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8, CQ-R *P. falciparum* IC_{50} = 160 nM
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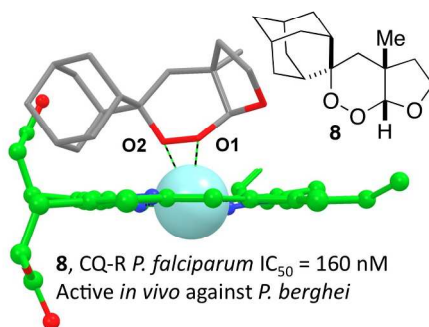
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Synthetic Spirocyclic Endoperoxides: New Antimalarial Scaffolds

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Here we report the development of a straightforward synthetic procedure for the preparation of spirocyclic endoperoxides as synthetic analogues of the natural product dihydroplakortin. Peroxides here presented are more potent antiplasmodials than dihydroplakortin itself and we proved for the first time their antimalarial activity in vivo.

Cyclic peroxides such as 1,2-dioxolanes, 1,2,4-trioxanes and 1,2-dioxanes are a class of organic compounds with interesting pharmacological properties and widely represented in nature. Artemisinin (ART) is an endoperoxide-based natural product, which is highly effective against clinically relevant *P. falciparum* strains responsible for human malaria. Currently, the so-called artemisinin-based combination therapies (ACT) are employed as first line treatment in most malaria endemic countries, adhering to WHO recommendations.[1-3] However, lower susceptibility to artemisinins is being reported from highly malaria endemic regions.[4] So, novel peroxides characterized by different structural features could delay the potential selection of *P. falciparum* resistant strains.[5] Moreover, cost associated to the extraction of this drug or to synthetic precursors from natural sources, prompted researcher at developing synthetic peroxides as low-cost alternatives to ART.[6]

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Since several years we are engaged in the design of suitable synthetic strategies for the preparation of antimalarial endoperoxides.[7-10] In particular we performed the first stereoselective synthesis of 9,10-dihydroplakortin (DHP, **1**, Figure 1),[10] a natural product endowed with interesting antiplasmodial properties.[11] We also reported the development of novel endoperoxides as synthetic analogues of DHP characterized by a bicyclic tetrahydrofuro[2,3-*c*][1,2]dioxane core. The synthetic peroxide **2** (Figure 1) was the most potent compound of the series and showed higher antimplasmodial potency than dihydroplakortin. The major limitation of these analogues was their synthesis as an inseparable mixture of diastereoisomers.[7] The structure-activity relationships (SARs) for this class of compounds were rationalized on the basis of the mechanism of action proposed for ART.
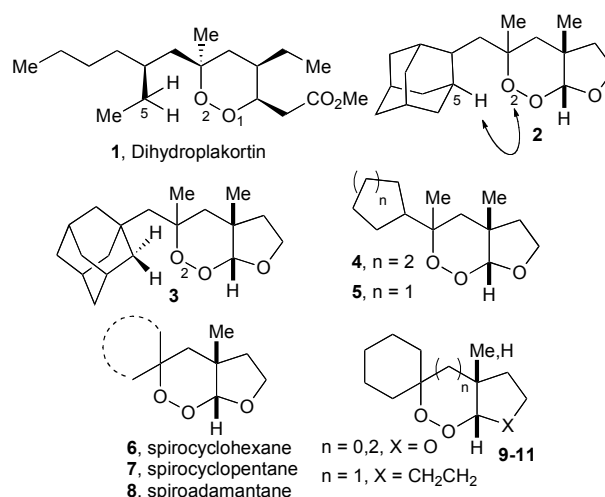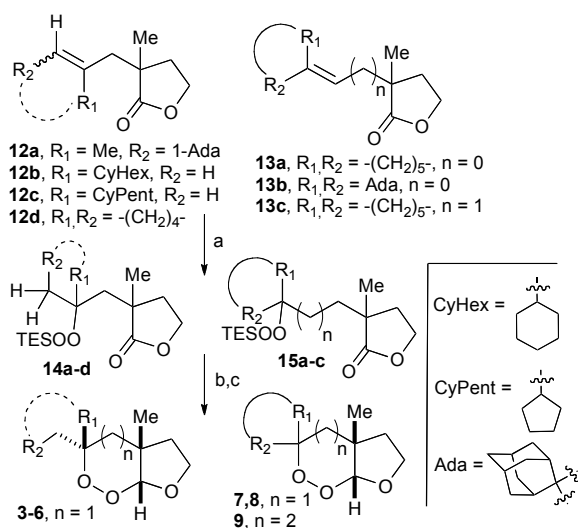

