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## Undecaprenol kinase: function, mechanism and substrate specificity of a potential antibiotic target

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1 **Undecaprenol Kinase: Function, Mechanism and Substrate Specificity of a Potential Antibiotic**  
2 **Target**

3

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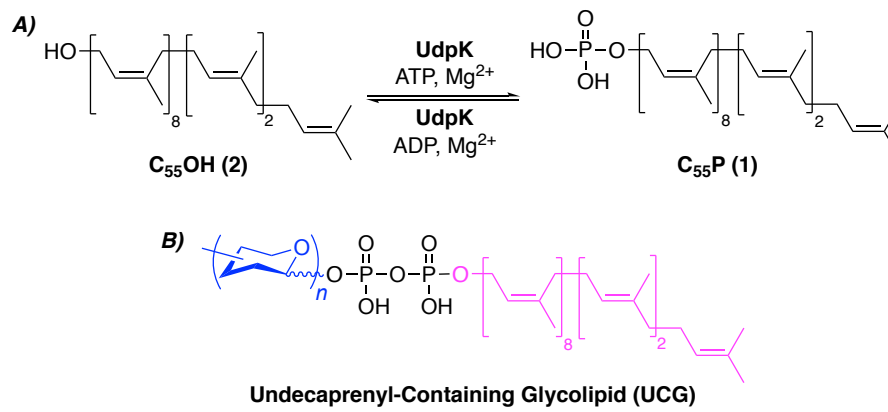
## 22 Abstract

23 The bifunctional undecaprenol kinase/phosphatase (UdpK) is a small, prokaryotic, integral  
24 membrane kinase, homologous with *Escherichia coli* diacylglycerol kinase and expressed by the  
25 *dgkA* gene. In Gram-positive bacteria, UdpK is involved in the homeostasis of the bacterial  
26 undecaprenoid pool, where it converts undecaprenol to undecaprenyl phosphate (C<sub>55</sub>P) and also  
27 catalyses the reverse process. C<sub>55</sub>P is the universal lipid carrier and critical to numerous glycopolymer  
28 and glycoprotein biosynthetic pathways in bacteria. *DgkA* gene expression has been linked to  
29 facilitating bacterial growth and survival in response to environmental stressors, as well being  
30 implicated as a resistance mechanism to the topical antibiotic bacitracin, by providing an additional  
31 route to C<sub>55</sub>P. Therefore, identification of UdpK inhibitors could lead to novel antibiotic treatments.  
32 A combination of homology modelling and mutagenesis experiments on UdpK have been used to  
33 identify residues that may be involved in kinase/phosphatase activity. In this review, we will  
34 summarise recent work on the mechanism and substrate specificity of UdpK.

35

## 36 1. Introduction

37 Undecaprenyl phosphate (C<sub>55</sub>P) (**1**) is a lipid phosphate composed of 11 isoprene units with  
38 the (*Z*<sub>8</sub>, *E*<sub>2</sub>, ω)-configuration (**Figure 1A**).[1–3] In Gram-positive bacteria, C<sub>55</sub>P can be synthesised  
39 from (*Z*<sub>8</sub>, *E*<sub>2</sub>, ω)-undecaprenol (C<sub>55</sub>OH) (**2**) through phosphorylation by undecaprenol kinase (UdpK),  
40 an enzyme that also catalyses the reverse process.[4] C<sub>55</sub>P is commonly referred to as the universal  
41 lipid carrier, as it is utilised in numerous essential bacterial biosynthetic pathways. However,  
42 exceptions are known, *Mycobacterium tuberculosis* for example uses a decaprenyl phosphate  
43 carrier[5–7]. Undecaprenol-containing glycolipids (UCGs), wherein a saccharide head group is  
44 linked to the C<sub>55</sub> chain through an anomeric pyrophosphate bond (**Figure 1B**), are key building blocks  
45 used in the biosynthesis of several important glycopolymers[8,9] and glycoproteins.[10]

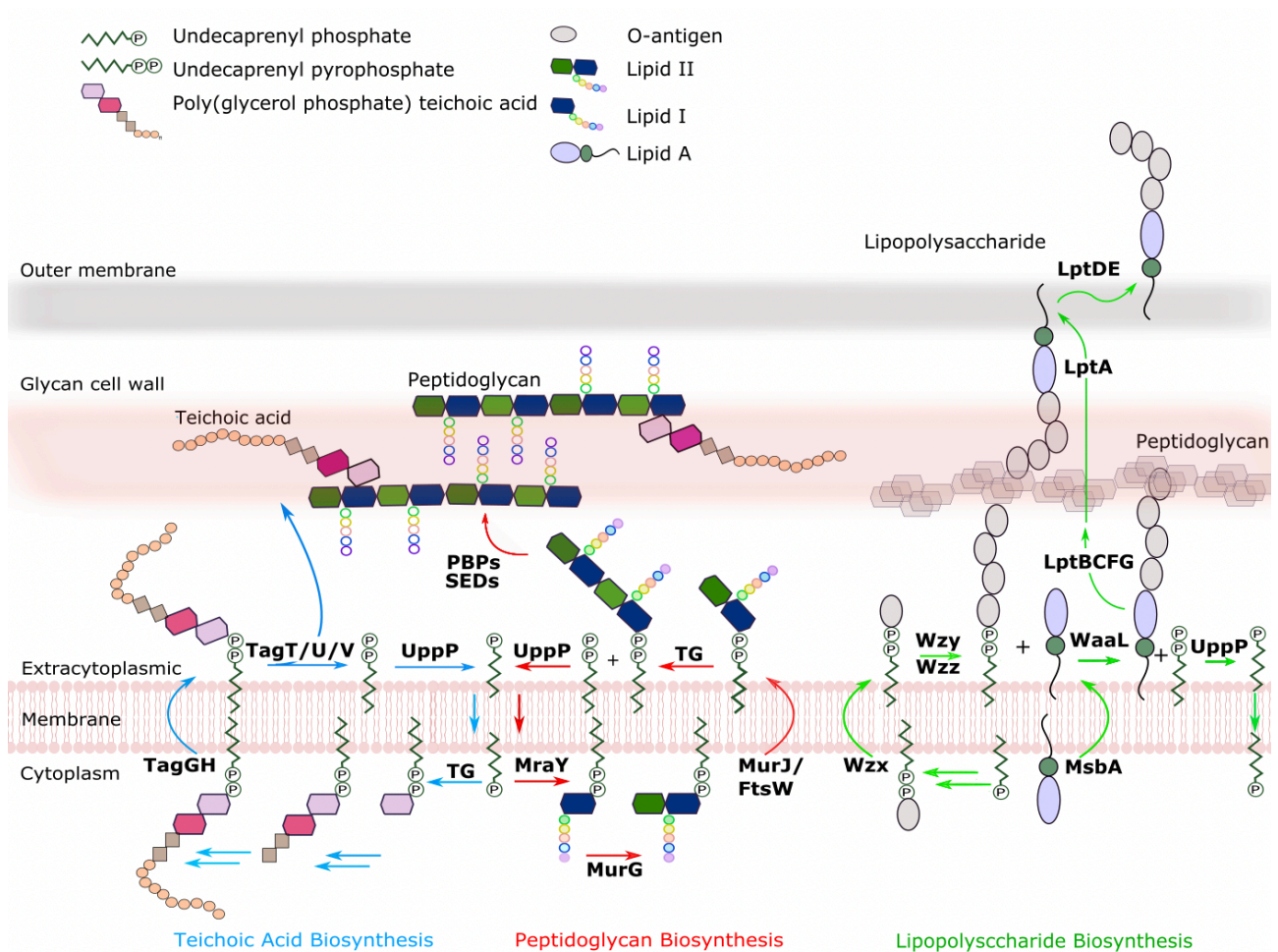


46

47 **Figure 1. A)** Phosphorylation of undecaprenol by UdpK. This enzyme can catalyse both phosphorylation and  
 48 dephosphorylation. **B)** Generic structure of an undecaprenol-containing glycolipid (UCG) with the saccharide  
 49 head group shown in blue and the undecaprenyl tail in purple. UCGs are used in bacterial glycopolymer and  
 50 glycoprotein synthesis. ATP = Adenosine triphosphate and ADP = adenosine diphosphate.

