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# Chemical structure and biological activity of a highly branched (1 → 3,1 → 6)-β-D-glucan from *Isochrysis galbana*

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ABSTRACT

A highly branched (1 → 3,1 → 6)-β-D-glucan was isolated from the microalga *Isochrysis galbana* Parke (Isochrysidales, Haptophyta). The polysaccharide structure was analyzed by methylation and Smith degradation, as well as by ESI and MALDI TOF mass spectrometry and NMR spectroscopy. The glucan was shown to contain a (1 → 6)-linked backbone, where every residue is substituted at position 3 by Glc, which in turn may be substituted at C-6 by a single Glc or by rather short (up to tetrasaccharide) oligosaccharide chains. All the 3-linked Glc residues are present in these side chains. In the biological activity experiments it was demonstrated that the polysaccharide directly inhibits the proliferation of U937 human leukemic monocyte lymphoma cells and therefore has potential anti-tumor activity.



## 1. Introduction

Microalgae are photosynthetic microorganisms with an important role as primary producers in most aquatic food webs. They are characterized by a high diversity and are potentially a great source of natural compounds for several biotechnological and therapeutic applications (Borowitzka, 2013; Guedes, Amaro, & Malcata, 2011; Raja, Hemaiswarja, Kumar, Sridhar, & Rengasamy, 2008). In fact, they convert inorganic substances into organic matter rich in lipids, proteins, carbohydrates and other molecules. Because of their high nutritional value, microalgae have been widely used in aquaculture as food for molluscs, zooplankton and early life-stages of crustaceans and small fishes (Hemaiswarja, Raja, Kumar, Ganesan, & Anbazhagan, 2011). Among microalgae, the species *Isochrysis galbana* is known to have good nutritional quality, and has received increasing interest in aquaculture mainly due to its polyunsaturated fatty acid content (Liu & Lin, 2001; Sánchez, Martínez, & Espinola, 2000; Yoshioka, Yago, Yoshie-Stark, Arakawa, & Morinaga, 2012). It also has beneficial effects on human health and provides a good source of fish oil substitution in the human diet (Batista, Gouveia, Bandarra Narcisa, Franco, & Raymundo, 2013; Yu et al., 2010). In addition, *I. galbana* showed promising curative effects inducing weight loss, decreased glucose, triacylglycerol and cholesterol levels in diabetic rats (Nuño et al., 2013). Cultivation of *I. galbana* has also been suggested for biodiesel production (Sanchez, Maceiras, Cancela, & Pérez, 2013).

*I. galbana* is a marine flagellated microalga belonging to the phylum Haptophyta, class Coccolithophyceae, subclass Prymnesiophycidae, order Isochrysidales, family Isochrysidaceae (Guiry & Guiry, 2013). Its cells are easily assimilated by larval animals because of their small size (4–7 μm) and absence of a tough cell wall (Jeffrey, Brown, & Volkman, 1994; Liu & Lin, 2001). *I. galbana* is represented by different strains (Durmaz et al., 2008), the most commonly cultured one is the Tahitian strain "T-iso" (Marchetti et al., 2012a). Generally, this microalga is characterized by a fast growth rate, wide temperature and salinity tolerance and absence of toxins ((Jeffrey et al., 1994). As all microalgae, the growth and biochemical composition of *I. galbana* cultures is dependent on variations of culture conditions (e.g. nutrients, temperature, salinity, and photoperiod) (Marchetti et al., 2012b).

*I. galbana* has a high content of soluble and insoluble polysaccharides and proteins as well as significant percentages of

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polyunsaturated fatty acids (Sánchez et al., 2000). The carbohydrate constituents of *I. galbana*, representing around 13% of dry matter, were reported to be a complex mixture of biopolymers containing glucose, galactose, mannose, xylose, arabinose, fucose and rhamnose in various proportions (Batista et al., 2013; Chu, Dupuy, & Webb, 1982).

Sun, Zhou, Xu, Li, and Yan (2012) were the first to obtain intracellular and extracellular polysaccharides from *I. galbana*. The polysaccharides were analyzed by UV and IR spectroscopy, and tested for their antibacterial properties. Yu et al. (2010) showed that a crude polysaccharide extract of *I. galbana* exerted immunomodulatory properties by the induction of IL-1 within murine macrophages. To date, there were no reports on the purification and characterization of individual polysaccharides from *I. galbana*. Here, we describe the isolation and structural analysis of a highly branched β-glucan from *I. galbana* cells and explore its immunostimulatory and cytotoxic effects in human monocytic U937 cells.

## 2. Materials and methods

### 2.1. Algae culture conditions

The starter culture of *I. galbana* was obtained from the Roscoff culture collection (Roscoff, France). The strain was maintained in the laboratory in 250 mL Erlenmeyer flasks and was shaken by hand daily. The culture was kept in an incubator (SANYO model MLR-351) at 18 °C and a photoperiod of 12L:12D cycle under a fluorescent light with an intensity of 2500 lux. Batch cultures in 2–6 l flasks were used to grow the microalgae for this study. The culture flasks were filled with autoclaved seawater of salinity 33 psu and enriched with Conway medium. The composition of Conway medium was: each liter of autoclaved seawater contained 100 mg $NaNO_3$, 20 mg $NaH_2PO_4$, 45 mg $Na_2EDTA$, 33.6 mg $H_3BO_3$, 0.36 mg $MnCl_2$, 1.3 mg $FeCl_3$, 0.021 mg $ZnCl_2$, 0.02 mg $CoCl_2{\cdot}6H_2O$, 0.02 mg $CuSO_4{\cdot}5H_2O$, 0.09 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.2 mg thiamine HCl (vit. $B_1$) and 0.01 mg cyanocobalamin (vit. $B_{12}$). Cultures were aerated with sterile air and incubated in the same condition as the strain inoculum. Alga growth was assessed daily by image analysis on Malassez slides under an inverted microscope (Olympus, IX71) after Lugol dying. *I. galbana* cultures used in this study were harvested at the 11th day during the advanced exponential growth phase with a concentration of more than ten million cells per milliliter.

### 2.2. Analysis of carbohydrate content of cells

Dry cells (3–4 mg) were defatted by extractions with $CHCl_3$–MeOH, 2:1 and 1:2 (v/v), and air-dried. The residue was hydrolyzed with 4 M TFA (110 °C, 3 h) in the presence of a known amount of *myo*-inositol (an internal standard) and the released monosaccharides were converted into alditol acetates by conventional methods and analyzed using GLC (Pettolino, Walsh, Fincher, & Bacic, 2012).

### 2.3. Polysaccharide extraction and fractionation

Polysaccharides from *I. galbana* were extracted essentially as described by Bobadilla, Rodriguez-Tirado, Imarai, Galotto, & Andersson (2013). Briefly, lyophilized cells (500 mg) were soaked with 85% ethanol (50 mL) for 10 min, the ethanolic solution was decanted, and a fresh portion of ethanol was added. Cells were soaked overnight at room temperature, then heated in a water bath at 70 °C for 10 min. The cell pellet was collected by centrifugation, suspended in aqueous 2% $CaCl_2$ (50 mL), and heated in a boiling water bath with stirring for 1 h. The mixture was cooled, centrifuged and the supernatant collected. The extraction was repeated twice with 20 mL of 2% $CaCl_2$ and for 30 min each. Pooled supernatants were cooled and extracted with 90% phenol followed by extraction with chloroform to remove proteins and lipophilic material. The aqueous phase was dialyzed and lyophilized to give a crude cell carbohydrate extract (63 mg).

