Supporting Information for

In vitro and *in vivo* trackable titanocene-based complexes using optical imaging or SPECT

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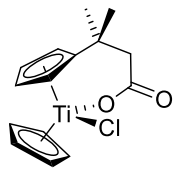
General Informations

All reactions were carried out under an atmosphere of argon using standard Schlenk glassware or in a glove box, unless otherwise stated. All commercially available reagents were used without any further purification. CH_2Cl_2, and DMF were dried using an MBRAUN SPS 800 solvent purification system or distilled under argon from appropriate drying agents and either used directly or stored under argon. DOTA derivative was obtained from CheMatech®.

All of the analyses were performed at the "Plateforme d'Analyse Chimique et de Synthèse Moléculaire de l'Université de Bourgogne" (PACSMUB). The identity and purity of the compounds were unambiguously established using high-resolution mass spectrometry and NMR. High resolution mass spectra were recorded on a Thermo LTQ Orbitrap XL ESI-MS spectrometer. NMR spectra (^{1}H, ^{11}B, ^{19}F, ^{13}C) were recorded on Bruker 300 Avance III, 500 Avance III, or 600 Avance II spectrometers. Chemical shifts are given relative to TMS and were referenced to the residual solvent signal ^{1}H, ^{13}C. The list of abbreviations for signals in NMR are the following: s = singlet, d = doublet, hept = heptuplet, m = multiplet, ps = pseudo-singlet, pt = pseudo-triplet, pq = pseudo-quadruplet, brs = broad singlet. Semi preparative HPLC were performed on a Thermo Scientific Dionex UltiMate 3000 system with an UltiMate 3000 diode array detector according to different methods that will be described thereafter. Analytical HPLC were performed using a Kinetex C18 2.6 µm-100 Å, 2.1*50 mm column with CH_3CN/0.1%TFA and H_2O/0.1%TFA eluents: linear gradient from 5% to 100% [CH_3CN/0.1%TFA] for 5 min, 100% [CH_3CN/0.1%TFA] for 2 min, return to initial conditions by linear gradient for 0 to 95% [H_2O/0.1%TFA] for 1 min. FT-IR spectra were recorded on IR FT BRUCKER Alpha with ALPHA-T module.

Compounds Chart



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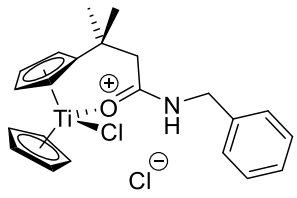
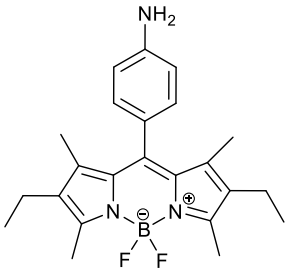


3

Data	Data



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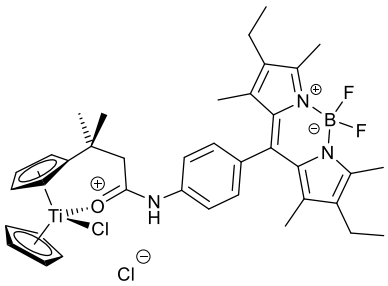
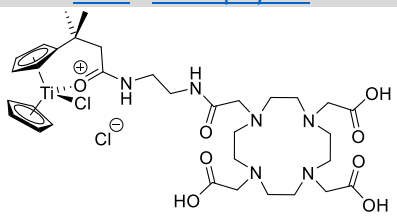


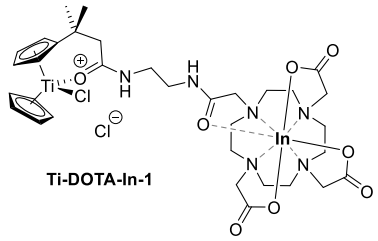
BODI-Ti-1

Data - Photophysics	Data - Photophysics

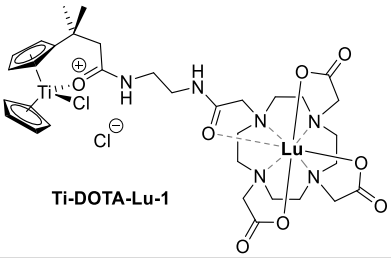


Ti-DOTA-1

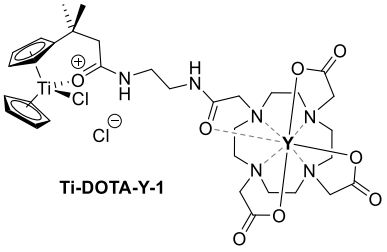
Data



Ti-DOTA-In-1



Ti-DOTA-Lu-1



Ti-DOTA-Y-1

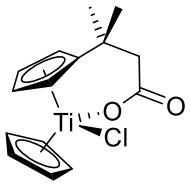
Data - Radiolabelling - Lipophilicity	Data	Data

Biological studies chart

***In vitro* evaluation**	**Imaging**
Stability studies	Optical imaging
Cell uptake of **Ti-DOTA-In-1**	Real time optical imaging
Cytotoxic assays	Biodistribution

Synthetic procedures

Compound 1



1

The compound **1** was synthesized following a reported procedure.[1] ^{1}H and ^{13}C NMR data are in agreement with literature data.

^{1}H NMR (300 MHz, $CDCl_3$, 300K) δ (ppm): 1.14 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 2.26 (d, ^{2}J = 14.8 Hz, 1H, CH_2^{disat}), 2.64 (d, ^{2}J = 14.8 Hz, 1H, CH_2^{disat}), 6.05 – 5.97 (m, 1H, Cp_{subst}), 6.11 – 6.05 (m, 1H, Cp_{subst}), 6.51 – 6.37 (m, 1H, Cp_{subst}), 6.47 (s, 5H, Cp_{free}), 6.68 – 6.56 (m, 1H, Cp_{subst}).

^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$, 300K) δ (ppm): 28.57 (CH_3), 29.46 (CH_3), 34.22 (C_q), 47.94 (CH_2), 105.85 (Cp_{subst}), 114.53 (Cp_{subst}), 119.44 (Cp_{subst}), 119.99 (Cp_{free}), 122.42 (Cp_{subst}), 147.89 (Cp_{subst}), 174.56 (CO).

IR (ATR) ν(cm^{-1}) = 3110, 3090, 2958, 1649 (strong, sharp, stretching, C=O), 1493, 1461, 1443 1415, 1362, 1313, 1295, 1276, 1193, 1158, 1017, 985, 951, 893, 864, 823 (medium, sharp, bending, C=C-H_{alkene}), 677, (medium, sharp, bending, C=C-H_{alkene}), 654, (medium, sharp, bending, C=C-H_{alkene}).

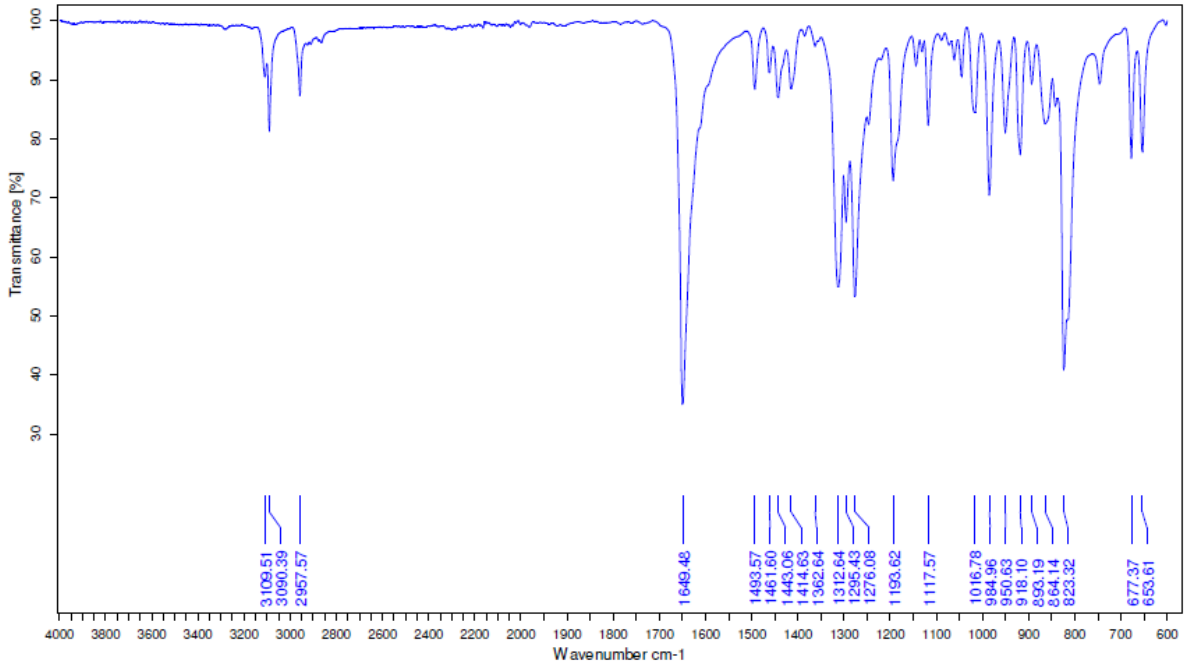
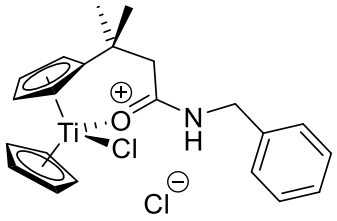


Figure 1: *IR spectrum of compound* ***1***

Compound 3



3

The compound **3** was synthesized adapting a reported procedure.[1] In a schlenk tube, titanocene-carboxylate **1** (80 mg, 0.256 mmol, 1 eq) was dissolved in $SOCl_2$ (0.8 mL, 11 mmol, 43 eq) and stirred for 1 h at room temperature. Excess $SOCl_2$ was removed under vacuum at 50°C for 3 h. The resulting titanocene-acyl chloride **2** was then dissolved in CH_2Cl_2 (1.5 mL) and added to a mixture of NaH (31 mg, 1.28 mmol, 5 eq) and benzylamine (41 mg, 0.384 mmol, 1.5 eq) in CH_2Cl_2 (2 mL). The solution was stirred at room temperature for 16 h. After filtration through celite the resulting solution was washed with 1M HCl containing 1 g of NaCl each 10 mL (3x2 mL). The organic layer was dried over $MgSO_4$ and the solvent removed under vaccum to give **3** as an orange powder(69mg, yield=62%).

^{1}H NMR (500 MHz, $CDCl_3$, 300K) δ (ppm) : 1.24 (s, 3H, CH_3) , 1.28 (s, 3H, CH_3), 2.91 (d, 1H, $^2J_{H-H}$ = 14.0 Hz, CO-CH_2^{diast}), 3.38 (d, 1H, $^2J_{H-H}$ = 13.3 Hz, CO-CH_2^{diast}), 4.36 (dd, 1H, $^2J_{H-H}$ = 14.5, ^3J = 6.1 Hz, N-CH_2^{diast}), 4.50 (dd, 1H, $^2J_{H-H}$ = 14.5, ^3J = 6.1 Hz, N-CH_2^{diast}), 6.03 (m, 1H, Cp_{subst}), 6.42 (s, 5H, Cp_{free}), 6.53 (m, 1H, Cp_{subst}), 6.81 (m, 1H, Cp_{subst}), 6.90 (m, 1H, Cp_{subst}), 7.26 (t, 1H, ^3J = 7.4 Hz, H^{Ar}_{para}), 7.34 (t, 2H, ^3J = 7.4 Hz, $H^{Ar}_{méta}$), 7.40 (d, 2H, ^3J = 7.0 Hz, H^{Ar}_{ortho}), 12.87 (s, 1H, NH).

^{13}C NMR (75 MHz, $CDCl_3$, 300K) δ (ppm) : 26.10 (CH_3), 29.81 (CH_3), 34.63 (C_q), 45.13 (CH_2), 46.75 (CH_2), 109.00 (Cp_{subst}), 115.98(Cp_{subst}), 119.43 (Cp_{subst}), 120.97 (Cp_{free}), 124.56 (C_{Ar}), 127.78 (C_{Ar} *2), 127.85 (C_{Ar}), 128.80 (C_{Ar} *2), 136.49(Cp_{subst}), 150.41 (Cp_{subst}), 176.38 (CO).

HRMS (ESI) : (MeOH), *positive mode exact mass for* $[M\text{-}Cl+OMe]^+$ ($[C_{23}H_{28}NO_2Ti_1]^+$) calculated : 398.16050Da / measured : 398.15751 Da.

IR (ATR) ν(cm^{-1}) = 3400 (medium, broad, bending, NH), 2962 (medium, broad, stretching, alkyle), 1604 (strong, sharp, stretching, C=O), 1551, 1433, 1370, 1259, 1017 (medium, sharp, stretching, C=C), 822 (medium, sharp, bending C=C), 731, 698.

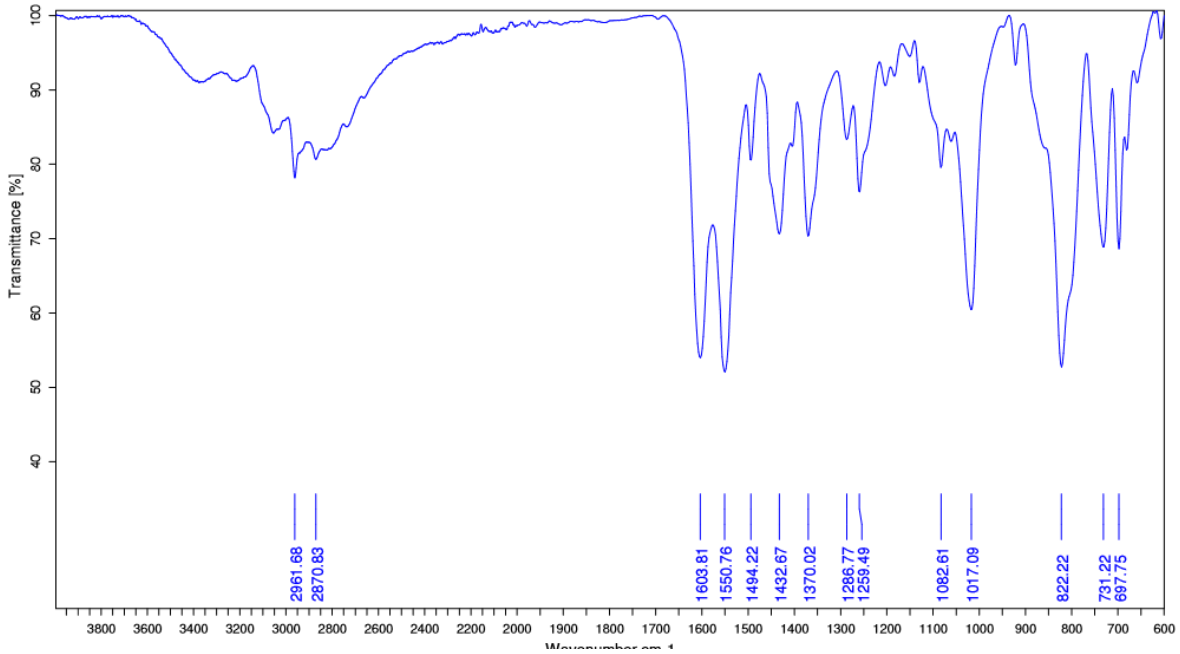
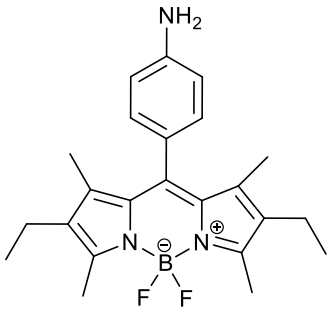


Figure 2: *IR spectrum of compound* ***3***

Compound 4



4

The compound **4** was synthesized following a reported procedure.[2]

^{1}H NMR ($CDCl_3$, 300 MHz, 300K) δ (ppm): 0.98 (t, $^3J_{H-H}$ = 7.4 Hz, 6H, CH_3 *2), 1.40 (s, 6H, CH_3 *2), 2.30 (q, $^3J_{H-H}$ = 7.4 Hz, 4H, CH_2 *2), 2.52 (s, 6H, 2 CH_3*2), 3.82 (s, 2H, NH_2), 6.77 (d, $^3J_{H-H}$ = 8.4 Hz, 2H, CH^{Ar} *2), 7.01 (d, $^3J_{H-H}$ = 8.5 Hz, 2H, CH^{Ar} *2).

