SUPPLEMENTARY INFORMATION

Modular assembly of multimodal imaging agents through an inverse electron demand Diels-Alder reaction

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MATERIALS AND ANALYTICAL METHODS

All chemicals were purchased from Sigma-Aldrich. Acros Organics and Alfa Aesar and used without further purification. (*R*)-NODAGA-NH_2, (*R*)-NODAGA(*t*Bu)$_3$, DOTAGA-NH_2, and DOTAGA(*t*Bu)$_4$ were provided by CheMatech (Dijon, France). The non-sulfonated and sulfonated Cyanine 5.0-COOH dyes, and the BODIPY-COOH dye were kindly given by Pr. A. Romieu and Dr. C. Goze respectively (ICMUB, Dijon, France). Trastuzumab (Herceptin, Roche, USA), and human serum (healthy male) were obtained through the Georges-Francois LECLERC Cancer Center - UNICANCER (Dijon, France).

HPLC-gradient grade MeCN was obtained from Biosolve or Carlo Erba. LC-HRMS grade MeCN was obtained from Fisher Scientific. All aqueous buffers used in this work and aqueous mobile-phases for HPLC and ionic chromatography were prepared using water purified with a PURELAB Ultra system from ELGA (purified to 18.2 MΩ cm).

Moisture sensitive reactions were performed under nitrogen or argon atmosphere.

^{1}H and ^{13}C NMR spectra were recorded on NMR 300 MHz Bruker Avance III NanoBay or NMR 500 MHz Bruker Avance III spectrometers under routine conditions at 298 K and were referenced to TMS or residual solvent signals. The following abbreviations and their combinations are used: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; bs, broad singlet; bt, broad triplet; bq, broad quadruplet. The ^{1}H and ^{13}C resonance signals were attributed by means of 2D COSY and HMQC experiments.

When possible, the number of TFA counterions was quantified by ionic chromatography. Otherwise, the number of TFA counterions was estimated, by assuming that all basic functions on the molecules formed TFA salts.
Ionic chromatography analyses were performed by Dr. Myriam Laly (PACSMUB, Dijon, France) on a Dionex ICS-5000 DC system equipped with a Dionex AS11-HC column and conductivity detector (Thermo Scientific). The separation was achieved with the following eluents: A: NaOH, 30 mM and B: H_2O by isocratic mode A/B (90 : 10, *v/v*). Analyses were performed in duplicates.

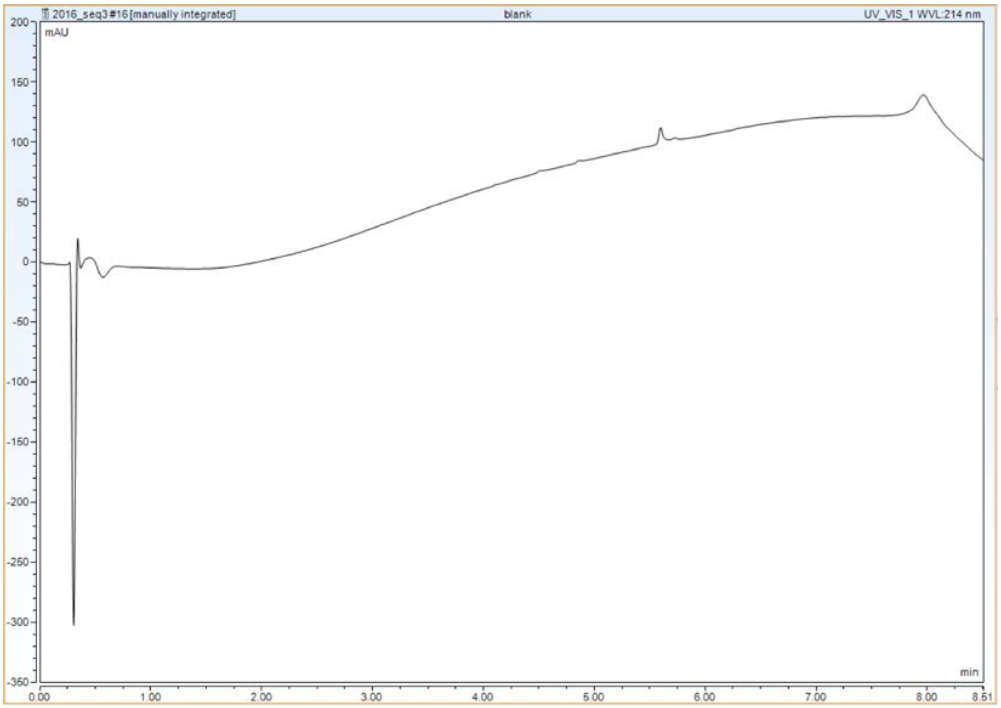
HRMS spectra of organic compounds were recorded on a mass spectrometer LTQ Orbitrap XL (Thermo Scientific) using an ESI source. If not specified, the spectrum was recorded in positive mode.

UV-Vis absorbance/fluorescence spectra were recorded on a CLARIOstar microplate reader (BMG LABTECH) with an Lvis plate for absorbance spectra measurements and black 96-wells microplates for excitation and emission spectra measurements.

Organic compounds were characterized by RP-HPLC-MS analyses performed on an UltiMate 3000 system Dionex (Thermo Scientific) equipped with a DAD detector and coupled to a low-resolution mass spectrometry detector MSQ Plus (Thermo Scientific), equipped with an ESI source. Separation was achieved using an RP Kinetex™ column (Phenomenex) (2.6 µm, 100 Å, 50 × 2.1 mm) with ultrapure water and HPLC-grade MeCN: A: H_2O 0.1% formic acid and B: MeCN 0.1% formic acid. Analyses were performed with the following gradient program: 5% to 100% of B in 5 min, 100% B for 1.5 min, 100% to 5% B in 0.1 min and 5% B for 1.9 min, at a flow rate of 0.5 mL/min.

The purity of title compounds was determined from the integration of HPLC-MS chromatograms at 214 nm. An example of blank chromatogram is shown below (Figure S1). Yields were calculated taking into account the purity determined from RP-HPLC-MS (yield = experimental mass × HPLC $purity_{214\ nm}$ / theoretical mass).

Figure S1. Illustrative RP-HPLC-MS chromatogram of a H_2O injection (blank).



Purifications by semi-preparative RP-HPLC were performed on an UltiMate 3000 system Dionex (Thermo Scientific) equipped with an UV-visible detector, on the following columns: BetaBasic-18 column (Thermo Scientific) (5 µm, 150 Å, 150 × 30 mm) at 15 mL/min or SiliaChrom® dt C18 column (Silicycle) (10 µm, 100 Å, 250 × 20 mm) at 20 mL/min, with HPLC grade eluents. The fractions of interest were analyzed by RP-HPLC-MS, pooled, concentrated under reduced pressure to remove organic solvents and freeze-dried.

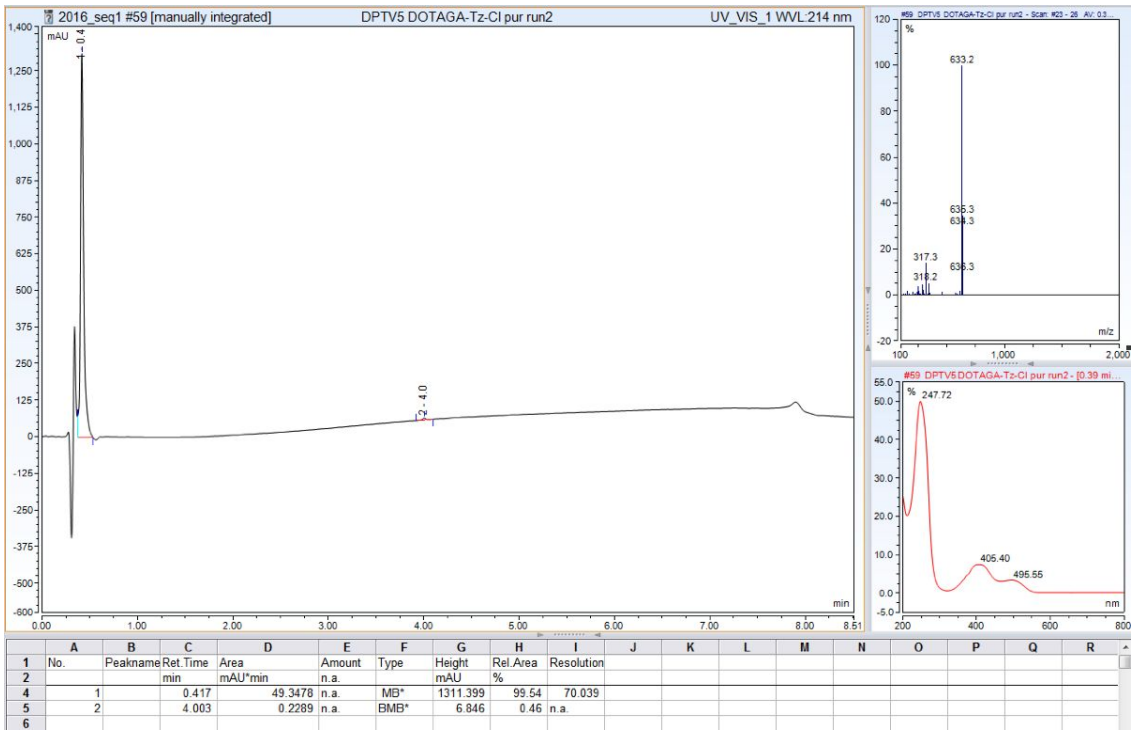
Thin layer chromatographies (TLC) were performed on silica plates $60F_{254}$ and revealed under UV light (254/365 nm).

Purifications by flash chromatography were performed on a Puriflash 430 system (Interchim) with prepacked silica columns (25 µm particles, Interchim) or on a Reveleris® X1 IES system (Grace) with prepacked GraceResolv Silica columns (20 µm particles, Grace). The fractions of interest were analyzed by RP-HPLC-MS or TLC, pooled and concentrated under reduced pressure to remove solvents.

SYNTHESIS OF COMPOUNDS 1 TO 8

2,2',2''-(10-(1-carboxy-4-((2-((6-chloro-1,2,4,5-tetrazin-3-yl)amino)ethyl)amino)-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid, DOTAGA-NH-Tz-Cl **1**.

Figure S2. RP-HPLC-MS analysis of DOTAGA-NH-Tz-Cl 1.



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1	No.	Peakname	Ret.Time	Area	Amount	Type	Height	Rel.Area	Resolution
2			min	mAU*min	n.a.		mAU	%	
4	1		0.417	49.3478	n.a.	MB*	1311.399	99.54	70.039
5	2		4.003	0.2289	n.a.	BMB*	6.846	0.46	n.a.

Figure S3. ^{1}H NMR spectrum of DOTAGA-NH-Tz-Cl 1.

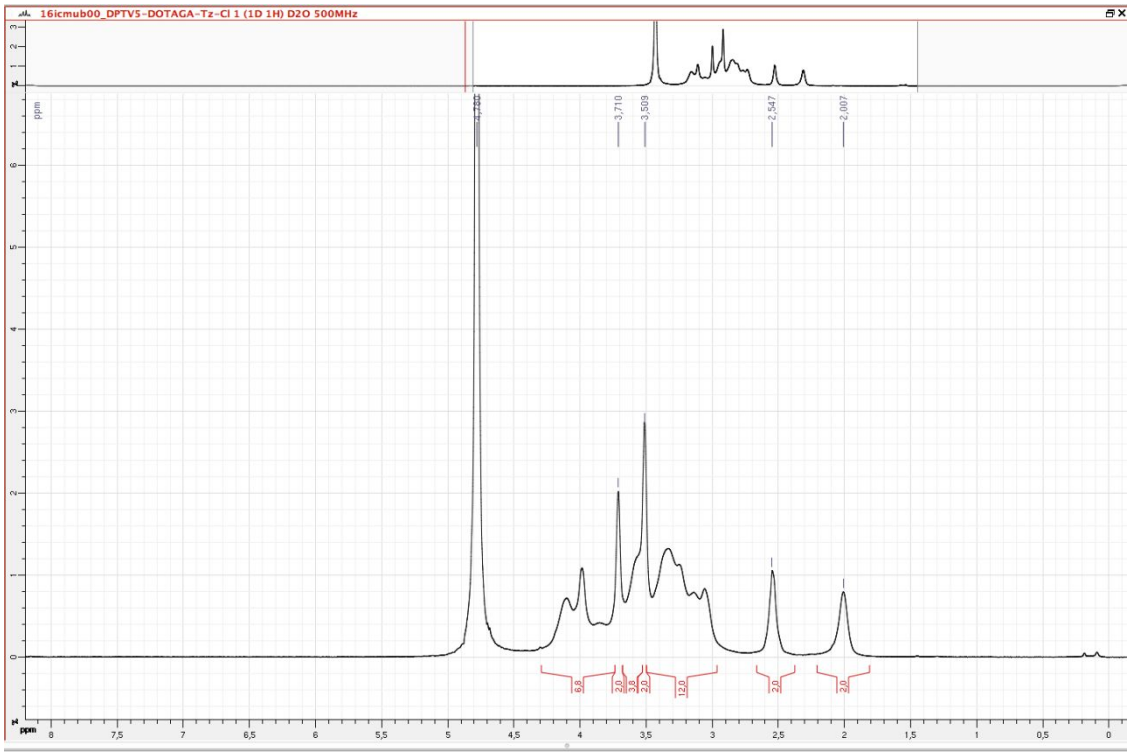
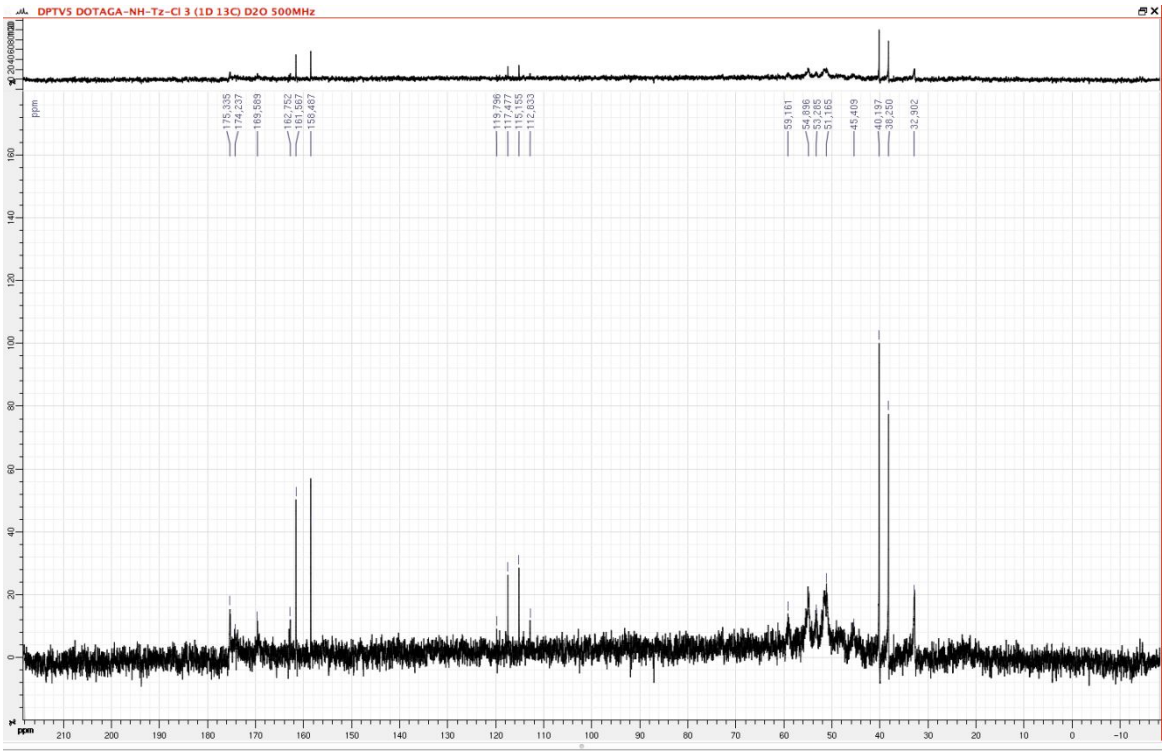


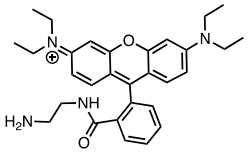
Figure S4. ^{13}C NMR spectrum of DOTAGA-NH-Tz-Cl 1.



(R)-2,2'-(7-(1-carboxy-4-((2-((6-chloro-1,2,4,5-tetrazin-3-yl)amino)ethyl)amino)-4-oxobutyl)-1,4,7-triazonane-1,4-diyl)diacetic acid, (R)-NODAGA-NH-Tz-Cl **2.**

The synthesis and analyses of (R)-NODAGA-NH-Tz-Cl **2** were reported earlier.[1]

***N*-(9-(2-((2-aminoethyl)carbamoyl)phenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium**, Rhodamine B-NH_2 **3**.



The chloride salt of *N*-(9-(2-carboxyphenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium (Rhodamine B·1Cl) (300 mg, 626 µmol), TSTU (225 mg, 748 µmol, 1.2 eq) and DIPEA (130 µL, 748 µmol, 1.2 eq) were dissolved in anhydrous DMF (2 mL). The solution was stirred 20 min at room temperature and ethylene diamine (416 µL, 6.24 mmol, 10 eq) and DIPEA (702 µL, 4.05 mmol, 6.5 eq) were added. After 1 h at room temperature, the reaction was placed at 4 °C. After 12h, the reaction was completed and solvents were eliminated under reduced pressure. The purification of the crude product by flash chromatography (eluents: H_2O 0.1% TFA, MeCN 0.1% TFA) yielded Rhodamine B-NH_2·1.9TFA as a purple powder (314 mg, 67%, purity: 94%).

Ionic chromatography analysis indicated an amount of 1.9 TFA per molecule.

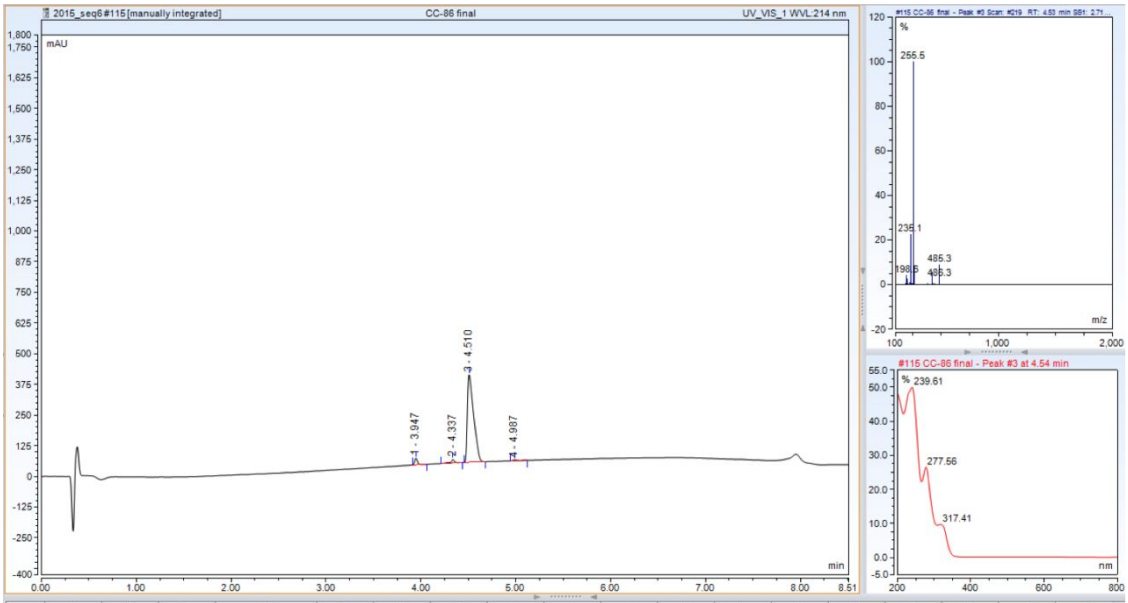
^1H NMR (300 MHz, CD_3OD) equilibrium between two forms, non-cyclic amide:lactam (1:0.1), non-cyclic amide form: ∂ 1.17 (t, ^3J = 7.1 Hz, 12H; CH_3), 2.65 (t, ^3J = 6.8 Hz, 2H; CH_2), 3.42 (t, ^3J = 6.8 Hz,

2H; CH_2), 3.47 (q, ^3J = 7.1 Hz, 8H; CH_2), 6.62 (m, 4H; CH), 6.76 (m, 2H; CH), 7.11 (m, 1H; CH), 7.60 (m, 2H; CH), 7.94 (m, 1H; CH), lactam form: ∂ 1.31 (t, ^3J = 7.1 Hz, 12H; CH_3), 2.94 (m, 2H; CH_2), 3.36 (m, 2H; CH_2), 3.68 (q, ^3J = 7.1 Hz, 8H; CH_2), 6.94 to 7.06 (m, 4H; CH), 7.21 (m, 2H; CH), 7.44 (m, 1H; CH), 7.79 (m, 2H; CH), 8.03 (m, 1H; CH).

