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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE FROM *Streptococcus pneumoniae* TYPE 18F

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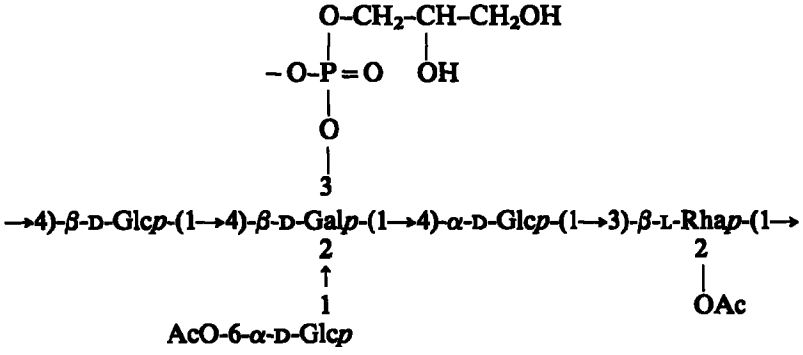
ABSTRACT

The structure of the capsular polysaccharide elaborated by *Streptococcus pneumoniae* type 18F (S18F) has been investigated by using n.m.r. spectroscopy, methylation analysis, and characterisation of oligosaccharides obtained on partial hydrolysis. It is concluded that the polysaccharide is composed of pentasaccharide repeating-units having the following structure.



In this structure, the absolute configuration of the glycerol phosphate moiety has not been determined, but is assumed to be D-glycerol 1-phosphate (*sn*-glycerol 3-phosphate). The location of an *O*-acetyl group at O-6 of the terminal α-D-glucopyranosyl groups is tentative only.

INTRODUCTION

Streptococcus pneumoniae group 18 consists of the types 18F, 18A, 18B, and 18C[1], each of which elaborates its own, type-specific capsular polysaccharide. Early studies of the polysaccharide from type 18F (S18F), referred to as S XVIII, by

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Heidelberger and his co-workers, are summarised in ref. 2. They showed that it was composed of D-glucose, D-galactose, L-rhamnose, glycerol, phosphate, and *O*-acetyl in the approximate proportions 3:1:1:1:1:1. They further demonstrated that the polymer was not of the teichoic acid type, but a polysaccharide proper with glycerol 1-phosphate attached as a substituent.

The structure of the polysaccharide elaborated by type 18C (S18C) has been determined by Lugowski and Jennings[3]. It consists of pentasaccharide repeating-units with the same composition as indicated for 18F by the earlier studies. The antigenic formulas[1] for 18F (18a, 18b, 18c, 18f) and for 18C (18a, 18b, 18c, and 18e) indicate that 18F and 18C have closely related structures. We now report structural studies of S18F.

RESULTS AND DISCUSSION

Sugar and phosphate analyses on S18F confirmed the results of the previous studies[2]. The ^{13}C-n.m.r. spectrum of *O*-deacetylated S18F showed, *inter alia*, signals for anomeric carbons at δ 103.4 ($^1J_{C,H}$ 166 Hz), 102.7 ($^1J_{C,H}$ 160 Hz), 101.3 ($^1J_{C,H}$ 162 Hz), 97.9 ($^1J_{C,H}$ 174 Hz), and 95.8 ($^1J_{C,H}$ 174 Hz), indicating a pentasaccharide repeating-unit with two α-linked and three β-linked pyranosidic sugar residues. This was confirmed by the ^{1}H-n.m.r. spectrum of *O*-deacetylated S18F, which showed, *inter alia*, signals for five anomeric protons at δ 5.40 (1 H, $J_{1,2}$ ~4 Hz), 5.13 (1 H, $J_{1,2}$ ~4 Hz), 4.89 (1 H, not resolved), 4.81 (1 H, $J_{1,2}$ 7.8 Hz), and 4.71 (1 H, $J_{1,2}$ 7.9 Hz). These results demonstrate that the L-rhamnopyranosyl residue and two other sugar residues are β-linked and that the remaining two residues are α-linked. Signals assigned to C-6 of the D-glucopyranosyl and D-galactopyranosyl residues at δ 62.0, 61.7, 61.6, and 60.9 indicate that all the primary positions in the sugars are unsubstituted. The ^{1}H-n.m.r. spectrum of S18F, in addition, showed signals for two

