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The thermoacidophilic archaeon *Sulfolobus acidocaldarius* contains an unusually short, highly reduced dolichol phosphate

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Abstract

Polyprenoids, polymers containing varied numbers of isoprene subunits, serve numerous roles in biology. In Eukarya, dolichyl phosphate, a phosphorylated polyprenol bearing a saturated α -end isoprene subunit, serves as the glycan carrier during N-glycosylation, namely that post-translational modification whereby glycans are covalently linked to select asparagine residues of a target protein. As in Eukarya, N-glycosylation in Archaea also relies on phosphorylated dolichol. In this report, LC-ESI/MS/MS was employed to identify a novel dolichyl phosphate (DoIP) in the thermoacidophilic archaeon, *Sulfolobus acidocaldarius*. The unusually short *S. acidocaldarius* DoIP presents a degree of saturation not previously reported. *S. acidocaldarius* DoIP contains not only the saturated α - and ω -end isoprene subunits observed in other archaeal DoIPs, but also up to five saturated intra-chain isoprene subunits. The corresponding dolichol and hexose-charged DoIP species were also detected. The results of the present study offer valuable information on the biogenesis and potential properties of this unique DoIP. Furthermore, elucidation of the mechanism of the α -isoprene unit reduction in *S. acidocaldarius* dolichol may facilitate the identification of the alternative, as yet unknown polyprenol reductase in Eukarya.

Keywords

Archaea; dolichol; electrospray ionization mass spectrometry; polyprenol; polyprenol reductase; *Sulfolobus acidocaldarius*

1. INTRODUCTION

Polyprenoids correspond to a group of hydrophobic polymers that comprise up to 100 isoprene subunits linked head-to-tail, with the resulting chain presenting a hydroxy group at the α -end [for review, see 1,2]. Reduction of the double bond in the α -end isoprene subunit gives rise to the dolichols. While polyprenoids serve a variety of biological roles [1, 3–5], phosphorylated polyprenols and dolichols are central components in pathways of N-

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glycosylation, a post-translational modification involving the covalent attachment of glycans to select asparagine residues of target proteins. Across evolution, asparagine-linked glycans are initially assembled on phosphoprenol carriers, namely dolichyl phosphate (DoIP)¹ in Eukarya and Archaea and polyprenol phosphate (typically undecaprenol phosphate (UndP)) in Bacteria [1,2,6].

The biosynthesis of polyprenoids begins with formation of the building blocks of isoprene-based molecules, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP and DMAPP are generated by either the classic mevalonate (MVA) pathway or by the mevalonate-independent deoxyxylulose phosphate (DOXP) pathway [7–10]. The subsequent union of DMAPP and several IPP subunits produces polyprenoids. These condensation reactions are mediated by prenyltransferases, which are also responsible for determining polyprenol *cis/trans* stereochemistry. In this manner, all-*trans* farnesyl (C₁₅) or geranylgeranyl (C₂₀) diphosphate are elongated into *cis/trans* polyprenol diphosphates.

Despite over 40 years of research and the resulting wealth of information available on polyprenol biosynthesis, many details remain unclear, particularly concerning those steps involved in the conversion of polyprenol diphosphate to DoIP. It is generally thought that polyprenol diphosphate undergoes two rounds of dephosphorylation to produce polyprenol. Reduction of the polyprenol α -position isoprene subunit yields dolichol, which is in turn phosphorylated to produce DoIP [11]. Most of the enzymes catalyzing these reactions have, however, yet to be identified. Recently, mammalian SRD5A3 and its yeast ortholog, DFG10, were shown to be polyprenol reductases responsible for converting polyprenol into dolichol [12]. Nonetheless, some dolichol is produced in cells lacking the encoding genes, indicating that additional polyprenol reductase(s) must exist. Moreover, those phosphatases responsible for transforming polyprenol diphosphate into polyprenol remain unknown.

The search for as yet unidentified enzymes involved in DoIP biogenesis may benefit from the study of Archaea. Archaea are a group of microorganisms that comprise a distinct branch of the tree of life that also includes eukaryal and bacterial limbs [13]. Although their ubiquitous distribution is now clear [14,15], Archaea remain best known as extremophiles, namely organisms able to thrive in some of the most physically challenging environments on Earth, being found at extremes of temperature, pH and salinity and other conditions seemingly inhospitable to life [16]. Possibly in response to their harsh surroundings, Archaea have often adopted unique biochemical strategies. For example, in the archaeal version of the MVA pathway, mevalonate-5-phosphate is decarboxylated to produce isopentenyl phosphate, which is in turn phosphorylated to generate IPP [17–19]. This differs from the classic version of the pathway, where mevalonate-5-phosphate is phosphorylated to yield mevalonate-5-pyrophosphate, which is subsequently decarboxylated to form IPP. Likewise, examples of archaeal DoIP structurally distinct from their eukaryal counterparts have been presented. In the halophilic archaeon, *Haloferax volcanii*, C₅₅ and C₆₀ DoIP shown to participate in N-glycosylation both the α - and the ω -position isoprene subunits are saturated [20–22]; in the eukaryal lipid, only the α -position isoprene subunit is saturated [1,2].

In the following, another unusual DoIP of archaeal origin is reported. *Sulfolobus acidocaldarius*, a thermoacidophilic archaeon that grows optimally at 80°C and pH 2 [23],

¹Abbreviations used: Acetate, Ac; chloroform, CHCl₃; *cis*-polyprenyl diphosphate synthase, CPDS; collision induced dissociation, CID; deoxyxylulose phosphate, DOXP; dimethylallyl diphosphate, DMAPP; dolichyl phosphate, DoIP; geranylgeranyl diphosphate, GGPP; geranylgeranyl reductase, GGR; isopentenyl diphosphate, IPP; methanol, MeOH; mevalonate, MVA; multiple reaction monitoring, MRM; tandem mass spectrometry, MS/MS; undecaprenol phosphate, UndP.

contains a version of the molecule that is both shorter than eukaryal or other known archaeal DolP and which presents a degree of saturation never seen before.