This extract was further fractionated on an anion exchange column Q-Sepharose fast flow ($Cl^-$-form, 1 cm × 10 cm, 21 mg/run), equilibrated in water, and eluted with water followed by a linear gradient of NaCl (0–1 M, 60 mL). Fractions were assayed for total sugars (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Carbohydrate positive fractions of the water eluate were pooled, concentrated, purified further on a Sephadex G-50 gel-filtration column (1 cm × 40 cm) by elution with 0.01% AcOH and lyophilized to give a neutral glucan (the total yield 44 mg). The charged material that eluted as a relatively sharp peak at ~0.2–0.35 M NaCl was dialyzed and lyophilized (yield, 4 mg).

### 2.4. Smith degradation

The glucan was subjected to Smith degradation essentially as described by Cordeiro, Reinhardt, and Iacomini (2012). Glucan (22 mg) was dissolved in 10 mL of 0.05 M $NaIO_4$ and kept for 72 h in the dark at 20 °C. To stop the reaction, 1,2-ethanediol (0.5 mL) was added, the solution was dialyzed and the resulting polyaldehyde was reduced with $NaBH_4$ overnight. The excess of $NaBH_4$ was destroyed with AcOH, the solution was dialyzed and concentrated. The material was partially hydrolyzed by AcOH (2%) at 100 °C for 2 h (Altman, Brisson, & Perry, 1988). The mixture was cooled, concentrated, and fractionated on a Sephadex G-50 column to give a HMW fraction (eluted close to the void volume, 'fraction 1', 5 mg) and a LMW fraction ('fraction 2', 2 mg).

### 2.5. General and analytical methods

#### 2.5.1. Monosaccharide analysis

Monosaccharides were detected as reduced and acetylated derivatives (alditol acetates). Polysaccharide samples (0.2–1 mg) with the *myo*-inositol internal standard were hydrolyzed with 4 M TFA (110 °C, 3 h), dried, and reduced with $NaBH_4$. The reagent was destroyed with 0.5 mL of AcOH, the solution was dried under the stream of air, dried twice with addition of MeOH (1 mL), acetylated with 0.4 mL $Ac_2O$–0.4 mL pyridine mixture for 1 h at 100 °C, dried, and analyzed by GC–MS.

#### 2.5.2. Methylation

Methylation was performed by the Ciucanu–Kerek procedure (Ciucanu & Kerek, 1984) as modified by Read, Currie, and Bacic (1996). The polysaccharide (0.5–1 mg) was dissolved in 1 mL of dry DMSO. Powdered NaOH (about 50 mg) was added and the mixture was stirred for 15 min, then 0.2 mL of MeI was added and the mixture was stirred for 1 h. The reaction was stopped by addition of 3 mL of 10% aq. $Na_2S_2O_3$. The permethylated product was extracted into $CHCl_3$ (2 mL). The organic phase was washed with water (5 × 2 mL) and evaporated. The product was hydrolyzed with 4 M TFA (110 °C, 3 h), dried, reduced with $NaBD_4$, converted into alditol acetates as described above, and analyzed by GC–MS (Lindberg & Lonngren, 1978).

#### 2.5.3. Mass spectrometry and polarimetry

GC–MS was performed on a Trace GC ULTRA system (Thermo Scientific) equipped with a capillary column NMTR-5MS (30 m × 0.25 mm) using a temperature gradient of 170 °C (3 min) → 250 °C at 5 °C/min and with a DSQ II MS detector.

High resolution mass spectra (HR MS) were measured on a Bruker maXis instrument using electrospray ionization (ESI). The measurements were done in a positive ion mode (interface capillary voltage – 4500 V); mass range from *m*/*z* 50 to *m*/*z* 3000 Da; external calibration was done with Electrospray Calibrant Solution (Fluka). A syringe injection was used for solutions in acetonitrile (flow rate 3 μL/min). Nitrogen was applied as a drying gas; the interface temperature was set at 180 °C.

MALDI-TOF mass spectra were obtained on an Ultraflex-III TOF/TOF instrument (Bruker Daltonics, Germany) equipped with a smartbeam laser of 355 nm wavelength using 2,5-dihydroxybenzoic acid as a matrix.

Optical rotation was measured for aqueous solutions of polysaccharides using a digital polarimeter PU-07 (Russia).

#### 2.5.4. NMR spectrometry

Samples were freeze-dried twice from 99.9% $D_2O$ and dissolved in 99.96% $D_2O$. $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer at 30 and/or 80 °C. Chemical shifts are reported with internal sodium 3-(trimethylsilyl)propanoate-2,2,3,3-$d_4$ ($\delta_H$ 0.0 and $\delta_C$ −1.6) as references. The NMR spectra were assigned by 2D $^1H$,$^1H$ COSY, TOCSY, ROESY, H-detected $^1H$,$^{13}C$ HSQC, HSQC-TOCSY and HMBC experiments, which were performed and analyzed using standard Bruker software. Mixing time of 100 ms was used in both TOCSY and HSQC-TOCSY experiments. The ROESY spectrum was acquired with 150 ms duration of spin-lock time. The HMBC experiment was optimized for coupling constant $J_{H,C}$ 8 Hz.

### 2.6. Biological activity

#### 2.6.1. Cell culture and treatments

U937 cells (ECACC) were maintained in a 37 °C, humidified 5% $CO_2$ incubator in RPMI 1640 media (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco) and 1% penicillin-streptomycin (P/S, Gibco). For basal and PMA experiments triplicate sets of cells were seeded at $1 \times 10^5$ per well in 24 well plates in serum-free medium (SFM) 2 h prior to treatment. For the treatments with the agonists LPS (*Escherichia coli*, Sigma) and PMA the cells were placed in 1% FCS-containing medium.

The glucan was reconstituted in 1 mL of sterile distilled water to a concentration of 6 mg/mL. The stock solution was aliquoted and stored at −20 °C and +4 °C for the aliquots not in use and in use, respectively. Directly prior to each experiment the glucan was diluted to the appropriate concentration in media containing 1% FCS.

For basal IL-8 expression studies, cells were left untreated or treated with glucan (0.01 pg–100 μg) for 24 h. To examine effects on LPS- or PMA-induced IL-8 expression cells were treated with the polysaccharide for 1 h prior to the addition of LPS (100 or 500 ng/mL) or PMA (100 or 10 ng/mL) for an additional 24 h. Cell supernatants were removed and stored at −20 °C until being used in IL-8 ELISAs.

#### 2.6.2. MTS and LDH assays

Cytotoxicity was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's instructions. This assay is a colorimetric method for determining the number of viable cells. It is based on the bioreduction by cells of MTS tetrazolium to a colored formazan product. The absorbance at 490 nm was measured on the multiscan plate reader.

