Synthesis

General

All chemicals and solvents were obtained from commercial sources and used without further purification. DMF and DCM used were dried with 4Å molecular sieves, unless stated otherwise. Column chromatography was performed with 40–63 µm silica (Silicycle). Dry column vacuum chromatography was performed as published by Pedersen *et al*. (*1*) with 15–40 µm silica (Merck) and Hyflo Supercell Celite (Sigma-Aldrich). HPLC was performed a Waters HPLC system using either a 1525EF or 2545 pump and a 2489 UV/VIS detector. For preparative HPLC either a Dr. Maisch GmbH Reprosil-Pur 120 C18-AQ 10 µm (250 × 20 mm) column or a XBridge Prep C8 10µm OBD 30x250mm column was used with a gradient of 0.1% TFA in H_2O/CH_3CN 95:5 to 0.1% TFA in H_2O/CH_3CN 5:95 in 40 minutes (12 or 25 mL/min, respectively) was employed. For semi-preparative HPLC a Dr. Maisch GmbH Reprosil-Pur C18-AQ 10 µm (250 × 10 mm) column was used and a gradient of 0.1% TFA in H_2O/CH_3CN 95:5 to 0.1% TFA in H_2O/CH_3CN 5:95 in 40 minutes (5 mL/min) was employed. For analytical HPLC a Dr. Maisch GmbH Reprosil-Pur C18-AQ 5 µm (250 × 4.6 mm) column was used and a gradient of 0.1% TFA in H_2O/CH_3CN 95:5 to 0.1% TFA in H_2O/CH_3CN 5:95 in 40 minutes (1 mL/min) was employed. Mass spectrometry was performed on a Bruker Microflex MALDI-TOF.

Merrifield resin synthesis

As performed by Lopalco *et al*. (2) Chloromethyl polystyrene resin (8.3 g, 15.0 mmol), N-Boc-aminophenol (9,4 g, 45.0 mmol), TBAI (1,7 g, 4.5 mmol) and $CsCO_3$ (14.7, 45.0 mmol) were dissolved in acetone, refluxed at 70 °C overnight under nitrogen atmosphere. Resin was then washed extensively with 100 mL DMF, H_2O, DMF, DCM and Et_2O and dried *in vacuo*. 4-(4-nitrobenzyl)pyridine test was used to determine reaction completion. 10.3 g (90% isolated yield) of Merrifield resin was formed.

(7R,10R,13R)-7,10,13-tris(hydroxymethyl)-2,5,8,11,14-pentaoxo-3-thia-6,9,12,15-tetraazahenicosan-21-oic acid (mas_3-Ahx-COOH)

mas_3-Ahx-COOH (1 mmol) was synthesised using standard (Fmoc) solid phase synthesis. After completion of the on-resin compound, it was cleaved by stirring in TFA for 3 h. The solution was drained and the solvent was evaporated *in vacuo*, re-dissolved in tBuOH/H_2O (1:1) and lyophilised. Lyophilisation yielded the title compound as a white, fluffy solid m/z $[M+Na]^+$ calcd. 531.532, found 530.998. The solid was used without further purification.

2,5-dioxopyrrolidin-1-yl (7R,10R,13R)-7,10,13-tris(hydroxymethyl)-2,5,8,11,14-pentaoxo-3-thia-6,9,12,15-tetraazahenicosan-21-oate (mas_3-Ahx-COOSu)

mas_3-Ahx-COOH (100 mg, 196.85 µmol) was dissolved in dry DMSO (2.5 mL) after which HSPyU (162 mg, 393.70 µmol) and DiPEA (171 µL, 984.25 µmol) were added. The solution was stirred under a N_2 atmosphere at room temperature for 30 minutes after which ethyl acetate (50 mL) was added. After centrifugation the supernatant was decanted and the white precipitate was washed 3 times with ethyl acetate (50 mL) and one time with diethyl ether (50 mL). The remaining solid was desiccated and purified by preparative HPLC. The correct fractions were pooled and lyophilised giving the title compound as a white solid in a 34% isolated yield (40 mg).

OtBu-Glu(OtBu)-urea-Lys(Z)-OtBu (EuK$(tBu)_3$-Z)

OtBu-Glu(OtBu)-urea-Lys(Z)-OtBu was synthesised as inspired by Khan *et al.* (3) H-Glu(OtBu)-OtBu.HCl (1.0 g, 3.38 mmol), 4-nitrophenyl chloroformate (682 mg, 3.38 mmol) and triethylamine (943 µL, 6.76 mmol) were dissolved in dry DCM (20 ml). The mixture was refluxed under a N_2 atmosphere for 25 minutes followed by stirring at r.t. for 60 minutes. A white precipitate was formed which disappeared when H-Lys(Z)-OtBu.HCl (1387 mg, 3.72 mmol) and triethylamine (943 µL, 6.76 mmol) were added. The solution turned yellow and was refluxed for 10 minutes before stirring at r.t. for 90 minutes. TLC showed full conversion of the starting material and thus the mixture was concentrated *in vacuo* to a small volume. Ethyl acetate (80 ml) was added and the suspension was stirred for 16 hours at r.t. after which it was filtered using a glass filter (P3). The white precipitate was washed with ethyl acetate, the filtrate was combined with the supernatant and concentrated *in vacuo* to obtain a yellow oil. Column chromatography

was performed using a gradient of ethyl acetate/hexane 1:5 to 1:2 over 6 column volumes, followed by 100% ethyl acetate for 1 column volume. The correct fractions were pooled and lyophilisation yielded the title compound as a slightly yellow oil in a 86% isolated yield (1.9 g, 95% pure). m/z $[M+Na]^+$ calcd. 644.8, found 644.6.

OtBu-Glu(OtBu)-urea-Lys(NH_2)-OtBu (EuK($tBu)_3$-NH_2)

OtBu-Glu(OtBu)-urea-Lys(NH_2)-OtBu was synthesised inspired Makowski *et al.* (4) OtBu-Glu(OtBu)-urea-Lys(Z)-OtBu (1.9 g, 3.06 mmol), ammonium formate (385 mg, 6.11 mmol) and Pd/C (19 mg) were refluxed (100°C) in absolute ethanol (40 ml) under a N_2 atmosphere for 120 minutes. The reaction mixture was allowed to cool down to r.t. before filtering the suspension over Celite. The Celite was subsequently rinsed with absolute ethanol (50 ml). The solvent was removed *in vacuo* resulting in a yellow oil. Purification by HPLC yielded the title compound in quantitative yield as a yellowish oil, (1.6 g, 94% pure). m/z $[M+H]^+$ calcd. 487.6, found 488.1 $[M+Na]^+$ calcd. 510.6, found 510.2. The oil was diluted with dry methanol for further use (94 mg/mL).

The (sulfo)indole-based building blocks for the Cy5-dyes were synthesised according to adjusted synthesis methods inspired by published procedures.(*5-7*) All products were directly used without further purification.

3-(4-(1,3-dioxoisoindolin-2-yl)butyl)-1,1-dimethyl-1H-benzo[e]indol-3-ium

1,1,2-trimethyl-1*H*-benzo[e]indole (6.3 g, 30 mmol) and 1-(4-bromobutyl)pyrrolidine-2,5-dione (25.4 g, 28.7 mmol) were added to sulfolane (60 mL) and stirred at 90 °C for 72 h under a N_2 atmosphere. The reaction mixture was precipitated in EtOAc and the resulting suspension was filtered. The residue was washed twice with Et_2O and EtOAc. Removing solvents *in vacuo* yielded a grey solid (9.2 g) which was used without any further purification.

6-(1,1-dimethyl-1,2-dihydro-3H-benzo[e]indol-3-yl)hexanoic acid

1,1,2-trimethyl-1*H*-benzo[e]indole (6.5 g, 31 mmol) and 6-bromohexanoic acid (12.1 g, 62 mmol) were heated at 70–95 °C in MeCN (60.0 mL) for 72 h under a N_2 atmosphere. The grey, solid product was washed twice with MeCN, Et_2O and EtOAc. Residual solvents were removed *in vacuo*, yielding a grey solid (6.6 g) that was used without further purification.

