Chemistry of Natural Compounds, Vol. 48, No. 3, July, 2012 [Russian original No. 3, May-June, 2012]

SECONDARY METABOLITES OF THE LICHEN *Cladonia stellaris*

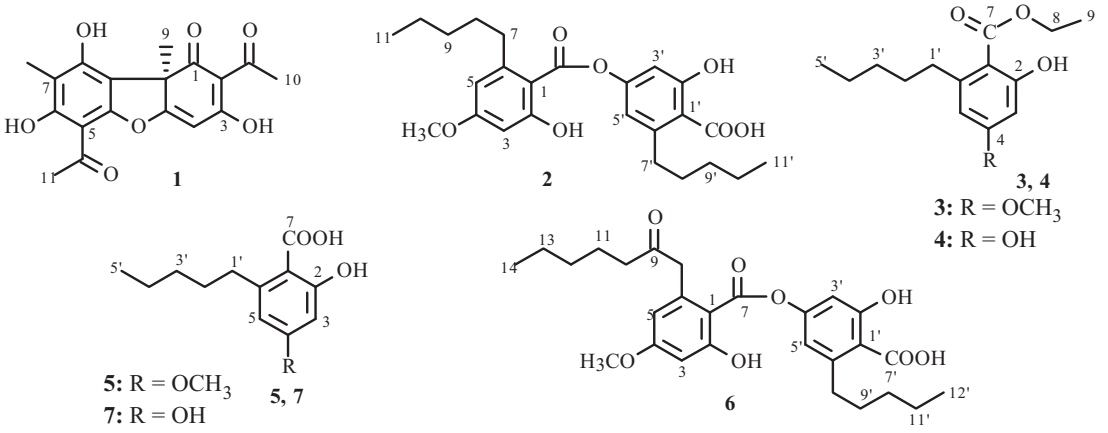
M. P. Polovinka,[1*] N. I. Komarova,[1] D. V. Korchagina,[1]
D. N. Sokolov,[1] O. A. Luzina,[1] N. G. Vlasenko,[2]
A. A. Malyuga,[2] E. V. Romanova,[3] and N. F. Salakhutdinov[1]

UDC 547.992

The composition of slightly polar extracts of the lichen Cladonia stellaris *(Opiz.) was studied. The major metabolites of the $CHCl_3$ and Me_2CO extracts were isolated. Lichen acids* ***6*** *and* ***7*** *in addition to* ***3–5****, derivatives of olivetolcarboxylic acid (****7****), were isolated for the first time from the lichen* C. stellaris*. The $CHCl_3$ extract was tested for antagonist activity against pathogens of the principal agricultural diseases.*

Keywords: (–)-usninic acid, secondary metabolites of *Cladonia stellaris* (Opiz.), fungicidal activity of lichen acids.

Secondary metabolites of lichens that are highly active against various bacteria and fungi were described in a review and a book [1, 2]. The genus *Cladonia* is one of the most numerous and varied and comprises greater than 400 lichen species [3]. The lichen *Cladonia stellaris* (Opiz.) (Cladoniaceae) is broadly distributed from the tundra to the deciduous forest zone and grows in large quantities in the Altai Mountains. According to the literature, the chemical composition of this lichen is practically unstudied [4]. It was noted that it is a dominant component of the terrestrial cover of boreal forests [5]. It was shown that the major components of *C. stellaris* extracts are usninic (**1**) and perlatolic (**2**) acids, which make up according to various researchers from 5 to 11% of the total lichen mass [6–9]. The most well studied metabolite is usninic acid (**1**), for which various researchers have reported antimicrobial, antiviral, antibiotic, anti-inflammatory, analgesic, and other types of activity [10–13]. For this reason, **1** is used in cosmetics, dentistry, and other areas of medicine. Russian scientists created the preparation Cladosent, which exhibits radioprotective properties, based on ground fronds of *C. stellaris* [14–17].



