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Authors: V. Narasimharao Thota, Michael J Ferguson, Ryan P Sweeney, and Todd Lowary

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COMMUNICATION

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Synthesis of the *Campylobacter jejuni* 81-176 strain capsular polysaccharide repeating unit reveals the absolute configuration of its *O*-methyl phosphoramidate motif

V. Narasimharao Thota, Michael J. Ferguson, Ryan P. Sweeney and Todd L. Lowary*[a]

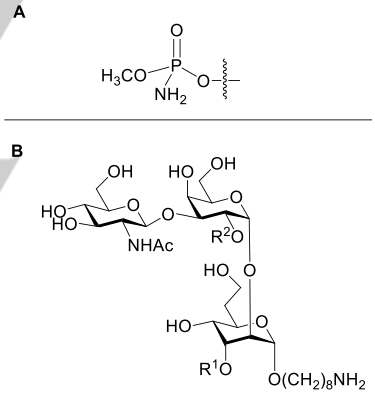
Abstract: The *O*-methyl phosphoramidate (MeOPN) motif is a non-stoichiometric modification of capsular polysaccharides (CPS) in ~70% of all *Campylobacter jejuni* strains. Infections by *C. jejuni* lead to food-borne illnesses and the CPS they produce are key virulence factors. The MeOPN phosphorus atom in these CPS is stereogenic and is found as a single stereoisomer. However, to date, the absolute stereochemistry at this atom has been undefined. We report the synthesis of the three repeating units found in *C. jejuni* 81-176 CPS; one of these possesses a MeOPN group. In the course of these studies we established that the stereochemistry of the phosphorus atom in this MeOPN group is *R*. These studies represent the first unequivocal proof of stereochemistry of this group in any *C. jejuni* CPS. The compounds produced are anticipated to be useful tools in investigations targeting the function and biosynthesis of this structurally-interesting modification, which so far has only been identified in campylobacter.

Campylobacter jejuni is a food-borne pathogen that causes significant gastrointestinal illness worldwide.[1] Although these infections usually resolve in a few days, they sometimes lead to the neurological disorder Guillain–Barré syndrome.[2] An important *C. jejuni* virulence factor is its capsular polysaccharide (CPS), which differentiates the 47 different serotypes identified to date.[3] These CPS possess a number of unusual structural motifs and prominent among these is the *O*-methyl phosphoramidate (MeOPN) group (**1**, Figure 1A). This motif is a non-stoichiometric substituent found in more than 70% of all *C. jejuni* strains. The MeOPN group is believed to contribute importantly to the virulence of the organism[4] and a vaccine currently in development for the prevention of *C. jejuni* infections incorporates CPS bearing this functionality.[5]

The phosphorus atom in the MeOPN substituent is stereogenic and it occurs as a single stereoisomer in the CPSs in which it is found. Recent work has established the biosynthesis of the MeOPN group: the immediate precursor is an activated CDP derivative and the amino group is derived from glutamine.[6] Despite these advances, the stereochemistry on phosphorus remains unknown. We report here the synthesis of the three repeating unit structures found in *C. jejuni* strain 81-176, including one that contains a MeOPN group. In executing these synthetic investigations, we established, for the first time, that the stereochemistry on phosphorus in the MeOPN motif is *R*.[16]

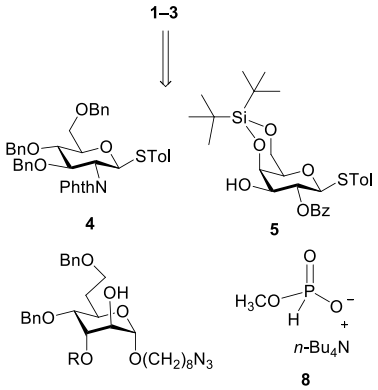
[a] V. N. Thota, Dr. M. J. Ferguson, Dr. R. P. Sweeney and Prof. Dr. T. L. Lowary
Department of Chemistry
University of Alberta
Edmonton, Alberta
Canada T6G 2G2
E-mail: tlowary@ualberta.ca
Supporting information for this article is given via a link at the end of the document.

