*Carbohydrate Research*, 142 (1985) 269–276
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

# STRUCTURE OF THE K95 ANTIGEN FROM *Escherichia coli* O75:K95:H5, A CAPSULAR POLYSACCHARIDE CONTAINING FURANOSIDIC KDO-RESIDUES

THOMAS DENGLER, BARBARA JANN, AND KLAUS JANN
*Max-Planck-Institut für Immunbiologie, D-7800 Freiburg-Zähringen (F.R.G.)*
(Received February 7th, 1985; accepted for publication, March 27th, 1985)

## ABSTRACT

The structure of the K95 antigenic capsular polysaccharide (K95 antigen) of *Escherichia coli* O75:K95:H5 was elucidated by determination of the composition, $^{1}H$- and $^{13}C$-n.m.r. spectroscopy, periodate oxidation, and methylation analysis. The K95 polysaccharide, which contains furanosidic 3-deoxy-D-*manno*-2-octulosonic acid (KDO*f*) residues, consists of →3)-β-D-Rib-(1→8)-KDO*f*-(2→ repeating units, has a molecular weight of ~25,000 (~65 repeating units), and is randomly *O*-acetylated (1 acetyl group per repeating unit at unknown positions).

## INTRODUCTION

Many invasive *Escherichia coli*, notably those causing urinary tract infections, have capsules which are composed of low-molecular-weight acidic polysaccharides[1–3]. Several of these capsular polysaccharides (K antigens) contain 3-deoxy-D-*manno*-2-octulosonic acid (KDO). Thus, the K6, K13, K20, and K23 antigens consist of KDO and ribose[4–7], the K12 antigen of KDO and rhamnose[8], the K14 antigen of KDO and 2-acetamido-2-deoxygalactose[9], and the K15 antigen of KDO and 2-acetamido-2-deoxyglucose[10]. In each of these polysaccharides, KDO is present in the pyranoid form, linked either at positions 5 (K12, K14, and K15 antigens) or 7 (K6, K13, K20, and K23 antigens), and, with the exception of one report[5], is β. In the course of studies of *E. coli* K antigens, we observed unusual n.m.r. spectra for the K95 antigen from uropathogenic and septicaemia-causing *E. coli* O75:K95:H5, indicative of the presence of furanosidic KDO residues. We now report on the K95 antigen.

## RESULTS AND DISCUSSION

The capsular (K95) polysaccharide was isolated from liquid cultures of *E. coli* E3b (O75:K95:H5) by a sequence[6,11] of precipitation with cetyltrimethylammonium bromide (Cetavlon), extraction with aqueous calcium chloride, precipitation with ethanol, and extraction with cold phenol (pH 6.5). The polysaccharide

0008-6215/85/$ 03.30 

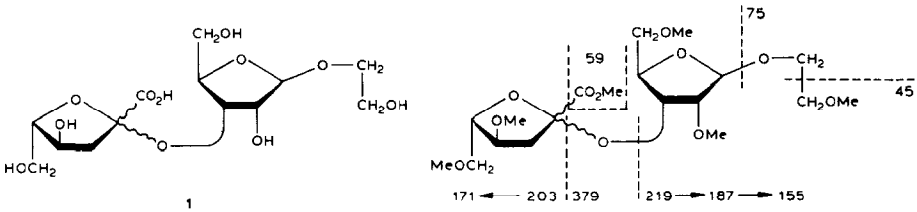
was obtained in a yield of 60 mg/L of culture medium and consisted of 3-deoxy-D-*manno*-2-octulosonic acid (KDO), ribose, and *O*-acetyl in the molar ratios 1:1:1.

In immune electrophoresis, the K95 polysaccharide exhibited a precipitation line with Cetavlon[12] and also with an anti-K95 serum, but not with an anti-O75 serum, indicating serological K95 specificity. The polysaccharide had $[\alpha]_D^{22}$ −30° (*c* 0.7, water) and an average molecular weight of ~25,000, as determined by the method of Yphantis[13].

