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

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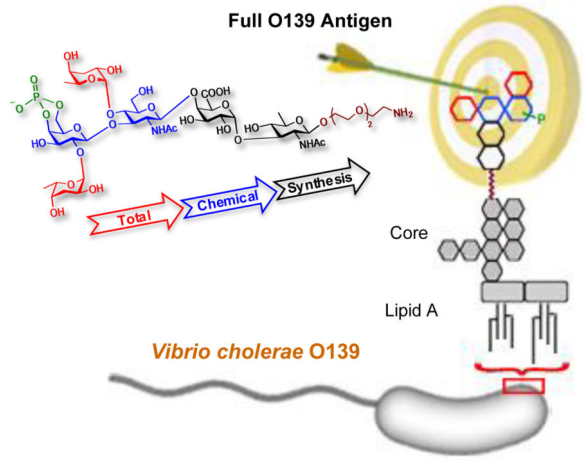
Abstract

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 which facilitated regioselectivity of the key transformations, stereoselective glycosylations, and allowed 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 the form ready for conjugation to suitable carriers, e.g. proteins, to yield immunogens.

Graphical abstract



Keywords

Oligosaccharides; O-Specific Antigen; Glycosylations; Stereoselectivity; *Vibrio cholerae* O139

Cholera in humans is caused by two strains of *Vibrio cholerae* O1 and *Vibrio cholerae* O139.[1–4] The disease is endemic in over 50 countries; it affects 3 to 5 million individuals

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each year, resulting in the deaths of over 100,000 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 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 structure of the O-specific polysaccharides (O-SP, O-antigen) 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→, Figure 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 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 on the first total synthesis of the complete protective O-antigen of *Vibrio cholerae* O139 in the form ready for conjugation.

The synthesis started with the preparation of the key disaccharide building blocks **3** and **8** (Scheme 1). Glycosidation of the α-glycosyl bromide **1**[13] with the 4,6-benzylidene acceptor **2**[14] was successfully carried out in the presence of AgOTf promoter using 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 (δ_H 4.75, d, $J_{1,2}$ = 8.1 Hz, H-1^{II}) and ^{13}C NMR (δ_C 99.4, $^{1}J_{C\text{-}1,H\text{-}1}$ = 165.2 Hz, C-1^{II}) spectra.

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** using benzyl bromide and sodium hydride in DMF-DME at low temperature (**6** to **7**, 86%), to minimize elimination at C-6^{I} in the presence of strong base, followed by regioselective reductive opening of the alkylidene ring in **7,** afforded the linker-equipped disaccharide acceptor **8** in 89% yield. Compared to the ^{13}C NMR spectrum of **7**, the signal for C-4^{II} in **8** (δ_C 68.2) shifted upfield (by ~6 ppm), which confirmed that the reductive opening of the *p*-methoxybenzylidene acetal led to the H*O*-4^{II}-free, 6^{II}-*p*-methoxybenzyl ether.

With the two building blocks **3** and **8** at hand, we focused on the 2+2 coupling. Accordingly (Scheme 2), NIS/AgOTf-promoted 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%). 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] Structural identification of the tetrasaccharide product **9** was provided 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** to **10**, 95%), and subsequent oxidative removal of the 6^{II}-*O*-*p*-methoxybenzyl group using DDQ in CH_2Cl_2-H_2O gave diol **11** (88%). Regioselective oxidation of the primary hydroxyl 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** using sodium cyanoborohydride and 2M HCl-Et_2O in THF at room temperature gave, with complete regioselectivity, the tetrasaccharide diol acceptor **13** (85%).

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 $CuBr_2/Bu_4NBr$.[19,20] However the reaction was largely incomplete. The activation of the freshly prepared α-colitosyl bromide **15** at halide-assisted 1,2-*cis* glycosylation conditions,[21] was successful (Scheme 3), and afforded the desired hexasaccharide **16** as the major product (66%) along with two isomeric pentasaccharides (~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 arrive at fragments of the O-antigen, and used in *Vibrio cholerae* O139-related antigenicity studies. The ^{13}C NMR spectra for **16** showed the expected downfield shift of the signal for C-4^{III} and C-2^{IV} as a result of colitosylation at these positions. In addition, signals for the two anomeric protons of the colitose moieties appeared as doublets (^{1}H NMR) at δ_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, ~91% combined yield). Global deprotection of **18** (by transforming 13 functional groups in 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 pure state (TLC, NMR[23]) by HPLC.