51

52  $\text{C}_{55}\text{P}$  effectively functions as a transmembranal carrier of hydrophilic saccharide subunits from the  
 53 location of their initial synthesis in the cytoplasm, across the cytoplasmic membrane and into the  
 54 extracytoplasmic space (**Figure 2**). [11,12] This is the site of further processing and polymerisation,  
 55 where complex glycopolymers and glycoproteins are formed. These form the scaffold of  
 56 peptidoglycan, a key component of the bacterial cell wall (**Figure 2**), and confers structural support  
 57 and environmental fortification to the cell. [8,13] Additionally, phylogenetic studies indicate that a  
 58 relationship exists between peptidoglycan biosynthetic genes present in plant chloroplasts and  
 59 bacteria. [14] Both Gram-positive and Gram-negative bacteria contain peptidoglycan in varied  
 60 quantities due to differing functional requirements. While Gram-negative bacteria have a  
 61 considerably thinner peptidoglycan layer than Gram-positive bacteria it performs essential structural,  
 62 chemical and biological roles in all bacterial species where it has been found. [15] Peptidoglycan is  
 63 the major constituent of the Gram-positive glycan cell wall, in which it forms a cross-linked  
 64 polymeric structure. Formation of this key component is reliant on  $\text{C}_{55}\text{P}$ , which is converted into lipid  
 65 I (by *MraY*) and then lipid II (by *MurG*), the final monomeric intermediate involved in peptidoglycan  
 66 biosynthesis. [16] Lipid II is transferred across the plasma membrane by a flippase (*MurJ*) [17] and/or



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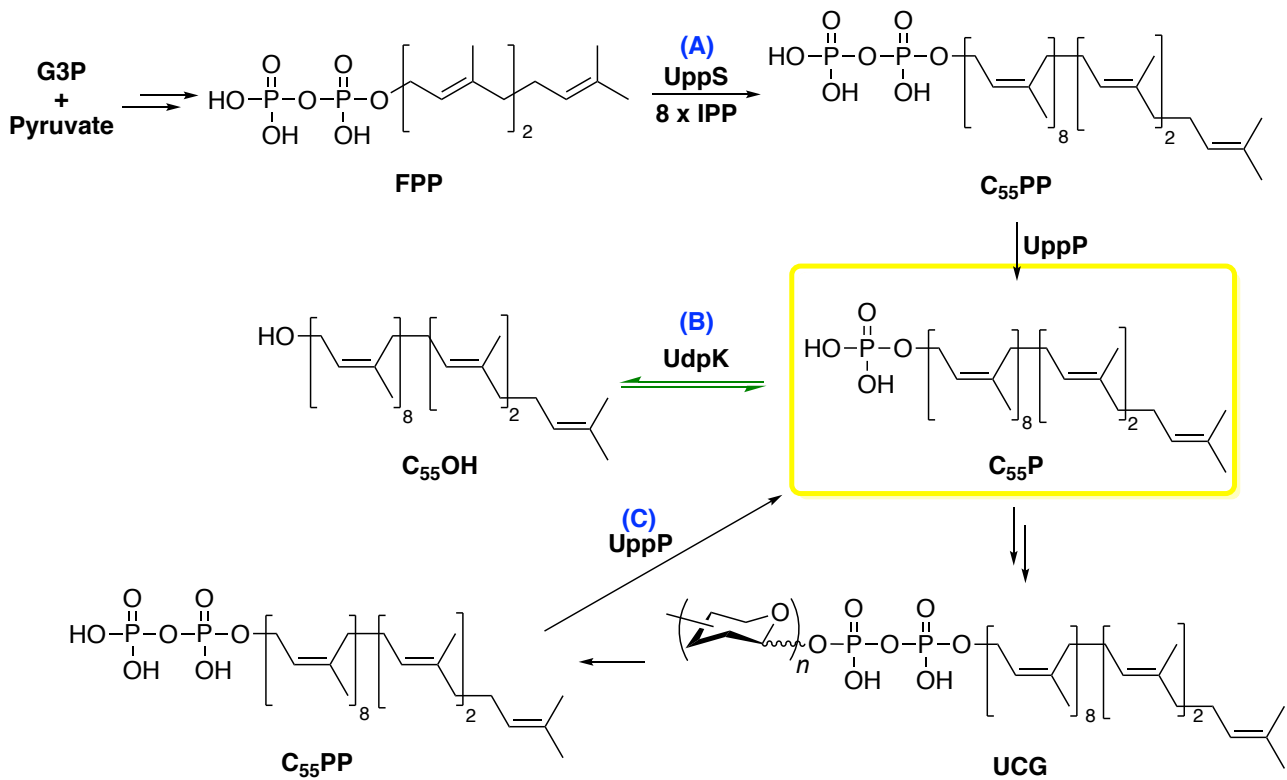
68 **Figure 2.** Schematic showing cellular processes in Gram-positive and/or Gram-negative bacteria that are  
69 reliant on C<sub>55</sub>P (Gram-positive bacteria do not have an outer membrane). Peptidoglycan (centre, red arrows)  
70 is an essential structural polymer in both Gram-positive and Gram-negative bacteria. C<sub>55</sub>P is sequentially  
71 converted to lipid I (by MraY) and then lipid II (by MurG), which is flipped across the membrane for  
72 polymerization.[12] Lipopolysaccharide (right, green arrows) is only found in Gram-negative bacteria and  
73 protects them from bile salts and lipophilic antibiotics. C<sub>55</sub>P is used in the synthesis of the O-antigen  
74 subunit.[20] This is then flipped across the membrane and polymerised by Wzz/Wzy, before the glycan head  
75 is transposed on to lipid A. Teichoic acid (left, blue arrows) is exclusive to Gram-positive bacteria and provides  
76 extra rigidity to the cell. After synthesis of the UCG and flipping across the membrane, the glycan is appended  
77 to the growing peptidoglycan chain. In all processes, undecaprenyl pyrophosphate (C<sub>55</sub>PP) is liberated into the  
78 outer leaflet of the cytoplasmic membrane. This is converted to C<sub>55</sub>P by undecaprenyl pyrophosphate  
79 phosphatase (UppP, BacA) before being recycled back to the inner leaflet of the membrane. PBP = penicillin-  
80 binding protein and SEDs = shape, elongation, division and sporulation. Polymerization is performed by class  
81 A PBPs and SEDs-class B PBP combinations. TG = transglycosylase. UdpK has been omitted for clarity.

82 FtsW[18][19]). Its polar head group extending into the extracytoplasmic space undergoes  
83 polymerisation and transfer reactions that result in its incorporation into the growing peptidoglycan  
84 layer. The peptidoglycan network of Gram-positive bacteria is further functionalised with teichoic  
85 acid and lipoteichoic acid, polymers which provide extra rigidity to the cell-wall by attracting divalent  
86 cations.[9] Teichoic acid biosynthesis also utilises C<sub>55</sub>P as a transmembrane shuttle to transport  
87 carbohydrate, carbohydrate-peptide and glycerol phosphate building blocks across the cytoplasmic  
88 membrane and is essential in both Gram-positive and Gram-negative bacteria.[21,22][23–25] As well  
89 as being involved in peptidoglycan biosynthesis, it is also required for the synthesis of  
90 lipopolysaccharide (LPS).[26,27] LPS are large molecular protrusions which are anchored in and  
91 extend from the outer leaflet of the outer membrane. They differ according to bacterial strain, relating  
92 differing bacterial toxicity and virulence.[28,29] These essential structures protect Gram-negative  
93 bacteria from bile salts and lipophilic antibiotics. Structurally, LPS is composed of lipid A  
94 (endotoxin), which anchors it to the outer membrane, a core oligosaccharide and the *O*-antigens. It is  
95 the synthesis of the *O*-antigen that is reliant on C<sub>55</sub>P, with *O*-antigen subunits synthesised as UCGs  
96 in the cytoplasm before being flipped across the membrane for further processing.[30–32] C<sub>55</sub>P also  
97 is employed in the synthesis of glycoproteins.[33] The appropriate UCGs are synthesised on the inner  
98 leaflet of the cytoplasmic membrane before being flipped to the outer leaflet for use in *O*- and *N*-  
99 glycosylation of proteins.[10,34–36] Peptidoglycan synthesis, teichoic acid synthesis and the *O*-  
100 linked mannose glycosylation of glycoproteins in Actinomycetes[37] are all essential processes in  
101 Gram-positive bacteria.

