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Mechanism and structure–activity relationships of norspermidine-based peptidic inhibitors of trypanothione reductase

Mark J. Dixon,[†] Richard I. Maurer, Cristina Biggi, Julen Oyarzabal,[‡] Jonathan W. Essex and Mark Bradley*

School of Chemistry, University of Southampton, Southampton SO17 1BJ, UK

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Abstract—A library of polyamine–peptide conjugates based around some previously identified inhibitors of trypanothione reductase was synthesised by parallel solid-phase chemistry and screened. Kinetic analysis of library members established that subtle structural changes altered their mechanism of action, switching between competitive and non-competitive inhibition. The mode of action of the non-competitive inhibitors was investigated in detail by a variety of techniques including enzyme kinetic analysis (looking at both NADPH and trypanothione disulfide substrates), gel filtration chromatography and analytical ultracentrifugation, leading to the identification of an allosteric mode of inhibition.

1. Introduction

Infection by *Trypanosoma* and *Leishmania* parasites is the cause of a number of tropical diseases in humans and domestic livestock that represent a major threat to Third World health. These diseases include African sleeping sickness (caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*), Chagas' disease (caused by *Trypanosoma cruzi*), mucocutaneous and cutaneous leishmaniasis (caused by various species of *Leishmania*) and Nagana (caused by *Trypanosoma congolense*). Currently available treatments suffer from resistance and severe side effects and the need for more effective drugs is acute.[1,2] The discovery of a significant difference in the redox defence mechanism of parasite and host has opened up a highly promising avenue of research into the development of novel drugs for the treatment of these diseases.[3,4]

The maintenance of an intracellular reducing environment is essential in protecting the cell from the highly reactive oxygen species that arise as a result of aerobic respiration and host immune response to infection. In most organisms, the abundant thiol glutathione (GSH) is utilised for this purpose, the level of which is maintained by the action of glutathione reductase (GR), which regenerates GSH from glutathione disulfide ($[GS]_2$). The GR/GSH system is replaced in trypanosomes and leishmania by an analogous system based on trypanothione (N^1,N^8-bis(glutathionyl)-spermidine, $T[SH]_2$) and trypanothione reductase (TR), which regenerates $T[SH]_2$ from trypanothione disulfide ($T[S]_2$). GR and TR are similar in many respects, both being NADPH-dependent homo-dimeric flavoproteins with approximately 40% homology in their amino acid sequence and essentially identical mechanisms.[5,6] However, the two enzymes display a remarkable degree of specificity for their respective substrates due to critical differences between their active sites.[7] In GR, the active site is positively charged to accommodate the carboxylate groups of $[GS]_2$, whereas the active site of TR is larger and has more hydrophobic character to

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* Corresponding author at present address: School of Chemistry, University of Edinburgh, Kings Buildings, West Mains Road, Edinburgh EH9 3JJ, UK. Tel.: +44 1316 504820; fax: +44 2380 596766; e-mail: mbradley@staffmail.ed.ac.uk
† Current address: Division of Biological Chemistry & Molecular Microbiology, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK.
‡ Current address: Molecular Informatics, Johnson & Johnson Pharmaceutical R&D, 45007 Toledo, Spain.

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accommodate the positively charged spermidine portion of $T[S]_2$.[6,8,9] The key role of TR in protecting the parasite from oxidative stress and the specificities of TR and GR make the enzyme a potential target for anti-trypanosomal drugs.

Numerous inhibitors of TR have been reported, based on tricyclic antidepressants,[10–14] polyamine conjugates[15–20] and substrate analogues[21,22] amongst others. Within our group, combinatorial and solid-phase chemistry has been utilised in the search for novel inhibitors of TR,[23–26] and one such investigation identified the spermidine-based compounds **1**–**3** (Fig. 1).[27] Compounds **1**–**3** represent promising lead structures with the most active, **2**, having a K_i of 100 nM. Surprisingly, **1**–**3** displayed non-competitive behaviour despite their structural similarity to $T[S]_2$ and other reported competitive inhibitors. Here, we describe our investigations into the mechanism of action of this class of inhibitors and the development of structure–activity relationships based on the screening of structurally simplified analogues.

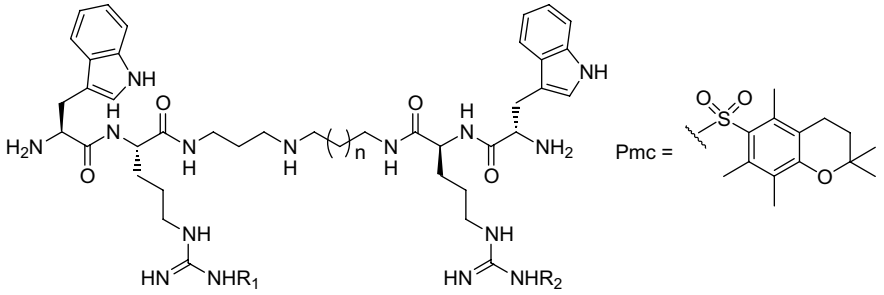
2. Results

In order to avoid the use of a mixture of regioisomers (as with **2**), norspermidine analogues of the lead compounds were used as direct replacements (**4**–**6**, Fig. 1). It was envisaged that this structural modification, whilst simplifying synthesis and purification, would have little effect on the inhibitory properties of the compounds, although it was necessary to confirm this experimentally. The lead compounds contain several structural components of potential importance to binding, including the arginine and tryptophan residues, the 2,2,5,7,8-pentamethylchroman-6-sulfonamide (Pmc) group and the polyamine. A library of simplified structural analogues of **4**–**6** was investigated with the aim of identifying key binding elements and developing SAR. The library consisted of norspermidine based compounds derivatised with structural elements on both arms (**10**–**12**, Scheme 1 and Table 1) and one arm only (**17**–**31**, Scheme 2 and Table 1). The one-armed compounds were derivatised on the second arm with either a simple amide group of various sizes or left as a primary amino group. The library was completed with the dipeptides H-Trp-Arg-OH (**32**) and H-Trp-Arg(Pmc)-OH (**33**), included to evaluate the importance of the polyamine backbone.

Compounds **4**–**6** and **10**–**12** were prepared from immobilised, bis-protected norspermidine **9**, attached to the resin via an acid labile, Wang-type linker (Scheme 1). Compound **9** was prepared by protection of the primary amino groups of norspermidine **7** by reaction with 2 equiv of 2-acetyl dimedone, prior to reaction with 4′-nitrophenyl-(4-hydroxymethyl-phenoxy-allylacetate)carbonate[28] to give **8**. The allyl ester of **8** was removed upon treatment with $Pd(PPh_3)_4$ and thiosalicylic acid, and the resulting carboxylic acid coupled to aminomethyl polystyrene resin via standard HOBt/DIC activation to give **9**. Synthesis of **4**–**6** and **10**–**12** was completed from **9** using standard solid-phase peptide chemistry and compounds were purified by semi-preparative HPLC. In order to avoid the total deprotection of the Pmc group during cleavage from the resin, a cocktail of TFA/thioanisole/DCM/water (10:2:7:1) for 4×15 min was used. HPLC analysis of the crude cleavage mixture (of **4**, **5** and **6)** is shown Figure 2.

Compounds **17**–**31** were prepared from immobilised, orthogonally protected norspermidine **13**[29]in a multiple parallel fashion (Scheme 2). Following Dde deprotection of **13**, blocking of the first arm was achieved by reaction with either di-*tert*-butyl dicarbonate, acetic anhydride or hexanoic anhydride to give **14**–**16**. The trifluoroacetyl group was removed and derivatisation on the second arm was accomplished using standard solid-phase chemistry.

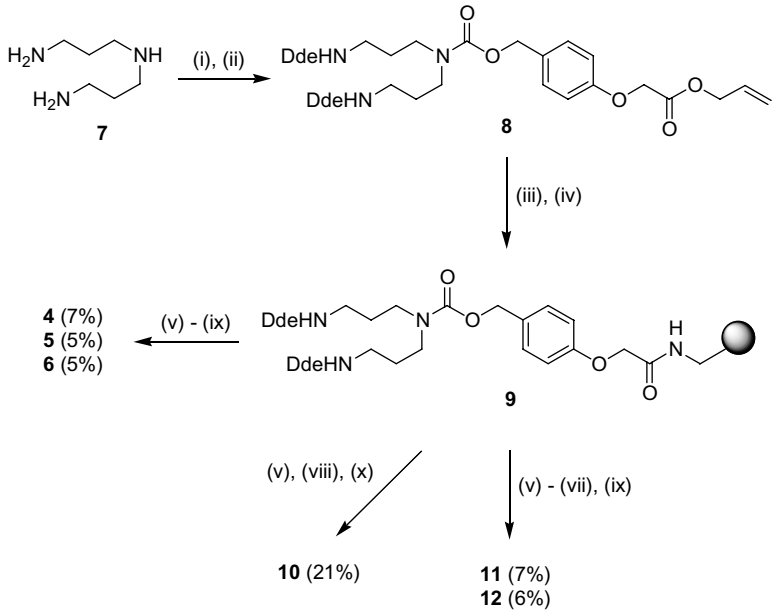
Initial screening of the entire library at a concentration of 100 μM revealed a range of activities. Compounds **17**–**22**, **32** and **33** showed little or no activity and were not investigated further. Compounds **4**–**6**, **10**–**12** and **23**–**31** exhibited significant activity and were subjected to full kinetic analysis (Table 1 and Fig. 3). The most potent inhibitors were two of the lead compounds, **5** and **6**, which had measured K_i values of 220 nM and 160 nM, respectively. The other library members exhibited varying potency with K_i values in the range 3–131 μM. As with the spermidine compounds **1**–**3**,



1 n =2, $R_1 = R_2 = H$
2 n = 2, $R_1 = H$, R_2 = Pmc/
n = 2, R_1 = Pmc, $R_2 = H$
3 n = 2, $R_1 = R_2$ = Pmc

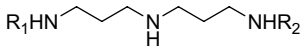
4 n =1, $R_1 = R_2 = H$
5 n = 1, $R_1 = H$, R_2 = Pmc
6 n = 1, $R_1 = R_2$ = Pmc

Figure 1. Previously identified spermidine-based TR inhibitors **1**–**3** and norspermidine analogues **4**–**6**.



Scheme 1. Preparation of **4**–**6** and **10**–**12**. Reagents and conditions: (i) 2-acetyldimedone, EtOH, reflux, 2 h, 99%; (ii) 4′-nitrophenyl-(4-hydroxymethylphenoxyallylacetate) carbonate, DMF, 16 h, 74%; (iii) $Pd(PPh_3)_4$, thiosalicylic acid, DCM, THF, 2 h, 70%; (iv) aminomethyl polystyrene resin, HOBt, DIC, DCM, DMF, 16 h; (v) 5% H_2NNH_2/DMF, 30 min; (vi) Fmoc-Arg(Pmc)-OH, HOBt, DIC, DCM, DMF, 2 h; (vii) 20% piperidine/DMF, 2 × 10 min; (viii) Boc-Trp(Boc)-OH, HOBt, DIC, DCM, DMF, 2 h; (ix) TFA/thioanisole/DCM/water (10:2:7:1), 4 × 15 min; (x) TFA/thioanisole/DCM/water (16:2:1:1), 2 h.

