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Natural product inhibitors of fatty acid biosynthesis: synthesis of the marine microbial metabolites pseudopyronines A and B and evaluation of their anti-infective activities

Anna C. Giddens [a], Lone Nielsen [a,†], Helena I. Boshoff [b], Deniz Tasdemir [c], Remo Perozzo [d], Marcel Kaiser [e], Feng Wang [f], James C. Sacchettini [f], Brent R. Copp [a,*]

[a] *Department of Chemistry, The University of Auckland, Private Bag 92019, Auckland, New Zealand*
[b] *Tuberculosis Research Section, LCID, NIAID, NIH, Bethesda, MD 20892, USA*
[c] *Centre for Pharmacognosy and Phytotherapy, University of London, London WC1N 1AX, United Kingdom*
[d] *School of Pharmaceutical Sciences, University of Geneva, 1211 Geneva 4, Switzerland*
[e] *Swiss Tropical Institute, 4002 Basel, Switzerland*
[f] *Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843, USA*

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Abstract

Total syntheses of the title natural products, pseudopyronines A (**1**) and B (**2**), have been achieved using methyl β-oxo carboxylic ester starting materials. The natural products and a small set of structurally related compounds were evaluated for growth inhibitory activity against a range of pathogenic microorganisms and were found to exhibit good potency ($IC_{50} \geq 0.46$ μg/mL) and selectivity towards *Leishmania donovani*. Several of the compounds inhibited recombinant fatty acid biosynthesis enzymes from both *Plasmodium falciparum* and *Mycobacterium tuberculosis*, validating these targets in the search for new anti-infective agents.

Keywords: Fatty acid biosynthesis; Marine natural product; *Leishmania*; *Mycobacterium*

1. Introduction

Fatty acids play crucial roles in the function and viability of cells providing components for biological membranes, acting as chemical messengers and facilitating the storage of energy. The biosynthesis of fatty acids follows an iterative process of condensation and elongation of acetate (in the form of malonate), which is undertaken by a sequence of enzymatic steps either in the context of a single multifunctional protein (Type I fatty acid synthase) in animals and fungi or a dissociable multienzyme system (Type II FAS) in plants, bacteria and protozoa.[1,2] The lack of homology between mammalian FAS-I and bacterial FAS-II systems makes it possible to discover and develop specific inhibitors that have therapeutic potential in the treatment of a number of diseases.[3]

A number of small molecule inhibitors of fatty acid biosynthesis (FAB) are known. Triclosan (an antiseptic) and isoniazid (an antituberculosis drug) both inhibit bacterial enoyl-ACP-reductase (FabI/InhA), the enzyme responsible for the final reduction step during FAB. New classes of inhibitors have been identified by screening synthetic compound libraries[4] and via bioassay guided studies of natural products. Examples of the latter include cerulenin,[5] thiolactomycin[6] and more recently bischloroanthrabenzoxocinone,[7] phomallenic acids A–C,[8] platensimycin[9] and flavonoids.[10,11] The thiolactomycin scaffold has been the subject of structure–activity studies, in particular focussing on growth inhibition of *Mycobacterium*

* Corresponding author. Tel.: +64 9 373 7599x88284; fax: +64 9 373 7422.
E-mail address: b.copp@auckland.ac.nz (B.R. Copp).
† Present address: Department of Chemistry, University of Aarhus, 8000 Aarhus C, Denmark.

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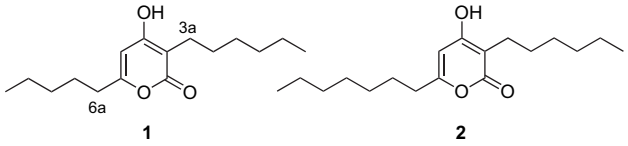
tuberculosis.[12] The presence of Type II fatty acid biosynthesis in the apicoplast of *Plasmodium falciparum* raises the possibility of FAB inhibitors also being useful templates for the discovery of antimalarial agents.[13,14] Studies involving thiolactomycin,[15] triclosan[16] and flavonoid[10] derivatives have been reported.

Arising from a search for new inhibitors of bacterial fatty acid biosynthesis, the 2-pyrone-containing natural products pseudopyronines A (**1**) and B (**2**) were recently reported as mild antibiotics.[17,18] The compounds, detected using a lacZ reporter cell-based assay, were isolated from fermentation of *Pseudomonas* sp. F92S91, which was itself isolated from a sponge collected in Fiji. Pyrone **2** had previously been reported as Sch 419560, identified from fermentation extracts of *Pseudomonas fluorescens*.[19] Pseudopyronines A and B inhibited the growth of Gram-positive bacteria, including *Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus*, *Moraxella catarrhalis* and *Enterococcus faecium* (MIC 1–64 μg/mL) with pseudopyronine B being the more active of the two compounds. Pseudopyronine B (**2**) inhibited the cellular uptake and incorporation of thymidine, uridine and amino acids suggestive of a mode of action related to the disruption of membrane function.[17] Notably, pseudopyronine B showed neither a membrane-damaging effect on human red blood cells nor caused haemolysis, indicating selectivity for bacterial membranes.



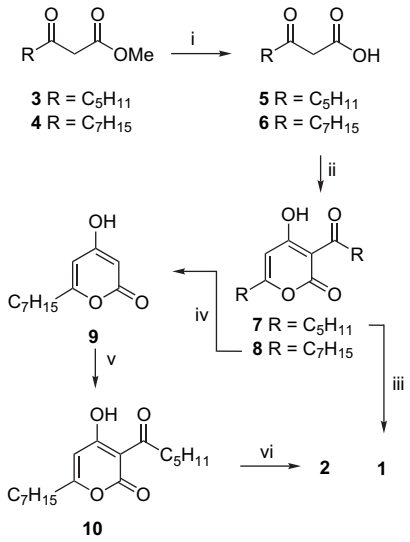
As part of our ongoing interest in the potential offered by natural products to act as leads in the development of new antituberculosis[20] and antimalarial agents,[10,21] we now report the synthesis of pseudopyronines A and B, the preparation of a number of analogues and the results of screening these compounds for activity against whole organisms and purified recombinant enzymes of the FAB pathway.