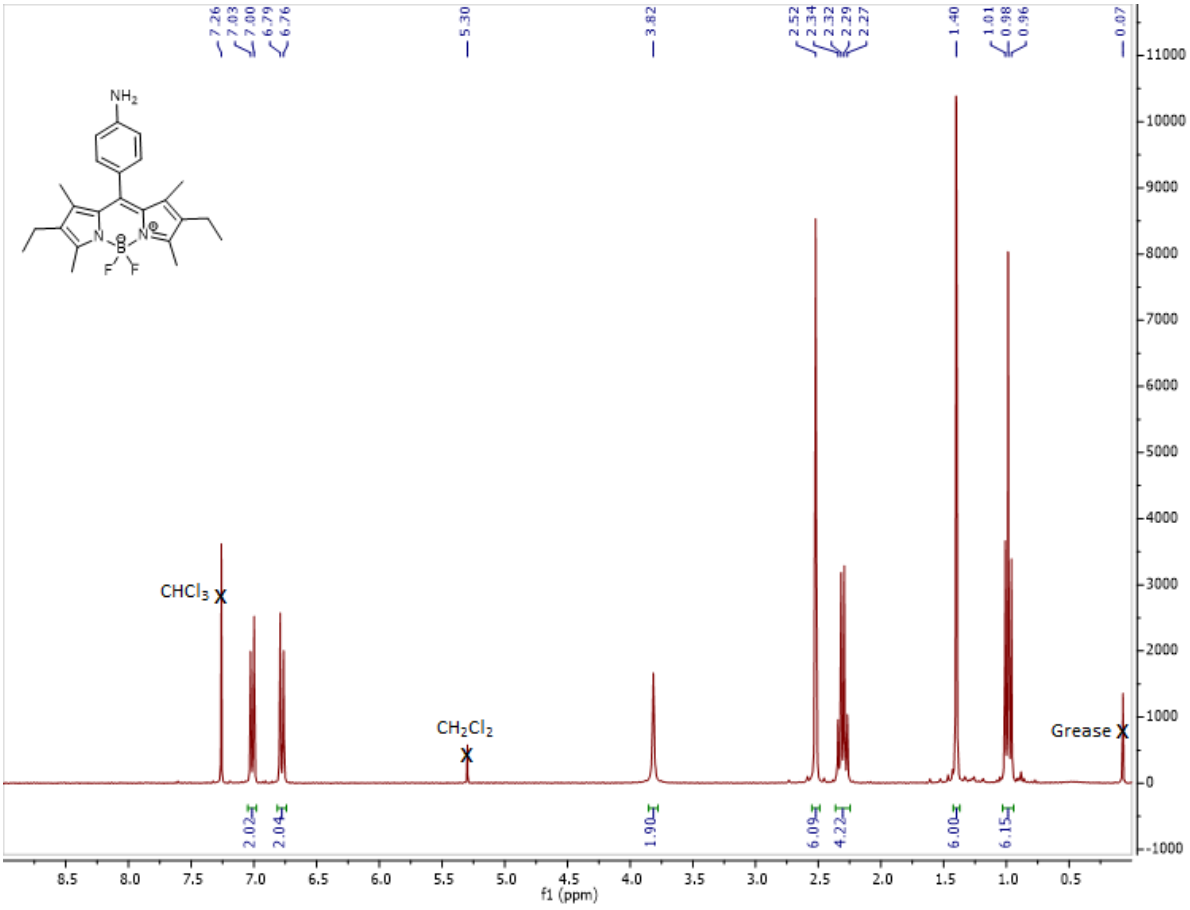
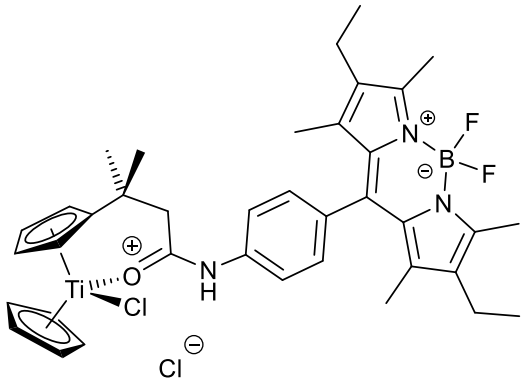


*Figure 3: ^{1}H-NMR spectrum of compound **4** ($CDCl_3$, 300 MHz)*

BODI-Ti-1



BODI-Ti-1

In a schlenk tube, titanocene-carboxylate **1** (40.6 mg, 0.130 mmol, 1 eq) was dissolved in $SOCl_2$ (0.39 mL, 5.454 mmol, 42 eq) and stirred for 1 h at room temperature. Excess $SOCl_2$ was removed under vacuum at 50°C for 3 h. The resulting titanocene-acyl chloride was then dissolved in $CHCl_3$ (0.65 mL) and added to a mixture of NaH (15.6 mg, 0.649 mmol, 5 eq) and BODIPY-aniline **4** (51.8 mg, 0.131 mmol, 1.01 eq) in $CHCl_3$ (0.65 mL). The solution was stirred at room temperature for 48 h. After filtration by canula, the resulting solution was evaporated and dry under vacuum, to obtain **BODI-Ti-1** as a red powder (54 mg, yield : 57 %).

^{1}H NMR ($CDCl_3$, 500 MHz, 300K) δ (ppm): 0.97 (t, $^3J_{H-H}$ = 7.6 Hz, 6H, 2 $CH_{3\ BODIPY}$), 1.32 (s, 6H, 2 $CH_{3\ BODIPY}$ *2), 1.41 (s, 3H, CH_3^{diast}), 1.44 (s, 3H, CH_3^{diast}), 2.29 (q, $^3J_{H-H}$ = 7.5 Hz, 4H, $CH_{2\ BODIPY}$ *2), 2.52 (s, 6H, $CH_{3\ BODIPY}$ *2), 3.37 (brs, 1H, CH_2^{diast}), 3.73 (brs, 1H, CH_2^{diast}) 6.06 (brs, 1H, Cp_{subst}), 6.67 (s, 5H, Cp_{free}), 6.70 (brs, 1H, Cp_{subst}), 7.06 (brs, 1H, Cp_{subst}), 7.36 (d, $^3J_{H-H}$ = 7.8 Hz, 2H, $CH_{aniline}$ *2), 7.41 (brs, 1H, Cp_{subst}), 7.89 (d, $^3J_{H-H}$ = 7.9 Hz, 2H, $CH_{aniline}$ *2), 13.83 (s, 1H, NH).

^{11}B{^{1}H} NMR ($CDCl_3$, 160 MHz, 300K) δ (ppm): 0.78 (t, $^{11}BF_{2\ BODIPY}$).

^{19}F{^{1}H} NMR ($CDCl_3$, 470.5 MHz, 300K) δ (ppm): -145.78 (q, $BF_{2\ BODIPY}$).

^{13}C{^{1}H} NMR ($CDCl_3$, 125.7 MHz, 300K) δ (ppm): 12.15 ($CH_{3\ BODIPY}$), 12.68 ($CH_{3\ BODIPY}$), 14.73 ($CH_{3\ BODIPY}$), 17.21 ($CH_{2\ BODIPY}$), 25.81 (CH_3^{diast}), 31.03 (CH_3^{diast}), 35.54 (C_q), 48.26 (CH_2^{diast}), 110.20 (Cp_{subst}), 116.78 (Cp_{subst}), 120.63 (Cp_{subst}), 121.73 (Cp_{free}), 123.00 (C_{Ar}), 125.07 (Cp_{subst}), 129.52 (C_{Ar}), 130.80 ($C_{q\ BODIPY}$), 133.13 ($C_{q\ BODIPY}$), 135.03 ($C_{q\ BODIPY}$), 135.94 ($C_{q\ BODIPY}$), 138.16 ($C_{q\ BODIPY}$), 138.94 ($C_{q\ BODIPY}$), 151.52 ($C_{q\ BODIPY}$), 154.26 ($C_{q\ BODIPY}$), 175.83 (CO).

HRMS (ESI): (DCM), *positive mode exact mass for* $[M]^+$ ($[C_{38}H_{44}BClF_2N_3OTi]^+$) calculated : 690.27080 Da / measured : 690.26988 Da.

IR (ATR) ν(cm^{-1}) = 3400 (medium, broad, bending, NH), 2961 (medium, broad, stretching, alkyle), 1537 (strong, sharp, stretching, C=O), 1473, 1317, 1185, 1059, 975, 822 (medium, sharp, bending C=C).

