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Biphenylquinuclidines as inhibitors of squalene synthase and growth of parasitic protozoa

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Abstract—In this paper we describe the preparation of some biphenylquinuclidine derivatives and their evaluation as inhibitors of squalene synthase in order to explore their potential in the treatment of the parasitic diseases leishmaniasis and Chagas disease. The compounds were screened against recombinant *Leishmania major* squalene synthase and against *Leishmania mexicana* promastigotes, *Leishmania donovani* intracellular amastigotes and *Trypanosoma cruzi* intracellular amastigotes. Compounds that inhibited the enzyme, also reduced the levels of steroids and caused growth inhibition of *L. mexicana* promastigotes. However there was a lower correlation between inhibition of the enzyme and growth inhibition of the intracellular parasites, possibly due to delivery problems. Some compounds also showed growth inhibition of *T. brucei rhodesiense* trypomastigotes, although in this case alternative modes of action other than inhibition of SQS are probably involved.

1. Introduction

The different forms of leishmaniasis and Chagas disease are caused by the protozoan parasites *Leishmania* spp. and *Trypanosoma cruzi*, respectively. The diseases cause high rates of mortality and morbidity, especially in tropical regions of the world. The current drugs available to treat these conditions suffer from poor clinical efficacy, toxicity and increasing problems due to resistance,[1] hence there is urgent need for new drugs in this area.

The enzymes of the sterol biosynthesis pathway are attractive targets for the specific treatment of these diseases, because the aetiological agents for these diseases require endogenous ergosterol and other 24-alkylated sterols for growth and survival and are unable to use the abundant supply of cholesterol present in the mammalian hosts. There are differences in the enzymes in the biosynthetic pathways of ergosterol and cholesterol, and a number of enzymes in the ergosterol biosynthetic pathway have been investigated as potential drug targets for these organisms and have shown to have great promise. Thus C14α-demethylase,[2–14] sterol 24-methyltransferase,[2,7,15–21] HMGCoA reductase,[22,23] squalene epoxidase,[4,24] squalene synthase and farnesyl pyrophosphate synthase,[25,26] have been studied both individually and in combination, with varying degrees of success. Inhibitors of different steps of the pathway can be used synergistically.

We are interested in squalene synthase, which catalyzes the condensation of two molecules of farnesyl pyrophosphate to produce squalene, the first committed step of the sterol pathway (Fig. 1). This enzyme has been of great interest as a potential drug target for inhibition of cholesterol biosynthesis in humans.[27] A number of

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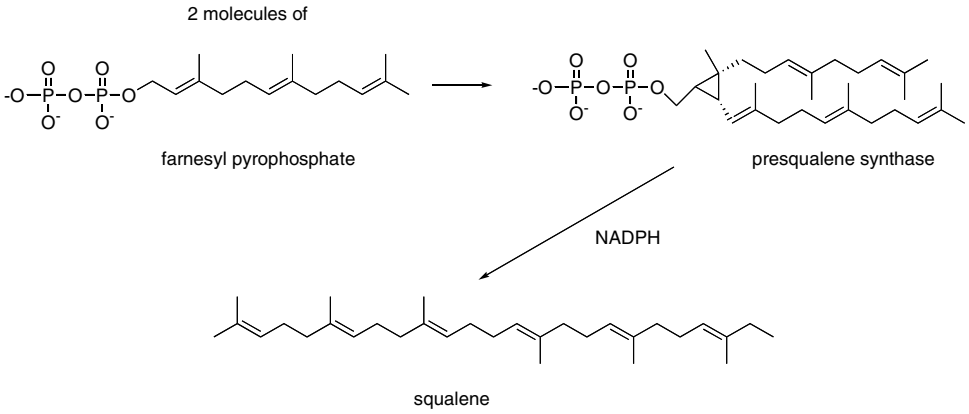


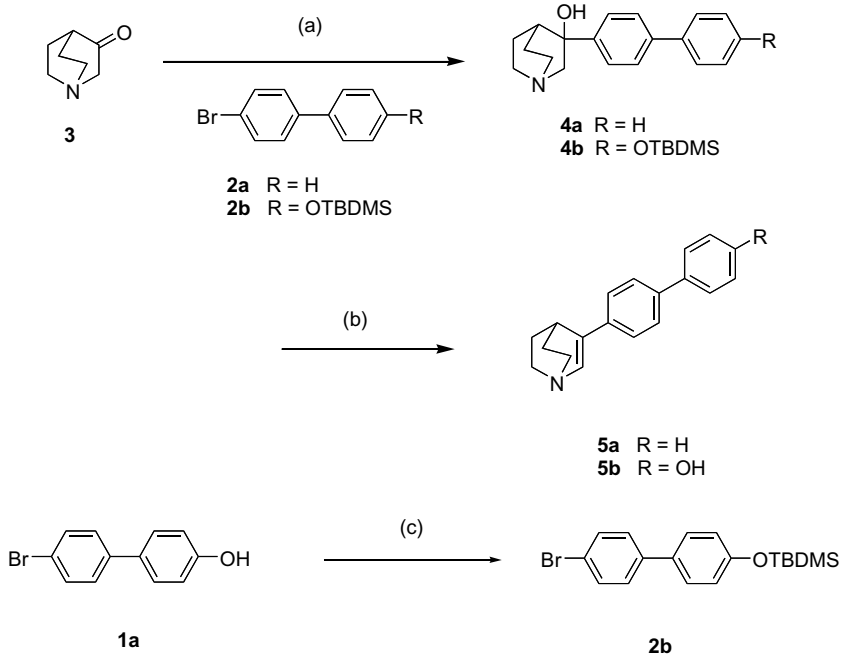
Figure 1. Condensation of two molecules of farnesyl pyrophosphate to produce squalene.

different classes of compounds have been investigated against mammalian enzymes, including bisphosphonates, benzylamines, squalestatins and quinuclidine derivatives.

