Synthesis and chemical characterisation of target identification reagents based on an inhibitor of human cell invasion by the parasite *Toxoplasma gondii*†

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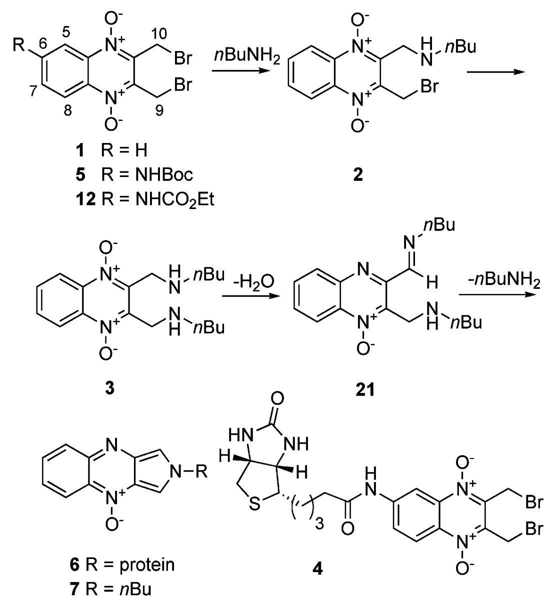
The use of phenotype-based screens as an approach for identifying novel small molecule tools is reliant on successful protein target identification strategies. Here we report on the synthesis and chemical characterisation of a novel reagent for protein target identification based on a small molecule inhibitor of human cell invasion by the parasite *Toxoplasma gondii*. A detailed ^{1}H NMR study and biological testing confirmed that incorporation of an amino-containing functional group into the aryl ring of this inhibitor was possible without loss of biological activity. Interesting chemical reactivity differences were identified resulting from incorporation of the new substituent. The amine functionality was then used to prepare a biotinylated reagent that is central to our current protein target identification studies with this inhibitor.

Introduction

Toxoplasma gondii is an intracellular parasite that causes the disease known as toxoplasmosis.[1] A key step in this parasite's lifecycle is the invasion of human cells. In the course of our efforts to develop novel chemical tools to help study this important biological process, we recently reported on the mechanism of reaction of 2,3-bis(bromomethyl)quinoxaline 1,4-dioxide (**1**) with primary and secondary amines (Scheme 1).[2] These studies provided the first experimental evidence in support of a mechanism that involves conversion of **1** *via* **2** to the disubstituted intermediate **3**. Computational studies were used to support our hypothesis that the reaction outcome stems from an electrostatic repulsion between the *N*-oxide functional group and the bromide leaving group which raises the energy of the transition state such that the expected cyclisation reaction of **2** does not occur.

Whilst our initial studies were chemically informative, it is difficult to assess their biological relevance or gain any real insight into the biological mode of action of compounds of this type without identifying a protein(s) that is covalently modified by **1**. We therefore decided to develop reagents and techniques to aid protein target identification studies using **1** (or its analogues). In our first approach we assessed whether **1** itself could be used as a means of detecting proteins labelled *via* the reaction pathway proposed in Scheme 1. In the second approach **4** (Scheme 1), a biotinylated version of **1**, was prepared. Here we report on the synthesis of **4** and compare the chemical reactivity and biological activity of **1** with **5**, a structurally related precursor of **4** (Scheme 1). This second approach is complicated by the fact that the incorporation of a substituent into **1** renders the two electrophilic carbon centres (C9 and C10) non-equivalent, with interesting chemical consequences.



Scheme 1 Reaction of **1** with an excess of a primary amine, *n*-butylamine.

Results and discussion

Fluorescence detection of the amine adduct 7

When carrying out protein target identification studies, it is essential to incorporate a means of detecting which proteins have been labelled by the chemical probe.[3] If **1** or analogues of **1** covalently modify a protein as described in Scheme 1, a protein-bound adduct **6** (or its analogues) would be formed. Due to the extended conjugation present in **6** and compounds like it, we predicted that it might be possible to detect proteins labelled by **1**

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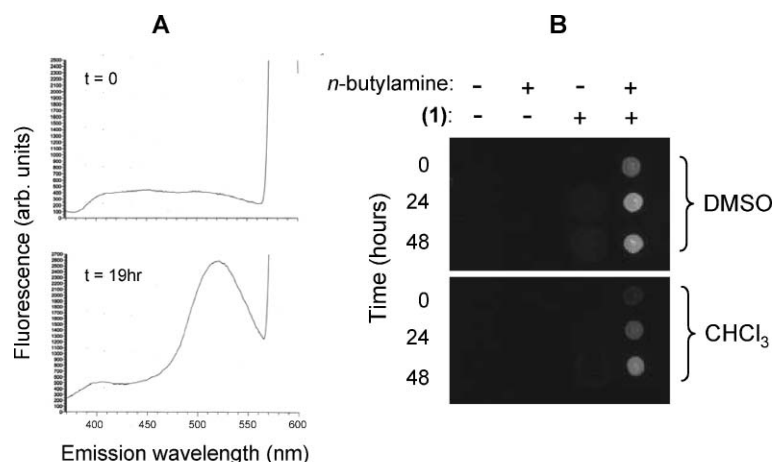


Fig. 1 Fluorescence characterisation of **7**. (A) Fluorescence emission spectra (a_{ex} = 292 nm) were determined after 0 or 19 h incubation of 5 mM **1** with 45 mM *n*-butylamine in DMSO. (B) **1** (20 mM) was incubated with or without *n*-butylamine (60 mM) in either DMSO or chloroform for 0, 24 or 48 h. At each time point, the reaction mixture was diluted 20-fold in phosphate buffered saline and 20 µl of this solution (approximately 20 nmol of **7**) was spotted onto Protran nitrocellulose membrane and visualised using a FluorSMax MultiImager (BioRad) with a UV epifluorescence excitation and a 520 nm long pass emission filter.

