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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.8b00148 • Publication Date (Web): 29 Jan 2018

Total Synthesis of a Densely-functionalized *Plesiomonas shigelloides* Serotype 51 Aminoglycoside Trisaccharide Antigen

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ABSTRACT: *Plesiomonas shigelloides,* a pathogen responsible for frequent outbreaks of severe traveler diarrhea, causes grave extraintestinal infections. Sepsis and meningitis due to *P. shigelloides* are associated with a high mortality rate as antibiotic resistance increases and vaccines are not available. Carbohydrate antigens expressed by pathogens are often structurally unique, and are targets for developing vaccines and diagnostics. Here, we report a total synthesis of the highly functionalized trisaccharide repeating unit **2** from *P. shigelloides* serotype 51 from three monosaccharides. A judicious choice of building blocks and reaction conditions allowed for the four amino groups adorning the sugar rings to be installed with two *N*-acetyl (Ac) groups, rare acetamidino (Am) and D-3-hydroxybutyryl (Hb) groups. The strategy for the differentiation of amino groups in trisaccharide **2** will serve well for the syntheses of other complex glycans.

1. INTRODUCTION

Plesiomonas shigelloides, a motile Gram-negative bacterium, is a common cause of severe travelers' diarrhea.[1] In children or patients with underlying diseases, *P. shigelloides* causes a variety of extraintestinal infections such as sepsis and meningitis that are associated with a high mortality rate.[1b] *P. shigelloides* infections are treated with various antibiotic regimes, although antibiotic resistance has been increased gradually.[1b, 2] A *P. shigelloides* vaccine would be highly desirable for travelers to subtropical and tropical regions.[2] *P. shigelloides* has been delineated into unique strains having 102 O-antigenic (lipopolysaccharide, LPS) and 51 H-antigenic serotypes.[3] LPS is involved in maintaining the effective barrier properties of the outer membrane and in the interaction between the bacteria and its environment due to their surface exposure.[4] It plays an important role during severe Gram-negative infections.[5] Carbohydrate antigens expressed by pathogens have been targets for developing vaccines and pathogen detection strategies.[6]

P. shigelloides serotype 51 O-antigen trisaccharide **1** [→4)-β-D-Glc*p*NAc3NHbA-(1→4)-α-L-Fuc*p*Am3OAc-(1→3)-α-D-Qui*p*NAc-(1→] (Figure 1) carries a host of functional groups: two aminodideoxyhexoses and one diaminodideoxyuronic acid are substituted with *N*- and *O*-acetyl groups, rare acetamidino (Am) and D-3-hydroxybutyryl (Hb) groups.[1a] Even for structurally diverse glycans, oligosaccharides such as **1** containing diacylated aminuronic acid, and functionalized with Am and/or Hb groups are very rare.[7] Such glycans have been identified primarily in the cell wall matrices of pathogenic bacteria (Figure 1), such as *Vibrio vulnificus* strain YJ016[8] and CECT 5198,[9] *Pseudomonas aeruginosa* serotype O5,[7a] and *Acinetobacter baumannii* ATCC 17961.[10] Genes responsible for the biosynthesis of 2,3-diaminuronic acids were found in over 50 pathogenic bacteria suggesting that these structures are more common than previously thought.[11] These results indicated that diacylated aminuronic acids may play an important role in pathogenicity as they are mainly found in pathogenic bacteria.[11] Chemical synthesis is an efficient means to procure sufficient amounts of pure oligosaccharides to understand the role of specific modifications and create potential vaccine candidates.[6a] Intense efforts have been devoted for the synthesis of bacterial, rare deoxy aminosugars, including 2,6-dideoxyhexosamines, 2,4-diamino-2,4,6-trideoxyhexoses and 2,6-dideoxy-4-ketohexosamines.[12] Kulkarni established an expedient protocol to synthesize orthogonally protected aminosugar building blocks for the assembly of complex bacterial glycans.[12b, c, 13]

Highly functionalized aminoglycoside trisaccharide **1** presents an intricate pattern of functional groups on a carbohydrate scaffold (Figure 2). Glycans related to **1** have not yet been synthesized since aminodideoxyhexoses and diaminodideoxyuronic acid are decorated with many functional groups. The Am and Hb groups influence the orthogonal installation of different functional groups and stereoselective glycosylations (*see* below). The retrosynthetic analysis of **1** thus relies on a judicious choice of protecting groups as well as considerations regarding the stereoselectivities of glycosylations. Here, we report the first chemical synthesis of *P. shigelloides* sero-

