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Supporting Information

Synthesis of RAFT Polymers as Bivalent Inhibitors of Cholera Toxin

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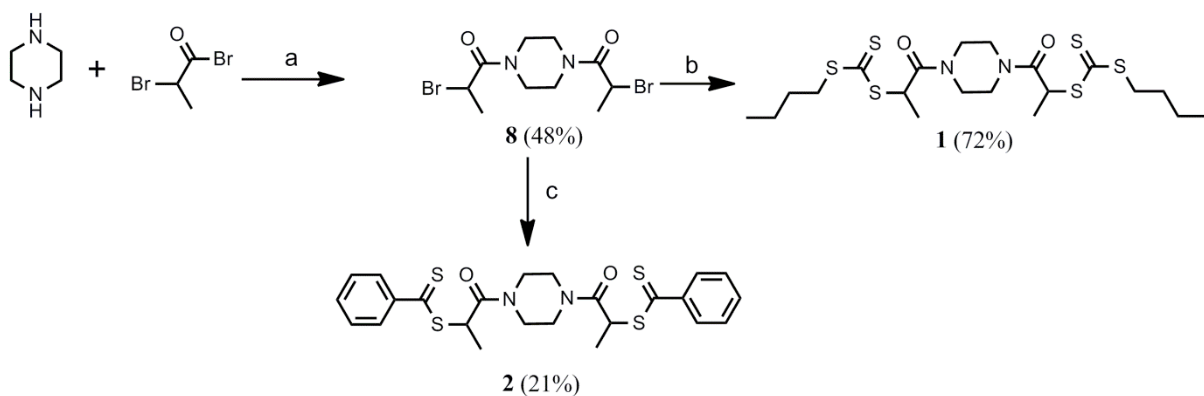
Experimental Section

General Methods

All non-aqueous reactions were performed under an atmosphere of dry nitrogen or argon, unless otherwise specified. DCM was distilled from CaH_2 in a recycling still. THF was dried with $LiAlH_4$ and then distilled from potassium metal in a recycling still. Et_3N was freshly distilled from CaH_2 under N_2. All reaction temperatures recorded are quoted as external temperatures. NAM was passed through an alumina plug before use. Reaction progress was monitored via thin-layer chromatography (TLC) using kieselgel 60 F_{254} plates (aluminium backed) and ethyl acetate/hexane or DCM/MeOH. Aluminium oxide 60 F_{254} neutral (Type E) plates were also used with ethyl acetate/hexane. TLC plates were visualised using a 254 nM UV lamp and/or a 10% w/v molybdophosphoric acid/EtOH stain or permanganate stain consisting of $KMnO_4$ (3.0 g), K_2CO_3 (20 g) and 5% w/v aqueous NaOH (5 mL) in H_2O (300 mL). "Flash column chromatography" was performed using silica gel (silica gel 60, 230-400 mesh ASTM) or aluminium oxide GF_{254} (Type 60/E) as the stationary phase and ethyl acetate/hexane or CH_2Cl_2/MeOH mixtures as the mobile phase. M_n NMR values were used to determine the amount of moles of RAFT polymers used in end-group modifications. ^{1}H, ^{13}C, DEPT, $^{1}H/^{13}C$-HSQC, $^{1}H/^{13}C$-HMBC and $^{1}H/^{1}H$-COSY Nuclear Magnetic Resonance (NMR) spectra were obtained on a Bruker Avance AV-300 spectrometer (^{1}H at 300.13 MHz and ^{13}C at 75.47 MHz, respectively), a Bruker Avance AV-400 spectrometer (^{1}H at 400.13 MHz and ^{13}C at 100.61 MHz, respectively), or a Bruker Avance AV-500 spectrometer (^{1}H at 500.19 MHz and ^{13}C at 125.78 MHz respectively). Proton and carbon chemical shifts were reported as δ values in parts per million (ppm) and are relative to residual solvent; coupling constants (J) are in Hz. Infrared (IR) spectra were recorded on a Shimadzu IR Prestige-21 Fourier-Transform Infrared Spectrometer. Samples

