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Further glucosides of lichens' acids from Central Asian lichens

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Abstract

Eight compounds isolated from an extract of Central Asian lichens comprised new glucosides having murolic, protoconstipatic and allo-murolic acids as the aglycones and a saccharide moiety linked at C-18 made up of four glucoses. The structures were elucidated using extensive spectroscopic analysis (1D and 2D NMR, MS, IR, UV and CD) and by chemical methods. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Glucosides; Lichens' acids; Lichens; Central Asia

1. Introduction

Lichens are symbiotic associations comprising fungal (mycobiont) and algal or cyanobacterial (photobiont) partners. They occur in a great variety of different habitats, and can survive severe environmental conditions such as drought, low temperatures, continuous light, or prolonged darkness. It has been suggested that in response to these stresses, natural selection has favoured species producing high concentrations of secondary metabolites (Ravinskaja, 1984).

Since ancient times some lichens have been used medicinally (Ichonose et al., 1994). Chemical and pharmacological investigations have shown that lichen secondary metabolites exhibit interesting biological activities and can be useful as antibiotics, UV absorbents, and antioxidants (Yamamoto et al., 1993).

As a continuation of our search for unusual bioactive compounds from Central Asian lichens (Řezanka and Dembitsky, 1998, 1999; Řezanka and Guschina, 1999, 2000), we examined the following nine lichen species: *Acarospora gobiensis* H. Magn.; *Cladonia furcata* (Huds.) Schrad., *Lecanora fructulosa* (Dicks.) Ach.; *Leptogium saturninum* (Dicks.) Nyl.; *Peltigera canina* (L.) Willd.; *Rhizoplaca peltata* Ram.; *Parmelia camtschadalis* Ach., *Parmelia tinctina* Mah. et Gill. and *Xanthoria elegans* (Link.) Th. Fr. These lichens were collected in the foothills of the Kungey-Alatau mountain range, Tien Shan Mountains, Issyk-Kul region (Republic of Kirghizstan). Prolonged periods of drought, high temperatures, and high levels of UV radiation characterize this region.

Although thousands of plant glycosides have been discovered in nature (Ikan, 1999), a much smaller number have been identified in lichens e.g. 1-(*O*-α-D-glucopyranosyl)-3 *S*,25*R*-hexacosanediol from *Solorina crocea* (Huneck and Yoshimura, 1996), and galapagin, lobodirin, mollin and roccellin (Huneck et al., 1992). The present study reports the structural elucidation of eight new glycosides (**1–8**), having murolic, protoconstipatic and allo-murolic acids as the aglycones and an oligosaccharide moiety linked at C-18 made up of two or three or four glucoses.

2. Results and discussion

In a recent paper (Řezanka and Guschina, 2000), we reported the isolation of several organic acids in lichens, murolic, protoconstipatic, allo-murolic acids and (18*R*)-18-*O*-β-D-glucopyranoside of murolic acid, and eight new glycosides from the water–methanolic extract of the Central Asian lichens collected in July 1996 along the shores of lake Issyk Kul, Tian-Shan mountain. Further fractionation of this extract by Sephadex LH-20 chromatography, followed by reversed-phase preparative HPLC was used to separate the new glucosides (**1–8**), together with the above four known compounds (Table 1). The structures of compounds were determined

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Table 1
Occurrence of lichens' acids and their glucosides from lichens of Tian Shan Mountains

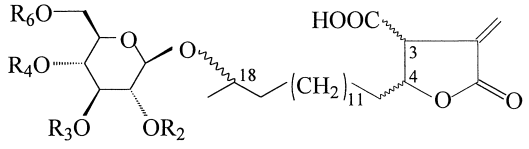
Name of lichen	Name of compounds; mg/100 g of dry weight												
	A	**B**	**C**	**D**	**E**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Acarospora gobiensis	22.5	–[a]	–	8.7	–	–	–	–	–	–	–	–	3.2
Cladonia furcata	–	24.3	–	–	11.4	0.5	–	–	2.9	0.8	–	7.8	–
Lecanora fructulosa	–	13.5	–	–	–	3.8	–	–	3.4	1.9	–	1.5	–
Leptogium saturninum	–	–	19.8	–	–	–	6.8	7.1	–	–	3.9	–	–
Parmelia camtschadalis	–	–	25.4	–	–	–	0.9	0.9	–	–	1.4	–	–
P. tinctina	–	19.1	–	–	12.0	4.2	–	–	6.3	1.4	–	4.3	–
Peltigera canina	–	22.7	–	–	–	0.2	–	–	5.7	3.1	–	0.7	–
Rhizoplaca peltata	13.8	–	–	1.8	–	–	–	–	–	–	–	–	6.1
Xanthoria elegans	–	–	5.4	–	–	–	2.9	6.3	–	–	4.1	–	–

[a] Less than 0.1 mg/100 g of dry wt.
A = 18-hydroxy-murolic acid, B = 18-hydroxy-protoconstipatic acid, C = 18-hydroxy-allo-murolic acid, D = (18*R*)-18-*O*-*β*-D-glucopyranoside of murolic acid, E = (18*S*)-18-*O*-*β*-D-glucopyranoside of protoconstipatic acid.

unambiguously using both 1D and 2D ^{1}H and ^{13}C NMR experiments in conjunction with the analysis of mass spectral and other spectroscopic data (UV, CD and IR).