The goal of the present study was to isolate extracted substances from *C. stellaris* and to study their structures, chemical properties, and the biological activity of the separate metabolites and total extracts. We previously published a patent on the isolation of usninic acid from various lichen species including from *C. stellaris* [18]. Herein we used samples of *C. stellaris* collected in various Altai regions (Semin gap, August 2004–2009 and Edigan, August 2005–2009).

1) N. N. Vorozhtsov Novosibirsk Institute of Organic Chemistry, Siberian Branch, Russian Academy of Sciences, Russia, 630090, Novosibirsk, Prosp. Akad. Lavrent′eva, 9, e-mail: polovina@nioch.nsc.ru; 2) Siberian Scientific Research Institute of Agronomy and Agricultural Chemicals, Russian Agricultural Academy, Russia, 630501, Krasnoobsk, Novosibirsk Oblast; 3) Central Siberian Botanical Garden, Siberian Branch, Russian Academy of Sciences, Russia, 630090, Novosibirsk, Ul. Zolotodolinskaya, 101. Translated from *Khimiya Prirodnykh Soedinenii*, No. 3, May–June, 2012, pp. 356–359. Original article submitted December 16, 2011.

 0009-3130/12/4803-0392

TABLE 1. Antagonist Activity of the $CHCl_3$ Extract Against Pathogens of Principal Agricultural Diseases

Dilution (extract:H_2O by mass)	Mycelium growth index, % vs. control			
	on day 3	on day 5	on day 7	on day 9
		Rhizoctonia solani		
1:100	–19.3	–21.5		
1:1000	–1.9	–10.3		
		Alternaria spp.		
1:100	–28.8	–34.3	–32.2	–27.7
1:1000	–5.6	–3.5	–3.9	–4.7
		Fusarium oxysporum		
1:100	–27.1	–38.3	–37.2	
1:1000	- 15.3	–18.3	–15.2	
		Phoma exiqua var *foveata*		
1:100	–20.3	–28.6	–34.1	–33.1
1:1000	–11.4	–8.9	–10.0	–7.4

All samples contained (–)-usninic acid (**1**) (1.1–1.4 mass% of dry lichen regardless of the collection site). The specific rotation differed for samples collected in different regions. The specific rotation for **1** from samples from Semin gap was $[\alpha]_D \sim -300–360°$; from samples from Edigan of higher optical purity, $[\alpha]_D \sim -430–490°$.

We selected the conditions for extracting substances from lichen fronds during the study of the chemical composition of *C. stellaris*. Fractional extraction by a succession of refluxing $CHCl_3$:Me_2CO solvents was most effective for isolating slightly polar metabolites from the lichens. Then, yellow crystals of **1** were precipitated from the $CHCl_3$ extract as described before [18]. Column chromatography of the filtrate over silica gel isolated compounds **2–7**. Compound **2** was perlatolic acid; **3–6**, derivatives of olivetolcarboxylic acid (**7**), which was the major component of the Me_2CO extract. Compounds **1–7** were reported [19] as lichen metabolites. However, PMR and ^{13}C NMR spectral data were not reported for **4**. Lichen acids **6** and **7** and derivatives of **7** (**3–5**) were isolated from *C. stellaris* for the first time.

Simultaneously with the study of the properties of *C. stellaris* extracts, we tested the $CHCl_3$ extract for antagonistic activity against pathogens of the principal agricultural diseases by the agar block method [20, 21]. A 1:100 dilution of the extract (extract:H_2O by mass) suppressed most effectively colony growth of *Fusarium oxysporum*, *Alternaria* spp., and *Phoma exiqua* var. *foveata* although the effect was less pronounced against *Phoma exiqua* var. *foveata* (Table 1). Its activity in 1:1000 dilution was still rather high against cultures of *F. oxysporum* and *P. exiqua* var. *foveata*, which cause root rot in many grain crops and potato blight.

Lichen extracts exhibiting fungicidal activity can be used to create new promising ecological preparations for controlling agricultural diseases. As an example, we present our patented preparation Bioclad [22], which is based on the total polar extract of *Cladonia* lichens and has successfully passed tests as an agent for controlling wheat diseases.

