# First application of triflic acid for selective cleavage of glycosidic linkages in structural studies of a bacterial polysaccharide from *Pseudoalteromonas* sp. KMM 634†

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A marine bacterium *Pseudoalteromonas* sp. KMM 634 produces a highly acidic, regular O-specific polysaccharide, containing D-glucuronic acid (D-GlcA), 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid (D-GlcNAc3NAcA), 2,3-diacetamido-2,3-dideoxy-D-mannuronoyl-L-alanine (D-ManNAc3NAcA6Ala) and 2-acetamido-2,4,6-trideoxy-4-[(*S*)-3-hydroxybutyramido]-D-glucose (D-QuiNAc4NAcyl). The polysaccharide was stable towards solvolysis with anhydrous HF but could be partially depolymerised by triflic acid. This new reagent for selective cleavage of carbohydrates split primarily 1,2-*trans*-β-glycosidic linkages and did not affect amide-linked substituents. As a result, a disaccharide and a trisaccharide with a GlcNAc3NAcA residue at the reducing end were obtained. Based on these data and studies of the initial polysaccharide and derived oligosaccharides by two-dimensional NMR spectroscopy, the following structure of the tetrasaccharide repeating unit was established:

→3)-α-D-Qui*p*NAc4NAcyl-(1→4)-β-D-Man*p*NAc3NAcA6Ala-(1→4)-β-D-Glc*p*NAc3NAcA-(1→4)-β-D-Glc*p*A-(1→

## Introduction

Cell-surface polysaccharides play an important role in functioning of the bacterial cell and interaction of bacteria with other biological systems, including the immune system of humans and animals. Chemotyping and serotyping on the basis of polysaccharides and lipopolysaccharides are widely used for classification of bacteria and elucidation of their phylogenetic relationships. Primary structures of a number of specific polysaccharides of bacteria have been established and most of them have been found to be built up of oligosaccharide repeating units.[1–4]

A modern approach to establishing polysaccharide structures involves sugar and methylation analyses in combination with NMR spectroscopy studies, including a sequence of two-dimensional homonuclear and heteronuclear ($^1$H,$^{13}$C and, when phosphate is present, $^1$H,$^{31}$P) shift-correlated experiments. When necessary, a selective cleavage of polysaccharides is performed in order to obtain oligosaccharide fragments. The most useful nonspecific cleavage method is partial acid hydrolysis.[5] Recently, partial solvolysis with anhydrous hydrogen fluoride has been introduced as well.[6]

In the present paper we report elucidation of the structure of the O-specific polysaccharide chain of the lipopolysaccharide (O-antigen) of a marine bacterium *Pseudoalteromonas* sp. KMM 634 which required elaboration of a new procedure for selective cleavage of glycosidic linkages using trifluoromethanesulfonic (triflic) acid.

## Results and discussion

Chemical analyses of the O-specific polysaccharide of *Pseudoalteromonas* sp. KMM 634 resulted in identification of D-glucuronic acid (D-GlcA), L-alanine and (*S*)-3-hydroxybutyric acid. However, in addition to typical signals for one *N*-acylated alanine residue[7] and one *N*-linked 3-hydroxybutyryl group,[8] the $^{13}$C NMR spectrum of the polysaccharide contained signals for four anomeric carbons at $\delta_C$ 97.9–104.8, thus indicating a tetrasaccharide repeating unit. As judged by the presence of seven signals for carbons bearing nitrogen at $\delta_C$ 51.0–56.2, the three monosaccharides that could not be detected were diamino sugars.