Synthesis of phthalimide dyes (general procedure A) (1a–1f)

As previously described (2). In short, the hemicyanine was prepared by heating the indole-based building block with N-((1E,3E)-3-(phenylimino)prop-1-en-1-yl)aniline in a mixture of AcOH/Ac_2O (1:1) to 90 °Covernight followed by stirring at 120 °C for 2 h. When the reaction was finished as determined by using UV/Vis spectroscopy (product at λ_{max} ~450 nm and starting material at λ_{max} ~390 nm), the mixture was cooled down and precipitated in diethyl ether. After repeated centrifugation, decanting and washing steps with diethyl ether and EtOAc the solid was dissolved in a 1:1 DCM/DMF mixture and directly added to the deprotected resin. The suspension was agitated at r.t. for 1 h. After this time, by-products were washed away with DCM/DMF mixtures and the cyanine dye was prepared by adding the corresponding second indole-based building block in a mixture of pyridine/Ac_2O (3:1). This mixture was agitated at r.t. for 18 h. Crude dyes were purified by means of DCVC (EtOAc/MeOH) and subsequent preparative HPLC.

Phth-Cy5-COOH (**1a**)

2-((1E,3E)-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(4-(1,3-dioxoisoindolin-2-yl)butyl)-3,3-dimethyl-3H-indol-1-ium was obtained from 1-(4-(1,3-dioxoisoindolin-2-yl)butyl)-2,3,3-trimethyl-3H-indol-1-ium (220 mg, 0.6 mmol), 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium (660 mg, 2.4 mmol) and N-((1E,3E)-3-(phenylimino)prop-1-en-1-yl)aniline (683 mg, 2.7 mmol) in 23% isolated yield (59 mg) as a blue, fluffy solid after lyophilisation. ^{1}H NMR (300 MHz, MeOD) δ 8.23 (td, J = 13.2, 6.7 Hz, 2H), 7.95–7.70 (m, 4H), 7.56–7.17 (m, 8H), 6.63 (t, J = 12.4 Hz, 1H), 6.31 (d, J = 13.6

Hz, 2H), 4.32–4.05 (m, 4H), 3.78 (t, J = 5.3 Hz, 2H), 2.35 (t, J = 7.2 Hz, 2H), 1.86 (s, J = 3.0 Hz, 6H), 1.73 (t, J = 7.6 Hz, 14H), 1.61–1.47 (m, 2H). m/z $[M]^+$ calcd. 670.873, found 670.295.

Phth-Cy5(SO_3)-COOH (**1b**)

(E)-1-(5-carboxypentyl)-2-((2E,4E)-5-(1-(4-(1,3-dioxoisoindolin-2-yl)butyl)-3,3-dimethyl-3H-indol-1-ium-2-yl)penta-2,4-dien-1-ylidene)-3,3-dimethylindoline-5-sulfonate was obtained from 1-(4-(1,3-dioxoisoindolin-2-yl)butyl)-2,3,3-trimethyl-3H-indol-1-ium (264 mg, 0.6 mmol), 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate (1100 mg, 2.4 mmol) and N-((1E,3E)-3-(phenylimino)prop-1-en-1-yl)aniline (678 mg, 2.6 mmol) in 13% isolated yield (65 mg) as a blue, fluffy solid after lyophilisation. δ 8.31 (td, *J* = 13.0, 8.4 Hz, 1H), 7.91 – 7.76 (m, 2H), 7.61 (dd, *J* = 13.4, 5.0 Hz, 1H), 7.41 (d, *J* = 4.0 Hz, 1H), 7.33 (d, *J* = 8.3 Hz, 1H), 7.29 – 7.22 (m, 1H), 6.55 (t, *J* = 12.4 Hz, 1H), 6.30 (dd, *J* = 18.1, 13.9 Hz, 1H), 4.11 (s, 2H), 3.62 (d, 11H), 2.20 (t, *J* = 7.2 Hz, 1H), 2.08 (s, 1H), 1.71 (s, 1H), 1.68 (d, *J* = 2.2 Hz, 5H), 1.61 – 1.49 (m, *J* = 14.6, 7.4 Hz, 1H), 1.44 – 1.33 (m, 1H), 1.28 (s, 1H), 1.23 (s, 1H), 1.14 (s, 1H), 0.87 (dd, *J* = 9.3, 5.5 Hz, 1H). m/z $[M+H]^+$ calcd. 750.930, found 750.600.

Phth-(SO_3)Cy5-COOH (**1c**)

2-((1E,3E)-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(4-(1,3-dioxoisoindolin-2-yl)butyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate was obtained from 1-(4-(1,3-dioxoisoindolin-2-yl)butyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate (440 mg, 1.0 mmol), 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium (1100 mg, 4.0 mmol) and N-((1E,3E)-3-(phenylimino)prop-1-en-1-yl)aniline (1200 mg, 4.4 mmol) in 5% isolated yield (39 mg) as a blue, fluffy solid after lyophilisation. δ 8.31 (t, *J* = 13.3 Hz, 1H), 7.92 – 7.76 (m, 2H), 7.68 – 7.56 (m, 1H), 7.46 – 7.29 (m, 1H), 7.23 (td, 1H), 6.56 (t, *J* = 12.5 Hz, 1H), 6.31 (d, *J* = 13.7 Hz, 1H), 4.12 (d, *J* = 5.8 Hz, 2H), 3.63 (s, 1H), 2.21 (t, *J* = 7.1 Hz, 1H), 1.68 (d, *J* = 8.6 Hz, 7H), 1.55 (t, 1H), 1.38 (t, 1H), 1.26 (d, *J* = 14.9 Hz, 2H), 1.11 (s, 1H), 0.87 (dd, *J* = 9.0, 5.0 Hz, 1H). m/z $[M+H]^+$ calcd. 750.930, found 750.778.

Phth-(SO_3)Cy5(SO_3)-COOH (**1d**)

2-((1E,3E)-5-((E)-1-(5-carboxypentyl)-3,3-dimethyl-5-sulfonatoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(4-(1,3-dioxoisoindolin-2-yl)butyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate was obtained from 1-(4-(1,3-dioxoisoindolin-2-yl)butyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate (264 mg, 0.6 mmol), 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium-5-sulfonate (1.7 g, 4.8 mmol) and N-((1E,3E)-3-(phenylimino)prop-1-en-1-yl)aniline (1.4 g, 5.3 mmol) in 16% isolated yield (86 mg) as a blue, fluffy solid after lyophilisation. δ 8.33 (td, *J* = 12.9, 5.6 Hz, 1H), 7.90 – 7.77 (m, 3H), 7.62 (td, *J* = 8.2, 1.5 Hz, 1H), 7.34 (d, *J* = 8.3 Hz, 1H), 6.55 (t, *J* = 12.4 Hz, 1H), 6.30 (dd, *J* = 13.9, 8.9 Hz, 1H), 4.14 – 4.00 (m, 9H), 3.96 (s, 1H), 3.62 (s, 1H), 3.55 (s, 1H), 2.29 (t, *J* = 7.2 Hz, 1H), 2.20 (t, *J* = 7.1 Hz, 1H), 2.08 (s, 1H), 1.68 (d, *J* = 5.4 Hz, 9H), 1.56 (dd, *J* = 14.9, 7.7 Hz, 1H), 1.38 (d, *J* = 6.4 Hz, 1H), 1.23 (s, 1H). m/z $[M+2H]^+$ calcd. 830.987, found 830.642.

Deprotection of the phthalimide (general procedure B) (2a–2f)

Compound **1a**–**1f** was dissolved in a solution of methylamine in EtOH (33 wt%) before stirring at r.t. for 4–7 hours. After completion, EtOH and residual methylamine were removed *in vacuo*. The crude was dissolved in H_2O/CH_3CN/TFA (75:25:0.1; 4.0 mL) and purified by preparative HPLC using a gradient of H_2O/CH_3CN/ TFA 75:25:0.1 to H_2O/CH_3CN/TFA 10:90:0.1 in 50 min.

2-((1E,3E)-5-((E)-1-(4-aminobutyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(5-carboxypentyl)-3,3-dimethyl-3H-indol-1-ium (**2a**). Blue, fluffy solid after lyophilisation, 59% isolated yield. m/z $[M]^+$ calcd. 540.771, found 540.600.

2-((1E,3E)-5-((E)-1-(4-aminobutyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1-(5-carboxypentyl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (**2b**). Blue, fluffy solid after lyophilisation, used without further purification. m/z $[M+H]^+$ calcd. 620.828, found 620.570.

(E)-1-(4-aminobutyl)-2-((2E,4E)-5-(1-(5-carboxypentyl)-3,3-dimethyl-3H-indol-1-ium-2-yl)penta-2,4-dien-1-ylidene)-3,3-dimethylindoline-5-sulfonate (**2c**). Blue, fluffy solid after lyophilisation, 21% isolated yield. m/z $[M+H]^+$ calcd. 620.828, found 620.400.

2-((1E,3E)-5-((E)-1-(4-aminobutyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3-(5-carboxypentyl)-1,1-dimethyl-1H-benzo[e]indol-3-ium (**2d**). Blue, fluffy solid after lyophilisation, 60% isolated yield. m/z $[M+H]^+$ calcd. 590.831, found 590.372.

