Supporting information

Cancer Immunoprofiling Strategy Using Mass Spectrometry Coupled with Bioorthogonal Cleavage

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I. Material and equipment

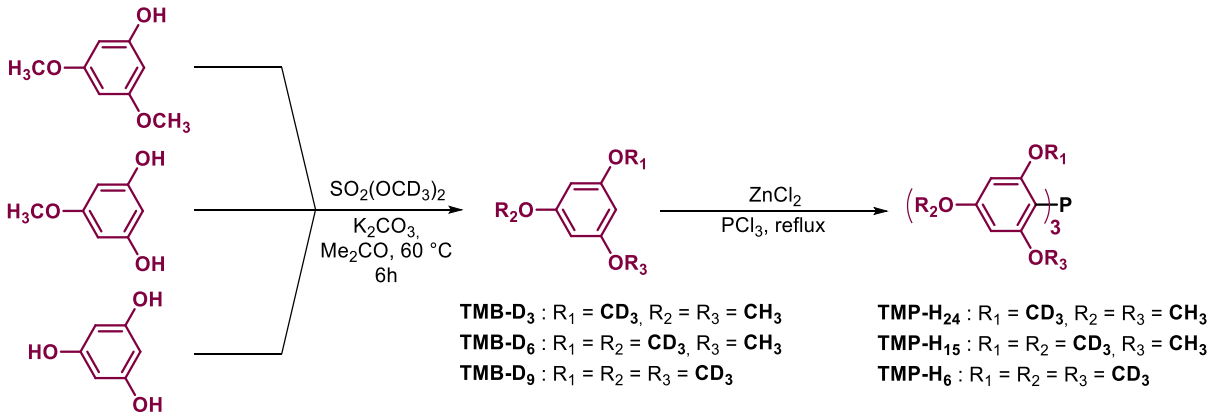
All chemical products commercially available were purchased from Sigma-Aldrich, Acros and Fluka and used without further purification. Anhydrous solvents: were purchased in anhydrous form and used without further purification. Dichloromethane was distilled form calcium hydride under nitrogen. Tris(2,4,6-trimethoxyphenyl)phosphine was commercial available and was purchased from Acros.

Reactions were monitored by TLC carried out on silica 0,25 mm (60 F254, Merck) using UV light as visualizing agent and basic aqueous permanganate as developing agent.
^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) were measured on a Brucker Advance 400 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from residual solvents peaks and coupling constants are reported as Hertz (Hz). Splitting patterns are designated as singlet (s), broad singlet (br. s), doublet (d), triplet (t), quartet (q), quintet (quint), heptuplet (hept), multiplet (m). Splitting patterns that could not be interpreted or easily visualized are designated as multiplet (m).
LCMS mass spectra were recorded using a single quadrupole mass spectrometer (SQD 2, Waters) with electrospray source coupled to Ultra-High Performance Liquid Chromatography (Acquity UPLC H-Class, Waters).
High resolution mass spectroscopy of the final compounds were determined using a Xevo® G2-XS QTof
Infrared spectra (IR) were obtained on a Perkin Elmer system 2000 FT-IR spectrophotometer or a Perkin Elmer UATR TWO FTIR spectrophotometer and are reported as wavelength numbers (cm-1).
Melting points (Mp) were obtained on a BÜCHI Melting Point B-545 and are reported in °C.
Absorbances were measured on a UV JASCO V-750 equipped with an injection module (GSP-909) and a Peltier (EHCS-760).

II. Synthetic Procedure and analytical Data for compounds

a. Synthesis of tris-trimethoxyphenylphosphines (TMP)

TMP-H33 is commercially available. The other TMP were synthesized in 2 steps according to scheme S1.

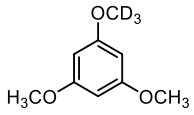


Scheme S1: Synthetic route to TMPs

General procedure for trimethoxybenzene (TMB) synthesis

The appropriate phenol (1.0 equiv.) was suspended in dry acetone (0.5 M) in an oven dry sealed tube, then K_2CO_3 and deuterated-dimethylsulfate (1.1 equiv./deuterable position) were successively added to the solution. The resulting mixture was then heated at 60 °C for six hours. After completion of the reaction, the crude product was evaporated and purified by column chromatography on silica gel (Heptane/AcOEt 90/10) to give the desired trimethoxybenzene.

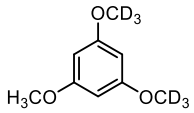
1,3-dimethoxy-5-(methoxy-d_3)benzene – **TMB-D$_3$**



$C_9H_9D_3O_3$
MW: 171.21 g/mol
White solid
88 %

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 6.09 (s, 3H), 3.77 (s, 6H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 161.6 (3C), 93.0 (3C), 55.5 (3C).
IR (cm^{-1}): 3001, 2961, 2939, 2840, 1590, 1477, 1460, 1427, 1194, 1167, 1151, 1107, 1069.
LCMS (ESI): *m/z* $[M+H]^+$ 172.
HRMS (ESI): calculated for $C_9H_{10}D_3O_3$ 172.1053 found 172.1055.

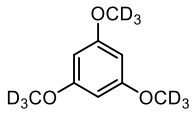
1-methoxy-3,5-bis(methoxy-d_3)benzene – **TMB-D_6**



$C_9H_6D_6O_3$
MW: 174.22 g/mol
White solid
81 %

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 6.09 (s, 3H), 3.77 (s, 3H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 161.6 (3C), 93.0(3C), 55.5 (3C).
IR (cm^{-1}): 3000, 2940, 2841, 2252, 2220, 2070, 1591, 1471, 1437, 1199, 1174, 1154, 1108, 1057.
LCMS (ESI): *m/z* $[M+H]^+$ 175
HRMS (ESI): calculated for $C_9H_7D_6O_3$ 175.1241 found 175.1242

1,3,5-tris(methoxy-d_3)benzene – **TMB- D_9**



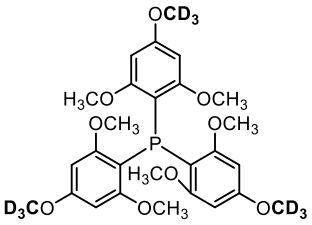
$C_9H_3D_9O_3$
MW: 177.24 g/mol
White solid
91 %

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 6.09 (s, 3H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 161.6 (3C), 93.0 (3C), 55.5 (m, 3C).
IR (cm^{-1}): 2252, 2220, 2130, 2070, 1590, 1466, 1170, 1107, 1054.
LCMS (ESI): *m/z* $[M+H]^+$ 178
HRMS (ESI): calculated for $C_9H_4D_3O_3$ 178.1429 found 178.1429

General procedure for tris-trimethoxyphenylphosphines TMP synthesis

Trimethoxybenzene (3.0 equiv.) and $ZnCl_2$ extra dry (1.0 equiv. stored in glovebox) were added into a sealed vial in glovebox. The vial was sealed, then PCl_3 (3.0 equiv.) was added. The reaction mixture was stirred at 80 °C for 16 h, then cooled to room temperature. Toluene (2.0 mL) was added dropwise very carefully to the mixture at 0 °C and then removed to afford a viscous residual complex of $ZnCl_2$. Aqueous ammonia was then added at 0 °C and the solution was extracted with toluene. The organic phases were combined, dried over $MgSO_4$ and evaporated under reduced pressure. The crude material was recrystallized in Et_2O to afford the desired phosphine.

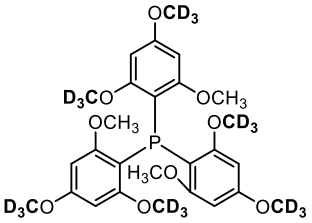
tris(2,6-dimethoxy-4-(methoxy-d3)phenyl)phosphine – **TMP-H_{24}**



$C_{27}H_{24}D_9O_9P$
MW: 541.58 g/mol
White solid
31 %

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 6.03 (d, $^4J_{P-H}$ = 2.5 Hz, 6H), 3.77 (s, 6H), 3.48 (s, 12H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 163.3, (d, $^4J_{P-C}$ = 9 Hz, 6C), 160.8 (3C), 108.4 (d, $^4J_{P-C}$ = 19 Hz, 3C), 91.31 (6C), 56.3 (6C), 55.2 (3C).
^{31}P NMR (163 MHz, $CDCl_3$, δ ppm): -70.04.
IR (cm^{-1}): 2072, 1712, 1590, 1572, 1452, 1415, 1347, 1290, 1230, 1191, 1168, 1125, 1106, 1002, 977.
LCMS (ESI): *m/z* $[M+H]^+$ 543
HRMS (ESI): calculated for $C_{27}H_{25}D_9O_9P$ 541.2427 found 541.2429

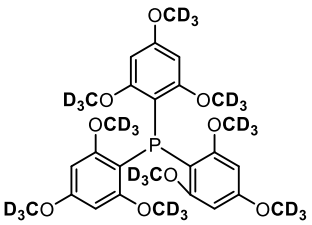
tris(6-methoxy-2,4-bis(methoxy-d3)phenyl)phosphine – **TMP-H_{15}**



$C_{27}H_{27}D_{18}O_9P$
MW: 550.30 g/mol
White solid
29 %

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 6.02 (d, *J* = 2.4 Hz, 6H), 3.77 (s, 3H), 3.48 (s, 6H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 167.3, (m, 6C), 160.8 (3C), 100.1 (3C), 91.6 (6C), 56.3 (6C), 55.2 (m, 3C).
^{31}P NMR (163 MHz, $CDCl_3$, δ ppm): -70.10.
IR (cm^{-1}): 3338, 2221, 2071, 1708, 1590, 1572, 1452, 1415, 1347, 1291, 1227, 1195, 1168, 1125, 1106, 1002.
LCMS (ESI) : *m/z* $[M+H]^+$ 551
HRMS (ESI) : calculated for $C_{27}H_{16}D_{18}O_9P$ 551.3070 found 551.3071

tris(2,4,6-tris(methoxy-d3)phenyl)phosphine – **TMP-H_6**

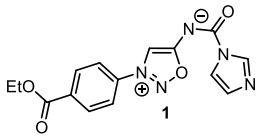


This compound was obtained as described in the literature. [1]

[1] Protopopov, Obshch. Khim.**1963**, *33*, 3050−3052.

b. Synthesis of the sydnonimine linker

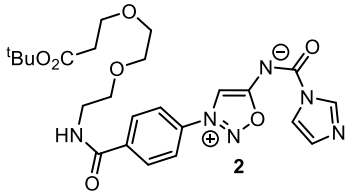
(3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)(1H-imidazole-1-carbonyl)amide – **1**



This compound was obtained as previously described by our group. [2]

(3-(4-((2-(2-(3-(tert-butoxy)-3-oxopropoxy)ethoxy)ethyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)(1H-imidazole-1-carbonyl)amide – **2**

$C_{24}H_{30}N_6O_7$
MW: 514.21 g/mol
Yellow moss
42 % (over two steps)

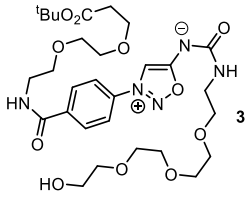


Compound **1** (2.0 g, 6.1 mmol, 1.0 equiv.) was dissolved in a mixture of THF (7.5 mL) and H_2O (7.5 mL). NaOH (0.269 g, 6.7 mmol, 1.1 equiv.) in water (1.0 mL) was added and the reaction mixture was stirred at room temperature for 2 h. THF was then evaporated, and the water solution was washed with DCM. Then, HCl (1 M) was added until pH reached 3 and the precipitate was collected by centrifugation and dried under vacuum. The yellow wish solid was solubilized in a mixture of MeCN (7.5 mL) and DMF (7.5 mL) and D*i*PEA (3.1 mL, 18.3 mmol, 3.0 equiv.) was added followed by HATU (2.8 g, 7.3 mmol, 1.2 equiv.) and $NH_2PEG_3CO_2{}^tBu$ (1.56 g, 6.7 mmol, 1.1 equiv.). Solvents were removed under vacuum and the crude mixture was purified by column chromatography (DCM 100 % to DCM/MeOH 94/06) to afford **2** as a yellow moss (1.3 g, 2.5 mmol, 42 % over two steps).

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.46 (s, 1H), 8.40 (s, 1H), 8.18-8.15 (m, 2H), 7.96-7.96 (m, 2H), 7.63 (t, *J* = 1.3 Hz, 1H), 7.05-7.04 (m, 1H), 3.76-3.61 (m, 11H), 2.48 (t, *J* = 6.1 Hz, 2H), 1.41 (s, 9H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 175.0, 171.1, 165.0, 154.5, 139.6, 137.5, 135.1, 129.8 (2C), 129.4, 121.7 (2C), 117.4, 105.6, 100.0, 80.8, 70.3, 69.4, 66.9, 40.1, 36.3, 28.1 (3C).
IR (cm^{-1}): 3317, 3135, 2977, 2872, 1723, 1660, 1572, 1502, 1470, 1434, 1414, 1366, 1351, 1291, 1254, 1223, 1207, 1157, 1093, 1051, 1008, 974, 843.
LCMS (ESI): *m/z* $[M+H\text{-imidazole}]^+$ 448
HRMS (ESI): calculated for $C_{24}H_{30}N_6NaO_7$ 537.2068; found 537.2070

[2] M. Riomet et al. *Org. Lett.* 2020, 22, 6, 2403–2408

(3-(4-((2-(2-(3-(tert-butoxy)-3-oxopropoxy)ethoxy)ethyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)carbamoyl)amide – **3**



$C_{29}H_{45}N_5O_{11}$
MW: 639.70 g/mol
Yellow oil
52 % over two steps

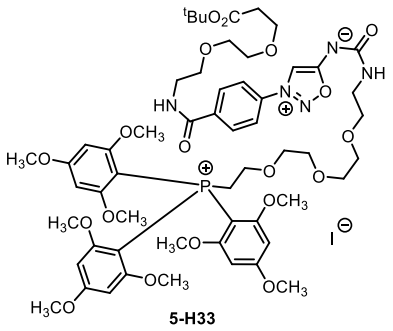
Compound **2** (0.800 g, 1.55 mmol, 1.0 equiv.) was dissolved in MeCN (8.0 mL). MeI (0.387 mL, 6.22 mmol, 4.0 equiv.) was added and the reaction mixture was stirred at room temperature for 16 h. Then, volatiles were removed and $CHCl_3$ (stabilized on amylene, 15.0 mL) and $NH_2PEG_4CO_2{}^tBu$ (0.330 g, 1.70 mmol, 1.1 equiv.) were successively added and stirred at room temperature for 16 h. Solvent was removed under vacuum and the crude mixture was purified by column chromatography (DCM/MeOH 94/06) to afford **3** as a yellow oil (0.582 g, 0.910 mmol, 52 % over two steps).

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.22 (s, 1H), 8.11 (d, *J* =8.4 Hz, 2H), 7.86 (d, *J* =8.4 Hz, 2H), 7.36 (brs, 1H), 6.23 (brs, 1H), 3.78-3.57 (m, 24H), 3.49-3.41 (m, 2H), 2.47 (t, *J* =6.2 Hz, 2H), 1.40 (s, 9H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 171.2 (2C), 165.4, 139.0, 135.7, 129.7 (2C), 121.8 (2C), 102.6, 81.0, 72.9, 70.7, 70.6, 70.4 (6C), 69.7, 67.0, 61.6, 40.5, 40.2, 36.4, 28.2 (3C).
IR (cm^{-1}): 3334, 2870, 1725, 1637, 1590, 1504, 1439, 1392, 1366, 1352, 1287, 1216, 1115, 957.
LCMS (ESI) : *m/z* $[M+H]^+$ 640
HRMS (ESI) : calculated for $C_{29}H_{45}N_5NaO_{11}$ 662.3001 found 662.3001

c. Synthesis of TMPP tagged linkers 5

General procedure for Tagged linker synthesis 5
Et_3N (0.527 mL, 3.91 mmol, 5.0 equiv.) and TsCl (0.297 g, 1.56 mmol, 2.0 equiv.) were added to a solution of **3** (0.500 mg, 0.78 mmol, 1.0 equiv.) in dry DCM (50.0 mL). The mixture was stirred overnight at room temperature. DCM was then removed under reduced pressure and the crude mixture was rapidly purified by flash chromatography DCM/MeOH 98/02, divided in 4 batches, and directly engaged in the next step. The tosylated intermediate (1.0 equiv.) was dissolved in dry DMF (0.2 M) and then $P(Ph(OMe)_3)_3$ (1.0 equiv.) and NaI (1.1 equiv.) were added to the solution and the mixture was heated overnight at 40 °C. Then, DMF was removed under reduced pressure and DCM 4.0 mL was added to precipitated impurities. The suspension was centrifuged and the supernatant was collected and evaporated under reduced pressure. The crude mixture was finally purified by column chromatography on silica gel (slowly DCM 100 % to DCM/MeOH 95/05) to give the desired product **5** as a yellow oil.

((2-(2-(2-(2-(bis(4-((l1-oxidaneyl)-l5-methyl)-2,6-dimethoxyphenyl)(2-((l1-oxidaneyl)-l5-methyl)-4,6-dimethoxyphenyl)phosphonio)ethoxy)ethoxy)ethoxy)ethyl)carbamoyl)(3-(4-((2-(2-(3-(tert-butoxy)-3-oxopropoxy)ethoxy)ethyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)amide iodide – **5-H$_{33}$**

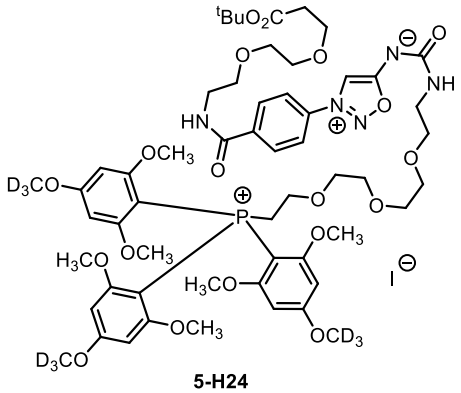


5-H33

$C_{56}H_{77}IN_5O_{19}P$
MW: 1282.12 g/mol
Yellow oil
28 %

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.29-8.24 (m, 2H), 7.87 (d, *J* = 8.8 Hz, 2H), 7.79 (s, 1H), 6.11 (d, *J* = 4.7 Hz, 6H), 3.87 (s, 9H), 3.73-3.32 (m, 44H), 2.48 (t, *J* = 6.3 Hz, 2H), 1.41 (s, 9H), NH unobserved.
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 171.2, 165.9 (d, *J* = 1 Hz, 3C), 165.4, 163.9 (d, *J* = 1 Hz, 6C), 139.2, 135.4, 130.1 (2C), 121.6 (2C), 102.4, 91.6 (d, *J* = 106 Hz, 3C), 91.2 (d, *J* = 7 Hz, 6C), 80.8, 70.6-70.3 (m, 8C), 69.5, 67.0, 66.6, 56.4 (6C), 56.2 (3C), 40.4, 40.0, 36.4, 28.2 (3C). (2C missing)
^{31}P NMR (163 MHz, $CDCl_3$, δ ppm): 0.1.
IR (cm^{-1}): 2938, 2726, 2640 , 1596, 1578, 1502, 1458, 1413, 1337, 1288, 1231, 1208, 1161, 1126, 1096, 1025, 951, 919, 815.
LCMS (ESI): *m/z* $[M+H]^{2+}$ 578
HRMS (ESI): calculated for $C_{56}H_{77}N_5O_{19}P$ 1154.4944 found 1154.4925

((2-(2-(2-(2-((2-((l1-oxidaneyl)-l5-methyl)-6-methoxy-4-(methoxy-d3)phenyl)bis(2,6-dimethoxy-4-(methoxy-d3)phenyl)phosphonio)ethoxy)ethoxy)ethoxy)ethyl)carbamoyl)(3-(4-((2-(2-(3-(tert-butoxy)-3-oxopropoxy)ethoxy)ethyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)amide iodide – **5-H$_{24}$**



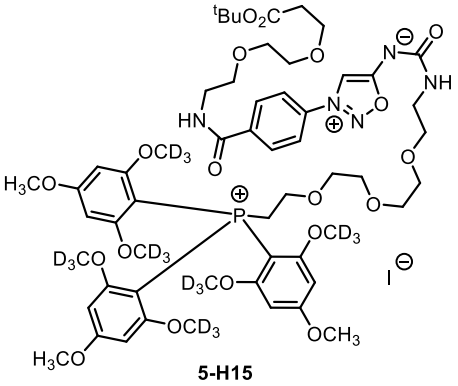
5-H24

$C_{56}H_{68}D_9IN_5O_{19}P$
MW: 1291.18 g/mol
Yellow oil
32 %

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.23 (d, *J* = 8.7 Hz, 2H), 8.15 (s, 1H), 7.84 (d, *J* = 8.7 Hz, 2H), 7.59 (t, *J* = 4.6 Hz, 1H), 6.11 (d, *J* = 4.7 Hz, 6H), 5.72 (t, *J* = 5.7 Hz, 1H), 3.87 (s, 6H), 3.72 – 3.56 (m, 30H), 3.49-3.35 (m, 8H), 2.48 (t, *J* = 6.3 Hz, 2H), 1.41 (s, 9H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 172.8, 171.2, 165.9 (3C), 165.4, 163.9 (6C), 161.8, 139.2, 135.4, 130.0 (2C), 121.5 (2C), 101.9, 91.6 (d, *J* = 106 Hz, 3C), 91.2 (d, *J* = 7 Hz, 6C), 80.8, 70.6--70.3 (m, 8C), 69.5, 67.0, 66.6, 56.4 (6C), 56.2 (3C), 40.4, 40.0, 36.4, 28.2 (3C).
^{31}P NMR (163 MHz, $CDCl_3$, δ ppm): 0.1.