2. MATERIALS AND METHODS

2.1. Strain and growth

S. acidocaldarius (DSM639) were grown in Brock's medium at 76°C, pH adjusted to 3 using sulphuric acid and supplemented with 0.1% (w/v) tryptone [23].

2.2. Lipid extraction

A *S. acidocaldarius* lipid extract was prepared according to Bligh and Dyer [24]. Briefly, 1.6 ml of PBS, 2 ml chloroform (CHCl₃) and 4 ml methanol (MeOH) were added to 0.5 g of lyophilized *S. acidocaldarius* cell powder in a 15 ml glass tube with a Teflon-lined cap to yield a single phase (CHCl₃:MeOH:PBS = 1:2:0.8, v/v) solution. Following intermittent mixing by vortex for 5 min, the mixture was sonicated in a water bath for 15 min at room temperature (25°C). The sample was centrifuged (3,000 rpm) for 5 min at room temperature and the supernatant was transferred to a fresh 15 ml glass tub with a Teflon-lined cap. Two milliliters CHCl₃ and 2 ml PBS were added to yield a two-phase Bligh-Dyer mixture (CHCl₃:MeOH:PBS = 2:2:1.8, v/v). After mixing, the sample was centrifuged (3,000 rpm) for 5 min at room temperature to separate the phases. The upper phase was removed and the lower CHCl₃ phase was dried under a stream of nitrogen. The dried lipid extract was stored at -20°C until further analyzed.

2.3. LC-ESI/MS

Normal phase LC-ESI/MS of the *S. acidocaldarius* lipid extract was performed using an Agilent 1200 Quaternary LC system coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA). An Ascentis Si HPLC column (5 µm, 25 cm × 2.1 mm) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v/v). Mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v/v/v). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v/v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 µl/min. The post-column splitter diverted ~10% of the LC flow to the ESI source of the Q-Star XL mass spectrometer, with MS settings as follows: Ion spray voltage (IS) = -4500 V, Curtain gas (CUR) = 20 psi, Ion source gas 1 (GS1) = 20 psi, De-clustering potential (DP) = -55 V, and FP = -150 V. Nitrogen was used as the collision gas for MS/MS experiments. Data acquisition and analysis were performed using the instrument's Analyst QS software.

Reverse phase LC-ESI/MS of the *S. acidocaldarius* lipid extract was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps and a SCL-10A system controller) coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (as above). LC was operated at a flow rate of 200 µl/min with a linear gradient as follows: 100% of mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 4 min. Mobile phase A consisted of methanol/acetonitrile/aqueous 1 mM ammonium acetate (60/20/20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 µm, 2.1 × 50 mm) was obtained from Agilent (Palo Alto, CA). The MS operating conditions were as above.

2.4. LC-Multiple reaction monitoring

Normal phase LC coupled with multiple reaction monitoring (MRM) was performed using an Agilent 1200 Quaternary LC system interfaced to a 4000 Q-Trap hybrid triple quadrupole linear ion-trap mass spectrometer equipped with a Turbo V ion source (Applied Biosystems). The LC conditions are same as described above. The post-column splitter diverted ~10% of the LC flow to the Turbo V ion source. MRM was performed in the negative ion mode with MS settings as follows: CUR = 20 psi; GS1 = 20 psi; Ion source gas 2 (GS2) = 30 psi; IS = -4500 V; Heater temperature (TEM) = 350 °C; Interface heater = ON; DP = -40 V; Entrance potential (EP) = -10 V; Collision cell exit potential (CXP) = -5 V. The MRM pairs are as follows: 719.6/79.0 (C₄₅ DolP from *S. acidocaldarius*), 917.8/79.0 (C₆₀ DolP from *Hfx. volcanii*), 1392.2/79.0 (C₉₅ DolP from human fibroblasts), and 845.6/79.0 (C₅₅ UndP from *Escherichia coli*).

3. RESULTS

3.1. *S. acidocaldarius* contains short, highly saturated DolP

To begin analysis of *S. acidocaldarius* DolP, normal phase LC-ESI/MS of a lipid extract was performed in the negative ion mode using a high resolution QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Fig 1A). The mass spectrum averaged from those acquired during the retention time of 20–21 min shows prominent monoisotopic ion peaks of m/z 651.559, 719.605 and 787.688, corresponding to the [M-H]⁻ ions of C₄₀, C₄₅ and C₅₀ DolP containing five saturated and three, four and five unsaturated isoprene units, respectively (Fig 1B). These measured ion masses are in agreement with the calculated values of the [M-H]⁻ ion of C₄₀, C₄₅ and C₅₀ DolP, namely m/z 651.549, 719.611 and 787.674, respectively. In addition, a m/z 731.632 peak corresponding to archaetidic acid (calculated value of m/z 731.632) was observed. The proposed structure of *S. acidocaldarius* C₄₅ DolP is presented in Fig 1C.

When MS/MS was performed on the [M-H]⁻ ion of C₄₅ DolP at m/z 719.63 (Fig 2A), a fragmentation pattern consistent with a DolP molecule presenting a saturated isoprene subunit at the α - as well as multiple saturated isoprene subunits along the polyisoprenoid chain, including that found at the ω -position. The same saturation patterns were obtained for C₄₀ and C₅₀ dolichyl phosphate, except that one less or one more isoprene unit was detected, respectively (not shown). As the site of some double bonds could not be defined based on the fragmentation data, these were tentatively assigned taking into consideration indirect evidence discussed below. The interpretation of the MS/MS data for *S. acidocaldarius* DolP was supported by the results of MS/MS analysis performed on the [M-H]⁻ ions of *Hfx. volcanii* DolP, human DolP and *E. coli* UndP (Fig 2B). Both the haloarchaeal and human lipids are known to contain a saturated α -position isoprene subunit [12,20,21,25], reflected here by the product ion peaks at m/z 165.026 and 165.030, respectively (Fig 2B, arrows, upper and middle panels, respectively). The same peak (m/z 165.038) was observed in the MS/MS spectrum of the [M-H]⁻ ion of *S. acidocaldarius* DolP (Fig 2A, arrow). By contrast, a m/z 163.012 peak was seen in the *E. coli* UndP MS/MS spectrum (Fig 2B, arrow, lower panel), corresponding to the unsaturated isoprene subunit at the α position expected for this molecule [26]. Confirmation of the mono-phosphorylated nature of the *S. acidocaldarius* molecule also came from a comparison of normal phase LC-MRM chromatographic peaks showing the retention time of *acidocaldarius* C₄₅ DolP (MRM pair: 719.6/79.0) with those of *Hfx. volcanii* DolP (MRM pair: 917.8/79.0), human DolP (MRM pair: 1392.2/79.0) and *E. coli* UndP (MRM pair: 845.6/79.0). The similar retention times of these species are reflective of them each carrying the same charge (-1) (Fig 3).

