Comprehensive screening of octopus amphiphiles as DNA activators in lipid bilayers: Implications on transport, sensing and cellular uptake

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Supplementary Information

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1. Supplementary methods

1.1. Materials and methods

As in ref. S1, Supporting Information. Briefly, reagents for synthesis were purchased from Fluka and Aldrich, amino acid derivatives from Novabiochem and Bachem, buffers and salts of the best grade available from Fluka or Sigma-Aldrich and used as received. 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) was from Sigma, and *p*-xylene-bis-pyridinium bromide (DPX) was from Invitrogen. Most of the aldehydes were purchased from Sigma, Fluka or Acros, and the non-commercially available aldehydes **T14**,[S2] **T16**,[S3] **T18**,[S4] **T18Δ9**[S5] and $\mathbf{T_{AOT}}$[S6] were synthesized from the corresponding alcohols using PCC oxidation protocols.[S7]

All reactions were performed under N_2 atmosphere. Unless stated otherwise, column chromatography was carried out on silica gel 60 (Fluka, 40-63 μm). Analytical (TLC) was performed in silica gel 60 (Fluka, 0.2 mm) and silica gel GF (Analtech, 1000 μm), respectively. $[\alpha]_D^{25}$ values were recorded on a Jasco P-1030 Polarimeter, melting points (Mp) on a heating table from Reichert (Austria). IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (ATR, Golden Gate, unless stated otherwise) and are reported as wavenumbers ν in cm^{-1} with band intensities indicated as s (strong), m (medium), w (weak), b (broad). ^1H, ^{13}C NMR and ^{31}P spectra were recorded (as indicated) either on a Bruker 300 MHz, 400 MHz or 500 MHz spectrometer and are reported as chemical shifts (δ) in ppm relative to TMS (δ = 0). Spin multiplicities are reported as a singlet (s), doublet (d), triplet (t), quartet (q) and quintet (quint), multiplet (m) and with the coupling constants (*J*) given in Hz. ^1H and ^{13}C resonances were assigned with the aid of additional information from 1D & 2D NMR spectra (H,H-COSY, DEPT 135, HSQC and HMBC). ESI-MS for the characterization of new compounds was performed on a Finnigan MAT SSQ 7000 instrument or a ESI API 150EX and are reported as mass-per-charge ratio *m/z* (intensity in %, [assignment]).

Vesicles were prepared with a Mini-Extruder from Avanti Polar Lipids (pore size 100 nm). Sample incubation was done on a Rotamax 120 (Heidolph). Fluorescence measurements were performed with a FluoroMax-2 spectrofluorometer (Jobin-Yvon Spex) equipped with a stirrer and a temperature controller, or a FluoroMax-3 spectrofluorometer equipped with a multiwell plate reader (MicroMax 384).

1.2. Abbreviations

Boc: *tert*-Butoxycarbonyl; Calculated: Calcd; Cbz: (Benzyloxy)carbonyl; CF: 5(6)-carboxyfluorescein; CH_3CN: Acetonitrile; DCM: Dichloromethane; DIEA: N-Ethyl-N,N-diisopropyl amine; DMSO: Dimethylsulfoxide; DPX: *p*-Xylene-bispyridinium bromide; EYPC: Egg yolk phosphatidylcholine; Et_2O: Diethyl Ether; Glu: L-Glutamic acid; Gly: Glycine; HBTU: O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate; HCl: Hydrochloric acid; HPTS: 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; HR-MS (ESI): High resolution mass spectrometry; LUVs: Large unilamellar vesicles; Tris: Tris(hydroxymethyl)aminomethane; MeOH: Methanol; rt: Room temperature; ^{t}Bu: *tert*-Butyl.