102

## 103 **2. Biosynthesis of Undecaprenyl Phosphate**

104 C<sub>55</sub>P can be biosynthesised via three different pathways (**Figure 3**).[4] In the *de novo*  
105 synthesis of C<sub>55</sub>P (**Figure 3A**), a multi-step cascade of reactions occurs in the cytoplasm that involves  
106 the initial generation of C<sub>55</sub>PP through a series of contiguous condensations between farnesyl



107

108 **Figure 3.** An overview of three different biosynthetic pathways that produce undecaprenyl phosphate (C<sub>55</sub>P).

109 **A)** *De novo* synthesis via the glyceraldehyde-3-phosphate (G3P) pathway; **B)** Phosphorylation of C<sub>55</sub>OH by

110 UdpK; and **C)** Recycling C<sub>55</sub>P from C<sub>55</sub>PP, a by-product of glycol-polymer/protein synthesis;.[4,39]

111

112 pyrophosphate and eight isopentenyl pyrophosphate units, catalysed by undecaprenyl pyrophosphate

113 synthase (UppS).[25,38] C<sub>55</sub>P is then generated through undecaprenyl pyrophosphate phosphatase

114 (UppP)-catalysed dephosphorylation of C<sub>55</sub>PP. Alternatively, in glyco-polymer/protein syntheses,

115 C<sub>55</sub>PP is a by-product left in the outer leaflet of the cytoplasmic membrane. UppP converts this C<sub>55</sub>PP

116 to C<sub>55</sub>P, before it is translocated back to the inner leaflet of the membrane for further use in UCG

117 synthesis (**Figure 3C**).[39] Analysis of X-ray crystal structures of *Escherichia coli* UppP lead both

118 the Caffrey and Strynadka groups to speculate that UppP may also function as a flippase for transport

119 of C<sub>55</sub>P back to the inner leaflet of the cytoplasmic membrane.[40,41] This is because its structure

120 resembles that of membrane channels (TagGH,[21] MsbA[42] and MurJ[43]), with the possibility of

121 alternating active sites on either side of the membrane. Additionally, C<sub>55</sub>P can be generated through

122 kinase mediated phosphorylation of C<sub>55</sub>OH (**Figure 3B**), a membrane bound lipid alcohol found

123 exclusively in Gram-positive bacteria. C<sub>55</sub>OH accounts for a high proportion of the total bacterial  
124 undecaprenoid pool found in Gram-positive bacteria but has not been found in Gram-negatives,  
125 suggesting this pathway is exclusive to Gram-positive organisms.[44] Knowledge of the active  
126 undecaprenol kinase and the gene(s) encoding it remained limited until a homologue of *E. coli*  
127 diacylglycerol kinase (DgK) was identified in *Streptococcus mutans* and the resulting *dgkA*-encoded  
128 protein was confirmed to be an active undecaprenol kinase.[45][46] More recently, Rock et al.  
129 identified the *dgkA* gene in *Bacillus subtilis* also operating as an undecaprenol kinase.[44] The *dgkA*  
130 gene codes for a membrane protein in both Gram-negative bacteria (DgkA) and in Gram-positive  
131 (UdpK).[46] Given the role that C<sub>55</sub>P plays in vital bacterial processes, the enzymes and numerous  
132 independent intermediates involved in its biosynthesis could make good antibiotic targets. Note,  
133 literature describing these enzymes can be confusing, as both the gene encoding undecaprenol kinase  
134 in Gram-positive bacteria and the gene encoding diacylglycerol kinase in Gram-negatives is called  
135 *dgkA*. For clarity, henceforth we will refer to the *udpk* gene when referencing the gene that encodes  
136 undecaprenol kinase and the *dgkA* gene when referring to the gene that encodes diacylglycerol kinase.

137

### 138 **3. Role of UdpK in Gram-Positive Bacteria**

139 In 2017, Wong and co-workers showed that UdpK has bifunctional enzymatic activities, in  
140 that it can catalyse both the phosphorylation of C<sub>55</sub>OH and the dephosphorylation of C<sub>55</sub>P.[4] The  
141 latter secondary function was certified by the recombinant expression of *S. mutans* UdpK in *E. coli*  
142 (which does not contain detectable levels of C<sub>55</sub>OH).[4] Upon expression of UdpK in *E. coli*, C<sub>55</sub>OH  
143 was detected, suggesting that UdpK converted native *E. coli* C<sub>55</sub>P to C<sub>55</sub>OH. UdpK offers an  
144 alternative pathway for bacterial cell wall homeostasis and function.

145 Under static conditions, biosynthesis of C<sub>55</sub>P by *de novo* synthesis and recycling pathways is  
146 favoured. However, the UdpK-mediated pathway is reserved for bacterial survival under an applied  
147 stressor, growth in inhospitable environments and/or to facilitate periods of rapid growth.[4,45]



148 Phosphorylation and dephosphorylation by UdpK are favoured at different points in cellular  
149 development. Under accelerated growth conditions, phosphorylation of C<sub>55</sub>OH is favoured as it  
150 generates larger quantities of C<sub>55</sub>P, facilitating rapid glycopolymer synthesis.[45] Phosphatase  
151 activity is favoured when growth is static to allow C<sub>55</sub>OH reserves to build up.[25] Additionally, *udpk*  
152 plays a role in phosphate homeostasis.[4] During periods of phosphate starvation, the gene is  
153 upregulated and promotes the generation of inorganic phosphate via the dephosphorylation of C<sub>55</sub>P  
154 to sustain cellular requirements.[4] UdpK is also instrumental to bacterial physiological regulation of  
155 bacterial biofilm formation and colony morphology.[47,48] Daugelat *et al.* described the influence  
156 UdpK has upon biofilm formation and bacterial infections.[47] *Mycobacterium smegmatis* UdpK  
157 deficient mutants form incomplete biofilms, characterised by isolated colonies of abnormal “caved-  
158 in” morphology.[47] Mice inoculated with *udpk*-deficient bacteria showed a reduction in penile  
159 smegma formation due to insufficient biofilm formation resulting from the lack of *udpk* gene  
160 expression.[47] This phenotypical influence was further noted in oral biofilm formation and the  
161 development of dental caries. Similar to *M. smegmatis* mutants, *S. mutans udpk*-knock-out strains  
162 exhibited reduced biofilm formation to the extent of approximately 40% when compared to wild-type  
163 bacteria. Additionally, UdpK was shown to be essential in maintaining bacterial function in  
164 cariogenic acidic environments[48] leading to the development of dental cavities and oral  
165 infections.[49,50] Yamashita *et al.* reported kinase activity to be critical for bacterial virulence at  
166 acidic pH.[49] Mutants of *S. mutans* grew at similar rates to wild-type bacteria at pH 7.4. The *udpk*  
167 gene reliance was seen when mediated at pH 5.5, as *S. mutans* mutants displayed restricted growth  
168 due to a heightened acid-sensitivity. Reintroduction of the *udpk* gene on a plasmid restored growth in  
169 acidic media by improving acid-tolerance.[45] A reduction in growth rate was further evident in  
170 systems doped with the eukaryotic DgkA inhibitor R59949 when incubated at pH 5.4 due to  
171 heightened acid-sensitivity imparted presumably by UdpK inhibition. This shows a correlation  
172 between *udpK* bacterial expression, bacterial acidic tolerance and virulence in acidic  
173 environments.[49]