The structures of the synthetic targets are shown in Figure 1B (**1**–**3**). Each consists of *N*-acetylglucosamine, galactose and an unusual 6-deoxyheptose[7] with the D-*altro*-stereochemistry. An aminooctyl aglycone was incorporated to allow facile conjugation to either proteins or surfaces. The heptose is present either in its unmethylated (**1**) or O-3 methylated form (**2**) and the MeOPN group is found on O-2 of the galactose residue (**3**).[8] The methyl and MeOPN groups are phase-variable modifications.[9] Trisaccharide **3**, lacking both of these functionalities, has been synthesized previously by Nam Shin and coworkers.[10] Our plan was to synthesize all three trisaccharides via a single strategy using building blocks **4**–**8** (Scheme 1).



1, R^1 = H, R^2 = H
2, R^1 = CH_3, R^2 = H
3, R^1 = CH_3, R^2 = $P(O)NH_2OCH_3$

Figure 1 A) MeOPN motif present in Campylobacter CPS; **B)** structures of the CPS repeating units present in *C. jejuni* strain 81-176 CPS (**1**–**3**).

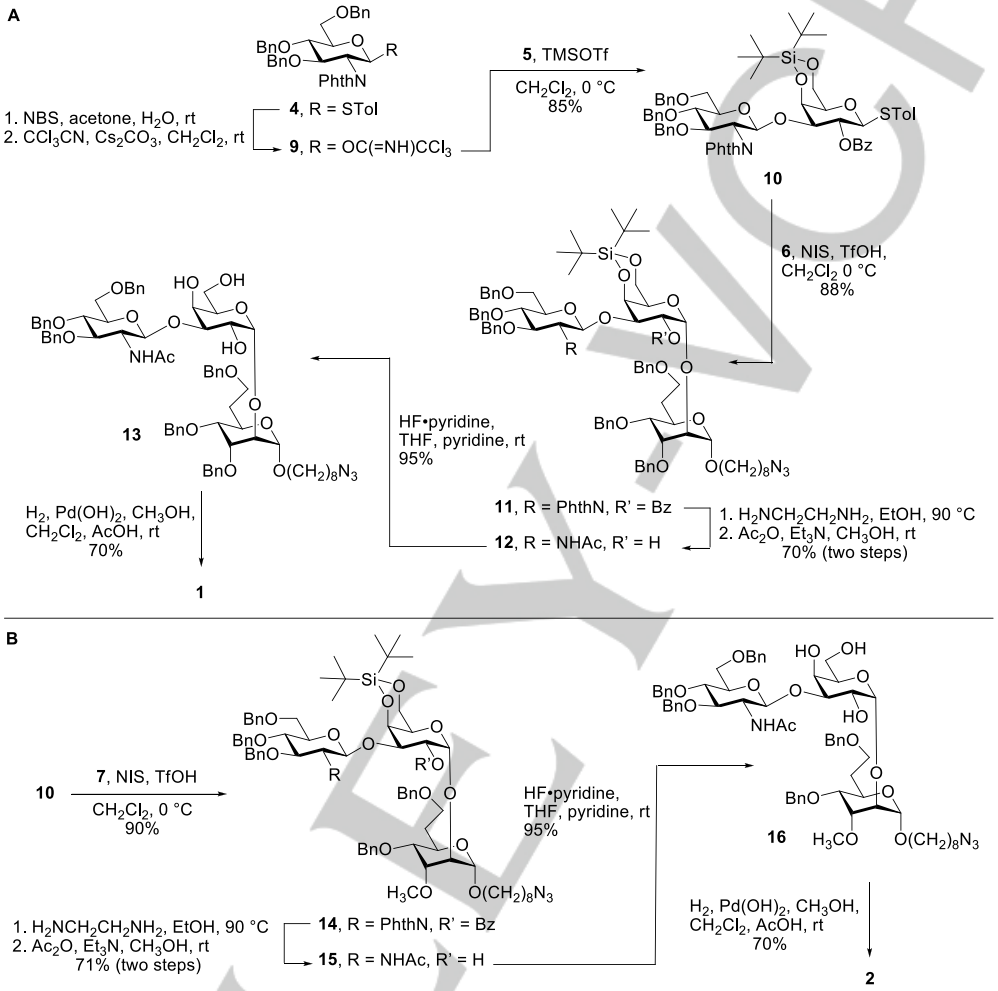


