Analogues of Thiolactomycin as Potential Antimalarial Agents

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Analogues of the natural antibiotic thiolactomycin (TLM), an inhibitor of the condensing reactions of type II fatty acid synthase, were synthesized and evaluated for their ability to inhibit the growth of the malaria parasite *Plasmodium falciparum*. Alkylation of the C4 hydroxyl group led to the most significant increase in growth inhibition (over a 100-fold increase in activity compared to TLM). To investigate the mode of action, the *P. falciparum* KASIII enzyme was produced for inhibitor assay. A number of TLM derivatives were identified that showed improved inhibition of this enzyme compared to TLM. Structure−activity relationships for enzyme inhibition were identified for some series of TLM analogues, and these also showed weak correlation with inhibition of parasite growth, but this did not hold for other series. On the basis of the lack of a clear correlation between inhibition of pfKASIII activity and parasite growth, we conclude that pfKASIII is not the primary target of TLM analogues. Some of the analogues also inhibited the growth of the parasitic protozoa *Trypanosoma cruzi*, *T. brucei*, and *Leishmania donovani*.

Introduction

Malaria is by far the world's most important tropical disease. In many developing countries and in sub-Saharan Africa especially, malaria exacts an enormous toll in lives, in medical costs, and in days of labor lost. At present, at least 300 million people are affected by malaria globally, and there are between 1 and 1.5 million malaria deaths annually.[1,2] Over the past several decades, the rapid development of drug-resistant strains has compounded the already serious health problems. Of the four known human malaria parasites, *Plasmodium falciparum* is the predominant cause of mortality, with 120 million new cases and 1 million deaths per year globally. It is this particular species that has given rise to formidable drug-resistant strains, resulting in the urgent need for new chemotherapeutic agents. The search for new agents has recently been aided with the completion of the *P. falciparum* genome sequence.[3] Detailed studies[4] of this genome have identified new potential drug and vaccine targets. One such target appears to be the fatty acid biosynthesis pathway of *P. falciparum*.

Fatty acid synthesis is a crucial function of living cells. The main steps in this process in animals are carried out by a single, multifunctional polypeptide fatty acid synthase (type I FAS). In contrast, plants and bacteria utilize a dissociable multienzyme system (type II FAS).[5] The structural differences between these systems are sufficient for the development of selective agents targeted against the β-ketoacyl acyl-carrier-protein synthases (KAS) and other individual enzymes of the type II FAS.[6,7]

In plants and bacteria, there appear to be three KAS enzymes, which are denoted KASI, KASII, and KASIII in plants (the *E. coli* equivalents are FabB, FabF, and FabH, respectively). The initial C2−C4 step of fatty acid biosynthesis is catalyzed by KASIII; thereafter, KASI and KASII are involved in chain elongation.[8] In most organisms with a type II fatty acid synthase, KASI and KASII show very high similarity at the protein level.

Recent discoveries reveal that *Plasmodium* synthesizes fatty acids in the apicoplast, which is a vestigial organelle thought to be derived from a chloroplast.[9−12] Not surprisingly, *Plasmodium* fatty acid synthase is a type II enzyme complex as found in plants and bacteria and, thus, differs markedly from human type I FAS. However, analysis of the recently published *P. falciparum* genome[3] reveals just two different KAS enzymes, with the KASI and KASII of typical type II systems replaced by a single enzyme that we and others have denoted KAS1/2, and a separate KASIII.[8]

A known inhibitor of the dissociable type II FAS enzymes is the naturally occurring thiolactone antibiotic thiolactomycin (TLM) **1**[13,14] (Figure 1). Because of its negligible toxicity toward mammals,[15] TLM or its analogues are leads for new drugs for malaria. Furthermore, because TLM and its derivatives target more than one enzyme (all the KAS enzymes, at least to some extent),[14] resistance due to point mutations should be less of a problem than for some drugs. Waller et al.[16] showed that thiolactomycin inhibits the proliferation of cultured *P. falciparum* with an IC_{50} value of 50 μM, and the antimalarial activity was attributed to the inhibition of the FAS condensing enzymes. However, the fact that there are several enzymatic targets may lead to complex

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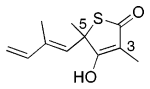

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Figure 1. Structure of thiolactomycin.

modes of action. Recently, Prigge et al. expressed and purified the three *P. falciparum* proteins required to initiate fatty acid biosynthesis (*P. falciparum* acyl carrier protein (pfACP), *P. falciparum* malonyl-CoA/ACP acyl transferase (pfMCAT), and pfKASIII) and revealed thiolactomycin **1** to be a poor inhibitor of the pfKASIII enzyme with an IC_{50} value greater than 330 μM.[17] Despite the low activity of TLM toward the enzyme, three derivatives (1,2-dithiole-3-ones) bearing similarities to the structure of TLM did show improved inhibition of pfKASIII activity (<10 μM). We were keen to discover if our TLM analogues (including derivatives from our earlier studies[18]) bearing the thiolactone ring would show improved activity against the pfKASIII enzyme and if there was any relationship between antimalarial activity and inhibition of this enzyme.

Previous studies have shown that analogues of TLM, with variations at the C5 position, have shown improved inhibition of pea (*Pisum sativum*) FAS[19]. Waller et al. also showed some TLM derivatives with variation at C5 showed encouraging antimalarial activity.[20] Indeed, recently we have shown that the introduction of hydrophobic alkyl groups at both the C3 and C5 positions of the thiolactone ring leads to increased inhibition toward the malaria parasite.[18] The best results were obtained with 10-carbon alkyl side chains at the C5 position accompanied by shorter alkyl groups (ethyl or propyl) at the C3 position. The most active compound (IC_{50} = 10 μM) showed a 14-fold improvement in activity against *P. falciparum* compared to TLM, which in our studies exhibited an IC_{50} of 143 μM.[18] Encouraged by these findings, we have since conducted detailed structure−activity relationships involving further derivatizations at the C3 and C5 positions, a variation in the type of heterocycle, and alkylation of the hydroxyl group at the C4 position. In this paper we present synthetic studies and findings in our attempts to improve the overall activity of TLM against *P. falciparum* cultured in red blood cells. In an attempt to shed more light on the mechanism of action of these compounds, we also assayed the compounds against one of the component enzymes of FAS, a recombinant pfKASIII.

Chemistry

Previously, we have reported the synthesis of a number of thiolactomycin analogues with variations at both the C3 and C5 positions of the ring.[18] We decided to undertake a more complete structure−activity relationship by looking at other positions around the ring. In particular, we proposed the following variations:

(1) Variation of the heterocyclic ring was carried out, in particular the change from a sulfur to an oxygen or nitrogen, which would vary the size and electronic properties of the ring.

(2) Addition of functionality to the substituents at the 5-position to these side chains was carried out with the aim of discovering new interactions between the side chain and the protein to increase potency. In addition, we wanted to increase the hydrophilicity of our compounds, which may be possible to achieve by addition of suitable functionality in the side chain.

(3) Addition of a second substituent to the 3-position of the thiolactone ring was carried out. As well as possible new interactions with the enzyme active site, this should also affect the electronic and steric properties of the ring, giving a carbonyl at the 4-position and removing the double bond between the 3 and 4 positions.

