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The colleflaccinosides, two chiral bianthraquinone glycosides with antitumor activity from the lichen *Collema flaccidum* collected in Israel and Russia

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Colleflaccinosides A and B, two chiral bianthraquinone glycosides from the two geographical varieties of lichen *Collema flaccidum* collected in Russia and Israel have been isolated as new natural products. Their structures were elucidated using UV, CD, IR, MS, 1D and 2D NMR spectral data, and chemical degradation. The colleflaccinosides B had significant antitumor activity in the crown gall tumor inhibition test.

Keywords: *Collema flaccidum*; Jelly lichen; Israel; Russia; Bianthraquinone glycosides; Colleflaccinosides A and B; Antitumor activity; Facility Mayak

1. Introduction

The chemistry of *Collema* is poorly studied, as are its biological activities. The scytonemin and mycosporine–glycine were identified in *C. coccophorum* [1], carotenoids in different *Collema* sp. [2], and the mannitol and 3-*O*-β-D-glucopyranosyl-D-mannitol were isolated from *C. leptosporum* [3]. Recently, collemin A was obtained from *C. cristatum* [4].

Anthraquinones are widely distributed pigments mainly in higher plants [5], fungi [6], and lichens [7]. Rhodocladonic acid was identified in lichen *Cladonia* sp. [8]; it is also the intermediate of synthetic product [9], i.e., paeciloquinone C. This compound, the common anthraquinone structurally comparable to our monomer compound, was also isolated from fungus *Paecilomyces carneus* [10]. Further anthraquinones with unusual isoprenyl (geranyl) chain(s) were isolated and identified in different plants [11] or synthesized [12,13]. On the contrary, vismiaquinone, the anthraquinone with isopentenyl side chain, was isolated from the leaves of *Vismia reichardtiana* [14].

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Whereas the 7,7′-coupled dimers are the most widespread group, the 5,5′- and 7,10′-dimers and the 5,10′-dimers are less common, being found among only a few species. Two atropisomeric forms of bianthraquinones have been recorded previously [15,16] but the stereochemistries in many further compounds are not known [17,18]. In general terms one can say that authors who measured high optical rotation, which were in many cases in hundreds of degrees, did not pay adequate attention to biphenyl stereochemistry.

The dimeric compound, 7,7′-bichrysophanol, was detected in soils [19,20]. The 7,7′-biphyscion, a dimer anthraquinone, also occurs in soil samples from Japan [21] and has been isolated from two toadstools [22]. Recently, cassiamin A and B were identified from *Smilax perfolata*, unfortunately again without their chirality. However, the optical rotation e.g., of cassiamin A was −193° [23].

In the course of continuous studies on lichens [24,25], new anthraquinone glycosides, colleflaccinosides A and B were isolated and their structures were elucidated using UV, CD, IR, MS, 1D and 2D NMR spectral data, and chemical degradation.

2. Results and discussion

A sample 761 g of *Collema flaccidum* (Ach.) Ach., jelly lichen, collected in September 2003, on Mount Carmel near Haifa, Israel, and 624 g of *C. flaccidum* collected in August 2001, Ural Mountains, Muslumovo Village, 50 km west from Chelyabinsk, Russia, were extracted separately by butanol and subsequently separated on Sephadex LH-20 columns. The fractions were further purified by RP-HPLC to give two glycosides, 13.6 mg of colleflaccinoside A (**1**) from Israel's lichen and 8.2 mg of colleflaccinoside B (**4**) from Russian lichen (see figure 1) which were identified by IR, UV, CD, optical rotation, MS, and ^{1}H and ^{13}C-NMR spectral data, and chemical degradation.

The colleflaccinoside A was isolated as an orange-red microcrystalline powder from *C. flaccidum* collected in Russia. The UV–Vis spectrum recorded in neutral ethanol showed the characteristic anthraquinonoid bands of 1,8-dihydroxy-substituted anthraquinones at 291 and 433 nm as well as two benzenoid bands at 262 and 369 nm [5]. In alkaline ethanol, the visible quinonoid band was shifted to 530 nm, characteristic of 1,8-dihydroxy-substituted anthraquinone nuclei. Further, IR absorption bands at 3469 (broad, OH stretch), 1667 (C=O stretch, non-chelated), and 1620 cm^{-1} (C=O stretch, chelated) spectra also suggested 1,8-dihydroxyanthraquinone chromophore [26], which was further supported by its ^{13}C NMR spectrum with signals at δ 186.3 and 182.3 for quinone carbonyl carbons.

The HRFABMS showed a $[M+Na]^+$ peak at m/z 951.2692 corresponding to the molecular formula $C_{48}H_{48}O_{19}$, deduced also by ^{13}C NMR and DEPT analyses. The negative-ion FABMS showed an $[M-H]^-$ ion at m/z 927 and fragment ions at m/z 765 and 747 corresponding to loss of $C_6H_{10}O_5$ ([M − H-dehydrated hexose]), and $C_6H_{12}O_6$ ([M − H-hexose]), respectively, from the [M − H] ion.

