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Total Synthesis of the Congested, Bisphosphorylated *Morganella morganii* Zwitterionic Trisaccharide Repeating Unit

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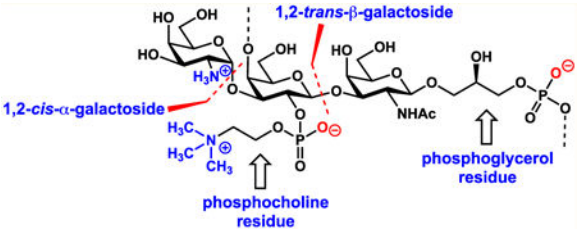
Abstract

Zwitterionic polysaccharides (ZPSs) activate T-cell-dependent immune responses by major histocompatibility complex class II presentation. Herein, we report the first synthesis of a *Morganella morganii* ZPS repeating unit as an enabling tool in the synthesis of novel ZPS materials. The repeating unit incorporates a 1,2-*cis*-α-glycosidic bond; the problematic 1,2-*trans*-galactosidic bond, Gal-β-(1 → 3)-GalNAc; and phosphoglycerol and phosphocholine residues which have not been previously observed together as functional groups on the same oligosaccharide. The successful third-generation approach leverages a first in class glycosylation of a phosphoglycerol-functionalized acceptor. To install the phosphocholine unit, a highly effective phosphocholine donor was synthesized.

Graphical abstract



INTRODUCTION

The capsule is a protective structure on the surface of a number of microbes. Its surface is coated with capsular polysaccharides (CPSs), incorporating residues that differ significantly in structure from those of the mammalian glycome.[1,2] Due to their incongruity, CPSs are ligands for the human immune system.[3,4] CPSs are antigens that work by activating B cells through cross-linking of cell surface receptors (Figure 1A). These B cells are partially activated and differentiate into plasma B cells that produce low-affinity IgM antibodies. In the absence of external mediators, only a *T-cell-independent* humoral response is induced.

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ASSOCIATED CONTENT

Supporting Information

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Experimental procedures, characterization data, and NMR spectra (PDF)

The authors declare no competing financial interest.

Unlike other carbohydrate antigens, zwitterionic polysaccharides (ZPSs) possess an alternating charge motif and elicit a *T-cell-dependent* response from the immune system (Figure 1B).[5,6] Mechanistically, an antigen-presenting cell (APC) consumes and lyses the ZPS. Next, antigenic fragments are loaded onto major histocompatibility class II (MHC-II) receptors. The APC then presents the resulting antigen/MHC-II complex on the cell surface. Helper T cells (T_h cells) bind to the antigen/MHC-II complex through the T-cell receptor (TCR), initiating cytokine release from the T_h cell which activates cytotoxic T cells (CTLs). B cells are also activated. In a parallel pathway, activated T_h cells bind to antigen-bound B cells to produce cytokines that fully activate B cells. This action results in immunoglobulin class switching and affinity maturation to produce high affinity IgG antibodies and memory B cells. *Memory B cell formation is central to a long- lasting immune response.*

Due to their immuno-stimulatory properties, ZPSs have been studied in a number of immunology settings. For example, ZPSs function as adjuvants when coadministered with poorly immunogenic antigens. In studies by the Wack group, ZPSs coadministered with the tetanus toxoid antigen were observed to increase antibody titers *in vivo*.[7]

The most common use of ZPSs is in cancer immunotherapy as substitutes for carrier proteins in vaccine development. ZPSs offer several advantages over carrier proteins such as keyhole limpet hemocyanin (KLH).[8–13] Carrier protein–antigen conjugates are difficult to synthesize and characterize. They also elicit low levels of IgG antibodies in patients.[14,15] It is believed this profile is due to the strong immune response elicited by carrier proteins themselves and their inadvertent suppression of immune responses to the antigen.[16] The most well-characterized ZPS is PS-A1 (**1**), a *Bacteroides fragilis* CPS (Figure 1C).[17] Foundational work by the Andreana lab has shown that, when administered to rodents, PS-A1 conjugates to tumor-associated carbohydrate antigens (TACAs) elicit high antibody titers.[18–21] Moreover, the antibodies are highly selective for their respective antigen. The Seeberger and Andreana laboratories have independently completed total syntheses of PS-A1 to better characterize its immunostimulatory properties.[22,23]

Recently, we took note of the *Morganella morganii* ZPS (MM-ZPS, **2**).[24] *In vitro* binding assays revealed that **2** engages the MHCII in a manner competitive with PS-A1. Moreover, cellular studies suggest that **2** activates CD4+ T-cells. Antibody binding assays of hydrolyzed fragments and oligomers of the repeating unit revealed that the phosphocholine functional group was the dominant element of the epitope. Isolating MM-ZPS from the microbial cell surface for further evaluation is challenged by two problems. First, extraction and purification are complex and result in the isolation of small, impure batches. Second, the isolation process can alter the ZPS. Thus, there is an unmet need to synthesize structurally defined antigens. While it is general practice in the field to synthesize repeating units that are ready for chemical polymerization, our strategy differs in that we plan to use the repeating unit as ink to “print” unnatural ZPS nanomaterials.[25] Indeed, three-dimensional (3D) printing is a frontier area of nanoscience due to its ability to produce 3D objects in advanced applications in areas ranging from tissue engineering to biomaterials. We hypothesize that nanoprinting can be used to synthesize ZPS materials, in the absence of traditional conjugation handles, through deposition of the ZPS onto polyelectrolyte (PE)

complexes with nanometer precision in all dimensions. Described herein is the first step in that process, the total synthesis of the MM-ZPS trisaccharide repeating unit.

