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Time-dependent inhibitors of trypanothione reductase: Analogues of the spermidine alkaloid lunarine and related natural products

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Abstract—The macrocyclic spermidine alkaloid lunarine **1** from *Lunaria biennis* is a competitive, time-dependent inhibitor of the protozoan oxidoreductase trypanothione reductase (TryR), a promising target in drug design against tropical parasitic diseases. Various molecules related to **1** and the alkaloid itself have been synthesized in racemic form and evaluated against TryR in order to determine the key features of **1** that are associated with time-dependent inhibition. Kinetic data are consistent with an inactivation mechanism involving a conjugate addition of an active site cysteine residue onto the C-24–C-25 double bond of the tricyclic nucleus of **1**. Comparison of data for synthetic (±)-**1**, the natural product, and other derivatives **7**–**10** from *L. biennis* confirms the importance of the unique structure of the tricyclic core as a motif for inhibitor design and reveals that the non-natural enantiomer may be a more suitable scaffold upon which thiophilic groups may be presented.

1. Introduction

The parasitic protozoa *Trypanosoma* and *Leishmania* are the causative agents of a number of life-threatening tropical diseases. These include human African sleeping sickness (*Trypanosoma brucei rhodesiense* and *T.b. gambiense*), Chagas' disease (*Trypanosoma cruzi*) and various forms of leishmaniasis (e.g., *Leishmania donovani*). Current treatments for these diseases suffer from several disadvantages such as lack of efficacy due to developing resistance, toxicity, cost of production, or practical problems of large-scale administration in Third World countries.[1] There is a pressing need to develop new chemotherapeutic agents, and to this end considerable attention has recently been devoted towards exploiting differences between some of the biochemical pathways of the parasites and their mammalian hosts.[2] An attractive target in this context is trypanothione reductase (TryR; EC 1.6.4.8), an NADPH-dependent oxidoreductase which plays an essential role in the parasites' defences against various reactive oxygen species (e.g., H_2O_2, O_2^- and OH) generated by aerobic metabolism and host macrophages.[3] TryR maintains an intracellular reducing environment by catalyzing the reduction of the N^1,N^8-bisglutathionyl spermidine conjugate trypanothione ($T[S]_2$) to dihydrotrypanothione ($T[SH]_2$) following reaction of the former with potentially harmful oxygen metabolites (Fig. 1). This redox defence mechanism is analogous to the mammalian glutathione-based system that employs glutathione disulfide reductase (GR; EC 1.6.4.2) The high degree of specificity shown by GR and TryR for their respective substrates[4] has encouraged the design of selective TryR inhibitors as a potential therapeutic strategy,[5] and this has been validated by the demonstration that disabling the function of TryR in *Leishmania*[6] and *T. brucei*[7] does indeed markedly increase their sensitivity towards oxidative stress.

Various small molecules have been identified as classical inhibitors of TryR, such as the naturally occurring bis(tetrahydrocinnamoyl)spermine derivative, kuko-

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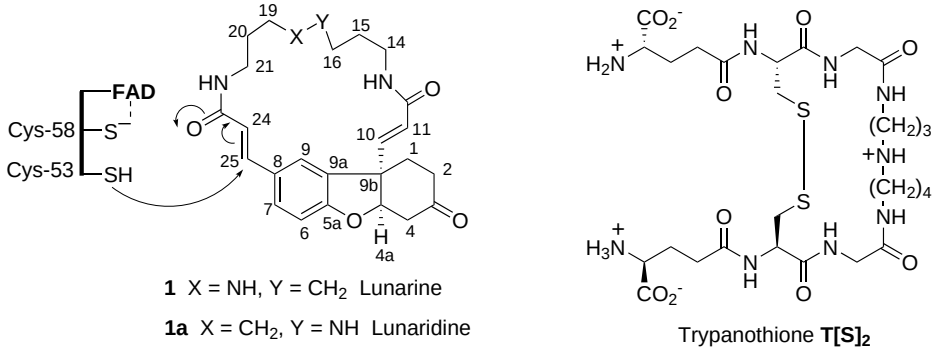


Figure 1. The time-dependent TryR inhibitor lunarine **1** and the native TryR substrate $T[S]_2$.

amine A[8] and several series of related *N*-alkyl or *N*-acyl-substituted linear polyamines.[9–11] However, it should be noted that for strictly competitive inhibitors, low nanomolar potencies may be required to maintain high levels of intracellular inhibition in the face of millimolar levels of $T[S]_2$ accumulating as a result of TryR inhibition.[1,12] For this reason, compounds which inactivate TryR in an irreversible or tight-binding manner may therefore present attractive alternatives as lead compounds.

Natural products are a rich potential source of new and structurally diverse antitrypanosomal leads.[13] The spermidine-based macrocyclic alkaloid lunarine **1**, originally isolated from *Lunaria biennis*,[14] was identified by virtual screening[15] as a potential lead TryR inhibitor and subsequently shown to inhibit the enzyme in a competitive, time-dependent fashion.[15,16] As shown in Figure 2, time-dependent (or slow binding) inhibition can occur by three basic mechanisms. In classical inhibition (mechanism A), 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 minutes. With an enzyme isomerisation mechanism (C), a rapid inhibitor binding step is followed by a slower enzyme/inhibitor conformational change. Irreversible inhibition proceeds by the same rapid binding step followed by a rate-limiting covalent modification of the enzyme (D).

The time-dependent inhibition of TryR by **1** is only evident when TryR is in the reduced state (i.e., when the redox-active disulfide pair Cys-53 and Cys-58 is in the dithiol form). We have therefore proposed an explanation for the time-dependent inactivation of $TryR_{red}$ by **1** which involves the conjugate addition of Cys-53 or Cys-58 to one of the α,β-unsaturated amide moieties of the lunarine macrocycle (Fig. 3).[15,16]

A more detailed understanding of this inhibition mechanism would facilitate the rational design of more effective, potentially irreversible, inhibitors based on the lunarine motif, and towards this goal we have now prepared a group of structural analogues of **1** (Figs. 4 and 6) which have been evaluated alongside some closely related alkaloids (Fig. 5) that were obtained during the isolation and classical structure determination of **1** itself.[14] In this study, we have attempted to address three key questions: (1) Is the time-dependent inactivation of TryR the result of a rate-limiting conformational change within the initial enzyme–inhibitor complex to form a more tightly bound complex or is it the result of covalent modification of the enzyme? (2) If a covalent TryR–lunarine complex is formed, is conjugate addition at either C-10 or C-25 of the natural product involved

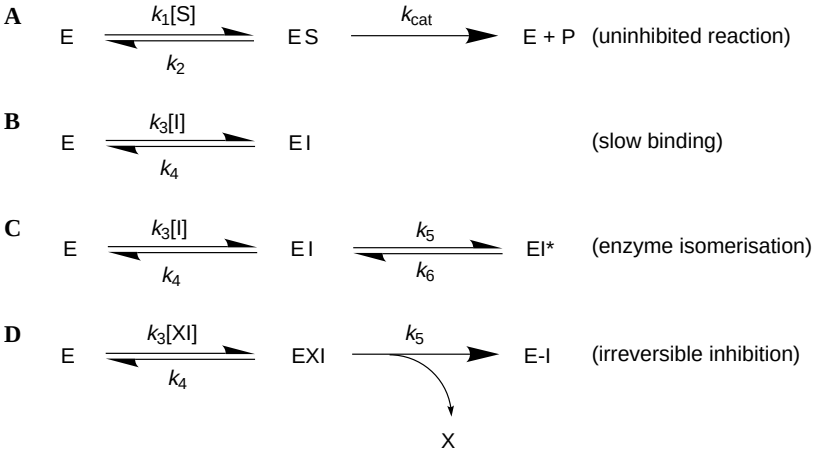


Figure 2. Mechanisms of time-dependent inhibition.

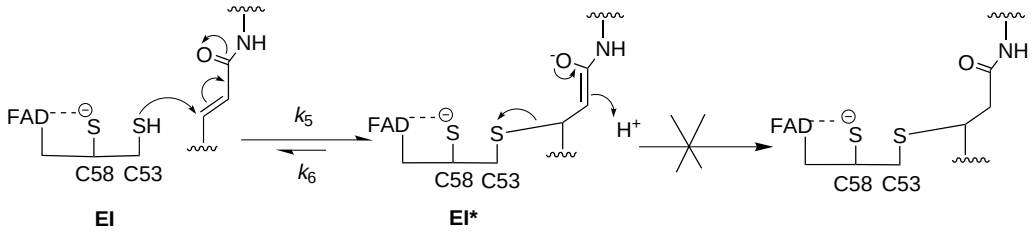
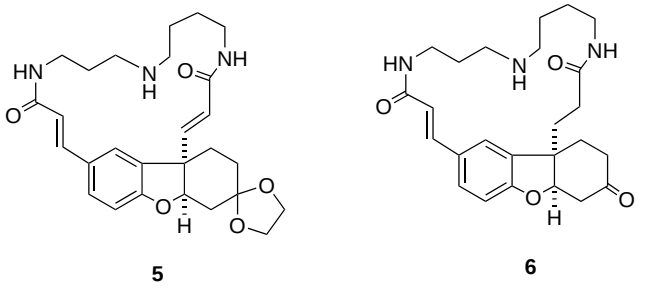


Figure 3. Proposed mechanism for time-dependent inactivation of TryR.



