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Fluorescent probes for investigation of isoprenoid configuration and size discrimination by bactoprenol-utilizing enzymes

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ABSTRACT

Undecaprenyl Pyrophosphate Synthase (UPPS) is an enzyme critical to the production of complex polysaccharides in bacteria, as it produces the crucial bactoprenol scaffold on which these materials are assembled. Methods to characterize the systems associated with polysaccharide production are non-trivial, in part due to the lack of chemical tools to investigate their assembly. In this report, we develop a new fluorescent tool using UPPS to incorporate a powerful fluorescent anthranilamide moiety into bactoprenol. The activity of this analogue in polysaccharide biosynthesis is then tested with the initiating hexose-1-phosphate transferases involved in Capsular Polysaccharide A biosynthesis in the symbiont *Bacteroides fragilis* and the asparagine-linked glycosylation system of the pathogenic *Campylobacter jejuni*. In addition, it is shown that the UPPS used to make this probe is not specific for *E*-configured isoprenoid substrates and that elongation by UPPS is required for activity with the downstream enzymes.

1. Introduction

Complex polysaccharides are critical to interactions between humans and bacterial pathogens as well as symbiotic microorganisms.[1–5] The wide array of roles that these molecules can play makes the development of new tools to understand their biosynthetic pathways crucial. Bactoprenol is an isoprenoid produced in bacterial systems for the assembly of complex carbohydrates, including teichoic acids, peptidoglycan, lipopolysaccharides and polysaccharide capsules.[6–12] The 55-carbon Bactoprenol (B-OH) is assembled as bactoprenyl diphosphate (BPP) from the 15-carbon precursor farnesyl diphosphate (FPP) and eight 5-carbon isopentenyl diphosphates (IPP), as shown in Scheme 1. This series of condensation reactions is catalyzed by Undecaprenyl Pyrophosphate Synthase (UPPS).[13]

Recently, a bactoprenyl probe was developed as a new tool to investigate complex bacterial oligosaccharide biosynthesis.[14] This probe was produced using UPPS from the symbiotic microbe *Bacteroides fragilis* as an enzymatic tool to incorporate a 4-nitroaniline moiety into BPP from the FPP analogue 4-nitroaniline geranyl diphosphate (4NA-G-PP, Fig. 1). However, the analogue was difficult to see in solution or on thin layer chromatography (TLC) plates. In addition, while the extinction coefficient of a 4-nitroaniline is high (9500 M^{-1} cm^{-1} at $\lambda_{395\ nm}$), other types of functionalities, such as fluorescent appendages, could allow for even more sensitive detection of products.

In order to design more powerful probes to study complex oligosaccharide biosynthetic pathways, it is imperative to know the limitations on what UPPS can and cannot accept as a substrate, and whether UPPS-catalyzed elongation of isoprenoid diphosphates is even necessary for downstream enzyme recognition. The X-ray crystal structure of UPPS from *Escherichia coli* has been solved as the apoenzyme and as the substrate or product analogue bound complex.[15–18] The FPP substrate is coordinated through its diphosphate moiety to essential Asn and Arg residues and is positioned in close proximity to IPP.[19] As the isoprenoid chain elongates, the farnesyl end is thought to travel through a hydrophobic tunnel until it reaches a length-determining helical floor. Once the product reaches this floor, it is released from the enzyme.

Synthetic analogues of FPP that are to be used as probes of downstream biosynthetic pathways must be able both to interact with UPPS and to travel through the hydrophobic tunnel below the enzyme active site. Other types of analogues have been used to study bacterial glycosylation with success.[12,20] However, the more these substrate alternatives resemble bactoprenol , the more likely they are to be accepted as a general probe for any bactoprenol-dependent bacterial glycosylation pathway. A clear distinction between analogue ability to interact with UPPS and ability to be elongated has been described with two analogues, MANT-O-GPP and Coumarin-GPP, shown in Figure 1.[21,22] The small appendage in MANT-O-GPP replacing the terminal (ω) isoprene of FPP was accepted as a substrate by UPPS, and elongated to a near full length

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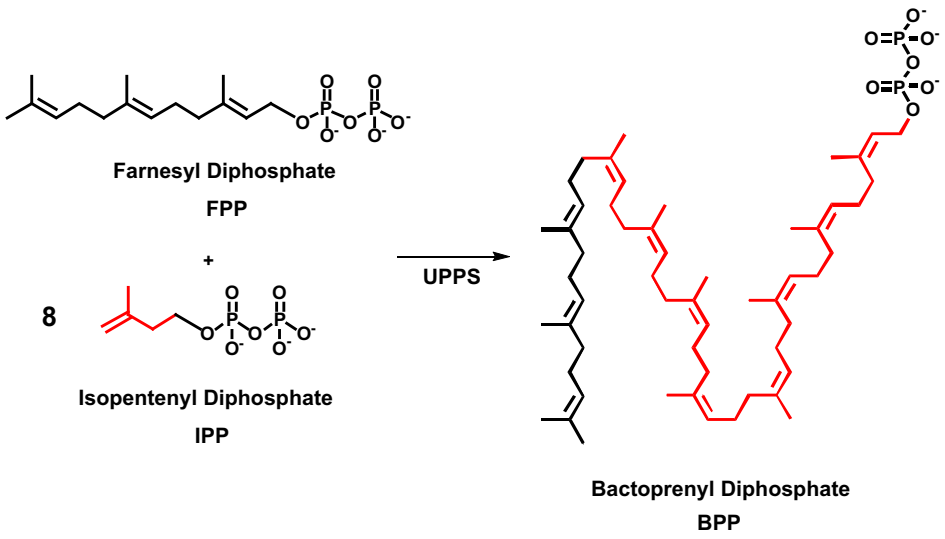


Scheme 1. Reaction catalyzed by UPPS probe.

