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In Vivo Antimalarial Activity and Mechanisms of Action of 4-Nerolidylcatechol Derivatives

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4-Nerolidylcatechol (1) is an abundant antiplasmodial metabolite that is isolated from *Piper peltatum* roots. *O*-Acylation or *O*-alkylation of compound 1 provides derivatives exhibiting improved stability and significant *in vitro* antiplasmodial activity. The aim of this work was to study the *in vitro* inhibition of hemozoin formation, inhibition of isoprenoid biosynthesis in *Plasmodium falciparum* cultures, and *in vivo* antimalarial activity of several 4-nerolidylcatechol derivatives. 1,2-*O*,*O*-Diacetyl-4-nerolidylcatechol (2) inhibited *in vitro* hemozoin formation by up to 50%. In metabolic labeling studies using [1-(*n*)-^{3}H]geranylgeranyl pyrophosphate, diester 2 significantly inhibited the biosynthesis of isoprenoid metabolites ubiquinone 8, menaquinone 4, and dolichol 12 in cultures of *P. falciparum* 3D7. Similarly, 2-*O*-benzyl-4-nerolidylcatechol (3) significantly inhibited the biosynthesis of dolichol 12. *P. falciparum in vitro* protein synthesis was not affected by compounds 2 or 3. At oral doses of 50 mg per kg of body weight per day, compound 2 suppressed *Plasmodium berghei* NK65 in infected BALB/c mice by 44%. This *in vivo* result for derivative 2 represents marked improvement over that obtained previously for natural product 1. Compound 2 was not detected in mouse blood 1 h after oral ingestion or in mixtures with mouse blood/blood plasma *in vitro*. However, it was detected after *in vitro* contact with human blood or blood plasma. Derivatives of 4-nerolidylcatechol exhibit parasite-specific modes of action, such as inhibition of isoprenoid biosynthesis and inhibition of hemozoin formation, and they therefore merit further investigation for their antimalarial potential.

Despite large investments of resources and scientific advances in molecular, cellular, and clinical research on malaria, clinically effective vaccines are still far from being available as tools for the control and eradication of malaria. Early diagnosis, case management, and especially drug-based therapy are important tools for the control of this disease (1). In 2013, an estimated 198 million cases of malaria were reported, and 584,000 deaths were attributed to this disease worldwide. Today, morbidity and mortality due to malaria remain at unacceptable levels, and great challenges must be surmounted in order to attain global targets set for malaria control (2).

The control of malaria has become gradually more complex due to the spread of *Plasmodium* spp. that are resistant to the antimalarials presently used in therapy (3). In recent years, *Plasmodium falciparum* resistance to artemisinin and its derivatives has been detected in four southeast Asian countries (2). *Plasmodium vivax* is the most important malaria parasite outside the African continent, and *in vivo* resistance and *in vitro* resistance of this parasite to chloroquine have been the subject of a growing number of recent reports (4, 5). Thus, new chemical entities that may overcome the mechanisms of resistance and offer significant advances over existing drug regimens are in urgent demand.

The sequencing of the *P. falciparum* and *P. vivax* genomes has led to the identification of a growing list of potential drug targets (6). A number of molecular targets are associated with the distinct functions of different organelles present in the asexual blood phases of *Plasmodium* spp. The parasite digestive vacuole and the apicoplast are among the important organelles, due to the intensities and specificities of their metabolic activities, which are absent in human beings, thus making the metabolic processes in these organelles interesting targets for antimalarial drugs (3).

The digestive vacuole is the organelle wherein the intense degradation of erythrocyte hemoglobin occurs, thus providing *Plasmodium* spp. with amino acids for protein synthesis. A by-product of hemoglobin digestion is heme, which is toxic to the parasite. Heme polymerizes rapidly forming hemozoin (malaria pigment) in the interior of the digestive vacuole (7). The inhibition of hemozoin formation is considered to be one of the main mechanisms of action of several antimalarial drugs in clinical use, such as chloroquine and artemisinin (8, 9).

The apicoplast is an organelle similar to the plastid and is pres-

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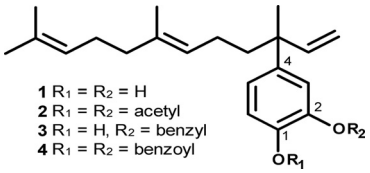

FIG 1 Structures of 4-nerolidylcatechol (**1**) and semisynthetic derivatives **2** to **4**.

ent in species of the phylum *Apicomplexa*, such as *Plasmodium* spp. In the apicoplast, unique metabolic pathways are present (biosynthesis of fatty acids, isoprenoids, iron-sulfur clusters, and heme) (10). One of the main metabolic activities of the apicoplast is the synthesis of isoprenoid compounds. In mammals, C_5 isoprene (hemi-terpene) precursors to isoprenoids are synthesized by the mevalonate pathway (MVA). *Plasmodium* spp. use the non-MVA (NMVA), or methylerythritol phosphate (MEP), pathway to synthesize these five-carbon isoprenoid precursors (11). Numerous intermediates and enzymes of this pathway have been characterized, and light has been shed on the physiological importance of the isoprenoid pathway and role of biosynthesized isoprenoid metabolites in *Plasmodium* survival (12–15). The NMVA (MEP) pathway is absent in humans and essential to *Plasmodium* survival. It is thus a potential source of targets for the development of novel antimalarials (16).

The apicoplast and digestive vacuole are distinct cellular compartments (organelles). Their physiological processes are targeted by different drugs. However, fosmidomycin exhibits dual mechanistic actions against *P. falciparum*. This compound inhibits the biosynthesis of isoprenoids in *Plasmodium* spp. and interferes in the prenylation of proteins of the digestive vacuole, thus affecting the overall process of hemozoin formation in the parasite (17).

Plant terpenes exhibiting *in vitro* antiplasmodial activity have been evaluated in *P. falciparum* to establish whether they may exert effects on the isoprenoid biosynthetic pathway. Thus, nerolidol causes total inhibition of *P. falciparum* trophozoite development at the schizont stage, and *in vitro* 50% inhibitory concentrations (IC_{50}s) in the range of 760 ± 23 nM (mean ± standard deviation) have been reported for nerolidol against *P. falciparum*. Terpenoid compounds like nerolidol, farnesol, and linalool have been shown to strongly inhibit the biosynthesis of both dolichol and the isoprene side chain of ubiquinones. Also, terpenes can inhibit the isoprenylation of proteins in the intraerythrocytic stages of *P. falciparum* in a specific manner that does not affect overall protein biosynthesis (18).

