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Structural studies on the capsular polysaccharide of *Klebsiella* serotype K40

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The capsular polysaccharide of *Klebsiella* serotype K40 contained D-mannose, D-glucuronic acid, D-galactose, and L-rhamnose in the approximate molar ratios 1:1:1:2. The primary structure of the capsular polysaccharide has been investigated mainly by methylation analysis, periodate oxidation, characterization of oligosaccharides, base degradation reaction, and ^{1}H and ^{13}CNMR spectroscopy. The polysaccharide does not contain any pyruvic acetal or *O*-acetyl substitution. It has a pentasaccharide repeating unit of the following primary structure:

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

The *Klebsiella* genus of the Enterobacteriaceae family has been classified [1, 2] into 81 serologically recognized strains [3]. Recently studies on the capsular polysaccharide of a new strain of *Klebsiella pneumonia* (typed as *Klebsiella* K82) have been reported [4]; the strain was typed as K82 on the grounds that it did not react with antiserum against the known K antigens. *Klebsiella* K40 belongs to the group of 20 chemotypes which also includes K53 and K80. The capsular polysaccharide of this chemotype contains glucuronic acid, mannose, galactose and rhamnose. The primary structures of the capsular antigens of K53 and K80, investigated by Dutton et al. [5, 6], indicate that there is a 1-carboxyethylidine substituent on the rhamnose residue of the polysaccharide of K80, but K53 does not contain any such substituent. The present investigation describes the complete primary structure of K40 capsular polysaccharide.

MATERIALS AND METHODS

Bacterial strain and isolation of the polysaccharide

Klebsiella K40, the serological test strain, was kindly supplied by Prof. S. Stirm of the University of Giessen, FRG. *Klebsiella* K40 was grown on nutrient medium in $D_{1.5}$ agar plates for 48 h at 37 °C, and then for another 48 h at room temperature. The capsular polysaccharide was isolated from the dry bacteria by the phenol/water/Cetavlon method [7]. From 100 large agar plates 28.2 g dry bacteria were obtained, which yielded 1.2 g (4.3%, w/w) of type-40 capsular polysaccharide.

The analytical ultracentrifugation was carried out with a 0.40% (w/v) solution of the polysaccharide in phosphate-buffered physiological saline in the Spinco model E instrument.

The sodium salt of the polysaccharide was converted into the acidic form by passing the aqueous solution over a column of Dowex-50 (H^+), and the equivalent weight of the acidic polysaccharide was determined by conductometric titration with a 0.1 M NaOH.

A portion of the polysaccharide was carboxyl-reduced with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene-sulfonate and sodium borohydride according to the method of Taylor et al. [8].

Qualitative and quantitative analysis of the constituent sugars

The polysaccharide (1%) was hydrolysed with 1 M H_2SO_4 at 100 °C for 24 h, neutralised with $BaCO_3$ and subjected to descending paper chromatography on Whatman no. 1 paper, and the following solutions were used in chromatography: (a) ethyl acetate/pyridine/water (4:1:1, v/v/v) and (b) ethyl acetate/glacial acetic acid/formic acid/water (18:3:1:4, v/v/v). Chromatograms were developed by alkaline silver nitrate reagent.

For quantitative analysis, 5 mg polysaccharide was hydrolysed with 2 ml of 0.5 M H_2SO_4 at 100 °C for 24 h. After neutralization with $BaCO_3$, constituent neutral sugars were analysed as alditol acetates [9] by gas/liquid chromatography (GLC) with the Chemito Gas Chromatograph-3800 using an ECNSS-M column. Glucuronic acid was estimated in the unhydrolysed sample colorimetrically by the carbazole/sulphuric acid method [9, 10]. The carboxyl-reduced product was also similarly analysed.

