Identification, molecular cloning and functional characterization of an octaprenyl pyrophosphate synthase in intra-erythrocytic stages of *Plasmodium falciparum*

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Isoprenoids play important roles in all living organisms as components of structural cholesterol, steroid hormones in mammals, carotenoids in plants, and ubiquinones. Significant differences occur in the length of the isoprenic side chains of ubiquinone between different organisms, suggesting that different enzymes are involved in the synthesis of these side chains. Whereas in *Plasmodium falciparum* the isoprenic side chains of ubiquinone contain 7–9 isoprenic units, 10-unit side chains are found in humans. In a search for the *P. falciparum* enzyme responsible for the biosynthesis of isoprenic side chains attached to the benzoquinone ring of ubiquinones, we cloned and expressed a putative polyprenyl synthase. Polyclonal antibodies raised against the corresponding recombinant protein confirmed the presence of the native protein in trophozoite and schizont stages of *P. falciparum*. The recombinant protein, as well as *P. falciparum* extracts, showed an octaprenyl pyrophosphate synthase activity, with the formation

of a polyisoprenoid with eight isoprenic units, as detected by reverse-phase HPLC and reverse-phase TLC, and confirmed by electrospray ionization and tandem MS analysis. The recombinant and native versions of the enzyme had similar Michaelis constants with the substrates isopentenyl pyrophosphate and farnesyl pyrophosphate. The recombinant enzyme could be competitively inhibited in the presence of the terpene nerolidol. This is the first report that directly demonstrates an octaprenyl pyrophosphate synthase activity in parasitic protozoa. Given the rather low similarity of the *P. falciparum* enzyme to its human counterpart, decaprenyl pyrophosphate synthase, we suggest that the identified enzyme and its recombinant version could be exploited in the screening of novel drugs.

Key words: antimalarial, isoprenoid, malaria, *Plasmodium falciparum*, prenyltransferase, terpene.

INTRODUCTION

Malaria, one of the most important infectious diseases in the world, kills over one million people each year, and its incidence is increasing in several regions of the world. Lethal forms of the disease are caused by *Plasmodium falciparum*, and the increasing resistance of this parasite to virtually all current drugs calls for the identification of new therapeutic targets and the development of new drugs [1]. In order to avoid the rapid emergence of resistance, combinations of antimalarial drugs acting on different points of the same metabolic pathway are believed to increase therapeutic success [2]. An important target for the development of new antimalarial drugs is isoprenoid biosynthesis, which occurs via the 2-C-methyl-D-erythritol-4-phosphate pathway [3–7] in *P. falciparum*; in contrast, mammalian cells, certain eubacteria, archaea and fungi synthesize isoprenoid precursors through the mevalonate pathway [8]. Several products of isoprenoid metabolism have also been identified in *P. falciparum*: prenylated proteins [3,5], dolichols [9] and ubiquinones [10]. All of these products are derived from linear prenyl diphosphates, which are synthesized by sequential condensations of IPP (isopentenyl pyrophosphate) with allylic prenyl diphosphates. The condensation is catalysed by a family of enzymes known as prenyltransferases or polyprenyl diphosphate synthases [11–13]. Short-chain isoprenoids (C_{10} to C_{20}) are used as precursors for many different

compounds, such as the farnesylated and geranylgeranylated proteins [14], while long-chain isoprenoids (C_{30} to C_{50}) seem to be used for the synthesis of the isoprenoid side chain of ubiquinone [13].

Amino acid sequence comparisons of polyprenyl diphosphate synthases have revealed the existence of similar, highly conserved regions containing two aspartate-rich domains (DDXXD) (domains II and VI), which are thought to be the binding sites for the diphosphate moieties of IPP and the allylic substrate [15–17]. Divergence in the N-terminal regions is assumed to represent signal sequences responsible for differential subcellular localizations [18,19].

OPPs (octaprenyl pyrophosphate synthase) belongs to a prenyltransferase family that catalyses the condensation reactions of FPP (farnesyl pyrophosphate) with five molecules of IPP to produce C_{40} OPP (octaprenyl pyrophosphate) [19]. OPPs enzymes are responsible for the biosynthesis of side chains attached to ubiquinones in *Escherichia coli* [20,21]. Other organisms contain ubiquinones with different side chain lengths that are synthesized by specific prenyltransferases. For example, the sizes of the ubiquinone side chain are C_{30} in *Saccharomyces cerevisiae*, C_{45} in rats and C_{50} in humans, and these are synthesized by hexaprenyl pyrophosphate synthase, solanesyl pyrophosphate synthase and decaprenyl pyrophosphate synthase respectively [22,23]. The presence of an active isoprenoid pathway for the biosynthesis

Abbreviations used: ESI, electrospray ionization; FPP, farnesyl pyrophosphate; FTS, S-farnesylthiosalicylic acid; GGPP, geranylgeranyl pyrophosphate; GST, glutathione S-transferase; IPP, isopentenyl pyrophosphate; OPP, octaprenyl pyrophosphate; OPPs, octaprenyl pyrophosphate synthase; PfOPPs, *Plasmodium falciparum* OPPs; TrfOPPs, truncated recombinant version of *P. falciparum* OPPs; Q-TOF, quadrupole-time-of-flight; RP-, reverse-phase; RT-PCR, reverse transcription-PCR.

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of isoprenic chains of CoQ in *P. falciparum* has also been demonstrated [10].

Herein, we cloned the gene encoding a putative *P. falciparum* OPPs and characterized its gene product. In parallel, we also characterized the partially purified native version of the enzyme from schizont stages, and compared its kinetic properties with those of the recombinant version. Finally, inhibition assays demonstrated the effects of the terpene nerolidol on the recombinant version of the enzyme.

EXPERIMENTAL

Materials

General molecular biology reagents were obtained from Gibco BRL/Invitrogen (Rockville, MD, U.S.A.). Nerolidol, biochemical reagents and standards were obtained from Sigma Chemical Co. Percoll® was purchased from Pharmacia (Uppsala, Sweden). [$1\text{-}^{14}\text{C}$]IPP ammonium salt ($55.0\text{ Ci}\cdot\text{mmol}^{-1}$), [$1\text{-}(n\text{-}^3\text{H})$]FPP triammonium salt ($17.0\text{ Ci}\cdot\text{mmol}^{-1}$) and [$1\text{-}(n\text{-}^3\text{H})$]GGPP (geranylgeranyl pyrophosphate) triammonium salt ($16.5\text{ Ci}\cdot\text{mmol}^{-1}$) were obtained from Amersham-Pharmacia Biotech. Geranyl pyrophosphate ammonium salt, FPP ammonium salt and GGPP ammonium salt were obtained from Sigma Chemical Co. Albumax I was from Gibco BRL Life Technologies. All solvents were of analytical grade or better. Hyperfilm™ MP films (Amersham-Pharmacia Biotech) were used for autoradiography.

Parasite cultures

Cultures of *P. falciparum* (isolate NF54; clone 3D7) were grown using a modification [7] of the method of Trager and Jensen [24]. Development and multiplication of cultures was monitored by microscopic evaluation of Giemsa-stained thin smears. Ring-infected (0–20 h forms), trophozoite-infected (20–40 h forms) and schizont-infected (40–48 h forms) erythrocytes were purified on 40/70/80 % discontinuous Percoll® gradients [25] (30 min at 25°C and $10\,000\text{ g}$), yielding an upper band of schizont stages (40 %), a band of trophozoite stages (70–80 % interface), and a pellet for the ring stage and uninfected erythrocytes.