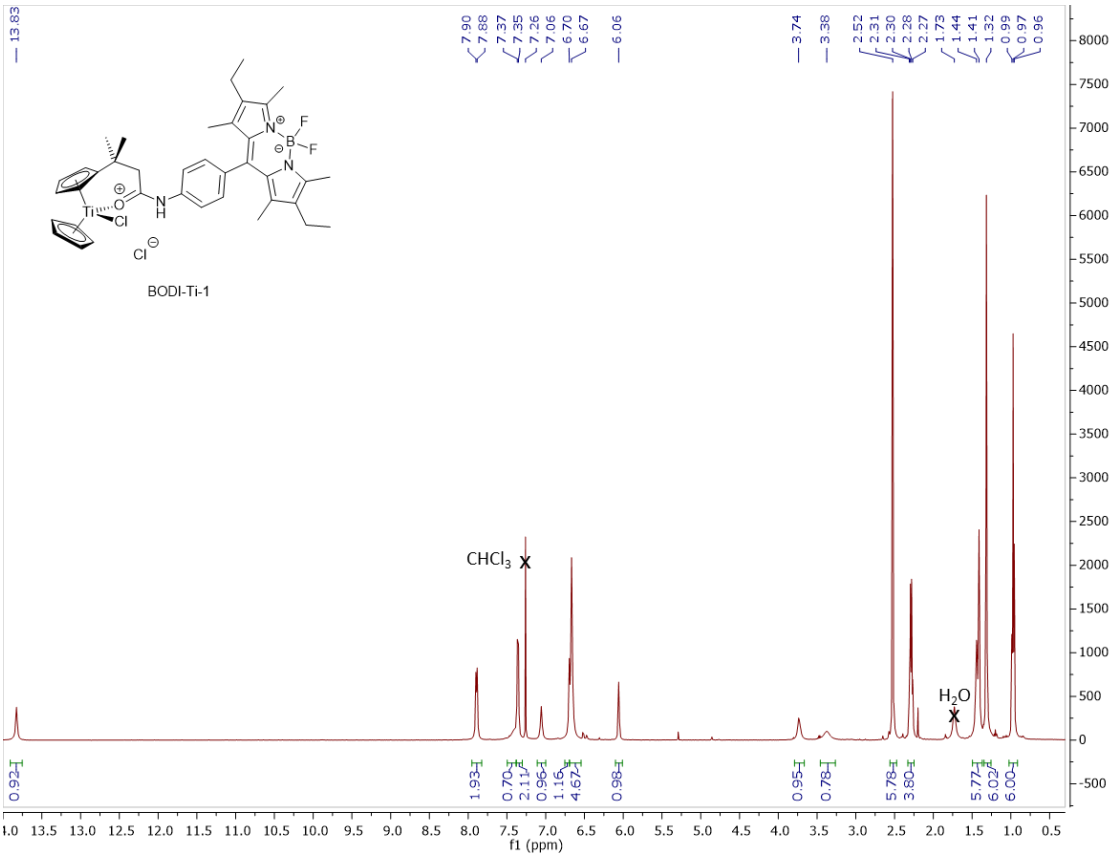


Figure 4: *^{1}H-NMR spectrum of* ***BODI-Ti-1*** *($CDCl_3$, 500 MHz)*

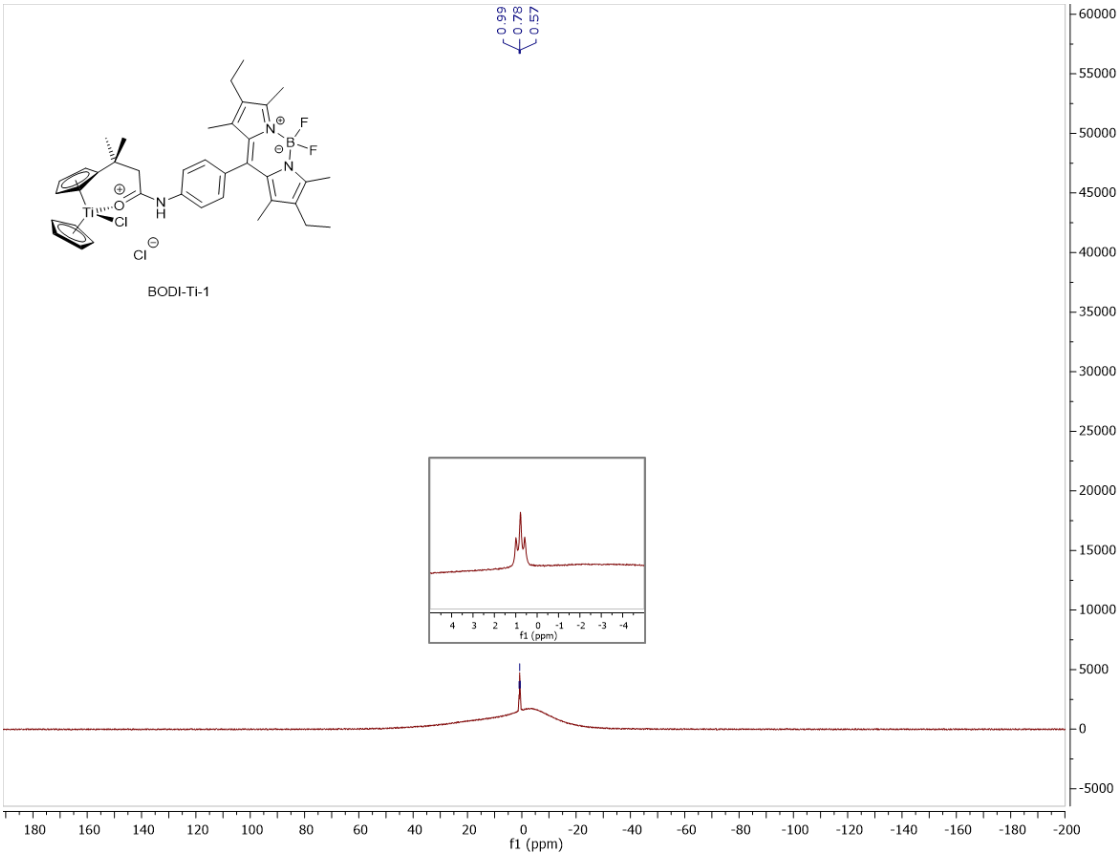


Figure 5: *^{11}B-NMR spectrum of* ***BODI-Ti-1*** *($CDCl_3$, 160 MHz)*

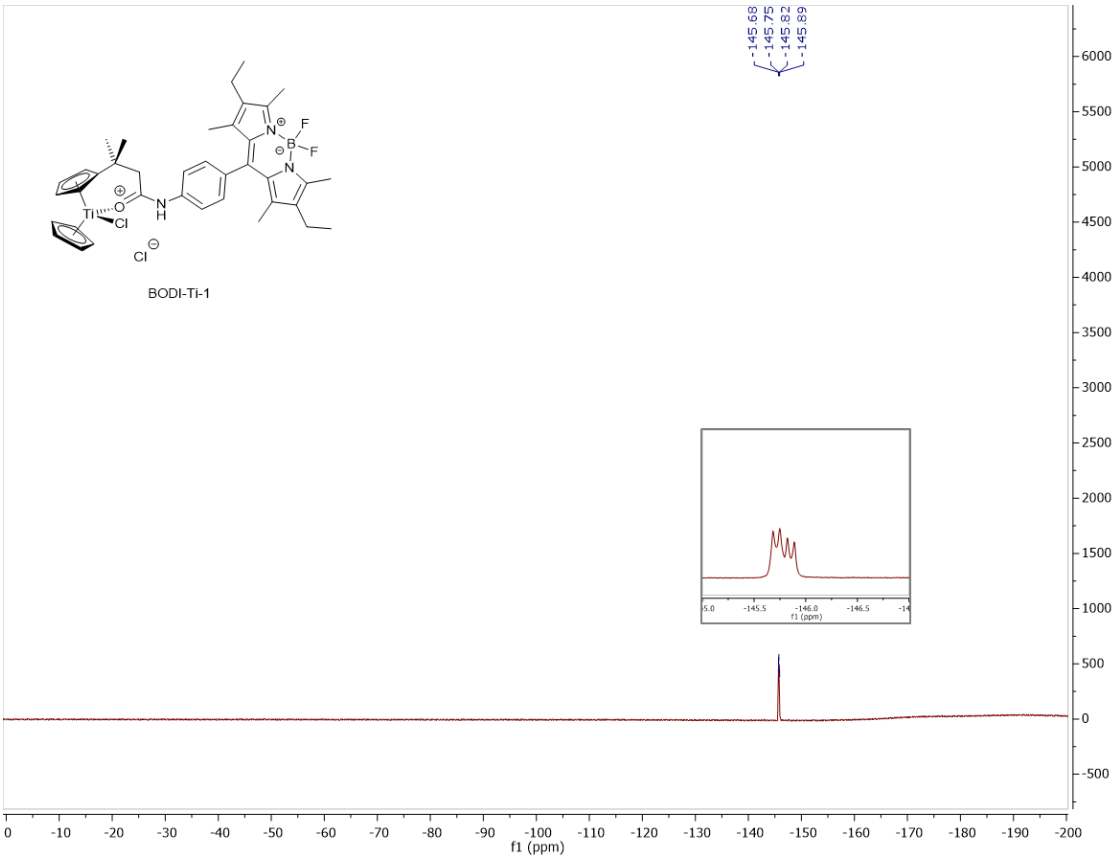


Figure 6: *^{19}F-NMR spectrum of* ***BODI-Ti-1*** *($CDCl_3$, 470.5 MHz)*

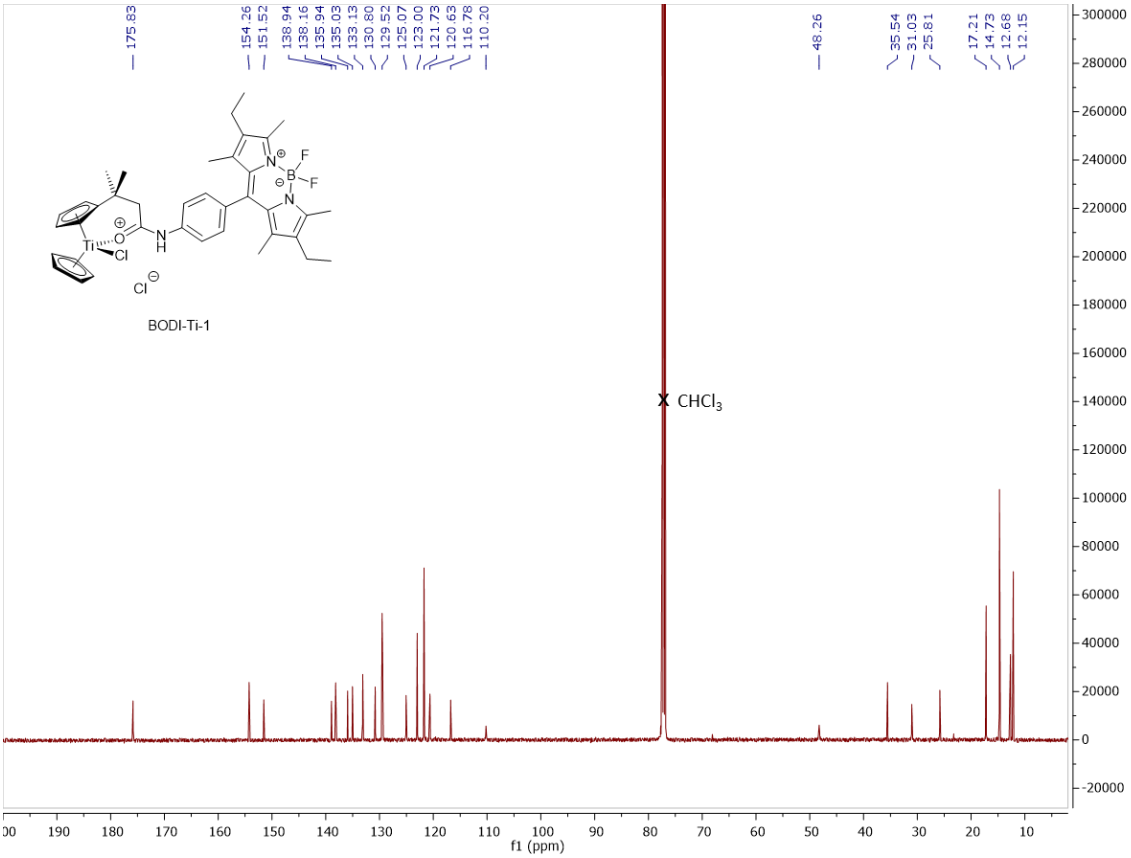


Figure 7: *^{13}C-NMR spectrum of* ***BODI-Ti-1*** *($CDCl_3$, 125 MHz)*

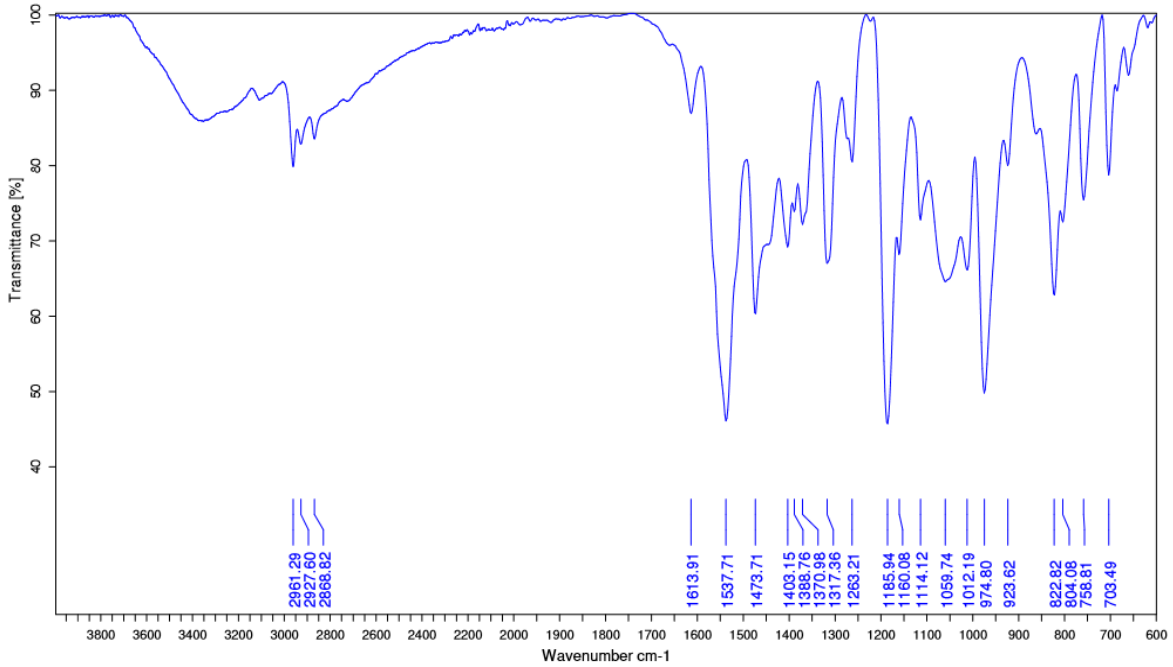
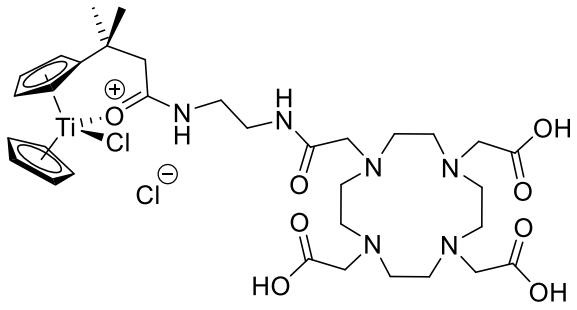


Figure 8: *IR spectrum of* ***BODI-Ti-1***

Ti-DOTA-1



Ti-DOTA-1

In a schlenk tube, titanocene-carboxylate **1** (102 mg, 0.326 mmol, 1 eq) was dissolved in $SOCl_2$ (1 mL, 14 mmol, 42 eq) and stirred for 1 h at room temperature. Excess $SOCl_2$ was removed under vacuum at 50°C for 3 h. The resulting titanocene-acyl chloride **2** was then dissolved in CH_2Cl_2 (2 mL) and added to a mixture of NaH (39 mg, 1.630 mmol, 5 eq) and DO3AtBu-N-(2-aminoethyl)ethanamide **5** (201 mg, 0.326 mmol, 1 eq) in CH_2Cl_2 (2 mL). The solution was stirred at room temperature for 48 h. After filtration through celite, the resulting solution was washed with HCl 1M (5mL) and stirred for 1 h in order to hydrolyse the protecting ester arms. The aqueous layer was then isolated and evaporated to yield **Ti-DOTA-1** as a red powder (212 mg, crude yield = 84%).

Semi-preparative HPLC purification was performed on a BetaBasic C18, 5 µm, 30*150 mm column with the following eluent system : 100% [H_2O/0.1%TFA] for 10 min, linear gradient from 0% to 40% [CH_3CN/0.1%TFA] for 40 min, 40% to 100% [CH_3CN/0.1%TFA] for 5 min, return to initial conditions by linear gradient from 0 to 100% [H_2O/0.1%TFA] for 1 min. Purification yield = 30%.

^{1}H NMR (600 MHz, DMSO, 300K) δ (ppm): 1.14 (s, 3H, CH_3), 1.17 (s, 3H, CH_3), 2.60 (d, 1H, ^2J = 15.2 Hz, CH_2^{diast}), 3.06 (d, 1H, ^2J = 15.2 Hz, CH_2^{diast}), 4.16 – 3.09 (m, 28H, DOTA), 6.09 – 6.04 (m, 1H, Cp_{subst}), 6.71 – 6.66 (m, 1H, Cp_{subst}), 6.85 (s, 5H, Cp_{free}), 6.99 – 6.93 (m, 1H, Cp_{subst}), 7.53 – 7.46 (m, 1H, Cp_{subst}), 9.06 (s, 1H, NH), 11.65 (s, 1H, NH).

^{13}C{^{1}H} NMR (151 MHz, DMSO, 300K) δ (ppm): 26.98 (CH_3), 28.71 (CH_3), 45.22 (CH_2), 33.64 (C_q), 54.73, 53.91, 52.67, 50.62, 48.39, 109.72 (Cp_{subst}), 117.63 (Cp_{subst}), 119.45 (Cp_{subst}), 121.66 (Cp_{free}), 123.87 (Cp_{subst}), 149.21 (Cp_{subst}), 168.56 (COOH), 171,52 (NH-CO-DOTA), 176.35 (NH-CO-Ti).

HPLC: t_r = 2.51 min, purity > 99%.

HR-MS (ESI): *positive mode exact mass for* $[M\text{-}Cl+OH]^+$ ($[C_{33}H_{48}N_6O_9Ti_1]^+$) calculated : 705.30859 Da / measured : 705.30707 Da.

IR (ATR) ν(cm^{-1}) = 3072, 2964, 1726 (strong, sharp, stretching, C=O), 1676 (strong, sharp, stretching, C=O), 1609 (strong, sharp, stretching, C=O), 1555 (strong, sharp, stretching, C=O), 1456, 1435, 1385, 1353, 1191, 1162, 1086, 831 (medium, broad, bending, $C=C\text{-}H_{alkene}$).
A broad band corresponding to the signal of water can be seen due to the high hygroscopic character of **Ti-DOTA-1**.

