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Design and Synthesis of Broad Spectrum Trypanosomatid Selective Inhibitors

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Neglected tropical diseases caused by parasitic infections are an ongoing and increasing concern that have a devastating effect on the developing world due to their burden on human and animal health. In this work, we detail the preparation of a focused library of substituted-tetrahydropyran derivatives and their evaluation as selective chemical tools for trypanosomatid inhibition and the follow-on development of photo-affinity probes capable of labeling target protein(s) *in vitro*. Several of these functionalised compounds maintain low micromolar activity against *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania major* and *Leishmania donovani*. In addition we demonstrate the utility of the photo-affinity probes for target identification through preliminary cellular localisation studies.

KEYWORDS

Trypanosomatid, Natural Products, Drug Design, Chemical Tools, Photo-affinity

Trypanosomatids are a family of parasites which cause life threatening, debilitating disease in humans.¹ The three major parasitic forms *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania spp.* are the causative agents of African sleeping sickness, Chagas disease and Leishmaniasis respectively.^{2,3,4} Combined, these parasites are responsible for a huge global health burden for millions of people in the worlds poorest countries. Unfortunately, the current treatment options are generally antiquated and many exhibit toxic side effects whilst being largely ineffective.^{5,6} Perhaps more significantly there are very few well-documented drug targets for these diseases.^{7,8,9}

The use of natural products in traditional medicinal practices dates back thousands of years and today natural products remain at the forefront of pharmaceutical discovery.¹⁰ While the structural diversity of natural products is remarkable, they frequently contain significant stereochemical complexity and a high degree of saturation (low Fsp³ count) - properties which are not well reflected in modern HTS screening libraries.^{10b} Annonaceous acetogenins are a series of fatty acid-derived natural products that have attracted interest as potential anti-cancer agents via their known inhibition of mitochondrial Complex I.^{11,12} Despite the common use of acetogenin-containing *Annonaceae* plant extracts as traditional medicines in trypanosomatid affected areas,¹³ acetogenins have only been tentatively explored as potential anti-parasitic agents, with initial promising results in several kinetoplastid cell lines.^{14,15}

Chamuvarinin (**1**, Figure 1), an acetogenin first synthesised by our group in 2011, was found to exhibit low micromolar activity towards bloodstream *T. brucei*.^{16,17} Using the chamuvarinin framework as a template, we designed chiral building blocks that mimic key structural features of chamuvarinin (stereochemical complexity, high degree of saturation) and can be rapidly combined to generate simplified non-natural analogues such as **2**, incorporating a 1,4-triazole

heterocyclic motif as a mimic of the central *cis*-substituted tetrahydrofuran moiety in chamuvarinin (Figure 1). These simplified structural analogues maintained anti-parasitic activity with increased, though modest, selectivity compared to mammalian cells.¹⁸ Herein, we detail further optimization of lead compound **2** using new tetrahydropyran building blocks with substitution on the flanking tetrahydropyran ring systems, as in **3**.¹⁹ We also report extended phenotypic screening of the related kinetoplastids *T. cruzi*, *L. major* and *L. donovani*.²⁰ Furthermore, as part of efforts to identify the protein target and establish the mode of action, we have adapted our core inhibitor scaffold to incorporate both a photo-reactive diazirine unit and an orthogonal reporter alkyne tag to produce photo-affinity labelling inhibitors.²¹ Direct incorporation of a small photo-affinity label was viewed as advantageous in terms of minimising disruption of ligand-protein binding interactions *in vitro*, and reducing non-specific labelling by retaining the structural features identified by phenotypic screening, thereby increasing the likelihood of successful target protein labelling.¹⁸

