Isolation and Structural Elucidation of Steroid Oligoglycosides from the Starfish *Asterias rubens* by Means of Direct Online LC-NMR-MS Hyphenation and One- and Two-Dimensional NMR Investigations

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LC-NMR-MS hyphenation has been applied in the screening of an asterosaponin subfraction obtained from the starfish *Asterias rubens* for unknown compounds. The suitability of this technique for characterizing compounds in the molecular weight range of 1200 to 1400 a.m.u. has been demonstrated. The additional information concerning the number of exchangeable protons provided by the LC-NMR-MS experiment proved to be particularly valuable for the structural elucidation. The LC-NMR-MS screening indicated the presence of hitherto unknown asterosaponins in the sample. Based on this information, four new compounds (ruberosides A–D; **2**–**4** and **6**) have been isolated and their structures have been elucidated using one- and two-dimensional NMR techniques. A minor component has tentatively been assigned the structure **5**.

Introduction

In the course of our study of biologically active compounds obtained from the Baltic starfish *Asterias rubens*, we focussed our efforts on subfractions containing mainly asterosaponins, which are $\Delta^{9(11)}$-3β,6α-dioxygenated steroids with a sulfate group attached at C-3 and an oligosaccharide chain containing five or six sugar units at C-6. Comprehensive review articles on these compounds have been provided by D'Auria et al.[1] and by Kornprobst et al.[2]

Individual compounds belonging to this class differ in their steroidal side chain and/or in their sugar moiety. As considerable effort is involved in the separation and characterization of saponin fractions, the recognition of unknown compounds at an early stage is a prerequisite for an efficient analysis of natural products.

In the case of asterosaponins, however, there are several complicating factors:

(i) Conventional screening methods such as column chromatography/TLC or HPLC-PDA are not sufficiently specific (as asterosaponins are difficult to separate and show only little UV absorbance).

(ii) HPLC-MS methods employing soft ionization techniques (e.g. ESI) are well-suited in principle, but yield little or no information on the various structural isomers. Furthermore, the response under ESI or APCI conditions strongly depends on the chemical nature of the compounds.

(iii) ^{1}H-NMR spectra can be used to check the purities of isolated compounds, but when applied to the analysis of mixtures even two-dimensional NMR spectra are difficult to interpret because of their complexity.

However, novel hyphenated techniques, such as LC-NMR and LC-NMR-MS, offer a new approach for the structural elucidation of these compounds.

Although LC-NMR[3] has frequently been used for analyses of mixtures of natural products during recent years,[4–11] this technique has not yet been applied to saponins in the molecular weight range of 1200–1400 a.m.u.

The hyphenation of the LC-NMR system to a mass spectrometer represents the next obvious step towards creating a comprehensive analytical system providing the complementary information of both NMR and MS in a single chromatographic run. Since the first reports on LC-NMR-MS in 1995,[12,13] the technique has mainly been applied to pharmaceutical drug metabolism research.[14] Very recently, however, the first applications concerning the analysis of natural products were published.[15,16]

On-flow ^{1}H-NMR chromatograms, recorded under isocratic conditions, offer three essential benefits for the screening of mixtures of closely related natural products:

(i) They permit a straightforward and rapid comparison of the spectra of individual compounds.

(ii) As each spectrum in the LC-NMR run is recorded under identical conditions (eluent composition, acquisition parameters), the recognition and interpretation of minor chemical shift differences is possible (Figure 1, boxes a, a′).

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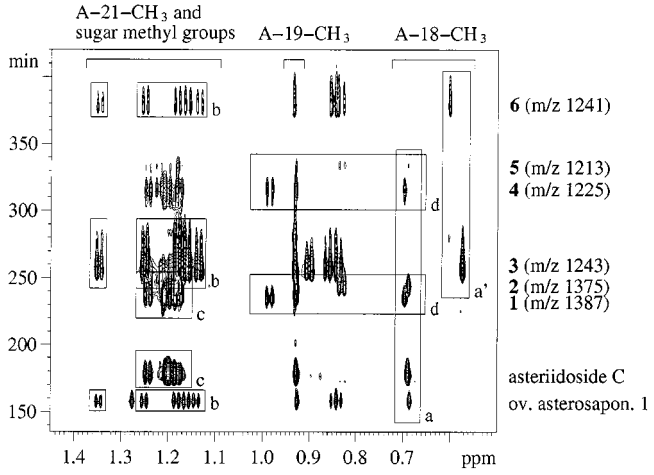


Figure 1. On-flow LC-NMR chromatogram of an asterosaponin subfraction from *Asterias rubens* showing expansion of the methyl proton resonances; ovarian asterosaponin **1** and asteriidoside C were added to the sample for comparison purposes; the boxes indicate structural analogies between the components

(iii) In many cases, the characterization of partly co-eluting compounds is possible.

Moreover, the hyphenation of the LC-NMR system to a mass spectrometer provides instant access to molecular weight data, fragmentation patterns (by MS^2 experiments), and the number of exchangeable protons of a compound (by comparing the MS data in deuterated and nondeuterated solvents).[17]

To some extent, two-dimensional NMR experiments acquired in stopped-flow mode represent a link between on-flow LC-NMR screening and the detailed structural elucidation of isolated compounds employing two-dimensional NMR techniques in conventional off-line probeheads. As the LC-NMR-MS hyphenation offers the possibility of triggering the stops of the LC pump by the MS signal, this technique is particularly well-suited for the reliable detection of compounds showing only weak UV absorbances. These MS-triggered stopped-flow experiments are preferably performed in the second LC-NMR-MS run carried out to determine the number of exchangeable protons by D/H back-exchange.

The main aim of this paper is to show how direct online LC-NMR-MS hyphenation provides preliminary structural information, which can be used in a second step for a target-guided selection of unknown compounds for a detailed two-dimensional NMR examination. This approach is illustrated here using an asterosaponin subfraction obtained following column chromatography. Another objective of this study has been to achieve complete structural elucidation of the components, in particular of their oligosaccharide moieties, by nondegradative spectroscopic methods. This not only saves time, but also material, which can be used more advantageously in tests of its biological activity.[18]

Thus, we describe herein the structural elucidation of four new asterosaponins obtained from *Asterias rubens*. Furthermore, a minor fifth component has tentatively been identified by recognition of appropriate proton resonances.

Since a complete structural elucidation of compounds as complex as asterosaponins cannot be achieved on the basis of the 1D proton NMR spectra provided by on-flow LC-NMR, there are two possible approaches for characterizing unknown compounds of this type by this method:

(i) Analysis of easy-to-assign resonances characteristic of certain structural features, e.g. olefinic protons, methyl groups, or anomeric protons of sugar units.

(ii) Comparison of subspectra of the unknowns with those of known compounds or with those of other unknowns, allowing the recognition of structural analogies.

The latter approach proved to be particularly effective in the case of asterosaponins as their identical aglycone or sugar moieties gave rise to virtually superimposable subspectra under isocratic conditions.

