Supporting Information

A new phosphoramidite enables orthogonal double labelling to form combination oligonucleotide probes

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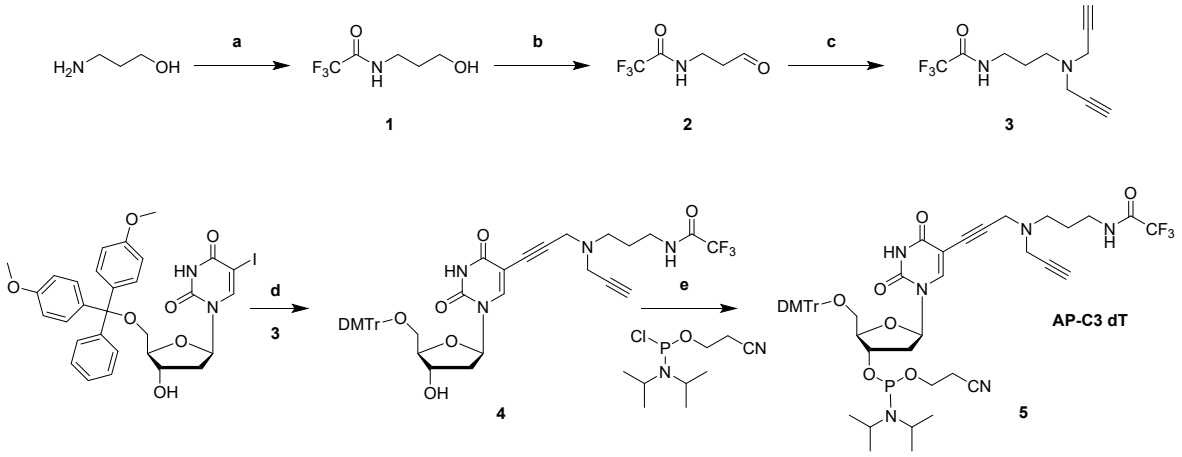
1. List of Abbreviations

Ac	Acetyl
CPG	controlled pore glass (solid support)
CuAAC	copper(I)-catalysed azide-alkyne 1,3-dipolar-cycloaddition
DIPEA	N,N-diisopropylethylamine
DMF	dimethylformamide
DMTr	4,4'-dimethoxytrityl
F_{ds}	fluorescence emission spectra of fluorescent probe-target complex
F_{ds}/F_{ss}	ratio of fluorescence emission at $\lambda_{em,\ max}$ of oligonucleotide duplexes to single stranded
F_{ss}	fluorescence emission spectra of single stranded complex of fluorescent probe
HPLC	High Performance Liquid Chromatography
HRMS	high resolution mass spectroscopy
$I_{0,\ max}$	fluorescence emission intensity at $\lambda_{em,\ max}$ of single stranded oligonucleotides
I_{max}	fluorescence emission intensity at $\lambda_{em,\ max}$ of probe-target duplexes
J	coupling constant in Hz (NMR)
LRMS	low resolution mass spectroscopy
NHS	N-hydroxysuccinimide
TCA	Trichloroacetic acid
TEAA	triethylammonium acetate (HPLC buffer)
TEAB	triethylammonium bicarbonate (HPLC buffer)
THPTA	tris(3-hydroxypropyltriazolylmethyl)amine
TLC	thin layer chromatography
T_m	DNA melting temperature
TO	thiazole orange
UV	ultraviolet

