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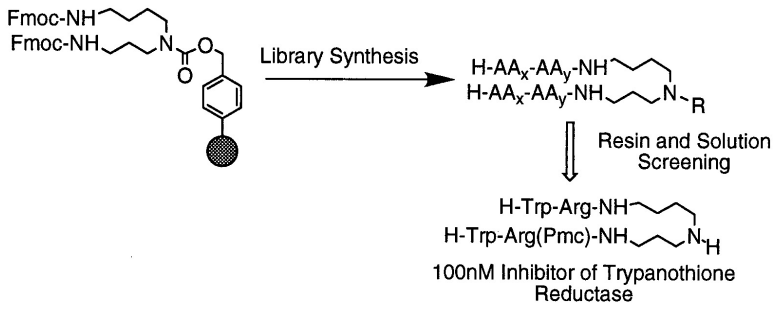

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Comparison of Resin and Solution Screening Methodologies in Combinatorial Chemistry and the Identification of a 100 nM Inhibitor of Trypanothione Reductase

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Two identical polyamine peptide conjugate libraries were screened against the parasitic enzyme trypanothione reductase. One of these libraries was in a solution format, while the other was resin-based and was used in two resin-based screens (a diminution assay and a direct bead screening). Potent inhibitors (100 nM) of trypanothione reductase were identified both in the solution screen and in the resin-based screens when using the PEGA resin of Meldal. Resin screening of both types failed to work with TentaGel resin. Importantly there was excellent agreement between the solution and resin-based assays, suggesting both methods are reliable for the screening of combinatorial libraries.

Combinatorial chemistry is proving to be a powerful tool in the arsenal of the medicinal and organic chemist.[1] There are a number of ways in which libraries can be prepared and screened; however, at the present time the predominant process is that of multiple parallel synthesis (MPS) in which large numbers of discrete compounds are synthesized for screening.[2] Compound mixtures are also still produced for solution screening but are less popular than they were previously and require deconvolution to identify the active component.[3] The direct screening of libraries on beads, which allows the library to be prepared using the powerful technique of split and mix synthesis, appears to have reduced in popularity and now seems to be used primarily for the screening of small synthetic receptors.[4] This is unfortunate when one considers the power and speed of resin screening methodologies,[5] but it has arisen due to the apparent capricious nature of resin-based screening and the difficulties associated with identifying the chemical structure on any given bead. Another method of direct bead screening that has been rarely used is diminution screening in which residual enzyme activity is measured after binding to the resin-bound library member.[6,7] This can be carried out using mixtures of beads and subsequent bead-based deconvolution to identify the active compound.

We wished to compare the screening results of two identical (peptide-based) polyamine conjugate libraries; one in a solution format and the other in two resin-based assays (a diminution type format and direct bead screening) with the enzyme trypanothione reductase,[8] which is believed to offer an attractive medicinal target in the treatment of trypanosomal diseases.[9] By using a defined polyamine template we selectively targeted the unique parasitic enzyme trypanothione reductase over the human counterpart glutathione reductase.[10] Three 576-compound peptide-based libraries were therefore prepared, using the polyamine linkers **1a,b,c**[11] as shown in Scheme 1, as 24 mixtures of 24 compounds, both in solution and on the solid phase (TentaGel[12] and PEGA[13]). The sublibraries were then screened for inhibition of the enzyme trypanothione reductase. The results of the solution screen and its deconvolution are shown in Figure 1. Thus in the initial mixture **(2)** three residues (tryptophan, phenylalanine, and arginine) were identified at the N-terminal position. Using the most active sublibrary (fixing the tryptophan residue), 24 single compounds were prepared **(3)** and screened and three potent inhibitors were identified **(3a,b,c)**. These three compounds were resynthesized individually on a larger scale, fully characterized chemically, and evaluated kinetically against trypanothione reductase. Interestingly, although in the deconvolution screen the most active compound was the arginine-containing compound **3a**, analysis of the purified compound showed reduced activity compared to **3c**. This was traced to the potency of the compounds **3d** and **3e** which still had one or two Pmc groups attached to the arginine side chain and which gave K_I values of 100 and 190 nM, respectively, following independent synthesis from **1a** and kinetic evaluation. The K_I values for all the inhibitors identified are shown in Scheme 1. Surprisingly, all were noncompetitive in nature (parallel lines in Eadie−Hofstee plots, Figure 3). However, a 100 nM inhibitor is a good hit from a library screen, although obviously this library was directed in nature. The N^4-Cbz compound **(3f)** also displayed good activity, an important observation for the subsequent resin screening since this mimics the attachment point of the library to the solid support and suggests the presence of the resin linker does not destroy binding.

Diminution resin screening, in which resin bead mixtures (24 sublibraries of 24 compounds) were added to the enzyme and filtered and the remaining activity determined, was totally unsuccessful in the case of TentaGel resin, with no

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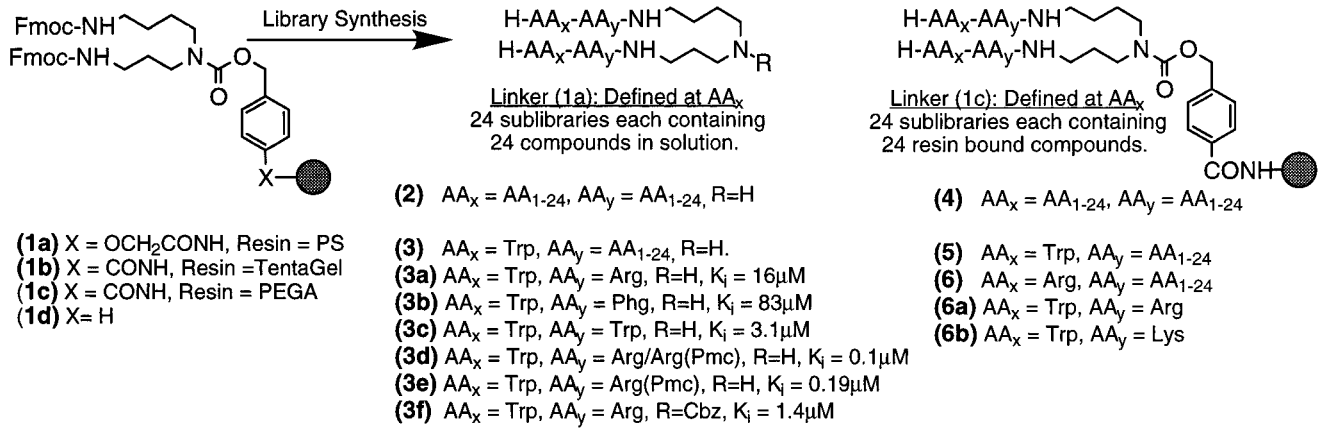
Scheme 1. Solid-Phase Synthesis of Polyamine Libraries and Identified Individual Hits (K_I, ±5%)



