Ethynyl benziodoxolones: Functional terminators for cell-penetrating poly(disulfide)s

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Supplementary Information

Table of Content

1. Materials and Methods

As in ref. S1, Supporting Information. Briefly, reagents for synthesis were purchased from Fluka, Sigma-Aldrich, TCI and Across, buffers and salts of the best grade available from Fluka or Sigma-Aldrich and used as received. Amicon Ultra 0.5 mL centrifugal filter units were purchased by Sigma-Aldrich.

Unless stated otherwise, column chromatography was carried out on silica gel 60 (Fluka, 40-63 μm). Analytical TLC was performed on silica gel 60 (Fluka, 0.2 mm). Fluorescence measurements were performed with a FluoroMax-4 spectrofluorometer (Horiba Scientific) equipped with a stirrer and a temperature controller (25.0 ± 0.1 ºC). Fluorescence spectra were corrected using instrument-supplied correction factors. UV-Vis spectra were recorded on a JASCO V-650 spectrophotometer equipped with a stirrer and a temperature controller (25.0 ± 0.1 ºC) and are reported as maximal absorption wavelength λ in nm (extinction coefficient ε in $M^{-1}cm^{-1}$). Gel-permeation chromatography (GPC) was performed using a JASCO LC-2000 Plus system equipped with a quaternary pump (JASCO PU-2089), photodiode array (JASCO MD-2018 Plus) and fluorescence (JASCO FP-2020 Plus) detectors. The chromatographic column used was a Superdex 75 10/300 GL (flow 0.4 mL/min, eluent: 30% ACN in 0.1 M acetate buffer pH = 6.5). Semi-preparative HPLC was performed using JASCO LC-2000 Plus system equipped with a quarternary pump (JASCO PU-2089) and UV/Vis detector (JASCO UV-2077 Plus). LC-MS were recorded using a Thermo Scientific Accela HPLC equipped with a Thermo C18 (5 cm x 2.1 mm, 1.9 μm particles) Hypersil gold column coupled with a LCQ Fleet three-dimensional ion trap mass spectrometer (ESI, Thermo Scientific) with a linear elution gradient from 95% H_2O 0.01% TFA to 90% ACN 0.01% TFA in 4.0 minutes at a flow rate of 0.75 mL/min. pH values were measured with a Consort C832 multi-parameter analyzer equipped with a VWR glass membrane pH electrode calibrated with Titrisol solution from Merck at pH 4.00 and 7.00. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (ATR, Golden Gate) and are reported as wavenumbers ν in cm^{-1} with band intensities indicated as s (strong), m (medium), w (weak), br (broad). ^1H and ^{13}C NMR spectra were recorded (as indicated) on a Bruker 400 MHz spectrometer and are reported as chemical shifts (δ) in ppm relative to TMS (δ = 0). Proton spin multiplicities are reported as a singlet (s), doublet (d), triplet (t),

quartet (q) and quintet (quint) with coupling constants (*J*) given in Hz, or multiplet (m). ^{1}H 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 analysis were performed on a Finnigan MAT SSQ 7000 instrument or an ESI API 150EX and are reported as mass-per-charge ratio *m/z* (intensity in %, [assignment]). ESI-HRMS analysis for the characterization of new compounds were performed on a QSTAR Pulsar (AB/MDS Sciex) and are reported as mass-per-charge ratio *m/z* calculated and observed. Fluorescence imaging was performed using Leica SP5 confocal microscope, equipped with 63x oil immersion objective lens.

Abbreviations. ACN: Acetonitrile; Calcd: Calculated; CF: 5(6)-Carboxyfluorescein; CH_2Cl_2: Dichloromethane; DMF: *N*,*N*-Dimethylformamide; DTT: 1,4-Dithio-DL-threitol; FBS: Fetal bovine serum; GPC: Gel permeation chromatorgraphy; HATU: 2-(7-Aza-1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; MEM: Minimum essential media; MeOH: Methanol; PBS: Phospate buffer saline; NEt_3: triethylamine; PDI: Polydispersity index; PS: Penicillin / streptomycin; rt: Room temperature; TAMRA: 5-carboxytetramethylrhodamine; TBTA: Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine; TEOA: Triethanolamine; TFA: Trifluoroacetic acid; THF: Tetrahydrofuran.