2. Experimental Section

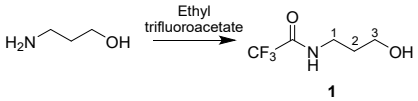
All reagents were purchased from Sigma-Aldrich, Acros Organics, Lonza, Invitrogen or Fisher Scientific and used without further purification, or dried as described below. 3 Å molecular sieves (beads, 4 – 8 mesh, Sigma-Aldrich) were used to dry MeOH and dry CH_2Cl_2 was collected from a Grubbs-type SPS. Thin layer chromatography (TLC) was performed using Merck TLC silica gel 60 F254 plates (0.22 mm thickness, aluminium backed) and the compounds were visualized by irradiation at 254/365 nm and stained with p-anisaldehyde or potassium permanganate. ^{1}H NMR spectra were measured at 400 MHz and 500 MHz on a Bruker DPX400 (AVIIIHD 400) and Bruker AVIIIHD 500 spectrometer. ^{13}C NMR spectra were measured at 101 MHz and 136 MHz on a Bruker DPX400 and Bruker AVIIIHD 500 spectrometer. ^{1}H were internally referenced to the appropriate residual undeuterated solvent signal; ^{13}C NMR spectra were referenced to the deuterated solvent. Assignment of the signals was aided by COSY (^{1}H - ^{1}H), HSQC-DEPT, HSQC (^{1}H - ^{13}C) and HMBC (^{1}H - ^{13}C) experiments. Low-resolution mass spectra (LRMS) were recorded using electrospray ionisation (ESI^+ or ESI^-) on a Waters ZMD quadrupole mass spectrometer in HPLC grade methanol. High-resolution mass spectra (HRMS) were recorded in HPLC grade methanol using electrospray ionisation (EI) on a Bruker APEX III FT-ICR mass spectrometer.

2.1. Synthesis of AP-C3 dT phosphoramidite



Scheme S 1 Synthesis of AP-C3 dT phosphoramidite. Reagents and conditions: (a) Neat ethyl trifluoroacetate, 3 h, 0 °C to RT, 97%; (b) CH_2Cl_2, Dess-Martin periodinane, 2 h, 0 °C to RT, 51%; (c) CH_2Cl_2, dipropargylamine, $NaBH(OAc)_3$, 2 h, RT, 95%; (d) DMF, compound 3, $PdCl_2(PPh_3)_2$, CuI, Et_3N, 12 h, RT, 59%; (e) CH_2Cl_2, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, DIPEA, 1.5 h, RT, 72%.

Synthesis of 2,2,2-trifluoro-N-(3-hydroxypropyl)acetamide (compound **1**)



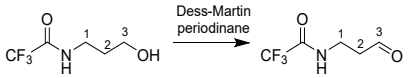
To a stirred 3-aminopropanol (6.0 g, 79.9 mmol, 1.0 eq.) ethyl trifluoroacetate (20.5 g, , 144 mmol, 1.8 eq.) was added dropwise under an argon atmosphere at 0 °C. The solution was stirred for 3 h, evaporated, followed by purification by column chromatography on silica gel (10% CH_3OH/CH_2Cl_2) to give the product as a pale-yellow liquid (13.3 g, 77.7 mmol, 97% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.80 (br s, 1H, NH), 3.72 (t, *J* = 5.6 Hz, 2H, H^3), 3.47 (app. q, *J* = 6.0 Hz, 2H, H^1), 3.19 (s, 1H, OH), 1.83 – 1.74 (m, 2H, H^2);

^{13}C NMR (101 MHz, $CDCl_3$): δ 158.0 (q, *J* = 36.7 Hz, $\underline{C}O\text{-}CF_3$), 116.1 (q, *J* = 287.3 Hz, CF_3), 60.7 (C3), 38.2 (C1), 30.6 (C2);

R_f = 0.60 ($MeOH:CH_2Cl_2$, *v*:*v* = 1:9), $KMnO_4$. Spectral data were in agreement with literature values.[1]

Synthesis of 2,2,2-trifluoro-N-(3-oxopropyl)acetamide (compound **2**)



1 **2**

To a stirred solution of compound 1 (3.2 g, 18.7 mmol, 1.0 eq.) in CH_2Cl_2 (60 mL) was added Dess-Martin periodinane (11.9 g, 28.1 mmol, 1.5 eq.) under an argon atmosphere at 0 °C. The suspension was stirred for 2 h at RT, quenched with sat. $NaHCO_3$ (120 mL), Et_2O (120 mL) was added and the biphasic mixture was stirred for 20 min. A precipitate formed which was removed by filtration. The phases were separated and the aqueous phase was extracted with Et_2O (60 mL). The combined organic layers were washed with sat. NaCl (50 mL), dried with $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography on silica gel (petroleum ether 40-60/EtOAc, 3:2, v:v) to give the product as a colourless liquid (1.6 g, 9.5 mmol, 51%). The product was used on the same day due to low stability.

