Carbohydrate Research, 134 (1984) 157–166
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Spectroscopic analysis of a 3-deoxy-D-*manno*-2-octulosonic acid (KDO)-disaccharide from the lipopolysaccharide of a *Salmonella godesberg* Re mutant

HELMUT BRADE*, ULRICH ZAHRINGER, ERNST T. RIETSCHEL,
Forschungsinstitut Borstel, D-2061 Borstel (F.R.G.)

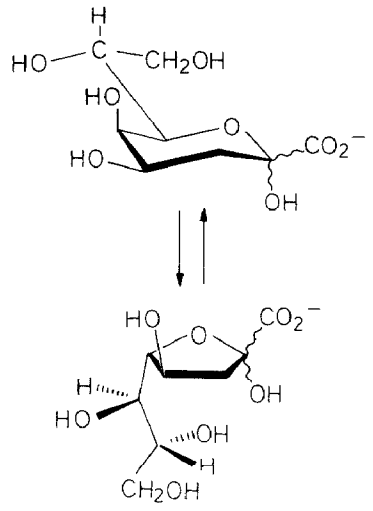
RUDOLF CHRISTIAN, GERHARD SCHULZ, AND FRANK M. UNGER
Sandoz-Forschungsinstitut Wien, A-1235 Wien (Austria)
(Received January 25th, 1984; accepted for publication, March 28th, 1984)

Recently, we have described the isolation, from the lipopolysaccharides (LPS) of several, rough mutants of *Salmonella minnesota*[1], of a disaccharide containing two 3-deoxy-D-*manno*-2-octulosonic acid (KDO) units. Following initial analysis of this material by colorimetric[2] and electrophoretic methods, we concluded that it is composed of a reducing KDO residue, and a (2→4 or 5)-linked, nonreducing KDO group[1]. Presently, we report further studies aimed at the spectroscopic elucidation of the structure of an analogous disaccharide from *S. godesberg*. As will be shown, the spectroscopic data confirm our initial assignments; moreover, they indicate that the interglycosidic linkage of the KDO disaccharide is α-(2→4).

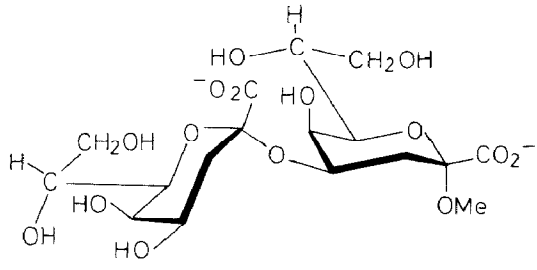
To obtain an appropriate sample, the LPS of *S. godesberg* Re mutant was subjected to mild acid-catalyzed hydrolysis in acetate buffer[1], and the fraction corresponding to a disaccharide (**3**) was isolated by preparative, high-voltage paper electrophoresis (hvpe) under the conditions previously described[1]. In this manner, a compound (yield 11.6 mg) was prepared which, in the different applications of the periodate–thiobarbituric acid (TBA) assay[2], and on hvpe with or without prior hydrolysis, or with reduction prior to hydrolysis, behaved as did the disaccharide from *S. minnesota*[1]. The sample from *S. godesberg* had a neutralization equivalent of 50 μmol, corresponding to 11.9 mg of KDO; by comparison with a calibration curve (prepared with the crystalline ammonium salt of KDO), a value of 11.7 mg was calculated from the TBA color yield obtained after hydrolysis of the disaccharide.

Mass spectrometry. — Disaccharide **3** was reduced with sodium borodeuteride and permethylated according to Hakomori[3]. The purified, permethylated derivative was subjected to combined g.l.c.–m.s. analysis, whereby one peak was observed, the two isomers expected after reduction not being resolved

