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Isolation and identification of poly-α-(1→4)-linked 3-*O*-methyl-D-mannopyranose from a hot-water extract of *Mycobacterium vaccae*

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Abstract

A polysaccharide around 3.6 kDa has been identified as the major carbohydrate moiety of a antineoplastic protein–polysaccharide complex (PS4A) obtained by boiling intact cells of *Mycobacterium vaccae* in water. ^{1}H and ^{13}C NMR spectra of this polysaccharide suggested it was a highly homogeneous polymer composed substantially of one monomer, probably an α-linked O-methylated mannose. Comparison of the COSY spectra of the original and acetylated polymer indicated that the glycosidic linkage and the methyl ether were interchangeable, at O-3 and O-4. Further study demonstrated that the benzyolated hydrolysate of the polymer was 1,2,4,6-tetra-*O*-benzoyl-3-*O*-methyl-β-mannopyranose. The hydrolysate was 3-*O*-methyl-α,β-mannopyranose and the polymer was therefore poly-α-(1→4)-linked 3-*O*-methyl-D-mannopyranose. This conclusion was further confirmed with an authentic sample of the monomer, which had spectral data identical to those of the hydrolyzate and co-eluted from an ion-exchange HPLC with the major sugar in the hydrolysate.

Keywords: Protein–polysaccharide complex; Poly-α-(1→4)-3-*O*-methyl mannopyranose; Synthesis; *Mycobacterium vaccae*

1. Introduction

The outmost cellular components of mycobacterial species have been found to consist mainly of polysaccharides and proteins [1,2]. Several investigators have confirmed that polysaccharides isolated from the surface of the mycobacterial cells by water extraction have antineoplastic activity [3–7]. The polysaccharides isolated from *Mycobacterium bovis* BCG Tice® substrain have been found to be associated with the extracellular integument, which is probably secreted by the bacterial cells in response to environmental stress, such as a change of oxygen tension [8]. These materials can also be removed by digestion with non-specific protease [9] and urea dissociation [10].

Mycobacterium vaccae, an avirulent and fast-growing mycobacterial species, has been found to be a potent immunostimulant. A suspension of heat-killed *M. vaccae* is currently being clinically tested as an adjuvant to tuberculosis chemotherapy and cancer immunotherapy [11–13]. We had previously

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demonstrated that the water-soluble exocellular proteoglycans extracted by boiling *M. vaccae* suspensions (PS4) also had significant antineoplastic activity [14,15]. In this report, we identify the major carbohydrate moiety of these antineoplastic proteoglycans as α-(1→4)-linked 3-*O*-methylated mannose of molecular weight about 3.6 kDa.

2. Experimental

Bacteria and materials.—*M. vaccae* (ATCC #15483, batch #93-12), purchased from ATCC (Rockville, MD), was cultivated in tryptic soy broth (TSB, Gibco Laboratories, Grand Island, NJ) at 37 °C for 5–7 days. The bacteria were collected by centrifugation and washed with double-distilled water. Washed cells were lyophilized in order to obtain a dry mass. All other reagents and materials were purchased from Fisher Scientific (Fairlawn, NJ), Sigma Chemical Company (St. Louis, Mo) or as noted.

Extraction and purification of the protein–polysaccharide complex.—Approximately 0.5 g of lyophilized cells were suspended in 500 mL of double-distilled water and boiled under reflux for 2 h. When cool, the suspension was passed through a 0.2-μm membrane filter and concentrated to a volume of 12–14 mL at 65 °C using a Büchi Rotavapor® (Brinkmann Instruments, Westbury, NY). The concentrate was dialyzed against distilled water overnight using a 3.5 kDa molecular weight cut-off dialysis membrane before lyophilization. This crude extract was labeled as PS4A.

Monosaccharide analysis of the PS4A.—PS4A samples (2.0 mg) were hydrolyzed in 1.0 mL of 2 M trifluoroacetic acid (TFA) by autoclave at 121 °C for 1 h in a sealed ampule. The autoclaved samples were dried under a flowing air stream and were analyzed with a DX500 high-performance anion-exchange chromatography (HPAEC) system (Dionex Corporation, Sunnyvale, CA) equipped with a CarboPac PA-10 column and a pulsed amperometric potentiometric detector (ED40) to determine the monosaccharide composition [16].