Traditionally used plants are sources of important antimalarial compounds, such as quinine and artemisinin and other secondary metabolites exhibiting potential as antimalarials (19, 20). The Amazon region, where malaria is endemic, is a rich source of antimalarial substances obtained from traditionally used antimalarial plants (21). 4-Nerolidylcatechol (**1**) is a major secondary metabolite of mixed terpene-phenylpropanoid biosynthetic origins found in the roots of *Piper peltatum*, a traditionally used antimalarial shrub from the Amazon region (Fig. 1). *P. peltatum* has been domesticated, and its roots (containing ca. 5% [wt/wt] compound **1**) can yield an estimated 27 kg of compound **1** per hectare. Thus, this compound is potentially available for large-scale applications (22, 23). It exhibits *in vitro* activity against the chloroquine-pyrimethamine-cycloguanil-resistant K1 strain of *P. falciparum* (IC_{50} = 0.67 μM) (24) and low oral and subcutaneous activity against *Plasmodium berghei* in mice (maximal effect was 63% inhibition at oral doses of 600 mg per kg of body weight per day) (25). Also, deactivated plasma taken from healthy mice that had orally ingested this compound exhibited high *in vitro* inhibition of *P. falciparum*, thus providing evidence for the presence of antiplasmodial metabolites in mouse blood as a result of oral ingestion of compound **1**.

Natural product **1** is unstable under ambient conditions or in a freezer. Mono- and di-*O*-alkyl and di-*O*-acyl derivatives of this compound have been introduced and exhibit improved chemical stability. These derivatives exhibit a range of *in vitro* inhibitory activities against the K1 strain of *Plasmodium falciparum* (IC_{50} = 0.67 to 23 μM) (26, 27), antioxidant activity comparable to food preservatives BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), and low toxicity to normal cells (28).

Derivatives of compound **1** exhibit catechol structures that can adopt planar conformations that *a priori* could make these derivatives excellent binders of heme (inhibitors of hemozoin formation). Also, like nerolidol, farnesol, and linalool and other terpenes that inhibit terpenoid synthesis in *P. falciparum*, derivatives of compound **1** have a linear terpenyl (nerolidyl) side chain that *a priori* could make these compounds promising inhibitors of isoprenoid synthesis in *Plasmodium* spp.

The aim of this work was to investigate the potential *in vivo* antimalarial activity of 4-nerolidylcatechol (**1**) derivatives and explore two independent mechanisms of possible antiplasmodial action of these compounds. Herein, derivatives **2** to **4** (Fig. 1) were assayed for (i) inhibition of *in vitro* hemozoin formation, (ii) *in vitro* inhibition of the biosynthesis of isoprenoid metabolites in *P. falciparum* cultures, and (iii) *in vivo* antimalarial activity in *P. berghei*-infected mice. The acute oral toxicity and presence of compound **2** in mouse blood after oral ingestion were investigated along with the *in vitro* recovery of this compound from human and mouse blood and plasma.

MATERIALS AND METHODS

Chemical substances. Natural product **1** makes up 5% or more of the dry weight of the mature roots of *P. peltatum* and was obtained by extraction of the dry, ground roots with chloroform and ethanol (1:1), evaporation of the solvents, and column chromatography on the resulting extract as previously described (25). The three semisynthetic derivatives **2** to **4** studied herein were synthesized from freshly purified compound **1** in straightforward synthetic procedures that have been published previously (26, 29). Briefly, 1,2-*O*,*O*-diacetyl-4-nerolidylcatechol (**2**) was prepared by diacetylation of compound **1** in acetic anhydride/pyridine. 2-*O*-Benzyl-4-nerolidylcatechol (**3**) was obtained by benzylation with benzyl bromide, and 1,2-*O*,*O*-dibenzoyl-4-nerolidylcatechol (**4**) was obtained by reaction with benzoyl chloride. After each of these reactions, column chromatography and preparative thin-layer chromatography were used for isolation and purification of the products whose structures were determined based on nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) techniques. The purities of the products were evaluated by thin-layer chromatography and liquid chromatography coupled to mass spectrometry, and they were considered to be >95% (26). The commercial drug standards chloroquine diphosphate (Sigma-Aldrich, Steinheim, Westphalia, Germany) and quinine sulfate (Sigma-Aldrich, Steinheim, Westphalia, Germany) were used as controls in the biological tests.

***In vitro* culture of *Plasmodium falciparum*.** The *in vitro* culture experiments were performed using the 3D7 clone of the NF54 strain (chloroquine sensitive) and the K1 *P. falciparum* strain. Parasites were cultured using the Trager and Jensen method (30) with modifications (24). The parasites were cultured at 37°C under a low-oxygen atmosphere (5% ox-

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ygen, 5% carbon dioxide, and the remainder nitrogen) in A+-type erythrocytes at a hematocrit of 3 to 5%. Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma-Aldrich) supplemented with 0.5% Albumax I (Gibco) was used. Synchronized ring-phase cultures were obtained by two consecutive treatments at intervals of 48 h with a 5% (wt/vol) solution of D-sorbitol (Sigma-Aldrich) as described by Lambros and Vanderberg (31). The development and growth of parasites were analyzed on smears of cultures stained with Panótico (Laborclin, Pinhais, Paraná, Brazil).

***In vitro* inhibition test.** A microtest was performed using the method introduced by Rieckmann et al. (32) with modifications (24). Briefly, 20 mM stock solutions of compounds **1** to **4** were initially prepared in dimethyl sulfoxide (DMSO) and then serially diluted (1:3) in culture medium (RPMI 1640) to obtain 7 final concentrations (100 to 0.14 μM). Chloroquine and quinine drug standards (7 final concentrations, 2.0 to 3.4×10^{-3} μM) were used as controls. Each sample of diluted compound was tested in triplicate in a 96-well plate containing a suspension of ring-stage (synchronized) parasitized red blood cells (pRBCs) with a hematocrit of 2% and 1% initial parasitemia. The final volume in each well was 200 μl. Wells containing pRBCs in culture medium and 2% DMSO were used as controls of parasite growth. The plates were incubated for 48 h at 37°C under the culture conditions described above. After incubation, smears of the contents of each well were prepared on microscope plates and colored with Panótico and examined using an optical microscope. The number of pRBCs present in a total of 2,000 red blood cells was counted. The parasitemia was expressed as a percentage. The half-maximal concentration (IC_{50}) responses were calculated using Origin 8.1 software (Origin Lab). All tests were performed in triplicate in a total of three independent experiments. One-way statistical analysis of variance (ANOVA) was performed, followed by Dunnett's *post hoc* test (Prism; Graph Pad, CA). A P value of <0.05 was considered statistically significant.