The $^1$H and $^{13}$C NMR signals for two monosaccharides (Tables 1 and 2) were assigned using two-dimensional shift-correlated experiments ($^1$H,$^1$H COSY, TOCSY, NOESY and H-detected $^1$H,$^{13}$C HMQC). Judging from the $^3J_{H,H}$ coupling-constant values[9] and $^{13}$C NMR chemical-shift data (Table 2), these were Glc*p*A and 2,4-diamino-2,4,6-trideoxy-α-glucopyranose (α-bacillosamine, α-Qui*p*N4N). Two other sugars were found to be β-linked diamino uronic acids, but their reliable identification was complicated by close positions of some signals in the $^1$H NMR spectrum (Table 1). Therefore, depolymerisation of the polysaccharide to mono- or/and oligosaccharide fragments was necessary.

Partial acid hydrolysis of polysaccharides enriched in amino sugars and uronic acids is known to be complicated by hydrolytic removal of *N*-acyl groups.[10] Anhydrous HF does not cleave amide linkages,[6] but the polysaccharide studied was stable towards solvolysis with HF. In a search for a new reagent for selective cleavage of glycosidic linkages, we tested triflic acid, a reagent which is used for deglycosylation of glycoproteins.[11] After aqueous work-up, two oligosaccharides, a disaccharide and a trisaccharide, were obtained and separated by gel chromatography. They were evidently produced by hydrolysis of unstable 1-*O*-triflyl derivatives, which were the primary solvolysis products.

Easy assignment of the $^1$H and $^{13}$C NMR spectra of the oligosaccharides and borohydride-reduced oligosaccharides

† Preliminary data were presented at the Xth European Carbohydrate Symposium (July 11–17th, 1999, Galway, Ireland).

**Table 1** $^{1}$H NMR chemical shifts ($\delta_H$). Additional signals for *N*-acetyl groups are at $\delta_H$ 1.81–2.10

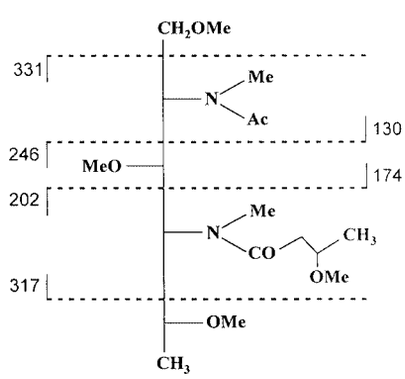
| Residue | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 |
|---|---|---|---|---|---|---|
| Polysaccharide | | | | | | |
| →3)-α-D-Qui*p*N4N-(1→ | 5.12 | 4.11 | 3.78 | 3.68 | 3.51 | 1.12 |
| →4)-β-D-Man*p*N3NA-(1→ | 4.89 | 4.33 | 4.35 | 4.03 | 4.06 | |
| →4)-β-D-Glc*p*N3NA-(1→ | 4.70 | 3.92 | 3.99 | 3.90 | 4.04 | |
| →4)-β-D-Glc*p*A-(1→ | 4.43 | 3.25 | 3.63 | 3.78 | 3.87 | |
| L-Alanine | | 4.37 | 1.51 | | | |
| *N*-(3-Hydroxybutyryl) | | 2.37 | 4.13 | 1.24 | | |
| Disaccharide (reduced) | | | | | | |
| β-D-Man*p*N3NA-(1→ | 5.02 | 4.49 | 4.10 | 3.72 | 4.01 | |
| →4)-D-Glc*p*N3NAol | 3.69 | 4.26 | 4.35 | 4.14 | 4.33 | |
| L-Alanine | | 4.47 | 1.47 | | | |
| Trisaccharide (reduced) | | | | | | |
| α-D-Qui*p*N4N-(1→ | 5.13 | 3.90 | 3.62 | 3.62 | 3.47 | 1.10 |
| →4)-β-D-Man*p*N3NA-(1→ | 5.00 | 4.37 | 4.34 | 3.98 | 4.10 | |
| →4)-D-Glc*p*N3NAol | 3.70 | 4.22 | 4.33 | 4.09 | 4.18 | |
| L-Alanine | | 4.23 | 1.52 | | | |
| *N*-(3-Hydroxybutyryl) | | 2.35 | 4.17 | 1.26 | | |