EXPERIMENTAL

High-performance liquid chromatography (HPLC) was performed in a Millichrom A-02 microcolumn chromatograph (ZAO EkoNova, Novosibirsk) using a standard chromatography column (2 × 75 mm) packed with reversed-phase sorbent (ProntoSIL, 120-5-C18, 5 μm, Bischoff, Germany). Gradient elution with simultaneous multi-wavelength detection at three wavelengths (230, 280, 360 nm) and MeOH eluent with 0.1% trifluoroacetic acid (TFA) were used.

PMR and ^{13}C NMR spectra were recorded on AM-400 and AV-300 spectrometers (Bruker) at operating frequencies 400.13 and 300.13 MHz (^1H) and 100.61 and 75.48 MHz (^{13}C). Column chromatography used silica gel (70–230 μm, Merck). The course of the chromatography was monitored by analyzing separate fractions by TLC over Silufol or Sorbfil plates using EtOH (2%) in $CHCl_3$.

A herbarium specimen of the plant is preserved in the Herbarium of the Central Siberian Botanical Garden, SB, RAS.

Extraction. Air-dried lichen *C. stellaris* (220 g) was extracted (3×) successively by $CHCl_3$ (1.5 L) under reflux for 4 h. The solvent was evaporated to afford a dry residue (6.80 g). The extract was dissolved under reflux in $CHCl_3$:EtOH (1:10, 100 mL). Upon cooling, yellow crystals of (–)-usninic acid (**1**; 2.90 g; 1.32%, here and henceforth mass% calculated

per mass of dry lichen is given for isolated substances), $[\alpha]_D^{25}$ –360° (*c* 1.60, $CHCl_3$) precipitated. The solvent was removed from the mother liquor in a rotary evaporator to afford a dry residue (3.24 g) that was chromatographed over a column of silica gel with elution by $CHCl_3$ with a gradient of EtOH from 2 to 30%. Several successive chromatographic separations produced (in order of elution from the column) **2** (0.05 g, 0.02%), **3** (0.03 g, 0.01%), **4** (0.02 g, 0.01%), **5** (0.07 g, 0.03%), **6** (0.05 g, 0.02%), and **7** (0.10 g, 0.05%).

The dried pulp of *C. stellaris* after $CHCl_3$ extraction was extracted (3×) with Me_2CO (1.5 L) under reflux for 4 h to afford a dry extract (2.52 g) that contained according to HPLC compound **7** as the major component.

(*R*)-1,1′-(3,7,9-Trihydroxy-8,9b-dimethyl-1-oxo-1,9b-dihydrodibenzo[*b*,*d*]furan-2,6-diyl)diethanone (1). Yellow crystals ($CHCl_3$:EtOH), mp 204°C. The PMR spectrum agreed with that published [19].

2-Hydroxy-4-(2-hydroxy-4-methoxy-6-pentylbenzoyloxy)-6-pentylbenzoic Acid (2). White crystals (Et_2O), mp 153–154°C (lit. [19] mp 107–108°C). The PMR spectrum agreed with that published [19]. Found: *m*/*z* 444.5249 $[M]^+$, $C_{25}H_{32}O_7$; calcd: MW 444.52488.

Ethyl-2-hydroxy-4-methoxy-6-pentylbenzoate (3). The PMR spectrum agreed with that published [19].

Ethyl-2,4-dihydroxy-6-pentylbenzoate (4). PMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.88 (3H, t, J = 7.0, H-5′), 1.24–1.40 (4H, m, H-3′,4′), 1.38 (3H, t, H-9), 1.59 (2H, m, H-2′), 2.82 (2H, t, H-1′), 4.35 (2H, q, H-8), 6.21 and 6.25 (2H, dd, J = 2.5, H-3,5). ^{13}C NMR spectrum ($CDCl_3$, δ, ppm): 171.1 (C-7), 164.9 (C-2), 160.1 (C-4), 148.4 (C-6), 104.5 (C-1), 110.4 (C-5), 100.9 (C-3), 60.8 (C-8), 36.5 (C-1′), 31.5 (C-3′), 31.2 (C-2′), 22.2 (C-4′), 13.6 (C-5′,9).