Animals and ethical approval. Adult female BALB/c mice (22 ± 3 g of body weight) were used for the acute toxicity assay and antimalarial *in vivo* tests and received water and food *ad libitum*. *In vivo* tests were performed using Guidelines for Ethical Conduct in The Care and Use of Animals of the National Institute for Amazon Research (INPA). This work was authorized by INPA's Commission of Ethics for the Use of Animals (CEUA 062/2012).

***In vivo* suppressive test with *Plasmodium berghei*.** Evaluation of the *in vivo* antimalarial activity was performed based on the Peters 4-day suppressive test (33) with modifications (25). Briefly, 0.2 ml of infected blood suspension containing 1×10^5 *P. berghei* NK65 pRBCs was inoculated intraperitoneally in mice. The animals were randomly divided into groups of 5 individuals. Animals in test groups were treated orally or subcutaneously with compound **2** or **4** at doses of 600 to 10 mg kg^{-1} day^{-1}. Positive-control group animals were treated orally or subcutaneously with 10 mg kg^{-1} day^{-1} of chloroquine, and negative-control group animals received 0.2 ml of vehicle (2% DMSO in water). Animals were treated for 4 days starting 24 h after inoculation with *P. berghei*. Parasitemia levels were assessed by examining Giemsa-stained thin blood smears from each animal via an optical microscope on days 5 and 7. Overall mortality was monitored daily in all groups during a period of 40 days following inoculation. The difference between the average parasitemia of negative-control groups (100%) and test groups was calculated as the percentage of parasite growth suppression (PGS) according to the following equation: $PGS = 100 \times [(A - B)/A]$, where A is the average parasitemia of the negative-control group and B corresponds to the parasitemia of the test group. Each sample was tested in 3 independent experiments. For comparisons of average parasitemia at different time points, analysis of variance was performed with a *post hoc* Mann-Whitney test for comparison of the means with Microcal Origin 8.1 software (Origin Lab, Northampton, MA).

***In vivo* acute toxicity assay.** Acute toxicity of 1,2-*O*,*O*-diacetyl-4-nerolidylcatechol (**2**) was determined in healthy mice based on the Organization for Economic Cooperation and Development (OECD) *Guidelines for the Testing of Chemicals for Acute Oral Toxicity* (34). Briefly, this involved gavage administration of compound **2** at doses of up to 2,000 mg kg^{-1} in groups of three mice. Compound **2** was diluted in 2% DMSO–distilled water solution and administered in a single 200-μl dose. The negative-control group received 200 μl of 2% DMSO solution. Animals were observed individually during the first 30 min and periodically over 24 h. Special attention was paid over the first 4 h and daily thereafter for 14 days.

Inhibition of hemozoin formation assay. The hemozoin formation inhibition assay was performed as described by Ncokazi and Egan (35) with modifications (36). Briefly, solutions with different concentrations (20 to 0.156 mg ml^{-1}) of compounds **2** and **4** were transferred (20 μl) in triplicate to a 96-well oval-bottomed plate. Next, bovine hematin (Sigma-Aldrich, Germany) solution (101 μl; 1.68 mM in 0.1 M sodium hydroxide) was added to each well followed by addition of pH 5 sodium acetate buffer (12 M; 58 μl) with constant stirring at 60°C. After incubation at 60°C for 60 min, the plate was centrifuged at 500 × *g* for 8 min. The supernatant was discarded and the crystals of hemozoin were redissolved in 200 μl of 0.1 M sodium hydroxide and transferred to a 96 flat-bottomed well plate. The reaction was monitored on a spectrophotometer (Spectra Max 340 PC384; Molecular Devices) at 405 nm, and the results were expressed as the percent inhibition of hemozoin formation. The optical density of the untreated controls corresponded to 100% hemozoin formation. Chloroquine was used as a positive control. Each substance was tested in three independent experiments.

Treatment of *P. falciparum* with compounds 2 and 3 and metabolic labeling. To evaluate the effects of compounds 2 and 3 on the biosynthesis of isoprenoids in *P. falciparum*, a protocol described by Rodrigues Goulart et al. (18) was used. Synchronized cultures of young trophozoites (ring form) of *P. falciparum* 3D7 exhibiting parasitemia of ca. 10% were treated or not treated for 48 h with compound **2** or **3** at a final concentration of 4 μM and metabolically labeled with 3.1 μCi ml^{-1} of [1-(*n*)-^{3}H]geranylgeranyl pyrophosphate triammonium salt {[1-(*n*)-^{3}H]GGPP; 16.5 μCi $mmol^{-1}$; Amersham} during the last 18 h. The *P. falciparum* schizonts obtained after the incubation period were purified using a discontinuous Percoll gradient (37). The schizonts (80 μl) were freeze-dried, and then lipid extraction was performed with hexane (three times, 0.5 ml). The extracts were combined and dried under a stream of nitrogen and resuspended in 500 μl of hexane. Each extract was divided into 2 aliquots for high-performance liquid chromatography (HPLC) analyses of dolichol 12 or isoprene chains linked to coenzyme Q as described below. In all experiments, the same quantities of treated and untreated parasites were analyzed.

Reverse-phase HPLC (RP-HPLC). Aliquots of the hexane extracts of treated and untreated schizonts were monitored for radioactivity. Samples of hexane extracts were suspended in 250 μl of methanol and analyzed by HPLC using a Phenomenex Luna C_{18} column (250 by 4.6 mm), Gilson HPLC 322 pump, and Gilson 152 variable UV-visible detector. Purified fractions were obtained on a Gilson fraction collector FC203B, and UNIPOINT System software was used to analyze chromatograms.