Performing the phosphorylation at a very late stage of the overall synthesis is an important feature in the design of this synthetic sequence. 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.

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

In conclusion, we have achieved the first synthesis of the full O-antigen of a human disease-causing pathogen (*Vibrio cholerae* O139, which is a complex, branched hexasaccharide consisting of five different monosaccharides and a cyclic phosphate, Figure 1). The highlights of the synthesis include: regio- and stereoselective transformations, and the 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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23. Data for compound **20**: ^{31}P NMR (162 MHz, D_2O): δ= −3.73 ($^3J_{P,H}$ = 21.9 Hz); ^{1}H NMR (600 MHz, D_2O): δ= 5.23 (d, $J_{1,2}$ = 3.8 Hz, H-1^{II}), 4.96 (d, $J_{1,2}$ = 3.4 Hz, H-1^{V}), 4.88 (d, $J_{1,2}$ = 3.3 Hz, H-1^{VI}), 4.66 (d, $J_{1,2}$ = 8.2 Hz, H-1^{IV}), 4.48 (d, $J_{1,2}$ = 8.3 Hz, H-1^{III}), 4.46 (d, $J_{1,2}$ = 8.5 Hz, H-1^{I}); ^{13}C NMR (150 MHz, D_2O): δ = 102.95 (C-1^{III}), 101.17 (C-1^{I}), 101.12 (C-1^{IV}), 100.88 (C-1^{II}), 99.56 (C-1^{V}) 97.76 (C-1^{VI}); HRMS (ESI-TOF): *m/z* [M – H]$^-$ calcd for $C_{47}H_{79}N_3O_{31}P$: 1212.4435; found: 1212.4446.
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26. Data for compound **21**: ^{31}P NMR (162 MHz, D_2O): δ = −3.72 ($^3J_{P,H}$ = 21.4 Hz); ^{1}H NMR (600 MHz, D_2O): δ = 5.26 (d, $J_{1,2}$ = 3.9 Hz, H-1^{II}), 4.96 (d, $J_{1,2}$ = 3.7 Hz, H-1^{V}), 4.77 (d, overlapped, H-1^{VI}), 4.67 (d, $J_{1,2}$ = 8.1 Hz, H-1^{IV}), 4.47 ($J_{1,2}$ = 8.7 Hz, H-1^{I}), 4.45 ($J_{1,2}$ = 8.5 Hz, H-1^{III}); ^{13}C NMR (150 MHz, D_2O): δ = 102.79 (C-1^{III}), 101.31 (C-1^{I}), 101.17 (C-1^{IV}), 100.22 (C-1^{II}), 99.45 (C-1^{V}) 97.94 (C-1^{VI}); HRMS (ESI-TOF): *m/z* [M – H]$^-$ calcd for $C_{46}H_{77}N_3O^{31}P$: 1198.4279; found: 1198.4282.