One class of compounds of particular interest are the arylquinuclidines. These compounds have been shown to be inhibitors of mammalian SQS and are good starting points for drug discovery. The arylquinuclidines are protonated at physiological pH and are thought to mimic a high energy intermediate carbocation intermediate in the reaction pathway. Recently the investigation of 3-(biphenyl-4-yl)-3-hydroxyquinuclidine (BPQ-OH) as an anti-parasitic has been reported.[28] This compound caused potent non-competitive inhibition of *Leishmania mexicana* and *T. cruzi* SQS (K_i 12–62 nM), inhibition of growth of *L. mexicana* promastigotes and *T. cruzi* epimastigotes, and blockade of sterol biosynthesis at the level of SQS. We decided to prepare some biphenylquinuclidines and investigate them as inhibitors of the *L. major* enzyme and for their anti-parasitic activity with the aim of carrying out some preliminary structure–activity relationships.

2. Chemistry

A series of arylquinuclidines was prepared according to the method of Brown et al. (Scheme 1).[29] The biphenylquinuclidines were prepared by condensation of quinuclidin-3-one with the corresponding lithium biphenyl. Two biphenyl moieties were used: an unsubstituted biphenyl moiety and a biphenyl moiety substituted with a TBDMS protected hydroxy group. The lithiated biphenyl reagents were obtained by halogen–metal exchange using *sec*-BuLi. The resulting alcohols **4a** and **4b** were then dehydrated under acidic conditions. Under these conditions, the TBDMS pro-



Scheme 1. Reagents and conditions: (a) **2a**,**b**, THF, *sec*-BuLi, −78 °C, 5 min, then **1**, −78 °C, 30 min, then rt, 12 h; (b) *p*-TsOH, toluene, Dean–Stark trap, reflux, 3 h; (c) TBDMSCl, imidazole, DMF, rt, 48 h.

Table 1. Inhibition of *L. major* SQS, and growth of *L. donovani* intracellular amastigotes, *T. cruzi* intracellular amastigotes and *T. brucei rhodesiense* trypomastigotes by biphenylquinuclidines

Compound	Inhibition of *L. major* SQS IC_{50} (μM)	*L. donovani* ED_{50} (μM)	*T. cruzi* ED_{50} (μM)	*T. brucei rhod.* ED_{50} (μM)	KB cells ED_{50} (μM)
4a	0.013	29.0	9.7	20.8	76.7
4b	>1	n.d.	0.36	0.098	3.1
5a	0.243	74.3	9.7	1.8	34.8
5b	0.096	>108	14.4	5.4	22.7

tecting group was removed from **4b** to give the corresponding hydroxy compound **5b**.

3. Biology

3.1. Cloning of the *Leishmania major* SQS

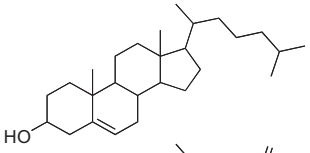
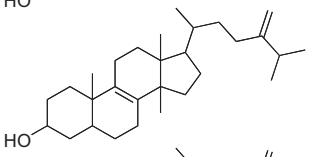
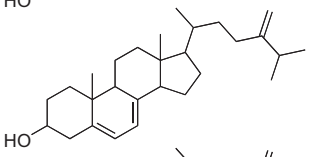
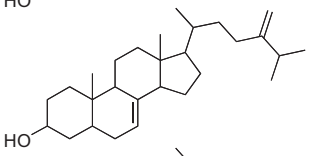
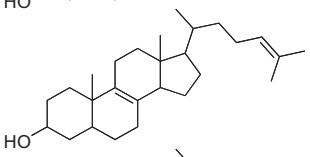
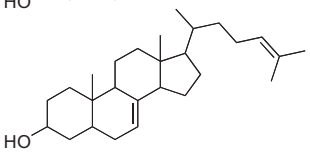
The *L. major* SQS was cloned and over-expressed in *Escherichia coli* for enzyme assays. Full details of the cloning and characterization of the enzyme will be reported elsewhere. A double-truncated protein was produced, lacking 16 residues at the N-terminus and 40 at the C-terminus. The DNA from the *LmSQS* gene was cloned into the pET28a vector and expressed in *E. coli* BL21 (DE3) RP cells as a His-tagged fusion protein.

3.2. Enzyme assays

The *E. coli* cells were lysed and the cell extract, enriched in *L. major* SQS, was used for the assays. The assay was conducted using radiolabelled FPP as substrate. Following extraction of the mixture, the product (newly formed squalene) was separated from unreacted substrate using TLC. The band corresponding to squalene was assessed for radioactivity, allowing an assessment of the amount of conversion of FPP to squalene.

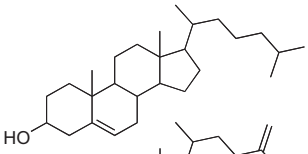
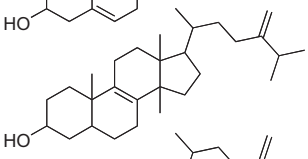
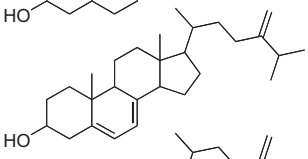
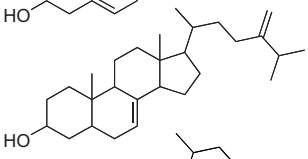
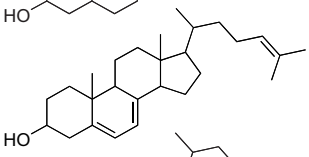
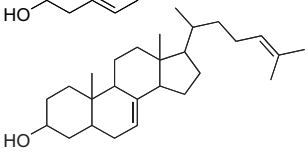
Compound **4a** (BPQ-OH) showed the most potent inhibition of the enzyme with a IC_{50} of 13 nM (Table 1). Dehydrating this compound to give **5a** reduced the activity by almost 20-fold, suggesting that either the hydroxyl group undergoes important interactions in the active site, or that in this more rigid conformation, the

Table 2. Effects of **4a** on the sterol composition of *L. mexicana* promastigotes

Sterol	Structure	Control	0.3 μM	1.0 μM	3.0 μM
Cholesterol		14.2	11.5	57.9	>99
Ergosta-8,24(24[1])-14-methyl-dien-3β-ol		9.4	4.1	n.d.	n.d.
Ergosta-5,7,24(24[1])-trien-3β-ol (5-dehydroepisterol)		56.8	70.3	17.4	n.d.
Ergosta-7,24(24[1])-dien-3β-ol (episterol)		15.6	14.1	24.7	n.d.
Cholesta-8,24-dien-3β-ol (zymosterol)		1.9	n.d.	n.d.	n.d.
Cholesta-7,24-dien-3β-ol		2.1	n.d.	n.d.	n.d.

