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Improving the dual anthelmintic activity

Schistosoma mansoni

Fasciola hepatica
Design, synthesis and anthelmintic activity of 7-keto-semperviroi analouges

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Abstract

The plant-derived, diterpenoid 7-keto-sempervirol was recently reported to display moderate activity against larval stages of *Schistosoma mansoni* (IC$_{50}$ = 19.1 µM) and *Fasciola hepatica* (IC$_{50}$ = 17.7 µM), two related parasitic blood and liver flukes responsible for the neglected tropical diseases schistosomiasis and fascioliasis, respectively. Here, we aimed to increase the potency of 7-keto-sempervirol by total synthesis of 30 structural analogues. Subsequent screening of these new diterpenoids against juvenile and adult lifecycle stages of both parasites as well as the human HepG2 liver cell line and the bovine MDBK kidney cell line revealed structure-activity relationship trends. The most active analogue, 7d, displayed improved dual anthelmintic activity over 7-keto-sempervirol (IC$_{50}$ ≈ 6 µM for larval blood flukes; IC$_{50}$ ≈ 3 µM for juvenile liver flukes) and moderate selectivity (SI ≈ 4 - 5 for blood flukes, 8 - 13 for liver flukes compared to HepG2 and MDBK cells, respectively). Phenotypic studies using scanning electron microscopy revealed substantial tegumental alterations in both helminth species, supporting the hypothesis that the parasite surface is one of the main targets of this family of molecules. Further modifications of 7d could lead to greater potency and selectivity metrics resulting in a new class of broad-spectrum anthelmintic.

**Keywords:** natural products, diterpenoids, schistosomiasis, fascioliasis, antiparasitic, anthelmintic
1. Introduction

Schistosomiasis, caused by infection with blood fluke schistosomes, is the most devastating human parasitic disease after malaria considering the number of people currently infected and at risk of infection [1]. It is a chronic disease of poverty characterized by pain and disability that, collectively, exacerbates the already compromised healthcare situation of developing tropical countries [2]. The number of people infected is approximately 600 million [3] and, along with 300 thousand deaths per year, schistosomiasis is considered as the most deadly neglected tropical disease (NTD) [4]. No vaccines are available to prevent infection and, therefore, treatment of infected individuals is predominantly facilitated by chemotherapy with praziquantel (PZQ) (Figure 1A). PZQ is active against adult worms of all *Schistosoma* species, but less effective against the immature forms, leading easily to reinfections [5]; moreover, due to its large scale administration, concerns about drug resistance are increasing [5,6].

Another NTD predominantly controlled by a single drug, triclabendazole, is fascioliasis. Ingestion of liver fluke parasites initiates fascioliasis and leads to up to 17 million human [7] and numerous livestock animal infections per annum, causing enormous economic losses in the global beef, lamb and milk industries [8]. In the United Kingdom alone, an estimated £23 million is lost annually due to fascioliasis [9]. However, recent reports suggest that this figure could rapidly increase due to changes in global climate and extensive animal movement [10,11]. Triclabendazole (Figure 1B) is the only commercially available drug that is active against both juvenile and adult liver fluke lifecycle stages. Unfortunately, triclabendazole resistant *Fasciola* parasites have been described throughout Asia, Africa, South America, North America, Australia and Europe [12], making the discovery of new drugs for combating this human and animal disease an urgent healthcare priority.

For these reasons, research into the discovery of new anti-flukicidal drugs is increasing with natural products being an exceptionally attractive resource for beginning such a process [13]. Indeed, plant-derived natural products are a well-recognised source of anti-infective compounds due to natural selection shaping the production of more toxic and protective
compounds to facilitate survival in a microorganisms-rich environment [14]. One example of a plant-derived compound isolated from *Lycium chinense* with bioactivity against both *Schistosoma mansoni* and *Fasciola hepatica* is the diterpenoid 7-keto-sempervirol (Figure 1C) [15]. While this diterpenoid was only moderately potent, it was effective in killing juvenile and adult forms of both fluke species. Considering the fact that some endemic areas for schistosomiasis are co-endemic for fascioliasis and that, in these areas, it is common to find people with both infections [16–18], a compound with potent dual anthelmintic activity against multiple lifecycle stages would be highly desirable.

![Figure 1. Anthelmintic compound structures discussed in this study.](image)

In this study, 7-keto-sempervirol was subjected to medicinal chemistry optimisation, with the aim of exploring whether different substitutions could increase the dual anthelmintic potency of this natural product. To achieve this, structural related analogues were obtained by total synthesis using the different synthetic pathways reported here. The compounds were subsequently screened against larval (schistosomula) and adult *S. mansoni* blood flukes as well as against newly excysted juvenile (NEJs), immature and adult *F. hepatica* liver flukes. The newly synthesised compounds were also screened against HepG2 liver human cells and MDBK kidney bovine cells to assess potency and selectivity as well as to deduce preliminary structure activity relationships (SARs). The results of compound synthesis and bioactivities are presented and discussed here.
2. Results and Discussion

2.1. Chemistry

Based on the previously reported anthelmintic activity of the diterpenoid 7-keto-sempervirol, we investigated the impact of chemical structure modification on activity to obtain more potent and selective analogues. To achieve this, six families of compounds containing the basic scaffold of 7-keto-sempervirol were synthesised with each family containing distinct aromatic substitutions (Figure 2).

![Figure 2. Description of the 7-keto-sempervirol analogues.](image)

Diverse synthetic strategies were adopted for each family. Non-cyclised analogues lacking bond C9-C10 were obtained from the coupling between commercially available beta-cyclocitral and substituted benzyl chlorides, as described by Huang et al. [19], to generate the first set of analogues as secondary alcohols (1a-f) (Scheme 1). Corresponding keto-analogues (2a-f) were then obtained by oxidation in the presence of tetrapropylammonium perruthenate (TPAP) and N-methylmorpholine N-oxide (NMO) (Scheme 1).
Scheme 1. Synthesis of diterpenoid analogues 1a-f and 2a-f. The scheme shows the synthesis of the non-cyclised analogues 1a-f and 2a-f from beta-cyclocitral using lithiation and oxidation chemistry.

From the synthesised keto-analogues, two different cyclization methods were performed to obtain the tricyclic diterpenoid scaffold with a keto-group in position six (Scheme 2). The first method (Scheme 2A) used the Lewis acid BBr$_3$ to facilitate the synthesis of the trans diastereoisomers (3a-c) as described by Huang et al. [19], while the second method (Scheme 2B) used RuCl$_3$ and AgOTf to obtain the cis diastereoisomer (4a-c), according to the method of Youn et al. [20]. However, this set of compounds showed instability when exposed to oxygen at room temperature and, therefore, their biological activity was not measured. Indeed, changes in colour and composition of the 6-keto compounds were observed as quickly as two (and up to seven) days post synthesis (See Supplementary information). Analysis of the resulting mixtures revealed high levels of autoxidation (cis > trans) that, upon subsequent purification and characterization, revealed the presence of a keto group in positions 6 and 7 (Scheme 2C). These 6,7-diketo derivatives (5a-c) were more stable than the original 6-keto compounds and, for this reason, are considered a new family of analogues. Literature precedent for spontaneous oxidation in terpene-based systems, including mechanistic studies, is known [21].
Scheme 2. Synthesis of diterpenoid analogues 3a-c and 5a-c. The scheme shows the synthesis of 6-keto (3a-c) and 6,7-diketo (5a-c) analogues based on diastereoselective ring closure and subsequent autoxidation of the cis-isomer.