(4) Investigation of a substituent at the 4-position was carried out.

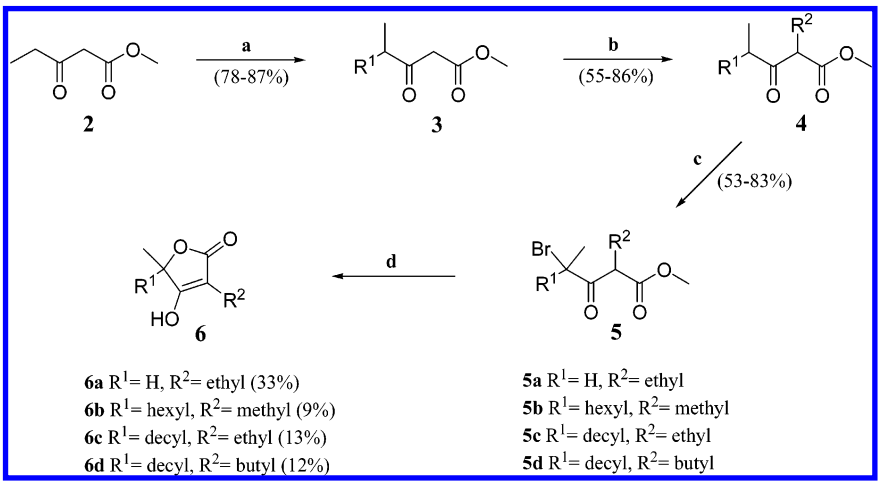
Using a synthetic strategy similar to that we previously reported,[18] we were able to synthesize a range of tetronic acid analogues (Scheme 1). Methyl propionylacetate **2** was treated with alkyl halides in the presence of sodium hydride and butyllithium to provide the alkylated products **3**. Subsequent alkylation at the C2 position with the relevant alkyl and benzyl halides employing potassium carbonate as base gave the desired β-ketoesters **4** in good yields. Treatment with pyridinium tribromide in acetic acid resulted in the formation of the bromides **5**. Following a literature procedure,[21] the bromides were duly converted into their respective tetronic acids **6** upon exposure to potassium hydroxide in water at 0 °C. Isolated yields following column chromatography were relatively low (9−33%). With the construction of the carbon skeleton prior to cyclization, the low-yielding alkylation reaction at the C5 position was avoided; this was especially important because the lactone intermediates were considered to be sensitive to the strong basic conditions required for alkylation.

The synthesis of the nitrogen analogue was accomplished as shown in Scheme 2. Glycine **7** was treated with thionyl chloride in dry dichloromethane at 0 °C, resulting in the formation of the methyl ester hydrochloride **8** in 89% yield. The ester was subsequently treated with benzophenone imine to afford the imine **9**. Following a literature procedure,[22] the imine was successfully alkylated to yield **10** (62%). Hydrolysis with 2 M HCl delivered the free amine **11**, which when reacted with diketene at 0 °C formed the desired cyclization precursor **12** in good yield (59%). Exposure of the amide **12** to sodium methoxide in refluxing methanol induced a Dieckman type cyclization,[23] resulting in the desired pyrole **13** in 10% yield.

In our previous studies we described the synthesis of a number of analogues with variations at the C5 position of the ring.[18] Recently, Douglas et al.[24] have reported improved methodology for the low-yielding alkylation reaction employing lithium bis(trimethylsilyl)amide (LTMSA) as base. Treatment of the thiolactone **14** with LTMSA and bromohexadecane in THF at −78 °C delivered the desired analogues **15** and **16** (Scheme 3). In a similar fashion, analogues **17** and **18** were also synthesized in 10% and 20% yields, respectively. Deprotection of the silyl ether **18** gave the primary alcohol **19** in 18% yield.

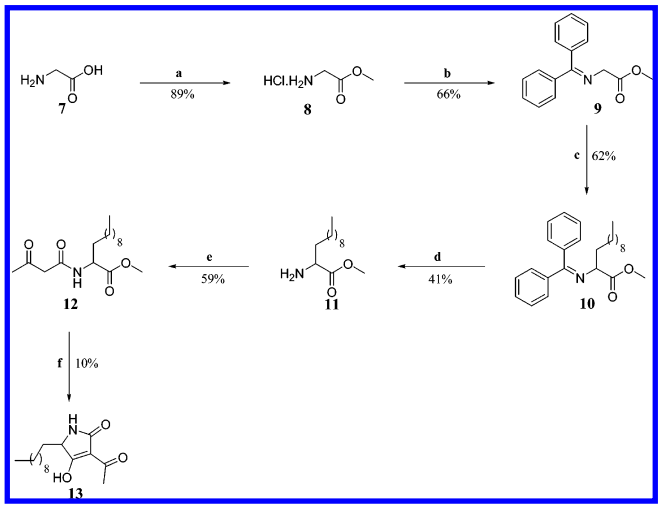
Derivitization of both the C3 position and the hydroxyl group was accomplished as shown in Scheme 4. Following a literature procedure,[24] the alkylated products **21** were obtained in varying yields by treatment of the thiolactone **20** with sodium hydride and the relevant halides. In the majority of cases the major

Scheme 1[a]



[a] Reagents and conditions: (a) NaH (1.2 equiv), BuLi (1.1 equiv), R^1I, 0 °C to room temp; (b) R^1I, K_2CO_3 (4 equiv), THF, reflux; (c) $PyBr_3$, AcOH, room temp; (d) KOH (2.5 equiv), H_2O, 0 °C to room temp.

Scheme 2[a]



[a] Reagents and conditions: (a) $SOCl_2$ (1.5 equiv), MeOH, 0 °C to room temp; (b) benzophenone imine (1 equiv), CH_2Cl_2, room temp; (c) LDA (1.1 equiv), iododecane (1 equiv), THF, −78 °C to room temp; (d) 2 M HCl, Et_2O, room temp; (e) diketene (1.2 equiv), $CHCl_3$, 0 °C to room temp; (f) NaOMe (1 equiv), MeOH, reflux.

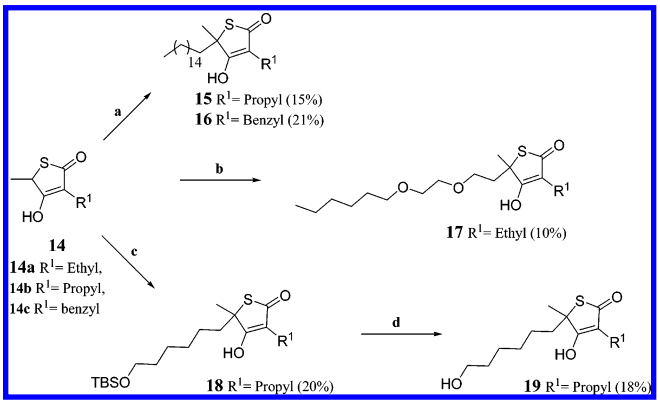
reaction appeared to be the desired alkylation at the C3 position of the ring, as reported in the literature. However, it was evident that a side reaction was occurring during the reaction, in which the hydroxyl group was also alkylated. Following flash column chromatography these compounds **22** were isolated, albeit in low yields.