Nucleic acid extraction, PCR amplification, and cloning and sequencing of the gene encoding PfOPP (P. falciparum OPPs)

Total RNA isolation was performed as described previously [26]. Parasite DNA was isolated from the parasite pellet by proteinase K digestion and phenol/chloroform extraction [27]. *P. falciparum* genomic DNA was submitted to PCR amplification using primers designed according to the sequence of the putative gene PFB0130w (<http://www.PlasmDB.org>; GenBank® accession number AAC71816), introducing BamHI restriction sites (sense, $5'\text{-CCGGATCCATGGTTCACCTAAGTAAAAG-3'}$; antisense, $5'\text{-CCGGATCCTCATTGAGGTTTCTTGATAAC-3'}$). PCR mixtures included 200 ng of extracted DNA, $0.1\text{ }\mu\text{M}$ of each primer, $200\text{ }\mu\text{M}$ dNTP, PCR buffer containing 1.5 mM MgCl_2 , 50 mM KCl and $0.05\text{ unit}/\mu\text{l}$ Taq DNA polymerase (Invitrogen). The amplification conditions were: initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 30 s, 52°C for 1 min and 72°C for 30 s; and a final incubation at 72°C for 10 min. The 1.6 kb amplicon obtained was cloned into pGEM®T-easy vector (Promega), according to the manufacturer's instructions. Three clones were sequenced in both directions using Big Dye terminator chemistry on an ABI3100 sequencer (Applied Biosystems). Identities were checked using the BLAST tool at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). Similarity be-

tween amino acid sequences was calculated using the Point program (<http://www.geocities.com/alvesjmp/software.html>).

RT-PCR (reverse transcription-PCR)

RT-PCR was performed on cDNAs from ring, trophozoite and schizont stages of *P. falciparum*. Total RNA from these stages was isolated by the Trizol method (Invitrogen) and treated sequentially three times with DNase I (Life Technologies/Invitrogen). An aliquot of each RNA sample was reverse-transcribed using SuperScript II (Invitrogen). To exclude the possibility of sample contamination with genomic DNA, an aliquot from each sample was treated in the same way, except that no reverse transcriptase was added. PCR conditions (30 cycles) were as described above. OPPs-specific primers (sense, $5'\text{-ATGGTTCACCTAAGTAAAAG-3'}$; inner antisense, $5'\text{-GGATTACACATAGGTATG-GC-3'}$) amplified a 400 bp fragment from cDNA. Primers specific for the *P. falciparum* myosin gene (sense, $5'\text{-TTACATG-TTGCATCTATGAGTG-3'}$; antisense, $5'\text{-TCTCAATTTTAA-ATCAATTGACATCTTTAATG-3'}$) were included in a control reaction. PCR products were separated on 1 % agarose/TAE (40 mM Tris/HCl, pH 7.8, 20 mM sodium acetate, 1 mM EDTA) gels. The RT-PCR products were cloned into the pGEM®T-easy vector (Promega) and sequenced as above.

Expression and purification of a recombinant version of PfOPP

A fragment of the PFB0130w gene was excised using EcoRI from the pGEM-PFB0130w plasmid and ligated into EcoRI-cut pGEX2T (Amersham Pharmacia Biotech). The resulting plasmid was transformed in BL21-CodonPlus™ (DE3)-Ril (Stratagene) bacteria. Protein expression was induced with 1 mM isopropyl β -D-thiogalactoside for 3 h at 37°C , after which bacterial cells were harvested and resuspended in lysis buffer [7 mM Na_2HPO_4 , 1 mM KH_2PO_4 , pH 7.2, 137 mM NaCl, 3 mM KCl (PBS), 0.1 % (v/v) Triton X-100, 0.05 mg/ml lysozyme and 0.2 mM PMSF]. Lysis was completed by three freeze/thaw cycles and genomic bacterial DNA was sheared by 10 passages through a G21 needle. Recombinant proteins were then purified using glutathione-Sepharose beads (Amersham-Pharmacia), following the manufacturer's instructions. The purified protein was dialysed extensively against PBS, checked for purity by SDS/PAGE [28] and quantified by the Bradford method [29].

Antibodies against the recombinant version of PfOPP

Three Balb/C mice were inoculated intraperitoneally with the purified recombinant version of PFB0130w–GST (glutathione S-transferase) fusion protein. As a control, three mice were inoculated with the same quantities of recombinant GST. The immunogen consisted of $60\text{ }\mu\text{g}$ of recombinant fusion protein in complete Freund's adjuvant (Sigma, St. Louis, MO, U.S.A.) for primary immunization, and the same concentration of recombinant polypeptide in Freund's incomplete adjuvant for two booster injections, 21 days and 42 days after the primary immunization. After 52 days, blood samples from the retro-ocular plexus were obtained, under anaesthesia, and sera were prepared.

Metabolic labelling and immunoprecipitation assays

Asynchronous cultures of *P. falciparum* with parasitaemias of approx. 10 % were labelled for 18 h with $25\text{ }\mu\text{Ci}/\text{ml}$ L-[^{35}S]methionine ($>1000\text{ Ci}/\text{mmol}$; Amersham) in methionine-depleted (10 mM) RPMI 1640 medium. Each stage (ring, trophozoite or schizont) was then purified as described above, followed by lysis of cells in two pellet volumes of ice-cold 10 mM Tris/HCl,

pH 7.2, 150 mM NaCl, 2 % (v/v) Triton X-100, 1 mM PMSF, 5 mM iodoacetamide, 1 mM tosyl-lysylchloromethane ('TLCK') and 1 µg/ml leupeptin. After incubation at 4°C for 15 min, lysates were centrifuged for 30 min at 4°C and 10000 g, and supernatants were stored in liquid nitrogen. The samples were immunoprecipitated as described by Kessler [30] and modified by Moura et al. [5]. Immunoprecipitated proteins were analysed by SDS/PAGE and autoradiography.

Partial purification of native PfOPPs

The partial purification of native PfOPPs was conducted only for schizont stage parasites. The parasite pellet obtained from 1 litre of culture (50 culture flasks with a parasitaemia of approx. 20 %) was purified on discontinuous Percoll® gradients as described above. The schizont pellet fraction was resuspended in 10 ml of buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.2 mM PMSF and 0.1 mM leupeptin at 4°C. The suspension was sonicated with a microtip for four cycles (15 s each). The lysate was centrifuged for 1 h at 4°C and 100 000 g. The supernatant (S-100) was subjected to 0–50 % ammonium sulphate precipitation. The precipitate was resuspended in 3 ml of buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 20 µM ZnCl₂ and 20 mM NaCl, and dialysed against the same buffer at 4°C. This fraction was then used immediately in enzymatic reactions or stored at –80°C.

