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A branched β-D-(1→3,1→6)-glucan from the marine diatom *Chaetoceros debilis* (Bacillariophyceae) characterized by NMR

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Abstract—The chrysolaminaran from the marine diatom *Chaetoceros debilis* was isolated and characterized by NMR spectroscopy. Cells were harvested in the stationary phase of growth after the medium had been depleted of nitrate when the chrysolaminaran content was expected to be at its highest. The chrysolaminaran was isolated with an yield of 17.5 mg/L, which corresponds to15.8 pg/cell. ^{1}H NMR indicated that the structure was similar to that of a β-(1→3) main chain with β-(1→6)-linked side chains. The degree of polymerization was found to be 30, corresponding to a molecular weight of ~4900. Thirty-three percent of the residues were found to be β-(1→6)-linked branches. The characteristics of the β-(1→6) branching were examined by NOESY NMR, which suggested pustulan-like branches, being β-(1→6) linked chains connected to the main chain with few branch points. Confirmation of the ^{1}H NMR data was done by ^{13}C-DEPT, TOCSY, COSY and HMQC NMR spectroscopy. The assignment of the resonances of the main β-(1→3) and β-(1→6) chains is presented. The structure proposed from our analyses is compared against other chrysolaminaran structures.

Keywords: Chrysolaminaran; *Chaetoceros debilis*; Diatom; Glucan; NMR

1. Introduction

β-(1→3)-Glucans are a class of compounds possessing a range of different biological activities, and are thus said to be biological response modifiers (BRMs).[1] One activity is the capability of stimulating the non-specific immune system of animals.[2–4] The activity of the β-(1→3)-glucans is dependent on molecular size and other structural traits, such as branching characteristics and solution conformation.[5,6] The literature available on structure–function relationships at present is extensive and in many cases contradictory, which is shown in reviews on the subject.[1,7]

Marine diatoms store β-(1→3)-glucans as an energy reserve,[8] and synthesis is facilitated upon depletion of nutrients in the growth medium.[9] Several structural studies have been performed on the β-(1→3)-glucans from diatoms, which are called chrysolaminarans due to their similarity with laminarans, which are found in most brown algae. The main difference between chrysolaminarans and laminarans is that the former are devoid of guluronic and mannuronic acid terminal end groups.[10] The chrysolaminaran from *Chaetoceros mülleri* has been characterized and found to be a β-(1→3)-glucan with a DP of 19–24 and a degree of β-(1→6)-branching (DB) of 0.005–0.009.[11] Laminaran from *Laminaria digitata* has DP/DB of 20–30/0.05.[12]

In a study made by Jamois et al., synthetic β-(1→3)-glucans with DPs of 4 and 5 have been found to have immunomodulatory effects.[13] The diatom *Thalassiosira weissflogii* has been found to have a DP of 5–13[14] without any branching, and could then be expected to possess the same activity as the synthetic tetra- and pentaglucosides. A chrysolaminaran from *Thalassiosira pseudonana* has been characterized in two independent

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works. Chiovitti et al. have used chromatography,[15] and in our laboratory NMR-spectroscopy has been used by Størseth et al. (unpublished data). This chrysolaminaran was found to be devoid of branching but was found to have a DP of 29 in the latter study, which is higher than the DP found for *T. weissflogii*. McConville et al. have characterized the chrysolaminarans from the ice diatom *Stauroneis amphioxys*,[16] which was found to have a molecular weight of 4000 (DP 24). Paulsen et al. found the chrysolaminaran of the marine diatom *Skeletonema costatum* to have a molecular weight of 6200 by gel filtration, and 13,000 by osmometry, with some branching in 1,2 and 1,6 positions.[17] The DP reported for chrysolaminarans has been in the range of 5–60.[8,10,15,18,19] Alekseeva found a fraction of the chrysolaminaran from the diatom *Stephanodiscus meyerii* to have a molecular weight of 40 kDa.[20] In a study made by Størseth et al., the chrysolaminarans from both *C. mülleri* and *T. weissflogii* were found to have a constant structure throughout the growth curve.[14]