Proteolysis of PS4A.—PS4A samples (10 mg/mL) dissolved in 0.1 M Tris–acetate buffer were digested with pronase, a type XIV protease from *Streptomyces griseus* (Sigma, St. Louis, MO) at 37 °C for 24 h in order to release the polysaccharides. The completion of digestion was determined with the gel-filtration HPLC system. The digestion was stopped by adjusting the pH to about 4.5 with 2% AcOH, followed by a brief centrifugation using a microcentrifuge (Fisher Scientific, Pittsburgh, PA). The supernatant was recovered for oligosaccharide purification.

Purification and NMR spectroscopy of the oligosaccharide.—Recovered supernatants were fractionated by Bio-Gel P-10 size-exclusion chromatography. The columns were equilibrated and eluted with double-distilled water. The eluent fractions were assayed using the phenol–sulfuric acid method for saccharide [17]. The major polysaccharide-containing fractions were recovered and deuterium-exchanged by lyophilization once in deuterium oxide (D_2O, Aldrich, Milwaukee, WI). ^1H NMR spectra were determined using a Bruker Avance 500 instrument operating at 500 MHz using D_2O as solvent. All chemical shifts were calculated from the spectrometer lock signal.

Acetylation of the oligomer.—Purified polysaccharide (10 mg) was dissolved in 1 mL of a 1:1 pyridine–Ac_2O mixture. The reaction was conducted at room temperature (rt) for 16 h, and the reaction mixture was dried under vacuum at rt.

Benzoylation of the hydrolysate of the oligomer: 1,2,4,6,-tetra-O-*benzoyl-3*-O-*methyl-β*-D-*mannopyranose* (**5**).—The purified oligomer (~4.0 mg) was subjected to hydrolysis in 1.0 mL of 2 M TFA by autoclave at 121 °C for 1 h in a sealed ampule. The recovered hydrolysate, containing an excess of sodium bicarbonate, was dissolved in pyridine (1 mL) and treated with benzoyl chloride (120 μL, 50 equiv) then stirred for 24 h at rt, after which thin-layer chromatography (TLC) showed completion. The major UV-active product (0.8 mg) was isolated by chromatography on preparative TLC plates (eluent: 2:1 $CHCl_3$–hexane, 5 times), and was identified from its spectral data as 1,2,4,6,-tetra-*O*-benzyl-3-*O*-methyl-β-D-mannopyranose (**5**). $[\alpha]_D^{20}$ −28.5° (*c* 1.3, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 3.46 (s, 3 H, O-Me), δ 3.90 (dd, 1 H,

$J_{3,4}$ 9.5 Hz, $J_{2,3}$ 3.2 Hz, H-3), δ 4.22 (m, 5 H, H-5), δ 4.48 (dd, 1 H, J_{gem} 12.2, $J_{5,6a}$ 4.8 Hz, H-6a), δ 4,74 (dd, 1 H, J_{gem} 12.2, $J_{5,6b}$ 4.8 Hz, H-6b), δ 5.89 (t, 1 H, $J_{3,4} = J_{4,5}$ 9.5 Hz, H-4), δ 6.05 (dd, 1 H, $J_{2,3}$ 3.2 Hz, $J_{1,2}$ 1.0 Hz, H-2), δ 6.27 (d, 1 H, $J_{1,2}$ 1.2 Hz, H-1), δ 7.10–7.65 (m, 12 H), 7.92 (d, 2 H, *J* 6.9 Hz), δ 8.06 (d, 2 H, *J* 7.0 Hz), 8.06 (d, 2 H, *J* 7.0 Hz), δ 8.16 (d, 2 H, *J* 7.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): 58.2 (Me), 63.1 (C-6), 67.7 (C-2), 68.1 (C-4), 73.5 (C-5), 79.8 (C-3), 91.9 ($^1J_{CH}$ 162.1 Hz, *C*-1), 128.7, 130.1, 130.3, 133.2, 133.7, 164.6, 165.5, 166.2, 166.4. Anal. Calcd for $C_{35}H_{30}O_{10}\cdot 0.5\ H_2O$: C, 67.85; H, 5.04. Found: C, 67.87; H, 5.28.