Measurement of enzymatic activity and product analysis of native PfOPPs

Enzymatic activity was measured by determination of the amount of [1-¹⁴C]IPP incorporated into butanol-extractable polyprenyl diphosphates. The standard assay mixture contained, in a final volume of 0.3 ml, HKMT buffer (100 mM Hepes, pH 7.5, 50 mM KCl, 0.5 mM MgCl₂, 0.1 % Triton X-100), 10 µM FPP, 0.33 µCi/ml [1-¹⁴C]IPP and a suitable amount of enzyme solution (recombinant or partially purified native PfOPPs), and was incubated at 30°C. The protein concentration used in all experiments was determined by its absorbance at 280 nm ($\epsilon_{280} = 20280 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Portions of the reaction mixture were withdrawn periodically (within 10 % substrate depletion) and the reaction was stopped by adding 10 mM EDTA (final concentration). The products were then extracted with 1 ml of butan-1-ol, and the radioactivity in the butan-1-ol extract was measured in c.p.m. mode with a Beckman 5000 β -radiation scintillation counter. The initial rate was calculated by determining the quantities of product formed or IPP consumed at each time point by counting the ¹⁴C radioactivity in the butanol phase (product) and in the aqueous phase (IPP) [31].

Kinetic parameters for recombinant and native PfOPPs

Each PfOPPs was assayed at eight different concentrations (1–200 µM) of IPP or FPP, while the other substrate (FPP or IPP respectively) was kept constant at 200 µM. K_m values (means $\pm$ S.E.M.) were obtained by the least-squares method using Enzfitter software (Elsevier-Biosoft, Cambridge, U.K.). Radiometric assays were performed by modifications of the methods of Hirooka et al. [32] and Sagami et al. [33].

Identification by RP (reverse-phase)-TLC of dephosphorylated polyisoprenoid products

Polyprenyl diphosphates were extracted from a 1 ml reaction and treated with potato acid phosphatase to convert the poly-prenyl pyrophosphates into their corresponding polyprenols. Hydrolysates were extracted with n-pentane and analysed by

RP-TLC (HPTLC RP-18; Merck) developed with acetone/water (19:1, v/v). Authentic standard polyprenols were visualized with iodine vapour, and the plates were exposed in the Storage phosphor screen for approx. 10 days and digitized using a Storm 820 apparatus (Amersham). Image analyses were performed using Image Quant software (Molecular Dynamics).

Purification and identification of the first intermediate (GGPP) by RP-HPLC

The n-butanol fraction was submitted to analytical RP-HPLC separations, which were performed on an Ultrasphere ODS Beckman column (4.6 mm $\times$ 26 cm) with a Gilson HPLC 322 pump connected to a Gilson 152 variable UV/visible detector at 214 nm. Fractions were collected in 1 ml/min intervals with a Gilson FC203B fraction collector, and UNIPONT™ System Software was used for the operation and registration of the chromatograms. Unless otherwise stated, 25 mM NH₄HCO₃, pH 8.0, was used to dissolve samples for analysis by RP-HPLC. The gradient elution system used was: solvent A, 25 mM NH₄HCO₃, pH 8.0; solvent B, 100 % (v/v) acetonitrile. A linear gradient was run from 0 % to 100 % B over a period of 40 min, after which 100 % B was then pumped through for an additional 5 min. All solvents were filtered and degassed before use, and the samples were passed through a 0.45 µm filter before injection, essentially as described by Zhang and Poulter [34].

The resulting fractions were vacuum evaporated and resuspended in scintillation liquid. Radioactivity quantification (c.p.m.) was performed in a Beckman 5000 β -radiation scintillation counter.

Purification and identification of dephosphorylated polyisoprenoid products ($\geq C_{40}$) by RP-HPLC

After the enzymatic reaction with either the recombinant or the native partially purified PfOPPs, the polyprenyl diphosphates were extracted with butan-1-ol and evaporated under a stream of nitrogen. The resulting polyprenyl diphosphates were treated with potato acid phosphatase according to the method of Fujii et al. [35], and submitted to analytical RP-HPLC separation performed in the same Gilson apparatus as described above, on a Phenomenex Luna C18 column (250 mm $\times$ 4.6 mm). Methanol/water (9:1, v/v) (solvent A) and hexane/propan-2-ol/methanol (1:1:2, by vol.) (solvent B) were used as solvents. A linear gradient was run from 5 % to 100 % B over a period of 25 min; 100 % B was then pumped through for an additional 5 min. The flow rate was 1.5 ml/min, using a method adapted from that of Low et al. [36].

Q-TOF (quadrupole–time-of-flight)-MS investigation of the first intermediate product (GGPP)

The samples purified by RP-HPLC with the retention time of the GGPP standard were analysed by ESI (electrospray ionization)-MS with direct infusion of an equimolar acetonitrile/water solution using a Harvard Syringe pump (model 11) and a Q-TOF mass spectrometer (Waters-Micromass) in the negative ion mode. Main conditions were as follows: flow rate, 5 µl/min; dissolution temperature, 100°C; block temperature, 100°C; capillary voltage, 3000 V; cone voltage, 30 V. The ESI-MS experiments were performed using the high-resolution TOF mass analyser scanning in the range m/z 50–1000. For the acquisition of ESI tandem mass spectra (ESI-MS/MS), the precursor ion was selected using the quadrupole analyser, and dissociated by collisions with argon in the hexapole collision cell at energies varying from 15 to 45 eV. The product ion mass spectra were acquired by TOF-MS analysis.

Figure 1 Alignment, transcription, expression and immunoprecipitation of PfOPPs

(A) Alignment of amino acid sequences of prenyltransferases. Black and white lettering on a grey background indicates identical and similar amino acids residues respectively. The two conserved DDXXD motifs are indicated. The star indicates a phenylalanine residue characteristic of OPPs activity. Aligned sequences are for the putative polyprenyl synthase of *P. falciparum* (Falciiparum; PlasmoDB gene PFB0130w), OPPs enzymes of *Thermotoga maritima* (Maritima; PDB code 1V4E) and *Escherichia coli* (Coli; GenBank® accession number P19641), solanesyl pyrophosphate synthase of *Mucor circinelloides* (Mucor; GenBank® accession number CAD42868.1), decaprenyl diphosphate synthase of fission yeast (Yeast; GenBank® accession number O43091), and GGPP synthase (GGPPsther; GenBank® accession number NP_394768.1) and FPP synthase (FPPsther; GenBank® accession number BAB59406.1) of *Thermoplasma volcanium*. (B) RT-PCR detection of putative PfOPPs transcripts. B1, control with myosin-specific oligonucleotides; B2, cDNA from different stages [ring (R), trophozoite (T) and schizont (S)] was amplified with sense and antisense

Ion-trap MS investigation of dephosphorylated polyisoprenoid products

Ion-trap ESI-MS and ESI-MS/MS data were collected using a Finnigan LCQ-Duo ion-trap mass spectrometer (Thermo-Finnigan, San Jose, CA, U.S.A.). The samples of dephosphorylated polyisoprenoids purified by RP-HPLC corresponding to the C₄₀, C₄₅ and C₅₅ standards were resuspended in 30 µl of chloroform/methanol (1:1, v/v) containing 2 mM lithium iodide. The samples (10 µl) were infused directly into the ESI probe across a 10 µl loop, using an Omnifit N₂ pressure system (Omnifit Ltd., Cambridge, U.K.) with 69 kPa (10 lb/in²) pressure at a flow rate of 10 µl/min, applying the same diluents as used for the sample. The C₄₀, C₄₅ and C₅₅ dephosphorylated polyisoprenoids were run in the ESI positive-ion mode, with spray voltage, capillary voltage and capillary temperature set at 4.52 kV, 17 V and 250.0 °C respectively, and mass spectra were acquired in SIM (selected ion monitoring) mode in the *m/z* ranges 570 ± 10 for C₄₀, 637 ± 3 for C₄₅ and 773 ± 5 for C₅₅. For ESI-MS/MS, a relative collision energy of 40% (2 eV) was applied, and the sheath (N₂) and collision helium gas pressures were 27.6 kPa (4 lb/in²) and 0.2 Pa (1.5 mtorr) respectively. The MS/MS spectra were acquired in full ion mode in the *m/z* ranges 500–600 for C₄₀, 500–700 for C₄₅ and 700–800 for C₅₅. These parameters were optimized for the highest intensity of the [M + Li]⁺ ion.

