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Structural and Sensory Characterization of Bitter Tasting Steroidal Saponins from Asparagus Spears (*Asparagus officinalis* L.)

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ABSTRACT: Application of sequential solvent extraction and iterative chromatographic separation in combination with taste dilution analysis recently revealed a series of steroidal saponins as the key contributors to the typical bitter taste of white asparagus spears (*Asparagus officinalis* L.). Besides six previously reported saponins, (25*R*)-furost-5-en-3*β*,22,26-triol-3-*O*-[*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside]-26-*O*-*β*-D-glucopyranoside, (25*R*)-furostane-3*β*,22,26-triol-3-*O*-[*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside]-26-*O*-*β*-D-glucopyranoside, and (25*S*)-furostane-3*β*,22,26-triol-3-*O*-[*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside]-26-*O*-*β*-D-glucopyranoside, and 3-*O*-[{*α*-L-rhamnopyranosyl-(1→2)}{*α*-L-rhamnopyranosyl-(1→4)}-*β*-D-glucopyranosyl]-(25*S*)-spirost-5-ene-3*β*-ol were identified for the first time as key bitter compounds in the edible spears of white asparagus by means of LC-MS/MS, LC-TOF-MS, 1D/2D-NMR spectroscopy, and hydrolysis experiments. This paper presents the isolation, structure determination, and sensory activity of these saponins. Depending on their chemical structure, the saponins identified showed human bitter recognition thresholds between 10.9 and 199.7 μmol/L (water).

KEYWORDS: *taste, bitter, asparagus, Asparagus officinalis L., saponins*

INTRODUCTION

Because of its alluring aroma and attractive taste profile, fresh and cooked asparagus spears (*Asparagus officinalis* L.) are highly appreciated by consumers. Compared to green asparagus, which is consumed worldwide, white asparagus is cultivated mainly in continental northwestern Europe by covering the plant with dirt while growing to keep it in the dark. This process, called "etiolation", produces the slightly bitter, but tender, fiberless, soft and more delicate flavor than the green one. If the bitter taste becomes too pronounced, it is considered an off-taste leading to consumer complaints and causing a major problem for vegetable producers.[1]

To answer the question as to which nonvolatile key taste compounds are responsible for the typical taste of asparagus spears, we recently applied the so-called taste dilution analysis on fractions isolated from raw and cooked asparagus, respectively, as reported in a companion paper.[2] This sensory-directed fractionation led to the identification of previously not reported 1,2-dithiolan-4-carboxylic acid 6-D-*α*/*β*-glucopyranose esters exhibiting an interesting buttery mouth-coating effect, besides a series of mono- and bidesmotic saponins **1a**/**b**-**5** (Figure 1) as the key players imparting the bitter taste of asparagus spears.[2] Because these saponins have as yet not been reported as key bitter taste compounds in asparagus spears, their isolation, structure determination, and sensory data are presented.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially from the sources given in parentheses: acetonitrile, ethanol, formic acid, methanol, glucose, xylose (Merck, Darmstadt, Germany); diosgenin, sarsapogenin, (Extrasynthése, Genay, France); galactose, rhamnose, and trifluoracetic acid (Fluka, Neu-Ulm, Germany); protodioscin (LGC Promochem, Wesel, Germany). Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France). Solvents were of HPLC grade (Merck Darmstadt, Germany). Water for HPLC separation was purified by means of a Milli-Q water advantage A 10 water system (Millipore, Molsheim, France). For sensory analysis, bottled water (Evian, low mineralization: 405 mg/L) was adjusted to pH 5.9 with trace amounts of formic acid prior to use. Freshly harvested, white asparagus spears (~21 cm in length) of the cultivar Grolim were obtained from local producers in Quedlinburg (May/June 2005), Münster (June 2007), and Schrobenhausen (May 2008), respectively. The fresh roots of *Asparagus officinals* L. were purchased from Maatschap Landers, Kessel (Netherlands) in 2007, washed with water and kept frozen at −20 °C until used.

Sensory Analyses. Seven female and five male panelists (25−40 years in age), who had given the informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, participated in panel training and sensory analyses using the sip-and-spit method and wearing nose clips as reported recently in a companion paper.[2] Closely following the procedure reported recently, bitter recognition threshold concentrations were determined in bottled water adjusted to pH 5.9 with trace amounts of 1% aqueous formic acid by means of a three-alternative forced choice test with ascending concentrations of the purified stimulus.[3−5] The threshold values of the sensory group were approximated by averaging the threshold values of the panelists in three independent sessions. Values between individuals and separate sessions differed by not more than plus/minus one dilution step; that is, a threshold value of 65.9 μmol/L for saponins **1a**/**b** (Figure 1) represents a range from 33.0 to 131.8 μmol/L.

Isolation and Identification of Bitter Saponins from Asparagus Spears. Following the fractionation scheme displayed in Figure 2, asparagus spears were ground, extracted with methanol and methanol/water (70/30, v/v; adjusted to pH 5.9 with trace amounts of aqueous formic acid (1% in water)), the combined organic layers were separated from solvent in vacuum and the lyophilized

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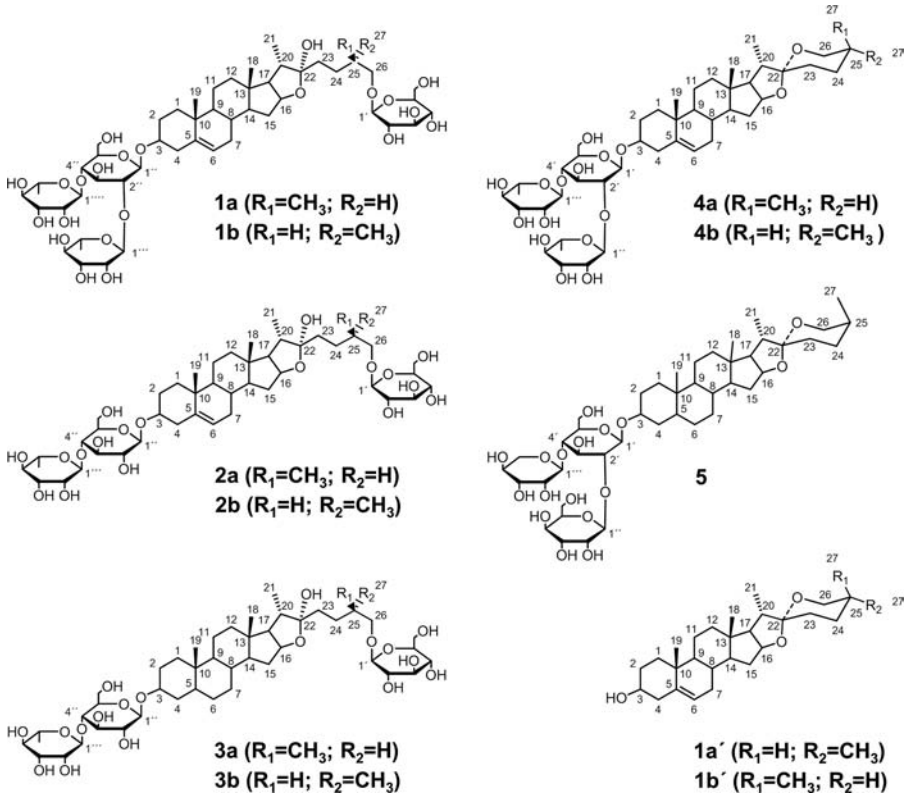

Figure 1. Chemical structures of bitter tasting saponins (**1a/b**-**5**) identified in white asparagus spears and the aglycons diosgenin (**1a′**) and yamogenin (**1b′**).

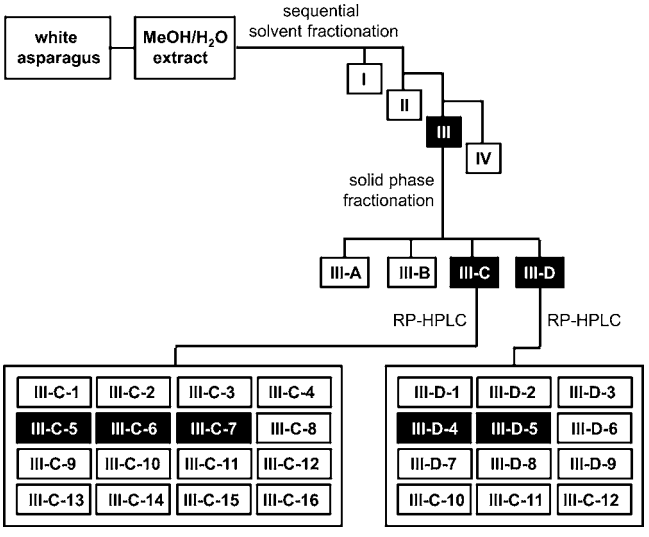

Figure 2. Separation scheme used to locate bitter tasting saponins in *Asparagus officinalis* L.

material obtained was extracted by pentane (I), followed by dichloromethane (II) and ethyl acetate (III) to obtain the solvent soluble fractions I–III and the water-solubles (fraction IV), as reported recently in a companion paper.[2] A solution of the lyophilized fraction III in methanol/water (30/70, v/v) was separated on a Strata C18-E SPE cartridge with a sequence of methanol/water mixtures to give fraction III-A (0/100, v/v, fraction III-B (30/70, v/v), fraction III-C (70/30, v/v), and fraction III-D (100/0, v/v), respectively. The two later fractions were separated by means of preparative RP-HPLC to give 16 and 12 subfractions, respectively, among which fractions III-C-5 to III-C-7, and III-D-4 showed the highest bitter impact. After rechromatography and verifying the purity of each fraction by means of analytical RP-HPLC, the key bitter components were separated from solvent in a vacuum, freeze-dried twice and then studied by LC-TOF-MS, LC-MS/MS, 1D/2D-NMR spectroscopy as well as sensory experiments.

