An anti-neoplastic glycan isolated from *Mycobacterium bovis* (BCG vaccine)

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Tice substrain BCG is used clinically as an immunotherapeutic agent against superficial bladder cancer. A boiling-water extract of this BCG showed anti-tumour activity against a murine S180 sarcoma model and was fractionated into three fractions, A, B and C, by the use of Sephadex LH-20 chromatography. An anti-tumour glucan, PS1A1, was isolated from fraction PS1A with Sephadex G-75. The molecular mass of PS1A1 was between 65 and 87 kDa by Sephadex G-100 chromatography. The structure of PS1A1 was investigated by one- and two-dimensional NMR spectroscopy and methylation analysis and was demonstrated to be primarily 1 → 6-α-linked glucose units. We postulate that the repeating unit is:

→6)-α-D-Glc*p*(1→6)-α-D-Glc*p*(1→4)-α-D-Glc*p*(1→6)-α-D-Glc*p*(1→6)-α-D-Glc*p*(1
6
↑
1
α-D-Glc*p*

INTRODUCTION

An attenuated strain of *Mycobacterium bovis*, Bacillus Calmette–Guérin (BCG vaccine), has been used as a tuberculosis vaccine for more than 70 years. The inverse relationship between cancer and tuberculosis was established as early as 1929 by Pearle [1] and the first trial of BCG vaccine against cancer was carried out by Holmgren [2] in Sweden in 1935. More recently, the anti-tumour activity of BCG has been demonstrated consistently [3–5] and commercial sources of BCG have been approved by the United States Food and Drug Administration as an immunotherapeutic agent for the treatment of superficial bladder cancer [6,7]. The vaccine has also shown efficacy against other tumours, such as melanoma [8], lung cancer [9] and leukaemia [10].

BCG is considered to be a general immunostimulant, and its anti-cancer activity has been attributed to a non-specific stimulation of the lymphoreticuloendothelial system [11]. The nature of the molecular components responsible for the anti-neoplastic properties of BCG vaccine remains uncertain [12].

Adverse effects of BCG immunotherapy, such as urethritis, disseminated mycobacterioses and enhancement of tumour growth, can be attributed to the use of whole, living, organisms [13], and it would be advantageous to isolate and identify any anti-neoplastic components for clinical application.

In this laboratory, the anti-tumour activity of BCG has been evaluated by the use of the S180 murine sarcoma model, and it was demonstrated that the anti-tumour activity of BCG persists in archived samples, irrespective of their age [14,15]. We have previously reported that a dialysed boiling-water extract of BCG, termed PS1, showed potent tumour inhibitory activity in the S180 sarcoma assay, and the initial characterization of PS1 has been carried out [16]. Here we report the results of structural analyses of an anti-neoplastic polysaccharide, PS1A1, a fraction isolated from this aqueous extract.

EXPERIMENTAL

S180 murine sarcoma assay

The method has been described by Klegerman et al. [14]. Female Swiss–Webster CFW mice (8 weeks old) were inoculated subcutaneously in the right flank with 3×10^5 viable S180 cells mixed with the preparation of materials to be tested. After 14 days, mice were killed, dissected and scored for tumour incidence relative to a control group receiving tumour cells plus PBS. One unit of inhibitory activity is defined as the minimum quantity of active principle sufficient to cause a significant (Fisher's exact test) decrease in the tumour incidence relative to controls, determined by assay of at least three dilutions of each fraction and extrapolation to the point of significance.

Extraction and preparation of PS1 from the Tice substrain of BCG

The contents of 12 ampoules of Tice BCG vaccine (lot 105x1, produced by Organon Teknika Inc., Chicago, IL, U.S.A.) were dispersed in 480 ml of PBS and centrifuged twice at 14000 *g* for 10 min. The washed BCG cells were extracted in a boiling-water flask by refluxing with distilled water (500 ml) for 2 h. The extract was passed through a 0.22 μm-pore-size membrane filter (Millipore Corp., Bedford, MA, U.S.A.) and concentrated by vacuum rotary evaporation. The concentrated water extract was dialysed through Spectra/Por 2 tubing against 2 litres of distilled water overnight. The lyophilized material retained in the tubing was termed PS1.