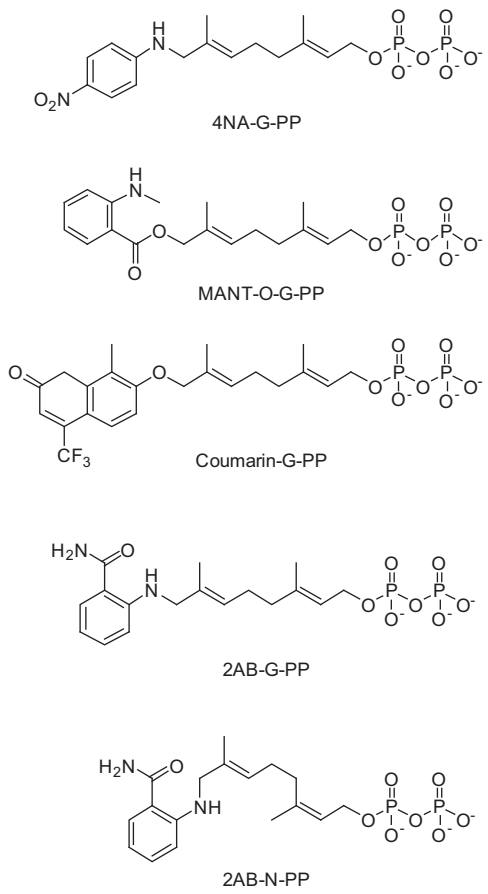


Figure 1. Analogues of FPP used to probe UPPS.

bactoprenol. However, the two-ring coumarin analogue was a poor substrate for UPPS and acted more like a potent, competitive inhibitor, possibly because its two-ring system was too bulky to fit through the hydrophobic tunnel. However, the structural limits of substrate activity for UPPS have not been systematically explored, so it is difficult to determine whether the difference in activity of these analogues was due to steric bulk or other properties of the substituents that interact unfavorably with the enzyme active site or tunnel.

A previously established method to analyze complex oligosaccharides linked to bactoprenyl diphosphates utilized a strongly fluorescent anthranilamide moiety that was incorporated into the free oligosaccharide following hydrolysis from the isoprenoid diphosphate.[8] After the oligosaccharide was cleaved and labeled, it was then purified by normal phase HPLC and identified by MALDI-MS.[8] We were interested in whether the anthranilamide could instead be linked directly to BPP to sensitively detect these linked sugars. Such a procedure would bypass the labeling and cleavage step and allow for further analysis of subsequent reactions through retention of the diphosphate linkage. This work describes a powerful new probe to study oligosaccharide assembly that incorporates this fluorescent tag into bactoprenol.

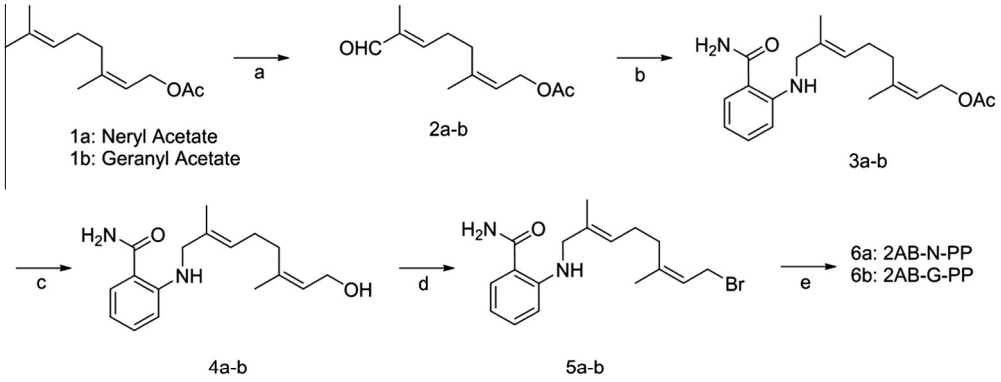
2. Results

2.1. Probe synthesis

Analogues of FPP can be used to incorporate new functionality into bactoprenol using UPPS. However, it is not clear how wide a range of functionality is acceptable to the enzyme. To probe the dependence of α-isoprene *E*/*Z*-configuration on substrate recognition, 2-aminobenzamide geranyl diphosphate (2AB-G-PP, Fig. 1) and 2-aminobenzamide neryl diphosphate (2AB-N-PP, Fig. 1) were designed to incorporate the easily detectable 2-aminobenzamide moiety replacing the terminal isoprene of FPP. The analogues were synthesized via a similar route (Scheme 2) from two allylic acetates, neryl acetate and geranyl acetate.[23–25] The allylic acetates were oxidized to aldehydes at the terminal methyl positions, then reductively aminated with 2-aminobenzamide. The resulting fluorescent acetate was then saponified, halogenated and coupled to the diphosphate, and purified by reverse phase (RP)-HPLC.

2.2. UPPS utilization of 2AB-G-PP

In order to test the activity of the 2-aminobenzamide analogues with UPPS, assays were initially performed with 2AB-G-PP, which was expected to be the preferred substrate due to its similarity to native FPP. Reaction mixtures were prepared containing UPPS, IPP and 2AB-G-PP, and long-chain isoprenoid products were phase separated from water-soluble 2AB-G-PP through extraction into



Scheme 2. Synthesis of 2AB-N-PP and 2AB-G-PP. Reagents and conditions: (a) *t*-BuOOH, salicylic acid, SeO_2, Et_2O; (b) 2-aminobenzamide, $NaBH(OAc)_3$, DCE; (c) K_2CO_3, MeOH, H_2O; (d) PBr_3, MeCN; (e) tris(tetra-*N*-butyl ammonium) diphosphate, MeCN.

chloroform. We found that an easily identifiable organic soluble fluorescent product was extracted into the organic layer when UPPS was present, and not when UPPS was left out of the reaction mixture (Fig. 2a). In addition, the aqueous layer was significantly more fluorescent when UPPS was left out of the reaction (Fig. 2b). TLC analysis of the reaction mixtures (Fig. 2c) indicated that a product was formed with increased hydrophobicity only when UPPS was present. Reactions could be easily monitored through this method by directly spotting the reaction mixture on TLC plates, drying the plates at 130 °C, then resolving the plate in a 73/53/11 chloroform/methanol/sodium borate buffer. Detection limits were on the order of 10 pmol through visualization over a 365 nm UV lamp.

