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THE CHEMICAL STRUCTURE OF THE D-GALACTO-D-MANNAN COMPONENT OF *Trypanosoma cruzi*: ^{13}C-N.M.R. SHIFT DEPENDENCE ON STRUCTURE OF D-GALACTOSE TO D-MANNOSE LINKAGE*

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ABSTRACT

Extraction of cells of *Trypanosoma cruzi* provided a D-galacto-D-mannan contaminated with a D-glucan, tentatively identified as glycogen. The galactomannan, on methylation, gave a number of methylated fragments which indicated, among other structures, α-D-mannopyranosyl units that were nonreducing ends, 2-*O*-, 2,3-di-*O*- and 3,4-di-*O*-substituted structures, and D-galactofuranosyl nonreducing end-units. ^{13}C-Nuclear magnetic resonance spectroscopy showed the presence of single-unit, nonreducing ends of β-D-galactofuranose. Their C-1 signal was at δ 106.6, differing from those of single-unit β-D-galactofuranosyl side-chains of D-galacto-furano-D-mannopyranans of *Sporothrix schenckii* (δ 109.3) and *Trichophyton interdigitale* (δ 109.4), and those of polysaccharides with longer side-chains, arising from *Aspergillus niger* (δ 108.4 and 109.2) and *Ceratocystis stenoceras* (δ 109.5, 107.6, and 107.2). Such a variation arises from a dependence on the position of substitution on the α-D-mannopyranosyl residues and was evident with synthetic methyl glycosides of 2-*O*- (C-1′, δ 107.7), 3-*O*- (C-1′, δ 106.5), and 6-*O*-β-D-galactofuranosyl-α-D-mannopyranose (C-1′, δ 109.7). Thus, the C-1 signal of the galactomannan of *T. cruzi*, at δ 106.6, is characteristic of a (1→3)-linkage between D-galactosyl and D-mannosyl units. The chemical structure of the *T. cruzi* galactomannan bore resemblances only to minor polysaccharide components of *Herpetomonas samuelpessoai* and *Crithidia fasciculata*, protozoa that stimulate resistance against Chagas' disease when injected into laboratory animals.

INTRODUCTION

Laboratory animals, when injected with live cells of the protozoa *Crithidia fasciculata*[1] and *Herpetomonas samuelpessoai*[2], develop resistance to infection by *Trypanosoma cruzi*, the causative agent of Chagas' disease. If the surface-polysaccharide

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components of the cells have a role in the immunostimulation process, it is of interest to determine whether the effect depends on similarities in chemical structures, as in a direct humoral antigen–antibody reaction, or is independent of fine chemical structure, as in a cell-mediated response. Thus far, the structures of polysaccharides of *Herpetomonas samuelpessoai*[3] and *Crithidia fasciculata*[4] have been elucidated. However, few structural components have yet been identified for polymers of *T. cruzi* cells. For example, polysaccharide extracts of cells of the epimastigote form of *T. cruzi*, grown on Chang's medium[5], have been reported by Gonçalves and Yamaha[6] to contain galactose, glucose, mannose, xylose, and glucosamine, and were antigenically active towards the serum of a patient having Chagas' disease and to rabbit immune serum. However, other antigenically active extracts consisted of a polysaccharide complex having galactose and mannose as component sugars[7]. Similarly, de Lederkremer *et al.*[8] found that galactose and mannose were components of an electrophoretically mobile complex obtained from epimastigote forms. Such carbohydrates can exist at the cell surface since trypo- and epi-mastigotes were reported[9] to agglutinate, in one series of experiments, with concanavalin A, consistent with the presence of either 2-*O*-substituted units or nonreducing ends of α-D-mannopyranose. However, in another study only epimastigotes agglutinated[10]. The present study concerns the determination of more detailed chemical structures of the D-galactose, D-mannose, and D-glucose components of epimastigotes of *T. cruzi*, grown on Warren's medium[11]. Also the component sugars of epimastigotes, grown on Chang's medium, were identified.

RESULTS

Parallel experiments were carried out to determine the sugar composition of *T. cruzi* cells grown on Warren's and Chang's media. Acid hydrolysis of whole cells provided a sugar mixture that was treated successively with sodium borohydride and acetylated to provide acetates of mannitol, galactitol, glucitol, and *myo*-inositol. The peak ratios obtained on g.l.c. were 18:9:2:71 (Warren's medium) and 9:6:1:84 (Chang's medium). In neither case was xylitol acetate detected, as would be expected from the work of Gonçalves and Yamaha[6]. The relative consistency of the galactose-to-mannose ratio suggests that they are components of the same molecule, in contrast to inositol the proportion of which varies.

