Oligosaccharides

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Total Synthesis of the Complete Protective Antigen of *Vibrio cholerae* O139

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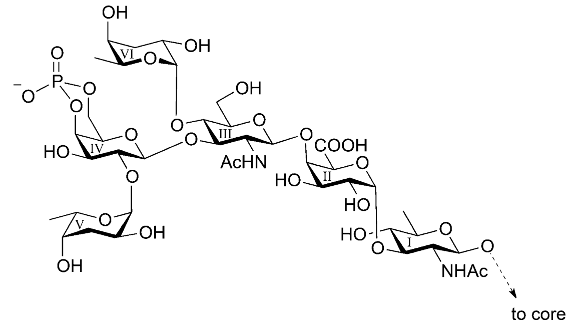
Abstract: *The first chemical synthesis of the complete protective O-antigen of a human-disease-causing pathogenic bacterium is described. The synthesis involved a protecting-group strategy that facilitated the regioselectivity of the key transformations, stereoselective glycosylation reactions, and enabled the one-step global deprotection of the completely assembled, fully protected, phosphorylated hexasaccharide by hydrogenation/hydrogenolysis. The final amino-group-functionalized, linker-equipped antigen was obtained in a form ready for conjugation to suitable carriers, for example, proteins, to yield immunogens.*



Scheme 1. Structure of the O-antigen of *Vibrio cholerae* O139.

Cholera in humans is caused by two strains of *Vibrio cholerae*: *Vibrio cholerae* O1 and *Vibrio cholerae* O139.[1–4] The disease is endemic in over 50 countries; it affects 3–5 million individuals each year and results in over 100000 deaths annually. Programs aimed at reducing the global burden of cholera by providing adequate sanitation and safe water have been unsuccessful. The field has realized that the development and deployment of an improved cholera vaccine will be a critical component in cholera control programs, until adequate sanitation and safe water are a reality for the most impoverished individuals on the planet.

We have been involved in developing a glycoconjugate vaccine for cholera from synthetic carbohydrates that mimic the structure of the O-specific polysaccharides (O-SPs, O-antigens) of bacterial pathogens for a number of years.[5–7] Generally, the prerequisite for developing such a vaccine is the availability of a large fragment of the O-specific polysaccharide characteristic of the bacterium, because O-SPs are the protective antigens.[8] *Vibrio cholerae* O139 is unique among bacteria that cause disease in humans in that the complete protective antigen is a single phosphorylated hexasaccharide consisting of five different monosaccharides (α-Col*p*-(1→2)-4,6-*P*-β-D-Gal*p*-(1→3)-[α-Col*p*-(1→4)]-β-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→3)-β-D-Qui*p*NAc-(1→; Scheme 1)[9,10] and not a chain of oligosaccharide repeating units, which is the usual scenario within the Gram-negative bacteria.[11] In the case of *Vibrio cholerae* O139, the work towards a synthetic vaccine has been hampered because the critical hexasaccharide has not been synthesized, although the structure was elucidated two decades ago. The lack of such a synthesis lies undoubtedly in the difficulties involved in the synthesis, isolation, and purification of the charged substance.

Having first verified the methodology that would be involved in the synthesis of the title antigen,[12,13] we now report the first total synthesis of the complete protective O-antigen of *Vibrio cholerae* O139 in a form ready for conjugation. The synthesis started with the preparation of the key disaccharide building blocks **3** and **8** (Scheme 2). Glycosidation of the α-glycosyl bromide **1**[13] with the 4,6-benzylidene acceptor **2**[14] was successfully carried out in the presence of the promoter AgOTf by the use of our improved protocol,[15] which avoids the use of molecular sieves, to afford exclusively the β-(1→3)-linked disaccharide **3** in excellent yield (90%). The configuration of the β-interglycosidic linkage was confirmed by both ^{1}H NMR ($\delta_H = 4.75$ ppm, d, $J_{1,2} = 8.1$ Hz, H-1^{II}) and ^{13}C NMR spectra ($\delta_C = 99.4$ ppm, $^1J_{C1,H-1} = 165.2$ Hz, C1^{II}).

