

Hydroxylation of a Hederagenin Derived Saponin by a Xylareaceous Fungus Found in Fruits of *Sapindus saponaria*

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Durante nossos estudos visando a obtenção de microorganismos endofíticos associados a plantas tropicais, um fungo do grupo Xylareaceae foi isolado das partes internas dos frutos de *Sapindus saponaria*. Os frutos de *S. saponaria* acumulam grande quantidade de saponinas triterpênicas e sesquiterpênicas. A saponina 3-O-(β-D-xilopiranosil)-(1→3)-α-L-rhamnopyranosil-(1→2)-α-L-arabinopyranosil-hederagenina foi isolada por métodos cromatográficos após saponificação do extrato bruto obtido dos frutos de *S. saponaria* e administrada junto ao meio de cultivo usado para crescimento do fungo. Após extração dos metabólitos obtidos, uma nova saponina foi purificada por CLAE em escala preparativa e caracterizada como um derivado 22α-hidroxilado. Essa saponina hidroxilada teve sua estrutura molecular elucidada através da interpretação de dados de EM/EM e RMN.

During our screening of tropical plants for endophyte microorganisms, a Xylareaceous fungus was found living on the internal part of *Sapindus saponaria* fruits. The fruits of *S. saponaria* accumulate great amounts of triterpenoidal and sesquiterpenoidal saponins. The saponin 3-O-(β-D-xylopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-hederagenin was isolated using chromatographic methods, after alkaline hydrolysis of the crude extract obtained from *S. saponaria* fruits and added to the culture medium used to grows the fungus. A new saponin was isolated from this experiment by preparative scale HPLC and characterized as a 22α-hydroxy derivative. The structure of this hydroxylated saponin was elucidated based on interpretation of MS/MS data and NMR spectra.

Keywords: *Sapindus saponaria*, saponin, *Xylaria*, biotransformation, hydroxylation

Introduction

As part of our investigations on the chemical aspects of tropical plants and microorganisms interactions, we have been studying the plant *Sapindus saponaria* (Sapindaceae). Following many other Sapindaceae, *S. saponaria* produces annually great amounts of small fruits where a sap is accumulated.¹ Studying the glycoside composition of this soap by means of mass spectrometry coupled with liquid chromatography (LC-MS), it was found that those fruits accumulate on their pericarp huge amounts of monodesmosidic triterpene (SAPs) and bisdesmosidic sesquiterpene (acyclic sesquiterpene oligoglycosides, ASOGs) acetylated saponins.² Saponins are usually referred

as antifungal and antibacterial compounds, being the monodesmosidic the most active ones.^{3,4} These compounds form a group of chemical weapons used by plants in the fight against fungi, and the protection against pathogenic microbes is probably their natural role in plants.⁴⁻⁶ Despite this status of saponins as antimicrobial compounds, during our chemical studies on *S. saponaria* fruits it was observed that a fungus grows from a hairy structure attached to the seeds. The seeds are in contact with the pericarps where saponins are accumulated. In our previous studies this fungus was isolated and tentatively classified as a member of Xylareaceae, based on morphological characteristics and secondary metabolite profile, and we have also reported that this fungus is able to metabolize the glycosides present in *S. saponaria* fruits.⁷ Thus, using LC-MS it was shown that the ASOGs are completely consumed by this Xylareaceous

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fungus, while the SAPs and some exogenous glycosides are hydroxylated at the aglycon part of the molecule, in a small scale fermentation experiment. In the present work, the saponin **1** (Figure 1) was purified from a saponificated extract obtained from fruits of *S. saponaria* and used for a scaled up biotransformation reaction. We describe here this biotransformation experiment and a complete structural characterization of the hydroxylated saponin produced by the fungus.

