Vol. 190, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1993 Pages 302-307

OCCURRENCE OF 2-*O*-METHYL-*N*-(3-DEOXY-*L*-*GLYCERO*-TETRONYL)-*D*-PEROSAMINE (4-AMINO-4,6-DIDEOXY-*D*-*MANNO*-PYRANOSE) IN LIPOPOLYSACCHARIDE FROM OGAWA BUT NOT FROM INABA O FORMS OF O1 *VIBRIO CHOLERAE*

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Received December 7, 1992

Summary: A structural study by GC-MS, methylation analysis, and ^{1}H and ^{13}C NMR was carried out on α(1→2)-linked linear *N*-(3-deoxy-*L*-*glycero*-tetronyl)-*D*-perosamine homopolymer constituting the O-polysaccharide chain of lipopolysaccharide from O1 *Vibrio cholerae* Ogawa and Inaba O forms. Occurrence of 2-*O*-methyl-*N*-(3-deoxy-*L*-*glycero*-tetronyl)-*D*-perosamine was demonstrated at the non-reducing terminus of the perosamine-homopolymer of lipopolysccharide from the Ogawa O form in contrast to the presence of *N*-(3-deoxy-*L*-*glycero*-tetronyl)-*D*-perosamine at the nonreducing terminus for the Inaba O form.

It was 100 years ago that Richard Pfeiffer (1) coined the term "Endotoxin" for a toxic, strongly cell-bound component of O1 *Vibrio cholerae* in order to distinguish it from "Exotoxin" which is released extracellularly from bacterial cells into liquid growth medium. In current terminology, this cell-bound substance (the endotoxin) precisely corresponds to lipopolysaccharide (LPS) residing in outer membranes of cell walls of this vibrio. O-Antigen of O1 *V. cholerae*, which is serologically divided into Ogawa and Inaba O forms, comprises three antigen factors, i.e., group antigen factor A, Ogawa antigen factor B and Inaba antigen factor C. O-Antigenic structures of the Ogawa and Inaba O forms are represented by antigenic formulae AB(c) and AC, respectively (2). Gram-negative bacterial LPS is a heteropolysaccharide consisting of three chemically and biologically distinct portions, i.e., lipid A which is the center of endotoxic activity of LPS, core polysaccharide and O polysaccharide chain (PS) which mainly determines serological specificity. The PS of O1 *V. cholerae* is known to be

Abbreviations: LPS, lipopolysaccharide; PS, O-polysaccharide chain; PerNTet, *N*-(3-deoxy-*L*-*glycero*-tetronyl)-*D*-perosamine; CH_3I, iodomethane; C^2H_3I, [^{2}H]-iodomethane.

0006-291X/93 $4.00

composed of α(1→2)-linked linear *N*-(3-deoxy-*L-glycero*-tetronyl)-*D*-perosamine (4-amino-4,6-dideoxy-*D-manno*-pyranose)-homopolymer (3,4). In this study, occurrence of 2-*O*-methyl-*N*-(3-deoxy-*L-glycero*-tetronyl)-*D*-perosamine was demonstrated at the non-reducing terminus of the PS of LPS from the Ogawa O form in contrast to presence of *N*-(3-deoxy-*L-glycero*-tetronyl)-*D*-perosamine at the non-reducing terminus of that from the Inaba O form of O1 *V. cholerae*.

Materials and Methods

Bacterial strains and LPS. Strains of O1 *Vibrio cholerae* NIH 41, NIH 90, P1418 (O form Ogawa, biotype classical), 35A3, 569B (O form Inaba, biotype classical), SLH22, 34D/13, Ubon 13, PE-1 (O form Ogawa, biotype eltor), V86, HP47, C5 (O form Inaba, biotype eltor) were used. Cultivation of the bacteria and preparation of LPS were described previously (5).

Preparation of PS. The PS of P1418 and NIH 41 (Ogawa O form) and 569B (Inaba O form) LPS were prepared from their mild acid hydrolysates (5% acetic acid, 100°C, 3 h) by Sephadex G-50 gel chromatography as described previously (6).

Analytical methods. Neutral and amino sugars and perosamine were determined by GLC as their alditol acetates prepared by the method of Kondo *et al.* (7). For determination of *N*-(3-deoxy-*L-glycero*-tetronyl)-*D*-perosamine (PerNTet), PS was subjected to solvolysis (8) in anhydrous hydrogen fluoride (HF) at room temperature for 3 h. The products were reduced by $NaBH_4$ after complete removal of HF under reduced pressure, peracetylated in pyridine : acetic anhydride (1:1) at 100°C for 30 min and analyzed by GLC.