174 Further studies of *B. subtilis* found that deletion of the *udpk* gene reduced their ability to  
175 correctly form an endospore.[51] The mutant endospores were shown to be unstable by observation  
176 with phase-contrast microscopy and to have a defective cortex structure as confirmed by electron  
177 microscopic examination. Additionally, the deletion was shown to have adverse effects upon bacterial  
178 adaption with a significant decrease in levels of dipicolinic acid being present in mutant spores, a  
179 compound involved in bacterial endospore heat resistance and dormancy.[52]

180 Due to its ability to offer an alternative mechanism for Gram-positive bacteria to access C<sub>55</sub>P,  
181 upregulation of UdpK-mediated C<sub>55</sub>P synthesis has also been implicated in antibiotic resistance.[53]  
182 Bacitracin is a topical antibiotic used in the treatment of minor injuries and skin infections that kills  
183 bacteria by sequestering C<sub>55</sub>PP, inhibiting dephosphorylation and recycling of C<sub>55</sub>P.[54] Colicin M,  
184 a polypeptide toxin produced by *E. coli*, also sequesters C<sub>55</sub>PP, disabling the regeneration of C<sub>55</sub>P  
185 and blocking C<sub>55</sub>P-reliant pathways.[55] In the presence of these antibiotics, upregulation of UdpK  
186 provides an alternative synthesis of the lipid carrier through phosphorylation of C<sub>55</sub>-OH. As the C<sub>55</sub>-  
187 OH pool is maintained on the inner-leaflet of the cell membrane, and neither bacitracin nor colicin M  
188 interact with C<sub>55</sub>OH, peptidoglycan synthesis can continue during antibiotic exposure. This resistance  
189 mechanism was observed by Kuramitsu and Lis, with *udpk*-deficient *S. mutans* strains displaying  
190 bacitracin hypersensitivity and abolished growth compared to wild type organisms.[45] Additionally,  
191 the *udpk* gene in Gram-positive organisms is part of a 3-gene operon. The operon consists of *Sgp*, a  
192 GTPase involved in ribosomal biogenesis and/or regulation and potential physiological membrane  
193 stress response pathways, and *ygfG*, a homologue of *E. coli* YbeY involved in ribosome regulation.  
194 The presence of the *udpk* gene in the operon suggests involvement in other stress-invoked response  
195 pathways potentially centred around ribosomal function.[56]

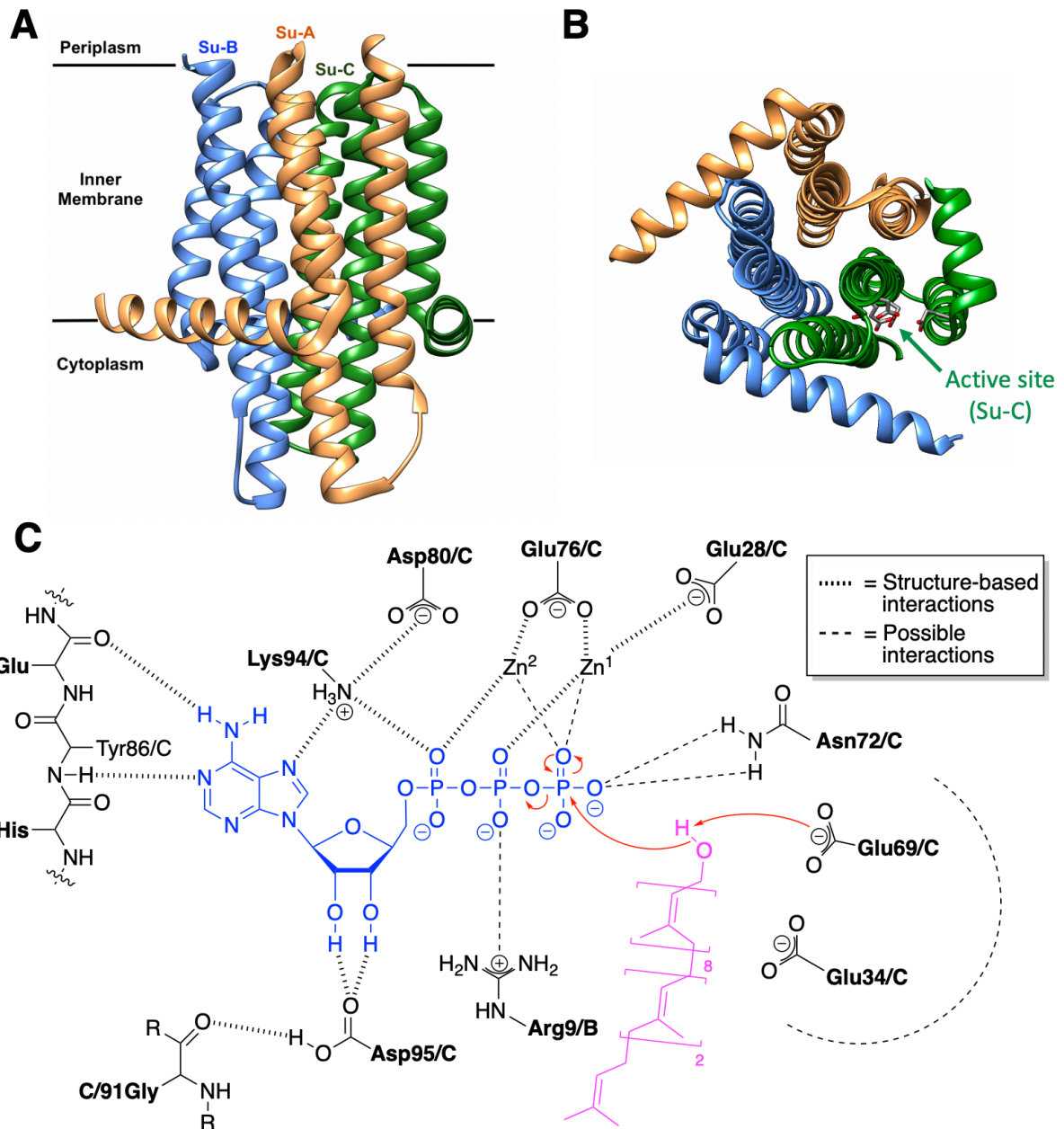
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199 **4. Structure and Mechanism of UdpK**

200 UdpK is an ATP-dependent enzyme that consists of 130 amino acids and is a homologue of  
201 DgkA from *E. coli*. [56] Both enzymes contain sequences with homologous regions and are distinct  
202 from other classes of kinase, which lead to unique biochemical functionality within this pair of  
203 enzymes. Due to there being no published structure of UdpK, DgkA has been used as a model for  
204 mechanistic studies on UdpK. [4] Caffrey and co-workers reported the crystal structure of DgkA up  
205 to 2.05 Å resolution. [57,58] The enzyme is a homotrimer, in which each monomer has three  
206 membrane-embedded helical domains and an *N*-terminal aliphatic  $\alpha$ -helix (**Figure 4A, B**). There are  
207 three active sites of the shared site type per kinase. The location of evolutionary conserved and  
208 essential residues when mapped to the crystal structure provided an explanation for the substrate  
209 docking mechanism which involves proximal alignment of the ATP  $\gamma$ -phosphate region and the  
210 primary hydroxyl group of the lipid (**Figure 4C**). Phosphorylation was deemed to proceed through  
211 direct phosphate transfer from nucleotide to lipid and not through an enzyme-phosphate intermediate.  
212 Glutamate and aspartate residues in the active site position the lipid substrate deprotonate the terminal  
213 hydroxy group to form an alkoxide. The alkoxide ion reacts with the adjacent  $\gamma$ -phosphate of the  
214 divalent metal-nucleotide complex, generating a pentavalent phosphate transition state, which  
215 subsequently collapses, releasing the phosphorylated lipid and ADP.



