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Metabolite Profiling of Triterpene Saponins in *Medicago truncatula* Hairy Roots by Liquid Chromatography Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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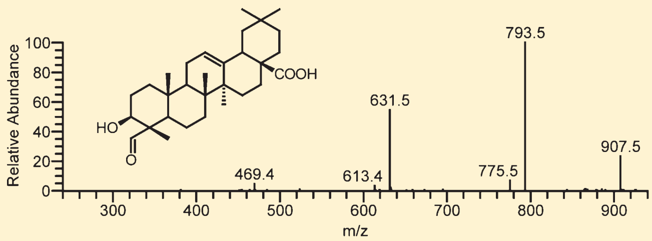
ABSTRACT: Triterpenes are one of the largest classes of plant natural products, with an enormous variety in structure and bioactivities. Here, triterpene saponins from hairy roots of the model legume *Medicago truncatula* were profiled with reversed-phase liquid chromatography coupled to negative-ion electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (LC ESI FT-ICR MS). Owing to the accuracy of the FT-ICR MS, reliable molecular formulas of the detected compounds could be predicted, which, together with the generated MS^n spectra, allowed the tentative identification of 79 different saponins, of which 61 had not been detected previously in *M. truncatula*. Upon collision-induced dissociation of saponins that contain a uronic acid residue in the sugar chain, fragment ions resulting from cross-ring cleavages of the uronic acid residues were observed. The identified saponins are glycosides of 10 different sapogenins, of which three were not detected before in *M. truncatula*. Zanhic acid glycosides, which are prevalent in the aerial parts of *M. truncatula*, were absent in the hairy root extracts. This metabolite compendium will facilitate future functional genomic studies of triterpene saponin biosynthesis in *M. truncatula*.



Legumes (Fabaceae) are important agricultural crops, characterized by the presence of root nodules that contain symbiotic nitrogen-fixing bacteria. Fixation of atmospheric nitrogen allows reduced fertilizer costs and makes legumes a key player in crop rotation to replenish nitrogen-depleted soil. Furthermore, legumes serve as a major source of proteins and oil for human and animal nutrition. Leguminous crops with a significant economical value include oilseed crops such as soybean (*Glycine max*) and peanut (*Arachis hypogaea*), forage and soil-conditioning crops such as alfalfa (*Medicago sativa*) and clover (*Trifolium* spp.), and pulses such as beans (*Phaseolus* spp.), peas (*Pisum* spp.), and lentils (*Lens* spp.).

The more than 19 400 leguminous species, distributed over 730 genera, produce a vast array of metabolites with diverse structures and biological activities. An important group of legume natural products are triterpene saponins, a class of secondary metabolites that display a wide range of biological activities.[1] In plants they serve as allelopathic, antipalatability, anti-insect, or antifungal agents.[2] Saponins also have a broad range of pharmaceutical properties, such as anti-inflammatory, antimicrobial, anticancer, or adjuvant activities.[3,4] Besides saponins, isoflavonoids, alkaloids, and nonprotein amino acids commonly occur as natural products in several legume species.[1,5]

As a model species for legume biology, barrel medic (*Medicago truncatula*), a close relative of alfalfa, is an excellent system to study secondary metabolism of legumes.[1,6] Consequently, several studies about the metabolite composition of *M. truncatula* have been published over the past decade. Metabolite profiling using LC-MS-based methods revealed the presence of a complex mixture of triterpene saponins in various *M. truncatula* tissues.[2,7−9] In accordance with other legumes from the Papilionoideae subfamily, *M. truncatula* is rich in isoflavonoids.[10,11]

Hairy roots are generated by an infection of healthy plant tissue, not necessarily roots, with *Agrobacterium rhizogenes*. They are characterized by fast growth rates and do not require growth regulators for their cultivation. Furthermore, they have a high genetic stability and a high biosynthetic potential for a longer time than natural roots.[12,13] Their potential to produce compounds that are also naturally occurring in wild-type roots in combination with their tolerance to cultivation in large-scale bioreactors makes hairy roots an appealing alternative for the destructive harvesting of wild-type roots, especially those of endangered plant species, to obtain valuable phytochemicals.[14,15] Hairy roots are also a valuable tool to investigate the secondary metabolism in *M. truncatula*. A prerequisite for the study of secondary metabolism and its application in metabolic engineering projects is the possibility to generate transgenic plant tissue. Although fertile transgenic *M. truncatula* plants can be obtained via *Agrobacterium tumefaciens*-mediated transformation,[16−19] the

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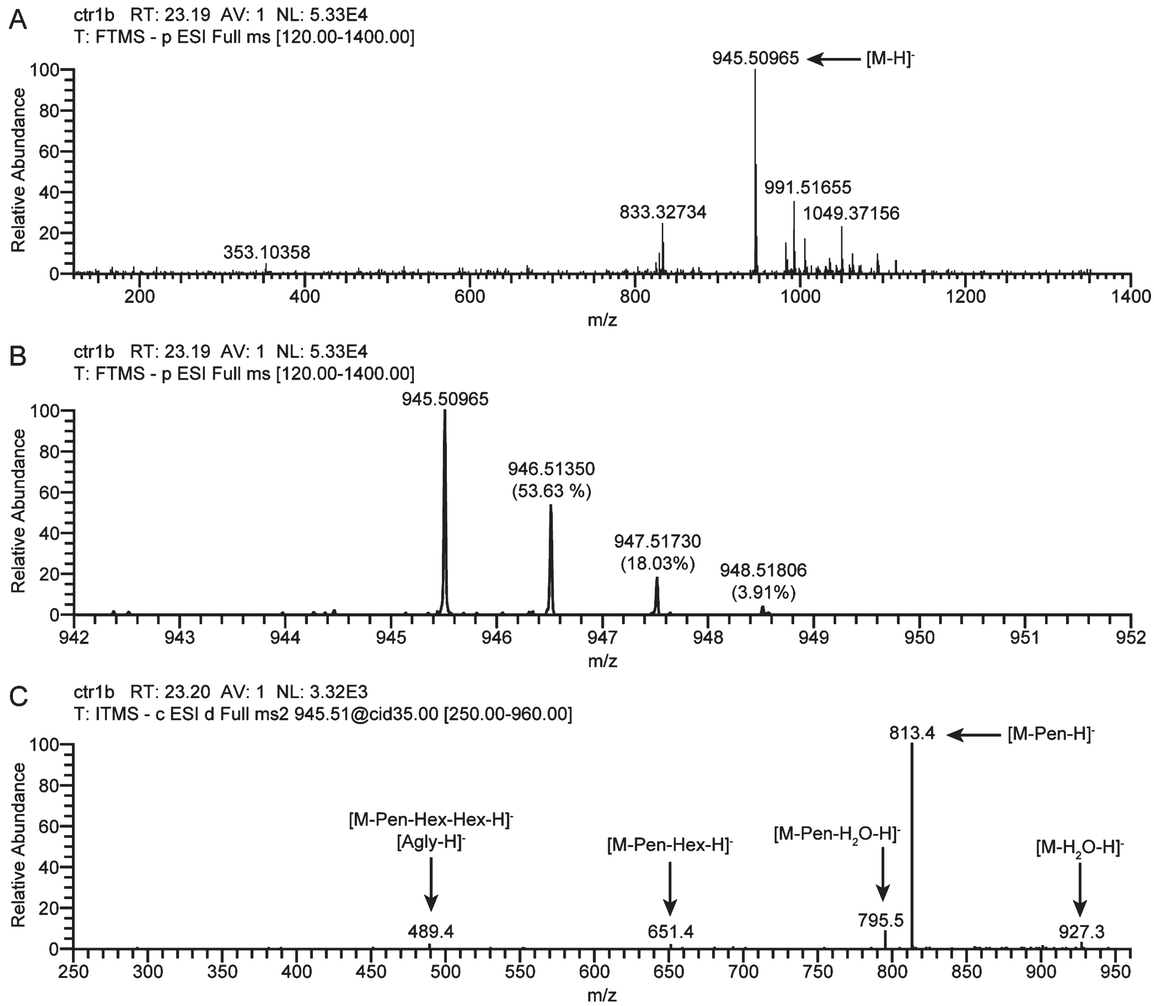

Figure 1. LC ESI FT-ICR MS chromatograms. (A) MS scan of peak at t_R 23.19 min. (B) Zoom of the MS scan of panel A, showing that the quasi-molecular ion is accompanied by additional isotope ions. The relative intensity of the isotope ions is indicated in brackets. (C) MS^2 fragmentation of the $[M - H]^-$ ion at m/z 945.51 (Pen-Hex-Hex-aglycone D).

published methods are time-consuming and lack the required throughput for simultaneous analysis of a significant number of genes. In contrast, *A. rhizogenes*-mediated transformation of *M. truncatula* seedlings allows a fast and efficient production of transgenic hairy roots;[20] the generation time needed from seed to a transgenic *M. truncatula* hairy root culture, ready for profiling or other analysis, is only three months.

For any metabolic engineering project targeting triterpene saponin biosynthesis, a detailed analysis of the saponin composition of the generated hairy roots is required. In the past decade, a few metabolic profiling studies on saponins in *M. truncatula* have been reported. In a first study, the presence of a large number of saponins in root extracts of *M. truncatula* was shown: based on fragmentation data under negative ionization, 27 *M. truncatula* saponins were tentatively identified.[2] Optimization of the latter method resulted in an extended list of 31 tentatively identified saponins.[7] In a later study, 12 additional saponins from the aerial parts of *M. truncatula* were characterized via spectroscopic methods, and their fragmentation behavior under negative ionization was reported.[8,9] Nevertheless, *M. truncatula* was postulated to synthesize many more triterpene saponins than identified to date.[2]

For identification purposes, LC-MS-based metabolomics studies benefit from the use of Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). FT-ICR MS provides highly accurate mass measurements, allowing a reliable prediction of the molecular formula of the detected ions. Here, we applied reversed-phase liquid chromatography (LC) coupled to negative-ion electrospray ionization (ESI) FT-ICR MS to investigate the saponin composition of hairy roots of *M. truncatula*. As a result, 79 saponins were detected and tentatively identified, of which soyasaponin I and 3-Glc-28-Glc-medicagenic acid were the most abundant compounds. The identified saponins are glycosides of 10 different sapogenins, of which three had hitherto not been detected before in *M. truncatula*.

RESULTS AND DISCUSSION

General Methodology. For metabolite profiling of triterpene saponins, the compounds from a methanol extract of *M. truncatula* hairy roots were separated by reversed-phase LC, followed by negative-ion ESI FT-ICR MS. The accurate m/z measurements of the FT-ICR MS, combined with the isotope abundances, allowed us to predict the molecular formula of the detected saponins. The structural information needed for the identification of the saponins was obtained by MS^n fragmentation using the linear ion trap (IT). Full FT-MS spectra were

Table 1. Observed Saponins from *M. truncatula* Hairy Roots, Tentative Identification, and FT-ICR MS^n Characteristics[a]