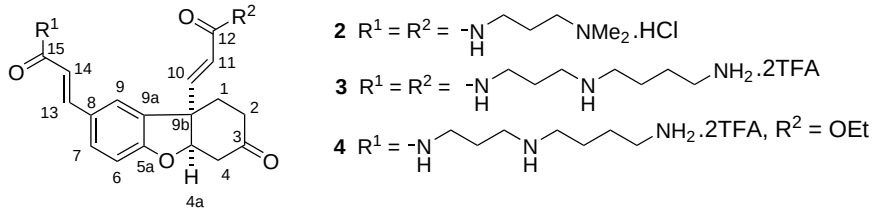


Figure 4. Synthetic lunarine analogues examined in this study and numbering scheme for compounds **2**–**4**. For these derivatives, potential loci for conjugate addition are C-10 or C-13.

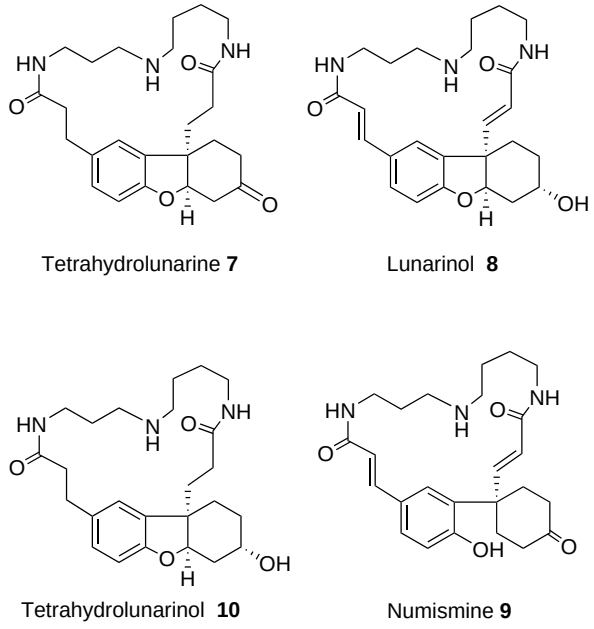


Figure 5. Lunarine derivatives of natural origin.[14]

(see lunarine numbering in Fig. 1)? (3) What structural features of lunarine can be modified/removed whilst still retaining time-dependent inhibition?

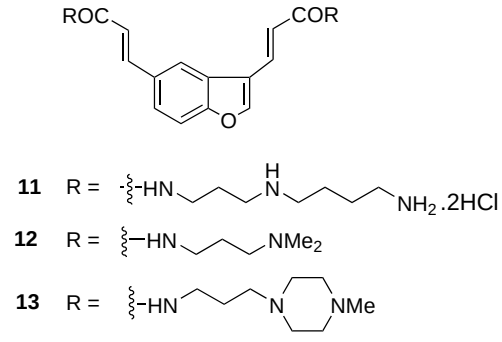
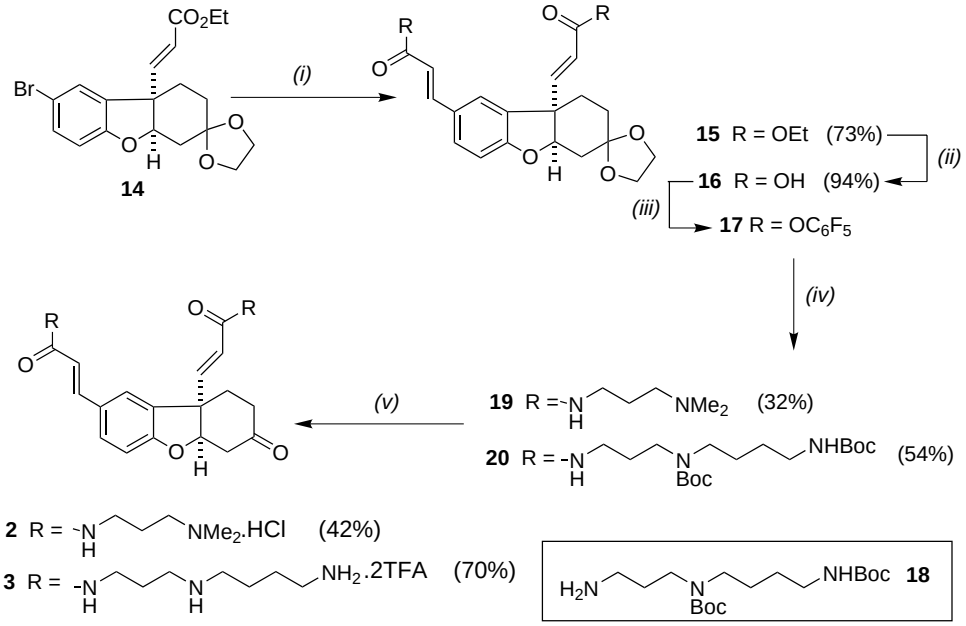


Figure 6. Previously prepared[23] lunarine-like molecules.

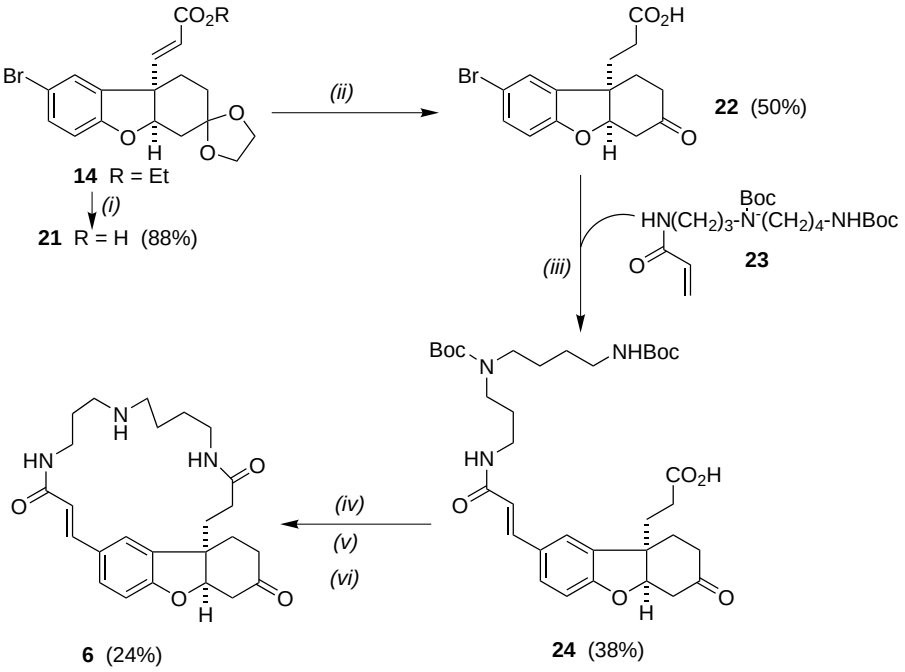
2. Results

2.1. Synthesis

We have recently reported a regioselective synthesis of racemic lunarine (±)-**1** via the differentially functionalised tricyclic intermediate **14**.[17] This synthetic route was designed to enable compound **14** to be utilised as a common intermediate for the preparation of the analogues **2**–**6**, **15**, **16** and **19**, described herein, also in racemic form. The bis-polyamine derivatives **2** and **3** were prepared from compound **14** as outlined in Scheme 1. Heck coupling of aryl bromide **14** with ethyl acrylate



Scheme 1. Reagents and conditions: (i) ethyl acrylate, $Pd(OAc)_2$, (*o*-Tol)$_3$P, Et_3N, 100 °C, 3 h; (ii) 1 M LiOH, 60% aq EtOH, 3 h; (iii) C_6F_5OH, EDC, DMAP, DMF, 3 h; (iv) RNH_2, CH_2Cl_2, DIPEA, 3 h; (v) 5% HCl in THF, 20 h or TFA, CH_2Cl_2 1 h.



Scheme 2. Reagents and conditions: (i) 1 M LiOH, 60% aq EtOH, 3 h; (ii) 5% Rh/alumina, EtOH, H_2 1 atm, 22 h; then 5% HCl in THF; (iii) **23**, $Pd(OAc)_2$, (*o*-Tol)$_3$P, Et_3N, DMF, 60 °C, 6 h; (iv) C_5F_5OH, EDC, DMAP, CH_2Cl_2, 5 h; (v) 4 M HCl–dioxane, 30 min; (vi) DIPEA, CH_2Cl_2, 2 days.

gave the bis-α,β-unsaturated ester **15** in 73% yield, which was then hydrolysed to diacid **16** under basic conditions. Compound **16** was then activated as the bis-pentafluorophenyl ester **17** prior to treatment with either 3-dimethylaminopropylamine or the selectively protected spermidine derivative **18**[18] to give compounds **19** and **20** in yields of 32% and 54%, respectively. Cleavage of the dioxolane protection of **19** was achieved using 5% HCl/THF to give **2** as the dihydrochloride salt in 32% yield. Treatment of **20** with TFA similarly gave **3** as the fully deprotected trifluoroacetate salt in 54% yield.