TABLE I

METHYLATION ANALYSIS OF S18F AND ITS HYDROLYSIS PRODUCTS[a]

Sugar[b]	T[c]	*Mole %*				
		A	B	C	D	E
1,2,4,5,6-Rha	0.36		15[d]		5[d]	2[d]
2,4-Rha	0.92	19				10
1,3,5,6-Gal	0.93			14[d]		
2,3,4,6-Glc	1.00	24	85	86	44	31
2,3,6-Glc	1.26	46			27	38
3,6-Gal	1.47	6			24	19
6-Gal	1.64	5				

[a] *A*, Native polysaccharide; *B*, $NaBD_4$-reduced disaccharide; *C*, $NaBD_4$-reduced trisaccharide; *D*, $NaBD_4$-reduced pentasaccharide; *E*, $NaBD_4$-reduced decasaccharide. [b] 2,4-Rha = 2,4-di-*O*-methyl-L-rhamnose, *etc.* [c] Retention time of the corresponding alditol acetate on an HP-54 column, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [d] Deuterium-labelled at C-1.

O-acetyl groups, at δ 2.17 (3 H) and 2.15 (3 H). The corresponding signals in the ^{13}C-n.m.r. spectrum appeared at δ 21.7 and 21.6.

Methylation analysis of S18F gave the sugars listed in Table I, column *A*. The D-glucose derivatives were identified from their mass spectra and retention times in g.l.c., the latter being the same as those of the authentic samples but different from those of the corresponding D-galactose derivatives. Thus, as three D-glucose residues were accounted for, the two slowest components were concluded to be D-galactose derivatives. The results demonstrate that the pentasaccharide repeating-unit of S18F contains one terminal D-glucopyranosyl group, one L-rhamnopyranosyl residue linked through O-3, and two D-glucopyranosyl residues linked through O-4. The D-galactose derivatives obtained indicate that the D-galactosyl residue is linked through O-2 and O-4, and, further, that the glycerol phosphate residue is linked to O-3 of the same residue but has been partially split off during the methylation.

On dephosphorylation of S18F by treatment with aqueous 48% hydrogen fluoride, extensive cleavage of glycosidic linkages also occcurred. The hydrolysate thus obtained was fractionated by gel-permeation chromatography. The smallest oligosaccharide was a disaccharide composed of D-glucose and L-rhamnose. Methylation analysis of its alditol (Table I, column *B*) demonstrated that D-glucose was terminal and L-rhamnose linked through O-3. The alditol showed, *inter alia*, signals in the ^{1}H-n.m.r. spectrum at δ 5.17 (1 H, $J_{1,2}$ 3.4 Hz) and 1.25 (3 H, $J_{5,6}$ 6.4 Hz). The combined evidence demonstrates that the disaccharide has structure **1**.

α-D-Glc*p*-(1→3)-L-Rha*p*
1

A trisaccharide, containing D-glucose and D-galactose in the ratio 2:1, was also obtained. Hydrolysis of its alditol, prepared by reduction with sodium borodeuteride, gave, *inter alia*, galactitol, deuterated at C-1. Methylation analysis (Table I, column *C*) showed that the D-galactosyl residue was linked through O-2 and O-4 and that the two D-glucopyranosyl residues were terminal. The ^{1}H-n.m.r. spectrum of the trisaccharide contained signals for anomeric protons at δ 5.45 (0.4 H, $J_{1,2}$ 3.4 Hz), 5.34 (0.5 H, $J_{1,2}$ 3.9 Hz), 5.09 (0.4 H, $J_{1,2}$ 3.9 Hz), 4.75 (0.5 H, $J_{1,2}$ 7.8 Hz), and 4.66 (1 H, $J_{1,2}$ 8.1 Hz). These signals demonstrate the presence of one α- and one β-linked D-glucopyranosyl residue, in addition to the reducing D-galactopyranose group. The signal for H-1 in the α-D-glucopyranosyl group is split but not that of the β-D-glucopyranosyl group, indicating that the former is linked to the 2-position of the reducing D-galactopyranose residue. In agreement with this, the α-D-glucopyranosyl H-1 signal in the spectrum of the alditol is moved to δ 5.21 (1 H, $J_{1,2}$ 3.4 Hz), whereas that of the β-D-glucopyranosyl group at δ 4.63 (1 H, $J_{1,2}$ 7.3) is virtually unaffected. The trisaccharide consequently has structure **2**.