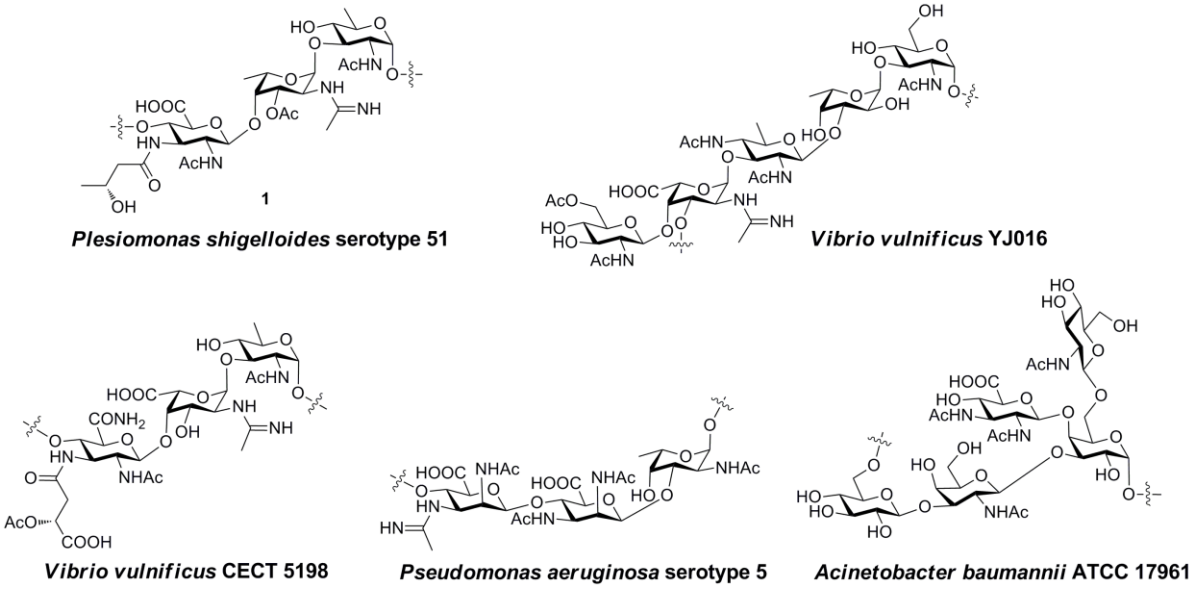


Figure 1. Representative structures of bacterial glycans containing dense aminoglycosides.

type 51 O-antigen trisaccharide **2** and acetamido derivative **3** (Figure 3). Including an amine-functionalized linker at the reducing end, each glycan carries no less than five functionalized amino groups that require orthogonal protecting group strategies and are thus an exceptional synthetic challenge. Both glycans will be instrumental for the development of a glycoconjugate *P. shigelloides* vaccine.

2. RESULTS AND DISCUSSION

2.1. Retrosynthetic analysis. An orthogonal linker has to be included in **2** and **3**, for subsequent conjugation or immobilization. The aminopropyl linker was placed at the reducing end of the synthetic oligosaccharides via an α-*O*-glycosidic linkage. Three building blocks **6**, **8** and **9** were identified for trisaccharide assembly in our first retrosynthetic attempt (Figure 3). Particularly important was the selection of protecting groups that allow for the selective unmasking of the functional groups. A D-3-hydroxybutyryl group was installed at the C3 amino group of diamino-D-glucuronate **6** before glycosylation. A trichloroacetamido (TCA) group was chosen to mask the acetamido group of **6** to enable the stereoselective formation of the β-glycosidic bond. The free hydroxyl and carboxylic acid groups were protected using benzyl (Bn) ethers, allowing for orthogonal removal in the presence of base-labile *O*-acetyl grou-

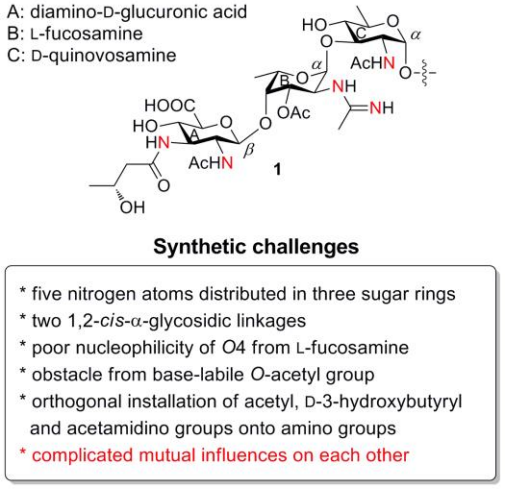


Figure 2. The synthetic challenges of *P. shigelloides* serotype 51 O-antigen trisaccharide **1**.

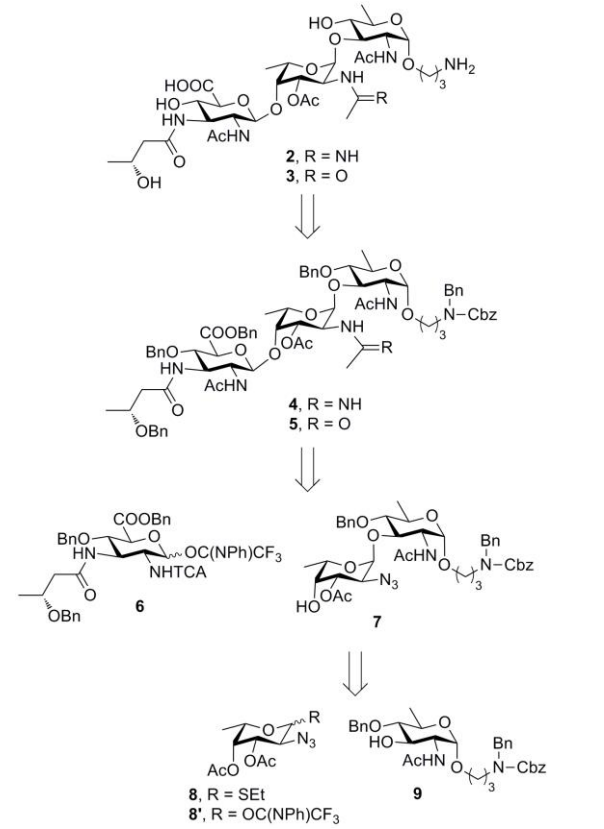
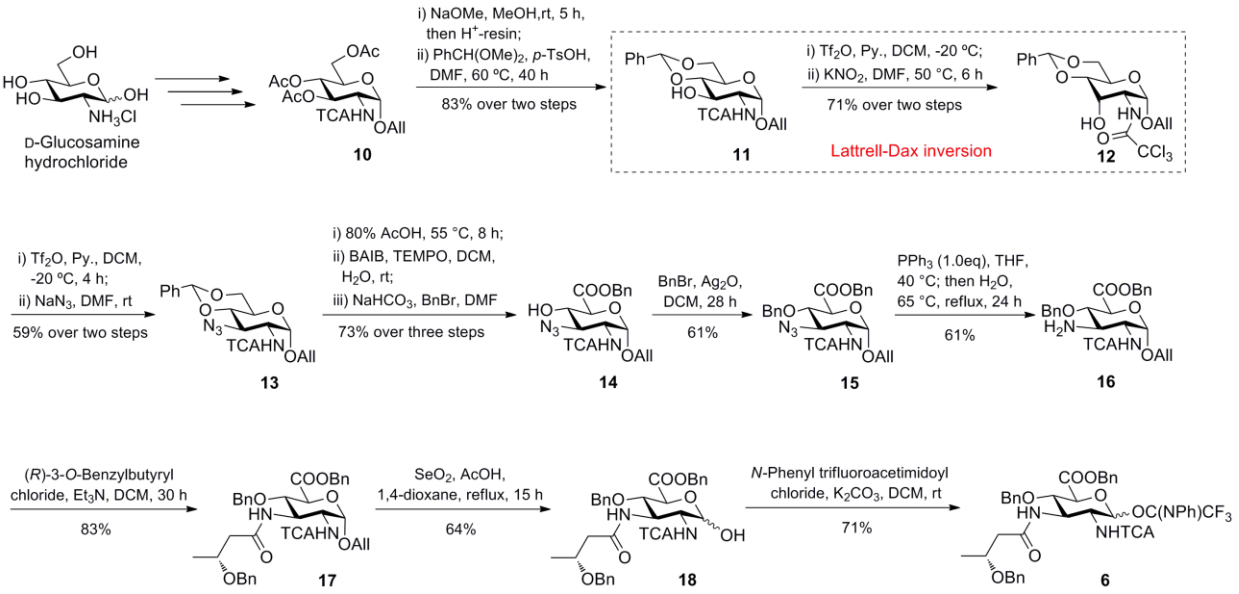


