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Isolation and characterization of methylated sugars from the tube of the hydrothermal vent tubiculous annelid worm *Alvinella pompejana*

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The tube of *Alvinella pompejana* contains in its carbohydrate fraction, 3 methylated monosaccharides: 2-mono-*O*-methyl-L-fucose, 3-mono-*O*-methyl-L-fucose and 2,4-di-*O*-methyl-L-fucose. The present work appears to be the first report of the occurrence of 2-mono-*O*-methyl-L-fucose and 3-mono-*O*-methyl-L-fucose in the animal kingdom. Moreover, it is the first time that 2,4-di-*O*-methyl-L-fucose is found in nature.

Hydrothermal vent; Methylated monosaccharide; *Alvinella pompejana*

1. INTRODUCTION

Alvinella pompejana [1], the Pompeii worm, is a polychaetous annelid found strictly around deep-sea hydrothermal vents, at a depth of 2600 m. It lives under very exceptional conditions (high temperatures, high pressures, metal-rich water), in tubes directly secreted on the white smockers whose temperatures can reach 250°C, while the surrounding water is 2°C. The tube is build out of organo-mineral material where minerals make up 45% of the total [2]. The carbohydrate part of the tube is made, largely, of unknown sugars which are probably methylated sugars according to their chromatographic mobilities. This kind of sugar is rare in nature. We report here the isolation and the characterization of 3 neutral methylated sugars from the tube of *Alvinella pompejana*.

2. MATERIALS AND METHODS

2.1. *Biological material*

Tubes from *Alvinella pompejana* were collected at 2600 m depth by the submersible *Cyana* in April 1984 during the Biocyarise cruise (12°48′ N, 103°56′ W). Tubes were preserved in formol. They were rinsed and air-dried before experiments.

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Abbreviatons: TFA, trifluoroacetic acid; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography-mass spectrometry; MeOH/HCl, methanol chloride

2.2. *Monosaccharide determinations*

Monosaccharide determinations were carried out after methanolysis (0.5 N MeOH/HCl, 24 h, 80°C) by gas-liquid chromatography of pertrimethyl-silylated methylglycosides [3,4] and after hydrolysis (4 N TFA, 4 h, 100°C), reduction with potassium borohydride, peracetylation (pyridine/acetic anhydride, v/v) and gas-liquid chromatography of alditol acetates.

2.3. *GLC analysis*

GLC was done with a Girdel series 300 or Spectra-Physics model 7100 gas chromatograph fitted with a flame-ionisation detector. A capillary column (0.3 mm inner diameter × 25 m) fused silica OV 101 was used with the following temperature programs: (a) alditol acetates, 110–240°C at 3°C/min; (b) pertrimethylsilylated methyl and butyl-glycosides, 120–240°C at 2°C/min; (c) permethylated sugars, 110–180°C at 2°C/min. Nitrogen was the carrier gas at 0.5 atm.

2.4. *Methylated monosaccharides isolation*

Tubes were reduced to powder, treated with chloroform (10 ml), and sonicated (three times). After centrifugation (10 000 × *g*, 5 min) the dry pellet was treated with 2 N TFA, 2 h at 100°C. After cooling, the hydrolysate was centrifuged for 15 min at 10 000 × *g*. The hydrolysate was evaporated under pressure with methanol to remove the acid. The extract was dissolved in distilled water and passed through a column of Dowex 50 × 8 resin (200–400 mesh, H^+ form, 20 × 2 cm) coupled with a column of Dowex 1 × 8 resin (200–400 mesh, CH_3COO^- form, 20 × 2 cm) and the eluate was freeze-dried. Preparative paper chromatography was carried out on Whatman no. 3 paper with *n*-butanol/acetic acid/water (4:1:5) as solvent [5]. Sugars were visualized with aniline oxalate [6].

2.5. *Dealkylation of methylated sugars [7,8]*

The experiments were performed on acetylated sugars in order to increase their solubilities in the reaction medium. The dry sugar (1 mg) is dissolved in 0.5 ml of pyridine and 0.5 ml of acetic anhydride during 24 h at room temperature. After elimination of the solvents under a stream of nitrogen, the acetylated sugar is dissolved in 0.5 ml of dichloromethane, and boron tribromide (50 µl) is added. After remaining for 2 min at room temperature, the mixture is evaporated to dryness. The product is deacetylated by treatment with sodium methoxide in methanol (15 min). After acidification with Dowex

50 × 8 (20–50 mesh, H^+ form), water is added and the resin is removed by filtration. Evaporation of the filtrate gives the free sugar.