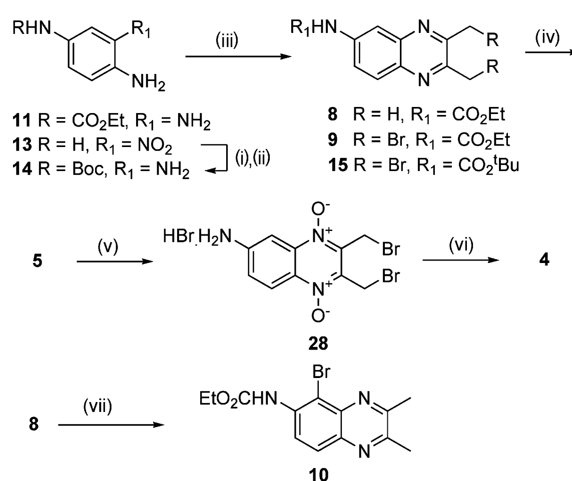
using fluorescence-based techniques. To evaluate this possibility, we incubated **1** for 19 hours in the presence or absence of the model substrate *n*-butylamine to give **7**. As expected, a strong fluorescence signal was detected at 515 nm when the crude reaction mixture was analysed following excitation at either 290 or 417 nm (Fig. 1A). No detectable fluorescence was seen at $t = 0$ (Fig. 1A) or in the absence of *n*-butylamine (Fig. 1B).

A solution of **1** was then incubated with a 3-fold molar excess of *n*-butylamine in DMSO or chloroform for varying lengths of time and spotted onto a nitrocellulose membrane in an analogous manner to experiments that would be carried out with protein lysates. The nitrocellulose was imaged using ultraviolet epifluorescence excitation resulting in the observation of fluorescence at all time points with a clear dependence on the presence of *n*-butylamine (Fig. 1B). Although these initial results were promising, when parasites were incubated with **1** and the parasite proteins extracted, resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, no fluorescent signal above background was detected (data not shown). This suggested that the inherent fluorescence of any protein adducts formed by reaction with **1** that were present on the nitrocellulose was too weak to be useful for target identification purposes. We therefore decided to chemically modify **1** to generate a new and more sensitive probe.

Incorporation of a linker unit into 1

Synthesis of a chemical probe for protein target identification studies usually requires the identification of a suitable attachment site to enable incorporation of, for example, a biotin tag without loss of the required biological activity. In addition, the chemistry used in conjunction with the attachment site must be compatible with the chemical functionality present in the active compound. The structure of **1** limits the choice of attachment site to the C6/7 and C5/8 positions, substitution at either of which typically renders the two electrophilic centres (C9 and C10) in the resulting analogue non-equivalent. In this case, the choice of linking chemistry provides a significant challenge due to the apparent incompatibility of the core structure with a nitrogen nucleophile (Scheme 1). Despite these concerns, **5** (Scheme 1) was selected for synthesis to assess whether attachment of a group at the C6/7 position in **1** affects its chemical reactivity and biological activity.

Our initial attempts to prepare C6/C7-substituted analogues of **1** proceeded *via* the known compound **8** (Scheme 2).[5] Oxidation of **8** with mCPBA (2.1 equiv.) led to a mixture of the two regio-isomeric quinoxaline mono-*N*-oxides and the required di-*N*-oxide that was difficult to separate using standard chromatographic techniques.[6] Additionally, an attempt to convert **8** to **9** using bromine, an analogous method to that reported in a literature synthesis of **1**,[7] was also unsuccessful leading to one major product **10** (Scheme 2) and a series of polybrominated compounds.[4] Our next attempt involved reaction of the known diamine



Scheme 2 Synthesis of **4** and **5**. *Reagents and conditions*: (i) Boc anhydride (1.1 equiv.), DCM, RT, 2 d, quant.; (ii) H_2, 10% Pd/C, MeOH, RT, 24 h, 99% of **14**; (iii) **14**, 1,4-dibromo-2,3-butanedione (1.05 equiv.), THF, 0 °C to RT, 17 h, 89% of **15**; (iv) mCPBA (5.0 equiv.), DCM, RT, 42 h, 80%; (v) 45% HBr in AcOH, AcOH, 10 min, 94%; (vi) (a) D-(+)-biotin (5 equiv.), $SOCl_2$ (xs), 30 min; (b) **28** (1 equiv.), pyridine (1 equiv.), DMF, 5 h, 24%; (vii) Br_2, DCM, 58%.

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11 with 1,4-dibromo-2,3-butanedione to give **9**.[4,8] Subsequent conversion of **9** to the corresponding di-*N*-oxide **12** (Scheme 1) was achieved using excess mCPBA in low yield[4] but attempts to remove the ethyl carbamate protecting group using 30% HBr in acetic acid were unsuccessful.[9] It was therefore decided to revisit this approach using a Boc protecting group. Compound **5** was successfully prepared by selective mono *N*-Boc protection of 2-nitro-*p*-phenylenediamine (**13**) in accordance with literature precedent.[10] Subsequent reduction of the nitro group gave diamine **14** in good overall yield.[11] Cyclocondensation with 1,4-bromo-2,3-butanedione gave quinoxaline **15** which was converted to the required di-*N*-oxide **5** using purified mCPBA.[11] X-Ray crystallographic analysis of **5** confirmed its structure.§

Comparison of the chemical reactivity of 1 and 5

We next studied the mechanism of reaction of **5** with *n*-butylamine using ^{1}H NMR techniques.[2,4,11] The disappearance of ^{1}H NMR signals attributed to **5** occurred significantly faster (t = 7000 s, Fig. 2A) than for **1** (t = 28 000 s), consistent with an increase in the rate of the first nucleophilic displacement reaction (Scheme 1). This was in line with the expected ability of the C6 NHBoc substituent to promote nucleophilic attack of an amine at the electrophilic centres through increased stabilisation of developing positive charge in the transition states.[12] It was envisaged that the C6 NHBoc substituent would promote attack at C9 leading to regioselective formation of **16** (*cf.* **17**, Fig. 2). Unfortunately, attempts to investigate this were not possible due to the complexity of the ^{1}H NMR spectrum at early time points. The relatively fast consumption of **5** also resulted in a modified reaction profile for intermediate **16/17** compared with **2** (Scheme 1 and Fig. 2A). Whilst both curves reach maxima at approximately the same time (t = 1200 and 1500 respectively), at t = 10 000 s intermediate **16/17** was present in only trace quantities whereas there remained a significant amount of **2**. This observation is consistent with faster nucleophilic displacement at both C9 and C10 in **5** (*cf.* **1**) due to the presence of the NHBoc substituent.

