Graphical Abstract

Synthesis and immunodetection of 6-O-methyl-phosphoramidyl- α -D-galactose: A *Campylobacter jejuni* antigenic determinant

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Note

Synthesis and immunodetection of 6-O-methyl-phosphoramidyl- α -D-galactose: A *Campylobacter jejuni* antigenic determinant

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Key words:

Campylobacter jejuni

Capsule polysaccharide

O-methyl phosphoramidate

Synthesis

Traveller's diarrhea vaccine

Abstract

Campylobacter jejuni is a leading cause of traveller's diarrhea. Previously, we have shown that a *C. jejuni* capsule polysaccharide (CPS) conjugate vaccine can fully prevent *C. jejuni* diarrhea in non-human primates. *C. jejuni* CPSs are decorated with non-stoichiometric amounts of O-methyl phosphoramidate (MeOPN) units that are key serospecific markers. In the case of *C. jejuni* serotype complex HS23/36, the MeOPN are at positions 2 and 6 of the CPS galactose (Gal). We describe here the synthesis of the p-methoxyphenyl glycoside of MeOPN→6- α -D-Galp, and its immunodetection by antisera raised by *C. jejuni* CPS conjugates with MeOPN at primary positions. The synthetic approach in this work served as the foundation for a similar MeOPN→6-Gal construct used in a conjugate vaccine, whose synthesis, immunogenicity and efficacy will be described elsewhere.

Campylobacter jejuni is a leading cause of bacterial gastroenteritis in humans worldwide.¹ This Gram-negative bacterium exposes surface capsular polysaccharides (CPSs) that are involved in colonization, invasion and against which serum antibodies are generated.² During the past decade, we designed a *C. jejuni* CPS conjugate vaccine that protected non-human primates against *C. jejuni* diarrhea³ and reduced cecal colonization of *C. jejuni* in chickens,⁴ the main source of *C. jejuni*. A CPS conjugate vaccine is currently undergoing human testing (ClinicalTrials.gov identifier: NCT02067676).

C. jejuni produces serotype-specific CPSs in which the expression of 6-deoxy-heptoses (6d-Hep) and O-methyl phosphoramidate (MeOPN) units, in non-stoichiometric amounts, are key antigenic markers.^{5,6} In *C. jejuni* strain 81-176 (serotype complex HS23/36), MeOPN has also been shown to be essential for complement resistance.^{7,8} The prototype *C. jejuni* vaccine in human trials is based on the CPS of strain 81-176 (serotype complex HS23/36), in which the MeOPN units are non-stoichiometrically attached at O-2 and O-6 of the galactose (Gal) in the repeating trisaccharide block:^{9,10}

Even though MeOPN is not present in each CPS trisaccharide repeating block, we

have observed that these MeOPN monosaccharide epitopes are highly immunogenic in CPS conjugate vaccines. We thus hypothesized that a conjugate containing solely MeOPN monosaccharide epitopes could be an effective *C. jejuni* vaccine. Towards this end, we describe here our first campaign in developing such a synthetic *C. jejuni* vaccine based on methyl phosphoramidate monosaccharide CPS epitopes. Our first target was MeOPN→6- α -D-Galp, as the p-methoxyphenyl (OMP) glycoside, expressed by *C. jejuni* HS23/36 CPSs.

For the synthesis of MeOPN→6- α -D-Galp-(1→OMP a suitable synthetic strategy circumventing mild acidic conditions was needed. We started with a previously reported compound, 4-methoxyphenyl- α -D-galactopyranoside **1**,¹¹ in which a trityl group was selectively introduced at the 6-position to yield **2**. Originally, benzylation was performed on compound **2**, but the extensive migration observed during the introduction of MeOPN forced us to find a more suitable protecting groups. Allyl groups were selected to protect the C-2, C-3 and C-4 positions which were resistant to migration. The trityl group was then removed from compound **3** exposing 6-OH for modification. The allyl groups would be later removed with catalytic hydrogenolysis, which proved to be compatible with the MeOPN modification.

The strategy for the introduction of MeOPN was inspired by a similar reaction proposed by Mara et al.¹² Compound **4** was treated with commercially available methyl dichlorophosphate in the presence of triethyl amine, followed by ammonolysis to furnish **5**. The ³¹P NMR spectrum of product **5** revealed two phosphorus signals indicative of two diastereoisomers resulting from the chiral nature of the newly

introduced MeOPN. The ^1H NMR spectrum also revealed a 1:1 mixture of two diastereoisomers with two sets of anomeric and OCH_3 resonances. The reaction yielded a mixture of side products, the most abundant being that in which the O-methyl group was replaced by an NH_2 .