β-D-Glc*p*-(1→4)-D-Gal*p*
2
↑
1
α-D-Glc*p* **2**

A pentasaccharide, containing D-glucose, D-galactose, and L-rhamnose in the proportions 3:1:1, was further obtained. Methylation analysis of its alditol (Table I, column *D*) demonstrated that it contained two terminal D-glucopyranosyl groups, a D-glucopyranosyl residue linked through the 4-position, a reducing L-rhamnopyranosyl residue linked through O-3, and a branching D-galactopyranosyl residue linked through O-2 and O-4. The ^{1}H-n.m.r. spectrum of the alditol showed, as expected, that two of the sugar residues were α-linked (δ 5.27, 1 H, $J_{1,2}$ 3.4 Hz; and δ 5.17, 1 H, $J_{1,2}$ 3.4 Hz) and that two were β-linked (δ 4.68, 2 H, $J_{1,2}$ 6.8 Hz). As oligosaccharides **1** and **2** should be integral parts of the pentasaccharide, it should have structure **3**. The signal at δ 5.17 ($J_{1,2}$ 3.4 Hz) in reduced **1** corresponded to two signals in the reducing pentasaccharide, at δ 5.11 ($J_{1,2}$ 3.9 Hz, 0.4 H) and δ 5.09 ($J_{1,2}$ 3.7 Hz, 0.6 H) and has consequently been assigned to the α-D-glucopyranosyl residue linked to the L-rhamnose in **3**.

β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-L-Rha*p*
2
↑
1
α-D-Glc*p*

3

From the results discussed above, two alternative structures (**4** and **5**) for the pentasaccharide repeating-unit of the polysaccharide backbone of S18F may be proposed.

→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→
2
↑
1
α-D-Glc*p*

4

→4)-α-D-Glc*p*-(1→2)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→
4
↑
1
β-D-Glc*p*

5

The highest oligosaccharide characterised was a decasaccharide, terminating with L-rhamnose and containing two repeating units. The isolation of this decasaccharide proves that S18F is a polysaccharide and not a polymer of the teichoic

acid type. N.m.r. spectra and methylation analysis of its alditol (Table I, column *E*) did not discriminate between the alternatives **4** and **5**. In order to do this, a more detailed analysis of the ^{1}H- and ^{13}C-n.m.r. spectra of *O*-deacetylated S18F was performed.

The ^{1}H-n.m.r. signals for most of the ring protons in the pentasaccharide repeating-unit of *O*-deacetylated S18F could be assigned (Table II). This was done by COSY, relayed COSY, double-relayed COSY, and C–H correlation experiments, as indicated in Table II. The five sets of chemical shifts given by the ring protons were assigned to the different sugar residues as follows. The signal at δ 4.89 was assigned to the β-L-rhamnopyranosyl group (β-L-Rha) because of its small coupling constant. The $J_{3,4}$ value is low for the residue giving a signal for H-1 at δ 4.71, demonstrating that this is the β-D-galactopyranosyl residue (β-D-Gal*p*). The remaining β-linked residue, with H-1 at δ 4.81, is consequently β-D-Glc. For one of the α-D-glucopyranosyl residues, with H-1 at δ 5.40, the shifts, relative to α-D-gluco-