The reaction profiles for **3** and **18** (Fig. 2B and Scheme 1), whilst difficult to interpret in detail, support another difference between

§ CCDC reference number 642107. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b704685e

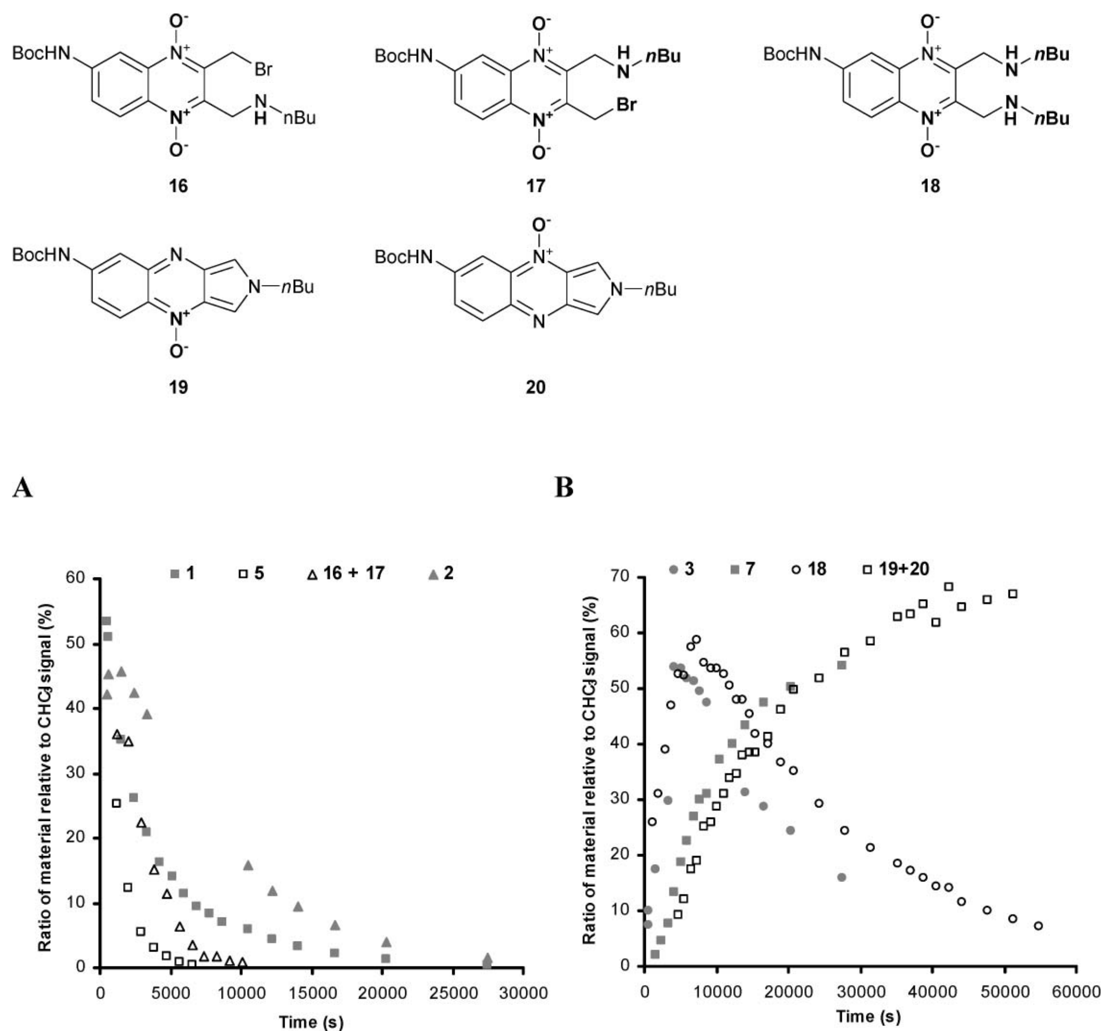
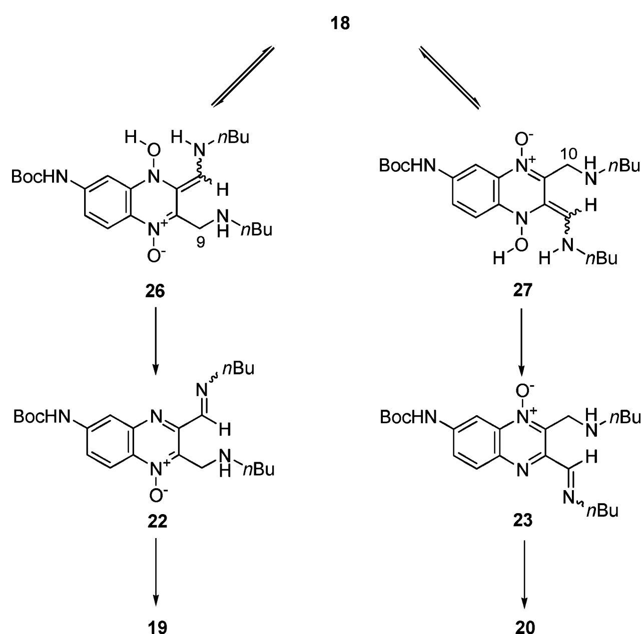


