Supporting Information

Synthetic analogs of the snail toxin 6-bromo-2-mercaptotryptamine dimer (BrMT) reveal that lipid bilayer perturbation does not underlie its modulation of voltage-gated potassium channels

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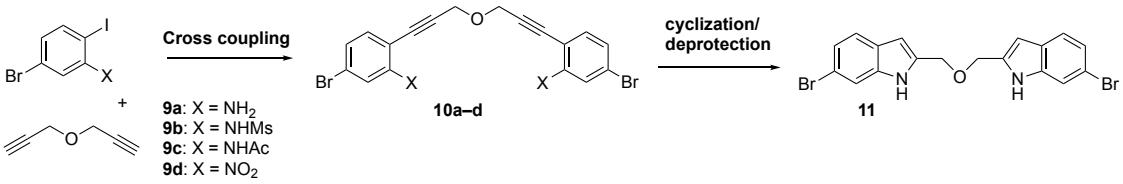
1. Supplementary Figures and discussion

Table S1. Optimization of bis-indole synthesis



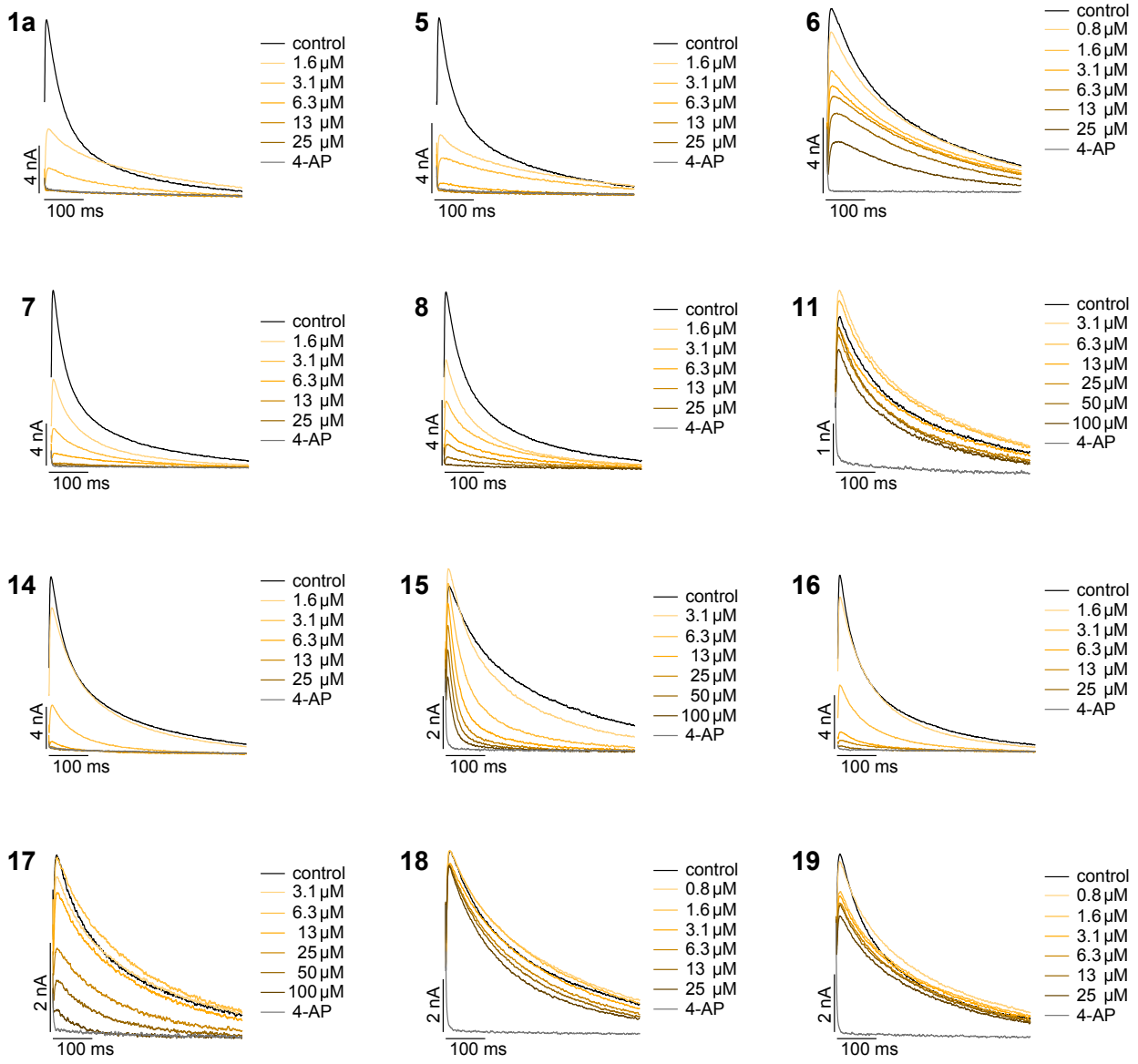
Entry	X	Cross Coupling Conditions[a]	Cyclization/ Deprotection Conditions[b]	Results[c]
1[d]	NH_2	$PdCl_2$ (5 mol%), PPh_3 (10 mol%), CuI (5 mol%), NEt_3, 60 ˚C, 14 h	–	Complex mixture
2	NO_2	$Pd(PPh_3)_2Cl_2$ (5 mol%), CuI (20 mol%); CH_3CN: NEt_3 (5:1), 2 h	$SnCl_2$–$2H_2O$ (6 eq.)	85% **10d**; Complex mixture obtained after attempted reduction.
3	NH_2	$Pd(PPh_3)_2Cl_2$ (3 mol%), CuI (5 mol%); NEt_3; 6 h	$NaAuCl_4$ (3 mol%)	90% **10a**; Cyclization reaction failed.
4[d]	NHMs	[$Pd(PPh_3)_2Cl_2$ (5 mol%), CuI (8 mol%); DMF: NEt_3 (1:1), 75 ˚C, 15 h	–	30% **11**
5[d]	NHAc	$Pd(PPh_3)_2Cl_2$ (10 mol%), CuI (27 mol%); CH_3CN: NEt_3 (5:1), 45 min.	TBAF (7.5 eq.)	Complex mixture
6	NHAc	$Pd(PPh_3)_2Cl_2$ (5 mol%), CuI (20 mol%); CH_3CN: NEt_3 (5:1), 45 min.	KO*t*-Bu (15 eq.)	90% **10c**; Attempted cyclization yielded a complex mixture.
7	NHAc	$Pd(PPh_3)_2Cl_2$ (5 mol%), CuI (20 mol%); CH_3CN: NEt_3 (5:1), 45 min.	TBAF in 0.1 M THF (7.5 eq.), 45 ˚C	90% **10c**; then 94% **11**

[a] Substrate **9** (0.40 mmol) was added to a 25 mL oven-dried flask with stir bar, sealed with a septum, and flushed with N_2. Dry CH_3CN or DMF (4 mL) that had been flushed with Ar in a sealed flask for 10 min. was transferred to the reaction flask by syringe. Pd(II) catalyst (3–10 mol%) and CuI (5–27 mol%) were then quickly added as solids, the flask was resealed, and NEt_3 (ratio to the solvent noted) followed by propargyl ether (0.21 mmol) were added by syringe. Reactions were performed at 20 ºC, unless otherwise noted. [b] Intermediate **10** (typically 0.2 mmol) was added to a 20 mL vial with stir bar and sealed under N_2, followed by the addition of solvent (2 mL) by syringe, and reducing agent and/or base. Reactions were performed at 20 °C, unless otherwise noted. [c] Refers to isolated yields where indicated. [d] Cross coupling, cyclization, and deprotection steps were performed in one pot.

Unprecedented double Larock reactions(*1, 2*) between several 2-iodoanilines and dipropargyl ether were attempted using several palladium (II) catalysts, but disappointingly these only gave complex, intractable mixtures of products (e.g., entry 1). Early attempts at an S_NAr reaction(*3*) between 2-fluoronitrobenzene and the anion of dipropargyl ether were unsuccessful (details not shown). A double Sonogashira cross coupling between 5-bromo-2-iodonitrobenzene **9d** and propargyl ether proceeded well, but attempts to reduce the nitro groups with subsequent ring closure gave an intractable mixture of products (entry 2). A similar double Sonogashira cross coupling with 5-bromo-2-iodoaniline **9a** was also successful (entry 3), but no reaction was observed when $NaAuCl_4$ was used to promote the ring closure according to a protocol from Zhang.(*4*) Using a protocol reported by Sakamoto,(*5*) we conveniently obtained a 30% yield of **11** via a one pot, double Sonogashira reaction, cyclization, and hydrolysis starting from the methanesulfonamide **9b** (entry 4). This yield was tolerable for a medicinal chemistry route to our desired compound, but the low yield and expense associated with the preparation of **9b** inspired us to explore alternatives. The analogous one pot reaction with acetamide **9c** gave a complex mixture (entry 5), though the double Sonogashira reaction without concurrent cyclization was effective (entry 6). The attempted cyclization of **10c** to **11** using KO*t*-Bu gave only a complex mixture (entry 6),