Finally, while the data presented here were obtained using *S. acidocaldarius* samples prepared from stationary-phase cells, consistent results were acquired from cells grown to mid-logarithmic phase (not shown).

3.2. *S. acidocaldarius* contains hexose-charged DolP

Like Eukarya and Bacteria, Archaea also perform N-glycosylation. In *S. acidocaldarius*, the surface layer glycoprotein is modified by a heterogeneous group of N-linked glycans, the largest of which corresponds to a tribranched hexasaccharide comprising two N-acetylglucosamine, two mannose, one glucose and one 6-sulfoquinovose (6-deoxy-6-sulfoglucose) residues [27]. The same N-linked glycan had been previously reported to decorate *S. acidocaldarius* cytochrome *b*_{558/566} [28]. Towards determining whether DolP participates in the *S. acidocaldarius* N-glycosylation pathway, the normal phase LC-ESI/MS/MS profile of the *S. acidocaldarius* Bligh-Dyer lipid extract was scanned for glycan-charged DolP species. Such examination revealed the presence of [M-H]⁻ ions at *m/z* 813.626, 881.687 and 949.695, respectively corresponding to C₄₀, C₄₅ and C₅₀ DolP modified by a hexose residue (Fig 4A). This assignment was confirmed upon MS/MS analysis of the C₄₅ DolP-hexose [M-H]⁻ peak at *m/z* 881.7 (Fig 4B). Furthermore, the extracted ion chromatogram of the *m/z* 881.7 revealed three distinct C₄₅ DolP-hexose species, retained at 16.45, 17.94 and 19.07 min, respectively (Fig 4C). Similarly, three DolP-hexose species were also found in *Hfx. volcanii* [22].

3.3. *S. acidocaldarius* contains both dolichol and DolP presenting varying degrees of reduction

The unusually high degree of double bond saturation in *S. acidocaldarius* DolP could be an adaptation to the high temperatures encountered by this organism, as suggested for *S. acidocaldarius* phospholipids, which contain almost fully saturated isoprenoid chains [29]. Hence, with the aim of gaining insight into the pathway of *S. acidocaldarius* DolP biosynthesis, the normal phase LC-ESI/MS profile of the *S. acidocaldarius* lipid extract was analyzed for the presence of precursors/derivatives of the C₄₅ species. In addition to the major C₄₅ DolP peak observed at *m/z* 719.605 and containing saturated isoprene subunits at the α -position, at the ω -position and at three additional internal positions, such analysis also revealed [M-H]⁻ ion peaks at *m/z* 711.565, 713.577; 715.585, 717.609 and 721.629 (Fig 5A). These correspond to versions of C₄₅ DolP containing saturated isoprene subunits at the α -position alone, at the α - and ω -positions and at the α -position, the ω -position and at one, two and four internal positions (Fig 5B). No fully unsaturated species was detected.

When the *S. acidocaldarius* lipid extract was subjected to reserve phase LC-ESI/MS analysis, acetate (Ac) adduct [M + Ac]⁻ ions of free C₄₅ dolichols (*m/z* 695.634, 697.652 and 699.655) and C₅₀ dolichols (*m/z* 763.707, 765.720 and 767.792) were observed (Fig 6, arrows in the top and bottom panels, respectively). The various versions of C₄₀ dolichol were undetectable, as overlapping peaks derived from other lipid groups in the sample obscured their signals.

4. DISCUSSION

One of the hallmarks that distinguish Archaea as a distinct domain of life is the composition of their membrane phospholipids. Rather than comprising fatty acyl groups ester-linked to *sn*-glycerol-3-phosphate as in Eukarya and Bacteria, archaeal phospholipids are composed of isoprenoid chains ether-linked to *sn*-glycerol-1-phosphate [30,31]. Moreover, the membranes of thermophilic archaea, such as *S. acidocaldarius*, contain membrane-spanning tetraether isoprenoid-based lipids [32, 33]. As such, the study of polyprenol biosynthesis in Archaea may reveal aspects of the process unique to this life form or alternatively, elucidate

aspects of the process not evident using other models. The present analysis of *S. acidocaldarius* DolP reveals this potential. *S. acidocaldarius* DolP is unlike its counterparts in other Archaea or in Eukarya. Comprising only eight to ten isoprene subunits, *S. acidocaldarius* DolPs are shorter than the C₇₀-C₁₁₀ species found in Eukarya [2] or the C₅₅ and C₆₀ species observed in *Hfx. volcanii* [20,21]. C₄₅-C₆₀ polyisoprenes are, however, detected in the Gram-negative bacterium, *Campylobacter jejuni*, where they serve as the platform upon which N-linked glycans are assembled [34]. Moreover, whereas *S. acidocaldarius* DolP contains saturated α - and ω -position isoprene subunits as do their *Hfx. volcanii* counterparts, the major *S. acidocaldarius* DolP species include an additional two-four saturated isoprene subunits, thus presenting a degree of saturation not previously observed elsewhere. This high degree of saturation may reflect the need for a molecule stable in the face of elevated temperatures and acidity, conditions naturally encountered by *S. acidocaldarius* DolP. By comparison, only the α -isoprene subunit is saturated in eukaryal DolP [1,2,25].