Figure 3. Retrosynthetic analysis of *P. shigelloides* serotype 51 trisaccharide **2** and **3**. Ac = acetyl; Bn = benzyl; Cbz = benzyloxycarbonyl; TCA = Trichloroacetyl.

Scheme 1. Synthesis of diamino-D-glucuronate 6



ps on the L-fucoside moiety. An azide group was placed at the 2-position of L-fucosamine **8** as a precursor of the basic acetamidino group to avoid complications during acid-mediated glycosylation reactions. A judicious choice of glycosylation partners and reaction conditions were important for the selective formation of the 1,2-*cis*-α-glyosidic bond between **8** and **9**. In addition, the C4 hydroxyl group in hexosamine derivatives such as **7** is a poor glycosyl acceptor due to its low nucleophilicity.[14] The C4 hydroxyl group of a 2-azido-2-deoxyglucose derivative was more nucleophilic than the corresponding hydroxyl group of 2-*N*-phthalimido-, *N*-acetyl-, *N*,*N*-diacetyl- and *N*-acetyl-*N*-benzyl-glucose derivatives.[14] Thus, the C2 azide of the L-fucoside in disaccharide **7** would be beneficial to the assembly of trisaccharide. Furthermore, the FucN *O*3-acetyl group in **7** had to be regioselectively installed before trisaccharide assembly. D-Quinovosamine **9** was designed to carry a permanent C4 benzyl ether, a C2 acetamide and an anomeric aminopropyl linker. Thus, the C3 hydroxyl group of **9** was ready for chain elongation.

2.2. Synthesis of diamino-D-glucuronate 6. Diamino-D-glucuronate **6** was synthesized starting from common D-glucosamine **10** (Scheme 1). Deacetylation and subsequent benzylidene protection of allyl glycoside **10** gave **11**. Triflation of **11** with triflic anhydride (Tf_2O) and pyridine yielded the C3 triflate derivative, as the initial part of a Lattrell-Dax inversion[15] from the *gluco*- to the *allo*-derivative **12** in 71% overall yield. Triflation of **12** followed by azide displacement gave rise to C3 azide derivative **13**. The 4,6-*O*-benzylidene protecting group of **13** was released quantitatively in 80% acetic acid solution. Subsequently, selective oxidation of the C6 hydroxyl group to the corresponding carboxylic acid was achieved efficiently using TEMPO/BAIB,[16] followed by treatment with benzyl bromide (BnBr) and sodium bicarbonate ($NaHCO_3$) furnished **14** in 73% overall yield. The 4-benzylation was performe-

Scheme 2. Synthesis of fucosides 8, 8' and 22

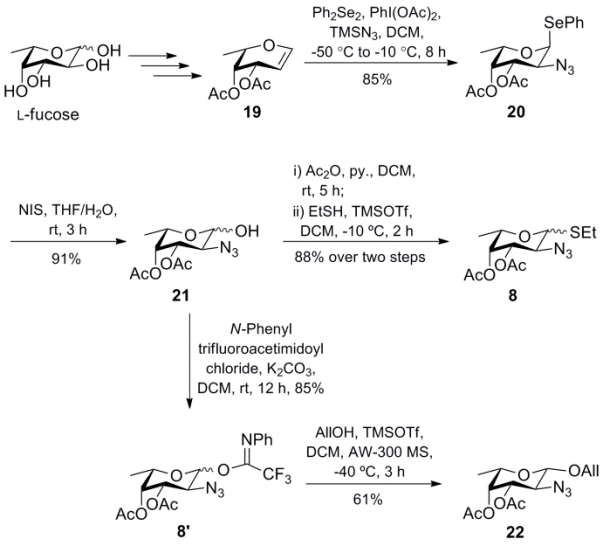
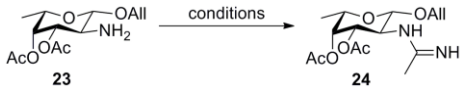


