Supporting Information

Fluorogenic squaraine dimers for the flow cytometry detection of urinary tract infections

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Table of Contents

1. Additional schemes, figures, and tables

Table S1. Photophysical properties of the dimeric and monomeric conjugates.

	dSQEt-Ubi			dSQC8-Ubi			dSQ⁺-Ubi			mSQC8-Ubi		
	λ_{abs}, nm[a]	λ_{fl}, nm[b]	QY, %[c]	λ_{abs}, nm[a]	λ_{fl}, nm[b]	QY, %[c]	λ_{abs}, nm[a]	λ_{fl}, nm[b]	QY, %[c]	λ_{abs}, nm[a]	λ_{fl}, nm[b]	QY, %[c]
DMSO	644	653	21 ± 0.7	645	654	26 ± 2.2	644	653	24 ± 0.7	645	653	31 ± 1.5
EtOH	632	641	12 ± 1.4	634	642	17 ± 1.6	632	641	17 ± 2.6	636	641	22 ± 1.7
MeOH	629	638	5.4 ± 0.3	631	640	7.6 ± 0.4	630	639	7.5 ± 0.5	631	639	12 ± 0.6
Water	585	640	0.5 ± 0.3	589 629	639	1.5 ± 0.7	584	639	0.6 ± 0.4	631	641	10 ± 2.8
PBS[d]	585	640	0.5 ± 0.4	591 633	639	0.7 ± 0.4	586	640	0.8 ± 0.3	631	641	10 ± 1.2
Turn-on[e]	42			37			31			3		

[a] Position of the maximum absorption wavelength. [b] Position of the maximum emission wavelength. [c] Fluorescence quantum yields expressed as mean from three independent experiments ± SD. [d] Phosphate-buffered saline. [e] Fluorescence turn-on, calculated as a ratio of fluorescence quantum yields in DMSO and PBS.

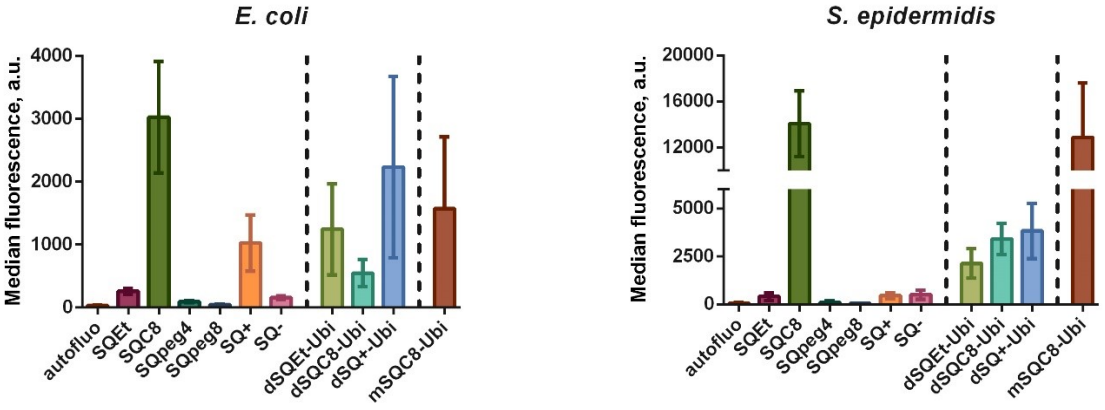


Figure S1. Median fluorescence intensities of the bacteria stained with 1 μM of squaraines **SQEt**, **SQC8**, **SQpeg4**, **SQpeg8**, **SQ⁺** and **SQ⁻**; dimeric probes **dSQEt-Ubi**, **dSQC8-Ubi** and **dSQ⁺-Ubi** and the monomeric probe **mSQC8-Ubi**; error bars represent standard deviation for n ≥ 3 biological replicates.

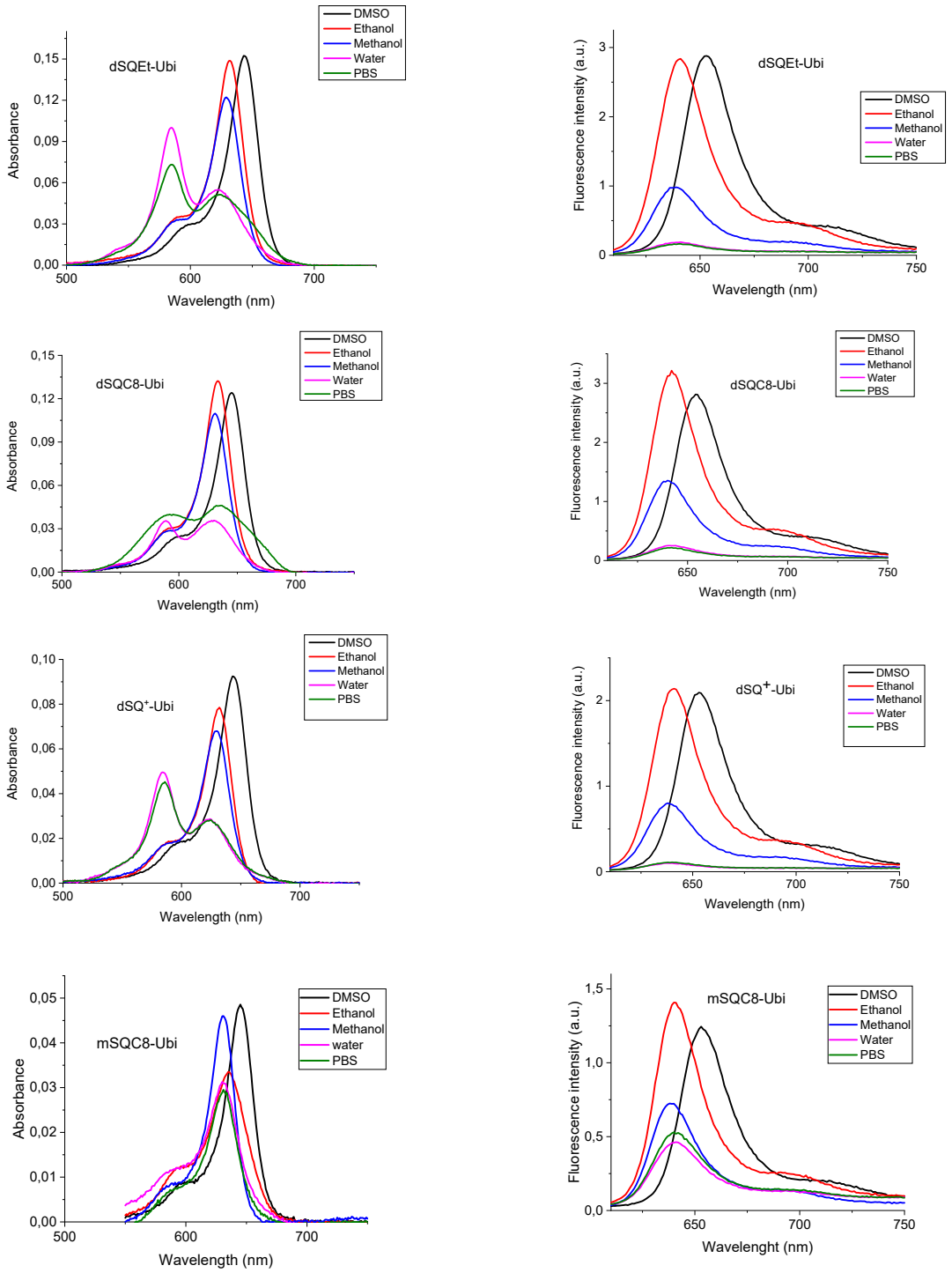


Figure S2. Absorption and fluorescence emission spectra of **dSQEt-Ubi**, **dSQC8-Ubi** and **dSQ⁺-Ubi** (200 nM) and **mSQC8-Ubi** (400 nM) in organic and aqueous solvents; λ_{ex} = 600 nm. PBS – phosphate-buffered saline.

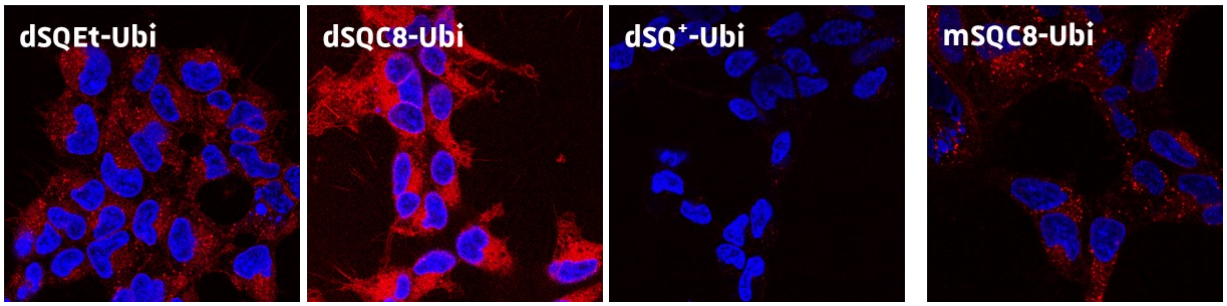


Figure S3. Confocal fluorescence microscopy images of live HEK293T cells stained with 1 μM of bacteria-targeting probes, retreated separately from the images of unconjugated squaraines using adjusted brightness and contrast settings. The images in each channel were recorded using identical instrumental settings.

Table S2. Characterization of the patient urine samples.

Sample code	RBC/mL[a]	WBC/mL[b]	Bacteria detected by the traditional culture-based approach [c]	Bacterial load (cells/μL) estimated by rapid flow cytometry with dSQ^+-Ubi
98AA	2000	1000	sterile	1738
101CL	4000	23000	sterile	965
109DJ	11000	1000	sterile	1334
71DJ	1000	2000	10^4 CFU/mL of *P. mirabilis*	3883
114SM	5000	41000	10^4 CFU/mL of *S. saprophyticus*	3304
111SS	30000	6586000	10^6 CFU/mL of *K. pneumoniae* + 10^6 CFU/mL of *E. coli*	7060
32TY	38000	1571000	10^7 CFU/mL of *E. coli*	16150
92YY	4000	33000	10^4 CFU/mL of *E. coli* + 10^5 CFU/mL of *S. gallolyticus*	10167
115PP	4319000	951000	10^6 CFU/mL of *K. pneumoniae*	6425
82PJ	6000	176000	10^7 CFU/mL of *K. pneumoniae*	17456
4AR	2025000	785000	10^4 CFU/mL of *E. faecium*	7133
87CM	13000	20000	10^7 CFU/mL of *E. faecalis*	2885
87DS	6000	182000	10^6 CFU/mL of *E. coli*	3786
44BM	4000	20400	10^7 CFU/mL of *E. coli*	8571
63DJ	554000	9292000	10^5 CFU/mL of *C. koseri*	10643
60DP	4000	444000	10^6 CFU/mL of *E. coli*	6369

[a] Red blood cells per mL. [b] White blood cells per mL. [c] Incubation on *Brilliance*™ UTI Clarity™ Agar plates for 24 hours followed by the detection of individual colony-forming strains by MALDI-TOF; CFU = colony-forming unit.