Although the pathway used for *S. acidocaldarius* DolP biogenesis has yet to be delineated, previous efforts point to several aspects of the process apparently unique to this organism. In contrast to almost all other known Archaea, *S. acidocaldarius* apparently relies on the classic MVA pathway to generate the building blocks of polyprenols, i.e. IPP and DMAPP, as this species lacks isopentenyl phosphate kinase, one of the two enzymes unique to the archaeal version of the pathway [35]. Secondly, *in vitro* analysis of *S. acidocaldarius* *cis*-polyprenyl diphosphate synthase revealed that GGPP is the preferred substrate of the enzyme [36]. Accordingly, the *S. acidocaldarius* DolP structures presented in this report contain the branching point after a geranylgeranyl moiety, although this remains to be confirmed experimentally. In most Eukarya and Bacteria, by contrast, farnesyl pyrophosphate serves as the primary substrate for *cis*-polyprenyl diphosphate synthase in DolP and UndP biogenesis [1,2].

Based on the present study, *S. acidocaldarius* DolP also presents a degree of saturation never seen before. Although the agents responsible for this high degree of saturation are not currently known, a geranylgeranyl reductase (GGR) thought to catalyze the hydrogenation of geranylgeranyl groups in *S. acidocaldarius* phospholipid precursors has been identified [37]. *In vitro* characterization revealed this *S. acidocaldarius* GGR to be capable of reducing only three of the four double bonds of geranylgeranyl diphosphate (GGPP), activity also seen in plant and bacterial GGRs. In this case, the α -isoprene subunit of GGPP is not reduced and thus remains accessible for further prenyl transfer reactions. It remains, however, to be determined whether this GGR, encoded by the *Saci_0986* gene, also participates in DolP biogenesis. It is conceivable that the multiple double bond reduction steps involved in *S. acidocaldarius* DolP biogenesis rely on another one or more of the six putative GGRs reportedly encoded by the *S. acidocaldarius* genome [19]. Since, as the various versions of *S. acidocaldarius* dolichol and DolP observed in the current study all presented a reduced isoprene subunit at the α -position, it is reasonable to assume that saturation at this position relies on a distinct enzyme not involved in the saturation of other double bonds found in *S. acidocaldarius* DolP precursors. While an α -isoprene reductase responsible for converting polyprenol to dolichol in Eukarya has only recently been identified, it is clear that more than one reductase can catalyze this activity [12]. Based on the results presented here as well as earlier findings, a proposed pathway for *S. acidocaldarius* DolP biosynthesis is presented in Fig 7.

Finally, the detection of *S. acidocaldarius* DolP modified by distinct hexoses implies that DolP serves as the glycan lipid carrier in N-glycosylation and that multiple glycan-modified DolP are involved in *S. acidocaldarius* N-glycosylation. This is the case in *Hfx. volcanii*, where the first four saccharide subunits of the S-layer glycoprotein N-linked pentasaccharide

(i.e. hexose, two hexuronic acids and a methyl ester of hexuronic acid [21]) are sequentially assembled on a common dolichyl phosphate carrier, whereas the final pentasaccharide residue, mannose, is derived from a distinct dolichyl phosphate [22]. In the present study, no DolP species bearing higher ordered glycans were detected. This could be because such species are present only at low levels at steady state conditions or that a different extraction protocol than the typical Bligh-Dyer method [24] used here is required for their detection. Indeed, short DolPs, such as detected here, are less hydrophobic than are longer DolPs. When linked to multiple sugars, the short *S. acidocaldarius* DolP may not be sufficiently hydrophobic to partition into the chloroform-based lower phase formed in the Bligh-Dyer extraction employed.

In conclusion, the thermacidophile *S. acidocaldarius* contains DolP that is not only shorter than what is found elsewhere but that also presents a degree of saturation not previously reported. With a completed genome sequence and protocols for gene deletion now available [38,39], it may be possible to fully delineate the pathway of *S. acidocaldarius* DolP biogenesis and identify those reductases responsible for the high degree of saturation detected in this molecule. Such findings may shed light on the enigmatic mechanisms involved in the reduction of polyprenol to dolichol in Eukarya, in particular, aiding in the identification of the alternative, as yet unknown eukaryal polyprenol reductase(s) [12].

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References

1. Swiezewska E, Danikiewicz W. Polyisoprenoids: Biosynthesis, structure and function. *Prog Lipid Res.* 2005; 44:235–258. [PubMed: 16019076]
2. Jones MB, Rosenberg JN, Betenbaugh MJ, Krag SS. Structure and synthesis of polyisoprenoids used in N-glycosylation across the three domains of life. *Biochim Biophys Acta.* 2009; 1790:485–494. [PubMed: 19348869]
3. Ericsson J, Dallner G. Distribution, biosynthesis, and function of mevalonate pathway lipids. *Subcell Biochem.* 1993; 21:229–272. [PubMed: 8256269]
4. McGarvey DJ, Croteau R. Terpenoid metabolism. *Plant Cell.* 1995; 7:1015–1026. [PubMed: 7640522]
5. Bajda A, Konopka-Postupolska D, Krzymowska M, Hennig J, Skorupinska-Tudek K, Surmacz L, Wójcik J, Matysiak Z, Chojnacki T, Skorzynska-Polit E, Drazkiewicz M, Patrzylas P, Tomaszewska M, Kania M, Swist M, Danikiewicz W, Piotrowska W, Swiezewska E. Role of polyisoprenoids in tobacco resistance against biotic stresses. *Physiol Plant.* 2009; 135:351–364. [PubMed: 19292825]
6. Burda P, Aebi M. The dolichol pathway of N-linked glycosylation. *Biochim Biophys Acta.* 1999; 1426:239–257. [PubMed: 9878760]
7. Bloch K. The biological synthesis of cholesterol. *Science.* 1965; 150:19–28. [PubMed: 5319508]
8. Miziorko HM. Enzymes of the mevalonate pathway of isoprenoid biosynthesis. *Arch Biochem Biophys.* 2011; 505:131–143. [PubMed: 20932952]
9. Rohmer M, Knani M, Simonin P, Sutter B, Sahm H. Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochem J.* 1993; 295:517–524. [PubMed: 8240251]
10. Rohmer M. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat Prod Rep.* 1999; 16:565–574. [PubMed: 10584331]