Compound **1** was obtained after HPLC as a white amorphous powder with $[\alpha]_D^{23} -14^{\circ}$ but without an exact melting point as the glycoside decomposed. The molecular weight determined by positive HRFABMS (high resolution fast atom bombardment mass spectrometry) was 693.8068 $(M+H)^+$, which corresponds to a molecular formula of $C_{33}H_{56}O_{15}$, and negative FABMS gave two ions at *m/z* 529 $(M\text{-}H\text{-}162)^-$ and *m/z* 367 $(M\text{-}H\text{-}2\times162)^-$. The exact disposition of the two-monosaccharide units and the position the interglycosidic linkage in compound **1** was determined using 2D NMR spectroscopy. HOHAHA (homonuclear Hartman–Hahn) experiments allowed resolution of the overlapped spectral region of the disaccharide moiety of **1** into a subset of individual monosaccharide spectra; 2D-COSY (correlated spectroscopy) experiments established the proton sequence with each sugar fragment starting from the well-resolved anomeric proton signals of a *β*-D-glucopyranoses (*δ* 4.71, *d*, *J* = 7.0 Hz and *δ* 5.45, *d*, *J* = 7.9 Hz) (Table 3). Chemical shifts, multiplicity of the proton signals, values of coupling constants, and chemical shifts of carbons indicated that the sugars must be in the *β*-D-glucopyranosyl forms (Tables 2 and 3) (Kasai et al., 1977).

In compound **1** a glycosidation shift (Tori et al., 1977) at C-2′ (ca +8.5 ppm) and the chemical shifts of H-1′ (*d* 4.71)



1 3S, 4S, 18R, R_2 = Glc, $R_3 = R_4 = R_6$ = H
2 3S, 4R, 18R, R_3 = Glc, $R_2 = R_4 = R_6$ = H
3 3R, 4S, 18S, R_6 = Glc, $R_2 = R_3 = R_4$ = H
4 3S, 4S, 18R, $R_2 = R_3$ = Glc, $R_4 = R_6$ = H
5 3S, 4S, 18R, $R_2 = R_6$ = Glc, $R_3 = R_4$ = H
6 3R, 4S, 18S, R_2 = Glc′′2-1Glc′′′, $R_3 = R_4 = R_6$ = H
7 3S, 4R, 18R, $R_3 = R_6$ = Glc, $R_2 = R_4$ = H
8 3R, 4S, 18S, R_2 = Glc′′6-1Glc′′′′, R_3 = Glc, $R_4 = R_6$ = H

Fig. 1. The structures of glucosides (**1–8**) from lichens.

Table 2
^{13}C NMR spectra of glucosides (**1–8**)

No. of atom	Compound (*δ* relative to TMS)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1′	101.4	103.8	104.6	104.4	104.3	104.8	106.3	105.2
2′	**82.0**	73.5	73.7	**81.1**	**82.3**	**82.4**	73.4	**81.8**
3′	77.9	**87.6**	76.7	**89.0**	77.4	77.2	**88.2**	**87.8**
4′	71.1	70.1	70.5	69.9	71.2	72.3	69.3	71.3
5′	77.4	78.2	75.4	78.0	77.2	77.4	76.7	75.5
6′	63.1	62.3	**68.5**	62.1	**68.3**	62.4	**68.9**	62.2
1″	106.8	104.9	103.4	104.0	103.5	103.5	105.6	104.4
2″	76.0	75.2	73.1	76.1	76.1	**85.9**	75.8	75.3
3″	78.5	78.4	77.5	78.9	78.5	77.9	78.0	77.8
4″	71.9	70.5	69.2	71.2	72.8	71.7	71.4	71.6
5″	76.9	78.0	77.0	77.7	78.3	79.4	78.8	77.3
6″	62.6	62.6	61.1	62.5	63.7	62.6	62.4	**70.3**
1‴				105.2	105.0	106.9	103.2	104.8
2‴				75.3	75.5	77.0	74.9	75.9
3‴				78.7	78.9	77.7	77.8	77.6
4‴				71.5	71.8	69.5	71.0	71.2
5‴				78.3	78.0	78.3	78.3	78.5
6‴				61.8	63.0	61.1	62.1	62.4
1⁗								104.1
2⁗								76.9
3⁗								78.1
4⁗								70.9
5⁗								78.0
6⁗								62.7

Table 3
^{1}H NMR spectra of glucosides (**1–3**)

No. of atom	Compound (δ; relative to TMS)		
	1	**2**	**3**
1′	5.07 (1H, *d*, $J=7.0$)	5.01 (1H, *d*, $J=7.0$)	5.12 (1H, *d*, $J=7.5$)
2′	3.98 (1H, *dd*, $J=7.0$; 9.0)	4.12 (1H, *dd*, $J=7.0$, 9.0)	4.24 (1H, *dd*, $J=7.8$, 8.8)
3′	3.94 (1H, *t*, $J=9.0$)	4.18 (1H, *dd*, $J=9.0$, 9.0)	4.32 (1H, *t*, $J=8.0$)
4′	3.64 (1H, *dd*, $J=9.3$; 9.3)	4.23 (1H, *dd*, $J=9.0$, 9.0)	4.30 (1H, *t*, $J=7.4$)
5′	3.68 (1H, *m*)	4.03 (1H, *m*)	4.15 (1H, *m*)
6′	4.09 (1H, *dd*, $J=12.0$, 5.0)	4.21 (1H, *dd*, $J=12.1$, 2.5)	4.38 (1H, *dd*, $J=11.1$, 7.0)
	4.38 (1H, *dd*, $J=12.0$, 1.4)	4.41 (1H, *dd*, $J=12.1$, 5.7)	4.72 (1H, *dd*, $J=11.1$, 3.0)
1″	5.45 (1H, *d*, $J=7.9$)	5.25 (1H, *d*, $J=7.5$)	5.16 (1H, *d*, $J=6.9$)
2″	4.01 (1H, *dd*, $J=8.7$, 7.9)	4.05 (1H, *dd*, $J=7.5$, 7.5)	3.95 (1H, *dd*, $J=8.2$, 7.4)
3″	4.15 (1H, *dd*, $J=8.7$, 8.7)	4.14 (1H, *dd*, $J=7.5$, 8.5)	4.07 (1H, *dd*, $J=7.4$, 9.0)
4″	4.11 (1H, *dd*, $J=8.8$, 8.7)	4.10 (1H, *dd*, $J=8.5$, 8.0)	4.01 (1H, *t*, $J=9.0$, 8.0)
5″	4.03 (1H, *m*)	4.00 (1H, *ddd*, $J=8.0$, 4.8, 2.1)	3.82 (1H, *dq*, $J=9.4$, 6.2)
6″	4.23 (1H, *dd*, $J=10.8$, 5.5)	4.25 (1H, *dd*, $J=12.3$, 4.8)	4.17 (1H, *dd*, $J=11.2$, 5.8)
	4.35 (1H, *dd*, $J=10.8$, 1.7)	4.50 (1H, *dd*, $J=12.3$, 2.1)	4.26 (1H, *dd*, $J=11.2$, 1.2)