The ^{1}H and ^{13}C NMR data (table 1) revealed a glycoside structure for **1**. The signals at δ 4.86 (d, $J=7.9$ Hz) and 100.8 (d) were assignable to C-1‴ of the glucopyranose moiety. Signals at δ 4.01–4.35 (6H) and δ 76.0, 78.5, 71.9, 76.9, and 62.6 were in good agreement with those of the D-glucopyranosyl moiety. Based on the J value 7.9 Hz of the doublet of H-1‴, the anomeric configuration of glucose was β. In order to locate

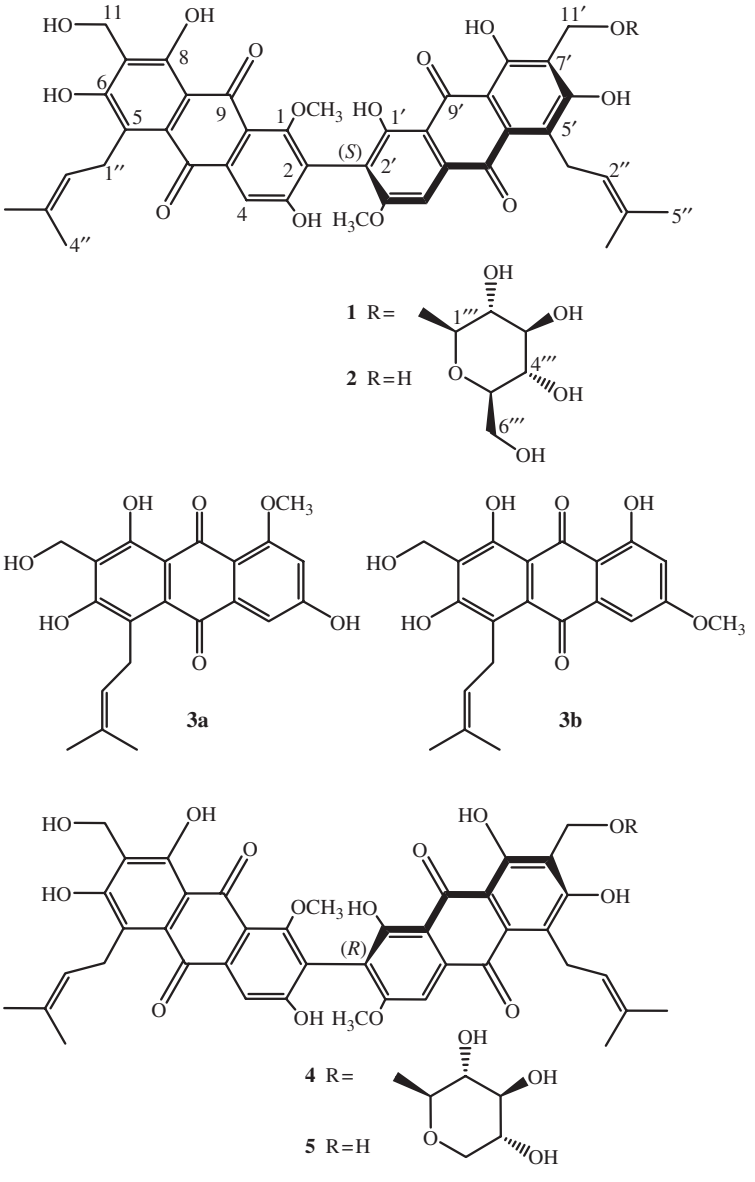

Figure 1. Structures of colleflaccinosides A and B (**1**, **4**), colleflaccines A and B (**2**, **5**) and reduction products 2,4,7-trihydroxy-3-hydroxymethyl-5-methoxy-1-(3′-methyl-but-2′-enyl)-anthraquinone (**3a**) and 2,4,5-trihydroxy-3-hydroxymethyl-7-methoxy-1-(3′-methyl-but-2′-enyl)-anthraquinone (**3b**).

the glucosylated hydroxy group (OH-1′, OH-3, OH-6, OH-6′, OH-8, OH-8′, OH-11, or OH-11′) the ^{13}C NMR data of **1** were compared with those of the reference compounds, described in literature i.e., positions 1 and 8 [27], position 6 [28], and positions 3 and 11 [29], where the position direct bound (without methylene) were excluded on the basis of the chemical shifts. The glucopyranose moiety was determined to be O-linked at position 11′ due to the downfield shift of this carbon signal to δ 58.1 ($\Delta + 0.4$)

Table 1. ^{1}H and ^{13}C NMR data of colleflaccinoside A (**1**) and colleflaccinoside B (**4**).