^{13}C-NMR (125.7 MHz, CD_3OD) equilibrium between two forms non-cyclic amide:lactam (1:0.1), non-cyclic amide form: ∂ 12.5, 39.2, 40.3, 47.6, 102.2, 112.0, 115.4, 124.2, 125.3, 129.4, 130.1, 130.2, 131.3, 148.6, 151.1, 154.7, 154.9, 171.3, lactam form signals were poorly resolved and were not attributed;

MS: $C_{30}H_{38}N_4O_2^+$ ([M+H]$^+$) m/z calculated: 486.3, found: 486.3.

Figure S5. RP-HPLC-MS analysis of Rhodamine B-NH_2 3.



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2			min	mAU*min	n.a.		mAU	%	
4	1		3.947	0.9315	n.a.	BMB*	28.182	3.24	7.325
5	2		4.337	0.6951	n.a.	BMB*	14.605	2.42	1.964
6	3		4.510	26.9817	n.a.	BMB*	355.956	93.79	4.884
7	4		4.987	0.1591	n.a.	BMB*	2.738	0.55	n.a.

Figure S6. ^{1}H NMR spectrum of Rhodamine B-NH_2 3.

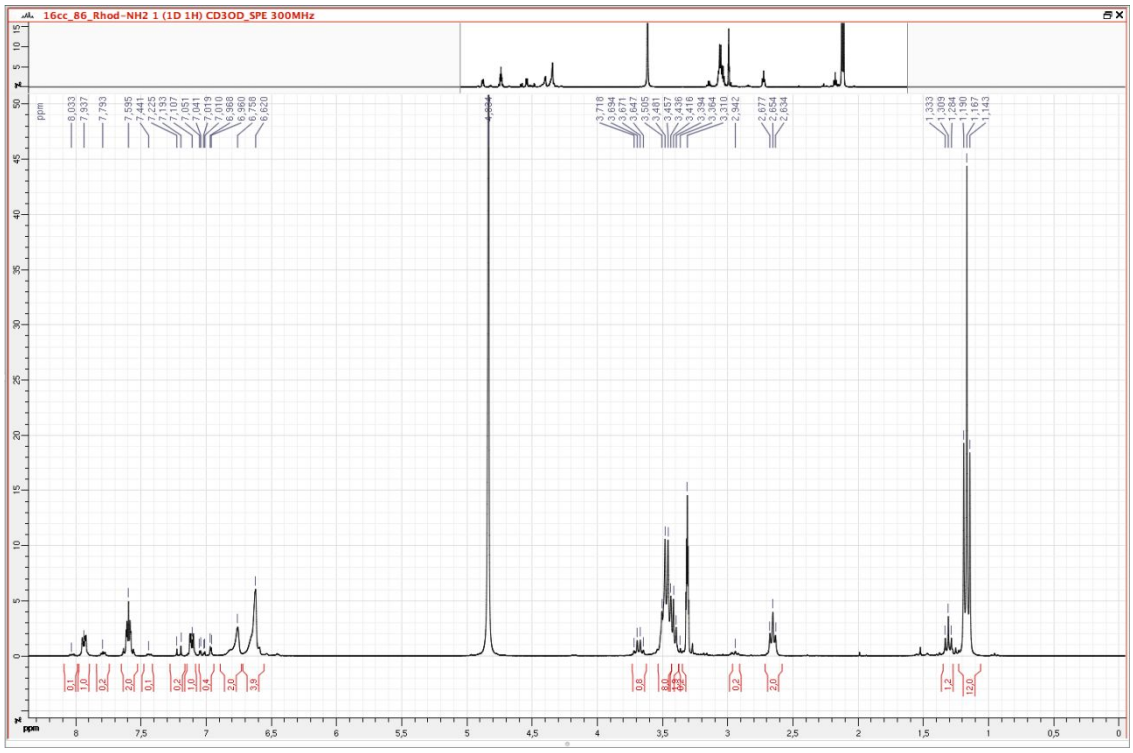
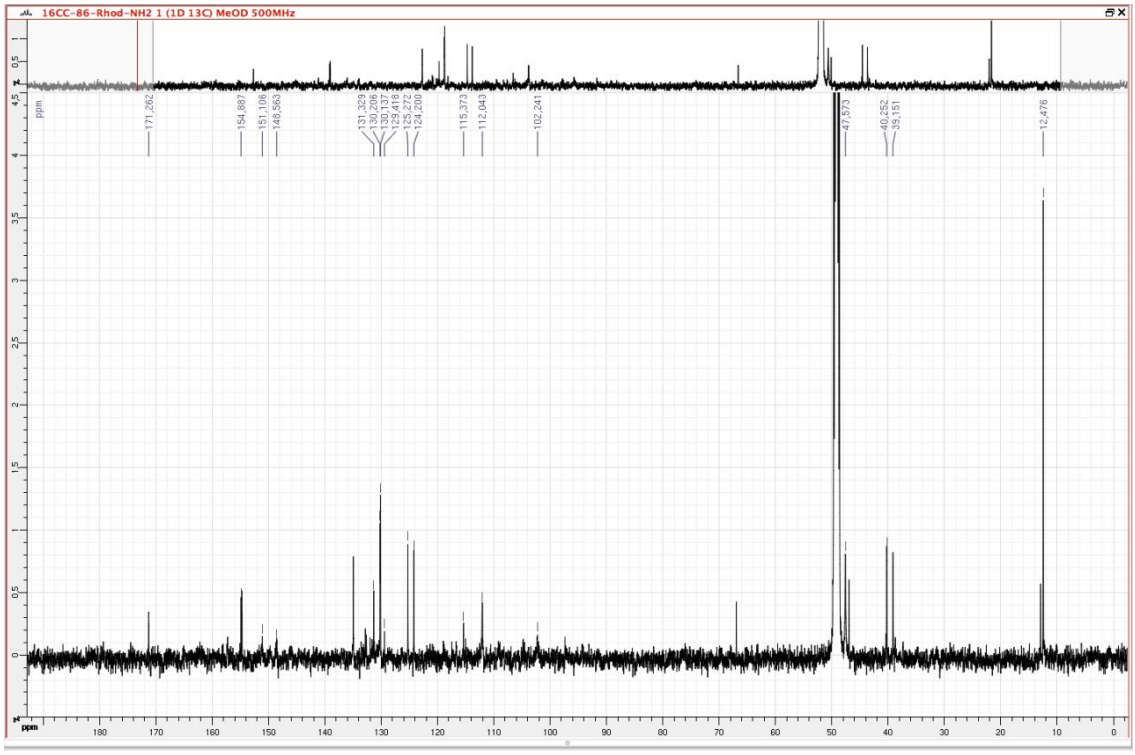
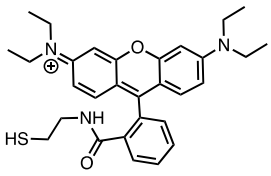


Figure S7. ^{13}C NMR spectrum of Rhodamine B-NH_2 3.



***N*-(6-(diethylamino)-9-(2-((2-mercaptoethyl)carbamoyl)phenyl)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium**, Rhodamine B-SH **4**.

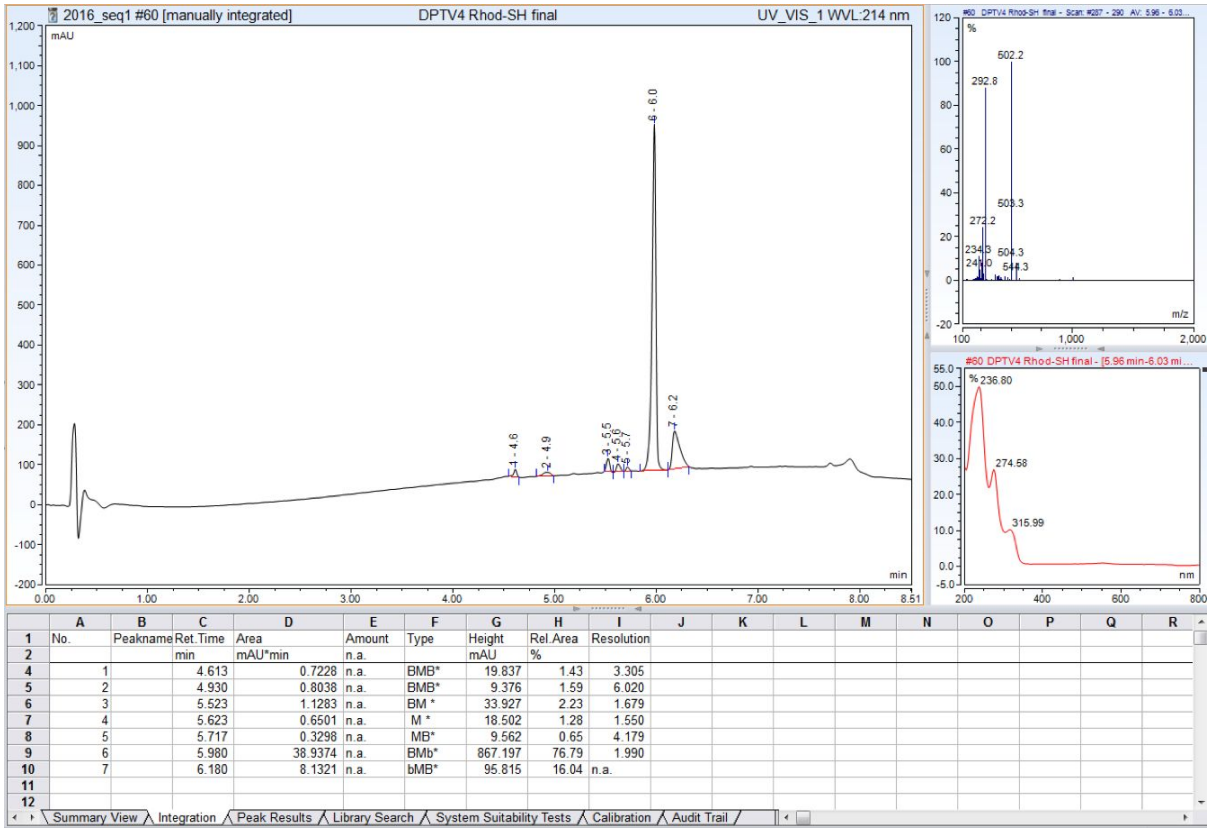


Rhodamine B·1Cl (200 mg, 418 µmol), HATU (191 mg, 502 µmol, 1.2 eq) and DIPEA (87 µL, 502 µmol, 1.2 eq) were dissolved in anhydrous DMF (2 mL). The solution was stirred 2 h at room temperature and cysteamine hydrochloride (95 mg, 836 µmol, 2.0 eq) and DIPEA (139 µL, 836 µmol, 2.0 eq) were added. After 2 h at room temperature, the DMF was eliminated under reduced pressure. Purification by flash chromatography on silica (eluents: DCM/MeOH) afforded Rhodamine B-SH·1Cl as a purple powder (179.3 mg, 61%, purity: 77%).

^{1}H NMR (300 MHz, CD_3OD) equilibrium between two forms, lactam/non-cyclic amide (1:0.4), non-cyclic amide form: ∂ 1.17 (t, ^3J = 7.1 Hz, 12H; CH_3), 2.19 (t, ^3J = 7.7 Hz, 2H; CH_2), 3.28 (t, ^3J = 7.7 Hz, 2H; CH_2), 3.54 (q, ^3J = 7.1 Hz, 8H; CH_2), 6.63 to 6.73 (m, 2H; CH), 6.74 to 6.84 (m, 2H; CH), 6.98 (m, 2H; CH), 7.11 (m, 1H; CH), 7.60 (m, 2H; CH), 7.91 (m, 1H; CH), lactam form: ∂ 1.31 (t, ^3J = 7.1 Hz, 12H; CH_3), 2.35 (t, ^3J = 6.5 Hz, 2H; CH_2), 3.23 (t, ^3J = 7.1 Hz, 2H; CH_2), 3.68 (q, ^3J = 7.1 Hz, 8H; CH_2), 6.92 to 6.96 (m, 2H; CH), 7.02 to 7.06 (m, 2H; CH), 7.23 (s, 1H; CH), 7.26 (s, 1H; CH), 7.46 (m, 1H; CH), 7.77 (m, 2H; CH), 7.94 (m, 1H; CH); some non-attributed impurities were detected at 7.52, 8.43 and 8.73.

MS: $C_{30}H_{36}N_3O_2S^+$ ($[M]^+$) m/z calculated: 502.3, found: 502.2.

Figure S8. RP-HPLC-MS analysis of Rhodamine B-SH 4.



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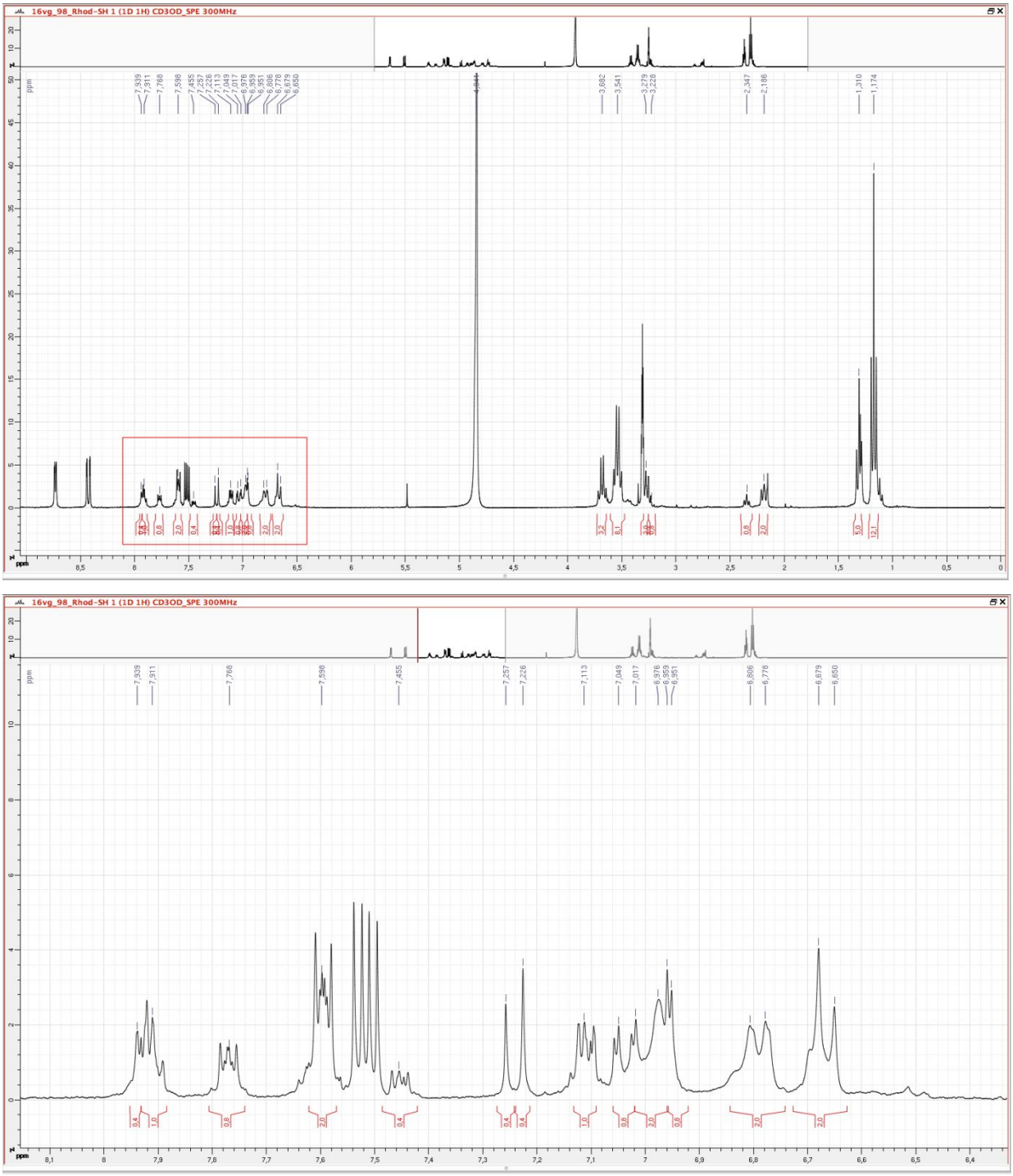
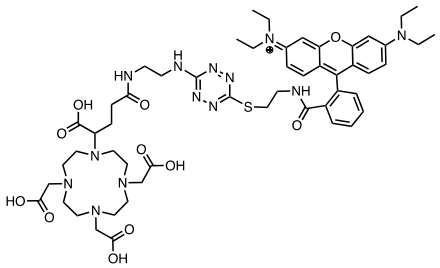
2			min	mAU*min	n.a.		mAU	%										
4	1		4.613	0.7228	n.a.	BMB*	19.837	1.43	3.305									
5	2		4.930	0.8038	n.a.	BMB*	9.376	1.59	6.020									
6	3		5.523	1.1283	n.a.	BM *	33.927	2.23	1.679									
7	4		5.623	0.6501	n.a.	M *	18.502	1.28	1.550									
8	5		5.717	0.3298	n.a.	MB*	9.562	0.65	4.179									
9	6		5.980	38.9374	n.a.	BMb*	867.197	76.79	1.990									
10	7		6.180	8.1321	n.a.	bMB*	95.815	16.04	n.a.									
11																		
12																		

Summary View | Integration | Peak Results | Library Search | System Suitability Tests | Calibration | Audit Trail

Figure S9. ^{1}H NMR spectrum of Rhodamine B-SH 4. Magnification of the region between 6.3 and 8.1 ppm.



***N*-(9-(2-((2-((6-((2-(4-carboxy-4-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)butanamido)ethyl)amino)-1,2,4,5-tetrazin-3-yl)thio)ethyl)carbamoyl)phenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium**, DOTAGA-NH-Tz-S-Rhodamine B **5**.



DOTAGA-NH-Tz-Cl·1.5TFA **1** (50.0 mg, 45.9 µmol) and rhodamine B-SH·Cl **4** (39.7 mg, 73.9 µmol, 1.6 eq) were dissolved in anhydrous DMF and DIPEA (65.7 µL, 395 µmol, 8.5 eq) was added. After 3 h at 75 °C, the solvent was removed under reduced pressure. Purification by semi-preparative RP-HPLC (eluents: H_2O 0.1% TFA, MeCN 0.1% TFA) afforded DOTAGA-NH-Tz-S-Rhodamine B·2.9TFA as a red oil (37.0 mg, 51%, purity: 91%).