The dihydrolunarine analogue **6** was prepared as shown in Scheme 2. Surprisingly, palladium on charcoal or palladium hydroxide-catalysed hydrogenation of **14** resulted in concomitant removal of the dioxolane protection and partial reduction of the aromatic bromide. Use of 5% rhodium on alumina allowed selective reduction of the double bond without affecting the aryl bromide, but still resulted in partial cleavage of the dioxolane. As the role of the dioxolane protection in **14** is to prevent opening of the tricyclic system via a base-induced retro-Michael reaction under the conditions required

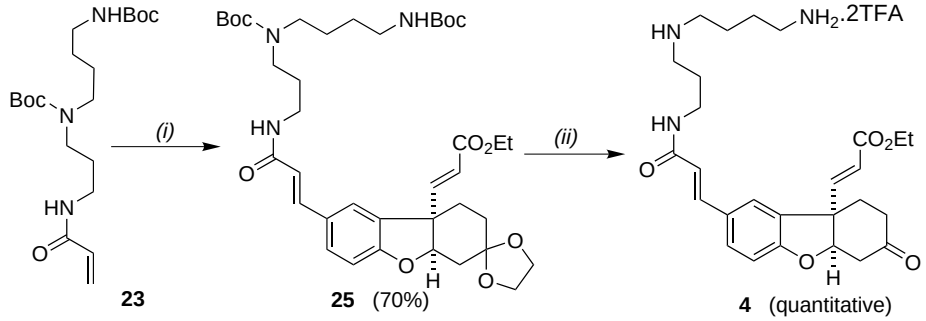
for subsequent ester hydrolysis,[19] this problem was addressed by converting **14** to acid **21** (88% yield) prior to the hydrogenation step. Selective rhodium-catalysed hydrogenation of the double bond of **21** was then successfully achieved, followed by cleavage of the dioxolane (5% HCl/THF) to give the saturated acid derivative **22**. Heck coupling between crude **22** and the *tert*-butoxycarbonyl-protected N^1-acryloylamidospermidine **23**[17] then gave the required cyclisation precursor **24** in 38% isolated yield. Compound **24** was activated as the pentafluorophenyl ester as previously, followed by removal of the Boc-protecting groups using 4 M HCl in dioxane. Macrocyclisation was then achieved by addition of DIPEA to a 0.6 mM solution of this hydrochloride salt to give **6** in 24% yield.

Monospermidine derivative **4** was also obtained from ester **14** via Heck coupling with **23** to give the protected polyamine adduct **25** in 70% yield, followed by deprotection with TFA as described previously (Scheme 3).

2.2. TryR inhibition assays

Compounds were initially screened for time-dependent inhibition of TryR using a high-throughput microplate assay previously developed for this purpose.[16] Active compounds were then subjected to a more detailed analysis (Table 1). Reaction progress curves for all the time-dependent inhibitors demonstrated the slow establishment of a final steady state velocity in agreement with either mechanism (B) or (C) (Fig. 2). The fact that both initial rates (v_i) and the steady state rates (v_s) varied as a function of inhibitor concentration indicates that a two-step isomerisation-type mechanism is operating (i.e., mechanism (C)—see Fig. 7). Here, a Michaelis-type EI-complex rapidly forms followed by the rate-limiting establishment of a more tightly bound EI*-complex. Two inhibition constants, K_i and K_i^*, must therefore be considered; these are the dissociation constants for the EI and EI*-complexes, respectively. Rates for the interconversion between EI and EI* (k_5 and k_6) and the k_5/k_6 ratios were also determined (Table 1).

All of the synthetic lunarine analogues, (±)-**1**, **2**–**6**, **15**, **16** and **19**, were evaluated as racemic mixtures. Racemic lunarine (±)-**1** has apparent K_i and K_i^* values that are significantly lower than those of the natural (+)-isomer **1**. Most of the synthetic lunarine analogues, (±)-**1**, **2**–**4**, **6** and **19**, are time-dependent inhibitors with comparable values for K_i and K_i^*. A notable exception is **5** (with the dioxolane replacing the carbonyl group of (±)-**1** at C-3), which along with **15** and **16**, does not inhibit TryR at a concentration of 100 μM. Whilst none of the natural product derivatives **7**–**10** show any time-dependent



Scheme 3. Reagents and conditions: (i) $Pd(OAc)_2$, **14**, Et_3N, $(o\text{-Tol})_3P$, DMF, 60 °C, 11 h; (ii) TFA, CH_2Cl_2, 50 min.

Table 1. Inhibition of TryR by **1** and related compounds

Compound	K_i (μM)	K_i^* (μM)	k_5 (min^{-1})	k_6 (min^{-1})	k_5/k_6
1[16]	304 (±2)	114 (±2)	0.0128	0.0077	1.7
(±)-**1**	53 (±10)	3.6 (±0.8)	0.0462	0.0034	13.6
2	93 (±6)	4.8 (±0.5)	0.1720	0.0093	18.4
3[16]	59 (±3)	3.5 (±0.9)	0.2320	0.0215	10.8
4	49 (±3)	4.9 (±0.8)	0.1538	0.0102	15.1
5	—[a]	—	—	—	—
6	79 (±3)	6.8 (±1.4)	0.0889	0.0084	10.6
7	96 (±10)	—	—	—	—
8	470 (±61)	—	—	—	—
9	160 (±22)	—	—	—	—
10	218 (±27)	—	—	—	—
11[23]	114 (±14)	—	—	—	—
12[23]	196 (±19)	—	—	—	—
13[23]	213 (±9)	34 (±6)	0.0329	0.0063	5.2
15	—[a]	—	—	—	—
16	—[a]	—	—	—	—
19	101 (±19)	4.0 (±0.7)	0.2612	0.0108	24.6

[a] No inhibition in a microplate assay[16] containing TryR (1 mU), NADPH (0.15 mM), DTNB (25 μM) and $T[S]_2$ (1 μM) incubated at 27 °C, pH 7.5, in the presence of 100 μM compound concentrations.

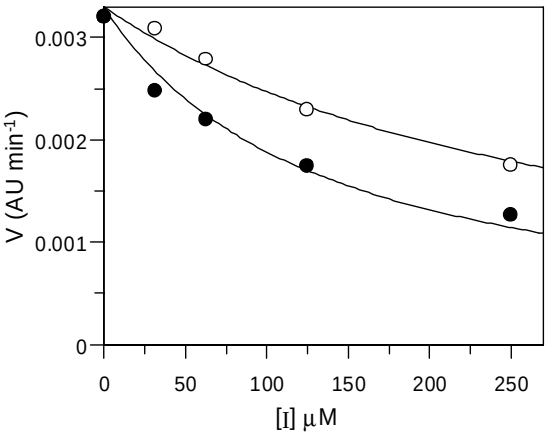


Figure 7. Variation in v_i (○) and v_s (●) with respect to concentration of (±)-**1**. These data were fitted to Eqs. 2 and 3 (see Section 5) to derive values for K_i and K_i^*, respectively (Table 1).

Table 2. Trypanocidal activities of synthetic lunarine derivatives

Compound[a]	IC_{50} (μM)
1	65 (±6)
3	29 (±3)
4	56 (±8)

[a] All racemic derivatives.

activity, they are all moderate linear competitive inhibitors of TryR with respect to $T[S]_2$.

2.3. Determination of antitrypanosomal activities

All the compounds in Table 1 were evaluated in vitro against bloodstream form trypomastigote *T. brucei rhodesiense* using the Alamar Blue® assay[20] at an initial concentration of 100 μM. Of the compounds tested, only racemic lunarine (±)-**1**, and the racemic derivatives **3** and **4**, showed any activity at this concentration. IC_{50} values were determined for these three derivatives as shown in Table 2. Neither natural lunarine **1** nor the other optically pure natural product derivatives, **7**–**10**, showed any activity in this screen.

2.4. Mass spectrometric analysis of the lunarine–TryR adduct

A sample of NADPH-reduced TryR was treated with 500 μM (±)-**1** under standard assay conditions (see Section 5) for 1 h, at which point the enzyme was 90% inactivated. Low molecular weight materials were removed from the reaction mixture by buffer exchange, and the latter was then analysed by electrospray mass spectrometry. The deconvoluted mass of TryR treated with (±)-**1** was 54,538 Da, compared to the untreated control of 54,100 Da.