11. Schenk B, Fernandez F, Waechter CJ. The ins(ide) and out(side) of dolichyl phosphate biosynthesis and recycling in the endoplasmic reticulum. *Glycobiology*. 2001; 11:61R–70R.
12. Cantagrel V, Lefeber DJ, Ng BG, Guan Z, Silhavy JL, Bielas SL, Lehle L, Hombauer H, Adamowicz M, Swiezewska E, De Brouwer A, Bluemel P, Cegielska J, Houliston SR, Swistun D, Ali BR, Babovic-Vuksanovic D, van Bokhoven H, Wevers RA, Raetz CRH, Freeze HH, Morava E, Al-Gazali L, Gleeson JG. SRD5A3 is required for converting polyprenol to dolichol and is mutated in a congenital glycosylation disorder. *Cell*. 2010; 142:1–15.
13. Woese CR, Kandler O, Wheelis ML. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci USA*. 1990; 87:4576–4579. [PubMed: 2112744]
14. Pace NR. A molecular view of microbial diversity and the biosphere. *Science*. 1997; 276:734–740. [PubMed: 9115194]
15. DeLong EF. Everything in moderation: archaea as ‘non-extremophiles’. *Curr Opin Genet Dev*. 1998; 8:649–654. [PubMed: 9914204]
16. Rothschild LJ, Mancinelli RL. Life in extreme environments. *Nature*. 2001; 409:1092–1101. [PubMed: 11234023]
17. Smit A, Mushegian A. Biosynthesis of isoprenoids via mevalonate in Archaea: the lost pathway. *Genome Res*. 2000; 10:1468–1484. [PubMed: 11042147]
18. Grochowski LL, Xu H, White RH. Methanocaldococcus jannaschii uses a modified mevalonate pathway for biosynthesis of isopentenyl diphosphate. *J Bacteriol*. 2006; 188:3192–3198. [PubMed: 16621811]
19. Matsumi R, Atomi H, Driessen AJ, van der Oost J. Isoprenoid biosynthesis in Archaea--biochemical and evolutionary implications. *Res Microbiol*. 2011; 162:39–52. [PubMed: 21034816]
20. Kuntz C, Sonnenbichler J, Sonnenbichler I, Sumper M, Zeitler R. Isolation and characterization of dolichol-linked oligosaccharides from *Haloferax volcanii*. *Glycobiology*. 1997; 7:897–904. [PubMed: 9363431]
21. Guan Z, Naparstek S, Kaminski L, Konrad Z, Eichler J. Distinct glycan-charged phosphodolichol carriers are required for the assembly of the pentasaccharide N-linked to the *Haloferax volcanii* S-layer glycoprotein. *Mol Microbiol*. 2010; 78:1294–1303. [PubMed: 21091511]
22. Kaminski L, Abu-Qarn M, Guan Z, Naparstek S, Ventura VV, Raetz CRH, Hitchen PG, Dell A, Eichler J. AglJ adds the first sugar of the N-linked pentasaccharide decorating the *Haloferax volcanii* S-layer glycoprotein. *J Bacteriol*. 2010; 192:5572–5579. [PubMed: 20802039]
23. Brock TD, Brock KM, Belly RT, Weiss RL. *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch Microbiol*. 1972; 84:54–68.
24. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959; 37:911–917. [PubMed: 13671378]
25. Ekström TJ, Chojnacki T, Dallner G. The alpha-saturation and terminal events in dolichol biosynthesis. *J Biol Chem*. 1987; 262:4090–4097. [PubMed: 3031061]
26. Wright A, Dankert M, Fennessey P, Robbins PW. Characterization of a polyisoprenoid compound functional in O-antigen biosynthesis. *Proc Natl Acad Sci USA*. 1967; 57:1798–1803. [PubMed: 4291948]
27. Peyfoon E, Meyer B, Hitchen PG, Panico M, Morris HR, Haslam SM, Albers SV, Dell A. The S-layer glycoprotein of the crenarchaeote *Sulfolobus acidocaldarius* is glycosylated at multiple sites with chitobiose-linked N-glycans. *Archaea*. 2010:754101. [PubMed: 20936123]
28. Zahringer U, Moll H, Hettmann T, Knirel YA, Schafer G. Cytochrome b558/566 from the archaeon *Sulfolobus acidocaldarius* has a unique Asn-linked highly branched hexasaccharide chain containing 6-sulfoquinovose. *Eur J Biochem*. 2000; 267:4144–4149. [PubMed: 10866817]
29. Dannenmuller O, Arakawa K, Eguchi T, Kakinuma K, Blanc S, Albrecht AM, Shmutz M, Nakatani Y, Ourisson G. Membrane properties of archaeal macrocyclic diether phospholipids. *Chemistry*. 2000; 6:645–54. [PubMed: 10807176]
30. Kates M. Biology of halophilic bacteria, Part II. Membrane lipids of extreme halophiles: biosynthesis, function and evolutionary significance. *Experientia*. 1993; 49:1027–1036. [PubMed: 8270029]