The other three families of compounds were obtained from one synthetic pathway according to the method described by Surendra and Corey [22] (Scheme 3). Substituted Grignard compounds were added to geraniol acetate in the presence of Li₂CuCl₄ (Scheme 3A) and the products obtained (6a-c) were then cyclized through an enantioselective reaction catalysed by the complex (R)-BINOL-SbCl₅; the resulting hydrophobic compounds (7a-d) lacked a keto group (Scheme 3B). Compounds 7a-d were then oxidized by CrO₃ to generate the corresponding 7-keto analogues 8a-d (Scheme 3C).
Scheme 3. Synthesis of diterpenoid analogues 6a-c, 7a-d, 8a-d. The scheme shows the synthesis of no-keto (7a-d), 7-keto analogues (8a-d) and their synthetic intermediates (6a-c) via addition of benzylic Grignard agent to geraniol acetate followed by enantioselective antimony-promoted cyclisation and chemical oxidation. Compounds 7d and 8d were obtained by demethylation of 7b and 8b, respectively, by BBr₃ in DCM.

For our studies we made use of the commercially available chiral ligand (R)-BINOL, as opposed to the o,o'-dichloro-BINOL employed by Surendra and Corey in their reported enantioselective synthesis [22]. Preliminary polarimeter analysis of our final compounds 8a-d indicated enantiomeric excesses (ee's) in the range of 20-30% (data not shown). For the purpose of this screening analysis, these compounds should be treated as racemic (as is the case of the standard of care drug praziquantel [5]). Enantiomeric synthesis and/or enantiomer resolution to study the contributory biological activity of single enantiomers will form the basis of future studies.

2.2. Biological activities

All stable compounds (1a-f, 2a-f, 5a-c, 6a-c, 7a-d, 8a-d) were first screened for anthelmintic activity on the larval stage (schistosomula) of *S. mansoni* using an integrated high-throughput, high content image (HCl) analysis platform called Roboworm [23,24]. Here, compounds are quantified according to their ability to affect both phenotype and motility of the treated larva. Any compound that leads to a score threshold that is equal to or falls below the values of -0.15 and -0.35 for phenotype and motility, respectively, in ≥70% of the
assayed larva is considered a hit [25]. As all compounds tested were hits at 50 µM (data not shown), subsequent schistosomula screens were performed at 25 and 10 µM (Figure 3).

Figure 3. Diterpenoid screening on *S. mansoni* schistosomula. Schistosome (n = 120) / diterpenoid co-cultures were incubated for 72 hr at 37 °C in a humidified atmosphere containing 5% CO₂. The effect that each of these diterpenoids had on schistosomula phenotype and motility at 25 µM (A) and 10 µM (B) is illustrated and compared with the negative (DMSO) and the positive controls (Auranofin). Compounds are scored by the high throughput platform Roboworm according to their effect on schistosomula phenotype and motility (indicated by change in red outlines on figures). Hit compounds (within the hit threshold) affect ≥ 70% of the larvae. Each point represents the average score of two replicates. Z'-scores [26] were calculated for these anti-schistosomula screens and included values corresponding to 0.69159 for phenotype and 0.48074 for motility. Images of the parasites after treatment with the controls and the six hits at 25 µM are also shown (C). *Result from a separate assay where the anthelmintic activity of 7-keto-sempervirol (7ks) was assessed.*
The gold thiolate anti-parasitic compound Auranofin was used as a positive control [27]. At 25 µM, six compounds (2c, 2d, 5a, 5b, 7d and 8c) were considered hits and at 10 µM, only compound 7d was still active. This compound was later screened at 1 µM but showed no anti-schistosomula activity (data not shown). The estimated IC50 values for 7d phenotype and motility were 6.8 and 6.3 µM, respectively and represent the most potent values when compared to all other analogues (Table 1).

Table 1. Diterpenoid anti-schistosomal activity and cell cytotoxicity summary.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 on schistosomula phenotype (µM)</th>
<th>IC50 on schistosomula motility (µM)</th>
<th>IC50 on adult worms (µM)</th>
<th>CC50 on HepG2 cells (µM)</th>
<th>CC50 on MDBK cells (µM)</th>
<th>Selectivity Index HepG2 cells (Phenotype / Motility)</th>
<th>Selectivity Index MDBK cells (Phenotype / Motility)</th>
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<td>&gt; 100</td>
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<td>NA</td>
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<tr>
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<td>&gt; 100</td>
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<td>4.8 / 5.2</td>
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<td>&gt; 100</td>
<td>2.1 / 5.7</td>
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<td>&gt; 100</td>
<td>2.3 / 3.0</td>
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<td>80.0</td>
<td>63.8</td>
<td>3.7 / 3.1</td>
<td>2.9 / 2.4</td>
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Estimated IC50s calculated from diterpenoid dose response curves for S. mansoni schistosomula phenotype and motility and S. mansoni adult worm motility. Estimated CC50s calculated from diterpenoid dose response curves for HepG2 and MDBK cells and selectivity index of activity on schistosomula when compared to both cell lines. The values are results of experiments in duplicate (for helminths) or triplicate (for cells).
A general observation was that most of the diterpenoids screened had a greater effect on schistosomula motility rather than phenotype. The lead compound 7-keto-sempervirol (7ks) was additionally screened by Roboworm and the anthelmintic activity, previously quantified by a different method [15], was comparable.

The six compounds active against schistosomula at 25 µM were subsequently screened on adult male and female worm pairs (Table 1). Amongst these six, the most active analogue was again 7d, able to kill all parasites at 50 µM and to significantly affect their motility until 12.5 µM (Figure 4). While there was no significant variation in the ability of 7d to differentially affect male and female worm motility, the compound inhibited egg production even at the lowest concentration tested, 6.25 µM (Figure 4A; for adult worm screening of the other compounds, see Supplementary Information, S1). At this concentration, only eggs with abnormal phenotypes were found; these phenotypes (when compared to eggs from control wells) included smaller eggs lacking both lateral spines and regular autofluorescence (Figure 4B and C). While the significance of abnormally produced eggs is currently unknown, the substantial ‘anti-fecundity’ activity is noteworthy as schistosome eggs are responsible for transmission in endemic areas and are the major cause of schistosomiasis-attributable chronic disease in infected humans [6].
Figure 4. Anthelmintic screening of compound 7d on adult S. mansoni worm pairs. (A) Adult schistosome worm pairs (3 pairs / well) were co-cultivated with 7d (50 µM - 6.25 µM) for 72 hr at 37 °C in a humidified atmosphere containing 5% CO₂. The effect that 7d had on schistosome motility and egg production was quantified according to the methodologies. Each bar represents the mean motility score ± SD of 12 adult worms (6 worms x 2 independent experiments) when treated with different concentrations and compared to the negative (DMSO) and positive control (Auranofin). The overlying line graph represents the mean egg count ± SD. * Significant difference compared to DMSO controls (p < 0.05).