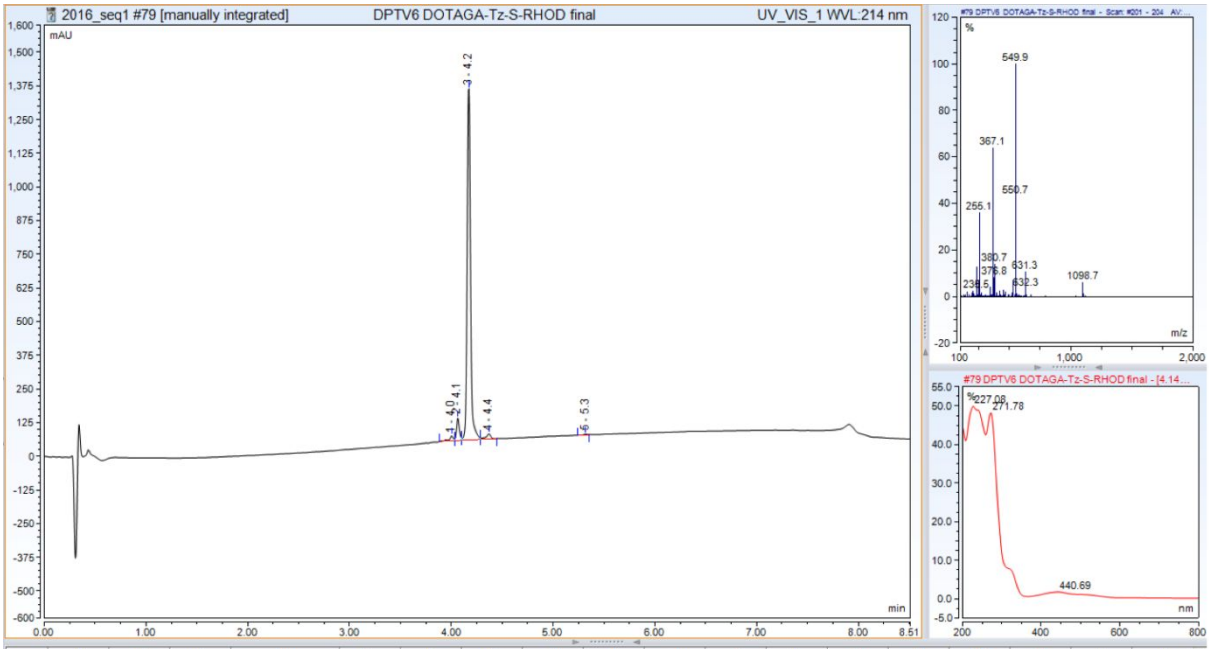
Ionic chromatography analysis indicated an amount of 2.9 TFA per molecule.

^{1}H NMR (500 MHz, D_2O) ∂ 1.13 (m, 12H; CH_3), 2.01 (m, 2H; CH_2), 2.56 (m, 2H; CH_2), 2.96 (m, 2H; CH_2), 3.00 to 3.48 (m, 12H; CH_2), 3.48 to 3.63 (m, 8H; CH_2), 3.69 (m, 10H; CH_2), 3.74 to 4.37 (m, 6H; CH_2), 3.99 (m, 1H; CH), 7.11 (m, 2H; CH), 7.24 (m, 3H; CH), 7.63 (m, 2H; CH), 7.72 (m, 2H; CH), 8.02 (m, 1H; CH);

^{13}C NMR (125.7 MHz, D_2O) ∂ 9.7, 28.3, 39.7, 40.1, 53.8, 64.4, 112.1, 116.4 (q, ^1J = 290.1 Hz, TFA), 118.2, 120.3, 123.4, 124.0, 129.2, 130.1, 131.2, 134.6, 138.5, 151.9, 152.1, 160.9, 162.9 (q, ^2J = 35.2 Hz, TFA), 164.4, 170.7, macrocycle CH_2 signals were not distinguishable;

HRMS: $C_{53}H_{72}N_{13}O_{11}S^+$ ($[M]^+$) m/z calculated: 1098.51895, found: 1098.52126; $C_{53}H_{71}N_{13}NaO_{11}S^+$ ($[M+Na]^+$) m/z calculated: 1120.50089, found: 1120.50291; $C_{53}H_{70}KN_{13}NaO_{11}S^+$ ($[M-H+Na+K]^+$) m/z calculated: 1158.45677, found: 1158.45431.

Figure S10. RP-HPLC-MS analysis of DOTAGA-NH-Tz-S-Rhodamine B 5.



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1	No.	Peakname	Ret.Time	Area	Amount	Type	Height	Rel.Area	Resolution									
2			min	mAU*min	n.a.		mAU	%										
4	1		4.003	0.8879	n.a.	BM *	18.726	1.65	1.133									
5	2		4.063	2.8010	n.a.	M *	83.913	5.21	2.073									
6	3		4.173	48.9241	n.a.	M *	1303.858	91.03	3.387									
7	4		4.370	1.0488	n.a.	MB*	19.475	1.95	17.405									
8	5		5.320	0.0813	n.a.	BMB*	2.909	0.15	n.a.									
9																		

Figure S11. ^{1}H NMR spectrum of DOTAGA-NH-Tz-S-Rhodamine B 5.

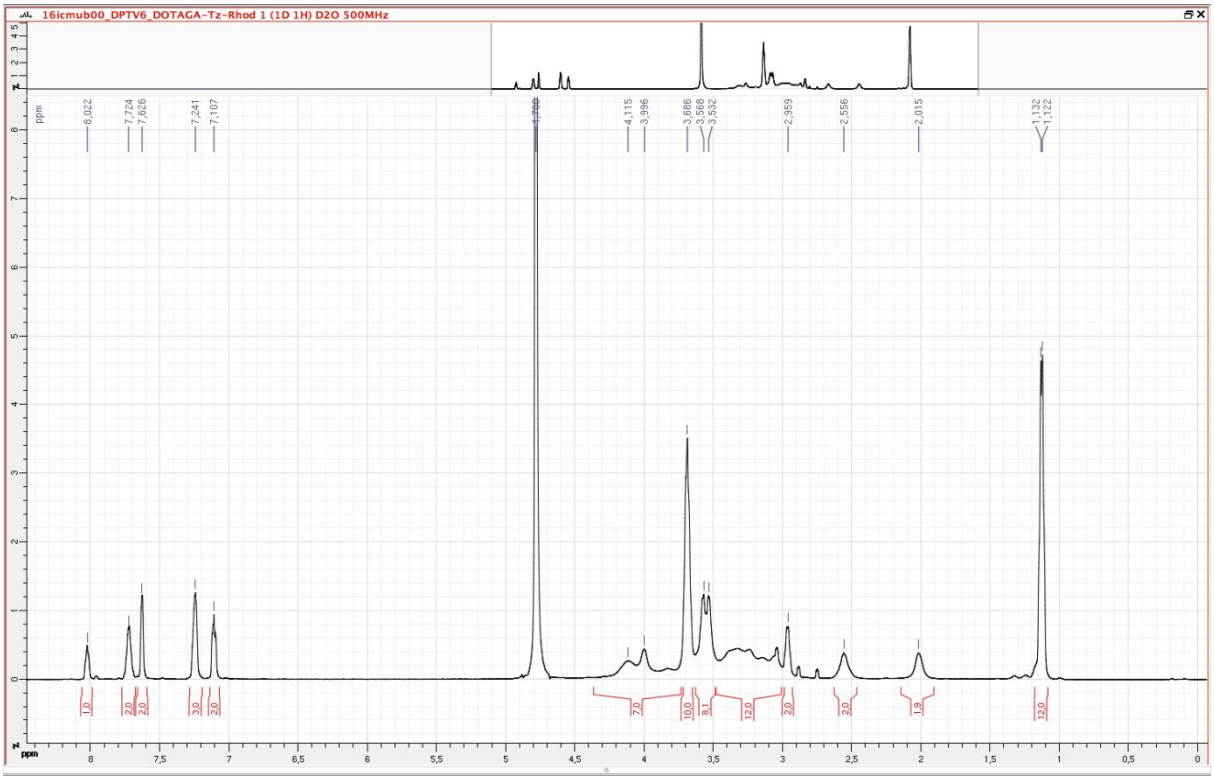


Figure S12. ^{13}C NMR spectrum of DOTAGA-NH-Tz-S-Rhodamine B 5.

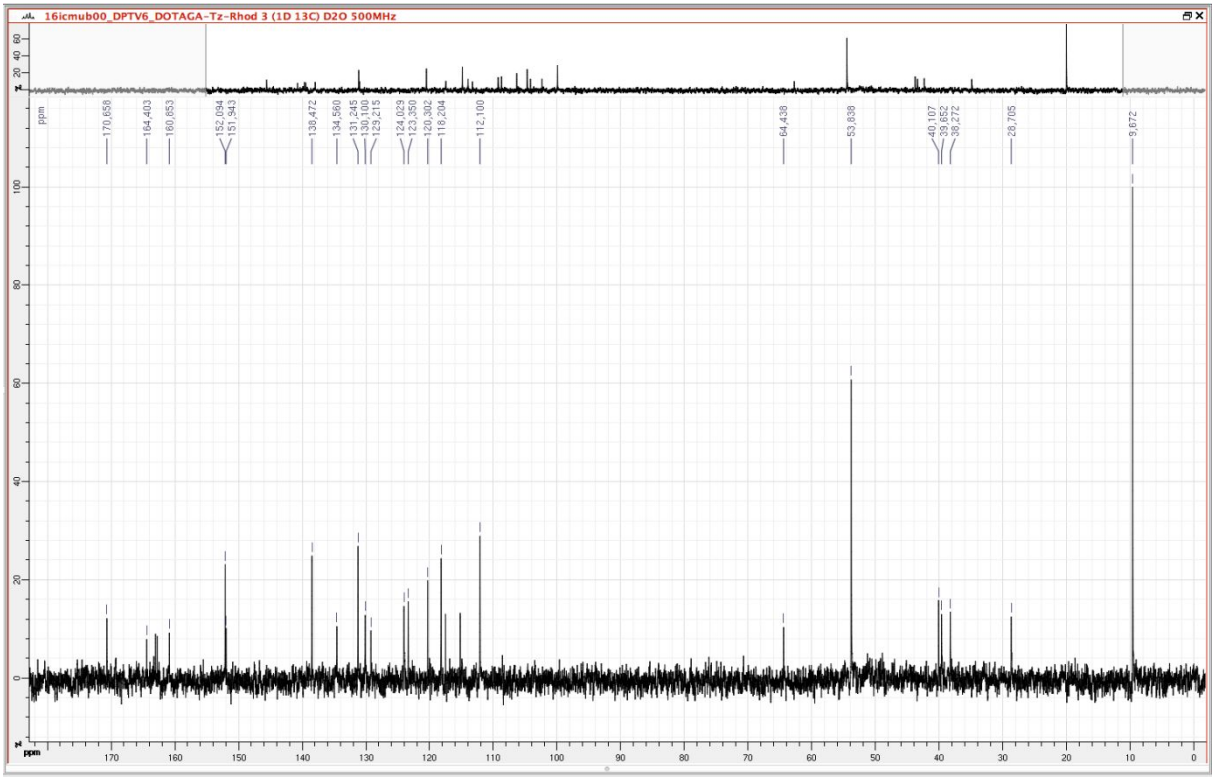
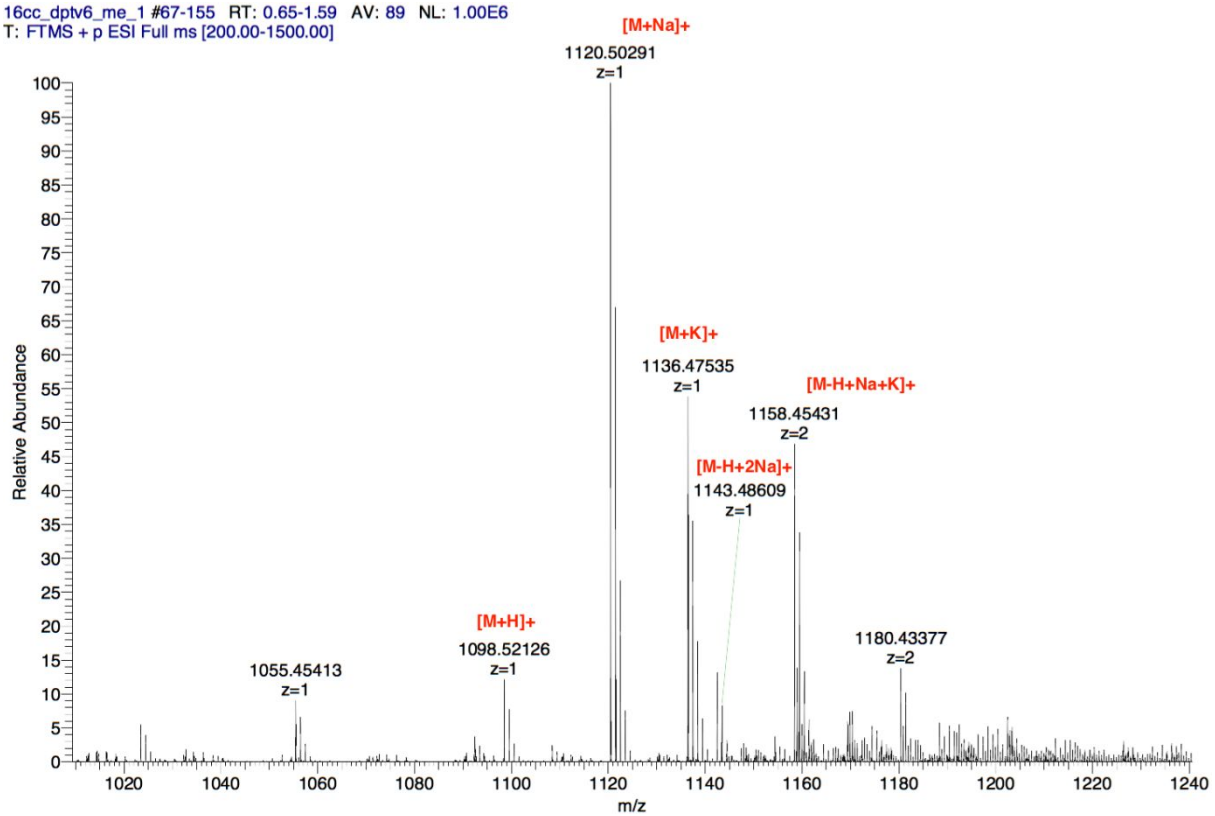
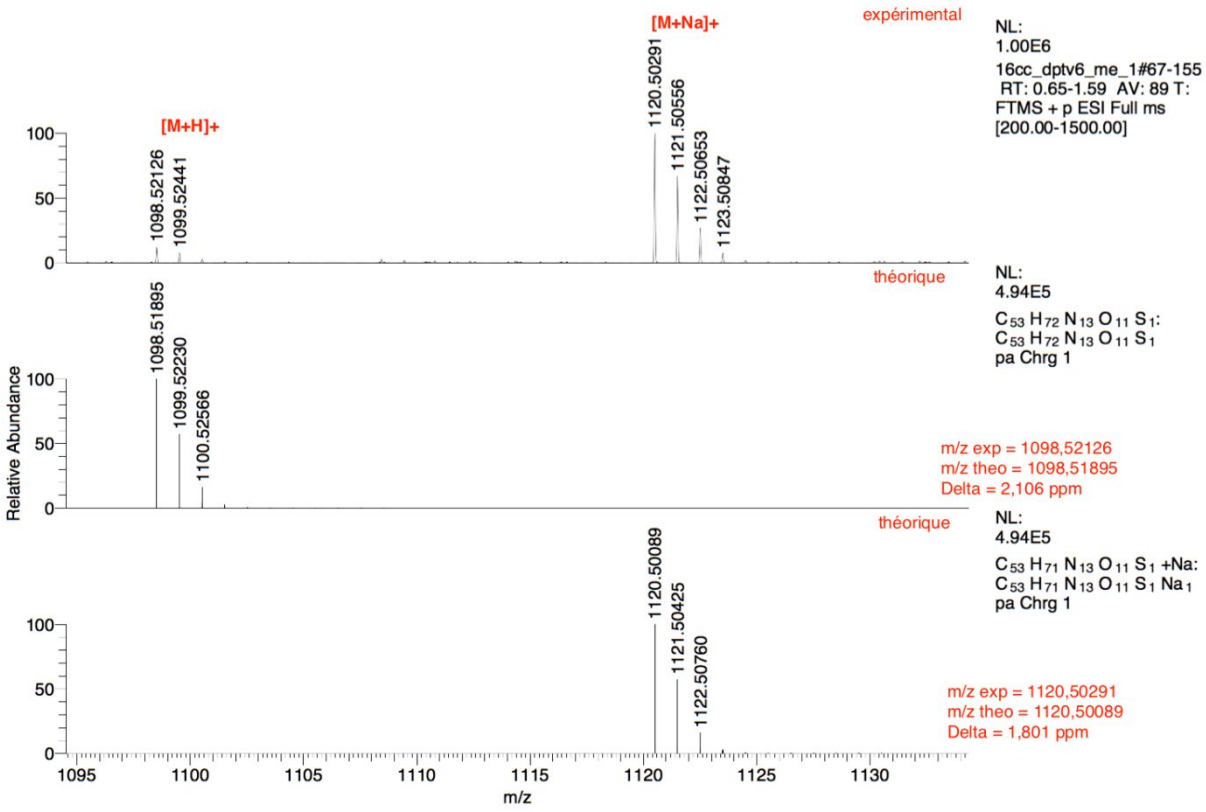


Figure S13. HRMS spectrum of DOTAGA-NH-Tz-S-Rhodamine B 5. Comparison with the simulated spectrum.




[M+Na]+
expérimental
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RT: 0.65-1.59 AV: 89 T:
FTMS + p ESI Full ms
[200.00-1500.00]
[M+H]+
1120.50291
1121.50556
1122.50653
1123.50847
1098.52126
1099.52441
théorique
NL:
4.94E5
C53 H72 N13 O11 S1:
C53 H72 N13 O11 S1
pa Chrg 1
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1099.52230
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Relative Abundance
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m/z theo = 1098,51895
Delta = 2,106 ppm
théorique
NL:
4.94E5
C53 H71 N13 O11 S1 +Na:
C53 H71 N13 O11 S1 Na1
pa Chrg 1
1120.50089
1121.50425
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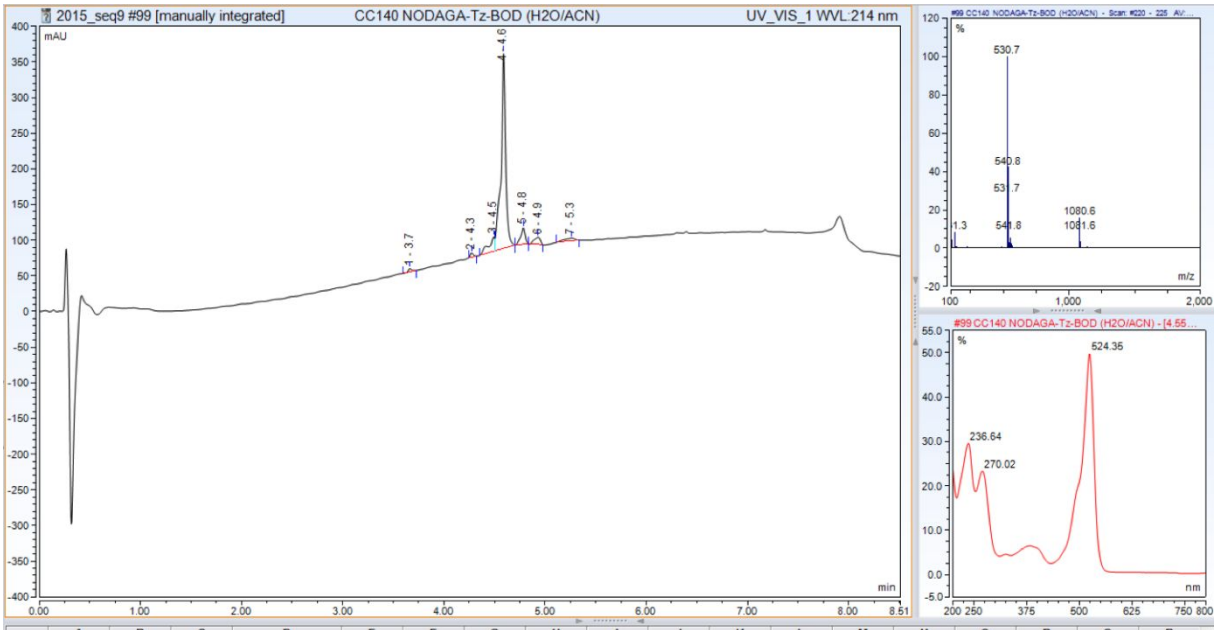
m/z theo = 1120,50089
Delta = 1,801 ppm
m/z

2,2',2''-(10-(1-carboxy-4-((2-((6-((2-(4-(2,8-diethyl-5,5-difluoro-1,3,7,9-tetramethyl-5*H*-4λ^4,5λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-10-yl)benzamido)ethyl)thio)-1,2,4,5-tetrazin-3-yl)amino)ethyl)amino)-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid, DOTAGA-NH-Tz-S-BODIPY **6**.