3. Discussion

All of the time-dependent inhibitors reported in Table 1 appear to inactivate TryR via a two-step isomerisation mechanism (C). Surprisingly, racemic lunarine (±)-**1** is significantly more potent than the natural stereoisomer **1** with K_i and K_i^* values that are 6- and 30-fold lower, respectively. The 'isomerisation constant,' k_5/k_6, is a benchmark of inhibitor efficacy where a greater k_5/k_6 value indicates a higher ratio for conversion between EI and EI*. For (±)-**1**, k_5/k_6 is 8-fold greater than for **1**. This difference is due to a 4-fold increase in k_5 and a 2-fold decrease in k_6. These results imply that the unnatural lunarine stereoisomer forms a more tightly bound EI-complex and subsequently converts to an EI*-adduct more rapidly than the natural product **1**.

With regard to the first question we intended to address in this study, **1** only exhibits time-dependent inhibition when incubated with TryR in the reduced state, when the active site cysteines are in the free thiol form.[15] This suggests that the active-site thiols play an important role in the time-dependent inhibition mechanism, and indeed when the conjugated double bonds in **1** are reduced, as in tetrahydrolunarine **7**, time-dependent inhibition is abolished, but this is retained when only the right-hand side double bond (C-10–C-11) is removed (compound **6**). With time-dependent inhibition only occurring when TryR is in the reduced (free thiol) state and the inhibitor requiring at least one Michael acceptor motif, a slowly reversible conjugate addition mechanism therefore seems plausible. Although based on the current experimental evidence, a non-covalent slow-binding mechanism cannot yet be ruled out entirely, the results above imply that if a covalent EI* complex is indeed formed between TryR and **1**, conjugate addition at C-25 (left-hand side double bond) by Cys53 is most likely to be involved. Support for the formation of a slowly dissociating, covalent EI* species is provided by mass spectrometric analysis of the enzyme inhibited by (±)-**1** where the 438 mass unit difference between native and inhibited proteins is consistent with the addition of one inhibitor molecule (M = 437) with an excellent level of agreement for an experiment of this type.[21]

Two criteria that were used to identify **1** as a potential TryR inhibitor were the presence of the spermidine-N^1,N^8-bis-amide moiety which is found in the natural substrate and also its macrocyclic nature (analogous to $T[S]_2$). However, the acyclic molecule N^1-glutathionylspermidine disulfide is a naturally occurring TryR substrate[4b] and a number of related synthetic TryR substrates have also been prepared.[22] In view of this, the non-cyclised linear bis-polyamines **2** and **3** were prepared, based on the core lunarine scaffold, along with the mono-polyamine derivative **4**, in order to establish whether the macrocyclic structure of **1** was an essential feature for inhibition. Comparing the bis-ethyl ester derivative **15** with compounds (±)-**1**, **2**–**4** and **19**, it is evident that the presence of some form of polyamine side-chain functionality is critical for inhibitor activity. Whether this is in the form of single or double linear polyamine chains or as part of a macrocyclic structure however is of only minor consequence to the overall inhibitor potency as there is only a marginal variation in K_i and K_i^* between (±)-**1**, **2**–**4** and **19**. The most significant differences can be seen in the rate constants k_5 and k_6 which differ by up to 6-fold, but an increase/decrease

in k_5 is usually accompanied by a comparable change in k_6, which is reflected by the much smaller variations in k_5/k_6.

The compounds prepared in this study and the natural product derivatives **7**–**10** also allow the importance of certain features of the core structure of lunarine itself to be assessed with regard to time-dependent inhibition. We have already shown[23] that simple bis-polyamines based on a benzofuranyl nucleus, analogous to **2** and **3**, are linear competitive inhibitors, although a further analogue bearing 4-methyl-piperazin-1-yl-propylamino substituents did show time-dependency. Compound **5** illustrates the profound effect of a more subtle structural variation as conversion of the carbonyl group at C-3 to a sp^3-centred dioxolane leads to total loss of activity. The disubstituted cyclohexanone ring of the lunarine nucleus preferentially adopts a twist-boat conformation,[24] presumably being converted to a chair form in **5** which then prevents the molecule from adopting a comparable bound conformation to lunarine in the TryR active site. This is supported by the finding that **8**, obtained by reduction of the C-3 carbonyl of **1**,[14] is not a time-dependent inhibitor and is indeed a substantially weaker competitive inhibitor of TryR than **1**. In contrast, the dioxolane derivative **19** (with two linear dimethylaminopropyl side chains) does inhibit TryR in a time-dependent manner, and in this case, it would appear that replacement of the polyamine bridge with two separate alkylamines introduces sufficient flexibility to offset any constraints imposed upon the inhibitor binding orientation by modification at C-3. The fact that activity is so completely abolished in **5** may therefore also indicate that larger C-3 substituents are not tolerated in compounds of this kind. Cleavage of the central bridging furan motif as in **9** also results in loss of time-dependency and once again (cf **8**) this may be due to the presence of the spermidine bridge that prevents a favourable enzyme-bound conformation from being adopted. The finding that **19** is a time-dependent inhibitor, in contrast to **8**, also leads us to discount the possibility that the mechanism of time-dependent inactivation for **1** involves the formation of a Schiff base species between the C-3 carbonyl and an active site Lys residue (e.g., Lys62), or indeed that this group plays a significant role, through hydrogen bonding interactions, in orientating the molecule with respect to Cys53 for the postulated conjugate addition step. It should also be noted that no evidence of internal Schiff base formation was observed with **3** and **4** on prolonged storage of these compounds as the trifluoroacetate salts in DMSO solution.

Based on these observations, a possible overall mechanistic explanation for the kinetic behaviour of **1** and the other compounds studied herein is as follows: when incubated with TryR in the reduced state, an equilibrium is rapidly established between the inhibitor 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) (Fig. 2), 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. Variations in the rate of time-dependent inactivation (k_5) with these compounds might therefore be dictated by the ease with which they are able to adopt an enzyme-bound conformation that is favourable for the nucleophilic attack by Cys53 at an α,β-unsaturated amide moiety. In these terms, the absence of time-dependent inhibition with **7**–**10** can be ascribed to their inability to bind in an orientation that is appropriate for the conjugate addition step as a result of minor modifications to the lunarine scaffold. Regarding the reversible nature of any covalent (EI*) adducts formed, in protic solvents conjugate addition reactions are normally considered to be essentially irreversible processes where, following the rate-limiting attack of a nucleophile, the intermediate enolate rapidly picks up a proton from solution and tautomerises to the final substituted product. It must be assumed that within the TryR active site, the amide enolate intermediate generated from a bound inhibitor is sufficiently stabilised and/or orientated such that protonation is disfavoured and the reverse reaction may occur (Fig. 3). As shown in Figure 7, when TryR is inactivated with increasing concentrations of (±)-**1**, v_s, the residual steady state rate does not tend to zero, which does not support a truly irreversible inactivation mechanism as embodied in mechanism D (Fig. 2), but rather is consistent with the proposed reversible EI → EI* conversion according to mechanism C. The values for the ratios k_5/k_6 (the so-called isomerisation constant) determined for those compounds that show time-dependent inhibition are also consistent with the proposed model, since in the limiting case of mechanism D, k_6 should tend to zero, leading to k_5/k_6 values several orders of magnitude higher than those observed.

The trypanocidal activities of all the lunarine analogues and spermidine natural products in this study were evaluated in vitro against *T. brucei* trypomastigotes. None of the natural product materials obtained either directly from *L. biennis* (**1** and **9**) or by simple chemical modification of natural lunarine (**7**, **8** and **10**) showed any trypanocidal activity at concentrations below 100 μM. It is noteworthy that these compounds all possess an optically pure 'Pummerer ketone' scaffold or a related core motif, whereas synthetic, racemic lunarine (±)-**1** and the racemic derivatives **3** and **4** were the only compounds in this study to show significant, albeit modest, in vitro activity. Encouragingly, **3**, which is perhaps the most promising TryR inhibitor to emerge from this study, also shows the best trypanocidal activity (IC_{50} = 29 μM). Further rational optimisation of the lunarine-derived 'Pummerer ketone' scaffold along with additional detailed studies on the mechanism of time-dependent TryR inhibition associated with molecules of this type may therefore be fruitful in terms of developing novel and effective antitrypanosomal compounds.

4. Conclusion

The data presented here further support the hypothesis that time-dependent inactivation of TryR by **1** and relat-

ed compounds occurs via a two-step 'enzyme isomerisation' process (mechanism C) which is associated with structural features that are potential sites for conjugate addition by active site cysteine residues. Initial indications suggest that the C-24–C-25 double bond of the lunarine nucleus is critical in this respect. While the polyamine moiety of **1** may be altered without compromising this novel mode of inhibition, modifications to the unique tricyclic nucleus appear to have a significant effect. For future inhibitor design, since analysis of racemic lunarine, (±)-**1**, shows that the unnatural stereoisomer is significantly more potent than the natural product, it should be profitable to use the unnatural stereoisomer of the tricyclic nucleus as the basis for high affinity ligand structures upon which alternative, correctly orientated Michael acceptor units might be presented. Such variations could address the need highlighted above to enhance the rate of EI → EI* conversion as a prerequisite for truly effective inhibition.