sublibrary showing reduction in residual activity, even with 25 000 resin site equivalents over enzyme stoichiometry. However, the screening was successful with PEGA. The results of the resin screen and its deconvolution are shown in Figure 2. The initial mixture screen identified four residues in the N-terminal position (tryptophan, phenylalanine, tyrosine, and arginine). Importantly, the three most active residues were also observed in the solution screen. Using the two most active sublibraries (fixing the tryptophan and arginine residues), 48 single-resin samples (**5** and **6**) were prepared and screened in the same manner. The most active resin sample in the tryptophan sublibrary corresponded to the original "hit" from the solution screen **(3a)**, although interestingly the introduction of a lysine residue in place of arginine also showed good activity **(6b)**. The other sublibrary (arginine fixed) was less defined, with many active samples, suggesting that the terminal arginine residue is essential for activity in this sublibrary with little predilection in the second position except for a hydrophobic nature.

Screening the 576-compound library **(4)** with biotinylated trypanothione reductase and an alkaline phosphatase-labeled antibody was followed by bead sequencing (see Figure 4). The amino terminal residues observed were 54% Phe and 46% Tyr, while the second residue was 100% Arg. Again the screening was only successful on PEGA resin. No binding was ever observed on TentaGel. The observation of arginine in the final position was absolute and agrees with the solution and resin screening results. The presence of the Phe and Tyr residues in the first position again correlates with the two

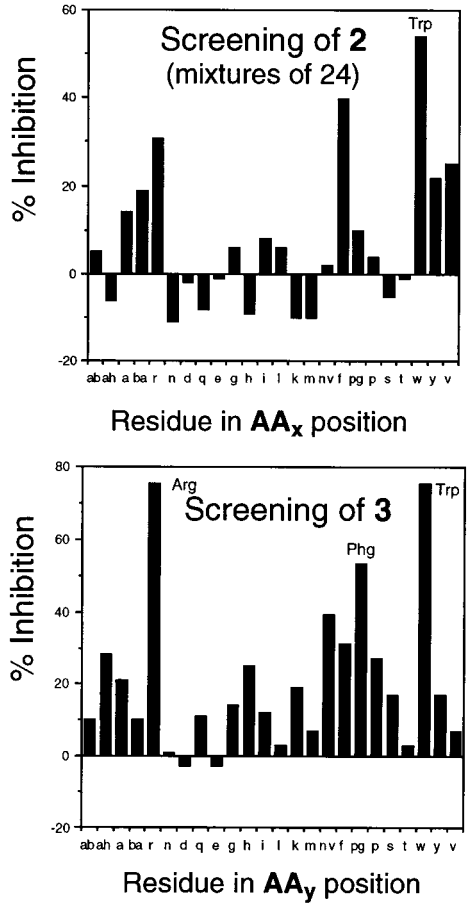


Figure 1. Solution screening and deconvolution of library **2**.[15]

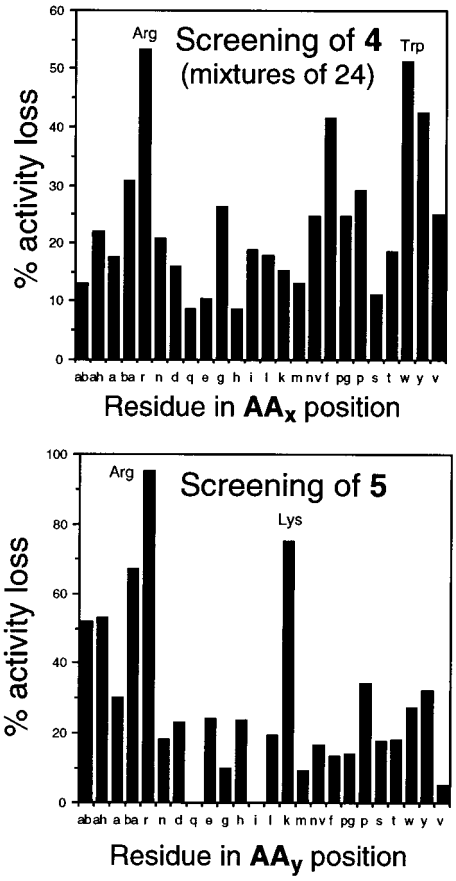


Figure 2. Dimunition screening and deconvolution of library **4**.[15]