IR (cm^{-1}): 3284, 2925, 2072, 1725, 1643, 1596, 1577, 1503, 1463, 1417, 1346, 1289, 1204, 1232, 1166, 1129, 1104, 1012, 955, 815, 728.
LCMS (ESI): *m/z* $[M+H]^{2+}$ 583
HRMS (ESI): calculated for $C_{56}H_{68}D_9N_5O_{19}P$ 1163.5509 found 1163.5503

((2-(2-(2-(2-(bis(4-((l1-oxidaneyl)-l5-methyl)-2,6-bis(methoxy-d3)phenyl)(4-methoxy-2,6-bis(methoxy-d3)phenyl)phosphonio)ethoxy)ethoxy)ethoxy)ethyl)carbamoyl)(3-(4-((2-(2-(3-(tert-butoxy)-3-oxopropoxy)ethoxy)ethyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)amide iodide – **5-H$_{15}$**

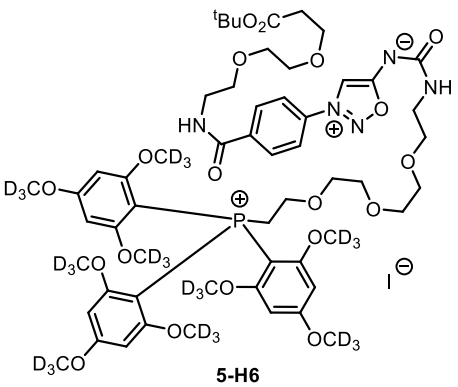


5-H15

$C_{56}H_{59}D_{18}IN_5O_{19}P$
MW: 1300.23 g/mol
Yellow oil
15 %

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.23 (d, *J* = 8.8 Hz, 2H), 8.15 (s, 1H), 7.84 (d, *J* = 8.8 Hz, 2H), 7.53 (t, *J* = 4.4 Hz, 1H), 6.11 (d, *J* = 4.7 Hz, 6H), 5.70 (t, *J* = 5.8 Hz, 1H), 3.88 (s, 3H), 3.75-3.53 (m, 25H), 3.48-3.33 (m, 7H), 2.48 (t, *J* = 6.3 Hz, 2H), 1.42 (s, 9H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 172.9, 171.2, 165.9 (3C), 165.4, 163.9 (6C), 161.8, 139.2, 135.4, 130.0 (2C), 121.5 (2C), 101.9, 91.6 (d, *J* = 106 Hz, 3C), 91.2 (d, *J* = 7 Hz, 6C), 80.8, 70.6-70.3 (m, 8C), 69.5, 67.0, 66.6, 56.4 (6C), 56.2 (3C), 40.4, 40.0, 36.4, 28.2 (3C).
^{31}P NMR (163 MHz, $CDCl_3$, δ ppm): 0.1.
IR (cm^{-1}): 2938, 2726, 2640 ,1596, 1578, 1502, 1458, 1413, 1337, 1288, 1231, 1208, 1161, 1126, 1096, 1025, 951, 919, 815.
LCMS (ESI): *m/z* $[M+H]^{2+}$ 586
HRMS (ESI): calculated for $C_{56}H_{59}D_{18}N_5O_{19}P$ 1172.6074 found 1172.6052

(3-(4-((2-(2-(3-(tert-butoxy)-3-oxopropoxy)ethoxy)ethyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((2-(2-(2-(2-(tris(2,4,6-tris(methoxy-d3)phenyl)phosphonio)ethoxy)ethoxy)ethoxy)ethyl)carbamoyl)amide iodide – **5-H$_6$**

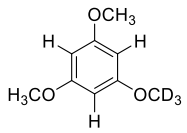


5-H6

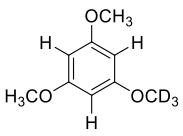
^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.23 (d, *J* = 8.7 Hz, 2H), 8.15 (s, 1H), 7.84 (d, *J* = 8.7 Hz, 2H), 7.53 (t, *J* = 4.6 Hz, 1H), 6.11 (d, *J* = 4.7 Hz, 6H), 5.70 (t, *J* = 5.7 Hz, 1H), 3.74 (s, 3H), 3.71-3.55 (m, 17H), 3.48 – 3.44 (m, 8H), 2.48 (t, *J* = 6.3 Hz, 2H), 1.48 (s, 9H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 172.8, 171.2, 165.8 (3C), 165.4, 163.9 (6C), 161.7, 139.0, 135.7, 130.0 (2C), 121.5 (2C), 101.9, 91.6 (d, *J* = 106.1 Hz, 3C), 91.2 (d, *J* = 7.1 Hz, 6C), 80.8, 70.6-70.3 (m, 8C), 69.5, 67.0, 66.6 (d, *J* = 3.9 Hz), 56.1-55.3 (m, 9C), 40.4, 40.0, 36.4, 28.2 (3C).
^{31}P NMR (163 MHz, $CDCl_3$, δ ppm): 0.1.
IR (cm^{-1}): 2938, 2726, 2640 ,1596, 1578, 1502, 1458, 1413, 1337, 1288, 1231, 1208, 1161, 1126, 1096, 1025, 951, 919, 815.
LCMS (ESI): *m/z* $[M+H]^{2+}$ 590
HRMS (ESI): calculated for $C_{56}H_{50}D_{27}N_5O_{19}P$ 1181.6644 found 1181.6646

III. NMR Spectra

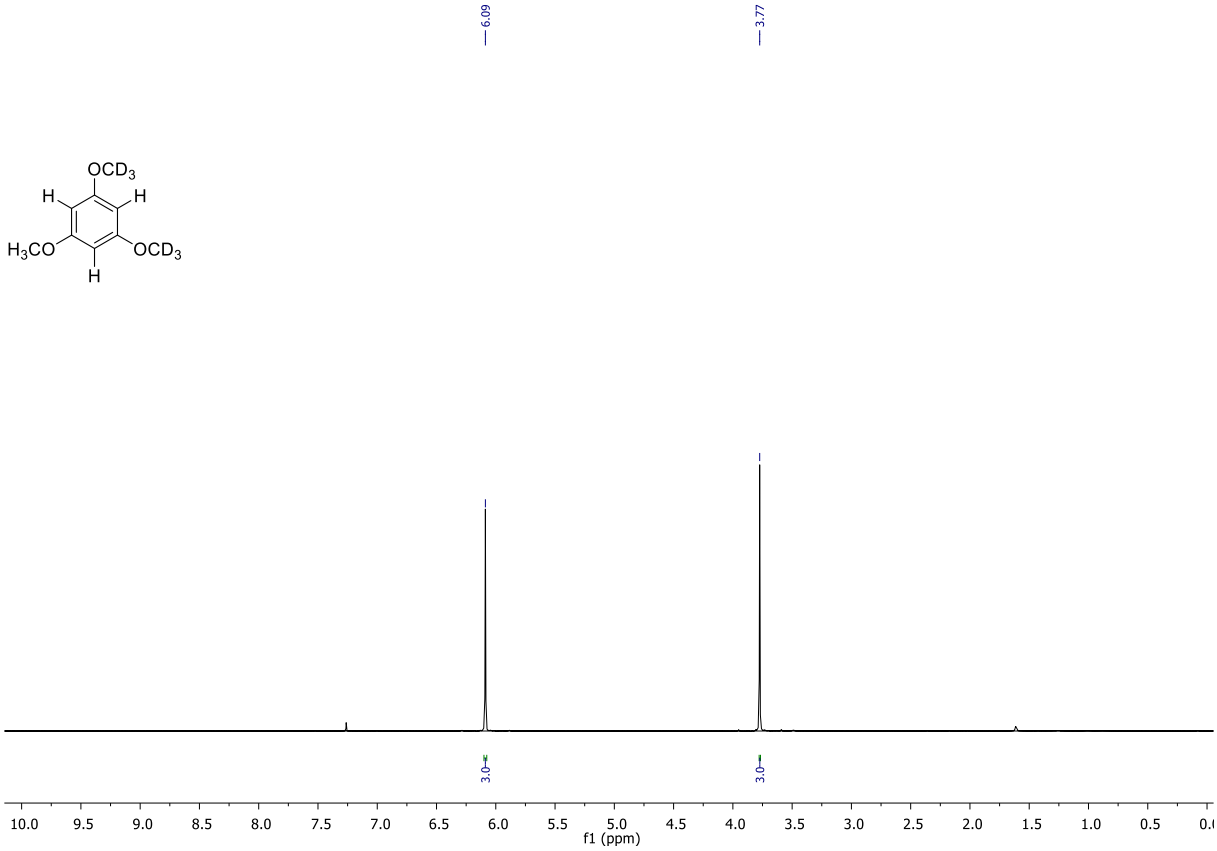
^{1}H NMR (400 MHz, $CDCl_3$, TMB-D3)



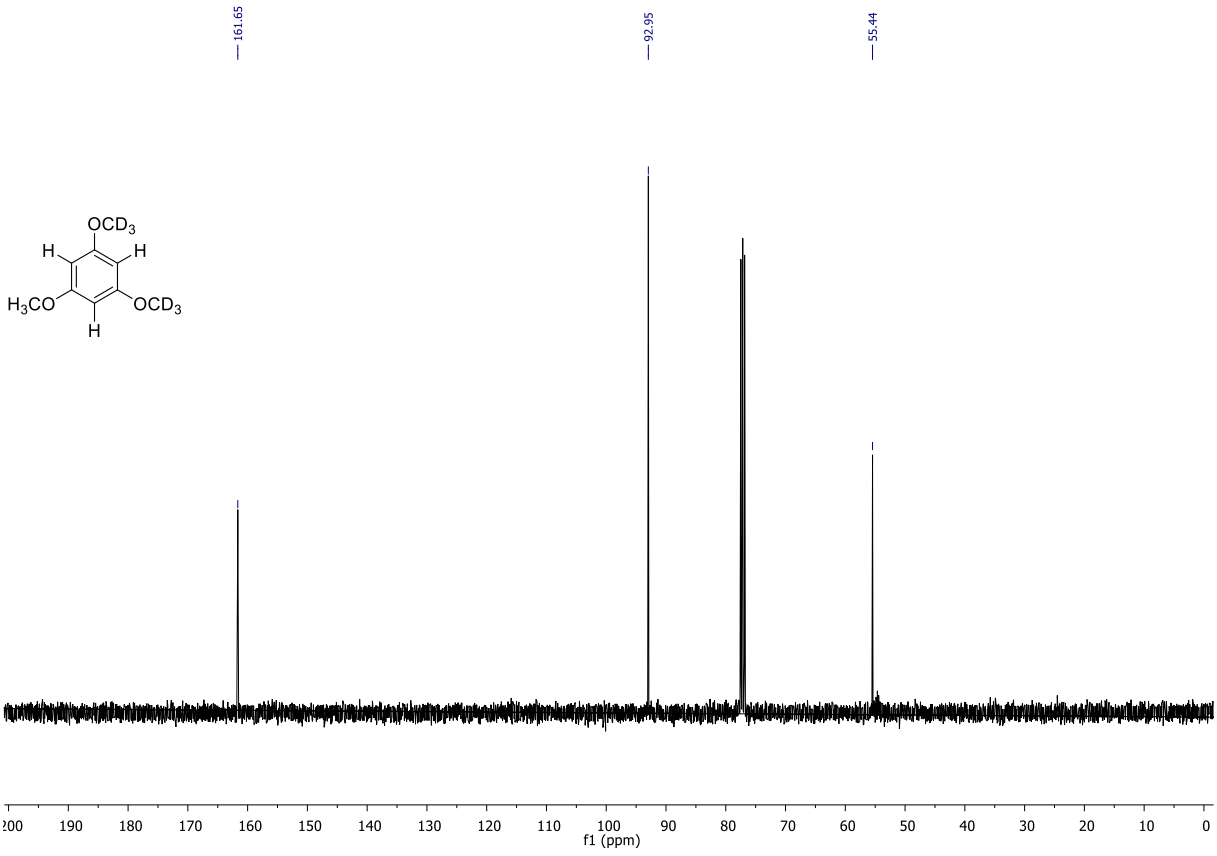
^{13}C NMR (100 MHz, $CDCl_3$, TMB-D3)



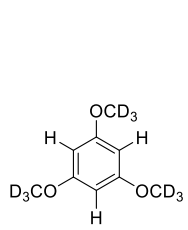
^{1}H NMR (400 MHz, $CDCl_3$, TMB-D6)



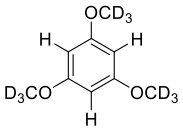
^{13}C NMR (100 MHz, $CDCl_3$, TMB-D6)



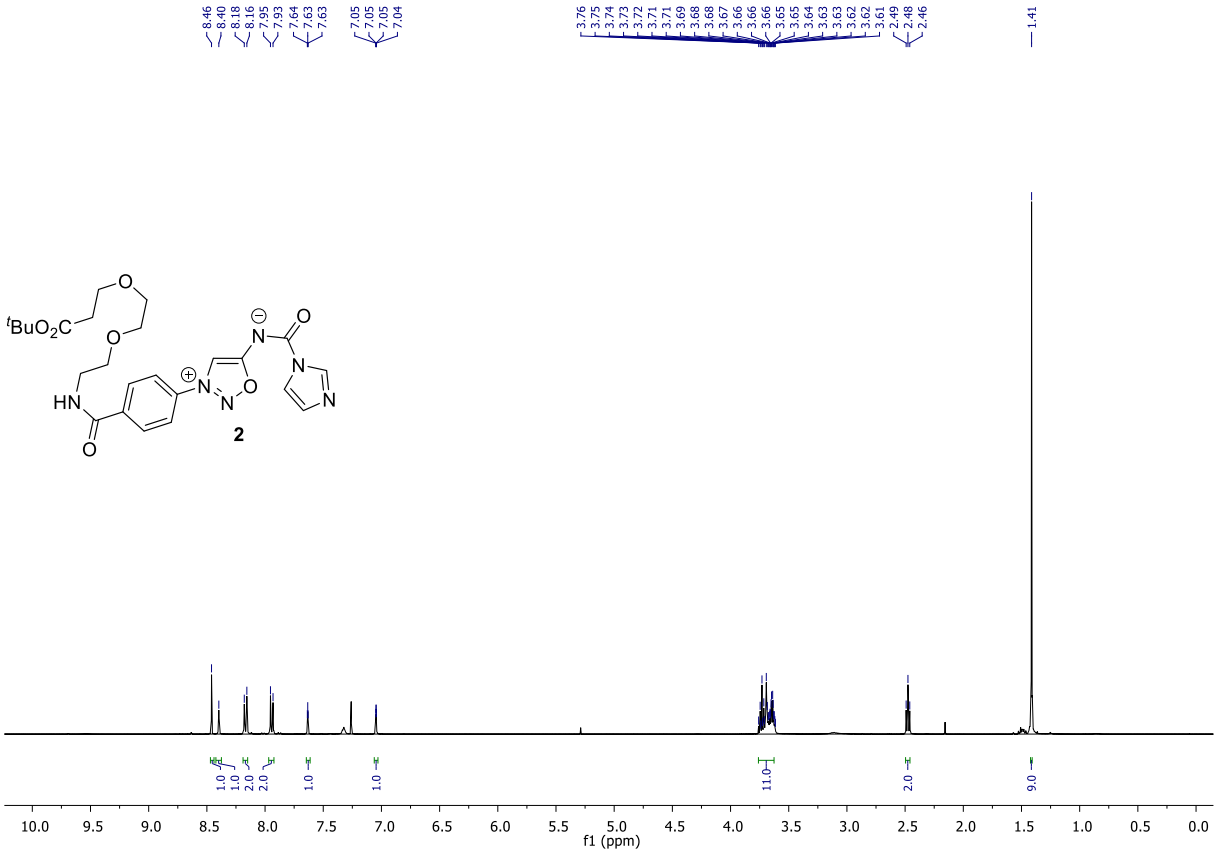
^{1}H NMR (400 MHz, $CDCl_3$, TMB-D9)



^{13}C NMR (100 MHz, $CDCl_3$, TMB-D9)

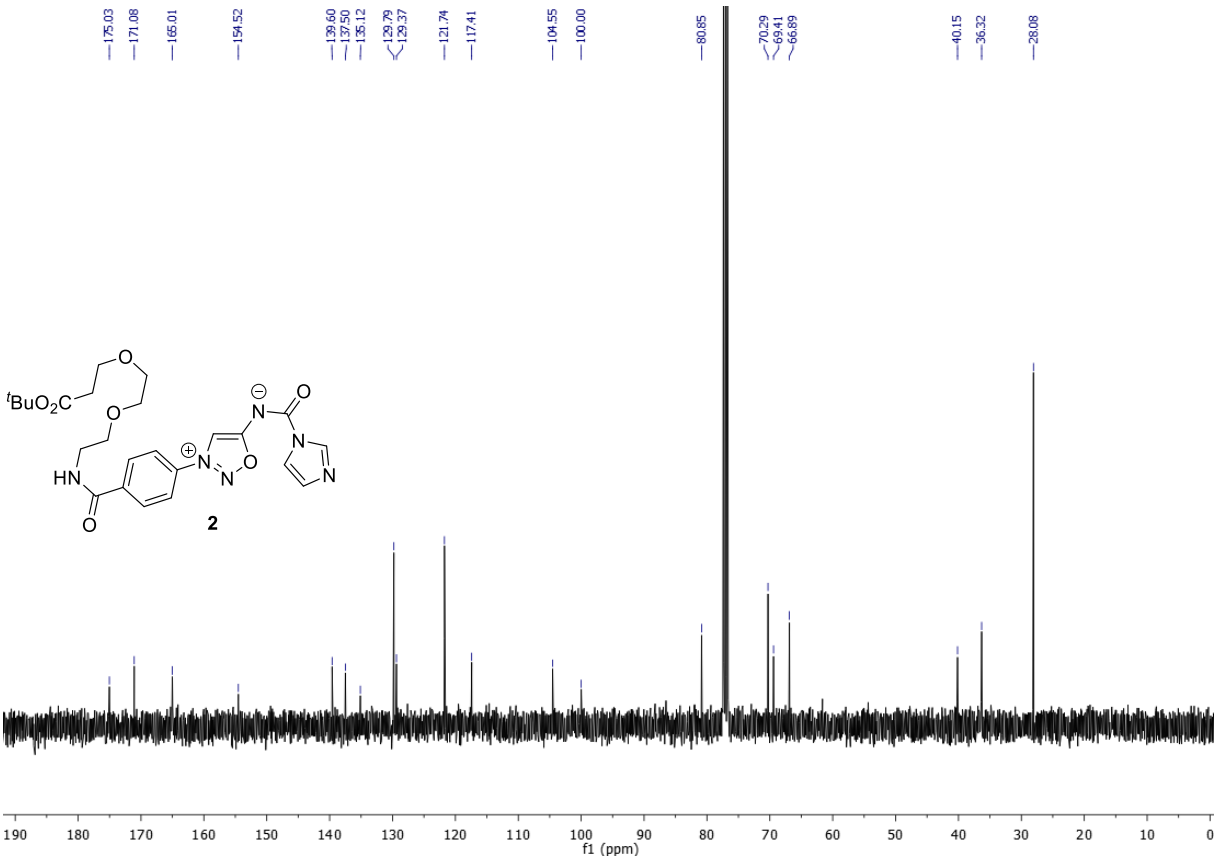


^{1}H NMR (400 MHz, $CDCl_3$, 2)



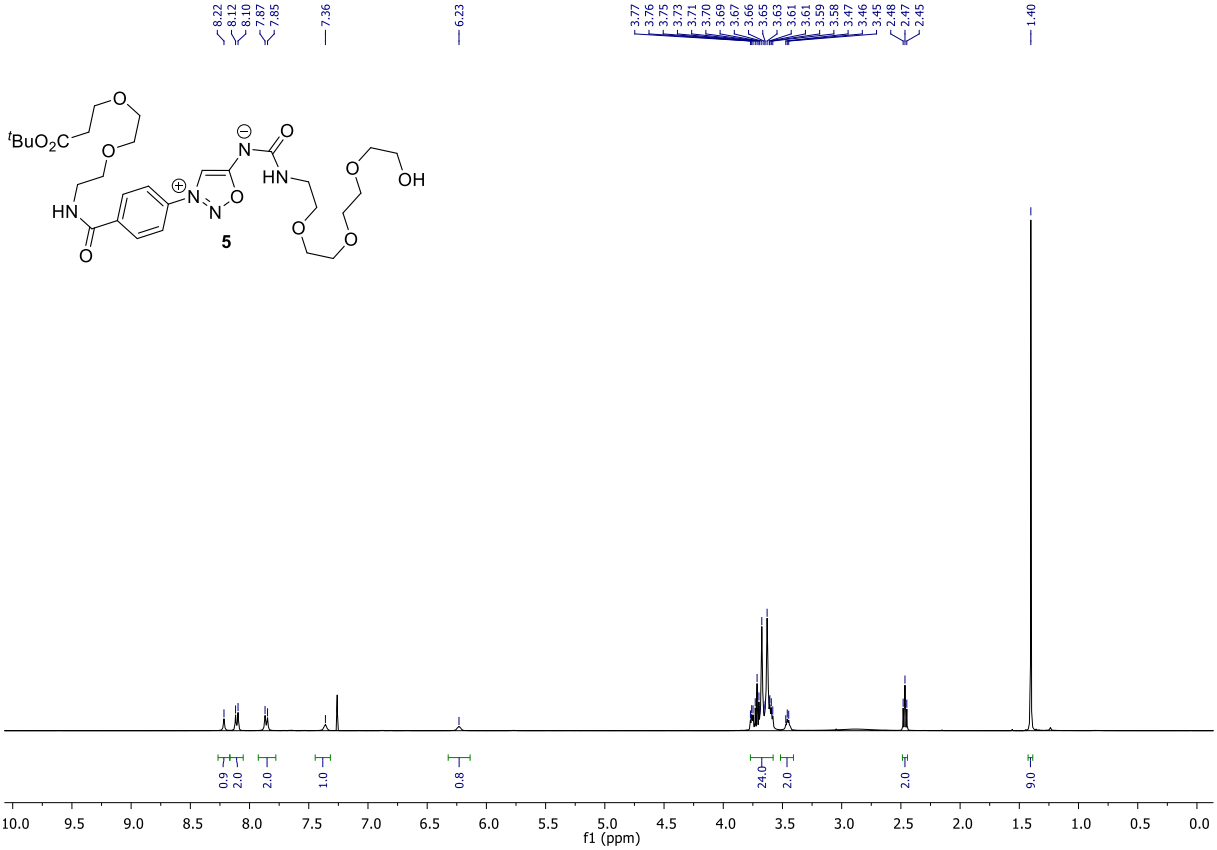
2

^{13}C NMR (100 MHz, $CDCl_3$, 2)

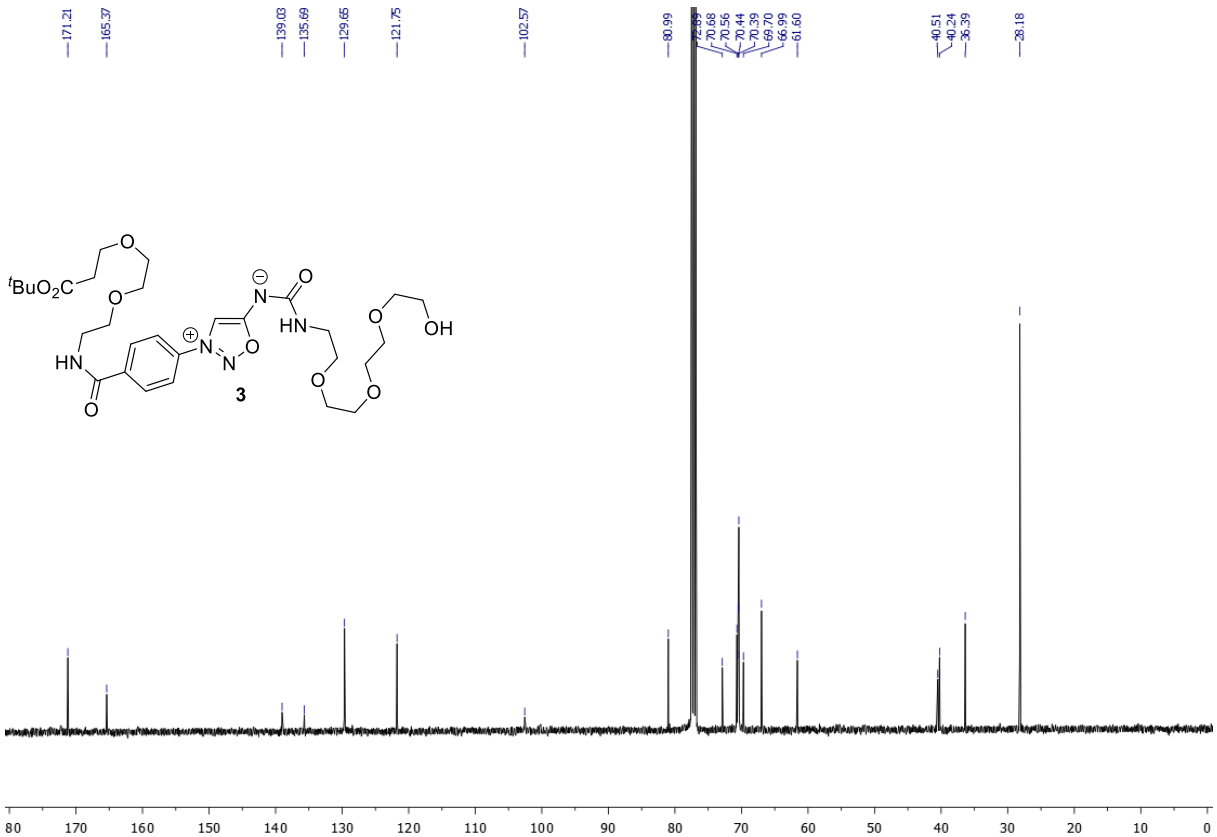


2

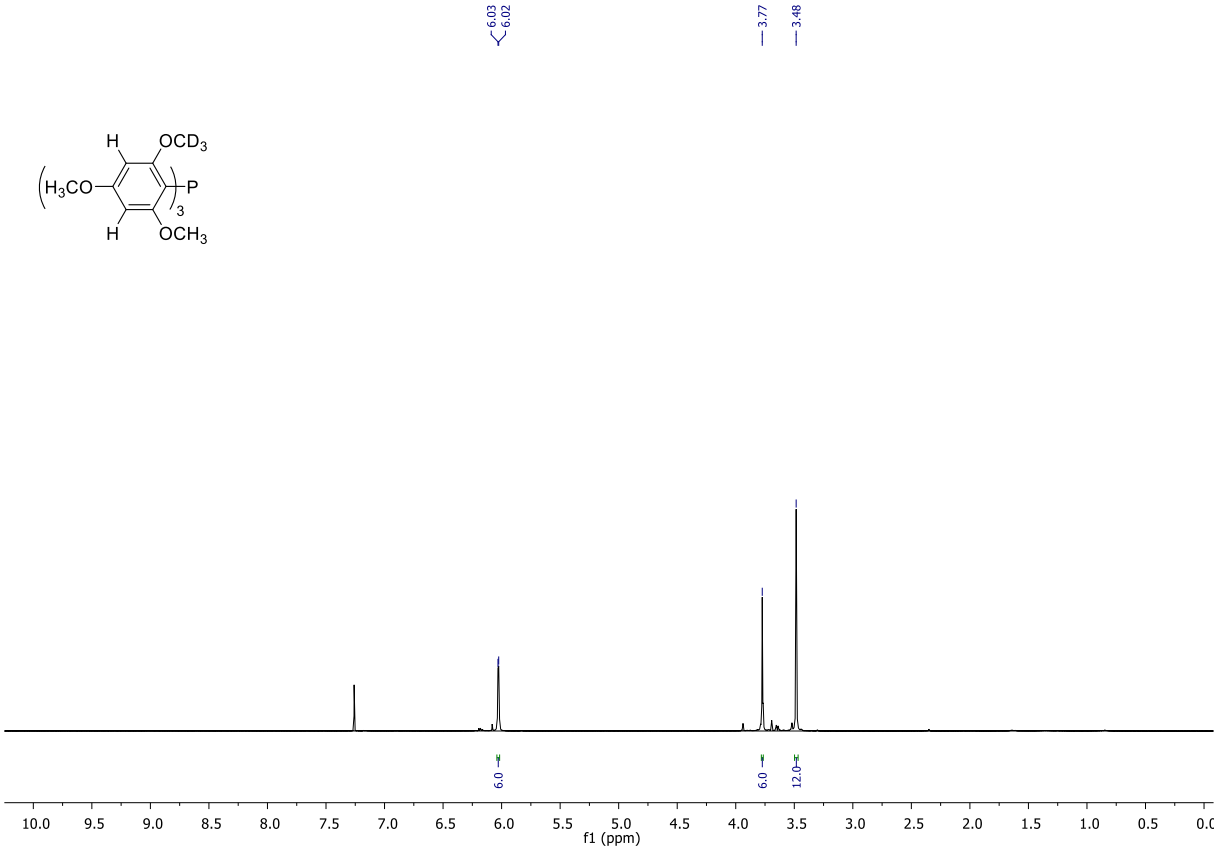
^{1}H NMR (400 MHz, $CDCl_3$, 3)



