Synthesis and biological evaluation of functionalised tetrahydro-β-carboline analogues as inhibitors of *Toxoplasma gondii* invasion†

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Techniques for the identification of the protein target(s) of small molecules are proving very important following an increase in the use of phenotype-based screening in chemical biology and drug discovery. One approach, known as the yeast-3-hybrid approach, has shown considerable potential. A key factor in the success of this approach is the preparation of a complex molecule referred to as a chemical inducer of dimerisation (CID). The synthesis of two CIDs based on a bioactive tetrahydro-β-carboline core structure is reported and evidence presented that shows the CIDs are of utility in this approach. A series of chemo- and bioinformatic studies coupled with SAR development inspired the choice of CIDs.

Introduction

Toxoplasma gondii, a protozoan parasite capable of infecting any warm-blooded animal, is one of the most common parasites of man.[1] Infection can be life threatening during pregnancy, or in the immunocompromised patient.[2] *T. gondii* is also considered a model for other related, but less experimentally tractable parasites in the phylum Apicomplexa, including *Plasmodium*, the causative agent of malaria, and *Cryptosporidium*, a significant world-wide cause of water-borne disease.[3] Apicomplexan parasites are only able to grow and replicate within cells of their hosts. The process of host cell invasion by *T. gondii* and other apicomplexans is therefore both a key step in the life cycle of the parasite and a potential point of therapeutic intervention.

The mechanism of host cell invasion by apicomplexan parasites has previously been studied in detail,[4] in part through the use of pharmacological inhibitors whose targets have been well characterised in other systems.[3b,5] However, little progress has been made towards the development of anti-parasitic drugs that target invasion. In a broader search for compounds that disrupt host cell invasion by *T. gondii*, we recently reported the use of a high-throughput cell-based assay to identify novel inhibitors of this process.[6] The assay delivered 24 non-cytotoxic compounds that reproducibly inhibit host cell invasion, including the functionalised tetrahydro-β-carboline **1** (Scheme 1A). However, the mode of action of **1** and the other inhibitors identified in this screen was unknown. The identification of a compound's protein target(s) is essential if it is to be used as a small molecule tool.[7,8] Here we report results from our programme to identify the protein target(s) of **1**. A chemoinformatic assessment of **1** led to several potential protein target candidates. However, SAR and bioinformatic investigations did not support the hypothesis that **1** inhibits *T. gondii* invasion by disrupting the function of a *T. gondii* homologue of a known mammalian protein target of **1**. As a result we were compelled to adopt genome/proteome-wide target identification approaches. In this context, we report the preparation of novel reagents based on **1** and present data that show these reagents are suitable for this purpose.

Results and discussion

Preliminary SAR

As a number of analogues of **1** are commercially available, initial studies focused on the biological assessment of 3 racemic compounds that can be viewed as truncated versions of **1**, compounds **2**, **3** and **4** (Scheme 1A). All 3 analogues were inactive in the invasion assay (no inhibition of invasion at final concentrations up to 100 μM) and we therefore concluded that the fused tetracyclic core structure of **1** represents the minimum bioactive pharmacophore.

Four stereoisomers of **1** exist and the next step focused on determining the influence that the stereochemistry at C5 and C11a in **1** had on the observed biological activity. Reacting either (*D*)- (**5a**) or (*L*)-tryptophan (**5b**) with *m*-hydroxybenzaldehyde under Pictet–Spengler reaction conditions led to formation of the corresponding *cis*-acid **2a** and its enantiomer **2b** respectively (Scheme 1B).[9a] In this and the other examples reported here, the major isomer formed in the Pictet–Spengler reaction had the *cis*-C5-C11a relative stereochemistry. Subsequent reaction of *cis*-**2a** or **2b** with ^{n}butylisocyanate gave *cis*-**6a** and **6b**, respectively in good yields. A NOESY experiment carried out on *cis*-**6b** showed a correlation between the C5 and C11a-hydrogens as expected for a *cis*-relationship.[10] This assignment is consistent with a previous literature investigation of the hydantoin ring-forming reaction in

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† Electronic supplementary information (ESI) available: Numbering scheme used for the β-carboline ring system, NOSEY spectrum of *cis*-**6b** and *trans*-**7a**, bioinformatic analysis of *T. gondii* kinesins, the yeast-3-hybrid approach, synthesis of C9-substituted analogues **7e–7j**, synthesis and biological activity of analogues **9ii**, **9iv**, **9x–xvii**, NMR characterisation of CID **23** and CID **15**. CCDC reference number 719462. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/b902319d

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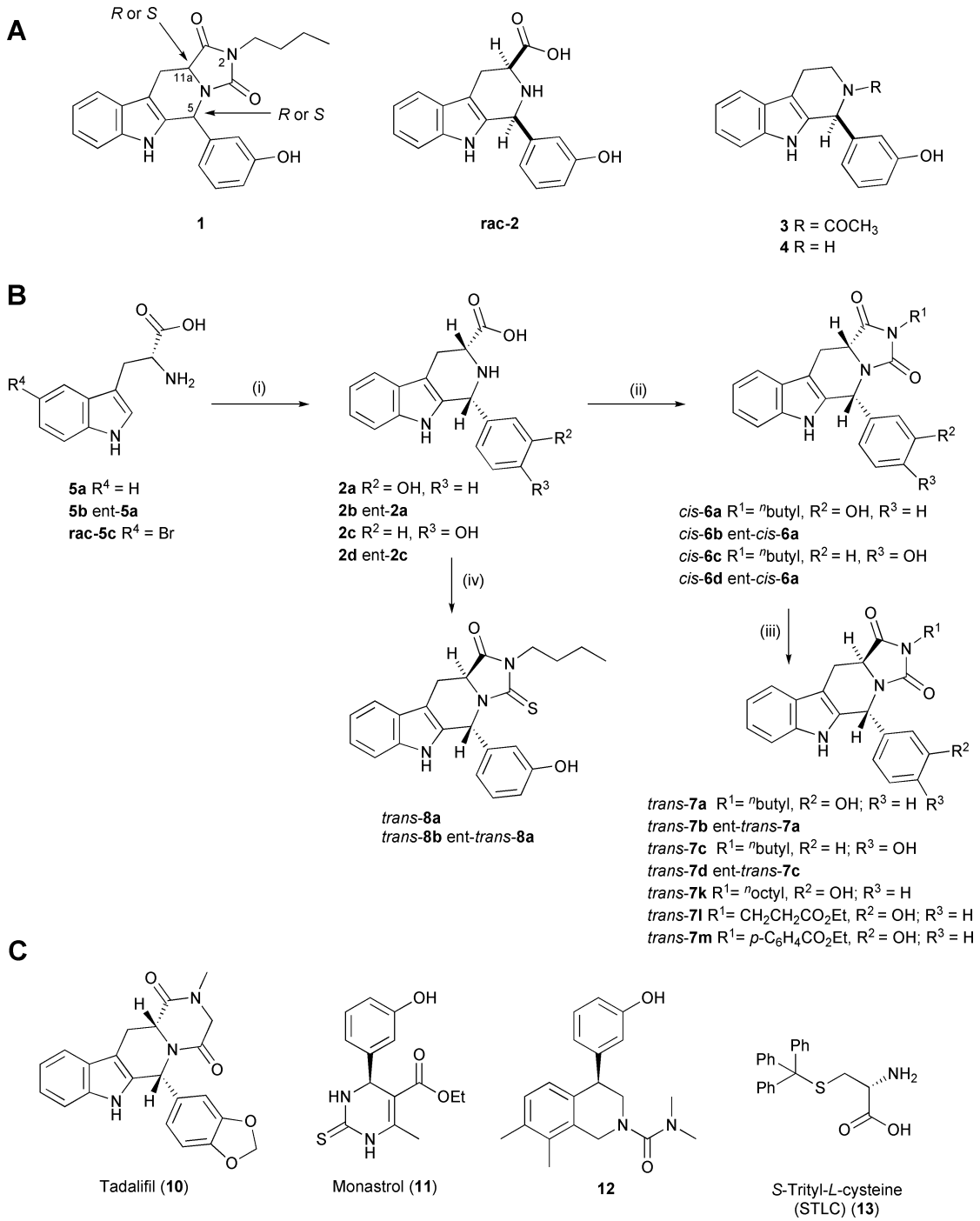


Scheme 1 **A** Tetrahydro-β-carboline analogues of **1**. **B** Synthesis of analogues of **1**. Reaction conditions: (i) H_2SO_4, H_2O, 100 °C, 8 hrs; 70% for **2a** (R^4 = H); 50% **2b** (R^4 = H); 34% **2c**; 52% **2d**; (ii) DMSO/acetone, R^1NCO, 56 °C, 2–4 hrs; 65% **6a**; 92% **6b**; 74% **6c**; 64% **6d**; (iii) K_2CO_3, MeCN, 82 °C, 30 min.; 80% **7a**; 99% **7b**; 53% **7c**; 77% **7d**; 87% **7k**; 82% **7l**; 63% **7m**; (iv) DMSO/acetone, R^1NCS, 56 °C, 2 hrs; 56% **8a**; 50% **8b**. **C** Known inhibitors of cGMP-specific 3′,5′-cyclic nucleotide phosphodiesterases and kinesins.

this heterocyclic system.[9a] In order to access the corresponding *trans*-isomers **7a** and **7b**, the C11a protons in *cis*-**6a** and *cis*-**6b** were epimerised by treatment with potassium carbonate in refluxing acetonitrile. X-Ray crystallographic analysis of *trans*-**7b** confirmed the relative stereochemical assignment at C5 and C11a.[10,11] Reaction of *cis*-**2a** and **2b** with ^{n}butylisothiocyanate led directly to the corresponding C3-thiocarbonyl analogues that, in line with literature precedent,[9] were assigned as *trans*-**8a** and *trans*-**8b** respectively (Scheme 1B).

Interestingly, the *trans*-(5*R*, 11a*S*)-isomer **7a**[12] proved to be the most potent inhibitor of *T. gondii* invasion with a minimum inhibitory concentration (MIC) of 12.5 μM (Table 1, entry 1). Its enantiomer *trans*-**7b** did not inhibit *T. gondii* invasion even at 100 μM, the highest concentration tested (Table 1, entry 2).

