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Enantiomeric 1,2,4-Trioxanes Display Equivalent in vitro Antimalarial Activity Versus *Plasmodium falciparum* Malaria Parasites: Implications for the Molecular Mechanism of Action of the Artemisinins

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The aim of this study was to synthesise pure enantiomers of potent antimalarial 1,2,4-trioxanes, which are related to the natural antimalarial artemisinin, and then to assay each against a panel of Plasmodium falciparum *strains. The working hypothesis was that if the artemisinin derivatives interact with a specific protein-target site, then there should be stereoselective differences in their activity. In five different* P. falciparum *isolates, however, the trioxane enantiomers (+)-**7a**, (−)-**7a** and (+)-**7b**, (−)-**7b**, showed the same level of in vitro antiparasitic activity.*

Introduction

Currently, most workers agree that the mechanism of action of endoperoxide-containing antimalarials, such as artemisinin (**1**), involves iron(II)-catalysed activation to afford one or more transient cytotoxic intermediates. The involvement of iron(II) was initially proposed because of the high selectivity of these drugs for malaria parasites (*Plasmodium falciparum*) during their erythrocytic stage of development. At this stage of the life cycle there is a high concentration of iron(II) in the form of haem.[1–4] Several research groups have recently challenged this assumption, and the identities of the "killing species" and source of iron required for bioactivation are areas of current debate.[5–7] Meshnick et al. have isolated several radiolabelled proteins from malaria-parasite infected erythrocytes that were treated with ^{14}C artemisinin, which suggests that parasite death could be brought about by alkylation of functional proteins.[8–10] Recently, iron(II) degradation of artemisinin in the presence of cysteine was found to result in the isolation of two artemisinin–cysteine adducts.[11] This led Wu and co-workers to propose that in the lethal event, reductive cleavage of the peroxide bond was brought about by intracellular iron–sulfur redox centres (rather than haem) that are common to many of the parasite's enzymes, and that alkylation of these enzymes could lead to parasite death.[12] In addition, artemisinin has been shown to be lethal to the parasite at an immature stage when it lacks a food vacuole and does not have high haematin/haem concentrations.[5]

Krishna and colleagues recently put forward a new biological target for the endoperoxide-containing antimalarials.[13,14] The study proposed that artemisinin and other endoperoxide-containing antimalarials specifically target a parasitic enzyme known as PfATP6. A sesquiterpene-lactone drug called thapsigargin (**2**) has been found to be a highly selective inhibitor of a mammalian Ca-ATPase (SERCA, sarco/endoplasmic reticulum membrane calcium ATPase; Scheme 1); the malaria parasite PfATP6 enzyme is a similar calcium-dependent ATPase. This enzyme was expressed in frogs' eggs and the inhibitory properties of both drugs against PfATP6 were assessed. It was found that both thapsigargin and artemisinin were able to inhibit the enzyme. Inhibition by both drugs in the heterologously expressed system was irreversible. Furthermore, both exhibited equivalent potency against PfATP6, whereas the artemisinins were significantly more effective as antimalarials

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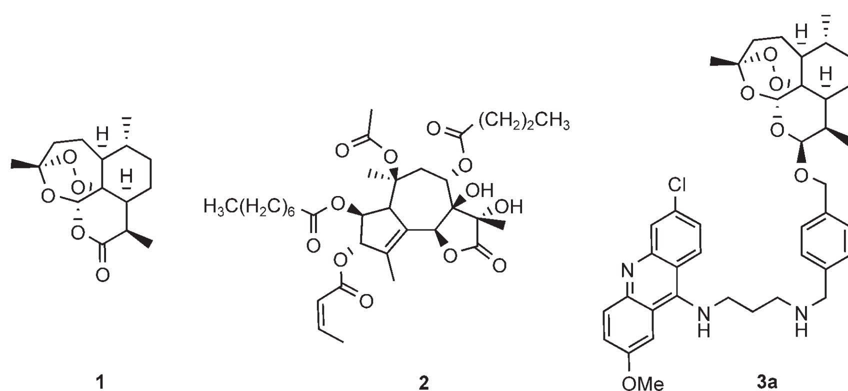


Scheme 1. Structures of artemisinin (**1**), thapsigargin (**2**) and a fluorescent, semisynthetic-artemisinin derivative **3 a**.

pare mirror-image pairs of enantiomerically pure 1,2,4-trioxanes that are related to artemisinin, and then to assay each enantiomer against a panel of *P. falciparum* isolates. As a critical and specific protein target, PfATP6 should like other enzymes, exhibit stereoselective preferences that translate into differences in the antimalarial activity of each enantiomer of a mirror-image pair.