Peak numbers and tentative identification of the observed saponins	[M − H]$^-$	Formula (δ ppm)	FT-ICR MS^n: *m/z* (% base peak)
1 dHex-Hex-HexA-Aglycone D	973.50386	$C_{48}H_{78}O_{20}$ (2.560)	**MS^2 [973.50]**: 955 (100)[M-H$_2$O-H]$^-$, 929 (10)[M-CO$_2$-H]$^-$, 911 (53)[M-H$_2$O-CO$_2$-H]$^-$, 827 (3)[M-dHex-H]$^-$, 809 (7)[M-dHex-H$_2$O-H]$^-$, 783 (6)[M-dHex-CO$_2$-H]$^-$, 765 (19)[M-dHex-H$_2$O-CO$_2$-H]$^-$, 665 (2)[M-dHex-Hex-H]$^-$, 647 (26)[M-dHex-Hex-H$_2$O-H]$^-$, 629 (14)[M-dHex-Hex-H$_2$O-H$_2$O-H]$^-$, 603 (7)[M-dHex-Hex-H$_2$O-CO$_2$-H]$^-$, 557 (13)[M-dHex-Hex-108-H]$^-$, 489 (11)[Agly-H]$^-$ **MS^3 [973.50→955]**: 911 (100)[M-H$_2$O-CO$_2$-H]$^-$, 765 (21)[M-dHex-H$_2$O-CO$_2$-H]$^-$, 679 (9), 629 (49)[M-dHex-Hex-H$_2$O-H$_2$O-H]$^-$, 557 (55)[M-dHex-Hex-108-H]$^-$, 489 (7)[Agly-H]$^-$
2 Pen-Hex-Hex-Aglycone D	945.50965	$C_{47}H_{78}O_{19}$ (3.380)	**MS^2 [945.51]**: 927 (3)[M-H$_2$O-H]$^-$, 813 (100)[M-Pen-H]$^-$, 795 (8)[M-Pen-H$_2$O-H]$^-$, 651 (2)[M-Pen-Hex-H]$^-$, 489 (2)[Agly-H]$^-$ **MS^3 [945.51→813]**: 651 (64)[M-Pen-Hex-H]$^-$, 489 (100)[Agly-H]$^-$
3 Hex-Hex-HexA-Bayogenin	987.48370	$C_{48}H_{76}O_{21}$ (3.106)	**MS^2 [987.48]**: 825 (4)[M-Hex-H]$^-$, 807 (100)[M-Hex-H$_2$O-H]$^-$, 645 (10)[M-Hex-Hex-H$_2$O-H]$^-$, 601 (18)[M-Hex-Hex-H$_2$O-CO$_2$-H]$^-$, 487 (2)[Agly-H]$^-$ **MS^3 [987.48→807]**: 645 (85)[M-Hex-Hex-H$_2$O-H]$^-$, 601 (100)[M-Hex-Hex-H$_2$O-CO$_2$-H]$^-$, 487 (12)[Agly-H]$^-$
4 Hex-dHex-Hex-Hex-Bayogenin dHex-Hex-Hex-Bayogenin[b]	1119.56348 957.51011[b]	$C_{54}H_{88}O_{24}$ (3.754) $C_{48}H_{78}O_{19}$ (3.818)	**MS^2 [1119.56]**: 957 (100)[M-Hex-H]$^-$, 811 (54)[M-Hex-dHex-H]$^-$, 793 (9)[M-Hex-dHex-H$_2$O-H]$^-$, 649 (33)[M-Hex-dHex-Hex-H]$^-$, 631 (13)[M-Hex-dHex-Hex-H$_2$O-H]$^-$, 487 (9)[Agly-H]$^-$ **MS^3 [1119.56→957]**: 811 (100)[M-Hex-dHex-H]$^-$, 793 (6)[M-Hex-dHex-H$_2$O-H]$^-$, 649 (11)[M-Hex-dHex-Hex-H]$^-$, 631 (3)[M-Hex-dHex-Hex-H$_2$O-H]$^-$, 487 (2)[Agly-H]$^-$ **MS^3 [1119.56→811]**: 649 (100)[M-Hex-dHex-Hex-H]$^-$, 631 (8)[M-Hex-dHex-Hex-H$_2$O-H]$^-$, 487 (68)[Agly-H]$^-$
5 Malonyl-Hex-Hex-HexA-Bayogenin	1073.48680	$C_{51}H_{78}O_{24}$ (5.378)	**MS^2 [1073.49]**: 1029 (100)[M-CO$_2$-H]$^-$ **MS^3 [1073.49→1029]**: 867 (15)[M-CO$_2$-Hex-H]$^-$, 849 (100)[M-CO$_2$-Hex-H$_2$O-H]$^-$, 807 (7)[M-malonyl-Hex-H$_2$O-H]$^-$, 789 (40)[M-malonyl-Hex-H$_2$O-H$_2$O-H]$^-$, 763 (11)[M-malonyl-Hex-H$_2$O-CO$_2$-H]$^-$, 643 (32)[M-CO$_2$-Hex-Hex-H$_2$O-CO$_2$-H]$^-$, 601 (45)[M-malonyl-Hex-Hex-H$_2$O-CO$_2$-H]$^-$, 583 (38)[M-malonyl-Hex-Hex-H$_2$O-H$_2$O-CO$_2$-H]$^-$, 565 (22), 515 (83), 487 (14)[Agly-H]$^-$
6 Hex-Hex-Hex-Bayogenin	973.50421	$C_{48}H_{78}O_{20}$ (2.919)	**MS^2 [973.50]**: 955 (10)[M-H$_2$O-H]$^-$, 811 (100)[M-Hex-H]$^-$, 793 (2)[M-Hex-H$_2$O-H]$^-$, 649 (20)[M-Hex-Hex-H]$^-$, 631 (7)[M-Hex-Hex-H$_2$O-H]$^-$, 487 (8) [Agly-H]$^-$ **MS^3 [973.50→811]**: 649 (100)[M-Hex-Hex-H]$^-$, 631 (20)[M-Hex-Hex-H$_2$O-H]$^-$, 487 (36) [Agly-H]$^-$ **MS^3 [973.50→649]**: 487 (100) [Agly-H]$^-$
7 dHex-Hex-HexA-Soyasapogenol A	957.50926	$C_{48}H_{78}O_{19}$ (2.931)	**MS^2 [957.51]**: 939 (100)[M-H$_2$O-H]$^-$, 895 (49)[M-H$_2$O-CO$_2$-H]$^-$, 811 (5)[M-dHex-H]$^-$, 767 (3)[M-dHex-CO$_2$-H]$^-$, 749 (21)[M-dHex-H$_2$O-CO$_2$-H]$^-$, 631 (25)[M-dHex-Hex-H$_2$O-H]$^-$, 613 (18)[M-dHex-Hex-H$_2$O-H$_2$O-H]$^-$, 541 (18)[M-dHex-Hex-108-H]$^-$, 473 (11)[Agly-H]$^-$, 453 (5) **MS^3 [957.51→939]**: 895 (83)[M-H$_2$O-CO$_2$-H]$^-$, 749 (19)[M-dHex-H$_2$O-CO$_2$-H]$^-$, 613 (61)[M-dHex-Hex-H$_2$O-H$_2$O-H]$^-$, 541 (100)[M-dHex-Hex-108-H]$^-$, 473 (4)[Agly-H]$^-$, 453 (2)
8 Hex-Hex-HexA-Aglycone A	985.46884	$C_{48}H_{74}O_{21}$ (3.914)	**MS^2 [985.47]**: 823 (100)[M-Hex-H]$^-$, 779 (2)[M-Hex-CO$_2$-H]$^-$, 761 (2)[M-Hex-CO$_2$-H$_2$O-H]$^-$, 643 (4)[M-Hex-Hex-H$_2$O-H]$^-$, 617 (2)[M-Hex-Hex-CO$_2$-H]$^-$, 599 (2)[M-Hex-Hex-CO$_2$-H$_2$O-H]$^-$, 485 (3)[Agly-H]$^-$ **MS^3 [985.47→823]**: 805 (1)[M-Hex-H$_2$O-H]$^-$, 779 (10)[M-Hex-CO$_2$-H]$^-$, 761 (3)[M-Hex-CO$_2$-H$_2$O-H]$^-$, 661 (2)[M-Hex-Hex-H]$^-$, 643 (100)[M-Hex-Hex-H$_2$O-H]$^-$, 617 (42)[M-Hex-Hex-CO$_2$-H]$^-$, 599 (28)[M-Hex-Hex-CO$_2$-H$_2$O-H]$^-$, 581 (3)[M-Hex-Hex-CO$_2$-H$_2$O-H$_2$O-H]$^-$, 567 (18), 485 (65)[Agly-H]$^-$, 467 (19)
9 Hex-Hex-Hex-Bayogenin	973.50407	$C_{48}H_{78}O_{20}$ (2.776)	**MS^2 [973.50]**: 955 (10)[M-H$_2$O-H]$^-$,811 (100)[M-Hex-H]$^-$, 649 (20)[M-Hex-Hex-H]$^-$, 631 (7)[M-Hex-Hex-H$_2$O-H]$^-$, 487 (8) [Agly-H]$^-$ **MS^3 [973.50→811]**: 649 (100)[M-Hex-Hex-H]$^-$, 631 (42)[M-Hex-Hex-H$_2$O-H]$^-$, 487 (86) [Agly-H]$^-$ **MS^3 [973.50→649]**: 487 (100) [Agly-H]$^-$ 409 (1), 393 (1), 391 (1)

Table 1. Continued

Peak numbers and tentative identification of the observed saponins	$[M-H]^-$	Formula (δ ppm)	FT-ICR MS^n: m/z (% base peak)
10 Hex-Hex-Aglycone C	823.41585	$C_{42}H_{64}O_{16}$ (4.481)	**MS^2 [823.42]**: 805 (4)[M-H_2O-H]$^-$, 661 (2)[M-Hex-H]$^-$, 617 (3)[M-Hex-CO_2-H]$^-$, 599 (7)[M-Hex-CO_2-H_2O-H]$^-$, 437 (100)[Agly-CO_2-H_2O-H]$^-$ **MS^3 [823.42→437]**: 391 (100), 375 (6)
11 Hex-HexA-Bayogenin	825.43079	$C_{42}H_{66}O_{16}$ (3.610)	**MS^2 [825.43]**: 807 (10)[M-H_2O-H]$^-$, 781 (3)[M-CO_2-H]$^-$, 765 (3), 763 (2)[M-H_2O-CO_2-H]$^-$, 705 (7), 663 (100)[M-Hex-H]$^-$, 645 (10)[M-Hex-H_2O-H]$^-$, 619 (41)[M-Hex-CO_2-H]$^-$, 601 (41)[M-Hex-CO_2-H_2O-H]$^-$, 587 (6)[M-Hex-CO_2-H_2O-H_2O-H]$^-$, 529 (6), 487 (34)[Agly-H]$^-$ **MS^3 [825.43→663]**: 587 (4)[M-Hex-CO_2-H_2O-H_2O-H]$^-$, 487 (100)[Agly-H]$^-$, 467 (5), 455 (6), 439 (5)
12 Hex-Hex-HexA-Hederagenin	971.48934	$C_{48}H_{76}O_{20}$ (3.728)	**MS^2 [971.49]**: 851 (4), 809 (100)[M-Hex-H]$^-$, 791 (7)[M-Hex-H_2O-H]$^-$, 747 (3)[M-Hex-CO_2-H_2O-H]$^-$, 629 (20)[M-Hex-Hex-H_2O-H]$^-$, 603 (3)[M-Hex-Hex-CO_2-H]$^-$, 585 (6)[M-Hex-Hex-CO_2-H_2O-H]$^-$, 539 (1)[M-Hex-Hex-108-H]$^-$, 471 (4) [Agly-H]$^-$ **MS^3[971.49→809]**: 747 (4)[M-Hex-CO_2-H_2O-H]$^-$, 647 (3)[M-Hex-Hex-H]$^-$, 629 (100)[M-Hex-Hex-H_2O-H]$^-$, 603 (9)[M-Hex-Hex-CO_2-H]$^-$, 585 (15)[M-Hex-Hex-CO_2-H_2O-H]$^-$, 553 (5), 539 (4)[M-Hex-Hex-108-H]$^-$, 471 (15)[Agly-H]$^-$, 469 (6), 439 (6)
13 Hex-Hex-Hex-Medicagenic acid	987.48516	$C_{48}H_{76}O_{21}$ (4.584)	**MS^2 [987.49]**: 825 (100)[M-Hex-H]$^-$, 781 (5)[M-Hex-CO_2-H]$^-$, 711 (3), 663 (4)[M-Hex-Hex-H]$^-$, 601 (4)[M-Hex-Hex-CO_2-H_2O-H]$^-$, 439 (53)[Agly-CO_2-H_2O-H]$^-$ **MS^3 [987.49→825]**: 439 (100)[Agly-CO_2-H_2O-H]$^-$
14 Malonyl-Hex-Hex-HexA-Hederagenin	1057.49199 1013.50072[b]	$C_{51}H_{78}O_{23}$ (5.558)	**MS^2 [1013.50]**: 971 (5)[M-malonyl-H]$^-$, 833 (100)[M-Hex-CO_2-H_2O-H]$^-$, 791 (8)[M-malonyl-Hex-H_2O-H]$^-$, 773 (20)[M-malonyl-Hex-H_2O-H_2O-H]$^-$, 729 (3)[M-malonyl-Hex-H_2O-CO_2-H]$^-$, 713 (3), 671 (37)[M-Hex-Hex-CO_2-H_2O-H]$^-$, 633 (4), 627 (25)[M-Hex-Hex-CO_2-CO_2-H_2O-H]$^-$, 611 (2), 601 (7), 585 (9)[M-malonyl-Hex-Hex-CO_2-H_2O-H]$^-$, 567 (43)[M-malonyl-Hex-Hex-CO_2-H_2O-H_2O-H]$^-$, 549 (6), 499 (70)[Hex-Hex-HexA-H]$^-$, 471 (2)[Agly-H]$^-$, 469 (5) **MS^3[1013.50→833]**: 773 (11)[M-malonyl-Hex-H_2O-H_2O-H]$^-$, 671 (100)[M-Hex-Hex-CO_2-H_2O-H]$^-$, 633 (11), 585 (15)[M-malonyl-Hex-Hex-CO_2-H_2O-H]$^-$, 567 (10)[M-malonyl-Hex-Hex-CO_2-H_2O-H_2O-H]$^-$, 471 (4)[Agly-H]$^-$
15 dHex-Hex-Hex-Hederagenin	941.51456	$C_{48}H_{78}O_{18}$ (3.209)	**MS^2 [941.51]**: 795 (100)[M-dHex-H]$^-$, 777 (10)[M-dHex-H_2O-H]$^-$, 633 (12)[M-dHex-Hex-H]$^-$, 615 (2)[M-dHex-Hex-H_2O-H]$^-$, 471 (1)[Agly-H]$^-$ **MS^3 [941.51→795]**: 633 (100)[M-dHex-Hex-H]$^-$, 615 (6)[M-dHex-Hex-H_2O-H]$^-$, 471 (25)[Agly-H]$^-$ **MS^3 [941.51→633]**: 471 (100)[Agly-H]$^-$, 405 (1), 393 (1)
16 Malonyl-Hex-HexA-Bayogenin	911.43120 867.44138[b]	$C_{45}H_{68}O_{19}$ (3.288)	**MS^2 [867.44]**: 825 (2)[M-malonyl-H]$^-$, 807 (37)[M-malonyl-H_2O-H]$^-$, 705 (3)[M-CO_2-Hex-H]$^-$, 645 (100)[M-malonyl-Hex-H_2O-H]$^-$, 601 (6)[M-malonyl-Hex-H_2O-CO_2-H]$^-$ **MS^3 [867.44→645]**: 469 (100)[Agly-H_2O-H]$^-$, 423 (10), 409 (40), 391 (8), 379 (21) **MS^3 [867.44→807]**: 601 (100)[M-malonyl-Hex-H_2O-CO_2-H]$^-$, 487 (2)[Agly-H]$^-$
17 Hex-Hex-HexA-Aglycone A	985.46931	$C_{48}H_{74}O_{21}$ (4.391)	**MS^2 [985.47]**: 823 (6)[M-Hex-H]$^-$, 805 (100)[M-Hex-H_2O-H]$^-$, 643 (10)[M-Hex-Hex-H_2O-H]$^-$, 599 (33)[M-Hex-Hex-H_2O-CO_2-H]$^-$ **MS^3 [985.47→805]**: 685 (4), 643 (45)[M-Hex-Hex-H_2O-H]$^-$, 599 (100)[M-Hex-Hex-H_2O-CO_2-H]$^-$ **MS^3 [985.47→599]**: 527 (100), 485 (2)[Agly-H]$^-$
18 Hex-Hex-Bayogenin	811.45241	$C_{42}H_{68}O_{15}$ (4.763)	**MS^2 [811.45]**: 649 (100)[M-Hex-Hex-H]$^-$, 631 (8)[M-Hex-Hex-H_2O-H]$^-$, 487 (62) [Agly-H]$^-$ **MS^3 [811.45→649]**: 487 (100) [Agly-H]$^-$ **MS^3 [811.45→487]**: 409 (100), 403 (21), 393 (38), 391 (74)
19 Hex-Hex-HexA-Hederagenin	971.48997	$C_{48}H_{76}O_{20}$ (4.377)	**MS^2 [971.49]**: 851 (3), 809 (100)[M-Hex-H]$^-$, 603 (5)[M-Hex-Hex-CO_2-H]$^-$, 585 (9)[M-Hex-Hex-CO_2-H_2O-H]$^-$, 569 (9), 499 (1), 471 (10)[Agly-H]$^-$ **MS^3 [971.49→809]**: 647 (32)[M-Hex-Hex-H]$^-$, 629 (27)[M-Hex-Hex-H_2O-H]$^-$, 611 (10)[M-Hex-Hex-H_2O-H_2O-H]$^-$, 603 (25)[M-Hex-Hex-CO_2-H]$^-$, 587 (12), 585 (15)[M-Hex-Hex-CO_2-H_2O-H]$^-$, 567 (58)[M-Hex-Hex-CO_2-H_2O-H_2O-H]$^-$, 553 (14), 499 (78), 471 (100)[Agly-H]$^-$, 469 (15), 439 (17) **MS^3 [971.49→471]**: 439 (37), 405 (30), 393 (100)

