucosyl-arabinosyl moiety. A significant peak at *m/z* 312 ($[294+NH_4]^+$) corresponds to this sugar moiety. Some aglycone fragments (*m/z* 455, 437, 248 and 204) are characteristic of an oleane-type triterpene [7]. Compound **2** shows a similar fragmentation pattern with a shift of 16 amu resulting from the substitution of the CH_2OH-group by a CH_3-group in the aglycone. These observations confirm the sequence glucose-arabinose-oleanolic acid.

Compound **3** was submitted to fast-atom-bombardment MS. (FAB.-MS.), a recently developed technique for highly polar or thermally labile compounds [11]. The following signals were observed: *m/z* 859 ($[(M+H)+92]^+$) corresponding to a cluster with glycerol used as solvent; *m/z* 767, the quasi-molecular ion $[M+H]^+$; *m/z* 455 and 437 which are fragments of the aglycone *(Fig., a)*. The sugar sequence was established by the negative-ion FAB.-MS. *(Fig., b)* with following signals: quasi-molecular ions at *m/z* 857 ($[(M-H)+92]^-$) and 765 ($[M-H]^-$) and a frag-

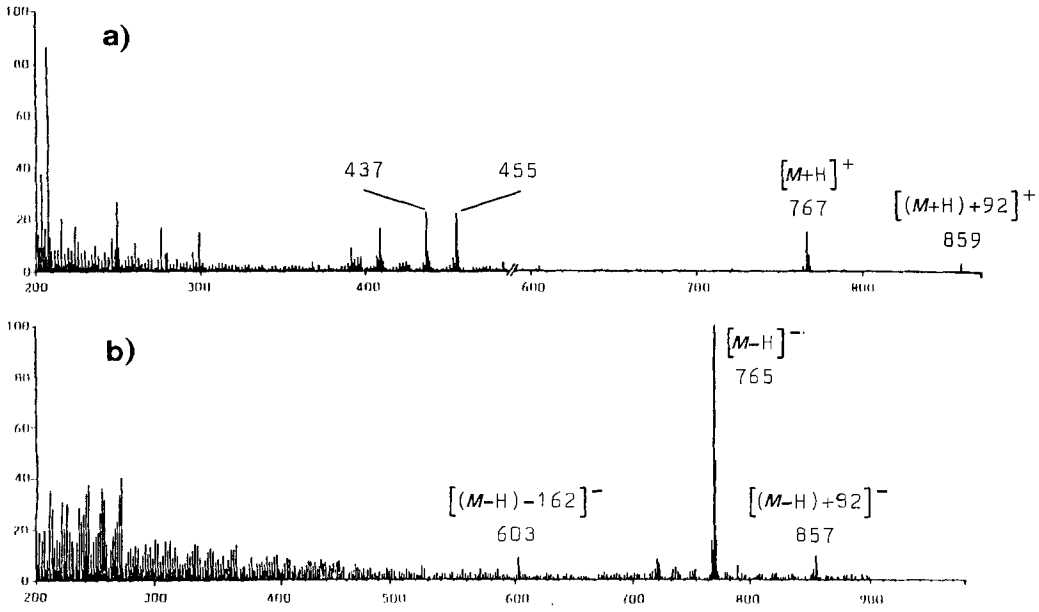


Figure. *FAB.-MS. of saponin* **3**. a) positive ion; b) negative ion

ment at 603 ($[(M-H)-162]^-$) corresponding to the loss of a glucosyl unit. FAB.-MS. was also used to confirm the structures of **4** and **5** which are glucuronosides of oleanolic acid and hederagenin, respectively. In the FAB.-MS. of **5**, following cluster and quasi-molecular ions were observed: *m/z* 779 ($[(M+K)+92]^+$); 763 ($[(M+Na)+92]^+$); 741 ($[(M+H)+92]^+$); 687 ($[M+K]^+$); 671 ($[M+Na]^+$) and 649 ($[M+H]^+$). The negative-ion FAB.-MS. showed a quasi-molecular ion at *m/z* 647 ($[M-H]^-$).