Fig. 2 Comparison of **A** initial displacement (left) and **B** total product formation in the reactions of **1** and **5** with *n*-butylamine. These studies were carried out using an initial concentration of **1** and **5** of 20 mM and *n*-butylamine of 60 mM in neutral $CDCl_3$ at 25 °C. 500 MHz ^{1}H NMR spectra were acquired at regular time points over 17 hours.

these two systems, the fact that **3** forms **7** more rapidly than **18** forms **19/20** (the sum of these two products is shown in Fig. 2B). For example, at $t = 14\,000$ s, the rate of production of **7** and **19/20** are approximately the same despite the fact that the concentration of **3** is 70% of that for **18**. This observation supports the view that **3** undergoes elimination of water to give imine **21** (Scheme 1) faster than **18** gives **22/23** (Fig. 3), possibly reflecting the relative ease of removal of a benzylic proton in **3** and **18**.[13]



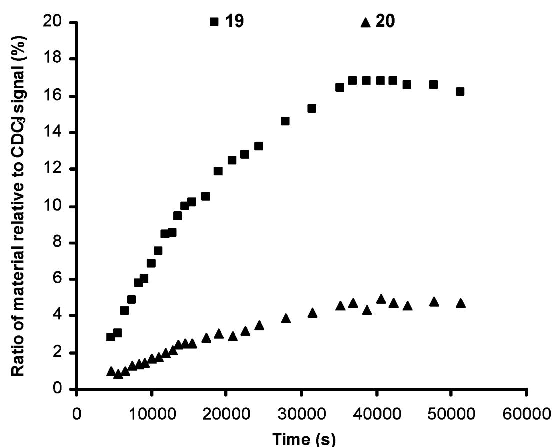
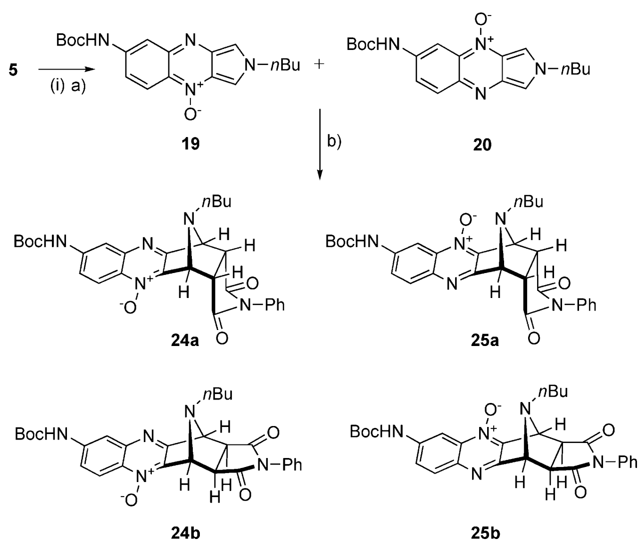


Fig. 3 Rationalisation of the observed product ratio (**19** : **20** 77 : 23).

The presence of a substituent at the C6 position in **5** has a second regiochemical consequence in this reaction as two isomeric products, **19** and **20**, can be formed on reaction of **5** with *n*-butylamine (Fig. 2 for structures). Careful analysis of the ^{1}H NMR spectrum of the crude reaction mixture showed that the final ratio of **19** : **20** was 77 : 23 (data not shown). However, attempts to isolate **19** and **20** proved difficult due to their limited stability to silica gel.[14] Further analytical evidence in support of the structures of **19** and **20** was obtained through their conversion



Scheme 3 Trapping of **19** and **20** in a Diels–Alder reaction with *N*-phenylmaleimide. *Reagents and conditions*: (i) (a) *n*-butylamine (3 equiv.), $CHCl_3$, RT, 3.5 h; (b) *N*-phenylmaleimide (4 equiv.), RT, 14 h. Isolated 36% (**24a/25a**), 18% (**24b/25b**). The dienophile, *N*-phenylmaleimide (4 equiv.), was added to the reaction mixture in chloroform at 25 °C, at $t = 12\,600$ seconds. This time point was selected as it corresponded to the point in the reaction profile at which **16/17** had been fully converted to **18** hence limiting the amount of *n*-butylamine available for undesired reaction with the dienophile (Fig. 2A).

to the corresponding Diels–Alder adducts by reaction with *N*-phenylmaleimide (Scheme 3).[15]

This reaction led to the formation of all four possible isomers **24a–25b** (Scheme 3) with the *endo*-adducts **24a/25a** being readily separable from the *exo*-adducts **24b/25b** by column chromatography. As expected, the *endo*-isomers were formed preferentially (*endo* : *exo* 71 : 29). Although it was not possible to isolate pure samples of each isomer, analysis of the ^{1}H NMR spectra of the two sets of adducts allowed their structural assignment.[4] The ratio of **24a** to **25a** and **24b** to **25b** was similar (**24a** : **25a** 76 : 24; **24b** : **25b** 75 : 25) and consistent with the ratio obtained on analysis of the crude sample of **19/20** (see above).

Rationalisation of the observed product ratio

The observed product ratio resulting from reaction of **5** with *n*-butylamine (**19** : **20** 77 : 23) can be rationalised based on a preferred formation of imine **22** (*cf.* **23** Fig. 3). Formation of **22/23** occurs *via* initial tautomerisation of **18** to form either **26** or **27**. The relative ease of this key tautomerisation step depends on the acidity of the protons at C9 and C10. In **18**, tautomerisation towards C10 would be preferred due to the more acidic nature of the protons at this position (*cf.* C9) leading to **19** as the major product, as observed.[13]

Effect of substitution on the desired biological activity

The studies presented above indicated that the incorporation of an NHBoc functional group at the C6/7 position of **1** to give **5** did not lead to a dramatic change in the mechanism of reaction with the primary amine *n*-butylamine. However, there were significant changes in several of the key chemical steps as

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assessed by ^{1}H NMR studies in chloroform. Subsequent biological studies showed that these modulations in chemical reactivity did not have a dramatic impact on the observed biological activity, as within the experimental error of the cell-based assay used,[1] **5** and **1** could be considered equipotent (lowest active concentration of **1** = 12.5 μM; of **5** = 25 μM).