For analysis of dolichol 12, the following gradient elution system was used at a flow rate of 1.5 ml min^{-1}: 9:1 methanol/water (solvent A) and 1:1:2 hexane/isopropanol/methanol (solvent B) were mixed in a linear gradient from 5 to 100% solvent B over 25 min. The column was further eluted for 5 min with 100% solvent B. The eluent was monitored at 210 nm. Fractions were each collected for 0.5 min (0.75 ml). The mobile phase was evaporated, scintillation liquid was added, and radioactivity was evaluated on a Beckman 5000β-radiation scintillation apparatus. Polyprenols were coinjected as standards in the same HPLC elution and fraction-collecting procedure (18).

For analysis of ubiquinones, the hexane extracts of treated and untreated schizonts were dried and resuspended in methanol and coinjected with Q_8 (ubiquinone with eight isoprene units) and menaquinone standards. An isocratic methanol/ethanol (1:1) elution system was used at a

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flow rate of 1.0 ml min^{-1} with fractions being collected at intervals of 0.5 min. The detector was operated at 275 nm. After evaporation of the mobile phase, scintillation liquid was added to each purified chromatographic fraction, and the fractions were analyzed for radioactivity by using a Beckman 5000β-radiation scintillation apparatus (15).

Data analysis. Comparative statistical analysis of peak areas from HPLC chromatograms of samples treated with compounds **2** and **3** versus untreated samples were performed for both dolichol 12 and ubiquinones. After evaluating the normality of the population, Student's *t* test was applied to the data, taking as the null hypothesis (H_0) the equality of the means between control and treated populations (with determination of 95% confidence limits). The average inhibition was then estimated with a significance of 95%.

Protein inhibition assay. For the protein synthesis inhibition assay, asynchronous cultures of *Plasmodium falciparum* were treated (4 μM) or not treated for 48 h with **2** and **3** and marked with [1-^{14}C]sodium acetate (3.1 μCi ml^{-1}; 56 mCi $mmol^{-1}$; Amersham) during the last 18 h. Then, the different intraerythrocytic stages of *P. falciparum* were purified as mentioned above. After purification, each stage (ring, trophozoite, and schizont) was lysed with twice its volume of an ice-cold solution made up of 10 mM Tris-HCl (pH 7.2), 150 mM sodium chloride, 2% (vol/vol) Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, 1 mM N-(*p*-tosyllysine) chloromethyl ketone, and leupeptin (1 μg ml^{-1}). After incubation for 15 min at 4°C, the lysates were centrifuged at 10,000 × *g* for 30 min and the supernatants were stored in liquid nitrogen for further analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gel electrophoresis. SDS-PAGE was performed in 12.5% gels as described elsewhere (38). The same number of substance-treated or untreated parasites as mentioned above were dissolved in sodium dodecyl sulfate sample buffer and applied to each well for analysis. All gels were treated with Amplify (Amersham), dried, and exposed to Kodak X-Omat film with intensifying screen sets at −70°C for 60 days.

UFLC conditions for pharmacokinetic evaluation. A Shimadzu ultrafast liquid chromatography (UFLC) Prominence system (Kyoto, Japan) consisting of a binary LC-20AT gradient pump, SPDM-20A diode-array detector (DAD), and SIL-20A automatic injector system was used to analyze blood, blood plasma, compound **2**, and mixtures of these prepared as described below. A Shim-Pack XR-ODS column (50 by 2.0 mm [inner diameter], 2 μm; Shimadzu) was used at room temperature. The mobile phase consisted of 0.1% aqueous (Milli-Q) mass spectral-grade formic acid (Fluka Analytical) (A) and 0.1% formic acid in acetonitrile (LiChrosolv, Merck, Darmstadt, Germany) (B) starting at 70% B for 2 min, then a linear increase to 100% B over 8 min, 100% B for 2 min, and a linear decrease to 70% B over 4 min (column reequilibration). Total run time was 16 min. The flow rate was 0.4 ml min^{-1} with a 15% split for mass spectral analysis (see below). The injector volume was 5 μl. Mass spectral-grade isopropanol (Chromasolve; Fluka Analytical) and acetonitrile (1:1) were used in the injector (C).

Mass spectrometer conditions for pharmacokinetic evaluation. A Bruker Daltonics MicroTOF-QII mass spectrometer (Bremen, Germany) with quadrupole-time of flight (TOF) analyzer exhibiting 17,500 full width at half maximum (FWHM) resolution and multichannel detector plate was used as the detector for the UFLC analysis described above to analyze blood, blood plasma, compound **2**, and mixtures of these prepared (below). An electrospray ionization (ESI) source in positive mode was operated using the following parameters: capillary voltage, 4,500 V; end plate offset, −500 V; nebulizer pressure, 2.0 × 10^5 Pa; dry heater temperature, 180°C; dry gas flow, 6.0 liters min^{-1}. The mass range analyzed was *m/z* 100 to 1,000. UFLC-MS analyses were controlled and processed, respectively, by using a Bruker Daltronics Hystar 3.2 system and Data Analysis 4.1 software.

Blood and blood plasma interactions with compound 2. The aim of this procedure was to evaluate potential metabolic reactions between diacetyl derivative **2** and human and mouse blood and blood plasma. It was performed by adapting recovery and analytical procedures from previous pharmacokinetic studies on compound **1** and other compounds (39–41). Mouse or human blood plasma was the supernatant liquid (500 μl) obtained after centrifuging noncoagulated, sodium citrate-treated blood at 1,750 × *g* for 10 min. A stock solution (1.0 mg ml^{-1}) of compound **2** in isopropanol was prepared. This solution (10 μl) was diluted 10-fold by addition to mouse or human blood plasma (90 μl). Next, isopropanol (1,000 μl) was added, and the resulting solution was vortexed (15 min) and then centrifuged (10 min, 1,750 × *g*). The supernatant liquid was transferred to a clean microcentrifuge tube and then was completely dried under a stream of nitrogen. The residue was dissolved in isopropanol (500 μl), filtered (0.22-μm porosity), and analyzed by UFLC-MS. This procedure was performed with human type A+ blood plasma and blood plasma from a healthy mouse. As a control for each experiment (blank), a sample of plasma was treated with vehicle (without compound **2**) and was otherwise processed as above.