RESULTS AND DISCUSSION

At the planning stage, we were drawn to repeating unit **3** which features a tetra-substituted galactose residue housing three of the repeating unit's four charges (Figure 2). This molecule also contains two sites of phosphorylation, including a phosphocholine residue which is rarely observed on carbohydrates.[26] Each residue is galactose derived, and one of the glycosidic bonds is a Gal-β-(1 → 3)-GalNAc linkage. Regardless of the conditions employed, installing this 1,2-*trans*-galactosyl bond is regularly accompanied by orthoester formation (when participating groups are located at C2).[27–32] Based on these considerations, our first-generation approach focused on stepwise functionalization of a central epoxygalactal residue **5**. After forming the 1,2-*cis*-α-glycosidic bond ($GalN_3$-α-(1 → 3)-Gal) (step *i*), we planned to install the phosphoglycerol arm using phosphoramidite **6** (step *ii*). Installation of the 1,2-*trans*-β-glycosidic bond and second oxygen–phosphorus bond would hinge on ring opening of the epoxide (steps *iii* and *iv*, respectively).[33,34]

The synthesis started from Schmidt trichloroacetimidate **8**,[35] prepared in seven steps from D-galactose (Scheme 1A). $BF_3{\cdot}Et_2O$-mediated glycosylation with known 6-*O*-TBS galactal acceptor[36] **9** proceeded selectively at C3 to provide α−**10** in 65% yield as the major product. The high α-selectivity can be attributed to solvent participation in a β-fashion by diisopropyl ether.[37] To avoid temporarily protecting the C3′ position, we installed the phosphoglycerol unit using phosphoramidite **6**.[38–40]

Subsequent oxidation of the intermediate phosphorus(III) species with *m*CPBA provided phosphate ester **11** in 87% yield as an inseparable mixture of phosphorus epimers. Next, epoxidation of the glycal with DMDO gave intermediate epoxide **12**.[34] Unfortunately, ring opening the epoxide was intractable. To circumvent this issue, glycal **11** was converted to thioglycoside donor **15** using a sequence of epoxidation, thiylation, and acylation of the resultant C2′ alcohol (Scheme 1B).[41] Interestingly, glycosylation of **15** with acceptor **13** provided orthoester **16** instead of glycoside **14**. Based on literature precedence, we hypothesized that the orthoacetate could be rearranged to the desired glycoside under Kochetkov conditions.[42,43] However, exposing **16** to acidic media resulted in hydrolysis and decomposition over prolonged reaction times. Derivatives containing electron-deficient acyl-protecting groups at C2 were also examined as donors (see SI) as electron-withdrawing groups have been shown to slow competitive orthoester formation.[37] Orthoester formation dominated regardless of the protecting group at C2.

As we terminated the first-generation approach, it was clear that the Gal-β-(1 → 3)-GalNAc bond was more difficult to install than we anticipated. In fact, with few exceptions, a large number of routine glycosylation procedures favor orthoester formation over glycosidic bond formation.[27–29,31,32,44–49] One obscure tactic used to generate this bond is to mask the C2 amine of the galactosamine acceptor as an azide.[27,50] We incorporated this maneuver into a second-generation approach targeting **3** (Figure 3). The plan called for Gal-β-(1 → 3)-GalNAc glycosidic bond formation early in the synthesis (step *i*). Following conversion of

the azide to an acetamide (step *ii*), we would install the α-glycosidic bond (step *iii*). The final steps would involve installation of the phosphoglycerol (step *iv*) and phosphocholine (step *v*) arms. Installing the phosphoglycerol unit in the latter stages of the synthesis would minimize the number of subsequent steps requiring handling and characterization of phosphorus epimers.

Donor **18** and acceptor **19** were synthesized from **d**-galactose in 6[51] and 12 steps, respectively (see SI). Glycosylation between donor **18** and **19** was followed by saponification to provide β-linked disaccharide **20** in 91% yield over two steps (Scheme 2). Subsequent acetonide protection of the C3′-C4′ alcohols and PMB protection of the C2′ alcohol (selected to allow for C4′ protecting group orthogonality in subsequent steps) gave fully protected disaccharide **21**. Exchange of the C2 amine-protecting group at this stage proved critical as the C2 and C2″ amines are differentially functionalized in the final product. Thus, we planned to use a $GalN_3$-based glycosyl donor to establish the trisaccharide core. $LiAlH_4$ reduction of the C2 azide and protection of the resulting amine as its diacetyl imide afforded **22** in quantitative yield.