Egg images obtained by fluorescence microscopy are also shown (under natural light in the left panels and using a GFP filter in the right panels). Eggs collected from control wells (B) are oval, contain a lateral spine and exhibit a homogeneous auto-fluorescent surface. Eggs collected from treatment wells (6.25 µM 7d) are abnormally shaped (C), smaller and are characterised by loss of the lateral spine and irregular auto-fluorescence.

The six most active compounds on schistosomula (2c, 2d, 5a, 5b, 7d and 8c) were also screened against the related trematode, F. hepatica (Figure 5). Initial screening on F. hepatica newly excysted juveniles (NEJs) was performed at 25 and 12.5 µM and demonstrated that, similarly to schistosomula, the most active anti-NEJ compounds were 7d and 8c (Figure 5A and 5B).
Figure 5. Anthelmintic screening of diterpenoids on *F. hepatica* newly excysted juveniles (NEJs). NEJs (n = 25) / diterpenoid co-cultures were incubated for 72 hr at 37 °C in a humidified atmosphere containing 5% CO₂. The effect that each of these diterpenoids had on NEJ phenotype and motility at 25 µM (A) and 12.5 µM (B) is illustrated and compared with the negative (DMSO) and the positive controls (Triclabendazole). Each bar graph represents the average score ± SD (n = 25) for phenotype (green) and motility (blue). Compounds 7d and 8c showed the greatest significant activity at the lowest test concentration. Images of the parasites after treatment with the controls and the 2 hits at 12.5 µM are also shown (C).

* Significant difference with DMSO control (*p* < 0.05).

Subsequently, titrations of these two compounds (and of 7ks as reference compound) allowed the calculation of the respective IC₅₀s, with 7d exhibiting values of 3.2 and 2.5 µM for phenotype and motility (Table 2). Both 7d and 8c were next screened on immature and mature liver fluke lifecycle stages (Figure 6; Supplementary Information, S2), with 7d again showing the most potent activity (IC₅₀ values of 10.4 and 28.4 µM, respectively (Table 2)).
Figure 6. Anthelmintic screening of compound 7d on *F. hepatica* immature and adult parasites. Immature (3 / well) and adult (2 / well) parasites were co-cultivated with 7d (40 µM - 4.4 µM) for 72 hr at 37 °C in a humidified atmosphere containing 5% CO₂. The effect that the compounds had on the immature (A) and adult (B) parasites was quantified according to the methodologies. Each bar represents the mean motility score ± SD of the worms when treated with different concentrations and compared to the negative (DMSO) and positive control (Triclabendazole). * Significant difference compared to DMSO controls (*p < 0.05).

Surrogate selectivity was assessed by screening all synthesised compounds on HepG2 liver human cells using the MTT assay. Here, all diterpenoids showed moderate selectivity, being on average 3-5 times more toxic on blood or liver flukes than human cells (Tables 1 and 2). Due to the veterinary importance of these parasitic diseases [9,17,28], compounds were also screened against MDBK kidney bovine cells and showed a toxicity comparable or lower than that observed for HepG2 cells (Tables 1 and 2).

Table 2. Diterpenoid anti-*Fasciola* activity and selectivity summary.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ on NEJs phenotype (µM)</th>
<th>IC₅₀ on NEJs motility (µM)</th>
<th>IC₅₀ on immatures (µM)</th>
<th>IC₅₀ on adults (µM)</th>
<th>Selectivity Index HepG2 cells (Phenotype / Motility)</th>
<th>Selectivity Index MDBK cells (Phenotype / Motility)</th>
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<tr>
<td>7d</td>
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<td>10.2 / 13.0</td>
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<tr>
<td>8c</td>
<td>9.9</td>
<td>8.4</td>
<td>14.6</td>
<td>39.9</td>
<td>3.3 / 3.9</td>
<td>7.1 / 8.3</td>
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</table>

Estimated IC₅₀s calculated from diterpenoid dose response curves for *F. hepatica* NEJ phenotype and motility, as well as motility for both *F. hepatica* immature and adult flukes. Selectivity index when compared to CC₅₀s on HepG2 and MDBK cells is also illustrated.
Looking at the difference in the anti-schistosomula activity of the analogues (where the most complete dataset has been collected), some trends in structure-activity relationship were found. Considering the non-cyclised analogues (1a-f and 2a-f), the alcohol series 1a-f was generally less active on schistosomula than the correspondent ketone series 2a-f. With regards to the aromatic portion, methoxy, iso-propyl and tert-butyl substitutions in position 12 (diterpenoid numeration from Figure 1) increased the anthelmintic activity when compared to the non-substituted analogues or to the ones with the methoxy group in 11 or 13. As a result, the most active compounds were 2c and 2d. This relationship was confirmed in the 7-keto series 8a-d with the most active compound being 8c. However, in the di-keto series 5a-c, the most active compound was 5b, which contained a methoxy group in position 13. This structure activity relationship, supported by different schistosomula phenotypes (Figure 3), could perhaps indicate a different mechanism of action. The strongest anti-schistosomal (both schistosomula and adult worms) activity was associated with a hydroxy group in position 12 as demonstrated by the most potent compound 7d. However, when other substituents were added along with the hydroxy group (like those found in 7ks and 8d), the anti-schistosomal activity was reduced. Similar findings were observed for *F. hepatica* NEJs, with the best activity against this liver fluke being associated with 7d and 8c.

To begin developing hypotheses regarding 7d’s mechanism(s) of action, Scanning Electron Microscopy (SEM) was used. Here, surface integrity of parasites treated with a sublethal concentration of 7d (25 µM for *S. mansoni* adults, 40 µM for *F. hepatica* adults, 13.3 µM for *F. hepatica* immatures) was specifically examined (Figure 7). In the case of adult blood fluke parasites, *S. mansoni* males treated with 7d displayed substantial tegumental disruption throughout the whole body, with the spinous tubercles losing their natural shape when compared to the control (Figure 7A and B). A similar phenotype, but less severe, was found in 7d-treated female schistosomes (not shown). Compound 7d’s apparent gender biased effect (with regards to surface architecture) is in agreement with other studies and could be associated with females having a less absorptive surface [29]. In the case of liver flukes, 7d
also caused noticeable damage to hermaphroditic adult *F. hepatica*, with the ventral surface decorated by swollen tegumental ridges and smaller spines (Figure 7C and D).