1.3. Synthesis

1.3.1. Synthesis of G1H6

Compound 8. HBTU (644 mg, 1.70 mmol) and DIEA (1.38 ml, 8.10 mmol) were sequentially added to a solution of **7** (228 mg, 0.81 mmol) in DCM (60 ml) under N_2. The reaction mixture was stirred for 5 min and a solution of **6** (1000 mg, 1.62 mmol)[S1] in DCM (5 ml) was added dropwise. The reaction mixture was stirred at rt for 2 hours and poured into a separation funnel containing DCM (100 ml) and $KHSO_4$ 1 M (150 ml). The aqueous phase was extracted with DCM (2 x 40 ml), and the organic layer was washed with HCl 1 M (200 ml), $NaHCO_3$ saturated aqueous solution (200 ml), water (150 ml) and brine (100 ml), dried over Na_2SO_4, filtered and evaporated under vacuum. The crude product was purified by column chromatography (DCM/AcOEt 9:1, R_f 0.55) to afford **8** (744 mg, 62%) as a colorless solid. Mp: 170-171 ^oC; $[\alpha]_D^{25}$: -39.4 (c = 0.1 in MeOH); IR (neat): 3265 (w, b), 2980 (w), 1720 (m), 1670 (m), 1634 (s), 1531 (m), 1367 (m), 1241 (s), 1158 (s), 1046 (s), 860 (w); ^{1}H NMR (400 MHz, CD_3OD): 7.50-7.22 (m, 5H), 5.20-5.02 (m, 2H), 4.66-4.24 (m, 4H), 4.16-3.87 (m, 1H), 2.57-1.80 (m, 20H), 1.48 (s, 45H), 1.46 (s, 9H). ^{13}C NMR (101 MHz, CD_3OD): 174.2-171.4 (10x, s), 157.2-155.6 (7x, s), 136.5 (s), 128.1 (2x, d), 127.7 (d), 127.6 (2x, d), 80.8 (2x, s), 80.67 (2x, s), 80.63 (2x, s), 66.5 (t), 53.5 (d), 52.9 (2x, d), 51.0 (2x, d), 31.1

(2x, t), 29.8 (t), 29.4 (2x, t), 29.3 (2x, t), 28.1 (t), 27.3 (18x, q), 26.9 (2x, t); MS (ESI, MeOH/H_2O 2:1): 1480 (35, $[M-H]^-$), 1381 (100, $[M-Boc]^-$).

Compound 9. Compound **8** (500 mg, 0.34 mmol) was dissolved in MeOH (30 ml) under N_2, and $Pd(OH)_2/C$ (20%) (14 mg, 0.02 mmol) was added in one portion. The reaction mixture was flushed with H_2 and stirred under H_2 atmosphere for 3 hours. The resulting suspension was filtered trough celite and the solvent was removed under vacuum to afford **9** (448 mg, quantitative) as a colorless solid. Mp: > 250 ^oC; $[\alpha]_D^{25}$: -44.9 (c = 0.1 in MeOH); IR (neat): 3247 (w, b), 2981 (w), 1722 (m), 1767 (s), 1502 (m), 1367 (m), 1243 (m, b), 1156 (s), 1014 (w), 863 (s); ^1H NMR (400 MHz, CD_3OD): 4.62-4.25 (m, 5H), 2.63-1.70 (m, 20H), 1.49 (s, 54H); ^{13}C NMR (101 MHz, CD_3OD): 176.1 (s), 175.9 (s), 174.2 (s), 173.7 (s), 173.5 (s), 173.2 (2x, s), 173.0 (s), 172.1 (s), 171.6 (s), 156.8 (2x, s), 156.4 (2x, s), 156.1 (2x, s), 80.8 (2x, s), 80.6 (4x, s), 53.10 (2x, d), 52.7 (d), 51.1 (2x, d), 31.4 (t), 29.9 (t), 29.3 (2x, t), 29.1 (2x, t), 27.3 (2x, t), 26.82 (18x, q), 26.79 (2x, t); MS (ESI, MeOH/H_2O 2:1): 1370 (66, $[M+Na]^+$), 1348 (75, $[M+H]^+$), 1270 (88, $[M-Boc+Na]^+$), 1248 (100, $[M-Boc+H]^+$), 770 (71, $[M-6Boc+Na]^+$).