Results and Discussion

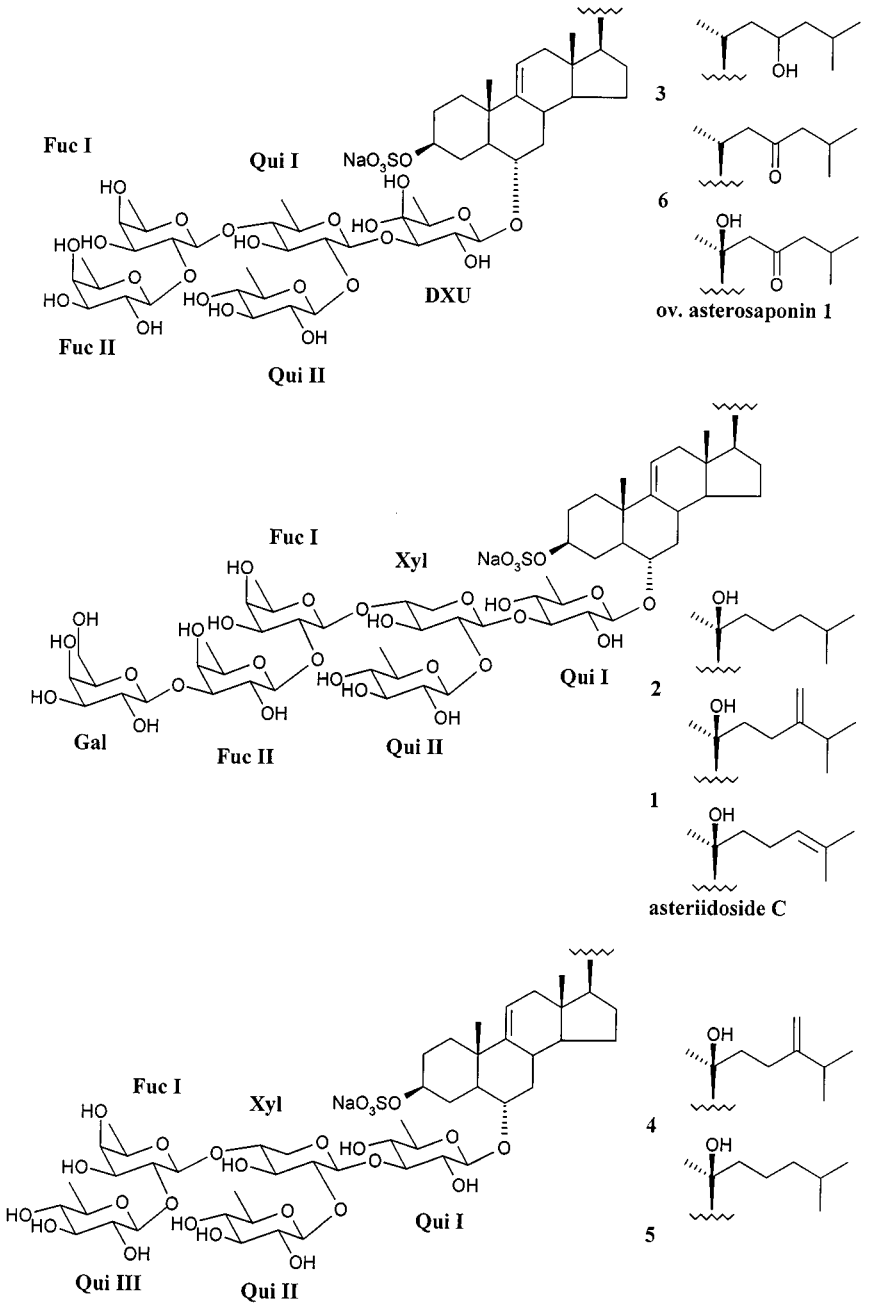
For the on-flow LC-NMR-MS experiments, 500 μg of the saponin subfraction was injected onto the column. For comparison purposes, 40 μg of the commonly occurring ovarian asterosaponin **1**[1,2] and 80 μg of the recently described asteriidoside C[19] (Scheme 1) were added to the sample. The NMR chromatogram revealed five main compounds (Figures 1–3, compounds **1**−**4**, **6**).

In Figure 1, the methyl proton region of the NMR chromatogram is shown, since the chemical shifts of the methyl groups are of high diagnostic value in interpreting the structure of the aglycone. Figure 2 shows the region corresponding to the anomeric protons, which are characteristic of the sugar moiety. Figure 3 shows the one-dimensional ^{1}H-NMR spectra of compounds **1**−**4** and **6**, as obtained from the on-flow NMR chromatogram.

The partial NMR chromatograms (Figure 1 and Figure 2) contain valuable information. Thus, the significant upfield shifts of the aglycone methyl groups 18-CH_3 (A-18-CH_3) of **3** and **6** suggest the absence of a hydroxy function at A-C-20 of the aglycone side chain (Figure 1, boxes a and a′). The conformity of the carbohydrate methyl signals and anomeric proton resonances of both **3** and **6** with those of ovarian asterosaponin 1 (Figure 1 and Figure 2, boxes b) suggest that all three compounds possess the same glycoside moiety having a 6-deoxy-*xylo*-hex-4-ulose unit directly attached to the aglycone. This is further corroborated by the conformity of the corresponding sugar-ring proton signals between δ_H = 3.00 and 3.90 (Figure 3).

In the same manner, comparison of the carbohydrate resonances of **1** and **2** suggested that the glycoside residue is identical to that of asteriidoside C (Figure 1 and Figure 2, boxes c).

Furthermore, comparison of resonances due to the aglycone methyl and olefinic protons led to the identification of corresponding aglycone side chains: compounds **1** and **4** clearly have the same aglycone unit (Figure 1 and Figure 2, boxes d, lower part). Moreover, the compounds eluting after **1** and **4**, i.e. **2** and **5**, show identical aglycone methyl signals (Figure 1 and Figure 2, boxes d, upper part). Al-



Scheme 1

though **5** is a minor component and thus only the methyl signals can be recognized in the on-flow NMR chromatogram, this information together with that from the MS led to its tentative assignment (vide infra).

Besides the molecular weight information, the additional information concerning the number of exchangeable protons provided by the LC-NMR-MS experiment proved to be especially useful, as is illustrated by a comparison between **3** and **6**. These compounds have the same oligosaccharide chain, but differ in the number of exchangeable protons (**3**: 13; **6**: 12). This is suggestive of the presence of an additional hydroxy group in the aglycone side chain of compound **3**.

The presence of a 23-hydroxy function in the side chain of **3** was also confirmed by an MS-triggered stopped-flow 2D-WET-TOCSY experiment carried out in the LC-NMR probehead. Figure 4 shows the correlation between a downfield methine proton at $\delta_H = 3.67$ and both the aglycone methyl groups A-26-CH_3 27-CH_3 ($\delta_H = 0.84/0.86$) and A-21-CH_3 ($\delta_H = 0.90$).

As these LC-NMR-MS experiments had shown the fraction to contain asterosaponins not previously described in the literature, compounds **1**−**6** were separated by RP-HPLC (water/ammonium hydrogen carbonate/acetonitrile) and submitted to extensive two-dimensional NMR examinations to corroborate the conclusions reached by LC-

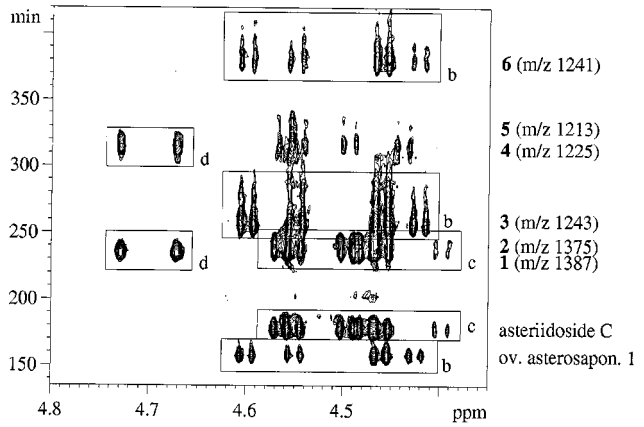


Figure 2. As Figure 1, showing expansion of the anomeric proton resonances; the boxes illustrate structural analogies between the components; at 300 K, the solvent suppression interfered with the fifth anomeric proton resonance of **4** expected at $\delta_H \approx 4.38$

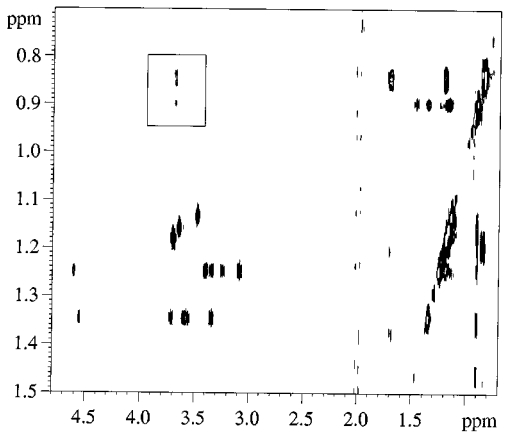


Figure 4. Stopped-flow 2D WET-TOCSY of **3**; magnetization transfer from the methyl groups A-21-CH_3 and A-26-CH_3/27-CH_3 to a resonance at $\delta_H = 3.67$ indicates the presence of a hydroxy function in the aglycone side chain.

