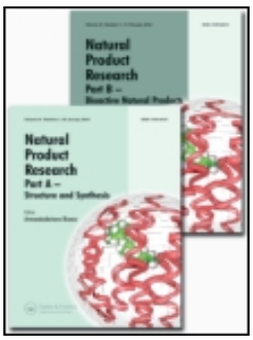

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Sugar composition of the moss *Rhodobryum ontariense* (Kindb.) Kindb.

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Although the second biggest terrestrial group of plants, bryophytes remain poorly known chemically compared to the angiosperms. In this article, the sugars of the moss *Rhodobryum ontariense*, an unstudied representative of the medicinally known genus, are reported. The chemical analysis revealed the usual plant sugar sucrose, and a new sugar, fructooligosaccharide 1-kestose, which is reported first not only for the genus *Rhodobryum*, but also for mosses. The trisaccharides have been scantily reported in bryophytes hitherto. This gives more significance to this study for further investigation of its role in the moss species. The health-promoting effect of 1-kestose is also briefly discussed.

Keywords: mosses; *Rhodobryum ontariense*; phytochemistry; fructooligosaccharides; 1-kestose

1. Introduction

Bryophyte chemistry is still poorly known compared to that of vascular plants (Asakawa, 1995, 2007). To date, *ca* 5% of total bryophytes have been studied chemically (A. Sabovljevic & M. Sabovljevic, 2008). Among bryophytes, liverworts have received significant attention due to the presence of oil bodies with terpenoid substances of high biological activities (Asakawa, 2008). Chemical properties of mosses are studied very poorly. The reasons for the low amount of bryophyte chemistry data are due to the problems of lack of availability of clean materials, taxonomic inconstancy and the problem with taxa recognition.

However, since 1960 interest in the analysis of chemicals isolated from bryophytes has increased. It has been confirmed that bryophytes possess a great number of different chemical constituents. Carbohydrates are widely presented as mono and polysaccharides (Chopra & Kumra, 1988).

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Up till now, only two species from the genus *Rhodobryum*, namely, *Rhodobryum roseum* and *Rhodobryum giganteum*, have been studied for their chemical and bioactive components (Dai, P. Liu, C. Liu, Wang, & Chen, 2006; Hu et al., 2009; Lei et al., 2001; Qiao, Ma, Lin, & Kong, 2004; Tan et al., 1983; Wang et al., 2006). For saccharides, the isolation and identification of fructose from *R. roseum* have been recently reported (Wang, P. Liu, & H. Liu, 2008). Data on *Rhodobryum ontariense* chemistry are lacking.

Rhodobryum ontariense is a widely distributed moss (Dierssen, 2001). In Serbia, *R. ontariense* is present in Deliblatska sands, in open steppic-woody dry areas, where the yearly temperature amplitude goes up to 60°C.

With the aim of studying the chemistry of *R. ontariense*, adequate screening of this moss was performed.

2. Results and discussion

The *n*-butanol extract obtained from the water residue of methanol extract of *R. ontariense* (Kindb.) Kindb., upon repeated reverse-phase chromatography afforded two pure compounds, which were classified as oligosaccharides by means of their spectroscopic data and typical chromatographic profile. The less polar compound was obtained as a colourless solid with a m.p. of 185–187°C on decomposition. Its molecular formula was determined to be $C_{12}H_{22}O_{11}$ by TOFMS and NMR data. The ^{13}C-NMR and DEPT spectra exhibited 12 carbon signals (1 quaternary, 8 methine and 3 methylene). These data suggested that it was sucrose, which was confirmed by comparison with an authentic sample.