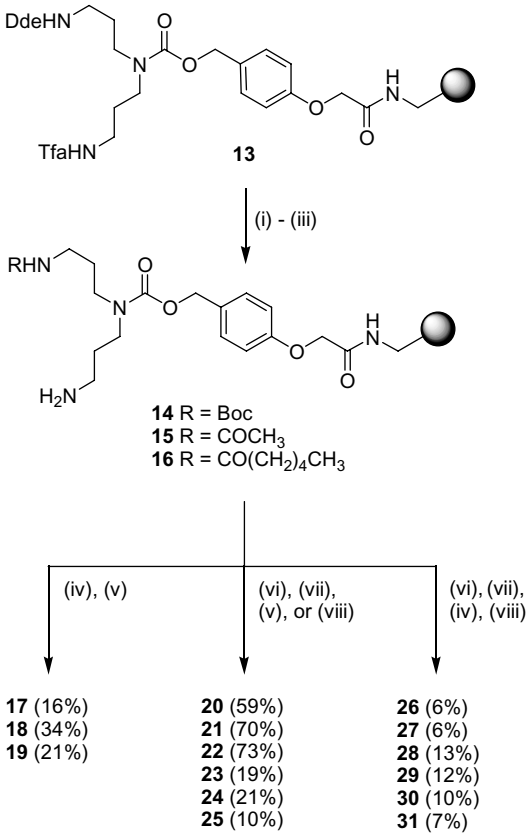
Table 1. Structures and results of kinetic analysis of **4**–**6**, **10**–**12** and **17**–**31**



Compound	R_1	R_2	Mode	K_i (μM)
4	Trp-Arg	Trp-Arg	Non-competitive	14 ± 0.5
5	Trp-Arg(Pmc)	Trp-Arg	Non-competitive	0.22 ± 0.02
6	Trp-Arg(Pmc)	Trp-Arg(Pmc)	Non-competitive	0.16 ± 0.01
10	Trp	Trp	Competitive	19 ± 1.1
11	Arg(Pmc)	Arg	Non-competitive	11 ± 0.5
12	Arg(Pmc)	Arg(Pmc)	Non-competitive	3 ± 0.3
17	Trp	H	Nd	>100
18	Trp	$COCH_3$	Nd	>100
19	Trp	$CO(CH_2)_4CH_3$	Nd	>100
20	Arg	H	Nd	>100
21	Arg	$COCH_3$	Nd	>100
22	Arg	$CO(CH_2)_4CH_3$	Nd	>100
23	Arg(Pmc)	H	Non-competitive	131 ± 7.2
24	Arg(Pmc)	$COCH_3$	Non-competitive	69 ± 3.2
25	Arg(Pmc)	$CO(CH_2)_4CH_3$	Non-competitive	7 ± 0.2
26	Trp-Arg	H	Competitive	83 ± 4.4
27	Trp-Arg(Pmc)	H	Non-competitive	9 ± 1.1
28	Trp-Arg	$COCH_3$	Competitive	69 ± 5.8
29	Trp-Arg(Pmc)	$COCH_3$	Non-competitive	12 ± 0.4
30	Trp-Arg	$CO(CH_2)_4CH_3$	Competitive	24 ± 2.0
31	Trp-Arg(Pmc)	$CO(CH_2)_4CH_3$	Non-competitive	6 ± 0.2

4–**6** exhibited non-competitive behaviour. Interestingly, this was not the case for all the library members, with several (**10**, **26**, **28** and **30**) exhibiting competitive behaviour.

The mechanism of action of **4**–**6** was investigated in more detail. Non-competitive behaviour of **5** was established with respect to NADPH by kinetic analysis (Fig. 3). The low K_m value of NADPH with TR (5 μM)[30] meant that extremely low concentrations of NADPH were required for this experiment, so reaction progression, as shown by NADPH consumption, was monitored using fluorescence rather than UV absorbance. Unfortunately, due to the difficult nature of these kinetic experiments, it was not possible to investigate all library members in this way. An alternative approach



Scheme 2. Preparation of **17**–**31**. Reagents and conditions: (i) 5% H_2NNH_2/DMF, 30 min; (ii) Boc_2O, DIPEA, DCM, 2 h or acetic anhydride/pyridine (1:1), 30 min or hexanoic anhydride/pyridine (1:1), 30 min; (iii) 1 M KOH/MeOH/THF (5:1:1), 2 × 2 h; (iv) Boc-Trp(Boc)-OH, HOBt, DIC, DCM, DMF, 2 h; (v) TFA/thioanisole/DCM/water (16:2:1:1), 2 h; (vi) Fmoc-Arg(Pmc)-OH, HOBt, DIC, DCM, DMF, 2 h; (vii) 20% piperidine/DMF, 2 × 10 min; (viii) TFA/thioanisole/DCM/water (10:2:7:1), 4 × 15 min (overall isolated yields).

was used to confirm these findings, whereby **4**–**6** were screened against glutamate dehydrogenase (GDH), an NADPH dependent enzyme that is involved in amino acid metabolism.[31] Although TR and GDH are unrelated enzymes, the NADPH binding sites are similar in both and a tight binding ligand in the TR NADPH pocket might be expected to show some activity with GDH. No inhibition was observed with **4**–**6** at a concentration of 100 μM.

TR is an α_2-homodimer and the active site is composed of residues from both subunits.[32] As such, an alternative possible mechanism of action involves binding of the inhibitors in the dimer interface region of the protein leading to the disruption of dimer formation, as has been reported with inhibitors of HIV-1 protease.[33–35] To investigate this possibility, analysis of TR by analytical ultracentrifugation (AUC) in the presence and absence of **4**–**6** was undertaken (AUC is a powerful technique for the determination of the stoichiometry of macromolecular complexes in their native, solution-phase state).[36] Analysis of TR in the absence of inhibitor gave a mass in good agreement with that reported for the TR dimer.[4] Analysis of TR in the presence of **4**–**6** ([I] > 5 × K_i) showed no significant change in the stoichiometry of the enzyme. The AUC findings were confirmed by analysis of TR in the presence and absence of **5** by gel filtration chromatography (Fig. 4).

3. Discussion

The solid-phase synthesis of both the one and two armed inhibitors was extremely clean as shown by the HPLC trace of the crude cleavage mixture of **4**–**6** (Fig. 2). The poor isolated yields (Scheme 2) were the result of instrumentally caused losses during semi-preparative HPLC purification. The non-competitive behaviour and K_i values obtained with **4**–**6** were in broad agreement with the results reported for the spermidine compounds **1**–**3** (K_i = 16 μM, 100 nM and 190 nM, respectively) and confirmed that the substitution of norspermidine for spermidine had minimal effect on the inhibitory properties of the compounds.

Kinetic analysis surprisingly showed both competitive and non-competitive inhibitors within the library. The competitive inhibitors were similar in structure to many reported TR inhibitors, containing both a hydrophobic group and a polyamine; however, this was also true of other compounds in this series which displayed non-competitive behaviour! No improvement in potency compared to **5** and **6** was observed with any of the

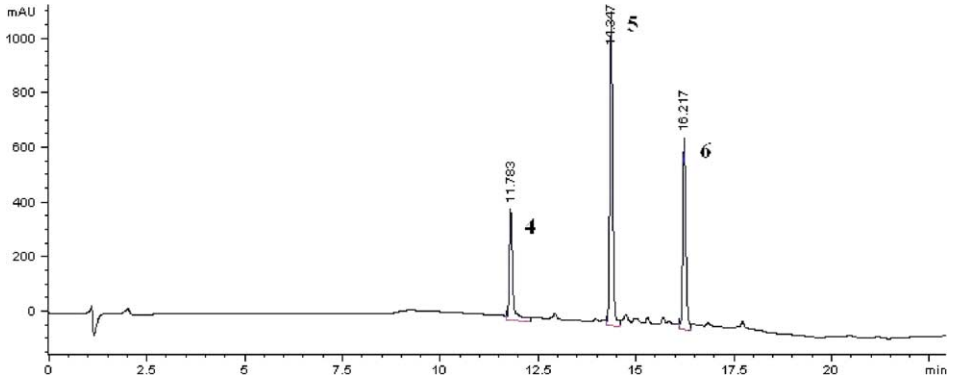


Figure 2. Analytical HPLC trace of a crude cleavage mixture giving **4**–**6**.

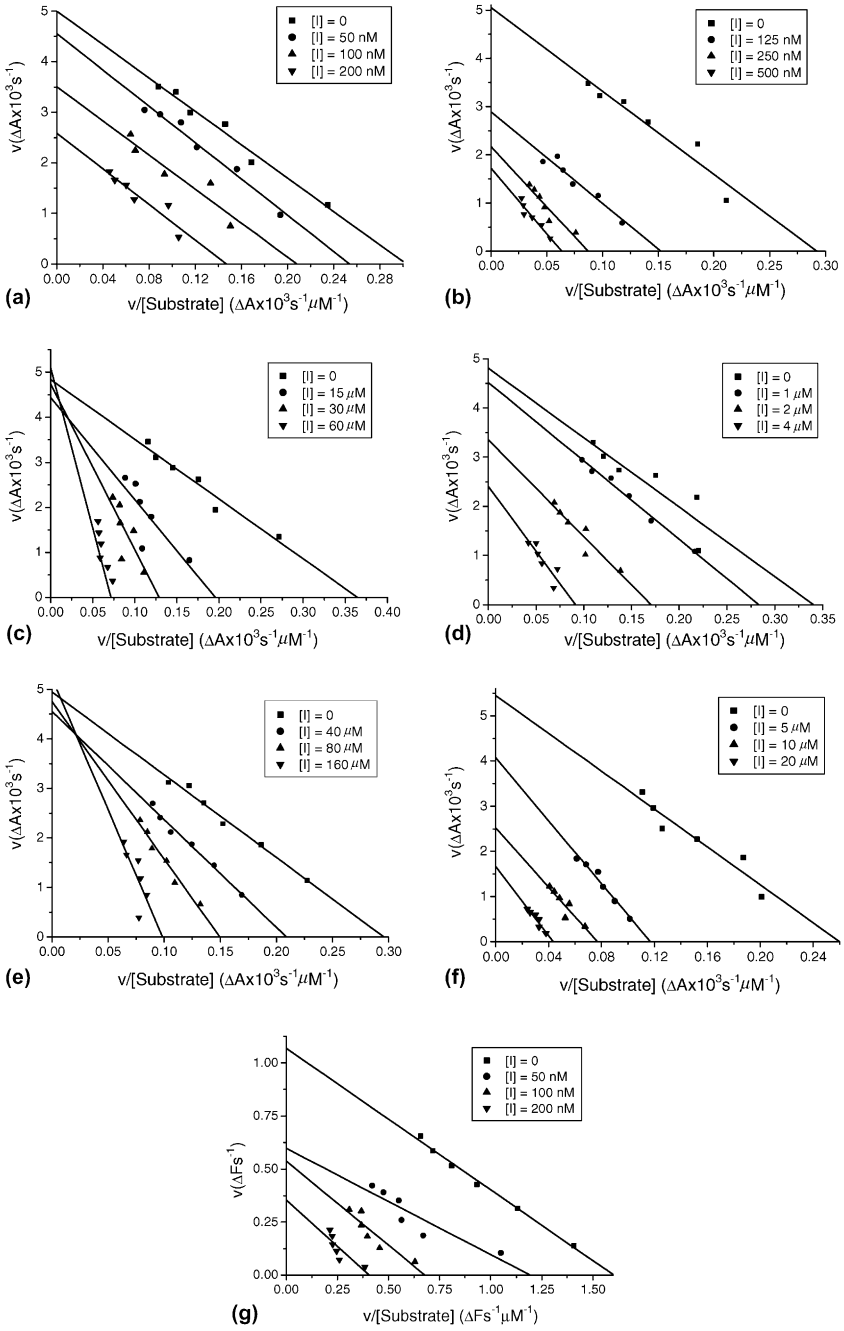


Figure 3. Eadie–Hofstee plots showing non-competitive behaviour with respect to disulfide substrate of (a) **5**, (b) **6**, (d) **12** and (f) **31** and competitive behaviour of (c) **10** and (e) **26**. Non-competitive behaviour of **5** with respect to NADPH is shown in (g). All assays were carried out in triplicate.

library members. Any change in structure was detrimental and resulted in an increase in K_i of at least an order of magnitude. In general, activity was related to structural similarity to **5** and **6** with the most active derivative, **12**, just lacking the tryptophan residues found in **6**, while structural simplification led to a corresponding loss of activity.