2. Chemical synthesis

2.1 General methods

Chemicals and reagents were obtained from commercial sources (MilliporeSigma, Fluorochem Ltd, TCI Europe N.V.) and used without further purification. Solvents were obtained from commercial sources (Carlo Erba Reagents GmbH, MilliporeSigma) and used as supplied. Fmoc-L-amino acids for peptide synthesis were purchased from Iris Biotech GmbH. Fmoc-protected Rink-amide resin was purchased from MilliporeSigma and the overall yields for the solid-phase synthesis were calculated based on the initial loading capacity provided by the supplier.

Reactions were monitored by analytical reverse-phase high-performance liquid chromatography (RP-HPLC), performed on an Agilent Technologies 1200 series HPLC system equipped with a C18 Kinetex column (5 µm, 4.6 mm ×150 mm) using a linear gradient (5% to 95% in 7.39 min, flow rate of 1.5 mL·min^{-1}) of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v) with detection at 220 nm, 254 nm, 365 nm and 630 nm.

Low-resolution mass spectra (LRMS) and high-resolution mass spectra (HRMS) were obtained on an Agilent Technologie 6520 Accurare-Mass Q-TOF LC/MS apparatus equipped with a Zorbax SB C18 column (1.8 µm, 2.1 × 50 mm) using electrospray ionization (ESI) and a time-of-flight analyzer (TOF).

Automated SPPS was carried out using a Liberty Blue synthesizer (CEM, France).

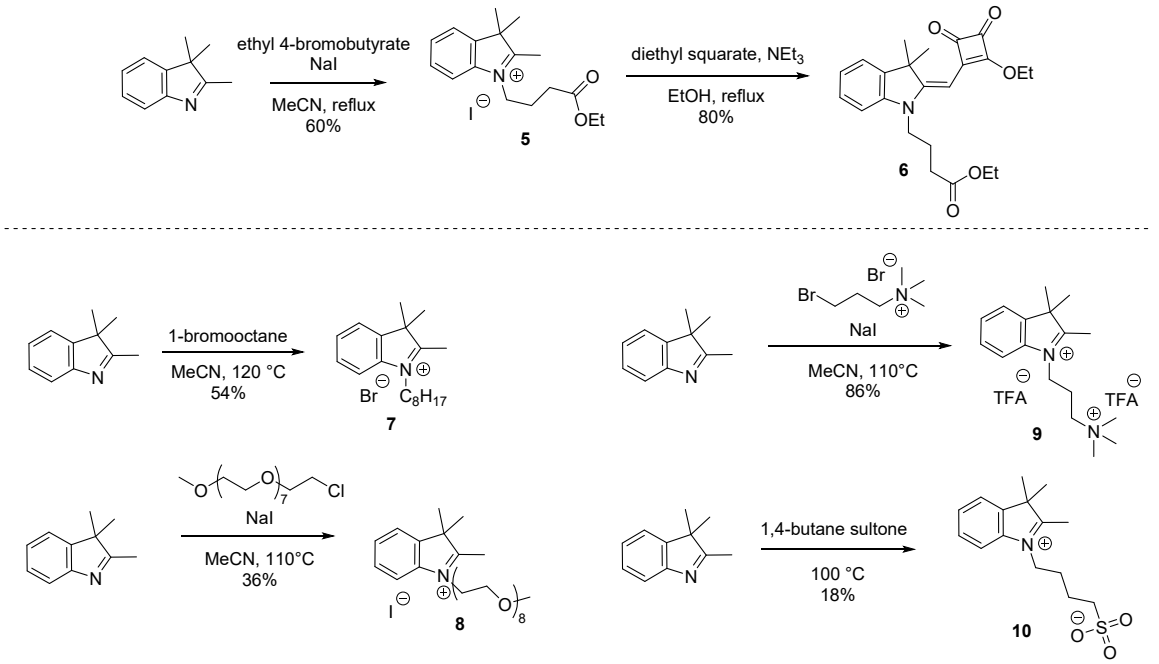
Semi-preparative reversed-phase HPLC chromatography was performed on a Gilson PLC2050 system equipped with a SunFire C18 column (5 µm, 19 × 150 mm) using a linear gradient (5% to 95% in 40 min, the flow rate of 18 mL·min^{-1}) of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v).

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker Avance III 400MHz, 500MHz or 700MHz BBFO+ probe spectrometer at 25°C. Deuterated solvents were purchased from Sigma-Aldrich. Chemical shifts are reported in ppm (δ), relatively to residual solvent.

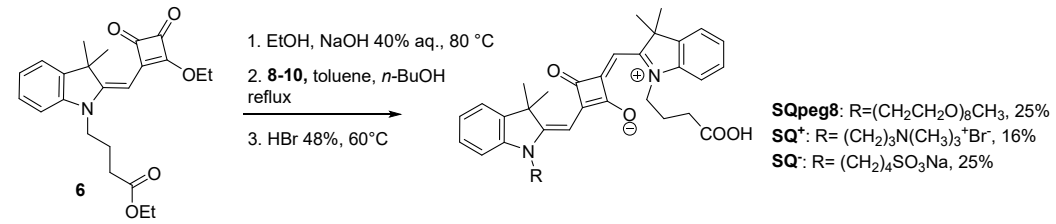
2.2 Synthesis of squaraines

SQEt and **SQpeg4** were synthesized following the protocol described by Karpenko, et al.[1] *N*-alkylated trimethylindolenines **5,**[2] **8,**[3] and **10**[4] were synthesized following the published protocols.

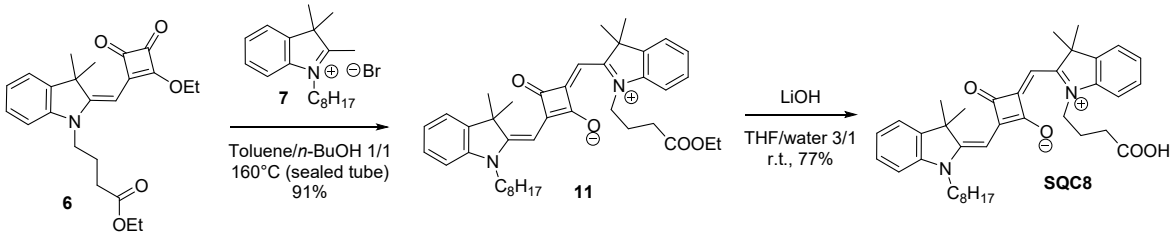
The synthesis of **SQpeg8**, **SQ$^+$,** and **SQ$^-$**, which followed the classical three-step approach (saponification, condensation, and acid hydrolysis), is described below. As for **SQC8**, its synthesis following the three-steps approach resulted in a very low yield (< 10%), so an alternative two-steps approach (condensation followed by saponification) was proposed as presented below.



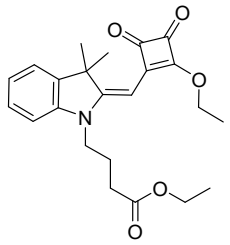
Three-steps synthetic approach



Two-steps synthetic approach



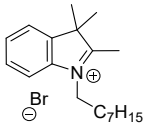
Ethyl 4-(2-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)methylene)-3,3-dimethylindolin-1-yl)butanoate (6)



N-alkylated trimethylindolenine **5** (1.0 g, 2.8 mmol, 1 eq.) and 3,4-diethoxy-3-cyclobutene-1,2-dione (1.9 g, 11 mmol, 4 eq.) were dissolved in absolute EtOH (33 mL). The reaction mixture was refluxed overnight and turned orange before becoming green. Solvent was removed under reduced pressure and the product was purified over a SiO_2 gel column with DCM. The orange solid was recrystallized from MeOH, yielding **6** as thin orange needles (900 mg, 80%). HPLC t_r = 5.17 min (> 95% purity [365 nm] [220 nm]). HRMS (ESI) calculated for $C_{23}H_{28}NO_5$ $[M+H]^+$: 397.1889; found: 397.1878. Calculated error: 2.8 ppm.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.22 (m, 2H), 7.07 (td, *J* = 7.4, 0.9 Hz, 1H), 6.98 – 6.92 (m, 1H), 5.42 (s, 1H), 4.90 (q, *J* = 7.1 Hz, 2H), 4.17 (q, *J* = 7.1 Hz, 2H), 3.95 – 3.86 (m, 2H), 2.43 (t, *J* = 6.9 Hz, 2H), 2.07 (quint, *J* = 7.0 Hz, 2H), 1.62 (s, 6H), 1.53 (t, *J* = 7.1 Hz, 3H), 1.27 (t, *J* = 7.1 Hz, 3H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 192.4, 187.70, 187.68, 173.8, 172.6, 168.2, 142.6, 140.8, 127.9, 122.8, 122.0, 108.4, 81.4, 70.0, 60.8, 48.0, 41.9, 30.9, 27.0, 21.4, 15.9, 14.2.

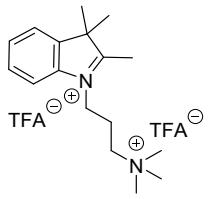
2,3,3-Trimethyl-1-octyl-3H-indol-1-ium bromide (7)



7 was synthesized following a modified procedure from the literature.[5] 2,3,3-Trimethylindolenine (250 mg, 1.6 mmol, 1 eq.) and 1-bromooctane (910 mg, 4.7 mmol, 3 eq.) were dissolved in dry acetonitrile (2 mL) in a sealed tube. The solution was heated at 120 °C for 48 h. The solvent was removed under vacuum and the crude reaction mixture was triturated using cold diethyl ether and cold ethyl acetate. The product was further dried under vacuum to yield **7** as a purple solid (300 mg, 54%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 – 7.53 (m, 4H), 4.75 (t, *J* = 7.8 Hz, 2H), 3.14 (s, 3H), 1.92 (m, 2H), 1.65 (s, 6H), 1.50 – 1.18 (m, 10H), 0.90 – 0.82 (m, 3H).