Results and Discussion

All compounds were assayed in vitro for their activity against the malarial parasite *P. falciparum* and the trypanosomatid parasites *T. brucei*, *T. cruzi*, and *L. donovani* (Tables 1−3). Compounds were screened against the clinically relevant form of the parasites: *P. falciparum* cultured in red blood cells, blood stream form *T. brucei rhodesiense*, *T. cruzi* amastigotes cultured intracellularly, and *L. donovani* axenic amastigotes.

Furthermore, the production of the recombinant maltose binding protein, *P. falciparum* KASIII (MBP-pfKASIII) fusion protein, has enabled us to screen our derivatives for enzyme inhibition against this condensing enzyme component of FAS (Tables 1−3). Previously we reported the activity of some thiolactomycin analogues (**23a**−**d**, **24a**−**k**, **25a**,**b**, **26a**,**b**, and **27**)[18] against *P. falciparum* in vitro. We have now assayed these compounds against the MBP-pfKASIII enzyme and report these data here for the first time to give a more comprehensive view of their activity against the enzyme (Table 1). The screening procedures for all assays are briefly described in the Experimental Section.

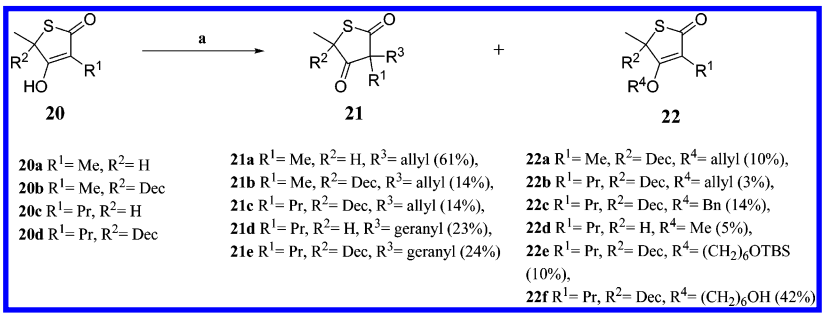
Scheme 3[a]



[a] Reagents and conditions: (a) LTMSA (2 equiv), bromohexadecane, THF, −78 °C to room temp; (b) LTMSA (2 equiv), alkyl bromide, THF, −78 °C to room temp; (c) LTMSA (2 equiv), alkyl bromide, THF, −78 °C to room temp; (d) 2 M HCl, MeOH, room temp.

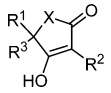
Growth Inhibition of *P. falciparum*. We have previously reported that analogues of the antibiotic thiolactomycin with aliphatic substituents at the C3 and C5 positions of the thiolactone ring have significantly enhanced activity against *P. falciparum* in vitro.[18] The best results were obtained with the longer alkyl chains (over 10 carbons in length) at the C5 position, supplemented with a smaller increase in chain length at the C3 position (two to three carbons). Following on from these initial structure−activity relationships, a new series of compounds has been designed. As discussed in the Introduction, thiolactomycin and its analogues may target a number of different enzymes, in this case KAS1/2 and/or KASIII, and possibly other molecular targets. This may give inhibitors complex modes of action and complex SARs. In addition, in studies of a

Scheme 4[a]



[a] Reagents and conditions: (a) NaH, R^3X, THF, reflux.

Table 1. Effect of Variation of the Heteroatom and Substituents at C3 and C5 on Parasite Growth and Inhibition of *P. falciparum* KASIII[a]



compd	R^1	R^2	R^3	X	*P. falc* IC_{50} (μM)	*T. cruzi* IC_{50} (μM)	*T. b. rhod.* IC_{50} (μM)	*L. don.* axenic IC_{50} (μM)	% inhib pfKASIII[c]
1[b]	Me	Me	isoprenoid	S	143	>427	256		
1a[b]	Me	Me	isoprenoid	S	>95	>142	182	56	30
6a	Me	Et	H	O	>351	>633	>633		
6b	Me	Me	hexyl	O	222	>424	247		NA
6c	Me	Et	decyl	O	94	146	62		
6d	Me	Bu	decyl	O	>15	70	13		60
13	Me	Ac	decyl	NH	63	153	7	3	NA
15	Me	Pr	hexadecyl	S	6	13	29	0.5	20
16	Me	Bn	hexadecyl	S	7	14	32	2	50
17	Me	Et	$-(CH_2)_2O(CH_2)_2O(CH_2)_5CH_3$	S	>60	195	119		NA
18	Me	Pr	$-(CH_2)_6$OTBS	S	37	150	2	1	NA
19	Me	Pr	$-(CH_2)_6$OH	S	157	64	64	88	NA
23a	Me	Me	H	S	>347	>624	>624		NA
23b	Me	Et	H	S	233	568	357		NA
23c	Me	Pr	H	S	>290	>522	288		NA
23d	Me	Bn	H	S	195	>408	408		NA
24a	Me	Me	hexyl	S	139	127	112		10
24b	Me	Me	octyl	S	153	>350	194		10
24c	Me	Me	geranyl	S	40	68	190		45
24d	Me	Me	decyl	S	36	68	171		30
24e	Me	Me	hexadecyl	S	25	28	62		55
24f	Me	Et	hexyl	S	61	195	71		5
24g	Me	Et	octyl	S	54	165	157		NA
24h	Me	Et	decyl	S	15	54	97		40
24i	Me	Et	hexadecyl	S	19	43	47		65
24j	Me	Pr	decyl	S	10	56	21		55
24k	Me	Bn	decyl	S	50	64	132		70
25a	Et	Me	H	S	>316	>568	>568		NA
25b	decyl	Me	H	S	72	164	6		30
26a	Et	Me	decyl	S	65	72	153		15
26b	Et	Me	hexadecyl	S	35	49	130		40
27	Me	Bu	decyl	S	71	77	8		60

[a] NA = no activity. Data for compounds **23**–**27** against *P. falciparum*, *T. cruzi*, and *T. b. rhodesiense* were reported previously and were included for comparative purposes. The inhibition data for **23**–**27** against pfKASIII are reported here for the first time. [b] Compound **1** is synthetic thiolactomycin prepared in our laboratories; compound **1a** is commercial thiolactomycin purchased from Sigma. [c] The percentage inhibition of MBP-pfKASIII at an inhibitor concentration of 100 μM.

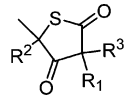
whole organism (in this case *P. falciparum*) there are further factors that complicate the SAR, such as uptake of compounds into cells and their metabolism.

There are currently no crystal structures available for any of the *Plasmodium* enzymes to assist the design process. Furthermore, there are no structures currently available for any KASIII in complex with TLM. However, there are crystal structures for various plant and bacterial enzymes, which may give some guidance to the design process, although of course care must be taken when interpreting data from these structures.