Zemplén de-O-acylation of the α-(1→3)-linked disaccharide **4**[12] gave tetraol **5** in virtually quantitative yield, and subsequent *p*-methoxybenzylidenation of **5** gave selectively the 4^{II},6^{II}-acetal derivative **6** (92%). Controlled benzylation of **6** with benzyl bromide and sodium hydride in DMF–DME at low temperature (**6**→**7**, 86%), to minimize elimination at C6^I in the presence of a strong base, followed by regioselective reductive opening of the alkylidene ring in **7**, afforded the linker-equipped disaccharide acceptor **8** in 89% yield. As compared to the ^{13}C NMR spectrum of **7**, the signal for C4^{II} in **8** ($\delta_C = 68.2$ ppm) was shifted upfield (by ca. 6 ppm), which confirmed that the reductive opening of the *p*-methoxybenzylidene acetal led to the HO-4^{II}-free, 6^{II}-*p*-methoxybenzyl ether.

With the two building blocks **3** and **8** in hand, we focused on the 2+2 coupling. Accordingly, NIS/AgOTf-promoted

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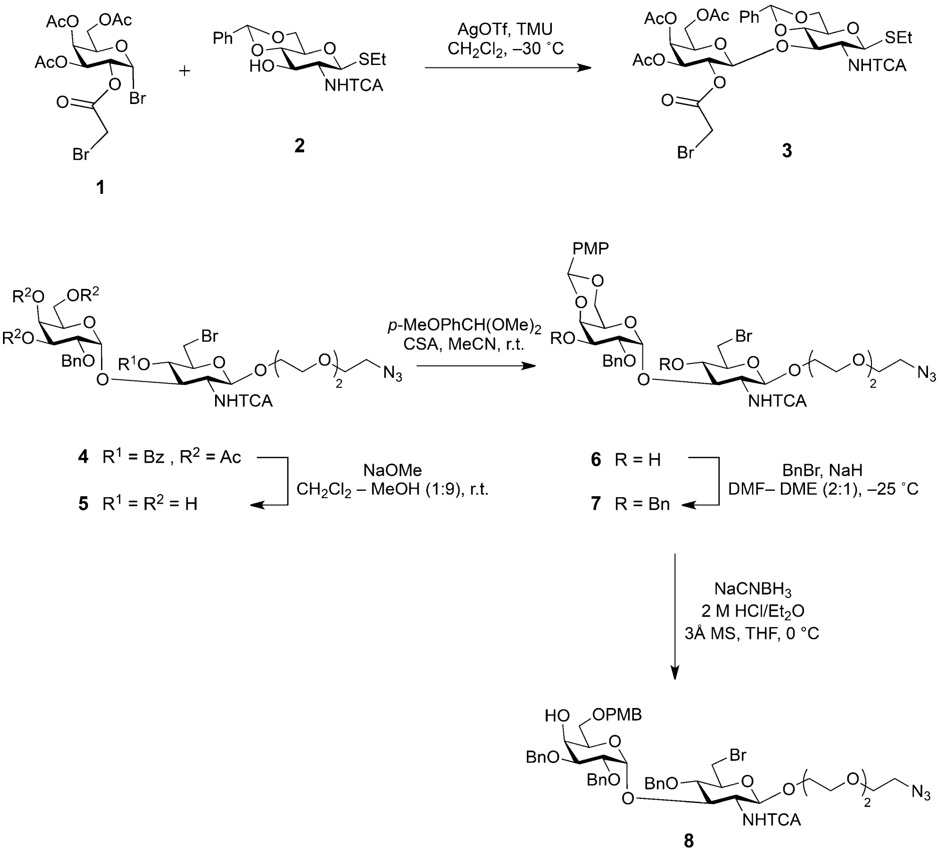


Scheme 2. Synthesis of the key disaccharide building blocks **3** and **8** (TCA = trichloroacetyl, Tf = trifluoromethanesulfonyl, TMU = 1,1,3,3-tetramethylurea, PMP = *p*-methoxyphenyl, PMB = *p*-methoxybenzyl, CSA = 10-camphorsulfonic acid, Bn = benzyl, DMF = *N*,*N*-dimethylformamide, DME = 1,2-dimethoxyethane, MS = molecular sieves).