Table 1 Minimum inhibitory concentrations (MIC) in the *T. gondii* cell invasion assay for analogues of **1**

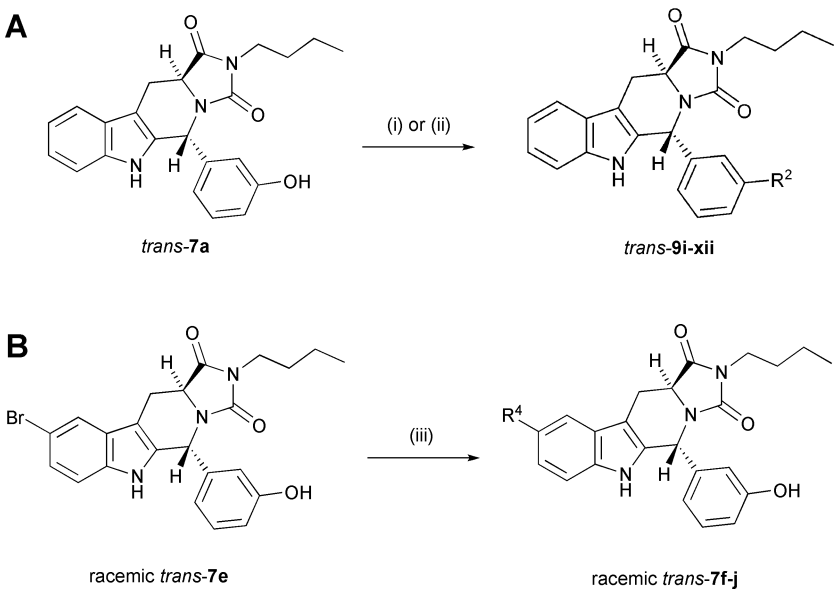
Entry #	Cpd #	C5	C11a	R^1	R^2	R^3	X	MIC (μM)
1	**7a**	*R*	*S*	^{n}Butyl	OH	H	O	12.5
2	**7b**	*S*	*R*	^{n}Butyl	OH	H	O	>100
3	**6a**	*R*	*R*	^{n}Butyl	OH	H	O	50
4	**6b**	*S*	*S*	^{n}Butyl	OH	H	O	50
5	**7c**	*R*	*S*	^{n}Butyl	H	OH	O	25
6	**7d**	*S*	*R*	^{n}Butyl	H	OH	O	25
7	**6c**	*R*	*R*	^{n}Butyl	H	OH	O	>100
8	**6d**	*S*	*S*	^{n}Butyl	H	OH	O	100
9	**8a**	*R*	*S*	^{n}Butyl	OH	H	S	3.1
10	**8b**	*S*	*R*	^{n}Butyl	OH	H	S	12.5
11	**9i**	*R*	*S*	^{n}Butyl	OCH_3	H	O	12.5

The other enantiomeric pair in this series, *cis*-**6a** and *cis*-**6b**, both exhibited weaker inhibition of *T. gondii* invasion compared to *trans*-**7a** with MICs of 50 μM (Table 1, entries 3 and 4). A similar activity trend was observed across a second series of four isomers, **6c**, **6d**, **7c**, **7d**, that differed from the **6/7a,b** series only in the position of the phenolic OH (Scheme 1B) with one exception. The activity associated with *trans*-**7d** was significantly increased compared to that of *trans*-**7b** (Table 1, entry 6 *vs.* entry 2). Unexpectedly, replacement of the C3 carbonyl in both *trans*-**7a** and *trans*-**7b** by a thiocarbonyl moiety[9b] to give *trans*-**8a** and *trans*-**8b** respectively led to a significant increase in potency (Table 1, entry 9 *vs.* 1 and 10 *vs.* 2). Conversion of the phenolic functional group in *trans*-**7a** to the corresponding methyl ether gave *trans*-**9i** (Scheme 2A) that had analogous activity to *trans*-**7a** (Table 1, entry 11 *vs.* entry 1).

Potential biological targets generated using chemoinformatics/bioinformatics

Substructure searches using the core structure of **1** revealed that *trans*-**7a** is an inhibitor of the mitotic kinesin Eg5[12] and **1** is structurally related to tadalafil (**10**) (Scheme 1C), a clinically-used inhibitor of the mammalian cGMP-specific 3′,5′-cyclic nucleotide phosphodiesterase PDE5.[13] A bioinformatic analysis of the *T. gondii* genome[14] revealed that the *T. gondii* genome encodes at least 15 kinesins (see also Foth and Soldati[15]) and 17 cyclic nucleotide PDEs.

Of the 15 kinesins encoded by *T. gondii*, conserved domain searches and BLASTP analyses suggested that four belong to kinesin family-5, of which human Eg5 is a member. Based on expression data,[14b] at least three of these four family-5 kinesins (TgME49_097110, TgME49_024880, TgME49_119710) are expressed in the tachyzoite stage of the parasite used in our invasion assays. *Trans*-**7a** inhibits human Eg5 allosterically and the amino acids that make up the allosteric inhibitor-binding pocket have recently been determined.[16] The primary sequences of these four *T. gondii* kinesins were aligned with several of the known family-5 kinesins, including human Eg5, and the corresponding inhibitor-binding regions were compared (see Figure S3[10]). Whilst comparisons based on inhibitor-binding region 1 (Y125-E145) were inconclusive, all four of the *T. gondii* kinesins appear to have reasonable sequence similarity to human Eg5 in inhibitor-binding region 2 (I202-L227). The observation that *trans*-**7a** is the most active isomer for both inhibition of Eg5[12] and inhibition of *T. gondii* invasion (Table 1) is also consistent with the hypothesis that *trans*-**7a** acts on a *T. gondii* Eg5 homologue. However, assay results obtained with other close structural analogues did not support this hypothesis. For both *trans*-**7a** and monastrol (**11**) (Scheme 1C), inhibition of Eg5 involves an important hydrogen bond between the phenol functional group and the backbone carbonyl oxygen of either Trp127 or Glu118 in Eg5.[17,18] In contrast, we observed that the phenolic OH in **7a** was not essential for inhibition of *T. gondii* invasion. The methyl ether analogue **9i** (Scheme 2A) was as potent in the *T. gondii* invasion assay as **7a** (Table 1, entry 11 *vs.* 1). In addition, previous studies on structurally related 4-aryl-tetrahydroisoquinoline inhibitors (e.g. **12**, Scheme 1C), which bind at the same site on human



Scheme 2 **A** Synthesis of *O*-substituted analogues of *trans*-**7a**. Reaction conditions: (i) PPh_3, DEAD, MeOH, THF; 85% for **9i**; (ii) RX, K_2CO_3, CH_3CN, 61–84%; for R^2 see Table 2. **B** Synthesis of analogues of *trans*-**7e** substituted at R^4 (see Table 2). Reaction conditions: (iii) $R^4B(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, DME, H_2O, 70 °C, 24 hrs. For details see ESI.[10]

Table 2 Minimum inhibitory concentrations (MIC) in the *T. gondii* cell invasion assay for analogues **7e**–**7m** and **9i**–**9xii**(see Schemes 1B and 2B for structures; R^3 = H in all cases). For the synthesis and testing of additional *O*-substituted analogues see supplementary material[10]

Entry #	Cpd #	C5	C11a	R^1	R^2	R^4	MIC (μM)
1	**7a**	*R*	*S*	^{n}Butyl	OH	H	12.5
2	**7e**	*S*/*R*	*R*/*S*	^{n}Butyl	OH	Br	25
3	**7f**	*S*/*R*	*R*/*S*	^{n}Butyl	OH	Ph	12.5
4	**7g**	*S*/*R*	*R*/*S*	^{n}Butyl	OH	4-Cl–C_6H_4	12.5
5	**7h**	*S*/*R*	*R*/*S*	^{n}Butyl	OH	4-Me–C_6H_4	25
6	**7i**	*S*/*R*	*R*/*S*	^{n}Butyl	OH	4-OMe–C_6H_4	25
7	**7j**	*S*/*R*	*R*/*S*	^{n}Butyl	OH	3,4-diCl-C_6H_4	100
8	**7k**	*R*	*S*	^{n}Octyl	OH	H	50
9	**7l**	*R*	*S*	$CH_2CH_2CO_2Et$	OH	H	>100
10	**7m**	*R*	*S*	*p*-C_6H_4-CO_2Et	OH	H	50
11	**9i**	*R*	*S*	^{n}Butyl	OMe	H	12.5
12	**9ii**	*R*	*S*	^{n}Butyl	OEt	H	6.25
13	**9iii**	*R*	*S*	^{n}Butyl	O^{n}Pr	H	12.5
14	**9iv**	*R*	*S*	^{n}Butyl	O^{n}Bu	H	6.25
15	**9v**	*R*	*S*	^{n}Butyl	Oallyl	H	12.5
16	**9vi**	*R*	*S*	^{n}Butyl	O^{i}Pr	H	1.6
17	**9vii**	*R*	*S*	^{n}Butyl	$OCH_2C(CH_3)=CH_2$	H	3.1
18	**9viii**	*R*	*S*	^{n}Butyl	Opropargyl	H	3.1
19	**9ix**	*R*	*S*	^{n}Butyl	OBn	H	12.5
20	**9x**	*R*	*S*	^{n}Butyl	OCH_2*p*-C_6H_4-OMe	H	100
21	**9xi**	*R*	*S*	^{n}Butyl	OCH_2*m*,*p*-C_6H_3-Cl_2	H	100
22	**9xii**	*R*	*S*	^{n}Butyl	CH_2CONH_2	H	25

Eg5 as **7a** and **11**, have demonstrated that analogues containing a *para*-phenol are inactive, whereas those containing the phenolic OH in the *meta*-position are active.[17] This is in contrast to our observations with **6c**, **6d**, **7c** and **7d** which all inhibit *T. gondii* invasion (Table 1, entries 5–8). Finally, the replacement of the C3 carbonyl in both *trans*-**7a** and **7b** with a thiocarbonyl had a significant effect on the observed biological activity in our system, whereas an identical change has previously been reported to have no impact on Eg5 inhibition.[12a] In the light of these observations and the fact that the known Eg5 inhibitors monastrol (**11**) and *S*-trityl-*L*-cysteine (**13**) (Scheme 1C) had no effect on parasite invasion (data not shown), it was concluded that the inhibition of *T. gondii* invasion by **1** probably occurs through a different target class or through a parasite kinesin with unique characteristics.

Of the 17 cyclic nucleotide PDEs encoded by *T. gondii*, at least nine are expressed in the tachyzoite stage of the parasite.[14] Tadalafil (**10**) targets a human PDE of the PDE5 family, but subclassification of the *T. gondii* cyclic nucleotide PDEs into their most likely families revealed no PDE5 family members within the *T. gondii* genome. PDE6 and PDE11 family members have been proposed as secondary targets of **10**[19,20] (and therefore, potentially of **1**), but these PDE families are also absent from the *T. gondii* genome. It is therefore unlikely that PDE5, 6 or 11 is the target of **1** in the parasite.