The extent of cell lysis was determined with a Cytotoxicity Detection Kit (LDH, Roche Applied Science, Meylan, France), according to manufacturer's instructions. This assay is based on measurement of activity of lactate dehydrogenase (LDH), a cytoplasmic enzyme released by damaged cells.

#### 2.6.3. IL-8 sandwich enzyme-linked immunosorbent assay (ELISA)

A sufficient number of wells on a 96 well Immulon® 2B ELISA micro-titer plate (Nunc, 3455) were coated with 100 μl of primary antibody MAB208 (R&D Systems) diluted to a concentration of 2 μg/mL in Voller's buffer ($Na_2CO_3$ 2.76 g/500 mL, $NaHCO_3$ 1.916 g/500 mL, pH 9.6). The plate was sealed and incubated overnight at 4 °C. Following incubation, the test wells were washed three times with 1X PBS and 0.1% Tween (PBS-T) (Sigma, P3818 and P5297, respectively) and tapped dry. Test wells were blocked with PBS-T containing 1% BSA, 200 μl per well, for 1 h at room-temperature. Wells were washed as before with PBS-T. A 1:2 dilution series, ranging from 2000–31.25 pg/mL of recombinant human IL-8 (R&D Systems, 208-IL-010) was prepared in serum free media to create a standard curve. 100 μl of standards or cell supernatant was added to test wells and incubated for 2 h at room temperature. Again, wells were washed as before with PBS-T. The corresponding secondary antibody BAF208 (R&D Systems) was diluted to the desired concentration in PBS-T (0.1 μg/mL) and 100 μl was added to the test wells. The plate was incubated for 2 h at room temperature, and was washed as before with PBS-T. A 1:2500 dilution of Streptavidin-horseradish peroxidase (Biolegend, 405210) was prepared in PBS-T and 100 μl was added to test wells. The plate was incubated for 30 min at room temperature, and washed as before. Finally 100 μl of ABTS (Zymed, 00-2024) was added to test wells and incubated for 20 min at room temperature covered in foil until a color change from clear to green was observed. Absorbance was then measured at 405 nm on a Wallac Victor$^2$ plate reader (Perkin Elmer). A standard curve of absorbance vs. concentration was plotted using GraphPad PRISM 4.0. The concentration of IL-8 in unknown samples was calculated by linear regression.

#### 2.6.4. Evaluation of apoptosis

For apoptosis evaluation, cell cultures were seeded at $1 \times 10^5$ cells/mL in 24 well plates in RPMI 1640 media (PAN Biotech, Dutscher, Brumath, France) supplemented with 10% (v/v) FCS (PAN Biotech), 1% penicillin-streptomycin (P/S, PAN Biotech) and 1% glutamine (PAN Biotech). Cells were treated with camptothecin at 5 μM (Sigma–Aldrich, St Quentin Fallavier, France) and with the glucan at 100 μg/mL for 8 h. Apoptosis was evaluated with a Cell Death Detection ELISA plus Kit (Roche Applied Science, Meylan, France) by determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates, according to manufacturer's instructions. All experiments were repeated 6 times. Mean values and standard deviations were calculated, and the statistical significance was determined using Student's *t*-test. Data with $p < 0.05$ were considered statistically significant.

#### 2.6.5. Cell proliferation

Cells were seeded in 24-well plates at $1 \times 10^5$ cells/mL in RPMI 1640 media (PAN Biotech) supplemented with 10% (v/v) fetal calf serum (PAN Biotech), 1% P/S (PAN Biotech) and 1% glutamine (PAN Biotech). Cells were maintained at 37 °C in a humidified atmosphere with 5% $CO_2$ in air. To analyze cell proliferation, cells were grown in the absence or presence of glucan (50 and 100 μg/mL) in growth medium. Cells were counted at 24 h, 48 h and 72 h with a hemacytometer (Brand Rudolph, Fisher Scientific). The experiments were repeated 4 times. Mean values and standard deviations were calculated, and the statistical significance was determined using Mann–Whitney *U*-test. Data with $p < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Polysaccharide composition of I. galbana

The alga was cultivated as described in Section 2. According to Durmaz et al. (2008), at 18 °C *I. galbana* can achieve both an intense specific growth rate and a high biomass productivity value. Algal cells were separated by centrifugation and then dehydrated and defatted by soaking in alcohol. Acid hydrolysis of defatted biomass afforded approximately 15% of Glc, and about 0.6% of each Ara, Xyl and Gal.

Polysaccharides were extracted from defatted biomass with hot water in the presence of $CaCl_2$, essentially as described by Bobadilla et al. (2013). The crude polysaccharide preparation was separated by anion exchange chromatography into a neutral fraction (approx. 70%) composed exclusively of Glc, and a negatively charged fraction, containing uronic acids (about 4%) and Ara, Xyl and Gal in an approximate molar ratio of 5:1:1.5. Quantitative determination of Glc in the neutral fraction by both colorimetry (Dubois et al., 1956) and GLC gave the results in a range of 90–95%, whereas the protein content was negligible.

### 3.2. Glucan purification and structural analysis

The detailed chemical structure of the neutral polysaccharide was established by NMR spectroscopy, methylation analysis, ESI and MALDI TOF MS before and after Smith degradation.

The native glucan gave viscous solutions in water, and was eluted as a broad peak close to the void volume of Sephadex G-50 (Fig. 1). Its $^1$H NMR spectrum showed a broad signal of anomeric protons at 4.6 ppm indicating the presence of β-linkages. The D-configuration of the Glc residues was proved by a rather low negative value of optical rotation of an aqueous solution of the polysaccharide, [$\alpha_D^{24}$] – 4.9 (c 1.0; water). Methylation revealed the presence in the native glucan of non-reducing terminal, 3-substituted, 6-substituted, and 3,6-disubstituted Glc*p*, indicating a highly branched structure (Table 1). The $^{13}$C NMR spectrum of the polysaccharide, which was similar to many other known β-glucans (Kim et al., 2000), coincided with this substitution pattern, but it was rather difficult to elucidate the mutual arrangement of differently substituted glucose residues from the spectral data. To get further insight into the chemical structure, the glucan was subjected to Smith degradation (periodate oxidation followed by borohydride reduction and mild acid hydrolysis), and the products were fractionated on Sephadex G-50 to give a HMW (1) and a LMW (2) fraction (Fig. 1). Both fractions were methylated and analyzed by ESI-MS, whereas fraction 1 was analyzed by MALDI-TOF MS (cf. Chizhov et al., 1998; Read et al., 1996).

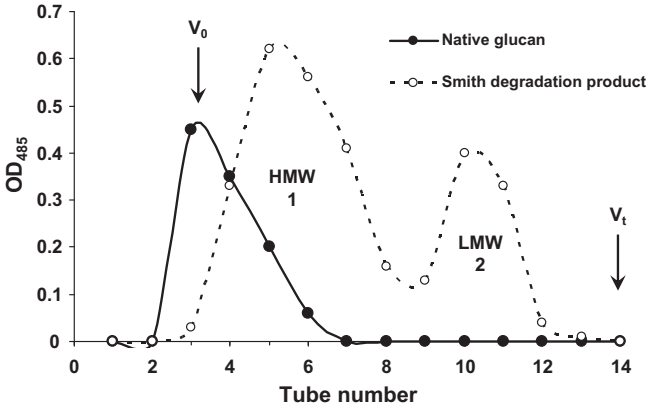



**Fig. 1.** Elution profiles of the native glucan from *Isochrysis galbana* and its product of Smith degradation on Sephadex G-50 column.

