Effects of an azasterol inhibitor of sterol 24-transmethylation on sterol biosynthesis and growth of *Leishmania donovani* promastigotes

Penny A. HAUGHAN,* Michael L. CHANCE† and L. John GOAD*‡

*Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.
and †Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, U.K.

Leishmania donovani promastigotes were cultured in the presence of an azasterol (20-piperidin-2-yl-5α-pregnane-3β,20-diol) to determine the effects on sterol biosynthesis and cell proliferation. Inhibition of growth increased gradually with azasterol concentrations up to 5 μg/ml; concentrations of azasterol exceeding 5 μg/ml were lethal. Sterol biosynthesis was affected by the azasterol when administered at concentrations as low as 100 pg/ml. The primary site of action was the alkylation at C-24 of a Δ^{24}-sterol precursor. The 24-alkylated sterols [ergosta-5,7,24(24[1])-trien-3β-ol and ergosta-5,7,22-trien-3β-ol] of the protozoan were replaced by Δ^{24}-cholesta-type sterols which then accumulated in the cells. Administration of the azasterol together with a bis-triazole inhibitor of the 14α-methylsterol 14-demethylase reaction, which operates in sterol biosynthesis, resulted in depletion of 24-alkylsterols and their replacement with predominantly 14α-methylsterols lacking a 24-alkyl group. Continuous subculture of promastigotes in the presence of the azasterol resulted in gradual depletion of 24-alkylsterols and their complete replacement by Δ^{24}-cholesta-type sterols. Transfer of the azasterol-treated cells to medium lacking azasterol resulted in a gradual restoration, after several subcultures, of the normal 24-alkylsterol pattern. The results indicate that, although 24-alkylsterols are normally produced by the protozoan, it can nevertheless survive with sterols possessing only the cholestane skeleton. Thus there is no absolute requirement for 24-alkylsterols to fulfil some essential 'sparking' role associated with cell growth in promastigotes.

INTRODUCTION

Leishmania species are pathogenic protozoa that infect man, and there is an urgent need to develop new chemotherapy for the treatment of leishmaniasis. In view of the success of antifungal drugs that are inhibitors of sterol biosynthesis, attention has been focused on the sterol metabolism of *Leishmania* as a potential target for drug therapy. The sterol compositions, sterol nomenclature and biosynthetic pathway of several species of *Leishmania* promastigotes and amastigotes have been described previously [1–4]; they consist of a complex mixture of ergostadienols, ergostatrienols and ergostatetraenols, often together with a smaller proportion of similar compounds with the stigmastane skeleton. The cells also contain a substantial amount of cholesterol and there is occasionally a trace of desmosterol; these sterols are not biosynthesized by the protozoan but are derived by the promastigotes from the foetal calf serum used to supplement their growth medium and by the amastigotes from the host macrophage cell. Sterol biosynthesis in *Leishmania* species is similar to that in fungi and involves lanosterol as an intermediate produced by cyclization of squalene-2,3-oxide. Subsequent steps result in demethylation at positions C-4 and C-14 and transmethylation of a Δ^{24}-sterol to eventually produce C-24 alkyl compounds such as ergosta-5,7,24(24[1])-trien-3β-ol and ergosterol (ergosta-5,7,22-trien-3β-ol) which are the typical major sterols of these protozoa [1,2].

On the basis of experiments with yeast [5–9], fungus [10], a plant tissue culture [11] and the protozoan *Paramecium tetraurelia* [12], it has been suggested that sterols may have two distinct functions within the cell. The first is as a structural component of membranes where they apparently stabilize the structure by interaction with the fatty acyl moieties of the membrane phospholipids and can affect membrane fluidity. In yeast this role is considered to be relatively non-specific with respect to sterol structure and may be fulfilled equally well in a fungal mutant sterol auxotroph by substitution of cholesterol or cholestanol for the naturally produced ergosterol [5,6]. The second function postulated for sterol is related to cell proliferation. There is evidence, largely from work on yeast sterol auxotrophs, that sterol is required in small amounts to allow cell growth to proceed (referred to as 'metabolic' or 'sparking' sterol or sterol synergism) [5–9]. The mode of action of the sterol in this context is unknown but the structural requirement appears from some investigations to be rather specific, and studies with a yeast sterol auxotroph revealed that the most effective sterol is one with a 24β-methyl group [8,9] and a Δ^5-double bond [6,13] such as ergosterol which is the most abundant yeast sterol [6]. It has been suggested that the trace sterol requirement may be related to promotion of cell-cycle events via involvement in the regulation of a protein kinase [14]. However, recent cloning and gene-disruption studies with *Saccharomyces cerevisiae* have seriously questioned these rather specific structural requirements for an essential sterol; genes encoding the Δ^5-sterol desaturase, Δ^8 to Δ^7-sterol isomerase and the C-24 sterol transmethylase were shown to be not essential for cell viability [15–18]. Other work with a plant cell suspension culture [11] has indicated that a 24α-ethyl sterol, such as stigmasterol, may be required to support growth and, similarly, in the ciliated protozoan *P. tetraurelia* [12] the requirement may be for either stigmast-22-en-3β-ol or stigmastan-3β-ol which are also 24α-ethyl sterols.

Abbreviation used: TMS, trimethylsilyl.

‡ To whom correspondence should be addressed.

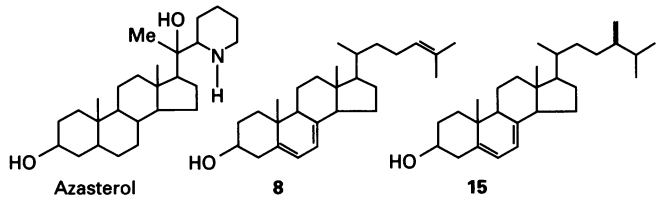

Figure 1 Structures of azasterol and the most predominant sterols in treated and untreated cultures

Azasterol, 20-piperidin-2-yl-5α-pregnane-3β,20-diol; **8**, cholesta-5,7,24-trien-3β-ol; **15**, ergosta-5,7,24(24[1])-trien-3β-ol.

Leishmania species produce sterols with the ergostane skeleton (i.e. with a 24-methyl or 24-methylene group) and thus resemble most fungi in this respect. However, the protozoa also produce in smaller amounts some sterols of the stigmastane type (with a 24-ethylidene group). Therefore, in an attempt to explore the need for specific sterols alkylated at the C-24 position to support the growth of the leishmanial parasites, we have now investigated the effects of an azasterol that inhibits the sterol C-24 alkylation reaction required in the production of the typical ergostane- and stigmastane-based sterols.