(a) Variation of the Heteroatom in the Five-Membered Ring. We decided to investigate variations of the heteroatom in the TLM ring (Table 1). Interestingly, in the case of the *Escherichia coli* KAS1 structure, the sulfur of the thiolactone ring does not appear to make any specific interactions with the active site.[25] Oxygen derivatives with longer alkyl chains at the C5 position lead to an increase in activity (**6a**–**c**) as with the analogous sulfur compounds[18] but were less active than the corresponding sulfur analogues (**23b**, **24a**, **24h**).

Owing to synthetic restraints, the synthesis of the nitrogen heterocycle **13** required the presence of an acetyl group at the C3 position of the ring. Earlier studies have shown that C3 acetyl analogues of TLM

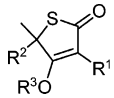
Table 2. Effect of 3,3-Disubstituted Compounds on Parasite Growth and Inhibition of *P. falciparum* KASIII



compd	R^1	R^2	R^3	*P. falc* IC_{50} (μM)	*T. cruzi* IC_{50} (μM)	*T. brucei* IC_{50} (μM)	*L. don.* axenic IC_{50} (μM)	% inhib pfKASIII[a]
21a	Me	H	allyl	62	>488	79		no activity
21b	Me	decyl	allyl	26	55	157		5
21c	Pr	decyl	allyl	19	47	9	82	no activity
21d	Pr	H	geranyl	9	21	55	12	90
21e	Pr	decyl	geranyl	77	>66	>200	>66	no activity

[a] The percentage inhibition of MBP-pfKASIII at an inhibitor concentration of 100 μM.

Table 3. Effect of 4-OH Substituted Compound on Parasite Growth and Inhibition of *P. falciparum* KASIII



compd	R^1	R^2	R^3	*P. falc* IC_{50} (μM)	*T. cruzi* IC_{50} (μM)	*T. brucei* IC_{50} (μM)	*L. don.* axenic IC_{50} (μM)	% inhib pfKASIII[a]
22a	Me	decyl	allyl	6	30	167		5
22b	Pr	decyl	allyl	1	80	14	66	no activity
22c	Pr	decyl	Bn	4	13	>223	>74	no activity
22d	Pr	H	Me	45	359	9	100	no activity
22e	Pr	decyl	$-(CH_2)_6OTBS$	3	14	149	7	10
22f	Pr	decyl	$-(CH_2)_6OH$	31	64	64	36	5

[a] The percentage inhibition of MBP-pfKASIII at an inhibitor concentration of 100 μM.

with C5 aryl or alkyl functionality displayed effective activities against *Staphylococcus aureus* and *Pasteurella multocida*.[26] Analogue **13** shows a 2-fold increase in activity (IC_{50} = 63 μM) over TLM **1** (IC_{50} = 143 μM) against *P. falciparum* in vitro (Table 1).

(b) Variation of the C5 Side Chain Functionality. To reduce the lipophilic nature of the analogues, we decided to increase the number of hydrogen bond donors/acceptors within the compounds, as either ether linkages (**17**) or a hydroxyl group (**19**), but neither modification showed advantage over TLM. However, as found previously, lipophilic substituents at the C5 position gave improved activity over TLM (**15**, **16**, **18**).

(c) Addition of a Second Substitutent on the 3-Position. Recently, Douglas et al.[24] reported effective inhibition by 3-alkylated 4-ketothiolactone analogues of TLM against *M. tuberculosis* H37Rv. The majority of our analogues (**21a**−**d**) containing the 4-ketothiolactone ring showed improved inhibition over TLM against the malaria parasite (Table 2), although a lipophilic substituent at either the 3- or 5-position was required for improved activity.

(d) Alkylation of the 4-Hydroxyl Group. The fact that the 4-ketothiolactones showed reasonable inhibition toward *P. falciparum* led us to question the influence the hydroxyl group has toward activity. Studies on the interactions of TLM with KAS I from *E. coli*[25] suggest the hydroxyl group at the C4 position binds to amino acid residues 270 and 305 through water-assisted hydrogen bonding. The water molecules are positioned at the base of the active site tunnel that would incorporate the malonyl-ACP substrate and the phosphopantetheine arm of ACP; similar features may possibly be found in *P. falciparum* FAS condensing enzymes such as the KASIII structure. We hoped that modification at the C4 hydroxyl group would exploit possible interactions within the active site tunnel and, hence, improve inhibition of growth if the condensing enzymes were key targets. Indeed, alkylation of the hydroxyl group (analogues **22a**−**f**) led to an increase in activity against the malaria parasite compared to TLM (Table 3). Our most active compounds belong to this series (**22b**, **22e**). A variety of substituents on the hydroxyl group was tolerated, but the presence of a hydrophobic substituent at the 5-position was important.

(e) Activity against Trypanosomatid Parasites. All our compounds were also assayed in vitro against the *Trypanosoma brucei rhodesiense* parasite, which is the causative agent of human African trypanosomiasis. Thiolactomycin has been identified as a promising lead for antitrypanosomal drug development because it inhibits trypanosomal myristate synthesis in vitro with a reported IC_{50} of ~150 μM.[27] Previously, we have shown that analogues of TLM demonstrated improved inhibition of the *T. brucei* parasite with the best analogue showing a 42-fold increase in activity (IC_{50} = 6 μM) compared to TLM (IC_{50} = 256 μM in our assays).[18] Once again, the majority of our compounds showed increased growth inhibition of *T. brucei* parasite compared to TLM (Tables 1−3). Analogue **18** registered the most notable increase in activity with an IC_{50} of 2 μM (Table 1). The most active analogue (**22b**) against the malaria parasite also showed improved inhibition against *T. brucei*, with an IC_{50} of 14 μM (Table 3).

Thiolactomycin **1** showed no in vitro activity (IC_{50} > 427 μM) against the causative organism of Chagas disease, *T. cruzi* (Table 1). However, a number of the analogues did show some improvement in inhibition toward the parasite. This was especially true for analogues **15** and **22c**, both of which showed IC_{50} values of 13 μM (Tables 1 and 3), a considerable improvement compared to TLM.

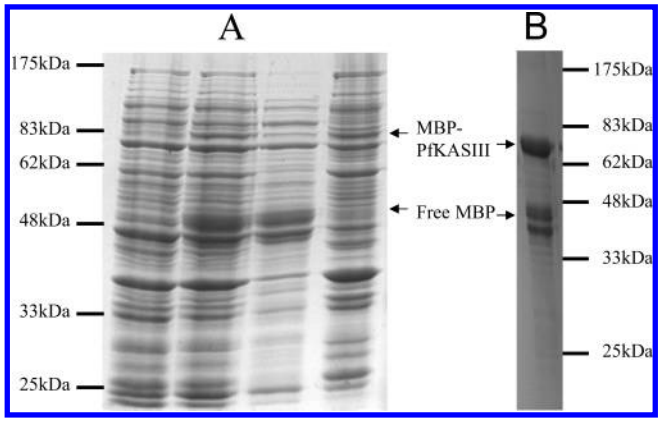


Figure 2. Production of MBP-pfKASIII. (A) A sample was removed from the culture before the addition of 0.4 mM IPTG (0 h), lane 1, and at 16 h postinduction, lane 2. Soluble (lane 3) and insoluble (lane 4) fractions from this 16 h sample were prepared by sonication of the cell suspension, followed by separation of soluble and insoluble fractions by centrifugation as described in the Experimental Section. The induced MBP-pfKASIII, expected size of 79 kDa, is indicated. (B) Sample derived following affinity chromatography. The proteins contained in each sample were separated by SDS−PAGE (12.5% acrylamide) and visualized by staining with Coomassie blue.

