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Research article

# Synthesis and evaluation of novel chromanone and quinolinone analogues of uniflorol as anti-leishmanial agents

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ABSTRACT

Within this work, we describe the design and synthesis of a range of novel chromanones and quinolinones, based on natural products reported to possess anti-leishmanial action. The core heterocycles were obtained either via classical or ionic liquid mediated Kabbe condensation in the case of chromanones, or aqueous Sonogashira based alkynylation followed by acid-catalysed cyclisation in the case of quinolinones. Upon testing in promastigotes, axenic amastigotes and *Leishmania*-infected macrophages, compound **13c** was identified as displaying interesting activity, inhibiting axenic amastigotes and intracellular amastigotes with $IC_{50}$s of 25.3 and 24.6μM respectively.

## 1. Introduction

Leishmaniasis is a group of diseases caused by protozoa of the genus *Leishmania*, transmitted through the bite of infected sandflies. Various clinical presentations exist, notably visceral, cutaneous, and mucosal forms, with visceral disease carrying the most serious consequences. Although treatments exist, they are often toxic, expensive, or both; thus the development of novel therapies remains an important strategy [1]. One approach is to investigate the composition and activity of natural medicines used by cultures in areas of disease endemicity. Diverse natural products have been shown to exhibit anti-leishmanial activity, with much interest focussed on small oxygenated heterocycles. Several examples of these metabolites have been isolated from species used by indigenous peoples in ethnomedicine (Figure 1). For example, the chromene encecalol angelate **1** was isolated from *Ageratum conyzoides*, a plant traditionally used for parasitic infections [2]. Upon isolation and testing, this compound showed weak activity, including an $IC_{50}$ of 14.6 μg/mL against axenic amastigotes of *L. donovani*. Within the genus *Calea*, several species have been investigated for anti-parasitic properties [3]. Among the bioactive constituents isolated from this genus are various oxygenated heterocycles including flavonoids, chromenes and chromanones. Of these, we were most interested in the chromanones known as uniflorols A and B (**2**–**3**), first isolated from the underground organs of *Calea uniflora*. An *E/Z* mixture of uniflorols inhibited *L. major* promastigote growth by 55–89% between 25-100 μg/mL, making them of interest as potential lead compounds [4]. Among other related structures, the biosynthetic precursors of oxygenated heterocycles, namely the chalcones, are well established as having diverse pharmacological potential, including as anti-leishmanial agents [5]. Both natural products, such as the licorice licochalcones, and their synthetic derivatives have been widely explored. Although biologically active, non-specificity of chalcones is often cited as a drawback of these compounds, which has been ascribed to their reactive α,β-unsaturated chalcone system [6]. Despite this, the pleiotropic effects of these compounds attract much research interest. In addition, the potentially problematic α,β-unsaturation may not actually be a pre-requisite for activity, as evidenced by retention of bioactivity in some dihydrochalcones. These include the chromene derivatives **4**–**6**, isolated from *Crotalaria ramosissima* [7]. Other natural products containing α,β-unsaturation also display interesting anti-leishmanial activity. One such example is the alkaloid piperine **7**, which displays concentration-dependent inhibition of *L. donovani* [8].

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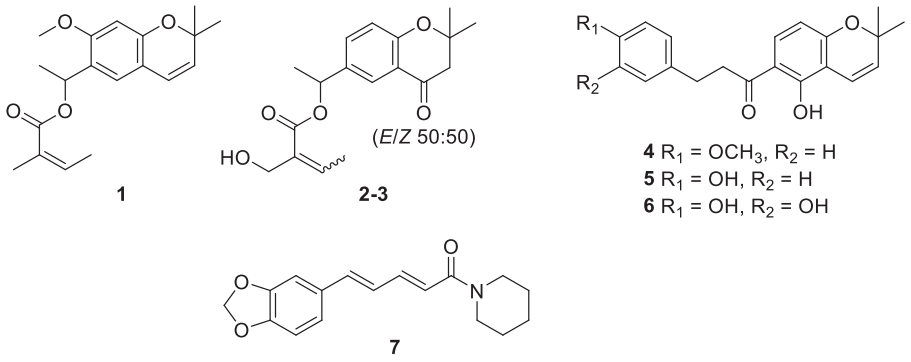



**Figure 1.** Natural products with activity against *Leishmania*.

Taking inspiration from natural product lead structures, several groups have developed analogues of such compounds (Figure 2). Drawing on the observed activity of the chromene glyasperin from *Smirnovia iranica*, Alizadeh et al prepared twelve chromenes, some of which, notably **8**, were moderately active against *L. major* [9]. Brenzan et al, in studying the structure activity relationships of coumarins ((-) mammea A/BB) isolated from *Calophyllum brasiliense*, prepared a series of analogues, of which the most active was chromanone **9**, inhibiting promastigote forms of *L. amazonensis* with an $IC_{50}$ of 0.9μM and a $CC_{50}$ of 85.4μM in macrophages [10]. Monzote et al prepared a number of synthetic chromanols, and some of these inhibited the growth of *Leishmania*. The observed activity of compounds **10a-b**, essentially vitamin E derivatives, was considered to be at least partly due to cyt bc(1) inhibition, in addition to antioxidative properties [11]. Notably however, in these latter two studies, activity was more prominent in 2-chromanones rather than in 4-chromanones. The importance of a chromene or chromanone system for anti-leishmanial activity in several natural products was underlined by the work of Sandjo et al, who tested a series of six natural products alone and in combination and found that the presence of these intact ring systems enhanced activity compared to their ring-opened analogues [12]. Chroman-4-one analogues of flavonols have also been evaluated against *L. major*, with **11** showing binding to the enzyme LmPTR1 and weak (31% at 50μM) inhibition of *L. infantum* [13]. Considering piperine as a lead compound, both the natural product and its analogues displayed toxicity to both *L. donovani* and *L. amazonensis* [14, 15]. This effect has been ascribed to alteration of parasite mitochondrial structure and function.

Interested in probing the structural requirements of **2-3** for anti-leishmanial activity, we designed three distinct target types **12**–**14**, as shown in Figure 3. Firstly, we envisaged **12** as more accessible, desmethyl analogues of uniflorols; **12a** representing the direct analogue of the natural product and **12b-c** constituting hybrid structures incorporating a lipophilic fragment. Knowing that encecalol angelate is unstable, and recognising similar potential within the uniflorols, we proposed to replace the labile ester in **12** with a more stable amine or amide functionality, with **13a** and **13b** the amide analogues of **12a** and **12c**, and **13c** featuring a linear alkyl fragment. Additionally, substitution of the heterocyclic oxygen for nitrogen was considered, to investigate the effect of additional H-bond donating capability and of altering the expected logP of the molecule, as depicted in **14** (and its analogues).

## 2. Results

### 2.1. Chemistry

Our synthetic approach towards compounds **12** is outlined in Scheme 1. Friedel-Crafts acylation of cresol **15** with acyl chloride in neat TfOH afforded the *o*-hydroxy acetophenone **16** [16]. Cyclisation to afford the chromanone core was accomplished using ionic liquid-promoted Kabbe condensation, which produced the desired product **17** in significantly higher yield than under standard conditions [17]. Oxidation of **17** with persulfate afforded **18**, lactarochromal, a known natural product [18]. Chemoselective bioreduction of **18** was achieved in excellent yield employing *D. carota* [19] with no evidence of diol formation, which occurred when we used other reportedly selective reduction protocols of ketoaldehydes, such as $Na_2CO_3/NaBH_4$ [20] or $SnCl_2/NaBH_4$ [21]. This reaction represents a route to the natural product 6-hydroxymethyl-2, 2-dimethylchroman-4-one **19**, previously reported from submerged cultures of a *Stereum* sp. [22].

Prior to esterification to afford **12a**, we were required to prepare a usable C5 synthon as the acid fragment. This is depicted in Scheme 2.

Baylis-Hillman reaction of methyl acrylate **22** with acetaldehyde, using 1,4-diazabicyclo-[2.2.2]octane (DABCO) as catalyst, provided the desired methyl 3-hydroxyl-2-methylenebutanoate **23** [23]. Reaction of **23** with *N*-bromosuccinimide/dimethylsulfide (NBS/$Me_2S$) [23] afforded **24**; alternatively, this transformation can be achieved using $LiBr/H_2SO_4$ in acetonitrile at room temperature [24], affording *Z*-**24** in similar yield in our hands. Reaction of **24** with sodium acetate in refluxing methanol [23] followed by $K_2CO_3$-mediated ester hydrolysis produced (2*E*)-2-(hydroxymethyl)-2-butenoate **25**. Methyl ester hydrolysis of **25** was expected to afford the free acid; however this reaction was unsatisfactory, affording only small quantities of the acid using either LiOH or $Ba(OH)_2$, and with dimerization as the primary reaction. In any case, attempted direct esterification of the free acid with alcohol **19** failed. We therefore protected the primary alcohol of **25** via tetrahydropyranylation catalysed by pyridinium *p*-toluenesulfonate [25].

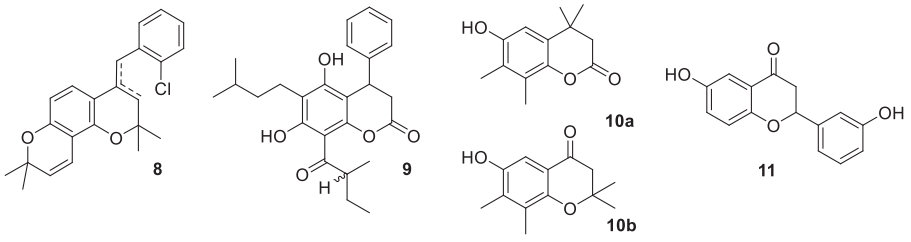



**Figure 2.** Synthetic derivatives of naturally occurring chroman, chromene, flavonoid and coumarin systems with activity against *Leishmania*.