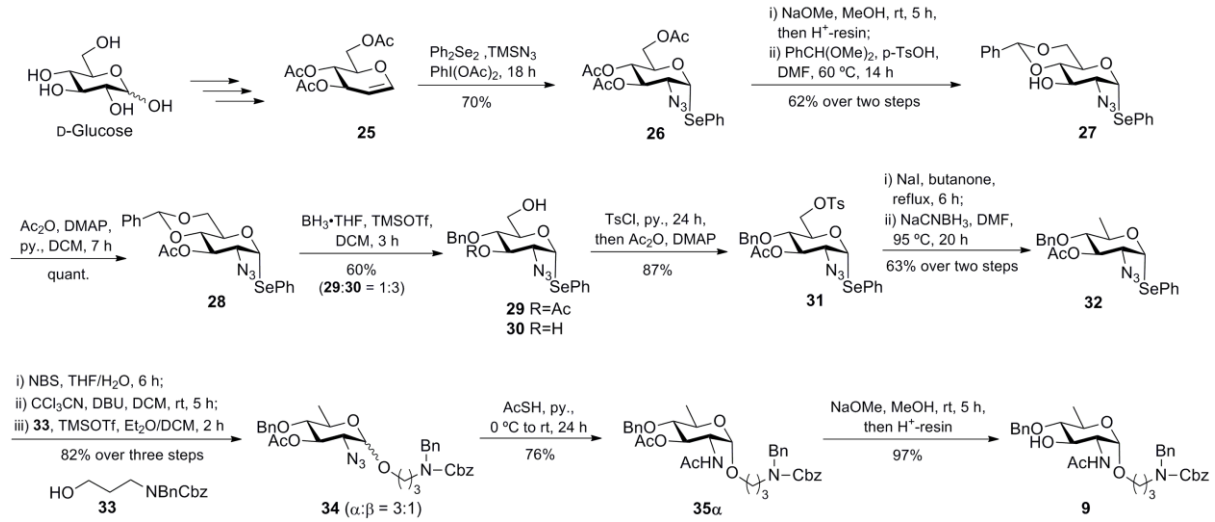
Table 1. A model introduction of an acetamidino group to 23



entry	conditions	yield
1	Ethylacetimidate hydrochloride (1.05 eq.), DMF, rt	ND[a]
2	Ethylacetimidate hydrochloride (1.05 eq.), EtOH, rt	ND
3	Ethylacetimidate hydrochloride (1.05 eq.), Et_3N (1.05 eq.), DMF, rt	ND
4	Ethylacetimidate hydrochloride (1.05 eq.), iPr_2NEt (1.05 eq.), DMF, rt	ND
5	*S*-Benzyl thioacetimidate hydrochloride (1.05 eq.), py., 0 °C	80%

[a]ND = not detected.

Scheme 3. Synthesis of D-quinovosamine 9



d using BnBr and silver oxide (Ag_2O) in dichloromethane (DCM) to give **15** in 61% yield. Next, the reduction of the azide group in **15** was targeted. Several strategies were tested: neither H_2/Lindlar's catalyst[17] nor sodium borohydride[18] were effective as reducing agents to produce the corresponding amine **16**. Using a Staudinger reduction condition with ten equivalents of triphenylphosphine (PPh_3) led to the undesired reduction of the TCA group to a dichloroacetyl group. A similar phenomenon had been reported in the presence of a tricyclohexylphosphine ligand of the Grubbs' catalyst.[19] Dehalogenation of the trichloroacetyl groups may be the result of the nucleophilic attack of PPh_3 on one of three chlorine atoms. Considering the reactivities of azide and TCA groups toward triphenylphosphine, the addition of just one equivalent of PPh_3 proved effective and completely suppressed the side reaction. Thus, treatment with one equivalent of PPh_3 in tetrahydrofuran at 40 °C, followed by the addition of water and reflux overnight gave **16** in 61% yield. (*R*)-3-*O*-benzylbutyryl chloride was coupled with **16** to give diamide **17**, which was finally converted to trifluoroacetimidate **6** in two steps and good yield.

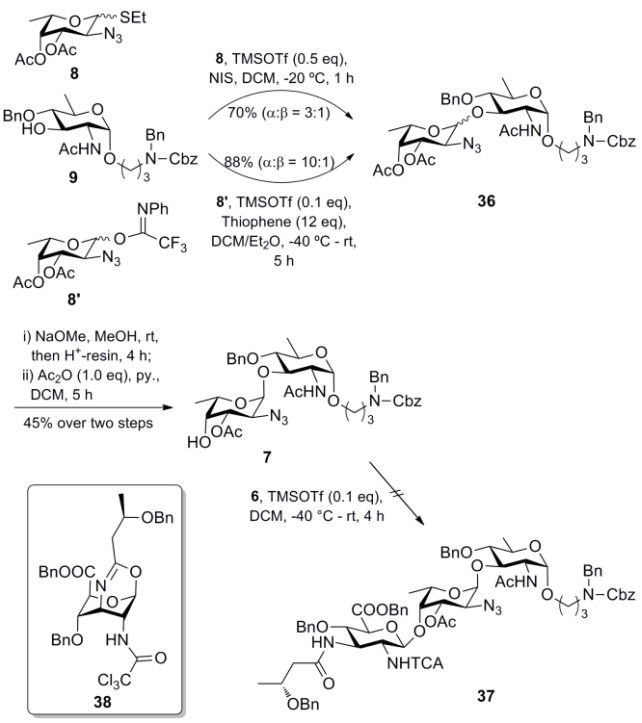
2.3. Synthesis of L-fucosamine 8. L-Fucose served as the starting material to access L-fucosamine **8** (Scheme 2). Conversion to fucal **19**[20] was followed by diphenyl diselenide (Ph_2Se_2)/trimethylsilyl azide ($TMSN_3$)-mediated azidoselenation to give C2 azide sugar **20** in 85% yield. After hydrolysis of the selenoglycoside, lactol **21** was further acetylated, and transformed into thioglycoside **8** in high yield over two steps. Introduction of trifluoroacetimidate into **21** produced fucosyl *Yu* donor **8'** as an alternative glycosylating agent.