The K95 polysaccharide was methylated (Hakomori) and then hydrolysed, and the products were converted into the alditol acetates. G.l.c. (140° on ECNSS-M) showed only one major component (*T* 0.93, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol) which m.s. showed to be 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methylribitol and indicated the presence of 3-linked ribose. No derivative of KDO could be detected by this method. Reduction of the carboxyl groups in the methylated polysaccharide[14] prior to hydrolysis did not give a partially methylated polyol derivative of the KDO moiety. Since linkage analysis of KDO by methylation had also previously failed with other polysaccharides[6,8,15], other methods were used (see below).

The K95 polysaccharide was *O*-deacetylated at pH 11 (4°, 16 h), oxidised with periodate, and treated with sodium borohydride. Chromatography on Biogel P-2 gave a major product **1**, eluted with water in the region of di- or tri-saccharides (Kd 0.53). Oligosaccharide **1** (90% yield) contained, in the molar ratio 1:1, ribose and a moiety which, in paper electrophoresis, stained like KDO with the thiobarbituric acid reagent and had $M_{KDO}$ 1.25. Reduction of the latter constituent of **1** with sodium borohydride followed by methylation[16,17] gave a pair of products which, in g.l.c.–m.s., showed peaks of equal intensity exhibiting identical mass spectra. The major fragments were *m/z* 103, 71 (103 − MeOH), 133, 101 (133 − MeOH), 161, 129 (161 − MeOH), 205, and 173 (205 − MeOH). From these data, the products of reduction were taken to be 3-deoxymannonic acid and 3-deoxygluconic acid. Since both were derived from KDO, following cleavage of the C-6–C-7 bond by periodate, they are assumed to be the L isomers. Methylation of **1** gave **2**, which was purified by chromatography on Sephadex LH-20. In g.l.c., **2** had *T* 13.1. C.i.-m.s. of **2**, with ammonia as the reagent gas, gave a peak for the quasi-molecular ion $(M + NH_4)^+$, with *m/z* 456, indicating a molecular weight of 438. The primary fragments obtained in e.i.-m.s. showed **2** to be methylated *O*-(3-deoxy-L-*erythro*-hexulofuranosylonic acid)-(2→3)-*O*-ribosyl-(1→1)-ethane-1,2-diol. This is in agreement with the fact that **1**, from which **2** was derived by methylation, was non-reducing. Fragmentation of the K95 polysaccharide by periodate oxidation indicated that the KDO residues were furanosidic and linked at position 8. Thus, in **1** and **2**, the aglyconic ethylene glycol residue was derived from C-7,8 of the KDO moiety of the K95 polysaccharide.

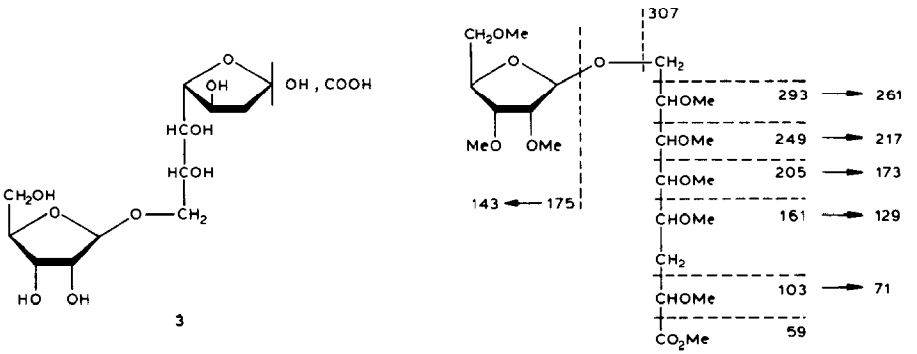
After periodate oxidation of the native polysaccharide, followed by borohydride reduction, ~10% of the starting material was recovered, whereas periodate oxidation of the *O*-deacetylated polysaccharide resulted in complete fragmentation,



1 2

indicating that ~10% of the KDO residues were *O*-acetylated at positions 6 and/or 7.