6, R = Bn
7, R = CH_3

Scheme 1. Retrosynthetic Analysis of **1**–**3**

The major challenges in the synthesis of **1**–**3** are: 1) the stereoselective installation of the α-galactoside residue; 2) the preparation of the 6-deoxy heptose with the D-*altro*-stereochemistry; and 3) introduction of the MeOPN moiety. We

chose to address the first challenge through the use of an α-selective donor with a di-*tert*-butyl silyl acetal on O-4 and O-6.[11] Triflation and inversion of an advanced intermediate (See Supporting Information) from the known 6-deoxy-D-*manno* heptopyranoside[12] was our approach in addressing the second. Finally, we envisioned that the MeOPN group could be introduced from **8**, or one of two other methods previously developed for introducing this structure into carbohydrates.[13] Monosaccharide **4**[14] is known and the synthesis of **5**–**7** is provided in the Supporting Information.

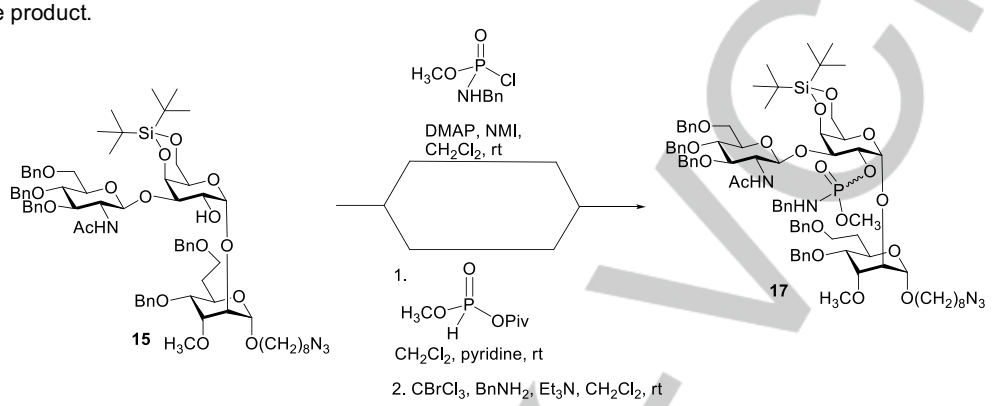


Scheme 2. A) Synthesis of **1**. **B)** Synthesis of **2**.