3.1. Tryptophan residue

Removal of the tryptophan residue resulted in a loss of potency (compare **5** and **6** with **11** and **12**; **27**, **29** and **31** with **23**–**25**; and **26**, **28** and **30** with **20**–**22**. The lack of activity of **20**–**22** is not surprising as previously screened polyamines show little activity against TR.[18]

3.2. Arginine residue

Removal of the arginine residue led to a change in mechanism (compare **4** with **10**) and a loss of potency (compare **26**, **28** and **30** with **17**–**19**). The lack of activity of **17**–**19** may simply be due to the arginine residue in **26**, **28** and **30** acting as a spacer and placing the polyamine and hydrophobic groups at an optimum distance apart for binding. Alternatively, the arginine side-chain in **26**, **28** and **30** may be involved in a

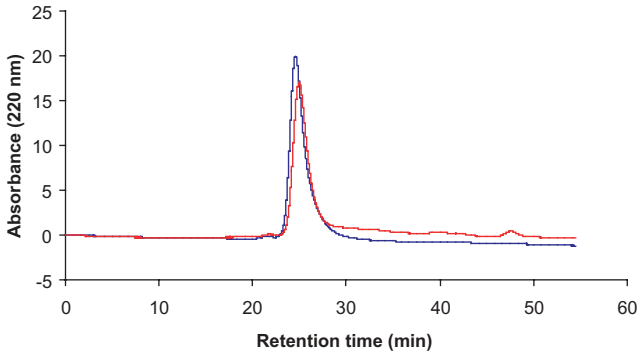


Figure 4. Gel filtration analysis of TR in absence (red) and presence (blue) of inhibitor **5** (10 μM).

binding interaction, increasing the potency of these compounds.

3.3. Pmc group

The Pmc group was clearly important for potency, as the eight top inhibitors all contained this structural feature. Removal of the group led to an increase in K_i (compare **5** and **6** with **4**; **27**, **29** and **31** with **26**, **28** and **30**; and **23**–**25** with **20**–**22**). A change in mechanism also resulted (compare **27**, **29** and **31** with **26**, **28** and **30**). Similar compounds containing other hydrophobic arginine protecting groups have been studied in our group and found to be potent TR inhibitors.[24]

3.4. One- and two-armed structures

The most potent compounds were derivatised on both arms of norspermidine, although significant activity was also observed with one-armed compounds. No correlation between the mode of action and either one- or two-armed structures was observed. In the one-armed series, the more hydrophobic hexanoyl capping group generally gave the best inhibition and the primary amino group the worst.

3.5. Norspermidine

The lack of activity of the dipeptides **32** and **33** demonstrated the importance of the polyamine portion of the inhibitors. The presence of the carboxylate moiety is almost certainly having a detrimental effect on the binding, as the TR active site is known to disfavour the binding of negatively charged over positively charged compounds.

Kinetic analysis of **5** showed non-competitive behaviour with respect to NADPH and the lack of activity against GDH of **4**–**6** supported this finding. Although NADPH and **4**–**6** share some structural similarities, such as hydrophobic portions (the adenine and nicotinamide in NADPH, the indole and PMC group in **4**–**6**), overall there is little similarity between the structures. At physiological pH, NADPH is negatively charged and the binding pocket in TR has a corresponding positive charge, whereas the inhibitors are positively charged. Given the non-competitive nature of **5**, the differences in structure and especially the differences in charge, the possibility of any of the non-competitive inhibitors binding at the NADPH pocket is small.

The possibility of inhibitor binding in the dimer interface region of the enzyme, hence causing disruption of the TR dimer, can be discounted as only dimeric enzyme was observed by both AUC and gel filtration chromatography. No monomeric enzyme was observed as would be expected in the case of dimer disruption. Clearly, inhibitor binding does not cause disruption to the dimeric form of the enzyme and therefore inhibitor binding presumably occurs at a site distinct from the dimer interface region.

A recent report described the action of a class of compounds termed aggregation inhibitors.[37] These inhibitors possess an unusual mechanism of action, whereby aggregates of many individual molecules form in solution and inactivate the target enzyme by absorption or adsorption. These inhibitors are typically highly sensitive to enzyme concentration and show little selectivity. This mechanism of action was discounted for the inhibitors in the present study as there was no evidence of aggregation by either AUC or gel filtration analysis, while activity was not enzyme-concentration dependent, nor were the compounds active against other enzymes.

4. Conclusions

In summary, multiple parallel solid-phase chemistry was utilised in the synthesis of a library of compounds that were screened against TR. No improvement in potency from the lead compounds was achieved, but SAR data were developed and both competitive and non-competitive behaviour was observed. The mechanism of action of the non-competitive inhibitors was investigated using a variety of techniques, and evidence for an allosteric mechanism involving inhibitor binding at a site distinct from the disulfide substrate active site, the NADPH active site, and the dimer interface region was obtained. The site of binding of the non-competitive inhibitors and specific interactions of the competitive inhibitors in the active site are currently under investigation by protein crystallography. The results presented strongly suggest that caution is needed when studying this protein in that simple assumptions about binding sites and mode of action may not always be justified as shown here for the clear distinction and change of mechanism from competitive to non-competitive with just small changes in structure.

5. Experimental

5.1. General information

NMR spectra were recorded on a Bruker DPX-400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. All coupling constants (J values) were measured in Hertz. ES mass spectra were recorded on a VG Platform Quadrupole Electrospray Ionisation mass

spectrometer. High resolution ES mass spectra were recorded on a Bruker Apex III FT-ICR mass spectrometer. Infra-red spectra were recorded on a BioRad FTS 135 Paragon 1000 spectrometer with a Golden Gate ATR accessory. Melting points were recorded on a Gallenkamp melting point apparatus and are uncorrected. Analytical RP-HPLC was performed on a Hewlett Packard HP 1100 chemstation equipped with a Phenomenex Prodigy 5 μm C_{18} (150 × 4.6 mm) column with a flow rate of 0.5 mL/min. Semi-preparative RP-HPLC was performed on a Hewlett Packard HP 1100 chemstation equipped with a Phenomenex Prodigy 5 μm C_{18} (250 × 10 mm) column with a flow rate of 2.5 mL/min. Mobile phase *A* was 0.1% TFA in water, mobile phase *B* was 0.042% TFA in acetonitrile. Gradient 1 was $T = 0$ min, $B = 10\%$, $T = 10$ min, $B = 90\%$, $T = 15$ min, $B = 90\%$. Gradient 2 was $T = 0$ min, $B = 0\%$, $T = 5$ min, $B = 0\%$, $T = 15$ min, $B = 60\%$, $T = 16$ min, $B = 100\%$, $T = 20$ min, $B = 100\%$. Gradient 3 was $T = 0$ min, $B = 0\%$, $T = 40$ min, $B = 50\%$, $T = 45$ min, $B = 90\%$, $T = 50$ min, $B = 90\%$. Thin layer chromatography (TLC) was performed on Alugram Sil G/UV/254 precoated plates and visualised under UV light. Column chromatography was performed using Sorbsil C60, 40–60 mesh silica. All reactions were performed at room temperature unless otherwise stated. UV/vis spectra and kinetics were recorded on a Hewlett Packard HP8452A diode array spectrophotometer. Fluorescence spectra were recorded on a Hitachi F2000 fluorescence spectrophotometer.

5.2. General procedures for solid-phase chemistry

Reactions were monitored by the use of a qualitative Kaiser test.[38]

5.3. Coupling of carboxylic acids

Acid (3 equiv relative to resin-bound amine) and *N*-hydroxybenzotriazole (3 equiv) were dissolved in DCM/DMF (4:1, 1 mL per 100 mg of resin) and the resultant solution shaken for 10 min. *N*,*N*′-Diisopropylcarbodiimide (3 equiv) was added and the resultant solution shaken for a further 10 min. The solution was added to the resin (pre-swollen in DCM (1 mL per 100 mg of resin) for 10 min) and the mixture shaken for 2 h. The resin was filtered and washed sequentially with DMF, DCM, MeOH and Et_2O (3 mL per 100 mg of resin, five times with each).

5.4. Dde deprotection

The resin was swollen in DCM (1 mL per 100 mg of resin) for 10 min, filtered and treated with 5% v/v hydrazine in DMF (1 mL per 100 mg of resin) for 30 min. The resin was filtered and washed sequentially with DMF, DCM, MeOH and Et_2O (3 mL per 100 mg of resin, five times with each).

5.5. Fmoc deprotection

The resin was swollen in DCM (1 mL per 100 mg of resin) for 10 min and treated with 20% v/v piperidine in DMF (1 mL per 100 mg of resin) for 2 × 10 min. The resin was filtered and washed sequentially with DMF, DCM, MeOH and Et_2O (3 mL per 100 mg of resin, five times with each).

5.6. Boc protection

The resin was swollen in DCM (1 mL per 100 mg of resin) for 10 min, filtered and treated with a solution of di-*tert*-butyl dicarbonate (5 equiv relative to resin-bound amine) and DIPEA (2 equiv) in DCM (1 mL per 100 mg of resin) for 2 h. The resin was filtered and washed sequentially with DMF, DCM, MeOH and Et_2O (3 mL per 100 mg of resin, five times with each).

5.7. *N*-Capping with acetic anhydride

The resin was swollen in DCM (1 mL per 100 mg of resin) for 10 min, filtered and treated with a mixture of acetic anhydride and pyridine (1:1, 1 mL per 100 mg of resin) for 30 min. The resin was filtered and washed sequentially with DMF, DCM, MeOH and Et_2O (3 mL per 100 mg of resin, five times with each).

5.8. *N*-Capping with hexanoic anhydride

The resin was swollen in DCM (1 mL per 100 mg of resin) for 10 min, filtered and treated with a mixture of hexanoic anhydride and pyridine (1:1, 1 mL per 100 mg of resin) for 30 min. The resin was filtered and washed sequentially with DMF, DCM, MeOH and Et_2O (3 mL per 100 mg of resin, five times with each).

5.9. Tfa deprotection

The resin was swollen in DCM (1 mL per 100 mg of resin) for 10 min, filtered and treated with a solution of 1 M KOH/THF/MeOH (5:1:1, 1 mL per 100 mg of resin) for 2 × 2 h. The resin was filtered and washed sequentially with DMF/water (1:1), MeOH/water (1:1), DMF, DCM, MeOH and Et_2O (3 mL per 100 mg of resin, five times with each).

5.10. Cleavage from the resin with TFA

The resin was swollen in DCM (1 mL per 100 mg of resin) for 10 min, filtered and treated with a mixture of TFA/thioanisole/DCM/water (16:2:1:1, 1 mL per 100 mg of resin) for 2 h or treated with a mixture of TFA/thioanisole/DCM/water (10:2:7:1, 1 mL per 100 mg of resin) for 4 × 15 min. The resin was filtered and washed with MeOH (3 mL per 100 mg of resin, five times), the filtrate and washings combined and concentrated in vacuo. The crude product was then dissolved in TFA, precipitated from ice-cold Et_2O, the precipitate collected by centrifugation and lyophilised.

5.11. Preparation of N^1,N^9-bis-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-norspermidine

Norspermidine (0.96 mL, 6.9 mmol) and 2-acetyl dimedone (2.50 g, 13.7 mmol) were dissolved in ethanol (47 mL) and heated at reflux for 2 h. The solvent was

removed in vacuo and the resulting yellow oil dissolved in EtOAc (50 mL), washed with water (3 × 50 mL), dried ($MgSO_4$) and concentrated in vacuo to give the title compound as a colourless oil (3.13 g, 99%).