(three-log orders) compared to thapsigargin. However, thapsigargin did antagonise the antimalarial activity of artemisinin which suggests a common target for both drugs (i.e., PfATP6).[13]

A fluorescent artemisinin derivative (**3**; Scheme 1) was synthesized by the O'Neill group and was used to image the distribution of the drug in infected erythrocytes by using confocal microscopy. In support of the PfATP6-inhibition hypothesis the drug was not localized to the food vacuoles but was distributed throughout the parasite cytosol.[13] In addition, Krishna and colleagues have demonstrated that the activity of artemisinin is not dependent on haem.[13]

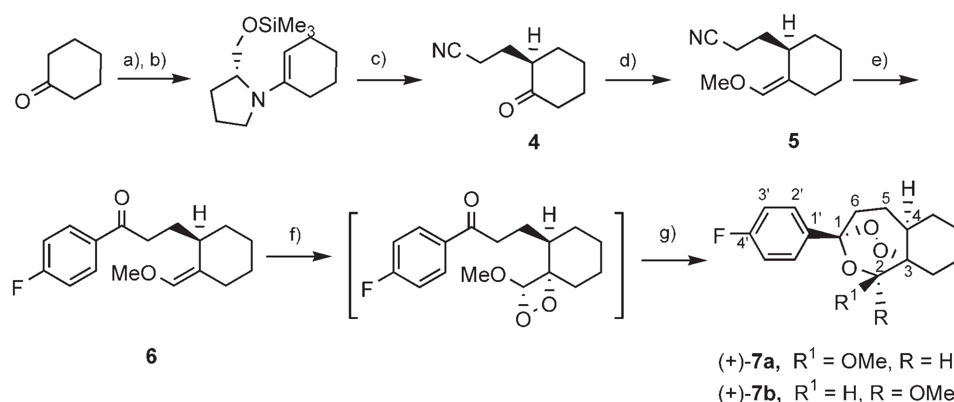
Recently, Haynes and co-workers have also provided some evidence against the "haem theory"[15] and "carbon-centred radicals" in mediating the antimalarial potency of trioxane-based compounds.[16] It has been proposed that the mechanism of action of the artemisinins involves a very specific non-covalent interaction between the protein and drug—a "recognition step"—prior to an irreversible modification of the protein. This mode of action has been proposed to explain the large body of structure–activity relationship (SAR) observations in this field where very minor structural changes lead to profound changes in antimalarial activity.[17,18]

Given the evidence in favour of PfATP6 as a principal target for the trioxane class of drug and also the proposal that a highly specific noncovalent interaction is involved prior to bioactivation, we reasoned that mirror-image pairs of enantiomerically pure 1,2,4-trioxanes could be used to test the "recognition hypothesis". Therefore, the aim of this study was to pre-

Results

Based on a previous expansive SAR study of 28 closely related C-3 aryl 1,2,4-trioxanes,[19] we selected enantiomers of **7 a** and **7 b** as our target molecules. This decision was guided by the fact that i) these analogues retain the A, B, C ring of the natural product,[20] ii) analogues in this class are not only potent in the low nanomolar region against *P. falciparum* in vitro, but are stable and have oral activity equivalent to artemisinin[21] and iii) several members of the C-3 aryl trioxane class have recently undergone extensive preclinical toxicological evaluation.[22]

Previous asymmetric approaches to simplified 1,2,4-trioxane analogues relied on synthetic methodology that delivers a product functionalized at the A–C ring junction in excellent yields and high enantiomeric excesses.[23] However, substitution at the C-5a position provides compounds that have very poor antimalarial activity in vitro.[23] Our improved approach to the synthesis of potent, enantiomerically enriched 1,2,4-trioxanes (**7**) is shown in Schemes 2 and 3. Studies by Ito et al. in 1985 revealed that enamines derived from *R* or *S*-pyrrolidinemethanol react with Michael acceptors, such as methyl acrylate, with



Scheme 2. Asymmetric synthesis of (+)-**7 a** and (+)-**7 b**. a) *R*-(−)-2-pyrrolidinemethanol, TsOH, Dean–Stark; b) DIPEA (1.3 equiv), TMSCl (1.3 equiv); c) acrylonitrile, $MgCl_2$, 20 h, 48%; d) $Ph_3P^+CH_2OMeBr^-$ (1.8 equiv), *n*BuLi (1.1 equiv), 74% from **4**, *Z/E* (1:1); e) 4-F-PhLi (1.1 equiv), Et_2O, 81% from **5**; f) methylene blue, CH_2Cl_2, −78°C, O_2, UV, 20–60 min; g) catalytic TBDMSOTf, CH_2Cl_2, 50 min, then Et_3N; (+)-**7 a**, R^1 = Me, R = H, beta isomer, 15% from **6** (85% *ee*); (+)-**7 b**, R = OMe, R^1 = H, alpha isomer, 25% yield from **6** (85% *ee*). DIPEA = diisopropylethylamine; LDA = lithium diisopropylamide; TBDMS = *tert*-butyldimethylsilyl triflate.

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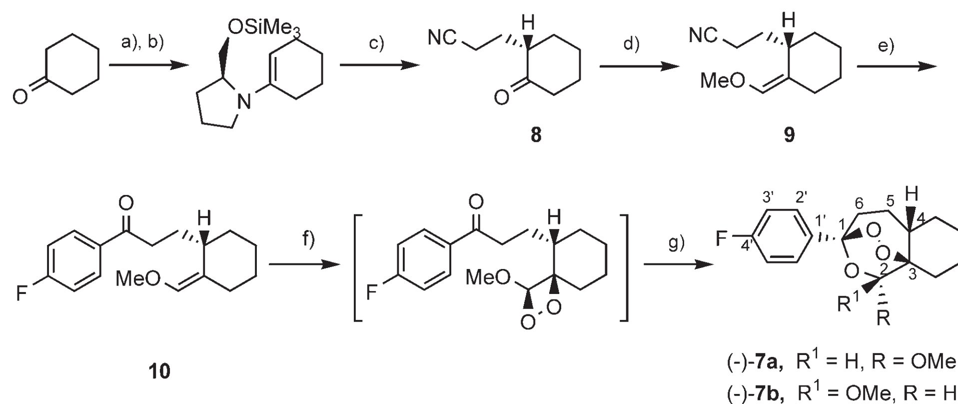