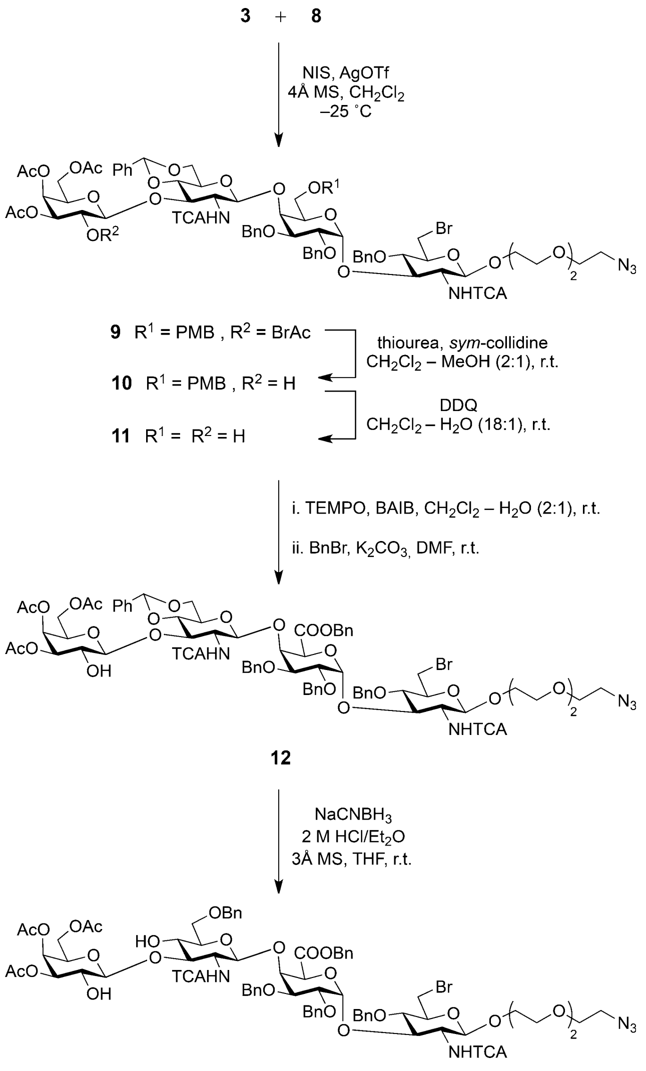
glycosylation of the spacer-equipped disaccharide acceptor **8** with the β-thioglycoside disaccharide donor **3** at −25 °C proceeded stereoselectively to afford the desired linear tetrasaccharide **9** (84 %; Scheme 3). The acidity of the reaction medium was optimized by using excess AgOTf to minimize the conversion of the donor into the corresponding stable oxazoline.[16] The structure of the tetrasaccharide product **9** was confirmed by its ^{1}H and ^{13}C NMR spectra, which showed signals characteristic of both the acceptor and the donor moieties.

Selective removal of the bromoacetyl ester in **9** by treatment with thiourea and *sym*-collidine[17] (**9**→**10**, 95 %), and subsequent oxidative removal of the 6^{II}-O-*p*-methoxybenzyl group with DDQ in CH_2Cl_2–H_2O, gave diol **11** (88 %). Regioselective oxidation of the primary hydroxy group in **11** with a combination of a catalytic amount of TEMPO free radical and a slight excess of BAIB in a diphasic CH_2Cl_2–H_2O solvent system, followed by benzylation (BnBr/K_2CO_3 in DMF) of the formed carboxylic acid, furnished uronate **12** (89 % over two steps). Reductive ring opening of the 4^{II}-6^{II}-O-benzylidene acetal in **12** with sodium cyanoborohydride and 2 M HCl–Et$_2$O in THF at room temperature gave the tetrasaccharide diol acceptor **13** (85 %) with complete regioselectivity.

The stereoselective installation of the two colitose residues at positions 2^{IV} and 4^{III} in diol **13** was first attempted by activation of ethyl 2,4-di-*O*-benzyl-3,6-dideoxy-1-thio-β-L-*xylo*-hexopyranoside (**14**)[18] with CuBr$_2$/Bu$_4$NBr.[19,20] However, the reaction was largely incomplete. Activation of the freshly prepared α-colitosyl bromide **15** under halide-assisted 1,2-*cis* glycosylation conditions[21] was successful (Scheme 4) and afforded the desired hexasaccharide **16** as the major product (66 %) along with two isomeric pentasaccharides (ca. 23 % combined yield), which were readily separable by chromatography. The two pentasaccharides can be either colitosylated to give more of the desired hexasaccharide **16**, or they can be deprotected to give fragments of the O-antigen and used in antigenicity studies related to *Vibrio cholerae* O139. The ^{13}C NMR spectra for **16** showed the expected downfield shift of the signals for C4^{III} and C2^{IV} as a result of colitosylation at these positions. Furthermore, signals for the two anomeric hydrogen atoms of the colitose moieties appeared as doublets in the ^{1}H NMR spectrum at $\delta_H = 5.24$ and 5.03 ppm ($J = 3.2$ and 3.7 Hz, respectively), which confirmed the formation of the desired α-glycosidic linkages.

