Synthesis and Antimalarial Activity of Trioxaquine Derivatives

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Abstract: Trioxaquines are dual molecules that contain a trioxane motif linked to an aminoquinoline entity. Among the different compounds of this series, trioxaquine *cis*-**15** (DU1302c), prepared from α-terpinene, a cheap natural product, showed efficient antimalarial activity in vitro on both sensitive and resistant strains of *Plasmodium falciparum* (IC_{50} = 5–19 nM). A stereochemical description of this stable, nontoxic, and non-genotoxic antimalarial agent is detailed. Mice infected with *P. vinckei* were successfully treated with *cis*-**15** in a four-day suppressive test. The doses required to decrease parasitemia by 50% (ED_{50}) were 5 and 18 mg kg^{-1} d^{-1} after intraperitoneal and oral administration, respectively. Parasitemia clearance was complete without recrudescence at an intraperitoneal dose of 20 mg kg^{-1} d^{-1}.

Keywords: aminoquinoline · peroxides · spiro compounds · trioxane · trioxaquine

Introduction

Malaria, the third most infectious cause of mortality, is prolific in more than 40% of the world's population and causes more than one million deaths each year, especially in Africa.[1] Given the spreading resistance of *Plasmodium falciparum* to most of the current antimalarial drugs (namely chloroquine, mefloquine, pyrimethamine, and proguanil), as well as the absence of a vaccine for protection against malaria, there is an urgent need for new effective, safe, and affordable drugs.[2] The use of artemisinin and related derivatives is increasing since they are among the few molecules active in the treatment of multidrug resistant *P. falciparum* malaria. They are well tolerated, and to date, significant resistance has not been reported either in clinical isolates or in laboratory experiments. These compounds are active against the early form of the malarial blood stages and induce a rapid clearance of the blood parasitemia. They also act against gametocytes, which are the sexual stages of the parasite that infect mosquitoes. As a result of a short half-time of elimination and appearance of recrudescence, artemisinin derivatives are now combined with more slowly eliminated drugs such as mefloquine or lumefantrine. This is done both to increase the efficiency of the treatment and to impede the development of resistance.[1,3] However, an erratic supply of the natural parent compound and its short half-life make it necessary to design cheap synthetic endoperoxide-based antimalarials.[4]

The action of artemisinin arises from the presence of an endoperoxide group that is able to produce radicals, which in turn damage the parasite.[4a] Our group contributed to demonstrating the alkylating activity of artemisinin derivatives and other related antimalarial trioxanes.[5–7] Subsequently, we decided to develop new molecules based on the "covalent bitherapy" concept in an effort to generate new affordable drugs that would be less prone to the development of resistance. These chimeric compounds, named trioxaquines, are obtained by covalent attachment of a trioxane entity, known to be responsible for the activity of artemisinin, to an aminoquinoline entity, which is responsible for the accumulation and activity of chloroquine in the parasite.

The synthesis and antimalarial activity of the first trioxaquine **5a**, named DU1102 (Figure 1), was recently reported.[8] Encouraged by the in vitro antimalarial activity of **5a** on laboratory strains,[8] as well as chloroquine- and pyri-

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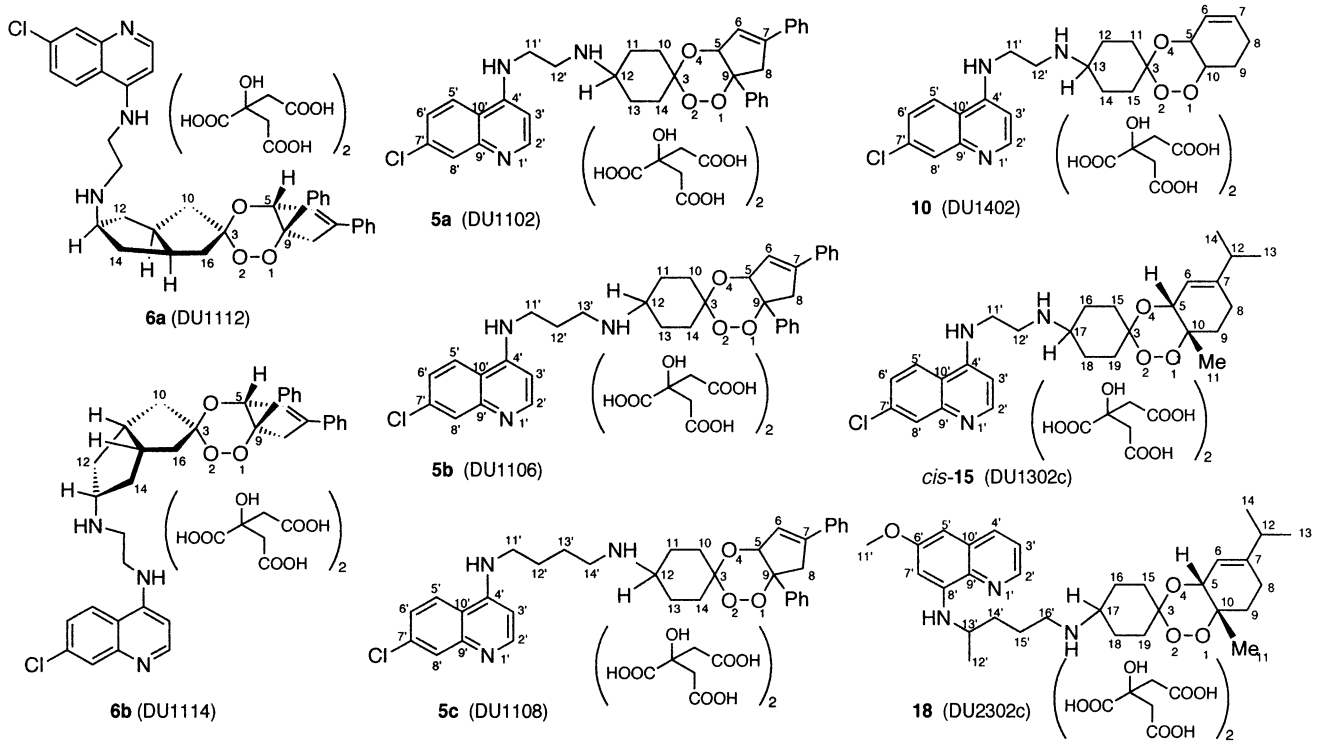


Figure 1. Structures of trioxaquine citrates **5a–c**, **6a**, **6b**, **10**, *cis*-**15**, and **18**. Trioxaquines **6a** and **6b** are racemates but only one enantiomer of each is depicted. Trioxaquines *cis*-**15** and **18** are 50:50 mixtures of two diastereomeric racemates but only one of the four possible stereoisomers is depicted for each trioxaquine.

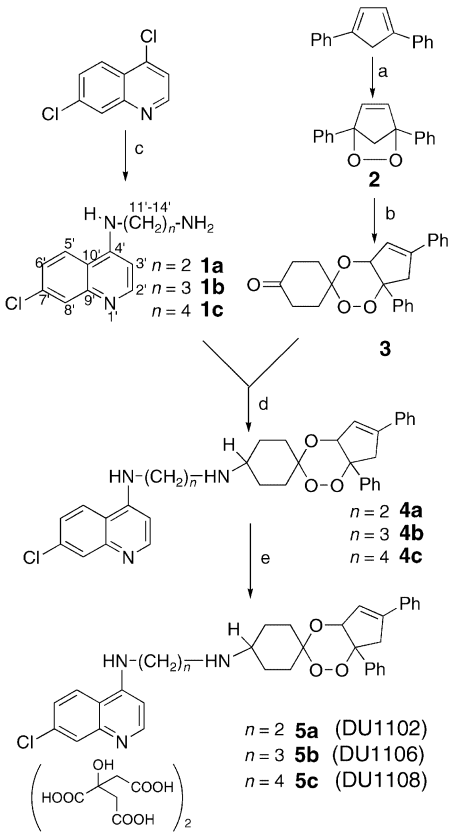
methamine-resistant human isolates of *P. falciparum*,[9] we aimed to synthesize a series of new trioxaquines (Figure 1) in an effort to increase the antimalarial activity of this series. In particular, we wanted to gain an understanding into the influence of several structural parameters, such as: 1) the length of the tether between the 4-aminoquinoline and the trioxane fragments [trioxaquines **5b** (DU1106) and **5c** (DU1108)]; 2) the use of different starting dienes [trioxaquines **10** (DU1402) and *cis*-**15** (DU1302c), obtained from 1,3-cyclohexadiene and α-terpinene, respectively]; and 3) the nature of the diketone used in the synthesis of the trioxane [trioxaquines **6a** (DU1112) and **6b** (DU1114) were obtained from *cis*-bicyclo[3.3.0]octane-3,8-dione,[10] rather than cyclohexane-1,4-dione, which has always been used previously]. Finally, trioxaquine **18** (DU2302c) was obtained from primaquine, an antimalarial drug based on 8-aminoquinoline instead of the 4-aminoquinoline motif.

In vitro, trioxaquine *cis*-**15** was found to be the most active, its inhibitory concentration (IC_{50}) being as low as 6 nM for the highly chloroquine-resistant *P. falciparum* strain FcM29-Cameroon. Given these results, as well as taking into consideration the feasibility of its synthesis, compound *cis*-**15** was chosen to be assessed for in vivo antimalarial activity on mice (*P. vinckei*) in a four-day suppressive test. The doses required to decrease parasitemia by 50% (ED_{50}) were 5 and 18 $mg\,kg^{-1}\,d^{-1}$ after intraperitoneal and oral administration, respectively. Moreover, parasitemia clearance was complete without recrudescence at an intraperitoneal dose of 20 $mg\,kg^{-1}\,d^{-1}$. Mice treated at 120 $mg\,kg^{-1}\,d^{-1}$ for four consecutive days (oral route) did not experience any toxic effects. Furthermore, it was found that trioxaquine *cis*-**15** was unable to induce the SOS response in *E. coli* even at a 20 μM concentration. This is 1000 times higher than its antimalarial IC_{50} values and indicates that in vitro *cis*-**15** is not genotoxic.

Results and Discussion

The parent trioxaquine **5a** was obtained by the convergent synthesis of building blocks that allowed for several modifications. Aminoquinoline **1a** was prepared by condensation of 1,4-diaminoethane with 4,7-dichloroquinoline. The trioxane–ketone **3** was obtained by condensation of the 1,4-endoperoxide of 1,4-diphenyl-1,3-cyclopentadiene **2** with cyclohexane-1,4-dione. Subsequent reductive amination of the trioxane–ketone **3** by the primary amine of aminoquinoline **1a** led to trioxaquine **4a**. To enhance its solubility, compound **4a** was protonated by citric acid to provide trioxaquine citrate **5a** (Scheme 1). As yet, a stereochemical study of compound **5a** has not been completed.

