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The tornabeatins, four tetrahydro-2-furanone derivatives from the lichenized ascomycete *Tornabea scutellifera* (With.) J.R. Laundon

Tomáš Řezanka [a,*], Marina Temina [b], Lumír Hanuš [c], Valery M. Dembitsky [d]

[a] *Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague, Czech Republic*
[b] *Biodiversity and Biotechnology Center of Cryptogamic Plants and Fungi, Institute of Evolution, Haifa University, Haifa, 31905, Israel*
[c] *Department of Medicinal Chemistry and Natural Products, School of Pharmacy, P.O. Box 12065, Hebrew University of Jerusalem, Jerusalem 91120, Israel*
[d] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 117 913 Moscow, Russia*

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Abstract

Tornabeatins A, B, C and D, have been isolated as new natural products from the lichenized ascomycete *Tornabea scutellifera*, and their structures elucidated using UV, IR, MS, 1D and 2D NMR spectral data and chemical degradation.

Keywords: *Tornabea scutellifera*; Tornabeatins; Lichenized ascomycete; Tetrahydro-2-furanones

1. Introduction

Unusual tetrahydro-2-furanone and γ-lactonic acid derivatives are widely distributed among lichen species and were reviewed in part in recently published books (Huneck and Yoshimura, 1996; Huneck, 2001) and some papers (Follmann and Huneck, 1980; Shrestha et al., 2002; Dembitsky, 1992). 3-Methylidene-tetrahydro-2-furanones (α-methylene-γ-butyro-lactones) constitute an important group of natural products and possess wide-ranging biological activities (Paquette and Mendez-Andino, 1999; Lee et al., 1999; Janecki et al., 2002). The 3-methylenetetrahydro-2-furanone ring is an internal building block of the sesquiterpene lactones, such as vernolepin, aromaticin, and others, which were isolated from some lichen and plant species. All these compounds have cytotoxic, antitumor, and often bactericidal properties (Janecki et al., 2002). The cytotoxic activity of 3-methylidene-tetrahydro-2-furanones is mainly associated with the exocyclic, conjugated double bond, which acts as an alkylating agent in a Michael-type reaction with thiol rich bionucleophiles. In addition, the 3-methylene-tetrahydro-2-furanone moiety is a characteristic component of a large number of biologically active natural products, especially the sesquiterpene lactones (Fukushi et al., 1998; Ortega et al., 1998; Fischer ettetrahydro-2-furanones (Chen et al., 1998) and even the parent 3-methylene-tetrahydro-2-furanone (Gette and Marks, 1990; van Rossum et al., 1998) have significant pharmacological activities. Tetrahydro-2-furanone diacylglycerol analogues are known to have activated protein kinase C, stimulated phosphorylation of the R-pseudosubstrate peptide, and inhibited binding of epidermal growth factor with an ED_{50} in primary mouse keratinocytes (Sharma et al., 1996).

The fruticose epiphytic lichen *Tornabea scutellifera* is widely distributed in the forest of northen Israel and

* Corresponding author. Tel.: +420-241-062-300; fax: +420-241-062-347.
E-mail address: rezanka@biomed.cas.cz (T. Řezanka).

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Cyprus (Littrski and Mayrhofer, 2003; Temina et al., 2002) and has a large biomass. Up to now, its secondary metabolites have not been studied.

The present work is continuation of our investigations of lichen metabolites (Dembitsky, 1992; Řezanka et al., 2003; Torres et al., 2003, 2004; Řezanka and Dembitsky, 2003) and describes the isolation of tornabeatins A, B, C and D from the lichenized ascomycete *T. scutellifera*.

2. Results and discussion

The pulverized lichen (845 g) was extracted with $CHCl_3$–MeOH and centrifuged. The organic layer was concentrated to dryness to afford 5.5 g of green-brown oily material. The crude extract was subjected to Sephadex LH-20 column and eluted with a $CHCl_3$–MeOH solvent system.

