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Sulfates as novel steroid metabolites in higher plants

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Dedicated to Dr. Peter Junior on the occasion of his 50th birthday

Abstract

Cardenolide sulfates were identified as a novel type of steroid conjugates representing the most polar 5α-configurated steroids of the cardenolide complex of *Adonis aleppica* BOISS. Detailed spectroscopic analysis including IR, DCI- and FAB-MS and high-field NMR measurements as well as chemical reactions resulted in the identification of the major sulfate as uzarigenin-3-*O*-sulfate (**1**) beside the minor components coroglaucigenin-3-*O*-sulfate (**2**) and corotoxigenin-3-*O*-sulfate (**3**). The inorganic sulfuric acid residue could be traced by applying trimethyl- and triethylammonia as DCI-MS reactant gases resulting in ammonium-sulfonate cleavage products. Comprehensive NMR studies led to the assignment of key ^{1}H resonances of these biogenetically closely related steroids. Definitive evidence for the unusual sulfuric acid semi-ester conjugation came from X-ray diffraction measurements confirming the structure and establishing the absolute configuration of uzarigenin-3-*O*-sulfate (**1**). Concerning the chemotaxonomy of the genus *Adonis*, due to these novel cardenolides an exceptional position of *Adonis aleppica* within the taxon has to be acknowledged.

Keywords: *Adonis aleppica*; Ranunculaceae; Cardenolides; Sulfates; Steroids; X-ray; NMR

1. Introduction

Cardenolides are prominent cardioactive secondary metabolites of many medicinal plants belonging to the Apocynaceae (*Nerium*, *Strophanthus*, *Thevetia*), Asclepiadaceae (*Periploca*, *Calotropis*, *Xysmalobium*), Scrophulariaceae (*Digitalis*), Ranunculaceae (*Adonis*) as well as to the Convallariaceae families (*Convallaria*, *Speirantha*). Depending on the A/B-ring fusion of the steroid core, the C_{23} aglycones of theses constituents are divided into the 5α- (A/B-trans) and the 5β-series (A/B-*cis*). The structural variety mostly arises from multiple hydroxylation of the steroid nucleus. In principle, this should open a great diversity within the group of glycosidic derivatives, but up to now all of them were characterized as 3-*O*-glycosides with unbranched sugar chains. Nevertheless, these higher plant metabolites are unique with respect to the carbohydrate moiety because the plants utilize rare (di-)desoxy sugars as building blocks. These typical desoxy sugars represent all the possible stereoisomers of the (2,)6-(di)desoxyhexoses and their 3-*O*-methylethers, respectively (Reichstein, 1951; Tamm, 1957). Within the 5α-series, structures with doubly-linked sugars i.e. hexosuloses were recently obtained from plants of the Asclepiadaceae (Cheung & Nelson, 1989 and subsequent publications). Further known types of derivatization are acetylation and formylation as observed in the lanatosides from *Digitalis lanata* (Wichtl, 1978) and the acetylrhamnosides from *Adonis vernalis* (Winkler, 1985). Representing a new entity of strongly polar conjugates, three cardenolide sulfates were now obtained from *Adonis aleppica* BOISS. (Ranunculaceae), an annual species (sect. Adonis) endemic to the Mesopotamian region (see Fig. 1).

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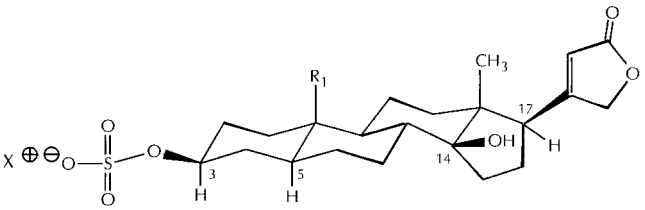

1 $R_1=CH_3$ uzarigenin–3–O–sulfate
2 $R_1=CH_2OH$ coroglaucigenin–3–O–sulfate
3 $R_1=CHO$ corotoxigenin–3–O–sulfate

Fig. 1. Structures of the isolated cardenolide sulfates **1**–**3**, given as salts (mostly K^+).

2. Results and discussion

Plants of the genus *Adonis* are well known for the occurrence of cardiac glycosides (Junginger, 1990; Pauli, 1993). In previous communications *A. aleppica* was shown to produce alepposides and adoligoses representing complex, long-chained strophanthidin oligoglycosides ($n = 4$–6) and oligosaccharides ($n = 3$–5), respectively, beside aleppotrioloside being a polar open-chained aliphatic alcohol glucoside (Pauli, 1993; Pauli, Berger, Matthiesen & Junior, 1993a; Pauli, Matthiesen & Junior, 1993b; Pauli, 1995). Right from the beginning of the phytochemical investigation of *A. aleppica*, a group of extremely polar compounds showing R_f values below 0.1 in the standard TLC solvent system $CHCl_3$–MeOH–H_2O (80:19:1) was recognized that yielded positive Kedde reactions. The main cardenolide **1** of this complex showed an intense, but slowly developing sea-blue color reaction when sprayed and heated with vanillin–H_2SO_4 reagent. Therefore, according to reactions performed with authentic cardenolides its structure should not be based on a 5β-configurated 19-CH_3 steroid such as digitoxigenin or periplogenin. Despite its polarity, **1** could be isolated in crystalline form by a combination of repeated CC, VLC and MPLC separations using many different adsorbents and solvent systems (see Section 3). Employing DCI-NH_3-MS as a routine method for the characterization of cardenolides and their glycosides (Pauli, 1993 and references therein), interpretation of the base peak at m/z 356 as being due to a $[MNH_4]^+$ quasimolecular ion of **1** would have led to a molecular mass being 36 a.m.u. lower than the most simple aglycones digitoxigenin or uzarigenin (**4**). While this could formally agree with the loss of two molecules of water liberated from C-3-OH and C-14-OH, all analytical data including ^{1}H- and ^{13}C-NMR measurements doubtlessly indicated the presence of an intact cardenolide without any skeletal double bonds (see Table 1).