Table 1. Continued

Peak numbers and tentative identification of the observed saponins	$[M-H]^-$	Formula (δ ppm)	FT-ICR MS^n: m/z (% base peak)
20 Malonyl-Hex-Hex-HexA-Aglycone A	1071.47137 1027.48023^b	$C_{51}H_{76}O_{24}$ (5.593)	**MS^2** [**1071.47**]: 1027 (100)[M-CO$_2$-H]$^-$ **MS^2** [**1027.48**]: 865 (14)[M-CO$_2$-Hex-H]$^-$, 847 (37)[M-CO$_2$-Hex-H$_2$O-H]$^-$, 823 (10)[M-malonyl-Hex-H]$^-$, 805 (7)[M-malonyl-Hex-H$_2$O-H]$^-$, 787 (100)[M-malonyl-Hex-H$_2$O-H$_2$O-H]$^-$, 779 (7), 761 (11), 641 (77)[M-CO$_2$-Hex-CO$_2$-H$_2$O-H]$^-$, 625 (9)[M-malonyl-Hex-Hex-H$_2$O-H$_2$O-H]$^-$, 581 (51)[M-malonyl-Hex-Hex-H$_2$O-H$_2$O-CO$_2$-H]$^-$, 513 (44), 485 (9)[Agly-H]$^-$ **MS^3** [**1027.48→787**]: 625 (54)[M-malonyl-Hex-Hex-H$_2$O-H$_2$O-H]$^-$, 581 (100)[M-malonyl-Hex-Hex-H$_2$O-H$_2$O-CO$_2$-H]$^-$, 513 (22), 485 (45)[Agly-H]$^-$ **MS^3** [**1027.48→641**]: 581 (100)[M-malonyl-Hex-Hex-H$_2$O-H$_2$O-CO$_2$-H]$^-$, 569 (6), 513 (11), 485 (38)[Agly-H]$^-$, 439 (2)
21 3-Glc-28-Glc-Medicagenic acid	825.42987	$C_{42}H_{66}O_{16}$ (2.494)	**MS^2** [**825.43**]: 663 (5)[M-Glc-H]$^-$, 619 (3)[M-Glc-CO$_2$-H]$^-$, 601 (35)[M-Glc-CO$_2$-H$_2$O-H]$^-$, 439 (100)[Agly-CO$_2$-H$_2$O-H]$^-$ **MS^3** [**825.43→439**]: 393 (51), 391 (100), 375 (8) **MS^3** [**825.43→601**]: 439 (100)[Agly-CO$_2$-H$_2$O-H]$^-$
22 Malonyl-Hex-HexA-Bayogenin	911.43371 867.44133^b	$C_{45}H_{68}O_{19}$ (6.041)	**MS^2** [**867.44**]: 825 (34)[M-malonyl-H]$^-$, 807 (25)[M-malonyl-H$_2$O-H]$^-$, 705 (9)[M-CO$_2$-Hex-H]$^-$, 645 (77)[M-malonyl-Hex-H$_2$O-H]$^-$, 601 (100)[M-malonyl-Hex-H$_2$O-CO$_2$-H]$^-$ **MS^3** [**867.44→601**]: 583 (3), 529 (100), 487 (7)[Agly-H]$^-$
23 dHex-Hex-Hex-Hederagenin	941.51536	$C_{48}H_{78}O_{18}$ (4.058)	**MS^2** [**941.51**]: 795 (100)[M-dHex-H]$^-$, 777 (12)[M-dHex-H$_2$O-H]$^-$, 633 (13)[M-dHex-Hex-H]$^-$, 615 (2)[M-dHex-Hex-H$_2$O-H]$^-$ **MS^3** [**941.51→795**]: 633 (100)[M-dHex-Hex-H]$^-$, 615 (5)[M-dHex-Hex-H$_2$O-H]$^-$, 471 (20)[Agly-H]$^-$ **MS^3** [**941.51→633**]: 471 (100)[Agly-H]$^-$, 393 (3)
24 Hex-Hex-HexA-Bayogenin	987.48378	$C_{48}H_{76}O_{21}$ (3.187)	**MS^2** [**987.48**]: 969 (3)[M-H$_2$O-H]$^-$, 825 (100)[M-Hex-H]$^-$, 645 (2)[M-Hex-Hex-H$_2$O-H]$^-$, 601 (2)[M-Hex-Hex-H$_2$O-CO$_2$-H]$^-$, 487 (3)[Agly-H]$^-$ **MS^3** [**987.48→825**]: 781 (4)[M-Hex-CO$_2$-H]$^-$, 645 (88)[M-Hex-Hex-H$_2$O-H]$^-$, 619 (24)[M-Hex-Hex-CO$_2$-H]$^-$, 601 (29)[M-Hex-Hex-H$_2$O-CO$_2$-H]$^-$, 569 (23), 487 (100)[Agly-H]$^-$, 467 (3)
25 Hex-dHex-Hex-HexA-Hederagenin	1117.54979	$C_{54}H_{86}O_{24}$ (5.515)	**MS^2** [**1117.55**]: 1099 (5)[M-H$_2$O-H]$^-$, 955 (100)[M-Hex-H]$^-$, 937 (4)[M-Hex-H$_2$O-H]$^-$, 893 (4)[M-Hex-H$_2$O-CO$_2$-H]$^-$, 791 (1)[M-Hex-dHex-H$_2$O-H]$^-$, 747 (1)[M-Hex- dHex-H$_2$O-CO$_2$-H]$^-$, 629 (2)[M-Hex-dHex-Hex-H]$^-$, 539 (2)[M-Hex-dHex-Hex-108-H]$^-$ **MS^2** [**1117.55→955**]: 937 (88)[M-Hex-H$_2$O-H]$^-$, 893 (67)[M-Hex-H$_2$O-CO$_2$-H]$^-$, 747 (67)[M-Hex-dHex-H$_2$O-CO$_2$-H]$^-$, 729 (21)[M-Hex-dHex-H$_2$O-H$_2$O-CO$_2$-H]$^-$, 629 (100)[M-Hex-dHex-Hex-H]$^-$, 611 (15)[M-Hex-dHex-Hex-H$_2$O-H]$^-$, 585 (14)[M-Hex-dHex-Hex-CO$_2$-H]$^-$, 539 (16)[M-Hex-dHex-Hex-108-H]$^-$, 471 (17)[Agly-H]$^-$, 453 (31)
26 3-Glc-Malonyl-28-Glc-Medicagenic acid	911.43233	$C_{45}H_{68}O_{19}$ (4.522)	**MS^2** [**911.43**]: 867 (100)[M-CO$_2$-H]$^-$, 705 (3)[M-Glc-CO$_2$-H]$^-$ **MS^3** [**911.43→867**]: 705 (4)[M-Glc-CO$_2$-H]$^-$, 661 (7)[M-Glc-CO$_2$-CO$_2$-H]$^-$, 601 (36)[M-Glc-malonyl-CO$_2$-H$_2$O-H]$^-$, 439 (100)[Agly-CO$_2$-H$_2$O-H]$^-$
27 Hex-Bayogenin	649.39774	$C_{36}H_{58}O_{10}$ (3.108)	**MS^2** [**649.40**]: 487 (100)[Agly-H]$^-$, 469 (15), 441 (28), 409 (36)
28 Hex-Medicagenic acid	663.37701	$C_{36}H_{56}O_{11}$ (3.051)	**MS^2** [**663.38**]: 645 (5)[M-H$_2$O-H]$^-$, 501 (13)[Agly-H]$^-$, 439 (100)[Agly-CO$_2$-H$_2$O-H]$^-$ **MS^3** [**663.38→439**]: 393 (83), 391 (100)
29 Hex-HexA-Aglycone A	823.41492	$C_{42}H_{64}O_{16}$ (3.352)	**MS^2** [**823.41**]: 661 (100)[M-Hex-H]$^-$, 617 (33)[M-Hex-CO$_2$-H]$^-$, 599 (52)[M-Hex-H$_2$O-CO$_2$-H]$^-$, 485 (15)[Agly-H]$^-$ **MS^3** [**823.41→661**]: 585 (5), 485 (100)[Agly-H]$^-$, 467 (34) **MS^3** [**823.41→599**]: 527 (100), 485 (3)[Agly-H]$^-$, 467 (2)
30 Malonyl-Hex-Hex-Medicagenic acid	911.43152 867.44123^b	$C_{45}H_{68}O_{19}$ (3.638)	**MS^2** [**867.44**]: 825 (33)[M-malonyl-H]$^-$, 807 (16)[M-malonyl-H$_2$O-H]$^-$, 705 (12)[M-Hex-CO$_2$-H]$^-$, 663 (10)[M-malonyl-Hex-H]$^-$, 645 (63)[M-malonyl-Hex-H$_2$O-H]$^-$, 601 (100)[M-malonyl-Hex-H$_2$O-CO$_2$-H]$^-$, 487 (9), 439 (82)[Agly-CO$_2$-H$_2$O-H]$^-$
31 dHex-Hex-HexA-Hederagenin	955.49433	$C_{48}H_{76}O_{19}$ (3.690)	**MS^2** [**955.49**]: 937 (100)[M-H$_2$O-H]$^-$, 911 (7)[M-CO$_2$-H]$^-$, 893 (35)[M-H$_2$O-CO$_2$-H]$^-$, 809 (8)[M-dHex-H]$^-$, 747 (24)[M-dHex-H$_2$O-CO$_2$-H]$^-$, 629 (28)[M-dHex-Hex-H$_2$O-H]$^-$, 611 (11)[M-dHex-Hex-H$_2$O-H$_2$O-H]$^-$, 539 (22)[M-dHex-Hex-108-H]$^-$, 471 (4)[Agly-H]$^-$ **MS^3** [**955.49→937**]: 893 (100)[M-H$_2$O-CO$_2$-H]$^-$, 859 (6), 747 (20)[M-dHex-H$_2$O-CO$_2$-H]$^-$, 611 (66)[M-dHex-Hex-H$_2$O-H$_2$O-H]$^-$, 539 (74)[M-dHex-Hex-108-H]$^-$, 471 (13)[Agly-H]$^-$, 451 (4), 441 (9) **MS^3** [**955.49→893**]: 747 (100)[M-dHex-H$_2$O-CO$_2$-H]$^-$, 729 (2), 585 (3), 567 (5), 471 (12)[Agly-H]$^-$