Polysaccharides from *T. cruzi* cell preparations were isolated following extraction with hot 6% aqueous potassium hydroxide, which decomposed protein and most of the nucleic acids. On neutralization with acetic acid and centrifugation, the aqueous layer was separated from less-dense lipids, and the polysaccharide was freed from nucleic acids by passage through a column of mixed-bed resin. From dry cells grown on Warren's medium, the polysaccharide yield was 3% and the derived acetates of mannitol, galactitol, glucitol, and inositol were detected with a g.l.c. peak-area ratio of 25:11:2:12. In contrast, these values were 1% and 42:21:3:32, respectively, for the polysaccharide derived from cells of Chang's medium. It would appear that the

galactose and mannose components arise by decomposition of the electrophoretically mobile complex previously described[8]. Detailed studies were only carried out on the polysaccharide isolated from cells grown in Warren's medium, because of their relative ease of growth and higher yield of polysaccharide.

The polysaccharide mixture was methylated by the successive procedures of Haworth[12] and Kuhn *et al.*[13], and the per-*O*-methylated product was degraded to a mixture of *O*-methylalditol acetates, which were identified by g.l.c.–m.s.[14]. The mixture was submitted to g.l.c. on a glass-capillary column containing SP2250 (OV-17), and the components were identified by their retention times and typical m.s. breakdown patterns obtained on electron impact. The presence of 2,3,6-tri-*O*- (27%) and 2,3-di-*O*-methylglucitol acetates (3%) indicated 4-*O*- and 4,6-di-*O*-substituted D-glucopyranose units, as present in glycogen, a known component of protozoa. However, this component was not positively identified. The 2,3,4,6-tetra-*O*-methyl derivative, arising from nonreducing end-units, unfortunately cochromatographed with the corresponding mannitol derivative (see later) and could not be identified. Of greater interest were the acetates of 2,3,4,6-tetra-*O*- (26%), 3,4,6-tri-*O*- (17%), 3,4-di-*O*- (4%), and 4,6-di-*O*-methyl (2%) derivatives of mannitol, and 2,3,5,6-tetra-*O*- (9%), 2,3,4,6-tetra-*O*- (1%), 2,3,6-tri-*O*- (5%), and 2,4-di-*O*-methyl

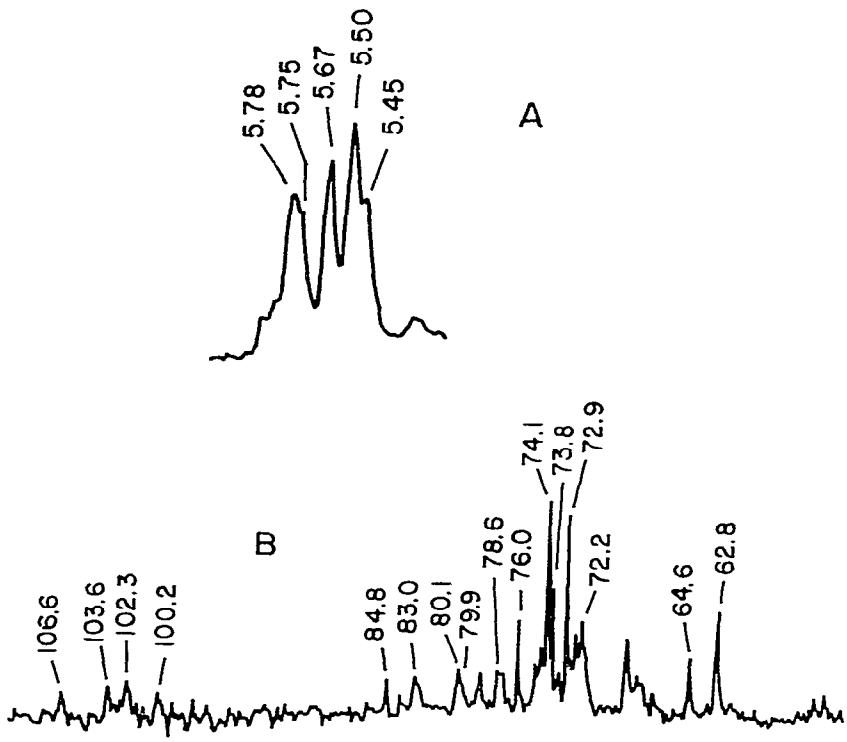


Fig. 1. (A) ^{1}H-N.m.r. spectrum of D-galacto-D-mannan of *T. Cruzi* (H-1 region). (B) ^{13}C-N.m.r. spectrum of D-galacto-D-mannan of *T. Cruzi*.