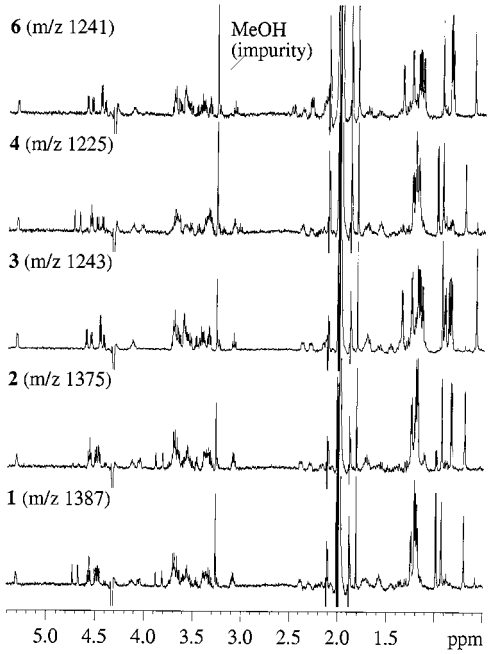


Figure 3. One-dimensional ^{1}H-NMR spectra of compounds **1**–**4** and **6** derived from the on-flow NMR chromatogram; note the conformity of the sugar-ring proton signals of **1** with those of **2** and of **3** with those of **6** in the region $\delta_H = 3.00$–3.90

NMR-MS and to provide the complete structural elucidations and signal assignments (Scheme 1, Table 1 and Table 2).

Ruberoside A (3)

The ESI-MS shows a molecular anion peak $[MD_{13}]^-$ at $m/z = 1256$ ($D_2O/^+ND_4$ formate/MeCN). In an MS^2 experiment, a major fragment was seen at $m/z = 1236$, corresponding to a loss of water (D_2O) from C-4 of the hydrated 6-deoxy-*xylo*-hex-4-ulose unit, as well as minor fragments at $m/z = 1108$ [1256 – 148]$^-$, 1088 [1236 – 148]$^-$, 960 [1108 – 148]$^-$, and 940 [1088 – 148]$^-$, indicating the sequential loss of two deoxyhexose units. A minor fragment at $m/z = 499$ [1256 – 757]$^-$ corresponded to complete loss of the glycosidic residue. A D/H back-exchange experiment indicated the molecular weight $[M]^-$ of the nondeuterated compound through a peak at $m/z = 1243$, demonstrating the presence of 13 exchangeable protons.

The ^{1}H-NMR spectrum of **3** reveals the presence of five anomeric protons in the downfield region between $\delta_H = 4.40$ and 4.60. Their large vicinal coupling constants ($^3J = 7.6$–7.9 Hz) indicate a *trans*-diaxial orientation of the anomeric protons with respect to their coupling partners (β-configuration). The overlapping carbohydrate resonances of the ring protons are found between $\delta_H = 3.00$ and 3.80. Five methyl doublets in the region between $\delta_H = 1.14$ and 1.36, which show coupling constants of $^3J = 6.1$–6.5 Hz suggest the presence of five 6-deoxy-sugar moieties.

The olefinic signal at $\delta_H = 5.30$, the multiplet at $\delta_H = 4.13$, and the remaining signals further upfield than $\delta_H = 2.50$, including five methyl proton resonances, could be assigned to the aglycone.

While the assignment of the signals attributable to the various sugar units was achieved by the application of two-dimensional NMR techniques (COSY, TOCSY, multiplicity-edited HSQC, HMBC, and HSQC-TOCSY), the identification of the sugar units was based mainly on analysis of the coupling patterns obtained from selective 1D TOCSY (*z*-filtered) subspectra of the individual sugars (data shown in Table 1).

1D TOCSY experiments performed by selective excitation of the anomeric protons at $\delta_H = 4.55$ and 4.59 produced two six-spin subspectra showing only large coupling constants ($^3J = 7.9$–9.4 Hz) for the first five protons and J couplings of about 6 Hz ($^3J_{5\text{-H},6\text{-Me}}$) with upfield methyl resonances at $\delta_H = 1.26$ and 1.36, respectively. This indicated the presence of two 6-deoxyhexoses with the 1-H to 5-H protons all in axial positions. Thus, these residues were identified as β-quinovose units.

Selective TOCSY experiments starting from the anomeric proton resonances at $\delta_H = 4.45$ and 4.46 resulted in two four-spin systems, each with two large coupling constants ($^3J_{1\text{-H},2\text{-H}}$, $^3J_{2\text{-H},3\text{-H}} > 5$ Hz) and two small coupling con-

Table 1. ^{1}H and ^{13}C-NMR signals of the sugar moiety of compounds **3**, **2**, and **4** in D_2O/CD_3CN (2:3)