The sugar sequence of **6** was determined by D./CI.-MS. At *m/z* 946 appears the quasi-molecular ion $[M+NH_4]^+$. Sugar-cleavage fragments are observed at *m/z* 814 ($[(M+NH_4)-132]^+$); 622 ($[(M+NH_4)-324]^+$) and 605 ($[(M+H)-324]^+$); 490 ($[(M+NH_4)-456]^+$) and 473 ($[(M+H)-456]^+$) corresponding to the loss of an arabinosyl unit, two glucosyl units and all the sugars, respectively. A signal at *m/z* 342 ($[324+NH_4]^+$) confirms the presence of a gentiobiosyl moiety. The D./CI.-MS. results support the observations made from the acidic and basic hydrolysis suggesting that an arabinosyl moiety is attached at C(3) and a gentiobiosyl moiety at C(28) of hederagenin.

The quasi-molecular ions of **7** and **8** are not observed in the D./CI.-MS. The fragmentation patterns of **7** and **8** correspond to those of **2** and **3**, respectively. However, an additional peak at *m/z* 342 ($[324+NH_4]^+$) can be observed. This signal corresponds to the gentiobiosyl moiety attached at C(28) of the aglycone. The structures of **7** and **8** were furthermore confirmed by FD.-MS. [12]. In the FD.-MS. of **8**, quasi-molecular ions are observed at *m/z* 1113 ($[M+Na]^+$) and 1129 ($[M+K]^+$). Signals at *m/z* 951 ($[(M+Na)-162]^+$); 789 ($[(M+Na)-324]^+$) and 495 ($[(M+Na)-618]^+$) correspond to the loss of one glucosyl unit, two glucosyl units and one arabinosyl and three glucosyl units, respectively. The signal at *m/z* 789 shows a high intensity because the sugar moiety attached on C(28) is more easily eliminated. Similar conclusions can be drawn from the FD.-MS. of **7**.

The interglycosidic linkages as well as the position of attachment of the sugar chains to the aglycones were established by ^{13}C-NMR. spectroscopy. In all the isolated saponins, a sugar chain was linked to the aglycone at C(3): C(3) of **1**, **3**, **6** and **8** appeared at 82.2 ppm[1]) whereas the chemical shift of this C-atom was 76.4 ppm in hederagenin [8]; C(3) of **2** and **7** appeared at 88.9 ppm[1]) whereas in oleanolic acid, this C-atom can be observed at 78.8 ppm.

In **2**, **3**, **7** and **8** which carry a glucosyl-arabinosyl moiety at C(3) of the aglycone, the anomeric C-atom of the arabinosyl moiety appears at 104.5 ppm[1]) and C(2′) at 81.1 ppm[1]). The chemical shifts of the anomeric C-atom and of C(2′) in compound **1** and **6** carrying only an arabinosyl unit are 106.5 ppm[1]) and 73.4 ppm[1]), respectively. These differences in chemical shifts can be explained by a substitution effect by another glycosyl linkage at C(2′) [13].

In the bidesmosidic saponins **6**, **7** and **8**, the two glucopyranosyl units forming the gentiobiosyl moiety attached at C(28) are linked in position 1→6. The C(6″)-signal (terminal glucose moiety) appears at 62.7 ppm[1]), whereas the chemical shift of C(6′) (inner glucose moiety) is 71.0 ppm[1]). The chemical shifts of the anomeric C-atoms C(1″) and C(1′) are 105.4 and 95.7 ppm[1]), respectively. The free carboxy

[1]) ±0.3 ppm.

group is observed at 180.2 ppm[1]) (compounds **1**, **2** and **3**) whereas, when esterified with a sugar moiety, the chemical shift is 176.5 ppm[1]) (compounds **6**, **7** and **8**). The β-D-pyranosyl configuration of glucose and glucuronic acid, as well as the α-L-pyranosyl configuration of arabinose, were deduced from ^{1}H- and ^{13}C-NMR. data.