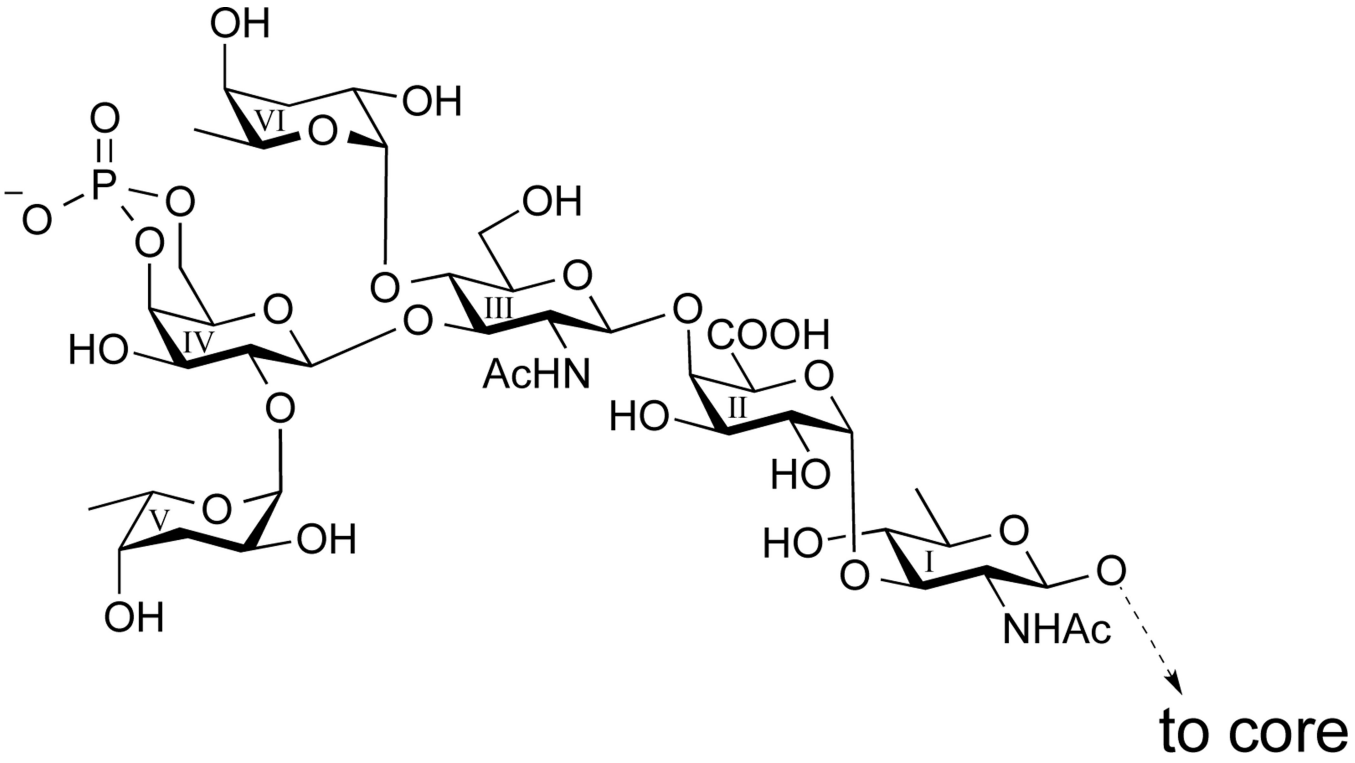
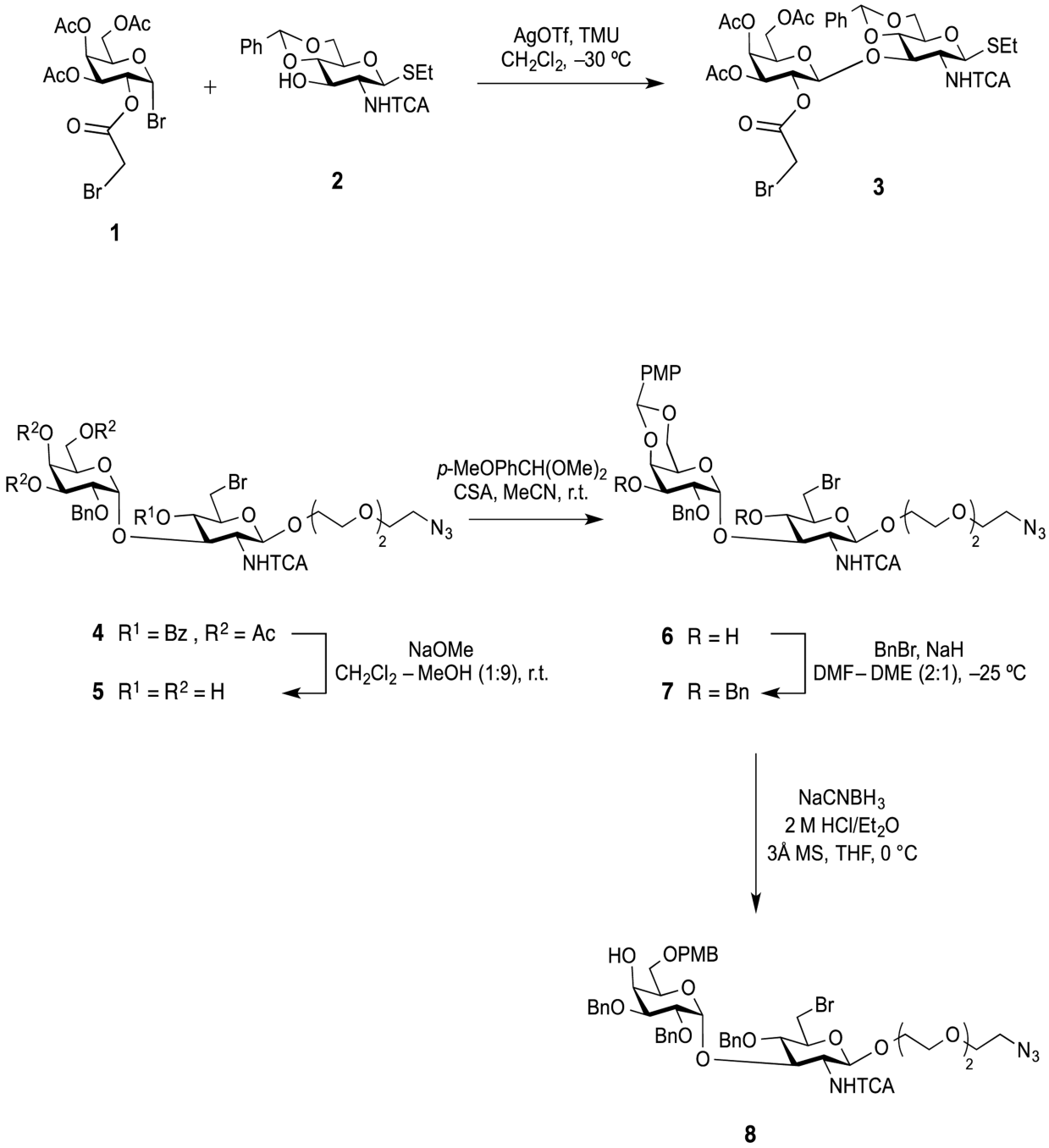