Synthesis of 4, a biotinylated analogue of 1

Having successfully identified a position in the structure of **1** that could be modified without loss of biological activity, we decided to replace the Boc group in **5** with the required biotin functionality. Treatment of **5** under standard Boc deprotection conditions, for example TFA, gave an intractable mixture of products. However, removal of the Boc group was achieved using HBr in acetic acid because the hydrobromide salt, **28**, precipitated from the crude reaction mixture in 94% yield (Scheme 2). Commercially available D-(+)-biotin was then converted to the corresponding acid chloride using thionyl chloride and reacted with **28** in the presence of pyridine in DMF to give **4** in moderate yield (Scheme 2). Having successfully completed the synthesis of **4**, this novel reagent is now the subject of studies that fall outside the scope of this paper to identify protein targets of **4** (and hence **1**).

Conclusion

Our high-throughput cell-based screen identified **1** as an inhibitor of human cell invasion by the parasite *Toxoplasma gondii*.[1] Subsequent studies demonstrated that **1** specifically inhibits extension of the parasite's conoid, an apical cytoskeletal structure of unknown function.[16] Compound **1** therefore represents a useful new experimental tool for studying conoid extension and invasion by this important human pathogen. Our previous studies showed that **1** can react rapidly and covalently with amine-based nucleophiles and unexpectedly gave rise to adducts of type **7**.[2] However, in order to link the interesting chemistry of **1** to its specific biological effects in *T. gondii*, it is crucial to identify its biological target(s). We had initially hoped to do so using the fluorescent adduct that is potentially generated when **1** reacts with protein-based amine nucleophiles. While experiments with 20 nmoles of a model substrate generated detectable fluorescence signals in a suitable format for use in target identification studies, no labelling of parasite proteins was observed, possibly because of insufficient signal-to-noise ratios.

To increase our detection sensitivity, we decided to explore whether a biotin group could be added to **1**. Biotin was chosen because highly sensitive detection reagents are commercially available and biotinylated probes have been successfully used in other systems for target identification.[3] The options of where to incorporate the biotin group in **1** (C6/7 or C5/8) were limited by several chemical constraints, including the fact that substitution at either of these positions renders the two electrophilic centres (C9 and C10) non-equivalent. Nonetheless, we decided to prepare **5** as a key synthetic intermediate. Subsequent analysis of the reaction of **5** with the model amine, *n*-butylamine, showed that whilst the underlying chemical reactivity was the same as **1**, several interesting differences resulted from the incorporation of the NHBoc substituent. This included the formation of regioisomeric final products **19** and **20**, the observed ratio of which was rationalised based on the relative acidity of the C9 and C10 protons. Importantly, the incorporation of an NHBoc functional group and the associated changes in chemical reactivity did not significantly alter the biological potency of **5** (*cf.* **1**). It was therefore decided to convert **5** to the biotinylated analogue **4**. This analogue of **1** is well suited for use in target identification studies that will be the topic of a future report.

Experimental

Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without further purification. Dichloromethane (DCM) was dried by heating under reflux over calcium hydride and distilled under an atmosphere of nitrogen. Thionyl chloride was freshly distilled under an atmosphere of nitrogen before use. mCPBA was purified by dissolving in DCM and washing with an aqueous solution of buffered KH_2PO_4 (1.0 M) at pH 7.4–7.5. The DCM phase was then treated as for a separation. Petroleum ether (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 ($CDCl_3$: $\delta_H = 7.26$, $\delta_C = 77.0$ ppm). Low and high-resolution mass spectral analysis were recorded using ES operating in positive or negative ion mode.

tert-Butyl 2,3-bis(bromomethyl)quinoxalin-6-ylcarbamate 1,4-dioxide, 5

To a solution of **15**[11] (1.04 g, 2.40 mmol) in anhydrous DCM (80 mL) was added purified mCPBA (2.07 g, 12.0 mmol) with stirring at room temperature. After 42 h the reaction mixture was diluted with DCM (250 mL) and washed with 10% w/v aqueous Na_2CO_3 solution (2 × 250 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow solid. Purification by flash column chromatography on silica gel (EtOAc : PE 40–60, 1 : 4) gave **5** as a bright yellow crystalline solid (0.89 g, 1.91 mmol, 80%); mp >180 °C dec. (recrystallised from EtOAc : PE 40–60 1 : 3). IR (KBr) ν_{max}/cm^{-1}: 3313, 1737 (CO), 1611, 1546, 1480, 1369 (N–O), 1237, 1145, 1030, 881–730 (ArC–H), 635 (C–Br); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.56 (1H, d, $^{3}J = 9.4$, H-8), 8.51 (1H, d, $^{4}J = 2.3$, H-5), 8.23 (1H, br d, $^{3}J = 9.4$, H-7), 7.37 (1H, br s, NH), 4.93 (2H, s, CH_2Br), 4.92 (2H, s, CH_2Br), 1.57 (9H, s, 3 × CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 151.8 (C), 143.5 (C), 140.1 (C), 138.1 (CH), 137.9 (C), 133.2 (C), 124.0 (C), 121.8 (CH), 106.2 (CH), 82.4 (C), 28.2 (3 × CH_3), 20.7 (CH_2), 20.6 (CH_2); MS-ES− (m/z) 464 ([M − H]$^-$, 2 × ^{81}Br, 21%), 462 ([M − H]$^-$, ^{79}Br + ^{81}Br, 100), 460 ([M − H]$^-$, 2 × ^{79}Br, 22); HRMS-ES− (m/z) calcd for $C_{15}H_{16}{}^{79}Br^{81}BrN_3O_4$ [M − H]$^-$: 461.9487, found 461.9494; anal. calcd for $C_{15}H_{17}Br_2N_3O_4$: C, 38.90; H, 3.70; N, 9.07. Found: C, 38.76; H, 3.54; N, 8.77%. The structure of **5** was confirmed by X-ray crystallographic analysis§.