(4%) derivatives of galactitol. The lack of correspondence of tetra-*O*- to di-*O*-methyl derivatives cannot at this time be explained. These results indicated a branched structure with nonreducing end-units of D-galactofuranose, D-galactopyranose (trace), and D-mannopyranose with 2-*O*-substituted D-mannopyranosyl units, 4-*O*-substituted D-galactopyranosyl (or 5-*O*-substituted galactofuranosyl) units, and a variety of di-*O*-substituted units. These branch points were 2,3-di-*O*- and 2,6-di-*O*-substituted D-mannopyranosyl and 3,6-di-*O*-substituted D-galactopyranosyl (or 2,6-di-*O*-substituted D-galactofuranosyl) units. The presence of D-galactofuranosyl and D-galactopyranosyl units agrees with the finding of Alves *et al.*[15] that, whereas some electrophoretically mobile fractions of *T. cruzi* were oxidized by D-galactose oxidase, specific for D-galactopyranose units, another fraction was unaffected, indicating D-galactofuranosyl units.

Partial acetolysis of the inositol-free polysaccharide preparation, followed by *O*-deacetylation gave, as indicated by paper chromatography, galactose, glucose, mannose, and a di- and tri-saccharide having mobilities corresponding to those of (1→2)-linked α-D-mannopyranosyl oligosaccharides. Thus, it appears that two consecutive (1→2) linkages are present, although a component (1→3) linkage is possible since 4,6-di-*O*-methylmannitol tetraacetate was formed in the methylation experiment. However, the ^{1}H-n.m.r. spectrum of the polysaccharide contains an H-1 signal at δ 5.78 (Fig. 1, A), typical of a chain of (1→2)-linked α-D-mannopyranosyl units[16].

The ^{13}C-n.m.r. spectrum of the undialyzed polysaccharide preparation (Fig. 1, B) shows many features consistent with the methylation, partial acetolysis, and ^{1}H-n.m.r. data. However, the sample was contaminated by *myo*-inositol, a product of alkaline hydrolysis of phosphatides, which co-precipitated on treatment with ethanol–acetone. It was the only low-molecular-weight carbohydrate detected by paper chromatography and gave four typical sharp signals at δ 76.0, 74.1, 73.8, and 72.9. (These signals were not present when a dialyzed sample was examined. The other signals, although present, were not as readily discerned, and the present spectrum is preferred). The polysaccharide signals were broader, as expected from a larger molecule with less segmental motion. Signals at δ 103.6, 102.3, and 100.2 were consistent with α-D-mannopyranosyl units since they correspond, respectively in shift, to C-1 signals of nonreducing end-units, 2-*O*-substituted, and 2,6-di-*O*-substituted units[17], like those present in the mannan of *Saccharomyces rouxii*, which contains a (1→6)-linked α-D-mannopyranosyl main-chain with each unit substituted in the 2-position by *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranose side-chains[18]. Signals were also present at δ 79.9 and 80.1, which correspond to C-2 atoms of 2-*O*-substituted and 2,6-di-*O*-substituted α-D-mannopyranosyl units, respectively. A signal at δ 79.4, characteristic of C-2 atoms in a linear D-mannan containing consecutive (1→2)-, and (1→2)- and (1→6)-linkages[19] was absent. Signals corresponding to glycogen were not identified.

β-D-Galactofuranosyl residues are indicated by ^{13}C signals at δ 106.6, 84.8, and 83.0, characteristic of C-1, C-4, and C-5 of methyl β-D-galactofuranoside, respectively[20,21]. Correspondence was noted with signals at δ 77.1 (C-3) and 64.6

(C-6), indicating that the residues were not *O*-substituted (except at C-1) and must exist principally as nonreducing ends.

The undialyzed polysaccharide preparation had $[\alpha]_D^{25}$ +10°, a value that can be interpreted in terms of component configurations, despite the presence of 24% of optically inactive *myo*-inositol and 4% of D-glucan. This optical rotation indicates that the β-D-galactofuranose residues are in the D form, in view of the positive contribution of the α-D-mannopyranose residues, which should be close to +88°, as reported for baker's yeast D-mannan[22]. This value should be diminished since −84° has been reported as the rotation of a β-D-linked D-galactofuranan of *Penicillium charlesii*[23].