Inhibition of PfOPPs activity

The terpene nerolidol was preincubated at 10, 50 or 100 nM with TPfOPPs (truncated recombinant PfOPPs) for 30 min at 30 °C (a parallel control reaction was assayed without nerolidol). After preincubation, the reaction was performed by fixing the [1-¹⁴C]IPP concentration at 200 µM (corresponding to 3.3 µCi/ml) and varying the FPP concentration (2, 5, 10 and 50 µM) in HKMT buffer. The reactions were performed as described above. The products were then extracted with 1 ml of butan-1-ol and the radioactivity in the extract was measured in the c.p.m. mode with a Beckman 5000 β-radiation scintillation counter. Inhibition data were fitted to the competitive inhibition model using the software Enzfitter for K_i calculations (mean ± S.E.M.) based on double-reciprocal Lineweaver–Burk plots.

A control assay with FTS (*S*-farnesylthiosalicylic acid) was performed at three different concentrations: 7 µM (*in vivo* IC₅₀ value), 14 µM and 21 µM. Nerolidol was diluted in methanol and FTS was diluted in chloroform at 0.1 M (stock solution) essentially as described in Rodrigues Goulart et al. [37].

RESULTS

Identification and cloning of a putative *P. falciparum* polyprenyl synthase

In the *P. falciparum* genome, a gene encoding a putative *P. falciparum* polyprenyl synthase was predicted and annotated on chromosome 2 (PFB0130w). The alignment of this sequence with those of OPPs enzymes of *Thermotoga maritima*, OPPs of *Escherichia coli*, solanesyl pyrophosphate synthase of *Mucor circinelloides*, decaprenyl diphosphate synthase of *Schizosaccharomyces pombe*, and GGPP synthase and FPP syn-

thase of *Thermoplasma volcanium* revealed a degree of similarity. Primers specific for the PFB0130w gene were designed and used to amplify by PCR the corresponding gene, and its sequence was confirmed by DNA sequencing. The deduced amino acid sequences of the putative *P. falciparum* polyprenyl synthase and several *trans*-prenyltransferases showed sequence identity, with two common DDXXD motifs. These conserved aspartate-rich regions have been associated with OPPs activity [18]. The *P. falciparum* polyprenyl synthase has also a phenylalanine at position 132, (corresponding to the position found in *T. maritima* OPPs), which was determined as being responsible for chain isoprenoid elongation to C₄₀ in the *T. maritima* enzyme (Figure 1A). The *P. falciparum* polyprenyl synthase sequence shows 18.67% similarity with the sequence of the corresponding human enzyme (similarity matrix not shown).

The putative PfOPPs is transcribed and expressed in intra-erythrocytic stage parasites

RT-PCR assays from purified ring, trophozoite and schizont stage parasites showed the presence of PFB0130w transcripts in ring and trophozoite stages, but not in schizont stages, suggesting that the gene is transcribed in a stage-specific manner (Figure 1B). To detect the protein corresponding to the PFB0130w gene, we expressed it as a recombinant GST fusion protein. We cloned a truncated version of the gene in the vector pGEX2T, and the resulting protein (putative TPfOPPs) was then used to immunize Balb/C mice. Notably, production of a full-length protein was impossible (results not shown). The truncated version showed a tendency for either premature arrest of translation or proteolysis, resulting in a co-purified GST portion (Figure 1C).

Using the putative TPfOPPs antiserum, we were able to immunoprecipitate a protein of the expected size of 63 kDa from parasites metabolically labelled with L-[³⁵S]methionine. As demonstrated in Figure 1(D), only the putative TPfOPPs antiserum, but not the anti-GST serum, was able to immunoprecipitate the 63 kDa protein from trophozoite and in schizont stages.

Demonstration of PfOPPs activity and analysis of its substrate specificity by RP-TLC

In order to identify if *P. falciparum* extracts or the recombinant peptide showed any enzymatic activity, *in vitro* assays were performed with 10 µM IPP and 50 µM [³H]GGPP (7.9 µCi/ml) or 50 µM [³H]FPP (0.39 µCi/ml) as an allylic substrate under the optimum conditions described above. The reaction products were extracted, dephosphorylated and submitted to RP-TLC. A polyprenol of eight isoprenic units (C₄₀), typical of OPPs activity, and another with nine isoprenic units (C₄₅) were observed when [³H]FPP was used as a substrate with either TPfOPPs or native PfOPPs (Figure 2, lanes 1 and 3). When [³H]GGPP was used as substrate, no products were observed (Figure 2, lanes 2 and 4). Similar results were obtained when [1-¹⁴C]IPP and FPP or GGPP were used for analysis of substrate specificity (results not shown). Importantly, no synthesis of polyprenols occurred when either TPfOPPs or native PfOPPs was added (Figure 2, lane 5), confirming an enzymatic activity of both the semi-purified *P. falciparum* extract (Figure 2, lane 3) and the recombinant

PfOPPs primers. Genomic DNA of *P. falciparum* (isolate NF54; clone3D7) was used as a positive control (g); +, and – denote the presence and absence respectively of reverse transcriptase. Fragment sizes are indicated on the right. (C) Expression assays of the putative PfOPPs by SDS/12.5%-PAGE. TPfOPPs, TPfOPPs–GST fusion protein; GST, control. (D) Immunoprecipitation of parasites metabolically labelled with L-[³⁵S]methionine using putative TPfOPPs antiserum. Proteins extracts from ring (R), trophozoite (T) and schizont (S) stages were separated by discontinuous Percoll® gradients, and immunoprecipitated using sera of mice immunized with GST alone as a control (1) or TPfOPPs (2), or mock-immunized as a negative control (3). The arrows indicate the protein with the expected molecular mass immunoprecipitated from trophozoite and schizont extracts using the putative TPfOPPs–GST antiserum.

Figure 2 Analysis of substrate specificity and demonstration of PfOPPs activity by RP-TLC

The enzymatic reaction, the acid hydrolyses and TLC were performed as described in the Experimental section. Lanes 1 and 3, [^3H]FPP as substrate; lanes 2 and 4, [^3H]GGPP as substrate; lane 5, negative control reaction without enzymes ([^3H]FPP as substrate). Lanes 1 and 2, TPfOPPs; lanes 3 and 4, native PfOPPs. The positions of various prenol plus geranylgeraniol and farnesol standards are indicated on the left.

polypeptide (Figure 2, lane 1), and also supporting the preference of the PfOPPs for FPP as the main substrate. No band was detected when geranyl pyrophosphate was used in the assay instead of FPP (results not shown). Importantly, the dephosphorylation procedure causes a loss of short-chain isoprenoids, since the non-elongated, radioactive substrate GGPP was not present even in lanes 2 and 4 of the RP-TLC (co-migrating with the standard geranylgeraniol).