Discussion. – By combination of different MS. methods of solid samples and ^{13}C-NMR. spectroscopy, eight saponins were identified in the fruits of *Lonicera nigra* L. The results of the biological tests confirm that monodesmosidic saponins show strong molluscicidal activity against *Biomphalaria glabrata* whereas bidesmosidic saponins are only weakly active or inactive [5]. Saponin **2** presented the highest activity and killed the snails within 24 h at a concentration of 2 ppm. Although *Lonicera nigra* L. does not occur in countries infected by schistosomiasis, the identification of its molluscicidal principles contributes to the study of the structure-activity relationship which should facilitate the selection of potential plant molluscicides.

This is the first report of the occurrence of saponins in *Lonicera nigra* L. Other species of the genus *Lonicera* such as *L. alpigena* L., *L. coerulea* L., *L. xylosteum* L. and *L. obovata* ROYLE have been tested. No activity was observed and no saponin could be detected in the crude extracts. According to *Hiller & Voigt* [15], compounds **5** and **7** have not been reported previously, whereas saponins **1**–**4**, **6** and **8** were found in several plant species [16].

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Experimental Part

General remarks. Melting points (m.p.) were determined on a *Kofler* block and are uncorrected. TLC. were carried out on silica-gel-precoated Al-sheets *(Merck)* with the lower layer of $CHCl_3$/MeOH/H_2O 65:35:10, detection with *Godin* reagent [14]. Hederagenin derivatives show a blue color whereas those of oleanolic acid are purple. Saponins **1**–**8** afforded following Rf-values: 0.88, 0.78, 0.69, 0.43, 0.38, 0.40, 0.31 and 0.26, respectively. For column chromatography (CC.) silica gel *60,* 63–200 µm *(Merck),* and *Sephadex LH-20 (Pharmacia, Fine Chemicals)* were used. Droplet counter-current chromatography (DCCC.) separations were achieved on a *Tokyo Rikakikai* apparatus, *Model A,* equipped with 300 glass tubes. ^{1}H-NMR. spectra were recorded on a *Bruker WP-200* apparatus at 200 MHz, ^{13}C-NMR. spectra on a *Bruker WP-200* spectrometer at 50.29 MHz in (D_5)pyridine as solvent and TMS as internal standard. Desorption/chemical ionization (D./CI.)-MS. were recorded on a *Ribermag R10-10B* quadrupole spectrometer with a *Ribermag* SIDAR data system, for details see [10]. Field desorption (FD.)-MS. were produced on a *Varian MAT 731* spectrometer equipped with a *SS-200* data system, using C-microneedles emitters, emitter heating current 20–40 mA. Fast-atom-bombardment (FAB.)-MS. were obtained on a *Finnigan MAT 212* spectrometer with a *SS-200* data system. The target was bombarded with 5 keV Xe-atoms, the ion source was at a temperature of 80° and at a pressure of $2 \cdot 10^{-5}$ Torr. Samples were suspended in glycerol.

Bioassays were made with snails of the species *Biomphalaria glabrata* as described previously [4].

Acidic hydrolysis. The saponin (2 mg) in 1 ml MeOH were refluxed in 10 ml of 4N HCl for 4 h. The aglycone was extracted with AcOEt and identified by TLC. on silica gel with diisopropyl ether/acetone 75:30. The aq. layer was then adjusted to pH 6 with $NaHCO_3$. After evaporation to dryness, the sugars were extracted from the residue with pyridine and analyzed by TLC. on silica gel with AcOEt/H_2O/MeOH/AcOH 65:15:15:20; detection with *p*-anisidine phtalate, and by HPLC. on a *μ-Bondapak*-NH_2-column, l = 30 cm, ∅ 4 mm *(Waters Assoc.)* using MeCN/H_2O, 4:1 at a flow rate of 0.8 ml/min and a refractive index detector.

Basic hydrolysis. The saponin (2 mg) in 1 ml MeOH were refluxed in 10 ml 0.5N aq. KOH for 1 h. The reaction mixture is adjusted to pH 6 with aq. HCl-solution, and then extracted with BuOH. The aq. phase has been treated as described above for the sugar analysis. The saponins in the org. phase were analyzed by TLC. on silica gel with $CHCl_3$/MeOH/H_2O 65:35:10 (lower layer).