Scheme 3. Asymmetric synthesis of (−)-**7a** and (−)-**7b**. a) *S*-(−)-2-pyrrolidinemethanol, TsOH, Dean–Stark; b) DIPEA (1.3 equiv), TMSCl (1.3 equiv); c) acrylonitrile, $MgCl_2$, 20 h, 45% from cyclohexanone; d) $Ph_3P^+CH_2OMeBr^-$ (1.8 equiv), LDA (1.1 equiv), 68% from **8**, *Z/E* (1:1); e) 4-F-PhLi (1.1 equiv), Et_2O, 36% from **9**; f) methylene blue, CH_2Cl_2, −78 °C, O_2, UV; g) catalytic TBDMSOTf, CH_2Cl_2, 50 min, then Et_3N; (−)-**7a**, R^1 = H, R = OMe, 20% from **10** (88% *ee*); (−)-**7b**, R = OMe, R^1 = H, 19% yield from **10** (87% *ee*).

excellent enantiomeric excesses. The presence of $MgCl_2$ as Lewis acid is crucial for the success of this reaction. We applied this methodology to the synthesis of the key nitrile, **4**.[24]

The enantiomerically enriched ketonitrile (**4**) was prepared from cyclohexanone in two steps without purification of the intermediate. *R*-(+)-Pyrrolidinemethanol was allowed to condense with a slight excess of cyclohexanone in refluxing benzene with a catalytic amount of tosic acid; the water formed by the reaction was removed by azeotropic distillation by using a Dean–Stark apparatus. When the appropriate amount of water was collected the solution was cooled to RT and chlorotrimethylsilane and diisopropylamine (DIPEA) were added. The resulting mixture was heated at gentle reflux to afford the intermediate enamine that was used crude in the next step. Following filtration of the precipitate and removal of benzene, the enamine was redissolved in THF and a slight excess of $MgCl_2$ and acrylonitrile were added. The reaction mixture was stirred at RT for 20 h and was then quenched at low temperature with a mildly acidic solution. The crude material was purified immediately by column chromatography on silica gel to afford the ketonitrile **4** in 48% yield. A Wittig reaction on ketone **4** with the ylide of methoxymethyl triphenylphosphonium chloride gave the enol ether **5** as a mixture of *E* and *Z* isomers in 74% yield. The ratio of isomers varied from 1.5:1 (*E:Z*) to 1:1, but was typically closer to the latter (as observed by the Posner group).[19] Jefford has also reported a 1:1 ratio of isomers obtained by a Horner–Wittig reaction. Only the *Z* isomer was found to undergo a [2+2] cyclo-addition reaction with singlet oxygen (used to introduce the peroxide bond in a subsequent step) so it was necessary to separate the isomers, which was readily achieved by chromatography on silica gel.[25] The next step was the introduction of the aryl ketone by 1,2-addition of a slight excess of 4-fluorophenyllithium (prepared from 4-fluorophenylbromide and *tert*-butyllithium at −78 °C in ether) to the nitrile. After an aqueous workup, aryl ketone **6** was isolated by chromatography on silica gel in good yield (80%). The final step of the synthesis was the introduction of the peroxide bond by photooxygenation of the enol-ether moiety of aryl ketone **6** in dichloromethane at −78 °C either by using rose bengal triethylamine salt or methylene blue as the sensitizer. Photooxygenation is facially selective and occurs only on the *si* face of the enol ether. The intermediate dioxetane was detectable by TLC after 20 min (*p*-anisaldehyde dip) but was not isolated since it is unstable and undergoes fragmentation when warmed to RT. Complete conversion of starting material takes 45–80 min. Following dioxetane formation, a solution of catalytic *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMSOTf) in dichloromethane was added to the reaction at low temperature. Consumption of the intermediate dioxetane was monitored closely by TLC and when the reaction was complete it was quenched with triethylamine and allowed to warm slowly to RT. The products were isolated in 40% yield as a 1.68:1 mixture of alpha and beta diastereomers **7a** and **7b** that were separated by chromatography.