When the K95 polysaccharide was hydrolysed with aqueous 1% acetic acid (100°, 1 h), preparative electrophoresis of the neutralised hydrolysate revealed one major saccharide (**3**, ~50%) with $M_{KDO}$ 0.75, which was purified by chromatography on Biogel P-2 (Kd 0.57). After reduction and methylation[16,17] of **3**, the product (**4**) was purified by chromatography on Sephadex LH-20. On c.i.-m.s. with ammonia as the reagent gas, **4** gave a peak for the quasi-molecular ion $(M + NH_4)^+$ at *m/z* 516, indicating a molecular weight of 498. The fragmentation pattern is shown in **4**. The data show that **3** was *O*-ribosyl-(1→8)-3-deoxy-D-*manno*-2-octulosonic acid, thus confirming the (1→8) linkage shown by the methylation analysis of **2**.



3 4

The K95 polysaccharide gave a complex $^{13}C$-n.m.r. spectrum (not shown) containing more than 30 signals, the anomeric region ($\delta$ 100–115) and that characteristic of carbonyl groups ($\delta$ 175–185) each containing at least 4 signals. This spectrum indicated that the polysaccharide was randomly *O*-acetylated. The proton-decoupled $^{13}C$-n.m.r. spectrum of the *O*-deacetylated polysaccharide contained 13 well resolved signals, the chemical shifts of which are shown in Table I. Practically the same spectrum was given by **1** (Table I), the main difference being the chemical shifts of the signals of C-6 and C-7 of KDO probably due to structural changes brought about by cleavage of the C-6–C-7 bond of the KDO residues during periodate oxidation.

TABLE I

$^{13}$C-N M R DATA FOR *O*-DEACETYLATED K95 POLYSACCHARIDE (A), *O*-DEACETYLATED K13 POLYSACCHARIDE (B), **1**, AND THE FURANOSE TAUTOMERS OF AMMONIUM KDO

| *Assignment* | | *A* | | **1** | | *B* | | *KDOf*[21] | | *KDOf*[22] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *δ* | *APT*[a] | *δ* | *APT*[a] | *δ* | *APT*[a] | *Major* | *Minor* | *Major* | *Minor* |
| KDO | C-1 | 176.5 | – | 176.4 | – | 174.0 | – | 178.4 | 177.8 | 178.3 | 177.7 |
| | C-2 | 110.0 | – | 110.2 | – | 102.3 | – | 105.1 | 103.8 | 105.1 | 104.1 |
| | C-3 | 45.1 | – | 45.5 | – | 35.1 | – | 45.5 | 44.5 | 45.5 | 44.5 |
| | C-4 | 73.3 | + | 73.2 | + | 68 0 | + | 73.3 | – | 73.5 | 71.0 |
| | C-5 | 87.2 | + | 90.0 | + | 65.8 | + | 86.4 | 85.8 | 86.2 | 86.2 |
| | C-6 | 71.1 | + | 64.3 | – | 70.8 | + | 71.2/72.3 | – | 72.0 | 72 0 |
| | C-7 | 71.6 | + | 61.8 | – | 75.7 | + | 72.3 | – | 72.7 | 72.6 |
| | C-8 | 70.1 | + | 70.3 | + | 59.0 | + | – | – | 64.0 | 63.8 |
| Ribose | C-1 | 108.6 | – | 108.5 | | 104.3 | + | | | | |
| | C-2 | 75.5 | + | 75.8 | + | 73.4 | + | | | | |
| | C-3 | 75.7 | + | 75.8 | + | 74.3 | + | | | | |
| | C-4 | 82.5 | + | 83.0 | + | 81.8 | + | | | | |
| | C-5 | 63.9 | – | 63.1 | – | 65.2 | – | | | | |

[a]The sign of signals obtained in APT spectra[23] is indicated.