5. Experimental

5.1. General

Melting points were recorded on an Electrothermal IA9000 series digital melting point apparatus, using open capillaries, and are quoted uncorrected. NMR spectra: ^{1}H NMR spectra were recorded on a Bruker Avance DPX 300 FT-spectrometer at 300 MHz, ^{13}C NMR were recorded on the same spectrometer at 75 MHz. All NMR spectra were taken at 300 K, except where specified. Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) are given in Hz. The numbering systems used for the macrocyclic and acyclic lunarine analogues in spectral assignments are as given in Figure 4.

EI mass spectra were recorded on a VC 70-S double focusing mass spectrometer. Low resolution electrospray mass spectra were carried out on a VG Quattro triple quadrupole mass spectrometer. High resolution electrospray mass spectra were obtained at the Michael Barber Centre for Mass Spectrometry (Department of Chemistry, UMIST, Manchester). Protein electrospray mass spectra were obtained using an ABI Mariner time-of-flight mass spectrometer in the University of Dundee Post-Genomics and Molecular Interactions Centre. Flash Chromatography was performed on columns of silica gel (Fluorochem, Silica Gel 60; 40–63 μ). Reversed-phase chromatography was performed using Supelco Discovery^{TM} DSC-18 solid phase extraction tubes. Triethylamine was distilled from CaH_2 and stored under nitrogen at room temperature. Anhydrous organic solvents were prepared using standard procedures.

Recombinant *T. cruzi* trypanothione reductase was purified from *Escherichia coli* as previously described.[25] The enzyme was stored as a suspension in 70% ammonium sulfate solution at 4 °C and extensively dialysed against the assay buffer before use. Trypanothione disulfide ($T[S]_2$) was purchased from Bachem and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from Sigma. Spectrophotometric assays of TryR were carried out in 1 mL cuvettes on a temperature-controlled Shimadzu UV–vis recording spectrophotometer. One unit of enzyme activity is defined as the amount of TryR required to reduce 1 μmol $T[S]_2$ in the presence of 150 μM NADPH under saturating substrate conditions at 27 °C and pH 7.5 in the absence of DTNB. Kinetic data analyses were performed using GraFit Version 5 (Erithacus Software Ltd). In vitro trypanocidal assays were carried out using a BioTek fluorescence microtitre plate reader at 480 nm excitation wavelength and 530 nm emission wavelength and the data were processed using GraFit Version 5 (see above).

5.2. Enzyme assays

Initially screening for time-dependent inhibition of TryR was carried out in a microplate format as reported previously.[16] 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 for 60 min by the increase in absorbance at 410 nm, due to formation of the 5-mercapto-2-nitrobenzoic acid (TNB) dianion. 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.

Compounds which inhibited TryR in a linear manner were analysed more closely to determine K_i values and the mode of inhibition. The standard assay mixture (1 mL) contained TryR (1 mU), 40 mM 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 1% 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 nonlinear regression analysis of the hyperbola plot of v against substrate concentration (S).

Compounds which exhibited time-dependent inactivation of TryR were studied further using a DTNB-coupled assay.[16] The assay mixture (1 mL) contained TryR (1 mU), 40 mM Hepes (pH 7.5), 1 mM EDTA, 150 μM NADPH, 1 μM $T[S]_2$, 25 μM DTNB and varying concentrations of inhibitor. Inhibitor stock solutions were made up using co-solvent mixtures of assay buffer and DMSO such that final assay mixtures contained no more than 1% DMSO. As in the microplate assays,

enzyme mixtures were pre-incubated with NADPH for 5 min at 27 °C prior to initiating the reaction by the addition of substrate, whereupon enzyme activity was monitored over 60 min by the increase in absorbance at 412 nm. To determine time-dependent inhibition constants, assays were carried out at five different inhibitor concentrations (0–2 K_i) and one substrate concentration (1 μM). For the purpose of curve-fitting, about 15–30 evenly spaced data points were taken from each progress curve. The concentration-dependent values for v_i, v_s, k' and the displacement of the curve from the vertical ordinate (d) were determined by nonlinear fitting of the above data to Eq. 1:[26]

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

Fitting of these data, by nonlinear regression, using Eqs. 2 and 3 gives values for K_i and K_i^*, respectively:

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

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

where S is the substrate concentration and K_m is the apparent Michaelis–Menten constant for TryR (determined in a separate experiment to be 9.1 ± 1.0 μM).[16] 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 can be determined from the relationship in Eq. 5:

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

Finally, the mean value for k_6 is then substituted into Eq. 4 to calculate k_5. For inhibition mechanism (C) (Scheme 1) the relationship between k' and inhibitor concentration can also be expressed by Eq. 6:

$$k' = k_6 + k_5(I/K_i)/(1 + S/K_m + I/K_i). \quad (6)$$

For the mass spectrometric analysis of the lunarine–TryR complex, a sample of TryR (18 μM) was reduced with NADPH (150 μM) and then incubated with and without (control) (±)-**1** (500 μM). Inactivation of TryR was monitored as above, and when 90% inactivation was reached, unbound low molecular components were washed away from the reaction mixture by buffer exchange (3×) using a Microcon YM-30 centrifugal filter with 20 mM, pH 7.7 ammonium bicarbonate. The buffer-exchanged sample was then analysed by electrospray mass spectrometry.

5.3. In vitro trypanocidal assays

Test compounds and a control drug (pentamidine) were dissolved in DMSO at 10 and 0.1 mM, respectively. Into the first column (A1–H1) of the 96-well microplate were added each compound solution (2 μL) and HMI-9 medium (198 μL), with an aliquot of HMI-9 medium containing 1% DMSO (100 μL) being added to the rest of the wells. A range of drug concentrations from 250 to 0.049 μM were prepared using a multichannel pipette to withdraw 100 μL from the first column and add into the adjacent column, repeating the process up to column 10. The aspirate from column 10 was added into column 12. HMI-9 medium (100 μL) containing 2000 *T. brucei rhodesiense* S427,117 bloodstream form trypomastigotes was added to each well apart from column 12 and the microplate was incubated for 3 days at 37 °C in 5% CO_2. Columns 11 and 12 therefore served as controls without drug or without trypanosomes, respectively. After the third day, Alamar Blue® (20 μL) was added to each well and the plate was incubated for a further 3 days under the same conditions. Finally, the fluorescence of the wells was read from the bottom of the plate. IC_{50} values were obtained by fitting of the data to the following equation:

$$y = \frac{\text{Range}}{1 + \left(\frac{x}{IC_{50}}\right)^s} + \text{Background}.$$

5.4. *cis*-3-Oxo-8,9b-bis-(*trans*-N^3-(dimethylamino)propylacrylamido)-1,2,3,4,4a,9b-hexahydrodibenzofuran (2)

Compound **19** (11 mg, 0.020 mmol) was dissolved in 5% HCl in THF (4 mL) and left to stir at room temperature for 20 h when all the starting material had been consumed (as determined by electrospray mass spectrometry). Solvents were removed and the residue was re-dissolved in H_2O (15 mL) and washed with EtOAc (3 × 5 mL). The aqueous layer was evaporated to give a crude yellow resin, which was purified by reverse-phase chromatography (10% aqueous MeOH eluent) to give **2** as the hydrochloride salt (5 mg, 42%). δ_H (300 MHz, CD_3OD) 1.69–2.13 (6 H, m, 3× CH_2) 2.78, 2.80, 2.82 (12H, overlapping singlets, 4× CH_3), 3.00–3.17 (4H, m, 2× CH_2), 3.19–3.39 (6H, m, 3× CH_2), 3.46–3.54 (2H, m, CH_2), 4.63 (1H, br s, H-4a), 5.75 (1H, d, J 15.6, CH-11), 6.47 (1H, d, J 15.6, CH-14), 6.65-6.83 (3H, m, H-6, H-9, CH-10), 7.28–7.40 (3H, m, 2× NH, H-7), 7.45 (1H, d, J 15.6, CH-13); δ_C (75 MHz, CD_3OD) 24.52 (CH_2), 24.64 (CH_2), 29.98 (C-1), 35.00 (CH_2), 36.55 (CH_2), 43.10 (CH_3), 49.05 (C-9b), 55.66 (CH_2NMe_2), 87.27 (C-4a), 110.62 (C-6), 118.03 (C-14), 121.92 (C-11), 125.92 (C-9), 129.03, 130.70, 131.26 (C-8, C-9a, C-7), 141.40 (C-13), 147.54 (C-10), 160.62 (C-5a), 168.68, 169.63 (2× amide CO); [Found: (ESI) 497.3123 $[M+H]^+$, $C_{28}H_{41}N_4O_4$ requires 497.3128]; *m/z* (ESI) 497 (100%, $[M+H]^+$), 249 (32, $[M+2H]^{2+}$).