216

217 **Figure 4.** **A)** View from the membrane and **B)** from the cytoplasm of the homotrimeric membrane embedded  
 218 DgkA from *E. coli* (PDB 4UXX). Subunit A (Su-A) is orange, subunit B (Su-B) is blue, and subunit C (Su-C)  
 219 is green. Enzyme active sites are found the cytoplasmic region of the protein. **C)** Proposed mechanism  
 220 for phosphorylation of undecaprenol by DgkA.

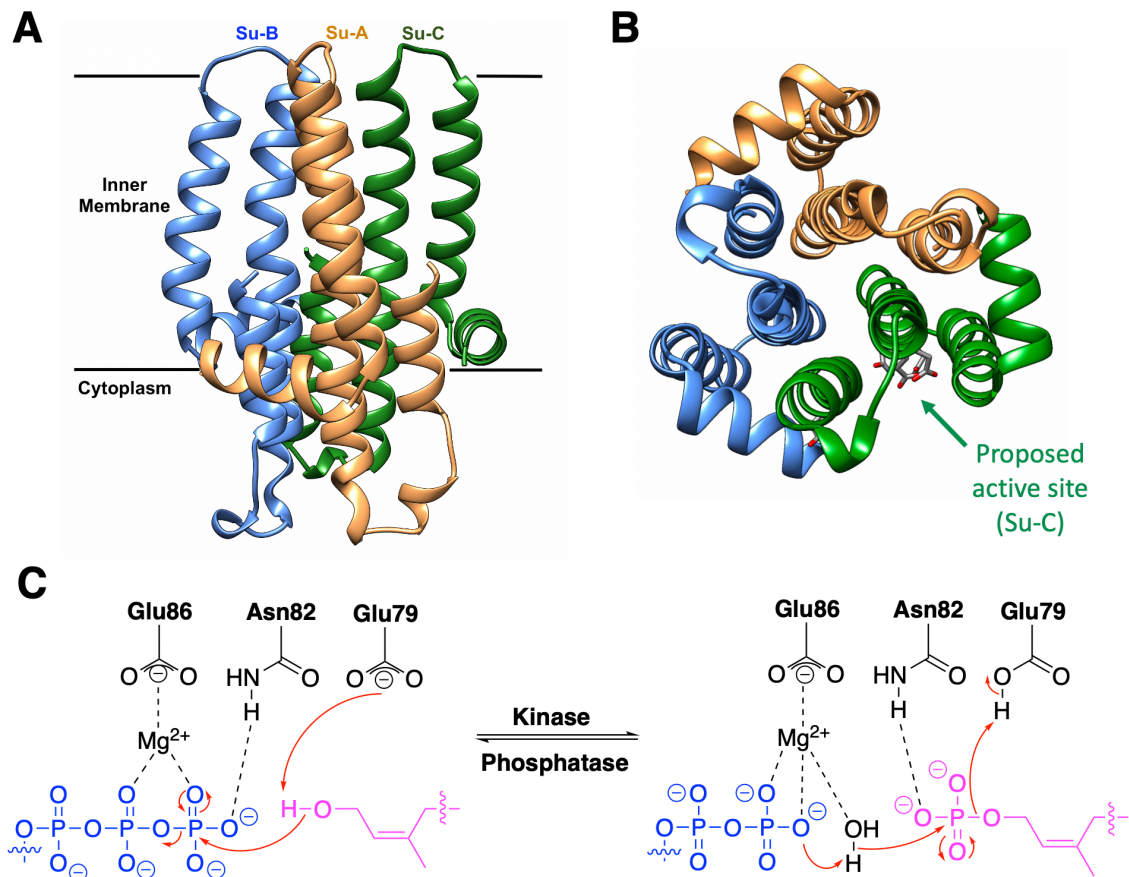
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223

224 In the absence of a crystal structure of UdpK, a homology model of the enzyme has been  
225 created using DgkA as a template (**Figure 5A, B**).[4] UdpK from *S. mutans* shares ~37% sequence  
226 identity with DgkA and predicted key amino acid residues are conserved between both enzymes. *S.*  
227 *mutans* UdpK was shown to require a number of amino acid residues for enzymatic function (**Figure**  
228 **5C**). These include Glu79, Asn82, Glu86, Asp90 and Asp106 in *S. mutans* UdpK.[4] Similar residues  
229 (e.g. Glu69, Asn72, Glu28, Asp76 and Asp80) are conserved in DgkA.[58] Phosphatase activity was  
230 also compromised in mutant strains, displaying a bi-functioning enzyme dependence on identical key  
231 residues.[4] No mutant strain was identified that could solely catalyse phosphorylation over  
232 dephosphorylation (or *vice versa*), lending credence to the conclusion that the two enzyme functions  
233 operate co-dependently and indispensably of each other, within a shared active site. Due to the  
234 structural and biochemical similarities between DgkA and UdpK, it is assumed that the  
235 phosphorylation mechanism is similar. In this proposed mechanism, Glu86 and Asn82 stabilise and  
236 position the nucleotide terminal phosphate (**Figure 5C**). Concurrently, a basic Glu79 initiates kinase  
237 activity through deprotonation of the alcohol to give an alkoxide, which then reacts with ATP to yield  
238 C<sub>55</sub>P and ADP. In the proposed phosphatase mechanism, Glu86 coordinates with the ADP β-  
239 phosphate, initiating deprotonation of water and subsequent attack of C<sub>55</sub>P by the resulting hydroxide  
240 ion. This releases the C<sub>55</sub>OH product, as well as ADP and inorganic phosphate. It is interesting to  
241 note that the proposed role of ADP is that of a nucleophilic catalyst.

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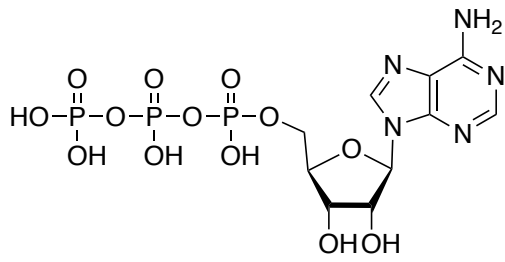


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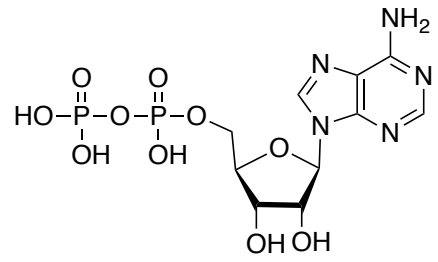
244 **Figure 5.** **A)** View from the membrane plane and **B)** from the cytoplasm of homology model of UdpK from  
 245 *S. mutans*. Model generated using SWISS-MODEL with *E. coli* DgKA-ACP complex (PDB 4UXX) as a  
 246 template. Subunit A (Su-A) is orange, subunit B (Su-B) is blue, and subunit C (Su-C) is green. Enzyme active  
 247 sites are found in the cytoplasmic region of the protein. **C)** Proposed active-site residues and mechanism of  
 248 UdpK for both kinase and phosphatase reactions.