R_f (DCM/MeOH, 17:3) 0.39. RP-HPLC (analytical, gradient 1, $\lambda = 220$ nm): $R_t = 6.3$ min. δ_H (400 MHz, $CDCl_3$): 13.40 (br s, 2H, 2 × C=C(CH_3)N*H*), 3.49 (m, 4H, 2 × C=C(CH_3)NHCH_2), 2.74 (t, 4H, $J = 7$, CH_2-NHCH_2), 2.56 (s, 6H, 2 × C=C(CH_3)NH), 2.35 (s, 8H, 4 × COCH_2), 1.83 (tt, 4H, $J = 7$, 7, 2 × CH_2C$H_2$$CH_2$), 1.50 (br s, 1H, CH_2N*H*CH_2), 1.02 (s, 12H, 2 × C(CH_3)$_2$). δ_C (100 MHz, $CDCl_3$): 197.8 (C), 173.8 (C), 108.2 (C), 53.2 (CH_2), 47.1 (CH_2), 41.6 (CH_2), 30.4 (C), 29.7 (CH_2), 28.5 (CH_3), 18.2 (CH_3). IR ν cm^{-1}: 2954, 2867, 2958, 1633, 1567. MS (ES+) *m/z*: 460.4 (100%) $[M+H]^+$, 919.8 (18%) $[2M+H]^+$. HRMS (ES+) $C_{26}H_{42}N_3O_4$ calcd: 460.3170. Found: 460.3167 $[M+H]^+$.

5.12. Preparation of N^1,N^9-bis-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-N^5-(4-(allyloxycarbonylmethoxy)-phenyl-methoxycarbonyl)-norspermidine (8)

N^1,N^9-bis-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-norspermidine (3.00 g, 6.5 mmol) and 4′-nitrophenyl-(4-hydroxymethylphenoxy-allylacetate) carbonate (3.03 g, 7.8 mmol) were dissolved in DMF (20 mL) and stirred for 16 h. The reaction mixture was poured into 1 M $KHSO_4$ (100 mL) and extracted with EtOAc (3 × 100 mL). The combined organic extracts were washed with water (5 × 100 mL), dried ($MgSO_4$) and concentrated in vacuo. The resulting oily residue was purified by column chromatography (eluting with EtOAc, then EtOAc/MeOH, 49:1) to give the title compound as a colourless oil (2.61 g, 74%).

R_f (EtOAc/MeOH, 49:1) 0.20. RP-HPLC (analytical, gradient 1, $\lambda = 220$ nm): $R_t = 10.0$ min. δ_H (400 MHz, $CDCl_3$): 13.50 (br s, 2H, 2 × N*H*), 7.28 (d, 2H, $J = 9$, 2 × ArC*H*), 6.86 (d, 2H, $J = 9$, 2 × ArC*H*), 5.91 (ddt, 1H, $J = 17$, 10.5, 5.5, C*H*=CH_2), 5.33 (dd, 1H, $J = 17$, 1, CH=CH*H*), 5.26 (dd, 1H, $J = 10.5$, 1, CH=C*H*H), 5.06 (s, 2H, OCH_2), 4.69 (d, 2H, $J = 5.5$, CH_2CH=CH_2), 4.65 (s, 2H, OCH_2), 3.43–3.30 (m, 8H, 2 × C$H_2$$CH_2CH_2$), 2.55–2.45 (m, 6H, 2 × C=C(CH_3)NH), 2.34 (s, 8H, 4 × CH_2C(CH_3)$_2$), 1.93–1.82 (m, 4H, 2 × CH_2C$H_2$$CH_2$), 1.01 (s, 12H, 2 × C(C$H_3$)$_2$). δ_C (100 MHz, $CDCl_3$): 197.3 (C),173.6 (C), 168.5 (C), 157.8 (C), 156.2 (C), 131.5 (CH), 130.2 (CH), 129.7 (C), 119.3 (CH_2), 114.7 (CH), 108.0 (C), 67.2 (CH_2), 66.0 (CH_2), 65.3 (CH_2), 53.6 (CH_2), 52.3 (CH_2), 45.1 (CH_2), 44.4 (CH_2), 40.7 (CH_2), 30.2 (C), 28.3 (CH_3), 17.9 (CH_3). IR ν cm^{-1}: 2955, 2859, 2958, 1758, 1636, 1570. MS (ES+) *m/z*: 708.2 (100%) $[M+H]^+$. HRMS (ES+) $C_{39}H_{54}N_3O_9$ calcd: 708.3855. Found: 708.3865 $[M+H]^+$.

5.13. Preparation of N^1,N^9-bis-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-N^5-4-((carboxy-methyl)phenyl-methoxy-carbonyl)-norspermidine

A solution of **8** (2.40 g, 3.4 mmol) in DCM/THF (1:1, 21 mL) was purged with N_2 for 1 h. Thiosalicylic acid (1.05 g, 6.8 mmol) and $Pd(PPh_3)_4$ (0.39 g, 0.3 mmol) were added and the reaction mixture stirred for 2 h. Solvent was removed in vacuo and the resulting oily residue purified by column chromatography (eluting with EtOAc, then EtOAc/MeOH, 3:2) to yield the title compound as a colourless oil (1.59 g, 70%).

R_f (EtOAc/MeOH, 2:1) 0.18. RP-HPLC (analytical, gradient 1, $\lambda = 220$ nm): $R_t = 8.5$ min. δ_H (400 MHz, $CDCl_3$): 13.40 (br s, 2H, 2 × N*H*), 7.30 (d, 2H, $J = 8.5$, 2 × ArC*H*), 6.88 (d, 2H, $J = 8.5$, 2 × ArC*H*), 5.08 (s, 2H, OCH_2), 4.67 (s, 2H, OCH_2), 3.43–25 (m, 8H, 2 × C$H_2$$CH_2CH_2$), 2.52–2.33 (m, 14H, 2 × C=C(CH_3)NH, 4 × CH_2C(CH_3)$_2$), 1.96–1.80 (m, 4H, 2 × CH_2C$H_2$$CH_2$), 1.03 (s, 12H, 2 × C(C$H_3$)$_2$). δ_C (100 MHz, $CDCl_3$): 197.3 (C), 172.8 (C), 169.8 (C), 156.9 (C), 155.8 (C), 129.3 (CH), 128.7 (C), 113.6 (CH), 107.0 (C), 66.0 (CH_2), 65.2 (CH_2), 51.7 (CH_2), 42.0 (CH_2), 40.1 (CH_2), 29.1 (C), 27.2 (CH_3), 25.2 (CH_2), 16.9 (CH_3). IR ν cm^{-1}: 2950, 1692, 1561. MS (ES+) *m/z*: 668.5 (100%) $[M+H]^+$, 690.5 (40%) $[M+Na]^+$. HRMS (ES+) $C_{36}H_{50}N_3O_9$ calcd: 668.3542. Found: 668.3563 $[M+H]^+$.

5.14. Preparation of polymer supported N^1,N^9-bis-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-norspermidine (9)

N^1,N^9-bis-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-N^5-4-((carboxy-methyl)phenyl-methoxy-carbonyl)-norspermidine (1.50 g, 2.3 mmol) was coupled to aminomethyl-polystyrene resin (1% DVB cross-linked, 1.11 mmol/g, 1.35 g, 1.5 mmol) according to the general procedure for carboxylic acid coupling with the exceptions that 1.5 equiv of reagents was used and the reaction was shaken for 16 h. The Dde groups of a small portion of resin were removed according to the general procedure and loading, measured by a quantitative Kaiser test, determined to be 1.32 mmol/g of amine (equivalent to 0.66 mmol/g of norspermidine).

IR ν cm^{-1}: 2928, 1697, 1637, 1573, 1452.

5.15. Preparation of N^1,N^9-bis-(L-tryptophanyl-L-arginyl)-norspermidine (4), N^1-L-tryptophanyl-L-arginyl-N^9-L-tryptophanyl-L-arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)-norspermidine (5), N^1,N^9-bis-(L-tryptophanyl-L-arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide))-norspermidine (6)

Compounds **4**–**6** were synthesised from **9** (0.60 g, 0.4 mmol) as outlined in Scheme 1 using the general procedures for solid-phase chemistry. The desired compounds were purified as their TFA salts by semi-prep HPLC (gradient 3). The desired compounds eluted at 22.4 min (**4**, 25 mg, 7%), 33.8 min (**5**, 23 mg, 5%) and 43.5 min (**6**, 28 mg, 5%).

Compound **4**: RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): $R_t = 11.8$ min. δ_H (400 MHz, CD_3OD): 7.71 (d, 2H, $J = 7.5$, 2 × ArC*H*), 7.44 (d, 2H, $J = 7.5$, 2 × ArC*H*), 7.30 (s, 2H, 2 × ArC*H*), 7.19 (t, 2H, $J = 7.5$, 2 × ArC*H*), 7.11 (t, 2H, $J = 7.5$, 2 × ArC*H*), 4.36–4.27 (m, 4H, 2 × Arg αC*H*, 2 × Trp αC*H*), 3.50 (dd, 2H, $J = 15$, 6, 2 × Trp βCH*H*), 3.40–3.17 (m, 10H,

2 × Trp βCH*H*, 2 × Arg δCH_2, 2 × CONHCH_2), 2.99–2.93 (m, 4H, CH_2NH CH_2), 1.94–1.59 (m, 12H, 2 × Arg βCH_2, 2 × Arg γCH_2, 2 × NHCH$_2$CH_2CH$_2$NH). δ_C (100 MHz, CD_3OD): 173.9 (C), 170.2 (C), 158.5 (C), 138.0 (C), 128.1 (C), 125.5 (CH), 122.8 (CH), 120.2 (CH), 119.0 (CH), 112.5 (CH), 107.7 (C), 54.6 (CH), 46.2 (CH_2), 41.7 (CH_2), 36.9 (CH_2), 30.0 (CH_2), 28.5 (CH_2), 27.2 (CH_2), 26.1 (CH_2). MS (ES+) *m/z*: 816.5 (100%) $[M+H]^+$. HRMS (ES+) $C_{40}H_{62}N_{15}O_4$ calcd: 816.5103. Found: 816.5103 $[M+H]^+$.

Compound **5**: RP-HPLC (analytical, gradient 2, λ = 220 nm): R_t = 14.2 min. δ_H (400 MHz, CD_3OD): 7.67 (d, 2H, *J* = 7.5, 2 × ArC*H*), 7.40 (d, 2H, *J* = 7.5, 2 × ArC*H*), 7.26 (s, 2H, 2 × ArC*H*), 7.15 (t, 2H, *J* = 7.5, 2 × ArC*H*), 7.06 (t, 2H, *J* = 7.5, 2 × ArC*H*), 4.40 (br s, 1H, Arg αC*H*), 4.33–4.22 (m, 3H, Arg αC*H*, 2 × Trp αC*H*), 3.50–3.42 (m, 2H, 2 × Trp βCH*H*), 3.31–3.11 (m, 10H, 2 × CONHCH_2, 2 × Trp βCH*H*, 2 × Arg δCH_2), 2.98–2.92 (m, 4H, CH_2NHCH_2), 2.65 (t, 2H, *J* = 6.5, ArCH_2), 2.59 (s, 3H, ArCH_3), 2.57 (s, 3H, ArCH_3), 2.11 (s, 3H, ArCH_3), 1.90–1.54 (m, 14H, 2 × Arg βCH_2, 2 × Arg γCH_2, 2 × NHCH$_2$CH_2CH$_2$NH, ArCH$_2$CH_2), 1.31 (s, 6H, C(CH_3)$_2$). δ_C (100 MHz, CD_3OD): 174.5 (C), 174.2 (C), 170.4 (C), 170.1 (C), 158.7 (C), 154.9 (C), 138.2 (C), 136.6 (C), 136.2 (C), 134.3 (C), 128.3 (C), 125.7 (CH), 125.1 (C), 123.0 (CH), 120.4 (CH), 119.5 (C), 119.2 (CH), 112.6 (CH), 107.9 (C), 74.9 (C), 54.8 (CH), 54.5 (CH), 46.5 (CH_2), 46.5 (CH_2), 41.9 (CH_2), 37.1 (CH_2), 33.7 (CH_2), 30.3 (CH_2), 30.2 (CH_2), 28.7 (CH_2), 27.4 (CH_2), 27.4 (CH_2), 26.9 (CH_3), 26.3 (CH_2), 22.3 (CH_2), 19.0 (CH_3), 17.9 (CH_3), 12.3 (CH_3). MS (ES+) *m/z*: 542.2 (100%) $[M+2H]^{2+}$, 1082.9 (13%) $[M+H]^+$.

