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Benzofuranyl 3,5-bis-Polyamine Derivatives as Time-Dependent Inhibitors of Trypanothione Reductase

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Abstract—The synthesis and evaluation of 3,5-disubstituted benzofuran derivatives as time-dependent inhibitors of the protozoan oxidoreductase trypanothione reductase are reported. These molecules were designed as simplified mimetics of the naturally occurring spermidine-bridged macrocyclic alkaloid lunarine **1**, a known time-dependent inhibitor of trypanothione reductase. In this series of compounds the bis-polyaminoacrylamide derivatives **2**–**4** were all shown to be competitive inhibitors, but only the bis-4-methyl-piperazin-1-yl-propylacrylamide derivative **4** displayed time-dependent activity. The kinetics of time dependent inactivation of trypanothione reductase by **1** and **4** have been determined and are compared and discussed herein.

Introduction

Parasitic protozoa belonging to the genera *Trypanosoma* and *Leishmania* cause a number of severe infectious diseases in humans and their domestic livestock in tropical countries. In humans, these include Chagas' disease (caused by *Trypanosoma cruzi*), African sleeping sickness (*Trypanosoma brucei rhodesiense* and *T.b. gambiense*), and visceral, mucocutaneous and cutaneous leishmaniasis (caused by various species of *Leishmania*).

A potential drug target in trypanosomes and leishmania has been identified through the discovery of a fundamental difference between the redox defence system of the trypanosomal/leishmanial parasite and the infected host.[1] The mammalian redox defence system, based on glutathione (L-γ-glutamyl-L-cysteinyl-glycine) and glutathione disulfide reductase (GR; EC 1.6.4.2), is replaced in trypanosomatids by an analogous system based on trypanothione [N^1,N^8-bis(glutathionyl)spermidine] and trypanothione disulfide reductase (TryR; EC 1.6.4.8). The structures of the disulfide substrates for TryR and GR are illustrated in Figure 1.

TryR is an NADPH-dependent flavoprotein oxidoreductase which maintains an intracellular reducing environment by the recycling of trypanothione disulfide ($T[S]_2$) to its dithiol form, $T[SH]_2$. Trypanothione is oxidised back to $T[S]_2$ following reaction with potentially damaging radicals and oxidants generated by aerobic metabolism and by host macrophages. By maintaining a high intracellular ratio of $T[SH]_2$ to $T[S]_2$, the TryR redox cycle is a primary line of defence for these parasites against respiratory burst responses from the mammalian host. Disabling the function of TryR in *Leishmania*[2] and *T. brucei*[3] has been shown to markedly increase the parasites' sensitivity to oxidative stress. $T[S]_2$ differs from GSSG by the presence of a spermidine cross-link between the two glycyl carboxyl groups (compare GSSG and $T[S]_2$ in Fig. 1). Due to structural and charge differences between $T[S]_2$ and GSSG, TryR and GR are mutually exclusive with respect to substrate specificity.[4] Thus the essential requirement for TryR, its absence in host metabolism and the potential to design specific inhibitors make it an attractive therapeutic target.

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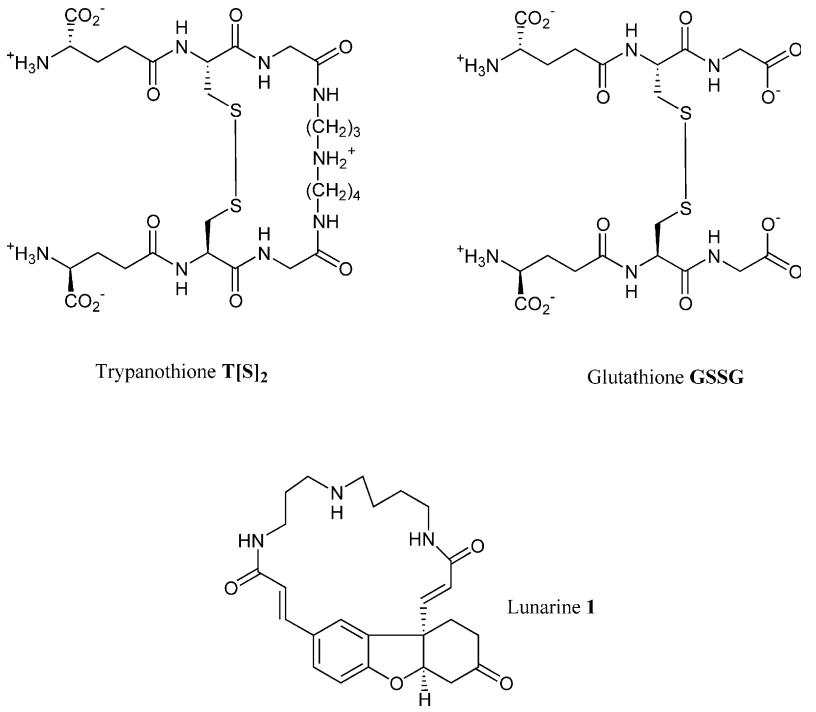


Figure 1. Native disulfide substrates for TryR ($T[S]_2$) and GR (GSSG) and the time dependent inhibitor of TryR lunarine **1**.

Various molecules have been identified as classical inhibitors of TryR such as hydrophobic linear polyamine derivatives[5] and the naturally occurring bis(tetrahydrocinnamoyl)spermine, kukoamine A.[6] Recently, the spermidine-based macrocyclic alkaloid lunarine **1** (Fig. 1) was identified as a competitive, time-dependent inhibitor of TryR in the reduced state ($TryR_{red}$).[7] This alkaloid is composed of a spermidine chain with the terminal nitrogen atoms forming amide linkages with two α,β-unsaturated carboxylic acid functions disposed upon an unusual 3-oxohexahydrodibenzofuranyl tricyclic scaffold.[8] A possible mechanism for this time-dependent inhibition involves the covalent modification of a redox-active cysteine residue in the active site of $TryR_{red}$ (C53) by conjugate addition to one of these unsaturated amide moieties in the lunarine macrocycle (Fig. 2). This is supported by both the requirement for the enzyme in its reduced form[7] and the presence of a potential Michael acceptor unit in the inhibitor.

As part of our ongoing investigations into the mechanistic details of TryR inhibition by the *Lunaria* alkaloids and the design of simplified analogues, we have prepared some benzofuranyl-based acyclic bis-polyamine analogues **2**–**4** of lunarine **1** where the skew boat cyclohexanone moiety[9] has been removed leaving a planar bicyclic benzofuranyl scaffold (Fig. 3). Acyclic bis-polyamine derivatives were chosen since bis-polyamine functionalised disulfides such as the naturally occurring N^1-glutathionylspermidine disulfide[4b] and the synthetic bis-dimethylaminopropyl- and bis-N^4-methylpiperazinyl amides of Ellman's reagent (DTNB)[10] are known TryR substrates. These three polyamine chains were chosen for functionalisation of a 3,5-disubstituted benzofuranyl template to give potential inhibitors **2**–**4**. Presented herein are the syntheses and inhibitory properties of these compounds.

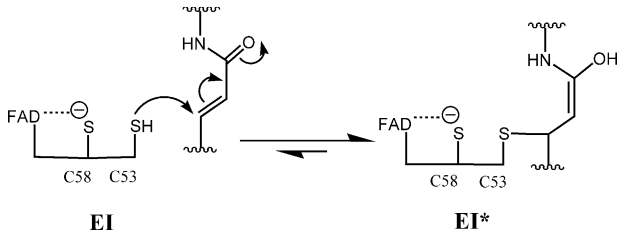


Figure 2. Proposed mechanism for time-dependent inactivation of TryR by the reversible formation of a covalent adduct between an active site thiol (C53) and one of the α,β-unsaturated amide groups of lunarine **1**.

Results

Synthesis

The common benzofuranyl derivative **11** that was required in this work was prepared as outlined in Schemes 1 and 2.