The spectrum of the K95 polysaccharide exhibited the same general pattern as that of the K13 polysaccharide (Table I), which also consists of equimolar amounts of KDO and ribose[6]. With respect to the signals of the K13 polysaccharide, which have all been assigned[4], most of the signals of the K95 polysaccharide appear 3–10 p.p.m. downfield. This type of shift difference is observed with furanoses, as compared to pyranoses[18–20]. We therefore analysed the spectrum in comparison with data available for the equilibrium forms of reducing KDO[21,22]. Since the anomeric forms of furanosidic KDO are dificult to differentiate, they have been termed the major (probably $\alpha$) and the minor (probably $\beta$) anomers, according to their relative equilibrium concentrations in aqueous solution. The values reported are included in Table I and support the interpretation that both ribose and KDO are present in the K95 polysaccharide as furanosides. The chemical shift values[4,20] indicate that the ribose is $\beta$.

Assignment of signals in the $^{13}$C-n.m.r. spectra of the K95 polysaccharide, the K13 polysaccharide, and **1** were facilitated through the use of the attached proton test (APT)[23,24]. In spectra recorded with this spin echo technique, signals due to =CH- and $CH_3$ groups are positive and those due to ≡C- and -$CH_2$ groups are negative. As shown in Table I, in the spectra of the K95 and K13 polysaccharides, the signals due to C-1,2,3,8 of KDO and that of C-5 of ribose are negative and all the others are positive. In the spectrum of **1**, the signals that were positive in the spectrum of the K95 polysaccharide, and have been assigned to C-6,7 of KDO, changed sign and were negative. This finding is in agreement with the conversion of the secondary hydroxyl groups at C-6,7 of KDO into primary hydroxyl groups

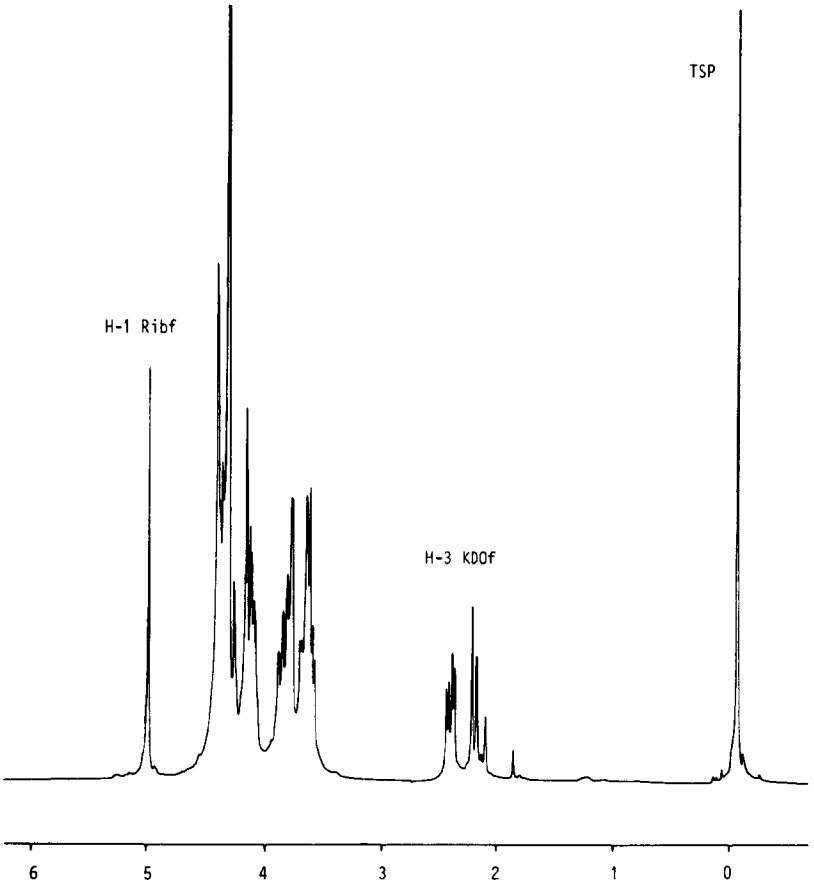



Fig. 1. $^1$H-N.m.r. spectrum (300 MHz) of the *O*-deacetylated K95 polysaccharide [$D_2O$, 70°, external sodium 4,4-dimethyl-4-sila-(2,2,3,3-$^2H_4$)pentanoate].

by periodate oxidation and borohydride reduction of the 8-linked furanosidic KDO residues in the K95 polysaccharide.