Removal of the allyl groups with palladium (II) chloride generated product **6**. Similar to **5**, a mixture of diastereoisomers was observed by ^1H and ^{31}P NMR spectroscopy (Fig. 1). ^1H NMR revealed that compound **6** was clean, but traces of the solvent, ethyl acetate, could still be detected at δ 1.05, 1.89 and 3.97. A 2D ^{31}H - ^{31}P HMBC NMR experiment was able to confirm that the MeOPN was introduced to the O-6 position through the correlation between the phosphorous of MeOPN and H-6,6' resonances of Gal. Subsequent work, that will be reported elsewhere, employed the same chemistry to produce **6** with an aminopentyl linker at C-1 (instead of OMP) in which higher yield (75%) was obtained for the last step (e). The differences between the two syntheses were a longer reaction time and a higher concentration of PdCl_2 .

Scheme 1. Synthesis of *O*-Me-phosphoramidate galactoside. Reagent and conditions: (a) TrCl , pyridine, 95%; (b) AllBr , NaH , DMF , $0\text{ }^\circ\text{C}$, 89%; (c) 80% AcOH , $80\text{ }^\circ\text{C}$, 78%;

(d) $\text{PCl}_2(\text{O})\text{OMe}$, Et_3N , CH_2Cl_2 , then $\text{NH}_3(\text{g})$, 19%; (e) PdCl_2 , MeOH , 39%.

Figure 1. ^{31}P NMR (top) and ^1H NMR (bottom) spectra of MeOPN→6- α -D-Galp-(1→OMP **6**.

The synthetic product MeOPN→6- α -D-Galp-(1→OMP **6** was tested for reactivity with antisera previously^{3,13,14} raised against *C. jejuni* CPS conjugates of serotypes HS1, HS3, HS4 and HS23/36. Figure 2 shows that **6** was strongly recognized by HS23/36 CPS conjugate antisera (CPS with a MeOPN at C-6 of Gal), by HS4 CPS

conjugate antisera (CPS with MeOPN at C-7 of *ido*-heptose), and, with lower intensity, by HS1 CPS conjugate antisera (CPS with low amounts of MeOPN at C-6 of Gal). The HS3 CPS conjugate antisera (CPS with MeOPN at C-2 of *ido*-heptose) did not react with **6**. No reaction was observed between α -D-Gal-(1 $\rightarrow$ OMP (devoid of MeOPN) and HS23/36 CPS conjugate antisera.

Figure 2. Immunodetection of MeOPN $\rightarrow$ 6- α -D-Galp-(1 $\rightarrow$ OMP **6** by *C. jejuni* CPS conjugate antisera of serotypes HS1 (1:500), HS3 (1:500), HS4 (1:2000) and HS23/36 (1:2000).

In summary, we have designed a synthetic route for the synthesis of the MeOPN $\rightarrow$ 6- α -D-Gal **6** HS23/36 CPS epitope and have shown that it can be recognized by antisera raised by conjugates that contain CPSs with methyl phosphoramidate at primary positions. Recently, Ashmus and Lowary¹⁵ have also described the syntheses of methyl phosphoramidate monosaccharides via the pre-fabrication of a phosphorylating reagent from methyl H-phosphonate ammonium salt and pivaloyl chloride (PivCl). The sugar H-phosphonate was then reacted with

benzylamine and subsequent deprotection yielded MeOPN. Initially, Ashmus and Lowary used POCl₃ as the phosphorylating reagent, followed by methanolysis and amination with benzylamine. However, this approach was discarded due to low yields and complications during purification. Our strategy here involved a two-step-one-pot reaction that started with a phosphorylation using commercially available methyl dichlorophosphate in the presence of triethyl amine, followed by ammonolysis with ammonia gas. Although our one-pot approach gave lower yields, it offers a relatively inexpensive and simpler alternative into phosphoramidyl glycans.

The detection of synthetic MeOPN→6-D-Gal by HS23/36, HS4 and HS1 CPS conjugate antisera pointed to the fact that these polyclonal preparations contain specific antibodies for MeOPN units at primary positions. The observed immunoreactions of **6** with CPS conjugate antisera were even more notable due to the fact that presumably only half of preparation **6** contained the MeOPN stereoisomer present in native CPSs. The stereochemistry of MeOPN in the CPSs is not yet known. The immunodetection of MeOPN→6-D-Gal was an indicator that this antigenic determinant could be used as an effective vaccine antigen and in which a single epitope could cross-protect across *C. jejuni* serotypes. The synthetic approach that yielded **6**, was used to design a MeOPN→6-α-D-Gal CRM₁₉₇ conjugate vaccine, whose synthesis, immunogenicity and efficacy will be described elsewhere.