TLC of PS1

PS1 was spotted on a TLC plate and the plate was developed in a solvent system (n-butanol/acetic acid/water, 7:2:3, by vol.). Two spray reagents were used for the detection of components on the TLC plate: (1) 0.2% ninhydrin in ethanol; (2) a mixture

Abbreviations used: BCG, Bacillus Calmette–Guérin, an attenuated *Mycobacterium bovis* vaccine; PS1, polysaccharide 1, an extract from the Tice substrain of BCG; PS1A1, PS1 component purified by size-exclusion chromatography; TFA, trifluoroacetic acid; GC-MS, gas chromatography–MS; TSP, 3-(trimethylsilyl)propionic 2,2,3,3-d_4 acid, sodium salt; DEPT, Distortionless Enhancement by Polarization Transfer; DQF-COSY, Double Quantum Filtered-COSY; RCT-COSY, Relayed Coherence Transfer-COSY; HOHAHA, homonuclear Hartmann Hahn; EI, electron-impact.

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of equal volumes of 20% H_2SO_4 and 0.2% 1,3-dihydroxynaphthalene in ethanol.

Sephadex LH-20 chromatography of PS1

PS1 (15 mg in 1 ml of distilled water) was applied to a column (1.5 cm × 45 cm) of Sephadex LH-20 gel (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) equilibrated with distilled water. The column was then eluted with distilled water. Eluent fractions (2 ml) were assayed for carbohydrate by both the phenol/sulphuric acid method [17] and by TLC. Three major peaks on the elution profile were identified (see Figure 1), and these were termed PS1A–PS1C.

Sephadex G-75 chromatography of PS1A

PS1A from the Sephadex LH-20 column in 1 ml of distilled water was applied to a column (1.5 cm × 45 cm) of Sephadex G-75 (Pharmacia) which was equilibrated and eluted with distilled water. Distilled water eluent fractions were assayed by the phenol/sulphuric acid method [17]. Four peaks from the elution profile of this fraction could be identified (see Figure 2) and were termed PS1A1–PS1A4.

Ethanol precipitation of PS1A1

PS1A1 (5 mg) from Sephadex G-75 chromatography was dissolved in 1 ml of distilled water, and 10 ml of ethanol (HPLC grade; Fisher Scientific) was added. The resultant suspension was centrifuged for 20 min at 14000 ***g***. The pellet was dissolved in distilled water, transferred to a vial and lyophilized. This lyophilized material, PS1A1, was used for structural studies.

Composition analysis of PS1A1

Alditol acetates of PS1A1 were prepared by the method of Blakeney et al. [18]. PS1A1 (2 mg) was dissolved in 1 ml of 2 M trifluoroacetic acid (TFA). The solution was sealed in an ampoule and autoclaved for 1 h at 121 °C. The autoclaved sample was transferred to a test tube and dried in a flowing air stream overnight. To the dried sample were added 0.1 ml of 1 M NH_4OH and 1 ml of $NaBH_4$/DMSO (0.1 g/5 ml), and the solution was incubated at 40 °C for 90 min. Acetic acid (0.1 ml; Fisher Scientific), 0.2 ml of 1-methylimidazole (Aldrich Chemical Co.) and 2 ml of acetic anhydride (Fisher Scientific) were added to the test tube, which was left at room temperature for 10 min, and 5 ml of distilled water was then added. After the solution in the test tube had cooled to ambient temperature, it was partitioned with 1 ml of dichloromethane. The dichloromethane phase was subsequently concentrated in a flowing air stream and used for gas chromatography (GC) analysis.

The alditol acetates were analysed on a Shimadzu GC-17A GC instrument using helium as carrier gas, a J & W Scientific DB-23 bonded phase capillary column (30 m × 0.25 mm) and a flame ionization detector. The temperature program used was initially set at 150 °C, held for 1 min after injection and then increased by 10 °C/min to 240 °C. The temperature was held constant at 240 °C for a further 10 min until all components had been eluted from the column. Injector and detector port temperatures were both set at 300 °C.