Various azasterols have been studied as inhibitors of the sterol 24-methyltransferase in yeast and other fungi. 23-Azacholesterol inhibits the conversion of zymosterol into ergosterol in *S. cerevisiae* [19] resulting in accumulation of the C_{27} sterol. The sterol compositions of mutants of *S. cerevisiae* deficient in Δ^{24}-sterol methyltransferase (EC 2.1.1.41) are similar to those seen in yeast cultures treated with azasterols such as 25-azacholesterol and 25-azacholestanol [20]. Azasterol treatment has a similar inhibitory effect on C-24 alkylsterol production in the protozoan *Crithidia fasiculata* [21] whereas 25-azacholesterol inhibits the sterol transmethylase enzyme in *Saprolegnia ferax* [22] as does 24-epi-iminolanosterol in *Gibberella fujikuroi* [10]. The inhibition of the sterol C-24 methyltransferase has been demonstrated [23] in a sunflower-cell culture treated with (24*R*,25*S*)-epi-iminolanosterol and in this case there was an accumulation of cycloartenol.

For the work described in this study we have use an azasterol (20-piperidin-2-yl-5α-pregnane-3β,20-diol, Figure 1) that is an effective inhibitor of sterol side-chain methylation in *Candida albicans* with an IC_{50} value of 20 nM [24]. This paper describes the effects of the azasterol on the growth, sterol composition and Δ^{24}-sterol transmethylase of the parasitic protozoan *Leishmania donovani*.

MATERIALS AND METHODS

Materials

The strain of *Leishmania* used in this study was *L. donovani* (MHOM/BL/67/ITMAP263). The azasterol and the bis-triazole, ICI 195739, were kindly provided by Dr. T. F. Boyle, ICI Pharmaceuticals, Alderley Edge, Cheshire, U.K.

Cell culture

Unless otherwise stated, cultures were grown in either HOMEM [25] supplemented with 15% (v/v) heat-inactivated foetal calf serum or in HOSMEM II [25]. Promastigotes were grown in 10 ml of medium at 27 °C for 48 h before harvesting by centrifugation. Growth was measured using a Coulter counter. The azasterol and the bis-triazole, ICI 195739, were added from stock solutions in dimethyl sulphoxide and ethanol respectively, such that organic solvent was less than 1% (v/v) of final total culture volume. Appropriate volumes of solvent (ethanol or dimethyl sulphoxide) were also added to control cultures. For the experiments on the effects of continuous exposure of the protozoan to azasterol, the cells were transferred every 3 days into fresh culture medium (10 ml) containing the indicated azasterol concentrations, harvested at the various intervals indicated and their sterol content and composition determined. To demonstrate restoration of the normal sterol profile, cells were taken after 23 days culture in the presence of 10 ng/ml azasterol and transferred to fresh azasterol-free medium and again subcultured at 3 day intervals into this medium; samples were withdrawn at intervals for sterol analysis.

Sterol extraction

After harvesting (10 ml of culture), cells were washed twice with Locke's solution (150 mM NaCl/3 mM $CaCl_2$/5 mM KCl/2 mM $NaHCO_3$/5.6 mM glucose) to remove medium remaining on the surface of the cells and then frozen until extraction. 5α-Cholestane (10 μg) was added to each culture as an internal standard; pellets were resuspended in 0.5 ml of water and then stirred in 10 ml of chloroform/methanol (2:1, v/v) for 3 h. The solution was concentrated under a stream of nitrogen until it turned cloudy. It was then extracted with 2 × 10 ml of petroleum ether (40–60 °C) and subsequently dried over anhydrous Na_2SO_4. The solvent extract was dried under a stream of nitrogen and the lipid stored at −20 °C until analysis.

Sterol analysis

Half the total lipid sample was placed in a small vial; 15 μl of pyridine and 15 μl of *NO*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane were added, and the vial was heated at 70 °C for 10 min. The sample was diluted by addition of 70 μl of cyclohexane and transferred to a 100 μl glass insert placed in an autosampler vial. The sterol trimethylsilyl (TMS) ethers were analysed by GC-MS on a Hewlett–Packard 5970 mass selective detector coupled to a HP 5890 gas chromatograph and Chemstation. Samples were injected on to a HP-1 GC capillary column (25 m × 0.22 mm internal diameter; 0.32 μm film thickness). Sample injection was at 50 °C; this temperature was held for 1 min and then increased at 25 °C/min to 150 °C and then at 6.5 °C/min to 285 °C. The final temperature was held for 30 min. Sterol identification was based on mass spectra [1,2,26]. Sterol compositions given are typical of a number of experiments in each case.

[*Me*-$^2H_3$]Methionine labelling studies

Cells were grown in low-methionine HOMEM supplemented with 30 mg/l [*Me*-$^2H_3$]methionine, synthesized as described previously [27]. Cultures were initiated from 15% (v/v) HOMEM-grown cells which were rigorously washed before resuspension in medium supplemented with [*Me*-$^2H_3$]methionine and azasterol (1 pg/ml–10 ng/ml). After 4 h of culture, the lipids were extracted from the cells as described above. Deuterium labelling of sterols was monitored by examining the following fragment ions where *M*1 = undeuteriated molecule and *M*2 = deuteriated molecule: ergosta-5,7,22-trien-3β-ol TMS ether, *m/z* 363 $[M1-TMSOH\text{-}CH_3]^+$, *m/z* 365 $[M2-TMSOH\text{-}CH_3]^+$; ergosta-5,7,24(24[1])-trien-3β-ol TMS ether, *m/z* 363 $[M1-TMSOH\text{-}CH_3]^+$, *m/z* 365 $[M2-TMSOH\text{-}CH_3]^+$; ergosta-5,7,22,24(24[1])-tetraen-3β-ol TMS ether, *m/z* 361 $[M1-TMSOH\text{-}CH_3]^+$, *m/z* 363 $[M2-TMSOH\text{-}CH_3]^+$; ergosta-7,24(24[1])-dien-3β-ol TMS ether,

m/z 455 $[M1-\text{TMSOH-CH}_3]^+$, m/z 457 $[M2-\text{TMSOH-CH}_3]^+$. In addition, stigmasta-5,7,24(24[1])-trien-3β-ol was also observed to be deuterated, but because of the second methylation at C-24[2], it was observed to have three deuterium-labelled species with molecular ions at m/z 485 $[M+1]^+$, 487 $[M+3]^+$ and 488 $[M+4]^+$.