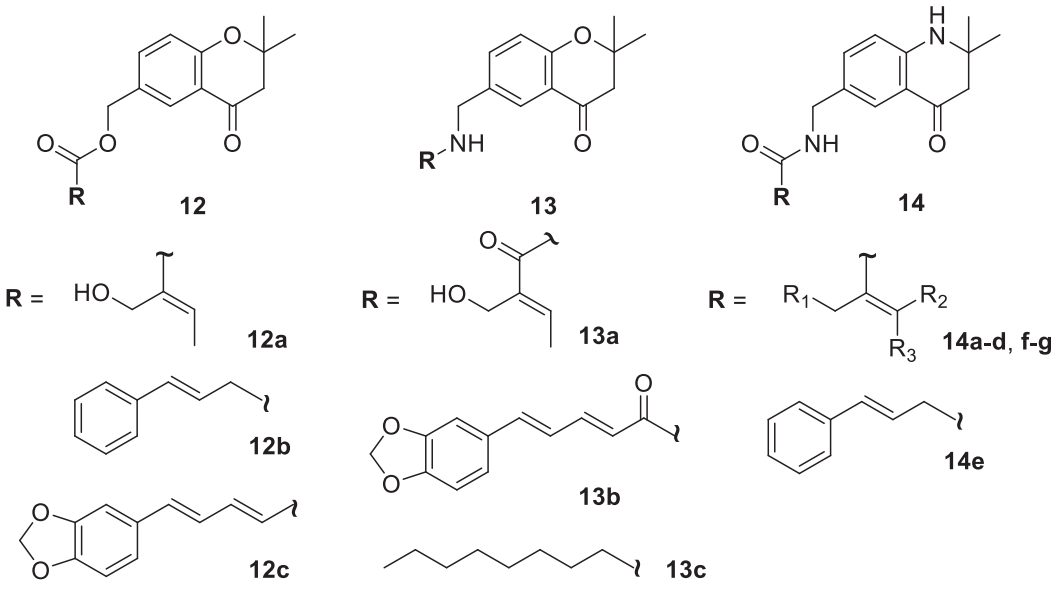
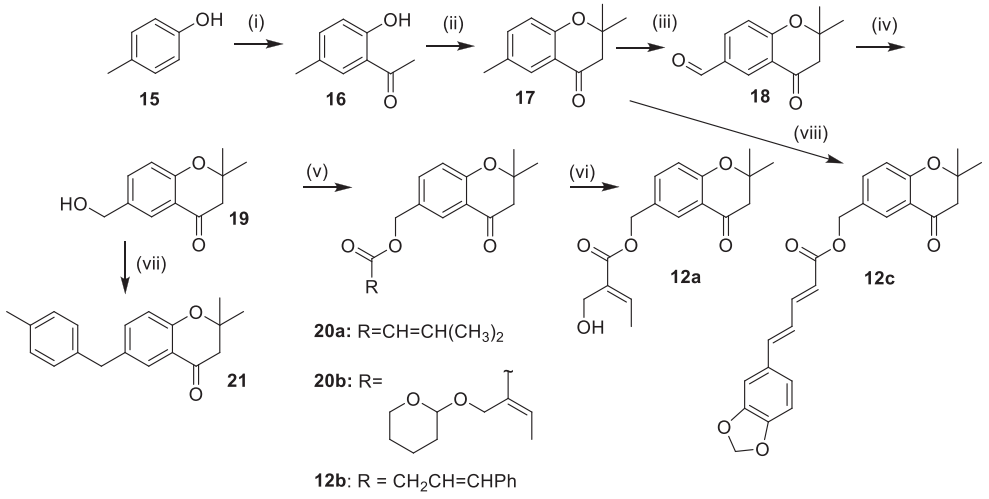



**Figure 3.** Synthetic targets based on lead compounds 2-3.



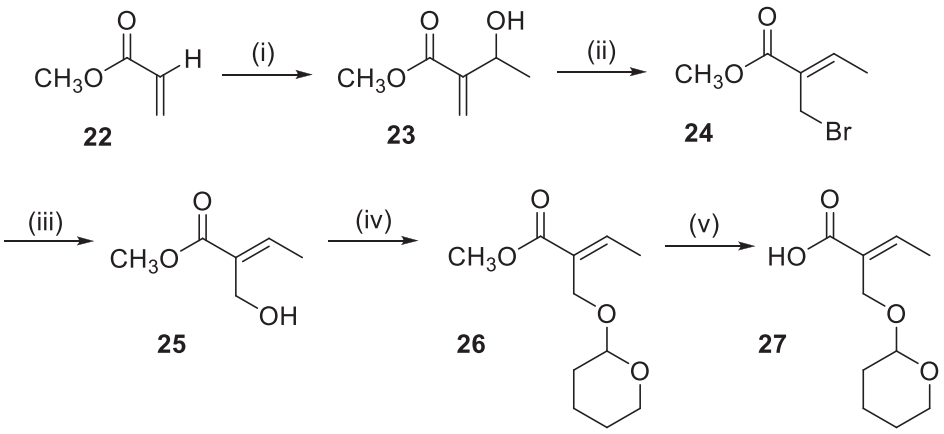

**Scheme 1.** Synthesis of compounds 12a-c. Reagents and conditions: (i) $CH_3COCl$, $CF_3SO_3H$, 0°C-RT, 24h; (ii) $CH_3COCH_3$, [bbim]Br, 95–100 °C, 8h; (iii) $K_2S_2O_8$, $CuSO_4$, $H_2O$/ACN (1:1), 75–80 °C, 1h; (iv) *D. carota*, $H_2O$, RT, 72h; (v) 27, appropriate acid, EDC, DMAP, RT; (vi) 20b, *p*-toluenesulfonic acid, MeOH, RT, 24h; (vii) acrylic acid, PFPAT, toluene, reflux; (viii) NBS, cyclohexane, Δ, 5h, then piperic acid, $K_2CO_3$, DMF, RT-80 °C, 8h.

Finally, LiOH-mediated hydrolysis of the protected **26** afforded **27** in good yield.

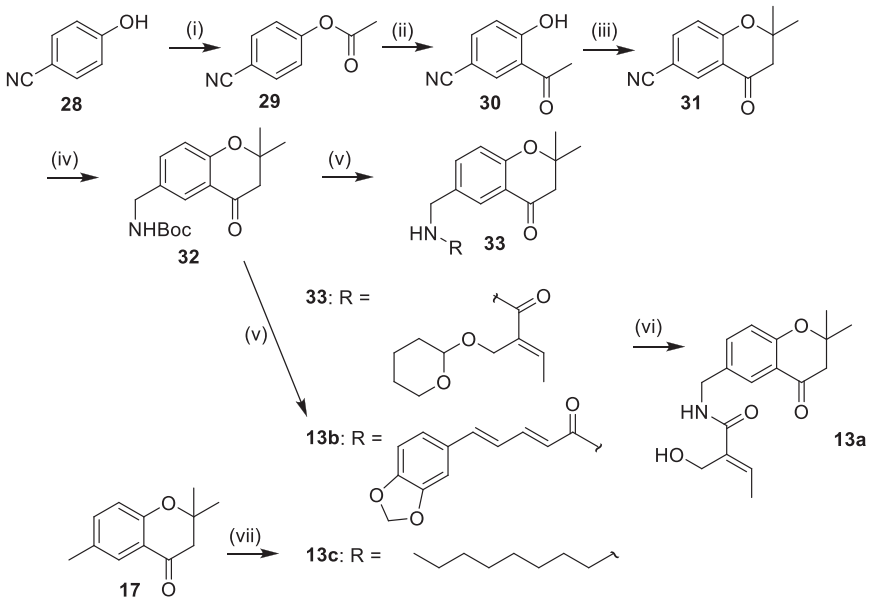
Prior to utilisation of valuable acid synthon **27**, we attempted to establish suitable conditions for reaction of the related commercial acrylic acid 3,3-dimethylacrylic acid with alcohol **19**. Using either $ZrOCl_2.8H_2O$ [26] or tosyl chloride/*N*-methylimidazole [27] resulted in no reaction. Interestingly, use of pentafluorophenylammonium triflate (PFPAT) in toluene [28] with various acrylic acids resulted in isolation of **21** in quantitative yield; this compound is novel to the literature. While the use of the inexpensive coupling reagent DCC under 4-dimethylaminopyridine (DMAP) catalysis resulted in approximately 10% of ester as estimated by NMR, it proved impossible to eliminate residual DCC contamination, despite careful chromatography. However, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) with DMAP catalysis under basic conditions afforded **20a** in acceptable yield. After analogous esterification of **19** with **27** to afford **20b**, deprotection of **20b** was accomplished using *p*-toluenesulfonic acid at room temperature, yielding **12a**. Compound **12b** was prepared analogously to **20a** via esterification of **19** with styrylacetic acid, while **12c** was prepared via bromination of **17** [29] and esterification with piperic acid.

Synthesis of compound **13a** is depicted in Scheme 3. Esterification of *p*-cyanophenol **28** followed by Fries rearrangement produced acetophenone **30**. Kabbe cyclisation under standard conditions afforded the known chromanone **31**. Reduction of the nitrile group was accomplished using nickel(II)/$NaBH_4$ [30] in the presence of di-*tert*-butyl dicarbonate; this reaction also reduced the benzylic ketone which was selectively re-oxidised using Jones reagent to afford the BOC-protected ketone **32**. Following carbamate cleavage with trifluoroacetic acid, EDC coupling was used to afford amide **33**. Removal of the tetrahydropyranyl group afforded **13a**. Compound **13b** was prepared analogously to **33** via amidation with piperic acid. **13c**, a secondary amine, was prepared by bromination of **17** followed by coupling with octylamine.

To produce quinolinone analogues (**14a-g**), an alternative strategy was adopted (Scheme 4). Iodination of cyanoaniline **34** afforded **35**,




**Scheme 2.** Synthesis of acid synthon 27. Reagents and conditions: (i) $CH_3CHO$, DABCO, RT, 7 days; (ii) NBS, $Me_2S$, DCM, RT, 17h; (iii) NaOAc, MeOH, Δ, 3.5h, then $K_2CO_3$, RT, 24h; (iv) 3,4-dihydropyran, DCM, pyridinium *p*-toluenesulfonate; (v) *p*-toluenesulfonic acid, MeOH, RT, 24h.




**Scheme 3.** Synthesis of compounds 13a-c. Reagents and conditions: (i) $CH_3COCl$, $Et_3N$, THF, 0°C-RT, 24h; (ii) $AlCl_3$, 160 °C, 3h; (iii) $CH_3COCH_3$, pyrrolidine, toluene, 90 °C, 3h; (iv) $NiCl_2.6H_2O$, $Boc_2O$, $NaBH_4$, MeOH, 15h, RT; then Jones reagent, $CH_3COCH_3$, 0°C-RT, 2h; (v) $CF_3CO_2H$, DCM, 0°C-RT, 3h, then appropriate acid, EDC, $Et_3N$, DCM, 0°C-RT, 3h; (vi) *p*-toluenesulfonic acid, MeOH, RT, 24h; (vii) NBS, cyclohexane, Δ, 5h, then octylamine, ACN, 100 °C, 4h.