and C-1′ (δ 101.4) of glucose indicated this monosaccharide was glycosidated at C-2 and linked at the aglycon. The one signal due to the anomeric proton of glucose (δ 5.45, *d*, $J=7.9$ Hz), correlating to the C-1″ resonance at δ 106.8 by HETCOR indicated that the glucose 2 unit was linked to a secondary alcoholic carbon (C-2′ of the glucose 1). By HOHAHA, this proton showed connectivities with a signal δ 4.01 (*dd*, $J=7.9$, 8.7 Hz, H-2″ by COSY), which was correlated by HETCOR (heteronuclear correlation) to a carbon resonance at δ 82.0 (C-2′) showing glycosylation at position 2′. The glucose 2 was determined to be terminal by the absence of a glycosylation shift. These deductions were confirmed by a COLOC (correlation via long range couplings) spectrum, which showed some diagnostic long-range correlations between H-1′ of glucose (δ 4.71) and C-18 (δ 61.0) of the aglycone (Rieser et al., 1992; Seo et al., 1978), between H-1″ (δ 5.45) of the glucose 2 unit and C-2′ (δ 82.0) of glucose 1, (see Tables 2 and 3). The structure of glucoside **1** was determined as the (18*R*)-18-*O*-β-D-glucopyranosyl-(1-2)-β-D-glucopyranoside of allo-murolic acid.

Acid methanolysis of **2** gave only glucose. Positive HRFABMS of **2** give a pseudomolecular ion at *m*/*z* 693.8064 $(M+H)^+$, ($C_{33}H_{56}O_{15}$) and showed the negative FABMS $(M-H)^-$ ion at *m*/*z* 691 with prominent fragments at *m*/*z* 529 $(M-H-162)^-$ and 513 $(M-H-178)^-$ (cleavage of a hexose unit with or without the glycosidic oxygen) and at *m*/*z* 367 $(M-H-162-162)^-$ due to the subsequent loss of two hexose units. The DEPT (distortionless enhancement by polarization transfer) ^{13}C NMR spectrum showed 33 signals, of which 12 were assigned to the saccharide portion and 21 to the aglycone moiety. The oligosaccharide structure was determined by 2D NMR. Even at high field (500 MHz) the 1D sugar spectral region of **2** was complex as most of the shifts were found between δ 4.00 and 5.25 and were overlapped by the aglycone signals. 1D and 2D TOCSY (total correlated spectroscopy) spectroscopy experiments allowed resolution of the overlapped spectrum of oligosaccharide into a subset of individual monosaccharide spectra. In the 1D and 2D TOCSY spectrum of **2** the anomeric proton signal ascribable to a β-D-glucopyranose (H-1′, δ 5.01, *d*, $J=7.0$ Hz) showed connectivities to four methine groups. This together with the 2D DQF-COSY (double quantum coherence-correlated spectroscopy) spectrum established the proton sequence within this sugar fragment as H-1′ (δ 5.01), H-2′ (δ 4.12), H-3′ (δ 4.18), H-4′ (δ 4.23) and H-5′ (δ 4.03), H-6a′ (δ 4.21), H-6b′ (δ 4.41) (Table 3).

Similar observations of the TOCSY and COSY experiments for second sugar residue (Tables 2 and 3) allowed complete sequential assignments for all proton resonances starting from the anomeric proton signal. HSQC experiments which correlated all proton resonances with those of each corresponding carbon (Tables 2 and 3) permitted assignments of the interglycosidic linkages by comparison of the ^{13}C shifts with those of the corresponding pyranosides, taking into account the known effects of glycosidation. The absence of any ^{13}C glycosidation shift for second glucopyranosyl residue suggested that this sugar was a terminal unit, while the glycosidation shift on C-3′ ($\delta+9.7$ ppm) of the glucopyranosyl unit linked at C-18 by a glycosidic bond allowed us to establish the presence of two glucopyranosyl residues at C-18. The position of each sugar unit was deduced from an HMBC experiment. The ^{1}H NMR and ^{13}C NMR data indicated the β configuration at the monomeric positions for glucopyranosyl units (Table 3). Therefore the structure (18*R*)-*O*-β-D-glucopyranosyl-(1-3)-β-D-glucopyranoside of murolic acid was assigned to **2**.