Compound 11. DIEA (256 μl, 1.5 mmol) and N,N'-Di-Boc-1-*H*-pyrazole-1-carboxamidine (**10**, 115 mg, 0.37 mmol) were sequentially added to a solution of **9** (400 mg, 0.30 mmol) in CH_3CN (35 ml) and water (1.5 ml) under N_2. The reaction mixture was stirred at 55 ^oC for 8 hours. The solvent was removed under vacuum and the residue was poured into a separation funnel containing AcOEt (25 ml) and HCl 1 M (25 ml), and the aqueous phase was extracted with AcOEt (2 x 15 ml). The combined organic layer was washed with water (25 ml) and brine (25 ml), dried over Na_2SO_4, filtered and evaporated under vacuum. The crude was purified by column chromatography (SiO_2, DCM/MeOH 9:1, R_f 0.47) to afford **11** (291 mg, 61%) as a colorless solid. Mp: > 250 ^oC; $[\alpha]_D^{25}$: -42.1 (c = 0.1 in MeOH); IR (neat): 3262 (w, b), 2980 (w), 1720 (m), 1648 (s), 1720 (m), 1648 (s), 1516 (w), 1367 (m), 1243 (m), 1152 (s), 1016 (w), 859 (w); ^1H NMR (400 MHz, CD_3OD): 4.60-4.23 (m, 5H), 2.55-1.99 (m, 20H), 1.55 (s, 9H), 1.48 (s, 63H); ^{13}C NMR (101 MHz, CD_3OD): 173.6 (2x, s), 173.2 (2x, s), 172.98 (2x, s), 172.94 (2x, s), 172.4 (2x, s), 171.7 (2x, s), 162.9 (s), 156.8 (s), 156.2 (2x, s), 155.3 (s), 152.6 (2x, s), 83.3 (2x, s), 80.8 (2x, s), 80.6 (2x, s), 79.3 (2x, s), 52.9 (2x, d), 51.0 (2x, d), 50.9 (d), 30.5 (2x, t), 29.9 (2x, t), 29.3 (14x, t),

28.3 (2x, t), 27.3 (21x, q), 26.9 (3x, q); MS (ESI, MeOH/H_2O 2:1): 1588 (100, $[M-H]^-$), 1489 (97, $[M-Boc]^-$).

Compound G1H6. HCl (1 M in Et_2O) (10 ml) was added to a solution of **11** (200 mg, 0.12 mmol) in DCM (5 ml) under N_2. The reaction mixture was stirred under reflux for 12 hours. The resulting suspension was then sonicated for 10 min and stirred again under reflux for 8 hours. Solvent was removed under vacuum and the colorless solid formed was dissolved in MeOH (0.5 ml) and Et_2O (20 ml) was added to obtain a colorless precipitate. The resulting suspension was centrifuged for 10 minutes and the solvent was discharged. The precipitate was washed with Et_2O (2 x 15 ml) and dried under high vacuum to afford **G1H6** (82 mg, 63%) (hydrochloric salt) as a colorless solid. Mp: > 250 ^oC; $[\alpha]_D^{25}$: -26.4 (c = 0.1 in H_2O); IR (neat): 3156 (m, b), 2901 (m, b), 2653 (m, b), 1652 (s), 1515 (s), 1181 (m, b); ^1H NMR (400 MHz, D_2O): 4.44-4.13 (m, 5H), 2.51-2.33 (m, 10H), 2.20-1.88 (m, 10H); ^{13}C NMR (101 MHz, D_2O): 176.8 (s), 175.4 (s), 174.8 (s), 173.6 (s), 173.0 (s), 172.5 (s), 172.3 (s), 171.9 (s), 170.9 (s), 170.8 (s), 156.6 (s), 54.2 (d), 53.1 (d), 52.9 (d), 52.8 (d), 51.2 (d), 30.8 (t), 29.8 (t), 29.0 (t), 28.9 (t), 28.8 (t), 27.6 (t), 26.0 (t), 25.76 (t), 25.66 (t), 25.59 (t); MS (ESI, MeOH/H_2O 2:1): 812 (48, $[M+Na]^+$), 790 (100, $[M+H]^+$).

1.4. General procedures for vesicle experiments

1.4.1. Vesicle preparation

Following the general procedures in ref. S1, a thin lipid film was prepared by evaporating a solution of 25 mg EYPC in 1 ml MeOH/$CHCl_3$ (1:1) on a rotary evaporator (rt) and then *in vacuo* overnight. The resulting film was hydrated with 1.0 ml buffer (5 mM HPTS, 16.5 mM DPX, 10 mM Tris, 72 mM NaCl, pH 7.4) for more than 30 min, subjected to freeze-thaw cycles (5x) and extrusions (15x) through a polycarbonate membrane (pore size, 100 nm). Extravesicular components were removed by gel filtration (Sephadex G-50) with 10 mM Tris, 107 mM NaCl, pH 7.4. Final conditions: ~5 mM EYPC; inside: 5 mM HPTS, 16.5 mM DPX, 10 mM Tris, 72 mM NaCl, pH 7.4; outside: 10 mM Tris, 107 mM NaCl, pH 7.4.