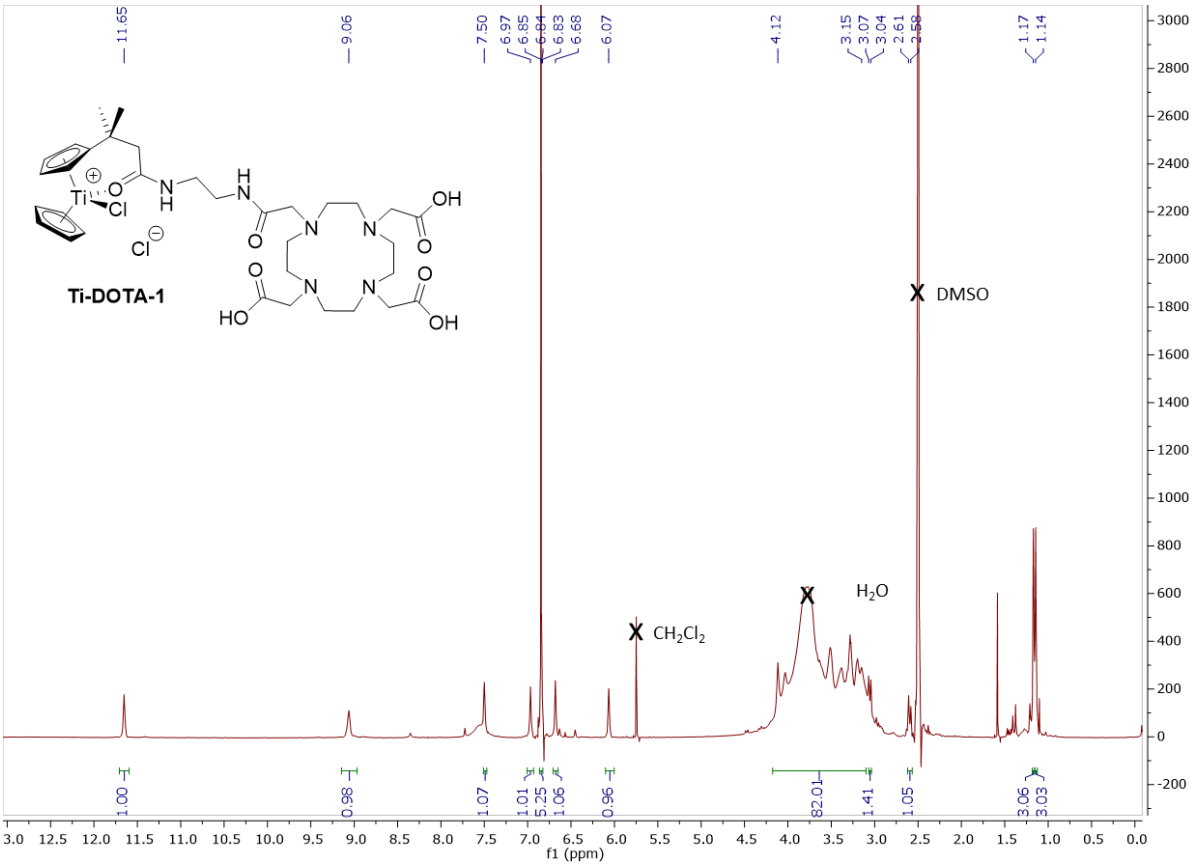


Figure 9: *^{1}H-NMR spectrum of **Ti-DOTA-1** (DMSO, 600 MHz)*

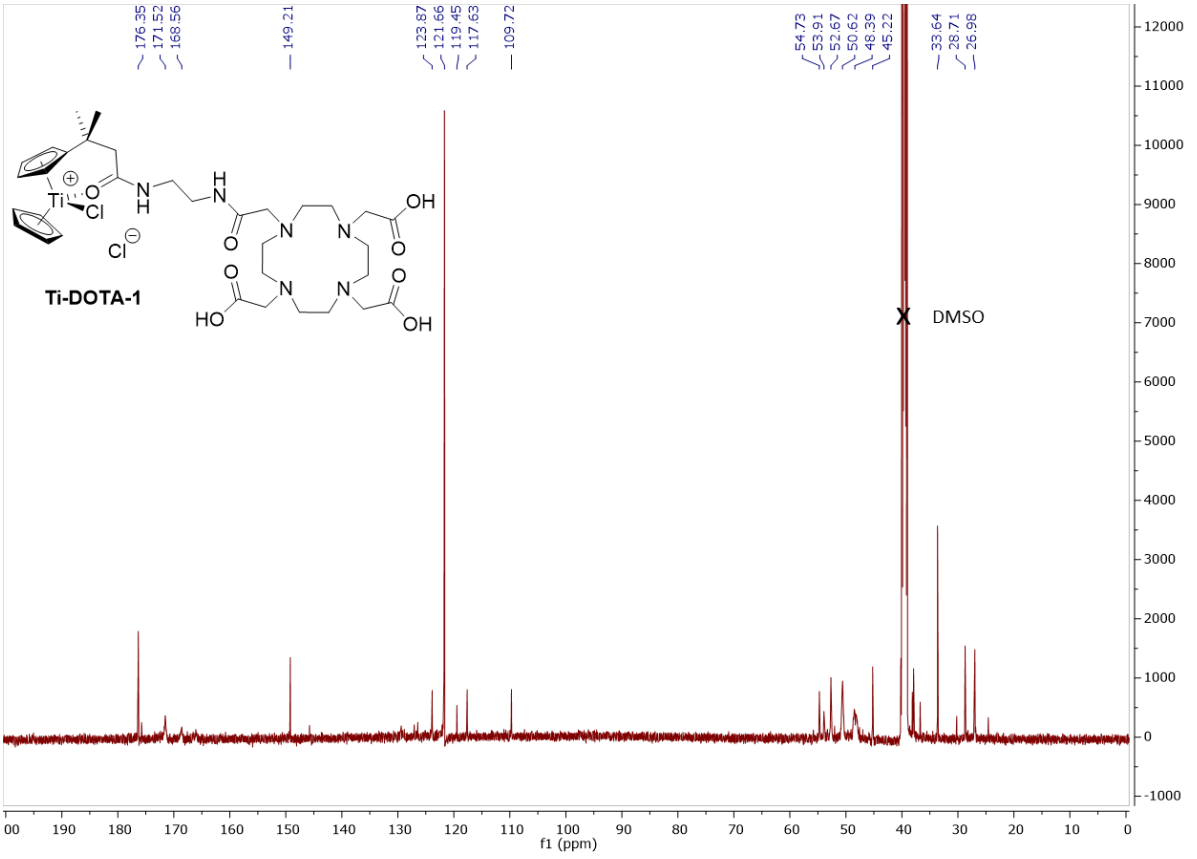
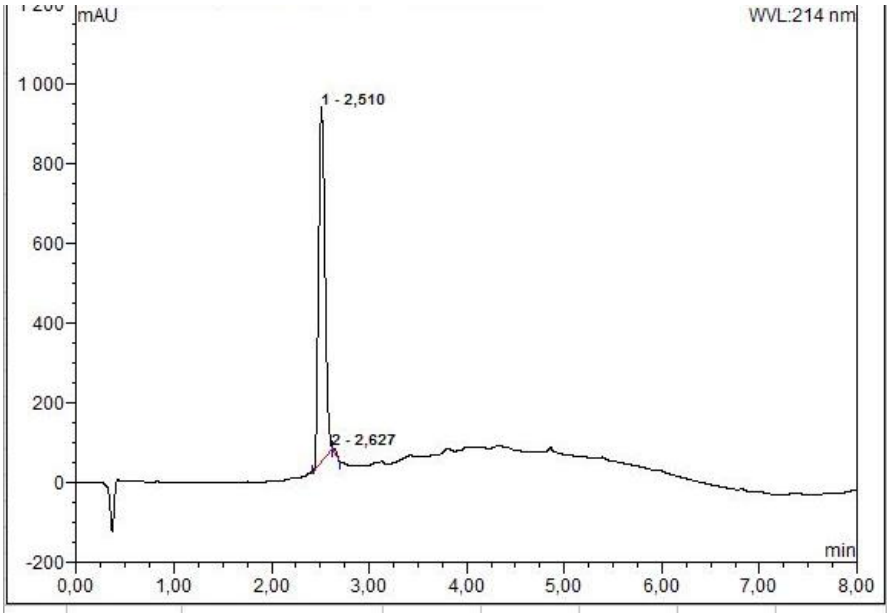


Figure 10: *^{13}C-NMR spectrum of **Ti-DOTA-1** (DMSO, 151 MHz)*



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	2,51	n.a.	889,107	66,861	99,50	n.a.	BMb*
2	2,63	n.a.	0,617	0,338	0,50	n.a.	bMB*
Total:			889,724	67,199	100,00	0,000	

Figure 11: *HPLC chromatogram of* ***Ti-DOTA-1***

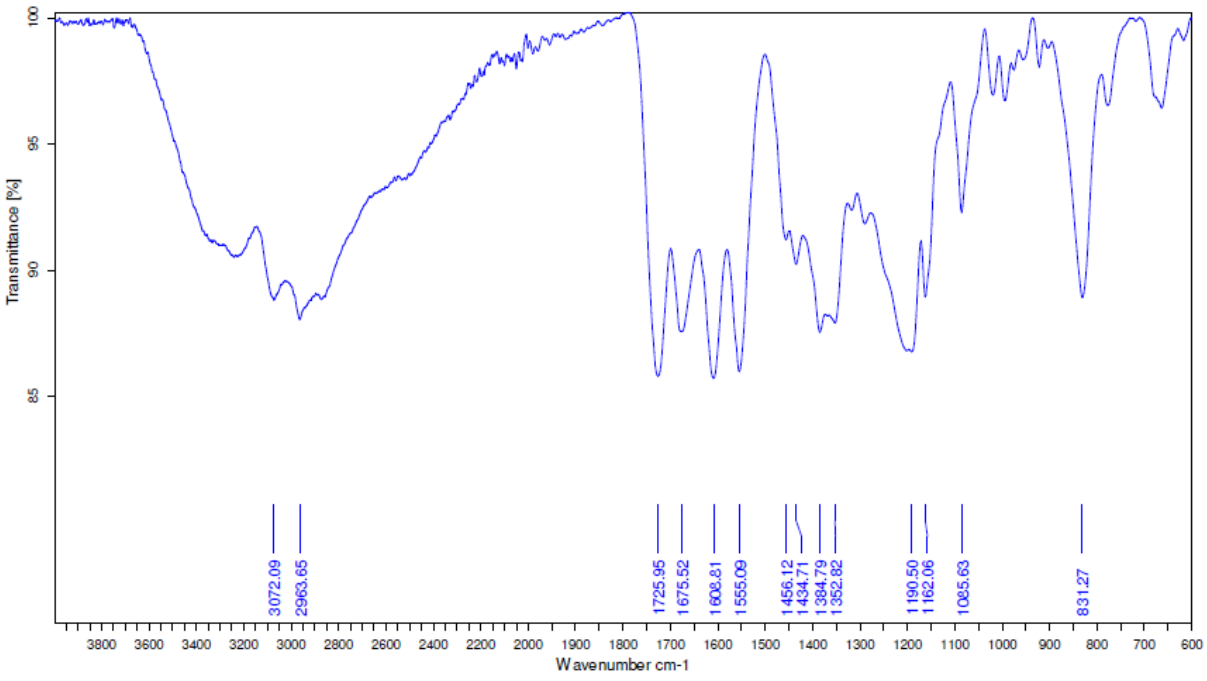
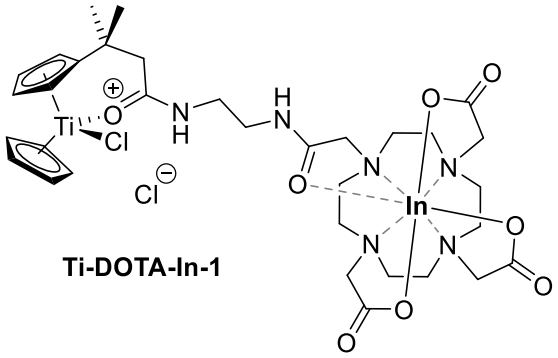


Figure 12: *IR spectrum of* ***Ti-DOTA-1***

Ti-DOTA-In-1



Ti-DOTA-In-1

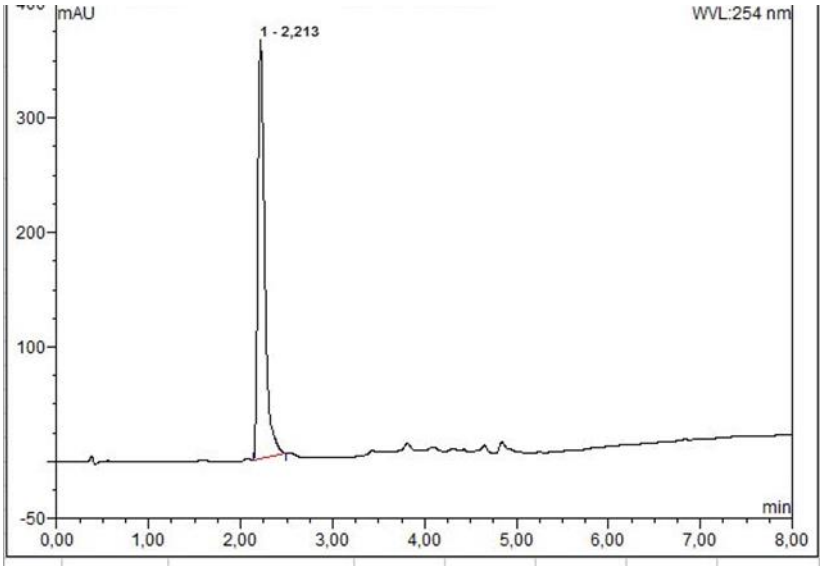
$InCl_3$ (6 mg, 0.0271 mmol, 1.05 eq) is dissolved in HCl 0.05M (4.5 mL). An aqueous solution of 1M NH_4OAc (0.5 mL) is added to reach the desired pH value (≈5.7). **Ti-DOTA-1** (20 mg, 0.0257 mmol, 1 eq) in 0.5mL H_2O is then added and the mixture is stirred at 37°C for 1h. The solvent is then evaporated under vacuum.
Semi-preparative HPLC purification was performed on a BetaBasic C18, 5 µm, 30*150 mm column with the following eluent system: 100% [H_2O/0.1%TFA] for 15 min, linear gradient from 0% to 40% [CH_3CN/0.1%TFA] for 40 min, 40% to 100% [CH_3CN/0.1%TFA] for 5 min, return to initial conditions by linear gradient from 0 to 100% [H_2O/0.1%TFA] for 1 min. (14% ACN)

Ti-DOTA-In-1 is obtained as an orange powder (10mg, 40%)

HPLC : t_r = 2.21 min, purity > 99%

HR-MS (ESI) : *positive mode exact mass for* $[M\text{-}Cl+OH]^+$($[C_{33}H_{48}In_1N_6O_9Ti_1]^+$) calculated : 835.19955 Da / measured : 835.19927 Da

IR (ATR) ν(cm^{-1}) = 3253, 3095, 2971, 1772, 1714, 1614 (strong, broad, stretching, C=O), 1441, 1402, 1372, 1320, 1174, 1132, 1084, 1014, 976, 925, 829, 795 (medium, broad, bending, C=C-H_{alkene}), 718, (medium, sharp, bending, C=C-H_{alkene}), 704 (medium, sharp, bending, C=C-H_{alkene}).