Methylation analysis. PS was methylated by the method of Hakomori (9) using CH_3I or C^2H_3I. The methylated materials were treated with anhydrous HF, reduced by $NaBH_4$, peracetylated as described above and analyzed by GLC and GC-MS.

GLC and GC-MS. GLC was carried out on a Shimadzu gas chromatograph 14A (Shimadzu, Kyoto, Japan) instrument equipped with a fused silica capillary column (0.25 mm x 35 m) coated with HR52 (Chromato Packing Center, Kyoto, Japan). The following temperature programmes were used; 150°C for 3 min increased to 320°C at 5°C/min for methylation analysis; 180°C for 3 min increased to 240°C at 4°C/min for perosamine, neutral and amino sugar analysis; 180°C for 3 min increased to 320°C at 5°C/min for determination of PerNTet. GC-MS was performed on a DX-300 (Nihon Denshi, Tokyo, Japan) instrument using the column described above. Electron-impact MS (EI-MS) were recorded at 70 eV and isobutane was used as reactant gas in chemical ionization MS (CI-MS).

NMR spectroscopy. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100.6 MHz) spectra were recorded on a Varian VXR-400S (Varian, USA) instrument for samples in ^2H_2O at 25°C. ^{1}H Chemical shifts are relative to the ^{2}HOH signal (4.75 ppm), and 1,4-dioxane (67.40 ppm) was used as an internal standard in ^{13}C-NMR. Assignments were made by ^{1}H,^{1}H-homonuclear and ^{1}H,^{13}C-heteronuclear COSY-NMR experiments. The anomeric configurations were determined in gated ^{13}C-NMR experiments. 2D-Heteronuclear spin lock purge pulse multiple bond correlation (HSMBC) spectroscopy was performed on a JEOL GXR-500 (Nihon Denshi, Tokyo, Japan) NMR spectrometer (5 mm probe) for sample in ^2H_2O at 25°C. The number of acquisitions (n) was 120 with 1024 x 512 data points (J = 8.3 Hz).

Results and Discussion

The PS isolated from P1418 (Ogawa) contained perosamine, quinovosamine, glucose, glucosamine and *L-glycero-D-manno*-heptose as reported (6). The sugar compositions of the PS of NIH 41 (Ogawa) and 569B (Inaba) were almost same as that of PS of P1418 (Ogawa). Besides these component sugars, a small amount

(0.063 μmol/mg) of an amino sugar having retention time (0.88) relative to that of perosamine was detected in the hydrolysates (10 *M* HCl, 90 °C, 15 min) of Ogawa (NIH 41 and P1418) PS which was not found in the same hydrolysate of Inaba (569B) PS. In the EI-mass spectra of the alditol acetates of the amino sugars (data not shown), fragment ion (*m/z* 158) corresponding to the C-4–C-6 unit of perosamine was observed, while fragment ions originating from C-1–C-2 and C-1–C-4 units (*m/z* 117 and 260, respectively) for the unknown sugar were both shifted to lower mass by 28 *Da* compared with those (*m/z* 145 and 288, respectively) for perosamine. The molecular mass of the compound (M_r=347) estimated by GC-MS (CI-mode) was 28 *Da* less than that of the perosamine derivative. The amino sugar was also detectable in peracetylated products obtained by HF-solvolysis of Ogawa (NIH 41 and P1418) PS as its *N*-3-deoxy-tetronylated aldtiol acetate having a retention time 0.94 relative to that of PerNTet. These results strongly suggested that the amino sugar detected in Ogawa (NIH 41 and P1418) PS was 2-*O*-methylated PerNTet. This compound was detected in all LPS from Ogawa O form (NIH 41, P1418, SLH22, 34D/13, Ubon 13 and PE-1) regardless of their biotype, but not in any LPS from Inaba O form (569B, 35A3, V86, HP47, and C5). The amount of the amino sugar in LPS of Ogawa O form was, on average, 1:20 relative to those of perosamine.