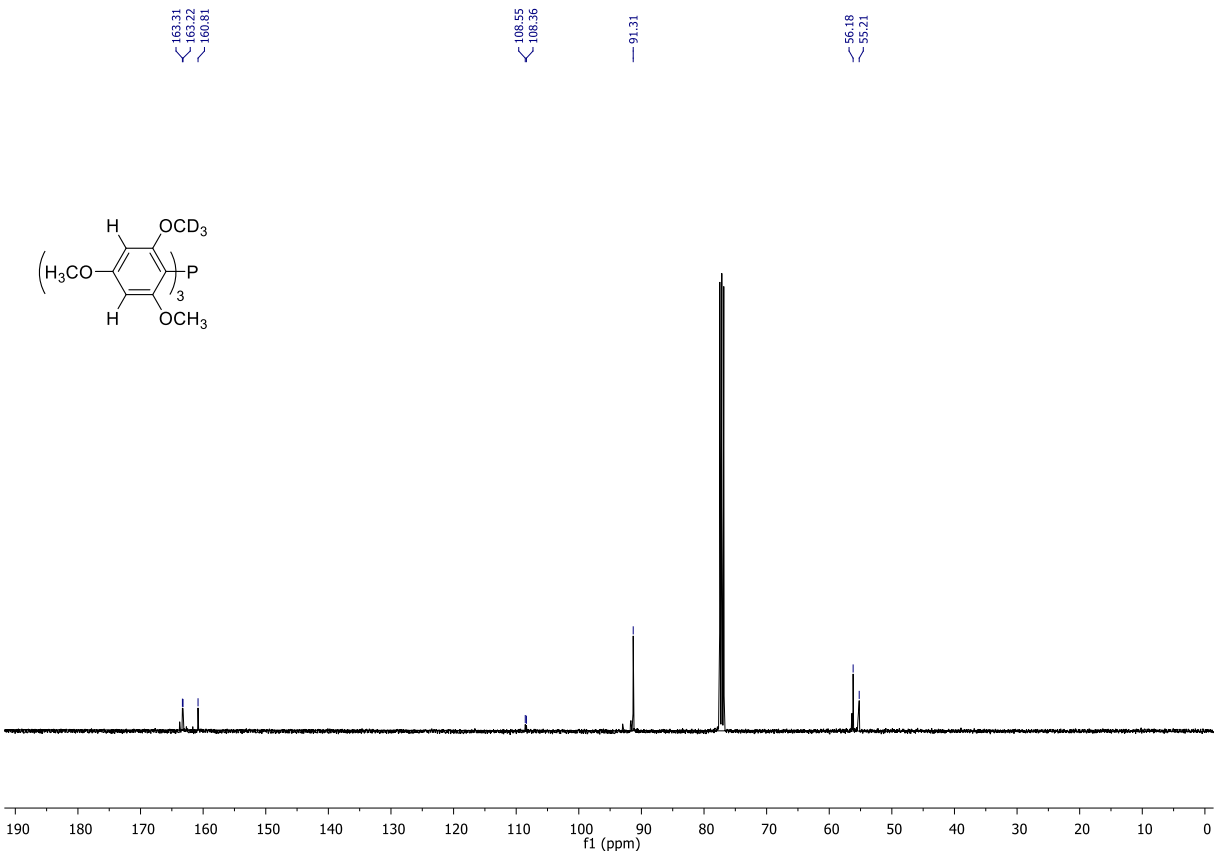
^{13}C NMR (100 MHz, $CDCl_3$, 3)



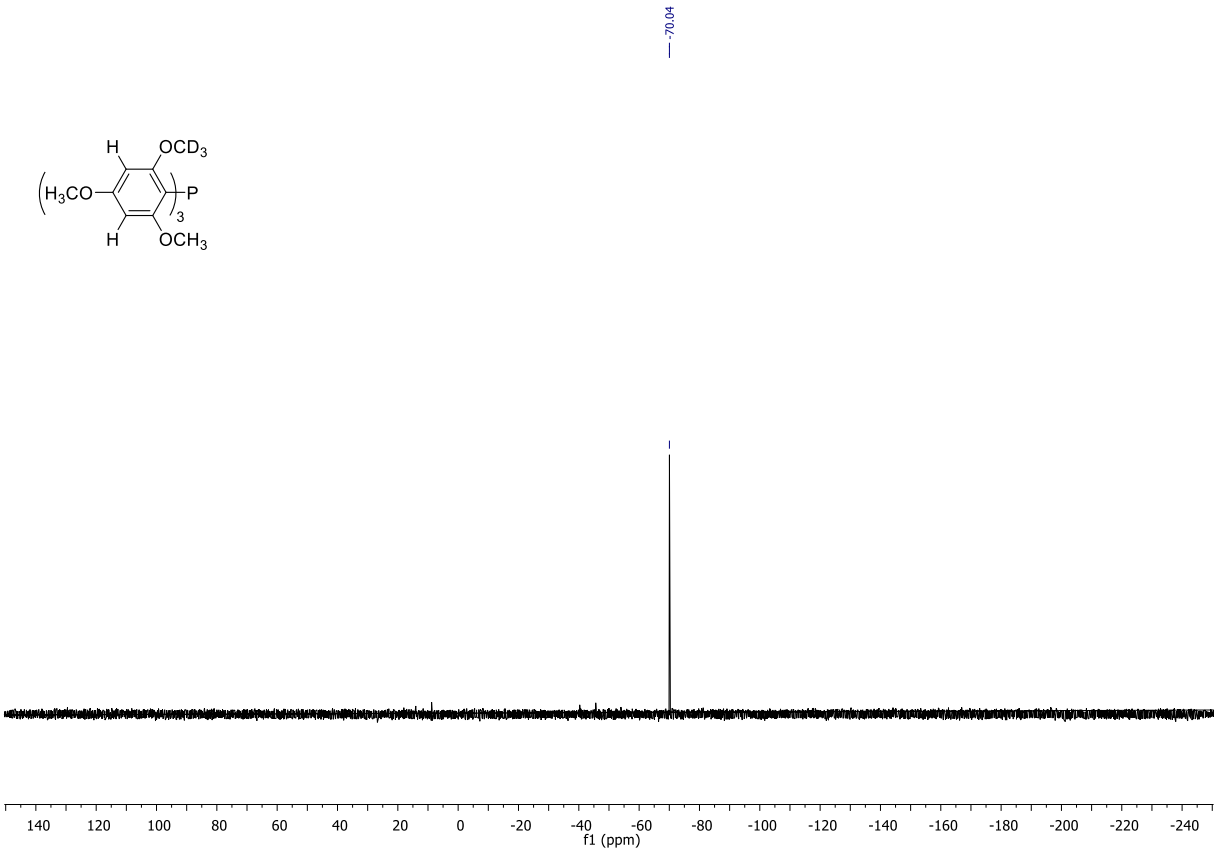
^{1}H NMR (400 MHz, $CDCl_3$, TMPP-H24)



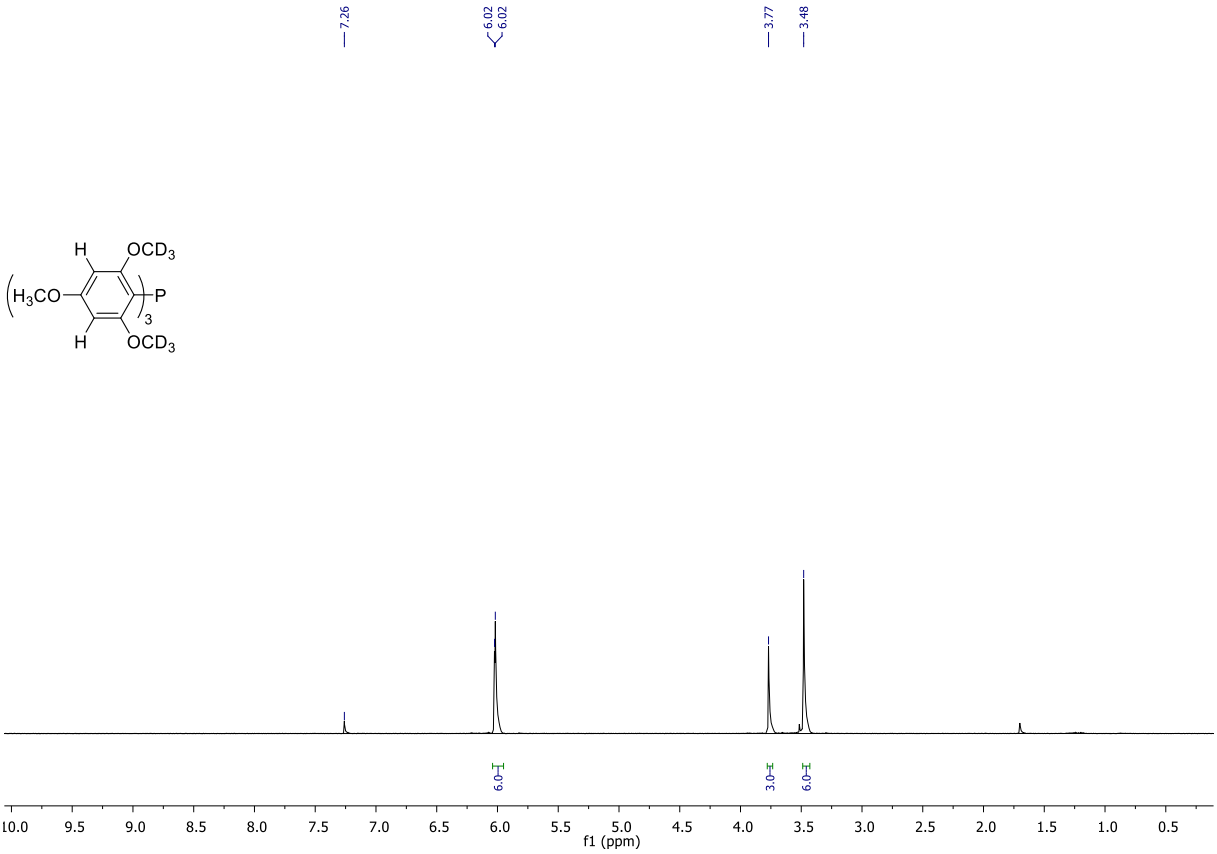
^{13}C NMR (100 MHz, $CDCl_3$, TMPP-H24)



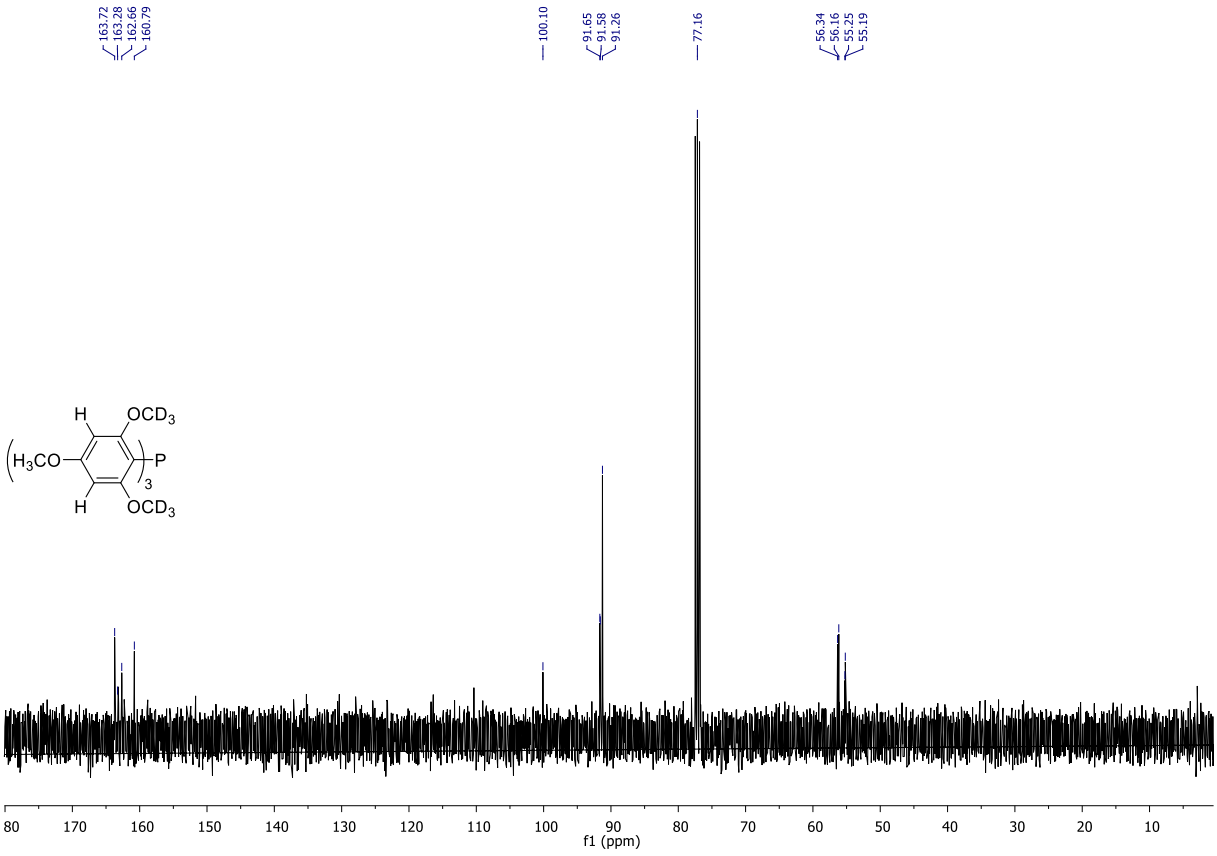
^{31}P NMR (163 MHz, $CDCl_3$, TMPP-H24)



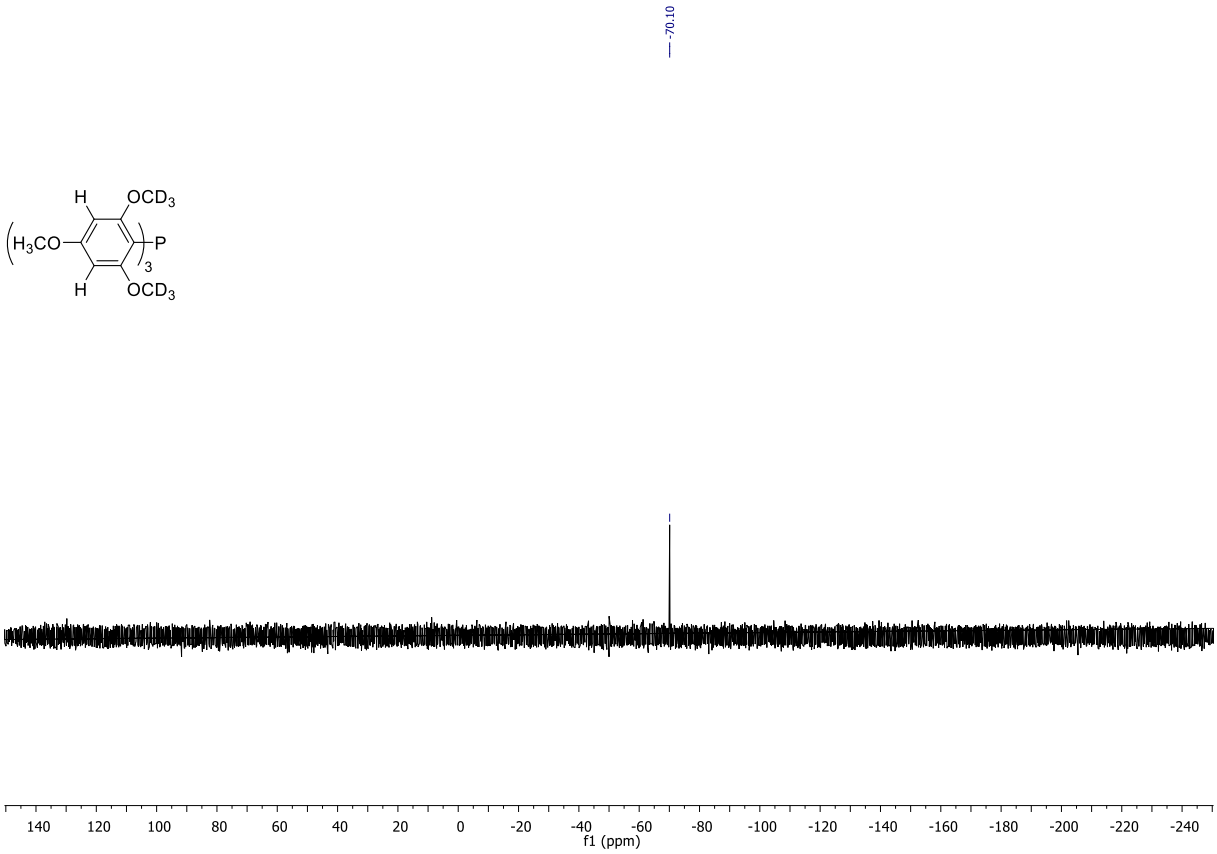
^{1}H NMR (400 MHz, $CDCl_3$, TMPP-H15)



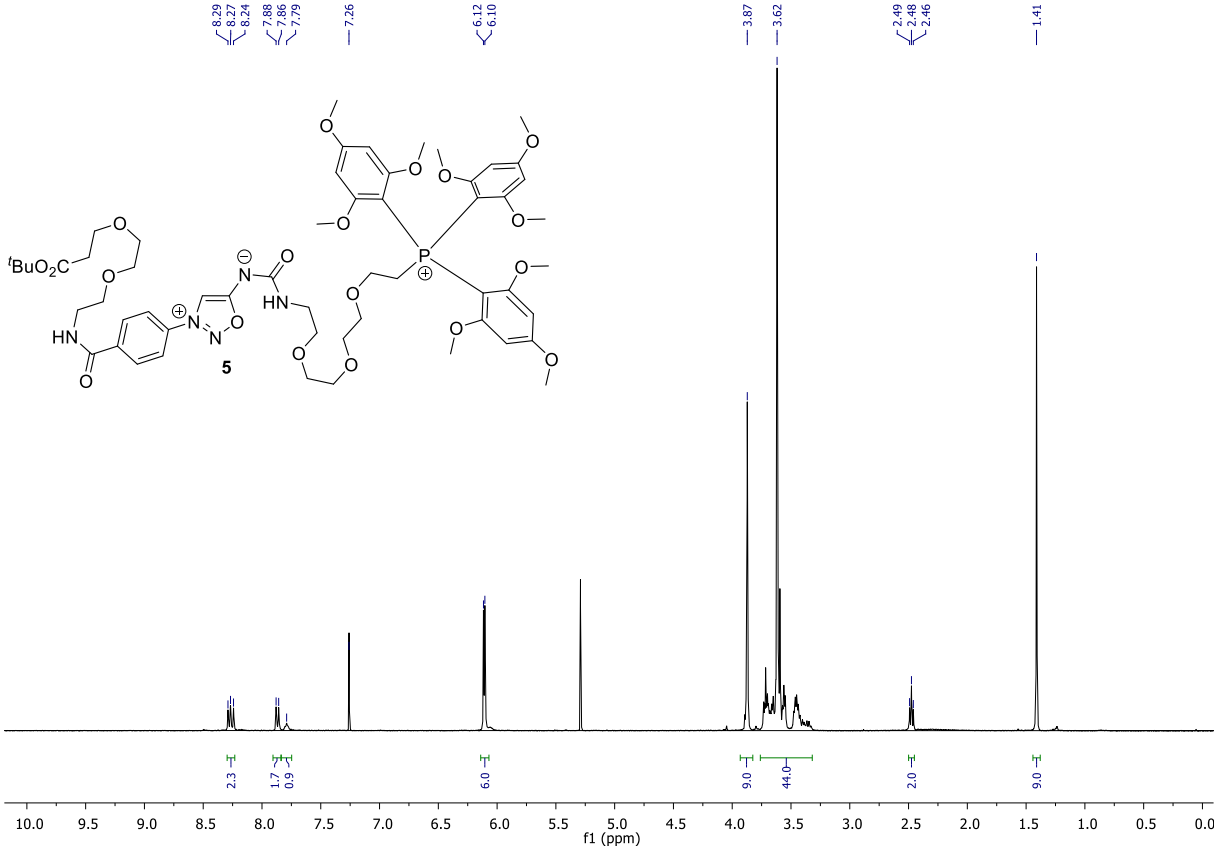
^{13}C NMR (100 MHz, $CDCl_3$, TMPP-H15)