No.	^{1}H of **1**	^{13}C of **1**	^{1}H of **4**	^{13}C of **4**
1a	–	112.3	–	112.3
1a′	–	113.8	–	113.8
1	–	161.7	–	161.7
1′	–	160.4	–	160.4
2, 2′	–	121.5	–	121.5
3	–	161.9	–	161.9
3′	–	163.6	–	163.6
4	7.01 (1H, s)	109.4	7.01 (1H, s)	109.4
4′	7.09 (1H, s)	108.0	7.09 (1H, s)	108.0
4a, 4a′	–	135.6	–	135.6
5a	–	137.1	–	137.1
5a′	–	138.4	–	138.4
5	–	114.2	–	114.2
5′	–	115.6	–	115.6
6	–	161.2	–	161.2
6′	–	161.9	–	161.9
7	–	115.7	–	115.7
7′	–	112.6	–	112.6
8	–	159.7	–	159.7
8′	–	160.1	–	160.1
8a	–	110.3	–	110.3
8a′	–	109.7	–	109.7
9, 9′	–	186.3	–	186.3
10, 10′	–	182.3	–	182.3
11	4.54 (2H, s)	57.7	4.54 (2H, s)	57.7
11′	4.65 (1H, d, $J=16.0$) 4.73 (1H, d, $J=16.0$)	58.1	4.65 (1H, d, $J=16.0$) 4.73 (1H, d, $J=16.0$)	58.1
1″	4.05 (1H, dd, $J=15.8$, 6.1) 4.30 (1H, dd, $J=15.8$, 6.1)	29.7	4.05 (1H, dd, $J=15.8$, 6.1) 4.30 (1H, dd, $J=15.8$, 6.1)	29.7
2″	5.13 (1H, t, $J=6.1$)	118.8	5.13 (1H, t, $J=6.1$)	118.8
3″	–	135.6	–	135.6
4″	1.84 (3H, s)	23.4	1.84 (3H, s)	23.4
5″	1.72 (3H, s)	18.3	1.72 (3H, s)	18.3
1-OMe	3.71 (3H, s)	55.7	3.71 (3H, s)	55.7
3′-OMe	3.74 (3H, s)	55.8	3.74 (3H, s)	55.8
1‴	4.86 (1H, d, $J=7.9$)	100.8	4.93 (1H, d, $J=7.5$)	101.4
2‴	4.01 (1H, dd, $J=8.7$, 7.9)	76.0	4.08 (1H, dd, $J=8.5$, 7.5)	72.9
3‴	4.15 (1H, t, $J=8.7$)	78.5	4.16 (1H, t, $J=8.5$)	76.5
4‴	4.11 (1H, dd, $J=8.8$, 8.7)	71.9	4.15 (1H, m)	69.2
5‴	4.03 (1H, m)	76.9	4.27 (1H, dd, $J=11.0$, 5.1) 3.67 (1H, dd, $J=11.0$, 9.8)	65.9
6‴	4.23 (1H, dd, $J=10.8$, 5.5) 4.35 (1H, dd, $J=10.8$, 1.7)	62.6	–	–

1 = 1′-OH 12.84, 8′-OH 12.15, 8-OH 11.42, 6-OH 10.15, 6′-OH 10.11.

and the change in the splitting pattern of the attached protons. While the enantiotopic C-11 protons of aglycone colleflaccine A (**2**) (δ 4.54, 2H) (table 2) appeared as a singlet, the diastereotopic C-11′ protons of compound **1** (δ 4.65, 1H and 4.73, 1H) were each a doublet ($J=16.0$ Hz). An HMBC correlation between 1‴-H (δ 4.86) of the glucose unit and the aglycone (δ 58.1, C-11′) can be observed (figure 2). Further evidence in support of the composition of compound **1** was obtained from enzymatic (β-D-glucosidase) hydrolysis that yielded aglycone **2** and D-glucopyranose based on co-TLC with authentic sugar samples. Furthermore, the δ of the anomeric proton of **1** was shifted to δ 4.86, which also strongly suggested that C-1‴ was linked to alcoholic

Table 2. ^{1}H and ^{13}C NMR data of colleflaccine A (**2**) and colleflaccine B (**5**).

	^{1}H of **2** or **5**	^{13}C of **2** or **5**
1a	–	112.3
1a′	–	113.8
1	–	160.8
1′	–	160.4
2, 2′	–	121.5
3	–	161.9
3′	–	163.6
4	7.01 (1H, s)	109.4
4′	7.09 (1H, s)	108.0
4a, 4a′	–	135.6
5a	–	137.1
5a′	–	138.4
5	–	114.2
5′	–	115.6
6	–	161.2
6′	–	161.9
7	–	115.7
7′	–	112.6
8	–	159.7
8′	–	160.1
8a	–	110.3
8a′	–	109.7
9, 9′	–	186.3
10, 10′	–	182.3
11, 11′	4.54 (2H, s)	57.7
1″	4.05 (1H, dd, $J=15.8, 6.1$) 4.30 (1H, dd, $J=15.8, 6.1$)	29.7
2″	5.13 (1H, t, $J=6.1$)	118.8
3″	–	135.6
4″	1.84 (3H, s)	23.4
5″	1.72 (3H, s)	18.3
OMe	3.71 (6H, s)	55.7

oxygen rather than a phenolic one [30]. The above data confirmed that OH-11′ was glycosylated and that all phenolic hydroxyls are free.