Fraction G was analyzed by LC–MS/APCI (liquid chromatography atmospheric pressure chemical ionization mass spectrometry) and TIC (total-ion chromatogram) composed of four peaks, all of which had maximal absorbance at 235 nm. The mass spectra of peaks 1–4 give pseudomolecular ions at *m*/*z* 351, 379, 353, and 353 revealed a ca. 5 second time lag in the retention times. Fraction G was purified by preparative RP-HPLC using detection at 234 nm. The eluate was concentrated to dryness to yield tornabeatin A (**1**), B (**2**), C (**3**), and D (**4**) (Fig. 1) as colorless oils.

The HREIMS of **1** showed a molecular ion at *m*/*z* 350.2826. These data are consistent with the molecular formula of $C_{22}H_{38}O_3$. The ^{13}C NMR spectrum of **1** showed 22 signals corresponding to 22 carbons of the lactone, and DEPT measurement revealed the presence of two methyl groups, 15 methylene (one bearing oxygen), three methine, and two oxygenated quaternary carbons at δ 85.7 and 165.0 which were assigned to tertiary alcohol and lactone carbons, respectively. The presence of an α-ylidene-γ-butyrolactone was indicated by the following NMR data.

The ^1H NMR spectrum of **1** (Table 1) shows signals due to three alkene protons in the isolated systems at δ 6.15 (1H, *t*, $J=7.6$ Hz, H-1′), 5.42 (1H, *m*, H-9′) and 5.42 (1H, *m*, H-10′) ppm; of a hydroxy-bearing methylene group at δ 3.75 (2H, *s*, H-6) ppm and a second methylene group as two *dt* at δ 2.26 and 2.73 (H-4), respectively; of a methyl group at δ 1.36 (3H, *s*, H-7) ppm and a terminal methyl group at δ 0.88 (3H, *t*, $J=7.0$, H-16′) ppm.

The geometrical configuration of the double bond between positions 3 and 7 was elucidated to be *Z* by the NOEs between H-4 and H-1′, and between H-6 and H-1′. The geometrical configuration of the second double bond was assumed to also be *Z* from the δ value (27.3 ppm) (Table 2) of the neighboring methylene carbon (Kling et al., 1993). The position of the *Z*-double bond in the side chain was deduced from the MS. The fragment peak at *m*/*z* 274 in the EIMS spectrum of tornabeatin A was intense, and two ions at 12-mass unit intervals were observed between *m*/*z* 234 and 246.

However, further confirmation of the position of the double bond in the side chain was obtained by analyzing the products obtained after ozonolysis of this compound. Compound **1** was treated with O_3 followed by oxidation with H_2O_2/HCOOH and esterification of the product with diazomethane. GC–MS separation afforded a dimethyl azelate, corresponding to the C-1′–C-9′ segment of **1** (Table 3) and methyl enanthate, corresponding to the C-10′–C-16′ segment. The relative retention time of both compounds from the natural specimen was identical with that of the commercial compounds, indicating that the position of disubstituted double bond was $\Delta^{9'}$.

The absolute configuration of **1** was determined to be *R* by means of CD spectroscopy. A comparison was then made between the CD spectrum of two model compounds, i.e., (2*R*) and (2*S*)-2-methyl-4-methylene-5-oxotetrahydrofuran-2-carboxylic acids (Pitacco et al., 2000).

Both the compound **1** and the model compound (2*R*)-2-methyl-4-methylene-5-oxo-tetrahydrofuran-2-carboxylic acids exhibited a negative Cotton effect for the