Figure 7. Scanning electron microscopy (SEM) images of *S. mansoni* and *F. hepatica* worms co-cultivated with 7d. SEM images of the adult blood flukes after treatment with control DMSO (A) and with compound 7d at 25 µM (B). The tubercles of the treated parasite appear damaged when compared to the control. Images of adult liver flukes after treatment with control DMSO (C) and 7d at 40 µM (D) are also shown. The ventral area of the parasite contains smaller spines within the swollen tegument. Finally, comparison of control (E) and
compound 7d at 13.3 µM (F) on immature liver flukes showed more substantial loss of spines and sloughing of the tegument on the ventral area of treated parasites.

Swelling of the tegument is likely due to an osmotic effect after changes in the permeability of the membrane surface and/or in the osmoregulatory system, often linked to unbalanced ion distribution [30]. Even more pronounced was the damage of 7d to F. hepatica immature flukes, where a complete loss of spines on the ventral surface was observed together with sloughing of the tegument and exposure of the basal lamina (Figure 7E and F). These findings, together with other data reported in the literature [2,31,32], support the hypothesis that one of the main targets of terpenoid like molecules is the tegument, a vital structure for anti-parasitic drugs because of its barrier function in host interactions [29]. However, the decreased motility and the inhibition of phenotypically normal egg production could also indicate other possible mechanisms of action as hypothesised for 7ks [15].

3. Conclusions

In conclusion, 30 analogues of the diterpenoid 7-keto-sempervirol were synthesised and 25 of them initially screened for anthelmintic activity on the larval stage of the parasite S. mansoni. Amongst these 25 compounds, the best was 7d having an IC$_{50}$ of 6.8 - 6.3 µM (phenotype and motility, respectively) and selectivity index of 3.8 - 4.1 when compared with HepG2 liver human cells and 4.8 - 5.2 when compared with MDBK kidney bovine cells. This compound was also active against adult schistosomes (IC$_{50}$ = 19.9 µM) and inhibited egg production at the lowest test concentration. More potent and selective activity was displayed on F. hepatica NEJs, with IC$_{50}$ of 2.5 - 3.2 µM (phenotype and motility, respectively) and selectivity index of 8 - 10.2 when compared with HepG2 cells and 10.2 – 13.0 with MDBK cells. This compound additionally displayed activity against immature and adult liver fluke parasites, with estimated IC$_{50}$ values of 10.4 µM and 28.4 µM. SEM studies of parasites treated with 7d, revealed tegumental alterations that could be the basis for the mechanism of action.
The results of this study showed the potential of this class of diterpenoids as promising new anthelmintic compounds and how a medicinal chemistry optimisation approach can lead to the identification of more potent molecules. Further studies deciphering mechanisms of action and improving the physicochemical properties should be pursued to help develop this promising class of molecules.

4. Experimental section

4.1. Chemistry

Commercial reagents and solvents were purchased from Sigma Aldrich or Fisher Scientific and used without further purification. 7-keto-sempervirol was obtained as previously described [15]. The synthesised compounds were characterized by high resolution mass HRMS, $^1$H, $^{13}$C and two-dimensional nuclear Magnetic Resonance (NMR) spectroscopy. NMR spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer in CDCl$_3$ solution referenced to the solvent residual peak. The reactions were monitored by Thin Layer Chromatography (TLC) on pre-coated TLC aluminium sheets of silica gel. The compounds were purified by column chromatography on silica gel (35-70 mesh) using the eluents indicated. Mass spectrometry was performed on a Bruker Daltonics microTof-LC system or on an Orbitrap Fusion Thermo Scientific with a Dionex UltiMate 3000 UHPLC system.

For each family of molecules, the general synthetic method is reported together with an example of compound characterization. All the compound characterization can be found in the Supplementary Information.

4.1.1. General method for synthesis of compounds 1a-f [19]

Under nitrogen, a mixture of naphthalene (3.75 eq) and lithium (3.75 eq) in anhydrous THF was stirred at room temperature for 2 h. Then, a solution of beta-cyclocitral (1.1 eq) and the substituted benzyl chloride (1 eq) was added dropwise via syringe at 0 °C. The reaction
mixture was stirred at room temperature for 2 h, then diluted with diethyl ether and treated
with saturated NH₄Cl. The organic phase was separated and the aqueous phase extracted
with ethyl acetate. The combined organic layers were washed with brine, dried over Na₂SO₄
and concentrated under reduced pressure. The crude product was purified by silica gel
column chromatography (4-10% ethyl acetate in petroleum ether).

4.1.1.1. 2-Phenyl-1-(2,6,6-trimethylcyclohex-1-en-1-yl)ethan-1-ol (1a)
Colourless oil, yield: 60%. ¹H NMR: δ 7.37-7.25 (5H, m, 5 x ArH), 4.48 (1H, dd, J = 10.4,
3.15 Hz, CH₂OH), 3.17 (1H, dd, J = 14.0, 10.6 Hz, CH₂Ph), 2.89 (1H, dd, J = 14 Hz, 3.2 Hz,
CH₂Ph), 2.05-2.00 (2H, m, CH₂), 2.01 (3H, s, CH₃C=C), 1.63-1.61 (2H, m, CH₂), 1.50-1.48
(2H, m, CH₂), 1.14 (3H, s, CH₃), 1.05 (3H, s, CH₃); ¹³C NMR: δ 139.92 (C), 139.14 (C),
131.87 (C), 129.33 (2 x ArCH), 128.60 (2 x ArCH), 126.44 (ArCH), 72.37 (CH₂OH), 43.23
(CH₂), 39.95 (CH₂), 34.86 (C(CH₃)₂), 34.12 (CH₂), 28.67 (CH₃), 28.08 (CH₃), 21.30 (CH₃),
19.34 (CH₂); HRMS-ESI m/z: [M + Na]⁺ calcd for C₁₁H₂₄ONa 267.1719, found 267.1720.

4.1.2. General method for synthesis of compounds 2a-f
The previously obtained alcohol (1 eq) and N-methylmorpholine N-oxide (NMO) (1.5 eq)
were dissolved in anhydrous dichloromethane under nitrogen. After stirring for 15 minutes,
tetrapropylammonium perruthenate (TPAP) (0.05 eq) dissolved in dichloromethane was
added and the mixture stirred overnight. The reaction mixture was then diluted with
dichloromethane, and washed with sodium sulphite solution and brine. The organic layer
was dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was
purified by silica gel column chromatography (4-10 % ethyl acetate in petroleum ether).