Table 1. Continued

Peak numbers and tentative identification of the observed saponins	$[M-H]^-$	Formula (δ ppm)	FT-ICR MS^n: m/z (% base peak)
32 Hex-dHex-Hex-HexA-Soyasapogenol E	1101.55386	$C_{54}H_{86}O_{23}$ (4.673)	**MS^2 [1101.55]**: 1083 (8)$[M\text{-}H_2O\text{-}H]^-$, 939 (100)$[M\text{-}Hex\text{-}H]^-$, 921 (6)$[M\text{-}Hex\text{-}H_2O\text{-}H]^-$, 877 (4)$[M\text{-}Hex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 731 (1)$[M\text{-}Hex\text{-}dHex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 613 (2)$[M\text{-}Hex\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H]^-$, 523 (3) **$MS^3$ [1101.55→939]**: 921 (100)$[M\text{-}Hex\text{-}H_2O\text{-}H]^-$, 877 (36)$[M\text{-}Hex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 793 (5), 731 (43)$[M\text{-}Hex\text{-}dHex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 713 (12)$[M\text{-}Hex\text{-}dHex\text{-}H_2O\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 613 (47)$[M\text{-}Hex\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H]^-$, 595 (26)$[M\text{-}Hex\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H_2O\text{-}H]^-$, 523 (58)$[M\text{-}Hex\text{-}dHex\text{-}Hex\text{-}108\text{-}H]^-$, 455 (7)$[Agly\text{-}H]^-$, 453 (4) **$MS^3$ [1101.55→1083]**: 921 (100)$[M\text{-}Hex\text{-}H_2O\text{-}H]^-$, 877 (43)$[M\text{-}Hex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 757 (22), 523 (50)$[M\text{-}Hex\text{-}dHex\text{-}Hex\text{-}108\text{-}H]^-$
33 Hex-Hex-HexA-Hederagenin	971.48921	$C_{48}H_{76}O_{20}$ (3.594)	**MS^2 [971.49]**: 809 (100)$[M\text{-}Hex\text{-}H]^-$, 791 (1)$[M\text{-}Hex\text{-}H_2O\text{-}H]^-$, 765 (1)$[M\text{-}Hex\text{-}CO_2\text{-}H]^-$, 629 (2)$[M\text{-}Hex\text{-}Hex\text{-}H_2O\text{-}H]^-$, 603 (1)$[M\text{-}Hex\text{-}Hex\text{-}CO_2\text{-}H]^-$, 585 (1)$[M\text{-}Hex\text{-}Hex\text{-}CO_2\text{-}H_2O\text{-}H]^-$, 539 (1), 471 (1)$[Agly\text{-}H]^-$ **MS^3 [971.49→809]**: 765 (6)$[M\text{-}Hex\text{-}CO_2\text{-}H]^-$, 747 (6)$[M\text{-}Hex\text{-}CO_2\text{-}H_2O\text{-}H]^-$, 647 (1)$[M\text{-}Hex\text{-}Hex\text{-}H]^-$, 629 (100)$[M\text{-}Hex\text{-}Hex\text{-}H_2O\text{-}H]^-$, 611 (2)$[M\text{-}Hex\text{-}Hex\text{-}H_2O\text{-}H_2O\text{-}H]^-$, 603 (27)$[M\text{-}Hex\text{-}Hex\text{-}CO_2\text{-}H]^-$, 585 (24)$[M\text{-}Hex\text{-}Hex\text{-}CO_2\text{-}H_2O\text{-}H]^-$, 567 (5)$[M\text{-}Hex\text{-}Hex\text{-}CO_2\text{-}H_2O\text{-}H_2O\text{-}H]^-$, 553 (17), 539 (9)$[M\text{-}Hex\text{-}Hex\text{-}108\text{-}H]^-$, 471 (100)$[Agly\text{-}H]^-$, 453 (17)
34 dHex-Hex-Hex-HexA-Soyasapogenol B	1103.57097	$C_{54}H_{88}O_{23}$ (5.987)	**MS^2 [1103.57]**: 1085 (100)$[M\text{-}H_2O\text{-}H]^-$, 1041 (60)$[M\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 957 (9)$[M\text{-}dHex\text{-}H]^-$, 913 (7)$[M\text{-}dHex\text{-}CO_2\text{-}H]^-$, 895 (33)$[M\text{-}dHex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 795 (2)$[M\text{-}dHex\text{-}Hex\text{-}H]^-$, 777 (31)$[M\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H]^-$, 759 (16)$[M\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H_2O\text{-}H]^-$, 620 (2), 571 (1), 457 (1)$[Agly\text{-}H]^-$ **MS^3 [1103.57→1085]**: 1067 (18)$[M\text{-}H_2O\text{-}H_2O\text{-}H]^-$, 1041 (100)$[M\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 895 (19)$[M\text{-}dHex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 759 (54)$[M\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H_2O\text{-}H]^-$, 619 (11)
35 Malonyl-Hex-Malonyl-Hex-Bayogenin	983.45391	$C_{48}H_{72}O_{21}$ (4.654)	**(MS)**: 983 (21)$[M\text{-}H]^-$, 895 (21)$[M\text{-}CO_2\text{-}CO_2\text{-}H]^-$, 734 (11)$[M\text{-}CO_2\text{-}CO_2\text{-}Hex\text{-}H]^-$, 691 (100)$[M\text{-}malonyl\text{-}Hex\text{-}CO_2\text{-}H]^-$, 649 (18)$[M\text{-}malonyl\text{-}Hex\text{-}malonyl\text{-}H]^-$, 631 (62)$[M\text{-}malonyl\text{-}Hex\text{-}malonyl\text{-}H_2O\text{-}H]^-$, 487 (39)$[Agly\text{-}H]^-$
36 Malonyl-Hex-Malonyl-Hex-Medicagenic acid	997.4345	$C_{48}H_{70}O_{22}$ (5.918)	**MS^2 [997.43]**: 953 (51)$[M\text{-}CO_2\text{-}H]^-$, 909 (100)$[M\text{-}CO_2\text{-}CO_2\text{-}H]^-$, 733 (6), 705 (24)$[M\text{-}malonyl\text{-}Hex\text{-}CO_2\text{-}H]^-$, 691 (26) **$MS^3$ [997.43→909]**: 891 (4)$[M\text{-}CO_2\text{-}CO_2\text{-}H_2O\text{-}H]^-$, 865 (6)$[M\text{-}CO_2\text{-}CO_2\text{-}CO_2\text{-}H]^-$, 849 (3)$[M\text{-}CO_2\text{-}CO_2\text{-}malonyl\text{-}H_2O\text{-}H]^-$, 703 (4)$[M\text{-}CO_2\text{-}CO_2\text{-}CO_2\text{-}Hex\text{-}H]^-$, 661 (6)$[M\text{-}CO_2\text{-}CO_2\text{-}malonyl\text{-}Hex\text{-}H]^-$, 643 (15)$[M\text{-}CO_2\text{-}CO_2\text{-}malonyl\text{-}Hex\text{-}H_2O\text{-}H]^-$, 439 (100)$[Agly\text{-}CO_2\text{-}H_2O\text{-}H]^-$
37 dHex-Hex-HexA-Soyasapogenol A	957.51003	$C_{48}H_{78}O_{19}$ (3.735)	**MS^2 [957.51]**: 939 (100)$[M\text{-}H_2O\text{-}H]^-$, 895 (50)$[M\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 811 (8)$[M\text{-}dHex\text{-}H]^-$, 767 (3)$[M\text{-}dHex\text{-}CO_2\text{-}H]^-$, 749 (29)$[M\text{-}dHex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 649 (1)$[M\text{-}dHex\text{-}Hex\text{-}H]^-$, 631 (29)$[M\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H]^-$, 613 (15)$[M\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H_2O\text{-}H]^-$, 541 (23)$[M\text{-}dHex\text{-}Hex\text{-}108\text{-}H]^-$, 473 (8)$[Agly\text{-}H]^-$ **MS^3 [957.51→939]**: 895 (100)$[M\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 749 (14)$[M\text{-}dHex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 613 (61)$[M\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H_2O\text{-}H]^-$, 541 (62)$[M\text{-}dHex\text{-}Hex\text{-}108\text{-}H]^-$ **MS^3 [957.51→895]**: 749 (100)$[M\text{-}dHex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 587 (5)$[M\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 473 (13)$[Agly\text{-}H]^-$
38 Malonyl-Hex-HexA-Hederagenin	895.43802 851.44613[b]	$C_{45}H_{68}O_{18}$ (5.283) $C_{44}H_{68}O_{16}$ (3.136)	**MS^2 [851.45]**: 809 (29)$[M\text{-}malonyl\text{-}H]^-$, 791 (27)$[M\text{-}malonyl\text{-}H_2O\text{-}H]^-$, 689 (82)$[M\text{-}Hex\text{-}CO_2\text{-}H]^-$, 629 (84)$[M\text{-}malonyl\text{-}Hex\text{-}H_2O\text{-}H]^-$, 585 (100)$[M\text{-}malonyl\text{-}Hex\text{-}CO_2\text{-}H_2O\text{-}H]^-$, 471 (4)$[Agly\text{-}H]^-$ **MS^3 [851.45→585]**: 513 (100), 471 (13)$[Agly\text{-}H]^-$ **MS^3 [851.45→629]**: 629 (100)$[M\text{-}malonyl\text{-}H_2O\text{-}H]^-$, 513 (54), 471 (78)$[Agly\text{-}H]^-$, 453 (45), 405 (64), 393 (74), 387 (22)
39 dHex-Hex-HexA-Hederagenin	955.49392	$C_{48}H_{76}O_{19}$ (3.261)	**MS^2 [955.49]**: 937 (100)$[M\text{-}H_2O\text{-}H]^-$, 911 (34)$[M\text{-}CO_2\text{-}H]^-$, 893 (68)$[M\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 809 (30)$[M\text{-}dHex\text{-}H]^-$, 747 (20)$[M\text{-}dHex\text{-}H_2O\text{-}CO_2\text{-}H]^-$, 689 (8), 629 (48)$[M\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H]^-$, 611 (8)$[M\text{-}dHex\text{-}Hex\text{-}H_2O\text{-}H_2O\text{-}H]^-$, 539 (14)$[M\text{-}dHex\text{-}Hex\text{-}108\text{-}H]^-$, 471 (17)$[Agly\text{-}H]^-$
40 Hex-HexA-Malonyl-Aglycone A	909.41850 865.42579[b]	$C_{45}H_{66}O_{19}$ (6.539) $C_{44}H_{66}O_{17}$ (3.543)	**MS^2 [865.43]**: 823 (3)$[M\text{-}malonyl\text{-}H]^-$, 821 (4)$[M\text{-}CO_2\text{-}CO_2\text{-}H]^-$, 805 (28)$[M\text{-}malonyl\text{-}H_2O\text{-}H]^-$, 761 (4)$[M\text{-}malonyl\text{-}CO_2\text{-}H_2O\text{-}H]^-$, 703 (8)$[M\text{-}CO_2\text{-}Hex\text{-}H]^-$, 643 (100)$[M\text{-}malonyl\text{-}Hex\text{-}H_2O\text{-}H]^-$, 599 (35)$[M\text{-}malonyl\text{-}Hex\text{-}CO_2\text{-}H_2O\text{-}H]^-$, 485 (1)$[Agly\text{-}H]^-$ **MS^3 [865.43→643]**: 509 (100), 485 (64)$[Agly\text{-}H]^-$, 467 (41), 421 (41)

Table 1. Continued

Peak numbers and tentative identification of the observed saponins	$[M-H]^-$	Formula (δ ppm)	FT-ICR MS^n: m/z (% base peak)
41 Hex-Hederagenin	633.40242	$C_{36}H_{58}O_9$ (2.547)	**MS^2 [633.40]**: 615 (33)[M-H_2O-H]$^-$, 513 (15), 471 (100)[Agly-H]$^-$, 453 (24), 405 (24), 393 (39)
42 Hex-Hex-HexA-Soyasapogenol E	955.49420	$C_{48}H_{76}O_{19}$ (3.554)	**MS^2 [955.49]**: 937 (2)[M-H_2O-H]$^-$, 835 (2), 793(100)[M-Hex-H]$^-$, 775 (2)[M-Hex-H_2O-H]$^-$, 731 (2)[M-Hex-H_2O-CO_2-H]$^-$, 613 (4)[M-Hex-Hex-H_2O-H]$^-$, 523 (5)[M-Hex-Hex-108-H]$^-$ **MS^3 [955.49→793]**: 749 (3)[M-Hex-CO_2-H]$^-$, 731 (29)[M-Hex-H_2O-CO_2-H]$^-$, 631 (2)[M-Hex-Hex-H]$^-$, 613 (100)[M-Hex-Hex-H_2O-H]$^-$, 595 (8)[M-Hex-Hex-H_2O-H_2O-H]$^-$, 587 (5)[M-Hex-Hex-CO_2-H]$^-$, 569 (8)[M-Hex-Hex-H_2O-CO_2-H]$^-$, 551 (6)[M-Hex-Hex-H_2O-H_2O-CO_2-H]$^-$, 537 (5), 523 (54)[M-Hex-Hex-108-H]$^-$, 455 (14)[Agly-H]$^-$, 453 (6)
43 Malonyl-Hex-Malonyl-Hex-Hederagenin	967.45877 879.47798[b]	$C_{48}H_{72}O_{20}$ (4.498)	**MS^2 [879,47]**: 861 (2)[M-CO_2-CO_2-H_2O-H]$^-$, 819 (6)[M-malonyl-CO_2-H_2O-H]$^-$, 777 (5)[M-malonyl-malonyl-H_2O-H]$^-$, 717 (27)[M-Hex-CO_2-CO_2-H]$^-$, 675 (100)[M-Hex-malonyl-CO_2-H]$^-$, 657 (3)[M-Hex-malonyl-CO_2-H_2O-H]$^-$, 615 (72)[M-Hex-malonyl-malonyl-H_2O-H]$^-$ **MS^3 [879.47→675]**: 657 (3)[M-Hex-malonyl-CO_2-H_2O-H]$^-$, 633 (20)[M-Hex-malonyl-malonyl-H]$^-$, 615 (100)[M-Hex-malonyl-malonyl-H_2O-H]$^-$, 513 (6)[M-Hex-malonyl-Hex-CO_2-H]$^-$, 485 (5), 471 (2)[Agly-H]$^-$, 453 (2), 425 (6), 393 (2) **MS^3 [879.47→615]**: 585 (26), 543 (6), 499 (6), 495 (8), 471 (23)[Agly-H]$^-$, 453 (9), 405 (16), 393 (100), 373 (7)
44 Hex-Pen-Hederagenin	765.44543	$C_{71}H_{66}O_{13}$ (3.088)	**MS^2 [765.45]**: 603 (100)[M-Hex-H]$^-$, 585 (7)[M-Hex-H_2O-H]$^-$, 471 (6)[Agly-H]$^-$ **MS^3 [765.45→603]**: 471 (100)[Agly-H]$^-$, 423 (14)
45 dHex-Hex-Hex-Soyasapogenol E	925.51905	$C_{48}H_{78}O_{17}$ (2.620)	**MS^2 [925.52]**: 779 (100)[M-dHex-H]$^-$, 761 (13)[M-dHex-H_2O-H]$^-$, 617 (7)[M-dHex-Hex-H]$^-$, 599 (1)[M-dHex-Hex-H_2O-H]$^-$, 455 (1)[Agly-H]$^-$ **MS^3 [925.52→779]**: 617 (100)[M-dHex-Hex-H]$^-$, 599 (4)[M-dHex-Hex-H_2O-H]$^-$, 455 (9)[Agly-H]$^-$
46 dHex-Hex-HexA-Hederagenin	955.49417	$C_{48}H_{76}O_{19}$ (3.523)	**MS^2 [955.49]**: 937 (100)[M-H_2O-H]$^-$, 911 (7)[M-CO_2-H]$^-$, 893 (50)[M-H_2O-CO_2-H]$^-$, 809 (15)[M-dHex-H]$^-$, 767 (8), 747 (8)[M-dHex-H_2O-CO_2-H]$^-$, 629 (23)[M-dHex-Hex-H_2O-H]$^-$, 611 (28)[M-dHex-Hex-H_2O-H_2O-H]$^-$, 539 (36)[M-dHex-Hex-108-H]$^-$, 471 (11)[Agly-H]$^-$
47 Malonyl-Hex-Hex-HexA-Soyasapogenol E	1041.49582 997.50288[b]	$C_{51}H_{78}O_{22}$ (4.438)	**MS^2 [997.50]**: 835 (3)[M-Hex-CO_2-H]$^-$, 817 (51)[M-Hex-H_2O-CO_2-H]$^-$, 775 (12)[M-Hex-H_2O-malonyl-H]$^-$, 757 (4)[M-Hex-H_2O-malonyl-H_2O-H]$^-$, 679 (3), 655 (100)[M-Hex-Hex-H_2O-CO_2-H]$^-$, 611 (5)[M-Hex-Hex-H_2O-CO_2-CO_2-H]$^-$, 595 (2)[M-Hex-Hex-H_2O-malonyl-H_2O-H]$^-$, 551 (3)[M-Hex-Hex-H_2O-CO_2-malonyl-H_2O-H]$^-$ **MS^3 [997.50→655]**: 595 (4)[M-Hex-Hex-H_2O-malonyl-H_2O-H]$^-$, 551 (20)[M-Hex-Hex-H_2O-CO_2-malonyl-H_2O-H]$^-$, 539 (100), 509 (65), 455 (15)[Agly-H]$^-$
48 dHex-Hex-Hex-Aglycone A	955.49454	$C_{48}H_{76}O_{19}$ (3.910)	**MS^2 [955.49]**: 809 (100)[M-dHex-H]$^-$, 791 (8)[M-dHex-H_2O-H]$^-$, 647 (5)[M-dHex-Hex-H]$^-$ **MS^3 [955.49→809]**: 647 (100)[M-dHex-Hex-H]$^-$, 485 (5)[Agly-H]$^-$
49 dHex-Hex-HexA-Bayogenin	971.48812	$C_{48}H_{76}O_{20}$ (2.473)	**MS^2 [971.49]**: 953 (66)[M-H_2O-H]$^-$, 927 (15)[M-CO_2-H]$^-$, 909 (100)[M-H_2O-CO_2-H]$^-$, 825 (4)[M-dHex-H]$^-$, 763 (6)[M-dHex-H_2O-CO_2-H]$^-$, 663 (3)[M-dHex-Hex-H]$^-$, 645 (47)[M-dHex-Hex-H_2O-H]$^-$, 627 (5)[M-dHex-Hex-H_2O-H_2O-H]$^-$, 601 (5)[M-dHex-Hex-H_2O-H_2O-CO_2-H]$^-$, 555 (15)[M-dHex-Hex-108-H]$^-$, 527 (5), 487 (5)[Agly-H]$^-$, 483 (3), 469 (6) **MS^3 [971.49→909]**: 837 (7), 763 (100)[M-dHex-H_2O-CO_2-H]$^-$, 745 (3)[M-dHex-H_2O-H_2O-CO_2-H]$^-$, 601 (24)[M-dHex-Hex-H_2O-H_2O-CO_2-H]$^-$, 583 (2), 573 (10), 487 (6)[Agly-H]$^-$, 485 (1), 459 (4), 439 (3) **MS^3 [971.49→953]**: 909 (100)[M-H_2O-CO_2-H]$^-$, 627 (11)[M-dHex-Hex-H_2O-H_2O-H]$^-$, 555 (40)[M-dHex-Hex-108-H]$^-$, 527 (2), 487 (2)[Agly-H]$^-$, 469 (6), 441 (1)
50 dHex-Hex-HexA-Hederagenin	955.49245	$C_{48}H_{76}O_{19}$ (1.723)	**MS^2 [955.49]**: 937 (100)[M-H_2O-H]$^-$, 893 (35)[M-H_2O-CO_2-H]$^-$, 747 (30)[M-dHex-H_2O-CO_2-H]$^-$, 629 (41)[M-dHex-Hex-H_2O-H]$^-$, 611 (22)[M-dHex-Hex-H_2O-H_2O-H]$^-$, 539 (21)[M-dHex-Hex-108-H]$^-$, 471 (18)[Agly-H]$^-$, 451 (4), 307 (4)
51 dHex-Hex-Hex-Bayogenin	957.50866	$C_{48}H_{78}O_{19}$ (2.304)	**MS^2 [957.50]**: 939(2)[M-H_2O-H]$^-$, 811 (100)[M-dHex-H]$^-$, 793 (6)[M-dHex-H_2O-H]$^-$, 649 (12)[M-dHex-Hex-H]$^-$, 631 (2)[M-dHex-Hex-H_2O-H]$^-$, 487 (2)[Agly-H]$^-$ **MS^3 [957.50→811]**: 649 (100)[M-dHex-Hex-H]$^-$, 631 (12)[M-dHex-Hex-H_2O-H]$^-$, 487 (56)[Agly-H]$^-$

