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Chemoenzymatic synthesis of an isoprenoid phosphate tool for the analysis of complex bacterial oligosaccharide biosynthesis

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Abstract

Undecaprenyl Pyrophosphate Synthase (UPPS) is a key enzyme that catalyzes the production of bactoprenols, which act as membrane anchors for the assembly of complex bacterial oligosaccharides. One of the major hurdles in understanding the assembly of oligosaccharide assembly is a lack of chemical tools to study this process, since bactoprenols and the resulting isoprenoid-linked oligosaccharides lack handles or chromophores for use in pathway analysis. Here we describe the isolation of a new UPPS from the symbiotic microorganism *Bacteroides fragilis*, a key species in the human microbiome. The protein was purified to homogeneity and utilized to accept a chromophore containing farnesyl diphosphate analogue as a substrate. The analogue was utilized by the enzyme and resulted in a bactoprenyl diphosphate product with an easy to monitor tag associated with it. Furthermore, the diphosphate is shown to be readily converted to monophosphate using a common molecular biology reagent. This monophosphate product allowed for the investigation of complex oligosaccharide biosynthesis, and was used to probe the activity of glycosyltransferases involved in the well characterized *Campylobacter jejuni* N-linked protein glycosylation. Novel reagents similar to this will provide key tools for the study of uncharacterized oligosaccharide assemblies, and open the possibility for the development of rapid screening methodology for these biosynthetic systems.

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Supplementary Material

Supporting information includes: standard curve for NA-B-PP integrals, PgIA time course with GalNAc incorporation into NA-B-PP-Bac, Full ¹H-NMR of NA-B-PP *cis*-7, NA-B-PP *cis*-6, and NA-B-P, ³¹P-NMR of crude NA-B-PPs in CDCl₃, NP-HPLC of crude NA-B-PP and NA-B-P products, EsiPred3D model of *B. fragilis* UPPS, primers used for UPPS PCR, high resolution-negative ion-ESI-MS of NA-B-PP *cis*-7, NA-B-PP *cis*-6, NA-B-P, NA-B-PP-Bac, NA-B-PP-Bac-GalNAc

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Keywords

bactoprenol; undecaprenol; undecaprenyl diphosphate synthase; *cis*-prenyl transferases; *Bacteroides fragilis*

Introduction

The biosynthetic routes to capsular polysaccharides, peptidoglycan and other important bacterial sugars have become a sought after targets for antibiotic development.^{1, 2} However, the production of these complex oligosaccharides is difficult to screen *in vitro*. One of the major drawbacks to studying sugar biosynthetic systems is the reliance of prokaryotic organisms on the 55-carbon isoprenoid bactoprenyl monophosphate (BP). There are no easily distinguishable chromophores associated with bactoprenol, therefore there are few rapid assays available to detect and quantify the activity of enzymes associated with BP production. In addition, the availability of BP is limited. Often a plant (*Magnolia kobus*) C55 undecaprenol is purchased or isolated and used to study oligosaccharide assembly systems.³ However, the plant isoprenoid is typically a 4-*trans* 7-*cis* isoprene, and the bacterial bactoprenol is 3-*trans*, 8-*cis*.⁴

Bactoprenyl diphosphate (BPP, **1**) is biosynthesized, as shown in figure 1, via a polymerization reaction catalyzed by the enzyme Undecaprenyl Pyrophosphate Synthase (UPPS). In this reaction a single farnesyl diphosphate (FPP, **2**) is condensed with eight isopentenyl diphosphates (IPP, **3**).⁵ The long chain hydrophobic isoprenoid product is then thought to be depyrophosphorylated and selectively monophosphorylated to provide BP. The isoprenoid monophosphate is the typical substrate of initiating glycosyltransferases in oligosaccharide biosynthesis.^{6, 7}

The biosynthesis of complex oligosaccharides in the prokaryotic pathogen *Campylobacter jejuni* is not affected by alternations in the polyisoprene structure, unless the changes to the structure are near the monophosphate moiety.⁸ Numerous analogues of the UPPS substrate, FPP **2**, have been designed that incorporate different functionalities into the molecule.^{9–18} However, few of these analogues have been used to probe the selectivity of UPPS enzymes, and none of these analogues have been used to produce alternative substrates for oligosaccharide assembly systems. Alternative products of UPPS formed by utilizing FPP analogues will provide an intriguing new set of tools that could be used to study bacterial glycobiology.

Understanding the substrate selectivity of UPPS towards FPP analogues is crucial, as this enzyme plays a key role in many important aspects of bacterial metabolism. In addition to oligosaccharide assembly, formation of BPP is a potential target for antibiotics.^{19, 20} It is widely believed that one of the major drawbacks to antibiotic use is the influence of antimicrobial compounds on symbiotic microbial systems.^{21, 22} Recently, it has been shown that UPPS from the pathogenic microbe *Helicobacter pylori* can be affected by an inhibitor that does not influence the activity of a similar *E. coli* UPPS.¹

The organism *Bacteroides fragilis* is a key microbe involved in the development of a normal mammalian immune system and growing evidence suggests that the clearance of this organism from gut microbiota may have an impact on the development of immune deficiency diseases including irritable bowel syndrome and potentially multiple sclerosis.^{22–26} The role of *B. fragilis* in immune system development is thought to be primarily due to the expression of a specific polysaccharide on its surface.^{22, 23, 27} Inhibition

of *B. fragilis* UPPS would therefore affect the immunomodulatory activity of this microbe even if it did not kill the bacteria.

In this report we have isolated UPPS from the symbiotic microbe *B. fragilis* to probe the substrate selectivity of this enzyme, and develop new tools for the study of oligosaccharide biosynthesis. We have found that the *B. fragilis* enzyme is able to utilize an alternative isoprenoid to build a novel bactoprenyl diphosphate. The analogue diphosphate was conveniently converted to monophosphate utilizing a common Alkaline Phosphatase. The new bactoprenyl monophosphate analogue was utilized in an HPLC based assay for initiating glycosyltransferase activity. The initiating glycosyltransferases studied were from the very well characterized enzymes associated with oligosaccharide production in the gram negative pathogen *C. jejuni*.^{6, 8, 28, 29} These results confirm that the analogue can be used as a tool for studying complex oligosaccharide assembly systems.