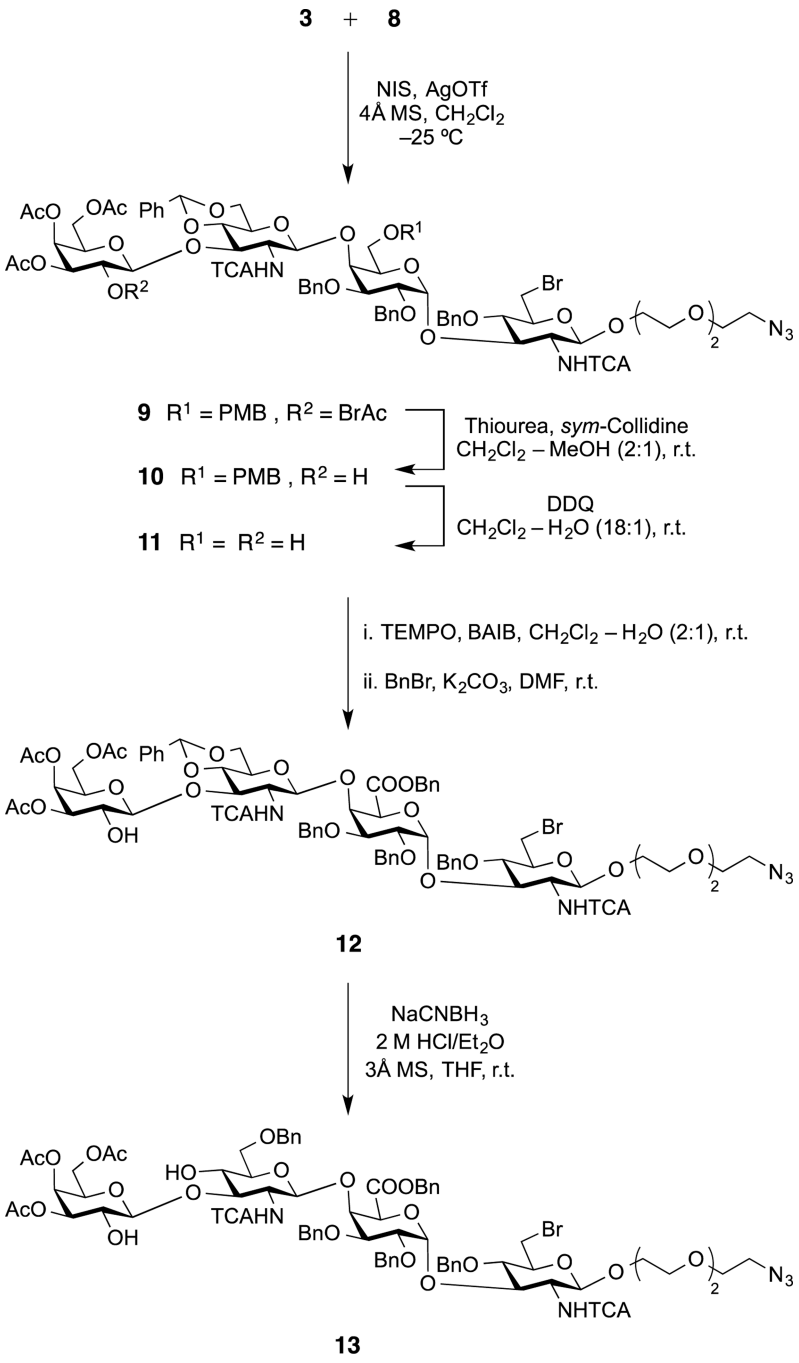