Table 1. Continued

Peak numbers and tentative identification of the observed saponins	$[M-H]^-$	Formula (δ ppm)	FT-ICR MS^n: m/z (% base peak)
52 Hex-HexA-Bayogenin	825.43024	$C_{42}H_{66}O_{16}$ (3.162)	**MS^2 [825.43]**: 645 (100)[M-Hex-H_2O-H]$^-$, 619 (28)[M-Hex-CO_2-H]$^-$, 601 (20)[M-Hex-H_2O-CO_2-H]$^-$, 569 (11), 487 (78)[Agly-H]$^-$, 455 (8) **MS^3 [825.43→645]**: 557 (11), 487 (11)[Agly-H]$^-$, 469 (47), 439 (100) **MS^3 [825.43→487]**: 467 (67), 455 (72), 437 (15), 409 (100), 393 (10), 391 (15)
53 Malonyl-dHex-Hex-HexA-Soyasapogenol B (Pisumsaponin I)	1027.51636	$C_{51}H_{80}O_{21}$ (4.308)	**MS^2 [1027.52]**: 983 (100)[M-CO_2-H]$^-$ **MS^3 [1027.52→983]**: 965 (100)[M-CO_2-H_2O-H]$^-$, 921 (54)[M-CO_2-CO_2-H_2O-H]$^-$, 837 (10)[M-CO_2-dHex-H]$^-$, 793 (10)[M-CO_2-dHex-CO_2-H_2O-H]$^-$, 775 (23)[M-CO_2-dHex-CO_2-H_2O-H_2O-H]$^-$, 675 (3)[M-CO_2-dHex-Hex-H]$^-$, 657 (24)[M-CO_2-dHex-Hex-H_2O-H]$^-$, 639 (20)[M-CO_2-dHex-Hex-H_2O-H_2O-H]$^-$, 613 (1)[M-CO_2-dHex-Hex-H_2O-CO_2-H]$^-$, 567 (21)[M-CO_2-dHex-Hex-108-H]$^-$, 499 (7)[M-CO_2-dHex-Hex-HexA-H]$^-$
54 Hex-HexA-Soyasapogenol E	793.43923	$C_{42}H_{66}O_{14}$ (1.575)	**MS^2 [793.44]**: 673 (8), 631 (100)[M-Hex-H]$^-$, 613 (13)[M-Hex-H_2O-H]$^-$, 569 (8)[M-Hex-H_2O-CO_2-H]$^-$ **MS^3 [793.44→631]**: 613 (4)[M-Hex-H_2O-H]$^-$, 555 (3), 497 (2), 455 (100)[Agly-H]$^-$
55 Malonyl-dHex-Hex-Medicagenic acid	895.43821 851.44654[b]	$C_{45}H_{68}O_{18}$ (5.496) $C_{44}H_{68}O_{16}$ (3.617)	**MS^2 [851.45]**: 809 (49)[M-malonyl-H]$^-$, 791 (8)[M-malonyl-H_2O-H]$^-$, 689 (37)[M-Hex-CO_2-H]$^-$, 629 (48)[M-malonyl-Hex-H_2O-H]$^-$, 585 (100)[M-malonyl-Hex-H_2O-CO_2-H]$^-$, 439 (9)[Agly-CO_2-H_2O-H]$^-$
56 Hex-Hex-Bayogenin	811.45028	$C_{42}H_{68}O_{15}$ (2.138)	**MS^2 [811.45]**: 649 (100)[M-Hex-H]$^-$, 631 (8)[M-Hex-H_2O-H]$^-$, 487 (58)[Agly-H]$^-$ **MS^3 [811.45→649]**: 487 (100)[Agly-H]$^-$, 439 (1)
57 dHex-Hex-Hex-Aglycone A	955.49230	$C_{48}H_{76}O_{19}$ (1.566)	**MS^2 [955.49]**: 937 (3)[M-H_2O-H]$^-$, 809 (100)[M-dHex-H]$^-$, 791 (6)[M-dHex-H_2O-H]$^-$, 647 (15)[M-dHex-Hex-H]$^-$, 629 (6)[M-dHex-Hex-H_2O-H]$^-$, 485 (8)[Agly-H]$^-$ **MS^3 [955.49→809]**: 647 (100)[M-dHex-Hex-H]$^-$, 629 (26)[M-dHex-Hex-H_2O-H]$^-$, 485 (92)[Agly-H]$^-$
58 Hex-Hex-Bayogenin	811.45207	$C_{42}H_{68}O_{15}$ (4.344)	**MS^2 [811.45]**: 649 (100)[M-Hex-H]$^-$, 631 (31)[M-Hex-H_2O-H]$^-$, 487 (98)[Agly-H]$^-$ **MS^3 [811.45→649]**: 487 (100)[Agly-H]$^-$, 439 (1)
59 Hex-HexA-Bayogenin	825.43143	$C_{42}H_{66}O_{16}$ (4.386)	**MS^2 [825.43]**: 645 (100)[M-Hex-H_2O-H]$^-$, 601 (17)[M-Hex-H_2O-CO_2-H]$^-$, 487 (6)[Agly-H]$^-$, 439 (1) **MS^3 [825.43→645]**: 487 (100)[Agly-H]$^-$, 467 (2), 455 (5), 439 (9) **MS^3 [825.43→601]**: 529 (100), 487 (8)[Agly-H]$^-$
60 3-Glc-Glc-Medicagenic acid	825.43148	$C_{42}H_{66}O_{16}$ (4.446)	**MS^2 [825.43]**: 663 (1)[M-Glc-H]$^-$, 601 (1)[M-Glc-H_2O-CO_2-H]$^-$, 439 (100)[Agly-CO_2-H_2O-H]$^-$ **MS^3 [825.43→439]**: 393 (61), 391 (100), 375 (25)
61 dHex-Hex-Hex-Hederagenin	941.51621	$C_{48}H_{78}O_{18}$ (4.961)	**MS^2 [941.51]**: 795 (100)[M-dHex-H]$^-$, 777 (10)[M-dHex-H_2O-H]$^-$, 633 (13)[M-dHex-Hex-H]$^-$, 615 (2)[M-dHex-Hex-H_2O-H]$^-$, 471 (1)[Agly-H]$^-$ **MS^3 [941.51→795]**: 633 (100)[M-dHex-Hex-H]$^-$, 615 (5)[M-dHex-Hex-H_2O-H]$^-$, 471 (24)[Agly-H]$^-$ **MS^3 [941.51→633]**: 471 (100)[Agly-H]$^-$
62 Malonyl-Hex-Hex-Bayogenin	897.45213 853.46093[b]	$C_{45}H_{70}O_{18}$ (3.556) $C_{44}H_{70}O_{16}$ (2.132)	**MS^2 [853.46]**: 811 (100)[M-malonyl-H]$^-$, 793 (85)[M-malonyl-H_2O-H]$^-$, 691 (6)[M-CO_2-Hex-H]$^-$, 673 (2)[M-CO_2-H_2O-Hex-H]$^-$, 649 (3)[M-malonyl-Hex-H]$^-$, 631 (6)[M-malonyl-Hex-H_2O-H]$^-$, 613 (2)[M-malonyl-Hex-CO_2-H]$^-$, 487 (10)[Agly-H]$^-$ **MS^3 [853.46→811]**: 649 (100)[M-malonyl-Hex-H]$^-$, 631 (33)[M-malonyl-Hex-H_2O-H]$^-$, 487 (40)[Agly-H]$^-$ **MS^3 [853.46→793]**: 715 (2), 691 (6), 649 (8)[M-malonyl-Hex-H]$^-$, 631 (51) [M-malonyl-Hex-H_2O-H]$^-$, 613 (8)[M-malonyl-Hex-CO_2-H]$^-$, 595 (4)[M-malonyl-Hex-H_2O-CO_2-H]$^-$, 583 (2), 515 (1), 487 (100)[Agly-H]$^-$, 469 (4), 439 (10)
63 Hex-HexA-Hederagenin	809.43693	$C_{42}H_{66}O_{15}$ (4.985)	**MS^2 [809.44]**: 747 (5)[M-H_2O-CO_2-H]$^-$, 647 (2)[M-Hex-H]$^-$, 629 (100)[M-Hex-H_2O-H]$^-$, 603 (11)[M-Hex-CO_2-H]$^-$, 585 (14)[M-Hex-H_2O-CO_2-H]$^-$, 567 (2)[M-Hex-H_2O-H_2O-CO_2-H]$^-$, 553 (5), 539 (2)[M-Hex-108-H]$^-$, 471 (15)[Agly-H]$^-$, 469 (6), 453 (1), 451 (1), 439 (8), 423 (2)
64 Hex-HexA-Aglycone A	823.41390	$C_{42}H_{66}O_{16}$ (2.113)	**MS^2 [823.41]**: 779 (12)[M-CO_2-H]$^-$, 761 (5)[M-H_2O-CO_2-H]$^-$, 643 (100) [M-Hex-H_2O-H]$^-$, 617 (40)[M-Hex-CO_2-H]$^-$, 599 (34)[M-Hex-H_2O-CO_2-H]$^-$, 581 (4)[M-Hex-H_2O-H_2O-CO_2-H]$^-$, 567 (12), 485 (72)[Agly-H]$^-$, 467 (21)