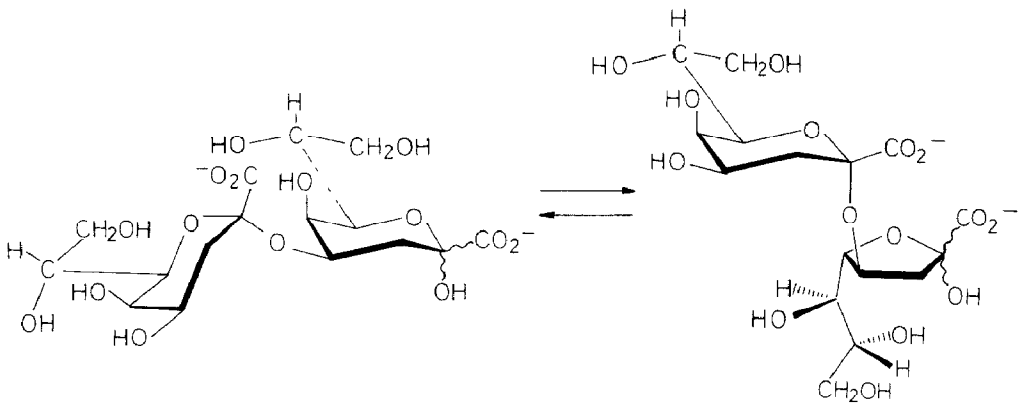
0008-6215/84/$ 03.00



1



2



3

under the conditions used. On chemical-ionization m.s. (c.i.–m.s.) with ammonia, a pseudomolecular ion-peak was observed at *m/z* 633 (615 + NH_4; spectrum not shown). When the same derivative was analyzed by electron-impact-ionization m.s. (e.i.–m.s.), high intensity fragments of *m/z* 177 and 145 were observed among others (Fig. 1). These fragments were interpreted as corresponding to the C-5–C-8 part of the (originally) reducing unit of the KDO-disaccharide, and to loss of methanol therefrom. The prominent occurrence of these fragments, as assigned in Scheme 1, indicated that the interglycosidic linkage of KDO disaccharide **3** is

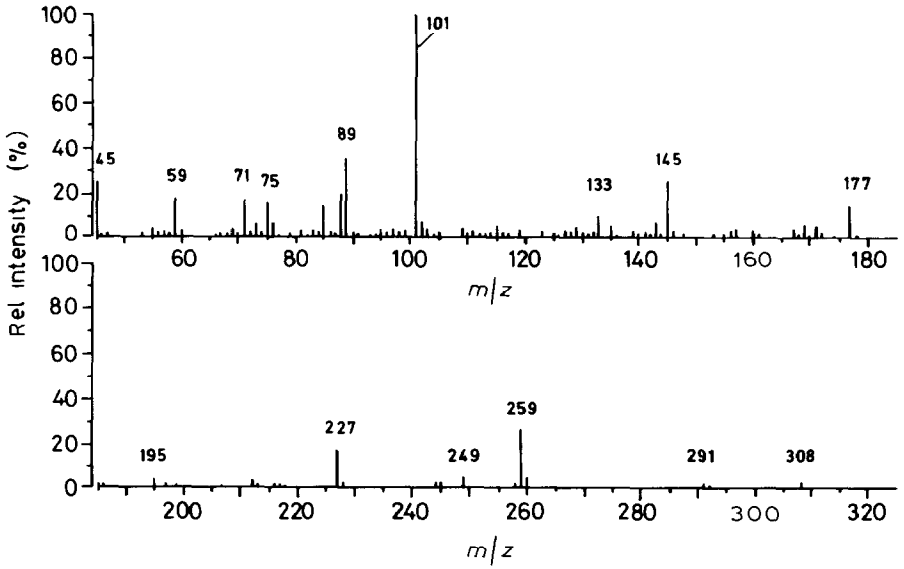
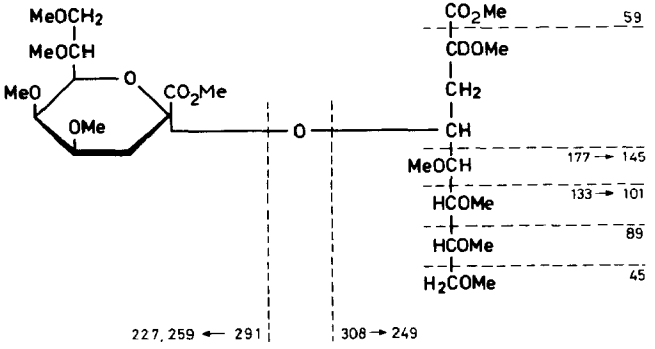


Fig. 1. Mass spectrum of the derivative obtained from the KDO disaccharide **3** *(S. godesberg)* by sequential reduction with sodium boro(^{2}H)hydride and per-*O*-methylation[3].