**Table 1**
Methylation analysis of the native glucan from *I. galbana* and the products of its Smith degradation.

| Methylated glucitol, [1–$^2$H] | Linkage indicated | Approx. molar ratio |
|---|---|---|
| *Native glucan* | | |
| 2,3,4,6-$Me_4$-*O*- | Terminal Glc*p* | 1.3 |
| 2,4,6-$Me_3$-*O*- | (1,3)-Glc*p* | 0.5 |
| 2,3,4-$Me_3$-*O*- | (1,6)-Glc*p* | 1 |
| 2,4-$Me_2$-*O*- | (3,6)-Glc*p* | 1.5 |
| *Smith degradation product, HMW* | | |
| 2,3,4,6-$Me_4$-*O*- | Terminal Glc*p* | 0.05 |
| 2,3,4-$Me_3$-*O*- | (1,6)-Glc*p* | 1 |
| *Smith degradation product, LMW* | | |
| 2,3,4,6-$Me_4$-*O*- | Terminal Glc*p* | 4 |
| 2,4,6-$Me_3$-*O*- | (1,3)-Glc*p* | 1 |
| 2,3,4-$Me_3$-*O*- | (1,6)-Glc*p* | 0.2 |

Surprisingly no 3-linked Glc residues were retained in the HMW fraction. 1D and 2D NMR analysis of the HMW product of Smith degradation showed linear chains of (1 → 6)-linked β-Glc*p* (Fig. 2 and Table 2). Methylation analysis was in agreement with this structure (Table 1). This result means that the native glucan contained a (1 → 6)-linked backbone, where every residue is substituted at position 3 by Glc*p*, which in turn may be substituted at C-6 by a single Glc*p* or by rather short oligosaccharide chains. All the 3-linked residues should be present in these side chains.

MALDI-TOF MS of the HMW product (fraction 1) of Smith degradation showed $[M+Na]^+$ ions consistent with composition of chains ($Glc_n$-Ara-$ol_x$), where Ara-ol is a residue of arabinitol formed at the reducing end of the molecule due to Smith degradation of a terminal 3-substituted or 3,6-disubstituted Glc residue, and $n$ = 7–29 and $x$ = 0 or 1 (Fig. 3). This is in agreement with the elution profile of the fraction on a Sephadex G-50 column (Fig. 1). The presence of chains having $x$ = 0 may be explained by partial splitting of chains having $x$ = 1 under acid hydrolysis conditions, and hence, a rather high polydispersity of the HMW fraction may be the result of this non-controlled degradation. In reality, the native glucan is also highly polydisperse, as it follows from its MALDI-TOF mass spectra. Peaks of ions up to $[Glc_{60}+Na]^+$, $m/z$ 9769.8, were observed in the spectrum registered using the reflectron mode, whereas even higher-molecular components up to $[Glc_{115}+Na]^+$ might be found in the spectrum registered using the linear mode.

HR ESI MS of methylated fraction 1 was in agreement with the above structure, but demonstrated a different DP, possibly due to discrimination of higher masses in the mass spectrometer. The main series of peaks corresponded to $[M+Na]^+$ ions of the expected permethylated oligoglucosylarabinitols, where M had the molecular formula $(C_9H_{16}O_5)_nC_{19}H_{38}O_{10}$, $n$ = from 0 to 8. The relative differences between experimental and calculated $m/z$ values were less than 5 ppm (Table 3).

Methylation analysis of the LMW product (fraction 2) indicated the presence of terminal and (1 → 3)-linked Glc in an approximate ratio of 4:1. The ESI MS spectrum of permethylated fraction 2 revealed the presence of permethylated mono-, di-, and trihexosylglycerols (Table 3). This means that the side chains of the native glucan were tri, tetra-, and pentasaccharides containing (1 → 3)-linkages between all the Glc*p* apart the "reducing" terminal residues, which should be substituted at position 6.

Taking into consideration the methylation and Smith degradation data, the NMR spectra of the native glucan were carefully reinvestigated. 1D $^1$H and $^{13}$C NMR spectra were assigned using 2D homonuclear $^1$H,$^1$H COSY, TOCSY and ROESY, as well as heteronuclear $^1$H,$^{13}$C HSQC, HSQC-TOCSY and HMBC experiments. Analysis of COSY, TOCSY, HSQC (Fig. 4) and HSQC-TOCSY spectra revealed the

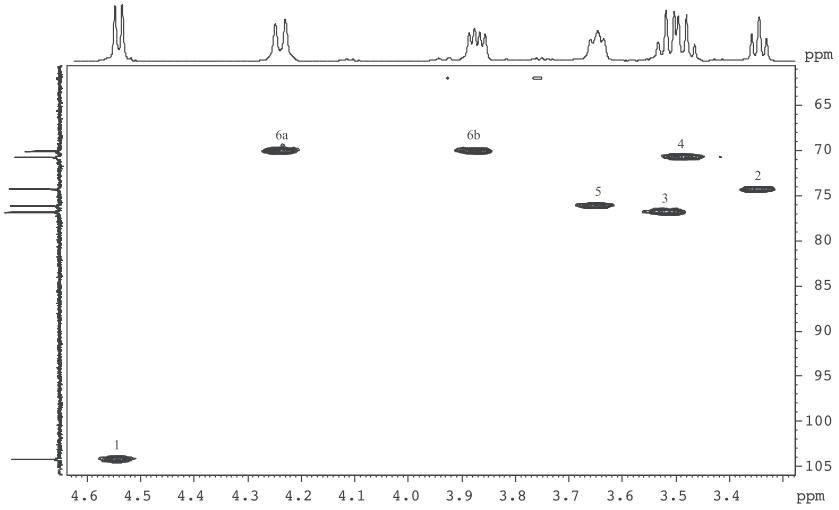


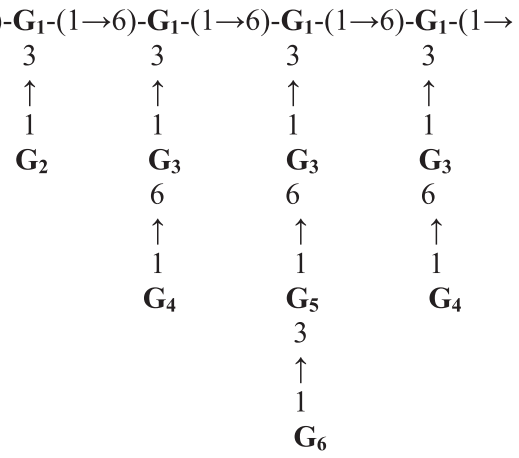

**Fig. 2.** $^{1}H–^{13}C$ HSQC NMR spectrum of the HMW product of Smith degradation of the β-glucan from *I. galbana*.

signals of 3,6-disubstituted ($G_1$), 6-substituted ($G_3$), 3-substituted ($G_5$) and terminal ($G_2$, $G_4$, $G_6$) β-glucopyranose residues (Table 2). The analysis of the ROESY spectrum (Fig. 5) provided details on the sequence of the residues. Intra-residue correlation peaks H-1($G_1$)/H-6,6′ ($G_1$) indicated that the polysaccharide core is built up of 1 → 6-linked residues. In turn, position 3 of $G_1$ is substituted by $G_3$ [an intense correlation peak H-1($G_3$)/H-3($G_1$) in Fig. 6] or by terminal residues $G_2$ [a less intense peak

**Table 2**
$^{1}H$ and $^{13}C$ NMR chemical shifts ($\delta$, ppm) of the HMW product of Smith degradation (**A**) and of the native β-glucan from *I. galbana* (**B**).