*Phenyl 4,6-*O*-benzylidene-3-*O*-methyl-1-thio-α-*D*-mannopyranoside* (**2**).—A mixture of S-phenyl 4,6-*O*-benzylidene-1-thio-α-D-mannopyranoside (**1**), prepared according to the literature method [18,19] (364 mg, 1.0 mmol) and dibutyltin oxide (300 mg, 1.2 equiv) in MeOH (10 mL) was refluxed with stirring until all of the dibutylin oxide dissolved. Methanol was then removed in vacuo, and the residue was dissolved under N_2 in dry DMF (10 mL) and heated to 120 °C. Iodomethane (120 μL, 2 equiv) was added with a syringe, and the mixture was stirred at the same temperature for 1 h. After cooling to rt, the mixture was poured into water (50 mL) and extracted with EtOAc (3 × 30 mL). The combined organics were dried over anhyd sodium sulfate and concentrated to a residue, which was purified by chromatography on silica gel with 2:1 $CHCl_3$–hexane as eluent to give the desired product **2** (236 mg, 63%), $[\alpha]_D^{20}$ +230° (*c* 0.3, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.82 (s, 1 H, OH), δ 3.60 (s. 3 H, OMe), 3.75 (dd, 1 H, $J_{2,3}$ 2.5 Hz, $J_{3,4}$ 9.5 Hz, H-3), δ 3.88 (t, 1 H, $J_{gem} = J_{5,6a}$ 10 Hz, H-6a), δ 4.12 (t, 1 H, $J_{3,4} = J_{4,5}$ 9.5 Hz, H-4), δ 4.20 (dd, 1 H, J_{gem} 10 Hz, $J_{5,6b}$ 3.6 Hz, H-6b), δ 4.40 (m, 2 H, H-2 and H-5), δ 5.65 (s, 1 H, Ph*CH*), δ 5.68 (br. s. 1 H, H-1), δ 7.20–7.60 (m, 10 H). Anal. Calcd for $C_{20}H_{22}O_5S$: C, 64.15; H, 5.92. Found: C, 63.81; H, 5.94.

*Phenyl 3-*O*-methyl-1-thio-α-*D*-mannopyranoside* (**3**).—Benzylidene acetal **2** (236 mg, 0.63 mmol) was dissolved in 1% H_2SO_4 in MeOH (10 mL) at 0 °C and then stirred at rt until TLC indicated consumption of the starting material. The solvent was then evaporated in vacuo to yield a residue, which was purified by chromatography on silica gel (eluent: 2:1 $CHCl_3$–MeOH) to give the title product **3** as a foam (173 mg, 97%). $[\alpha]_D^{20}$ +177.3° (*c* 3.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.45 (m, 4 H, OMe, H-3), δ 3.70 (br. d, 1 H, J_{gem} 12 Hz, H-6a), δ 3.95 (br.d, 1 H, J_{gem} 12 Hz, H-6b), δ 4.00–4.20 (m, 2 H, H-4 and H-5), δ 4.30 (m, 1 H, H-2), δ 4.60 (br. s, 1 H, OH), δ 4.70 (m, 2 H, 2 × OH), δ 5.58 (br. s, 1 H, H-1), δ 7.20–7.35 (m, 3 H), δ 7.40–7.50 (m, 2 H); ^{13}C NMR ($CDCl_3$, 75 MHz): 57.3 (Me), 61.2 (C-6), 65.1 (C-4), 68.9 (C-2), 73.6 (C-5), 81.7 (C-3), 88.2 (C-1), 127.7, 129.2, 131.8, 134.0. Anal. Calcd for $C_{13}H_{18}O_5S$: C, 54.53; H, 6.34. Found: C, 54.56; H, 6.40.