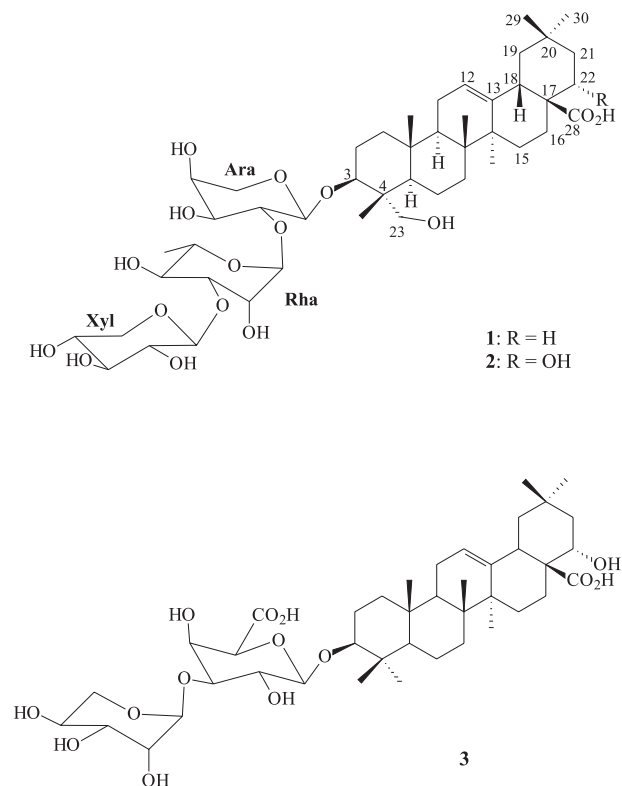


Figure 1. Molecular structure of the saponin starting material (**1**), the hydroxylated product (**2**), and a model compound (**3**).

Results and Discussion

In a previous work, we have shown that the saponins produced by *S. saponaria* occur as a complex mixture of naturally non-regioselective acetylated glycosides.² Alkaline hydrolysis of these natural glycosides produced simplified mixtures of compounds formed of only four SAPs and five ASOGs. Preparative scale HPLC separation of the SAP fraction led to isolation of pure saponin **1**, which was characterized as 3-O-(β -D-xylopyranosyl)-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-hederagenin by comparison of both MS and NMR spectra with those published by Grover and co-workers.⁸

Saponin **1** (100 mg) was submitted to biotransformation experiment and, after the conclusion of this experiment,

it was isolated 18 mg (18% yield) of the hydroxylated compound **2**. The MS data indicated that **2** was produced by the addition of an oxygen atom in **1**. The ESI mass spectrum of **1** shows a deprotonated molecule at m/z 881 ($[M-H]^-$) in agreement with the molecular formula $C_{46}H_{74}O_{16}$ (882 Da) of SAP **1**. Analyses of the compound produced by the fungus revealed a compound with a peak at m/z 897 ($[M-H]^-$) in its mass spectrum, probably associated with a compound whose molecular weight is 898 Da. The product ion spectrum obtained from m/z 881 (from **1**) shows ions detected at m/z 749 ($[SAP-xyl]^-$), 603 ($[SAP-xyl-ara]^-$) and 471 ($[SAP-xyl-ara-gly]^- = [Aglycone]^-$) which correspond to the losses of the sugar chain from the saponin. The same fragmentation experiment, using the precursor ion of m/z 897 ($[M-H]^-$) generated from **2**, produced exactly the same type of fragmentation but with the peaks in the mass spectrum shifted by 16 Da. For example, the peak at m/z 765 corresponds to the fragmentation that produced m/z 749 from **1**, plus 16 Da; m/z 747 corresponds to loss of water (18 Da) from m/z 765. Therefore the presence of m/z 487 ($[Aglycon]^-$) in the product ion spectrum of m/z 897 is an indicative that the addition of the 16 mass unit (oxygen) have occurred at the aglycon of the saponin **1** moiety.

The 1D 1H NMR spectra of saponins **1** and **2** are very complex and contains numerous overlapping signals. Thus, direct comparison of the 1D spectra did not allow visualization of an extra hydrogen atom bounded to an oxygen bearing carbon in **2**. On the other hand, the 2D NMR spectra (1H - 1H COSY) were efficient to localize this hydrogen signal. Figure 2 shows a comparison of a sector from the COSY spectra obtained for **1** and **2**. The partial spectrum of **1** (Figure 2A) shows correlations of hydrogen H-12 (δ 5.49) with H-11 (δ 1.92) and of the H-3 (δ 4.29) with H-2 (δ 2.01). These correlations are also seen in compound **2** spectrum (Figure 2B) which shows an extra contour due H-22 (δ 4.55) / H-21 (δ 1.81) coupling. In the HSQC spectrum of **2**, the hydrogen at δ 4.55 (H-22) is correlated with the ^{13}C NMR signal at δ 70.9 (C-22), corroborating the MS data and confirming the hydroxylation at the aglycone in saponin **1**.