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2,3-Bis(bromomethyl)quinoxalin-6-ylamine 1,4-dioxide hydrobromide, 28

To a solution of **5** (580 mg, 1.25 mmol) in AcOH (5.0 mL) was added a solution of 45% HBr in AcOH (4.0 mL) and the reaction mixture stirred at room temperature for 10 min. The resulting solution was added dropwise to cold dry diethyl ether (20.0 mL) with stirring. The resulting precipitate was collected by suction filtration washing with diethyl ether (20.0 mL). The desired product **28** was collected as a pink solid (521 mg, 1.17 mmol, 94%); mp > 200 °C dec. IR (NaCl, nujol) ν_{max}/cm^{-1}: 3387 (NH_2), 2576, 1329 (N–O), 1159, 1026, 834 and 736 (ArC–H); ^{1}H NMR (300 MHz, CD_3OD): δ 8.24 (1H, d, ^3J = 9.4, *H*-8), 7.42 (1H, d, ^4J = 2.4, *H*-5), 7.28 (1H, dd, ^3J = 9.4, ^4J = 2.4, *H*-7), 4.94 (2H, s, CH_2), 4.93 (2H, s, CH_2); MS-ES+ (*m*/*z*) 366 ([M − Br]$^+$, 2 × ^{81}Br, 39%), 364 ([M − Br]$^+$, ^{79}Br + ^{81}Br, 100), 362 ([M − Br]$^+$, 2 × ^{79}Br, 44), 316 (55), 314 (54), 300 (14), 298 (13); HRMS-ES+ (*m*/*z*) calcd for $C_{10}H_{10}{}^{79}Br^{81}BrN_3O_2$ [M − Br]$^+$: 363.9119, found 363.9124; HRMS-ES+ (*m*/*z*) calcd for $C_{10}H_{10}{}^{79}Br_2N_3O_2$ [M − Br]$^+$: 361.9140, found 361.9139; anal. calcd for 1.03HBr.$C_{10}H_9Br_2N_3O_2$: C, 27.87; H, 2.34; N, 9.75. Found: C, 27.88; H, 2.06; N, 9.41%.

***N*-[2,3-bis(bromomethyl)quinoxalin-6-yl 1,4-dioxide]-5-[(3a*S*,4*S*,6a*R*)-2-oxo-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanamide, 4**

D-(+)-Biotin (500 mg, 2.05 mmol) and thionyl chloride (10.0 mL) were stirred together under nitrogen. The biotin dissolved and gave a homogeneous yellow solution after stirring at room temperature for 15 min. After a further 15 min at room temperature the excess thionyl chloride was removed *in vacuo*. DMF (8.0 mL) was added to the residue followed by pyridine (32 mg, 0.41 mmol) with stirring under nitrogen. Compound **28** (182 mg, 0.41 mmol) was added and the reaction mixture stirred at room temperature for 5 h. The solvent was removed *in vacuo*. Purification by flash column chromatography on silica gel (DCM : MeOH 1 : 19) gave **4** as a yellow solid (58 mg, 0.10 mmol, 24%); mp >180 °C dec. IR (thin film, DCM) ν_{max}/cm^{-1}: 3500–2800, 1745, 1718 (CO amide), 1688, 1654 (CO urea), 1561, 1539, 1459, 1433, 1332 (N–O), 1264, 1037, 733 and 699 (ArC–H); ^{1}H NMR (300 MHz, CD_3OD): δ 9.01–8.96 (1H, m, *H*-5), 8.51–8.46 (1H, m, *H*-8), 8.06–7.99 (1H, m, *H*-7), 5.14 (2H, s, CH_2Br), 4.99 (2H, s, CH_2Br), 4.56 (2H, s), 4.49–4.44 (1H, m, C*H*CH$_2$S of biotin unit), 4.31–4.27 (1H, m, C*H*CHS of biotin unit), 3.24–3.18 (1H, m, C*H*S of biotin unit), 2.90 (1H, dd, ^2J = 12.7, ^3J = 4.9, one of the CH_2S of biotin unit), 2.67 (1H, d, ^2J = 12.7, one of the CH_2S of biotin unit), 2.48 (2H, t, ^3J = 7.2, CH_2CON), 1.84–1.43 (6H, m, (CH_2)$_3$CH$_2$ON); ^{13}C NMR (125.5 MHz, $CDCl_3$) δ 175.1 (C), 166.1 (C), 144.6 (C), 142.3 (C), 139.9 (C), 139.7 (C), 135.3 (C), 126.6 (CH), 122.1 (CH), 108.4 (CH), 63.4 (CH), 61.8 CH), 57.1 (CH), 41.0 (CH_2), 37.8 (CH), 36.6 (CH_2), 27.8 (CH_2), 29.6 (CH_2), 26.5 (CH_2), 21.9 (CH_2); MS-ES+ (*m*/*z*) 614 ([M + Na]$^+$, 2 × ^{81}Br, 5%), 612 ([M + Na]$^+$, ^{79}Br + ^{81}Br, 31), 610 ([M + Na]$^+$, 2 × ^{79}Br, 4), 570 ([M − CONH$_2$ + Na]$^+$, 2 × ^{81}Br, 3), 568 ([M − CONH$_2$ + Na]$^+$, ^{79}Br + ^{81}Br, 100), 566 ([M − CONH$_2$ + Na]$^+$, 2 × ^{79}Br, 49); HRMS-ES+ (*m*/*z*) calcd for $C_{20}H_{23}{}^{79}Br^{81}BrN_5O_4NaS$ [M + Na]$^+$: 611.9715, found 611.9731; HRMS-ES+ (*m*/*z*) calcd for $C_{20}H_{23}{}^{79}Br_2N_5O_4NaS$ [M + Na]$^+$: 609.9735, found 609.9717.