Results

Cloning, expression and purification of *Bacteroides fragilis* Undecaprenyl Pyrophosphate Synthase

The UPPS gene was amplified from *B. fragilis* strain 25285 genomic DNA and ligated into a pET-24a vector encoding an N-terminal T7-tag and a C-terminal His₆-tag. The vector was transformed into C41, RP, and Rosetta BL-21 *E. coli* cells for over-expression. The C41 BL-21 cells provided the largest amount of protein based on western blot analysis of lysed cells. Ponceau staining and gel filtration were consistent with a highly pure monodispersed protein after Ni-NTA purification. Western blot detection of the T7-tag confirmed that the protein was the UPPS recombinant protein as shown in figure 2a. The protein oligomeric state was estimated to be consistent with a dimer to a tetramer of the predicted 32 kDa protein based on gel filtration as shown in figure 2b. Dynamic Light Scattering studies confirmed that the product was a dimer, which is consistent with the crystallized structure of *E. coli* UPPS³⁰. After gel filtration the final protein yield was approximately 55 mg from a one liter expression culture.

Confirmation of recombinant *B. fragilis* Undecaprenyl Pyrophosphate Synthase function

In order to determine if the purified UPPS protein was functional, pure UPPS was mixed with IPP **3** and [³H]-labeled FPP **2** for two hours. The product of the reaction was extracted into chloroform. The extract was then loaded onto a normal phase thin layer chromatography (TLC) plate, in parallel with a control [³H]-labeled FPP. After TLC separation of the mixture the plate was analyzed for radioactivity. As shown in figure 3a, a new spot with high mobility was formed in the reaction. No FPP was observed in the product extract. These results confirmed the expected function of the recombinant *B. fragilis* UPPS.

Utilization of an FPP analogue by *B. fragilis* UPPS

Radiolabel based methods for detecting polyisoprenoids are expensive and require specialized equipment. The analogue shown in figure 4, *p*-nitro-anilinogeranyl diphosphate (**4**, NAGPP), initially designed by the Spielmann group,¹² is a substrate for the prenyltransferase enzyme Protein Farnesyl Transferase.¹² We were interested in whether this strongly ultraviolet light absorbing compound ($E_{395nm}=9500 \text{ M}^{-1} \cdot \text{cm}^{-1}$) could also be utilized by UPPS. The *p*-nitro-aniline moiety is simple to detect visually and through spectroscopic methods, and is significantly less expensive than tritium labeled substrates. In addition, use of the *p*-nitro-aniline analogue has none of the hazards associated with it relative to the radioactive isoprene.

In order to determine if NAGPP **4** could be utilized by UPPS we mixed it with IPP **3** and the enzyme overnight and extracted polyisoprenoid product into chloroform. As shown in figure 3b, TLC analysis of the aqueous and organic extracts indicated that a product was formed, which was chloroform soluble and had a TLC retention factor (R_f) nearly identical to the product with the native substrate. No *p*-nitro-aniline-linked compounds were observed in the chloroform layer without IPP **3** present, suggesting that only UPPS products would be extracted and not the reaction substrate

Multiple *p*-nitroaniline-bactoprenyl diphosphates are formed by UPPS

It was not possible to determine whether the product of the UPPS reaction was full length with eight isopentene additions or if it was a smaller chain from TLC analysis alone. To isolate the individual components of the product we utilized a reverse phase (RP) HPLC method with a C18 column. UPPS was assayed for products formed over time using an isocratic mobile phase of 1:1 ammonium bicarbonate buffer to *n*-propanol, as shown in Figure 5a. The two major peaks associated with the reaction had retention times of 15.3 and 23 minutes. Each of these peaks were characterized by negative ion mode electrospray ionization mass spectrometry ((-)ESI-MS) where we found an m/z of 924.9 amu and 462.0 amu for the 23 minute peak and an m/z of 856.9 amu and 427.9 amu for the 15.3 minute peak. These values were consistent with the H_2A^- and HA^- forms of a product with seven isoprenoid additions (*cis*-7NA-B-PP) for the 23.0 minute peak and six isoprenoid additions (*cis*-6NA-B-PP) for the 15.3 minute peak respectively.

After isolation and chemical characterization of each of the products, as discussed later in this report, we developed a standard curve based on the RP-HPLC integrals of a known quantity of the *cis*-7NA-B-PP to analyze the rate of product formation (supporting figure 1). Interestingly, as shown in figure 5b, we found that the *cis*-6NA-B-PP was formed at a rate of about 4 nM/sec while the *cis*-7NA-B-PP was formed at a rate of about 1 nM/sec, with only limited other intermediates detected. The rate of the *cis*-6NA-B-PP slowed after consumption of most of the NAGPP starting material. The steady state rate of the reaction to form the *cis*-7NA-B-PP appeared the same throughout the reaction. These results suggested that the final isoprene addition in the reaction was rate limiting under the conditions of this assay.

Alkaline Phosphatase catalyzes diphosphate cleavage

Previous studies have utilized a potato acid pyrophosphatase to cleave the diphosphate group from long chain isoprenoid diphosphates.³¹ The alcohol products of these reactions were then characterized by reverse phase TLC analysis. A major interest of our laboratory is the isolation of bactoprenyl monophosphates that could be used to study the typical initiating step of oligosaccharide biosynthesis in bacteria. Currently, the simplest method available to obtain this material would be to cleave the diphosphate to alcohol then use a non-specific kinase to phosphorylate the allylic alcohol.^{3, 32}

Alkaline Phosphatase is a common reagent utilized in protein isolation and molecular biology laboratories. We were interested to see if this phosphatase could be used to prepare monophosphate or alcohol forms of bactoprenol. BPP and *p*-nitro-aniline BPP (NA-B-PP) were extracted as described above and treated with commercially available alkaline phosphatase linked to an anti-rabbit antibody. Surprisingly, as shown in figure 6a, a new product was formed with a TLC R_f that would be expected for the monophosphate product of each. The antibody-linked enzyme appeared to catalyze the cleavage of the polyprenyl diphosphate quantitatively. RP-HPLC analysis of the *cis*-7NA-B-PP reaction with the alkaline phosphatase shown in figure 6b suggested that the reaction was 96% complete after one hour. (-)ESI-MS analysis of the new peak formed in the *cis*-7NA-B-PP reaction

confirmed that the RP-HPLC peak at 40.7 minutes contained a product of 844.9 amu which was consistent with the HA- form of the isoprenoid monophosphate (NA-B-P).