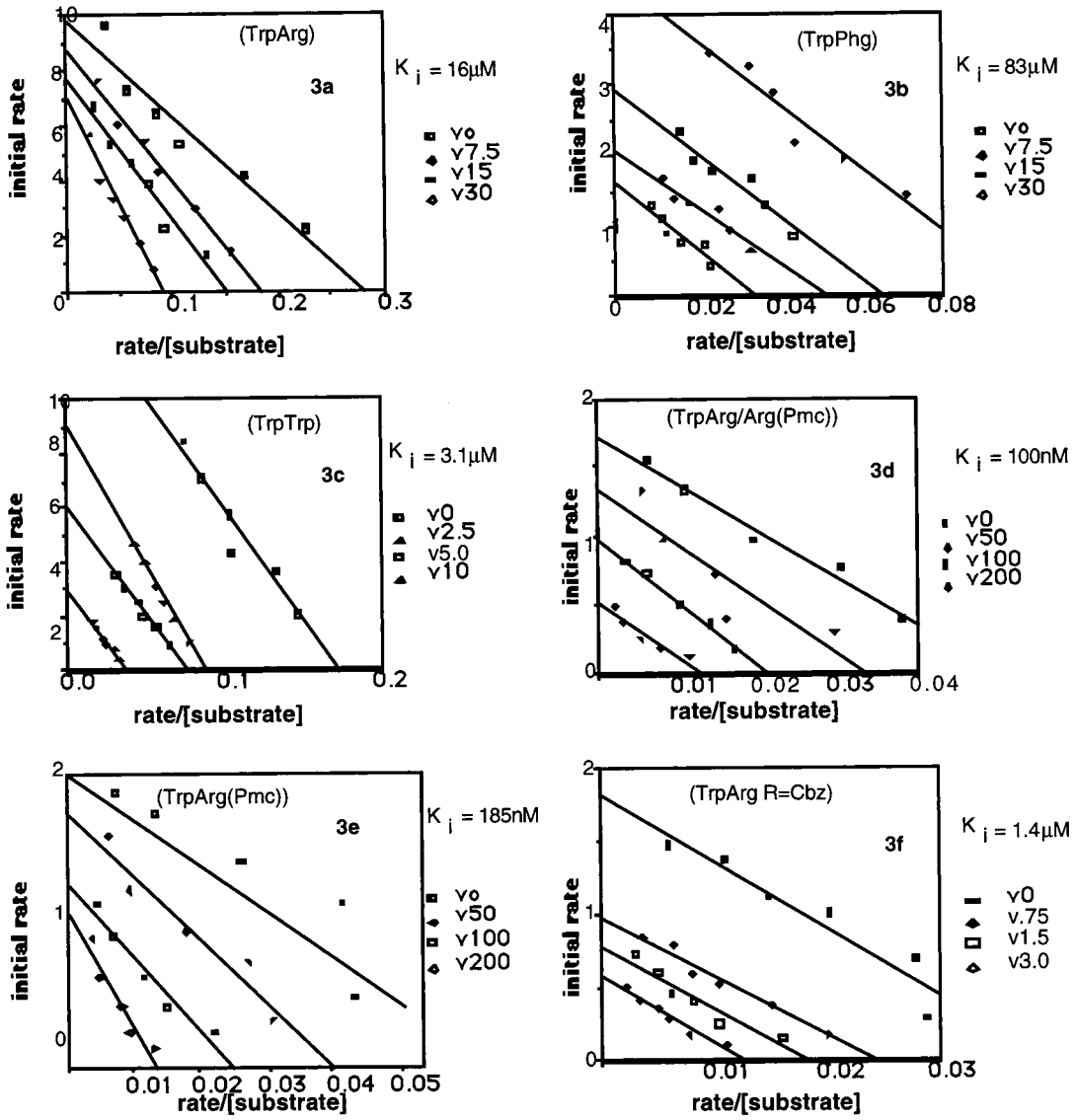


Figure 3. Eadie−Hofstee plots. Kinetic analysis of compounds **3a**−**f.**

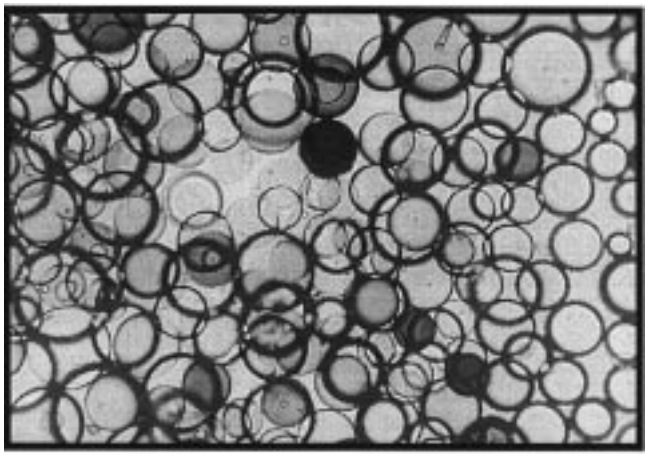


Figure 4. Resin screen of **4** with biotinylated trypanothione reductase (see Experimental Section).

other screens, both of which show the presence of these residues. However, and surprisingly, there was no evidence for Trp residues in the first position, which was seen in the other two screens, maybe indicating an alkylation problem with this residue during the resin-based deprotection.

Clearly there is consensus between the three screening methods: all identified Arg in one position and an aromatic residue in the second, although the nature of this second group varied. These experiments show that resin screening of either the diminution type or directly on beads produces valuable information for inhibitor design and synthesis and that this correlates with solution screening data. These experiments lend weight to the value of resin screening in drug discovery projects. Importantly we have determined that the nature of the resin is very important for screening since TentaGel was not suitable for screening this particular enzyme even in the simple affinity screen mode and with thousands of ligand equivalents available, presumably due to enzyme accessibility, although from previously reported screening results we expected surface groups would provide sufficient ligands for binding. The rationale for failure in this case is unclear, but it should be noted that the size exclusion limits for 1% and 2% cross-linked polystyrene are 14 000 and 2700, respectively,[16] and 1% cross-linked polystyrene is the base resin for TentaGel. Trypanothione reductase is also an α_2 homodimer with subunits of approximately 55 kDa. PEGA resin worked exceptionally well,

although it was more difficult to handle synthetically. The identification of a 100 nM inhibitor of trypanothione reductase is a good starting point for future optimization studies and the most potent inhibitor to date. However, it should also be noted that this inhibitor would not have been identified using single pure compounds and does show, in this case at least, the advantage of testing small compound mixtures, even those which contain some impurities or reaction side products but whose structures can be deduced from the synthetic process.

Experimental Procedures

Resin Screening.[14] (a) Solution. Trypanothione reductase activity was assayed at 25 °C in 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 100 μM NADPH, and 100 μM trypanothione (saturating) in a final volume of 1 mL. Inhibitors were added, making a final concentration of 2.4 μM per component in both the initial mixture screen and in the deconvolution screen. Initial rates were measured, and percent inhibition was related to a control reaction with no inhibitor present.

(b) Diminution Assay. Trypanothione reductase (0.5 μg) was incubated with samples of resin-bound libraries (sublibraries of 24 resin-bound compounds for the initial screen and single-resin-based compounds for the subsequent deconvolution) using both TentaGel and PEGA resins (2.5 mg preswollen in buffer) in 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The resin and enzyme were mixed at 4 °C for 15 h in small Eppendorf tubes, when a small hole was punched in the base. This allowed the remaining enzyme solution to be removed by gentle centrifugation with retention of the beads. The supernatant was assayed and compared to a control reaction with base resin to give percent inhibition or percent enzyme removal.