were analysed using either sodium chloride plates (NaCl) or attenuated total reflection (ATR) and absorptions are given in wavenumbers (cm^{-1}). Low resolution mass spectra were obtained on a Bruker Daltronics Esquire 6000 Ion-Trap mass spectrometer employing Electrospray Ionisation (ESI) with 40 eV cone voltage; samples were run in 0.1% formic acid. High resolution mass spectra were obtained on a Waters Q-TOF II, employing Electrospray Ionisation (ESI) with 35 eV cone voltage, using lock spray and sodium iodide as a reference sample. Low and high resolution mass spectra were also obtained on a ThermoQuest MAT95XP mass spectrometer, employing Electron Impact (EI) with 70eV cone voltage and employing PerFluoroKerosene (PFK) as a reference sample. Mass spectrometric analysis for the RAFT polymers was performed using a Bruker Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Data were acquired using reflector-positive ion mode, with an acceleration voltage of 20 kV and delayed extraction. Data acquisition was performed using Bruker Daltonics FlexControl 3.0 software and data analysis was carried out with Bruker Daltonics FlexAnalysis 3.0 software. Gel permeation chromatography (GPC) was performed on a Waters 2695 Separations Module, with a Waters 2414 Refractive Index Detector and a Waters 2996 Photodiode Array Detector, a series of four Polymer Laboratories PLGel columns (3 × 5 μm Mixed-C and 1 × 3 μm Mixed-E), Empower Pro Software and using THF (1.0 mL/min) as the mobile phase. The GPC was calibrated with narrow polydispersity polystyrene standards (Polymer Laboratories EasiCal PS-1, MW from 264 to 7500000 – Batch No. PS-1-43) and molecular weights are reported as polystyrene equivalents.



Scheme S1 *Reagents and conditions*: **a** $NaHCO_3$, $CHCl_3$, 5 °C; **b** CS_2, Et_3N, CH_2Cl_2, butanethiol; **c** bromobenzene, Mg, I_2, THF, CS_2, 75 °C/-5 °C/rt.

2-Bromo-1-[4-(2-bromopropanoyl)piperazin-1-yl]propan-1-one (8)

Compound **8** was prepared as described in the literature.[1] Compound **8** was obtained as a white solid (10.1 g, 48%) after recrystallisation with ethyl acetate and cyclohexane. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.54 (2H, q, J = 6.6 Hz), 4.07 (2H, br d, J = 6.9 Hz), 3.69-3.51 (4H, m), 3.35 (2H, d, J = 9.6 Hz), 1.81 (6H, t, J = 6.6 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.5, 45.1, 41.5, 37.4, 21.1.

2-Butylsulfanylcarbothioylsulfanyl-1-[4-(2-butylsulfanylcarbothioylsulfanylpropanoyl)piperazin-1-yl]propan-1-one (1)

Butanethiol (1.50 mL, 13.8 mmol), carbon disulfide (1.67 mL, 27.6 mmol), triethylamine (3.85 mL, 27.6 mmol) and DCM (10 mL) were combined and stirred overnight at room temperature (not under an inert atmosphere). Compound **8** (2.05 g, 5.8 mmol) was dissolved in DCM (8 mL) and the solution was added to the previously mentioned reaction mixture, which was then stirred overnight again at room temperature. The reaction mixture was then diluted with DCM, washed with water, brine, dried over $MgSO_4$ and then concentrated *in vacuo*. The crude material was purified by column chromatography on silica gel eluting with 0-50% EtOAc:Hexane to yield compound **1** as a bright yellow solid (2.17 g, 72%). IR (NaCl, cm^{-1}) 2957, 2928, 2868, 1647, 1456, 1427, 1373, 1203, 1088, 1047, 816; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.16 (2H, q, J = 6.6 Hz), 3.88-3.33 (11H, br m), 1.72-1.62 (5H, m), 1.52 (6H, d, J = 6.9 Hz), 1.42 (4H, sex., J = 7.2 Hz), 0.92 (6H, t, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 222.3, 168.8, 45.4, 45.2, 42.0, 36.8, 29.5, 21.7, 17.2, 3.2; MS-EI: m/z [M]$^+$ for