Campylobacter jejuni glycosyltransferases utilize NA-B-P as a substrate

The *p*-nitro-aniline bactoprenyl analogue was designed as a proof of principle compound that would enhance the convenience of monitoring glycosyltransferase reactions, and open the door to new assay systems for studying bacterial glycosylation. The analogue was tested with a set of well characterized glycosyltransferases involved in the asparagine-linked glycosylation of proteins in the gram negative pathogen *Campylobacter jejuni*.⁶ Importantly, we chose the *C. jejuni* system to test this analogue because the activity of all the enzymes involved has been previously characterized in detail.^{6, 8, 28, 29, 34} As shown in scheme 1, the enzyme PglC is an initiating glycosyltransferase that transfers N,N'-diacetylbacillosamine(Bac)-phosphate from uridine diphosphate-linked Bac (UDP-Bac) to BP.⁶ A crude preparation of NA-B-P (containing multiple isoprenoid lengths) was mixed with UDP-Bac, biosynthesized as described by Olivier et al.,²⁸ and over-expressed PglC. We observed conversion of the NA-B-P to a lower R_f material by TLC (Figure 7a) suggesting that Bac-phosphate was incorporated into the NA-B-P analogue by PglC.

Often the activity of *C. jejuni* PglC is monitored using a coupled reaction with the next glycosyltransferase in the pathway PglA.^{6, 33} This is in part due to easier accessibility to a tritium-labeled UDP-linked N-AcetylGalactosamine (UDP-GalNAc) substrate for PglA. Here we were interested in how well the NA-B-P reaction compared with the previous method for following the first steps in the *C. jejuni* oligosaccharide biosynthetic pathway.^{3, 4, 33} To do this we mixed undecaprenol, an *S. mutans* diacylglycerol kinase, ATP, PglC, PglA, UDP-Bac and UDP-[³H]GalNAc in a single reaction mixture. Alternatively, we prepared a reaction without kinase and ATP, but with the crude NA-B-P preparation instead of undecaprenol. Reactions were monitored by the incorporation of tritium-label into the organic soluble isoprenoid. We found that both NA-B-P and undecaprenol were substrates for the PglC and PglA enzymes, as radioactivity was only found in the organic extracts when the isoprenoids were present. Importantly, when UDP-Bac, or PglC was left out of the mixture, no organic soluble radioactive product was observed. The extent of sugar transfer was the same between the two isoprenoids (data not shown). In addition a new spot was observed by TLC analysis in the presence of PglC/PglA, UDP-GalNAc and UDP-Bac with the NA-B-P analogue (Figure 7a).

Confirmation that [³H]GalNAc is incorporated into the photoactive isoprenoid

TLC plates with the resolved crude NA-B-P and undecaprenol product were analyzed for radioactivity to determine if the new product formed included the radioactive sugar. A separate plate was spotted at the origin and solvent front region to ensure appropriate localization of peaks with plate position (Figure 7b). We found that the new yellow *p*-nitro-aniline product was associated with the radioactivity observed on the plate, and that this spot was at the same location as the radioactivity observed with undecaprenol. No significant organic soluble tritium counts were observed in any of these reactions without UDP-Bac present.

RP-HPLC confirmation of bacterial glycosyltransferase activity

We were next interested in whether the *cis*-7NA-B-P isolated by RP-HPLC could be utilized by PglC as an acceptor for Bac-P. A reaction was prepared containing the purified analogue, PglC and UDP-Bac and was analyzed by RP-HPLC. As shown in figure 8a, a new peak at 20.6 minutes was observed from this reaction where 85% of the NA-B-P was consumed after fifty-three minutes of incubation to give the presumed NA-B-PP-Bac. In addition, when the product of the PglC reaction was treated with PglA and UDP-GalNAc

another new peak at 18.8 minutes was observed that likely represented NA-B-PP-Bac-GalNAc. As shown in figure 8b, we were able to monitor the stable incorporation of the last sugar into the isoprenoid over several hours. The reaction continued for five hours until 60% of the NA-B-PP-Bac had been consumed (supporting figure 2). Isolation of the analytical scale components of these reactions and (–)ESI-MS analysis confirmed the designation of each of the products as we observed a m/z peak at 576.4 amu for the NA-B-PP-Bac which was consistent with the molecular weight of the A^{2-} anion. We also observed an m/z peak at 677.3 amu for the NA-B-PP-Bac-GalNAc which was consistent with the A^{2-} form of that isoprenoid.

In order to compare our RP-HPLC method of analysis with the classic radiolabel based methods we tested the incorporation of a $[^3H]$ GalNAc into the NA-B-PP-Bac-GalNAc peak. Using RP-HPLC analysis of a reaction containing UDP- $[^3H]$ GalNAc, UDP-Bac, PglC, PglA and NA-B-P we determined the extent of incorporation of $[^3H]$ GalNAc based on standard curve analysis of the integrals associated with the NA-B-PP-Bac and NA-B-PP-Bac-GalNAc peaks. No leftover NA-B-P was observed in the analysis. We determined that 15.8 pmols of NA-B-PP-Bac-GalNAc was present in the 10 μ L aliquot analyzed by RP-HPLC based on the standard curve. In addition, simply determining the percent conversion based on peak integrals and input NA-B-P gave a similar value. The RP-HPLC chromatogram is shown in figure 8c. Chloroform extraction of the same amount of the reaction mixture and scintillation counting led to an estimate of 11.5 pmols of organic soluble radiolabel. Fractions from the RP-HPLC analysis were collected from 3 minutes (the approximate dead time of the system) to 22 minutes. These fractions were then counted for radioactivity. Finally, as shown in figure 8c, we found that tritium label that did not pass straight through the column, as would be expected for UDP- $[^3H]$ GalNAc, was associated with the UV active NA-B-PP-Bac-GalNAc peak. In addition, the amount of radioactivity in the flow through relative to the amount in the peak was consistent with what was expected from the other analyses. These results with the well characterized *C. jejuni* system strongly support the use of this analogue in the analysis of complex carbohydrate assembly systems.