Sterols were extracted from cells exposed to the indicated compound concentration for 96 h; they were separated by silicic acid column chromatography and analyzed by quantitative capillary gas–liquid chromatography and mass spectrometry. Composition is expressed as mass percentages. n.d. is not detected.

Table 3. Effects of **4b** on the sterol composition of *L. mexicana* promastigotes

Compound	Structure	Control	1.0 μM	3.0 μM
Cholesterol		14.2	11.9	14.9
Ergosta-8,24(24^1)-14-methyl-dien-3β-ol		9.4	6.9	4.1
Ergosta-5,7,24(24^1)-trien-3β-ol (5-dehydroepisterol)		56.8	64.7	63.5
Ergosta-7,24(24^1)-dien-3β-ol (episterol)		15.6	14.6	12.9
Cholesta-5,7,24-trien-3β-ol		n.d.	n.d.	2.0
Cholesta-7,24-dien-3β-ol		2.1	1.9	2.6

Sterols were extracted from cells exposed to the indicated compound concentration for 96 h; they were separated by silicic acid column chromatography and analyzed by quantitative capillary gas–liquid chromatography and mass spectrometry. Composition is expressed as mass percentages. n.d. is not detected.

biphenyl group is held in a less optimal conformation. We also investigated the effect of adding a lipophilic substituent to give **4b**. This compound had essentially no activity suggesting that there is a limitation to the size of substituent that can be appended to compound **4a**. The analogue of **5a** in which there is a hydroxyl group on the end of the biphenyl substituent, compound **5b**, showed increased enzyme inhibition compared to **5a**.

3.3. Studies on lipid composition

The effects of the compounds on the sterol composition of *L. mexicana* promastigotes were investigated (Tables 2–5). By monitoring the sterol composition, it should be possible to establish the effect of the compounds on the sterol composition in parasites and hence investigate the mechanism of action of compounds in cellular systems. For compound **4a** (Table 2), there was a dose-dependent reduction in the relative content of the parasites endogenous sterols (episterol and 5-dehydroepisterol). There were no significant effects seen on sterol composition at 0.3 μM concentration, some effect at 1 μM and almost complete loss of endogenous sterols at 3 μM. This is consistent with inhibition of ergosterol biosynthesis at the stage prior to formation of lanosterol (in this case suggesting inhibition of squalene synthase). A more significant effect was seen with compound **5a** (Table 4), where there was complete loss of endogenous sterols at 1 μM.

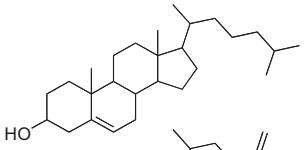
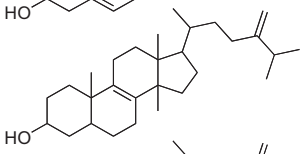
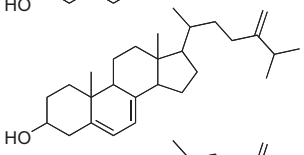
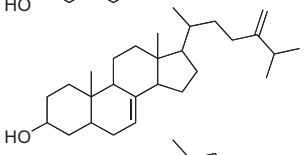
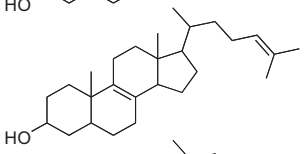
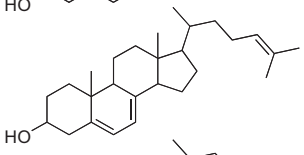
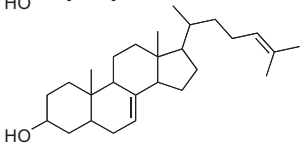
Compound **4b** (Table 3) showed no effect on sterol composition at 3 μM, suggesting no inhibition of SQS at these concentrations, whilst compound **5b** showed an activity comparable to that of compound **5a** (Table 5).

3.4. Growth inhibition

Compounds were also investigated for growth inhibition of various parasites (Fig. 2A–D). Against *L. mexicana* promastigotes (the same species used for the studies on sterol composition), compound **4a**, had an MIC (minimum inhibitory concentration defined as that required for complete growth arrest) of 1 μM and caused cell lysis after 24 h (Fig. 2A). Compound **5a**, was more potent as a growth inhibitor, consistent with a more significant effect on sterol composition than compound **4a**, with an MIC of <1 μM (Fig. 2C). The TBDMS protected compound **4b** had no effect on *L. mexicana* cell growth, which was again consistent with no effect on the sterol composition (Fig. 2B). Compound **5b** had a similar effect on cell growth as compound **5a** (Fig. 2D).