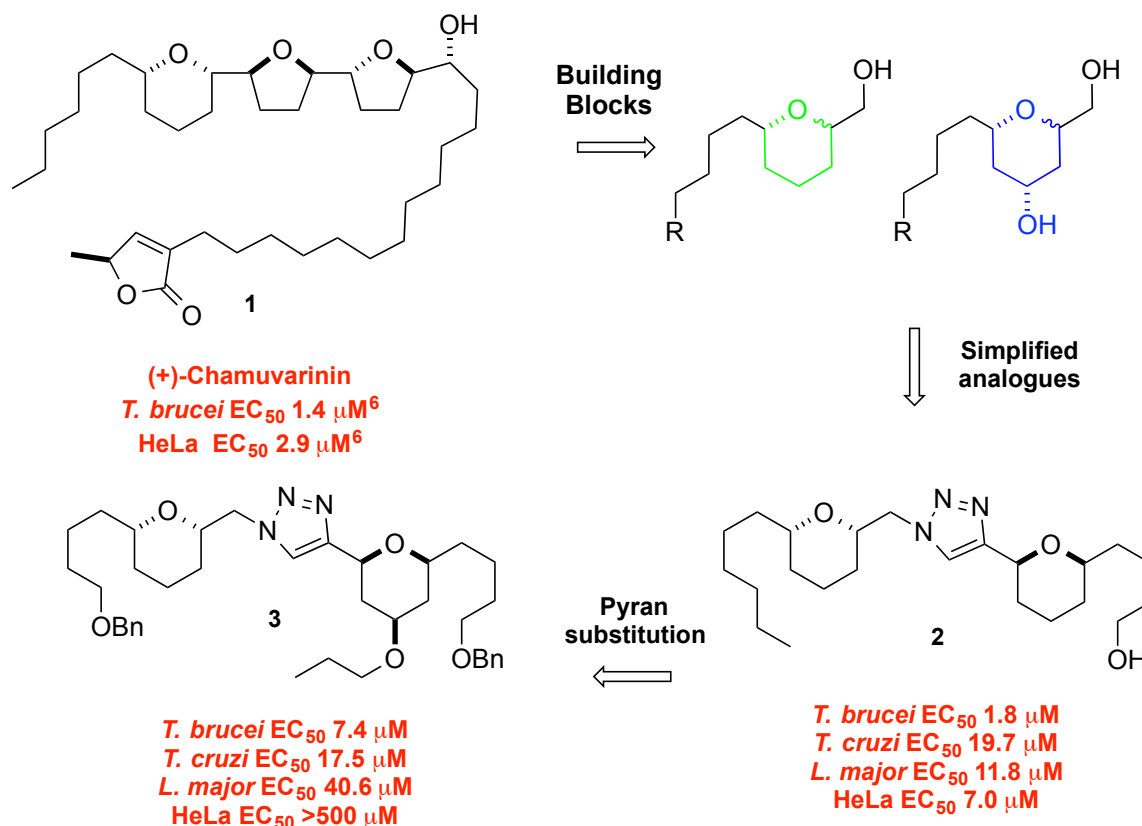


Figure 1. Chamuvarinin and simplified analogues

RESULTS AND DISCUSSION

Synthesis of Functionalised Inhibitors. Our original triazole-based inhibitors were limited as lead compounds due to the limited scope for substitution, preventing further exploration of structure-activity relationships and probing of three-dimensional fragment space.¹⁸ In order to expand the number of strategic attachment/diversification points we viewed that additional chemical handles could be incorporated on either of the tetrahydropyran (THP) ring systems. In particular modification of the 4-position in the tetrahydropyran rings was attractive as these two sites are directed spatially away from the central tri-heterocyclic core that is most likely to be involved in crucial inhibitor-protein binding interactions (Figure 2).

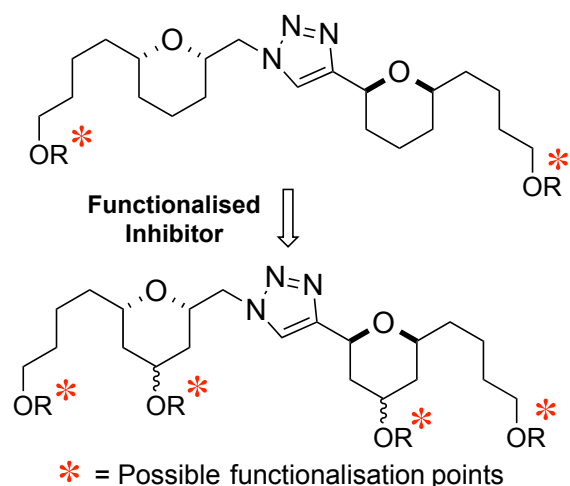
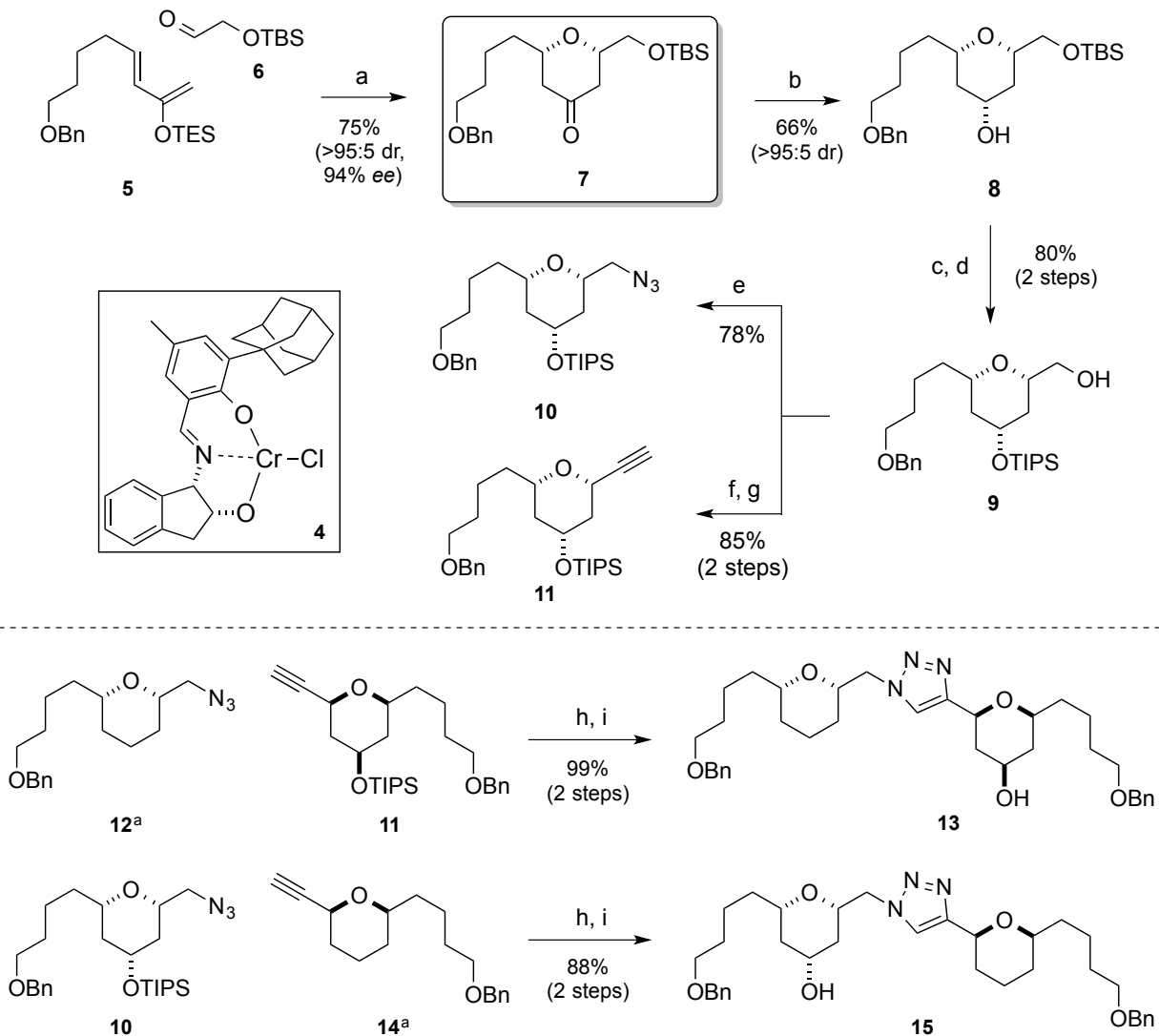