31. Koga Y, Morii H. Biosynthesis of ether-type polar lipids in archaea and evolutionary considerations. *Microbiol Mol Biol Rev.* 2007; 71:97–120. [PubMed: 17347520]
32. de Rosa M, Gambacorta A, Bu'lock JD. Extremely thermophilic acidophilic bacteria convergent with *Sulfolobus acidocaldarius*. *J Gen Microbiol.* 1975; 86:156–164. [PubMed: 234504]
33. Langworthy TA. Comparative lipid composition of heterotrophically and autotrophically grown *Sulfolobus acidocaldarius*. *J Bacteriol.* 1977; 130:1326–1332. [PubMed: 863856]
34. Reid CW, Stupak J, Szymanski CM, Li J. Analysis of bacterial lipid-linked oligosaccharide intermediates using porous graphitic carbon liquid chromatography-electrospray ionization mass spectrometry: Heterogeneity in the polyisoprenyl carrier revealed. *Anal Chem.* 2009; 81:8472–8478. [PubMed: 19772334]
35. Boucher Y, Kamekura M, Doolittle WF. Origins and evolution of isoprenoid lipid biosynthesis in archaea. *Mol Microbiol.* 2004; 52:515–527. [PubMed: 15066037]
36. Hemmi H, Yamashita S, Shimoyama T, Nakayama T, Nishino T. Cloning, expression, and characterization of cis-polyprenyl diphosphate synthase from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*. *J Bacteriol.* 2001; 183:401–404. [PubMed: 11114943]
37. Sato S, Murakami M, Yoshimura T, Hemmi H. Specific partial reduction of geranylgeranyl diphosphate by an enzyme from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* yields a reactive prenyl donor, not a dead-end product. *J Bacteriol.* 2008; 190:3923–3929. [PubMed: 18375567]
38. Chen L, Bruegger K, Skovgaard M, Redder P, She Q, Torarinsson E, Greve B, Awayez M, Zibat A, Klenk HP, Garrett RA. The genome of *Sulfolobus acidocaldarius*, a model organism of the Crenarchaeota. *J Bacteriol.* 2005; 187:4992–4999. [PubMed: 15995215]
39. Wagner M, Berkner S, Ajon M, Driessen AJM, Albers SV. Expanding and understanding the genetic toolbox of the hyperthermophilic genus *Sulfolobus*. *Biochem Soc Trans.* 2009; 37:97–101. [PubMed: 19143610]

Highlights

- A novel dolichol phosphate was identified in the thermoacidophilic archaeon, *Sulfolobus acidocaldarius* using LC-ESI/MS/MS.
- The unusually short *S. acidocaldarius* dolichol phosphate presents a degree of saturation not previously reported.
- *S. acidocaldarius* dolichol phosphate - and -position isoprenes are saturated, as are intra-chain isoprenes.
- Hexose-charged dolichol phosphate species were also detected.

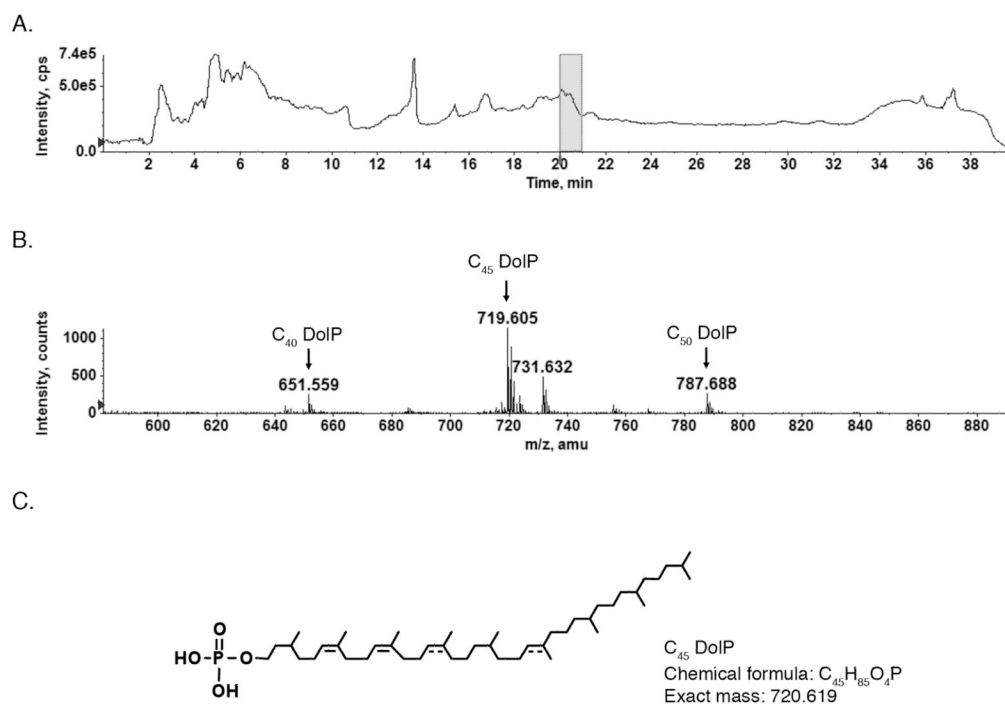


Fig 1. Identification of a novel DolP by normal phase LC/ESI-MS/MS analysis of a *S. acidocaldarius* lipid extract. A. The total ion chromatogram of normal phase LC-ESI/MS analysis performed in the negative ion mode. B. The $[M-H]^-$ ions of C₄₀, C₄₅ and C₅₀ DolP detected at m/z 651.559, 719.605 and 787.688, assigned as C₄₀ DolP, C₄₅ DolP and C₅₀ DolP, respectively. The mass spectrum shown is averaged from spectra acquired during the 20–21 min window, indicated by the shaded area in A. C. The proposed structure of *S. acidocaldarius* C₄₅DolP. Where the position of a double bond is only speculated, a dotted line is drawn.

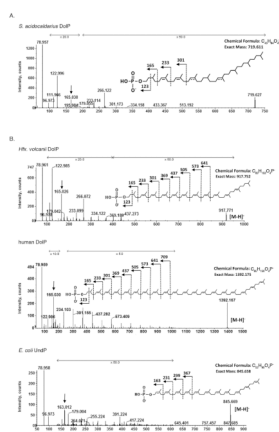


Fig 2. Structural elucidation of *S. acidocaldarius* DolP by MS/MS. A. Collision-induced dissociation (CID) MS/MS analysis of the m/z 719.6 $[M-H]^-$ ion peak corresponding to *S. acidocaldarius* C_{45} DolP. B. CID MS/MS analysis of the $[M-H]^-$ ion peaks at m/z 917.8, 1392.2, and 845.6, corresponding to *Hfx. volcanii* C_{55} DolP (top panel), human C_{95} DolP (middle panel) and *E. coli* C_{55} UndP (bottom panel), respectively. In each MS/MS spectrum, the arrow denotes the mass of the α -position isoprene subunit-containing fragment ion, while the inset shows the fragmentation scheme and lists the chemical formula and expected mass of the starting material. The arrows marked x5.0, x10.0, x20.0, x25.0 and x50.0 reflect magnification of ion peaks in the corresponding m/z region.

