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Synthesis of spacer-equipped phosphorylated di-, tri- and tetrasaccharide fragments of the O-specific polysaccharide of *Vibrio cholerae* O139

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Abstract—The synthesis of oligosaccharide fragments of the O-specific polysaccharide of *Vibrio cholerae* O139 containing a 4,6-cyclic phosphate galactose residue linked to GlcNAc is described. 8-Azido-3,6-dioxaoctyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside, obtained by condensation of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide and 8-azido-3,6-dioxaoctyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside, was converted to 8-azido-3,6-dioxaoctyl 3-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2-acetamido-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (**6**) by reductive opening of the acetal, followed by deacetylation and selective benzylation. Phosphorylation of **6** furnished two isomeric 4,6-cyclic 2,2,2-trichloroethyl phosphates. Glycosylation of the (*S*)-phosphate with 2,4-di-*O*-benzyl-3,6-dideoxy-α-L-*xylo*-hexopyranosyl bromide under halide-assisted conditions gave the desired tetrasaccharide, together with a trisaccharide. Global deprotection and reduction of the azide to an amine was effected by catalytic hydrogenation/hydrogenolysis to give the deprotected tetrasaccharide, which is functionalized for conjugation.

Keywords: Glycosylation; 4,6-Cyclic phosphate; Oligosaccharides; Glycoconjugates; *Vibrio cholerae* O139

Cholera is an enteric disease, caused by *Vibrio cholerae*, marked by severe diarrhea that can lead to dehydration, hypotensive shock and death.[1] Until *V. cholerae* O139 emerged in 1992, *V. cholerae* O1 was the only strain associated with epidemic and pandemic cholera. Unlike other known non-O1 serotypes, this new strain causes serious illness, and the lack of protective immunity against it in *V. cholerae* O1 endemic regions quickly made it an additional threat to public health.[2] Although *V. cholerae* O139 evolved from *V. cholerae* O1,[3] the constitution of the cell wall is completely different. Most notably, the O139 strain acquired a capsular polysaccharide (CPS) whose repeating unit is also expressed as the O-specific polysaccharide (O-PS).[4] Both the CPS and the O-PS have been shown to be virulence factors,[4] which indicates that the O-antigen or its fragments could be haptens suitable as antigenic components of a conjugate vaccine against *V. cholerae* O139. However, the O-PS is a complex branched hexasaccharide containing five different monosaccharides, including two rare deoxy sugar moieties, and a 4,6-cyclic phosphate (Fig. 1).[5] Synthesis of such a structure is a formidable

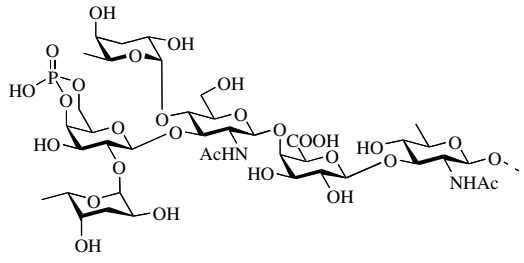

Figure 1. Structure of the O-specific polysaccharide of *V. cholerae* O139.