Figure 2. Functionalisation points on original inhibitors compared to new inhibitors

Access to substituted building block **7** was established by application of a Jacobsen hetero-Diels-Alder reaction with excellent diastereo- and enantioselectivity (Scheme 1).²² This intermediate was key to the synthesis of a series of substituted THP building blocks. Alcohol functionality was installed by reduction with NaBH_4 to give equatorial alcohol **8** with high diastereocontrol.²³ TIPS protection, followed by selective TBS deprotection gave alcohol **9**, which could be independently elaborated to the azide **10** under Mitsunobu condition or to alkyne **11** by Swern oxidation and Ohira-Bestmann homologation.^{24,25,26} To install the desired triazole heterocycle the Cu(I)-mediated Huisgen cycloaddition was utilized with combinations of suitable azide and alkyne fragments followed by fluoride deprotection to rapidly access **13** and **15**, respectively, in excellent overall yields.²⁷

Scheme 1. Synthesis of inhibitors **13** and **15**

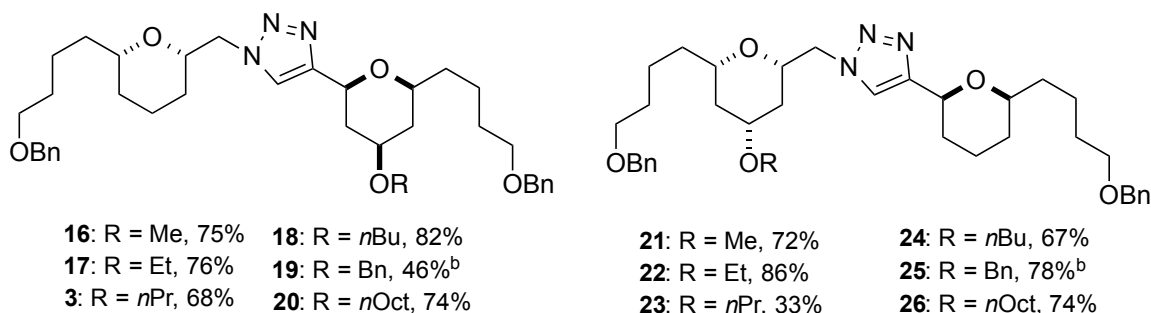
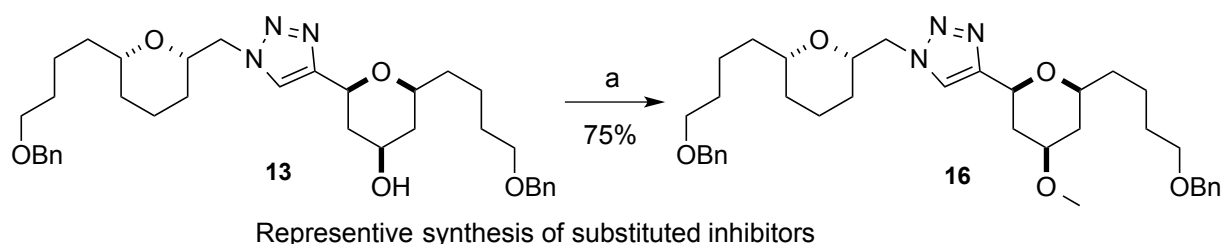


Reagents and conditions: a) Cr(III) cat. **4**, 4Å MS, MTBE, 0 °C – rt, 24 h, then acidified CHCl₃, 20 min, b) NaBH₄, MeOH, 0 °C, 2 h, c) TIPS-triflate, 2,6-lutidine, CH₂Cl₂, -78 °C, 4 h, d) CSA, CH₂Cl₂, MeOH, 0 °C, 4 h, e) PPh₃, DIPEA, DIAD, DPPA, 0 °C – rt, 16 h, f) (COCl)₂, DMSO, Et₃N, CH₂Cl₂, -78 °C – rt, 2 h, g) dimethyl diazo-2-oxopropylphosphonate, K₂CO₃, MeOH/THF, 21 h, h) CuSO₄·5H₂O, sodium ascorbate, *t*BuOH, H₂O, 20 h, i) TBAF, THF, 0 °C – rt, 2 h.