Linkage analysis of PS1A1

Methylation of the intact polysaccharide was accomplished with dimsyl lithium according to the procedure of Kvernheim [19]. After methylation, the methylated polysaccharide was purified and isolated using Waters Sep-Pak C18 cartridges as described by Waeghe et al. [20]. The purified isolate from this procedure was reacted with 300 μl of 'Superdeuteride' (1.0 M solution of lithium triethylborodeuteride in tetrahydrofuran; Aldrich Chemical Co., Milwaukee, WI, U.S.A.) for 90 min to selectively deuterate any carboxyl groups in the polysaccharide. The superdeuteride was destroyed at the end of this time with TFA (50 μl; neat) and evaporated to dryness. This was followed by hydrolysis, reduction and acetylation according to the procedure of Harris et al. [21].

GC-MS analysis of partially methylated alditol acetates was carried out on a Finnegan 4510 GC-MS instrument. The GC temperature program was initiated at 90 °C and held for 1 min, after which the temperature was increased at 10 °C/min up to a final temperature of 240 °C. This was held constant for a further 10 min until all the components had been eluted from the column.

NMR spectroscopy of PS1A1

The sample was initially deuterium-exchanged by lyophilization from ^2H_2O, followed by dissolution in 99.98% ^2H_2O (0.5 ml; Aldrich). Spectra were recorded on a Bruker AM 400 instrument operating at 400.135 MHz for proton and 100.62 MHz for carbon. Sample concentration was approx. 5 mg/ml with typical sweep widths for normal one-dimensional experiments of 4800 Hz (12 p.p.m.) for ^1H and 23000 Hz (225 p.p.m.) for ^{13}C. Chemical shifts were referenced to internal TSP [3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid; sodium salt]. Multipulse experiments that were carried out included ^{13}C Distortionless Enhancement by Polarization Transfer (DEPT) (135°), Double Quantum Filtered (DQF)-COSY, Relayed Coherence Transfer (RCT)-COSY and homonuclear Hartmann Hahn (HOHAHA) experiments. The published spectra were generated using the tools available in NMR Pipe (distributed by Molecular Simulations, Inc.).

RESULTS

S180 mouse sarcoma assay

The effect of progressive purification of PS1 on the anti-neoplastic activity measured using the murine S180 sarcoma assay is summarized in Table 1.

Table 1 Murine S180 sarcoma assay

Female Swiss–Webster CFW mice (8 weeks old) were inoculated subcutaneously in the right flank with 3×10^5 viable S180 cells mixed with the material to be tested. After 14 days mice were killed, dissected and scored for tumour incidence relative to a control group receiving tumour cells plus PBS. The titre is the lowest quantity of sample producing significant inhibition of tumour formation. One unit of inhibitory activity is defined as the minimum quantity of active principle sufficient to cause a significant decrease in the tumour incidence relative to controls, determined by assay of at least three dilutions of each fraction and extrapolation to the point of significance.

Fractions	Titre (mg/kg)	Specific activity (units/mg)
PS1	0.080	500
PS1A	< 0.038	> 1000
PS1B + PS1C	–	0
PS1A1	$\ll 2 \times 10^{-5}$	$\gg 2 \times 10^6$
PS1A2	$< 2 \times 10^{-5}$	$> 2 \times 10^6$
PS1A3	0.014	3170
PS1A4	0.006	7096

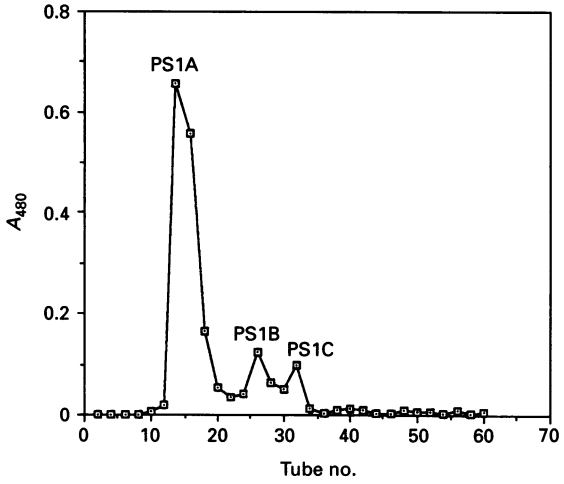

Figure 1 Elution profile of PS1 on a Sephadex LH-20 column

PS1 was applied to a Sephadex LH-20 column (1.5 cm × 45 cm) which was equilibrated and eluted with distilled water at a flow rate of 1 ml/min, and 2 ml fractions were collected. The eluate was assayed for carbohydrates by the phenol/sulphuric acid method.