The $^1$H-n.m.r. spectrum of the native K95 polysaccharide (not shown) exhibited several signals in the anomeric region between $\delta$ 5.01 and 4.98, as well as multiplets at $\delta$ 2.42 and 2.15. The spectrum of the *O*-deacetylated polysaccharide (Fig. 1, Table II) exhibited distinct signals in these regions, indicating that the spectral complexity is due to variously located *O*-acetyl groups. The anomeric region of the *O*-deacetylated polysaccharide contained one singlet at $\delta$ 4.98. Comparison with n.m.r. data obtained for the K13 polysaccharide (not shown) indicated this signal to be due to H-1 of $\beta$-ribose. The signals of H-3*a* and H-3*e* of KDO are indicative of the anomeric KDO linkages[25]. For methyl $\alpha$-KDO*p*, these values are $\delta$ 1.79 (dd) and 2.04 (dd); and for methyl $\beta$-KDO*p* $\delta$ 1.74 (dd) and 2.38 (dd)[21,25]. Similar values were reported for the sodium and ammonium salts of free reducing KDO and for KDO-containing polysaccharides[21,22,25]. However, chemical and $^{13}$C-

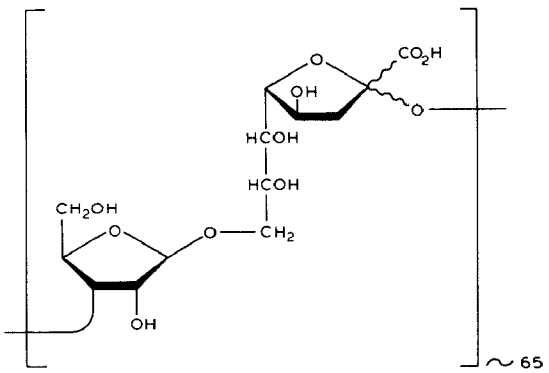
TABLE II

CHEMICAL SHIFTS FOR H-3 OF KDO IN THE $^1$H-N M R SPECTRA OF THE *O*-DEACETYLATED K95 POLYSACCHARIDE (K95 PS) AND **1**

| *Sample* | $\delta^a$ | $J_{3,3}$ | $J_{3,4}$ |
|---|---|---|---|
| K95 PS | 2.19 (dd) | 14.4 | 1.7 |
| | 2.32 (dd) | 14.5 | 6.6 |
| **1** | 2.15 (dd) | 14.6 | 1.9 |
| | 2.40 (dd) | 14.5 | 6.3 |

[a]Internal $Me_4Si$.

n.m.r. data showed the presence of furanosidic KDO in the K95 polysaccharide, but since there are no n.m.r. data avilable on furanosidic α- and β-KDO glycosides, the anomeric form of the furanosidic KDO residues in the K95 polysaccharide cannot be determined. On the basis of the foregoing results, the K95 polysaccharide can be formulated as **5**. From the results of periodate oxidation, it was concluded that only ~10% of the KDO is acetylated at positions 6 and 7. The position of the remaining acetyl groups is not known. The complexity of the n.m.r. spectra, however, indicated a random substitution of the K95 polysaccharide with *O*-acetyl groups. To our knowledge, the capsular K95 polysaccharide of *E. coli* is the only example of a polysaccharide which contains furanosidic KDO.




**5**

The K95 and K13 polysaccharides cross react serologically. The K13 polysaccharide also contains ribose and KDO. The serological cross-reactivity is possibly due to 3-linked β-ribose as common determinant. Comparative immunochemical studies of the K13 and K95 polysaccharides are in progress.

EXPERIMENTAL

*Bacteria and cultivation*. — *E. coli* E3b (O75:K95:H5) was obtained from

Drs. I. and F. Ørskov (Copenhagen) and grown to the late log phase (5–7 h) in a fermenter at 37° in 10-L batches, which contained, per L, $K_2HPO_4 \cdot 3\ H_2O$ (9.7 g), $KH_2PO_4$ (2 g), sodium citrate · 5 $H_2O$ (0.5 g), $MgCl_2 \cdot 7\ H_2O$ (0.1 g), casamino acids (20 g), and the dialysable part of yeast (100 mL from 500 g in 5 L of deionised water).