Table 1. Continued

Peak numbers and tentative identification of the observed saponins	[M − H]$^-$	Formula (δ ppm)	FT-ICR MS^n: *m/z* (% base peak)
65 Pen-Hex-Hex-Aglycone B	925.48149	$C_{47}H_{74}O_{18}$ (1.352)	**MS^2 [925.48]**: 907 (25)[M-H_2O-H]$^-$, 793 (100)[M-Pen-H]$^-$, 775 (12)[M-Pen-H_2O-H]$^-$, 631 (54)[M-Pen-Hex-H]$^-$, 613 (7)[M-Pen-Hex-H_2O-H]$^-$, 469 (5)[Agly-H]$^-$ **MS^3 [925.48→793]**: 775 (9)[M-Pen-H_2O-H]$^-$, 631 (100)[M-Pen-Hex-H]$^-$, 613 (10)[M-Pen-Hex-H_2O-H]$^-$, 469 (9)[Agly-H]$^-$ **MS^3 [925.48→631]**: 613 (29)[M-Pen-Hex-H_2O-H]$^-$, 469 (100)[Agly-H]$^-$, 451 (3)
66 Hex-Hex-Aglycone A	809.43647	$C_{42}H_{66}O_{15}$ (4.417)	**MS^2 [809.44]**: 647 (99)[M-Hex-H]$^-$, 629 (27)[M-Hex-H_2O-H]$^-$, 485 (100)[Agly-H]$^-$ **MS^3 [809.44→485]**: 467 (100), 455 (21), 439 (11), 423 (40), 421 (61), 403 (48), 391 (11), 363 (10), 347 (18) **MS^3 [809.44→647]**: 485 (100)[Agly-H]$^-$
67 Hex-Medicagenic acid	663.37661	$C_{36}H_{56}O_{11}$ (2.448)	**MS^2 [663.38]**: 487 (6)[Agly-H_2O-H]$^-$, 439 (100)[Agly-CO_2-H_2O-H]$^-$ **MS^3 [663.38→439]**: 393 (61), 391 (100), 375 (11)
68 dHex-Hex-HexA-Soyasapogenol E	939.49979	$C_{48}H_{76}O_{18}$ (4.152)	**MS^2 [939.50]**: 921 (100)[M-H_2O-H]$^-$, 877 (24)[M-H_2O-CO_2-H]$^-$, 793 (11)[M-dHex-H]$^-$, 749 (5)[M-dHex-CO_2-H]$^-$, 731 (18)[M-dHex-H_2O-CO_2-H]$^-$, 613 (14)[M-dHex-Hex-H_2O-H]$^-$, 595 (17)[M-dHex-Hex-2H_2O-H]$^-$, 523 (11)[M-dHex-Hex-108-H]$^-$, 465 (24), 455 (8)[Agly-H]$^-$ **MS^3 [939.50→921]**: 877 (100)[M-H_2O-CO_2-H]$^-$, 731 (44)[M-dHex-H_2O-CO_2-H]$^-$, 595 (35)[M-dHex-Hex-2H_2O-H]$^-$, 523 (14)[M-dHex-Hex-108-H]$^-$, 465 (45)
69 dHex-Hex-HexA-Hederagenin	955.49569	$C_{48}H_{76}O_{19}$ (5.114)	**MS^2 [955.49]**: 937 (100)[M-H_2O-H]$^-$, 893 (63)[M-H_2O-CO_2-H]$^-$, 809 (14)[M-dHex-H]$^-$, 765 (23)[M-dHex-CO_2-H]$^-$, 747 (19)[M-dHex-H_2O-CO_2-H]$^-$, 629 (18)[M-dHex-Hex-H_2O-H]$^-$, 611 (19)[M-dHex-Hex-2H_2O-H]$^-$, 539 (21)[M-dHex-Hex-108-H]$^-$, 471 (8)[Agly-H]$^-$, 469 (3)
70 Hex-Bayogenin	649.39741	$C_{36}H_{58}O_{10}$ (2.599)	**MS^2 [649.39]**: 487 (100)[Agly-H]$^-$, 409 (3), 391 (2) **MS^3 [649.39→487]**: 467 (29), 455 (14), 421 (35), 409 (100), 403 (17), 393 (34), 391 (52), 379 (17)
71 Hex-HexA-Hederagenin	809.43534	$C_{42}H_{66}O_{15}$ (3.021)	**MS^2 [809.44]**: 629 (100)[M-Hex-H_2O-H]$^-$, 585 (11)[M-Hex-H_2O-CO_2-H]$^-$, 471 (2)[Agly-H]$^-$ **MS^3 [809.44→629]**: 499 (12), 471 (100)[Agly-H]$^-$, 469 (11), 439 (12) **MS^3 [809.44→585]**: 513 (100), 499 (2), 471 (5)[Agly-H]$^-$
72 Malonyl-Hex-Medicagenic acid	749.37838 705.38773[b]	$C_{39}H_{58}O_{14}$ (4.003) $C_{38}H_{58}O_{12}$ (3.089)	**MS^2 [749.38]**: 705 (100)[M-CO_2-H]$^-$ **MS^2 [705.39]**: 439 (100)[Agly-CO_2-H_2O-H]$^-$ **MS^3 [705.39→439]**: 393 (70), 391 (100), 375 (9)
73 Pen-Hex-HexA-Soyasapogenol B	927.49925	$C_{47}H_{76}O_{18}$ (3.624)	**MS^2 [927.50]**: 909 (100)[M-H_2O-H]$^-$, 865 (14)[M-CO_2-H_2O-H]$^-$, 795 (50)[M-Pen-H]$^-$, 733 (34)[M-Pen-CO_2-H_2O-H]$^-$, 633 (3)[M-Pen-Hex-H]$^-$, 615 (44)[M-Pen-Hex-H_2O-H]$^-$, 597 (65)[M-Pen-Hex-H_2O-H_2O-H]$^-$, 571 (4)[M-Pen-Hex-CO_2-H_2O-H]$^-$, 525 (72)[M-Pen-Hex-108-H]$^-$, 457 (15)[Agly-H]$^-$
74 Hex-HexA-Aglycone A	823.41450	$C_{42}H_{66}O_{16}$ (2.842)	**MS^2 [823.41]**: 643 (100)[M-Hex-H_2O-H]$^-$, 599 (50)[M-Hex-H_2O-CO_2-H]$^-$, 485 (4)[Agly-H]$^-$, 467 (4) **MS^3 [823.41→643]**: 599 (26)[M-Hex-H_2O-CO_2-H]$^-$, 515 (13), 487 (12), 485 (87)[Agly-H]$^-$, 469 (12), 467 (100) **MS^3 [823.41→599]**: 581 (1), 527 (100), 485 (4)[Agly-H]$^-$, 439 (2)
75 Malonyl-Hex-Bayogenin	735.39924 691.40869[b]	$C_{39}H_{60}O_{13}$ (4.003) $C_{38}H_{60}O_{11}$ (3.477)	**MS^2 [691.41]**: 649 (10)[M-malonyl-H]$^-$, 631 (100)[M-malonyl-H_2O-H]$^-$, 487 (2)[Agly-H]$^-$ **MS^3 [691.41→631]**: 601 (100), 487 (10)[Agly-H]$^-$, 469 (20), 421 (6), 409 (18), 393 (10), 391 (10) **MS^3 [691.41→649]**: 487 (100)[Agly-H]$^-$, 439 (1)
76 Malonyl-Hex-HexA-Hederagenin	895.43874 851.44633[b]	$C_{45}H_{68}O_{18}$ (6.088) $C_{44}H_{68}O_{16}$ (3.371)	**MS^2 [851.45]**: 809 (1)[M-malonyl-H]$^-$, 791 (2)[M-malonyl-H_2O-H]$^-$, 671 (39)[M-Hex-C_2H_2O-H_2O-H]$^-$, 627 (14)[M-Hex-malonyl-H_2O-H]$^-$, 611 (11)[M-Hex-malonyl-CO_2-H]$^-$, 585 (9), 567 (13), 499 (100), 471 (4)[Agly-H]$^-$
77 3-Rha-Gal-GlcA-Soyasapogenol B	941.51540	$C_{48}H_{78}O_{18}$ (4.101)	**MS^2 [941.52]**: 923 (100)[M-H_2O-H]$^-$, 879 (43)[M-H_2O-CO_2-H]$^-$, 795 (8)[M-Rha-H]$^-$, 751 (5)[M-Rha-CO_2-H]$^-$, 733 (24)[M-Rha-H_2O-CO_2-H]$^-$, 633 (2)[M-Rha-Gal-H]$^-$, 615 (28)[M-Rha-Gal-H_2O-H]$^-$, 597 (17)[M-Rha-Gal-2H_2O-H]$^-$, 525 (25)[M-Rha-Gal-108-H]$^-$, 457 (8)[Agly-H]$^-$ **MS^3 [941.52→923]**: 879 (100)[M-H_2O-CO_2-H]$^-$, 733 (19)[M-Rha-H_2O-CO_2-H]$^-$, 597 (54)[M-Rha-Gal-2H_2O-H]$^-$, 525 (70)[M-Rha-Gal-108-H]$^-$, 457 (4)[Agly-H]$^-$ **MS^3 [941.52→879]**: 733 (100) [M-Rha-H_2O-CO_2-H]$^-$, 457 (7)[Agly-H]$^-$

Table 1. Continued

Peak numbers and tentative identification of the observed saponins	$[M - H]^-$	Formula (δ ppm)	FT-ICR MS^n: m/z (% base peak)
78 Rha-Gal-GlcA-Soyasapogenol E	939.49939	$C_{48}H_{76}O_{18}$ (3.726)	**MS^2 [939.50]**: 921 (100)[M-H_2O-H]$^-$, 877 (42)[M-H_2O-CO_2-H]$^-$, 793 (8)[M-Rha-H]$^-$, 749 (1)[M-Rha-CO_2-H]$^-$, 731 (41)[M-Rha-H_2O-CO_2-H]$^-$, 613 (47)[M-Rha-Gal-H_2O-H]$^-$, 595 (29)[M-Rha-Gal-2H_2O-H]$^-$, 523 (64)[M-Rha-Gal-108-H]$^-$, 455 (5)[Agly-H]$^-$ **MS^3 [939.50→921]**: 877 (78)[M-H_2O-CO_2-H]$^-$, 731 (26)[M-Rha-H_2O-CO_2-H]$^-$, 595 (36)[M-Rha-Gal-2H_2O-H]$^-$, 523 (100)[M-Rha-Gal-108-H]$^-$, 455 (6)[Agly-H]$^-$ **MS^3 [939.50→523]**: 455 (91)[Agly-H]$^-$, 453 (100), 439 (13)
79 HexA-Hederagenin	647.38199	$C_{36}H_{56}O_{10}$ (3.726)	**MS^2 [647.38]**: 629 (5), 587 (7), 571 (18), 527 (4), 499 (3), 471 (100)[Agly-H]$^-$, 439 (7) **MS^3 [647.38→471]**: 439 (18), 405 (43), 393 (100)

[a] HexA, uronic acid, such as glucuronic acid or galacturonic acid; Hex, hexose, such as glucose or galactose; dHex, 6-deoxyhexose, such as rhamnose or furanose; Pen, pentose, such as arabinose or xylose; GlcA, glucuronic acid; Glc, glucose; Gal, galactose; Rha, rhamnose. [b] Ion resulting from in-source fragmentation.

interchanged with dependent IT-MS^n scan events, existing of an MS^2 and two MS^3 scans. In the MS^2 scan, the most abundant ion in the previous full MS scan was fragmented. The two most abundant daughter ions in every MS^2 scan event were subjected to a dependent MS^3 scan event. In addition, in-source fragmentation data were included in the identification process.

Identification of Saponins. Saponins are amphipathic glycosides consisting of a lipophilic aglycone or sapogenin covalently linked to one (monodesmosidic) or more (di- or tridesmosidic) sugar chains via an ether or ester glycosidic bond. The sugar residues are mainly hexoses (e.g., glucose and galactose), 6-deoxyhexoses (e.g., rhamnose and furanose), pentoses (e.g., arabinose and xylose), and uronic acids (e.g., glucuronic acid and galacturonic acid). As reported before, upon collision-induced dissociation (CID) under negative ESI-MS, saponins undergo glycosidic cleavages, retaining the charge at the reducing terminus (i.e., the fragment that contains the aglycone).[21,22] The detected (grand)daughter ions in the MS^n spectra provide structural information about the sugar residues and aglycone of the fragmented saponin.

Figure 1 illustrates the identification of a hitherto unknown saponin. As indicated in Figure 1A, the unknown saponin showed an $[M - H]^-$ anion at m/z 945.50965. Because saponins consist of the elements C, H, and O, three different molecular formulas are possible within an error range of 5 ppm: $C_{65}H_{69}O_6$ (δ ppm = −0.331), $C_{40}H_{81}O_{24}$ (δ ppm = −2.830), and $C_{47}H_{77}O_{19}$ (δ ppm = 3.380). To determine the correct molecular formula, the abundance of the isotopes was considered. In nature, stable elemental isotopes occur in a stable ratio. For instance, ^{13}C occurs at approximately 1.11% of the most frequent carbon isotope, ^{12}C. Similarly, ^2H and ^{17}O occur at 0.015 and 0.038% of ^1H and ^{16}O, respectively. Hence, the observed monoisotopic quasi-molecular ions (M_0) are always accompanied by additional isotope ions ($M+1$, $M+2$, ...) (Figure 1B), of which the relative abundance depends on the elemental composition of the compound. The predicted relative abundance of the $M + 1$ ions of the three possible molecular formulas was 73.41, 46.53, and 54.05%, respectively. For the unknown saponin, the observed relative abundance of the $M + 1$ ion was 53.63% (Figure 1B), indicating that the molecular formula corresponding to the $[M - H]^-$ anion is $C_{47}H_{77}O_{19}$ (δ ppm = 3.380). The sugar residues and the aglycone could be identified from the MS^n spectra. For this saponin, MS^2 fragmentation led to the generation of five daughter ions, resulting from the loss of water (−18 Da) and/or one or more hexose (−162 Da) and/or pentose (−132 Da) residues. The smallest daughter ion, at m/z 489, represents the aglycone ion, $[Agly - H]^-$, resulting from the loss of a pentose and two hexose moieties (Figure 1C). Based on this rationale, tens of other saponins present in the extract of *M. truncatula* hairy roots could tentatively be identified. All identified saponins and their MS^n data are listed in Table 1.