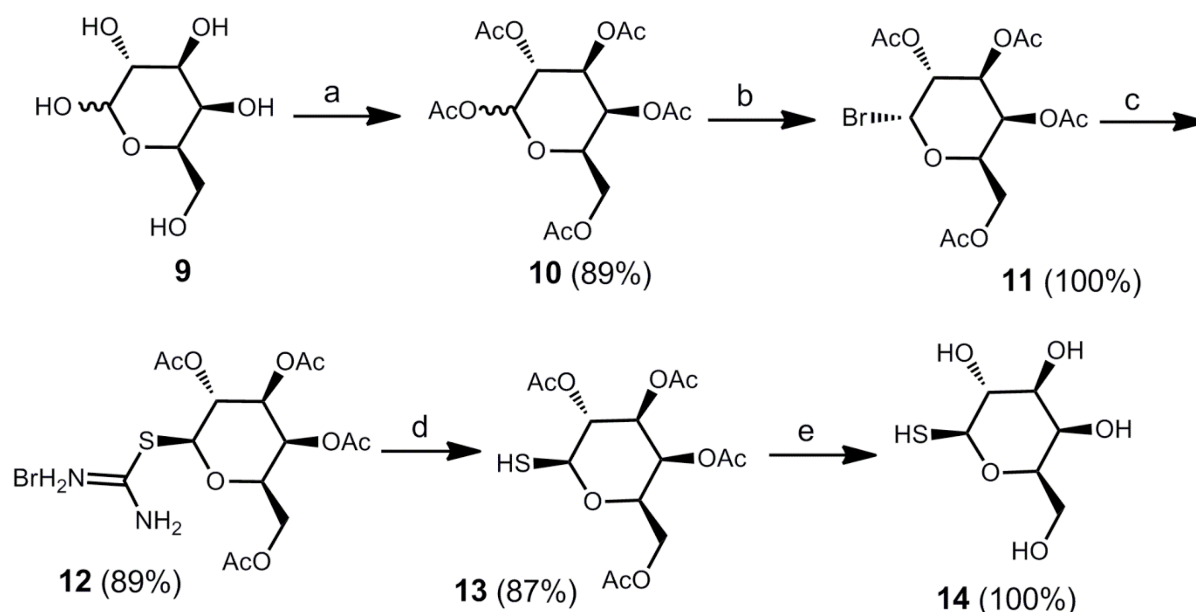
$C_{20}H_{34}N_2O_2S_6^+$ calculated 526.0, observed 526.0; HRMS-EI: *m/z* $[M]^+$ for $C_{20}H_{34}N_2O_2S_6^+$ calculated 526.0939, observed 526.0915.

[2-[4-[2-(Benzenecarbonothioylsulfanyl)propanoyl]piperazin-1-yl]-1-methyl-2-oxo-ethyl] benzenecarbodithioate (2)

The dithioester Grignard reagent was prepared by placing magnesium turnings (213 mg, 8.8 mmol) and dry THF (20 mL) in a three-necked round bottom flask that was fitted with a dropping funnel and a water condenser. Bromobenzene (854 μL, 8.2 mmol) in dry THF (10 mL) was added to the dropping funnel and approximately half of this solution was added dropwise to the previously mentioned reaction mixture. A catalytic amount of iodine was added to the reaction mixture, which was then heated to reflux. The remaining bromobenzene in THF was added to the gently refluxing solution and the solution was allowed to reflux for a further 20 min. The Grignard solution was then cooled to –5 °C and carbon disulfide was added dropwise, which immediately turned the colour of the solution from colourless to orange/red. The reaction was allowed to proceed at –5 °C for 35 min, after which compound **8** (1.32 g, 3.7 mmol) was added in one portion and the resulting mixture was allowed to stir at room temperature for 45 min. The reaction mixture was then diluted with DCM and washed with water. The aqueous phase was then re-extracted with DCM and the combined organic extracts were dried over $MgSO_4$ and concentrated *in vacuo*. The crude material was purified by column chromatography on silica gel eluting with 20-91% EtOAc:Hexane to yield compound **2** as a pale red solid (382 mg, 21%). IR (NaCl, cm^{-1}) 3055, 2974, 2926, 2860, 1709, 1643, 1589, 1445, 1310, 1175, 1043; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.95 (4H, br d, J = 7.2 Hz), 7.51 (2H, br d, J = 6.6 Hz), 7.37 (4H, br d, J = 7.2 Hz), 5.09 (2H, q, J = 6.9 Hz), 3.92-3.42 (8H, m), 1.58 (6H, d, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 225.4, 168.9, 143.7, 128.1, 126.7, 46.1, 45.5, 45.2, 42.0, 16.7; MS-ESI: *m/z* $[M + Na]^+$ for $C_{24}H_{26}N_2NaO_2S_4^+$ calculated 525.1, observed 525.1; HRMS-ESI: *m/z* $[M + Na]^+$ for $C_{24}H_{26}N_2NaO_2S_4^+$ calculated 525.0775, observed 525.0750.

1-Thio-β-D-galactopyranoside (14)

Compound **14** was prepared as described[2] (**Scheme S2**), using compound **13** (3.47 g) in a quantitative yield. Data for compound **14** were identical to literature values.[2] Compound **10** was prepared as described from D-galactose (**9**).[3] Data for compound **10** were identical to literature values.[3] Compounds **11-13** were prepared as described[2] and the data were identical to literature values.[2]



Scheme S2: *Reagents and conditions*: **a** Ac_2O, pyridine, DMAP, 0 °C/rt **b** HBr/AcOH; **c** thiourea, acetone, 60 °C; **d** $Na_2S_2O_5$, CH_2Cl_2/H_2O; **e** Na, MeOH.