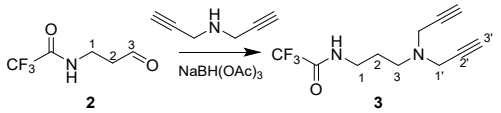
^{1}H NMR (400 MHz, d_6-DMSO): δ 9.63 (t, *J* = 1.4 Hz, 1H, H^3), 9.46 (s, 1H, NH), 3.49 – 3.42 (m, 2H, H^1), 2.70 (td, *J* = 6.6, 1.4 Hz, 2H, H^2);

^{13}C NMR (101 MHz, d_6-DMSO): δ 201.9 (C3), 156.7 (q, *J* = 36.2 Hz, $\underline{C}O\text{-}CF_3$), 116.3 (q, *J* = 288.0 Hz, CF_3), 42.3 (C1), 33.7 (C3);

^{19}F NMR (376 MHz, d_6-DMSO): δ -74.5 (CF_3);

R_f = 0.43 (EtOAc:*n*-hexane, *v*:*v* = 2:3), $KMnO_4$. Spectral data of the compound were reported in $CDCl_3$.[2]

Synthesis of N-(3-(N,N-dipropargyl)propyl)-2,2,2-trifluoroacetamide (compound **3**)



2 **3**

To a stirred solution of compound 2 (1.78 g, 10.5 mmol, 1.5 eq.) and dipropargylamine (0.72 mL, 7.0 mmol, 1.0 eq.) in an anhydrous CH_2Cl_2 (23 mL) under an argon atmosphere at RT, was added $NaBH(OAc)_3$ (2.97 g, 14.0 mmol, 2.0 eq.). The solution was stirred for 2 h, quenched with sat. $NaHCO_3$ (10 mL) and stirred for 20 min. The mixture was extracted with CH_2Cl_2 (2 x 15 mL), the organic phase was washed with water, saturated aqueous NaCl. The organic layer was collected and dried with Na_2SO_4. The crude product was purified by column chromatography on silica gel (EtOAc/petroleum ether 40-60, 1:10 to 7:3, v:v) to give the product as a white solid (1.63 g, 6.6 mmol, 95% yield).

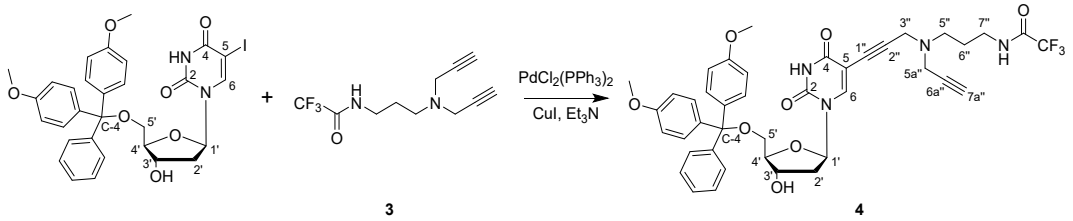
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (br s, 1H, NH), 3.42 (app. q, *J* = 5.8 Hz, 2H, H^{1}), 3.37 (d, *J* = 2.4 Hz, 4H, $H^{1'}$), 2.71 (app. t, *J* = 2.7 Hz, 2H, H^{3}), 2.19 (t, *J* = 2.4 Hz, 2H, $H^{3'}$), 1.71 – 1.63 (m, 2H, H^{2});

^{13}C NMR (101 MHz, $CDCl_3$): δ 157.1 (q, *J* = 36.7 Hz, $\underline{C}O\text{-}CF_3$), 117.4 (q, *J* = 287.9, CF_3), 77.9 (C2'), 73.6 (C3'), 51.5 (C3), 42.2 (C1'), 39.9 (C1), 24.4 (C2);

^{19}F NMR (376 MHz, $CDCl_3$): δ -76.10 (CF_3);

HRMS (ESI$^+$) *m/z* $[M+H]^+$ for $C_{11}H_{14}F_3N_2O$ calc. 247.1053 found 247.1052; R_f = 0.60 (MeOH:CH_2Cl_2, *v*:*v* = 1:9), $KMnO_4$.