Compound **3**, obtained as an amorphous white powder, presents in its positive HRFABMS spectrum a pseudomolecular peak $(M+H)^+$ at *m*/*z* 693.8066, corresponding to the molecular formula $C_{33}H_{56}O_{15}$. In the

negative FABMS spectrum there is, besides the molecular peak $(M\text{-}H)^-$ at m/z 691, a peak due to the loss of the glucose $(M\text{-}H\text{-}162)^-$ at m/z 529 and of gentiobiose $(M\text{-}H\text{-}324)^-$ at m/z 367. Its ^{13}C NMR spectrum shows 33 resonances, sorted by DEPT experiments in to 1 CH_3, 14 CH_2, 3 CH, and 3 quaternary C. The remaining 12 resonances of the ^{13}C NMR spectrum of **3** are generated by two hexoses, and indicate exactly two β-D-glucopyranoses 1–6 linked to form the disaccharide gentiobiose, as demonstrated by the downfield shift of the inner sugar methylene (C-6′). The ^1H NMR spectrum pattern of the aglycone moiety is very similar to that of protoconstipatic acid. Similar to (18 *S*)-18-*O*-β-D-glucopyranoside of protoconstipatic acid (Řezanka and Guschina, 2000), the aglycone was determined as protoconstipatic acid, and the sugar moiety as gentiobiose with the site of glycosylation at the 18-OH. So compound **3** can be identified as the (18 *S*)-18-*O*-β-D-glucopyranosyl-(1-6)-β-D-glucopyranoside of protoconstipatic acid.

Acid hydrolysis of **4** afforded only D-glucose, confirmed by specific rotation $[\alpha]_D^{23}-52.7$. The white powder of glucoside **4** gave positive HRFABMS m/z 855.9503 $(M+H)^+$, corresponding to formula $C_{39}H_{66}O_{20}$; in negative FABMS ions at m/z 691 $(M\text{-}H\text{-}162)^-$, m/z 529 $(M\text{-}H\text{-}2\times162)^-$ and 367 $(M\text{-}H\text{-}3\times162)^-$ occurred. In the ^1H NMR spectrum of **4**, three anomeric proton signals appeared at δ 5.64, 5.60, and 5.34, indicating that each a glucose has a β-configuration. The corresponding three-anomeric carbons were observed at δ 104.4, 104.0, and 105.2. Also, the downfield shifted ^{13}C NMR resonances among the sugar units were observed at δ 81.1 and 89.0, indicating the probable points of glycosidic linkage in the oligosaccharide to be at C-2′ and C-3′. ^1H-^1H COSY and HMQC experiments revealed the glycosidic attachments at C-2′ (δ 81.1) and C-3′ (δ 89.0) for glucose (glucose 1). Further, the HMBC spectrum showed connectivities between the H-1′ proton of glucose 1 and C-18 of the aglycone, the H-1″ proton of glucose 2 and C-2′ of glucose 1, and the H-1‴ proton of glucose 3 and C-3′ of glucose 1. Thus, **4** was formulated as (18*R*)-18-*O*-β-D-glucopyranosyl-(1-3)-[β-D-glucopyranosyl-(1-2)]-β-D-glucopyranoside of allo-murolic acid.

FABMS in the negative-ion mode were run for compound **5**, which gave $(M\text{-}H)^-$ ions at m/z 853, and in positive HRFABMS the corresponding ion at 855.9509 $(M+H)^+$ gave the molecular formula $C_{39}H_{66}O_{20}$. The ^1H NMR spectrum of **5** was typical for a glycoside, with the sugar region showing three anomeric proton doublets, with coupling constants characteristic of β-anomers ($J_{1,2}$ ca 8 Hz). The glycosidic protons were resolved well enough to allow most of the coupling constants to be measured. Assignment of the different resonances to each one of the three residues was achieved through COSY and TOCSY experiments. The large coupling constants found for protons H-2, H-3, and H-4 of the spin-systems of each residue were consistent with glucose moieties (see Table 4). The ROESY (rotating frame nuclear Overhauser effect spectroscopy) spectrum of **5**, in addition to the expected intraresidue signals, gave the cross peaks H-1″−H-2′, H-1‴−H-6a′, and H-1‴−H-6b′, suggesting that the glucose-2 and glucose-3 units are linked to glucose-1 at C-2′ and C-6′, respectively.

The ^{13}C NMR spectrum of **5** gave 39 carbons and supported the deductions derived from the interpretation of the ^1H NMR spectrum. Compound **5** showed one carbonyl peak, i.e. δ 173.8 (carboxylic). The rest of the ^{13}C NMR chemical shifts were assigned from the HMQC spectrum (see Table 2). The values for the aglycone moiety agreed with those published for similar aglycones (Řezanka and Guschina, 2000), and the value of the anomeric carbon for glucose-1 δ 104.3 demonstrated that it was linked as an acetal to the C-18 hydroxyl group of the aglycon. In addition, C-2′ and C-6′ of the same unit gave values shifted downfield from those expected for unsubstituted glucopyranose, which indicated that glucose-1 was glycosylated at these positions by the other two glucose units (glucose-2 and glucose-3), thus supporting the ROESY results. Unequivocal demonstration of the type of glycosidic linkages was obtained from a HMBC experiment, which, in addition to expected intraresidue peaks for all different moieties (the aglycon and the three sugar residues), gave the cross peaks H-1″−C-2′, H-1‴−C-6′ and H-18−H-1′. Accordingly, glycoside **5** was assigned as the (18*R*)-18-*O*-β-D-glucopyranosyl-(1-2)-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranoside of allo-murolic acid.