1.4.2. Preparation of odorant hydrazones

As in ref. S1, 10 μl of 100 mM reactive counterion (**G1H6**, **G1H4**, **G1H3**, **G1H2** and **G1H1**) in DMSO was mixed with 2 equivalent of odorant for each tail of reactive counterion (e.g., 40 μl of 200 mM odorant in DMSO for reactive counterion with four tails) and the total volume was adjusted to 100 μl with DMSO. The mixture was stirred at 60˚C for 1 hour, and the resulting hydrazone was then used for DNA activation experiments. Hydrazone formation was confirmed by mass spectrometry [S1]

1.4.3. DNA activation experiments

Screening at constant [DNA] with variable [Activator].

As in ref. S1, EYPC-LUV stock solutions (5 μl) were diluted with a buffer (10 mM Tris, 107 mM NaCl, pH 7.4) and placed in a thermostated fluorescence cuvette (25 ˚C) and gently stirred (total volume in the cuvette, ~2000 μl; final lipid concentration, ~13 μM). HPTS efflux was monitored at λ_{em} 511 nm (λ_{ex} 413 nm) as a function of time after addition of activator (odorant hydrazones, 10 μL in DMSO, variable final concentration: 100-0.10 μM) at t = 0 s, transporter (calf thymus DNA, 20 μl stock solution in buffer, constant final concentration: 1.25 μg/ml) at t = 40 s and 1.2% aqueous triton X-100 (40 μl, 0.024% final concentration) at t = 200 s.

Screening at constant [Activator] with variable [DNA].

As in ref. S1, EYPC-LUV stock solutions (25 μl) were diluted with a buffer (10 mM Tris, 107 mM NaCl, pH 7.4) and placed in a thermostated fluorescence cuvette (25 ˚C) and gently stirred (total volume in the cuvette, ~2000 μl; final lipid concentration, ~65 μM). HPTS efflux was monitored at λ_{em} 511 nm (λ_{ex} 413 nm) as a function of time after addition of activator (odorant hydrazones, 10 μL in DMSO, constant final concentration: 6.15 μM) at t = 0 s, transporter (calf thymus DNA, 20 μl stock solution in buffer, variable final concentration: 5000-0.125 ng/ml) at t = 40 s and 1.2% aqueous triton X-100 (40 μl, 0.024% final concentration) at t = 200 s.

Fluorescence intensities were normalized to fractional emission intensity I(t) using equation (S1)

$$I(t) = (I_t - I_0) / (I_\infty - I_0) \qquad \text{(S1)}$$

where $I_0 = I_t$ at DNA addition, $I_\infty = I_t$ at saturation after lysis. Effective concentration for hydrazone EC_{50} and Hill coefficient n were determined by plotting the fractional activity Y (= I(t) at saturation just before lysis, t = ~190 s) as a function of varied concentration $c_{variable}$ of activator or DNA and fitting them to the Hill equation (S2)

$$Y = Y_0 + (Y_{MAX} - Y_0) / \{1 + (EC_{50} / c_{variable})^n\} \qquad \text{(S2)}$$

where Y_0 is Y without hydrazone, Y_{MAX} is Y with an excess hydrazone at saturation, EC_{50} is the concentration of hydrazone required to reach 50% activity and n is the Hill coefficient. All dose response curves for hydrazones are shown in Figs. S2 and S3, all data from Hill analysis in Tables S1 and S2.