Methylation analysis of PS of 569B (Inaba), NIH 41 (Ogawa) and P1418 (Ogawa) using CH_3I revealed the presence of 1,5-di-*O*-acetyl-4,6-dideoxy-4-[*N*-methyl(2',4'-di-*O*-methyl-3'-deoxy-*L-glycero*)-tetronamido]-2,3-di-*O*-methyl-mannitol (1,5-Ac_2PerNTet, from non-reducing terminal PerNTet) and 1,2,5-tri-*O*-acetyl-4,6-dideoxy-4-[*N*-methyl(2',4'-di-*O*-methyl-3'-deoxy-*L-glycero*)-tetronamido]-3-*O*-methyl-mannitol (1,2,5-Ac_3PerNTet, from 2-substituted PerNTet) in the products obtained from the three PS. The retention times (*t*=22.33 min for 1,5-Ac_2PerNTet and *t*=23.88 min for 1,2,5-Ac_3PerNTet) and the EI-mass spectra of these compounds obtained from Ogawa PS were identical to those of corresponding derivatives obtained from Inaba PS. Upon GC-MS, the characteristic prominent fragment ions *m/z* 117 (C-1–C-2), 161 (C-1–C-3), 334 (C-1–C-4), 260 (C-4–C-6) and 304 (C-3–C-6) were observed for 1,5-Ac_2PerNTet and *m/z* 189 (C-1–C-3), 362 (C-1–C-4), 260 (C-4–C-6) and 304 (C-3–C-6) were observed for 1,2,5-Ac_3PerNTet. No other derivative originating from 3- or 2,3-substituted perosamine was detectable and no difference was recognized in the methylation analysis between PS of Ogawa and Inaba. On the other hand, the methylation analysis of PS of NIH 41 (Ogawa) and P1418 (Ogawa) using C^2H_3I revealed the presence of compounds A (*t*=22.33 min) and B (*t*=23.88 min) (Fig. 1). In compound A, the methyl group on position C-2 was not replaced by a deuterio-methyl group whereas the methyl groups present on positions C-3, C-2' and C-4' and that on the amino group were replaced by deuterio-methyl groups. Table 1 shows the results obtained by methylation analysis using C^2H_3I. In GC-MS of A, fragment ions *m/z* 117, 164, 346, 269 and 316

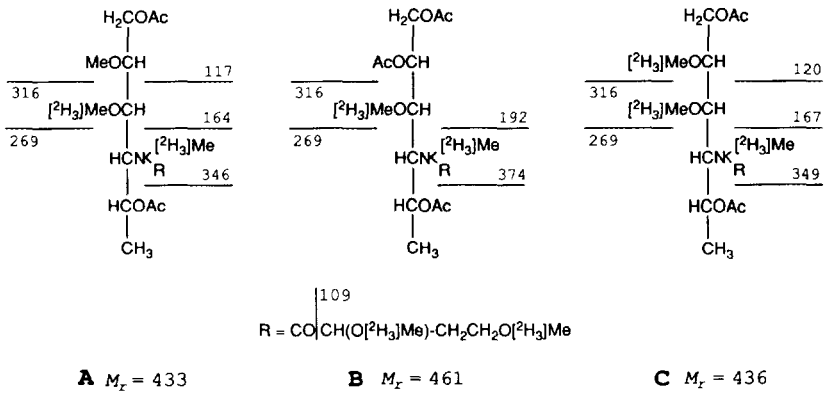

Fig.1. Structures and fragmentation patterns of A: 1,5-di-*O*-acetyl-4,6-dideoxy-4-[*N*-methyl(2',4'-di-*O*-methyl-3'-deoxy-*L-glycero*-)-tetronamido]-2,3-di-*O*-methyl-mannitol carrying [$^2H_3$]methyl groups on positions C-3, 2', 4' and on the amino group, B: 1,2,5-tetra-*O*-acetyl-4,6-dideoxy-4-[*N*-methyl(2',4'-di-*O*-methyl-3'-deoxy-*L-glycero*)-tetronamido]-3-*O*-methyl-mannitol carrying [$^2H_3$]methyl groups on positions C-3 2', 4' and on the amino group, C: 1,5-di-*O*-acetyl-4,6-dideoxy-4-[*N*-methyl(2',4'-di-*O*-methyl-3'-deoxy-*L-glycero*)-tetronamido]-2,3-di-*O*-methyl-mannitol carrying [$^2H_3$]methyl groups on positions C-2, 3, 2', 4' and on the amino group detected by methylation analysis of O-polysaccharide of P1418 (Ogawa) and 569B (Inaba) using C^2H_3I.