(c) Bead Screening. (+)-Biotin-*N*-succinimidyl ester (0.1 mg, 0.3 mmol) in pH 8 phosphate buffer (50 mM K_2HPO_4, 100 mM NaCl, 5 mM EDTA) (1.5 mL) was added to trypanothione reductase (1 mg) and left at 30 °C for 2 h. Excess reagent was removed by dialysis into pH 7.5 phosphate buffer (50 mM K_2HPO_4, 1 mM EDTA) (3 × 200 mL; 2 × 6 h, 1 × 15 h). Successful modification was verified by Western blotting. Treatment of the nitro-cellulose membrane with streptavidin-alkaline phosphatase conjugate, followed by the substrate for alkaline phosphatase (BCIP/NBT), resulted in staining of the modified enzyme, which was shown to be fully active following modification.

The PEGA-bound library (50 mg of beads) was treated with BSA blocking buffer (150 mM NaCl, 25 mM Tris, 1% BSA, 0.1% Tween-20, pH 7.4) (1 mL) for 3 h. The resin was filtered and resuspended in fresh blocking buffer (1 mL). Biotinylated TR (BTR) (3 μg) was added along with streptavidin-alkaline phosphatase conjugate (0.25 μg) and left overnight. The beads were filtered and washed twice with PBS washing buffer (140 mM NaCl, 27 mM KCl, 10.1 mM K_2HPO_4, 1.8 mM KH_2PO_4, 0.3% Tween-20, pH 7.3), followed by addition of the alkaline phosphatase substrate (BCIP/NBT). After 30 min, a differentiation could be seen with approximately 20 beads stained blue−black and several others displaying a gradient of color. Thirteen of the very dark beads were removed and Edman sequenced, giving the results shown in Table 1. Screening with TentaGel did not work in this case.

Table 1. Results of Edman Sequencing[a]

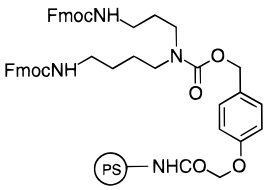
sample	residue aa_X	residue aa_Y
1	Tyr	Arg
2	Tyr	Arg
3	Tyr	Arg
4	Tyr	Arg
5	Tyr	Arg
6	Tyr	Arg
7	Phe	Arg
8	Phe	Arg
9	Phe	Arg
10	Phe	Arg
11	Phe	Arg
12	Phe	Arg
13	Phe	Arg

[a] Residue aa_X: 54% Phe, 46% Tyr. Residue aa_Y: 100% Arg.

(d) Inhibitor Characterization. All single compounds were purified to homogeneity by reverse phase HPLC and gave the expected NMR and HRMS data. Inhibitors were assayed using initial rate measurements, at least in duplicate, at six or more substrate concentrations ranging typically between $0.25K_m$ and $5K_m$ and at a range of inhibitor concentrations. The inhibition constants (K_I) were calculated using this initial rate data using Eadie−Hofstee plots.

$$V_{max}(app) = V_{max} - (V_{max}(app))(I)/K_I$$

Library Synthesis. Preparation of N^1,N^8-bis(9-Fluorenylmethoxycarbonyl)-N^4-(4-benzyloxycarbonyl-1-oxyacetamidomethylresin) Spermidine (1a). N^1,N^8-bis(9-Fluorenylmethoxycarbonyl)-N^4-(4-benzyloxycarbonyl-1-oxyacetic acid) spermidine (0.53 g, 0.66 mmol) was dissolved in DCM (10 mL) and cooled on ice. HOBt (0.11 g, 0.73 mmol) was added with DIC (0.11 mL, 0.73 mmol). After 10 min the solution



was added to aminomethyl resin (1.0 g, 0.66 mmol NH_2) and the suspension was shaken overnight. Coupling was not complete thus DIC (30 μL) was added and the mixture warmed to ~35 °C. After 4 h the reaction was complete as shown by a qualitative ninhydrin test, and the resin was filtered and washed with DCM (1 × 15 mL), DMF (2 × 15 mL), and DCM (2 × 15 mL). Yield: 1.49 g, with linker substitution of 0.31 mmol g^{-1} as determined by Fmoc analysis.

Preparation of N^1-aa^1-aa^2-N^8-aa^1-aa^2 Spermidine: Libraries 2 and 4. Compounds **1a**−**c** (1.2 g, 0.36 mmol) were treated with 20% piperidine in DMF (15 mL) for 2 × 10 min, then washed with DMF (2 × 15 mL) and DCM (2 × 15 mL). The beads were split equally by weight into 24 polypropylene tubes with sinters at the bottom. Twenty-four Fmoc amino acids (0.15 mmol) were preactivated as described below. Arg(Pmc)-OH (99 mg), His(Trt)-OH (93

mg), Lys(Boc)-OH (70 mg), Ser(^{t}Bu)-OH (58 mg), Thr(^{t}Bu)-OH (60 mg), and Val-OH (51 mg) were dissolved in DCM (0.5 mL). γ-Abu-OH (49 mg), Ala-OH (47 mg), Asp(OBu)-OH (62 mg) and Trp-OH (62 mg) were dissolved in DCM and DMF (3:1), and ϵ-Ahx-OH (53 mg), β-Ala-OH (47 mg), Glu(^{t}Bu)-OH (55 mg), Gly-OH (45 mg), Ile-OH (53 mg), Leu-OH (53 mg), Met-OH (56 mg), Nva-OH (51 mg), Phe-OH (58 mg), Phg-OH (56 mg), Pro-OH (51 mg) and Tyr(^{t}Bu)-OH (69 mg) were dissolved in DCM (0.5 mL) and 1 drop of DMF, while Asn-OH (53 mg) and Gln-OH (64 mg) were dissolved in DMF (0.5 mL). The solutions were cooled on ice, and a solution of HOBt in DMF (23 mg, 0.15 mmol in 0.2 mL) was added to each amino acid. DIC (24 μL, 0.15 mmol) was added, and the mixtures were left on ice for a further 10 min. After addition to each reaction syringe, the couplings were left for 2.5 h. Amide formation was not complete as shown by individual qualitative ninhydrin tests, thus the process was repeated once more for 3 h. After capping with Ac_2O (0.2 mL) in DMF (0.5 mL) and pyridine (0.5 mL) and washing, two-thirds of each sample was combined and one-third retained. The combined resin was split into 24 and treated with 20% piperidine in DMF (1.5 mL, 2 × 15 min). After washing, a second coupling was performed. After 2 h, couplings were repeated for Asn, Glu, Leu, Phg, and Thr with 2 equiv of amino acid for 1 h. Capping with Ac_2O was followed by Fmoc deprotection. Cleavage from the resin **(2)** or side chain deprotection **(4)** on the resin was achieved with TFA, TIS, EDT, and H_2O (37:1:1:1, 10 mL) for 5 h (the thiol scavengers were not used for the resin-based libraries). The solutions were dripped into 1:1 ether and hexane to precipitate the products. The compounds were collected by centrifugation, the solvent was decanted, and the residue was shaken with ether and hexane. After recentrifugation and decanting of the solvent, 1 mL of water was added to each tube and the compounds were lyophilized overnight. Sublibraries were analyzed by HPLC ESMS, and the results indicated that all the expected components were present.

