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Synthesis of Zwitterionic 1,1′-Glycosylphosphodiester: A Partial Structure of Galactosamine-Modified *Francisella* Lipid A

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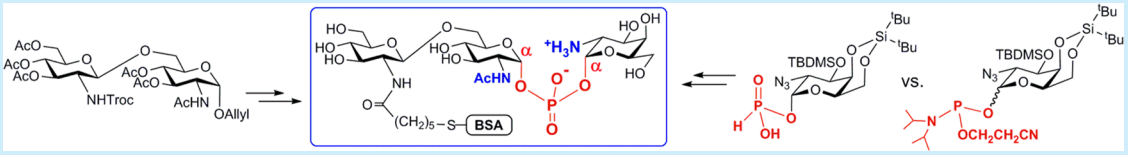
Supporting Information



ABSTRACT: Synthesis of a "double glycosidic" phosphodiester comprising anomeric centers of two 2-amino-2-deoxy-sugars is reported. The carbohydrate epitope of *Francisella* lipid A modified with α-D-galactosamine at the anomerically linked phosphate has been stereoselectively prepared and coupled to maleimide-activated bovine serum albumin via an amide-linked thiol-terminated spacer group. H-Phosphonate and phosphoramidite approaches have been explored for the coupling of 4,6-DTBS-2-azido-protected GalN lactol and peracetylated spacer-equipped reducing βGlcN(1→6)GlcN disaccharide via phosphodiester linkage. Deprotection conditions preserving the integrity of the labile glycosidic zwitterionic phosphodiester were elaborated.

Francisella is a highly infectious Gram-negative intracellular zoonotic bacterium that can cause tularemia, an extremely contagious lethal pulmonary disease in mammals.[1] *F. tularensis*, which is a causative agent of tularemia in humans, provokes special anxiety due to its classification as a bioterrorism agent.[2] Lipopolysaccharide (LPS) is one of the principal virulence factors of Gram-negative bacteria, whereas the lipid A portion of LPS is primarily responsible for eliciting the innate immune response through Toll-like Receptor 4 (TLR4)−myeloid differentiation-2 (MD-2) complex. The major lipid A of *Francisella* has an unusual tetraacylated structure, which lacks the 4′-phosphate and a 3′-acyl chain and contains an α-D-GalN residue at the glycosidically connected 1-phosphate (Figure 1).[3−6] The four subspecies of genus *F. tularensis*, highly infectious

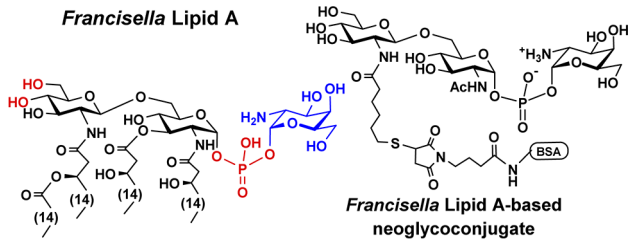


Figure 1. Structure of GalN-modified lipid A from *Francisella* and a related synthetic neoglycoconjugate.

type A strain (Schu S4, *tularensis*), less virulent type B strain (*holartica*), attenuated type B strain, designated as live vaccine strain (LVS), *F. mediasiatica*, and a nonvirulent laboratory strain *F. novicida* share a similar lipid A structure having a GalN modification at the 1-phosphate.[3,7−9] *Francisella* LPS was shown to be not recognized by TLR4/MD-2 complex which is attributed to the hypoacylated structure of its lipid A lacking a 4′-phosphate,[10−12] though *F. tularensis* LPS was shown to provide protection against LVS infection in mice.[13] The biological significance of GalN modification of *Francisella* lipid A has not yet been fully clarified, though it is associated with enhanced bacterial virulence. *F. novicida* mutants which are deficient in GalN modification have been shown to have attenuated pathogenicity in mice and are capable of stimulating the innate immune system.[14]

One of the most studied *Francisella* strains, *F. novicida*, was shown to possess a truncated lipopolysaccharide form composed of 90% of the α-D-GalN-modified lipid A portion, which is deprived of the core sugars and polymeric O-antigen.[7,15] The diglucosamine backbone of lipid A modified by α-D-GalN at the 1-phosphate is, in this instance, the exposed and, possibly, the antigen-presenting part of LPS. A novel system of LPS remodelling enzymes involving a bifunctional Kdo-hydrolase has been implicated in the synthesis of the partially truncated *Francisella* LPS structures.[16−18] To assess the antigenic potential of the GalN modification, a lipid A - based epitope βGlcN(1→6)-αGlcN(1 →P←1)-αGalN which is conserved in all *Francisella* strains, has been synthetically prepared and coupled to maleimide-activated bovine serum albumin (BSA) via thiol-terminated spacer group.