**Figure 1** Structure of reference (**1-2**) and title (**3-11**) endoperoxides

Scheme 1 General synthesis of endoperoxides **3-9**. [Reaction conditions: a) Co(thd)$_2$, Et$_3$SiH, O$_2$, t-BuOOH (5 M in nonane), 1,2-dichloroethane (1,2-DCE), 25 °C, 4 h; b) DIBAL, DCM, -78 °C, 1.5 h; d) TMSOTf, DCM, -78 °C, 5 min.]

Although the mechanism of action of ART is still debated,[12] it seems that the peroxide bond is activated within the food vacuole of the parasite, a specialized organelle accommodating the hydrolysis of hemoglobin, a necessary source of amino acids for the parasites. During this process, free Fe(II)-heme is released and reacts with the peroxide bond of ART to form an O-centered radical, that probably undergoes a 1,5-H shift to form a highly toxic C-centered radical,[13,14] ultimately responsible of the antiplasmodial activity. The formation of C-centered radicals has also been proposed for several synthetic endoperoxides, comprising DHP.[15-17] For our synthetic peroxides, we found that subtle modifications of the substituent at C3, affecting the distance between the C5 of the lateral chain and the O2 of the peroxide bridge (C$_5$-O$_2$ distance < 3 Å for optimal activity), had strong impact on the antiplasmodial potency, in line with the mode of action of ART and DHP.[7] As a continuation of our previous work, we were interested in developing suitable synthetic strategies aimed at modifying the lateral chain at C3 of the bicyclic skeleton (**3-8**) and the size of the rings forming the bicyclic system (**9-11**) reducing the number of chiral centers. In particular, we were interested in the synthesis of spiro-derivatives **6-8** that lack the stereogenic center at C3 and represent a further simplification of this class of compounds.

As described in Scheme 1, a three-step reaction protocol was employed for the assembly of the bicyclic core starting from olefin intermediates **12a-d** and **13a-c**. The first step of the protocol consisted in the Mukaiyama peroxysilylation reaction[18,19] of the double bond of intermediates **12a-d** and **13a-c** to afford peroxides **14a-d** and **15a-c**, respectively. The Mukaiyama reaction proceeds regioselectively at the more substituted carbon of the double bond. Subsequently the lactone functionality was reduced to the corresponding lactol by using diisobutylaluminum hydride (DIBAL). Finally, trimethylsilyl triflate (TMSOTf)-promoted simultaneous deprotection and cyclization of the resulting lactols afforded the cyclised endoperoxides **3-5**, isolated as inseparable
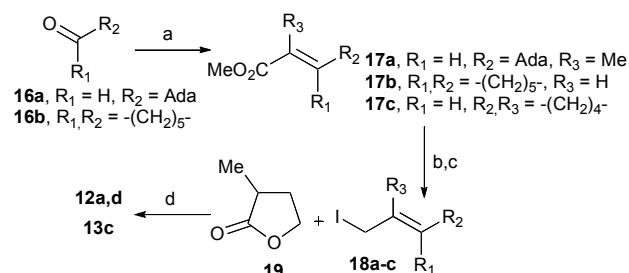