1. Experimental

1.1. General Procedures

All chemicals were purchased from commercial suppliers and used as received. Molecular sieves were activated by heating with heating mantle under reduced pressure. Thin layer chromatography (TLC) was carried out on TLC silica gel F₂₅₄. Sugar compounds were visualized by UV light or by charring with 10% H₂SO₄ in ethanol. Flash chromatography was performed with silica gel P60, (43-60 μ m, 230-400 mesh). ¹H NMR and ¹³C NMR spectra were recorded with Bruker 400 or 600 MHz spectrometers. The proton signal of residual, non-deuterated solvent (δ 7.24 ppm for CHCl₃) was used as internal reference for ¹H spectra. For ¹³C spectra, the chemical shifts are reported relative to the solvent (δ 77.1 ppm for CDCl₃). For ¹H NMR data, diastereomer proton signals were indicated by an asterisk (*). Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter and concentration (c) is expressed in g/100 ml. High-resolution mass spectra (HRMS) for the synthetic compounds were recorded by electron spray ionization mass spectroscopy (time-of-flight analyzer) at the mass spectrometry facility, university of Guelph, Ontario.

1.2. 4-Methoxyphenyl 6-O-trityl- α -D-galactopyranoside (2)

To a solution of **1** (2.7 g, 9.3 mmol) dissolved in pyridine (40 mL), trityl chloride (3.1 g, 11 mmol) was added and the reaction mixture was stirred at 60 °C for 3 days. The reaction mixture was then concentrated and purified with flash chromatography (1:1 EtOAc-hexanes) to yield **2** (4.7 g, 95%). $[\alpha]_{\text{D}}^{25} = +91.2^\circ$ (c = 0.21, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 7.44-7.20 (m, 15H, Ar-H); 7.11-6.83 (m, 4H, MeOC₆H₄);

5.51 (d, 1H, $J = 3.6$ Hz, H-1); 4.05-3.93 (m, 4H, H-2, H-3, H-4, H-5); 3.79 (s, 3H, OCH₃); 3.54-3.32 (m, 2H, H-6). ¹³C NMR (100 MHz, CDCl₃): δ 155.3, 151.2, 150.6, 144.3, 143.8, 143.7, 143.6, 129.1, 128.6, 128.0, 127.9, 127.8, 127.5, 127.3, 127.1, 127.0, 118.5, 117.9, 114.6, 114.5, 114.4 (Ar); 98.4 (C-1); 87.0, 71.2, 70.0, 69.3 (C-2, C-3, C-4, C-5); 63.6 (C-6); 55.6 (CH₃). HRMS (ESI): Calcd for C₃₂H₃₂NaO₇ [M+Na]⁺: 551.2046, found: 551.2021.

1.3. 4-Methoxyphenyl 2,3,4-tri-*O*-allyl-6-*O*-trityl- α -D-galactopyranoside (**3**)

A solution of **2** (4.7 g, 8.8 mmol) dissolved in DMF (60 mL) with allyl bromide (4.6 mL, 53 mmol) was cooled to 0 °C. Sodium hydride, 60% dispersion in mineral oil (1.2 g, 29 mmol) was added and the reaction mixture was stirred for 1 h at 0 °C. The reaction was then quenched with MeOH (10 mL), poured into ice-cold water (100 mL) and extracted with EtOAc (3 $\times$ 100 mL). The organic layer was dried over Na₂SO₄ and concentrated. Purification by flash chromatography eluting with 1:7 EtOAc-hexanes gave **4** (5.1 g, 89%). $[\alpha]_D^{25} = +132.6^\circ$ ($c = 0.1$, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 7.38-7.18 (m, 15H, Ar-H); 7.10-6.75 (m, 4H, MeOC₆H₄); 6.00-5.53 (m, 3H, CH₂-CH=CH₂); 5.42 (d, 1H, $J = 3.2$ Hz, H-1); 5.33-4.98 (m, 6H, CH₂-CH=CH₂); 4.37-3.72 (m, 13H, CH₂-CH=CH₂, H-2, H-3, H-4, H-5, OCH₃); 3.38 (m, 1H, H-6a); 3.01 (m, 1H, H-6b). ¹³C NMR (100 MHz, CDCl₃): δ 155.0, 151.0, 143.9 (Ar); 135.2, 135.1, 135.0 (CH₂-CH=CH₂); 128.6, 127.8, 127.0, 119.0, 117.4, 117.3, 116.4, 114.4 (CH₂-CH=CH₂, Ar); 97.5 (C-1); 86.8; 78.2 (C-2); 77.4 (C-4); 76.1 (C-5); 73.9, 72.5, 71.9 (CH₂-CH=CH₂); 70.4 (C-3) 63.3 (C-6); 55.6 (OCH₃). HRMS

(ESI): Calcd for $C_{41}H_{44}NaO_7$ $[M+Na]^+$: 671.2985, found: 671.2970.