Moreover, the proton resonances were fully compatible with the basic structure of a H-5α and H-17α configurated cardenolide aglycone bearing a 3β-OH

Table 1
Carbon NMR data of the isolated cardenolide sulfates **1**–**3** and uzarigenin (**4**) as well as substituent chemical shifts (s.c.s. = $\Delta\delta$) arising from sulfatation and different degrees of oxidation of C-19. It is noteworthy that the s.c.s. observed with the 5α-cardenolides are quite different to those in the 5β-series (δ in ppm, 100/125 MHz, CD_3OD as solvent, ' < ' given in cases when $|\Delta\delta| < 0.35$

C-atom	1	2	3	4	s.c.s. ($\Delta\delta$) sulfatation	s.c.s. ($\Delta\delta$) 19-OH	s.c.s. ($\Delta\delta$) 19-oxo
1	38.20	32.67	31.97	38.27	<	−5.53	−6.23
2	29.61	30.09	29.46	32.04	−2.43	+0.48	<
3	79.71	79.74	78.75	71.76	+7.95	<	−0.96
4	36.12	36.58	37.69	38.74	−2.62	+0.46	+1.57
5	45.79	46.04	44.22	45.79	<	<	−1.57
6	29.78	29.30	30.80	29.95	<	−0.48	+1.02
7	28.67	28.70	28.56	28.74	<	<	<
8	42.45	43.04	43.82	42.53	<	+0.59	+1.37
9	50.96	51.38	50.14	51.10	<	+0.42	−0.82
10	36.68	40.28	52.56	36.87	<	+3.60	+15.88
11	22.28	23.96	22.89	22.34	<	+1.68	+0.61
12	40.79	41.13	40.36	40.83	<	<	−0.43
13	51.01	51.15	50.76	50.99	<	<	<
14	86.43	86.45	85.76	86.29	<	<	−0.67
15	33.29	33.39	32.76	33.34	<	<	−0.53
16	28.00	28.04	27.87	28.00	<	<	<
17	51.97	52.17	51.88	52.03	<	<	<
18	16.41	16.50	16.16	16.38	<	<	<
19	12.52	59.90	210.22	12.52	<	+47.38	+197.70
20	178.45	178.58	178.26	178.45	<	<	<
21	75.49	75.39	75.33	74.34	<	<	<
22	117.67	111.75	117.90	117.79	<	<	<
23	177.58	177.30	177.23	177.25	<	<	<

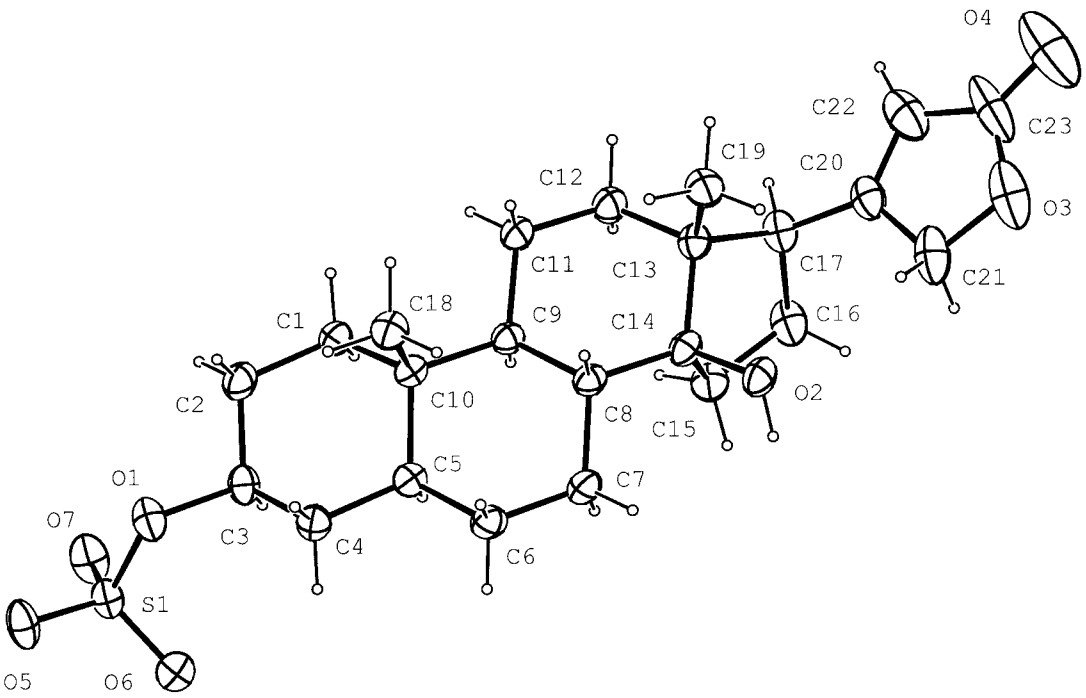

Fig. 2. The absolute configuration of uzarigenin-3-*O*-sulfate (**1**) was established through X-ray diffraction analysis and is represented here by an ORTEP molecule plot.

function and two angular methyl substituents CH_3-18/19. When compared with the resonances of authentic uzarigenin (**4**) and uzarigenin-3-*O*-*β*-glucoside (Pauli, 1993), it became obvious that only the dddd signal of H-3α at 4.283 ppm was affected by a marked substituent chemical shift (s.c.s.) of +0.771 and +0.444 ppm, respectively. At the same time all high-field (fingerprint) resonances were nearly unshifted. In analogy, the assignment of the majority of ^{13}C resonances of **1** perferctly matched with the exception of the A-ring carbons only. These observations were readily explained by the presence of a strongly electronegative C-3-OR substitution other than a sugar residue. Its inorganic nature was suggested by the lack of further carbon resonances. In addition, the symmetry of the carbon s.c.s. observed for the α- and *β*-nuclei (+7.95 versus −2.62 and −2.43 ppm, respectively) pointed towards a non-chiral group *R* because of the lack of diastereotopism.