Compound **6** was obtained as an amorphous powder. Acid hydrolysis of **6** afforded only protoconstipatic acid as the aglycon and D-glucose as the sugar components. The positive FAB mass spectrum of **6** showed a protonated molecular ion at m/z 855.9503 $(M+H)^+$ as a base peak (corresponding to formula $C_{39}H_{66}O_{20}$) together with a fragment ion at m/z 529 $(M\text{-}H\text{-}2(\text{hexose})^-$ (negative FABMS). The ^{13}C and DEPT NMR spectra of **6** showed 39 signals, of which 18 were assigned to the saccharide portion and 21 to the aglycon moiety. The ^1H NMR spectrum showed three anomeric proton signals at δ 5.05 (1 H, *d*, $J=7.7$ Hz), δ 5.58 (1 H, *d*, $J=8.4$ Hz), and δ 5.28 (1H, *d*, $J=8.2$ Hz). The ^{13}C signals of **6** were superimposable onto those of glycosides **4** and **5**, except for the signals due to the sugar moiety. In the ^{13}C NMR spectrum the presence of three anomeric signals at δ 104.8, 103.5, and 106.9 confirmed the three sugar residues. Assignments of the sugar proton resonances were achieved by a ^1H-^1H COSY spectrum, and the ^{13}C data were assigned by a HMQC (heteronuclear multiple quantum coherence) spectrum. Interglycosidic linkages were established by HMBC techniques. The HMBC spectrum of **6** showed cross peaks between the signals at δ 5.05 (glucose H-1′) and δ 61.0 (C-18 of the

Table 4
^{1}H NMR spectra of glucosides (**4–7**)

	Compound (δ relative to TMS)			
No. of atom	**4**	**5**	**6**	**7**
1′	5.64 (1H, *d*, J=8.0)	5.61 (1H, *d*, J=8.2)	5.05 (1H, *d*, J=7.7)	5.18 1H, *d*, J=7.9)
2′	4.56 (1H, *t*, J=8.0)	4.06 (1H, *dd*, J=8.2; 9.2)	4.31 (1H, *dd*, J=7.7, 8.7)	4.30 (1H, *dd*, J=7.9, 8.9)
3′	4.36 (1H, *dd*, J=9.0, 9.0)	3.97 (1H, *dd*, J=9.2, 9.7)	4.70 (1H, *dd*, J=9.1, 8.7)	4.25 (1H, *dd*, J=9.0, 8.9)
4′	4.16 (1H, *dd*, J=9.0, 9.0)	3.84 (1H, *dd*, J=9.7; 9.2)	4.01 (1H, *dd*, J=9.1, 10.0)	4.33 (1H, *dd*, J=9.0, 9.4)
5′	4.05 (1H, *ddd*, J=9.0, 5.0, 2.0)	3.74 (1H, *ddd*, J=9.2, 4.9, 2.0)	3.77 (1H, *td*, J=10.0, 8.1)	4.10 (1H, *ddd*, J=9.4, 4.5, 1.8)
6′	4.29 (1H, *dd*, J=12.0, 2.0)	4.17 (1H, *dd*, J=11.6, 4.9)	4.08 (1H, *dd*, J=11.3, 6.9)	4.28 (1H, *dd*, J=9.4, 4.5)
	4.58 (1H, *dd*, J=12.0, 5.0)	4.27 (1H, *dd*, J=11.6, 2.2)	4.37 (1H, *dd*, J=11.3, 4.1)	4.51 (1H, *dd*, J=9.4, 4.5)
1″	5.60 (1H, *d*, J=8.0)	5.48 (1H, *d*, J=7.7)	5.58 (1H, *d*, J=8.4)	5.35 (1H, *d*, J=8.2)
2″	4.10 (1H, *dd*, J=8.5, 8.0)	3.92 (1H, *dd*, J=7.7, 8.7)	4.76 (1H, *dd*, J=8.4, 8.7)	4.08 (1H, *dd*, 8.2, 8.7)
3″	4.27 (1H, *dd*, J=8.5, 8.5)	4.10 (1H, *dd*, J=8.7, 8.8)	3.94 (1H, *dd*, J=8.7, 8.8)	4.17 (1H, *dd*, J=8.7, 8.6)
4″	4.31 (1H, *dd*, J=9.5, 8.5)	4.19 (1H, *dd*, J=8.8, 10.8)	4.39 (1H, *dd*, J=8.8, 9.3)	4.12 (1H, *dd*, J=8.6, 8.3)
5″	3.85 (1H, *ddd*, J=9.0, 4.0, 2.5)	4.08 (1H, *ddd*, J=1.6, 7.0, 10.8)	3.97 (1H, *m*)	3.98 (1H, *ddd*, J=8.3, 4.1, 1.9)
6″	3.99 (1H, *dd*, J=12.5, 2.5)	4.12 (1H, *dd*, J=11.8, 7.0)	4.35 (1H, *dd*, J=9.0)	4.20 (1H, *dd*, J=8.3, 4.1)
	4.21 (1H, *dd*, J=12.5, 4.0)	4.32 (1H, *dd*, J=11.8, 1.6)	4.67 (1H, *dd*, J=9.0)	4.38 (1H, *dd*, J= 8.3, 1.9)
1‴	5.34 (1H, *d*, J=8.0)	5.30 (1H, *d*, J=7.6)	5.28 (1H, *d*, J=8.2)	5.12 (1H, *d*, J=7.7)
2‴	4.08 (1H, *dd*, J=8.0, 8.0)	4.03 (1H, *dd*, J=7.6, 9.1)	4.05 (1H, *dd*, J=8.2, 8.0)	4.05 (1H, *dd*, J=7.7, 8.4)
3‴	4.25 (1H, *dd*, J=8.0, 8.0)	4.24 (1H, *dd*, J=9.1, 9.2)	4.22 (1H, *dd*, J=8.0, 8.1)	4.23 (1H, *dd*, J=8.4, 8.3)
4‴	4.13 (1H, *dd*, J=8.1, 8.0)	4.15 (1H, *dd*, J=9.2, 9.4)	4.10 (1H, *dd*, J=8.1, 9.5)	4.14 (1H, *dd*, J=8.3, 10.2)
5‴	3.95 (1H, *ddd*, J=8.1, 5.2, 2.3)	4.00 (1H, *ddd*, J=9.4, 4.9, 2.0)	3.74 (1H, *ddd*, J=9.5, 5.7, 2.4)	3.92 (1H, *ddd*, J=10.2, 4.2, 2.1)
6‴	4.19 (1H, *dd*, J=12.0, 5.0)	4.29 (1H, *dd*, J=2.2, 4.9)	3.74 (1H, *dd*, J=10.7, 5.7)	4.01 (1H, *dd*, J=10.2, 2.1)
	4.41 (1H, *dd*, J=12.0, 2.5)	4.48 (1H, *dd*, J=12.2, 4.0)	4.28 (1H, *dd*, J=10.7, 2.4)	4.35 (1H, *dd*, J=10.2, 4.2)