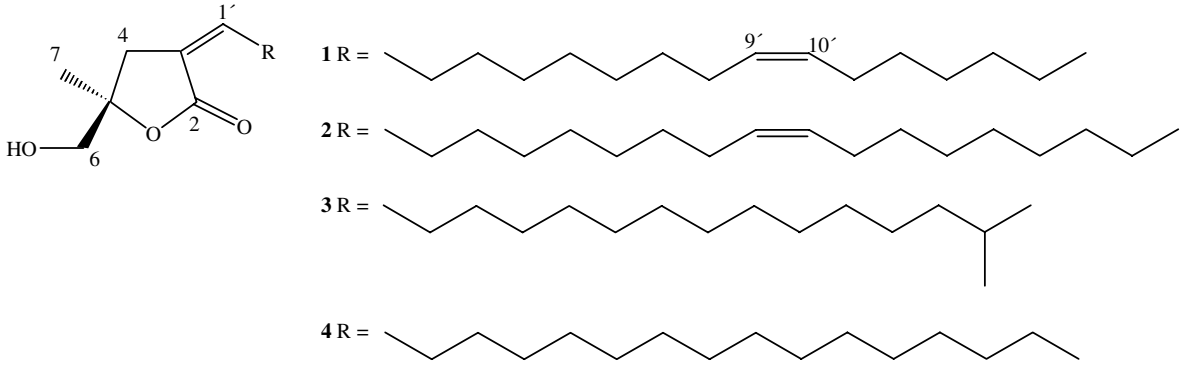

Fig. 1. Structures of tornabeatins A, B, C and D (**1**–**4**).

Table 1
^{1}H NMR data of lactones (**1–4**)

No.	1	2	3	4
4	2.26 (1H, *dt*, *J*=2.6,15.0),	2.26 (1H, *dt*, *J*=2.6,15.0),	2.26 (1H, *dt*, *J*=2.6,15.0),	2.26 (1H, *dt*, *J*=2.6,15.0),
	2.73 (1H, *dt*, *J*=2.6,15.0)	2.73 (1H, *dt*, *J*=2.6,15.0)	2.73 (1H, *dt*, *J*=2.6,15.0)	2.73 (1H, *dt*, *J*=2.6,15.0)
6	3.75 (2H, *s*)	3.75 (2H, *s*)	3.75 (2H, *s*)	3.75 (2H, *s*)
7	1.36 (3H, *s*)	1.36 (3H, *s*)	1.36 (3H, *s*)	1.36 (3H, *s*)
1′	6.15 (1H, *t*, *J*=7.6)	6.15 (1H, *t*, *J*=7.6)	6.15 (1H, *t*, *J*=7.6)	6.15 (1H, *t*, *J*=7.6)
2′	2.10 (2H, br *d t*, *J*=7.5, 7.5)	2.10 (2H, br *d t*, *J*=7.5, 7.5)	2.10 (2H, br *d t*, *J*=7.5, 7.5)	2.10 (2H, br *d t*, *J*=7.5, 7.5)
3′	1.43 (2H, br *tt*, *J*=7.1, 7.1)	1.43 (2H, br *tt*, *J*=7.1, 7.1)	1.43 (2H, br *tt*, *J*=7.1, 7.1)	1.43 (2H, br *tt*, *J*=7.1, 7.1)
4′–6′	1.3 (6H, *m*)	1.3 (6H, *m*)	1.3 (6H, *m*)	1.3 (6H, *m*)
7′, 12′	1.33 (4H, *m*)	1.33 (4H, *m*)	1.3 (12H, *m*)	1.3 (16H, *m*)
8′, 11′	1.96 (4H, *m*)	1.96 (4H, *m*)		
9′, 10′	5.42 (2H, *m*)	5.42 (2H, *m*)		
13′	1.3 (4H, *m*)	1.3 (8H, *m*)	1.15 (2H, br *d t*, *J*=6.6, 6.8)	
14′			1.51 (1H, *sept*, *J*=6.6)	
15′	1.23 (2H, *m*)		0.86 (3H, *d*, *J*=6.6)	1.23 (2H, *m*)
16′	0.88 (3H, *t*, *J*=7.0)		0.86 (3H, *d*, *J*=6.6)	0.88 (3H, *t*, *J*=7.0)
17′	–	1.23 (2H, *m*)	–	–
18′	–	0.88 (3H, *t*, *J*=7.0)	–	–

$\pi \rightarrow \pi^*$ transition $\Delta\varepsilon_{229}$ −4.5 (MeOH) and $\Delta\varepsilon_{227}$ −3.9 (MeOH) (Pitacco et al., 2000). Vice versa the second model compound, i.e., (2*S*)-2-methyl-4-methylene-5-oxo-tetrahydrofuran-2-carboxylic acids had $\Delta\varepsilon_{227}$ +3.2 (MeOH) (Pitacco et al., 2000). Although the γ-carbon atom in **1** bears a hydroxymethyl group in place of a carboxyl group, the order of polarizability of the substituents is the same in both compounds, thus allowing a comparison between the curves to be made (Uchida and Kuriyama, 1974; Cain et al., 1996).