The enantiomers of methoxy diastereomers (+)-**7a** and (+)-**7b** were prepared as shown in Scheme 3 by using *S*-(+)-pyrrolidinemethanol in the first step of the sequence. The chiral trioxanes (−)-**7a** and (−)-**7b** were obtained in 40% yield from **10** and 88% *ee*. For the purposes of this study, we principally focused on the **7a** pair of enantiomers as probes since in the racemic series these isomers express higher antimalarial activity than their C-12 epimeric diastereomers, **7b**.[14] Both of the enantiomers (+)-**7a** and (−)-**7a** were purified to 100% *ee* by using a Chiralcel OJ semipreparative column. Data presented in Table 1 also include antiparasitic activities for the less potent

Table 1. IC_{50} values of enantiomeric 1,2,4-trioxane analogues (+)-**7a**, (−)-**7a** and (+)-**7b**, (−)-**7b** and racemic trioxanes against different strains of *P. falciparum*

Compound	IC_{50} [nM]				
	3D7[a]	Dd2[a]	K1[a]	HB3[a]	NF54[c]
(+)-**7a**	29.7 ± 3.1	24.5 ± 2.2	21.5 ± 1.9	19.20 ± 2.4	29
(−)-**7a**	25.2 ± 3.8	26.4 ± 1.8	24.2 ± 3.4	20.45 ± 1.8	36
rac-**7a**	27.3 ± 2.5	26.7 ± 2.90	n.d.[b]	n.d.[b]	30[d]
(+)-**7b**	62.3 ± 4.9	69.02 ± 1.0	74.4 ± 7.8	n.d.[b]	n.d.[b]
(−)-**7b**	65.3 ± 3.6	68.02 ± 6.0	70.1 ± 5.8	n.d.[b]	n.d.[b]
rac-**7b**	63.2 ± 6.1	61.34 ± 8.7	n.d.[b]	n.d.[b]	60[d]
artemisinin	15.2 ± 4.5	14.2 ± 3.7	n.d.[b]	n.d.[b]	11.7

[a] 3D7 = chloroquine sensitive; DdD2 = multidrug resistant (mefloquine and chloroquine); K1 = chloroquine resistant; HB3 = chloroquine sensitive. [b] n.d. = not determined. [c] Antimalarial-parasite activity was determined as reported previously.[33] The standard deviation for each set of quadruplicates was an average of 8.5 (≤20%) of the mean. R^2 values for the fitted curves were ≥0.985. [d] Data taken from ref. [19].

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diasteromers, (+)-**7b** and (−)-**7b**, against three strains of *P. falciparum*, along with activities obtained for racemic **7a** and **7b**.

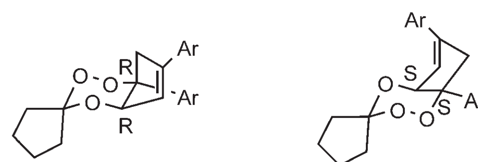
The pure enantiomers were assayed against five different strains of *P. falciparum*. These strains included the highly multidrug resistant Dd2 strain and the chloroquine resistant K1 strain.

As can be seen in Table 1 pairs of enantiomers (+)-**7a**, (−)-**7a** and (+)-**7b**, (−)-**7b** display statistically the same levels of antimalarial potency against chloroquine resistant, sensitive and multidrug resistant strains of *P. falciparum*. As seen for the racemic series,[19] the enantiomerically pure beta methoxy isomers (+)-**7a** and (−)-**7a** have superior activity to their alpha methoxy diastereomers (+)-**7b** and (+)-**7b**.

Discussion

We have prepared both enantiomers of 1,2,4-trioxane **7a** and **7b**, which are related to the natural antimalarial artemisinin, by a novel asymmetric synthesis and have assayed each against a panel of *P. falciparum* malaria parasite strains. The selection of targets **7a** and **7b** was made on the basis that these analogues retain the tricyclic A, B, C ring that is present in the natural product and that racemic **7a** has excellent activity, both in vitro and in vivo, against *Plasmodium beghei* (N-strain) in mice.[19] There was no statistical difference in the level of in vitro activity of trioxane enantiomers (+)-**7a** and (−)-**7a** with the five strains of *P. falciparum* tested. This was also the case for the less potent alpha diastereomers (+)-**7b** and (−)-**7b**. In general, activities of the purified enantiomers were similar to those obtained for racemic mixtures of **7a** and **7b**. These results argue against a specific interaction at an enzyme active site. If chiral recognition by a protein is required before trioxane activation, then each enantiomer of a mirror-image pair of enantiomers should inhibit this enzyme or undergo protein–chiral recognition in a stereospecific manner. These results are consistent with the theory that an achiral-ferrous species triggers the bioactivation of antimalarial trioxanes. The same conclusion was reached in 1995 by Jefford and colleagues based on data obtained for the in vitro activity of enantiomeric pairs of synthetic cyclopento-trioxanes, **11a** and **11b** (Scheme 4);[26] **11a**/**11b** were obtained by chromatography of the racemic mixture on a Chiracel OG column and it was shown that both enantiomers have similar levels of in vitro activity. In addition, in vivo studies in mice confirmed that configuration was not important in determining antimalarial activity; the racemate and individual enantiomers expressed very similar ED_{50} and ED_{90} values regardless of the route of administration. Recently, studies by Najjar and co-workers have also demonstrated that enantiomeric benzyl-ether analogues (**12a** and **12b**) of the natural endoperoxide G3 factor (Scheme 4) have identical activity against *P. falciparum*, in vitro.[27]

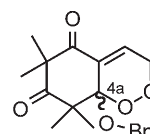
Thus, the conclusions of our study are not just limited to close structural analogues of artemisinin (i.e., **7a** and **7b**) but also extend to structurally distinct 1,2,4-trioxane analogues in the fenozan series and simple semisynthetic endoperoxides in the G3 series.