Figure 1.
Structure of the O-Antigen of *Vibrio cholerae* O139.



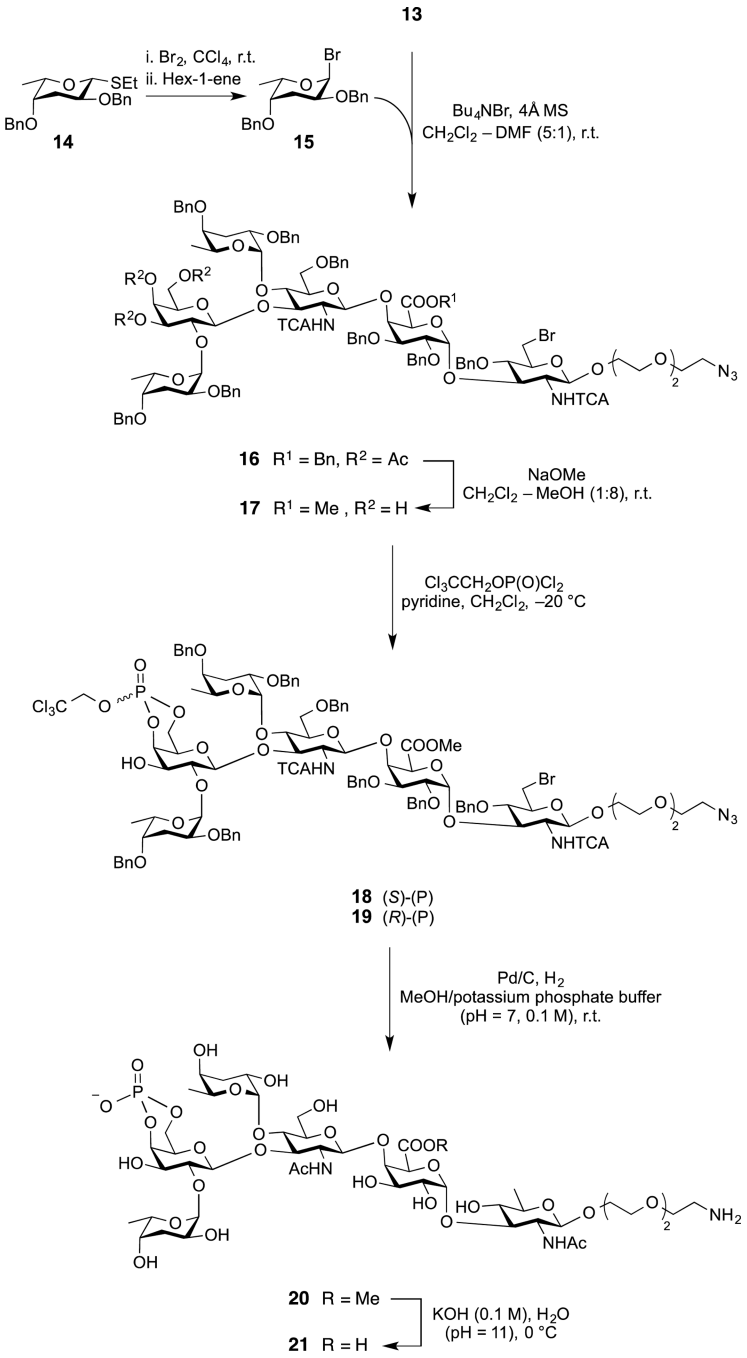
Scheme 1.

Synthesis of the Key Disaccharide Building Blocks **3** and **8**. (TCA = trichloroacetyl, AgOTf = silver trifluoromethanesulfonate = silver triflate, TMU = 1,1,3,3-tetramethylurea, PMP = *p*-methoxyphenyl, PMB = *p*-methoxybenzyl, CSA = 10-camphorsulfonic acid, MS = molecular sieves, DMF = *N,N*-dimethylformamide, DME = 1,2-dimethoxyethane, THF = tetrahydrofuran).



Scheme 2.

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).



Scheme 3.

Synthesis of the Spacer-Equipped, Phosphorylated Hexasaccharide **21**.