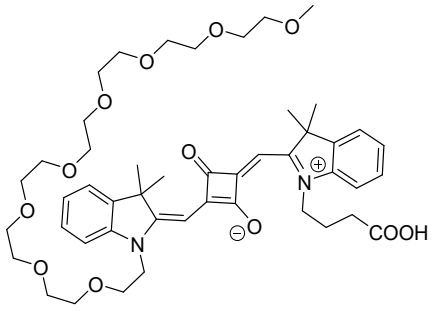
2,3,3-Trimethyl-1-(3-(trimethylammonio)-propyl)-3*H*-indolium iodide (9)



9 was synthesized according to a modified procedure from the literature.[4,6] In a sealed tube, to a solution of 2,3,3-trimethylindolenine (0.5 g, 3.1 mmol, 1 eq.) and NaI (2.3 g, 16 mmol, 5 eq.) in MeCN (4 mL) was added (3-bromopropyl)trimethylammonium bromide (1.2 g, 4.6 mmol, 1.5 eq.). The reaction mixture was stirred at 110 °C for 70 h. MeCN was removed under reduced pressure. The crude product was dissolved in DMSO and was purified by reverse phase semi-preparative chromatography using a constant 5% gradient for 10 min then a linear gradient of 5-40% v/v MeCN (0.1% v/v TFA) in H_2O (0.1% v/v TFA) in 30 min. The combined fractions were freeze-dried to afford **9** as a yellow oil (1.2 g, 86%). HPLC t_r = 1.56 min (> 95% purity [220 nm] [254 nm]).

^{1}H NMR (400 MHz, CD_3OD) δ 8.10 – 8.01 (m, 1H), 7.84 – 7.75 (m, 1H), 7.72 – 7.64 (m, 2H), 4.62 (t, *J* = 8.3 Hz, 2H), 3.85 – 3.73 (m, 2H), 3.24 (s, 9H), 2.61 – 2.44 (m, 2H), 1.65 (s, 6H).

4-((1-(3-carboxypropyl)-3,3-dimethyl-3*H*-indol-1-ium-2-yl)methylene)-2-((3,3-dimethyl-1-(2,5,8,11,14,17,20,23-octaoxapentacosan-25-yl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate (SQpeg8)

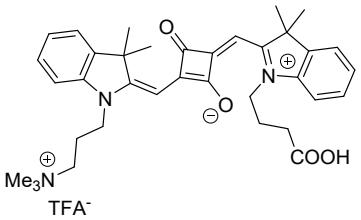


To a solution of **6** (32 mg, 0.08 mmol, 1 eq.) in ethanol (5 mL) was added NaOH (40% in water, 500 µL, 5 mmol, 62 eq.). The solution was heated at 80 °C for 3 h, cooled, then neutralized with

6 M HCl. The solution was filtered over silica gel, washed with DCM and MeOH (90:10) and the solvents were evaporated. The crude product was dissolved in a toluene/*n*-butanol mixture (1:1, 4 mL) and **8** (52 mg, 0.08 mmol, 1 eq.) was added. The reaction mixture was refluxed overnight in a flask equipped with a Dean-Stark apparatus. Solvents were evaporated and the crude product was taken in water and 48% HBr (1:1, 10 mL) and heated at 60 °C for 1 h. The solution was cooled and then extracted with DCM until the aqueous phase was no longer blue. The organic layer was dried over anhydrous $MgSO_4$. Solvents were removed under vacuum. The product was purified by reverse phase HPLC using a C18 column and a linear gradient of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v) at 20% for 3 min then 20% to 90% in 30 min with a flow rate of 20 $mL.min^{-1}$. Detection was set at 600 nm. Fractions with the desired compound were freeze-dried to yield **SQpeg8** as a blue solid (17 mg, 25%). HPLC t_r = 5.08 min (> 95% purity [630 nm]). HRMS (ESI) calculated for $C_{47}H_{65}N_2O_{12}$ $[M+H]^+$: 849.4538 ; found: 849.4543. Calculated error: 0.6 ppm.

^{1}H NMR (500 MHz, CD_3OD) δ 7.44 (t, *J* = 6.6 Hz, 2H), 7.39 – 7.27 (m, 4H), 7.19 (td, *J* = 7.4, 3.6 Hz, 2H), 6.03 (s, 1H), 5.98 (s, 1H), 4.36 (t, *J* = 5.3 Hz, 2H), 4.18 (t, *J* = 7.8 Hz, 2H), 3.91 (t, *J* = 5.3 Hz, 2H), 3.68 – 3.47 (m, 28H), 3.33 (s, 3H), 2.52 (t, *J* = 6.9 Hz, 2H), 2.08 (quint, *J* = 7.1 Hz, 2H), 1.82 – 1.71 (m, 12H).
^{13}C NMR (126 MHz, CD_3OD) δ 184.5, 176.9, 176.7, 176.1, 172.9, 172.1, 144.2, 143.6, 143.2, 143.0, 129.3, 129.0, 125.4, 125.3, 123.3, 123.1, 112.2, 111.4, 87.7, 87.0, 72.9, 72.2, 71.65, 71.62, 71.50, 71.48, 71.3, 69.0, 59.1, 50.58, 50.56, 45.2, 43.9, 31.5, 27.4, 27.4, 23.2.

4-((1-(3-carboxypropyl)-3,3-dimethyl-3*H*-indol-1-ium-2-yl)methylene)-2-((3,3-dimethyl-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate (SQ^+)



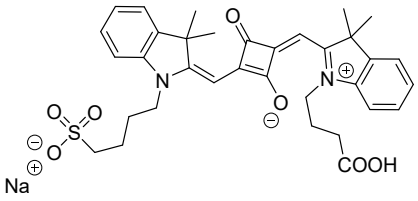
To a solution of **6** (145 mg, 0.36 mmol, 1.0 eq.) in ethanol (5 mL) was added NaOH (40% in water, 500 µL). The solution was heated at 80 °C for 3 h and cooled before neutralizing the solution with 6 M HCl. The solution was filtered over silica gel, washed with DCM and MeOH (90:10) and the solvents were evaporated. The crude product was dissolved in a toluene/*n*-butanol mixture (1:1, 4 mL) and **9** (133 mg, 0.50 mmol, 1.4 eq.) was added. The reaction mixture was refluxed overnight

in a flask equipped with a Dean-Stark apparatus. Solvents were evaporated and the crude product was taken in water and 48% HBr (2:1, 10 mL) and heated at 60 °C for 1 h. The solution was cooled and then extracted with DCM until the aqueous phase was no longer blue. The organic layer was dried over anhydrous $MgSO_4$. Solvents were removed under vacuum. The crude product was purified by reverse phase HPLC using a C18 column and a linear gradient of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v) at 20% for 3 min then 20% to 90% in 30 min with a flow rate of 20 mL.min^{-1}. Detection was set at 600 nm. Fractions with the desired compound were freeze-dried to yield **SQ+** as a blue solid (33 mg, 16%). HPLC t_r = 4.67 min (> 95% purity [630 nm]). HRMS (ESI) calculated for $C_{36}H_{44}N_3O_4$ [M] $^+$: 582.3326; found: 582.3318. Calculated error: 1.4 ppm.

^{1}H NMR (400 MHz, CD_3OD) δ 7.55 – 7.43 (m, 2H), 7.43 – 7.33 (m, 3H), 7.33 – 7.14 (m, 3H), 6.05 (s, 1H), 5.88 (s, 1H), 4.31 – 4.08 (m, 4H), 3.66 – 3.51 (m, 2H), 3.19 (s, 9H), 2.54 (t, *J* = 6.9 Hz, 2H), 2.38 – 2.27 (m, 2H), 2.10 (quint, *J* = 7.1 Hz, 2H), 1.85 – 1.68 (m, 12H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 181.2, 180.3, 174.2, 170.4, 168.9, 142.5, 142.0, 128.5, 128.4, 124.5, 124.1, 122.8, 110.9, 110.5, 86.8, 63.1, 52.9, 49.4, 49.1, 42.9, 31.0, 27.1, 26.9, 22.5, 20.9.

4-(2-((3-((1-(3-carboxypropyl)-3,3-dimethyl-3*H*-indol-1-ium-2-yl)methylene)-2-oxido-4-oxocyclobut-1-en-1-yl)methylene)-3,3-dimethylindolin-1-yl)butane-1-sulfonate (SQ$^-$)



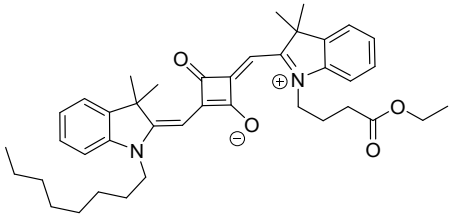
To a solution of **6** (105 mg, 0.26 mmol, 1 eq.) in ethanol (5 mL) was added NaOH (40% in water, 500 µL). The solution was heated at 80 °C for 3 h and cooled before adding 6 M HCl to neutralize the solution. The solution was filtered over silica gel, washed with DCM and MeOH (90:10) and the solvents were evaporated. The crude product was dissolved in a toluene/*n*-butanol mixture (1:1, 4 mL) and **10** (78 mg, 0.26 mmol, 1 eq.) was added. The reaction mixture was refluxed overnight in a flask equipped with a Dean-Stark apparatus. Solvents were evaporated and the crude product was heated at 60 °C for 3 h in water and 48% HBr (1:1, 5 mL). The solution was cooled and then extracted with DCM until the aqueous phase was no longer blue. The organic layer was dried over anhydrous $MgSO_4$. Solvents were removed under vacuum. The product was purified by semi-preparative HPLC using a C18 column and a linear gradient of MeCN with 0.1%

TFA (v/v) in H_2O with 0.1% TFA (v/v) at 20% for 3 min then 20% to 90% in 30 min with a flow rate of 20 mL.min^{-1}. Detection was set at 600 nm. Fractions with the desired compound were freeze-dried to yield **SQ-** as a blue solid (17 mg, 25%). HPLC t_r = 4.60 min (> 95% purity [630 nm]). HRMS (ESI) calculated for $C_{34}H_{39}N_2O_7S^-$ $[M+2H]^+$: 619.2478; found: 619.2483. Calculated error: 0.8 ppm.

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.52 (dd, *J* = 7.4, 3.4 Hz, 2H), 7.45 – 7.30 (m, 4H), 7.24 – 7.13 (m, 2H), 5.90 (s, 1H), 5.88 (s, 1H), 4.32 – 4.04 (m, 4H), 2.66 (t, *J* = 7.3 Hz, 2H), 2.41 (t, *J* = 7.1 Hz, 2H), 1.91 (quint, *J* = 7.1 Hz, 2H), 1.84 – 1.71 (m, 4H), 1.70 – 1.56 (m, 12H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 174.3, 170.4, 170.1, 159.0, 158.7, 142.5, 141.99, 141.96, 128.5, 124.6, 124.5, 122.74, 122.72, 116.6, 111.2, 110.9, 86.8, 86.5, 51.1, 49.3, 43.5, 42.8, 40.7, 30.9, 26.8, 26.1, 22.7, 22.4.