## 249 5. UdpK Nucleotide Specificity

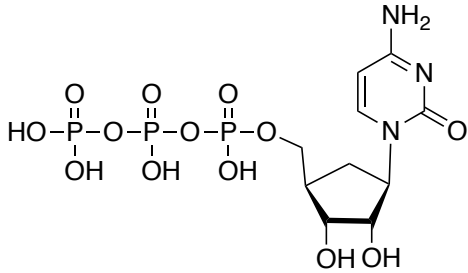
250 The kinase and phosphatase reactions catalysed by UdpK use different nucleotides (ATP (**3**)  
 251 for kinase, ADP (**4**) for phosphatase). Whether the kinase or phosphatase reaction dominates partly  
 252 depends on the ratio of ATP and ADP.[4] For the kinase reaction, only ATP analogues with the  
 253 adenine base are tolerated (**Figure 6**).[59] This selectivity can be rationalised through analysis of the  
 254 DgkA structure reported by Caffrey and co-workers, which can operate as a putative model for UdpK.  
 255 The purine base of adenine nucleotides are optimally aligned within the active site via hydrogen  
 256 bonds



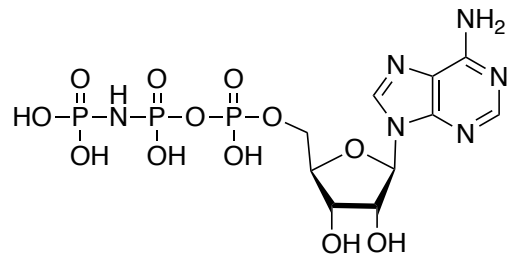
ATP (3)  
Active



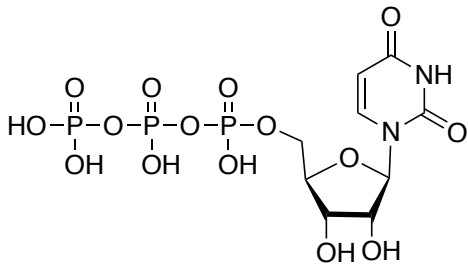
ADP (4)  
Active



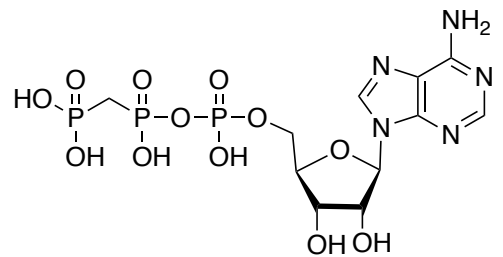
CTP (5)  
Not active



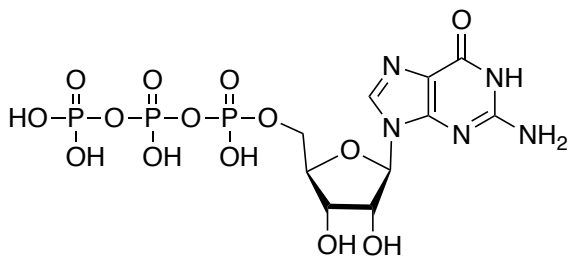
AMPPNP (6)  
Not active



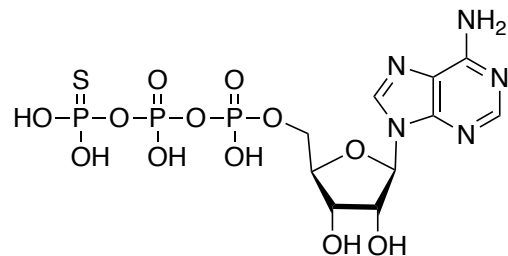
UTP (7)  
Not active



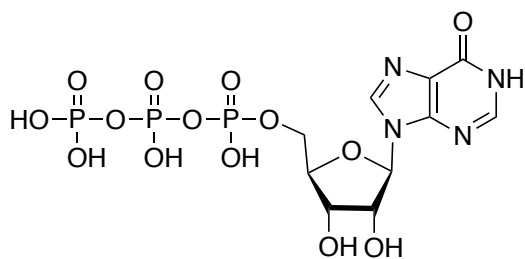
AMPPCP (8)  
Not active



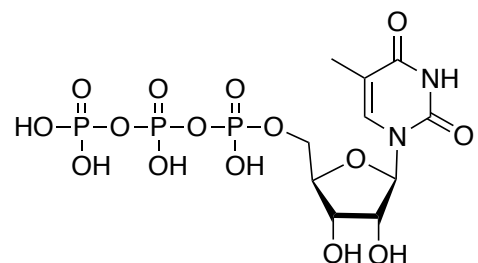
GTP (9)  
Not active



Adenosine 5'-(γ-Thio)triphosphate (10)  
Active



ITP (11)  
Active (DgK)



TTP (12)  
Not active (DgK)

257

258 **Figure 6.** Nucleotides tolerated by UdpK.

259

260 with backbones of amino acid residues His87C and Glu85C (**Figure 4**). Additionally, Tyr86C located  
261 between the binding residues further embeds the nucleotide via a  $\pi$ -stacking interaction between the  
262 adenylyl core and the tyrosyl ring. Changing adenine to guanine in the nucleotide triphosphate results  
263 in replacement of the amino group at position 6 with a carbonyl. Given that the adenine amino group  
264 is involved in a hydrogen bonding interaction with the Glu85 backbone carbonyl in DgkA, this  
265 substitution with oxygen likely leads to a repulsive interaction between the guanine ring and protein,  
266 explaining the decreased binding affinity.[4] Under similar conditions, crystal soaking with non-  
267 adenine containing nucleotides (GTP, thymidine triphosphate (TTP), CTP or UTP) had no effect on  
268 the DgkA crystal.[57] In another study with DgkA, a reduction in  $k_{cat}$  was observed when GTP (**9**) or  
269 inosine triphosphate (ITP) (**11**) were used, compared to the optimal ATP system[58]. Screening ATP  
270 analogues has provided insight into the phosphoryl transfer mechanism and more information on  
271 substrate selectivity. Adenosine-5'-( $\gamma$ -thio)triphosphate (**10**) was shown to be an active substrate of  
272 UdpK but is turned over at a slower rate than ATP,[4] presumably due to the reduced electrophilicity  
273 and sterics of the thiophosphate.[60] The activity of this substrate reinforces the proposal that  
274 phosphate transfer occurs at the  $\gamma$ -phosphate position of ATP.[4,57] Analogues containing a different  
275 functional linkage between the  $\beta$ - and  $\gamma$ -phosphorus atoms, such as adenosine-5'-( $\beta,\gamma$ -  
276 imido)triphosphate (AMPPNP) (**6**) and adenosine- $\beta,\gamma$ -methyleneadenosine-5'-triphosphate  
277 (AMPPCP) (**8**), are not substrates as the terminal phosphate cannot be cleaved.[4] Both UdpK and  
278 DgkA are ATP dependent, with the most significant sequence conservation found in the ATP binding  
279 regions. The phosphatase activity of UdpK was shown to be ADP (**4**) dependent.