Further, the absolute configuration of the C-5 was determined by hydrolysis and ozonolysis. For this purpose, we used oxidative splitting, oxidation of the reaction products and chromatography of appropriate methyl esters on a chiral capillary column to determine the absolute configuration of compound **1**. As shown in Table 3, two non-chiral and one chiral compounds were isolated from the reaction mixture, i.e., (2*R*)-2-methyl-2-hydroxybutanedioic acid (citramalic acid). Baseline separations were obtained on the chiral capillary column (conditions are described in Section 3) with an appropriate phase. First, the standards of both enantiomers of citramalic acid were chromatographed as methyl esters. Second, the oxidation products were chromatographed (as methyl esters) and the retention times of the corresponding peaks were compared (Table 3). Thus, the full structure of **1** was (5*R*,$\Delta^{3,1'Z}$,7′*Z*)-3-hexadec-7′-enylidene-5-hydroxymethyl-5-methyl-dihydrofuran-2-one (Fig. 1). This structure is supported by the NMR data of the known synthetic compounds with similar structures (Janecki et al., 2002). To the best of our knowledge, natural products having this moiety have been not reported previously.

The structure of tornabeatin B (**2**) was shown to be the same as that of tornabeatin A except that one ethylene (C_2H_4=28 mass units) moiety was inserted in the hexadecyl side chain of tornabeatin A.

All the spectra of tornabeatin C (**3**) indicated, that the structure of this compound was also very similar to that of **1**, only difference in side chain were observed. The doublet signal at δ 0.86 with the intensity of six protons showed the presence of an iso-branched moiety (*gem*-dimethyl) and no signals corresponding to the double bond(s) were observed. That the isohexadecyl side chain is attached to the butyrolactone moiety at position 3 was indicated by the HMBC (^{1}H–^{13}C) and NOESY (^{1}H–^{1}H) spectra, as is depicted in Fig. 2. This is also supported by the observation of a series of fragmentations of the isoalkyl chain from *m*/*z* 319 $[M]^+$ to 55 (base peak), with weak relative intensity at *m*/*z* 290 $[M-29]^+$ in the EIMS spectrum.

The compound **4** (tornabeatin D) exhibited NMR and mass spectra consistent with a saturated C_{16} side chain.

Monotetrahydrofuranic acetogenins represent a large class of natural compounds (ca. 500) (Alali et al., 1999;

Table 2
^{13}C NMR data of lactones (**1–4**)

	1	2	3	4
2	165.0	165.0	165.0	165.0
3	134.4	134.4	134.4	134.4
4	34.2	34.2	34.2	34.2
5	85.7	85.7	85.7	85.7
6	73.0	73.0	73.0	73.0
7	20.4	20.4	20.4	20.4
1′	138.8	138.8	138.8	138.8
2′	26.5	26.5	26.5	26.5
3′	30.3	30.3	30.3	30.3
4′	30.3	30.3	30.3	30.3
5′	30.3	30.3	30.3	30.3
6′	30.3	30.3	30.3	30.3
7′	30.3	30.3	30.3	30.3
8′	27.3	27.3	30.3	30.3
9′	131.7	131.7	30.3	30.3
10′	131.7	131.7	30.3	30.3
11′	27.3	27.3	30.6	30.3
12′	30.3	30.3	27.5	30.3
13′	30.3	30.3	39.4	30.3
14′	32.5	30.3	28.5	32.5
15′	23.1	30.3	27.3	23.1
16′	14.0	32.5	27.3	14.0
17′	–	23.1	–	–
18′	–	14.0	–	–