2-((3,3-dimethyl-1-octylindolin-2-ylidene)methyl)-4-((1-(4-ethoxy-4-oxobutyl)-3,3-dimethyl-3*H*-indol-1-ium-2-yl)methylene)-3-oxocyclobut-1-en-1-olate (11)

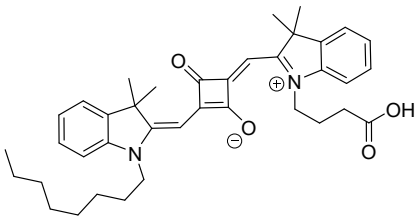


In a microwave-type tube, **6** (409 mg, 1.03 mmol, 1 eq.) and **7** (363 mg, 1.03 mmol, 1 eq.) were dissolved in a 1:1 mixture of toluene and *n*-butanol (20 mL). The tube was sealed and heated at 160 °C overnight. The solvents were removed under vacuum and the crude product was purified by column chromatography over silica gel (DCM to DCM/MeOH 90:10) to afford **11** as a blue solid (586 mg, 91%). R_f = 0.8 (SiO_2, DCM/MeOH 95:5). HRMS (ESI) calculated for $C_{40}H_{51}N_2O_4$ $[M+H]^+$: 623.3849, Found: 623.3847. Calculated error 0.3 ppm.

^{1}H NMR (500 MHz, CD_3OD) δ 7.44 (t, *J* = 7.0 Hz, 2H), 7.39 – 7.33 (m, 2H), 7.31 – 7.26 (m, 1H), 7.26 – 7.15 (m, 3H), 5.97 (s, 1H), 5.94 (s, 1H), 4.23 – 4.05 (m, 6H), 2.59 – 2.45 (m, 2H), 2.10 (quint, *J* = 7.1 Hz, 2H), 1.82 (quint, *J* = 7.5 Hz, 2H), 1.74 (s, 12H), 1.52 – 1.25 (m, 12H), 1.25 – 1.18 (m, 3H), 0.94 – 0.81 (m, 3H).

^{13}C NMR (126 MHz, CD_3OD) δ 185.2, 177.6, 176.8, 174.9, 173.2, 172.5, 144.3, 144.2, 143.9, 143.7, 129.83, 129.81, 126.1, 125.9, 112.1, 111.9, 87.8, 87.5, 62.3, 51.2, 51.1, 50.2, 50.1, 45.3, 44.4, 33.5, 32.3, 31.0, 30.9, 28.8, 28.5, 28.0, 27.9, 24.3, 23.7, 15.1, 15.0.

4-((1-(3-carboxypropyl)-3,3-dimethyl-3*H*-indol-1-ium-2-yl)methylene)-2-((3,3-dimethyl-1-octylindolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate (SQC8)

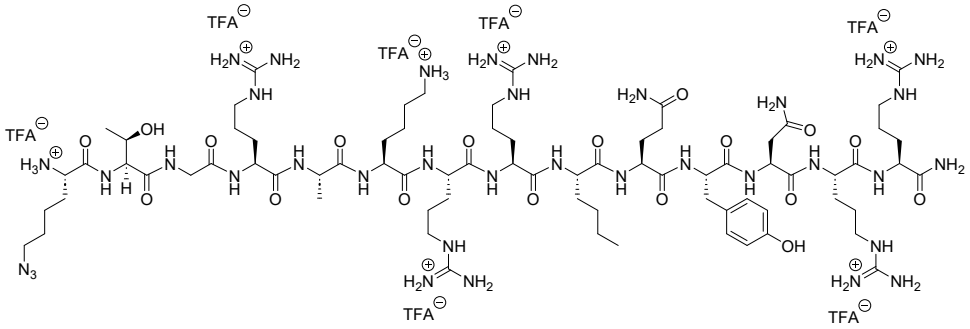


To a solution of **11** (560 mg, 0.9 mmol, 1 eq.) in THF (30 mL) and water (10 mL), lithium hydroxide monohydrate (76 mg, 1.8 mmol, 2 eq.) was added. The mixture was stirred overnight at room temperature. Solvents were removed under vacuum and the crude product was taken in DCM and washed with 0.5 M HCl. The organic layer was dried over $MgSO_4$. The solvent was removed under vacuum. The product was purified by column chromatography over silica gel (DCM to DCM/MeOH 90:10) to afford the desired compound **SQC8** as a blue solid (410 mg, 77%). HPLC t_r = 6.26 min (> 95% purity [650 nm] [220 nm]).

HRMS (ESI) calculated for $C_{38}H_{47}N_2O_4^+$ $[M+H]^+$: 594.3458, Found: 594.3476. Calculated error 3.0 ppm.

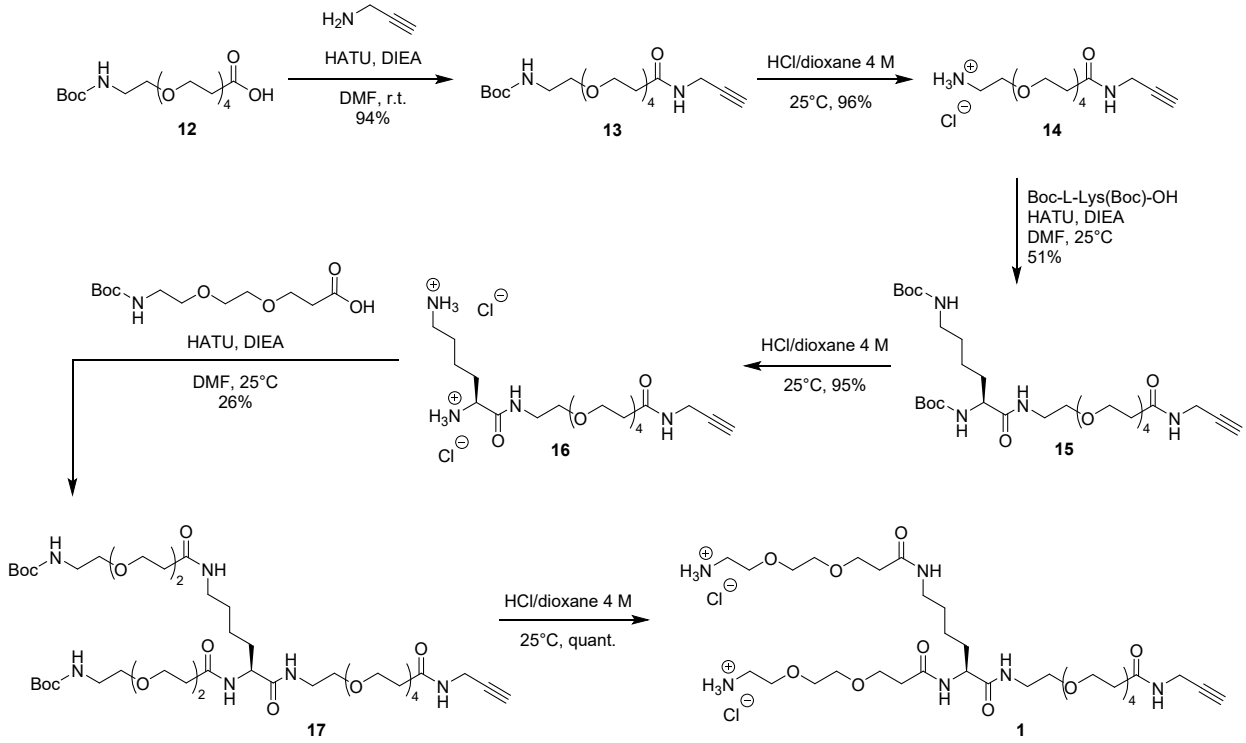
^{1}H NMR (500 MHz, DMSO) δ 7.52 (d, *J* = 7.3 Hz, 2H), 7.40 – 7.30 (m, 4H), 7.20 – 7.12 (m, 2H), 5.84 (s, 1H), 5.80 (s, 1H), 4.17 – 4.03 (m, 4H), 2.38 (q, *J* = 8.0 Hz, 2H), 1.91 (t, *J* = 7.5 Hz, 2H), 1.68 (s, 12H), 1.40 – 1.19 (m, 12H), 0.83 (t, *J* = 6.6 Hz, 3H). The product was not soluble enough to obtain a proper ^{13}C NMR spectrum.

2.3 Solid-phase peptide synthesis of LysN3-Ubi

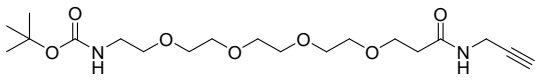


LysN3-Ubi was synthesized by automated solid-phase peptide synthesis using the standard Fmoc/tBu chemistry on a Fmoc-Rink Amide AM resin (loading 0.40 mmol/g) starting from 0.1 mmol of resin. The Fmoc groups were cleaved using a 20% (v/v) solution of piperidine in DMF. Fmoc-protected amino acids (10 eq., 2 M solutions) were coupled in DMF in the presence of DIC (0.5 M) and Oxyma (1 M) for 7 minutes using microwave activation. Washing steps were performed using DMF. The peptide was cleaved from the resin using TFA/TIS/H_2O 95/2.5/2.5 (v/v) (10 mL) for 4 hours. The solution was filtered under vacuum, and the peptide was precipitated with cold diethyl ether (7 volumes per volume of the cleavage mixture). The precipitated peptide was centrifuged at 4°C at 3000 x g for 2 min. The supernatant was removed by decantation, and the crude precipitated peptides were dried *in vacuo*. Crude peptide was re-dissolved in water and purified by semi-preparative reversed-phase HPLC. Fractions containing the desired peptide were collected and freeze-dried, affording **LysN3-Ubi** as white solid (91 mg, 36 %). Analytical HPLC t_R= 2.02 min (>95% purity at 220 nm); HRMS (ESI-TOF): Calculated for $C_{75}H_{134}N_{64}O_{18}$ $[M+3H]^{3+}/3$: 610.0304; found: 610.0309. Calculated error: 0.9 ppm.

2.4 Synthesis of the linker 1



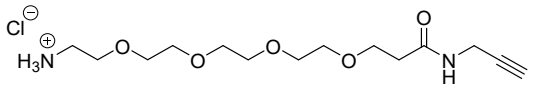
***tert*-Butyl (15-oxo-3,6,9,12-tetraoxa-16-azanonadec-18-yn-1-yl)carbamate (13)**



13 was synthesized according to a modified procedure from the literature.[7] HATU (880 mg, 3.3 mmol, 1.2 eq.), DIEA (1.68 mL, 9.6 mmol, 5 eq.) and then propargylamine (0.25 mL, 3.9 mmol, 2 eq.) were added to a solution of Boc-15-amino-4,7,10,13-tetraoxapentadecanoic acid **12** (704 mg, 1.9 mmol, 1 eq.) in DMF (15 mL). The reaction mixture was stirred at room temperature for 2 h. Solvent was removed under reduced pressure, and the crude product was dissolved in DCM. The organic phase was washed twice with a 0.1 M HCl solution. The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The product was purified over a silica gel column using a MeOH in DCM gradient (0:100 up to 10:90). Compound **13** was obtained as a sticky yellow solid (726 mg, 94%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.86 (s, 1H), 5.04 (t, *J* = 6.0 Hz, 1H), 4.02 (dd, *J* = 5.4, 2.5 Hz, 2H), 3.73 (t, *J* = 5.6 Hz, 2H), 3.70 – 3.58 (m, 12H), 3.54 (t, *J* = 5.1 Hz, 2H), 3.31 (q, *J* = 5.5 Hz, 2H), 2.51 (t, *J* = 5.6 Hz, 2H), 2.19 (t, *J* = 2.6 Hz, 1H), 1.43 (s, 9H).