**Table 2** $^{13}$C NMR chemical shifts ($\delta_C$). Additional signals for *N*-acetyl groups are at $\delta_C$ 23.6 (Me) and 169.7–177.9 (CO)

| Residue | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|
| Polysaccharide | | | | | | |
| →3)-α-D-Qui*p*N4N-(1→ | 97.9 | 54.4 | 77.9 | 56.2 | 68.9 | 18.2 |
| →4)-β-D-Man*p*N3NA-(1→ | 100.1 | 52.7 | 54.4 | 70.1 | 77.8 | |
| →4)-β-D-Glc*p*N3NA-(1→ | 102.8 | 54.4 | 54.4 | 79.1 | 76.5 | |
| →4)-β-D-Glc*p*A-(1→ | 104.8 | 73.6 | 75.0 | 82.0 | 75.8 | |
| L-Alanine | | 51.0 | 18.8 | | | |
| *N*-(3-Hydroxybutyryl) | | 46.4 | 66.4 | 23.7 | | |
| Disaccharide (reduced) | | | | | | |
| β-D-Man*p*N3NA-(1→ | 100.6 | 52.6 | 55.0 | 67.6 | 79.0 | |
| →4)-D-Glc*p*N3NAol | 62.0 | 53.1 | 51.3 | 79.6 | 71.9 | |
| L-Alanine | | 50.4 | 18.7 | | | |
| Trisaccharide (reduced) | | | | | | |
| α-D-Qui*p*N4N-(1→ | 98.0 | 55.3 | 69.5 | 58.1 | 68.7 | 18.2 |
| →4)-β-D-Man*p*N3NA-(1→ | 99.9 | 52.8 | 54.8 | 70.4 | 78.0 | |
| →4)-D-Glc*p*N3NAol | 61.8 | 53.0 | 51.2 | 78.7 | 72.3 | |
| L-Alanine | | 52.0 | 18.7 | | | |
| *N*-(3-Hydroxybutyryl) | | 46.2 | 66.6 | 24.0 | | |

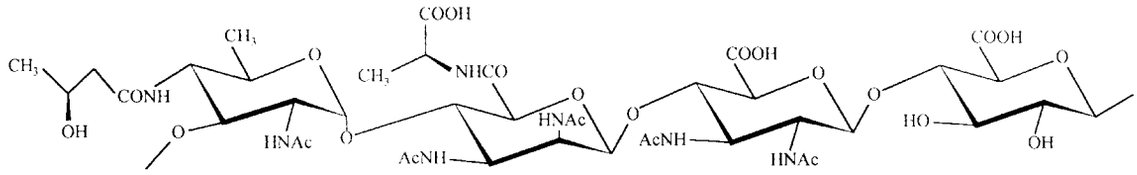
(Tables 1 and 2) allowed identification of the diamino uronic acids as 2,3-diamino-2,3-dideoxyglucopyranuronic acid (Glc*p*N3NA) and 2,3-diamino-2,3-dideoxymannopyranuronic acid (Man*p*N3NA). Like alanine, they were present in both oligosaccharides obtained, and in both oligosaccharides GlcN3NA was at the reducing end. The signals for H-2 of Ala and H-5 of GlcN3NA shifted upfield by 0.18 and 0.28 ppm, respectively, with an increase of pD from 3 to 11; hence, these components had the free carboxy group.[12] In contrast, no significant pD dependence was observed for the H-5 signal of ManN3NA, thus showing amidation of the carboxy group with alanine.