11a, Ar = 4-F-Ph
IC_{50} = 2.07 ng ml^{-1} (D6 Clone)
ED_{50} = 2.1 mg kg^{-1} (sc)
ED_{50} = 2.6 mg kg^{-1} (po)

11b, Ar = 4-F-Ph
IC_{50} = 3.95 ng ml^{-1} (D6 Clone)
ED_{50} =1.8 mg kg^{-1} (sc)
ED_{50} = 2.1 mg kg^{-1} (po)

racemic **11a**/**11b** IC_{50} = 1.85 ng ml^{-1}



12a, C4a -α-OBn } equipotent in vitro
12b, C4a -β-OBn

racemic **12a**/**12b** IC_{50} = 330 nM

Scheme 4. Structures and antimalarial activities of fenozan enantiomers **11a**/**11b** and semisynthetic G3-factor enantiomers **12a**/**12b**.

Other enantiomeric pairs of antimalarial agents that target haemoglobin derived oxidised haem (i.e., haematin) have also been shown to express virtually the same activity against *P. falciparum*, in vitro. These include enantiomers of chloroquine,[28,29] benflumetol,[30] halofantrine[30] and mefloquine.[31] Although it is widely accepted that haematin is the target for the 4-aminoquinoline class of antimalarial, that the achiral iron species responsible for artemisinins bioactivation is also haem (reduced haematin) has been questioned by several pieces of research and discussed in detail elsewhere.[5,6] By employing the iron chelator desferrioxamine (DFO) it has been demonstrated that there is a definitive iron-dependent mechanism of action for several artemisinin derivatives.[13] The results reported here for close structural analogues of artemisinin are significant since they indicate that this iron-dependent trioxane-bioactivation process and expression of antimalarial effects occurs without stereospecific interactions at the biological target(s).

Experimental Section

Unless otherwise noted, all solvents and reagents were obtained from commercial suppliers and used without further purification. Analytical TLC was performed on aluminium sheets that were precoated with silica gel (Merck). Visualisation was accomplished by UV light (254 nm). Column chromatography was carried out on Merck 938S silica gel. IR spectra were recorded in the range 4000–600 cm^{-1} by using a Perkin–Elmer 298 IR spectrometer. Solid samples were run neat on sodium chloride discs as Nujol mulls and liquids. Proton NMR spectra were recorded by using Bruker NMR spectrometers (400, 250 and 200 MHz). Spectra were referenced to the residual-solvent peak and chemical shifts were expressed in

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ppm from the internal-reference peak. Significant ^{1}H NMR data are written in order: number of protons, multiplicity (br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiple; br s, broad singlet, br m, broad multiplet), coupling constants in Hertz, assignment. (An arbitary numbering system for trioxanes **7a** and **7b** is used in the NMR assignments; see Schemes 1 and 2). Mass spectra were recorded at 70 eV by using a VG7070E and/or micromass LCT mass spectrometers. The molecular ion M^+, with intensities in parentheses is given followed by peaks that correspond to major fragment losses. Melting points were performed by using a Gallemkamp apparatus and are reported uncorrected. Elemental analyses were performed in the microanalysis laboratory at the Department of Chemistry, University of Liverpool (UK). A Chiracel OJ semipreparative column, ethanol (2%)/hexane (98%) and Chiralpak AD column (0.5% IPA in hexane) were used to purify enantiomers described in this study.

***R*-2[2-(Cyanoethyl)]cyclohexanone (4)**: *R*-(−)-2-pyrrolidinemethanol (4.90 g, 4.78 mL, 48.4 mmol) and *p*TsOH (10 mg, 5×10^{-5} mmol) were added to a solution of cyclohexanone (5.17 g, 5.46 mL, 52.7 mmol) in anhydrous benzene (50 mL). The flask was equipped with a Dean–Stark head and the mixture was refluxed vigorously for 3 h with azeotropic distillation of water. Following the collection of sufficient water (~0.9 mL) the reaction was then cooled to 0 °C. TMSCl (12.3 mL, 96.6 mmol) and DIPEA (12.6 mL, 72.6 mmol) were then added which caused immediate formation of a precipitate. The mixture was refluxed gently with vigorous stirring for a further 2 h and cooled to RT. The reaction mixture was then filtered to eliminate the precipitate and the solvent was removed in vacuo to afford the crude enamine, a brown solid which was easily hydrolysed and therefore handled under nitrogen. The crude enamine was dissolved in anhydrous tetrahydrofuran (70 mL) and anhydrous $MgCl_2$ (5.86 g, 61.5 mmol) was added slowly followed by acrylonitrile (3 mL, 47.1 mmol). The mixture was stirred at RT for 20 h and was then cooled to 0 °C by using an ice/water bath. Finally, it was quenched with water (20 mL) and HCl (1.0 M, 10 mL). The organic layer was separated and the solvent was removed in vacuo. The crude material was purified by flash-column chromatography on silica gel (3:7; ethyl acetate/hexane) to give the product as a pale-yellow oil (3.51 g, 48%). In order to prevent racemisation the product was stored in the freezer at −30 °C. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=1.29$–1.97 (6H, m), 1.99–2.17 (3H, m), 2.34–2.51 (4H, m); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=15.8$, 25.7, 26.2, 28.5, 34.8, 42.8, 49.4, 120.2 (CN), 212.2 (CO); ν_{max} (thin film) 2941, 2245 (CN), 1737, 1733 (C=O); CIMS: $m/z=[M+NH_4]^+$ 169 (100); HRMS $[M+NH_4]^+$ calcd: 169.13398; found: 169.13409; $[\alpha]_D=-6°$ ($c=0.7$, $CHCl_3$)