2-((1E,3E,5E)-5-(3-(4-aminobutyl)-1,1-dimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)penta-1,3-dien-1-yl)-1-(5-carboxypentyl)-3,3-dimethyl-3H-indol-1-ium (**2e**). Blue, fluffy solid after lyophilisation, 68% isolated yield. m/z $[M+H]^+$ calcd. 590.831, found 590.319.

2-((1E,3E,5E)-5-(3-(4-aminobutyl)-1,1-dimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)penta-1,3-dien-1-yl)-3-(5-carboxypentyl)-1,1-dimethyl-1H-benzo[e]indol-3-ium (**2f**). Blue, fluffy solid after lyophilisation, 76% isolated yield. m/z $[M+H]^+$ calcd. 640.891, found 640.451.

Coupling of the mas$_3$ chelate (general procedure C) (3a–3f)

The deprotected cyanine dye as prepared above (**2a**–**2f**) was dissolved in DMSO before basifying with DiPEA (3.0 eq) and a solution of mas$_3$-Ahx-COOSu in DMSO (1.0 eq, 20 mg/mL). The resulting mixture was stirred at r.t. for 60–90 minutes before adding DMSO (500 µL) and H_2O/TFA (99.9:0.1; 3.0 mL) and purifying by preparative HPLC using a gradient of H_2O/CH_3CN/TFA 75:25:0.1 to H_2O/CH_3CN/TFA 10:90:0.1 in 50 min.

(**3a**). Blue, fluffy solid after lyophilisation, used without any further purification. m/z $[M]^+$ calcd. 1030.532, found 1031.001.

(**3b**). Blue, fluffy solid after lyophilisation, used without further purification. m/z $[M+H]^+$ calcd. 1111.356, found 1111.086.

(**3c**). Blue, fluffy solid after lyophilisation, used without any further purification. m/z $[M+H]^+$ calcd. 1111.356, found 1111.539.

(**3d**). Blue, fluffy solid after lyophilisation, 77% isolated yield. m/z $[M]^+$ calcd. 1081.359, found 1081.188.

(**3e**). Blue, fluffy solid after lyophilisation, 57% isolated yield. m/z $[M]^+$ calcd. 1081.359, found 1081.501.

(**3f**). Blue, fluffy solid after lyophilisation, 71% isolated yield. m/z $[M]^+$ calcd. 1131.419, found 1131.094.

Coupling of the EuK(tBu)$_3$ moiety (general procedure D) (4a–4f)

3a–3f was dissolved in DMSO (1.0 mL) before adding PyBOP (4.0 eq) and NMM (2.0 eq). The resulting mixture was stirred at r.t. for 20 minutes before the addition of $EuK(tBu)_3$ (2.0 eq). This mixture was stirred at r.t. for 5–6 hours before adding H_2O/TFA (99.1:0.1; 3.0 mL) and where appropriate, the mixture was further acidified to pH = 1–3 with a few drops of TFA. The crude material was then purified by preparative HPLC using a gradient of H_2O/CH_3CN/ TFA 75:25:0.1 to H_2O/CH_3CN/TFA 10:90:0.1 in 50 min.

(**4a**). Blue, fluffy solid after lyophilisation, 89% isolated yield. m/z $[M]^+$ calcd. 1500.922, found 1500.995.

(**4b**). Blue, fluffy solid after lyophilisation, 35% isolated yield. m/z $[M+H]^+$ calcd. 1580.979, found 1580.529.

(**4c**). Blue, fluffy solid after lyophilisation, 56% isolated yield. m/z $[M+H]^+$ calcd. 1580.979, found 1580.829.

(**4d**). Blue, fluffy solid after lyophilisation, 56% isolated yield. m/z $[M]^+$ calcd. 1550.982, found 1151.119.

(**4e**). Blue, fluffy solid after lyophilisation, 67% isolated yield. m/z $[M]^+$ calcd. 1550.982, found 1551.559.

(**4f**). Blue, fluffy solid after lyophilisation, 57% isolated yield. m/z $[M]^+$ calcd. 1601.042, found 1600.914.

Deprotection of the EuK moiety (general procedure E) (5a–5f)

4a–**4f** was dissolved in a solution of TFA/triisopropylsilane (95:5; 1.5 mL) before stirring under a N_2 atmosphere for 3.5 hours. After this time, solvents were removed *in vacuo* and the resulting crude was then purified by preparative HPLC using a gradient of H_2O/CH_3CN/ TFA 75:25:0.1 to H_2O/CH_3CN/TFA 10:90:0.1 in 50 min.

(**5a; EuK-Cy5-mas$_3$**). Blue, fluffy solid after lyophilisation, 61% isolated yield, 99% pure as assessed by HPLC. m/z $[M]^+$ calcd. 1332.598, found 1332.439.

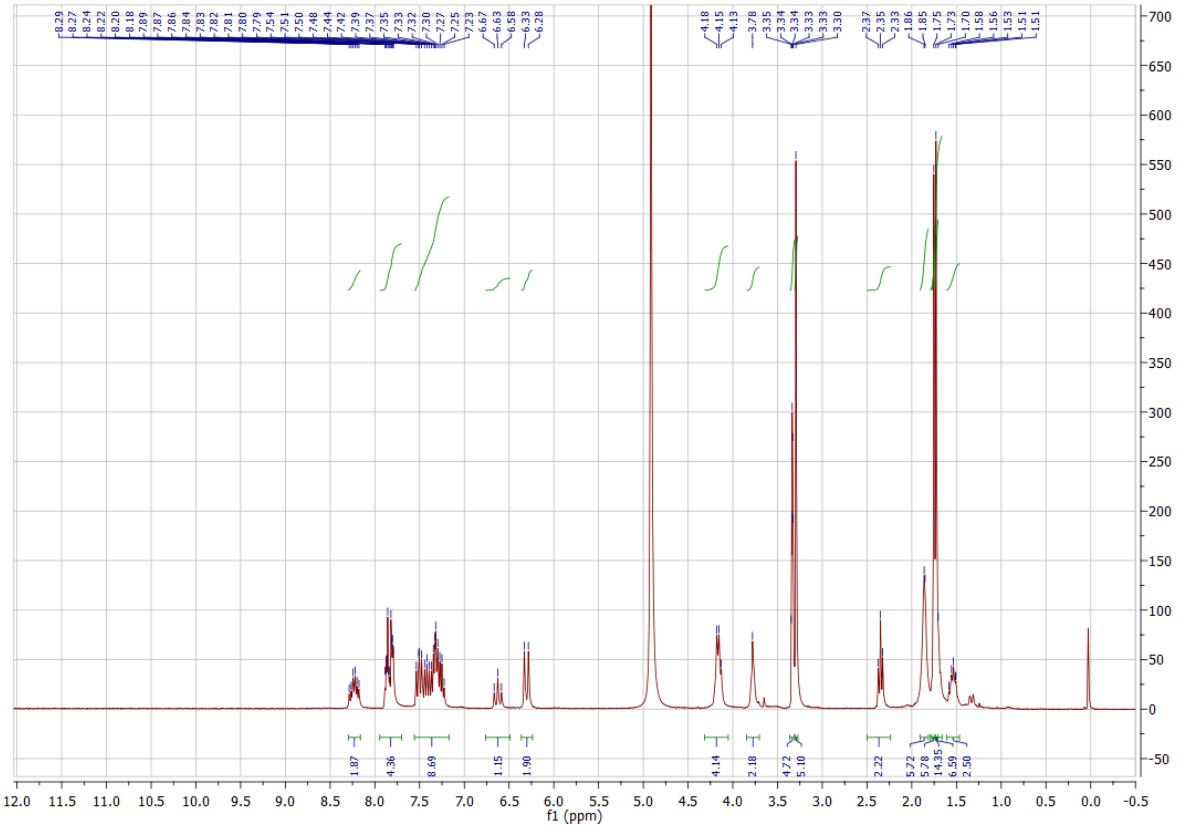
(**5b; EuK-(SO_3)Cy5-mas$_3$**). Blue, fluffy solid after lyophilisation, 18% isolated yield. m/z $[M+H]^+$ calcd. 1412.655, found 1412.528.

(**5c; EuK-Cy5(SO_3)-mas$_3$**). Blue, fluffy solid after lyophilisation, 28% isolated yield. m/z $[M+H]^+$ calcd. 1412.655, found 1412.461.