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	2,21	n.a.	364,761	31,887	100,00	n.a.	BMB
Total:			364,761	31,887	100,00	0,000	

Figure 13: *HPLC chromatogram of* ***Ti-DOTA-In-1***

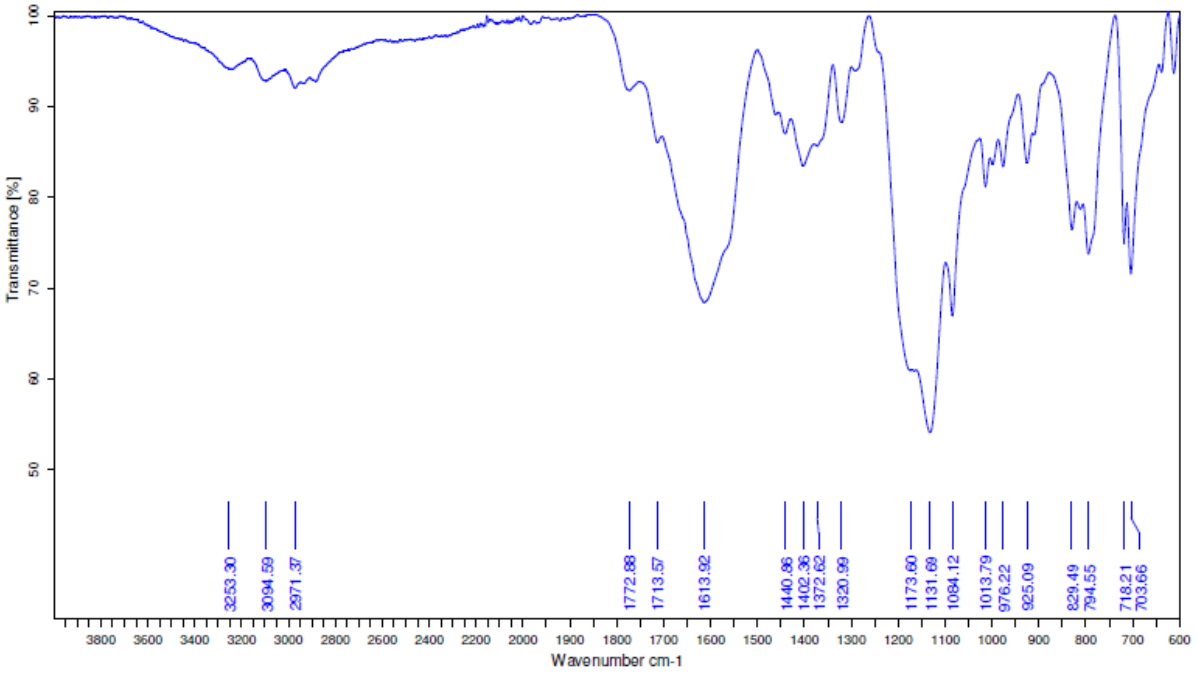
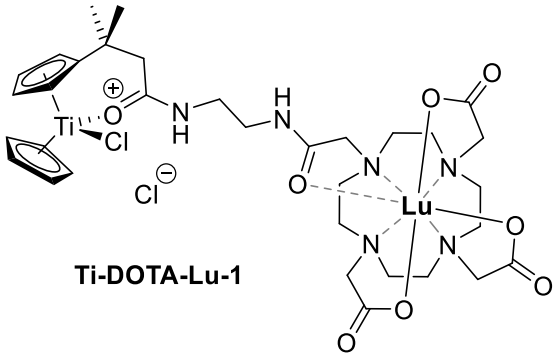


Figure 14: *IR spectrum of* ***Ti-DOTA-In-1***

Ti-DOTA-Lu-1



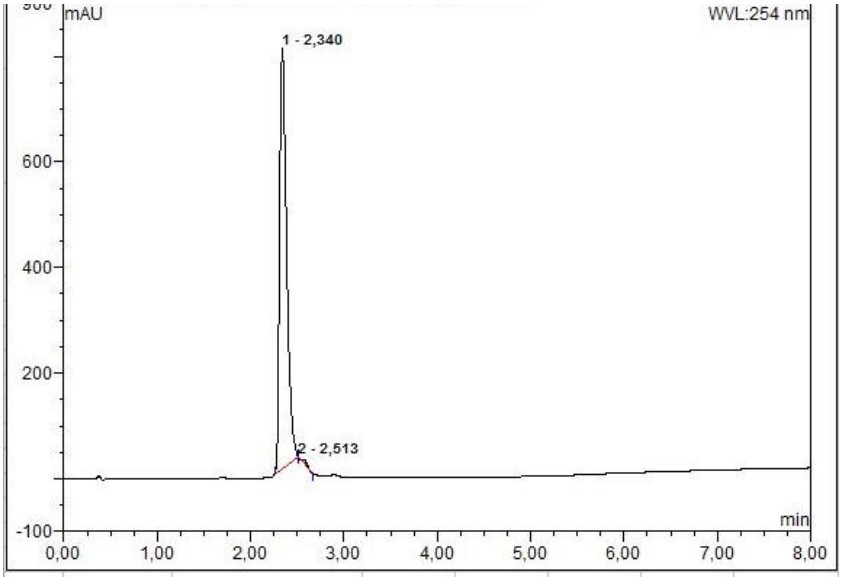
Ti-DOTA-Lu-1

$Lu(NO_3)_3$ (9.8 mg, 0.0271 mmol, 1.05 eq) is dissolved in HCl 0.05M (4.5 mL). An aqueous solution of 1M NH_4OAc (0.5 mL) is added to reach the desired pH value (≈5.7). **Ti-DOTA-1** (20 mg, 0.0257 mmol, 1 eq) in 0.5mL H_2O is then added and the mixture is stirred at 37°C for 1h. The solvent is then evaporated under vacuum.
Semi-preparative HPLC purification was performed on a BetaBasic C18, 5 µm, 30*150 mm column with the following eluent system: 100% [H_2O/0.1%TFA] for 15 min, linear gradient from 0% to 40% [CH_3CN/0.1%TFA] for 40 min, 40% to 100% [CH_3CN/0.1%TFA] for 5 min, return to initial conditions by linear gradient from 0 to 100% [H_2O/0.1%TFA] for 1 min. (18% ACN).

Ti-DOTA-Lu-1 is obtained as an orange powder (5.9mg, 19%)

HPLC: t_r = 2.34 min, purity > 99%

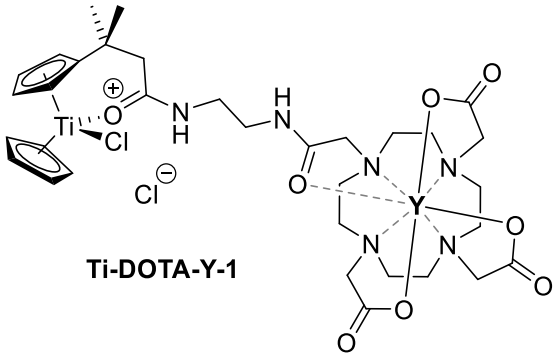
HR-MS (ESI): *positive mode exact mass for* $[M\text{-}HCl]^+$ ($[C_{33}H_{48}Lu_1N_6O_9Ti_1]^+$) calculated : 878.23237 Da / measured : 878.23408 Da



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	2,34	n.a.	796,809	69,608	99,51	n.a.	BMb*
2	2,51	n.a.	0,633	0,339	0,49	n.a.	bMB*
Total:			797,441	69,947	100,00	0,000	

Figure 15: *HPLC chromatogram of* ***Ti-DOTA-Lu-1***

Ti-DOTA-Y-1



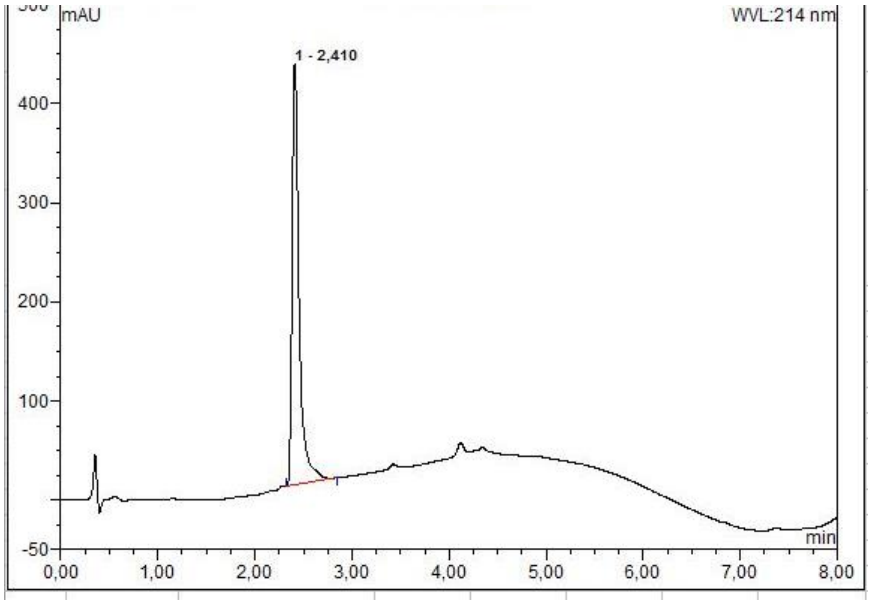
Ti-DOTA-Y-1

YCl_3 (8.2 mg, 0.0271 mmol, 1.05 eq) is dissolved in HCl 0.05M (4.5 mL). An aqueous solution of 1M NH_4OAc (0.5 mL) is added to reach the desired pH value (≈5.5). **Ti-DOTA-1** (20 mg, 0.0257 mmol, 1 eq) in 0.5mL H_2O is then added and the mixture is stirred at 37°C for 1h. The solvent is then evaporated under vacuum.
Semi-preparative HPLC purification was performed on a BetaBasic C18, 5 µm, 30*150 mm column with the following eluent system: 100% [H_2O/0.1%TFA] for 15 min, linear gradient from 0% to 40% [CH_3CN/0.1%TFA] for 40 min, 40% to 100% [CH_3CN/0.1%TFA] for 5 min, return to initial conditions by linear gradient from 0 to 100% [H_2O/0.1%TFA] for 1 min. (18% ACN)

Ti-DOTA-Y-1 is obtained as an orange powder (4.5mg, 19%)

HPLC: t_r = 2.41 min, purity >99%

HR-MS (ESI): *positive mode exact mass for* $[M\text{-}Cl+OH]^+$([$C_{33}H_{48}Y_1N_6O_9Ti_1]^+$) calculated: 809.20152 Da / measured : 809.20287 Da



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	2,41	n.a.	424,679	35,838	100,00	n.a.	BMB
Total:			424,679	35,838	100,00	0,000	

Figure 16: *HPLC chromatogram of* ***Ti-DOTA-Y-1***

Photophysical measurements

UV-Visible absorption spectra were recorded on a JASCO V630BIO spectrometer. The steady-state fluorescence emission spectra were obtained by using a JASCO FP8500 spectrofluorometer instrument. All fluorescence spectra were corrected for instrument response. The fluorescence quantum yields (Φ_F) were calculated from equation:

$$\frac{\Phi_F}{\Phi_{FR}} = \frac{n^2}{n_R^2} \times \frac{\int_0^\infty I_F(\lambda_E, \lambda_F) d\lambda_F}{\int_0^\infty I_{FR}(\lambda_E, \lambda_F) d\lambda_F} \times \frac{1-10^{-A_R(\lambda_E)}}{1-10^{-A(\lambda_E)}}$$

Φ_F and Φ_{FR} are fluorescence quantum yields of the compound and the reference respectively. $A(\lambda_E)$ and $A_R(\lambda_E)$ are the absorbance at the excitation wavelength, and n is the refractive index of the medium. I_F and I_{FR} are fluorescent intensities of the compound and the reference respectively. Rhodamine 6G ($\Phi_F = 0.92$ in water) was used as standard.[3] In all Φ_F determinations, correction for the solvent refractive index (η) was applied.

Photophysical data of the different compounds in DMSO at 293K

Compound	λ_{abs} (nm)	λ_{em} (nm)	ε ($M^{-1}.cm^{-1}$)	ϕ (%)[a]
4	522	539	31,266	0.4
BODI-Ti-1	526	539	26,462	82

a: rhodamine 6G (Φ_f = 92 %, λ_{exc} = 530 nm, in water).