^aSynthesis of THP azide **12** and alkyne **14** are detailed in our previous publication.¹⁸

With these modified inhibitor scaffolds in hand, a range of alkyl ethers were prepared (Scheme 2) in order to probe the tolerance for substitution on individual THP rings whilst maintaining anti-parasitic activity. Williamson etherification of **13** with the specified alkyl halides (NaH, THF) proceeded smoothly to provide ether derivatives (**3** and **16 – 26**).

Scheme 2. Synthesis of alkylated derivatives



Reagents and conditions: a) NaH, RI, THF, 0 °C – reflux, 24 – 48 h. Note: Reaction time varied depending on length of alkyl substituent being added. ^bAlkyl bromide was used.

Biological Screening. Following the synthesis of **3**, **16–26** we screened against our selected parasite cell lines: *T. brucei* (bloodstream and procyclic form), *T. cruzi* (epimastigotes) and *L. major* (promastigotes) in the first instance and counter-screened against the mammalian cell lines HeLa and Vero (Table 1). Addition of a substituent on the alkyne THP ring was well tolerated with alcohol **13** having no loss of potency across all cell lines when compared to the activity of the parent compound **27** and only a small decrease from our previously reported lead compound

2.⁸ Results for the analogous alcohol **15** (with the alcohol on the azide side) were very similar to that of **13** and this proved to be a general result for this series of compounds with inhibition largely independent of which ring the substituent was located on.

However, the length of the alkyl substituent had a dramatic effect on the type of activity displayed. In bloodstream *T. brucei* the length of the alkyl substituent had a minimal effect on potency, an *n*-butyl substituent (**18**) maintained activity when compared to **27**. Inhibition activity is only moderately reduced with a bulky *n*-octyl substituent (**20**). The potency in other parasitic cell lines (insect forms of *T. brucei*, *T. cruzi* and *L. major*) were more sensitive to alkyl ether chain length. However, all tolerated an *n*-propyl ether (**3**) with potency being maintained in both *T. brucei* (procyclic) and *T. cruzi* (epimastigote) with only a small decrease observed in *L. major* (promastigote), when compared to **27**. Interestingly, while inhibition activity in mammalian HeLa cells was unaffected by an ethyl substituent (**17**), both the *n*-propyl and *n*-butyl substituted variants (**3** and **18**) had no effect at doses of 500 μM . Importantly, this means **3** selectively targeted all trypanosomatid cell lines tested with no detrimental activity towards mammalian HeLa cells. Additionally, this series of compounds were evaluated in the mammalian Vero cell line to gauge their activity in non-cancerous cells. This assay demonstrated an even more pronounced effect on cell inhibition activity than that seen for HeLa. The methyl ether variant (**16**) displayed decreased inhibition activity, while the ethyl ether variant **17** had no effect at 500 μM . An anomalous result was found with compound **24** having an inhibitory effect on Vero cells. However, it should be noted that the assay indicated an inhibitory effect on respiration, yet the cells were visibly still viable at the highest concentration tested (>500 μM), suggesting static rather than cidal activity.

Table 1. Biological profiles for substituted inhibitors

Analogue	R	<i>T. brucei</i> (BSF) EC ₅₀ (μM)	<i>T. brucei</i> (insect) EC ₅₀ (μM)	<i>T. cruzi</i> EC ₅₀ (μM)	<i>L. major</i> EC ₅₀ (μM)	HeLa EC ₅₀ (μM)	Vero EC ₅₀ (μM)
2	-	1.8 ± 0.1 ^a	0.8 ± 0.1 ^a	19.7 ± 1.3	11.8 ± 0.7	7.0 ± 1.0 ^a	ND
27	H	4.0 ± 0.1	3.8 ± 0.2	28.6 ± 1.3	29.8 ± 3.2	23.0 ± 3.8	ND
Alkyne-side							
13	OH	8.1 ± 0.5	5.3 ± 0.3	16.2 ± 0.5	18.8 ± 0.9	9.0 ± 0.8	14.4 ± 0.6
16	OMe	9.3 ± 0.7	11.6 ± 0.3	24.4 ± 1.0	31.4 ± 1.3	>40	111.1 ± 45
17	OEt	5.7 ± 0.5	10.2 ± 0.3	13.2 ± 0.9	22.7 ± 1.5	53.6 ± 5.3	>500
3	OnPr	7.4 ± 0.4	12.3 ± 0.5	17.5 ± 1.3	40.6 ± 3.6	>500	>500
18	OnBu	4.8 ± 0.4	11.2 ± 0.5	>500	58.5 ± 5.5	>500	>500
19	OBn	5.0 ± 0.2	72.6 ± 4.9	>500	>500	>500	>500
20	OnOct	33.1 ± 2.1	>500	>500	>500	>500	>500
Azide-side							
15	OH	4.6 ± 0.3	3.5 ± 0.1	29.8 ± 0.7	15.6 ± 0.1	12.1 ± 0.8	16.5 ± 0.6
21	OMe	4.5 ± 0.4	6.7 ± 0.4	17.4 ± 1.6	29.3 ± 1.1	18.9 ± 2.9	50.6 ± 15.2
22	OEt	5.1 ± 0.3	10.7 ± 0.3	12.7 ± 0.7	15.3 ± 0.4	21.1 ± 5.3	>500
23	OnPr	6.3 ± 0.5	10.8 ± 0.5	11.2 ± 0.6	63.4 ± 8.7	43.2 ± 7.3	>500
24	OnBu	3.8 ± 0.1	12.0 ± 0.4	>500	72.0 ± 12.6	>500	16.2 ± 0.7
25	OBn	5.2 ± 0.5	84.6 ± 5.4	>500	>500	>500	>500
26	OnOct	39.2 ± 5.2	>500	>500	>500	>500	>500
Nifurtimox	-	2.4 ± 0.1 ^b	ND	1.5 ± 0.0	0.67 ± 0.1	91.2 ± 11.3 ^b	37.3 ± 4.1
Pentamidine	-	0.0012	ND	0.9 ± 0.3	24.4 ± 0.9	44.1 ± 5.9 ^c	6.4 ± 1.3