Next, deprotection of the C3′–C4′ acetonide with $CuCl_2{\cdot}H_2O$ gave the C3′–C4′ diol acceptor **23** in modest yield along with recovered starting material which could be recycled. Conventional methods of acetonide removal (80% AcOH, *p*-TsOH/MeOH, aq. TFA, etc.) proved unsuccessful and resulted in hydrolysis of the glycosidic bond or conversion of the diacetyl imide to the acetamide. Leveraging the enhanced nucleophilicity of the equatorial C3′ alcohol over the axial C4′ alcohol, we examined glycosylation with imidate donor **8**. While optimized conditions gave a complex mixture of every possible glycosylation product, the desired α-anomer **24** was the major product in 40% yield. Efforts to improve this reaction by esterifying the C4′ position proved futile as the modification curtailed the nucleophilicity of the C3′ alcohol. Having established the trisaccharide core, we next focused on phosphorylation. The C4′ phosphoglycerol residue was installed by coupling **24** with phosphoramidite **6** and subsequently oxidizing at phosphorus using mCPBA. **25** was isolated in 85% yield. At this stage, completion of the MM-ZPS **2** required installation of the C2′ phosphocholine residue and global deprotection. Unfortunately, oxidative removal of the C2′ PMB ether was unsuccessful. Both traditional (DDQ,[52] CAN) and nontraditional (HCl/HFIP,[53] TfOH,[54,55] CBr_4-TPP,[56,57] silver(I),[58] thermolysis,[59] $MgBr_2$,[59] and homogeneous electron transfer[60]) conditions proved ineffective. Reassessing the route, we hypothesized the central galactose residue was too hindered to allow for installation of all substituents. Accordingly, we identified a new target repeating unit where the phosphoglycerol residue was shifted to the reducing end (Figure 4). This modification transfers a substituent away from the central residue and eliminates synthesis of a repeating unit with an unnatural *O*-alkyl capping unit at the reducing end.

At the planning stage (Figure 5), it was clear that the synthesis of this new target (**2**) would maintain its novelty as the strategy would now feature an unprecedented glycosylation reaction, use of a phosphoglycerol-containing acceptor (step *i*). We opted to use thioglycoside donors to form each glycosidic bond due to their stability and scalability. The trisaccharide backbone would be constructed from its nonreducing end to its reducing end to minimize waste of the more valuable acceptor (steps *ii* and *iii*). The final challenge would be

installation of the phosphocholine unit (step *iv*), which has thus far proved elusive. Thus, we anticipated developing a new reagent to install this motif.

The third-generation approach commenced with an NIS-TMSOTf-promoted coupling of thioglycoside **31** to phosphoglycerol acceptor **32** (see SI). After glycosylation, $NaOCH_3$-mediated removal of the C3 acetate gave β–34 in 93% yield over two steps (Scheme 3). Presumably, neighboring group participation from the C2 trichloroacetamide assisted in exclusive formation of the β-anomer. Subsequent NIS-TMSOTf-promoted glycosylation with thioglycoside **30** occurred in 91% yield to give **35**.

Again, the high β-selectivity can be attributed to neighboring group participation, this time from the C2′ acetate. Next, we moved forward with liberating the C3′ alcohol, which would serve as the acceptor in the final glycosylation event. To our surprise, we encountered difficulties while removing the silyl ether. The most productive conditions, HF-pyridine buffered by additional pyridine, provided acceptor **36** in 69% yield. A third NIS-TMSOTf-promoted glycosylation with thioglycoside **29** (see SI) gave trisaccharide **37** in 71% yield as a 2.4 to 1 mixture of α and β anomers. A number of α-selective additives (DMF, thioethers, and ethers) were evaluated in the reaction. Ultimately, 25 equiv of diisopropyl ether was the most α-selective additive. Finally, $NaOCH_3$-mediated removal of the C2′ acetate provided trisaccharide **38** in 95% yield.

With the trisaccharide core in hand, we arrived at the most critical step of the synthesis, installation of the phosphocholine residue (Scheme 4). The initial approach to phosphorylation focused on using the well-established cyclic phospholanes **40** and **41**. In theory, following phosphorylation, the 5-membered ring of **42** can be opened by trimethylamine to provide the phosphocholine unit. Unfortunately, this line of inquiry was met by two challenges. First, the C2′ alcohol of **38** proved to be both too sterically hindered and poorly nucleophilic to couple with either phospholane. Second, cyclic phospholanes are vulnerable to heat, moisture, and silica gel chromatography.

To introduce this residue, we synthesized a new phosphocholine donor, choline-2-cyanoethyl *N*,*N*-diisopropyl-phosporamidate tetraphenylborate **44**, which functioned very well when using tetrazole as an activator. After formation of the oxygen–phosphorus bond (**33** + **38**), subsequent oxidation to the phosphate was achieved using *tert*-butyl hydroperoxide (TBHP). Finally, exposure to DBU led to removal of the cyanoethyl group, via β-elimination, to provide the desired phosphodiester **45** in 75% yield over the three steps.