Isolation. The berries of *Lonicera nigra* L. were collected in Champex (VS), Switzerland. Fresh berries (500 g) were extracted with MeOH. The crude extract (15 g) was chromatographed on a silica gel column (∅ = 6 cm, l = 100 cm) with $CHCl_3$/MeOH/H_2O 75:23:2 (3 l); then the polarity of the solvent was increased by changing the ratio to 64:32:4 (5 l), 50:46:4 (4 l) and finally 31:65:4 (4 l). The eluate was analyzed by TLC. The bioactive fraction *I* (900 mg) contained the less polar saponins, two other fractions (*II:* 1.3 g and *III:* 1.2 g) also containing saponins were collected. These three fractions were further studied. Fraction *I* was submitted to two consecutive DCCC. separations using $CHCl_3$/MeOH/H_2O 25:45:30 in the descending mode.

Five saponins were isolated by this way. The final purification of each compound was achieved on a *Sephadex LH-20* column with MeOH as eluant and afforded **1** (70 mg), **2** (10 mg), **3** (50 mg), **4** (15 mg) and **5** (10 mg).

Fraction *II* was submitted to DCCC. with $CHCl_3$/MeOH/H_2O 25:45:30 in the descending mode to elute the less polar compounds. The mobile phase was collected and analyzed by TLC. Two fractions (*IIa* and *IIb*) containing **6** and 7, respectively, were obtained. Further purification of *IIa* (170 mg) by chromatography on a silica gel column with $CHCl_3$/MeOH/H_2O 65:35:10 (lower phase) afforded **6** (50 mg).

Fraction *IIb* (55 mg) was submitted to DCCC. with BuOH/acetone/H_2O 35:10:55 in the ascending mode and yielded saponin **7** (10 mg) after purification on a *Sephadex LH-20* column with MeOH.

Purification of fraction *III* on a silica gel column with the lower layer of $CHCl_3$/MeOH/H_2O 65:35:10 yielded a mixture of **7** and **8**. A further separation on silica gel with $CHCl_3$/MeOH/H_2O 65:28:7 (lower phase) afforded 7 (15 mg) and **8** (25 mg).

Structure elucidation. – a) *Saponin* **1** *(hederagenin-3-O-α-L-arabinopyranoside).* $C_{33}H_{56}O_8$, white powder, m.p. 225–228°, in all respects identical with a sample previously isolated [4].

b) *Saponin* **2** *(oleanolic acid-3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranoside).* $C_{41}H_{66}O_{12}$, white powder, m.p. 261–264°. Acidic hydrolysis afforded oleanolic acid, D-glucose and L-arabinose. – ^{13}C-NMR. (50.29 MHz, (D_5)pyridine): chemical shifts of the aglycone correspond to those previously described for oleanolic acid [8]. Sugar signals: 104.5 (C(1′)); 81.1 (C(2′)); 73.5 (C(3′)); 68.3 (C(4′)); 65.0 (C(5′)); 106.1 (C(1″)); 76.4 (C(2″)); 78.0 (C(3″)); 71.7 (C(4″)); 78.0 (C(5″)) and 62.7 (C(6″)). – D./CI.-MS. (NH_3): 768 ($[M+NH_4]^+$); 606 ($[(M+NH_4)-162]^+$); 474 ($[(M+NH_4)-294]^+$); 312 ([glucosyl-arabinosyl + NH_4]$^+$).

c) *Saponin* **3** *(hederagenin-3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranoside).* $C_{41}H_{66}O_{13}$, white cristals, m.p. 269–271°. Acidic hydrolysis afforded hederagenin, D-glucose and L-arabinose. – ^{13}C-NMR. chemical shifts of the aglycone correspond to those of hederagenin [8], sugar C-signals are identical with those of saponin **2**. – D./CI.-MS. (NH_3): 784 ($[M+NH_4]^+$); 767 ($[M+H]^+$); 622 ($[(M+NH_4)-162]^+$); 490 ($[(M+NH_4)-294]^+$); 312 ([glucosyl-arabinosyl + NH_4]$^+$). – FAB.-MS. (positive ion): 859 ($[(M+H)+92]^+$); 767 ($[M+H]^+$); 455, 437 and 409 (aglycone fragments). – FAB.-MS. (negative ion): 765 ($[M-H]^-$); 603 ($[(M-H)-162]^-$).