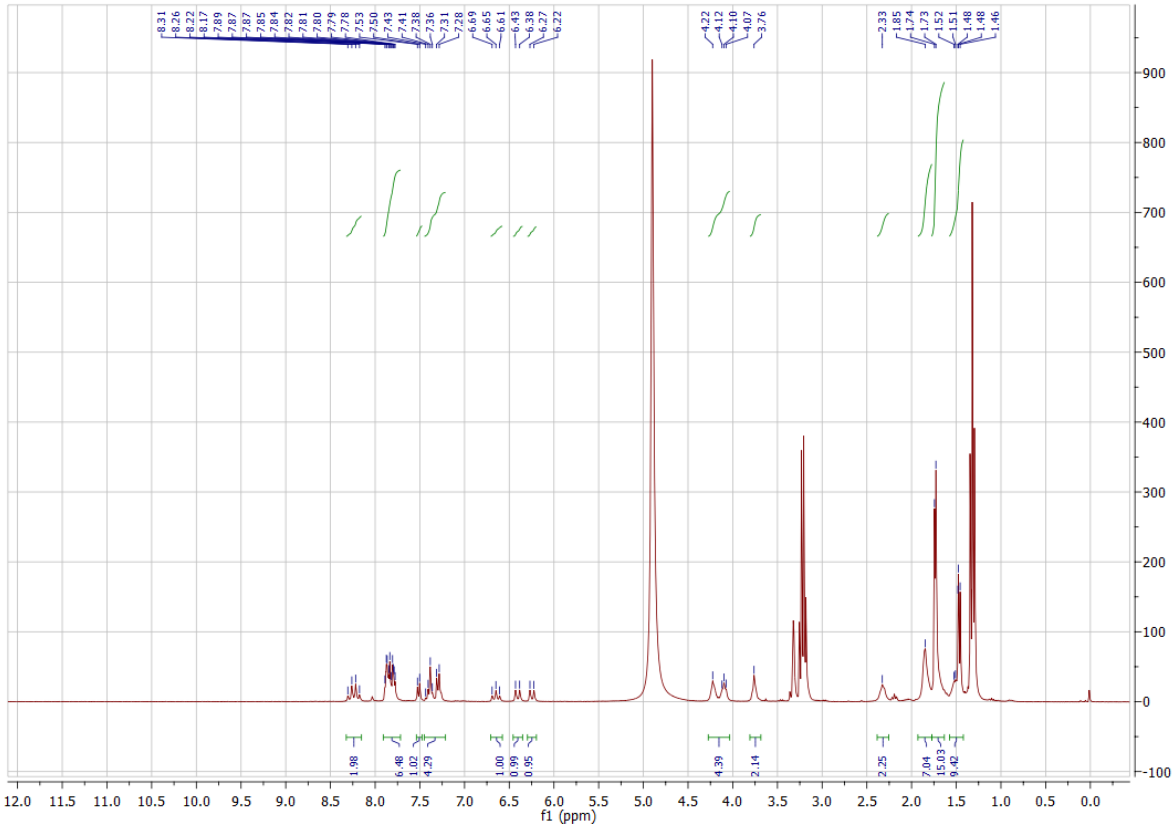
(**5d; EuK-(Ar)Cy5-mas$_3$**). Blue, fluffy solid after lyophilisation, 63% isolated yield, 98% pure as assessed by HPLC. m/z $[M+H]^+$ calcd. 1382.658, found 1382.526.

(**5e; EuK-Cy5(Ar)-mas$_3$**). Blue, fluffy solid after lyophilisation, 98% isolated yield, 97% pure as assessed by HPLC. m/z $[M+H]^+$ calcd. 1382.658, found 1382.388.

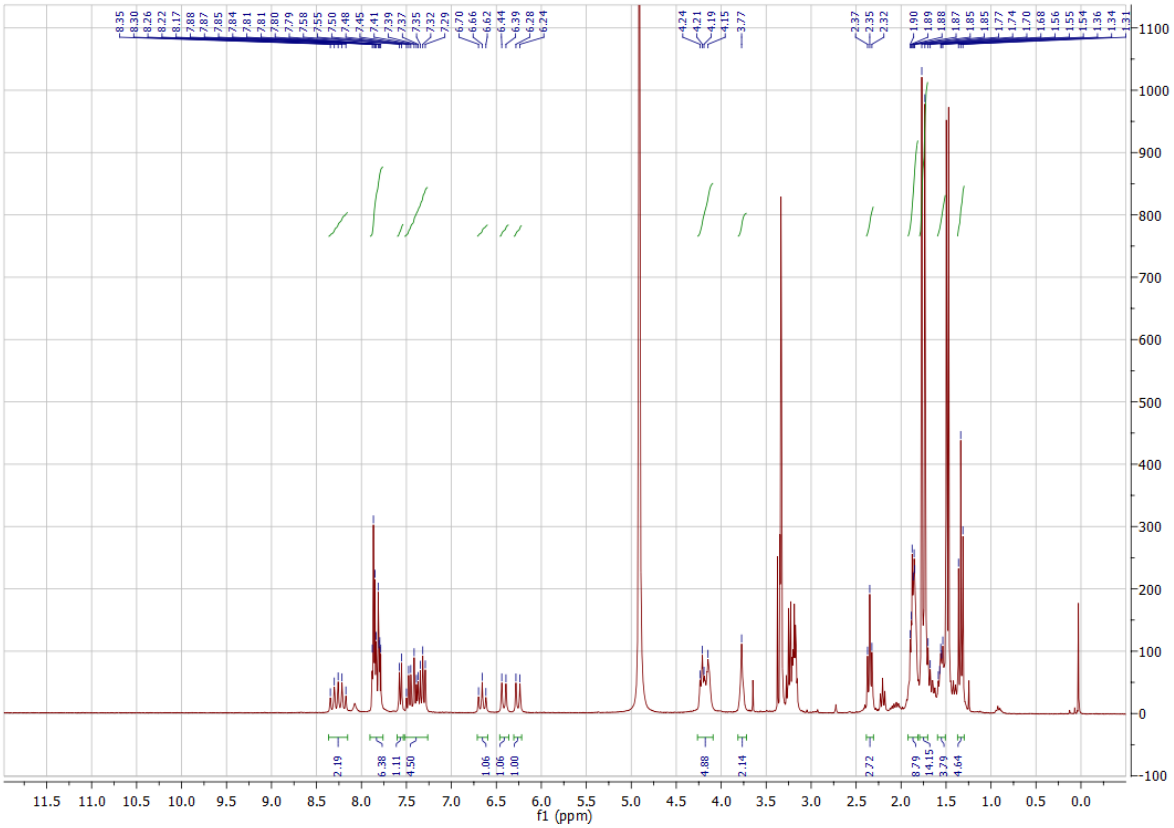
(**5f; EuK-(Ar)Cy5(Ar)-mas$_3$**). Blue, fluffy solid after lyophilisation, 73% isolated yield, 98% pure as assessed by HPLC. m/z $[M+H]^+$ calcd. 1432.718, found 1432.544.



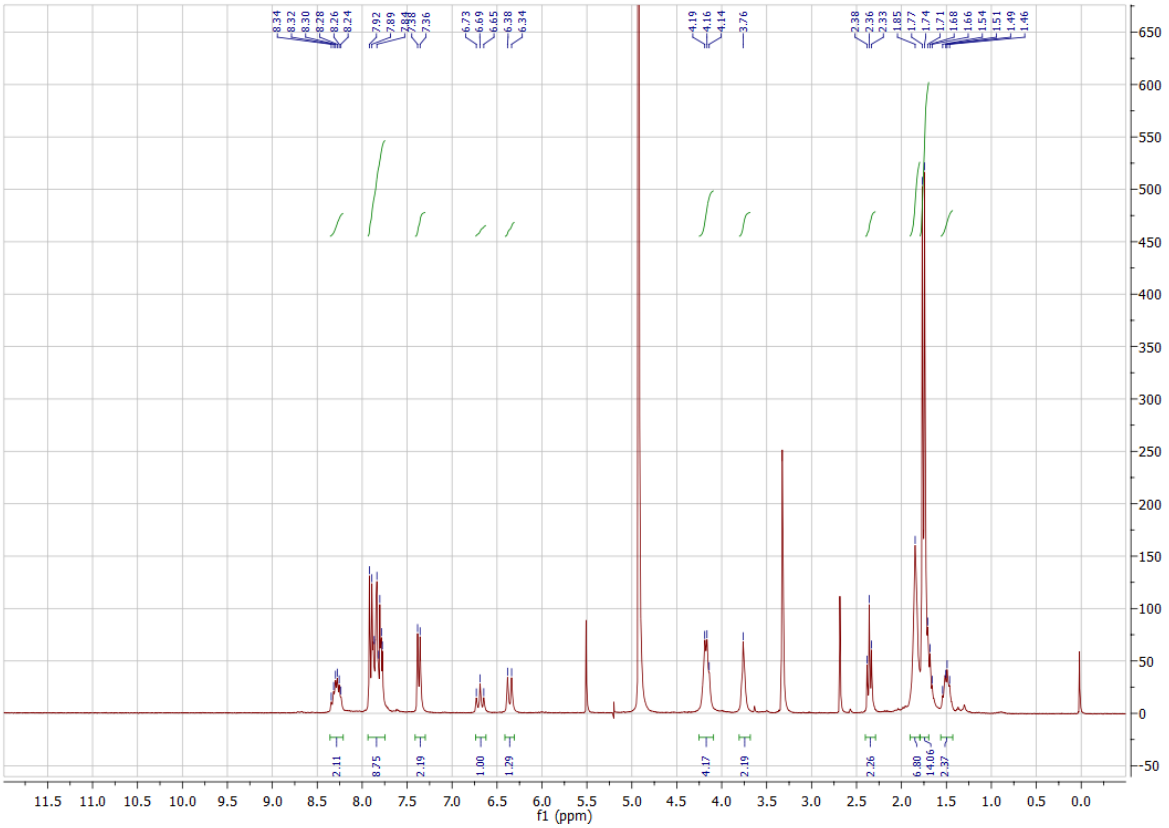
Supplemental Figure 1. ^{1}H NMR spectrum of Phth-Cy5-COOH (**1a**)