Table 3
The presence of products degradation (determined by chiral capillary GC) after oxidation of lactones (**1–4**)

Compound	RT of products after degradation (min^{-1})				
	Standards	1	2	3	4
Methyl enanthate	6.38	6.40	–	–	–
Methyl pelargonate	9.54	–	9.56	–	–
Dimethyl azelate	13.52	13.57	13.55	–	–
Dimethyl 2*R*-citramalate	19.08	19.03	19.04	19.06	19.07
Dimethyl 2*S*-citramalate	19.61	–	–	–	–

Rodriguez-Saona and Trumble, 2000). Discovered tornabeatins A–D (Fig. 1) from lichenized ascomycete *T. scutellifera* contain a double bond in position 3 and have a similar structure to **5**, and **6** (see Fig. 3). Acetogenins with structure **5** were isolated from hexane extract of leaves of *Mollinedia marliae* (Claros et al., 2000), and from the stem bark of *Litsea akoensis* (Chen et al.,

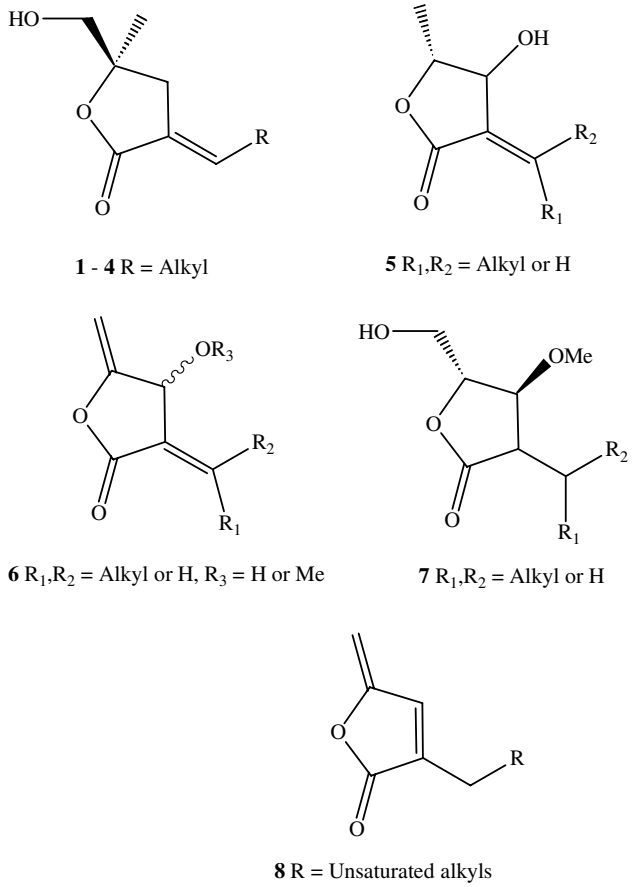

Fig. 3. Comparative structures of monotetrahydrofuran acetogenins isolated from *T. scutellifera*, and also found in lichenized ascomycete plant species.

1998); acetogenins belonging to group **6** were isolated from the leaves of *Lindera glauca* (Seki et al., 1994); from the trunk wood of *Clinostemon mahuba* (Martinez et al., 1979; Martinez et al., 1981); from the roots of *L. glauca* (Seki et al., 1994), and also from the aerial parts of *Artabotrys hexapetalus* (Wong and Brown, 2002). Acetogenins belonging to group **7** with hydroxy group were found in petroleum ether extract of the stem bark of *Miliusa elutina* (Jumana et al., 2000) and in the aerial parts of *Goniothalamus gardneri* (Seidel et al., 1999). The isolation of all four tornabeatins (A–D) from lichen for

