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Structure determination of a novel uronic acid residue isolated from the exopolysaccharide produced by a bacterium originating from deep sea hydrothermal vents

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Abstract

The exopolysaccharide produced by the bacterium *Alteromonas sp.* strain 1644 originating from deep sea hydrothermal vents was shown to contain a novel glucuronic acid derivatives: 3-*O*-[(*R*)-1-carboxyethyl]-D-glucuronic acid. The structure of this compound was established on the basis of mass spectrometric data, methylation analysis, preparation of derivatives, and chemical synthesis of references compounds. © 1996 Elsevier Science Ltd.

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1. Introduction

The present work is part of a project of the National Centre for Scientific Research (CNRS; GDR Bactocean program) aiming at collecting new microorganisms as new sources of thermostable enzymes and polysaccharides. In that respect, deep sea hydrothermal vents present a particular interest since they are characterized by extreme conditions such as high pressures, step temperature gradients, and high concentrations of divalent cations. In 1987 the French organization IFREMER (Institut Français de

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Recherche pour l'Exploitation de la Mer) led an expedition on the East Pacific Rise at a depth of 2600 m. Among the bacteria collected, *Alteromonas sp.* strain 1644, isolated from the epidermis of *Alvinella caudata* (Polychaeta: Annelida) [1], was of particular interest since it was shown to secrete large amounts of polysaccharide even under milder conditions (such as room temperature and atmospheric pressure). During the study of the physico-chemical properties of this product, interesting rheological behaviour was observed, namely the formation of elastic and resistant gels in the presence of divalents cations [2,3]. In order to produce this polysaccharide on a large scale, the bacterium *Alteromonas sp.* was grown in vitro and the culture conditions were optimized to produce it selectively and in high yield. At this stage, the structural characterization also became an important issue and prompted the present study. A preliminary sugar composition determination showed a high content of negatively charged residues, namely uronic acids, as well as an unusual sugar unit. This work deals with the structural characterization of this sugar residue supported by mass spectrometry, methylation analysis, preparation of derivatives, and chemical synthesis of reference compounds.

2. Experimental

Bacteria and culture conditions.—*Alvinella caudata* tissues were collected in 1987 during the 'Hydronaute' expedition on the East Pacific Rise (12°48′56″ N, 103°56′72″ W) at a depth of 2600 m.

Alteromonas sp. strain 1644 was isolated from this invertebrate [4]. Bacterium culture was obtained by using a zinc enriched Oppenheimer and Zobell medium. The polysaccharide was produced on a large scale in a 2 L fermenter containing 1.2 L of medium at atmospheric pressure and a temperature of 25 °C. Bacteria were grown on synthetic marine broth which contained peptone 4 g/L, yeast extract 1 g/L, sea salt 30 g/L, and 3-(*N*-morpholino)-propanesulfonic acid 5 g/L at pH 7.2.

Isolation and purification of the polysaccharide.—The bacterial cells were removed from the liquid culture by centrifugation at 20,000 g for 2 h. The supernatant was successively filtered on Sartorius membranes of decreasing porosity: 8 μm, 3 μm, 1.2 μm, 0.8 μm, and 0.45 μm. The polysaccharide was precipitated by addition of one volume of ethanol and was washed with ethanol/water mixtures (ethanol 5% step gradient from 70% to 100%).

The polysaccharide (50 mg) was purified by anion exchange chromatography on a diethylaminoethyl–trisacryl column (36.2 × 3.3 cm) equilibrated with 0.05 M 2-amino-2-hydroxymethyl-1,3-propandiol/hydrochloride at pH 8.6. Elution was performed using the same buffer (500 mL) followed by a step gradient of 0.1, 0.2, 0.3, 0.5, and M sodium chloride in the same buffer. Gel filtration was performed on a Sepharose 2B column (90 × 1.8 cm) using 0.5% acetic acid as eluent.

Gas chromatography / mass spectrometry.—Gas chromatography (GC) analyses were performed on Delsi DI 700 gas chromatograph equipped with a fused silica OV 101 capillary column (0.3 mm I.D. × 25 m), using helium as carrier gas at pressure of 0.5 atm. The following temperature program (120–240 °C at 2 °C/min) was used for alditol acetates, pertrimethylsilylated methyl, and butyl glycosides, and permethylated sugars.

GC/MS analyses were carried out on a Nermag R10-10 mass spectrometer (Rueil Malmaison, France). MS analyses were carried out in electron impact (Ei, 70 eV) or chemical ionization (Ci) mode using ammonia as reactant gas.