RESULTS AND DISCUSSION

Effect of azasterol on growth

Cultures of *L. donovani* promastigotes were grown for 48 h in the presence of azasterol at concentrations ranging from 1 pg/ml to 100 μg/ml (Figure 2). There was a gradual decrease in growth rate compared with the control cultures in the presence of azasterol at concentrations up to 5 μg/ml. At this concentration, cell numbers were about 50% of control values. As the concentration was increased from 5 μg/ml to 10 μg/ml the growth of the promastigote cultures was abolished. In cultures treated with concentrations of 10 μg/ml and above, the promastigotes appeared rounded and many were aflagellate, indicating that they were no longer viable. There was thus an apparent two-stage effect of the azasterol on the organism. Firstly, a gradual dose-dependent inhibition of culture growth and secondly a cytotoxic inhibition occurring at a concentration of the inhibitor in excess of about 5 μg/ml.

Sterol composition of azasterol-treated promastigotes

The sterol compositions of *L. donovani* promastigotes treated with 5 ng/ml–50 μg/ml azasterol were examined (Table 1). In control cultures the predominant sterol was ergosta-5,7,24(24[1])-trien-3β-ol (**15**, 35.8%), with cholesterol (**1**, 18.3%), ergosta-5,7,22-trien-3β-ol (**12**, 12.9%) and ergosta-5,7-dien-3β-ol (**16**, 11.8%) as the other major sterol components. On exposure to azasterol, even at concentrations as low as 5 ng/ml, there was an appreciable accumulation of cholesta-5,7,24-trien-3β-ol (**8**, Figure 1) and cholesta-7,24-dien-3β-ol (**9**) which together accounted for over 50% of the total sterol at azasterol concentrations in the range 5–500 ng/ml. There was a corresponding decline in the proportion of 24-alkyl sterol (**12**, **15** and **16**), providing evidence that the Δ^{24}-sterol transmethylase step of sterol biosynthesis was being inhibited by azasterol. In a further series of incubations, the very high sensitivity of the Δ^{24}-sterol transmethylase to inhibition by azasterol was revealed (Table 2); maximal accumulation of sterols **8** and **9** and diminution of the content of sterols **12**, **15** and **16** was observed at concentrations of azasterol as low as 100 pg/ml (Tables 1 and 2). At an azasterol concentration of 500 ng/ml (Table 1), cholesta-8,24-dien-3β-ol (**7**) also began to accumulate and in the culture treated with 5 μg/ml azasterol, 28% of the sterol was cholesta-8,24-dien-3β-ol (**7**) and 22.5% was cholesta-5,7,24-trien-3β-ol (**8**). With azasterol at a concentration that caused cell morbidity (10 or 50 μg/ml) the recovered sterols consisted of only the two major C_{28} sterols (**12** and **15**) seen in the control culture together with a somewhat higher proportion of cholesterol. The former two sterols may be derived from the original inoculum of cells, and, as the cells had not grown, there had been no production and accumulation of sterols such as **7** or **8**.

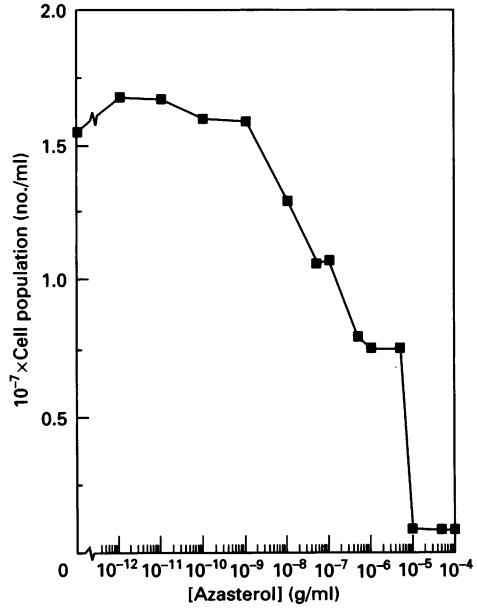

Figure 2 Effect of increasing concentrations of azasterol on a population of promastigotes

Combined azasterol and bis-triazole treatment of promastigotes

When azasterol was used in combination with a bis-triazole inhibitor (ICI 195739) [28] of the sterol 14α-demethylase reaction, no synergism between the two compounds was observed. Azasterol was added at concentrations of 5 pg/ml, 5 ng/ml and 5 μg/ml, but only at 5 μg/ml was there any significant effect on cell growth. The bis-triazole was added at concentrations of 0.1, 1 and 10 μg/ml which reduced cell numbers to 81, 47 and 26% of control values respectively. Combinations of the two drugs inhibited growth of cultures on an additive basis. All cells treated with 5 μg/ml azasterol appeared rounded, and, when the bis-triazole was added, the cells were apparently so fragile that they were unable to survive the harvesting procedure.

Analysis of the sterols produced by cultures treated with azasterol alone (Table 3) showed, as before, an accumulation of cholesta-5,7,24-trien-3β-ol (**8**), cholesta-7,24-dien-3β-ol (**9**) and also, in the 5 μg/ml treated cultures, cholesta-8,24-dien-3β-ol (**7**). Cultures treated with the bis-triazole alone accumulated 14α-methylcholesta-8,24-dien-3β-ol (**19**), 4α,14α-dimethylcholesta-8,24-dien-3β-ol (**20**), 14α-methylergosta-8,24(24[1])-dien-3β-ol (**21**), 14α-methylergosta-8,22,24(24[1])-trien-3β-ol (**22**) and 4α,14α-dimethylergosta-8,24(24[1])-dien-3β-ol (**23**). As the concentration of bis-triazole was increased, the proportions of these sterols changed. At the lowest concentration, 14α-methylcholesta-8,24-dien-3β-ol (**19**), 4α,14α-dimethylcholesta-8,24-dien-3β-ol (**20**) and 14α-methylergosta-8,24(24[1])-dien-3β-ol (**21**) were the major sterols. At 1 μg/ml, 4α,14α-dimethylcholesta-8,24-dien-3β-ol (**20**) and 4α,14α-dimethylergosta-8,24(24[1])-dien-3β-ol (**23**) had increased, and 14α-methylcholesta-8,24-dien-3β-ol (**19**) and 14α-methylergosta-8,24(24[1])-dien-3β-ol (**21**) decreased. At 10 μg/ml, 4α,14α-dimethylcholesta-8,24-dien-3β-ol (**20**) and 4α,14α-dimethylergosta-8,24(24[1])-dien-3β-ol (**23**) were the most significant sterols with only a small amount of 14α-methylergosta-8,24(24[1])-dien-3β-ol (**21**). However, all the cultures retained at least a small amount of ergosta-5,7,22-trien-3β-ol (**12**) and also about 25–35% cholesterol, which was apparently taken up from the culture medium. These effects of the ICI 195739 on the sterol composition of *L. donovani* are in line with similar effects of other bis-triazoles and triazoles on the sterol compositions of several *Leishmania* and *Trypanosoma* species [2,3,29–32].