Compound **6**: RP-HPLC (analytical, gradient 2, λ = 220 nm): R_t = 16.2 min. δ_H (400 MHz, CD_3OD): 7.63 (d, 2H, *J* = 8, 2 × ArC*H*), 7.36 (d, 2H, *J* = 8, 2 × ArC*H*), 7.23 (s, 2H, 2 × ArC*H*), 7.11 (t, 2H, *J* = 8, 2 × ArC*H*), 7.03 (t, 2H, *J* = 8, 2 × ArC*H*), 4.39 (br s, 2H, 2 × Arg αC*H*), 4.22 (m, 2H, 2 × Trp αC*H*), 3.43 (dd, 2H, *J* = 15, 6, 2 × Trp βCH*H*), 3.33–3.16 (m, 8H, 2 × Trp βCH*H*, 2 × Arg δCH*H*, 2 × CONHCH_2), 3.13–3.07 (m, 2H, 2 × Arg δCH*H*), 2.98–2.94 (m, 4H, CH_2NHCH_2), 2.62 (t, 4H, *J* = 6.5, 2 × ArCH_2CH$_2$), 2.56 (s, 6H, 2 × ArCH_3), 2.54 (s, 6H, 2 × ArCH_3), 2.08 (s, 6H, 2 × ArCH_3), 1.90–1.75 (m, 10H, 2 × NHCH$_2$CH_2CH$_2$-NH, 2 × ArCH$_2$CH_2, 2 × Arg βCH*H*), 1.74–1.63 (m, 2H, 2 × Arg βCH*H*), 1.60–1.51 (m, 4H, 2 × Arg γCH_2), 1.29 (s, 12H, 2 × C(CH_3)$_2$). δ_C (100 MHz, CD_3OD): 174.4 (C), 170.2 (C),158.2 (C), 155.0 (C), 138.2 (C), 136.5 (C), 136.1 (C), 134.3 (C), 128.4 (C), 125.7 (CH), 125.2 (C), 122.9 (CH), 120.4 (CH), 119.5 (C), 119.2 (CH), 112.6 (CH), 107.9 (C), 75.0 (C), 54.8 (CH), 54.5 (CH), 46.6 (CH_2), 37.1 (CH_2), 33.7 (CH_2), 30.3 (CH_2), 28.8 (CH_2), 27.5 (CH_2), 26.9 (CH_3), 22.4 (CH_2), 19.1 (CH_3), 18.0 (CH_3), 12.3 (CH_3). MS (ES+) *m/z*: 675.4 (100%) $[M+2H]^{2+}$, 1349.0 (22%) $[M+H]^+$.

5.16. Library characterisation

All compounds were characterised by ^{1}H NMR, LRMS, HRMS and RP-HPLC. A representative sample (approximately 40%) of library compounds was also characterised by ^{13}C NMR.

5.17. Preparation of N^1,N^9-bis-(L-tryptophanyl)-norspermidine (10)

Compound **10** was synthesised from **9** (0.20 g, 0.13 mmol) as outlined in Scheme 1 using the general procedures for solid-phase chemistry. The desired compound was purified as the TFA salt by semi-prep HPLC (gradient 3), elution time = 21.4 min (17 mg, 21%).

RP-HPLC (analytical, gradient 2, λ = 220 nm): R_t = 12.3 min. δ_H (400 MHz, CD_3OD): 7.62 (d, 2H, *J* = 7.5, 2 × ArC*H*), 7.40 (d, 2H, *J* = 7.5, 2 × ArC*H*), 7.22 (s, 2H, 2 × ArC*H*), 7.16 (t, 2H, *J* = 7.5, 2 × ArC*H*), 7.08 (t, 2H, *J* = 7.5, 2 × ArC*H*), 4.11 (t, 2H, *J* = 7.5, 2 × Trp αC*H*), 3.42–3.16 (m, 8H, 2 × Trp βCH_2, 2 × CONHCH_2), 2.65 (t, 4H, *J* = 7, CH_2NH CH_2), 1.71 (tt, 4H, *J* = 7, 7, 2 × CH$_2$CH_2CH$_2$). δ_C (100 MHz, CD_3OD): 170.9 (C), 138.2 (C), 128.4 (CH), 125.5 (CH), 123.0 (CH), 120.3 (CH), 119.1 (CH), 112.7 (C), 108.1 (C), 55.3 (CH), 46.2 (CH_2), 37.4 (CH_2), 28.8 (CH_2), 27.1 (CH_2). MS (ES+) *m/z*: 504.4 (100%) $[M+H]^+$, 526.4 (11%) $[M+Na]^+$. HRMS (ES+) $C_{28}H_{38}N_7O_2$ calcd: 504.3082. Found: 504.3087 $[M+H]^+$.

5.18. Preparation of N^1-L-arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)-N^9-L-arginyl-norspermidine (11) and N^1,N^9-bis-(L-arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide))-norspermidine (12)

Compounds **11** and **12** were synthesised from **9** (0.40 g, 0.26 mmol) as outlined in Scheme 1 using the general procedures for solid-phase chemistry. The desired compounds were purified as the TFA salts by semi-prep HPLC (gradient 3), elution time = 31.0 min (**11**, 14 mg, 7%) and 40.7 min (**12**, 18 mg, 6%).

Compound **11**: RP-HPLC (analytical, gradient 2, λ = 220 nm): R_t = 13.3 min. δ_H (400 MHz, CD_3OD): 3.97–3.87 (m, 2H, 2 × Arg αC*H*), 3.50–3.16 (m, 8H, 2 × Arg δCH_2, 2 × CONHCH_2), 3.10 (t, 4H, *J* = 7.5, CH_2NHCH_2), 2.69 (t, 2H, *J* = 7, ArCH_2CH$_2$), 2.59 (s, 3H, ArCH_3), 2.58 (s, 3H, ArCH_3), 2.12 (s, 3H, ArCH_3), 2.03–1.83 (m, 10H, 2 × Arg βCH_2, 2 × NHCH$_2$CH_2CH$_2$NH, ArCH$_2$CH_2), 1.74–1.61 (m, 4H, 2 × Arg γCH_2), 1.33 (s, 6H, C(CH_3)$_2$). MS (ES+) *m/z*: 356.1 (100%) $[M+2H]^{2+}$, 710.7 (23%) $[M+H]^+$. HRMS (ES+) $C_{32}H_{60}N_{11}O_5S$ calcd: 710.4494. Found: 710.4500 $[M+H]^+$.

Compound **12**: RP-HPLC (analytical, gradient 2, λ = 220 nm): R_t = 15.5 min. δ_H (400 MHz, CD_3OD): 3.96 (t, 2H, *J* = 6.5, 2 × Arg αC*H*), 3.50–3.08 (m, 12H, 2 × NHCH_2CH$_2$CH_2NH, 2 × Arg δCH_2), 2.67 (t, 4H, *J* = 7, 2 × ArCH_2CH$_2$), 2.58 (s, 6H, 2 × ArCH_3), 2.56 (s, 6H, 2 × ArCH_3), 2.11 (s, 6H, 2 × ArCH_3), 2.03–1.81 (m, 12H, 2 × Arg βCH_2, 2 × ArCH$_2$CH_2, 2 × NHCH$_2$CH_2-CH$_2$NH), 1.64 (tt, 4H, *J* = 7, 7, 2 × Arg γCH_2), 1.32 (s, 12H, 2 × C(CH_3)$_2$). MS (ES+) *m/z*: 489.1 (100%) $[M+2H]^{2+}$, 998.6 (12%) $[M+Na]^+$. HRMS (ES+) $C_{46}H_{78}N_{11}O_8S_2$ calcd: 976.5471. Found: 976.5475 $[M+H]^+$.

5.19. Preparation of 17–25

Compounds **17**–**25** were synthesised from **13** (0.20 g, 0.1 mmol) as outlined in Scheme 2 using the general procedures for solid-phase chemistry.

5.20. N^1-L-Tryptophanyl-norspermidine (17)

The desired compound was isolated as the TFA salt by semi-prep HPLC (gradient 3), elution time = 20.8 min (7 mg, 16%).

RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 10.8 min. δ_H (400 MHz, CD_3OD): 7.63 (d, 1H, $J = 8$, ArC*H*), 7.41 (d, 1H, $J = 8$, ArC*H*), 7.23 (s, 1H, ArC*H*), 7.17 (t, 1H, $J = 8$, ArC*H*), 7.09 (t, 1H, $J = 8$, ArC*H*), 4.11 (t, 1H, $J = 7.5$, Trp αC*H*), 3.44–3.17 (m, 4H, Trp βCH_2, CONHCH_2), 3.10–2.98 (m, 4H, CH_2CH$_2$CH_2-NH$_2$), 2.81 (t, 2H, $J = 7.5$, CONHCH$_2$CH$_2$CH_2), 2.13–2.02 (m, 2H, CH_2CH$_2$NH$_2$), 1.84–1.73 (m, 2H, CONHCH$_2$CH_2). MS (ES+) *m*/*z*: 318.2 (100%) $[M+H]^+$. HRMS (ES+) $C_{17}H_{28}N_5O$ calcd: 318.2288; (found) 318.2287 $[M+H]^+$.

5.21. N^1-L-Tryptophanyl-N^9-acetyl-norspermidine (18)

The desired compound was isolated as the TFA salt by semi-prep HPLC (gradient 3), elution time = 22.4 min (16 mg, 34%).

RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 11.2 min. δ_H (400 MHz, CD_3OD): 7.63 (d, 1H, $J = 8$, ArC*H*), 7.42 (d, 1H, $J = 8$, ArC*H*), 7.24 (s, 1H, ArC*H*), 7.15 (t, 1H, $J = 8$, ArC*H*), 7.09 (t, 1H, $J = 8$, ArC*H*), 4.13 (t, 1H, $J = 7.5$, Trp αC*H*), 3.39 (dd, 1H, $J = 14.5$, 7.5, Trp βCH*H*), 3.35–3.21 (m, 5H, Trp βC*H*H, 2 × CONHCH_2), 2.88 (t, 2H, $J = 7$, CH_2NHCH$_2$), 2.69 (t, 2H, $J = 7$, CH_2NHCH$_2$), 1.99 (s, 3H, CH_3), 1.85 (tt, 2H, $J = 7$, 7, CH$_2$CH_2CH$_2$), 1.75 (tt, 2H, $J = 7$, 7, CH$_2$CH_2CH$_2$). δ_C (100 MHz, CD_3OD): 174.4 (C), 170.8 (C), 138.2 (C), 128.4 (C), 125.5 (CH), 122.9 (CH), 119.2 (CH), 112.7 (CH), 108.2 (C), 55.3 (CH), 46.4 (CH_2), 46.1 (CH_2), 37.3 (CH_2), 36.9 (CH_2), 28.7 (CH_2), 27.6 (CH_2), 27.1 (CH_2), 22.5 (CH_3). MS (ES+) *m*/*z*: 360.2 (100%) $[M+H]^+$, 382.2 (25%) $[M+Na]^+$. HRMS (ES+) $C_{19}H_{30}N_5O_2$ calcd: 360.2394. Found: 360.2392 $[M+H]^+$.