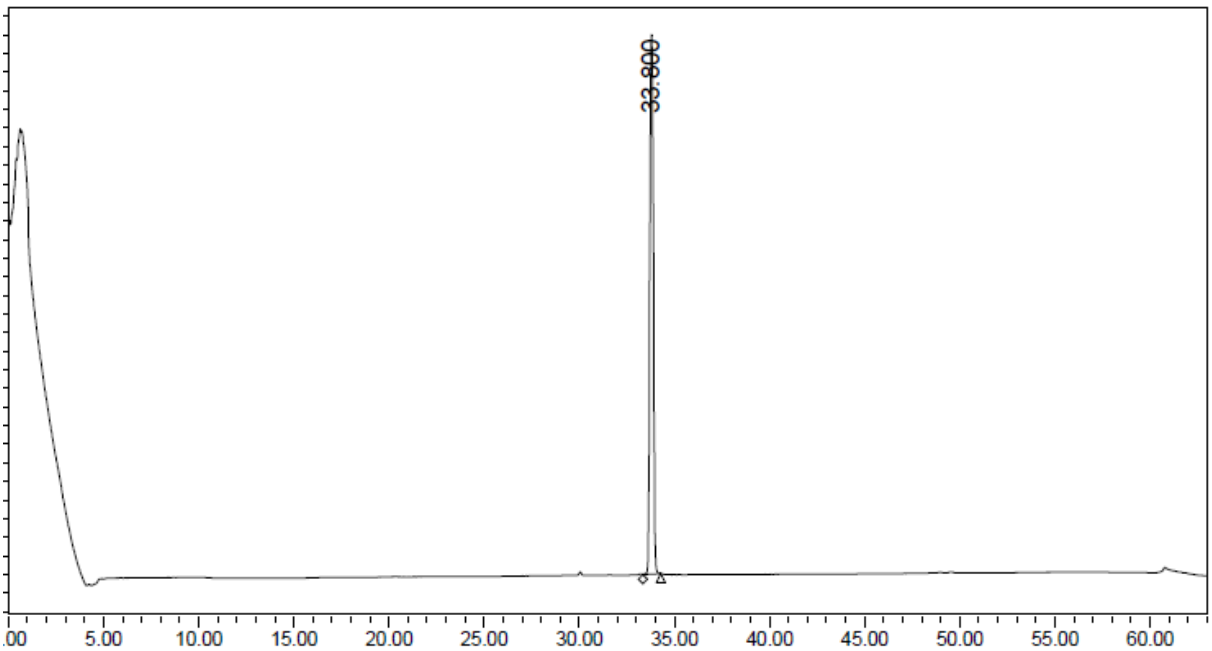
Supplemental Figure 2. ^{1}H NMR of Phth-Cy5-(SO_3)COOH (**1b**)



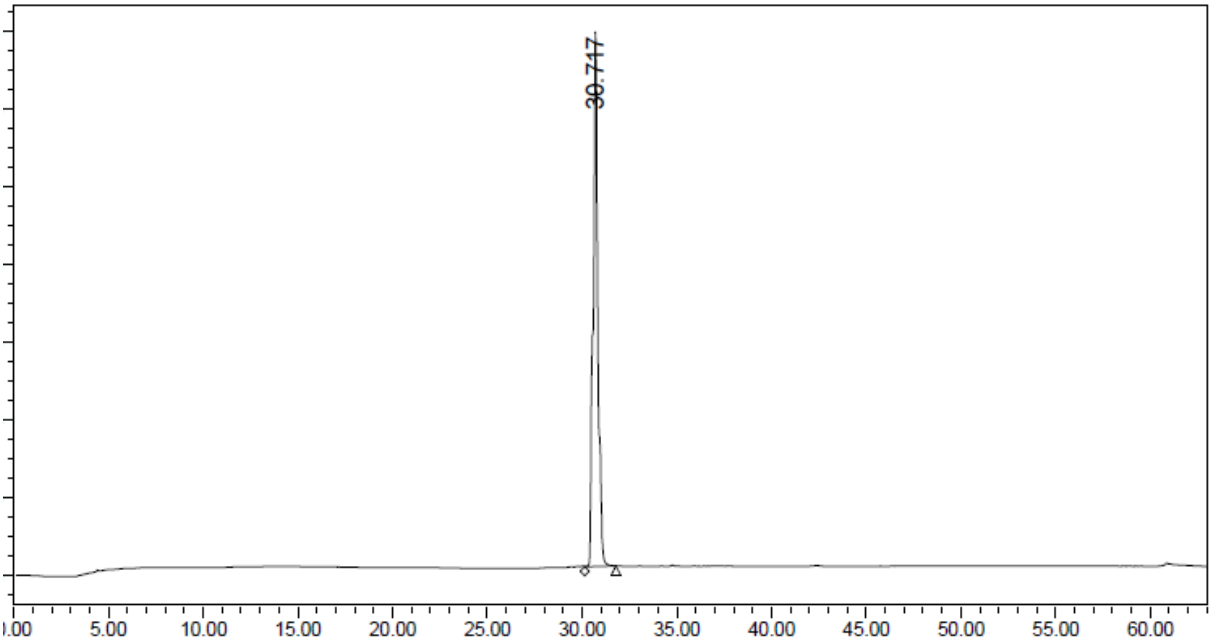
Supplemental Figure 3. ^{1}H NMR of Phth-(SO_3)Cy5-COOH (**1c**)



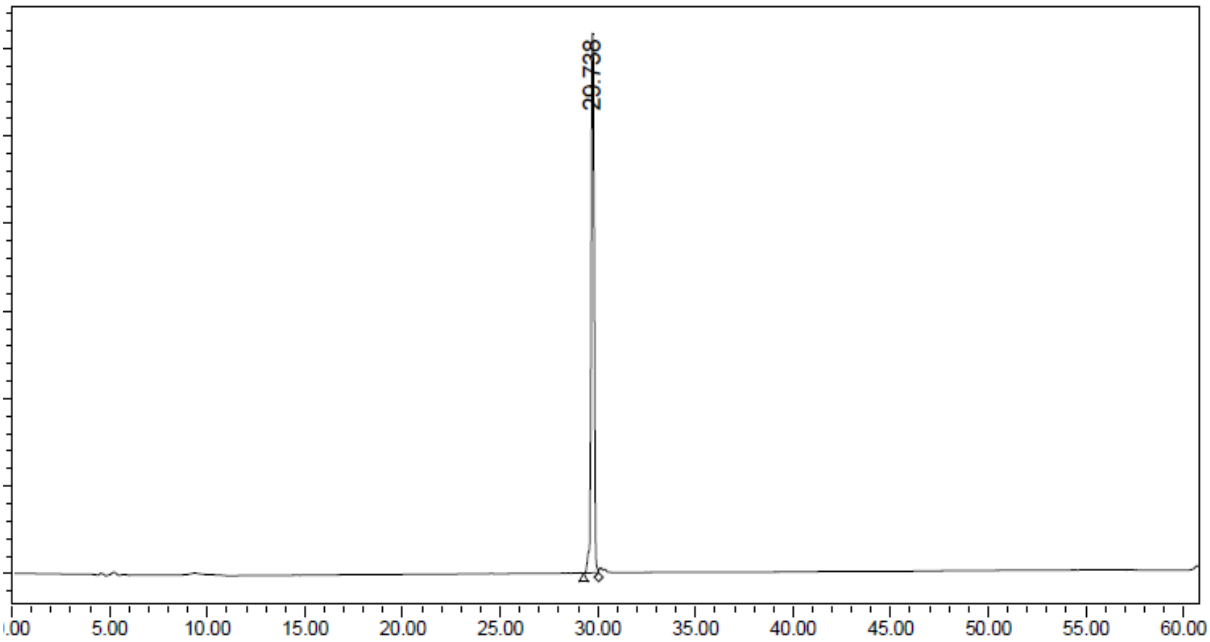
Supplemental Figure 4. ^{1}H NMR of Phth-(SO_3)Cy5-(SO_3)COOH (**1d**)



