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Bisanthraquinone glycosides of *Hypericum perforatum* with binding inhibition to CRH-1 receptors

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Abstract

Four new bisanthraquinone glycosides, *S*-(+)-skyrin-6-*O*-β-glucopyranoside (**1**), *R*-(−)-skyrin-6-*O*-β-glucopyranoside (**2**), *S*-(+)-skyrin-6-*O*-β-xylopyranoside (**3**) and *S*-(+)-skyrin-6-*O*-β-α-arabinofuranoside (**4**), have been isolated from an ethanol–water (1:1, v/v) dry extract of the aerial parts of *Hypericum perforatum* L. The structures were elucidated by spectroscopic methods, mainly NMR and mass spectrometry. Circular dichroism was used to determine their axial stereochemistry revealing **1** and **2** to be atropisomers. **1** and **2** inhibited [^{125}I]sauvagine binding to corticotropin releasing hormone (CRH-1) receptors. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Hypericum perforatum*; Hypericaceae; Bisanthraquinone glycosides; *S*-(+)-skyrin-6-*O*-β-glucopyranoside; *R*-(−)-skyrin-6-*O*-β-glucopyranoside; *S*-(+)-skyrin-6-*O*-β-xylopyranoside; *S*-(+)-skyrin-6-*O*-α-arabinofuranoside; CRH-1 receptor

1. Introduction

Hypericum perforatum L. is widely used for the treatment of mild to moderate depression. The pharmacological activity of its extracts has been verified in several clinical studies (for references see Linde and Mulrow, 1999), but still there are questions about the active principles and the mode of action. It can be assumed that the antidepressant effect results from several active constituents including hypericins, hyperforins and others such as procyanidins and flavonoids exerting different modes of action (Baureithel et al., 1997; Gobbi et al., 1999; Meier, 1999; Simmen et al., 1999).

On the search for further potentially active compounds and pharmacological principles we isolated the four new bisanthraquinone glycosides **1** to **4** from an ethanol–water dry extract. The aglycone of the four glycosides is skyrin. It has been found in various fungi in pure culture (e.g. *Penicillium islandicum* and *P. rugulosum*), in fruit bodies of *Cortinarius atrovirens* and *Dermocybe austroveneta*, in lichens (e.g. *Acroscyphus sphaerophoroides*) and in the insect *Pseudococcus albizziae*. Up to now, no compounds of this class have been isolated from *Hypericum* species, although the presence of both skyrin and hypericin in *H. perforatum* is not surprising, since their biosynthesis was proposed to follow a similar pathway (Gill and Giménez, 1991).

Both in vitro and in vivo studies with *Hypericum* extracts have demonstrated reduced corticosteroid levels in tissues and rats suggesting an additional mechanism for antidepressant actions (Thiele et al., 1993). Two of the new bisanthraquinone glycosides have therefore been studied for their potency to inhibit [^{125}I]sauvagine binding to corticotropin releasing hormone-1 (CRH-1) receptors.

2. Results and discussion

An ethanol–water (1:1, v/v) dry extract of *Hyperici herba* (Ze117, Zeller company) was fractionated by liquid–liquid–partitioning and subsequent RP-18

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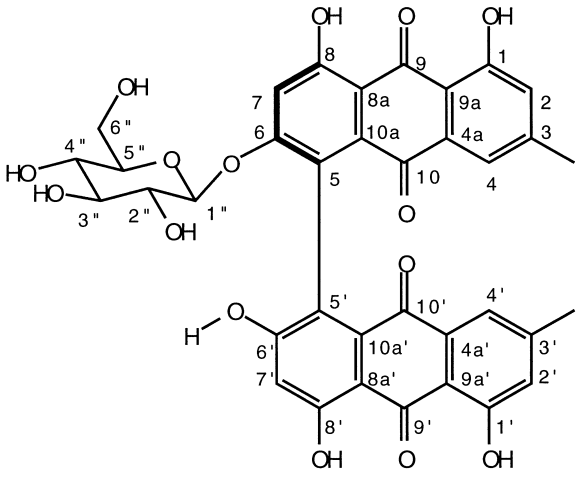
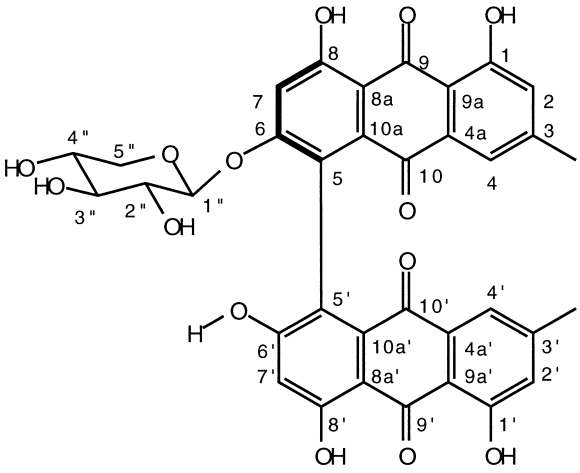
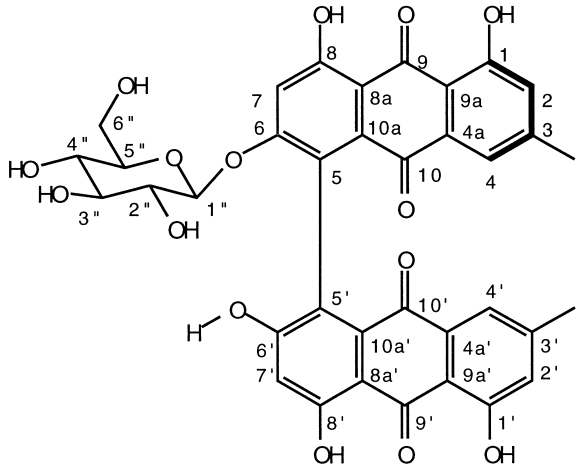