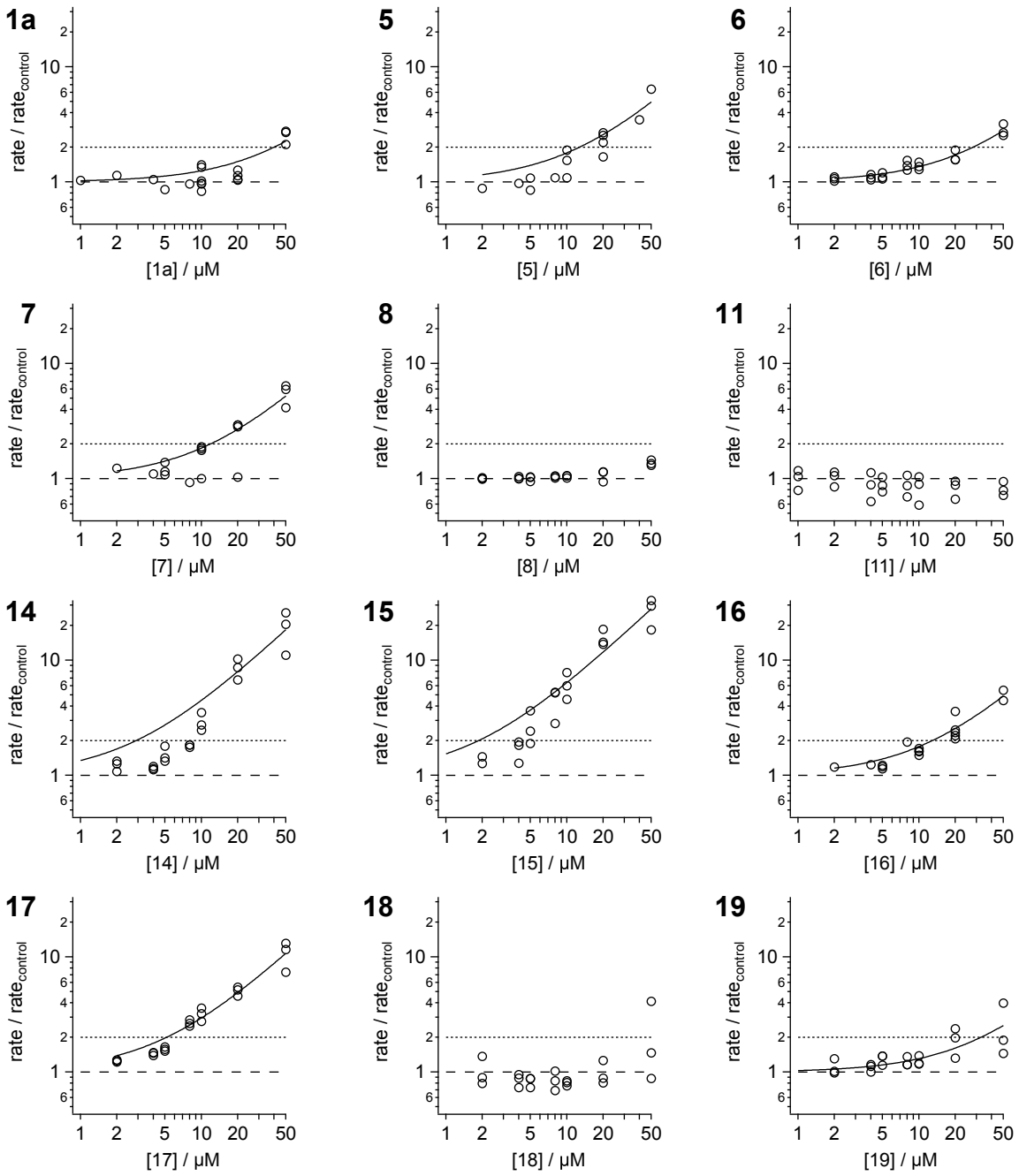
but switching to excess TBAF at 45 °C permitted isolation of the desired bis-indole **11** in excellent yield after ring closure (indole formation) and hydrolysis (entry 7). Several solvents (DMF, THF) and catalyst loadings were also tested briefly, with the optimal result (5 mol% $Pd(PPh_3)_2Cl_2$, 20 mol% CuI, and 5:1 CH_3CN/NEt_3) given in entry 7. We noticed that the ring closure and hydrolysis steps were sensitive to the water content of the solution. Commercially available 1M TBAF solution in THF worked well, but a freshly prepared solution made from solid TBAF did not work unless water was added to the reaction.

Figure S1. Representative Kv1.4 current responses to modulators



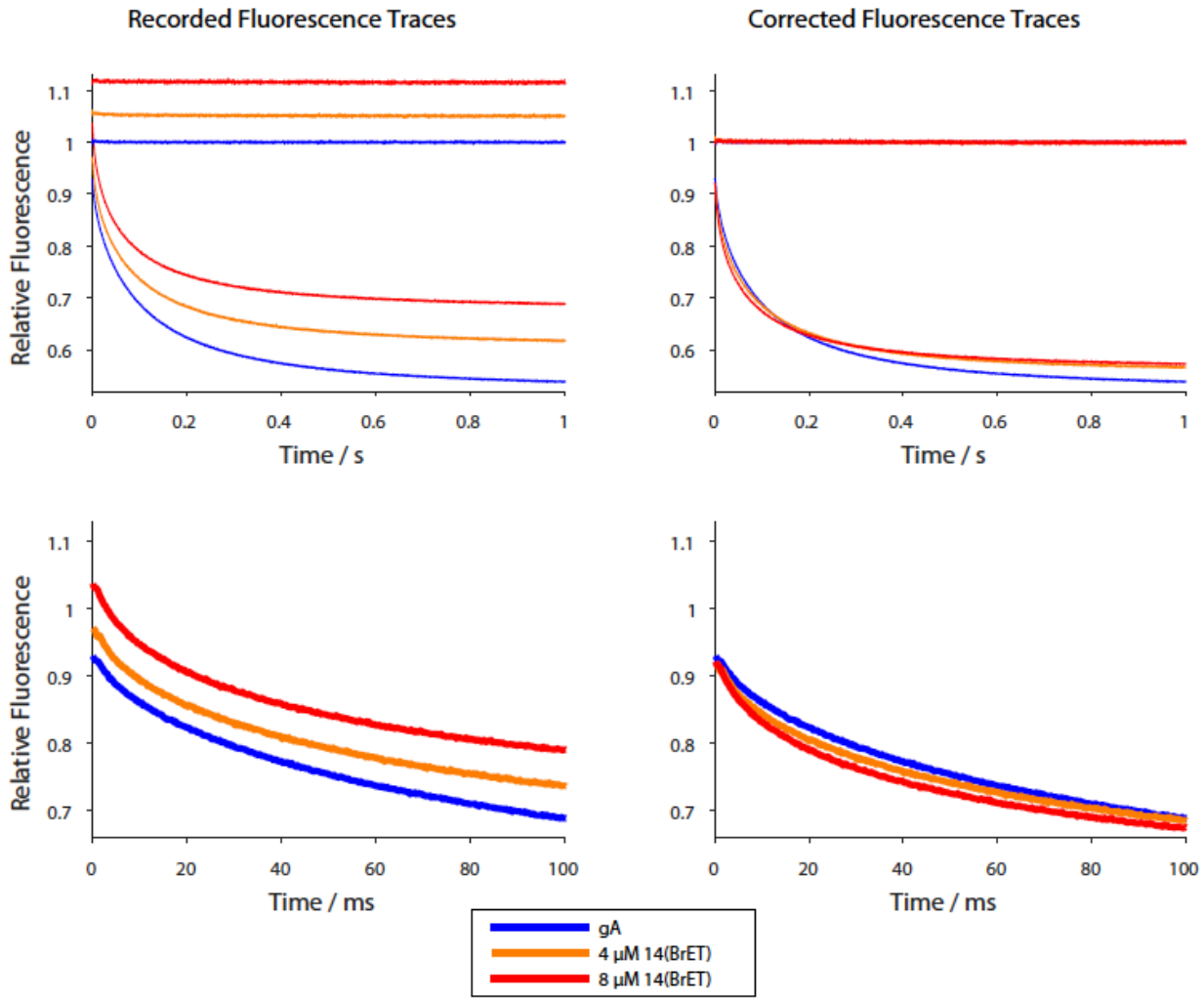
Current measured during voltage steps from -100 mV to 0 mV. Compound tested is indicated by number to left of each panel. Concentration tested is indicated in legend. 4-AP indicates currents remaining during application of 10 mM 4-aminopyridine.

Figure S2. Gramicidin A assay data



In panels, circles indicate Tl^+ quench rates normalized to quench rate under control conditions. Each circle at same concentration is an independent experiment. Lines are fits of Equation 6 (see SI Section 8). Fit values are reported in Table 1 of the manuscript.

Figure S3. Representative normalization of fluorescence in Gramicidin A assay



The effect of BrMT on the time course of intravesicular quenching of ANTS as a measure of gramicidin A (gA) activity (see Section 8 for full protocol). Samples were excited at 352 nm and measurements of the emission were recorded at 455 nm. Traces in blue are the quenching of ANTS loaded LUVs without the presence of BrMT; traces in orange are of ANTS loaded LUVs in the presence of 4 µM BrMT; and traces in red are of ANTS loaded LUVs in the presence of 8 µM BrMT LUVs. The figure shows fluorescence traces obtained with BrET at two different time resolutions (top panels 0 – 1 s; bottom panels 0 –100 ms). In each panel, the top three traces (clearly visible only in the left panels) show the fluorescence signal recorded in the absence of Tl^+, whereas the lower three traces show the fluorescence signal in the presence of Tl^+. Left panels, the fluorescence time courses recorded in the absence of BrET and the presence of 4 and 8 µM BrET, normalized the signal recorded in the absence of Tl^+ and BrET. The difference in the traces recorded in the absence of Tl^+ reflects the intrinsic BrET fluorescence. Right panels, the corrected fluorescence time courses obtained by subtracting the intrinsic fluorescence signal due to BrET (determined from the traces recorded in the absence of Tl^+).