***tert*-Butyl-2-butyl-2*H*-pyrrolo[3,4-*b*]quinoxalin-7-ylcarbamate 4-oxide, 19 and *tert*-butyl-2-butyl-2*H*-pyrrolo[3,4-*b*]quinoxalin-6-ylcarbamate 4-oxide, 20**

Prepared according to the kinetic NMR procedure using **5** and *n*-butylamine (see reference 2 for a general protocol). Also, on a preparative scale: to a solution of **5** (112 mg, 0.24 mmol) in $CDCl_3$ (6 mL) was added a solution of *n*-butylamine (53 mg, 0.73 mmol) in $CDCl_3$ (6 mL) with stirring at room temperature. After 15 h the reaction mixture was quenched with silica and the solvent removed *in vacuo*. Purification by flash column chromatography on silica gel (EtOAc) gave **19** and **20** as a mixture of regioisomers. **19** and **20** were collected as an unstable dark red oil (35 mg, 0.10 mmol, 41%) and were observed to decompose rapidly in the absence of solvent. Compounds **19** + **20**: MS-ES+ (*m*/*z*) 357 ([M + H]$^+$, 100), 301 (56); HRMS-ES+ (*m*/*z*) calcd for $C_{19}H_{25}N_4O_3$ [M + H]$^+$: 357.1927, found 357.1925. Compound **19**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.48 (1H, d, ^3J = 9.8 Hz, *H*-5), 7.91 (1H, d, ^4J = 2.1, *H*-8), 7.69 (1H, dd, ^3J = 9.8, ^4J = 2.1, *H*-6), 7.61 (1H, d, ^4J = 2.5, *H*-3), 7.51 (1H, d, ^4J = 2.5, *H*-1), 6.83 (1H, br s, N*H*), 4.35 (2H, t, ^3J = 7.1, NCH_2), 2.04–1.94 (2H, m, NCH$_2$CH_2), 1.55 (9H, s, 3 × CCH_3), 1.41–1.28 (2H, m, CH_2CH$_3$), 0.97 (3H, t, ^3J = 7.4, CH_3). Compound **20**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.41 (1H, d, ^4J = 2.5, *H*-5), 8.18 (1H, d, ^3J = 9.5, *H*-8), 7.79 (1H, dd, ^3J = 9.5, ^4J = 2.5, *H*-7), 7.61 (1H, d, ^4J = 2.5, *H*-3), 7.58 (1H, d, ^4J = 2.5, *H*-1), 6.97 (1H, br s, N*H*), 4.42 (2H, t, ^3J = 7.1, NCH_2), 2.06–2.00 (2H, m, NCH$_2$CH_2), 1.55 (9H, s, 3 × CCH_3), 1.47–1.36 (2H, m, CH_2CH$_3$), 0.99 (3H, t, ^3J = 7.4, CH_3).

Diels–Alder adducts with *N*-phenylmaleimide

To a solution of **5** (0.56 g, 1.20 mmol) in degassed $CHCl_3$ (60.0 mL) was added *n*-butylamine (0.26 g, 3.60 mmol) with stirring at room temperature. After 3.5 h, *N*-phenylmaleimide (0.83 g, 4.80 mmol) was added and the reaction continued stirring at room temperature for 14 h. The solvent was removed *in vacuo* to give a brown solid, which by ^{1}H NMR analysis of the crude reaction contained *endo* : *exo* products in a ratio of 71 : 29 and showed four regioisomeric products. Purification by flash column chromatography on silica gel (EtOAc : PE 40–60, 1 : 1) gave the desired compounds, **24a/25a** as a pink crystalline solid (0.23 g, 0.43 mmol, 36%); and **24b/25b** as a yellowy brown solid (0.12 g, 0.22 mmol, 18%).

***endo* Regioisomers, 24a and 25a**

Compounds **24a** and **25a** mixed mp > 80 °C dec. to red crystals; IR (NaCl, nujol) ν_{max}/cm^{-1}: 1776, 1717 (CO), 1239, 1156, 1052, 833, 734 (ArC–H) and 690; MS-ES+ (*m*/*z*) 1081 ([2M + Na]$^+$, 53%), 552 ([M + Na]$^+$, 7), 530 ([M + H]$^+$, 40), 411 (8), 379 (100), 357 (36), 247 (24); MS-ES− (*m*/*z*) 528 ([M − H]$^-$, 100%), 355 (24); HRMS-ES− (*m*/*z*) calcd for $C_{29}H_{30}N_5O_5$ [M − H]$^-$: 528.2247, found 528.2232. Major regioisomer **24a**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.47 (1H, d, ^3J = 9.3, *H*-8), 8.08 (1H, d, ^4J = 2.3, *H*-5), 7.84 (1H, dd, ^3J = 9.3, ^4J = 2.3, *H*-7), 7.14–7.05 (4H, m, *H*-3′, *H*-5′, N*H* and *H*-4′), 6.37–6.32 (2H, m, *H*-2′ and *H*-6′), 5.40 (1H, dd, ^3J = 5.3, ^4J = 1.6, *H*-9), 4.82 (1H, dd, ^3J = 5.6, ^4J = 1.6, *H*-15), 4.13–4.08 (1H, m, *H*-10), 4.08–4.04 (1H, m, *H*-14), 2.37 (2H, t, ^3J = 7.5, NCH_2), 1.54 (9H, s, 3 × CCH_3), 1.50–1.43 (2H, m, NCH$_2$CH_2), 1.32–1.24 (2H, m, CH_2CH$_3$), 0.87 (3H, t, ^3J = 7.3, CH_3); ^{13}C NMR (125.5 MHz, $CDCl_3$) δ; 172.5 (C), 172.0 (C),