Beside the signals for the aglycone moiety, the ^{1}H NMR spectral data revealed signals for two methoxyl groups at δ 3.71 and 3.74 (each 3H, s), three *peri* hydroxyl protons at δ 12.84, 12.15, and 11.42 (each 1H, br s), as well as only two aromatic protons, both singlets. The ^{13}C NMR spectrum (table 1) showed 39 carbon signals (thereof nine duplicate) and the DEPT spectrum demonstrated the presence of six methyls, five methylenes, nine methines, and 28 quaternary carbons in which there were four carbonyls. In the aromatic region, signals in the ^{1}H NMR spectrum for an isolated aromatic proton at δ 7.01 (1H, s, H-4) and 7.09 (1H, s, H-4′) were observed. Two sets of resonances indicated the presence of 3″-methyl-2″-butenyl (isoprenyl) groups. The HMBC spectrum clearly demonstrated the ^{3}J correlations of H-1″ to C-6, C-5a, and C-2″; hydroxy proton at C-6′ to C-7′. These results indicated that two isoprenyl groups were connected to C-5 of ring A and C-5′ of ring C′. The NOESY spectral data also supported this substitution pattern for compound **3b** as shown in figure 2.

The structure of bianthrachinone (**2**) was confirmed by reductive cleavage of the natural product **2** with alkaline sodium dithionite. The reaction mixture so obtained

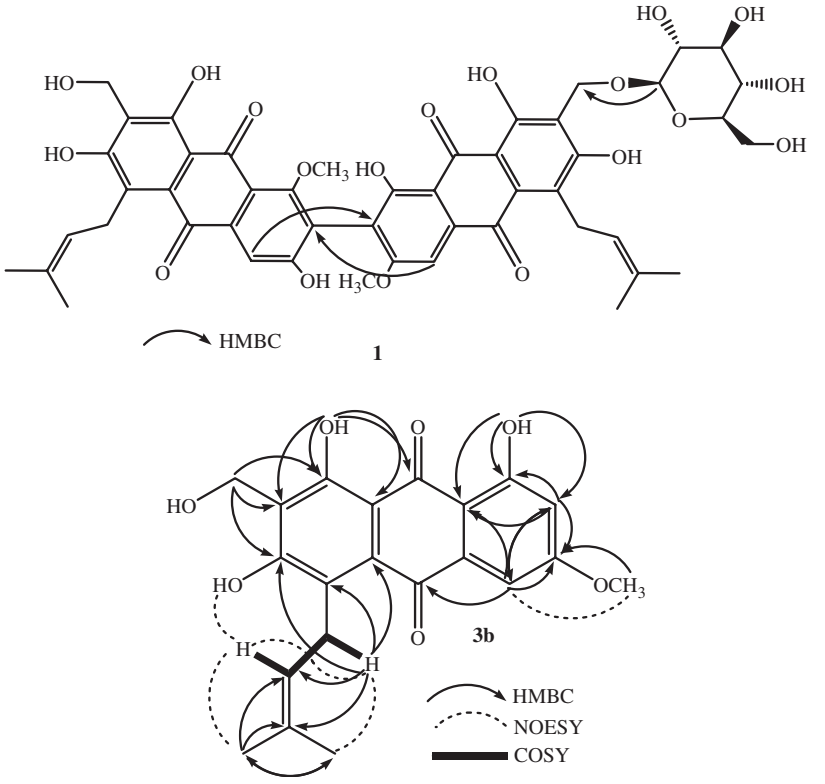

Figure 2. HMBC, ^{1}H–^{1}H COSY, and NOE correlations of compounds **1** and **3b**.

was purified by TLC to afford compounds **3a** and **3b**, which were identified by spectroscopic comparison (see table 3).

The location at C-2/C-2′ of the biaryl bond in **1** followed from (i) the absence of a signal arising from H-2, H-2′ and the appearance of H-4, H-4′ as a singlet, (ii) the characteristic upfield shift of H-4 from δ 7.25 in the spectrum of anthraquinones (**3a** and **3b**) to δ 7.01 and/or δ 7.09 in the spectrum of **1** due to *meta* arylation, and (iii) a small upfield shift for OMe probably due to the twisted conformation of the dimer which would result in the shielding of these protons by the other anthraquinone moiety.

Thus, the ^{1}H and ^{13}C assignments of **1** were made by a combination of COSY, HMBC, and NOESY experiments and by comparison with the degradation product. Therefore, the structure of **1** was assigned to be 3,6,8,1′,6′,8′-hexahydroxy-7-hydroxymethyl-1,3′-dimethoxy-5,5′-bis-(3′′-methyl-but-2′′-enyl)-7′-β-D-glucopyranosyloxymethyl-[2,2′]bianthracenyl-9,10,9′,10′-tetraone.