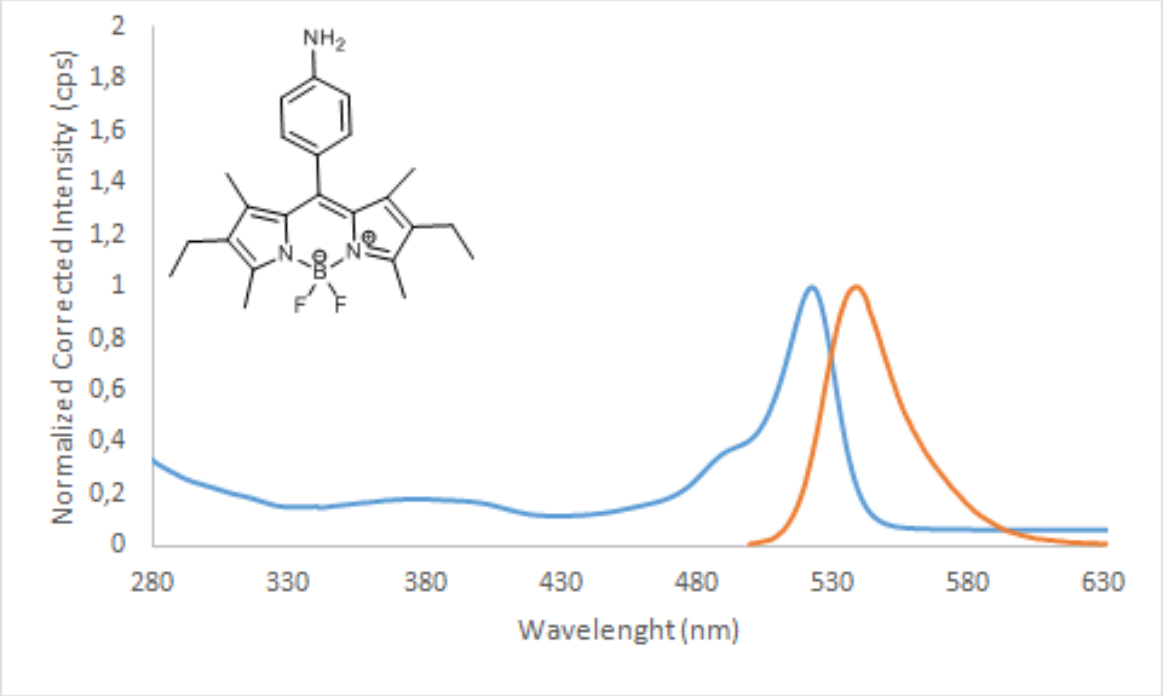


Figure 17: *Spectra of **4** in DMSO, at 293 K (Absorption (blue) and emission (red, λ_{ex} = 488 nm)).*

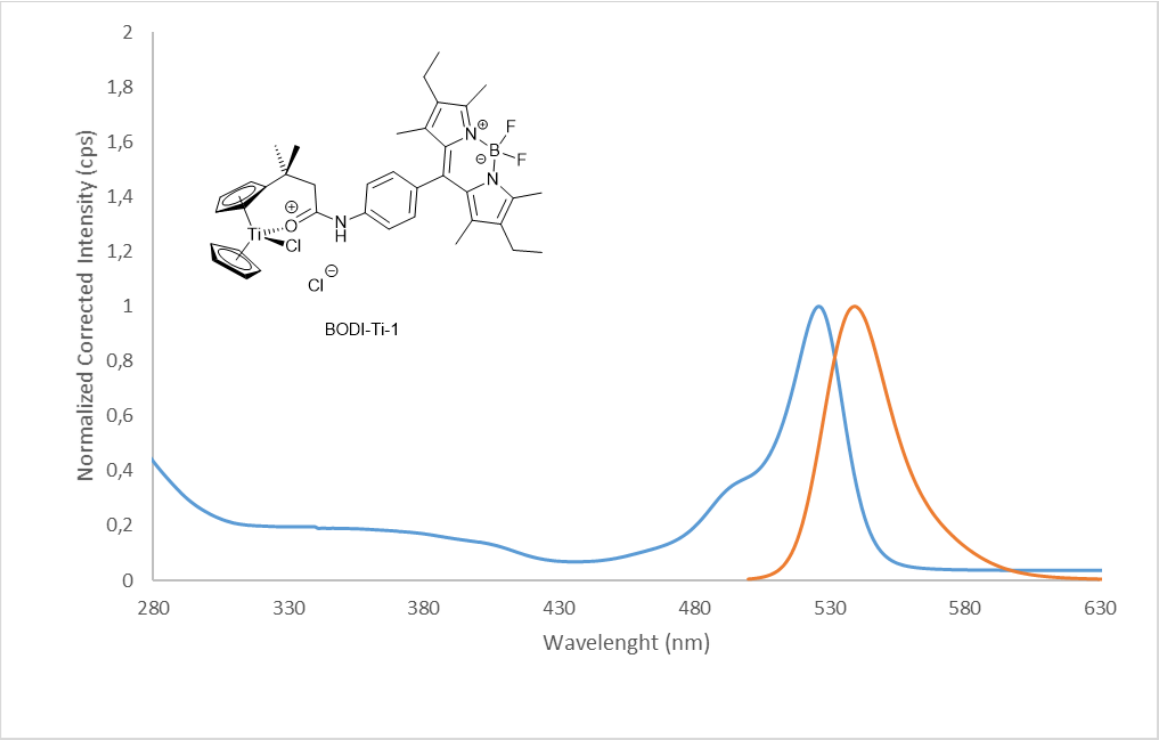


Figure 18: *Spectra of **BODI-Ti-1** in DMSO, at 293 K (Absorption (blue) and emission (red, λ_{ex} = 488 nm)).*

In vitro confocal microscopy experiments

Cells (PC3, B16F10)

B16F10 and PC3 were routinely cultured in 75 cm^2 tissue culture flasks (Nunc™) at 37 °C in a humidified, 5% CO2 atmosphere respectively in respectively RPMI and DMEM supplemented with 10% foetal bovine serum (Dutscher). Cells were sub cultured twice weekly using standard protocols.

Cells were seeded on chambered coverglass (12 Well Chamber-ibidi) and allowed to recover. After 24h, cells were incubated with 1 µM of **BODI-Ti-1** during 4 h, then washed with PBS1X. For co-localization experiments, cells were fixed and permeabilized with iced methanol for 10 min at room temperature. Methanol was eliminated, then, cells were incubated with a 5 µM DRAQ5 solution 5-10min then mounted with Fluoromount-G® (Southern Biotech). A co-staining with DRAQ5 was preferred to DAPI one to identify cells nuclei to prevent any problem of superposition of the emissions of the nuclei stain and the BODIPY derivative.

Confocal imaging

Confocal imaging was performed using a confocal laser-scanning microscope (Leica TCS SP8) with a × 63 HCX PL APO oil immersion (ON 1.4) objective lens, that allowed to simultaneously obtain DIC (Differential Interference Contrast) and fluorescent images (1024 pixels × 1024 pixels), and LASX software (Leica Microsystems, Ltd).

The samples were excited using internal microscope lasers and emission intensity was recorded at the appropriate emission wavelength. Fluorescence images were sequentially acquired. For co-localization experiments, **BODI-Ti-1** (green) was excited at 488 nm and its emission was recorded from 493 to 600 nm, whereas DRAQ5 (red) was excited at 638 nm and its emission was recorded from 661nm to 778 nm.

Image processing and analyses were carried out using Fiji/ImageJ

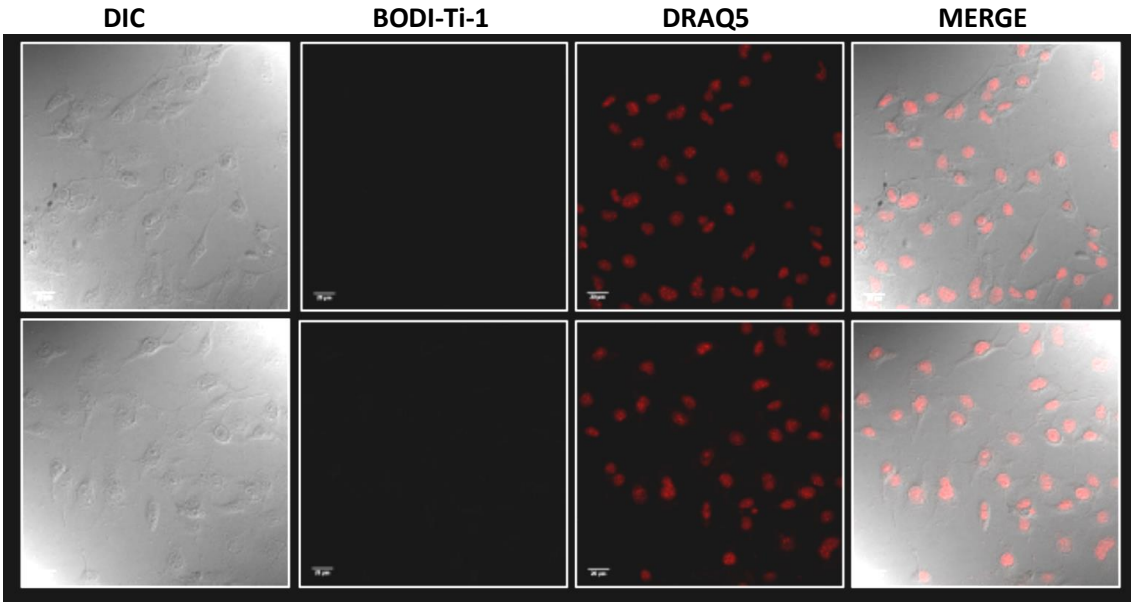


Figure 19: *Confocal immunofluorescent analysis of B16F10 untreated. Nuclei are counterstained with DRAQ5 (red, fluorescent DNA dye).*

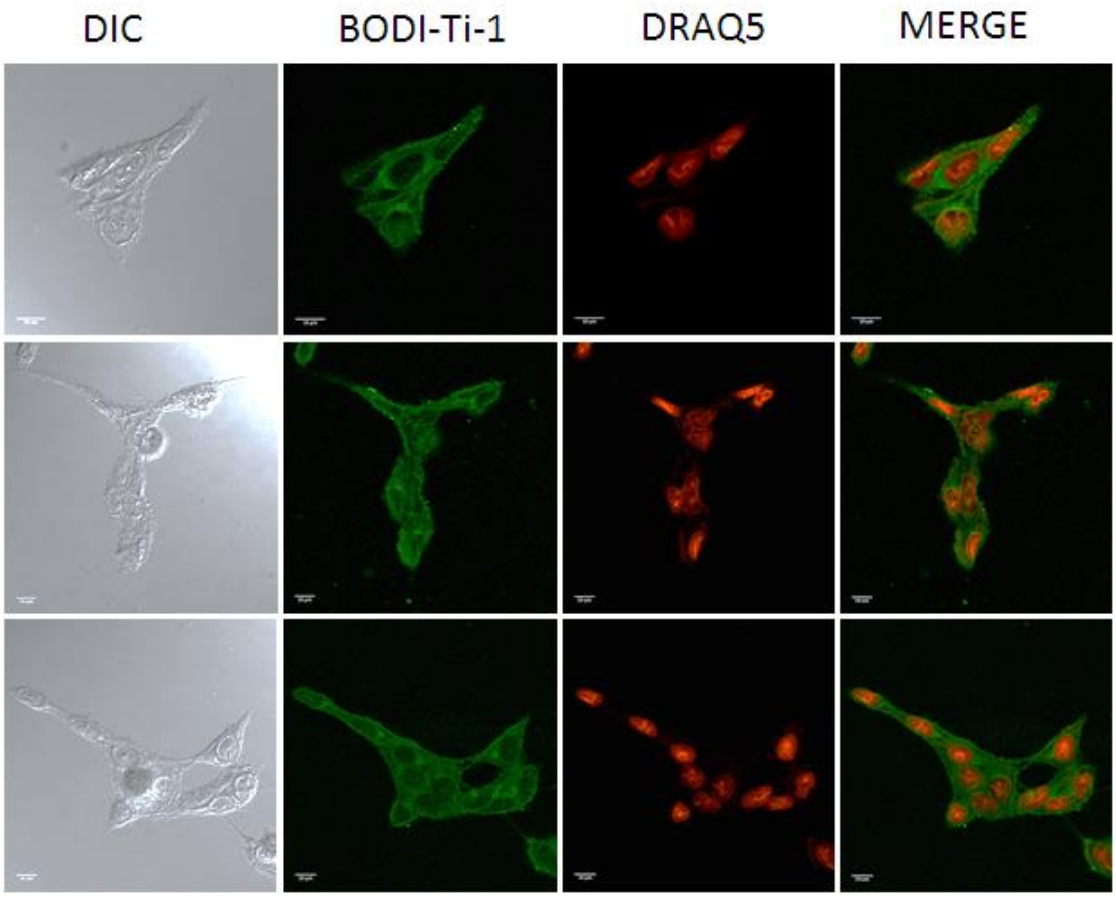


Figure 20: *Confocal immunofluorescent analysis of PC3 labelled with **BODI-Ti-1**. Cells are incubated with 1 µM **BODI-Ti-1** (green) for 4h at 37°C and nuclei are counterstained with DRAQ5 (red, fluorescent DNA dye).*

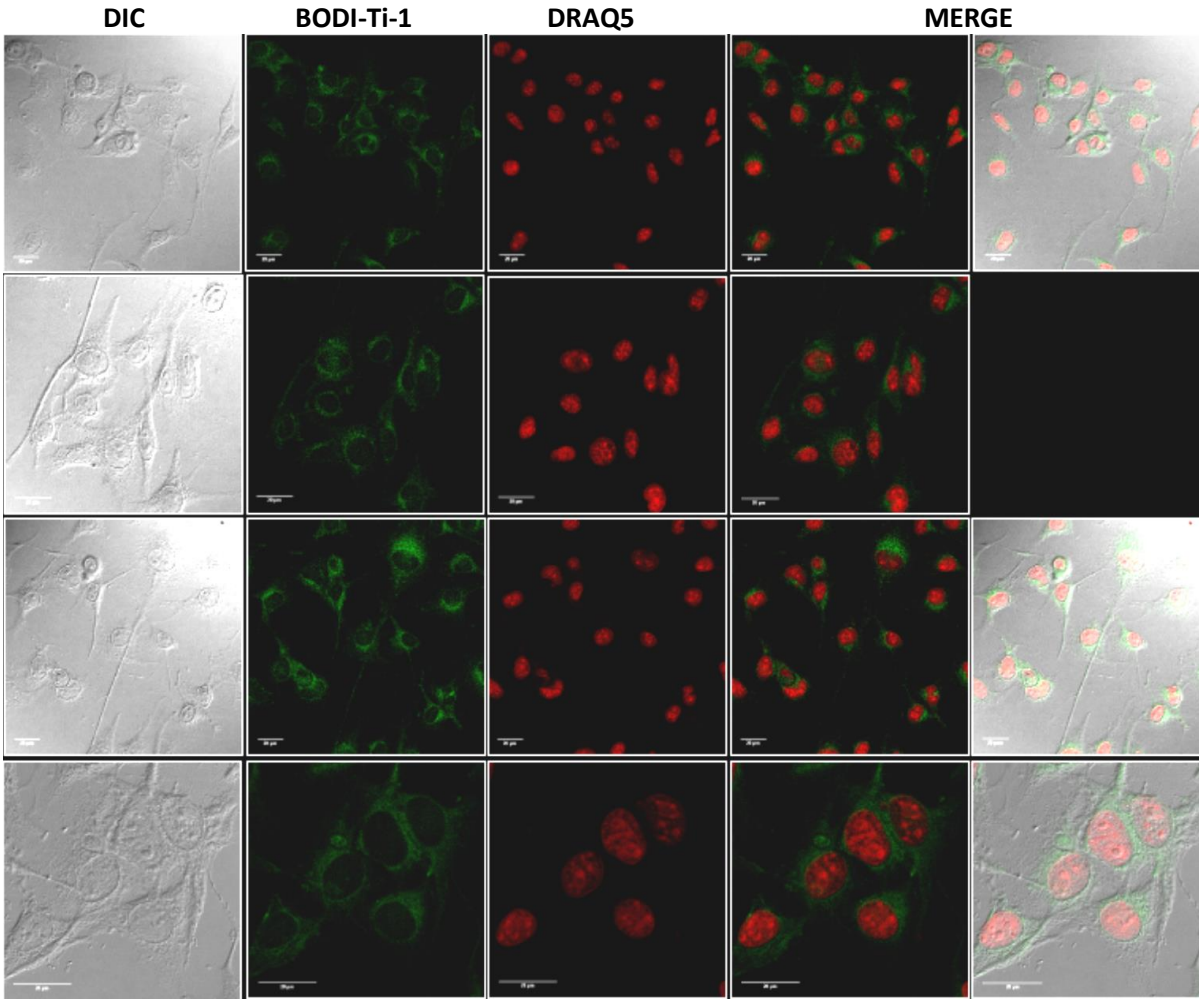


Figure 21: *Confocal immunofluorescent analysis of B16F10 labelled with* ***BODI-Ti-1****. Cells are incubated with 1 µM* ***BODI-Ti-1*** *(green) for 4h at 37°C and nuclei are counterstained with DRAQ5 (red, fluorescent DNA dye).*

Live Imaging

For the live imaging, cells were seeded on a 35mm cell imaging dish with a glass bottom, suitable for cell culture and fluorescence microscopy (ibidi) and allowed to recover.