As previously documented for the condensation of endoperoxide **2** with cyclopentanone,[11] in trioxane–ketone **3** (Scheme 1), both the trioxane and the cyclopentene are *cis*-fused, and H5 and the phenyl C9 are contiguous. Therefore, rather than a diastereomeric racemate (four stereoisomers), as is theoretically expected for a molecule that has two chiral carbons, compound **3** was obtained as a racemate. Moreover, the reductive amination step in the trioxaquine synthesis created a pseudoasymmetric center at the C12 spiro carbon. Therefore, two diastereomeric racemates were obtained in which the amine and the endoperoxide were



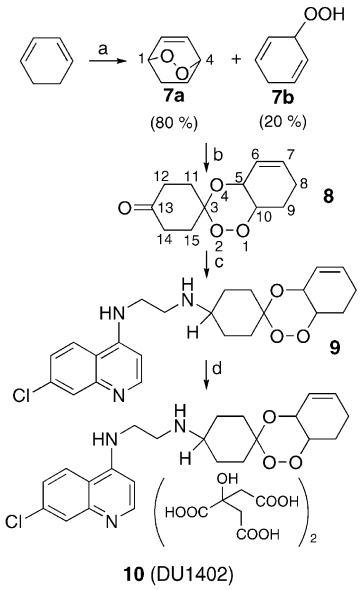
Scheme 1. Synthesis of trioxaquines **5a–c** from 1,4-diphenyl-1,3-cyclopentadiene: a) O_2, *hν*, TPP, CH_2Cl_2, 5 °C; b) cyclohexane-1,4-dione, Me_3SiOTf, CH_2Cl_2, −78 °C; c) $H_2N(CH_2)_nNH_2$, 85 °C; d) $NaBH(OAc)_3$, CH_2Cl_2; e) citric acid, acetone.

either *trans* or *cis* with respect to the mean plane of the cyclohexyl moiety. As a result, compound **4a** (and consequently **5a**) was isolated as an equimolecular mixture of two diastereomeric racemates (four stereoisomers). This stereochemical aspect is further discussed with respect to compounds **12** and *cis*-**14**.

Trioxaquines synthesized from 4,7-dichloroquinoline

Variations of the diaminoalkyl tether: Changes in the length of the lateral chloroquine chain influence antimalarial activity, and are able, in some cases, to circumvent chloroquine-resistance of *Plasmodium falciparum*.[12,13] Several trioxaquines with diaminoalkyl tethers of different lengths were synthesized to determine the effect this had on antimalarial activity. Substitution of 4,7-dichloroquinoline with different α,ω-diaminoalkanes is generally easy, but poor yields have been reported in the case of long diaminoalkanes because of the formation of bisquinoline derivatives.[14] Substitution of 4,7-dichloroquinoline with 1,3-diaminopropane and 1,4-diaminobutane gave the expected aminoquinolines **1b** and **1c** in 75 and 60% yield, respectively (Scheme 1). The subsequent steps were then performed under conditions similar to those used for **5a**.[8] Finally, reductive amination of the trioxane–ketone **3** by **1b** and **1c** produced trioxaquines **4b** and **4c** in 95 and 70% yield, respectively. As could be assessed from the doubled-up ^1H NMR signals assigned to H5 and the quinoline protons, **4b** and **4c** were also obtained (and not separated) as the *trans* and *cis* diastereomeric racemates. These were subsequently protonated with citric acid in quantitative yields to give the corresponding trioxaquine dicitrates **5b** and **5c**, respectively. The chemical shifts of the quinoline and diaminoalkane tether protons indicate that in a solution of DMSO, **5a–c** are monoprotonated trioxaquine citrates. This has also recently been reported for trioxaquines **6a** and **6b**[10] (Figure 1).

Trioxaquine from 1,3-cyclohexadiene: Trioxaquines **4a–c** were obtained from endoperoxide **2**, which in turn is prepared by photo-oxygenation of 1,4-diphenyl-1,3-cyclopentadiene. Unfortunately, this diene, which is synthesized in four steps from the sodium salt of ethyl acetoacetate and phenacyl bromide,[11,15] is obtained in an overall yield of only 20%. To reduce the number of steps and to enhance the total yield of the synthesis, we decided to use a commercially available diene. Therefore, as depicted in Scheme 2 and



Scheme 2. Synthesis of trioxaquine **10** from 1,3-cyclohexadiene: a) O_2, *hν*, TPP, CH_2Cl_2, 5 °C; b) cyclohexane-1,4-dione, Me_3SiOTf, CH_2Cl_2, −78 °C; c) $NaBH(OAc)_3$, **1a**, CH_2Cl_2; d) citric acid, acetone.

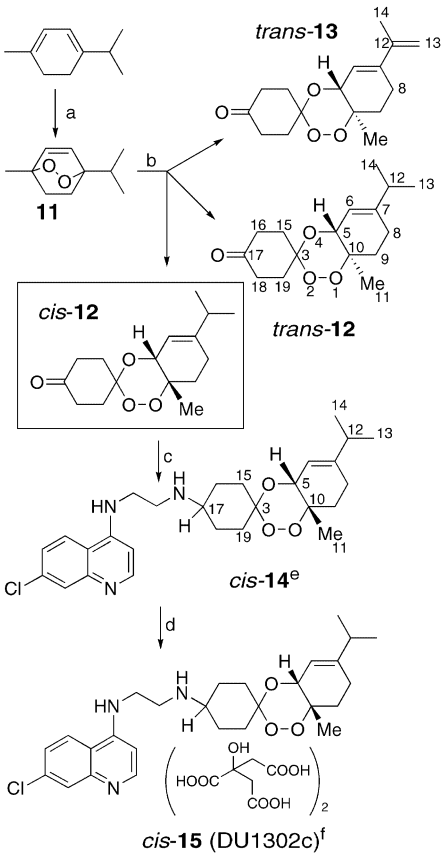
Scheme 3, respectively, trioxaquine **10** was prepared from 1,3-cyclohexadiene, while *cis*-**15** was synthesized from α-terpinene. Photo-oxygenation of 1,3-cyclohexadiene afforded a mixture of the desired endoperoxide **7a** (80% yield) and 3-hydroperoxo-1,4-cyclohexadiene **7b** (20% yield). Compound **7a** results from the [4+2]-cycloaddition of $^1O_2$, while **7b** arises from an ene reaction of $^1O_2$. Attempts to purify **7a** by column chromatography were unsucessful. Subsequent condensation of this mixture with cyclohexane-1,4-dione in dichloromethane at −78 °C in the presence of Me_3SiOTf as catalyst gave, after silica-gel chromatography, the expected trioxane–ketone **8** in low yield (<10%). The low yield obtained for **8** arises because the 1,4-endoperoxide does not contain any substituents. Therefore, in contrast to 1,4-diphenyl-1,3-cyclopentadiene, when this endoperoxide is opened by Me_3SiOTf, an unstabilized carbocation is formed.

In addition, in trioxane–ketone **8** the trioxane and cyclohexene ring can be either *cis* or *trans* fused; this gives rise to a mixture of two diastereomeric racemates, which correspond to four different possible configurations at C5 and C10. This is not the case for trioxane–ketone **3**, as the short-ring constraint of cyclopentene does not allow a *trans* fusion. The four stereoisomers of compound **8** could not be separated (this problem will be further discussed for trioxane–ketone **12** and trioxaquine *cis*-**14**, see below, Scheme 3). Reductive amination of **8** by aminoquinoline **1a** produced trioxaquine **9**. The aminoquinoline fragment can be introduced at C13 on the same side as the peroxide bond with respect to the mean plane of the cyclohexane ring, or on the opposite side. Thus, this gives rise to two diastereomeric trioxaquines for each starting trioxane–ketone. Compound **9** (which refers to the mixture of eight stereoisomers) was then further protonated to give trioxaquine citrate **10** in 40% overall yield from trioxane–ketone **8**. The use of 1,3-cyclohexadiene did reduce the number of steps in the synthesis but did not improve on the overall yield (7% for **5a** from the sodium salt of ethyl acetoacetate compared to 2% for **10** from 1,3-cyclohexadiene). Moreover, the latter synthesis doubled the number of stereoisomers obtained.

Trioxaquine from α-terpinene: Given the low yields obtained when an unsubstituted diene such as 1,3-cyclohexadiene was used, we turned our attention to the cheap and commercially available disubstituted diene α-terpinene (1-methyl-4-isopropyl-1,3-cyclohexadiene). We hereby report a detailed study that includes stereochemical considerations, of the synthesis of trioxaquine *cis*-**15**. Photo-oxygenation of α-terpinene afforded the desired endoperoxide **11**. Compound **11** is in fact the natural product ascaridole, a racemic endoperoxide that has a (1*R*,4*R*) and (1*S*,4*S*) configuration, and which exhibits weak antimalarial activity (Scheme 3).[16] Condensation of cyclohexane-1,4-dione with **11** in dichloromethane at −78°C in the presence of Me_3SiOTf as catalyst gave, after separation by silica gel chromatography, three different trioxane–ketones, namely *trans*-**12**, *cis*-**12**, and *trans*-**13**. Both *trans*-**12** and *cis*-**12** have the expected trioxane–ketone structure and consist of two racemic diastereomers. In *cis*-**12**, the trioxane and cyclohexene rings are *cis* fused, that is, H5 and the 11-methyl group are contiguous. This corresponds to a 5*S*,10*R* and 5*R*,10*S* configuration. Therefore, *cis*-**12** is a racemate that should be noted as (5*RS*,10*SR*)-**12** according to CIP convention.[17] However, to shorten this notation, we use the *cis*/*trans* descriptor because it is also accepted for cyclanes. In *trans*-**12**, the trioxane and cyclohexene rings are *trans* fused, that is, H5 and the 11-methyl group are on opposite planes of the C5−C10 bond. In turn, this gives rise to the 5*S*,10*S* and 5*R*,10*R* configuration (*trans*-**12** should, therefore, be noted as (5*RS*,10*RS*)-**12** according to CIP convention).

Although the formation of a trioxane that bears an angular isopropyl residue at C10 cannot in priciple be ruled out during the condensation of ascaridole **11** with cyclohexane-1,4-dione, this compound was not detected.

The structures of *cis*-**12** and *trans*-**12** were unambiguously determined by X-ray diffraction of crystals obtained by the slow evaporation of a solution of the corresponding compound from a hexane/EtOAc (80:20, v/v) mixture (Figure 2). It should be noted that in the case of trioxane–ketone **3**, a *trans* fusion of the trioxane and cyclopentene ring is not possible because of steric constraints.[11] Therefore, the only stereoisomers obtained for **3** were enantiomers that had a 5*S*,9*S* and 5*R*,9*R* configuration.



Scheme 3. Synthesis of trioxaquine *cis*-**15** from α-terpinene: a) O_2, *hv*, TPP, CH_2Cl_2, 5°C; b) cyclohexane-1,4-dione, Me_3SiOTf, CH_2Cl_2, −78°C; c) $NaBH(OAc)_3$, **1a**, CH_2Cl_2; d) citric acid, acetone; e) *cis*-**14** is a 50:50 mixture of the two diastereomers *trans*,*cis*-**14** and *cis*,*cis*-**14**; f) *cis*-**15** is a 50:50 mixture of the two diastereomers *trans*,*cis*-**15** and *cis*,*cis*-**15**.