**(A)** →6)-**G**-(1→6)-**G**-(1→6)-**G**-(1→6)-**G**-(1→6)-**G**-(1→6)-**G**-(1→

**(B)**




| Monosaccharide unit | Symbol | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|---|
| | | *H-1* | *H-2* | *H-3* | *H-4* | *H-5* | *H-6,6′* |
| →6)-β-D-Glc*p*-(1 → 6)- | **G** | 104.23<br>*4.54* | 74.31<br>*3.35* | 76.82<br>*3.52* | 70.75<br>*3.49* | 76.13<br>*3.65* | 70.07<br>*4.24; 3.87* |
| →6)-β-D-Glc*p*-(1→6)-<br>3)<br>↑ | **$G_1$** | 104.26<br>*4.59* | 74.69<br>*3.42* | 87.57<br>*3.70* | 70.14<br>*3.52* | 75.43<br>*3.82* | 70.91<br>*4.17, 3.93* |
| β-D-Glc*p*-(1 → 3)- | **$G_2$, $G_6$** | 104.18<br>*4.72* | 74.84<br>*3.41* | 77.0<br>*3.52* | 71.03<br>*3.44* | 77.31<br>*3.52* | 62.17<br>*3.92, 3.75* |
| →6)-β-D-Glc*p*-(1 → 3)- | **$G_3$** | 104.48<br>*4.68* | 74.04<br>*3.56* | 76.89<br>*3.55* | 70.90<br>*3.52* | 76.12<br>*3.68* | 69.88<br>*4.19, 3.88* |
| β-D-Glc*p*-(1 → 6)- | **$G_4$** | 103.88<br>*4.51* | 74.50<br>*3.36* | 77.07<br>*3.53* | 71.03<br>*3.44* | 77.13<br>*3.45* | 62.28<br>*3.92, 3.75* |
| →3)-β-D-Glc*p*-(1 → 6)- | **$G_5$** | 103.67<br>*4.54* | 74.04<br>*3.56* | 86.32<br>*3.74* | 79.86<br>*3.54* | 77.0<br>*3.54* | 62.28<br>*3.92, 3.75* |

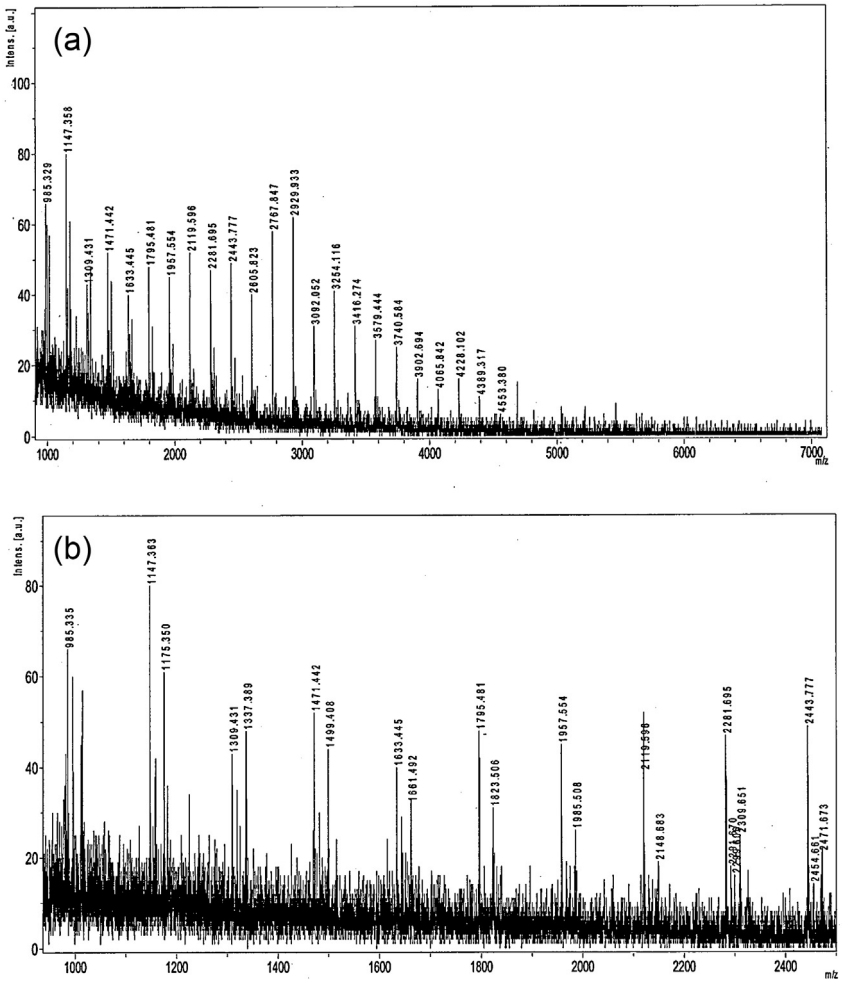



**Fig. 3.** (a) MALDI-TOF mass spectrum of the HMW product of Smith degradation of the β-glucan from *I. galbana*. (b) Fragment of the mass spectrum showing pairs of signals differing in 28 Da.

H-1($G_2$)/H-3($G_1$)]. Another intense signal in the anomeric region of the $^1$H NMR spectra belongs to H-1 of terminal residues $G_4$ linked to C-6 of residues $G_3$ [correlation peaks H-1($G_4$)/H-6,6′ ($G_3$)]. The remaining correlation peaks of anomeric protons belonging to $G_5$ (4.54 ppm) and $G_6$ (4.72 ppm) were masked by more intense main peaks having similar chemical shifts. The main details of the polymer structure were confirmed by the HMBC spectrum (Fig. 6), where the following inter-residue peaks were observed:

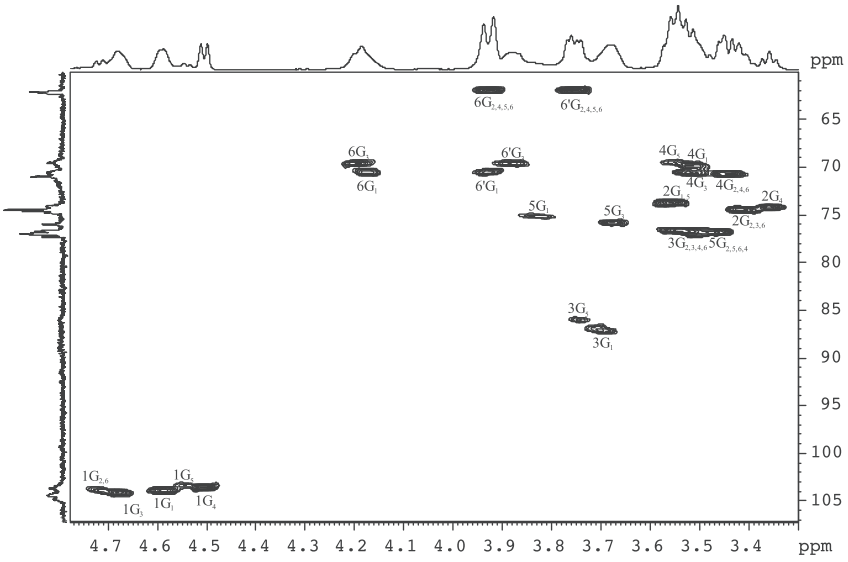



**Fig. 4.** $^1$H–$^{13}$C HSQC NMR spectrum of the native β-glucan from *I. galbana*.

**Table 3**
High-resolution ESI mass spectral data ([M+Na]$^+$ ions) of methylated fractions of Smith-degraded glucan from *I. galbana*.

| *n* | Molecular formula | Experimental, *m*/*z* | Calculated, *m*/*z* |
|---|---|---|---|
| | Methylated HMW | | |
| 1 | $C_{19}H_{38}O_{10}$ | 449.2359 | 449.2357 |
| 2 | $C_{28}H_{54}O_{15}$ | 653.3348 | 653.3355 |
| 3 | $C_{37}H_{70}O_{20}$ | 857.4339 | 857.4353 |
| 4 | $C_{46}H_{86}O_{25}$ | 1061.5326 | 1061.5350 |
| 5 | $C_{55}H_{102}O_{30}$ | 1265.6319 | 1265.6348 |
| 6 | $C_{64}H_{118}O_{35}$ | 1469.7315 | 1469.7346 |
| 7 | $C_{73}H_{134}O_{40}$ | 1673.8310 | 1673.8344 |
| 8 | $C_{82}H_{150}O_{45}$ | 1877.9305 | 1877.9311 |
| 9 | $C_{91}H_{166}O_{50}$ | 2082.0307 | 2082.0339 |
| | Methylated LMW | | |
| 1 | $C_{15}H_{30}O_8$ | 361.1835 | 361.1833 |
| 2 | $C_{24}H_{46}O_{13}$ | 565.2841 | 565.2831 |
| 3 | $C_{33}H_{62}O_{18}$ | 769.3834 | 769.3828 |

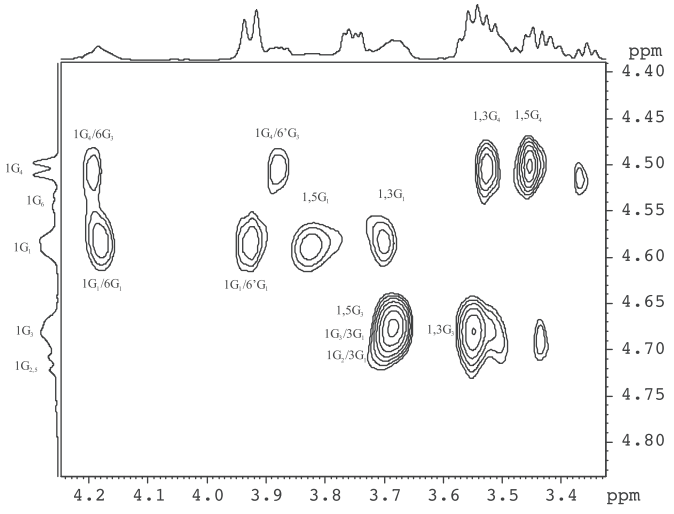
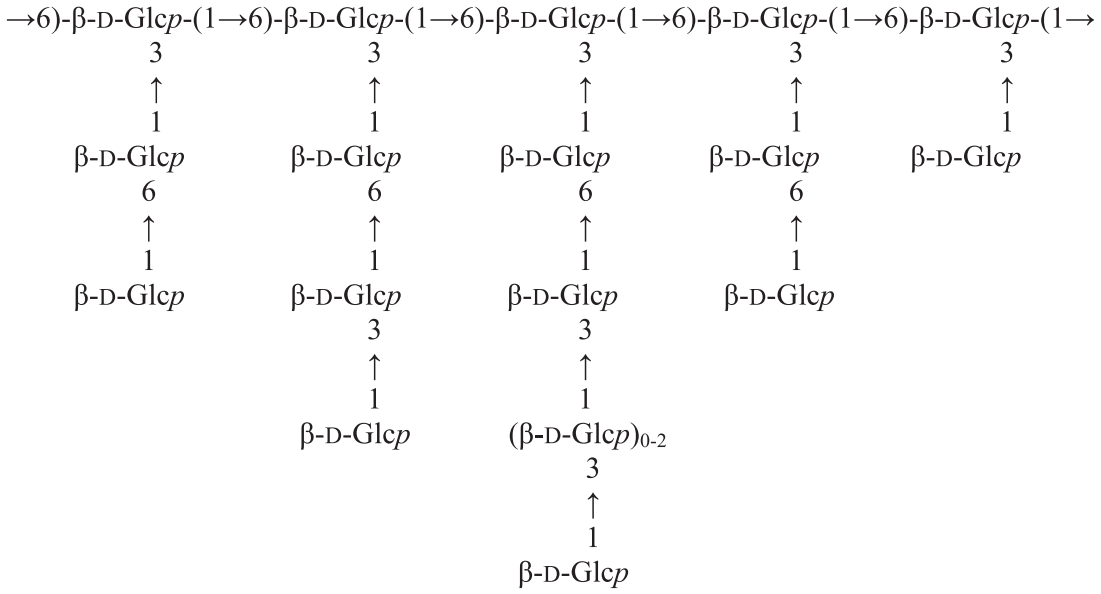



**Fig. 5.** $^1$H–$^1$H ROESY NMR spectrum of the native β-glucan from *I. galbana*.

H-6′($G_1$)/C-1($G_1$), H-6,6′($G_3$)/C-1($G_4$) and H-3($G_1$)/C-1($G_3$). The final proton and carbon-13 signal assignments are given in Table 2. The highly branched structure of the β-glucan from *I. galbana* may be depicted as follows:




### 3.3. Biological studies

In order to determine whether the β-glucan from *I. galbana* possesses immunomodulatory properties, its effect on interleukin-8 (IL-8) expression by the human U937 monocytic cell line was evaluated. This was tested on untreated cells and cells that were stimulated with either lipopolysaccharide (LPS, 100 ng/mL, 24 h) or phorbol 12-myristate 13-acetate (PMA, 1 ng/mL, 24 h). Initial experiments confirmed that the glucan does not bind directly to LPS or IL-8 (data not shown). The effect on basal IL-8 expression in U937 cells is shown in Fig. 7A. There was no significant effect on IL-8 expression over the dose range tested (1–200 μg/mL). The effects on LPS- and PMA-induced IL-8 expression are shown in Fig. 7B and C. LPS and PMA caused significant increases in IL-8 protein expression from the U937 cells. The glucan had no marked inhibitory or stimulatory effects on LPS- or PMA-induced IL-8 expression.