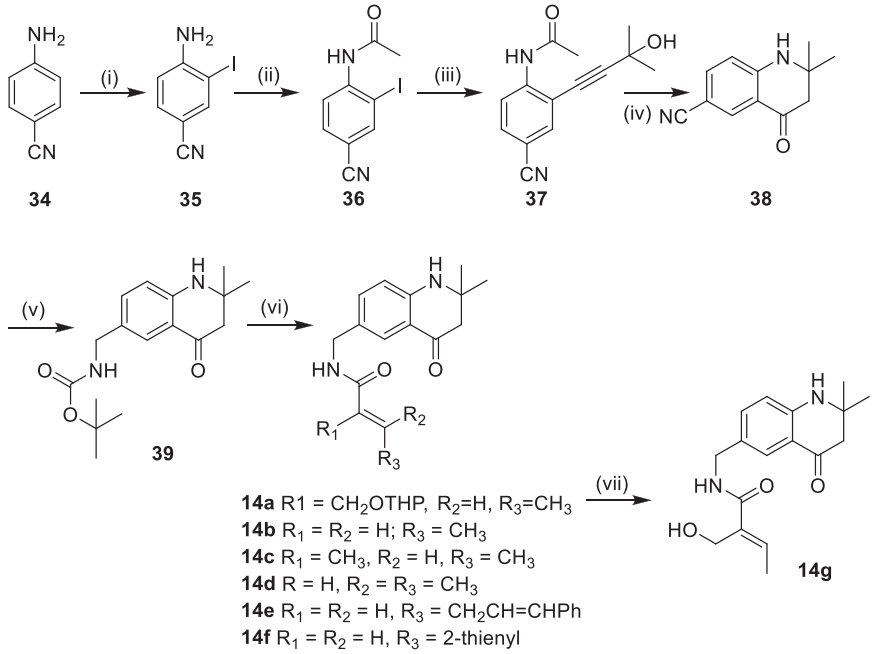
which was acetylated prior to Sonogashira coupling under aqueous conditions to afford alkyne **37** [31]. Acid-mediated cyclisation gave quinolinone **38**, whose nitrile group was reduced analogously to that of **31**, and similarly, any over-reduced alcohol could be selectively oxidised to **39** using Jones reagent. Following carbamate hydrolysis of **39**, amidation with various acids was facilitated by EDC to give **14a-f**. Compound **14g** was obtained via terminal deprotection of **14a**.

### 2.2. Pharmacological activity

Selected compounds were evaluated for their leishmanicidal ability against *Leishmania infantum* infected macrophages (Table 1) and also against promastigote or axenic stages of different *Leishmania* species (Table 2). In addition, compounds were tested for their cytotoxicity against bone marrow-derived macrophages (BMDM) (Table 1). Treatment was performed for 24h and results obtained through evaluation of cell viability upon treatment when compared to control.

## 3. Discussion

Our target compounds were designed based on reported activity of uniflorols A and B and related structures against *Leishmania*, with the intent of improving activity through functional group variation and hybridisation, while improving likely pharmacokinetic behaviour. Based on the results of screening, we identified compound **13c**, a secondary amine derivative, as displaying interesting activity. $IC_{50}$s recorded for all tested compounds against intracellular amastigotes exceeded 5μM, with **12**, **13**, **21** and **14g** having lower $IC_{50}$s than quinolinones **14b-f**. Introduction of the nitrogenated quinolinone heterocycle did not enhance activity, which may point towards a chroman or chromanone pharmacophore as optimal. None of the tested compounds showed gross toxicity towards uninfected macrophages, with **13c** displaying some cytotoxicity and a selectivity index of 1.6. Only compound **14e**, the styryl derivative, showed any indicative activity against *L. amazonensis* promastigotes. This result in itself is interesting however, as Harel et al synthesised chromane




**Scheme 4.** Synthesis of compounds 14a-g. Reagents and conditions: (i) ICl, $CH_3CO_3H$, RT, 30min; (ii) $Ac_2O$, $H_2SO_4$, 70 °C, 10min; (iii) $H_2O$, $PdCl_2$, 2-methylbut-3-yn-2-ol, pyrrolidine, 110 °C, 5h; (iv) conc. HCl/$H_2O$, 80 °C, 5h; (v) $NiCl_2.6H_2O$, $Boc_2O$, $NaBH_4$, MeOH, 15h, RT; then Jones reagent, $CH_3COCH_3$, 0°C-RT, 2h; (vi) $CF_3CO_2H$, DCM, 0°C-RT, 3h, then RCOOH, EDC, $Et_3N$, DCM, 0°C-RT, 3h; (vii) 14a, *p*-toluenesulfonic acid, MeOH, RT, 24h.

**Table 1.** Activity[a] of chromanones and quinolinones against *Leishmania*-infected macrophages.

| Compound | $IC_{50}$ (μM) | $CC_{50}$ (μM) |
|---|---|---|
| | *L. infantum* | BMDM |
| **12a** | 5–10 | ND[b] |
| **12b** | NT[c] | ND |
| **12c** | NT | ND |
| **13a** | 5–10 | ND |
| **13b** | NT | ND |
| **13c** | 24.6 ± 0.4 | 40.2 ± 5.7 |
| **21** | 5–10 | ND |
| **14b** | >10 | >40 |
| **14c** | >10 | >40 |
| **14d** | >10 | >40 |
| **14e** | >10 | >40 |
| **14f** | >10 | >40 |
| **14g** | 5–10 | ND |
| Amphotericin B | 0.22 ± 0.22 | 4.37 ± 0.97 |

[a](μM +/- SD); [b]ND = non-determinable; [c]NT = not tested.

and chromene derivatives of another bioactive natural product, encecalin, and highlighted several chromane derived amines and amides as novel lead compounds, finding greater activity among the amines, and in particular, a phenylbutylamine derivative showed potent anti-leishmanial activity [32]. Our chromanones share some similarity with these structures but, in addition to the differential oxidation state of the heterocycle, they also lack hydroxyl or methoxyl substitution at C7. Whether our compounds may be further optimised to improve activity is the subject of ongoing work. It may well be that increased hydrophobicity may be important, as evidenced by the impact of isoprenyl and phenyl substituents in molecules such as **9**, shown to display significantly greater activity than a synthetic coumarin lacking these functionalities [10]. To this end, variation of the α,β unsaturated side chain of the compounds **12** and **13** may yield derivatives of interest. We are particularly interested in pursuing compounds without the α,β unsaturated Michael acceptor functionality found within many of these natural product analogues, and in this regard are encouraged by the activity of **14e** and **13c**, which lack this functionality.

## 4. Conclusion

In this paper, we have demonstrated a novel synthetic route towards ester and amide derivatives of the natural chromanones uniflorol A and B, and also a parallel route towards quinolinone analogues. We also demonstrate a synthetic approach to the natural product 6-hydroxymethyl-2,2-dimethylchroman-4-one. These synthetic pathways allows access to a range of compounds, some of which show potential as anti-leishmanial targets.

## 5. Experimental

### 5.1. Chemistry

All required chemicals, solvents, and reagents were purchased from Sigma-Aldrich and were of reagent grade. Reaction progress was monitored on pre-coated thin layer chromatographic aluminum sheets (Silica Gel Merck 60 $F_{254}$), and TLC visualization was done using a UV lamp. Fourier transform infrared spectra were carried out with neat film coated samples on diamond using a Nicolet™ iS™ 10 FT-IR spectrophotometer (Thermo Fisher). Significant absorption peak (νmax) values are given in $cm^{-1}$. $^1H$ and $^{13}C$ NMR spectra were recorded on Bruker Avance 400 spectrometer at 400MHz and 100MHz, respectively, in $CDCl_3$ and $CD_3OD$ using tetramethylsilane (TMS) as the internal standard. Chemical shift values are given on the δ (ppm) scale, with signals are described as follows: s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), br. (broad signal), m (multiplet), with coupling constants (*J*) expressed in Hz. Mass spectral analyses were recorded using a Waters LCT Premiere XE (ESI-TOF MS) instrument. All calculated exact mono isotopic mass distributions were calibrated against internal reference standards.

**Table 2.** Activity of chromanones and quinolinones against *Leishmania* promastigotes (P) or axenic amastigotes (AA).

| Compound | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|
| | *L. amazonensis* (P) | *L. major* (P) | *L. infantum* (P) | *L. infantum* (AA) |
| **12a** | >20 | >20 | >20 | ND |
| **13a** | >20 | >20 | >20 | ND |
| **13c** | NT[b] | NT | NT | 25.3 +/-2.1 |
| **21** | >20 | >20 | >20 | ND |
| **14b** | >20 | >20 | >20 | ND |
| **14c** | >20 | >20 | >20 | ND |
| **14d** | >20 | >20 | >20 | ND |
| **14e** | 10–20 | >20 | ND[a] | ND |
| **14f** | >20 | >20 | >20 | ND |
| **14g** | >20 | >20 | >20 | ND |
| Amphotericin B | 0.2 ± 0.06 | 0.17 ± 0.08 | 0.18 ± 0.09 | 0.4 ± 0.3 |

[a]ND = not determined; [b]NT = not tested.

**1-(2-hydroxy-5-methylphenyl)ethan-1-one** (**16**) The compound was prepared and characterized as previously described in Ref. [16]. Yield: 50%.

**2,2,6-trimethylchroman-4-one** (**17**) A mixture of **16** (3.68g; 24.5mmol), acetone (3.6mL, 49.0mmol), and morpholine (1.1mL, 12.6mmol) was added to 5g of the ionic liquid[bbim]Br [33], and stirred at 95–100 °C for 8 h. The reaction mixture was then extracted with EtOAc (3 × 10mL). The combined EtOAc extracts were concentrated *in vacuo* and the resulting product was purified by flash column chromatography to yield **17**, in accordance with literature data for the compound [18] (2.21g, 47%).

**2,2-dimethyl-4-oxochromane-6-carbaldehyde** (**18**) The compound was prepared and characterized as previously described in Ref. [18]. Yield: 47%.