Scheme 2 Synthesis of intermediates **12a,d** and **13c**. [Reaction conditions: a) triethyl 2-phosphonopropionate (for **17a**), or triethyl phosphonoacetate (for **17b**), NaH, THF, 0 °C, 1 h, then 25 °C, 2 h; b) DIBAL, DCM, -78 °C, 1.5 h; c) I$_2$, PPh$_3$, imidazole, 1:3 MeCN/Et$_2$O, 0 °C, 30 min (from **17a,c**) or KI, BF$_3$•Et$_2$O, dioxane, 25 °C, 3 h (from **17b**); d) i. **19**, LiHMDS, THF, -78 °C, 30 min; ii. **17a-c**, -78 °C, 30 min, then 25 °C, 2 h.]
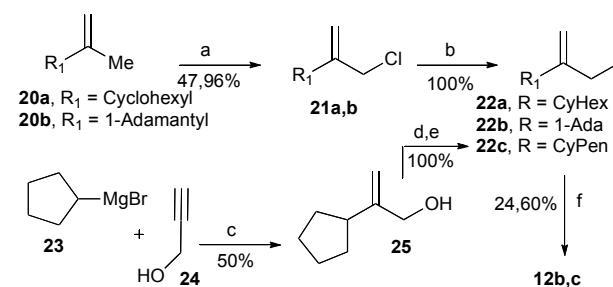
Scheme 3 Synthesis of intermediates **12b,c**. [Reaction conditions: a) CeCl$_3$x7H$_2$O, 10-13% NaOCl, 1:1 DCM/H$_2$O, 25 °C, 14 h; b) NaI, acetone, 25 °C, 18 h; c) CuI, THF, 25 °C 24 h, 80 °C, 5 h; d) MsCl, Et$_3$N, Et$_2$O, 0 °C, 12 h; (e) LiI, Et$_2$O, sonication, 25 °C, 3 h; (f) i. **19**, LiHMDS, THF, -78 °C, 30 min; ii. **22a,c**, -78 °C, 30 min, then 25 °C, 2 h.]
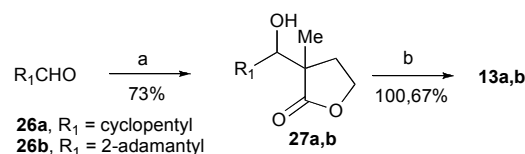
Scheme 4 Synthesis of intermediates **13a,b**. [Reaction conditions: a) **19**, LDA, -78 °C, 20 min, then 25 °C, 8 h; (b) SOCl$_2$, pyridine, 25 °C, 2 h.]
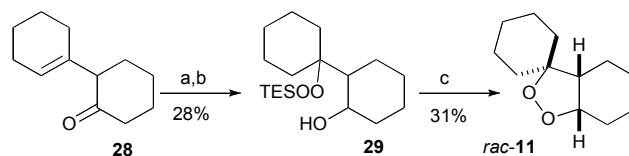
Scheme 5 Synthesis of rac-**11**. [Reaction conditions: a) NaBH$_4$, EtOH, 0 °C, 1 h, then 25 °C, 12 h; b) Co(thd)$_2$, Et$_3$SiH, O$_2$, t-BuOOH (5 M in nonane), 1,2-DCE, 25 °C, 4 h; c) MsCl, Et$_3$N, DCM, 0 °C, 2 h.

mixtures of diastereoisomers, and spiroperoxides **6-9** obtained as racemates. Following a slightly modified protocol, the synthesis of endoperoxide **10** is reported as Supplementary Information (SI). The synthesis of intermediates **12a,d** and **13c** is described in Scheme 2. 1-Adamantylaldehyde **16a** and cyclohexanone **16b** were submitted to the Horner–Wadsworth–Emmons olefination reaction to afford the α,β-unsaturated esters **17a,b**. DIBAL-mediated reduction of above esters **17a,b** and of commercially available ester **17c** afforded the corresponding primary alcohols that were