All these studies, when seen together, suggest that chrysolaminarans from different diatoms span a great number of different molecular weights from ~1 to 40 kDa and branching characteristics with 0–20% of the residues being part of linkages. Further, the structure from one species may be expected to be constant. As such, chrysolaminarans are interesting for use as BRMs. Laminaran from *L. digitata* has been found to be a BRM,[21] and the similarities in structure between laminarans and chrysolaminarans have led to studies on the effects of the chrysolaminaran from *C. mülleri* on the survival and growth of cod larvae in our laboratories.[22] Enhancement in both growth and survival was found in this study. In the present study, we wanted to examine the structure of the chrysolaminaran from the same genus as *C. mülleri*, the marine diatom *Chaetoceros debilis*.

2. Experimental

2.1. Cultivation and isolation

C. debilis Cleve was cultivated with use of the Guillard f/2 medium.[23] The alga (1 L) was grown to the late log phase and diluted to 1.5 L at 18 °C and continuous light (~200 μE/s^2 m). The pH was kept in the range of 7.5–9.0 by adjusting the amount of CO_2 added (0–2%). Growth was monitored by measuring the absorbance at 750 nm and the growth rate was expressed as

$$\mu = \ln(N_1/N_0)/t \quad (1)$$

where N_0 is the biomass as time t_0 and N_t is the biomass at time t.

The cells were harvested in the late stationary phase, when the growth rate was close to zero and the medium was depleted of nitrate (analyzed using a Skalar autoanalyzer using the standard methods provided with the instrument), by collection of the cells on filters (Whatman GF/C). Culture (1.2 L) was collected and extracted with 0.05 M H_2SO_4.[24] The extract was neutralized with NaOH and freeze dried to give 210 mg of crude extract, which after dialysis of (MWCO 1000) gave the chrysolaminaran in 10% yield, corresponding to 17.5 mg/L and 15.8 pg/cell, which was used directly for NMR analysis.

2.2. NMR analysis

A NMR sample was prepared by the method of Kim et al.,[12] by dissolving 5 mg of the chrysolaminaran in Me_2SO-d_6/D_2O 6:1. All spectra were recorded on a Bruker DRX-500 spectrometer fitted with a TXI probe at a temperature of 353 K, using standard pulse programs from the Bruker library (Bruker Biospin GMBH, Rheinstetten, Germany). All processing was done using the Bruker Topspin 1.3 program.

For ^{1}H NMR a 45° observe pulse was used to record 256 transients of 32k data points. The acquisition time (AQ) was 3.32 s and the interscan delay (d1) was 3 s. Zero filling and exponential line broadening (0.3 Hz) was applied before Fourier transform. DP was calculated as shown by Kim et al. The ^{13}C DEPT135 spectrum was recorded by collecting 15k scans of 64k data points with an AQ of 1.74 s and d1 of 2.5 s. Zero filling and exponential line broadening (1 Hz) was applied before Fourier transform. All ^{1}H spectra were referenced to the Me_2SO peak, which was set to 2.55 ppm. For carbon the corresponding peak was set to 39.5 ppm. All 2D homonuclear spectra were collected as 2048×512 data points in the F2 × F1 directions, zero filled and apodized by a square sine bell window function (SSB). TOCSY and NOESY spectra with mixing times of 50–300 ms were acquired. The HMQC-spectrum was acquired by recording 16 scans into 2048×1024 data points in the F2 × F1 directions. The spectral width was 6×16 kHz. Zero filling and SSB was applied.

3. Results and discussion

The resolution of the 500 MHz ^{1}H NMR spectrum of the chrysolaminaran from *C. debilis* allowed for the assignment of the anomeric resonances, which are found in the 5.10–4.20 ppm region by comparison with laminaran and gentiobiose standards purchased from Sigma, as well as with chrysolaminarans previously described in our laboratories. Homo- and hetero-nuclear correlation spectroscopy was used to verify assignments. These included ^{1}H, ^{1}H-COSY, NOESY, TOCSY and ^{1}H,^{13}C-HMQC. A ^{13}C NMR spectrum was recorded using the DEPT135 pulse sequence.