3-(2-Methoxymethylidene-cyclohexyl)-*R*-propionitrile (5): A flame dried round-bottomed flask was charged with flame-dried methoxymethyl triphenylphosphonium chloride (12.2 g, 35.5 mmol) and anhydrous THF (48 mL). The mixture was cooled to 0 °C and then *n*-butyllithium (22.1 mL of 1.6 M in hexane, 35.3 mmol) was added dropwise. The resulting deep-red mixture was warmed to RT and stirred for 1 h. The mixture was cooled to −78 °C and **4** (3.5 g, 23.2 mmol) was added as a solution in anhydrous THF (15 mL) via canula. The reaction mixture was allowed to warm slowly to RT and was stirred for a further 10 h. The mixture was then cooled to 0 °C and quenched cautiously with water/ether (1:1, 100 mL) and was then allowed to warm to RT. The product was extracted into ethyl acetate (3×50 mL), dried over magnesium sulfate and the solvent removed in vacuo. The crude product, a brown solid, was triturated under ether/hexane (1:9) to remove most of the triphenylphosphine oxide and the washings were concentrated. The resulting residue was purified by flash-column chromatography on silica gel (1:24; ethyl acetate/hexane) to afford two isomeric products, yellow oils (1.52 g (*Z*) and 1.52 g (*E*), 74%). Compound **5** ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta=1.21$–1.27 (1H, m), 1.42–1.74 (6H, m), 1.84–1.99 (3H, m), 2.23–2.28 (2H, m), 2.88 (1H, m), 3.51 (3H, s), 5.86 (1H, d, $J=1.75$ Hz); ^{13}C NMR ($CDCl_3$,100 MHz): $\delta=15.6$, 22.1, 26.8, 28.0, 28.4, 31.7, 33.0, 59.6, 117.4, 120.9, 141.8; ν_{max} (thin film) 2932, 2856, 2244 (CN); CIMS $m/z=[M+NH_4]^+$ 197 (32), $[M+H]^+$ 180 (10), 138 (35), 125 $[M\text{-}CH_2CH_2CN]^+$ (14); HRMS $[M+NH_4^+]$ calcd: 197.16539; found: 197.16591; $[\alpha]_D=+58°$ ($c=0.2$, $CHCl_3$). *E* isomer of **5** ^{1}H NMR (400 MHz): $\delta=1.41$–1.24 (1H, m) 1.69–1.49 (6H, m), 2.02–1.78 (3H, m), 2.14–2.09 (1H, m), 2.44–2.18 (2H, m), 3.56 (3H, s), 5.80 (1H); ^{13}C NMR (100 MHz): $\delta=15.8$, 22.4, 22.9, 27.3, 27.5, 33.2, 38.0, 59.8, 118.0, 120.3, 140.8; $[\alpha]_D=-3°$ ($c=0.3$, $CHCl_3$)

1-(4-Fluorophenyl)-3-(2-methoxymethylene-cyclohexyl)-*R*-propan-1-one (6): A solution of 4-fluorophenyllithium was prepared by the addition of 4-fluorophenyl bromide (0.24 mL, 2.15 mmol) to a solution of *tert*-butyllithium (1.48 mL of 1.33 M in pentane, 1.97 mmol) in anhydrous ether (8 mL) at −78 °C. The solution was stirred for 1 h and then a solution of **5** in anhydrous ether (1 mL) was added via canula and the pale-yellow solution turned a dark-yellow colour. The solution was warmed to 0 °C over 0.5 h, recooled to −78 °C and water (3 mL) was added. The mixture was then warmed to RT with vigorous stirring. It was then transferred to a separating funnel and diluted with water (20 mL) and ether (20 mL). The organic layer was removed, dried over magnesium sulfate and the solvent removed in vacuo. The product was purified by flash-column chromatography on silica gel (1:9; ethyl acetate/hexane) to afford a pale-yellow oil (0.40 g, 81%). ^{1}H NMR (200 MHz): $\delta=1.53$–2.05 (10H, m), 2.85–2.94 (3H, m), 3.41 (3H, s, H_7), 5.79 (1H, d, $J=1.66$ Hz), 7.12 (2H, m), 7.98 (2H, m, Ar); ^{13}C NMR (75 MHz): $\delta=21$, 25, 26, 28, 31, 32, 36, 59, 115, 116, 119, 130, 131, 140, 164, 199; ν_{max} (thin film) 2930, 2856, 1682 (C=O); CIMS $m/z=[M+NH_4]^+$ 277 (100), 245 (13), 217 (26); HRMS $[M+NH_4]^+$ calcd: 277.16037; found: 277.16071; $[\alpha]_D=+72°$ ($c=0.07$, $CHCl_3$).