*3-*O*-Methyl-*D*-mannose* (**4**). Thioglycoside **3** (138 mg, 0.48 mmol) was dissolved in a mixture of acetone (40 mL) and water (2 mL) and treated with *N*-bromosuccinamide (188 mg, 2.2 equiv). The mixture was stirred in the absence of light at rt until TLC indicated complete consumption of **3** (15 h). The volatile material was then removed in vacuo, and the residue was purified by chromatography on silica gel (eluent: 5:1 $CHCl_3$–MeOH) to afford compound **4** (78 mg, 84% yield) [23,24]. Major anomer: ^{1}H NMR (CD_3OD, 300 MHz): δ 3.42 (dd, 1 H, $J_{2,3}$ 3.0 Hz, $J_{3,4}$ 8.9 Hz, H-3), 3.45 (m, 3 H, OMe), δ 3.65–3.85 (m, 4 H, H-4, H-5, 2 × H-6), δ 3.86 (m, 1 H, H-2), δ 5.10 (m, 1 H, H-1); ^{13}C NMR (CD_3OD, 75 MHz): 57.5 (Me), 63.1 (C-6), 67.7 (C-4), 68.9 (C-2), 74.1 (C-5), 82.2 (C-3), 95.9 (C-1). The minor anomer was characterized by ^{13}C NMR, δ (CD_3OD, 75 MHz): 57.4, 63.0, 67.4, 69.2, 78.2, 85.0, 95.8; ^{13}C NMR, δ (D_2O, 75 MHz): 58.9, 63.6, 68.2, 69.6, 78.8, 84.8, 96.4.

*1,2,4,6,-Tetra-*O*-benzoyl-3-*O*-methyl-β-*D*-mannopyranose* (**5**) *and 1,2,4,6,-tetra-*O*-benzoyl-3-*O*-methyl-α-*D*-mannopyranose* (**6**). To a solution of tetrol **4** (30 mg, 0.15 mmol) in pyridine (10 mL) was added benzoyl chloride (104 μL, 0.9 mmol). The mixture was stirred at ambient temperature overnight, then aq sodium bicarbonate (20 mL) was added, and the whole was extracted with EtOAc (3 × 20 mL). The combined organic solvents were dried and concentrated to a residue, which

was purified by chromatography on preparative TLC plates (developing system: $CHCl_3$) to give the two anomers **5** (55 mg, 60%, identical to the above-described sample that originated from *M. vaccae*) and **6** (18 mg, 20%). Data for **6**: $[\alpha]_D^{20}$ −3.3° (*c* 2.6, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 3.48 (s, 3 H, O-Me), δ 4.13 (dd, 1 H, $J_{2,3}$ 3.0 Hz, $J_{3,4}$ 9.9 Hz, H-3), δ 4.44 (m, 2 H, H-5 and H-6a), δ 4.70 (br. d, 1 H, $J_{5,6b}$ 9.6 Hz, H-6b), δ 5.85 (dd, 1 H, $J_{2,3}$ 3.0, $J_{1,2}$ 1.9 Hz, H-2), δ 5.99 (t, 1 H, $J_{3,4} = J_{4,5}$ 10 Hz, H-4), δ 6.58 (d, 1 H, $J_{1,2}$ 1.9 Hz, H-1), δ 7.25–7.60 (m, 12 H), δ 8.05–8.15 (m, 8 H); ^{13}C NMR ($CDCl_3$, 75 MHz): 57.9 (Me), 62.3 (C-6), 67.1 (C-2), 67.5 (C-4), 70.8 (C-5), 76.3 (C-3), 91.5 ($^1J_{CH} = 177.0$ Hz, C-1), 128.0, 128.1, 128.2, 128.6, 128.9, 129.1, 1129.4, 129.5, 129.6, 129.7, 132.7, 133.1, 133.2, 133.7, 163.5, 165.0, 165.1, 165.8. Anal. Calcd for $C_{35}H_{30}O_{10}$: C, 68.85; H, 4.95. Found: C, 68.83; H, 5.07.