Subsequent de-O-acetylation (Zemplén) of **16**, followed by selective phosphorylation with 2,2,2-trichloroethyl phosphorodichloridate[22] at −20 °C, gave predominantly the (S_P)-4^{IV},6^{IV}-cyclic 2,2,2-trichloroethyl phosphate **18** (*S*/*R* 9:1, ^{31}P NMR, ca. 91 % combined yield). Global deprotection of **18** (by the transformation of 13 functional groups in a one-pot reaction) was successfully carried out by catalytic hydrogenation/hydrogenolysis (Pd/C, H$_2$, 1 atm) at pH 7 (0.1 M potassium phosphate buffer, to neutralize HCl formed). Compound **20** was obtained (87 %) in a pure state (TLC, NMR[23]) by HPLC.

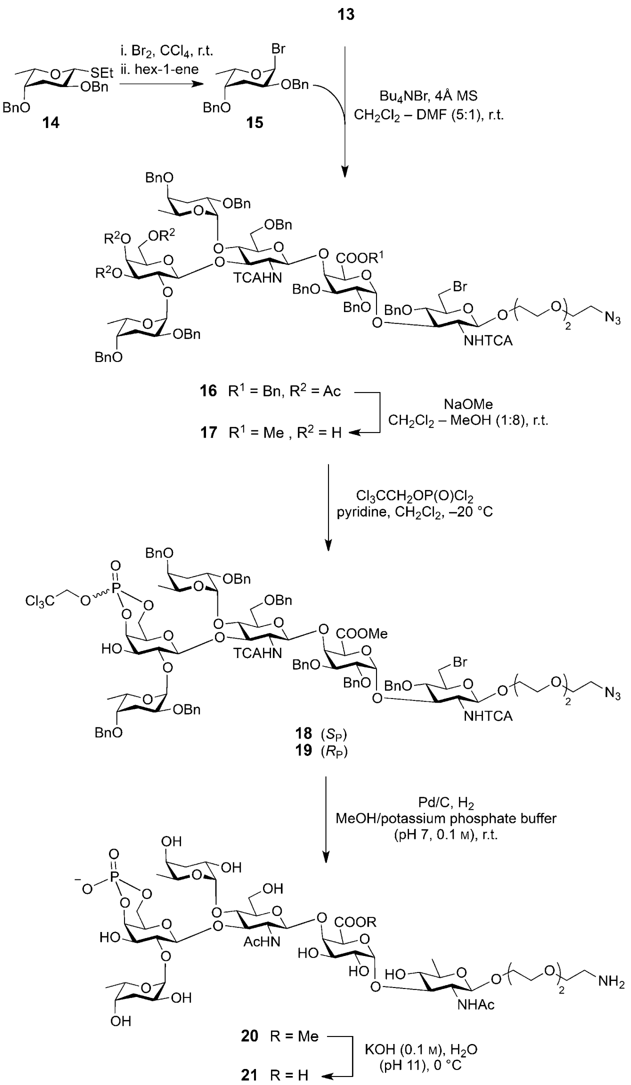
An important feature of the design of this synthetic sequence is that the phosphorylation is performed at a very late stage of the overall synthesis. Although the two isomeric (*S*,*R*) cyclic phosphates can be separated by chromatography, a mixture of the isomeric phosphates formed can be used directly for the reductive deprotection step. Because the phosphorus atom is no longer asymmetric after removal of the trichloroethyl group,[24,25] the same product is formed from *S* and *R* isomers, and the amount of the product can be increased. Accordingly, similar treatment of a mixture of the two isomeric cyclic phosphates **18** and **19** gave, as expected, the desired compound **20** in comparable yield.



Scheme 3. Synthesis of the linear tetrasaccharide diol acceptor **13** (NIS = *N*-iodosuccinimide, DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, TEMPO = 2,2,6,6-tetramethyl-1-piperidinyloxy free radical, BAIB = [bis(acetoxy)iodo]benzene).