3-O-[α-L-Rhamnopyranosyl-(1→2)-{α-L-rhamnopyranosyl-(1→4)}-β-D-glucopyranosyl]-26-O-[β-D-glucopyranosyl]-(25R/S)-22-hydroxyfurost-5-ene-3β,26-diole, ***1a/b****, Figure 1.* LC-MS (ESI$^-$): *m/z* 1047 (100, [M − H]$^-$), 1083 (26, [M + Cl]$^-$); MS/MS (DP = −175 V): *m/z* (%) 1047 (26), 902 (33), 755 (26), 575 (20), 413 (14), 402 (20), 260 (20), 204 (20), 163 (20), 143 (26), 130 (26), 119 (52), 113 (54), 101 (85), 88 (75), 85 (42), 71 (66), 59 (100); LC-TOF-MS: *m/z* 1071.5360 ([M + Na]$^+$, measured), *m/z* 1071.5352 ([M + Na]$^+$, calcd. for $C_{51}H_{84}O_{22}Na$). ^{1}H and ^{13}C NMR data are given in Tables 1 and 2.

(25R/S)-Furost-5-ene-3β,22,26-triol-3-O-[α-L-rhamnopyranosyl-(1→4)-β-D-gluco-pyranosid]-26-O-β-D-glucopyranoside, ***2a/b****, Figure 1.* LC-MS (ESI$^-$): *m/z* 901 (100, [M − H]$^-$), 947 (68, [M + Cl]$^-$); MS/MS (DP = −185 V): *m/z* (%) 901 (100), 755 (6), 205 (1), 161 (1), 143 (1), 131 (1), 119 (2), 115 (1), 112 (3), 103 (2), 101 (8), 89 (8), 87 (2), 85 (5), 73 (4), 71 (6), 59 (18); LC-TOF-MS: LC-TOF-MS: *m/z* 901.4805 ([M − H]$^-$, measured), *m/z* 901.4797 ([M − H]$^-$, calcd. for $C_{45}H_{73}O_{18}$). ^{1}H and ^{13}C NMR data are given in Tables 1 and 2.

(25R/S)-Furostane-3β,22,26-triol-3-O-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside]-26-O-β-D-glucopyranoside, ***3a/b****, Figure 1.* LC-MS (ESI$^-$): *m/z* 903 (100, [M − H]$^-$), 939 (21, [M + Cl]$^-$); MS/MS (DP = −195 V): *m/z* (%) 903 (100), 757 (10), 739 (2), 595 (3), 433 (3), 119 (1), 113 (3), 103 (1), 101 (8), 89 (1), 87 (1), 85 (5), 83 (2), 73 (3), 71 (5), 59 (16), 55 (1); LC-TOF-MS: *m/z* 903.4929 ([M − H]$^-$, measured), *m/z* 903.4953 ([M − H]$^-$, calcd. for $C_{45}H_{75}O_{18}$). ^{1}H and ^{13}C NMR data are given in Tables 1 and 2.

3-O-[α-L-Rhamnosypyranosyl-(1→2)-α-L-rhamnosypyranosyl-(1→4)-β-D-glucopyranosyl]-(25S)-spirost-5-en-3β-ole, ***4a/b****, Figure 1.* LC-MS (ESI$^-$): *m/z* 867 [100, M − H]$^-$, 903 [49, M + Cl]$^-$, 913 (22, [M + HCOO]$^-$); MS/MS (DP = −200 V): *m/z* (%) 867 (100), 721 (29), 575 (3), 509 (3), 289 (3), 265 (2), 246 (14), 205 (25), 179

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Table 1. ^{1}H and ^{13}C NMR Data (500 MHz; pyridine-d_5 plus 5% MeOD) of the Aglycon Moiety of Saponins 1a/b-3a/b

	1a/b				2a/b				3a/b			
position	δ_C/ppm	HSQC	δ_H/ppm	M [J/Hz]	δ_C/ppm	HSQC	δ_H/ppm	M [J /Hz]	δ_C/ppm	HSQC	δ_H/ppm	M [J/Hz]
1	37.4	[CH_2]	0.90−0.97	m (o)	37.0	[CH_2]	0.89−0.96	m	30.7	[CH_2]	1.34−1.58	m
			1.67−1.76	m (o)			1.66−1.73	m			1.59−1.78	m
2	30.0	[CH_2]	1.77−1.93	m (o)	30.0	[CH_2]	1.78−1.94	m	26.8	[CH_2]	1.34−1.58	m
			1.95−2.10	m (o)			1.94−2.11	m			1.78−2.10	m
3	78.3	[CH]	3.77−3.93	m	78.0	[CH]	3.76−3.89	m	74.5	[CH]	4.13−4.24	m
4	38.9	[CH_2]	2.62−2.71	m	38.8	[CH_2]	2.60−2.70	m	30.2	[CH_2]	1.59−1.78	m
			2.72−2.80	m			2.70−2.77	m			1.59−1.78	m
5	140.7	[C]			140.8	[C]			36.7	[CH]	1.95−2.11	m
6	121.8	[CH]	5.09−5.31	m	121.6	[CH]	5.08−5.52	m	26.5	[CH_2]	1.01−1.12	m
											1.59−1.78	m
7	32.1	[CH_2]	1.34−1.48	m	32.0	[CH_2]	1.29−1.46	m	26.8	[CH_2]	0.86−0.98	m
			1.77−1.93	m			1.78−1.94	m			1.12−1.34	m
8	31.6	[CH]	1.77−1.93	m	31.7	[CH]	1.46−1.57	m	35.2	[CH]	1.34−1.58	m
9	50.2	[CH]	0.81−0.91	m	50.2	[CH]	0.82−0.87	m	40.2	[CH]	1.12−1.34	m
10	37.0	[C]			37.2	[C]			35.3	[C]		
11	21.0	[CH_2]	1.34−1.48[a]	m	21.0	[CH_2]	1.31−1.46	m	21.1	[CH_2]	1.12−1.34	m
			1.34−1.48[a]	m			1.31−1.46	m			1.12−1.34	m
12	39.8	[CH_2]	0.98−1.15	m	39.9	[CH_2]	1.05−1.14	m	40.3	[CH_2]	1.59−1.78	m
			1.67−1.76	m			1.66−1.78	m			2.17−2.24	m
13	40.7	[C]			40.7	[C]			41.2	[C]		
14	56.5	[CH]	0.97−1.14	m	56.5	[CH]	0.96−1.05	m	56.2	[CH]	1.02−1.12	m
15	32.2	[CH_2]	1.34−1.48	m	32.3	[CH_2]	1.31−1.46	m	32.3	[CH_2]	1.34−1.58	m
			1.95−2.10	m			1.94−2.12	m			1.95−2.11	m
16	81.0	[CH]	4.84−4.94	m	81.0	[CH]	4.84−4.97	m	81.2	[CH]	4.90−4.98	m
17	63.7	[CH]	1.77−1.93	m	63.6	[CH]	1.78−1.94	m	63.9	[CH]	1.78−1.95	m
18	16.3	[CH_3]	0.85	s	16.3	[CH_3]	0.83	s	16.7	[CH_3]	0.84	s
19	19.3	[CH_3]	1.01	s	19.3	[CH_3]	1.00	s	23.6	[CH_3]	0.81	s
20	40.5	[CH]	2.16−2.23	m	40.5	[CH]	2.14−2.21	m	40.6	[CH]	2.17−2.25	m
21	16.3	[CH_3]	1.27	d [J = 7.0]	16.1	[CH_3]	1.29	d [J = 7.0]	16.4	[CH_3]	1.30	d [J = 7.0]
22	110.5	[C]			110.6	[C]			110.5	[C]		
23	37.0	[CH_2]	1.60−1.75	m	37.0	[CH_3]	1.66−1.78	m	36.9	[CH_2]	1.59−1.78	m
			1.95−2.10	m			1.78−1.94	m			1.95−2.11	m
24	28.1	[CH_2]	1.60−1.75	m	28.2	[CH_3]	1.57−1.66	m	28.1	[CH_2]	1.59−1.78	m
			1.95−2.10	m			1.94−2.12	m			1.95−2.11	m
	28.15[b]	[CH_2]	1.60−1.75[b]	m	28.2[a,b]	[CH_3]	1.57−1.66[b]	m	28.1[a,b]	[CH_2]	1.59−1.78[b]	m
			1.95−2.10[b]	m			1.94−2.12[b]	m			1.95−2.11[b]	m
25	34.2[c]	[CH]	1.67−1.76	m	34.0	[CH]	1.78−1.94	m	34.2	[CH]	1.78−2.10	m
	34.3[b,c]	[CH]	1.77−1.93[b]	m	34.0[a,b]	[CH]	1.78−1.94[b]	m	34.4[b]	[CH]	1.78−2.10[b]	m
26	74.9	[CH_2]	3.93−3.99	m	75.1	[CH_2]	3.44−3.48	m	75.2	[CH_2]	3.93−4.02	m
			4.00−4.07	m			4.03−4.07	m			4.02−4.13	m
	75.0[b]	[CH_2]	3.93−3.99[b]	m	75.1[a,b]	[CH_2]	3.44−3.48[b]	m	75.3[b]	[CH_2]	3.93−4.02[b]	m
			4.00−4.07[b]	m			4.03−4.07[b]	m			4.02−4.13[b]	m