The compounds were also investigated for their effect on *Leishmania donovani* and *T. cruzi* intracellular amastigotes and for toxicity to a mammalian cell line. None of the compounds showed significant inhibition of *L. donovani* intracellular amastigotes. In contrast all the compounds showed at least moderate growth inhibition of *T. cruzi*. In particular, compound **4b** showed a potent

Table 4. Effects of **5a** on the sterol composition of *L. mexicana* promastigotes

Compound	Structure	Control	0.3 μM	1.0 μM
Cholesterol		14.2	14.3	>99
Ergosta-8,24(24^1)-14-methyl-dien-3β-ol		9.4	3.6	n.d.
Ergosta-5,7,24(24^1)-trien-3β-ol (5-dehydroepisterol)		56.8	51.6	n.d.
Ergosta-7,24(24^1)-dien-3β-ol (episterol)		15.6	9.5	n.d.
Cholesta-8,24-dien-3β-ol (zymosterol)		1.9	n.d.	n.d.
Cholesta-5,7,24-trien-3β-ol		n.d.	14.2	n.d.
Cholesta-7,24-dien-3β-ol		2.1	6.8	n.d.

Sterols were extracted from cells exposed to the indicated compound concentration for 96 h; they were separated by silicic acid column chromatography and analyzed by quantitative capillary gas–liquid chromatography and mass spectrometry. Composition is expressed as mass percentages. n.d. is not detected.

inhibition of *T. cruzi* amastigote proliferation (ED_{50} = 0.36 μM).

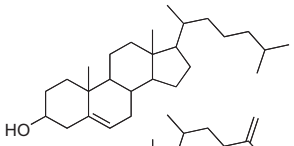
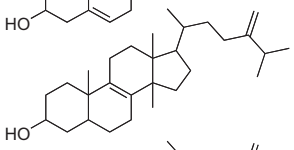
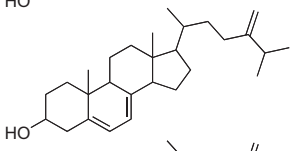
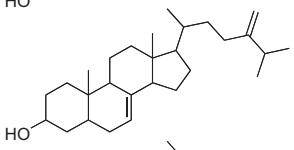
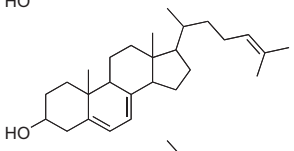
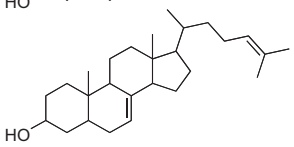
As a part of routine screening, compounds were also assayed for their effect on the growth of *T. brucei rhodesiense* blood stream form trypomastigotes. These compounds showed growth inhibition of this parasite; in particular **4b** was a very potent inhibitor of growth (ED_{50} = 0.098 μM).

4. Discussion

In this paper we report the synthesis of several biphenylquinuclidines, with activity against trypanosomatid parasites. Some of the compounds were potent inhibitors of *L. major* SQS. The compounds also showed effects on the sterol composition of *L. mexicana* parasites (consistent with inhibition of SQS in the parasite) and on the growth of the *L. mexicana* promastigotes. In general there was very good correlation between the effect on sterol composition and growth of the promastigotes. Thus compounds **5a** and **5b** showed the most pronounced effects on both the sterol composition and on the growth of *L. mexicana* promastigotes, whilst compound **4a** showed a slightly smaller effect on both sterol composition and promastigote growth. Interestingly compound **4a** gave slightly more potent inhibition of *L. major* SQS than **5a** or **5b**. Compound **4b** had no effect on sterol composition, promastigote growth or showed no inhibition of the recombinant enzyme.

Despite the potent inhibition of SQS and of strong inhibition of the growth of *L. mexicana* promastigotes, none of the experimental compounds was able to inhibit the growth of intracellular *L. donovani* amastigotes. There could be several reasons for this. First the intracellular forms of the Leishmania parasites are found within a parasitophorous vacuole inside the host macrophages. This means that to access the parasite, the compounds have to cross the cell membrane of the host cell, the membrane of the parasitophorus vacuole and then the membrane of the parasites. In addition the parasitophorous vacuole has a pH of about 5.5. Possibly the compounds are fully charged at that pH and cannot cross the final barrier constituted by the parasite's membrane. A second possibility is that the parasite could scavenge required sterols from the host;

Table 5. Effects of **5b** on the sterol composition of *L. mexicana* promastigotes

Compound	Structure	Control	0.1 μM	0.3 μM	1.0 μM
Cholesterol		10.1	9.9	14.2	>99
Ergosta-8,24(24^1)-14-methyl-dien-3β-ol		n.d.	1.8	n.d.	n.d.
Ergosta-5,7,24(24^1)-trien-3β-ol (5-dehydroepisterol)		70.4	70.3	70.8	n.d.
Ergosta-7,24(24^1)-dien-3β-ol (episterol)		19.5	13.7	15.0	n.d.
Cholesta-5,7,24-trien-3β-ol		n.d.	2.3	n.d.	n.d.
Cholesta-7,24-dien-3β-ol		n.d.	2.0	n.d.	n.d.

Sterols were extracted from cells exposed to the indicated compound concentration for 96 h; they were separated by silicic acid column chromatography and analyzed by quantitative capillary gas–liquid chromatography and mass spectrometry. Composition is expressed as mass percentages. n.d. is not detected.

however many sterol biosynthesis inhibitors can prevent growth of intracellular *Leishmania* amastigotes, implying that scavenging of sterols from the host is not a significant process or at least is not capable of compensating for the inhibition of the synthesis of endogenous sterols.

All compounds had significant inhibitory activity on the growth of *T. cruzi* intracellular amastigotes. These intracellular amastigotes are more accessible as whilst they are intracellular parasites, they are not contained within a vacuole, but develop freely in the host cell's cytoplasm. Compound **4b** was the most potent growth inhibitor against this stage, although this compound does not inhibit the *L. major* SQS. There are a number of possible explanations for this: this specific compound may have alternative mechanisms of action independent of SQS; or that it can penetrate into the host cells and be transformed in the intracellular environment to the parental compound **4a**; or there may be subtle differences in the structure of the *L. major* and *T. cruzi* SQS.