BODIPY-SH [1] (11.7 mg, 24.2 µmol, 1.7 eq) and DOTAGA-NH-Tz-Cl·1.5TFA **1** (15.3 mg, 14.0 µmol, 1.0 eq) were dissolved in anhydrous DMF (1 mL), and DIPEA (22.8 µL, 121.0 µmol, 8.6 eq) was added. The reaction was completed in 1.5 h at 75 °C and the solvent was eliminated under reduced pressure. Purification by semi-preparative RP-HPLC on reverse-phase (eluents: H_2O 0.1% FA, MeCN 0.1% FA) yielded DOTAGA-NH-Tz-S-BODIPY·0.1TFA as a pink powder (18.0 mg, 68%, purity: 78%).
Ionic chromatography analysis indicated an amount of 0.1 TFA per molecule.
^{1}H NMR (500 MHz, MeOD) ∂ 0.99 (bt, 6H; CH_3), 1.32 (s, 8H; CH_2, CH_3), 2.01 (m, 2H; CH_2), 2.35 (bq, 4H; CH_2), 2.48 (s, 6H; CH_3), 3.03 to 3.25 (m, 7H; CH, CH_2), 3.34 to 4.16 (m, 24H; CH_2), 7.46 (m, 2H; CH), 8.01 (m, 2H; CH).
^{13}C-NMR (125.7 MHz, DMSO) ∂ 11.5, 12.3, 14.5, 16.4, 29.7, 32.4, 37.6, 49.8, 50.7, 52.8, 54.3, 55.2, 61.9, 128.1, 128.3, 129.7, 132.8, 134.6, 137.6, 138.0, 139.6, 153.5, 160.9, 164.1, 165.7, 170.4, 172.3, 172.8.
HRMS: $C_{49}H_{69}BF_2N_{13}O_{10}S^+$ ($[M+H]^+$) m/z calculated: 1080.50667, found: 1080.50639; $C_{49}H_{68}BF_2N_{13}NaO_{10}S^+$ ($[M+Na]^+$) m/z calculated: 1102.48861, found: 1102.48834; $C_{49}H_{67}BF_2N_{13}Na_2O_{10}S^+$ ($[M-H+2Na]^+$) m/z calculated: 1124.47056, found: 1124.46975.

Figure S14. RP-HPLC-MS analysis of DOTAGA-NH-Tz-S-BODIPY 6.



	A	B	C	D	E	F	G	H	I
1	No.	Peakname	Ret.Time	Area	Amount	Type	Height	Rel.Area	Resolution
2			min	mAU*min	n.a.		mAU	%	
4	1		3.663	0.1537	n.a.	BMB*	4.441	0.81	12.064
5	2		4.273	0.1960	n.a.	BMB*	6.161	1.04	n.a.
6	3		4.490	1.4969	n.a.	BM *	20.312	7.94	n.a.
7	4		4.590	14.6502	n.a.	MB*	273.269	77.66	2.856
8	5		4.783	1.1834	n.a.	BMb*	23.699	6.27	1.487
9	6		4.933	0.7011	n.a.	bMB*	10.026	3.72	1.830
10	7		5.263	0.4824	n.a.	BMB*	3.840	2.56	n.a.

Figure S15. ^{1}H NMR spectrum of DOTAGA-NH-Tz-S-BODIPY 6.

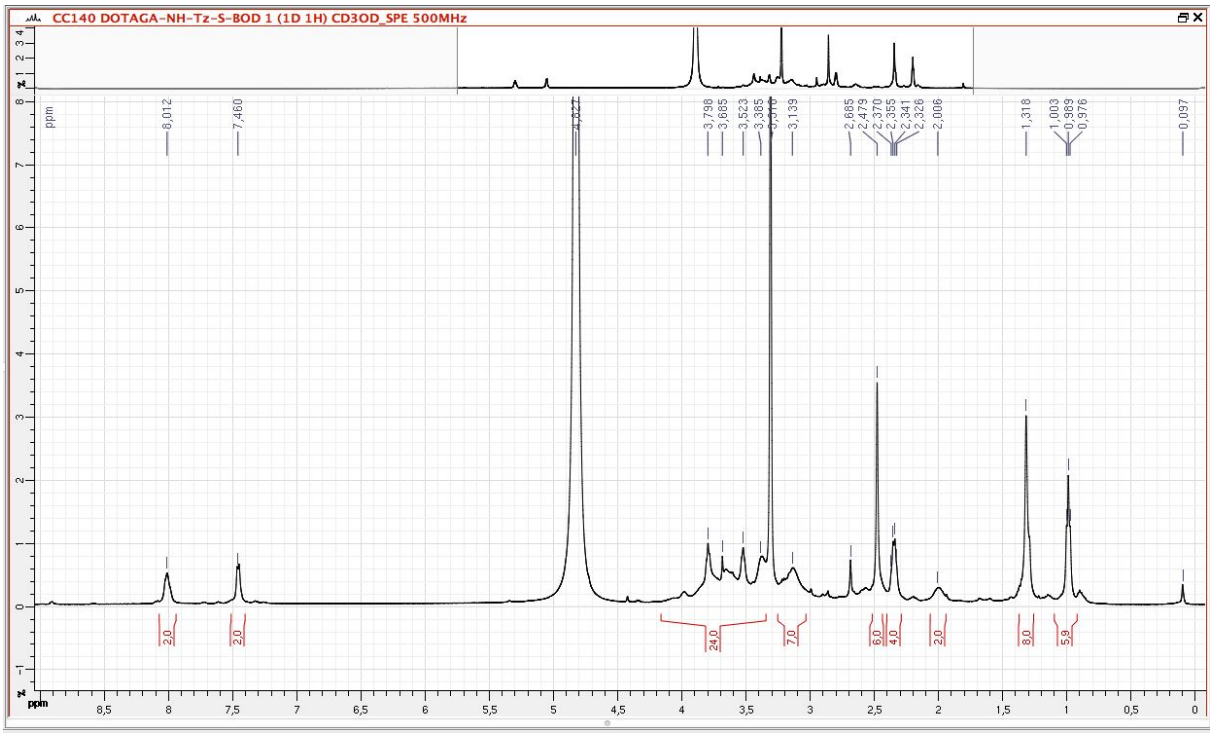


Figure S16. ^{13}C NMR spectrum of DOTAGA-NH-Tz-S-BODIPY 6.

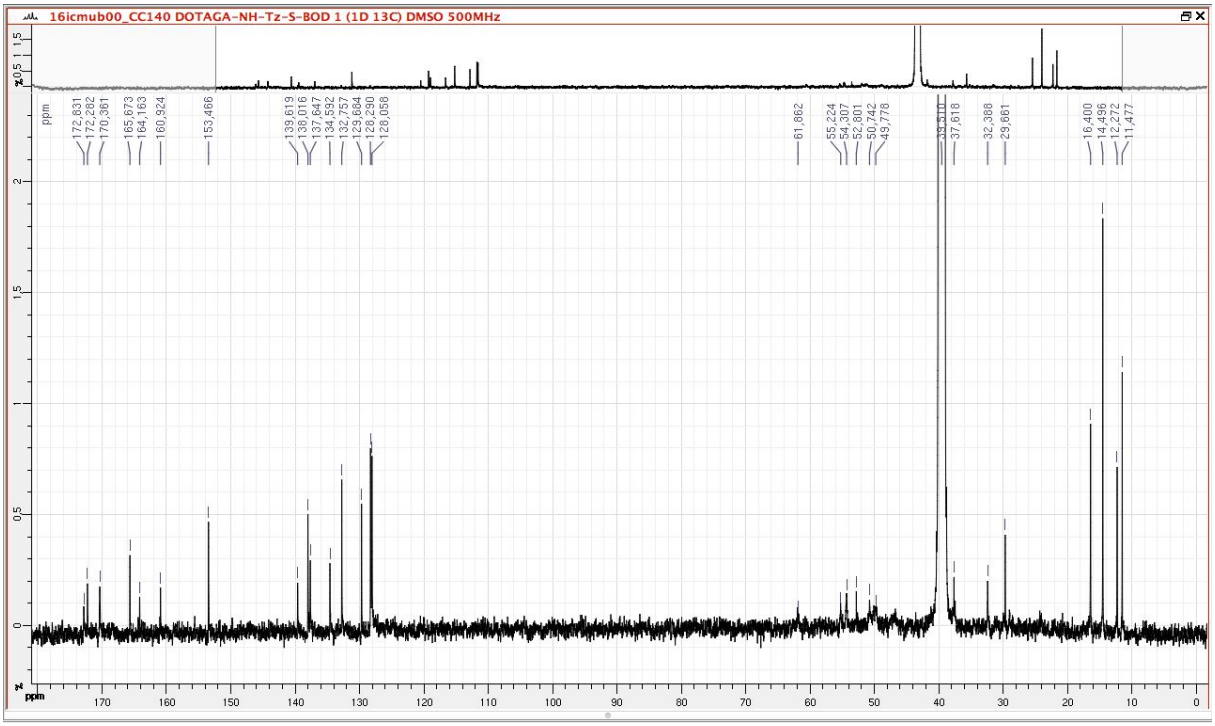
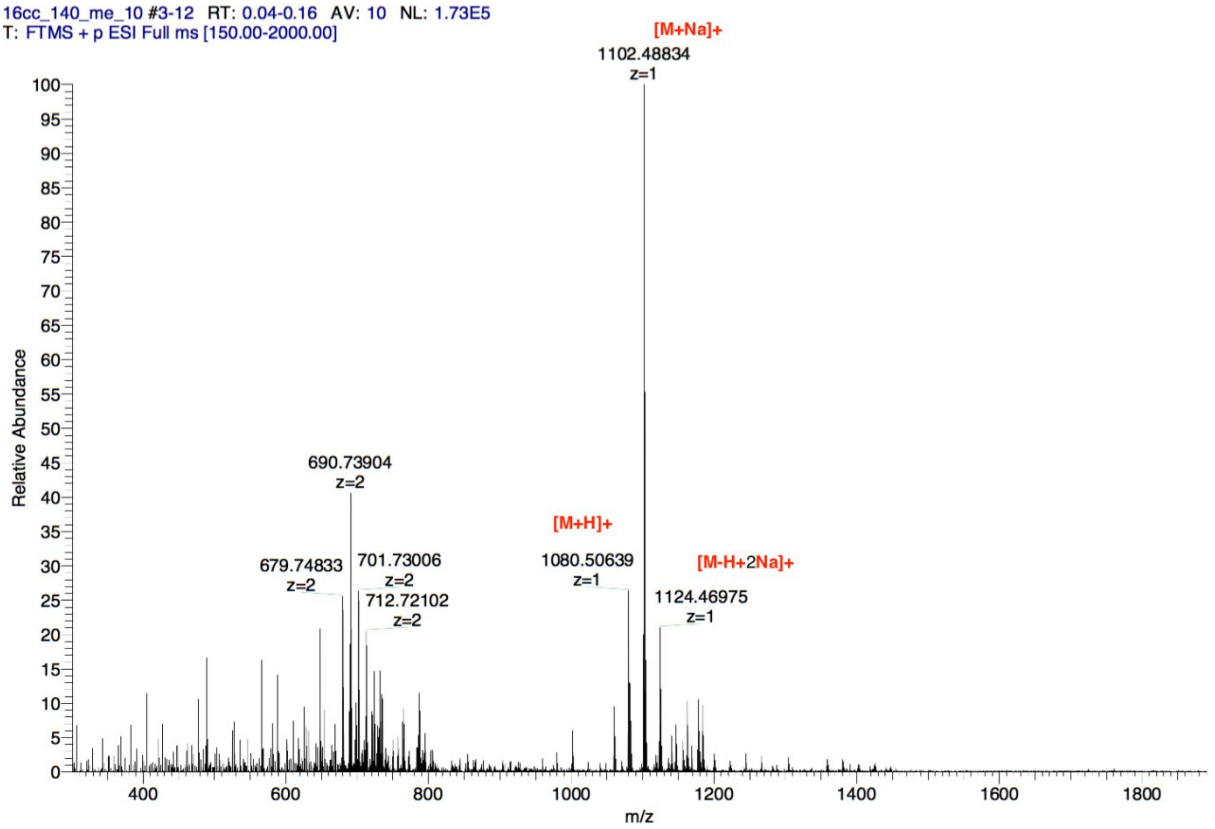
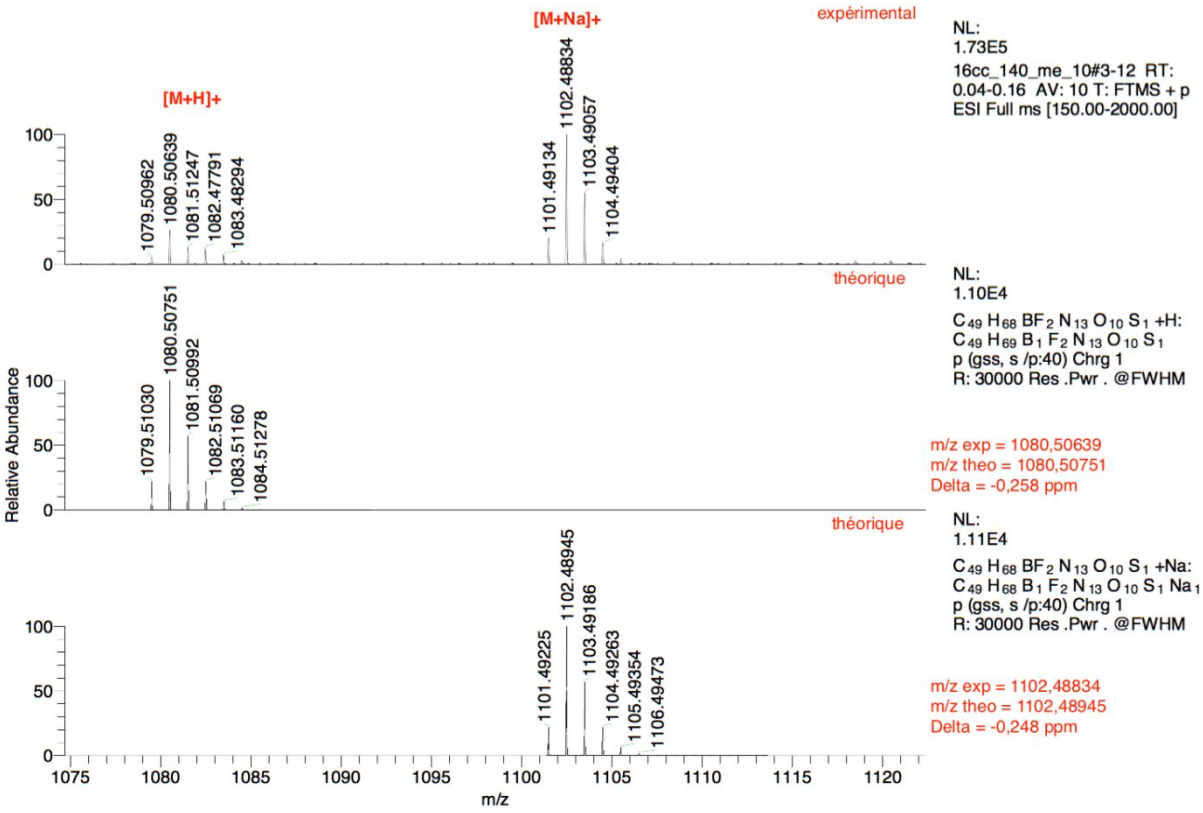
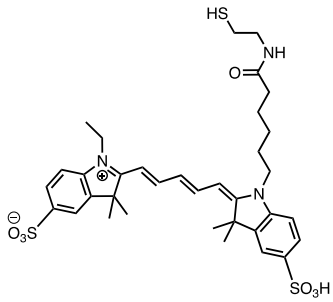


Figure S17. HRMS spectrum of DOTAGA-NH-Tz-S-BODIPY 6. Comparison with simulated spectrum.




[M+Na]+
expérimental
NL:
1.73E5
16cc_140_me_10#3-12 RT:
0.04-0.16 AV: 10 T: FTMS + p
ESI Full ms [150.00-2000.00]
[M+H]+
1079.50962
1080.50639
1081.51247
1082.47791
1083.48294
1101.49134
1102.48834
1103.49057
1104.49404
théorique
NL:
1.10E4
C49 H68 BF2 N13 O10 S1 +H:
C49 H69 B1 F2 N13 O10 S1
p (gss, s /p:40) Chrg 1
R: 30000 Res .Pwr . @FWHM
Relative Abundance
1079.51030
1080.50751
1081.50992
1082.51069
1083.51160
1084.51278
m/z exp = 1080,50639
m/z theo = 1080,50751
Delta = -0,258 ppm
théorique
NL:
1.11E4
C49 H68 BF2 N13 O10 S1 +Na:
C49 H68 B1 F2 N13 O10 S1 Na1
p (gss, s /p:40) Chrg 1
R: 30000 Res .Pwr . @FWHM
1101.49225
1102.48945
1103.49186
1104.49263
1105.49354
1106.49473
m/z exp = 1102,48834
m/z theo = 1102,48945
Delta = -0,248 ppm
m/z

1-ethyl-2-((1*E*,3*E*)-5-((*E*)-1-(6-((2-mercaptoethyl)amino)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3*H*-indol-1-ium-5-sulfonate, sulfonated Cyanine 5.0-SH.



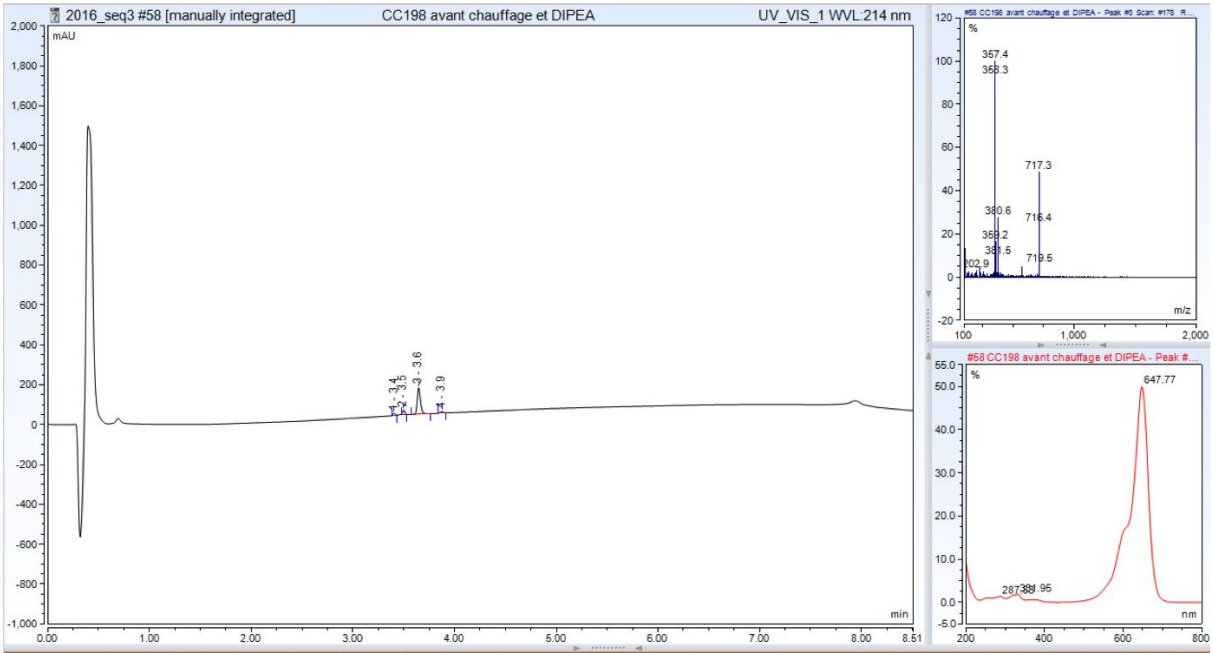
2-((1*E*,3*E*)-5-((*E*)-1-(5-carboxypentyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3*H*-indol-1-ium-5-sulfonate (sulfonated Cyanine 5.0-COOH) (16.0 mg, 24.4 µmol), TSTU (8.8 mg, 29.2 µmol, 1.2 eq) and DIPEA (15.3 µL, 87.6 µmol, 3.6 eq) were dissolved in anhydrous DMF (500 µL). The solution was stirred 5 min at room temperature and cystamine dihydrochloride (8.2 mg, 36.5 µmol, 1.5 eq), dissolved in 500 µL of borate buffer (2.5 M, pH 8) and DIPEA (3.2 µL, 18.4 µmol) was added. After 1 h at room temperature, DTT (6.8 mg, 43.8 µmol, 1.8 eq) was added and the solution was stirred overnight at room temperature. Solvents were eliminated under reduced pressure. Purification by semi-preparative RP-HPLC (eluents: H_2O 0.1% TFA, MeCN 0.1% TFA) yielded sulfonated Cyanine 5.0-SH as a blue powder (7.3 mg, 34%, purity: 82%).