QuiN4N was present in the trisaccharide only. In contrast to the initial polysaccharide, acid hydrolysis of the trisaccharide released smoothly a QuiN4N derivative carrying one *N*-acetyl and one *N*-(3-hydroxybutyryl) group. The mass spectrum of the corresponding *O*-acetylated alditol displayed prominent peaks for the C-1–C-4 and C-2–C-6 fragment ions, but no significant peaks for smaller fragments essential for determination of the position of the *N*-acyl groups. However, when the *N*,*O*-methylated derivative **1** was analysed, clear peaks were observed for the C-1–C-2 and C-4–C-6 fragment ions at *m*/*z* 130 and 202, thus indicating the location of the *N*-acetyl and *N*-(3-hydroxybutyryl) groups at positions 2 and 4 of QuiN4N, respectively. As followed from the $^{1}$H and $^{13}$C NMR data, the amino groups of the other amino sugars were *N*-acetylated.




**1**

The data obtained showed that the disaccharide and trisaccharide obtained by partial solvolysis of the polysaccharide with triflic acid have the following structures:



**2**

β-Man*p*NAc3NAcA6Ala-(1→4)-GlcNAc3NAcA and
α-Qui*p*NAc4NAcyl-(1→4)-β-Man*p*NAc3NAcA6Ala-(1→4)-GlcNAc3NAcA,

where QuiNAc4NAcyl is 2-acetamido-2,4,6-trideoxy-4-[(*S*)-3-hydroxybutyramido]glucose and ManNAc3NAcA6Ala is 2,3-diacetamido-2,3-dideoxymannuronoyl-L-alanine. The structures of the oligosaccharides were confirmed by two-dimensional NMR spectroscopy studies, including $^{1}H$,$^{13}C$ HMQC and NOESY experiments (data not shown).

With known composition and structures of the oligosaccharide fragments, the assignment of the $^{1}H$ and $^{13}C$ NMR spectra of the polysaccharide could be completed (Tables 1 and 2). In the $^{13}C$ NMR spectrum of the polysaccharide, as compared with the spectrum of the trisaccharide, the signal for C-3 of QuiN4N was shifted downfield by 8.4 ppm; hence, QuiN4N is substituted at position 3. Similarly, the signal for C-4 of GlcA was shifted downfield by ≈9.3 ppm, as compared with its position in the spectrum of free β-D-GlcpA.[13] Therefore, GlcA was substituted at position 4, and the polysaccharide is linear.

The NOESY spectrum of the polysaccharide showed typical intraresidue NOE patterns for α-linked (H-1/H-2) and β-linked (H-1/H-3 and H-1/H-5) sugars, which confirmed the configurations of the glycosidic linkages. In addition, interresidue cross-peaks were observed between the following transglycosidic protons: QuiN4N H-1/ManN3NA H-4, ManN3NA H-1/GlcN3NA H-4, GlcN3NA H-1/GlcA H-4, and GlcA H-1/QuiN4N H-3. These data were consistent with the glycosylation pattern and the linear character of the polysaccharide. Analysis of the $^{13}C$ NMR chemical shifts using known regularities[14] showed that the three diamino sugars have the same absolute configuration as D-GlcA.

Therefore, the tetrasaccharide repeating unit of the O-specific polysaccharide of *Pseudoalteromonas* sp. KMM 634 has structure **2**.

Comparison of the structures of the polysaccharide and the derived oligosaccharides showed that triflic acid cleaved primarily the 1,2-*trans*-β-glycosidic linkages of β-GlcA and β-GlcN3NA, whereas the 1,2-*cis*-β-glycosidic linkage of β-ManN3NA was stable. The glycosidic linkage of α-QuiN4N, which was expected to be much more labile towards solvolysis compared with the linkage of uronic acids,[6] was cleaved only partially. Therefore, triflic acid splits selectively glycosidic linkages without any significant sugar destruction. Another advantage of triflic acid is that it does not cleave amide linkages and thus allows isolation of *N*-acylated amino sugars and amides of uronic acids. Our preliminary studies on the O-specific polysaccharides of *Proteus* and *Vibrio cholerae* showed that triflic acid is applicable to selective cleavage of various polysaccharides. These data will be published elsewhere.