With the complete core structure in hand, the final maneuver in the synthesis was a remarkable, exhaustive catalytic hydrogenation of **45**. This reaction served to remove three benzylidene acetals, one PMB ether, and three benzyl ethers. The reductive environment also converted the C2 trichloroacetamide to the acetamide and the C2″ azide to an amine. Starting from 330 mg (0.2 mmol) of **45**, purification using size exclusion chromatography (P2-Biogel) gave 155 mg of MM-ZPS **2** in 89% isolated yield. Charge-deconvoluted ESI FT ICR (electron spray ionization Fourier transformation ion cyclotron resonance) mass spectrometry revealed molecular mass spectral peaks in agreement with the desired calculated mass (calcd [M + H] for $C_{28}H_{55}N_3O_{23}P_2$ = 864.2780, found 864.2772). ^{1}H NMR

coupling constants revealed the presence of one α glycosidic bond at C1″ (J = 5.33, 3.6 Hz) and two β glycosidic bonds at C1′ (J = 4.70, 7.9 Hz) and C1 (J = 4.46, 7.6 Hz). 2-D NMR experiments, including ^{1}H–^{31}P HSQC, enabled full assignment of the ZPS's glycosidic bonds and phosphorus functionality. Additionally, the NMR data from the synthetic repeating unit **2** are in complete agreement with the data reported for the naturally occuring MM-ZPS polymer (Table S1).

CONCLUSION

In summary, we have completed the first total synthesis of the repeating unit of MM-ZPS **2**. Key steps include: (1) early stage phosphoglycerol glycosylation occurring in high yield and excellent β-selectivity, (2) formation of the challenging Gal-β- (l → 3)-GalNAc bond without generating any undesired orthoacetate byproduct, and (3) installation of the phosphocholine group using a new choline donor. As studies with the naturally occurring MM-ZPS demonstrated that single repeating units do not elicit an immune response, current efforts are focused on using the synthetic repeating unit to synthesize non-natural ZPS materials. Studies regarding the synthesis and immunological properties of these materials are currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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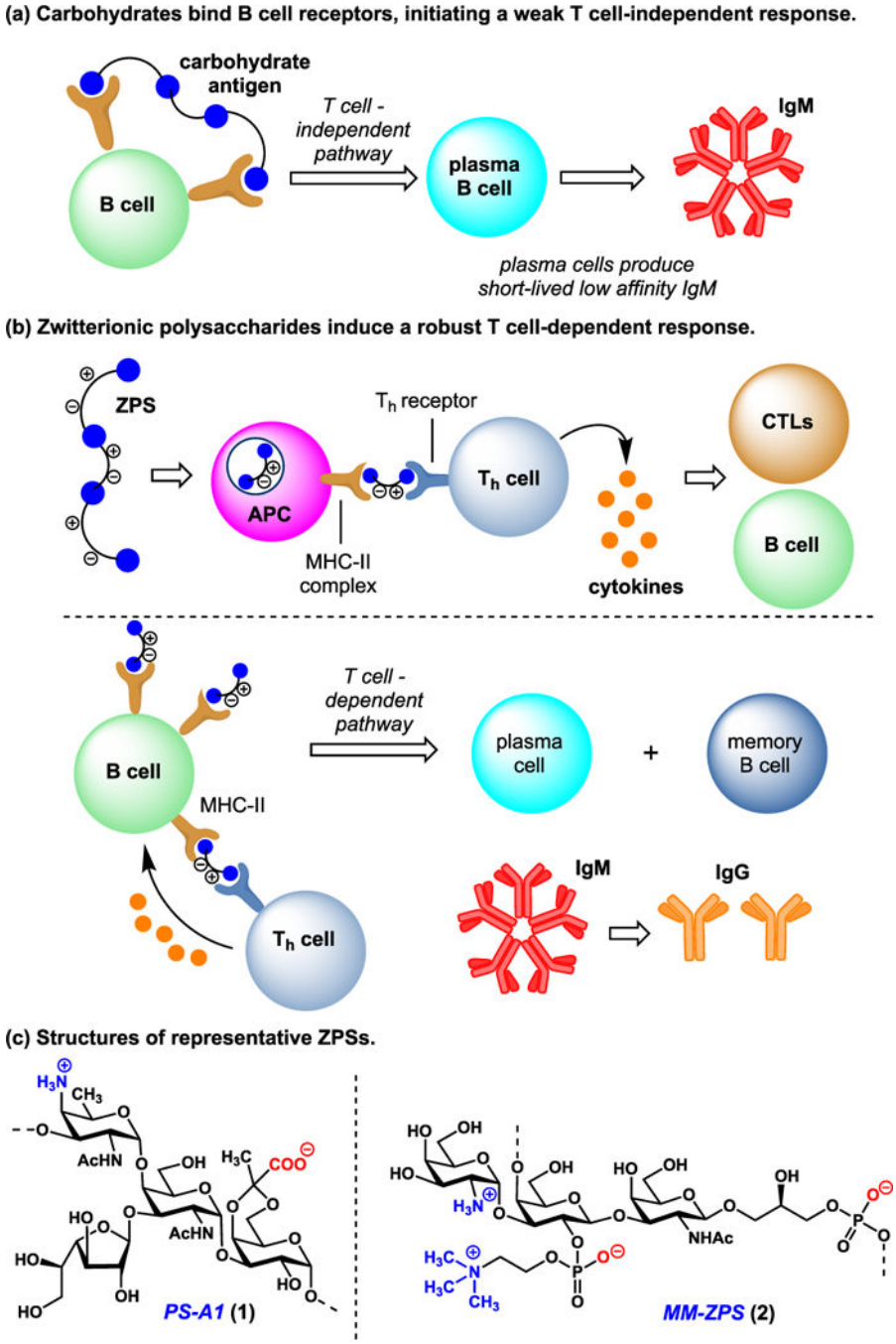


Figure 1.