2.3. α-Isoprene configuration is not critical for UPPS recognition

In order to determine the effect of the α-isoprene configuration on substrate recognition by UPPS, a RP-HPLC assay was utilized with 2AB-G-PP and 2AB-N-PP. Identical reaction mixtures were prepared containing each substrate and UPPS, which were quenched with *n*-propanol after 1 hour then separated by RP-HPLC (Fig. 3a). The starting material flowed rapidly through the column with very little interaction, while three hydrophobic products resulted in major peaks detected by fluorescence (ex. 350 nm em. 450 nm) at 5.6, 9.2 and 16.5 min. Interestingly, we observed identical activity with 2AB-N-PP, where levels of product were nearly the same as with the 2AB-G-PP (Fig. 3b). These results suggested that there was little effect on UPPS turnover with a change in the *E*/*Z* configuration of the analogues. In addition, since retention

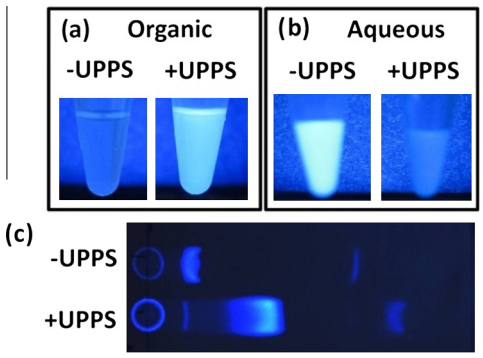


Figure 2. Reactions of 2AB analogues. (a) organic extracts of reactions without (−UPPS) or with (+UPPS), (b) aqueous extracts of reactions in a, (c) TLC analysis of 2AB-G-PP reaction with or without UPPS. 2AB-B-PP R_f = 0.5, 2AB-G-PP R_f = 0.13 in 73/53/11 $CHCl_3$/MeOH/NaBorate buffer.

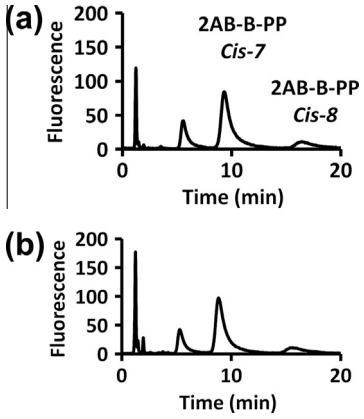


Figure 3. (a) HPLC analysis of 2AB-G-PP reactions; (b) HPLC analysis of 2AB-N-PP.

times of the products were identical, it is likely that the number of isoprene units incorporated into each were identical.

2.4. UPPS can incorporate eight isoprene units into 2AB-G-PP

In order to confirm the identity of the material formed in the 2AB-G-PP reactions, large scale (>1 μmol) reaction products were isolated by RP-HPLC (Supplementary Fig. 1) and characterized by ESI-MS. The longest HPLC retention time products were the 2-aminobenzamide bactoprenyl diphosphates (2AB-B-PP, Fig. 4a–c), which included seven (*cis-7*) or eight (*cis-8*) isoprene additions. These results were surprising, as previously, we had observed a maximum of seven isoprene additions with 4NA-G-PP.[14] Another peak was observed in the reaction at 5.6 min and is presumed to be 2AB-B-PP *cis-6* however enough material was not collected to confirm this.

2.5. ^{1}H NMR characterization of 2AB-B-PP *cis-7*

The 2AB-B-PP *cis-7* purified as described above was next characterized by ^{1}H NMR (Fig. 5). The chemical shifts of the 2AB-G-PP were identical to chemical shifts in the bactoprenyl diphosphate with additional protons associated with the newly incorporated isoprene units. Integration of the spectrum confirmed nine total olefins as would be expected for the *cis-7* isoprenoid. The integral for the methylene and methyl protons were expected to be 36 and 27, respectively. The observed number of protons associated with the methylene and methyl groups were 34 and 29, which were very close to the expected value. The slight difference in the number of protons is likely due to error propagated from integration

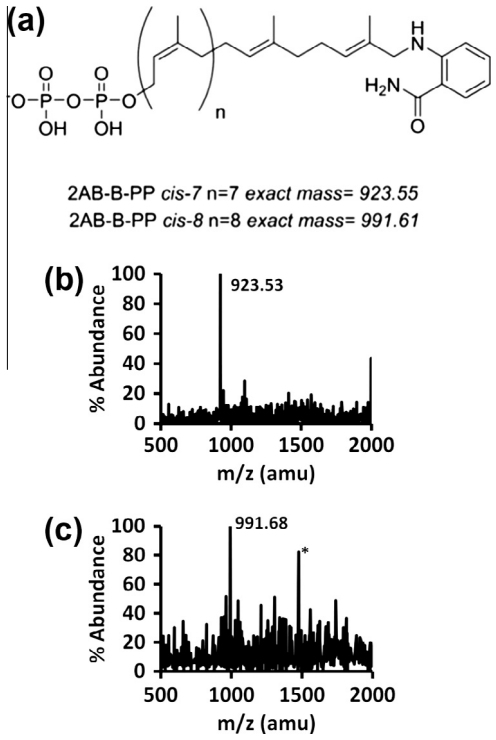


Figure 4. Characterization of 2AB reaction products. (a) Structure of primary 2AB-B-PP products; (b) MS analysis of *cis-7*; (c) MS analysis of *cis-8*. *unidentified contaminating peak at ~1477 amu.