Colleflaccinoside B (**4**) was obtained as an amorphous orange-red powder from *C. flaccidum* collected in Israel with a molecular formula $C_{47}H_{46}O_{18}$, as determined by the positive-ion HRFABMS; m/z 921.2586 $[M+Na]^{+}$. The spectral data of **4** were essentially analogous with those of **1** and suggestive of a bianthraquinone glycoside of the same type. However, slight differences were recognized in the signals from the sugar moiety. The signal of the anomeric proton at δ 4.93 (d, $J = 7.5$ Hz) was shown to be associated with the five sugar carbon signals at δ 101.4 (d), 72.9 (d), 76.5 (d), 69.2

Table 3. ^{1}H and ^{13}C NMR data of compounds **3a** and **3b**.

	^{1}H of **3a**	^{13}C of **3a**	^{1}H of **3b**	^{13}C of **3b**
1a	–	112.3	–	114.1
1	–	161.4	–	161.6
2	6.63 (1H, d, $J=2.0$)	114.6	6.63 (1H, d, $J=2.0$)	114.6
3	–	161.5	–	163.8
4	7.25 (1H, d, $J=2.0$)	109.4	7.18 (1H, d, $J=2.0$)	106.7
4a	–	135.6	–	135.1
5a	–	137.1	–	137.7
5	–	114.2	–	114.6
6	–	161.6	–	161.2
7	–	115.9	–	115.7
8	–	159.2	–	159.7
8a	–	111.5	–	110.3
9	–	186.9	–	186.3
10	–	182.8	–	182.3
11	4.55 (2H, s)	57.3	4.55 (2H, s)	57.7
1′	4.17 (1H, d, $J=6.1$)	29.1	4.17 (1H, d, $J=6.1$)	29.7
2′	5.12 (1H, t, $J=6.1$)	119.3	5.12 (1H, t, $J=6.1$)	118.2
3′	–	135.1	–	135.9
4′	1.84 (3H, s)	23.8	1.84 (3H, s)	23.0
5′	1.69 (3H, s)	18.5	1.69 (3H, s)	18.1
OMe	3.98 (3H, s)	55.2	3.94 (3H, s)	55.9

(d), and 65.9 (t), which were consistent with the presence of a β-D-xylopyranosyl unit of **4** [31]. Enzymatic hydrolysis of **4** with xylosidase (EC 3.2.1.37) liberated a genuine aglycon colleflaccine B (**5**), identified as 3,6,8,1′,6′,8′-hexahydroxy-7,7′-bis-hydroxymethyl-1,3′-dimethoxy-5,5′-bis-(3″-methyl-but-2″-enyl)-[2,2′]bianthracenyl-9,10,9′,10′-tetraone by the optical rotation, CD, MS, and ^{1}H and ^{13}C NMR data (see section 3 and table 1), and D-xylose. Long-range correlations from the signals due to the anomeric proton of the xylosyl moiety at δ 4.93 and the H-11′ protons at δ 4.65 and/or 4.73 to the carbon signal (C-11′) at δ 58.1 in the HMBC spectrum indicated the location of the xylose at C-11′. Thus, the structure of **4** was shown to be 3,6,8,1′,6′,8′-hexahydroxy-7-hydroxymethyl-1,3′-dimethoxy-5,5′-bis-(3″-methyl-but-2″-enyl)-7′-β-D-xylopyranosyloxymethyl-[2,2′]bianthracenyl-9,10,9′,10′-tetraone.

The shielding of ca $\sim$0.25 ppm observed for 1,3′ methoxyl hydrogens in **2** may be due to the twisted conformation of the dimer about the 2,2′ bond, which would place them in the shielding zone of the other anthraquinone moiety. Models of bianthraquinones (**2** and **5**) showed (figure 3) that rotation about the 2,2′ bond is hindered, which should give rise to the biphenyl type chirality. This is confirmed by the optical activity of both the compounds (see section 3).

The very strong CD-values show clearly that they are derived from the typical CD-coupling between the two practically identical achiral chromophores. At the longest wavelength such a CD-couplet can be found with maxima at 435 nm (ε 5.3) and 395 nm ($\varepsilon+6.8$). Cotton effects due to the long axis of the two anthraquinone moieties showed that the two long axes of the anthraquinone nuclei in **2** are twisted in an anticlockwise manner. The exciton theory when applied to this system gives a positive CD-couplet for P-helicity according to Cahn–Ingold–Prelog nomenclature [32,33].

Hence, the difference between compounds **2** and **5** was evident only in the stereochemistry. This was confirmed from the CD spectrum, in which opposite Cotton