The shift value of the C-1 signal at δ 106.6 was used as a means of suggesting the position of substitution of single-unit side-chains of β-D-galactofuranose on adjacent α-D-mannopyranosyl units. These were preferred over galactose-to-galactose linkages since there were only minor proportions of di-*O*- and tri-*O*-substituted D-galactosyl units, according to the methylation data. A shift dependency of the substitution position on the D-mannosyl unit was strongly suggested by comparison of low-field C-1 shifts of β-D-galactofuranosyl signals of various β-D-galactofurano-α-D-mannopyranans (Table I). These vary widely, being δ 109.3 for single-unit side-chains of polysaccharides of *Sporothrix schenckii*[24] and *Trichophyton interdigitale*[25],

TABLE I

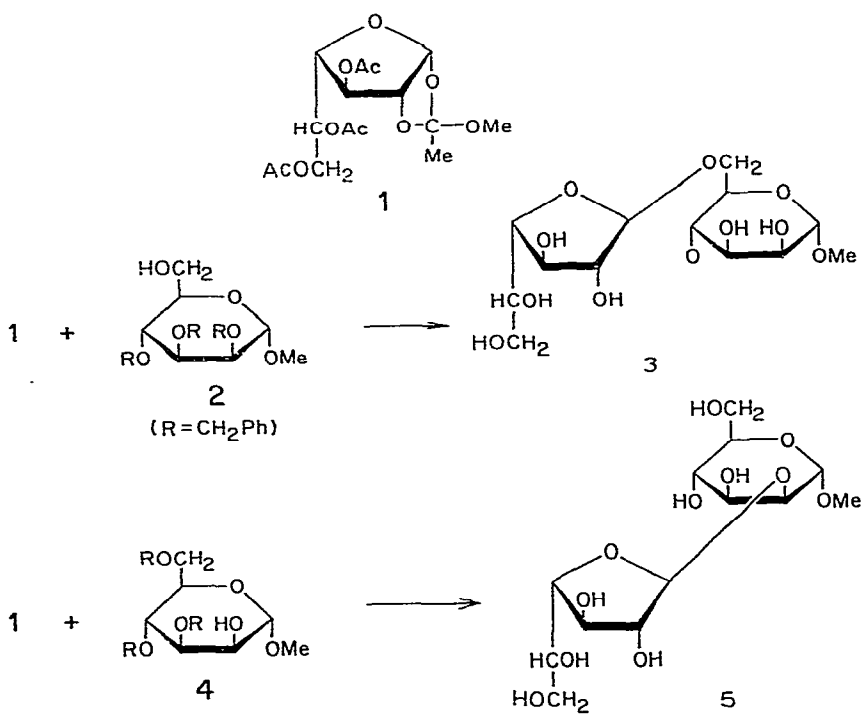
CHEMICAL SHIFTS OF C-1′ FOR VARIOUS STRUCTURES CONTAINING β-D-GALACTOFURANOSYL UNITS ATTACHED TO α-D-MANNOPYRANOSYL OR β-D-GALACTOFURANOSYL UNITS

Chemical shift, δ	*Chemical structure*
106.5	β-D-Gal*f* of β-D-Gal*f*-(1→3)-Me-α-D-Man*p*
109.7	β-D-Gal*f* of β-D-Gal*f*-(1→6)-Me-α-D-Man*p*
107.7	β-D-Gal*f* of β-D-Gal*f*-(1→2)-Me-α-D-Man*p*
109.3	Single unit of β-D-galactofuranosyl side-chains of D-galacto-D-mannan of *S. schenckii*, attached (1→2), (1→3), or (1→6) to α-D-mannopyranosyl units[24]
109.4	Single unit of β-D-galactofuranosyl side-chains of D-galacto-D-mannan of *T. interdigitale*, attached (1→2), (1→3) and/or (1→6) to α-D-mannopyranosyl units[25]
108.4 (major) 109.2	Approximately 4-unit side-chains of β-D-(1→5)-linked D-galactofuranosyl units in D-galacto-D-mannan of *A. niger*, attached (1→2) or (1→6) to α-D-mannopyranosyl units[26]
109.5 107.6 107.2	D-Galacto-D-mannan of *C. stenoceras* containing a single unit and β-D-(1→6)-linked D-galactofuranosyl side-chains[27]
106.6	β-D-Galactofuranosyl units of D-galacto-D-mannan of *T. cruzi*
106.5	β-D-Galactofuranosyl units of polysaccharide of *C. fasciculata*[1]

and δ 106.5 for that of *Crithidia fasciculata* polysaccharide[4]. A D-galacto-D-mannan from *Aspergillus niger* gave a major C-1 signal at δ 108.4 corresponding to (1→5)-linked β-D-galactofuranosyl units, and a minor one at δ 109.2 arising from the unit concerned in the galactose-to-mannose linkage[26]. Also the D-galacto-D-mannan of *Ceratocystis stenoceras*, which contains some (1→6)-linked β-D-galactofuranosyl units, gave[27] three C-1 signals at δ 109.5, 107.6, and 107.2. Of the three, the signal at δ 109.5 arises, at least partly, from (1→6)-linked β-D-galactofuranosyl units.