^{1}H NMR (500 MHz, D_2O) ∂ 1.51 (m, 5H; CH_2, CH_3), 1.70 (m, 2H; CH_2), 1.78 (bs, 6H; CH_3),1.83 (s, 6H; CH_3), 1.96 (m, 2H; CH_2), 2. 40 (m, 2H; CH_2), 2.71 (m, 2H; CH_2), 3.44 (m, 2H; CH_2), 4.21 (m, 4H; CH_2), 6.71 (m, 1H; CH), 7.48 (m, 2H, CH), 8.02 (m, 4H, CH), 8.18 (m, 2H, CH).

The low amount of product available did not allow us to perform proper ^{13}C NMR.

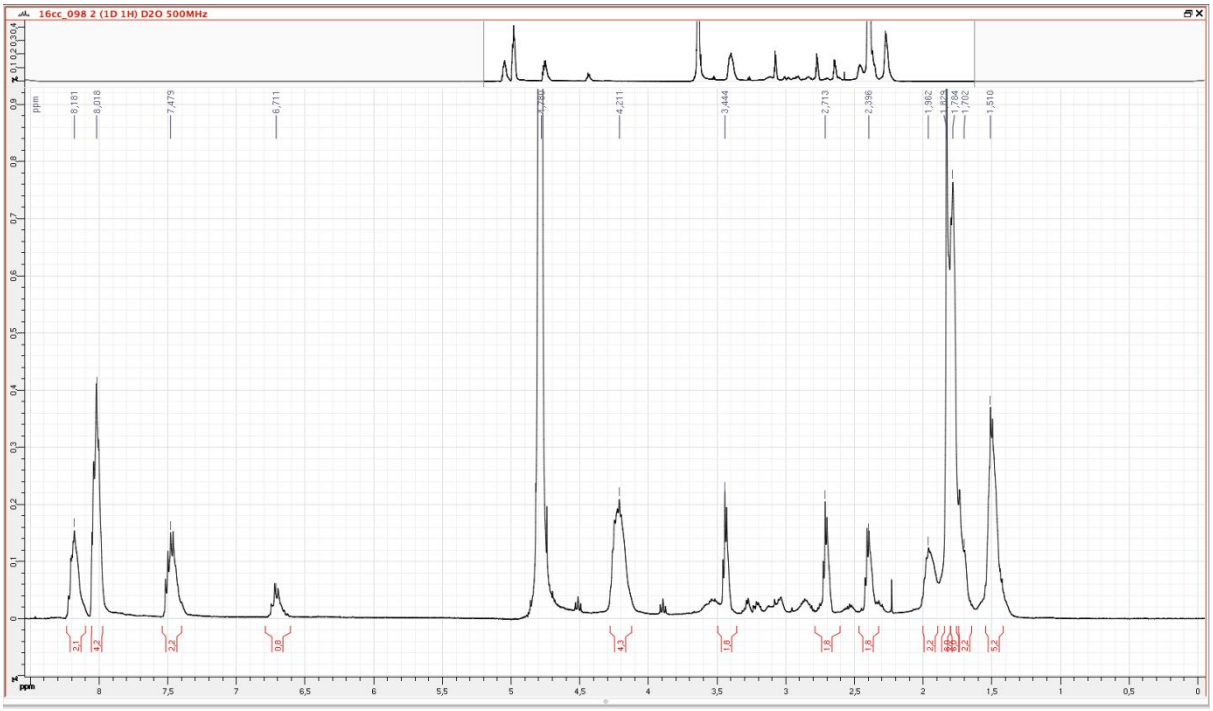
MS: $C_{35}H_{46}N_3O_7S_3^+$ ([M]$^+$) m/z calculated: 716.2, found: 716.3.

Figure S18. RP-HPLC-MS analysis of sulfonated Cyanine 5.0-SH.



	A	B	C	D	E	F	G	H	I
1	No.	Peakname	Ret.Time	Area	Amount	Type	Height	Rel.Area	Resolution
2			min	mAU*min	n.a.		mAU	%	
4	1		3.407	0.3224	n.a.	BMB*	13.104	5.22	2.367
5	2		3.500	0.5285	n.a.	BMB*	22.538	8.56	3.024
6	3		3.647	5.0690	n.a.	BMB*	134.152	82.13	4.042
7	4		3.873	0.2517	n.a.	BMB*	7.540	4.08	n.a.
8									

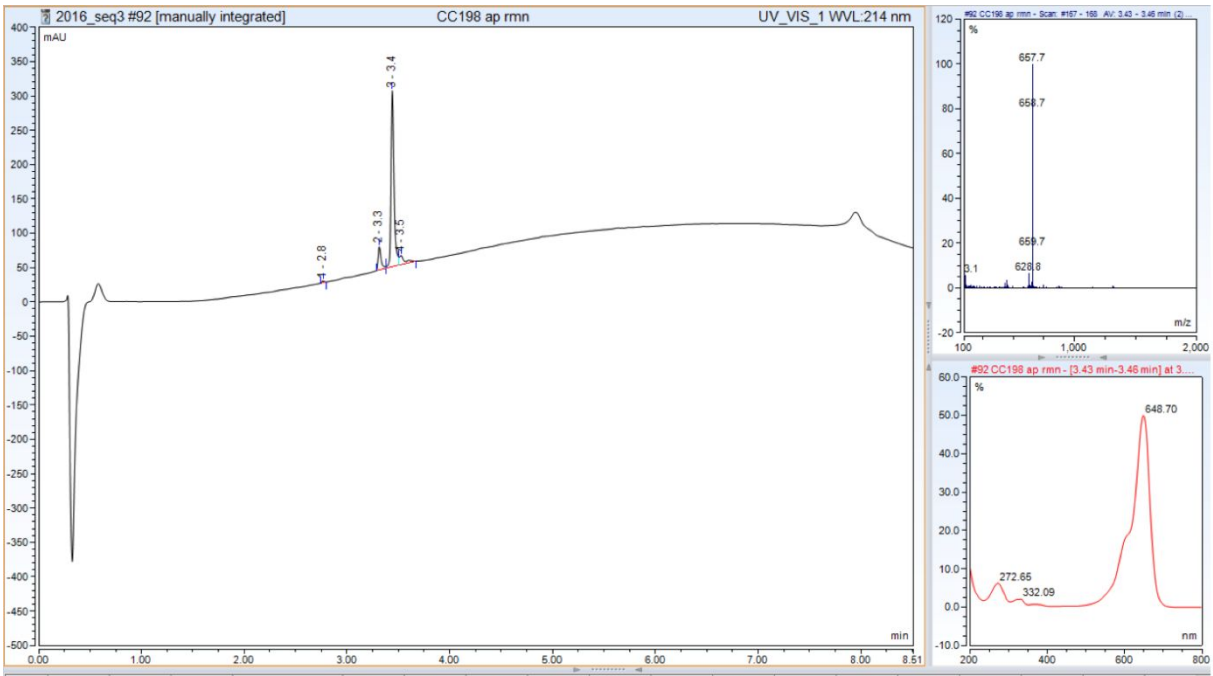
Figure S19. ^{1}H NMR spectrum of sulfonated Cyanine 5.0-SH.



2-((1*E*,3*E*)-5-((*E*)-1-(6-((2-((6-((2-(4-carboxy-4-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)butanamido)ethyl)amino)-1,2,4,5-tetrazin-3-yl)thio)ethyl)amino)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3*H*-indol-1-ium-5-sulfonate, DOTAGA-NH-Tz-S-sulfonated Cyanine 5.0 **7**.

The synthesis of DOTAGA-NH-Tz-S-sulfonated Cyanine 5.0 **7** is described in the manuscript.

Figure S20. RP-HPLC-MS analysis of DOTAGA-NH-Tz-S-sulfonated Cyanine 5.0 7.



	A	B	C	D	E	F	G	H	I
1	No.	Peakname	Ret.Time	Area	Amount	Type	Height	Rel.Area	Resolution
2			min	mAU*min	n.a.		mAU	%	
4	1		2.767	0.0703	n.a.	BMB*	2.519	0.65	11.777
5	2		3.313	1.1319	n.a.	BM *	34.005	10.46	2.589
6	3		3.440	8.8413	n.a.	M *	256.248	81.70	n.a.
7	4		3.527	0.7778	n.a.	MB*	13.539	7.19	n.a.
8									

Figure S21. ^{1}H NMR spectrum of DOTAGA-NH-Tz-S-sulfonated Cyanine 5.0 7.

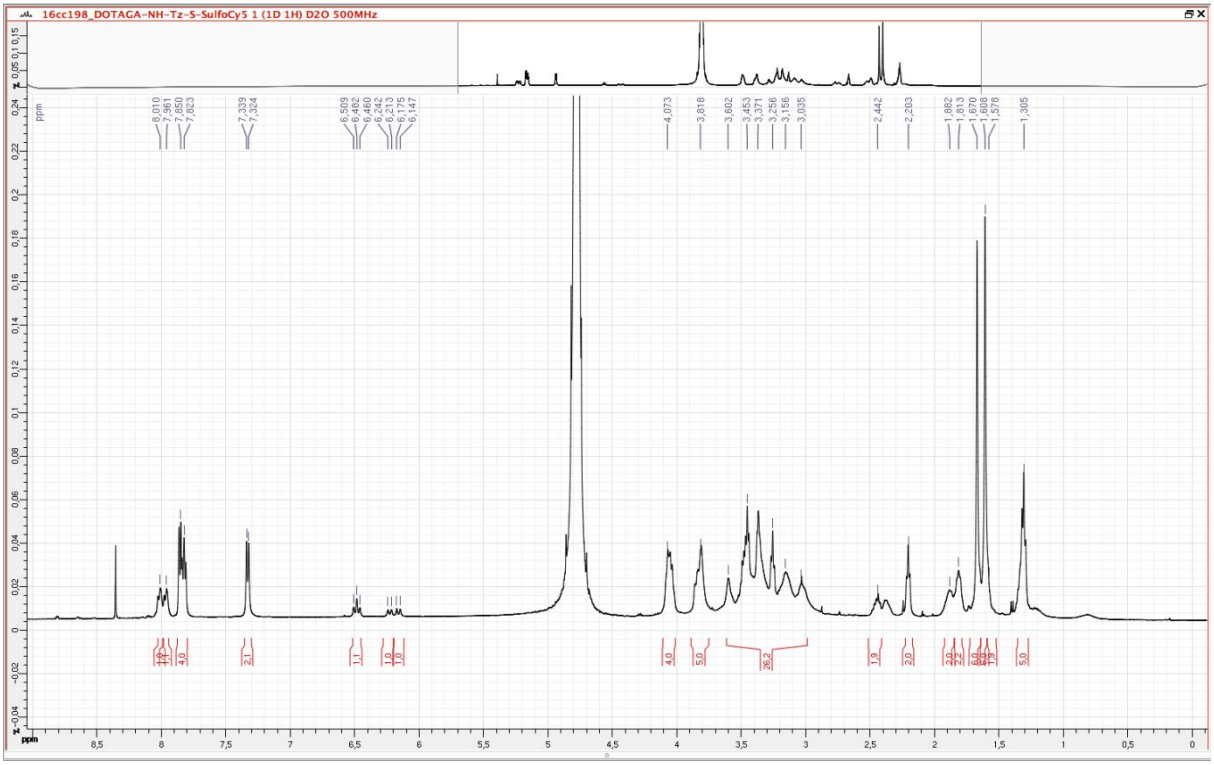
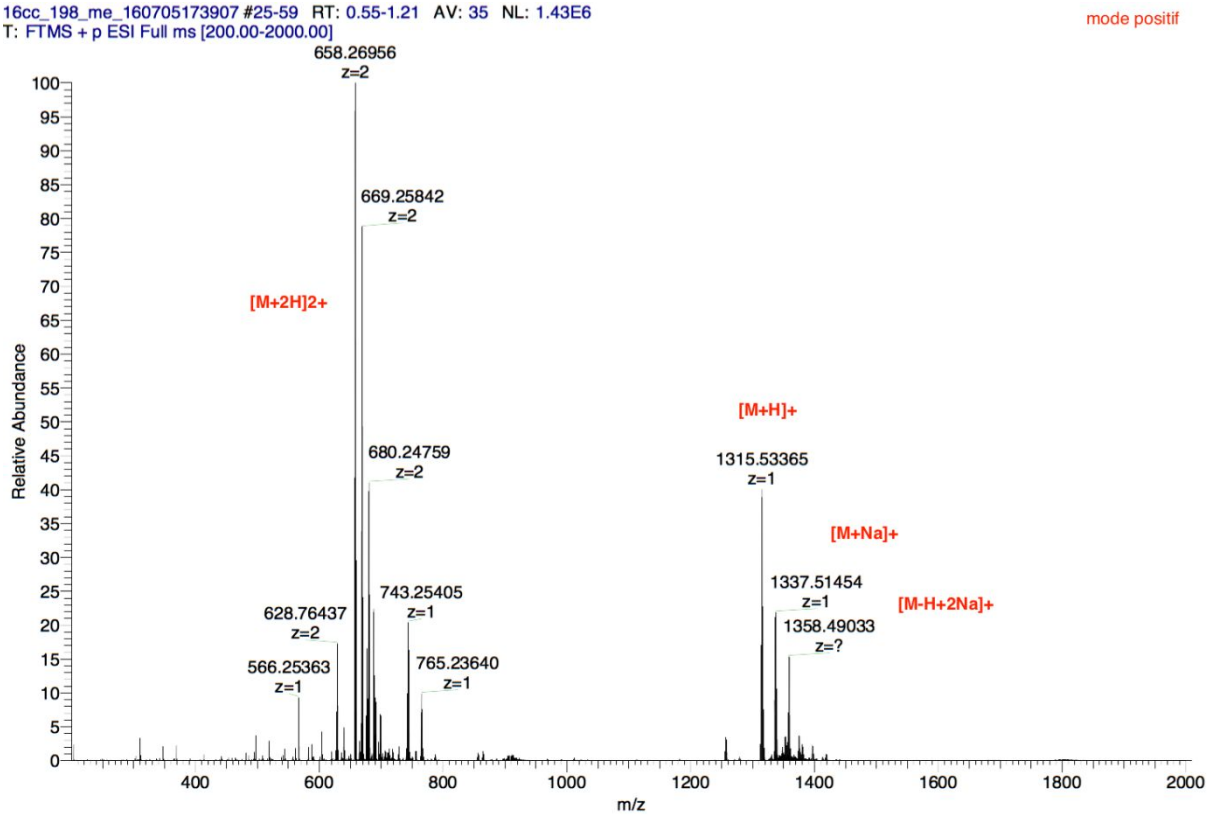
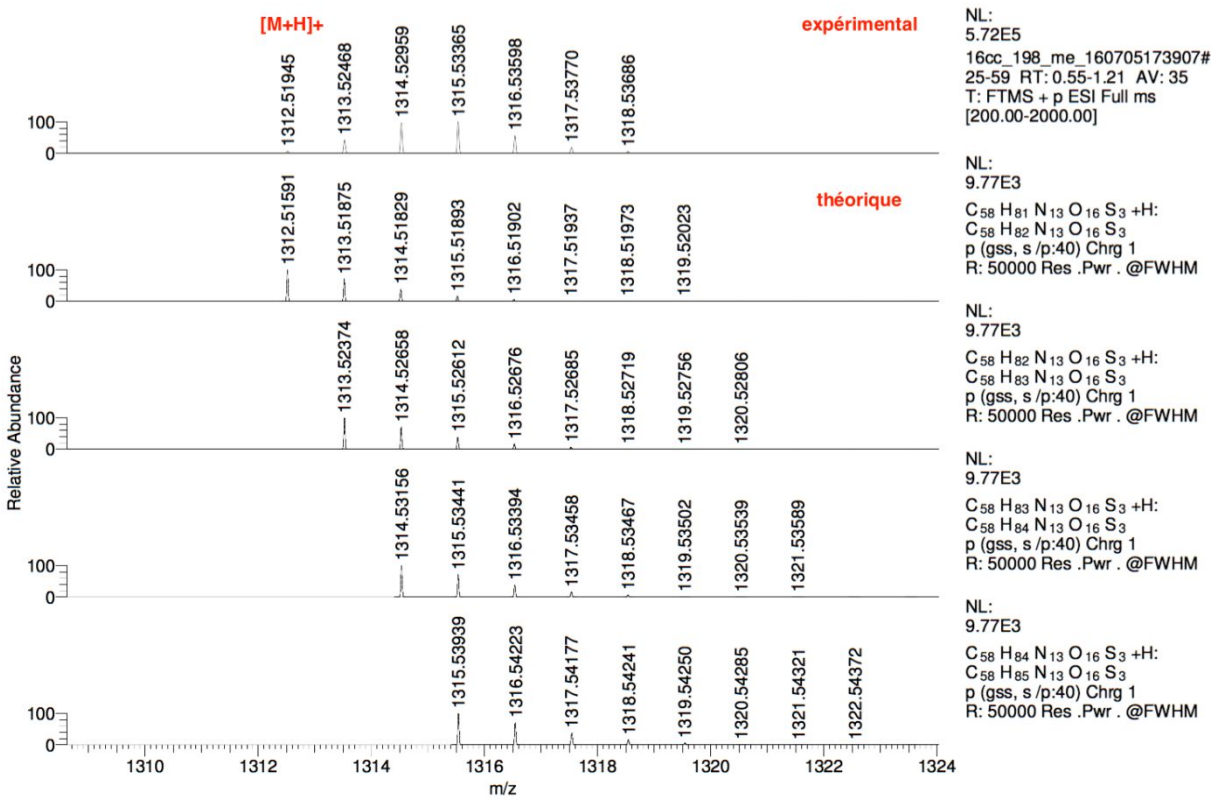


Figure S22. HRMS spectrum of DOTAGA-NH-Tz-S-sulfonated Cyanine 5.0 7. Comparison with simulated spectrum.





1-ethyl-2-((1*E*,3*E*)-5-((*E*)-1-(6-((2-mercaptoethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3*H*-indol-1-ium, Cyanine 5.0-SH.