3.1. ^{1}H NMR spectroscopy

The resonances from the β-(1→3)-linked chain of the chrysolaminaran were easily assigned on the basis of their chemical shifts and comparison with the previously mentioned standards. The anomeric region of the ^{1}H NMR spectrum is presented in Figure 1. At 5.03 ppm the only α-anomeric resonance was found, corresponding to the α-reducing end group (RT). A resonance at 4.57 ppm was assigned to the β-(1→3) linked backbone chain (BC). This resonance was found in partial overlap with the resonances assigned to the units positioned second to the RT group (SRT) at 4.53 and 4.51 ppm, attached to the β- and α-RT positions, respectively. At 4.46 and 4.43 ppm, the non-reducing end group (NRT) and β-RT resonances of a β-(1→3) chain were found. In the laminaran standard, ^{1}H NMR spectrum resonances of β-(1→6) linked residues are found at 4.34 and 4.28, and these have been assigned by Kim et al.[12] to be from terminal side chain and internal side chain groups, respectively. In the chrysolaminaran from *C. debilis*, one broad peak was observed in this region from 4.35 to 4.27 ppm. A closer look at the peak shape suggested that it consisted of several peaks, thus indicating that β-(1→6) side chains are of different lengths and not uniform as those found in laminaran. Only one set of resonances belonging to reducing end groups was observed, which belonged to the RT of the β-(1→3) chain. No resonances from reducing groups of β-(1→6)-linked resonances were observed. This is the same situation as with laminaran. From the ^{1}H-resonances, the DP was calculated to be 30. To obtain the DB, the resolved resonance of the terminal side chain (TSC) anomer is needed.[12] This was not the case for the ^{1}H spectrum obtained for the *C. debilis* chrysolaminaran, and it would thus be wrong to describe the branched residues in terms of degree of branching (DB), as this term would refer to the frequency of the branches. The ratio between β-(1→3) and β-(1→6)-linked residues could however be obtained by the resonances and approximately 10 of the 30 residues in the chrysolaminaran were thus found to be β-(1→6)-linked. The DP corresponds to a molecular weight of ∼4900. Previously reported chrysolaminarans have had branching percentages ranging from 0.5% to 20% of the linkages that have been β-(1→6), and several reports have been made of chrysolaminarans devoid of branching. To our knowledge, the *C. debilis* chrysolaminaran has the highest percentage of β-(1→6)-linked residues being part of branches, either as part of branch points, or as residues being part of the branch. From the anomeric resonances, it was possible to assign the ^{1}H chemical shifts of the other positions in the β-(1→3) chain and the β-(1→6) linked residues using ^{1}H,^{1}H-COSY and TOCSY. These results are shown in Table 1.

3.2. ^{13}C DEPT and ^{1}H,^{13}C NMR spectroscopy

The ^{13}C DEPT spectrum supported the linkage information from the ^{1}H spectrum (the HMQC spectrum with the DEPT spectrum as a projection is shown in