As the concentration of azasterol was increased in the presence of a constant bis-triazole concentration, the 14α-methylergosta

Table 1 Effect of azasterol on the sterol composition of *L. donovani* promastigotes

L. donovani promastigotes were treated with azasterol over the concentration range 0.005–50 μg/ml, and the sterol composition at each concentration was determined. Sterol identifications: **1**, cholest-5-en-3β-ol; **2**, cholesta-5,24-dien-3β-ol; **6**, cholestatrien-3β-ol; **7**, cholesta-8,24-dien-3β-ol; **8**, cholesta-5,7,24-trien-3β-ol; **9**, cholesta-7,24-dien-3β-ol; **10**, cholesta-5,7,22,24-tetraen-3β-ol; **11**, ergosta-5,7,9(11),22-tetraen-3β-ol; **12**, ergosta-5,7,22-trien-3β-ol; **13**, ergosta-5,7,9(11),24(24[1])-tetraen-3β-ol; **14**, ergosta-5,7,22,24(24[1])-tetraen-3β-ol; **15**, ergosta-5,7,24(24[1])-trien-3β-ol; **16**, ergosta-5,7-dien-3β-ol; **17**, ergosta-7,24(24[1])-dien-3β-ol; **20**, 4α,14α-dimethylcholesta-8,24-dien-3β-ol; **23**, 4α,14α-dimethylergosta-8,24(24[1])-dien-3β-ol. For structures, see ref. [4]. Structures of sterols **8** and **15** are also given in Figure 2. tr, Trace.

	Sterol composition (%)									
Sterol	Control	0.005	0.01	0.05	0.1	0.5	1.0	5.0	10	50
1	18.3	19.6	19.0	18.4	19.4	16.2	21.6	15.2	57.7	78.4
2	0.4	2.6	3.5	4.8	7.9	4.7	1.7	2.5	–	–
6	–	–	–	–	–	–	6.1	5.4	–	–
7	–	2.7	–	–	–	2.6	11.2	28.1	–	–
8	–	47.9	49.3	46.5	48.6	46.4	34.9	22.5	–	–
9	–	7.7	5.6	4.3	6.8	4.6	7.3	7.4	–	–
10	1.6	–	–	–	–	–	–	–	–	–
11	4.6	–	–	–	–	–	–	–	–	–
12	12.9	8.9	8.9	10.4	8.4	9.2	8.0	5.3	16.7	10.9
13	5.6	–	–	–	3.5	–	–	–	–	–
14	7.6	4.3	3.9	5.7	–	5.5	3.0	5.6	–	–
15	35.8	5.3	7.4	7.1	4.1	7.0	5.6	7.0	25.6	10.7
16	11.8	0.9	2.3	2.8	1.2	2.2	0.6	0.9	–	–
17	1.4	–	–	–	–	–	–	–	–	–
20	–	–	tr	tr	–	tr	–	–	–	–
23	–	–	–	–	–	1.7	–	–	–	–
Total sterol (μg/culture)	51.75	83.31	79.79	70.14	56.63	77.62	59.27	66.71	10.21	5.05

Table 2 Effect of non-growth-inhibitory concentrations of azasterol on the sterol profile of *L. donovani* promastigotes

L. donovani promastigotes were treated with azasterol over the concentration range 1 pg/ml–10 ng/ml, and the sterol composition at each concentration was determined. The percentage of the total sterol with the cholestane skeleton but excluding cholesterol (%C) was also determined. Abbreviation: tr, trace.

	Sterol composition (%)					
Sterol	Control	1 pg/ml	10 pg/ml	100 pg/ml	1 ng/ml	10 ng/ml
1	5.8	6.7	7.7	7.5	9.8	7.9
2	1.4	–	1.8	2.7	–	–
4	–	–	–	–	6.0	4.9
5	–	–	–	–	tr	tr
6	–	2.4	–	–	–	–
7	0.7	2.0	–	2.2	tr	tr
8	4.5	9.9	9.1	45.9	46.5	52.6
9	4.6	7.1	4.8	16.3	18.1	14.1
10	6.2	4.5	6.3	3.0	2.1	2.1
12	21.0	19.5	19.3	10.6	9.6	9.7
15	48.3	41.2	45.2	8.3	7.7	6.9
16	4.5	3.6	4.4	tr	tr	tr
18	1.3	1.2	–	–	–	–
23	1.7	1.7	1.3	3.4	–	1.8
Total sterol (μg/culture)	48.40	49.85	35.68	32.42	30.68	44.54
%C	17.0	28.1	23.2	74.6	80.4	79.5

compounds were replaced by 14α-methylcholesta compounds (Table 3). The major sterols present in the dual-treated cultures were 4α,14α-dimethylcholesta-8,24-dien-3β-ol (**20**) with a smaller amount of 14α-methylcholesta-8,24-dien-3β-ol (**19**) together with cholesterol from the culture medium. 14α-Methylcholesta derivatives accumulated in the presence of the two inhibitors but there was some build-up of the 14α-methylergosta derivative **23** in three of the cultures (**j**, **l** and **m**). In only one culture (**k**) was there evidence of a sterol with the cholestane skeleton and lacking a 14α-methyl group (except for cholesterol itself which was derived from the medium). Thus it appeared that both the inhibition of C-24 alkylation and the inhibition of C-14 demethylation by these aza-compounds were very effective. However, again a minor amount of ergosta-5,7,22-trien-3β-ol (**12**) remained in all cultures; this may be carried over from the cells used in the inoculum to set up the cultures. No data could be obtained for cultures treated with 5 μg/ml azasterol in the presence of bis-triazole as the cells were too delicate to survive harvesting.