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Table 1. continued

	1a/b				2a/b				3a/b			
position	δ_C/ppm	HSQC	δ_H/ppm	M [J/Hz]	δ_C/ppm	HSQC	δ_H/ppm	M [J/Hz]	δ_C/ppm	HSQC	δ_H/ppm	M [J/Hz]
27	17.3[a]	[CH$_3$]	0.99	d [J = 6.6]	17.3	[CH$_3$]	0.95	d [J = 6.7]	17.4[a]	[CH$_3$]	0.95	d [J = 6.6]
	17.3[a,b]	[CH$_3$]	0.95[b]	d [J = 6.6]	17.3[a,b]	[CH$_3$]	1.00[b]	d [J = 6.7]	17.4[a,b]	[CH$_3$]	1.00[b]	d [J = 6.6]

[a]Fall together. [b]Belongs to the epimere. [c]Interchangeable.

(3), 163 (20), 161 (7); LC-TOF-MS: m/z 867.4711 ([M − H]$^-$, measured), m/z 867.4742 ([M − H]$^-$, calcd. for $C_{45}H_{71}O_{16}$). ^{1}H and ^{13}C NMR data are given in Tables 3 and 4.

*3-O-[β-D-Glucopyranosyl-(1→2)-β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl]-(25S)-5β-spirostan-3β-ole, **5**, Figure 1.* LC-MS/MS and LC-TOF-MS were identical to those found for saponin **5** isolated from asparagus roots.

Isolation of Saponin 5 from Asparagus Roots. Asparagus roots (1 kg) were ground in a laboratory blender (Retsch, Haan, Germany) for 60 s at 3500 U/min, extracted with methanol (2 L) for 1 h upon vigorous stirring under an atmosphere of argon and, after filtration, the residue was extracted with a methanol/water mixture (7/3, v/v; pH 5.9; 4 × 2 L) for 1 h. The combined organic extracts were freed from solvent in a vacuum, the crude extract was taken up in water (1 L) and then it was sequentially extracted with *n*-pentane (4 × 500 mL), dichloromethane (4 × 500 mL), followed by ethyl acetate (4 × 500 mL). The corresponding extracts were combined, freed from solvent in a vacuum and freeze-dried to obtain the pentane extract (yield: 8.7%), the dichloromethane extract (yield: 0.7%), and the ethyl acetate extract (yield: 1.9%), the latter of which was dissolved in acetonitrile/water (70/30, v/v; 50 mg/2 mL) and separated by preparative RP-HPLC using a 250 × 21.2 mm i.d., 5 μm, HyperClone ODS C18 column (Phenomenex, Aschaffenburg, Germany) equipped with a guard column of the same type. Using a flow rate of 20 mL/min chromatography was performed, starting with a mixture (95/5, v/v) of 1% formic acid in water and 1% formic acid in acetonitrile. Thereafter the acetonitrile concentration was first increased to 50% within 50 min and then within 10 min to 100%, and, after maintaining the acetonitrile content for 10 min, it was decreased within 10 min to 5% acetonitrile and, finally, maintaining it at this acetonitrile content for additional 10 min. The effluent of the peak showing the pseudo molecular ion m/z 871.4733 ([M − H]$^-$) in an UPLC-TOF-MS experiment was collected individually in several runs and the eluates of the corresponding fractions were combined. After the solvent was separated in a vacuum and freeze-dried, the bitter saponin **5** (>98% purity) was analyzed by means of LC-MS/MS, UPLC-TOF-MS, and 1D/2D-NMR experiments.

*3-O-[β-D-Glucopyranosyl-(1→2)-β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl]-(25S)-5β-spirostan-3β-ole, **5**, Figure 1.* LC-MS (ESI$^-$): m/z (%) 871 (100, [M − H]$^-$), 917 (34, [M + HCOO]$^-$), 907 (27, [M + Cl]$^-$); MS/MS (DP = −195 V): m/z (%) 871 (100), 739 (26), 709 (6), 577 (10), 541 (2), 457 (2), 305 (5), 221 (4), 184 (2), 179 (4), 161 (33), 158 (15), 155 (2); LC-TOF-MS: m/z 871.4733 ([M − H]$^-$, measured), m/z 871.4691 ([M − H]$^-$,calcd for $C_{44}H_{71}O_{17}$). ^{1}H and ^{13}C NMR data are summarized in Tables 3 and 4.

Acidic Hydrolysis of Saponins. A solution of the saponin (2 mg) in methanol (5 mL) was added to a solution of aqueous trifluoracetic acid (3 mol/L in water; 4 mL) and, then, heated at 100 °C for 6 h. The reaction mixture was then cooled to r.t., neutralized with an aqueous KOH solution (4 mol/L) and, after adding water (20 mL), the solution was extracted with ethyl acetate (3 × 10 mL). The aqueous fraction was diluted with water (30 mL) and analyzed by means of high-performance ion chromatography (HPIC) for soluble carbohydrates, whereas the combined organic layers were separated from solvent in vacuum and, then, analyzed for the saponin aglycon by means of LC-MS/MS, LC-TOF-MS and 1D/2D-NMR analysis.

*Diosgenin, **1a**′, and Yamogenin, **1b**′, Figures 1 and 3.* LC-MS (ESI$^+$): m/z (%) 415 (100, M + H]$^+$), 437 (7, M + Na]$^+$), MS/MS (DP = −130 V): m/z (%) 413 (100), 243 (2), 57 (4); LC-TOF-MS: m/z 415.3204 ([M + H]$^+$, measured), 415.3207 ([M + H]$^+$, calcd for $C_{27}H_{42}O_3$). ^{1}H and ^{13}C NMR data were identical with those reported in the literature.[6−8]

High Performance Ion Chromatography (HPIC). Anion exchange chromatography was performed on an ICS-2500 ion chromatography system (Dionex, Idstein, Germany) consisting of a GS 50 gradient pump, an AS 50 autosampler, an AS 50 thermal compartment, and an ED 50 electrochemical detector as reported in a companion paper.[2] Carbohydrates released upon acidic hydrolysis of saponins were identified by comparing the retention times with that of

Table 2. ^{1}H and ^{13}C NMR Data (500 MHz; pyridine-d_5 plus 5% MeOD) of the Sugar Moieties of Saponins 1a/b−3a/b

	1a/b				2a/b				3a/b			
position	δ_C/ppm	HSQC	δ_H/ppm	M [J/Hz]	δ_C/ppm	HSQC	δ_H/ppm	M [J /Hz]	δ_C/ppm	HSQC	δ_H/ppm	M [J/Hz]
1′	105.0	[CH]	4.98	d [J = 7.8]	104.8	[CH]	5.06	d [J = 7.8]	105.0	[CH]	5.12	d [J = 7.8]
	104.8[d]	[CH]	4.76[d]	d [J = 7.6]	104.6[d]	[CH]	4.77[d]	d [J = 7.7]	104.7[d]	[CH]	4.78[d]	d [J = 7.6]
2′	74.9	[CH]	3.93−4.00	m	74.9	[CH]	3.88−3.98	m	74.9	[CH]	3.93−4.02	m
3′	78.3[a]	[CH]	3.53−3.66	m	78.3	[CH]	3.75−3.88	m	78.4	[CH]	3.89−3.93	m
4′	71.4	[CH]	4.12−4.23	m	71.5	[CH]	4.07−4.21	m	71.5	[CH]	4.13−4.26	m
5′	78.3[a]	[CH]	4.24−4.36	m	78.3	[CH]	4.21−4.35	m	78.4	[CH]	4.26−4.38	m
6′	62.5	[CH_2]	4.24−4.36	m	62.5	[CH_2]	4.21−4.35	m	62.6	[CH_2]	4.26−4.38	m
			4.44−4.51	m			4.44−4.48	m			4.54−4.58	m
1″	100.1	[CH]	4.90	br. s [J = 7.7][b]	102.1	[CH]	4.94	d [J = 7.7]	102.9	[CH]	4.83	d [J = 7.6]
2″	78.3[a]	[CH]	4.12−4.23	m	74.9	[CH]	3.88−3.98	m	74.9	[CH]	4.03−4.13	m
3″	76.8	[CH]	3.56−3.66	m	76.9	[CH]	4.07−4.21	m	76.5	[CH]	4.13−4.26	m
4″	78.0	[CH]	4.24−4.36	m	78.2	[CH]	4.21−4.35	m	78.2	[CH]	4.42−4.48	m
5″	77.7	[CH]	4.12−4.23	m	77.9	[CH]	3.55−3.68	m	77.1	[CH]	3.65−3.70	m
6″	60.9	[CH_2]	4.00−4.07	m	61.3	[CH_2]	4.03−4.07	m	61.4	[CH_2]	4.03−4.13	m
			4.12−4.24	m			4.21−4.35	m			4.13−4.26	m
1‴	101.9	[CH]	6.33	br. s [J = 2.6][b]	101.6	[CH]	6.29	br. s [J = 2.5][b]	102.6	[CH]	5.88	br. s [J = 2.8][b]
2‴	72.2	[CH]	4.70−4.75	m	72.3[c]	[CH]	4.54−4.64	m	72.5[c]	[CH]	4.67−4.69	m
3‴	72.4	[CH]	4.59−4.64	m	72.4[c]	[CH]	4.49−4.54	m	72.6[c]	[CH]	4.54−4.58	m
4‴	73.3	[CH]	4.24−4.36	m	73.9	[CH]	4.21−4.35	m	73.9	[CH]	4.26−4.38	m
5‴	69.4	[CH]	4.84−4.95	m	70.3	[CH]	4.91−4.97	m	70.2	[CH]	4.98−5.04	m
6‴	18.3	[CH_3]	1.70−1.75	m	18.5	[CH_3]	1.68	d [J = 6.4]	18.5	[CH_3]	1.69	d [J = 6.2]
1⁗	102.7	[CH]	5.78	br. s [J = 2.6][b]								
2⁗	72.5[c]	[CH]	4.59−4.64	m								
3⁗	72.2[c]	[CH]	4.52−4.58	m								
4⁗	73.5	[CH]	4.24−4.36	m								
5⁗	70.2	[CH]	4.84−4.95	m (o)								
6⁗	18.5	[CH_3]	1.58	d [J = 6.1]								