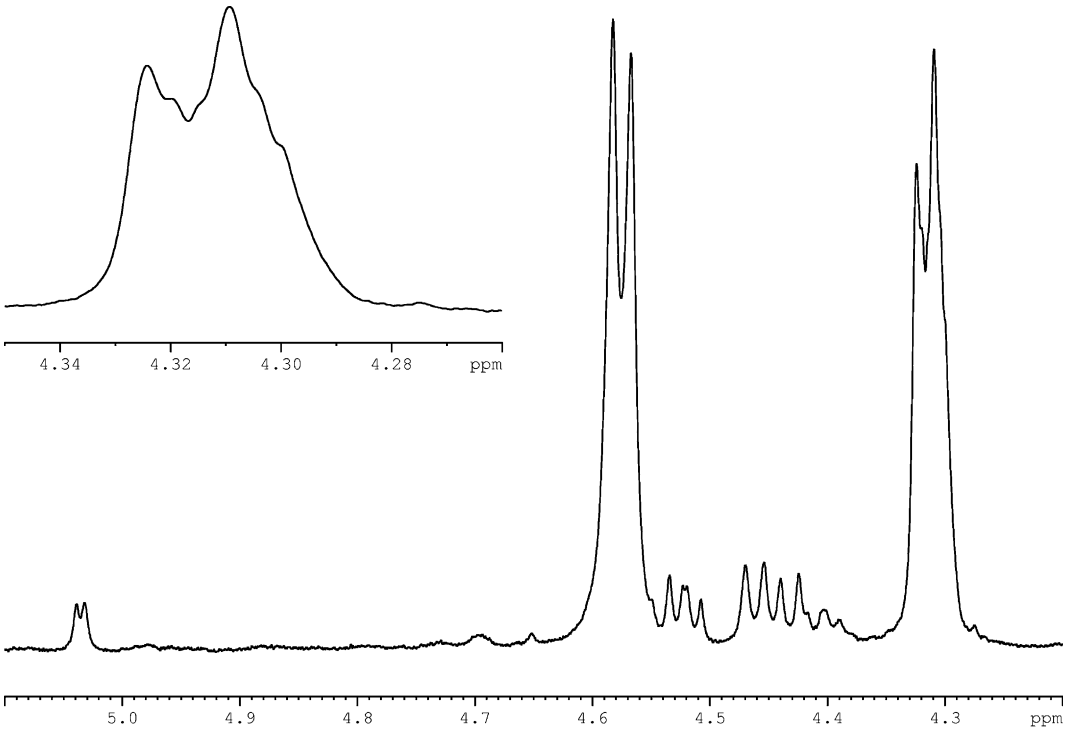

Figure 1. The anomeric region of the ^{1}H NMR spectrum of the chrysolaminaran isolated from *C. debilis* with excerpt of the resonance(s) found at 4.35–4.27.

Table 1. The chemical shift values and correlations of the chrysolaminaran isolated from *C. debilis*

Position	^{1}H	^{1}H–^{1}H COSY correlations (correlated nuclei)	^{1}H–^{1}H NOE correlations (correlated nuclei)	^{13}C-correlation
(1→3):1	4.57	3.35 (2)	3.54 (3) 3.27–3.39 (5)	103.34
(1→3):2	3.35	4.57 (1) 3.54 (3)		73.43
(1→3):3	3.54	3.35 (2) 3.30 (4)	4.57 (1) 3.32 (5)	86.34
(1→3):4	3.30	3.54 (3) 3.32 (5)		68.94
(1→3):5	3.32	3.75 (6) 3.55 (6′) 3.30 (4)	4.57 (1) 3.75 (6) 3.54 (3)	76.74
(1→3):6 and 6′	3.75 (6) 3.55 (6′)	3.75 (6) 3.55 (6′) 3.32 (5)	3.32 (5)	61.38
(1→6):1	4.31	3.09 (2)	4.03 (6) 3.65 (6′) 3.38 (5) 3.25 (3) 3.09 (2)	103.63
(1→6):2	3.09	4.31 (1) 3.25 (3)	3.25 (3)	73.85
(1→6):3	3.25	3.22 (4) 3.09 (2)	3.09 (2)	76.69
(1→6):4	3.22	3.25 (3) 3.38 (5)	3.38 (5)	70.51
(1→6):5	3.38	4.03 (6) 3.65 (6′) 3.22 (4)	4.31 (1) 3.65 (6) 3.22 (4)	75.96
(1→6):6	4.03 (6) 3.65 (6′)	4.03 (6) 3.65 (6′) 3.38 (5)	4.31 (1) 3.38 (5)	69.07