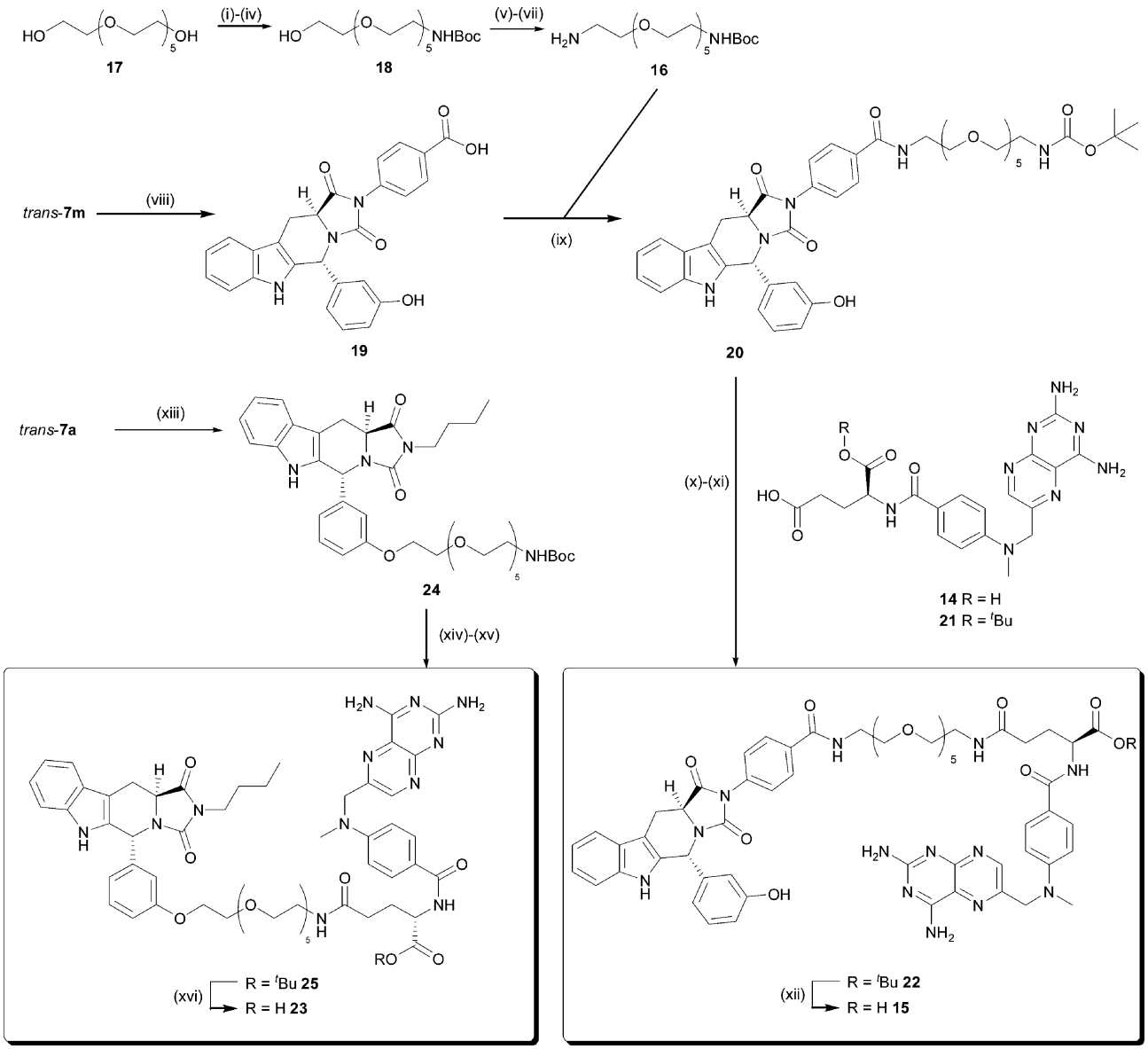
Genome/proteome-wide target identification techniques

As no clear leads to a putative target of **1** in *T. gondii* could be reached from the chemo/bioinformatic analyses and initial SAR studies, we focused on the synthesis of analogues of *trans*-**7a** that could be used for more general target identification techniques.[7] In particular, we decided to prepare reagents that were applicable to the yeast-3-hybrid (Y3H) approach (see Figure S4).[10,21] This relatively new technique shows considerable promise for identifying protein binding partners of small molecules. Amongst many challenges in the Y3H approach is the synthesis of a key compound referred to as a chemical inducer of dimerisation (CID). This requires linkage of methotrexate (Mtx (**14**), Scheme 3) to the molecule under study in a manner that preserves its ability to bind to its target(s). Confidence that an appropriate position for Mtx (**14**) attachment has been chosen comes from SAR studies.

Extended SAR studies

The effect of incorporating a substituent in the indole aromatic ring of *trans*-**7a** was explored to assess whether this position may provide a potential site for linker attachment or whether incorporation of an R^4-substituent (Scheme 2B) would lead to an increase in potency. An analogue of **7a** containing a bromine at R^4 (**7e**) was prepared from commercially available 5-bromo-(*DL*)-tryptophan (**5c**) (Schemes 1B and 2B).[10] Racemic *trans*-**7e** was active in the *T. gondii* invasion assay (Table 2, entry 2) and since the large bromo-substituent was tolerated, a Topliss[22] series of aryl ring-containing analogues, **7f–j**, was prepared from **7e** using Suzuki coupling methodology (Scheme 2B).[10] With the exception of the incorporation of an electron-withdrawing *meta*-chlorine atom (c.f. Table 2, entry 4 *vs.* entry 7), the nature of the substituent on the phenyl ring had little effect on the observed biological activity of these analogues. Given the flat SAR for **7f–j** and the relatively low yields associated with substitution at R^4, alternative sites for attachment of the linker were explored and the R^4 = H substitution was retained.

Variation of the *N*2-substituent of the hydantoin ring in *trans*-**7a** was considered first as a possible linker attachment site (Scheme 1A for numbering system). Exchange of the ^{n}butyl substituent for an ^{n}octyl group was achieved by reaction of **2a** with ^{n}octylisocyanate followed by epimerisation at C11a to give *trans*-**7k** (Scheme 1B). This analogue retained the desired biological activity, albeit at a reduced level (Table 2, entry 8). This



Scheme 3 Synthetic routes to chemical inducers of dimerisation (CIDs) **15** and **23**. Reagents and conditions: (i) TsCl, Ag_2O, KI, DCM, 84%; (ii) NaN_3, DMF, 110 °C, 93%; (iii) PPh_3, THF, H_2O, 98%; (iv) Boc_2O, DCM, 89%; (v) MsCl, DIPEA, DCM, 75%; (vi) NaN_3, DMF, 110 °C, 89%; (vii) PS-PPh_3, THF, H_2O, quant.; (viii) LiOH, THF, H_2O, 66%; (ix) **16**, TPTU, Et_3N, DMF, 45%; (x) TFA, DCM; (xi) **21**, TPTU, DIPEA, DMF, 65% over 2 steps; (xii) TFA, DCM, 55%; (xiii) **18**, DEAD, PPh_3, THF, 62%; (xiv) TFA, DCM; (xv) **21**, EDC, HOBt, DIPEA, DCM, DMF, 63% over 2 steps; (xvi) TFA, DCM, 66%.

result suggested that the *N*2-position could be a suitable point of attachment for a linker unit and led to the synthesis and testing of *trans*-**7l**, an analogue containing a shorter alkyl chain terminating in an ester functionality (Scheme 1B). It was envisaged that hydrolysis of the ester would provide a carboxylic acid functional group that could be used to attach the linker unit using a standard amide bond-forming reaction. Unfortunately, **7l** proved inactive in the parasite invasion assay. Before abandoning this approach, however, an alternative ester analogue, *trans*-**7m**, was prepared that contained an aryl spacer between *N*2 (Scheme 1B) and the required ester functionality. **7m** did inhibit *T. gondii* invasion (Table 2, entry 10), thereby providing a suitable structure for synthetic transformation to the desired CID **15** (Scheme 3). The different activities of **7l** and **7m** may be due to differences in the levels to which the esters are hydrolysed under the assay conditions in live parasites.

Synthesis of CID 15

Scheme 3 shows the route used to access **15**. A key component of this reaction sequence was synthesis of the PEG linker unit **16**, which contained both a primary amine functional group and a second suitably protected amine allowing for sequential amide bond-forming reactions. Previous reports[23] described the synthesis of **16** in relatively low yield (17% yield over 5 steps from hexaethylene glycol (**17**)). We initially converted **17** to the Boc-protected amine **18** in a four step sequence. This involved mono-tosylation of **17** and substitution of the resulting tosylate using

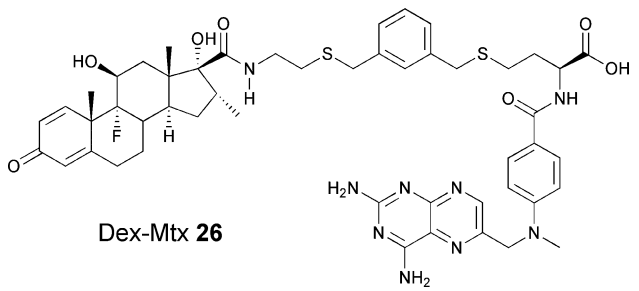
sodium azide, followed by Staudinger reduction of the azide and *N*-protection.[23a] Subsequent conversion of **18** to the corresponding mesylate, followed by reaction with sodium azide and reduction of the azide functionality using polymer supported triphenyl phosphine led to a significant improvement in the overall yield of **16** (46% over 7 steps from **17**). The use of the solid phase reagent in the final step circumvented purification challenges associated with **16**. Coupling of the linker unit **16** to *trans*-**7m** was then carried out by hydrolysis of the ethyl ester functionality in **7m** to give the corresponding acid **19**. TPTU-mediated coupling of **19** with **16** afforded **20** in acceptable yield (Scheme 3). The preparation of acid **21**,[24] a suitably protected version of methotrexate (**14**), allowed the synthesis of **15** to be completed. Boc-deprotection of **20** followed by a second TPTU-mediated coupling step, this time with **21**, gave the desired CID **15** after hydrolysis of the ^{t}butyl ester in **22**. Extensive characterisation of **15** using a range of NMR techniques and mass spectroscopic analysis were consistent with the structure of **15** as drawn.[10]