Table 3 Sterol compositions of *L. donovani* promastigote cultures treated with combinations of azasterol and bis-triazole

The amount of drug(s) added to the culture (azasterol/ml: bis-triazole (ICI 195739)/ml) is indicated as follows; a = 0:0 (control); b = 5 pg:0; c = 5 ng:0; d = 5 μg:0; e = 0:0.1 μg; f = 0:1 μg; g = 0:10 μg; h = 5 pg:0.1 μg; i = 5 pg:1 μg; j = 5 pg:10 μg; k = 5 ng:0.1 μg; l = 5 ng:1 μg; m = 5 ng:10 μg. Sterol identifications: **1**, cholest-5-en-3β-ol; **2**, cholesta-5,24-dien-3β-ol; **3**, cholesta-5,7,9(11)-trien-3β-ol; **4**, cholesta-5,7,9(11),24-tetraen-3β-ol; **7**, cholesta-8,24-dien-3β-ol; **8**, cholesta-5,7,24-trien-3β-ol; **9**, cholesta-7,24-dien-3β-ol; **10**, cholesta-5,7,22,24-tetraen-3β-ol; **11**, ergosta-5,7,9(11),22-tetraen-3β-ol; **12**, ergosta-5,7,22-trien-3β-ol; **13**, ergosta-5,7,9(11),24(24^1)-tetraen-3β-ol; **14**, ergosta-5,7,22,24(24^1)-tetraen-3β-ol; **15**, ergosta-5,7,24(24^1)-trien-3β-ol; **16**, ergosta-5,7-dien-3β-ol; **17**, ergosta-7,24(24^1)-dien-3β-ol; **18**, stigmasta-5,7,24(24^1)-trien-3β-ol; **19**, 14α-methylcholesta-8,24-dien-3β-ol; **20**, 4α,14α-dimethylcholesta-8,24-dien-3β-ol; **21**, 14α-methylergosta-8,24(24^1)-dien-3β-ol; **22**, 14α-methylergosta-8,22,24(24^1)-trien-3β-ol; **23**, 4α,14α-dimethylergosta-8,24(24^1)-dien-3β-ol. Abbreviation: tr, trace.

	Sterol composition (%)												
	Azasterol				Bis-triazole			Azasterol + bis-triazole					
Sterol	a	b	c	d	e	f	g	h	i	j	k	l	m
1	42.3	31.9	45.4	27.6	36.5	24.7	31.0	28.8	28.2	26.2	32.1	27.5	40.9
2	–	1.5	–	–	–	–	–	2.6	–	–	2.6	0.6	–
3	–	–	–	3.3	–	–	–	–	–	–	–	–	–
4	–	–	4.6	–	–	–	–	–	–	–	–	–	–
7	–	–	–	37.6	–	–	–	–	–	–	7.6	–	–
8	–	19.8	26.6	10.6	–	–	–	–	–	–	–	–	–
9	–	4.8	8.8	8.3	–	–	–	–	–	–	0.4	–	–
10	0.5	–	–	–	–	–	–	–	–	–	–	–	–
11	1.5	–	–	–	–	–	–	–	–	–	–	–	–
12	12.3	13.6	6.1	3.4	7.9	6.2	2.3	10.9	5.8	2.4	2.8	3.0	–
13	1.9	3.2	0.3	2.6	–	–	–	–	–	–	1.0	–	–
14	4.9	–	–	–	–	–	–	–	–	–	–	–	–
15	30.7	22.7	5.6	4.7	3.2	1.0	–	1.3	1.9	0.4	2.2	3.4	–
16	3.3	1.4	0.8	0.9	–	–	–	–	–	–	–	–	–
17	2.7	0.9	–	–	–	–	–	0.8	–	–	–	–	–
18	–	–	–	–	3.6	1.5	–	–	–	–	–	–	–
19	–	–	–	–	9.0	3.2	tr	20.4	9.4	1.8	30.2	12.2	–
20	–	–	1.6	1.0	15.9	41.1	46.2	21.8	46.2	50.2	20.8	49.0	45.0
21	–	–	–	–	21.0	10.4	3.6	10.9	5.9	1.6	–	–	–
22	–	–	–	–	1.4	3.2	–	2.5	2.6	tr	–	tr	3.1
23	–	–	–	Tr	1.5	8.7	17.4	–	–	15.4	0.4	4.3	11.0
Total sterol (μg/culture)	23.7	62.6	31.5	58.1	54.2	51.5	38.3	35.3	36.3	34.0	35.7	42.5	13.2

Table 4 Percentage composition of endogenous sterols from *L. donovani* cultured in the presence of azasterol and bis-triazole in terms of methylation at C-24, C-4 and C-14

The amount of drug(s) added to the culture (azasterol/ml: bis-triazole (ICI 195739)/ml) indicated as follows; a = 0:0 (control); b = 5 pg:0; c = 5 ng:0; d = 5 μg:0; e = 0:0.1 μg; f = 0:1 μg; g = 0:10 μg; h = 5 pg:0.1 μg; i = 5 pg:1 μg; j = 5 pg:10 μg; k = 5 ng:0.1 μg; l = 5 ng:1 μg; m = 5 ng:10 μg. C, C_{27}-cholestane-based sterols excluding cholesterol; E, C_{28}-ergostane-based sterols; 14MeC, cholestane-based sterols with a 14α-methyl and, in some cases, a 4α-methyl substituent; 14MeE, ergostane-based sterols with a 14α-methyl and, in some cases, a 4α-methyl substituent. Abbreviation: tr, trace.

	Sterol composition (%)												
	Azasterol				Bis-triazole			Azasterol + bis-triazole					
	a	b	c	d	e	f	g	h	i	j	k	l	m
C	1	38	73	82	–	–	–	4	–	–	15	1	–
E	99	62	24	16	23	11	3	17	11	4	9	9	tr
14MeC	–	–	3	1	39	59	67	60	77	72	75	84	76
14MeE	–	–	–	–	38	30	30	19	12	24	1	6	24

Table 4 presents an overview of the effects of the azasterol and bis-triazole on the nature of the sterols in the treated cells. The results for cholesterol, which is taken up from the foetal calf serum in the culture medium, have been omitted, and the results for the remaining sterols produced *de novo* by the protozoan have been summed with respect to the nature of the carbon skeletons (cholestenols, ergostenols, 14α-methylcholestenols and 14α-methylergostenols). In control cultures virtually all the sterol was of the ergostenol type. When azasterol was added, the ergostenols were partially replaced by a quantity of cholestenols; in the presence of bis-triazole, 14α-methylcholestenols and 14α-methylergostenols were predominant. When the two inhibitors were added together, there was an increase, as expected, in the 14α-methylcholestenols, which is the result of azasterol inhibition of 24-methylation of the precursor sterols. However, cells treated with the highest concentrations of both inhibitors exhibited a higher proportion of 4α,14α-dimethylergostenols than might be expected.