The most polar compound was obtained as amorphous solid. Its molecular formula was determined to be $C_{18}H_{32}O_{16}$ from TOFMS and NMR data. The ^{13}C-NMR and DEPT spectra exhibited 18 carbon signals (2 quaternary, 11 methine and 5 methylene). These data suggested that it was a trisaccharide with one pyranose and two furanose moieties, which was confirmed by the presence of only one anomeric proton at δ 5.41 (d, $J = 3.8$ Hz) in its ^{1}H-NMR spectrum. Its acid hydrolysis gave D-glucose and D-fructose (ratio 1 : 2). A combination of 2-D NMR experiments (COSY, TOCSY, HMQC and HMBC) allowed us to assign all signals in the ^{1}H- and ^{13}C-NMR spectra and to define the spin system of the glucose unit. The HMBC showed the correlation between the anomeric proton at δ 5.41 and the quaternary carbon at δ 104.9, which allowed for the connection between the glucose and a fructose moiety, like sucrose. It was difficult to establish the connection of the second fructose unit, because the chemical shifts of both quaternary carbons were very close. In order to establish this connection, the isolated trisaccharide was acetylated. The correlation between the anomeric proton of glucose unit (δ 5.71) and the quaternary carbon at δ 104.3, observed in the HMBC experiment of the full acetylated derivative, identified the C-2 of the fructose moiety linked to glucose. Furthermore, the difference of chemical shifts of the quaternary carbon atoms (δ 104.3 and 103.1), observed in the ^{13}C-NMR spectrum of the full acetylated trisaccharide, suggested that the second fructose was (2→1)-linked to fructose 1, because only this connection was in accordance with all ^{13}C chemical shifts (assigned by 2-D NMR), and the less high field shift of C-2′ due to only one β-acetylation shift. Thus, the structure of isolated trisaccharide was elucidated as β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*

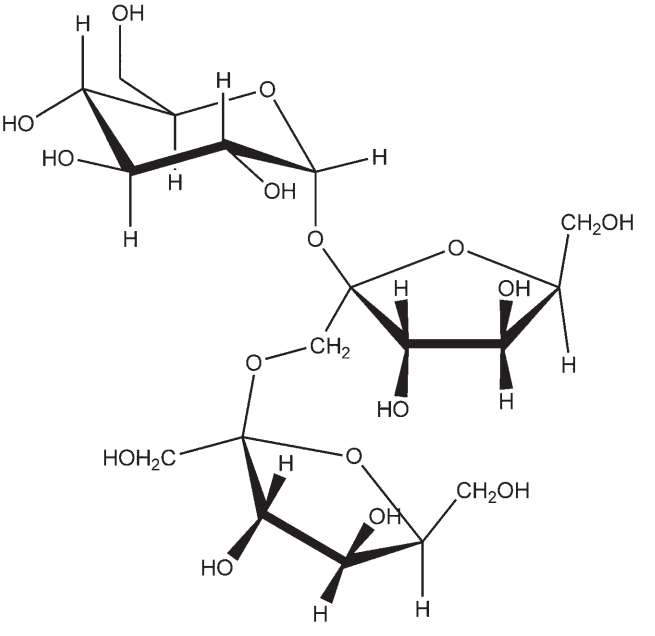

Figure 1. 1-Kestose.

(1-kestose) (Figure 1). The obtained NMR data for 1-kestose compare well with those from literature (Calub, Waterhouse, & Chatterton, 1990).

The fructooligosaccharide 1-kestose is first recorded in the genus *Rhodobryum* and mosses as a whole. Marschall, Proctor, and Smirnoff (1998) found this trisaccharide in the liverwort *Porella platyphylla* (L.) Lindb.

In nature, about 15% of flowering plant species belonging to selected families of both monocots and dicots synthesise fructooligosaccharides (FOS) from sucrose. These FOS are generally shorter and have diverse structures (Banguela & Hernández, 2006).

1-Kestose is potentially attractive from an ecological point of view: there is strong evidence that FOS protect the plant against drought and freezing (Hisano et al., 2004), most likely by stabilising cell membranes (Hincha, Zuther, Hellewege, & Heyer, 2002; Vereyken, Chupin, Demel, Smeekens, & De Kruijff, 2001).