Scheme 1. Fragmentation pattern corresponding to the mass spectrum shown in Fig. 1.

(2→4). The fragments of *m/z* 291, 259, and 227 were assigned to the nonreducing KDO group and to loss of one or two molecules of methanol therefrom.

For comparison, the heptosyl→KDO disaccharide isolated from the LPS of *S. minnesota* R4 (Rd_2 mutant) was analyzed in parallel. The interglycosidic linkage of this disaccharide has been determined to be α-(1→5) by degradation to a metasaccharinic acid derivative (with sodium periodate–sodium borohydride), followed by treatment with α-D-mannosidase[4]. Upon c.i.–m.s. with ammonia, the derivative obtained by sequential reduction and permethylation of heptosyl→KDO gave a pseudomolecular ion peak at *m/z* 605 (587 + NH_4; spectrum not shown). In the e.i. mass spectrum, prominent peaks were observed at *m/z* 162 and 130. These correspond to the fragment comprising C-1–C-4 of the (originally) reducing KDO

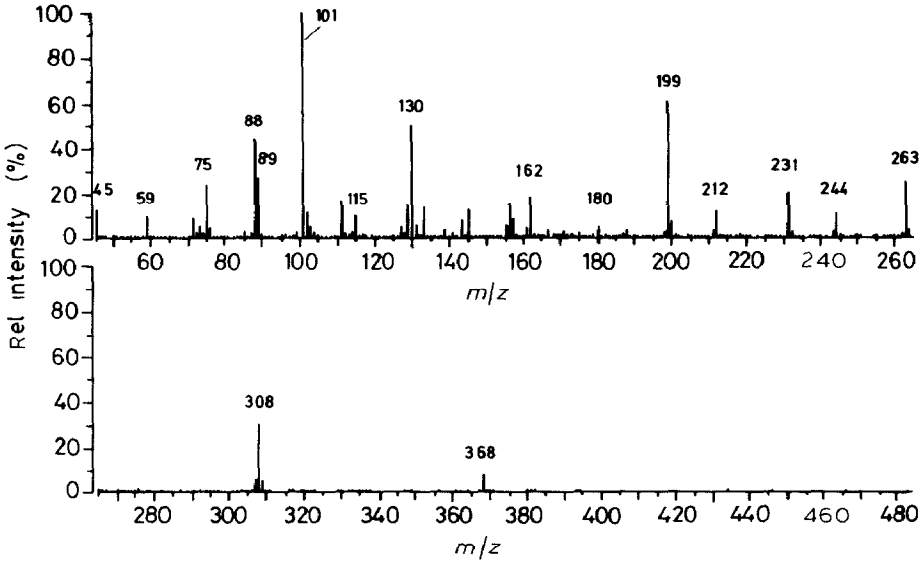
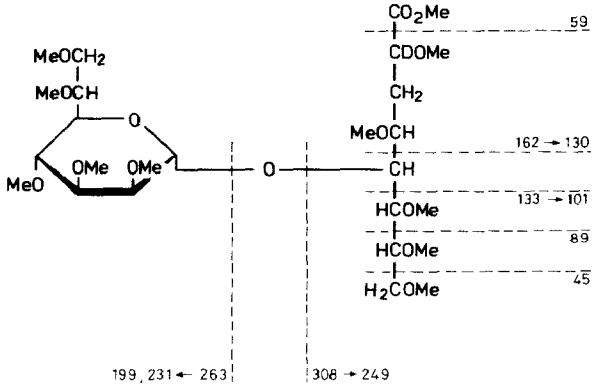


Fig. 2. Mass spectrum of the derivative obtained from heptosyl→KDO[4] by sequential reduction with sodium boro(^{2}H)hydride and per-*O*-methylation[3].