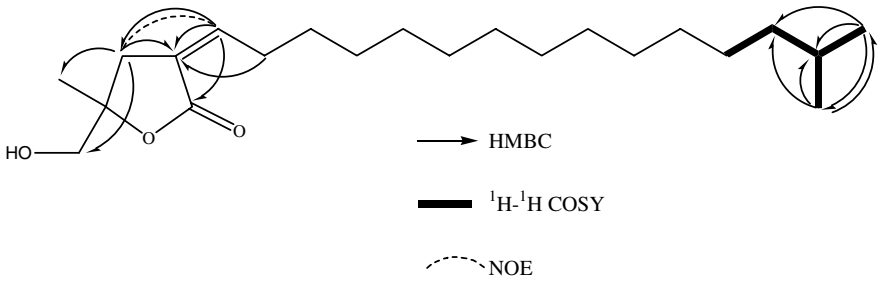

Fig. 2. HMBC, ^{1}H-^{1}H COSY, and NOE correlations of tornabeatin C **(3)**.

the first time is of phylogenic interest, although the closely related compounds (**5**–**8**) have already been isolated from higher plants, as well as from liverwort (Toyota et al., 1997).

Among the monotetrahydrofuran acetogenins belonging to the types **5**–**7** have been found biological active compounds. Those, four new butanolides, akolactones A, B, litseakolides A, B, along with four known butanolides, litsenolides B, C_2, and hamabiwalactone A (type **5**) were isolated from the stem bark of *L. akoensis*, and showed cytotoxic activity against P-388, KB16, A 549, and HT-29 cancer cell lines (Chen et al., 1998). Various (3*S*)-2-alkylidene-3-hydroxy-4-methylene-butanolides (type **6**) have cytotoxic activity (Niwa et al., 1975a,b; Baussanne et al., 1997). Goniothalamusin and acetogenins-A and -B (type **7**) isolated from *M. elutina* exhibited significant antibacterial and cytotoxic activities against Gram-positive (*Bacillus cereus*, *Staphylococcus aureus*, *Streptomyces* β-*haemoliticus*), and Gram-negative (*Salmonella typhi*, *Shigella flexneri*, *Shigella dysenteriae*) bacterial species (Jumana et al., 2000; Seidel et al., 1999). According to these suggestions were by us discovered new cyclopentenones, namely tornabeatins A–D **1**–**4**, which show a modest activity against different microorganisms (see Table 4). They are active against *S. aureus* and *Bacillus subtilis*, but inactive against the Gram-negative bacterium *Escherichia coli*.

The two most popular primary screening bioassays are the brine shrimp lethality test and the crown gall tumor inhibition test. The first 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. All compounds showed significant 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, ranging from 29% for compound **2** to 65% for compound **1**.

Table 4
Bioactivities of cyclopentenones (**1**–**4**)

Test organism	**1**	**2**	**3**	**4**
S. aureus[a]	28	35	21	24
B. subtilis[a]	32	24	27	20
E. coli[a]	0	0	0	0
S. cerevisiae[a]	14	17	5	6
A. salina[b,c]	1.8	2.4	3.8	5.1
P. lividus[c,d]	2.2	3.4	1.6	1.6
A. tumefaciens[c,e]	35 ± 4[f]	71 ± 5	49 ± 8	53 ± 6

[a] Samples (10 μg) were applied on 50.8 mm paper disks, values are diameters (mm) of inhibitory zones.
[b] in μg/ml (minimum lethal doses).
[c] the details in Experimental Section.
[d] in μg/ml (IC_{50}).
[e] presented values are means of three determinations.
[f] % of crown gall tumor inhibition (± S.D.).

3. Conclusions

We have isolated four new compounds, which contain a tetrahydro-2-furanone unit, and having the appropriate pharmacologically activity, e.g., as the monotetrahydrofuran acetogenins belonging to types **5**–**7**. This is the first report of the isolation of long-chain tetrahydrofuranones from lichens, although they have been isolated previously form higher plant or liverworts.

4. Experimental

4.1. General experimental procedures

UV-VIS spectra were measured in MeOH within the range 220–550 nm in a Cary 118 (Varian) apparatus. A Perkin–Elmer (Perkin–Elmer, Norwalk, CT) 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–Elme) 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. Gas chromatography analysis was in a Hewlett–Packard HP 5980 gas chromatograph (Hewlett Packard, Czech Republic).