Fig. 2). Correlation of the ^{13}C and ^{1}H resonances was done using the HMQC spectrum. The C-1 of the β-(1→3) BC and β-(1→6) resonances were well separated at 103.34 and 103.64, respectively. Although care should be taken when integrating non-quantitative ^{13}C NMR spectra, the percentage of β-(1→6)-linked residues was found to be 37% upon integration of the resonances of the anomeric region, and thus in good correlation with the ^{1}H NMR spectrum. The negative resonances in the DEPT135 spectrum from C-6 positions, found at 69.07 and 61.38 ppm, show the typical downfield placement of carbon shifts having linked, as compared to free, hydroxyl groups,[25,26] which is also seen in the ^{1}H spectrum for the protons on the C-6 position. Correlations from two ^{1}H to these ^{13}C resonances show the different frequencies of the two methylene protons of the C-6 position. This also supports the linkage results from the ^{1}H spectrum. Assignments of ^{13}C-resonances with ^{1}H correlations are given in Table 1.

3.3. NOE NMR

To further support the structure found from the through-bond coupling information and chemical shifts from ^{1}H, ^{1}H,^{1}H-COSY and TOCSY, it is desirable to have evidence of inter-residue NOEs.[27] In chrysolaminarans where the only building block is the glucopyranosyl-unit, this may be somewhat difficult as intra- and inter-residual NOEs may overlap. Figure 3 shows the TOCSY and NOESY spectra combined graphically. In the β-(1→3)-linked backbone of the chrysolaminaran from *C. debilis*, NOE cross peaks are seen from the anomeric resonance of the BC at 4.57 ppm to the ^{1}H on C-3 at 3.54 ppm and on C-5 at 3.32 ppm. It was not possible to ascertain whether the 1–3 cross peaks originated from an inter- or intra-residual NOE correlation. NOE's between the BC and the anomeric resonances of the NRT, or from the BC to the ^{1}H on C-3 of the SRT or RT, which are resolved from the BC, were not observed. Observation of a NOE correlation between an anomeric resonance and a C-6 ^{1}H resonance would support the assignment of those resonances belonging to β-(1→6) linked glucopyranosyl residues. This was observed in our spectra where NOE correlation peaks were found between the anomeric resonances at 4.31 and one of the two ^{1}H on the C-6 of the residues having an anomeric resonance at 4.31. Monteiro et al. have used NOE NMR to describe two different conformations in the linkages of the β-(1→6)-linked glucan from *Actinobacillus suis*,

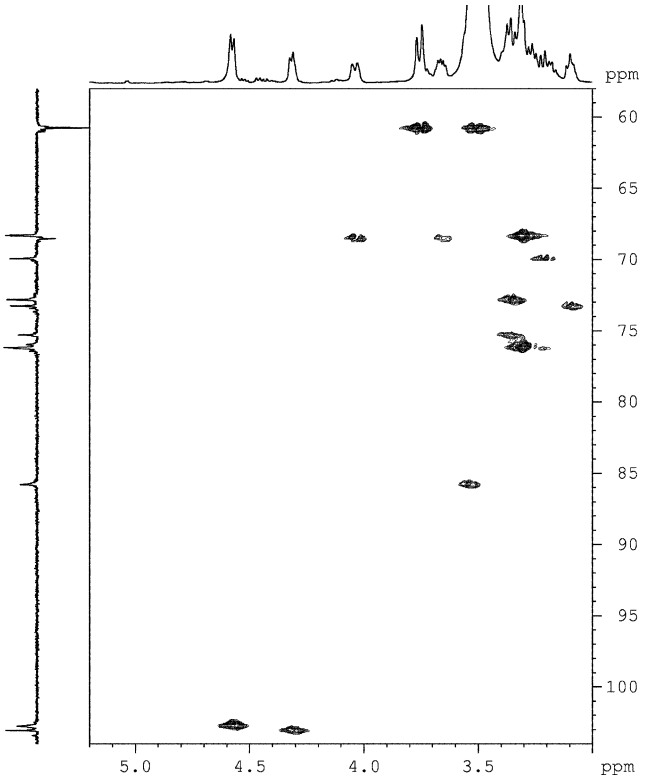

Figure 2. The HMQC spectrum of the chrysolaminaran isolated from *C. debilis* with the ^{13}C DEPT135 spectrum as a projection for the F1 direction. The complete ^{1}H spectrum (not shown elsewhere) is shown as the projection of the F2 direction.