TABLE II

^{1}H- AND ^{13}C-N.M.R. CHEMICAL SHIFT DATA[a] FOR *O*-DEACETYLATED S18F

	H/C					
Sugar residue	*1*	*2*	*3*	*4*	*5*	*6*
α-D-Glc*p*-(1- (α-D-GlcII)	5.40 (0.17) 97.9 (174)	3.52[b] (−0.02)	3.90[b,c] (0.18)	3.40[b,d] (−0.02)		
-4)-α-D-Glc*p*-(1- (α-D-GlcI)	5.13 (−0.10) 95.8 (174)	3.64[b] (0.10)	3.97[b,c] (0.25)	3.80[d] (0.38)		
-3)-β-L-Rha*p*-(1-	4.89 (0.04) 101.3 (162)	4.29[b] (0.36) 68.6[e]	3.68[b] (0.09)		3.38[b] (−0.01)	1.32 (0.02) 17.4
-4)-β-D-Glc*p*-(1-	4.81 (0.17) 103.4 (166)	3.35[b] (0.10)	3.71[b,c] (0.21)	3.65[d] (0.23)	3.54[d] (0.08)	
-4)-β-D-Gal*p*-(1- 2 |	4.71 (0.13) 102.7 (160)	4.04[b] (0.53)	4.33[b,c] (0.67)	4.43[b,d] (0.49) 76.5		
O ‖ O–P–O–Glycerol | O⁻	4.02[e] 3.92[e] 67.4[e] (~5 Hz)		3.60[e] 3.70[e] 63.7[e]			

[a]Chemical shift displacements are given in parenthesis and, for anomeric carbons, one-bond coupling constants are given in brackets. [b]COSY. [c]Relayed COSY. [d]Double-relayed COSY. [e]CH-Correlation experiment.

pyranose, are small, except for H-3. For the other α-D-glucopyranosyl residue, with H-1 at δ 5.13, as well as for the β-D-glucose residue, the shifts are much larger. The latter α-D-glucose derivative is therefore assigned to the 4-linked α-D-glucopyranosyl residue (α-D-GlcI) and the former to the α-D-glucopyranosyl group (α-D-GlcII). The assignment of α configuration to the terminal D-glucopyranosyl group is in agreement with structure **4**. The chemical shift for H-3 of α-D-GlcII is possibly affected by the proximity of this proton to the phosphate group. It is also evident from Table II that linkage sites are not readily derived from the ^{1}H-n.m.r. glycosylation shifts.

The sequence of the sugar residues was inferred from the 2D-n.O.e. spectrum of *O*-deacetylated S18F (Table III). In addition to inter-residue contacts, there is generally an n.O.e. contact between an anomeric proton and the proton on the corresponding glycosyloxylated carbon. Protons vicinal to the latter proton may also be close to the anomeric proton. Such additional proton–proton contacts further result in upfield shifts of the proton signals[4]. Thus, α-D-GlcI shows n.O.e. contact with H-2 and H-3 of β-L-Rha and its H-1 signal is shifted upfield by 0.10 p.p.m. An n.O.e. contact between H-1 of β-L-Rha with H-4 of β-D-Glc demonstrates that **4** is the correct structure of the pentasaccharide repeating-unit. An intra-residue n.O.e. contact between H-1 and H-2,3, as expected for a β-L-rhamnopyranosyl residue, is also observed. No cross-peak for an n.O.e. contact between β-L-Rha and α-D-GlcII was observed. An n.O.e. contact between H-1 of α-D-GlcII and H-2 of β-D-Gal is also observed. For β-D-Glc, but not for β-D-Gal, the expected n.O.e. contacts were observed.

In the ^{13}C-n.m.r. spectrum of the *O*-deacetylated S18F, signals of full intensity at δ 63.7 and 67.4 ($^2J_{C,P}$ ~5 Hz) indicated that all phosphate occurs as glycerol 1-phosphate. The proton signals corresponding to the carbon signals at δ 67.4 also appeared at low fields, namely, δ 3.92 and 4.02. The absolute configuration of the glycerol 1-phosphate moiety has not been determined. It most probably derives from cytidine diphosphate glycerol, in which the glycerol phosphate moiety has the 1-D (*sn*-3) configuration[5], and it is assumed that it has the same configuration in the