[a]Signals fall together. [b]Determined from J-resolved experiment. [c]Interchangeable. [d]Belongs to the epimere.

reference compounds (glucose, rhamnose, and xylose) and by means of cochromatography.

High Performance Liquid Chromatography (HPLC). For analytical as well as for semipreparative analyses of fraction III−B a HPLC apparatus (Shimadzu, Duisburg, Germany) consisting of a LC-20AT pump, a SIL-20A autosampler, a DGU-20A3 degaser, and a Sedex LT-ELSD detector Model 75 (Sedere, Alfortville, France) was used. Chromatographic separations were performed on an analytical 250 × 4.6 or a semipreparative 250 × 10 mm i.d., 5 μm, HyperClone ODS 120 column (Phenomenex, Aschaffenburg, Germany) operated with a flow rate of 1 mL/min or 3.5 mL/min, respectively. When semipreparative separation was performed, the split ratio was set to 0.8 mL/min for the ELSD detector. Data acquisition was done by means of LabSolutions LCsolutions V. 1.21 (Shimadzu, Duisburg, Germany).

For preparative analyses of fraction III−B a HPLC apparatus equipped with two S 1122 pumps (Sykam, Eresing, Germany), a Sedex LT-ELSD detector Model 85 (Sedere, Alfortville, France), and a Rh 7725i type Rheodyne injection valve (Rheodyne, Bensheim, Germany) was used. Chromatography was done using a preparative 250 × 21.2 mm i.d., 5 μm, Microsorb column (Varian, Darmstadt, Germany) operated with a flow rate of 18.0 mL/min, respectively. The split ratio was set to 1.0 mL/min for the ELSD detector. Data acquisition was done by means of Chromstar V. 6.2 (SCPA, Weyhe, Germany).

For analytical up to preparative chromatography of fractions III-C and III-D, the HPLC apparatus (Jasco, Groß-Umstadt, Germany) was equipped with two PU-2087 Plus pumps, an AS-2055 Plus autosampler, a Sedex LT-ELSD detector Model 85 (Sedere, Alfortville, France; split ratio was set to 1 mL/min for the ELSD), and a Rh 7725i type Rheodyne injection valve (Rheodyne, Bensheim, Germany). Data acquisition was performed by means of Chrompass 1.8.6.1 (Jasco, Groß-Umstadt, Germany). Chromatographic separations were performed on a 250 × 4.6, a 250 × 10, or a 250 × 21.2 mm i.d, 5 μm, HyperClone ODS 120 column (Phenomenex, Aschaffenburg, Germany) operated with a flow rate of 1, 3.5, or 20 mL/min, respectively.

Preparative separation of fractions obtained from asparagus roots was performed on a HPLC apparatus (Gilson, Limburg-Offheim, Germany) equipped with two 322 pumps, a GX-281 liquid handler, a QuickSplit semipreparative adjustable flow splitter (Analytical Scientific Instruments, El Sobrante, USA), and a Sedex Model 85 Low Temperature ELSD (Sedere, Alfortville, France; split ratio was set to 1 mL/min for the ELSD). Automated peak detection and data processing were performed by using Trilution LC Media software (Version 2.0; Gilson, Limburg-Offheim, Germany).

Thin Layer Chromatography (TLC). Following a literature procedure,[9] TLC was performed with 5 × 10 cm silica gel 60 F_{254} HPTLC-plates (Merck, Darmstadt, Germany) and chloroform/methanol/water (8/4/1; v/v/v) as the mobile phase in a vertical chamber. After development, the HPTLC plate was sprayed with anisaldehyde reagent comprising a mixture (85/10/5/0.8; v/v/v/v) of methanol, acetic acid, sulphuric acid, and 4-methyl-benzaldehyde and was, then, heated in an oven for 2 min at 110 °C.

Liquid Mass Spectrometry (LC-MS). An API 4000 QTRAP mass spectrometer (Applied Biosystems, Darmstadt, Germany) with direct flow infusion was used to acquire electrospray ionization (ESI) mass spectra and product ion spectra. The MS system was operated in the

Table 3. ^{1}H and ^{13}C NMR Data (500 MHz; pyridine-d_5 plus 5% MeOD) of the Aglycone Part of Saponins 4a/b and 5

	4a/b				5			
position	δ_C/ppm	HSQC	δ_H/ppm	M [J/Hz]	δ_C/ppm	HSQC	δ_H/ppm	M [J /Hz]
1	37.4	[CH_2]	0.88−0.94	m	30.7	[CH_2]	1.36−1.50	m
			1.66−1.76	m			1.70−1.82	m
2	30.2	[CH_2]	1.76−1.86	m	26.8	[CH_2]	1.36−1.48	m
			2.01−2.11	m			1.82−1.89	m
3	78.3	[CH]	3.75−3.85	m	75.3	[CH]	4.11−4.22	m
4	39.0	[CH_2]	2.58−2.67	m	30.7	[CH_2]	1.70−1.82	m
			2.68−2.75	m			1.70−1.82	m
5	140.9	[C]			36.5	[CH]	2.05−2.17	m
6	121.9	[CH]	5.23−5.29	m	26.6	[CH_2]	1.11−1.20	m
							1.70−1.82	m
7	32.4	[CH_2]	1.28−1.44	m	26.8	[CH_2]	0.88−0.94	m
			1.76−1.86	m			1.18−1.36	m
8	31.8	[CH]	1.44 1.58	m	35.5	[CH]	1.50−1.61	m
9	50.4	[CH]	0.79−0.88	m	40.2	[CH]	1.18−1.36	m
10	37.6	[C]			35.8	[C]		
11	21.2	[CH_2]	1.28−1.44	m	21.0	[CH_2]	1.18−1.36	m
			1.28−1.44	m			1.36−1.50	m
12	40.0	[CH_2]	0.95−1.11	m	40.1	[CH_2]	1.11−1.18	m
			1.61−1.66	m			1.70−1.82	m
13	40.5	[C]			40.7	[C]		
14	56.7	[CH]	0.88−1.10	m	56.2	[CH]	1.01−1.15	m
15	32.2	[CH_2]	1.28−1.44	m	32.0	[CH_2]	1.36−1.48	m
			1.93−2.01	m			2.05−2.17	m
16	81.2	[CH]	4.38−4.48	m	81.3	[CH]	4.49−4.54	m
	81.3[a]	[CH]	4.07−4.18	m				
17	62.8	[CH]	1.70−1.76	m	62.9	[CH]	1.82−1.89	m
	62.9[a]	[CH]	1.70−1.76	m				
18	16.4[b]	[CH_3]	0.76	s	16.4	[CH_3]	0.83	s
19	19.4	[CH_3]	0.99	s	23.6	[CH_3]	0.98	s
20	42.0	[CH]	1.86−1.92	m	42.2	[CH]	1.92−1.99	m
	42.5[a]	[CH]	1.76−1.86	m				
21	14.9	[CH_3]	1.04−1.11	m	14.4	[CH_3]	1.10	d [J = 6.9]
	15.0[a]	[CH_3]	1.04−1.11	m				
22	109.4	[C]			109.6	[C]		
	109.8[a]	[C]						
23	31.9	[CH_2]	1.28−1.44	m	26.3	[CH_2]	1.18−1.36	m
			1.91−2.02	m			1.82−1.89	m
	26.4[a]	[CH_2]	1.28−1.44	m				
			1.76−1.86	m				
24	29.3	[CH_2]	1.44−1.58	m	26.1	[CH_2]	1.36−1.48	m
			1.70−1.76	m			2.05−2.17	m
	26.2[a]	[CH_2]	1.28−1.44	m				
			2.02−2.11	m				
25	30.7	[CH]	1.44−1.58	m	27.5	[CH]	1.50−1.61	m
	27.6[a]	[CH]	1.44−1.58	m				
26	67.0	[CH_2]	3.37−3.46	m	64.8	[CH_2]	3.37	br. d [J = 11.1]
			4.13−4.24	m			4.08	dd [J = 3.1, 11.1]
	65.1[a]	[CH_2]	3.28−3.34	m				
			3.96−4.02	m				
27	17.2	[CH_3]	0.65	d [J = 5.9]	15.9	[CH_3]	1.03	d [J = 7.2]
	16.3[a,b]	[CH_3]	1.02	d [J = 7.1]				
28								