Synthesis of 5-propargylamino(N-propargyl-N-propyl-2,2,2-trifluoroacetamide)-5'-*O*-(4,4'-dimethoxytritryl)-2'-deoxythymidine (compound **4**)



3 **4**

To a stirred solution of 5-iodo-5'-DMTr-deoxythymidine (4.20 g, 6.40 mmol, 1.0 eq.), compound 3 (3.2 g, 12.8 mmol, 2.0 eq.), $PdCl_2(PPh_3)_2$ (0.494 g, 0.704 mmol, 0.11 eq.) and CuI (0.072 g, 0.378 mmol, 0.059 eq.) in degassed DMF (60.6 mL) under an argon atmosphere at RT, degassed Et_3N (5.35 ml) was added. The mixture was stirred for 12 h, evaporated to dryness and the resulting residue was dissolved in EtOAc (100 mL). The organic layer was washed with an EDTA sodium salt solution (0.15 molar, 50 mL), H_2O (50 mL) and sat. NaCl (50 mL). The organic phase was dried with Na_2SO_4 and evaporated, followed by purification by chromatography on silica gel (17-100% EtOAc/CH_2Cl_2) to give the product as a pale-yellow foam (2.92 g, 3.76 mmol, 59% yield).

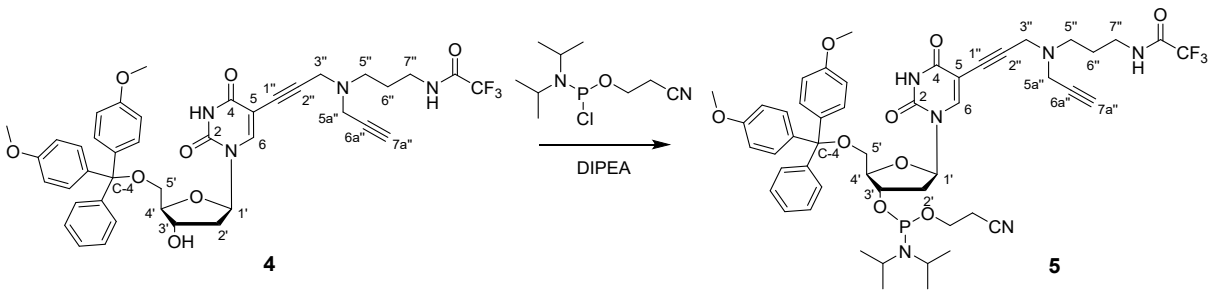
^{1}H NMR (500 MHz, $CDCl_3$): δ 9.66 (s, 1H, NH-thymidine), 8.26 (t, *J* = 5.3 Hz, 1H, NH-chain), 8.12 (s, 1H, H^{6}), 7.46 – 7.42 (m, 2H, Ar-H), 7.37 – 7.33 (m, 4H, Ar-H), 7.33 – 7.29 (m, 2H, Ar-H), 7.26 – 7.21 (m, 1H, Ar-H), 6.88 – 6.84 (m, 4H, Ar-H), 6.34 (t, *J* = 7.5 Hz, 1H, $H^{1'}$), 4.56 – 4.52 (m, 1H, $H^{4'}$), 4.11 (app. q, *J* = 3.1 Hz, 1H, $H^{3'}$), 3.80 (s, 6H, OCH_3), 3.46 (dd, *J* = 10.8, 3.1 Hz, 1H, $H^{5'a}$), 3.38 – 3.29 (m, 5H, $H^{5'b}$, $H^{3''}$and $H^{7''}$), 3.19 (d, *J* = 2.5 Hz, 2H, $H^{5a''}$), 2.56 – 2.51 (m, 3H, $H^{2'a}$ and $H^{5''}$), 2.30 (ddd, *J* = 13.6, 7.6, 6.0 Hz, 1H, $H^{2'b}$), 2.21 (t, *J* = 2.5 Hz, 1H, $H^{7a''}$), 1.54 (app. p, *J* = 6.1 Hz, 2H, $H^{6''}$).

^{13}C NMR (126 MHz, $CDCl_3$): δ 162.1 (C4), 158.6 (Ar), 157.2 (q, *J* = 36.5 Hz, $\underline{C}O\text{-}CF_3$), 149.4 (C2), 144.5 (Ar), 142.3 (C6), 135.6 (Ar), 130.0 (Ar-H), 128.1 (Ar-H), 127.9 (Ar-H), 127.0 (Ar-H), 116.2 (q, *J* = 287.8 Hz, CF_3), 113.3 (Ar-H), 99.9 (C5), 88.8 (C2''), 87.0 (C-4), 86.6 (C4'), 85.7 (C1'), 78.6 (C6a''), 76.0 (C1''), 73.3 (C7a''), 72.2 (C3'), 63.4 (C5'), 55.3 (OCH_3), 50.8 (C5''), 43.1 (C3''), 42.7 (C5a''), 41.6 (C2'), 39.4 (C7''), 24.6 (C6'').