Although several synthetic transformations have been developed for the preparation of amidines,[21] few methods are known to introduce acetamidines into complex synthetic glycans. To test these transformations on model systems, monosaccharide 1-*O*-allyl-2-azido fucoside **22** and amino derivative **23** were produced from imidate **8'** (*see* Supporting Information). Ethylacetimidate hydrochloride, a well-known Pinner salt, may react with amines to give the corresponding acetamidines.[22-23] However, treatment of **23** with ethylacetimidate hydrochloride failed to give the desired product in the absence (Table 1, entries 1-2) or presence (entries 3-4) of different bases. In contrast, the reaction of **23** with *S*-benzyl thioacetimidate hydrochloride[24] in pyridine proceeded smoothly to afford desired acetamidine **24** in 80% yield under mild conditions (entry 5).

2.4. Synthesis of D-quinovosamine 9. Triacetyl glucal **25** served as starting material for the synthesis of D-quinovosamine **9** (Scheme 3). The C2 azide derivative **26** was prepared by azidoselenation of **25**.[25] Deacetylation and subsequent benzylidene acetal formation gave alcohol **27**, which was acetylated to give **28**. A regioselective, reductive ring-opening of the 4,6-*O*-benzylidene acetal using borane tetrahydrofuran complex (BH_3·THF)/trimethylsilyl trifluoromethanesulfonate (TMSOTf) converted **28** to the corresponding 4-*O*-benzyl-6-hydroxy derivative **29** and deacetylated phenylseleno glycoside **30**. Moreover, tosylation of **29** and **30** by using 4-toluenesulfonyl chloride (TsCl) in the presence of pyridine followed by treatment with acetic anhydride gave desired 6-*O*-tosyl derivative **31** in 87% yield. Subsequent nucleophilic tosylate substitution with NaI in refluxing butanone,[26] followed by reduction with sodium cyanoborohydride ($NaCNBH_3$) yielded quinovosamine derivative **32** in good overall yield. For insertion of an aminopropyl linker, the hydrolyzed product of **32** was transformed to known *Schmidt* donor followed by acid-catalyzed glycosylation with *N*-Bn-*N*-Cbz-3-aminopropan-1-ol **33** in diethyl ether/DCM to give an inseparable mixture **34** (α:β = 3:1) in 82% overall yield. Subsequent conversion of the C2 azide to the corresponding acetamide group in AcSH/pyridine produced the separable amides **35α** and **35β** in 76% yield. Deacetylation of **35α** afforded D-quinovosamine acceptor **9**.

2.5. Attempt to prepare fully protected trisaccharide 37. The initial attempt at trisaccharide assembly started from the reducing to the non-reducing end (Scheme 4). The union of thioglycoside donor **8** and acceptor **9** in the presence of TMSOTf and *N*-iodosuccinimide (NIS) at -20 °C led to an α/β mixture of the disaccharide **36** in a ratio of 3:1. TMSOTf-catalyzed glycosylation of **9** with *Yu* donor **8'** in a blended solvents system including DCM, diethyl ether and thiophene afforded **36** in 88% yield and better stereoselectivity (α:β = 10:1). Next, the synthesis of trisaccharide

Scheme 4. Attempt to prepare fully protected trisaccharide 37



37 was attempted by glycosylation of disaccharide acceptor **7** obtained in two steps from **36** with donor **6** using TMSOTf as promoter. However, instead of expected trisaccharide **37**, the bicyclic dihydro-1,3-oxazine **38** was obtained. A HMBC correlation between H1 and C2' (carbonyl carbon of C3 butyrylamide) indicates the formation of bicyclic dihydro-1,3-oxazine through connectivity between C1 and carbonyl oxygen of C3 butyrylamide. The small $^{3}J_{H,H}$ coupling constants of five sugar ring protons and a W-coupling between H1 and H3 indicate that this diaminoglucopyranoside derivative turns its five equatorial substituents into axial orientations through a $^{4}C_{1}$ to $^{1}C_{4}$ ring flip in response to intramolecular cyclization. The similar oxazine byproduct was also found when phenyl 3-amide-thioglycoside[27] and 3-amide-glycal[28] were used as glycosyl donors. Although bicyclic dihydro-1,3-oxazine have proven to be useful for stereospecific proton-assisted glycosylations,[29] the intramolecular cyclization reaction was obviously a significant obstacle for preparing β-glycosidic linkage in the target trisaccharide. To avoid any complications during the glycosylation due to the butyrylamido and other acylamido groups, it was, at this point, decided to preserve the azide group in the diamino-D-glucuronate moiety throughout trisaccharide assembly.