2.6. *GC-MS analysis*

Capillary GC-MS was carried out with a Riber-Mag R10-10 (Rueil Malmaison, France) mass spectrometer using a capillary column of silicone OV 101 (0.3 mm inner diameter × 30 m). For analysis of alditol acetates, pertrimethyl-silylated methyl and butyl-glycosides or permethylated sugars, a program of 100–240°C (3°C/min) was used. Compounds were characterized by electron impact and chemical ionization (ammonia) mass spectroscopy.

2.7. *Methylation analysis*

Permethylation of free sugars obtained by paper chromatography was carried out on methylglycosides (0.5 N MeOH/HCl, 24 h, 80°C) in dimethylsulfoxide solution using sodium hydroxyde and methyl iodide as reagents [9]. To a solution of the methylglycoside (1 mg) in dimethylsulfoxide (0.5 ml), finely powdered NaOH (5 mg) and methyl iodide (0.5 ml) was added. The mixture was sonicated for 1 h. The reaction was stopped by addition of water (10 ml) and crystals of sodium thiosulfate were added to decolorize the solution. The fully methylated sugar was extracted with chloroform (3 × 1 ml). The chloroform extract was washed 10 times with 20 ml of water, dried

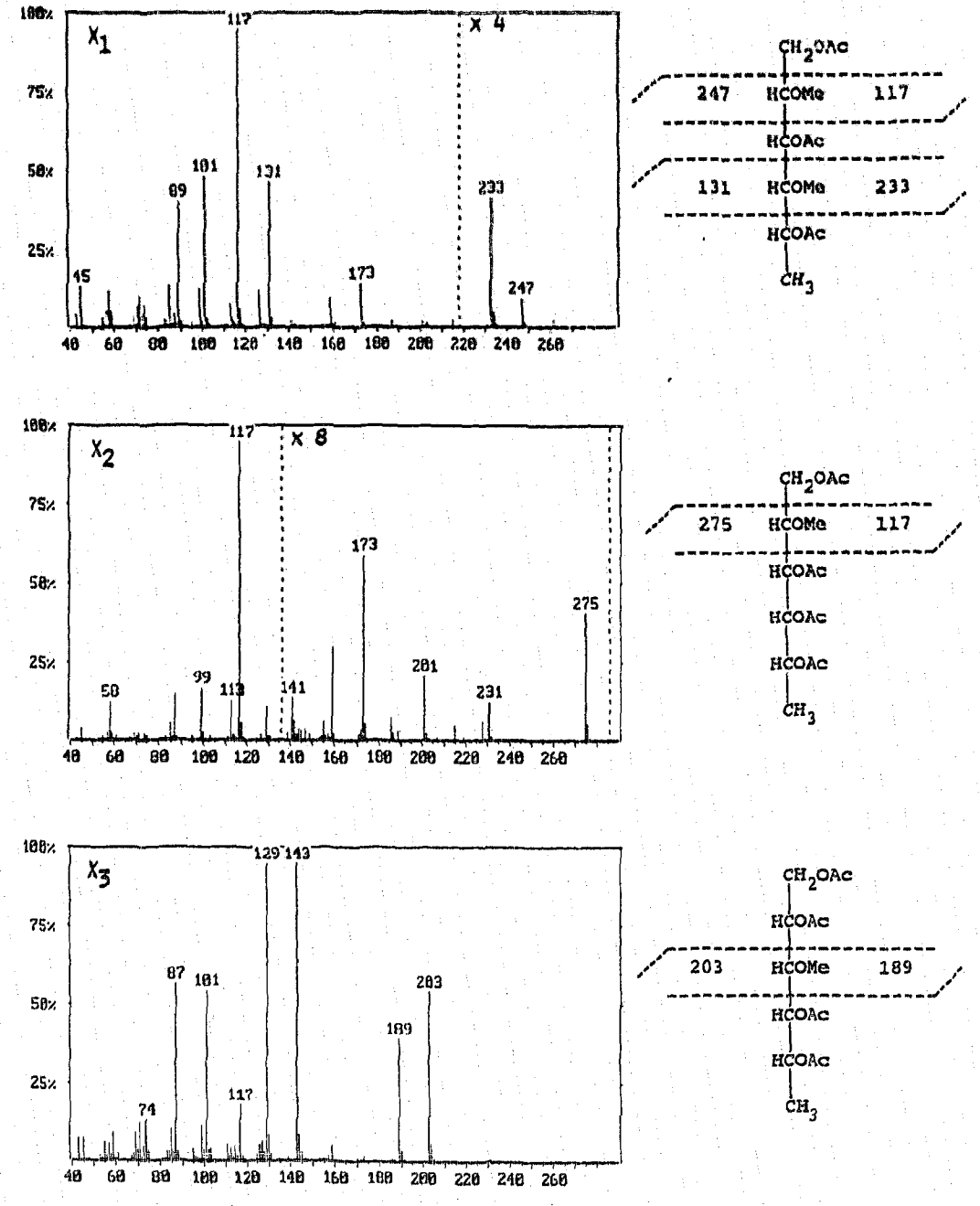

Fig. 1. Paper chromatography in Partridge solvent [5], of monosaccharides obtained after acid hydrolysis of tubes. (1) glucose, (2) galactose, (3) fucose, (4) *Alvinella pompejana*'s tube hydrolysate (2 N TFA, 2 h), X_1, 2,4-di-*O*-methyl-L-fucose; X_2, 2-mono-*O*-methyl-L-fucose; X_3, 3-mono-*O*-methyl-L-fucose.

with anhydrous sodium sulfate and finally evaporated under a stream of nitrogen.

2.8. *Absolute configuration of sugars*

A solution of the demethylated methylglycoside in (-)2-butanol and a catalytic amount of trifluoroacetic acid was kept at 80°C overnight and then concentrated [10]. The resulting butylglycoside is pertrimethylsilylated [11] and analysed by gas-liquid chromatography.

3. RESULTS