280

## 281 **6. UdpK Lipid Substrate Specificity**

282 UdpK has been shown to phosphorylate a broad range of lipid alcohol substrates (**Table 1**).  
283 UdpK and DgkA, while both structurally similar, display different substrate selectivity, with *B.*  
284 *subtilis* UdpK catalysing the phosphorylation of undecaprenol but not diacylglycerol.[44] In DgkA



285 and UdpK, the largest proportion of amino acids are conserved in the active site between the  
286 transmembrane domains 2 and 3, which is associated with nucleotide selectivity[4]. However, in the  
287 N-terminal helix and the C-terminal transmembrane domains 1 and 3, which are associated with lipid  
288 binding, there is less conservation, which is likely responsible for the differing lipid selectivity.[4]

289 The first significant analysis of UdpK's lipid substrate-specificity was performed by Wong  
290 and coworkers.<sup>39</sup> UdpK processes polyprenols of varied lengths, with shorter isoprenols such as  
291 nerols (**14**, **15**), farnesols (**16** – **18**), geranylgeraniol (**19**) and heptaprenol (**20**) performing better than  
292 the larger polyprenols.[59] (*E*<sub>8</sub>, *ω*)-solanesol (**22**) is two isoprene units shorter than bacterial (*Z*<sub>7</sub>, *E*<sub>3</sub>,  
293 *ω*) undecaprenol and all of its alkenes are in the *trans*-configuration. It is processed by UdpK at a  
294 similar rate to (*Z*<sub>7</sub>, *E*<sub>3</sub>, *ω*)-undecaprenol, suggesting that the double bond stereochemistry does not  
295 significantly impact enzyme activity. Polyprenols containing a saturated  $\alpha$ -isoprene (dolichol, **25**) or  
296 all saturated isoprene units (phytol, **24**) are processed at similar rates to undecaprenol but aliphatic  
297 alcohols (**26** – **28**) are poorer substrates, suggesting that hydrocarbon branching is important.  
298 Synthetic pentaprenols containing a dabsyl group at the *ω*-terminus (**29** – **32**) are also readily  
299 processed by UdpK. Wong and co-workers also found that common hydroxy-containing surfactants  
300 such as Tween 20 (**33**), Triton X-100 (**34**) and Tergitol NP-40 (**35**) were phosphorylated by UdpK.  
301 This was qualitatively determined by checking for the presence of phosphorylated surfactants by TLC  
302 and LCMS. In another study, Rock and co-workers found that UdpK was incapable of  
303 phosphorylating ceramide (**36**), D-erythro-sphingosine (**37**), 1-oleoyl-*rac*-glycerol (**38**), 1,2-dioleoyl-  
304 *sn*-glycerol (**39**) and phosphatidylinositol (**40**). These respective studies by Wong and Rock suggest  
305 that UdpK preferentially processes linear primary alcohols. It appears that additional hydrophilic  
306 moieties close to the alcohol group and/or increased steric hinderance, i.e., secondary alcohols, are  
307 poorer substrates.

308 The Cochrane lab recently reported the semi-synthesis of a library of novel labelled  
309 undecaprenol analogues.[61] *d*<sub>1</sub>-(*Z*<sub>7</sub>, *E*<sub>3</sub>, *ω*)-Undecaprenol (**41**) was synthesised from (*Z*<sub>7</sub>, *E*<sub>3</sub>, *ω*)-  
310 undecaprenol (which is extracted from bay leaves) and found to be readily processed at similar rates

311 to (*Z*<sub>7</sub>, *E*<sub>3</sub>,  $\omega$ )-undecaprenol. Additionally, the terminal  $\omega$ -isoprene unit was modified with a variety  
312 of different chemical labels or functional groups, including azides (**42**, **48**), thioesters (**43**), alcohols  
313 (**44**), epoxides (**45**), alkynes (**46**, **47**), spin-labels (**49**), fluorophores (**50** – **52**) and photoaffinity labels  
314 (**53**). Rates were broadly comparable for all of these  $\omega$ -modified substrates, showing that this position  
315 is an excellent site for chemical labelling on undecaprenol.

316

## 317 **7. Conclusions and Future Outlook**

318 Due to the bacterial adaptations imparted by UdpK expression and the number of unique  
319 biosynthetic pathways reliant on undecaprenyl phosphate, this enzyme could make a good antibiotic  
320 target in Gram-positive bacteria. Inhibition of this kinase could impede bacterial survival by  
321 inhibiting the synthesis of essential glycopolymers and glycoproteins in already stressed bacteria (for  
322 example, by another antibiotic), as well as restore sensitivity to antibiotics such as bacitracin, whose  
323 efficacy is reduced by UdpK-mediated C<sub>55</sub>P synthesis. At present the only structure of UdpK  
324 available is a homology model and although mechanistic hypotheses have been derived from this,  
325 rational drug-design would be better aided by a high-resolution X-ray crystal structure. Given the  
326 promiscuity of UdpK towards lipid alcohols, substrate-mimics or novel scaffolds that bind to the  
327 kinase active site may offer new antibiotic candidates and in turn may aid in the generation of a crystal  
328 structure of undecaprenol kinase via complexation with these novel inhibitors. To date all UdpK  
329 assays have relied on either a coupled pyruvate kinase/lactate dehydrogenase coupled enzyme assay  
330 and/or thin-layer chromatography (TLC). High-throughput screening of large compound libraries  
331 using the coupled enzyme assay system is problematic as there are three enzymes that could be  
332 inhibited. TLC provides a clear result but is not high throughput. Chelation-enhanced fluorescence  
333 (CHEF) screening of kinases, which was first reported by the Imperiali group,[62,63] allows the high-  
334 throughput screening of protein kinases.[64,65] This method was adapted by the Anslyn group for  
335 the differential sensing of MAP Kinases using chemically labelled peptides.[66] If analogous lipid

336 substrates could be prepared that allow CHEF-monitoring of UdpK, such a system could enable high-  
337 throughput screening of large compound libraries for identification of new antibiotic candidates. The  
338 studies reported herein highlight UdpK as a vital enzyme in Gram-positive bacteria under  
339 environmental or antibiotic stressors, and emphasise its potential as an antibiotic target.