Proof for *R* being a sulfuric acid semi-ester residue could be gleaned from various directions. Solvolysis in dioxane-pyridine according to McKenna and Norymberski (McKenna & Norymberski, 1957) performed under TLC control deliberated uzarigenin (**4**), while **1** could not be hydrolyzed with alkaline but yielded isocardenolide artifacts. This represents the typical behavior of sulfated sugars and triterpenes being quite resistant to solvolysis (Kitagawa, Kobayashi & Sugawara, 1978). In addition, **1** was preparatively obtained by sulfatation of authentic **4** using SO_3/pyridine reagent (McKenna & Norymberski, 1957; Hertzberg, 1983). The product was characterized by TLC and spectroscopic measurements (MS, NMR) and found to be identical with **1**. Furthermore, **1** like the two other compounds **2** and **3** showed the typical IR bands of S=O bond stretching and deformation at 820 and 1230 cm^{-1}, respectively (Bruno, Minale & Riccio, 1989; Minale, Riccio, Pizza & Zolo, 1986). Finally, the anion of the free acid $[M–H]^-$ of **1** gave rise to a base-peak at *m*/*z* 453 in a negative-mode FAB-MS spectrum supporting the molecular formula of the free acid being $C_{23}H_{34}O_7S$ and thus the presence of sulfur as being the unusual heteronucleus in **1**.

Consequently, the structure of **1** was shown to be the sulfuric acid semi-ester of uzarigenin, i.e., uzarigenin-3-*O*-sulfate. Definitive evidence for this structure came from X-ray crystallographic studies. The compound was found to be the Na^+ salt of the sulfate ester, with both water and ethanol solvent molecules. The structure of the anion is illustrated in Fig. 2, which also indicates the absolute configuration determined in the X-ray experiment. The *trans*-fusion of the A and B rings (C-5/10), the *β*-configuration of the sulfate substituent at C-3, the *β*-oriented OH at C-14, and the butenolide substituent attached to C-17*β* provide evidence for the uzarigenin skeleton. The S1–O1 distance is 1.580(3) Å, indicative of a single bond, while the other three S–O distances fall within the range 1.438(3)–1.447(3) Å. The Na^+ cation is approximately octahedrally coordinated by two water mol-

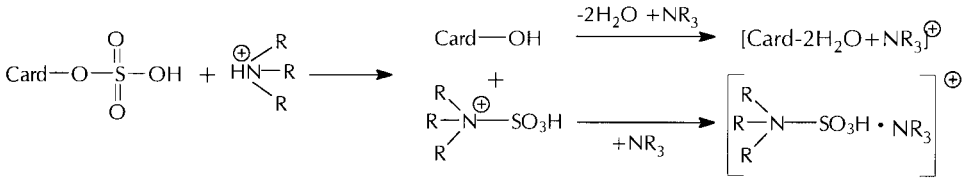

Fig. 3. Proposed reaction scheme explaining the most prominent ions that were observed in the DCI-MS spectra of the cardenolide sulfates. The formation of ammonium-sulfonate species gives rise to intense or base peaks at *m/z* 199 and 283 when using trimethylammonia and triethylammonia as reactant gases, respectively. Notably, the DCI plasma of triethylammonia also contains $[CH_2{=}NEt_2]^+$ species that are capable of ionizing the sulfates giving rise to additional low intensity peaks shifted by −16 a.m.u. (Card = cardenolide aglycone).

ecules, two ethanol oxygen atoms, and two sulfate oxygen atoms (both O5), with Na–O distances in the range 2.266(4)–2.576(5) Å.

In view of the MS data of the other two isolated polar cardenolides **2** and **3**, their successful structure elucidation was in need for a better understanding of the DCI-NH_3-MS behavior of cardenolide sulfates. Although weaker lines (<1% relative intensity) were observed for intact ester species of **1** at *m/z* 436 $[M–2H_2O + NH_4]^+$, 453 $[M–2H_2O + NH_4 + NH_3]^+$, and 471 $[M–H_2O + NH_4 + NH_3]^+$, the base-peak obviously was due to the loss of sulfuric acid and water leading to *m/z* 356 $[M–H_2SO_4–H_2O + NH_4]^+$. Evidence for the cleavage of the ester bond in the DCI plasma came from experiments using trimethylammonia and triethylammonia as reactant gases. Because of the *N*-alkylation, stable ammonium-sulfonate species can be formed giving rise to very prominent lines at *m/z* 199 and 283, respectively. Therefore we propose a general DCI reaction scheme for cardenolide sulfates as depicted in Fig. 3. Accordingly, the DCI mass spectra of **2** and **3** were interpreted as summarized in Table 2. Consequently, the structure of **2** was differing from **1** by an aldehyde function instead of a Me group (+14 a.m.u.), while **3** should bear a primary alcohol (+16 a.m.u.). This was supported by the presence of a typical ^1H aldehyde singlet at 9.997 ppm for **3** as well as two AB-type 12.1 Hz doublets t 3.849 and 3.715 ppm arising from the primary alcohol in **2**, respectively. Both spectra exhibited the same multiplet that can be interpreted in terms of nuclei first order spin systems as a dddd signal (5.0/5.1/11.0/11.1 Hz) being due to H-3ax of a 5α-configurated steroid (for a more detailed analysis see Pauli, 1993). Comparison of their chemical shift at 4.296 (**3**) and 4.324 (**2**) with 4.283 ppm for **1** pointed to an equal s.c.s. due to sulfatation as discussed above.

Table 2
DCI-MS behavior of the cardenolide sulfates **1**–**3**. When NH_3 is used as the reactant gas, the base-peak is observed at *m/z* [MW(aglycone)-18] due to $[M–H_2SO_4–H_2O + NH_4]^+$, while weaker lines are found for species with intact sulfuric acid esters such as $[M–2H_2O + NH_4 + NH_3]^+$ at *m/z* [MW(sulfate)-1]. On the other hand, sulfonated ammonium species are the corresponding cleavage products as observed with trimethylammonia and triethylammonia and are indicative of the sulfonic acid group bound to the steroids

Ion composition	**1** *m/z*	**2** *m/z*	**3** *m/z*
$[M–H_2SO_4–H_2O + NH_4]^+$ (base-peak)	356	372	370
$[M–H_2SO_4 + NH_4]^+$	374	390	388
$[M–SO_3 + NH_4]^+$	392		
$[M–2H_2O + NH_4]^+$	436	452	450
$[M–2H_2O + NH_4 + NH_3]^+$	453	469	467
$[M–H_2O + NH_4 + NH_3]^+$	471	487	485
$[M–H_2SO_4–H_2O + NMe_3H]^+$ (base-peak)	398	414	412
$[M–H_2SO_4 + NMe_3H]^+$	416	432	430
$[M + H]^+$	455	471	469
$[Me_3N–SO_3H + NMe_3]^+$	199	199	199
$[M–H_2SO_4–H_2O + H_2C{=}NEt_2]^+$	424	440	438
$[M–H_2SO_4–H_2O + NEt_3H]^+$	440	456	454
$[M–H_2SO_4 + NEt_3H]^+$	458	474	472
$[M–SO_3 + NEt_3H]^+$	476		
$[Et_2NCH_2–SO_3H + NEt_3]^+$	267	267	267
$[Et_3N–SO_3H + NEt_3]^+$ (base-peak)	283	283	283