Table 5 Extent of incorporation of [*Me*-$^2H_3$]methionine into sterols of *L. donovani* in the presence of azasterol

The percentage of the sterol containing deuterium at position C-24[1] (and C-24[2] in the case of sterol **18**) was determined on exposure to azasterol of various concentrations as shown. nd, Deuterium labelling of the sterol was not detected. Sterol identifications: **12**, ergosta-5,7,22-trien-3β-ol; **13**, ergosta-5,7,9(11),24(24[1])-tetraen-3β-ol; **14**, ergosta-5,7,22,24(24[1])-tetraen-3β-ol; **15**, ergosta-5,7,24(24[1])-trien-3β-ol; **17**, ergosta-7,24(24[1])-dien-3β-ol; **18**, stigmasta-5,7,24(24[1])-trien-3β-ol.

	Percentage of sterol with ^2H at C-24[1]					
Sterol	Control	1 pg/ml	10 pg/ml	100 pg/ml	1 ng/ml	10 ng/ml
12	30	23	22	14	5	nd
13	67	64	62	0	nd	nd
14	60	58	57	0	nd	nd
15	65	60	63	23	nd	nd
17	63	31	53	nd	nd	nd
18	16	15	nd	nd	nd	nd

Azasterol inhibition of [*Me*-$^2H_3$]methionine incorporation into sterols

In all the experiments with azasterol alone, or in combination with the bis-triazole, the cells retained some 24-alkylsterol, and compounds **12** and **15** were never less than about 5% of the total sterol mixture (see comment above). This amount of 24-alkylsterol may have been sufficient to maintain growth if it were acting in some 'sparking' or 'metabolic' capacity as suggested for other organisms such as yeasts (see the Introduction). We therefore investigated the origin of the 24-alkylsterol that persisted in inhibitor-treated cells and determined whether azasterol completely or only partially inhibited the Δ^{24}-sterol transmethylase. A commercially available low-methionine medium (HOMEM) was supplemented with [*Me*-$^2H_3$]methionine and used to examine further azasterol inhibition of the sterol C-24 methylation process in *L. donovani* promastigotes. Cells were incubated in this medium for 48 h, over a range of azasterol concentrations, and ^2H incorporation into the sterols was measured after that time. The C-24 alkylsterols synthesized during exposure to the deuterium-labelled methionine had molecular masses 2 mass units higher than normal, as the *Me*-$^2H_3$ group was added by a mechanism that gave rise to a 24-methylenesterol intermediate which is converted into the various C-24 alkylsterols typical of the organism [33].

The effect of azasterol on cell growth over the concentrations used in this labelling experiment was negligible. Analysis of the sterols showed that, with cultures exposed to azasterol at concentrations exceeding 100 pg/ml, there was an accumulation of cholesta-5,7,24-trien-3β-ol (**8**) and, to a lesser extent, cholesta-7,24-dien-3β-ol (**9**), at the expense of ergosta-5,7,24(24[1])-trien-3β-ol (**15**) and ergosta-5,7-dien-3β-ol (**16**). The amount of ergosta-5,7,22-trien-3β-ol (**12**) was also reduced to about half its control value (see also data in Table 2). Thus, even though the growth of the cultures was unaffected, the proportion of sterol with the cholestane skeleton as opposed to the ergostane-based structure rose dramatically on exposure to azasterol at concentrations in excess of 100 pg/ml.

Deuterium incorporation from [*Me*-$^2H_3$]methionine into these cells confirmed azasterol inhibition of C-24 alkylsterol synthesis at inhibitor concentrations above 100 pg/ml (Table 5). In the control, with the exception of ergosta-5,7,22-trien-3β-ol (**12**), about 60% of the C_{28} sterols (**13–17**) contained two deuterium atoms. However, ergosta-5,7,22-trien-3β-ol (**12**) was only about 30% dideuteriated. This may be explained by the fact that ergosta-5,7,22-trien-3β-ol is the final biosynthetic product and the amount of deuterium reaching this compound during the experiment may be less than that reaching its precursors. There was also evidence of deuteriation of the sigmasta-5,7,24(24[1])-trien-3β-ol (**18**), and three different deuteriated species were detected (MS ions for $[M+1]^+$, $[M+3]^+$ and $[M+4]^+$) representing the incorporation of one, three and four deuterium atoms from the two methyl groups derived from methionine and consistent with the proposed mechanism for elaboration of a 24-ethylidene sterol such as **18**. It was notable that deuterium labelling of sterol **18** was eliminated on exposure to 10 pg/ml azasterol, but this concentration of inhibitor had little or no effect on the incorporation of deuterium into the 24-methylene sterols (**13–15**, **17**). This may indicate that the second transmethylation reaction required for the conversion of a 24-methylene sterol (e.g. **17**) into a 24-ethylidene (**18**) is more sensitive to inhibition by this particular azasterol.

At 100 pg/ml azasterol the only sterols containing deuterium were ergosta-5,7,22-trien-3β-ol (**12**) and ergosta-5,7,24(24[1])-trien-3β-ol (**15**) but the extent of deuteriation was significantly reduced in both. With the azasterol concentration at 1 ng/ml there was only a very small incorporation of deuterium into sterol **12**, and, at 10 ng/ml, labelling of the sterols was eliminated. It is therefore clear that the azasterol was inhibiting the formation of 24-alkylsterols, and any present in cells cultured in the presence of azasterol at concentrations in excess of 1 ng/ml must have been derived from the initial inoculum of *L. donovani* cells used to start the culture. It thus appears that 24-alkylsterols have a relatively long life in protozoan cells and may not be easily depleted in two or three generations by culture in the presence of the azasterol inhibitor of the sterol C-24 transmethylation reaction.