Chemical characterization of bactoprenyl diphosphates

The isolated *cis*-6 NA-B-PP, *cis*-7 NA-B-PP and NA-B-P were diluted into d_6 DMSO for detailed NMR characterization. Interestingly, we found upon integration of the *cis*-6 NA-B-PP spectrum and normalization to the clear aromatic protons (7.9 and 6.6 ppm) in the spectrum that there were approximately six new olefin protons (5.1 ppm) associated with the spectrum that were distinct from the *trans*- protons (5.3 ppm) observed for the NAGPP **4** starting material (figure 9a). These easily distinguished peaks are consistent with the presence of the *Z*-form isoprenoids added by the UPPS enzyme. In addition, we were also able to observe an increase in the number of protons associated with the *cis*- proton resonance frequency by one in the *cis*-7 NA-B-PP (figure 9b). This same number of protons was observed in this position for the *cis*-7 NA-B-P product of alkaline phosphatase (supporting figure 3a). Upon further inspection of the 1H -NMR spectrum we observed an increase in the number of methylene protons in the 2 ppm region and an increase in a specific methyl peak at 1.58 ppm (supporting figure 3b–c). These results were consistent with the (–) ESI-MS spectroscopic identification of the *cis*-6 and *cis*-7 products. In addition, a triplet peak from 4.17 ppm was consistent with previous observations of a through oxygen splitting of the methylene nearest the diphosphate by the phosphorous atom and splitting by the olefin proton nearest to the diphosphate group. All chemical formulas were verified by high resolution mass spectrometry.

^{31}P -NMR was less conclusive for the diphosphate product as it appeared that the diphosphate peaks in d_6 DMSO overlapped with one another at –9.0 ppm (figure 9c) as a

broad base can be observed in the peak consistent with the phosphorous atom nearest the isoprenoid chain. However, the crude product of the UPPS reaction with NAGPP was analyzed in CDCl_3 and we were able to observe two clear peaks one at -6 ppm and the other at -9 ppm (supporting figure 4). The structure of the phosphorous peak most closely associated with the isoprenoid chain (-6 ppm) is not a clear doublet and is much more broad than the -9 ppm peak. The decrease in signal to noise for this -6 ppm peak along with solvent effects may account for the fact that it was not observed in the *cis*-7NA-B-PP spectrum. Importantly, when the *cis*-7NA-B-PP was treated with alkaline phosphatase and the product purified by RP-HPLC we observed a shift in the major phosphorous peak of *cis*-7NA-B-PP to 0 ppm for the *cis*-7NA-B-P (figure 9d).

NP-HPLC analysis of *p*-nitroaniline-bactoprenyl diphosphate

Normal phase (NP)-HPLC was performed on an amine bonded resin using solvent conditions identical to the TLC analysis. We found that in the NAGPP **4** reaction with IPP **3**, that multiple products were observed with similar retention times utilizing this method (supporting figure 5). However, attempts to isolate material failed due to decomposition of the product upon drying in the chloroform/methanol/sodium borate mobile phase. Attempts to use ammonium acetate and ammonium bicarbonate buffers in place of the sodium borate buffer failed to resolve sharp HPLC peaks, and extractions to remove sodium borate from the HPLC fractions were cumbersome and unreliable.

Discussion

Limits of natural substrate selectivity of UPPS

The X-ray crystal structure of the UPPS enzyme from *E. coli* has been solved (PDB code 3QAS) ^{35–37}. FPP binds to the active site and is positioned adjacent to the IPP substrate and coordinated to a Mg^{2+} ion. It is thought that as the isoprenoid chain is elongated it travels through a hydrophobic hole just below the catalytic site. The length of the isoprenoid is believed to be controlled by the presence of a hydrophobic barrier at the bottom of the hole.³⁵ Mutation studies of *E. coli* UPPS suggest that alterations in the hydrophobic hole lead to increased chain length of BPP **1** while mutations in the FPP/IPP binding site result in decreased product length.^{38, 39}

The fluorescent FPP analogue **5** shown in figure 10 is a substrate for *E. coli* UPPS,³¹ and has been used to measure the kinetics of UPPS catalyzed reactions. The FPP analogue appeared to be a substrate with properties similar to the native FPP **2**, where seven isopentyl groups were incorporated into the polyisoprenoid chain. It is believed that the smaller fluorescent moiety incorporated into the analogue allowed for better substrate recognition than was observed with the coumarin analogue **6** synthesized previously by the same group. The coumarin analogue **6** is known to bind *E. coli* UPPS and undergo one to two isoprene additions before it is released.⁴⁰ It was presumed that the hydrophobic hole was responsible for restricting the activity of this analogue. Our work is consistent with this interpretation of the previous data, where our relatively small analogue was incorporated into the bactoprenyl diphosphate product. The NAGPP **4** is about the same size as analogue **5**, but the electronic properties are quite different. Many FPP analogues have been synthesized by other groups^{9, 13, 31, 41–44} and the use of these analogues to further probe the substrate selectivity of UPPS from either organism would greatly enhance our understanding of the limits on substrate structure modification. New analogues could lead to improved assay systems as well as potential new inhibitors of UPPS enzymes.

Symbiont and pathogen UPPS targeting

The amino acid sequence of the *B. fragilis* and the structurally characterized *E. coli* UPPS are 43.9% identical and 67% similar. The EsyPred3D software⁴⁵ was able to thread the sequence of the *B. fragilis* enzyme into the crystal structure of the homologous *E. coli* protein (supporting figure 6). Importantly, several key areas of the protein appeared to be quite similar with respect to location in the protein and sequence identities. The FPP binding site was clearly conserved between the two organisms along with a catalytic aspartate, IPP binding site and the hydrophobic hole in the protein. However, there was a flexible loop region adjacent to the active site that could impart some differences in the specificity of the two enzymes with respect to alternative substrates or inhibitors.

H. Pylori UPPS was previously shown to be specifically inhibited by compounds that did not affect the activity of *E. coli* UPPS.¹ It is possible that such inhibitors may not affect other commensal bacteria, including *B. fragilis*. However, no other UPPS enzymes from bacteria have been isolated until now. This work shows that UPPS from a new organism can be easily isolated and tested with different analogue diphosphates. Screening inhibitors or substrate analogues with this protein in addition to proteins from other organisms could lead to profiling the UPPS enzyme for inhibitors that are directed at pathogenic organisms preferentially over symbiotic. This would provide an important new tool in understanding the relationships between symbiotic and pathogenic organisms with antibiotic usage.