The LC–MS/APCI was realized as mentioned previously (Řezanka and Dembitsky, 2003), briefly: the HP 1090 series (HP 1090 series, Hewlett–Packard, USA) was used with two columns (HIRPB-250AM 250×2.1 mm ID, 5 μm phase particle). A quadruple mass spectrometer system Navigator (Finnigan MAT, San Jose, CA) was used: vaporizer temperature 400 °C, capillary heater temperature 220 °C, corona current 5 μA, sheath gas high-purity nitrogen, pressure ca. 380 kPa, and auxiliary gas (also nitrogen) flow rate 1500 ml/min. Ions with *m*/*z* 50–1500 were scanned with a scan time of 0.5 s, flow 0.37 ml/min. Compounds were separated using a solvent program with water–acetonitrile (50:50) to 10 min and linear gradient from 10 to 40 min (100% acetonitrile).

4.2. Plant material

The lichenized ascomycete *T. scutellifera* (With.) J.R. Laundon (a genus of fruticose lichens in the family

Physciaceae) was collected in September 2003 on Mount Meron from sun exposed wood surfaces around Haifa at approximately 600 m above sea level. It was identified by Dr. M. Temina and has its voucher, HAI-032441, deposited in the Herbarium of the Institute of Evolution.

4.3. Extraction, isolation and identification

The aqueous-MeOH layer (Blight and Dyer, 1959) of lipid extracts from 100 g of air-dried lichens (Řezanka and Dembitsky, 2003) was separated on a Sephadex LH-20 column eluted with MeOH–H_2O (9:1) yielding a yellow oil. This oil was further fractionated by RP-HPLC on a C18-Bondapak column (30 cm×7.8 mm, flow rate 2.0 ml/min) eluted with MeCN–H_2O (1:2) to yield compounds **1** (3.5 mg), **2** (2.3 mg), **3** (0.6 mg), and **4** (0.5 mg).

4.3.1. Oxidative splitting (Kroft et al., 1981)

A stream of 4% ozone was passed through a solution of the given compound (~1 mg) in dichloromethane (2 ml) at −78 °C for 5 min. The solution was flushed with nitrogen and concentrated. The residue was dissolved in 90% HCOOH (0.7 ml) and 30% hydrogen peroxide (0.3 ml) was added. After gentle heating, the mixture was heated under reflux for 70 min. The mixture was concentrated and the residue was dissolved in methanol (0.5 ml) and treated with etheral diazomethane.

4.3.2. Chiral chromatography

FS capillary column HYDRODEX β-3P ID 0.25 mm, length 25 m, with the stationary phase [heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrine] from Macherey-Nagel GmbH & Co. KG, Düren, Germany was used. Oven temperature: 50–150 °C at 2 °C/min, then to 240 °C at 5 °C/min, carrier gas helium, 20 cm/s, detector FID, 300 °C, injection of 1 μl mixture in methylene chloride (for standards: containing 0.5 mg/ml of each analyte), split (100:1), 300 °C.

4.3.3. Tornabeatin A (*1*)

Yield 3.5 mg, colorless oil; $[\alpha]_D^{23}$ − 15.5 (c 0.12, MeOH); UV (MeOH) λ_{max} 209 (log ε 4.24), 229 (4.15) nm; IR (film) ν_{max} 3490–3400 (OH), 1760 (C═O), 1664 (C═C)1455, 1380, 1270, 1210, 1110, 1050, 995, 940 cm^{-1}; CD (MeOH) λ_{ext} nm (Δε) 202 (+5.9), 229 (−4.5); LC–MS/APCI: *m/z* 351 $[M+H]^+$; HREIMS *m/z* 350.2826 $C_{22}H_{38}O_3$ $[M]^+$, calculated for $[C_{22}H_{38}O_3]^+$ 350.2821; ^1H and ^{13}C NMR data, see Tables 1 and 2.