	3					**2**					**4**				
Pos.	δ_C		δ_H		J[a]	δ_C		δ_H		J[a]	δ_C		δ_H		J[a]
	Qui I					**Qui I**					**Qui I**				
1	103.0	CH	4.55	$J_{1,2}$	7.9	103.7	CH	4.39	$J_{1,2}$	7.8	103.6	CH	4.39	$J_{1,2}$	7.9
2	83.2	CH	3.56	$J_{2,3}$	9.2	73.9	CH	3.30	$J_{2,3}$	9.0	73.8	CH	3.30	$J_{2,3}$	8.3
3	74.3	CH	3.73	$J_{3,4}$	9.2	89.3	CH	3.37	$J_{3,4}$	9.2	89.2	CH	3.37	$J_{3,4}$	9.0
4	83.9	CH	3.34	$J_{4,5}$	9.4	74.2	CH	3.07	$J_{4,5}$	9.4	74.2	CH	3.07	$J_{4,5}$	9.2
5	71.7	CH	3.60	$J_{5,6}$	6.1	71.8	CH	3.34	$J_{5,6}$	6.1	71.8	CH	3.34	$J_{5,6}$	6.1
6	17.7	CH_3	1.36			18.0	CH_3	1.21			17.9	CH_3	1.21		
	Qui II					**Qui II**					**Qui II**				
1	105.0	CH	4.59	$J_{1,2}$	7.9	105.0	CH	4.57	$J_{1,2}$	7.8	105.0	CH	4.56	$J_{1,2}$	7.9
2	75.3	CH	3.25	$J_{2,3}$	9.2	75.3	CH	3.25	$J_{2,3}$	9.4	75.3	CH	3.25	$J_{2,3}$	9.4
3	75.9	CH	3.35	$J_{3,4}$	9.4	76.1	CH	3.33	$J_{3,4}$	9.4	76.0	CH	3.33	$J_{3,4}$	9.2
4	75.2	CH	3.09	$J_{4,5}$	9.4	75.3	CH	3.10	$J_{4,5}$	9.4	75.3	CH	3.09	$J_{4,5}$	9.3
5	73.4	CH	3.40	$J_{5,6}$	6.3	73.4	CH	3.38	$J_{5,6}$	6.1	73.3	CH	3.38	$J_{5,6}$	6.1
6	17.6	CH_3	1.26			17.6	CH_3	1.25			17.6	CH_3	1.25		
	Fuc I					**Fuc I**					**Qui III**				
1	101.5	CH	4.46	$J_{1,2}$	7.6	100.8	CH	4.47	$J_{1,2}$	7.9	104.7	CH	4.48	$J_{1,2}$	7.9
2	80.9	CH	3.60	$J_{2,3}$	9.6	81.3	CH	3.57	$J_{2,3}$	9.9	75.6	CH	3.20	$J_{2,3}$	9.4
3	73.8	CH	3.70	$J_{3,4}$	3.4	73.7	CH	3.70	$J_{3,4}$	3.3	76.0	CH	3.34	$J_{3,4}$	9.2
4	71.7	CH	3.66	$J_{4,5}$	<1	71.7	CH	3.66	$J_{4,5}$	<1	75.7	CH	3.02	$J_{4,5}$	9.2
5	71.4	CH	3.71	$J_{5,6}$	6.5	71.5	CH	3.68	$J_{5,6}$	6.5	73.0	CH	3.36	$J_{5,6}$	6.0
6	16.2	CH_3	1.19			16.3	CH_3	1.19			17.8	CH_3	1.22		
	Fuc II					**Fuc II**					**Fuc**				
1	105.3	CH	4.45	$J_{1,2}$	7.8	105.0	CH	4.50	$J_{1,2}$	7.8	101.0	CH	4.44	$J_{1,2}$	7.9
2	72.8	CH	3.45	$J_{2,3}$	9.9	71.8	CH	3.61	$J_{2,3}$	9.9	81.2	CH	3.53	$J_{2,3}$	9.4
3	73.6	CH	3.53	$J_{3,4}$	3.5	83.3	CH	3.69	$J_{3,4}$	3.3	73.7	CH	3.68	$J_{3,4}$	3.5
4	72.0	CH	3.60	$J_{4,5}$	<1	71.7	CH	3.88	$J_{4,5}$	<1	71.7	CH	3.65	$J_{4,5}$	<1
5	71.8	CH	3.64	$J_{5,6}$	6.5	71.5	CH	3.70	$J_{5,6}$	≈6	71.5	CH	3.67	$J_{5,6}$	6.5
6	16.4	CH_3	1.17			16.7	CH_3	1.21			16.2	CH_3	1.19		
	DXU					**Xyl**					**Xyl**				
1	103.6	CH	4.40	$J_{1,2}$	7.9[b]	103.8	CH	4.54	$J_{1,2}$	7.7	103.7	CH	4.53	$J_{1,2}$	7.9
2	72.8	CH	3.39	$J_{2,3}$	9.2[b]	83.1	CH	3.46	$J_{2,3}$	9.0	83.1	CH	3.46	$J_{2,3}$	8.3
3	89.5	CH	3.42			74.8	CH	3.71	$J_{3,4}$	9.4	74.8	CH	3.71	$J_{3,4}$	8.9
4	92.6	C				77.4	CH	3.74	$J_{4,5ax}$	9.5	77.4	CH	3.73	$J_{4,5ax}$	9.9
5	73.0	CH	3.46	$J_{5,6}$	6.4	63.9	CH_2	4.06/	$J_{4,5eq}$	4.4	63.9	CH_2	4.04/	$J_{4,5eq}$	4.4
								3.37	$J_{5ax,5eq}$	11.7			3.36	$J_{5ax,5eq}$	11.7
6	12.5	CH_3	1.14												
						Gal									
1						105.1	CH	4.48	$J_{1,2}$	7.6[b]					
2						72.0	CH	3.52	$J_{2,3}$	9.4[b]					
3						73.6	CH	3.53	$J_{3,4}$	2.9[b]					
4						69.3	CH	3.81	$J_{4,5}$	<1					
5						75.9	CH	3.56							
6						61.7	CH_2	3.67–3.64		br. m					

[a] J values determined by *z*-filtered 1D TOCSY spectra, except for anomeric and methyl resonances, which were determined directly from the ^{1}H-spectrum. – [b] J values confirmed by simulation of the subspectrum.

stants ($^3J_{3\text{-H},4\text{-H}}$, $^3J_{4\text{-H},5\text{-H}} < 5$ Hz). These coupling patterns are in accordance with a β-*galacto*-configuration of the sugar residues. The magnetization transfer of the TOCSY experiment stopped at 4-H due to the very small J coupling ($^3J_{4\text{-H},5\text{-H}} < 1$ Hz).

To identify these β-*galacto*-configured sugars, information provided by further NMR experiments was used. The presence of five carbohydrate methyl groups suggested these residues were β-fucose units, which was confirmed by HMBC correlations between Fuc-C-6 and Fuc-4-H (δ_C = 16.4 → δ_H = 3.60 and 16.2 → 3.66) as well as between Fuc-6-H and Fuc-C-4 (δ_H = 1.17 → δ_C = 72.0 and 1.19 → 71.7). Finally, a strong NOESY correlation between the anomeric protons and 5-H established the axial orientation of the latter.

Following selective excitation of the anomeric proton at δ_H = 4.40, magnetization was transferred to two further protons, revealing a three-spin system with vicinal coupling constants ^3J of about 7.9 and 9.2 Hz. The presence of a 6-deoxy-*xylo*-hex-4-ulose unit (DXU) was indicated by a HMBC correlation between δ_H = 1.14 (DXU-6-CH_3, the fifth carbohydrate methyl resonance to be assigned) and a quaternary carbon at δ_C = 92.6 (DXU-C-4). On the other hand, a HMBC correlation between DXU-C-4 and δ_H = 3.42 (DXU-3-H) and 3.46 (DXU-5-H) established the connectivity to the three-spin system DXU-1-H to 3-H. A strong NOESY correlation between DXU-1-H and 5-H indicated the axial orientation of the latter.

The common D-configurations of the sugar units were assigned by analogy with the sugar configurations in the

Table 2. ^{1}H and ^{13}C-NMR signals of the aglycone of compounds **3**, **6**, **2**, and **4** in D_2O/CD_3CN (2:3)

	3				**6**				**2**				**4**			
Pos.	δ_C	m	δ_H[c]		δ_C	m	δ_H		δ_C	m	δ_H		δ_C	m	δ_H	
1	36.0	CH_2	1.39/	α	36.1	CH_2	1.38/	α	36.0	CH_2	1.38/	α	36.0	CH_2	1.38/	α
			1.69	β			1.69	β			1.69	β			1.70	β
2	28.9	CH_2	2.07/	α	28.9	CH_2	2.07/	α	28.9	CH_2	2.07/	α	28.9	CH_2	2.07/	α
			1.59	β			1.59	β			1.59	β			1.59	β
3	79.3	CH	4.13		79.3	CH	4.12		79.5		4.12		79.5	CH	4.13	
4	30.2	CH_2	2.40/	α	30.3	CH_2	2.40/	α	30.2	CH_2	2.42/	α	30.2	CH_2	2.41/	α
			1.28	β			1.29	β			1.28	β			1.29	β
5	49.0	CH	1.21		49.0	CH	1.22		49.1		1.21		49.0	CH	1.20	
6	80.3	CH	3.56		80.3	CH	3.56		80.4		3.56		80.3	CH	3.56	
7	41.1	CH_2	0.91/	α	41.1	CH_2	0.91/	α	41.3	CH_2	0.89/	α	41.1	CH_2	0.89/	α
			2.30	β			2.30	β			2.29	β			2.30	β
8	36.0	CH	2.05		36.1	CH	2.06		35.8	CH	2.04		35.8	CH	2.05	
9	146.2	C			146.2	C			146.1	C			146.1	C		
10	38.7	C			38.7	C			38.6	C			38.6	C		
11	117.2	CH	5.30		117.2	CH	5.30		117.3		5.30		117.3	CH	5.30	
12	42.4	CH_2	1.97/	α	42.2	CH_2	1.96/	α	42.7	CH_2	1.99/	α	42.6	CH_2	2.01/	α
			2.15	β			2.13	β			2.18	β			2.18	β
13	41.7	C			41.7	C			41.7	C			41.6	C		
14	54.0	CH	1.26		54.1	CH	1.27		54.3		1.29		54.3	CH	1.24	
15	25.6	CH_2	1.73/		25.7	CH_2	1.74/		23.0[b]	CH_2	1.73/		22.9[b]	CH_2	1.74/	
			1.13				1.14				1.63				1.65	
16	29.0	CH_2	1.88/		28.9	CH_2	1.80/		25.3[b]	CH_2	1.74/		25.2[b]	CH_2	1.74/	
			1.26				1.25				1.15				1.16	
17	57.6	CH	1.18		56.7	CH	1.20		58.4		1.53		58.5	CH	1.56	
18	11.8	CH_3	0.58		11.7	CH_3	0.61		13.5	CH_3	0.70		13.4	CH_3	0.71	
19	19.5	CH_3	0.94		19.5	CH3	0.94		19.5	CH_3	0.94		19.4	CH_3	0.93	
sidechain																
20	34.2	CH	1.37		33.1	CH	1.91		75.6	C			75.5	CH		
21	19.2	CH_3	0.91	d (6.6)	19.6	CH_3	0.85	d (6.5)	25.7	CH_3	1.18	s	25.3	CH_3	1.21	s
22	45.3	CH_2	1.46/		50.7	CH_2	2.46/		44.5	CH_2	1.40/		43.0	CH_2	1.54/	
			1.16				2.13				1.25				1.45	
23	68.4	CH	3.66		214.7	C			22.6	CH_2	1.24		29.5	CH_2	1.99	
24	46.5	CH_2	1.19		52.6	CH_2	2.27	ABX (15/6.5)	40.1	CH_2	1.11		157.7	C		
25	24.8	CH	1.73		25.1	CH	2.02		28.4	CH	1.50		34.4	CH	2.22	
26	21.8	CH_3	0.85	d (6.6)	22.5[a]	CH_3	0.84	d (6.6)	22.8	CH_3	0.84	d (6.7)	22.1	CH_3	0.99	d (6.6)
27	24.1	CH_3	0.87	d (6.6)	22.7[a]	CH_3	0.86	d (6.6)	22.8	CH_3	0.84	d (6.7)	22.1	CH_3	0.99	d (6.6)
28													106.7	CH_2	4.72/	br s
															4.66	br s