Final elucidation of the structures of **2**/**3** was based on extensive NMR studies including (DQF-)COSY, NOESY, HMQC, and HMBC measurements. As already evident from the ^{13}C-NMR data summarized in Table 1, the only difference of the isolated cardenolides **1**–**3** was the oxidation status of C-19 ($CH_3 \rightarrow CH_2OH \rightarrow CHO$, respectively). Accordingly, significant sets of ^{13}C s.c.s. could be obtained that should be helpful tools in the future structure assignment of A/B-*trans* linked steroids. At this point it has to be mentioned, that the s.c.s. values calculated from analogous 5β-configurated compounds (e.g. periplogenin [CH_3], strophanthidol [CH_2OH], and strophanthidin [CHO]) are quite different and show manifold deviations up to as much as 1.0 ppm.

The identification of the steroid aglycones as well as supporting evidence for the sulfate substitution came from detailed proton NMR studies. Table 3 divides the steroid signals into two groups: The prominent cardenolide resonances appearing at lower field >2.5 ppm on one hand, and the more complex partially overlapping CH/CH_2 resonances of the steroid nucleus above 2.5 ppm on the other. Assignment of the latter became possible by combining the mentioned 2D techniques with resolution enhanced 1D spectra. Unresolved signals and the presence of higher order coupling pattern was figured for 2H-11 and 2H-12. In

Table 3
^{1}H-NMR resonances of the cardenolide sulfates **1**–**3** sorted into two groups (see discussion for details) as well as s.c.s. values for the sulfatation of uzarigenin (**4**, $\Delta\delta = \delta_H[\mathbf{1}]-\delta_H[\mathbf{4}]$). It has to be noted that the given J values are not always matching the direct peak-to-peak distances usually measured in case of first order spin systems, but they rather reflect the true couplings resulting from spectral simulation calculations (δ in ppm, J in Hz, 400/500 MHz, CD_3OD as solvent)

H-atom	Multiplicity	H,H-coupling	Compound (δ[ppm])			s.c.s. ($\Delta\delta$) sulfatation
		J (Hz)	**1**	**2**	**3**	
> 2.5 ppm						
3α	dddd	4.8, 5.1, 11.0, 11.3	4.283	4.324	4.296	0.770
17α	dd	5.4, 9.5	2.840	2.819	2.811	<
18	s		0.877	0.841	0.841	<
19[A/B]	br s[2xbr d][a]	[< 1[a], 12.1]	0.836	3.849 (19A), 3.717 (19B)	9.997	0.033
21A	ddd	0.5, 1.8, 18.5	5.022	5.030	5.009	
21B	dd	1.8, 18.5	4.898	4.907	4.894	
22	br t/dt	0.5, 1.8, 1.8	5.890	5.885	5.885	
< 2.5 ppm						
1A = eq	ddd	3.7, 3.7, 13.6	1.771	2.332	2.420	0.055
1B = ax	(d)ddd	0.9[19][a], 3.7, 13.6, 13.6	1.033	0.815	1.060	0.057
2A = eq	ddddd	2.4, 3.7, 3.7, 5.1, 13.5	2.014	2.010	2.10	0.253
2B = ax	dddd	3.7, 11.3, 13.5, 13.7	1.52	1.580	1.45[b]	0.125
4A = eq	dddd	2.8, 3.0, 4.8, 12.3	1.827	1.887	2.027	0.274
4B = ax	ddd	11.0, 12.0, 12.3	1.412	1.529	1.405	0.130
5(ax)	dddd	~ 0.5[6eq], 3.0, 12.0, 12.3	1.134	1.241	1.35[b]	0.059
6A = eq	(br)ddd	2.8, 4.2, 12.4	1.362	1.287	1.494	<
6B = ax	dddd	3.0, 9.9, 12.3, 12.4	1.238	1.203	1.45[b]	<
7A = eq	dddd	2.8, 3.0, 3.7, 12.8	2.034	2.050	2.209	<
7B = ax	dddd	4.2, 12.1, 12.4, 12.8	1.097	1.118	1.288	<
8 (ax)	ddd/dt	3.7, 11.7, 12.1	1.583	1.769	1.607	<
9 (ax)	(br)ddd/dt[a]	< 1[19][a], 3.8, 8.9, 11.7	0.979	1.010	1.237	<
11A [=eq]	m (dddd)	2, 3, 6, 13	1.50	1.625	1.982 [ax]	<
11B [=ax]	m (dddd)	3.5, 11.8, 12.4, 12.8	1.269	1.603	1.719 [eq]	<
12A = eq	m (ddd)	3.4, 3.4, 13.5	1.52	1.492	1.594	<
12B = ax	m (ddd)	3.0, 12.3, 13.5	1.45	1.383	1.35[b]	<
15$A = \alpha$	ddd	9.6, 10.0, 13.4	2.090	2.129	2.085	<
15$B = \beta$	ddd	1.3, 9.3, 13.4	1.702	1.699	1.676	<
16$A = \alpha$	dddd	1.3, 9.5, 10.0, 13.1	2.157	2.162	2.151	<
16$B = \beta$	dddd	5.4, 9.3, 9.6, 13.1	1.857	1.850	1.860	<

[a] At least one small W-type coupling (^4J) between 1H-19 (H-19B in **2**) and H-9.
[b] Unresolved signals.