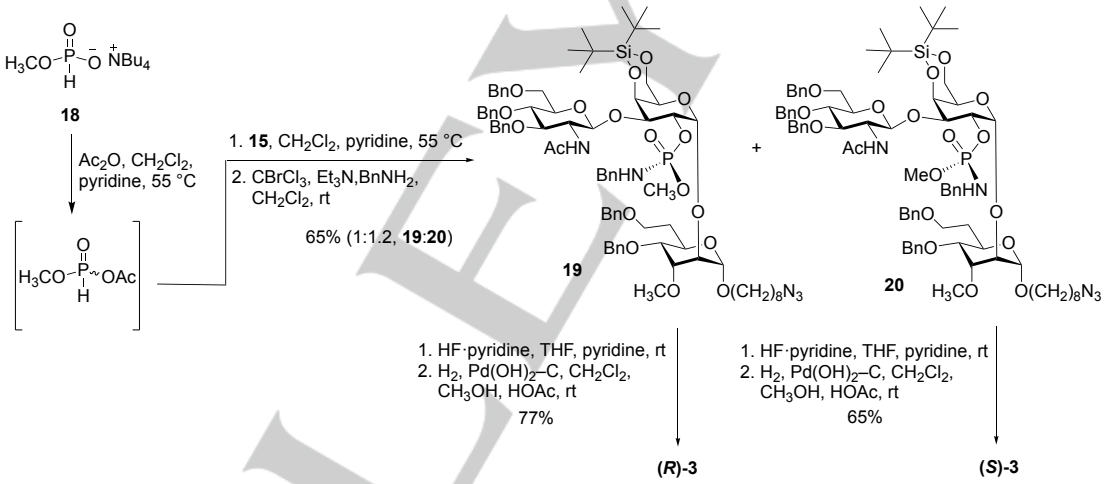
Accessing the parent trisaccharide **1** and the derivative containing the phase-variable methyl group (**2**) began with the synthesis of disaccharide **10** (Scheme 2A). Thus, thioglycoside **4** was converted into trichloroacetimidate **9**, which was then used immediately to glycosylate alcohol **5** employing trimethylsilyl trifluoromethanesulfonate as the activator. This reaction furnished the β-linked disaccharide **10** in 85% yield. Subsequent glycosylation of the 6-deoxy *altro*-heptoside acceptor **6** with **10**, promoted by *N*-iodosuccimide and triflic acid, afforded an 88% yield of trisaccharide **11**. Treatment of **11** with ethylenediamine at 90 °C cleaved both the phthalimide and the benzoate ester; selective *N*-acetylation of the intermediate product was achieved by reaction with acetic anhydride, triethylamine and methanol. This sequence yielded trisaccharide **12** in 70% yield over the two steps. Deprotection of **12** was achieved by first treatment with hydrogen fluoride in pyridine, giving **13**, which was then subjected to hydrogenolysis and azide reduction, providing target **1** in 67% yield over the two steps. With this route established, the preparation of **2** was achieved (Scheme 2B) by glycosylation of **6** with **10** and then application of the same functional group transformations and deprotection reactions used for synthesizing **1**.

Next, we turned our attention to the synthesis of **3**, which contains both of the phase-variable modifications: the methyl and MeOPN groups. We initially explored two previously reported methods (Scheme 3) to carry out this transformation. We first attempted to introduce the MeOPN onto **15** using methyl benzylphosphoramidochloridate,[13b] but under these conditions, no product was formed; only the starting material was isolated.

The use of a two-step approach[13a] involving an activated *O*-methyl *H*-phosphonate pivalate ester (obtained by treatment of the parent *O*-methyl *H*-phosphonate with pivaloyl chloride immediately before addition of the alcohol) and subsequent oxidation via the Atherton–Todd reaction,[15] again resulted only in the re-isolation of **15**. We also explored two derivatives of **15** in which the silyl acetal was replaced with other groups (See Supporting Information, Scheme S4), but these substrates too gave, at best, only very low yields of the desired methyl phosphoramidate product.



Scheme 3. Attempted synthesis of phosphoramidate **17** from **15** using two reported methods[13]



Scheme 4. Successful introduction of the methyl phosphoramidate into **15** and elaboration into target trisaccharide **3**.

We postulated that the lack of success in introducing the phosphoramidate onto **15** was due to steric hindrance near the C-2 hydroxyl group of the galactose residue. We further surmised that using a reagent smaller than those shown in Scheme 3, and more forcing conditions, would provide the desired target. With this approach in mind, **15** was subjected to a modified version of the H-phosphonate formation (Scheme 4).

Activation of the precursor H-phosphonate (**18**) was done using acetic anhydride instead of pivaloyl chloride, and the coupling was done at 55 °C instead of room temperature. Under these conditions, a new product was formed that, when subjected to the Atherton–Todd reaction,[15] furnished the desired *O*-methyl phosphoramidate in 65% yield. As in other examples using this approach,[13a] products corresponding to both diastereomers on phosphorus were formed (**19** and **20**). While often these two diastereomers are inseparable, in this case they could be separated. Fortuitously, one of the diastereomers (**19**) was a solid and we obtained an X-ray structure of the compound (Figure 3).[16] Analysis of the structure allowed us to assign the stereochemistry at phosphorous in **19** as *R*.[17] By inference then, the phosphorus atom in **20** has the *S* stereochemistry.