The tribromo derivative **6** was easily prepared from 5-bromobenzofuran **5**[11] by treatment with bromine in dichloromethane (Scheme 1)[12] and, due to its instability, was immediately treated with KOH[13] to give the 3,5-dibromobenzofuran **7** in excellent yield (98%). Compound **7** was then treated with copper(I) cyanide in DMF under reflux[14] to obtain the dinitrile **8** in 52% yield.[15] The relatively low yield of this reaction was due to significant mechanical loss during the treatment of the reaction products with acidic $FeCl_3$, which was a necessary step as residual copper(I) salts complex with the aromatic nitriles making separation difficult [$FeCl_3$

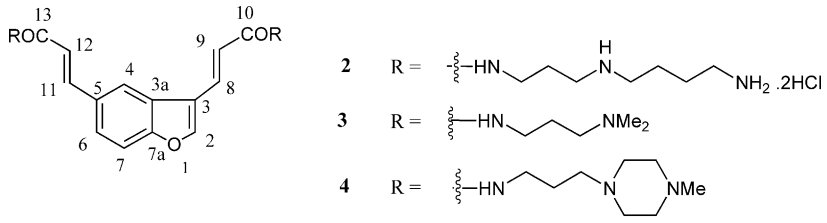
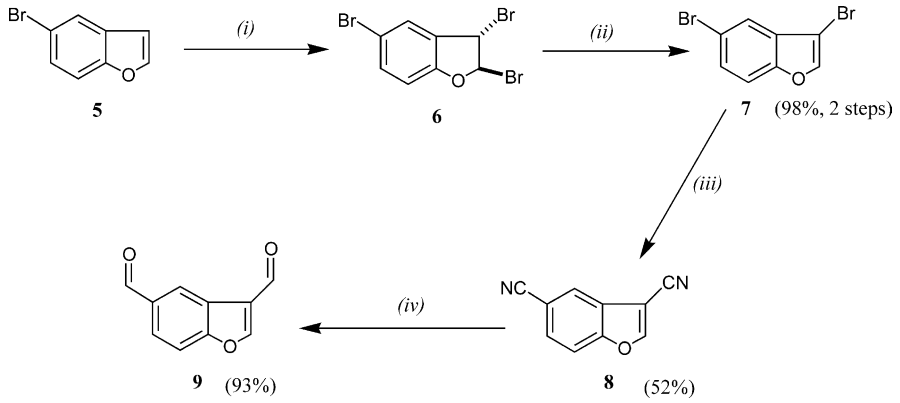
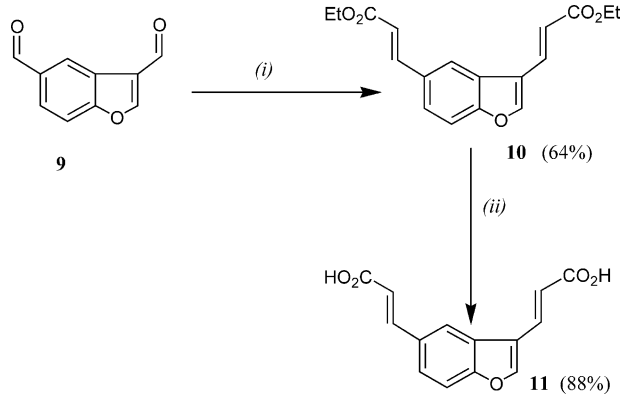


Figure 3. Structure of target compounds **2–4**.



Scheme 1. Reagents and conditions: (i) Br_2, CH_2Cl_2, 40 min; (ii) KOH, EtOH, reflux, 15 min; (iii) CuCN, DMF, reflux, 16 h, then $FeCl_3$, 1.5 M HCl, 70 °C, 30 min; (iv) DIBAL, CH_2Cl_2, −15 °C, 3 min, then 1 M $HCl_{(aq)}$, 0 °C.



Scheme 2. Reagents and conditions: (i) $Ph_3P{=}CHCO_2Et$, DMF, reflux, 4 h; (ii) LiOH, $THF/H_2O/MeOH$, 4 h.

treatment oxidises them to the non-complexing copper(II) species].[15] Compound **8** was then reduced to the dialdehyde **9** by brief treatment with DIBAL at −15 °C. Lower reaction temperatures could not be employed in this case due to precipitation of the starting material.

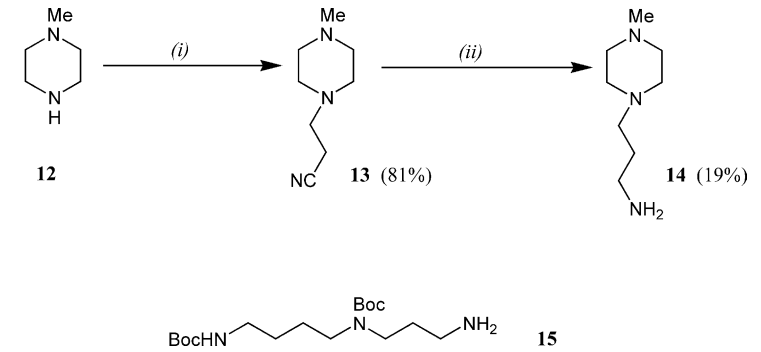
To complete the preparation of diacid **11**, dialdehyde **9** was first converted to the bis-α,β-unsaturated ester **10** by reaction with (ethoxycarbonylmethylene)-triphenylphosphorane in 64% yield (Scheme 2). Saponification of **10** by treatment with aqueous LiOH then gave the di-carboxylate **11** in 88% isolated yield after ion exchange chromatography. Although the dilithium salt of **11** was readily soluble in water, the free acid was only sparingly soluble in both water and most organic solvents, and some product was therefore lost through precipitation on the cation exchange column that was used to isolate **11**.

Three polyamines were required for preparation of the acyclic benzofuranyl bis-polyamine derivatives **2–4**. 3-Dimethylaminopropylamine was commercially available, while the selectively masked spermidine **15** was prepared as previously described.[8] *N*-Methylpiperazinylpropylamine **14** was prepared as shown in Scheme 3, nitrile derivative **13** being prepared from *N*-methylpiperazine **12** using a modification of a previously reported procedure,[16] then hydrogenated over a rhodium catalyst to give the free amine **14**,[17] albeit in low yield (19%).

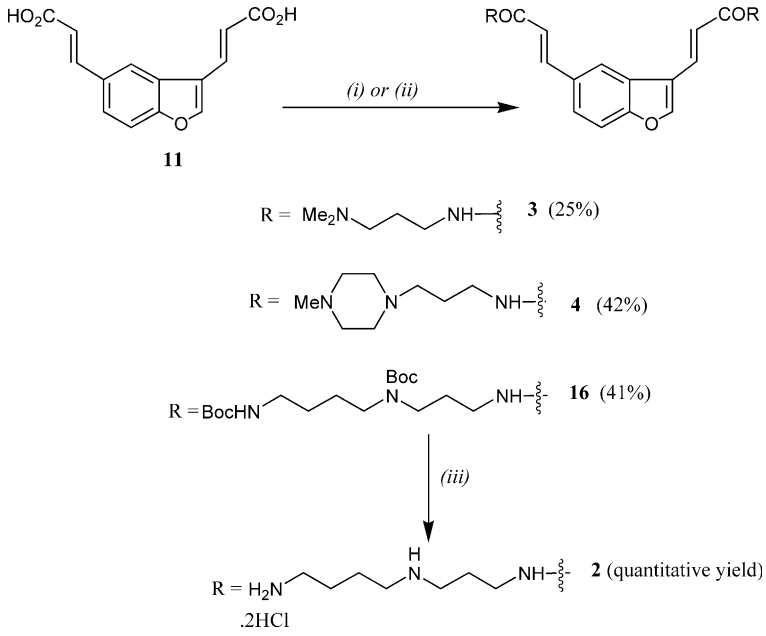
The bis-polyamine derivatives were prepared from the diacid **11** using two different amide bond-forming procedures (Scheme 4). Compounds **3** and **4** were prepared via the in situ formation of the mixed anhydride of **11** and diphenylphosphinic chloride,[18] which was then treated with 3-dimethylaminopropylamine or **14** to give **3** and **4** in yields of 35 and 42%, respectively. Activation of **11** as the bis-pentafluorophenyl ester followed by treatment with the selectively protected spermidine derivative **15**[8] gave the *tert*-butoxycarbonyl-protected bis-spermidine analogue **16** in 41% yield. Cleavage of the *tert*-butoxycarbonyl protecting groups with 4 M HCl in dioxane then gave **2** in essentially quantitative yield as the hydrochloride salt.