5.22. N^1-L-Tryptophanyl-N^9-hexanoyl-norspermidine (19)

The desired compound was purified as the TFA salt by semi-prep HPLC (gradient 3), elution time = 28.9 min (11 mg, 21%).

RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 13.0 min. δ_H (400 MHz, CD_3OD): 7.51 (d, 1H, $J = 8$, ArC*H*), 7.29 (d, 1H, $J = 8$, ArC*H*), 7.12 (s, 1H, ArC*H*), 7.03 (t, 1H, $J = 8$, ArC*H*), 6.97 (t, 1H, $J = 8$, ArC*H*), 3.99 (t, 1H, $J = 7.5$, Trp αC*H*), 3.32–3.08 (m, 6H, Trp βCH_2, 2 × CONHCH_2), 2.76 (t, 2H, $J = 7$, CH$_2$NHCH_2), 2.59 (t, 2H, $J = 7$, CH$_2$NHCH_2), 2.11 (t, 2H, $J = 7.5$, COCH_2), 1.78–1.47 (m, 6H, COCH$_2$CH_2, 2 × NHCH$_2$-CH_2), 1.29–1.14 (m, 4H, CH_2CH_2CH$_3$), 0.81 (t, 3H, $J = 7$, CH_3). MS (ES+) *m*/*z*: 416.3 (100%) $[M+H]^+$. HRMS (ES+) $C_{23}H_{38}N_5O_2$ calcd: 416.3020. Found: 416.3025 $[M+H]^+$.

5.23. N^1-L-Arginyl-norspermidine (20) (17 mg, 59%)

δ_H (400 MHz, CD_3OD): 4.11 (q, 1H, $J = 6.5$, Arg αC*H*), 3.57–3.14 (m, 10H, Arg δCH_2, CH_2CH$_2$CH_2NHCH_2-CH$_2$CH_2), 2.29–1.99 (m, 6H, Arg βCH_2, CH$_2$CH_2-CH$_2$NHCH$_2$CH_2CH$_2$), 1.84–1.74 (m, 2H, Arg γCH_2). δ_C (100 MHz, CD_3OD): 170.7 (C), 158.1 (C), 53.9 (CH), 46.3 (CH_2), 45.7 (CH_2), 41.4 (CH_2), 37.6 (CH_2), 37.4 (CH_2), 29.1 (CH_2), 26.6 (CH_2), 26.3 (CH_2), 24.9 (CH_2). MS (ES+) *m*/*z*: 288.4 (100%) $[M+H]^+$. HRMS (ES+) calcd: 288.2506. Found: 288.2506 $[M+H]^+$.

5.24. N^1-L-Arginyl-N^9-acetyl-norspermidine (21) (23 mg, 70%)

δ_H (400 MHz, CD_3OD): 3.97 (br s, 1H, Arg αC*H*), 3.46–3.23 (m, 6H, Arg δCH_2, 2 × CONHCH_2), 3.11–3.01 (m, 4H, CH_2NHCH_2), 2.05–1.87 (m, 9H, Arg βCH_2, 2 × NHCH$_2$CH_2CH$_2$NH, CH_3), 1.77–1.67 (m, 2H, Arg γCH_2). δ_C (100 MHz, CD_3OD): 174.2 (C), 170.7 (C), 158.7 (C), 54.0 (CH), 46.6 (CH_2), 41.5 (CH_2), 37.4 (CH_2), 37.1 (CH_2), 29.6 (CH_2), 27.5 (CH_2), 27.2 (CH_2), 25.3 (CH_2), 22.5 (CH_3). MS (ES+) *m*/*z*: 330.4 (100%) $[M+H]^+$, 352.4 (8%) $[M+Na]^+$. HRMS (ES+) $C_{14}H_{32}N_7O_2$ calcd: 330.2612. Found: 330.2605 $[M+H]^+$.

5.25. N^1-L-Arginyl-N^9-hexanoyl-norspermidine (22) (28 mg, 73%)

δ_H (400 MHz, CD_3OD): 3.98 (br s, 1H, Arg αC*H*), 3.48–3.24 (m, 6H, Arg δCH_2, 2 × CONHCH_2), 3.12–3.05 (m, 4H, CH_2NHCH_2), 2.25 (t, 2H, $J = 7.5$, COCH_2), 2.08–1.89 (m, 6H, Arg βCH_2, 2 × NHCH$_2$CH_2CH$_2$NH), 1.79–1.58 (m, 4H, Arg γCH_2, COCH$_2$CH_2), 1.42–1.29 (m, 4H, CH$_3$CH_2CH_2), 0.95 (t, 3H, $J = 7$, CH_3). δ_C (100 MHz, CD_3OD): 177.3 (C), 170.7 (C), 158.8 (C), 54.0 (CH), 46.6 (CH_2), 46.5 (CH_2), 41.4 (CH_2), 37.4 (CH_2), 37.0 (CH_2), 32.6 (CH_2), 29.6 (CH_2), 27.6 (CH_2), 27.2 (CH_2), 26.7 (CH_2), 25.3 (CH_2), 23.4 (CH_2), 14.3 (CH_3). MS (ES+) *m*/*z*: 386.5 (100%) $[M+H]^+$.

5.26. N^1-L-Arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)-norspermidine (23)

The desired compound was purified as the TFA salt by semi-prep HPLC (gradient 3), elution time = 32.4 min (13 mg, 19%).

RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 13.7 min. δ_H (400 MHz, CD_3OD): 3.98 (t, 1H, $J = 6.5$, Arg αC*H*), 3.52–3.32 (m, 4H, Arg δCH_2, CONHCH_2), 3.23–3.08 (m, 6H, H$_2$NCH_2CH$_2$CH_2NHCH_2), 2.72 (t, 2H, $J = 6.5$, ArCH_2), 2.63 (s, 3H, ArCH_3), 2.61 (s, 3H, ArCH_3), 2.18–2.09 (m, 5H, ArCH_3, NHCH$_2$CH_2CH$_2$NH), 2.08–1.86 (m, 6H, Arg βCH_2, NHCH$_2$CH_2CH$_2$NH, ArCH$_2$CH_2), 1.67 (tt, 2H, $J = 7$, 7, Arg γCH_2), 1.36 (s, 6H, C(CH_3)$_2$). MS (ES+) *m*/*z*: 554.4 (100%) $[M+H]^+$, 576.4 (70%) $[M+Na]^+$. HRMS (ES+) $C_{26}H_{48}N_7O_4S$ calcd: 554.3483. Found: 554.3498 $[M+H]^+$.

5.27. N^1-L-Arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)-N^9-acetyl-norspermidine (24)

The desired compound was purified as the TFA salt by semi-prep HPLC (gradient 3), elution time = 34.3 min (15 mg, 21%).

RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 14.1 min. δ_H (400 MHz, CD_3OD): 3.99 (t, 1H, $J = 6.5$, Arg αC*H*), 3.50–3.19 (m, 6H, 2 × CONHCH_2, Arg δCH_2), 3.12–2.95 (m, 4H, CH_2NHCH_2), 2.73 (t, 2H, $J = 7$, ArCH_2), 2.62 (s, 3H, ArCH_3), 2.60 (s, 3H, ArCH_3), 2.15 (s, 3H, ArCH_3), 2.04–1.86 (m, 11H, COCH_3, ArCH$_2$CH_2, Arg βCH_2, 2 × NHCH$_2$CH_2CH$_2$NH), 1.68 (t, 2H, $J = 7$, Arg γCH_2), 1.36 (s, 6H, C(CH_3)$_2$). MS (ES+) *m/z*: 596.5 (100%) $[M+H]^+$, 618.5 (35%) $[M+Na]^+$. HRMS (ES+) $C_{28}H_{50}N_7O_5S$ calcd: 596.3589. Found: 596.3581 $[M+H]^+$.

5.28. N^1-L-Arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)-N^9-hexanoyl-norspermidine (25)

The desired compound was purified as the TFA salt by semi-prep HPLC (gradient 3), elution time = 39.3 min (8 mg, 10%).

RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 15.0 min. δ_H (400 MHz, CD_3OD): 4.01 (t, 1H, $J = 6.5$, Arg αC*H*), 3.54–3.18 (m, 6H, Arg δCH_2, 2 × CONHCH_2), 3.13–3.05 (m, 4H, CH_2NHCH_2), 2.72 (t, 2H, $J = 6.5$, ArCH_2), 2.62 (s, 3H, ArCH_3), 2.61 (s, 3H, ArCH_3), 2.24 (t, 2H, $J = 7.5$, COCH_2), 2.15 (s, 3H, ArCH_3), 2.07–1.86 (m, 8H, Arg βCH_2, ArCH$_2$CH_2, 2 × NHCH$_2$CH_2CH$_2$NH), 1.73–1.59 (m, 4H, COCH$_2$CH_2, Arg γCH_2), 1.40–1.30 (m, 10H, CH$_3$CH_2CH_2, C(CH_3)$_2$), 0.95 (t, 3H, $J = 7$, CH_3CH$_2$). MS (ES+) *m/z*: 652.5 (100%) $[M+H]^+$, 674.5 (90%) $[M+Na]^+$, 326.9 (70%) $[M+2H]^{2+}$. HRMS (ES+) $C_{32}H_{58}N_7O_5S$ calcd: 652.4215. Found: 652.4213 $[M+H]^+$.

5.29. Preparation of 26–31

Compounds **26–31** were synthesised from **13** (0.40 g, 0.2 mmol) as outlined in Scheme 2 using the general procedures for solid-phase chemistry.

5.30. N^1-L-Tryptophanyl-L-arginyl-norspermidine (26) and N^1-L-tryptophanyl-L-arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)-norspermidine (27)

The desired compounds were purified as the TFA salts by semi-prep HPLC (gradient 3), elution time = 18.2 min (**26**, 7 mg, 6%) and 35.6 min (**27**, 11 mg, 6%).

Compound **26**: RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 10.8 min. δ_H (400 MHz, CD_3OD): 7.69 (d, 1H, $J = 7.5$, ArC*H*), 7.41 (d, 1H, $J = 7.5$, ArC*H*), 7.27 (s, 1H, ArC*H*), 7.17 (t, 1H, $J = 7.5$, ArC*H*), 7.09 (t, 1H, $J = 7.5$, ArC*H*), 4.36–4.23 (m, 2H, Arg αC*H*, Trp αC*H*), 3.49 (dd, 1H, $J = 15$, 6, Trp βCH*H*), 3.38–2.97 (m, 11H, Trp βCH*H*, Arg δCH_2, NHCH_2CH$_2$CH_2NHCH_2 CH$_2$CH_2NH$_2$), 2.13–2.05 (m, 2H, CH_2CH$_2$NH$_2$), 1.93–1.56 (m, 6H, Arg βCH_2, Arg γCH_2, NHCH$_2$CH_2CH$_2$NH). MS (ES+) *m/z*: 474.4 (100%) $[M+H]^+$. HRMS (ES+) $C_{23}H_{40}N_9O_2$ calcd: 474.3299. Found: 474.3291 $[M+H]^+$.