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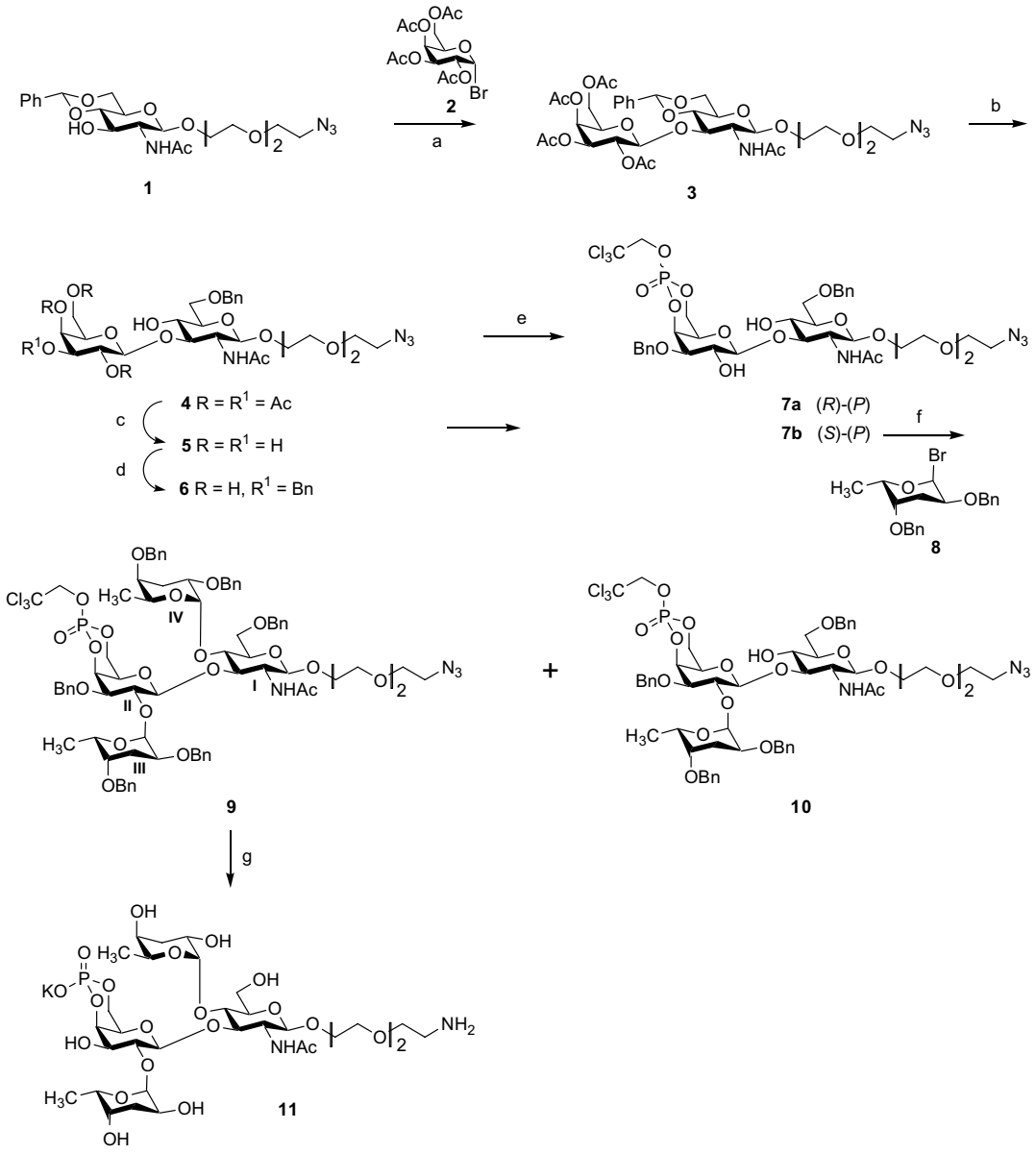

Scheme 1. Reagents and conditions: (a) $Hg(CN)_2$, 4 Å MS, 1:1 CH_3NO_2–benzene, 40 °C; (b) Et_3SiH, CF_3SO_3H, 4 Å MS, CH_2Cl_2, −78 °C; (c) anhydrous K_2CO_3, MeOH, rt; (d) (i) Bu_2SnO, toluene, reflux; (ii) CsF, BnBr, DMF, rt; (e) $Cl_3CCH_2OP(O)Cl_2$, pyridine, CH_2Cl_2, −15 °C; (f) Bu_4NBr, 4 Å MS, 5:1 CH_2Cl_2–DMF, rt; (g) Pd–C, H_2 (150 psi), 1:1 *i*-PrOH–potassium phosphate buffer, rt.

challenge.[†] As an initial step towards a conjugate vaccine for cholera, serotype O139, we focused on the synthesis of fragments of the aforementioned hexasaccharide, to be used for mapping the epitopes that are crucial for eliciting protective immunity. Since the most important antigenic determinants in bacterial oligosaccharides usually can be found at the upstream[6] end, and because two dideoxy residues (3,6-dideoxy-L-*xylo*-hexopyranose, coli- tose), which are often immuno-dominant, are located there, we started with fragments from that region. Oscarson and co-workers first prepared O139-related upstream tri- and tetrasaccharide fragments,[7] but those structures lacked the 4,6-cyclic phosphate on the galactose residue, and required further derivatization to be amenable to conjugation.

Recently, we described the synthesis of a spacer-equipped phosphorylated Col-(1→2)-Gal fragment.[8] Here, we report the synthesis of three other oligosaccharide fragments of the O-PS of *V. cholerae*

[†]Note added in proof: While this paper was in preparation, an independent synthesis of the sequence shown in Figure 1 was reported. See Turek, D.; Sundgren, A.; Lahmann, M.; Oscarson, S. *Org. Biomol. Chem.* **2006**, *4*, 1236–1241; DOI: 10.1039/b518125a.