The structure of the third, minor trioxane–ketone *trans*-**13** was determined by NMR experiments. Compound **13** is an analog of *trans*-**12** in which the isopropyl substituent at C7 has been oxidized to an isopropenyl group (Scheme 3). In the ^1H NMR spectrum of *trans*-**13**, the signal for the 11-methyl group appeared, as it did for *trans*-**12**, as a sharp signal, whereas this resonance was a very broad singlet in the case of *cis*-**12**. This analysis indicates that *trans*-**13** has a (5*RS*,10*RS*) configuration. In addition, NOE experiments showed a spatial proximity between one of the H13 protons and the two H8 protons; this is compatible with an s-*trans* conformation of the diene.

The trioxane–ketone *cis*-**12** was the major product isolated for all the different experimental conditions used. For example, when the reaction was carried out with 6 molar equivalents of diketone for 2 h, the *trans*-**12**/*cis*-**12**/*trans*-**13** ratio was 12:78:10, and *cis*-**12** was isolated in 26% yield with respect to the α-terpinene. Unfortunately, changes in reaction time, temperature, quantity of catalyst, or quality

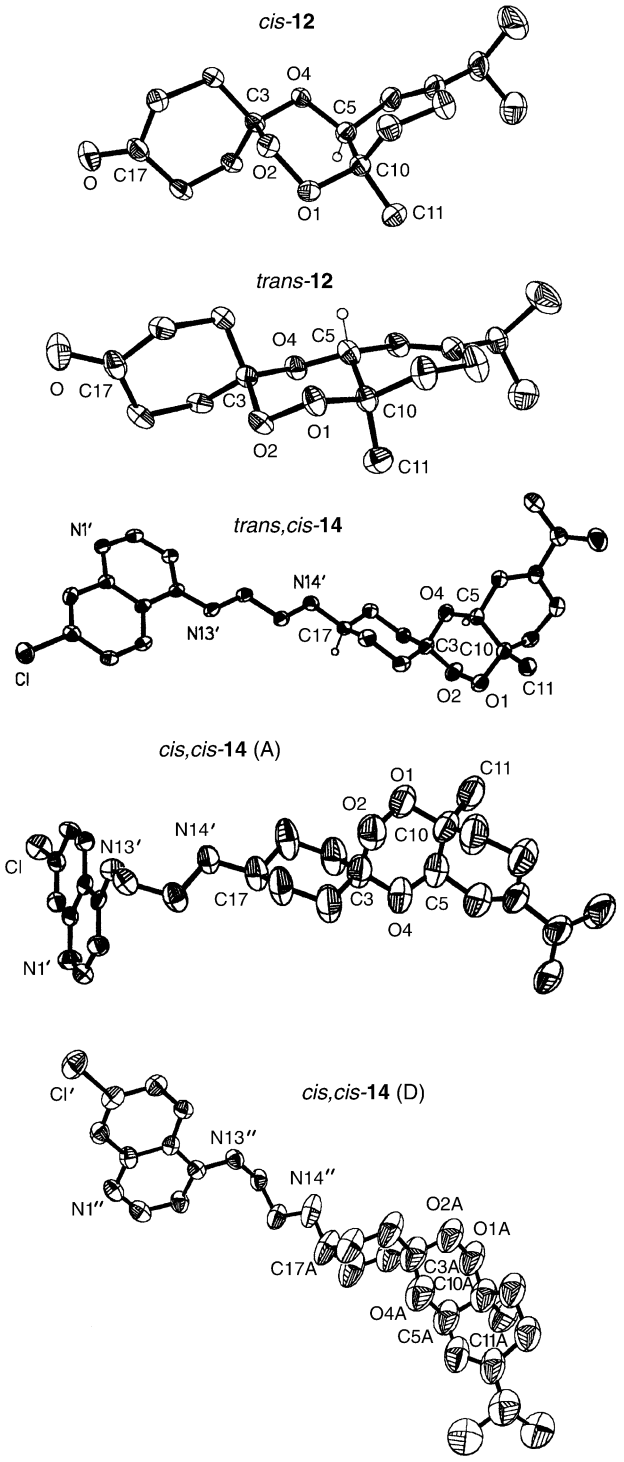


Figure 2. Crystal structures of trioxanes *cis*-**12** and *trans*-**12**, as well as trioxaquines *trans*,*cis*-**14** and *cis*,*cis*-**14**. In *cis*,*cis*-**14**, all conformers and enantiomers co-crystallized but only A and D are represented.

of solvent (distilled or not) did not improve the yield of *cis*-**12**. Thus, it was necessary to separate and purify the major diastereomer *cis*-**12** by chromatography.

Reductive amination of *cis*-**12** by aminoquinoline **1a** afforded trioxaquine *cis*-**14** in 60–80 % yield (Scheme 3). From analysis of the ^{1}H NMR spectra, *cis*-**14** was found to be a 50:50 mixture of two trioxaquine diastereomeric racemates (i.e. four stereoisomers). In principle, non-stereoselective reductive amination at C17 should afford compounds that have the amine group of the aminoquinoline at C17 and the peroxide at C3 either in an equatorial or axial position with respect to the cyclohexane ring. Therefore, when a racemate of the trioxane–ketone *cis*-**12** (5*R*,10*S* + 5*S*,10*R*) is used as the starting material, reductive amination is expected to provide eight compounds. In particular, there should be four stereoisomers, each of which exist as two conformers because of the conformational equilibrium of the cyclohexane ring (Figure 3).

In B and C (and B′ and C′), the amine and the peroxide substituents are *trans* with respect to the cyclohexane ring. For this reason, the mixture of racemates BB′ + CC′ is named *trans*,*cis*-**14**. Here *trans* refers to the relative position of the aminoquinoline and peroxide with respect to the cyclohexane ring, while *cis* refers to the trioxane–cyclohexene ring junction. In A and D (and A′ and D′), the amine and the peroxide substituents are *cis*, that is, on the same side of the mean plane of the cyclohexane ring. As a result, this diastereomeric racemate is named *cis*,*cis*-**14**.

To enable structure elucidation and biological evaluation, the two diastereomers of *cis*-**14** were partially separated by selective precipitation (dichloromethane/cyclohexane, 1:8, v/v); this led to an enriched *trans*,*cis*-**14**/*cis*,*cis*-**14** mixture (70:30). The enriched mixture was then subjected to column chromatography on silica gel (EtOAc/triethylamine, 95:5, v/v). The first diastereomeric racemate eluted was *trans*,*cis*-**14**. Its structure was unambiguously determined [X-ray diffraction of a crystal obtained by the slow evaporation of an ethanol/EtOAc (50:50, v/v) solution (Figure 2)] to be conformer C (and C′), in which the aminoquinoline is in an equatorial position. Conformer B (and B′), which is less stable because the aminoquinoline residue is axial, was not observed.

The second diastereomeric racemate eluted was *cis*,*cis*-**14**. This diastereomeric racemate was crystallized by the slow diffusion of diethyl ether into a dichloromethane solution. In this compound, the asymmetric unit contains both the A and D conformer. It should be noted that a fast equilibrium between conformers A (and A′) and D (and D′) exists in solution, and therefore, did not allow the two to be distinguished by NMR spectroscopy at room temperature. Moreover, this conformational equilibrium makes the chromatographic purification of *cis*,*cis*-**14** tedious and erratic. The recovery of *trans*,*cis*-**14** was much easier.

Each of the two diastereomeric racemates were protonated with citric acid to afford the corresponding trioxaquine citrates *trans*,*cis*-**15** and *cis*,*cis*-**15** in quantitative yield. These citrates were then tested for in vitro antimalarial activity.

Since *trans*,*cis*-**14** and *cis*,*cis*-**14** displayed similar in vitro antimalarial activity (see below), they were not separated any further. Compound *cis*-**14**, which consisted of a 50:50 mixture (quantified on the basis of its ^{1}H NMR analysis) of the two diastereomeric racemates *trans*,*cis*-**14** and *cis*,*cis*-**14** (four stereoisomers) was thereby protonated with citric acid to afford *cis*-**15**. Subsequently, this mixture of *trans*,*cis*-**15** and *cis*,*cis*-**15** (50:50 ratio) was used for in vivo antimalarial tests.

Trioxaquine synthesized from primaquine: The quinoline entity of all the trioxaquines described up to this point was

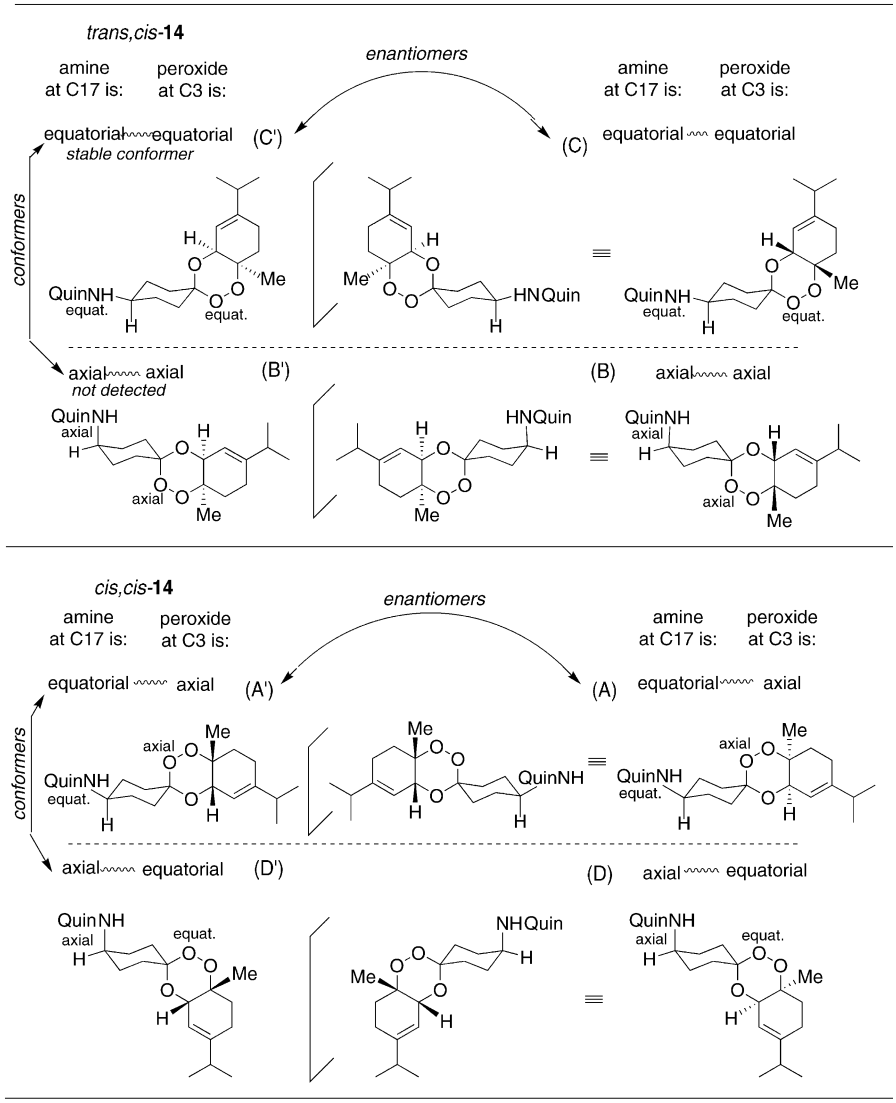


Figure 3. The eight possible structures of trioxaquine *cis*-**14**, in which the amine and the peroxide functions are either axial or equatorial with respect to the cyclohexane residue. (Quin–NH refers to the aminoquinoline **1a**, see Scheme 1).