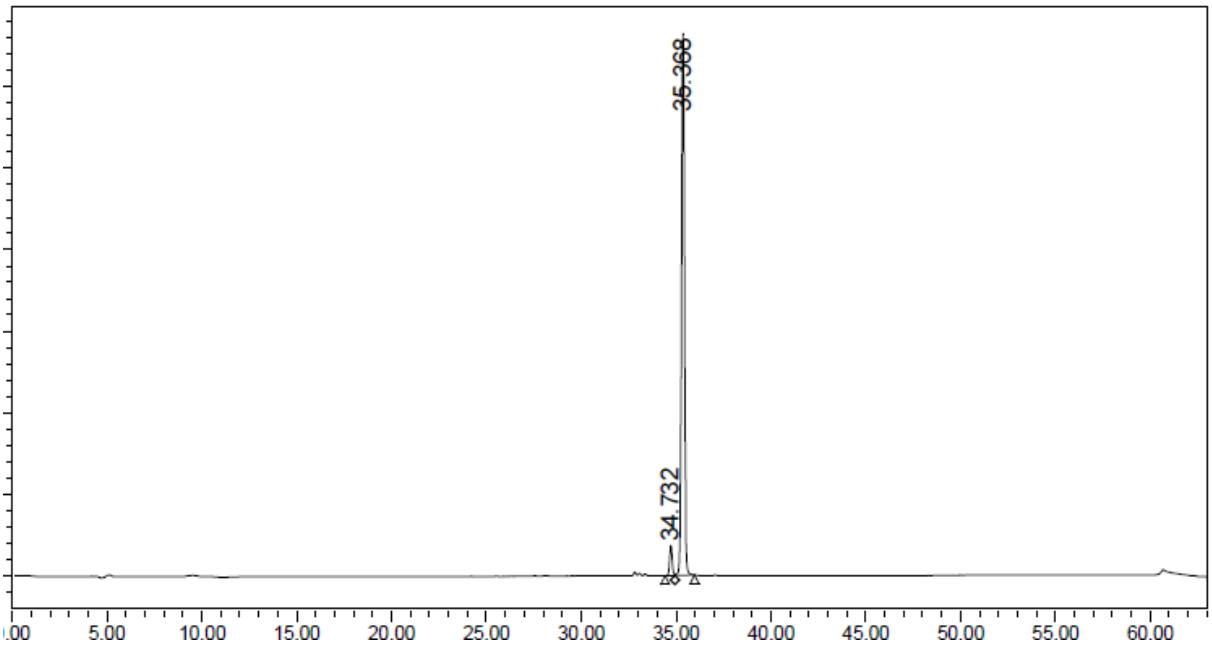
Supplemental Figure 5. Chromatogram (650 nm) of EuK-Cy5-mas_3



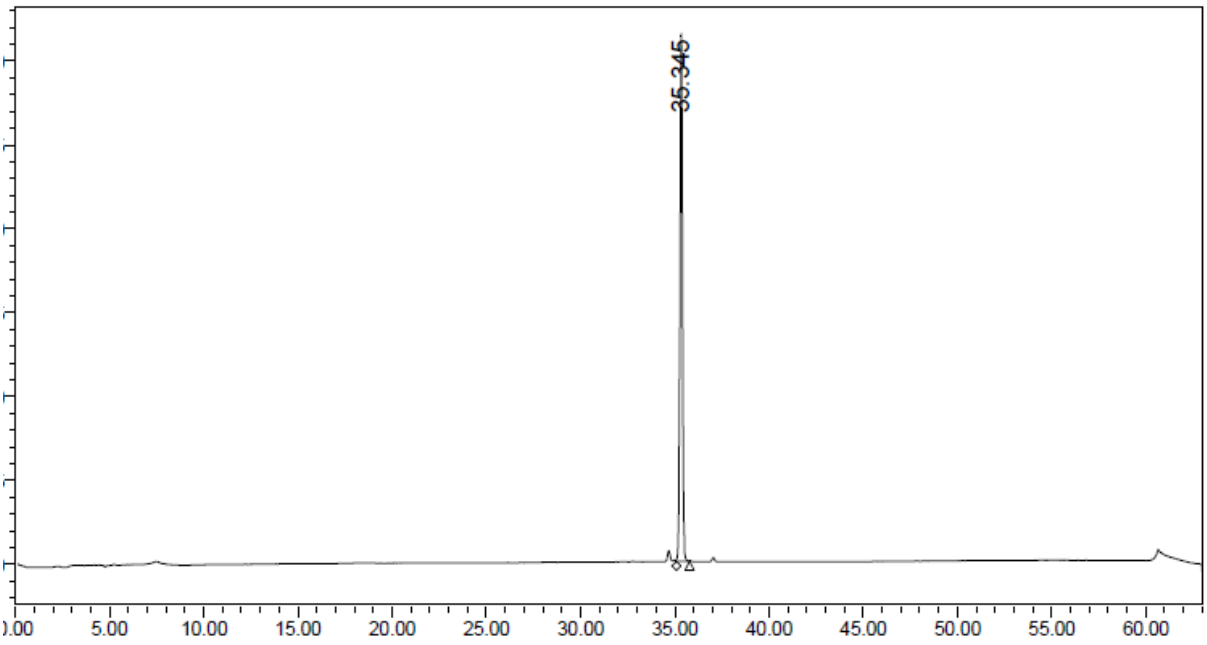
Supplemental Figure 6. Chromatogram (650 nm) of EuK-(SO_3)Cy5-mas_3