addition to the above mentioned set of ^{13}C s.c.s. values arising from 3-*O*-sulfatation (see Table 1), the complete assignment of the proton resonance now allows the determination of distinguishing ^{1}H shift effects (see Fig. 4). Calculation of the shift differences ($\Delta\delta$) of uzarigenin-3-*O*-sulfate (**1**) and the free alcohol uzarigenin (**4**) led to a set of ^{1}H s.c.s. included in Table 3. As expected, the sulfuric acid moiety mostly influences ring A of the steroid. Interestingly, all s.c.s. are positive values i.e. sulfatation solely causes downfield shift effects. At the same time they are symmetrical like already found in the ^{13}C domain which indicates the non-chiral nature of the introduced residue. While the equatorial β-protons at C-2/4 are shifted by 0.264 ± 0.015 ppm, the corresponding axial ^{1}H nuclei exhibit significantly smaller s.c.s. of only 0.128 ± 0.004 ppm. This can be perfectly understood because the three equatorial substituents at C-2(H)/3(OH)/4(H) share relatively close spatial interactions as obvious from a molecular mechanics calculation. At the same time, the signals of the γ-nuclei H-1A/B and H-5 are displaced by identical amounts of 0.057 ± 0.002 ppm. In the C-19-OH series, comparison of **2** and coroglaucigenin (**5**) yields an identical picture with slightly higher s.c.s. for the axial neighbours: 0.262 ± 0.031 ppm for C-2/4eq, 0.172 ± 0.006 ppm for C-2/4eq, and 0.047 ± 0.012 ppm for the γ-nuclei H-1/5.

Fig. 5 shows the most important NOE interactions observed in a 2D NOESY map of **2**. The experiment allowed the determination of the α- and the β-face

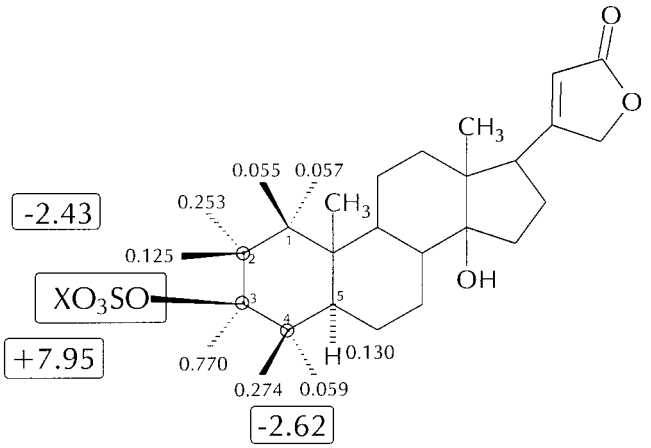

Fig. 4. Representative substituent chemical shifts (s.c.s.) arising from 3-*O*-sulfatation of the steroid skeleton as calculated from uzarigenin (**4**) in both the ^{13}C and the ^{1}H domain symbolized by boxed values plus circled centers and by smaller legends, respectively.

orientation of important core ^{1}H nuclei. The NOEs further indicated the preferred 14,21-conformation of the butenolide side-chain which also resulted from a systematic conformational search using torsion-forcing calculations. Furthermore, it was possible to unequivocally assign the α/β-orientation of the A/B methylene protons at C-15/16 as well as the relative stereochemistry of the diastereotopic (AB) hydroxymethylene protons of C-19. The latter showed both a distinctive NOE pattern (H-19A = pro-R ↔ H-8 [2.08 Å], H-19B = pro-S ↔ H-4ax [2.04 Å] and H-2ax [2.48 Å]) as well as a conclusive $^{3}J_{C,H}$ coupling behavior in the HMBC experiment: H-19A shares a coupling with C-1 (dihedral angle $\theta = 150°$) but not with C-5 ($\theta = 270°$); H-19B interacts with C-5 ($\theta = 30°$) but not with C-1 ($\theta = 270°$). According to the Karplus relationship, this fully agrees with the same spatial arrangement following from the NOE measurements, for a conformation

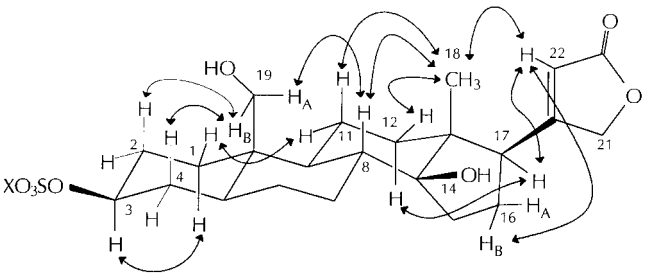

Fig. 5. Selected NOE interactions in **2** as observed in a 2D NOESY map providing proof for the relative stereochemistry of key protons on both α- and β-face of the molecule. Furthermore, the diastereotopic methylene protons at C-19 can be clearly assigned to be H-19A = pro-R and H-19B = pro-S which is in excellent agreement to their long-range C,H-coupling behavior (see discussion for details). Finally, it gives evidence for the 14,21-rotamer of the butenolide side-chain which is in agreement with molecular mechanics calculations.

structure refer to Fig. 5. An exciting finding that also supports the stereochemistry of the whole framework is the observation of a $^{4}J_{H,H}$ long-range coupling between H-19B and H-9: Being detected in the DQF-COSY of **2** as well as resulting from the shape of the H-9 signal, it is mediated by the nearly planar, W-type arrangement along the four connecting bonds, but does not effect in a detectable fine splitting of the H-18 signal. Therefore, we can conclude that there is additional broadening caused by other long-range coupling(s). In fact, optimized gaussian resolution enhancement of the ^{1}H-NMR spectrum of **3** yielded a *t*-like signal for the aldehyde proton H-19 split by 0.9 Hz which indicates two active long-range couplings involving H-9 and H-1(!). Nevertheless, since we could not locate equivalent reports in the literature, these findings should not only be able to serve as detailed references for the structure assignment of analogues but also demonstrate the power of ^{1}H-NMR in the elucidation of steroid stereochemistry.

In order to summarize the structure elucidation discussion, **2** was shown to be coroglaucigenin-3-*O*-sulfate, while **3** is corotoxigenin-3-*O*-sulfate. In principle, the structure of steroid sulfates is known from animal source, e.g., the bufadienolide-3-*O*-sulfates from *Bufo marinus* (Butler et al., 1996). However, this is the first report of cardenolide sulfates as genuine plant constituents and, to our best knowledge, represents the first finding of steroid sulfates as secondary metabolites produced by higher plants. Other than in the group of flavonoids, sulfatation is rarely found as a metabolization pathway in the biosynthesis of plant secondary constituents. Marine organisms are the main source of such sulfuric acid semi-esters and are well known for producing sulfated carotenoids (Hertzberg, 1983). Furthermore, from members of the Asteroideae and Holothurioideae (for a review see Minale et al., 1986), several triterpenes and steroids have been identified that either bear sulfated sugar residues or show direct 3-*O*-sulfatation (Bruno et al., 1989). Examples of lately discovered nitrogen-free sulfates from higher plants include the naphthopyrane cassiapyrone (Messana, Ferrari, Salete, Cavalcanti & Gacs-Baitz, 1990), the xanthone selinetin-O-sulfate (Lemmich & Shabana, 1984), 5′-hydroxysulfonyloxy jasmonic acid (Achenbach, Hübner, Brandt & Reiter, 1994), the benzylglucoside derivative salvadoside (Kamel et al., 1992), the cyanogenic glycoside cardiospermin sulfate (Hübel & Nahrstedt, 1979), and ellagic acid 2-O-sulfate (Terashima et al., 1990).