Biosynthesis of complex oligosaccharides

A major interest of our laboratory is the development of new probes to study oligosaccharide assembly in prokaryotes. Typically reactions in these pathways first require a bactoprenyl monophosphate as a substrate.³⁴ However, isolation of such monophosphates is difficult and monitoring reactions with isoprenoids is cumbersome and expensive. This work provides the first new tool to be used to study these biosynthetic systems with a strongly absorbing UV active probe as an isoprenoid substrate for these enzymes. Such a system removes much of the restrictions on the study of these proteins and may allow for the development of rapid assay systems needed for isoprenoid/sugar transferases. The quantities of bactoprenyl monophosphate analogue obtained from the described procedures will be more than sufficient for use with complex downstream enzymes such as glycosyltransferases involved in peptidoglycan and polysaccharide biosynthesis. Our laboratory is currently developing additional probes with increased detection sensitivity and improved photophysical properties.

Materials and Methods

General

Synthetic procedures for NAGPP **4** preparation were performed as described by Chehade et al.^{12, 46} IPP **3** tris-tetra-N-butyl ammonium form was synthesized as described by Poulter and co-workers.⁴⁷ FPP **2** was purchased from Sigma-Aldrich and [³H]-FPP was purchased from American Radiolabeled Chemicals. *p*-nitro-aniline containing species concentrations were measured by UV absorbance at 395 nm using an extinction coefficient of 9500 M⁻¹ cm⁻¹. All general solvents and reagents were purchased from Sigma-Aldrich, VWR or Fisher unless stated otherwise. Custom primers were purchased from Invitrogen. Reverse phase preparative HPLC was performed on a Polaris C18 250 × 21.2 column for the purification of IPP and NAGPP. All HPLC analysis was performed on an Agilent 1100 system equipped with a diode array detector, autoinjector, degasser and quaternary pump. HPLC flow through was typically collected on a Gilson FC-205 fraction collector

UPPS cloning

The UPPS gene was amplified using the polymerase chain reaction (PCR) from *B. fragilis* strain 25285 genomic DNA (ATCC). Primers (supporting figure 7) were designed to insert a BamHI and XhoI restriction site on the 5' and 3' end of the gene respectively. The PCR reaction was performed using a Promega PCR core system kit on a thermocycler with a 55° C annealing temperature and 35 cycles. After amplification, the gene was purified on an agarose gel, digested with BamHI and XhoI (New England Biolabs), and ligated (Promega ligase) into a double digested pET-24a (Novagen) vector using standard protocols. Competent DH5 α *E. coli* cells were then transformed with the vector and positive clones were selected by lack of sensitivity to Kanamycin..

UPPS expression

UPPS plasmid was isolated and purified using standard methods (Fermentas plasmid purification kit) from the transformed DH5 α cells then used to transform protein expression cells including BL-21 -RP (Stratagene), -C41 (Lucigen), and -Rosetta (EMD Millipore). Cells were grown overnight in 5 mL starter cultures, of which 1 mL was used to inoculate 1 L of Luria Broth at 37° C. At an O.D. of 0.4–0.6, isopropyl thiogalactoside was added to a final concentration of 1 mM and the incubation temperature was reduced to 17° C. Cultures were left shaking overnight. Cells were harvested by centrifugation for 30 minutes at 4° C, 5000 X g. The cell pellet was then washed with 75 mL of 0.9% NaCl then centrifuged again for 30 minutes at 4° C, 5000 X g. Pellets were stored at –80° C or immediately lysed in 20 mL of lysis buffer containing 50 mM Tris-HCl (pH=8.0), 200 mM NaCl, 20 mM imidazole. Cells were lysed on ice via sonication at 25% power for 2 minutes with a pulse of 1 sec on and 1 sec off. Cells were then centrifuged for 1 hour and 15 minutes at 4° C, 135,000 $\times$ g. The supernatant was mixed with 3 mL of Ni-NTA (PerfectoPro, 5 Prime Inc) for one hour then poured over a column. The flow through was collected and the Ni-NTA column was washed with 50 mM Tris-HCl/200 mM NaCl/50 mM imidazole 4 $\times$ 3 column volumes and the protein was eluted off the column with 6 $\times$ 0.5 column volumes of elution buffer (50 mM Tris-HCl/200 mM NaCl/ 500 mM imidazole). Elutions containing UPPS were identified by SDS-PAGE and transfer Ponceau analysis, and finally by western blot utilizing an anti-T7 alkaline phosphatase conjugated primary antibody. Fractions containing protein were dialyzed in 50 mM Tris-HCl/200 mM NaCl/0.5% glycerol 3 $\times$ 1L then frozen at –80° C for later use. Protein was further purified by Gel filtration chromatography performed on a Hi-Load 16/60 Superdex 200 column (GE Healthcare) using an Akta prime plus Fast Protein Liquid Chromatography system (GE Healthcare). Protein standards purchased from Sigma-Aldrich were analyzed using identical conditions. Dynamic Light Scattering was performed on a Dawn Helios II system from Wyatt. Purified UPPS was functional for more than one year when stored at – 80° C under the above conditions.

Bactoprenyl diphosphate general preparation

Crude bactoprenyl diphosphates were prepared in 50 mM HEPES (pH=7.4), 0.1% Triton X-100 hydrogenated, 5 mM KCl, and 0.5 mM MgCl₂ in a total volume of 200 μ L. Reactions were left at 37° C for one to two hours, after which 100 μ L aliquots were quenched with the addition of 0.8 mL of 2:1 CHCl₃/MeOH. The Organic phase was washed 3X with 0.2 mL of pure solvent upper phase (PSUP: 20 g KCl/240 mL MeOH/240 mL Water). The organic layer was dried on a centri-vap concentrator, or analyzed by TLC or NP-HPLC directly.

[³H]-bactoprenyl diphosphate—Tritium-labeled bactoprenyl diphosphate was prepared as follows. FPP (6 nmols) and [³H]-FPP (3 pmols, specific activity 57,000 dpm/pmol) were mixed with UPPS (3.4 nmols) and IPP (13 μ mol). Reaction product was extracted as described above and used directly for TLC analysis.

Crude p-nitro-aniline bactoprenyl diphosphate preparation (NA-B-PP crude)—

Crude product analyzed by TLC and NP-HPLC was prepared with 1.3 μmol s of NAGPP mixed with 13 μmol s of IPP and 1.7 nmols of UPPS in a final volume of 200 μL . ^1H -NMR and ^{31}P -NMR analysis were performed on a 500 MHz Jeol spectrometer. The ^{31}P spectrum is available in supporting figure 2.