Monosaccharide composition determination.—Monosaccharides resulting from methanolysis (0.5 M methanolic HCl, 24 h, 80 °C) were analyzed by GC/MS as pertrimethylsilylated methyl glycoside derivatives [5]. Alternatively, hydrolysis of the polysaccharide with 4 M TFA (4 h, 100 °C), reduction of monosaccharides with potassium borohydride (KBH_4), peracetylation with pyridine/acetic anhydride (1:1) yielded alditol acetates which were also analyzed by GC/MS.

Absolute configuration of sugars [6,7].—The free monosaccharides obtained by hydrolysis with 4 M TFA (4 h, 100 °C) were converted into (*R*)-2-butyl glycosides using M HCl in (*R*)-2-butanol for 12 h at 80 °C. The resulting butyl glycosides were trimethylsilylated prior to GC analysis.

Isolation of the unknown sugar residue.—In order to isolate the monosaccharide constituents, the polysaccharide was treated with 4 M TFA at 100 °C for 4 h. The dried hydrolysate was subjected to preparative paper chromatography (Whatman No. 3) using ethyl acetate/pyridine/acetic acid/water (5:5:1:3) as eluent. Sugars were detected with aniline oxalate [8]. The main fraction was purified by high performance anion exchange chromatography (HPAEC, DIONEX, Sunnyvale USA) using a CarboPac PA1 column (9 × 250 mm). Elution was performed with A: 0.1 M NaOH and B: M sodium acetate in 0.1 M NaOH as eluent at a flow rate of 2 mL/min. The following gradient program was used 0–10 min: 0–30% B; 10–30 min: 30–50% B; 30–40 min: 50–100% B.

Carboxyl reduction.—The oligosaccharide was treated with M methanolic HCl for 6 h at room temperature in order to convert the carboxylic acid groups into methyl ester. The derivatized oligosaccharide was dissolved in methanol and the methyl esters were reduced using KBH_4 at room temperature for 12 h or alternatively sodium borodeuteride ($NaBD_4$). After acidification with Dowex 50 × 8 resin (H^+ form), which was removed by filtration, the dried carboxyl reduced oligosaccharide (**II**) was subjected independently to methanolysis, hydrolysis, and butanolysis for monosaccharide determination as described above.

Methylation.—The carboxyl reduced oligosaccharide (200 μg) was dissolved in 200 μL of a sodium/hydroxide dimethyl sulfoxide suspension, prepared by stirring anhydrous dimethyl sulfoxide with powdered sodium hydroxide [9]. After sonication for 30 min, 400 μL methyl iodide was added and the suspension was sonicated for another 2 h. The methylated product was extracted into chloroform and back-washed with water. The methylated product was subjected to hydrolysis by 4 M TFA (6 h, 100 °C) and analyzed as partially methylated alditol acetate (**VII**) by GC/MS [10].

Dealkylation of the novel sugar [11,12].—The carboxyl reduced oligosaccharide (**II**, 1 mg) was acetylated prior to the dealkylation with 1 mL pyridine/acetic anhydride (1:1) for 24 h. Boron tribromide (50 μL) was added to a solution of acetylated sugar in 500 μL dichloromethane and the reaction mixture was stirred for 2 min. The resulting dried product was deacetylated by treatment with 0.5 M sodium methoxide in methanol for 15 min and neutralized with Dowex 50 × 8 resin (H^+ form).

Synthesis of 3-O-[(R)-1-carboxyethyl]-D-glucose and 3-O-[(S)-1-carboxyethyl]-D-glucose [13].—1,2:5,6-Di-*O*-isopropylidene-α-D-glucose (250 mg) was dissolved in

17 mL 1,4-dioxane and NaH (145 mg) was added under vigorous stirring at 95 °C for 1 h. After cooling to 65 °C, 350 mg 2-chloropropionic acid (either *R* or *S* enantiomer) were added, and the mixture was stirred for 1 h. A second portion of 570 mg NaH and 7.5 mL 1,4-dioxane were added, and the mixture was stirred for another 14 h at 65 °C. After cooling, 25 mL water were added carefully to destroy the excess NaH. Dioxane was evaporated and the resulting aqueous solution was extracted with $CHCl_3$. The aqueous phase was acidified to pH 3 with 2.5 M HCl and reextracted with chloroform. Evaporation of the organic layer yielded the product which was deprotected by treatment with M TFA (4 h, 100 °C).

Reduction of 3-O-*(1-carboxyethyl)*-D-*glucose.*—3-*O*-(1-carboxyethyl)-D-glucose (500 μg) was esterified by treatment with 1 mL of M methanolic HCl at room temperature for 12 h. The dried product in solution in 1 mL methanol was reduced with KBH_4 as described above.