In addition, the effect of the glucan on cell death was tested using an MTS assay. There was no effect on cell death after 24 h at doses of 1 or 10 μg/mL (Fig. 7D). Above this concentration (100 and 200 μg/mL) there was up to ~30% cytotoxicity ($P = 0.0286$). As there was no parallel increase in IL-8 release from the cells at these doses of β-glucan, it was first hypothesized that cell death was likely to have occurred via an apoptotic rather than a necrotic process, in contrast to the absence of apoptosis (Battle et al., 1998), and even to the anti-apoptotic activity of laminarans (Kim, Kim, Kim, Lee, & Lee, 2006).

Apoptosis was assessed by release of mono- and oligonucleosomes in the cytoplasm using the Cell Death Detection ELISA plus Kit, after incubation of cells with glucan at 100 μg/mL, using camptothecin as a positive control. Surprisingly, no detectable apoptosis was observed (Fig. 8). In order to verify if cytotoxicity may be associated with cell lysis, we performed a lactate dehydrogenase (LDH) assay. No difference was observed for untreated cells and cells treated with glucan at 100 μg/mL for 24 h (data not shown).

Thus, the observed cytotoxicity in the MTS assay could be due to the inhibition of cell proliferation rather than cell mortality. In agreement with this hypothesis, a significant inhibition of cell proliferation was observed after 72 h of incubation in the presence of glucan at 50 μg/mL, and after 48 and 72 h in the presence of glucan at 100 μg/mL (Fig. 9). These results suggest that the β-glucan from *I. galbana* directly inhibits the proliferation of U937 human leukemic monocyte lymphoma cells, similarly to other (1 → 3,1 → 6)-β-D-glucans enriched in (1 → 6)-linkages (cf. Ermakova et al., 2013; Pang et al., 2005), and therefore has potential anti-tumor activity.

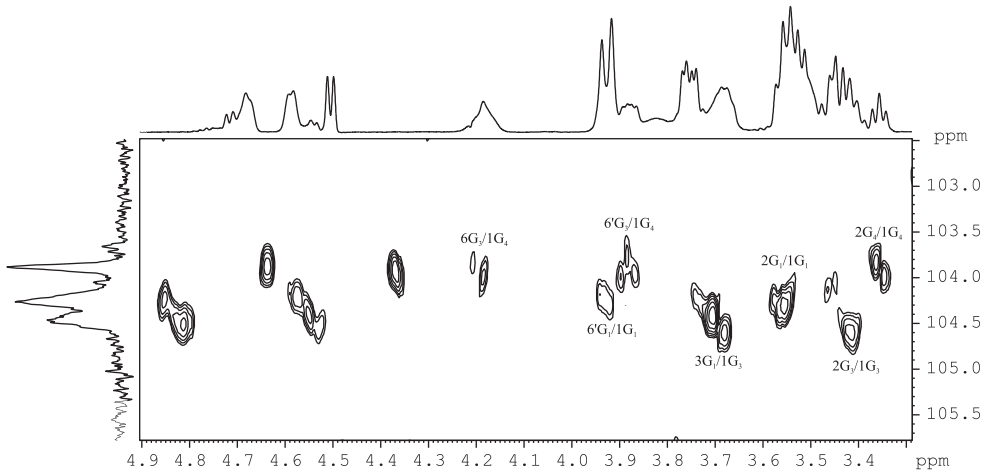



**Fig. 6.** $^{1}H–^{13}C$ HMBC NMR spectrum of the native β-glucan from *I. galbana* (Arabic numerals before slash denote protons and those after slash denote carbon atoms in residues $G_1$, $G_3$ and $G_4$).

## 4. Discussion

Reserve $(1\rightarrow 3,1\rightarrow 6)$-β-D-glucans are common polysaccharides of the algae belonging to the kingdom Chromista. The best known glucans of this type are laminarans of the brown seaweeds. The majority of laminarans contain linear backbones built up of 3-linked β-D-glucopyranose residues with several single β-D-glucopyranose residues attached as side substituents to C-6 of the

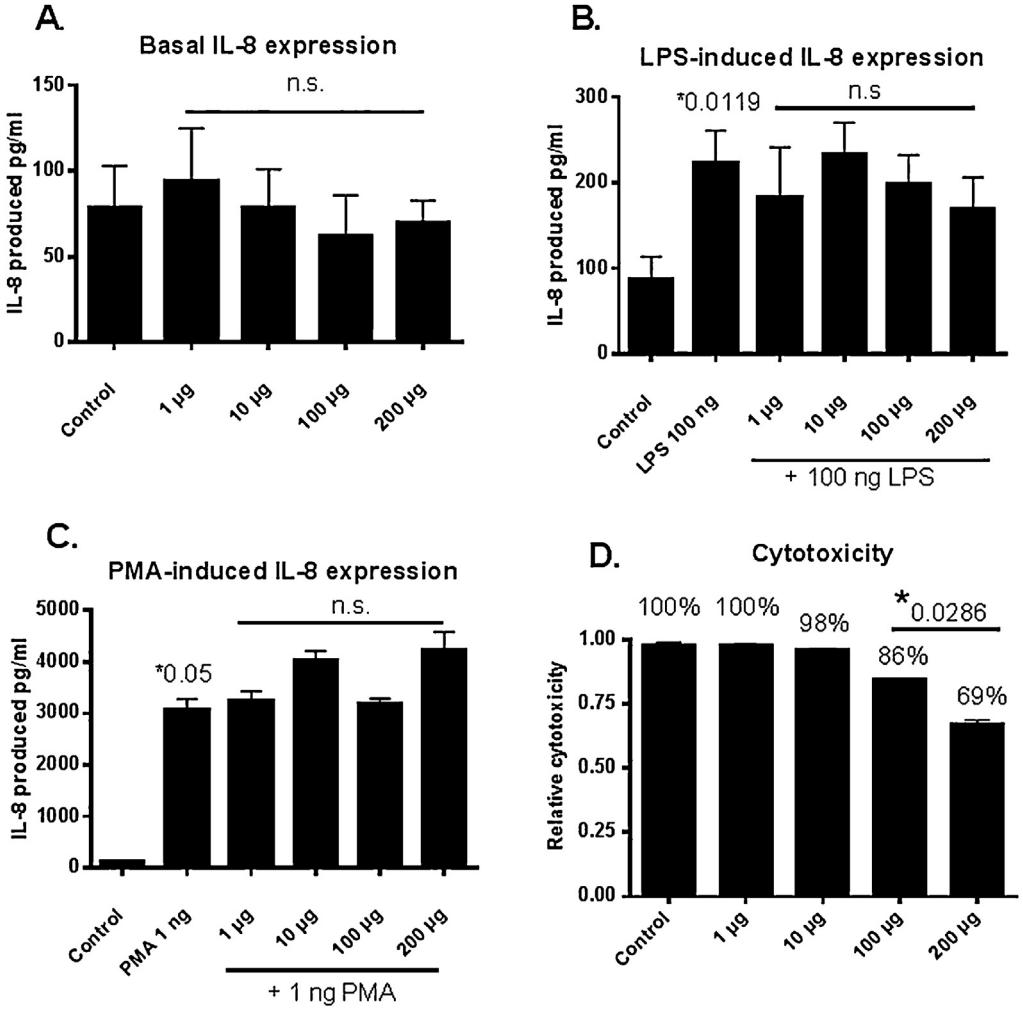



**Fig. 7.** IL-8 levels in human monocytic U937 cells that were left untreated (A) or stimulated with (B) LPS (100 ng/mL) or (C) PMA (1 ng/mL) in the absence or presence of β –glucan (doses as indicated) for 24 h. (D) Cytotoxicity assay measured by MTS test after 24 h. Assays were performed in triplicate, *P* values marked by asterisk were calculated using non-parametric, two-tailed *t* test; n.s. – non-significant.