Supplemental Figure 7. Chromatogram (650 nm) of EuK-Cy5(SO_3)-mas_3



Supplemental Figure 8. Chromatogram (650 nm) of EuK-(Ar)Cy5-mas$_3$



Supplemental Figure 9. Chromatogram (650 nm) of EuK-Cy5(Ar)-mas$_3$

Results

Chemistry

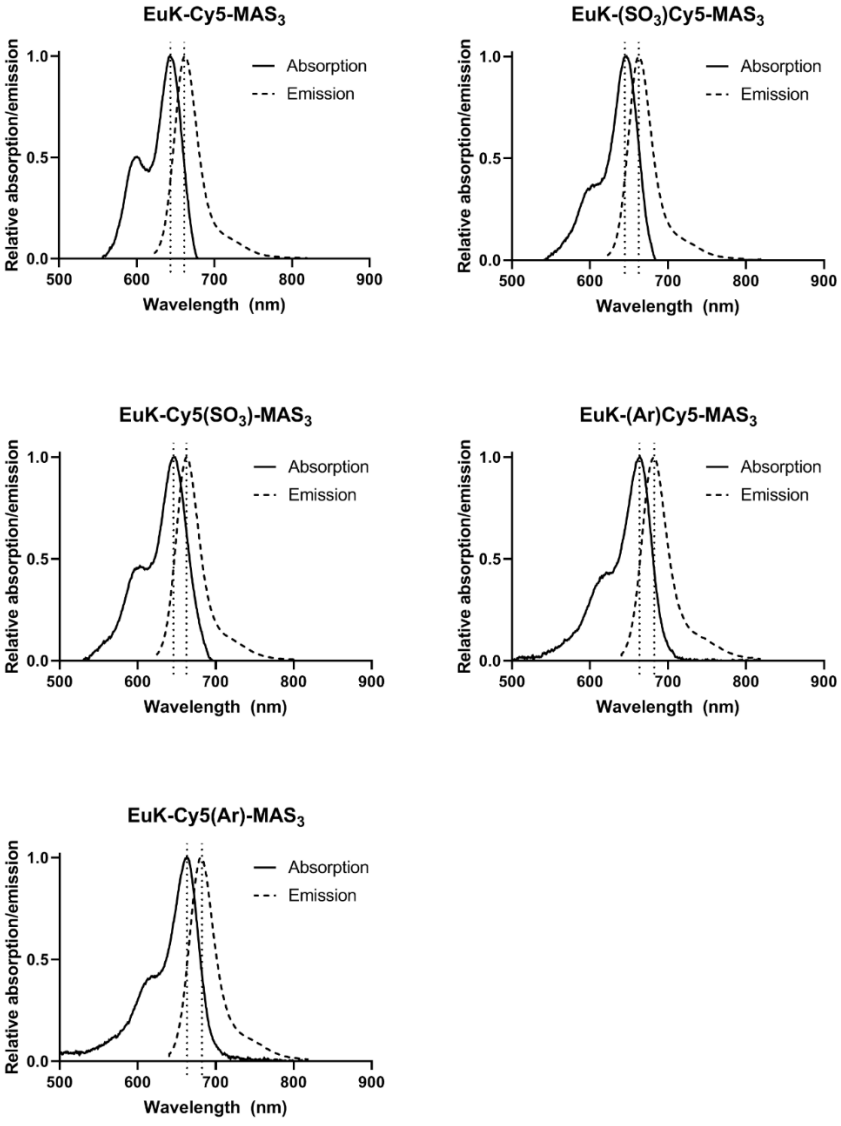
To allow identification of the most optimal interactions between the cyanine backbone and the amphipathic funnel, a series of five structurally related hybrid PSMA analogues was synthesised: EuK-Cy5-mas_3, EuK-(SO_3)Cy5-mas_3, EuK-Cy5(SO_3)-mas_3, EuK-(Ar)Cy5-mas_3, and EuK-Cy5(Ar)-mas_3 (Figure 1A and 1B). The 2-mercaptoacetyl-seryl-seryl-seryl chelate (mas_3; including a six-carbon spacer; Figure 1C) was conjugated to the respective indole of Cy5 dyes in 57–77% isolated yield. Consecutive addition of the tri-*tert*-butyl-protected (EuK$(tBu)_3$) targeting moiety to the orthogonal indole (35–89% isolated yield) followed by deprotection (18–98% isolated yield; Figure 1C) resulted in the final products. Comparing the yields of the four synthetic steps combined indicated that the introduction of a substituent on an indole and the position thereof influenced the synthetic ease: unsubstituted (32% yield) > benzene-containing (16–25% yield) > sulfonate-containing (3–6% yield).

Photophysical Properties

Compared to the unsubstituted EuK-Cy5-mas_3, the introduction of a sulfonate moiety on either indole (EuK-(SO_3)Cy5-mas_3, EuK-Cy5(SO_3)-mas_3) led to a slight increase in maximum excitation wavelength ($\lambda_{ex,max}$) and maximum emission wavelength ($\lambda_{em,max}$; bathochromic shift; Table 1). The introduction of an extra benzene moiety on either indole (EuK-(Ar)Cy5-mas_3, EuK-Cy5(Ar)-mas_3) increased this bathochromic shift to around 20 nm (Table 1, Supplemental Figure 2), which is in line with literature (*16*). However, the fluorescence brightness—the product of a fluorophore's molar extinction coefficient (ε; derived from the corresponding free fluorophore, Table SI1) and quantum yield (Φ_F)—of the tracer analogues with an extra benzene moiety on either indole was reduced by nearly 50%. This finding, which is in line with literature,(*15*) brings about a reduction in visibility compared to the sulfonate-containing tracers while using the same camera system and settings, thus making them less suitable for image-guided surgery. The

brightness of EuK-(SO_3)Cy5-mas_3 was in the same range as the brightness described in previous literature on PSMA-targeting hybrid tracers ($0.8 \cdot 10^4$–$2.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) (*17-20*).

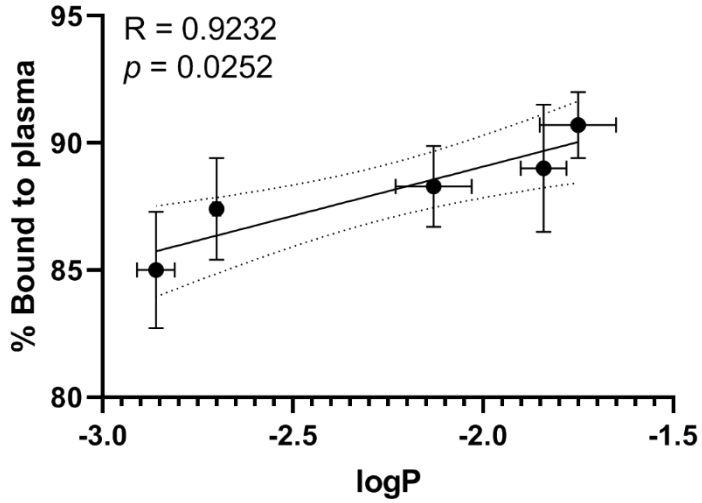
Absorption and emission spectra



Supplemental Figure 10. Absorption (solid lines) and emission (dashed lines) spectra for all hybrid PSMA-targeting tracers. Dotted vertical lines represent the maximum absorption and emission wavelengths for each corresponding spectrum.

Compound	Molar extinction coefficient in PBS (ε; $M^{-1} \cdot cm^{-1}$)
Phth-Cy5-COOH	$9.30 \cdot 10^4$
Phth-(SO_3)Cy5-COOH	$1.46 \cdot 10^5$
Phth-Cy5(SO_3)-COOH	$9.50 \cdot 10^4$
Phth-(Ar)Cy5-COOH	$6.00 \cdot 10^4$
Phth-Cy5(Ar)-COOH	$3.70 \cdot 10^4$

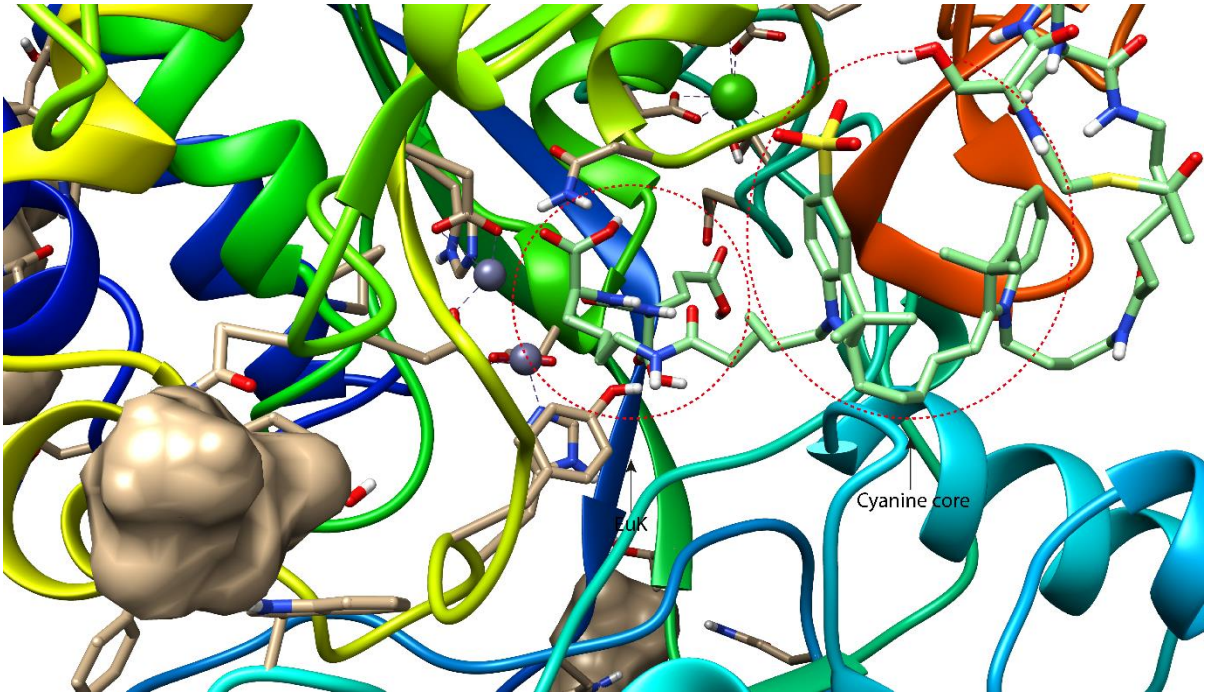
Supplemental Table 1. Molar extinction coefficient of the free fluorophores $\varepsilon_{fluorophore}$ for the determination of the brightness of hybrid tracers.