The procedure described above was also performed by adding compound **2** to whole (complete) mouse or human blood. A solution of compound **2** in isopropanol (40 μl, 1.0 mg ml^{-1}) was mixed with whole blood (360 μl), and after gentle manual homogenizing in a clean microcentrifuge tube (10 min), the mixture was centrifuged (10 min, 1,750 × *g*). The supernatant liquid (100 μl) was transferred to a new clean microcentrifuge tube. Isopropanol (1,000 μl) was added, and the resulting solution was vortexed (10 min) and then centrifuged (10 min, 1,750 × *g*). The supernatant liquid was transferred to a new clean microcentrifuge tube and completely dried under a stream of nitrogen. The residue was dissolved in isopropanol (500 μl), filtered (0.22-μm porosity), and analyzed by UFLC-MS (39). As a control (blank) for each experiment, a sample of complete blood was treated with vehicle (without compound **2**) and was otherwise processed as above. Three independent experiments were performed.

UFLC-HRMS analysis of mouse blood after oral administration of compound 2. To study alterations in the composition of mouse blood after oral ingestion of compound **2**, 50 and 600 mg kg^{-1} of compound **2** diluted in 2% DMSO–distilled water solution were administered to healthy mice (200 μl) by gavage. One hour after administration, mice were anesthetized with 200 μl of xylazine-ketamine (1 ml kg^{-1}), and blood (500 μl) was removed from mice by cardiac puncture and stabilized with sodium citrate (anticoagulant). The citrated blood was centrifuged (10 min, 1,750 × *g*). The plasma (100 μl) was transferred to a clean microcentrifuge tube and isopropanol (1.0 ml) was added. The mixture was vortexed (10 min) and then centrifuged (10 min, 1,750 × *g*). The supernatant liquid was transferred to another microcentrifuge tube and was completely dried under a stream of nitrogen. The residue was dissolved in isopropanol (500 μl), filtered (0.22-μm porosity), and analyzed by UFLC-HRMS. Two independent experiments were performed.

RESULTS

***In vitro* antiplasmodial activity.** IC_{50}s and standard deviations for 4-nerolidylcatechol (**1**) and derivatives **2** to **4** against *P. falciparum* K1 and 3D7 strains are summarized in Table 1. This is the first report on the *in vitro* antiplasmodial activity (IC_{50} = 4.8 and 5.5 μM against the K1 and 3D7 strains, respectively) of compound **2**. Compounds **1**, **3**, and **4** were evaluated against the 3D7 strain here for the first time. In general, derivatives **2** to **4** were less active *in vitro* than natural compound **1**. The cytotoxicity/antiplasmodial selectivity indices of compounds **1**, **3**, and **4** were calculated based on previously published cytotoxicity data against normal cells (28). The concentration (4.0 μM) of compounds **2** and **3** used to evaluate the effects on the *in vitro* incorporation of isoprene precursors into dolichols, ubiquinones, or proteins was established based on these *in vitro* inhibition results.

***In vivo* antimalarial activities.** Compounds **2** and **4** were evaluated for *in vivo* antimalarial activity using the Peter's suppression

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TABLE 1 *In vitro* IC_{50} values of 4-nerolidylcatechol (**1**) and semisynthetic derivatives **2** to **4** against *Plasmodium falciparum* K1 and 3D7 strains and selectivity indices

Compound	IC_{50}[a] (μM)			
	P. falciparum[a]		Mouse embryonic fibroblasts[b]	SI[c]
	K1	3D7		
1	0.67 ± 0.1	0.59 ± 0.2	31.5	47/53
2	4.85 ± 1.2	5.57 ± 1.0	<15.7	<3.2/2.8
3	7.05 ± 2.0	5.94 ± 1.8	>124	>17/>21
4	28.73 ± 10.3	38.07 ± 15.6	95.8	3.3/2.5
Chloroquine diphosphate	0.13 ± 0.1	0.05 ± 0.02	ND[d]	ND
Quinine sulfate	0.16 ± 0.1	0.11 ± 0.1	ND	ND

[a] IC_{50} values were calculated by probit analysis as described in Materials and Methods. Student's *t* test was applied to results. Values are expressed as means ± standard deviations ($n = 3$ experiments).
[b] Mouse embryonic fibroblasts were of the 3T3L1 line. Data in this column for compounds **1**, **3**, and **4** were previously published (28).
[c] SI, selectivity index, calculated with the formula IC_{50} (fibroblasts)/IC_{50} (*P. falciparum*) and is reported for the K1 and 3D7 strains.
[d] ND, not determined.

test. The results are presented in Table 2. At 50 mg kg^{-1} day^{-1}, a dose often used to identify potential new drugs in murine malaria (42, 43), compound **2** was considered partially active (44, 45), as it suppressed *P. berghei* growth by >30% on days 5 and 7 for both routes of administration. At the highest dose (600 mg kg^{-1} day^{-1}), subcutaneous and oral administration of compound **2** led to 72 and 64% suppression of parasitemia, respectively, on the fifth day after treatment began. Compound **2** was inactive at doses of 10 mg kg^{-1} day^{-1}. In general, compound **4** exhibited lower suppression of parasitemia than compound **2**. Thus, maximal inhibition by compound **4** was 48% on the fifth day when administered subcutaneously. A decrease in activity on the seventh day was in general observed compared to the corresponding activity on the fifth day at all doses tested and for both routes of administration. Survival times of infected animals were not significantly increased compared to untreated controls in any of the groups that were treated with compound **2** or **4**, although animals treated orally with 600 mg kg^{-1} day^{-1} of compound **2** exhibited an average survival time of 22 ± 2 days. Control groups treated with 10 mg kg^{-1} day^{-1} of chloroquine diphosphate exhibited 99 to 100% suppression of parasitemia on both days of observation and survival rates of >40 days. The median lethal dose (LD_{50}) of compound **2** was >2 g kg^{-1}, since no animal death or sign of intoxication was observed at any of the assayed doses.

Compound **2** was not detected by LC-MS in the plasma of healthy mice 1 h after administration of 50 or 600 mg kg^{-1} by gavage. This compound was also not detected by UFLC-HRMS after *in vitro* dilution (0.1 mg ml^{-1}; 0.25 mM) in whole blood or blood plasma of mice. Also, no metabolites of compound **2** were discernible by UFLC-HRMS in human or mouse blood plasma, human or mouse complete blood, or in the blood of mice 1 h after oral ingestion of compound **2**. Compound **2** was detected by UFLC-HRMS after *in vitro* dilution in human blood and human blood plasma as a chromatographic peak having a retention time of 6.3 min and characteristic $[M + H - C_2H_2O]^+$, $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$, and $[M + K]^+$ adduct/fragment ions (data not presented).