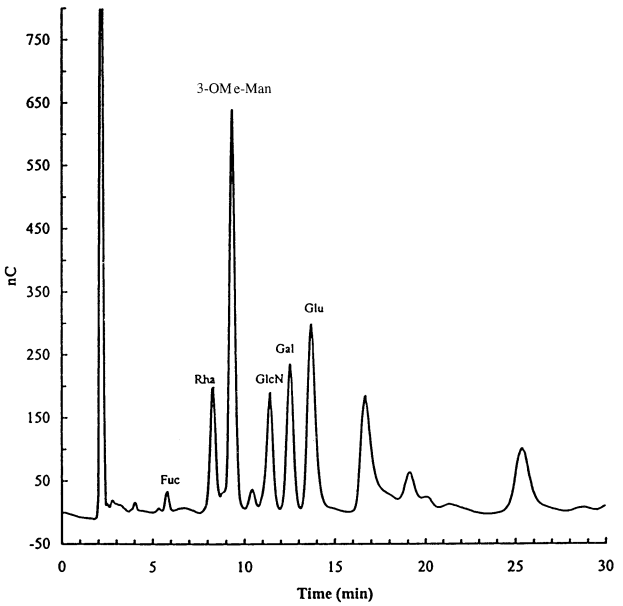


Fig. 1. Monosaccharide profiles of PS4A. The monosaccharide peaks separated by high-performance anion-exchange HPLC were identified using internal standards.

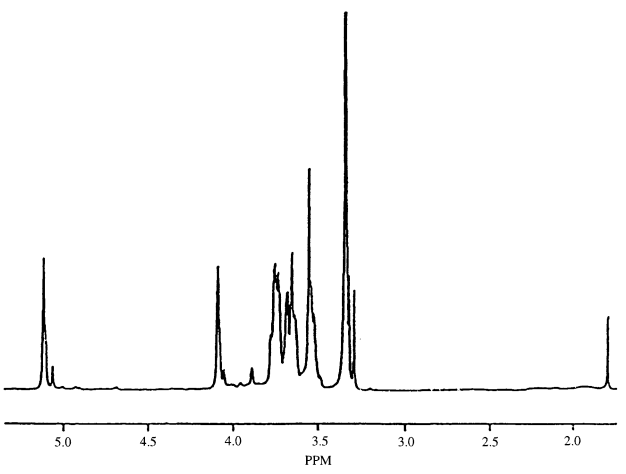


Fig. 2. ^1H NMR spectrum of the oligomer. The ^1H NMR spectrum was obtained in D_2O with a Bruker Avance 500 spectrometer.

3. Results

The boiling-water extract (PS4A) comprised about 5–6% (w/w) of the dried *M. vaccae* cell mass and proved to be predominantly a mixture of proteins and polysaccharides with a protein-to-sugar ratio of 1.5:1. Lipids were not detected, and only trace amounts of phosphate were detected in the organic phase. The monosaccharide profiles of PS4A were determined from the hydrolysates by a HPAEC system with known monosaccharide standards (Fig. 1). 3-*O*-Methyl-D-mannose was identified as the most abundant sugar (37.65%), along with glucose (25.35%), glucosamine (18.43%), rhamnose (13.62%), fucose (3.6%) and small amounts ($< 1.35\%$) of other sugars. Additional studies indicated that the *O*-methylated mannose was the predominant component of the polysaccharidic fraction isolated by gel-filtration chromatography, suggesting it was not covalently linked to the proteins.