1-Amino-*N*-(prop-2-yn-1-yl)-3,6,9,12-tetraoxapentadecan-15-amide (14)



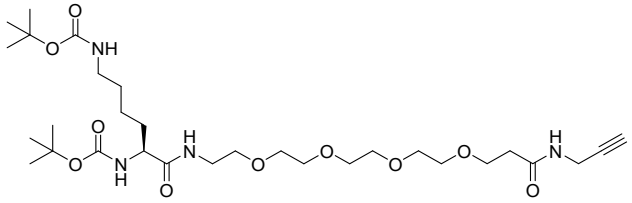
13 (600 mg, 1.6 mmol, 1 eq.) was dissolved in a solution of 4 M HCl in dioxane (11 mL) and stirred at room temperature for 4 h. A precipitate was formed after 15 min. The precipitate was filtered and washed with cold diethyl ether to give **14** as a light orange solid (550 mg, 96%).

HRMS (ESI) calculated for $C_{14}H_{27}N_2O_5$ $[M+H]^+$: 303.1920; found: 303.1915. Calculated error: 1.6 ppm.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (s, 1H), 7.97 (s, 2H), 4.03 (d, *J* = 2.5 Hz, 2H), 3.91 (t, *J* = 4.9 Hz, 2H), 3.83 (t, *J* = 5.9 Hz, 2H), 3.77 – 3.59 (m, 17H), 3.21 (q, *J* = 5.2 Hz, 2H), 2.69 (t, *J* = 5.9 Hz, 2H), 2.22 (t, *J* = 2.5 Hz, 1H). Traces of dioxane.

^{13}C NMR (126 MHz, $CDCl_3$) δ 171.4, 80.4, 71.1, 70.44, 70.36, 70.30, 70.1, 70.0, 69.97, 67.5, 67.0, 40.0, 36.2, 28.9.

Di-*tert*-butyl (5,21-dioxo-8,11,14,17-tetraoxa-4,20-diazahexacos-1-yne-22,26-diyl)(*S*)-dicarbamate (15)



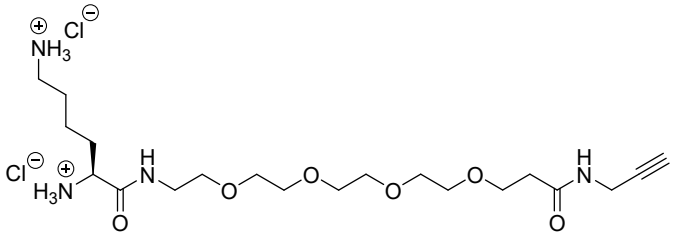
HATU (764 mg, 2.0 mmol, 1.1 eq.) and DIEA (960 µL, 5.4 mol, 3.0 eq.) were added to a solution of **14** (550 mg, 1.8 mmol, 1.0 eq.) in DMF (10 mL). Commercial Boc-Lys(Boc)-OH·DCHA (697 mg, 2.0 mmol, 1.1 eq.) was then added and the reaction mixture was stirred at room temperature for 2 h. Solvents were removed under reduced pressure and the mixture was stirred in AcOEt (10 mL) and a 10% aqueous solution of citric acid (5 mL) until the solution became clear. The organic layer was washed with water and dried over $MgSO_4$. The product was purified over a silica gel column using a gradient of MeOH in DCM (0:10 to 1:9) to yield **15** as an oil (586 mg, 51%).

HRMS (ESI) calculated for $C_{30}H_{55}N_4O_{10}$ $[M+H]^+$: 631.3918; found: 631.3915. Calculated error: 0.5 ppm.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.10 (s, 1H), 6.96 (s, 1H), 5.31 (d, *J* = 8.1 Hz, 1H), 4.73 (t, *J* = 5.9 Hz, 1H), 4.08 (s, 1H), 4.03 (dt, *J* = 4.5, 2.1 Hz, 2H), 3.81 – 3.58 (m, 14H), 3.54 (t, *J* = 5.1 Hz, 2H), 3.50 – 3.36 (m, 2H), 3.09 (q, *J* = 6.7 Hz, 2H), 2.51 (t, *J* = 5.9 Hz, 2H), 2.20 (t, *J* = 2.6 Hz, 1H), 1.88 – 1.25 (m, 29H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.3, 171.4, 156.3, 155.8, 80.2, 79.9, 79.2, 71.2, 70.7, 70.6, 70.4, 70.3, 70.0, 67.2, 54.4, 40.3, 39.4, 36.7, 32.8, 29.8, 29.0, 28.6, 28.5, 22.7.

(*S*)-1-(2,6-Diaminohexanamido)-*N*-(prop-2-yn-1-yl)-3,6,9,12-tetraoxapentadecan-15-amide (16)



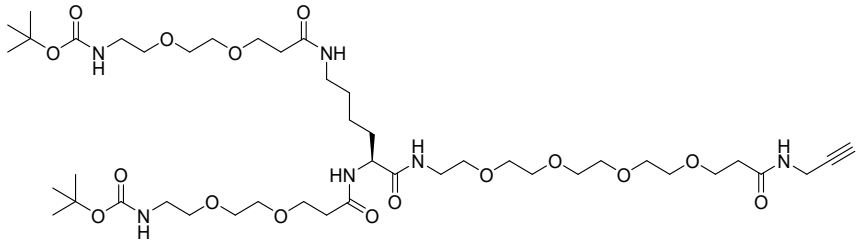
15 (530 mg, 0.84 mmol) was dissolved in MeOH (2 mL) and a solution of 4 M HCl in dioxane (5 mL) was added. The reaction mixture was stirred at room temperature for 3 h. Solvents were removed under vacuum to afford **16** as a colorless oil (400 mg, 95%) that was used without further purification.

HRMS (ESI) calculated for $C_{20}H_{39}N_4O_6$ $[M+H]^+$: 431.2870; found: 431.2873. Calculated error: 0.8 ppm.

^{1}H NMR (500 MHz, CD_3OD) δ 3.99 (d, *J* = 2.6 Hz, 2H), 3.94 (t, *J* = 6.6 Hz, 1H), 3.79 – 3.73 (m, 2H), 3.73 – 3.50 (m, 17H), 3.42 – 3.35 (m, 1H), 3.00 (t, *J* = 6.8 Hz, 2H), 2.64 (t, *J* = 2.5 Hz, 1H), 2.50 (t, *J* = 6.0 Hz, 2H), 1.99 – 1.89 (m, 2H), 1.80 – 1.71 (m, 2H), 1.58 – 1.48 (m, 2H). Traces of dioxane.

^{13}C NMR (126 MHz, CD_3OD) δ 173.6, 170.0, 80.7, 72.3, 71.6, 71.5, 71.39, 71.37, 71.2, 71.0, 70.2, 68.12, 68.07, 54.2, 40.4, 40.3, 37.2, 31.9, 29.5, 28.0, 22.8.

Di-*tert*-butyl(9,17-dioxo-11-((15-oxo-3,6,9,12-tetraoxa-16-azanonadec-18-yn-1-yl)carbamoyl)-3,6,20,23-tetraoxa-10,16-diazapentacosane-1,25-diyl)(*S*)-dicarbamate (17)



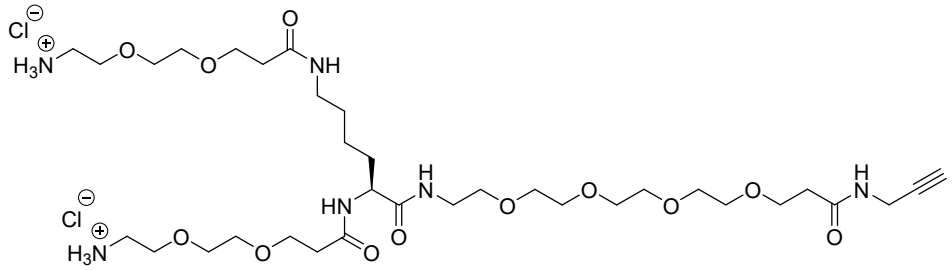
HATU (374 mg, 0.98 mmol, 2 eq.) and DIEA (430 µL, 2.46 mmol, 5 eq.) were added to a solution of *t*-Boc-*N*-amido-PEG2-propionic acid (273 mg, 0.98 mmol, 2 eq.) and **16** (318 mg, 0.49 mmol, 1 eq.) in DMF (10 mL). The reaction mixture was stirred at room temperature for 2 h. Solvent was removed under reduced pressure, and the crude product was dissolved in DCM. The organic phase was washed twice with 0.1 M HCl and with brine. The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The product was purified over a silica gel column using a MeOH in DCM gradient (from 0:100 up to 10:90) to give **17** as a sticky pale-yellow oil (120 mg, 26%).

HRMS (ESI) calculated for $C_{44}H_{81}N_6O_{16}$ $[M+H]^+$: 949.5709; found: 949.5714. Calculated error: 0.5 ppm.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (s, 1H), 7.47 (s, 1H), 7.16 (s, 1H), 6.59 (s, 1H), 5.38 (s, 1H), 5.17 (s, 1H), 4.50 – 4.37 (m, 1H), 4.00 (dt, *J* = 5.5, 2.8 Hz, 2H), 3.82 – 3.67 (m, 7H), 3.67 – 3.54 (m, 19H), 3.52 (t, *J* = 5.2 Hz, 6H), 3.47 – 3.35 (m, 2H), 3.35 – 3.22 (m, 4H), 3.19 (q, *J* = 6.7 Hz, 2H), 2.51 (dt, *J* = 12.4, 6.0 Hz, 4H), 2.43 (t, *J* = 5.9 Hz, 2H), 2.21 (d, *J* = 5.1 Hz, 1H), 1.88 – 1.73 (m, 1H), 1.63 (dq, *J* = 14.6, 7.6 Hz, 1H), 1.56 – 1.26 (m, 23H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 171.8, 171.53, 171.46, 171.2, 156.2, 156.1, 80.2, 79.3, 79.1, 71.1, 70.5, 70.3-69.84 (multiple broad signals, PEG), 67.3, 67.2, 67.1, 52.8, 39.3, 38.9, 36.9, 36.4, 32.4, 29.0, 28.9, 28.44, 28.42, 22.5.