Inhibition assays

Compounds **2–4** and **10**, **11** and **16** were initially screened for time-dependent inhibition of TryR using a microplate assay which we have developed for this purpose.[19] The inhibition assays were carried out using 100 μM inhibitor concentrations in the presence of 1 μM trypanothione disulfide and excess NADPH.



Scheme 3. Reagents and conditions: (i) CH_2=CHCN, MeOH, 2.5 h; (ii) 5% Rh/C, H_2 (2–3 atm), EtOH saturated with NH_3, 20 h.



Scheme 4. Reagents and conditions: (i) $Ph_2P(O)Cl$, Et_3N, DMF, −10 °C 1 h then add RNH_2, 6 h; (ii) C_6F_5OH, EDCI, DMAP, DMF, 3 h then RNH_2, CH_2Cl_2, 2 h; (iii) 4 M HCl/dioxane, 1 h.

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was included in the assay mixture to mediate the rapid re-oxidation of the product $T[SH]_2$ back to $T[S]_2$. In this manner, the continuous recycling of $T[SH]_2$ ensured that the concentration of substrate remained constant for the duration of these prolonged assays such that any decrease in enzyme activity over time could be assigned to time-dependent inhibition rather than substrate depletion. In order to screen for time-dependent inactivation of TryR, these continuous assays were monitored for 60 min following the addition of inhibitor.

With the absence of any polyamine side chains, neither the diester **10** nor the diacid **11** showed any inhibitory activity towards TryR at 100-μM concentrations. **11** was also inactive against human GR at 100-μM concentrations in the presence of 80 μM GSSG substrate (K_m = 64 μM).

Inhibition of TryR by the *tert*-butoxycarbonyl protected bis-spermidine derivative **16** was also found to be negligible, but in the initial microplate assay both **2** and **3** inhibited TryR in a linear manner. A more detailed analysis of these compounds showed them to be simple competitive inhibitors of TryR, with respect to $T[S]_2$, with respective K_i values of 114 (±14) and 196 (±19) μM (compared to a substrate K_m value of 28 (±4) μM (Fig. 4). The methylpiperazine functionalised bis-polyamine **4** displayed time-dependent activity against TryR over a 60-min incubation period in the microplate assay. A further detailed analysis of this compound was therefore carried out alongside lunarine **1** to deduce both the kinetic parameters and likely mechanism of time-dependent inhibition of TryR with **4** as outlined below.

Time-dependent inhibition can arise from three different mechanisms (Fig. 5). In classical inhibition mechanisms, the steady state equilibrium between enzyme and inhibitor is rapidly established (usually within milliseconds). Simple slow binding (mechanism B) occurs when the inhibitor binds to the enzyme like a classical inhibitor but with a slower binding rate such that the steady state is attained over a much longer timescale of several seconds or even min. With an enzyme isomerisation mechanism (C), the Michaelis–Menten complex (EI) is rapidly formed followed by slower conformational

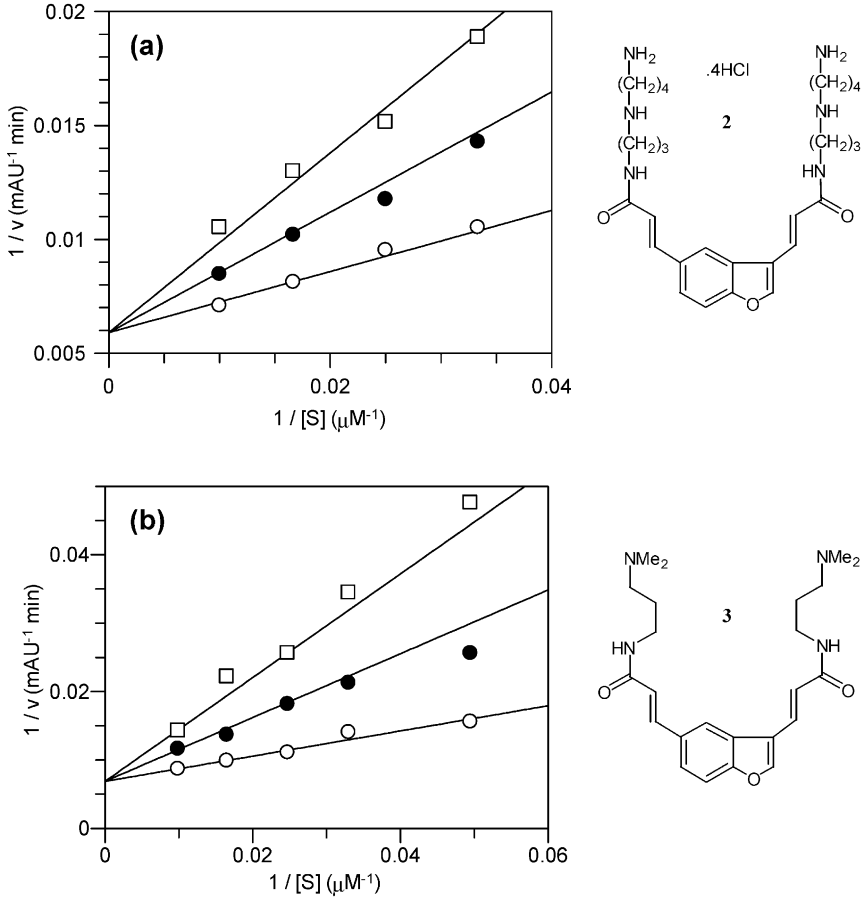


Figure 4. (a) Double reciprocal plots of initial rate against substrate concentration ($T[S]_2$) in the presence of compound **2** at 0 μM (○); 100 μM (●); and 200 μM (□) concentrations. (b) Double reciprocal plots of initial rate against substrate concentration ($T[S]_2$) in the presence of compound **3** at 0 μM (○); 293 μM (●); and 596 μM (□) concentrations.

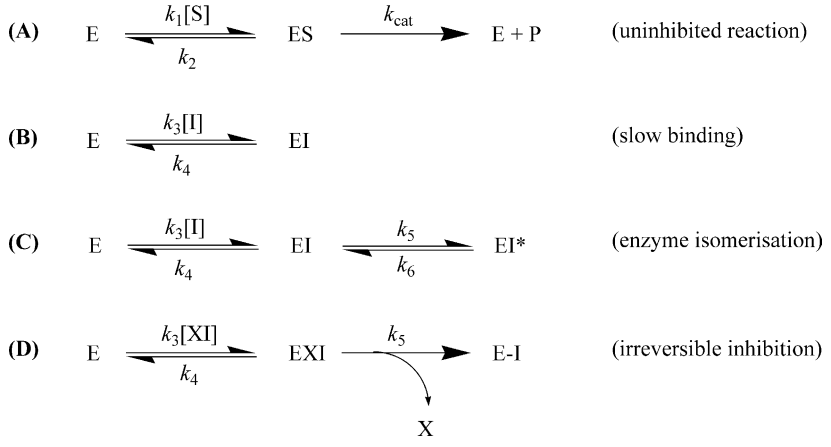


Figure 5. The basic mechanisms of time-dependent inhibition.

changes in either the enzyme or inhibitor resulting in a more tightly bound complex (EI*). Irreversible inhibition proceeds by the same rapid binding step followed by a rate-limiting covalent modification of the enzyme (D).