RAFT polymerisation of *N*-acryloylmorpholine using RAFT agent 1

A stock solution (30 mL) was made up comprising NAM (7.20 g, 51.0 mmol), AIBN (2.51 mg, 2.4 mol%), trioxane (768 mg, 8.5 mmol) and the remaining volume was filled with dioxane. Two aliquots (5 mL) of the stock solution were added to ampoules containing the appropriate amount of RAFT agent **1** (36 mg for **NAM 126** and 27 mg for **NAM 166**). The solutions were degassed through four freeze-thaw-evacuate cycles, sealed under vacuum, and then placed in a thermostatted oil bath at 60 °C for 16 h and 10 min. The ampoules were then cooled, opened, and precipitated into ether. RAFT polymers (**NAM 126-166**) were obtained as fluffy pale yellow/very pale yellow solids. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.25-5.15 (2H, **CH**S(C=S)$SCH_2CH_2CH_2CH_3$), 4.25-2.87 (4H, S(C=S)S$\mathbf{CH_2}CH_2CH_2CH_3$; 8H, piperidine CH_2; *n* morpholine CH_2), 2.85-2.15 (2H, piperazineN(C=O)**CH**CH_3; *n*CH_2**CH**(C=O)N backbone), 1.92-1.45 (4H, S(C=S)$SCH_2\mathbf{CH_2}CH_2CH_3$; *n*$\mathbf{CH_2}$CH(C=O)N backbone), 1.43-

1.09 (4H, S(C=S)SCH$_2$CH$_2$**CH$_2$**CH$_3$; *n***CH$_2$**CH(C=O)N backbone), 1.05 (6H, br s, piperazineN(C=O)CH**CH$_3$**), 0.92 (6H, t, *J* = 2.2 Hz, S(C=S)SCH$_2$CH$_2$CH$_2$**CH$_3$**). *n* is the degree of polymerisation. GPC and NMR M_n values are shown in **Table** S1.

RAFT polymerisation of *N*-acryloylmorpholine using RAFT agent 2.

A stock solution (15 mL) was made up comprising NAM (5.62 g, 39.8 mmol), AIBN (3.3 mg, 10 mol%), trioxane (597 mg, 6.63 mmol), compound **2** (100 mg, 0.2 mmol) and the remaining volume was filled up with dioxane. One aliquot (4.0 mL) of the stock solution was added to an ampoule and the solution was degassed through four freeze-thaw-evacuate cycles, sealed under vacuum, and then placed in a thermostatted oil bath at 60 °C for 24 h. The ampoule was then cooled, opened, and precipitated into ether. **NAM 130** was obtained as strawberry/pale pink solid. ^{1}H NMR (CDCl$_3$, 400 MHz) δ 7.94-7.85 (4H, m, Ph), 7.54-7.50 (2H, t, *J* = 7.4 Hz, Ph), 7.40-7.37 (4H, t, *J* = 7.6 Hz, Ph), 5.17-5.05 (2H, **CH**S(C=S)Ph), 4.15-2.90 (8H, piperidine CH$_2$; *n* morpholine CH$_2$), 2.85-2.15 (2H, piperazineN(C=O)**CH**CH$_3$; *n*CH$_2$**CH**(C=O)N backbone), 2.05-1.45 (*n***CH$_2$**CH(C=O)N backbone), 1.45-1.24 (*n***CH$_2$**CH(C=O)N backbone), 1.04 (6H, br s, piperazineN(C=O)CH**CH$_3$**). *n* is the degree of polymerisation. GPC and NMR M_n values are shown in **Table** S1.

General Procedure for RAFT end group cleavage and disulfide coupling with Ellman's reagent. NAM 130 is used as an illustrative example.

NAM 130 (103.4 mg, 1 eq.) and Ellman's reagent (46 mg, 24 eq.) were both dissolved in deionised water (2 mL) and the reaction mixture was purged with N_2 for 15 min. *N*-Butylamine (24 μL, 50 eq.) was then added to the resulting pale red/pink solution and the reaction mixture was allowed to stir at room temperature for 16 h. The resulting orange/red/yellow mixture was then dialysed against deionised water in a 10 kDa membrane. The remaining solution in the membrane was then freeze-dried to yield **NAM 130 E** as a white solid (99.8 mg). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 7.95-7.55 (4H, br s, Ph), 7.53-7.32 (2H, br s, Ph), 4.55-3.05 (8H, piperidine CH$_2$; *n* morpholine CH$_2$), 3.05-2.15 (2H, piperazineN(C=O)**CH**CH$_3$; *n*CH$_2$**CH**(C=O)N backbone), 2.15-1.45 (*n***CH$_2$**CH(C=O)N backbone), 1.45-1.10 (*n***CH$_2$**CH(C=O)N backbone), 1.04 (6H, br s,

piperazineN(C=O)CH**CH_3**). GPC and NMR M_n values for **NAM 20-166 E** are shown in **Table S2**. *n* is the degree of polymerisation

General Procedure for disulfide exchange of Ellman's reagent with 1-thio-β-*D*-galactopyranoside. NAM 130 E is used as an illustrative example.