Isolation and purification of anti-tumour polysaccharide

TLC analysis of PS1 showed that there were four spots on the TLC plate. One spot on the origin of the plate gave a blue colour when sprayed with 1,3-dihydroxynaphthalene (carbohydrate detection reagent), but did not show colour when sprayed with ninhydrin. Three other spots, with R_F values of 0.31, 0.43 and 0.54, showed a pink colour when sprayed with ninhydrin and a blue colour with 1,3-dihydroxynaphthalene.

PS1 was first fractionated by Sephadex LH-20 chromatography. The elution profile of Sephadex LH-20 chromatography obtained by the analysis of carbohydrate in each fraction showed three peaks (Figure 1). When the fractions from the LH-20 chromatography were analysed by TLC, it was found that the first peak on the elution profile contained only the spot at the origin of the TLC plate as described above, while peaks 2 and 3 contained the other three spots. Therefore the bioassay was performed using the material from the first peak (PS1A) and the materials from the combined peaks 2 (PS1B) and 3 (PS1C).

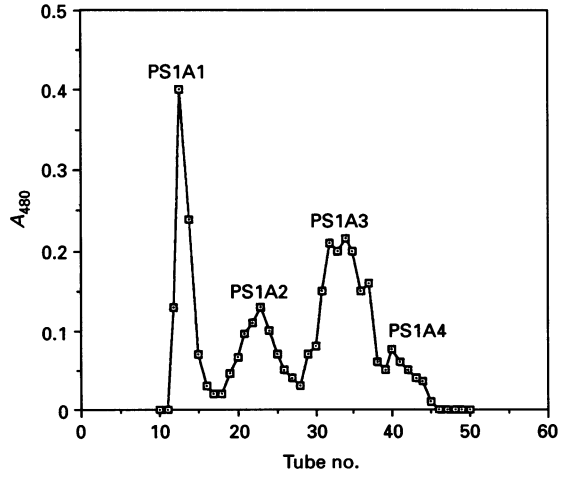

Figure 2 Elution profile of PS1A on a Sephadex G-75 column

PS1A was applied to a Sephadex G-75 column (1.5 cm × 45 cm) which was equilibrated and eluted with distilled water at a flow rate of 0.5 ml/min, and 2 ml fractions were collected. The eluate was assayed for carbohydrates by the phenol/sulphuric acid method.

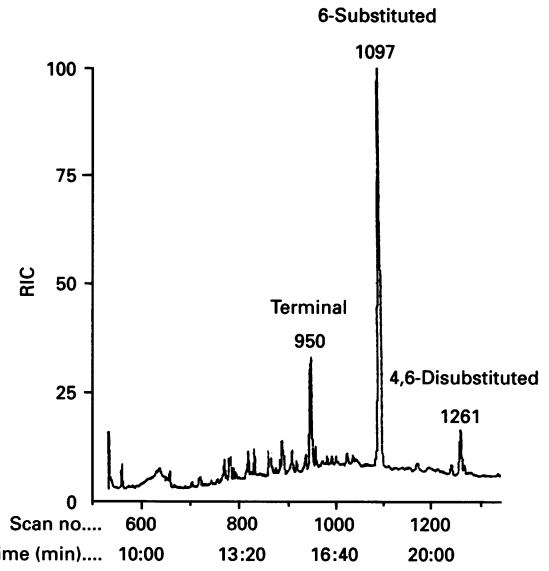

Figure 3 Total ion chromatogram of partially methylated alditol acetates of PS1A1

Partially methylated alditol acetates of PS1A1 were analysed using a Finnegan 4510 GC-MS. The peaks labelled 950, 1097 and 1261 represent terminal, 6-substituted and 4,6-substituted glucose derivatives respectively. Peaks with retention times less than peak labelled 950 are impurities. RIC, reconstructed ion current.