2. Synthesis

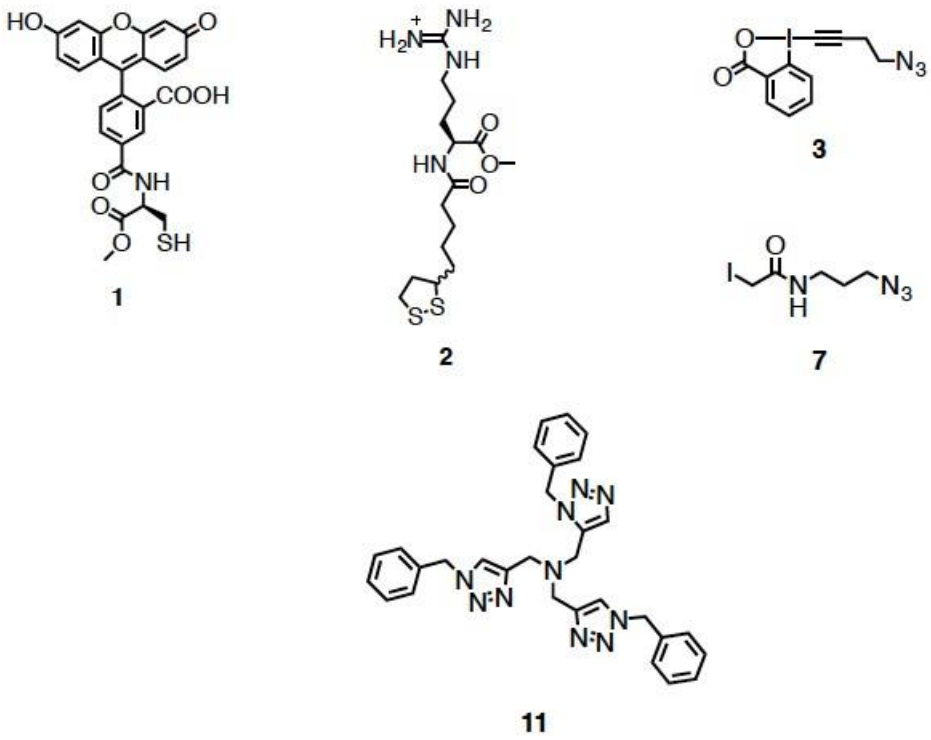


Chart S1.

Compound 1. This compound was prepared as reported in ref. S1.

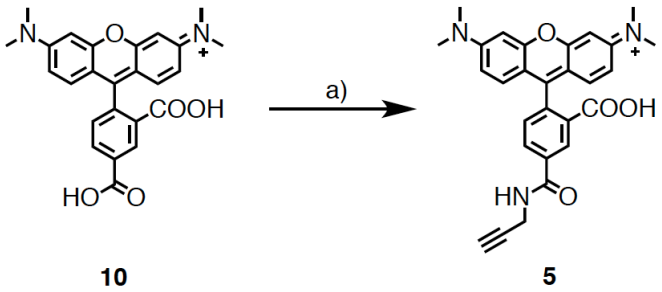
Compound 2. This compound was prepared as reported in ref. S2.

Compound 3. This compound was prepared as reported in ref. S3.

Compound 7. This compound was prepared as reported in ref. S4.

Compound 10. This compound was prepared as reported in ref. S5.

Compound 11. This compound was prepared as reported in ref. S6.



Scheme S1. a) propargylamine, NEt_3, HATU, dry DMF, rt, 1.3 h, 40%.