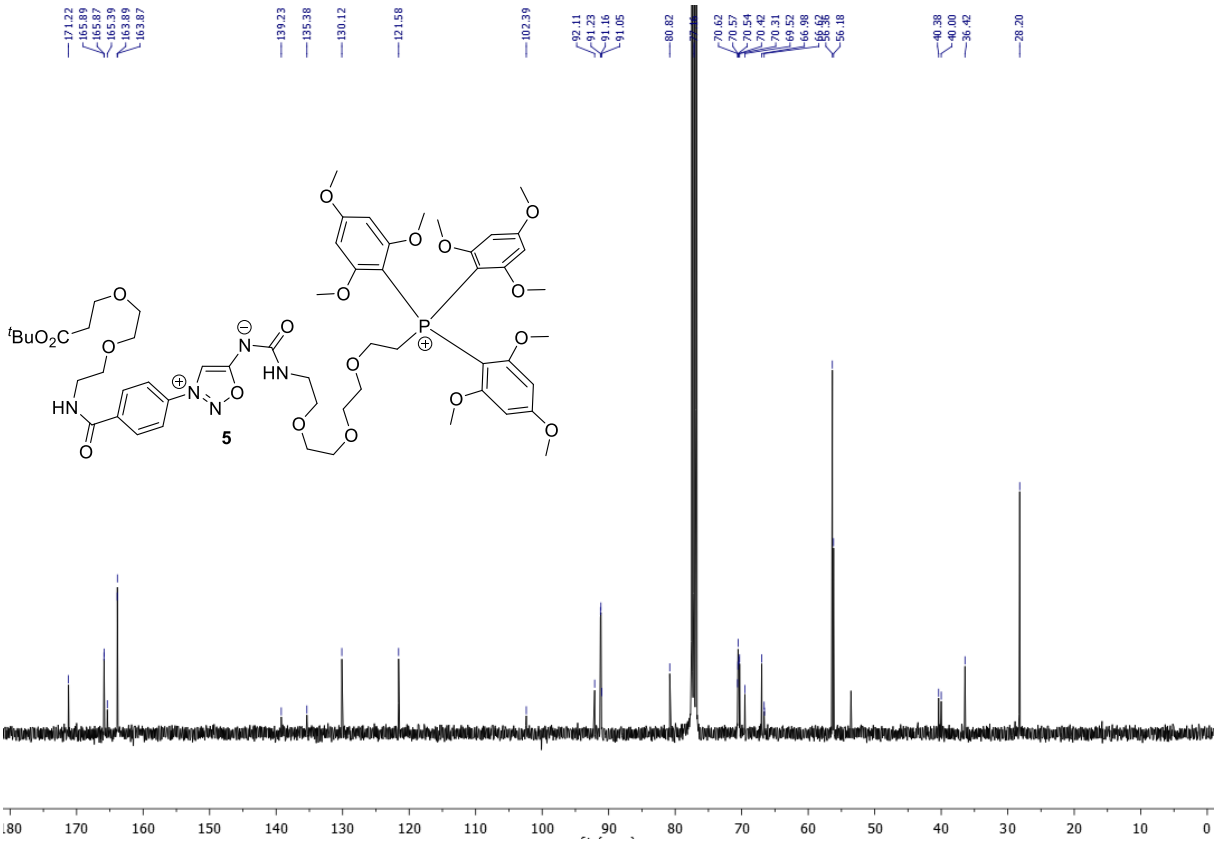
^{31}P NMR (163 MHz, $CDCl_3$, TMPP-H15)



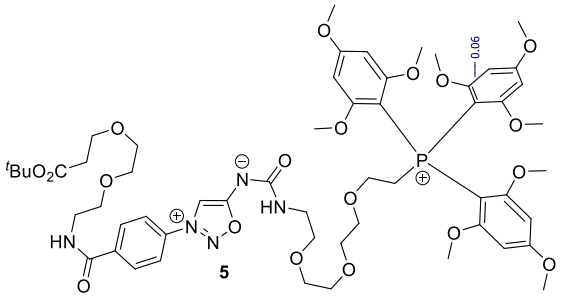
^{1}H NMR (400 MHz, $CDCl_3$, 5-H33)

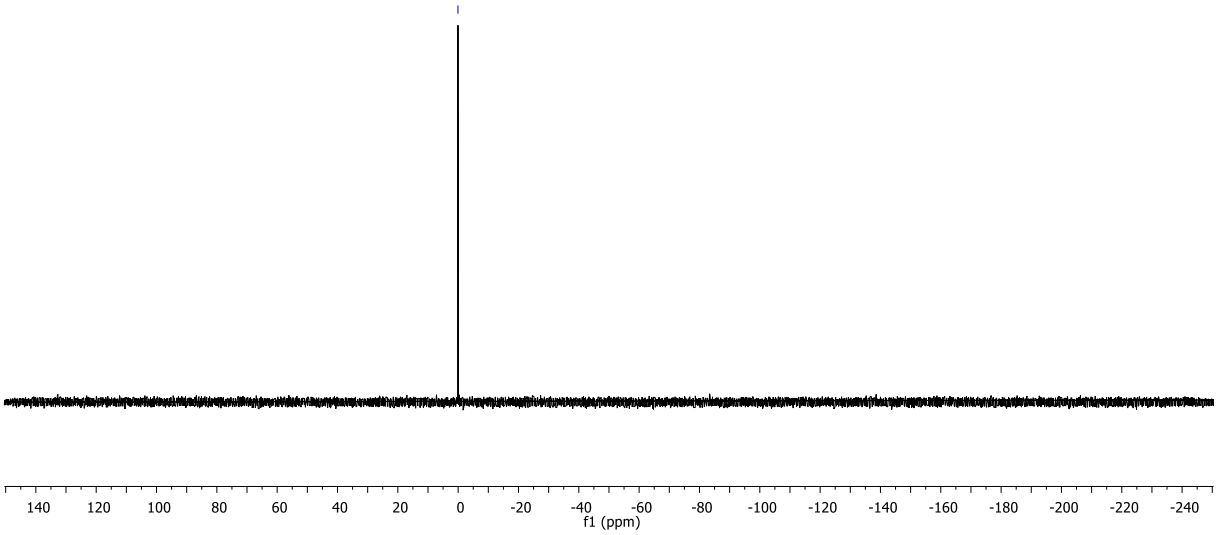


^{13}C NMR (100 MHz, $CDCl_3$, 5-H33)

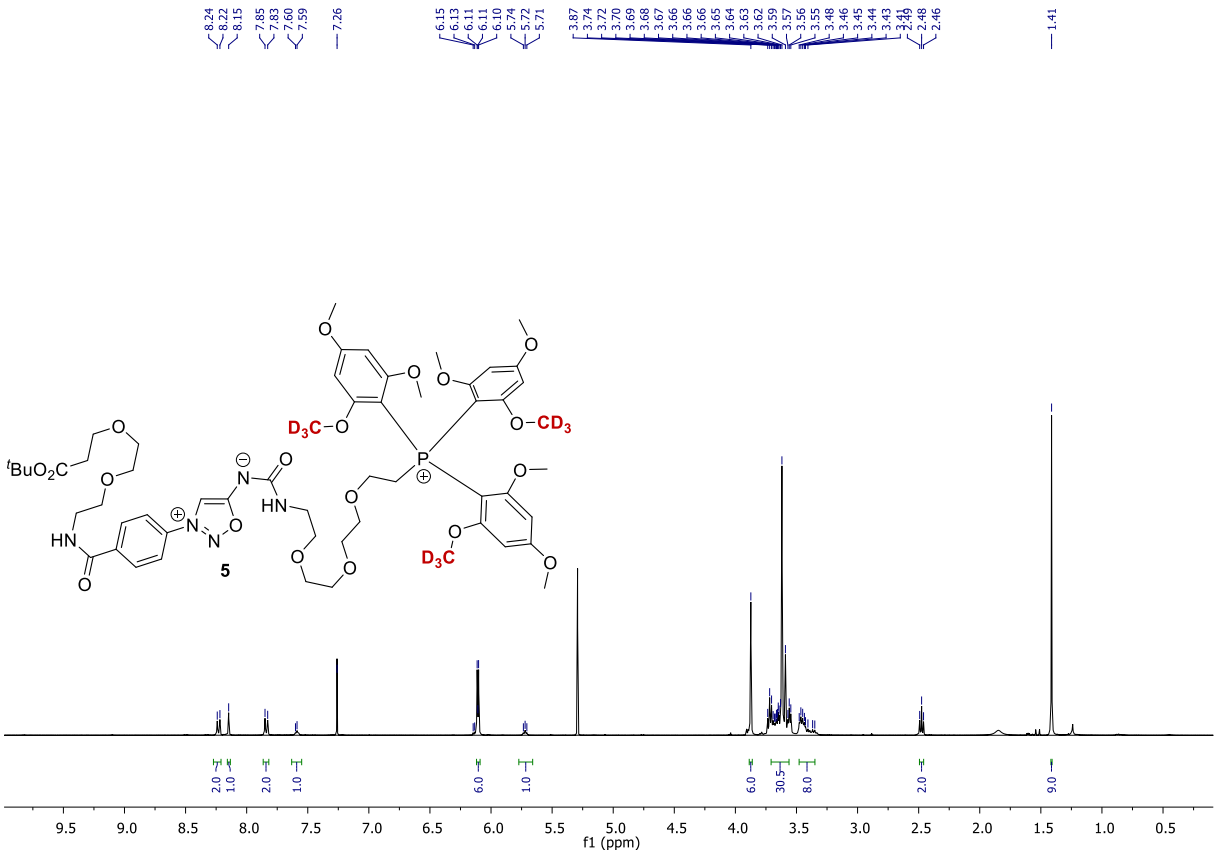


^{31}P NMR (163 MHz, $CDCl_3$, 5-H33)

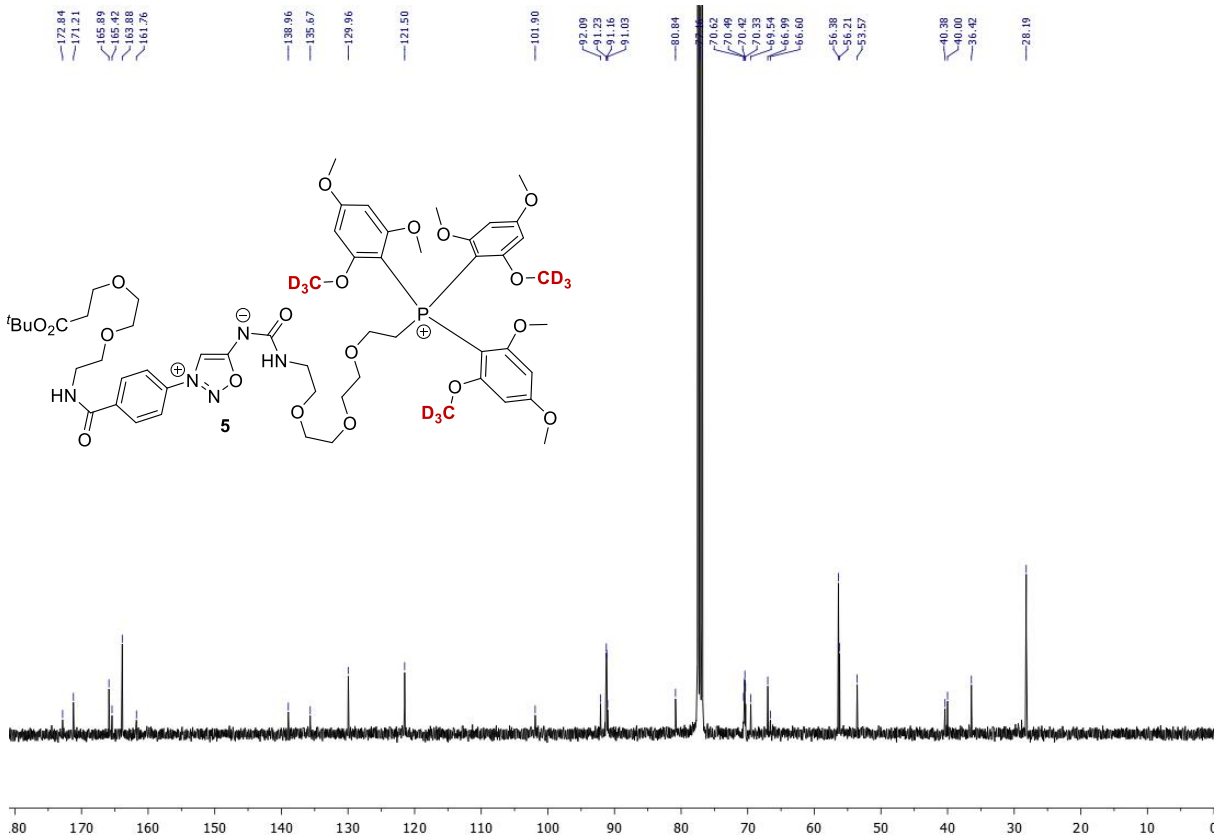




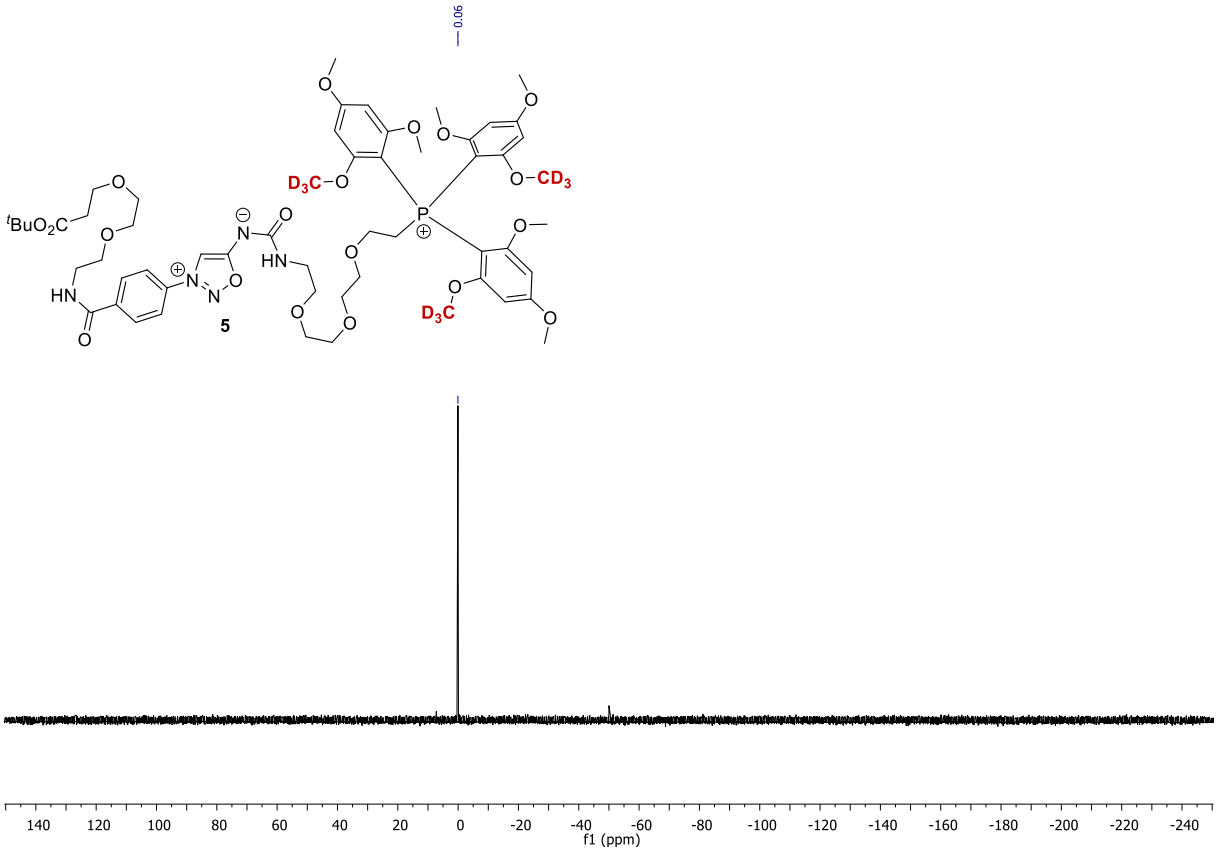
^{1}H NMR (400 MHz, $CDCl_3$, 5-H24)



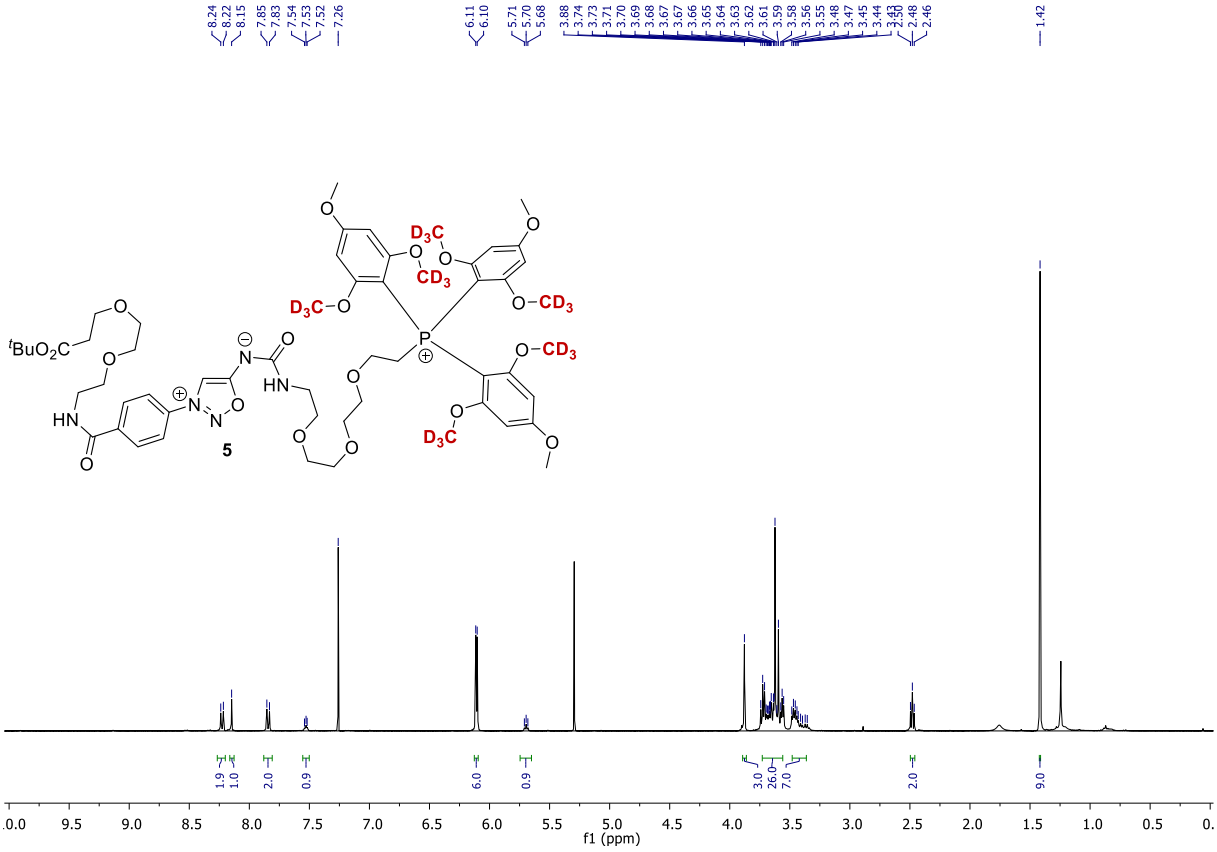
^{13}C NMR (100 MHz, $CDCl_3$, 5-H24)



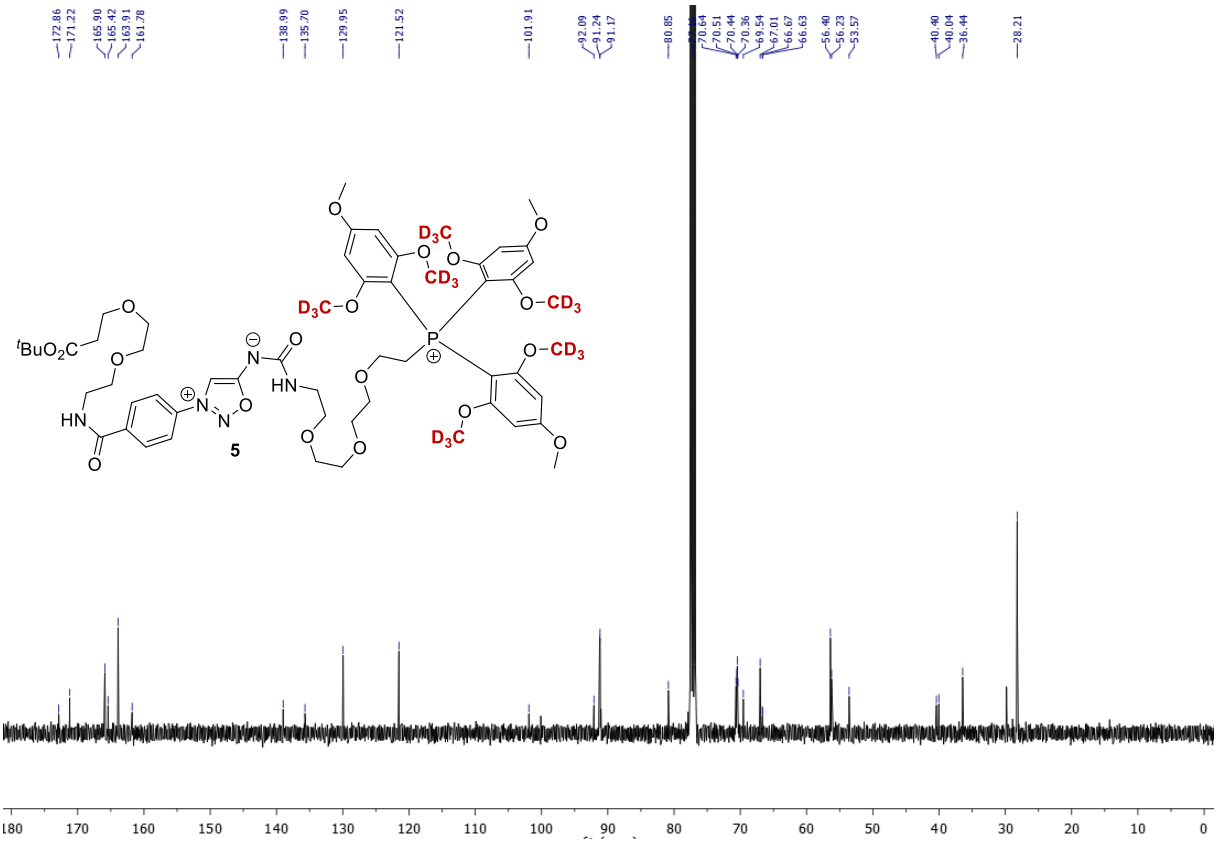
^{31}P NMR (163 MHz, $CDCl_3$, 5-H24)



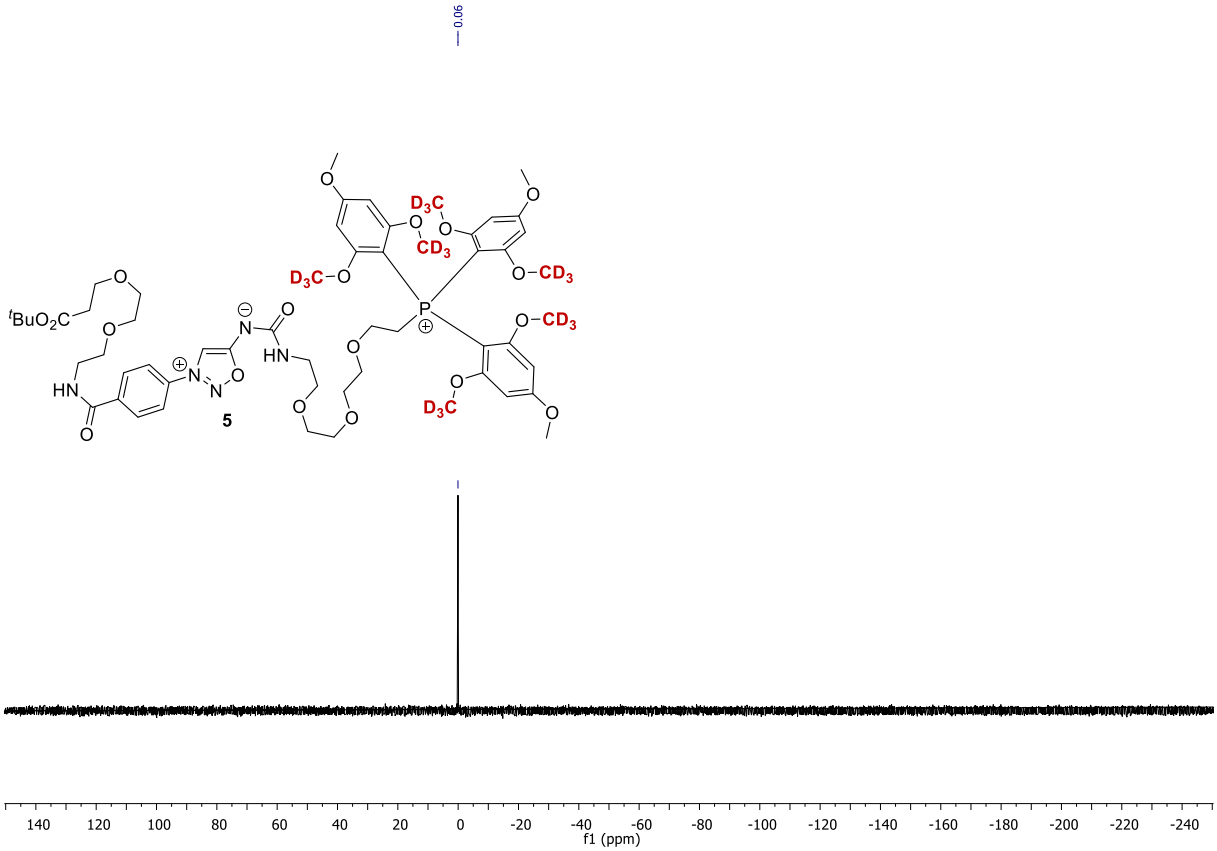
^{1}H NMR (400 MHz, $CDCl_3$, 5-H15)