2-Hydroxy-4-methoxy-6-pentylbenzoic Acid (5). White crystals (Et_2O), mp 116–120°C. PMR spectrum (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.89 (3H, t, J = 7.0, H-5′), 1.29–1.38 (4H, m, H-4′,3′), 1.53–1.63 (2H, m, H-2′), 2.90 (2H, m, H-1′), 3.81 (3H, s, H-8), 6.32 and 6.33 (2H, dd, J = 2.5, H-3,5). ^{13}C NMR spectrum ($CDCl_3$, δ, ppm): 13.0 (C-5′), 22.6 (C-4′), 31.5 (C-3′), 32.0 (C-2′), 36.8 (C-1′), 98.8 (C-3), 102.7 (C-5), 113.8 (C-1), 149.2 (C-6), 166.5 (C-4), 174.0 (C-7); agreed with the literature [19]. Found: *m*/*z* 238.2835 $[M]^+$, $C_{13}H_{18}O_4$; calcd: MW 238.2835.

2-Hydroxy-4-(2-hydroxy-4-methoxy-6-(2-oxoheptyl)benzoyloxy)-6-pentylbenzoic Acid (6). PMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.80 (3H, t, J = 7.0, H-14), 0.88 (3H, t, J = 7.0, H-12′), 1.10–1.23 (4H, m, H-12,13), 1.27–1.39 (4H, m, H-10′,11′), 1.51(2H, m, H-11), 1.58 (2H, m, H-9′), 2.39 (2H, t, J = 7.0, H-10), 2.94 (2H, m, H-8′), 3.82 (3H, s, H-15), 4.03 (2H, s, H-8), 6.27 (1H, d, J = 2.5, H-5), 6.44 (1H, d, J = 2.5, H-3), 6.49 (1H, d, J = 2.2, H-5′), 6.61 (1H, d, J = 2.2, H-3′), 11.25 and 11.32 (2H, s and br.s, 2OH). ^{13}C NMR spectrum ($CDCl_3$, δ, ppm): 13.67 (C-14), 13.87 (C-122), 22.25 (C-13), 22.31 (C-11′), 23.19 (C-11), 31.22 (C-12), 31.31 (C-9′), 31.83 (C-10′), 36.35 (C-8′), 42.32 (C-10), 51.10 (C-8), 55.40 (C-15), 100.05 (C-3), 104.18 (C-1), 108.82 (C-1′), 108.92 (C-3′), 113.34 (C-5), 115.97 (C-5′), 138.88 (C-6), 149.85 (C-6′), 154.33 (C-4′), 164.88 and 166.53 (C-2,4), 165.11 (C-2′), 168.59 (C-7), 174.18 (C-7′), 207.23 (C-9).

2,4-Dihydroxy-6-pentylbenzoic Acid (7). White crystals (Et_2O), mp 148–149°C. The PMR spectrum agreed with that published [19].

Screening of experimental preparations for fungicidal and fungistatic properties was performed under laboratory conditions by the agar block method [20, 21]. The fungal pathogens of the main diseases of grain and potato in western Siberia were screened. These included *Alternaria* spp., *Penicillium* spp., *Rhizoctonia solani*, *Phoma exigua* var. *foveata*, and *Fusarium oxysporum*. Extract (0.50 g) was ground with a spatula in emulsifier (liquid soap). The resulting substance was dissolved in warm water (~40–50°C, 49.30 g H_2O) calculated for 0.01 g of extract per milliliter of solution (1:100 dilution). The tested preparations were inoculated into agar PDA medium (potato-dextrose agar) as an aqueous solution with 1:100 and 1:1000 dilutions (extract:H_2O). The resulting media (20 mL each) was poured into Petri dishes (9 cm diameter) into which pure fungal culture causing plant mycosis was then inoculated (disks with mycelium 5-mm in diameter). The control was medium without added compounds. The incubation temperature was +24°C. The dimensions of colonies were measured when they reached 9 cm in the control. Growth suppression of colonies was calculated using the Abbot formula [23]. Experiments were carried out in triplicate.

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