(*S*)-*N,N'*-(5,21-Dioxo-8,11,14,17-tetraoxa-4,20-diazahexacos-1-yne-22,26-diyl)bis(3-(2-(2-aminoethoxy)ethoxy)propanamide) (1)



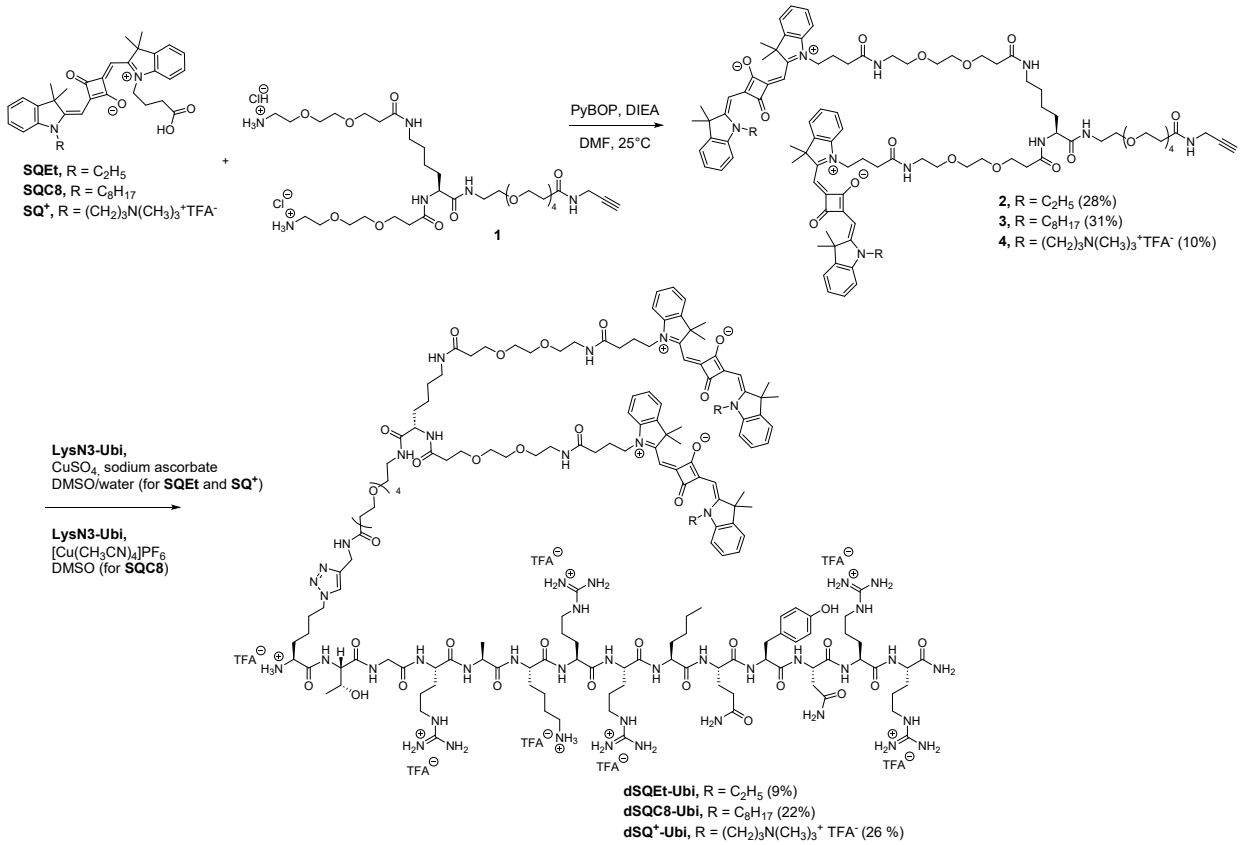
17 (110 mg, 0.12 mmol, 1 eq.) was dissolved in a solution of 4 M HCl in dioxane (5 mL) and MeOH (2 mL) and stirred at room temperature for 5 h. Solvents were removed under vacuum to afford **1** as a transparent oil (97 mg, quantitative) that was used without further purification.

HRMS (ESI) calculated for $C_{34}H_{66}N_6O_{12}$ $[M+2H]^{2+}/2$: 375.2370; found: 375.2369. Calculated error: 0.1 ppm.

^{1}H NMR (500 MHz, CD_3OD) δ 4.30 (dd, *J* = 8.8, 5.3 Hz, 1H), 3.97 (d, *J* = 2.5 Hz, 2H), 3.86 – 3.59 (m, 32H), 3.57 (t, *J* = 5.6 Hz, 2H), 3.41 – 3.36 (m, 2H), 3.35 (s, 1H), 3.19 (t, *J* = 6.9 Hz, 3H), 3.16 – 3.10 (m, 4H), 2.63 (t, *J* = 2.6 Hz, 1H), 2.62 – 2.52 (m, 3H), 2.48 (q, *J* = 6.2 Hz, 4H), 1.88 – 1.75 (m, 1H), 1.75 – 1.61 (m, 1H), 1.49 (quint, *J* = 7.6 Hz, 2H), 1.49 – 1.33 (m, 2H).

^{13}C NMR (126 MHz, CD_3OD) δ 173.1, 172.6, 172.3, 172.2, 79.4, 71.0, 70.1-69.8 (multiple broad signals, PEG), 69.2, 66.5-66.37 (multiple broad signal, PEG), 53.5, 39.39, 39.36, 39.0, 38.7, 36.1, 35.9, 34.3, 34.2, 31.5, 28.6, 28.1, 22.8.

2.5 Synthesis of the dimeric probes



General procedure for the synthesis of dimers 2, 3, and 4

To a solution of **1** (1.0 eq, typically 5 mg) and the corresponding squaraine (1.9 eq) in dry DMF (0.5 mL), PyBOP (3.0 eq) and DIEA (10 eq) were added. The reaction mixture was stirred at room temperature for 2 h. The resulting mixture was directly purified by semi-preparative reverse phase HPLC using a C18 column and a gradient of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v) with a flow rate of 20 mL.min^{-1}. Detection was set at 600 nm. Fractions containing the desired compound were freeze-dried to yield the dimers as blue solids.

2: Yield - 28%, HPLC t_r = 5.96 min (> 95% purity [650 nm] [530 nm]). HRMS (ESI) calculated for $C_{98}H_{131}N_{10}O_{18}$ $[M+3H]^{3+}/3$: 578.6548; found: 578.6542. Calculated error: 0.9 ppm.

3: Yield - 31%, HPLC t_r = 7.69 min (> 95% purity [650 nm] [220 nm]). HRMS (ESI) calculated for $C_{110}H_{152}N_{10}O_{18}Na_2$ $[M+2Na]^{2+}/2$: 973.5541; found: 973.5528. Calculated error: 1.3 ppm.

4: Yield - 10%, HPLC t_r = 4.79 min (> 90% purity [650 nm] [530 nm]). HRMS (ESI) calculated for $C_{106}H_{148}N_{12}O_{18}^{2+}$ $[M]^{2+}/2$: 938.5517; found: 938.5507. Calculated error: 1.1 ppm.

dSQEt-Ubi

DMF and water were bubbled with argon before use. **LysN3-Ubi** (4.2 mg, 2.4 µmol, 2.0 eq.) was dissolved in water (200 µL) and added to a solution of **2** (2 mg, 1.2 µmol, 1.0 eq.) in DMF (150 µL). A freshly prepared solution of sodium ascorbate (10 mg·mL^{-1}, 52 µL, 4.5 eq.) in water was added to a freshly prepared solution of $CuSO_4 \cdot 5H_2O$ (10 mg·mL^{-1}, 17 µL, 1.5 eq.) in water. The resulting solution was immediately added to the peptide and dye mixture. The reaction solution was stirred under argon for 1 h at 37 °C. The solution was purified by semi-preparative reverse phase HPLC on a C18 column using a linear gradient of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v) (20% to 30% over 5 min then 30% to 60% over 30 min at the flow rate of 20 mL.min^{-1}). Detection was set at 600 nm. Fractions with the desired compound were freeze-dried to yield **dSQEt-Ubi** as a blue solid (0.4 mg, 9%). HPLC t_r = 2.49 min (> 95% purity [650 nm] [220 nm]). HRMS (ESI) calculated for $C_{173}H_{268}N_{46}O_{36}$ $[M+6H]^{6+}/6$: 594.3426; found: 594.3439. Calculated error: 2.2 ppm.

dSQ$^+$-Ubi

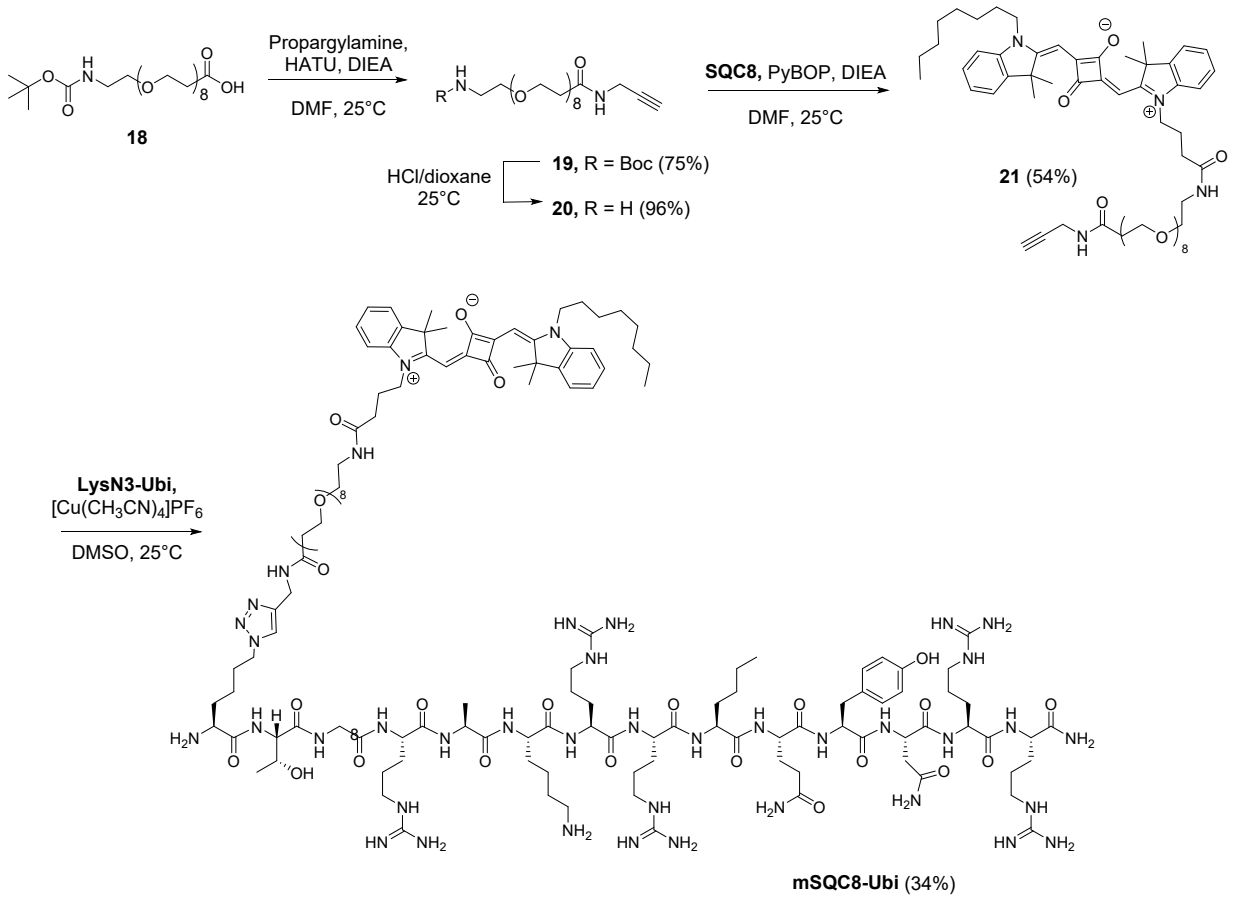
LysN3-Ubi (2.7 mg, 1.5 µmol, 1.1 eq.) and **4** (2.5 mg, 1.3 µmol, 1.0 eq.) were dissolved in DMSO (300 µL), which was bubbled with argon. A freshly prepared solution of sodium ascorbate in water (10 mg·mL^{-1}, 119 µL, 4.5 eq.) was added to a freshly prepared solution of $CuSO_4 \cdot 5H_2O$ in water (10 mg·mL^{-1}, 50 µL, 1.5 eq.). The resulting solution was immediately added to the peptide and dye mixture. The reaction solution was stirred under argon for 30 min at 37 °C. The mixture was directly purified by semi-preparative reverse phase HPLC on a C18 column using a linear gradient of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v) (20% to 30% over 5 min then 30% to 60% over 30 min at the flow rate of 20 mL.min^{-1}). Detection was set at 600 nm. Fractions with the desired compound were freeze-dried to yield **dSQ$^+$-Ubi** as a blue solid (1.3 mg, 26%). HPLC t_r = 3.79 min (> 90% purity [650 nm] [530 nm]). HRMS (ESI) calculated for $C_{181}H_{287}N_{48}O_{36}^{2+}$ $[M+5H]^{7+}/7$: 618.0337; found: 618.2009. Calculated error: 2.7 ppm.

