ELSEVIER

Carbohydrate Research 267 (1995) C3–C7

CARBOHYDRATE RESEARCH

Preliminary communication 7-*O*-Carbamoyl-L-*glycero*-D-*manno*-heptose: a new core constituent in the lipopolysaccharide of *Pseudomonas aeruginosa* ☆

Frank Beckmann [a], Hermann Moll [a], Karl-Erich Jäger [b], Ulrich Zähringer [a,*]

[a] *Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, Parkallee 22, D-23845 Borstel, Germany*

[b] *Lehrstuhl für Biologie der Mikroorganismen, Ruhr-Universität Bochum, D-44780 Bochum, Germany*

Received 28 October 1994; accepted 30 November 1994

Keywords: Lipopolysaccharide; Core oligosaccharide; *Pseudomonas aeruginosa*; 7-*O*-Carbamoylheptose

Lipopolysaccharides (LPS) of several *Pseudomonas aeruginosa* rough mutant strains appear to have a common core oligosaccharide containing Glc, GalN, L-*glycero*-D-*manno*-heptose (L,D-Hep), 3-deoxy-D-*manno*-octulosonic acid (Kdo), and, as a very peculiar feature, the amino acid alanine [1,2]. Recently, structural analysis of the complete core oligosaccharide of the R5 mutant has been reported by Masoud et al. [3]. Based on a general NMR strategy and in contrast to a previous finding [2], the authors have identified two L-*glycero*-D-*manno*-heptose residues in the inner core region. By using GLC–MS and chemical derivatization procedures we also identified two heptosyl residues in the LPS of the the rough mutant strain *P. aeruginosa* PAC605. In addition, we have now found the hitherto unknown 7-*O*-carbamoyl-L-*glycero*-D-*manno*-heptose in all rough mutant and wild type LPS of *P. aeruginosa* investigated. 7-*O*-Carbamoyl-L-*glycero*-D-*manno*-heptose was also identified in LPS from related *Pseudomonas* species, such as *P. fluorescens* and *P. syringae*, and, thus, appears to be a characteristic and diagnostic marker for *Pseudomonas* spp. of a newly classified RNA group I [4].

For the GLC–MS [carried out on a 30-m HP-5® fused silica capillary column

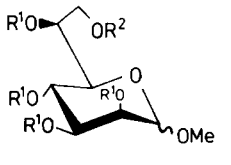
☆ Presented at the XVIIth International Carbohydrate Symposium, Ottawa, Canada, July 17–22, 1994. This paper is dedicated to Professor Harrald Tschesche on the occasion of his 60th birthday (7 June 1995).

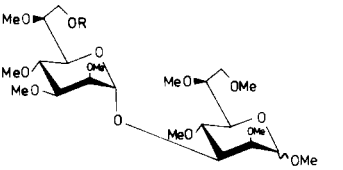
* Corresponding author.

SSDI 0008-6215(94)00371-8

(Hewlett–Packard) which was operated at 150°C for 3 min, then raised to 320°C at 5°C/min] analysis of core sugars, *P. aeruginosa* PAC605 LPS (20 mg) was dephosphorylated (aq 48% HF, 48 h, 4°C) to give LPS–HF from which lipid A was cleaved using mild acid hydrolysis (0.1 M sodium acetate buffer, pH 4.4, 14 h, 100°C) and removed by centrifugation. The saccharides in the supernatant solution were fractionated on Sephadex G-10 to give 7.2 mg (59% w/w) of a dephosphorylated core oligosaccharide.

Methanolysis (2 M HCl–MeOH, 85°C, 30 min) and peracetylation (Ac_2O–pyridine, 60°C, 30 min) of the core revealed, in addition to the well known core constituents of *P. aeruginosa* PAC605 [2], two peracetylated methyl heptosides differing in retention time and MS fragmentation pattern. One heptoside, having the shorter retention time (t_R 17.4 min) and giving by chemical ionization (CI) MS a pseudomolecular ion of $[M + NH_4]^+$ at m/z 452, was identified as a methyl 2,3,4,6,7-penta-*O*-acetylheptopyranoside (**1**), whereas the second (t_R 21.5 min, $[M + NH_4]^+$ m/z 453) was, as will be shown below, a derivative of a so far unknown 7-*O*-carbamoylheptose (**2**).