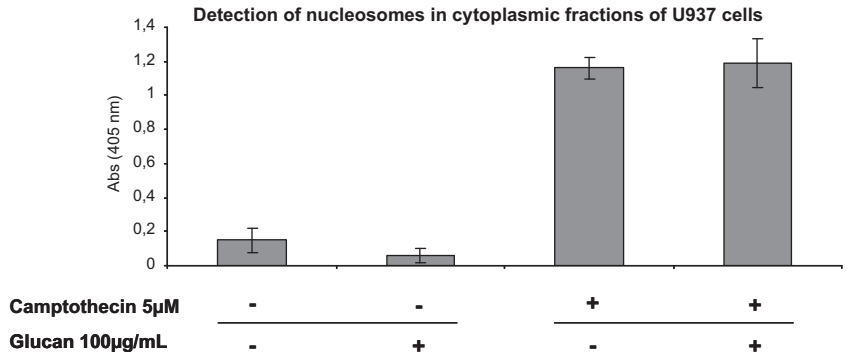



**Fig. 8.** Detection of nucleosomes in cytoplasmic fractions of U937 cells. Camptothecin (5 μM) induced oligonucleosome formation in U937 cells (=positive control). Glucan at 100 μg/mL had no effect on oligonucleosome formation after 8 h of treatment.

backbone (Percival & McDowell, 1967), although there are examples of laminarans containing both 1 → 3 and 1 → 6 linkages in the backbone (Maeda & Nisizawa, 1968). Representatives of other algal phyla may contain β-glucans, which differ considerably from laminarans in the ratio and arrangement of 3- and 6-linked residues (Usov, 2013). In particular, two haptophytes belonging to the class Coccolithophyceae were shown to contain β-glucans, where 6-linked residues predominate (Hirokawa et al., 2008; Vårum, Kvam, & Myklestad, 1986). The corresponding polysaccharide isolated from *Emiliania huxleyi* contained a backbone of 6-linked β-D-Glc*p* residues bearing side chains of gentiobiose residues attached to position 3 of every residue of the backbone (Vårum et al., 1986). The glucan from *I. galbana* has a similar structure with more complex side chains.

Numerous β-glucans built up mainly of 3-linked and 6-linked Glc*p* have been obtained from bacteria, algae and fungi and studied as biological response modifiers (Bacic, Fincher, & Stone, 2009). Their biological activity was shown to depend on the ratio of 1 → 3 and 1 → 6 linkages (degree of branching) and chain length (molecular mass distribution). Especially important is the ability of high-molecular weight linear glucans to adopt a triple helical conformation in solution (Sletmoen & Stokke, 2008). Thus, scleroglucan and schizophyllan were shown to be more effective immunomodulators than laminaran due to more effective binding to specific (1 → 3)-β-D-glucan receptors of a human monocyte-like cell line, U937 (Mueller et al., 2000). At the same time, laminaran oligomers with an average DP of 13 and 1 → 3 and 1 → 6 linkages ratio of 1.3:1 can effectively stimulate monocytes to inhibit the proliferation of U937 cells (Pang et al., 2005). Since the β-glucan from *I. galbana* differs from most of the known analogs by the structure of its backbone, side chains and degree of branching, it was interesting to characterize its possible biological activity.

We tested the immunomodulatory properties of the purified β-glucan from *I. galbana* by evaluating its effect on interleukin-8 expression by the human U937 monocytic cell line. Although these cells are known to express both dectin-1 and the TLR2/6 heterodimer (Ikewaki, Fujii, Onaka, Ikewaki, & Inoko, 2007; Jin et al., 2011), the glucan had no significant inhibitory or stimulatory effects on LPS- or PMA-induced IL-8 expression. This suggests that the previously reported proinflammatory effects of crude β-glucan preparations from *I. galbana* are not due to the glucan itself (Yu et al., 2010). However, it is also important to note that glucan-induced responses may differ in human and murine monocytic cells. This may explain the observed lack of an immunomodulatory effect here.

In addition, we observed the ability of the glucan to inhibit the proliferation of U937 cells, a human leukemic monocyte lymphoma cell line. This property resembles the inhibitory action of an oligosaccharide fragment of laminaran from *Eisenia bicyclis*, containing a high proportion of 1 → 6 linkages (Pang et al., 2005), as well as the anti-tumor activity of native laminaran from this alga, which can inhibit growth of human melanoma SK-MEL-28 and

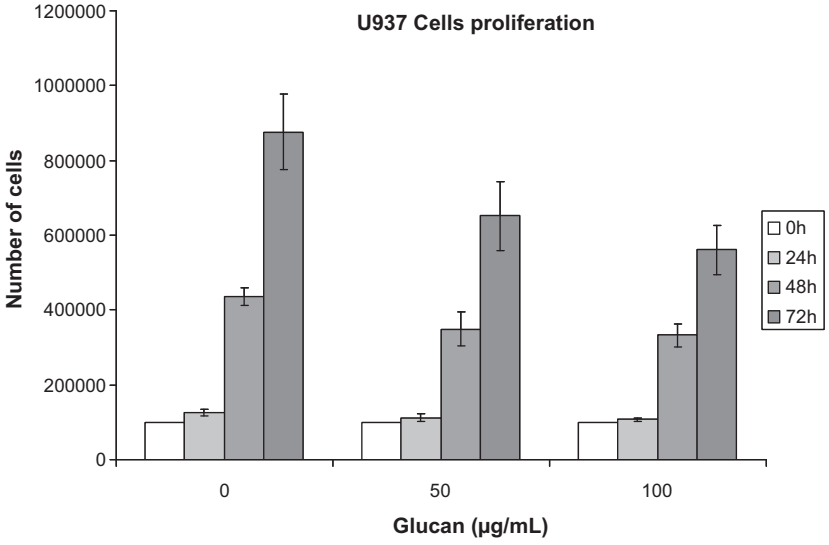



**Fig. 9.** U937 cell growth at different concentrations of glucan (0, 50, and 100 μg/mL).

DLD-1 colon cancer cells (Ermakova et al., 2013). This observation suggests that *I. galbana* β-glucan could have potential as an anti-tumor agent for myeloid cell cancers.

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