dSQC8-Ubi

LysN3-Ubi (3.6 mg, 2.0 µmol, 1.1 eq.) and **3** (3.4 mg, 1.8 µmol, 1.0 eq.) were dissolved in DMSO bubbled with argon (400 µL), then tetrakis(acetonitrile)copper(I) hexafluorophosphate (0.6 mg, 1.8

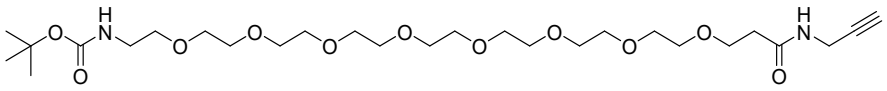
µmol, 1 eq.) was added. The reaction mixture was stirred at room temperature for 3 h. The crude mixture was purified by semi-preparative reverse phase flash chromatography on a C8 column using a linear gradient of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v) (15% to 80% over 30 min at the flow rate of 10 mL.min^{-1}). Detection was set at 600 nm. Fractions with the desired compound were freeze-dried to yield **dSQC8-Ubi** as a blue solid (1.5 mg, 22%). HPLC t_r = 3.51 min (> 95% purity [650 nm] [220 nm]). HRMS (ESI) calculated for $C_{185}H_{286}N_{46}O_{36}$ $[M+3H]^{3+}/3$: 1243.7399; found: 1243.7370. Calculated error: 2.4 ppm.

2.6 Synthesis of the monomeric probe mSQC8-Ubi



LysN3-Ubi = Lys(N_3)-Thr-Gly-Arg-Ala-Lys-Arg-Arg-Nle-Gln-Tyr-Asn-Arg-Arg-NH_2

***tert*-butyl (27-oxo-3,6,9,12,15,18,21,24-octaoxa-28-azahentriacont-30-yn-1-yl) carbamate (19)**



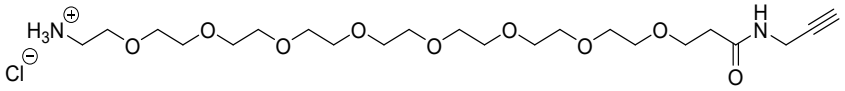
Propargylamine (12 µL, 180 µmol, 5 eq.) was added to a solution of Boc-NH-PEG8-CH2CH2COOH **18** (20 mg, 37 µmol, 1 eq.) in DMF (0.5 mL). HATU (21 mg, 55 µmol, 1.5 eq.) and DIEA (32 µL, 180 µmol, 5 eq.) were then added and the mixture was stirred at room temperature for 1 hour. Solvent was removed under reduced pressure. The crude was purified by column chromatography over silica gel (DCM to DCM/MeOH 90:10 to yield **19** as a colorless oil (16 mg, 75%).

HRMS (ESI) calculated for $C_{34}H_{66}N_6O_{12}$ $[M+2H]^{2+}/2$: 375.2370; found: 375.2369. Calculated error: 0.1 ppm.

^{1}H NMR (500 MHz, CD_3OD) δ 4.30 (dd, *J* = 8.8, 5.3 Hz, 1H), 3.97 (d, *J* = 2.5 Hz, 2H), 3.86 – 3.59 (m, 32H), 3.57 (t, *J* = 5.6 Hz, 2H), 3.41 – 3.36 (m, 2H), 3.35 (s, 1H), 3.19 (t, *J* = 6.9 Hz, 3H), 3.16 – 3.10 (m, 4H), 2.63 (t, *J* = 2.6 Hz, 1H), 2.62 – 2.52 (m, 3H), 2.48 (q, *J* = 6.2 Hz, 4H), 1.88 – 1.75 (m, 1H), 1.75 – 1.61 (m, 1H), 1.49 (quint, *J* = 7.6 Hz, 2H), 1.49 – 1.33 (m, 2H).

^{13}C NMR (126 MHz, CD_3OD) δ 173.1, 172.6, 172.3, 172.2, 79.4, 71.0, 70.1-69.8 (multiple broad signals, PEG), 69.2, 66.5-66.37 (multiple broad signal, PEG), 53.5, 39.39, 39.36, 39.0, 38.7, 36.1, 35.9, 34.3, 34.2, 31.5, 28.6, 28.1, 22.8.

1-Amino-*N*-(prop-2-yn-1-yl)-3,6,9,12,15,18,21,24-octaoxaheptacosan-27-amide (20)

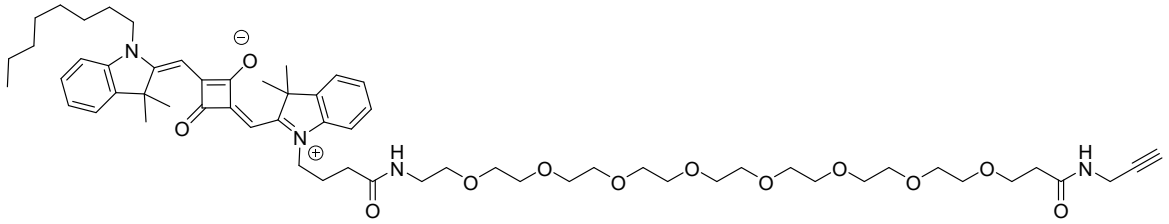


Compound **19** (16 mg, 28 µmol) was dissolved in a solution of 4 M HCl in dioxane (4 mL). The solution was stirred at room temperature for 2 hours. Volatiles were removed under vacuum to give **20** as a colorless oil (14 mg, 96%) that was used without further purification.

^{1}H NMR (500 MHz, CD_3OD) δ 3.97 (d, *J* = 2.6 Hz, 2H), 3.81 – 3.62 (m, 31H), 3.17 (t, *J* = 5.1 Hz, 2H), 2.61 (t, *J* = 2.6 Hz, 1H), 2.47 (t, *J* = 6.1 Hz, 2H).

^{13}C NMR (126 MHz, MeOD) δ 173.4, 80.7, 72.3, 71.5-71.1 (broad PEG signal), 71.0, 70.7, 68.0, 67.9, 49.5, 49.3, 40.6, 37.3, 29.4.

(*E*)-2-(((*E*)-3,3-dimethyl-1-octylindolin-2-ylidene)methyl)-4-((1-(5,33-dioxo-8,11,14,17,20,23,26,29-octaoxa-4,32-diazahexatriacont-1-yn-36-yl)-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene)-3-oxocyclobut-1-en-1-olate (21)



A mixture of **SQC8** (8 mg, 14 µmol, 1 eq.), **20** (7 mg, 14 µmol, 1 eq.), PyBOP (11 mg, 20 µmol, 1.5 eq.) and DIEA (24 µL, 140 mmol, 10 eq.) in DMF (1 mL) was stirred at room temperature for 1 hour. The crude was purified by reverse phase HPLC on a C18 column using a linear gradient of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v) (30% to 50% in 5 min, then 50% to 95% over 30 min at the flow rate of 20 mL.min^{-1}). Detection was set at 600 nm. Fractions with desired compound were freeze-dried to yield product as a blue solid (8 mg, 54%). HPLC t_r = 5.17 min (> 95% purity [650 nm] [220 nm]. HRMS (ESI) calculated for $C_{60}H_{88}N_4O_{12}$ $[M+2H]^{2+}/2$: 528.3189; found: 528.3189. Calculated error: 2.0 ppm.

mSQC8-Ubi

Tetrakis(acetonitrile)copper(I) tetrafluoroborate (0.4 mg, 1 µmol, 1 eq.) was added to a solution of **LysN3-Ubi** (2 mg, 1.1 µmol, 1.2 eq.) and **21** (1 mg, 1 µmol, 1 eq.) in DMSO (400 µL) bubbled with argon. The reaction mixture was stirred under argon at room temperature overnight. The crude mixture was purified by semi-preparative reverse phase flash chromatography on a C8 column using a linear gradient of MeCN with 0.1% TFA (v/v) in H_2O with 0.1% TFA (v/v) (20% to 90% over 40 min at the flow rate of 10 mL.min^{-1}). Detection was set at 600 nm. Fractions with the desired compound were freeze-dried to yield **mSQC8-Ubi** as a blue solid (1 mg, 34%). HPLC t_r = 2.65 min (> 95% purity [650 nm] [220 nm]). HRMS (ESI) calculated for $C_{135}H_{220}N_{40}O_{30}$ $[M+5H]^{5+}/5$: 577.3421; found: 577.3463. Calculated error: 0.2 ppm.

3. UV-vis absorption and fluorescence measurements

Spectroscopy-grade solvents were purchased from TCI or Merck. Hellma SUPRASIL 114F-QS 10 mm quartz cuvettes were used for spectroscopy measurements in organic solvents. Plastic Eppendorf semi-micro Vis Cuvettes were used for measurements in aqueous solutions.