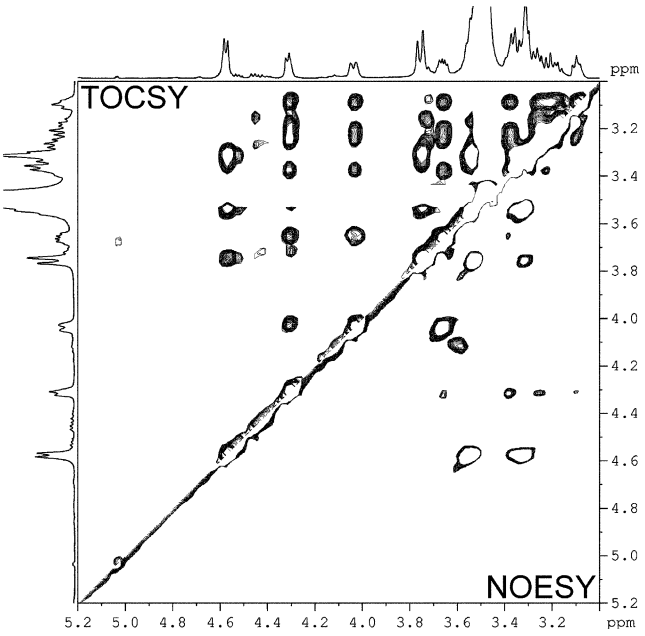

Figure 3. The TOCSY and NOESY spectra of the chrysolaminaran isolated from *C. debilis* graphically edited into one figure. Mixing times are 300 ms for both experiments.

where the presence of 6,1, 6′,1 and 6′,2 inter-residue NOEs was detected.[28] From the β-(1→6) branches in our study of the *C. debilis* chrysolaminaran, only 6′,1 inter-residue NOEs were found (Fig. 4). This could indicate that only one conformer is present. The NOESY spectrum gave NOE cross peaks from the β-(1→6) anomer resonance to protons on C-3, C-5 positions in addition to the C-6′ position. The C-3 and C-5 ^{1}H resonances were found at 3.25 and 3.38 ppm, respectively. Other NOE peaks observed are reported in Table 1.

The broadness of the anomeric β-(1→6) resonance, the fact that it is situated at 4.32 ppm, which is the same as is reported for the BC of β-(1→6) polymer pustulan in the same solvent system,[12] and the presence of the inter residue 1–6 NOE all suggest that the β-(1→6) linkages are present in pustulan-like β-(1→6) chains and not as single residues. This is also supported by comparing the spectrum of the *C. debilis* chrysolaminaran with laminaran and the chrysolaminaran from *C. mülleri*. Laminaran has been reported to have short β-(1→6) linked branches[12] and the ^{1}H spectra give resolved peaks at 4.34 and 4.28, which are assigned to side chain (SC) and terminal side chain residues (TSC). The chrysolaminaran from *C. mülleri* has been found to have single glucopyranosyl residues in β-(1→6) linkages. The resonance from these residues is found at 4.28 ppm. The *C. debilis* chrysolaminaran has the same percentage of β-(1→6)-linked residues as scleroglucan found by Kim et al. to be 33% by the method used in this study. In scleroglucan there is one β-(1→6) branch point in every third β-(1→3) BC residue.[4,12] This has consequences for the β-(1→3) BC anomeric resonances, which are spilt up into overlapping doublets compared to the single doublet at 4.57 ppm from glucans with few or no branch points.[12] In the spectrum from *C. debilis*, the BC resonance is one doublet, which is in partial overlap with the SRT resonances. From this we propose that the branching characteristics of the *C. debilis* chrysolaminaran are those of a glucan with few long β-(1→6) side chains linked to a β-(1→3) backbone with relatively few branch points. A resolved TSC from the β-(1→6) linked residues was lacking in the *C. debilis* chrysolaminaran and because of this the average chain length and number of branch points could not be calculated.