Compound 5. To a solution of **10** (24 mg, 0.050 mmol) in dry DMF (10 mL), NEt_3 (15 μL, 0.10 mmol) was added and the resulting mixture was stirred at rt under Ar atmosphere. After 10 min, HATU (22 mg, 0.060 mmol) was added and the resulting mixture was stirred at rt under Ar atmosphere. After 10 min, propargylamine (5 μL, 0.080 mmol) was added and the solution stirred at rt under Ar atmosphere. After 60 min, solvent was removed *in vacuo* and the purple crude was purified first by flash column chromatography (CH_2Cl_2/MeOH 8:2, R_f 0.29) and then by semi-preparative HPLC (R_t 10.3 min), yielding compound **5** as a dark purple solid (10.3 mg, 40%). Mp: 166 – 167 °C; IR (neat): 1649 (m), 1597 (s), 1535 (w), 1493 (m), 1411 (w), 1348 (s), 1187 (s), 1134 (w), 928 (w), 824 (w), 701 (w); ^{1}H NMR (400 MHz, CD_3OD): 8.79 (d, ^4J (H,H) = 1.8 Hz, 1H), 8.28 (dd, ^3J (H,H) = 7.9 Hz, ^4J (H,H) = 1.8 Hz, 1H), 7.55 (d, ^3J (H,H) = 7.9 Hz, 1H), 7.16 (d, ^3J (H,H) = 9.5 Hz, 2H), 7.08 (dd, ^3J (H,H) = 9.5 Hz, ^4J (H,H) = 2.4 Hz, 2H), 7.00 (d, ^4J (H,H) = 2.4 Hz, 2H), 4.25 (d, ^4J (H,H) = 2.5 Hz, 2H), 3.29 (s, 12H), 2.68 (t, ^4J (H,H) = 2.5 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): 167.7 (C), 167.3 (C), 160.6 (C), 159.1 (C), 159.0 (C), 138.4 (C), 137.2 (C), 132.9 (C), 132.4 (CH), 132.0 (CH), 131.9 (CH), 131.4 (CH), 115.6 (CH), 114.7 (C), 97.4 (CH), 80.5 (C), 72.3 (CH), 40.9 (CH_3), 30.2 (CH_2); MS (ESI, MeOH): 468 (100, $[M]^+$).

3. Polymerization

3.1. General Procedure

The procedure described in ref. S2 was adapted to obtain each polymer. Stock solutions of propagator **2**, CF-initiator **1** and terminators **3** and **7** were freshly prepared. To 8 μL of propagator solution (3 M in DMF) in an Eppendorf tube, 8 μL of initiator solution (50 mM in DMF) and 64 μL of TEOA buffer (1 M, pH = 7.0) were added. The sample was kept at 25 °C with vigorous agitation at 1000 rpm for 30 minutes. After polymerization, 10 μL of terminator solution (40 mM in DMF) were added and the obtained solution was immediately purified by GPC.

3.2. Purification and Characterization

Polymers were purified by GPC using Superdex 75 10/300GL (10×300 mm) chromatographic column and 30% ACN in 0.1 M acetate buffer (pH = 6.5) as eluent. 100 μL of sample were loaded on the column and the flow rate was set to 0.4 mL/min from 0 to 40 min, 0.6 mL/min from 41 to 80 min. Commercial molecular weight standards were used for calibration (Table S1, Fig. S1). Quantification of the polymers was achieved by UV-Vis measurements based on CF-initiator absorption.

Table S1. GPC data for polymers **4** and **8**.

Polymer	M_w[a]	M_n[b]	PDI
4	10.4 kDa	9.7 kDa	1.07
8	10.3 kDa	9.9 kDa	1.04

[a]Mass average molecular weight. [b]Number average molecular weight.

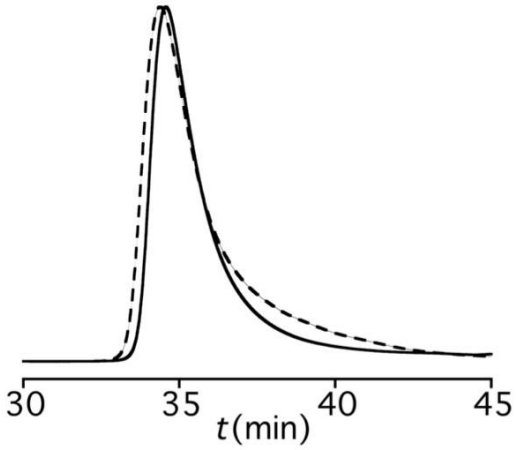


Figure S1. GPC profile of polymers **4** (solid) and **8** (dashed) after purification detected at λ_{em} = 517 nm (λ_{ex} = 492 nm).

4. Terminator Functionalization

4.1. CuAAC Reaction and Purification

Stock solutions of alkyne **5** and TBTA **11** were freshly prepared in THF. Stock solutions of sodium ascorbate and $CuSO_4 \cdot 5H_2O$ were freshly prepared in water.

CuAAC reaction to obtain polymer **6** was carried out in an Eppendorf tube by mixing 73 μL of polymer **4** (220 μM in water) with 10 μL of alkyne **5** solution (10 mM in THF) and then adding 10 μL of sodium ascorbate solution (10 mM in water), 10 μL of $CuSO_4 \cdot 5H_2O$ solution (1 mM in water) and 2 μL of TBTA solution (10 mM in THF).