In addition, FOS of short and medium sizes are prebiotics that are now in increasing demand in the functional market. According to the concepts, functional foods have an added health value above their nutritive properties (Roberfroid, 2000). They are attractive because of their health-promoting effect. Although the simple sugars fructose and glucose are quickly absorbed into the body by the intestines, FOS are indigestible for the most part and therefore act as non-digestible fibres in the diet. Actually, these sugars have a low caloric value and dietary fibre like properties due to the fact that the β-fructosyl linkages cannot be hydrolysed by the digestive enzyme in the upper part of the human gastrointestinal tract. The commercially available FOS are the typical examples of prebiotics for bifidobacteria, the predominant group of the colonic micro flora. Short-chain FOS are more convenient substrates for the rapid growth of bifidobacteria (Van der Meulen, Avonts, & De

Vuyst, 2004), whereas branched FOS are claimed to provide for a long-lasting source of energy (Weyens et al., 2004).

Although FOS with low and medium degrees of polymerisation are important primarily because of their functional properties, they have additional applications in the food industry (Banguela & Hernández, 2006). The trisaccharide 1-kestose has a natural sweet taste, and in blend with other low calorie sweeteners, it can replace sucrose in certain specific uses.

3. Experimental

3.1. *General*

Melting points were measured on a Kofler apparatus and are uncorrected. Optical rotations were measured on a Jasco DIP 370 polarimeter, using a 10-cm microcell. HRESI-MS spectra were recorded with Micromass Q-TOF Micro Mass Spectrometer (Waters) in the electrospray-ionisation mode. ^{1}H- and ^{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker Avance-400 spectrometer, using an inverse probe fitted with a gradient along the z-axis, using the solvent signal as an internal standard. The 2-D NMR spectra were obtained using Bruker's microprograms. Si gel chromatography was performed using pre-coated Merck F_{254} plates and Merck Kieselgel 60 powder. Reverse-phase chromatography was performed using Lichroprep RP-18 (Merck) and Lobar C-18 column (Merck).

3.2. *Plant material*

The *R. ontariense* sample was collected from Deliblatska sands, NE Serbia in March 2007. A voucher specimen has been deposited in the Herbarium of the Institute of Botany, University of Belgrade, Serbia (bryophyte collection BEOU4708).

3.3. *Extraction and isolation*

Before extraction, the moss was carefully inspected for contaminants: soil and other plant materials were completely removed. The gametophyte tips were used for the extraction. Air-dried parts of *R. ontariense* (12 g) were ground and extracted with 90% methanol at room temperature. The extract was filtered and concentrated under reduced pressure to give 0.92 g of residue, which was dissolved in water and extracted with petroleum ether, ethyl acetate and *n*-butanol. The crude *n*-butanol extract (0.39 g) obtained after evaporation of the solvent *in vacuo* was fractioned by RP-18 flash chromatography, eluting with increased gradient of methanol in water. Sugar-rich fractions were then purified on Lobar RP-18 columns (water/methanol gradient) to yield 1-kestose (92 mg; 0.77% of dry weight of plant) and sucrose (62 mg; 0.57% of dry weight).

3.4. *Sucrose*

Colourless solid; m.p. 185–187°C with decomposition; $[\alpha]_D + 66.5°$ (c 0.02, MeOH); HRESIMS (positive) m/z: 365.1095 $[M + Na]^+$, calculated for $C_{12}H_{22}O_{11}Na$, 365.1072; NMR data were identical to those of an authentic sample.

3.5. *1-Kestose*

Amorphous solid, $[\alpha]_D + 13.6^\circ$ (c 0.02, MeOH); ^{1}H-NMR (400 MHz, CD_3OD): δ 5.41 (1H, d, $J = 3.8$ Hz, H-1), 4.17 (1H, d, $J = 8.5$ Hz, H-3′), 4.15 (1H, d, $J = 8.5$ Hz, H-3″), 4.12 (1H, t, $J = 8.5$ Hz, H-4′), 4.10 (1H, t, $J = 8.5$ Hz, H-4″), 4.00 (2H, d, $J = 10.8$ Hz, H-1′), 3.95 (1H, dd, $J = 9.7$ and 4.7 Hz, H-5), 3.87–3.62 (11H, overlapped signals), 3.51 (1H, dd, $J = 9.7$ and 3.8 Hz, H-2) and 3.46 (1H, t, $J = 9.7$ Hz, H-4); ^{13}C-NMR (100.6 MHz, CD_3OD): δ 104.95 (s, C-2″), 104.90 (s, C-2′), 93.1 (d, C-1), 82.8 (d, C-5″), 82.7 (d, C-5′), 78.3 (d, C-3″), 78.1 (d, C-3′), 75.9 (d, C-4″), 75.1 (d, C-4′), 73.9 (d, C-3), 73.1 (d, C-5), 72.5 (d, C-2), 70.8 (d, C-4), 63.8 (t, C-1″), 63.3 (t, C-6″), 63.2 (t, C-6′), 61.8 (t, C-6) and 61.5 (t, C-1′); HRESIMS (positive) m/z: 527.1625 $[M + Na]^+$, calculated for $C_{18}H_{32}O_{16}Na$, 527.1600.