The compounds were also investigated against *T. brucei rhodesiense* bloodstream form trypomastigotes, as part of routine screening. These forms do not appear to biosynthesize their own sterols and are thought to scavenge cholesterol from the human host,[30,31] whilst procyclics (present in the insect vector) contain significant amounts of ergosterol and other 24-alkyl sterols, which they produce themselves. The compounds tested produced a significant inhibition in the growth of *T. brucei* blood stream forms. In particular compound **4b** was very potent as a growth inhibitor, yet showed no activity against the recombinant enzyme. This observation suggests that these compounds have some other mode of action against these parasites, rather than inhibition of SQS. This merits further investigation, as compound **4b**, in particular, showed very potent growth inhibition of *T. brucei rhodesiense*.

In conclusion, the biphenylquinuclidines had potent inhibitory activity of *L. major* SQS, and block de novo sterol synthesis in *L. mexicana* promastigotes associated with growth inhibition of these cells. The analogues prepared here showed weak or no inhibition of growth of intracellular *L. donovani* amastigotes, but they were active against the intracellular stages of *T. cruzi*, probably due to differences in parasitic strategies of these organisms.

5. Experimental

5.1. Chemistry

Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. Infrared

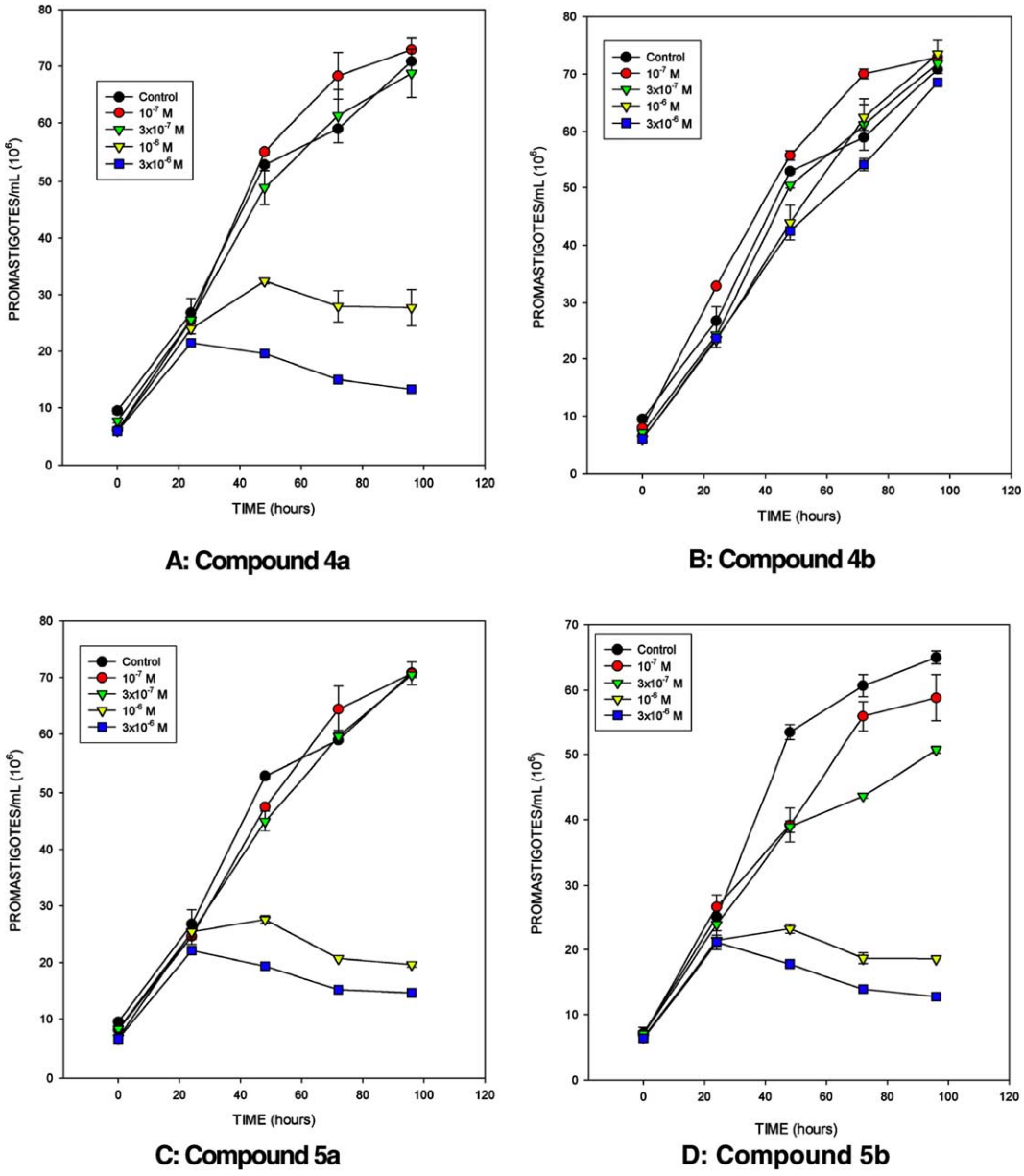


Figure 2. Growth inhibition of *L. mexicana* promastigotes (A) inhibition by compound **4a**; (B) inhibition by compound **4b**; (C) inhibition by compound **4c**; (D) inhibition by compound **5b**.

spectra were recorded as thin films for liquid samples, or as Nujol mulls for solid samples, on a Perkin–Elmer 1600 series FTIR spectrophotometer using sodium chloride plates. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance DPX300 spectrometer operating at 300 and 75 MHz, respectively, with tetramethylsilane as internal standard, using deuterated chloroform purchased from Goss unless stated otherwise. Assignments for ^{13}C spectra were made with the aid of the ACD Labs software. Low resolution mass spectra that is electrospray, were recorded using a fisons VG Platform II spectrometer. High resolution spectra were obtained on a VG ZAB spectrometer from the EPSRC Mass Spectrometry Service at Swansea University, UK. Microanalyses were obtained from the analytical and chemical consultancy services MEDAC LTD. All reactions were performed in pre-dried apparatus under an atmosphere of nitrogen unless otherwise stated. Solvents and reagents were purchased from chemical companies and used without further purification. Dry solvents were generally purchased in sure sealed bottles stored over molecular sieves. Thin layer chromatography (TLC) was performed on Merck silica gel $60F_{254}$ plates. Column chromatography was carried out using Fisons matrix silica 60 (35–70 μm).