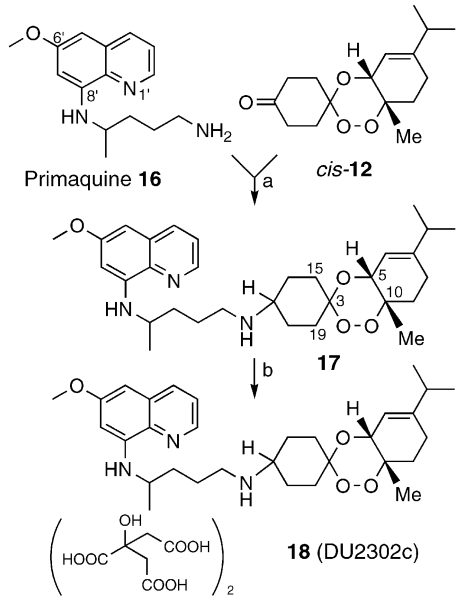
similar to chloroquine, a 7-chloro-4-aminoquinoline, and was used on the basis of many structure–activity relationship studies.[18] Primaquine **16** was considered as a suitable alternative as it has been widely used for eradication of the dormant hepatic forms of the parasite responsible for relapses in *P. vivax* or *P. ovale* infections,[19] and because it is also active against the sexual stages of the parasite involved in the transmission of the disease. Therefore, incorporation of a primaquine moiety and a trioxane fragment into a single compound could potentially lead to new drugs that have both gametocidal and schizontocidal activities. Primaquine **16** (Scheme 4) is an 8-aminoquinoline that contains a primary amine that can be directly utilized in the reductive amination of a trioxane–ketone. Reductive amination of trioxane *cis*-**12** with primaquine gave trioxaquine **17** in 63% yield (Scheme 4). Subsequent protonation was carried out in acetone with 2.0 molar equivalents of citric acid to afford trioxaquine citrate **18** after evaporation of the solvent. This new trioxaquine is highly soluble in acetone as well as in water. It should be noted that the diastereomers from the *cis* or *trans* disposition of the peroxide and primaquine substituent with respect to the cyclohexane ring were not separated. Compound **18** consisted of an equimolecular mixture of eight stereoisomers, just like *cis*-**15** because it contains an additional chiral center at C13′ of primaquine.

Characteristic data for the trioxaquine citrates: Table 1 presents several characteristic features of the different trioxaquines: molecular weight, overall yield for the syntheses from commercially available reactants, number of steps, and solubility. The trioxaquine prepared from primaquine is the only one that is soluble in pure water. All the other trioxaquines have to be previously dissolved in DMSO prior to the addition of water, physiological serum, or culture medium. The reported data indicate the maximum trioxaquine concentration in DMSO that can be diluted by an infinite amount of physiological serum without precipitation. Among the trioxaquines synthesized from 4-aminoquino-

Table 1. Molecular weights, yields, and solubilities of the different trioxaquines.

Trioxaquine	Molecular weight [base (citrate form)]	Global yield[a] [%]	Number of steps[a]	Solubility[b] [mg mL^{-1}]
5a (DU1102)	568.2 (952.4)	7	9	1.0 ± 0.2
5b (DU1106)	582.2 (966.4)	5	9	1.0 ± 0.2
5c (DU1108)	596.2 (980.4)	5	9	1.0 ± 0.2
6a (DU1112)	594.2 (978.4)	2	9	0.6 ± 0.2
6b (DU1114)	594.2 (978.4)	2	9	0.6 ± 0.2
10 (DU1402)	430.0 (814.2)	2	5	70 ± 10
cis-**15** (DU1302c)	485.1 (870.3)	20	5	50 ± 10
18 (DU2302c)	523.0 (907.2)	20	4	soluble in water[c]

[a] Calculated with respect to the starting materials (sodium salt of ethyl acetoacetate for **5a–c** and **6a** and **6b**, 1,3-cyclohexadiene for **10**, and α-terpinene for *cis*-**15** and **18**). [b] These trioxaquines are not directly soluble in water or physiological serum. The reported solubility is the maximum concentration of the trioxaquine citrate in DMSO that can be diluted with an infinite amount of physiological serum (0.9 wt% NaCl in water) without precipitation. [c] Solubility in water: 1 mg mL^{-1}.



Scheme 4. Synthesis of trioxaquine **18** from primaquine: a) $NaBH(OAc)_3$, CH_2Cl_2; b) citric acid, acetone. *cis*-**12** is a racemate but only one enantiomer is depicted. Trioxaquines **17** and **18** are 50:50 mixtures of two diastereomeric racemates but only one of the four possible stereoisomers is depicted for each trioxaquine.

lines, those that contain a cyclohexene (**10**) or terpinene moiety (*cis*-**15**) had a solubility of 70 and 50 mg mL^{-1} in DMSO, respectively. This is much higher than that found for trioxaquines **5** and **6**, which have a diphenylcyclopentene residue (1 mg mL^{-1}). A molecular weight below 500 is one of the "five's rule" points reported by Lipiski et al. in order for a drug to have good oral availability.[20] With a molecular mass of 485 for its base form, and in light of the high overall yield (20%) obtained, *cis*-**15** is the most promising compound.

Table 2. IC_{50} values [nM] for the trioxaquine citrates against *Plasmodium falciparum*.

Trioxaquine citrates	IC_{50} [nM][a] Nigerian CQS[b]	FcB1-Columbia CQR[b]	FcM29-Cameroon CQR+[b]
5a (DU1102)	22 (1)	27 (3)	27 (10)
5b (DU1106)	181 (59)	41 (4)	133 (102)
5c (DU1108)	87 (20)	41 (4)	41 (14)
6a (DU1112)	112[c]	31 (17)	39 (22)
6b (DU1114)	108 (43)	63 (2)	85 (7)
10 (DU1402)	36 (12)	71 (52)	49 (0)
cis-**15** (DU1302c)	17(6); [7[c]][d]	15 (2)	6 (1)
trans,cis-**15**	[9[c]][d]	19 (2)	7 (2)
cis,cis-**15**	[5[c]][d]	11 (4)	5 (1)
18 (DU2302c)	176 (39)	112 (17)	108 (16)
Chloroquine[e,f]	62 (10)	116 (57)	174 (26)
Artemisinin[e]	6 (1)	5 (1)	8 (1)
Primaquine[e,g]	875 (200)	1075 (17)	1400 (300)

[a] IC_{50} values are the mean of at least 2–5 independent experiments, except where noted (the SEM value is indicated in parentheses, SEM = standard error of the mean). [b] CQS = chloroquine sensitive, CQR = chloroquine resistant, CQR+ = highly chloroquine resistant. [c] Single experiment. [d] Measured on the chloroquine-sensitive F32-Tanzania strain instead of the Nigerian strain. [e] Measured as reference. [f] Chloroquine diphosphate was used. [g] Primaquine diphosphate was used.

Biological activity

In vitro antimalarial activity:
We tested the in vitro antimalarial activity of the eight synthesized trioxaquine citrates to determine the influence that the different structural modifications had on their biological activity. Four different strains of *Plasmodium falciparum* were used: a Nigerian strain and F32-Tanzania, which are chloroquine sensitive (CQS); and FcB1-Columbia and FcM29-Cameroon, which are chloroquine resistant (CQR) and highly chloroquine resistant (CQR+), respectively. The threshold IC_{50} for in vitro resistance to chloroquine is usually estimated to be 100 nM.[21] The results are summarized in Table 2.

First of all, it should be highlighted that the antimalarial activities are high, especially for trioxaquines **5a** and *cis*-**15**, which have IC_{50} values in the range of 22–27 nM and 5–19 nM, respectively. In addition, these drugs are as active on chloroquine resistant as on chloroquine sensitive *P. falciparum* strains. For *cis*-**15**, the IC_{50} values are 7 and 17 nM for F32-Tanzania and the Nigerian (sensitive) strains, and 15 and 6 nM for the FcB1 and FcM29 (resistant) strains, respectively.

Variations in the length of the diaminoalkyl tether $(-CH_2-)_n$ between the trioxane and the 4-aminoquinoline moieties (**5a**, $n=2$; **5b**, $n=3$; **5c**, $n=4$) did not significantly modify the antimalarial activity. In some cases, lower activities were observed when the diaminoalkyl chain was longer than two carbon atom units: $IC_{50}=181$ and 41 nM for the Nigerian and FcB1 strains, respectively, for **5b** ($n=3$), while for **5a** ($n=2$) the values obtained were 22 and 27 nM, respectively. As a result, a two-carbon atom tether was used for most of the trioxaquines that contained a 4-aminoquinoline fragment.

The influence of certain stereochemical features was also evaluated. Trioxaquines **6a** and **6b** (Figure 1) are diastereomeric racemates, which were prepared from *cis*-bicyclo[3.3.0]octane-3,8-dione. They differ only in the stereochemistry at the C3 spiro junction. That is, the peroxide function is either on the same side or on the opposite side of H11 and H15 with respect to the C10-C12-C14-C16 plane; this gives rise to the *exo* and *endo* racemates **6a** and **6b**, respectively. The structure of each diastereomer has been proposed from a detailed NMR study.[10] The antimalarial activity of these two diastereomers was not significantly different. For example, IC_{50} values of 112 and 108 nM were obtained for **6a** and **6b**, respectively, against the chloroquine-sensitive Nigerian strain. For the chloroquine-resistant strain a difference was observed (factor of 2), but it was low and did not allow us to conclude that the C3 stereochemistry of this trioxaquine has any real influence on its antimalarial activity.

Changes in the starting diene, that is, from 1,4-diphenyl-1,3-cyclopentadiene in **5a** to 1,3-cyclohexadiene in **10** or α-terpinene in *cis*-**15** also did not effect the antimalarial activities of these compounds and they too were quite active. Of the two, trioxaquine *cis*-**15** is the more active against both the CQS and CQR strains, with IC_{50} values of 7 and 17 nM for the sensitive F32-Tanzania and Nigerian strains, respectively, and 15 and 6 nM for the resistant FcB1 and FcM29 strains, respectively (Table 2). These results indicate that trioxaquine *cis*-**15** is efficient on a wide range of strains. Furthermore, each of the two diastereomers of *cis*-**15** (*trans*,*cis*-**15** and *cis*,*cis*-**15**) were independently tested, and each were found to have activities in the same range. For the highly resistant strain FcM29, the IC_{50} values were 6(1) nM for the 50:50 mixture of diastereomers, and 7(2) and 5(1) nM for *trans*,*cis*-**15** and *cis*,*cis*-**15**, respectively. On the other hand, for the chloroquine-sensitive F32 strain, the IC_{50} values were 9 nM for *trans*,*cis*-**15** and 5 nM for *cis*,*cis*-**15**, while the value for *cis*-**15** was 7 nM. These IC_{50} values fall below the range expected (10–20 nM) for efficient antimalarials based on a trioxane moiety.[4,22,23] Since the two diastereomers of *cis*-**15** displayed similar antimalarial activity in vitro, they were not separated for further in vivo evaluation in mice. The high efficacy and solubility of trioxaquine *cis*-**15**, as well as the ease with which it was synthesized, make *cis*-**15** an attractive trioxaquine.