The majority of naturally occurring glycosidically - linked phosphodiesters contain phosphoester bonds connecting one anomeric and one nonanomeric hydroxyl group. The formation of this type of phosphodiesters is generally carried out in conjunction with the well-established phosphotriester, phosphoramidite or H-phosphonate methodologies.[19−23] Herein we report on the first synthesis of a 1,1′-glycosylphosphodiester wherein the anomeric centers of two 2-amino-2-deoxy-sugars

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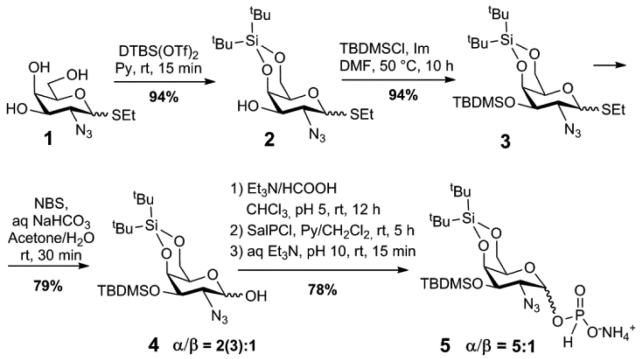
(αGlcN and αGalN as in *Francisella* lipid A) are involved. The assembly of such "double glycosidic" phosphodiester represents a formidable synthetic challenge with respect to the requirements for the anomeric stereocontrol and intrinsic instability of the 2-amino-2-deoxy-glycosylphosphate derivatives under a variety of chemical conditions.

Two classes of P(III) phosphorus compounds could serve as intermediates in the synthesis of double anomeric phosphodiesters, namely phosphoramidite (three-coordinated) or H-phosphonate (tetra-coordinated). Both possess an electrophilic phosphorus center capable of easily reacting with various nucleophiles. The advantage of the phosphoramidite procedure lies in the mildness of the phosphitylation and oxidation conditions, whereas the instability of the intermediary glycosyl phosphoramidites and glycosyl phosphites, which represent perfect glycosyl donors,[24] count as drawbacks. The merits of the H-phosphonate methodology include substantial stability of the intermediate glycosidic H-phosphonate monoesters as well as the absence of protecting groups at phosphorus. However, the oxidation of the H-phosphonate phosphodiesters into P(V) species often requires harsh conditions which can eventually lead to side-reactions. Herein we explored the applicability of both methods toward the synthesis of a *Francisella* lipid A - related epitope containing αGlcN(1→P←1)αGalN fragment.

The synthesis of αGalN-H-phosphonate **5** commenced with regioselective introduction of the 4,6-*O*-di-*tert*-butylsilylene (DTBS) group into triol **1**[25] to furnish compound **2** (Scheme 1). The DTBS group was expected to enhance the α/β ratio in

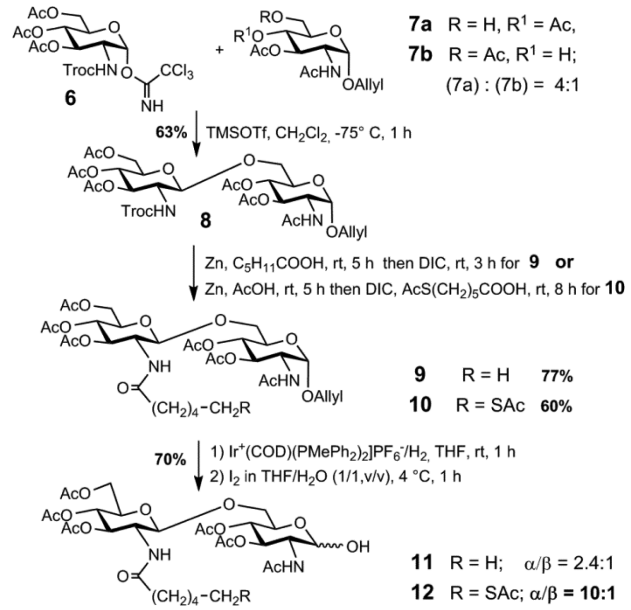
Scheme 1. Synthesis of the Glycosyl-H-phosphonate