Scheme 2. Fragmentation pattern corresponding to the mass spectrum shown in Fig. 2.

residue and subsequent loss of methanol (Fig. 2 and Scheme 2). The fragments of *m/z* 263, 231, and 199 derived from the nonreducing heptosyl group, which confirms that the interglycosidic linkage of this disaccharide is (1→5). A similar fragmentation pattern was recently observed for another KDO derivative substituted at O-5, namely deuterium-reduced, permethylated 5-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-3-deoxy-D-*manno*-2-octulopyranosonic acid[5].

Thus, the fragment pairs *m/z* 177–145 and 162–130 are characteristic markers for differentiating 4-*O*- and 5-*O*-glycosylated, deuterium-reduced, permethylated KDO residues, respectively. Both fragment pairs do not occur together in the same spectrum.

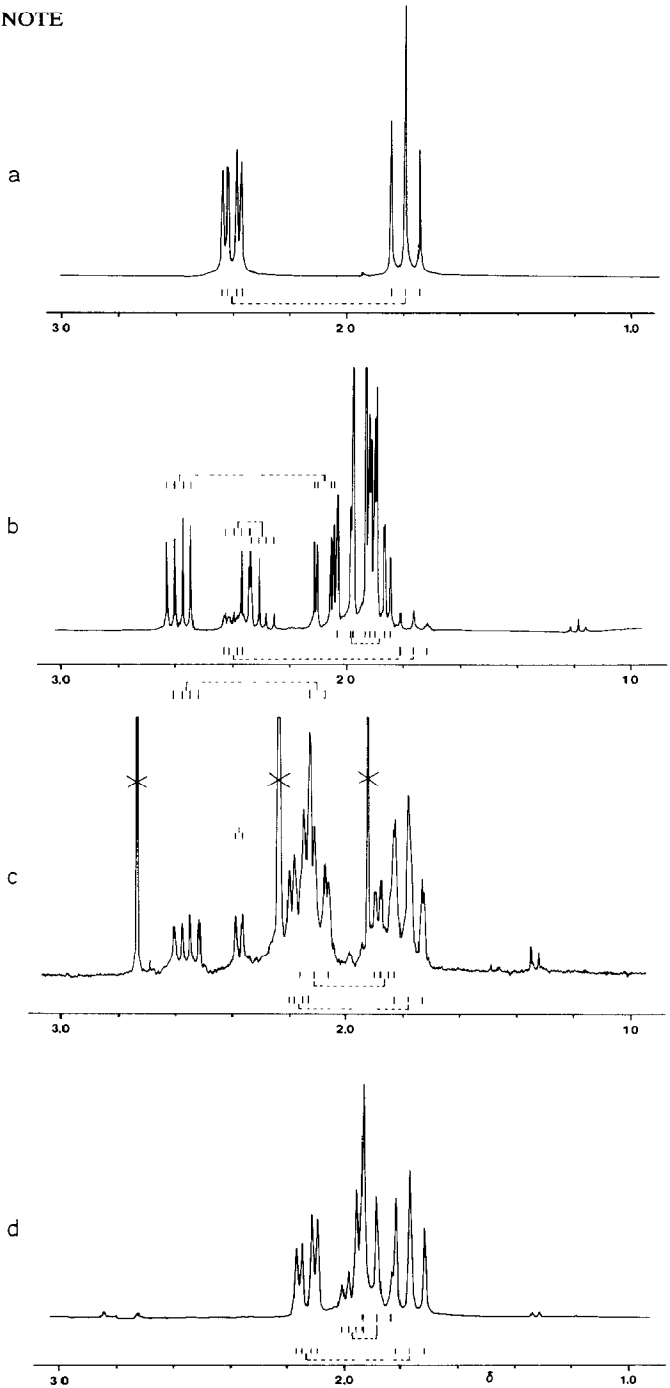


Fig. 3. Partial ^{1}H-n.m.r.-spectra (δ 1.0–3.0) recorded at 250 MHz with a Bruker WH 250 instrument, for a solution in D_2O at room temperature (internal standard, Me_4Si) of: (a) sodium (methyl 3-deoxy-β-D-*manno*-2-octulopyranosid)onate; (b) ammonium salt of KDO (**1**); (c) KDO disaccharide **3** derived from LPS of *S. godesberg* (the crossed-out lines are due to contaminants); and (d) synthetic KDO disaccharide derivative **2**.