^{19}F NMR (376 MHz, $CDCl_3$): δ -75.91 (CF_3);

HRMS (ESI$^+$) *m/z* $[M+H]^+$ for $C_{41}H_{42}F_3N_4O_8$ calc. 775.2949 found: 775.2940; R_f = 0.20 (EtOAc:CH_2Cl_2, *v*:*v* = 7:3), *p*-anisaldehyde, UV.

Synthesis of 5-propargylamino(N-propargyl-N-propyl-2,2,2-trifluoroacetamide)-5'-*O*-(4,4'-dimethoxytritryl)-2'-deoxythymidine diisopropylamino cyanoethyl phosphoramidite (compound **5**)



To a solution of compound 4 (2.01 g, 2.60 mmol, 1.0 eq.) and anhydrous degassed DIPEA (1.27 mL, 7.28 mmol, 2.8 eq.) in anhydrous degassed CH_2Cl_2 (60 mL) under an argon atmosphere, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.76 mL, 3.41 mmol, 1.3 eq.) was added . The reaction was stirred for 1.5 h, quenched with degassed saturated KCl solution (30 mL) and the organic phase was passed through a layer of anhydrous Na_2SO_4 followed by evaporation to give a yellow foam. The crude product was purified by chromatography on silica gel (degassed 60-85% EtOAc/hexane) pre-equilibrated with pyridine, under an atmosphere of argon to give the product as white foam (1.82 g, 1.87 mmol, 72% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (s, 1H, NH-chain), 8.08 (s, 0.5H, H^6 isomer a), 8.04 (s, 0.5H, H^6 isomer b), 7.39 – 7.33 (m, 2H, Ar-H), 7.30 – 7.20 (m, 6H, Ar-H), 7.19 – 7.10 (m, 1H, Ar-H), 6.80 – 6.73 (m, 4H, Ar-H), 6.23 (dt, *J* = 7.3, 5.9 Hz, 1H, $H^{1'}$), 4.57 – 4.48 (m, 1H, $H^{3'}$), 4.15 – 4.05 (m, 1H, $H^{4'}$), 3.72 (s, 6H, OCH_3), 3.79 – 3.33 (m, 5H, $H^{5'a}$ and ^{i}Pr-CH and $OC\underline{H}_2CH_2$-CN), 3.30 – 3.15 (m, 5H, $H^{3''}$and $H^{5'b}$ and $H^{7''}$), 3.06 (app. dd, *J* = 6.1, 2.4 Hz, 2H, $H^{5a''}$), 2.55 (t, *J* = 6.3 Hz, 1H, CH_2^b-CN), 2.48 (ddd, *J* = 13.7, 5.9, 2.8 Hz, 1H, $H^{2'a}$), 2.44 – 2.37 (m, 2H, $H^{5''}$), 2.35 (t, *J* = 6.3 Hz, 1H, CH_2^a-CN), 2.25 (dt, *J* = 13.7, 7.3 Hz, 1H, $H^{2'b}$), 2.11 (app. q, *J* = 2.4 Hz, 1H, $H^{7a''}$), 1.45 – 1.34 (m, 2H, $H^{6''}$), 1.12 – 1.07(m, 9H, iPr-CH_3), 0.98 (d, *J* = 6.8 Hz, 3H, iPr-CH_3).

^{31}P NMR (162 MHz, $CDCl_3$): δ 149.08 (s), 148.68 (s);

HRMS (ESI^+) *m/z* $[M+H]^+$ for $C_{50}H_{59}F_3N_6O_9P$ calc. 975.4028 found 975.4018; HRMS (ESI^-) *m/z* $[M-H]^-$ for $C_{50}H_{57}F_3N_6O_9P$ calc. 973.3882 found 973.3872; R_f = 0.31 and 0.48 for two isomers (EtOAc:hexane, *v*:*v* = 7:3), *p*-anisaldehyde, UV.