NAM 130 E (44.9 mg, 1 eq.) and 1-thio-β-D-galactopyranoside (29.7 mg, 59 eq.) were both dissolved in a phosphate buffer (2 mL, pH ~8; prepared from NaH_2PO_4 and Na_2HPO_4) and the reaction mixture was purged with N_2 for 5 min. The reaction mixture was then allowed to stir at room temperature for 18 h. The resulting reaction mixture was then dialysed against deionised water in a 10 kDa membrane. The remaining solution in the membrane was then freeze-dried to yield **NAM 130 S** as a white solid (40.7 mg, 93% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.35 (2H, br s, anomeric galactose H), 4.35-2.90 (8H, piperidine CH_2; *n* morpholine CH_2; 12H, galactose H), 2.90-2.00 (2H, piperazine N(C=O)**CH**CH_3; *n*CH_2**CH**(C=O)N backbone), 2.00-1.40 (*n*$\mathbf{CH_2}$CH(C=O)N backbone), 1.40-1.12 (*n*$\mathbf{CH_2}$**CH**(C=O)N backbone), 1.06 (6H, br s, piperazine N(C=O)CH$\mathbf{CH_3}$). *n* is the degree of polymerisation.

Table S1: Properties of PNAM with trithiocarbonate, dithioester and piperazine end-group functionality.

Polymers	$[NAM]_O$/ $[RAFT]_O$	Time (h)	Convn (%)	Theor M_n	NMR M_n	GPC (THF) M_p	M_w/M_n	λ_{max} (nm)
NAM 126	126.0/1.0	16	>99	18300	16300	13100	1.20	308
NAM 130	200.0/1.0	24	65	18900	15300	13300	1.19	265
NAM 166	166.0/1.0	16	>99	24000	22200	16700	1.18	308

Table S2: Properties of PNAM with Ellman's end-group functionality.

Polymers	Theor M_n	NMR M_n	GPC (THF) M_p	M_w/M_n	λ_{max}
NAM 126 E	18500	20800	12100	1.08	313
NAM 130 E	19000	19000	12900	1.13	318
NAM 166 E	24100	27700	15800	1.08	309

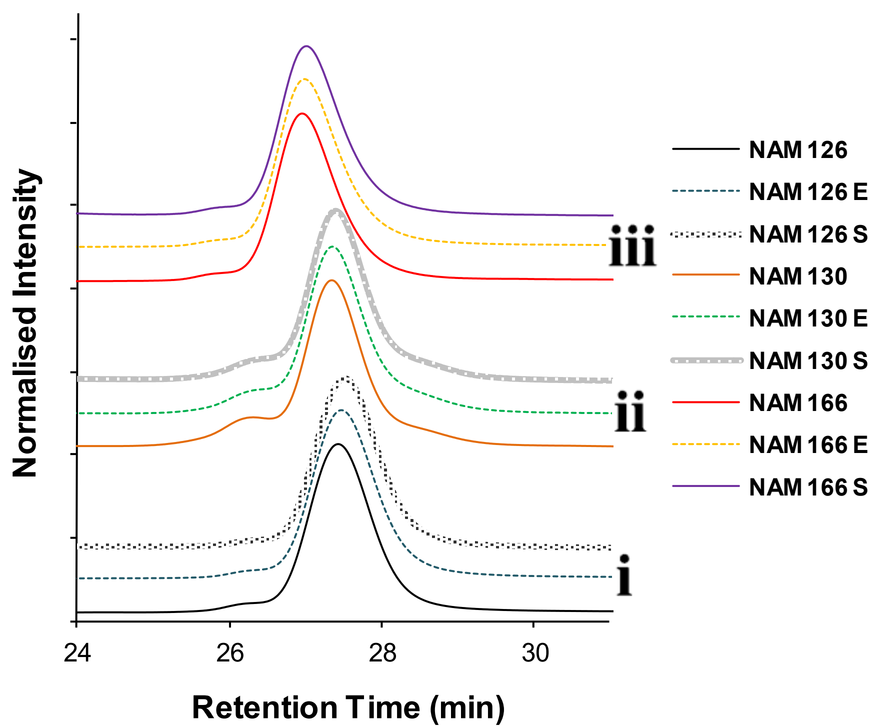


Figure S1. Comparison of GPC traces for the end-group modifications that were performed with PNAM. **i NAM 126**; **ii NAM 130**; **iii NAM 166**.