The position where the oxygen atom was added in the saponin **1** was suggested by interpretation of NMR spectral data analyzed in comparison with the saponin starting material and model compounds found in the literature. The 1H NMR spectrum of **2** shows five signals for methyl groups (δ 1.34, 3H-27; δ 1.15, 3H-24; δ 1.09, 3H-30; δ 1.09, 3H-26; δ 1.00, 3H-25; δ 0.98, 3H-29) like found in spectrum of **1**.

The 2D 1H - 1H TOCSY spectrum of **2** showed three continuous spin systems for rings A (H-1 to H-3), B (H-5 to H-7) and C (H-9 to H-12). These correlations are

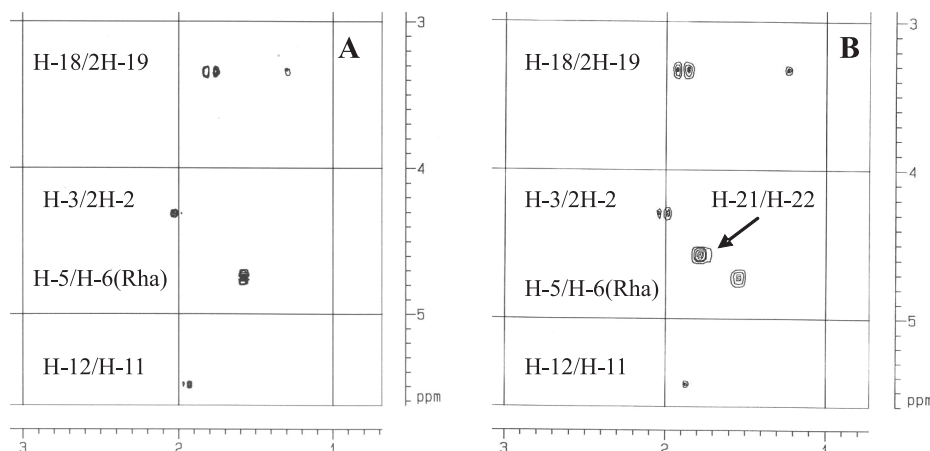


Figure 2. Sections of the ^1H - ^1H COSY spectra of **1** (A) compared to **2** (B) showing an additional correlation due H-22 (δ 4.55) coupled with H-21 (δ 1.81).

Table 1. ^{13}C NMR data^a of aglicons in saponins **1** and **2** and calculated ^{13}C chemical shift difference indicating the positioning of the additional OH group in **2** and comparison with a model compound (**3**)

C	1	2	$\Delta\delta^{1b}$	3 ¹¹	$\Delta\delta^{2c}$
1	39.3	38.8	-0.5	38.4	-0.4
2	26.5	26.6	+0.1	26.4	-0.2
3	81.3	80.9	-0.4	89.1	+8.2
4	43.8	43.1	-0.7	39.4	-3.7
5	47.9	47.9	0.0	55.5	+7.6
6	18.4	17.9	-0.4	18.3	+0.4
7	33.2	33.2	0.0	32.9	-0.3
8	40.0	39.8	+0.2	39.8	0.0
9	48.5	47.4	-1.1	47.8	+0.4
10	37.2	36.7	-0.5	36.8	+0.1
11	24.0	23.7	-0.3	23.6	-0.1
12	122.6	122.7	+0.1	122.7	0.0
13	145.5	144.0	-1.5	144.0	0.0
14	42.4	42.3	-1.1	42.4	+0.1
15	28.7	27.8	-0.9	27.8	0.0
16	24.0	16.7	-7.3	16.8	+0.1
17	46.9	53.0	+6.1	53.0	0.0
18	42.4	43.3	+0.9	43.4	+0.1
19	47.0	45.7	-1.3	45.8	+0.1
20	31.2	31.4	+0.2	31.4	0.0
21	34.6	43.4	+8.8	43.1	-0.3
22	33.6	70.9	+37.3	71.3	+0.4
23	64.4	63.7	-0.7	27.9	-35.8
24	14.4	14.0	-0.4	16.8	-2.9
25	16.4	15.9	-0.5	15.2	-0.7
26	17.9	17.2	-0.7	17.2	0.0
27	26.4	26.3	-0.1	26.6	+0.3
28	182.0	179.3	-2.7	179.3	0.0
29	33.1	32.5	-0.6	33.3	+0.8
30	23.9	25.0	+1.1	25.0	0.0