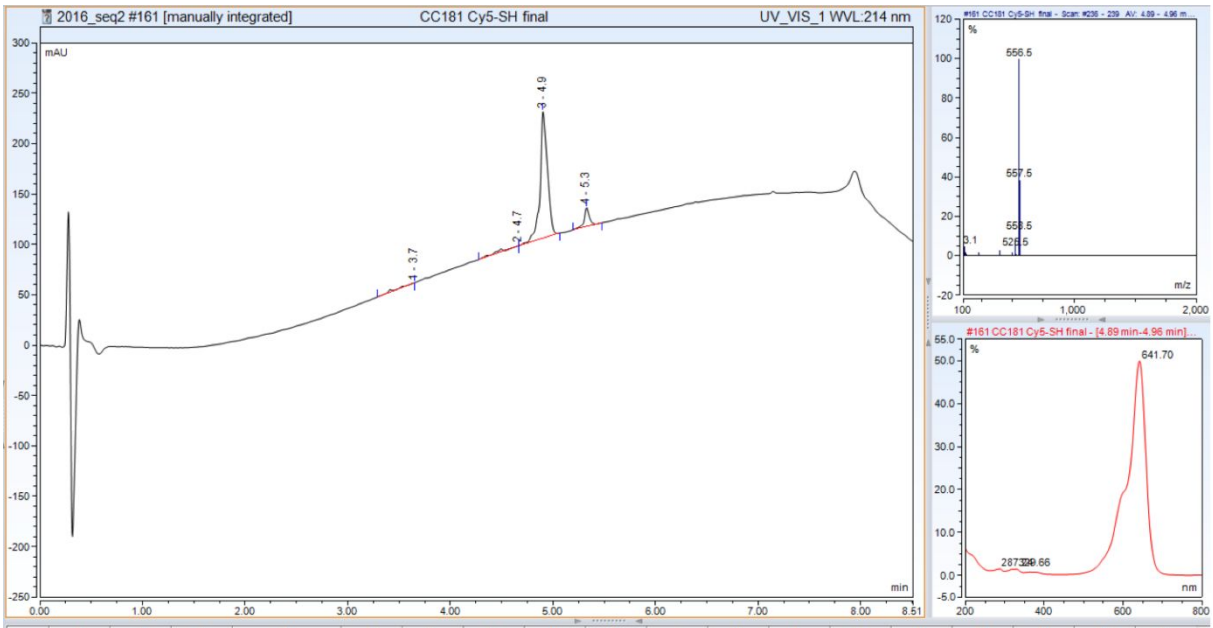
The TFA salt of 2-((1*E*,3*E*)-5-((*E*)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3*H*-indol-1-ium (Cyanine 5.0-COOH·1TFA) (20.0 mg, 32.7 µmol), TSTU (11.8 mg, 39.3 µmol, 1.2 eq) and DIPEA (6.9 µL, 39.3 µmol, 1.2 eq) were dissolved in anhydrous DMF (800 µL). The solution was stirred 10 min at room temperature and cysteamine hydrochloride (7.4 mg, 65.4 µmol, 2.0 eq) and DIPEA (11.4 µL, 65.4 µmol, 2.0 eq) were added. After 1 h at room temperature, DTT (13.1 mg, 85.0 µmol, 2.6 eq) and water (400 µL) were added and the solution was stirred 7 h at room temperature. The DMF was eliminated under reduced pressure. Purification by semi-preparative RP-HPLC (eluents: H_2O 0.1% TFA, MeCN 0.1% TFA) gave Cyanine 5.0-SH·1TFA as a blue powder (22.2 mg, 89%, purity: 88%).

^{1}H NMR (500 MHz, CD_3CN+D_2O) ∂ 1.34 (t, ^3J = 7.0 Hz, 3H; CH_3), 1.42 (m, 2H; CH_2), 1.60 (m, 1H; SH),1.63 (m, 2H; CH_2), 1.66 (s, 12H; CH_3), 1. 78 (m, 2H; CH_2), 2.13 (t, ^3J = 6.9 Hz, 2H; CH_2), 2.54 (m, 2H, CH_2), 2.72 to 3.22 (non-identified impurity) 3.27 (m, 2H, CH_2), 4.00 (t, ^3J = 7.5 Hz, 2H; CH_2), 4.06 (q, ^3J = 7.0 Hz, 2H, CH_2), 6.22 (d, ^3J = 13.7 Hz, 2H; CH), 6.55 (t, ^3J = 12.5 Hz, 1H; CH), 6.74 (bs, 1H, NH), 7.25 (m, 4H, CH), 7.40 (m, 2H, CH), 7.48 (m, 2H, CH), 8.08 (dd, ^3J = 13.7, 12.5 Hz, 2H, CH).

^{13}C NMR (125.7 MHz, CD_3CN+D_2O) ∂ 13.0, 25.2 (2C), 26.4, 27.4, 28.1, 28.2, 36.8, 40.5, 43.6, 45.3, 50.6 50.7, 104.1, 104.4, 112.2, 112.4, 123.6, 123.7, 125.9, 126.4 (2C), 129.9, 130.0, 142.8, 143.0, 143.3, 143.7, 155.2, 155.3, 174.1, 174.4, 174.8.

MS: $C_{35}H_{46}N_3OS^+$ ([M]$^+$) m/z calculated: 556.3, found: 556.5.

Figure S23. RP-HPLC-MS analysis of Cyanine 5.0-SH.



	A	B	C	D	E	F	G	H	I
1	No.	Peakname	Ret.Time	Area	Amount	Type	Height	Rel.Area	Resolution
2			min	mAU*min	n.a.		mAU	%	
4	1		3.650	0.1651	n.a.	BMB*	0.000	1.38	n.a.
5	2		4.663	0.2501	n.a.	BMB*	0.000	2.09	n.a.
6	3		4.903	10.4712	n.a.	BMB*	125.584	87.65	4.428
7	4		5.327	1.0600	n.a.	BMB*	18.831	8.87	n.a.
8									

Figure S24. ^{1}H NMR spectrum of Cyanine 5.0-SH.

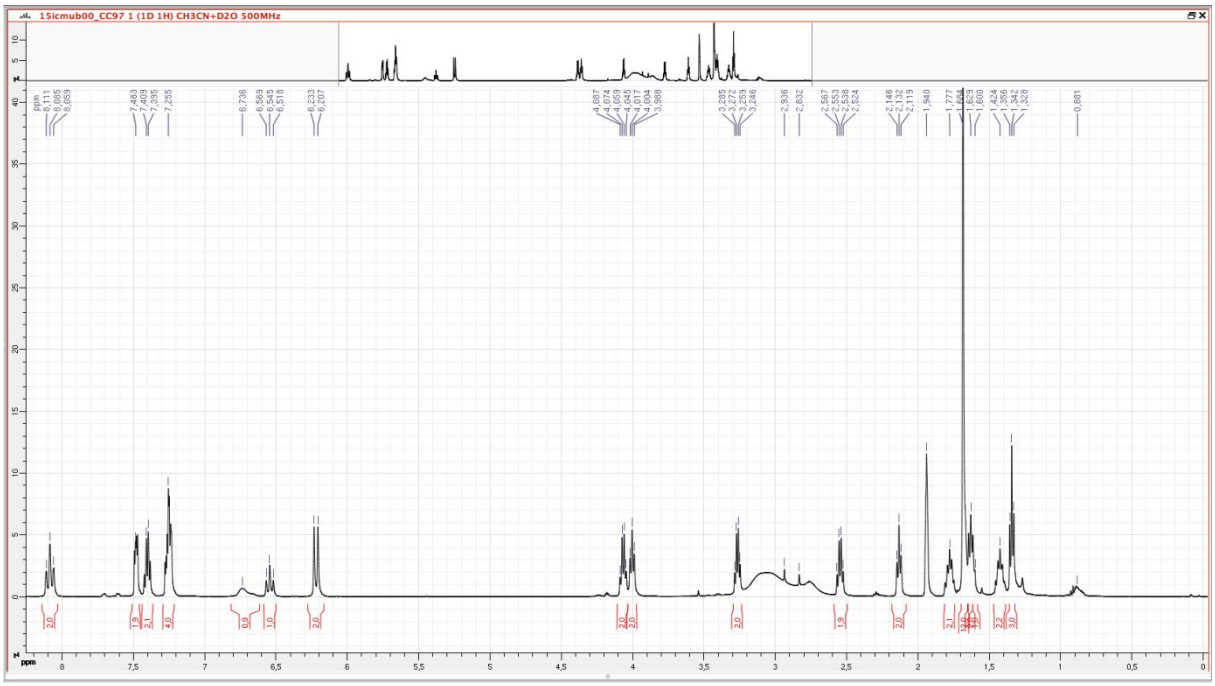
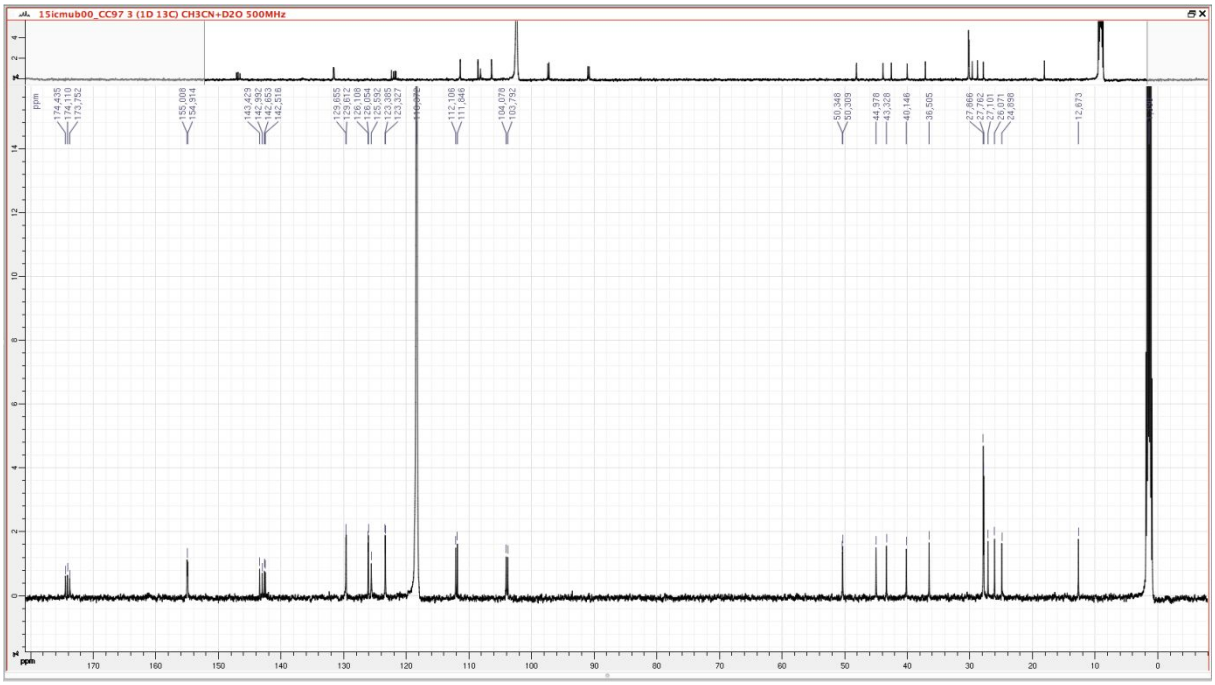


Figure S25. ^{13}C NMR spectrum of Cyanine 5.0-SH.



2-((1*E*,3*E*)-5-((*E*)-1-(6-((2-((6-((2-(4-carboxy-4-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)butanamido)ethyl)amino)-1,2,4,5-tetrazin-3-yl)thio)ethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3*H*-indol-1-ium, DOTAGA-NH-Tz-S-Cyanine 5.0 **8**.

DOTAGA-NH-Tz-Cl·1.5TFA **1** (19.5 mg, 24.3 µmol, 1.1 eq) and Cyanine 5.0-SH·1TFA (14.9 mg, 22.2 µmol, 1.0 eq) were dissolved in anhydrous DMF (1 mL) and DIPEA (28.0 µL, 149.5 µmol, 6.7 eq) was added. After 1 h at 75 °C, the solvent was removed under reduced pressure. Purification by semi-preparative RP-HPLC (eluents: H_2O 0.1% FA, MeCN 0.1% FA) gave DOTAGA-NH-Tz-S-Cyanine 5.0·2.2TFA **11** as a blue powder (6.3 mg, 18%, purity: 90%).

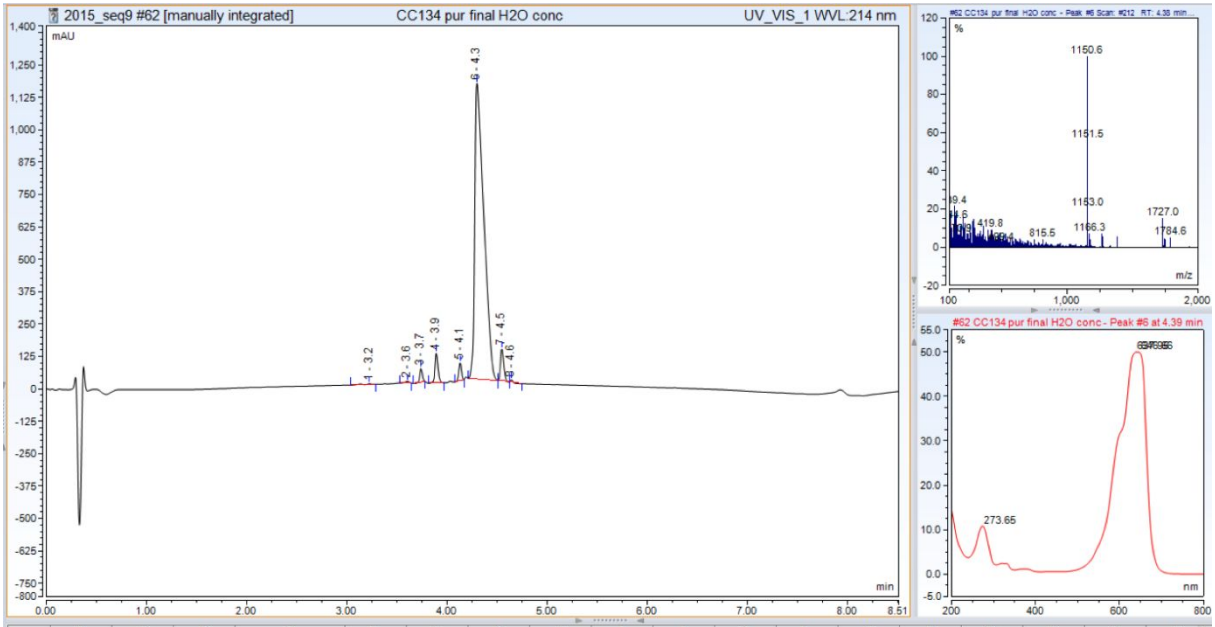
Ionic chromatography analysis indicated an amount of 2.2 TFA per molecule.

^{1}H NMR (500 MHz, CD_3CN) ∂ 1.33 (t, ^3J = 7.0 Hz, 3H; CH_3), 1.42 (m, 2H; CH_2), 1.61 (m, 2H; CH_2), 1.67 (s, 12H; CH_3), 1. 76 (m, 2H; CH_2), 2.12 (t, ^3J = 7.2 Hz, 2H; CH_2), 2.17 to 2.75 (non-identified impurity), 2.76 to 3.97 (m, 35H; macrocycle + CH_2), 4.00 (bt, 2H; CH_2), 4.06 (q, ^3J = 7.0 Hz, 2H, CH_2), 6.21 (d, ^3J = 13.0 Hz, 2H; CH), 6.53 (t, ^3J = 13.0 Hz, 1H; CH), 7.25 (m, 4H, CH), 7.40 (m, 2H, CH), 7.47 (m, 2H, CH), 8.07 (t, ^3J = 13.0, 2H, CH).

^{13}C NMR (125.7 MHz, CD_3CN) ∂ 13.0, 26.4, 27.4, 28.1, 28.2, 31.5, 36.8, 39.5, 39.7, 40.5, 42.4, 45.3, 50.6, 50.7, 104.1, 104.3, 112.2, 112.4, 123.6, 123.7, 125.9, 126.4, 126.5, 129.9, 130.0, 142.8, 143.0, 143.3, 143.7, 155.2, 155.3, 160.8, 161.1, 162.5, 162.8, 166.7, 174.2, 174.5, 174.7, macrocyclic signals in the range of 20.0 to 60.0 were not clearly distinguishable.

HRMS: $C_{58}H_{82}N_{13}O_{10}S^{+}$ ($[M]^{+}$) m/z calculated: 1152.60228, found: 1152.60445; $C_{58}H_{82}N_{13}NaO_{10}S^{2+}$ ($[M+Na]^{2+}$) m/z calculated: 587.79575, found: 587.79558.

Figure S26. RP-HPLC-MS analysis of DOTAGA-NH-Tz-S-Cyanine 5.0 8.



	A	B	C	D	E	F	G	H	I
1	No.	Peakname	Ret.Time	Area	Amount	Type	Height	Rel.Area	Resolution
2			min	mAU*min	n.a.		mAU	%	
4	1		3.223	0.2933	n.a.	BMB*	3.563	0.22	6.078
5	2		3.603	0.2734	n.a.	BMB*	5.822	0.21	2.372
6	3		3.740	1.4752	n.a.	BMB*	53.270	1.12	3.114
7	4		3.893	4.0362	n.a.	BMB*	114.939	3.07	4.643
8	5		4.130	2.0531	n.a.	BMB*	68.755	1.56	1.545
9	6		4.297	118.9203	n.a.	BMB*	1140.435	90.32	2.192
10	7		4.547	4.6018	n.a.	BMB*	124.200	3.50	1.973
11	8		4.643	0.0121	n.a.	BMB*	7.358	0.01	n.a.

Figure S27. ^{1}H NMR spectrum of DOTAGA-NH-Tz-S-Cyanine 5.0 8.

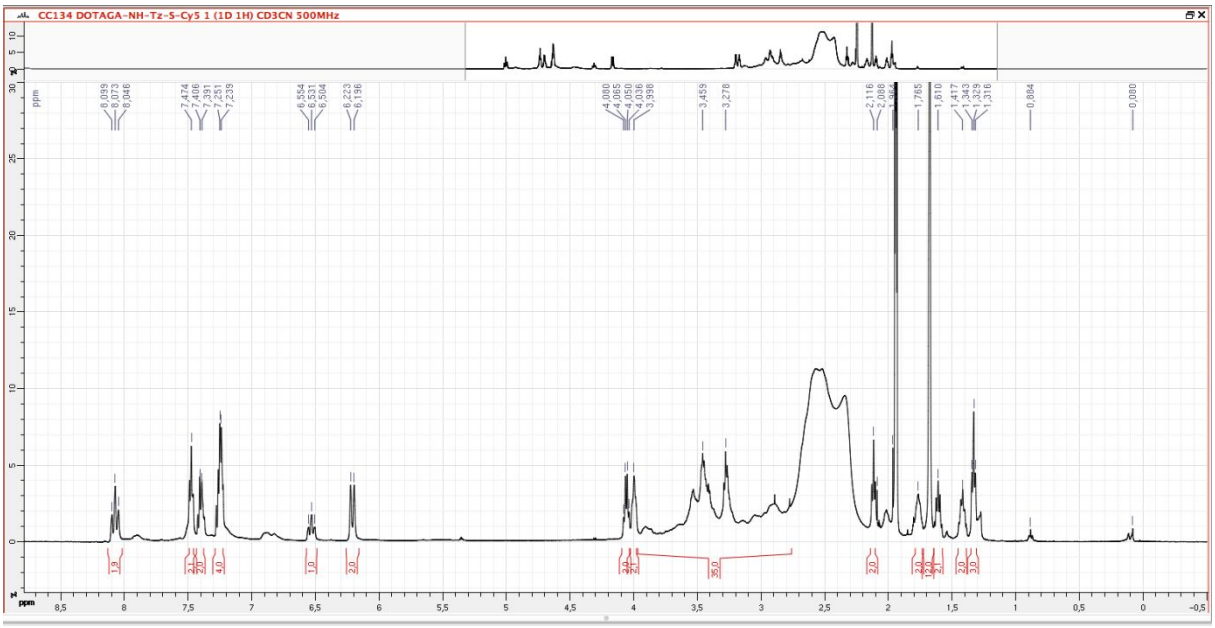


Figure S28. ^{13}C NMR spectrum of DOTAGA-NH-Tz-S-Cyanine 5.0 8.