CuAAC reaction to obtain polymer **9** was carried out in an Eppendorf tube by mixing 73 μL of polymer **8** (45 μM in water) with 10 μL of alkyne **5** solution (10 mM in THF) and then adding 10 μL of sodium ascorbate solution (10 mM in water), 10 μL of $CuSO_4 \cdot 5H_2O$ solution (1 mM in water) and 2 μL of TBTA solution (10 mM in THF).

The resulting solutions were kept at 25 °C and vigorously agitated at 1000 rpm for 17h. Purification of the mixtures was achieved by washing with water (12 x 200 μL) using centrifugal filter units with a molecular weight cutoff of 3 kDa, yielding polymers **6** and **9** in water. The efficiency of the purification method was verified by LC-MS where no peak due to alkyne **5** and TBTA was visible. Moreover, GPC analysis of the purified polymer **6**

confirmed that the polymer was not affected by CuAAC conditions (Fig. S2). Fluorescence in GPC detected at λ_{em} = 582 nm (λ_{ex} = 552 nm) confirmed that TAMRA was successfully conjugated to the terminator.

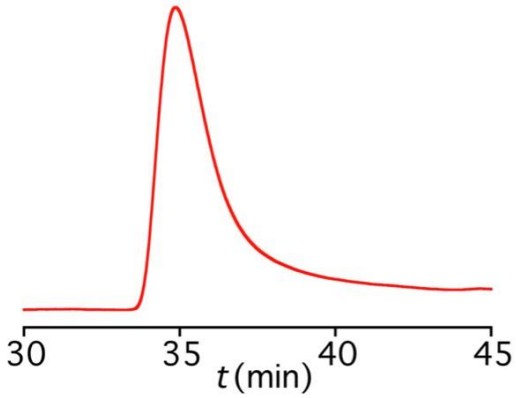


Figure S2. GPC profile of polymer **6** detected at λ_{em} = 582 nm (λ_{ex} = 552 nm).

4.2. FRET Measurement and Depolymerization

Stock solutions of polymers **6** and **9** (8 μM) in TEOA buffer (1 M, pH 7) were freshly prepared and their absorption measured by UV-Vis spectrometry. Fluorescence emission spectra were recorded of the same stock solutions by excitation at 488 nm (Fig. S3). The stock solution of DTT (1 M, water) was freshly prepared. Depolymerization was carried out on the same stock solutions by addition of 10 μL of DTT (final concentration of 50 mM). The resulting solutions were kept at 25 °C and vigorously agitated at 1000 rpm. After 20 min, fluorescence emission spectra (λ_{ex} = 488 nm) of the depolymerized mixtures were recorded (Fig. S3).

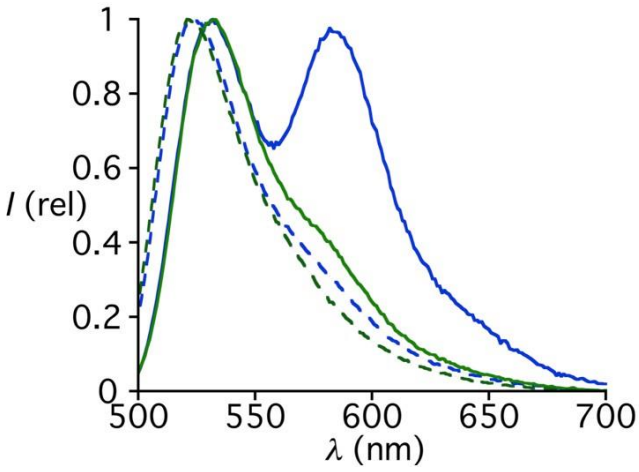


Figure S3. Emission spectra of polymers **6** (blue) and **9** (green), before (solid) and after (dashed) depolymerization.