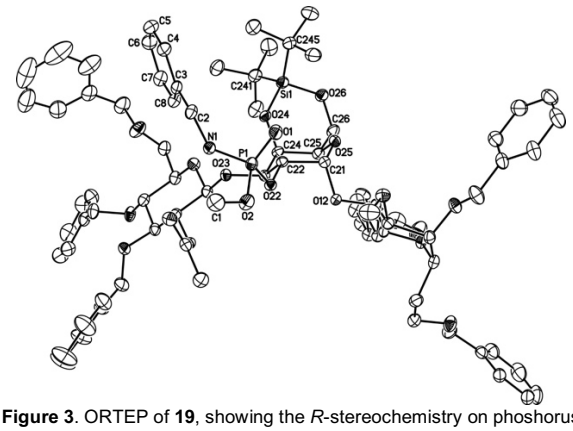


Figure 3. ORTEP of **19**, showing the *R*-stereochemistry on phoshorus

In further characterizing **19** we were surprised to find that the ^{31}P NMR spectrum of the compound showed two signals instead of the anticipated single peak (ratio 2:1); a similar thing was seen in the ^{31}P spectrum of **20** (ratio 4:1). Given the difficulty we had in installing the MeOPN group, presumably due to steric congestion, we hypothesized that these two resonances arise from restricted rotation around one of the phosphoramidate bonds. To test this, we measured the ^{31}P spectrum on **19** and **20** at 60 °C. Under these conditions, the two ^{31}P signals for both compounds changed in relative intensity, leading to spectra that contained one major signal; in the case of **20** a single resonance was observed (See Supporting Information, Page S92). This experiment provides support for restricted rotation leading to the doubling of the signals. Unfortunately, the solvent used ($CDCl_3$) limited the temperature at which we could heat the sample and thus it was never possible to obtain spectra of **19** that had a single resonance, indicative of free rotation in this molecule. Nevertheless, this observation, and the difficulty with which it is install the MeOPN group onto the molecule, provides insight into the sterically hindered nature of this region of the molecule.

To allow the determination of the phosphorous stereochemistry in the natural CPS, both **19** and **20** were deprotected (Scheme 4). Treatment of **19** with HF•pyridine furnished a diol that was then subjected to hydrogenolysis using $Pd(OH)_2$ to afford **(*R*)-3** in 77% yield over the two steps. Similar treatment of **20** provided an 65% yield of **(*S*)-3**. We found that the hydrogenolysis needed significant optimization (See Supporting Information, Table S1) and the best results were obtained when a small amount of acetic acid was added to the reaction mixture. Without acetic acid we found that the majority of the MeOPN group was cleaved. The deprotected compounds were also found to have limited stability in D_2O; most of the MeOPN group was cleaved within few hours during storage in D_2O. The lability of this group in the native CPS was not reported previously,[8c] although we note that the NMR spectra of the polysaccharide were recorded in the presence of 1% acetic acid. It thus appears that the presence of a small amount of acid is required for the molecule to be stable in solution. This is also consistent with our finding (above) that in the absence of acetic acid in the solvent mixture, the hydrogenolysis led to cleavage of the MeOPN group. The extraction of these CPS was also done using hot phenol, an acidic medium.[8c]

With both **(*R*)-3** and **(*S*)-3** in hand, comparison of the NMR data with that previously reported for the CPS[5b,8c] was carried out (Table 1). It should be noted that for these deprotected compounds the restricted rotation seen in **19** and **20** was not observed; thus in all NMR spectra only single resonances were present.

Table 1. Comparison of the NMR data for ***(R)*-3** and ***(S)*-3** with natural CPS

	^{31}P NMR[a]	^{1}H NMR[a],[b]
***(R)*-3**	14.291	4.54
***(S)*-3**	13.849	4.48
CPS	14.257[8c]	4.52[5b]

[a] Chemical shifts in ppm

[b] Chemical shift of H-2 in galactose residue

The ^{31}P NMR spectrum of **(*R*)-3** showed a signal at 14.291 ppm and the resonance for this atom in the CPS is in close agreement (14.257 ppm). On the other hand, the ^{31}P NMR spectrum of **(*S*)-3** showed a resonance at 13.849 ppm. Both spectra contained a single resonance, which suggests that there was no loss of stereochemical integrity on phosphorus during the deprotection. Had scrambling of the phosphorus stereochemistry occurred, a mixture of the two diastereomers would be expected, not complete conversion to the opposite stereoisomer. When considering the H-2 signal of galactose residue in the ^{1}H NMR spectra, the data for **(*R*)-3** (4.54 ppm) is in close agreement with that reported for the CPS (4.52 ppm), whereas the data for **(*S*)-3** differs. Based on these comparisons, we conclude that MeOPN group in the *C. jejuni* 81-176 CPS is *R*.