Absorption spectra were recorded using a Shimadzu UV-2700i spectrometer. Fluorescence spectra were recorded using a FluoroMax4 spectrofluorometer (Jobin Yvon, Horiba). The excitation wavelength was 600 nm, with the recorded emission spectral ranges of 610–850 nm. Unless specified, all fluorescence spectra were corrected for instrumental factors. The absorbance of sample solutions at the excitation wavelength was kept below 0.05 to avoid inner filter effects. Determination of relative fluorescence quantum yields (Φ_F) was performed using DiD in MeOH as a reference (Φ_F = 33%).[8] Data treatment was performed using OriginPro 2021.

The molar absorption coefficients of the dyes were determined using a serial dilution approach. Three independent samples of each dye were accurately weighed and dissolved in DMSO to prepare stock solutions at a concentration of 10 mM. Each stock solution was then diluted three times in MeOH to obtain a range of concentrations with absorbance values between 0.1 and 1. The absorbance of each solution was measured at the maximum absorption wavelength. Finally, the molar absorption coefficients were determined by calculating the slope of the absorbance versus concentration plot.

4. Bacterial strains

Escherichia coli K-12 *(*ATCC-10798) and *Staphylococcus epidermidis* (ATCC-14990) were purchased from LGC Standards S.a.r.l. (Molsheim, France). Luria-Bertani (LB) medium and agar from Athena Enzyme Systems were used for culturing bacteria.

Bacterial stocks were prepared as follows. Aliquots of the rehydrated bacteria were streaked on LB-agar plates and incubated for 18 hours at appropriate temperatures. Fresh LB medium (5 mL) was inoculated with single bacterial colonies and incubated at 30 °C, 220 rpm for 28 h. Glycerol stocks were prepared by adding a 50% v/v glycerol solution to the liquid bacterial culture (for a final concentration of glycerol 25% v/v) and stored at –80 °C.

5. Mammalian cell culture

HEK 293T cells were cultured in Eagle's minimal essential medium (MEM, Invitrogen, cat. no. 21090) supplemented with 10% FBS, 100 $U \cdot mL^{-1}$ of penicillin, 100 $\mu g \cdot mL^{-1}$ of streptomycin, 2 mM of glutamine, at 37 °C in a humidified 5% CO_2 atmosphere. A total of 70−80% maximal cell confluence was maintained by passing cells twice a week using the standard protocol.

6. Fluorescence confocal microscopy

HEK 293T cells. For microscopy experiments, cells were seeded into 35 mm ibiTreat µ-dishes (IBiDi) at 50,000 cells per dish 3 days before imaging. The day of the experiment, the culture medium was aspirated, and the cells were washed with DMEM/F-12 medium without phenol red. Cells were co-labeled with the squaraine fluorophores or conjugated probes (final concentration 1 µM) and Hoechst 33342 (final concentration 1 $\mu g \cdot mL^{-1}$) in DMEM/F-12 medium without phenol red for 15 min at 37 °C. Fluorescence confocal microscopy experiments were performed on a Leica TCS SP8 microscope equipped with a HCX PL APO CS2 63x/1.40 OIL objective. The imaging was performed at 22 °C. Squaraines and dimeric probes were excited with a 638 nm 30 mW laser at 0.1% or 1% intensity, and fluorescence was detected at 645–850 nm. Hoechst 33342 was excited with a 405 nm 50 mW laser, and its fluorescence was detected at 430−480 nm. The pinhole was set to 1 airy unit. The images were acquired at 1.5× magnification as a mean of 2 scans of 1024 × 1024 size. All images were processed with ImageJ. The same acquisition settings, brightness, and contrast parameters were applied to all the images.

E. coli and S. epidermidis. Two days before the experiment, glycerol stocks were streaked on LB-agar plates and incubated at 37 °C overnight. Fresh LB medium (5 mL) was inoculated with single bacterial colonies and incubated overnight at 37 °C on a rotary shaker (250 rpm). Overnight cultures were diluted in fresh LB to OD_{600} of 0.05 (which corresponds to approximately $5.40*10^8$–$1.12*10^9$ CFU/mL for *E. coli* and to $1.13–2.30*10^9$ CFU/mL for *S. epidermidis*[9]) and then incubated at 37 °C, 250 rpm, until OD_{600} reached 0.5–1.0. **dSQ^+-Ubi** was added to the bacterial suspension in LB from 0.5 mM stock solutions in DMSO to the desired final concentrations of 1 µM. Samples were incubated for 10 min at 37 °C, then 5 µL of labeled bacterial culture were deposed in the middle of the well of a µ-Slide 8 Well Glass Bottom. The bacteria were covered with 40 µL of 1 % low-melting agarose in PBS kept at 42°C and then kept at room temperature. Fluorescence confocal microscopy experiments were performed on a Leica TCS SP8 microscope equipped with a HCX PL APO CS2 63x/1.40 OIL objective. The imaging was performed at 22 °C. **dSQ^+-Ubi** was

excited with a 638 nm 30 mW laser at 1% (*E. coli*) or 3% (*S. epidermidis*) intensity and detected at 645−850 nm. The pinhole was set to 1 airy unit. The images were acquired as means of 2 scans of 1024 × 1024 size. All images were processed with ImageJ.

7. Flow cytometry analysis of bacteria and HEK 293T cells

Model E. coli and S. epidermidis. Bacteria in the logarithmic growth phase were used for the flow cytometry experiments. Two days before the experiment, glycerol stocks were streaked on LB-agar plates and incubated at 37 °C overnight. Fresh LB medium (5 mL) was inoculated with single bacterial colonies and incubated overnight at 37 °C on a rotary shaker (250 rpm). Overnight cultures were diluted in fresh LB to OD_{600} of 0.05 (which corresponds to approximately 5.40×10^8–1.12×10^9 CFU/mL for *E. coli* and to $1.13–2.30\times10^9$ CFU/mL for *S. epidermidis*[9]) and then incubated at 37 °C, 250 rpm, until OD_{600} reached 0.5–1.0. Fluorophores and probes were added to the bacterial suspension in LB from 0.1 mM stock solutions in DMSO to the desired final concentrations of 1 µM. Samples were incubated for 10 min with shaking at 37 °C, then fixed by adding an equal volume of 8% solution of PFA in PBS to reach a final concentration of PFA 4% w/v. Samples were then diluted 10–100 times in PBS and analyzed on a BD FACSymphony/A1 flow cytometer. 20,000 cells per sample were counted at a low flow rate using a 640 nm laser and the APC detection filter (670/30 nm).

HEK 293T cells. HEK 293T cells grown to 60-80% confluence were detached with Accutase and resuspended in DMEM/F-12 medium without phenol red at a concentration of 10^6 cells·mL^{-1}. Fluorophores and probes were added to the cell suspension from 0.1 mM stock solutions in DMSO to the desired final concentrations of 1 µM. Samples were incubated for 10 min at 37 °C, then directly analyzed on a BD FACSymphony/A1 flow cytometer. 20,000 cells per sample were counted at a low flow rate using a 640 nm laser and the APC detection filter (670/30 nm).

Bacterial clinical isolates. Clinical isolates of *K. oxytoca*, *E. cloacae*, *E. coli* and *S. aureus* from the collection of the GIPAM team at the Saint-Etienne University Hospital Center (France) were inoculated onto Columbia blood agar plates and incubated overnight at 37°C. Three colonies of each isolated strain were resuspended in 1 mL of PBS to reach an OD_{600} of 0.5. To 200 µL of each suspension were added 2 µL of the 0.1 mM DMSO stock solution of **dSQ$^+$-Ubi** to achieve a final concentration of 1 µM. The samples were incubated for 10 min at room temperature, then diluted 100 times in PBS and analyzed on a Cytek® Aurora flow cytometer. 30,000 cells per sample were counted at a low flow rate (15 µL/min) using a 640 nm laser and the R2 detection channel (670–688 nm).

Data analysis. Flow cytometry data were analyzed using RStudio (version 2022.7.2.576) with the *flowCore* package (version 2.14.0).[10] Plots were created using the *ggplot2* package (version 3.4.4).[11]

8. Flow cytometry analysis of patient urine samples

16 urine samples from patients with suspected UTIs at the Saint-Etienne University Hospital Centre (France) were enrolled in the study. Urine samples were collected from patients in BD Vacutainer Urine Culture & Sensitivity tubes (Becton, Dickinson and Co, USA). Cytological parameters such as hematuria, leucocyturia (significant positive threshold ≥ 10^4 cells·ml^{-1}) were measured using the Iris iQ200 select Automated Urine Microscopy Analyzer.[12] 10 µL calibrated bacteriological loop was used to inoculate a chromogenic agar plate (UTI Clarity Brilliance, ThermoScientific, UK). Plates were then incubated at 37°C for 24 hours. The rest of the urine samples were kept overnight at +4°C. MALDI-TOF MS System (MALDI Biotyper Sirius, Bruker Daltonics, Germany) was used for bacterial identification of suspected uropathogen colonies. The complete characterization of the collected urine samples (the parameters of hematuria and leucocyturia, the quantities and the nature of pathogens) is provided in Table S2.

The next day, 1 µL of the 0.1 mM DMSO stock solution of **dSQ^+-Ubi** was added to 100 µL of each urine sample to achieve a final concentration of 1 µM. The samples were stained with the probe for 10 min at room temperature, then directly analyzed on a Cytek® Aurora flow cytometer. 5 µL of each sample were analyzed at a low flow rate (15 µL/min) using a 640 nm laser and the R2 detection channel (670–688 nm). The same protocol was applied to the analysis of the unstained control urine samples.

Flow cytometry data were analyzed using RStudio (version 2022.7.2.576) with the *flowCore* package (version 2.14.0).[10] Plots were created using the *ggplot2* package (version 3.4.4).[11]

Initial gating was performed on an fsc_h versus ssc_h density plot with thresholds set to ssc_h > 2000, ssc_h < 300,000, fsc_h > 2000, and fsc_h < 300,000 to exclude debris and artifacts. Next, second gating was performed on an R2_a versus ssc_h density plot to separate stained and unstained cells. The optimal thresholds were identified for each sample by comparing the fluorescence intensities of stained and unstained control samples. Typically, the thresholds were set to R2_a > 3,000; 5,000; 10,000 or 20,000.

Finally, we calculated the number of cells in the selected cell population and divided the number by 5, which gave us the estimate of the number of fluorescent bacteria per µL of urine sample.

9. Ethics

All experiments were performed in accordance with French bioethics law and were approved by the Ethics Committee of the University Hospital of Saint-Étienne (reference number IRBN492025/CHUSTE). Opt-out consent was obtained from all human participants involved in this study.

10. Generative AI statement

During the preparation of this work the authors used ChatGPT for polishing the initial draft of the manuscript, namely for correcting grammar errors, refining phrasing for clarity and conciseness, and suggesting alternative word choices. The authors reviewed and edited the generated content as needed and take full responsibility for the content of the publication.

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