A lower antimalarial activity was observed for the compounds that contained 8-aminoquinoline as the quinoline moiety (i.e. **18**), and confirmed that the 4-aminoquinoline entity is vital for activity. However, the results obtained for **18** also showed that covalent coupling of a trioxane moiety with primaquine leads to a large increase in the intraerythrocytic antimalarial activity in comparison to primaquine itself, particularly for the CQR strains (FcB1 strain: IC_{50} = 112 and 1075 nM for trioxaquine **18** and primaquine, respectively). This result indicates that **18**, as a "modified primaquine", may have potential usage against both sexual and asexual erythrocytic stages. In addition, since primaquine is the only drug active against hepatic malaria stages, it would be of interest to test **18** on hepatic parasite stages.

In vivo antimalarial activity of *cis*-15 (DU1302c) against *P. vinckei*: Compound *cis*-**15** (DU1302c) was tested in mice infected with *P. vinckei* according to a modified version of Peters' four day suppressive test, and was evaluated both after intraperitoneal and oral administration using the intravenous route for parasite inoculation. Parasitemia in control mice generally reached as high as 20–40% on day 5, and death occurred within the following four days. After once daily intraperitoneal administration for four days, the first dose being administered 1 day after parasite inoculation, compound *cis*-**15** exhibited potent antimalarial activity with an ED_{50} of 5 mg kg^{-1} d^{-1}. Moreover, parasitemia was completely cured without recrudescence over 60 days when 20 and 50 mg kg^{-1} d^{-1} doses were administered.

By oral administration, an ED_{50} value of 18 mg kg^{-1} d^{-1} was obtained for *cis*-**15**; this corresponds to 21 μmol kg^{-1} d^{-1}, and is in the range reported for artemisinin antimalarial activity (18–30 μmol kg^{-1} d^{-1} against *P. berghei*).[24,25] It should be noted that there was an absence of in vivo toxicity at doses of 120 mg kg^{-1} d^{-1}. Infected mice treated at this dose orally for four days did not experience any toxic effects over 60 days of being monitored, and weight loss was not observed for cured mice. The absence of toxicity at high doses administered orally has also been observed in non-infected mice (100 mg kg^{-1} d^{-1} for three consecutive days).

In addition, it should be noted that the ED_{50} value for oral treatment with *cis*-**15** was only three to fourfold higher than that obtained when treatment was intraperitoneal; this suggests that this drug has a rather good bioavailability by oral administration.

In vitro genotoxicity evaluation of cis-15 (DU1302c): The genotoxicity of trioxaquine *cis*-**15** was also evaluated. Agents that damage DNA, such as alkylating or oxidative agents, or UV radiation, also induce systems of DNA repair, one of which involves a complex system of cellular changes known as the SOS response. The SOS response has been exhaustively studied for *Escherichia coli*, and it has been shown that a correlation exists between the ability of a drug to induce the SOS response in *E. coli* and the genotoxicity that it will display in humans.[26] To evaluate the potential genotoxicity of *cis*-**15**, we measured its ability to induce the SOS response in a modified strain of *E. coli* (GE 864). This strain shows an increased expression of β-galactosidase upon induction of the SOS response.[27] The mutagen drug mitomycin C was used as a standard. The β-galactosidase activity was measured through hydrolysis of the *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and was monitored spectrophotometrically at 420 nm.[28] The ability to induce the SOS response was then compared for trioxaquine *cis*-**15** (5 μM in DMSO) and mitomycin C (3 μM in H_2O) (pure DMSO and H_2O were tested as blank experiments). The results are presented in Figure 4. Only baseline β-galactosidase activity was detected when *E. coli* was treated with *cis*-**15**, either in H_2O or DMSO. Therefore, in contrast to mitomycin C, trioxaquine *cis*-**15** was unable to induce the SOS response. As a result, higher concentrations of *cis*-**15** were tested, and even at a concentration of 20 μM, which is 1000 times more than what was used to obtain the antimalarial IC_{50} values (results not shown), *cis*-**15** was unable to induce the SOS response.

Stability of the trioxaquine cis-15: An antimalarial drug candidate should be stable under most storage conditions. Indeed, as determined by ^{1}H NMR spectroscopy, crystalline trioxaquine *cis*-**15** underwent less than 5% decomposition upon storage in air at room temperature for more than six months, or in acidic solution (HCl 0.01 M) for three days. The thermal stability of *cis*-**15** is also very good. Upon heating the crystalline trioxaquine at 60 °C in air for 24 h, less than 5% decomposition was observed (^{1}H NMR spectroscopy).

Conclusions

A family of new antimalarial drugs has been prepared by modification of the convergent synthesis of trioxaquine **5a**.

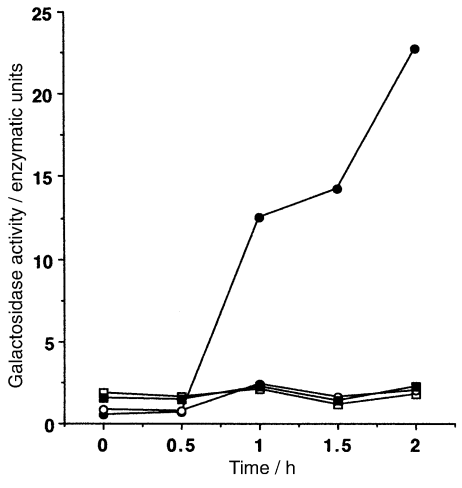


Figure 4. Genotoxicity test: trioxaquine *cis*-**15**-mediated induction of β-galactosidase expression by *E. coli* [5 μM in DMSO (■)]. Mitomycin C [3 μM in H_2O (●)], H_2O (□), and DMSO (○) are given for comparison.

Compound **5a** was the first dual antimalarial drug to combine both a trioxane entity that acts as a potential alkylating agent, as in artemisinin, and a 4-aminoquinoline moiety similar to chloroquine. Therefore, trioxaquines are composed of two fragments that act on a common target, namely heme released from parasite hemoglobin digestion, but do so through two different mechanisms. This concept of "covalent bitherapy" is better than a simple combination of an aminoquinoline with a trioxane, as it allows potent antimalarial agents to be prepared, while at the same time considerably reducing the risk of drug resistance.

Trioxanes functionalized by a ketone were synthesized by reaction of a diketone with the photo-oxygenation product of a diene, namely 1,4-diphenyl-1,3-cyclopentadiene or α-terpinene. The described trioxaquines were obtained by reductive amination of the resultant trioxane–ketones with a 4-aminoquinoline that bears a primary amine side chain. Synthetic modifications (quinoline, diamine, diene, diketone) of the parent trioxaquine afforded compounds with excellent in vitro antimalarial activities against different CQS and CQR strains of *P. falciparum*. The majority of IC_{50} values obtained were in the range 5–40 nM.

Trioxaquine *cis*-**15** (DU1302c) was selected for in vivo evaluation. Mice infected with *P. vinkei* were successfully treated in a four-day suppressive test with a once daily intraperitoneal or oral administration. The doses required to decrease parasitemia by 50% (ED_{50}) were 5 and 18 $mg\,kg^{-1}\,d^{-1}$ for intraperitoneal and oral administration, respectively. Parasitemia clearance was complete without recrudescence at an intraperitoneal dose of 20 $mg\,kg^{-1}\,d^{-1}$. In addition, trioxaquine *cis*-**15** did not induce any toxic effects in mice treated by the oral administration of 120 $mg\,kg^{-1}\,d^{-1}$ over four consecutive days.

In conclusion, trioxaquine *cis*-**15** meets the main criteria of pharmacological activity necessary for further biological evaluation, namely the absence of genotoxicity, as well as good chemical stability. Therefore, *cis*-**15** can be considered as a promising antimalarial drug candidate.

Experimental Section

Chemical materials and methods: NMR spectra were recorded on Bruker AM250, DPX300, or AMX400 spectrometers. Chemical shifts are given with respect to external trimethylsilane (TMS). DCI mass spectra were acquired on a Nermag R10-10H instrument and electrospray (ES) mass spectra were obtained on an API 365 Sciex Perkin Elmer instrument. Chromatography columns were conducted on silica gel 60 ACC Chromagel, 70–200 μm granulometry (SDS, Peypin, France), and 60 F_{254} plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography. Chloroquine diphosphate, primaquine diphosphate, artemisinin, and mitomycin C were purchased from Sigma Aldrich, France.

Crystallographic data

***cis*-12**: $C_{16}H_{24}O_4$, $M_r = 280.35$, triclinic, $P\bar{1}$, $a = 5.9954(5)$, $b = 9.3036(8)$, $c = 14.2689(12)$ Å, $\alpha = 106.335(1)$, $\beta = 90.329(2)$, $\gamma = 100.609(2)°$, $V = 744.32(11)$ Å^3, $Z = 2$, $T = 193(2)$ K, 5064 reflections (3617 independent, $R_{int} = 0.0143$), largest electron density residue: 0.410 e Å^{-3}, R_1 [for $I > 2\sigma(I)$] = 0.0518 and $wR_2 = 0.1493$ (all data) with $R_1 = \Sigma \| F_o | - | F_c \| / \Sigma | F_o |$ and $wR_2 = [\Sigma w(F_0^2 - F_c^2)^2 / \Sigma w(F_0^2)^2]^{1/2}$.

***trans*-12**: $C_{16}H_{24}O_4$, $M_r = 280.35$, triclinic, $P\bar{1}$, $a = 6.056(2)$, $b = 9.230(3)$, $c = 14.055(4)$ Å, $\alpha = 104.992(6)$, $\beta = 90.626(6)$, $\gamma = 100.614(6)°$, $V = 744.5(3)$ Å^3, $Z = 2$, $T = 193(2)$ K, 2431 reflections (1824 independent, $R_{int} = 0.0201$), largest electron density residue: 0.277 e Å^{-3}, R_1 [for $I > 2\sigma(I)$] = 0.0642 and $wR_2 = 0.1905$ (all data).

***trans*,*cis*-14**: $C_{27}H_{36}ClN_3O_3$, $M_r = 485.04$, monoclinic, $P2_1/c$, $a = 17.429(2)$, $b = 11.143(1)$, $c = 12.932(1)$ Å, $\beta = 91.443(2)°$, $V = 2510.7(5)$ Å^3, $Z = 4$, $T = 153(2)$ K, 8518 reflections (3206 independent, $R_{int} = 0.0492$), largest electron density residue: 0.255 e Å^{-3}, R_1 [for $I > 2\sigma(I)$] = 0.0594 and $wR_2 = 0.1464$ (all data).