were assigned to C-1–C-2, C-1–C-3, C-1–C-4, C-4–C-6 and C-3–C-6 fragments, respectively. In contrast, compound A was not detectable from 569B (Inaba) PS, instead, compound C was identified as a terminal sugar which had the same

Table 1. Results of GC-MS of compounds A, B and C detected by methylation analysis of the O-polysaccharide chain of lipopolysaccharide isolated from O1 *Vibrio cholerae* Ogawa (P1418) and Inaba (569B) O forms

Compound	M_r[a]	Characteristic fragment ions *m/z* (%)[b]
A	433	74(22.5), 104(19.8), 109(100.0), 117(31.8), 120(15.5), 133(64.9), 164(10.6), 210(13.4), 230(2.8), 256(4.7), 269(65.7), 282(5.5), 316(9.5), 325(1.2), 346(4.7)
B	461	74(22.9), 90(9.4), 109(100.0), 118(7.8), 133(54.2), 178(5.5), 192(11.0), 238(12.9), 269(67.1), 310(2.6), 316(1.6), 353(5.3), 374(3.4)
C	436	74(26.1), 107(36.2), 109(100.0), 120(45.7), 133(62.5), 167(13.4), 213(9.9), 256(5.2), 269(64.6), 285(7.9), 316(10.5), 328(18.0), 349(7.3), 358(11.8), 375(3.1)

[a] Determined based on molecular ions [$(M+1)^+$] obtained by CI-MS.
[b] EI-mass spectra were recorded at 70 eV.

retention time (t=22.33 min) as that of A, however, easily distinguishable from A by the shifted fragment ions m/z 120 (117+3, C-1–C-2), 167 (164+3, C-1–C-3), and 349 (346+3, C-1–C-4) produced by an additional introduction of a deuterio-methyl group at position C-2. The molecular masses of A, B and C (M_r=433, 461 and 436, respectively) estimated by GC-MS (CI-mode) were in good agreement with those calculated for the respective derivatives. These results indicated that deuterio-methyl group was not introduced by permethylation using C^2H_3I on the hydroxyl group on position C-2 of the terminal PerNTet residue present in PS of Ogawa, i.e., 2-*O*-methyl-PerNTet was present as a terminal sugar of perosamine homopolymer constituting the PS of Ogawa LPS, while the terminal PerNTet present in Inaba PS was not substituted.

In the ^{1}H-NMR spectra of both Ogawa and Inaba PS, the signals corresponding to the protons in a linear homopolymer of α(1→2) linked PerNTet were observed at 1.19 ppm (*d*, *J* 5.13 Hz, 3H, H-6), 1.88 ppm (broad *ddd*, 1H, H-3'a), 2.05 ppm (broad *ddd*, 1H, H-3'b), 3.76 ppm (*t*, *J* 6.41 Hz, 2H, H-4'), 3.96 ppm (broad *d*, 2H, H-4 and H-5), 4.17 ppm (broad *d*, 2H, H-2 and H-3), 4.32 ppm (*dd*, J_1 3.85 Hz, J_2 8.59 Hz, 1H, H-2') and 5.18 ppm (*s*, 1H, H-1). It was noted that a signal of methoxy-protons was detected at 3.51 ppm (*s*, 0.2H) in the spectrum of Ogawa PS but not in that of Inaba. In the ^{13}C-NMR sepctrum of Ogawa PS, the weak signals of methoxycarbon and C-2 carbon of perosamine substituted by the methoxy group were observed at 59.66 ppm and 79.61 ppm, respectively, in addition to the strong 10 signals at 17. 65 ppm (C-6), 36.80 ppm (C-3'), 53.75 ppm (C-4), 58.69 ppm (C-4'), 68.38 ppm (C-3), 69.06 ppm (C-5), 69.82 ppm (C-2'), 77.98 ppm (C-2), 101.52 ppm (C-1: $J_{C1\text{-}H1}$ 174.1 Hz indicating α-linkage) and 178.19 ppm (C-1') originating from PerNTet homopolymer. These two weak signals at 59.66 ppm and 79.61 ppm were not seen in the spectrum of Inaba PS. 2D-HSMBC spectroscopy (Fig. 2) of Ogawa PS clearly showed that the position C-2 (tC-2) of non-reducing end perosamine residue in the homopolymer was *O*-methylated, in which the significant C,H-long range coupling was observed between methoxyprotons and the *O*-methylated C-2 carbon in addition to the typical cross-peaks such as H-1–C-2, H-1–C-3 and so on.