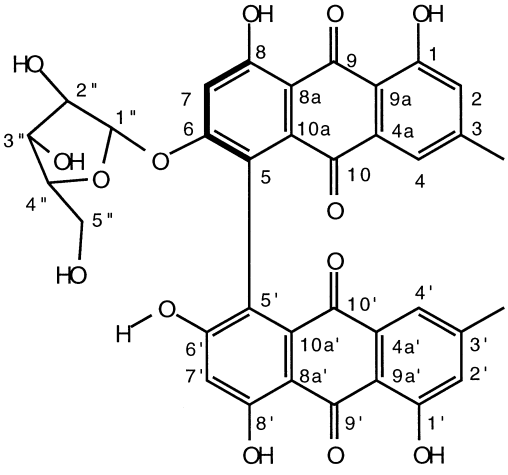
S-(+)-skyrin-6-*O*-β-glucopyranoside (**1**).



S-(+)-skyrin-6-*O*-β-xylopyranoside (**3**).



R-(−)-skyrin-6-*O*-β-glucopyranoside (**2**).



S-(+)-skyrin-6-*O*-α-arabinofuranoside (**4**).

vacuum liquid chromatography. Preparative thin layer chromatography (TLC) on silica gel led to the isolation of the new skyrin glycosides **1**–**4**.

S-(+)-skyrin-6-*O*-β-glucopyranoside (**1**) was isolated as an orange-red powder. The ^{1}H NMR of **1** (see Table 1) revealed signals for six aromatic protons resonating as singlets at δ 7.32, 7.16, 7.06, 7.05, 6.95 and 6.28. It also showed the presence of a hexose and two signals for aromatic methyl protons at δ 2.30 and 2.35 (each 3H). The anomeric proton of the hexose was observed as a doublet at δ 5.06. The coupling constant of 7.5 Hz indicated the linkage to be β. The location of the biaryl bond followed from the absence of a signal arising from H-5 and H-5′.

The ^{13}C NMR spectrum (see Table 3) of **1** revealed 36 discrete signals that could be assigned to individual carbon atoms on the basis of two-dimensional NMR techniques (HMQC and HMBC). The carbonyl carbons C-9 and C-10 resonated at δ 192.3 and δ 184.0, respectively. Acetylation led to a strong upfield shift of C-9 (δ 179.9). The HMBC spectra of **1** in CD_3OD showed signals at δ 164.2 and δ 166.2, which correlated both with H-7 (δ 7.06). The signal at δ 164.2 showed an additional correlation with the anomeric proton of the sugar H-1″ (δ 5.06). These data did not allow a definite assignment of C-6 and C-8. The signals at δ 179.0 and δ 168.1 showed both correlations with H-7′ (δ 6.28) not making it clear, which was C-6′ and C-8′.