Since the structures of the just mentioned polysaccharides were only partly determined, a number of model methyl glycosides of β-D-galactofuranosyl-α-D-mannopyranoses were prepared and their C-1′ shifts measured (Table I), in order to confirm the shift dependence. Variation was found between those of the 2-*O*- (δ 107.7), 3-*O*- (δ 106.5), and 6-*O*-isomer (δ 109.5). (The C-1′ shift of 2-*O*-β-D-galactofuranosyl-α-D-mannopyranose was at δ 107.8, close to that of its methyl glycoside.) These shifts are distinguishable from that of δ 108.4 observed for C-1 of a (1→5)-linked β-D-galactofuranosyl chain. However, the presence of a signal at δ 109.7 is ambiguous and could signify the presence of β-D-galactofuranosyl units attached to the 6-positions of β-D-galactofuranosyl[27] or α-D-mannopyranosyl units, or both. It, thus, appears that the C-1 signal of the D-galacto-D-mannan of *T. cruzi*, which is at δ 106.6, arises from a (1→3)-linkage between β-D-galactofuranosyl and α-D-mannopyranosyl units.

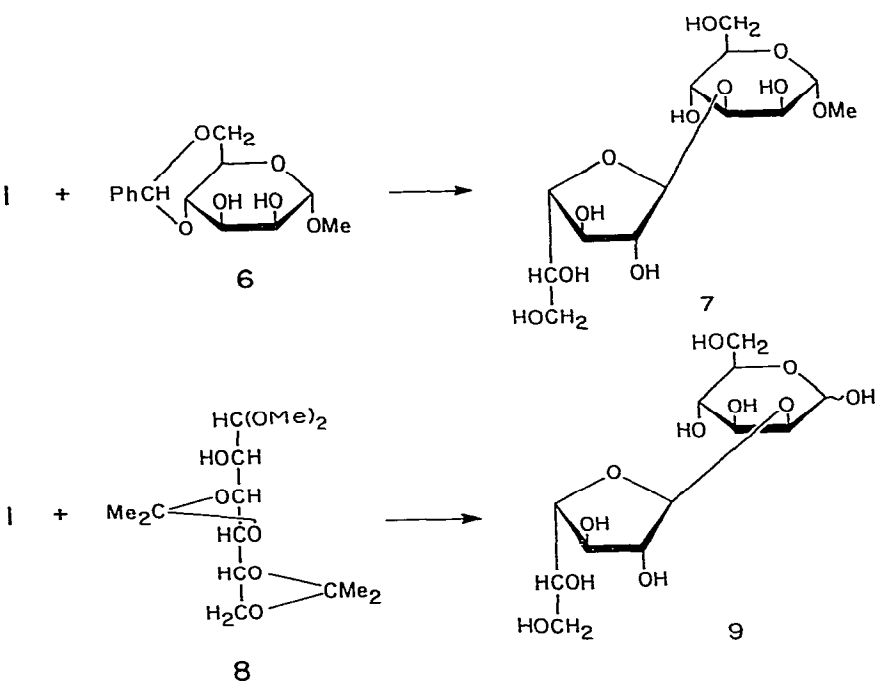
The methyl glycoside of 3-*O*-β-D-galactofuranosyl-α-D-mannopyranose (7)



was prepared by reaction of 3,5,6-tri-*O*-acetyl-α-D-galactofuranose 1,2-(methyl orthoacetate) (**1**) with methyl 4,6-*O*-benzylidene-α-D-mannopyranoside (**6**) in the presence of mercuric bromide. Substitution took place exclusively at OH-3 since the product (C-1′, δ 106.5) had 13 signals and the C-2 and C-4 resonances underwent β-shifts of −3.2 and −1.7 p.p.m., respectively, when compared with those of methyl α-D-mannopyranoside. The C-3 signal was displaced downfield by 5.8 p.p.m.

An attempt to synthesize the 2-*O*-isomer (**5**) by reaction of 3,5,6-tri-*O*-acetyl-α-D-galactofuranose 1,2-(methyl orthoacetate) (**1**) with methyl 3-*O*-acetyl-4,6-*O* benzylidene-α-D-mannopyranoside[31] was unsuccessful. The required material, however, was isolated in low yield following condensation with methyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**4**). It was contaminated by a minor β-D-isomer, which arose from an impurity in the acceptor, but a successful interpretation of ^{13}C-n.m.r. signals was still possible. 2-*O*-Galactosylation occurred since comparison of the C-1 resonance of methyl α-D-mannopyranoside with that of the disaccharide revealed a strong *O*-substitution shift of −2.6 p.p.m., corresponding to substitution adjacent to an axial C-O linkage[32]. The corresponding α-shift of the C-2 signal was +4.8 p.p.m.