**6-(hydroxymethyl)-2,2-dimethylchroman-4-one** (**19**) To **18** (100mg, 0.49mmol) in distilled water (50mL) was added freshly cut slices of *D. carota* (10g). The resulting mixture was stirred vigorously at room temperature for 72h. The reaction was filtered, and the filtrate washed with ethyl acetate (50mL). The water/ethyl acetate mixture was separated, and the ethyl acetate extract dried over $Na_2SO_4$. The crude orange oil was purified by flash column chromatography to afford the benzylic alcohol **19**, in accordance with literature data for the compound [22] (76mg, 75%).

**(2,2-dimethyl-4-oxochroman-6-yl)methyl 3-methylbut-2-enoate** (**20a**) To a solution of 3,3-dimethylacrylic acid (100mg, 1.0mmol) in dichloromethane (10mL) was added EDC HCl (327mg, 1.71mmol) and DMAP (5mg). To this solution was added **19** (206mg, 1.0mmol). The reaction was stirred for 1 week at room temperature. The residual solvent was removed *in vacuo*, and the crude residue purified by flash column chromatography to afford ester **20a** as a colourless oil (125mg, 43%). $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.38 (6H, s, ($CH_3$)$_3$), 1.82 (3H, s, ($CH_3$), 2.11 (3H, s, $CH_3$), 2.64 (2H, s, $CH_2CO$), 4.99 (2H, s, $ArCH_2O$), 5.64 (1H, m, CH = C), 6.85 (1H, d, $J$ = 8.3Hz, H8), 7.41 (1H, dd, $J$ = 8.5, 2.3Hz, H7), 7.79 (1H, d, $J$ = 2.3Hz, H5). $^{13}$CNMR $\delta_C$ 20.3, 26.6 (2C), 27.5, 48.8, 64.6, 79.4, 115.7, 118.7, 119.9, 126.4, 128.9, 136.3, 157.6, 159.8, 166.3, 192.3.

**(2,2-dimethyl-4-oxochroman-6-yl)methyl (*E*)-2-(((tetrahydro-2H-pyran-2-yl)oxy)methyl)but-2-enoate** (**20b**) To a solution of **27** (60mg, 0.3mmol) in dichloromethane (10mL) was added EDC HCl (100mg, 0.52mmol) and DMAP (2mg). To this solution was added **19** (60mg, 0.29mmol). The reaction was stirred for 2 weeks at room temperature. The residual solvent was removed *in vacuo*, and the crude residue purified by flash column chromatography to afford ester **20** as a colourless oil (37mg, 33%). IR $\nu_{max}$ (neat) 1232, 1490, 1618, 1692, 1712, 2938cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.46 (6H, s, ($CH_3$)$_3$), 1.48–1.83 (6H, m, ($CH_2$)$_3$), 1.94 (3H, d, $J$ = 7.2Hz, $CH_3C$ = C), 2.72 (2H, s, $CH_2CO$), 3.50 (1H, m, H of $OCH_2$ pyran), 3.88 (1H, m, H of $OCH_2$ pyran), 4.23 (1H, d, $J$ = 11Hz, 1H of $OCH_2$ = C), 4.51 (1H, d, $J$ = 11Hz, 1H of $OCH_2$ = C), 4.66 (1H, m, OCHO), 5.14 (2H, br. s, $OCH_2Ar$), 6.92 (1H, d, $J$ = 8.4Hz, H8), 7.13 (1H, q, $J$ = 7.2Hz, CH = C), 7.51 (1H, dd, $J$ = 8.5, 2.4Hz, H7), 7.86 (1H, d, $J$ = 2.3Hz, H5). $^{13}$CNMR $\delta_C$ 14.6, 19.4, 25.4, 26.6 (2C), 30.5, 48.8, 60.5, 62.1, 65.6, 79.4, 98.4, 118.7, 119.9, 126.4, 128.7, 129.8, 136.4, 143.6, 159.8, 166.8, 192.3. HRMS $(M+Na)^+$ 411.1786, $C_{22}H_{28}O_6Na$ requires 411.1784.

**2,2-dimethyl-6-(4-methylbenzyl)chroman-4-one** (**21**) To a solution of **27** (49mg, 0.24mmol) in toluene (10mL) was added **18** and a few crystals of PFPAT. The reaction was heated to reflux for 5h, cooled and allowed to stir at room temperature overnight. Excess solvent was removed *in vacuo*, and the crude residue purified by flash column chromatography to afford a white crystalline solid (34mg). IR $\nu_{max}$ (neat) 1372, 1484, 1610, 1684, 2924cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.48 (6H, s, ($CH_3$)$_2$), 2.28 and 2.34 (3H, 2 x s, $ArCH_3$), 2.73 (2H, s, $CH_2CO$), 3.91 and 3.96 (2H, 2 x s, $ArCH_2Ar$), 6.87 (1H, d, $J$ = 8.3Hz, ArH), 7.09–7.21 (4H, m, 4 x ArH), 7.25 and 7.31 (1H, 2 x dd, $J$ = 8.4, 1.9Hz, ArH), 7.70 and 7.75 (1H, 2 x d, $J$ = 1.5Hz, ArH). $^{13}$CNMR $\delta_C$ 19.8 & 21.1, 26.7 (2C), 38.5 & 40.6, 48.9, 79.1, 118.4 & 118.5, 119.9, 126.1 × 2, 126.2 & 126.7, 128.7, 129.3, 129.9, 130.4, 132.8 & 133.9, 135.7 & 136.5, 136.7 & 136.9, 137.8 & 138.6, 158.4 & 158.5, 192.7 × 2. HRMS $(M+H)^+$ 281.1547, $C_{19}H_{21}O_2$ requires 281.1542.

**(2,2-dimethyl-4-oxochroman-6-yl)methyl (*E*)-2-(hydroxymethyl)but-2-enoate** (**12a**) *p*-Toluenesulfonic acid monohydrate (5mg, 0.026mmol) was added to a solution of **20b** (10mg, 0.026mmol, and MeOH (5mL) at room temperature. This solution was maintained for 24 h at room temperature, quenched with saturated aqueous $NaHCO_3$ (10mL), and then concentrated. The resulting mixture was extracted with EtOAc (2 × 10mL). The combined organic extracts were washed with brine (10mL), dried over $Na_2SO_4$, and concentrated. The residue was purified by flash column chromatography to give 6.1mg of the corresponding alcohol (78%). IR $\nu_{max}$ (neat) 1129, 1193, 1262, 1490, 1690, 2852, 2922, 3454cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.47 (6H, s, C($CH_3$)$_2$), 1.9 (3H, d, $J$ = 7.3Hz, $CH_3CH$ = C), 2.73 (2H, s, $CH_2C$ = O), 4.37 (2H, br. s, $HOCH_2$), 5.15 (2H, s, $OCH_2Ar$), 6.94 (1H, d, $J$ = 8.4Hz, H8), 7.02 (1H, q, $J$ = 7.2Hz, C=CH), 7.50 (1H, dd, $J$ = 8.5, 2.4Hz, H7), 7.87 (1H, d, $J$ = 2.4Hz, H5). $^{13}$CNMR $\delta_C$ 14.3 ($CH_3C$ = C), 26.6 (($CH_3$)$_2$), 48.8 ($CH_2C$ = O), 57.0 ($CH_2OH$), 65.8 ($COOCH_2$), 79.5 (C2), 118.8 (ArCH), 120.0 (ArC), 126.7 (ArCH), 128.3 (ArC), 131.6 (ArC), 136.4 (ArCH), 141.3 (ArCH), 160.0 (ArC), 167.2 (OC = O), 192.3 (C4).

HRMS $(M+Na)^+$ 327.1193, $C_{17}H_{20}O_5Na$ requires 327.1208.

**(2,2-dimethyl-4-oxochroman-6-yl)methyl (*E*)-4-phenylbut-3-enoate** (**12b**)

Prepared as for **20a** using *trans*-Styrylacetic acid. Colourless oil (32mg, 30%). IR $\nu_{max}$ (neat) 1132, 1154, 1258, 1437, 1490, 1619, 1690, 1734, 2926cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.46 (6H, s, C($CH_3$)$_2$), 2.72 (2H, s, $CH_2C$ = O), 3.28 (2H, dd, $J$ = 7.1, 1.2Hz, $OCOCH_2$), 5.09

(2H, s, OC**H**$_2$Ar), 6.30 (1H, dt, $J$ = 7.1Hz, CH$_2$C**H** = CH), 6.48 (1H, br. d, CH = C**H**Ar), 6.93 (1H, d, $J$ = 8.6Hz, **H**8), 7.22–7.37 (5H, m, 5 x Ar**H**), 7.48 (1H, dd, $J$ = 8.4, 2.3Hz, **H**7), 7.87 (1H, d, $J$ = 2.3Hz, **H**5). $^{13}$CNMR $\delta_C$ 25.6 ((**C**H$_3$)$_2$), 37.3 **C**H$_2$CH = CH), 47.7 (**C**H$_2$C = O), 64.8 (COOC**H**$_2$), 78.5 (**C**2), 117.8 (CH = **C**H), 118.9 (Ar**C**), 120.4 (CH = **C**H), 125.3 (2 x CH = **C**H), 125.7 (CH = **C**H), 126.6 (CH = **C**H), 127.2 (Ar**C**), 127.5 (2 x CH = **C**H), 132.6 (CH = **C**H), 135.4 (CH = **C**H), 135.7 (Ar**C**), 158.9 (Ar**C**), 170.4 (O**C** = O), 191.2 (**C**4). HRMS $(M+NH_4)^+$ 368.1975, $C_{22}H_{26}NO_4$ requires 368.1856.