Identification by RP-HPLC and MS of the first intermediate product of PfOPPs catalysis

In the next step, we characterized early intermediates of the syntheses reactions. To this end, enzymatic reactions were performed with $10\ \mu\text{M}$ [$1\text{-}^{14}\text{C}$]IPP and $50\ \mu\text{M}$ FPP in the presence of TPfOPPs (Figure 3A1) or native PfOPPs (Figure 3A2). The products produced by both versions of PfOPPs were then submitted to RP-HPLC purification. After extraction and RP-HPLC analysis, a major peak with a retention time of 23 min, coinciding with standard GGPP, was observed for the labelled products. To confirm that FPP was the substrate for OPPs, we conducted the same reaction with geranyl pyrophosphate as substrate. Under these conditions no peak was detected at this retention time for either TPfOPPs or native PfOPPs (results not shown), confirming the RP-TLC results (Figure 2).

In order to identify the molecules with a retention time of 23 min in the RP-HPLC analysis, we conducted the same enzymatic reaction with non-radioactive substrates (IPP and FPP) in the presence of TPfOPPs, and analysed the products by ESI-Q-TOF-MS. As indicated by the great similarity between the ESI tandem mass spectra shown in Figures 3(B1) and 3(B2), the eluting compound was confirmed to be GGPP. As seen in both spectra, deprotonated GGPP of m/z 449.5 dissociated upon 35 eV energy collisions mainly by processes driven by the pyrophosphate group (Figure 3B3). Under the same conditions, the MS/MS spectrum of GGPP presented the same fragments (Figure 3B1), confirming its identity with the standards (Figure 3B2).

Identification by RP-HPLC and MS of the production of polyisoprenoids of $\geq \text{C}_{40}$ by PfOPPs

To determine if PfOPPs synthesizes products of C_{40} or larger, assays were carried out with $10\ \mu\text{M}$ [$1\text{-}^{14}\text{C}$]IPP and $50\ \mu\text{M}$ FPP

in the presence of TPfOPPs (Figure 4A1) or partially purified native PfOPPs (Figure 4A2). The products were analysed in their dephosphorylated forms. Three major peaks with retention times of 25, 26 and 30 min coincided with the authentic C_{40} , C_{45} and C_{55} polyisoprenoid standards respectively. Peaks with retention times between 5 and 20 min may correspond to smaller polyisoprenoids, and were not characterized further. The structures of the purified compounds detected as peaks 1, 2 and 3 were then investigated by MS. The ESI mass and tandem mass spectra shown in Figures 4(B1) and 4(B2) reveal the presence of a C_{40} polyisoprenoid (monoisotopic mass 562.7 Da), represented by the major single-charged lithium adduct ($[M + \text{Li}]^+$) ion species at m/z 569.4 in reactions with both TPfOPPs (Figure 4B1-II) and native PfOPPs (Figure 4B1-III). This same ion species was detected when the authentic C_{40} polyisoprenoid standard (Figure 4B1-I) was analysed. The molecular identity was confirmed by comparing the ESI-MS/MS spectrum of the ion of m/z 568.9 $[M + \text{Li}]^+$ produced by TPfOPPs (Figure 4B2-II) and native PfOPPs (Figure 4B2-III) with the ESI-MS/MS spectrum of the standard (Figure 4B2-I), revealing the same dissociation profile, with a major fragment ion at m/z 551.2 $[M + \text{Li} - \text{H}_2\text{O}]^+$. Accordingly, Figures 4(C1) and 4(C2) show the presence of a C_{45} polyisoprenoid (monoisotopic mass 631.0 Da), represented by the major single-charged lithium adduct ($[M + \text{Li}]^+$) ion species at m/z 637.6 in reactions with TPfOPPs (Figure 4C1-II). This same ion species was detected when the authentic C_{45} polyisoprenoid standard (Figure 4C1-I) was analysed. The molecular identity was confirmed by comparing the ESI-MS/MS spectrum of the ion of m/z 637.3 of $[M + \text{Li}]^+$ from the TPfOPPs reaction (Figure 4C2-II) with the ESI-MS/MS spectrum of the standard (Figure 4C2-I), revealing the same dissociation profile with the major fragment ion of m/z 619.4 $[M + \text{Li} - \text{H}_2\text{O}]^+$. Probably due to the low quantities of the C_{45} polyisoprenoid in the reaction with native PfOPPs, we were unable to detect this molecule.

Similarly, in Figures 4(D1) and 4(D2) the presence of a C_{55} polyisoprenoid (monoisotopic mass 767.2 Da) is shown, represented by the peak of m/z 773.2 $[M + \text{Li}]^+$ for the reactions with both TPfOPPs (Figure 4D1-II) and PfOPPs (Figure 4D1-III), as well as the similar ionization of an authentic polyisoprenoid C_{55} standard (Figure 4D1-I). The molecular identity was confirmed by comparison of the MS/MS spectrum of the ion of m/z 773.4 of $[M + \text{Li}]^+$ of TPfOPPs (Figure 4D2-II) and PfOPPs (Figure 4D2-III) with the MS/MS spectrum of the standard (Figure 4D2-I), revealing the same dissociation in the major fragment ion of m/z 755.6 $[M + \text{Li} - \text{H}_2\text{O}]^+$ and another minor ion of m/z 743.5 $[M + \text{Li} - 30]^+$.

Kinetic parameters of TPfOPPs and native PfOPPs

To characterize the enzymatic activity of TPfOPPs and partially purified native PfOPPs, we conducted kinetic characterization and measured the products of reactions catalysed by the two enzymes. Addition of TPfOPPs or PfOPPs to reaction mixtures containing [$1\text{-}^{14}\text{C}$]IPP and FPP led to the formation of polyisoprenoids, whereas without this addition no product was observed.

The Lineweaver–Burk plot in Figure 5 shows the similarity between the Michaelis constants for TPfOPPs (IPP, $2.4 \pm 0.2\ \mu\text{M}$; FPP, $1.1 \pm 0.1\ \mu\text{M}$) and PfOPPs (IPP, $3.1 \pm 0.4\ \mu\text{M}$; FPP, $2.9 \pm 0.7\ \mu\text{M}$). Given the kinetic similarity between TPfOPPs and native PfOPPs, subsequent inhibition assays were conducted only with the recombinant version.

Inhibition of PfOPPs by the terpene nerolidol

To elucidate if PfOPPs can be inhibited by allylic isoprene analogues, we conducted enzymatic reactions using FPP and

Figure 3 Identification by RP-HPLC and MS of the first intermediates in the reaction catalysed by PfOPPs

HPLC was used to purify the first product (GGPP) in the reaction catalysed by TPfOPPs (**A1**) and partially purified native PfOPPs (**A2**). GGPP, detected as a peak with the retention time of 23 min in the RP-HPLC analysis, was then characterized by Q-TOF MS. The ESI mass spectrum (**B1**) confirms the detection of the polyisoprenoid GGPP (as the deprotonated molecule of m/z 449.5) as the first intermediate in reactions catalysed by TPfOPPs. As shown by ESI tandem mass spectra (**B1** and **B2** for the standard), GGPP dissociates upon 35 eV collisions by routes driven by the pyrophosphate group (**B3**).