***cis*,*cis*-14**: $C_{27}H_{36}ClN_3O_3$, $M_r = 485.04$, orthorhombic, $Pca2_1$, $a = 13.079(4)$, $b = 11.157(3)$, $c = 35.171(10)$ Å, $V = 5132(3)$ Å^3, $Z = 8$, $T = 193(2)$ K, 11630 reflections (5093 independent, $R_{int} = 0.1790$), largest electron density residue: 0.318 e Å^{-3}, R_1 [for $I > 2\sigma(I)$] = 0.0921 and $wR_2 = 0.2488$ (all data). Very small, weak diffracting crystals and high disorder problems account for the high R values obtained, as well as a data/parameter ratio of 6.16.

Data for all the structures were collected at low temperatures using an oil-coated shock-cooled crystal on a Bruker-AXS CCD 1000 diffractometer with $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å). The structures were solved by direct methods (SHELXS-97)[29] and all non-hydrogen atoms were refined anisotropically using the least-squares method on F^2.[30] CCDC-219910–219913 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK; fax: (+44) 1223-336033; e-mail: deposit@ccdc.cam.ac.uk).

Synthesis of the aminoquinolines: In a typical procedure,[8] a mixture of 4,7-dichloroquinoline (5.0 g, 25 mmol) and α,ω-diaminoalkane (5.0 mol equiv) was stirred at 85 °C for 5 h. Sodium hydroxide (40 mmol) was then added (1 M aqueous solution), and the resultant solid was extracted with EtOAc (250 mL) at 50 °C. The organic layer was washed with distilled water, brine, distilled water again, dried over anhydrous sodium sulfate, and the solvent was evaporated under vacuum to dryness. Aminoquinolines **1a–c** were used without further purification. Proton numbering is reported in Figure 1 (the same numbering applies for both aminoquinolines **1a–c** and the aminoquinoline moiety of trioxaquines **5a–c**). The synthesis of compounds **1a**, **4a**, and **5a** was reported in ref. [8].

7-Chloro-4-[*N*-(3-aminopropyl)amino]quinoline (1b): Light-yellow powder; yield: 75%; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 1.58$ (br s, 2H; H_2NC13'), 1.87 (m, 2H; H12′), 3.03 (t, 2H; H13′), 3.39 (m, 2H; H11′), 6.30 (d, $^3J = 5.5$ Hz, 1H; H3′), 7.29 (dd, $^3J = 8.9$ and $^4J = 2.2$ Hz, 1H; H6′), 7.50 (br s, 1H; HNC4′), 7.70 (d, $^3J = 8.9$ Hz, 1H; H5′), 7.90 (d, $^4J = 2.2$ Hz, 1H; H8′), 8.48 ppm (d, $^3J = 5.5$ Hz, 1H; H2′); MS (DCI/NH_3^+): *m/z* (%): 235 (2), 236 (100) $[M+H]^+$, 237 (14), 238 (34), 239 (5).

7-Chloro-4-[*N*-(4-aminobutyl)amino]quinoline (1c): Light-yellow powder; yield: 60%; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 1.45$ (br s, 2H; H_2NC14'), 1.64 (m, 2H; H13′), 1.85 (m, 2H; H12′), 2.81 (t, 2H; H14′), 3.29 (m, 2H; H11′), 6.04 (br s, 1H; HNC4′), 6.36 (d, $^3J = 5.5$ Hz, 1H; H3′), 7.31 (dd, $^3J = 8.9$ and $^4J = 2.2$ Hz, 1H; H6′), 7.72 (d, $^3J = 8.9$ Hz, 1H; H5′), 7.92 (d, $^4J = 2.2$ Hz, 1H; H8′), 8.50 ppm (d, $^3J = 5.5$ Hz, 1H; H2′);

MS (DCI/NH_3^+): *m/z* (%): 249 (2), 250 (100) $[M+H]^+$, 251 (18), 252 (36), 253 (5).

Synthesis of the trioxane–ketones: In a typical procedure, the endoperoxide (25 mmol) was first obtained by photo-oxygenation (irradiation with a 600 W visible lamp at 0–5 °C under bubbling oxygen with tetraphenylporphyrin as photosensitizer) of the corresponding diene in dichloromethane (50 mL). Formation of the endoperoxide was monitored by ^{1}H NMR spectroscopy of the crude mixture (1–5 h from the starting diene). When photo-oxygenation was complete, the crude solution was cooled to −78 °C. Cyclohexane-1,4-dione (6.0 mol equiv with respect to the starting diene) and Me_3SiOTf (0.4 equiv) were added, and the reaction was stirred at −78 °C for 2 h. Triethylamine (1 equiv) was then added and the solution was allowed to warm up to room temperature. The organic layer was washed with distilled water, dried over anhydrous sodium sulfate, and the solvent was evaporated. The light-brown powder isolated was purified by column chromatography. Proton and carbon atom numbering is reported in Scheme 2 and 3 for compounds **8** and **12**, respectively.

When 1,3-cyclohexadiene was used, the photo-oxygenation product consisted of a mixture of the desired 1,4-endoperoxide (80%) and 3-hydroperoxo-1,4-cyclohexadiene (20%). This mixture was then used without purification.

Trioxane–ketone 8: Chromatography: SiO_2, hexane/EtOAc, 70:30, v/v; white solid; yield: 7%. Two diastereomeric racemates **8a** and **8b** were obtained and were not separated. Characterization was performed on the mixture, but for clarity, the ^{1}H NMR signals of each diastereomeric racemate are described separately. ^{1}H NMR (250 MHz, $CDCl_3$): **8a** $\delta = 1.55$ (m, 1H; H9), 1.92 (m, 1H; H9), 2.02 (m, 2H; cyclohexanone), 2.32 (m, 2H; H8), 2.41 (m, 5H; cyclohexanone), 2.68 (m, 1H; cyclohexanone), 4.25 (ddd, $^3J = 13.0$, 8.5, and 3.5 Hz; 1H; H10), 4.43 (m, 1H; H5), 5.53 (m, 1H; H7), 5.70 ppm (m, 1H; H6); **8b** $\delta = 1.70$–2.70 (m, 12H; H9, H8, and cyclohexanone), 4.33 (m, 1H; H10), 4.52 (m, 1H; H5), 5.70 (m, 1H; H7), 5.92 ppm (m, 1H; H6); MS (DCI/NH_3^+): *m/z* (%): 242 (100) $[M+NH_4]^+$, 243 (40), 244 (17).

Trioxane–ketone 12: Chromatography: SiO_2, hexane/EtOAc, 70:30, v/v; then SiO_2, pentane/diethyl ether, 75:25, v/v. Three trioxane–ketones were separated. In order of elution they were *trans*-**12**, *cis*-**12**, and *trans*-**13**. The ratio of purified compounds *trans*-**12**/*cis*-**12**/*trans*-**13** was 12:78:10. Yield of *cis*-**12** was 26% with respect to α-terpinene.

***trans*-12**: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.00$ (d, $^3J = 6.8$ Hz, 6H; H13 and H14), 1.41 (s, 3H; H11), 1.63 (m, 2H; H9), 2.05 (m, 2H; cyclohexanone), 2.15 (m, $^3J = 6.8$ Hz, 1H; H12), 2.24 (m, 2H; H8), 2.30–2.55 (m, 5H; cyclohexanone), 2.65 (m, 1H; cyclohexanone), 4.61 (m, 1H; H5), 5.29 ppm (m, 1H; H6); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 15.3$ (C11), 21.7 and 21.8 (C13 and C14), 26.2 (C8), 27.5 (CH_2 cyclohexanone), 29.6 (C9), 34.2 (C12), 34.2, 36.8, and 37.1 (CH_2 cyclohexanone), 73.1 (C5), 81.2 (C10), 103.0 (C3), 118.4 (C6), 145.3 (C7), 210.5 ppm (C17); MS (DCI/NH_3^+): *m/z* (%): 298 (100) $[M+NH_4]^+$, 299 (27), 300 (11). Slow evaporation of a solution of *trans*-**12** in a mixture of hexane/EtOAc 80:20 v/v, afforded monocrystals that were subsequently analyzed by X-ray diffraction.

***cis*-12**: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.02$ and 1.04 (2×d, $^3J = 6.4$ Hz, 6H; H13 and H14), 1.10 (brs, 3H; H11), 1.55 (m, 1H; H9), 2.03 (m, 2H; cyclohexanone), 2.10–2.35 (m, 4H; H12, H8, and 1H cyclohexanone), 2.35–2.55 (m, 4H; cyclohexanone), 2.75 (m, 2H; 1H cyclohexanone and 1H C9), 4.06 (m, 1H; H5), 5.44 ppm (m, 1H; H6); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 19.8$ (C11), 21.5 and 21.8 (C13 and C14), 25.7 (C9), 26.4 (C8), 27.8 (CH_2 cyclohexanone), 34.2 (CH_2 cyclohexanone), 35.0 (C12), 37.0 and 37.2 (CH_2 cyclohexanone), 68.3 (C5), 79.3 (C10), 101.4 (C3), 116.4 (C6), 150.5 (C7), 210.8 ppm (C17); MS (DCI/NH_3^+): *m/z* (%): 298 (100) $[M+NH_4]^+$, 299 (22), 300 (7). Slow evaporation of a solution of isomer *cis*-**12** in a mixture of hexane/EtOAc 80:20 v/v, afforded monocrystals that were subsequently analyzed by X-ray diffraction.

***trans*-13**: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.35$ (s, 3H; H11), 1.74 (m, 1H; H9), 1.94 (m, 4H; 1H H9 and H14), 2.01 and 2.08 (m, 4H; H19 and H15), 2.16 (m, 1H; H8), 2.42 (m, 1H; H8), 2.51 (m, 4H; H18 and H16), 4.34 (d, $^3J = 4.4$ Hz, 1H; H5), 5.03 (s, 1H; H13), 5.13 (s, 1H; H13), 5.89 ppm (d, $^3J = 4.4$ Hz, 1H; H6); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta =$ 21.0 (C14), 24.1 (C8), 24.5 (C11), 32.9 (C9), 36.0 and 37.2 (C19 and C15), 38.6 and 38.8 (C18 and C16), 78.1 (C5), 79.0 (C10), 107.4 (C3), 114.2 (C13), 120.5 (C6), 142.1 (C7), 142.8 (C12), 211.2 ppm (C17); MS (DCI/NH_3^+): *m/z* (%): 280 (100), 281 (22), 282 (8).

Synthesis of the trioxaquines: In a typical procedure,[8] the trioxane–ketone (1.0 mol equiv, 50 mM) and aminoquinoline (1.25 equiv) were mixed in dichloromethane. Sodium triacetoxyborohydride (1.25 equiv) was then added. The reaction mixture was stirred at room temperature for 15–20 h and was then washed twice with distilled water. The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated under vacuum to dryness. For the preparation of trioxaquine **17**, the commercially available primaquine diphosphate was initially deprotonated with aqueous sodium hydroxide (1 M), and the primaquine base was then extracted with dichloromethane. This step was quantitative.