Table 1. ^{1}H and ^{13}C chemical shifts (δ) for tetrasaccharide **11**[a]

Residue[b,c]	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6
I	4.39	3.81	4.03	3.62–3.73	3.47	3.91–4.01, 3.86
	101.97	55.86	75.44	72.60	75.63	59.46
II	4.69	3.59	3.88	4.54	3.60	4.30–4.41
	101.19	76.32	72.50[d]	76.60[d]	67.53[d]	68.85[d]
III	4.97	3.91–4.01	1.80–1.89	3.76	4.28	1.19
	99.68	63.66	32.84	68.87	66.27	15.61
IV	4.89	3.91–4.01	2.05, 1.80–1.89	4.21	4.75	1.19
	97.90	63.41	32.68	68.55	66.96	15.72

[a] Measured at 600 MHz (^{1}H) and 150 MHz (^{13}C) for solutions in D_2O at 5 °C.
[b] Sugar residues are numbered as shown for structure **9** in Scheme 1. Nuclei from the linker are denoted with a prime.
[c] $\delta_{H\text{-}1a'}$ 3.91–4.01; $\delta_{H\text{-}1b'}$, $\delta_{H\text{-}2'}$, $\delta_{H\text{-}3'}$, $\delta_{H\text{-}4'}$, $\delta_{H\text{-}5'}$ 3.62–3.73; $\delta_{H\text{-}6'}$ 3.15; δ_{CH_3CO} 2.02; δ_{CO} 173.99; $\delta_{C\text{-}2'}$, $\delta_{C\text{-}3'}$, $\delta_{C\text{-}4'}$, $\delta_{C\text{-}5'}$, 70.05, 70.00, 69.76, 66.93, $\delta_{C\text{-}1'}$ 69.32, $\delta_{C\text{-}6'}$ 39.35, δ_{CH_3CO} 22.33.
[d] $J_{C\text{-}3,P}$ 7.0 Hz; $J_{C\text{-}4,P}$ 5.2 Hz; $J_{C\text{-}5,P}$ 4.9 Hz; $J_{C\text{-}6,P}$ 4.2 Hz (partial overlap).

O139. They contain the Gal-(1→3)-GlcNAc sequence with 4,6-cyclic phosphate on the galactose residue and are equipped with a linker functionalized for conjugation.

The stepwise synthesis of the phosphorylated tri- and tetrasaccharide fragments (Scheme 1) was designed based on our finding that a 4,6-cyclic phosphate can be regioselectively introduced in excellent yield, even on carbohydrates with several free secondary hydroxyl groups.[8] This selectivity reduces the required number of protecting group manipulations, thus considerably shortening the synthesis of the targeted phosphorylated sugars. Glycosylation of **1**[9] [mp 184–187 °C, $[\alpha]_D^{25}$ −78 (*c* 1.63, $CHCl_3$); lit.[9] 193–194 °C, $[\alpha]_D^{25}$ −76 (*c* 1.6, $CHCl_3$)] with **2** under Helferich conditions gave β-linked disaccharide **3**‡ [85%, mp 129.0–132.5 °C, $[\alpha]_D^{25}$ −16.8 (*c* 0.51, $CHCl_3$)], equipped with a triethylene glycol spacer whose terminal azide serves as a latent amine. Reductive cleavage of the benzylidene acetal to 6-*O*-benzyl derivative **4** was effected with Et_3SiH–CF_3SO_3H at −78 °C.[10] Compound **4** [87%, mp 103.5–104.0 °C, $[\alpha]_D^{25}$ +1.7 (*c* 0.54, $CHCl_3$)] was formed together with a small amount of 4,6-O-deprotected material (8%). Both products exist as a mixture of conformers (NMR), with the ratio depending on the solvent of measurement. This phenomenon is most prominent in benzene-d_6 (4:1) and chloroform-*d* (85:15), but is almost absent in hydrogen bond-accepting solvents such as pyridine-d_5, methanol-d_4, dimethyl sulfoxide-d_6 and acetic acid-d_4. These observations are consistent with a recent report that protected trisaccharides containing an *N*-acetylglucosamine residue can adopt unexpected conformations as a result of hydrogen bond formation involving the amide group.[11] Liao et al. studied the coupling constants for the *N*-acetylglucosamine residue and found that they were inconsistent with a $^{4}C_1$ chair in non-hydrogen bond-accepting solvents because of a rapid equilibrium between different conformers. It has to be assumed that, in our case, the long triethylene glycol spacer slows down the equilibrium between conformations enough to lead to the existence of different conformers in solution on the NMR time scale. Deacetylation of **4** [→**5**, 96%, $[\alpha]_D^{25}$ −13.8 (*c* 0.55, MeOH)] and subsequent selective benzylation of the formed polyol furnished **6** [80%, $[\alpha]_D^{25}$ −1.90 (*c* 0.59, MeOH)]. Phosphorylation of **6** gave an isomeric mixture (3.8:1) of 4,6-cyclic 2,2,2-trichloroethyl phosphates (86%);[8] the (*S*)-phosphate **7b** [68%, mp 166–168 °C (dec), $[\alpha]_D^{25}$ −2.5 (*c* 0.5, $CHCl_3$), ^{31}P NMR (121 MHz, $CDCl_3$): δ −8.71 (relative to H_3PO_4)] (predominating) and (*R*)-phosphate **7a** [18%, $[\alpha]_D^{25}$ −9.0 (*c* 0.54, $CHCl_3$), ^{31}P NMR (121 MHz, $CDCl_3$): δ −10.61]. Phosphate acceptor **7b** was subjected to halide-assisted[12] glycosylation with colitosyl bromide **8**, which was obtained from the corresponding ethyl 1-thio-β-colitoside[13] by treatment with Br_2. Serendipitously, these glycosylation conditions furnished not only the desired tetrasaccharide **11** [34%, $[\alpha]_D^{25}$ −36.8 (*c* 0.55, $CHCl_3$), ^{31}P NMR (121 MHz, $CDCl_3$): δ −2.30], but also the useful trisaccharide **10** [48%, $[\alpha]_D^{25}$ −9.0 (*c* 0.55, $CHCl_3$), ^{31}P NMR (121 MHz, $CDCl_3$): δ −9.41]. The structure of both compounds was confirmed by mass spectrometry, combustion and NMR analysis. For trisaccharide **10**, the significant downfield shift of H-2^{II} (0.32 ppm, compared to **7b**; for numbering of sugar residues, see Scheme 1) and the COSY-crosspeak between H-4^{I} and the hydroxyl group confirmed attachment of the colitose residue to galactose, while the coupling constant of the H-1^{III} doublet (δ 5.50, $J_{1,2}$ 3.3 Hz) indicated formation of an α-colitosidic bond. As expected, downfield shifts were observed for H-2^{II} and H-4^{I} (0.24 and 0.48 ppm, respectively, compared to **7b**) for tetrasaccharide **9**, and the coupling constants of H-1^{III} (δ 5.62, $J_{1,2}$ 3.4 Hz) and H-1^{IV} (δ 5.02,