Supplemental Figure 11. Correlation between logP and PPB. Dotted lines represent 95% confidence interval

Compound	Serum stability after 24 h (% remaining; ^{99m}Tc-mas$_3$ complex; n = 2)
^{99m}Tc-EuK-Cy5-mas$_3$	88.5 ± 2.0%
^{99m}Tc-EuK-(SO_3)Cy5-mas$_3$	>99%
^{99m}Tc-EuK-Cy5(SO_3)-mas$_3$	>99%
^{99m}Tc-EuK-(Ar)Cy5-mas$_3$	>99%
^{99m}Tc-EuK-Cy5(Ar)-mas$_3$	>99%

Supplemental Table 2. ^{99m}Tc–mas$_3$ complex stability in serum after 24 h.



Supplemental Figure 12. Docking of EuK-(SO3)Cy5-mas3 in human glutamate carboxypeptidase II, or rather the prostate-specific membrane antigen (PSMA). PDB ID 2oot, docking with Autodock Vina, visualisation with UCSF Chimera. Binuclear zinc depicted as purple spheres.

Supplemental Table 3. *In vivo* biodistribution data at 2 h.

	^{99m}Tc-EuK-Cy5-mas$_3$	^{99m}Tc-EuK-(SO$_3$)Cy5-mas$_3$	^{99m}Tc-EuK-Cy5(SO$_3$)-mas$_3$	^{99m}Tc-EuK-(Ar)Cy5-mas$_3$	^{99m}Tc-EuK-Cy5(Ar)-mas$_3$
Blood	1.10 ± 0.15	0.24 ± 0.10	0.18 ± 0.07	2.32 ± 0.67	6.91 ± 1.08
Brain	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.03 ± 0.03
Lungs	1.77 ± 2.03	0.02 ± 0.01	0.02 ± 0.01	1.95 ± 1.35	0.82 ± 0.75
Heart	0.55 ± 0.08	0.17 ± 0.10	0.12 ± 0.06	1.07 ± 0.32	2.64 ± 0.80
Liver	1.82 ± 0.80	0.56 ± 0.45	0.45 ± 0.14	3.25 ± 2.18	9.64 ± 6.78
Kidneys	8.98 ± 5.78	18.35 ± 8.51	10.45 ± 12.83	15.64 ± 19.90	11.90 ± 12.26
Spleen	1.03 ± 0.72	0.97 ± 1.22	0.18 ± 0.19	0.39 ± 0.20	0.39 ± 0.20
Stomach	1.51 ± 1.19	1.74 ± 1.32	0.19 ± 0.11	0.82 ± 0.87	3.16 ± 2.11
Intestines	2.80 ± 2.35	1.61 ± 0.29	1.11 ± 0.68	6.62 ± 4.56	21.24 ± 13.40
Tumour	3.0 ± 2.4	15.27 ± 2.85	0.00 ± 0.00	3.47 ± 0.81	4.14 ± 3.33
Muscle	0.15 ± 0.16	0.17 ± 0.19	0.00 ± 0.00	0.37 ± 0.30	0.12 ± 0.16
Fat	0.05 ± 0.02	0.01 ± 0.00	0.00 ± 0.00	0.10 ± 0.08	0.20 ± 0.09
Salivary gland	0.08 ± 0.03	0.02 ± 0.01	0.02 ± 0.02	0.13 ± 0.07	0.23 ± 0.12
Prostate	0.71 ± 0.59	0.10 ± 0.06	0.03 ± 0.03	0.42 ± 0.40	1.37 ± 1.23
Tumour:blood	2.68 ± 2.16	70.15 ± 50.66	0.02 ± 0.02	0.65 ± 0.42	1.16 ± 0.01
Tumour:muscle	18.95 ± 14.66	5157.50 ± 949.17	0.46 ± 0.28	172.0 ± 84.95	5.79 ± 0.86
Tumour:prostate	4.22 ± 3.31	154.73 ± 28.48	0.02 + 0.00	10.36 ± 8.32	2.53 ± 0.59
Tumour:fat	60.07 ± 42.33	2578.75 + 474.59	0.65 ± 0.52	18.83 ± 1.30	46.50 ± 34.78
Tumour:kidney	2.75 ± 3.78	0.56 ± 0.14	0.00 ± 0.00	1.79 ± 2.40	1.61 ± 2.00

References

1. Pedersen DS, Rosenbohm C. Dry column vacuum chromatography. *Synthesis-Stuttgart.* 2001;2001:2431-2434.

2. Lopalco M, Koini EN, Cho JK, Bradley M. Catch and release microwave mediated synthesis of cyanine dyes. *Org Biomol Chem.* 2009;7:856-859.

3. Khan TH, Eno-Amooquaye EA, Searle F, Browne PJ, Osborn HM, Burke PJ. Novel inhibitors of carboxypeptidase G2 (CPG2): potential use in antibody-directed enzyme prodrug therapy. *J Med Chem.* 1999;42:951-956.

4. Makowski M, Rzeszotarska B, Smelka L, Kubica Z. Synthesis of Peptides with α,β-Dehydroamino Acids, III. Debenzyloxycarbonylation and Detrifluoroacetylation of Dehydroalanine and Dehydrophenylalanine Peptides. *Liebigs Annalen der Chemie.* 1985;1985:1457-1464.

5. Bunschoten A, van Willigen DM, Buckle T, et al. Tailoring Fluorescent Dyes To Optimize a Hybrid RGD-Tracer. *Bioconjug Chem.* 2016;27:1253-1258.

6. Mujumdar RB, Ernst LA, Mujumdar SR, Lewis CJ, Waggoner AS. Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. *Bioconjug Chem.* 1993;4:105-111.

7. Hensbergen AW, van Willigen DM, Welling MM, et al. Click Chemistry in the Design and Production of Hybrid Tracers. *ACS Omega.* 2019;4:12438-12448.

8. Spa SJ, Hensbergen AW, van der Wal S, Kuil J, van Leeuwen FWB. The influence of systematic structure alterations on the photophysical properties and conjugation characteristics of asymmetric cyanine 5 dyes. *Dyes and Pigments.* 2018;152:19-28.

9. van der Wal S, Kuil J, Valentijn ARPM, van Leeuwen FWB. Synthesis and systematic evaluation of symmetric sulfonated centrally C C bonded cyanine near-infrared dyes for protein labelling. *Dyes and Pigments.* 2016;132:7-19.

10. Weineisen M, Simecek J, Schottelius M, Schwaiger M, Wester HJ. Synthesis and preclinical evaluation of DOTAGA-conjugated PSMA ligands for functional imaging and endoradiotherapy of prostate cancer. *EJNMMI Res.* 2014;4:63.

11. Buckle T, van Willigen DM, Spa SJ, et al. Tracers for Fluorescence-Guided Surgery: How Elongation of the Polymethine Chain in Cyanine Dyes Alters the Pharmacokinetics of a Dual-Modality c[RGDyK] Tracer. *J Nucl Med.* 2018;59:986-992.

12. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem.* 2010;31:455-461.

13. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem.* 2004;25:1605-1612.

14. Marques RB, van Weerden WM, Erkens-Schulze S, et al. The human PC346 xenograft and cell line panel: a model system for prostate cancer progression. *Eur Urol.* 2006;49:245-257.

15. van Willigen DM, van den Berg NS, Buckle T, et al. Multispectral fluorescence guided surgery; a feasibility study in a phantom using a clinical-grade laparoscopic camera system. *Am J Nucl Med Mol Imaging.* 2017;7:138-147.

16. Sauer M, Hofkens J, Enderlein J. Basic Principles of Fluorescence Spectroscopy. In: Sauer M, Hofkens J, Enderlein J, eds. *Handbook of Fluorescence Spectroscopy and Imaging*. Weinheim, Germany: Wiley; 2011:1-30.

17. Banerjee SR, Pullambhatla M, Byun Y, et al. Sequential SPECT and optical imaging of experimental models of prostate cancer with a dual modality inhibitor of the prostate-specific membrane antigen. *Angew Chem Int Ed Engl.* 2011;50:9167-9170.

18. Baranski AC, Schafer M, Bauder-Wust U, et al. Improving the Imaging Contrast of (68)Ga-PSMA-11 by Targeted Linker Design: Charged Spacer Moieties Enhance the Pharmacokinetic Properties. *Bioconjug Chem.* 2017;28:2485-2492.

19. Kommidi H, Guo H, Nurili F, et al. (18)F-Positron Emitting/Trimethine Cyanine-Fluorescent Contrast for Image-Guided Prostate Cancer Management. *J Med Chem.* 2018;61:4256-4262.

20. Schottelius M, Wurzer A, Wissmiller K, et al. Synthesis and Preclinical Characterization of the PSMA-Targeted Hybrid Tracer PSMA-I&F for Nuclear and Fluorescence Imaging of Prostate Cancer. *J Nucl Med.* 2019;60:71-78.