Preparation of N^1,N^8-bis(tryptophanylargininyl) Spermidine (3a). The above synthetic procedure was repeated, coupling with Fmoc-Arg(Pmc)-OH and Fmoc-Trp-OH using 0.2 g of **1a**. Cleavage was performed using TFA, phenol, EDT, and TIS (37:1:1:1, 1.5 mL) for 4 h. The mixture was dripped into ether and hexane (1:1, 25 mL) and the resin washed with TFA (0.5 mL). The white precipitate was collected by centrifugation for 15 min at 4 krpm. The supernatant was discarded, the pellet was resuspended in ether and hexane (1:1, 25 mL), the suspension was spun down, and the process was repeated. The product was dissolved in water and lyophilized to give a white TFA salt. Purification was achieved via semipreparative RP-HPLC using a gradient of H_2O, 0.1% TFA (A) and MeCN, 0.1% TFA (B) with a flow rate of 2 mL min^{-1} (λ = 254 nm): t = 0 (100% A); t = 30 (40% A, 60% B); t = 35 (100% B); t = 40 (100% B); t = 50 (100% A); t = 55 (100% A). The product eluted at 23.5 min (103 mg, 56%).

δ_H (360 MHz, D_2O) (All coupling constants (J) are recorded in hertz.): 7.54 and 7.52 2× (1H, d, J = 8, indole C^4), 7.46 and 7.44 2× (1H, dd, J = 8, 1, indole C^7), 7.26 and 7.25 2× (1H, s, indole C^2), 7.21 and 7.19 2× (1H, ddd, J = 8, 8, 1, indole C^6), 7.11 and 7.09 2× (1H, ddd, J = 8, 8, 1, indole C^5), 4.27 and 4.26 2× (2H, t, J = 7, Trp αH), 4.12 and 4.09 2× (2H, t, J = 7, Arg αH), 3.33 (4H, d, J = 7, Trp βH), 3.10 (2H, t, J = 7, C^{1}H$_2$), 3.07 (4H, m, Arg δH), 2.98 (2H, t, J = 7, C^{8}H$_2$), 2.90 (2H, tt, J = 8, 8, C^{5}H$_2$), 2.88 (2H, tt, J = 7, 7, C^{3}H$_2$), 1.76 (2H, tt, J = 7, 7, C^{2}H$_2$), 1.70−1.51 (4H, m, Arg βH), 1.57 (2H, tt, J = 8, 8, C^{6}H$_2$), 1.49−1.33 (6H, m, Arg γH and C^{7}H$_2$), 1.39 (2H, m). δ_C (90 MHz, D_2O): 175.2, 174.6, 171.9, 171.8 (amide C═O), 159.2 (Arg guanidine), 138.7 (indole C^{7a}), 129.1 (indole C^{3a}), 127.8 (indole C^2), 124.7 (indole C^6), 122.1 (indole C^5), 2 × 120.5 (indole C^4), 114.5 (indole C^7), 108.8 (indole C^3), 56.1 (Trp αC), 56.0 (Arg αC), 49.7 (C^{5}H$_2$), 47.5 (C^{3}H$_2$), 43.0 (Arg δC), 41.2 (C^{8}H$_2$), 38.8 (C^{1}H$_2$), 31.3 and 31.2 (Trp βC), 29.3 (Arg βC), 2 × 28.0 (C^{2,7}H$_2$), 26.7 (Arg γC), 25.6 (C^{6}H$_2$). IR υ cm^{-1} (Nujol mull): 3284, 3190 (m, NH), 1671, 1621 (s, amide C═O). MS (ES+) m/z: 277.7 (100%) [M + 3H]$^{3+}$, 416.1 (50%) [M + 2H]$^{2+}$. HRMS: $C_{41}H_{64}O_4N_{15}$ (calcd) 830.5266; (found) 830.5269 [M + H]$^+$.