5.1.1. 4-Bromo-4′[(*tert*-butyldimethylsilyl)oxy]biphenyl (2b). A mixture of 4-bromo-4′-hydroxybiphenyl (2 g, 8.03 mmol), *tert*-butyldimethylsilyl chloride (1.88 g, 12.04 mmol) and imidazole (0.82 g, 12.4 mmol) in DMF (20 mL) was stirred at room temperature for 48 h. The reaction mixture was diluted with chloroform, washed with water (3 × 50 mL), dried over sodium sulfate and reduced in vacuo to yield a white solid

(2.23 g, 76%); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.20 (6H, s, $SiCH_3$), 0.99 (9H, s, $C(CH_3)_3$), 6.85 (2H, d J = 9 Hz, 7-CH), 7.34–7.50 (9H, m, ar–CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ = −3.9 ($SiCH_3$), 18.7 (10-C), 26.1 (11-CH_3), 120.9 (7-CH), 121.2 (7′-CH), 128.4 (CH), 128.8 (CH), 132.2 (CH), 133.4 (CH), 140.2 (4-C), 156.0 (8-C).

5.1.2. 3-(Biphenyl-4-yl)-3-hydroxyquinuclidine (4a). ^{s}BuLi in cyclohexane (5 mL, 6.4 mmol) was added to a stirred solution of 4-bromobiphenyl (1.27 g, 5.4 mmol) in THF (20 mL) at −78 °C. The mixture was stirred for 5 min, and a solution of quinuclidin-3-one (0.62 g, 4.9 mmol) in THF (10 mL) was added during 20 min. Stirring was continued at −78 °C for 30 min and the mixture allowed to reach room temperature overnight; 2 N HCl (30 mL) was added below 10 °C and the aqueous layer washed with Et_2O (2 × 50 mL) before the addition of excess 10 M NaOH to pH 14. The mixture was extracted with ethyl acetate, which had been heated to 50 °C and the extract allowed to cool, dried and evaporated. TLC of the ethyl acetate residue showed mainly presence of the starting material. Quinuclidine **2** had remained in the diethyl ether layer. Chromatography over silica gel with MeOH/$CHCl_3$ (0%→25%) as the eluent afforded **2** as a white solid (0.343 g, 25%); R_f 0.32 (20% MeOH/$CHCl_3$); mp 165–166 °C; m/z (ES^+) 280.1 ($M+H^+$, 100%); HRMS calculated for $C_{19}H_{22}NO$ ($[M+H]^+$) 280.1701, found: 280.1699; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.47 (3H, m, 4-CH, 8-CH_2), 2.18 (2H, m, 5-CH_2), 2.72 (2H, t, J = 8 Hz, 6-CH_2), 3.01 (2H, m, 7-CH_2), 3.16 (1H, s), 3.45 (1H, d, J = 14 Hz, –OH), 7.24–7.52 (9H, m, ar–CH); ^{13}C NMR (75 MHz, $CDCl_3$) 21.4 (CH_2), 22.9 (CH), 32.7 (4-CH), 46.2 (CH_2), 47.1 (CH_2), 61.8 (2-CH_2), 72.5 (3-C), 126.8 (ar–CH), 127.4 (ar–CH), 127.8 (ar–CH), 129.2 (ar–CH), 140.5 (10-C), 140.8 (11-C), 144.9 (9-C).

5.1.3. 3-(Biphenyl-4-yl-4′[(*tert*-butyldimethylsilyl)oxy)]-3-hydroxyquinuclidine (4b). ^{s}BuLi in cyclohexane (3.5 mL, 4.1 mmol) was added to a stirred solution of 4-bromo-4′[(*tert*-butyldimethylsilyl)oxy]biphenyl **1** (0.99 g, 2.7 mmol) in THF (20 mL) at −78 °C. The mixture was stirred for 5 min, and a solution of quinuclidin-3-one (0.26 g, 2.0 mmol) in THF (10 mL) was added during 20 min. Stirring was continued at −78 °C for 30 min and the mixture allowed to reach room temperature overnight. The reaction mixture was reduced to vacuum. Flash chromatography with MeOH/$CHCl_3$ (0%→20%) as the eluent afforded **3** as a white solid (0.43 g, 50%); R_f 0.52 (40% MeOH/$CHCl_3$); m/z (ES^+) 279 (M^+-OTBDMS, 100%), 410 (M^+, 58%); HRMS calculated for $C_{25}H_{36}NO_2Si$ ($[M+H]^+$) 410.2510, found: 410.2504; ^{1}H NMR (300 MHz, CD_3OD) δ 0.19 (6H, s, 13-CH_3), 0.98 (9H, s, 14-CH_3), 1.78 (1H, m, 4-CH), 1.94 (2H, m, 8-CH_2), 2.50 (2H, m, 5-CH_2), 3.29 (2H, m, 6-CH_2), 3.40 (2H, m, 7-CH_2), 3.49 (2H, m, –OH), 3.99 (1H, m, –OH), 6.88 (2H, d, J = 9 Hz, 12-CH), 7.49 (2H, d, J = 9 Hz, ar–CH), 7.60 (4H, m, ar–CH); ^{13}C NMR (75 MHz, CD_3OD) δ −3.8 (14-CH_3), 19.5 (15-C), 20.2 (CH_2), 21.4 (CH_2), 26.6 (15-CH_3), 33.2 (4-CH), 47.2 (CH_2), 48.1 (CH_2), 60.9 (2-CH_2), 72.6 (3-C), 122.0 (12-CH), 127.9 (ar–CH), 128.2 (ar–CH), 129.4 (ar–CH), 135.2 (ar-C), 142.1 (ar-C), 143.5 (ar-C), 157.3 (13-C).