‡ All new compounds produced correct analytical figures by combustion analysis, except **11**. Copies of NMR spectra of compound **11** are available as Supplementary data (see Supplementary data section for details).

$J_{1,2}$ 3.5 Hz) were consistent with α-linkage of the colitose residues. The coupling constants for H-1^{I} ($J_{H\text{-}1,H\text{-}2}$ 6.7 Hz, $J_{C\text{-}1,H\text{-}1}$ 166.7 Hz) differ from those usually found for a β-glucosamine residue in a $^{4}C_{1}$ conformation,[14] but are in accordance, in particular $J_{C\text{-}1,H\text{-}1}$, with a distorted conformation of the β-glucosamine unit due to hydrogen-bond formation of the acetamido group.[11] Bush and co-workers[15] analyzed the conformation of the tetrasaccharide portion of the hexasaccharide repeating unit of the CPS of *V. cholerae* O139 by NMR and molecular modeling. Their experiments showed that the tetrasaccharide adopts a compact and tightly folded conformation in which the cyclic phosphate is in close contact with the colitose residue linked to the β-glucosamine unit. Though protected, synthetic tetrasaccharide fragment **9** reflects these findings. Deshielding, as a result of the interaction between the phosphate and the colitose residue attached to β-GlcNAc, leads to a downfield shift (7.11 ppm) of the ^{31}P-signal, compared to trisaccharide **10**, and downfield shifts of H-3_{ax}^{IV} (0.09 ppm), H-4^{IV} (0.1 ppm), H-5^{IV} (0.4 ppm) and H-6^{IV} (0.05 ppm), compared to H-3_{ax}^{III}, H-4^{III}, H-5^{III} and H-6^{III}, respectively. Unusually high hydrogen pressure was required for hydrogenolysis/hydrogenation of **9** [→**11**, ∼50%, ^{31}P NMR (121 MHz, D_2O): δ −3.68, ESITOFMS (neg.): 835.3127 ($[M-K]^-$; calcd 835.3113), ESITOFMS (pos.): 837.3282 ($[M-K+2\ H]^+$; calcd 837.3270)] over palladium-on-charcoal catalyst, a fact that can be explained by hampered interaction of reaction sites with the catalyst surface because of the aforementioned compact conformation. NMR data for the deprotected tetrasaccharide **11** are summarized in Table 1. Full experimental details and deprotection of disaccharide **7a** and trisaccharide **10** will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.04.007.

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