TABLE III

OBSERVED N.O.E. CONTACTS FROM ANOMERIC PROTONS OF *O*-DEACETYLATED S18F

Anomeric proton	*N.O.e. contacts to*
5.40 (α-D-GlcII)	4.04 (β-D-Gal, H-2), 3.52 (α-D-GlcII, H-2)
5.13 (α-D-GlcI)	4.29 (β-L-Rha, H-2), 3.64 (α-D-GlcI, H-2), 3.68 (β-L-Rha weak)
4.89 (β-L-Rha)	4.29 (β-L-Rha, H-2), 3.68 (β-L-Rha, H-3), 3.71 (β-D-Glc, H-3), 3.63 (β-D-Glc, H-4)
4.81 (β-D-Glc)	4.43 (β-D-Gal, H-4), 3.71 (β-D-Glc, H-3), 3.54 (β-D-Glc, H-5)

TABLE IV

^{1}H-N.M.R. CHEMICAL SHIFTS FOR NATIVE S18F AND THE CHEMICAL SHIFT DISPLACEMENTS RELATIVE TO *O*-DEACETYLATED S18F

	H-1	*H-2*	*H-3*	*H-4*
α-D-Glc*p*-(1-	5.37 (−0.03)	3.51[a] (0.01)	3.90[b] (0.00)	
-4)-α-D-Glc*p*-(1-	5.04 (−0.09)	3.58[a] (−0.06)	3.91[b] (−0.06)	
-3)-β-L-Rha*p*-(1-	5.04 (0.15)	5.63[a] (1.33)	3.85[a,b] (0.17)	3.52[b]
-4)-β-D-Glc*p*-(1-	4.76 (−0.05)	3.32[a] (−0.03)	3.65[b] (−0.06)	
-4)-β-D-Gal*p*-(1-	4.68 (−0.03)	4.00[a] (−0.04)	4.32[b] (−0.01)	

[a]Assigned from COSY. [b]Assigned from relayed COSY.

polysaccharide.

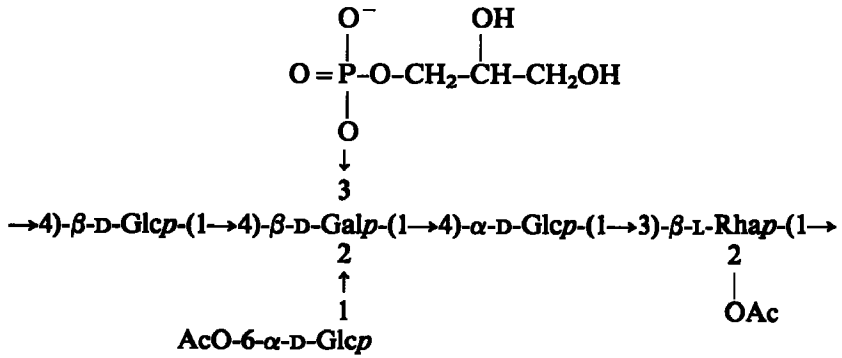
^{1}H-N.m.r. studies of monoacetates of methyl D-gluco- and D-galacto-pyranosides[6] have shown that, for ring protons, the signal for the methine proton of the acetoxylated carbon is shifted 1.1–1.5 p.p.m. downfield and that smaller downfield shifts (~0.20 p.p.m.) are observed for the adjacent protons. The chemical shifts of some protons in native S18F, and their shifts relative to the signals in the corresponding *O*-deacetylated polysaccharide, are given in Table IV. The large downfield shift of H-2 and the smaller downfield shifts of H-1 and H-3 of β-L-Rha demonstrate that one of the *O*-acetyl groups is linked to C-2 of this residue. *O*-Acetyl groups linked to secondary positions in sugars readily migrate, but this is excluded here because of the substitution of O-1 and O-3. The signals of C-2 and C-1 for rhamnose are shifted 1.4 and −1.5 p.p.m., respectively, in the native polysaccharide, also in agreement with an *O*-acetyl group on C-2 of rhamnose.

As no other shifts of this magnitude were observed in the ^{1}H-n.m.r. spectrum, it is concluded that the second *O*-acetyl group is linked to a 6-position. In agreement with this assumption, a signal at 63.9 in the ^{13}C-n.m.r. spectrum of native S18F moves to δ 61.6 on deacetylation.