TABLE I

PARTIAL ^{1}H-N M R SPECTRA OF THE AMMONIUM SALT OF KDO (**1**), SYNTHETIC DISACCHARIDE **2**, AND KDO DISACCHARIDE FROM *S. godesberg* (**3**)

Compound	*δ*	*J(Hz)*	*Interpretation*
1	1.88 (dd)	13; 5	H-3*e*, *α p*
	1.99 (dd)	13; 11	H-3*a*, *α p*
	2.08 (dd)	14; 3	H-3, major *f*
	2.30 (dd)	13; 7.5	H-3, minor *f*
	2.38 (dd)	13; 7	H-3, minor *f*
	2.60 (dd)	14; 7	H-3, major *f*
2	1.78 (dd)	12.5; 12.5	H-3′*a*
	2.14 (dd)	12.5; 5	H-3′*e*
3	1.78 (dd)	12.5; 12.5	H-3′*a*
	1.86 (dd)	12.5; 5	H-3*e*, *α p*
	2.10 (br. d)	15	H-3, major *f*
	2.11 (t)	12.5	H-3*a*, *α p*
	2.16 (dd)	12.5; 5	H-3′*e*
	2.38 (m)		H-3, minor *f*
	2.56 (dd)	15; 7.5	H-3, major *f*

^{1}H-Nuclear-magnetic-resonance spectroscopy. — The resonances of the H-3's (deoxy-protons) of the octulosonic residues of KDO disaccharide **2** in the range *δ* ~1.0–3.0 were observed for a solution in deuterium oxide (see Fig. 3c). These signals are diagnostic for the ring form and anomeric configuration of KDO residues[6–9]. For the KDO disaccharide **3**, they appeared as a complex pattern that was examined by comparison with the spectra of synthetic model compounds (see Fig. 3 and Table I).

On the basis of the mass-spectral evidence, which indicated that O-4 of the reducing KDO residue is involved in the interglycosidic linkage, the presence of several, tautomeric disaccharides having the reducing residue in pyranose or furanose form was expected. As a first approximation, it was assumed that the distribution of tautomers in the KDO disaccharide would be similar to that found[10] for free KDO. For comparison, the appropriate region of the spectrum of the ammonium salt of KDO (**1**) is shown in Fig. 3b. An interpretation of the H-3 signals recorded for free KDO (tautomeric mixture) is given in Table I. The distribution of tautomers appears to be ~65 for the *α*-pyranose form, 25 and 8 for the major and minor furanose forms, and 2% or less for the *β* pyranose form. The small proportion of *β*-ketopyranose form was supported by a comparison with the spectrum of sodium (methyl 3-deoxy-*β*-D-*manno*-2-octulopyranosid)onate[7,8] (Fig. 3a). Empirical assignments of the H-3 resonances due to the reducing residues of the tautomeric KDO disaccharides are reported in Table I. Even though there is considerable overlap among the different multiplets, it may be concluded that the tautomeric forms of the disaccharide that are present contain the reducing residue