The structure of the isolated oligomer was characterized in detail by one- and two-dimensional NMR spectroscopy. The simplicity of the ^1H (Fig. 2) and ^{13}C NMR spectra of PS4A3 suggested a material with a high degree of structural homogeneity. In particular, the ^{13}C NMR spectrum (125 MHz, D_2O) comprised only six sharp resonances at δ: 58.8, 63.6, 68.8, 75.0, 83.4, and 103.8 with minor peaks of much lower intensity (Fig. 3). The signal at δ 75.0 was considerably broader than the others, suggesting that it contained the unresolved resonances of two or more carbons. The gated decoupled ^{13}C NMR spectrum revealed both the anomeric $^1J_{C,H}$ coupling to be 173 Hz, consisting of an α-glycosidic linkage [25] and the signal at δ 58.8 to

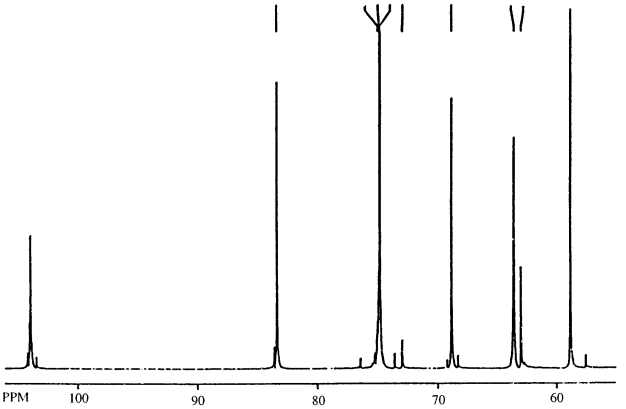


Fig. 3. ^{13}C NMR spectrum of the oligomer.

be a quartet indicating a methyl ether. The ^1H NMR spectrum (500 MHz, D_2O) was correspondingly simple (Fig. 2). It consisted of a broad, anomeric singlet at δ 5.10, a further broader singlet at δ 4.10, a series of multiplets centered on δ 3.75, 3.60, and 3.55 and a sharp singlet at δ 3.35, confirming the existence of the methyl ether moiety. A COSY spectrum revealed the coupling between the broad singlets at δ 5.10 and 4.10, leading to the assignment of the latter as H-2 (data not shown).

In order to determine the site of linkage and methylation, the polymer was next exposed to acetic anhydride and a catalytic amount of 4-(dimethylamino)pyridine in pyridine at room temperature overnight, followed by concentration to dryness under vacuum. The ^1H NMR spectrum of the reaction was considerably more complex than that of the polymer itself, which indicated that some decomposition had accompanied acetylation. Nevertheless, it still consisted of one major component. A COSY spectrum (500 MHz, $CDCl_3$) revealed that H-2 of the major component, still a broad singlet, now resonated at 5.40 and the two H-6s at δ 4.25 and 4.35, whereas H-3 and H-4 were part of a multiplet at δ 3.60 to 3.65. H-5 of the major component of this acetylated mixture resonated at δ 3.85. This pattern of chemical shift changes suggested that O-2 and O-6 had undergone acetylation and that the positions of the linkage and the methyl ether were, interchangeably, at O-3 and O-4. Finally, the broad singlet nature of H-1 and H-2, together with the axial nature of the glycosidic bond, strongly suggested the axial orientation for O-2, as in mannose.

The ^1H and ^{13}C NMR spectra of the crude hydrolysate were also simple and indicated the presence of one major component, consistent with the hypothesis of a highly homogenous polymer, present as a major and a minor anomer. The ^1H NMR spectrum (500 MHz, D_2O), aside from the broad singlets δ 5.09 and 4.05 assigned, respectively to H-1 and H-2 of the major anomer, was poorly resolved, which prevented interpretation of the coupling pattern and assignment of stereochemistry.

A sample of the hydrolysate, containing $NaHCO_3$, was recovered from the NMR experiment, lyophilized and then treated with benzoyl chloride and pyridine for 24 h at room temperature. Concentration and preparative TLC over silica gel enabled isolation of the major UV-active product, whose ^1H NMR spectrum (300 MHz, $CDCl_3$) was fully resolved (Fig. 4) and permitted its unambiguous identification as 1,2,4,6-tetra-*O*-benzoyl-3-*O*-methyl-β-D-mannopyranose (**5**). Consequently, the hydrolysate is 3-*O*-methyl-D-mannopyranose (**4**) [23,24], and the oligomer poly-α-(1→4)-linked 3-*O*-methylmannopyranose (Fig. 5). We note that a similar