The time-dependent inhibition mechanism for **4** and **1** was investigated using an extended continuous assay[19] to monitor the time-dependent effect of different inhibitor concentrations on enzyme activity (Figs 6 and 7, respectively). The reaction progress curves for TryR in the presence of a range of concentrations of **4** (Fig. 6a) indicate a slow establishment of a final steady state velocity as a result of either mechanism (B) or (C). The concentration-dependent values for the initial rate (v_i), steady-state rate (v_s), the apparent rate constant for establishing the steady state equilibrium between EI and EI* (k') and the displacement of the curve from the vertical ordinate (d) were determined by non-linear fitting of these data to eq 1.[20]

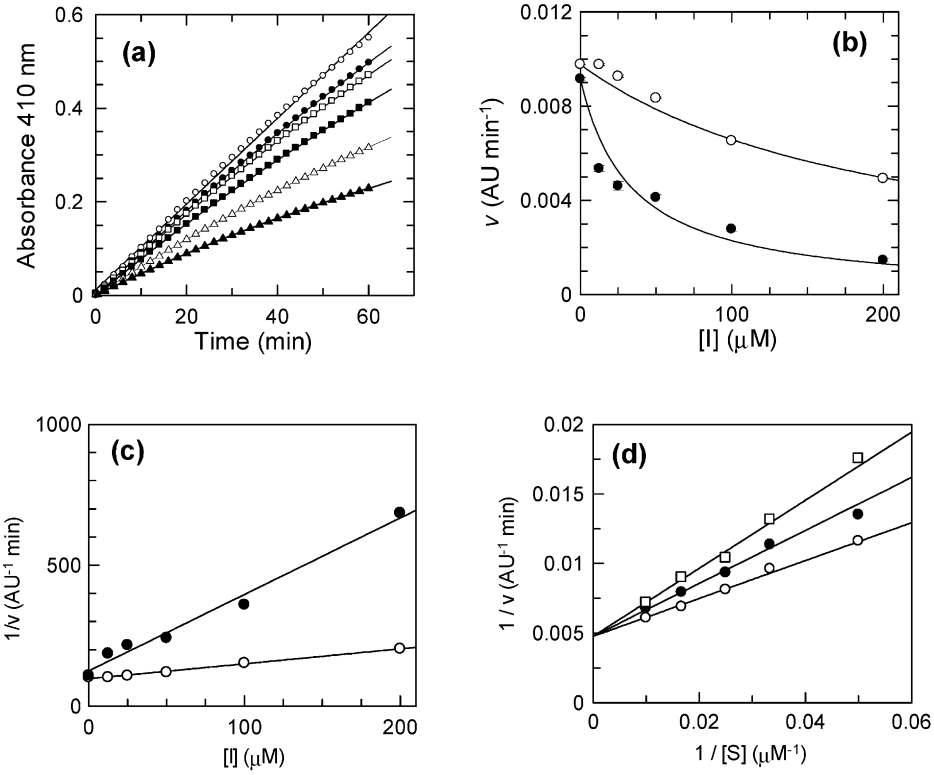


Figure 6. Inhibition of TryR by **4**. (a) Reduction in enzyme activity over time as a function of inhibitor concentration: no inhibitor (○); plus inhibitor: 12.5 μM (●); 25 μM (□); 50 μM (■); 100 μM (△); 200 μM (▲). (b) Re-plot of variation in v_i (○) and v_s (●) with respect to concentration of **4**. These data were fitted to equations (2) and (3) to derive values for K_i and K_i*, respectively (Table 1). (c) Variation of the reciprocal of v_i (○) and v_s (●) as a function of concentration of **4**. (d) Double reciprocal plot of v_i against substrate concentration in the presence of no inhibitor (○); plus inhibitor: 100 μM (●); 200 μM (□) to demonstrate the competitive nature of inhibition by **4**.

$$P = v_s t + (v_i - v_s)(1 - e^{-k't})/k' + d \quad (1)$$

The fact that both v_i and v_s vary as a function of inhibitor concentration for **4** (Fig. 6a and b) clearly indicates that a two-step isomerisation mechanism is operating [i.e., mechanism (C)] where a Michaelis-type inhibitor complex (EI) forms as an intermediate and two inhibition constants must therefore be considered. The first (K_i) is the dissociation constant for the initial EI complex and the second (K_i*) is the dissociation constant for the EI* complex that is subsequently formed. Non-linear curve fitting of these data using eqs 2 and 3 (Fig. 6b) was used to derive values for K_i and K_i*, respectively for **4**.

$$v_i = v_{i=0}/(1 + I/K_i(1 + S/K_m)) \quad (2)$$

$$v_s = v_{i=0}/(1 + I/K_i^*(1 + S/K_m)) \quad (3)$$

where S is the substrate concentration and K_m the apparent Michaelis–Menten constant for TryR. The variation in initial and steady state rates as a function of inhibitor concentration is shown in Fig. 6c where the slopes of the two lines illustrate the difference in the magnitudes of K_i and K_i*. The ratio between the forward and reverse isomerisation steps (k_5/k_6) for the interconversion between EI and EI* was calculated using eq 4.

$$k_5/k_6 = K_i/K_i^* - 1 \quad (4)$$

For each inhibitor concentration, the value for k_6 was determined from the relationship in eq 5.

$$k_6 = v_s k'/v_i \quad (5)$$

Finally, the mean value for k_6 was substituted into eq 4 to calculate k_5.

An identical analysis was performed for **1**[21] using the above procedures (see Fig 7) in order to extract the same kinetic parameters for time-dependent inhibition of TryR. This data is summarised in Table 1.

Discussion

Comparing **4** with **1**, it is apparent that the quite substantial structural modifications made actually have relatively little effect on the initial dissociation constant for this inhibitor with TryR. On the other hand, a 3-fold reduction in K_i* does result; a difference which is also reflected in the isomerisation constants k_5/k_6 (see Table 1). It should be noted that the differences in K_i* are a consequence of differences in the forward isomerisation constants k_5 as the reverse isomerisation constants for **4** and **1** are effectively the same. As the absolute k_5 values are relatively low it takes a long time (many min) to establish the steady state equilibrium between EI and

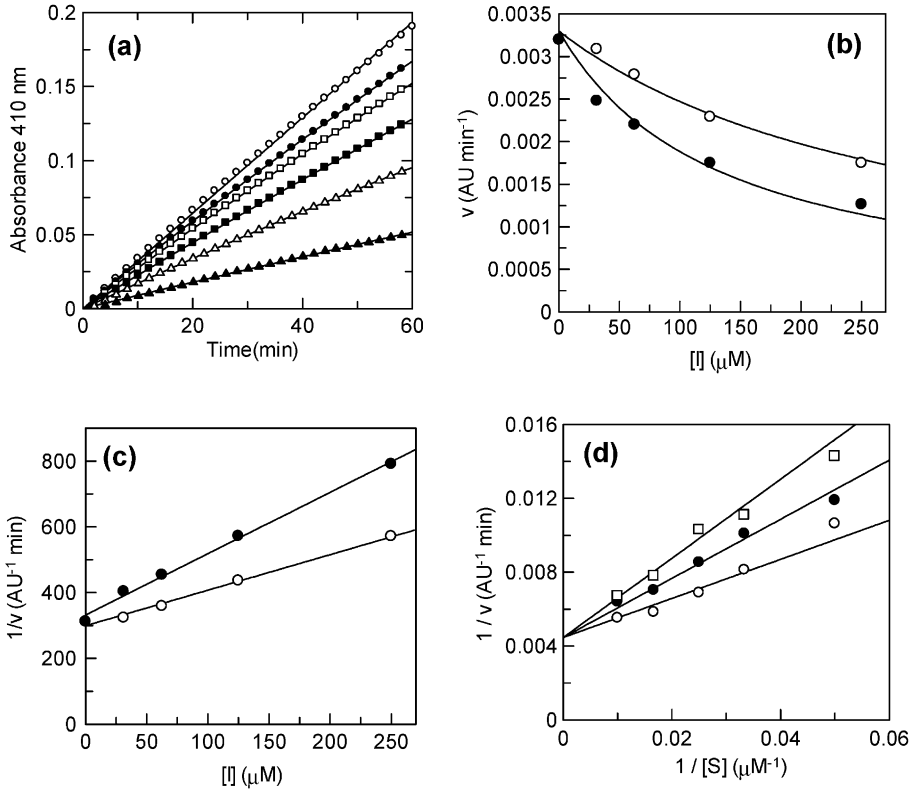


Figure 7. Inhibition of TryR by **1**. (a) Reduction in enzyme activity over time as a function of inhibitor concentration: no inhibitor (○); plus inhibitor: 31.25 μM (●); 62.5 μM (□); 125 μM (■); 250 μM (△); 500 μM. (b) Re-plot of variation in v_i (○) and v_s (●) with respect to concentration of **1**. These data were fitted to eqs 2 and 3 to derive values for K_i and K_i*, respectively (Table 1). (c) Variation of the reciprocal of v_i (○) and v_s (●) as a function of concentration of **1**. (d) Double reciprocal plot of v_i against substrate concentration in the presence of no inhibitor (○); plus inhibitor: 100 μM (●); 200 μM (□) to demonstrate the competitive nature of inhibition by **1**.