From the above data, we concluded that, in the Ogawa PS, 2-*O*-methyl-*N*-(3-deoxy-*L*-*glycero*-tetronyl)-*D*-perosamine is present at the non-reducing terminus in contrast to the Inaba PS where *N*-(3-deoxy-*L*-*glycero*-tetronyl)-*D*-perosamine is present at the non-reducing terminus, thus representing the only structural difference so far found between the O-polysaccharide chains of LPS from the Ogawa and the Inaba O forms of O1 *V. cholerae*. Based on the antigenic formulae of the antigenic structures of the Ogawa and the Inaba O forms, this conclusion leads to the contention that the Ogawa antigen factor B of O1 *V. cholerae* is substantially related to the presence of the 2-*O*-methyl-*N*-(3-deoxy-*L*-*glycero*-tetronyl)-*D*-perosamine residue in LPS from the Ogawa O form. An immunochemical study is now in progress in author's laboratory to confirm this hypothesis.

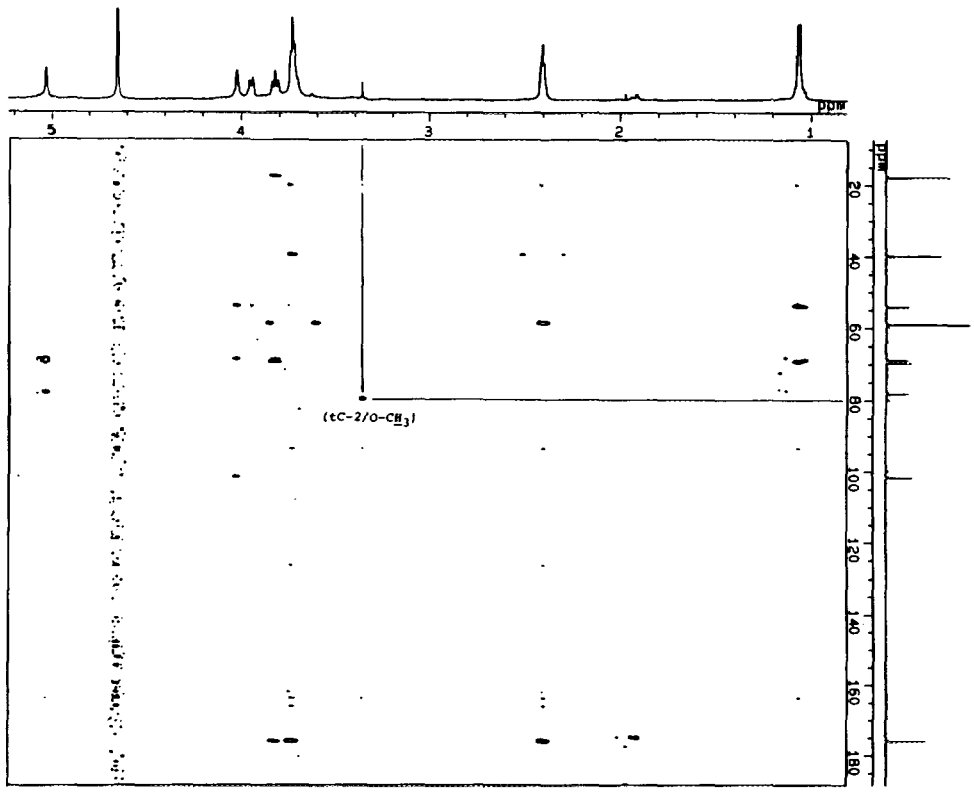

Fig. 2. $^{1}H,^{13}C$-Heteronuclear spin lock purge pulse multiple bond correlation (HSMBC) spectrum of O-polysaccharide from P1418 (Ogawa) lipopolysaccharide. The spectrum was recorded for sample in $^{2}H_2O$ at 25°C using a JEOL GXR-500 NMR spectrometer (5 mm probe). The significant C,H-long range coupling was observed between methoxyprotons (3.51 ppm) and the *O*-methylated C-2 carbon (tC-2, 79.61 ppm) of the perosamine residue at the nonreducing terminus.

Acknowledgment

Professor Stephen G. Wilkinson, School of Chemistry, The University Hull, England, is gratefully acknowledged for his invaluable advice and encouragement.

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