2.6. General retrosynthetic considerations to optimize protecting group strategies. To consider mutual influences and operational complexities associated with the two azide groups (Figure 4, red part in **36** and green part in **15**) during the consecutive reactions, the azide group of **36** had to be reduced to the corresponding amine and then temporarily protected. The temporary protecting group would later on have to be easily removed in anticipation of acetamidination. Accordingly, a general, orthogonally protected trisaccharide **A** was designed that would allow for selective deprotection of the C2 amine, C3 hydroxyl of the fucoside and the C3 azide of the non-reducing terminal glucuronate. The synthesis of

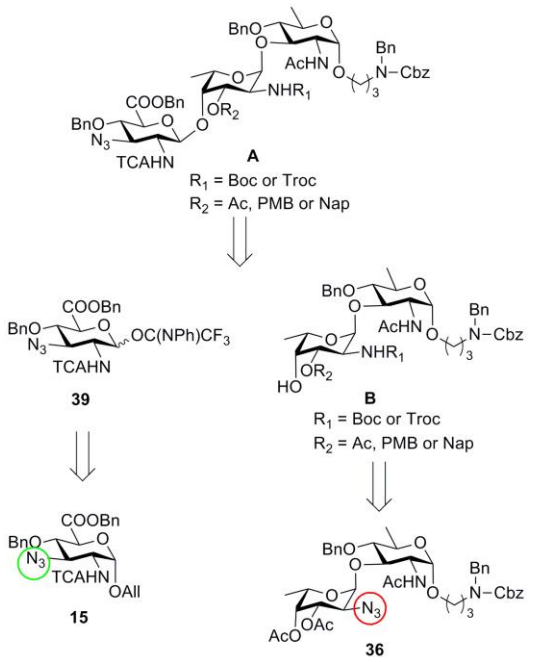


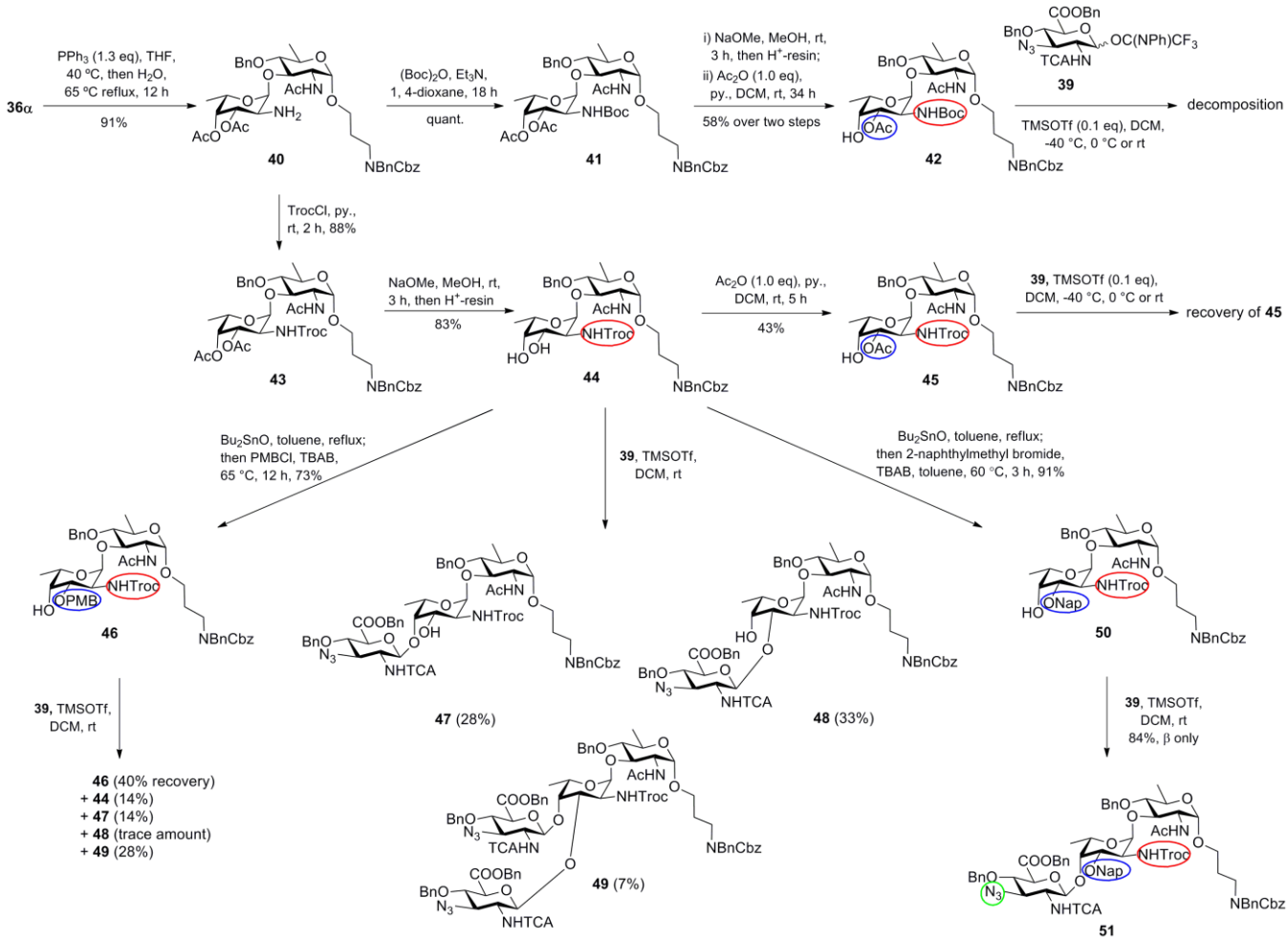
Figure 4. Design and retrosynthetic analysis of a trisaccharide **A** toward **2** and **3**.

trisaccharide **A** would start with **15** and **36** by optimizing the amino and hydroxyl protecting groups of a general disaccharide **B**. The temporary FucN amino protecting group in **B** should allow for orthogonal removal in the presence of base-labile ester groups. Thus, tert-butyloxycarbonyl (Boc) and 2,2,2-trichlorethoxycarbonyl (Troc) were chosen as amino protecting group candidates. To minimize the FucN C3' protection/deprotection steps, an acetyl group was the first option for C3′ protection in the disaccharide acceptor. In view of the disarming effect of the C3′ acetate on glycoside reactivity of the disaccharide acceptor, the electron-donating 4-methoxybenzyl ether (PMB) and 2-naphthylmethyl ether (Nap) allowing for orthogonal removal in the presence of base-labile esters were considered as alternative temporary protecting groups for **B**.