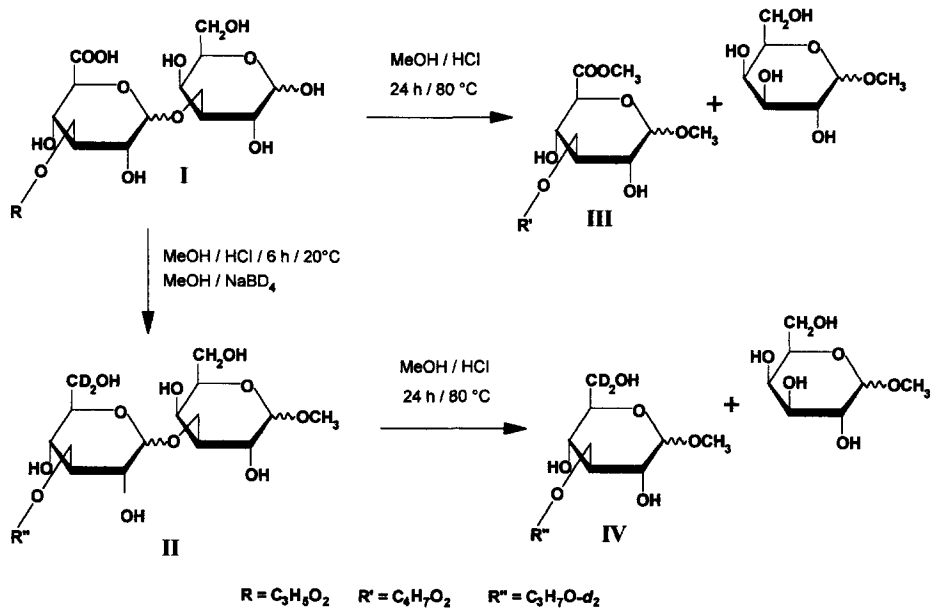
3. Results and discussion

The polysaccharide isolated and purified by ion exchange chromatography on a diethylaminoethyl–trisacryl column using 300 mM NaCl as eluent was shown to be homogeneous by gel filtration chromatography (Sepharose 2B column).

A preliminary sugar composition analysis was carried out by subjecting the polysaccharide to methanolysis and trimethylsilylation. GC/MS analysis of the mixture showed presence of glucose, galactose, glucuronic acid, and galacturonic acid as well as an unknown sugar residue, which is characterized by two signals ($t_R/t_{R_{myo-inositol}} = 0.91$ and 0.92) corresponding to the two anomers. The mass spectra associated to these peaks indicated in both cases a $[M + NH_4]^+$ molecular species at m/z 470, which may correspond to a tris(trimethylsilylated) hexuronic acid methyl ester bearing a $C_4H_7O_2$ substituent.

In order to isolate this unknown sugar residue for detailed structural characterization, the native polysaccharide was subjected to an extensive acidic hydrolysis with 4 M TFA (100 °C, 4 h). The resulting mixture was subjected to a preparative paper chromatography using ethyl acetate/pyridine/acetic acid/water (5:5:1:3) as eluent. A late eluting fraction was obtained ($R_{Glc} = 0.18/R_{GlcUA} = 0.31$), next to the fractions containing hexoses and uronic acids, which was submitted to high performance anion exchange chromatography and showed the presence of two peaks. Both components were collected in a series of successive HPAE chromatography runs. The GC/MS sugar composition analysis of the two fractions after methanolysis and pertrimethylsilylation showed the presence of galactose and an unknown residue in the first one, galacturonic acid and glucuronic acid in the second one. The presence of disaccharides reflects the stability of the glycosyl uronic bond towards acidic hydrolysis.

A detailed structural study was performed on the disaccharide constituted of a galactose unit and the unknown residue (**I**, see Scheme 1). In a first step this disaccharide was treated with methanolic hydrochloric acid in order to convert carboxylic groups into methyl esters, and was then reduced with $NaBD_4$ to yield the labelled product (**II**), which showed the presence of four deuterium atoms by MS

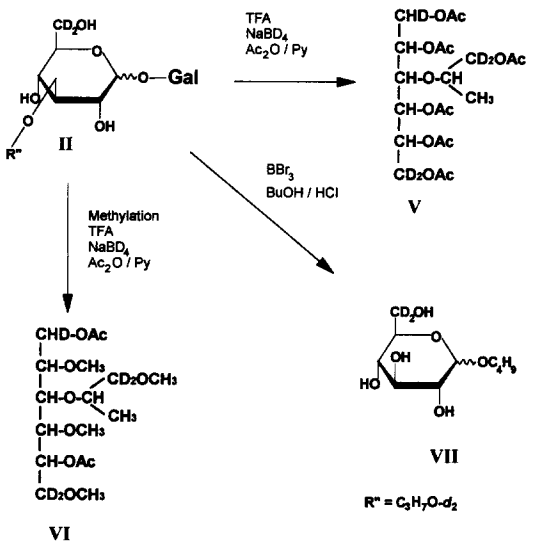


Scheme 1.