2. General information for synthetic work

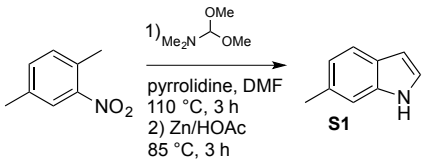
All reagents and solvents were purchased from commercial vendors and used as received, and water for reactions and workups was deionized and purified by charcoal filtration. Saturated ammonia in dichloromethane (DCM) solution was obtained by shaking 1 volume of 40% aq. NH_4OH with 3 volumes of DCM in a separatory funnel for 1 minute, then drying in a bottle over excess Na_2SO_4. NMR spectra were recorded on Varian 300 MHz or 400 MHz spectrometers as indicated. Proton and carbon chemical shifts are reported in parts per million (ppm; δ) relative to tetramethylsilane (^{1}H δ 0), $(CD_3)_2CO$ (^{1}H δ 2.05, ^{13}C δ 29.84), d_6-DMSO (^{1}H δ 2.50, ^{13}C δ 39.5), or CD_3OD (^{1}H δ 3.31, ^{13}C δ 49.00). NMR data are reported as follows: chemical shifts, multiplicity (obs = obscured, app = apparent, br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, comp = complex overlapping signals); coupling constant(s) in Hz; integration. Unless otherwise indicated, NMR data were collected at 25 °C. Flash chromatography was performed using Biotage SNAP cartridges filled with 40-60 μm silica gel on Biotage Isolera systems, with photodiode array UV detectors. Analytical thin layer chromatography (TLC) was performed on Agela Technologies 0.25 mm glass plates with 0.25 mm silica gel. Visualization was accomplished with UV light (254 nm) and $KMnO_4$ stain followed by heating, unless otherwise noted. Tandem liquid chromatography/mass spectrometry (LC-MS) was performed on a Shimadzu LCMS-2020 with autosampler, photodiode array detector, and single-quadrupole MS with ESI and APCI dual ionization, using a Peak Scientific nitrogen generator. Unless otherwise noted, a standard LC-MS method was used to analyze reactions and reaction products: Phenomenex Gemini C18 column (100 x 4.6 mm, 3 μm particle size, 110 A pore size); column temperature 40 °C; 5 μL of sample in MeOH at a nominal concentration of 1 mg/mL was injected, and peaks were eluted with a gradient of 15−95% MeOH/H_2O (both with 0.1% formic acid) over 3 min., then 95% MeOH/H_2O for 2.25 min. Purity was measured by UV absorbance at 210 or 254 nm. Chemical names were generated and select chemical properties were calculated using ChemAxon Marvin suite (https://www.chemaxon.com). NMR data were processed using ACD/NMR Processor Academic Edition (http://www.acdlabs.com).

Abbreviations: DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; EtOAc, ethyl acetate; HOAc, acetic acid; HPLC, high-performance liquid chromatography; LAH, lithium aluminum hydride; MTBE, methyl *tert*-butyl ether; NBS, *N*-bromosuccinimide; TBAF, tetrabutylammonium fluoride; THF, tetrahydrofuran; TLC, thin layer chromatography.

3. Synthesis of tryptamine building blocks

Tryptamine (from Alfa Aesar), 6-methyoxytryptamine (Acros), 5-bromoindole (Alfa Aesar), 6-fluoroindole (TCI), and 6-chloroindole (Alfa Aesar) were purchased and used as received. 1-Dimethylamino-2-nitroethylene and 6-bromotryptamine were synthesized according to Nicolaou et al., (*6*) and purified by column chromatography.

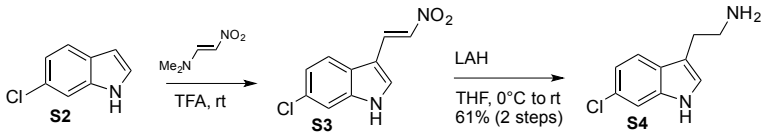
Synthesis of 6-methyl indole via Leimgruber-Batcho reaction(*7*) (unoptimized)



2,5-Dimethylnitrobenzene (4.53 g, 30.0 mmol) and DMF (60 mL) were added to a 1 L flask, which was sealed and flushed with nitrogen. DMF-dimethylacetal (12.0 mL, 90.3 mmol) and pyrrolidine (2.50 mL, 30.0 mmol) were then added by syringe, and the reaction was heated at 110 °C for 3 h. The flask was cooled to rt and diluted with Et_2O (300 mL) and water (300 mL). The aqueous layer was back-extracted with Et_2O (3 x 100 mL), and the combined organics were dried over Na_2SO_4, filtered, concentrated, and dried under high vacuum. The resulting oil was dissolved in 4:1 HOAc/H_2O (100 mL) and heated at 75 °C. Zn powder (8.83 g, 135 mmol) was added portionwise over 1 h while stirring vigorously, then the mixture was heated at 85 °C for 2 h. The reaction was cooled, filtered, and neutralized by slowly adding aqueous $NaHCO_3$ in a large flask with vigorous stirring. The resulting phases were separated, and the combined organics were washed with water, then brine, before drying over $MgSO_4$, filtering, and concentrating to a brown oil. The crude material was dissolved with toluene and purified by flash chromatography (100 g SiO_2 cartridge; 0–25% EtOAc/hexanes gradient) to give 6-methylindole (**S1**) as a brown liquid (463 mg, 12%),[1] consistent with literature characterization data.(*8*)

[1] We suspect that improved results may be achieved by performing the reduction/cyclization step under nitrogen and minimizing the exposure of the crude product to air/light (especially when dissolved in non-polar solvents). A significant amount of highly-colored byproducts were observed, which are presumably derived from indole oxidation.

Representative procedure for tryptamine synthesis via 3-(2-nitrovinyl)indoles: Synthesis of 6-chlorotryptamine.

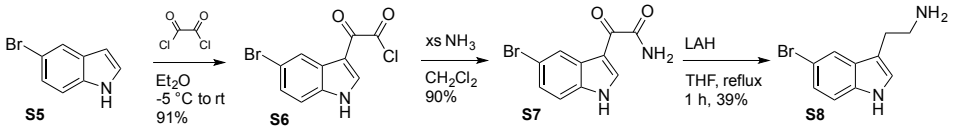


A flask containing 6-chloroindole (100 mg, 0.660 mmol) and 1-dimethylamino-2-nitroethylene (77 mg, 0.663 mmol) was sealed under nitrogen and cooled on ice. Trifluoroacetic acid (1 mL) was added and the suspension was removed from the ice bath, after which time the mixture gradually dissolved. The reaction was stirred for 20 h, after which time TLC analysis (50% EtOAc/hexanes) showed complete conversion to a single more polar product (R_f = 0.3) appearing as a bright yellow spot on the TLC plate. The reaction was quenched by slowly pipetting into a flask containing half-saturated aqueous $NaHCO_3$ (20 mL) and EtOAc (10 mL). The aqueous layer was re-extracted with EtOAc, then the combined organics were dried over $MgSO_4$, filtered, concentrated, and dried under high vacuum.

The crude nitrovinylindole **S3** was then dissolved with dry THF and transferred dropwise by syringe (using total 6 mL THF) to a flask containing LAH (125 mg, 3.29 mmol) and THF (3.3 mL) sealed under nitrogen on ice, with a vent line to an oil bubbler. The reaction was stirred for 5.5 h, then quenched by adding dropwise a saturated solution of aqueous Na_2SO_4 (1 mL) and stirring for 0.5 h. The resulting supernatant was transferred to a separatory funnel, and the remaining salts were washed with EtOAc, which was also transferred to the funnel. The combined organics were washed with water, then brine, before drying over Na_2SO_4, filtering, and concentrating to a red oil. The crude material was dissolved with DCM/EtOAc and purified by flash chromatography (~2.5 x 10 cm SiO_2). The product was eluted with DCM saturated with NH_3 (100 mL), then 1–3% MeOH/DCM (saturated with NH_3) to give a red oil (78 mg, 61% from 6-chloroindole), consistent with literature characterization data for 6-chlorotryptamine (**S4**).(*9*)

In this manner, 6-fluorotryptamine was also made (39% yield from 6-fluoroindole).

Representative procedure for tryptamine synthesis via Speeter-Anthony protocol: Synthesis of 5-bromotryptamine.



5-Bromoindole (**S5**) (588 mg, 3.0 mmol) was added to a 40 mL vial with stir bar, and also a screw cap with silicone septum. The vial was sealed under nitrogen and dry Et_2O was added by syringe before the vial was cooled in an ice bath. Oxalyl chloride (0.8 mL, 9.2 mmol) was added dropwise with rapid stirring, which led to the formation of a yellow precipitate. The reaction was removed from the bath and stirred for 16 h before filtering the solid with a Hirsch funnel and washing with Et_2O (5 mL).