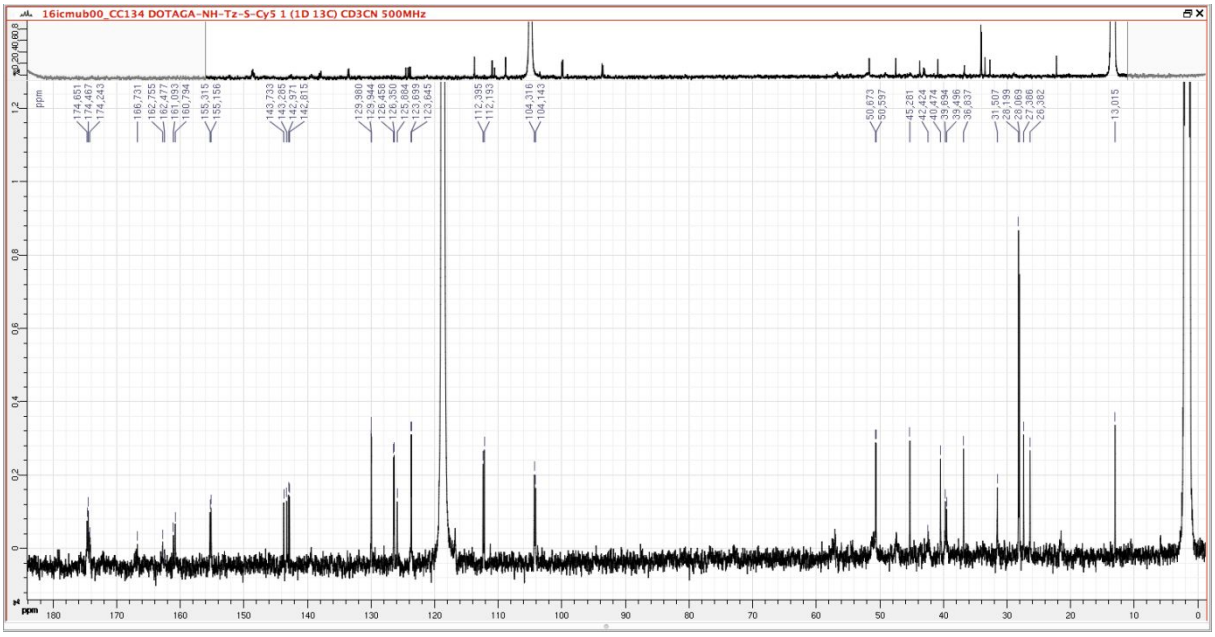
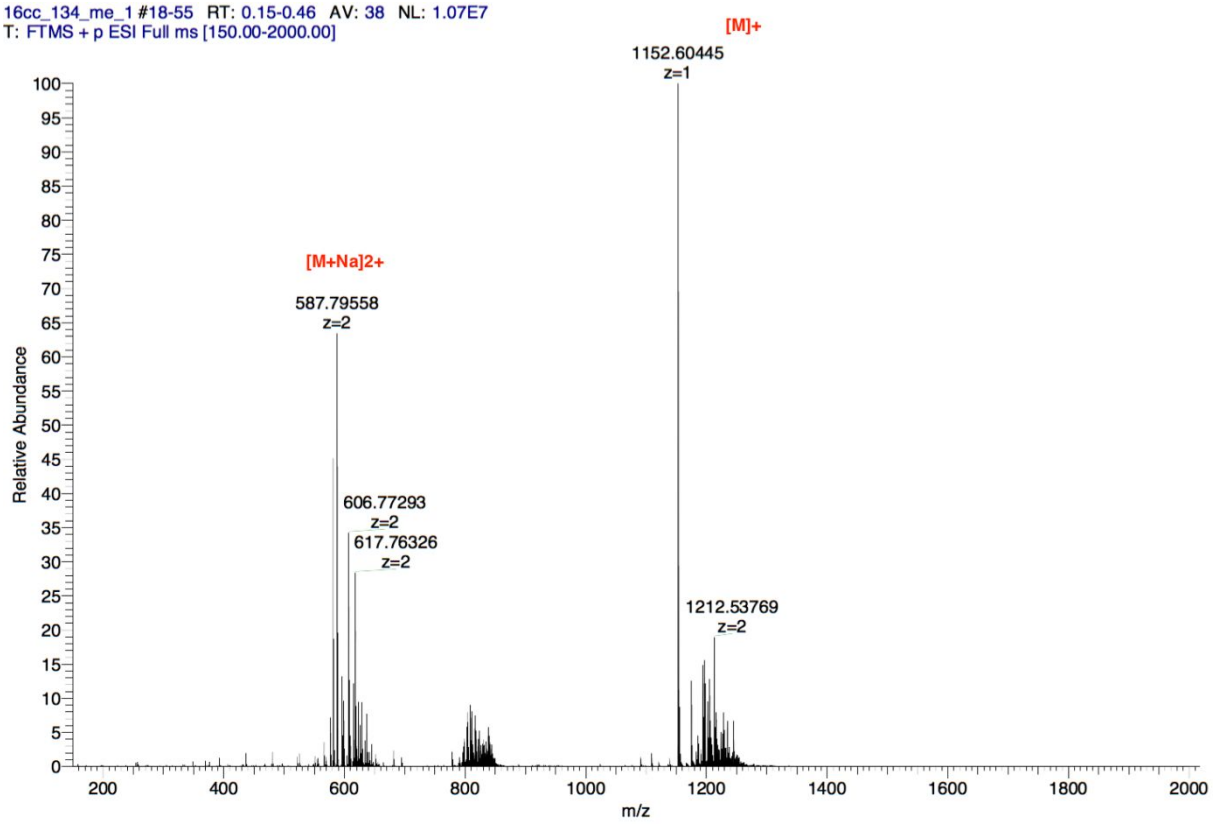
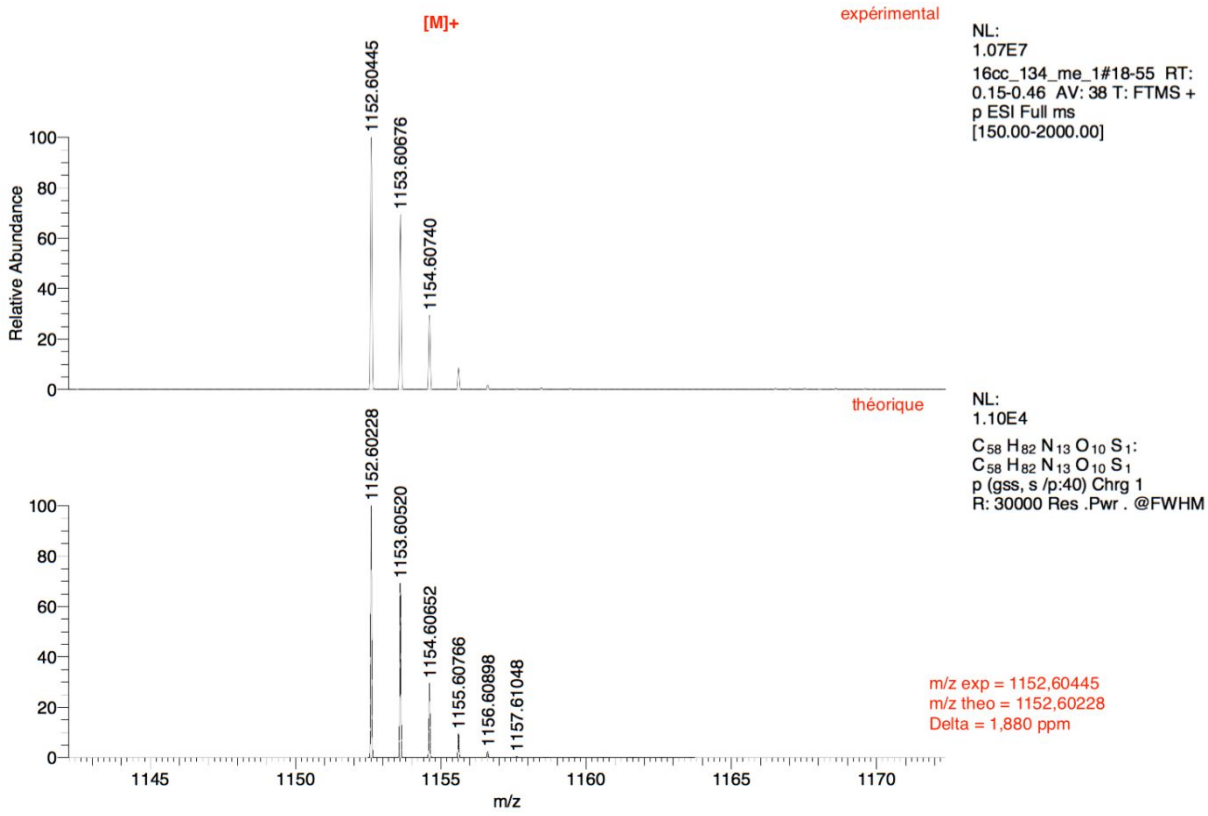


Figure S29. HRMS spectrum of DOTAGA-NH-Tz-S-Cyanine 5.0 8. Comparison with simulated spectrum.

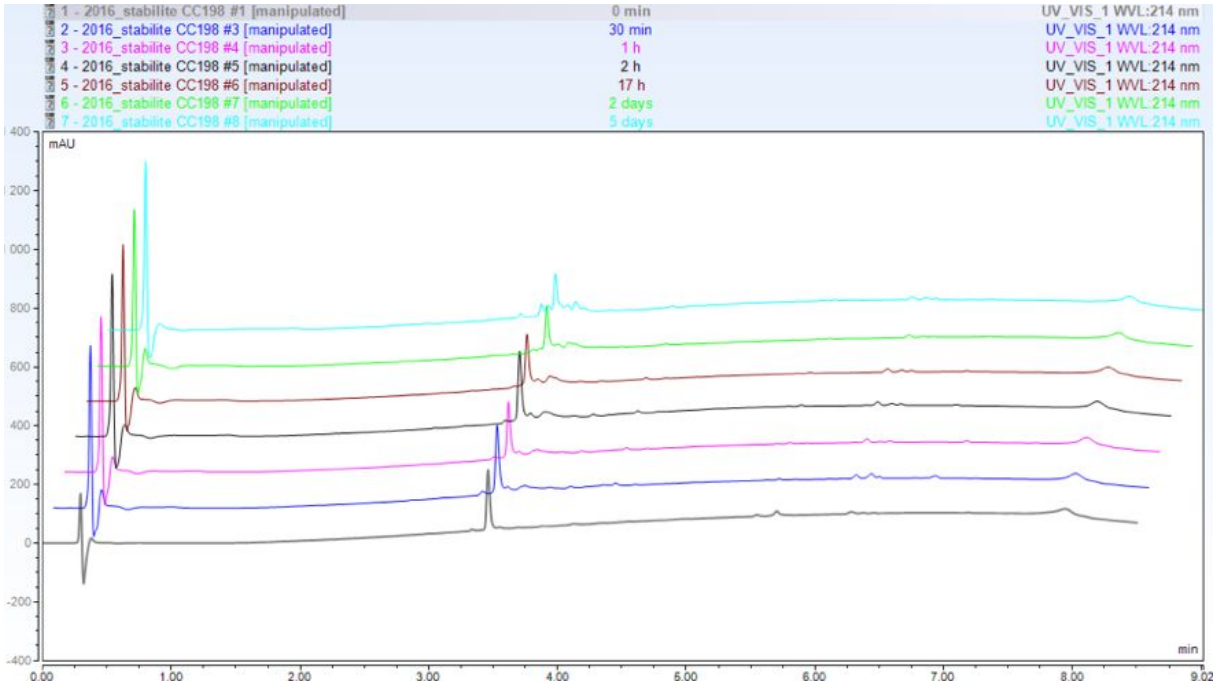




IN VITRO STABILITY OF THE BIMODAL PROBE 7 IN FBS/RPMI

25 µL of a stock solution of DOTAGA-NH-Tz-S-sulfonated Cyanine 5.0 **7** at 5 mM in H_2O (125 nmol) were stirred in a thermomixer (400 rpm, 37 °C, dark) with 100 µL of FBS/RPMI (25 : 75, *v/v*). Final concentration in **7** was 1 mM. After 30 min, 1 h, 2 h, 17 h, 2 days, and 5 days, a sample of 10 µL of solution was collected and diluted with 40 µL of absolute EtOH. Samples were vortexed and centrifuged (11700 rpm, 30 min) to remove precipitated proteins. The supernatant was analyzed by RP-HPLC-MS (UV detection at 214 nm).

Figure S30. Study of the stability of 7 in FBS/RPMI by RP-HPLC. Top: RP-HPLC-MS analysis of the initial compound **7** of the samples collected after incubation in FBS/RPMI (25 : 75, *v/v*) during 0 min, 30 min, 1 h, 2 h, 17 h, 2 d, 5 d.



PREPARATION OF TRASTUZUMAB-BCN 9

26.7 µL of a stock solution of ((1*R*,8*S*,9*s*)-bicyclo[6,1,0]non-4-yn-9-yl)methyl 2,5-dioxopyrrolidine-1-carboxylate (BCN-NHS) at 2 mM in DMSO (53.3 nmol, 4.0 eq) was added to a 2 mg/mL solution of trastuzumab (2 mg, 13.3 nmol) in carbonate-bicarbonate buffer (0.2 M, pH 8.5) with 10% DMSO. The solution was stirred in a thermomixer (800 rpm, 25 °C) during 1 h 45. Excess of BCN was removed by ultrafiltration on an Amicon Ultra Ultracel-30 kDa (Merck Millipore). This ultrafiltration step allowed us to concentrate the product and exchange the buffer from carbonate-bicarbonate buffer to PBS (1 M, pH 7.4). The pure BCN-modified trastuzumab **9** was obtained as a 35.5 mg/mL* solution in PBS (protein recovery = 91%). Based on MALDI-TOF analysis, the degree of labeling (amount of BCN per BSA) was determined to be 3.3.

* the concentration of the trastuzumab in mg/mL was calculated by UV spectrophotometry by using the extinction coefficient of the trastuzumab at 280 nm (1.48 $L.g^{-1}.cm^{-1}$, value from ExPASy).

CHARACTERIZATION OF THE TRASTUZUMAB CONJUGATES 9-10

Figure S31. MALDI-TOF mass spectrum of native trastuzumab.

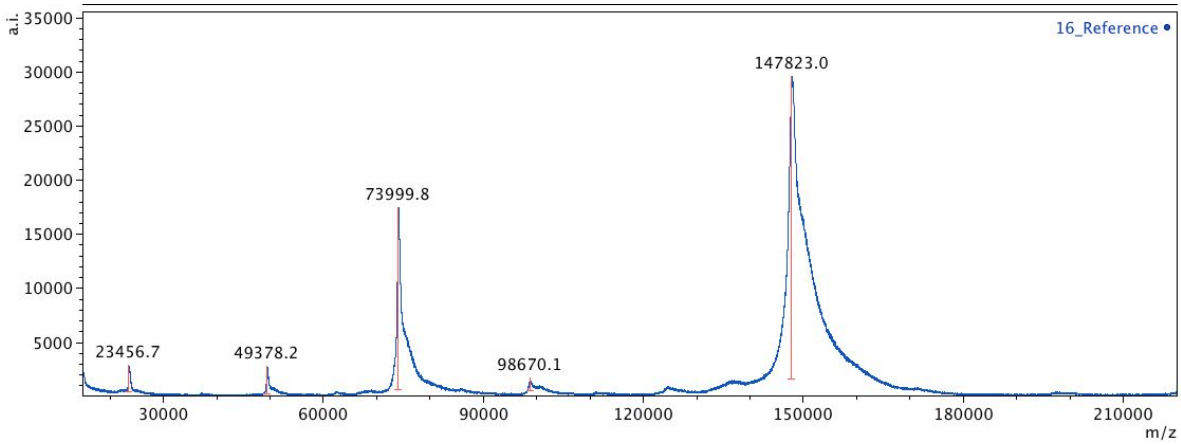


Figure S32. MALDI-TOF mass spectrum of trastuzumab-BCN. 9.

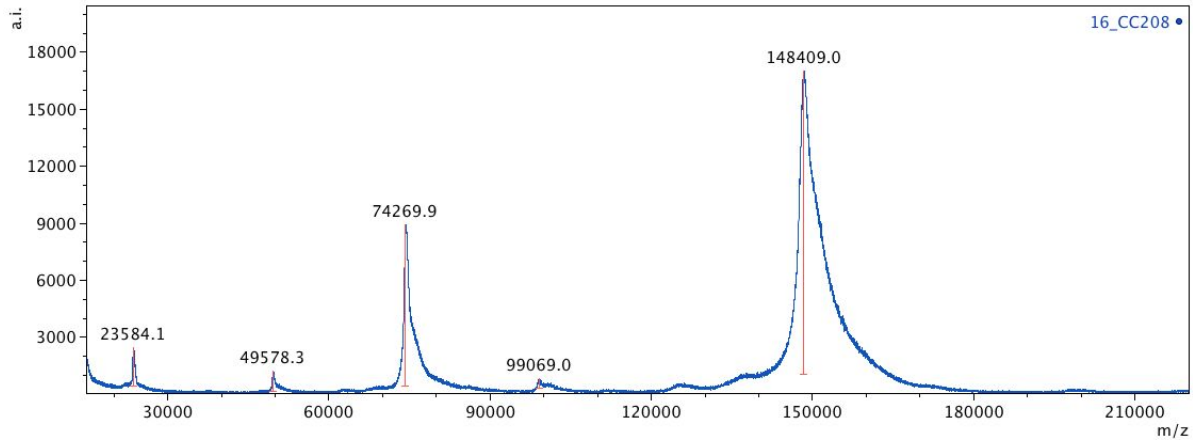


Figure S33. MALDI-TOF mass spectrum of the dually-modified trastuzumab 10.