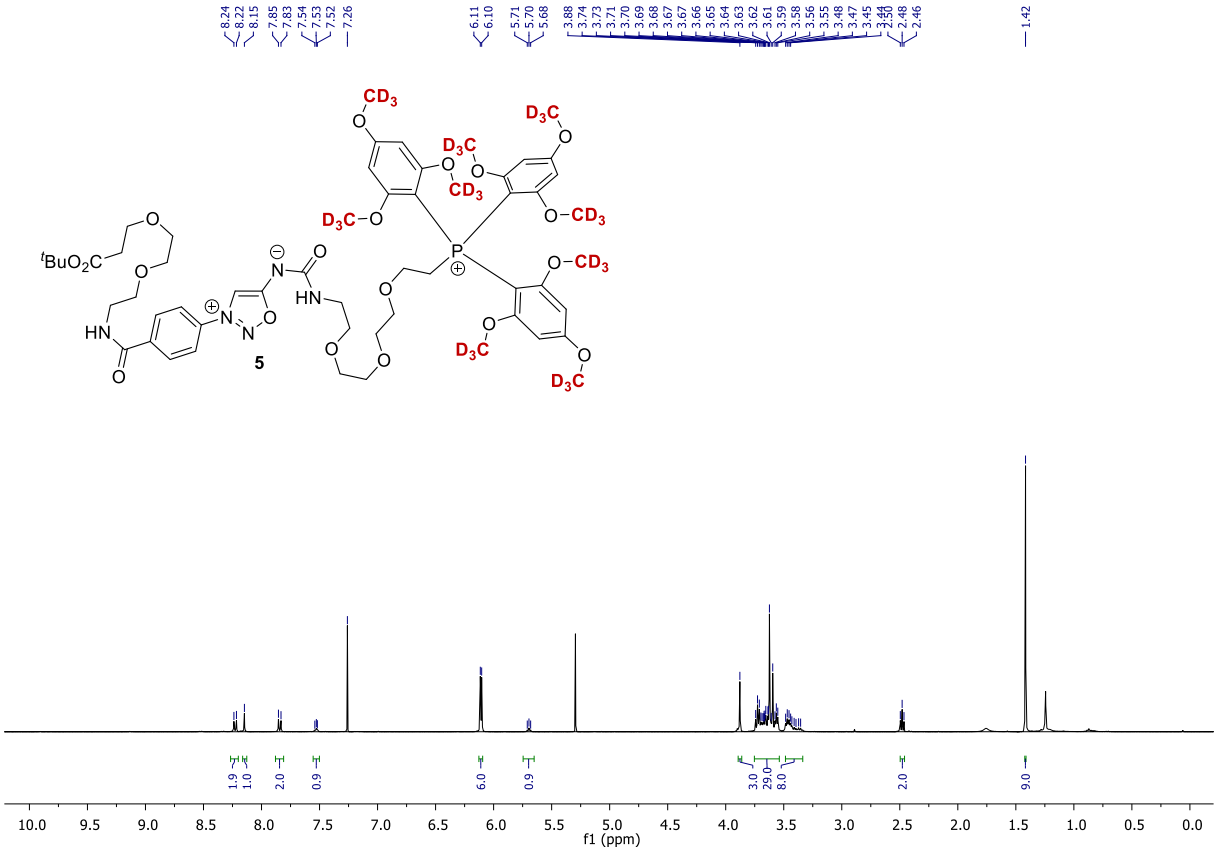
^{13}C NMR (100 MHz, $CDCl_3$, 5-H15)



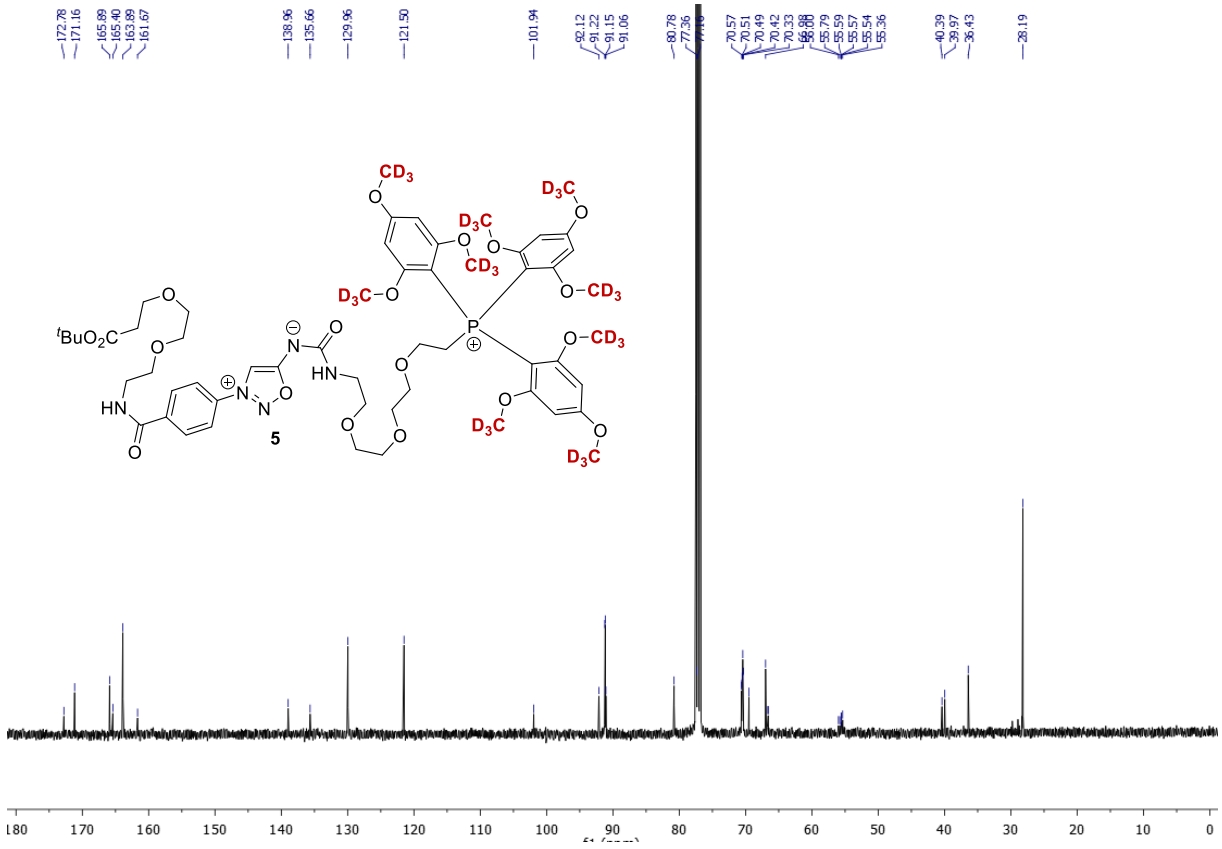
^{31}P NMR (163 MHz, $CDCl_3$, 5-H15)



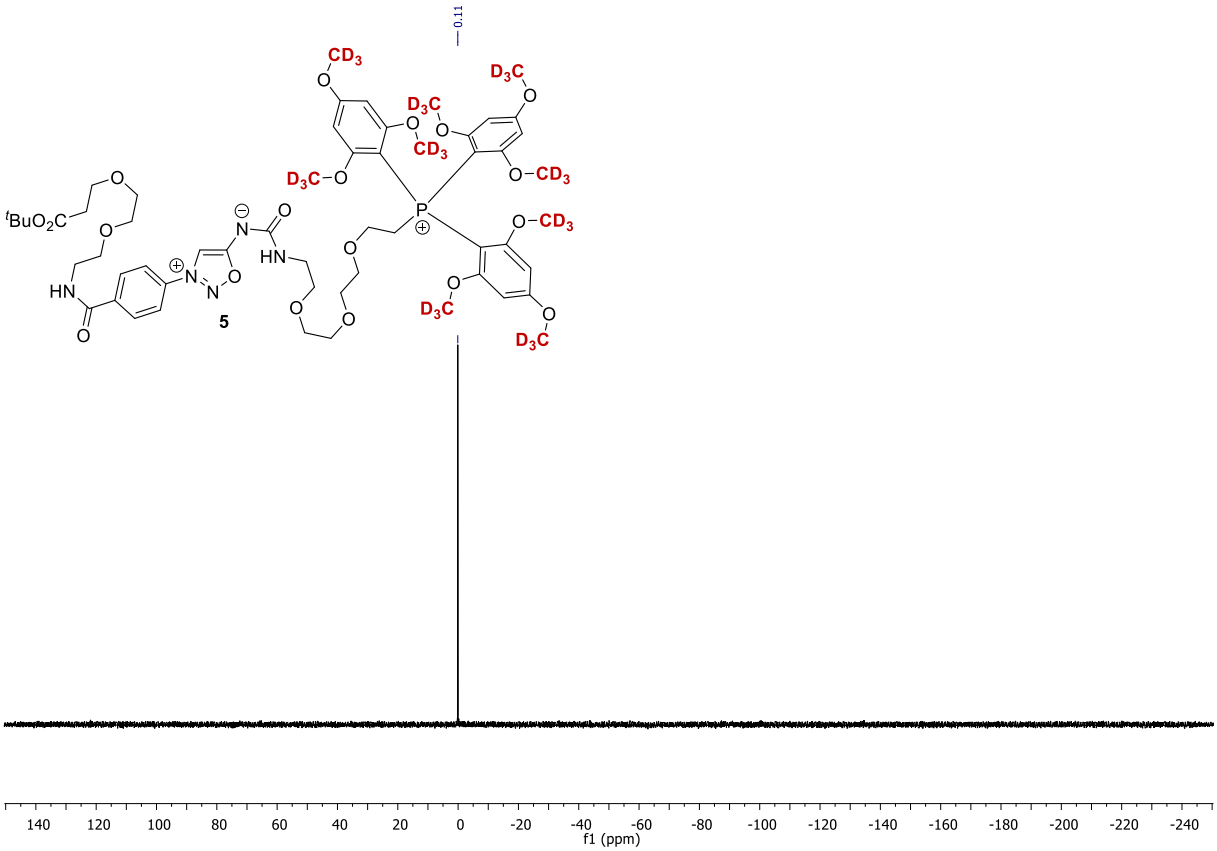
^{1}H NMR (400 MHz, $CDCl_3$, 5-H6)



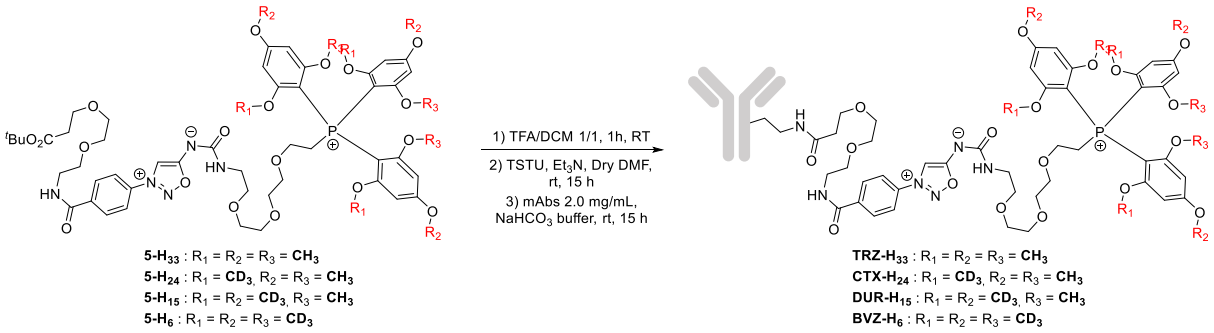
^{13}C NMR (100 MHz, $CDCl_3$, 5-H6)



^{31}P NMR (163 MHz, $CDCl_3$, 5-H6)



IV. General procedure for antibody-Tag conjugates preparation



General procedure for TMPP-antibody bioconjugation

5 was dissolved in a solution of DCM/TFA 1/1 (0.05 M) and the solution was stirred for 2 hours. After completion of the reaction, the crude mixture was evaporated under high vacuum to give the corresponding carboxylic acid quantitatively. To this acid (1.0 equiv.), TSTU (1.0 equiv.) and Et_3N (1.0 equiv.) were added in dry DMF and stirred at 25 °C for 3 hours. Then 5-10 µL, depending of the mAb, of this solution (10-20 equiv.) were then added to 1.0 mL of a solution of mAb at 2.0 mg/mL in 0.1 M $NaHCO_3$ pH 8.3. The mixture was then incubated at 25°C for 4 hours and the resulting mAbs-H_{xx} were purified *via* a minitrap® cartridge (1000 µL loading, 1000 µL elution of 0.9 % NaCl in DI water).

Table S1. Preparation and characterization of TMPP-mAbs

TMPP-mAb	**5**-Hxx (equiv.)	**Concentration** (BSA assay)	**TAR**
TRZ-H33	**5**-H33 (20 equiv.)	1,26 mg/mL	4.16
CTX-H24	**5**-H24 (20 equiv.)	1.06 mg/mL	2.49
DUR-H15	**5**-H15 (10 equiv.)	1.28 mg/mL	2.35
BVZ-H6	**5**-H6 (20 equiv.)	0.54 mg/mL	3.44

General procedure for BCA assay

The concentration of antibodies functionalized with TMPP tags was determined by BCA assay. Briefly in a 96-well plate, 90 µL of the BCA Working Reagent solution were added to 10 µL of the antibody solution and incubated 30 min at 37 °C. After 5 min, the temperature was cooled down to room temperature; the absorbance of the solution at 562 nm was measured on a microplate spectrophotometer (CLARIO Start Plus, BM6 LABTECH). The antibody concentration was determined by comparison to a standard calibration curved made with BSA protein at concentration ranging from 0.2 to 1 g/L. (Figure S1).

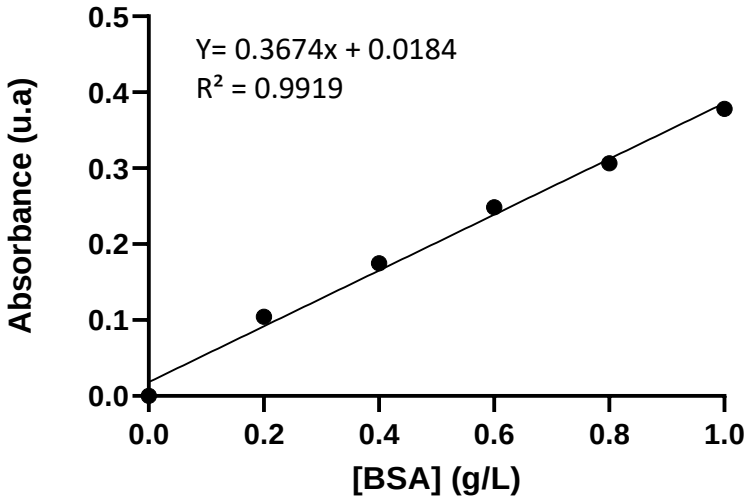
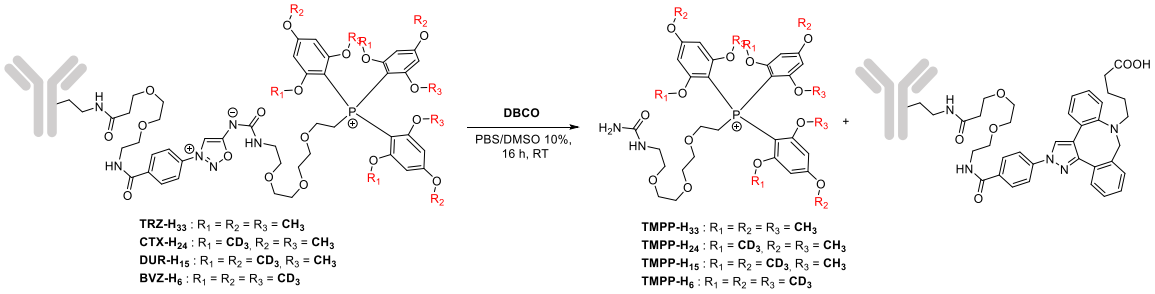


Figure S1: BSA standard calibration curve. BSA absorption at 562 nm versus protein concentration (g/L).

General procedure for TAR (Tag-Antibody-Ratio) determination

Due to the permanent positive charge of TMPP tags, the antibody-conjugates are multicharged making impossible a direct analysis of the conjugates by MALDI. The determination of TAR was therefore carried out by analyzing the amount of released TMPP tags after click and release SPSIC reaction with DBCO.



DBCO (10 µL, 100 µM, 100 equiv./mAbs) was added to a solution of mAbs-H_{xx} (100 µL, 1 equiv.) in 0.9 % NaCl solution. The mixture was agitated overnight, and the released Tag was quantified in this solution by HPLC. Knowing the concentration of both the antibody and the tag, the TAR was calculated by dividing the amount the TAG-H_{xx} by the amount of antibody.

V. Stability of antibody-Tag conjugates

General procedure for stability studies

Stability in blood plasma of bioconjugates was determined by measuring the release of their tags upon reaction of the sydnonimine linker with DBCO. The four antibody-tag conjugates were incubated in blood plasma for 4, 24 and 48 hours at 37 °C. After click-and-release SPSIC reaction with DBCO, the released TMPP tags were analysed by LC-HRMS and quantified relative to a blank (non-incubated) sample.

Experimental protocol:

3 µL of TMPP-mAbs conjugate solutions (TRZ-H33 8.4 µM, CTX-H24 7.1 µM, DUR-H15 8.5 µM, BVZ-H6 5.6 µM) were added to 27 µL of mice blood plasma. The solution were incubated at 37 °C for 4, 24 or 48 hours before addition of 3 µL of DBCO-Acid (10 equiv. in HPLC grade DMSO) and stirred for 30 min at 37 °C. Plasma proteins were then precipitated with 6 volumes (200 µL) of an ice-cold solution of methanol/acetonitrile (1:1). The obtained suspensions were centrifuged 15 min at 16000g, 4 °C. 150 µL of supernatant were then transferred to a 2 mL vial for LC-MS analysis. The same protocol was used for blank samples (t0, no incubation). Each experiment was performed in duplicate.

Relative quantification was performed by comparing the integration of the MS signal of the released TMPP tags. All analyses were performed on a Xevo G2-XS Q-Tof mass spectrometer, coupled with ultra-high-performance liquid chromatography (Acquity I-Class), both from Waters. The separation of released TMPP tags was achieved on a BEH C18 column (50 x 2.1 mm ; 1.7 µm) at 40 °C. The mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 600 µL.min^{-1} with a gradient from 5 to 100% B in 1.5 min. The injection volume was set to 2 µL and the autosampler temperature at 15 °C. The parameters of the electrospray ion source (ESI) were: Capillary voltage 3.0 kV ; sampling cone voltage 75 V ; source temperature 140 °C ; desolvatation gas (N_2) temperature and flow rate 550 °C, 1000 L/H ; cone gas flow rate 100 L/H. MS data were acquired with the software Masslynx (Waters) in the positive ionization mode over a mass range of m/z 50 - 1200 Da. Collision energy was set to 6 V.

Mass accuracy within 3 ppm was reached using a solution of leucine enkephalin for internal calibration. TargetLynx (Waters) software was used for automatic integrations of the released TMPP tags signal. Integrations were performed on TMPP-tags XIC with a mass window of 50 mDa (TMPP-H33, TMPP-H24, TMPP-H15 and TMPP-H6, respectively m/z 751.32, 760.38, 769.44, 778.49).

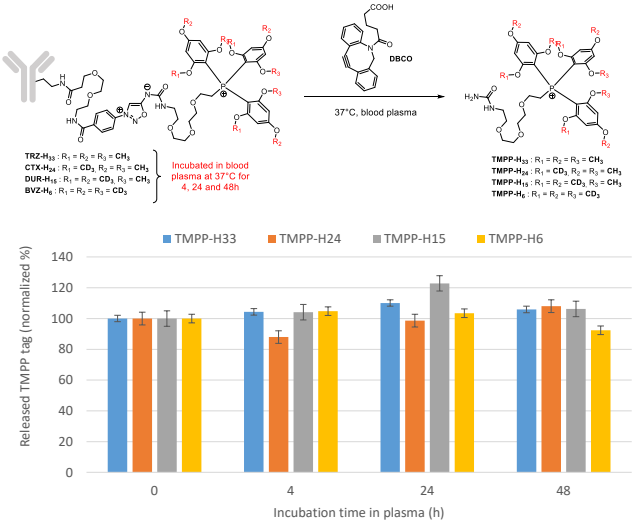


Figure S2: Stability of bioconjugates in blood plasma.

VI. Kinetic studies

General procedure for kinetic studies with TMPP-mAbs conjugates

Reactions were monitored by measuring the concentration of the released TMPP tags using the appearance of fragment ions Frag-H6, 15, 24 and 33 by LC-HRMS/MS (Figure S3).

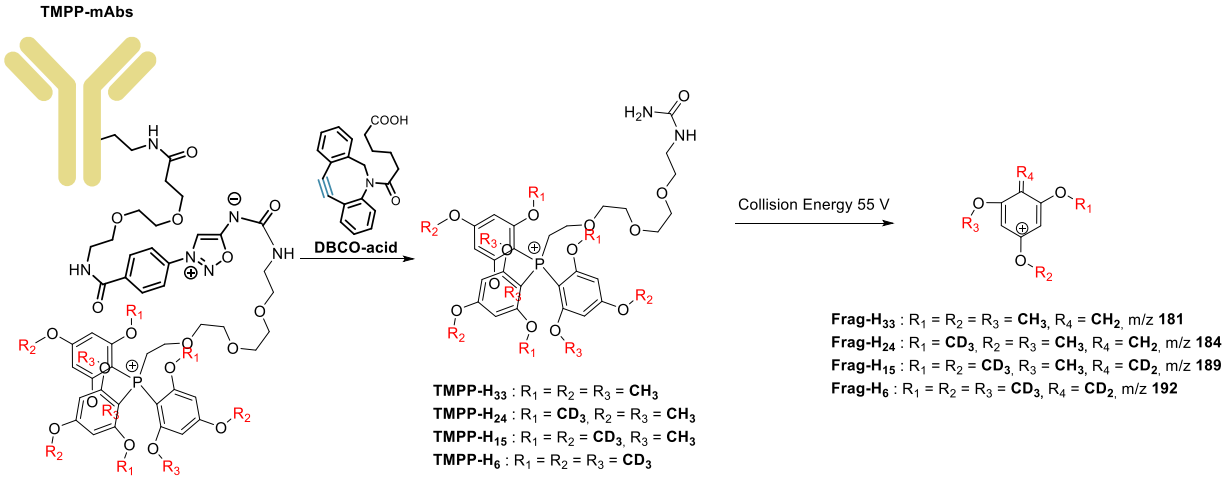


Figure S3. Monitoring of TMPP tag release upon addition of DBCO-acid on TMPP-mAbs.