a) EC₅₀ values were determined against bloodstream (BSF) and procyclic (insect) *T. brucei brucei*, *T. cruzi* (epimastigotes), *L. major* (promastigotes), mammalian HeLa cells and mammalian Vero cells. Data represents the mean ± SD of n = 3 independent experiments performed in triplicate. **2** and **27** shown for comparison. For synthesis of **2** see Ref.(18). For synthesis of **27** see supplementary information. ND = Not determined. ^aTaken from Ref.(18). ^bTaken from Ref.(28). ^cTaken from Ref.(29).

With these promising results, we evaluated selected compounds in the clinically relevant, intracellular forms of *T. cruzi* and *L. donovani* cultured in mammalian THP-1 cells (results for these compounds with bloodstream *T. brucei* are also included, Table 2). Interestingly, intracellular amastigote *T. cruzi* (AMA) were more sensitive to the alkyl ether substituent than the free swimming epimastigote form, with compounds **21** and **22** displaying good anti-parasitic activity, while compounds **3**, **17** and **23** showed no inhibitory effect. By contrast, the alkyl ether substituent appeared to have negligible influence on intracellular *L. donovani* amastigote (INMAC AMA), with all compounds screened having low to moderate micromolar inhibitory activity, compound **21** being particularly potent. Counter-screening against the host THP-1 mammalian cell line showed that compounds with an alkyl substituent had moderate to no observable inhibitory activity – however the SAR of this effect does not hold a clear trend.

The lack of inhibitory activity in THP-1 cells gave rise to selective compounds towards bloodstream *T. brucei*, in particular compounds **21** and **23** were highly selective when compared to host THP-1 cells (>56 and >79 fold, respectively). The same compounds were also highly selective towards *L. donovani*. When considering selectivity towards intracellular *T. cruzi*, only **21** displayed good selectivity compared to the host THP-1 cells (>9 fold). Importantly many of these compounds also had limited inhibitory activity in Vero/HeLa cells (see Table 1), which is significant when considering overall general toxicity towards the host organism. In sum, these results demonstrated that alkyl ether substitution of the THP scaffold was valuable as a method to increase selectivity while maintaining anti-parasitic activity. We have now generated a series of selective chemical tools, with structures of type **21-23** being, in particular, useful in guiding future inhibitor design and development due to their promising activity and selectivity profiles towards the clinically relevant parasite forms.

Table 2. Biological profiles for selected inhibitors against clinically relevant forms