[a] Signal assignments may be interchanged. – [b] Signal assignment by comparison with literature values. – [c] Coupling constants (in Hz) are given in parentheses.

previously isolated saponins.[1] The sites of glycosidic linkage were determined from interglycosidic HMBC correlations between the anomeric protons and carbon nuclei of the adjacent sugar rings. The assignments were confirmed by NOESY correlations.

Figure 5 shows the corresponding anomeric traces of the HMBC and HSQC-TOCSY spectra. From the HSQC-TOCSY anomeric traces, the ^{13}C chemical shifts of the sugar-ring nuclei can be discerned. Note that the resonances between δ_C = 80 and 90 are shifted downfield as a result of the glycosidation. Thus, it is apparent that Qui I bears two glycosidic linkages. On the other hand, the anomeric traces of the HMBC spectrum reveal the interglycosidic long-range correlations between anomeric protons and ring-carbon nuclei of the adjacent sugar units. As these correlations are not apparent in the HSQC-TOCSY experiment, they must clearly arise from interglycosidic ^3J (COCH) couplings.

From the HMBC correlations Fuc II-1-H → Fuc I-C-2, Fuc I-1-H → Qui I-C-4, Qui II-1-H → Qui I-C-2, Qui I-1-H → DXU-C-3 and DXU-1-H → A-C-6, the pentasaccharide chain shown in Scheme 1 could be established. The interglycosidic NOEs between the anomeric protons and the sugar-ring protons beyond the glycosidic linkage support these assignments.

Both the ^{1}H and ^{13}C chemical shift data of the steroidal nucleus (Table 2), obtained from the multiplicity-edited HSQC and HMBC, are in very good agreement with literature data[18–23] for previously identified asterosaponins, thus indicating a 3β,6α-dihydroxysteroid aglycone sulfated at C-3 with the saccharide moiety at C-6. The stereochemistry of the steroidal skeleton was confirmed by NOESY correlations between A-19-CH_3 and A-6β-H/8β-H, A-18-CH_3 and A-8β-H, A-12α-H and A-14α-H/17α-H, as well as between A-3α-H and A-1α-H/5α-H. The stereochemistry at A-C-20 was deduced by analogy with previously isolated asterosaponins.[1,2]

The aglycone side chain resonances (Table 2) were assigned by tracing the COSY connectivities starting from the methyl groups of the side chain A-21-CH_3, 26-CH_3, and 27-CH_3. The chemical shifts of the A-23 resonances (δ_H =

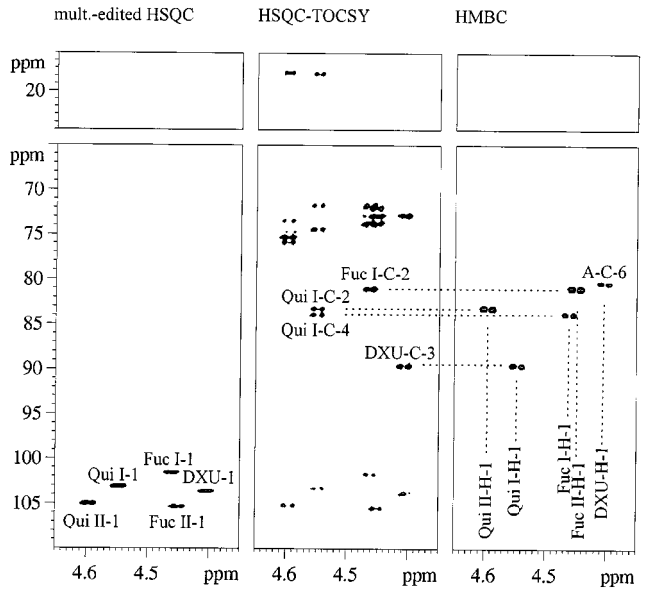


Figure 5. Traces of the anomeric proton and carbon resonances in the multiplicity-edited HSQC, HSQC-TOCSY, and HMBC spectra of **3**; the interglycosidic correlations of the anomeric protons with a carbon nucleus of an adjacent sugar residue establish the linkage sites in the glycoside chain

3.66, δ_C = 68.4) are supportive of the presence of a 23-hydroxy function in the side chain.

The ^{13}C chemical shifts of the aglycone are in very good agreement with literature data for nipoglycoside D.[20]

The structure of compound **3** can thus be defined as 6α-*O*-{β-D-fucopyranosyl-(1→2)-β-D-fucopyranosyl-(1→4)-[β-D-quinovopyranosyl-(1→2)]-β-D-quinovopyranosyl-(1→3)-β-D-6-deoxy-*xylo*-hex-4-ulosyl}-5α-cholest-9(11)-en-23-ol-3β-yl sulfate. This compound has not previously been isolated from starfish. We propose the name ruberoside A for this novel compound.

The ^1H- and ^{13}C-NMR resonances of the following compounds were assigned in the same manner as described above (data shown in Table 1 and Table 2).

Ruberoside B (6)

The ESI-MS shows a molecular anion peak $[MD_{12}]^-$ at *m/z* = 1253 ($D_2O/^+ND_4$ formate/MeCN). An MS^2 experiment revealed a fragmentation pattern identical to that of compound **3**, with a major fragment at *m/z* = 1233 corresponding to a loss of water (D_2O) from C-4 of the hydrated 6-deoxy-*xylo*-hex-4-ulose unit, and minor fragments at *m/z* = 1105 [1253 – 148]$^-$, 1085 [1233 – 148]$^-$, 957 [1105 – 148]$^-$, and 937 [1085 – 148]$^-$ indicating the sequential loss of two deoxyhexose units. A minor fragment at *m/z* = 496 [1253 – 757]$^-$ corresponded to complete loss of the glycosidic residue. A D/H back-exchange experiment indicated the weight of the molecular anion $[M]^-$ of the nondeuterated compound through a peak at *m/z* = 1241, thereby revealing the presence of 12 exchangeable protons in compound **6** (as opposed to 13 in compound **3**).