in the α-pyranose and in the two furanose forms. The relative proportions of the tautomers were estimated, from the integration curve, as ~47 for the α-pyranose, and ~37 and ~16% for the furanose forms. This estimate is based on the assumptions that: (a) the multiplet at δ 1.70–1.95 contains the resonances of H-3*a* of the nonreducing group of all tautomers, and H-3*e* of the reducing residue of the α-pyranose tautomer; (b) the multiplet at δ 2.05–2.25 contains the resonances of H-3*e* of the nonreducing group of all tautomers, of H-3*a* of the reducing residue of the α-pyranose tautomer, and of one H-3 of the reducing residue of the major furanose tautomer; (c) the multiplet at δ 2.35–2.40 corresponds to H_2-3 of the reducing residue of the minor, furanose tautomer; and (d) the dd centered at δ 2.56 represents the resonance of one H-3 of the reducing residue of the major, furanose tautomer. These assumptions are substantiated by the analogous spectrum of the ammonium salt of KDO (**1**) (Fig. 3b) wherein the signals are readily assigned to the corresponding tautomeric forms by first-order analysis. The assignment of the signals (Table I) to the H-3's of the nonreducing group of KDO disaccharide **3** was made by comparison with the corresponding shifts and multiplicities of the H-3's in the nonreducing KDO group of synthetic disodium [methyl 3-deoxy-4-*O*-(3-deoxy-α-D-*manno*-2-octulopyranosyl)onate-α-D-*manno*-2-octulopyranosid]onate[11] (**2**) (Table I). The H-3 signals of the nonreducing octulopyranosylonic group of the KDO disaccharide from *S. godesberg* (**3**) are practically superimposable upon those of the nonreducing group of the synthetic, α-(2→4)-linked KDO disaccharide derivative **2**. Together with the evidence for the presence of furanose tautomers, which could not be formed from a (2→5)-linked disaccharide, this similarity strongly supports the assignment of a (2→4)-interglycosidic linkage for **3**, as based on the mass spectral data. Furthermore, in conjunction with the shift value (δ 2.34) of H-3*e* of the nonreducing KDO group of synthetic, β-(2→4)-linked KDO disaccharide[11] **2**, the ^{1}H-n.m.r.-data suggest the α anomer configuration for the nonreducing octulopyranosylonic group of KDO disaccharide **3**.

^{13}C-N.m.r.-spectroscopy. — On the basis of the ^{1}H-n.m.r. data, the preparation under study was assumed to consist of several tautomer forms of a KDO disaccharide **3**, each of which being composed of (a) an α-(2→4)-linked, nonreducing octulopyranosylonic group, corresponding to the nonreducing group of the synthetic KDO disaccharide derivative **2**, and (b) the respective tautomer of the reducing KDO residue, the distribution of tautomers deviating from that of the ammonium salt of KDO in that larger proportions of the furanose form are present in the KDO disaccharide.

As seen in Fig. 4, a comparison of the proton-decoupled, 62.5 MHz, Fourier-transform, ^{13}C-n.m.r.-spectrum of KDO disaccharide **3** (Fig. 4b) with the analogous spectra of the synthetic disaccharide derivative **2** (Fig. 4a) and of the ammonium salt of KDO **1** (Fig. 4c) is in agreement with the aforementioned assumption. Considering firstly the nonreducing KDO group common to all tautomeric forms of disaccharide **3**, it may be seen that the signals attributable to the nonreducing KDO group of the model disaccharide **2** (Fig. 4a) all correspond to signals of closely