2. Results and discussion

2.1. Chemistry

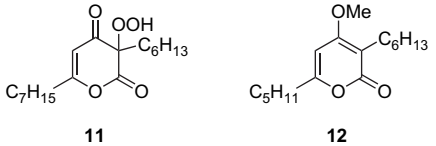
2.1.1. Pseudopyronines A and B

While a number of methodologies have been reported for the preparation of 4-hydroxy-2-pyrones, the carbonyldiimidazole-mediated condensation of β-keto carboxylic acids[22] was particularly attractive for the synthesis of the pseudopyronines as it could also lead to the preparation of a number of related compounds for structure–activity studies. In the case of pseudopyronine A (**1**), the required β-oxo carboxylic acid **5** was prepared via saponification of the known methyl β-oxo ester **3** (Scheme 1).[23] Cyclisation of **5** using 1.1 equiv of carbonyldiimidazole in THF afforded the target acylpyrone skeleton **7**. Reduction of the α-acyl group in **7** utilising $NaCNBH_3$ in THF/aq HCl[24] afforded pseudopyronine A (**1**), which exhibited spectroscopic data identical to those reported for the natural product.[18] The synthesis of pseudopyronine B (**2**) started with ester **4**[23] and progressed to pyrone **8** in similar fashion. Deacylation of **8** by heating at 90 °C in 90% H_2SO_4 yielded 6-heptyl-4-hydroxy-2-pyrone (**9**) with subsequent acylation by hexanoyl chloride in TFA affording the pyrone skeleton with the appropriate length carbon chain at C-3. Reduction with $NaCNBH_3$ in THF/aq HCl afforded pseudopyronine B (**2**). Our sample of **2** exhibited identical spectroscopic data to those reported for the natural product[18] and also co-eluted on analytical HPLC with an authentic sample of pseudopyronine B.



Scheme 1. Reagents and conditions: (i) NaOMe, MeOH, H_2O, rt; (ii) 1,1′-carbonyldiimidazole, THF, rt, 24 h; (iii) $NaCNBH_3$, THF, 2 M HCl, rt, 2.5 h; (iv) 90% H_2SO_4, 130 °C, 1 h; (v) hexanoyl chloride, TFA, reflux, 3 h; (vi) $NaCNBH_3$, THF, 2 M HCl, rt, 19 h.

The original report of **1** and **2** demonstrated the lack of stability of **2** in the $CDCl_3$ NMR solvent, with **2** undergoing reaction with oxygen to form hydroperoxide **11**.[18] We also observed similar instability for pseudopyronine A (**1**), which converted over 7 days standing in $CDCl_3$ in an NMR tube to form the analogous 3-peroxy derivative. New NMR signals were observed at δ 5.68 (s, H-5) and δ 1.92 (m, 2H-3a) consistent with those reported for the 3-peroxy derivative of pseudopyronine B.[18] This instability should not be observed if the 4-hydroxyl group is trapped as the methyl ether. Indeed, 4-*O*-methyl pseudopyronine A (**12**), prepared in 53% yield from **1** by reaction with excess trimethylphosphate, was stable in $CDCl_3$ for many weeks with no observed degradation.



2.1.2. Analogues

To be in a position to study aspects of the structure–activity requirements of pseudopyronines A and B, the preparation

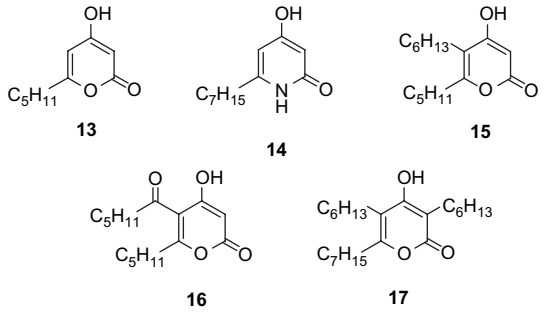


Figure 1. Analogues prepared.

of a number of related compounds was also undertaken (Fig. 1).

The known pyrone **13**[22] was prepared by deacylation of intermediate **7** (Scheme 1) using 90% H_2SO_4 in 43% yield. Conversion of pyrone **9** to pyridone **14** proceeded smoothly by reaction with aq NH_3 in 50% yield.[25] The pseudopyronine A regioisomer **15** was prepared via triethylsilane reduction of the known 5-acylpyrone **16**,[26] and 5-hexyl substituted pseudopyronine B **17** was prepared by a published procedure.[27]

2.2. Biological evaluation

Pyrones **1**, **2**, **7**–**10**, **12**, **13**, **15**–**17** and pyridone **14** were initially evaluated for growth inhibition of *M. tuberculosis*, *P. falciparum*, *Leishmania donovani*, *Trypanosoma brucei rhodesiense* and *Trypanosoma cruzi*, and for cytotoxicity towards the mammalian L6 and P388 cell lines (Table 1). Pseudopyronines A and B exhibited modest activity towards *M. tuberculosis* grown in nutrient deficient GAST media.[28] When assayed against *M. tuberculosis* grown in 7H9 media significantly less antibiotic activity was observed for all compounds, perhaps as a consequence of nutrient abundance in this media making the organism less stressed and less susceptible to antibiotic action or the presence of albumin, binding to which would reduce drug availability. The 3-acyl precursor to pseudopyronine A (**7**) was the only pyrone tested that exhibited comparable antimycobacterial activity to the natural products. In the parasitic assays, the majority of compounds tested exhibited slightly more potent activity towards *L. donovani* than towards the other parasites. In general, the compounds appeared to be more toxic towards P388 leukaemia cells than towards primary mammalian L6 cells. 3-Acylpyrones **7** and **10** were the most active leishmanicidal compounds detected in the present study (IC_{50}s 0.46 and 0.55 μg/mL, respectively). These compounds exhibited modest selectivity (therapeutic) indices (IC_{50} value for cytotoxicity divided by IC_{50} value for antiparasitic activity) of up to 10. This was followed by compounds **2**, **8**, **1**, **15** and **12**, with less potent antileishmanial activities (IC_{50} values 1.38–3.37 μg/mL), but higher selectivity indices against mammalian cells. Similar levels of selectivity were observed for the mono-alkyl substituted pyrones **9** and **13**, which exhibited modest potency towards *L. donovani* (IC_{50} 7–13 μg/mL) but with poor or no