Methylation analysis

Methylation of the polysaccharide was carried out by the method of Hakomori [11] following the experiment details of Hellerqvist et al. [12]. The polysaccharide (10 mg) was dissolved in dimethylsulfoxide, and freshly prepared methylsulfinyl carbanion was added with constant stirring. Methyl iodide was then added slowly in an ice-cold bath and

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Abbreviation. GLC, gas/liquid chromatography.

the solution was stirred well for several hours. The methylated product was purified by passing it through a Sephadex LH-20 column and eluted with chloroform. A portion of the methylated product was carboxyl-reduced and dideuterated [13, 14] with calcium borodeuteride. After stirring overnight at room temperature, the solution was dialysed against distilled water and lyophilized. The isolated oligosaccharides were also methylated in the same way except that the permethylated product was not dialysed. The purified methylated product was hydrolysed successively with 90% formic acid (100 °C, 3 h) and 0.125 M H_2SO_4 (100 °C, 14 h), and converted to alditol acetates. The products were analysed by GLC and mass spectrometry (MS). Mass spectrometry was performed with the Finningen combined GLC/MS instrument.

Uronic acid degradation [15, 16]

The permethylated polysaccharide (20 mg) was dissolved in a mixture (5 ml) of dimethylsulfoxide and 2,2-dimethoxypropane (20:1) containing a trace of *p*-toluenesulfonic acid. The solution was stirred well under nitrogen. Methylsulfinyl carbanion (2 M, 2.5 ml) was then added, and the mixture was kept at room temperature overnight. It was neutralized with 50% acetic acid then diluted with water and extracted with chloroform. The recovered material was subjected to mild hydrolysis with 50% acetic acid for 1 h at 100 °C. The methylated degraded product was isolated by partition between chloroform and water. The dried product was hydrolysed and the derived alditol acetates were analysed by GLC and mass spectrometry as described under methylation analysis.

Isolation of the oligosaccharides

The K40 polysaccharide was partially hydrolysed with 0.5 M H_2SO_4 at 100 °C (1 mg dissolved in 1 ml acid solution, hydrolysed 2 h for aldobiouronic acid and 1 h for other oligosaccharides). After neutralisation with $BaCO_3$, the acidic oligosaccharides were separated by high-voltage electrophoresis [17] (45 V/cm, 2 h) in pyridine/glacial acetic acid/water (10:4:86) at pH 5.3 using Whatman 3MM paper. The isolated aldobiouronic acid (H2), aldotriouronic acid (H3), and aldotetrauronic acid (H4) were analysed by the usual methods.

Determination of reducing end-group [18]

The reducing end-group of the oligosaccharide was determined according to the method of Morrison. The oligosaccharide (2 mg in 1 ml water) was reduced with $NaBH_4$ (2 mg) overnight. The reduced product was successively treated with 0.1 M HCl (16 h, 100 °C), and then with 5% hydroxyammonium chloride in pyridine (2 ml, boiled at 100 °C, 15 min). The cooled solution was boiled with acetic anhydride (3 ml, 100 °C, 1 h). The reducing end-group was converted to acetylated alditol and the remaining part of the oligosaccharide was available as monomers of acetylated aldononitriles. The chloroform extract of the mixture was analysed by GLC using a OV-17 column.

Periodate oxidation

To the K40 polysaccharide solution (20 mg in 5 ml H_2O) was added sodium metaperiodate solution (0.1 M, 5 ml), and the solution was kept for 100 h at room temperature in the dark. An excess of ethylene glycol was then added to the solution to destroy the excess of added periodate. After dialysis, the product was recoverd by lyophilization. The oxidised polyaldehyde was reduced with sodium borohydride (25 mg), decationised with Dowex-50 (H^+), and evaporated to dryness. The reduced polyol was hydrolysed with 0.5 M H_2SO_4 (100 °C, 8 h), and the products were analysed by GLC as alditol acetates.

NMR spectroscopic analysis

NMR spectroscopic analysis (^1H and ^{13}C) was carried out with the Bruker WM-300 spectrometer with D_2O as the solvent. The dissolution of the polysaccharide in absolute D_2O and subsequent lyophilization were carred out three times to exchange H by ^2H before using it for spectroscopic measurements. In the ^{13}C NMR experiment a solution of the polysaccharide (30 mg/ml D_2O) was run at room temperature at 75.47 MHz using sodium 2,2,3,3-tetradeutero-4,4-dimethyl-4-silapentanoate (TSP) as the external standard. ^1H NMR experiments were carried out with the polysaccharide and its derived oligosaccharides (10 – 20 mg/ml D_2O) using the same external standard at room temperature at 300.13 MHz.