O-Acetyl groups in polysaccharides have been located by comparison of the methylation analysis data using the Hakomori procedure[7], when these groups are split-off, and a similar analysis using methylation with methyl trifluoromethanesulfonate and 4-methyl-2,6-di-*tert*-butylpyridine in trimethyl phosphate[8] during which they are stable. Methylation analysis of S18F using the latter procedure (Table I, column F) was not entirely successful, as part of the polysaccharide did not dissolve and was not methylated, and as some deacetylation also occurred. Thus, in addition

to the expected 4-*O*-methyl-L-rhamnose, 2,4-di-*O*-methyl-L-rhamnose was obtained in an approximately equal amount. In addition to 2,3,4,6-tetra-*O*-methyl-D-glucose, a substantial amount of 2,3,4-tri-*O*-methyl-D-glucose was obtained, strongly indicating that the second *O*-acetyl group is linked to O-6 of the terminal α-D-glucopyranosyl group.

From the combined evidence, structure **6** is suggested for the pentasaccharide repeating-unit of S18F. In this structure, the configuration of the glycerol 1-phosphate has not been proved but is assumed to be D for biosynthesis reasons, and the location of an *O*-acetyl group at O-6 of the α-D-glucopyranosyl group is tentative.



6

The structure of the pentasaccharide repeating-unit of S18C[3] is, as expected, closely similar. The main difference is that the L-rhamnopyranosyl residue is α-linked. It also contains only one *O*-acetyl group, which was not located.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° (bath) or by flushing with air. For g.l.c., a Hewlett-Packard 5830 instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates were performed on an HP-54 column, using a temperature programme from 200° (3 min) → 250° at 2°/min. Partially methylated alditol acetates were separated on an SE-54 column, using a temperature programme from 150° (2 min) → 220° at 2°/min. G.l.c.-m.s. was performed on a Hewlett-Packard 5970 MSD instrument. Absolute configurations of the sugars were determined according to the procedure of Gerwig *et al.*[9].

N.m.r. spectroscopy. — N.m.r. spectra of solutions in deuterium oxide were recorded at 70° (^{13}C) and 85° (^{1}H) with a JEOL GX-400 instrument. Chemical shifts are reported in p.p.m., using internal acetone (δ 31.07) and internal sodium 3-trimethylsilylpropanoate-d_4 for ^{13}C- and ^{1}H-n.m.r. spectroscopy, respectively. COSY, relayed COSY, NOESY, and C–H correlation spectroscopy experiments were per-

formed according to JEOL standard pulse sequences, and the double-relayed COSY experiment was performed according to Bax and Drobny[10].

For the correlation spectroscopy, a 90° mixing pulse was used. Relaxation times (T_1) were determined by the inversion recovery method. The NOESY experiment was performed using a mixing time of 375 ms.

Preparation of native polysaccharide. — Crude polysaccharide (200 mg) in water (100 mL) was extracted with phenol[11]. The product from the aqueous phase was applied to a column of DEAE-Trisacryl (1.6 × 20 cm), which was irrigated first with water (150 mL) and then with a linear gradient of aqueous sodium chloride (0→0.5M, 500 mL). The fractions were analysed for carbohydrate by the anthrone test, and the middle section of the main fraction was dialysed and freeze-dried to give the pure polysaccharide (70 mg).

O-Deacetylation. — The polysaccharide was treated with 0.1M sodium hydroxide for 16 h at 20°, neutralised, and dialysed. The product in 0.01M citrate buffer of pH 3 was filtered through Dowex 50 (H^+) and purified by chromatography on a column of DEAE-Trisacryl as described above.

Partial hydrolysis. — *O*-Deacetylated S18F (30 mg) was dissolved in aqueous 48% hydrofluoric acid (3 mL) and kept for 44 h at 4°. After removal of the acid, the residue was applied to a column (2.5 × 90 cm) of Bio-Gel P-2 eluted with mM formic acid. The fractionation was monitored by using a differential refractometer. The total amount of recovered mono- and oligo-saccharides was 22 mg.

Methylation analysis. — This was performed as previously described[7]. The methylated products were purified by absorbing them on Sep-Pak C_{18}-cartridges[12]. Native S18F was also methylated with methyl trifluoromethanesulfonate, as described by Prehm[8].

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