Continuous culture of promastigotes with azasterol

A long-term culture of the protozoan in the continuous presence of a sublethal concentration of the azasterol was undertaken to determine whether the cells could be totally depleted of 24-alkylated sterol and still remain viable. Table 6 presents the sterol compositions of cultures grown continuously in the presence of 100 pg/ml or 1 ng/ml azasterol, which did not cause any reduction in the growth rate compared with controls. Exposure of the cells to 100 pg/ml azasterol caused a gradual appearance of cholesta-5,7,24-trien-3β-ol (**8**) and a corresponding decrease in the proportion of ergosta-5,7,24(24[1])-trien-3β-ol (**15**) up to 30 days of culture. This reveals incomplete inhibition of the C-24 sterol transmethylation reaction, thereby enabling the cells to maintain the concentration of 24-alkylsterols at a relatively high

Table 6 Effect on the sterol composition of continuous culture of *L. donovani* promastigotes in the presence of azasterol at 100 pg/ml or 1 ng/ml concentrations

A, cells were cultured with 100 pg/ml azasterol for 2, 7, 16 or 30 days; B, cells were cultured with 1 ng/ml azasterol for 2, 7, 16 or 35 days. Sterol identifications: **1**, cholest-5-en-3β-ol; UnCt, unidentified cholestatetraenol; **8**, cholesta-5,7,24-trien-3β-ol; **9**, cholesta-7,24-dien-3β-ol; **12**, ergosta-5,7,22-trien-3β-ol; **15**, ergosta-5,7,24(24^1)-trien-3β-ol; UnEt, unidentified ergostatetraenol; UnC_{29}, unidentified C_{29} sterol. Abbreviation: tr, trace.

	Sterol composition (%)								
		A				B			
Sterol	Control	2	7	16	30	2	7	16	35
1	18.8	12.2	23.5	17.1	14.0	17.1	27.2	15.8	12.9
UnCt	–	–	–	–	–	–	6.6	–	2.2
8	–	–	10.0	13.0	20.2	21.1	53.1	83.7	74.6
9	–	–	–	–	–	–	7.4	–	7.6
12	13.1	7.9	10.1	12.8	13.3	13.2	–	–	tr
15	64.7	51.6	51.9	52.8	47.5	45.9	5.6	0.5	2.7
UnEt	3.4	5.0	4.3	4.3	5.0	2.5	–	–	–
UnC_{29}	–	23.3	–	–	–	–	–	–	–
Total sterol (μg/culture)	17.6	16.5	15.7	16.1	15.5	22.2	10.4	18.6	27.1

level. It is notable that after an initial dip at 2 days, the concentration of ergosta-5,7,22-trien-3β-ol (**12**) was restored to a value close to that in the untreated control. Extending the concept of a sparking role for sterol in yeast, this could be interpreted as sterol **12** being the preferred product sterol needed to fulfil some particular function in the protozoan. At an azasterol concentration of 1 ng/ml there was a rapid and massive build-up of the cholesta-5,7,24-trien-3β-ol (**8**) and depletion of the C_{28} 24-alkylsterols, **12** and **15**. However, a small amount of these sterols still persisted even after 35 days of culture and it could be argued that this may be sufficient to fulfil any 'sparking' role played by such sterols in these protozoa. Therefore, *L. donovani* was cultured continuously with the azasterol concentration raised to 10 ng/ml which had no retarding effect on growth. It was now found that the content of C_{28} 24-alkylsterols (**15**, **16**) declined rapidly to trace levels at 8–32 days of exposure (Table 7), and, after 65 days, sterols **15** and **16** were not detectable by GC–MS analysis. At 65 days the putative precursor sterol, cholesta-5,7,24-trien-3β-ol (**8**), was now the major component of the sterol mixture together with cholesterol (derived from the medium) which showed an increase compared with the control and also with cells cultured for a shorter period of time in the presence of azasterol (Table 7).

After depleting *L. donovani* cells of 24-alkylsterols by continuous culture in the presence of azasterol, we investigated whether the ability to produce 24-alkylsterols was retained if the

Table 7 Effect on the sterol composition of *L. donovani* of continuous culture in the presence of 10 ng/ml azasterol followed by transfer to an azasterol-free medium and continuous culture for a further 28 days

A, cells cultured for 2, 8, 16, 32 or 65 days in the presence of 10 ng/ml azasterol; B, cells cultured in medium containing 10 ng/ml azasterol for 23 days before transfer to azasterol-free medium and culture for 0, 9, 19 or 28 days. Sterol identifications: **1**, cholest-5-en-3β-ol; UnCt, unidentified cholestatetraenol; **8**, cholesta-5,7,24-trien-3β-ol; **9**, cholesta-7,24-dien-3β-ol; **12**, ergosta-5,7,22-trien-3β-ol; **15**, ergosta-5,7,24(24^1)-trien-3β-ol; **16**, ergosta-5,7-dien-3β-ol; **17**, ergosta-7,24(24^1)-trien-3β-ol; UnEt, unidentified ergostatetraenols; **18**, unidentified stigmasta derivatives; **25**, 4α,4β,14α-trimethyl and 4α,14α-dimethylcholesta derivates. Abbreviation: tr, trace.

	Sterol composition (5)									
		A					B			
Sterol	Control	2	8	16	32	65	0	9	19	28
1	18.8	21.8	19.6	18.6	17.1	32.5	27.7	18.2	21.0	35.4
UnCt	–	3.7	–	3.7	2.6	4.1	–	2.8	–	–
8	–	49.1	79.2	71.4	71.5	63.4	72.3	73.3	0.8	–
9	–	–	1.2	6.4	5.7	–	–	5.7	–	–
12	13.1	–	–	tr	–	–	tr	–	12.0	11.2
15	64.7	4.0	tr	tr	tr	–	tr	–	48.9	48.7
16	–	–	–	–	–	–	–	–	5.2	tr
17	–	–	–	–	–	–	–	–	3.8	tr
UnEt	3.4	–	–	–	–	–	–	–	4.2	4.7
18	–	21.5	–	tr	–	–	–	–	2.1	–
25	–	–	–	–	3.0	–	–	–	2.0	–
Total sterol (μg/culture)	17.6	17.9	24.5	22.1	18.5	22.9	5.6	19.27	24.8	13.8

azasterol was withdrawn. Cells exposed to azasterol for 23 days, which were growing normally but showed only a trace of C_{28} sterol, were transferred to fresh medium containing no azasterol, and the subculturing procedure was continued for a further 28 days. For the first 9 days on the new medium there was little change in sterol composition, with C_{28} sterols being undetectable and the C_{27} triene, **8**, still being the major sterol. This could be explained by the carry-over of sufficient azasterol to maintain inhibition through several generations of cells, as it is active at 50–100 pg/ml in the medium (Tables 1, 2 and 6). Between 9 and 19 days of culture, there was a progressive change in the sterol composition (results not shown) from predominantly C_{27} sterols, with the 24-alkylsterols, **15** and **16**, appearing in amounts comparable with those seen in control cultures. The last trace of the C_{27} triene, **8**, had disappeared by 28 days and, although the amount of cholesterol (**1**) was enhanced, the sterol pattern was now essentially the same as that of the untreated controls (Table 7).