Compound **27**: RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 14.2 min. δ_H (400 MHz, CD_3OD): 7.67 (d, 1H, $J = 8$, ArC*H*), 7.39 (d, 1H, $J = 8$, ArC*H*), 7.25 (s, 1H, ArC*H*), 7.15 (t, 1H, $J = 8$, ArC*H*), 7.07 (t, 1H, $J = 8$, ArC*H*), 4.44 (br s, 1H, Arg αC*H*), 4.23 (dd, 1H, $J = 8$, 6, Trp αC*H*), 3.46 (dd, 1H, $J = 15$, 6, Trp βCH*H*), 3.38–3.19 (m, 3H, Trp βCH*H*, CONHCH_2), 3.17–3.00 (m, 8H, Arg γCH_2, CH_2NHCH_2CH$_2$CH_2), 2.68 (t, 2H, $J = 6.5$, ArCH_2), 2.59 (s, 3H, ArCH_3), 2.57 (s, 3H, ArCH_3), 2.13–2.04 (m, 5H, ArCH_3, CH_2CH$_2$NH$_2$), 1.95–1.79 (m, 5H, Arg βCH*H*, ArCH$_2$CH_2, NHCH$_2$CH_2CH$_2$NH), 1.77–1.55 (m, 3H, Arg βCH*H*, Arg γCH_2), 1.31 (s, 6H, C(CH_3)$_2$). MS (ES+) *m/z*: 740.5 (100%) $[M+H]^+$, 370.9 (90%) $[M+2H]^{2+}$. HRMS (ES+) $C_{37}H_{58}N_9O_5S$ calcd: 740.4276. Found: 740.4285 $[M+H]^+$.

5.31. N^1-L-Tryptophanyl-L-arginyl-N^9-acetyl-norspermidine (28) and N^1-L-tryptophanyl-L-arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)-N^9-acetyl-norspermidine (29)

The desired compounds were purified as the TFA salts by semi-prep HPLC (gradient 3), elution time = 19.4 min (**28**, 16 mg, 13%) and 37.0 min (**29**, 21 mg, 12%).

Compound **28**: RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 11.1 min. δ_H (400 MHz, CD_3OD): 7.66 (d, 1H, $J = 8$, ArC*H*), 7.39 (d, 1H, $J = 8$, ArC*H*), 7.26 (s, 1H, ArC*H*), 7.15 (t, 1H, $J = 8$, ArC*H*), 7.06 (t, 1H, $J = 8$, ArC*H*), 4.30–4.24 (m, 2H, Arg αC*H*, Trp αC*H*), 3.45 (dd, 1H, $J = 15$, 6.5, Trp βCH*H*), 3.38–3.16 (m, 7H, Trp βCH*H*, 2 × CONHCH_2, Arg δCH_2), 2.96–2.88 (m, 4H, CH_2NHCH_2), 1.95 (s, 3H, CH_3CO), 1.90–1.80 (m, 4H, 2 × NHCH$_2$CH_2CH$_2$NH), 1.78–1.57 (m, 4H, Arg βCH_2, Arg γCH_2). δ_C (100 MHz, CD_3OD): 174.3 (C), 174.2 (C), 170.4 (C), 158.7 (C), 138.1 (C), 128.4 (C), 125.8 (CH), 122.9 (CH), 120.4 (CH), 119.2 (CH), 112.6 (CH), 107.9 (C), 54.9 (CH), 54.8 (CH), 46.5 (CH_2), 46.3 (CH_2), 41.9 (CH_2), 37.0 (CH_2), 36.9 (CH_2), 30.1 (CH_2), 28.7 (CH_2), 27.6 (CH_2), 27.4 (CH_2), 26.3 (CH_2), 22.5 (CH_3). MS (ES+) *m/z*: 258.9 (100%) $[M+2H]^{2+}$, 516.5 (46%) $[M+H]^+$. HRMS (ES+) $C_{25}H_{42}N_9O_3$ calcd: 516.3405. Found: 516.3410 $[M+H]^+$.

Compound **29**: RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): R_t = 14.7 min. δ_H (400 MHz, CD_3OD): 7.70 (d, 1H, $J = 8$, ArC*H*), 7.42 (d, 1H, $J = 8$, ArC*H*), 7.29 (s, 1H, ArC*H*), 7.18 (t, 1H, $J = 8$, ArC*H*), 7.10 (t, 1H, $J = 8$, ArC*H*), 4.46 (br s, 1H, Arg αC*H*), 4.26 (t, 1H, $J = 7$, Trp αC*H*), 3.49 (dd, 1H, $J = 15$, 6, Trp βCH*H*), 3.37–3.23 (m, 5H, Trp βCH*H*, 2 × CONHCH_2), 3.20–3.12 (m, 2H, Arg δCH_2), 3.05–2.97 (m, 4H, CH_2NHCH_2), 2.71 (t, 2H, $J = 6.5$, ArCH_2CH$_2$), 2.62 (s, 3H, ArCH_3), 2.61 (s, 3H, ArCH_3), 2.14 (s, 3H, ArCH_3), 1.98 (s, 3H, COCH_3), 1.92–1.82 (m, 6H, 2 × NHCH$_2$ CH_2CH$_2$NH, ArCH$_2$CH_2), 1.80–1.69 (m, 2H, Arg βCH_2), 1.67–1.58 (m, 2H, Arg γCH_2), 1.35 (s,

6H, C(CH_3)$_2$). δ_C (100 MHz, CD_3OD): 174.5 (C), 174.3 (C), 170.2 (C), 158.6 (C), 154.9 (C), 138.3 (C), 136.5 (C), 136.1 (C), 134.4 (C), 128.4 (C), 125.7 (CH), 125.1 (C), 123.0 (CH), 119.5 (CH), 119.2 (C), 119.0 (CH), 112.6 (CH), 107.9 (C), 75.0 (C), 54.8 (CH), 54.4 (CH), 46.6 (CH_2), 46.5 (CH_2), 37.0 (CH_2), 33.8 (CH_2), 30.3 (CH_2), 28.8 (CH_2), 27.7 (CH_2), 27.5 (CH_2), 26.9 (CH_3), 22.5 (CH_2), 22.4 (CH_3), 19.0 (CH_3), 17.9 (CH_3), 12.3 (CH_3). MS (ES+) *m/z*: 782.7 (100%) $[M+H]^+$, 804.7 (55%) $[M+Na]^+$. HRMS (ES+) $C_{39}H_{60}N_9O_6S$ calcd: 782.4382. Found: 782.4357 $[M+H]^+$.

5.32. N^1-L-Tryptophanyl-L-arginyl-N^9-hexanoyl-norspermidine (30) and N^1-L-tryptophanyl-L-arginyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)-N^9-hexanoyl-norspermidine (31)

The desired compounds were purified as the TFA salts by semi-prep HPLC (gradient 3), elution time = 26.6 min (**30**, 14 mg, 10%) and 41.7 min (**31**, 14 mg, 7%).

Compound **30**: RP-HPLC (analytical, gradient 2, λ = 220 nm): R_t = 12.8 min. δ_H (400 MHz, CD_3OD): 7.70 (d, 1H, *J* = 8, ArC*H*), 7.42 (d, 1H, *J* = 8, ArC*H*), 7.28 (s, 1H, ArC*H*), 7.18 (t, 1H, *J* = 8, ArC*H*), 7.10 (t, 1H, *J* = 8, ArC*H*), 4.33 (dd, 1H, *J* = 8, 6, Arg αC*H*), 4.27 (dd, 1H, *J* = 8, 6, Trp αC*H*), 3.49 (dd, 1H, *J* = 15, 6, Trp βC*H*H), 3.38–3.20 (m, 7H, Trp βCH*H*, Arg δCH_2, 2 × CONHCH_2), 3.03–2.90 (m, 4H, CH_2NHCH_2), 2.22 (t, 2H, *J* = 7.5, COCH_2), 1.90–1.59 (m, 10H, Arg βCH_2, Arg γCH_2, 2 × NHCH$_2$CH_2CH$_2$ NH, CH_2-CH$_2$CO), 1.39–1.27 (m, 4H, CH$_3$CH_2CH_2), 0.92 (t, 3H, *J* = 7, CH_3CH$_2$). MS (ES+) *m/z*: 286.9 (100%) $[M+2H]^{2+}$, 572.4 (70%) $[M+H]^+$, 594.4 (15%) $[M+Na]^+$. HRMS (ES+) $C_{29}H_{50}N_9O_3$ calcd: 572.4031. Found: 572.4041 $[M+H]^+$.

Compound **31**: RP-HPLC (analytical, gradient 2, λ = 220 nm): R_t = 15.5 min. δ_H (400 MHz, CD_3OD): 7.71 (d, 1H, *J* = 8, ArC*H*), 7.44 (d, 1H, *J* = 8, ArC*H*), 7.29 (s, 1H, ArC*H*), 7.19 (t, 1H, *J* = 8, ArC*H*), 7.11 (t, 1H, *J* = 8, ArC*H*), 4.50 (br s, 1H, Arg αC*H*), 4.26 (t, 1H, *J* = 7, Trp αC*H*), 3.49 (dd, 1H, *J* = 15, 6, Trp βC*H*H), 3.39–3.14 (m, 7H, Trp βCH*H*, Arg δCH_2, 2 × CONHCH_2), 3.09–2.97 (m, 4H, CH_2NHCH_2), 2.72 (t, 2H, *J* = 7, ArCH_2CH$_2$), 2.63 (s, 3H, ArCH_3), 2.61 (s, 3H, ArCH_3), 2.22 (t, 2H, *J* = 7.5, COCH_2), 2.15 (s, 3H, ArCH_3), 1.97–1.83 (m, 6H, ArCH$_2$CH_2, 2 × NHCH$_2$CH_2CH$_2$NH), 1.82–1.71 (m, 2H, Arg βCH_2), 1.70–1.57 (m, 4H, CH_2CH$_2$CO, Arg γCH_2), 1.39–1.28 (m, 10H, C(CH_3)$_2$, CH$_3$CH_2CH_2), 0.94 (t, 3H, *J* = 7, CH_3CH$_2$). MS (ES+) *m/z*: 420.0 (100%) $[M+2H]^{2+}$, 860.7 (50%) $[M+Na]^+$, 838.7 (45%) $[M+H]^+$. HRMS (ES+) $C_{43}H_{68}N_9O_6S$ calcd: 838.5008. Found: 838.5002 $[M+H]^+$.

5.33. Preparation of Boc-L-tryptophanyl-(Boc)-N^G-2,2,5,7,8-pentamethylchroman-6-sulfonamide-L-arginine

To a stirred solution of Boc-Trp(Boc)-OH (0.25 g, 0.6 mmol) and *N*-hydroxy succinimide (0.71 g, 0.6 mmol) in THF (5 mL) at 0 °C was added *N*,*N*′-dicyclohexylcarbodiimide (0.13 g, 0.6 mmol). After 2 h, the reaction mixture was filtered and the filtrate concentrated in vacuo to yield a white foam. The crude product was dissolved in DMF (5 mL) and a solution of H-Arg(Pmc)-OH (0.27 g, 0.6 mmol) and NEt_3 (86 μL, 0.6 mmol) in DMF (2 mL) was added. The reaction mixture was stirred for 16 h and concentrated in vacuo. The residue was taken into water (50 mL), extracted with EtOAc (4 × 50 mL), the organic extracts combined, dried ($MgSO_4$) and concentrated in vacuo to yield the title compound as a white solid (0.39 g, 77%).