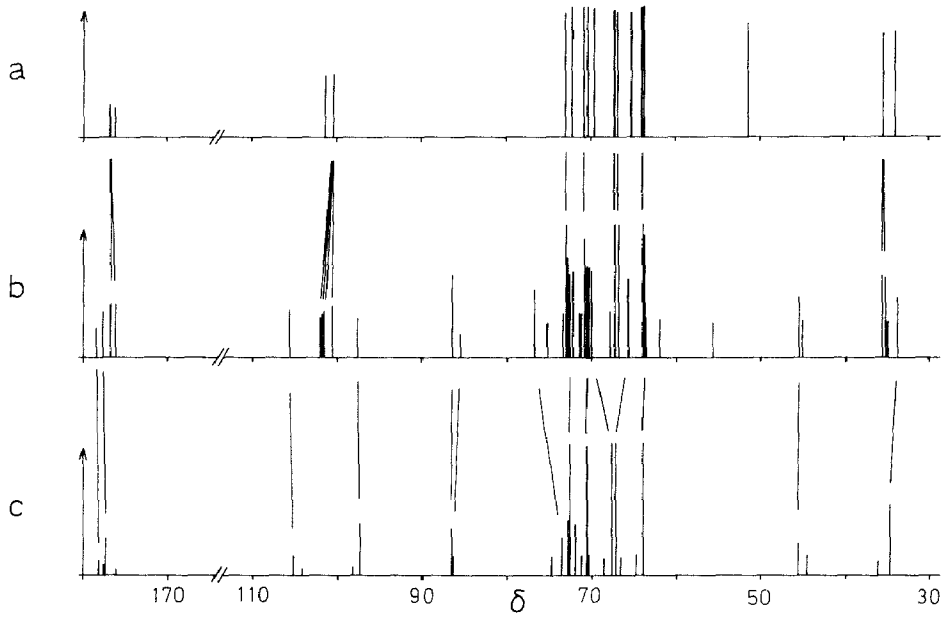


Fig. 4. Bar diagrams corresponding to the ^{13}C-n m.r.-spectra recorded at 62.9 MHz with a Bruker WH 250 instrument for a solution in D_2O at room temperature (external standard, 1,4-dioxane, δ 67.4) of: (a) synthetic KDO disaccharide derivative **2**; (b) KDO disaccharide **3** derived from the LPS of *S. godesberg*; and (c) ammonium salt of KDO (**1**).

similar shift values in the spectrum of **3** (Fig. 4b). As would be expected, several of these signals (*e.g.*, those at δ 35–36, 100–102, and 176–177) occur in multiple form due to the different effects of the tautomeric, reducing residues upon the respective carbon atoms (C-3′,-2′, and -1′) of the nonreducing KDO residue. The chemical shift values corresponding[7,11] to C-4, -6, and -1 of the octulopyranosylonic group (Table II) confirm the α anomer configuration indicated by the ^{1}H-n.m.r.-spectrum. The signals due to C-3 (δ ~45), -5 (85–86), and -2 (~105) of the furanose tautomers of the ammonium salt of KDO (**1**) have corresponding counterparts in the spectrum of **3** (Fig. 4 and Table II). The lines attributable to the preponderant α-ketopyranose tautomer of the ammonium salt of KDO (for C-3 at δ ~35, -2 at ~97, and -1 at ~177) correspond to those at δ 33.9, 97.5, and 177 in Fig. 4b; the β-shift of the C-3 signal (~1 p.p.m.) is in agreement with the presence of a (2→4)-linked, nonreducing octulopyranosylonic group in KDO disaccharide **3**.

The signal intensities corresponding to C-3, -2, and -1 of the reducing KDO residues (Fig. 4b) agree with the proportions of tautomeric forms estimated from the integration of the ^{1}H-n.m.r.-spectrum. Clearly, the relative intensities of the lines attributable to the major, furanose tautomer are comparable to those of the lines assigned to the α-pyranose form. By contrast, in the spectrum of the ammonium salt of KDO (Fig. 4c), the α-pyranose form is by far the preponderant tautomer. In addition, a multitude of signals in the range δ 70–75 is present (Fig.

TABLE II

^{13}C-N M R CHEMICAL SHIFTS (δ) EMPIRICALLY ASSIGNED TO THE AMMONIUM SALT OF KDO, THE AMMONIUM SALT OF KDO METHYL α-GLYCOSIDE, AND TO THE INDIVIDUAL KDO RESIDUES OF NATURAL AND SYNTHETIC KDO DISACCHARIDES[a]

Compound[b]	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-7*	*C-8*
α-KDOp-(2→4)-α-KDO*p*	176.67	100.48	35.43	67.18	66.77	72.89	70.66	63.96
methyl α-glycopyranoside	176.68	100.33	35.34	67.13	66.67	73.00	70.72	63.85
α-KDO-(2→4)-KDO*f* (major)	176.06	101.55	35.18	[c]	[c]	[c]	[c]	[c]
α-KDO*p*-(2→4)-*α-KDOp*	177.43	97.44	33.74	69.91	65.59	72.12	70.25	63.84
methyl α-glycopyranoside	176.08	101.32	33.99	69.57	65.12	72.08	70.21	63.80
KDOp	177.46	97.33	34.60	67.57	67.09	72.18	70.32	63.97
methyl α-glycopyranoside	176.11	101.29	34.87	67.11	66.77	72.16	70.16	63.89
α-KDO*p*-(2→4)-*KDOf* (major)	178.30	105.45	45.25	76.72	86.31	72.57	72.76	63.84
KDOf (major)	178.26	105.13	45.47	73.47	86.57	71.93	72.66	63.97

[a]Recorded with a Bruker Spectrospin instrument at 62.5 MHz in the Fourier-transform mode; 297° K; standard external 1,4-dioxane δ 67.40. [b]The residues to which the signals are assigned are designated by italic letters. [c]The lines attributable to C-4′–C-8′ of this tautomer apparently coincide with the corresponding lines attributed to the other tautomer(s).