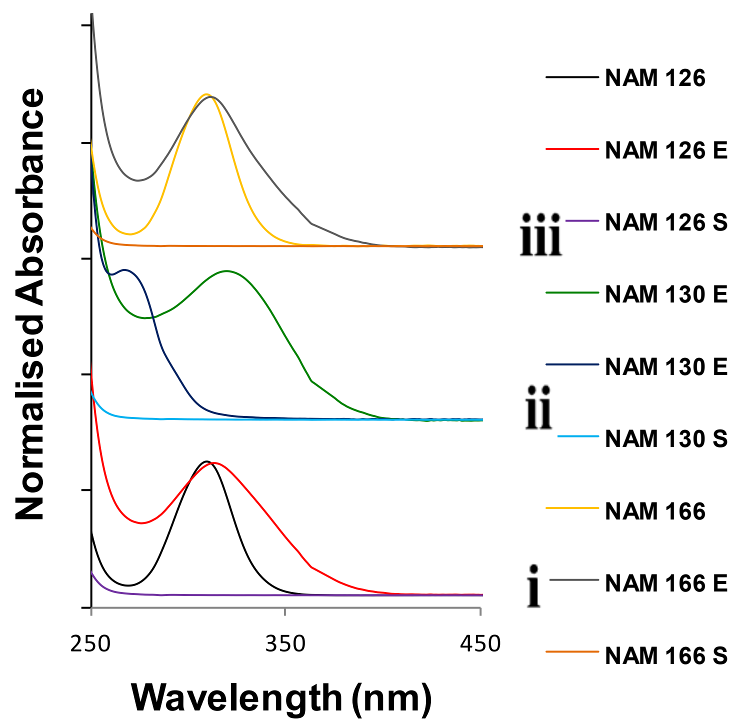


Figure S2. Comparison of the UV traces for the end-group modifications that were performed with PNAM: **i NAM 126**; **ii NAM 126 E**; **iii NAM 126 S**.