2.7. Efforts toward trisaccharide formation. Trisaccharides that fulfill the general formula **A** were then targeted via several intermediates **B** (Scheme 5). Reduction of the azido group in **36** using the Staudinger method gave amine **40**, which was Boc protected. The diol product derived from Boc protected disaccharide **41** was regioselectively acetylated to produce acceptor **42**. Unfortunately, glycosylation of acceptor **42** with donor **39** (prepared in two steps from **15**, *see* Supporting Information) failed to proceed at different reaction temperatures (-40 °C, 0 °C or room temperature). Decomposition of acceptor **42** due to cleavage of the Boc group was detected during these reactions. Even attempts to glycosylate *N*-Boc-fucosamine monosaccharide model with the corresponding thioglycoside using the milder activating agent DMTST[16] as a promoter also failed.

The instability of the Boc group under glycosylation conditions forced us to retreat to a Troc group as alternative and prepare disaccharide acceptor **45** in three steps from **40** (Scheme 5). Donor **39** and acceptor **45** did not react to form the desired trisaccharide

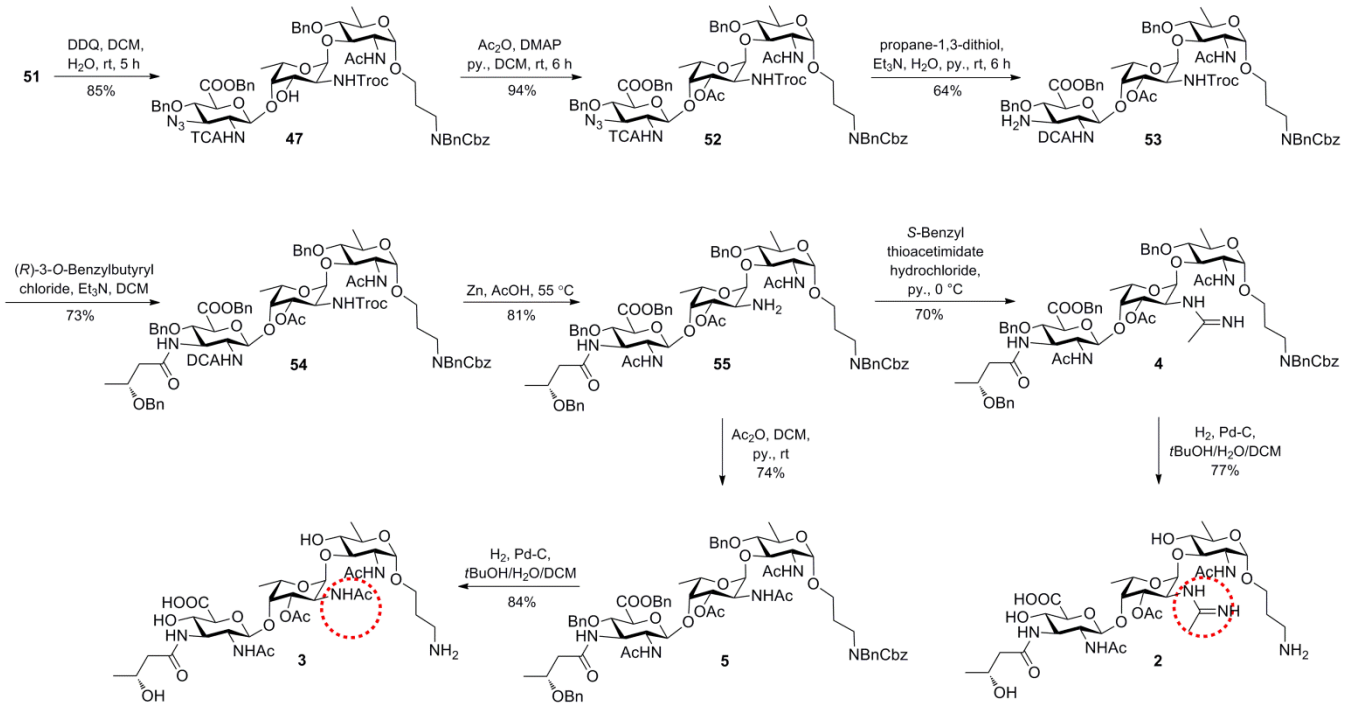
Scheme 5. Attempted assembly of trisaccharides



but rather acceptor **45** was isolated unchanged. The Troc group is thus stable under glycosylation conditions but the electron-withdrawing acetyl group (blue part in **45**) may reduce the nucleophilicity of the adjacent C4' hydroxyl group too drastically. Therefore, the acetyl group was replaced by the electron-donating PMB group to afford disaccharide acceptor **46** from **44**. Surprisingly, the PMB group, previously reported to withstand trichloroacetimidate glycosylation conditions using 0.8 eq. TMSOTf,[30] was partially cleaved during glycosylation of **46** with donor **39** using 0.1 eq. TMSOTf. Trisaccharide **47** (14%, β-only, the anomeric proton of the donor residue: 5.12 ppm, $^3J_{H1/H2}$ = 6.63 Hz), **48** (trace amount) and tetrasaccharide **49** (28%, β-only) were obtained, alongside with recovered acceptor **46** (40%) and disaccharide **44** (14%). The β-configuration in **47** was confirmed by C3'-*O*-acetylated product **52** (Scheme 6, for the anomeric proton of the donor residue in **52**: 4.89 ppm, $^3J_{H1/H2}$ = 8.1 Hz). The β-configuration of two newly-formed glycosidic bonds in **49** was clearly confirmed by the anomeric proton $J_{C,H}$ coupling of 164 Hz and 166 Hz. Therefore, disaccharide **44** bearing two free hydroxyl groups was next used for a stochastic glycosylation with donor **39**, whereby **47** (28%, β-only) and **48** (33%, β-only, the anomeric proton of the donor residue: 5.06 ppm, $^3J_{H1/H2}$ = 8.0 Hz) were obtained in similar yields alongside tetrasaccharide **49** (7%, β-only). Apparently, the C4' and C3´ hydroxyl groups in **44** are of similar reactivity. The formation of tetrasaccharide **49** demonstrated that the low reactivity of the C4' hydroxyl group can be mainly attributed to electronic rather than steric factors. To address this challenge, we employed electron-donating Nap ether as C3´ protecting group. Target trisaccharide **51** was obtained by glycosylation of disaccharide acceptor **50** with donor **39** in 84% yield and complete stereoselectivity (β-only, the anomeric proton of the donor residue: 5.14 ppm, $^3J_{H1/H2}$ = 7.2 Hz).