Finally, saponification (0.1 M KOH in H_2O at pH 11) of the methyl ester **20** was followed by HPLC purification to give the pure spacer-equipped, phosphorylated hexasaccharide **21** (83% yield), and its structure was confirmed by NMR and HRMS data.[26]

In conclusion, we have completed synthesis of the full O-antigen of the human-disease-causing pathogen *Vibrio cholerae* O139, which is a complex, branched hexasaccharide consisting of five different monosaccharides and a cyclic phosphate (Scheme 1). The highlights of the synthesis include regio- and stereoselective transformations and a protecting-group strategy that allows global deprotection. The final hexasaccharide is equipped with a linker, which is functionalized for conjugation to yield a vaccine. Both conjugation and related antigenicity studies with the hexasaccharide antigen and a wide spectrum of fragments thereof are in progress.



Scheme 4. Synthesis of the spacer-equipped, phosphorylated hexasaccharide **21**.

Acknowledgements

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Keywords: glycosylation · oligosaccharides · O-specific antigens · pathogenic bacteria · stereoselectivity

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[1] J. B. Kaper, J. G. Morris, M. M. Levine, *Clin. Microbiol. Rev.* **1995**, *8*, 48–86.
[2] T. Ramamurthy, S. Garg, R. Sharma, S. K. Bhattacharya, G. B. Nair, T. Shimada, T. Takeda, T. Karasawa, H. Kurazano, A. Pal, Y. Takeda, *Lancet* **1993**, *341*, 703–704.
[3] M. J. Albert, A. K. Siddique, M. S. Islam, A. S. G. Faruque, M. Ansaruzzaman, S. M. Faruque, R. B. Sack, *Lancet* **1993**, *341*, 704.

[4] M. J. Albert, M. Ansaruzzaman, P. K. Bardhan, A. S. G. Faruque, S. M. Faruque, M. S. Islam, D. Mahalanabis, R. B. Sack, M. A. Salam, A. K. Siddique, M. D. Yunus, K. Zaman, *Lancet* **1993**, *342*, 387 – 390.

[5] P. Kováč in *Protein – Carbohydrate Interactions in Infectious Diseases* (Ed.: C. Bewley), Royal Society of Chemistry, London, **2006**, pp. 175 – 220.

[6] A. Chernyak, S. Kondo, T. K. Wade, M. D. Meeks, P. M. Alzari, J. M. Fournier, R. K. Taylor, P. Kováč, W. F. Wade, *J. Infect. Dis.* **2002**, *185*, 950 – 962.

[7] A. A. Tarique, A. Kalsy, M. Arifuzzaman, S. M. Rollins, R. C. Charles, D. T. Leung, J. B. Harris, R. C. LaRocque, A. Sheikh, M. S. Bhuiyan, R. Saksena, J. D. Clements, S. B. Calderwood, F. Qadri, P. Kováč, E. T. Ryan, *Clin. Vaccine Immunol.* **2012**, *19*, 594 – 602.

[8] J. B. Robbins, R. Schneerson, S. C. Szu, V. Pozsgay, *Pure Appl. Chem.* **1999**, *71*, 745 – 754.

[9] A. D. Cox, J. R. Brisson, V. Varma, M. B. Perry, *Carbohydr. Res.* **1996**, *290*, 43 – 58.

[10] A. D. Cox, M. B. Perry, *Carbohydr. Res.* **1996**, *290*, 59 – 65.

[11] Y. A. Knirel in *Microbial Glycobiology: Structures, Relevance and Applications* (Eds.: A. Moran, P. Brennan, O. Holst, M. von Itzstein), Elsevier, Amsterdam, **2009**, pp. 57 – 73.

[12] S. E. Soliman, P. Kováč, *J. Org. Chem.* **2015**, *80*, 4851 – 4860.

[13] S. E. Soliman, P. Kováč, *J. Org. Chem.* **2015**, *80*, 11227 – 11232.

[14] A. A. Sherman, O. N. Yudina, Y. V. Mironov, E. V. Sukhova, A. S. Shashkov, V. M. Menshov, N. E. Nifantiev, *Carbohydr. Res.* **2001**, *336*, 13 – 46.

[15] S. E. Soliman, P. Kováč, *Synthesis* **2014**, *46*, 748 – 751.