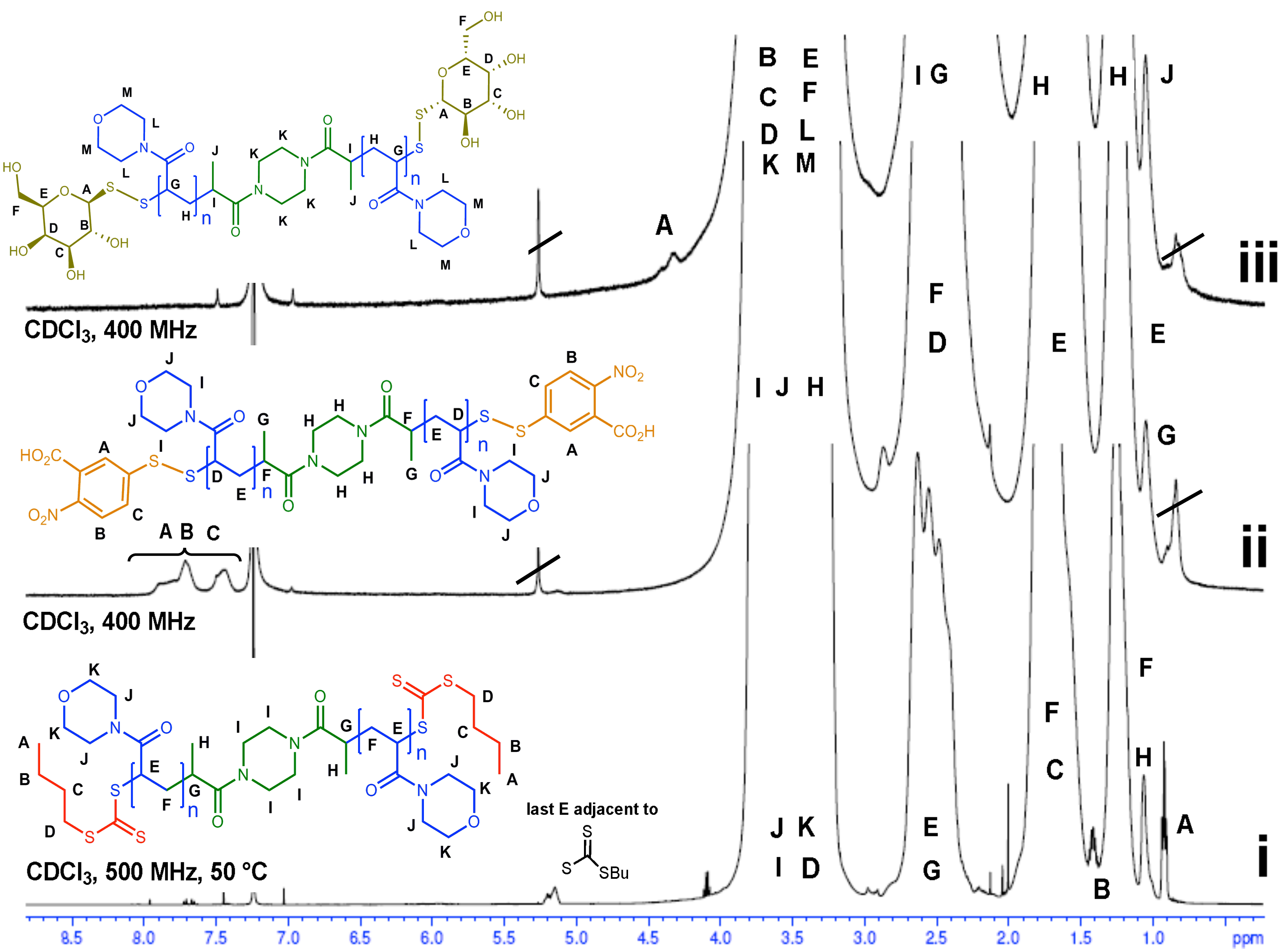


Figure S3. Overlapped ^{1}H-NMR spectra of: i NAM 126; ii NAM 126 E; iii NAM 126 S.

Biological studies

The binding of cholera toxin (CT) to its cell surface receptor (G_{M1}) was determined as described previously.[4,5] In brief, a solution of G_{M1} in phosphate-buffered saline (PBS) was used to coat wells of a Nunc-Immuno Maxisorp 96-well plate (100 µl per well) overnight at room temperature. After this and subsequent incubations, the plate was washed 3 times with PBS. The wells of the plate were initially incubated one hour with 360 µl blocking buffer (0.25% bovine serum albumin in PBS-0.05% Tween 20) to block non-specific binding sites, and then with 100 µl CT (1-100 ng/ml in blocking buffer), 100 µl goat anti-CT antibody in blocking buffer, and 100 µl rabbit anti-goat alkaline phosphatase conjugate in blocking buffer. These incubations were at room temperature for one hour, except for CT (1.5 hours). Finally, the plate received an extra wash with distilled water, and 100 µl alkaline phosphatase substrate (para-nitro phenol) in Tris buffer was added. The rate of change of absorbance at 405 nm was measured over 10 minutes using a Bio-Tek Synergy HT plate reader (Bio-Tek, Winooski, VT, USA). Inhibition of the binding of CT to immobilised G_{M1} was determined by incubating the inhibitor with CT (total volume 130 µl) for 1 hour in a disposable glass tube with gentle rocking on a rocking platform Mixer (Ratek Instruments, Melbourne, Australia) during the incubation of the assay plate with 360 blocking buffer. A 100 µl aliquot from each test tube was then added to a well of the 96-well plate, after which the assay proceeded as normal. The amount of unbound CT was determined by reference to a control plot of rate of absorbance change *vs* CT concentration that was performed on the same plate. The amount of unbound CT was plotted against the concentration of inhibitor. If the values of percent inhibition of binding approached or spanned 50%, the IC_{50} for inhibition of binding was calculated from a second-order polynomial fit to the data. Control plots were obtained by plotting [(CT concentration) divided by (10^3 × rate of absorbance change)] *vs* (CT concentration). A typical example is shown in Figure S3, which illustrates the linear

nature of the plot. Knowing the intercept and gradient of this plot, the concentration of free CT (i.e., CT not bound to immobilised G_{M1}) could be determined from the rate of absorbance change at any given concentration of inhibitor of CT binding to immobilised G_{M1}. An example of a polynomial fit to a plot of free [CT] *vs* concentration of inhibitor (in this case **NAM 130**) is shown in Figure S4.