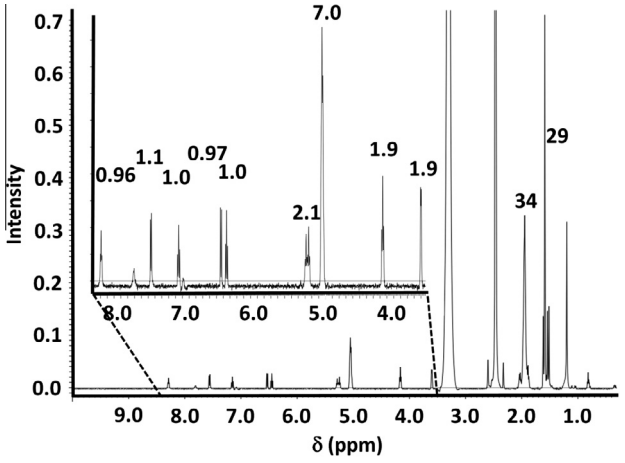


Figure 5. ^{1}H NMR of 2AB-B-PP *cis-7* on a 500 MHz spectrophotometer in d_6 DMSO. Integral values are given above each peak.

normalization. Coupled with the mass spectrometry data, these results confirmed the formation of the long chain isoprenoid with 2AB-G-PP as the UPPS substrate. Our previous analogue, 4NA-G-PP, had been elongated using a tetra-*N*-butylammonium-linked IPP, which resulted in a relatively complex ^{1}H NMR spectrum due to the counterion butyl group protons. Importantly, in this $^{+}NH_4$ form IPP was used which greatly reduced the complexity of the spectrum and allowed for facile integration and analysis.

2.6. Initiating hexose-1-phosphate transferase, WcfS

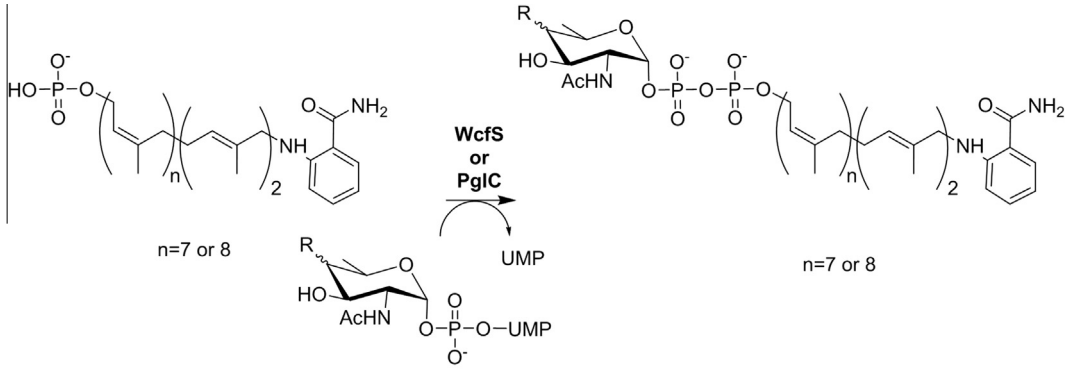
Recently, our group has isolated the key initiating hexose-1-phosphate transferase, WcfS, involved in capsule biosynthesis for the mammalian symbiont *B. fragilis*.[26] This enzyme catalyzes the transfer of *N*-acetyl-4-amino-galactosamine-1-phosphate (AADGal-1-P) from uridine diphosphate (UDP)-linked AADGal to bactoprenyl monophosphate (Scheme 3). HPLC analysis indicated that as low as 20 fmols of 2AB-B-PP *cis-8* could be detected, which was more than three orders of magnitude better than the nitroaniline analogue. This improved detection sensitivity prompted us to test the analogue with WcfS. The 2AB-B-PP *cis-7* was first converted to the monophosphate (2AB-B-P) with an alkaline phosphatase.[14] Reactions were then prepared with a WcfS containing *E. coli* membrane fraction and UDP-AADGal, biosynthesized as previously reported.[26] Products of these reactions were analyzed by RP-HPLC (Fig. 6a). We found that the retention was decreased with the incorporation of AADGal-phosphate. All of the limiting 2AB-B-P appeared to be consumed, in the reaction. Similar results were observed for the *cis-8* compound (data not shown). The isolated WcfS product had the expected molecular weight of 2AB-B-PP-AADGal based on ESI-MS (Fig. 6b). These results suggest that the new 2AB probe could be utilized to provide highly sensitive detection for this initiating hexose-1-phosphate transferase, and will provide a remarkable probe for studying downstream glycosylation reactions.

2.7. Short chain neryl analogue is not a substrate for WcfS

Our analysis of the UPPS reaction suggests that smaller isoprenoids than the *cis-8* and *cis-7* can be made, and we have previously shown that a *cis-6* 4-nitroaniline bactoprenyl phosphate is a substrate for WcfS.[26] We were interested in whether a short chain, two isoprene, chemically synthesized neryl phosphate could be utilized for these studies to allow us to bypass the use of UPPS in synthesizing our probes. A 2AB-neryl phosphate was synthesized through methods similar to the diphosphates. 2AB-N-P was mixed with WcfS and UDP-AADGal and analyzed by RP-HPLC. No product was observed using HPLC gradient conditions similar to those used to purify 2AB-N-P (data not shown). These results suggested that the longer chain isoprenoids were critical to recognition by the initiating hexose-1-phosphate transferase, and extension by UPPS is crucial to the development of these tools.