4.3.4. Tornabeatin B (*2*)

Yield 2.3 mg, colorless oil; $[\alpha]_D^{23}$ − 6.9 (c 0.11, MeOH); UV (MeOH) λ_{max} 210 (log ε 4.37), 230 (4.42) nm; IR (film) ν_{max} 3500–3400 (OH), 1760 (C═O), 1665 (C═C) cm^{-1}; CD (MeOH) λ_{ext} nm (Δε) 201 (+6.1), 230 (−4.6); LC–MS/APCI: *m/z* 379 $[M+H]^+$; HREIMS *m/z* 378.3138 $C_{24}H_{42}O_3$ $[M]^+$, calculated for $[C_{24}H_{42}O_3]^+$ 378.3134; ^1H and ^{13}C NMR data, see Tables 1 and 2.

4.3.5. Tornabeatin C (*3*)

Yield 0.6 mg, colorless oil; $[\alpha]_D^{23}$ − 12.5 (c 0.14, MeOH); UV (MeOH) λ_{max} 209 (log ε 4.12), 228 (3.95) nm; IR (film) ν_{max} 3500–3400 (OH), 1762 (C═O), 1668 (C═C) cm^{-1}; CD (MeOH) λ_{ext} nm (Δε) 203 (+5.6), 228 (−4.4); LC–MS/APCI: *m/z* 353 $[M+H]^+$; HREIMS *m/z* 352.2981 $C_{22}H_{40}O_3$ $[M]^+$, calculated for $[C_{22}H_{40}O_3]^+$ 352.2977; ^1H and ^{13}C NMR data, see Tables 1 and 2.

4.3.6. Tornabeatin D (*4*)

Yield 0.5 mg, colorless oil; $[\alpha]_D^{23}$ − 11.9 (c 0.09, MeOH); UV (MeOH) λ_{max} 205 (log ε 4.03), 229 (3.67) nm; IR (film) ν_{max} 3500–3400 (OH), 1764 (C═O), 1666 (C═C) cm^{-1}; CD (MeOH) λ_{ext} nm (Δε) 206 (+5.2), 229 (−4.1); LC-MS/APCI: *m/z* 353 $[M+H]^+$; HREIMS *m/z* 352.2979 $C_{22}H_{40}O_3$ $[M]^+$, calculated for $[C_{22}H_{40}O_3]^+$ 352.2977; ^1H and ^{13}C NMR data, see Tables 1 and 2.

4.3.7. Antibacterial tests

The test organisms were *B. subtilis*, *S. aureus*, *E. coli* and *Saccharomyces cerevisiae* (Czechoslovak Collection of Microorganisms, Brno). Antibacterial assays were carried according to the literature (Řezanka and Guschina, 2000). The amounts used were 50 μg of compound per test disk (see Table 3).

4.3.8. Brine shrimp toxicity bioassay

The sample (~0.05 mg) was dissolved in 50 μl of DMSO and added to a test vial of artificial seawater (3.0 ml). Approximately 20 brine shrimp, *Artemia salina*, were added to the vial. The brine shrimp were observed periodically over a 24 h period. A positive assay was the death of all brine shrimp.

4.3.9. Sea urchin eggs test

Sea urchin eggs and sperms were collected from mature specimens of *Paracentrotus lividus*. Soon after fertilization, the eggs were dropped onto the test media which were previously prepared from a graded concentration of compounds **1** and **2** dissolved in EtOH and filtered seawater. After allowing the test media to stand, the embryos were observed with a light microscope at certain intervals. In an inhibitory test against the development of sea urchin eggs (Jacobs et al., 1981) **1** and **2** were effective at levels of IC_{50} 2.5 and 0.2 μg/ml, respectively.

4.3.10. Crown gall tumors on potato disks test

The *A. tumefaciens* potato disc assay for tumor/antitumor induction was performed according to the procedure

described in the literature (McLaughlin, 1991; McLaughlin et al., 1993). The potatoes were sterilized by immersion in ethanol 70% during 2 min and in 50% sodium hypochlorite solution (active chlorine 30 g/l) during 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 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), 1.5 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) ($n = 15$ to 25) was 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) KI (2 g) in 300 ml distilled H_2O. Significant activity is indicated when two independent assays give 20% or greater inhibition.

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