(a),(b) Immunological response to carbohydrate antigens. (c) Structures of PS-A1 (**1**) and MM-ZPS (**2**).

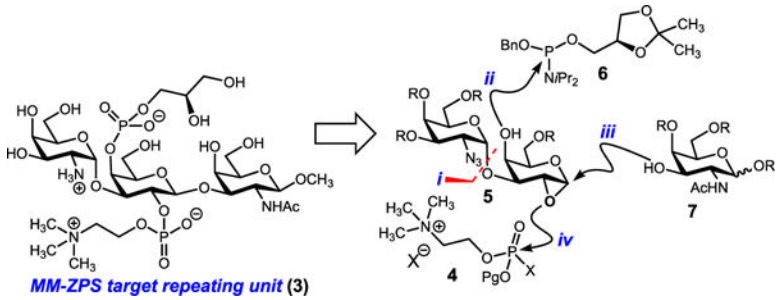


Figure 2.
First-generation analysis.

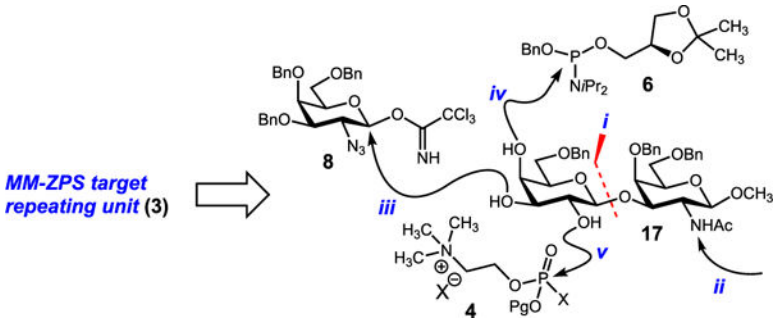


Figure 3.
Second-generation analysis.

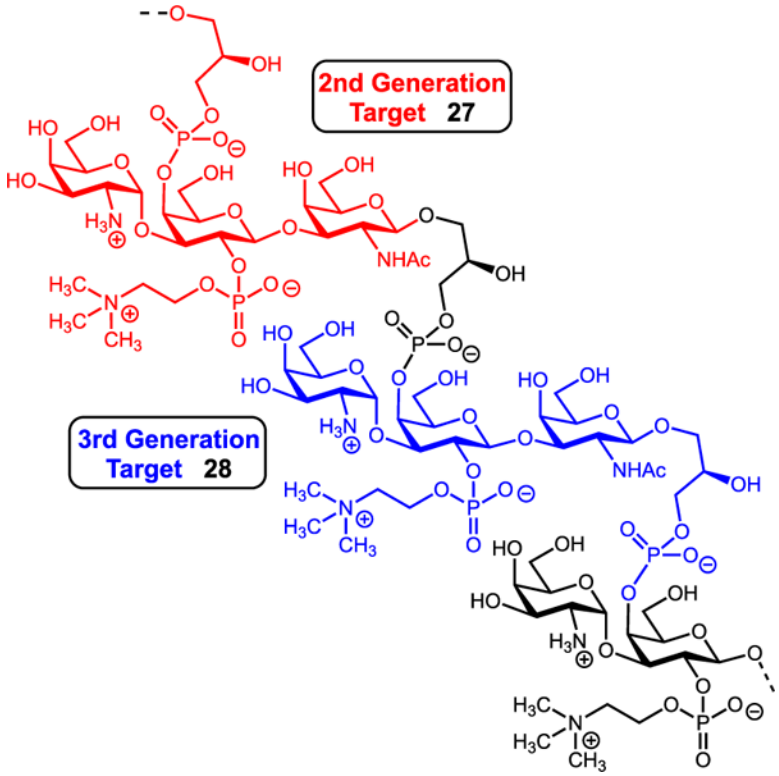


Figure 4.
Target repeating unit frameshift.