Synthesis of CID 23

Early studies had demonstrated that substitution of the phenolic oxygen in *trans*-**7a** with a methyl group led to an analogue **9i** that retained the desired biological activity (Table 1, entry 11). Substitution at this position was therefore further investigated using a parallel synthesis approach (Scheme 2A). Our initial alkylation protocol used the Mitsunobu reaction giving **9i** in good yield. However, this method was impractical for parallel synthesis applications. Instead, analogues **9ii–9xii** were prepared by treating *trans*-**7a** with one equivalent of the appropriate alkyl halide in the presence of excess potassium carbonate. This method allowed the facile isolation of the desired compounds following filtration through a short plug of silica gel. Variation of the phenolic substitutent by extending the alkyl chain from methyl up to ^{n}butyl (**9i–9iv**) had little effect on activity (Table 2 entries 11–14). However, the incorporation of either unsaturated and/or branched *O*-alkyl chains led to either retention (**9v**, Table 1, entry 15) or more frequently an increase in activity. For example, the *O-iso*-propyl analogue **9vi** consistently inhibited *T. gondii* host cell invasion at a final concentration of 1.6 μM (Table 2, entry 16) and **9vii**, an analogue containing a 2-methylallyl substituent, was active at 3.1 μM (Table 2, entry 17). The incorporation of an *O*-propargyl group in **9viii** also led to an increase in potency (Table 1, entry 18) but plans to access a series of 1,2,3-triazole-containing analogues from **9viii** were abandoned when it was determined that incorporation of a structurally related *O*-benzyl substituent was detrimental to activity (Table 2, entries 20 and 21).

The ability to substitute the phenolic oxygen in *trans*-**7a** with a range of different alkyl groups whilst retaining activity suggested to us that this may also be an appropriate place to incorporate a linker unit. However, as the introduction of an amide group at this position led to decreased activity (Table 2, entry 22), we decided to use the previously prepared linker unit **18** (Scheme 3). Mitsunobu reaction of *trans*-**7a** with **18** gave the phenolic ether **24**, which was converted to the desired CID **23** *via* the ^{t}butyl ester **25** in 26% yield over 3 steps. Extensive analysis of **23** using a range of NMR techniques supported the structural assignment of **23**.[10]

Table 3 Structure of control CID Dex-Mtx **26**. Strain V1019Y was incubated for 48 hrs at 30 °C in medium with and without leucine, in the presence of the CID and competitors indicated. Entry 3 confirms that Dex-Mtx can support the growth of V1019Y in selective medium (compare to entry 2). This growth was competitively inhibited by methotrexate, (**14**) (entry 4), **15** and **23** (entries 5–6)



Dex-Mtx **26**

Entry	Medium	CID	Competitor	OD_{600} (48 hrs)
1	+ Leu	—	—	1.30
2	– Leu	—	—	0.04
3	– Leu	10 μM **26**	—	0.93
4	– Leu	10 μM **26**	100 μM Mtx (**14**)	0.05
5	– Leu	10 μM **26**	100 μM **15**	0.00
6	– Leu	10 μM **26**	100 μM **23**	0.00
7	+ Leu	—	100 μM **15**	1.31
8	+ Leu	—	100 μM **23**	1.31
9	– Leu	—	100 μM **15**	0.03
10	– Leu	—	100 μM **23**	0.05

Chemical inducers of dimerisation (CID) 15 and 23 are suitable for use as target identification reagents

In order to perform a Y3H screen, it is a prerequisite that the synthesised CIDs are cell-permeable and can gain access to the yeast nucleus, the location of the transcription factor fusion proteins that allow selection of positive small molecule-target interactions (see Figure S4[10]). The cell- and nuclear-permeability of **15** and **23**, our two CIDs based on **1**, were assessed by a competition assay using a positive control strain of yeast, V1019Y. This strain has previously been used to validate other CIDs in a similar manner to the one described here.[21b] V1019Y is a leucine (Leu) auxotroph and, in the absence of any CID, is only able to grow in medium containing leucine. However, V1019Y is engineered to activate expression of a *LEU2* reporter when there is a positive interaction between a control CID, Dex-Mtx **26** (Mtx (**14**) linked to dexamethasone, Table 3), and a control target fusion protein (the rat glucocorticoid receptor fused to the B42 activation domain) resulting in CID-dependent leucine prototrophy and restoration of growth in the absence of exogenous leucine.

Strain V1019Y was inoculated into a 96-well plate and growth was assessed under a variety of conditions by measuring the OD_{600} after incubation for 48 hours at 30 °C. As expected, in the absence of any CID, strain V1019Y only grew when leucine was provided exogenously (Table 3, entry 1; compare to no growth in the absence of leucine, entry 2). In agreement with previous reports,[21b] we showed that incubation of V1019Y with Dex-Mtx **26** (10 μM) was sufficient to induce a transcriptional response and supported growth in the absence of leucine (Table 3, entry 3). Also as previously reported,[21b] the addition of excess Mtx (**14**) to the medium was found to abrogate the transcriptional activation

of the *LEU2* reporter through competition with Dex-Mtx **26** for binding to the dihydrofolate reductase (DHFR)-containing fusion protein present in V1019Y (see Table 3, entry 4 and Figure S4[10]) We then proceeded to test whether **15** and **23** could compete with Dex-Mtx **26** in an analogous manner.

Like Mtx (**14**), both **15** and **23** (100 μM) are able to inhibit the CID-dependent growth of V1019Y in the presence of Dex-Mtx **26**, indicating that they do indeed compete for binding to DHFR (Table 3, entries 5–6). Further control experiments demonstrated that the observed growth inhibition is not due to cytotoxicity associated with **15** or **23** (Table 3, entries 7 and 8) and that neither **15** or **23** was able to autoactivate the *LEU2* reporter in the absence of the rat glucocorticoid receptor-B42 activation domain fusion (Table 3, entries 9 and 10). These data strongly suggest that both **15** and **23** are cell- and nuclear-permeable and are therefore suitable for Y3H screening experiments.

Conclusion

The ability of cell-based high-throughput assays to deliver compounds that elicit the desired response in cells is very powerful.[25] For example, our previous studies led to the identification of 24 non-cytotoxic inhibitors of host cell invasion by the parasite *Toxoplasma gondii*.[6] However, this approach is limited by the challenges inherent in identifying the mode of action of the bioactive compounds. Here we describe our follow-up studies on the *T. gondii* invasion inhibitor **1**. Our initial studies identified *trans*-**7a** as the most active isomer of **1**, a result that led us to propose that parasite kinesins or phosphodiesterases may be the relevant target(s) following chemoinformatic analysis. A more detailed analysis of these possibilities using bioinformatic approaches and a comparison of our SAR data with that existing in the literature suggested that the mechanism of action of **1** is most likely through interaction with a different class of protein. A range of genome/proteome wide approaches to target identification was therefore considered culminating in a focus on the use of the recently reported yeast-3-hybrid (Y3H) method.[21] A key challenge in this approach is the synthesis of the required CIDs. Here we report the synthesis of two such CIDs, **15** and **23**, based on **1**. The CIDs differ in the point of attachment of the linker and methotrexate units. We also demonstrate that **15** and **23** have the properties necessary to access the nucleus of yeast cells, an absolute requirement for their use in this approach. Y3H screening using **15** and **23** has recently been initiated and will be the subject of a future report.

Experimental

Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without further purification. PE 40–60 refers to the fraction of light PE 40–60 boiling in the range 40–60 °C. Melting points were recorded using an Electrothermal 9100 capillary melting point apparatus. Values are quoted to the nearest 0.5 °C. IR spectra were measured on a Perkin-Elmer Paragon 1000 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a Brüker Advance 300/500 instrument. *J* values are quoted in Hz. Chemical shifts are calibrated with reference to the residual proton and carbon resonances of the solvent ($CDCl_3$: δH = 7.26, δC = 77.0 ppm). In general, the ^{1}H and ^{13}C NMR spectra of several of the compounds reported were found to be concentration dependent. Low and high-resolution mass spectral analysis were recorded using ES operating in positive or negative ion mode. Details of the synthesis of additional compounds discussed here are included in the supplementary material including an improved spectroscopic characterisation of several literature compounds.

(5*R*, 11a*S*)-2-(4-Benzoic acid ethyl ester)-5-(3-hydroxyphenyl)-6*H*-1,2,3,5,11,11a-hexahydro-imidazo[1,5-*b*]-β-carboline-1,3-dione (7m). **7m** was prepared according to the general protocols described in the ESI from **5a** *via* **2a** and the corresponding *cis*-isomer.