2.8. Assay development by thin layer chromatography

While HPLC is clearly the method of choice for detailed quantitative reaction analysis and isolation of products, TLC can often be a quicker method for screening reactions to identify the function of an unknown protein, or for screening reaction conditions for known proteins. Using the *Campylobacter jejuni* enzyme, PglC, which catalyzes transfer of 4-*N*-Acetyl Bacillosamine (AcBac)-phosphate to a bactoprenyl phosphate (Scheme 3), we tested the ability to screen assay conditions by TLC. Duplicate reactions were prepared with 96 μM 2AB-B-P *cis-8* and either 160 μM or 80 μM UDP-AcBac. The sub-stoichiometric UDP-linked sugar was expected to give less product than when the sugar was in excess. After a 1 h incubation period with PglC, 2 μL of the reaction (~200 pmols of anthranilamide) was removed and separated by TLC (Fig. 7). We found that we could easily distinguish between the two reactions and that the fluorescence was similar for the duplicate measurements. The size and intensity of each of the separated TLC spots was also quantified and subtracted from background to determine if this was a viable method for estimating reaction turnover. We observed 20 ± 2% turnover for the 160 μM reaction and 7.7 ± 0.5% turnover for the 80 μM reaction. These values were consistent with expected values and with the appearance of the spots on the plate. Identical reactions were prepared and analyzed by RP-HPLC to determine the accuracy of these measurements (Supplementary Fig. 2). We found that the 160 μM reaction turnover based on fluorescent peak integrals was 14%, while the



Scheme 3. Reactions catalyzed by WcfS and PglC.

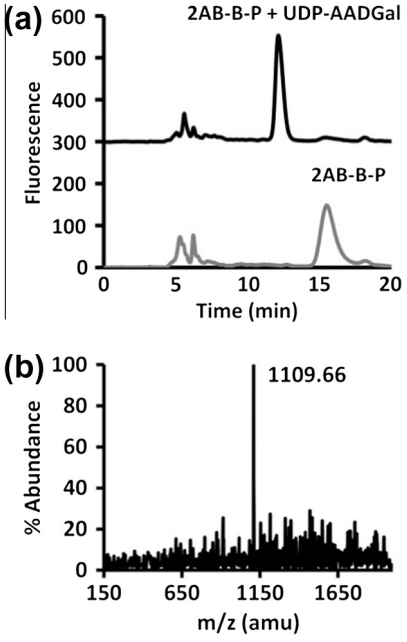


Figure 6. (a) HPLC analysis of 2AB-B-P *cis-7* reactions with UDP-AADGal catalyzed by WcfS; (b) ESI-MS analysis of WcfS reaction product. Expected molecular weight 1109.65 amu.

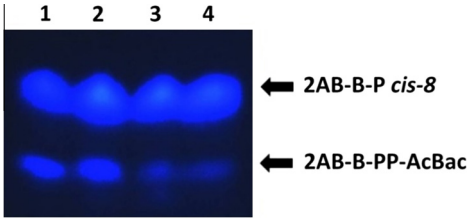


Figure 7. TLC analysis of PglC reactions. Reactions containing 96 μM HPLC purified 2AB-B-P *cis-8* were spotted on TLC plates and resolved in 73/53/11 $CHCl_3$/MeOH/sodium borate buffer. (1–2) duplicate spots of 160 μM UDP-AcBac reaction, (3–4) duplicate spots of 80 μM UDP-AcBac reactions. 2AB-B-P R_f = 0.58 2AB-B-PP-AcBac R_f = 0.51.

80 μM reaction turnover was 6%. These results suggest that the TLC assay results were useful for approximate turnover not perfect quantification. The TLC assays were very quick to perform and visualization of the 2AB substrate and product over a UV lamp was straightforward. Overall, these TLC based assays should be readily usable for the analysis of other complex oligosaccharide biosynthetic systems. Reactions with the analogue were also performed in which full turnover was observed with PglC (data not shown). Importantly, since both substrate and product can be observed on the TLC plates, each lane can be monitored for relative turnover, and does not require perfect spotting of the plates. However, it is clear that an HPLC-based analysis will be much more reliable with respect to quantitative accurate turnover measurements.

3. Discussion

3.1. Alternative substrates and UPPS

Numerous analogues of FPP have been designed to probe the substrate specificity of other prenyltransferases, particularly those involved in the farnesylation of critical mammalian signaling proteins.[23,24,27–31] These analogues included a diverse array of aromatic moieties replacing multiple isoprenes of FPP for the investigation of enzymes associated with those pathways. Application of such systematic studies to the structure function relationships between FPP and UPPS would greatly benefit our ability to both design new probes and gain better insight into the mechanism of substrate recognition by the protein. The relatively small sampling of analogues that have been tested with UPPS thus far makes it difficult to discern why the 2AB-G-PP analogue accepts eight isoprene additions while the 4NA-G-PP does not. However, it is clear that a fair degree of freedom is available for functionalization of an aromatic system replacing the terminal isoprene of FPP.

3.2. Conclusions

The fluorescent bactoprenyl diphosphate provides an intriguing new tool for the analysis of complex oligosaccharide biosynthetic systems. The enormous detection sensitivity with this molecule makes the analysis of these pathways much more tractable as very low turnover reactions can be observed readily even by TLC analysis. In addition, the ability to readily isolate products from these reactions for characterization by ESI-MS is also an important feature of these probes. Of critical interest to us is whether these types of molecules or precursors can be used for cellular analysis of oligosaccharide assembly systems in vivo. In addition, we are conducting ongoing studies to probe how well 2AB-G-PP acts as a substrate relative to FPP. Both of these areas have broad significance with respect to utilizing these probes to investigate uncharacterized polysaccharide biosynthesis pathways.