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subsequently converted into the iodides **18a-c.** While iodides **18a,c** were obtained by treatment of the alcohols with iodine, triphenylphosphine, and imidazole, iodide **18b** could not be obtained by using the same protocol. **18b** was instead obtained by treatment of the alcohol precursor with potassium iodide in the presence of boron trifluoride. Iodides **18a-c** were next used to alkylate lactone **19** using lithium hexamethyldisilylamide (LiHMDS) as the base to afford **12a,d** and **13c.** Intermediates **12b,c,** bearing cyclohexyl and cyclopentyl substituents, were prepared as reported in Scheme 3. The synthesis of an analogue bearing an adamantyl-radical at the same position was also attempted without success. Accordingly, cyclohexyl and 1-adamantyl derivatives **20a,b** were treated with cerium chloride as a Lewis acid and sodium hypochlorite as the oxidizing agent to afford chloromethyl derivatives **21a,b** in reasonable yields. Intermediates **21a,b** were in turn converted into the corresponding iodides **22a,b.** Intermediate **22c** was synthesized starting from **25,** in turn

prepared following a different synthetic approach by reacting the Grignard reagent **23,** formed in situ, with propargyl alcohol **24.**[20] The resulting primary alcohol **25** was then converted to **22c** via a mesyl ester intermediate that was sonicated in the presence of lithium iodide to afford the desired iodide **22c** in good overall yield. Iodides **22a-c** were used as alkylating agents for the enolate of lactone **19.** While **22a,c** smoothly furnished the corresponding alkylation products, the same reaction performed with the adamantyl derivative **22b** failed, probably due to its higher steric hindrance. For the synthesis of intermediates **13a,b** (Scheme 4), alcohol intermediates **27a,b,** in turn obtained by aldol condensation of aldehydes **26a,b** with **19** in the presence lithium diisolpropylamide (LDA), were dehydrated to the corresponding olefins. Finally, dioxolane *rac-***11** was synthesized as described in Scheme 5. The carbonyl group of the commercially available ketone **28** was reduced using sodium borohydride and the double bond

Table 1 Antiplasmodial activity, calculated O_1, O_2---Fe(II)-heme distances[b], and ΔG_{bind} (kcal/mol)[c]

Cpd	Structure	D10	W2	O_2-Fe[b]	O_1-Fe[b]	ΔG_{bind}[c] (kcal/mol)
		IC_{50} (µM)[a]				
3		0.85	0.39	2.27 (E1)[d] 2.21 (E2)[e]	2.13 (E1) 2.16 (E2)	-29.33 -30.48
4		0.39	0.14	2.25 (E1) 2.20 (E2)	2.22 (E1) 2.22 (E2)	-30.28 -29.25
5		1.2	0.8	2.31 (E1) 2.34 (E2)	2.24 (E1) 2.32 (E2)	-27.30 -26.55
6		5.3	1.1	3.89	3.03	-23.82
7		0.84	0.47	2.81	2.24	-28.43
8		0.61	0.16	2.18	2.16	-30.69
9		>20	>17.0	3.36	3.90	-19.76
10		8.5	>25	2.40	2.99	-24.46
11		7.2	3.2	2.34	3.22	-22.64
1[7]		0.86	0.44	2.20	2.27	-31.04
ART		0.029	0.012	2.14	2.10	-43.13
CQ		0.015	0.228	-	-	-

[a]IC_{50} values are the mean of at least three determinations in duplicate. Standard errors were all within 10% of the mean. [b]The distances were calculated and reported in Å by means of measurement tool implemented in Maestro graphical interface from the poses obtained by docking calculation; [c]The binding energy was calculated by means of Prime MM-GSBA[21]; [d]E1 = epimer 1: 3*R**,4a*S**,7a*R**; [e]E2 = epimer 2: 3*S**,4a*S**,7a*R**.

was hydroperoxysilylated to **29** employing Mukaiyama reaction conditions. Treatment of **29** with methanesulfonyl chloride in the presence of triethylamine directly afforded the cyclization product *rac*-**11**.