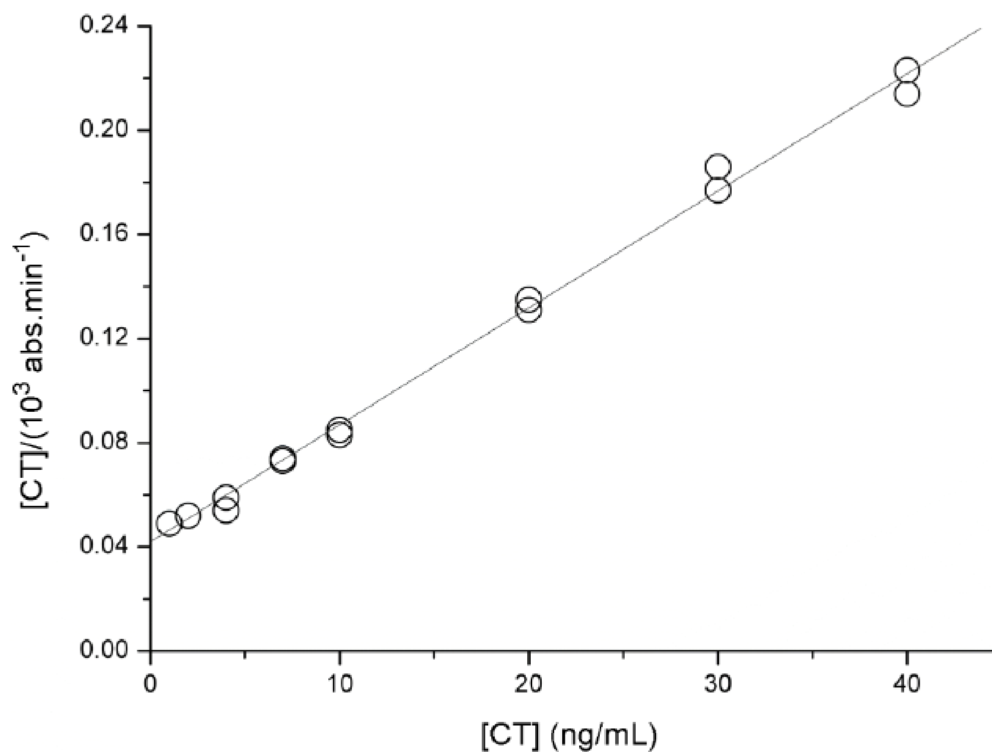


Figure S3: Linear form of the relationship of rate of absorbance change with concentration of CT in control wells.

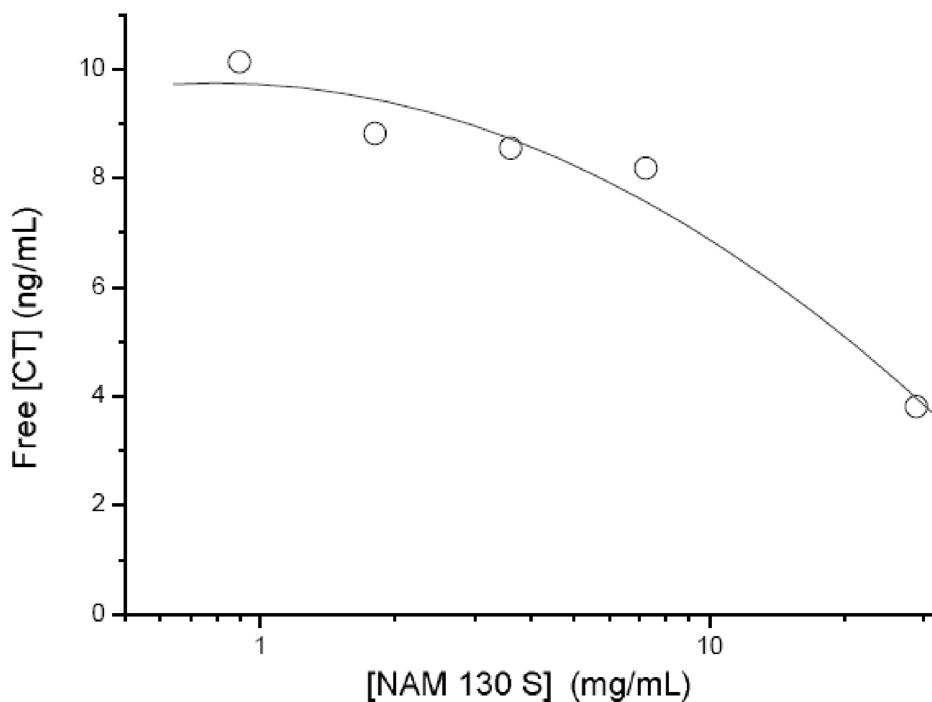


Figure S4: Inhibition of the binding of 10 ng/ml CT to immobilised G_{M1} by **NAM 130 S**.

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