1.4. 4-Methoxyphenyl 2,3,4-tri-O-allyl- α -D-galactopyranoside (4)

A solution of **3** (300 mg, 0.46 mmol) in 80% aqueous AcOH (5 mL) was stirred at 80 °C for 1.5 h. The reaction mixture was concentrated before purification by flash chromatography (1:6 EtOAc-hexanes) giving **5** (147 mg, 78%). $[\alpha]_D^{25} = +68.0^\circ$ (c = 0.1, $CHCl_3$); 1H NMR (400 MHz, $CDCl_3$): δ 7.02-6.78 (m, 4H, $MeOC_6H_4$); 5.95-5.89 (m, 3H, $CH_2-CH=CH_2$); 5.50 (d, 1H, $J = 3.5$ Hz, H-1); 5.35-5.12 (m, 6H, $CH_2-CH=CH_2$); 4.42 (dd, 1H, $J_1 = 3.2$ Hz, $J_2 = 9.3$ Hz, H-3); 4.27-3.89 (m, 10H, $CH_2-CH=CH_2$, H-2, H-4, H-5, OH); 3.81 (m, 1H, H-6a); 3.74 (s, 3H, OCH_3); 3.70 (m, 1H, H-6b). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.1, 150.9 (Ar); 135.0, 134.9 ($CH_2-CH=CH_2$); 118.6, 118.0, 117.4, 116.6, 114.5 ($CH_2-CH=CH_2$, Ar); 97.5 (C-1); 78.2, 75.9, 74.0, 72.6, 72.0, 71.0 ($CH_2-CH=CH_2$, C-2, C-3, C-4, C-5); 62.7 (C-6); 55.6 (OCH_3). HRMS (ESI): Calcd for $C_{22}H_{30}NaO_7$ $[M+Na]^+$: 429.1890, found: 429.1891.

1.5. 4-Methoxyphenyl

2,3,4-tri-O-allyl-6-O-methyl-phosphoramidyl- α -D-galactopyranoside (5)

To a solution of **4** (65 mg, 0.16 mmol) and methyl dichlorophosphate (150 μ L, 1.3 mmol) dissolved in CH_2Cl_2 (3 mL) with molecular sieves (4Å), Et_3N (175 μ L, 1.3 mmol) was added drop-wise. The reaction mixture was stirred at room temperature for 5 hours. Upon completion of the reaction as judged by TLC, ammonia gas was

injected into the reaction mixture through a needle. After 10 min, the reaction mixture was filtered and concentrated. Purification with column chromatography (1:1 EtOAc-hexanes) yielded product **5** (15 mg, 19%). $[\alpha]_{\text{D}}^{25} = +100.5^{\circ}$ ($c = 0.2$, CHCl_3); ^1H NMR (400 MHz, CDCl_3): δ 7.04-6.77 (m, 8H, MeOC_6H_4); 5.99-5.85 (m, 6H, $\text{CH}_2\text{-CH=CH}_2$); 5.48 (d, 1H, $J = 3.6$ Hz, H-1); 5.47 (d, 1H, $J = 3.5$ Hz, H-1*); 5.36-5.10 (m, 12H, $\text{CH}_2\text{-CH=CH}_2$); 4.41 (m, 2H, H-3, H-3*); 4.29-4.10 (m, 16H, $\text{CH}_2\text{-CH=CH}_2$, H-2, H-2*, H-4, H-4*); 3.95-3.86 (m, 6H, H-5, H-5*, H-6, H-6*); 3.73 (s, 6H, OCH_3); 3.62 (d, 3H, $^3J_{\text{PH}} = 11.4$ Hz, POCH_3); 3.53 (d, 3H, $^3J_{\text{PH}} = 11.4$ Hz, POCH_3^*); 2.75 (d, 2H, NH_2); 2.56 (d, 2H, NH_2^*). ^{13}C NMR (100 MHz, CDCl_3): δ 155.2, 155.0, 150.9 (Ar); 135.0, 134.9 ($\text{CH}_2\text{-CH=CH}_2$); 128.9, 128.3, 118.8, 118.5, 117.7, 117.5, 117.4, 116.6, 114.5, 114.4 ($\text{CH}_2\text{-CH=CH}_2$, Ar); 97.6, 97.2 (C-1); 78.1, 75.8, 74.4, 74.0, 72.7, 71.9, 70.5, 70.4, 70.0, 69.9, 68.5, 65.5, ($\text{CH}_2\text{-CH=CH}_2$, C-2, C-3, C-4, C-5, C-6); 55.7, 53.3, 53.2 (OCH_3). ^{31}P NMR (162 MHz, CDCl_3): δ 10.58, 10.35. HRMS (ESI): Calcd for $\text{C}_{23}\text{H}_{35}\text{NO}_9\text{P}$ $[\text{M}+\text{H}]^+$: 500.2050, found: 500.2035.