The anti-tumour fraction PS1A from Sephadex LH-20 chromatography was further separated on a Sephadex G-75 column. The elution profile of Sephadex G-75 chromatography, determined by the phenol/sulphuric acid method, showed four peaks, PS1A1–PS1A4 (Figure 2). PS1A1 exhibited the greatest anti-tumour activity, and identification of this anti-tumour component was therefore attempted.

Structural studies of PS1A1

The GC chromatogram of alditol acetates from PS1A1 showed only glucitol, indicating that PS1A1 is substantially a glucan.

The molecular mass of PS1A1 was estimated relative to dextran standards (average molecular masses 9.3, 39.2 and 73 kDa, Sigma, St. Louis, MO, U.S.A.; 25 and 150 kDa, Fluka Biochimica, Basle, Switzerland) on a Sephadex G-100 column. The results indicated that the molecular mass of PS1A1 was between 65 and 87 kDa.

The total ion chromatogram of partially methylated alditol acetates of PS1A1 generated from GC-MS analysis is shown in Figure 3. The electron-impact (EI) mass spectra of the peaks in the ion chromatogram indicated that peaks with retention times less than that of peak 950 were impurities. The EI mass spectra of the peaks labelled 950, 1097 and 1261 showed that they represented terminal, 6-substituted and 4,6-substituted monosaccharide derivatives respectively by comparison with standard mass spectra [22]. Authentic glucose standards were also analysed and corresponded with the retention times given above. This is consistent with our observation that only glucose is detected by GC of the alditol acetates. The proton NMR spectrum of PS1A1

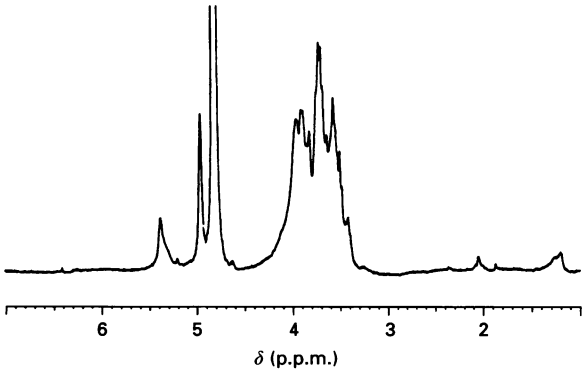

Figure 4 Proton NMR spectrum of PS1A1

The proton NMR spectrum of PS1A1 in ^2H_2O was measured on a Bruker AM 400 spectrometer. Chemical shifts were referenced to internal standard TSP.

(Figure 4) showed only signals assigned to carbohydrate. The resonances at 4.97 and 5.39 p.p.m. were assigned to anomeric protons, while the other sugar protons are seen overlapped between 3.2 and 4.0 p.p.m.

The chemical shifts of protons in the individual glucose residues in PS1A1 were assigned by the analysis of several two-dimensional NMR experiments [23], including DQF-COSY (Figures 5a and 5b), RCT-COSY (Figure 6) and HOHAHA spectroscopy (Figure 7). This is illustrated from examination of the DQF-COSY spectrum, where it is seen that the anomeric resonance at 4.96 p.p.m. shows an off-diagonal element to a signal at 3.56 p.p.m., assigned as H2. H2 then shows a correlation at 3.71 p.p.m. to H3, and this procedure is repeated from H3 through to H6 and H6′. These assignments were then confirmed, or new assignments made, through the use of RCT-COSY and HOHAHA, where additional off-diagonal elements to the anomeric resonance can be seen such that, in the HOHAHA spectrum (Figure 7), the signals of H2 to H5 show correlations to the anomeric resonance at 4.96 p.p.m. As can be seen in the RCT spectrum, there are some additional intense cross-peaks other than those from the main component, which we ascribe to some slight impurity. These signals are also evident in the one-dimensional proton spectrum but their size is far smaller than would be suggested by the intensity of the cross-peaks in the RCT spectrum. The nature of this component would appear to be carbohydrate in origin, possibly monosaccharide glucose from slight decomposition, as the chemical shifts of the various correlations correspond approximately to those of α and β glucose, cf. α-H1 = 5.21 p.p.m and β-H1 = 4.6 p.p.m. The chemical shift assignments for the individual glucose residues are shown in Table 2.