Table 1
Summary of biological activities observed for compounds **1**, **2**, **7**–**10**, **12**–**17**

Compound	*M. tuberculosis*[a]	*M. tuberculosis*[b]	*T. b. rhodesiense*[c]	*T. cruzi*[d]	*L. donovani*[e]	*P. falciparum*[f]	P388[g]	L6[h]
1	3.125	>20	13.09	17.27	2.63	14.89	4.7	23.2
2	0.78–1.56	25	12.46	12.03	1.38	14.2	5.4	17.9
7	0.78	5	12.12	6.61	0.46	16.41	1.9	5.53
8	Insol[i]	Insol[i]	12.88	10.19	2.1	>5	3.3	41.8
9	12.5	n.t.[j]	44.16	>30	6.77	>50	>25	>90
10	Insol[i]	Insol[i]	10.77	6.05	0.55	17.37	3.1	5.58
12	12.5	50	4.15	6.35	3.37	3.1	7.1	57.5
13	6.25	>50	50.77	>30	13.5	>5	>25	>90
14	12.5	>50	11.6	>30	>30	>5	>25	>90
15	6.25	>50	14.73	>30	3.3	>5	>25	82.6
16	12.5	>50	28.88	>30	9.0	>5	11.4	>90
17	>50	>50	11.34	19.68	11.18	>5	9.3	53.8
Isoniazid	0.01	0.01						
Melarsoprol			0.003					
Benznidazole				0.28				
Miltefosine					0.15			
Chloroquine						0.043		
Mitomycin C							0.18	
Podophyllotoxin								0.006

[a] *Mycobacterium tuberculosis* H37Rv grown in GAST medium. MIC (μg/mL).
[b] *Mycobacterium tuberculosis* H37Rv grown in 7H9 medium. MIC (μg/mL).
[c] *Trypanosoma brucei rhodesiense* (strain STIB 900), trypomastigote stage. IC_{50} (μg/mL).
[d] *Trypanosoma cruzi* (strain Tulahuen C4), amastigote stage. IC_{50} (μg/mL).
[e] *Leishmania donovani* (strain MHOM-ET-67/L82), amastigote/axenic stage. IC_{50} (μg/mL).
[f] *Plasmodium falciparum* (strain K1), IEF stage. IC_{50} (μg/mL).
[g] P388 murine leukaemia cell line. IC_{50} (μg/mL).
[h] L6 rat skeletal myoblast cell line. IC_{50} (μg/mL).
[i] Insoluble in media.
[j] Not tested.

Table 2
In vitro activity of compounds **1**, **2**, **7**–**10**, **12**–**17** against fatty acid biosynthesis enzymes PfFabG, PfFabI, PfFabZ and InhA

Compound	PfFabG[a]	PfFabI[a]	PfFabZ[a]	InhA[b]
1	>100	12	>100	35%
2	12.8	4	>100	61% (3.8)
7	>100	80	100	37%
8	n.t.[c]	n.t.	n.t.	65% (3.1)
9	28.4	>100	100	39%
10	>50	>50	>100	24%
12	n.t.	n.t.	n.t.	68% (2.9)
13	n.t.	n.t.	n.t.	42%
14	n.t.	n.t.	n.t.	40%
15	n.t.	n.t.	n.t.	12%
16	n.t.	n.t.	n.t.	47%
17	n.t.	n.t.	n.t.	43%

[a] IC_{50} (μg/mL).
[b] Percentage inhibition at 10 μM drug dose or IC_{50} (μg/mL) in parenthesis.
[c] Not tested.

cytotoxicity at the highest test concentrations. While all compounds showed some antitrypanosomal activity against African *T. brucei rhodesiense*, only half of the tested compounds were active towards American *T. cruzi*. Compound **12** appeared to be the most promising trypanocidal agent as it inhibited both *Trypanosoma* species with IC_{50} values of 4.15 and 6.35 μg/mL.

A selection of the available pyrones (**1**, **2**, **7**, **9**, **10**) were evaluated for their ability to inhibit the function of three recombinant enzymes (PfFabG, PfFabI and PfFabZ) of *P. falciparum* fatty acid biosynthesis while the full set of compounds were evaluated against one enzyme from *M. tuberculosis* (InhA=MtFabI) (Table 2). Both natural products **1** and **2** inhibited PfFabI, the enoyl-ACP-reductase of *P. falciparum* with modest IC_{50}s of 12 and 4 μg/mL, respectively. Pseudopyronine B (**2**) and pyrone **9** inhibited PfFabG, a β-ketoacyl-ACP-reductase, while none of the compounds tested inhibited PfFabZ (β-hydroxyacyl-ACP-dehydratase). The enzyme inhibitory activities of compounds **1** and **2** were well correlated with their in vitro growth inhibitory potential on *P. falciparum* whole cells, providing evidence that the PfFab pathway may be the potential cellular target of these compounds. The antimalarial activity of compound **7**, however, is possibly off-target, as the IC_{50} values for PfFabI and PfFabZ enzymes are much higher in comparison to its antiplasmodial activity. Compound **9** appeared to be less potent against PfFabG and PfFabZ enzymes and it was less efficacious against cultured parasites. The natural product pseudopyronine B (**2**) and the methyl ether of pseudopyronine A (**12**) exhibited activity against the InhA enzyme with IC_{50} values of 3.8 and 2.9 μg/mL, respectively. There was no apparent correlation, however, between the ability to inhibit the InhA enzyme and whole organism (*M. tuberculosis*) activity for most of the remaining synthetic compounds.