d) *Saponin* **4** *(oleanolic acid-3-O-β-D-glucuronopyranoside).* $C_{36}H_{48}O_9$, white powder, m.p. 242–244°. Acidic hydrolysis afforded oleanolic acid and D-glucuronic acid. – FAB.-MS.: 655 ($[M+Na]^+$); 633 ($[M+H]^+$); 457 ($[(M+H)-176]^+$).

e) *Saponin* **5** *(hederagenin-3-O-β-D-glucuronopyranoside).* $C_{36}H_{48}O_{10}$, white powder, m.p. 282–284° (dec.). Acidic hydrolysis afforded hederagenin and D-glucuronic acid. – FAB.-MS.: 687 ($[M+K]^+$); 671 ($[M+Na]^+$); 649 ($[M+H]^+$); 455, 437 and 409 (hederagenin fragments).

f) *Saponin* **6** *(3-O-α-L-arabinopyranosyl-hederagenin-28-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside).* $C_{47}H_{76}O_{18}$, white powder, m.p. 220–222°. Acidic hydrolysis afforded hederagenin, D-glucose and L-arabinose, and basic hydrolysis led to saponin **1** and gentiobiose. – ^{13}C-NMR. (50.29 MHz, (D_5)pyridin): sugar signals of the α-L-arabinosyl unit: 106.5 (C(1)); 73.4 (C(2)); 74.7 (C(3)); 69.6 (C(4)); 66.8 (C(5)); gentiobiosyl moiety: 95.7 (C(1′)); 73.9 (C(2′)); 78.4 (C(3′)); 69.6 (C(4′)); 78.0 (C(5′)); 71.1 (C(6′)) (inner unit); 105.6 (C(1″)); 75.2 (C(2″)); 78.8 (C(3″)); 71.7 (C(4″)); 78.4 (C(5″)) and 62.8 (C(6″)) (terminal unit). – D./CI.-MS. (NH_3): 946 ($[M+NH_4]^+$); 814 ($[(M+NH_4)-132]^+$); 622 ($[(M+NH_4)-294]^+$); 490 ($[(M+NH_4)-456]^+$); 342 ([glucosyl-glucosyl $+NH_4]^+$). No signal is observed at 312 ([glucosyl-arabinosyl $+NH_4]^+$).

g) *Saponin* **7** *(3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl oleanolic acid-28-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside).* $C_{53}H_{86}O_{22}$, white powder, m.p. 201–203°. Basic hydrolysis afforded saponin **2** and gentiobiose. – ^{13}C-NMR.: the chemical shifts of the β-D-glucosyl-α-L-arabinosyl moiety are identical with those observed for saponin **2**. C-signals of the gentiobiosyl unit correspond to those of compound **6**. – FD.-MS.: 1113 ($[M+K]^+$); 1097 ($[M+Na]^+$); 935 ($[(M+Na)-162]$); 773 ($[(M+Na)-324]^+$); 611 ($[(M+Na)-486]^+$); 479 ($[(M+Na)-618]^+$).

h) *Saponin* **8** *(3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-hederagenin-28-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside).* $C_{53}H_{86}O_{23}$, white powder, m.p. 180–182°. Basic hydrolysis afforded saponin **3** and gentiobiose. – ^{13}C-NMR. chemical shifts of the sugar C-atoms are identical with those of compound **7**. – FD.-MS.: 1129 ($[M+K]^+$); 1113 ($[M+Na]^+$); 951 ($[(M+Na)-162]$); 789 ($[(M+Na)-324]^+$); 627 ($[(M+Na)-486]^+$); 495 ($[(M+Na)-618]^+$).

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