[16] J. Banoub, P. Boullanger, D. Lafont, *Chem. Rev.* **1992**, *92*, 1167 – 1195.

[17] *sym*-Collidine was used as a weak non-nucleophilic organic base to prevent any acyl-group migration during the selective O-debromoacetylation reaction.

[18] B. Ruttens, P. Kováč, *Synthesis* **2004**, 2505 – 2508.

[19] S. Sato, M. Mori, Y. Ito, T. Ogawa, *Carbohydr. Res.* **1986**, *155*, C6 – C10.

[20] S. E. Soliman, R. W. Bassily, R. I. El-Sokkary, J. Banoub, M. A. Nashed, *Carbohydr. Res.* **2009**, *344*, 395 – 399.

[21] R. U. Lemieux, K. B. Hendriks, R. V. Stick, K. James, *J. Am. Chem. Soc.* **1975**, *97*, 4056 – 4062.

[22] K. Grześkowiak, *Synthesis* **1980**, 831 – 833.

[23] Data for compound **20**: ^{31}P NMR (162 MHz, D$_2$O): $\delta = -3.73$ ppm ($^3J_{\mathrm{P,H}} = 21.9$ Hz); ^{1}H NMR (600 MHz, D$_2$O): $\delta = 5.23$ (d, $J_{1,2} = 3.8$ Hz, H-1$^{\mathrm{II}}$), 4.96 (d, $J_{1,2} = 3.4$ Hz, H-1$^{\mathrm{V}}$), 4.88 (d, $J_{1,2} = 3.3$ Hz, H-1$^{\mathrm{VI}}$), 4.66 (d, $J_{1,2} = 8.2$ Hz, H-1$^{\mathrm{IV}}$), 4.48 (d, $J_{1,2} = 8.3$ Hz, H-1$^{\mathrm{III}}$), 4.46 ppm (d, $J_{1,2} = 8.5$ Hz, H-1$^{\mathrm{I}}$); ^{13}C NMR (150 MHz, D$_2$O): $\delta = 102.95$ (C1$^{\mathrm{III}}$), 101.17 (C1$^{\mathrm{I}}$), 101.12 (C1$^{\mathrm{IV}}$), 100.88 (C1$^{\mathrm{II}}$), 99.56 (C1$^{\mathrm{V}}$), 97.76 ppm (C1$^{\mathrm{VI}}$); HRMS (ESI-TOF): m/z $[M-\mathrm{H}]^-$ calcd for $C_{47}H_{79}N_3O_{31}P$: 1212.4435; found: 1212.4446.

[24] K. Grześkowiak, R. W. Adamiak, M. Wiewiorowski, *Nucleic Acids Res.* **1980**, *8*, 1097 – 1105.

[25] A. Paquet, *Int. J. Pept. Protein Res.* **1992**, *39*, 82 – 86.

[26] Data for compound **21**: ^{31}P NMR (162 MHz, D$_2$O): $\delta = -3.72$ ppm ($^3J_{\mathrm{P,H}} = 21.4$ Hz); ^{1}H NMR (600 MHz, D$_2$O): $\delta = 5.26$ (d, $J_{1,2} = 3.9$ Hz, H-1$^{\mathrm{II}}$), 4.96 (d, $J_{1,2} = 3.7$ Hz, H-1$^{\mathrm{V}}$), 4.77 (d, overlapped, H-1$^{\mathrm{VI}}$), 4.67 (d, $J_{1,2} = 8.1$ Hz, H-1$^{\mathrm{IV}}$), 4.47 ($J_{1,2} = 8.7$ Hz, H-1$^{\mathrm{I}}$), 4.45 ppm ($J_{1,2} = 8.5$ Hz, H-1$^{\mathrm{III}}$); ^{13}C NMR (150 MHz, D$_2$O): $\delta = 102.79$ (C1$^{\mathrm{III}}$), 101.31 (C1$^{\mathrm{I}}$), 101.17 (C1$^{\mathrm{IV}}$), 100.22 (C1$^{\mathrm{II}}$), 99.45 (C1$^{\mathrm{V}}$) 97.94 ppm (C1$^{\mathrm{VI}}$); HRMS (ESI-TOF): m/z $[M-\mathrm{H}]^-$ calcd for $C_{46}H_{77}N_3O_{31}P$: 1198.4279; found: 1198.4282.

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