All data recorded of the chrysolaminaran isolated from *C. debilis* are supportive of each other and of the assignment made in the ^{1}H NMR spectrum. The NOE spectrum was found to be necessary to elucidate the nature of the β-(1→6)-chain, as was also the comparison of the spectrum with literature data from scleroglucan. The percentage of β-(1→6)-linked residues found for this chrysolaminaran is to our knowledge the highest reported, and is an example of the variety that may be expected from chrysolaminarans. The *C. mülleri* chrysolaminaran, as mentioned previously, has a DB of 0.005–0.009 and this exemplifies the variations, which may occur inside the same genus. Another example of variations within the same genus is the chrysolaminarans isolated from two *Thalassiosira* diatoms: *T. pseudo-*

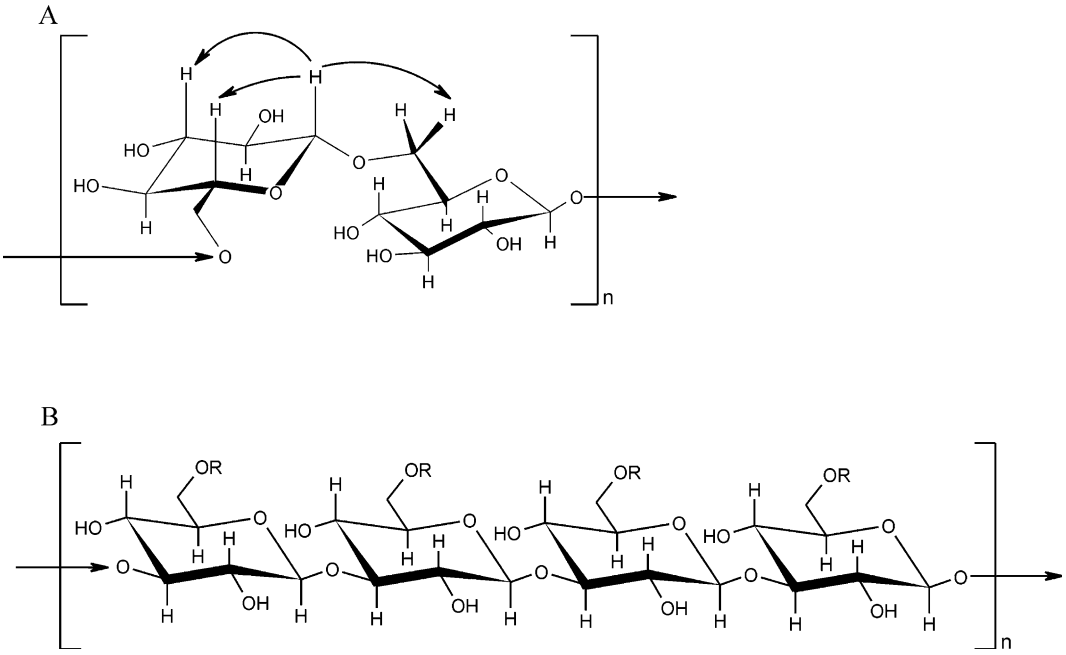

Figure 4. Structure elements of the chrysolaminaran isolated from *C. debilis*. (A) Linkage and NOE correlations found in the β-(1→6)-chain. (B) Structure of the β-(1→3) main chain, R: H or the β-(1→6)-chain in (A).

nana and *T. weissflogii*, which have been shown to be β-(1→3)-linked and to have DPs of 29 (Størseth et al., unpublished data) and 5–13,[14] respectively. These two diatoms were found to be devoid of branching by NMR, and as the DPs were shown only to vary in length. The two *Chaetoceros* diatoms discussed here both have β-(1→6)-linkages in various degrees. The linkages of diatoms of different genera should be examined in further studies, as these results indicate that diatoms of the same genus could provide structurally different chrysolaminarans differing in the ratios of DP/DB, and not in the types of linkages present.

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