the anomeric lactol **4**, since this cyclic protection has been shown to exert a remote α-directing effect in the glycosylation reactions involving 4,6-*O*-DTBS-protected galactose donors.[26] Next, the 3-OH group in compound **2** was protected by reaction with TBDMS-chloride in DMF in the presence of imidazole to give **3**. *N*-Bromo-succinimide-mediated hydrolysis of the thioethyl glycoside provided the anomeric lactol **4** as a mixture of anomers (α/β = 2(3):1). Since the stereoselectivity of the ensuing phosphitylation with P(III) reagents generally depends on the α/β ratio in the starting hemiacetal, we attempted to increase the proportion of the α-configured lactol in **4** by *in situ* anomerisation. Experiments in the NMR tube confirmed that lactol **4** could be enriched in the α-anomer under acidic conditions. Therefore, a solution of **4** in chloroform was treated with triethylammonium formate - formic acid buffer (pH 5) for 12 h, followed by the traceless removal of buffer by coevaporation with toluene. The reaction of **4** (α/β = 4:1) with 2-chloro-1,3,2-benzodioxaphosphorin-4-one (salicyl-chlorophosphite, SalPCl)[27] was performed in DCM in the presence of a 2-fold excess of pyridine (over SalPCl) which afforded glycosyl-H-phosphonate **5** (α/β = 4:1, according to the ^{1}H NMR spectra of the crude product), isolated with a higher proportion of the α–anomer (α/β = 5:1) as the ammonium salt in 78% yield.

Thereafter, the β–(1→6)-linked diglucosamine **12**, equipped with a 6-thioacetylhexanoylamino spacer group at C-2′, as well as its 2′-hexanoylamino counterpart **11** as a model compound, were prepared (Scheme 2). To this end, the βGlcN(1→6)GlcN

Scheme 2. Preparation of (1→6) Diglucosamine Lactol



disaccharide was assembled by a TMSOTf-assisted glycosylation of the 6-OH acceptor **7a**[28] with peracetylated trichloroacetimidate donor **6**.[29] The acceptor was applied as a 4:1 mixture of positional isomers, 4-*O*-acetyl-6-OH compound **7a** and 4-OH-6-*O*-acetyl compound **7b**, which inevitably arise due to the known propensity of the 4-*O*-acetyl group in glucopyranoses to undergo (4→6) migration in a variety of chemical conditions.[30] Owing to the diminished reactivity of **7b** compared with **7a** in a (1→6) glycosylation, the target (1→6) disaccharide **8** could be efficiently prepared and readily isolated in 63% yield. Reductive cleavage of the 2′*N*-Troc protecting group with Zn in hexanoic acid followed by addition of *N*,*N*′-diisopropylcarbodiimide (DIC) afforded amide **9**. To install the thiol-terminated spacer, the 2′*N*-Troc group was reduced by Zn in acetic acid, the resulting amine was *in situ* acylated with 6-thioacetyl-hexanoic acid/DIC which afforded compound **10**. Anomeric deprotection by isomerization of allyl- to propenyl- group, followed by hydrolysis with aqueous I_2 provided anomeric lactols **11** (α/β = 2.4:1) and **12** (isolated with a high preponderance of α-anomer, α/β = 10:1).

To explore the stereoselectivity and efficiency of phosphitylation with GalN-H-phosphonate **5**, a pivaloyl chloride (PivCl)-mediated coupling of **5** to the thiol-free model compound **11** was first performed (Scheme 3). A diluted solution of PivCl had to be gradually added to the reaction mixture to avoid a formation of P-acyl byproducts and possible over-reaction of the intermediate H-phosphonate phosphodiester with an excess of PivCl.[31]

The H-phosphonate coupling was monitored by ^{31}P NMR (162 MHz, $CDCl_3$) spectroscopy which indicated gradual disappearance of the H-phosphonate **5** (δ: 1.06 ppm, J_{P-H} =