2a (0.5 g, 1.62 mmol) and 4-(ethoxycarbonyl)phenyl isocyanate (0.47 g 2.44 mmol) were stirred at reflux for 3 hours in anhydrous DMSO (0.5 mL) and anhydrous acetone (2 mL). The solution was diluted with water (5 mL) and extracted into EtOAc (3 × 5 mL). The combined organic layers were dried (Na_2SO_4) and reduced *in vacuo*. Purification of the residue by chromatography on silica gel (EtOAc:Hex 20:80) afforded (5*R*,11a*R*)-2-(4-benzoic acid ethyl ester)-5-(3-hydroxyphenyl)-6*H*-1,2,3,5,11,11a-hexahydro-imidazo[1,5-*b*]-β-carboline-1,3-dione as a yellow solid (0.51 g, 1.05 mmol, 65%). m.p. 176.8–177.4 °C; ν_{max} (KBr) 3349, 2981, 1773, 1729 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_4) δ 7.99 (2H, d, *J* 8.8, H3′), 7.52 (1H, d, *J* 6.7, H10), 7.47 (2H, d, *J* 8.8, H2′), 7.18–7.06 (4H, m, H7, 5″, 9, 8), 6.82 (1H, d, *J* 7.8, H6″), 6.70 (1H, s, H2″), 6.63 (1H, d, ^3J 8.3, H4″), 5.73 (1H, s, H5), 4.50 (1H, dd, *J* 11.7, 4.8, H11a), 4.30 (2H, q, *J* 7.17, H7′), 3.45 (1H, dd, *J* 15.6, 4.8, H11$_{syn}$), 3.12 (1H, dd, *J* 15.6, 11.7, H11$_{anti}$), 1.31 (3H, t, *J* 7.2, H8′); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.0 (C=O), 165.9 (C=O), 156.2 (C), 153.2 (C=O), 140.1 (C), 137.3 (C), 136.8 (C), 135.4 (C), 133.0 (C), 130.3 (CH), 130.2 (CH), 126.1 (C), 125.4 (CH), 122.9 (CH), 120.3 (CH), 119.9 (CH), 118.5 (CH), 115.9 (CH), 114.7 (CH), 111.3 (CH), 106.6 (C), 61.2 (CH_2), 57.8 (CH), 56.8 (CH), 22.6 (CH_2), 14.3 (CH_3); LRMS (ES$^+$) *m/z*: 482.30 [M + H]$^+$; HRMS (ES$^+$) [M + H]$^+$ *m/z* expected for $C_{28}H_{24}N_3O_5$ 482.1716 found 482.1733 $[\alpha]_D^{20} = +0.8$ ($c = 0.007$, EtOAc). The *cis*-isomer (0.49 g, 1.01 mmol) was then dissolved in anhydrous MeCN (12 mL) and K_2CO_3 (0.182 g, 1.32 mmol) was added. After stirring at reflux for 5 hours, the mixture was filtered and the solvent was removed *in vacuo*. Purification of the residue by chromatography on silica gel (EtOAC:Hex 20:80) gave **7m** as a yellow solid (0.31 g, 0.64 mmol, 63%). m.p. 178.2–179.1 °C; ν_{max} (KBr) 3349, 2981, 1773, 1729 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.90 (1H, s, NH), 9.50 (1H, s, OH), 8.05 (2H, d, *J* 8.5, H3′), 7.85 (2H, d, *J* 8.5, H2′), 7.57 (1H, d, *J* 8.0 H10), 7.31 (1H, d, *J* 8.2, H7), 7.18 (1H, m, H5″), 7.13–7.08 (1H, m, H9), 7.06–7.00 (1H, m, H8), 6.82 (1H, d, *J* 8.04, H6″), 6.78 (1H, s, H2″), 6.73 (1H, d, *J* 7.7, H4″), 6.21 (1H, s, H5), 4.60 (1H, dd, *J* 10.9, 5.6, H11a), 4.33 (2H, q, *J* 10.4, H7′), 3.42 (1H, dd, *J* 14.8, 5.02, H11$_{syn}$), 3.08 (1H, dd, *J* 10.65, 4.53, H11$_{anti}$), 1.31 (3H, t, *J* 7.1, H8′); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7 (C=O), 165.8 (C=O), 165.5 (C), 158.1 (C=O), 156.1 (C), 144.4 (C), 136.8 (C), 136.5 (C), 131.4 (C), 130.8 (CH), 130.2 (CH), 129.9 (CH), 126.1 (C), 122.9 (CH), 119.3 (CH), 118.6 (CH), 117.9 (CH), 115.6 (CH), 115.4 (CH), 111.8 (CH), 106.5 (C), 61.3 (CH_2), 53.2 (CH), 52.1 (CH), 22.9 (CH_2), 14.7 (CH_3); LRMS (ES$^+$) *m/z*: 482.29 [M + H]$^+$; HRMS (ES$^+$) [M + H]$^+$ *m/z* expected for $C_{28}H_{24}N_3O_5$ 482.1716 found 482.1709 $[\alpha]_D^{20} = -258.0$ ($c = 0.003$, EtOAc).

General procedure for the alkylation of 7a

To a solution of **7a** (200 mg, 0.51 mmol) in anhydrous MeCN (2 mL) was added K_2CO_3 (105.7 mg, 0.77 mmol) and the appropriate alkyl halide (1.0 eq, 0.51 mmol). After stirring at reflux for 18 hrs, the reaction mixture was diluted with H_2O (2 mL). The mixture was extracted with DCM (2 × 2 mL) and the combined extracts were dried (Na_2SO_4) and concentrated *in vacuo.* Purification of the residue by filtration through a plug of silica (EtOAc:Hex 1:9) afforded the desired product.

(5*R*,11a*S*)-2-Butyl-5-(3-propoxyphenyl)-6*H*-1,2,3,5,11,11a-hexahydro-imidazo[1,5-*b*]-β-carboline-1,3-dione (9iii). Yield 63%, yellow oil. ν_{max} (KBr) 2960, 1713, 1490, 1222 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.49 (1H, d, *J* 7.5, H10), 7.25–7.07 (4H, m, H8, 9, 5″, 7), 6.87–6.78 (3H, m, H4″, 2″, 6″), 6.19 (1H, s, H5), 4.25 (1H, dd, *J* 11.0, 5.5, H11a), 3.80 (2H, t, *J* 6.6, H8″), 3.51–3.39 (3H, m, H1′, 11_{syn}), 2.81 (1H, dd, *J* 15.3, 11.0, $H11_{anti}$), 1.75–1.64 (2H, m, H9″), 1.59–1.51 (2H, m, H2′), 1.30–1.22 (2H, m, H3′), 0.93 (3H, t, *J* 7.6, H10″), 0.86 (3H, t, *J* 7.4, H4′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.8 (C=O), 160.2 (C), 155.0 (C=O), 140.7 (C), 136.6 (C), 130.4 (C), 130.2 (CH), 126.2 (C), 122.9 (CH), 120.3 (CH), 120.2 (CH), 118.4 (CH), 114.1 (CH), 114.0 (CH), 111.2 (CH), 108.1 (C), 69.5 (CH_2), 53.4 (CH), 51.9 (CH), 38.6 (CH_2), 30.2 (CH_2), 23.5 (CH_2), 22.5 (CH_2), 20.0 (CH_2), 13.6 (CH_3), 10.5 (CH_3); LRMS (ES^+) *m*/*z*: 432.33 (100%) $[M + H]^+$. $[\alpha]_D^{20} = -204.2$ (*c* = 0.00034, DCM) HRMS (ES^+) $[M + H]^+$ *m*/*z* expected for $C_{26}H_{30}N_3O_3$ 432.2107 obtained 432.2110.

(5*R*,11a*S*)-2-Butyl-5-(3-allyloxyphenyl)-6*H*-1,2,3,5,11,11a-hexahydro-imidazo[1,5-*b*]-β-carboline-1,3-dione (9v). Yield 74%, yellow solid. m.p. 85.4–86.0 °C; ν_{max} (KBr) 2963, 1710, 1499, 1235 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.49 (1H, d, *J* 7.5, H10), 7.25–7.07 (4H, m, H8, 9, 5″, 7), 6.87–6.78 (3H, m, H4″, 2″, 6″), 6.19 (1H, s, H5), 5.99–5.87 (1H, m, H9″), 5.30 (1H, d, *J* 17.3, $H10''_{trans}$), 5.12 (1H, d, *J* 10.5, $H10''_{cis}$), 4.45–4.39 (2H, m, H8″), 4.25 (1H, dd, *J* 11.0, 5.5, H11a), 3.51–3.39 (3H, m, H1′, 11_{syn}), 2.81 (1H, dd, *J* 15.3, 11.0, $H11_{anti}$), 1.59–1.51 (2H, m, H2′), 1.30–1.22 (2H, m, H3′), 0.86 (3H, t, *J* 7.4, H4′); ^{13}C NMR (100 MHz, $CDCl_3$), δ 172.8 (C=O), 160.2 (C), 155.0 (C=O), 140.7 (C), 136.6 (C), 130.2 (CH), 130.4 (C), 130.2 (CH), 126.2 (C), 122.9 (CH), 120.3 (CH), 120.2 (CH), 118.4 (CH), 118.0 (CH_2), 114.1 (CH), 114.0 (CH), 111.2 (CH), 108.1 (C), 68.9 (CH_2), 53.4 (CH), 51.9 (CH), 38.6 (CH_2), 30.2 (CH_2), 23.5 (CH_2), 20.0 (CH_2), 13.6 (CH_3); LRMS (ES^+) *m*/*z*: 430.33 (100%) $[M + H]^+$. $[\alpha]_D^{20} = -206.5$ (*c* = 0.00441, DCM) HRMS (ES^+) $[M + H]^+$ *m*/*z* expected for $C_{26}H_{28}N_3O_3$ 430.1577 obtained 430.1585.

(5*R*,11a*S*)-2-Butyl-5-(3-*iso*propoxyphenyl)-6*H*-1,2,3,5,11,11a-hexahydro-imidazo[1,5-*b*]-β-carboline-1,3-dione (9vi). Yield 61%, yellow solid. m.p. 105–106 °C; ν_{max} (KBr) 3320, 2957, 1766, 1701, 1454, 1236 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.79 (1H, s, NH), 7.56 (1H, d, *J* 7.7, H10), 7.31–7.15 (4H, m, H8, 9, 5″, 7), 6.89–6.84 (3H, m, H6″, 4″, 2″), 6.27 (1H, d, *J* 1.3, H5), 4.52 (1H, sept, *J* 6.1, H8′), 4.33 (1H, dd, *J* 11.1, 5.6, H11a), 3.57–3.48 (3H, m, H1′, 11_{syn}), 2.88 (1H, ddd, *J* 15.3, 11.1, 1.8, $H11_{anti}$), 1.65–1.57 (2H, m, H2′), 1.39–1.29 (8H, m, H9, H3′), 0.92 (3H, t, *J* 7.4, H4′); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9 (C=O), 158.5 (C), 155.0 (C=O), 140.7 (C), 136.6 (C), 130.5 (C), 130.2 (CH), 126.2 (C), 122.9 (CH), 120.2 (CH), 120.1 (CH), 118.5 (CH), 115.9 (CH), 115.5 (CH), 111.2 (CH), 108.1 (C), 69.9 (CH), 53.3 (CH), 52.0 (CH), 38.6 (CH_2), 30.3 (CH_2), 23.6 (CH_2), 22.0 (CH_3), 21.9 (CH_3), 20.0 (CH_2), 13.7 (CH_3); LRMS (ES^+) *m*/*z*: 454.13 (100%) $[M + Na]^+$; HRMS (ES^+) $[M + Na]^+$ *m*/*z* expected for $C_{26}H_{29}N_3O_3Na$ 454.2107 obtained 454.2110; $[\alpha]_D^{20} = -198.2$ (*c* = 0.0011, $CHCl_3$).