4.1.2.1. 2-Phenyl-1-(2,6,6-trimethylcyclohex-1-en-1-yl)ethan-1-one (2a)
Colourless oil, yield: 72%. ¹H NMR: δ 7.25-7.22 (2H, m, 2 x ArH), 7.17-7.13 (3H, m, 3 x
ArH), 3.77 (2H, s, CH₂Ph), 1.90 (2H, t, J = 6.3 Hz, CH₂), 1.62-1.57 (2H, m, CH₂), 1.51 (3H, s,
CH₃, C=C), 1.39-1.37 (2H, m, CH₂), 1.01 (6H, s, 2 x CH₃); ¹³C NMR: δ 208.29 (C=O), 143.31
(C), 134.03 (C), 129.98 (2 x ArCH), 129.62 (C), 128.42 (2 x ArCH), 126.86 (ArCH), 52.16 (CH₂Ph), 38.96 (CH₂), 33.46 (C(CH₃)₂), 31.23 (CH₂), 28.87 (2 x CH₃), 21.18 (CH₃), 18.93 (CH₂); HRMS-ESI m/z: [M + Na]⁺ calcd for C₁₇H₂₂ONa 265.1563, found 265.1564.

4.1.3. General method for synthesis of compounds 3a-c [19]

To a solution of the previously obtained ketone (1 eq) in anhydrous dichloromethane was added a solution of BBr₃ 1 M (6 eq) in dichloromethane dropwise at -78 °C, under nitrogen. The resulting mixture was stirred for 30 minutes and then allowed to reach 0 °C before continued stirring for 3 h. The reaction was quenched carefully with NaHCO₃ solution and the aqueous phase was extracted with ethyl acetate. Organic layers were washed with brine and dried over Na₂SO₄. The crude product was purified by silica gel column chromatography (5-30% ethyl acetate in petroleum ether).

4.1.3.1. Trans 6-oxopodocarpa-8,11,13-triene (3a)

Colourless oil, yield: 82%. ¹H NMR: δ 7.35 (1H, d, J = 7.7 Hz, ArH), 7.26 (1H, td, J = 7.7, 0.9 Hz, ArH), 7.20 (1H, td, J = 7.4 Hz, 1.3 Hz, ArH), 7.07 (1H, dd, J = 7.4 Hz, 0.9 Hz, ArH), 3.64 (2H, s, CH₂-7), 2.41 (1H, s, CH-5), 2.41-2.36 (1H, m, CH₂), 1.80-1.67 (4H, m, 2 x CH₂), 1.46-1.43 (1H, m, CH₅), 1.34 (3H, s, CH₃C=C), 1.19 (3H, s, CH₃), 1.12 (3H, s, CH₆); ¹³C NMR: δ 209.47 (C=O), 149.00 (ArC), 132.44 (ArC), 128.47 (ArCH), 126.95 (ArCH), 126.45 (ArCH), 123.68 (ArCH), 126.45 (CH₃), 62.55 (CH₅), 45.12 (CH₂), 42.91 (CH₂), 40.58 (CH), 38.68 (CH₂), 33.08 (CH₃), 32.76 (C), 24.62 (CH₃), 21.65 (CH₃), 18.76 (CH₂); HRMS(ESI) m/z: [M + Na]⁺ calcd for C₁₇H₂₂ONa 265.1563, found 265.1565.

4.1.4. General method for synthesis of compounds 4a-c [20]

A mixture of AgOTf (0.4 eq) and RuCl₃ x H₂O (0.2 eq) in dry dichloroethane was stirred for 1 h. The previously obtained ketone (1 eq) was then added and the resulting solution was stirred overnight at 80 °C. The solvent was evaporated and crude product purified by silica
gel column chromatography using dichloromethane and petroleum ether (5-10% ethyl acetate in petroleum ether).

4.1.4.1. Cis 6-oxopodocarpa-8,11,13-triene (4a)

Colourless oil, yield: 51%. $^1$H NMR: δ 7.34 (1H, d, J = 7.8 Hz, Ar$H$), 7.26 (1H, t, J = 7.4 Hz, Ar$H$), 7.19 (1H, t, J = 7.4 Hz, Ar$H$), 7.07 (1H, d, J = 7.4 Hz, Ar$H$), 3.74-3.51 (2H, m, C$H_2$-7), 2.56-2.53 (1H, m, C$H_2$), 2.12 (1H, s, C$H$-5), 1.55-1.53 (1H, m, C$H_2$), 1.33-1.25 (4H, m, 2 x C$H_2$), 1.08 (3H, s, C$H_3$), 0.95 (3H, s, C$H_3$), 0.31 (3H, s, C$H_3$); $^{13}$C NMR: δ 212.38 (C=O), 141.65 (ArC), 134.22 (ArC), 128.69 (ArCH), 127.24 (ArCH), 126.50 (ArCH), 66.55 (CH-5), 44.17 (CH$_2$), 42.32 (CH$_2$), 38.90 (C), 36.13 (CH$_2$), 34.40 (C), 33.42 (CH$_3$), 32.28 (CH$_3$), 22.58 (CH$_3$), 18.94 (CH$_2$); HRMS(ESI) m/z: [M + Na]$^+$ calcd for C$_{17}$H$_{22}$ONa 265.1563, found 265.1565.

4.1.5. General method for synthesis of compounds 5a-c

After exposure to air and light at room temperature for 2-7 days, changes in colour, from colourless to yellow, were noticed on the cis-6-keto compounds. The new di-keto compounds generated were isolated after purification by column chromatography (8-15% ethyl acetate in petroleum ether). The oxidation procedure was repeated using the procedure followed for compounds 8a-c (see below).

4.1.5.1. Cis 6,7-dioxopodocarpa-8,11,13-triene (5a)

Yellow solid, yield: 95%. $^1$H NMR: δ 8.13 (1H, dd, J = 7.8 Hz, 1.3 Hz, Ar$H$), 7.68-7.65 (1H, m, Ar$H$), 7.48 (1H, d, J = 7.9 Hz, Ar$H$), 7.41 (td, J = 7.8, 1.0 Hz, Ar$H$), 2.68 (1H, s, CH-5), 2.62-2.59 (1H, m, CH$_2$), 1.63-1.52 (2H, m, CH$_2$), 1.45-1.37 (1H, m, CH$_2$), 1.37-1.32 (2H, m, CH$_2$), 1.23 (3H, s, CH$_3$), 0.97 (3H, s, CH$_3$), 0.38 (3H, s, CH$_3$); $^{13}$C NMR: δ 198.94 (C=O), 181.48 (C=O), 150.00 (ArC), 135.73 (ArCH), 133.84 (ArC), 130.36 (ArCH), 127.55 (ArCH), 124.82 (ArCH), 68.97 (CH-5), 42.05 (CH$_2$), 39.83 (C), 38.83 (CH$_3$), 36.18 (CH$_2$), 35.62 (C), 31.45
4.1.6. General method for synthesis of compounds 6a-c [22]

To a solution of geraniol acetate (1 eq) in THF at 0 °C, 0.1M Li$_2$CuCl$_4$ solution in THF (0.1 eq) was added dropwise and then a solution of Grignard reagent (2 eq) was added dropwise at 0 °C. After 2 h at 0 °C, the mixture was quenched with saturated aqueous NH$_4$Cl solution and the aqueous layer extracted with diethyl ether. Combined organic layers were washed with brine, dried over Na$_2$SO$_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (0-1% ethyl acetate in petroleum ether) to afford the coupling product.