5.1.4. 3-(Biphenyl-4-yl)-2,3-dehydroquinuclidine (5a). 4-Toluenesulfonic acid (0.492 g, 2.6 mmol) and **2** (0.24 g, 0.86 mmol) were heated under reflux in toluene (50 mL) for 3 h. using a Dean–Stark water separator. The toluene was evaporated and the residue dissolved in 1 M NaOH (50 mL). The aqueous mixture was extracted with chloroform (2 × 50 mL) and the organic layer was dried and concentrated to yield a white solid (0.083 g, 37%); R_f 0.52 (20% MeOH/$CHCl_3$); m/z (ES^+) 261 (M^+, 100%); HRMS calculated for $C_{19}H_{20}N$ ($[M+H]^+$) 262.1595, found 262.1596; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.65 (2H, m, 8-CH_2), 1.85 (2H, m, 5-CH_2), 2.72 (2H, m, 6-CH_2), 3.01 (2H, m, 7-CH_2), 3.27 (1H, s, 4-CH), 6.94 (1H, s, 2-CH), 7.31–7.67 (9H, m, ar–CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.7 (CH_2), 29.6 (4-CH), 49.6 (CH_2), 126.6 (2-CH), 127.4 (ar–CH), 127.8 (ar–CH), 129.2 (ar–CH), 136.2 (9-C), 141.1 (10-C), 146.7 (11-C).

5.1.5. 3-(Biphenyl-4-yl-4′-hydroxy)-2,3-dehydroquinuclidine (5b). 4-Toluenesulfonic acid (0.400 g, 2.1 mmol) and **3** (0.286 g, 0.70 mmol) were heated under reflux in toluene (30 mL) for 10 h. The toluene was evaporated and the residue dissolved in 1 M NaOH (50 mL). The aqueous mixture was extracted with chloroform (5 × 50 mL) and the organic layer was dried and concentrated to yield a white solid (0.093 g, 48%); R_f 0.30 (40% MeOH/$CHCl_3$); m/z (ES^+) 278 ($M+H^+$, 100%); HRMS calculated for $C_{19}H_{20}NO$ ($[M+H]^+$) 278.1539, found 278.1538; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.54 (2H, m, 8-CH_2), 1.75 (2H, m, 5-CH_2), 2.59 (2H, m, 6-CH_2), 2.95 (2H, m, 7-CH_2), 3.17 (1H, s, 4-CH), 3.74 (1H, s, –OH), 6.69 (1H, s, 2-CH), 6.83 (2H, d, J = 9 Hz, 12-CH), 7.26–7.47 (6H, m, ar–CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.9 (CH_2), 29.4 (4-CH), 49.3 (CH_2), 116.1 (2-CH), 125.6 (ar–CH), 127.1 (ar–CH), 128.4 (ar–CH), 132.4 (11-C), 134.6 (ar–CH), 134.8 (ar–CH), 140.9 (10-C), 147.0 (9-C), 157.0 (13-C).

6. Enzyme assays

6.1. Cloning of enzyme

To test the potential for inhibition of *L. major* SQS by the different compounds, protein extracts of *E. coli* cells containing the plasmid pET28a-LmSQS2 were used as enzyme source. A double truncated *L. major* SQS protein, that lacks 16 residues at the N-terminus and 40 at the C-terminus, was expressed in *E. coli* BL21 (DE3) RP cells. Briefly, the DNA fragment was amplified from the *LmSQS* gene and cloned in the pET28a vector (Novagen). The resulting plasmid was introduced in bacterial cells; the recombinant truncated *L. major* SQS is expressed as a His-tagged fusion protein when cells are induced with 1 mM IPTG during 2 h at 25 °C. After induction, cells were disrupted by sonication in a buffer containing 20 mM phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 500 mM NaCl, 10 mM CHAPS, 10% glycerol, 10 mM β-mercaptoethanol and protease

inhibitors (0.02 mg/mL leupeptine, 0.05 mg/mL aprotinine, 10 mM phenanthroline, 0.05 mg/mL trypsine inhibitor, 1 mM benzamidine and 50 μM PMSF). Soluble extracts were used as enzyme source (3 μg).

6.2. Enzyme assay

A standard SQS activity assay contained 50 mM phosphate buffer (pH 7.4), 20 mM $MgCl_2$, 5 mM CHAPS, 1% Tween 80, 10 mM DTT, 0.025 mg/mL BSA, 0.25 mM NADPH, 2.1 mM 6-phosphate glucose, 0.125 mg/mL 6-phosphate glucose dehydrogenase and 0.5 μM FPP (10080 dpm/pmol) as substrate. The reaction was started with the protein extract and the final volume of the reaction was 200 μL. After incubation at 37 °C for 10 min, 40 μL of 10 M NaOH were added, followed by 10 μL of a (50:1) mixture of 70% EtOH and squalene. Resulting mixtures were mixed vigorously by vortexing, then 20 μL aliquots were applied to 2.5×10 cm channels of a silica gel thin layer chromatogram, and newly formed squalene was separated from unreacted substrate by chromatography in toluene–EtOAc (9:1). The region of the squalene band was removed, immersed in Hydrofluoro liquid scintillation fluid, and assessed for radioactivity using a Pharmacia LKB liquid scintillation counter. Negative controls were reactions containing soluble extracts of *E. coli* BL21 (DE3) RP cells transformed with pET28a (not overexpressing *L. major* SQS). No activity was observed using this extract as an enzyme source. IC_{50} values were calculated from the hyperbolic plot of percentage of inhibition versus concentration of inhibitor.

7. Effect on steroid composition

L. mexicana amazonensis promastigotes were cultivated in LIT medium supplemented with lactoalbumin and 10% foetal calf-serum (Gibco) (3) at 26 °C, without agitation. The cultures were initiated with a cell density of 2.10^6 cells/mL and the drug was added at a cell density of 0.5–1.10^7 cells/mL. Cell densities were measured with an electronic particle counter (model ZBI; Coulter Electronics Inc., Hielah, Fla.) and by direct counting with a haemocytometer. Cell viability was followed by Trypan blue exclusion using light microscopy.