Inhibition of hemozoin formation. Figure 2 presents inhibition data for compounds **2** and **3** and chloroquine. Compound **2** inhibited hemozoin formation by 50% at a concentration of 20 mg ml^{-1} (50 mM), and inhibitory activity decreased as a function of concentration. Compound **3** exhibited low inhibition of hemozoin formation (maximum of 20% inhibition at the highest concentration tested). At concentrations of 40 mg ml^{-1} (ca. 100 mM), these compounds were insoluble and could not be tested.

TABLE 2 *In vivo* suppression of *Plasmodium berghei* parasitemia in mice after oral and subcutaneous treatment with compound **2** or **4**[a]

Compound and dose (mg/kg/day)	% parasitemia ± SEM (% inhibition of parasite growth)[b]				Avg survival time ± SD (days)	
	Oral		Subcutaneous			
	Day 5	Day 7	Day 5	Day 7	Oral	Subcutaneous
Compound **2**						
600	0.9 ± 0.3 (64)	1.2 ± 0.73 (56)	0.7 ± 0.4 (72)	0.9 ± 0.3 (70)	22 ± 2	22 ± 3
200	1.0 ± 0.5 (62)	1.43 ± 0.7 (48)	1.0 ± 0.3 (60)	1.0 ± 0.2 (66)	21 ± 3	19 ± 3
50	1.5 ± 0.6 (44)	1.9 ± 0.4 (32)	1.8 ± 0.8 (33)	1.9 ± 0.7 (37)	18 ± 4	19 ± 4
10	2.1 ± 0.6 (23)	2.4 ± 0.7 (12)	2.7 ± 0.4 (0)	3.8 ± 0.7 (0)	20 ± 3	17 ± 2
Compound **4**						
200	1.5 ± 0.6 (46)	2.4 ± 0.6 (28)	1.4 ± 0.7 (48)	2.7 ± 0.8 (17)	21 ± 3	20 ± 2
50	2.0 ± 0.7 (27)	2.5 ± 1.0 (23)	1.9 ± 0.9 (32)	3.4 ± 0.9 (0)	20 ± 2	19 ± 4
10	3.0 ± 0.8 (0)	3.4 ± 0.9 (0)	2.8 ± 0.6 (0)	3.6 ± 1.2 (0)	19 ± 1	17 ± 4
Chloroquine diphosphate						
10	0.00 (100)	0.02 (99)	0.03 (99)	0.03 (99)	>40	>40
Control	2.7 ± 0.9	3.3 ± 0.7	2.6 ± 0.6	3.0 ± 0.9	19 ± 4	21 ± 3

[a] The experiment was performed following the Peters protocol (33).
[b] The percentage (mean ± standard error of the mean) of parasitized red blood cells of a total of 5 mice in three independent experiments. The percent inhibition of parasite growth (parasitemia reduction) was compared to results for untreated control mice. The Mann-Whitney test was used to evaluate statistical differences between groups. A *P* value of <0.05 was considered statistically significant.

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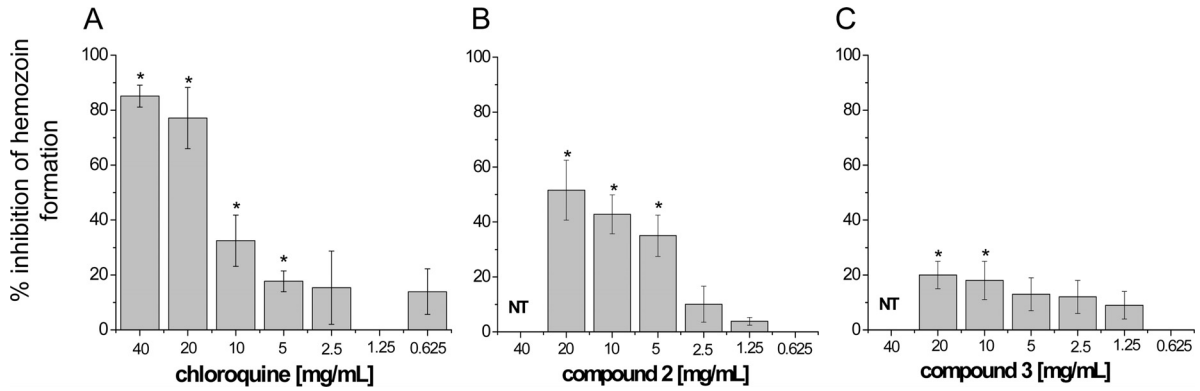

FIG 2 Inhibition of hemozoin formation by chloroquine (A), 1,2-*O*,*O*-diacetyl 4-nerolidylcatechol (compound **2**) (B), and 2-*O*-benzyl 4-nerolidylcatechol (compound **3**) (C). Significant differences compared to drug-free controls are indicated in each graph by an asterisk ($P \leq 0.05$). NT, not tested (insoluble).

Effects on the biosynthesis of isoprenoids and proteins in *P. falciparum*. The effects of compounds **2** and **3** on the biosynthesis of isoprenoids in *P. falciparum* were determined by metabolic labeling with the precursor [1-(*n*)-^{3}H]GGPP in treated parasites (with 4 μM compound **2** or **3**) or untreated parasites for 48 h. The schizont extracts were analyzed by RP-HPLC for the presence of dolichol containing 12 isoprene units and ubiquinones. Figure 3 presents the radioactivities of the fractions corresponding to the retention times of dolichol 12, ubiquinone 8, and menaquinone 4 of the hexane extract obtained from the cultures in the schizont stage. The radioactivities of fractions from the tests were compared with those of the corresponding fractions from untreated controls. The latter fractions were considered to have 100% (maximal) incorporation of the radiolabeled precursor [1-(*n*)-^{3}H]GGPP. The statistical analysis applied is described in Material and Methods.

Biosynthesis of dolichol 12 was inhibited by 41 and 45% by compounds **2** and **3**, respectively, in the schizont stage. Also, compound **2** significantly inhibited the biosynthesis of ubiquinone and menaquinone by 36 and 41%, respectively. When the cultures were treated with compound **3**, no significant difference in the biosynthesis of prenylated quinones compared to untreated controls was observed. SDS-PAGE analysis of proteins from ring stages, trophozoites, and schizonts from cultures labeled with [1-^{14}C]sodium acetate and treated or not treated with compound **2** or **3** revealed that there was no inhibition of protein synthesis by these compounds (see Fig. S1 in the supplemental material).