Interestingly, the three sulfates **1**–**3** are biogenetically very closely related compounds. Considering the fact that the cardenolide complex of *A. aleppica* is dominated by 5β-cardenolides, sulfatation can be understood as a unique and efficient way of producing polar conjugates of 5α-cardenolides. In this context it

has to be marked out that this type of conjugation has yet not been observed in any other of the more than 40 5β-cardenolides isolated from the same plant. However, while a few minor very polar *glycosides* of 5α-steroids were also detected, the production of sulfates can be interpreted in terms of solubilization (detoxification?) of biosynthetical side products. This hypothesis is supported by the observation that the glycosidic diversity in the 5β series of *A. aleppica* steroids is much greater than in the 5α group of steroids in which the sulfates represent the majority of derivatives (Pauli, 1998). Concerning the formation of water-soluble sulfates, an interesting field observations is the advanced development of *A. aleppica* with respect to plant size, branching, and number of flowers of individuals growing on wet soil due to rain and snow water drainage sites. While the investigated plants were collected from one large population growing on rich Mesopotamian soil with some specimens measuring up to 100 cm, elevated sites with dry soil hosted miniaturized forms of *A. aleppica* with only a few cm of height.

Due to the fact that **1** represents one of the major cardenolides accumulated by *A. aleppica*, this finding will place this species into an exceptional position within the genus *Adonis*. At the same time it has to be acknowledged that only with the help of an optimized isolation procedure and modern spectroscopy methods it became possible to isolate and characterize these highly polar compounds two of them being accumulated in 1 ppm quantities only.

3. Experimental

3.1. Plant material

Authentic plant material of *Adonis aleppica* BOISS. (total plants, air-dried, 3.5 kg dry wt) was collected in April 1990 near Urfa (Turkey) and identified by the author. Voucher specimens are deposited at the Heinrich-Heine-Universität Düsseldorf, Germany, and the Pharmacognosy Department of Haçettepe University in Ankara, Turkey.

3.2. Extraction, pre-purification, and isolation

The plants were successively extracted with 23 l $CHCl_3$, 49 l MeOH and 24 l MeOH–H_2O (70%) using an Ultra-Turrax apparatus. The combined extracts (1145 g) were evaporated in vacuo at 40°C to give a brown gummy residue, divided into seven portions, redissolved in H_2O and exhaustively extracted with 27.2 l $CHCl_3$-*iso*-PrOH (3:2) and 14.7 l *n*-BuOH.

The combined $CHCl_3$-*iso*-PrOH layers (162 g) were divided into three portions and each chromatographed on XAD-2 (1 kg) in two portions by stepwise elution with H_2O, MeOH and acetone. Gel chromatography of the 7.0 l MeOH eluates (38 g upon evaporation) on Sephadex LH-20 (Pharmacia, 1 kg, elution with MeOH) in two portions led to a fraction A2C2 (3.4 g) containing the cardenolide sulfate **1** beside polar cardenolide glycosides and flavonoid glycosides/phenolics (see Pauli, 1993).

The combined *n*-BuOH layers (105 g) were divided into two portions and each chromatographed on XAD-2 (1 kg) in two portions by stepwise elution with H_2O, MeOH–H_2O 1:1 and MeOH. VLC of the 8.0 l MeOH–H_2O eluates (33 g upon evaporation) on cellulose (Avicel Merck, 100 g, stepwise elution with EtOAc–MeOH–H_2O) led to a fraction A2C3 (20.3 g) containing the cardenolide sulfates **2** and **3** beside polar cardenolide glycosides and phenolics (see Pauli, 1993).

3.3. Isolation of *1*

Further separation of fraction A2C2 was achieved by vacuum chromatography (VLC) with silicagel 60 using a stepwise gradient elution with petrol ether–EtOAc–MeOH–H_2O (500 ml fractions) and yielded a polar fraction A2C2b (370 mg) containing **1** beside flavonoid glycosides. Another Sephadex LH-20 gel-filtration (MeOH) allowed enrichment of **1** (fr. A2C2bb, 87 mg) which was finally chromatographed by MPLC on RP-18 silicagel (9 g LiChroprep, 24–40 μm, flow 5.5 ml min^{-1}) with a MeCN–H_2O gradient. Upon crystallization from EtOH **1** was obtained in pure from as both colorless crystals (25 mg) and a yellowish-white amorphous powder (29 mg).

3.4. Isolation of *2* and *3*

Fraction A2C3 represented a mixture of mainly polar phenolics and a few Kedde reagent positive compounds as minor components. Their separation was achieved by VLC with 170 g silicagel 60 using a stepwise gradient elution with EtOAc–MeOH–H_2O (300 ml fractions) and yielded a polar fraction A2C3d (11020 mg) containing **2** and **3** beside flavonoid glycosides. Prior to gel-filtration, the latter were further removed by another VLC step under the same conditions but collecting multiple 300 ml fractions with the same solvent mixtures. Thus, A2C3dc (2140 mg) was passed over Sephadex LH-20 (120 g, MeOH) and led to a enriched fraction A2C3dca of **3** (540 mg). The latter was chromatographed by MPLC on 33 g RP-18 silicagel 60 (25–40 μm, MeOH–H_2O gradient, flows at 6.0 ml min^{-1}) to give fraction A2C3dca2 (38 mg) containing mainly **2** and **3**.

Isolation of both **2** and **3** was achieved by each twice repeated MPLC on RP-18 silica 60 (13 g 15–25 μm, and MeCN–H_2O gradient, flow at 4.5 ml min^{-1})

to yield 3.0 mg of **2** and 4.0 mg of **3** as colorless amorphous solids.