[a]Belongs to the epimere. [b]Interchangeable.

full-scan mode detecting positive or negative ions at an ion spray voltage at −4500 V in the negative mode and at 5500 V in the positive mode. The MS/MS parameters were tuned for each compound to record fragmentation of the $[M + H]^+$ or $[M - H]^-$ molecular ions into specific product ions. Data acquisition and instrumental control were performed with Analyst 1.4.2 software (Applied Biosystems, Darmstadt, Germany).

UPLC/Time-of-Flight Mass Spectrometry (UPLC/TOF-MS). Aliquots (1−5 μL) of the analytes (**1a/b**-**5**) dissolved in methanol/water (8/2, v/v; 1 mL) were injected into an Acquity UPLC core

Table 4. ^{1}H and ^{13}C NMR Data (500 MHz; pyridine-d_5 plus 5% MeOD) of the Sugar Moieties of Saponins 4a/b and 5

	4a/b				5			
position	δ_C/ppm	HSQC	δ_H/ppm	M [J/Hz]	δ_C/ppm	HSQC	δ_H/ppm	M [J /Hz]
1′	100.2	[CH]	4.86	d [J = 7.6]	101.4	[CH]	4.78	d [J = 7.6]
2′	78.3	[CH]	4.18−4.28	m	81.0	[CH]	4.11−4.22	m
3′	76.9	[CH]	3.56−3.63	m	75.5	[CH]	4.11−4.22	m
4′	78.6	[CH]	4.18−4.28	m	80.3	[CH]	4.11−4.22	m
5′	77.9	[CH]	4.07−4.18	m	76.2	[CH]	3.70−3.75	m
6′	61.2	[CH_2]	3.96−4.01	m	61.9	[CH_2]	4.38−4.43	m
			4.13−4.20	m			4.45−4.49	m
1″	102.1	[CH]	6.12	d [J = 1.3]	105.1	[CH]	5.35	d [J = 7.6]
2″	72.4	[CH]	4.70−4.72	m	77.6	[CH]	3.98−4.09	m
3″	72.5	[CH]	4.54−4.56	m	77.5	[CH]	4.11−4.22	m
4″	73.9	[CH]	4.18−4.28	m	71.6	[CH]	4.11−4.22	m
5″	69.6	[CH]	4.86−4.91	m	78.3	[CH]	3.84−3.94	m
6″	18.6	[CH_3]	1.66−1.76	m	62.7	[CH_2]	4.38−4.43	m
							4.45−4.49	m
1‴	102.9	[CH]	5.71	d [J = 1.2]	105.3	[CH]	4.96	d [J=7.6]
2‴	72.4	[CH]	4.66−4.68	m	74.7	[CH]	3.84−3.94	m
3‴	72.6	[CH]	4.46−4.52	m	77.9	[CH]	3.98−4.09	m
4‴	73.9	[CH]	4.18−4.28	m	70.4	[CH]	3.98−4.09	m
5‴	70.4	[CH]	4.76−4.86	m	67.2	[CH_2]	3.56−3.61	m
	18.4	[CH_3]	1.44−1.58	m			4.11−4.22	m

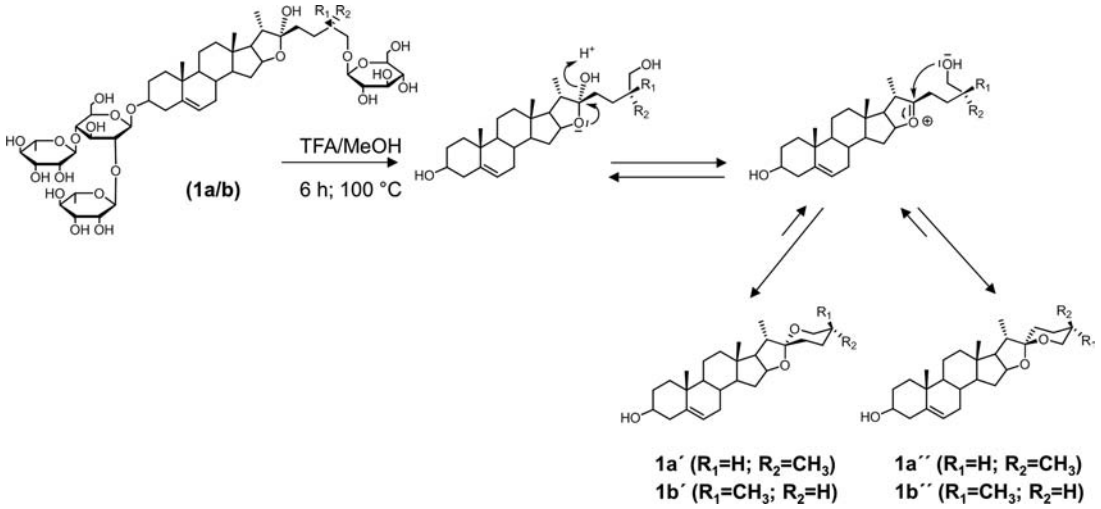

Figure 3. Formation of 22(*R*),25(*R*)-configured diosgenin (**1a′**), 22(*R*),25(*S*)-configured yamogenin (**1b′**), (22*S*,25*S*)-(20α)-spirost-5-en-3β-ole (**1a″**), and (22*S*,25*R*)-(20α)-spirost-5-en-3β-ole (**1b″**) as candidate aglycons upon acidic hydrolysis of saponin **1a/b**.

system (Waters) connected to a SYNAPT G2 HDMS spectrometer (Waters UK Ltd., Manchester, UK) operating in the positive or negative electrospray (ESI) modus with the following parameters: capillary voltage +2.5 kV or −3.0 kV, sampling cone 30, extraction cone 4.0, source temperature 150 °C, desolvation temperature 450 °C, cone gas 30 L/h, and desolvation gas 850 L/h. Chromatographic separations were performed on a 2 × 150 mm, 1.7 μm, BEH C18 column (Waters) operated at 40 °C with a solvent gradient (flow rate 0.3 mL/min) of acetonitrile (solvent A) and 0.1% aqueous formic acid (solvent B): 0 min, 0% B; 7 min, 100% B; 9 min, 100% B; 10 min, 0% B. The instrument was calibrated over a mass range from m/z 100 to 1200 using a solution of sodium formate (0.5 mmol/L) in 2-propanol/water (9/1, v/v). All data were lock mass corrected using leucine enkephaline as the reference (m/z 556.2771 for $[M + H]^+$; m/z 554.2615 for $[M - H]^-$). Data acquisition and analysis was done performed by using the MassLynx software (version 4.1; Waters).

LC/Time-of-Flight Mass Spectrometry (LC/TOF-MS). High-resolution mass spectra of analytes (**1a′**/**1b′**) were recorded on a Bruker Micro-TOF-Q spectrometer (Bruker Daltronics, Bremen, Germany) with flow injection referenced on sodium formiate (5 mmol/L). Data acquisition was performed by using Daltonics DataAnalysis software (version 3.4; Bruker).

Nuclear Magnetic Resonance Spectroscopy (NMR). One- and two-dimensional ^{1}H and ^{13}C NMR spectra were acquired on a Bruker 500 MHz Avance III spectrometer (Bruker, Rheinstetten, Germany). Pyridine-d_5 and $MeOD_4$ (20 μL) for hydroxyproton exchange were used as solvent and chemical shifts are reported in parts per million relative to the pyridine-d_5 solvent signals: ^{1}H NMR: 7.19, 7.55, and 8.71 ppm; ^{13}C NMR: 123.5, 135.5, and 149.5 ppm). 2D NMR experiments (COSY, TOCSY, *J*-RESOLVE, HMQC/HSQC, DEPT, ROESY, HMBC) were carried out using the pulse sequences taken from the Bruker software library. Data processing was performed by using Topspin NMR software (version 3.1 and 2.1; Bruker, Rheinstetten, Germany) and Mestre-C (Mestrelab Research, A Coruña, Spain).