Table 1. Kinetic parameters for time-dependent inhibition of TryR by **1** and **4**

Compd	K_i (μM)	K_i* (μM)	k_5/k_6	k_5 (min^{-1})	k_6 (min^{-1})
4	213 (±9)	34 (±6)	5.2	0.0329	0.0063 (±0.0006)
1	304 (±2)	114 (±2)	1.7	0.0128	0.0077 (±0.0024)

EI*. This made it possible to determine the type of slow binding inhibition (e.g., competitive, non-competitive, etc.) by taking initial rate measurements (the first 60 s) over a range of substrate and **4** concentrations. The subsequent double reciprocal plot shows **4** to be a competitive inhibitor of TryR with respect to $T[S]_2$ (Fig. 6d).

The initial dissociation constants (K_i) for **2** and **4** are comparable to that of lunarine **1**, despite the simultaneous replacement of both the fused tricyclic scaffold of **1** with a planar bicyclic benzofuranyl ring system and also the bridging spermidine macrocycle with two linear alkylamines. The bis-polyamine derivatives **2**–**4** are all competitive inhibitors of TryR (with respect to $T[S]_2$) which may be explained by the affinity of their polyamine 'side chains' for the negatively charged spermidine binding pocket in the enzyme active site. Neither the neutral ethyl diester **10** nor the anionic diacid **11** are inhibitors of TryR, while the extremely bulky side chains on the *tert*-butoxycarbonyl protected bis-spermidine derivative **16** are also detrimental to inhibitor potency. Whilst the structural differences between the polyamine units of **2**–**4** appear to have little effect on their potency as competitive inhibitors of TryR, their efficacy as time-dependent inhibitors is much more sensitive to such alterations. Neither the bis-spermidine **2** nor the bis-dimethylamino-propylamine derivative **3** exhibit time-dependent inhibition kinetics with TryR during the timescale of the continuous enzyme inhibition experiments (60 min) in contrast to bis-methylpiperazinyl derivative **4**.

One possible explanation for the observed time-dependence of **4** is as follows: first an equilibrium is rapidly established between **4** and TryR where a Michaelis-type complex (EI) is formed. This may be followed by a much slower conformational change in order to position one of the α,β-unsaturated amide units in line for nucleophilic attack by an active site thiol to give a reversible covalent adduct (EI*). Relating this to mechanism (C), k_5 would reflect the conformational reorientation of the inhibitor and if the conjugate addition step were effectively instantaneous, k_6 would reflect the reverse reaction. For **4**, k_5 is 3 times greater than that determined for lunarine **1**, and so this might be attributed to the greater flexibility of the open chain structure of **4** that enables it to undergo a more rapid conformational change during the EI to EI* transition. In contrast, the conformational flexibility of **1** is more restricted by the spermidine bridge. The off rates (k_6) for **4** and **1** are effectively the same which is to be expected

if k_6 relates to a retro-Michael addition process since its rate will be governed by the leaving group ability of the β-carbon substituent (the same C53 residue in both cases).

Alternatively, if **4** or **1** were to undergo conjugate addition without any further change to the conformation of the initial EI complex, then the rate of nucleophilic attack by C53 alone should be what dictates the rate of EI* formation (reflected by k_5). That is, if the inhibitor were to dock in the active site such that a Michael acceptor unit and C53 are well aligned for conjugate addition, then the rate of conversion from EI to EI* will be faster than for the case where inhibitor binding leads to a less favourable mutual alignment. The 3-fold reduction in rate of time-dependent inactivation with **1** might therefore be simply because it is docked in the active site in a comparatively less favourable orientation for Michael addition relative to **4**. In these terms, the absence of time-dependent inhibition with **2** and **3** may then again be ascribed to their particular polyamine moieties that do not allow them to adopt an active-site-bound orientation that is favourable for reaction with C53.

Conclusion

The results presented herein demonstrate that a greatly simplified achiral analogue of the natural product lunarine **1** may exhibit comparable properties as a time-dependent inhibitor of TryR. Such results are encouraging as the synthetic complexity of **1** is an obstacle towards its further development as a lead chemotherapeutic against the range of orphan diseases associated with trypanosomal parasites. Further detailed investigations of the mechanism and minimal structural and functional requirements of time dependent-inhibitors for **1** and related compounds are ongoing and will be reported in due course.

Experimental

General

Melting points were recorded on an Electrothermal IA9000 series digital melting point apparatus, using open capillaries and are quoted uncorrected. IR spectra were recorded on a Nicolet 205 FT spectrophotometer. UV spectra were recorded on a Perkin-Elmer Lambda 16 spectrophotometer.

NMR Spectra: ^{1}H NMR spectra were recorded on a Bruker Avance DPX 300 FT-spectrometer at 300 MHz, ^{13}C NMR spectra were recorded on the same spectrometer at 75 MHz. Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) are given in Hz. Low resolution mass spectra were recorded on a VC 70-S double focusing mass spectrometer in Electron Impact (EI) mode, or on a VG Quattro triple quadrupole electrospray mass spectrometer (ESI). Accurate mass measurements were obtained on the VC 70-S instrument, or at the EPSRC National Mass Spectrometry Service (University of Wales Swansea). Flash chromatography was performed on columns of silica gel (Fluorochem, Silica Gel 60; 40–63 μ). Reverse phase chromatography was performed using Supelco Discovery™ DSC-18 solid phase extraction tubes. Petroleum ether bp 40–60 °C (referred to as petrol) was distilled through a vigreux column prior to use. All other solvents were dried using standard procedures.

Recombinant *T. cruzi* TryR was expressed in *Escherichia coli* and purified, as previously described.[22] The enzyme was stored as a suspension in 70% ammonium sulfate solution at 4 °C and extensively dialysed against the assay buffer before use. Glutathione reductase was purified to homogeneity from human erythrocytes.[23] Trypanothione disulfide was purchased from Bachem and other biochemical reagents from Sigma. Spectrophotometric assays of TryR and GR were carried out on a temperature controlled Shimadzu UV–vis recording spectrophotometer. Microtitre plate assays were carried out in 96-well plates on a Molecular Devices ThermoMax microplate reader.

Enzyme assays

For TryR, the standard assay mixture (1 mL) contained TryR (1 mU), 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 1 mM EDTA, 0.14 mM NADPH and varying concentrations of inhibitor/substrate. Inhibitor stock solutions were made up using co-solvent mixtures of assay buffer and DMSO; final assay mixtures contained 2% DMSO. Enzyme mixtures were pre-incubated with NADPH for 5 min at 27 °C prior to initiating the reaction by the addition of substrate. Enzyme activity was monitored by the decrease in absorbance at 340 nm due to NADPH oxidation. For K_i determinations, inhibition assays were carried out at three different inhibitor concentrations and five substrate concentrations (100, 60, 40, 30 and 20 μM). Initial rates (*v*) were measured from the linear region of product formation and K_i values were determined by weighted non-linear regression analysis of the hyperbola plot of *v* against substrate concentration (S) using the equation for linear competitive inhibition in GraFit (Erithacus Software Ltd). GR assays were carried out in a similar manner using a buffer system composed of 100 mM KH_2PO_4 (pH 7.0), 200 mM KCl and 1 mM EDTA.[24]

For the microtitre plate assays, the final assay mixtures (0.25 mL) contained TryR (1 mU) in the presence of 40 mM HEPES (pH 7.5), 1 mM EDTA, 0.15 mM NADPH, 25 μM DTNB, 1 μM $T[S]_2$ and inhibitor (100 μM). Enzyme mixtures were pre-incubated with NADPH for 5 min at 27 °C prior to initiating the enzyme reaction by the addition of substrate followed by inhibitor. Following inhibitor addition, enzyme activity was followed by the increase in absorbance at 410 nm, due to formation of the 5-thio-2-nitrobenzoic acid di-anion. Substrate depletion did not contribute to any deviation from linearity in these experiments as $T[S]_2$ concentrations were kept at a constant level by

the rapid DTNB-mediated re-oxidation of the $T[SH]_2$ product.