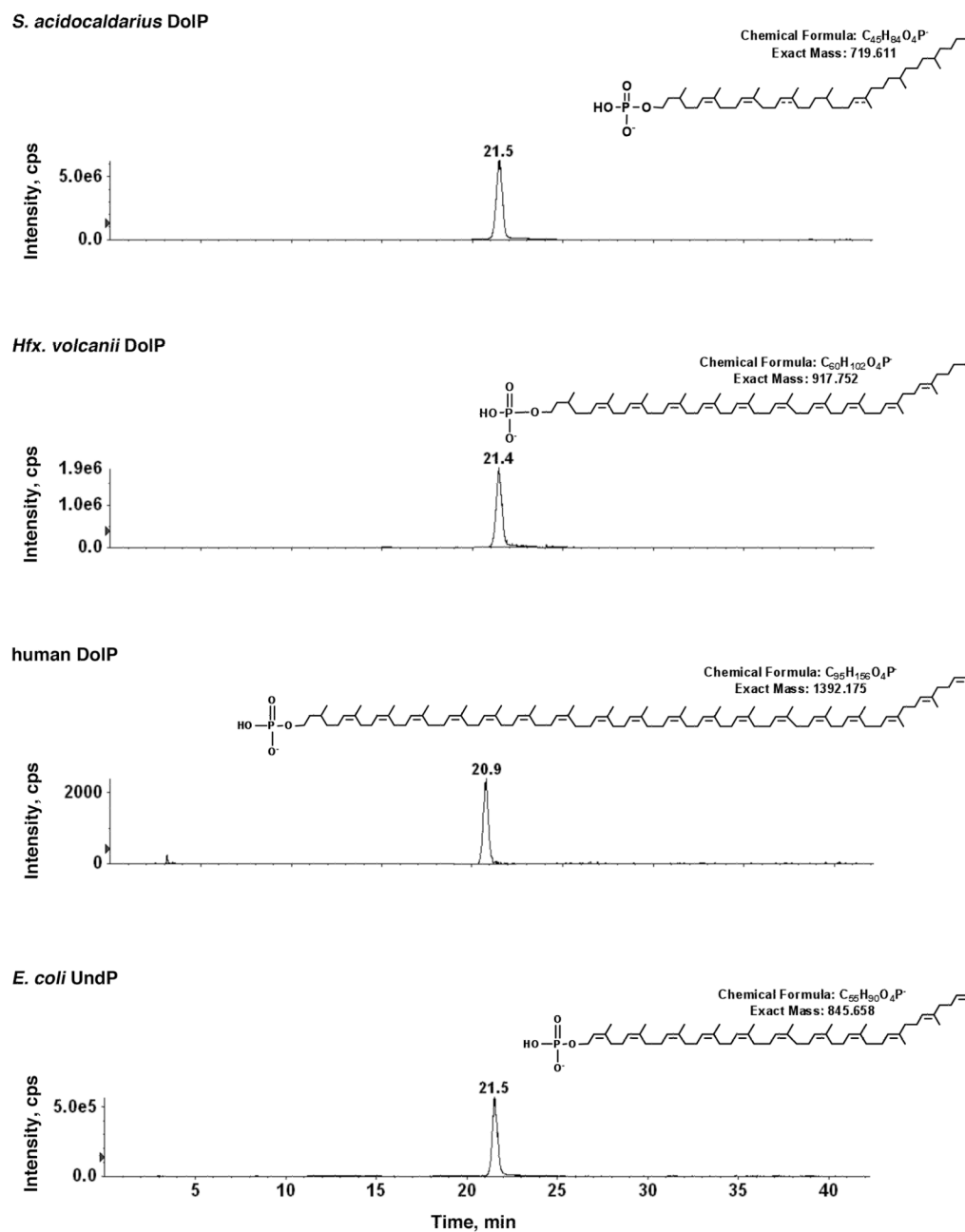


Fig 3. Normal phase LC of *S. acidocaldarius* DoIP and other mono-phosphorylated polyisoprenoids. LC-MRM chromatographic peaks portraying the LC retention times of *S. acidocaldarius* C₄₅ DoIP, *Hfx. volcanii* C₅₅ DoIP, human C₉₅ DoIP and *E. coli* C₅₅ UndP are presented as listed. In each panel, the inset shows the chemical structure, chemical formula and expected mass of each [M-H]⁻ ion.

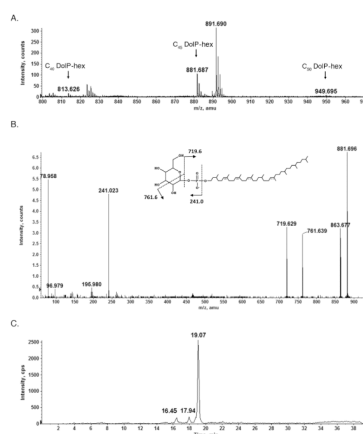
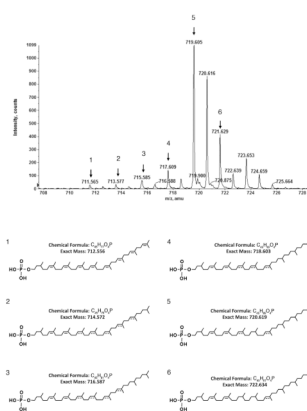


Fig 4. Normal phase LC/ESI-MS analysis of a *S. acidocaldarius* lipid extract reveals DolP-hexose. A. The $[M-H]^-$ ions of C₄₀, C₄₅ and C₅₀ DolP-hexose detected at m/z 813.626, 881.687 and 949.695, indicated by C₄₀ DolP-hex, C₄₅ DolP-hex and C₅₀ DolP-hex, respectively. The mass spectrum shown is averaged from spectra acquired during the 18.9–19.3 min window. B. CID MS/MS of the $[M-H]^-$ ion of hexose-charged C₄₅ DolP (m/z 881.687). The inset shows the predicted chemical structure of DolP-hexose and the MS/MS fragmentation scheme. Where the position of a double bond is only speculated, a dotted line is drawn. C. Normal phase LC-extracted ion chromatogram of the hexose-charged C₄₅ DolP $[M-H]^-$ ion at m/z 881.7, revealing three stereoisomeric species.