Chromium trioxide oxidation [19]

A mixture of the polysaccharide (10 mg and *myo*-inositol (10 mg) was dissolved in formamide (3 ml), and acetylated with acetic anhydride and pyridine. The isolated freeze-dried product was dissolved in acetic acid (3 ml) and powdered chromium trioxide (200 mg) was added. The solution was heated for 3 h at 50 °C. The mixture was partitioned between water and chloroform. The product isolated from the chloroform extract was evaporated to dryness. The oxidised product was then analysed for the constituent sugars as alditol acetates by GLC. Another portion of the polysaccharide was carboxyl-reduced and then subjected to chromium trioxide oxidation.

Enzymic hydrolysis [20]

The aldobiouronic acid (1 mg) of K40 polysaccharide was dissolved in acetate buffer (pH 4.5, 2 ml) and incubated with β-D-glucuronidase from *Helix pomatia* at 37 °C for 24 h. The liberated sugars were detected by paper chromatography.

RESULTS

Composition

Klebsiella strain K40, grown in nutrient agar plates, yielded 4.5% (w/w) of acidic polysaccharide. Homogeneity of the polysaccharide was tested by measuring the sedimentation coefficient in an analytical ultracentrifuge, where a single peak ($s^\circ_{20} = 3.2 \times 10^{-13}$ s) was obtained. The relative molecular mass of the acidic polysaccharide, determined by conductometric titration, was found to be 840. Optical rotations of the sugars, isolated from the hydrolysate, indicated that mannose, galactose and glucuronic acid had D configuration and rhamnose L. The sugar composition of the polysaccharide is shown in Table 1.

Methylation analysis

The results of methylation analysis are recorded in Table 2. The methylated monomers were analysed by GLC/MS of the

alditol acetates. The dideuterated fragment, obtained from the permethylated glycan after reduction/dideuteration, was identified by the appearance of the peak at $m/z = 263$ in the mass spectra, which was observed instead of the normal peak at $m/z = 261$.

Characterization of oligosaccharides

Some properties of the acidic oligosaccharides obtained from the polysaccharide by partial hydrolysis are shown in Table 3, and the results of methylation analysis are given in Table 2. The dideuterated fragment from 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol was identified by the mass spectral peaks at $m/z = 191$ and 235 instead of normal peaks at $m/z = 189$ and 233.

Uronic acid degradation

The results of uronic acid degradation in the permethylated polysaccharide are shown in Table 2, column VII. It was observed that the β-elimination caused the loss of 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol. This indicated that the 2-substituted rhamnose (one unit) was attached to *O*-4 of the glucuronic acid.

Periodate oxidation

Periodate oxidation of the polysaccharide yielded rhamnose and galactose as the main components in the molar ratios 0.9:1.0. As expected, the results indicated that the branched rhamnosyl and the 3-linked galactosyl residues survived periodate oxidation.

Anomeric configurations

The results of ^{1}H NMR and ^{13}C NMR spectra are summarised in Table 4. The ^{1}H NMR spectrum of the polymer showed the presence of five anomeric protons, of which three were α linkages and two β linkages. From the appearance of the signal at $\delta = 4.64$ ppm ($J = 7.5$ Hz) in the spectrum of H2, a β linkage was assigned to glucuronic acid. Liberation of glucuronic acid by the treatment of the aldobiouronic acid with β-D-glucuronidase also confirmed that glucuronic acid was β-linked. In the spectrum of H3, the anomeric signals at $\delta = 5.22$ ppm and 5.15 ppm were integrated together for 1.5 integrals for protons, and hence it was obvious that the rhamnose was α-linked. The signal at $\delta = 4.85$ ppm ($J = 7.5$ Hz) in the spectrum of H4 was accounted for by the β-linkage of the galactose attached to rhamnose. From the signals at $\delta = 5.05$ ppm ($J = 2.5$ Hz) and 5.32 ppm ($J = 3.0$ Hz) in the spectrum of the polymer, the mannose and the second rhamnose of the repeating unit were deduced to be α-linked.

In accordance with the assignment of anomeric configurations by the ^{1}H NMR spectral data, the signals of the spectrum of the polymer in the anomeric region of the ^{13}C NMR were also assigned.