**(2,2-dimethyl-4-oxochroman-6-yl)methyl (2*E*,4*E*)-5-(benzo[d][1,3]dioxol-5-yl)penta-2,4-dienoate** (**12c**) Piperic acid (100mg, 0.46mmol) was dissolved in DMF (3mL) and $K_2CO_3$ (70mg, 0.51mmol) was added. The mixture was stirred at room temperature for 30 min. The bromide of **17** [25] (140mg, 0.52mmol) was added and the mixture was stirred at 80 °C for 8h, protected from light. Upon completion, the reaction was quenched with water (25mL) and extracted with dichloromethane (3 × 10mL) and dried ($Na_2SO_4$). The solvent was removed *in vacuo*, and the residue purified by flash chromatography to give **12c** as a golden oil (111mg, 60%). IR $\nu_{max}$ (neat) 1130, 1229, 1256, 1489, 1501, 1607, 1618, 1693, 1713, 2977cm$^{-1}$. $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.47 (6H, s, (C**H**$_3$)$_2$, 2.73 (2H, s, C**H**$_2$CO), 5.15 (2H, s, ArC**H**$_2$), 5.99 (OC**H**$_2$O), 5.97 (1H, d, *signal overlap*, C=C**H**), 5.99 (2H, s, OC**H**$_2$O), 6.68 & 6.72 (1H, 2 x d, $J$ = 10.9Hz, C**H** = CH), 6.81 (2H, m, 2 x C**H** = C), 6.93 (2H, m, 2 x C**H** = C), 6.99 (1H, s, C**H** = C), 7.44 (1H, 2 x d, $J$ = 10.9 Hz, C**H** = C), 7.52 (1H, dd, $J$ = 8.4, 2. Hz, **H**7), 7.89 (1H, br. s, $J$ = 2Hz, **H**5); $^{13}$CNMR $\delta_C$ 26.6 ((**C**H$_3$)$_2$), 48.8 (**C**H$_2$), 65.4 (**C**H$_2$), 79.5 (**C**(CH$_3$)$_2$), 101.4 (O**C**H$_2$O), 105.9 (C**H** = C), 108.6 (C**H** = C), 118.7 (C**H** = C), 119.8 (C**H** = C), 119.9 (Ar**C**), 123.1 (C**H** = C), 124.4 (C**H** = C), 126.6 (C**H** = C), 128.7 (Ar**C**), 130.5 (Ar**C**), 136.4 (C**H** = C), 140.6 (C**H** = C), 145.5 (C**H** = C), 148.3 (Ar**C**), 148.6 (Ar**C**), 159.9 (Ar**C**), 166.9 (O**C** = O), 192.3 (**C**=O).

**methyl 3-hydroxy-2-methylenebutanoate** (**23**) The compound was prepared and characterized as previously described in Ref. [23]. Yield: 45%.

**methyl (*Z*)-2-(bromomethyl)but-2-enoate** (**24**) The compound was prepared and characterized as previously described in Ref. [23]. Yield: 79%.

**methyl (*E*)-2-(hydroxymethyl)but-2-enoate** (**25**) The compound was prepared and characterized as previously described in Ref. [23]. Yield: 77%.

**methyl (*E*)-2-(((tetrahydro-2H-pyran-2-yl)oxy)methyl)but-2-enoate** (**26**) To a solution of **25** (0.24g, 1.85mmol) in DCM (10mL) was added 3,4-dihydropyran (0.47g, 5.60mmol) and a few crystals of pyridine *p*-toluenesulfonate. The reaction was allowed to stir at room temperature for 72 h, after which time the reaction was partitioned between saturated sodium bicarbonate/ethyl acetate, the organic solvent evaporated, and the crude residue purified by flash chromatography to yield the product as a pale oil (0.27g, 68%): IR $\nu_{max}$ (neat) 905, 974, 1022, 1117, 1235, 1436, 1716, 2946cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.42–1.78 (6H, m, 3 x C**H**$_2$), 1.87 (d, $J$ = 7.0Hz, C**H**$_3$CH = C), 3.44–3.50 (1H, m, H of OC**H**$_2$CH$_2$), 3.69 (3H, s, OC**H**$_3$), 3.82–3.88 (1H, m, H of OC**H**$_2$CH$_2$), 4.18 (1H, d, $J$ = 11Hz, H of C=CC**H**$_2$O), 4.43 (1H, d, $J$ = 11Hz, H of C=CC**H**$_2$O), 4.60 (1H, dd, $J$ = 3.9, 3.1Hz, OC**H**O), 7.04 (1H, q, $J$ = 7.2Hz, C=C**H**).

**(*E*)-2-(((tetrahydro-2H-pyran-2-yl)oxy)methyl)but-2-enoic acid** (**27**) To a solution of **26** (1.163g, 5.43mmol) in THF/$H_2O$ (2:1, 15mL) was added lithium hydroxide (0.13g, 5.43mmol). The reaction was stirred at room temperature overnight, diluted with 1M HCl and extracted twice with ethyl acetate (2 × 50mL). The organic layers were combined, dried over $Na_2SO_4$ and concentrated, to afford the acid as a colourless oil (0.83g, 76%). IR $\nu_{max}$ (neat) 1021, 1438, 1689, 2941cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.40–1.81 (6H, m, 3 x C**H**$_2$), 1.89 (d, $J$ = 7.3Hz, C**H**$_3$CH = C), 3.44–3.50 (1H, m, H of OC**H**$_2$CH$_2$), 3.81–3.87 (1H, m, H of OC**H**$_2$CH$_2$), 4.17 (1H, d, $J$ = 11Hz, H of C=CC**H**$_2$O), 4.42 (1H, d, $J$ = 11Hz, H of C=CC**H**$_2$O), 4.62 (1H, dd, $J$ = 3.5Hz, OC**H**O), 7.15 (1H, q, $J$ = 7.3Hz, C=C**H**). HRMS $(M+H)^+$ 199.0962, $C_{10}H_{17}O_4$ requires 199.0970.

**4-cyanophenyl acetate** (**29**) The compound was prepared and characterized as previously described in Ref. [34]. Yield: 100%.

**3-acetyl-4-hydroxybenzonitrile** (**30**) The compound was prepared and characterized as previously described in Ref. [34]. Yield: 55%.

**2,2-dimethyl-4-oxochromane-6-carbonitrile** (**31**) To a solution of **30** (2.74g, 17.0mmol) in toluene (30mL) was added pyrrolidine (0.5mL, 5.99mmol) and acetone (1.7mL, 23.2mmol). The resulting solution was stirred at 90 °C for 3 h in a Dean Stark apparatus and then cooled. The cold mixture was evaporated *in vacuo*, and the residue purified by flash column chromatography to afford **31**, as an off-white solid, in accordance with literature data for the compound [35] (1.95g, 57%).

***tert*-butyl ((2,2-dimethyl-4-oxochroman-6-yl)methyl)carbamate** (**32**) To a stirred solution of **31** (1.58g, 7.86mmol) in dry methanol (30mL) at 0 °C and under $N_2$, were added $Boc_2O$ (3.43g, 15.7mmol) and $NiCl_2{\cdot}6H_2O$ (190mg, 0.80mmol). $NaBH_4$ (2.08g, 55.0mmol) was then added in small portions over 20min. The reaction was exothermic and effervescent. The resulting reaction mixture containing a finely divided black precipitate was allowed to warm to room temperature and left to stir for 15hr, at which point diethylenetriamine (0.75mL, 6.94mmol) was added. The mixture was allowed to stir for 30 min before solvent evaporation. The purple residue was dissolved in EtOAc (50mL) and extracted with saturated $NaHCO_3$ (2 × 50mL). The organic layer was dried ($Na_2SO_4$) and the solvent removed *in vacuo* to yield a mixture of products, with upon purification by flash column chromatography afforded as the predominant product the carbamate alcohol: $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.47 (3H, s, C(C**H**$_3$), 1.60 (3H, s, C(C**H**$_3$), 1.62 (9H, s, C(C**H**$_3$)$_3$), 2.02 (1H, dd, $J$ = 13.3, 8.8Hz, 1H of **H**3), 2.33 (1H, dd, $J$ = 13.3, 6.1Hz, 1H of **H**3), 4.40 (2H, d, $J$ = 6.0Hz, NC**H**$_2$), 4.94 (1H, br. s, N**H**), 4.99 (1H, m, H**4**), 6.91 (1H, d, $J$ = 8.5Hz, **H**8), 7.26 (1H, br. d, **H**7), 7.53 (1H, d, $J$ = 2.0Hz, **H**5). $^{13}$CNMR $\delta_C$ 25.9, 28.4, 28.9, 42.7, 63.6, 75.4, 117.5, 124.3, 128.8, 128.7, 130.6, 152.5, 155.9). To a stirred solution of this alcohol (0.5g, mmol) in acetone (30mL) at 0 °C, was added the Jones reagent (2mL). The resulting reaction was allowed to warm to room temperature and left to stir for 2hr. On appearance of the green $Cr_2(SO_4)_3$, anhydrous sodium sulphate (0.30g, 2.12mmol) was added. After 2 h, the solvent was evaporated, and the reaction separated using ether/water. After three washings with ether (3 × 20mL), the combined organic extracts were filtered and the solvent removed *in vacuo*. The residue was purified by flash column chromatography on silica gel to yield the amide as a clear oil, which solidified on standing (0.41g, 83%). IR $\nu_{max}$ (neat) 1165, 1250, 1270, 1492, 1525, 1685, 2975, 3392cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.38 (6H, s, C(C**H**$_3$)$_2$), 1.39 (9H, s, C(C**H**$_3$)$_3$), 2.64 (2H, s, C**H**$_2$), 4.20 (3H, d, $J$ = 6.0Hz, NHC**H**$_2$), 4.80 (1H, br. s, N**H**), 6.83 (1H, d, $J$ = 8.5Hz, **H**8), 7.35 (1H, br. d, $J$ = 8.5, **H**7), 7.67 (1H, d, **H**5). $^{13}$CNMR $\delta_C$ 26.6 ((**C**H$_3$)$_2$), 28.4 C(C**H**$_3$)$_3$), 43.8 (**C**H$_2$), 48.8 (**C**H$_2$), 79.3 (**C2**), 118.8 (Ar**C**H), 119.9 (Ar**C**), 124.9 (Ar**C**H), 131.4 (Ar**C**), 135.7 (Ar**C**H), 155.8 (Ar**C**), 159.3 (NH**C** = O), 192.5 (**C**4).