The DEPT-135° spectrum of PS1A1 shows strong signals typical of an α-1 → 6-linked glucan, with the anomeric carbon resonance at 100.5 p.p.m. and that of C6 at 68.7 p.p.m. In addition to the signals of the α-1 → 6-linked glucan there are three other weaker signals at 63.5 p.p.m. (CH_2), 74.5 and 75.9 p.p.m. (CH) which can be assigned to the terminal glucose residue.

DISCUSSION

The results of both GC analysis and NMR spectroscopy indicate that the principal monosaccharide residues of PS1A1 are glucose. On examination of the chemical shift of the anomeric protons it seems that the residues are α-linked, since α-linkages commonly

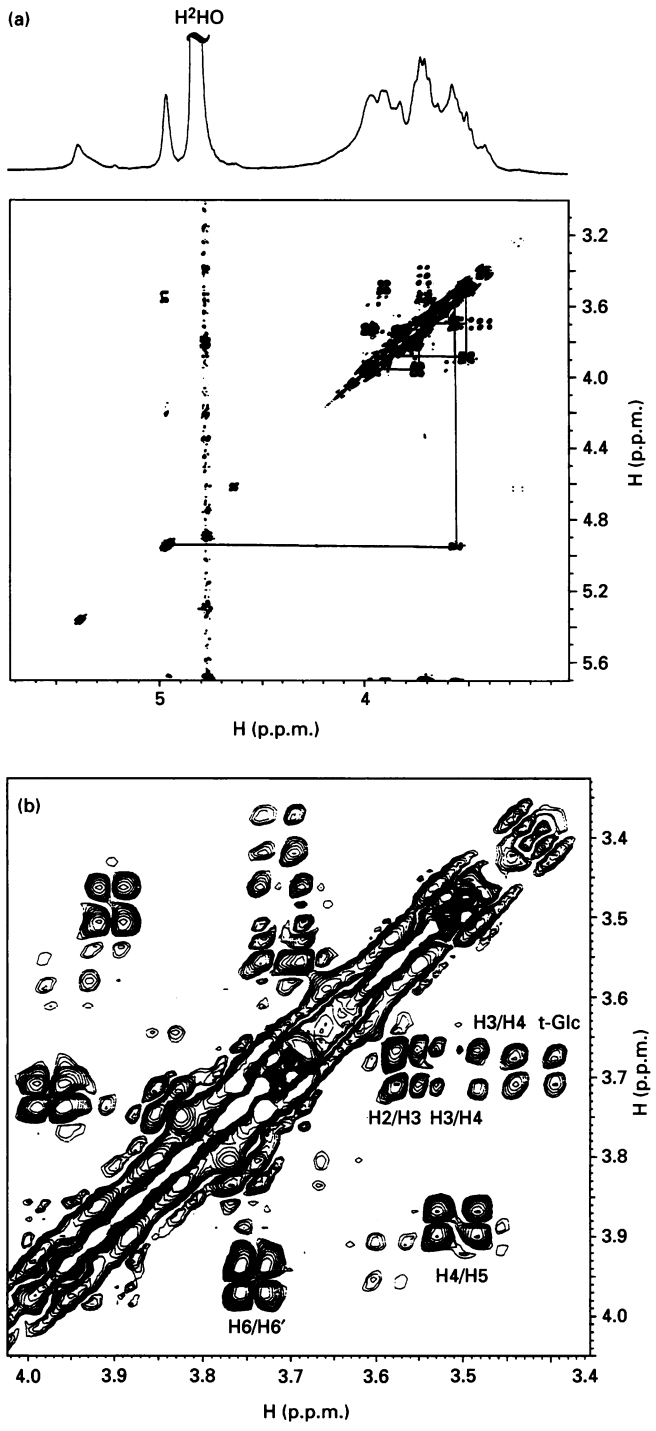

Figure 5 (a) DQF-COSY spectrum of PS1A1, and (b) expansion of the ring proton region to clearly show the correlations and assignments

t-Glc indicates terminal glucose.

show chemical shifts above 4.9 p.p.m. This is confirmed by the value of the H1–H2 coupling constant (approx. 3–4 Hz), which is indicative of an α-linkage by consideration of the Karplus relationship (i.e. Eq–Ax = 3–4 Hz; Ax–Ax = 8–9 Hz, where Eq is equatorial and Ax is axial) in monosaccharides. Other coupling constants also confirm that PS1A1 is a glucose homopolymer, since H3–H4 shows a large coupling constant ($\approx$10 Hz), indicative of the trans-diaxial arrangement in glucose.