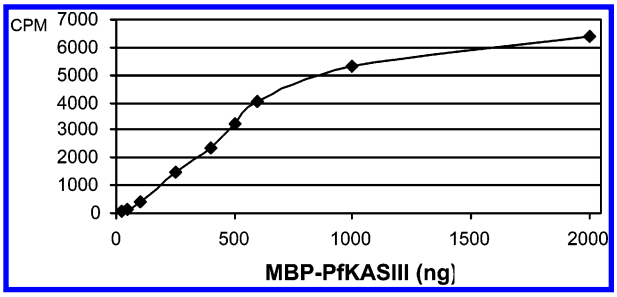


Figure 3. Incorporation of radiolabel with varying amounts of MBP-pfKASIII.

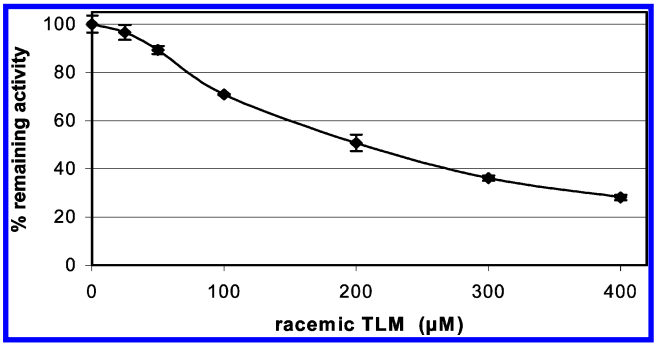


Figure 4. Determination of IC_{50} for thiolactomycin.

Several compounds were also tested in vitro against axenic *L. donovani* amastigotes. Encouragingly, a few compounds showed good activity against the parasite. Analogues **13**, **16**, and **18** all showed IC_{50} values between 1 and 3 μM. However, the best compound (**15**) with a hexadecyl side chain at the C5 position and a propyl substituent at C3 registered an IC_{50} of 0.5 μM (Table 1).

Preparation of Enzyme. To investigate the mode of action of TLM analogues further, we decided to isolate one of the possible target sites for activity, the *P. falciparum* KASIII condensing enzyme. We could then evaluate if there was any correlation between inhibition of the enzyme and inhibition of the growth of *P. falciparum*. The KASIII protein was obtained as a fusion protein with maltose binding protein (MBP-pfKASIII) from a clone in *E. coli*, following a 16 h induction. The protein was present mainly in the insoluble fraction of the *E. coli* cells (Figure 2A). However, sufficient fusion protein (along with some free MBP) was obtained from the soluble fraction following affinity chromatography for subsequent enzyme assay (Figure 2B).

Attempts to separate the MBP from the pfKASIII using the vector encoded factor Xa proteinase cut site located between the two regions proved to be unsuccessful, presumably because of inaccessibility of the cleavage site. However, assays of the fusion protein indicated condensing enzyme activity for the MBP-pfKASIII protein. To determine a suitable concentration of enzyme for use in inhibition assays, the amount of MBP-pfKASIII in the assays was varied while keeping the concentrations of all other components constant (Figure 3). It can be seen from this experiment that the incorporation increases in a linear fashion up to about 600 ng of MBP-pfKASIII. Assessment of enzyme activity at a range of concentrations below 600 ng showed a range of 74−93 pmol min^{-1} μg^{-1} protein. For further assays, 500 ng of fusion protein was used to ensure sufficient radioactive incorporation into product without working outside the linear range of the assay.

The initial inhibitor assay was performed using racemic thiolactomycin **1a** (Sigma, Poole, U.K.) at a range of concentrations and with a final concentration of DMSO of 2%. This DMSO concentration was shown to have no effect on enzyme activity (results not shown). These experiments indicated an IC_{50} for the racemic thiolactomycin under these conditions of approximately 200 μM (Figure 4).

Enzyme Assays. In screening our thiolactomycin derivatives, inhibitor concentrations of 100 μM (at which TLM **1a** gives approximately 30% inhibition) were used in order to assess improvements in activity over the parent compound. A number of the analogues showed increased inhibition of MBP-pfKASIII activity compared to commercial TLM **1a** (Table 1). Notably, as with the in vitro results against the malaria parasite, the derivatives containing the longer alkyl groups at the C5 position tended to show improved activity against the enzyme (Table 1). Analogue **24e**, with a hexadecyl side chain at the C5 position, shows an inhibitory activity of 55%. Likewise, the geranyl derivative **24c** also shows improved activity (45%) compared to TLM (30%).

From the in vitro results against the malaria parasite we had observed that increasing the chain length at the C3 position had a positive effect on efficacy. In the case of the enzyme assays, when there was a hydrophobic substituent in the 5-position, hydrophobic substituents at the 3-position also showed inhibition comparable to or greater than that of TLM (see compounds **24e**, **24i**, **15**, **16**, which have a hexadecyl substituent at the 5-position, and compounds **24h**, **24j**, **27**, **24k**, which have a decyl substituent at the 5-position). For the benzyl derivative **24k**, an IC_{50} against MBP-PfKASIII of 50 μM was determined, showing a 4-fold increase in potency compared to TLM (200 μM). This change in

activity is similar to that observed against *P. falciparum* (IC_{50} = 50 μM for analogue **24k**; IC_{50} = 143 μM for TLM).

The tetronic acid derivative (**6b**) showed suppressed activity against MBP-pfKASIII (Table 1), whereas **6d** with the butyl substituent at C3 inhibited 60% of the enzyme activity. The nitrogen analogue **13** registered no activity against the enzyme. No activity was observed for the analogues in which the long aliphatic side chain at C5 had been functionalized (analogues **17**−**19**), implying that the main mode of action of these series of compounds is not inhibition of MBP-pfKASIII.

Despite showing encouraging inhibition of cultures of *P. falciparum* compared to TLM, the 4-ketothiolactones (**21a**−**e**) mainly showed negligible inhibitory effects against MBP-pfKASIII (Table 2). However, the most active compound against the malaria parasite (**21d**, IC_{50} = 9 μM) for this particular series of analogues also showed strong (90%) inhibition of the enzyme at 100 μM. For the best compound we have produced against MBP-PfKASIII, an IC_{50} for enzyme inhibition was determined as 18 μM, an increase in potency of approximately 10-fold compared to TLM (200 μM). In contrast, the 4-OH substituted compounds (**22a**−**f**) showed negligible inhibition of the enzyme, despite some of the compounds showing potent inhibition of the parasite (Table 3). These inhibitors (**21a**−**e** and **22a**−**f**) thus appear to target another enzyme or alternative pathways.

The lack of general correlation between inhibition of PfKASIII and inhibition of the growth of *P. falciparum* led us to investigate the activity of these compounds against the *P. falciparum* KAS1/2. This enzyme was cloned and overexpressed, and assays were carried out. None of the compounds showed significant inhibition of the PfKAS1/2. (The cloning, characterization, and assay data will be published separately.) This indicates that the inhibitory activity of these compounds on the growth of *P. falciparum* is not due to inhibition of PfKAS1/2.