In conclusion, we have successfully synthesized the three repeating units present in CPS produced by *C. jejuni* 81-176, including one that contains a MeOPN motif. While carrying out these syntheses we obtained a crystal structure of a key intermediate that, following its deprotection and comparison with data for the polysaccharide, established the phosphorous stereochemistry of the MeOPN in the native CPS as *R*. This represents the first unambiguous determination of the stereochemistry of a campylobacter CPS MeOPN group, which exists naturally as a single stereoisomer. It remains to be determined if MeOPN-functionalized CPS produced by other campylobacter have the same stereochemistry. However, we note that the biosynthesis of the group is done enzymatically,[6] which, depending on the similarities of enzymes across species, could suggest it is the same in all campylobacters. The use of ***(R)*-3** and its enantiomer ***(S)*-3** in probing the specificity of sera raised against vaccination with the native CPS is ongoing and these compounds will be useful probes in studies of MeOPN function and assembly. We anticipate this study will also motivate the development of methods for the diastereoselective introduction of MeOPN groups into carbohydrates.

Acknowledgements

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Keywords: Capsular polysaccharide • 6-deoxyheptose • glycosylation • NMR spectroscopy • phosphoramidate

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[16] Crystallographic data for **19**: $C_{81}H_{110}N_5O_{18}PSi$, M_r = 1500.79, colourless needle, 0.38 x 0.06 x 0.05 mm, orthorhombic, $P2_12_12_1$ (No. 19), a = 15.1429(3) Å, b = 20.0680(4) Å, c = 26.8195(6) Å, V = 8150.1(3) Å^3, Z = 4, ρ_{calcd} = 1.223 g cm^{-3}, μ = 1.008 mm^{-1}, Cu Kα (1.54178), T = –100 °C, 2θ limit = 144.77°, no. of measured (48945) and independent (15989) reflections, R_{int} = 0.0513, R_1 = 0.0436, wR_2 = 0.1137, largest difference peak and hole 0.282 and –0.333 e Å^{-3}. Data were collected on a Bruker D8 diffractometer, equipped with a Cu microfocus source and an APEX II CCD detector, using 1.0° ω and ϕ scans and 15 s exposures. Face-indexed absorption corrections were performed using SADABS-2016. The structure was solved using intrinsic phasing method of SHELXT-2014 and refined with the program SHELXL-2018. The CIF has been deposited at the Cambridge Crystallographic Data Centre (CCDC 1866111) and can be obtained free of charge via https://www.ccdc.cam.ac.uk/structures/

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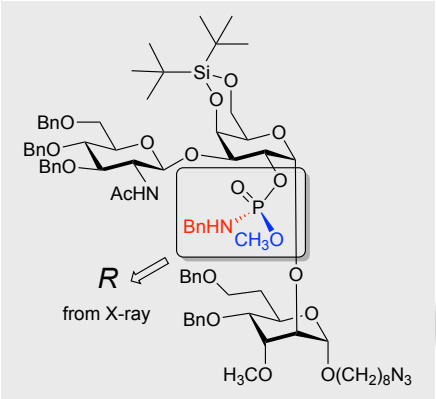
A single approach enabled the synthesis of three trisaccharide fragments of the capsular polysaccharide produced by *C. jejuni* 81-176. The targets all include 6-deoxy-D-*altro*-heptose and one is functionalized with an *O*-methyl phosphoramidate motif, which contains a stereogenic phosphorus atom. X-ray analysis of an advanced intermediate allowed, for the first time, the phosphorus stereochemistry in the natural glycan to be determined.



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