4. Methods

4.1. General

All general reagents and solvents were purchased from Sigma–Aldrich, VWR or Fisher unless stated otherwise. 2-Aminobenzamide-containing species were quantified at 350 nm using an extinction coefficient of 4500 M^{-1} cm^{-1}. All HPLC was performed on an Agilent 1100 system equipped with a diode array and fluorescence detector and Gilson fraction collector. TLC was performed on K6f non-fluorescent plates (Whatman). Synthetic protocol TLC was performed in 1:1 hexanes:ethyl acetate, while bactoprenol TLC was performed in 73/53/11 $CHCl_3$/MeOH/0.1 M sodium borate. NMR spectroscopy was performed on a 500 MHz or 300 MHz Jeol for ^1H NMR and ^{13}C NMR, and a 300 MHz Jeol spectrometer for ^{31}P NMR. ESI-MS analysis was performed in negative ion mode on a Thermo Electron Finnigan LTQ ESI-MS with injection volumes of 200 μL and concentrations ranging from 50 to 300 μM. UPPS, PglC, WcfS, and substrates were prepared as described previously.[14,26,32,33] Synthetic protocols generally followed those established by Spielmann and co-workers including **2b** previously reported.[24]

4.2. 2AB-G-PP and 2AB-N-PP analytical scale reactions

Reactions were prepared containing 6.8 μM 2AB-G-PP or 2AB-N-PP, 0.1% *n*-dodecyl-β-D-maltoside, 50 mM HEPES (pH 7.4), 5 mM KCl, 0.5 mM $MgCl_2$, 0.2 mM IPP, 344 nM UPPS in 250 μL total volume. After 1 h at 37 °C, reactions were quenched with the addition of 250 μL of *n*-propanol then analyzed by RP-HPLC. Isocratic analytical HPLC on bactoprenol-linked materials was performed on a C18 column (Agilent Eclipse XDB-C18 5 μm 4.6 × 150 mm) in 50% *n*-propanol/50% ammonium bicarbonate (25 mM), with fluorescence detection at ex. 350 nm, em. 450 nm.

4.3. 2AB-B-PP synthesis

2AB-B-PP was prepared in 50 mM HEPES (pH 7.4), 0.5 mM $MgCl_2$, 5 mM KCl, and 0.1% DDM, with 5.7 μM UPPS, 20 μmol of IPP and 3 μmol of 2AB-G-PP in a total volume of 1.5 mL. The reaction was incubated at 37 °C for 6 h. Products were purified using an isocratic method 75% *n*-propanol/25% ammonium bicarbonate (25 mM) on a C18 Varian 250 × 21.2 mm column (flow rate 4.0 mL/min).

4.4. 2-Amideaniline-bactoprenyl diphosphate *cis*-7 characterization

^1H NMR (500 MHz): δ 8.29 (t, 1H, J = 5.9 Hz), 7.56 (d, 1H, J = 8.2 Hz), 7.15 (t, 1H, J = 7.5 Hz), 6.53 (d, 1H, J = 8.5 Hz), 6.45 (t, 1H, J = 7.5 Hz), 5.29 (t, 1H, J = 6.5 Hz), 5.25 (t, 1H, J = 6.5 Hz), 5.05 (m, 7H), 4.16 (t, 2H, J = 6.5 Hz), 3.60 (d, 2H, J = 4.9 Hz), 2.04 (q, 2H, J = 7 Hz), 1.95 (am, 32H), 1.62 (s, 3H), 1.59 (as, 20H), 1.54 (s, 3H), 1.51 (s, 3H). Expected mass (923.55 amu) ESI-MS (M^{-1}): 923.53 amu.

2-Amideaniline-bactoprenyl diphosphate *cis*-8 Expected mass (991.61 amu) ESI-MS (M^{-1}) 991.68 amu.

4.5. 2AB-B-P synthesis

The 2AB bactoprenyl monophosphate was prepared by dissolving 2AB-B-PP in 20% *n*-propanol in 25 mM ammonium bicarbonate. Sample was then treated with 2 μL of goat anti-rabbit alkaline phosphate (Pierce) and incubated for 2 h. Product was purified by HPLC as described for 2AB-B-PP.

2-Amideaniline-bactoprenyl monophosphate *cis*-8 expected mass (911.64 amu) ESI-MS (M^{-1}) 911.73 amu.

4.6. TLC and extraction analysis of 2AB-G-PP reactions

Reactions were prepared with 367 μM 2AB-G-PP, 10 mM IPP, 4.3 μM UPPS, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.1% DDM 50 mM HEPES (pH 7.4). Reactions and control without UPPS were incubated for 2 h at 37 °C. A 2.5 μL aliquot of sample and control was then spotted onto a non-fluorescent TLC plate. The plate was dried for 3 min in a 130 °C oven, then product was separated in 73/53/11 $CHCl_3$/MeOH/0.1 M sodium borate over 50 mm. A 20 μL aliquot was extracted into 400 μL of 2:1 $CHCl_3$/methanol then washed with 3 × 100 μL Pure Solvent Upper Phase (PSUP, 4% KCl in 1:1 methanol/water). Tubes containing the organic or aqueous extracts and TLC plates were visualized over a UV transilluminator (365 nm).

4.7. WcfS reactions with 2AB-B-P and 2AB-N-P

The 2AB-B-PP-AADGal was prepared and 2AB-N-P was tested by adding 23 μg total protein in a membrane fraction of WcfS to a 200 μL solution containing 0.1 nmol 2AB-B-P *cis*-7 or short chain 2AB-N-P, 19.6 nmol UDP-AADGal, 1% Triton X-100 hydrogenated, 10 mM $MgCl_2$, 0.5% DMSO, and 50 mM Tris–HCl (pH 8.0). The 2AB-B-P reaction was incubated at room temperature for 30 min, after which it was analyzed using the RP-HPLC method described above. 2AB-N-P was analyzed by RP-HPLC using the same RP-C18 column, but loaded onto the column in 20% acetonitrile/80% 25 mM ammonium bicarbonate, then separated with a linear gradient over 30 min to 100% acetonitrile. Two negative controls were used, one in the absence of UDP-AADGal and the other without WcfS protein.

2-Amideaniline-bactoprenyl diphosphate AADGal *cis*-7 Expected mass (1109.65 amu) ESI-MS (M^{-1}) 1109.66 amu.