5. Cellular Uptake

5.1. Cell Culture

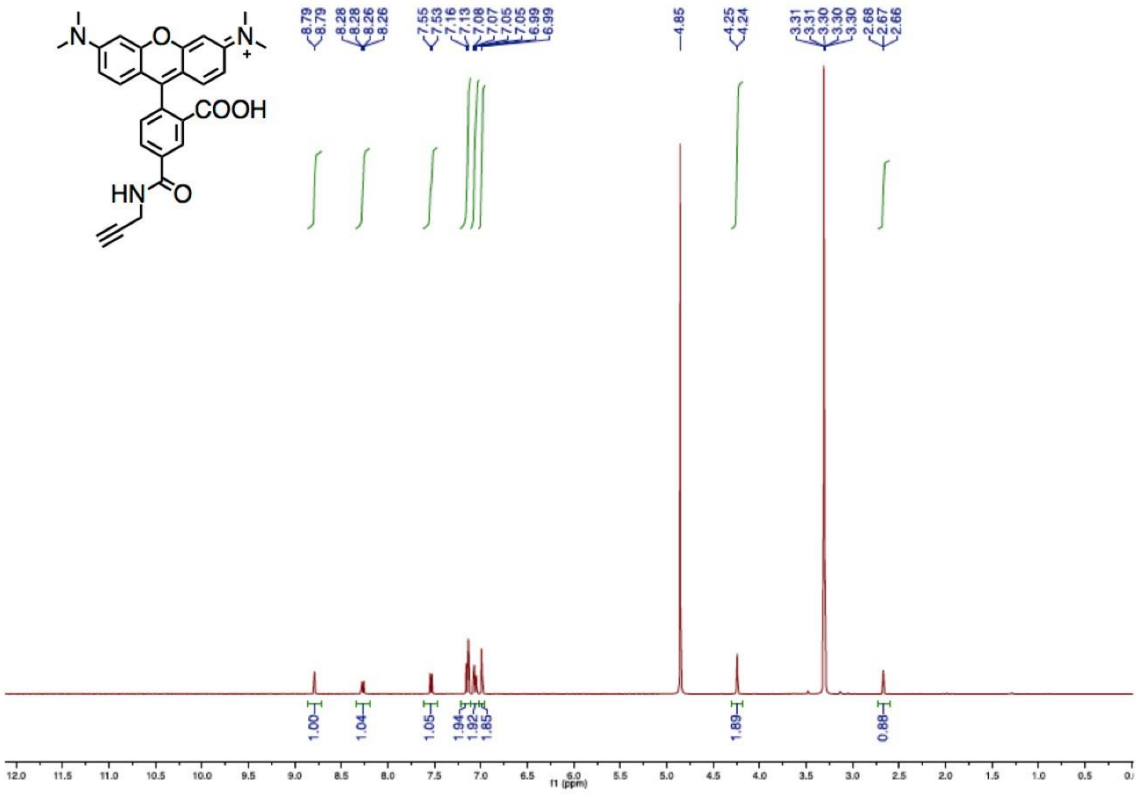
Human cervical cancer-derived HeLa Kyoto cells were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 1% penicillin / streptomycin (PS) and 1% L-glutamine. The cells were grown on a 25 cm^3 tissue culture flask (TPD corporation) at 37 °C under 5% CO_2.

5.2. Confocal Microscopy

HeLa Kyoto cells were seeded at 1 x 10^5 cells/well on 35 mm glass-bottomed dishes (MatTek Corporation) and cultured overnight. After removing the medium, the cells were washed 3 times with Liebovitz's medium and treated with 1 mL of polymer **6** solution (500 nM in Liebovitz's medium). The cells were incubated for 15 min at 37 °C, then the media was removed by aspiration. Cells were washed 3 times with PBS containing 0.1 mg/mL heparin, twice with Liebovitz's medium and then kept in Liebovitz's medium. Distribution of fluorescent polymer was analyzed without fixing using a confocal laser scanning microscope (Leica SP5) equipped with 63x oil immersion objective lens. Ar laser was used as light

source (4% laser power) with excitation wavelength 488 nm, emission 498 ~ 535 nm for CF and emission 571 ~ 650 nm for TAMRA (Leica HyD^{TM} detector); DPSS laser (4%) with excitation wavelength 561 nm and emission 571 ~ 650 nm for TAMRA (Leica HyD^{TM} detector). During CLSM analysis, the samples were kept at 37 °C.