3. Conclusion

The 3,6-dialkyl-4-hydroxy-2-pyrone marine metabolites pseudopyronines A and B have been synthesized via a methodology based upon the condensation of β-oxo carboxylic acids. We have shown that the natural products and a small set of structurally related compounds exhibit good potency and reasonable selectivity against a panel of parasitic protozoa, in particular towards *L. donovani*. Several of the compounds, including the natural products **1** and **2**, were found to inhibit the FAB enzymes PfFabG (β-ketoacyl-ACP-reductase), PfFabI (enoyl-ACP-reductase) and InhA (enoyl-ACP-reductase) and thereby inhibit the growth of *P. falciparum* and *M. tuberculosis*. These results provide further validation of fatty acid biosynthesis as a target for the discovery of new anti-infective agents.

4. Experimental section

4.1. General methods (chemistry)

All reagents were used as supplied. Tetrahydrofuran was dried over sodium wire. All solvents were distilled prior to use. Analytical thin layer chromatography was carried out on Merck DC-plastikfolien Kieselgel 60 F_{254} plates and products were visualised by UV fluorescence. Flash column chromatography was performed using Merck 40–63 μm silica gel. Mass spectra were recorded on a VG-70SE mass spectrometer. High-resolution mass spectra were recorded at a nominal resolution of 5000. Infrared spectra were recorded on a Perkin–Elmer 1600 FTIR spectrophotometer, with all samples examined as films on a KBr disk. Melting points were measured on an Electrothermal melting point apparatus and are uncorrected. Analytical reversed phase HPLC was run on a Waters 600 HPLC photodiode array system using an Alltech Rocket™ C18 column (3 μm Econosphere, 33×7 mm) and eluting with a linear gradient of H_2O (0.05% TFA) through to MeCN. NMR spectra were recorded at 298 K at either 300 or 400 MHz for ^1H and 75 or 100 MHz for ^{13}C on Bruker Avance 300 or 400 spectrometers. Chemical shifts are given in parts per million on the δ scale referenced to the residual solvent peaks ($CHCl_3$: ^1H 7.25, ^{13}C 77.0 ppm; DMSO-d_6: ^1H 2.50, ^{13}C 39.4 ppm; MeOH-d_4: ^1H 3.30, ^{13}C 49.0 ppm). Assignments were aided by DEPT135, HSQC and HMBC experiments. Microanalyses were carried out by the Campbell Laboratory, University of Otago, Dunedin, New Zealand.

Pyrones **16** and **17** were prepared by the literature methods.[26,27]

4.1.1. 3-Oxo-octanoic acid (*5*)

Methyl 3-oxo-octanoate[23] (1.12 g, 6.50 mmol) and sodium methoxide (0.457 g, 8.46 mmol) were dissolved in methanol (17 mL). Water (17 mL) was added and the mixture left stirring for 18 h at room temperature. Methanol and water were removed under reduced pressure to give an orange oil. HCl (10%, v/v, 15 mL) was added and a yellow precipitate formed. Dichloromethane (30 mL) was added and the precipitate dissolved to give a yellow solution. The aqueous phase was extracted with dichloromethane (2×20 mL). The combined organic phases were washed with water (2×15 mL), dried over anhydrous magnesium sulfate and the solvent removed

in vacuo. The crude solid was washed with hexane (2×15 mL) to give **5** as a keto/enol* (4.2:1) mixture (yellow solid; 0.513 g, 50%) (enol NMR resonances are indicated by asterisk). Mp 70–72 °C; IR ν_{max} 3420, 1723, 1703 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 11.79* (1H, s, OH enolic, 12%), 9.23 (1H, br s, OH), 5.02* (1H, s, H-2 enolic, 12%), 3.50 (2H, s, H-2, 76%), 2.55 (2H, t, J=7.4 Hz, H-4, 76%), 2.21* (2H, t, J=7.4 Hz, H-4 enolic, 24%), 1.60 (2H, m, H-5), 1.30 (4H, m, H-6, H-7), 0.88 (3H, t, J=6.9 Hz, H-8); ^{13}C NMR (75 MHz, $CDCl_3$) δ 204.1 (quat., C-3), 182.0* (quat., C-3 enolic), 171.7 (quat., C-1), 88.2* (CH, C-2 enolic), 47.9 (CH_2, C-2), 43.2 (CH_2, C-4), 35.3* (CH_2, C-4 enolic), 31.1 (CH_2, C-6), 25.9* (CH_2, C-5 enolic), 23.1 (CH_2, C-5), 22.3 (CH_2, C-7), 13.8 (CH_3, C-8); MS (EI, 70 eV) *m/z* 158 ($M^{+\bullet}$, 1%), 114 (15), 71 (22), 58 (100), 43 (100); HRMS (EI): found $M^{+\bullet}$, 158.0943. $C_8H_{14}O_3$ requires 158.0943.

4.1.2. 3-Hexanoyl-4-hydroxy-6-pentyl-2-pyrone (*7*)