1 $R^1 = R^2 = Ac$

2 $R^1 = Ac$, $R^2 = CNH_2$

3 $R^1 = Me$, $R^2 = CNMe_2$

4 $R^1 = Me$, $R^2 = H$

5 $R^1 = Me$, $R^2 = Ac$

6 R = $CNMe_2$

7 R = H

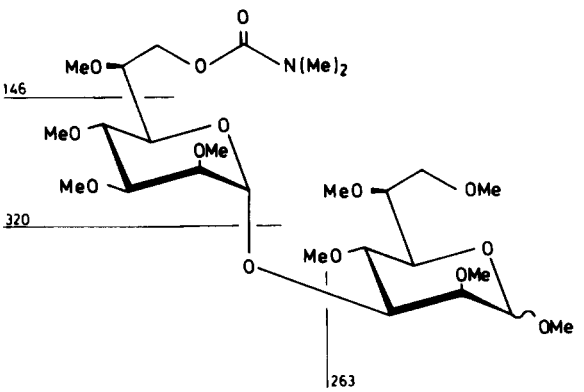
8 R = Ac

Permethylation [5] of the glycosides obtained by methanolysis revealed methyl *O*-(*N*,*N*-dimethylcarbamoyl)-tetra-*O*-methylheptopyranoside (**3**, t_R 17.8 min) which showed a characteristic fragmentation in the electron impact (EI) MS with a peak $[M - OMe]^+$ at m/z 320 and an even more diagnostic peak at m/z 146 assigned to the fragment $[Me_2NCOOCH - CH_2OMe]^+$ or $[MeOCH - CH_2OCONMe_2]^+$ indicating attachment of the *N*,*N*-dimethylcarbamoyl residue at O-6 or O-7 of the heptose.

Perethylation (NaOH–Me_2SO–EtI) [5] of the methyl *O*-carbamoylheptoside converted it into the corresponding perethylated derivative carrying 6 ethyl groups. This was proved by MS analysis showing a mass increment of $\Delta m/z$ 14 amu for each alkyl residue. When CD_3I was used for methylation, GLC–MS analysis revealed a monosaccharide having a characteristic $[M - OMe]^+$ fragment with m/z 338 corresponding to m/z 320 in **3** and a fragment with m/z 155 which was derived from bond cleavage between C-5 and C-6 and supported again the idea of a 6-*O*- or 7-*O*-carbamoyl substituted heptose. The expected molecular masses of all the heptose derivatives investigated were further confirmed by the CI (ammonia) MS data.

Reduction with $LiAlH_4$ in diethyl ether was found to be a mild and selective method for cleaving the *N,N*-dimethylcarbamoyl residue from **3** to give **4** carrying a free hydroxyl group in the position originally occupied by the carbamoyl residue. Resubstitution of the free hydroxyl group using *N,N*-dimethylcarbamoyl chloride (Sigma) in pyridine yielded a heptose derivative, which showed t_R and mass fragmentation pattern upon EIMS and CIMS identical with those of the original compound **3**. *O*-Acetylation of **4** yielded compound **5** which gave, upon GLC–MS analysis, a characteristic fragment $[MeOCH - CH_2OAc]^+$ with m/z 117, derived from bond cleavage between C-5 and C-6 and thus proving carbamoylation at O-7.

In order to assign the configuration of the 7-*O*-carbamoylheptose (L,D or D,D), LPS–HF was treated with mild alkali (0.1 *M* NaOH, 30 min, room temperature) aiming at selective removal of the 7-*O*-carbamoyl residue. After hydrolysis (0.1 M HCl, 48 h, 100°C), reduction ($NaBH_4$), and peracetylation (Ac_2O–pyridine), the derived alditol



6

M = 599

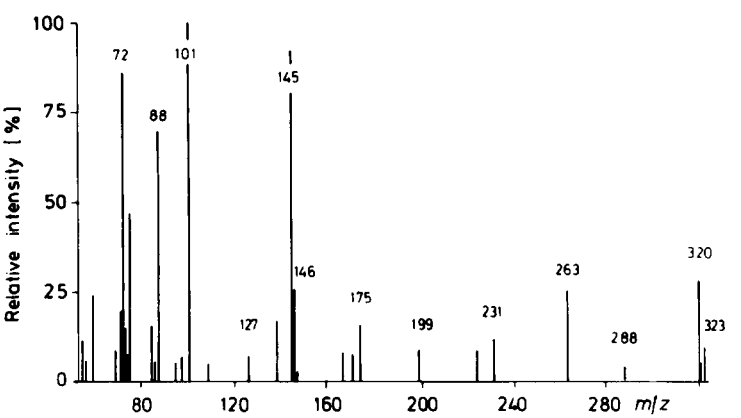


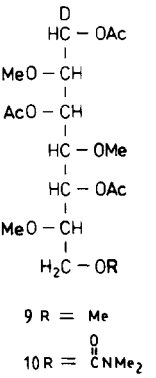
Fig. 1. Structure and EIMS of the permethylated methyl 3-*O*-(7-*O*-carbamoylheptopyranosyl)heptopyranoside **6**. The α linkage is shown according to ^1H NMR data [3].

acetates were analyzed by GLC–MS. The retention time (21.9 min) of the heptitol derivative was compared with that of reference samples of L-*glycero*-D-*manno*-heptitol (t_R 21.9 min) and D-*glycero*-D-*manno*-heptitol (t_R 21.4 min), thus indicating L-*glycero*-D-*manno*-heptose to be the only heptose present in the core oligosaccharide. These data are also in agreement with the ^{1}H NMR data [3].