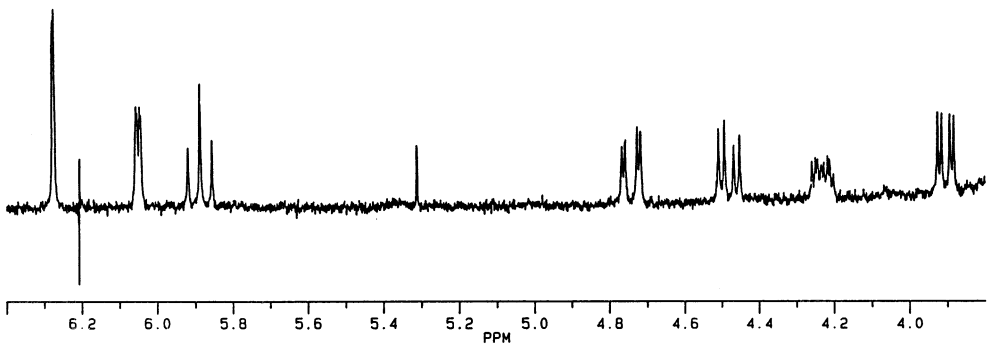


Fig. 4. Partial ^1H NMR spectrum of 1,2,4,6-tetra-*O*-benzoyl-3-*O*-methyl-β-D-mannose (**5**) in $CDCl_3$.

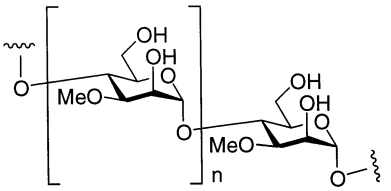
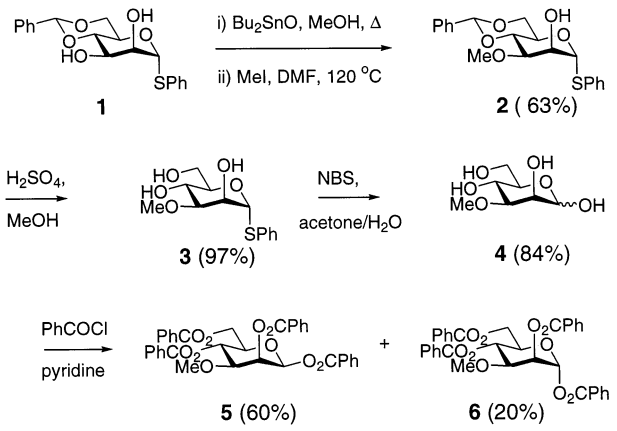


Fig. 5. Structure of the oligomer.



Scheme 1. Synthesis of the authentic monomer and its tetrabenzoates.

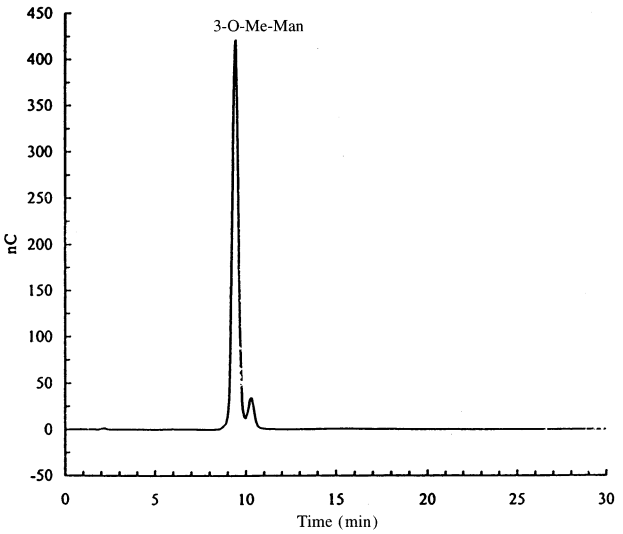


Fig. 6. HPLC analysis of synthetic 3-*O*-methyl-D-mannose. This chromatogram showed that the authentic sample had an identical retention time with the major sugar identified in PS4A (Fig. 1).

oligomer has been previously reported from *M. smegmatis* and that its ^{1}H NMR spectrum bears a very strong resemblance to that of the present sample [26].