Cross-Ring Cleavage of Uronic Acid Residues. Saponins are reported to undergo only glycosidic cleavages upon CID under negative ESI-MS.[21] However, in this study, we observed a recurrent fragmentation pattern for saponins that contain a uronic acid residue, indicating a cross-ring cleavage of the uronic acid residue upon CID, as illustrated with the MS^2 spectrum observed for 3-Rha-Gal-GlcA-soyasapogenol B (Figure 2A,B). The anions with the highest relative intensity, observed at m/z 923 and 879, correlated to the neutral losses of 18 (H_2O) and 62 Da ($H_2O + CO_2$), respectively. A similar pattern was observed after the loss of the rhamnose residue (m/z 795), and the ions observed at m/z 751 and 733 correlated to an additional loss of 44 (CO_2) and 62 Da ($H_2O + CO_2$), respectively. Furthermore, three more characteristic peaks were observed at m/z 615, 597, and 525, corresponding to a loss of the galactose residue, and an additional loss of 18 (H_2O), 36 ($H_2O + H_2O$), and 108 Da. The latter loss can only be explained by a cross-ring cleavage of the uronic acid residue. Cross-ring cleavages of glycosides are charge-remote fragmentations, i.e., fragmentations that are not initiated by the charge position. Because of the low internal energy of negative ions, cross-ring cleavages of glycosides do not readily occur.[21] An exception are those that are linked via an ester bond as in, for example, feruloylated oligosaccharides[23,24] or sinapoyl glucose.[25] Sinapoyl glucose serves as a UV protectant in *Arabidopsis*, and upon CID in the negative ionization mode, losses of 60, 90, and 120 Da are observed,[25] which correspond to a $^{0,4}X$, $^{0,3}X$, and $^{0,2}X$ cross-ring cleavage, respectively (see legend of Figure 2 for explanation on nomenclature). However, in this study, the cross-ring cleavage of the uronic acid residue should be charge-driven, because the carboxylic group of the uronic acid bears the negative charge. The loss of 108 Da might be due to the loss of water followed by an additional loss of a $C_3H_6O_3$ fragment of 90 Da or by the loss of a $C_3H_8O_4$ fragment involving a $^{1,4}X$ cross-ring cleavage of the uronic acid residue (Figure 2C). This typical fragmentation pattern was observed in 20 compounds and aided in the tentative identification of hitherto unknown saponins. By means of this methodology, a total of 79 saponins present in the *M. truncatula* hairy root extracts were identified (Table 1). However, MS^n does not provide enough information for absolute structural characterization of the metabolites; hence, the identifications remain tentative.

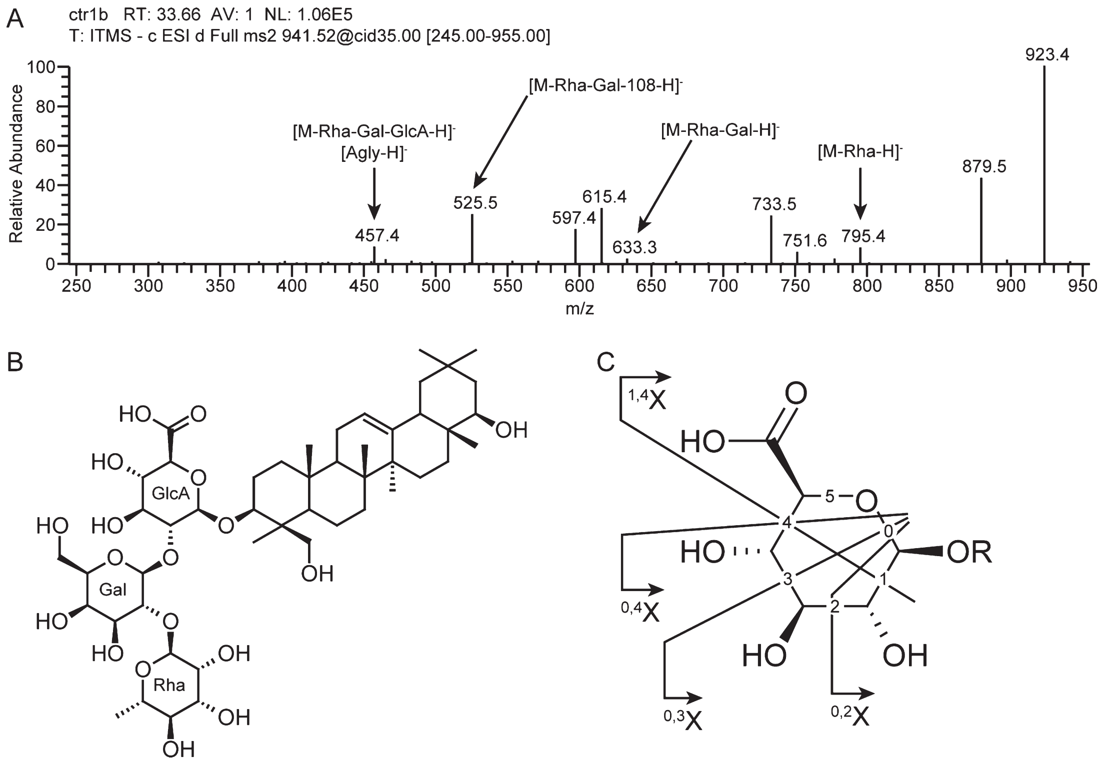

Figure 2. Cross-ring cleavage of uronic acid residues. (A) MS^2 fragmentation of 3-Rha-Gal-GlcA-soyasapogenol B. (B) Structure of 3-Rha-Gal-GlcA-soyasapogenol B. (C) Nomenclature of the cross-ring fragmentation of glucuronic acid.

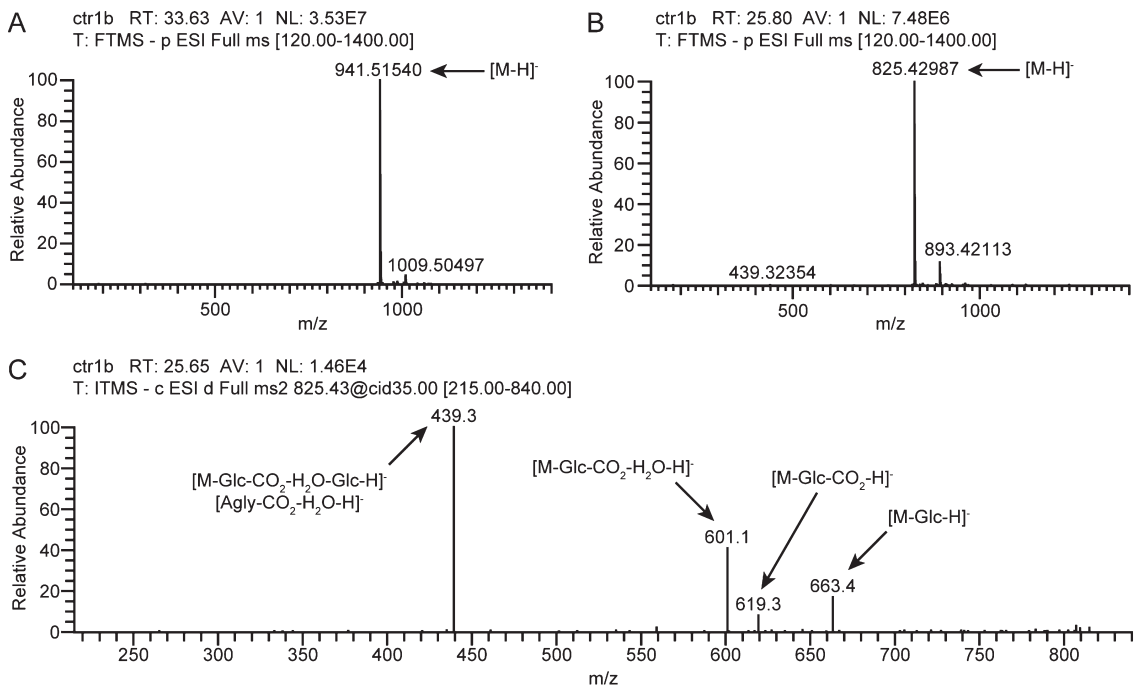

Figure 3. LC ESI FT-ICR MS chromatograms. (A) MS scan of peak at t_R 33.63 min (3-Rha-Gal-GlcA-soyasapogenol B). (B) MS scan of peak at t_R 25.80 min (3-Glc-28-Glc-medicagenic acid). (C) MS^2 fragmentation of 3-Glc-28-Glc-medicagenic acid.

Most Abundant Compounds in *M. truncatula* Hairy Roots. In the metabolite extracts of *M. truncatula* hairy roots, the major peak, at a retention time (t_R) of 33.63 min, yielded an $[M - H]^-$ anion at m/z 941.51540 ($C_{48}H_{78}O_{18}$, δ ppm = 4.101) (Figure 3A). In *M. truncatula*, two saponins have been described with the same molecular formula, Rha-Hex-Hex-hederagenin and soyasaponin I (3-Rha-Gal-GlcA-soyasapogenol B).[7] The MS^2 spectrum (Figure 2A and Table 1) corresponded to

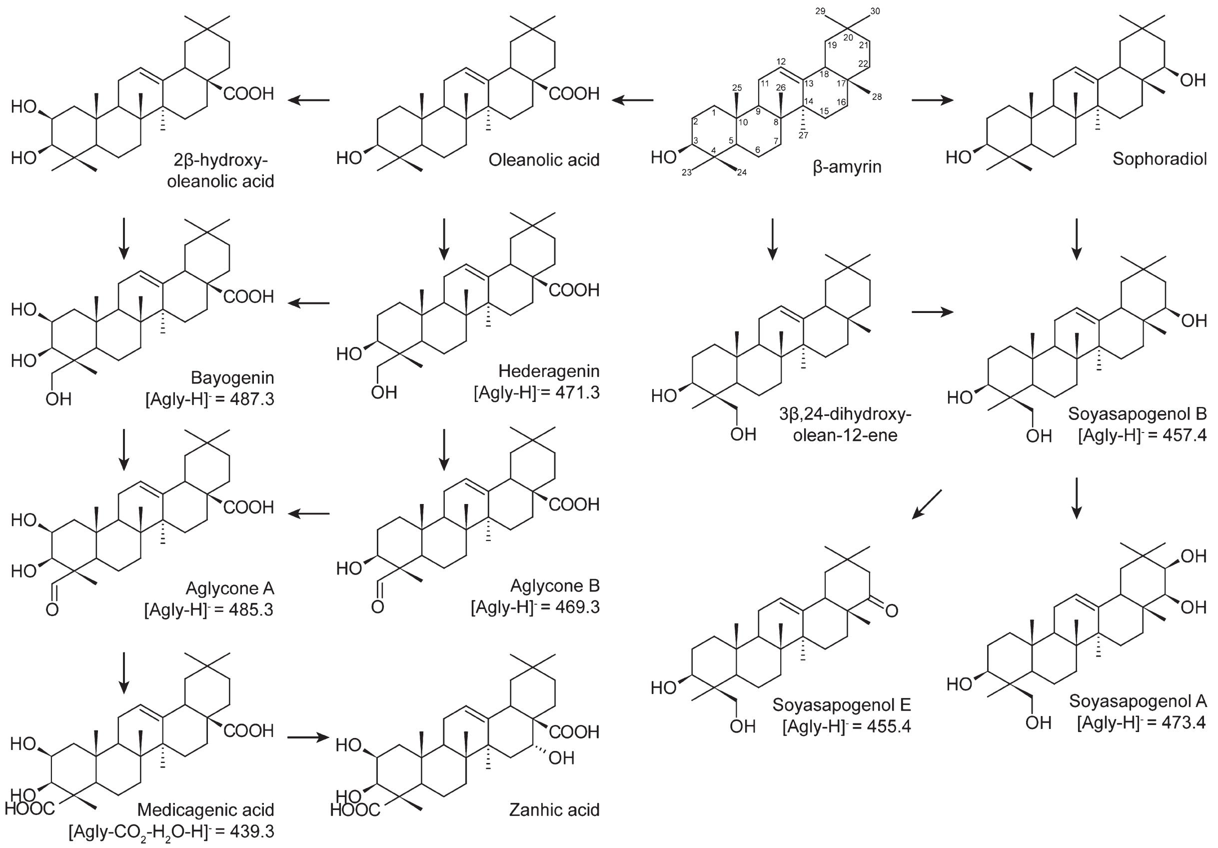

Figure 4. Theoretical biosynthetic pathway of sapogenins in *M. truncatula* and m/z values of the observed aglycones in the hairy roots extract.

previously observed MS/MS data of soyasaponin I,[26] allowing us to conclude that soyasaponin I is the most abundant saponin in the *M. truncatula* hairy roots extract. This is consistent with the previous observation that the major saponins in the roots of *M. truncatula* are soyasapogenol conjugates.[7] The second most abundant compound, eluting at 25.80 min, yielded an $[M - H]^-$ anion at m/z 825.42987 ($C_{42}H_{66}O_{16}$, δ ppm = 2.494) (Figure 3B). In the MS^2 spectrum (Figure 3C), the base peak occurs at m/z 439, a fragment ion resulting from the decarboxylation and dehydration of the medicagenic acid aglycone ion.[2] Additional anions were observed at m/z 663, 619, and 601, correlating to the sequential loss of a hexose (162 Da), a CO_2 (44 Da), and a H_2O (18 Da) molecule from the parent ion. This fragmentation pattern corresponded to the previously reported MS data of 3-Glc-28-Glc-medicagenic acid and 3-Glc-Glc-medicagenic acid.[2] To annotate the detected compound, we scanned for other peaks containing an anion with an m/z value between 825.40 and 825.45. Four additional isomers were detected, but only one (t_R 32.15 min) yielded a fragment ion at m/z 439 associated with medicagenic acid. As 3-Glc-28-Glc-medicagenic acid has been reported to have a lower retention time than 3-Glc-Glc-medicagenic acid,[2] the peak at t_R 25.80 min has been annotated as 3-Glc-28-Glc-medicagenic acid, and the peak at t_R 32.15 min as 3-Glc-Glc-medicagenic acid.