(+)-9-(4-Fluorophenyl)-12α-methoxy-10,11,13-trioxa-tricyclo-[7.2.2.0^{0,0}]tridecane (+)-(7b) and (+)-9-(4-fluorophenyl)-12β-methoxy-10,11,13-trioxa-tricyclo[7.2.2.0^{0,0}] tridecane (+)-(7a): A small amount of methylene blue (<10 mg) was added to a solution of ketone **6** (230 mg, 0.83 mmol) in anhydrous dichloromethane (35 mL) in a two-necked flask that was equipped with inlet and outlet tubes. The solution was cooled to −78 °C and oxygen was bubbled through it for 20 min. The flask was then sealed and equipped with a large oxygen balloon. The stirred solution was irradiated by using a 500 W tungsten-filament lamp and the reaction was monitored by TLC until complete consumption of the starting material was observed (typically 45–80 min). At this stage, the oxygen balloon was replaced with a nitrogen balloon and a solution of TBDMSOTf (0.14 mL, 0.62 mmol) in anhydrous dichloromethane (1 mL) was added via canula to the stirred reaction that was still at −78 °C. The solution was stirred at low temperature for 5 h, quenched by the addition of NEt_3 (0.35 mL, 2.49 mmol) and then allowed to warm slowly to RT. The solvent was removed in vacuo and the residue was purified immediately by chromatography on silica gel (1:49; ethyl acetate/hexane) to afford the two-isomeric products as colourless-crystalline solids (64 mg (α) and 38 mg (β), 40% combined yield).

*α-isomer (**7b**)*: ^{1}H NMR (400 MHz, $CDCl_3$), m.p. 85–87 °C: $\delta=1.14$–1.33 (4H, m), 1.58–1.82 (5H, m), 1.90 (1H, m), 2.25 (1H, H-5, ddd, 14.4, 4.8, 2.44 Hz), 2.40 (1H, H_6, m), 2.82 (1H, ddd, $J=14.4$, 13.2, 3.6 Hz), 3.62 (3H, OCH_3, s), 5.17 (1H, H-2, s), 7.03 (2H, m, Ar), 7.54 (2H, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=23.5$, 25.6 (C-6), 27.5 (C-5),

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32.9, 33.8, 38.0, 45.8 (C-4), 56.4 (C-7), 84.0 (C-3), 96.6 (C-2), 104.1 (C-1), 115.3 (C-3′, d, J_{C_F}=22.0 Hz), 127.8 (C-2′, d, J_{C_F}=8.3 Hz), 137.0 (C-1′, d, J_{C_F}=3.0 Hz), 164.5 (C-4′, d, J_{C_F}=250 Hz); ν_{max} (thin film) 2933, 2863, 1602, 1513, 835; EIMS *m/z*=309 $[M+H]^+$ (7), 276 (7), 123 (100); HRMS $[M+H]^+$ calcd: 309.15021; found: 309.14997; $[\alpha]_D=+27°$ (*c*=1.0, $CHCl_3$). This compound was purified to 100% *ee* by using a Chiralpak AD HPLC column, t_R=28.5 min (0.5% IPA in hexane). $[\alpha]_D=+31°$ (c, 1.0, $CHCl_3$).

*β-isomer (**7a**)*: m.p. 82–84°C, ^1H NMR (400 MHz, $CDCl_3$): δ=1.23–1.29 (2H, m), 1.64–1.76 (7H, m), 1.78–1.91 (2H, m), 2.30 (1H, H_6, ddd, *J*=14.4, 4.8, 3.2 Hz), 2.78 (1H,H_5, ddd, *J*=14.4, 13.2, 3.6 Hz), 3.64 (3H, s, OCH_3), 5.14 (1H, H-2, s), 7.04 (2H, m, Ar), 7.54 (2H, m, Ar); ^{13}C NMR (100 MHz, $CDCl_3$): δ=24.2, 25.4, 27.2, 31.2, 36.0, 39.5, 47.9 (C-4), 57.5 (C-7), 84.2 (C-3), 105.1 (C-1), 105.5 (C-2), 115.3 (C-2′, d, J_{C_F}=21.2 Hz), 127.6 (C-3′, d, J_{C_F}=8.4 Hz), 137.2 (C-1′, J_{C_F}=3.0 Hz), 164.5 (C-4′, d, J_{C_F}=246 Hz); ν_{max} (thin film) 2934, 1600, 875, 832; EIMS *m/z*=309 $[M+H]^+$ (100), 276 (92), 151 (92), 126 (72), 109 (17); HRMS $[M+H]^+$ calcd: 309.15021; found: 309.14997; $[\alpha]_D=+96°$ (*c*=1.0, $CHCl_3$). This material was purified to 100% *ee* by using a Chiracel OJ semipreparative column, ethanol (2%)/hexane (98%), t_R=34.6 min (254 nm), $[\alpha]_D^{24.3}=+125.1°$.

***S*-2[2-(Cyanoethyl)]cyclohexanone (8)**: The procedure described for the synthesis of **4** was employed but *S*-(−)-2-pyrrolidinemethanol instead of *R*-(−)-2-pyrrolidinemethanol was used. The product was obtained after column chromatography (**8**, 3.27 g, 45% yield) in 90% *ee*. $[\alpha]_D=+7.4°$ (*c*=1.6, MeOH) and was spectroscopically identical to **4**.