5 µL of TMPP-mAbs conjugate solutions (TRZ-H33 8.4 µM, CTX-H24 7.1 µM, DUR-H15 8.5 µM, BVZ-H6 5.6 µM) were diluted in 1485 µL of PBS 0.1 M. The solution was equilibrated at 37 °C in the LC autosampler before adding 10 µL of DBCO-Acid (50 equiv. in HPLC grade DMSO). To ensure selective measurement of the TMPP tag concentrations, LC-MS/MS analysis were performed. TMPP-H6, 15, 24 and 33 were selected as precursor ions and signals of fragment ions Frag-H6, 15, 24 and 33 were detected. 5 µL of the reaction mixture were regularly injected in the LC-MS instrument to follow the reaction. The peak of fragments Frag-H6, 15, 24 and 33 were integrated in the chromatograms. Calibration curves were generated from different standard concentrations of TMPP tags to establish the correlation between the integrated signal of fragments Frag-H6, 15, 24 and 33 and the concentration of the released TMPP tags in the reaction mixture, thus permitting to follow the release of TMPP tags from the mAbs. Example of tag release kinetic is given in Figure S4.

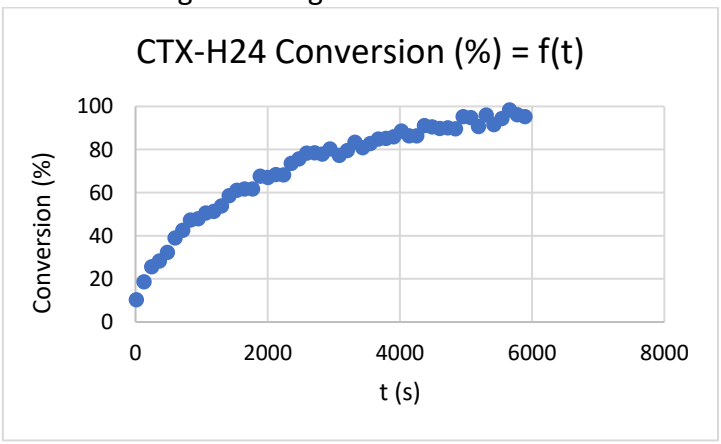


Figure S4. Release of tag TMPP-H24 from bioconjugate CTX-H24 over time. Reaction conditions: [CTX-H24] = 58.65 nM; [DBCO] = 2932.5 nM; PBS 0.1M.

Kinetic constants k were determined using the integrated rate equation for second order reaction:

$$ln\left(\frac{[A_0][B]}{[B_0][A]}\right) * \frac{1}{([B_0]-[A_0])} = k * t$$

with $[A_0]$ and $[B_0]$ = initial concentrations of TMPP-mAbs conjugates and DBCO-Acid respectively, [A] and [B] = concentration at time t of TMPP-mAbs conjugates and DBCO-Acid respectively.

[A] and [B] are calculated using the equations:

$$[A] = [A_0] - x \text{ and } [B] = [B_0] - x,$$

with x = measured concentration of released TMPP tag.

The calculated kinetic constant k is the slope of the linear plot of $ln\left(\frac{[A_0][B]}{[B_0][A]}\right) * \frac{1}{([B_0]-[A_0])}$ versus time. An example is given in Figure S5 for CTX-H24.

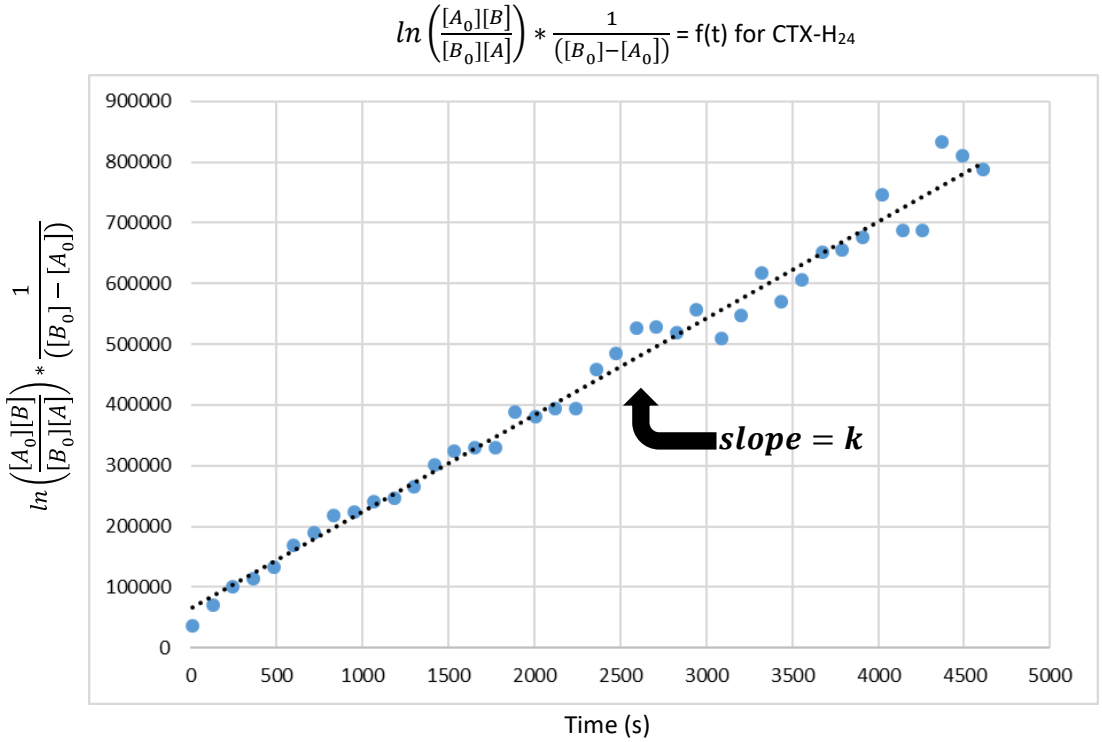


Figure S5. Release of tag TMPP-H24 from bioconjugate CTX-H24 over time. A = CTX-H24; B = DBCO-acid. Reaction conditions: [CTX-H24] = 58.65 nM; [DBCO] = 2932.5 nM; PBS 0.1M.

Kinetic constants were calculated by plotting $ln\left(\frac{[A_0][B]}{[B_0][A]}\right) * \frac{1}{([B_0]-[A_0])}$ versus time for the first 1000 seconds of the reaction. Each experiment was performed in triplicate.

All analyses were performed on a Xevo G2-XS Q-Tof mass spectrometer, coupled with ultra-high-performance liquid chromatography (Acquity I-Class), both from Waters. The separation of released TMPP tags and antibodies was achieved on a BEH C18 column (50 x 2.1 mm, 1.7 µm) at 40 °C. The mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 800 µL.min^{-1}. The elution program was set in an isocratic mod with 65% solvent A for a duration of 90 seconds. The injection volume was set to 5 µL and the autosampler temperature at 37 °C.

The parameters of the electrospray ion source (ESI) were: Capillary voltage 3.5 kV, sampling cone voltage 15 V, source temperature 140 °C, desolvatation gas (N_2) temperature and flow rate 550 °C, 1000 L/H, cone gas flow rate 100 L/H.

MS/MS data were acquired with the software Masslynx (Waters) in the positive ionization mode over a mass range of m/z: 50 - 1200 Da. Collision energy was set to 55 V. Mass accuracy within 3 ppm was reached using a solution of leucine enkephalin for internal calibration. TargetLynx (Waters) software was used to construct calibration curves and for automatic calculation of the released TMPP tag concentrations. Integrations were automatically performed on fragments Frag-H6, 15, 24 and 33 m/z XIC with a mass window of 20 mDa.

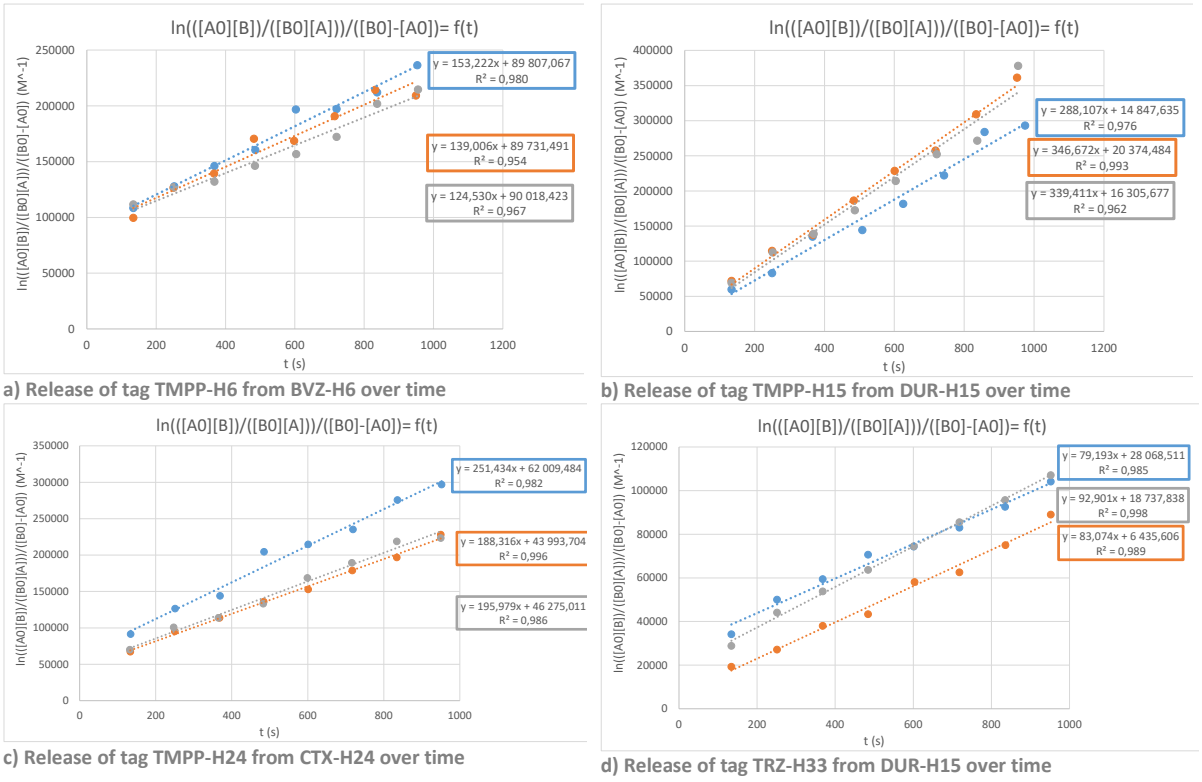


Figure S6. Release of TMPP tags from mAb-TMPP conjugates. Reaction conditions: a) [BVZ-H6] = 27.8 nM, [DBCO] = 1392 nM, PBS 0.1M. b) [DUR-H15] = 66.8 nM, [DBCO] = 3342 nM, PBS 0.1M. c) [CTX-H24] = 58.65 nM, [DBCO] = 2932.5 nM, PBS 1X. d) [TRZ-H33] = 116.5 nM, [DBCO] = 5824 nM, PBS 0.1M.

Table S2. Kinetic rate constants values

mAb-TMPP	**BVZ-H6**	**DUR-H15**	**CTX-H24**	**TRZ-H33**
k ($M^{-1}.sec^{-1}$)	138.9 ± 14.3	324.7 ± 31.9	211.9 ± 34.4	85.1 ± 7.1

VII. In vitro Experiments

Materials

Bovin serum albumin (BSA), ammonium Chloride (NH_4C l≥ 99.5%), phosphate buffered saline (PBS) tablette, bovin serrum albumin (BSA), tween 20, triton, paraformaldéhyde, acetone, Bicinchoninic Acid Protein Assay Kit, 4',6-diamidino-2-phénylindole (DAPI, $C_{16}H_{15}N_5$, 50.25 $g.mol^{-1}$), Dibenzocyclooctyne-PEG'-Fluor 545 (DBCO-Tamra, $C_{54}H_{57}N_50_{10}$, 936.09g/mol), Dibenzocyclooctyne-acid (DBCO-acid, $C_{21}H_{19}N0_3$, 333.38g/mol) were purchased from Sigma-Aldrich (France). Methanol and 2-Methylbutan were purchased from Carlo Erba (France) and Honeywell (France), respectively. CellBrite Fixe 640 Kit were purchased from Biotium (USA). SuperFrost Ultra Plus TM slides and 96-well plate were obtained from FisherScientific (France) and Greiner bio one (France), respectively. Normal (0.9%) saline and isoflurane were purchased from Baxter (France). Dulbecco's phosphate buffered saline (D-PBS, 10mM), HEPES buffer, Trypsin-EDTA, Antibiotic-antimycotic, fetal bovine serum (FBS) and cell culture medium (DMEM, Dulbecco's Modified Eagle's medium or McCoy's 5A) were purchased from Gibco (Thermo Fisher Scientific, France). Matrigel matrix basement membrane was purchased from Discovery Labware. Erbitux (Cetuximab, CTX, 5 mg/mL), Bevacizumab (BVZ, 25 mg/mL), Durvalumab (DUR, 50 mg/mL) and Herceptin (trastuzumab, TRZ, 150 mg) were purchased from Merck (Belgium), Pfizer (USA), AstraZeneca (United Kingdom) and Roche (Switzerland).
All products were used as received without further purification. Distilled water was purified using a Milli-Q system greater than 18MΩ cm resistance (Millipore, France) for all immunohistological buffers.

Detection and quantification limits of TMPP-tags by UPLC-MS/MS

50 µL of the cell media were evaporated to dryness under nitrogen using a Turbovap LV evaporator maintained at 40°C (Biotage, UK). Dried residue was then resuspended with 50 µL of a mixture of water/methanol (70/30) with 0.1% formic acid. The whole was vortexed 15 sec. and centrifuged 10 min at 20.000 g and supernatant was transferred into a vial for analysis.
Ten µL of supernatant maintained at + 4°C was injected into the LC-MS/MS system which consist on a Waters ACQUITY I-Class UPLC® System with an Acquity BEH C18 2.1 x 50 mm, 1.7 µm, 130 Å pore size column and a reversed phase gradient over a run time of 5.5 min. A mix of mobile phase A (water with 0.1% formic acid) and mobile phase B (methanol with 0.1% formic acid) were used at a flow rate of 0.5 mL/min and a column temperature of 60°C. The gradient conditions ramped from 0% to 100% B between 0.5 and 3.0 min and then return to 0% B at 3.51 and were maintained up to 5.5 min for re-equilibration. In these conditions mean retention time was around 2.20 min for all TMPP-tags.
The mass spectrometry analysis was performed on a Waters XEVO™ TQ-S Mass Spectrometer operating in positive ion electrospray MRM mode. Three transitions were monitored for each TMPP-tags. Quantification was performed by averaging the 3 concentratons thus obtained. Multiple transitions monitored were *m/z* 750.95>180.0, 708.1, 734.15, 760.46>184.1, 717.26, 743.29, 769.46>189.13, 726.29, 752.22 and 778.12>191.87, 733.81, 759.46 for TMPP-H33, TMPP-H24, TMPP-H15 and TMPP-H6, respectively.

Quantification were performed using linear regression with 1/X^2 weighing. Lower limit of quantification (LLOQ) was determined as the lower concentration calculated by linear regression showing a maximum deviation of ± 20%. Limit of detection was determined as concentration which gave a signal-to-noise ratio of about 3.

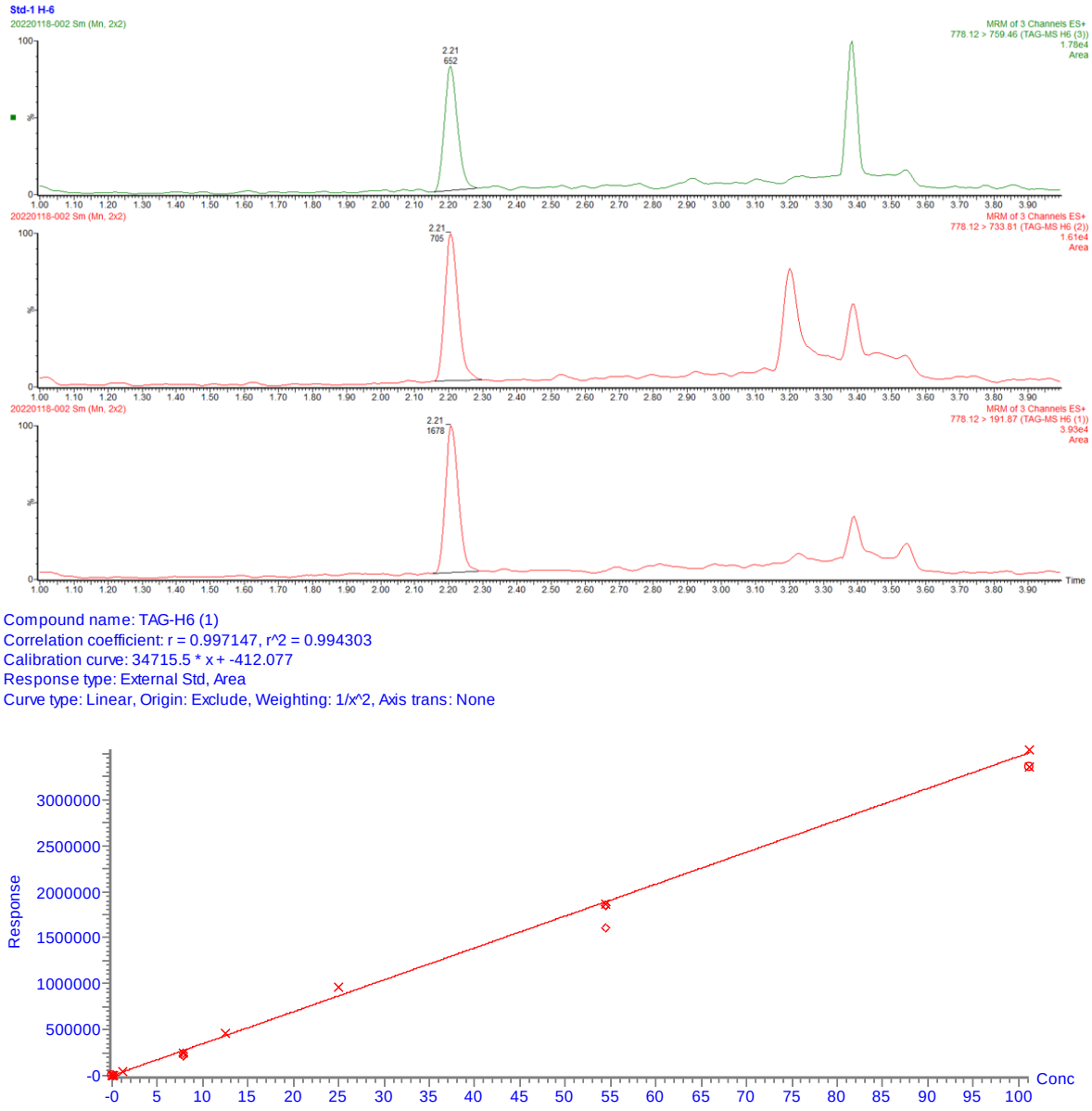


Figure S7. Typical chromatogram at the LLOQ level and calibration curve of TMPP tags (here TMPP-H6)

Table S3. Limits of detection and of quantification of TMPP tags

	Detection limit (pM)	Quantification limit (pM)
TMPP-H33	20	65.3
TMPP-H24	25	73.6
TMPP-H15	20	66.3
TMPP-H6	25	74.5

Cell culture

Human epidermoid carcinoma (A431), lung adenocarcinoma (H1975) and glioblastoma (U87MG) were purchased from Sigma-Aldrich. Human breast adenocarcinoma (SkBr3) was furnished by the laboratory of Molecular and Cellular Therapeutic Engineering & Glycosyltransferases from the University of Lorraine (France). Cells were cultured in a humidified incubator (Sanyo, Japan) at 37 °C in an atmosphere containing 5% of CO_2 in complete medium *i.e.* DMEM or McCoy's 5A supplemented with 10% of heat-inactivated FBS (Fetal Bovine Serum) and 1% antibiotic-antimycotic (Streptomycine, amphotéricine B, pénicilline). Mycoplasma absence was confirmed using MycoAlert™ kit (Lonza, USA).

Quantification of the receptors expressions by Western Blot analysis

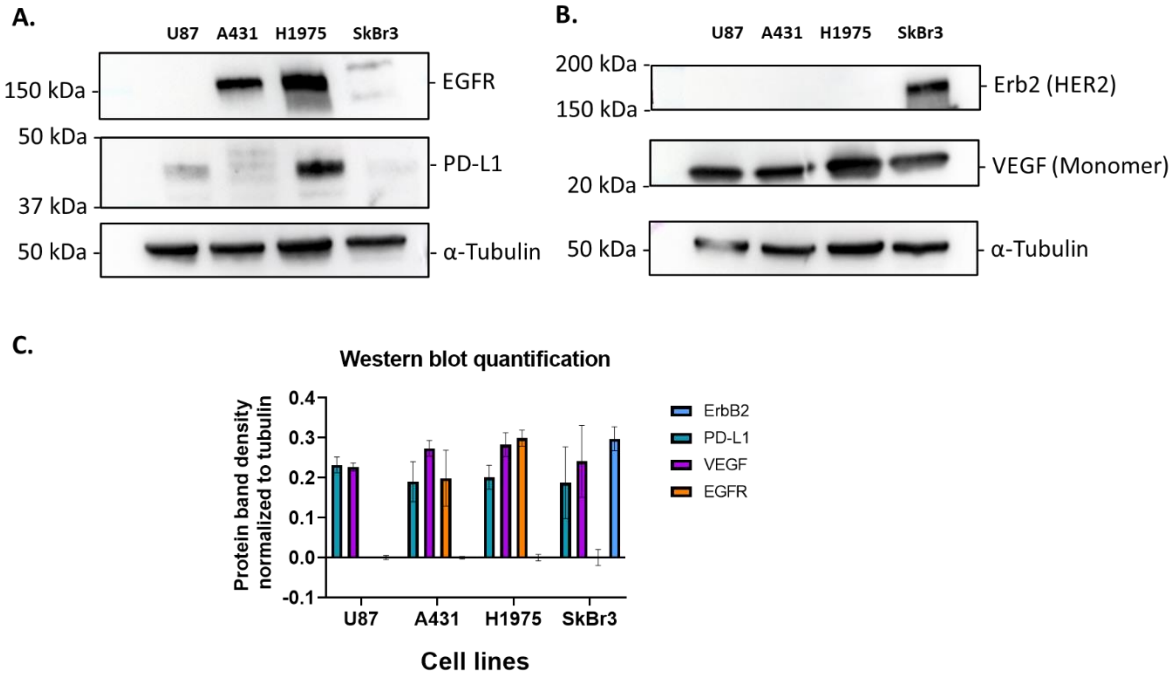


Figure S8 : Expression of PD-L1, VEGF, EGFR and Erb2 in the cell lines U87, A431, H1975, SkBr3. **A.** Western blot of the EGFR and PD-L1 proteins with the housekeeping protein α-Tubulin. The two bands observed for EGFR, particularly for SkBr3, can be explained by the glycosylation in EGFR ectodomain ligand binding that helps maintaining the dimeric interface.[3] **B.** Western Blot of the Erb2 and VEGF proteins with the housekeeping protein α-Tubulin. **C.** Western blot quantification analysis using ImageJ sofware. The protein expressions are normalized to the α-tubulin expression.

Competitive binding assay

Binding assay were conducted to determine the impact of the TMPP-tags functionalization on the antibody affinity to their cell membrane receptors. The binding assay was performed via two technics: by fluorescence analysis and by mass spectrometry analysis.