1.6. 4-Methoxyphenyl 6-O-methyl-phosphoramidyl- α -D-galactopyranoside (**6**)

To a solution of **5** (17.0 mg) in dissolved in MeOH (1 mL), PdCl_2 (5.0 mg) was added and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was then filtered and concentrated. Purification with column chromatography (EtOAc) yielded product **6** (5.1 mg, 39%). $[\alpha]_{\text{D}}^{25} = +63.5^{\circ}$ ($c = 0.1$, H_2O); ^1H NMR (400 MHz, D_2O): δ 6.98-6.80 (m, 8H, MeOC_6H_4); 5.41 (d, 1H, $J = 3.6$ Hz, H-1); 5.38 (d, 1H, $J = 3.6$ Hz, H-1*); 4.13 (m, 2H, H-3, H-3*); 4.01-3.85 (m, 8H, H-4, H-4*, H-5,

H-5*, H-6, H-6*); 3.78 (m, 2H, H-2, H-2*); 3.63 (s, 6H, OCH₃); 3.41 (d, 3H, ³J_{PH} = 11.4 Hz, POCH₃); 3.33 (d, 3H, ³J_{PH} = 11.4 Hz, POCH₃*). ¹³C NMR (100 MHz, D₂O): δ 154.6, 150.0, 149.9, 119.3, 119.1, 114.9 (Ar); 98.1, 97.9 (C-1); 70.3, 70.2, 70.0, 69.1, 68.8, 67.8, 65.8 (C-2, C-3, C-4, C-5, C-6); 55.6 (OCH₃); 53.6, 53.5, 53.4 (OCH₃). ³¹P NMR (162 MHz, CDCl₃): δ 14.58, 14.43. HRMS (ESI): Calcd for C₁₄H₂₄NO₉P [M+H]⁺ 380.1111; found 380.1110.

1.7 Immunodot blots

MeOPN→6-α-D-Galp-(1→OMP **6** was adjusted to 1 mg/ml and 2 ul was spotted onto nitrocellulose membranes. The individual spots were immunodetected with four different polyclonal antisera made against different conjugate vaccines in which different *C. jejuni* polysaccharide capsules were conjugated to CRM₁₉₇; serum against an HS23/36 conjugate (final dilution 1:1000), against an HS4 conjugate (final dilution 1:1000), against an HS1 conjugate (final dilution 1:500); and against an HS3 conjugate (final dilution 1:500). Secondary antibodies were either goat anti-rabbit (for HS23/36 and HS4) or goat anti-mouse (HS1 and HS3). Rabbit antibodies were generated by Harlan Laboratories (Indianapolis, IN) and mouse antibodies were generated at NMRC. Immunoblots were developed using a chemiluminescence kit (SuperSignal West Femto Maximum Sensitivity Substrate, ThermoFisher).

Supplementary data

NMR of new compounds

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada through a Discovery grant to MAM. CPE and PG were supported by Navy Work Unit 6000.RAD1.DA3.A0308. The views expressed in this paper are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense nor the US government. PG is an employee of the US government. This work was prepared as part of her official duties. Title 17 USC 105 provides that ‘Copyright protection under this title is not available for any work of the United States Government.’ Title 17 USC 101 defines a US government work as a work prepared by an employee of the US government as part of that person’s official duties.

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Figure legends

Figure 1. ^{31}P NMR (top) and ^1H NMR (bottom) spectra of MeOPN $\rightarrow$ 6- α -D-Galp-(1 $\rightarrow$ OMP **6**.

Figure 2. Immunodetection of MeOPN $\rightarrow$ 6- α -D-Galp-(1 $\rightarrow$ OMP **6** by *C. jejuni* CPS conjugate antisera of serotypes HS1 (1:500), HS3 (1:500), HS4 (1:2000) and HS23/36 (1:2000).