4.8. PglC TLC reactions

Reaction mixtures were prepared in duplicate containing 96 μM 2AB-B-P *cis*-8, 160 μM or 80 μM UDP-AcBac (synthesized as previously described[32]), 2% DMSO, 0.9 mg/mL PglC, 50 mM TrisOAc (pH 8.0), 101% Triton-X-100, 10 mM $MgCl_2$ in a total volume of 25 μL. Reactions were incubated at room temperature for 1 h then 2 μL was spotted on TLC plates and product was separated in 73/53/11 $CHCl_3$/MeOH/0.1 M sodium borate buffer. Plates were visualized over a transilluminator. Peak quantification was performed using Adobe Photoshop where plate images were converted to black and white then spot area and gray value were measured. Percent turnover was determined from the gray value and pixel area of the product minus background over the total for substrate and product. RP-HPLC was performed as described with the WcfS reactions except separations were performed in 60% *n*-propoanl/40% 25 mM ammonium bicarbonate.

4.9. Synthetic protocols

4.9.1. (6*Z*)-3,7-Dimethyl-8-acetoxyl-2,6-octadien-1-al (2a)

6.983 g (0.035 mol) of **1a** was added to a stirring solution of 0.491 g (3.5 mmol) of salicylic acid, 0.665 g (3.5 mmol) of SeO_2 and 14.82 g (0.164 mol) of *tert*-butylhydroperoxide (70%) in 35 mL of diethyl ether at 0 °C and left to stir for 48 h. The volume of the mixture was then reduced to one half and was washed with 15 mL of 5% $NaHCO_3$, saturated $CuSO_4$, repeatedly washed with saturated $Na_2S_2O_3$, water, brine and then dried over $MgSO_4$.

Solvent was removed by rotary evaporation and the crude oil was then purified by flash chromatography (5% EtOAc/hexanes) to afford 3.53 g (47%) of **2a**. TLC: (R_f 0.7). ^{1}H NMR (300 MHz): δ 9.28 (s, 1H), 6.37 (t, 1H), 5.32 (t, 1H), 4.49 (d, 2H), 2.39 (q, 2H), 2.25 (t, 2H), 1.91 (s, 3H) 1.69 (s, 3H), 1.58 (s, 3H). ^{13}C NMR (76 MHz): δ 195.2, 171.0, 153.2, 140.8, 139.7, 120.5, 60.7, 30.4, 27.3, 25.8 (contaminant), 23.2, 21.0, 9.2.

4.9.2. (6*Z*)-8-(2-Amideaniline)-3,7-dimethyl-1-acetoxyl-2,6-octadiene (3a)

3.53 g (0.0168 mol) of aldehyde **2a** was added to a stirring solution of 2.51 g (0.0184 mol) of anthranilamide and 1.41 g (0.0235 mol) of acetic acid in 75 mL of dichloroethane in a flame dried flask. After the solution became homogenous, 5.34 g (0.0250 mol) of $NaBH(OAc)_3$ was added and left to stir for 24 h at room temperature. The reaction mixture was then dried via rotary evaporation to one half the initial volume and quenched with 200 mL of 5% $NaHCO_3$. The mixture was then extracted with diethyl ether three times (100 mL), dried with $MgSO_4$, and concentrated via rotary evaporation. The crude oil was then purified by flash chromatography (15% EtOAc/hexanes) to yield 0.9086 g (22%) of **3a**. TLC: (R_f 0.32) ^{1}H NMR (300 MHz): δ 7.97 (t,1H), 7.38 (d, 1H), 7.26 (t, 1H), 6.62 (d, 1H), 6.54 (t, 1H), 5.78 (br s, 2H), 5.34 (m, 2H), 4.47 (d, 2H), 3.70 (d, 2H), 2.13 (m, 4H), 2.02 (s, 3H), 1.73 (s, 3H), 1.65 (s, 3H). ^{13}C NMR (76 MHz): δ 172.2, 171.2, 150.4, 142.6, 133.5, 132.2, 128.3, 124.6, 119.5, 114.3, 113.2, 112.3, 61.0, 50.5, 31.8, 26.4, 23.4, 21.3, 14.5. Expected mass (329.19 amu). Found: M^{-1}: 329.3 amu.

4.9.3. (6*E*)-8-(2-Amideaniline)-3,7-dimethyl-1-acetoxyl-2,6-octadiene (3b)

Yield (44%). TLC: (R_f 0.37) ^{1}H NMR (300 MHz): δ 8.00 (t, 1H), 7.37 (d, 1H), 7.28 (t, 1H), 6.62 (d, 1H), 6.56 (t, 1H), 5.84 (br s, 2H), 5.35 (t, 1H), 5.29 (t, 1H), 4.55 (d, 2H), 3.69 (d, 2H), 2.16 (m, 2H), 2.06 (m, 5H), 1.67 (s, 3H), 1.65 (s, 3H). ^{13}C NMR (76 MHz): δ 172.4, 164.8, 150.4, 137.9, 133.8, 131.6, 128.4, 124.5, 121.6, 114.8, 114.2, 112.9, 112.1, 59.3, 50.1, 38.9, 25.5, 16.4, 15.0. Expected mass (329.19 amu). Found: M^{-1} 329.2 amu.

4.9.4. (6*Z*)-8-(2-Amideaniline)-3,7-dimethyl -2,6-octadien-1-ol (4a)

0.500 g (2.016 mmol) of **3a** was dissolved in 21 mL of methanol and introduced into a flask containing 0.834 g (6.04 mmol) of K_2CO_3 dissolved in 1.5 mL of water. The reaction was refluxed at 70 °C for 24 h. The solution was then dissolved in water and extracted four times (50 mL) with chloroform, washed with brine and dried with $MgSO_4$. The crude solution was then concentrated via rotary evaporation and purified by flash chromatography (20% EtOAc/hexanes) to yield 0.200 g (40%) of **4a**. TLC: (R_f 0.1) ^{1}H NMR (300 MHz) δ 7.94 (t,1H), 7.39 (d,1H), 7.24 (t, 1H), 6.61 (d,1H), 6.51 (t,1H), 6.37 (br s, 2H), 5.35 (m,2H), 4.07 (d, 2H), 3.64 (d,2H), 2.96 (br s, 1H), 2.09 (m,4H), 1.68(s, 3H), 1.64 (s, 3H). ^{13}C NMR (76 MHz) δ 172.8, 150.6, 138.19, 133.3, 132.1, 128.7, 125.3, 124.3, 114.5, 113.3, 112.1, 58.9, 50.3, 31.6, 25.7, 23.3, 15.2 Expected mass (287.18 amu). Found: M^{-1} 287.3 amu.