2-*O*-β-D-Galactofuranosyl-α,β-D-mannose (**9**) was isolated following reaction of 3,5,6-tri-*O*-acetyl-α-D-galactofuranose 1,2-(methyl orthoacetate) (**1**) with 3,4:5,6-di-*O*-isopropylidene-D-mannose dimethyl acetal (**8**). The C-1′ signal of the α-D anomer was at δ 107.8, close to that of the methyl glycoside. Methyl 6-*O*-β-D-galactofuranosyl-α-D-mannopyranoside (**3**) was obtained in several steps after condensation of 3,5,6-tri-*O*-acetyl-α-D-galactofuranose 1,2-(methyl orthoacetate) (**1**) with methyl 2,3,4-tri-



O-benzyl-α-D-mannopyranoside (**2**). The structure of the disaccharide was confirmed by the ^{13}C-n.m.r. spectrum, which contained 13 signals, and by the absence of a C-6 signal at δ 62.8, corresponding to a free OH-6 group. It was displaced downfield by 5.9 p.p.m.

DISCUSSION

The present data show that, although the D-galacto-D-mannan of *T. cruzi* is different from the major polysaccharide components of *C. fasciculata* and *H. samuelpessoai*, some of the minor components are similar.

The major polysaccharides of *C. fasciculata* are a β-D-(1→2)-linked D-mannopyranan and one having a β-D-(1→3)-linked D-galactopyranan main-chain with some of the units being substituted at O-2 by single D-arabinopyranosyl groups[4]. However, minor amounts of α-D-mannopyranosyl side-chains are present, since degradation to D-mannose was observed following the action of an *exo*-α-D-mannosidase. Also, methylation data are consistent with the presence of β-D-galactofuranosyl nonreducing end-units which gave rise to a minor ^{13}C-n.m.r. signal at δ 106.5, tentatively suggesting the presence of a structure equivalent to that of the *T. cruzi* D-galacto-D-mannan.

The major components of *H. samuelpessoai* are a series of low-molecular-weight oligomers containing β-D-(1→2)-linked D-mannopyranosyl residues and a branched-chain D-glucurono-D-xylan with D-glucopyranosyluronic acid nonreducing end units connected to α- and β-linked D-xylopyranosyl units[3]. However, the surface of the cell in the flagellar regions reacts[28] strongly with concanavalin A, which is consistent with α-D-mannopyranosyl nonreducing end-units, or 2-*O*-substituted α-D-mannopyranosyl units, or both. This side-chain structure was confirmed by the liberation of D-mannose from polysaccharide extracts of flagella by the action of an *exo*-α-D-mannosidase[3]. It is also of interest that flagellar suspensions of both *H. samuelpessoai* and *C. fasciculata* give rise to resistance against infection with *T. cruzi*, the effect being stronger with the former preparation[29]. Thus, comparison of the chemical structures of the polysaccharides from the three protozoans shows partial resemblances and an inconclusive result in respect to the exact role of polysaccharides, if any, in the immunostimulation process. Perhaps a more definite conclusion would be reached if laboratory animals were injected with purified polysaccharide preparations of *C. fasciculata* and *H. samuelpessoai*, and their resistance tested towards infection with *T. cruzi*. Success in immunization has been achieved by use of a cell-surface glycoprotein from *T. cruzi* that does not appear to carry the antigen causing autoimmune antibody-formation[30].

EXPERIMENTAL

Microorganism and growth conditions. — *Trypanosoma cruzi* Y strain was grown on Warren's and Chang's media at 28° in stationary culture. After 5 days, the cells were centrifuged and washed three times with a saline solution.

Isolation of polysaccharide. — The sugar compositions of cells of *T. cruzi* were determined by hydrolysis with 0.5M sulfuric acid for 18 h at 100°, followed by sodium borohydride reduction and conversion of alditols to their acetates. These gave g.l.c. peaks[33] corresponding to acetates of mannitol, galactitol, glucitol, and inositol in ratios of 18:9:2:71 (Warren's medium) and 9:6:1:84 (Chang's medium).

T. cruzi cells (1.5 g), grown on Warren's medium, were extracted for 6 h in 6% aqueous potassium hydroxide (20 mL) at 100°, the suspension was neutralized with acetic acid and then centrifuged, and the supernatant evaporated to a small volume. Excess ethanol was added and the resulting precipitate filtered off, washed with ethanol, and dried, providing 122 mg of crude polysaccharide.