The antiplasmodial activity of the novel endoperoxides **3-11** was tested against two laboratory *P. falciparum* strains, the chloroquine-sensitive (CQ-S) D10 and the chloroquine-resistant (CQ-R) W2, accordingly to described procedures.[7] The resulting IC_{50} are reported in Table 1.

To understand the modulation of antiplasmodial potency observed for the synthesized compounds, we in depth analyzed the interaction of the peroxide moiety of compounds **4-11** with Fe(II)-heme (which is the first and the key step for the formation of the toxic radical species) by molecular docking analysis employing Glide software, after structure optimization performed by using *ab initio* calculation employing the Jaguar software.[22,23] Endoperoxides here reported have been produced and tested as mixtures of diastereoisomers (**3-5**) or as racemates (**6-11**), while docking studies were performed for each enantiomer. As expected, heme is unable to discriminate between enantiomers[24] and no differences in the binding mode of each enantiomeric couple were observed. Based on our molecular docking calculations, we found a good correlation between the distance of oxygen atoms from the reactive heme iron center and the antimalarial activity (Table 1). In fact, the compounds that display a larger distance of the peroxide moiety from the iron center such as compound **6**, **9-11** were found less potent *in vitro* than compounds **3-5,7,8** exhibiting a shorter distance. For example, peroxide **4** (one of the most active compound in the set) displaying a very short distance between the peroxide oxygen atoms and the iron center (Table 1) and both atoms could be able to coordinate the heme iron. On the contrary, the inactive analogue **9** does not possess the appropriate distance to interact with heme iron.

For comparison, distances calculated for the reference compounds ART and DHP are also in good agreement with a correct interaction with heme (Table 1 and Figure S1 in the Supporting Information). Also the free-binding energy[21] (ΔG_{bind}) calculated for all complexes (Table 1), displayed a good correlation with antiplasmodial potency, being around -20 kcal/mol for inactive compounds (**9**), comprised between -20 and -25 kcal/mol for compounds in the micromolar range (**6,10,11**), and < -25 kcal/mol for compounds in the low micromolar range (**1**, **3-5,7,8**). It is worth noticing that ART presents a calculated ΔG_{bind} well below the value found for the spiroperoxides here presented, consistent with its nanomolar antiplasmodial potency. Compound **8**, displaying an estimated ΔG_{bind} of -30.69 kcal/mol, is the most potent plakortin-related endoperoxide described to date.[11] Accordingly, both epimers of compounds **4** and **5** (Figure 2A-D) interact with heme in a similar fashion. However, the larger steric hindrance of the cyclohexyl ring of **4** with respect to the cyclopentyl ring of **5** results in a stronger hydrophobic interaction with heme for **4**. For the couple of analogues **6** and **7** (Figure 3A,B), the steric hindrance of the cyclohexyl ring physically hampers the accommodation of **6** for a strong interaction with Fe(II)-heme (Figure 2A), while the cyclopentyl ring of **7** (Figure 3B) has a reduced steric hindrance and is well tolerated allowing both O_2 and O_1 of the peroxide
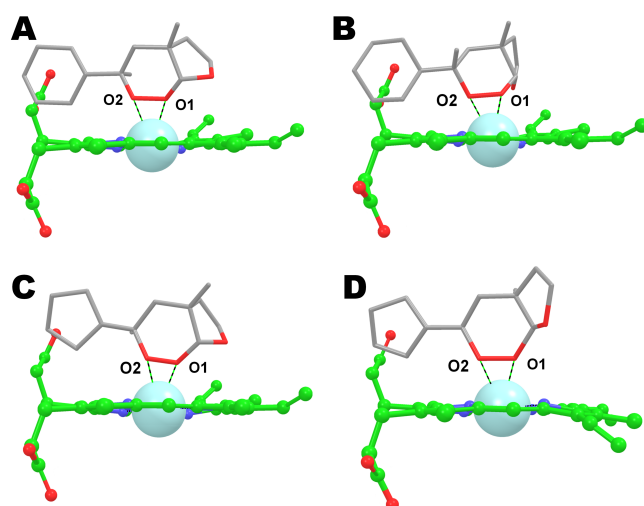