4.1.6.1. Trans (4,8-Dimethylnona-3,7-dien-1-yl)benzene (6a)

Colourless oil, yield: 90%. $^1$H NMR: δ 7.32 (2H, t, J = 7.5 Hz, 2 x ArH), 7.25-7.21 (3H, m, 3 x ArH), 5.24 (1H, t, J = 7.0 Hz, C=H), 5.15 (1H, t, J = 7.0 Hz, C=H), 2.69 (2H, t, J = 8.0 Hz, CH$_2$), 2.38-2.34 (2H, m, CH$_2$), 2.14-2.10 (2H, m, CH$_2$), 2.05-2.02 (2H, m, CH$_2$), 1.74 (3H, s, CH$_3$), 1.66 (3H, s, CH$_3$), 1.61 (3H, s, CH$_3$); $^{13}$C NMR: δ 142.54 (C), 135.87 (C), 131.42 (C), 128.61 (2 x CH), 128.33 (2 x CH), 125.78 (CH), 124.50 (CH), 123.75 (CH), 39.86 (CH$_2$), 36.29 (CH$_2$), 30.11 (CH$_2$), 26.87 (CH$_2$), 25.84 (CH$_3$), 17.83 (CH$_3$), 16.09 (CH$_3$); HRMS-ESI m/z: [M + Na]$^+$ calcd for C$_{17}$H$_{22}$O$_2$Na 258.1620, found 258.1625.

4.1.7. General method for synthesis of compounds 7a-c [22]

A solution of R-BINOL (0.5 eq) in dry dichloromethane was cooled to -78 °C and then 1M SbCl$_5$ solution in dichloromethane (0.5 eq) was added and the mixture was stirred for 15 min at -78 °C. A precooled solution of the previously obtained alkene (1 eq) in dry dichloromethane was added at -78 °C and the reaction mixture was stirred at the same temperature for 5 h. The reaction mixture was quenched with saturated NaHCO$_3$ solution and the aqueous layer was extracted with diethyl ether. Combined organic layers were
washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by silica gel chromatography (1-2% ethyl acetate in petroleum ether) to afford the cyclized product.

4.1.7.1. Trans 13-methoxypodocarpa-8,11,13-triene (7b)
White solid, yield: 63%. ¹H NMR: δ 7.18 (1H, d, J = 8.7 Hz, ArH), 6.71 (1H, dd, J = 8.7, 2.8 Hz, ArH), 6.58 (1H, d, J = 2.7 Hz, ArH), 3.77 (3H, s, OCH₃), 2.95-2.82 (2H, m, CH₂), 2.28-2.26 (1H, m, CH₂), 1.90-1.86 (1H, m, CH₂), 1.74-1.67 (2H, m, CH₂), 1.62-1.58 (1H, m, CH₂), 1.50-1.48 (1H, m, CH₂), 1.41-1.37 (1H, m, CH₂), 1.35-1.32 (1H, m, CH), 1.26-1.23 (1H, m, CH₂), 1.18 (3H, s, CH₃), 0.96 (3H, s, CH₃), 0.94 (3H, s, CH₃); ¹³C NMR: δ 157.08 (ArC), 142.86 (ArC), 136.70 (ArC), 125.60 (ArCH), 113.31 (ArCH), 111.95 (ArCH), 55.24 (OCH₃), 50.72 (CH), 41.84 (CH₂), 39.16 (CH₂), 37.39 (C), 33.53 (C), 33.48 (CH₃), 30.83 (CH₂), 25.09 (CH₃), 21.73 (CH₃), 19.47 (CH₂), 19.17 (CH₂); HRMS-ESI m/z: [2M + Na]⁺ calcd for C₃8H₅₂O₂Na 539.3860, found 539.3863.

4.1.8. General method for synthesis of compounds 8a-c
The previously obtained no-keto diterpenoid 7a-c was added dropwise to a solution of CrO₃ (1 eq) in acetic acid at room temperature. After overnight stirring, the reaction mixture was neutralized with NaHCO₃ and the aqueous phase extracted with diethyl ether. The organic layer was washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (2-5% ethyl acetate in petroleum ether).

4.1.8.1. Trans 7-oxopodocarpa-8,11,13-triene (8a)
Colourless oil, yield: 20% (2 steps). ¹H NMR: δ 8.01 (1H, dd, J = 7.8, 1.6 Hz, ArH), 7.52 (1H, dd, J = 8.0, 1.6 Hz, ArH), 7.38 (1H, d, J = 7.9 Hz, ArH), 7.30-7.26 (1H, m, ArH), 2.76-2.62 (2H, m, CH₂), 2.36-2.34 (1H, m, CH₂), 1.90 (1H, dd, J = 13.8, 4.2 Hz, CH), 1.79 (1H, tt, J = 13.7, 3.3 Hz, CH₂), 1.72-1.68 (1H, m, CH₂), 1.58-1.55 (2H, m, CH₂), 1.30-1.25 (1H, m, CH₂),
1.25 (3H, s, CH₃), 1.01 (3H, s, CH₃), 0.95 (3H, s, CH₃); ¹³C NMR: δ 199.74 (C=O), 156.29 (ArC), 134.17 (ArCH), 131.00 (ArC), 127.46 (ArCH), 126.24 (ArCH), 123.82 (ArCH), 49.42 (CH), 41.49 (CH₂), 38.30 (C), 38.06 (CH₂), 36.36 (CH₂), 33.46 (C), 32.69 (CH₃), 23.58 (CH₃), 21.48 (CH₃), 19.03 (CH₂); HRMS(ESI) m/z: [M + Na]^+ calcd for C₁₇H₂₂ONa 265.1563, found 265.1565.

4.1.9. General method for synthesis of compounds 7d and 8d

To a solution of the previously obtained methoxy-diterpenoids (7b, 8b), a solution of 1M BBr₃ (6 eq) in dichloromethane at -78 °C was added dropwise under nitrogen. After stirring for 30 minutes, the reaction mixture was warmed to 0°C and stirred for 3 hours. The mixture was then neutralized with aqueous NaHCO₃ and the aqueous phase extracted with ethyl acetate. The organic layer was washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (10-15% ethyl acetate in petroleum ether) to afford the hydroxy-diterpenoids.