The crude polysaccharide was dissolved in water and the solution treated with mixed Amberlite IR-120 (H^+) and Dowex 1-X8 (OAc^-) ion-exchange resins to remove nucleic acids, and the suspension was filtered and lyophilized. The product (15 mg) was precipitated from water with an excess of 2:1 (v/v) acetone–ethanol and had $[\alpha]_D^{25}$ +10° (*c* 0.1, water). Hydrolysis gave acetates of mannitol, galactitol, glucitol, and inositol with a peak ratio of 25:11:2:12 (for the polysaccharide preparation from cells grown on Chang's medium the ratio was 42:21:3:32).

Methylation of polysaccharides. — The purified polysaccharide (5 mg) was methylated by the method of Haworth[12], and the methylation was completed with the procedure of Kuhn *et al.*[13]. The per-*O*-methyl derivative was converted to *O*-methylalditol acetates, which were examined by g.l.c.–m.s.[14]. The products were characterized by their mass spectra, obtained by electron impact, and by their retention times in a 30-meter, glass-capillary column containing SP2250(OV-17), maintained at 170° for the first 30 min, and then programmed at 2°/min up to 220°, maximum. The acetate fragments observed were (retention times in minutes and percentages of the total peak area): 2,3,4,6-tetra-*O*-methylmannitol (and glucitol) (26.0, 26%); 2,3,5,6-tetra-*O*-methylgalactitol (27.4, 9%); 2,3,4,6-tetra-*O*-methylgalactitol (29.2, 1%); 3,4,6-tri-*O*-methylmannitol (37.4, 17%); 2,3,6-tri-*O*-methylgalactitol (39.3, 5%); 2,3,6-tri-*O*-methylglucitol (40.0, 27%); 4,6-di-*O*-methylmannitol (46.4, 3%); 3,4-di-*O*-methylmannitol (48.3, 4%); 2,3-di-*O*-methylglucitol (49.0, 3%); and 2,4-di-*O*-methylgalactitol (50.5, 4%). The high proportion of 2,3,6-tri-*O*-methylglucitol cannot be explained satisfactorily.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectrum of the polysaccharide was obtained as previously described[34] from a sample (10 mg) of nucleic acid-free product, dissolved in deuterium oxide (1 mL) at 70°, and contained in a coaxial-cylinder cell within a 12-mm diameter tube. Chemical shifts are expressed as δ relative to an external standard of tetramethylsilane, the shift of which was determined in a separate experiment. Determinations for chloroform-*d* solutions were carried out at ambient temperatures, shifts being based on internal tetramethylsilane.

Methyl 6-O-β-D-galactofuranosyl-α-D-mannopyranoside (3). — 3,5,6-Tri-*O*-acetyl-α-D-galactofuranose 1,2-(methyl orthoacetate) (1) was prepared from 2,3,5,6-tetra-*O*-acetyl-β-D-galactofuranosyl chloride by the method of Kochetkov *et al.*[35], except that silica gel was used to remove traces of 2,6-lutidine from the product[36].

The halide was prepared from 1,2,3,5,6-penta-*O*-acetyl-β-D-galactofuranose by the action[37] of acetic acid–hydrogen chloride. The orthoester (0.40 g) was added to a solution of methyl 2,3,4-tri-*O*-benzyl-α-D-mannopyranoside[38] (**2**, 0.75 g) and mercuric bromide (50 mg) in nitromethane (2 mL), and the mixture maintained for 1 h at 100°. After addition of pyridine (0.2 mL), the solution was evaporated to a syrup. This was *O*-deacetylated with sodium methoxide in methanol and the product *O*-debenzylated by hydrogenation in the presence of palladium in acetic acid. The product was fractionated by cellulose column chromatography in 10:1 (v/v) acetone–water, which removed methyl hexoside, and 7:1 (v/v) acetone–water, which removed the methyl glycoside of the disaccharide (**3**, 122 mg), $[\alpha]_D^{25}$ −13° (*c* 1.2, water). On a paper chromatogram prepared with 40:11:19 (v/v) 1-butanol–ethanol–water (solvent *A*) and sprayed with ammoniacal silver nitrate, it had (R_{Rib}) 0.76; ^{13}C-n.m.r. (D_2O at 70°): δ 109.7 (C-1'), 102.6 (C-1), 84.8, 82.7, 78.5, 73.3, 72.6, 72.3, 71.6, 68.8, 68.7, 64.6, and 56.6.