3.5. General procedures

TLC monitoring of the fractionation was done in $CHCl_3$–MeOH–H_2O 80:19:1 (solvent system 1 = standard), 70:29:1 (solvent system 2), and EtOAc–MeOH–H_2O 72–25–8 (solvent system 3), detection was performed by subsequent spraying of 2% 3.5-$DiNO_2$-benzoic acid and 2 N KOH alcoholic solutions (Kedde test) and vanillin–H_2SO_4 reagent.

3.6. Reference compound and partial synthesis

Authentic uzarigenin (50 mg) for spectroscopic reference measurements and partial synthesis of uzarigenin-3-*O*-sulfate was purchased from Roth, Karlsruhe. Sulfatation was performed in 5 ml anhydrous pyridine using 220 mg SO_3/pyridine complex reagent as described in (McKenna & Norymberski, 1957). The mixture was stirred for 30 min and then filtered through a 5 g cellulose column. The latter was washed with 5 ml pyridine, 30 ml $CHCl_3$ and 50 ml MeOH 50% to yield 290 mg of crude sulfatation product upon evaporation. Purification steps included gel-filtration on 120 g Sephadex LH-20 in MeOH and repeated MPLC on RP-18 silica using MeOH-H_2O gradients. Thus, 37 mg of uzarigenin-3-*O*-sulfate were obtained which according to all analytical data including ^{1}H- and ^{13}C-NMR was identical with **1**.

3.7. Instrumentation

For NMR spectroscopy the samples were dissolved in methanol-d_4 with an isotopic purity of 99.8% D (Aldrich, Milwaukee, no. 15194) to give a final sample volume of 0.7 ml corresponding to a filling height of 40 mm in 5 mm tubes (Wilmad 528-8). In case of **1**, the addition of three drops of D_2O (99.8% D. Merck) was necessary.

The spectra were recorded at 300 K on Bruker AC 400 and AMX 500 spectrometers (all equipped with 5 mm dual inverse probes) operating at 400/500 MHz for ^{1}H, and 100/125 MHz for ^{13}C, respectively. Chemical shifts are reported in ppm on the δ scale with the solvent as internal standard (3.300 and 49.00 ppm, respectively), the coupling constants (J) are given in Hz; s = singlet, d = doublet, dd = double doublet etc., t = triplet. APT (Attached Proton Test), DEPT, [(PS-)DQF]-COSY, NOESY, and HMBC/HMQC experiments were measured using the Bruker standard software. The spectra were obtained under the following conditions: ^{1}H 10°–30° pulse, delay after acquisition (D1) 1.5–3 s processed with 0.2 Hz line broadening (LB) or with Lorentz–Gauss resolution enhancement and zero-filling. Acquisition using 16 k (SI) (32 k at 500 MHz) data points in a spectral window of ca. 6 ppm yielded a digital resolution better than 0.2 Hz (or 0.0004 ppm). Water peak suppression was achieved by presaturation setting the HDO signal on resonance (O1). ^{1}H 2D COSY/NOESY with SW 6 or 9 ppm, D 1.2 s, 1 k × 512 (PS-DQF) or 256 increments, 90° shifted sinebell-squared apodization, zero-filled in t_1 dimension during processing; the NOESY mixing times were 700–850 ms. Inverse correlations were optimized for 140 Hz (HMQC) and 8 Hz (HMBC), respectively. Off-line data processing was done with the manufacturers NMR data processing software (Bruker DISNMR and WinNMR/UXNMR packages).

DCI mass spectra were run on a Finnigan INCOS 50 System with ammonia, trimethylammonia and triethylammonia as reactant gases. The emitter heating rate was 10 mA s^{-1}, calibration was done with FC43 and methane as reactant gas.

Optical rotations were measured with a Perkin-Elmer 241 polarimeter and UV spectra taken with a Beckman DB-G instrument. Middle pressure liquid chromatography (MPLC) preparations were carried out on Latek 9 mm and self-built glass columns (20 cm × 16 mm I.D.) with a Knauer HPLC pump Model 64, a DuPont detector and additional TLC detection. Gradient elution was performed using corresponding vessels as described by Pauli (1993) or by using a Knauer gradient control unit. Droplet counter current chromatography (DCCC) was run on a Büchi 670 apparatus. Molecular mechanics calculations (systematic conformational search and minimization) were performed using the MOBY program of Dr. U. Höweler, Münster.

3.8. X-ray diffraction analysis

X-ray diffraction data were collected on an Enraf-Nonius CAD4 diffractometer equipped with CuK_α ($\lambda = 1.54184$ Å) radiation and a graphite monochromator. Two Friedel related octants of data were collected. Data reduction included corrections for background, Lorentz, polarization, and absorption effects. Absorption corrections were based on ψ scans. The structure was solved by direct methods and refined using the MolEN programs (Fair, 1990). Refinement was by full-matrix least squares, with neutral-atom scattering factors and anomalous dispersion corrections. Weights were $w = 4F_o^2[\sigma^2(I) + (0.02F_o^2)^2]^{-1}$. All non-hydrogen atoms were refined anisotropically. H atoms on the OH group and one water molecule were placed from difference maps, while most others were placed in calculated positions. Hydrogen atoms on water molecule O2W and that of the ethanol OH group were not located. Crystal data, final R values,

Table 4
Crystal data and X-ray data collection parameters of uzarigenin-3-*O*-sulfate (**1**)

Formula	Na $[C_{23}H_{23}SO_7]\cdot 2H_2O\cdot C_2H_5OH$
Crystal shape	Fragment
Formula weight	558.7
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Temperature, °C	23
a, Å	6.3871(3)
b, Å	18.645(1)
c, Å	23.438(2)
Cell volume, Å^3	2791.1(6)
Z	4
D_{calc}, g cm^{-3}	1.329
μ_{calc}, cm^{-1}	16.0
Radiation	CuK_α
Crystal dimensions, mm	$0.27 \times 0.38 \times 0.45$
Minimum transmission, %	93.3
Decay of standards	< 2%
Reflections measured	6479
2θ range, degree	$4 < 2\theta < 150$
Range of h, k, l	7, 23, ±29
Unique data	5742
Observed data	4427
Criterion for observations	$\mathbf{I} > 3\sigma(I)$
No. of parameters	335
R	0.064
R_w	0.074
Extinction	$1.38(12) \times 10^{-6}$
Maximum final diff. map	0.70e$^-$ Å^{-3}

and other details are included in Table 4. Refinement with the reported absolute configuration for **1** yielded R values in Table 5, while the opposite configuration yielded $R = 0.071$, $R_w = 0.082$. Thus, the absolute configuration is determined to be that illustrated. A complete set of crystallographic data (CIF) has been deposited at the Cambridge Crystallographic Data Centre (CCDC 125672).