^{1}H-NMR and HMBC spectra of **1** in DMSO-d_6 exhibited only one sharp OH-signal (δ 12.45) which showed correlations with C-8a′, C-7′, C-9′ and δ 166.6 (C-6′ or C-8′) in the HMBC spectrum, pointing the signal at δ 166.6 to be the resonance of C-8′. Consequently, the signal resonating at δ 178.0 has to be C-6′. The assignment of C-6 and C-8 has been done by comparison of the ^{1}H NMR data of the acetyl derivative of **1**(=**1a**, 1,1′,8,8′,2″,3″,4″,6″-octaacetyl-*S*-(+)-skyrin-6-*O*-β-glucopyranoside, see Table 2) with literature data of hexaacetylskyrin (Takeda et al., 1973). The acetylation of **1** was not complete. This finding was confirmed by the positive FABMS, which gave a $[M+2H]^{+}$ peak at *m*/*z* 1038 for **1a** being consistent with the molecular

Table 1
^{1}H NMR spectral data (δ ppm, J in Hz) of **1**, **2**, **3**, and **4** (500.13 MHz for **1**, **2** and **4**; 600.13 MHz for **3**; CD_3OD)

	1		**2**		**3**		**4**	
H	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)
2	7.05 *s*		7.05 *s*		7.03 *s*		7.04 *s*	
4	7.32 *s*		7.35 *s*		7.31 *s*		7.34 *s*	
7	7.06 *s*		7.09 *s*		6.99 *s*		7.01 *s*	
Me-3	2.35 *s*		2.36 *s*		2.34 *s*		2.35 *s*	
2′	6.95 *s*		6.93 *s*		6.94 *s*		6.93 *s*	
4′	7.16 *s*		7.21 *s*		7.17 *s*		7.20 *s*	
7′	6.28 *s*		6.25 *s*		6.25 *s*		6.23 *s*	
Me-3′	2.30 *s*		2.30 *s*		2.23 *s*		2.30 *s*	
1″	5.06 *d*	7.5	4.85 *d*	7.8	5.03 *d*	6.8	5.53 *d*	1.1
2″	3.16*dd*	7.5/9.2	3.14*dd*	7.87/8.8	3.17*dd*	6.8/8.6	3.91*dd*	1.5/4.1
3″	3.40*dd*	9.2/9.2	3.28*dd*	9.2/9.1	3.37–3.33 *m*		3.78*dd*	4.1/6.7
4″	3.24*dd*	9.4/9.4	3.23*dd*	9.3/9.2	3.37–3.33 *m*		3.70 *m*	2.9/4.6/5.9
5A″	3.47*ddd*	2.3/6.0/9.6	3.39*ddd*	2.3/6.1/9.1	3.77*dd*	2.9/9.9	3.67*dd*	2.9/13.7
5B″					3.37–3.33 *m*		3.52*dd*	4.7/12.2
6A″	3.83*dd*	2.3/12.1	3.84*dd*	2.3/12.2				
6B″	3.62*dd*	5.8/12.5	3.63*dd*	6.1/12.2				

formula $C_{52}H_{44}O_{23}$. In literature published shifts for $COC\underline{H}_3$-8/8′ and $COC\underline{H}_3$-1/1′ were δ 2.50 and δ 2.47, respectively. Corresponding signals could be found in the ^{1}H NMR spectrum of compound **1a**: δ 2.50/δ 2.48 (3H each, s) and δ 2.45 (6H, s). The resonances at δ 2.50/δ 2.48 were assigned to $COC\underline{H}_3$-8/8′. The HMBC experiment revealed correlations of $COC\underline{H}_3$-8/8′ with the resonances at δ 152.0 and δ 151.95; they were concluded to be the signals of C-8 and C-8′. The signal at δ 152.0 correlated with H-7 (δ 7.20) in the HMBC spectrum indicating to be the resonance of C-8. Correspondingly, δ 151.95 correlated with H-7′ (δ 6.90). The ^{1}H NMR spectrum of **1a** lacks signals of $COC\underline{H}_3$-6/6′ usually resonating near δ 1.93 (Takeda et al., 1973). Therefore it can be concluded that OH-C-6′ has not been acetylated and **1a** is 1,1′,8,8′,2″,3″,4″,6″-octaacetyl-*S*-(+)-skyrin-6-*O*-β-glucopyranoside (for ^{13}C NMR data of compound **1a** see Table 3). In the HMBC spectrum C-6 (δ 158.5) revealed a correlation with the anomeric proton (δ 5.14, *d*, J = 7.6 Hz) confirming 6-OH to be the binding site of the sugar. The hydrogen bond between C-6′ and the sugar moiety probably hindered the acetylation of OH-C-6′. Knowing 6-OH is the binding site of the sugar, the assignment was accomplished for compound **1**. The assignment of the carbons 4a (δ 135.7) and 9a (δ 115.0) has been done by comparison with literature data of other anthraquinone derivatives (Toma et al., 1975).

The sugar moiety was identified as a β-glucopyranose by the ^{1}H and ^{13}C NMR spectral data supported by DQF-COSY as well as ROESY experiments and published data (De Tommasi et al., 1993).