4.1.9.1. Trans podocarpa-8,11,13-trien-13-ol (7d)

White solid, yield: 59%. ¹H NMR: δ 7.12 (1H, d, J = 8.6 Hz, ArH), 6.61 (1H, dd, J = 8.5, 2.8 Hz, ArH), 6.50 (1H, d, J = 2.8 Hz, ArH), 4.56 (3H, br s, OH), 2.90-2.77 (2H, m, CH₂), 2.26-2.23 (1H, m, CH₂), 1.88-1.85 (1H, m, CH₂), 1.76-1.67 (2H, m, CH₂), 1.61-1.58 (1H, m, CH₂), 1.49-1.47 (1H, m, CH₂), 1.37 (1H, td, J = 13.2, 3.5 Hz, CH₂), 1.31 (1H, dd, J = 12.5, 2.3 Hz, CH), 1.22 (1H, td, J = 13.5, 4.0 Hz, CH₂), 1.16 (3H, s, CH₃), 0.95 (3H, s, CH₃), 0.93 (3H, s, CH₃); ¹³C NMR: δ 152.92 (ArC), 143.08 (ArC), 137.05 (ArC), 125.81 (ArCH), 115.00 (ArCH), 113.01 (ArCH), 50.71 (CH), 41.86 (CH₂), 39.19 (CH₂), 37.43 (C), 33.54 (C), 33.46 (CH₃), 30.60 (CH₂), 25.11 (CH₃), 21.74 (CH₃), 19.47 (CH₂), 19.12 (CH₂); HRMS-ESI m/z: [2M + Na]^+ calcd for C₃₄H₄₈O₂Na 511.3547, found 511.3547.

4.2. Biological evaluation
4.2.1. Compound handling and storage

In preparation for biological assays conducted, all compounds were solubilised in DMSO (Fisher Scientific, Loughborough, UK) and stored at -20°C at a stock concentration of 16mM.

4.2.2. *Schistosoma mansoni* schistosomula culture and compound screening

*S. mansoni* (Puerto Rican Strain, Naval Medical Research Institute - NMRI) cercariae were collected from infected *Biomphalaria glabrata* (NMRI) snails after exposure to 2 hours of light at 26 °C and then mechanically transformed into schistosomula as described [33]. Newly transformed schistosomula were prepared for 72 hour high throughput screening (HTS) in 384-well black-sided microtiter plates (Perkin Elmer, MA, USA) as described in Nur-e-Alam [24], with a final DMSO concentration of 0.625%. The effect of compounds on 72 hour cultured schistosomula was deduced by analysing the effect on both motility and phenotype of treated schistosomula using the image analysis model described by Paveley [25].

4.2.3. *Schistosoma mansoni* adult worms culture and compound screening

MF-1 mice (Harlan, UK) were infected by percutaneous exposure to 200 cercariae. Mature adult parasites were recovered from hepatic portal veins by perfusion as described by Smithers and Terry [34] seven weeks post infection. Three adult male and three adult female worms (i.e. three worm pairs) were cultured per well in a 48-well tissue culture plate (Fisher Scientific, Loughborough, UK) containing 1 ml of modified DMEM (Gibco, Paisley, UK) media (containing 10% v/v Hepes (Sigma-Aldrich, Gillingham, UK), 10% v/v Foetal Calf Serum (Gibco, Paisley, UK), 0.7 % v/v 200 mM L-Glutamine (Gibco, Paisley, UK), 1% v/v Antibiotic/antimycotic (Gibco, Paisley, UK). Worms were incubated for 1 hour at 37 °C in a humidified atmosphere containing 5% CO₂ before being dosed with test compounds obtaining final concentrations of 50, 25, 12.5 and 6.25 µM (0.3% DMSO final concentration). While all worms were scored manually after 24hr, 48 hr and 72hr using microscopic methods described in the literature [35], only motility metrics at 72hr are reported. At 72hr, eggs were
also collected and counted from each well. After enumeration, eggs were finally subjected to fluorescence microscopy as described below.

4.2.4. *Fasciola hepatica* newly excysted juvenile (NEJ) culture and screening

*F. hepatica* (Italian strain) metacercariae were obtained from Ridgeway Research, Gloucestershire, UK. During our experiments, two procedures were used to generate newly excysted juveniles (NEJs) from metacercariae. In the first procedure, NEJs were produced according to Dixon *et al.* [36] and Wilson *et al.* [37] with minor modifications. These modifications included overnight incubation of cysts in distilled water before treating them in 5ml solution containing 1% w/v pepsin (Sigma-Aldrich, St Louis, USA) and 0.4% v/v 1M HCl for 1 hr at 37°C. After pepsin treatment, the cysts were washed with distilled water before suspending them in 5 ml Na₂S₂O₄ solution (0.035g Na₂S₂O₄ (Fisher Scientific, UK) + 0.1g NaHCO₃ (Acros Organics, USA) + 0.08g of NaCl (Acros Organics, USA) and 1% v/v 1M HCl) for 2 hr at 37°C. Subsequently, the parasites were washed with distilled water and DMEM containing 1% v/v Antibiotic/antimycotic solution, respectively. Finally, the cysts were incubated in 5ml DMEM solution containing 0.02g of Sodium Tauroglycocholate (Fisher Scientific, UK) for 1 hr at 37°C to facilitate parasite excystment. The second method used to prepare NEJs from metacercariae involved initial rupturing of cyst outer walls with a dissecting needle instead of pepsin digestion. Following cyst rupturing, excystment of NEJs was performed essentially as described above.

Post-excystment, the NEJs were distributed into 48-well tissue culture plates at a density of 25 parasites per well in RPMI-1640 (Gibco, Paisley, UK) containing 1% v/v Foetal Calf Serum. Test compounds were initially screened at 25 µM and 12.5 µM (0.16% DMSO) before a dose response titration of the most active ones was performed (10 µM, 5 µM, 2.5 µM, 1.25 µM and 0.625 µM; all in 0.16% DMSO). Control wells included parasites incubated with 0.16% DMSO (negative) and 10 µM Triclabendazole (positive, Sigma-Aldrich, UK). All parasite/compound co-cultures were performed in a total volume of 1 ml. Parasites were scored for both motility and phenotype at 24, 48 and 72 hours as previously described [38],
but only results at 72hr are reported. Motility was scored from 1 to 5, with 1 signifying normal movement and 5 no movement; phenotype was scored from 1 to 6, with 1 representing a normal phenotype and 6 a dissolved parasite [15].