aglycon), 5.58 (inner glucose H-1″) and 82.4 (glucose C-2′), and 5.28 (terminal glucose H-1‴) and 85.9 (inner glucose C-2″). The anomeric configurations of the glucoses were determined as β based on the large coupling constants ($J_{1,2}$~8 Hz) in the ^{1}H NMR spectrum. Furthermore, acid hydrolysis of **6** gave protoconstipatic acid and D-glucose. Thus, the structure of glycoside **6** was determined to be protoconstipatic acid (18 *S*)-18-*O*-β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl-(1-2)-β-D-glucopyranoside.

Glycoside **7** had the same molecular formula $C_{39}H_{66}O_{20}$ as **4–6** from its positive HRFABMS (m/z 855.9508; i.e. $[M+H]^+$), negative FABMS (give ion at m/z 853 $(M\text{-}H)^-$ and from ^{13}C DEPT NMR data. Its ^{1}H and ^{13}C NMR spectra indicated that **7** possessed the same aglycon as **5** but differed in the sugar (Tables 2 and 4). Three sugar units in **7** were indicated, as there were three anomeric protons and carbons (Tables 2 and 4). It was apparent that the three sugars were present in one saccharide unit attached to C-18. The overall structure assignment was accomplished using the same protocol as for the above-mentioned glucosides, which permitted the full assignment of the protons and carbons, and the sugar components were identified as glucoses. The linkage positions for the sugar units were established using the HMBC and NOESY correlations. Compound **7** had nearly the same sugar arrangement as **5** except that the terminal glucose at the C-2′ in **5** was replaced by glucose and linked to the C-3′ instead. The linkage was also supported from fragmentation patterns observed in the FABMS experiment. FABMS analysis of the deprotonated molecular ion $(M\text{-}H)^-$ at m/z 853 gave the splitter ions at m/z 691 $(M\text{-}H\text{-}162)^-$ and m/z 673 $(M\text{-}H\text{-}H_2O\text{-}162)^-$ by the loss of one of the terminal hexose units. Furthermore, subjected to MS analysis, the prominent fragment at m/z 367 is formed by loss of the trisaccharide chain linked to C-18 of glycoside. The β configuration for the sugars was determined from their $^3J_{1,2}$ (Table 4). Based on the above information, glycoside **7** is the (18 *R*)-18-*O*-β-D-glucopyranosyl-(1-3)-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranoside of murolic acid.

An elemental formula of $C_{45}H_{76}O_{25}$ was suggested for **8** from its FABMS (both positive- and negative-ion) and ^{13}C NMR data. In the ^{13}C NMR spectrum, olefinic carbons at δ 126.1 and 133.7 were observed and gave evidence for the presence of one double bond. A characteristic CH_2 doublet of protons in the ^{1}H NMR spectrum at δ 6.02 and δ 6.37 (J=3.0 Hz), indicated that the aglycon included a methylene group and hence might be γ-lactonic acid, as was seen for the methylene group in protoconstipatic acid. The saccharide composition of **8** was identified by GC and the sugars were identified as glucose. In the ^{1}H NMR spectrum of **8**, four anomeric proton signals at δ 4.93, 5.58, 5.27, and 5.14 were observed, corresponding to signals at δ 105.2, 104.4, 104.8, 104.1, respectively, and indicating that, **8** possesses four sugar units. All four anomeric protons showed β glycosidic linkages according to the coupling constants of their anomeric protons (J= 7.6–7.8 Hz). From the ^{1}H-^{1}H COSY and TOCSY spectra, all proton signals belonging to each sugar moiety in **8** were identi-

Table 5
^{1}H NMR spectra of glucoside (**8**)

No. of atom	Compound (δ relative to TMS) **8**
1′	4.93 (1H, *d*, J=7.6)
2′	4.65 (1H, *dd*, J=7.7, 8.7)
3′	4.20 (1H, *dd*, J=9.1, 8.7)
4′	3.78 (1H, *dd*, J=9.1, 10.0)
5′	4.04 (1H, *dd*, J=10.0, 8.1)
6′	4.57 (1H, *dd*, J=11.3, 6.9)
	4.22 (1H, *dd*, J=11.3, 1.4)
1″	5.58 (1H, *d*, J=7.7)
2″	4.12 (1H, *dd*, J=8.4, 8.7)
3″	4.32 (1H, *dd*, J=8.7, 8.8)
4″	4.28 (1H, *dd*, J=8.8, 9.3)
5″	3.99 (1H, *ddd*, J=2.0 7.0, 10.8)
6″	4.62 (1H, *dd*, J=11.8, 7.0)
	4.73 (1H, *dd*, J=11.8, 1.6)
1‴	5.27 (1H, *d* , J=7.8)
2‴	4.05 (1H, *dd*, J=7.6, 9.1)
3‴	4.53 (1H, *dd*, J=9.1, 9.2)
4‴	4.17 (1H, *dd*, J=9.2, 9.4)
5‴	4.07 (1H, *dd*, J=9.4, 4.9, 2.0)
6‴	4.41 (1H, *dd*, J=12.2, 4.9)
	4.30 (1H, *dd*, J=12.2, 2.0)
1⁗	5.14 (1H, *d*, J=7.8)
2⁗	4.02 (1H, *dd*, J=8.2, 9.2)
3⁗	4.25 (1H, *dd*, J=9.2, 9.7)
4⁗	4.26 (1H, *dd*, J=9.7, 9.2)
5⁗	4.09 (1H, *ddd*, J=9.2, 4.9, 2.0)
6⁗	4.24 (1H, *dd*, J=11.6, 4.9)
	4.38 (1H, *dd*, J=11.6, 2.2)