R-(-)-skyrin-6-*O*-β-glucopyranoside (**2**) was isolated as an orange-red powder. **1** and **2** showed different *Rf* values (**1**: 0.20, **2**: 0.31) on TLC (silica gel) employing EtOAc–acetonitrile–H_2O (10:1:1) as eluent, small deviations in their ^{1}H and ^{13}C NMR spectral data and opposite CD curves. The two dimers gave almost

Table 2
^{1}H NMR spectral data (δ ppm, J in Hz) of **1a** and **2a** (500.13 MHz, $CDCl_3$) and published data of hexaacetylskyrin ($CDCl_3$)

	1a		**2a**		Takeda et al. (1973)
H	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	δ (ppm)
2	7.15 *s*		7.14 *s*		7.15
4	7.67 *s*		7.69 *s*		7.69
7	7.20 *s*		7.15 *s*		7.38
Me-3	2.40 *s*		2.37[a]*s*		2.42
2′	7.14 *s*		7.13 *s*		7.15
4′	7.65 *s*		7.64 *s*		7.69
7′	6.90 *s*		6.99 *s*		7.38
Me-3′	2.40 *s*		2.36[a]*s*		2.42
1″	5.14 *d*	7.6	5.32 *d*	6.03	
2″	4.89*dd*	7.7/8.5	4.88*dd*	6.1/6.5	
3″	5.17*dd*	9.3/9.3	5.09*dd*	7.0/9.1	
4″	4.98*dd*	9.5/9.7	5.21*dd*	9.3/9.6	
5″	3.88*ddd*	2.9/5.7/9.9	3.92*ddd*	2.6/5.1/10.1	
6A″	4.14 *m*		4.19*dd*	2.5/12.2	
6B″			4.12*dd*	5.1/12.4	
$COCH_3$-1	2.45 *s*		2.45 *s*		2.47
$COCH_3$-1′	2.45 *s*		2.44 *s*		2.47
$COCH_3$-8	2.50[a]*s*		2.49[b]*s*		2.50
$COCH_3$-8′	2.48[a]*s*		2.48[b]*s*		2.50
$COCH_3$-6					1.93
$COCH_3$-6′					1.93
$COCH_3$-2″	1.94 *s*		1.59 *s*		
$COCH_3$-3″	1.91 *s*		1.90 *s*		
$COCH_3$-4″	2.00 *s*		2.00 *s*		
$COCH_3$-6″	2.11 *s*		1.97 *s*		

[a,b] Assignments in the same column bearing the same superscript may be interchanged.

identical IR spectra, MS spectra and UV absorption maxima, showing slightly higher ε values for **2**. Consequently, **1** and **2** seem to differ in their axial stereochemistry. The CD spectra of **1** and **2** showed antipodal split bands around 255 nm suggesting them to be atropisomers. The spectrum of **1** revealed strong positive first ($[\Delta\varepsilon]+30.47$ (263 nm)) and negative second ($[\Delta\varepsilon]-10.12$ (247 nm)) Cotton effects. On the other hand, the spectrum of **2** revealed strong negative first ($[\Delta\varepsilon]-40.94$ (263 nm)) and positive second ($[\Delta\varepsilon]+12.90$ (248 nm)) Cotton effects.

Table 3
^{13}C NMR spectral data (δ ppm) of **1**, **2** (75.47 MHz, CD_3OD), **3**, **4** (125.77 MHz, CD_3OD) and **1a** and **2a** (125.77 MHz, $CDCl_3$)