Proton and carbon atom numbering is reported in Figure 1 (the same numbering is used for each trioxaquine and the corresponding citrate salt, for instance for trioxaquine **4a** and its citrate **5a**, for **9** and **10**, for *cis*-**14** and *cis*-**15**, as well as for **17** and **18**).

Trioxaquine 4b: Light-yellow oil; yield: 95%; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 1.25$–2.10 (m, 10H; H10, H11, H13, H14, and H12′), 2.42 (m, 1H; H12), 2.60 (m, 1H; HNC12), 2.95 (m, 3H; 1H H8 and 2H H13′), 3.29 (d, 1H; H8), 3.39 (q, 2H; H11′), 5.10 and 5.17 (2×brs, 2×0.5H; H5), 6.30 (m, 2H; H6 and H3′), 7.25–7.65 (m, 11H; phenyl and H6′), 7.75 and 7.83 (2×d, $^3J = 10.0$ Hz, 2×0.5H; H5′), 7.93 (2×d, $^4J = 3.0$ Hz, 2×0.5H; H8′), 8.01 (brs, 1H; HNC4′), 8.50 ppm (2×d, $^3J = 6.0$ Hz, 2× 0.5H; H2′); MS (DCI/NH_3^+): *m/z* (%): 580 (5), 582 (100) $[M+H]^+$, 583 (39), 584 (39), 585 (15).

Trioxaquine 4c: Light-yellow oil; yield: 69%; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 1.25$–2.10 (m, 12H; H10, H11, H13, H14, H12′, and H13′), 2.46 (m, 1H; H12), 2.61 (m, 1H; HNC12), 2.74 (q, 2H; H14′), 3.00 (d, 1H; H8), 3.30 (m, 3H; H8 and H11′), 5.18 (2×brs, 2×0.5H; H5), 5.96 (brs, 1H; HNC4′), 6.35 (m, 2H; H6 and H3′), 7.25–7.65 (m, 11H; phenyl and H6′), 7.78 (2×d, 2×0.5H; H5′), 7.89 (2×d, 2×0.5H; H8′), 8.50 ppm (2×d, 2×0.5H; H2′); MS (DCI/NH_3^+): *m/z* (%): 596 (100) $[M+H]^+$.

Trioxaquine 9: Light-yellow oil; yield: 40%; the two diastereomeric racemates were not separated. ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 1.25$–2.40 (m, 12H; H10, H11, H13, H14, H8, and H9), 2.65 (m, 2H; HNC13 and H13), 3.00 (m, 2H; H12′), 3.27 (m, 2H; H11′), 4.05–4.25 (m, 1H; H10), 4.42 (m, 1H; H5), 5.45–5.90 (m, 2H; H6 and H7), 6.03 (brs, 1H; HNC4′), 6.32 (m, 1H; H3′), 7.30 (m, 1H; H6′), 7.66 (m, 1H; H5′), 7.89 (m, 1H; H8′), 8.46 ppm (m, 1H; H2′); MS (DCI/NH_3^+): *m/z* (%): 430 (100) $[M+H]^+$, 431 (29), 432 (45).

Trioxaquine *cis*-14: Light-yellow oil; yield: 60%; MS (DCI/NH_3^+): *m/z* (%): 486 (100) $[M+H]^+$, 487 (36), 488 (42), 489 (12), 490 (2).

Separation of the two diastereomeric racemates of *cis*-14: The 50:50 mixture of *trans*,*cis*-**14** and *cis*,*cis*-**14** was diluted (12 g L^{-1}) at room temperature in a mixture of dichloromethane/cyclohexane (1:8, v/v). Upon cooling to −20 °C, a 70:30 mixture of *trans*,*cis*-**14** and *cis*,*cis*-**14** precipitated. The precipitate was recovered by filtration. Further purification on silica gel (EtOAc/triethylamine, 95:5, v/v) provided quantitative recovery of the pure *trans*,*cis*-**14**, which eluted first. *cis*,*cis*-**14** was then eluted, but chromatography resulted in the loss of about 65% of this latter compound. The supernatant solution, which contained a mixture of *trans*,*cis*-**14** and *cis*,*cis*-**14** (30:70), was evaporated and the two diastereomeric racemates were purified in the same manner as the precipitate. The overall yield of trioxaquine *cis*-**14** isolated, as calculated from the commercially available α-terpinene starting material was 20%.

***trans*,*cis*-14**: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.02$ (d, $^3J = 6.9$ Hz, 3H; H14), 1.04 (d, $^3J = 6.9$ Hz, 3H; H13), 1.05 (brs, 3H; H11), 1.52 (m, 2H; 1H H9 and 1H cyclohexyl), 1.59 (m, 2H; cyclohexyl), 1.70–1.90 (m, 5H; cyclohexyl), 2.10–2.30 (m, 4H; H8, 1H H12, and HNC17), 2.60–2.80 (m, 2H; H17 and 1H H9), 3.07 (m, 2H; H12′), 3.36 (m, 2H; H11′), 3.98 (brs, 1H; H5), 5.42 (d, 1H; H6), 6.20 (brs, 1H; HNC4′), 6.35 (d, $^3J = 5.4$ Hz, 1H; H3′), 7.35 (dd, $^3J = 8.9$ and $^4J = 2.1$ Hz, 1H; H6′), 7.76 (d, $^3J = 8.9$ Hz, 1H; H5′), 7.91 (d, $^4J = 2.1$ Hz, 1H; H8′), 8.48 ppm (d, $^3J = 5.4$ Hz, 1H; H2′); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 19.8$ (C11), 21.5 (C13), 21.8 (C14), 25.7 (C9), 26.6 (C8), 28.0–35.0 (C15, C16, C18, and C19), 35.0 (C12), 42.8 (C11′), 45.1 (C12′), 55.3 (C17), 67.4 (C5), 79.0 (C10), 99.5 (C3′), 102.5 (C3), 116.8 (C6), 117.7 (C10′), 122.1 (C5′), 125.7 (C6′), 128.6 (C8′), 135.3 (C7′), 149.1 (C9′), 150.0 (C7), 150.4 (C4′), 152.1 ppm (C2′).

Slow evaporation of a solution of *trans,cis*-**14** in a mixture of EtOH/EtOAC 50:50 v/v afforded monocrystals that were subsequently analyzed by X-ray diffraction.

***cis,cis*-14**: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.98$ (d, $^3J = 6.9$ Hz, 3H; H14), 0.99 (d, $^3J = 6.9$ Hz, 3H; H13), 0.99 (brs, 3H; C11), 1.30–1.60 (m, 4H; 1H C9 and 3H cyclohexyl), 1.70–1.90 (m, 5H; cyclohexyl), 2.00–2.30 (m, 4H; H8, H12, and HNC17), 2.40–2.70 (m, 2H; 1H H9 and H17), 2.97 (m, 2H; H12′), 3.24 (m, 2H; H11′), 4.02 (brs, 1H; H5), 5.37 (d, 1H; H6), 6.12 (brs, 1H; HNC4′), 6.30 (d, $^3J = 5.4$ Hz, 1H; H3′), 7.29 (dd, $^3J = 9.0$ and $^4J = 2.1$ Hz, 1H; H6′), 7.68 (d, $^3J = 9.0$ Hz, 1H; H5′), 7.88 (d, $^4J = 2.1$ Hz, 1H; H8′), 8.44 ppm (d, $^3J = 5.4$ Hz, 1H; H2′); ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 20.5$ (C11), 21.0 (C13), 21.2 (C14), 23.0–35.0 (C8, C9, C15, C16, C18H_2, and C19), 34.5 (C12), 42.4 (C11′), 44.6 (C12′), 55.4 (C17), 67.2 (C5), 78.0 (C10), 98.9 (C3′), 102.0 (C3), 116.3 (C6), 117.3 (C10′), 121.8 (C5′), 125.1 (C6′), 127.9 (C8′), 134.7 (C7′), 148.7 (C9′), 149.5 (C7), 150.1 (C4′), 151.6 ppm (C2′).

Slow diffusion of diethyl ether into a dichloromethane solution afforded monocrystals that were subsequently analyzed by X-ray diffraction.

Trioxaquine 17: Column chromatography: SiO_2, hexane/EtOAc/Et_3N, 60:35:5, v/v/v). Although several stereoisomers were present, they were not separated. Light-yellow oil; yield: 63%; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.90$–1.10 (m, 9H; H13, H14, and H11), 1.28 (2×d, $^3J = 6.3$ Hz, 3H; H12′), 1.30–1.90 (m, 13H; 1H H9, H15, H16, H18, H19, H14′, and H15′), 2.00–2.30 (m, 3H; H8 and H12), 2.40–2.80 (m, 5H; 1H H9, H16′, H17, and HNC17), 3.60 (m, 1H; H13′), 3.86 (s, 3H; H11′), 3.98 (2×brs, 1H; H5), 5.39 (brs, 1H; H6), 6.03 (d, $^3J = 8.1$ Hz, 1H; HNC8′), 6.27 (d, $^4J = 2.4$ Hz, 1H; H7′), 6.30 (d, $^4J = 2.4$ Hz, 1H; H5′), 7.28 (dd, $^3J = 4.2$ and $^3J = 8.3$ Hz, 1H; H3′), 7.90 (brd, $^3J = 8.0$ Hz, 1H; H4′), 8.50 ppm (dd, $^3J = 4.2$ and $^4J = 1.2$ Hz, 1H; H2′); MS (DCI/NH_3^+): *m/z* (%): 524 (100) $[M+H]^+$, 525 (62), 526 (17), 527 (2).

Synthesis of the trioxaquine citrates: In a typical procedure,[8] the relevant trioxaquine (20–200 mg) was solubilized in acetone (0.5–5 mL) before a solution of anhydrous citric acid (2.5 mol equiv) in acetone (0.5–5 mL) was added. The trioxaquine dicitrate spontaneously precipitated, and after being centrifuged, the precipitate was washed with diethyl ether and dried under vacuum. Elemental analysis and NMR spectroscopy confirmed the presence of two citric acid or citrate molecules per trioxaquine. In DMSO solution, monoprotonation of the trioxaquine was attested by the chemical shifts of the quinoline and diaminoalkane tether protons. Proton numbering is reported in Figure 1.

Trioxaquine citrate 5b: White powder (quantitative yield from **4b**); ^{1}H NMR (250 MHz, [D_6]DMSO): $\delta = 1.40$–2.20 (m, 10H; H12′, H10, H11, H13, and H14), 2.65 (d, $^2J = 15.1$ Hz, 4H; citric acid/citrate), 2.76 (d, $^2J = 15.1$ Hz, 4H; citric acid/citrate), 3.00–3.80 (m, 7H; H8, H11′, H13′, and H12), 5.43 (2×brs, 1H; H5), 6.61 (2×dd, 1H; H6), 6.72 (2×d, 1H; H3′), 7.45 (m, 6H; phenyl), 7.65 (m, 5H; 4H phenyl and H6′), 7.93 (2×d, 1H; H8′), 8.42 (2×d, 1H; H5′), 8.60 ppm (2×d, 1H; H2′); MS (ES$^+$): *m/z* 582.3 (monoprotonated base); elemental analysis calcd (%) for ($C_{47}H_{52}ClN_3O_{17}$, 1H_2O): C 57.35, H 5.53, N 4.27; found: C 57.09, H 5.20, N 4.24.