[1- 14 C]IPP as substrates in the presence of 10, 50 and 100 nM nerolidol. The products of these reactions were then analysed by HPLC and β -scintillation counting. In a parallel reaction, FTS was used as a control at concentrations of 7 μ M (the *in vivo* IC₅₀ value), 14 μ M and 21 μ M (results not shown). Catalysis by TPfOPPs in the presence of 200 μ M [1- 14 C]IPP and 1 μ M FPP was inhibited up to 68 % when 100 nM nerolidol was added. A maximal inhibition of 57 % was observed at an inhibitor concentration of 50 nM, whereas when nerolidol was present at 10 nM, no consistent inhibition was observed (results not shown). The K_i of nerolidol, as calculated from Lineweaver–Burk plots, was 15 ± 6 nM (Figures 6A and 6B) and the type of inhibition was clearly competitive within this inhibitor concentration range.

DISCUSSION

Due to their absence in humans, the enzymes of the 2-C-methyl-D-erythritol-4-phosphate pathway of isoprenoid precursor biosynthesis have been identified as an interesting target for the development of novel antibiotics, as well as antimalarial and herbicidal agents. In view of the differences observed in the length

of the isoprenoid side-chain compounds encountered in humans and the malaria parasite *Plasmodium falciparum*, we asked if the enzymes that finally lead to the synthesis of medium- and/or long-chain isoprenoids could also be exploited as drug targets. Herein, we demonstrate the existence of an OPPs in *P. falciparum*. This is the first report that directly demonstrates an OPPs activity in parasitic protozoa and the inhibition of this activity by the terpene nerolidol.

The deduced amino acid sequences of PfOPPs and several *trans*-prenyltransferases showed some amino acid sequence similarity, with the presence of two common DDXXD motifs that are characteristic of OPPs activity among various organisms. There was significant divergence in N-terminal regions, which are assumed to be signal sequences responsible for different subcellular localizations [20].

The first DDXXD motif is responsible for binding of and interaction with FPP, and the second motif for IPP binding [15,38]. In the archaeon *T. maritima*, several key amino acids were identified, on the basis of the conformational structure of OPPs, that play critical roles in regulating the chain length of the final products. These include Ala-76 and Ser-77 for C₂₀ products. Phe-132 was

Figure 4 Identification by RP-HPLC and MS of polyisoprenoids of C_{40} , C_{45} and C_{55} formed in the reaction catalysed by PfOPPs

RP-HPLC was used to purify the dephosphorylated products (C_{40} , C_{45} and C_{55}) of the reactions catalysed by TPfOPPs (**A1**) and partially purified native PfOPPs (**A2**). Arrows indicate the elution positions of authentic isoprenoid standards: 1, C_{40} ; 2, C_{45} ; 3, C_{55} . ESI ion-trap MS was then used to analyse the dephosphorylated polyisoprenoid products (C_{40} , C_{45} and C_{55}). (**B1**, **B2**) ESI mass spectra show a C_{40} polyisoprenoid as a major singly charged $[M + Li]^+$ ion of m/z 568.3 in both the TPfOPPs (**B1-II**) and native PfOPPs (**B1-III**) reactions, as for the C_{40} polyisoprenoid standard (**B1-I**). The molecular structure of this product was confirmed by comparison of the tandem mass spectrum of the $[M + Li]^+$ ions of m/z 568.9 formed in both the TPfOPPs (**B2-II**) and native PfOPPs (**B2-III**) reactions with that of the standard (**B2-I**). The major fragment ion in these spectra is $[M + Li - H_2O]^+$ of m/z 551.2. Panels (**C1**) and (**C2**) show the presence of a C_{45} polyisoprenoid, represented by the major singly charged lithium adduct ($[M + Li]^+$) ion species at m/z 637.6 in reactions with TPfOPPs (**C1-II**). This same ion species was detected when the authentic polyisoprenoid standard of C_{45} (**C1-I**) was analysed. The molecular identity was confirmed by comparing the ESI-MS/MS spectrum of the ion of m/z 637.3 of $[M + Li - H_2O]^+$ formed in the TPfOPPs reaction (**C2-II**) with that of the standard (**C2-I**), revealing the same dissociation profile with a major fragment ion of m/z 619.4 $[M + Li - H_2O]^+$. (**D1**, **D2**) ESI mass spectra show a C_{55} polyisoprenoid as a major singly charged $[M + Li]^+$ ion of m/z 773.2 from both the TPfOPPs (**D1-II**) and native PfOPPs (**D1-III**) reactions, as for the C_{55} polyisoprenoid standard (**D1-I**). The molecular structure of the product was confirmed by comparison of the tandem mass spectrum of the $[M + Li]^+$ ions of m/z 773.5 formed in both the TPfOPPs (**D2-II**) and native PfOPPs (**D2-III**) reactions with that of the standard (**D2-I**). The major fragment ion in these spectra is $[M + Li - H_2O]^+$ of m/z 755.6.

found to be crucial for the limited elongation of isoprenic chains to the correct C_{40} product. In the *P. falciparum* version of the enzyme, only Phe-132 seems to be conserved. Despite limited identity between *T. maritima* OPPs and PfOPPs, Phe-132 may also play this role in PfOPPs, since de Macedo et al. [10] and Rodrigues Goulart et al. [37] detected isoprenoids from C_{35} to C_{45} from parasites metabolically labelled with $[1-^{14}C]$ sodium acetate in *in vitro* cultures. Notably, the *P. falciparum* version of the protein sequence shows only 18.67% similarity with the human version, perhaps explaining the differences in isoprenic side-chain lengths between *P. falciparum* and humans.

The transcription of PfOPPs was found to occur in the ring and trophozoite stages of the parasite, and the polypeptide was mainly found in schizont-stage parasites, as detected by immunoprecipitation assays. These results are in accordance with previous transcriptome data from Le Roch et al. [39] and proteomics

data published by Nirmalan et al. [40] and Florens et al. [41]. Additionally, our group demonstrated that CoQ7–CoQ9 are biosynthesized predominantly in the trophozoite and schizont stages [10,37].

To confirm that the recombinant and native *P. falciparum* proteins possessed OPPs-like enzymatic activity, we characterized both versions. To define substrate specificity, the condensation products of the reaction of $[^3H]$ FPP with unlabelled IPP were analysed by RP-TLC, and corresponded to a C_{40} polyisoprenoid and longer polymerization products. These products were observed when using both versions of PfOPPs. Similar to the *T. maritima* and *E. coli* enzymes, no products were detected when $[^3H]$ GGPP was used as a substrate [18].

The OPPs activity was confirmed by purification of the synthesized products by RP-HPLC. When the products were analysed in their dephosphorylated forms, three major peaks,

Figure 5 Lineweaver-Burk plots of activity data for native PfOPPs and TPfOPPs with different concentrations of IPP or FPP

Intercepts at the x-axis denote similar Michaelis constants for the native and recombinant forms of the enzyme, for binding of both substrates.