4b), as must be expected when a significant proportion of furanose tautomers is present. Obviously, it is impossible to assign with certainty individual resonances in this portion of the spectrum (Fig. 4b). On the other hand, these lines are much less numerous in the spectrum of the ammonium salt of KDO (Fig. 4c).

The (2→4)-interglycosidic linkage of KDO disaccharide **3** derived from the LPS of *S. godesberg* is in agreement with the earlier findings by Prehm *et al.*[12] on the core segment of the LPS from the "deep-rough" *Escherichia coli* mutant BB 12, and with the assignment made by Munson *et al.*[13] for the product synthesized, *in vitro*, from CMP-KDO and a "lipid-A-precursor" in the presence of CMP-3-deoxyoctulosonate:3-deoxyoctulosylono-lipid A 3-deoxyoctulosylonotransferase (KDO-transferase). Finally, disaccharide **3** corresponds to the "core segment" of the LPS from *S. minnesota* Re 595, as shown by the ^{1}H- and ^{13}C-n.m.r.-spectra of a pseudotrisaccharide obtained by sequential hydrazinolysis, deamination with nitrous acid, and reduction with sodium borohydride[14]. The disaccharide structure of these Re "core segments" and the α anomeric configuration of their constituent KDO residues were previously postulated on the basis of a partial interpretation of the ^{13}C-n.m.r.-spectra of undegraded LPS from a heptoseless mutant of *E. coli*[15].

ACKNOWLEDGMENTS

The authors thank Mr. H. Moll for the mass-spectroscopy experiments. H.B. was the recipient of a grant from the Deutsche Forschungsgemeinschaft (Br 731/3).

REFERENCES

1 H. BRADE, C. GALANOS, AND O. LUDERITZ, *Eur. J. Biochem.*, 131 (1983) 201–203.
2 H. BRADE, C. GALANOS, AND O. LUDERITZ, *Eur. J. Biochem.*, 131 (1983) 195–200.
3 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
4 W. DROGE, V. LEHMANN, O. LUDERITZ, AND O. WESTPHAL, *Eur. J Biochem.*, 14 (1970) 175–184.
5 B. JANN, P. HOFMANN, AND K. JANN, *Carbohydr. Res.*, 120 (1983) 131–141.
6 J. HAVERKAMP, L. DORLAND, J. F. G. VLIEGENTHART, J. MONTREUIL, AND R. SCHAUER, *Abstr. Int. Symp. Carbohydr. Chem.*, 9 (1978) D-7.
7 A. K. BHATTACHARJEE, H. J. JENNINGS, AND C. P. KENNY, *Biochemistry*, 17 (1978) 645–651.
8 F. M. UNGER, D. STIX, AND G. SCHULZ, *Carbohydr. Res.*, 80 (1980) 191–195.
9 F. M. UNGER, *Adv. Carbohydr. Chem. Biochem.*, 38 (1981) 323–388.
10 R. CHERNIAK, R. G. JONES, AND D. S. GUPTA, *Carbohydr. Res.*, 75 (1979) 39–45.
11 P. WALDSTATTEN, R. CHRISTIAN, H. PAULSEN, G. SCHULZ, AND F. M. UNGER, unpublished results.
12 P. PREHM, S. STIRM, B. JANN, AND K. JANN, *Eur. J. Biochem.*, 56 (1975) 41–55.
13 R. S. MUNSON, JR., N. S. RASMUSSEN, AND M. J. OSBORN, *J. Biol. Chem.*, 253 (1978) 1503–1511.
14 R. CHRISTIAN, G. SCHULZ, AND F. M. UNGER, *Tetrahedron Lett.*, in press.
15 S. M. STRAIN, S. W. FESIK, AND I. M. ARMITAGE, *J. Biol. Chem.*, 258 (1983) 2906–2910.