**(*E*)-*N*-((2,2-dimethyl-4-oxochroman-6-yl)methyl)-2-(((tetrahydro-2H-pyran-2-yl)oxy)methyl)but-2-enamide** (**33**) To a solution of **32** (542mg, 1.78mmol) in DCM (5ml) at 0 °C was added trifluoroacetic acid (5mL). The reaction was stirred overnight, allowing the reaction to reach room temperature. When TLC analysis showed completion of the reaction, the solvent was removed *in vacuo*, removing any remaining TFA azeotropically. After washing with base, extracting with ether and removing the solvent gave the free amine, a dark oil, which was reacted without further purification: 0.05g, 0.24mmol of this amine was added to a solution of **27** (50mg, 0.25mmol) in dichloromethane (10mL) at 0 °C containing EDC HCl (0.07g, 0.37mmol) and triethylamine (0.046mL, 0.33mmol). The reaction was allowed to reach room temperature and stirred for 24 h under a $N_2$ atmosphere. The residual solvent was removed *in vacuo*, and the crude residue purified by flash column chromatography to afford amide **33** as a colourless oil (45mg, 38%). IR $\nu_{max}$ (neat) 1189, 1257, 1300, 1487, 1532, 1616, 1667, 2975, 3338cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.33–1.49 (4H, m, C**H**$_2$C**H**$_2$ pyran), 1.38 (6H, s, C(C**H**$_3$)$_2$), 1.57–1.69 (2H, m, C**H**$_2$ pyran), 1.80 (3H, d, $J$ = 7.3Hz, C**H**$_3$CH = C), 2.63 (2H, s, C**H**$_2$C = O), 3.40 (1H, m, H of OC**H**$_2$ pyran) 3.68 (1H, m, H of

OC$\underline{H}_2$ pyran), 4.33 (2H, q, $J$ = 10.5Hz, OC$\underline{H}_2$C = C), 4.39 (2H, dd, $J$ = 5.8, 1.8Hz, NHC$\underline{H}_2$), 4.52 (1H, dd, $J$ = 5.1, 2.6Hz, OC$\underline{H}$O), 6.82 (1H, d, $J$ = 8.5Hz, $\underline{H}$8), 6.95 (1H, q, $J$ = 7.3Hz, C=C$\underline{H}$), 7.21 (1H, br., N$\underline{H}$), 7.38 (1H, dd, $J$ = 8.5, 2.5Hz, $\underline{H}$7), 7.69 (1H, d, $J$ = 2.3Hz, $\underline{H}$5).

**(*E*)-*N*-((2,2-dimethyl-4-oxochroman-6-yl)methyl)-2-(hydroxymethyl)but-2-enamide** (**13a**) *p*-Toluenesulfonic acid monohydrate (5mg, 0.026mmol) was added to a solution of **33** (10mg, 0.026mmol, and MeOH (5mL) at room temperature. This solution was maintained for 7h at room temperature, quenched with saturated aqueous $NaHCO_3$ (10 mL), and then concentrated. The resulting mixture was extracted with EtOAc (2 × 10mL). The combined organic extracts were washed with brine (10mL), dried over $Na_2SO_4$, and concentrated. The residue was purified by flash column chromatography to give the alcohol (5.2mg, 66%). IR $\nu_{max}$ (neat) 1257, 1299, 1488, 1531, 1616, 1667, 1686, 2925, 3334cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.38 (6H, s, C(C$\underline{H}_3$)$_2$), 1.77 (3H, d, $J$ = 7.0Hz, C$\underline{H}_3$CH = C), 2.63 (2H, s, C$\underline{H}_2$C = O), 2.76 (1H, br., O$\underline{H}$), 4.34 (2H, br. s, HOC$\underline{H}_2$), 4.38 (2H, d, $J$ = 5.8Hz, NHC$\underline{H}_2$), 6.58 (1H, q, $J$ = 7Hz, C=C$\underline{H}$), 6.82 (1H, d, $J$ = 8.5Hz, $\underline{H}$8), 6.88 (1H, br., N$\underline{H}$), 7.37 (1H, dd, $J$ = 8.5, 2.3Hz, $\underline{H}$7), 7.66 (1H, d, $J$ = 2.3Hz, $\underline{H}$5). $^{13}$CNMR $\delta_C$ 13.6 (C$\underline{H}_3$C = C), 26.6 ((C$\underline{H}_3$)$_2$), 42.7 (NHC$\underline{H}_2$), 48.8 (C$\underline{H}_2$C = O), 57.6 (C$\underline{H}_2$OH), 79.3 (C$\underline{2}$), 118.9 (ArC$\underline{H}$), 119.9 (ArC), 125.2 (ArC$\underline{H}$), 130.9 (ArC), 134.0 (ArC), 134.7 (ArC$\underline{H}$), 136.0 (ArC$\underline{H}$), 159.4 (ArC), 168.7 (NHC = O), 192.6 (C4). HRMS $(M+H)^+$ 302.1404, $C_{17}H_{22}NO_4$ requires 302.1392.

**(2*E*,4*E*)-5-(benzo[d] [1,3] dioxol-5-yl)-*N*-((2,2-dimethyl-4-oxochroman-6-yl)methyl)penta-2,4-dienamide** (**13b**) To a solution of piperic acid (21mg, 0.096mmol) in dichloromethane (5mL) at 0 °C under a $N_2$ atmosphere was added EDC (27mg, 0.14mmol) and triethylamine (12mg, 0.12mmol). To this solution was added de-BOC **32** (19mg, 0.093mmol). The reaction was allowed to reach room temperature and stirred for 24 h. The residual solvent was removed *in vacuo*, and the crude residue purified by flash column chromatography to afford amide **13b** as a colourless oil (22mg, 56%). IR $\nu_{max}$ (neat) 982, 1035, 1249, 1486, 1614, 1690, 2922cm$^{-1}$. $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.45 (6H, s, (C$\underline{H}_3$)$_2$, 2.71 (2H, s, C$\underline{H}_2$), 4.49 (2H, d, $J$ = 5.9Hz, NC$\underline{H}_2$), 5.81 (1H, t, $J$ = 5.7Hz, NH), 5.92 (1H, d, $J$ = 14.8Hz, COC$\underline{H}$ = C) 5.98 (2H, s, OC$\underline{H}_2$O), 6.66 & 6.69 (1H, 2 x d, $J$ = 10.8Hz, C$\underline{H}$ = CH), 6.79 (2H, m, 2 x C$\underline{H}$ = C), 6.90 (2H, m, 2 x C$\underline{H}$ = C), 6.98 (1H, br. s, C$\underline{H}$ = C), 7.40 (1H, dd, $J$ = 14.9, 10.7Hz, C$\underline{H}$ = C), 7.47 (1H, dd, $J$ = 8.5, 2.1Hz, $\underline{H}$7), 7.76 (1H, d, $J$ = 2Hz, $\underline{H}$5); $^{13}$CNMR $\delta_C$ 26.6 ((C$\underline{H}_3$)$_2$), 42.9 (C$\underline{H}_2$), 48.8 (C$\underline{H}_2$), 79.4 (C(CH$_3$)$_2$), 101.4 (OC$\underline{H}_2$O), 105.8 (C$\underline{H}$ = C), 108.5 (C$\underline{H}$ = C), 119.0 (C$\underline{H}$ = C), 119.9 (ArC), 122.5 (C$\underline{H}$ = C), 122.7 (C$\underline{H}$ = C), 124.5 (C$\underline{H}$ = C), 125.3 (C$\underline{H}$ = C), 130.8 × 2 (2 x ArC), 136.2 (C$\underline{H}$ = C), 139.3 (C$\underline{H}$ = C), 141.8 (C$\underline{H}$ = C), 148.2 (ArC), 148.3 (ArC), 159.4 (ArC), 166.0 (NC = O), 192.5 (C=O). HRMS $(M+H)^+$ 406.1654, $C_{24}H_{24}NO_5$ requires 406.1654.

**2,2-dimethyl-6-((octylamino)methyl)chroman-4-one** (**13c**) 100mg (0.37mmol) of 6-(bromomethyl)-2,2-dimethylchroman-4-one [25] was dissolved in acetonitrile (25mL) under aerobic conditions. To this stirred solution was added octylamine (45mg, 0.35mmol), and the reaction mixture was stirred at 100 °C for 4h. Upon completion, the solvent was removed *in vacuo*, and the residue purified by flash chromatography to give **13c** as a pale golden oil (32mg, 27%). IR $\nu_{max}$ (neat) 1196, 1256, 1297, 1486, 1615, 1690, 2854, 2924cm$^{-1}$. $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 0.87 (3H, t, $J$ = 6.5Hz, C$\underline{H}_3$), 1.19–1.55 (12H, m, (C$\underline{H}_2$)$_6$), 1.45 (6H, m, (C$\underline{H}_3$)$_2$, 2.63 (2H, t, $J$ = 7.3Hz, NHC$\underline{H}_2$CH$_2$, 2.71 (2H, s, C$\underline{H}_2$CO), 3.77 (2H, s, ArC$\underline{H}_2$), 6.91 (1H, d, $J$ = 8.6Hz, $\underline{H}$8), 7.54 (1H, d, $J$ = 8.4, $\underline{H}$7), 7.77 (1H, br. s, $\underline{H}$5); $^{13}$CNMR $\delta_C$ 14.1 (C$\underline{H}_3$), 22.7 (C$\underline{H}_2$), 26.6 ((C$\underline{H}_3$)$_2$), 27.3 (C$\underline{H}_2$), 29.3 (C$\underline{H}_2$), 29.5 (2 x C$\underline{H}_2$), 31.8 (C$\underline{H}_2$), 48.8 (C$\underline{H}_2$), 49.0 (C$\underline{H}_2$), 52.7 (C$\underline{H}_2$), 79.3 (C(CH$_3$)$_2$), 118.7 (ArC$\underline{H}$), 119.9 (ArC), 126.1 (ArC$\underline{H}$), 136.7 (ArC$\underline{H}$), 159.3 (ArC), 192.6 (C=O). HRMS $(M+H)^+$ 318.2431, $C_{20}H_{32}NO_2$ requires 318.2433.

**4-amino-3-iodobenzonitrile** (**35**) The compound was prepared and characterized as previously described in Ref. [36].

***N*-(4-cyano-2-iodophenyl)acetamide** (**36**) The compound was prepared and characterized as previously described in Ref. [36].

**N-(4-cyano-2-(3-hydroxy-3-methylbut-1-yn-1-yl)phenyl)acetamide** (**37**) To 5g (17.5mmol) of **36** were added water (25mL), $PdCl_2$ (30mg, 1mol%) and pyrrolidine (7.3mL, 87.5mmol) under aerobic conditions. The resulting mixture was stirred at 50 °C for 5 min. To this mixture was added 2-methylbut-3-yn-2-ol (1.7mL, 21mmol), and the reaction mixture was stirred at 110 °C for 5h. The reaction was then extracted with EtOAc (3 × 10 mL), and the combined organic layers dried with anhydrous sodium sulfate. The solvent was removed *in vacuo* to give a crude residue, which was reacted as such in the next step. IR $\nu_{max}$ (neat) 1454, 1508, 1614, 2217, 2876, 2978, 3341cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.65 (6H, s, (C$\underline{H}_3$)$_2$), 2.26 (3H, s, C$\underline{H}_3$CO), 7.52 (1H, dd, $J$ = 8.7, 1.9Hz, $\underline{H}$5), 7.60 (1H, d, $J$ = 2Hz, $\underline{H}$3), 8.44 (1H, d, $J$ = 8.5Hz, $\underline{H}$6), 8.60 (1H, br. s, N$\underline{H}$).