To a stirred solution of 3-oxo-octanoic acid (**5**) (0.500 g, 3.16 mmol) in dry tetrahydrofuran (6 mL) under nitrogen at room temperature was added 1,1′-carbonyldiimidazole (0.697 g, 4.30 mmol) in dry tetrahydrofuran (11 mL). The resulting mixture was left stirring at room temperature for 24 h. The mixture was then acidified to pH 1 with 0.5 M HCl and extracted with ethyl acetate (60 mL). The organic layer was washed with brine (1×60 mL), dried over anhydrous magnesium sulfate and solvent removed in vacuo. The crude solid was recrystallised from methanol to yield **7** as pale yellow crystals (0.184 g, 21%). Mp 42–43 °C (lit.[22] mp 59.5–60 °C); IR ν_{max} 3430, 1720, 1635 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.90 (1H, s, H-5), 3.05 (2H, t, J=7.5 Hz, H-3b), 2.46 (2H, t, J=7.4 Hz, H-6a), 1.65 (4H, m, H-3c, H-6b), 1.33 (8H, m), 0.89 (6H, t, J=7.0 Hz, H-3f, H-6e); ^{13}C NMR (75 MHz, $CDCl_3$) δ 208.0 (quat., C-3a), 181.3 (quat., C-4), 172.5 (quat., C-6), 161.1 (quat., C-2), 100.7 (CH, C-5), 99.6 (quat., C-3), 41.6 (CH_2, C-3b), 34.2 (CH_2, C-6a), 31.4 (CH_2, C-3d), 31.0 (CH_2, C-6c), 26.0 (CH_2, C-6b), 23.7 (CH_2, C-3c), 22.5 (CH_2, C-3e or C-6d), 22.2 (CH_2, C-6d or C-3e), 13.9 (CH_3, C-3f or C-6e), 13.8 (CH_3, C-3f or C-6e); MS (EI, 70 eV) *m/z* 280 ($M^{+\bullet}$, 17%), 237 (98), 224 (100), 209 (90), 168 (68), 99 (35), 69 (32), 43 (73); HRMS (EI): found $M^{+\bullet}$, 280.1674. $C_{16}H_{24}O_4$ requires 280.1675. Anal. Calcd for $C_{16}H_{24}O_4$: C, 68.54; H, 8.63. Found: C, 68.66; H, 8.77.

4.1.3. 3-Hexyl-4-hydroxy-6-pentyl-2-pyrone (pseudopyronine A) (*1*)

To a stirred solution of acylpyrone **7** (0.100 g, 0.357 mmol) in tetrahydrofuran (2.1 mL) was added 2 M HCl (1.8 mL). Sodium cyanoborohydride (0.056 g, 0.891 mmol) was added portionwise to the resulting solution. The mixture was left stirring at room temperature for 2.5 h. The solvent was then removed in vacuo, the resulting solid dissolved in dichloromethane (5 mL) and washed with water (2×5 mL), dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo to give a yellow solid. Purification by column chromatography (dichloromethane) afforded **1** as a yellow solid (0.078 g, 82%). Spectroscopic data agreed with those reported in the literature.[18] Mp 106–108 °C; analytical HPLC t_R 7.11 min; IR ν_{max} 3430, 1634 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4) δ 5.97 (1H, s, H-5), 2.46 (2H, t, J=7.6 Hz, H-6a), 2.36 (2H, t, J=7.4 Hz, H-3a), 1.64 (2H, q, J=7.4 Hz, H-6b), 1.43 (2H, m, H-3b), 1.35 (4H, m, H-6c, H-6d), 1.30 (6H, m, H-3c, H-3d, H-3e), 0.90 (6H, m, H-3f, H-6e); ^{13}C NMR (100 MHz, MeOH-d_4) δ 168.8 (quat., C-2), 167.8 (quat., C-4), 165.1 (quat., C-6), 103.9 (quat., C-3), 101.0 (CH, C-5), 34.3 (CH_2, C-6a), 32.9 (CH_2, C-3d), 32.2 (CH_2, C-6c), 30.2 (CH_2, C-3c), 29.0 (CH_2, C-3b), 27.6 (CH_2, C-6b), 23.9 (CH_2, C-3a), 23.7 (CH_2, C-3e), 23.4 (CH_2, C-6d), 14.4 (CH_3, C-3f), 14.3 (CH_3, C-6e); MS (EI, 70 eV) *m/z* 266 ($M^{+\bullet}$, 18%), 209 (15), 195 (100), 168 (15), 99 (13), 44 (17); HRMS (EI): found $M^{+\bullet}$, 266.1872. $C_{16}H_{26}O_3$ requires 266.1882.

4.1.4. 3-Oxo-decanoic acid (*6*)

Methyl 3-oxo-decanoate (**4**)[23] (7.05 g, 35.2 mmol) and sodium methoxide (2.88 g, 53.3 mmol) were dissolved in methanol (100 mL). Water (100 mL) was added and the mixture left stirring 18 h at room temperature. Methanol and water were removed in vacuo to give an orange oil. HCl (10%, v/v, 100 mL) was added and a yellow precipitate formed. Dichloromethane (200 mL) was added and the precipitate dissolved to give a yellow solution. The aqueous phase was extracted with dichloromethane (2×100 mL). The combined organic phases were washed with water (1×100 mL), dried over anhydrous magnesium sulfate and concentrated in vacuo to give an orange solid. The crude solid was washed with hexane (2×100 mL) to afford **6** as a keto/enol* (4.4:1) mixture (yellow solid; 3.22 g, 49%) (enol NMR resonances are indicated by asterisk). Mp 74–75.5 °C (lit.[29] mp 76–77 °C); IR ν_{max} 3431, 1723, 1702 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 11.78* (1H, s, OH enolic, 11%), 5.02* (1H, s, H-2 enolic, 11%), 3.50 (2H, s, H-2, 78%), 2.55 (2H, t, J=7.6 Hz, H-4, 78%), 2.21* (2H, t, J=7.6 Hz, H-4 enolic, 22%), 1.57 (2H, m, H-5), 1.27 (8H, m), 0.86 (3H, t, J=7.0 Hz, H-10); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.5 (quat., C-3), 182.2* (quat., C-3 enolic), 171.5 (quat., C-1), 88.2* (CH, C-2 enolic), 47.9 (CH_2, C-2), 43.3 (CH_2, C-4), 35.3* (CH_2, C-4 enolic), 31.6 (CH_2, C-8), 28.9 (CH_2, C-6 or C-7), 28.9 (CH_2, C-6 or C-7), 26.2* (CH_2, C-5 enolic), 23.4 (CH_2, C-5), 22.6 (CH_2, C-9), 14.0 (CH_3, C-10); MS (EI, 70 eV) *m/z* 186 ($M^{+\bullet}$, 1%), 142 (19), 71 (36), 58 (100), 43 (35); HRMS (EI): found $M^{+\bullet}$, 186.1259. $C_{10}H_{18}O_3$ requires 186.1256.