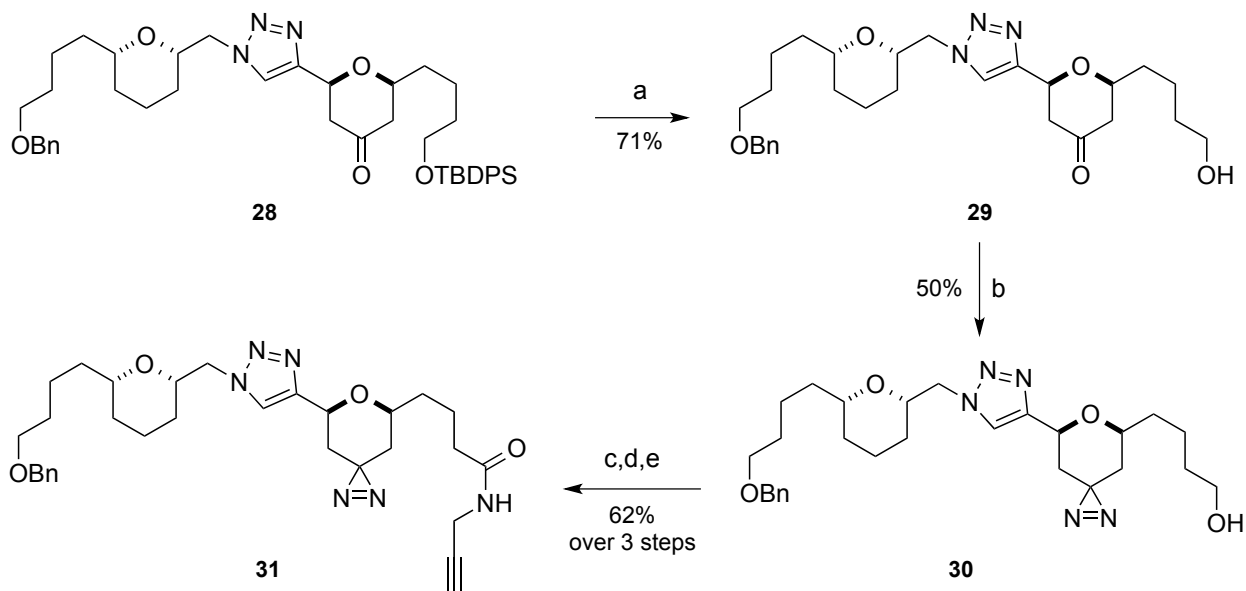
Analogue	R	<i>T. brucei</i> (BSF) EC ₅₀ (μM)	<i>T. brucei</i> Selectivity Index ^a	<i>T. cruzi</i> AMA EC ₅₀ (μM)	<i>T. cruzi</i> Selectivity Index ^b	<i>L. donovani</i> INMAC AMA EC ₅₀ (μM)	<i>L. donovani</i> Selectivity Index ^c	THP-1 EC ₅₀ (μM)
2	-	1.8 ± 0.1 ^a	25	26.1 ± 3.2	>1.7	32.5 ± 2.0	>1.3	44.8 ± 2.9
Alkyne-side								
17	OEt	5.7 ± 0.5	12	>250	-	20.7 ± 2.5	>3.3	69.7 ± 10.1
3	OnPr	7.4 ± 0.4	12	>100	-	32.8 ± 5.1	>2.6	86.7 ± 4.7
Azide-side								
15	OH	4.6 ± 0.3	5.2	ND	-	17.7 ± 2.4	>1.3	23.9 ± 2.6
21	OMe	4.5 ± 0.4	>56	27.0 ± 3.3	>9.2	3.8 ± 0.8	>65	>250
22	OEt	5.1 ± 0.3	12	37.2 ± 10.7	>1.5	21.7 ± 2.8	>2.7	59.2 ± 6.3
23	OnPr	6.3 ± 0.5	>79	>100	-	27.3 ± 9.6	>18	>500
Nifurtimox	-	2.4 ± 0.1 ^b	>100	4.7 ± 1.2	>53	ND	-	>250
Pentamidine	-	0.0012	47000	ND	-	0.27 ± 0.18	210	56.7 ± 5.9

a) EC₅₀ values were determined against mammalian THP-1 cells, *T. brucei* (BSF), amastigote *T. cruzi* (AMA) in host mammalian THP-1 cells, *L. donovani* (promastigote) and *L. donovani* (AMA INMAC) in host THP-1 cells. Data represents the mean ± SD of n = 3 independent experiments performed in triplicate. ^aThe selectivity index refers to the EC₅₀ values of *T. brucei* (BSF) compared to mammalian THP-1 cells. ^bThe selectivity index refers to the EC₅₀ values of *T. cruzi* (AMA) compared to mammalian THP-1 cells. ^cThe selectivity index refers to the EC₅₀ values of *L. donovani* (AMA INMAC) compared to mammalian THP-1 cells.

Synthesis of Photo-Affinity Labelling Probes. With lead analogues against our three kinetoplastid cell lines, we have initiated investigations into the mode of action of our natural product-like inhibitors, particularly the development of suitable photo-affinity labelling probes for protein target elucidation. Our SAR studies showed parasite inhibition was sensitive to increasing sterics on the core THP scaffolds, particularly in *T. cruzi* and *L. major*. Thus, when considering established methods³⁰ to incorporate photo-reactive tags we were concerned by the additional steric demands and the resultant structural deviation causing a loss of affinity and/or selectivity for relevant protein targets. In an effort to minimise such structural changes, compound **31** was targeted with the appropriate diazirine and alkyne groups incorporated directly onto the inhibitor backbone (Scheme 3).