DISCUSSION

In previous work, natural compound **1** administered subcutaneously in mice at high doses (200 to 600 mg kg^{-1} day^{-1}) suppressed *P. berghei* by 0 and 41 to 61%, respectively, on days 5 and 7 after inoculation. Also, at oral doses of 200 to 600 mg kg^{-1} day^{-1} compound **1** suppressed *P. berghei* on the fifth and seventh days by 15 to 63 and 49 to 60%, respectively (25). The subcutaneous and oral activities observed for derivative **2** (Table 2) represent improved *in vivo* antimalarial activities compared to those reported previously for compound **1**. The suppression of parasitemia observed for compound **2** (≥30%) is indicative of partial antimalarial activity (42, 43). The development of derivatives of **1** exhibiting greater *in vivo* antimalarial activity would therefore be desirable. In this vein, it is important to keep in mind the possible limitations

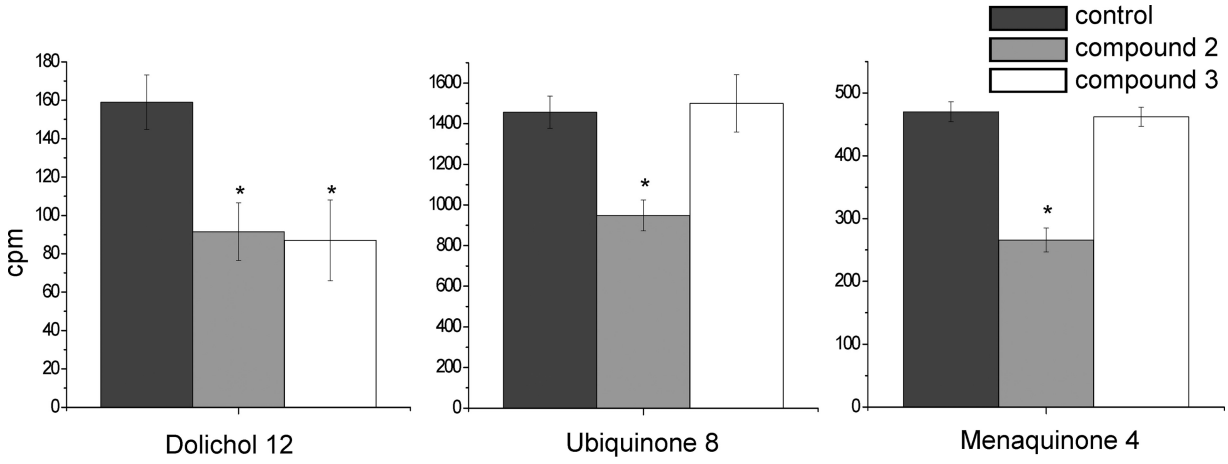

FIG 3 The radioactive peaks corresponding to the retention times of dolichol 12 (18), ubiquinone 8, and menaquinone 4 (15) from the hexane extract of the schizont stage, untreated or treated for 48 h with 4.0 μM 1,2-*O*,*O*-diacetyl 4-nerolidylcatechol (compound **2**) or 2-*O*-benzyl-4-nerolidylcatechol (compound **3**) after purification by RP-HPLC as described in Materials and Methods. Metabolic labeling was performed with [1-(*n*)-^{3}H]geranylgeranyl pyrophosphate. A one-way ANOVA was applied, and *P* values of <0.05 were considered statistically significant. An asterisk indicates a significant difference compared to untreated controls.

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of optimizing activity based on rodent malaria as a model for human malaria infections. For example, clinically viable drugs, such as quinine (against *P. berghei* ANKA, 50% effective dose [ED_{50}] = 34 mg kg^{-1} day^{-1} [46] and slow clearance [47]) and artemisinin derivatives, exemplified by artesunate (recrudescence below 80 mg kg^{-1} day^{-1} via oral against *P. vinckei*) (48) are not optimal for treating rodent malaria parasites but are of unquestionable therapeutic value in humans.

The pharmacokinetic profile of natural compound **1** has been determined in Sprague-Dawley rats (49), and bioavailability was found to be 2.7% based on blood analyses 10 to 180 min after oral administration of 10 mg kg^{-1}. Plasma concentrations of compound **1** after intravenous injection (100 mg kg^{-1}) exhibited a large distribution and rapid elimination rate, as expected for highly lipophilic drugs such as compound **1**. Here, the pharmacokinetics of compound **2** could not be determined, presumably due to metabolism and or strong interactions of compound **2** with mouse blood and mouse blood plasma components. Interestingly, the *in vitro* tests demonstrated that compound **2** could be recovered from human blood and human blood plasma.

Several classes of antimalarial drugs are known to cause an increase in the concentration of free heme (toxic to *Plasmodium* spp.) through the inhibition of hemozoin formation. Chloroquine is the classic drug known to inhibit hemozoin formation (9, 50). The inhibition of hemozoin formation observed for derivative **2** may be related to structural features of this compound and direct interactions with heme. Compound **2** may adopt highly planar conformations and undergo noncovalent van der Waals and or π-stacking interactions with heme (50). Furthermore, the catechol oxygen atoms in derivatives of compound **1** may interact strongly with iron in heme (as is believed to occur with the endoperoxide oxygen atoms of artemisinin [8]) and undergo complexation and redox reactions (28). The plausibility of these interactions needs corroboration through docking studies and spectroscopic analysis of heme/hemozoin-drug intermediates.

In *Plasmodium* spp., isoprenoids are biosynthesized via the MEP pathway in the interior of the apicoplast. *P. falciparum* biosynthesizes essential intermediates and final products important to its survival via the isoprenoid pathway, such as carotenoids, vitamin E, ubiquinones, menaquinones, and dolichols, as well as geranylated and farnesylated proteins (13–17). Through the incorporation of the radioactive metabolic precursor [1-(*n*)-^{3}H]G-GPP in cultures that were treated or untreated with compounds **2** or **3**, it was possible to determine the inhibitory effects of these substances on the biosynthesis of dolichol, menaquinone, and ubiquinone metabolites in schizonts of the 3D7 strain of *P. falciparum*. The inhibition of isoprenoid biosynthesis in *P. falciparum* by compounds **2** and **3** was considered good because it occurred at concentrations (4 μM) lower than the IC_{50}s, while overall protein biosynthesis was unaffected. No significant inhibition of isoprenoid biosynthesis by compounds **2** or **3** was observed in ring and trophozoite stages of *P. falciparum* (data not presented). This result is related to the relatively low metabolic production of isoprenoids at these early stages in parasite development (12, 18).