4.1.5. 6-Heptyl-4-hydroxy-3-octanoyl-2-pyrone (*8*)

To a stirred solution of 3-oxo-decanoic acid (**6**) (0.367 g, 1.97 mmol) in dry tetrahydrofuran (4 mL) under nitrogen at room temperature was added 1,1′-carbonyldiimidazole (0.431 g, 2.66 mmol) in dry tetrahydrofuran (8 mL). The resulting mixture was left stirring at room temperature for 24 h after which time it was acidified to pH 1 with 0.5 M HCl, and extracted with ethyl acetate (1×50 mL). The organic layer was washed with brine (1×50 mL), dried over anhydrous magnesium sulfate and concentrated in vacuo to give an orange solid. The crude solid was recrystallised from methanol to afford **8** as a pale yellow crystals (0.232 g, 35%). Mp

58–59 °C (lit.[22] mp 64.5–65 °C); IR ν_{max} 3435, 1727, 1639 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.89 (1H, s, H-5), 3.04 (2H, t, J=7.6 Hz, H-3b), 2.45 (2H, t, J=7.5 Hz, H-6a), 1.64 (4H, m, H-3c, H-6b), 1.31 (16H, m), 0.86 (6H, m, H-3h, H-6g); ^{13}C NMR (75 MHz, $CDCl_3$) δ 208.0 (quat., C-3a), 181.2 (quat., C-4), 172.5 (quat., C-6), 161.0 (quat., C-2), 100.7 (CH, C-5), 99.6 (quat., C-3), 41.6 (CH_2, C-3b), 34.3 (CH_2, C-6a), 31.7 (CH_2, C-3f or C-6e), 31.6 (CH_2, C-3f or C-6e), 29.2 (CH_2, C-3d or C-3e), 29.1 (CH_2, C-3e or C-3d), 28.9 (CH_2, C-6c or C-6d), 28.8 (CH_2, C-6d or C-6c), 26.3 (CH_2, C-6b), 24.0 (CH_2, C-3c), 22.6 (CH_2, C-3g or C-6f), 22.5 (CH_2, C-6f or C-3g), 14.0 (CH_3, C-3h), 14.0 (CH_3, C-6g); MS (EI, 70 eV) m/z 336 ($M^{+\bullet}$, 14%), 265 (85), 252 (92), 237 (55), 168 (53), 69 (30), 57 (84), 41 (93), 40 (100); HRMS (EI): found $M^{+\bullet}$, 336.2300. $C_{20}H_{32}O_4$ requires 336.2301. Anal. Calcd for $C_{20}H_{32}O_4$: C, 71.39; H, 9.59. Found: C, 71.64; H, 9.75.

4.1.6. 6-Heptyl-4-hydroxy-2-pyrone (**9**)

Sulfuric acid (90%, 6 g) was added to pyrone **8** (0.880 g, 2.62 mmol) and the solution was heated at 130 °C for 1 h. The reaction mixture was then cooled to room temperature and a few pieces of ice were added with stirring. The oily residue was extracted with ethyl acetate (3×20 mL) and the combined organic layers washed with water (3×20 mL), dried over anhydrous magnesium sulfate and concentrated in vacuo to give a brown oil. Purification by column chromatography (dichloromethane/methanol 9:1) afforded **9** as a yellow solid (0.379 g, 69%). Mp 72–73 °C (lit.[22] mp 71–71.5 °C); R_f (10% MeOH/CH_2Cl_2) 0.33; IR ν_{max} 3433, 1646 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.97 (1H, d, J=2.0 Hz, H-5), 5.57 (1H, d, J=2.0 Hz, H-3), 2.47 (2H, t, J=7.5 Hz, H-6a), 1.63 (2H, q, J=7.6 Hz, H-6b), 1.27 (8H, m, H-6c-f), 0.86 (3H, m, H-6g); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6 (quat., C-4), 168.4 (quat., C-2), 167.4 (quat., C-6), 101.3 (CH, C-5), 89.7 (CH, C-3), 33.6 (CH_2, C-6a), 31.6 (CH_2, C-6e), 28.9 (2×CH_2, C-6c, C-6d), 26.6 (CH_2, C-6b), 22.6 (CH_2, C-6f), 14.0 (CH_3, C-6g); MS (EI, 70 eV) m/z 210 ($M^{+\bullet}$, 14%), 139 (34), 126 (100), 111 (78), 98 (44), 84 (78), 69 (77), 41 (41); HRMS (EI): found $M^{+\bullet}$, 210.1257. $C_{12}H_{18}O_3$ requires 210.1256.

4.1.7. 6-Heptyl-3-hexanoyl-4-hydroxy-2-pyrone (***10***)

A mixture of pyrone **9** (0.150 g, 0.713 mmol) and hexanoyl chloride (0.20 mL, 1.42 mmol) in trifluoroacetic acid (0.5 mL) was refluxed under nitrogen for 3 h. The mixture was cooled to room temperature and ice water (1.5 mL) added. The resulting precipitate was dissolved in dichloromethane (5 mL) and the solution washed with water (3×10 mL). The organic layer was dried over anhydrous magnesium sulfate and concentrated in vacuo. Purification of the crude solid by column chromatography (dichloromethane) afforded **10** as a pale yellow solid (0.143 g, 65%). Mp 63.5–64.5 °C; R_f (33% EtOAc/hexane) 0.74; IR ν_{max} 3432, 1727, 1637 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.89 (1H, s, H-5), 3.05 (2H, t, J=7.4 Hz, H-3b), 2.46 (2H, t, J=7.5 Hz, H-6a), 1.63 (4H, m, H-3c, H-6b), 1.32 (12H, m, H-3d–f), 0.87 (6H, m, H-3f, H-6g); ^{13}C NMR (75 MHz, $CDCl_3$) δ 208.0 (quat., C-3a), 181.3 (quat., C-4), 172.5 (quat., C-6), 161.1 (quat., C-2), 100.7 (CH, C-5), 99.6 (quat., C-3), 41.6 (CH_2, C-3b), 34.3 (CH_2, C-6a), 31.6 (CH_2, C-6e), 31.4 (CH_2, C-3d), 28.9 (CH_2, C-6c), 28.8 (CH_2, C-6d), 26.4 (CH_2, C-6b), 23.7 (CH_2, C-3c), 22.5 (CH_2, C-6f), 22.5 (CH_2, C-3e), 14.0 (CH_3, C-3f or C-6g), 13.9 (CH_3, C-6g or C-3f); MS (EI, 70 eV) m/z 308 ($M^{+\bullet}$, 19%), 265 (100), 252 (95), 237 (73), 168 (54), 69 (25), 43 (64); HRMS (EI): found $M^{+\bullet}$, 308.1994. $C_{18}H_{28}O_4$ requires 308.1988. Anal. Calcd for $C_{18}H_{28}O_4$: C, 70.10; H, 9.15. Found: C, 70.39; H, 9.23.