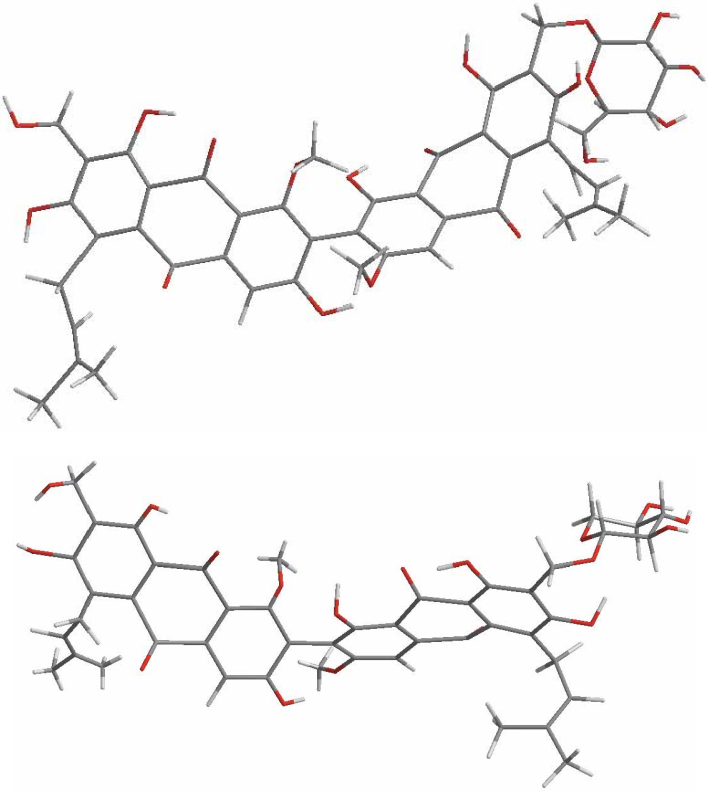

Figure 3. Models of bianthraquinones – colleflaccinoside A (**1**) and colleflaccinoside B (**4**).

effects allowed the absolute configuration of compound **2** to be proposed as *S* instead of *R* as in compound **5**. Therefore, the aglycone (**2**) of glycoside (**1**) is atropisomer of aglycone (**5**) liberated from glycoside (**4**), as shown in figure 1.

Unfortunately, the values of both optical rotations are not identical and also the CD spectra demonstrated a moderate difference. Accordingly, we suppose that the aglycone (**5**) is a mixture of two atropisomers, which are not separable at the present time. Identification of an anisochiral mixture (approximately in the ratio 3 : 1) of two enantiomers flavomannin was described previously [34].

A number of anthraquinones and bianthrones have been shown to possess antiviral activities [35]. Unfortunately only in one paper [36] the antiviral activity of skyrin (bianthraquinone) was determined but it was inactive. Further, paper [37] described that anthraquinone monomers showed higher anti-cancer activity than bianthraquinones. The cytotoxic activities between different phenylanthraquinones were described [38]. Compounds with *P* biaryl bond and the sugar moiety at C-4′ did not show apparent cytotoxicity but compounds with the *M* biaryl bond and the sugar moiety at C-6′ exhibited cytotoxicity. The various diastereoisomers of the bianthrones were observed [39] to be significantly active but did not differ very much from each other in terms of cytotoxic potency. Meso bianthrone was active, whereas its isomer was six times less active. Consequently, a careful determination of the relative configuration of the dimers being impossible, it was also not possible to correlate

Table 4. Bioactivities of colleflaccinoside A and B (**1** and **4**).

Test organism	**1**	**4**
S. aureus[a]	0	0
B. subtilis[a]	0	0
E. coli[a]	0	0
S. cerevisiae[a]	0	0
Agrobacterium tumefaciens[b,c]	7 ± 3[d]	84 ± 6

[a] Samples (10 μg) were applied on 50.8 mm paper disks, values are diameters (mm) of inhibitory zones.
[b] The details in section 3.
[c] Presented values are means of three determinations.
[d] % of crown gall tumor inhibition (±SD).

the weak differences of activity with stereochemistry at C-10 and C-10′. In the paper of El-Gamal *et al*. [40] it is mentioned that the free anthraquinones showed stronger activity than the anthraquinone glycosides. The cytotoxicity and anticancer activity of anthraquinone glycosides was published [41]. Interaction between oligonucleotide and anthraquinone derivatives was proved by shielding protons and 2D NOESY spectrum. From the above-mentioned text it is obvious that the information about cytotoxicity of anthraquinones is very confused.

According to these suggestions, we discovered two new chiral bianthraquinone glycosides, namely colleflaccinosides A and B, (**1**) and (**4**), which show no activity against different microorganisms (see table 4).

The crown gall tumor inhibition test has been used for the active antitumor agents produced *in vivo* by organisms and is also used to evaluate extracts for different pharmacological activities. The isolated compounds were evaluated by their ability to inhibit the growth of crown gall tumors on potato discs inoculated with *Agrobacterium tumefaciens* carrying a tumor-inducing plasmid. Both compounds showed inhibition of the growth of crown gall tumors on potato disks, suggestive of *in vivo* antitumor activity. All the extracts assayed demonstrated crown gall tumor inhibition, having 7% for compound **1** and 84% for compound **4**. Different antitumor activities of our compounds get viewed in margin spatial interactions of aromatic rings of anthraquinone glycosides and DNA.