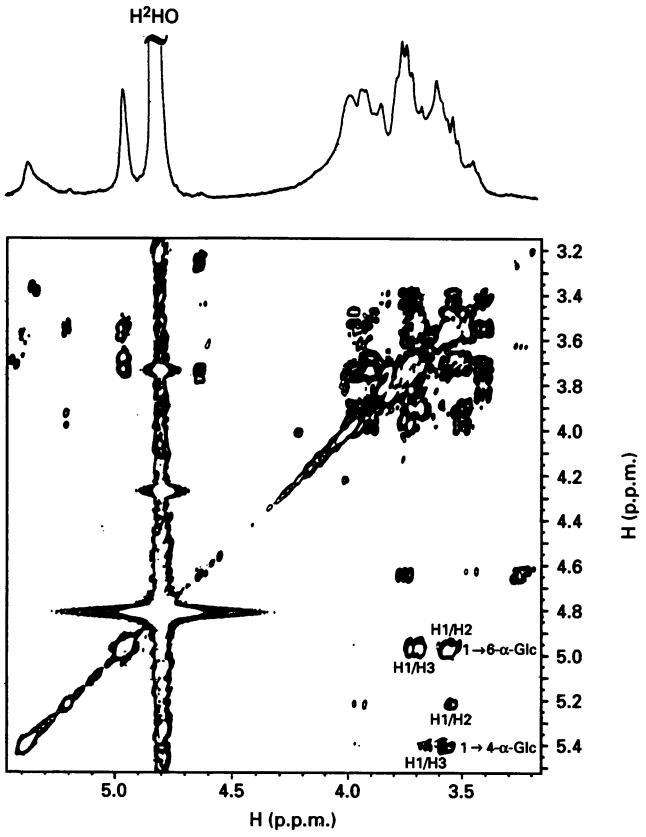

Figure 6 Homonuclear RCT-COSY spectrum of PS1A1

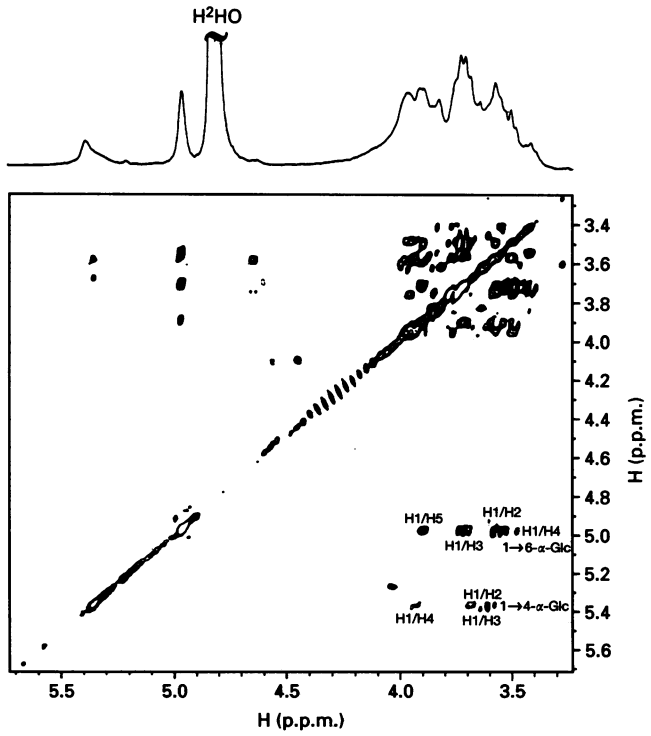

Figure 7 HOHAHA spectrum of PS1A1

Table 2 Proton chemical shifts of PS1A1

The proton NMR spectrum of PS1A1 (5 mg/0.5 ml of ^2H_2O) was measured on a Bruker AM400 spectrometer. Chemical shifts (p.p.m.) were referenced to the internal standard TSP at room temperature. t- indicates terminal; ND, not determined.