2.2. Synthesis of oligonucleotides

Standard DNA phosphoramidites and solid supports were purchased from Link Technologies and Applied Biosystems Ltd. Oligonucleotides were synthesized using an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 1.0 µmol phosphoramidite cycle of acid-catalyzed using trichloroacetic acid (TCA) detritylation, coupling, capping and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by an automated trityl cation conductivity monitoring facility and in all cases were >98.0%. β-Cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C and T monomers was 50 seconds and the coupling time for the modified monomers (AP-C3) was 600 seconds. Cleavage of the oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution (1 h, RT) followed by heating in a sealed tube (5 h, 55 °C). After evaporation of the ammonia under vacuo, the fully

deprotected oligonucleotides were purified by reverse-phase HPLC (RP-HPLC) on a Gilson system using a Luna 10 µm C8 100 Å pore Phenomenex 10x250 mm column with a gradient of acetonitrile (HPLC grade) in water (Sterile, Millipore system) with TEAA buffer (buffer A: 0.1 M triethylammonium acetate, pH 7.0; buffer B: 0.1 M triethylammonium acetate with 50% acetonitrile, pH 7.0; gradient: 0% to 100% buffer B over 20 min, flow rate: 4 mL/min) or TEAB buffer (buffer A: 0.1 M triethylammonium bicarbonate, pH 7.5; buffer B: 0.1 M triethylammonium bicarbonate, pH 7.5, with 50% acetonitrile) were used (0% to 100% buffer B over 20 min). Elution was monitored by UV absorption at 260-299 nm. The purified oligonucleotides were desalted with GE Healthcare Life Sciences illustra NAP 25 then NAP 10 gel filtration columns. After RP-HPLC purification, all oligonucleotides were characterised by electrospray mass spectrometry using a Bruker microTOF II focus ESI-TOF MS or UPLC-MS Waters XEVO G2-QTOF instrument in ESI^- mode. Data were processed using MaxEnt or MassLynx v4.1.

For the synthesis of 2'-OMe RNA oligonucleotides, phosphoramidite, solid supports and additional reagents were purchased from Link Technologies, the procedure was identical to the DNA oligonucleotides apart from extended coupling time to 10 min for each step including modifications.

2.3. General procedure of copper-click 1,3-dipolar cycloaddition (CuAAC)

To a solution of AP-C3 modified oligonucleotide (40 nmol, 1.0 eq.) in H_2O (600 µL), TEAA buffer (2 M, pH = 7.0; 200 µL), DMSO (590 µL) and stock of TO azide (10 nmol/µL in DMSO, 12 µL, 7.5 eq. per modification) and freshly prepared sodium ascorbate (0.9 mg/ 1.0 mL of H_2O; 200 µL) were vortexed together. Mixture was degassed by bubbling argon gas for 5 min followed by addition of Cu^{II}:THPTA (tris(3-hydroxypropyltriazolylmethyl)amine) complex (10 mM in 55%/45% DMSO/H_2O; 100 µL, Cu source: $CuSO_4$). Reaction was shaken for 3 hours at 30 °C, followed by double ethanol precipitation with NaOAc (3 M) and was purified by reverse-phase HPLC with C18 column (RP-HPLC) to obtain pure product with full conversion.

2.4. NHS ester labelling

A solution of ROX NHS ester (20 eq. per modification) in DMF (80 µL) was added to an oligonucleotide (200 nmol) dissolved in carbonate buffer ($NaHCO_3$/Na_2CO_3, 0.5 M, pH = 8.75, 120 µL) and shaken for 3 h at 36 °C. The mixture was desalted by NAP gel filtration column and purified by RP-HPLC to obtain the desired labelled oligonucleotide.

2.5. Steady state fluorescence measurements

Fluorescence studies were performed on a Perkin Elmer LS50B luminescence spectrometer fitted with Perkin Elmer PTP-1 Peltier temperature controller. FLWinlabTempScan software was used with settings of 400 nm/s scan speed. For TO the emission wavelength was recorded from 510 nm to 700 nm, excitation wavelength 484 nm or 510 nm, gain high (900V), excitation slit width 7.0 nm, emission slit width 7.0 nm. For ROX the range of

emission was 578 nm to 750 nm at excitation wavelength of 570 nm; the gain and slits width were identical as TO.