3-(2-Methoxymethylidene-cyclohexyl)-*S*-propionitrile (9): A flame dried round bottom flask (200 mL) was cooled to 0°C, charged with dry THF (50 mL), diisopropylamine (5.6 mL, 40.0 mmol) and treated with a solution of *n*-butyllithium (1.6 M) in hexane (25.0 mL, 40.0 mmol) with a gas-tight syringe under a dry argon atmosphere. The lithium diisopropylamide (LDA) solution was cannulated into the THF slurry of methoxymethyltriphenyl phosphonium chloride, stirred for 1.0 h at RT and cooled to −78°C. The resulting red ylide was treated with a THF solution of ketone **8** (3.27 g, 21.6 mmol) via cannula over 5 min, allowed to stir at −78°C for 1 h and then warmed to RT over 2 h. After a further 10 h, the reaction was cooled to 0°C, slowly treated with water (50 mL) and ether (50 mL). Following standard workup, the resulting yellow oil was chromatographed on silica gel by using ethyl acetate/hexane (3:97) to give the *Z*- (1.26 g, 33%) and *E*-enol ethers (1.37 g, 35%). The spectroscopic characteristics of **9** were identical to **5**.

1-(4-Fluorophenyl)-3-(2-methoxymethylene-cyclohexyl)-*S*-propan-1-one (10): *t*BuLi (1.7 M) in pentane (8.3 mL, 14.1 mmol) was added to a solution of 4-fluorophenylbromide (1.85 g, 10.5 mmol) in ether (40 mL) at −78°C with a syringe, over 1 min. This solution was stirred at −78°C for 1 h and then nitrile **9** (1.26 g, 7.05 mmol) in ether (5 mL) was added to it dropwise, via a cannula. The reaction was stirred at −78°C for 0.2 h, warmed to 0°C over 0.5 h, cooled to −78°C and finally quenched with water (10 mL). The crude product was obtained by standard workup. Silica-gel chromatography with ethyl acetate/hexane (3:97) as eluent gave the desired ketone **10** (0.70 g, 36%) in 86% *ee* $[\alpha]_D=-76.9°$ (*c*=12, $CHCl_3$).

(−)-9-(4-Fluorophenyl)-12α-methoxy-10,11,13-trioxa-tricyclo-[7.2.2.0^{0,0}]tridecane (−)-(7b) and (−)-9-(4-fluorophenyl)-12β-methoxy-10,11,13-trioxa-tricyclo[7.2.2.0^{0,0}] tridecane (−)-(7a): The two trioxanes (−)-**7b** and (−)-**7a** were prepared from ketone **10** (0.660 g, 2.39 mmol) by using the same procedure employed for the corresponding (+)-enantiomers. Workup and silica-gel chromatography with ethyl acetate/hexane (3:97) as eluent provided (−)-**7a** (51 mg, 20%) as a white solid that was spectroscopically identical to (+)-**7a**; m.p. 82–84°C (lit. 87–88°C). Further chromatography provided the corresponding alpha isomer (−)-**7b** (49 mg, 19%); m.p. 95°C (lit. 97–98°C). This compound was spectroscopically identical to (+)-**7b**. The beta isomer (−)-**7a** was purified to 100% *ee* by using a Chiracel OJ semipreparative column, ethanol (2%)/hexane (98%), t_R=24.6 min (254 nm), $[\alpha]_D^{24.1}=-115°$ (c, 1.2, $CHCl_3$). The alpha diastereomer was purified to 100% *ee* by using a Chiralpak AD semipreparative column. $[\alpha]_D=-33°$ (*c*=1.2, $CHCl_3$).

Antiparasitic activity: For in vitro antimalarial-parasite assessment against the 3D7, Dd2, K1 and HB3 strains of *P. falciparum* the following protocol was employed. Parasites were maintained in continuous culture by using the method of Jensen and Trager.[32] Cultures were grown in flasks that contained human erythrocytes (2–5%) with parasitemia in the range of 1% to 10%. Cultures were suspended in RPMI 1640 medium that was supplemented with HEPES (25 mM), $NaHCO_3$ (32 mM) and human serum (10%; complete medium). Cultures were incubated at 37°C in a mixture of O_2 (3%), CO_2 (4%) and N_2 (93%). Antiparasitic activity was assessed with an adaptation of the 48 h sensitivity assay of Desjardins et al. which uses [^{3}H]-hypoxanthine incorporation as a measurement of parasite growth.[33] Stock drug solutions were prepared in DMSO (100%) and diluted to the appropriate concentration with complete medium. Assays were performed in sterile 96-well microtitre plates. Each plate contained parasite culture (200 μL; 2% parasitemia, 0.5% haematocrit) with or without drug dilutions (10 μL). Each drug was tested in triplicate and parasite growth compared to control wells, which were defined as having 100% parasite growth. After 24 h incubation at 37°C, hypoxanthine (0.5 μCi) was added to each well. Cultures were incubated for a further 24 h before they were harvested onto filter mats, dried for 1 h at 55°C and counted by using a Wallac 1450 Microbeta Trilux liquid scintillation and luminescence counter. IC_{50} values were calculated by interpolation of the probit transformation of the log dose-response curve.

Antiparasitic-activity data recorded for the beta enantiomers (+)-**7a** and (−)-**7a** and racemic **7a** and **7b** against the NF54 strain, were determined by using the procedures detailed in a previous publication.[34]

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