fied, starting from the anomeric protons. All sugar connectivities were established using NOESY and HMBC experiments. In the NOESY spectrum cross-peak signals were observed between H-1′ and H-18, H-1″ and H-2′, H-1‴ and H-3′, H-1⁗ and H-6″. The HMBC experiment showed long range correlations between H-1′ and C-18, H-1″ and C-2′, H-1‴ and C-3′, H-1⁗ and C-6″. Thus, the structure of **8** was assigned as the (18 *S*)-18-*O*-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl-(1-3)-β-D-glucopyranoside of protoconstipatic acid.

To summarize the present study, the chemical structures and structural relationships among the 8 component glucosides produced by lichens from Central Asia are shown in Fig. 1.

We have isolated new glucosides containing from one to four glucose units. The above-mentioned glycosides were identified in lichens for the first time.

In contrast to glucosidic compounds from higher plants, the first glucosides identified from lichens (with different amount of glucoses) exhibit more variability of aglycones and saccharides. This may be caused by the very stressful environment, in which lichens grow, namely temperatures as high as 80°C, desiccation by wind and saline substrates.

3. Experimental

3.1. General experimental procedures

UV spectra were measured in heptane within the range of 200–350 nm by a Cary 118 (Varian) apparatus. Circular dichroism (CD) measurement was carried out under a dry N_2 on a Jasco-500A spectropolarimeter at 24°C. A Perkin–Elmer Model 1310 (Perkin–Elmer, Norwalk, CT, USA) IR spectrophotometer was used for scanning IR spectroscopy of acids and glycosides as neat films. 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.

3.2. Plant material

The specimens of *Acarospora gobiensis* H. Magn., *Cladonia furcata* (Huds.) Schrad, *Lecanora fructulosa* (Dicks.) Ach., *Leptogium saturninum* (Dicks.) Nyl., *Peltigera canina* (Neck.) Hoffm., *Rhizoplaca peltata* Ram., *Parmelia camtschadalis* Ach., *P. tinctina* Mah. et Gill., and *Xanthoria elegans* (Link.) Th. Fr. were collected in July 1996 along the shore of Lake Issyk Kul, Tian-Shan mountain.

3.3. Extraction and isolation

The aqueous-MeOH layer obtained from the Blight and Dyer (1959) method of lipid extracts from 100 g of air-dried lichens, (see Řezanka and Guschina, 2000) was separated on a Sephadex LH-20 column eluting with MeOH–H_2O (9:1) yielding three fractions. Fraction A was further fractionated by RP-HPLC on a C18-Bondapak column (30 cm×7.8 mm, flow rate 2.0 ml/min) with ACN–H_2O (1:2) to yield compounds **1–3**. Fraction B was separated by RP-HPLC with ACN–H_2O (2:5) to yield the compounds **4–7**. Fraction C using ACN–H_2O (1:3), yielded only compound **8**.

3.4. Acid hydrolysis of glycosides

Glycosides (~0.5 mg) were refluxed in 2 N HCl (0.5 ml) for 2 h. Aglycone were extracted three times with EtOAc (10 ml). After separating the organic layer, the aqueous phase was neutralized with $NaHCO_3$ and lyophilized.

The identification and the D or L configuration of sugars was determined using gas chromatography according to the method of Gerwig et al. (1978), with some modifications, as previously described (Řezanka and Guschina, 2000).

3.5. Enzymatic hydrolysis and determination of the glycosides

A solution of glycoside (2 mg) in acetate buffer (pH 4.4, 10 ml) was treated with β-glucosidase (20 mg) for 48 h at 37°C. The reaction solution was evaporated to dryness, and the residue was chromatographed on a column of silica gel (10 g), using CH_2Cl_2–MeOH–H_2O (90:10:1) to provide lichen acids for ^{1}H NMR and CD analysis.

3.6. (S)-MTPA and (R)-MTPA esters

(S)-MTPA ester of aglycones from glucosides **1–8** (Ohtani et al., 1991a,b). To a CH_2Cl_2 solution (100 μl) of aglycone (0.3 mg), DMAP (1.0 mg), and Et_3N (2 μl) was added (R)-(−)-MTPACl (2.0 mg) at room temperature, and stirring was continued for 3 h. After evaporation of solvent, the residue was purified by Si gel TLC (hexane–AcOEt, 2:1) to provide the (S)-MTPA ester as a colorless oil.

(R)-MTPA Ester of aglycones from glucosides **1–8**. Each aglycone (0.3 mg) was treated with (S)-(+)-MTPACl (2.0 mg) by the same procedure as described above to provide the (R)-MTPA ester as colorless oil.