[3] M. A. Irani, S. Kannan, C. Verma. *Proteins* **2017**, *85 (8)*, 1529-1549.

➢ Fluorescence analysis

Briefly, a low concentration of CTX-H24 (10 µg, 133.33 nM, 0.066 nmol) with or without a pharmacologic dose of CTX (100-fold excess of CTX) were added to 750 000 cells suspended in 500 µL of DMEM. After 2 hours incubation at 37 °C or 4 °C, the cells were centrifugated and washed three times with 1 mL of DMEM to remove unbound antibody. The cells were re-suspended in 500 µL of DMEM and incubated with 10 equiv. of DBCO-TAMRA (1.4 µM in DMEM containing 1.4% DMSO) at 37 °C for 5 minutes under stirring. Then, cells were washed again 3 times with PBS. Finally, the fluorescence of the pellets was measured in a UV-visible spectrophotometer (λ_{ex} = 450 nm). Control samples without CTX-H24 was also measured. Samples were done in triplicate.

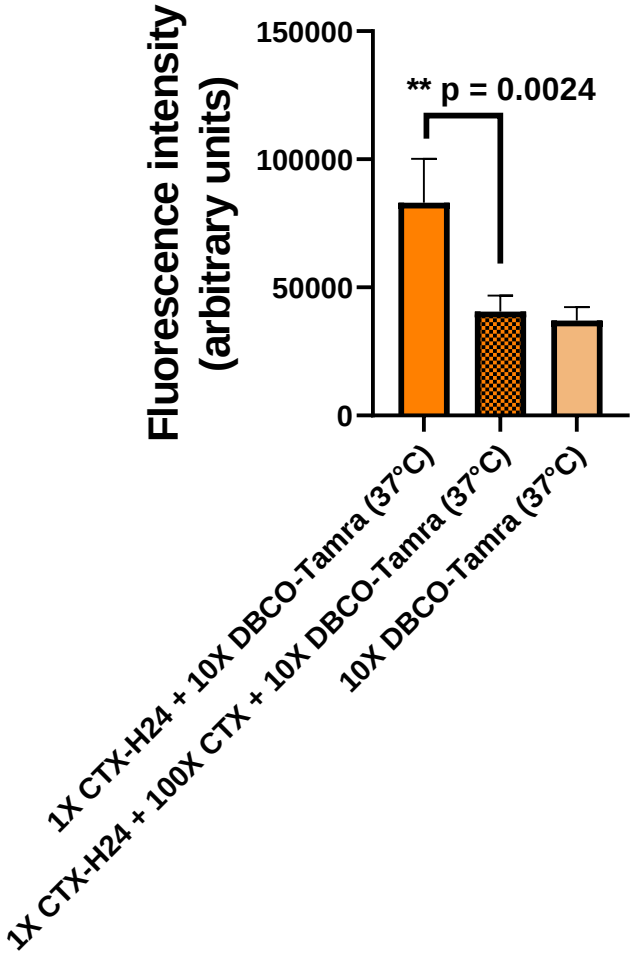
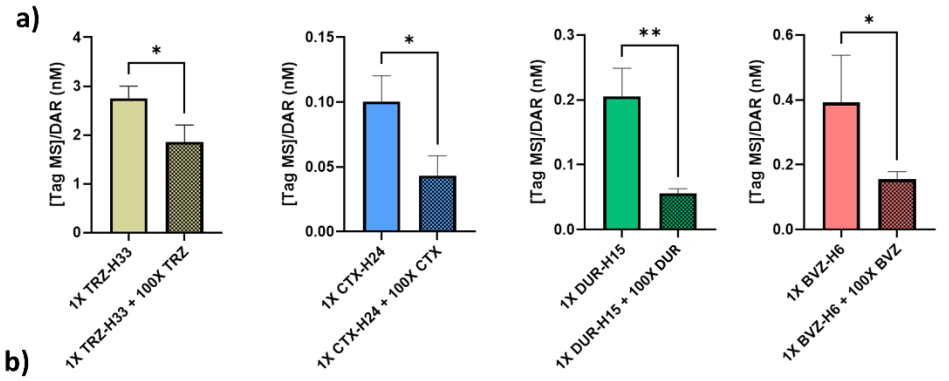


Figure S9 : Fluorescent evaluation of the specific and unspecific binding of CTX-H24 and DBCO-TAMRA probes by incubated A431 cells with or without excess of CTX in at 37°C. Results are represented as mean ± standard deviation (n = 3, 750 000 cells).

➢ Mass spectrometry analysis

Briefly, low concentration of TRZ-H33, CTX-H24, DUR-H15, BVZ-H6, (1 µg, 13.33 nM, 0.0066 nmol), with or without a pharmacologic dose of CTX (100-fold excess of CTX) were added to 250 000 cells suspended in 500 µL of DMEM (For TRZ: SkBr3, for CTX: A431, for DUR: H1975, for BVZ: U87MG). After 2 hours incubation at room temperature with continuous stirring, the cells were centrifugated and washed three times with 1 mL of DMEM to remove unbound

antibody. The cells were re-suspended in 500 µL of DMEM and incubated with large excess of DBCO-acid (75 757 equiv., 1 mM in DMEM containing 1% DMSO) at 37°C for 20 minutes under stirring. Then, cells were harvested by centrifugation and the supernatants were stored at -20°C until mass spectrometry analysis. Samples were done in triplicate.



b)

	TRZ-H33	TRZ-H33 + 100xTRZ	CTX-H24	CTX-H24 + 100xCTX	DUR-H15	DUR-H15 + 100xDUR	BVZ-H6	BVZ-H6 + 100xBVZ
MS-Tag Name	TMPP-H33	TMPP-H33	TMPP-H24	TMPP-H24	TMPP-H15	TMPP-H15	TMPP-H6	TMPP-H6
[MS-Tag] released	2753±248 pM	1859±347 pM	103±20 pM	43±15 pM	206±43pM	56±7pM	392±145pM	155±24pM

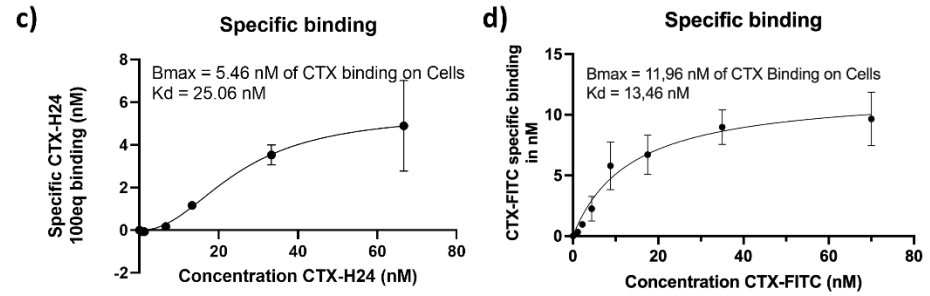


Figure S10 : Binding assay using mass spectrometry analysis a) Concentrations of released tags from left to rigth: TRZ-H33 (0.8 mg.mL^{-1}), CTX-H24 (1.06 mg.mL^{-1}), DUR-H15 (1.281 mg.mL^{-1}) and BVZ-H6 (0.54 mg.mL^{-1}) associated to the H1975, A431, U87 and SkBr3 cells, respectively with or without an excess of themselves (100 x). Results are represented as mean ± standard deviation (n = 3, 750 000 cells). **b)** Table summarizing the concentrations of released Tags. **c)** In the saturation binding assay using CTX-H24, the Bmax and Kd values were determined through specific binding with hill slope analysis from GraphPad. These results were then compared under identical conditions with CTX conjugated with fluorescein (FITC). **d)** FITC isothiocyanate was mixed with CTX and incubated at room temperature for 60 minutes to achieve covalent conjugation. Unreacted FITC was subsequently removed using a PD-10 column. The FITC-to-antibody ratio was assessed through fluorescence measurement (BMG, Clariostar), yielding a ratio of 2.13. Subsequently, CTX-FITC was incubated at various concentrations in A431 cells, mirroring the conditions used in c). Non-specific binding was subtracted from the total binding, resulting in the specific binding presented in nM. The Kd values for both CTX-FITC and CTX-H24 were found to be in the same range, approximately 10 to 20 nM, indicating a minimal impact of H24 conjugation on CTX binding.

Fluorescent microscopy experiments

In vitro: targeting CTX-H24 with DBCO-TAMRA in live cells.

A431 cells were seeded in 8-well Nunc Lab-Tek I chambers (150 000 cells per well in 500 µL of DMEM) one day before the experiment. The medium was then changed, and cells were incubated with CTX-H24 (5 µg, 66.67 nM, 0.033 nmol) in 500 µL of DMEM for 2 h at 37°C. The cells were washed three times with DMEM, re-suspended in 500 µL of DMEM and incubated with 100 equivalents of DBCO-TAMRA (6 µM in 500 µL of DMEM containing 0.6% DMSO) at 37 °C for 15 min. Cells were washed again three times with DMEM and cells were incubated with DAPI (10 µg/mL ou 30 µM in DMEM) to stain the nucleus during 15 min at 37 °C. Cells were washed one time with PBS. CellBrite Fix 640 was used to stain the membrane 1X PBS 15 min at 37°C. Cells were washed three times with PBS. Cells were fixed in neutral buffer formalin 10 % for 20 min at RT. Cells were washed three times with PBS before imaging. Control samples without CTX-H24 or without CellBrite Fix 640 were also imaged.

Fluorescence microscopy was performed on an AxioObserver Z1 microscope (Zeiss) using two objectives (X20 o X40) with relevant filters :

- Long pass filter ($\lambda_{exc.}$ = 405 nm and $\lambda_{em.}$ = 454 nm) for DAPI
- Band Pass filter ($\lambda_{exc.}$ = 553 nm and $\lambda_{em.}$ = 575 nm) for TAMRA
- Band Pass filter ($\lambda_{exc.}$ = 638 nm and $\lambda_{em.}$ = 667 nm) for CellBrite Fix 640.

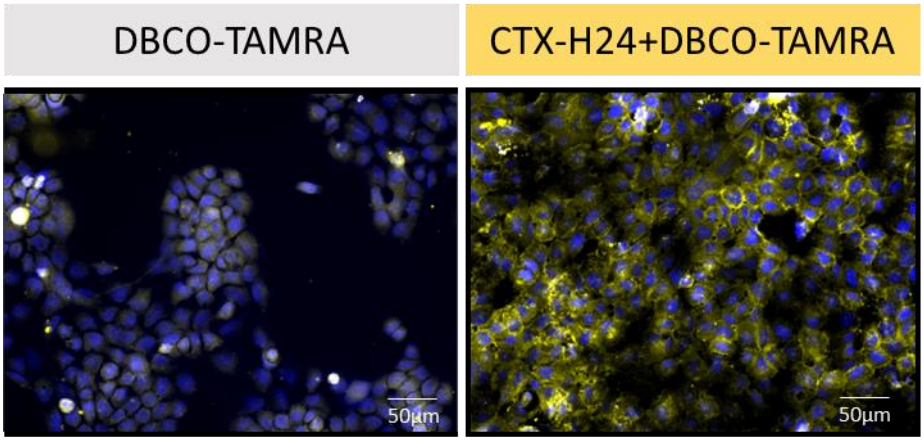


Figure S11 : DBCO-TAMRA (yellow) merged with DAPI staining of the nucleus (blue) of A431 cells incubated without CTX-H24 or with CTX-H24 (66.67 nM).

DBCO toxicity

AlamarBlue Cell Viability assay

To determine the IC_{50} (half-maximal inhibitory concentration) of DBCO on the HeLa cell line (human cervical cancer), the AlamarBlue Cell Viability Assay (ThermoFisher) was employed. Cells were seeded at a density of 5000 cells per well in 96-well plates. After 24 hours, reaching 40–50% confluence, cells were treated with various conditions: no medium, untreated control, vehicle control (2% DMSO), and increasing concentrations of DBCO ranging from 0.005 mM to 2 mM. 10 µL of AlamarBlue reagent was added to each well, followed by 3 hours of incubation. Fluorescence was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The fluorescence values were plotted against DBCO concentration, and the IC50 was determined using dose-response curve fitting via GraphPad software.

MTS assay

To evaluate cell viability and proliferation following DBCO incubation, the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was employed. Four cell lines (U87, A431, H1975, SkBr3) were seeded in 96-well plates at a density of 5000 to 10000 cells per well in culture medium. Control wells with untreated cells and blank wells containing only medium were included. After 24 hours of incubation at 37°C with 5% CO_2 to allow cell attachment, cells were treated with vehicle (DMSO) or DBCO (0.1 mM) for 1 hour and 24 hours. The MTS reagent was then added at room temperature, and absorbance was measured at 490 nm using a microplate reader, with blank values subtracted from all measurements. Absorbance readings were normalized to untreated controls and presented as a percentage of the control in graphical form.

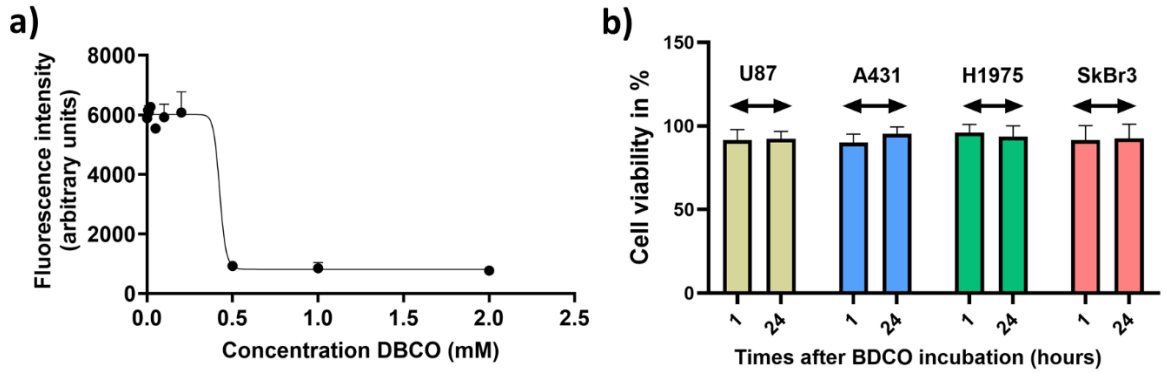


Figure S12. DBCO toxicity. a) Cytotoxicity curves of DBCO on HeLa cells via AlamarBlue Cell Viability assay. The IC_{50} was determined at 0.4 mM. **b)** Viability of the different cell lines after BDCO incubation at 0.1 mM compared to the cells without DBCO incubation. The assay was performed on MTS kit assay.

VIII. Experiment on Tissues

Serial tumor sections (14 µm thick) were cut at -20°C with a cryostat (Leica) on SuperFrost Ultra Plus TM slides. Frozen tumor slides were fixed in neutral buffer formalin 10 % for 15 min at RT, then washed 5 min with PBS buffer. The neutral buffer formalin 10 % effect were inactivated by incubating the sections in NH_4Cl solution (50 mM in PBS), then washed 5 min with PBS buffer. Sections were successively immersed during 5 min at RT in permeabilized solutions: MeOH/acetone (50/50) and Triton solution (0.1% in PBS), then washed 5 min with PBS buffer.

Sections were immersed in blocking solution (5% BSA in Tween-20 solution of 0.5 % in DMEM without phenol red) for 1h at RT. Sections were incubated 2 h at RT with a low concentration of CTX-H24 alone (1 µg, 13.33 nM, 0.0066 nmol) or in the co-presence of 100-fold excess of CTX in Tween-20 solution of 0.5 % in DMEM without phenol red. After washing three times with DMEM without phenol red, sections were incubated 20 min at 37°C with DBCO-acid (75 757, 10 mM in DMEM containing 10% DMSO). The supernatant of three adjacent tumor sections was pulled together and stored at -20°C until mass spectrometry analysis.

Statistical analysis

All data are presented as mean ± standard deviation (SD). The statistical analyses were performed using GraphPad Prism software (Graph Pad software Inc., San Diego, USA). Normality was assessed using the d'Agostino-Pearson test. Unpaired t-test were used for statistical significance of intergroup comparisons (*$p<0.05$).

Ex vivo: proteins of interest expression on the A431 implanted cells by immunofluorescence.
Snap-Frozen A431 tumor were cut in 10µm slices. The slices have been fixed in 10% formalin (Sigma-Aldrich # HT501128-4L) during 15 minutes at room temperature (RT) for immunofluorescence for protein analysis. All the slices were incubated during 5 minutes in PBS Triton buffer to permeabilized the tissue. After bovine serum albumin blocking (BSA, ThermoFisher), the slices were washed with cold PBS and then incubated with the different antibodies of interest:

- EGFR : 43B2 monoclonal Rabbit from Abcam (dilution: 1/100)
- ErbB2 : MA514509 monoclonal Rabbit from Fisher Scientific (dilution: 1/250)
- PD-L1 : E1L3N monoclonal Rabbit from CellSignaling (dilution: 1/100)
- VEGF : ABS82 Polyconal Rabbit from Sigma Aldrich (dilution: 1/200)

Slices were washed three times with PBS buffer between each step. The protein expression was determined with a secondary antibody (2-Goat anti-Rabbit, Alexa Fluor™ 546, dilution: 1/1000). Sections were mounted using Prolong Diamond Antifade Moutain with DAPI to label cell's nucleus. An Axio Observer Z1 microscope (Zeiss, Germany) was used with a 20x objective to scan the full sections. Post processing qualitative analysis was performed with ZEN software (v2.6, Zeiss).

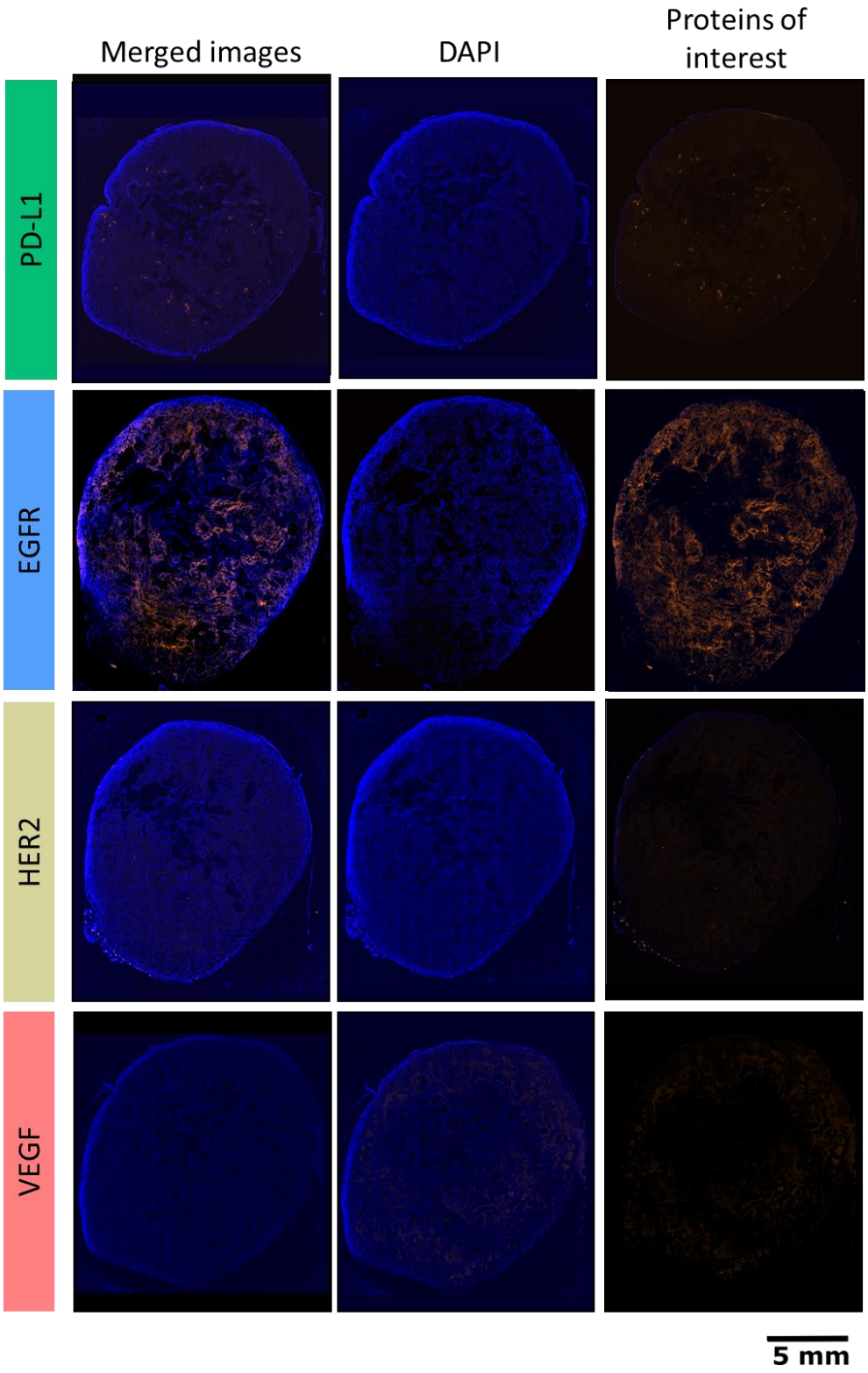


Figure S13. Immunofluorescence analysis on *ex vivo* A431 tumor slices. Immunofluorescence was performed on 10µm adjacent section. PD-L1, EGFR, HER2, VEGF have been revealed with secondary antibody goat anti rabbit Alexa Fluor 546 (Fisher Scientist). EGFR are mainly expressed on the A431 tumor. A secondary expression can be observed slightly with PD-L1.

IX. In vivo Experiments

Ethics and animals

Animal experiments were conducted in agreement with the European Directive 2010/63/EU on the protection of laboratory animals (French law transposition: Decree No. 2013-118). They were performed at the imaging facility from BioMaps and DMTS with protocols approved by the Ethical Committee of CEA (CEtEA, authorization CEEA N° 44).

Subcutaneous tumor model

Mice anesthetized with isoflurane in a mixture of N_2/O_2 [80:20] (4% for induction and 2% for maintenance) received a subcutaneous injection of A431 cells at 5.0 x 10^6 tumor cells suspended in 100 µL of PBS: Matrigel (50%/50% v/v) in both right and left flanks for heterotopic establishment of tumors. Animal weight and tumors growth was monitoring three times a week. When the tumor reached 1 cm in diameter after approximately 20 days, mice were euthanized by cervical dislocation under isoflurane (5%). Tumors were removed, immersed in 2-Methylbutan and frozen in liquid nitrogen.

Biodistribution of DBCO [^{18}F]-6 after intratumoral injection

In total, 8-female athymic NMRI nude mice five-week-old were purchased from Janvier laboratories (Le Genet sur Isle, France, Mus musculus, NMRI-FOXN1 Nu/Nu). Mice were housed four per cage with food and water *ad libitum* in an environmental enrichment (polycarbonate cottages and wooden stocks), in a temperature (22 ± 2 °C) and humidity (40%) controlled room and were maintained under specific pathogen-free conditions.

[^{18}F]-DBCO was prepared according to our previously reported protocol.[4] Briefly, the radiosynthesis was carried out using TRACERlab FXFN or FXNPro synthesizer starting with [^{18}F]fluoride trapping on an anion exchange cartridge, then eluted with a solution of K_2CO_3 (2 mg) and K222 (12-15 mg) in a water/MeCN 30:70 solution (1 mL). After evaporation, the corresponding tosylate precursor (5 mg) in MeCN (1 mL) was added and the mixture heated to 90 °C for 15 minutes. The crude mixture was purified by semi-preparative HPLC (Zorbax SB C18 column, water/MeCN 35:65, 0.1% TFA) and the eluate was reformulated in ethanol (1.5 mL) with a C18 cartridge. The final molar activity was 193 GBq.µmol^{-1}. Before injection, the DBCO concentration was reajusted to 100 mM. The dose injected by mouse was 9.5±2.3 MBq, 10 µL.

[4] Richard, M.; Truillet, C.; Tran, V. L.; Liu, H.; Porte, K.; Audisio, D.; Roche, M.; Jego, B.; Cholet, S.; Fenaille, F.; Kuhnast, B.; Taran, F.; Specklin, S. *Chem. Commun.* **2019**, *55*, 10400–10403.