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160.5 (C), 152.0 (C), 146.0 (C), 141.8 (C), 136.9 (C), 132.5 (C), 130.5 (C), 128.9 (2 × CH), 128.5 (CH), 125.7 (2 × CH), 121.5 (CH), 119.6 (CH), 115.8 (CH), 81.7 (C), 67.7 (CH), 63.2 (CH), 48.2 (CH_2), 47.1 (CH_2), 47.0 (CH), 30.5 (CH_2), 28.2 (CH_3), 20.3 (CH_2), 13.8 (CH_3). Minor regioisomer **25a**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.38 (1H, d, $^4J = 2.4$, *H*-5), 8.15 (1H, br m, *H*-7), 8.02 (1H, d, $^3J = 9.1$, *H*-8), 7.14–7.05 (4H, m, *H*-3′, *H*-5′, N*H* and *H*-4′), 6.37–6.32 (2H, m, *H*-2′ and *H*-6′), 5.42 (1H, dd, $^3J = 5.3$, $^4J = 1.6$, *H*-9), 4.83 (1H, dd, $^3J = 5.6$ $^4J = 1.6$, *H*-15), 4.13–4.08 (1H, m, *H*-10), 4.08–4.04 (1H, m, *H*-14), 2.37 (2H, t, $^3J = 7.5$, NCH_2), 1.54 (9H, s, 3 × CCH_3), 1.50–1.43 (2H, m, NCH$_2$CH_2), 1.32–1.24 (2H, m, CH_2CH$_3$), 0.87 (3H, t, $^3J = 7.3$, CH_3); ^{13}C NMR (125.5 MHz, $CDCl_3$) δ; 172.6 (C), 171.9 (C), 157.7 (C), 152.1 (C), 141.0 (C), 140.8 (C), 138.4 (C), 137.3 (C), 130.5 (C), 130.7 (CH), 128.9 (2 × CH), 128.5 (CH), 125.7 (2 × CH), 123.3 (CH), 105.2 (CH), 81.7 (C), 67.6 (CH), 63.3 (CH), 48.2 (CH_2), 47.1 (CH), 47.0 (CH), 30.5 (CH_2), 28.2 (CH_3), 20.3 (CH_2), 13.8 (CH_3).

exo Regioisomers, 24b and 25b

Compounds **24b** and **25b** mixed mp 113.0–115.0 °C dec.; IR (NaCl, nujol) ν_{max}/cm^{-1}: 1779, 1712 (CO), 1238, 1154, 1052, 840, 757 (ArC–H), and 692; MS-ES+ (*m/z*) 552 ([M + Na]$^+$, 6%), 379 (100), 269 (59), 247 (14); MS-ES− (*m/z*) 528 ([M − H]$^-$, 100%), 355 (14); HRMS-ES− (*m/z*) calcd for $C_{29}H_{30}N_5O_5$ [M − H]$^-$: 528.2247, found 528.2259. Major regioisomer **24b**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.51 (1H, d, $^3J = 9.3$, *H*-8), 8.07 (1H, d, $^4J = 2.3$, *H*-5), 7.84 (1H, dd, $^3J = 9.3$, $^4J = 2.3$, *H*-7), 7.53–7.40 (3H, m, *H*-3′, *H*-5′and *H*-4′), 7.32–7.29 (2H, m, *H*-2′ and *H*-6′), 6.93 (1H, br s, N*H*), 5.27 (1H, s, *H*-9), 4.76 (1H, s, *H*-15), 3.32 (1H, d, $^3J = 7.1$, *H*-14), 3.22 (1H, d, $^3J = 7.1$, *H*-10), 2.33 (2H, t, $^3J = 7.0$, NCH_2), 1.56 (9H, s, 3 × CCH_3), 1.46–1.34 (2H, m, NCH$_2$CH_2), 1.34–1.20 (2H, m, CH_2CH$_3$), 0.85 (3H, t, $^3J = 7.3$, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 174.7 (C), 174.2 (C), 163.0 (C), 152.0 (C), 146.1 (C), 141.6 (C), 138.4 (C), 132.6 (C), 131.9 (C), 129.3(2 × CH), 128.9 (CH), 126.4 (2 × CH), 121.4 (CH), 119.5 (CH), 115.8 (CH), 81.8 (C), 68.8 (CH), 63.8 (CH), 47.4 (CH_2), 47.0 (CH_2), 46.9 (CH), 30.5 (CH_2), 28.2 (CH_3), 20.0 (CH_2), 13.7 (CH_3). Minor regioisomer **25b**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.43 (1H, d, $^4J = 2.3$, *H*-5), 8.07 (1H, br d, $^3J = 9.1$, *H*-7), 8.02 (1H, d, $^3J = 9.1$, *H*-8), 7.53–7.40 (3H, m, *H*-3′, *H*-5′and *H*-4′), 7.30 (2H, m, *H*-2′ and *H*-6′), 7.06 (1H, br s, N*H*), 5.28 (1H, s, *H*-9), 4.76 (1H, s, *H*-15), 3.32 (1H, d, $^3J = 7.1$, *H*-10/14), 3.22 (1H, d, $^3J = 7.1$, *H*-10/14), 2.33 (2H, t, $^3J = 7.0$, NCH_2), 1.56 (9H, s, 3 × CCH_3), 1.46–1.34 (2H, m, NCH$_2$CH_2), 1.34–1.20 (2H, m, CH_2CH$_3$), 0.85 (3H, t, $^3J = 7.3$, CH_3).

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