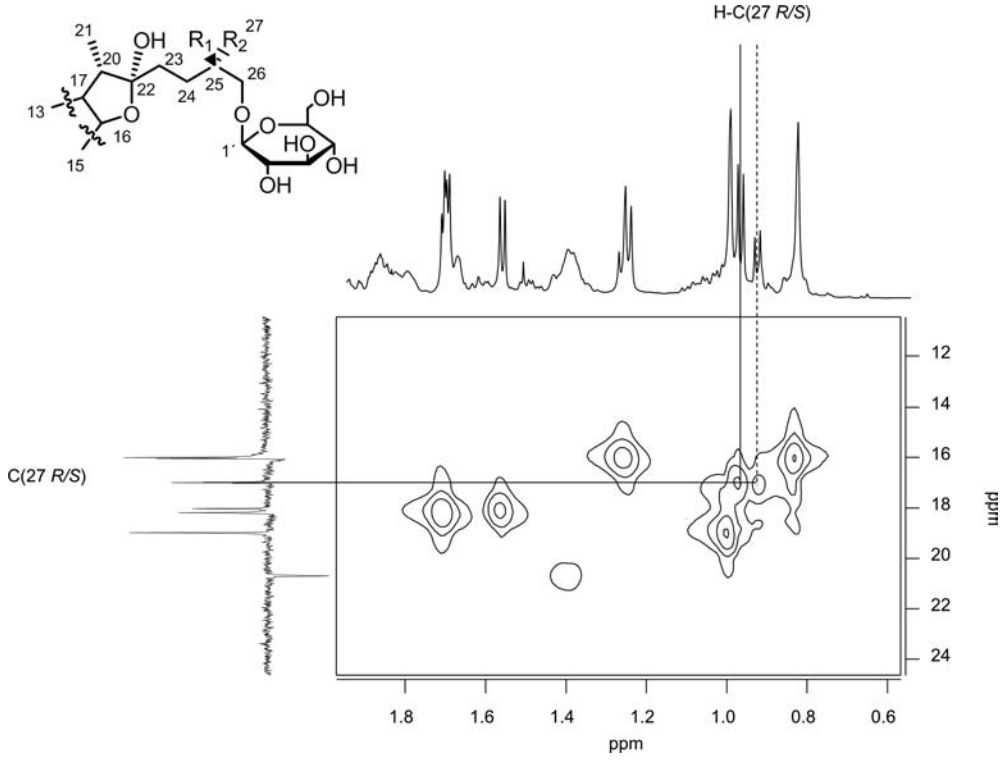

Figure 4. Excerpt of the HMQC spectrum of the purified saponin **1a/b**.

RESULTS AND DISCUSSION

Aimed at determining the chemical structure of the key bitter tasting saponins, a molecular sensory science approach was recently applied on a methanol/water extract prepared from white asparagus spears following the fractionation strategy displayed in Figure 2.[2] As HPLC fractions III-C-5 to III-C-7, and III-D-4 were found with the highest bitter impact, the following investigation was focused on the preparative isolation and structure determination of the saponins evoking the bitter taste of these fractions.

Identification of Bitter Tasting Saponins (1a/b-5) in Asparagus Spears. Preparative HPLC, followed by solvent removal in vacuum and degustation experiments revealed compound **1** as the bitter key tastant in fraction III-C-5. Analysis of the isolate by TLC showed an intensely green spot after spraying with anisaldehyde/sulfuric acid as expected for saponin structures.[9] LC-MS (ESI^-) analysis of **1** revealed m/z 1047.2 as the pseudo molecular ion ($[M - H]^-$), thus suggesting a molecular mass of 1048 Da. This was confirmed by LC-TOF-MS indicating an empirical formula of $C_{51}H_{84}O_{22}$. Additional LC-MS/MS experiments, performed in the ESI^+ mode, led to the identification of the daughter ions m/z 869.4 $[M-18-162+H]^+$, 723.5 $[M-18-162-146+H]^+$, 577.6 $[M-18-162-2\times146+H]^+$, and 415.2 $[M-18-162-2\times146-162+H]^+$, thus demonstrating the presence of two hexose and two methylpentose moieties in the target saponin.

For unequivocal identification of the glycosidically bound carbohydrates, an aliquot of **1** was hydrolyzed with aqueous trifluoracetic acid, and the hydrolysate was extracted with ethyl acetate to separate the saponin aglycone in the organic layer from the aqueous layer containing the monosaccharides. High performance ion chromatography of the aqueous fraction revealed D-glucose and L-rhamnose in a ratio of 1:1 as the target monosaccharides by comparison of retention times with the corresponding reference compounds, followed by cochromatography. LC-TOF-MS (ESI^+) analysis of the saponin aglycon in the ethyl acetate fraction showed a pseudo molecular ion m/z 415.3204 ($[M + H]^+$), thus suggesting an empirical formula of $C_{27}H_{42}O_3$ fitting to that of diosgenin or yamogenin as candidate aglycones.

The ^1H NMR and ^{13}C NMR spectra of the bitter isolate showed several duplicate signals, suggesting the presence of a mixture of isobaric saponin isomers with the 25(*R*)- and 25(*S*)-steroidal saponins **1a** and **1b** (Figure 1) as the quantitatively predominant isomers in III-C-5. Attempts to further separate these isomers by means of chromatography were however not successful, being well in line with literature findings.[10,11] To gain insight into the stereochemistry of these bidesmosidic saponins, also the aglycones released after acidic hydrolysis were analyzed by means of NMR-spectroscopy. Considering the stereo centers of **1a/b** at position C(22) and C(25), four sapogenins were to be expected after acid hydrolysis (Figure 3). Next to the 22(*R*),25(*R*)-configured diosgenin (**1a′**) and the 22(*R*),25(*S*)-configured yamogenin (**1b′**), also the two 22(*S*)-isomers (22*S*,25*S*)-(20α)-spirost-5-en-3β-ole (**1a″**) and (22*S*,25*R*)-(20α)-spirost-5-en-3β-ole (**1b″**) could be suggested as putative hydrolysis products (Figure 3). Among these structures, 1D/2D-NMR experiments allowed the identification of diosgenin and yamogenin in the hydrolysate of **1a/b**. Being well in agreement with literature data,[6,7] rather similar chemical shifts were found for proton H−C(27) and carbon atom C(27) of yamogenin (1.08 ppm for H−C(27), 16.6 ppm for C(27)) and diosgenin (0.81 ppm for H−C(27), 16.9 ppm for C(27)). Literature studies reported that yamogenin and diosgenin are generated upon acidic hydrolysis of 3-*O*-[{α-L-rhamnopyranosyl-(1→2)}{α-L-rhamnopyranosyl-(1→4)}-β-D-glucopyranosyl]-(25*S*)-spirost-5-ene-3β-ol, coined AS-2-I, and postulated the formation of yamogenin by acidic isomerization of diosgenin.[12,13] However, NMR studies on highly purified

diosgenin performed prior to and after acidic hydrolysis using aqueous trifluoracetic acid did not show the generation of the isomeric yamogenin. Taking all these data into consideration, the 22(*R*),25(*S*)-configured yamogenin was concluded to exist natively in asparagus spears.

After identification of the aglycones of **1a**/**b**, the following spectroscopic experiments were focused on the structure determination of the intact saponins. The ^{13}C NMR spectrum showed a total of 51 signals for each isomer (**1a** and **1b**) resonating between 17.2 and 140.6 ppm, among which 27 signals were well in alignment with the aglycone structure of diosgenin and yamogenin, respectively. Moreover, ^{1}H NMR experiments showed five steroidal methyl groups at 0.87 (H−C(18)), 0.95 (H−C(27)), 0.99 (H−C(27)), 1.03 (H−C(19)), and 1.27 ppm (H−C(21)), two secondary methyl groups at 1.70−1.75 and 1.58 ppm corresponding to protons H−C(6‴) and H−C(6⁗) of the rhamnose moieties, as well as the olefinic proton H−C(6) resonating at 5.09−5.31 ppm.

For an unequivocal signal assignment, homonuclear (COSY, TOCSY, *J*-RESOLVED) and heteronuclear 2D NMR experiments (HMQC, HMBC) were performed. Moreover, the stereochemistry of the aglycones was studied by rotating frame nuclear overhauser and exchange spectroscopy (ROESY). ROESY-correlations of the rings A−E as well as the side chains of **1a**/**b** were well in line with those expected for yamogenin and diosgenin aglycones and confirmed the NOE-correlations reported in the literature.[11,14−19] In particular, ROESY correlations could be observed between the proton pairs H−C(19)/H$_\beta$−C(1), H−C(19)/H$_\beta$−C(4), H$_\alpha$−C(1)/H$_\alpha$−C(3), H$_\alpha$−C(4)/H$_\alpha$−C(3) in rings A/B, between H$_\alpha$−C(3) of ring A and H$_\alpha$−C(1′) of the chalcotriose moiety, as well as between the proton pairs H−C(8)/H−C(18), H−C(8)/H−C(19), H−C(14)/H−C(16), and H−C(16)/H−C(17) in rings C−E.