C	1	2	3	4	1a	2a
1	163.6 *s*	163.1	163.3	163.1	149.6 *s*	149.6
2	124.5 *d*	124.1	124.3	124.1	130.2 *d*	130.88
3	149.5 *s*	149.6	149.5	149.5	146.2[a]*s*	146.0[a]
4	121.3 *d*	121.6	121.3	121.5	126.1 *d*	126.1
4a	135.7 *s*	135.7	135.8	135.8	134.7[b]*s*	134.8[b]
5	129.0 *s*	130.4	129.5	130.2	124.6 *s*	125.4
6	164.2 *s*	164.4	164.1	164.0	158.5 *s*	157.7
7	108.9 *d*	110.3	109.0	108.6	115.9 *d*	115.3
8	166.2 *s*	165.8	166.1	165.9	152.0 *s*	151.66[c]
8a	113.2 *s*	112.9	113.1	112.2	122.1 *s*	121.7
9	192.3 *s*	192.6	188.2	192.6	179.9 *s*	180.1
9a	115.0 *s*	114.8	115.0	114.9	122.7 *s*	122.7
10	184.0 *s*	183.6	183.8	183.8	182.4 *s*	182.4
10a	133.8[a]*s*	133.0[a]	133.8[a]	133.1[a]	132.7[c]*s*	132.7
Me-3	22.0 *q*	22.1	22.0	22.0	21.6 *q*	21.6
1′	162.4 *s*	162.4	162.4	162.8	149.7 *s*	149.7
2′	124.0 *d*	123.7	123.9	123.9	130.3 *d*	130.9
3′	147.1 *s*	146.8	147.0	147.0	145.4[a]*s*	145.4[a]
4′	120.6 *d*	120.8	120.5	120.6	125.8 *d*	125.8
4a′	135.4 *s*	135.5	135.5	135.5	134.8[b]*s*	134.7[b]
5′	130.7 *s*	130.4	130.7	131.0	121.9 *s*	122.9
6′	179.0 *s*	178.1	179.2	178.7	157.8 *s*	158.2
7′	110.1 *d*	109.5	110.0	109.8	116.6 *d*	116.9
8′	168.1 *s*	168.1	168.2	168.2	151.95 *s*	151.7[c]
8a′	106.4 *s*	106.4	106.2	106.2	120.8 *s*	120.6
9′	187.9 *s*	187.9	187.8	187.7	179.9 *s*	180.0
9a′	115.6 *s*	115.8	115.8	115.8	122.9 *s*	122.9
10′	186.3 *s*	186.1	186.4	186.2	183.0 *s*	183.2
10a′	132.3[a]*s*	132.0[a]	132.3[a]	132.0[a]	134.2[c]*s*	133.4
Me-3′	21.9 *q*	21.9	21.8	21.9	21.5 *q*	21.5
1″	103.0 *d*	102.4	103.3	108.0	98.4 *d*	96.6
2″	75.1 *d*	74.3	74.4	83.4	70.5 *d*	70.9
3″	77.5 *d*	78.0	76.7	77.8	72.3 *d*	72.5
4″	70.8 *d*	71.1	70.5	86.2	68.0 *d*	67.9
5″	78.4 *d*	78.4	66.7	62.2	72.3 *d*	72.1
6″	62.4 *t*	62.6			62.1 *t*	61.6
$\underline{C}OCH_3$-1					169.62 *s*	169.6
$\underline{C}OCH_3$-1′					169.55 *s*	169.6
$\underline{C}OCH_3$-8					170.1[d]*s*	169.2[d]
$\underline{C}OCH_3$-8′					169.0[d]*s*	168.9[d]
$\underline{C}OCH_3$-2″					168.4 *s*	168.2
$\underline{C}OCH_3$-3″					170.2 *s*	170.1
$\underline{C}OCH_3$-4″					169.2 *s*	170.0
$\underline{C}OCH_3$-6″					170.5 *s*	170.9
CO$\underline{C}H_3$-1					21.23[e]*q*	21.2[e]
CO$\underline{C}H_3$-1′					21.20[e]*q*	21.2[e]
CO$\underline{C}H_3$-8					21.14[e]*q*	21.2[e]
CO$\underline{C}H_3$-8′					21.11[e]*q*	21.1[e]
CO$\underline{C}H_3$-2″					20.6[f]*q*	19.8
CO$\underline{C}H_3$-3″					20.5[f]*q*	20.5
CO$\underline{C}H_3$-4″					20.4[f]*q*	20.7
CO$\underline{C}H_3$-6″					20.3[f]*q*	20.4

[a–f] Assignments in the same column bearing the same superscript may be interchanged.

The absolute configuration was determined by comparison of the CD spectrum of **1** with that of (+)-skyrin (Jägers, 1980), to which *S* axial stereochemistry has been assigned by kinetic resolution studies (Billen et al., 1988). The CD spectrum of **1** (Fig. 1) resembled that of (+)-skyrin indicating *S*-configuration for **1** and therefore *R*-configuration for **2**. The NMR assignment of **2** has been done according to **1** (see Tables 1 and 2). Shift differences in the ^{13}C NMR were biggest around the biaryl linkage at the carbons C-5 ($\Delta\delta$ 1.4), C-7 ($\Delta\delta$ 1.4), C-10a ($\Delta\delta$ 0.8), C-6′ ($\Delta\delta-0.9$) and C-7′ ($\Delta\delta-0.6$) and within the sugar moiety at the carbon C-2″ ($\Delta\delta-0.8$) indicating different anisotropic effects in the two atropisomeric forms. Hydrolysis of **1** and **2** on a TLC (silica gel) plate (Kartnig and Wegschaider, 1971) and comparison to authentic β-glucose confirmed that the sugar moiety of **1** and **2** was β-glucose.