Preparation of N^1,N^8-bis(tryptophanylphenylglycinyl) Spermidine (3b). N^1,N^8-bis(9-Fluorenylmethoxycarbonyl)-N^4-(4-benzyloxycarbonyl-1-oxyacetamido-methylresin) spermidine (0.45 g, 0.12 mmol) was treated with 20% piperidine in DMF (2 × 10 mL) for 2 × 10 min and washed with DMF (4 × 10 mL) and DCM (2 × 10 mL). The resin was suspended in a solution of DIPEA (0.10 mL, 0.6 mmol) and a catalytic amount of DMAP in DCM (10 mL). Fmoc-Phg-OH (0.22 g, 0.6 mmol) and PyBrop (0.28 g, 0.6 mmol) were added, and the resin was agitated for 4 h. A qualitative ninhydrin test proved positive. The resin was drained and the procedure repeated using Fmoc-Phg-OH (82 mg, 0.22 mmol), PyBrop (0.21 g, 0.45 mmol), DIPEA (0.15 mL, 0.86 mmol), and a catalytic amount of DMAP. The reaction was left overnight, and the resin was washed with DMF (4 × 10 mL) and DCM (2 × 10 mL). Fmoc removal was achieved using 20% piperidine in DMF (6 mL, 2 × 2 min). The beads were washed with DMF (4 × 10 mL) and DCM (2 × 10 mL) and resuspended in a solution of DIPEA (0.15 mL, 0.86 mmol) and a catalytic amount of DMAP in DCM (10 mL). Boc-Trp-OH (0.14 g, 0.46 mmol) and PyBrop (0.21 g, 0.46 mmol) were added, and the resin was agitated for 3 h. The resin was washed with DMF (4 × 10 mL) and DCM (2 × 10 mL) and treated with TFA and water (39:1, 8 mL) for 2 × 4 h. The cleavage mixture was filtered and the resin washed with MeOH (2 × 10 mL). The solvent was removed in vacuo and the pale brown oil taken up in water and lyophilized. The mixture was separated by semipreparative RP-HPLC using a gradient of H_2O, 0.1% TFA (A) and MeCN, 0.1% TFA (B) with a flow rate of 2 mL min^{-1} (λ = 254 nm): t = 0 (90% A, 10% B); t = 20 (50% A, 50% B); t = 25 (100% B); t = 30 (100% B); t = 40 (100% A). The major peaks at 19.9 min (LL 25.6 mg), 25.6 and 25.9 min (LD and DL, 13.1 mg), and 27.7 min (DD 2.1 mg) were confirmed as the desired products by ESMS (43% overall yield).

LL Diastereoisomer (3b). δ_H (360 MHz, CD_3OD): 7.66 and 7.65 2× (1H, dd, J = 8,3, indole C^4), 7.45−7.39 (5H, m, aryl), 7.39−7.29 (7H, m, aryl + indole C^7), 7.29 and

7.26 2× (1H, s, indole C^2), 7.14 and 7.13 2× (1H, ddd, J = 8, 8, 3, indole C^6), 7.06 and 7.04 2× (1H, ddd, J = 8, 8, 3, indole C^5), 5.36 and 5.32 2× (1H, s, L-Phg αH), 4.25 (2H, dd, $J_{AX} + J_{BX}$ = 14, Trp αH_X), 3.45 (2H, dd, J_{AB} = 15, J_{AX} = 6, Trp βH_A), 3.29 (2H, dd, J_{AB} = 15, J_{BX} = 8, Trp βH_B), 3.23 and 3.12 2× (2H, t, J = 7, $C^{1,8}H_2$), 2.81 and 2.77 2× (2H, t, J = 7, $C^{3,5}H_2$), 1.77 (2H, tt, J = 7, 7, C^2H_2), 1.56, 1.49 2× (2H, tt, J = 7, 7, $C^{6,7}H_2$). δ_C (90 MHz, CD_3OD): 173.2, 172.2, 170.2, 170.0 (amide C═O), 163.3 (q, J_{CF} = 35, CF_3CO_2), 138.9, 138.6, 138.5 (indole C^{7a} + aryl C^4), 130.6, 130.2 (aryl C^3), 130.0, 129.8 (aryl C^4), 129.0, 128.9 (aryl C^2), 128.7 (indole C^{3a}), 126.1, 126.0 (indole C^2), 123.2 (indole C^6), 120.7, 120.6 (indole C^5), 119.4 (indole C^4), 118.5 (q, J_{CF} = 279, CF_3CO_2), 113.0, 112.9 (indole C^7), 108.3, 108.2 (indole C^3), 59.6, 59.4 (Phg αC), 55.1 (Trp αC), 48.7 (C^5H_2), 46.3 (C^3H_2), 40.0 (C^8H_2), 37.3 (C^1H_2), 29.2, 29.1 (Trp βC), 27.7 (C^2H_2), 27.5 (C^7H_2), 24.7 (C^6H_2). MS (ES+) *m*/*z*: 393.0 (100%) $[M + 2H]^{2+}$, 784.5 (30%) $[M + H]^+$, 806.4 (15%), $[M + Na]^+$. HRMS: $C_{45}H_{54}O_4N_9$ (calcd) 784.4299; (found) 784.4479 $[M + H]^+$.

Preparation of N^1,N^8-bis(tryptophanyltryptophanyl) Spermidine (3c). The above procedure was repeated, coupling Fmoc-Trp(Boc)-OH.

RP-HPLC (analytical): flow rate = 1 mL min^{-1} (λ = 254 nm); H_2O, 0.1% TFA (A) and MeCN, 0.1% TFA (B): t = 0 (80% A, 20% B); t = 20 (50% A, 50% B); t = 30 (100% B); t = 35 (100% B); t = 45 (100% A); t = 50 (100% A). R.t.= 15.2 min. δ_H (360 MHz, D_2O): 7.51 and 7.49 2× (2H, d, J = 7, indole C^4), 7.42 and 7.40 2× (2H, d, J = 7, indole C^7), 7.24 and 7.23 2× (1H, s, indole C^2), 7.20−7.13 (4H, m, indole C^6), 7.11 and 7.10 2× (1H, s, indole C^2), 7.12−7.07 (4H, m, indole C^5), 4.44 and 4.38 2× (1H, dd, $J_{AX} + J_{BX}$ = 14, "B" Trp αH), 4.27 (2H, dd, $J_{AX} + J_{BX}$ = 14, "A" Trpαh), 3.33 (4H, d, J_{AX} = 6, "A" Trp βH_A), 3.09 (4H, d, J_{BX} = 8, "B" Trp βH_B) (no AB splitting observed), 2.70−2.60 (4H, m, $C^{1,8}H_2$), 2.28 (2H, t, J = 7, C^5H_2), 2.10 (2H, t, J = 7, C^3H_2), 1.30 (2H, tt, J = 7, 7, C^2H_2), 1.00 (2H, tt, J = 7, 7, C^6H_2), 0.88 (2H, tt, J = 7, 7, C^7H_2). δ_C (90 MHz, D_2O): 174.8, 174.2, 2 × 171.6 (amide C═O), 138.7, 138.6, 138.5 (indole C^{7a}), 129.6, 129.5, 129.1 (indole C^{3a}), 127.6, 126.9 (indole C^2), 124.7, 124.5, 124.4 (indole C^5), 122.1, 121.9, 121.8 (indole C^6), 121.0, 120.5 (indole C^4), 114.5, 114.4, 114.3 (indole C^7), 111.2, 108.9, 108.8 (indole C^3), 57.5 and 57.4 ("B" Trp αC), 56.1 ("A" Trp αC), 49.2 (C^5H_2), 46.7 (C^3H_2), 41.0 (C^8H_2), 38.5 (C^1H_2), 30.1 and 29.9 ("B" Trp βC), 29.3 ("A" Trp βC), 2 × 27.4 ($C^{2,7}H_2$), 25.0 (C^6H_2). MS (ES+) *m*/*z*: 446.1 (100%) $[M + 2H]^{2+}$, 890.3 (10%) $[M + H]^+$. HRMS: (glycerol−thioglycerol−DMSO matrix) $C_{51}H_{60}O_4N_{11}$ (calcd) 890.4830; (found) 890.4847 $[M + H]^+$.