2.8. Synthesis of target trisaccharides 2 and 3. The final steps commenced with removal of the *O*3'-Nap in **51** using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in aqueous DCM in 85% yield, and acetylation to obtain ester **52** in 94% yield (Scheme 6). Azide reduction in **52** using the Staudinger conditions that had worked well for monosaccharide **15** failed to give desired trisaccharide amine **53**. Reduction of the azide in **52** using 1,3-propanedithiol and *i*Pr$_2$NEt in MeOH[31] gave an inseparable mixture of 2''-dichloroacetamido-3''-aminosugar **53** and the corresponding 2''-trichloroacetamido-3''-aminosugar. Notably, treatment of **52** with 1,3-propanedithiol, Et$_3$N and water in pyridine afforded pure 2''-dichloroacetamido-3''-aminosugar **53**. The installation of the 3-benzyloxybutyryl group onto amine **53** with (*R*)-3-*O*-benzylbutyryl chloride gave **54** in 73% yield. Trisaccharide **54** was treated with a large excess of zinc powder in acetic acid at 55 °C

Scheme 6. Final assembly of two fully deprotected trisaccharides 2 and 3



to remove the Troc group and reduce the *N*-dichloroacetamide to an *N*-acetamide. The reaction of amine **55** with *S*-benzyl thioacetimidate hydrochloride in pyridine proceeded smoothly to afford desired acetamidine trisaccharide **4** in 70% yield. Treatment of amine **55** with acetic anhydride in a mixture of DCM and pyridine gave desired acetamide trisaccharide **5** in 74% yield. Global deprotection of **4** with Pd/C hydrogenation gave target trisaccharide **2** in 77% yield. Global deprotection of **5** by hydrogenation afforded target trisaccharide **3** in 84% yield after purification by size-exclusion chromatography. The NMR data (^{1}H and ^{13}C NMR spectra) of synthetic **2** are in agreement with those of the natural isolate (details *see* Supporting Information). The slight spectral differences are most evident toward the reducing end and probably arise due to the aminopropyl glycoside in the synthetic material in place of the glycosidic bond in the natural isolate.

CONCLUSION

An elaborate synthetic strategy was developed to accomplish the synthesis of *P. shigelloides* serotype 51 O-antigen trisaccharide **2** and its acetamido derivative **3**. The 2,3-diamino-D-glucuronate relied on a double inversion at C3 and was first used in the assembly of a dense aminoglycoside bearing heterogeneous substitutions. The 1,2-*cis*-α-selectivity for glycosylation between **8'** and **9** relied on a non-participating C2 azide group and solvent effects. Moreover, the orthogonally protected disaccharide **50** was designed and prepared for glycosylation with C3 azide donor **39**, thus avoiding the formation of bicyclic dihydro-1,3-oxazine byproduct that formed when a C3 amide donor was utilized. In contrast to an *O*3′-acylated, disarmed disaccharide, an armed disaccharide **50** equipped with an *O*3'-Nap was highly reactive toward diamino-D-glucuronate **39** and the formation of desired trisaccharide **51**. This orthogonally protected trisaccharide **51** allows for selective unmasking of the C2' amine, C3' hydroxyl group and C3'' azide, thus providing an efficient route to **2** and **3**. The rare acetamidino group was successfully introduced into trisaccharide **2** using *S*-benzyl thioacetimidate hydrochloride. An aminopropyl linker installed at the reducing end of **2** and **3** enables the further conjugation or immobilization of complex aminoglycosides, which may facilitate identification of the immunologically important domains of *P. shigelloides* serotype 51 O-antigen. This challenging synthesis taught valuable lessons for the synthesis of other complex aminoglycosides.

ASSOCIATED CONTENT

Supporting Information.

This material is available free of charge via the Internet at http://pubs.acs.org.

Experimental procedures, characterization data of synthetic compounds, and 1D and 2D NMR spectra (PDF)

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Notes

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We are grateful for financial support from National Natural Science Foundation of China (21302068), the Max Planck Society International Partner Group Program, the High-end Foreign Experts Recruit-

ment Program, the Thousand Talents Plan (Young Professionals), Deutscher Akademischer Austauschdienst (DAAD) and China Scholarship Council (CSC). P.H.S. thanks the Max-Planck Society for generous financial support.

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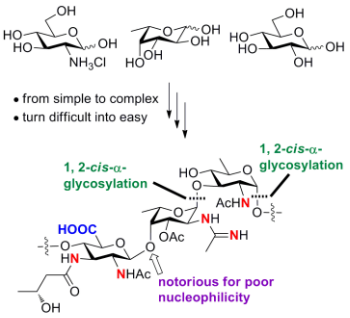
Total Synthesis of a Densely-functionalized *Plesiomonas shigelloides* Serotype 51 Aminoglycoside Trisaccharide Antigen



Total synthesis of O-antigen trisaccharide from *P. shigelloides* serotype 51