The solid was transferred to a 50 mL flask and dried under high vacuum, to give 780 mg (91%) of the crude glyoxyl chloride **S6**. A saturated solution of NH_3 (~0.45 M) in DCM (33 mL, 15 mmol) was added to the flask, which was sealed with a plastic cap (secured by tape) and stirred vigorously for 1 h. The resulting suspension (containing both glyoxamide product and NH_4Cl) was concentrated, filtered, washed with water (to remove NH_4Cl), then Et_2O. The resulting waxy solid was dried under high-vacuum to yield 653 mg (90%) of glyoxamide **S7** as an off-white powder. ^1H NMR (d_6-DMSO, 400 MHz): δ 11.50 (br s, 1

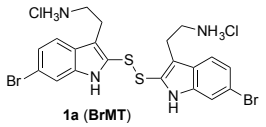
H), 8.71 (s, 1 H), 8.32 (d, *J* = 2.0 Hz, 1 H), 8.08 (br s, 1 H), 7.74 (br s, br, 1 H), 7.49 (d, *J* = 8.6 Hz, 1 H), 7.38 (dd, *J* = 8.6, 2.0 Hz, 1 H).

Glyoxamide **S7** (300 mg, 1.12 mmol) was added to an oven-dried 50 mL 2-neck flask with stopcocked sidearm and stir bar. An oven-dried water condenser was attached, and the apparatus was sealed with septa and evacuated and backflushed with nitrogen three times before dry THF (3 mL) was added to form a suspension. LAH (213 mg, 5.6 mmol) was added to a large oven-dried vial and sealed under nitrogen. Dry THF (6 mL) was added to form a slurry, which was then transferred by syringe and 18-gauge, 3" steel needle slowly through the stopcock of the flask to the rapidly stirring suspension of **S7**. Additional THF (2 mL total) was used to rinse the vial and syringe. The reaction was then heated at 70 ºC for 1 h, then cooled on ice and quenched by the dropwise addition of a saturated solution of aqueous Na_2SO_4 (1.5 mL). The resulting salts were filtered with Celite and washed well with Et_2O (70 mL). The combined organics were washed with water, then the tryptamine product was extracted with 1M aq. HCl (2 x 25 mL). The acidic extracts were basified with 30% aq. NaOH, then the resulting free base was extracted with EtOAc (4 x 20 mL) after saturating with NaCl. The combined organics were dried over Na_2SO_4, filtered, and concentrated to an orange oil. The crude material was dissolved with EtOAc and purified by flash chromatography (~3 x 12 cm SiO_2). The product was eluted with DCM saturated with NH_3 (50 mL), then 1–4% MeOH/CH_2Cl_2 (DCM saturated with NH_3) to give a red oil which slowly crystallized (105 mg, 39%), consistent with literature characterization data for 5-bromotryptamine (**S8**).(*10*)

In this manner, 6-methyltryptamine was also prepared.(*11*)

4. Protocol for bistryptamine–disulfide formation

Representative procedure for bistryptamine-2-disulfide synthesis: Synthesis of BrMT (1a).



1a (BrMT)

6-Bromotryptamine (110 mg, 0.46 mmol) was added to a 5 mL oven-dried flask with stir bar. Trichloroacetic acid (150 mg, 0.92 mmol) was added to the flask in a glove bag, then sealed with a septum. The flask was flushed with nitrogen, then dry THF (1 mL) was added and the reaction was cooled in an ice bath before freshly-distilled (via U-tube under nitrogen) S_2Cl_2 (~18.4 µL, 0.23 mmol) was added slowly via microsyringe. The reaction was left in the bath to warm slowly, stirring for 18 h before it was quenched with saturated aqueous $NaHCO_3$ (2 mL). The mixture was diluted with EtOAc and water, separated, and the combined organics were dried over Na_2SO_4, filtered, and concentrated to a red oil (177 mg). LC-MS analysis indicated that the desired product was present (M+1 = 541), along with bistryptamine monosulfide and trisulfide byproducts. The crude oil was dissolved with MeOH and treated with HCl in dioxane to form the dihydrochloride salt, which is more stable to storage.

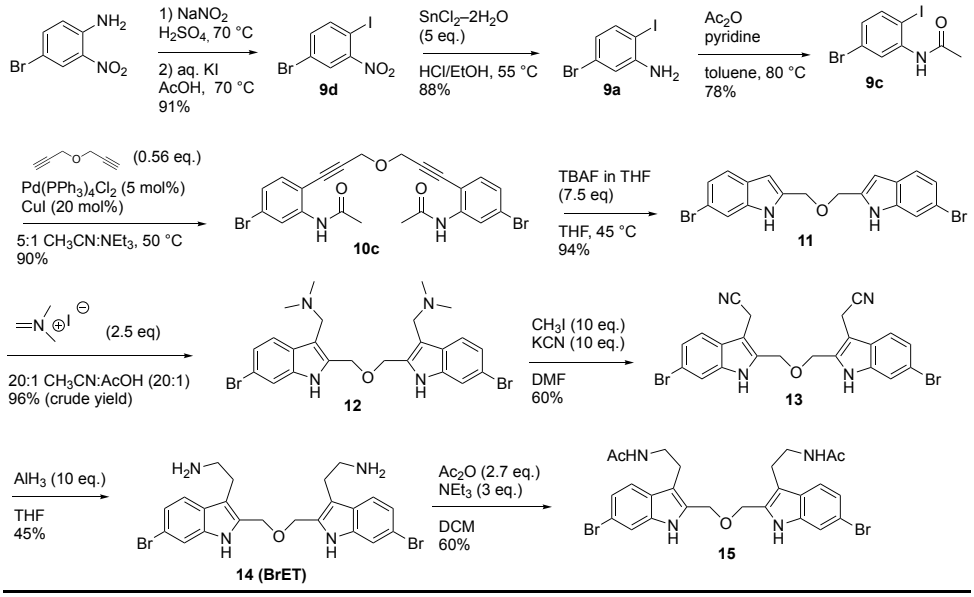
A portion of this material (88 mg, ~0.14 mmol) was purified by a reductive/oxidative work-up. The oil was transferred in MeOH (total 2 mL) to a 2-dram vial with stir bar. The vial was cooled on ice and $NaBH_4$ (54 mg, 1.4 mmol) was added portionwise with stirring. The reaction was sealed with a septum and flushed with nitrogen before water (2 mL) and Et_2O (2 mL) were added by syringe. The layers were mixed well by stirring rapidly, then the ether layer was carefully siphoned off by syringe. This process was repeated twice, then EtOAc (2 mL) and aq. H_2O_2 (3 wt%, 146 µL, 0.14 mmol) were added and the biphasic mixture stirred well. A bright yellow color indicative of the disulfide was immediately visible. The top organic layer was pipetted off and the aqueous layer was re-extracted twice with EtOAc (2 mL).

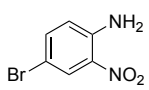
The combined organics were dried over Na_2SO_4, filtered, concentrated, re-dissolved in methanol, and treated with HCl in dioxane to reform the dihydrochloride salt. This was finally concentrated to give 25 mg (~35%) of a bright yellow oil/solid. LC-MS analysis (5–100% CH_3CN/H_2O + 0.1% formic acid, 17 min. run) showed the desired product in 90–95% purity without mono- and trisulfide byproducts.

The purity of a portion of this BrMT was improved slightly by preparative HPLC. An optimized method was developed: A Shimadzu semi-preparative HPLC with autosampler, photodiode array UV detector, and fraction collector was used; Phenomenex Gemini C_{18} 250 x 10 mm column (10 µm particle size); 5 mL/min flow rate; maximum 100 µL injections of 10 mg/mL BrMT in MeOH; 10–35% CH_3CN/H_2O + 0.1% TFA over 5 min., 35% for 7 min., 35–100% over 2 min., 100–10% over 2 min., and 10% for 4 min. prior to the next injection. Under these conditions, the major peak was eluted at 9.8 min. Clean fractions containing the major peak were combined and the solution was basified with aq. $NaHCO_3$ and extracted twice with EtOAc. The extracts were dried well over Na_2SO_4, filtered, concentrated, re-dissolved in methanol, and treated with HCl in dioxane to reform the dihydrochloride salt. This was finally concentrated to give purified BrMT as a yellow solid. 2.4 mg was obtained in this manner from ~6 mg of crude material, with ^{1}H NMR consistent with the published data.

Similar protocols were repeated with 6-chlorotryptamine, 6-fluorotryptamine, 6-methyltryptamine, 6-methoxytryptamine, and 5-bromotryptamine.