For the analysis of the effects of drugs on the lipid composition of promastigotes, total lipids from control and drug-treated cells were extracted and fractionated into neutral and polar lipid fractions by silicic acid column chromatography and gas–liquid chromatography.[6,7,12,13] The neutral lipid fractions were first analyzed by thin layer chromatography (on Merck 5721 silica gel plates with heptane–isopropyl ether–glacial acetic acid [60:40:4] as developing solvent) and conventional gas–liquid chromatography (isothermic separation in a 4-m glass column packed with 3%OV-1 on Chromosorb 100/200 mesh, with nitrogen as carrier gas at 24 mL/min and flame ionization detection in a Varian 3700 gas chromatograph). For quantitative analysis and structural assignments the neutral lipids were separated in a capillary high resolution column (25 m × 0.20 mm i.d. Ultra-2 column, 5% phenyl-methyl-siloxane, 0.33 μm film thickness) in a Hewlett–Packard 6890 Plus gas chromatograph equipped with a HP5973A mass sensitive detector. The lipids were injected in choloroform and the column was kept a 50 °C for 1 min, then the temperature was increased to 270 °C at a rate of 25 °C/min and finally to 300 °C at a rate of 1 °C/min. The carrier gas (He) flow was kept constant at 0.5 mL/min. Injector temperature was 250 °C and the detector was kept at 280 °C.

8. Growth inhibition studies

8.1. *L. mexicana* promastigotes

L. mexicana promastigotes were cultivated in LIT medium supplemented with lactabulmin and 10% foetal calf-serum (Gibco)[28] at 26 °C, without agitation. The cultures were initiated with a cell density of 2.10^6 cells/mL and the drug was added at a cell density of 0.5–1.10^7 cell/mL. Cell densities were measured with an electronic particle counter (model ZBI; Coulter Electronics Inc., Hielah, Fla.) and by direct counting with an haemocytometer. Cell viability was followed by Trypan blue exclusion.

8.2. *L. donovani* amastigotes

Peritoneal exudate macrophages were harvested from CD1 mice, 24 h after starch induction. After washing the macrophages were dispensed into Lab-tek™ 16-well tissue culture slides and maintained in RPMI1640 + 10% heat-inactivated foetal calf serum (HIFCS) at 37 °C, 5% CO_2/air mixture for 24 h. *L. donovani* (MHOM/ET/67/L82) amastigotes were harvested from an infected Golden hamster spleen and were used to infect the macrophages at a ratio of 5 parasites:1 macrophage. Infected cells were left for a further 24 h and then exposed to drug[32] for a total of 5 days, with the overlay being replaced on day 3.[33] The top concentration for the test compounds was 30 μg/mL and all concentrations were carried out in quadruplicate. On day 5 the overlay is removed, the slides fixed (100% methanol) and stained (10% Giemsa, 10 min) before being evaluated microscopically. ED_{50} values were calculated using Ms*xl*fit. The ED_{50} value for the positive control drug, Pentostam©, is usually 3–8 μg Sb^V/mL.

8.3. *T. cruzi* amastigotes

Murine (CD1) peritoneal macrophages were harvested 24 h after starch induction. One hundred microlitre was dispensed into 96-well plates at a concentration of 4×10^5/mL. After 24 h the cells were infected with *T. cruzi* Tulahuan LAC-Z trypomastigotes, harvested from L6 feeder layer cultures. Twenty-four hour later the infected cells were exposed to the drug[20] for 3 days. Fifty microlitres of 500 μM CPRG:1% nonidet P-40 was added to each well. The plates were read after 2–5 h, λ570.[34] ED_{50} (ED_{90}) values were calculated using Ms*xl*fit. L6 fibroblasts are also used as host cells.

8.4. *T. brucei rhodesiense* trypomastigotes

T. brucei rhodesiense STIB900 blood stream form (bsf) trypomastigotes were maintained in HMI-18 medium[35] with 15% heat-inactivated foetal calf serum (HIFCS) [Harlan-SeraLab, UK] at 37 °C, 5%, CO_2/air mixture. Trypomastigotes were washed and resuspended in fresh medium at a concentration of 2×10^5/mL. The top concentration for the test compounds was 30 μg/mL. The ED_{50} for pentamidine is usually between 1.0 and 0.1 ng/mL. Plates were incubated for 72 h at 37 °C in 5% CO_2/95% air mixture. At 72 h the plates were assessed microscopically before Alamar Blue was added.[36] Plates were read after 5–6 h on a Gemini Fluorescent plate reader (Softmax Pro. 3.1.1, Molecular Devices, UK) at EX/EM 530/585 nm with a filter cut-off at 550 nm. ED_{50} values were calculated with Ms*xl*fit (IDBS, UK).

8.5. Cytotoxicity against vertebrate cells

Plates were seeded with 100 μL KB cells @ 4×10^4/mL, RPMI 1640 + 10% HIFCS and incubated at 37 °C, 5% CO_2 for 24 h. The overlay was removed and replaced by test drugs[20] in fresh medium @ 300, 30, 3 and 0.3 μg/mL. The positive control drug was podophyllotoxin (Sigma, UK). Dilutions were carried out in triplicate. Plates were incubated for a further 72 h, at 37 °C, 5% CO_2. The wells assessed were microscopically for cell growth. The overlay was removed and wells washed with PBS (pH 7.0) × 3. Then 100 μL PBS + 10 μL AlamarBlue™ were added per well and plates incubated for 2–4 h (37 °C, 5% CO_2) before reading at EX/EM 530/585 nm (cut-off 550 nm) in a Gemini plate reader. ED_{50} (ED_{90}) values were calculated compared to blanks and untreated controls.

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