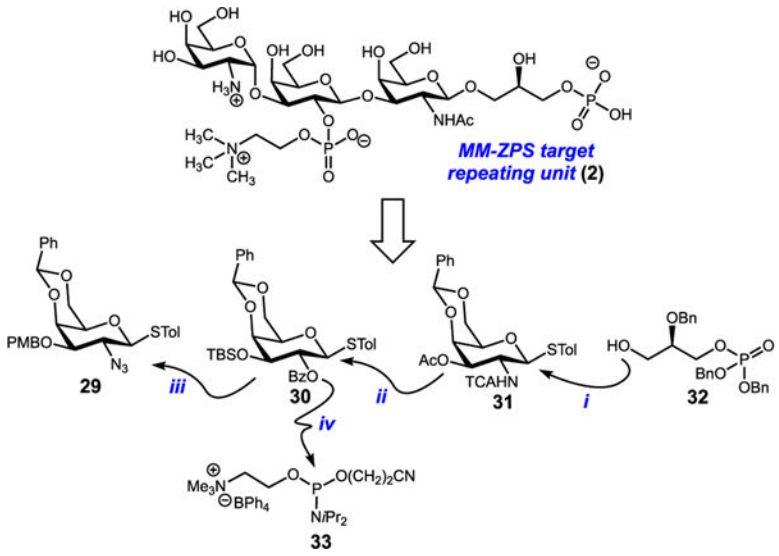
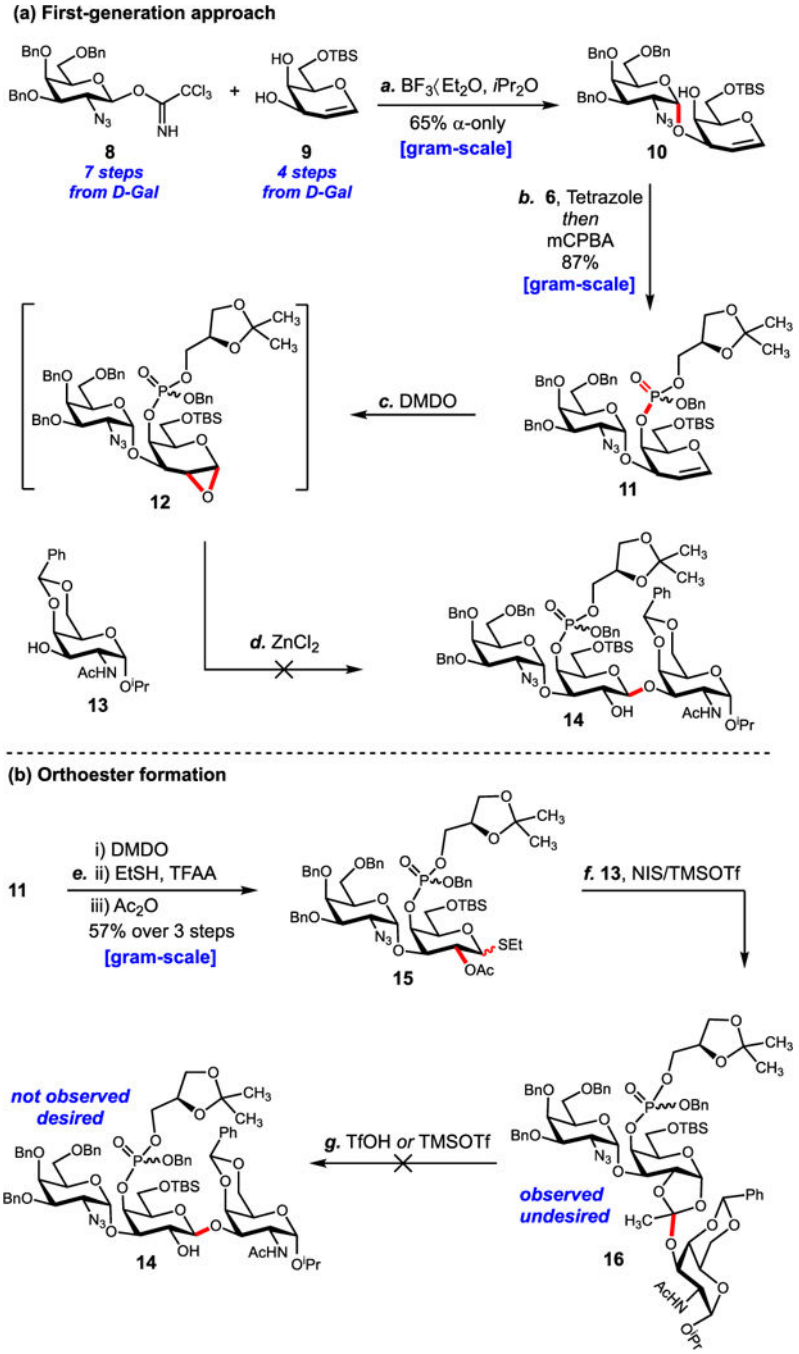