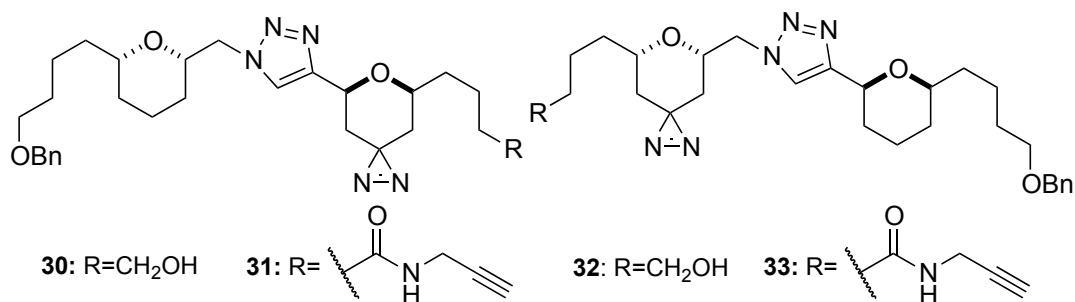
Following significant route optimisation it was found that initial deprotection of silyl ether **28**³¹ with TBAF was preferential prior to the installation of the diazirine unit. With the free alcohol **29** in hand, installation of the diazirine group could be effected in a one-pot synthesis utilising a modified literature procedure.³² Treatment with liquid ammonia in a sealed tube at elevated temperature was necessary to form the imine, which after addition of hydroxylamine sulfonic acid yielded the intermediate diaziridine. Subsequent oxidation using molecular iodine gave the desired diazirine compound **30** in good yield. Mild conditions for installation of the alkyne chemical handle were sought due to the temperature sensitivity of the diazirine group. Optimal conditions involved sequential Swern oxidation followed by Pinnick oxidation to the carboxylic acid.^{25,33} Alkyne functionality was installed using simple amide coupling with propargyl amine to deliver the desired photo-affinity labelling probe **31**. The azide-side photo-affinity labelling inhibitor **32** was synthesised in an analogous manner.³¹

Scheme 3. Synthesis of photo-affinity labelling probe 31



Reagents and conditions: a) TBAF, THF, 0 °C – rt, 2 h, b) $\text{NH}_3(\text{l})$, 80 °C, 3 h, then $\text{H}_2\text{NOSO}_3\text{H}$, 18 h, then I_2 , MeOH, c) $(\text{COCl})_2$, DMSO, Et_3N , CH_2Cl_2 , -78 °C – rt, 2 h, d) NaClO_2 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, *t*BuOH, H_2O , 0 °C – rt, 1.5 h, e) Propargylamine, EDCI.HCl, DMAP, CH_2Cl_2 , 18 h.

As detailed in Table 3, the diazine containing probes were assessed for trypanocidal activity in *T. brucei*, *T. cruzi* and *L. major*. Pleasingly diazine analogues **30** and **32** maintain good inhibition profiles, while **31** and **33** incorporating the alkyne reporter also maintains good levels of inhibition in all parasitic cell lines with the exception of *T. cruzi* which displayed reduced levels of inhibition. Photo-affinity probes suitable for interrogation of *T. cruzi* are currently being sought. The minimal structural differences between these compounds and the substituted ether inhibitors validate our design principles for the apparent continuation of binding to the target protein(s).

Table 3. Biological profiles for photo-affinity labelling probes

Analogue	<i>T. brucei</i> (BSF) EC ₅₀ (μ M)	<i>T. brucei</i> (insect) EC ₅₀ (μ M)	<i>T. cruzi</i> EC ₅₀ (μ M)	<i>L. major</i> EC ₅₀ (μ M)
21	4.6 \pm 0.4	6.7 \pm 0.4	17.4 \pm 1.6	29.3 \pm 1.1
30	10.1 \pm 0.5	8.5 \pm 0.3	29.9 \pm 0.8	32.6 \pm 1.2
31	17.5 \pm 1.1	25.7 \pm 4.5	51.2 \pm 2.0	20.3 \pm 0.4
32	11.8 \pm 0.8	8.9 \pm 0.2	44.2 \pm 3.4	25.1 \pm 1.1
33	13.0 \pm 1.0	16.1 \pm 0.6	59.6 \pm 7.0	31.5 \pm 1.0

a) EC₅₀ values were determined against bloodstream (BSF) and procyclic (insect) *T. brucei*, *T. cruzi* (epimastigotes), *L. major* (promastigotes). Data represents the mean \pm SD of n = 3 independent experiments performed in triplicate.

We finally looked to obtain proof of principle that our photo-affinity labelling derivatives were capable of selective protein binding through cell localisation studies in *T. brucei* and *L. major*. Incubation of **31** with *L. major* was followed by cross-linking with UV irradiation (365 nm) to label target protein(s) within the cell. Subsequent cell permeabilisation and ‘click’ reaction with commercially available Cy5.5 azide fluorescent tag allowed visualization of inhibitor-protein conjugates by fluorescence microscopy (Figure 3a). Co-localisation with MitoTracker RedCMXros indicated that inhibitors primarily target the mitochondrion. Similar mitochondrial labelling was obtained with procyclic *T. brucei* cells and compound **33** (Figure 3b). Control experiments with cells exposed to compound **2**, lacking the diazirine group and therefore unable

to UV-crosslink to its target(s), showed no protein labeling, suggesting that the labeling with Cy5.5 reporter is specific to the photo-affinity probe. Further details of the isolation and identification of the mitochondrial target as the F1 α /F1 β subunits of TbATPase (Complex V) in procyclic *T. brucei* using **31** are detailed elsewhere.³⁴ Ongoing studies are underway to establish whether the ATPase is a common target in bloodstream *T. brucei*, intracellular *T. cruzi* and *Leishmania sp.* using **31** and details will be reported in due course.