5. Protocols for synthesis of BrET (14) and 15

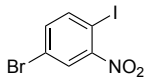




4-bromo-2-nitroaniline

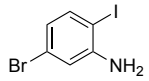
A suspension of NBS (11.9 g. 6.69 mmol) in HOAc (40 mL) was prepared and transferred slowly to a solution of 2-nitroaniline (9.00 g, 6.51 mmol) in HOAc (53 mL) at 60 °C, at such a rate as to maintain the temperature of the reaction in the range of 60–65 °C. The reaction mixture was heated at 60 °C for 3.5 h,

then ~35 mL of HOAc was removed by rotary evaporation. Crystallization was induced by gradual addition of water (97.5 mL) after cooling the reaction to 40 °C. The resulting suspension was slowly cooled to 5 °C and filtered, and the solid was washed with water (18 mL) and dried under vacuum to yield the title compound (10.6 g, 75%) as an orange solid. ^{1}H NMR (400 MHz, d_6-acetone): δ = 8.16 (d, J = 2.4 Hz, 1 H), 7.52 (dd, J = 2.4, 9.2 Hz, 1 H), 7.08 (d, J = 8.7 Hz, 1 H). (CAS No. 875-51-4)



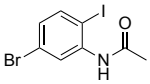
4-bromo-1-iodo-2-nitrobenzene (9d)

$NaNO_2$ (6.00 g, 87.0 mmol) was added slowly to conc. H_2SO_4 (100 mL) at 20 °C in a 500 mL flask with stir bar, and the mixture was heated to 70 °C for 15 min., then cooled to 10 °C before aniline **13** (15.0 g, 69.1 mmol) in glacial acetic acid (120 mL) was added slowly while maintaining the temperature in the range of 8–10 °C. The reaction was stirred at 20 °C for 50 min., then added to a preheated solution of potassium iodide (15.0 g, 90.4 mmol) in water (75 mL), and heated at 55 °C for 1 h. A sample was taken from the reaction, quenched with aqueous sodium thiosulfate solution, neutralized to pH 7 with 10 M NaOH solution, and extracted with EtOAc. TLC analysis (40% EtOAc/hexanes) of the organic layer indicated that the starting material was consumed. The reaction was cooled to 20 °C, diluted with water (25 mL), and treated with saturated aqueous sodium thiosulfate solution (15 mL). The resultant mixture was neutralized to pH 7 with 10 M NaOH solution, then extracted with DCM (3 x 75 mL). The organic layers were combined and dried over sodium sulfate, filtered, and dried under high vacuum to yield the title compound (22.0 g, 97% yield) as an orange solid. R_f = 0.51 (40% EtOAc/hexanes); ^{1}H NMR (300 MHz, d_6-DMSO): δ = 8.18 (d, J = 2.2 Hz, 1 H), 7.99 (d, J = 8.4 Hz, 1 H), 7.59 (dd, J = 2.3, 8.4 Hz, 1 H); ^{13}C NMR (75 MHz, d_6-DMSO): δ = 143.2, 137.1, 128.1, 122.4, 87.6. (CAS No. 112671-42-8)



5-bromo-2-iodoaniline (9a)

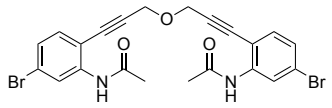
A solution of $SnCl_2$ (10.32 g, 45.75 mmol) in conc. HCl (15.0 mL, 37%) was prepared in a 100 mL flask with stir bar. A suspension of nitrobenzene **14** (3.00 g, 9.15 mmol) in ethanol (30 mL, 200 proof) was added at once, and the reaction was heated at 60 °C for 45 min. The reaction mixture was then cooled to 20 °C and basified to pH 9 with saturated aqueous KOH solution, then extracted with DCM (2 x 75 mL). The organic layers were combined and dried over sodium sulfate, filtered, and dried under high vacuum for 30 min. The crude oil was treated with 0.9 M HCl in ether (20 mL), and the resulting solid was filtered and washed with MTBE, to yield the aniline hydrochloride salt of **9a** as an off-white solid (2.72 g, 89%). ^{1}H NMR (300 MHz, d_6-DMSO): δ = 8.18 (d, J = 2.2 Hz, 1 H), 7.99 (d, J = 8.4 Hz, 1 H), 7.59 (dd, J = 2.3, 8.4 Hz, 1 H). (CAS No. 64085-52-5)



***N*-(5-bromo-2-iodophenyl)acetamide (9c)**

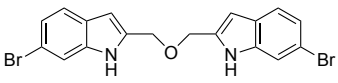
Aniline salt **10a** (2.00 g, 6.71 mmol) was suspended in dry toluene (25.0 mL) in a 100 mL flask with stir bar sealed under nitrogen at 20 °C. Pyridine (0.71 mL, 8.8 mmol) and acetic anhydride (0.82 mL, 8.7 mmol) were added dropwise to the suspension, then the mixture was stirred at 70 °C for 5 h. A sample was taken from the reaction, washed with sodium bicarbonate solution, and extracted with EtOAc. TLC analysis (4% MeOH/DCM) of the organic layer indicated that the starting material was consumed. The reaction was cooled to 20 °C, diluted with DCM (30 mL), and washed twice with aqueous 1M HCl (30 mL). The organic layer was washed with brine solution (30 mL) and dried over sodium sulfate, filtered,

and dried under high vacuum to yield 2.1 g of a pale yellow oil. The crude oil was dissolved in 1% MeOH/DCM and purified by flash chromatography (100 g SiO_2 cartridge; 0–10% MeOH/DCM gradient) to yield the title compound as a white solid (1.83 g, 80%). R_f = 0.84 (4% MeOH/DCM); ^{1}H NMR (300 MHz , d_6-DMSO): δ = 9.48 - 9.40 (m, 1 H), 7.75 (d, J = 8.4 Hz, 1 H), 7.61 (d, J = 2.3 Hz, 1 H), 7.12 (dd, J = 2.4, 8.4 Hz, 1 H), 2.02 (s, 3 H); ^{13}C NMR (75 MHz , d_6-DMSO): δ = 169.2, 141.9, 141.0, 130.7, 130.0, 121.7, 95.3, 23.9.



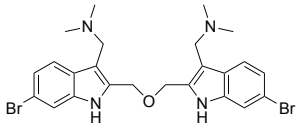
_N_-[5-bromo-2-(3-{[3-(4-bromo-2-acetamidophenyl)prop-2-yn-1-yl]oxy}prop-1-yn-1-yl)phenyl]acetamide (10c)

Anilide **9c** (490 mg, 1.24 mmol) was added to a 25 mL flask with stir bar and sealed under argon. Dry acetonitrile (10 mL), $(PPh_3)_2PdCl_2$ (50.0 mg, 0.071 mmol), copper (I) iodide (58.0 mg, 0.31 mmol), NEt_3 (2 mL) and propargyl ether (90 µL, 0.87 mmol) were added respectively at 20 ˚C and the mixture was heated at 50 °C for 2 h. A sample was taken by syringe, diluted with EtOAc, and washed with water. LC-MS analysis of the concentrated organic sample indicated that the starting material was consumed. The reaction was cooled to 20 ˚C, diluted with water (50 mL), and extracted with EtOAc (50 mL). The organic layer was separated and washed with brine solution (40 mL), then dried over sodium sulfate, filtered, and dried under high vacuum to yield 440 mg of a brown solid. The crude was dissolved in DCM and purified by flash chromatography (10 g SiO_2; 0–10% MeOH/DCM gradient) to yield the title compound (340 mg, 90% yield) as a yellow solid. R_f = 0.45 (50% EtOAc/hexanes); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.67 - 8.58 (m, 1 H), 7.85 - 7.76 (m, 1 H), 7.27 (d, *J* = 1.5 Hz, 1 H), 7.18 (dd, *J* = 2.0, 8.4 Hz, 1 H), 4.61 (s, 2 H), 2.23 - 2.19 (m, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 168.4, 140.2, 133.2, 126.9, 124.6, 122.7, 92.4, 82.0, 58.0, 25.1; MS (ESI^+) calculated for $C_{22}H_{18}Br_2N_2O_3$ [M+Na] 540.97, found 540.90.



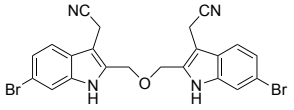
6-bromo-2-{[(6-bromo-1*H*-indol-2-yl)methoxy]methyl}-1H-indole (11)

Bis-alkyne **10c** (114 mg, 0.22 mmol) was stirred with 1M TBAF in THF (1.5 mL) in a 4 mL vial with stir bar at 20 °C under nitrogen for 1 h, after which time LC-MS analysis of a sample from the reaction indicated that the starting material was consumed. The reaction was diluted with EtOAc (25 mL) and washed with deionized water (3 x 25 mL), then the organic layer was dried over sodium sulfate, filtered, and dried under high vacuum to yield 40 mg of a brown solid. The crude material was dissolved in DCM and purified by flash chromatography (10 g SiO_2; 20–100% EtOAc/hexanes gradient) to yield the title compound **11** as a yellow solid (30 mg, 90%). R_f = 0.49 (70% EtOAc/hexanes); ^{1}H NMR (400 MHz , CD_3OD): δ = 7.50 - 7.47 (m, 2 H), 7.40 (d, *J* = 8.4 Hz, 2 H), 7.08 (dd, *J* = 1.8, 8.4 Hz, 2 H), 6.41 - 6.38 (m, 2 H), 4.66 (s, 4 H); ^{13}C NMR (101 MHz, CD_3OD): δ = 137.7, 136.1, 127.0, 121.9, 121.2, 114.3, 113.4, 101.2, 64.4; MS (ESI^+) calculated for $C_{18}H_{14}Br_2N_2O$ [M+H] 432.95, found 433.00.



({6-bromo-2-[({6-bromo-3-[(dimethylamino)methyl]-1*H*-indol-2-yl}methoxy)methyl]-1*H*-indol-3-yl}methyl)dimethylamine (12)

Bis-indole **11** (225 mg, 0.58 mmol) was added to a 20 mL vial with stir bar and sealed under nitrogen. 20:1 acetonitrile : acetic acid (10.5 mL) and Eschenmoser's salt (240 mg, 1.30 mmol) were then added, and the resulting beige suspension was stirred vigorously for 5 h, after which time the reaction was basified with aqueous NaOH to pH 11. The resulting mixture was extracted with EtOAc (2 x 30 mL), and the combined organics were dried over sodium sulfate, filtered, and concentrated to a yellow solid. The crude bis-gramine **12** (220 mg, 77%) was used in the subsequent step without purification.