***N*-[3-(4-Amino-butylamino)-propyl]-3-(3{2-[3-(4-amino-butylamino)-propylcarbamoyl]-vinyl}-benzofuran-5-yl)-acrylamide *(tetra-hydrochloride salt)* (2).** A solution of **16** (26 mg, 0.028 mmol) dissolved in 4 M HCl in dioxane (10 mL) was stirred at room temperature for 1 h. The solvent was then evaporated and the resulting residue was coevaporated several times with ether to give **2** as a white solid (18.5 mg, 100%). δ_H (300 MHz, D_2O); 1.52–1.74 (8H, m, $4\times CH_2$), 1.77–1.93 (4H, m, $2\times CH_2$), 2.83–3.08 (12H, m, $6\times CH_2$), 3.23–3.36 (4H, m, $2\times CH_2$), 6.29 (1H, d, $J=16.0$) and 6.34 (1H, d, $J=15.6$, H-9 and H-12), 7.22 (2H, d, $J=15.8$, H-8 and H-11), 7.30 (1H, d, $J=9.0$, H-7), 7.35 (1H, d, $J=9.3$, H-6), 7.46 (1H, s, H-2), 7.82 (1H, s, H-4); δ_C (75 MHz, D_2O); 24.09, 25.21, 27.07, 37.58, 40.07, 46.50, 48.25 ($7\times CH_2$), 113.51 (C-7), 118.63 (C-3), 120.10, 121.04, 122.38 (C-4, C-9, C-12), 126.17 (C-6 overlapping C-3a), 131.35 (C-11), 131.81 (C-5), 142.54 (C-8), 150.49 (C-2), 157.76 (C-7a), 170.11 (C=O), 170.20 (C=O); [Found: (ESI^+) 513.3563 $[M+H]^+$, $C_{28}H_{45}N_6O_3$ requires 513.3547]; m/z (ESI^+) 513 (60%, $[M+H]^+$), 484 (100).

***N*-(3-Dimethylaminopropyl)-3-{3-[2-(3-dimethylamino-propylcarbamoyl)-vinyl]-benzofuran-5-yl}-acrylamide (3).**
Method A. A solution of the bis-pentafluorophenyl ester of **11** (38 mg, 0.064 mmol) and DIPEA (60 mg, 0.464 mmol) in CH_2Cl_2 (0.5 mL), was treated with a solution of dimethylaminopropylamine (19 mg, 0.186 mmol) in CH_2Cl_2 (0.5 mL), added dropwise, and the mixture was left to stir at room temperature for 3 h. The reaction mixture was then diluted with CH_2Cl_2 (10 mL) washed with saturated $NaHCO_3$ (10 mL) and the organics were dried ($MgSO_4$). The solvent was evaporated to give a solid. This was purified by chromatography eluting with a 4:1 mixture of $CHCl_3$ and EtOH (saturated with ammonia) to give **3** as a white solid (4 mg, 15%). δ_H (300 MHz, $CDCl_3$) 1.72–1.83 (4H, m, $2\times CH_2$), 2.28 (12H, s, $4\times CH_3$), 2.45 (4H, t, $J=6.4$, $2\times CH_2$), 3.47–3.55 (4H, m, $2\times NHCH_2$), 6.45 (1H, d, $J=15.6$, H-9), 6.60 (1H, d, $J=15.8$, H-12), 7.42–7.58 (4H, m, H-6, H-7, 2×NH), 7.68 (1H, d, $J=15.8$, H-11), 7.71 (1H, d, $J=15.6$, H-8), 7.81 (1H, s, H-2), 7.89 (1H, br, H-4); δ_C (75 MHz, $CDCl_3$) 26.66, 26.96 ($NHCH_2CH_2$), 39.54, 39.82 ($NHCH_2$), 45.84 (CH_3), 58.60, 58.88 (CH_2NMe_2), 112.59 (C-7), 118.43 (C-3), 121.16, 121.29 (C-9, C-4), 122.51 (C-12), 124.88 (C-6), 126.23 (C-3a), 129.92 (C-11), 131.18 (C-5), 140.83 (C-8), 147.65 (C-2), 156.95 (C-7a), 166.37 (C=O); [Found: (ESI^+) 427.2707 $[M+H]^+$, $C_{24}H_{35}N_4O_3$ requires 427.2703]; m/z 427 (100%, $[M+H]^+$).

Method B. A solution of **11** (36 mg, 0.062 mmol) and triethylamine (14 mg, 0.139 mmol) in a 1:1 mixture of THF/pyridine (2 mL), cooled to −12 °C, was treated with a solution of diphenylphosphinic chloride (31 mg, 0.131 mmol) in THF (0.5 mL), added dropwise. This was stirred at −12 °C for 60 min giving a cloudy mixture. A solution of dimethylaminopropylamine (13 mg, 0.127 mmol), and triethylamine (14 mg, 0.139 mmol) in THF (0.5 mL) was then added dropwise and a precipitate slowly began to form whilst stirring at room temperature for 14 h. The reaction was filtered and the solids washed with EtOAc. The combined filtrates were washed with saturated aqueous Na_2CO_3 (10 mL) and the organics were dried ($MgSO_4$). The solvent was evaporated to give a residue that was purified by reverse phase chromatography [MeOH/MeCN (9:1) as eluent] to give **3** (6 mg, 25%).

***N*-[3-(4-Methyl-piperazine-1-yl)-propyl]-3-(3-{2-[3-(4-methyl-piperazin-1-yl)-propylcarbamoyl]-vinyl}-benzofuran-5-yl)-acrylamide (4).** A solution of the bis-pentafluorophenol ester of **11** (38 mg, 0.064 mmol) and DIPEA (60 mg, 0.464 mmol) in CH_2Cl_2 (0.5 mL), was treated with a solution of **14** (25 mg, 0.159 mmol) in CH_2Cl_2 (0.5 mL) added dropwise and was left to stir at room temperature for 3 h. The reaction mixture was diluted with CH_2Cl_2 then washed with saturated aqueous $NaHCO_3$ (10 mL), dried ($MgSO_4$) and the solvent was evaporated to give a solid. This was purified by chromatography eluting with a 4:1 mixture of $CHCl_3$ and EtOH (saturated with ammonia) to give the desired product as a white solid (14 mg, 42%). δ_H (300 MHz, $CDCl_3$) 1.77–1.83 (4H, m, $2\times CH_2CH_2CH_2$) 2.28 (3H, s, CH_3), 2.29 (3H, s, CH_3), 2.38–2.83 (20H, m, $10\times CH_2$), 3.48 (2H, q, $J=6.2$, $NHCH_2$), 3.50 (2H, q, $J=6.4$, $NHCH_2$), 6.45 (1H, d, $J=15.6$, =CH-9), 6.63 (1H, d, $J=15.8$, =CH-12), 7.43 (1H, d, $J=8.6$) and 7.48 (1H, d, $J=8.6$, H-6 and H-7), 7.67 (1H, d, $J=15.8$, =CH-11), 7.69, d, $J=15.6$, =CH-10), 7.62–7.69 (1H, br, NH overlapping H-10), 7.80 (1H, s, H-2), 7.87 (1H, s, H-4), 7.84–7.92 (1H, br, NH overlapping H-4); δ_C (75 MHz, $CDCl_3$) 25.70, 25.82 ($NHCH_2CH_2$), 39.81, 39.89 ($NHCH_2$), 46.36, 46.45 (CH_3), 53.38, 53.43 ($NHCH_2CH_2CH_2$), 55.70 (NCH_2CH_2NMe), 57.47, 57.53 (NCH_2CH_2NMe), 112.69 (C-7), 118.40 (C-3), 121.27, 121.55 (C-9, C-4), 122.58 (C-12), 124.50 (C-6), 126.17 (C-3a), 129.92 (C-11), 131.14 (C-5), 140.73 (C-8), 147.88 (C-2), 156.96 (C-7a), 166.33 (C=O); [Found: (ESI^+) 537.3547 $[M+H]^+$, $C_{30}H_{44}N_6O_3$ requires 537.3547]; m/z 537 (100%, $[M+H]^+$).