^a. At 100 MHz in pyridine- d_5 . ^b. $\Delta\delta^1 = \delta\mathbf{2} - \delta\mathbf{1}$. ^c. $\Delta\delta^2 = \delta\mathbf{3} - \delta\mathbf{2}$. $\Delta\delta < 0.5$ is considered not significant variation.

indicative that the hydroxylation did not occur at rings A, B and C. Couplings of 2H-15 with 2H-16 were not clearly seen due severe signal overlapping although analysis of HSQC data allows the ascribing of C-15 (δ 27.8) and C-16 (δ 16.7) signals. The ^1H NMR signal for H-18 (δ 3.38, dd, J 3.4/8.7 Hz), showing typical couplings with vicinal methylene hydrogens, exclude the possibility of hydroxylation at C-19. Therefore, by exclusion, the hydroxyl group was added by the fungus at C-21 or C-22. The comparative analysis of the ^{13}C NMR data for **1** and **2** (Table 1) also shows that the chemical shifts for the carbons at rings A, B and C are very similar. When compared with **1**, the ^{13}C chemical shift difference ($\Delta\delta$) becomes important only for some carbons (C-16 to C-22) at rings D and E.

A literature survey covering the recent publications on saponin and triterpene molecular structures revealed three compounds interesting for comparison with **2**. Thus, the ^{13}C NMR spectrum of **2** was compared with a 16-hydroxyoleanolic acid derived saponin isolated from *Albizia procera*,⁹ a triterpene 21-hydroxyhederagenin,¹⁰ and finally with scoparinoside (**3**), a 22-hydroxyolenolic acid derived saponin, isolated from fruits of *Kochia scoparia*.¹¹ The best match found indicated compound **2** to be a 22-hydroxy saponin. The ^{13}C NMR data of **2** (Table 1) shows good agreement ($\Delta\delta$ close to zero) with those of **3** considering the signals for carbons at rings D and E. The chemical shift of C-17 (δ 53.0) appears to be a good guide for the assignment of the hydroxyl group at C-22, since it is at *ca.* δ 46.0 – 48.0 in other rings D/E hydroxylated compounds.⁹⁻¹¹ In the same way observed for scoparinoside (**3**)¹¹, it was not detected H-22/C-28 HBMG long-range correlation. Probably, the long relaxation time of C-28 and the *cis* H-22/C-28 spatial relationship (Figure 3) difficult this observation. The shielding effect ($\Delta\delta = -7.3$)

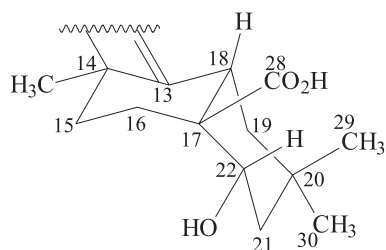


Figure 3. 3D visualization of rings D and E of the hydroxylated saponin **2**.

of the C-22-hydroxyl over C-16 (δ 16.7) and a nOe between H-22 and 3H-29 observed in ROESY spectrum indicates that compound **2** is a 22 α -hydroxylated product of **1**.

In general, deglycosylations (hydrolysis at the sugar chain) are frequently reported as saponin metabolisms done by microorganisms.¹²⁻¹⁴ Hydroxylation (monooxygenase expression) is the reaction that occurred in saponin **1**. This biochemistry process may find important technological application since expression of monooxygenase from fungi in saponins is not commonly found in the literature.

Experimental

Equipment

Preparative scale HPLC separations were performed using a Shimadzu equipment (LC-8A pump, CBM-10A communication module) equipped with an UV detector (SPD-6AV) set at 206 nm. HPLC grade acetonitrile (ACN) was obtained from Mallinckrodt, and H₂O was purified in a Milli-Q system (Millipore). ESI-MS/MS spectra were acquired in negative ion mode on a triple quadrupole Micromass Quattro LC spectrometer, equipped with a Z-Spray API ion source and a megaflo electrospray probe. NMR spectra were recorded in C₅D₅N (Aldrich) on a Bruker DRX 400 spectrometer operating at 400 MHz for hydrogen and 100 MHz. Some analysis were also performed using a Bruker AV 500 Ultrashield spectrometer operating at 500 MHz for ¹H and 125 MHz for ¹³C.