3.6. *Acetylation of 1-kestose*

The trisaccharide (20 mg) was dissolved in 5 mL of pyridine and 1 mL of acetic anhydride. The mixture was refluxed at 160°C for 2 h. After evaporating to dryness, the residue was chromatographed on a Si gel column and eluted with chloroform to yield 1-kestose hendecaacetate (18 mg), as colourless syrup, $[\alpha]_D - 13.5^\circ$ (c 0.018, MeOH); ^{1}H-NMR ($CDCl_3$): δ 5.71 (1H, d, $J = 3.4$ Hz, H-1), 5.46 (1H, d, $J = 8.6$ Hz, H-3″), 5.42 (1H, t, $J = 10.2$ Hz, H-3), 5.37 (1H, d, $J = 8.6$ Hz, H-3′), 5.22 (1H, t, $J = 10.2$ Hz, H-4), 4.88 (1H, dd, $J = 10.2$ and 3.4 Hz, H-2), 4.44–4.20 (13H, overlapped signals), 2.16 (6H, s, $COCH_3$), 2.12 (3H, s, $COCH_3$), 2.11 (3H, s, $COCH_3$), 2.10 (3H, s, $COCH_3$), 2.09 (3H, s, $COCH_3$), 2.08 (3H, s, $COCH_3$), 2.07 (3H, s, $COCH_3$), 2.05 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$) and 2.00 (3H, s, $COCH_3$); ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ 170.4–169.7 (11 s, $COCH_3$), 104.3 (s, C-2′), 103.1 (s, C-2″) 90.3 (d, C-1), 79.1 (d, C-5″), 78.1 (d, C-5′), 76.6 (d, C-3″), 76.2 (d, C-3′), 76.0 (d, C-4″), 75.3 (d, C-4′), 70.3 (d, C-2), 70.1 (d, C-3), 69.0 (d, C-5), 68.0 (d, C-4), 64.4 (t, C-1″), 63.7 (t, C-1′), 62.9 (t, C-6″), 62.5 (t, C-6′), 59.7 (t, C-6) and 21.5–20.8 (11 q, $COCH_3$); HRESIMS (positive) m/z: 989.2695 $[M + Na]^+$, calculated for $C_{40}H_{54}O_{27}Na$, 989.2662.

3.7. *Hydrolysis of 1-kestose*

1-Kestose (5 mg) dissolved in 0.5 mL of 2N HCl was refluxed for 1 h. The reaction mixture was neutralised, and analysed by TLC, using acetone–butanol–H_2O (8 : 1 : 1) as the solvent, in comparison with glucose and fructose, which are used as standards. The sugar spots were visualised by reaction with α-naphthol. Afterwards, monosaccharide composition was established by high-pressure anion exchange-pulsed amperometric detector (HPAE-PAD, Dionex) equipped with a Carbopac PA1 column eluted with 15 mM NaOH (1 ml min^{-1}), giving D-glucose and D-fructose (ratio 1 : 2) identified by their retention times in comparison with those of authentic samples.

4. Conclusion

The saccharide 1-kestose was isolated for the first time from mosses. It is the first record for FOS in this biggest group of bryophytes. The compound is particularly

interesting regarding the investigation of moss physiological adaptations to drought and freezing as well as for its health value.

However, more studies should be done for obtaining a better understanding of the *R. ontariense* sugar chemistry. A possible way of producing large amounts is to develop adequate micropropagation technology, *in vitro* culture. This method can enable easier and larger production of the plant material that can be used for new investigations.

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