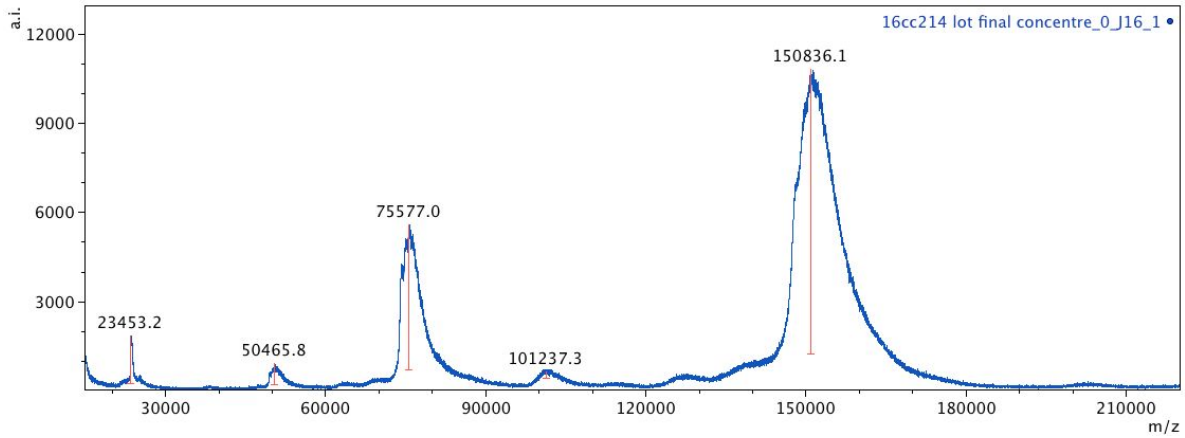
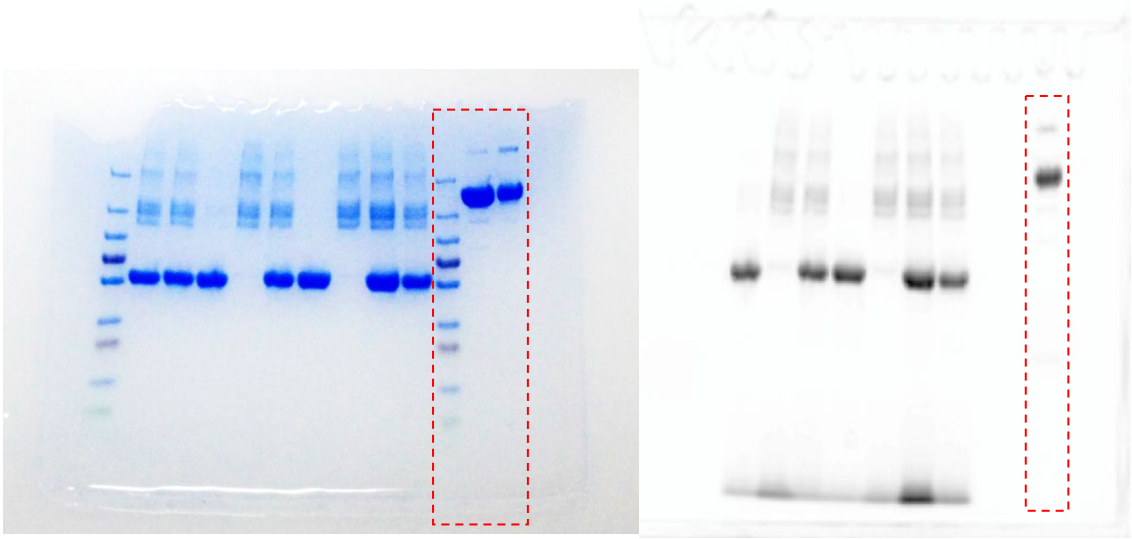


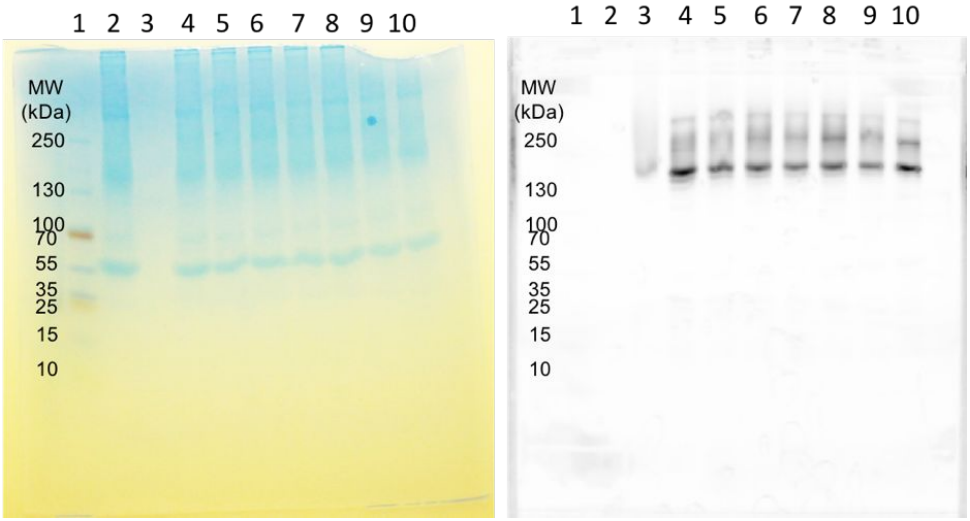
Figure S34. SDS-PAGE analysis of native trastuzumab and 10. Uncropped photos corresponding to the gels shown in Fig. 4c. (left) Coomassie blue revelation; (right) Fluorescence acquisition in pair filter mode (620/670 nm).



Coomassie Fluorescence

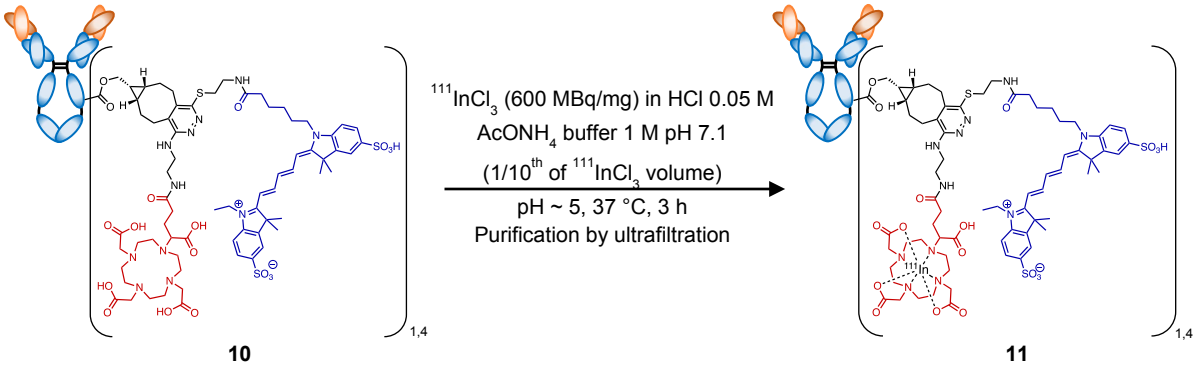
IN VITRO STABILITY OF THE CONJUGATE 10

Figure S35. SDS-PAGE analysis of 10. Uncropped photos corresponding to the gel shown in Fig. 4d. Line 1: Protein ladder, line 2: human plasma, line 3: bioconjugate **10** without incubation in human plasma, lines 4 to 10: bioconjugate **10** with incubation in human plasma during respectively 0 h, 1 h, 2 h, 4 h, 8 h, 24 h and 48 h. (left) Coomassie staining; (right) Fluorescence analysis, pair filter mode (620/670 nm).



RADIOLABELING OF THE CONJUGATE 10 WITH [^{111}In]-$InCl_3$

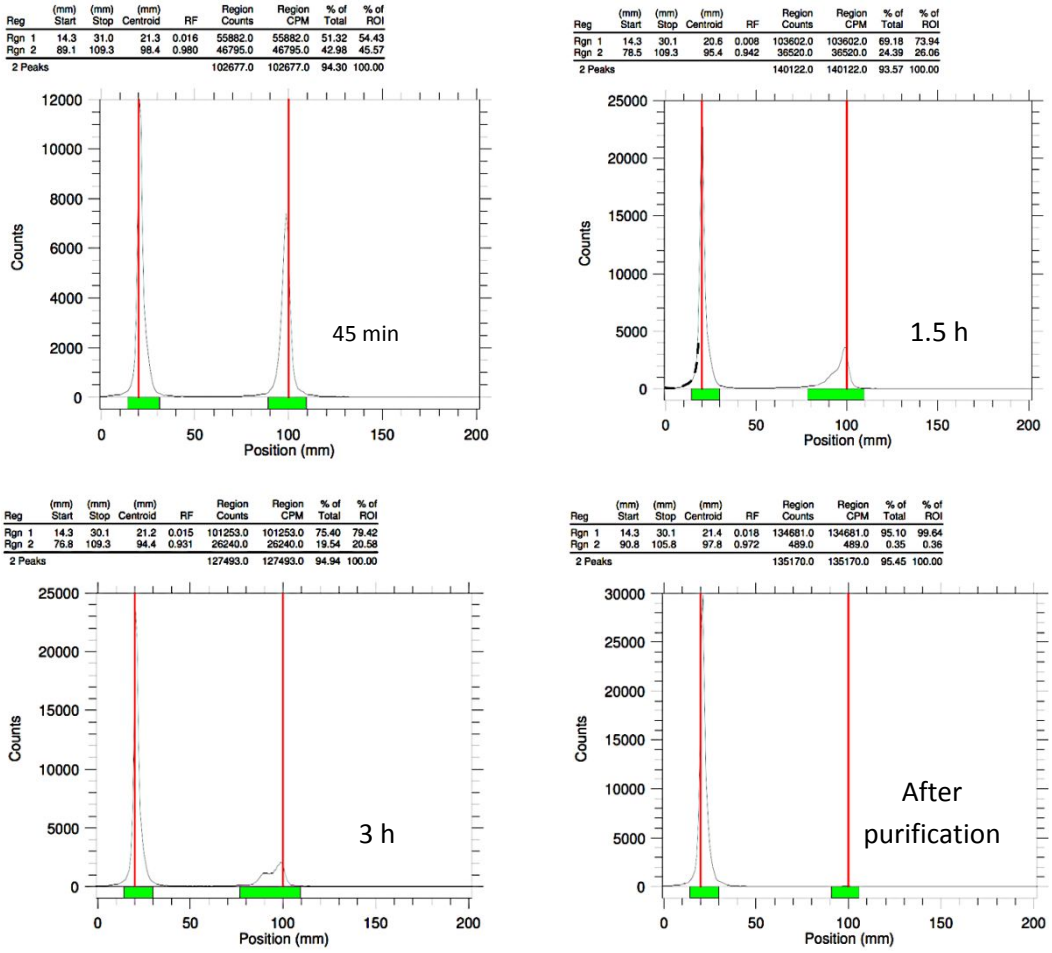
Optimization of the radiolabeling conditions



19.5 µL of a $^{111}InCl_3$ solution at 513 MBq/mL (10 MBq) in HCl was added to 16.7 µg of the bioconjugate **10**. To compensate for the acidity originating from the $^{111}InCl_3$ solution, 1.95 µL of ammonium acetate buffer (1 M, pH 5.7) were added to reach a final pH around 5. The solution was stirred in a thermomixer at 37 °C. Instant thin layer chromatographies (iTLCs) were performed at 45 min, 1 h 30 and 3 h time points to determine the ratio of ^{111}In complexed. To 1 µL samples of solution, 1 µL of 50 mM EDTA in 0.1 M ammonium acetate was added in order to chelate free indium-111. The 2 µL mixtures were deposited on ITLC-SG strips at 2 cm of the bottom. The eluent (sodium citrate 0.1 M, pH 5) was allowed to rise to 10 cm from the bottom of the strips. Radiolabeled antibody remained at the application point while free indium-111 or ^{111}In-chelates migrated with solvent front. The strips were then analyzed using a γ-radiochromatograph.

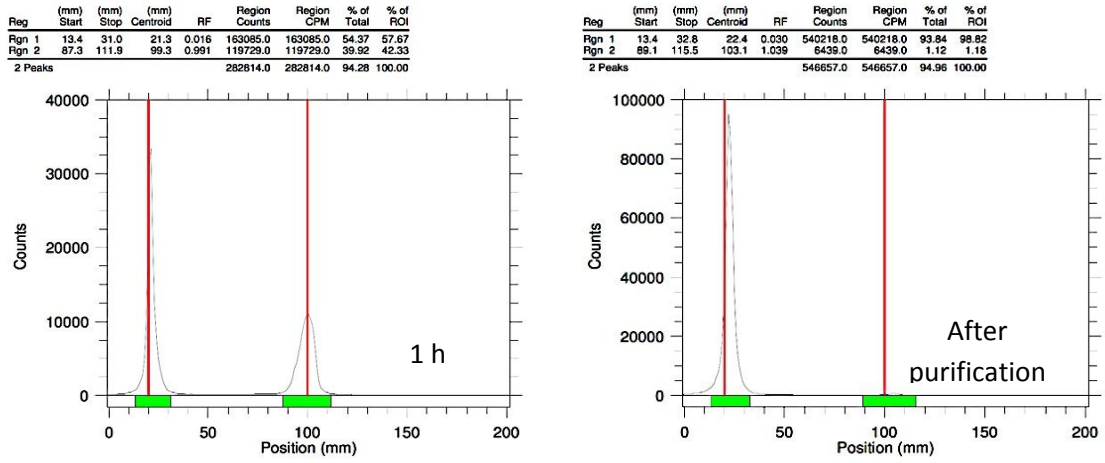
After 3 h of incubation, 3.0 µL of 50 mM EDTA in 0.1 M ammonium acetate was added to the reaction mixture. The resulting ^{111}In-EDTA was then removed by ultrafiltration on an Amicon Ultra Ultracel-30 kDa (Merck Millipore) and ITLC was performed, that showed a radiochemical purity >99%. Measure of radioactivity introduced in the Amicon Ultra Ultracel-30 kDa and obtained after the ultrafiltration allowed us to calculate a purification yield of 76%, a global ratio of recovered protein of 70% and a specific activity of 496 MBq/mg.

Figure S36. Monitoring of the radiolabeling of 10 by iTLC. iTLC profiles of the crude reaction mixture at 45 min, 1.5 h, 3 h and of the pure radiolabeled conjugate **11**. The integrated areas are displayed as green bars. The chart above each iTLC profile shows the signal quantification, expressed in CPM and in percentage (radiochemical purity).



Radiolabeling of 10 for *in vivo* studies

Figure S37. iTLC profiles of the crude reaction mixture at 1 h, and of the pure radiolabeled conjugate **11**. The integrated areas are displayed as green bars. The chart above each iTLC profile shows the signal quantification, expressed in CPM and in percentage (radiochemical purity).



IN VIVO EVALUATION OF THE RADIOLABELED CONJUGATE 11 BY SPECT-CT/FLUORESCENCE IMAGING

Figure S38. Biodistribution data for each mouse, by gamma counting. Values are expressed in percentage of injected dose per gram (%ID/g) for each collected organ (tumor, liver, kidneys, blood, muscle, bladder/urine, spleen). Graphs were obtained with Prism 7 software (GraphPad).

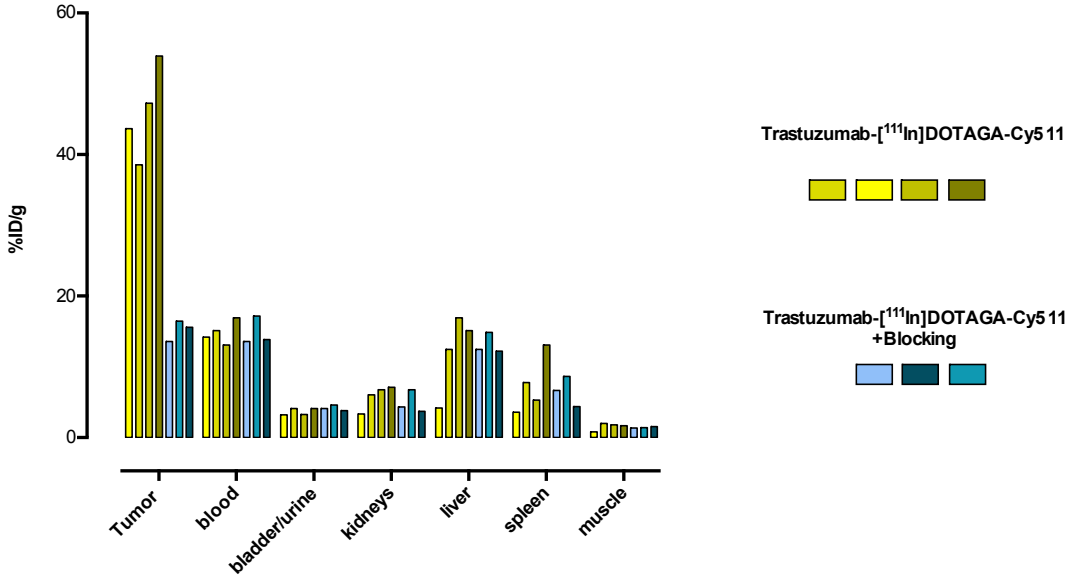
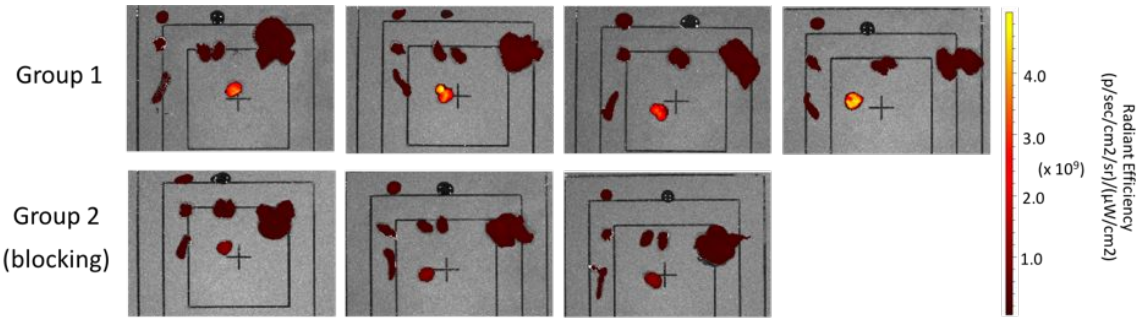


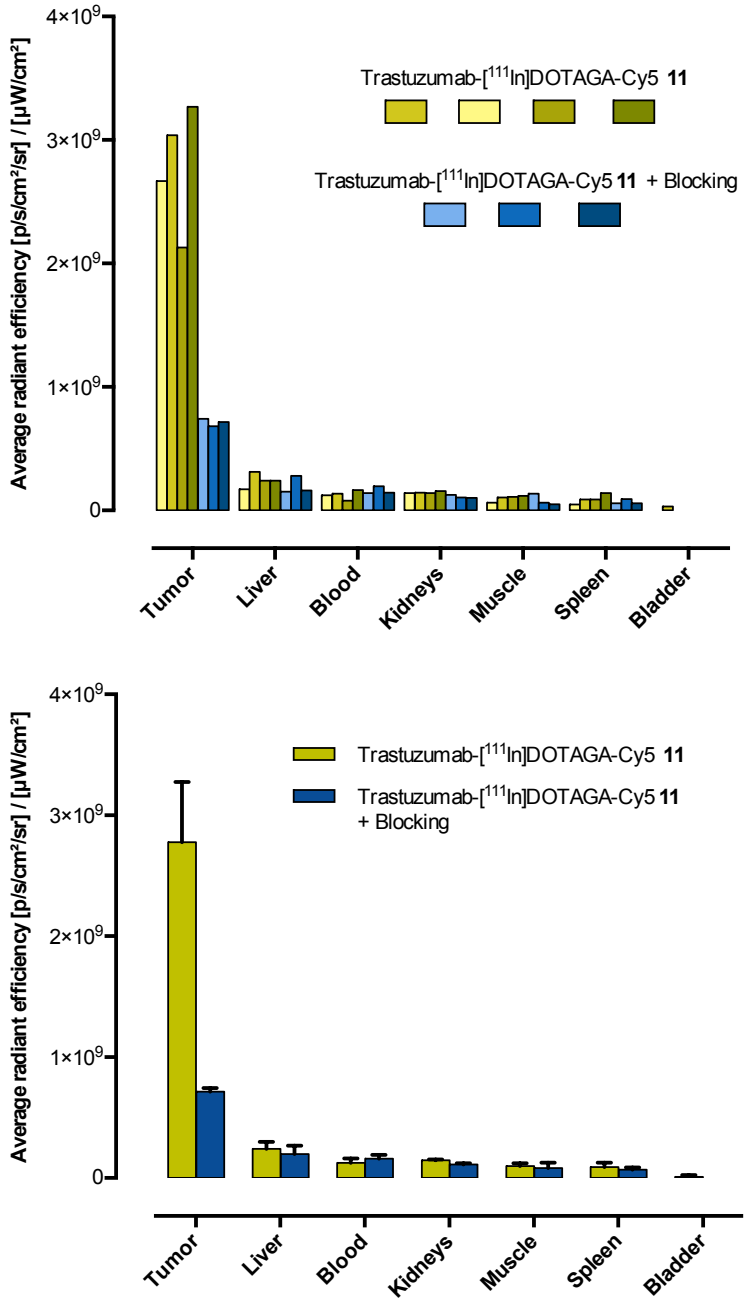
Figure S39. Fluorescence imaging of organs after euthanasia of the mice at 24 h p.i. The fluorescence signal of different isolated organs (tumor, liver, kidneys, blood, muscle, urine, spleen), displayed as radiant efficiency ([p/s/cm²/sr]/[µW/cm²]), is superimposed to white light images (filter mode Ex./Em. 620/670 nm).



Organs position:

blood/plasma	urine	
muscle	kidneys	liver
spleen	tumor	

Figure S40. Biodistribution data for each mouse, by fluorescence imaging. Values are expressed as percentages, related to the sum of average radiant efficiency ([p/s/cm²/sr] / [µW/cm²]) for each collected organ. Diagrams were produced with Prism 6 or 7 software (Graph Pad). (Top) values for each mouse; (bottom) means and SD for the two groups with n = 4 for group 1 and n = 3 for group 2.



REFERENCES

1. Canovas, C., Moreau, M., Bernhard, C., Oudot, A., Guillemin, M., Denat, F., and Goncalves, V. (2018) Site-Specific Dual Labeling of Proteins on Cysteine Residues with Chlorotetrazines. *Angew. Chem. Int. Ed. 57*, 10646–10650.