Plant material

Fruits of *S. saponaria* were collected in São Carlos, São Paulo State, Brazil. A voucher specimen (No. 003651) was deposited in the herbarium of the Botanic Department of Universidade Federal de São Carlos, Brazil.

Extraction and isolation of SAP I

Fruits of *S. saponaria* were dried under circulating air at a temperature of 50 °C during 48 h. Finely powdered

pericarpe (451.3 g) of dried fruits separated from seeds was extracted with methanol. The methanol extract was concentrated by distillation under reduced pressure and finally lyophilized to eliminate residual water. Part of the lyophilized extract (15.0 of 101.3 g) was stirred with 130 mL of aqueous NaOH 2 mol L⁻¹ for 30 min. The reaction solutions were acidified with 23 mL of HCl 2 mol L⁻¹. The resulting solution was passed through an octadecylsilyl (ODS) open column, which was further eluted with pure MeOH. The MeOH solution was evaporated and then part of the extract obtained (*ca.* 3 g) was repetitively subjected to preparative scale reverse phase HPLC (*ca.* 150 mg per injection), on an ODS column (Shimadzu Prep K, 2.0×25.0 m), eluted with ACN:H₂O (1:1 v/v) at a flow rate of 10 mL min⁻¹, with UV detection at 206 nm.

3-O-(β -D-xylopyranosyl)-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-hederagenin (**1**)

White amorphous powder; $[\alpha]_D^{25} + 113.4^\circ$ (MeOH, *c* 0.001); ¹H NMR (400 MHz, C₅D₅N): Aglicon: δ 5.49 (br s, H-12), 4.29 (m, H-3), 4.28 (d, *J* 10 Hz, H-23a), 3.93 (d, *J* 10 Hz, H-23b), 3.32 (dd, *J* 3.7/9.0 Hz, H-12), 1.26 (s, 3H-27), 1.14 (s, 3H-24), 1.06 (s, 3H-30), 1.04 (s, 3H-26), 1.01 (s, 3H-25), 0.93 (s, 3H-29), Arabinose: 5.07 (d, *J* 7 Hz, H-1), 4.62 (m, H-2), 4.00 (dd, *J* 3.9/8.1 Hz, H-3), 4.14 (m, H-4), 4.30 (m, H-5a), 3.67 (br d, *J* 10.8 Hz, H-5b), Rhamnose: 6.35 (br s, H-1), 4.92 (m, H-2), 4.83 (dd, *J* 2.9/9.5 Hz, H-3), 4.46 (t, *J* 9.5 Hz, H-4), 4.85 (dq, *J* 6.2/9.5 Hz, H-5), 1.55 (d, *J* 6.2 Hz, 3H-6), Xylose: 5.32 (d, *J* 7.3 Hz, H-1), 3.94 (t, *J* 8.8 Hz, H-2), 3.99 (t, *J* 9.5 Hz, H-3), 4.00 (dt, *J* 9.5/5.1 Hz, H-4), 4.13 (dd, *J* 11.0/5.1 Hz, H-5a), 3.56 (dd, *J* 11.0/9.5 Hz, H-5b); ¹³C NMR (100 MHz, C₅D₅N): see Table 1; ESIMS (daughter ion scan, 35 eV.): 881 [M-H]⁻ (100), 749 [M-H-Xyl]⁻ (23), 603 [M-H-Xyl-Rha]⁻ (29), 471 [Hederagenin - H]⁻ (16).

Isolation of fungus

Details of fungus isolation procedures and tentative identification are described elsewhere.⁷ After their isolation, the fungus was labeled with the code LaBioMMi217 and deposited at the Laboratory Biochemistry and Micromolecular of Microorganisms – LaBioMMi – of the Chemistry Department of the Federal University of São Carlos, São Carlos, Brazil.