Preparation of cis-6 and cis-7 NA-B-PP—NA-B-PP reactions were prepared as described above on a 65 μM NAGPP with 613 μM IPP and 1 μM UPPS with all other components the same as the crude preparation. After four hours of incubation at 37° C, n-propanol was added to give a 50% n-propanol solution. Product was isolated by RP-HPLC as described below.

NA-B-PP-cis-6 • 3N⁺Bu₄—RP-HPLC Rt: 15.3 min. ^1H -NMR (d_6DMSO , 500 MHz): δ 7.88 (d, 2H), 6.54-6.60 (m, 2H), 5.19-5.29 (m, 2H), 4.97-5.08 (m, 6H), 4.15-4.20 (t, 2H), 3.61-3.64 (bs, 2H), 3.06-3.16 (22H N⁺Bu₄ expected 24H), 3.04-3.59 (H₂O), 2.27-2.63 (d_6DMSO), 1.85-2.07 (m, 30H; expected 28H), 1.60 (s, 3H), 1.58 (s, 14H, expected 15H), 1.48-1.58 (N⁺Bu₄ and last CH₃ 32H expected 30H), 1.24 (h, N⁺Bu₄ 28H expected 24H), 0.89 (t, N⁺Bu₄ 41H, expected 36H). ^{31}P -NMR (202 MHz): δ -8.45 ppm; HR-(−)ESI-MS: expected m/z 857.4635 [M-H][−]; found: 857.4639

NA-B-PP-cis-7 • 2N⁺Bu₄—RP-HPLC Rt: 23.0 min ^1H -NMR (500 MHz) : δ 7.90 (d, 2H), 6.53-6.59 (m, 2H), 5.20-5.30 (m, 2H), 4.96-5.10 (m, 7H), 4.16-4.23 (t, 2H), 3.62-3.66 (bs, 2H), 3.06-3.16 (N⁺Bu₄ 22H expected 24H), 3.04-3.59 (H₂O), 2.27-2.63 (d_6DMSO), 1.82-2.08 (m, 31H; expected 32H), 1.60 (s, 3H), 1.58 (s, 17H, expected 18H), 1.48-1.58 (N⁺Bu₄ and remaining methyls 32H, expected 30H), 1.24 (h, N⁺Bu₄ 28H, expected 24H), 0.89 (t, N⁺Bu₄ 41H expected 36H). ^{31}P -NMR (202 MHz): δ -8.66 (2P), 0.15 ppm (0.2P); HR-(−)ESI-MS: expected m/z 925.5261 [M-H][−] found 925.5286

Crude bactoprenyl monophosphate biosynthesis

Monophosphate analogue and bactoprenyl monophosphate were typically prepared as follows. Dried organic extracts from the crude diphosphate preparations above were dissolved in 98 μL of 5% DMSO, 18% n-Propanol and 50 mM Tris-HCl (pH=8.0). The mixture was sonicated in a bath for 3 minutes. Next, 2 μL of goat anti-rabbit alkaline phosphatase (Pierce) was added and the mixture was incubated for 1 hour to overnight. Products from the reaction were extracted as described above for the diphosphates, and either directly analyzed by TLC or dried on a centri-vap system.

Preparation of cis-7 NA-B-P • 4N⁺Bu₄—NA-B-PP cis-7 was diluted into 200 μL of 20% n-propanol in 25 mM ammonium bicarbonate. The mixture was then treated with 1 μL of alkaline phosphatase and the reaction was monitored by RP-HPLC with a 1 μL injection immediately after enzyme was added and another injection after 70 minutes. Injections were then scaled up to 100 μL and product with a retention time of 39–42 minutes was isolated. The NA-B-P was dried then characterized by NMR and MS. RP-HPLC Rt: 40.4 min ^1H -NMR (Jeol 300 MHz) : δ 7.92 (d, 2H), 6.58-6.64 (m, 2H), 5.18-5.35 (m, 2H), 5.00-5.14 (m, 7H), 4.02-4.13 (t, 2H), 3.64-3.69 (bs, 2H), 3.06-3.16 (N⁺Bu₄ 41H expected 32H), 3.04-3.59 (H₂O), 2.27-2.63 (d_6DMSO), 1.88-2.06 (m, 30H; expected 32H), 1.47-1.66 (N⁺Bu₄ remaining methyls 60H expected 59H), 1.24 (h, N⁺Bu₄ 36H expected 32H), 0.89 (t, N⁺Bu₄ 49H, expected 48H). ^{31}P -NMR (Jeol 122 MHz): δ 0.15 ppm (s); HR-(−)ESI-MS: expected m/z 845.5598 [M-H][−] found 845.5621

TLC analysis of bactoprenyl diphosphates and monophosphates

Thin Layer Chromatography was performed on normal phase K6f non-fluorescent plates (Whatman). Generally, dried diphosphate or monophosphate products from above were dissolved in 50 μ L of 2:1 $\text{CHCl}_3/\text{MeOH}$. Oven dried TLC plates were spotted and placed in a chamber containing 73/53/11 $\text{CHCl}_3/\text{MeOH}/0.1$ M Sodium Borate. The plates were removed after solvent had traveled 50 mm. [^3H]-labeled product was analyzed on a Bioscan AR-2000 radio-TLC imaging scanner. Unlabeled *p*-nitro-aniline products were analyzed by placing the plates on a transilluminator and direct visualization. Detection of the *p*-nitro-aniline spot was enhanced by photographing the plate under UV illumination using a Canon G11 digital camera.

NMR characterization of analogue diphosphate and monophosphate crude

The crude *p*-nitro-aniline bactoprenyl diphosphate or monophosphate was dissolved in 300 μ L of CDCl_3 . ^1H -NMR spectra were taken on a 500 MHz Jeol Spectrometer in a CDCl_3 Shigemi tube. ^{31}P -NMR spectra were recorded on the same spectrometer with a 1 sec relaxation delay overnight. Re-analysis of crude NMR samples suggested product decomposition in CDCl_3 over time. It is important to note that d_6DMSO used with pure samples led to an overlap in the phosphorous peaks that we were unable to resolve. This peak shifted significantly in d_6DMSO for the purified NA-B-P *Cis-7* analogue.

High Resolution mass spectral analysis

HR-MS was performed by the David H. Murdock Research Institute (DHMRI) Metabolomics Laboratory, by direct injection coupled to quadrapole time-of-flight mass spectrometry operating in the negative mode. All samples were supplied to the DHMRI dissolved in 20% *n*-propanol in 25 mM ammonium bicarbonate buffer at 0.1 g/L.