5.5. *cis*-3-Oxo-8,9b-bis-(*trans*-N^1-(acrylamidospermidyl))-1,2,3,4,4a,9b- hexahydrodibenzofuran (3)

A solution of **20** (37 mg, 0.036 mmol) in CH_2Cl_2 (1 mL) was treated with TFA (1 mL), added in one portion, and the resulting pale brown solution was stirred at room temperature for 1 h. The solution was then diluted with acetone (4 mL) and was then co-evaporated several times with a 1:1 co-solvent mixture of CH_2Cl_2/acetone. The residue was then dissolved in water (15 mL) and washed with EtOAc (3 × 5 mL). The aqueous layer was evaporated and the crude residue was purified by

reverse-phase chromatography (H_2O eluent) to give **3** as the trifluoroacetate salt (26 mg, 70%). δ_H (300 MHz, D_2O); 1.70–3.50 (34 H, m, 17× CH_2), 5.15 (1H, dd, J 7.1, 2.7, H-4a), 6.15 (1H, d, J 15.7, H-11), 6.53 (1H, d, J 15.7, H-14), 6.76–6.90 (2H, m, H-9, H-6), 7.11 (1H, m, J 15.7, H-10), 7.14–7.60 (4H, m, H-7, H-13, 2× NH); δ_C (75 MHz, CD_3OD); 24.72 (CH_2), 25.99 (CH_2), 28.01 (C-1), 28.18 (C-2), 32.02 (C-4), 37.48 (CH_2), 40.40 (CH_2), 46.83 (CH_2), 48.48 (CH_2), 51.54 (C9b), 88.94 (C-4a), 111.70 (C-6), 119.50 (C-14), 124.63 (C-9), 125.96 (C-11), 130.62 (C-8), 131.79 (C-7), 133.01 (C-9a), 139.26 (C-13), 148.65 (C-10), 162.87 (C-5a), 169.19, 170.29 (2× amide CO), 211.29 (ketone CO); [Found: (ESI) 292.2032 $[M+2H]^{2+}$, $C_{32}H_{52}N_6O_4$ requires 292.2025]; *m/z* (ESI) 583 (65%, $[M+H]^+$), 292 (100, $[M+2H]^{2+}$).

5.6. *cis*-3-Oxo-8-*trans*-(N^1-acrylamidospermidyl)-9b-*trans*-ethylacrylyl-1,2,3,4,4a,9b-hexahydrodibenzofuran (4)

A solution of **25** (33 mg, 0.045 mmol) in CH_2Cl_2 (1 mL) was treated with TFA (1 mL) and the resulting pink solution was stirred at room temperature for 50 min. The reaction mixture was then diluted with acetone (4 mL), solvents were evaporated and the residue was co-evaporated several times with acetone to remove the excess acid (in order to avoid reformation of dioxolane and to facilitate removal of ethylene glycol as the isopropyl dioxolane by extraction into organic solvent). The crude residue was re-dissolved in H_2O (15 mL) and washed with EtOAc (3 × 5 mL). The aqueous layer was evaporated and the crude residue was purified by C_{18} reversed-phase chromatography (10% aqueous MeOH eluent) to give **4** as the trifluoroacetate salt (25 mg, quantitative). δ_H (300 MHz, D_2O); 1.05 (3H, t, *J* 7.0, CH_3), 1.52–1.94 (9H, m), 2.10–2.23 (2H, m), 2.53–3.0 (7H, m), 3.18–3.28 (2H, m), 3.98 (2H, q, *J* 7.1, CH_2CH$_3$), 4.78–4.83 (1H, br s, H-4a), 5.70 (1H, d, *J* 16.9, CH-11), 6.16 (1H, d, *J* 15.7, CH-14), 6.50 (1H, d, *J* 8.3, H-6), 6.88 (1H, d, *J* 15.9, CH-13), 7.00–7.20 (3H, m, H-7, H-9, CH-10) ; δ_C (75 MHz, D_2O); 13.74 (CH_3), 23.12 (CH_2), 24.25 (CH_2), 26.10, (C-1), 30.04 (C-2), 35.22 (CH_2), 36.61 (CH_2), 39.10 (CH_2), 41.95 (CH_2), 45.50 (CH_2), 47.28 (CH_2), 49.98 (C-9b), 62.13 (CH_2CH$_3$), 86.91 (C-4a), 110.90 (C-6), 118.06 (C-14), 120.98 (C-11), 124.81 (C-9), 128.86 (C-8), 130.86 (C-7), 130.83 (C-9a), 140.98 (C-13), 151.38 (C-10), 160.47 (C-5a), 168.32 (ester CO), 169.07 (amide CO), 214.57 (ketone CO); [Found: (ESI) 484.2807 $[M+H]^+$, $C_{27}H_{38}N_3O_5$ requires 484.2811]; *m/z* (ESI); 484 (100%, $[M+H]^+$).

5.7. (±)-10,11-Dihydrolunarine (6)

A solution of **24** (25 mg, 0.038 mmol), in CH_2Cl_2 (1 mL), cooled to 0 °C, was treated with a solution of EDC (8 mg, 0.42 mmol), DMAP (1 mg, 0.008 mmol) and pentafluorophenol (8 mg, 0.043 mmol) in CH_2Cl_2 (0.5 mL), and the mixture was left to stir at room temperature for 5 h. The reaction mixture was then diluted with CH_2Cl_2 (20 mL), washed with saturated aqueous $NaHCO_3$ (3 × 10 mL), followed by brine (10 mL), dried ($MgSO_4$) and solvents were evaporated to give the pentafluorophenyl ester as a pale brown solid. The *tert*-butoxycarbonyl groups were then removed by treatment with a 4 M solution of HCl in anhydrous dioxane (2 mL) at room temperature for 30 min. Solvents were then evaporated and the residue was co-evaporated several times with CH_2Cl_2 to give a pale yellow/brown foam, which was thoroughly dried in vacuo over 5 h. The hydrochloride salt was then re-dissolved in CH_2Cl_2 (3 mL) before slowly adding dropwise, over 20 min, to a solution of DIPEA (0.2 mL, 1.148 mmol) in CH_2Cl_2 (60 mL) and the mixture was left to stir at room temperature for 2 days. The reaction mixture was then washed with saturated aqueous $NaHCO_3$ (20 mL) followed by brine (20 mL), dried (Na_2SO_4) and the solvents were removed to give a crude white solid. This was purified by chromatography over a thin bed of silica (eluting with a 4:1 mixture of EtOH (saturated with NH_3) and $CHCl_3$) to give **6** as a white solid (4 mg, 24%). δ_H (300 MHz, CD_3OD/D_2O (4:1)); 1.36–3.05 (24H, m, 12× CH_2), 4.85–5.00 (H-4a is masked by the broad D_2O solvent peak), 6.43 (1H, d, *J* 15.7, CH-24), 6.77 (1H, *J* 8.2, H-6), 7.32 (1H, dd, *J* 8.3, 1.6, H-7), 7.48 (1H, d, *J* 1.6, H-9), 7.50 (1H, d, *J* 15.6, H-25); δ_C (75 MHz, CD_3OD/D_2O (4:1)); 28.46, 28.51, 28.63 (C-1, C-15, C-16), 32.14 (C-10), 34.08 (C-11), 35.34 (C-20), 36.92 (C-2), 41.24 (C-14 overlapping C-21), 49.98, 50.23 (C-17, C-19), 58.75 (C-4), 87.15 (C-4a), 110.97 (C-6), 120.52 (C-24), 123.95 (C-9), 130.39 (C-8), 132.40 (C-7), 133.90 (C-9a), 142.06 (C-25), 163.42 (C-5a), 169.29 (CO-23), 175.69 (CO-12), 212.42 (ketone-CO); [Found: (ESI) 440.2538 $[M+H]^+$, $C_{25}H_{34}N_3O_4$ requires 440.2549]; *m/z* (ESI); 440 (100%, $[M+H]^+$).