A region around Chelyabinsk in the Southern Urals has been heavily contaminated due to operational and accidental releases from the first Soviet plutonium production facility Mayak. Environmental samples from this area were affected by significant radioactive releases into the Techa River, which started in 1948, and by fallout from the explosion of a fission product storage tank in 1957. Soil, sediment, and water samples were contained ^{90}Sr (37,000 Bq kg^{-1}), ^{137}Cs (5600 Bq kg^{-1}), and plutonium (9.9 Bq kg^{-1}) [42]. The highest contamination was found in the vicinity of the Techa River around Muslumovo village. It therefore remains to be established whether the unique radioactive environment, where *C. flaccidum* was grown might have some bearing on the ability of this lichen to biosynthesize opposite chiral isomers of scarce lichen pigments.

Regioselectivity was clearly observed in this article, as the lichen from the Ural Mountains was capable of generating P-anthraquinone (**1**), while the lichen from Mount Carmel near Haifa biosynthesized the M-atropisomer (**4**). Colleflaccinoside A and B are new bianthraquinone glycosides, and this is the first report on the isolation of the bianthraquinone derivatives from genus *Collema*.

3. Experimental

UV–Vis spectra were measured in MeOH within the range of 220 to 550 nm in a Cary 118 (Varian) apparatus. A Perkin-Elmer (Perkin-Elmer, Norwalk, CT, USA) model 1310 IR spectrophotometer was used for scanning IR spectroscopy as neat films. Circular dichroism (CD) measurement was carried out under dry N_2 on a Jasco-500A spectropolarimeter at 24°C. A Perkin-Elmer Model 1310 (Perkin-Elmer, Norwalk, CT, USA) IR spectroscope was used. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H), 125.7 MHz (^{13}C). High- and also low-resolution MS were recorded using a VG 7070E-HF spectrometer (70 eV). HRFABMS (positive and/or negative ion mode) were obtained with a PEG-400 matrix. All chemicals were purchased from Sigma-Aldrich.

The lichenized ascomycetes *C. flaccidum* (Ach.) Ach. (Collemataceae; Lecanorales), jelly lichen, collected in September 2003 on Mount Carmel near Haifa, Israel, and 624 g of *C. flaccidum* collected in August 2001, Ural Mountains, Muslumovo Village, 50 km west from Chelyabinsk, Russia were further used. The voucher specimens are deposited in the collection of the second author.

Lichens were separately extracted by butanol (triplicate) and the extracts were further chromatographed by means of the Sephadex LH-20 columns with chloroform–methanol 1 : 2 and then separated by RP-HPLC on a Discovery C18 column (Supelco) particle size 5 μm, length × I.D. (250 mm × 21.2 mm) using a linear gradient from 10% H_2O and 90% acetonitrile to 10% water and 90% acetonitrile over 50 min, with a flow rate of 9.0 mL min^{-1} and monitored by a variable wavelength detector at 450 nm. The yields were 13.6 mg of compound **1** and 8.2 mg of compound **4** in the crude extracts.

Compound **1** (11.1 mg) was subjected to enzymatic hydrolysis with 40 mg β-D-glucosidase (EC 3.2.1.21) in AcOH–AcONa buffer (pH 5.0, 10 mL) at room temperature for 20 h. The reaction mixture was passed through a combination of a Sep-Pak C18 cartridge eluting with 20% MeOH and followed by MeOH. The MeOH eluted fraction was purified by silica gel chromatography eluting with $CHCl_3$–MeOH (9 : 1) to yield **2** (8.9 mg). The 20% MeOH eluted fraction containing D-glucose was also analyzed by direct TLC comparison and optical rotation with an authentic sample: R_f 0.30 (*n*-PrOH–H_2O, 85 : 15).

Compound **4** (5.7 mg) was hydrolyzed by β-xylosidase (EC 3.2.1.37) (35.0 mg) and processed as above. Yield of **5** was 3.4 mg. The 20% MeOH eluted fraction containing D-xylose (R_f 0.39).

A mixture of compound **2** (2 mg), 1N NaOH (2 mL), and $Na_2S_2O_4$ (0.1 g) was heated at 70°C for 20 min. After extraction with $CHCl_3$, followed by acidification with HCl, the $CHCl_3$ layer was evaporated *in vacuo*. The residue was chromatographed on a TLC plate by developing with petroleum diethylether–EtOAc–H_2O (72.5 : 24 : 3.5). The major bands were identified as compounds **3b** and **3a** by NMR and MS data.

Colleflaccinoside A (1). Yield 13.6 mg, orange-red microcrystalline powder; $[\alpha]_D^{23}$ −255 ($c = 0.09$, MeOH); UV (MeOH) λ_{max} (log ε) 291 (4.24), 262 (4.15), 369 (3.56), 433 (3.08) nm; UV (alkaline MeOH) λ_{max} (log ε) 530 (4.0) nm; IR (film) ν_{max} 3490–3400 (OH), 1667 (C=O stretch, non-chelated), 1620 (C=O stretch, chelated) cm^{-1}; CD

(MeOH) λ_{ext} nm ($\Delta\varepsilon$) 272 (−8.7), 295 (+6.9), 315 (−4.1), 395 (+6.8), 435 (−5.3); HRFABMS *m/z* 951.2692 $C_{48}H_{48}O_{19}$ $[M+Na]^+$, calculated for $[C_{48}H_{48}O_{19}+Na]^+$ 951.2687; negative-ion LRFABMS *m/z* 927 $[M-H]^-$, *m/z* 765 $[M–H–C_6H_{11}O_5]^-$, *m/z* 747 $[M–H–C_6H_{11}O_5–H_2O]^-$; ^1H and ^{13}C NMR data, see table 1.