3.9. Uzarigenin-3-O-sulfate (*1*)

Colorless crystals; $C_{23}H_{34}O_7S$ (free acid); R_f 0.07 in standard solvent system 1; mp 242°C (uncorrected); UV (λ_{max}, log ε): 217 nm (4.07); IR ν_{max}^{KBr} cm^{-1}: 3465 and 2930 (OH), 1740 and 1615 (butenolide), 1230 and 820 (sulfate); $\alpha_D^{20} + 11°$ (MeOH, $c = 2.00$) DCI-NH_3-MS m/z (relative intensity): 356 (100) $[M-H_2SO_4-H_2O + NH_4]^+$, for further data see Table 2; FAB-MS m/z (relative intensity): 453 (100) $[M-H]^-$; ^{1}H-NMR: see Table 3; ^{13}C-NMR: see Table 1.

3.10. Coroglaucigenin-3-O-sulfate (*2*)

White amorphous solid; $C_{23}H_{34}O_8S$ (free acid); R_f 0.04 in standard solvent system 1, R_f 0.78 in solvent system 3; mp 173–178°C (uncorrected); UV (λ_{max}, log ε): 217 nm (3.99); IR ν_{max}^{KBr} cm^{-1}: 3460 and 2930 (OH), 1740 and 1620 (butenolide), 1230 and 820 (sulfate); $\alpha_D^{20} + 60°$ (MeOH, $c = 0.26$) DCI-NH_3-MS m/z (relative intensity): 372 (100) $[M-H_2SO_4-H_2O + NH_4]^+$, for further data see Table 2; ^{1}H-NMR: see Table 3; ^{13}C-NMR: see Table 1.

Table 5
Coordinates and equivalent isotropic thermal parameters for **1**

Atom	x	y	z	B_{eq} (Å^2)
S1	0.4188(2)	0.62327(6)	0.58249(5)	3.51(2)
Na1	0.7227(3)	0.7370(1)	0.4997(1)	6.59(5)
O1	0.5404(5)	0.5604(2)	0.5518(1)	4.11(6)
O2	1.2557(4)	0.1967(2)	0.7085(1)	3.76(6)
O3	1.4588(6)	−0.0258(2)	0.7331(2)	7.7(1)
O4	1.398(1)	−0.1340(3)	0.7024(3)	12.2(2)
O5	0.4493(5)	0.6821(2)	0.5431(1)	4.50(7)
O6	0.5157(6)	0.6337(2)	0.6375(1)	5.22(8)
O7	0.2028(5)	0.6024(2)	0.5869(2)	5.04(7)
C1	0.5538(6)	0.3595(2)	0.5711(2)	3.45(8)
C2	0.5115(6)	0.4331(3)	0.5440(2)	3.84(9)
C3	0.5671(6)	0.4929(2)	0.5836(2)	3.37(8)
C4	0.7915(7)	0.4880(2)	0.6035(2)	3.58(9)
C5	0.8296(6)	0.4150(2)	0.6314(2)	3.17(8)
C6	1.0462(6)	0.4090(2)	0.6579(2)	3.54(8)
C7	1.0713(7)	0.3398(2)	0.6915(2)	3.81(9)
C8	1.0168(6)	0.2738(2)	0.6561(2)	3.06(8)
C9	0.8013(6)	0.2807(2)	0.6274(2)	3.07(8)
C10	0.7801(6)	0.3505(2)	0.5918(2)	2.97(7)
C11	0.7499(7)	0.2130(2)	0.5942(2)	3.88(9)
C12	0.7664(7)	0.1455(2)	0.6305(2)	3.97(9)
C13	0.9825(6)	0.1358(2)	0.6588(2)	3.33(8)
C14	1.0398(6)	0.2049(2)	0.6917(2)	3.17(8)
C15	0.9029(7)	0.1990(3)	0.7453(2)	4.02(9)
C16	0.9154(8)	0.1200(3)	0.7619(2)	4.7(1)
C17	0.9585(7)	0.0782(2)	0.7070(2)	3.83(9)
C18	0.9270(7)	0.3490(2)	0.5396(2)	3.76(8)
C19	1.1428(7)	0.1156(3)	0.6136(2)	4.0(1)
C20	1.1338(7)	0.0248(3)	0.7123(2)	4.1(1)
C21	1.3440(8)	0.0398(3)	0.7370(2)	6.0(1)
C22	1.132(1)	−0.0428(3)	0.6968(3)	6.5(1)
C23	1.328(1)	−0.0755(3)	0.7084(3)	7.9(1)
O1W	1.3201(6)	0.2603(2)	0.8224(2)	6.5(1)
O2W	0.4671(6)	0.7609(3)	0.4168(2)	8.7(1)
O1E	0.9912(7)	0.6541(3)	0.4813(3)	10.6(2)
C1E	0.968(2)	0.6014(7)	0.4437(3)	16.4(4)
C2E	1.118(2)	0.5562(6)	0.4306(4)	16.1(3)

3.11. Corotoxigenin-3-O-sulfate (*3*)

White amorphous solid; $C_{23}H_{32}O_8S$ (free acid); R_f 0.06 in standard solvent system 1, R_f 0.85 in solvent system 3; mp 173–178°C (uncorrected); UV (λ_{max}, log ε): 217 nm (3.89); IR ν_{max}^{KBr} cm^{-1}: 3460 and 2930 (OH), 1740 and 1620 (butenolide), 1230 and 820 (sulfate); $\alpha_D^{20} + 32°$ (MeOH, $c = 0.35$) DCI-NH_3-MS m/z (relative intensity): 370 (100) $[M-H_2SO_4-H_2O + NH_4]^+$, for further data see Table 2; ^{1}H-NMR: see Table 3; ^{13}C-NMR: see Table 1.

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