5.8. *cis*-3-(1,3-Dioxolan-2-yl)-8,9b-bis-(*trans*-ethylacrylyl)-1,2,3,4,4a,9b-hexahydrodibenzofuran (15)

A solution of **14** (191 mg, 0.466 mmol), palladium(II) acetate (5 mg, 0.022 mmol), tri-*o*-tolylphosphine (20 mg, 0.066 mmol), triethylamine (100 μL, 0.717 mmol) and ethyl acrylate (70 μL, 0.647 mmol) in DMF (1 mL) was heated to 100 °C under argon for 3 h. The reaction was quenched with H_2O and extracted with diethyl ether (3 × 15 mL). The combined organics were washed with H_2O (10 mL), dried ($MgSO_4$) and the solvents were evaporated to give a crude yellow oil. This was purified by chromatography (petrol/EtOAc (3:1) eluent) to give **15** as a colourless gum (145 mg, 73%). δ_H (300 MHz, $CDCl_3$); 1.22 (3H, t, *J* 7.1, CH_3) 1.26 (3H, t, *J* 7.1, CH_3), 1.48–1.70 (2H, m, CH_2), 1.94–2.14 (4H, m, 2× CH_2), 3.81–3.96 (4H, –OCH_2CH_2O–), 4.13 (2H, q, *J* 7.1, CH_2), 4.18 (2H, q, *J* 7.1, CH_2), 4.73 (1H, t, *J* 5.5, H-4a), 5.75 (1H, d, *J* 15.9, CH-11), 6.25 (1H, d, *J* 15.9, CH-14), 6.82 (1H, d, *J* 8.3, H-6), 6.99 (1H, d, *J* 15.9, H-10), 7.20 (1H, d, *J* 1.0, H-9), 7.31 (1H, dd, *J* 8.3, 1.3, H-7), 7.57 (1H, d, *J* 15.9, H-13); δ_C (75 MHz, $CDCl_3$); 14.57 (CH_3), 14.70 (CH_3), 29.38 (C-1), 30.65 (C-2), 36.48 (C-4), 49.78 (C-9b), 60.65 (CH_2), 61.00 (CH_2), 64.50 (CH_2), 65.01 (CH_2), 87.90 (C-4a), 107.55 (C-3), 111.73 (C-6), 116.16 (C-14), 121.92 (C-11), 123.25 (C-9), 128.53 (C-8), 130.82 (C-7), 133.46 (C-9a), 144.57 (C-13), 150.29 (C-10), 161.02 (C-5a), 166.47 (CO), 167.50 (CO); [Found: (EI) 428.18742, $C_{24}H_{28}O_7$ requires 428.18351];

m/z 428 (50%, M^+), 383 (12, M–OEt), 99 (100, cyclic ethylene ketal fragment $C_5H_7O_2^+$).

5.9. *cis*-3-(1,3-Dioxolan-2-yl)-1,2,3,4,4a,9b-(hexahydrodibenzofuranyl)-8,9b-di-acrylic acid (16)

A solution of **15** (130 mg, 0.318 mmol) in EtOH/H_2O (3:1, 8 mL) was treated with a solution of LiOH (336 mg, 8.01 mmol) in H_2O (2 mL) and the mixture was stirred at room temperature for 3 h. The reaction mixture was passed through a column of Amberlyst IR-120 (plus) cation exchange resin (60% aqueous EtOH eluent) and acidic fractions were collected. Solvents were removed and the residue was dissolved in CH_2Cl_2 (30 mL), washed with brine (10 mL) and dried ($MgSO_4$). Final removal of the solvent gave **16** as a white solid (111 mg, 94%). δ_H (300 MHz, $CDCl_3$); 1.60–1.80 (2H, m, CH_2), 2.03–2.24 (4H, m, 2× CH_2), 3.88–4.09 (4H, m, –OCH_2CH_2O–), 4.83 (1H, t, *J* 5.3, H-4a), 5.86 (1H, d, *J* 15.8, CH-11), 6.32 (1H, d, *J* 15.9, CH-14), 6.91 (1H, d, *J* 8.3, H-6), 7.17 (1H, d, *J* 15.8, CH-10), 7.30 (1H, s, H-9), 7.42 (1H, d, *J* 8.2, H-7), 7.73 (1H, d, *J* 15.8, H-13), 13.0–13.4 (2H, br s, 2× CO_2H); δ_C (75 MHz, $CDCl_3$); 29.29 (C-1), 30.66 (C-2), 36.50 (C-4), 49.92 (C-9b), 64.56, 65.06 (2× CH_2), 87.86 (C-4a), 107.61 (C-3), 111.96 (C-6), 115.24 (C-14), 121.42 (C-11), 123.73 (C-9), 128.27 (C-8), 131.43 (C-7), 133.35 (C-9a), 147.02 (C-13), 152.58 (C-10), 161.43 (C-5a), 171.42 (CO), 172.87 (CO); [Found: (EI) 372.11906, $C_{20}H_{20}O_7$ requires 372.12090]; *m/z* 372 (70%, M^+).

5.10. *cis*-3-(1,3-Dioxolan-2-yl)-8,9b-bis-(*trans*-pentafluorophenylacrylyl)-1,2,3,4,4a,9b-hexahydrodibenzofuran (17)

A solution of **16** (110 mg, 0.295 mmol) in CH_2Cl_2 (6 mL) was cooled to 0 °C and EDC (130 mg, 0.678 mmol) and DMAP (2 mg, 0.016 mmol) were added, followed by a solution of pentafluorophenol (130 mg, 0.706 mmol) in CH_2Cl_2 (0.5 mL). The mixture was stirred at room temperature overnight. The reaction mixture was diluted with CH_2Cl_2 (20 mL), washed with saturated $NaHCO_3$ (2 × 10 mL), followed by brine (10 mL), dried ($MgSO_4$) and the solvents were evaporated to give **17** as a pale brown oil (227 mg). This material was stored at 4 °C and used without further purification.

5.11. *cis*-3-(1,3-Dioxolan-2-yl)-8,9b-bis-(*trans*-N^3-(dimethylamino)propylacrylamido)-1,2,3,4,4a,9b-hexahydrodibenzofuran (19)

A solution of **17** (0.067 mmol) in CH_2Cl_2 (0.5 mL) was treated with a solution of 3-(dimethylamino)propylamine (17 mg, 0.168 mmol) and DIPEA (70 μL, 0.402 mmol) in CH_2Cl_2 (1 mL), added dropwise. The mixture was stirred at room temperature for 3 h, then the solvents were removed and the crude residue was purified by chromatography (5% ammonium hydroxide in MeOH as eluent) to give **19** as a white foam (11 mg, 32%). δ_H (300 MHz, $CDCl_3$); 1.52–1.77 (6H, m, 3× CH_2), 1.94–2.11 (4H, m, 2× CH_2), 2.19 (6H, s, 2× CH_3), 2.25 (6H, m, 2× CH_3), 2.27–2.43 (4H, m, 2× CH_2), 3.30–3.48 (4H, m, 2× CH_2), 3.86–4.02 (4H, m, OCH_2CH_2O), 4.72 (1H, t, *J* 5.1 H-4a), 5.73 (1H, d, *J* 15.6, CH-11), 6.21 (1H, d, *J* 15.6, CH-14), 6.82 (1H, d, *J* 8.3, H-6), 6.91 (1H, d, *J* 15.6, CH-10), 7.18 (1H, d, *J*1.5, H-9), 7.19–7.30 (3H, m, H-7, 2× NH), 7.49 (1H, d, *J* 15.6, CH-13); δ_C (75 MHz, $CDCl_3$); 26.11, 26.34 (2× CH_2), 29.71 (C-1), 30.28 (C-2), 35.99 (C-4), 39.27 (CH_2), 45.34, 44.45 (2× CH_3), 49.05 (C-9b), 58.30, 58.40 (2× CH_2), 64.07, 64.64 (OCH_2CH_2O), 87.49, (C-4a), 107.29, (C-3), 111.04 (C-6), 118.83 (C-14), 122.28 (C-11), 124.14 (C-9), 128.56 (C-8), 129.87 (C-7), 133.63 (C-9a), 139.97 (C-13), 145.10 (C-10), 159.98 (C-5a), 165.29 (CO), 166.18 (CO); [Found: (ESI) 541.3369 $[M+H]^+$, $C_{30}H_{45}N_4O_5$ requires 541.3390]; *m/z* (ESI); 563 (6%, $[M+Na]^+$), 541 (100, $[M+H]^+$).

5.12. *cis*-3-(1,3-Dioxolan-2-yl)-8,9b-bis-(*trans*-N^4,N^8-bis-(*tert*-butoxycarbonyl)-N^1-acrylamidospermidyl)-1,2,3,4,4a,9b-hexahydrodibenzofuran (20)