4.1.8. 6-Heptyl-3-hexyl-4-hydroxy-2-pyrone (pseudopyronine B) (***2***)

To a stirred solution of acylpyrone **10** (0.070 g, 0.227 mmol) in tetrahydrofuran (4.6 mL) was added 2 M HCl (3.7 mL). Sodium cyanoborohydride (0.041 g, 0.652 mmol) was added portionwise to the resulting solution. The reaction was monitored by TLC and after 4 h there was mostly starting material present. More sodium cyanoborohydride (0.040 g, 0.637 mmol) was added and the reaction mixture was left stirring for 19 h. The solvent was then removed in vacuo and the resulting solid dissolved in dichloromethane (10 mL). The organic layer was washed with water (2×10 mL), dried over anhydrous magnesium sulfate and concentrated in vacuo to give a yellow solid. Purification by column chromatography (dichloromethane) afforded **2** as a pale yellow solid (0.035 g, 52%). Spectroscopic data agreed with those reported in the literature[18] and the product co-eluted on analytical HPLC with an authentic sample. Mp 84–86 °C; analytical HPLC t_R 7.59 min; IR ν_{max} 3429, 1633 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 10.99 (1H, br s, OH), 5.95 (1H, s, H-5), 2.39 (2H, t, J=7.5 Hz, H-6a), 2.24 (2H, t, J=7.7 Hz, H-3a), 1.52 (2H, q, J=7.0 Hz, H-6b), 1.35 (2H, m, H-3b), 1.27 (14H, m), 0.84 (6H, m, H-3f, H-6g); ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.70 (quat., C-2), 164.68 (quat., C-4), 162.7 (quat, C-6), 101.3 (quat., C-3), 99.1 (CH, C-5), 32.5 (CH_2, C-6a), 31.1 (CH_2, C-6e), 31.0 (CH_2, C-3d), 28.4 (CH_2, C-3c), 28.2 (CH_2, C-6d), 28.1 (CH_2, C-6c), 27.4 (CH_2, C-3b), 26.1 (CH_2, C-6b), 22.6 (CH_2, C-3a), 22.0 (CH_2, C-6f), 21.9 (CH_2, C-3e), 13.81 (CH_3, C-6g), 13.78 (CH_3, C-3f); MS (EI, 70 eV) m/z 294 ($M^{+\bullet}$, 8%), 224 (35), 195 (15), 55 (74), 53 (81), 43 (100); HRMS (EI): found $M^{+\bullet}$, 294.2197. $C_{18}H_{30}O_3$ requires 294.2195.

4.1.9. 4-Hydroxy-6-pentyl-2-pyrone (***13***)

Sulfuric acid (90%, 0.5 g) was added to acylpyrone **7** (0.017 g, 0.061 mmol) and the solution was heated at 130 °C for 1 h. The reaction mixture was then cooled to room temperature and a few pieces of ice were added with stirring. The oily residue was extracted with ethyl acetate (3×5 mL) and the combined organic layers washed with water (1×5 mL). The organic layer was dried over anhydrous magnesium sulfate and concentrated in vacuo. The crude oil was purified by column chromatography (dichloromethane/methanol 9:1) to afford **13** as a yellow solid (0.0047 g, 43%). Mp 47–49 °C (lit.[22] mp 48.0–50.0 °C); R_f (10% MeOH/CH_2Cl_2) 0.30; IR ν_{max} 3401,

1655 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.98 (1H, d, J=2.0 Hz, H-5), 5.57 (1H, d, J=2.0 Hz, H-3), 2.46 (2H, t, J=7.5 Hz, H-6a), 1.63 (2H, q, J=7.4 Hz, H-6b), 1.30 (4H, m, H-6c, H-6d), 0.87 (3H, m, H-6e); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.6 (quat., C-4), 168.3 (quat., C-2), 167.4 (quat., C-6), 101.3 (CH, C-5), 89.8 (CH, C-3), 33.6 (CH_2, C-6a), 31.0 (CH_2, C-6c), 26.3 (CH_2, C-6b), 22.3 (CH_2, C-6d), 13.8 (CH_3, C-6e); MS *m/z* (EI, 70 eV) *m/z* 182 ($M^{+\bullet}$, 27%), 126 (100), 111 (89), 98 (48), 84 (79), 69 (89), 43 (36); HRMS (EI): found $M^{+\bullet}$, 182.0942. $C_{10}H_{14}O_3$ requires 182.0943.