The resonances of the steroidal side chain were assigned by tracing the COSY connectivities starting from the methyl groups of the side chain. While the connectivity pathway starting at A-21-CH_3 proceeds via A-20-H and extends to A-22-CH_2, the spin system starting from A-26-CH_3/27-CH_3 proceeds via A-25-H to A-24-CH_2. The presence of the carbonyl function at A-C-23 was established from HMBC correlations between A-C-23 (δ_C = 214.7) and A-22-CH_2 (δ_H = 2.46/2.13), 24-CH_2 (δ_H = 2.27) and 25-H (δ_H = 2.02).

The resonances of the steroidal skeleton are identical to those found for the other asterosaponins. The signals attributable to the glycoside chain match those of compound **3**, as already indicated by the on-flow LC-NMR spectra. An additional examination of the sugar units by 1D TOCSY, as well as an analysis of the glycosidic linkages by HMBC, confirmed the structure of the glycoside residue.

The ^{13}C chemical shifts are in very good agreement with literature data for saponins possessing a 24,25-dihydromarthasterone aglycone.[21]

Compound **6** was thus identified as 6α-*O*-{β-D-fucopyranosyl-(1→2)-β-D-fucopyranosyl-(1→4)-[β-D-quinovopyranosyl-(1→2)]-β-D-quinovopyranosyl-(1→3)-β-D-6-deoxy-*xylo*-hex-4-ulosyl}-5α-cholest-9(11)-en-23-one-3β-yl sulfate. We propose the name ruberoside B for this novel asterosaponin.

Compound 1

Compound **1** was identified as asteriidoside B, an asterosaponin possessing a 24(28)-ene function in the side chain, which has recently been isolated from *Asteriidae*.[19] Both the MS {ESI-MS: *m/z* = 1402 $[MD_{15}]^-$ ($D_2O/^+ND_4$ formate/MeCN) MS^2: *m/z* = 1237 [1402 – 165]$^-$, 1089 [1237 – 148]$^-$, and 941 [1089 – 148]$^-$; D/H back-exchange: *m/z* = 1387; no. of exchangeable protons = 15} and NMR data are in good agreement with that reported in the literature.[19]

This was confirmed by a 1D TOCSY and HMBC examination of the glycosidic residue. Selective TOCSY experiments revealed two β-*gluco*-configured spin systems showing magnetization transfer to high-field resonances at δ_H = 1.21 and 1.25, respectively (quinovose units), a pentapyranose system with axially configured 1-H to 4-H protons (xylose), as well as three β-*galacto*-configured spin systems. HMBC correlations between C-6 and 4-H (δ_C = 16.7 → δ_H = 3.88 and 16.3 → 3.66) established the connectivities of two carbohydrate methyl signals to their corresponding β-*galacto* fragments, indicating the presence of two fucose units. The remaining β-*galacto* unit was assigned to a CH_2OH group at δ_C = 61.7, as befits a galactose unit. This is in accordance with the MS data, which are indicative of a terminal hexose unit.

Ruberoside C (2)

The ESI-MS shows a molecular anion peak $[MD_{15}]^-$ at *m/z* = 1390 ($D_2O/^+ND_4$ formate/MeCN). An MS^2 experiment revealed fragments at *m/z* = 1225 [1390 – 165]$^-$, 1077 [1225 – 148]$^-$, and 929 [1077 – 148]$^-$ corresponding to the sequential loss of a hexose and two deoxyhexose units. A

D/H back-exchange experiment indicated the molecular weight $[M]^-$ of the nondeuterated compound through a peak at m/z = 1375, revealing the presence of 15 exchangeable protons.

The LC-NMR chromatogram (Figure 1 and Figure 2) had already indicated that **2** has the same glycoside chain as **1** and the reference compound asteriidoside C. The multiplicity-edited HSQC shows carbohydrate resonances which are virtually superimposable on those of **1**.

The aglycone side chain proton resonances were assigned by tracing the COSY connectivity pathway beginning at the terminal methyl groups A-26-CH_3/27-CH_3. The ^{13}C chemical shifts obtained for the aglycone side chain are in very good agreement with the values reported for latespinoside B.[22]

The structure of compound **2** was thus identified as 6α-*O*-{β-D-galactopyranosyl-(1→3)-β-D-fucopyranosyl-(1→2)-β-D-fucopyranosyl-(1→4)-[β-D-quinovopyranosyl-(1→2)]-β-D-xylopyranosyl-(1→3)-β-D-quinovopyranosyl}-20-hydroxy-α-cholest-9(11)-en-3β-yl sulfate, for which we suggest the name ruberoside C.

Ruberoside D (4)

The ESI-MS shows a molecular anion peak $[MD_{12}]^-$ at m/z = 1237 ($D_2O/^+ND_4$ formate/MeCN). An MS^2 experiment revealed fragments at m/z = 1089 $[1237 - 148]^-$ and 941 $[1089 - 148]^-$, corresponding to the sequential loss of two deoxyhexose units. A D/H back-exchange experiment indicated the molecular weight $[M]^-$ of the nondeuterated compound through a peak at m/z = 1225, which is consistent with the presence of 12 exchangeable protons.

Selective excitation of the anomeric doublets at δ_H = 4.39, 4.48, and 4.56 revealed 1D TOCSY subspectra of β-*gluco*-configured six-spin systems (with large axial/axial coupling constants in all cases) showing magnetization transfer to a high-field 6-H resonance (δ_H = 1.21, 1.22, and 1.25, respectively). Thus, these sugar moieties were readily identified as β-quinovose units.

Following selective excitation of the anomeric proton at δ_H = 4.53, a 1D TOCSY subspectrum showing a six-spin system with four axial/axial couplings (^3J = 7.9−8.9 Hz) and one 11.7 Hz geminal coupling constant was obtained. This sugar unit was thus identified as xylose in its pyranose form. The methylene resonance at δ_C = 63.9 in the multiplicity-edited HSQC was assigned to its Xyl-C-5 position.

The fifth sugar unit (anomeric proton at δ_H = 4.44) has a β-*galacto*-configured pyranose ring ($^3J_{3,4}$ = 3.5 Hz, $^3J_{4,5}$ < 1 Hz). The presence of four carbohydrate methyl groups suggested this residue to be a β-fucose unit, which was confirmed by a HMBC correlation between Fuc-6-CH_3 and Fuc-C-4 (δ_H = 1.18 → δ_C = 71.7). Finally, a NOESY correlation between Fuc-1-H and Fuc-5-H established the axial orientation of the latter.

The striking similarity between the aglycone methyl proton resonances and the two broad olefinic singlets downfield from the anomeric protons in the LC-NMR chromatogram suggests that the aglycone side chain is the same as that in compound **1**, possessing a 24(28)-ene function (Figure 1 and 2, boxes d).

The presence of an olefinic methylene signal in the multiplicity-edited HSQC (A-28: δ_C = 106.7, δ_H = 4.72/4.66) in conjunction with HMBC correlations between A-26-CH_3/27-CH_3 and A-C-24 (δ_C = 157.7) and between A-28-CH_2, A-C-23 (δ_C = 29.5), and C-25 (δ_C = 34.4) confirmed this assignment. The ^{13}C chemical shifts for the aglycone are in very good agreement with literature data for asteriidoside B[19] and asteroside D.[23]

Thus, compound **4** was identified as 6α-*O*-{β-D-quinovopyranosyl-(1→2)-β-D-fucopyranosyl-(1→4)-[β-D-quinovopyranosyl-(1→2)]-β-D-xylopyranosyl-(1→3)-β-D-quinovopyranosyl}-20-hydroxy-5α-cholest-9(11),24(28)-dien-3β-yl sulfate, for which we propose the name ruberoside D.

Compound 5

The LC-NMR chromatogram showed a minor compound that eluted shortly after **4**. The molecular anion peak $[MD_{12}]^-$ was seen at m/z = 1225 ($D_2O/^+ND_4$ formate/MeCN), i.e. 12 units lower than that of **4**. An MS^2 experiment revealed fragments at m/z = 1077 $[1225 - 148]^-$ and 929 $[1077 - 148]^-$, corresponding to the sequential loss of two deoxyhexose units. A D/H back-exchange showed a molecular ion peak $[M]^-$ at m/z = 1213, indicating the presence of 12 exchangeable protons.