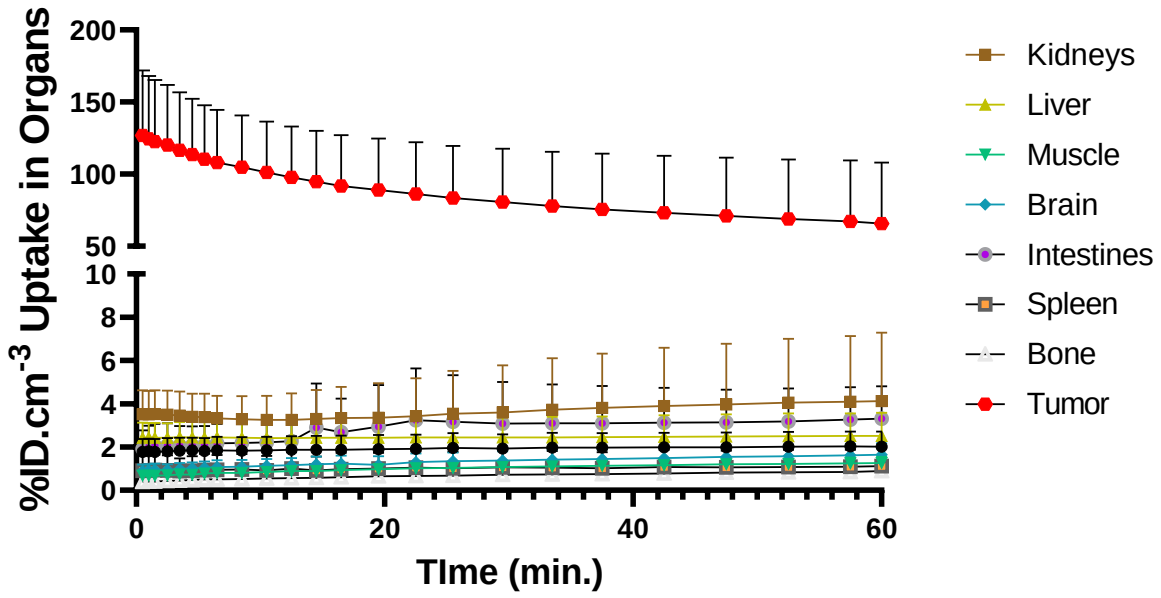


Figure S14. Time Activity Curves (TACs) of DBCO [^{18}F]-**6** in organs of interest during the first hour post-injection.

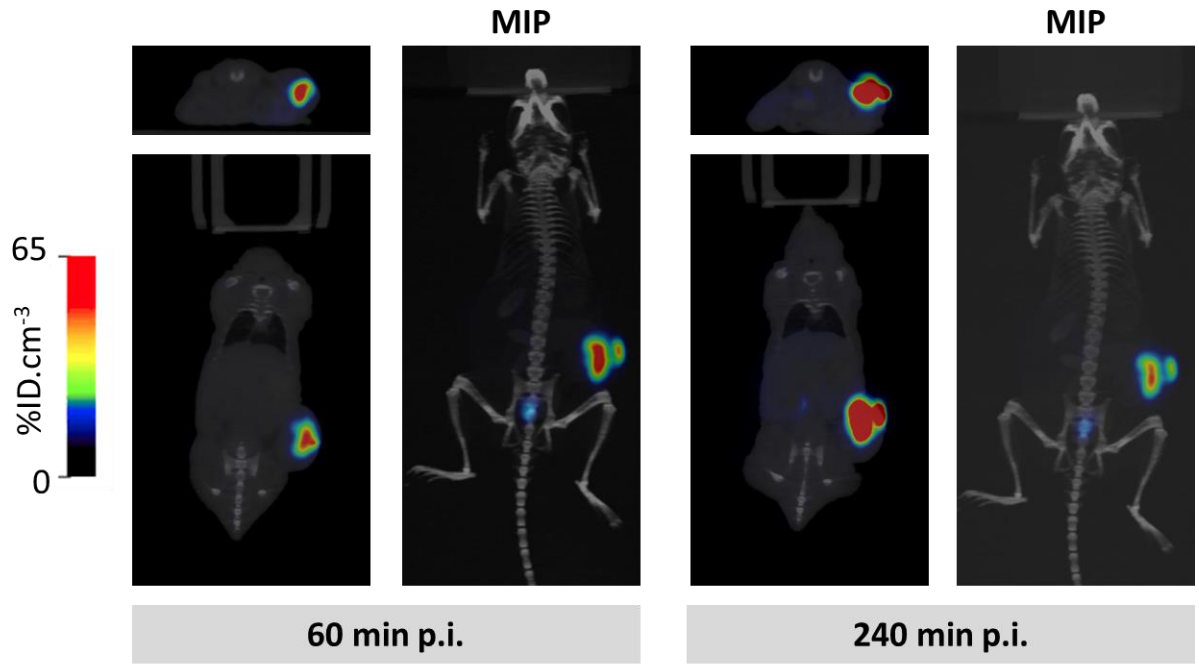


Figure S15. *In vivo* representation of DBCO [^{18}F]-**6** distribution at 1 hour and 4 hours post-injection. Representative micro-PET/CT images of mice bearing A431 tumor after intratumoral administration of DBCO [^{18}F]-**6** over time after injection with representative MIP images.

***In vivo* pharmacokinetics of TMPP tags**

6 NMRI nude mice (female, 5 weeks) were implanted with the A431 in each flank (rigth and left), with 5 5.0 x 10^6 cells per injection. The weight of the mice and the size of the tumors were measured regularly over time. 21 days post implantation after the tumour growth, the mice were injected intravenously with the cocktail of four distinct tags at 0.2 mg/kg for each tag (H33, H24, H15, H6, total volume: 120 µL). Then, blood samples were collected (retro-

orbital) at 30 min, 1 h, 2 h, 4 h, and 24 h after injection, on three distinct mice at each time. The plasma was separated thanks to a centrifugation of the blood in heparin tube at 20°C, 15000 g, 15 min, and conserved at -20°C before LC-MS analysis. Urine samples were collected after 24 h in metabolic cage on three mice, and conserved at -20°C before LC-MS analysis. Quantification of tags contained in plasma and urine was performed using UPLC-ESI(+)-MS/MS method. Animals were euthanized 24 h after tags administration.

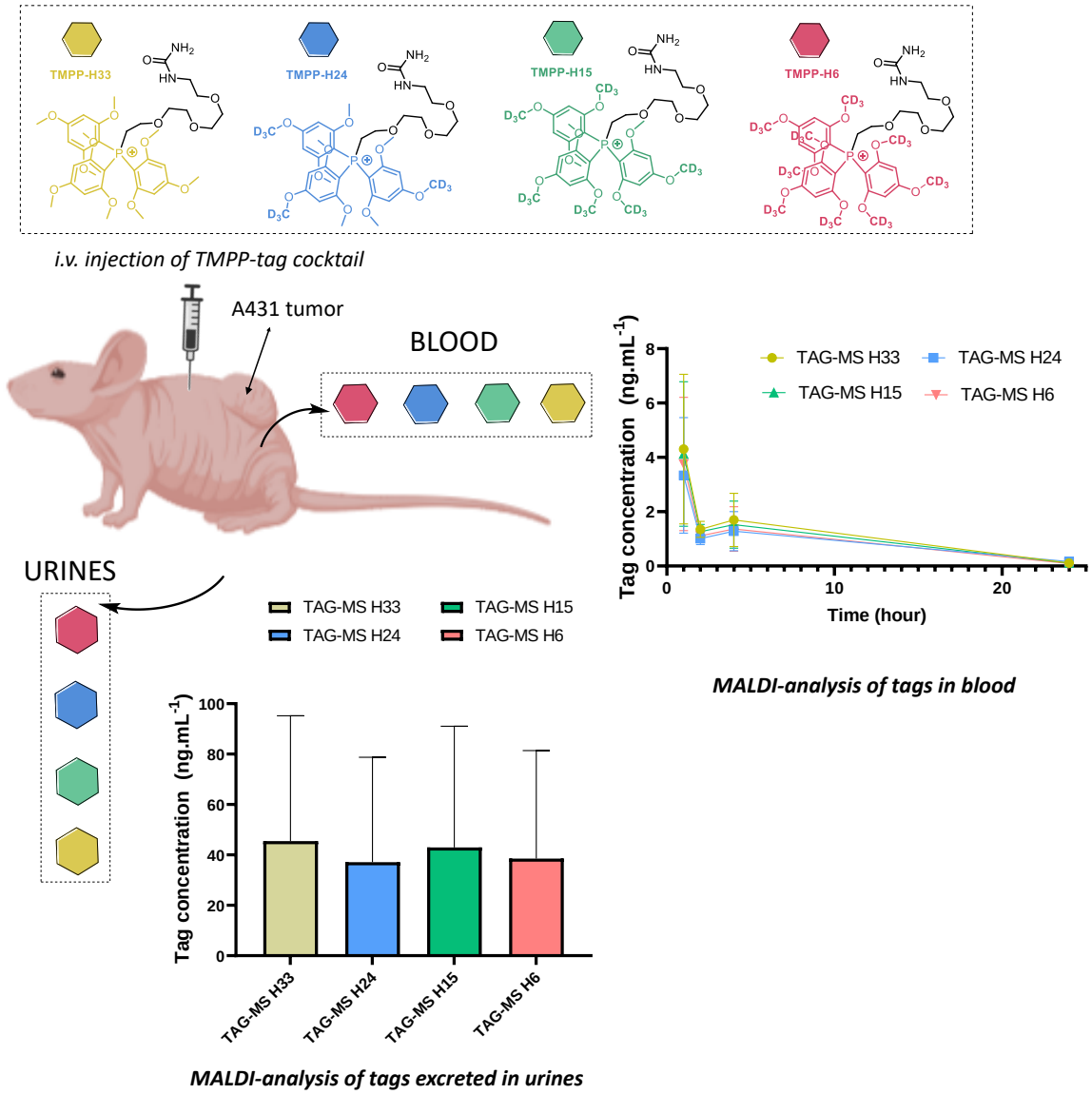


Figure S16: Pharmacokinetic data of TMPP-Tags after injection of the 4 Tags. Blood sampling was performed at different time points from 30 min to 2 hours. Urine was sampling during 24 h. Twenty-four hours after injection, the mice were euthanized and the organs of interest (blood, kidneys, liver and tumors) were extracted for MS analysis.

In vivo **immunoprofiling**

Two experiments have been performed to assess Tags release in plasma, blood and tumors at various time after injection.

For the first experiments, 6 NMRI nude mice (female, 5 weeks) were implanted with the A431 in each flank (rigth and left), with 5.0 x 10^6 cells per injection. The weight of the mice and the size of the both left and right tumors were measured regularly over time. 21 days post implantation after the tumour growth, the mice were injected intravenously with the cocktail of four antibodies, TRZ-H33, CTX-H24, DUR-H15, BVZ-H6 (100 µg per antibody, total volume: 120 µL). 3 days post-injection, the tumors were injected with 10 µL of a DBCO [^{19}F]-**6** solution (100 mM in DMSO/H_2O-NaCl 0.9% 30/70). Blood sampling via retro-orbital protocole was performed under anesthesia (2% of isofluorane, in O_2) at 30 min, 1 h, 2 h and 24 h post-injection, on three distinct mice at each time. The plasma was separated thanks to a centrifugation of the blood in heparin tube at 20°C, 15000 g, 15 min. The urine of three mice was collected during 24 h thanks to individual metabolic cages. The plasma and urine were stored at -20°C before LC-MS processing. At 24 h post DBCO [^{19}F]-**6** injection, all the mice were euthanized for *ex vivo* MS analysis on various shredded organs of interest (blood, kidneys, liver and tumors).

For the second experiments, 4 NMRI nude mice (female, 5 weeks) were implanted with the A431 in each flank (rigth and left), with 5.0 x 10^6 cells per injection. The weight of the mice and the size of the tumors were measured regularly over time. 21 days post implantation after the tumour growth, the mice were injected intravenously with the cocktail of four antibodies, TRZ-H33, CTX-H24, DUR-H15, BVZ-H6 (100 µg per antibody, total volume: 120 µL). The urine was collected thanks to metabolism cages during the 3 days before DBCO [^{19}F]-**6** injection. 3 days after mAb cokctail injection, only the left tumor of each mice was injected with 10 µL of a DBCO [^{19}F]-**6** solution (100 mM in DMSO/H_2O-NaCl 0.9% 30/70). The urine of three mice was collected during 24 h thanks to individual metabolic cages. Blood sampling via retro-orbital protocole was performed under anesthesia (2% of isofluorane, in O_2) before the mAb cocktail injection, 48 h after mAb injection and after DBCO [^{19}F]-**6** injection at 30 min and 24 h post-injection, on the four distinct mice at each time. The plasma was separated thanks to a centrifugation of the blood in heparin tube at 20°C, 15000 g, 15 min. The plasma and urine were stored at -20°C before LC-MS processing. At 24 h post DBCO [^{19}F]-**6** injection, all the mice were euthanized for *ex vivo* MS analysis on various shredded organs of interest (blood, kidneys, liver and tumors).

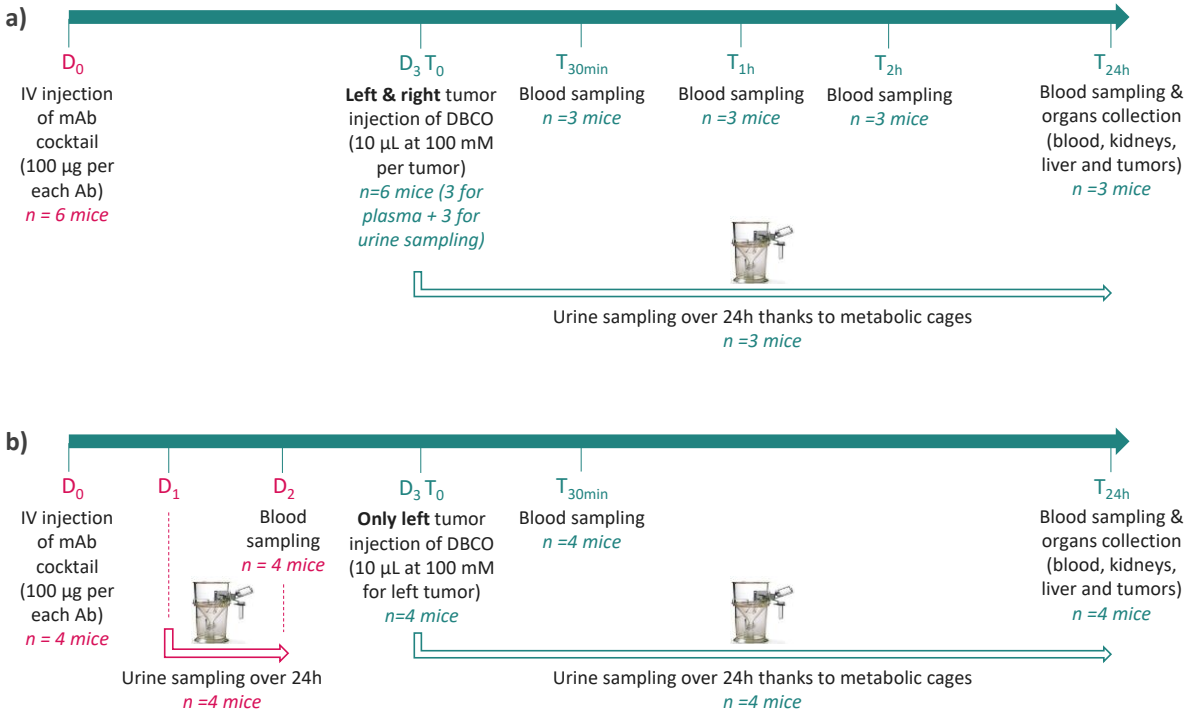


Figure S17. Implemented protocol for the *in vivo* immunoprofiling proof of concept with the experimental timeline. **a)** For the first experiments, day 3 after antibody cocktail injection, DBCO [^{19}F]-**6** was injected in both the rigth and left tumors of the mice (10 µL of DBCO [^{19}F]-**6** per tumor) and the blood was sampling at different time points (30 min to 24 hours). Urine was sampling over 24 h into metabolic cages. Twenty-four after injection of DBCO [^{19}F]-**6** the mice were euthanized and the organs of interest extracted for *ex vivo* analysis. **b)** For the second experiments, day 3 after antibody cocktail injection, DBCO was injected only in the left tumor of the mice (10 µL of DBCO [^{19}F]-**6**) and the blood was sampling at different time points (30 min and 24 hours). Urine was sampling over 24 h into metabolic cages at two different time points: i) after mAB injection and before DBCO [^{19}F]-**6** injection, and ii) after DBCO [^{19}F]-**6** injection. Twenty-four after injection of DBCO [^{19}F]-**6** the mice were euthanized and the organs of interest extracted for *ex vivo* analysis.

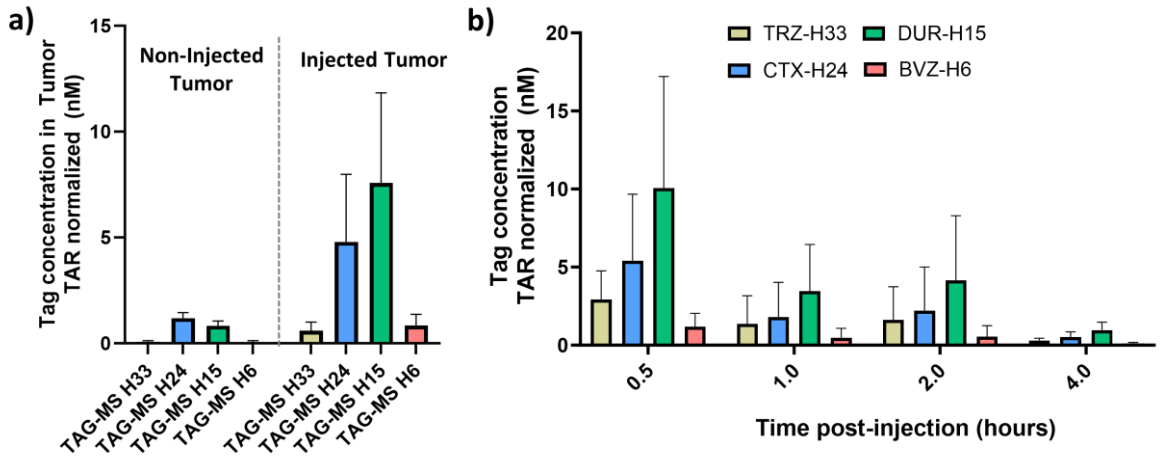


Figure S18. a) Comparison between injected and non-injected tumor samples with the Tag. Significantly higher uptake of H24 and H15 is observed in comparison to H33 and H6 within the injected tumor ($p < 0.01$, Kruskal-Wallis test). Conversely, in the non-injected tumor, the tag concentration is markedly lower ($p < 0.0001$, Kruskal-Wallis test) for each tag, indicating a

limited circulation of the DBCO [^{19}F]-**6** after tumoral injection, consistent with the expected PET imaging distribution**. b)** Blood kinetics were assessed through MS analysis of blood samples after DBCO [^{19}F]-**6** injection (Experiments 1 & 2). The kinetics reveal a consistent ratio of Tag elimination over time. Notably, before DBCO [^{19}F]-**6** injection no Tag was detected in the blood or urine, underscoring the absence of release (below the detection limits of the MS technique).

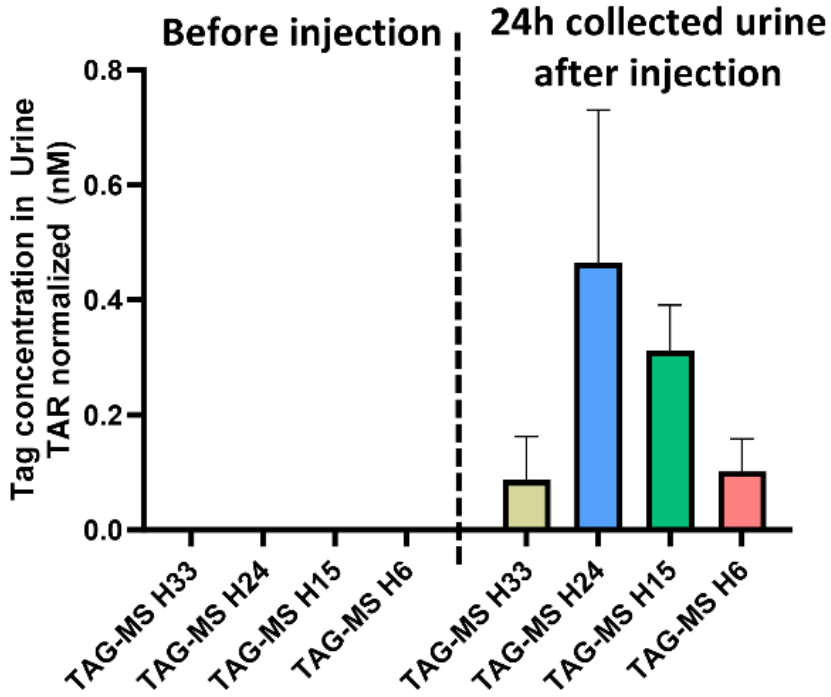


Figure S19. The assessment of Tag concentration was conducted both before intratumoral DBCO [^{19}F]-**6** injection (following the administration of cocktail antibodies) and after DBCO [^{19}F]-**6** injection, utilizing urine collected over a 24-hour period (Experiment 2). Remarkably, in absence of DBCO [^{19}F]-**6** injection no detectable Tag was observed in the urine, indicating a robust and stable conjugation of the Tag with the antibodies.

LC-MS/MS analysis of samples from *in vivo* experiments: Tags H33, H24, H15 and H6 quantification in growth medium and in mouse plasma, kidney, liver, urine and tumor.

All solvents (VWR France) were LC/MS grade. 50 µL of growth medium was evaporated to dryness using a Turbovap LV evaporator maintained at 40°C (Biotage, UK). Extract was then resuspended with 50 µL of water/acetonitrile (70/30, V/V) with 0.1% formic acid. At last, debris were removed by spinning 5 min at 20,000 g and 5°C and supernatant was transferred into a new vial for analysis.

Kidney, liver and tumor samples were homogenized with three volumes of water using a Bertin Technologies Precellys homogenizer before analysis. Then, 200 µL of acetonitrile was added in 50 µL of tissue homogenate (kidney, liver or tumor), plasma for the protein precipitation or urine. The whole was vortexed 15 s and centrifuged 15 min at 20,000 g and

5°C. Extract was then resuspended with 50 µL of water/acetonitrile (70/30, V/V) with 0.1% formic acid. At last, debris were removed by spinning 10 min at 20,000 *g* and 5°C and supernatant was transferred into a new vial for analysis.

Ten µL of extract maintained at +5°C was injected into the LC-MS/MS system which consist on a Waters ACQUITY UPLC® System with an Acquity UPLC BEH C18 2.1*50 mm column and a reversed phase gradient over a run time of 5.5 minutes. A mix of mobile phase A (water and 0.1% formic acid) and mobile phase B (methanol and 0.1% formic acid) were used at a flow rate of 0.5 mL/min and a column temperature of 60°C. The gradient conditions ramped from 0% B to 100% B between 0.5 and 3 min and maintained up between 3 and 3.5 min, ramped to 5% B at 3.51 min and maintained up to 5.5 min for re-equilibration. The mass spectrometry analysis was performed on a Waters XEVO™ TQ-S Mass Spectrometer operating in positive ion electrospray MRM mode. Multiple transitions monitored were *m/z* 751.0 > 180.01, 760.5 > 184.1, 769.5 >189.1 and 778.1 > 733.8 for TAG H33, H24, H15 and H6, respectively. In these conditions mean retention time was around 2.2 min for each Tags.

Quantification was performed using linear regression with $1/X^2$ weighing and calibration ranges from 0.5 to 100 ng/mL, for each Tags.