Anal. Calc. for $C_{13}N_{24}O_{11}$: C, 43.82; H, 6.79. Found: C, 43.31; H, 6.58.

Methyl 2-O-β-D-galactofuranosyl-α-D-mannopyranoside (**5**). — Orthoester **1** (2.0 g) was similarly treated, but with a 4-h reaction period, with methyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside[39] [**4**, 3.5 g; ^{13}C-n.m.r. ($CDCl_3$ at 32°): δ 100.6 (C-1) and 101.0 (minor, 16%, β-D anomer)]. The product was chromatographed on a cellulose column in 1-butanol containing 5% of water as eluent to give a mixture (89 mg) containing ~60% of the α-D-glycoside and ~40% of the β-D-glycoside.

Anal. Calc. for $C_{13}H_{24}O_{11}$: C, 43.82; H, 6.79. Found: C, 43,36; H, 6.43.

α-D-Glycoside. P.c. (solvent *A*): R_{Rib} 0.91; ^{13}C-n.m.r.: δ 107.7 (C-1'), 100.0 (C-1), 84.9 (C-4'), 82.7 (C-2'), 78.3 (C-3'), 76.6 (C-2), 74.4, 72.5, 71.5, 69.0, 64.5 (C-6'), 62.6 (C-6), and 56.5 (OCH_3).

β-D-Glycoside. P.c. (solvent *A*): R_{Rib} 0.81; ^{13}C-n.m.r.: δ 109.6 (C-1'), 103.3 (C-1), 85.1 (C-4'), 82.6 (C-2'), 78.5 (C-3'), 78.2 (C-5), 76.9 (C-2), 73.9, 72.6, 69.2, 64.5 (C-6'), 62.9 (C-6), and 58.7 (OCH_3).

Methyl 3-O-β-D-galactofuranosyl-α-D-mannopyranose (**7**). — Orthoester **1** (0.40 g) was condensed with methyl 4,6-*O*-benzylidene-α-D-mannopyranoside[40] (**6**, 0.30 g) as described for the synthesis of **3**. The product was *O*-deacetylated and the *O*-benzylidene groups removed with 80% aqueous acetic acid for 30 min at 100°. The mixture was chromatographed on a cellulose column. 1-Butanol containing 5% of water eluted **6** and 1-butanol containing 10% of water eluted **7** (61 mg), $[\alpha]_D^{25}$ −37° (*c* 0.6, water); p.c. (solvent *A*): R_{Rib} 0.94; ^{13}C-n.m.r. (D_2O at 70°): δ 106.5 (C-1'), 102.4 (C-1), 84.9 (C-4'), 82.9 (C-2'), 78.6 (C-3'), 77.6 (C-3), 74.3 (C-5), 72.5 (C-5'), 68.6 (C-2), 66.9 (C-4), 64.5 (C-6'), 62.7 (C-6), and 56.5 (OCH_3).

Anal. Calc. for $C_{13}H_{24}O_{11}$: C, 43.82; H, 6.79. Found: C, 43.40; H, 6.83.

2-O-β-D-Galactofuranosyl-α,β-D-mannose (**9**). — A mixture of orthoester **1** (1.5 g) and 3,4:5,6-di-*O*-isopropylidene-D-galactose dimethyl acetal[41] (**8**, 1.5 g) in nitromethane (4 mL) containing mercuric bromide (80 mg) was maintained for 3 h at 100°. Pyridine (0.1 mL) was then added, the solution evaporated, and the residue dissolved in 0.1M sodium methoxide in methanol (3 mL). After 1 h, the solution was

evaporated and the product treated with barium hydroxide (2.0 g) in water (100 mL) for 3 h at 100° to destroy reducing sugars. The solution was neutralized with carbon dioxide, the suspension filtered, and the filtrate de-ionized with mixed ion-exchange resins. The solution was evaporated and the resulting syrup partially hydrolyzed, to remove *O*-isopropylidene and dimethyl acetal groups, in 80% aqueous acetic acid (10 mL) for 30 min at 100°. The product was fractionated on a cellulose column with 7:1 (v/v) acetone–water as eluent in order to free the resulting disaccharide from D-mannose to give syrupy **9** (115 mg), $[\alpha]_D^{25}$ −30° (*c* 0.8, water); p.c. (solvent A): R_{Gal} 1.0; ^{13}C-n.m.r. for preponderant α-D anomer; (D_2O at 70°): δ 107.8 (C-1′) and 93.2 (C-1).

Anal. Calc. for $C_{12}H_{22}O_{11}$: C, 42.10; H, 6.48. Found: C, 41.95; H, 6.81.

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