(5*R*,11a*S*)-2-Butyl-5-(3-(2-methyl-allyloxy)phenyl)-6*H*-1,2,3,5,11,11a-hexahydro-imidazo[1,5-*b*]-β-carboline-1,3-dione (9vii). Yield 71%, yellow solid. m.p. 94–95 °C; ν_{max} (KBr) 3332, 2956, 1765, 1702, 1454, 1237 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.78 (1H, s, NH), 7.56 (1H, d, *J* 7.5, H10), 7.32–7.15 (4H, m, H8, 9, 5″, 7), 6.93–6.88 (3H, m, H6″, 4″, 2″), 6.27 (1H, d, *J* 1.2, H5), 5.03, (1H, s, H10″), 4.96 (1H, s, H10″), 4.38 (2H, s, H8″) 4.32 (1H, dd, *J* 11.0, 5.5, H11a), 3.56–3.48 (3H, m, H1′, 11_{syn}), 2.88 (1H, ddd, *J* 15.3, 11.0, 1.8, $H11_{anti}$), 1.79 (3H, s, H11″), 1.65–1.57 (2H, m, H2′), 1.40–1.27 (2H, m, H3′), 0.93 (3H, t, *J* 7.4, H4′); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.8 (C=O), 159.2 (C), 154.9 (C=O), 140.6 (C), 140.5 (C), 136.6 (C), 130.4 (CH), 130.1 (C), 126.1 (C), 122.8 (CH), 120.4 (CH), 120.1 (CH), 118.4 (CH), 114.7 (CHx2), 113.1 (CH_2), 111.2 (CH), 107.9 (C), 71.7 (CH_2), 53.3 (CH), 51.9 (CH), 38.6 (CH_2), 30.2 (CH_2), 23.5 (CH_2), 20.0 (CH_2), 19.2 (CH_3), 13.6 (CH_3); LRMS (ES^+) *m*/*z*: 466.13 (100%) $[M + Na]^+$; HRMS (ES^+) $[M + Na]^+$ *m*/*z* expected for $C_{27}H_{29}N_3O_3Na$ 466.2107 obtained 466.2087; $[\alpha]_D^{20} = -194.4$ (*c* = 0.00125, $CHCl_3$).

(5*R*,11a*S*)-2-Butyl-5-(3-prop-2-ynyloxyphenyl)-6*H*-1,2,3,5,11,11a-hexahydro-imidazo[1,5-*b*]-β-carboline-1,3-dione (9viii). Yield 72%, yellow solid. m.p. 92–94 °C; ν_{max} (KBr) 3329, 2973, 1767, 1700, 1456, 1238 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.80 (1H, s, NH), 7.56 (1H, d, *J* 7.5, H10), 7.32–7.16 (4H, m, H8, 9, 5″, 7), 7.01–6.94 (3H, m, H6″, 4″, 2″), 6.29 (1H, d, *J* 1.4, H5), 4.65 (2H, d, *J* 2.4, H8″), 4.32 (1H, dd, *J* 11.0, 5.5, H11a), 3.56–3.49 (3H, m, H1′, 11_{syn}), 2.88 (1H, ddd, *J* 15.3, 11.0, 1.8, $H11_{anti}$), 2.46 (1H, t, *J* 2.4, H10″), 1.65–1.58 (2H, m, H2′), 1.40–1.29 (2H, m, H3′), 0.93 (3H, t, *J* 7.4, H4′); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.8 (C=O), 158.0 (C), 154.9 (C=O), 140.7 (C), 136.6 (C), 130.2 (CH), 130.2 (C), 126.1 (C), 122.9 (CH), 121.3 (CH), 120.1 (CH), 118.4 (CH), 114.9 (CH), 114.8 (CH), 111.2 (CH), 108.1 (C), 75.8 (C), 75.6 (CH), 55.8 (CH_2), 53.3 (CH), 51.8 (CH), 38.6 (CH_2), 30.2 (CH_2), 23.5 (CH_2), 20.0 (CH_2), 13.6 (CH_3); LRMS (ES^+) *m*/*z*: 450.11 (100%) $[M + Na]^+$; HRMS (ES^+) $[M + Na]^+$ *m*/*z* expected for $C_{26}H_{25}N_3O_3Na$ 450.1794 obtained 454.1806; $[\alpha]_D^{20} = -178.3$ (*c* = 0.0012, $CHCl_3$).

(5*R*,11a*S*)-2-Butyl-5-(3-benzyloxyphenyl)-6*H*-1,2,3,5,11,11a-hexahydro-imidazo[1,5-*b*]-β-carboline-1,3-dione (9ix). Yield 84%, yellow solid. m.p. 101–102 °C; ν_{max} (KBr) 3328, 2932, 1767, 1701, 1456, 1238 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.01 (1H, s, NH), 7.56 (1H, d, *J* 7.5, H10), 7.39–7.16 (9H, m, H8, 9, 5″, 7, 10″, 11″, 12″), 6.96–6.60 (3H, m, H6″, 4″, 2″), 6.26 (1H, d, *J* 1.4, H5), 5.01 (2H, d, *J* 1.9, H8″), 4.25 (1H, dd, *J* 11.0, 5.5, H11a), 3.56–3.45 (3H, m, H1′, 11_{syn}), 2.87 (1H, ddd, *J* 15.3, 11.0, 1.8, $H11_{anti}$), 1.66–1.56 (2H, m, H2′), 1.37–1.30 (2H, m, H3′), 0.93 (3H, t, *J* 7.4, H4′); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.8 (C=O), 159.2 (C), 154.9 (C=O), 140.6 (C), 136.6 (C), 136.5 (C), 130.3 (C), 130.2 (CH), 128.6 (CH x2), 128.1 (CH), 127.5 (CH x2), 126.1 (C), 122.8 (CH), 120.6 (CH), 120.1 (CH), 118.4 (CH), 115.0 (CH), 114.7 (CH), 111.2 (CH), 108.0 (C), 70.0 (CH_2), 53.2 (CH), 51.9 (CH), 38.6 (CH_2), 30.2 (CH_2), 23.5 (CH_2), 20.0 (CH_2), 13.6 (CH_3); LRMS (ES^+) *m*/*z*: 502.09 (100%) $[M + Na]^+$; HRMS (ES^+) $[M + Na]^+$ *m*/*z*

expected for $C_{30}H_{29}N_3O_3Na$ 502.2107 obtained 502.2102; $[\alpha]_D^{20} = -181.7$ ($c = 0.0012$, $CHCl_3$).

(5*R*,11a*S*)-2-(4-Benzoic acid)-5-(3-hydroxyphenyl)-6*H*-1,2,3,5,11,11a-hexahydro-imidazo[1,5-*b*]-β-carboline-1,3-dione (19). To a solution of **7m** (0.200 g, 0.42 mmol) in THF (10 mL) was added LiOH (0.101 g, 0.42 mmol) in H_2O (5 mL), and left to stir overnight. The solvent was reduced, and the residue was partitioned between EtOAc (10 mL) and H_2O (10 mL). The aqueous layer was separated, adjusted to pH 4 with 2M HCl, and extracted with EtOAc (3 × 10 mL). These combined extracts were washed with brine, dried (Na_2SO_4) and reduced *in vacuo.* Purification of the residue by chromatography on silica gel (DCM:MeOH 98:2) afforded **19** as a yellow solid (0.132 g, 0.29 mmol, 66%). m.p. 155.7–158.3 °C; ν_{max} (KBr) 3352, 1726, 1245, 1123 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 10.97 (1H, s, NH), 9.49 (1H, s, OH), 8.03 (2H, d, *J* 8.7, H3′), 7.62–7.55 (3H, m, H7, 2′), 7.31 (1H, d, *J* 8.0, H10), 7.20–7.15 (1H, m, H5″), 7.13–7.08 (1H, m, H9), 7.06–7.00 (1H, m, H8), 6.82 (1H, d, *J* 7.8, H6″), 6.77 (1H, s, H2″), 6.74 (1H, d, *J* 8.0, H4″), 6.22 (1H, s, H5), 4.60 (1H, dd, *J* 10.3, 5.8, H11a), 4.05 (1H, s, OH), 3.41 (1H, dd, *J* 15.1, 5.8, $H11_{syn}$), 3.12–3.05 (1H, m, $H11_{anti}$); ^{13}C NMR (100 MHz, DMSO-d_4) δ 171.8 (C=O), 167.2 (C=O), 158.1 (C), 153.3 (C=O), 141.5 (C), 136.8 (C), 136.5 (C), 136.3 (C), 131.4 (C), 130.2 (CH), 130.2 (CH), 126.4 (CH), 122.2 (CH), 119.3 (CH), 119.1 (CH), 118.7 (CH), 115.7 (CH), 115.4 (CH), 111.9 (CH), 106.6 (C), 53.3 (CH_2), 52.2 (CH_2), 23.0 (CH_2); LRMS (ES^+) *m/z*: 454.24 $[M + H]^+$; HRMS (ES^+) $[M + H]^+$ *m/z* expected for $C_{26}H_{20}N_3O_5$ 454.1403 found 454.1437 $[\alpha]_D^{20} = -114.5$ ($c = 0.00031$, MeOH).

Synthesis of 20. TPTU (32.8 mg, 0.11 mmol) was added to a solution of **19** (50 mg, 0.11 mmol) and Et_3N (15.4 uL, 0.11 mmol) in anhydrous DMF (2 mL) and stirred for 18 hrs. A solution of **16** (41.91 mg, 0.11 mmol) in anhydrous DMF (2 mL) was added and stirred for 3 days. The solvent was reduced and the residue was partitioned between EtOAc (10 mL) and H_2O (10 mL). The organic layer was washed with sat. $NaHCO_3$ (2 × 5 mL), brine (5 mL), dried (Na_2SO_4) and reduced *in vacuo.* Purification of the residue by chromatography on silica gel (DCM:MeOH 97:3) afforded **20** as a yellow solid (40.3 mg, 0.049 mmol, 45%). m.p. 64.1–64.3 °C; ν_{max} (KBr) 3334, 2872, 1720, 1507, 1247 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.86 (2H, d, *J* 8.7, H3′), 7.51–7.45 (3H, m, H7, 2′), 7.26–7.08 (4H, m, H8, 9, 5″, 7), 6.91–6.80 (3H, m, H4″, 2″, 6″), 6.30 (1H, s, H5), 3.63–3.37 (25H, m, H_{linker}, 11_{syn}), 3.05–2.93 (1H, m, $H11_{anti}$), 1.35 (9H, s, H9′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6 (C=O), 167.0 (C=O), 159.6 (C), 153.7 (C=O), 140.7 (C), 137.1 (C), 137.0 (C), 136.7 (C), 134.7 (CH), 130.7 (CH), 130.5 (C), 128.4 (CH x2), 126.5 (C), 125.9 (CH x2), 123.4 (CH), 121.1 (CH), 120.6 (CH), 118.9 (CH), 115.4 (CH), 115.3 (CH), 111.8 (CH), 108.2 (C), 70.3–70.9 (CH_2 x10), 70.5(C), 53.6 (CH) 52.6 (CH) 40.3 (CH_2) 28.8 (CH_3), 24.1 (CH_2); LRMS (ES^+) *m/z*: 815.20 $[M + H]^+$; HRMS (ES^+) $[M + H]^+$ *m/z* expected for $C_{43}H_{54}N_5O_{11}$ 816.3820 found 816.1753 $[\alpha]_D^{20} = -104.3$ ($c = 0.00015$, DCM).