NP-HPLC monitoring of NA-B-PP production

HPLC analysis of BP and BPP was performed on a Pursuit amine bonded silica column (Varian) using an isocratic mobile phase of 73/53/11 $\text{CHCl}_3/\text{MeOH}/0.1$ M Sodium Borate. Reaction monitoring was performed by injecting 0.5 μ L aliquots from a reaction containing 1.3 mM NAGPP, 10 mM IPP, 860 nM UPPS, 0.1% Triton X-100, 50 mM HEPES (pH=7.4), 1 mM MgCl_2 , and 10 mM KCl in a total volume of 100 μ L.

Sugar transfer by PglC to NA-BP for TLC—*Campylobacter jejuni* PglC was prepared as described previously from overexpressing BL-21 cells.⁶ Uridine diphosphate-linked N-N'-diacetyl-bacillosamine (UDP-Bac) was prepared as described by Olivier et al.²⁸ For TLC analysis sugar transfer assays were performed in solutions containing 1% Triton X-100, 10 mM MgCl_2 , 50 mM Tris-HCl (pH=8.0), 0.1 mM NA-BP, 0.4 mM UDP-Bac and 10% DMSO plus 10 μ L of a crude PglC preparation in a final volume of 100 μ L. After two hours the entire mixture was extracted into 2:1 $\text{CHCl}_3/\text{MeOH}$ and washed with PSUP three times. The organic extract was dried on a centrivap system, dissolved in minimum 2:1 $\text{CHCl}_3/\text{MeOH}$ then analyzed by TLC as described above. A negative control reaction was prepared identically to the above without UDP-Bac present.

Sugar transfer by PglC and PglA to Undecaprenol and NA-BP for TLC—

Streptococcus mutans diacylglycerol kinase, and *C. jejuni* PglA were prepared as described previously.^{28, 32} Assays were performed in a solution containing 10% DMSO, 1% Triton X-100, 10 mM MgCl_2 , 50 mM Tris-HCl (pH=8.0), 0.27 mM NA-B-P 0.8 mM UDP-Bac, and 0.4 mM UDP- ^3H GalNAc (specific activity=86 dpm/pmol) plus 10 μ L of a crude PglA and PglC preparation each in a final volume of 100 μ L. A positive control reaction was prepared with the above components except NABP was replaced with 0.1 mM

undecaprenol, 10 μ L of *S. mutans* diacylglycerol kinase containing membrane fractions, and 100 mM ATP. Negative control reactions were prepared without NABP. After an overnight incubation a 10 μ L aliquot of the reaction was extracted into CHCl_3 and dried under a stream of air. Organic extract was dissolved in DMSO and 4.8 mL of ECOLITE scintillation fluid was added to both the organic extract and aqueous washes. Samples were counted on a Perkin-Elmer Tricarb scintillation counter. The remaining reaction material was extracted into chloroform and washed then analyzed by TLC, visual detection of product and detection of incorporated tritium-labeled sugar using the Bioscan TLC plate imager.

RP-HPLC UPPS and AP reaction analysis and product isolation

NA-B-PP and NA-B-P products were isolated using a 25 mM ammonium bicarbonate/n-propanol (1:1) isocratic mobile phase at 0.5 mL/min on a Polaris C18 5 μ m 250 $\times$ 4.6 mm analytical column. Products (15.3 minutes *cis*-6 and 23 minutes *cis*-7) were isolated dried on a centri-vap system then characterized by NMR and Mass Spectrometry. The time course analysis of the NAGPP reaction was performed on a 13 nmol scale with 1 μ L/200 μ L injections of the sample every 47 minutes. Product formed was determined based on a standard curve developed with pure NA-B-PP *cis*-7 (supporting figure 1) that was derived from injecting multiple concentrations and multiple volumes from known concentrations of an NA-B-PP *cis*-7 standard. After isolation and NMR characterization, the *cis*-7 product from the preparative reaction was dissolved in 20% n-Propanol/25 mM ammonium bicarbonate, converted to monophosphate as described above and isolated using conditions identical to the NA-B-PP *cis*-7 RP-HPLC analysis.

RP-HPLC glycosyltransferase analysis

A PglC reaction was prepared with 1% Triton-X-100, 10 mM MgCl_2 , 50 mM Tris-HCl (pH=8.0), 12.5 μ M NA-B-P, 100 μ M UDP-Bac, and 10 μ L of crude PglC in a final volume of 200 μ L. At 1 minute and 53 minutes 10 μ L was analyzed by RP-HPLC using the conditions described above. Percent turnover was determined based on the integrals of the NA-B-P and new NA-B-P-Bac peak at 20.9 minutes. The sample was then treated with UDP-GalNAc to a concentration of 3.3 mM, and 10 μ L of crude PglA. The reaction was then monitored by RP-HPLC at the time points indicated in supporting figure 2.

Radioactive sugar detection by RP-HPLC and scintillation counting

A reaction was prepared with 5 nmols NA-B-P, 80 nmols UDP-Bac and UDP-[^3H]GalNAc (3 nmols, specific activity= 566 dpm/pmol), PglC and PglA as above. 10 μ L was injected onto a C18 column and was separated using the isocratic RP-HPLC method described above. Fractions were collected from 3 minutes to 22 minutes. 4.8 mL of Ecolite scintillation fluid was added to each fraction and tritium in those fractions was detected by scintillation counting. In addition, 20 μ L of the reaction was extracted into CHCl_3 and counted as described above. Percent turnover was determined by inspection of the HPLC peak integrals (6% turnover from NA-B-PP-Bac $\rightarrow$ 15.7911 pmols NA-B-PP-Bac in 10 μ L), comparison of peak integrals to a standard curve (NA-B-PP-Bac-GalNAc area=26 $\rightarrow$ 15.79 pmols in 10 μ L) and radioactivity in the mixture (6500 dpm in 10 μ L $\rightarrow$ 11.47 pmols).

PglC and PglA product isolation

PglC and PglA reactions were prepared as described above on a 5 nmol NA-B-P scale with 80 nmols UDP-Bac and/or 1.3 μ mols of UDP-GalNAc with twice the enzyme used in the analysis above. Final reaction volume was 200 μ L. After an overnight incubation at room temperature the reactions were quenched with 200 μ L of n-propanol and the products were isolated by RP-HPLC. Products were collected and dried for further characterization. Low Resolution Mass Spectrometry was performed on a Mariner ESI-MS in negative ion mode.