Conclusion

Following from our previous SAR studies,[18] we have reported the design and synthesis of further thiolactomycin analogues as agents against *P. falciparum*, the causative organism of malaria that produces most fatalities. The compounds were designed with the intention of targeting the type II FAS utilized for fatty acid biosynthesis. Compounds showed complex SAR data against both the parasite and the recombinant MBP-pfKASIII enzyme. There was a weak correlation between antiparasitic activity and enzyme inhibition, suggesting that inhibition of this enzyme is not the primary mode of action. Indeed, it can be noted that effective analogues appeared to be significantly more potent toward growth of *P. falciparum* than toward enzyme inhibition.

When considering inhibition of the parasite growth, several analogues showed inhibitory effects greater than thiolactomycin **1**. Despite showing increased activity compared to TLM, a variation of heteroatom within the ring system did not significantly increase the efficacy of the compounds compared to analogous sulfur derivatives from previous studies.[18] Likewise, the introduction of hydrophilic functionality within the hydrophobic side chain at the C5 position appeared to diminish activity relative to compounds bearing saturated aliphatic side chains. The 4-ketothiolactones showed enhanced inhibition of the malaria parasite compared to TLM. However, it was alkylation of the C4 hydroxyl group that brought about the most significant increases in activity, with the best compound (**22b**) showing a 143-fold increase in activity (IC_{50} = 1 μM) compared to TLM.

The inhibitory effects of our compounds against the first condensing enzyme identified in *P. falciparum* (MBP-pfKASIII) are also reported. A number of compounds showed improved inhibition of the enzyme compared to commercial TLM **1a**, which produced 30% inhibition at 100 μM. As with the in vitro results against the malaria parasite, generally, compounds bearing the longer hydrophobic side chains at the C5 position tended to exhibit increased inhibition of pfKASIII compared to TLM **1a**. The presence of a benzyl moiety at C3 (analogue **24k**) has a further positive effect toward inhibition of the enzyme (70%; IC_{50} = 50 μM). Varying the heteroatom within the ring system resulted in mixed levels of inhibition, with the tetronic acid **6d** showing 60% inhibition but the pyrole derivative **13** exhibiting no activity against pfKASIII. Most other analogues showed weak or negligible inhibition of the enzyme. The 4-ketothiolactone **21d** proved to be the most effective, inhibiting 90% of the enzyme activity (IC_{50} = 18 μM).

When the inhibition of the MBP-pfKASIII and the inhibition of growth of *P. falciparum* in cell culture are compared, there is no general direct correlation, although within a certain series of compounds there is some correlation. This suggests that inhibition of the MBP-pfKASIII is not the primary mode of action of these compounds. Indeed, from studies of type II FAS enzymes from bacteria[25] or plants,[19] the KASIII component is the least sensitive of the condensing enzymes toward TLM or its analogues.[19] Furthermore, the compounds did not show significant inhibition of KAS1/2. Our data would suggest, therefore, that other enzymes or other proteins are more important for the activity of TLM analogues in *P. falciparum*. It is possible that for some series of compounds (e.g., those substituted at the C5 position with long alkyl chains) inhibition of pfKASIII could contribute to their efficacy. However, detailed evaluation of modes of action requires identification of other possible enzyme targets and detailed studies of the uptake and metabolism of the analogues in *P. falciparum*.

In addition to work with *P. falciparum*, all compounds were assayed against two other parasitic protozoa, *T. cruzi* and *T. brucei*. Varied activities were found against the *T. brucei* and *T. cruzi* parasites with the best compounds registering IC_{50} values in the low-micromolar range. A series was also tested in vitro against *L. donovani*, the causative organism of leishmaniasis, of which analogue **15** showed good activity (IC_{50} = 0.5 μM). Thus, our study has further emphasized the effectiveness of thiolactomycin **1** analogues as active compounds against the parasites that cause malaria, African trypanosomiasis, Chagas disease, and leishmaniasis. We hope also that the recognition of new inhibitors such as **21d**, which shows good activity against both the pfKASIII enzyme and growth of *P. falciparum*, will

prove to be valuable in designing further chemicals for controlling malaria.

Experimental Section

Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. ^{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. Low-resolution mass spectra, i.e., electrospray (ES), 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, U.K. Microanalyses were obtained from the analytical and chemical consultancy services Medac Ltd. All reactions were performed in predried 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).

Cyclization. General Procedure A for Compounds 6a−d. Potassium hydroxide (2.5 equiv) in water (20 mL) was added to the bromide **5** (1.0 equiv) at 0 °C. The resulting solution was then vigorously stirred at ambient temperature for 15 h. The aqueous layer was washed with diethyl ether (2 × 30 mL) and acidified to pH 1 with the addition of 2 M HCl (∼20 mL). The aqueous layer was then extracted with diethyl ether (3 × 40 mL), and the combined organic solutions were washed with brine (100 mL), then dried over magnesium sulfate. The solvent was removed in vacuo. The crude residue was purified by flash column chromatography (5−30% ethyl acetate in hexanes). Spectral data for compounds **6a−d** are provided in the Supporting Information.

1-(5-Decyl-4-hydroxy-2-oxo-2,5-dihydro-1*H*-3-pyrrolyl)-1-ethanone (13). Amide **12** (0.24 g, 0.76 mmol) dissolved in methanol (2 mL) was added dropwise to a stirred solution of sodium methoxide (41 mg, 0.76 mmol) in dry methanol (5 mL) at ambient temperature. The resulting solution was heated to reflux and stirred for 15 h. The reaction mixture was allowed to cool, and the solvent was evaporated. The crude residue was dissolved in water (∼10 mL), and the aqueous layer was acidified to pH 1 with 2 M HCl (10 mL). The aqueous layer was extracted with diethyl ether (3 × 10 mL). The organic solutions were washed with water (2 × 20 mL) and brine (2 × 20 mL), then dried and evaporated. Column chromatography (0−30% ethyl acetate in hexanes) of the crude residue gave the desired tetramic acid **13** as a colorless solid (20 mg, 10%), mp 100−102 °C; ^{1}H NMR ($CDCl_3$) δ_H 0.93 (3H, t, J = 6.9, 15-CH_3), 1.31 (16H, m, 8 × CH_2), 1.59−1.70 (1H, m, CH), 1.85−1.92 (1H, m, CH), 2.52 (3H, s, CH_3), 3.87 (1H, dd, J = 7.6 and 4.1, 5-CH), and 6.06 (1H, s, NH); ^{13}C NMR δ_C 14.5 and 19.9 (both CH_3), 23.0, 25.6, 29.7, 29.8, 29.9, 29.9, 32.3 (all CH_2), 62.8 (5-CH), 101.9 (3-C), 175.6 (4-C), 185.4 (CO), and 196.0 (CO); MS (ES^-) *m/z* 280.0 ($M - H,^-$ 100%). Anal. ($C_{16}H_{27}O_3N$) C, H, N.