**2,2-dimethyl-4-oxo-1,2,3,4-tetrahydroquinoline-6-carbonitrile** (**38**) To crude **37** (3g, 12.4mmol) was added a 1:1 mixture of conc. HCl/$H_2O$ (20mL). The reaction was heated at 80 °C for 5 h, after which the reaction was extracted with ethyl acetate/water (3 × 50 mL), and the combined organic layers dried with anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the crude residue purified by flash chromatography to give **38** as a brown oil (1.1g, 44%). IR $\nu_{max}$ (neat) 804, 1514, 1612, 1669, 2219, 2922, 3322cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.29 (6H, s, C$\underline{H}_3$CC$\underline{H}_3$), 2.55 (2H, s, C$\underline{H}_2$), 6.57 (1H, d, $J$ = 8.8Hz, $\underline{H}$8), 7.39 (1H, dd, $J$ = 8.5, 2Hz, $\underline{H}$7), 8.03 (1H, d, $J$ = 2Hz, $\underline{H}$5). $^{13}$CNMR $\delta_C$ 27.7 (C$\underline{H}_3$C$\underline{H}_3$), 49.9 (C$\underline{3}$), 53.7 (C$\underline{2}$), 100.0, 116.3 (ArC$\underline{H}$), 117.5, 119.1, 132.8 (ArC$\underline{H}$), 137.3 (ArC$\underline{H}$), 151.7, 191.8 (C=O).

***tert*-butyl ((2,2-dimethyl-4-oxo-1,2,3,4-tetrahydroquinolin-6-yl)methyl)carbamate** (**39**) To a stirred solution of **38** (0.5g, 2.5mmol) in dry methanol (20mL) at 0 °C, were added $Boc_2O$ (0.6g, 2.75mmol) and $NiCl_2{\cdot}6H_2O$ (0.06g, 0.25mmol). $NaBH_4$ (0.47g, 12.4mmol) was then added in small portions over 10 min. The reaction was exothermic and effervescent. The resulting reaction mixture containing a finely divided black precipitate was allowed to warm to room temperature and left to stir for 15hr, at which point diethylenetriamine (0.26mL, 2.4mmol) was added. The mixture was allowed to stir for 30 min before solvent evaporation. The purple residue was dissolved in EtOAc (50mL) and extracted with saturated $NaHCO_3$ (2 × 50mL). The organic layer was dried ($Na_2SO_4$) and the solvent removed *in vacuo* to yield a mixture of products, including carbamate **39** (0.25g, 33%). IR $\nu_{max}$ (neat) 645, 802, 1162, 1274, 1515, 1623, 1651, 1695, 3316cm$^{-1}$. $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.24 (6H, s, C$\underline{H}_3$CC$\underline{H}_3$), 1.38 (9H, s, (C$\underline{H}_3$)$_3$C), 2.50 (2H, s, C$\underline{H}_2$), 4.12 (3H, m, NC$\underline{H}_2$ and N$\underline{H}$(CH$_3$)$_2$), 4.73 (1H, br. s, N$\underline{H}$CO), 6.52 (1H, d, $J$ = 8.5Hz, $\underline{H}$8), 7.18 (1H, br. d, $J$ = 8.5Hz, $\underline{H}$7), 7.61 (1H, d, $J$ = 2Hz, $\underline{H}$5). $^{13}$CNMR $\delta_C$ 27.6 (2C), 28.4 (3C), 44.0, 50.5, 53.6, 79.4, 116.3, 117.6, 125.8, 127.8, 135.2, 149.3, 155.9, 193.8.

#### 5.1.1. *General method (14a-f)*

To a solution of appropriate acid (0.49mmol) in dichloromethane (10mL) at 0 °C was added EDC (0.08g, 0.52mmol) and triethylamine (0.08mL, 0.57mmol). After 30 min, the primary amine liberated via trifluoroacetic acid treatment of **39** (100mg, 0.49mmol) was added to this solution. The reaction was allowed to reach room temperature and stirred for 24 h. The residual solvent was removed *in vacuo*, the residue extracted with saturated bicarbonate/DCM, and the crude organic residue purified by flash column chromatography (Pet. Ether:EtOAc 1:1) to afford the amide.

**(*E*)-*N*-((2,2-dimethyl-4-oxo-1,2,3,4-tetrahydroquinolin-6-yl)methyl)-2-(((tetrahydro-2H-pyran-2-yl)oxy)methyl)but-2-enamide** (**14a**) Yellow oil, (89mg, 47%). IR $\nu_{max}$ (neat) 1021, 1506, 1619, 1662, 2923, 3306cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.31 (6H, s, C$\underline{H}_3$CC$\underline{H}_3$), 1.40–1.72 (6H, m, (C$\underline{H}_2$)$_3$), 1.86 (3H, d, $J$ = 7.2Hz, C$\underline{H}_3$), 2.57 (2H, s, C$\underline{H}_2$CO), 3.47 (1H, m, H of OC$\underline{H}_2$ pyran), 3.74 (1H, m, H of OC$\underline{H}_2$ pyran), 4.12 (1H, m, N$\underline{H}$), 4.34–4.42 (4H, m, OC$\underline{H}_2$C = C and NHC$\underline{H}_2$, 4.59 (1H, m, OC$\underline{H}$O), 6.58 (1H, d, $J$ = 8.4Hz, $\underline{H}$8), 7.01 (1H, q, $J$ = 7.2Hz, CH = C$\underline{H}$), 7.19 (1H, br., N$\underline{H}$), 7.29 (1H, dd, $J$ = 8.4, 2.1Hz, $\underline{H}$7), 7.71 (1H, d, $J$ = 2Hz, $\underline{H}$5). $^{13}$CNMR $\delta_C$ 13.8, 19.7, 25.2, 27.7 (2C), 30.5, 43.0, 50.6,

53.6, 60.7, 63.0, 97.7, 116.3, 117.7, 126.2, 127.6, 130.4, 135.7, 138.7, 149.2, 167.5, 193.8. HRMS $(M+H)^+$ 387.2265, $C_{22}H_{31}N_2O_4$ requires 387.2284.

**(*E*)-*N*-((2,2-dimethyl-4-oxo-1,2,3,4-tetrahydroquinolin-6-yl) methyl)but-2-enamide** (**14b**) Yellow oil, (88mg, 66%); $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.31 (6H, s, C**H**$_3$CC**H**$_3$), 1.85 (3H, dd, $J$ = 6.9, 1.7Hz, CHC**H**$_3$), 2.57 (2H, s, C**H**$_2$), 4.37 (2H, d, $J$ = 5.7Hz, NHC**H**$_2$, 5.77 (1H, br. s, N**H**), 5.79 (1H, m, C**H** = CHCH$_3$), 6.59 (1H, d, $J$ = 8.3Hz, **H**8), 6.87 (1H, m, CH$_3$C**H** = C), 7.28 (1H, dd, $J$ = 2.2Hz, **H**7), 7.68 (1H, d, $J$ = 2.3Hz, **H**5). $^{13}$CNMR $\delta_C$ 17.8, 27.6 (2**C**), 42.8, 50.5, 53.6, 116.4, 117.6, 124.9, 126.1, 127.3, 135.7, 140.3, 149.4, 165.8, 193.9. HRMS $(M+H)^+$ 273.1601, $C_{16}H_{21}N_2O_2$ requires 273.1603.

**(*E*)-*N*-((2,2-dimethyl-4-oxo-1,2,3,4-tetrahydroquinolin-6-yl) methyl)-2-methylbut-2-enamide** (**14c**) Yellow solid, (87mg, 62%). IR $\nu_{max}$ (neat) 634, 1190, 1278, 1289, 1516, 1604, 1656, 3263, 3347cm$^{-1}$. $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.32 (6H, s, C**H**$_3$CC**H**$_3$), 1.74 (3H, dd, $J$ = 7.0, 1.0Hz, CHC**H**$_3$), 1.84 (3H, m, C**H**$_3$C = CH), 2.58 (2H, s, C**H**$_2$), 4.37 (2H, d, $J$ = 5.6Hz, NHC**H**$_2$, 5.96 (1H, br. s, N**H**), 6.45 (1H, m, CH$_3$C**H** = C), 6.59 (1H, d, $J$ = 8.4Hz, **H**8), 7.29 (1H, dd, $J$ = 8.4, 2.2Hz, **H**7), 7.69 (1H, d, $J$ = 2.3Hz, **H**5). $^{13}$CNMR $\delta_C$ 12.5, 13.9, 27.7 (2**C**), 43.1, 50.5, 53.6, 116.5, 117.6, 126.2, 127.4, 131.0, 131.6, 135.7, 149.4, 169.2, 193.9. HRMS $(M+H)^+$ 287.1707, $C_{17}H_{23}N_2O_2$ requires 287.1760.

***N*-((2,2-dimethyl-4-oxo-1,2,3,4-tetrahydroquinolin-6-yl) methyl)-3-methylbut-2-enamide** (**14d**) Yellow oil, (77mg, 55%). IR $\nu_{max}$ (neat) 599, 654, 683, 1163, 1252, 1517, 1618, 3251cm$^{-1}$. $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.31 (6H, s, C**H**$_3$CC**H**$_3$), 1.83 (3H, d, $J$ = 0.9Hz, C=CC**H**$_3$), 2.17 (3H, d, $J$ = 0.9Hz, C=CC**H**$_3$), 2.56 (2H, s, C**H**$_2$), 4.33 (2H, d, $J$ = 5.7Hz, NHC**H**$_2$, 5.57 (1H, br., C=C**H**), 5.75 (1H, br. s, N**H**), 6.59 (1H, d, $J$ = 8.4Hz, **H**8), 7.27 (1H, dd, $J$ = 8.4, 2.2Hz, **H**7), 7.67 (1H, d, $J$ = 2.2Hz, **H**5). $^{13}$CNMR $\delta_C$ 19.9, 27.2, 27.6 (2**C**), 42.5, 50.5, 53.6, 116.4, 117.5, 118.3, 126.0, 127.5, 135.6, 149.4, 151.4, 166.8, 193.9. HRMS $(M+H)^+$ 287.1751, $C_{17}H_{23}N_2O_2$ requires 287.1760.