4.9.5. (6*E*)-8-(2-Amideaniline)-3,7-dimethyl-2,6-octadien-1-ol (4b)

Yield (60%). TLC: (R_f 0.16) ^{1}H NMR (300 MHz): δ 7.86 (br t, 1H), 7.31(d, 1H), 7.22 (t, 1H), 6.58 (d, 1H), 6.48 (t, 1H), 5.77 (br s, 2H), 5.32 (m, 2H), 4.06 (d, 2H), 3.60 (d, 2H), 2.11 (q, 2H), 1.99(t, 2H), 1.60 (s, 3H), 1.57 (s, 3H). ^{13}C NMR (76 MHz): δ 172.2, 164.8, 150.4, 137.9, 133.8, 131.6, 128.4, 124.5, 121.3, 114.8, 112.9, 112.1, 59.2, 50.1, 38.9, 25.5, 16.4, 15.0. Expected mass (287.18 amu). Found: M^{-1} 287.3 amu.

4.9.6. (6*Z*)-8-(2-Amideaniline)-3,7-dimethyl-1-bromo-2,6-octadiene (5a)

0.100 g (0.4013 mmol) of **4a** in 5 mL of MeCN was added to 0.0436 g (0.161 mmol) of PBr_3 and allowed to stir for 15 min at 0 °C. The reaction mixture was then concentrated via rotary evaporation and sent through a short pad of silica with a 50% ethyl acetate/hexanes solution. Solvent was removed by rotary evaporation to yield a crude mixture of **5a**. TLC: (R_f 0.43).

4.9.7. (6*E*)-8-(2-Amideaniline)-3,7-dimethyl-1-bromo-2,6-octadiene (5b)

TLC: (R_f 0.43).

4.9.8. (6*Z*)-8-(2-Amideaniline)-3,7-dimethyl-2,6-octadiene diphosphate (6a)

The crude mixture of (**5a**) was immediately added to 1.10 g (1.20 mmol) of dry tris(tetra-*N*-butylammonium) hydrogen diphosphate and allowed to stir for 24 h at room temperature. The crude mixture was then dissolved in 50 mL of 25 mM ammonium bicarbonate buffer and passed through an ion exchange column (ammonium form). The column was eluted with 200 mL of buffer and that mixture was frozen and lyophilized overnight. The solid material was dissolved in minimal buffer and purified via reverse phase HPLC to yield 0.009 g (5%). ^{1}H NMR (300 MHz): δ 7.39 (d, 1H), 7.23 (m, 2H), 6.68 (d, 1H), 6.60 (t, 3H), 5.24 (m, 2H), 4.23 (t, 2H), 3.58 (s, 2H), 2.00 (m, 4H), 1.52 (s, 3H), 1.47 (s, 3H). ^{31}P NMR (122 MHz): δ −7.51 (d, 1P), −9.93 (d, 1P). Expected mass (447.11 amu). ESI-MS (M^{-1}) 447.25 amu.

4.9.9. (6*E*)-8-(2-Amideaniline)-3,7-dimethyl-2,6-octadiene diphosphate (6b)

Yield 14%. ^{1}H NMR (300 MHz) δ 7.41 (d, 1H, J = 7.5 Hz), 7.24 (t, 1H, J = 7.2 Hz), 6.68 (d, 1H, J = 8.7 Hz), 6.63 (t, 1H, J = 7.8 Hz), 5.18 (m, 2H, J = 7.5 Hz), 4.25 (t, 2H, J = 6.3 Hz), 3.58 (s, 2H), 2.00 (t, 2H, J = 7.2 Hz), 1.89 (q, 2H, J = 6.6 Hz) 1.49 (s, 3H), 1.46 (s, 3H). ^{13}C NMR (76 MHz): 183.1, 148.6, 142.6, 133.5, 132.0, 128.8, 125.8, 119.6, 116.8, 115.9, 113.8, 62.8, 50.0, 38.3, 25.1, 15.5, 13.5. ^{31}P NMR (122 MHz) δ −7.5 (d, 1P), −10.5 (d, 1P). Expected mass (447.11 amu). ESI-MS (M^{-1}) 447.22 amu.

4.9.10. (6*Z*)-8-(2-Amideaniline)-3,7-dimethyl-2,6-octadiene monophosphate

A crude mixture of **5a** was immediately added to 0.634 g (0.690 mmol) of dry di (tetra-*N*-butyl ammonium) hydrogen monophosphate and allowed to stir at room temperature for 24 h. The crude mixture was then dissolved in 50 mL of 25 mM ammonium bicarbonate buffer and passed through an ion exchange column (ammonium form). The column was eluted with 200 mL of buffer and that mixture was frozen and lyophilized overnight. The solid material was dissolved in minimal buffer and purified via reverse phase HPLC to afford 3.0 mg (2.3%) of monophosphate product. ^{1}H NMR (300 MHz): δ 7.41 (d, 1H), 7.27 (t, 1H), 6.70 (d, 1H), 6.63 (t, 1H), 5.24 (m, 2H), 4.13 (t, 2H), 3.61 (s, 2H), 2.01 (m, 4H), 1.55 (s, 3H), 1.50 (s, 3H). ^{31}P NMR (122 MHz): δ 2.17 (s). Expected mass (367.14 amu). ESI-MS (M^{-1}) 367.33 amu.

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Supplementary data

Supplementary data (RP-HPLC of large scale HPLC preparation of 2AB-B-PP, PglC reactions with 2AB-B-P *cis-8*, ESI-MS of 2AB-B-P *cis-8*, full chemical spectra of BPP precursors) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.06.007.

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