Alkylation. General Procedure B for Compounds 15−18. Lithium bis(trimethylsilyl)amide (1.0 M solution in THF, 2.0 equiv) was added dropwise to a stirred solution of thiolactone **14** (1.0 equiv) in dry THF (6 mL) at −78 °C. After the mixture was stirred for 0.5 h at −78 °C, the halide (1.0 equiv) was added dropwise. After being stirred for a further 0.5 h, the mixture was allowed to warm to ambient temperature and stirred overnight. The reaction was quenched with the addition of saturated aqueous ammonium chloride (∼10 mL), and the organic layer was separated. The aqueous layer was extracted with diethyl ether (3 × 10 mL). The organic layers were washed with water (3 × 20 mL) and brine (40 mL) and dried over anhydrous magnesium sulfate, and the solvent was removed in vacuo. The crude residue was purified by flash column chromatography (0−30% gradient of ethyl acetate in hexanes). Spectral data for compounds **15−18** is provided in the Supporting Information.

4-Hydroxy-5-(6-hydroxyhexyl)-5-methyl-3-propyl-2(5*H*)-thiophenone (19). Hydrochloric acid (10 M, 1.0 mL, 10.03 mmol) was added dropwise to the silyl ether **18** (0.40 g, 1.03 mmol) in methanol (6 mL) at ambient temperature. The reaction mixture was stirred vigorously for 1 h, after which time the solvent was removed in vacuo. The crude residue was purified using flash column chromatography (0−5% methanol in dichloromethane) to give the title compound **19** as a pale-yellow oil (50 mg, 18%); ^{1}H NMR δ_H 0.83 (3H, t, J = 7.3, 3′-CH_3), 1.31−1.47 (10H, m, 5 × CH_2), 1.62 (3H, s, CH_3), 1.82−1.89 (2H, m, 1′-CH_2), 2.15 (2H, t, J = 7.4, 6-CH_2), and 3.51 (2H, t, J = 6.4, 11-CH_2); ^{13}C NMR δ_C 14.3 (CH_3), 22.5 (CH_2), 25.4 (CH_3), 26.1, 26.8, 27.1, 27.3, 29.6, 33.8, 39.3 (all CH_2), 57.9 (5-C), 62.8 (11-CH_2), 115.5 (3-C), 180.8 (4-C), and 194.3 (2-CO); MS (ES^+) *m/z* 273.2 ($M + H,^+$ 100%); HRMS (ES^+) $(M + H)^+$ $C_{14}H_{25}O_3S$ requires 273.1519, found 273.1517. Anal. ($C_{14}H_{24}O_3S·0.05H_2O$) C, H, N.

Alkylation. General Procedure C for Compounds 21a, 21b/22a, 21c/22b, 21d, 21e, 22c−e. Thiophenone **20** (1.0 equiv) was added portionwise to a stirred suspension of sodium hydride (1.2 eqiuv) in dry THF (10 mL) at ambient temperature. After the mixture was stirred for 0.5 h, the halide (1.1 equiv) was added dropwise. The resulting solution was then heated to reflux and stirred for 15−20 h. The mixture was allowed to cool, and the reaction was quenched with the addition of aqueous saturated ammonium chloride (10 mL). The organic layer was separated, and the aqueous layer was extracted with diethyl ether (3 × 10 mL). The organic layers were washed with water (2 × 20 mL) and brine (2 × 20 mL) and dried over anhydrous magnesium sulfate, and the solvent was removed in vacuo. The crude residue was purified by flash column chromatography (0−20% ethyl acetate in hexanes). Spectral data for compounds **21a**, **21b/22a**, **21c/22b**, **21d**, **21e**, **22c**, **22d**, **22e** are provided in the Supporting Information.

4-[(6-Hydroxyhexyl)oxy]-5-methyl-3-propyl-2(5*H*)-thiophenone (22f). Hydrochloric acid (0.33 mL, 10 M, 3.36 mmol) was added dropwise to a stirred solution of the silyl ether **22e** (0.13 g, 0.33 mmol) in methanol (2 mL) at ambient temperature. The resulting solution was allowed to stir for 0.5 h. The methanol was then removed in vacuo, and the resulting residue was dissolved in diethyl ether (∼10 mL). Water (10 mL) was added, and the aqueous layer was extracted with diethyl ether (3 × 5 mL). The organic solutions were washed with water (3 × 10 mL) and brine (3 × 10 mL), then dried and evaporated to give the title compound **22f** as a colorless oil (38 mg, 42%); ^{1}H NMR δ_H 0.92 (3H, t, J = 7.0, CH_3), 1.37−1.48 (6H, m, 3 × CH_2), 1.54−1.57 (2H, m, CH_2), 1.58 (3H, d, J = 7.1, CH_3), 1.68−1.77 (2H, m, CH_2), 2.24 (2H, t, J = 6.9, CH_2), 3.65 (2H, t, J = 7.0, CH_2OH), 4.11 (1H, q, J = 7.1, 5-CH), and 4.17−4.26 (2H, m, OCH_2); ^{13}C NMR δ_C 14.2 (CH_3), 20.6, 22.3 (both CH_2), 25.8 (CH_3), 25.9, 30.2, 32.9, 41.9 (all CH_2), 51.1 (5-CH), 63.0 (CH_2OH), 71.1 (OCH_2), 119.9 (3-C), 179.1 (4-C), and 196.1 (2-CO); MS (ES^+) *m/z* 295.1 ($M + Na,^+$ 100%); HRMS (ES^+) $(M + Na)^+$ $C_{14}H_{24}O_3NaS$ requires 295.1344, found 295.1342. Anal. ($C_{14}H_{24}O_3S$) C, H, N.

Biological Assays. Cloning the *pfkasIII* Gene. A plasmid containing the full length gene encoding pKASIII was kindly provided by Dr. Sean Prigge (Johns Hopkins School of Public Health, Baltimore, MD) for use as a PCR template. In earlier studies, Prigge's group had demonstrated that when produced in *E. coli* as a maltose binding protein (MBP) fusion, the full-length protein was insoluble whereas a truncated version of the gene, lacking the sequences encoding the bipartite signal peptide, was produced in soluble form.[28] As a result, forward (GGTGGT*GAATTC*CATGTCCGGAGTAAAATAATAGGAC) and reverse (GGTGGT*GTCGAC*TTAATATTTAAGTATAACGCATCCATATG) oligonucleotide primers (with *Eco*RI and *Sal*I restriction sites incorporated, respectively (in italics)) were used to generate an equivalent truncated form of the gene. PCR was carried out using the proofreading *Pfu* turbo DNA polymerase (Stratagene, Amsterdam, The Netherlands) under the following conditions: 95 °C, 45 s; 55 °C,

45 s; 65 °C, 2.5 min for 30 cycles followed by a final extension period of 10 min at 65 °C. To facilitate cloning of the resultant amplicon (~1000 base pairs) into the pGEM-T vector (Promega, Southampton, U.K.), a further two cycles of amplification were carried out after the addition of *Taq* polymerase (Sigma, Poole, U.K.), which, in a template-independent manner, adds the A overhangs necessary for cloning into this vector. After ligation into pGEM-T, a colony containing the gene was identified by PCR screening using M13 forward and reverse primers. The inserted DNA was sequenced to confirm that no mutations had occurred, and the insert was subsequently excised with *Eco*RI and *Sal*I and recloned into the MBP expression vector pMALc2x (New England Biolabs, Beverly, MA) that had previously been cut with the same enzymes and treated with shrimp alkaline phosphatase (Roche, Basel, Switzerland). Clones containing the insert in the correct orientation were identified by DNA sequencing and were transferred to *E. coli* BL21 (DE3) codon + RIL cells (Stratagene, Amsterdam, The Netherlands) for protein production.