*Isolation and purification of the capsular polysaccharide.* — The acidic capsular polysaccharide and the bacterial cells were precipitated from the liquid culture by the addition of 1 vol. of aqueous 2% hexadecyltrimethylammonium bromide (Cetavlon). All of the following operations were carried out at 4°. The polysaccharide was extracted from the precipitate with M calcium chloride, purified by three cycles of precipitation from aqueous solution with ethanol (80% final concentration), followed by repeated extractions with cold phenol (80%, buffered to pH 6.5 with sodium acetate)[6,11]. The combined, final aqueous phases were centrifuged for 4 h at 105,000*g* and the supernatant solution was lyophilised. The residue was further purified by chromatography on Sephadex G-50.

*Analytical methods.* — KDO was determined, after hydrolysis of the polysaccharide with aqueous 1% acetic acid for 2 h at 100°, by the thiobarbituric acid assay[26], and ribose was determined, after hydrolysis with 0.1M hydrochloric acid for 48 h at 100°, by g.l.c. of the alditol acetate on ECNSS-M. Acetate was determined[27] by g.l.c. on Porapack QS. For the quantification of the components, $^1H$- and $^{13}C$-n.m.r. spectroscopy of the polysaccharide was also used. High-voltage paper electrophoresis was performed on Whatman No. 1 paper (analytical runs) or on Schleicher and Schüll 2043b paper (preparative runs) at pH 5.3 for 90 min with 42 V/cm. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. G.l.c. was performed with a Varian Aerograph Series 1400 instrument, equipped with an autolinear temperature programmer and a Hewlett–Packard 3380 integrator, and g.l.c.–m.s. was performed with a Finnigan MAT 1020B automatic system at 70 eV on a CB CP SIL 5 (25 m × 0.25 mm) column, using helium as the carrier gas. N.m.r. spectra were recorded with a Bruker WM 300 spectrometer in the F.t. mode at 70° ($^1H$) and 33° ($^{13}C$) [external sodium 4,4-dimethyl-4-sila-(2,2,3,3-$^2H_4$)pentanoate]. The $^{13}C$ values were corrected (−1.31 p.p.m.) by using 1,4-dioxane ($\delta$ 67.4 based on $Me_4Si$), and the $^1H$ values by −0.07 p.p.m. (1,4-dioxane signal at $\delta$ 3.7).

O-*Deacetylation.* — A solution of the K95 polysaccharide (100 mg) in dilute aqueous ammonium hydroxide (5 mL) of pH 11 was kept for 16 h at 4°, then neutralised, chromatographed on Sephadex G-50, and lyophilised.

*Isolation of* **1**. — A solution of the K95 polysaccharide (100 mg) in 0.05M sodium metaperiodate (10 mL of phosphate-buffered saline, pH 7.5) was left at 4° for 90 h. After the addition of ethylene glycol (0.5 mL), the solution was concentrated to ~2 mL and chromatographed on a column (10 × 900 mm) of Biogel P-2. Fractions containing carbohydrate were combined and concentrated to ~5 mL. Sodium borohydride (100 mg) was added and the mixture was kept at 4° for 16 h. To this solution was added Dowex 50W-X12 to pH 4–5. Methanol was added and

the mixture was concentrated to near dryness *in vacuo*. This process was repeated three times. The residue was taken up in water (~2 mL) and chromatographed on a column (10 × 900 mm) of Biogel P-2. Fractions cotaining carbohydrate were combined, concentrated, and lyophilised. The yield of **1** was 85–90%.

*Isolation of* **3**. — A solution of the K95 polysaccharide (100 mg) in aqueous 1% acetic acid (10 mL) was heated at 100° for 1 h and then concentrated *in vacuo* to ~1 mL. Subsequent high-voltage paper electrophoresis yielded **3**, which was purified by chromatography on a column (10 × 900 mm) of Biogel P-2. The yield of purified **3** was ~50%.