2,3,5-Tribromo-2, 3-dihydrobenzofuran (6). A solution of bromine (0.87 g, 16.92 mmol) in CH_2Cl_2 (20 mL) was added, dropwise over 5 min, to a solution of 5-bromobenzofuran **5**[11] (3.03 g, 15.38 mmol) in CH_2Cl_2 (20 mL) and the mixture was stirred at room temperature for 40 min. The reaction mixture was washed with 0.05 M sodium thiosulfate (20 mL) followed by water (20 mL) and the organics were then dried ($MgSO_4$) and the solvent was evaporated to give **6** as a white solid (5.95 g). This was immediately carried through the next step without further purification. δ_H (300 MHz, $CDCl_3$) 5.69 (1H, s, 3-H), 6.89 (1H, s, 2-H), 6.96 (1H, d, $J=8.6$, 7-H), 7.49 (1H, dd, $J=8.6$, 2.1, 6-H), 7.64 (1H, d, $J=2.1$, 4-H).

3,5-Dibromobenzofuran (7). Potassium hydroxide powder (1.75 g, 31.19 mmol) was added in one portion to a suspension of **6** (5.95 g, 15.38 mmol) in absolute EtOH (25 mL). The solids immediately dissolved, and a fine white precipitate (KBr) began to form. The reaction

mixture was heated under reflux for 15 min then allowed to cool before the solids were filtered off and washed with EtOH. The combined filtrates were evaporated and the residue obtained was then partitioned between petrol/EtOAc (1:1, 60 mL) and water (15 mL). The organics were washed with water (2×15 mL) and the aqueous layers back-extracted with petrol (3×10 mL). The combined organics were dried ($MgSO_4$) and the solvent was evaporated to give the crude product. This was then purified by chromatography (petrol eluent) to give **7** as a white solid (4.16 g, 98% over two steps). δ_H (300 MHz, $CDCl_3$) 7.38 (1H, d, $J=8.8$, 7-H), 7.46 (1H, dd, $J=8.7$, 1.9, 6-H), 7.66 (1H, s, 2-H), 7.70 (1H, d, $J=1.9$, 4-H); δ_C (75 MHz, $CDCl_3$) 97.50 (C-3), 113.69 (C-7), 117.05 (C-5), 123.05 (C-4), 128.90 (C-6), 129.44 (C-3a), 144.24 (C-2), 153.60 (C-7a); [Found: (EI) 273.86516, $C_8H_4Br_2O$ requires 273.86289]; *m/z* 274, 276, 278 (100%, 1:2:1, M^+), 197 (10, M−Br).

Benzofuran-3,5-dicarbonitrile (8). A mixture of **7** (221 mg, 0.80 mmol) and copper(I) cyanide (184 mg, 2.05 mmol) in anhydrous DMF (2 mL) was heated under reflux overnight (the mixture changed from a green slurry to a deep red/brown solution during this time). The reaction mixture was allowed to cool before pouring into a solution of 1 M $FeCl_3$ in 1.5 M aqueous HCl (5 mL). After heating at 70 °C for 30 min, the slurry was extracted with dichloromethane (4×8 mL) and the combined organics were washed with water, 2 M HCl, water and then 2 M NaOH (10 mL each) The organics were dried ($MgSO_4$) and the solvent was evaporated to give a brown solid. The crude product was adsorbed onto silica and then purified by chromatography (CH_2Cl_2/petrol eluent, 8:2) to give **8** as a white solid (67 mg, 50%) which was sufficiently pure for use in the next step. ν_{max} ($CDCl_3$)/cm^{-1}; 2238 (C≡N); δ_H (300 MHz, $CDCl_3$) 7.73 (1H, d, $J=8.8$, 7-H), 7.77 (1H, dd, $J=8.7$, 1.5, 6-H), 8.12 (1H, s, 4-H), 8.30 (1H, s, 2-H); δ_C (75 MHz, $CDCl_3$) 95.41 (C-3), 109.26, 110.73 (CN, C-5), 113.65 (C-7), 117.89 (CN), 125.28 (C-4), 125.42 (C-3a), 130.20 (C-6), 153.70 (C-2), 155.69 (C-7a); *m/z* 168 (100%, M^+).

Benzofuran-3, 5-dicarbaldehyde (9). A solution of **8** (598 mg, 3.56 mmol) in anhydrous CH_2Cl_2 (40 mL) at −15 °C was treated with a solution of 1.5 M DIBAL (5.8 mL, 8.70 mmol) in toluene. The reaction was complete within 3 min (by TLC). The reaction mixture was warmed to 0 °C and 2 M HCl (20 mL) was added dropwise. The reaction mixture was then allowed to warm to room temperature, the organic layer was separated and the aqueous layer was then extracted with CH_2Cl_2 (10 mL). The combined organics were washed with water, dried ($MgSO_4$) and the solvent was evaporated to give a pale yellow solid. This was purified by chromatography (CH_2Cl_2 eluent) to give **9** as a pale yellow solid (576 mg, 93%). ν_{max} ($CDCl_3$)/cm^{-1}; 1698 (C=O); δ_H (300 MHz, $CDCl_3$) 7.69 (1H, d, $J=8.6$, 7-H), 8.02 (1H, dd, $J=8.6$, 1.2, 6-H), 8.39 (1H, s, 4-H), 8.71 (1H, s, 2-H), 10.11 (1H, s, CHO), 10.22 (1H, s, CHO); δ_C (75 MHz, DMSO-d_6) 113.34 (C-7), 123.05, 123.66 (C-3, C-3a), 125.05 (C-4), 127.38 (C-6), 133.91 (C-5), 158.44 (C-7a), 159.81 (C-2), 186.75 (CHO), 192.94 (CHO); [Found: (EI) 174.03058, $C_{10}H_6O_3$ requires 174.03170]; *m/z* 174 (100%, M^+), 173 (100, M−H), 145 (37, M−CHO).

3-[3-(2-Ethoxycarbonyl-vinyl)-benzofuran-5-yl]-acrylic acid ethyl ester (10). A solution of **9** (107 mg, 0.614 mmol) and (carbethoxymethylene)triphenyl-phosphorane (540 mg, 1.55 mmol) in DMF (6 mL) was heated under reflux for 4 h. The reaction mixture was then diluted with EtOAc (20 mL) and washed with water (10 mL). The organics were dried ($MgSO_4$) and the solvent was evaporated to give a brown solid. The crude product was adsorbed onto silica and purified by chromatography (CH_2Cl_2 eluent) to give **10** as a white solid (124 mg, 64%). Mp (EtOAc) 98–99 °C; λ_{max} ($CHCl_3$)/nm 258 (ε 37,722), 270 (34,897), 294 (28,652); δ_H (300 MHz, $CDCl_3$) 1.37 (3H, t, $J=7.1$, CH_3), 1.38 (3H, t, $J=7.1$, CH_3), 4.29 (2H, q, $J=7.1$, CH_2), 4.31 (2H, q, $J=7.1$, CH_2), 6.48 (1H, d, $J=16.0$, H-9), 6.56 (1H, d, $J=16.1$, H-12), 7.52 (1H, d, $J=8.6$, H-7), 7.57 (1H, dd, $J=8.7$, 0.9, H-6), 7.77 (1H, d, $J=16.1$, H-11), 7.81 (1H, d, $J=16.0$, H-8), 7.90 (1H, s, H-2), 7.97 (1H, s, H-4); δ_C (75 MHz, $CDCl_3$) 14.55, 14.75 (2×CH_3), 60.95, 61.07 (2×CH_2), 112.91 (C-7), 118.36 (C-3), 118.40 (C-9), 119.44 (C-12), 121.58 (C-4), 125.64 (C-6), 125.92 (C-3a), 131.02 (C-5), 134.17 (C-11), 144.79 (C-8), 148.89 (C-2), 157.42 (C-7a), 167.26 (C=O); [Found: (EI) 314.11726, $C_{18}H_{18}O_5$ requires 314.11543]; *m/z* 314 (100%, M^+), 269 (42, M−OEt).