analysis. A portion of this carboxyl reduced disaccharide was hydrolyzed and converted into an alditol acetate (**V**) for GC/MS. The ammonia chemical ionization mass spectrum showed a $[M + NH_4]^+$ species at m/z 403, which indicates presence of five deuterium atoms, four of them resulting from the reduction of two carboxyl groups. Alditol acetates exhibit the presence of fragments at m/z 103, 278, 351, 378 suggesting an hexitol which corresponds to the reduced hexuronic acid unit bearing a 1,1-d_2-1,2-dihydroxy-propyl group in position 3, deriving from a lactic acid residue (see Scheme 3).

Another portion of product **II** was permethylated, and subsequently hydrolyzed with 4 M TFA (100 °C, 4 h), reduced with sodium borodeuteride and acetylated to yield two partially methylated alditol acetates, galactitol and **VI** (see Scheme 2). The analysis of these products by GC/MS corroborated the presence of a hydroxyl-propionic acid residue linked in position 3 of a terminal non reductive hexuronic acid. It indicates furthermore a substitution of the galactose moiety in position 3 (cf. Scheme 3). The nature of the hexuronic acid unit constituting the unknown sugar unit was determined by treatment of the carboxyl reduced disaccharide **II** with boron tribromide. The reaction mixture was methanolyzed and pertrimethylsilylated and the GC/MS analysis of the derivatized methyl glycosides showed presence of galactose, 6,6-d_2-glucose, and residual reduced unknown sugar.

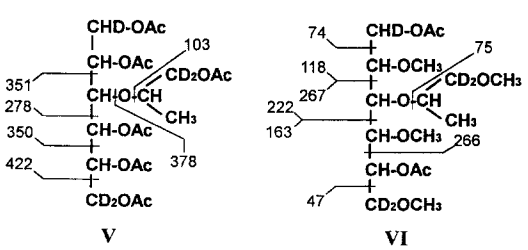
The absolute configuration of the 6,6-d_2-glucose unit was determined by treatment of the disaccharide **II** with boron tribromide followed by solvolysis with M hydrochloric acid in (*R*)-2-butanol. The GC/MS analysis of the pertrimethylsilylated butyl glycosides allowed assignment of a D-configuration to the galactose and the 6,6-d_2-glucose (**VII**) residues by comparison with authentic references.



Scheme 2.

In order to specify the absolute configuration of the lactic acid residue, the *R* and *S* diastereoisomers of 3-*O*-(2-(1-hydroxy)propyl]-D-glucose were synthetized. 1,2:5,6-Di-*O*-isopropylidene-D-glucofuranose was alkylated with (*S*)-2-chloropropionic acid. Chloropropionic acid when condensed with an alcoholate through an SN_2 mechanism undergoes an inversion of configuration thus yielding 3-*O*-[(*R*)-1-carboxyethyl]-D-glucose. In a similar way, the (*S*) diastereoisomer was obtained by reacting (*R*)-2-chloropropionic acid with 1,2:5,6-di-*O*-isopropylidene-D-glucose. The products were subsequently treated with methanolic HCl and reduced with $NaBH_4$. The pertrimethylsilylated references and the pertrimethylsilylated derivative of the reduced unknown sugar (**IV**) were analyzed by GC. The retention times of the two anomers of derivatized 3-*O*-[(*R*)-2-(1-hydroxy)propyl]-D-glucose and the reduced unknown sugar (**VI**) showed perfect agreement (38.4 and 39.3 min). The other isomer, 3-*O*-[(*S*)-2-(1-hydroxy)propyl]-D-glucose showed retention times of 37.4 and 38.7 min.

The new sugar residue identified in the exopolysaccharide produced by the bacterium *Alteromonas sp.* strain 1644, is a 3-*O*-[(*R*)-1-carboxyethyl]-D-glucuronic acid. Bacterial



Scheme 3.

polysaccharides quite often exhibit original constituents, and a similar compound, 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic acid, was reported for the *Klebsiella Type 37* polysaccharide [14]. However, this is the first report of the occurrence of 3-*O*-[(*R*)-1-carboxyethyl]-D-glucuronic acid.

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