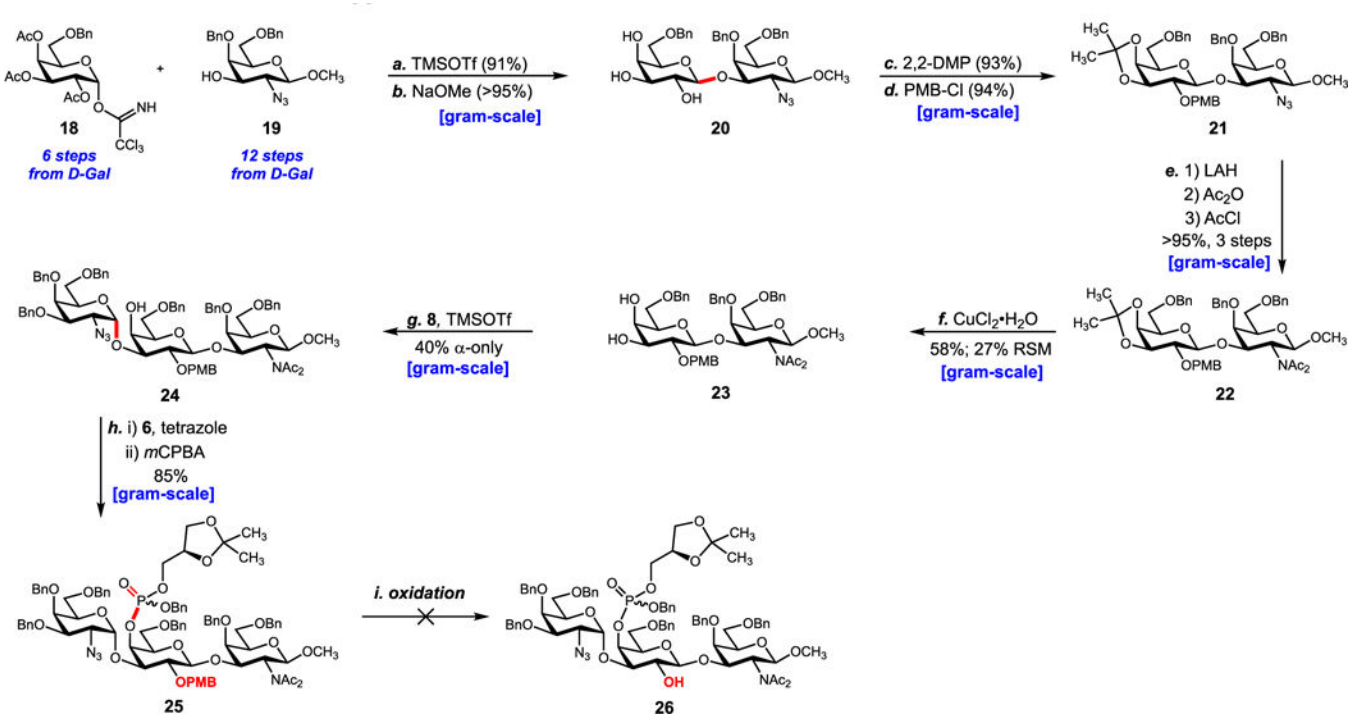
Figure 5.
Third-generation analysis.



Scheme 1.

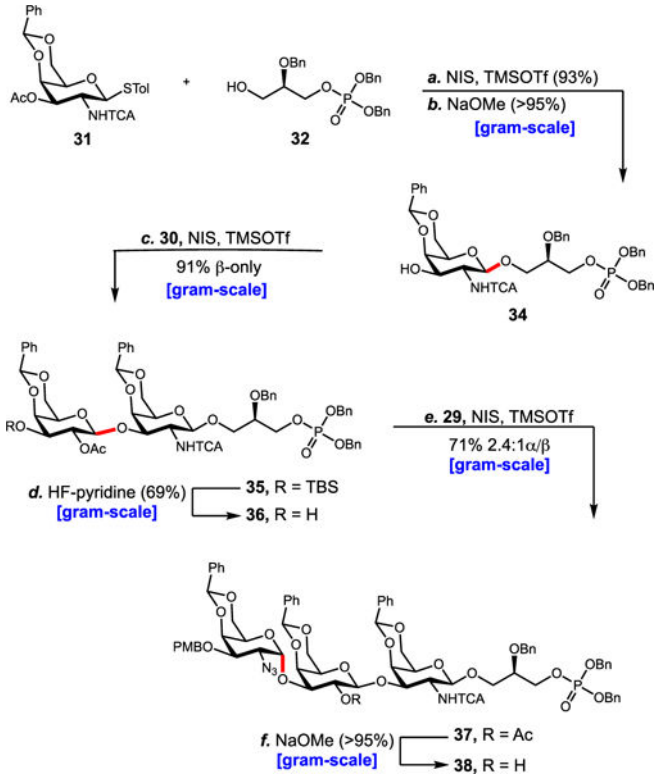
(a) First-Generation Approach and (b) Alternative Donor Leading to an Undesired Orthoester Product[a] [a]Reagents and conditions: (a) $BF_3{\cdot}Et_2O$, 4:1 iPr_2O/CH_2Cl_2, −40 °C → 25 °C, 1 h, 4 Å MS, 65% α-only; (b) **6** (1.5 equiv), 1*H*-tetrazole (1.5 equiv), CH_3CN, 0 °C → 25 °C, 0.5 h then *m*CPBA (1.5 equiv), CH_2Cl_2, −78 °C → 0 °C, 2 h, 87%; (c) DMDO (1.2 equiv), CH_2Cl_2, −78 °C → 0 °C, 1 h, then **13** (1.2 equiv), $ZnCl_2$ (2.5 equiv), −78 °C → 25 °C, >3 days, <5%; (d) DMDO (1.2 equiv), CH_2Cl_2, 0 °C, 20 min, then EtSH (28 equiv), TFAA (0.1 equiv), CH_2Cl_2, −78 °C → 0 °C, 1 h then Ac_2O (10 equiv), Et_3N (10 equiv),

DMAP (0.1 equiv) CH_2Cl_2, 0 °C, 10 h, 57% over 3 steps; (e) **13** (2.5 equiv), NIS (1.2 equiv), TMSOTf (0.15 equiv), CH_2Cl_2, −78 °C → 25 °C, 2.5 h, <5%; (f) TMSOTf (0.15 equiv) or TfOH (0.15), CH_2Cl_2, −78 °C→ 25 °C, 2.5 h.