Moreover, the NMR experiments allowed a clear differentiation of **1a** and **1b**. While the methyl group at position C(25) of **1a** was (*R*)-configured, the same carbon atom in **1b** exhibited an 25(*S*)-configuration. Following descriptions in the literature,[15,18] the 25(*R*/*S*)-orientation of the methyl-groups C(27) could be explained by means of two doublets at 0.95 and 0.99 ppm [m, H−C(27*R*/*S*)] (Figure 4). Especially, after a comparative study of ^{13}C and ^{1}H NMR chemical shifts for the assignment of the configuration (25*R*/*S*) of the methyl group C(27), Agrawal et al. [18] suggested that resonances for H−C(27) in 25(*S*)-configuration occurs at a lower field (ca. 0.3 ppm) than in the 25(*R*)-configuration. Moreover, according to Wang et al.[15] two sets of ^{13}C NMR signals for position C(24), C(25), C(26), and C(1′) differing by less than 0.2 ppm were noticed for the epimeric mixture of **1a**/**1b**. An unequivocal assignment of all chemical shifts for both the *R*/*S*-isomers was not possible.

The ^{1}H NMR spectrum of **1a**/**b** showed resonances at 4.98, 4.76, 4.90, 5.78, and 6.33 ppm corresponding to the anomeric protons of the two hexose and two methylpentose moieties in the molecules. The coupling constant of 1.6 Hz found for the anomeric protons of both rhamnose moieties indicated an α-configuration each, while all glucosyl moieties showing coupling constants of 7.6−7.8 Hz, respectively, corresponded to the β-configuration. The full assignment of the carbohydrates moieties as well as their linkage to the aglycone backbone was achieved by means of COSY, TOCSY, and HMBC experiments. For example, the HMBC spectrum of **1a**/**b** showed long-range correlations between H−C(1″) and C(3), H−C(1‴) and C(2″), H−C(1⁗) and C(4″), and H−C(1′) and C(26), respectively. Taking all these findings into account, the bitter tasting saponins in fraction III−C-5 were identified as the epimeric saponins 3-*O*-[α-L-rhamnopyranosyl-(1→2)-{α-L-rhamnopyranosyl-(1→4)}-β-D-glucopyranosyl]-26-*O*-[β-D-glucopyranosyl]-(25*R*)-22-hydroxyfurost-5-ene-3β,26-diole (**1a**, Figure 1) and 3-*O*-[α-L-rhamnopyranosyl-(1→2)-{α-L-rhamnopyranosyl-(1→4)}-β-D-glucopyranosyl]-26-*O*-[β-D-glucopyranosyl]-(25*S*)-22-hydroxyfurost-5-ene-3β,26-diole (**1b**). Whereas saponin (**1b**) has been reported earlier as protoneodiscin in the bottom cuts of *Asparagus officinalis* L., the epimeric protodioscin (**1a**) was previously isolated from asparagus seeds and identified in spears by means of LC-MS/MS.[9,12,20−23]

Rechromatography of key bitter compounds located in fraction III-C-6 led to the identification of the saponins **2a** and **2b**. LC-TOF-MS analysis of the saponin mixture (**2a**/**b**) revealed an empirical formula of $C_{45}H_{74}O_{18}$ for both compounds, thus differing from protodioscin (**1a**) and protoneodiscin (**1b**) by a loss of a L-rhamnose moiety. Acidic hydrolysis of the bitter isolate with trifluoracetic acid, followed by HIPIC analysis revealed D-glucose and L-rhamnose in a ratio of 2:1. Comparison of the ^{1}H, ^{13}C, DEPT-135, homonuclear (COSY, TOCSY), heteronuclear (HMQC, HMBC), as well as ROESY data of target compounds **2a**/**b** with those of **1a**/**b** confirmed the lack of the rhamnose moiety linked at C(2) of the aglycone-connected glucose. Missing correlations of an anomeric proton to C(2″) in the HMBC spectrum as well as the upfield chemical shift of C(2″) from 74.9 (**2a**/**b**) to 78.3 ppm (**1a**/**b**) underlined these structural differences between both bidesmosidic saponins and led to the identification of the bitter saponins in fraction III-C-6 as (25*R*)-furost-5-en-3β,22,26-triol-3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside]-26-*O*-β-D-glucopyranoside (**2a**, Figure 1) and (25*S*)-furost-5-en-3β,22,26-triol-3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside]-26-*O*-β-D-glucopyranoside (**2b**). Although **2a** was identified earlier to be released from protodioscin by enzymatic hydrolysis using an enzyme from *Curvularia lunata*,[23] to the best of our knowledge the presence of this bidesmoside in asparagus spears as well as its bitter taste activity has not been reported. Without any NMR spectroscopic proof, compound **2b** was previously suggested as a tasteless saponin in asparagus bottom cuts.[9,12,21,22] In comparison, saponin **2b** isolated in the present study showed a distinct bitter taste.

Purification of fraction III-C-7 by means of RP-HPLC led to an epimeric mixture of saponins (**3a**/**b**) showing an elemental composition of $C_{45}H_{76}O_{18}$ (LC-TOF-MS) and the pseudo molecular ion *m*/*z* 887.5 ([M−18+H]$^+$) by means of LC-MS (ESI$^+$). MS/MS analysis of the ion *m*/*z* 887.5 revealed the neutral loss of 146 and 2 × 162 amu to give the daughter ions *m*/*z* 417.5 suggesting a sarsapogenin or smilagenin aglycone, respectively, and *m*/*z* 579.5 and 725.5, thus collaborating with the presence of one methylpentose and two hexose moieties for **3a** as well as for **3b**. For further structure elucidation and NMR signal assignment, COSY, DEPT, TOCSY, *J*-RESOLVED, ROESY, HMQC, and HMBC experiments were performed. Analysis of the carbohydrate moiety in **3a**/**b** by means of homo- and heteronuclear 2D-NMR experiments revealed almost identical ^{1}H and ^{13}C NMR signals of the C(3)- and C(27)-linked glycosidic residues as found for **2a**/**b**. Scrutiny of the NMR data of **3a**/**b** and **2a**/**b** (Tables 1 and 2) showed that **3a**/**b** differed from **2a**/**b** mainly in the A and B-ring. Compared to **1a**/**b** and **2a**/**b**, the NMR spectra of the aglycon of **3a**/**b** was lacking the double bond C(5)═C(6). Taking all spectroscopic

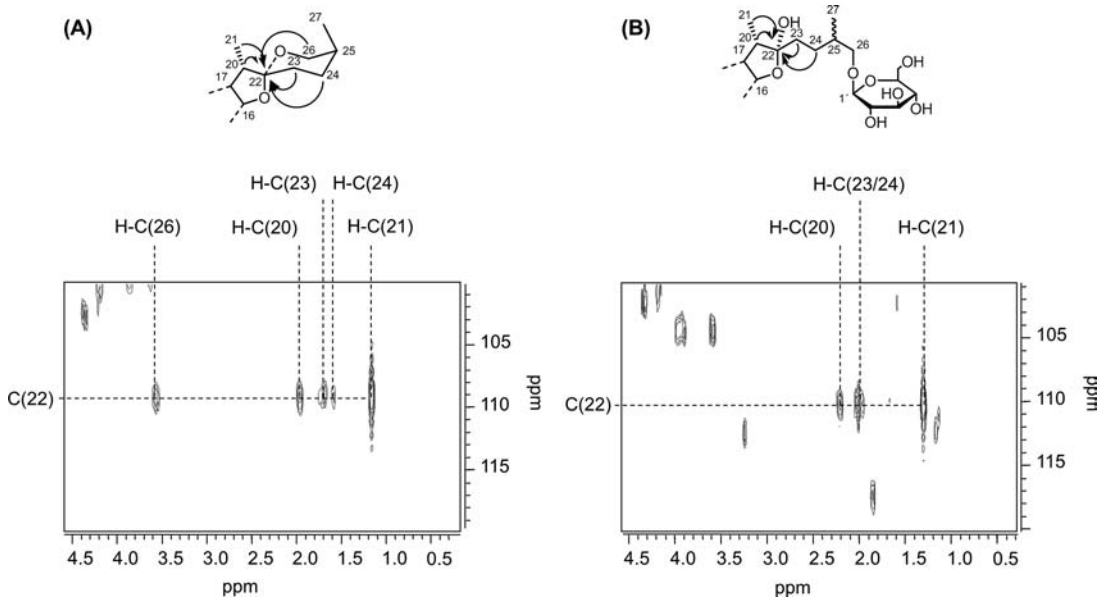

Figure 5. Excerpt of HMBC spectra of the purified saponins **5** (A) and **1a/b** (B).