Murolic acid, colorless plates, m.p. 112.5°C, $[\alpha]_D^{24}+11.3$ (*c* 0.15, $CHCl_3$), UV, CD, and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

Protoconstipatic acid, white flakes, m.p. 104°C, $[\alpha]_D^{24}-12.8$ (*c* 0.16, $CHCl_3$), UV, CD and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

allo-Murolic acid, white plates, m.p. 89.5°C, $[\alpha]_D^{24}-15.9$ (*c* 0.11, $CHCl_3$), UV, CD and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

(18*R*)-18-*O*-β-D-Glucopyranoside of murolic acid, colorless powder, mp 146.5°C, $[\alpha]_D^{24}-188°$ (*c* 0.15, MeOH); UV 219 nm (log ε 3.88); HRFABMS *m/z* 531.6645 $[MH]^+$ and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

(18*S*)-18-*O*-β-D-Glucopyranoside of protoconstipatic acid, colorless powder, mp 157°C, $[\alpha]_D^{24}-179°$ (*c* 0.14, MeOH); UV 218 nm (log ε 3.82); HRFABMS *m/z* 531.6648 $(MH)^+$, and ^{1}H NMR spectra, (see Řezanka and Guschina, 2000).

(18*R*)-18-*O*-β-D-Glucopyranosyl-(1-2)-β-D-glucopyranoside of allo-murolic acid (**1**), white amorphous powder, $[\alpha]_D^{23}-14°$; HRFABMS *m/z* 693.8068 $(M+H)^+$, calculated for $[C_{33}H_{56}O_{15}+H]^+$ 693.8063; negative FABMS *m/z* 691 $(M-H)^-$, 529 $(M-H-162)^-$ and 367 $(M-H-162-162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 3.

(18*R*)-*O*-β-D-Glucopyranosyl-(1-3)-β-D-glucopyranoside of murolic acid (**2**), white powder, $[\alpha]_D^{23}-35°$; HRFABMS *m/z* 693.8064 $(M+H)^+$, calculated for $[C_{33}H_{56}O_{15}+H]^+$ 693.8062; negative FABMS *m/z* 691 $(M-H)^-$, 529 $(M-H-162)^-$, 513 $(M-H-178)^-$, 367 M-H-162-162)$^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 3.

(18*S*)-18-*O*-β-D-Glucopyranosyl-(1-6)-β-D-glucopyranoside of protoconstipatic acid (**3**), white powder, $[\alpha]_D^{23}-18°$; HRFABMS *m/z* 693.8066 $(M+H)^+$, calculated for $[C_{33}H_{56}O_{15}+H]^+$ 693.8070; negative FABMS *m/z* 691 $(M-H)^-$, 529 $(M-H-162)^-$, 367 $(M-H-162-162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 3.

(18*R*)-18-*O*-β-D-Glucopyranosyl-(1-3)-[β-D-glucopyranosyl-(1-2)]-β-D-glucopyranoside of allo-murolic acid (**4**), white powder, $[\alpha]_D^{23}-52.7°$; HRFABMS *m/z* 855.9503 $(M+H)^+$, calculated for $[C_{39}H_{66}O_{20}+H]^+$ 855.9499; negative FABMS *m/z* 691 $(M-H-162)^-$, 529 $(M-H-2\times162)^-$ and 367 $(M-H-3\times162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 4.

(18*R*)-18-*O*-β-D-Glucopyranosyl-(1-2)-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranoside of allo-murolic acid (**5**), colorless powder, $[\alpha]_D^{23}-42.0°$; HRFABMS *m/z* 855.9509 $(M+H)^+$, calculated for $[C_{39}H_{66}O_{20}+H]^+$ 855.9504; negative FABMS *m/z* 853 $(M-H)^-$, 691 $(M-H-162)^-$, 529 $(M-H-2\times162)^-$ and 367 $(M-H-3\times162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 4.

(18*S*)-18-*O*-β-D-Glucopyranosyl-(1-2)-β-D-glucopyranosyl-(1-2)-β-D-glucopyranoside of protoconstipatic acid (**6**), colorless powder, $[\alpha]_D^{23}-62.0°$; HRFABMS *m/z* 855.9503 $(M+H)^+$, calculated for $[C_{39}H_{66}O_{20}+H]^+$ 855.9500; negative FABMS *m/z* 529 (M-H-162-162)$^-$ and 367 (M-H-3×-162)$^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 4.

(18*R*)-18-*O*-β-D-Glucopyranosyl-(1-3)-[β-D-glucopyranosyl-(1-6)]-β-D-glucopyranoside of murolic acid (**7**), colorless powder, $[\alpha]_D^{23}-49.0°$; HRFABMS *m/z* $C_{39}H_{66}O_{20}$ $(M+H)^+$, calculated for $[C_{39}H_{66}O_{20}+H]^+$ 855.9508; negative FABMS *m/z* 853 $(M-H)^-$, 691 $(M-H-162)^-$, 673 (M-H-H2O-162)$^-$, 529 $(M-H-2\times162)^-$ and 367 $(M-H-3\times162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 4.

(18*S*)-18-*O*-[β-D-Glucopyranosyl-(1-6)]-β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl-(1-3)-β-D-glucopyranoside of protoconstipatic acid (**8**), white powder, $[\alpha]_D^{23}-91.0°$; HRFABMS *m/z* $C_{45}H_{76}O_{25}$ $(M+H)^+$, calculated for $[C_{45}H_{76}O_{25}+H]^+$ 1018.0914; negative FABMS *m/z* 1015 $(M-H)^-$, 853 $(M-H-162)^-$ and 691 $(M-H-2\times162)^-$; 529 $(M-H-3\times162)^-$; 367 $(M-H-4\times162)^-$; ^{1}H and ^{13}C NMR spectra, see Tables 2 and 5.

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