Synthesis of 22. **20** (26 mg, 0.032 mmol) was dissolved in DCM (3 mL) and TFA (50 μL, 0.7 mmol) was added. The solution was stirred for 5 minutes then the solvent was removed *in vacuo.* The crude reaction product **21** was used in the next step without purification. (^1H NMR (400 MHz, $CDCl_3$) δ 7.86 (2H, d, *J* 8.7, H3′), 7.53–7.45 (3H, m, H7, 2′), 7.20–7.03 (4H, m, H8, 9, 5″, 7), 6.87–6.55 (3H, m, H4″, 2″, 6″), 6.15 (1H, s, H5), 3.63–3.37 (25H, m, H_{linker}, 11_{syn}), 2.97–2.83 (1H, m, $H11_{anti}$); MS (ES^+) *m/z*: 716 $[M + H]^+$).

21 (16.4 mg, 0.032 mmol), TPTU (9.31 mg, 0.032 mmol) and DIPEA (209.2 μL, 1.28 mmol) in anhydrous DMF (1 mL) was stirred for 18 hrs. A solution of the above amine (23 mg, 0.032 mmol) in anhydrous DMF (1 mL) was added and stirred for 3 days. The solvent was reduced *in vacuo* and the residue purified by chromatography on silica gel (0–10% MeOH in DCM) to afford **22** as a bright yellow solid (25.11 mg, 0.021 mmol, 65%). m.p. 74.1–75.5 °C; ν_{max} (KBr) 3449, 1715, 1509, 1364, 1224, 1093 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 10.97 (1H, s, NH), 9.50 (1H, s, OH), 8.56 (1H, s, H_{mtx}), 8.24 (1H, d, *J* 7.7, H_{mtx}), 7.85 (2H, d, *J* 8.4, H3′), 7.71 (2H, d, *J* 8.4, H2′), 7.58–7.51 (3H, m, H7, H_{mtx}), 7.31 (1H, d, *J* 7.7, H10), 7.17 (1H, dd, *J* 7.5, 8.0, H5″), 7.10 (1H, dd, *J* 7.7, 7.0, H9), 7.03 (1H, dd, *J* 7.7, 7.0, H8), 6.84–6.72 (5H, m, H6″, H2″, H4″, H_{mtx}), 6.20 (1H, s, H5), 4.77 (2H, s, H_{mtx}), 4.70 (1H, dd, *J* 10.5, 5.6, H11a), 4.23–4.16 (1H, m, H_{mtx}), 3.66–3.06 (26H, m, H_{linker}, 11_{anti}, 11_{syn}), 3.20 (3H, s, H_{mtx}), 2.24–2.11 (2H, m, H_{mtx}), 2.04–1.82 (2H, m, H_{mtx}), 1.37 (9H, s, H_{mtx}); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.1 (C=O), 171.6 (C=O), 171.5 (C=O), 171.3 (C=O), 166.2 (C=O), 165.5 (C), 164.1 (C), 162.9 (C), 162.6 (C), 157.6 (C), 155.2 (C), 152.8 (C=O), 150.8 (C), 149.0 (CH), 141.05 (C), 136.6 (C), 134.2 (C), 133.5 (C), 130.9 (C), 129.7 (CH), 128.8 (CH x2), 127.5 (CH x2), 126.2 (CH x2), 125.7 (C), 121.7 (CH), 121.1 (C), 118.8 (CH), 118.6 (CH), 118.2 (CH), 115.2 (CH), 114.9 (CH), 111.3 (CH), 110.9 (CH x2), 106.0 (C), 80.2 (C), 69.7–68.8 (CH_2), 54.8 (CH_2), 53.4 (CH), 52.9 (CH), 51.6 (CH), 39.5 (CH_3), 31.7 (CH_2), 27.6 (CH_3), 26.4 (CH_2), 22.5 (CH_2); LRMS (ES^+) *m/z*: 1230.76 (100%) $[M + Na]^+$, 1208.87 (80%) $[M + H]^+$.

Synthesis of CID 15. **22** (150 mg, 0.12 mmol) was dissolved in DCM (3 mL) and TFA (500 μL, 1:6 TFA:DCM) was added. The solution was stirred at room temperature for 5 hrs. Toluene (5 mL) was added and the solvent was removed *in vacuo.* Purification of the residue by chromatography on silica gel (0–30% MeOH in DCM) afforded **15** as a yellow solid (75 mg, 0.065 mmol, 55%). m.p. 76.5–77.0 °C; ν_{max} (KBr) 3433, 2925, 1638, 1421, 1204, 1099 cm^{-1}; ^1H NMR (400 MHz, $(CD_3)_2SO$) δ 10.97 (1H, s, NH), 9.50 (1H, s, OH), 8.56 (1H, s, H23′), 8.24 (1H, d, *J* 7.4, NH), 7.96 (2H, d, *J* 8.8, H3′,), 7.74 (2H, d, *J* 8.8, H2′), 7.60–7.53 (3H, m, H7, H_{mtx}), 7.31 (1H, d, *J* 8.1, H10), 7.17 (1H, dd, *J* 7.7, H5″), 7.13 (1H, dd, *J* 8.1, 7.7, H9), 7.03 (1H, dd, *J* 7.6, 7.4, H8), 6.84–6.72 (5H, m, H6″, H2″, H4″, H_{mtx}), 6.23 (1H, s, H5), 4.79 (2H, s, H_{mtx}), 4.72 (1H, dd, *J* 10.5, 5.6, H11a), 4.33–4.26 (1H, m, H_{mtx}), 3.66–3.26 (26H, m, H_{linker}, 11_{syn}), 3.22 (3H, s, H_{mtx}), 3.09 (1H, m, $H11_{anti}$), 2.24–2.18 (2H, m, H_{mtx}), 2.09–1.87 (2H, m, H_{mtx}); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 173.8 (C=O), 171.8 (C=O), 171.6 (C=O),171.4 (C=O), 166.2 (C=O), 165.5 (C), 164.1 (C), 162.9 (C), 162.8 (C), 157.7 (C), 155.2 (C), 153.0 (C=O), 151.0 (C), 149.2 (C), 141.2 (C), 136.8 (C), 134.4 (C), 133.7 (C), 131.1 (C), 129.8 (CH), 128.9 (CHx2), 127.7 (CHx2), 126.3 (CHx2), 125.8 (C), 121.7 (CH), 121.2 (C), 118.9 (CH), 118.6 (CH), 118.3 (CH), 115.2 (CH), 115.0 (CH), 111.5 (CH), 111.1 (CHx2), 106.1 (C), 68.8–69.7 (CH_2), 54.9 (CH_2), 52.9 (CH), 52.3 (CH), 51.7 (CH), 38.5 (CH_3), 32.0 (CH_2), 26.6 (CH_2), 22.6 (CH_2); LRMS (ES^-) *m/z*: 1150.57 (100%) $[M-H]^-$.

Synthesis of 24. Diethyl azidocarboxylate (154.3 μL, 0.98 mmol) was added dropwise to a solution of triphenylphosphine (257 mg, 0.98 mmol) in anhydrous THF (12 mL) and stirred for 30 minutes. A solution of **7a** (303.1 mg, 0.78 mmol) and **18** (373.4 mg, 0.98 mmol) in anhydrous THF (15 mL) was added, and the mixture stirred for 48 hrs. The solvent was reduced, and the residue partitioned between EtOAc (30 mL) and H_2O (30 mL). The aqueous layer was extracted with EtOAc (2 × 10 mL) and the combined organics were dried over Na_2SO_4 and reduced. Purification of the residue by chromatography on silica gel (Hex:EtOAc 1:1 to 1:9 as the eluent) gave **24** as a yellow oil (366.2 mg, 0.49 mmol, 62.4%). m.p. 153–154 °C; ν_{max} (KBr) 3332, 2936, 1760, 1695, 1456, 1231 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.38 (1H, s, NH), 7.56 (1H, d, *J* 7.0, H10), 7.32–7.13 (4H, m, H8, 9, 5″, 7), 6.95 (1H, d, *J* 7.7, H6″), 6.88–6.85 (2H, m, H4″, 2″), 6.27 (1H, d, *J* 0.8, H5), 5.08–4.98 (1H, br s, NH), 4.23 (1H, dd, *J* 11.0, 5.5, H11a), 4.08–4.06 (2H, m, H_{linker}), 3.82–3.78 (2H, m, H_{linker}), 3.69–3.46 (21H, m, H_{linker}, $H11_{syn}$, H4′), 3.25–3.20 (2H, m, H_{linker}), 2.88 (1H, ddd, *J* 15.3, 11.0, 1.8, $H11_{anti}$), 1.66–1.56 (2H, m, H2′), 1.43 (9H, s, H_{linker}), 1.37–1.29 (2H, m, H3′), 0.92 (3H, t, *J* 7.4, H4′); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.8 (C=O x2), 159.2 (C), 154.8 (C=O), 140.8 (C), 136.7 (C), 130.5 (C), 129.9 (CH), 126.4 (C), 122.5 (CH), 120.5 (CH), 119.7 (CH), 118.2 (CH), 114.7 (CH), 114.2 (CH), 111.3 (CH), 107.4 (C), 77.1 (C), 70.7 (CH_2), 70.6 (CH_2), 70.6 (CH_2), 70.5 (CH_2), 70.4 (CH_2), 70.1 (CH_2), 69.7 (CH_2), 69.6 (CH_2), 67.3 (CH_2), 53.2 (CH), 51.8 (CH), 40.2 (CH_2), 38.5 (CH_2), 30.1 (CH_2), 28.3 (CH_3), 23.4 (CH_2), 19.9 (CH_2), 13.5 (CH_3); LRMS (ES^+) *m/z*: 753.5 $[M + H]^+$; HRMS (ES^+) $[M + H]^+$ *m/z* expected for $C_{40}H_{57}N_4O_{10}$ 753.4075 obtained 753.4081.