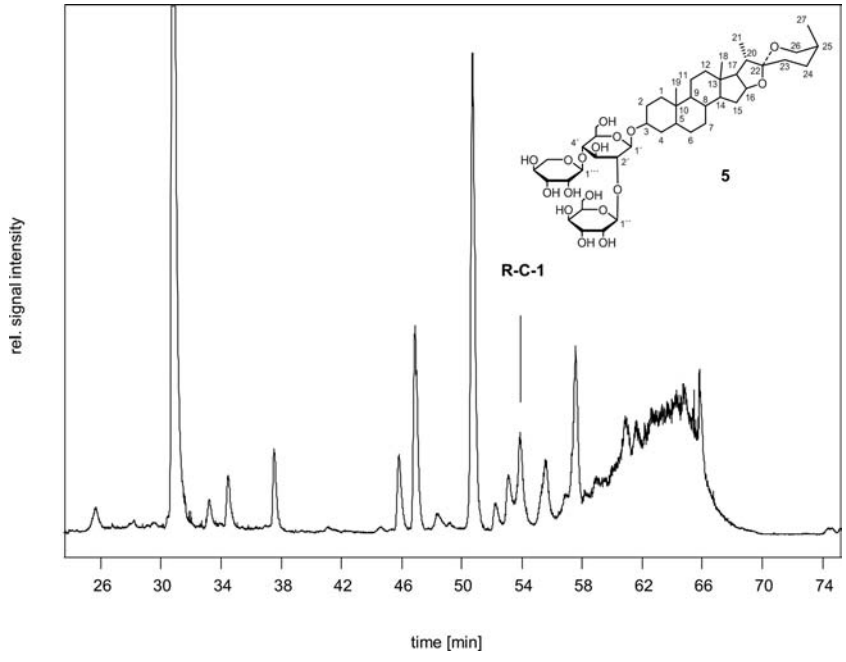

Figure 6. HPLC/ELSD chromatogram of the ethyl acetate fraction prepared from asparagus roots. Location of the saponin **5** is indicated by an arrow.

data into consideration, the bitter compounds isolated from fraction III-C-7 were identified as the previously not reported (25*R*)-furostane-3*β*,22,26-triol-3-*O*-[*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside]-26-*O*-*β*-D-glucopyranoside (**3a**; Figure 1) and (25*S*)-furostane-3*β*,22,26-triol-3-*O*-[*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside]-26-*O*-*β*-D-glucopyranoside (**3b**, Figure 1).

LC-TOF-MS analysis of the rather hydrophobic bitter fraction III-D-4 revealed an epimeric mixture of more than two bitter saponins. LC-MS and LC-TOF-MS analysis of the major ingredients **4a/b** isolated from III-D-4 revealed an epimeric mixture of two bitter saponins exhibiting the same molecular weight of 868 Da and an elemental composition of $C_{45}H_{72}O_{16}$. The ^{1}H NMR spectrum showed anomeric proton signals at 4.86 ppm for H−C(1′), at 6.12 ppm for H−C(1″) and at 5.17 ppm for H−C(1‴), thus suggesting the presence of one glucose and two rhamnose moieties as part of a chalcotriose unit. These monosaccharides were verified by HPIC analysis of an acidic hydrolysate of **4a/b**. Moreover, a heteronuclear correlation between the resonance signal at 4.86 ppm, assigned as the proton H−C(1′) of the chalcotriose unit, and the carbon atom C(3) resonating at 78.3 ppm demonstrated that the chalcotriose unit to be linked to the aglycon at C(3). The ^{1}H and ^{13}C NMR signals of the E- and F-ring of the aglycone suggested **4a/b** to be a steroidal mixture of 25(*R*)- and 25(*S*)-spirostanol monodesmosides. Comparison of the HMBC data of **4a/b** with those obtained for **1a/b** confirmed the spirostanol instead of the furostanol aglycon

(Figure 5). In particular, the $^4J_{C,H}$ correlation observed for **4a/b** between H−C(26) of the F-ring and C(22) of the E-ring across the oxygen atom in the HMBC spectra was well in line with data published earlier [8] (Figure 5A). Taking all spectroscopic data into consideration, LC-MS/MS and 1D/2D NMR experiments and comparison with literature data[6,14,19,24,25] led to the unequivocal identification of the bitter compounds in fraction III-D-4 as 3-*O*-[{*α*-L-rhamnopyranosyl-(1→2)}{*α*-L-rhamnopyranosyl-(1→4)}-*β*-D-glucopyranosyl]-(25*S*)-spirost-5-ene-3*β*-ol (4a, Figure 1) and 3-*O*-[{*α*-L-rhamnopyranosyl-(1→2)}{*α*-L-rhamnopyranosyl-(1→4)}-*β*-D-glucopyranosyl]-(25*R*)-spirost-5-ene-3*β*-ol (4b, Figure 1). Although the occurrence of **4a** in asparagus was assumed in the literature,[9,21,22] its bitter taste activity was not previously reported. Although saponin **4b** has been identified in plants of the *Dioscorea* family, neither its presence in *Asparagus officinalis* L., nor its sensory activity has been previously reported in the literature.[25]

Next to the epimers **4a/b**, LC-MS and LC-TOF-MS analysis of the key bitter fraction III-D-4 revealed a minor saponin exhibiting a molecular weight of 872 Da and an elemental composition of $C_{44}H_{72}O_{17}$. Fitting well with the structure of the monodesmosidic saponin 3-*O*-[{*β*-D-glucopyranosyl-(1→2)}{*β*-D-xylopyranosyl-(1→4)} *β*-D-glucopyranosyl] (25*S*),5*β*-spirostan-3*β*-ol (**5**), which was previously isolated from white asparagus bottom cuts.[24,26] In order to obtain suitable amounts for NMR spectroscopic structure verification, saponin **5** was isolated in preparative amounts from Asparagus roots by sequential solvent extraction, followed by HPLC purification (Figure 6). After structure verification by means of LC-MS/MS, TOF-MS, and NMR spectroscopy, the identity of the isolated reference material with that of saponin **5** (Figure 1) detected in fraction III-D-4 of Asparagus spears was performed by comparison of retention times, mass spectroscopic data, as well as cochromatography.

Sensory Activity of Saponins. In order to evaluate the sensory activity of the previously identified compounds, the taste threshold of each epimeric saponin mixture (**1a/b**−**4a/b**) as well as of the monodesmoside No. **5** were determined. Prior to sensory analysis, the purity of all compounds was checked by HPLC-MS as well as ^{1}H NMR spectroscopy. In order to overcome the limited water-solubility of the saponins, the human bitter recognition threshold concentrations of compounds **1a/b**−**5** were determined in 2% aqueous ethanol (pH 5.9) by means of a triangle test. The sensory analysis revealed a clear bitter impact for all tested saponins and, depending on their chemical structure, low recognition thresholds between 10.9 and 199.7 *μ*mol/L (Table 5). The lowest threshold concentration was determined for the bidesmosidic diosgenyl saponin **2a/b** exhibiting a glucose as well as a 4-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside moiety attached to the aglycon at carbon C(3) and C(26), respectively. These data are in contradiction to literature reports postulating that this saponin does exhibit any bitter taste activity.[12] Comparison of the diosgenin saponin **2a/b** and the sarsapogenin saponin **3a/b** revealed that the aglycon did not strongly influence the taste thresholds of these compounds. Interestingly, most of the bitter saponins identified in the present study have a chalcotriose sugar chain attached at C(3). As the threshold concentrations of **1a/b** and **4a/b** did not differ significantly, the glucose moiety attached to the aglycon at position C(26) seems to play a minor role for bitter perception. Compared to the saponins containing a chalcotriose (**1a/b**, **4a/b**) or a 4-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside moiety (**2a/b** and **3a/b**), saponin **5** bearing a xylose moiety showed a somewhat higher threshold concentration of 199.7 *μ*mol/L.

Table 5. Human Taste Recognition Thresholds of Bitter Compounds Identified In Asparagus

compound (no.[a])	bitter taste threshold [*μ*mol/L][b]
3-*O*-[*α*-L-Rha*p*-(1→2)-{*α*-L-rha*p*-(1→4)}-*β*-D-glc*p*]-26-*O*-[*β*-D-glc*p*]-(25*R*/*S*)-22-hydroxyfurost-5-ene-3*β*,26-diole (**1a/b**)	65.9
(25*R*/*S*)-furost-5-en-3*β*,22,26-triol-3-*O*-[*α*-L-rha*p*-(1→4)-*β*-D-glc*p*]-26-*O*-*β*-D-glc*p* (**2a/b**)	10.9
(25*R*/*S*)-furostane-3*β*,22,26-triol-3-*O*-[*α*-L-rha*p*-(1→4)-*β*-D-glc*p*]-26-*O*-*β*-D-glc*p* (**3a/b**)	25.5
3-*O*-[{*α*-L-Rha*p*-(1→2)}{*α*-L-rha*p*-(1→4)}-*β*-D-glc*p*]-(25*R*/*S*)-spirost-5-ene-3*β*-ol (**4a/b**)	70.6
3-*O*-[{*β*-D-Glc*p*-(1→2)}{*β*-D-xyl*p*-(1→4)}-*β*-D-glc*p*]-(25*S*),5*β*-spirostan-3*β*-ol (**5**)	199.7

[a]The structures of the numbered epimeric mixtures are given in Figure 1. [b]Taste recognition threshold concentrations were determined in aqueous solution (pH 5.9; 2% EtOH) by means of a triangle test.

In conclusion, activity-guided fractionation of asparagus spears allowed the identification of the saponins **1a/b-5** as the phytochemicals showing the highest bitter taste impact. Aimed at demonstrating the contribution of the individual saponins to the bitterness of fresh and cooked asparagus spears, quantitative studies are currently in progress and will be published elsewhere.

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Notes

The authors declare no competing financial interest.

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