Cells were imaged in suitable medium without red phenol. The time-lapse images were acquired using xyt mode of the confocal laser-scanning microscope (Leica TCS SP8) with a × 63 HCX PL APO oil immersion (ON 1.4) objective lens, that allowed to simultaneously obtain DIC (Differential Interference Contrast) and fluorescent images (1024 pixels × 1024 pixels). The temperature on the microscope stage was held stable during time-lapse sessions using an electronic temperature-controlled airstream incubator, H301 T UNIT Bold Line Top Stage Incubator (OKOLab).

The samples were excited using internal microscope lasers and emission intensity was recorded at the appropriate emission wavelength. Fluorescence images were sequentially acquired. **BODI-Ti-1** (green) was excited at 488 nm and its emission was recorded from 493 to 600 nm. Cells were used for real-time data collection for a maximum of 4 h. Images and movies were generated and analyzed using LASX software (Leica Microsystems, Ltd) and ImageJ software. For presentation purposes, the time-lapse images were exported in avi and TIFF: "**Time-lapse images-B16F10-25uM.avi**" part-1, 2, and 3.

Cytotoxicity assays

The cell lines PC3 prostate, B16F1 melanoma (ATCC) and A2780 ovarian (Sigma-Aldrich) were cultured in medium RPMI 1640 (ovarian, prostate) or DMEM + GlutaMAX™-I (melanoma) supplemented with 10% Fetal Bovine Serum (Gibco) at 37 °C, 5% CO_2 in a humidified atmosphere. All cells were adherent in monolayers and when confluent were subcultured with 0.05% trypsin-EDTA (Gibco) and splitted. The cytotoxicity of the compounds was evaluated by using the yellow tetrazolium salt MTT that is reduced by viable cells to yield purple formazan. For the cytotoxicity studies, cells (10^4-$2x10^4$ cells/well) were seeded in 96 well plates and incubated overnight. Stock solutions (20 mM) of the compounds were made in water or DMSO (BODI-Ti-1) then diluted in medium to the desired concentrations (0.1-200 µM). For the higher concentration of the Bodipy compound in medium (50 µM) the percentage of DMSO was 0.25%, which induced no cytotoxic effect. Cells were incubated with the compounds in media for 24 and 48h. At the end of the incubation period, the compounds were removed and the cells were incubated with 0.2 mL of MTT solution (0.5 mg/mL phosphate buffered saline (PBS)). After 3 h at 37°C, 5% CO_2, the medium was removed and the purple formazan crystals were dissolved in 0.2 mL of DMSO by shaking. The cellular viability was evaluated by measuring the absorbance at 570 nm as previously described.[4] Each experiment was repeated at least twice and each concentration tested in at least six replicates.

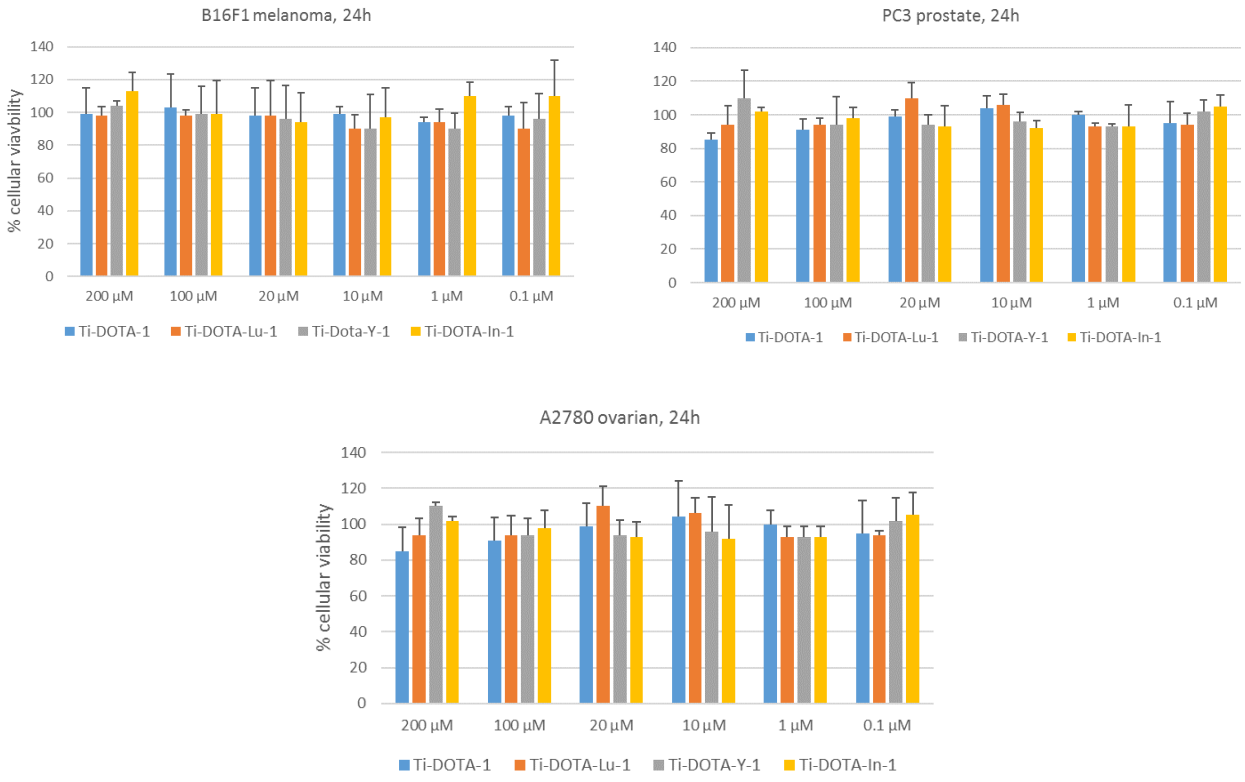


Figure 22: *In vitro cytotoxic activity of complexes after 24 h incubation against B16F1, PC3, and A2780 cells. Results are the mean (±SEM).*

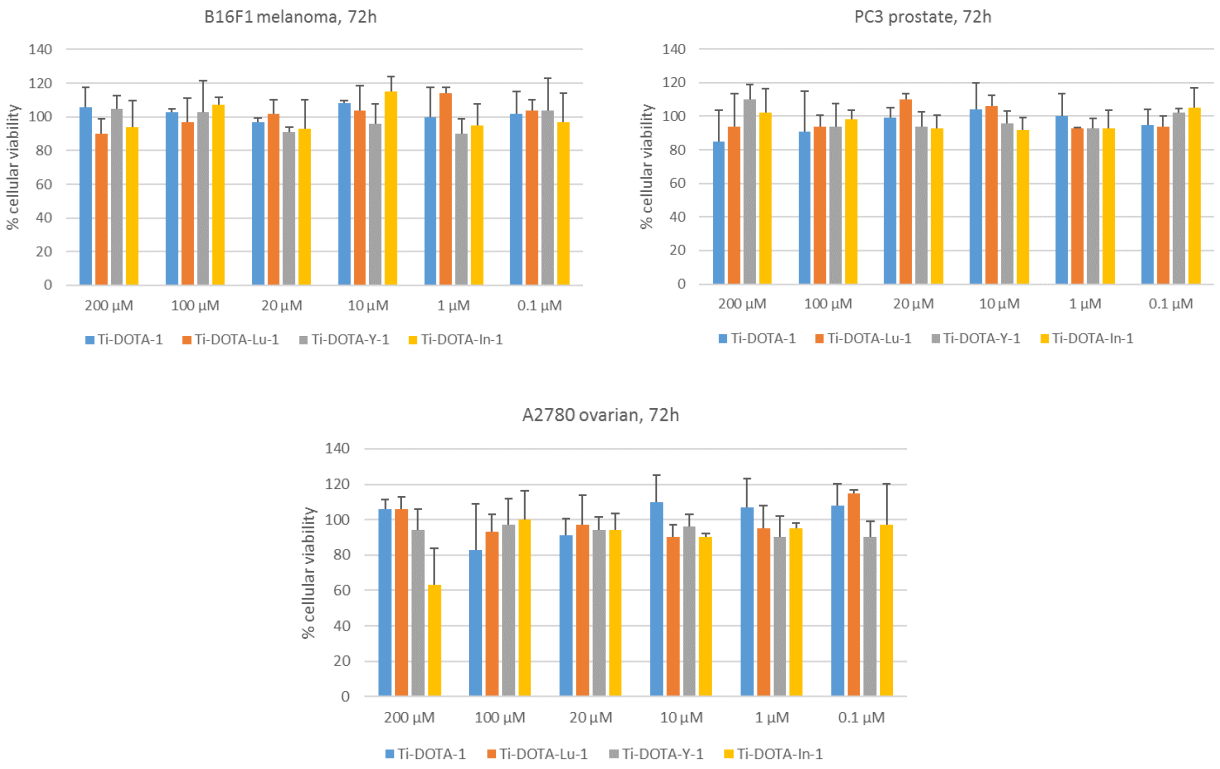


Figure 23: *In vitro cytotoxic activity of complexes after 72 h incubation against B16F1, PC3, and A2780 cells. Results are the mean (±SEM).*

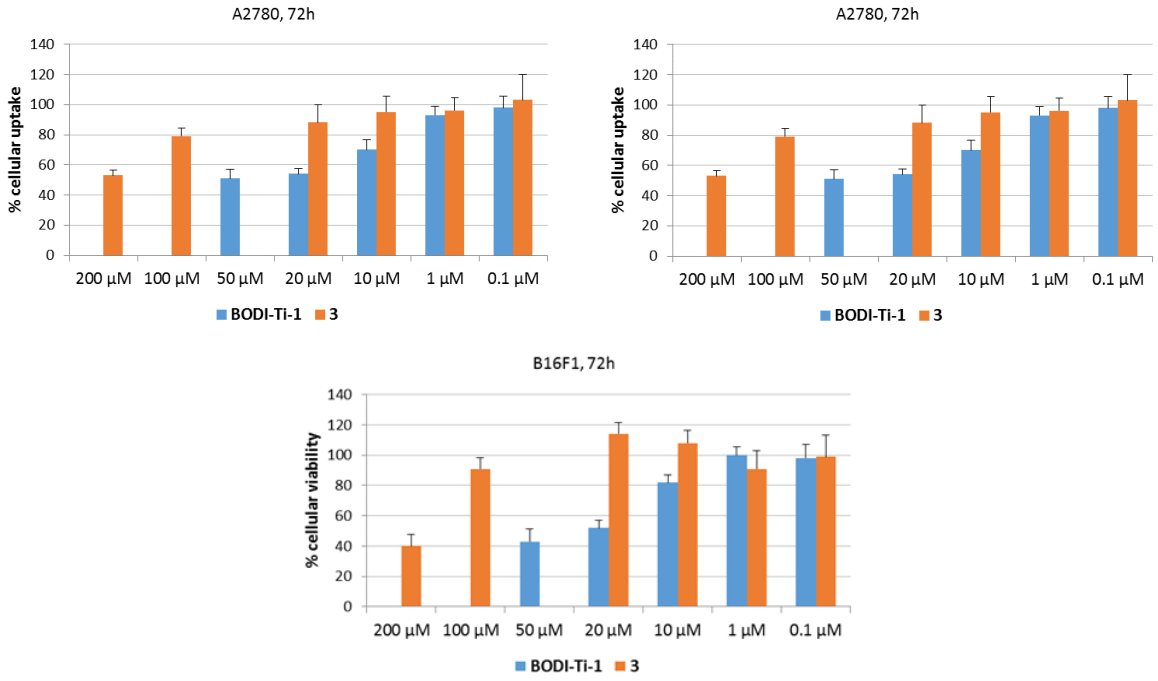
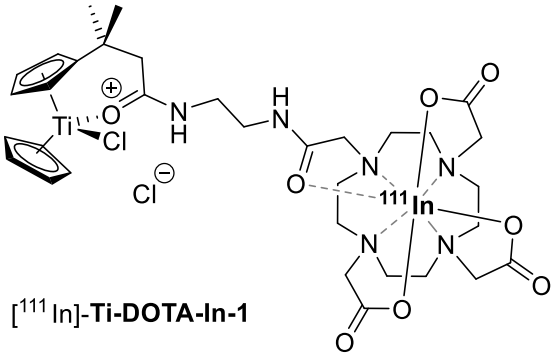


Figure 24: *In vitro cytotoxic activity of **3** and **BODI-Ti-1** after 72 h incubation against A2780, B16F1, and PC3 cells. Results are mean±SD.*

Radiolabelling

Ti-DOTA-^{111}In-1



[^{111}In]-**Ti-DOTA-In-1**

Radiolabeling studies:

Our initial radiolabeling procedure was performed using the Ti-DOTA-1 ligand in solution (aq), mixed in acetate buffer (pH ≈ 5,0) and then the $^{111}InCl_3$ was added. The mixture was heated for 30 min at 95°C. When we analysed the solution by HPLC we observed the presence of three different species and free ^{111}In. By comparison with the Ti-DOTA-In-1 chromatogram we verified that none of these three species corresponded to the ^{111}In congener. Verious attempts to optimize the radiolabeling procedure were made including : temperature (65 – 100 °C), pH (4.5 – 5.5), ligand concentration (0.5 – 2.5 mM) and buffers (ammonium acetate, sodium acetate). However, none of these modifciations allowed us to obtain the desired ^{111}In complex.

From these studies we have reasoned that the radiolabeling conditions, namely the high temperatures, most likely lead to the degradation of the **Ti-DOTA-1**. Since in these radiolabeling studies the molar amount of $^{111}InCl_3$ is very low, compared with that of the ligand, the high temperatures are essential to ensure a successful radiolabeling of DOTA-like chelators. Hence, we decided to explore a different approach which would provide a more favorable reaction kinetics for complexation. We performed a synthesis similar to the one of the cold **Ti-DOTA-In-1**, but spiked the $InCl_3$ solution with $^{111}InCl_3$. This would ensure a higher amount of In in the medium and enhance the reaction kinetics.

Radiolabeling procedure: 40 µL of $^{111}InCl_3$ (700 µCi) were added to 200 µL of $InCl_3$ (6 mg/ 4.5 mL, 0.05 M HCl). A CH_3COONH_4 solution (1 M, aq) was added until pH ≈ 5.5 (≈ 100 µL) and then **Ti-DOTA-1** (22.5 µL, 20 mg/500 µL, aq) was mixed. The solution was heated at 37°C for 1 h.