3-[3-(2-Carboxy-vinyl)-benzofuran-5-yl]-acrylic acid (11). A solution of **10** (122 mg, 0.388 mmol), in THF (4 mL) was treated with an aqueous solution of 3.5 M lithium hydroxide (2 mL) to give a biphasic mixture. MeOH was then added dropwise (approx. 2 mL) until a homogeneous mixture was obtained which was stirred at room temperature for 4 h. The solvents were evaporated to give a white solid, then lithium salts were removed by passing the crude product through a column of Amberlite IR120 (plus) cation exchange resin (50% aqueous THF as eluent). Acidic fractions were collected and the solvent was evaporated to give **11** as a white solid (88 mg, 88%). δ_H (300 MHz, DMSO-d_6) 6.70 (1H, d, $J=16.2$, H-9), 6.76 (1H, d, $J=16.5$, H-12), 7.70–7.85 (4H, m, H-6, H-7, H-8, H-11), 8.42 (1H, s, H-2), 8.57 (1H, s, H-4), 12.25–12.50 (2H, br, 2×CO_2H); δ_C (75 MHz, DMSO-d_6) 112.40 (C-7), 117.61 (C-3), 118.86 (C-9), 119.77 (C-12), 121.9 (C-4), 125.07 (C-3a), 125.50 (C-6), 130.70 (C-5), 133.45 (C-11), 144.19, 150.12 (C-2, C-8), 156.32 (C-7a), 167.74 (C=O); *m/z* (ESI$^+$) 282.1 (100%, $[M+Na]^+$).

Preparation of the bis-pentafluorophenyl ester of 11. A solution of **11** (32 mg, 0.128 mmol) in DMF (4 mL) at 0 °C was treated with EDC (58 mg, 0.302 mmol), DMAP (1 mg, 0.008 mmol) and a solution of pentafluoro-phenol (58 mg, 0.318 mmol) in DMF (0.5 mL) to give a yellow mixture that was stirred at room temperature for 3 h. The reaction mixture was diluted with EtOAc (20 mL), washed with saturated aqueous $NaHCO_3$ (10 mL) and then brine (10 mL). The organics were dried ($MgSO_4$) and the solvent evaporated to give the bis-pentafluorophenyl ester as a pale brown solid. This material was then used without further purification.

3-(4-Methyl-piperazin-1-yl)-propionitrile (13).[16] Acrylonitrile (0.67 mL, 10.18 mmol) was added dropwise to a solution of *N*-methyl-piperazine **12** (1.02 g, 10.18 mmol) in MeOH (6 mL) and was left to stir at room temperature for 2.5 h. The solvent was evaporated to give an oily residue that was purified by elution through a thin bed of silica (EtOAc/MeOH, 9:1 as eluent) to give **13** as a pale yellow oil (1.26 g, 81%). δ_H (300 MHz, $CDCl_3$); 2.26 (3H, s, CH_3), 2.34–2.60 (8H, m, 4×NCH_2), 2.48 (2H, t, $J=6.8$, NCH_2CH_2), 2.67 (2H, t, $J=6.8$, CH_2CN).

3-(4-Methyl-piperazin-1-yl)-propylamine (14).[17] A mixture of 3-(4-methyl-piperazin-1-yl)-propionitrile 13 (583 mg, 3.80 mmol) and 5% Rh/C (98 mg, 0.19 mmol) in 2.77 M ethanolic ammonia (8 mL) was stirred under a hydrogen atmosphere (5 atm pressure) for 20 h. The reaction mixture was then filtered through Celite and the solvent was evaporated to give an oily residue which was purified by short-path distillation to give **14** as a colourless oil (110 mg, 19%). bp 34 °C (0.05 mmHg) (lit.,[16] 52 °C, 0.3 mmHg); δ_H (300 MHz, $CDCl_3$); 1.63–1.73 (2H, m, $CH_2CH_2CH_2$), 2.30 (3H, s, CH_3), 2.35–2.59 (8H, m, 4×NCH_2), 2.66 (2H, t, $J=6.8$, $NCH_2CH_2CH_2$), 2.76 (2H, t, $J=6.8$, $NCH_2CH_2CH_2NH_2$).

{4-[*tert*-Butoxycarbonyl-(3-{3-[3-(2-{3-[*tert*-butoxycarbonyl-(4-*tert*-butoxycarbonylamino-butyl)-amino]-propyl-carbamoyl} - vinyl) - benzofuran - 5 - yl] - acryloylamino} - propyl)-amino]-butyl}-carbamic acid *tert*-butyl ester (16). A solution of **11** (21 mg, 0.081 mmol) and triethylamine (25 μL, 0.180 mmol) in DMF (1 mL) at −10 °C was treated with a solution of diphenylphosphinic chloride (31 μL, 0.163 mmol) in DMF (0.5 mL) added dropwise. This was stirred at −10 to 0 °C for 60 min giving a cloudy green mixture. A solution of **15** (62 mg, 0.179 mmol), and triethylamine (25 μL, 0.180 mmol) in DMF (0.5 mL) was then added dropwise and the reaction was stirred at room temperature for 6 h. The solvent was evaporated and the residue was then partitioned between water (5 mL) and EtOAc (5 mL). The aqueous layer was extracted with EtOAc (3×5 mL), and the combined organics were then dried ($MgSO_4$) and the solvent evaporated to give an oily residue that was purified by chromatography (EtOAc eluent) to give **16** as a colourless oil (30 mg, 41%).

δ_H (300 MHz, 323 K, $CDCl_3$); 1.43 (9H, s, 3×CH_3), 1.44 (9H, s, 3×CH_3), 1.47 (18H, s, 6×CH_3), 1.46–2.05 (12H, m, 6×CH_2), 3.08–3.51 (16H, m, 8×CH_2), 4.60–4.90 (2H, br, 2×NH), 6.49 (1H, d, $J=15.6$, H-9), 6.68 (1H, d, $J=15.8$, H-12), 6.80–7.22 (2H, br, 2×NH), 7.44 (1H, d, $J=8.5$, H-7), 7.49 (1H, d, $J=8.3$, H-6), 7.69 (1H, d, $J=15.8$, H-11), 7.72 (1H, d, $J=15.6$, H-8), 7.81 (1H, s, H-2), 7.92 (1H, s, H-4); δ_C (75 MHz, $CDCl_3$); 26.12 (CH_2), 27.76 (CH_2), 27.85 (CH_2), 28.11 (CH_2), 28.84 (CH_3), 36.31 (CH_2), 36.71 (CH_2), 40.47 (CH_2), 43.86 (CH_2), 44.32 (CH_2), 45.36 (CH_2), 47.06 (CH_2), 79.62, 79.66, 80.08, 80.27 (4×C), 112.55 (C-7), 118.54 (C-3), 121.04 (C-9, C-4, overlapping [as determined by HETCOR)], 122.46 (C-12), 125.44 (C-6), 126.13 (C-3a), 129.93 (C-11), 131.23 (C-5), 141.05 (C-8), 147.93 (C-2), 156.49 (C-7a), 157.01 (carbamate–C=O), 166.61 (amide–C=O); *m/z* (ESI^+) 913 (100%, $[M+H]^+$).

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