To the best of our knowledge, the substitution of a sugar by a carbamoyl residue is unique in bacterial LPS. The only carbamoylated sugar identified so far in Nature is a terminal *N*-acylated and *N*-methylated GlcN residue in the Nod factor of *Azorhizobium caulinodans*, where the carbamoyl residue is also located at the primary hydroxyl group (OH-6) comparable to OH-7 of heptose; however, in this case, the carbamoylation was found to be non-stoichiometric ($\sim$ 60%) [6].

EIMS of the disaccharide **6** obtained from the dephosphorylated core oligosaccharide by milder methanolysis (0.5 M HCl–MeOH, 65°C, 30 min) and permethylation revealed diagnostic fragments with m/z 320 and 263 which were assigned to two heptosyl residues as shown in Fig. 1. The terminal "non-reducing" heptose residue (Hep II) was found to carry the *N*,*N*-dimethylcarbamoyl substituent whereas the "reducing" Hep I was fully *O*-methylated. The presence of the diagnostic $\mathbf{J}_1$ fragment [7] (m/z 323 = 263 + 60) derived from Hep I established that the carbamoyl group was localized at Hep II in **6**. As described above for the monosaccharides, reduction ($LiAlH_4$) of **6** afforded **7** which was reacylated (*N*,*N*-dimethylcarbamoyl chloride) to give a heptose disaccharide derivative showing t_R and MS fragmentation pattern identical with those of the original compound **6**. As expected, *O*-acetylation of **7** yielded disaccharide **8**, which, upon GLC–MS analysis, gave the same characteristic fragment $[MeOCH-CH_2OAc]^+$ with m/z 117 as monosaccharide **5** and the same $\mathbf{J}_1$ fragment with m/z 323 as disaccharide **6**.

The linkage between Hep II and Hep I in **6** was determined by methylation analysis, whereby partially methylated heptitols, derived by hydrolysis (2 M CF_3CO_2H, 1 h, 120°C) and reduction ($NaBD_4$), were acetylated (Ac_2O–pyridine) and analyzed by GLC–MS. As a result, 1,3,5-tri-*O*-acetyl-2,4,6,7-tetra-*O*-methylheptitol (**9**, t_R 15.1 min) was identified as the heptitol derived from Hep I, indicating the linkage in the disaccharide **6** as Hep*p*-(1 → 3)-Hep. The (1 → 3) linkage between the two heptose residues in the *P. aeruginosa* rough mutant (strain R5) has also been proved by ^{1}H NMR spectroscopy [3].



Methylation analysis of the dephosphorylated core oligosaccharide revealed 1,3,5-tri-*O*-acetyl-7-*O*-(*N*,*N*-dimethylcarbamoyl)-2,4,6-tri-*O*-methylheptitol **10** (t_R 24.5 min). The occurrence of a 3-*O*-acetylated 7-*O*-carbamoylheptitol derivative can be accounted for by the substitution at position 3 of 7-*O*-carbamoylheptose (Hep II) in the core oligosaccharide. This conclusion fits in with the attachment of GalN(Ala) to Hep II in LPS of *P. aeruginosa* rough mutant strains shown by Rowe and Meadow [2] and Masoud et al. [3].

After 7-*O*-carbamoyl-L-*glycero*-D-*manno*-heptose had been identified, LPS of various bacteria were tested for the presence of this core component. These were rough mutant strains of *P. aeruginosa* (PAC605, PAC557, PAC608, and R5), various wild type strains (Fischer immunotypes 1, 2, and 7), and some other *Pseudomonas* species, such as *P. fluorescens*, *P. syringae*, and *P. wieringae*. In all these cases 7-*O*-carbamoyl-L-*glycero*-D-*manno*-heptose was unambiguously identified, supporting the idea that this compound may be a suitable chemical marker for *Pseudomonas* species. Since, according to a new classification scheme [4], all tested strains belong to the genetically defined RNA group I of so called "authentic" *Pseudomonas*, this fact is highly remarkable from the point of view of improvement of the taxonomical classification of Pseudomonadaceae which is known as one of the most heterogeneous groups among Gram-negative bacteria.

References

[1] S.F. Koval and P.M. Meadow, *J. Gen. Microbiol.*, 98 (1977) 387–398.
[2] P.S.N. Rowe and P.M. Meadow, *Eur. J. Biochem.*, 132 (1983) 329–337.
[3] H. Masoud, E. Altman, J.C. Richards, and J.S. Lam, *Biochemistry*, 33 (1994) 10568–10578.
[4] N.J. Palleroni, *Antonie van Leeuwenhoek; J. Microbiol. Serol.*,, 64 (1993) 231–251.
[5] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
[6] P. Mergaert, M. Van Montagu, J.C. Promé, and M. Holsters, *Proc. Natl. Acad. Sci. U.S.A.*, 90 (1993) 1551–1555.
[7] J. Lönngren and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.