Synthesis of 25. **24** (339.7 mg, 0.45 mmol) was dissolved in DCM (8 mL) and TFA (2 mL, mmol) was added. The solution was stirred for 5 minutes then the solvent was removed *in vacuo.* The crude reaction product was used in the next step without purification. (^{1}H NMR (300 MHz, $CDCl_3$) δ 9.35–9.34 (2H, m, NH), 8.44 (1H, s. NH), 7.55 (1H, d, *J* 7.2, H10), 7.32–7.14 (4H, m, H8, 9, 5″, 7), 6.93–6.84 (3H, m, H6″, 4″, 2″), 6.21 (1H, d, *J* 0.6, H5), 4.42 (1H, dd, *J* 11.0, 5.5, H11a), 4.14–4.12 (2H, m, H_{linker}), 3.86–3.83 (2H, m, H_{linker}) 3.75–3.72 (2H, m, H_{linker}), 3.68–3.49 (19H, m, H_{linker}, $H11_{syn}$, H1′), 3.18–3.13 (2H, m, H_{linker}), 2.92 (1H, ddd, *J* 15.3, 11.1, 1.7, $H11_{anti}$), 1.65–1.55 (2H, m, H2′), 1.40–1.28 (2H, m, H3′), 0.92 (3H, t, *J* 7.4, H4′)).

The residue was dissolved in DCM (8 mL) and DIPEA (374.5 μL, 2.15 mmol) was added. To this was added a solution of **21** (219.5 mg, 0.43 mmol), EDC (92.0 mg, 0.48 mmol) and HOBt (64.9 mg, 0.48 mmol) in anhydrous DMF (8 mL). After stirring for 42 hours, the solvent was removed and the residue partitioned between DCM (20 mL) and H_2O (20 mL). The organic layer was washed with sat $NaHCO_3$ (20 mL), sat NH_4Cl (20 mL), dried (Na_2SO_4) and reduced *in vacuo.* Purification of the residue by chromatography on silica gel (DCM:MeOH 99:1 to 9:1 as the eluent) gave **25** as an orange solid (309.0 mg, 0.27 mmol, 62.7%). m.p. 146–147 °C; ν_{max} (KBr) 3338, 2928, 1765, 1708, 1452, 1244 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.76 (1H, s, NH), 8.57 (1H, s, NH), 7.79 (2H, d, *J* 8.7, H_{mtx}), 7.52 (1H, d, *J* 7.9, H10), 7.37 (1H, d, *J* 7.9, H7), 7.18–7.04 (4H, m, H8, 9, 5″, 6″), 6.85 (1H, s, H_{mtx}), 6.76–6.71 (2H, m, H2″, H4″), 6.69 (2H, d, *J* 8.7, H_{mtx}), 6.30 (1H, s, H5), 4.70 (2H, s, H_{mtx}), 4.51–4.47 (1H, m, H_{mtx}), 4.29 (1H, dd, *J* 11.0, 5.6, H11a), 4.07–3.98 (2H, m, H_{linker}), 3.77–3.75 (2H, m, H_{linker}), 3.69–3.39 (21H, m, H_{linker}, $H11_{syn}$, H_{mtx}), 3.30–3.22 (2H, m, H_{linker}), 3.15 (3H, s, H_{mtx}), 2.87 (1H, dd, *J* 14.0, 11.0, $H11_{anti}$), 2.37–2.32 (2H, m, H_{mtx}), 2.22–2.12 (2H, m, H_{mtx}), 1.62–1.56 (2H, m, H2′), 1.45 (9H, s, H_{mtx}), 1.34–1.24 (2H, m, H3′), 0.91 (3H, t, *J* 7.4, H4′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0 (C=O x2), 172.9 (C=O), 171.5 (C=O), 162.7 (C), 158.9 (C), 154.8 (C=O), 153.1 (C), 152.9 (C), 151.3 (C), 149.5 (CH), 149.3 (C), 141.0 (C), 136.8 (C), 130.7 (C), 129.7 (CH), 128.8 (CH x2), 126.0 (C), 122.3 (CH), 121.8 (C), 121.7 (C), 120.3 (CH), 119.5 (CH), 118.1 (CH), 114.6 (CH), 113.9 (CH), 111.5 (CH x2), 111.4 (CH), 107.0 (C), 81.9 (C), 70.5 (CH_2), 70.5 (CH_2), 70.4 (CH_2), 70.3 (CH_2), 70.0 (CH_2), 69.6 (CH_2), 69.5 (CH_2), 67.2 (CH_2), 55.7 (CH_2), 53.2 (CH), 51.8 (CHx2), 39.3 (CH_2), 39.3 (CH_3), 38.5 (CH_2), 32.4 (CH_2), 30.1 (CH_2), 28.0 (CH_3), 27.8 (CH_2), 23.5 (CH_2), 19.9 (CH_2), 13.6 (CH_3); LRMS (ES^+) *m/z*: $C_{59}H_{76}N_{12}O_{12}Na$ $[M + Na]^+$ 1168.21 (100%), $[M + H]^+$ 1146.27 (7%), $[M + Na]^{2+}$ 584.58 (40%).

Synthesis of CID 23. **25** (179.9 mg, 0.16 mmol) was dissolved in DCM (2 mL) and TFA (0.6 mL, 7.8 mmol) was slowly added. The solution was stirred at RT for 5 hours. Toluene (5 mL) was added and the solvent was removed *in vacuo.* Purification of the residue by chromatography on silica gel (DCM:MeOH 99:1 to 4:1 as the eluent) gave **23** as an orange solid (115.8 mg, 0.11 mmol, 66.4%). m.p. 132–134 °C; ν_{max} (KBr) 3410, 2942, 1761, 1706, 1456, 1232 cm^{-1}; ^{1}H NMR (300 MHz, DMSO) δ 10.88 (1H, s, NH), 8.57 (1H, s, H_{mtx}), 8.32 (1H, s, OH), 8.26 (1H, d, *J* 7.4, NH), 7.89 (1H, t, *J* 5.4, NH), 7.85–7.79 (1H, br s, NH), 7.73 (2H, d, *J* 8.9, H_{mtx}), 7.66–7.58 (1H, br s, NH), 7.54 (1H, d, *J* 7.6, H10), 7.31–7.25 (2H, m, H7, 4″), 7.11–7.06 (1H, m, H5″), 7.04–6.99 (1H, m, H9), 6.97–6.87 (3H, m, H8, 6″, 2″), 6.82 (2H, d, *J* 8.9, H_{mtx}), 6.80–6.72 (2H, br s, NH), 6.17 (1H, s, H5), 4.79 (2H, s, CH_{mtx}), 4.62 (1H, dd, *J* 10.6, 5.4, H11a), 4.31–4.24 (1H, m, H_{mtx}), 4.12–3.98 (2H, m, H_{linker}), 3.70 (2H, pseudo t, *J* 4.5, H_{linker}), 3.60–3.38 (21H, m, H_{linker}, $H11_{syn}$), 3.21 (3H, s, H_{mtx}), 3.20–3.13 (2H, m, H1′), 2.83–2.71 (1H, m, $H11_{anti}$), 2.22–2.17 (2H, m, H_{mtx}), 2.05–1.86 (2H, m, H_{mtx}), 1.55–1.45 (2H, m, H2′), 1.28–1.17 (2H, m, H3′), 0.86 (3H, t, *J* 7.4, H4′); ^{13}C NMR (125 MHz, DMSO) δ 173.7 (C=O), 172.6 (C=O x2), 171.6 (C=O), 166.1 (C), 162.6 (C), 162.4 (C=O), 158.6 (C), 154.3 (C), 150.8 (C), 149.0 (CH), 146.1 (C), 141.5 (C), 136.6 (C), 131.1 (C), 129.8 (CH), 128.8 (CH x2), 125.6 (C), 121.6 (CH), 121.3 (C), 121.0 (C), 119.8 (CH), 118.7 (CH), 118.1 (CH), 114.0 (CH), 113.6 (CH), 111.3 (CH), 110.9 (CH x2), 105.8 (C), 69.8 (CH_2), 69.6 (CH_2), 69.6 (CH_2), 69.4 (CH_2), 68.9 (CH_2), 68.8 (CH_2), 67.0 (CH_2), 54.7 (CH_2), 52.9 (CH), 52.2 (CH), 51.4 (CH), 38.4 (CH_2), 37.6 (CH_2), 31.8 (CH_2), 31.5 (CH_3), 29.5 (CH_2), 26.4 (CH_2), 22.6 (CH_2), 19.3 (CH_2), 13.4 (CH_3); LRMS (ES^-) *m/z*: $C_{55}H_{67}N_{12}O_{12}$ $[M - H]^-$ 1087.33.

Biology experimental

Parasite growth and invasion assay. *T. gondii* tachyzoites (RH strain) were cultured at 37 °C in confluent monolayers of human foreskin fibroblasts (HFFs) grown in Dulbecco's Modified Eagle's medium (DMEM) (HyClone) containing 1% fetal calf serum (Invitrogen). Freshly egressed parasites were prepared for invasion assays by passage through a 26 gauge needle followed by filtration through a 3.0 μm Nuclepore track etch membrane filter (Whatman). Invasion assays were performed as previously described[6] except that samples were scored manually rather than by automated fluorescence microscopy.

Yeast growth and CID competition assays. Yeast strain V1019Y[26] was grown in synthetic complete (SC) medium (lacking histidine and tryptophan, and supplemented with 2% galactose and 2% raffinose) ± leucine at 30 °C with shaking. For CID competition experiments, CIDs were diluted into the SC medium prior to inoculation with yeast. 2.0×10^5 yeast cells were inoculated into a total volume of 150 µl of SC medium (± leucine, ± CID) in the wells of a 96-well tissue culture dish (Corning). The 96-well plate was sealed with parafilm and incubated at 30 °C with shaking for 48 hours. OD_{600} measurements were taken with a plate reader (Biotek Instruments). CID **26** was used at 10 µM and competing CIDs were tested at a 10-fold molar excess (100 µM).

Bioinformatics analyses. The *Toxoplasma gondii* genome database (ToxoDB release 5.0) was searched by BLAST using the 4 signature motifs of the kinesin heavy chain motor domain[26,27] or the catalytic domain of cGMP-specific 3′, 5′ cyclic nucleotide phosphodiesterases[26,28] to identify putative targets of **1**. The sequence of each of the identified candidates was further analyzed by performing a BLAST search and a conserved domain search against GenBank. Putative classification of *T. gondii* kinesins was performed using known family placement of the closest homolog identified and according to previous reports.[29] The identified cGMP-specific 3′,5′ cyclic nucleotide phosphodiesterases were similarly classified into putative families according to previous reports.[30] Expression data (EST, SAGE, microarray) available in ToxoDB were also analyzed for each candidate gene.

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