Preparation of N^1-(Tryptophanylargininyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide))-N^8-(tryptophanylargininyl) Spermidine and N^1-(Tryptophanylargininyl)-N^8-(tryptophanylargininyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)) Spermidine (3d), and N^1,N^8-bis(tryptophanylargininyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide)) Spermidine (3e). The above procedure was repeated, coupling Fmoc-Arg(Pmc)-OH and Fmoc-Trp-OH onto 0.5 g of **1a**. Cleavage was performed using 7.5% TFA in DCM (6 mL, 10 × 1 h). The cleavage mixture was filtered into water each time and the resin washed with methanol (5 mL). The fractions were combined, and the solvent was removed in vacuo. The products were lyophilized to give a pale brown foam. The mixture was separated by semi-preparative RP-HPLC using a gradient of H_2O, 0.1% TFA (A) and MeCN, 0.1% TFA (B) with a flow rate of 2 mL min^{-1} (λ = 254 nm): t = 0 (90% A, 10% B); t = 10 (90% A, 10% B); t = 25 (50% A, 50% B); t = 35 (100% B); t = 40 (100% B); t = 50 (100% A). The desired products, as verified by ESMS, eluted at 29.6 min (mono-Pmc protected **(3d)**) (18.5 mg, 13%) and 33.2 min (di-Pmc protected **(3e)**) (48.7 mg, 28%).

3d. δ_H (360 MHz, CD_3OD): 7.67 (2H, dd, J = 8,2, indole C^4), 7.39 (2H, dd, J = 8,2, indole C^7), 7.24 and 7.22 (4 H, 4 × s, indole C^2), 7.12 (2H, ddd, J = 8, 8, 2, indole C^6), 7.03 (2H, m, indole C^5), 4.49−4.37 (1H, m, Arg αH), 4.34 and 4.32 2× (0.5H, t, J = 6, Arg αH), 4.26 and 4.24 2× (1H, dd, $J_{AX} + J_{BX}$ = 14, Trp αH_X), 3.49 (2H, dd, J_{AB} = 15, J_{AX} = 6, Trp βH_A), 3.29−3.05 (10H, m, Trp βH_B + Arg δ CH_2 + $C^{1,8}H_2$), 3.00−2.88 (4H, m, $C^{3,5}H_2$), 2.63 (2H, t, J = 7, Pmc-C^4H_2), 2.57 and 2.54 2× (6H, s, Pmc-C^5 and C^7 CH_3), 2.09 (6H, s, Pmc-C^8 CH_3), 1.95−1.76 (2H, m, C^2H_2), 1.83 (2H, t, J = 7, Pmc-C^3H_2), 1.76−1.62 (4H, m, Argβ CH_2), 1.62−1.48 (6H, m, $C^{6,7}H_2$ + Arg γ CH_2), 1.30 (6H, s, Pmc-$C^{2a,b}$ CH_3). δ_C (90 MHz, CD_3OD): 158.3 (Arg guanidine), 155.2 (Pmc aryl C^{8a}), 138.5 (indole C^{7a}), 136.8, 136.4, 134.7 (Pmc aryl $C^{5,6,7}$), 128.7 (indole C^{3a}), 126.0 (indole C^2), 125.4 (Pmc aryl C^{4a}), 123.2 (indole C^6), 120.7 (indole C^5), 119.8 (Pmc aryl C^8), 119.5 (indole C^4), 112.9 (indole C^7), 108.2 (indole C^3), 75.2 (Pmc C^2), 55.1, 55.0 (Trp αC + Arg αC), 48.9 (C^5H_2), 46.6 (C^3H_2), 42.2 (Arg δC), 39.8 (C^8H_2), 37.3 (C^1H_2), 34.1 (Pmc-C^4H_2), 30.9, 30.7 (Arg βC), 29.0 (Trp βC), 27.7 (C^2H_2), 27.5 (C^7H_2), 27.2 (Pmc-$C^{2a,b}$ CH_3), 26.5 (C^6H_2), 24.8 (Arg γC), 22.7 (Pmc-C^3H_2), 19.4, 18.2 (Pmc-C^5 and C^7 CH_3), 12.6 (Pmc-C^8 CH_3). IR υ cm^{-1} (Nujol mull): 1686, 1672 (s, amide C═O). MS (ES+) *m*/*z*: 549.2 (100%) $[M + 2H]^{2+}$, 1096.4 (45%) $[M + H]^+$.