Absence of Zanhic Acid Glycosides. A remarkable observation was the absence of zanhic acid glycosides in the list of identified saponins. In previous studies, zanhic acid was shown to be one of the major aglycone moieties in aerial parts of *M. truncatula*,[8,9] and it was also reported to be present in other *M. truncatula* plant tissues, including roots.[7] In a more recent study, however, zanhic acid could not be detected in *M. truncatula* roots.[27] Similarly, no zanhic acid-containing compounds were detected in the hairy roots. Hairy root cultures are known to accumulate secondary metabolites that are normally produced in "natural" roots of differentiated plants.[13,14,28] Hence, the absence of zanhic acid residues in hairy roots may indicate that this compound is produced only in the aerial parts of the plant. Consequently, the occurrence of the enzyme responsible for the 16α-hydroxylation of the saponin backbone (Figure 4) might be restricted to the aerial parts of the plant. This finding might further support a tissue-specific role for the saponins *in planta*. Indeed, the saponin mixture from the aerial parts of the plant has a protective effect against herbivores, whereas that from the roots rather shows allelopathic and antimicrobial activities.[29] Thus, the *in vivo* role of the zanhic acid glycosides might be related to antinutritional effects in the aerial parts of the plant. In addition to zanhic acid glycosides, aerial parts are particularly rich in medicagenic acid glycosides.[7−9] Notably, none of the major medicagenic acid glycosides occurring in the aerial parts of the plant were found in the hairy roots, further underscoring the unique saponin composition of roots and aerial parts. In root extracts of greenhouse-grown *M. truncatula* plants, the presence of 31 saponins, mainly soyasapogenol conjugates, was shown.[7] Here, we could confirm the presence of 18 of these 31 previously described saponins. For some of the remaining saponins of this list, we could detect the corresponding nominal masses, but the observed accurate masses and fragmentation data did not unambiguously support the proposed structures. Hence, we did not include them in Table 1. However, in addition to the 18 previously described saponins, 61 other saponins were

Table 2. Observed Aglycones in *M. truncatula*

m/z [observed ion]	Molecular formula	Tentative identification
455 $[\text{Agly-H}]^-$	$C_{30}H_{48}O_3$	Soyasapogenol E
457 $[\text{Agly-H}]^-$	$C_{30}H_{50}O_3$	Soyasapogenol B
469 $[\text{Agly-H}]^-$	$C_{30}H_{46}O_4$	Aglycone B (3β-hydroxy-23-oxo-olean-12-en-28-oic acid)
471 $[\text{Agly-H}]^-$	$C_{30}H_{48}O_4$	Hederagenin
473 $[\text{Agly-H}]^-$	$C_{30}H_{50}O_4$	Soyasapogenol A
485 $[\text{Agly-H}]^-$	$C_{30}H_{46}O_5$	Aglycone A (2β,3β-dihydroxy-23-oxo-olean-12-en-28-oic acid)
487 $[\text{Agly-H}]^-$	$C_{30}H_{48}O_5$	Bayogenin
489 $[\text{Agly-H}]^-$	$C_{30}H_{50}O_5$	Aglycone D
437 $[\text{Agly-CO}_2\text{-H}_2\text{O-H}]^-$	$C_{30}H_{44}O_6$	Aglycone C
439 $[\text{Agly-CO}_2\text{-H}_2\text{O-H}]^-$	$C_{30}H_{46}O_6$	Medicagenic acid

encountered in this study, all of which had not been detected before in *M. truncatula*. This result underscores the power of the applied FT-ICR MS platform in metabolome analysis.

Ten Different Aglycones. One prerequisite we applied to include the detected compounds in the list of tentatively identified saponins was the occurrence of an aglycone ion in the MS^n fragmentation data. As such, we could show that the 79 identified saponins present in the extract of *M. truncatula* hairy roots were glycosides of 10 different aglycones. The structures of the aglycones are given in Figure 4, and the observed aglycone ions, their predicted molecular formulas, and tentative identifications are listed in Table 2. For five of the aglycones, i.e., hederagenin, bayogenin, medicagenic acid, and soyasapogenols B and E (Figure 4), saponins containing the aglycone have been described before.[2,7−9] A sixth aglycone yielded an $[\text{Agly} - \text{H}]^-$ ion at *m/z* 473 (Figure 5A). Although this aglycone was only detected as a (grand)daughter ion with nominal *m/z* values, its molecular formula could be predicted by the accurate prediction of the molecular formula of the parent ion and the observed loss of sugar residues. With this method, the molecular formula was predicted to be $C_{30}H_{50}O_4$, which corresponds to the molecular formula of soyasapogenol A. No saponins of *M. truncatula* that contain soyasapogenol A as the aglycone had been described, but the compound had been shown to occur in *M. truncatula* as a sapogenin.[27] Hence, the two saponins containing this aglycone were tentatively identified as soyasapogenol A glycosides.

Furthermore, four other, unknown, aglycones were observed. Aglycone A was shown to occur in 10 of the tentatively identified saponins. It had an observed $[\text{Agly} - \text{H}]^-$ ion at *m/z* 485 (Figure 5C), and its molecular formula was calculated to be $C_{30}H_{46}O_5$. An aglycone with this mass had been previously observed in *M. truncatula*, but its structure was not elucidated.[2,7,30] Only one sapogenin with this molecular formula is known to occur in the genus *Medicago*, namely, 2β,3β-dihydroxy-23-oxo-olean-12-en-28-oic acid. This aglycone contains a C-23 formyl group, which is thought to be the biosynthetic intermediate in the enzymatic oxidation of the C-23 hydroxy group of bayogenin, eventually leading to the carboxylation of medicagenic acid (Figure 4).[31] Thus, compounds containing aglycone A as the sapogenin could be 2β,3β-dihydroxy-23-oxo-olean-12-en-28-oic acid glycosides. Another identified saponin yielded an aglycone anion at *m/z* 469 (Figure 5D) and a predicted molecular formula of $C_{30}H_{46}O_4$ (aglycone B). Similar to aglycone A, this compound may contain a C-23 formyl group and may be the result of an enzymatic oxidation of hederagenin, leading to 3β-hydroxy-23-oxo-olean-12-en-28-oic acid (Figure 4).

Aglycone C, a third unknown aglycone encountered in one of the saponins of *M. truncatula* hairy roots, yielded a first product ion at *m/z* 437. Similar to medicagenic acid, this ion is the result of the decarboxylation and dehydration of aglycone C, which has a calculated molecular formula of $C_{30}H_{44}O_6$. Decarboxylation and dehydration of aglycones are observed only in medicagenic acid[2] and zanhic acid[8] and, thus, could be related to the presence of a C-23 carboxylic group on the aglycone. Consequently, we assume that aglycone C contains a C-23 carboxylic group. Two saponins contain an aglycone (aglycone D) yielding an anion at *m/z* 489 (Figure 1C) and a calculated molecular formula of $C_{30}H_{50}O_5$. No aglycones with this molecular formula have been described in *Medicago* to date, but, assuming a β-amyrin-derived triterpene skeleton, the molecular formula points toward an aglycone containing five hydroxy groups. Thus, we postulate that aglycone D could be an oxidation product of soyasapogenol A. However, since we cannot postulate a structure based on these assumptions, we did not include the aglycones C and D in Figure 3.

EXPERIMENTAL SECTION

Generation and Cultivation of Transgenic *M. truncatula* Hairy Roots. *A. rhizogenes*-mediated transformation of *M. truncatula* (ecotype Jemalong J5) hairy roots was carried out as reported[20] with the following modifications. After scarification by treatment with sulfuric acid for 5 min, seeds were surface sterilized with 12% NaOCl during 2 min and washed with sterile H_2O. Subsequently, seeds were treated with 1 μM 6-benzylaminopurine for 3 h and thereafter allowed to germinate on wet, sterile 3 mm Whatman paper at room temperature in the dark. After 2 days of germination, the seedlings were wounded by cutting approximately 2 mm from the root tip of the radicle. The wounded seedlings were infected with *A. rhizogenes* and placed on slanted agar plates containing Murashige and Skoog (MS) medium (pH 5.8) supplemented with vitamins (Duchefa). The plates were sealed with micropore tape and placed vertically. Cocultivation was allowed for 10 days under a 16 h/8 h light/dark regime at 22 °C. After 10 days, the plants were transferred to new plates, containing 100 mg/L cefotaxime to prevent *A. rhizogenes* growth, and incubated under identical conditions. After 10 days, hairy roots were excised from the plants and transferred to liquid MS medium (pH 5.8) supplemented with vitamins, 1% (w/v) sucrose, and 100 mg/L cefotaxime to eliminate *A. rhizogenes* contamination. The hairy roots were grown for 7 days in the dark at 24 °C and shaking at 300 rpm. Subsequently, the roots were transferred to horizontal Petri dishes containing solid MS medium (pH 5.8) supplemented with 1% (w/v) sucrose and 100 mg/L cefotaxime and grown in the dark at 24 °C. After 3 weeks, young hairy root tissue was subcultured to solid medium without antibiotics. For maintenance, the

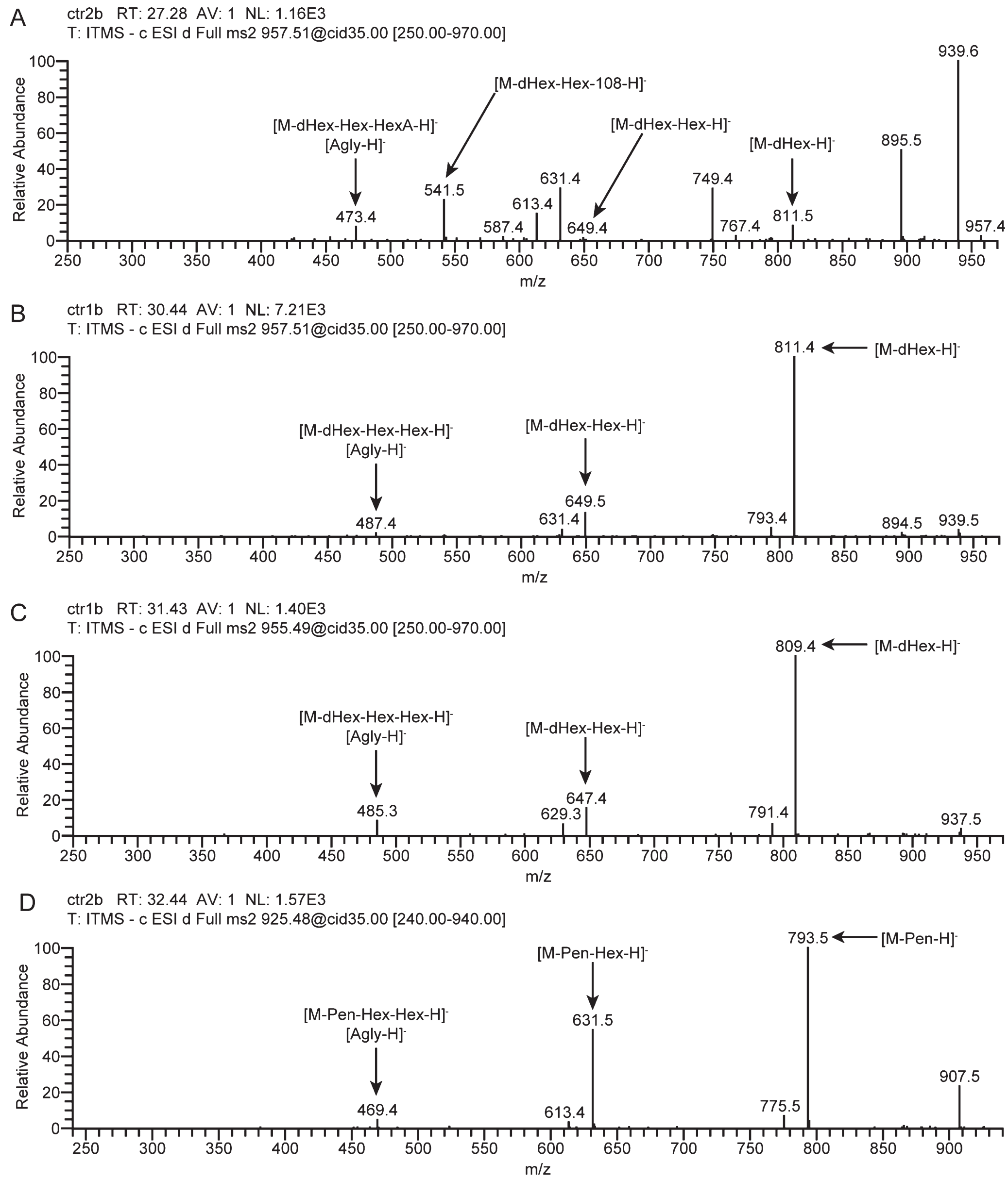

Figure 5. LC ESI FT-ICR MS chromatograms leading to the detection of the various aglycones. (A) MS^2 fragmentation of dHex-Hex-HexA-soyasapogenol A. (B) MS^2 fragmentation of dHex-Hex-Hex-bayogenin. (C) MS^2 fragmentation of dHex-Hex-Hex-aglycone A. (D) MS^2 fragmentation of Pen-Hex-Hex-aglycone B.

axenic hairy roots cultures were subcultured every 3 weeks onto fresh plates. Hairy roots for metabolic profiling were grown for 21 days in liquid MS medium (pH 5.8) supplemented with vitamins and 1% (w/v) sucrose.

Metabolite Extractions. *M. truncatula* hairy roots, grown for 21 days in liquid medium, were harvested and rinsed with purified H_2O under vacuum filtration. Subsequently, the roots were frozen and ground in liquid N_2. Of the ground material, 400 mg was extracted with 1 mL of MeOH at room temperature for 10 min, followed by centrifugation for 10 min at 20800*g*. Under vacuum, 500 μL of the supernatant was evaporated to dryness, and the residue was dissolved in 600 μL of H_2O/cyclohexane (2:1, v/v). Samples were centrifuged (10 min at 20800*g*), and 200 μL of the aqueous phase was retained for analysis.

LC ESI FT-ICR MS. For reversed-phase LC, an Acquity UPLC BEH C18 column (150 × 2.1 mm, 1.7 μm; Waters, Milford, MA) was serially coupled to an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm) and mounted on an ultra-high-performance LC system consisting of a Accela pump (Thermo Electron Corporation, Waltham, MA, USA) and Accela autosampler (Thermo Electron Corporation). The Accela LC system was hyphenated to a LTQ FT Ultra (Thermo Electron Corporation) via an electrospray ionization source. The following gradient was run using water/MeCN (99:1, v/v) acidified with 0.1% (v/v) HOAc (solvent A) and MeCN/water (99:1, v/v) acidified with 0.1% (v/v) HOAc (solvent B): time 0 min, 5% B; 30 min, 55% B; 35 min, 100% B. The loop size, flow, and column temperature were 25 μL, 300 μL/min, and 80 °C, respectively. Full loop injection was applied. Negative ionization was obtained with the following parameter values: capillary temperature 150 °C, sheath gas 25 (arbitrary units), aux. gas 3 (arbitrary units), and spray voltage 4.5 kV. Full FT-MS spectra between *m*/*z* 120−1400 were recorded at a resolution of 100 000. For identification, full MS spectra were interchanged with a dependent MS^2 scan event in which the most abundant ion in the previous full MS scan was

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fragmented, and two dependent MS^3 scan events in which the two most abundant daughter ions were fragmented. The collision energy was set at 35%.

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