**(*E*)-*N*-((2,2-dimethyl-4-oxo-1,2,3,4-tetrahydroquinolin-6-yl) methyl)-4-phenylbut-3-enamide** (**14e**) Yellow oil, (129mg, 75%). IR $\nu_{max}$ (neat) 693, 966, 1163, 1302, 1506, 1620, 1645, 3297cm$^{-1}$. $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.30 (6H, s, C**H**$_3$CC**H**$_3$), 2.55 (2H, s, C**H**$_2$), 3.18 (2H, d, $J$ = 7.2Hz, C**H**$_2$C = C), 4.24 (1H, br., N**H**), 4.31 (2H, d, $J$ = 5.6Hz, NHC**H**$_2$, 5.99 (1H, br. s, N**H**), 6.29 (1H, m, C**H** = CH), 6.52 (1H, d, $J$ = 15.9Hz, CH = C**H**), 6.58 (1H, d, $J$ = 8.4Hz, **H**8), 7.21–7.37 (6H, m, 6 x Ar**H**), 7.66 (1H, d, $J$ = 2.1Hz, **H**5). $^{13}$CNMR $\delta_C$ 27.7 (2C), 40.9, 43.1, 50.5, 53.6, 116.5, 117.6, 122.3, 126.2, 126.4 (2**C**), 127.0, 127.8, 128.6 (2C), 134.7, 135.6, 136.6, 149.4, 170.6, 193.9. HRMS $(M+H)^+$ 349.1902, $C_{22}H_{25}N_2O_2$ requires 349.1916.

**(*E*)-*N*-((2,2-dimethyl-4-oxo-1,2,3,4-tetrahydroquinolin-6-yl) methyl)-3-(thiophen-2-yl)acrylamide** (**14f**) Yellow solid, (110mg, 69%). IR $\nu_{max}$ (neat) 704, 820, 959, 1211, 1526, 1614, 3252cm$^{-1}$. $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.25 (6H, s, C**H**$_3$CC**H**$_3$), 2.51 (2H, s, C**H**$_2$), 4.37 (2H, d, $J$ = 5.6Hz, NHC**H**$_2$, 5.76 (1H, br. s, N**H**), 6.12 (1H, d, $J$ = 15.3Hz, C**H** = CH), 6.53 (1H, d, $J$ = 8.4Hz, **H**8), 6.96 (1H, m, Ar**H**), 7.13 (1H, d, $J$ = 3.7Hz, Ar**H**), 7.22–7.25 (2H, m, 2 x Ar**H**), 7.65 (1H, d, $J$ = 2.0Hz, **H**5), 7.70 (1H, d, $J$ = 15.2Hz, CH = C**H**). $^{13}$CNMR $\delta_C$ 26.6 (2**C**), 28.7, 42.1, 49.5, 52.6, 115.5, 116.6, 118.3, 125.2, 126.1, 126.3, 127.0, 129.3, 133.1, 134.7, 138.9, 148.4, 164.4, 192.8. HRMS $(M+H)^+$ 341.1310, $C_{19}H_{21}N_2O_2S$ requires 341.1324.

**(*E*)-*N*-((2,2-dimethyl-4-oxo-1,2,3,4-tetrahydroquinolin-6-yl) methyl)-2-(hydroxymethyl)but-2-enamide** (**14g**) Prepared as for **13a** using 50mg (**14a**); Yellow oil, (22mg, 56%). IR $\nu_{max}$ (neat) 1272, 1294, 1506, 1600, 1664, 2921, 3276cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400MHz) $\delta_H$ 1.30 (6H, s, C**H**$_3$CC**H**$_3$), 1.80 (3H, d, $J$ = 7.2Hz, C**H**$_3$), 2.54 (2H, s, C**H**$_2$CO), 4.34 (2H, d, $J$ = 5.7Hz, NHC**H**$_2$, 4.38 (2H, s, C**H**$_2$OH), 4.44 (1H, br. s, N**H**), 6.58 (1H, d, $J$ = 8.4Hz, **H**8), 6.64 (1H, q, $J$ = 7.1Hz, C=C**H**), 7.16 (1H, br., N**H**), 7.24 (1H, dd, $J$ = 8.5, 2.3Hz, **H**7), 7.64 (1H, d, $J$ = 2.3Hz, **H**5). $^{13}$CNMR $\delta_C$ 13.6, 27.6 (2**C**), 42.8, 50.5, 53.5, 57.3, 116.5, 117.4, 125.9, 127.2, 134.0, 134.8, 135.6, 149.5, 168.8, 194.2. HRMS $(M+Na)^+$ 325.1517, $C_{17}H_{22}N_2O_3Na$ requires 325.1528.

### 5.2. Bioassay procedures

#### 5.2.1. Ethics statement

BALB/c mice were obtained from the animal breeding facility at the Instituto de Investigação e Inovação em Saúde (i3S), University of Porto. The animals were maintained in pathogen free conditions, in individually ventilated cages and were fed with sterilized food and water *ad libitum*. All animal procedures were performed in compliance with the Local Animal Ethics Committee of i3S, licensed by DGAV (Direção Geral de Alimentação e Veterinária, Govt. of Portugal – DGAV). Animals were handled in strict accordance with good animal practice as defined by national authorities (DGAV, directive 113/2013 from 7th August) and European legislation (directive 2010/63/EU, revising directive 86/609/EEC).

#### 5.2.2. Parasite culture

*Leishmania* parasites used in this work were obtained from infected mice splenocytes by amastigote to promastigote differentiation as described by Sereno *et al* [37]. To ensure infectivity, promastigotes were cultured at 25 °C for a maximum of 10 passages. The insect stage of *L. infantum* (strain MHOM/MA/67/ITMAP263) was maintained in RPMI 1640 GlutaMAX™-I medium (Gibco® Life Technologies Thermo Fisher) supplemented with 10% heat inactivated Fetal Bovine Serum (FBSi, Gibco® Life Technologies Thermo Fisher), 50U/mL penicillin and 50 μg/mL streptomycin (Gibco® Life Technologies Thermo Fisher) and 5 mM HEPES sodium salt pH 7.4. Promastigotes from both *L. major* (strain MHOM/SA/85/JISH118) and *L. amazonensis* (strain MHOM/BR/LTB0016) were kept in Schneider's medium (Sigma-Aldrich) supplemented with 10% FBSi, 100U/mL penicillin and 100 μg/mL streptomycin, 2% human urine, 5 mg/mL phenol red and 5mM HEPES sodium. For infection assays, *L. infantum* promastigotes were kept in culture at 25 °C for 5–7 days without medium renewal to promote differentiation from the exponential to the stationary phase. Axenic amastigotes of *L. infantum* were differentiated from parasites recently recovered from the spleen of infected mice, as described before [37], and maintained at 37 °C, 5% CO2, in MAA medium supplemented with 20% (v/v) iFBS, 2 mM Glutamax (Gibco), and 0.023 mM hemin (Sigma-Aldrich).

#### 5.2.3. Determination of $IC_{50}$ against Leishmania intracellular amastigotes

Macrophages were obtained from BALB/c mice bone-marrow precursors by differentiation with L929 cell conditioned medium (LCCM) as described in Vale-Costa *et al.* [38]. Briefly, bone-marrow cells were seeded onto 96-well plates at a density of $3 \times 10^5$ cells/mL and maintained in culture for 7 days at 37 °C with 5% $CO_2$ in DMEM medium with GlutaMAX™-I (Gibco® Life Technologies Thermo Fisher) supplemented with 10% FBSi, 50U/mL penicillin and 50 μg/mL streptomycin, 1% MEM-NEAA and 10% LCCM. Macrophages were then co-incubated with a rate of 10:1 stationary-phase promastigotes for 3 h at 37 °C with 5% $CO_2$, after which non-phagocytosed parasites were removed by washing. Twenty four hours later, test compounds, diluted in DMEM medium, were added to the infected monolayers, and cells were maintained in culture for a further 24 h. Infected macrophages subjected to 0.5% DMSO were used as a negative control and amphotericin B was used as a standard drug. Activity against *L. infantum* intracellular amastigotes was determined according to Gomes-Alves *et al.* [39]. For this, cells were fixed, stained with a DAPI (Sigma-Aldrich)/HCS CellMask™ (Invitrogen) solution and photographed in a IN Cell Analyzer 2000 microscope (GE Healthcare). Upon image acquisition, both the total number of macrophages as well as the number of infected cells was determined with the IN Cell Investigator Developer Toolbox v. 1.9.2 software (GE Healthcare).

The percentage of infected cells was then used to assess the $IC_{50}$ values determined with Graph Pad Prism software.

#### 5.2.4. Cytotoxicity in mammalian cells

Bone marrow-derived macrophages, obtained as above, were incubated at 37 °C and 5% $CO_2$ with test compounds, up to a highest concentration of 40 μM for 24 h. Cell viability was determined using the resazurin assay [38]. The 50% cytotoxic concentration ($CC_{50}$) was calculated from viability percentage plots in relation to control using Graph Pad Prism software.

#### 5.2.5. Evaluation of compound activity against promastigotes of different Leishmania species and axenic amastigotes of L. infantum

Promastigotes in the late exponential phase were seeded at $3 \times 10^6$ cells/mL in a final volume of 100 μL of either RPMI (*L. infantum*) or Schneider's medium (*L. major* and *L. amazonensis*). *L. infantum* axenic amastigotes were seeded at $1.5 \times 10^5$ cells in 100 μL of MAA20. Test compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluted in a final volume of 100 μL of RPMI, Schneider 's medium or MAA20. Treatment with 0.5% DMSO and amphotericin B were used as negative and positive controls, respectively. All experimental conditions were performed in triplicate. Parasite viability was evaluated upon 24h exposure to each compound and was assessed using the resazurin assay [38]. The fluorescence intensity was read in a Sinergy™2 Microplate Reader (BioTek Instruments). The 50% inhibitory concentration ($IC_{50}$) values obtained were determined with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA).

## Declarations

### Author contribution statement

Helena Castro, Tânia Cruz, Ahmed Alsaffar, Patrick Farrell: Performed the experiments; Analyzed and interpreted the data.

Patrícia de Aguiar Amaral: Conceived and designed the experiments.

Paula da Silva Cardoso: Performed the experiments.

Ana M. Tomás: Conceived and designed the experiments; Analyzed and interpreted the data.

James W. Barlow: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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