The strong inhibition of dolichol 12 biosynthesis in schizonts (Fig. 3) can be explained by the interference of compounds **2** and **3** in the isoprene chain elongation mechanism in dolichol biosynthesis, as has been suggested for the isoprene chains attached to the benzoquinone ring (51). The reduction in the availability of dolichol 12 produced by these derivatives can presumably interfere directly in posttranslational protein modifications (12, 16) and other metabolic processes that require the presence of dolichols.

Compound **2** strongly inhibited the biosynthesis of the prenylated metabolites ubiquinone and menaquinone in *P. falciparum* (Fig. 3). This is significant, as ubiquinones and menaquinones are linked to the survival of *Plasmodium* spp. in the host. Ubiquinone, also known as coenzyme Q, is an important electron carrier which is actively synthesized in the mitochondria of *Plasmodium* parasites and exerts an important protective antioxidant effect in the parasite (52, 53). An octaprenyl pyrophosphate synthase has been found in the intraerythrocytic stages of *P. falciparum* that is responsible for the biosynthesis of the isoprene side chains attached to the benzoquinone ring of ubiquinones. The recombinant version of this synthase exhibited marked similarity to that of the native, partially purified octaprenyl pyrophosphate synthase from schizont-stage parasites and was inhibited by nerolidol with a K_i of 10 nM (51). Vitamin K_2, also known as menaquinone (MQ), is in a class of fat-soluble vitamins that regulates metabolic pathways. In radiolabeling experiments of the direct precursor, Tonhosolo et al. (15) demonstrated that vitamin K_2 is synthesized by *P. falciparum* and acts as an important electron receiver of the respiratory chain. Also, inhibition of menaquinone production by Ro 48-8071, a known 1,4-dihydroxy-2-naphthoate prenyltransferase inhibitor, resulted in decreased parasite growth. Given the importance of menaquinone and ubiquinone to parasite survival, future work should focus on the specific molecular targets upon which derivative **2** directly acts during the biosynthesis of these compounds.

The inhibition of hemozoin formation and the inhibition of isoprenoid biosynthesis in *Plasmodium* generally involve independent molecular targets (6, 7, 16). However, work by Howe et al. (17) and others has demonstrated that the prenylation of the small ATPase Rab5 is associated with small hemoglobin-containing vacuoles that collectively represent >50% of hemoglobin uptake in trophozoites and schizonts (54). Disruption of posttranslational prenylation of Rab5 by fosmidomycin in turn disrupts the localization of Rab5 in vacuolar membranes and is associated with abnormal food vacuole morphologies. *P. falciparum* is believed to perform *de novo* synthesis of the terpenoid fragments it requires for ubiquinone and prenylated protein synthesis (17). It is conceivable that besides interfering in the biosynthesis of terpenoids in the apicoplast, 4-nerolidylcatechol derivatives **2** and **3** may interrupt the posttranslational prenylation of Rab5 and other proteins associated with normal vacuolar membrane formation in *P. falciparum*. However, the action of **2** and **3** on prenyltransferases or digestive vacuole formation mechanisms in *P. falciparum* was not demonstrated herein. 4-Nerolidylcatechol derivatives biochemically inhibited the synthesis of isoprenoids by inhibiting the incorporation of precursors in the metabolic pathway in the live parasite. Also, these derivatives chemically inhibited hemozoin formation *in vitro*, especially compound **2**, which exhibits inhibition similar to chloroquine, thus providing indirect evidence for this mechanism of action. Derivatives of 4-nerolidylcatechol potentially could act on different targets, in the apicoplast and in the food vacuole, in distinct mechanistic processes. Future work should investigate the possibility that derivatives of **1** are inhibitors of prenyl transferases that are important to digestive vacuole physiology and function.

4-Nerolidylcatechol (**1**) is a major component of infusions and

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other extracts prepared from the roots and leaves (22-25, 55) of the traditionally used caapeba plant (*Piper peltatum* and *P. umbellata*) (56). Caapeba extracts are active *in vitro* against *P. falciparum* and inactive or variably active against *P. berghei* in mice (57–62). Interestingly, the deactivated blood plasma taken from healthy rodents after oral ingestion of caapeba extracts inhibits *P. falciparum in vitro* (59). We isolated compound **1** from *P. peltatum* extracts and demonstrated that it exhibits good *in vitro* inhibition against *P. falciparum* (24, 25, 29) and, after oral ingestion, activates murine blood plasma against *P. falciparum* (25). However, as discussed above, *P. berghei* exhibits low sensitivity to caapeba extracts and 4-nerolidylcatechol and, as seen here, to derivatives of **1**. In similar circumstances, diamidines were tested in the *P. vinckei* model because *P. berghei* is almost insensitive to these drugs. Further studies on 4-nerolidylcatechol derivatives should explore *P. vinckei*-infected mouse or *P. falciparum*-infected humanized mouse (HuMouse) models (63).

Natural compound **1** exhibits *in vitro* antiplasmodial activity that is desirable as a starting point for a chemistry program for the development of new antimalarials; however, its *in vivo* antimalarial activity and stability are not satisfactory. In general, derivatives of compound **1** exhibit decreased *in vitro* antiplasmodial activity and improved stability and *in vivo* antimalarial activity compared to **1**. Nonetheless, the levels of *in vivo* activity observed for compound **2** are not satisfactory and may be negatively influenced by poor pharmacokinetics.

Derivatives of 4-nerolidylcatechol inhibit *in vitro* biosynthesis of vital isoprenoid metabolites in *P. falciparum*. New derivatives with improved activity, solubility, and pharmacokinetics are needed. Poor pharmacokinetics associated with low water solubility (and fast clearance) were also problems identified for the low-polarity natural antimalarial artemisinin, and these properties were markedly improved by the development of the water-soluble derivative sodium artesunate (64). Also, formulations of derivatives of compound **1** may help to improve bioavailability. In this vein, an inclusion complex of compound **1** and derivatized cyclodextrin has been prepared that exhibits improved water solubility (65). The development of more soluble, structurally diverse 4-nerolidylcatechol derivatives and formulations could be important strategies for increasing bioavailability and the *in vivo* antimalarial activities of these compounds.

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