1.5. References

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2. Supplementary Figures

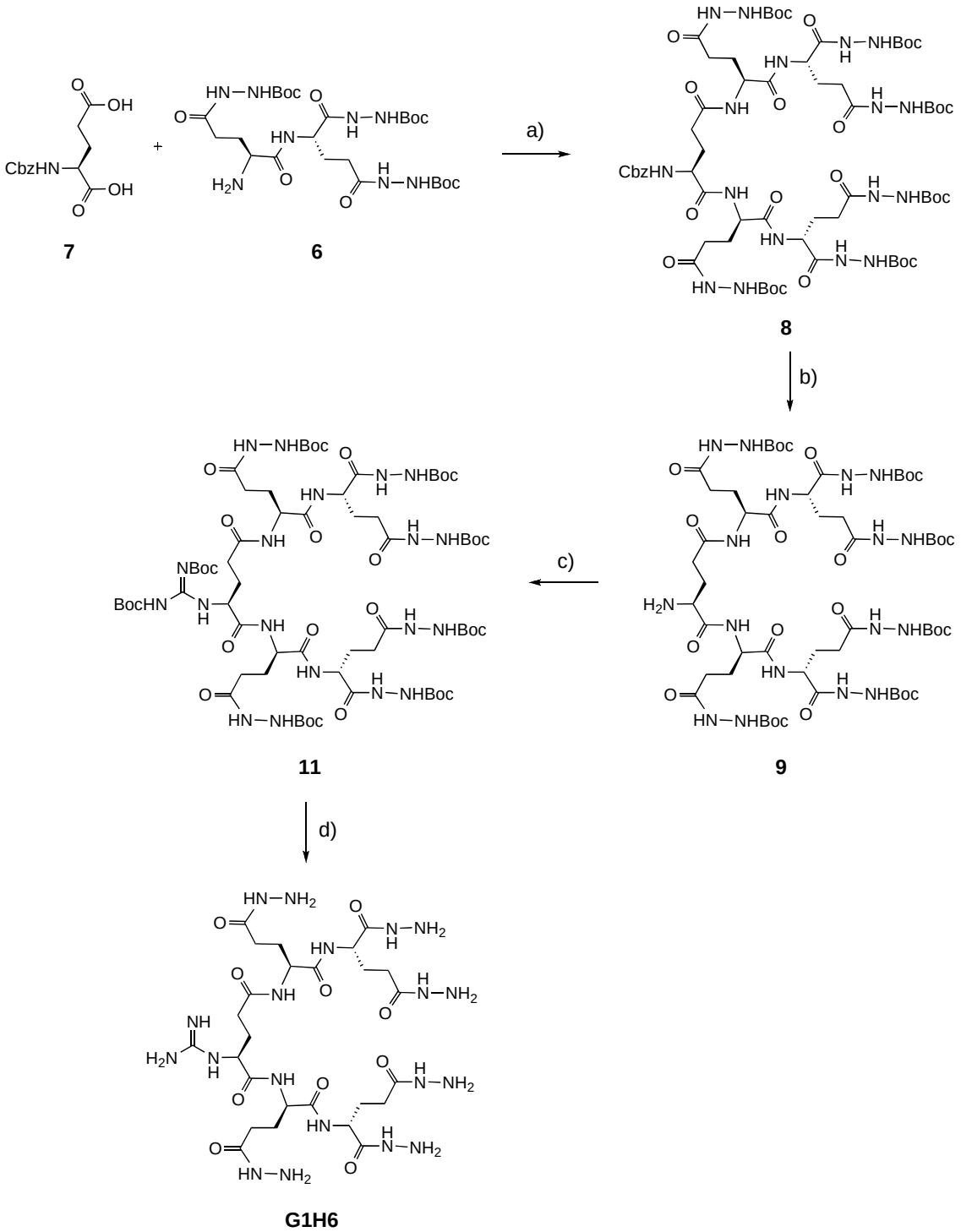
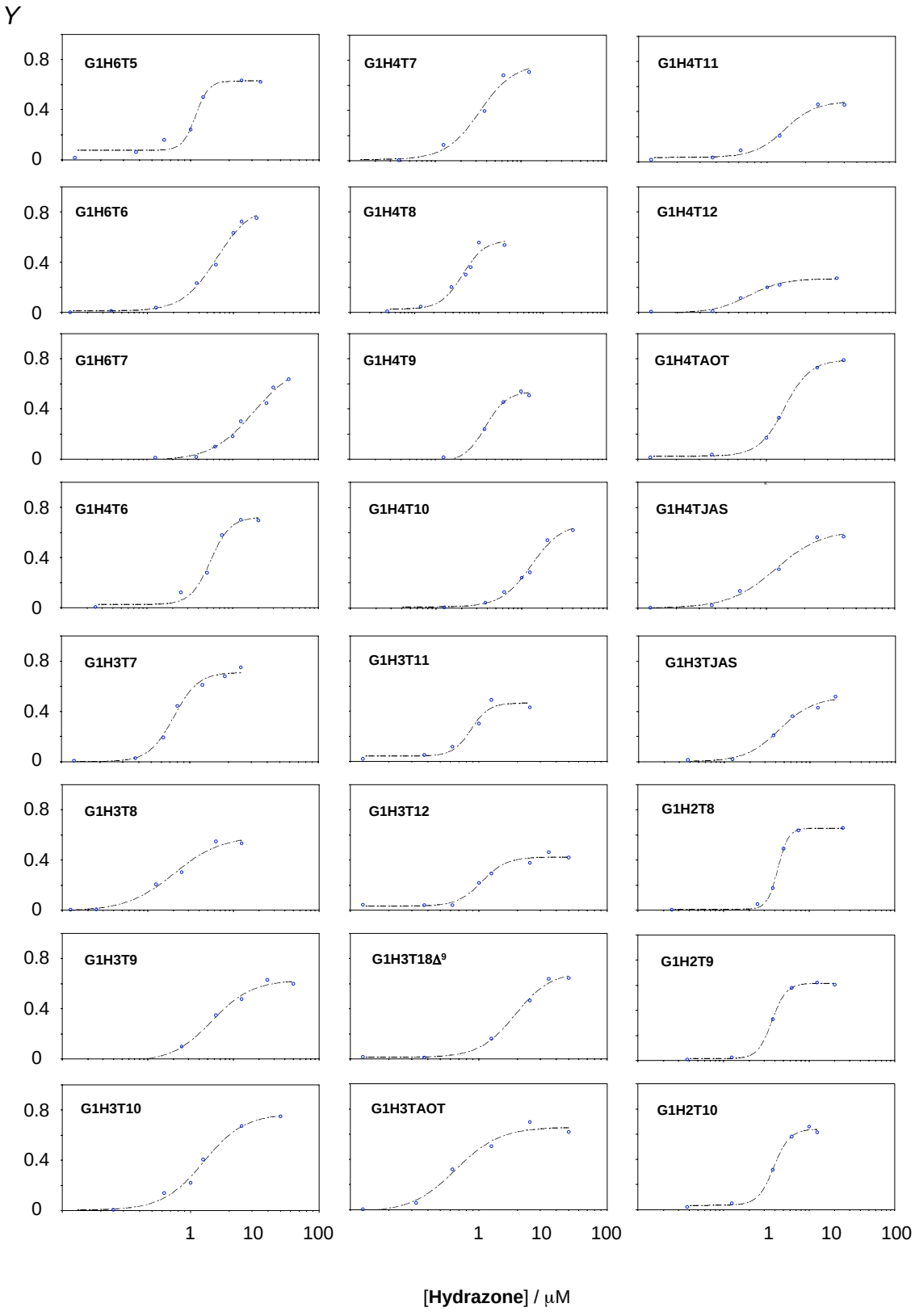