NA-B-PP-Bac characterization—RP-HPLC Rt: 20.9 minutes, HR-(−)ESI-MS: expected m/z 1153.6376 [M-H][−] found 1153.6415

NA-B-PP-Bac-GalNAc characterization—RP-HPLC Rt: 18.9 minutes, HR-(−)ESI-MS: expected m/z 1356.7170 [M-H][−] found 1356.7252

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

TLC	thin layer chromatography
GalNAc	N-acetylgalactosamine
UDP	uridine diphosphate
BPP	bactoprenyl diphosphate
BP	bactoprenyl monophosphate
NA	<i>p</i> -nitro-aniline
Bac	N,N'-diacetylbacillosamine
HPLC	high performance liquid chromatography
NMR	nuclear magnetic resonance spectroscopy
FPP	farnesyl diphosphate
IPP	isopentenyl diphosphate
Und	undecaprenol
PSUP	pure solvent upper phase
ATP	adenosine triphosphate
NAGPP	<i>p</i> -nitro-aniline geranyl diphosphate
UPPS	undecaprenyl pyrophosphate synthase
(−)ESI-MS	negative ion mode electrospray ionization mass spectrometry
HR	High Resolution
LR	Low resolution
amu	atomic mass units

m/z mass charge ratio

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HIGHLIGHTS

- *B. fragilis* Undecaprenyl Pyrophosphate Synthase (UPPS) isolation
- Bactoprenyl diphosphate analogue preparation with UPPS
- Bactoprenyl monophosphate analogue preparation with alkaline phosphatase
- Monitoring a UPPS reaction by HPLC
- Analysis of *C. jejuni* oligosaccharide assembly steps by TLC and HPLC

Figure 1.
Reaction catalyzed by Undecaprenyl Pyrophosphate Synthase

Figure 2. Purification of UPPS. A) Ponceau staining and anti-T7 western blot of recombinant T7-UPPS-His6 purified from C41 expression cells. B) Gel filtration analysis of UPPS with overlaid standards β -amylase, carbonic anhydrase and cytochrome c.

a)

b)

Figure 3.

Recombinant UPPS is functional. TLC products from a reaction of [^3H]FPP with IPP in the presence of UPPS and control [^3H]-FPP alone. B) TLC plate illuminated from underneath of aqueous (1) and organic (2) extracts of NAGPP and IPP reactions catalyzed by UPPS. TLC plate is shown in black and white and is contrast enhanced.

Figure 4.
FPP analogue NAGPP

Figure 5.

UPPS catalyzes the formation of multiple *p*-nitro-aniline-linked products. A) Reactions were prepared containing NAGPP, IPP and UPPS and aliquots were monitored by RP-HPLC. Peak identities were confirmed by negative ion mode ESI-MS as described in the text. B) A standard curve was made with purified *cis*-7NA-B-PP for quantification of HPLC peak integrals. Integrals were used to plot the formation in pmols of the *cis*-7 and *cis*-6 products over time. The assay detection limit was 10 pmols of NABPP

Figure 6.

Conversion of bactoprenyl diphosphate to monophosphate. A) TLC plate scan of bactoprenyl diphosphate reaction relative to [^3H]-BPP above a TLC image of *p*-nitro-aniline-bactoprenyl diphosphate (1) and *p*-nitro-aniline-bactoprenyl monophosphate (2). B) RP-HPLC analysis of *cis*-7 NABPP treated with commercial alkaline phosphatase.

a)

b)

Figure 7.

Formation of *p*-nitro-aniline bactoprenyl mono- and di-saccharides A) TLC organic extract analysis of 1) NABP and PglC without UDP-Bac, 2) NABP/ UDP-Bac/PglC reaction 3) NABP/UDP-Bac/UDP-[³H]GalNAc/PglC/PglA reaction. B) TLC plate organic extract radiation analysis of reactions containing undecaprenol or NABP with UDP-Bac/UDP-[³H]GalNAc/PglC/and PglA. Undecaprenol reactions also contained ATP and the *S. mutans* kinase. The plate shown in A3 was the same plate scanned for radioactivity. Radioactive sample was spotted at the origin and solvent front to calibrate the position on the TLC plate where the origin was at 0 mm and the solvent front was at 50 mm. The marker data was overlaid with the Und-PP-Bac-GalNAc data. NA-B-P data is offset by 1000 tritium counts.

All TLC was performed in 73/53/11 CHCl₃/MeOH/0.1M sodium borate. TLC plates are in black and white and contrast enhanced.

a)

Figure 8.

RP-HPLC analysis of PglC and PglA reactions. A) A reactions was prepared containing NA-B-P, UDP-Bac and PglC and analyzed by RP-HPLC at 1 minute and 53 minutes. Integrals associated with NA-B-P and NA-B-PP-Bac are given in parenthesis and percent yield is provided on the right. B) Reactions from (a) were treated with UDP-GalNAc and PglA then monitored at the given time points. A new peak with an m/z ratio consistent with NA-B-PP-Bac-GalNAc was observed to increase over time in the reaction. C) A reaction was prepared with PglC, PglA, UDP-Bac, UDP-[^3H]GalNAc and NA-B-P. The reaction was monitored by RP-HPLC and product turnover calculated from percent turnover based solely on integrals in this reaction analysis and by comparison with a standard curve. The 395 nm

chromatogram trace is shown with the solid line. Fractions containing every 0.5 minutes of flow through from 3 minutes to 22 minutes were collected and counted on a scintillation counter. Background gray bar graph represents tritium label in each fraction. Radioactivity in the dead volume flow through is cutoff to enhance viewing of the radioactivity associated with the new peak.

a)

b)

Figure 9.

Chemical characterization of bactoprenyl analogues ^1H -NMR of olefin and aromatic regions of A) *cis*-6 NA-B-PP and NAGPP reference and B) *cis*-7 NA-B-PP. Expected integrals are given in parenthesis and actual normalized integral is given below. All spectra integration was normalized off of the 7.9 ppm aromatic protons C) ^{31}P -NMR of *cis*-7 NA-B-PP and D) *cis*-7 NA-B-P.

Figure 10.
Previously known UPPS active analogues of FPP

Scheme 1.

Campylobacter jejuni biosynthesis of a bactoprenyl diphosphate-linked disaccharide