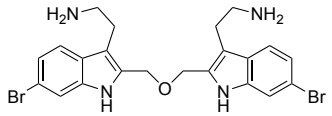


2-[6-bromo-2-({[6-bromo-3-(cyanomethyl)-1*H*-indol-2-yl]methoxy}methyl)-1*H*-indol-3-yl]acetonitrile (13)

The bis-gramine **12** (70.0 mg, 0.128 mmol) was added to a 15 mL flask with stir bar and sealed under nitrogen. Dry DMF (5 mL), KCN (85 mg, 1.31 mmol) and CH_3I (80 μL, 1.3 mmol) were added respectively under nitrogen and the reaction was stirred at 20 °C for 16 hrs. LC-MS analysis of a sample from the reaction indicated that the starting material was consumed. The reaction was diluted with EtOAc (50 mL) and washed with water (50 mL). The organic layer was separated and dried over sodium sulfate, filtered, and concentrated to 80 mg of a crude brown oil, which was dissolved in DCM and purified by flash chromatography (10 g SiO_2; 0–70% EtOAc/hexanes gradient) to yield the bis-nitrile **13** as a pale yellow solid (50 mg, 76%). ^{1}H NMR (400 MHz , CD_3OD): δ = 7.51 (d, *J* = 1.7 Hz, 2 H), 7.49 (s, 1 H), 7.47 (s, 1 H), 7.18 (d, *J* = 2.0 Hz, 1 H), 7.16 (d, *J* = 1.8 Hz, 1 H), 4.76 - 4.72 (m, 4 H), 3.92 - 3.89 (m, 4 H); ^{13}C NMR (101 MHz, CD_3OD): δ = 136.6, 133.3, 125.7, 122.4, 119.1, 118.3, 115.4, 113.9, 102.3, 62.7, 11.4; MS (ESI$^+$) calculated for $C_{22}H_{16}Br_2N_4O$ [M+H] 510.97, found 510.85.

Synthesis of Alane (AlH_3):

LAH (780 mg, 10.3 mmol) was added to an oven-dried 25 mL 2-necked sidearm flask with stopcock containing a stir bar, with a mark indicating the 20 mL level. The flask was sealed with a septum and flushed with nitrogen, then cooled on ice under slow nitrogen flow. Dry THF (15 mL) was added to form a slurry, then sulfuric acid (96%, 555 μL) was added slowly over ~10 min. from a glass syringe with steel needle, with vigorous stirring. Caution: Strong gas (H_2) evolution—a large gauge needle and tubing with vent to an oil bubbler were used to minimize pressure build-up. The resulting suspension was removed from the ice bath and stirred for 2 h, before it was topped up to the 20 mL mark with THF. Finally, the septum was quickly replaced with a greased glass stopper secured with electrical tape. The solids (primarily lithium sulfate) were left to settle and the resulting supernatant (~1 M in AlH_3) was used by attaching a small septum to the stopcock and drawing out the desired quantity via plastic syringe and needle under slightly positive nitrogen pressure. The alane can be stored in the freezer and used for several months with a gradual decreasing of titer.

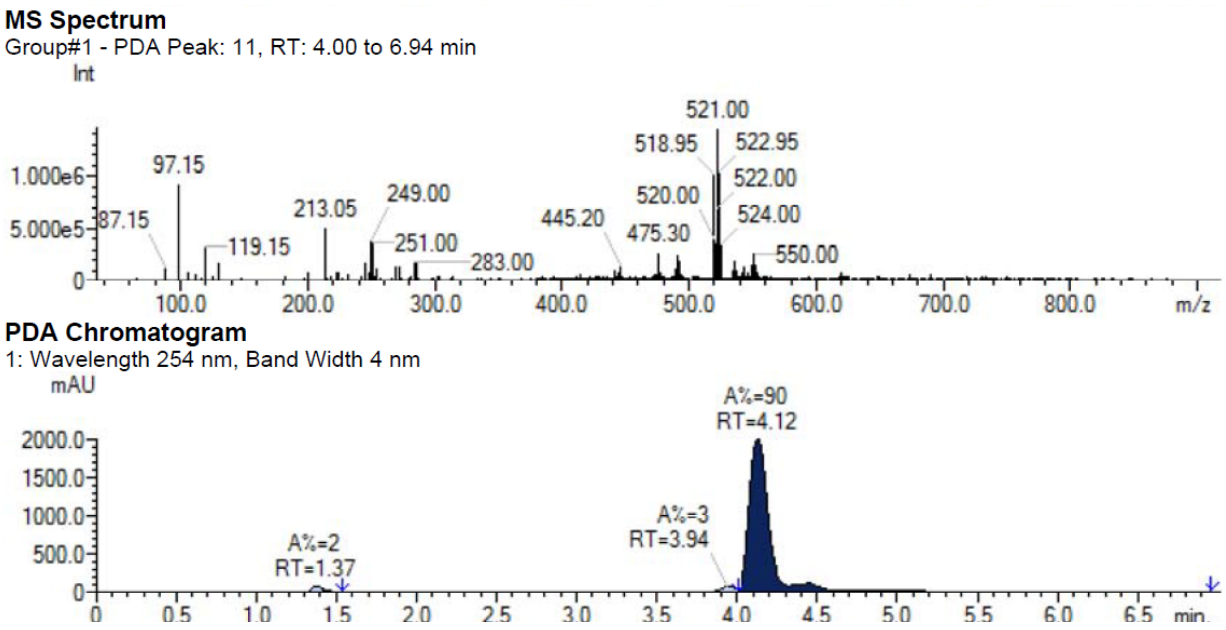


2-[2-({[3-(2-aminoethyl)-6-bromo-1*H*-indol-2-yl]methoxy}methyl)-6-bromo-1*H*-indol-3-yl]ethan-1-amine (14, BrET)

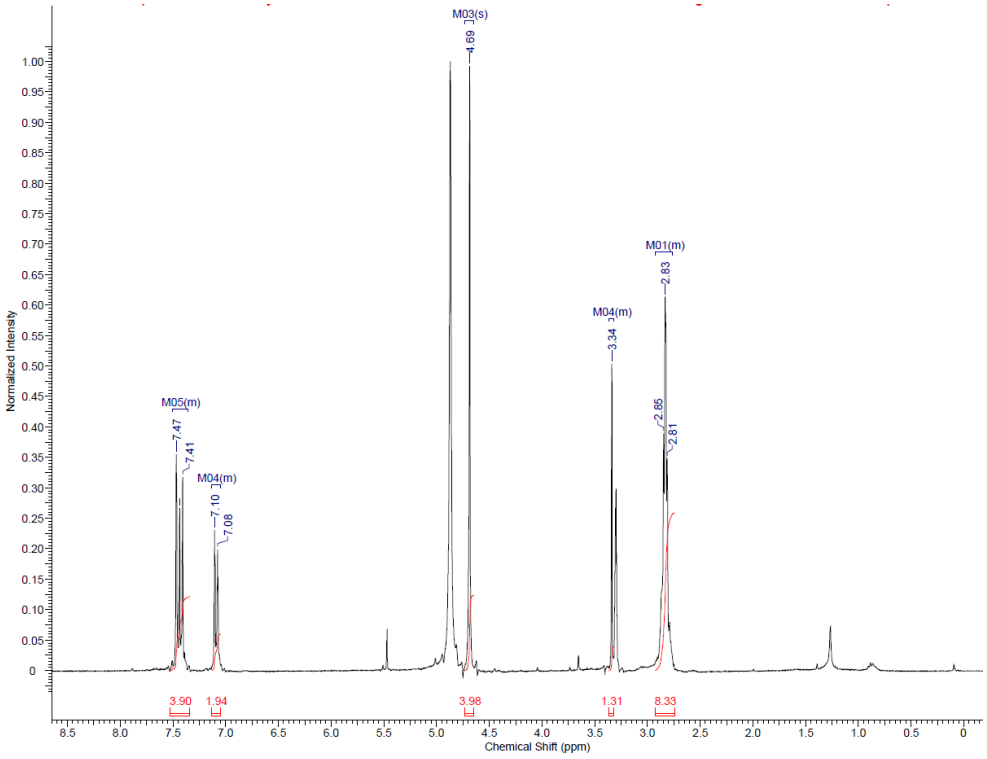
The bis-nitrile **13** (70.0 mg, 0.137 mmol) was added to a 25 mL flask with stirrer bar and sealed under nitrogen. Dry THF (4 mL) was added and the flask was cooled to 0 °C. Freshly prepared AlH_3 solution (~1 M in THF, 1.4 mL, 1.4 mmol) was added dropwise at 0 °C under nitrogen. The reaction mixture was warmed to 20 °C and stirred for 3 h with a vent line to an oil bubbler only, to permit release of gas without the reaction running dry. After 3 h, the reaction was quenched by adding dropwise saturated aqueous sodium sulfate solution (1 mL) under nitrogen and stirring for 10 min. The resulting mixture was diluted with EtOAc (10 mL) and washed with water (10 mL) and brine solution (10 mL). The combined organics were dried over sodium sulfate, filtered, and concentrated to a colorless oil (76 mg), which was dissolved in DCM and purified by flash chromatography (10 g SiO_2, 0–16% MeOH/DCM (DCM saturated with ammonia) gradient) to yield **14** (BrET) as a white solid (60 mg, 45%). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.53 - 7.35 (m, 4 H), 7.09 (dd, *J* = 1.6, 8.4 Hz, 2 H), 4.69 (s, 4 H), 2.89 - 2.73 (comp, 8 H); ^{13}C NMR (75 MHz, CD_3OD): δ = 137.2, 133.0, 127.0, 121.8, 119.8, 115.0, 113.7, 111.3, 62.9, 42.2, 26.9; MS (ESI$^+$) calculated for $C_{22}H_{24}Br_2N_4O$ [M+H] 519.03, found 518.95.