R_f (EtOAc/hexane, 1:1) 0.37. Mp 203–206 °C. RP-HPLC (analytical, gradient 1, λ = 220 nm): R_t = 12.0 min. δ_H (400 MHz, CD_3OD): 8.05 (d, 1H, *J* = 7.5, ArC*H*), 7.59 (d, 1H, *J* = 7.5, ArC*H*), 7.47 (s, 1H, ArC*H*), 7.24 (t, 1H, *J* = 7.5, ArC*H*), 7.17 (t, 1H, *J* = 7.5, ArC*H*), 4.41–4.32 (m, 2H, Arg αC*H*, Trp αC*H*), 3.22–3.10 (m, 3H, Trp βCH*H*, Arg δCH_2), 2.92 (dd, 1H, *J* = 14.5, 9, Trp βCH*H*), 2.61 (t, 2H, *J* = 7, ArCH_2), 2.53 (s, 3H, ArCH_3), 2.51 (s, 3H, ArCH_3), 2.06 (s, 3H, ArCH_3), 1.90–1.73 (m, 3H, Arg βCH*H*, ArCH$_2$CH_2), 1.70–1.58 (m, 10H, Arg βCH*H*, CO$_2$C(CH_3)$_3$), 1.55–1.45 (m, 2H, Arg γCH_2), 1.34–1.23 (m, 15H, CO$_2$C(CH_3)$_3$, C(CH_3)$_2$). δ_C (100 MHz, CD_3OD): 174.7 (C), 174.3 (C), 157.6 (C), 154.7 (C), 151.0 (C), 136.8 (C), 136.5 (C), 136.1 (C), 134.7 (C),131.8 (C), 125.4 (CH), 125.0 (C), 123.6 (CH), 120.1 (CH), 119.3 (C), 117.5 (C), 116.0 (CH), 84.6 (C), 80.7 (C), 74.8 (C), 55.9 (CH), 53.2 (CH), 41.5 (CH_2), 34.7 (CH_2), 33.8 (CH_2), 30.1 (CH_2), 28.7 (CH_3), 28.4 (CH_3), 27.0 (CH_3), 22.3 (CH_2), 19.0 (CH_3), 17.9 (CH_3), 12.3 (CH_3). IR ν cm^{-1}: 3300, 1728, 1547, 1453, 1368, 1252, 1157. MS (ES+) *m/z*: 827.5 (100%) $[M+H]^+$, 849.5 (92%) $[M+Na]^+$. HRMS (ES+) $C_{41}H_{58}N_6O_{10}SNa$ calcd: 849.4008. Found: 849.3797 $[M+Na]^+$.

5.34. Preparation of L-tryptophanyl-L-arginine (32) and L-tryptophanyl-N^G-2,2,5,7,8-pentamethylchroman-6-sulfonamide-L-arginine (33)

Boc-L-tryptophanyl-(Boc)-N^G-2,2,5,7,8-pentamethylchroman-6-sulfonamide-L-arginine (0.10 g, 0.1 mmol) was treated with a solution of TFA/thioanisole/DCM/water (10:2:7:1, 5 mL) at room temperature for 30 min. The reaction mixture was concentrated in vacuo, the resulting oily residue dissolved in TFA, precipitated from ice-cold Et_2O, collected by centrifugation and lyophilised. The desired compounds were purified as the TFA salts by semi-prep HPLC (gradient 3), elution time = 22.0 min (**32**, 12 mg, 33%) and 42.6 min (**33**, 15 mg, 24%).

Compound **32**: RP-HPLC (analytical, gradient 2, λ = 220 nm): R_t = 11.6 min. δ_H (400 MHz, CD_3OD): 7.76 (d, 1H, *J* = 7.5, ArC*H*), 7.56 (d, 1H, *J* = 7.5, ArC*H*), 7.42 (s, 1H, ArC*H*), 7.30 (t, 1H, *J* = 7.5, ArC*H*), 7.21 (t, 1H, *J* = 7.5, ArC*H*), 4.52–4.37 (m, 2H, Arg αC*H*, Trp αC*H*), 3.60–3.42 (m, 2H, Trp βCH_2), 3.34–3.25 (m, 2H, Arg δCH_2), 2.16–1.64 (m, 4H, Arg βCH_2, Arg γCH_2). δ_C (100 MHz, CD_3OD): 175.0 (C), 170.9 (C), 158.1 (C), 137.7 (C), 128.1 (C), 126.0 (CH), 122.9

(CH), 120.3 (CH), 119.0 (CH), 112.7 (CH), 107.4 (C), 54.7 (CH), 53.6 (CH), 41.7 (CH_2), 29.5 (CH_2), 28.1 (CH_2), 25.5 (CH_2). MS (ES+) *m*/*z*: 361.1 (100%) $[M+H]^+$. HRMS (ES+) $C_{17}H_{25}N_6O_3$ calcd: 361.1982. Found: 361.1978 $[M+H]^+$.

Compound **33**: RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): $R_t = 15.8$ min. δ_H (400 MHz, CD_3OD): 7.73 (d, 1H, $J = 7.5$, ArC*H*), 7.41 (d, 1H, $J = 7.5$, ArC*H*), 7.27 (s, 1H, ArC*H*), 7.17 (t, 1H, $J = 7.5$, ArC*H*), 7.09 (t, 1H, $J = 7.5$, ArC*H*), 4.46 (dd, 1H, $J = 8.5$, 4.5, Arg αC*H*), 4.24 (dd, 1H, $J = 9$, 5.5, Trp αC*H*), 3.50 (dd, 1H, $J = 15$, 5.5, Trp βCH*H*), 3.29–3.15 (m, 3H, Trp βCH*H*, Arg δCH_2), 2.70 (t, 2H, $J = 7$, ArCH_2), 2.59 (s, 3H, ArCH_3), 2.57 (s, 3H, ArCH_3), 2.13 (s, 3H, ArCH_3), 2.02–1.91 (m, 1H, Arg βCH*H*), 1.86 (t, 2H, $J = 7.0$, ArCH_2CH_2), 1.82–1.71 (m, 1H, Arg βCH*H*), 1.69–1.59 (m, 2H, Arg γCH_2), 1.34 (s, 6H, C(CH_3)$_2$). δ_C (100 MHz, CD_3OD): 174.4 (C), 170.2 (C), 158.0 (C), 154.8 (C), 138.3 (C), 136.6 (C), 136.2 (C), 134.3 (C), 128.3 (C), 125.8 (CH), 125.1 (C), 122.9 (CH), 120.3 (CH), 119.5 (C), 119.1 (CH), 112.6 (CH), 107.8 (C), 75.0 (C), 54.8 (CH), 53.6 (CH), 41.5 (CH_2), 33.8 (CH_2), 29.9 (CH_2), 28.8 (CH_2), 26.9 (CH_3), 22.3 (CH_2), 18.9 (CH_3), 17.8 (CH_3), 12.2 (CH_3). MS (ES+) *m*/*z*: 627.4 (100%) $[M+H]^+$, 649.4 (48%) $[M+Na]^+$. HRMS (ES+) $C_{31}H_{43}N_6O_6S$ calcd: 627.2960. Found: 627.2972 $[M+H]^+$.

5.35. Trypanothione reductase enzymatic assays

Kinetic analysis was performed in assays of a total volume of 1 mL which contained ammonium acetate (50 mM NH_4OAc, 1 mM EDTA, pH 7.5), NADPH (100 μM), *T. cruzi* TR (9.3 nM) and various known concentrations of nortrypanothione disulfide. Initial rates were recorded at 340 nm at inhibitor concentrations estimated to be 0.5, 1 and 2 K_i. NADPH kinetic analysis was performed in assays of a total volume of 1 mL which contained ammonium acetate buffer (50 mM NH_4OAc, 1 mM EDTA, pH 7.5), *N*-benzyloxycarbonyl-L-cysteinylglycine-3-dimethylaminopropylamide disulfide[39] (300 μM), TR (9.3 nM) and various known concentrations of NADPH. Initial rates were recorded by monitoring the fluorescence of NADPH ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 460$ nm) at inhibitor concentrations estimated to be 0.5, 1 and 2 K_i. Data were collected in triplicate and the type of inhibition deduced from Eadie–Hofstee and Lineweaver–Burke plots. K_i values were generated using the appropriate model with the commercial program GraFit (Erithacus Software Ltd). Brief sonication was necessary to effect dissolution of some of the inhibitors in the assay buffer.

5.36. Single-point library screening

Kinetic analyses were performed in assays of a total volume of 1 mL, containing ammonium acetate buffer (50 mM NH_4OAc, 1 mM EDTA, pH 7.5), NADPH (100 μM), nortrypanothione (30 μM), TR (9.3 nM) and inhibitor (100 μM). Initial rates were recorded at 340 nm, data were collected in triplicate and compared to a control containing no inhibitor.

5.37. Glutamate dehydrogenase assay

Kinetic analyses were performed in assays of a total volume of 1 mL, containing sodium phosphate buffer (100 mM NaH_2PO_4, pH 7.5), EDTA (1 mM), NADPH (100 μM), α-ketoglutarate (250 μM), NH_4Cl (50 mM) and glutamate dehydrogenase (10 μM). Initial rates were recorded at 340 nm at an inhibitor concentration of 100 μM. Data were collected in triplicate and compared to a control containing no inhibitor.

5.38. Analytical ultracentrifugation

Sedimentation equilibrium experiments were conducted on a Beckman Optima XL/A analytical ultracentrifuge, using 12 mm path length Beckman cells with six channel charcoal-filled Epon centrepieces and quartz windows, in an AN-50Ti rotor (three cells plus counterbalance) at 20 °C. Reference buffer (120 μL) and sample (110 μL) were loaded into the cells and absorbance scans ($\lambda = 280$ nm) recorded at 3000 rpm to check loading concentrations and uniform distribution of cell contents. The rotor speed was increased to 8000 rpm and absorbance scans recorded approximately every 4 h until sedimentation equilibrium was achieved (as judged by absence of change in overlays or subtractions of successive scans). Scans were conducted in step mode with 10 replicates per data point. The rotor speed was increased to 13,000 rpm and the analysis repeated. Samples were prepared in ammonium acetate buffer (50 mM NH_4OAc, 1 mM EDTA, pH 7.5) and consisted of TR (0.5 mg/mL) alone, TR (0.5 mg/mL) with **4** (70 μM), TR (0.5 mg/mL) with **5** (15 μM) and TR (0.5 mg/mL) with **6** (70 μM). Data from all samples were very similar and no change in the protein oligomerisation state was observed. Molecular weight estimates were generated by fitting the data from each scan using Beckman Origin software to a one-species model. The estimated molecular mass obtained for each sample were TR alone (114 kDa, 109 kDa), TR+**4** (106 kDa, 103 kDa), TR+**5** (114 kDa, 109 kDa) and TR+**6** (110 kDa, 105 kDa) at 8,000 and 13,000 rpm, respectively, in good agreement with the reported mass of TR dimer (107.8 kDa).[4]

5.39. Gel filtration chromatography

Gel filtration chromatography was performed on a Gilson HPLC system (234 autoinjector, 321 pump, 155 UV/vis detector) equipped with a Sephadex G-200 column (Amersham Pharmacia Biotech), eluted with phosphate buffer (50 mM Na_3PO_4, 150 mM NaCl, pH 7.0) at a flow rate of 0.5 mL/min with UV detection at 220 and 280 nm. Column calibration was achieved by plotting $Log_{10}M_r$ against R_t for six protein standards (4 mg/mL, 20 μL): horse myoglobin (17 kDa, 32.3 min), carbonic anhydrase (29 kDa, 30.8 min), chicken egg albumin (43 kDa, 28.2 min), bovine serum albumin (66 kDa, 25.2 min), β-amylase (200 kDa, 21.9 min) and blue dextran (2000 kDa, 14.6 min). TR (1 mg/mL, 20 μL) was then analysed and a retention time of 25.0 min, equating to a M_r of 103.0 kDa, recorded. The column was then equilibrated with buffer containing **5** (10 μM) and column calibration repeated. Analysis of

TR (1 mg/mL) incubated for 30 min with **5** (10 μM) gave a R_t of 24.7 min, equating to a M_r of 105.3 kDa.

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