Figure 6 Inhibition of TPfOPPs by nerolidol

(A) Fractional inhibition by 0, 50 and 100 nM nerolidol in the presence of different concentrations of FPP (1, 2, 5, 10 and 50 μ M). The data are shown as Lineweaver-Burk plots. (B) Replot of inhibition data. Points are experimental data, and curves were obtained by fitting the data to competitive inhibition equations (least-squares method).

corresponding to C_{40} , C_{45} and C_{55} polyisoprenoids, were detected. These results were confirmed by MS and tandem MS analysis. In this analysis we used lithium iodide for the ionization of polyisoprenoids instead of sulphates or derivatization to *t*-butyldimethylsilyl ethers [42,43], since the yield of derivatized dolichols and polyisoprenoids using the latter derivatization compounds is very low.

The atypical C_{55} product detected in enzymatic reactions may have been synthesized due to the presence of an excess of precursor molecules. Similar products were also found during *in vitro* experiments with native OPPs from *T. maritima* [19]. Again, both the recombinant and native versions of the *P. falciparum*

enzyme showed the same product spectrum of C_{40} , C_{45} and C_{55} isoprenoids.

We tried to express the complete sequence of PfOPPs in several expression vectors, but the polypeptide was not expressed, suggesting that the 3'-end of the gene contains regulatory sequences, as seen in the dihydrofolate reductase gene [44]. The portion of the polypeptide expressed as a GST-fusion protein contains the important regions for substrate binding and catalytic activity. Additionally, parameters such as the spectrum of products formed by the recombinant version showed marked similarity to that of the native, partially purified PfOPPs from schizont-stage parasites.

The chemical structure of the terpene nerolidol resembles that of the substrate FPP. Previously, our group demonstrated that *P. falciparum* parasites treated with nerolidol showed a reduced ability to synthesize CoQ in all intra-erythrocytic stages, and inhibited *P. falciparum* growth *in vitro* by decreasing the progression of the ring to the trophozoite stage [10]. This drug also suppressed *Leishmania amazonensis* promastigotes *in vitro* and intracellular amastigote growth by inhibiting the synthesis of dolichol and ergosterol [45]. In the present study, a clear competitive inhibitory action of nerolidol on PfOPPs was detected. In contrast, when we used FTS at a concentration three times higher than the IC_{50} , product formation was the same as in the control reactions without this drug. These results reinforce the specificity of PfOPPs for FPP.

Another aspect of the inhibition assay was to verify the viability of the enzyme as an antimalarial target. Several observations support this idea. First, the isoprenoid side chains attached to the benzoquinone ring in mammalian cells are longer than those in *P. falciparum*; thus the enzyme involved in side-chain synthesis must be different. Accordingly, the sequence similarity between the human decaprenyl pyrophosphate synthase and the plasmodial OPPs is very low. Secondly, the CC_{50} (concentration at which the number of cells or cell proliferation is reduced by 50 %) of nerolidol in mammalian cells was determined to be approx. 180 times higher than the IC_{50} in *P. falciparum* [45], and the free plasmodial enzyme can be inhibited efficiently by low concentrations of this terpene.

In conclusion, the present study demonstrated that *P. falciparum* has an OPPs activity. Further structural studies to compare the active sites of human and malarial enzymes may reveal possible inhibitors specific for PfOPPs.

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REFERENCES

- 1 Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. and Hay, S. I. (2005) The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* (London) **434**, 214–217.
- 2 Vial, H. J. (2000) Isoprenoid biosynthesis and drug targeting in the Apicomplexa. *Parasitol. Today* **16**, 140–141.
- 3 Chakrabarti, D., Da Silva, T., Barger, J., Paquette, S., Patel, H., Patterson, S. and Allen, C. M. (2002) Protein farnesyltransferase and protein prenylation in *Plasmodium falciparum*. *J. Biol. Chem.* **277**, 42066–42073.