It should be noted that attempts to prepare the HCl salt of BrET in organic solvents were unsuccessful, and led to decomposition of the material.

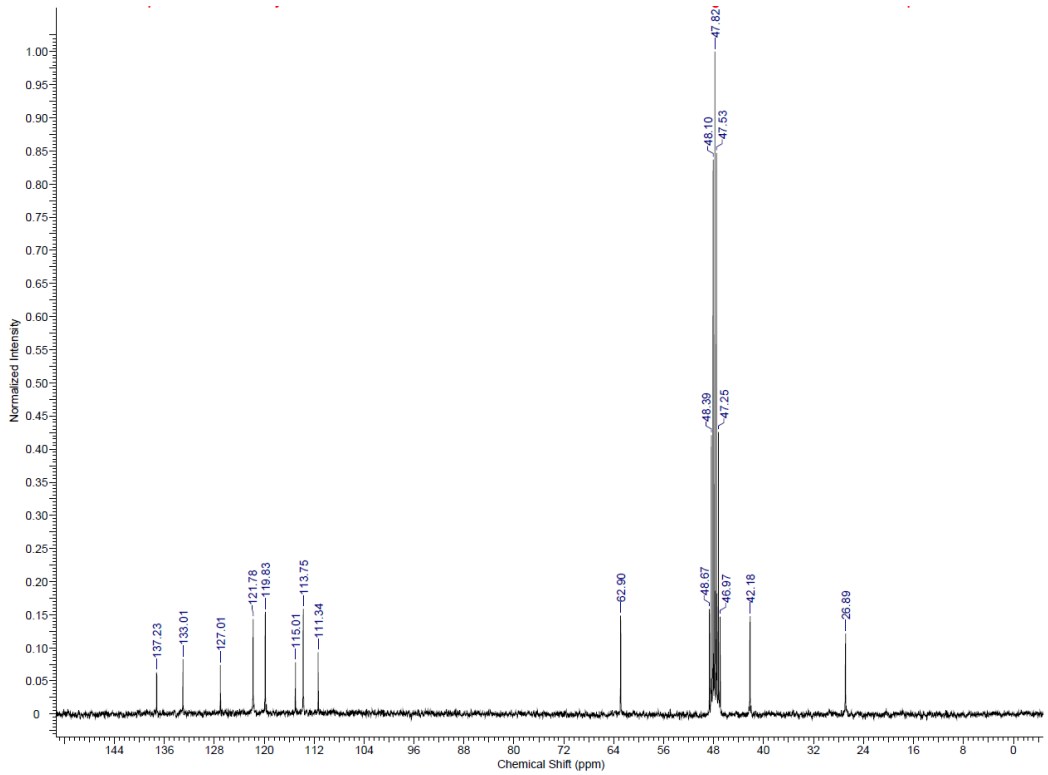
LC-MS Chromatogram of 14 (BrET)

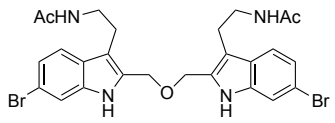


^{1}H NMR of 14 (BrET)



^{13}C NMR of 14 (BrET)





***N*-{2-[6-bromo-2-({[6-bromo-3-(2-acetamidoethyl)-1*H*-indol-2-yl]methoxy}methyl)-1*H*-indol-3-yl]ethyl}acetamide (15)**

BrET **14** (12.0 mg, 0.023 mmol) was added to a 8 mL vial with stir bar and sealed under nitrogen. Dry DCM (3 mL) was added and the vial was cooled at 0 °C. NEt_3 (10 µL, 0.07 mmol) and acetic anhydride (6 µL, 0.06 mmol) were added by microsyringe. The resulting mixture was warmed to 20 °C and stirred for 30 min. A sample was taken from the reaction, diluted with EtOAc and washed with sodium bicarbonate solution. TLC analysis (5% MeOH/DCM) of the organic layer indicated that the starting material was consumed. The reaction was diluted with DCM (2 mL) and washed with aqueous sodium bicarbonate solution (2 x 5 mL). The combined organics were dried over sodium sulfate, filtered, and concentrated to a colorless solid (10 mg), which was dissolved in DCM and purified by flash chromatography (10 g SiO_2; 0–10% MeOH/DCM gradient) to yield the bis-amide **15** as a white solid (7 mg, 60%). R_f = 0.37 (5% MeOH/DCM); ^{1}H NMR (300 MHz, CD_3OD) δ = 7.48 - 7.45 (m, 3 H), 7.44 (s, 1 H), 7.14 - 7.08 (m, 2 H), 4.70 (br. s, 4 H), 3.37 - 3.32 (comp, 4 H), 2.92 (t, *J* = 7.1 Hz, 4 H), 1.80 - 1.77 (comp, 6 H)*;* ^{13}C NMR (75 MHz, CD_3OD): δ = 172.1, 137.2, 132.9, 127.0, 121.8, 119.8, 115.0, 113.8, 111.3, 63.0, 40.5, 23.6, 21.3; MS (ESI^+) calculated for $C_{26}H_{28}Br_2N_4O_3$ [M+H] 603.05, found 602.90.

6. Electrophysiology methods

Cell culture

A tetracycline-inducible Kv1.4 cell line was established in CHO-K1 cells stably expressing a tetracycline repressor (T-REx-CHO, ThermoFisher), and a Kvβ2 subunit to increase surface expression. The T-REx-CHO cells were maintained in tissue-culture treated polystyrene dishes 37°C in a 5% CO_2 atmosphere in Ham's F12 media containing 10% FBS (Hyclone Fetal Bovine Serum Characterized (cat. SH30071.03, lot AXM55317), 1% penicillin/streptomycin (Life Technologies 15140-122), and 10 μg/mL blasticidin. Cells were transfected with a Kvβ2/RBG4 REF and GFP (EGFP-C1, Clontech) using Lipofectamine 2000 (ThermoFisher). GFP fluorescence was used to confirm transfection and the vector confers G418 resistance. Two days after transfection, 500 μg/mL G418 was added to media. After emergence of G418-resistant cell colonies, cells were subcloned by dilution into 96-well plates. Wells containing single clones were expanded and screened for Kvβ2 expression by immunoblotting with an anti-Kvβ2 monoclonal antibody (UC Davis/NIH NeuroMab Facility Cat# 73-021, RRID:AB_10673520). The CHO-K1/T-REx/ Kvβ2 H7 clonal cell line was expanded for further use. Rat Kv1.4 cDNA was excised with EcoRI from a pRC/CMV expression construct (*12*), and ligated into a Zeocin-resistance expression vector containing tetracycline operator sequences (pcDNA4/TO, ThermoFisher) at its EcoRI site. The Kv1.4/pcDNA4/TO vector and a dsRed vector (pDsRed-monomer-C1, Clontech) were transfected using Lipofectamine 2000. dsRED fluorescence was used to confirm transfection. One day after transfection 250 μg/mL zeocin was added to media. After emergence of zeocin-resistant cell colonies, cells were subcloned by dilution into 96-well plates. Wells containing single clones were expanded, incubated overnight with 1 μg/mL tetracycline and screened for large inactivating K^+ currents by manual patch clamp electrophysiology. Cell line clone E8, which also had dsRed fluorescence was selected for electrophysiology assays, and maintained with 1 μg/ml blasticidin, 25 μg/ml zeocin to retain Kv1.4 and the tetracycline repressor. Frozen aliquots of 5-10 million cells were prepared for electrophysiology. Cells were expanded for freezing in 175 cm^2 flasks in media without selection agents. 4-8 hours before freezing, 1 μg/ml tetracycline was added to media to induce a desirable amount of channel expression. Cells were washed with divalent-free Dulbecco's Phosphate Buffered Saline (Gibco 14190144), dissociated with Detachin (Gelantis), centrifuged at 500g for 2 minutes then resuspended to 5-10 million cells/mL in Recovery media (Gibco 12648010), frozen in cryogenic tubes in an insulated container (CoolCell, Biocision) at -80°C overnight, and stored in liquid N_2 until the day of use.

Electrophysiology

To measure currents from Kv1.4 channels, ensemble voltage-clamp recordings were performed on an automated IonFlux system (Fluxion Biosciences). Cells were thawed and resuspended in CHO-SFMII media (Life Technologies 12052-114) supplemented with 25 mM HEPES (pH 7.3), and shaken at 300 rpm in a 25 cm^2 polypropylene flask at room temperature for 30 minutes prior to use. Cells were centrifuged at 200 *g* for 3 minutes, then resuspended in extracellular solution (below) to a concentration of $5x10^6$ cells/mL and pipetted along with test compounds and intracellular solution into 384 well IonFluxHT plates immediately before recording, according to manufacturer's protocols. The extracellular solution contained (in mM): 50 HEPES, 20 KOH, 150 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 0.1 Mg-EDTA, adjusted to pH 7.3 with HCl. The intracellular solution contained (in mM): 50 KF, 70 KCl, 35 KOH, 5 EGTA, 50 HEPES, 2 TCEP adjusted to pH 7.3 with HCl. Temperature was approximately 20°C. The IonFluxHT plates contain 96 cell 'traps': electrically isolated wells with 20 holes to capture cells and establish patch clamp seals with individual cells. These allow ensemble voltage clamp recordings from up to 20 cells per trap. Trap pressure was held at 6 mmHg during cell positioning and data acquisition with a step to 12 mmHg for 15 s during break-in. Initial resistance of each well trap with these solutions was 0.5-0.7 MΩ. Currents were recorded at a sampling rate of 5 kHz. Holding potential was -120 mV. To activate Kv1.4, 1 s test pulses to 0 mV were repeated every 10 s.