*Methylation of the K95 polysaccharide,* **1**, *and* **3**. — The procedure has been described in detail[8,16,17]. Prior to methylation, **3** was reduced with sodium borohydride.

ACKNOWLEDGMENTS

We thank Dr. K. Himmelspach and Miss H. Kochanowski for the n.m.r. spectra, and Mr. D. Borowiak for the mass spectra.

REFERENCES

1 F. ØRSKOV, I. ØRSKOV, B. JANN, AND K. JANN, *Acta Pathol. Microbiol. Scand., Sect. B*, 79 (1971) 142–152.
2 I. ØRSKOV, F. ØRSKOV, B. JANN, AND K. JANN, *Bacteriol. Rev.*, 41 (1977) 667–710.
3 K. JANN AND B. JANN, *Prog. Allergy*, 33 (1983) 53–79.
4 A. NESZMELYI, K. JANN, P. MESSNER, AND F. UNGER, *J. Chem. Soc., Chem. Commun.*, (1982) 1017–1019.
5 H. J. JENNINGS, K. G. ROSELL, AND K. G. JOHNSON, *Carbohydr. Res.*, 105 (1982) 45–56.
6 W. F. VANN AND K. JANN, *Infect. Immun.*, 25 (1979) 85–92.
7 W. F. VANN, T. SØDERSTRØM, W. EGAN, F. P. TSUI, R. SCHNEERSON, I. ØRSKOV, AND F. ØRSKOV, *Infect. Immun.*, 39 (1982) 623–629.
8 M. A. SCHMIDT AND K. JANN, *Eur. J. Biochem.*, 31 (1983) 509–517.
9 B. JANN, P. HOFMANN, AND K. JANN, *Carbohydr. Res.*, 120 (1983) 131–141.
10 W. F. VANN, personal communication.
11 E. C. GOTSCHLICH, M. REY, C. ETIENNE, W. R. SANDBORN, R. TRIAUS, AND B. CVEJANOVIC, *Prog. Immunobiol. Stand.*, 5 (1972) 485–491.
12 F. ØRSKOV, *Acta Pathol. Microbiol. Scand., Sect. B*, 84 (1976) 319–320.
13 D. A. YPHANTIS, *Ann. N.Y. Acad. Sci.*, 88 (1960) 586–601.
14 B. LINDBERG, *Methods Enzymol.*, 28 (1972) 178–195.
15 B. PREHM, *Ph. D. Dissertation*, University of Freiburg, 1974.
16 K. JANN AND K. RESKE, *Eur. J. Biochem.*, 31 (1972) 320–328.
17 B. LINDBERG AND J. LÖNNGREN, *Methods Enzymol.*, 50 (1978) 3–33.
18 S. J. ANGYAL AND V. A. PICKLES, *Aust. J. Chem.*, 25 (1972) 1695–1710.
19 S. J. ANGYAL AND V. A. PICKLES, *Aust. J. Chem.*, 25 (1972) 1710–1718.
20 K. BOCK AND C. PEDERSEN, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–65.
21 R. CHERNIAK, R. G. JONES, AND D. S. GUPTA, *Carbohydr. Res.*, 75 (1979) 39–49.
22 H. BRADE, U. ZAHRINGER, E. T. RIETSCHEL, R. CHRISTIAN, G. SCHULZ, AND F. M. UNGER, *Carbohydr. Res.*, 134 (1984) 157–166.
23 S. L. PATT, *J. Magn. Reson.*, 46 (1982) 535–539.
24 R. BENN AND H. GUNTHER, *Angew. Chem.*, 95 (1983) 381–411.
25 F. M. UNGER, *Adv. Carbohydr. Chem. Biochem.*, 38 (1981) 323–388.
26 V. S. VARAVDEKAR AND L. D. SASLAW, *J. Biol. Chem.*, 234 (1959) 1945–1950
27 I. FROMME AND H. BEILHARZ, *Anal. Biochem.*, 84 (1978) 347–353.