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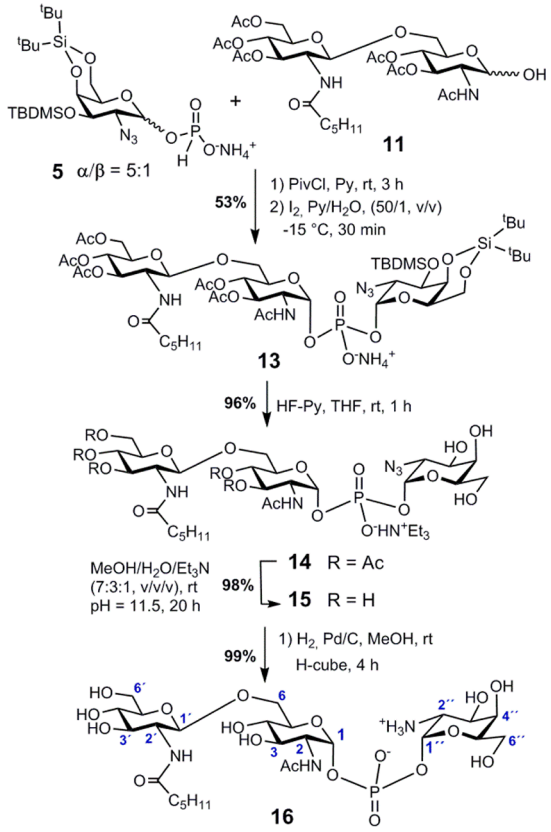
Scheme 3. Assembly of the "Double Anomeric" Phosphodiester Entailing Two 2-Amino-2-deoxy-sugars



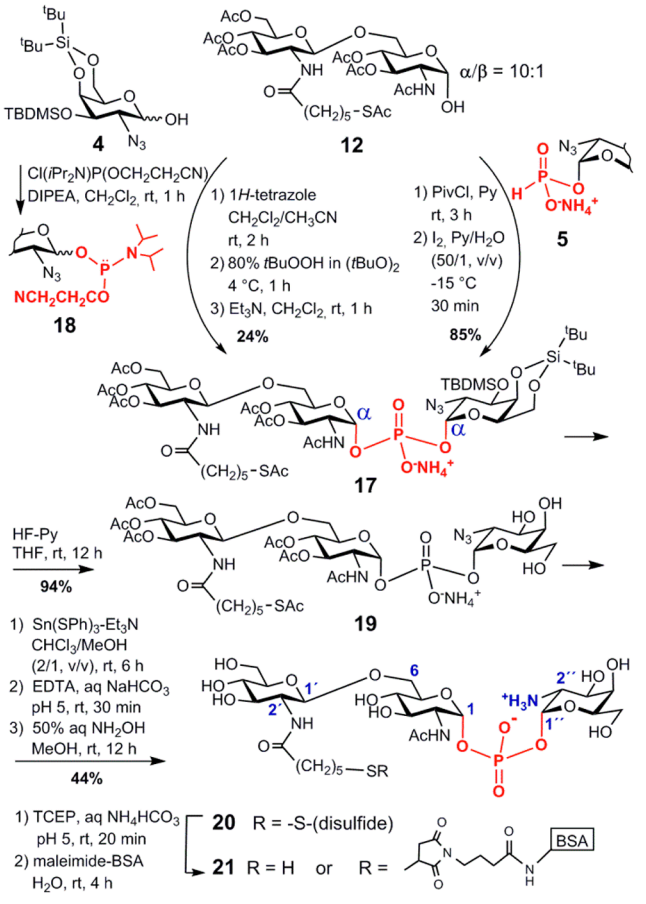
640 Hz) and the formation of the intermediate H-phosphonate diesters (δ: 4.21 ppm, J_{P-H} = 737 Hz and δ: 4.32 ppm, J_{P-H} = 728 Hz for the 1-α- and 1-β-configured compounds, respectively, both diastereomers at phosphorus). Oxidation by aqueous I_2 in pyridine at −15 °C and successive isolation by silica gel chromatography afforded anomerically pure **13** as ammonium salt in 53% yield. The α,α-(1→P←1) anomeric configuration was confirmed by the downfield shifts of the H-1 (GlcN→P) and H-1″ (GalN→P) signals and by $J_{1,2}$ and $J_{1,P}$ coupling constants ($J_{1,2}$ = 3.5 Hz, $J_{1,P}$ = 7.3 Hz and $J_{1'',2''}$ = 3.2 Hz, $J_{1'',P}$ = 7.9 Hz). Desilylation of the GalN moiety by treatment with HF·Py provided triol **14** which was subsequently deacetylated at pH 11.5 (MeOH-H_2O-Et_3N) to afford a "double anomeric" phosphodiester **15** possessing an azido group at the GalN unit. Hydrogenation over Pd/C in methanol followed by purification by chromatography on Sephadex LH-20 resulted in a quantitative formation of the zwitterionic trisaccharide **16**. The NMR spectra were fully assigned by 1D and 2D-spectroscopy and were in excellent agreement with the data reported for the carbohydrate backbone of *Francisella* lipid A isolated by alkaline hydrolysis,[15] thus confirming the structure of the α-GalN(1→P)-modified diglucosamine backbone.