Test compounds were dissolved in acetonitrile (ACN)/water solutions to 10 mM. Compound stocks were diluted serially to test concentrations in extracellular solution including 1% (v/v) FL reagent (Fluxion) and 5μM tetrodotoxin (Abcam Biochemicals ab120054). Vehicle controls included 1% FL reagent, 5 μM tetrodotoxin, and acetonitrile for each compound replicate. Compounds were perfused in series from low to high concentration. All experiments were concluded with 10 mM 4-aminopyridine (Sigma A0152) in vehicle to inhibit all Kv1.4 current.

The range of analog concentrations that were tested in voltage clamp assays were limited by experimental considerations. At high concentrations, solvent concentrations posed a concern, and BrMT destabilizes the patch clamp seal at concentrations >20 μM.(*13, 14*) Concentrations of BrMT <1 μM resulted in very slow onset of channel inhibition, which required long equilibration times (>1000 s), that were incompatible with the solution reservoirs of the IonFlux system. While another voltage clamp method (e.g. manual patch clamp or *Xenopus* oocyte voltage clamp) might enable extension of the concentration range tested, the hydrophobicity of BrMT leads to different effective concentrations in different cell preparations,(*13, 14*) and we limited the concentration range tested rather than attempt to merge datasets from different preparations. As noted in the main text, this experimental system dependence is consistent with BrMT's interacting with membranes.

Electrophysiology data analysis

Linear leak currents were subtracted using the IonFlux data analysis software (Fluxion) from a 10 ms voltage step from the holding potential to -110 mV preceding 40 ms at the holding potential and the depolarizing step to 0 mV. Inactivating current was measured as the difference from the peak (6 to 25 ms) and final (990-1000 ms) current during a 1000 ms voltage step from -120 mV to 0 mV. Currents were exported from time points 15 s before each solution change and 85 s after 4-AP addition. Leak-subtracted currents were zeroed to values after 4-AP addition and normalized to maximum current. Records that did not exhibit current with Kv1.4-like activation and deactivation kinetics prior to the addition of the lowest concentration of test compound were excluded from the analysis. Records that exhibited substantial run-up, or inconsistent resistance at holding potential were also excluded. A time-dependent run-down in current was observed in nearly all vehicle control experiments. To correct for this run down, values were divided by the normalized time-matched mean current from vehicle controls.

Graphing and analysis of electrophysiology were performed in IgorPro 7 (Wavemetrics). Concentration response and kinetics curves were fit by non-linear least-squares methods employing the Levenberg-Marquardt algorithm in IgorPro. After transformation to fraction of maximal current, the concentration-response of each compound was fit with a Hill equation:

$$F_{inhib} = \frac{1}{\left\{1+\left(\frac{IC50}{[compound]}\right)^{h}\right\}} \qquad (1)$$

where F_{inhib} is the fraction of maximal current inhibited during a 0 mV voltage step, *IC*50 is the current at which 50% of current is inhibited, [compound] is the concentration of test compound, and h is the Hill slope.

Time constants of activation and inactivation were determined by fitting a double exponential equation:

$$I_K = I_0 + A_1 \exp\left\{\frac{-(t-t_0)}{\tau_1}\right\} + A_2 \exp\left\{\frac{-(t-t_0)}{\tau_2}\right\} \qquad (2)$$

to Kv1.4 currents after a step to 0 mV.

Plotting and correlation of IC_{50}, $K_P^{W\rightarrow L}$, and gA_2 values were performed in IgorPro 7. Regressions were standard linear correlations with correlation coefficients and p-values for two-tailed tests reported. Compounds that were not effective at the highest concentration tested were excluded from the correlations.

7. Isothermal calorimetry methods

Isothermal titration calorimetry

Heats of partitioning were measured using an TA Instruments (Newcastle, DE) low volume nano ITC with 170 µL sample cell volume and 50 µL syringe volume. The experiments were conducted as described previously.(*15*) $DC_{22:1}PC$ LUVs without ANTS or gA were rehydrated in the same $NaNO_3$ buffer as in the fluorescence assay. The BrMT derivatives were diluted from DMSO stock with $NaNO_3$ buffer. The DMSO concentration was kept ≤ 1%, and was matched between the lipid and drug samples to minimize any effects of the heats of dilution.

The BrMT analogs (50 µM) were added from the syringe into the lipid sample in the cell (10 mM lipid); the concentrations of each drug-lipid titration were optimized to give a large signal with a wide dynamic range that eventually saturated. An initial injection of 0.2 µL was discarded during the analysis to allow for cell-syringe equilibration artifacts. Then nineteen 2 µL injections of drug into lipid were spaced sufficiently apart (≥200 s) to ensure that the signal returned to baseline between injections. The results were baseline corrected using the TA Instruments NanoAnalyze software and the final heat generated per injection was integrated using MatLab (Mathworks, Natick, MA) to determine the enthalpy per injection. Assuming that the process can be described as partitioning between two immiscible phases, the partition constant ($K_P^{W\rightarrow L}$) and total enthalpy of partitioning ($H_P^{W\rightarrow L}$) were determined from fitting the plot of injection enthalpies versus injection number using Eq. 3 (derived following (*16*)):

$$\delta h_A(i) = \frac{\delta V \cdot C_L \cdot v_L \cdot n_{tot}^A \cdot V_W(0) \cdot \Delta H_A^{W\rightarrow L} \cdot K_A^{W\rightarrow L}}{\left(V_W(0) + (i-1)\cdot\delta V \cdot (1 + C_L \cdot v_L \cdot K_A^{W\rightarrow L})\right)^2} \quad (3)$$

where i denotes the injection number, $\delta h_A(i)$ the injection enthalpy, δV the injection volume, C_L the lipid concentration, v_L the lipid molar volume, n_{tot}^A the amount of amphiphile in moles, and $V_W(0)$ the initial cell volume. Eq. 3 was fit to each binding isotherm using the non-linear least-squares routine with the Trust-region algorithm implemented in Matlab.

8. Gramicidin assay methods

The gramicidin-based fluorescence assay has been described previously. (*17, 18*) In brief, large unilamellar vesicles (LUVs), loaded with intravesicular ANTS were prepared from $DC_{22:1}PC$ and gramicidin (weight ratio 1000:1, corresponding to a ~2000:1 molar ratio) using freeze-drying, extrusion and size-exclusion chromatography; the final lipid concentration was 4-5 mM, and the suspension was stored in the dark at 12.5°C for a maximum of 7 days. The size distribution was determined using dynamic light scattering using an Anton Paar LitesizerTM 500 instrument; the average diameter was 133 nm, with an average Polydispersity index of 7.6 % indicating that the samples are monodisperse. Before use, the LUV-ANTS stock was diluted to 200-250 µM lipid with $NaNO_3$ buffer (140 mM $NaNO_3$, 10 mM HEPES, pH 7).

The BrMT derivatives (dissolved in DMSO) or DMSO (as control) were added to a LUV-ANTS sample and equilibrated at 25 °C in the dark for 10 min. before the mixture was loaded into a stopped-flow spectrofluorometer (Applied Photophysics SX.20, Leatherhead, UK) and mixed with either $NaNO_3$

buffer or $TlNO_3$ buffer; Tl^+ (thallous ion) is a gramicidin channel-permeant quencher of the ANTS fluorescence. Samples were excited at 352 nm and the fluorescence signal above 455 nm was recorded in the absence (4 successive trials) or presence (9 successive trials) of the quencher. All the BrMT derivatives fluoresce to varying degrees and addition of these drugs to LUVs in control experiments without gA or Tl^+ increased the fluorescence signal, and each signal was normalized to account for the compound's fluorescence (Figure S3). The instrument has a dead time of < 2 ms, and the next 2-100 ms segment of each fluorescence quench trace was fitted to a stretched exponential, which is a computationally efficient way to represent a sum of exponentials with a distribution of time constants, reflecting the LUV size distribution, e.g. eq. 4: (*19*)

$$F(t) = F(\infty) + \left(F(0) - F(\infty)\right) \cdot \exp\left\{-(t/\tau)^{\beta}\right\} \quad (4)$$

where F(t) denoted the fluorescence intensity as a function of time, t; τ was a parameter with units of time; and β ($0 < \beta \leq 1$, where $\beta = 1$ denotes a homogenous sample) was a measure of the LUV dispersity. The rate of Tl^+ influx was determined at 2 ms (eq. 5) (*19*)

$$k(t) = \frac{\beta}{\tau} \cdot \left(\frac{t}{\tau}\right)^{\beta-1}\Bigg|_{2\text{ms}} \quad (5)$$

The quench rate for each experiment represents the average influx rate of the trials with Tl^+. The quench rate was normalized to the rate in control experiments without any drug, and the reported values represent averages from three or more experiments. The analog concentration that elicits a doubling of the rate of gramicidin-dependent quenching (gA_2) was determined from a global fit of

$$\frac{rate}{rate_{control}} = 1 + \frac{[compound]}{gA_2} \quad (6)$$

to all individual data points.

9. References

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