A solution of **17** (0.067 mmol) in CH_2Cl_2 (0.5 mL) was treated with a solution of **18**[17] (58 mg, 0.168 mmol) and DIPEA (70 μL, 0.402 mmol) in CH_2Cl_2 (1 mL), added dropwise. The mixture was stirred at room temperature for 3 h. The reaction mixture was then diluted with CH_2Cl_2 (20 mL), washed with 5% aqueous citric acid (2 × 8 mL) followed by saturated $NaHCO_3$ (8 mL), dried ($MgSO_4$) and solvents were evaporated to give a yellow gum. This was purified by chromatography (EtOAc/MeOH, 98:2) to give **20** as a white foam (38 mg, 54%). Some of the ^1H NMR signals for this compound were very broad at room temperature so ^1H NMR analysis was carried out at 50 °C. δ_H (300 MHz, $CDCl_3$, 323 K); 1.43 (36H, s, 12× CH_3), 1.44–1.79 (12H, m, 6× CH_2), 1.88 (2H, dd, *J* 6.9, 1.7, CH_2), 1.98–2.11 (4H, m, 2× CH_2), 3.04–3.39 (16H, m, 8× CH_2), 3.85–3.97 (4H, m, OCH_2CH_2O), 4.60–4.80 (2H, 2× NH), 4.75 (1H, t, *J* 5.5, H-4a), 5.80–5.87 (1H, m, CH-11), 6.25–6.33 (1H, m, CH-14), 6.82 (1H, d, *J* 8.3, H-6), 6.96 (1H, d, *J* 15.1, CH-10), 7.21 (1H, d, *J* 1.5, H-9), 7.30 (1H, dd, *J* 8.3, 1.6, H-7), 7.53 (1H, d, *J* 15.6, CH-13); δ_C (75 MHz, $CDCl_3$); 25.61 (CH_2), 27.31 (CH_2), 27.63 (CH_2), 28.34 (CH_3), 29.60 (C-1), 30.25 (C-2), 35.86, 35.88 (C-4, CH_2), 39.99, (CH_2), 43.53 (CH_2), 46.59 (CH_2), 49.05 (C-9b), 64.00, 64.58 (OCH_2-CH_2O), 79.14 (C), 79.82 (C), 107.31 (C-3), 111.01 (C-6), 118.67 (C-14), 122.48 (C-11), 123.93 (C-9), 128.52 (C-8), 129.86 (C-7), 133.62 (C-9a), 140.30 (C-13), 146.13 (C-10), 156.03, 156.41 (2× carbamate-CO), 160.01 (C-5a), 165.52, 166.49 (2× amide-CO); *m/z* (ESI); 1049 (25%, $[M+Na]^+$), 1027 (100, $[M+H]^+$), 927 (28, $[M\text{-}Bu^tOCO+2H]^+$).

5.13. *cis*-3-(1,3-Dioxolan-2-yl)-8-bromo-9b-(*trans*-acrylic acid)-1,2,3,4,4a,9b-hexahydrodibenzofuran (21)

A solution of **14** (220 mg, 0.538 mmol) in EtOH/H_2O (3:2, 6 mL) was treated with LiOH (225 mg, 5.38 mmol) and the mixture was stirred at room temperature for 3 h. The reaction mixture was then neutralised with 5% aqueous citric acid and extracted with EtOAc (3 × 20 mL). The combined organics were washed with H_2O (20 mL), dried ($MgSO_4$) and the solvent was evaporated to give **21** as a white foam (181 mg, 88%). δ_H

(300 MHz, $CDCl_3$); 1.57–1.77 (2H, m, CH_2), 2.00–2.18 (4H, m, 2× CH_2), 3.89–4.05 (4H, m, OCH_2CH_2O), 4.78 (1H, t, *J* 5.6, H-4a), 5.84 (1H, d, *J* 15.9, CH-11), 6.79 (1H, d, *J* 8.5, H-6), 7.15 (1H, d, *J* 15.9, CH-10), 7.18 (1H, d, *J* 2.1, H-9), 7.31 (1H, dd, *J* 8.4, 2.1, H-7), 13.4–13.9 (1H, br s, CO_2H); δ_C (75 MHz, $CDCl_3$); 28.55 (C-1), 30.02 (C-2), 35.84 (C-4), 49.79 (C-9b), 63.94, 64.42 (OCH_2CH_2O), 86.90 (C-4a), 107.01 (C-3), 112.56 (C-Br), 112.86 (C-6), 120.68 (CH-11), 126.33 (C-9), 131.83 (C-7), 133.79 (C-9a), 152.05 (CH-10), 157.55 (C-5a), 170.66 (CO); [Found: (EI) 380.01799, $C_{17}H_{17}BrO_5$ requires 380.04594]; *m/z* 380, 382 (14% M^+), 99 (100, cyclic ethylene ketal fragmentation $C_5H_7O_2^+$).

5.14. *cis*-3-Oxo-8-bromo-9b-(2-carboxyethyl)-1,2,3,4,4a, 9b-hexahydrodibenzofuran (22)

A mixture of **21** (148 mg, 0.388 mmol) and 5% rhodium on alumina (10 mg) in EtOH (10 mL) was stirred at room temperature under hydrogen (1 atmosphere) for 22 h. The mixture was filtered through Celite and the solvent was evaporated to give a crude solid, which was dissolved in a 50% aqueous THF solution of 2 M HCl (8 mL) and stirred at room temperature for 90 min. The reaction mixture was neutralised (2 M aq NaOH) and then extracted with CH_2Cl_2 (4 × 10 mL). The combined organics were dried ($MgSO_4$) and solvents were evaporated to give a crude oil, which was purified by chromatography (EtOAc/petroleum ether (7:3) eluent) to give **22** as a white resin (70 mg, 50%). The product contained traces of impurities (by ^1H and ^{13}C NMR), which could not be separated by chromatography, and so the material was carried through the subsequent Heck coupling reaction prior to further purification. δ_H (300 MHz, $CDCl_3$); 1.70–2.44 (8H, m, 4× CH_2), 2.61 (1H, dd, *J* 16.9, 3.5, CH_AH_B-4), 2.80 (1H, dd, *J* 17.0, 3.2, CH_AH_B-4), 4.82 (1H, d, *J* 3.3, H-4a), 6.57 (1H, d, *J* 8.5, H-6), 7.11 (1H, d, *J* 2.0, H-6), 7.19 (1H, dd, *J* 8.5, 2.1, H-7), 9.00 (1H, br s, CO_2H); δ_C (75 MHz, $CDCl_3$); 29.86 (C-10), 33.27 (C-1), 34.17 (C-11), 35.95 (C-2), 42.14 (C-4), 47.90 (C-5a), 85.20 (C-4a), 111.99 (C-6), 126.94 (C-9), 132.60 (C-7), 133.22 (C-9a), 158.97 (C-5a), 178.57 (CO_2H), 209.37 (CO); [Found: (EI) 338.00999, $C_{15}H_{15}BrO_4$ requires 338.01537]; *m/z* 340, 338 (88%, M^+), 267, 265 (88, M–$CH_2CH_2CO_2H$), 187 (100, M-Br-$CH_2CH_2CO_2H$).

5.15. *cis*-3-Oxo-8-*trans*-(N^4,N^8-bis-(*tert*-butoxycarbonyl)-N^1-acrylamidospermidyl)-9b-(2-carboxyethyl)-1,2,3,4, 4a,9b-hexahydrodibenzofuran (24)

A mixture of **22** (67 mg, 0.198 mmol), **23** (87 mg, 0.218 mmol), palladium(II) acetate (4 mg, 0.020 mmol), tri-*o*-tolylphosphine (18 mg, 0.053 mmol) and triethylamine (138 μL, 0.988 mmol) in DMF (1 mL) was stirred at 60 °C under argon for 6 h. The reaction was then quenched with brine (10 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organics were washed with 5% aqueous citric acid (10 mL), dried (Na_2SO_4) and the solvents were removed to give a crude brown resin. This was purified by chromatography (EtOAc/MeOH 95:5 as eluent) to give a colourless resin (70 mg) containing unidentifiable impurities which proved to be inseparable by chromatography. When the impure resin was triturated with diethyl ether, a white precipitate formed. The ethereal layer was removed and the precipitate was washed several times with diethyl ether to give **24** as a white solid (49 mg, 38%). δ_H (300 MHz, $CDCl_3$) 1.42 (9H, s, 3× CH_3), 1.45 (9H, s, 3× CH_3), 1.50–2.36 (14H, m, 7× CH_2), 3.04–3.38 (8H, m, 4× CH_2), 2.67 (1H, d, *J* 15.3, CH_AH_B-4), 2.82 (1H, d, *J* 15.3 ($CHAH_B$-4), 4.70–5.00 (2H, br s, 2× NH), 4.86 (1H, br s, H-4a), 6.35 (1H, d, *J* 15.6, H-14), 6.67 (1H, d, *J* 8.2, H-6), 7.13–7.38 (2H, br s, H-7, H-9), 7.51 (1H, d, *J* 15.6, H-13); δ_C (75 MHz, $CDCl_3$); 26.13, 27.79, 28.14 (3× CH_2), 28.82 (CH_3), 30.73 (C-10), 33.17 (C-1), 34.72 (C-11), 36.04 (CH_2), 36.49 (C-2), 40.50 (CH_2), 42.16 (C-4), 44.00 (CH_2), 47.13 (C-9b), 47.47 (CH_2), 79.61 (C), 80.31 (C), 85.69 C-4a), 110.41 (C-6), 119.26 (C-14), 123.88 (C-9), 129.09 (C-8), 129.78 (C-7), 132.28 (C-9a), 140.64 (C-13), 156.55, 156.87 (2× carbamate CO), 161.23 (C-5a), 167.06 (amide CO), 177.83 (carboxyl CO), 209.49 (ketone CO); *m/z* (ESI); 680 (40%, $[M+Na]^+$), 658 (36, $[M+H]^+$), 558 (100, $[M\text{-}Bu^tOCO+2H]^+$).

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