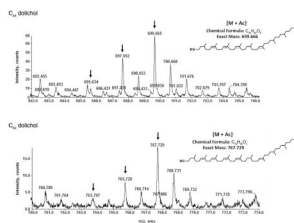


Fig 6.

Detection of *S. acidocaldarius* dolichol by reverse phase LC-ESI/MS. The acetate adduct $[M + Ac]^-$ ions of C₄₅ dolichols (m/z 695.634, 697.652 and 699.655) and C₅₀ dolichols (m/z 763.707, 765.720 and 767.792) are observed. The mass spectra shown are averaged from spectra acquired during the 15.1–15.6 min window.

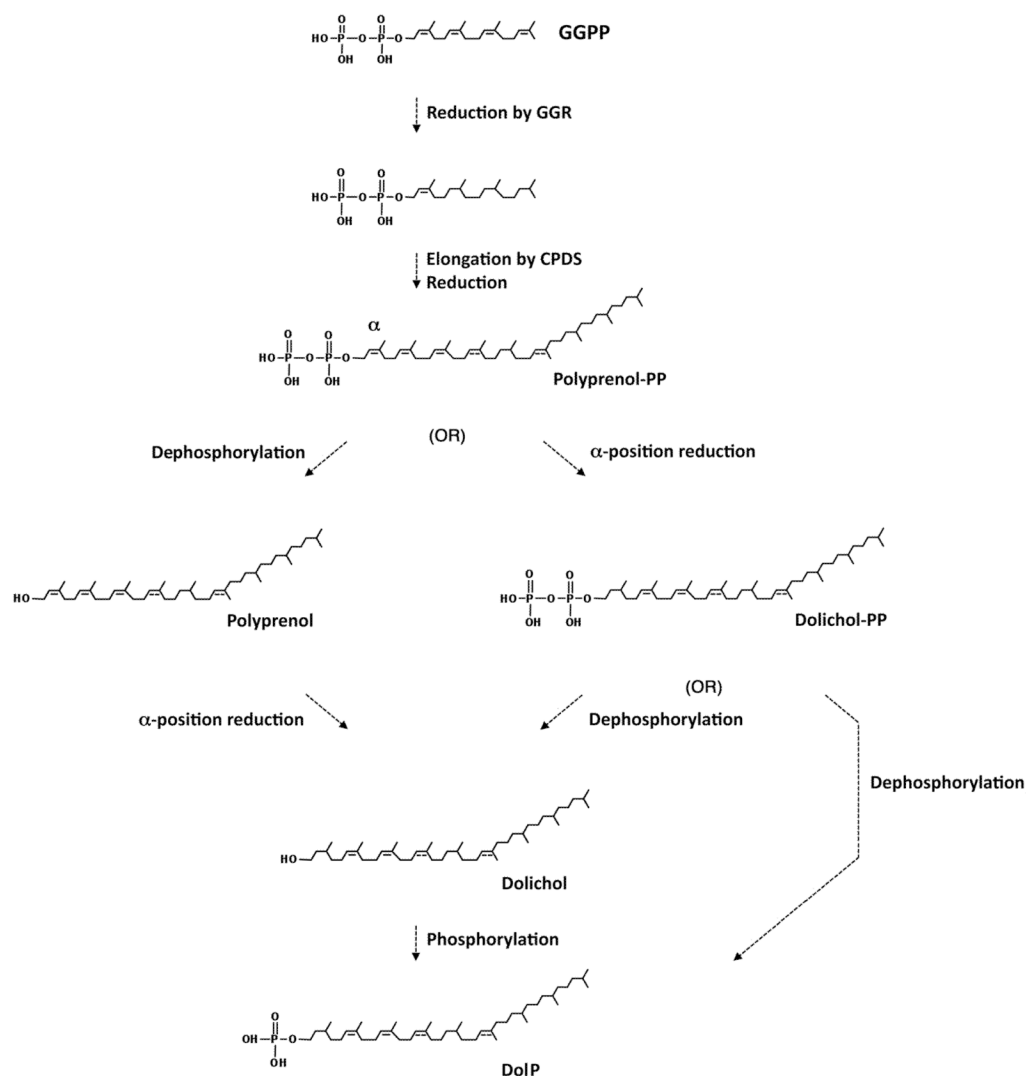


Fig 7.

A proposed *S. acidocaldarius* DolP biosynthesis pathway. In *S. acidocaldarius*, three IPP subunits and DMAPP, all synthesized via the classic MVA pathway [34], combine to form GGPP (not shown). Three of the four double bonds in this species are reduced by GGR; the α -position isoprene subunit remains unsaturated [36]. Four-six isoprene subunits are then added at the α -position of the GGPP backbone by *cis*-polyprenyl diphosphate synthase(s) (CPDS) [35] to generate C₄₀, C₄₅ and C₅₀ polyprenol diphosphate (Polyprenol PP). The polyprenol diphosphate α position isoprene subunit is indicated. At this point, removal of the two phosphate groups, followed by multiple reduction reactions and phosphorylation may occur to yield DolP, in a process reminiscent of what is proposed to occur in Eukarya [1,2]. Alternatively, C₄₀, C₄₅ and C₅₀ polyprenol diphosphate may undergo multiple reduction reactions followed by removal of one phosphate group or removal of both phosphate groups and re-phosphorylation, in both cases yielding DolP. The order in which the α -position and other isoprene subunits of the polyprenol diphosphate precursor are reduced is not known. Furthermore, where the position of a double bond is only speculated, a dotted line is drawn.