S-(+)-skyrin-6-*O*-β-xylopyranoside (**3**) was obtained as orange-red amorphous powder. The assignment of its NMR resonances has been done by comparison with NMR spectral data of **1** involving two-dimensional experiments (HMQC and HMBC). The sugar moiety was determined as a β-xylopyranose by comparison of the ^{1}H and ^{13}C NMR spectral data as well as ^{1}H–^{1}H spin coupling constants with published data (Hirota et al., 1990; Kusano et al., 1995). This finding was confirmed by 2D-NMR analysis. The HMBC displayed a long-range correlation between C-6 (δ 164.1) and the anomeric proton (δ 5.03, *d*, $J=6.8$ Hz), revealing a 6-*O*-β glycosidation. Hydrolysis on a TLC plate confirmed the sugar moiety of **3** to be β-xylose. The ESIMS (negative mode) was consistent with the molecular formula $C_{35}H_{36}O_{14}$ giving the $[M-H]^-$ ion peak at m/z 669. The CD spectrum of **3** indicated *S* chirality at the axis. *S*-(+)-skyrin-6-*O*-α-arabinofuranoside (**4**) was isolated as orange-red powder. The assignment of the NMR spectral data was done in analogy to **3**. The sugar moiety was determined as α-arabinofuranose by ^{1}H and ^{13}C NMR spectral data and ^{1}H–^{1}H spin coupling constants being in agreement with reported data (Iorizzi et al., 1996). ^{1}H–^{1}H COSY and off-resonance ROESY spectra supported the result. The HMBC displayed a long-range correlation between C-6 (δ 164.0) and the anomeric proton (δ 5.53, *d*, $J=1.1$ Hz), revealing the site of glycosidation to be 6-*O*-α. The ESIMS gave the $[M-H]^-$ ion peak at m/z 669 being consistent with the molecular formula $C_{35}H_{36}O_{14}$. From the CD spectrum, it was derived that **4** has the same stereochemistry at the axis as **1** and **3**.

A more detailed description of the structural elucidation of compound **1** to **4** can be found in Wirz (2000). Bisanthraquinone glycosides **1** and **2** were tested for their potency to inhibit [^{125}I]sauvagine binding to CRH-1 receptors. Both compounds showed moderate inhibition of the binding to CRH-1 receptors with IC_{50} values of 1 and 4 μmol/l, respectively. IC_{50} values of hyperforin and hypericin were 10 and 6 μmol/l (unpublished data). This indicated a possible antidepressant action via the hypothalamic–hypophyseal–adrenal axis. However the fact that only small amounts of these compounds (0.0065–0.029%) are present in the dried plant (Wirz, 2000) implies that these bisanthraquinone glycosides play only a minor role in the antidepressant effect of *Hypericum*.

3. Experimental

3.1. General

NMR spectra were recorded on a Bruker AMX-300 (operating at 300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C), a Bruker AMX-500 (operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C) and a Bruker AMX-600 spectrometer (operating at 600.13 MHz for ^{1}H and 150.92 MHz for ^{13}C). The skyrin glycosides were measured in CD_3OD and DMSO-d_6 , their acetylated derivatives in $CDCl_3$. All spectra were referenced to residual hydrogen or carbon resonances of the respective solvents. ESIMS spectra were measured on a Finnigan TSQ 7000 mass spectrometer. FAB mass spectra were obtained on a ZAB 2-SEQ (VG) spectrometer at 8.3 keV either in the positive-ion mode or in the negative-ion mode, using 3-NOBA (3-nitrobenzyl alcohol) as a matrix. IR spectra were recorded from **1** and **2** on a Perkin–Elmer system 2000 FT IR spectrophotometer (Perkin–Elmer, Rotkreuz, Switzerland) using KBr pellets. UV/VIS spectra were measured in MeOH from 200–500 nm on a Kontron-Uvikon 930 spectrophotometer (Kontron Instruments, Zürich, Switzerland). CD spectra were recorded in MeOH from 200–400 nm on a Jasco J-710/720 spectropolarimeter (Jasco Corporation, Tokyo) at 23°C using a 0.1 cm cell. Regenerated cellulose syringe filters (0.2 μm, 13 mm) were from Schleicher & Schuell (D-Dassel). RP material (C-Gel C 18 HL, particle size 0.04–0.063 mm) was obtained from Chemie Uetikon AG (Uetikon, Switzerland). RP-18 F_{254s} pre-coated plates (layer thickness 0.25 mm) were purchased from Merck (Darmstadt, Germany) and silica gel 60 F_{254} pre-coated aluminium sheets (layer thickness 0.2 mm) from Merck (Dietikon, Switzerland).