	Chemical shift (p.p.m.)		
Proton	1 → 6-α-Glc*p*	1 → 4-α-Glc*p*	t-Glc*p*
H1	4.96	5.39	4.96
H2	3.56	3.58	3.55
H3	3.71	3.68	3.71
H4	3.50	3.94	3.41
H5	3.90	ND	3.83*
H6	3.98	ND	3.83*
H6′	3.74	ND	3.76*

* Chemical shifts assigned on the basis of their comparison with α-glucose.

From the aforementioned GC-MS results, it would appear that the polysaccharide PS1A1 is a glucan with predominantly 1 → 6 linkages. Moreover, some terminal glucose and a branched residue, namely 4,6-substituted glucose, were also observed, suggesting that there are occasional branch points along this main chain at position 4. This is consistent with the NMR results in that a large signal (4.96 p.p.m.) was observed in the ^1H NMR spectrum which is assigned to the anomeric proton of α-linked 1 → 6 glucose [cf. dextran (39.2 or 73 kDa) α-1 → 6-linked glucose at 4.96 p.p.m.].

An additional smaller signal at 5.38 p.p.m. is assigned to the anomeric resonance of a glucose residue which is linked to position 4 by consideration of the chemical shift of the corresponding proton in similar molecules such as glycogen and maltose [24].

Integration of the GC and NMR peaks corresponding to these branch points indicates that there is approximately one branch point every 5–6 residues of the 1 → 6 main chain. There are two possible structures of PS1A1 that are consistent with these observations, shown as A and B in Figure 8. To differentiate between the two molecules, the chemical shifts of the unique features of each molecule have to be taken into consideration. For example, in structure A the substitution of the terminal glucose occurs at position 4, so one might anticipate that this residue would show chemical shifts very similar to those of the non-reducing glucose of maltose, whereas structure B shows the terminal glucose substituted at position 6 and the residue substituted to position 4 within the main chain.

Examination of the chemical shifts of the glucose residue substituted at position 4 (Table 2, residue 2) is ambiguous, since a complete assignment has not been made, although the chemical shift of H4 of 3.94 p.p.m. (cf. 3.41 p.p.m. for α-glucose) is suggestive that structure A is not correct. Further evidence to support this conclusion can be seen in the DQF-COSY spectrum (Figure 5) where a single intense cross-peak is observed at 3.71/3.41 p.p.m., i.e. the expected position of an H3–H4 cross-peak in α-glucose. Moreover, this cross-peak also has the same chemical shift for H3 and H3 at the bulk structure, namely 1 → 6-linked α-glucose, as might be expected if the terminal glucose were also substituted at position 6. Finally, as observed in the RCT-COSY spectrum, the aforementioned signal at 3.41 p.p.m. shows additonal cross-peaks which also show chemical shifts similar to those of H5, H6 and H6′ of glucose. Therefore we assigned these signals to the terminal glucose residue and consider

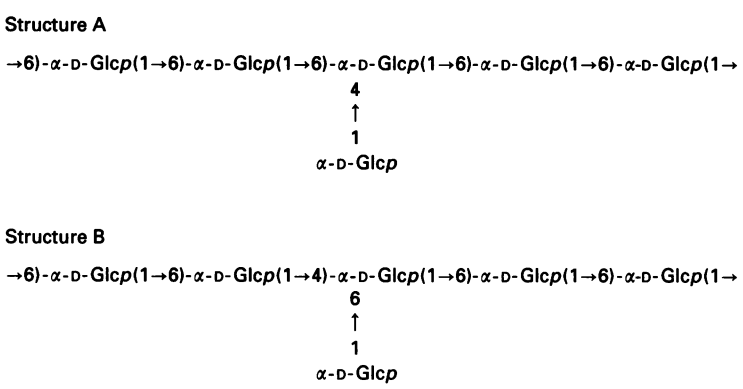

Figure 8 Possible structures of PS1A1

See the text for details.

that structure B is most likely to be the basic structural element of PS1A1.

The results of this and previous studies [16] indicate that a significant proportion of the BCG anti-neoplastic activity is due to a previously undescribed mycobacterial dextran possessing immunomodulatory properties. Further investigation of the microstructural features that are responsible for the observed biological properties of this molecule is required.

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