Production of Recombinant MBP-pfKASIII Fusion Protein. LB medium (**2l**) containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) was inoculated with an overnight culture of the above cells containing the *pfkasIII* clone. The MBP-pfKASIII fusion protein was induced essentially as described in Waters et al.,[28] by the addition of isopropyl β−D-thiogalactopyranoside to a final concentration of 0.4 mM followed by incubation at 20 °C for a further 16 h. At this stage, cell pellets could be stored at −20 °C until required, with no adverse effect on the purification of the MBP-pfKASIII protein. The harvested cells were then resuspended in 40 mL of column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA). Lysis of the cells was achieved by three rounds of freeze/thawing, and the intracellular contents were disrupted by sonication (60% amplitude, 30 s) using a Soniprobe sonicator (Lucas Dawe Ultrasonics Ltd, London, U.K.). The soluble fraction of the lysed cells was collected by centrifugation (20000*g*, 20 min, 4 °C) and filter-sterilized to remove any remaining intact cells. In an attempt to reduce the amount of proteolysis, protease inhibitor cocktail (Sigma Chemicals Ltd., Poole, Dorset, U.K.) was added in accordance with the manufacturer's instructions and the sample was stored on ice prior to further processing. Soluble MBP-pfKASIII fusion protein was purified by affinity chromatography on amylose resin (New England Biolabs, Beverly, MA). Typically, 1 mg of purified, soluble MBP-pfKASIII protein suitable for enzymatic studies was produced from each liter of culture.

Assay of KASIII Enzyme Activity and Inhibitor Studies. The KASIII condensing enzyme activity was assayed using a filter disk assay.[25,29] The final assay contained 100 mM NaH_2PO_4 buffer, pH 6.6, 25 μM *E. coli* acyl carrier protein (ACP), 1 mM β−mercaptoethanol, 1 μg of FabD enzyme from *E. coli*, 65 μM malonyl-CoA, 500 ng of MBP-pfKASIII, and 45 μM [1-^{14}C]acetyl-CoA in a final 40 μL reaction volume. To ensure complete reduction of ACP, it was preincubated with the β-mercaptoethanol and the sodium phosphate buffer at 37 °C for 30 min. The remaining components were then added to the reduced ACP mix. Inhibitors were dissolved in 20% DMSO, and 2 μL was added to the reaction tubes. Control reactions contained 2% DMSO, which had no detectable effect on enzyme activity.

The reaction was started by the addition of 2 μL of MBP-pfKASIII, followed by incubation at 37 °C for 15 min. To stop the reaction, 35 μL of the assay was spotted onto a Whatman 21 mm filter disk. Each filter disk was washed with gentle shaking in 20 mL of successive solutions of 10% trichloroacetic acid (TCA), 5% TCA, and 1% TCA at 4 °C for 20 min each. To establish the total number of counts added to each tube, a control reaction was spotted onto a filter disk but was not washed in TCA. Filter disks were dried and placed in scintillation vials containing 3 mL of scintillation cocktail (Fisher, Loughborough, U.K.). The decays per minute were measured using a Beckman 1209 Rackbeta liquid scintillation counter (EG&G Wallac, Milton Keynes, U.K.).

Growth of Organisms (Parasites). ***Plasmodium falciparum.*** In vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay,[30,31] using the chloroquine and pyrimethamine resistant K1 strain and the standard drugs chloroquine (Sigma C6628) and artemisinin (arteannuin, qinghaosu; Sigma 36,-159-3). Compounds were dissolved in DMSO at 10 mg/mL and added to parasite cultures incubated in RPMI 1640 medium without hypoxanthine, supplemented with HEPES (5.94 g/L), $NaHCO_3$ (2.1 g/L), neomycin (100 U/mL), AlbuMAX (5 g/L), and washed human red cells A^+ at 2.5% hematocrit (0.3% parasitemia). Serial doubling dilutions of each drug were prepared in 96-well microtiter plates and incubated in a humidified atmosphere at 37°C; 4% CO_2, 3% O_2, 93% N_2.

After 48 h, 50 μL of [^{3}H]hypoxanthine (=0.5 μCi) in medium was added to each well of the plate. The plates were incubated for a further 24 h under the same conditions. The plates were then harvested with a Betaplate cell harvester (Wallac, Zurich, Switzerland), and the red blood cells were transferred onto a glass fiber filter and then washed with distilled water. The dried filters were inserted into a plastic foil with 10 mL of scintillation fluid and counted in a Betaplate liquid scintillation counter (Wallac, Zurich, Switzerland). IC_{50} values were calculated from sigmoidal inhibition curves using Microsoft Excel.

Trypanosoma cruzi. Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 (cells/well)/100 μL in RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 h, 5000 trypomastigotes of *T. cruzi* (Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene) were added in 100 μL per well with 2× of a serial drug dilution. The plates were incubated at 37 °C in 5% CO_2 for 4 days. Then the substrate CPRG/Nonidet was added to the wells. The color reaction, which developed during the following 2−4 h, was read photometrically at 540 nm. From the sigmoidal inhibition curve, IC_{50} values were calculated.

Trypanosoma brucei rhodesiense. Minimum Essential Medium (50 μL) supplemented according to Baltz et al.[32] with 2-mercaptoethanol and 15% heat-inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were added to the wells. Then 50 μL of trypanosome suspension (*T. b. rhodesiense* STIB 900) was added to each well and the plate was incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Resazurin (12.5 mg in 100 mL of distilled water) was then added to each well, and incubation was continued for a further 2−4 h (Raz et al.[33]). The plates are read in a microplate fluorescence scanner (Spectramax Gemini XS by Molecular Devices) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. From the sigmoidal inhibition curve, IC_{50} values were calculated.

L. donovani. Amastigotes of *Leishmania donovani* strain MHOM/ET/67/L82 were grown in axenic culture at 37 °C in SM medium[34] at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO_2 in air. An amount of 100 μL of culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution was seeded in 96-well microtiter plates. Seven 3-fold dilutions were used covering a range from 30 to 0.041 μg/mL. Each drug was tested in duplicate, and each assay was repeated at least once. After 72 h of incubation, the plates were inspected under an inverted microscope to ensure growth of the controls and sterile conditions. An amount of 10 μL of Alamar Blue (12.5 mg of resazurin dissolved in 100 mL of distilled water) was then added to each well, and the plates were incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA). Decrease of fluorescence (=inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves, the IC_{50} values were calculated.

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Supporting Information Available: Spectral data for compounds **6a**−**d**, **15**−**18**, **21a**−**e**, **22a**−**e** and results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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