Table 1. *Sugar composition of* Klebsiella *K40 capsular polysaccharide*
Hexoses were determined by GLC as alditol acetates [9], and hexuronic acid by the carbazole/sulphuric acid method [10]; polysaccharide was carboxyl-reduced by using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and $NaBH_4$ according to the method of Taylor et al. [8]

Polysaccharide	Molar ratios of constituent sugars				
	D-Man	D-Gal	L-Rha	D-GlcA	D-Glc
Native	1.00	1.02	1.75	1.15	–
Carboxyl-reduced	1.00	1.05	1.85	0.25	0.85

Table 3. *Acidic oligosaccharides from* Klebsiella *serotype 40 capsular polysaccharide obtained by partial acid hydrolysis*
H2 indicates aldobiouronic acid; H3, aldotriouronic acid; H4, aldotetrauronic acid. Ratio of reducing/non-reducing hexoses was obtained from the ratio of acetylated alditol/aldononitriles; the GlcA derivative not recorded in GLC analysis

Determination	Oligosaccharides		
	H2	H3	H4
Yield	12.5	8.5	24.4
Molar ratios of sugars:			
L-Rha	0.8	0.9	1.7
D-Gal	–	1.0	1.0
D-GlcA	1.0	1.0	1.0
Reducing-end sugar	Rha	Gal	Rha
Ratio of reducing/non-reducing hexoses	–	1:1	1:1.8
Mobility in paper electrophoresis (relative to GlcA)	0.62	0.46	0.36

Table 2. *Identification and molar ratios of* O-*acetyl*-O-*methyl alditols obtained from* Klebsiella *K40 capsular polysaccharide and its derivatives*
3,4-RhaOH etc. indicates 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol etc. t_r indicates retention time relative to 2,3,4,6-GlcOH ($t_r = 1.00$) and 2,3-GlcOH ($t_r = 5.39$) in GLC on an ECNSS-M column at 170 °C. I, type-40 polysaccharide, permethylated; II, type-40 polysaccharide, permethylated and reduced with $Ca(BD_4)_2$; III, aldobiouronic acid, permethylated; IV, aldotriouronic acid, permethylated; V, aldotetrauronic acid, permethylated; VI, aldotetrauronic acid, permethylated and carboxyl-reduced with $Ca(BD_4)_2$; VII, type-40 polysaccharide, base-degraded

Peracetyl derivative of	t		Ratio of peak integrals obtained in GLC						
	literature	found	I	II	III	IV	V	VI	VII
3,4-RhaOH	0.92	0.90	0.88	0.80	1.00	0.80	1.80	1.80	–
2,3,4,6-ManOH	1.00	1.00	1.00	1.00	–	–	–	–	1.00
3-RhaOH	1.94	1.90	0.80	1.20	–	–	–	–	0.80
2,4,6-GalOH	2.28	2.23	1.00	1.05	–	1.00	1.00	1.00	1.05
2,3,4-GlcOH	2.49	2.48	–	–	–	–	–	0.80	–
2,3-GlcOH	5.39	5.39	–	0.88	–	–	–	–	–

Table 4. *NMR data for* Klebsiella *K40 capsular polysaccharide and its oligosaccharides*
^{1}H NMR spectra were run at 300.13 MHz at room temperature, and ^{13}C at 75.47 MHz, room temperature; TSP was the external standard used. In ^{1}H NMR chemical shifts were measured relative to acetone (δ = 2.22 ppm), and coupling constants, when available, in Hz; in ^{13}C NMR chemical shifts were measured relative to acetone (31.07 ppm)