Since the tube of *Alvinella pompejana* contains sulfur [2,12], it has been removed with chloroform. 2 g of tubes, reduced to powder, were treated with chloroform. After extraction of sulfur, we obtained 1.9 g of tubes which was hydrolysed with 2 N TFA for 2 h at 100°C. After centrifugation of the solution the supernatant was reduced under vacuum and freeze-dried. The hydrolysate was deionized on Dowex columns and the unknown sugars were isolated by preparative paper chromatography on Whatman No. 3 paper in Partridge solvent [5] (Fig. 1). We can observe 3 monosaccharides (X_1, X_2, X_3) which move faster than fucose. Their R_{fuc} values are: $R_{fuc}X_1 = 2.05$; $R_{fuc}X_2 = 1.6$; $R_{fuc}X_3 = 1.4$. The preparative paper chromatography yielded 3.5 mg of X_1, 1.2 mg of X_2 and 2.7 mg of X_3, from 28 mg of starting material. The 3 compounds were converted into alditol acetates for GLC and GLC-MS studies since alditol acetates have relatively simple fragmentation patterns [13–15]. Mass spectra of alditol acetates from each unknown sugar and their fragmentation (electron impact mode) schemes are given in Fig. 2. GLC-MS of the alditol acetate derivative of the X_1 sugar gave primary fragments at *m/z* 117, 131, 233 and 247 allowing to establish the sugar as a 2,4-di-*O*-methyl-6-deoxy-hexose. GLC-MS of the alditol acetate derivative of X_2 sugar gave major primary fragments at *m/z* 117 and 275 allowing to establish this alditol acetate as a 2-*O*-methyl-6-deoxy-hexitol. From the fragmentation scheme of the alditol acetate derivative of X_3 sugar, we observe two primary fragments *m/z* 203 and *m/z* 189. By elimination of acetic (mol. wt = 60) and a subsequent loss of ketene (mol. wt = 42) secondary fragments *m/z* 143 and *m/z* 101 arise from the primary fragment *m/z* 203.

In analogy, secondary fragments *m/z* 129 and *m/z* 87 arise from the primary fragment *m/z* 189. This mass spectrometric analysis thus allows the identification of the X_3 unknown sugar as a 3-*O*-methyl-6-deoxy-hexose. The mass spectra in a chemical ionization mode confirm the mono-*O*-methyl-6-deoxy-hexose nature for X_2 and X_3 sugars ($M+NH_4^+ = 366$, in peracetylated form) and the di-*O*-methyl-6-deoxy-hexose nature for X_1 sugar ($M+NH_4^+ = 338$, in peracetylated form).