### 4.2.5. Fasciola hepatica adult and immature culture and screening

*F. hepatica* adult (8 weeks post-infection) and immature (4-5 weeks post-infection) parasites were collected from infected livers of cattle and springer lambs (Randall Parker Foods, Llanidloes, Wales) and washed three times in phosphate buffered saline (PBS). Over the next 24 hr, parasites were subsequently washed in RPMI 1640 containing 1% v/v Antibiotic solution and 10% v/v Foetal Bovine Serum (Gibco, Paisley, UK) (changing the wash media every hour for the first three hours and then every 6 hours). After washing, adult parasites were moved into 50 ml falcon tubes (2 parasites/tube) containing 6 ml of media (RPMI 1640, 2.5% v/v HEPES, 1% Antibiotic/antimycotic, 1% v/v Foetal Bovine Serum) whereas immature parasites (3 parasites/well) were moved into 6-well tissue culture plates (Thermo Scientific, Denmark) containing 3 ml of the same media. All parasites were placed at 37°C in a humidified environment containing 5% CO\(_2\). Test compounds were added to parasite cultures at 40, 13.3 and 4.4 µM final concentrations (in 0.25% DMSO). Control wells included parasites incubated with 0.25% DMSO (negative) and 10 µM Triclabendazole (positive, Sigma-Aldrich, UK). Both mature and immature parasites were then scored for motility at 24, 48 and 72 hours, but only results at 72 hours showed for adult worms, motility was scored from 1 to 6 where 1 equates to good movement (curled, sticking on wall, movement on petri plate or conical flask), 2 equates to moderate movement (less vigour but more than 10 sec pulses or peristaltic waves), 3 equates to resting (less than 10 sec pulses in head and body), 4 equates to lethargy (less than 2 second pulses in head and body), 5 equates to faint movement of suckers (movement of oral or ventral suckers only, whole body paralysed) and 6 equates to no movement at all (or dead). The movement of immature parasites was scored from 1 to 5 (1 good or normal, 2 moderate, 3 low, 4 very little and 5 none).
4.2.6. HepG2 and MDBK cell culture and MTT assay

Cells were grown to ~80% confluency in culture media (BME with phenol red for HepG2 cells or EMEM for MDBK cells, 10% v/v Foetal Bovine Serum, 1% v/v MEM non-essential amino acid solution, 1% v/v 200 mM L-Glutamine, 1% v/v antibiotic/antimycotic). Confluent cells were prepared for cytotoxicity assays in the same manner as stated by Nur-e-Alam [24]. Briefly, 2.5 x10^4 were seeded in a black walled 96-well microtiter plate (Fisher Scientific, Loughborough, UK) and incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO₂. Test compounds were then added at final concentrations of 100, 75, 50, 25, 10, 1, 0.1, 0.01 µM (1.25 final % DMSO) in parallel to negative (DMSO; 1.25%) and positive (1% v/v Triton X-100) [39] controls. Following a further incubation for 24 hours, the MTT assay was performed as previously described [24].

4.2.7. Scanning Electron Microscopy and Fluorescence Microscopy

Following 72 hr co-culture with compounds at sub-lethal concentrations, parasites were processed for SEM analysis alongside those parasites derived from positive (Auranofin) and negative (DMSO) control wells. To separate male and female schistosomes for fixing and further SEM processing, several steps from Collins et al. were adapted [40]. Briefly, schistosomes were collected following treatment and washed in 1mL of anaesthetic (1% ethyl 3-aminobenzoate methane (Sigma-Aldrich, Gillingham, UK) dissolved in DMEM) to relax and separate male and female worms. Gender separated schistosome worms were then further relaxed and killed in a solution of 1mL of 0.6mM MgCl₂ (Fisher Scientific, Loughborough, UK) and then briefly washed in PBS before being placed in SEM fixative (0.1M sodium cacodylate, 2.5% v/v glutaraldehyde (Agar Scientific, Stansted, UK) in ultra-pure water). Following fixation, adult schistosomes were stored at 4°C until ready for SEM analysis. The same fixation procedure was adopted for all F. hepatica immature and adult hermaphroditic worms.
In preparation for SEM analysis, all blood and liver fluke samples were then exposed to a number of wash, staining and dehydration steps before mounting for SEM. Initially, worms were washed twice in 0.1M sodium cacodylate for 30 minutes, and then stained in 1% osmium tetroxide solution (Agar Scientific, Stansted, UK) for 2 hours. Following staining, worms were then washed in 0.1M sodium cacodylate for 1 hour and then ultra-pure water for a further 30 minutes. Worms were then dehydrated using a 30, 50, 70, 95, 100% ethanol series (Fisher Scientific, Loughborough, UK) for 30 minutes each and then dried using hexamethyldisilazane critical drying point agent (Agar Scientific, Stansted, UK). Initially, worms were left in the drying agent for 3 hours. This was then removed and replaced with further drying agent, which was then allowed to evaporate off over night.

Once worm samples were fully dried, they were carefully mounted on self-adhesive conductive carbon tabs, on aluminium specimen stubs (both Agar Scientific, Stansted, UK) and then coated with gold using a Polaron E5000 SEM Coating Unit. Coated worms could then be stored in a desiccation jar until imaging. SEM imaging was conducted using a Hitachi S-4700 FESEM microscope (Ultra High Resolution, an accelerating voltage of 5.0kV with a working distance of 5.0mm). Images were captured at 2560x1920 resolution.

After collection of schistosome worms for SEM analyses, media left in the wells were collected in separated Eppendorf tubes, spun down (200 x g for 2 minutes) and then carefully removed leaving a thin pellet of eggs at the bottom. Eggs were then fixed with 500 µl of 10% formaldehyde in PBS and stored at 3 - 4 °C until use. Eggs were then transferred onto glass slides and visualised using a Leica LMD 6000 Laser Microdissection Microscope under natural light and GFP filter conditions.

### 4.2.8. Statistics

Multiple $t$-tests and Bonferroni post hoc test were used to determine any significant difference between each compound treatment and the DMSO control. Statistical tests and $IC_{50}$ calculations were performed using GraphPad Prism 7.02.
4.2.9. Ethics statement

All procedures performed on mice adhered to the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986 (project license PPL 40/3700) as well as the European Union Animals Directive 2010/63/EU and were approved by Aberystwyth University’s (AU) Animal Welfare and Ethical Review Body (AWERB).

Author contribution

Conceived and designed the experiments: AC, ADW, KFH. Performed the experiments: AC (compound synthesis), AC, CB (compound characterization), AC, KCLW, HW (*S. mansoni* screening), AC, ACh, KCLW (*F. hepatica* screening), AC (cell screening). Manuscript preparation: AC, ADW, KFH.

The authors declare no competing financial interest.

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Appendix A. Supplementary information
Supplementary information include HRMS, $^1$H-NMR and $^{13}$C-NMR chemical shifts and spectra of the compounds and bar graphs of *S. mansoni* and *F. hepatica* adult fluke screening.
References


Design, synthesis and anthelmintic activity of 7-keto-sempervirol analogues

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Highlights
- 30 analogues of the anthelmintic diterpenoid 7-keto-sempervirol were synthesised
- Analogues were screened against \textit{S. mansoni} and \textit{F. hepatica} juvenile and adult stages
- Compound 7d showed improved dual anthelmintic activity over 7-keto-sempervirol
- Compound 7d inhibited egg production and affected surface tegument integrity