These experiments show that 24-alkylsterols such as **15** and **16** are not an absolute requirement for the survival and growth of *L. donovani* and that the organism can adapt to the sole use of C_{27} sterols such as the accumulated endogenous product cholesta-5,7,24-trien-3β-ol (**8**) and cholesterol (**1**) taken up from the medium. With regard to a bulk role in membranes, the C_{27} sterols are structurally suitable and may play this role as they do in many other organisms, including the mutant yeast sterol autotroph [5,6]. However, if sterols play a 'sparking' role in *Leishmania*, as suggested for yeast [5–9], it must be concluded on the present evidence that a 24-alkyl group is not an absolutely essential structural feature. Moreover, even if 24-alkylsterols are the best suited to play this role, the present results show that the protozoan has the ability to adapt its sterol requirements and substitute available C_{27} sterols, although this may be true only if the change in sterol composition occurs gradually in response to the lower concentrations of azasterol. Certainly we found that high concentrations of azasterol (5–10 μg/ml) were lethal to the organism but other factors may contribute to the toxicity, such as incorporation of the azasterol into the cell membranes at a sufficiently high concentration to cause malfunction. As the experimental protocol used did not allow quantification of the azasterol in the cells, further experiments are required to verify the incorporation of the drug into the cell membranes. To extrapolate these observations to the *in vivo* situation, experiments will also have to be performed on the amastigote form of the parasite.

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REFERENCES

1 Goad, L. J., Holz, G. G. and Beach, D. H. (1984) Mol. Biochem. Parasitol. **10**, 161–170
2 Beach, D. H., Goad, L. J. and Holz, G. G. (1988) Mol. Biochem. Parasitol. **31**, 149–162
3 Hart, D. T., Lauwers, W. J., Willemsens, G., Vanden Bosch, H. and Operdoes, F. R. (1989) Mol. Biochem. Parasitol. **33**, 123–134
4 Haughan, P. A. and Goad, L. J. (1991) in Biochemical Protozoology as a Basis for Drug Design (Coombes, G. H. and North, M. J., eds.), pp. 312–328. Taylor and Francis, London
5 Rodriguez, R. J. and Parks, L. W. (1983) Arch. Biochem. Biophys. **225**, 861–871
6 Rodriguez, R. J., Low, C., Bottema, C. D. K. and Parks, L. W. (1985) Biochim. Biophys. Acta **837**, 336–341
7 Ramgopal, M. and Bloch, K. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 712–715
8 Pinto, W. J. and Nes, W. R. (1983) J. Biol. Chem. **258**, 4472–4476
9 Pinto, W. J., Lozano, R., Sekula, B. C. and Nes, W. R. (1983) Biochem. Biophys. Res. Commun. **112**, 47–54
10 Nes, W. D. and Heupel, R. C. (1988) Arch. Biochem. Biophys. **244**, 211–217
11 Haughan, P. A., Lenton, J. R. and Goad, L. J. (1988) Phytochemistry **27**, 2491–2500
12 Whitaker, B. D. and Nelson, D. L. (1988) J. Gen. Microbiol. **134**, 1441–1447
13 Lorenz, R. T., Casey, W. M. and Parks, L. W. (1989) J. Bacteriol. **171**, 6169–6173
14 Dahl, C., Biemann, H. and Dahl, J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4012–4016
15 Gaber, R. F., Copple, D. M., Kennedy, B. K., Vidal, M. and Bard, M. (1989) Mol. Cell. Biol. **9**, 3447–3456
16 Athrington, B. A., Bennett, L. G., Skatrud, P. L. et al. (1991) Gene **102**, 39–44
17 Ashman, W. H., Barbuch, R. J., Ulbright, C. E., Jarrett, H. W. and Bard, M. (1991) Lipids **26**, 628–632
18 Bard, M., Lees, N. D., Turi, T. et al. (1993) Lipids **28**, 963–967
19 Pierce, H. D., Pierce, A. M., Srinvasan, R., Unrau, A. M. and Oehlschlager, A. C. (1978) Biochim. Biophys. Acta **529**, 429–437
20 Pierce, A. M., Unrau, A. M., Oehlschlager, A. C. and Woods, R. A. (1979) Can. J. Biochem. **57**, 201–208
21 Rahman, M. D. and Pascal, R. A. (1990) J. Biol. Chem. **265**, 4989–4996
22 Nes, W. D. and Le, P. H. (1988) Pesticide Biochem. Physiol. **30**, 87–95
23 Tal, B. and Nes, W. D. (1987) Plant Physiol. Suppl. **83**, 161
24 Boyle, F. T. (1990) in Chemotherapy of Fungal Diseases (Ryley, J. F., ed.), pp. 3–29, Springer Verlag, Berlin
25 Berens, R. T., Brun, R. and Krassner, S. M. (1976) J. Parasitol. **62**, 360–365
26 Goad, L. J. (1991) in Methods in Plant Biochemistry (Harborne, J. B., ed.), vol. 7, pp. 369–434, Academic Press, London
27 Dolphin, D. and Endo, K. (1970) Anal. Biochem. **36**, 338–342
28 Boyle, T., Gilman, T. J., Gravestock, M. B. and Wardleworth, J. M. (1988) Ann. N.Y. Acad. Sci. **544**, 86–100
29 Goad, L. J., Holz, G. G. and Beach, D. H. (1985) Mol. Biochem. Parasitol. **15**, 257–279
30 Berman, J. D., Goad, L. J., Beach, D. H. and Holz, G. G. (1986) Mol. Biochem. Parasitol. **20**, 85–92
31 Haughan, P. A., Chance, M. C. and Goad, L. J. (1992) Biochem. Pharmacol. **44**, 2199–2206
32 Van Den Bossche, H. (1993) Microbiol. Eur. **Nov.-Dec.**, 20–28
33 Haughan, P. A., Chance, M. C. and Goad, L. J. (1993) Exp. Parasitol. **77**, 147–154