6. NMR Spectra



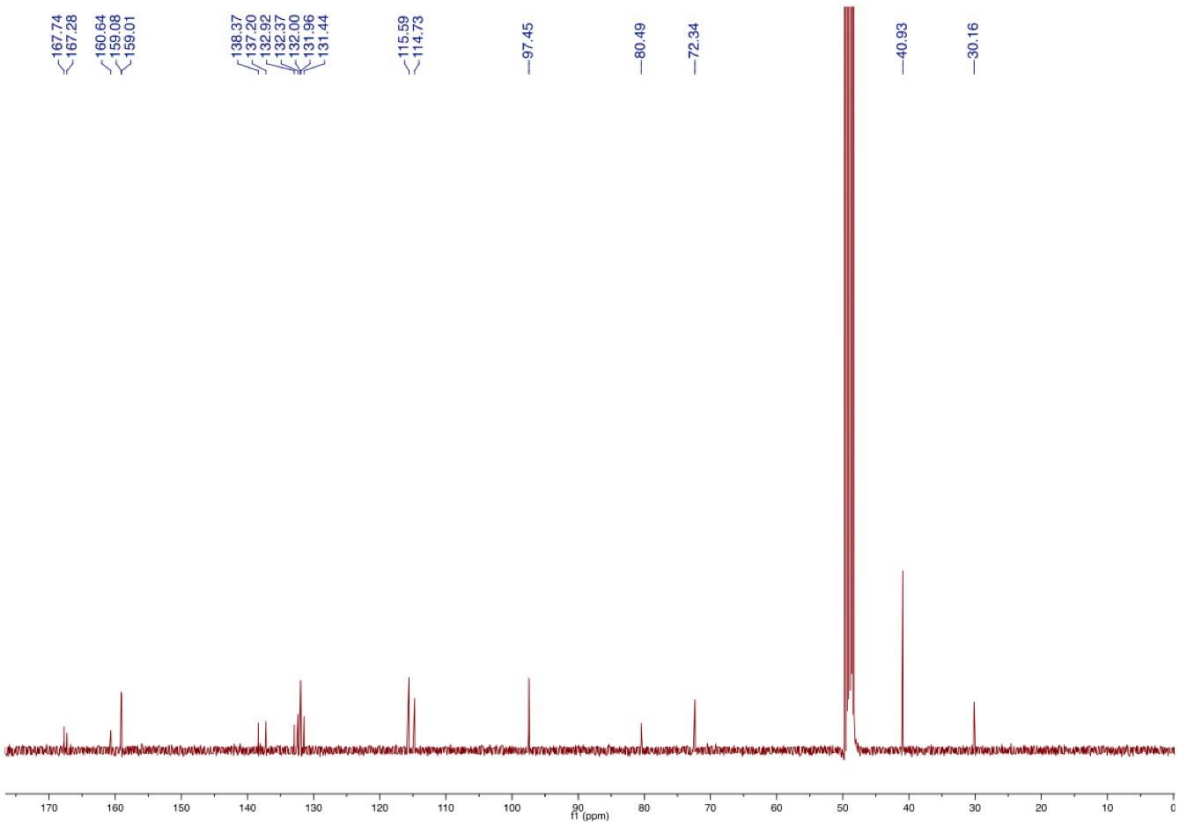


Fig. S4. ^{1}H NMR and ^{13}C NMR spectra of **5** in CD_3OD.

7. Supplementary Reference

S1 G. Gasparini, G. Sargsyan, E.-K. Bang, N. Sakai and S. Matile, *Angew. Chem. Int. Ed.*, 2015, **54**, 7328-7331.

S2 E.-K. Bang, G. Gasparini, G. Molinard, A. Roux, N. Sakai and S. Matile, *J. Am. Chem. Soc.*, 2013, **135**, 2088-2091.

S3 R. Frei, M. D. Wodrich, D. P. Hari, P. A. Borin, C. Chauvier and J. Waser, *J. Am. Chem. Soc.*, 2014, **136**, 16563-16573.

S4 N. Kotoku, C. Nakata, T. Kawachi, T. Sato, X.-H. Guo, A. Ito, Y. Sumii, M. Arai and M. Kobayashi, *Bioorg. Med. Chem.*, 2014, **22**, 2102-2112.

S5 M. V. Kvach, I. A. Stepanova, I. A. Prokhorenko, A. P. Stupak, D. A. Bolibrukh, V. A. Korshun and V. V. Shmanai, *Bioconjugate Chem.*, 2009, **20**, 1673-1682.

S6 B. Vonhoeren, S. Dalgleish, L. Hu, M. M. Matsushita, K. Awaga and B. J. Ravoo, *ACS Appl. Mater. Interfaces*, 2015, **7**, 7049-7053.