The carbohydrate methyl proton resonances of this minor compound are identical to those of **4**, while the aglycone methyl proton resonances are identical to those of **2**, thus suggesting a saponin consisting of the pentasaccharide chain of **4** and the unsubstituted aglycone side chain of **2**.

The molecular mass 12 units lower than that of **2** and the striking resemblance of the signal patterns between the pairs **4/5** and **1/2** in the on-flow LC-NMR run (Figure 1, boxes d) supported this assignment, even though only the methyl groups of compound **5** could be observed in the NMR chromatogram.

A fraction of this compound was also obtained in the course of the preparative isolation and a ^1H-NMR spectrum as well as 1D TOCSY spectra of the sugar units were recorded. These experiments supported the tentative structural assignment of **5** as 6α-*O*-{β-D-quinovopyranosyl-(1→2)-β-D-fucopyranosyl-(1→4)-[β-D-quinovopyranosyl-(1→2)]-β-D-xylopyranosyl-(1→3)-β-D-quinovopyranosyl}-20-hydroxy-5α-cholest-9(11)-en-3β-yl sulfate.

Experimental Section

Animal Material: The crude extracts of *Asterias rubens* were provided by the Institut für Angewandte Biologie und Landschaftsplanung - Bioplan GmbH, Groß Stove/Rostock, Germany. Specimens of *Asterias rubens* were collected at Fredericia, Denmark (Baltic Sea) in September 1997. The following extraction procedure was used: The animal material, frozen prior to storage, was cut into small pieces and soaked in acetonitrile for 16 h. The supernatant was then decanted and the remaining solid mass was again soaked in acetonitrile and exposed to ultrasonic treatment. The combined acetonitrile extracts were subsequently concentrated to dryness. The

concentrated extract was then taken up in water and centrifuged. Finally, the supernatant was lyophilized. From 500 g of fresh animal material, about 7 g of crude extract was obtained.

Extraction and Isolation: The crude extract (20 g) from *Asterias rubens* was submitted to preparative chromatography on a 24 × 4 cm RP-18 (40 μm particle size) column. The fractions were eluted using a gradient from 100% H_2O to 100% MeOH. The asterosaponin fraction (1688 mg) eluted at a 30:70 composition of H_2O/MeOH.

600 mg of the collected asterosaponin fraction was further chromatographed on an RP-18 column eluting with H_2O/MeOH (50:50 to 10:90) yielding, besides pure asterosaponins, several subfractions containing unseparated saponins. 179 mg of the saponin subfraction investigated in this article was obtained at an eluent composition of 40:60 H_2O/MeOH. This saponin subfraction was then submitted to LC-NMR-MS analysis.

As this examination indicated the presence of new compounds in the sample, the subfraction was further separated by HPLC, employing the following conditions: A solution of 20 mg of the saponin subfraction in 800 μL of eluent was repeatedly chromatographed on an analytical HPLC column (YMC J'sphere ODS L 80, 4.6 × 250 mm) using 20 mmol ammonium hydrogen carbonate in H_2O/CH_3CN (65:35) as eluent. A 20 μL injection loop was used, the flow was 1 mL/min., and the UV detection wavelength was 210 nm. Compounds **1**−**6** were collected and dried by repeated co-evaporation.

LC-NMR-MS Analysis: The LC system consisted of a Bruker LC-22 pump, a Rheodyne 7725 i injection valve, a Bischoff Lambda-1010 UV detector at 210 nm, and a Bruker BPSU-36 peak sampling unit. At the outlet of the BPSU, an LC packings ICP-4–20 flow splitter was attached, which allowed the transfer of 95% of the flow to the NMR spectrometer and 5% to the MS. Additional buffer was added to the MS flow in a ratio of 4:1 by means of a PEEK T-piece (Upchurch) and a syringe pump (Cole Parmer 74900). For MS experiments in deuterated solvents, 20 mmol of deuterated ammonium formate in D_2O was used; D/H back-exchange experiments employed 20 mmol of ammonium formate in H_2O. Due to the residual D_2O in the eluent, the most abundant peak in the D/H back-exchange experiments was $[M + 2D]^-$. However, $[M]^-$ was clearly distinguishable. A YMC J'sphere ODS L80 HPLC column (4.6 × 250 mm) was used with 20 mmol ammonium formate in D_2O/CH_3CN (64:36) as eluent. The eluent flow rate was 0.05 mL/min for on-flow LC-NMR-MS experiments and 0.5 mL/min for the MS-triggered stopped-flow experiments.

A 25 mg/mL solution of the sample in the eluent was injected using a 20 μL injection loop (corresponding to an absolute amount of 500 μg injected onto the column). For comparison purposes, 2 mg/mL (40 μg absolute) of ovarian asterosaponin **1**[1,2] and 4 mg/mL (80 μg absolute) of asteriidoside C[19] were added to the sample. These reference compounds were isolated from other fractions of *Asterias rubens* and their structures have been determined employing the same NMR methods as described here.

A Bruker Avance DRX 600 spectrometer equipped with a 4 mm *z*-gradient LC probe head was used. On-flow spectra were recorded using the following conditions: ^1H frequency: 600 MHz; temperature: 300 K; sweep width, 8400 Hz; 24 k data points, zero-filled to 32 k; exponential multiplication LB 1 Hz; 108 scans per row, pulse repetition time 2 s. Solvent suppression was achieved by the WET sequence with ^{13}C decoupling during the WET pulse train. Chemical shifts were referenced to CH_3CN: δ_H = 2.00. To extract 1D ^1H-NMR spectra from the on-flow NMR chromatogram, up to four rows of a peak were added. 2D WET-TOCSY spectra were recorded in the MS-triggered stopped-flow mode. ^{13}C decoupling was applied during the WET-pulse train and acquisition. Spectral size was 4k × 512 data points, zero-filled to 4k × 2k data points; 100 ms TOCSY mixing time.

The mass spectrometer used was a Bruker Esquire-LC ion-trap MS equipped with an ESI ionization source. Ionization parameters: negative-ion mode; capillary voltage 3150 V, end-plate voltage 2350 V; dry gas: 11 L/min nitrogen at 300 °C; nebulizing gas: nitrogen at 30 psi. Scan from *m*/*z* = 50 to 1600; accumulation cut-off *m*/*z* = 85; 125 to 150 averages per spectrum in the on-flow LC-NMR-MS mode; 20 averages in the stopped-flow mode.

NMR: For the further two-dimensional and selective NMR experiments, a 2.5 mm *z*-gradient micro probe head was used and samples were placed in 2 mm OD NMR capillaries (Wilmad). Standard Bruker microprograms using gradient selection (except in TOCSY experiments) were applied.

The operating conditions were as follows: frequencies: ^1H 600 MHz; ^{13}C 150 MHz; solvent: D_2O/CD_3CN, (2:3); temperature: 300 K. Chemical shifts were referenced to CD_3CN: δ_H = 1.975; δ_C = 1.3. A typical MLEV-spinlock time for TOCSY experiments was 100 ms; a typical NOESY mixing time 200 ms. The HMBC long-range coupling $^{2/3}J_{CH}$ was set to 7 Hz. With the exception of HMBC, all spectra were recorded in phase-sensitive mode. Typical spectral size was 4k × 512 data points, processed to 4k × 2k points using zero-filling in the homonuclear and linear prediction in the heteronuclear experiments. Shifted squared sine window functions were applied in both dimensions.

In the selective 1D TOCSY experiments, a 100 ms half-gaussian-shaped pulse was used for selective excitation. The standard Bruker microprogram applying a *z*-filter was used. The mixing time varied between 25 and 150 ms. The sweep width was set at 7200 Hz, 32 k data points were acquired, and an exponential multiplication of LB 0.3 Hz was applied to the FID. Spectra simulations were performed using the Bruker NMRsim software.