Figure S1. Synthesis of **G1H6**. a) HBTU, DIEA, DCM, rt, 62%; b) Pd/C, H_2, rt, quantitative; c) N,N'-Di-Boc-1-*H*-pyrazole-1-carboxamidine (**10**), DIEA, CH_3CN, 50 ^oC, 61%; d) 1M HCl in Et_2O, DCM, reflux 63%.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G1H3T18Δ9
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0
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[Hydrazone] / μM

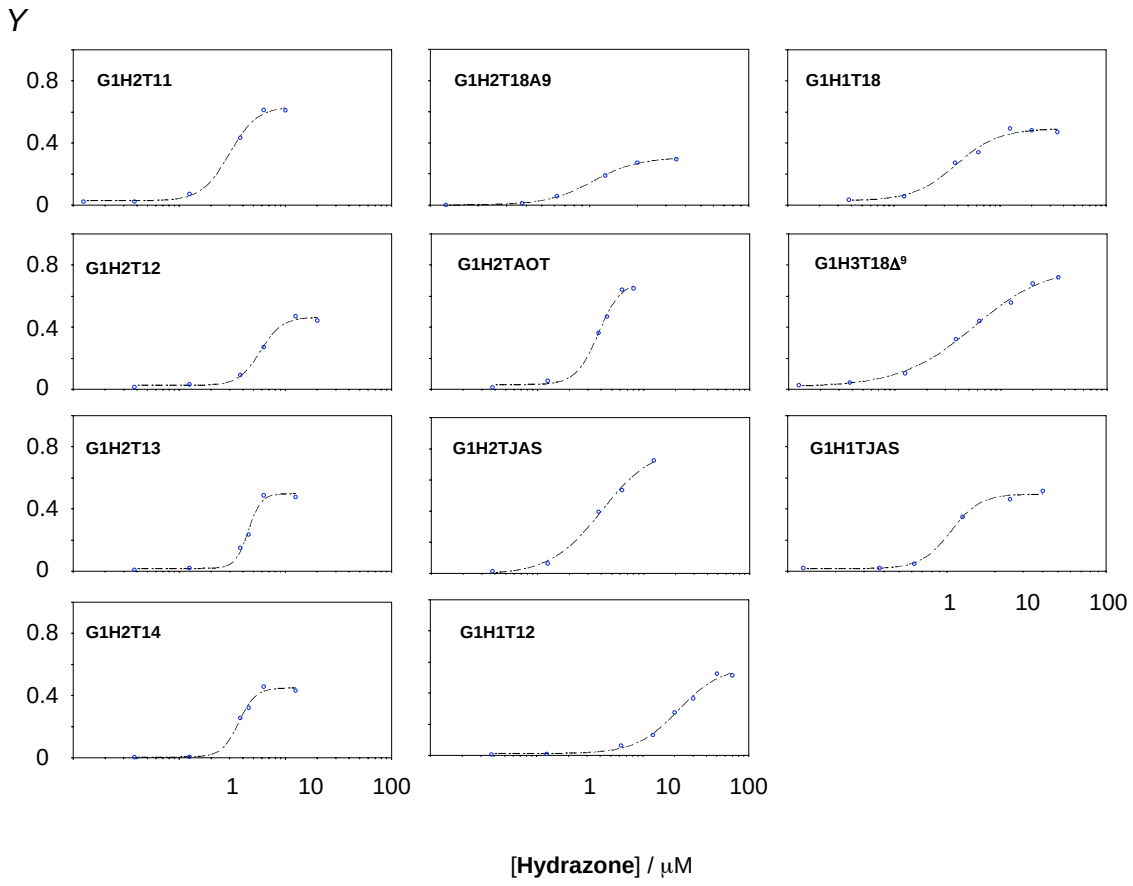

Figure S2. Dose-response curves for aldehydes with different chain length (**T5-T18**) plus the branched examples ($\mathbf{T_{AOT}}$, $\mathbf{T_{JAS}}$, $\mathbf{T18\Delta^{9}}$) derivatized with five different reactive counterions (**G1H1**, **G1H2**, **G1H3**, **G1H4** and **G1H6**).

3. Supplementary Tables

Table S1. EC_{50} and Y_{max} for hydrazones at a constant concentration of DNA and a variable concentration of activator (1st screening).[a]

	G_1H_1		**G_1H_2**		**G_1H_3**		**G_1H_4**		**G_1H_6**	
	Y_{max}[b]	EC_{50}[c]	Y_{max}[b]	EC_{50}[c]	Y_{max}[b]	EC_{50}[c]	Y_{max}[b]	EC_{50}[c]	Y_{max}[b]	EC_{50}[c]
T_4	NA[d]		NA[d]		NA[d]		NA[d]		NA[d]	
T_5	NA[d]		NA[d]		NA[d]		NA[d]		63.1±4.1	11.0±1.6
T_6	NA[d]		NA[d]		NA[d]		72.1±4.6	5.5±0.4	84.7±7.0	6.36±0.7
T_7	NA[d]		NA[d]		71.9±3.4	7.4±0.4	77.6±9.1	3.3±0.7	76.5±1.4	17.7±4.4
T_8	NA[d]		65.4±1.5	4.3±0.1	59.0±9.1	2.0±0.5	57.6±7.6	5.6±1.0	NA[d]	
T_9	NA[d]		61.5±0.7	3.6±0.1	62.9±3.5	5.6±0.7	54.0±3.8	3.8±0.3	NA[d]	
T_{10}	NA[d]		64.1±2.4	3.9±0.2	77.2±4.9	12.6±1.7	67.3±5.4	13.0±1.3	NA[d]	
T_{JAS}	49.7±1.8	10.6±0.7	81.2±10.6	4.1±0.7	51.3±4.6	4.4±0.6	62.1±6.1	11.8±1.6	NA[d]	
T_{AOT}	NA[d]		69.3±5.0	3.7±0.1	65.6±5.6	6.6±1.1	79.3±1.3	13.8±0.3	NA[d]	
T_{11}	NA[d]		63.7±2.6	2.9±0.2	46.6±6.1	8.7±1.2	48.0±4.6	13.7±1.6	NA[d]	
T_{12}	57.2±4.5	21.6±2.3	46.5±2.0	5.8±0.3	42.4±1.9	10.5±0.7	26.8±1.9	7.1±0.7	NA[d]	
T_{13}	D[e]		49.9±3.5	4.5±0.2	NA[d]		D[e]		NA[d]	
T_{14}	D[e]		44.9±2.7	3.6±0.2	NA[d]		NA[d]		NA[d]	
T_{16}	D[e]		NA[d]		NA[d]		NA[d]		NA[d]	
T_{18}	49.3±2.7	3.8±0.5	NA[d]		NA[d]		NA[d]		NA[d]	
$T_{18\Delta 9}$	79.4±4.6	5.5±0.6	30.5±0.6	10.5±0.3	69.6±5.1	19.2±1.7	NA[d]		NA[d]	