Compound	^{1}H NMR				^{13}C NMR	
	δ	(J)	ratio of integrals	proton assignment	chemical shift	carbon assignment
	ppm	H_2			ppm	
GlcAβ1 – 2RhaOH	5.18	(2.0)	0.5	α-RhaOH		
aldobiouronic acid	4.64	(7.5)	1.0	β-GlcA		
	4.55		0.6	β-RhaOH		
	1.32	(7.0)	3.0	CH_3 of Rha		
GlcAβ1 – 2Rhaα1 – 3GalOH	5.22		1.5	α-GalOH		
aldotriouronic acid	5.15			α-Rha		
	4.75	(7.0)	0.5	β-GalOH		
	4.60	(7.0)	1.0	β-GlcA		
	1.30	(6.0)	3.0	CH_3 of Rha		
GlcAβ1 – 2Rhaα1 – 3Galβ1 – 2RhaOH	5.20	(2.0)	0.5	α-RhaOH		
aldotetrauronic acid	5.08		1.0	α-Rha		
	4.85	(7.5)	1.0	β-Gal		
	4.56		1.5	β-GlcA		
	4.50			β-RhaOH		
	1.32	(6.0)	6.0	CH_3 of Rha		
– 4GlcAβ1 – 2Rhaα1-3Galβ1 – 2Rhaα1 –	5.32	(3.0)	1.0	α-Man	176.16	C-6 of GlcA
4	5.20	(3.0)	1.0	α-Rha	102.19	β-GlcA
α|	5.05	(2.5)	1.0	α-Rha	101.64	β-Gal
1 Man	4.80	(7.0)	1.0	β-Gal	99.90	α-Man
native polysaccharide	4.48	(7.5)	1.0	β-GlcA	95.71	α-Rha
	1.32	(6.0)	6.0	CH_3 of Rha	95.31	α-Rha
					61.76	C-6 of
					61.23	Gal and Man
					17.79	C-6 of Rha
					17.46	C-6 of Rha

In order to obtain further information on the anomeric configurations, the acetylated products of the native and carboxyl-reduced polysaccharide were subjected to chromium trioxide oxidation [19]. The oxidised products were analysed as alditol acetates for the neutral sugars. The molar ratios of mannose, galactose and rhamnose in the native polysaccharide were 1.00:0.25:1.40, and in the carboxyl-reduced product the molar ratios of mannose, galactose, rhamnose and glucose were 1.00:0.30:1.50:0.25. Galactose was considerably degraded in both the samples, and glucose derived from glucuronic acid in the carboxyl-reduced product was also degraded during oxidation. The survival of mannose and rhamnose indicated that they were all α-linked.

DISCUSSION

The quantitative constituent analysis (Table 1), the results of methylation (Table 2) and NMR spectrosocopy of the polymer (Table 4) indicate that the polysaccharide consists of pentasaccharide repeating units containing one residue each of unsubstituted D-mannose, 4-substituted D-glucuronic acid, 3-substituted D-galactose, 2-substituted L-rhamnose, and 2,4-disubstituted L-rhamnose. The repeating unit must be branched with a chain L-rhamnosyl residue carrying the branch that terminates in a D-mannosyl unit.

The ^{13}C NMR spectrum of K40 polysaccharide showed five signals (Table 4) in the anomeric region with an approximately equal ratio of integrals. The signals at 17.46 ppm and 17.79 ppm were assigned to CH_3 groups of the two rhamnose units. The signal at 176.16 ppm was attributed to C-6 of the D-glucuronic acid residue [21]. In the ^{1}H NMR spectrum of the polysaccharide, five signals were observed for five anomeric protons (Table 4). The ratio of integrals for the signal at δ = 1.32 ppm indicated the presence of six protons in two methyl groups of the two rhamnose units. The absence of signals at δ = 1.5 ppm and 2.2 ppm indicated the absence of pyruvate [22] and acetate [23] in the polysaccharide. Results of chromium trioxide oxidation and the enzymic reaction also support the assigned anomeric configurations. The spectral results also indicated that the repeating unit was a pentasaccharide.

From the sugar composition and other properties (Table 3), and methylation data (Table 2), the following structures of the aldobiouronic acid (H2), aldotriouronic acid (H3), and aldotetrauronic acid (H4) are established:

H2: D-GlcA-(1→2)-L-Rha

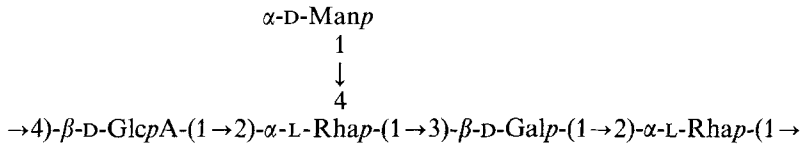
H3: D-GlcA-(1→2)-L-Rha-(1→3)-D-Gal

H4: D-GlcA-(1→2)-L-Rha-(1→3)-D-Gal-(1→2)-L-Rha

The position of mannose in the repeating unit could be located from the results of base degradation reaction. When the permethylated polysaccharide is subjected to base degradation, the 4-substituent on the glycosyluronic acid residue is eliminated along with the uronic acid. The loss of 1,2,5-tri-*O*-

acetyl-3,4-di-*O*-methylrhamnitol (Table 4, column VII) indicated that the 2-substituted rhamnose was attached to c-4 of the glucuronic acid, and hence it could be concluded that the glucuronic acid was linked to the other rhamnose unit in the chain, which was branched with mannose residue occupying the terminal position. The possibility of placing the 4-substituted glucuronic acid in the branch at the rhamnose residue of the chain could also be eliminated on the grounds that there was no loss of 2,3,4,6-tetra-*O*-methylmannitol after the β-elimination reaction. After locating the position of mannose, the sequence of all the sugar residues of the pentasaccharide repeating unit was established.

From the discussion of the results reported in the present investigation, the complete primary structure of the capsular polysaccharide from *Klebsiella* K40 has been established. The K40 glycan is composed of pentasaccharide repeating units having the following structure:



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REFERENCES

1. Nimmich, W. (1968) *Z. Med. Mikrobiol. Immunol. 154*, 117–131.
2. Nimmich, W. & Münter, W. (1975) *Z. Allg. Mikrobiol. 15*, 127–129.
3. Nimmich, W. (1979) *Z. Allg. Mikrobiol. 19*, 343–347.
4. Dutton, G. G. S. & Lim, A. V. S (1983) *Carbohydr. Res. 123*, 247–257.
5. Dutton, G. G. S. & Paulin, M. (1980) *Carbohydr. Res. 87*, 107–117.
6. Dutton, G. G. S. & Karunaratne, D. N. (1984) *Carbohydr. Res. 131*, 103–114.
7. Westphal, O. & Jann, K. (1965) *Methods Carbohydr. Chem. 5*, 83–91.
8. Taylor, R. L. & Conrad, H. E. (1972) *Biochemistry 11*, 1383–1388.
9. Sawardeker, J. S., Sloneker, J. H. & Jeanes, A. (1965) *Anal. Chem. 12*, 1602–1604.
10. Bitter, T. & Muir, M. H. (1962) *Anal. Biochem. 4*, 330–334.
11. Hakomori, S., (1964) *J. Biochem. (Tokyo) 55*, 205–207.
12. Hellerqvist, C. G., Lindberg, B., Svensson, S., Holme, T. & Lindberg, A. A. (1968) *Carbohydr. Res. 8*, 43–55.
13. Kollonitsch, J., Fuchs, O. & Gabör, V. (1955) *Nature (Lond.) 175*, 346.
14. Chakraborty, A. K. (1982) *Makromol. Chem. 183*, 2881–2887.
15. Lindberg, B., Lönngren, J. (1976) *Methods Carbohydr. Chem. 7*, 142–148.
16. Aspinall, G. O. & Rosell, K. G. (1977) *Carbohydr. Res. 57*, c-53–c-26.
17. Niemann, H., Frank, N. & Stirm, S. (1977) *Carbohydr. Res. 59*, 165–177.
18. Morrison, I. M. (1975) *J. Chromatogr. 108*, 361–364.
19. Hoffman, J. & Lindberg, B. (1980) *Methods Carbohydr. Chem. 8*, 117–122.
20. Thurow, H., Choy, Y. M., Frank, N., Niemann, H. & Stirm, S. (1975) *Carbohydr. Res. 41*, 241–255.
21. Joseleau, J. P., Michon, F. & Vignon, M. (1982) *Carbohydr. Res. 101*, 175–182.
22. Choy, Y. M., Dutton, G. G. S, Stephen, A. M. & Yang, M. T. (1972) *Anal Lett. 5*, 675–681.
23. Bebault, G. M., Choy, Y. M., Dutton, G. G. S., Funnell, N., Stephen, A. M. & Yang, M. T. (1973) *J. Bacteriol. 113*, 1345–1347.