340

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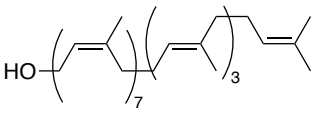
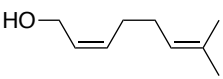
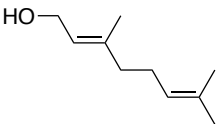
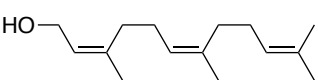
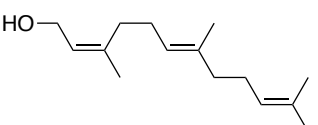
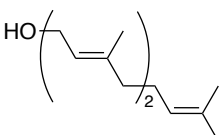
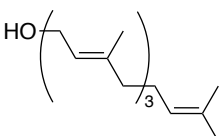
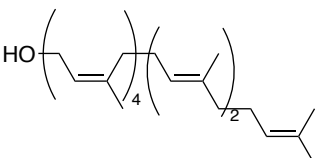
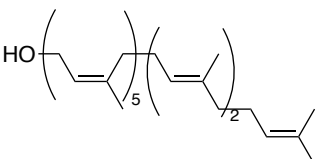
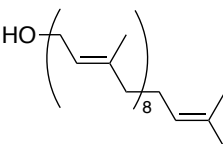
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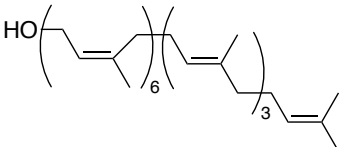
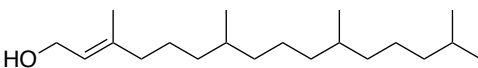
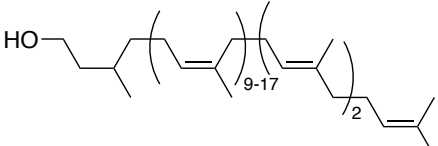
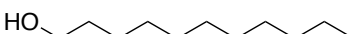
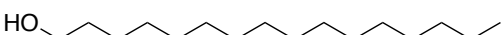
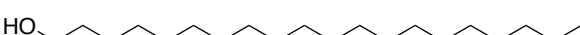
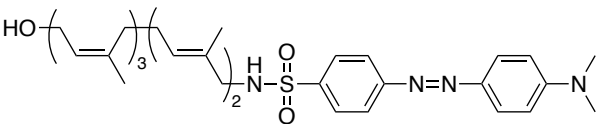
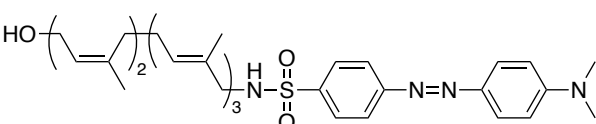
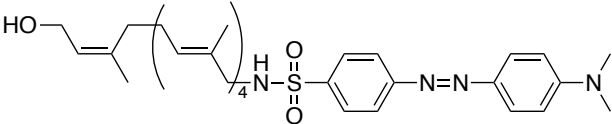
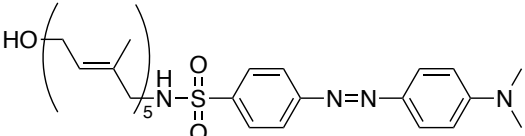
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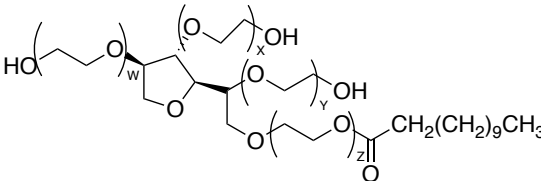
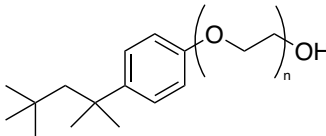
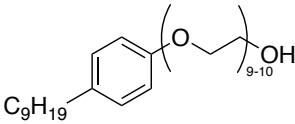
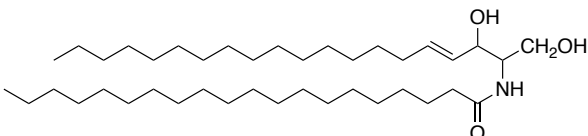
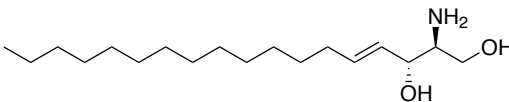
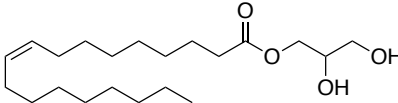
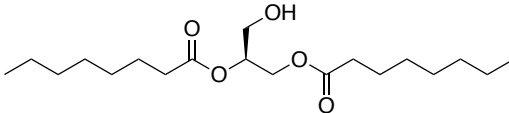
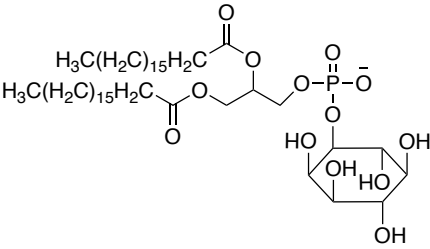
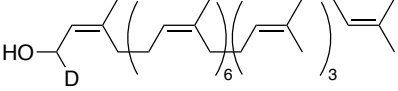
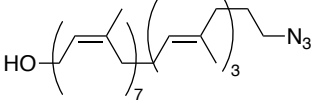
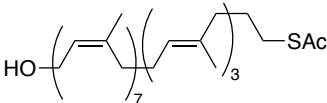
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Structure	Substrate	Activity <sup>[a]</sup> [ $\mu\text{mol}$ $\text{mg}^{-1} \text{min}^{-1}$ ]	Ref
13 	( <i>Z</i> <sub>7</sub> , <i>E</i> <sub>3</sub> , $\omega$ )-Undecaprenol	5.1 $\pm$ 0.2	[59]
14 	( <i>Z</i> , $\omega$ )-Nerol	8.7 $\pm$ 0.3	[59]
15 	( <i>E</i> , $\omega$ )-Nerol	6.2 $\pm$ 0.1	[59]
16 	( <i>Z</i> <sub>2</sub> , $\omega$ )-Farnesol	21.2 $\pm$ 0.4	[59]
17 	( <i>Z</i> , <i>E</i> , $\omega$ )-Farnesol	5.4 $\pm$ 0.4	[59]
18 	( <i>E</i> <sub>2</sub> , $\omega$ )-Farnesol	11.5 $\pm$ 0.2	[59]
19 	( <i>E</i> <sub>3</sub> , $\omega$ )-Geranylgeraniol	9.3 $\pm$ 1.0	[59]
20 	( <i>Z</i> <sub>4</sub> , <i>E</i> <sub>2</sub> , $\omega$ )-Heptaprenol	8.8 $\pm$ 0.2	[59]
21 	( <i>Z</i> <sub>5</sub> , <i>E</i> <sub>2</sub> , $\omega$ )-Octaprenol	3.8 $\pm$ 0.1	[59]
22 	( <i>E</i> <sub>8</sub> , $\omega$ )-Solanesol	4.9 $\pm$ 0.3	[59]

23		( <i>Z</i> <sub>6</sub> , <i>E</i> <sub>3</sub> , ω)-Decaprenol	3.8 ± 0.1	[59]
24		Phytol	5.6 ± 0.8	[59]
25		Dolichol (α-dihydro, <i>Z</i> <sub>9-17</sub> , <i>E</i> <sub>2</sub> )	4.5 ± 0.2	[59]
26		Undecanol	2.1 ± 0.1	[59]
27		Hexadecanol	1.9 ± 0.1	[59]
28		Eicosanol	1.3 ± 0.1	[59]
29		( <i>Z</i> <sub>3</sub> , <i>E</i> <sub>2</sub> )-Pentaprenol (dabsyl)	4.1 ± 0.1	[59]
30		( <i>Z</i> <sub>2</sub> , <i>E</i> <sub>3</sub> )-Pentaprenol (dabsyl)	4.0 ± 0.4	[59]
31		( <i>Z</i> , <i>E</i> <sub>4</sub> )-Pentaprenol (dabsyl)	5.9 ± 0.0	[59]
32		( <i>E</i> <sub>5</sub> )-Pentaprenol (dabsyl)	3.3 ± 0.1	[59]

33		Tween 20	Active <sup>[b]</sup>	[59]
34		Triton X-100	Active <sup>[b]</sup>	[59]
35		Tergitol NP-40	Active <sup>[b]</sup>	[59]
36		Ceramide	Inactive <sup>[b]</sup>	[44]
37		D-Erythro-sphingosine	Inactive <sup>[b]</sup>	[44]
38		1-Oleoyl- <i>rac</i> -glycerol	Inactive <sup>[b]</sup>	[44]
39		1,2-dioleoyl- <i>sn</i> -glycerol	Inactive <sup>[b]</sup>	[44]
40		Phosphatidylinositol	Inactive <sup>[b]</sup>	[44]
41		( <i>Z</i> <sub>7</sub> , <i>E</i> <sub>3</sub> )-Undecaprenol ( <i>α</i> - <i>d</i> <sub>1</sub> )	9.4 ± 0.5	[61]
42		( <i>Z</i> <sub>7</sub> , <i>E</i> <sub>3</sub> )-Undecaprenol ω-azide	10.8 ± 0.6	[61]
43		( <i>Z</i> <sub>7</sub> , <i>E</i> <sub>3</sub> )-Undecaprenol ω-thioacetate	11.8 ± 0.1	[61]

44		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω-tetrahydropyran, ω-OH	3.4 ± 0.3	[61]
45		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω- epoxide	8.1 ± 0.7	[61]
46		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω- propargylamine	7.1 ± 0.5	[61]
47		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω- (3-ethynylaniline)	9.0 ± 0.5	[61]
48		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω- (4-azidoaniline)	9.4 ± 0.2	[61]
49		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω- (4-amino-TEMPO)	5.1 ± 0.3	[61]
50		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω-(2-aminobenzamide)	6.9 ± 0.8	[61]
51		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω-(pyrenemethylamine)	6.2 ± 0.1	[61]
52		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω-(4-nitroaniline)	9.0 ± 0.9	[61]
53		(Z <sub>7</sub> ,E <sub>3</sub> )-Undecaprenol ω- diazirine	5.1 ± 0.3	[61]

359 <sup>[a]</sup>UdpK activity determined colorimetrically using a pyruvate kinase/lactate dehydrogenase coupled  
360 assay unless otherwise stated. <sup>[b]</sup> Actual rate unavailable.

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