Demethylation of each sugar with boron tribromide, followed by methylglycosylation and trimethylsilylation allows to identify (by GLC-MS) each sugar as a methyl fucoside. These results were confirmed by the identification, by GLC-MS of the permethyl derivatives obtained after permethylation of each sugar. For all components, we identified the 2,3,4-tri-*O*-methyl fucoside. Absolute configurations of demethylated sugars have been determined by GLC and GLC-MS using a chiral alcohol. The three sugars, analysed as butyl-pertrimethyl-silylated glycosides have the same retention time as L-fucose.

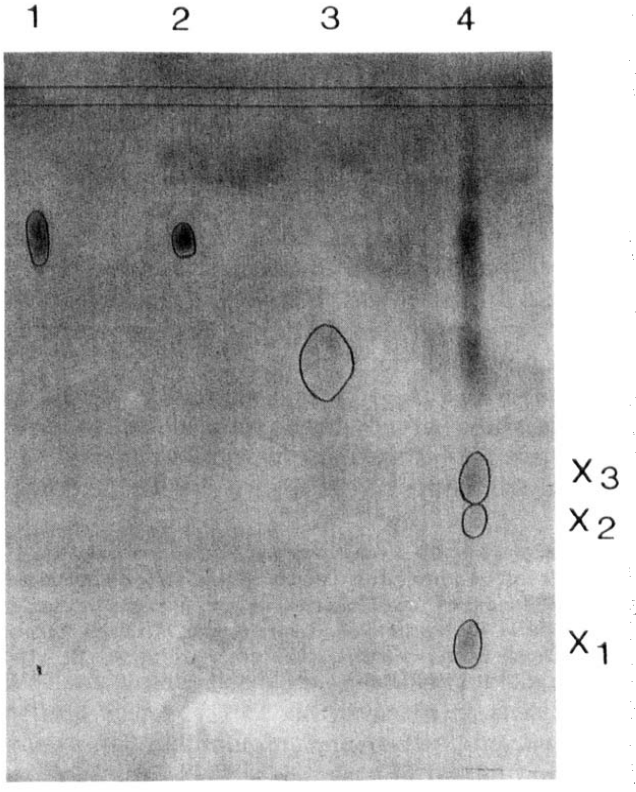

Fig. 2. EI-mass spectra of alditol acetates from X_1, X_2 and X_3 sugars.

In conclusion, we have identified in the tube of *Alvinella pompejana*, the three unknown sugars as: X_1, 2,4-di-*O*-methyl-L-fucose; X_2, 2-mono-*O*-methyl-L-fucose; X_3, 3-mono-*O*-methyl-L-fucose.

4. DISCUSSION

Naturally occurring methyl ethers of sugars are relatively rare in nature. 3-*O*-methyl-fucose (digitalose) is known as a constituent of cardiac glycosides [16], as a free sugar from the brown seaweed *Desmaresta acateata* [17], in glycolipids from shellfishes [18–20] and from *Mycobacterium* [21]. The configuration of the sugar from the last 3 sources was not determined. The L configuration of 3-*O*-methyl-fucose was found as constituent of *Rhizobium* extracellular polysaccharides [22]. 2-*O*-methyl-fucose has been identified in *Desmaresta acateata* [17], in glycolipids from *Mycobacterium* [21,23]. The L isomer of 2-*O*-methyl-fucose was found in lipopolysaccharides from photosynthetic prokaryotes [24]. The present work ap-

pears to be the first report of the occurrence of 2-*O*-methyl-L-fucose and 3-*O*-methyl-L-fucose in the animal kingdom. 3-*O*-Methyl-fucose was found in glycolipids from *Corbicula sendai* [18], from oyster gills [19] and from *Hyriopsis schlegelii* [20] but the authors did not argue whether it was the D- or L-isomer. Moreover, it is the first time that 2,4-di-*O*-methyl-L-fucose is found in nature. The D configuration of this sugar was found in the antibiotic Labilomycin [25]. 2,4-di-*O*-methyl-fucose was found in glycolipids from *Mycobacterium avium* [26], but the anomeric configuration was not determined.

Unknown sugars, with high paper chromatographic mobilities, have already been reported in the tube of a non-deep-sea polychete annelid *Myxicola infundibulum* [27]. But the nature of the sugars was not determined.

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