Colleflaccinoside B (4). Yield 8.2 mg, amorphous orange-red powder; $[\alpha]_D^{23}$ +192 ($c = 0.08$, MeOH); UV (MeOH) λ_{max} (log ε) 290 (4.20), 265 (4.18), 370 (3.60), 435 (3.10) nm; UV (alkaline MeOH) λ_{max} 525 (3.9) nm; IR (film) ν_{max} 3490–3400 (OH), 1665 (C=O stretch, non-chelated), 1622 (C=O stretch, chelated) cm^{-1}; CD (MeOH) λ_{ext} nm ($\Delta\varepsilon$) 270 (+5.9), 293 (−4.7), 318 (+2.8), 397 (−4.2), 436 (+3.4); HRFABMS *m/z* 921.2586 $C_{47}H_{46}O_{18}$ $[M+Na]^+$, calculated for $[C_{47}H_{46}O_{18}+Na]^+$ 921.2581; negative-ion LRFABMS *m/z* 897 $[M-H]^-$, *m/z* 765 $[M–H–C_5H_9O_4–H_2O]^-$; ^1H and ^{13}C NMR data, see table 1.

Colleflaccine A (2). Yield 8.9 mg, orange-red crystals; $[\alpha]_D^{23}$ −247 ($c = 0.10$, MeOH); UV (MeOH) λ_{max} (log ε) 290 (4.15), 261 (4.08), 366 (3.51), 434 (3.12) nm; IR (film) ν_{max} 3490–3400 (OH), 1665 (C=O stretch, non-chelated), 1621 (C=O stretch, chelated) cm^{-1}; CD (MeOH) λ_{ext} nm ($\Delta\varepsilon$) 271 (−8.6), 292 (+6.7), 316 (−4.2), 393 (+5.7), 435 (−4.9); HRFABMS *m/z* 789.2159 $C_{42}H_{38}O_{14}$ $[M+Na]^+$, calculated for $[C_{40}H_{34}O_{14}+Na]^+$ 789.2159; ^1H and ^{13}C NMR data, see table 2.

Colleflaccine B (5). Yield 3.4 mg, orange-red crystals; $[\alpha]_D^{23}$ +184 ($c = 0.011$, MeOH); CD (MeOH) λ_{ext} nm ($\Delta\varepsilon$) 273 (+5.8), 294 (−4.6), 316 (+2.9), 397 (−4.3), 435 (+3.3); the other spectral data were identical with compound **2**.

Compound 3a. HRFABMS *m/z* 407.1111 $C_{21}H_{20}O_7$ $[M+Na]^+$, calculated for $[C_{21}H_{20}O_7+Na]^+$ 407.1107; ^1H and ^{13}C NMR data, see table 3.

Compound 3b. HRFABMS *m/z* 407.1109 $C_{21}H_{20}O_7$ $[M+Na]^+$, calculated for $[C_{21}H_{20}O_7+Na]^+$ 407.1107; ^1H and ^{13}C NMR data, see table 3.

The test organisms were *Bacillus subtilis*, *Staphyloccocus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae* (Czechoslovak Collection of Microorganisms, Brno). Antibacterial assays were carried out according to the literature [31]. The amounts used were 50 μg of compound per test disk (see table 4).

The *Agrobacterium tumefaciens* potato disc assay for tumor/antitumor induction was performed according to the procedure described in literature [43]. The potatoes were sterilized by immersion in ethanol 70% for 2 min and in 50% sodium hypochlorite solution (active chlorine 30 g L^{-1}) for 30 min. Then, the potatoes were rinsed several times with sterilized distilled water, in the laminar flow hood. A core of tissue was extracted from each tuber with a sterilized 1.5 cm cork borer. Discs of 0.5 cm were cut with a scalpel. The potato discs were placed in 1.5% agar Petri dishes. To each potato disc was applied 0.05 mL of a solution containing 2 mL of a broth culture of *A. tumefaciens* (48 h culture of ca 109 cells mL^{-1}), 1.5 mL of sterile H_2O and 0.5 mL of the solution test extract (8 mg of extract in 2 mL of DMSO filtered through 0.22 mm filters). Control discs were prepared with sterile DMSO instead of test extract. A minimum of three Petri dishes (5 disks/dish) ($n = 15$–25) were used for each test compound and the control. Following preparation, the Petri dishes were placed in an incubator at 27°C for 12–21 days. To determine the number of tumors, the potato discs were stained with a solution of I_2 (1 g) and KI (2 g) in 300 mL distilled H_2O.

Significant activity was indicated when two independent assays gave 20% or greater inhibition.

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