Finally, authentic samples of the monomer (**4**) and its corresponding benzoylated derivatives (**5**) were prepared (Scheme 1). The all-important regioselective 3-O-monomethylation was achieved by sequential treatment of known **1** [18,19] with dibutyltin and iodomethane [20]. The regioselectivity of closely related alkylations has previously been adequately demonstrated in one of our laboratories [21,22]. The regioselectivity of this alkylation is further adequately supported by the spectral data for the perbenzoylated 3-*O*-methylmannose derivatives **5** and **6**. The authentic sample (**4**) had spectral data identical to those of the major component of the hydrolysate. The synthesized monomer (**4**) also had an identical retention time to that of the monosaccharide in the hydrolysate of the PS4A sample in HPLC analysis (Fig. 6). With the structure of the oligomer fully elucidated, it was possible to return to the spectral data and assign the ^{13}C NMR spectrum as follows using standard ^{1}H–^{13}C correlation methods: δ (D_2O) 58.8 (Me), 63.6 (C-6), 68.8 (C-2), 75.0 (C-4 and C-5), 83.4 (C-3) and 103.8 (C-1).

4. Discussion

Ultrastructural studies have demonstrated that the mycobacterial cell wall is a triple-layered structure [27]. An inner layer of moderate electron density probably contains peptidoglycans. A wider (9–10 nm) electron-transparent middle layer appears to consist of the hydrophobic lipids of the cell wall. The outermost electron-opaque layer varies in thickness, electron density and appearance among species, and under different growth conditions. Kobatake et al. [3] first reported that the exocellular materials extracted by boiling *M. tuberculosis* Aoyama B strains were polysaccharide-rich mixtures composed of glucan ($M_w > 75$ kDa), arabinomannan (M_w 13 kDa) and mannan (M_w 4 kDa). Previous studies by our group have demonstrated that much of the antineoplastic activity of *M. bovis* BCG Tice substrain is associated with a glucan having a molecular weight of $\sim$65 kDa [7]. The Connaught substrain, also clinically employed in the treatment of superficial bladder cancer, has a glycan component that probably differs in structure, but, on a weight-in-weight basis, is equivalent in biological activity [10]. These

studies confirmed the existence of high-molecular-weight glycans on the exocellular surface of slow-growing mycobacteria. Moreover, in the case of *M. bovis*, the biologically active components are associated with the cellular integument surrounding the bacterial cells [10].

Although extracts of the rapid growing *M. vaccae* had similar biological activity to materials obtained from *M. bovis*, it was evident that the components responsible for the immunostimulating activity in this case were structurally different. Hot-water extracts of intact cells of *M. vaccae* contained polysaccharides that were associated with a proteinaceous component [14]. The major polysaccharide recovered from PS4A was identified as poly-α-(1→4)-3-*O*-methyl-D-mannose with a molecular weight of around 3.6 kDa [28]. *O*-Methyl sugars are not widely distributed in nature but have been frequently isolated from mycobacteria. Poly-α-(1→4)-3-*O*-methyl-D-mannose, composed of 10 to 13 monomers, has previously been isolated from *M. smegmatis* and was considered as a biosynthetic precusor of the larger methylmannose polysaccharide [26]. Similar sugars have also been reported in the lipopolysaccharide of *Klebsiella* and *Escherichia coli*, which seems to be of immunological importance even though it is present only in small amount [29].

Although *O*-methyl sugars are frequently found in mycobacteria, the distribution and biological activity of such sugars, especially 3-*O*-methyl-D-mannose, is still not clear. This study is the first report of the isolation of homogeneous poly-3-*O*-methylmannose in *M. vaccae*. Our study also found that this sugar is associated with water-soluble glycoproteins that have immunostimulating activity [15]. This information is of great importance for the further study and use of *M. vaccae*, which is currently being tested as a nonspecific immunostimulant.

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