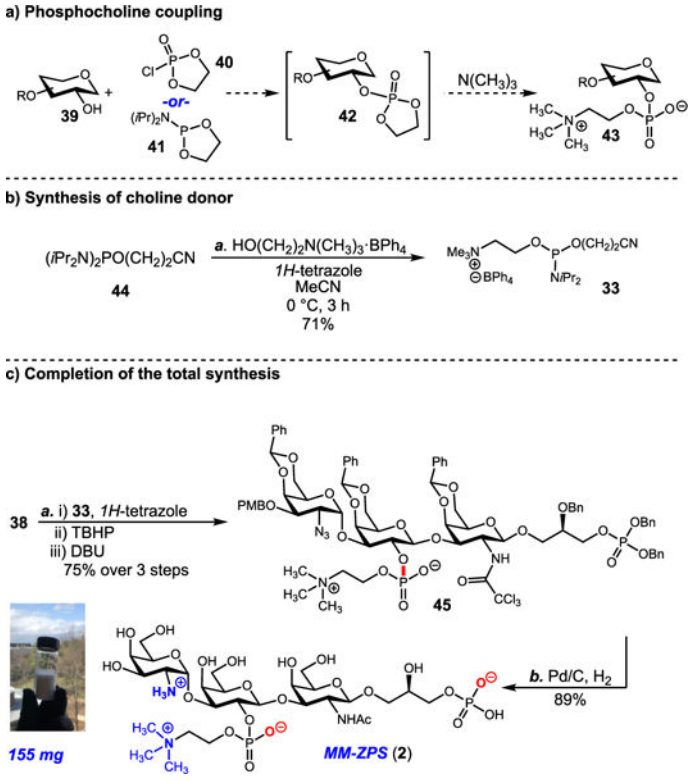
Scheme 2.

Second-Generation Approach[a] [a]Reagents and conditions: (a) TMSOTf (0.15 equiv), CH_2Cl_2, 0 °C → 25 °C, 1 h, *β*-only, 91%; (b) $NaOCH_3$, CH_3OH, 25 °C, 2 h, > 95%; (c) 2,2-DMP, *p*-TsOH, CH_2Cl_2, 25 °C, 0.5 h, 93%; (d) PMBCl (1.2 equiv), NaH (2.0 equiv), DMF, 0 °C → 25 °C, 1 h, 94%; (e) $LiAlH_4$, THF, 0 °C → 25 °C, 1 h then Ac_2O, pyr., DMAP, 0 °C → 25 °C, 2 h then AcCl, DIPEA, 2:3 CH_2Cl_2/CH_3CN, *μ*wave 85 °C, 3 h, >95% over three steps; (f) $CuCl_2{\cdot}H_2O$, CH_3CN, 0 °C → 25 °C, 3 h, 58% with 27% RSM; (g) 8 (2.5 equiv), TMSOTf (0.15 equiv), CH_2Cl_2, −78 °C→ −40 °C, 2.5 h, *α*-only, 40%; (h) 6 (1.5 equiv), 1*H*-tetrazole (1.5 equiv), CH_3CN, 0 °C → 25 °C, 0.5 h then *m*CPBA (1.5 equiv), CH_2Cl_2, −78 °C → 0 °C, 2 h, 85%.



Scheme 3.

Third-Generation Approach[a] [a]Reagents and conditions: (a) NIS (2.0 equiv), TMSOTf (0.15 equiv), CH_2Cl_2, 4 Å MS, 0 °C → 25 °C, 1.0 h, *β*-only, 93%; (b) $NaOCH_3$, CH_3OH, 25 °C, 2 h, >95%; (c) 30 (1.5 equiv), NIS (1.5 equiv), TMSOTf (0.10 equiv), CH_2Cl_2, 4 Å MS, −78 °C, 1.0 h, *β*-only, 91%; (d) HF-pyridine (10.0 equiv), pyridine, 25 °C, 3 h, 69%; (e) 29 (1.4 equiv), NIS (2.0 equiv), TMSOTf (0.15 equiv), 2.4:1 CH_2Cl_2/*i*Pr_2O, 4 Å MS, −60 °C → 0 °C, 5.0 h, 2.4:1 *α*/*β*, 71%; (f) $NaOCH_3$, CH_3OH, 25 °C, 2 h, >95%.



Scheme 4.

(a) Phosphocholine Coupling and (b) Completion of the Total Synthesis[a] [a]Reagents and conditions: (a) **33** (1.2 equiv), 1*H*-tetrazole (1.2 equiv), CH_3CN, 0.5 h, 25 °C then TBHP (1.2 equiv), CH_3CN, 0 °C, 1 h then DBU (5.0 equiv), CH_2Cl_2, 25 °C, 18 h, 78% over three steps; (b) Pd/C (15% w/w), H_2 (balloon), CH_3OH, 25 °C, 88 h, 89%.