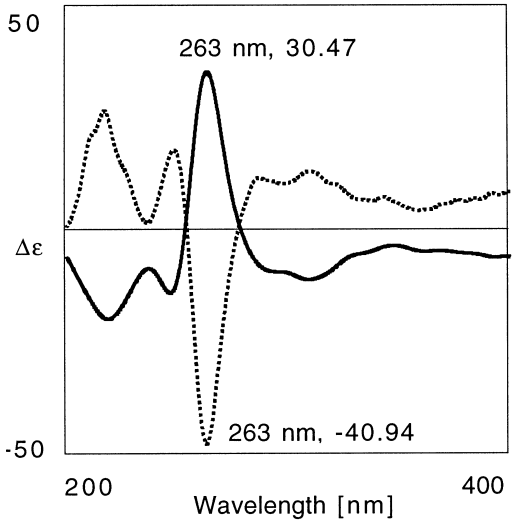


Fig. 1. CD spectra of — (S)-(+)-Skyrin-6-*O*-β-glucopyranoside (**1**) and ··· (R)-(−)-Skyrin-6-*O*-β-glucopyranoside (**2**).

3.2. Plant material

The source material was an EtOH–H_2O (1:1, v/v) dry extract (Ze117 from Zeller company, Romanshorn, Switzerland) of *Hyperici herba*.

3.3. Isolation

Dry extract of *Hyperici herba* (20 g) were partitioned in the solvent system hexane–toluene–H_2O–EtOAc–HCO_2H (75:225:135:120:15). The residue of the upper level was dissolved in 20 ml MeOH and stored at 8°C. After 2 h the formed precipitate was removed by filtration. The filtrate was evaporated under vacuum to yield 320 mg extract. The procedure was repeated several times. Extract (4.32 g) was dissolved in 50 ml acetonitrile–H_2O–THF (5:4.9:0.1) and stored at 8°C for two h. Again the precipitate was removed by filtration. The residue of the filtrate was dissolved in 10 ml acetonitrile–H_2O–TFA (5:4.9:0.1) and applied to RP-18 VLC employing the same eluent. After TLC (RP-18) control with the eluent acetonitrile–H_2O–TFA (5:4.9:0.1), the fractions containing **1** and **2** (340–500 ml) and the fractions enriched with **3** and **4** (615–815 ml) were combined. Evaporation of the solvent left 382.3 mg and 111.0 mg, respectively. The samples were dissolved in EtOH absolute and partly subjected to TLC (silica gel) over a distance of approx. 15 cm employing EtOAc–acetonitrile–H_2O (10:1:1) as eluent. The *Rf* values of the compounds were 0.20 (**1**), 0.31 (**2**), 0.38 (**3**) and 0.51 (**4**). The relevant bands were cut out and macerated twice with absolute EtOH for 20 min. After filtration, the EtOH was evaporated under reduced pressure below 30°C. This led to the isolation of **1** (18.1 mg), **2** (29.8 mg), **3** (3.4 mg) and **4** (7.3 mg).

*3.3.1. S-(+)-Skyrin-6-O-β-glucopyranoside (**1**)*

Red-orange amorphous powder (18.1 mg), melting point > 300°C, FABMS (negative) *m/z* 700 $[M]^-$, 722 $[M+Na-H]^-$, 537 $[M-C_6O_5H_{11}]^-$, 519 $[M-C_6O_5H_{11}-H_2O]^-$, ESIMS (negative) *m/z* 699 $[M-H]^-$, UV–Vis (MeOH) λ_{max} (log ε): 219 (4.53), 258 (4.49), 298 (4.21), 455 (4.03) nm, CD (MeOH) λ_{max} ($\Delta\varepsilon$): 309 (−7.67), 263 (+30.47), 247 (−10.12), 219 (−14.85) nm, IR (KBr) ν_{max} 3396, 2967, 2926, 1672, 1625, 1603, 1552, 1487, 1453, 1385, 1361, 1272, 1244, 1194, 1134, 1118, 1072, 1039, 926, 913, 865, 849, 801, 784, 754, 657, 625, 584, 563, 485 cm^{-1}, ^{1}H-NMR spectral data see Table 1, ^{13}C NMR data see Table 3.

*3.3.2. R-(−)-Skyrin-6-O-β-glucopyranoside (**2**)*

Red-orange amorphous powder (29.8 mg), melting point > 300°C, FABMS (negative) *m/z* 699 $[M-H]^-$, 722 $[M+Na-H]^-$, 537 $[M-C_6O_5H_{11}]^-$, 519 $[M-C_6O_5H_{11}-H_2O]^-$, ESIMS (negative) *m/z* 699 $[M-H]^-$, UV-Vis (MeOH) λ_{max} (log ε): 222 (4.59), 258 (4.54), 296 (4.28), 455 (4.14) nm, CD (MeOH) λ_{max} ($\Delta\varepsilon$): 309 (+9.02), 263 (−40.94), 248 (+12.90) nm, IR (KBr) ν_{max} identical to compound **1**, ^{1}H NMR spectral data see Table 1, ^{13}C NMR data see Table 3.