4.1.10. 3-Hexyl-4-methoxy-6-pentyl-2-pyrone (*12*)

Trimethylphosphate (0.5 mL, 4.25 mmol) was added to pseudopyronine A (**1**) (0.030 g, 0.113 mmol) and potassium carbonate (0.019 g, 0.135 mmol) and the resulting mixture was heated at 140 °C, under nitrogen, for 2 h. The mixture was then cooled to room temperature, diluted with water (5 mL) and extracted with ethyl acetate (4×3 mL). The combined organic layers were dried over anhydrous magnesium sulfate and concentrated in vacuo to give an orange oil. Purification by column chromatography (hexane/ethyl acetate 3:1) afforded **12** as a yellow oil (0.017 g, 53%). R_f (CH_2Cl_2) 0.56; IR ν_{max} 1644 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.95 (1H, s, H-5), 3.84 (3H, s, OCH_3), 2.45 (2H, t, J=7.6 Hz, H-6a), 2.37 (2H, t, J=7.6 Hz, H-3a), 1.65 (2H, q, J=7.6 Hz, H-6b), 1.40 (2H, m, H-3b), 1.29 (10H, m), 0.87 (6H, m, H-3f, H-6e); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.8 (quat., C-4), 165.6 (quat., C-2), 164.7 (quat., C-6), 105.8 (quat., C-3), 94.1 (CH, C-5), 56.1 (CH_3, OCH_3), 34.2 (CH_2, C-6a), 31.7 (CH_2, C-3d), 31.2 (CH_2, C-6c), 29.2 (CH_2, C-3c), 28.0 (CH_2, C-3b), 26.7 (CH_2, C-6b), 23.3 (CH_2, C-3a), 22.6 (CH_2, C-3e), 22.3 (CH_2, C-6d), 14.1 (CH_3, C-3f), 13.9 (CH_3, C-6e); MS (EI, 70 eV) *m/z* 280 ($M^{+\bullet}$, 7%), 210 (48), 209 (100), 181 (16), 43 (13); HRMS (EI): found $M^{+\bullet}$, 280.2033. $C_{17}H_{28}O_3$ requires 280.2038.

4.1.11. 6-Heptyl-4-hydroxy-2-pyridone (*14*)

Ammonia (28%, 2 mL) was added to 6-heptyl-4-hydroxy-2-pyrone (**9**) (0.10 g, 0.476 mmol) and the solution heated at 100 °C for 6 h. The reaction was diluted with water (4 mL) and cooled to room temperature. The resulting solid was filtered and dried in vacuo. Purification by column chromatography (dichloromethane/methanol 9:1) afforded **14** as a pale brown solid (0.050 g, 50%). Mp 255–257 °C; R_f (10% MeOH/CH_2Cl_2) 0.27; IR ν_{max} 3268, 3106, 1633 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 10.86 (1H, br s), 10.31 (1H, br s), 5.58 (1H, d, J=2.0 Hz, H-5), 5.33 (1H, d, J=2.1 Hz, H-3), 2.33 (2H, t, J=7.8 Hz, H-6a), 1.51 (2H, q, J=6.6 Hz, H-6b), 1.24 (8H, br s, H-6c–f), 0.85 (3H, m, H-6g); ^{13}C NMR (75 MHz, DMSO-d_6) δ 167.4 (quat., C-4 or C-2), 164.7 (quat., C-2 or C-4), 150.0 (quat., C-6), 97.2 (CH, C-5), 95.8 (CH, C-3), 32.0 (CH_2, C-6a), 31.0 (CH_2, C-6e), 28.2 (2×CH_2, C-6c, C-6b), 27.9 (CH_2, C-6b), 21.9 (CH_2, C-6f), 13.8 (CH_3, C-6g); MS (EI, 70 eV) *m/z* 209 ($M^{+\bullet}$, 14%), 152 (12), 138 (48), 125 (100), 97 (16), 69 (11), 41 (13); HRMS (EI): found $M^{+\bullet}$, 209.1412. $C_{12}H_{19}NO_2$ requires 209.1416. Anal. Calcd for $C_{12}H_{19}NO_2 \cdot 1/6H_2O$: C, 67.89; H, 9.18; N, 6.60. Found: C, 67.81; H, 9.00; N, 6.62.

4.1.12. 5-Hexyl-4-hydroxy-6-pentyl-2-pyrone (*15*)

5-Hexanoyl-4-hydroxy-6-pentyl-2-pyrone (**16**)[26] (0.050 g, 0.178 mmol), triethylsilane (0.125 mL, 0.774 mmol) and $LiClO_4$ (0.2 mg, 0.00188 mmol) were dissolved in TFA (1.2 mL) and left stirring at room temperature for 23 h. The solvent was removed in vacuo to give an orange oil. Purification by column chromatography (dichloromethane/methanol 9.5:0.5) afforded **15** as a yellow solid (0.034 g, 71%). Mp 71.5–73 °C; R_f (10% MeOH/CH_2Cl_2) 0.38; IR ν_{max} 3444, 1704, 1639 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 11.40 (1H, br s, OH), 5.66 (1H, s, H-3), 2.50 (2H, t, J=7.6 Hz, H-6a), 2.35 (2H, t, J=7.5 Hz, H-5a), 1.63 (2H, q, J=7.4 Hz, H-6b), 1.43 (2H, m, H-5b), 1.31 (10H, m), 0.87 (6H, m, H-5f, H-6e); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7 (quat., C-4), 167.7 (quat., C-2), 163.1 (quat., C-6), 113.5 (quat., C-5), 90.0 (CH, C-3), 31.6 (CH_2, C-6c), 31.4 (CH_2, C-5d), 30.7 (CH_2, C-6a), 29.5 (CH_2, C-5b), 29.2 (CH_2, C-5c), 27.2 (CH_2, C-6b), 24.2 (CH_2, C-5a), 22.6 (CH_2, C-6d), 22.3 (CH_2, C-5e), 14.0 (CH_3, C-5f or C-6e), 13.9 (CH_3, C-6e or C-5f); MS (EI, 70 eV) *m/z* 266 ($M^{+\bullet}$, 12%), 223 (24), 195 (100), 153 (30), 99 (29), 71 (18), 43 (58); HRMS (EI): found $M^{+\bullet}$, 266.1880. $C_{16}H_{26}O_3$ requires 266.1882. Anal. Calcd for $C_{16}H_{26}O_3$: C, 72.14; H, 9.84. Found: C, 72.32; H, 9.81.

4.2. General methods (biological assays)

Details of the whole organism parasite[30,31] and *M. tuberculosis*[20] and purified enzyme[10,31,32] biological assay protocols have been reported elsewhere.

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