3e. δ_H (360 MHz, CD_3OD): 7.64 (2H, dd, J = 8, 3, indole C^4), 7.46 (2H, dd, J = 8, 3, indole C^7), 7.23 (2H, d, J = 7, indole C^2), 7.11 and 7.12 2× (1H, ddd, J = 8, 8, 3, indole C^6), 7.10 (2H, ddd, J = 8, 8, 3, indole C^5), 4.34−4.45 (2H, m, Arg αH), 4.23 2× (1H, dd, $J_{AX} + J_{BX}$ = 14, Trp αH_X), 3.43 + 3.42 2× (1H, dd, J_{AB} = 15, J_{AX} = 6, Trp βH_A), 3.23 (2H, dd, J_{AB} = 15, J_{BX} = 8, Trp βH_B), 3.28−3.03 (8H, m, Arg δH + $C^{1,8}H_2$), 2.90−3.00 (4H, m, $C^{3,5}H_2$), 2.62 (4H, t, J = 7, Pmc-C^4H_2), 2.59 + 2.56 2× (6H, s, Pmc-C^5 and C^7 CH_3), 2.09 (6H, s, Pmc-C^8 CH_3), 1.91−1.80 (2H, m, C^2H_2), 1.80 (4H, t, J = 7, Pmc-C^3H_2), 1.68 (4H, m, Arg βH), 1.60−1.49 (8H, m, $C^{6,7}H_2$ + Arg γH), 1.30 (12H, s, Pmc-$C^{2a,b}$ CH_3). δ_C (90 MHz, CD_3OD): 174.8, 173.8, 170.5, 170.4 (amide C═O), 163.2−162.2 (m, CF_3CO_2), 158.3 (guanidine), 155.2 (Pmc aryl C^{8a}), 138.5 (indole C^{7a}), 136.9, 136.5, 134.8 (Pmc aryl $C^{5,6,7}$), 128.6 (indole C^{3a}), 126.0 (indole C^2), 125.4 (Pmc aryl C^{4a}), 123.2 (indole C^6), 120.7 (indole C^5), 119.8 (Pmc aryl C^8), 119.5 (indole C^4), 112.9 (indole C^7), 108.2 (indole C^3), 75.2 (Pmc C^2), 55.1 (Trp αC), 54.9 and 54.8 (Arg αC), 48.9 (C^5H_2), 46.6 (C^3H_2), 41.5 (Arg δC), 39.8 (C^8H_2), 37.2 (C^1H_2), 34.0 (Pmc-C^4H_2), 30.8 and 30.6 (Arg βC), 29.0 (Trp βC), 27.7 (C^2H_2), 27.5 (C^7H_2), 27.2 (Pmc-

$C^{2a,b}$ CH_3), 26.5 (C^6H_2), 24.8 (Arg γC), 22.6 (Pmc-C^3H_2), 19.3 and 18.2 (Pmc-C^5 and C^7 CH_3), 12.6 (Pmc-C^8 CH_3). IR υ cm^{-1} (Nujol mull): 3319 (m, NH), 1681, 1672, 1622 (s, amide C═O). MS (ES+) *m*/*z*: 681.9 (100%) $[M + 2H]^{2+}$, 1362.7 (55%) $[M + H]^+$, 1384.6 (40%) $[M + Na]^+$.

Preparation of N^1,N^8-bis(argininyltryptophanyl)-N^4-benzyloxycarbonyl Spermidine (3f). N^1,N^8-Bis(*tert*-butyloxycarbonyl-tryptophanylargininyl(2,2,5,7,8-pentamethylchroman-6-sulfonamide))-N^4-benzyloxycarbonyl spermidine (50 mg, 0.03 mmol) was dissolved in DCM, TFA, thioanisole, and water (25:23:1:1, 2 mL) and left to stir at room temperature for 2 h. The mixture was dripped into cold ether and hexane (1:1, 20 mL), and the precipitate was recovered by centrifugation (4000 rpm) and decanting the solvent. The white solid was shaken with ether and hexane (1:1, 20 mL), and the process was repeated. Excess solvent was removed in vacuo and the crude product purified by semipreparative RP-HPLC using a gradient of H_2O, 0.1% TFA (A) and MeCN, 0.1% TFA (B) with a flow rate of 2.5 mL min^{-1} (λ = 254 nm): t = 0 (75% A, 25% B); t = 10 (75% A, 25% B); t = 30 (25% A, 75% B); t = 35 (100% B); t = 40 (100% B); t = 50 (75% A, 25% B). The major products were the mono and di Pmc protected compounds eluting at 24.6 min and 29.5 min, respectively. These were combined and treated twice with DCM, TFA, water, thioanisole, and trisopropylsilane (25:22:1:1:1, 2 × 1 mL) for 2 × 2 h. Purification was achieved via semipreparative RP-HPLC using a gradient of H_2O, 0.1% TFA (A) and MeCN, 0.1% TFA (B) with a flow rate of 2 mL min^{-1} (λ = 254 nm): t = 0 (80% A, 20% B); t = 10 (80% A, 20% B); t = 30 (30% A, 70% B); t = 40 (100% B); t = 45 (100% B); t = 55 (80% A, 20% B). The desired product eluted at 22.2 min (21 mg, 52%). δ_H (360 MHz, CD_3OD): 7.62−7.54 (2H, m, indole C^4), 7.49 (2H, d, J = 8, indole C^7), 7.45−7.33 (5H, m, Ph), 7.32 (2H, s, indole C^2), 7.24 (2H, t, J = 8, indole C^6), 7.19−7.11 (2H, m, indole C^5), 5.10 (2H, s, benzylic CH_2), 4.34 (2H, t, J = 6, Trp αH), 4.24−4.08 (2H, m, Arg αH), 3.49−3.35 (4H, m, Trp βH), 3.35−3.20 (4H, m, Arg δH), 3.20−2.90 (8H, m, $C^{1,3,5,8}H_2$), 1.80−1.54 (6H, m, C^2H_2, Arg βH), 1.59−1.03 (8H, m, $C^{6,7}H_2$, Arg γH). δ_C (90 MHz, CD_3OD): 174.3, 171.8 (amide C═O), 159.2 (guanidine), 139.0, 138.7 (aryl C^4 + indole C^{7a}) 131.3, 130.9, 130.0 (aryl $C^{1,2,3}$), 129.2 (indole C^{3a}), 127.6 (indole C^2), 124.7 (indole C^6), 122.1 (indole C^5), 120.2 (indole C^4), 114.5 (indole C^7), 108.8 (indole C^3), 69.9 (Cbz CH_2), 56.1 (Trp + Arg αC), 48.8 (C^5H_2), 44.1 (C^3H_2), 43.0 (C^8H_2), 41.7 (Arg δC), 39.4 (C^1H_2), 31.4, 29.4, 28.2 (Arg βC + $C^{2,7}H_2$), 27.7 (Arg γC), 26.7 (C^6H_2). IR υ cm^{-1} (Nujol mull): 3307, 3201 (m, NH), 1671 (s, urethane + amide C═O). MS (ES+) *m*/*z*: 1568.4 (60%) $[M + H]^+$, 1590.4 (100%) $[M + Na]^+$, 1606.4 (30%) $[M + K]^+$.

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