*3.3.3. R-(−)-Skyrin-6-O-β-xylopyranoside (**3**)*

Red-orange amorphous powder (3.4 mg), ESIMS (negative) *m/z* 669 $[M-H]^-$, UV–Vis (MeOH) λ_{max} (log ε): 219 (4.41), 258 (4.37), 294 (4.10), 456 (3.87) nm, CD (MeOH) λ_{max} ($\Delta\varepsilon$): 310 (−5.42), 264 (+23.28), 251 (−7.25) nm, ^{1}H NMR spectral data see Table 1, ^{13}C NMR data in see Table 3, ^{13}C NMR spectral data of the β-xylopyranose moiety of **3** in pyridine-d_5: δ 105.2 (C-1″), 74.7 (C-2″), 77.7 (C-3″), 70.3 (C-4″), 67.4 (C-5″).

*3.3.4. R-(−)-Skyrin-6-O-α-arabinofuranoside (**4**)*

Red-orange amorphous powder (7.3 mg), ESIMS (negative) *m/z* 669 $[M-H]^-$, UV–Vis (MeOH) λ_{max} (log ε): 219 (4.44), 258 (4.39), 294 (4.15), 459 (3.92) nm, CD (MeOH) λ_{max} ($\Delta\varepsilon$): 310 (−7.82), 263 (+25.57), 250 (−7.71), ^{1}H-NMR spectral data see Table 1, ^{13}C NMR data see Table 3.

*3.4. Acetylation of compounds **1** and **2***

Anhydrous pyridine (0.5 ml), **1** (3.2 mg) and acetic anhydride (0.5 ml) were kept in the dark at room temperature for 18 h. The reaction mixture was diluted with 2 ml H_2O and stored at 8°C for 1 h. It was then applied to a Sep-Pak® tC18 cartridge, which had been washed with 10 ml of MeOH and pre-conditioned with 10 ml H_2O. Pyridine, acetic anhydride and not acetylated **1** were eluted with H_2O, followed by the elution of the acetylated compound with $CHCl_3$. Evaporation of $CHCl_3$ under reduced pressure below 30°C left 3.1 mg of **1a** (1,1′,8,8′,2″,3″,4″,6″-octaacetyl-*S*-(+)-skyrin-6-*O*-β-glucopyranoside). **2** (6.5 mg) was treated as described above. As the reaction product of **2** was not homogenous, acetylation was repeated followed by a chromatography on a silica gel column (320×10 mm internal diameter) with benzene–acetone (9:1) for further purification. This led to the isolation of 3.5 mg pure compound **2a** (1,1′,8,8′,2″,3″,4″,6″-octaacetyl-*R*-(−)-skyrin-6-*O*-β-glucopyranoside).

*3.4.1. Compound **1a** (1,1′,8,8′,2″,3″,4″,6″-octaacetyl-S-(+)-skyrin-6-O-β-glucopyranoside)*

FABMS (positive) *m/z* 1038 $[M+2H]^+$, ^{1}H NMR spectral data see Table 2, ^{13}C NMR data see Table 3.

*3.4.2. Compound **2a** (1,1′,8,8′,2″,3″,4″,6″-octaacetyl-R-(−)-skyrin-6-O-β-glucopyranoside)*

FABMS (positive) *m/z* 1038 $[M+2H]^+$, ^{1}H NMR spectral data see Table 2, ^{13}C NMR data see Table 3.

*3.5. Hydrolysis of **1–3** on a TLC plate*

Methanolic solutions of **1**–**3** were applied on a silica gel plate together with the reference substance glucose and xylose. The TLC plate was kept in a double TLC chamber saturated with hydrochloric acid gas for 10 min at 100°C. The plate was cooled down for 30 min in the chamber, dried for 60 min at room temperature and then 20 min at 80°C. The plate was developed in EtOAc–MeOH–CH_3COOH–H_2O (60:15:15:10) over 8 cm. Detection was done with 0.5 g thymol and 5 ml H_2SO_4 in 95 ml EtOH. After spraying the plate was heated for 10 min at 120°C.

3.6. Binding studies with the corticotropin releasing hormone-1 (CRH-1) receptor

Cloning and expression of the CRH-1 receptor was performed according to Gottowik et al. (1997).

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