The radiolabeling procedure leads to the formation of the desired compound with a radiochemical yield of ≈ 62%. After HPLC purification, the overall yield was ≈ 28%. CH_3CN and TFA of the HPLC solvents were removed with N_2. t_r = 8.9 min (Method 1), t_r = 6.4 min (Method 2).

HPLC Method 1: Solvent A – TFA 0.1%(aq), B – CH_3CN (0.1% TFA). 5% to 70% B for 15 min, 70% to 100% B for 1 min, 100% B for 4 min.

HPLC Method 2: Solvent A – TFA 0.1%(aq), B – CH_3CN (0.1% TFA). 5% to 100% B for 5 min, 100% B for 5 min.

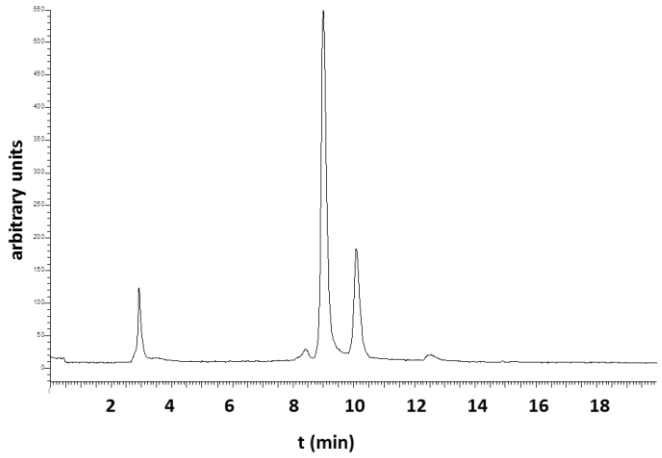


Figure 25: *Chromatogram of* ***Ti-DOTA-1*** *labelling with* ^{111}*In (HPLC Method 1)*

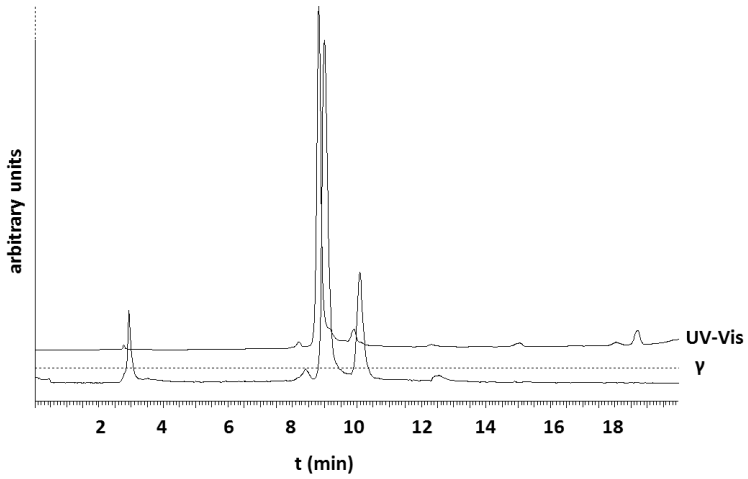


Figure 26: *Chromatograms of* ***Ti-DOTA-1*** *labelling with* ^{111}*In (γ), and* ***Ti-DOTA-In-1*** *(UV-Vis) (HPLC Method 1).*

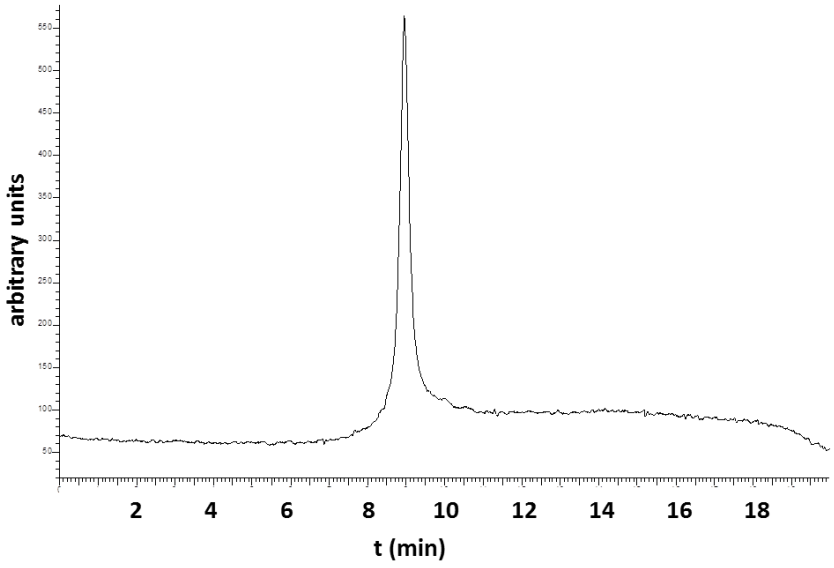


Figure 27: *Chromatogram of [*^{111}*In]-****Ti-DOTA-In-1*** *(HPLC Method 1)*

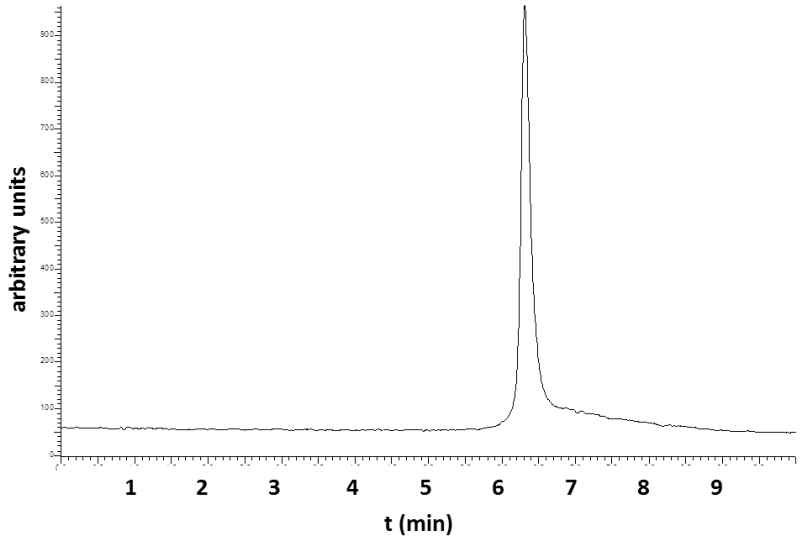


Figure 28: *Chromatogram of [^{111}In]-**Ti-DOTA-In-1** (HPLC Method 1)*

Stability evaluation

Stability studies indicate that compound [^{111}In]-**Ti-DOTA-In-1** is stable in cell culture medium until 24 h, but at 48 h we verify the formation of another species. The compound is more unstable in the presence of human serum since we can see that after only 1 h there is already the presence of another peak in the chromatogram. This new peak grows in intensity with time in incubation and at 48 h there is a higher amount of this new species than our initial compound.

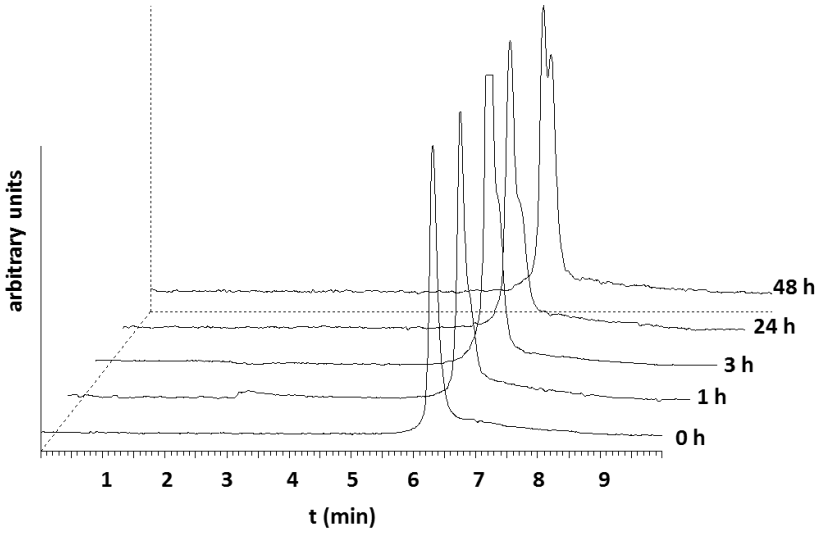


Figure 29: *Chromatograms of [^{111}In]-**Ti-DOTA-In-1** in the presence of cell culture medium incubated at 37°C, injected at different time points (HPLC Method 2)*

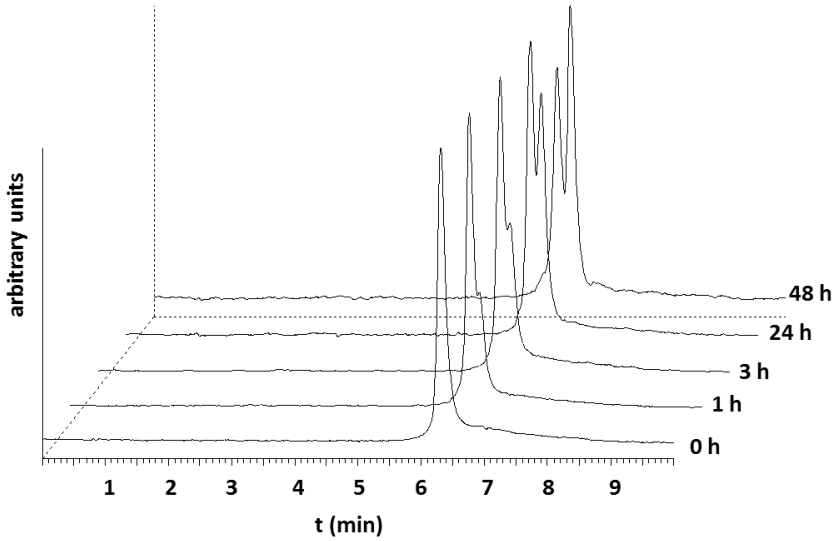


Figure 30: *Chromatograms of [^{111}In]-**Ti-DOTA-In-1** in the presence of cell culture medium incubated at 37°C, injected at different time points (HPLC Method 2)*

Lipophilicity studies:

Lipophilicity studies were perfomed using the octanol-water methodology. In a mixture of 500 µL of PBS (0.01 M) and 500 µL of Octanol, 10 µL of [^{111}In]-**Ti-DOTA-In-1** solution was added. The mixture was vortexed and then centrifuged to separate both phases. Four 100 µL aliquots of both phases were removed and analyzed in a gamma counter:

PBS (cpm): 454149.3, 48675.5, 486726.2, 508291.4
Octanol (cpm): 16.6, 79.7, 53.5, 119.5
Calculation of the partition coefficient Log $D_{o/w}$ = -3.86 ± 0.11
This result indicates that the compound is very hydrophilic.

Cellular uptake studies

We have performed cellular uptake studies with [^{111}In]-**Ti-DOTA-In-1** in three different cell lines: mousse melanoma (B16F1), human ovarian carcinoma (A2780) and human prostate cancer (PC3).

Cellular uptake studies show no significant difference between the different cell lines. Also the overall uptake is very low (< 1 %) even after 48 h incubation. This is most likely the reason why these compounds have also a low cytotoxic activity.

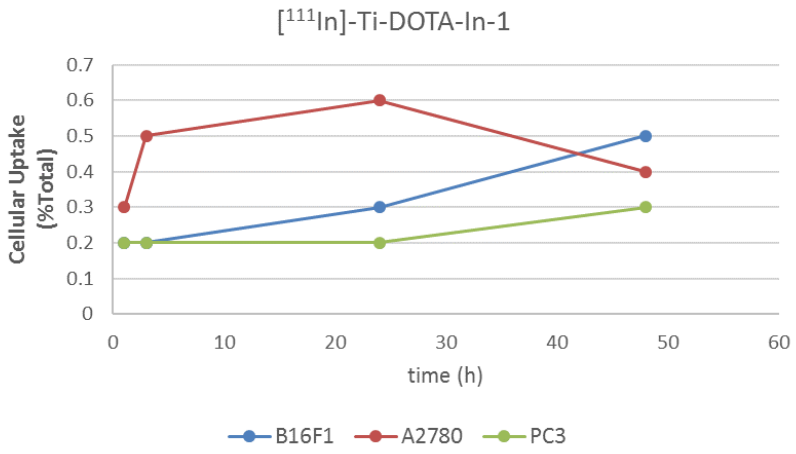


Figure 31: *Cellular uptake of [^{111}In]-**Ti-DOTA-In-1** in the A2780, B16F1, and PC3 cells at several time points*

Biodistribution

The *in vivo* behaviour of the [^{111}In]-**Ti-DOTA-In-1** was evaluated in groups of 3 female CD-1 mice (randomly bred, Charles River, Spain) weighting approximately 25 g each. Animals were intravenously injected with 100 µL (0.9-1.1 MBq) of the complex via the tail vein and were maintained on normal diet ad libitum. At 15 minutes and 1 h mice post-injection (p.i.) were sacrificed by cervical dislocation. The radioactive dosage administered and the radioactivity in the sacrificed animal were measured in a dose calibrator (Capintec, CRC25R). The difference between the radioactivity in the injected and sacrificed animal was assumed to be due to excretion, mainly urinary excretion. Blood samples were taken by cardiac puncture at sacrifice. Tissue samples of the main organs were then removed, weighted and counted in a gamma counter (Berthold LB2111). Biodistribution results were expressed as percent of injected activity per organ or g organ (% I.A./ g organ).

Results of the biodistribution studies, expressed as % I.A./g, at 15 min and 1 h p.i. are summarized for the most relevant organs in the Table S1.

Table S 1: *Biodistribution of the [^{111}In]-**Ti-DOTA-In-1** at 15 min and 1 h after administration in CD-1 mice.*

Organ	**% I.A./g**	
	15 min	**1 h**
Blood	7.5 ± 0.5	4.6 ± 0.6
Liver	1.4 ± 0.3	1.5 ± 0.4
Intestine	0.4 ± 0.1	0.5 ± 0.2
Spleen	0.7 ± 0.2	0.6 ± 0.2
Heart	1.2 ± 0.5	0.9 ± 0.4
Lung	1.8 ± 0.4	1.7 ± 0.6
Kidney	11.9 ± 5.0	11.0 ± 2.2
Muscle	0.9 ± 0.2	0.7 ± 0.2
Skeletal	1.3 ± 0.6	1.7 ± 0.4
Stomach	0.6 ± 0.1	0.6 ± 0.2
Pancreas	1.0 + 0.5	0.7 ± 0.3
Brain	0.21 + 0.07	0.2 + 0.1
Excretion (% I.A.)	38.6 ± 4.2	59.5 ± 4.7

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