[a]All data were obtained from Hill analysis of dose response curves for hydrazones **G1H*n*T*m*** (*n* = 1-6, *m* = 4-18 and **$T18\Delta^9$**, **T_{JAS}** & **T_{AOT}**; variable final concentrations: 100-0.10 μM) and detected with calf thymus DNA (constant final concentration: 1.25 μg/ml) in HPTS/DPX-LUVs. [b]Maximal activity (%). [c]Effective concentration for hydrazones needed to reach $Y_{max}/2$ (μM). [d]NA, no activity (Y_{max} < 20% at 25 μM hydrazone). [e]D, only detergent effect.

Table S2. EC_{50} and Y_{max} for hydrazones at a constant concentration of activator and a variable concentration of DNA (2nd screening).[a]

	G_1H_2		**G_1H_3**		**G_1H_4**		**G_1H_6**	
	Y_{max}[b]	EC_{50}[c]	Y_{max}[b]	EC_{50}[c]	Y_{max}[b]	EC_{50}[c]	Y_{max}[b]	EC_{50}[c]
T_4	NA[d]		NA[d]		NA[d]		NA[d]	
T_5	NA[d]		NA[d]		NA[d]		NA	
T_6	NA[d]		NA[d]		NA[d]		44.4±5.2	0.71±0.16
T_7	NA[d]		NA[d]		25.6±3.0	0.18±0.07	NA[d]	
T_8	NA[d]		51.0±3.8	0.36±0.07	17.1±1.2	0.45±0.06	NA[d]	
T_9	NA[d]		29.3±0.06	0.53±0.07	NA[d]		NA[d]	
T_{10}	NA[d]		NA[d]		NA		NA[d]	
T_{JAS}	NA[d]		17±0.05	0.55±0.03	NA[d]		NA[d]	
T_{AOT}	NA[d]		NA[d]		NA[d]		NA[d]	
T_{11}	18.0±1.3	0.49±0.08	NA[d]		NA[d]		NA[d]	
T_{12}	18.7±1.6	0.44±0.07	NA[d]		NA[d]		NA[d]	
T_{13}	NA[d]		NA[d]		NA[d]		NA[d]	
T_{14}	NA[d]		NA[d]		NA[d]		NA[d]	
T_{16}	NA[d]		NA[d]		NA[d]		NA[d]	
T_{18}	NA[d]		NA[d]		NA[d]		NA[d]	
$T_{18\Delta 9}$	NA[d]		NA[d]		NA[d]		NA[d]	

[a]All data were obtained from Hill analysis of dose response curves for hydrazones **G1H*n*T*m*** (*n* = 1-6, *m* = 4-18 and **T18Δ^9**, **T_{JAS}** & **T_{AOT}**; constant final concentration: 6.15 μM) and detected with calf thymus DNA (variable final concentration: 0.125-5000 ng/ml) in HPTS/DPX-LUVs. [b]Maximal activity (%). [c]Effective DNA concentration needed to reach $Y_{max}/2$ (μM). [d]NA, no activity (Y_{max} < 20% at 1.25 μg/ml DNA).