Figure 2 Docked poses of both epimers of **4** (panels A and B in gray sticks) and **5** (panels C and D) in complex with heme (green balls and sticks), charged iron was colored cyan and represented by CPK model. The potential ligand-metal coordination bonds are reported as black-green dotted lines. The picture was generated by Maestro.[25]

Figure 3 Docked poses of **6-9** (gray sticks; A, B, C, and D, respectively) in complex with heme (green balls and sticks), charged iron was colored cyan and represented by CPK model. The potential ligand-metal coordination bonds are reported as black-green dotted lines. The picture was generated by Maestro.[25]
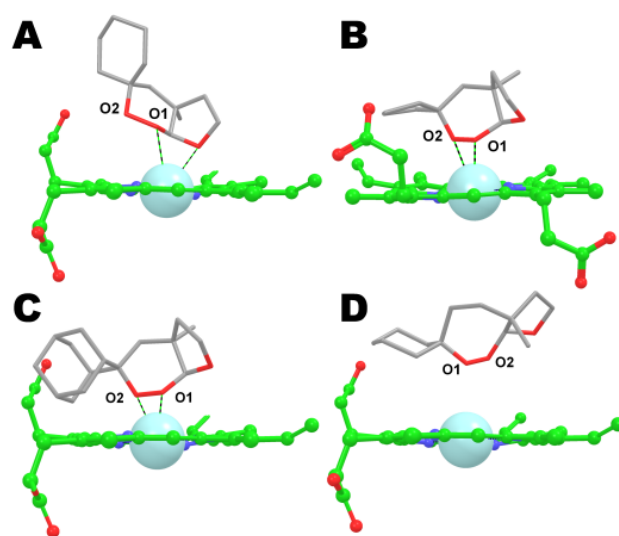
system to interact with Fe(II)-heme. Docking studies (Figures 2,3) highlighted that subtle differences in the structure of the peroxide and in particular of the size and shape of their side chains are responsible for a fine-tuning of the antiplasmodial activity. On the other hand, the bulky adamantane ring (**8**, Figure 3C) is able to maximize hydrophobic interactions with the planar protoporphyrin-IX ring at the same time maintaining a correct orientation of both the peroxide oxygens for their interaction with Fe(II)-heme, thus resulting in an overall improved ΔG_{bind} and, as a consequence, higher antiplasmodial potency. Finally, the seven-membered ring of compound **9** (Figure 3D) is not tolerated since it hampers a favourable conformation for reaching the right distance to iron atom contained in the protoporphyrin ring (Figure 3D).

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Spiroperoxide **8**, the most potent antiplasmodial agent of the series, was submitted to a preliminary *in vivo* evaluation in the *P. berghei* mouse model of malaria using the Peters 4-day test.[26] After administration of **8** at a daily dose of 100 mg/kg (i.p.), mice showed a 72.5% reduction in parasitaemia. Notably, compound **8** did not elicit, at the concentration used in the *in vivo* study, any sign of toxicity in mice consistently with its determined in vitro toxicity against mouse fibroblasts NIH3T3 (TC$_{50}$ = 150 μM).

In conclusion, we herein presented the development of a straightforward synthetic procedure for the preparation of spiroperoxides as racemic mixtures endowed with antiplasmodial activity against *P. falciparum* CQ-S and CQ-R strains. Being heme an achiral target, compounds were tested as racemates since no difference on antiplasmodial activity for each enantiomer could be expected.[24] Molecular modelling studies highlighted key molecular features responsible for activity and potency, and will be useful for further optimizing their in vitro potency. Moreover, we have herein for the first time provided evidence that plakortin-derived synthetic analogues do have antimalarial activity *in vivo*. The straightforward synthetic approach to spirocyclic endoperoxides may pave the way to the development of more potent analogues in vivo.

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