## Experimental

### Bacterium, growth, isolation of lipopolysaccharide and O-specific polysaccharide

*Pseudoalteromonas* sp. KMM 634 is a ubiquitous, aerobic, marine, Gram-negative, heterotrophic prokaryote which produces restrictase. It was isolated from a sponge *Hexactinellida* taken at 400 m depth in the Indian Ocean near Australia during the expedition of the research ship Academic Oparin in 1990. The strain is maintained in the collection of the Pacific Institute of Bioorganic Chemistry (KMM). The bacterium was grown in modified Youshimizu–Kimura medium.[15]

A lipopolysaccharide was isolated from dry bacterial cells by phenol–water extraction[16] followed by treatment with DNAse and RNAse and ultracentrifugation. The lipopolysaccharide was degraded with 2% aq. AcOH at 100 °C until a lipid precipitate had been formed, and a high-molecular mass O-specific polysaccharide was obtained by gel chromatography of the supernatant on Sephadex G-50 (S).

### NMR spectroscopy

NMR spectra were run on a Bruker DRX-500 spectrometer for solutions in 99.96% $D_2O$ at 50 °C for the polysaccharide and 40 °C for the oligosaccharides, using acetone as internal standard ($\delta_H$ 2.225, $\delta_C$ 31.45). Two-dimensional spectra were obtained using standard Bruker software, and the XWINNMR 2.1 program (Bruker) was used to acquire and maintain NMR data. Mixing times of 200 and 100 ms were used in TOCSY and NOESY experiments, respectively.

### Chromatography and mass spectrometry

Gel chromatography was performed on a column (56 × 2.6 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate, pH 4.5, or a column (52 × 1.6 cm) of TSK HW-40 (S) in 1% aq. AcOH and monitored using a Knauer differential refractometer. Cation-exchange chromatography was performed on a Biotronik LC-2000 amino acid analyser using an Ostion LG AN B resin and 0.2 M aq. sodium citrate, pH 3.28, at 55 °C. GLC was performed on a Hewlett-Packard 5880 instrument equipped with a capillary column of Ultra 2 stationary phase and a temperature program of 160 to 290 °C at 10 °C $min^{-1}$. GLC-MS was performed on a Hewlett Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer.

### Chemical analyses

L-Alanine was identified after hydrolysis with 2 M TFA (120 °C; 2 h) by cation-exchange chromatography and GLC of the acetylated (+)-*sec*-butyl ester.[17] (*S*)-3-Hydroxybutyric acid was analysed by GLC of the trifluoroacetylated (+)-*sec*-octyl ester.[18] D-GlcA was identified by GLC after methanolysis of the polysaccharide with 1 M HCl–MeOH at 80 °C for 16 h followed by acetylation. The absolute configuration of D-GlcA was determined by GLC of the acetylated (+)-*sec*-butyl ester (+)-*sec*-butyl glycosides.[19,20] QuiNAc4NAcyl was analysed by GLC-MS of the *N*,*O*-methylated alditol derived by hydrolysis of the trisaccharide with 4 M HCl (65 °C; 4 h) followed by *N*-acetylation with acetic anhydride in saturated aq. $NaHCO_3$, conventional reduction with $NaBH_4$, and methylation.[21] The MS data (*m*/*z*, relative intensity in parentheses): 331 (1), 317 (10), 299 (0.2), 285 (5.5), 246 (22), 214 (18), 202 (22), 174 (32), 156 (5.5), 142 (24), 130 (38), 102 (86), 88 (100).

### Solvolysis with triflic acid

The polysaccharide (30 mg) was treated with triflic acid (0.5 ml) at 4 °C for 16 h under anhydrous conditions. After neutralisation with 25% aq. ammonia at 4 °C, the reaction products were

fractionated by gel chromatography on TSK HW-40 (S) to give a trisaccharide (7.5 mg) and a disaccharide (6 mg).

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