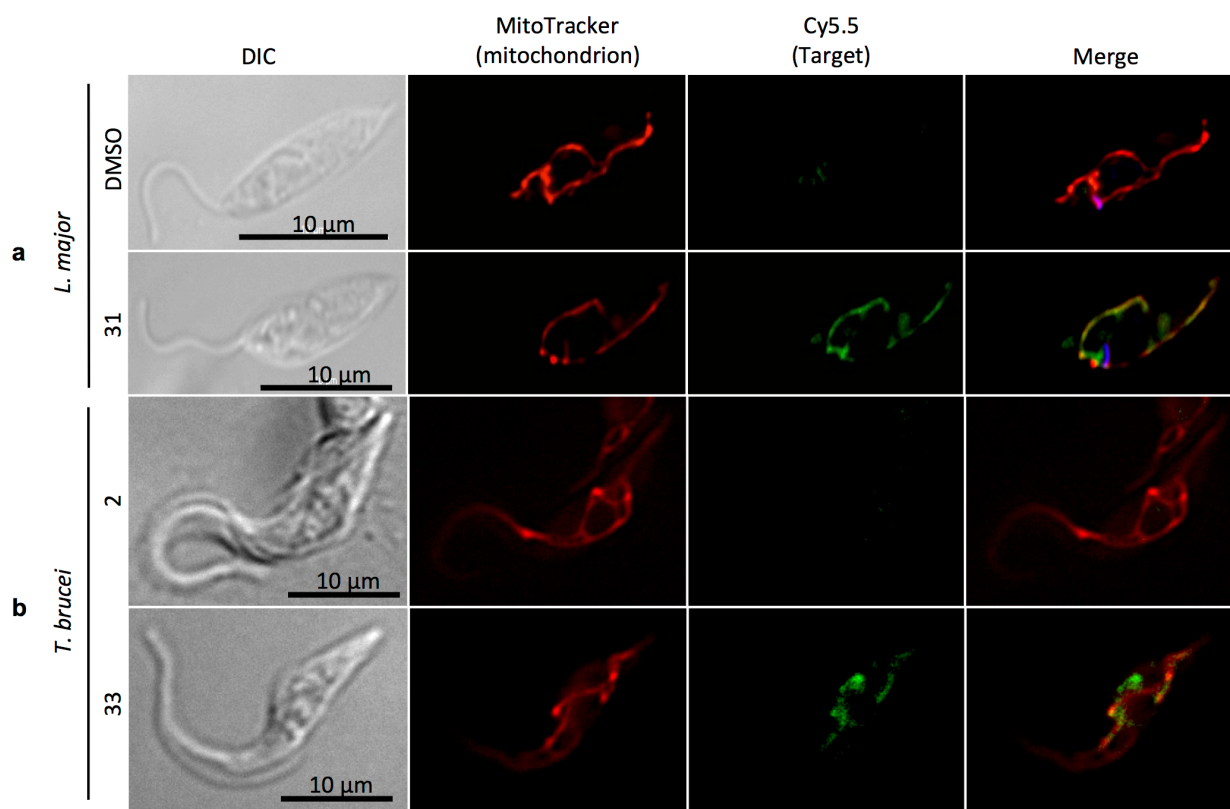


Figure 3. a) Co-localisation between MitoTracker Red CMXros (red) stained *L. major* cells labelled with compound **31** and Cy5.5 fluorescent tag (green). DMSO is shown as a negative control, b) Co-localisation between MitoTracker Red CMXros (red) stained procyclic *T. brucei* cells labelled with compound **33** and Cy5.5 fluorescent tag (green). Cells exposed to compound **2** (not capable of photo-crosslinking) shown as a negative control.

CONCLUSION

Building on our initial natural product based *T. brucei* inhibitors, a novel series of bis-tetrahydropyran compounds were synthesised and screened against a panel of trypanosomatids. Members of this novel class of compound display broad-spectrum kinetoplastid inhibition with minimal toxicity towards several mammalian cell lines. A systematic investigation of alkyl substitution on the tetrahydropyran rings revealed the key steric demands across each cell line, creating a ‘window of selectivity’ where low micromolar parasite inhibition can be maintained and mammalian cells remain viable. Extension into the clinically relevant forms of *T. cruzi* and *L. major* also showed promising activity and selectivity profiles. We have successfully functionalized our chemical probes with dual photo-affinity and reporter capabilities and shown that they target the mitochondria in both *T. brucei* and *L. major*.³⁴ Identification and analysis of the mitochondrial protein target in conjunction with further biological validation will now enable further structural simplification and lead optimization in terms of potency and selectivity, with the view to the development of natural product-inspired therapies for trypanosomatid associated diseases.

ASSOCIATED CONTENT

Supporting Information

Supporting Information includes details of; i) chemical synthesis and compound characterization; and ii) biological methods, cell cultures, cytotoxic assays and subcellular localization studies.

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ABBREVIATIONS

MS, molecular sieves, TIPS triisopropylsilyl, CSA, (\pm)-camphorsulfonic acid, DIPEA, N,N-diisopropylethylamine, DIAD, diisopropyl azodicarboxylate, DPPA, diphenyl phosphoryl azide, EDCI.HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, RI, alkyl iodide [where R = respective alkyl substituent], *spp.*, species, TBDPS, tert-butyldiphenylsilyl, dr, diastereomeric ratio, cat., catalyst, SD, standard deviation, BSF, blood stream form, ND, not determined, PBS, phosphate-buffered saline, SDS, sodium dodecyl sulfate, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, AMA INMAC, amastigote in macrophages.

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