Biotransformation experiments and chemical analysis

The fungus was seeded in a Petri dish containing PDA (potato-dextrose-agar) and allowed to grow for 12

days. The saponin **1** (100 mg) was dissolved in 20 mL of distilled water and equally distributed in twenty 0.5-liter Erlenmeyer flasks, each containing 150 mL of liquid medium (45 g glucose, 0.48 g NH_4NO_3 , 5.0 g KH_2PO_4 , 1.0 g MgSO_4 , 0.1 g FeSO_4 , 0.015 g CuSO_4 , 0.161 g ZnSO_4 , 0.01 g MnSO_4 , and 0.1 g $(\text{NH}_4)_2\text{MoO}_4$ dissolved in 1.5 L of distilled water). These Erlenmeyer flasks were autoclaved (121 °C, 25 min) and then pieces of the PDA (potato-dextrose-agar) culture containing mycelium were added to fifteen of the twenty flasks (five were kept as control) and were allowed at 25 °C standing in the dark during 15 days. The mycelium was separated by gravity filtration and the liquid phase was concentrated under reduced pressure and finally lyophilized. The extract was repetitively subjected to preparative scale separations using the same conditions described above for the isolation of **1**. These procedures led to isolation of 18.0 mg of pure saponin **2**. The ESI-MS/MS spectra were acquired in negative ion mode on a triple quadrupole Micromass Quattro LC spectrometer. The desolvation and ion source block temperatures were set, respectively, at 300 and 125°C. Gaseous N_2 was used to nebulize (80 L/H) and desolvate (350 L/H). The optimal voltages found for the probe and ion source components to produce maximum intensity of the ions $[\text{M}-\text{H}]^-$ were 3.3 kV for the stainless steel capillary, 38V for the sample cone, and 6V for the extractor cone. The parent/daughter runs (MS/MS) were performed by adding Ar in the collision cell to produce a pressure of 1.5×10^{-3} mBar for collisional induced dissociation (CID). The optimal collisional energy used to decompose the ions $[\text{M}-\text{H}]^-$ was 25-30 eV.

3-O-(β-D-xylopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-22α-hydroxyhederagenin (2)

White amorphous powder; $[\alpha]_D^{25} + 98.1^\circ$ (MeOH, *c* 0.001); $^1\text{H NMR}$ (400 MHz, $\text{C}_5\text{D}_5\text{N}$): Aglicon: δ 5.50 (br s, H-12), 4.55 (m, H-22), 4.30 (m, H-3), 4.26 (d, *J* 10 Hz, H-23a), 3.91 (d, *J* 10 Hz, H-23b), 3.38 (dd, *J* 3.4/8.7 Hz, H-12), 1.34 (s, 3H-27), 1.15 (s, 3H-24), 1.09 (s, 3H-30), 1.09 (s, 3H-26), 1.00 (s, 3H-25), 0.98 (s, 3H-29), Arabinose: 5.06 (d, *J* 7 Hz, H-1), 4.63 (m, H-2), 4.02 (dd, *J* 3.9/8.1 Hz, H-3), 4.13 (m, H-4), 4.33 (m, H-5a), 3.65 (br d, *J* 10.8 Hz, H-5b), Rhamnose: 6.31 (br s, H-1), 4.91 (m, H-2), 4.80 (dd, *J* 2.9/9.5 Hz, H-3), 4.43 (t, *J* 9.5 Hz, H-4), 4.87 (dq, *J* 6.2/9.5 Hz, H-5), 1.58 (d, *J* 6.2 Hz, 3H-6), Xylose: 5.29 (d, *J* 7.3 Hz, H-1), 3.91 (t, *J* 8.8 Hz, H-2), 3.97 (t, *J* 9.5 Hz, H-3), 4.01 (dt, *J* 9.5/5.1 Hz, H-4), 4.11 (dd, *J* 11.0/5.1 Hz, H-5a), 3.55 (dd, *J* 11.0/9.5 Hz, H-5b); $^{13}\text{C NMR}$ (100 MHz,

$\text{C}_5\text{D}_5\text{N}$): see Table 1; ESIMS (daughter ion scan, 35 eV.): 897 $[\text{M}-\text{H}]^-$ (100), 765 $[\text{M}-\text{H}-\text{Xyl}]^-$ (29), 747 $[\text{M}-\text{H}-\text{Xyl}-\text{OH}]^-$ (8), 619 $[\text{M}-\text{H}-\text{Xyl}-\text{Rha}]^-$ (63), 601 $[\text{M}-\text{H}-\text{Xyl}-\text{Rha}-\text{OH}]^-$ (52), 487 $[\text{22-Hydroxy-hederagenin} - \text{H}]^-$ (78).

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