The same methodology was successfully employed for the synthesis of the α,α-(1→P←1) trisaccharide **17** equipped with an acetyl-protected sulfhydryl-containing spacer (Scheme 4). Application of the nearly pure α-anomeric form (α/β = 10:1) of the (1→6) diglucosamine lactol **12** allowed an enhancement of the yield of the H-phosphonate coupling up to 85%.

Alternatively, to exploit the merits of the phosphoramidite procedure, the *N,N*-diisopropyl-2-cyanoethyl phosphoramidite **18** (^{31}P NMR, δ, ppm: 151.2, 150.5, 150.4, 149.0, α/β anomeric mixture, (*R*),(*S*)-diastereomers at phosphorus) was prepared in situ by treatment of GalN lactol **4** with *N,N*-diisopropyl cyanoethylchlorophosphite in the presence of DIPEA.[32] 1*H*-Tetrazole-mediated coupling of the latter to lactol **12** (α/β = 10:1) afforded a mixture of the intermediate anomeric phosphite−triesters (^{31}P NMR, major products δ, ppm: 138.9, 138.7). After in situ oxidation with *tert*-butyl hydroperoxide (80% in di-*tert*-butyl peroxide) and treatment with Et_3N to remove the cyanoethyl protecting group from the phosphotriester by β-elimination, extensive purification on silica gel furnished the target phosphodiester **17** in 24% yield. This poor outcome could be explained by the necessity to perform four sequential transformations as a "one-pot" procedure owing to the inherent instability and the impossibility of isolation of the glycosyl phosphite/phosphate triester intermediates, such that the residual reagents could become involved in side-reactions during each following step.

Stepwise deprotection of **17** involved treatment with HF·Py to remove the silyl protecting groups, reduction of the azido group in **19** and final deacetylation to afford **21** (Scheme 4). The presence of the terminal thiol in **19** precluded application of the Pd-catalyzed hydrogenation for the reduction of the azido group, so that the alternative procedures were investigated. Classical Staudinger conditions did not result in any transformation, apparently due to the steric inaccessibility of the 2″-N_3-group adjacent to the α,α-(1↔1)-phosphodiester linkage. Application of the less sterically demanding trimethylphosphine (instead of PPh_3) in THF/aq NaOH[33] was unsuccessful as well. Treatment

Scheme 4. Synthesis of βGlcN(1→6)-αGlcN(1→P←1)-αGalN Epitope and BSA Conjugate



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of **19** with a reducing tin(II) reagent, $[Et_3NH][Sn(SPh)_3]$ complex,[34,35] resulted in a clean reduction of the 2″-N_3-group into the amino group. Prolonged reaction times in the presence of an excess of tin(II) reagent caused a loss of GalN moiety, presumably by a nucleophilic attack of the newly formed 2″-NH_2-group on the adjacent 1″-phosphate. To suppress this side reaction, an excess of the tin(II) complex was trapped by the treatment with the chelating agent, ethylenediaminetetraacetic acid (EDTA), immediately after the N_3-reduction was completed. Final deacetylation was performed under basic conditions (50% aqueous NH_2OH) to afford, after isolation on Superdex Peptide column, a zwitterionic phosphodiester **21** (as a mixture with disulfide **20**) in 44% yield. Reduction of the disulfide bond in **20** was performed by tris(2-carboxyethyl)-phosphine (TCEP)[36] and monitored by ^{1}H and HSQC NMR. Coupling of **21** to a maleimide-activated BSA provided the βGlcN(1→6)-αGlcN(1→P←1)-αGalN-containing neoglycoconjugate comprising up to an average of 13 pseudotrisaccharide units per BSA molecule (according to MALDI-TOF mass spectrometry data).

The unique structure of GalN-modified *Francisella* lipid A renders a "double anomeric" phosphodiester αGlcN(1→P←1)αGalN an attractive synthetic target. The lipid A-based neoglycoconjugate containing an epitope βGlcN(1→6)αGlcN-(1→P←1)αGalN, which is conserved in all *Francisella* strains, might be of considerable use for the generation of specific diagnostic antibodies which could be employed in immunoaffinity diagnostic assays for the prompt detection of *Francisella* infection by the direct antigen determination in medical samples.[37] The epitope can potentially be attached to different surfaces via its thiol-terminated spacer and utilized in diagnostic immunoassays as a capture antigen.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and ^{1}H, ^{13}C, and ^{31}P NMR spectra (partially HSQC/COSY spectra) of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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