Ruberoside A (3): Negative LC-ESI-MS (D_2O/$^+ND_4$ formate/MeCN): *m*/*z* = 1256 $[MD_{13}]^-$; MS^2: *m*/*z* = 1236, 1108, 1088, 960, 940, 499. – Negative LC-ESI-MS (D/H back-exchange): *m*/*z* = 1243 $[M]^-$. – ^1H and ^{13}C NMR: δ_H, δ_C (D_2O/CD_3CN, 2:3), see Table 1 and 2.

Ruberoside B (6): Negative LC-ESI-MS (D_2O/$^+ND_4$ formate/MeCN): *m*/*z* = 1253 $[MD_{12}]^-$; MS^2: *m*/*z* = 1233, 1105, 1085, 957, 937, 496. – Negative LC-ESI-MS (D/H back-exchange): *m*/*z* = 1241 $[M]^-$. – ^1H and ^{13}C NMR: δ_H, δ_C (D_2O/CD_3CN, 2:3) of the aglycone, see Table 2. ^1H- and ^{13}C-NMR data of the sugar moiety are identical to those of compound **3**.

Ruberoside C (2): Negative LC-ESI-MS (D_2O/$^+ND_4$ formate/MeCN): *m*/*z* = 1390 $[MD_{15}]^-$; MS^2: *m*/*z* = 1225, 1077, 929. – Negative LC-ESI-MS (D/H back-exchange): *m*/*z* = 1375 $[M]^-$. – ^1H and ^{13}C NMR: δ_H, δ_C (D_2O/CD_3CN, 2:3), see Table 1 and 2.

Ruberoside D (4): Negative LC-ESI-MS (D_2O/$^+ND_4$ formate/MeCN): *m*/*z* = 1237 $[MD_{12}]^-$; MS^2: *m*/*z* = 1089, 941. – Negative LC-ESI-MS (D/H back-exchange): *m*/*z* = 1225 $[M]^-$. – ^1H and ^{13}C NMR: δ_H, δ_C (D_2O/CD_3CN, 2:3), see Table 1 and 2.

Compound 1: Negative LC-ESI-MS (D_2O/$^+ND_4$ formate/MeCN): *m*/*z* = 1402 $[MD_{15}]^-$; MS^2: *m*/*z* = 1237, 1089, 941. – Negative LC-ESI-MS (D/H back-exchange): *m*/*z* = 1387 $[M]^-$. – ^1H and ^{13}C-NMR data (δ_H, δ_C; D_2O/CD_3CN, 2:3) of the aglycone are identical

with those of compound **4**; NMR data of the sugar moiety identical with those of compound **2**. Thus, this compound was found to be structurally identical to asteriidoside B.[19]

Compound 5: Negative LC-ESI-MS ($D_2O/^+ND_4$ formate/MeCN): m/z = 1225 $[MD_{12}]^-$; MS^2: m/z = 1077, 929. – Negative LC-ESI-MS (D/H back-exchange): m/z = 1213 $[M]^-$. – ^{1}H-NMR spectrum of the aglycone unit superimposable on that of compound **2**; ^{1}H-NMR spectrum of the sugar moiety identical to that of compound **4**. ^{1}H NMR: δ_H (D_2O/CD_3CN, 2:3) = 5.30 (br. d, A-11-H), 4.56 (d, J = 7.9 Hz, Qui II-1-H), 4.53 (d, J = 7.9 Hz, Xyl-1-H), 4.48 (d, J = 7.9 Hz, Qui III-1-H), 4.44 (d, J = 7.9 Hz, Fuc-1-H), 4.39 (d, J = 7.9 Hz, Qui I-1-H), 2.40 (m, A-4-H), 2.29 (m, A-7-H), 1.25 (d, J = 6.1 Hz, Qui II-6-CH_3), 1.22 (d, J = 6.2 Hz, Qui III-6-CH_3), 1.20 (d, J = 6.2 Hz, Qui I-6-CH_3), 1.19 (d, Fuc-6-CH_3), 1.18 (s, A-21-CH_3), 0.93 (s, A-19-CH_3), 0.84 (d, J = 6.6 Hz, A-26-CH_3/27-CH_3), 0.70 (s, A-18-CH_3).

1D TOCSY subspectra of the individual sugar units superimposable on those of compound **4**.

Acknowledgments

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[1] M. V. D'Auria, L. Minale, R. Riccio, *Chem. Rev.* **1993**, *93*, 1839–1895.
[2] J. M. Kornprobst, C. Sallenave, G. Barnathan, *Comp. Biochem. Physiol*. **1998**, *119B*, 1–51.
[3] J. C. Lindon, J. K. Nicholson, I. D. Wilson, *Adv. Chromatogr.* **1996**, *36*, 315–382.
[4] J. L. Wolfender, S. Rodriguez, K. Hostettmann, *J. Chromatogr. A* **1998**, *794*, 299–316.
[5] D. Hölscher, B. Schneider, *Phytochemistry* **1999**, *50*, 155–161.
[6] G. Bringmann, C. Günther, J. Schlauer, M. Rückert, *Anal. Chem.* **1998**, *70*, 2805–2811.
[7] B. Vogler, I. Klaiber, G. Roos, C. U. Walter, W. Hiller, P. Sandor, W. Kraus, *J. Nat. Prod.* **1998**, *61*, 175–178.
[8] I. D. Wilson, E. D. Morgan, R. Lafont, B. Wright, *J. Chromatogr. A* **1998**, *799*, 333–336.
[9] K. Pusecker, K. Albert, E. Bayer, *J. Chromatogr. A* **1999**, *836*, 245–252.
[10] S. Strohschein, C. Rentel, T. Lacker, E. Bayer, K. Albert, *Anal. Chem.* **1999**, *71*, 1780–1785.
[11] A. E. Bäcker, S. Thorbert, O. Rakotonirainy, E. C. Hallberg, A. Olling, M. Gustavsson, B. E. Samuelsson, B. Soussi, *Glycoconj. J.* **1999**, *16*, 45–58.
[12] F. S. Pullen, A. G. Swanson, M. J. Newman, D. S. Richards, *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1003–1006.
[13] J. P. Shockor, S. E. Unger, I. D. Wilson, P. J. Foxall, J. K. Nicholson, J. C. Lindon, *Anal. Chem.* **1996**, *68*, 4431–4435.
[14] I. D. Wilson, J. C. Lindon, J. K. Nicholson, *LC-GC* **1998**, *16*, 842–852.
[15] I. D. Wilson, E. D. Morgan, R. Lafont, J. P. Shockor, J. C. Lindon, J. K. Nicholson, B. Wright, *Chromatographia* **1999**, *49*, 374–378.
[16] S. H. Hansen, A. G. Jensen, C. Cornett, I. Bjornsdottir, S. Taylor, B. Wright, I. D. Wilson, *HPLC '99*, Granada, Spain, **1999**.
[17] R. M. Holt, M. J. Newman, F. S. Pullen, D. S. Richards, A. G. Swanson, *J. Mass Spectrom.* **1997**, *32*, 64–70.
[18] J. A. Findlay, M. Jaseja, D. J. Burnell, J.-R. Brisson, *Can. J. Chem*. **1987**, *65*, 1384–1391.
[19] S. De Marino, M. Iorizzi, E. Palagiano, F. Zollo, C. Roussakis, *J. Nat. Prod.* **1998**, *61*, 1319–1327.
[20] M. Iorizzi, L. Minale, R. Riccio, T. Yasumoto, *J. Nat. Prod.* **1993**, *56*, 1786–1798.
[21] M. J. Vazquez, E. Quinoa, R. Riguera, A. San Martin, J. Darias, *Tetrahedron* **1992**, *48*, 6739–6746.
[22] R. Higuchi, M. Fujita, S. Matsumoto, K. Yamada, T. Miyamoto, T. Sasaki, *Liebigs Ann.* **1996**, 837–840.
[23] R. Riccio, M. Iorizzi, L. Minale, Y. Oshima, T. Yasumoto, *J. Chem. Soc., Perkin Trans. 1* **1988**, 1337–1347.

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