Trioxaquine citrate 5c: White powder (quantitative yield from **4c**); ^{1}H NMR (250 MHz, [D_6]DMSO): $\delta = 1.50$–2.10 (m, 12H; H12′, H13′, H10, H11, H13, and H14), 2.65 (d, 4H; citric acid/citrate), 2.76 (d, 4H; citric acid/citrate), 3.10–3.70 (m, 5H; H8, H14′, and H12), 3.50 (m, 2H, H11′), 5.43 (2×brs, 1H; H5), 6.61 (2×dd, 1H; H6), 6.75 (2×d, 1H; H3′), 7.53 (m, 6H; phenyl), 7.71 (m, 5H; 4H phenyl and H6′), 7.96 (m, 2H; H8′ and HNC4′), 8.47 (2×d, 1H; H5′), 8.57 ppm (2×d, 1H; H2′); MS (ES$^+$): *m/z* 596.2 (monoprotonated base); elemental analysis calcd (%) for ($C_{48}H_{54}ClN_3O_{17}$, 1H_2O): C 57.74, H 5.65, N 4.21; found: C 57.91, H 5.59, N 4.33.

Trioxaquine citrate 10: White powder (quantitative yield from **9**); ^{1}H NMR (250 MHz, [D_6]DMSO): $\delta = 1.50$–2.60 (m, 12H; H8, H9, H11, H12, H14, and H15), 2.68 (d, 4H; citric acid/citrate), 2.78 (d, 4H; citric acid/citrate), 3.35 (m, 3H, H11′ and H13), 3.77 (m, 2H; H12′), 4.05–4.40 (m, 1H; H10), 4.50–4.65 (m, 1H; H5), 5.55–6.10 (m, 2H; H6 and H7), 6.78 (m, 1H; H3′), 7.70 (m, 1H; H6′), 7.98 (m, 1H; H8′), 8.38 (m, 1H; H5′), 8.63 ppm (m, 1H; H2′); MS (ES$^+$): *m/z* 430.3 (monoprotonated base); elemental analysis calcd (%) for ($C_{35}H_{44}ClN_3O_{17}$, 1H_2O): C 50.51, H 5.57, N 5.05; found: C 50.87, H 5.20, N 5.09.

Trioxaquine citrate *cis*-15: White powder (quantitative yield from *cis*-**14**); ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta = 0.97$ (m, 9H; H11, H13, and H14), 1.10–2.50 (m, 13H; H8, H9, H12, H15, H16, H18, and H19), 2.57 (d, $^2J = 15.2$ Hz, 4H; citric acid/citrate), 2.67 (d, $^2J = 15.2$ Hz, 4H; citric acid/citrate), 3.24 (m, 3H; H12′ and HC17), 3.69 (m, 2H; H11′), 3.98 and 4.06 (2×brs, 1H; H5), 5.32 (m, 1H; H6), 6.70 (d, $^3J = 5.8$ Hz, 1H; H3′), 7.59 (dd, $^3J = 9.0$ and $^4J = 2.1$ Hz, 1H; H6′), 7.88 (d, $^4J = 2.1$ Hz, 1H; H8′), 8.29 (d, $^3J = 9.0$ Hz, 1H; H5′), 8.50 ppm (d, $^3J = 5.8$ Hz, 1H; H2′); MS (ES$^+$): *m/z* 486.2 (monoprotonated base); elemental analysis calcd (%) for ($C_{39}H_{52}ClN_3O_{17}$, 1H_2O): C 52.73, H 6.13, N 4.73; found: C 52.66, H 6.30, N 4.52.

Trioxaquine citrates *trans,cis*-**15** and *cis,cis*-**15** were prepared in the same way, that is, from the separated trioxaquine diastereoisomers *trans,cis*-**14** and *cis,cis*-**14**, respectively.

Trioxaquine citrate 18: Trioxaquine **17** (164 mg) was dissolved in acetone (4 mL) and a solution of 2.0 equivalents of citric acid (120 mg) in acetone (4 mL) was added with stirring. The trioxaquine citrate **18** was obtained by evaporation of the solvent. Orange powder (quantitative yield from **17**); ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta = 0.96$ (m, 9H; H11, H13, and H14), 1.23 (d, $^3J = 6.3$ Hz, 3H; H12′), 1.30–2.30 (m, 16H; H14′, H15′, H8, H9, H15, H16, H18, and H19), 2.57 (d, $^2J = 15.2$ Hz, 4H; citric acid/citrate), 2.66 (d, $^2J = 15.2$ Hz, 4H; citric acid/citrate), 2.94 (m, 2H; H16′), 3.07 (m, 1H; H17), 3.37 (brs, 1H; HN), 3.70 (m, 1H; H13′), 3.83 (s, 3H; H11′), 4.00 and 4.04 (2×brs, 2×0.5H; H5), 5.33 (d, 1H; H6), 6.18 (d, $^3J = 8.9$ Hz, 1H; HNC8′), 6.31 (d, $^4J = 2.4$ Hz, 1H; H7′), 6.50 (d, $^4J = 2.4$ Hz, 1H; H5′), 7.44 (dd, $^3J = 8.3$ and $^3J = 4.2$ Hz, 1H; H3′), 8.09 (dd, $^3J = 8.3$ and $^4J = 1.6$ Hz, 1H; H4′), 8.30 (brs, HNC17), 8.55 ppm (dd, $^3J = 4.2$ and $^4J = 1.6$ Hz, 1H; H2′); MS (ES$^+$): *m/z* 524.4 (monoprotonated base); elemental analysis calcd (%) for ($C_{43}H_{61}N_3O_{18}$, 1H_2O, 1C_3H_6O): C 56.15, H 7.07, N 4.27; found: C 55.74, H 6.78, N 4.12.

Biological activity of the trioxaquines

Biological materials: Four strains of *P. falciparum* were cultured according to a modified Trager and Jensen method in a 5% CO_2 atmosphere at 37 °C.[31,32] The chloroquine-sensitive strains, Nigerian ($IC_{50} = 62$ nM) and F32-Tanzania ($IC_{50} = 25$ nM), and both chloroquine-resistant strains, FcB1-Columbia ($IC_{50} = 116$ nM) and FcM29-Cameroon ($IC_{50} = 174$ nM), were chosen for this study. The parasites were maintained in vitro in human red-blood cells ($O^{\pm}$) that were diluted to 1% hematocrit in RPMI 1640 medium (BioMedia, Boussens, France) and supplemented with 25 mM Hepes complemented with 5% human AB serum (Centre de Transfusion Sanguine, Toulouse, France). Parasite cultures tested were not synchronized in vitro. Male Swiss albino mice, which weighed 30–40 g, were obtained from C.E.R Janvier (France). *P. vinckei petteri* (279BY) was provided by Dr. I. Landau (Museum National d'Histoire Naturelle, Paris, France).[33]

In vitro antimalarial activity: The antiplasmodial activity of the trioxaquines was evaluated by the radioactive microdilution method described by Desjardins et al. and modified as follows.[34,35] Drug dilutions were tested several times in triplicate in 96-well plates (TPP, Switzerland) that contained cultures at various stages of 1% parasitemia, and that had a 1% hematocrit.[35] For each test, the plates of parasite culture were incubated with drugs at decreasing concentrations for 48 h, and radioactive hypoxanthine was added to the medium 24 h after the beginning of incubation. The stock solutions of the trioxaquines (5 mg mL^{-1}), primaquine, and artemisinin (1 mg mL^{-1}) were prepared in DMSO (Acros Organics, Belgium), while the stock solution of chloroquine was prepared in RPMI 1640 (1 mg mL^{-1}). All dilutions were done in RPMI 1640, and it was ensured that the trioxaquines did not reprecipitate under these conditions. Parasite growth was estimated by [^{3}H]hypoxanthine (Amersham Pharmacia Biotech, France) incorporation. Concentrations of trioxaquines that inhibited 50% of the parasite growth (IC_{50}) were graphically determined by plotting the drug concentration versus percent of parasite growth inhibition at 48 h of incubation.[36] The IC_{50} values given in the text represent the mean value of 2–5 independent experiments (as mentioned in the footnotes of Table 2). The chloroquine diphosphate, artemisinin, and primaquine diphosphate (Sigma Aldrich, France) sensitivities for the four strains were routinely tested.

In vivo antimalarial activity: In vivo antimalarial activity was determined against the rodent strain *P. vinckei petteri* according to a modified version of the four-day Peters' suppressive test.[37] Swiss mice were inoculated in-

travenously with 10^7 parasitized red-blood cells (resuspended in 0.2 mL saline medium). Thereafter, the drug was administered to the animals once daily in DMSO for four consecutive days. Drug treatment was initiated 24 h after parasite inoculation to study the curative properties of the compounds and was administered intraperitoneally (100 μL) or orally (100 μL). Parasitemia levels were determined on the day following the last treatment. ED_{50}, which is the dose that leads to 50% parasite growth inhibition in comparison to the control test (treated with an equal volume of vehicle) was evaluated from a plot of activity (expressed as % of control) versus the log dose. For each dose, three animals were treated, while six control animals were considered. Treatment was considered as curative when no parasites were detected after 60 days.[37]

Genotoxicity evaluation: An exponential culture of the *Escherichia coli* modified strain GE864 was grown in an M9 liquid medium (6 $g L^{-1}$ Na_2HPO_4, 3 $g L^{-1}$ KH_2PO_4, 0.5 $g L^{-1}$ NaCl, 1 $g L^{-1}$ NH_4Cl autoclaved for 20 min at 120 °C, then supplemented with 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM 0.2% glucose, 1 $\mu g mL^{-1}$ thiamine, and 0.1% "casaminoacid"). This *E. coli* culture was treated with *cis*-**15** at concentrations of 5–20 μM in DMSO or with 3 μM mitomycin C in H_2O. The culture was incubated at 37 °C and aliquots were withdrawn after 0, 30, 60, 90, and 120 min of incubation. The absorbance of the aliquots was measured at 600 nm to evaluate the quantity of bacteria. Bacteria were lysed with chloroform and a phosphate buffer that contained β-mercaptoethanol (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol). To measure the β-galactosidase activity the lysed cultures were incubated at 28 °C and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added. After a determined time of incubation, Na_2CO_3 (1 M) was added to quench the reaction, the bacterial fragments were centrifuged, and absorbance of the supernatant at 420 nm was measured to quantify the *o*-nitrophenate. The enzymatic activity *u* was calculated according to the following formula and plotted against time: $u = 1000 \times$ OD(420 nm)/reaction time × reaction volume × OD(600 nm).[28]

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