- 4 Borrmann, S., Issifou, S., Esser, G., Adegnik, A. A., Ramharter, M., Matsigui, P. B., Oyakhrome, S., Mawili-Mbumba, D. P., Missinou, M. A., Kun, J. F. et al. (2004) Fosmidomycin-clindamycin for the treatment of *Plasmodium falciparum* malaria. *J. Infect. Dis.* **190**, 1534–1540
- 5 Moura, I. C., Wunderlich, G., Uhrig, M. L., Couto, A. S., Peres, V. J., Katzin, A. M. and Kimura, E. A. (2001) Limonene arrests parasite development and inhibits isoprenylation of proteins in *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* **45**, 2553–2558
- 6 Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., Turbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H. K., Soldati, D. and Beck, E. (1999) Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science* **285**, 1573–1576
- 7 Cassera, M. B., Gozzo, F. C., D'Alexandri, F. L., Merino, E. F., del Portillo, H. A., Peres, V. J., Almeida, I. C., Eberlin, M. N., Wunderlich, G., Wiesner, J. et al. (2004) The methylerythritol phosphate pathway is functionally active in all intraerythrocytic stages of *Plasmodium falciparum*. *J. Biol. Chem.* **279**, 51749–51759
- 8 Goldstein, J. L. and Brown, M. S. (1990) Regulation of the mevalonate pathway. *Nature (London)* **343**, 425–430
- 9 Couto, A. S., Kimura, E. A., Peres, V. J., Uhrig, M. L. and Katzin, A. M. (1999) Active isoprenoid pathway in the intra-erythrocytic stages of *Plasmodium falciparum*: presence of dolichols of 11 and 12 isoprene units. *Biochem. J.* **341**, 629–637
- 10 de Macedo, C. S., Uhrig, M. L., Kimura, E. A. and Katzin, A. M. (2002) Characterization of the isoprenoid chain of coenzyme Q in *Plasmodium falciparum*. *FEMS Microbiol. Lett.* **207**, 13–20
- 11 Liang, P. H., Ko, T. P. and Wang, A. H. (2002) Structure, mechanism and function of prenyltransferases. *Eur. J. Biochem.* **269**, 3339–3354
- 12 Tachibana, A., Yano, Y., Otani, S., Nomura, N., Sako, Y. and Taniguchi, M. (2000) Novel prenyltransferase gene encoding farnesylgeranyl diphosphate synthase from a hyperthermophilic archaeon, *Aeropyrum pernix*. Molecular evolution with alteration in product specificity. *Eur. J. Biochem.* **267**, 321–328
- 13 Ogura, K. and Koyama, T. (1998) Enzymatic aspects of isoprenoid chain elongation. *Chem. Rev.* **98**, 1263–1276
- 14 Danesi, R., McLellan, C. A. and Myers, C. E. (1995) Specific labeling of isoprenylated proteins: application to study inhibitors of the post-translational farnesylation and geranylgeranylation. *Biochem. Biophys. Res. Commun.* **206**, 637–643
- 15 Joly, A. and Edwards, P. A. (1993) Effect of site-directed mutagenesis of conserved aspartate and arginine residues upon farnesyl diphosphate synthase activity. *J. Biol. Chem.* **268**, 26983–26989
- 16 Song, L. and Poulter, C. D. (1994) Yeast farnesyl-diphosphate synthase: site-directed mutagenesis of residues in highly conserved prenyltransferase domains I and II. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3044–3048
- 17 Tarshis, L. C., Yan, M., Poulter, C. D. and Sacchettini, J. C. (1994) Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-Å resolution. *Biochemistry* **33**, 10871–10877
- 18 Guo, R. T., Ko, T. P., Chou, C. C., Shr, H. L., Chu, H. M., Tsai, Y. H., Liang, P. H. and Wang, A. H. (2003) Preliminary X-ray diffraction analysis of octaprenyl pyrophosphate synthase crystals from *Thermotoga maritima* and *Escherichia coli*. *Acta Crystallogr. D Biol. Crystallogr.* **59**, 2265–2268
- 19 Guo, R. T., Kuo, C. J., Chou, C. C., Ko, T. P., Shr, H. L., Liang, P. H. and Wang, A. H. (2004) Crystal structure of octaprenyl pyrophosphate synthase from hyperthermophilic *Thermotoga maritima* and mechanism of product chain length determination. *J. Biol. Chem.* **279**, 4903–4912
- 20 Okada, K., Suzuki, K., Kamiya, Y., Zhu, X., Fujisaki, S., Nishimura, Y., Nishino, T., Nakagawa, T., Kawamukai, M. and Matsuda, H. (1996) Polyprenyl diphosphate synthase essentially defines the length of the side chain of ubiquinone. *Biochim. Biophys. Acta* **1302**, 217–223
- 21 Okada, K., Kamiya, Y., Zhu, X., Suzuki, K., Tanaka, K., Nakagawa, T., Matsuda, H. and Kawamukai, M. (1997) Cloning of the sdsA gene encoding solanesyl diphosphate synthase from *Rhodobacter capsulatus* and its functional expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *J. Bacteriol.* **179**, 5992–5998
- 22 Ashby, M. N. and Edwards, P. A. (1990) Elucidation of the deficiency in two yeast coenzyme Q mutants. Characterization of the structural gene encoding hexaprenyl pyrophosphate synthetase. *J. Biol. Chem.* **265**, 13157–13164
- 23 Teclebrhan, H., Olsson, J., Swiezewska, E. and Dallner, G. (1993) Biosynthesis of the side chain of ubiquinone:trans-prenyltransferase in rat liver microsomes. *J. Biol. Chem.* **268**, 23081–23086
- 24 Trager, W. and Jensen, J. B. (1976) Human malaria parasites in continuous culture. *Science* **193**, 673–675
- 25 Braun-Breton, C., Jendoubi, M., Brunet, E., Perrin, L., Scaife, J. and Pereira da Silva, L. (1986) *In vivo* time course of synthesis and processing of major schizont membrane polypeptides in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **20**, 33–43
- 26 Cui, L., Rzomp, K. A., Fan, Q., Martin, S. K. and Williams, J. (2001) *Plasmodium falciparum*: differential display analysis of gene expression during gametocytogenesis. *Exp. Parasitol.* **99**, 244–254
- 27 Snounou, G., Viriyakosol, S., Zhu, X. P., Jarra, W., Pinheiro, L., do Rosario, V. E., Thaitong, S. and Brown, K. N. (1993) High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol. Biochem. Parasitol.* **61**, 315–320
- 28 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 29 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 30 Kessler, S. W. (1975) Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**, 1617–1624
- 31 Pan, J. J., Kuo, T. H., Chen, Y. K., Yang, L. W. and Liang, P. H. (2002) Insight into the activation mechanism of *Escherichia coli* octaprenyl pyrophosphate synthase derived from pre-steady-state kinetic analysis. *Biochim. Biophys. Acta* **1549**, 64–73
- 32 Hirooka, K., Bamba, T., Fukusaki, E. and Kobayashi, A. (2003) Cloning and kinetic characterization of *Arabidopsis thaliana* solanesyl diphosphate synthase. *Biochem. J.* **370**, 679–686
- 33 Sagami, H., Ishii, K. and Ogura, K. (1985) Geranylgeranylpyrophosphate synthetase of pig liver. *Methods Enzymol.* **110**, 184–188
- 34 Zhang, D. and Poulter, C. D. (1993) Analysis and purification of phosphorylated isoprenoids by reversed-phase HPLC. *Anal. Biochem.* **213**, 356–361
- 35 Fujii, H., Koyama, T. and Ogura, K. (1982) Hexaprenyl pyrophosphate synthetase from *Micrococcus luteus* B-P 26. Separation of two essential components. *J. Biol. Chem.* **257**, 14610–14612
- 36 Low, P., Dallner, G., Mayor, S., Cohen, S., Chait, B. T. and Menon, A. K. (1991) The mevalonate pathway in the bloodstream form of *Trypanosoma brucei*. Identification of dolichols containing 11 and 12 isoprene residues. *J. Biol. Chem.* **266**, 19250–19257
- 37 Rodrigues Goulart, H., Kimura, E. A., Peres, V. J., Couto, A. S., Aquino Duarte, F. A. and Katzin, A. M. (2004) Terpenes arrest parasite development and inhibit biosynthesis of isoprenoids in *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* **48**, 2502–2509
- 38 Marrero, P. F., Poulter, C. D. and Edwards, P. A. (1992) Effects of site-directed mutagenesis of the highly conserved aspartate residues in domain II of farnesyl diphosphate synthase activity. *J. Biol. Chem.* **267**, 21873–21878
- 39 Le Roch, K. G., Zhou, Y., Blair, P. L., Grainger, M., Moch, J. K., Haynes, J. D., De La Vega, P., Holder, A. A., Batalov, S., Carucci, D. J. and Winzler, E. A. (2003) Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* **301**, 1503–1508
- 40 Nirmalan, N., Sims, P. F. and Hyde, J. E. (2004) Quantitative proteomics of the human malaria parasite *Plasmodium falciparum* and its application to studies of development and inhibition. *Mol. Microbiol.* **52**, 1187–1199
- 41 Florens, L., Liu, X., Wang, Y., Yang, S., Schwartz, O., Peglar, M., Carucci, D. J., Yates, III, J. R. and Wub, Y. (2004) Proteomics approach reveals novel proteins on the surface of malaria-infected erythrocytes. *Mol. Biochem. Parasitol.* **135**, 1–11
- 42 Griffiths, W. J., Hjertman, M., Wejde, J., Sjövall, J. and Larsson, O. (1996) Analysis of dolichol and polyprenols and their derivatives by electron ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **10**, 663–675
- 43 Hsu, F. F. and Turk, J. (2001) Structural determination of glycosphingolipids as lithiated adducts by electrospray ionization mass spectrometry using low-energy collisional-activated dissociation on a triple stage quadrupole instrument. *J. Am. Soc. Mass Spectrom.* **12**, 61–79
- 44 Rathod, P. K., Ganesan, K., Hayward, R. E., Bozdech, Z. and DeRisi, J. L. (2002) DNA microarrays for malaria. *Trends Parasitol.* **18**, 39–45
- 45 Arruda, D. C., D'Alexandri, F. L., Katzin, A. M. and Uliana, S. R. (2005) Antileishmanial activity of the terpene nerolidol. *Antimicrob. Agents Chemother.* **49**, 1679–1687

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