Experiment: samples were prepared in 300 µL cuvettes with 0.25 µM probe containing TO, 200 mM NaCl, 10 mM Na-phosphate buffer pH = 7.0 at 37 °C. Spectra were recorded for the single stranded oligonucleotide probe, and then titrated with 1.1 eq. of the desired target oligonucleotide and recorded again. F_{ds}/F_{ss} was calculated at the maximum emission wavelength (λ_{em}) of double strand probes (from 510 to 700 nm unless stated otherwise), each probe-target pair was recorded at least in duplicate and values are given in the charts as an averaged value.

2.6. UV melting studies

UV melting measurements were made on a Varian Cary 4000 UV-Vis spectrophotometer with a Cary temperature controller. Cary Win UV Thermal software was used with an absorbance wavelength of 260 nm. Samples were analysed in 1 mL quartz cuvettes (1 cm path length) and were made to 1.0 µM oligonucleotide probe concentration (X = TO, ROX, TO/ROX strand) and 1.1 µM oligonucleotide target concentration in phosphate buffer (NaH_2PO_4, 10 mM, NaCl 200 mM at pH 7.0, unless stated otherwise). The samples were initially denatured by heating to 85 °C at 10 °C min^{-1} then cooled to 15 °C at -1 °C min^{-1} and then maintained at 15 °C for 2 min before heating to 85 °C at 1 °C min^{-1}. UV absorption was recorded every 0.1 °C. The melting temperature (Tm) values were derived from the derivatives of the melting curves and calculated at 260 nm using Cary Win UV Thermal application software. Four successive melting curves were measured and averaged.

2.7. RT-PCR and Fluorescence melting studies

A polymerase master mix consisting of Vent (exo-) DNA polymerase (2 U/uL, 0.25 uL, NEB, cat. No. M0257S), ThermoPol buffer (10 x, 2 uL), heat-activated CleanAmp® dNTPs (10 mM, 0.4 uL, tebu-bio, cat. No. 040N-9506-2) and water (14.15 uL) was prepared in the 0.2 mL white 8-tube PCR strips (Bio-rad, cat. No. TLS0851). The mix was then added to a solution of d2(PCR)-TO/RX probe (10 uM, 0.3 uL/0.6 uL, to make a different probe concentration), forward primer (10 uM, 1.6 uL), reverse primer (10 uM, 0.3 uL) and diluted template (1 uL). Total reaction volume is 20 uL. After sealing the PCR tubes the reaction mixture was mixed thoroughly by vortexing and centrifuged before RT-PCR amplification.

After initial thermal activation (120 s, 95 °C), 45 cycles of denaturation (15 s, 95 °C) and annealing/extension (60 s, 57.5 °C) were performed. At the end of each extension step, samples were excited at 560-590 nm and the emission at 610-650 nm was monitored. For melt curves analysis, samples were heated from 25 °C to 85 °C and fluorescence was recorded every 0.1 °C in ROX channel.

Fluorescence melting were undertaken using a BioRad CFX96 Real-Time PCR Instrument, with CFX Manager software (BioRad), monitoring in the following channels: TET channel (excitation range 515–535 nm, detector range 560–580 nm), ROX channel (excitation range 560–590 nm, detector range 610–650 nm. Reactions were run in 0.2 mL low-profile white 8-tube strips with optically clear lids (BioRad). Samples were made up to 0.4 µM oligonucleotide probes against 0.44 µM targets in buffer (NaH_2PO_4, 10 mM; NaCl 200 mM) at pH 7.0. The samples

were initially denatured by heating to 85 °C at 1 °C min^{-1} then cooled to 25 °C at 1 °C min^{-1} and fluorescence was recorded every 0.1 °C in TET channel and ROX channel.

2.8. Fluorescence lifetime measurements

Fluorescence lifetime decays were determined with a time-correlated single photon counting (TCSPC) technique on a FS5 spectrofluorometer (Edinburgh Instruments). Picosecond pulsed diode laser (EPL-475) at 475 nm with 10 MHz repetition rate was used as excitation source for TO labelled samples. The cuvette holder (qpod, Quantum Northwest) was temperature controlled by a Peltier device (TC 125, Quantum Northwest) and decays were acquired at 298 K. The emission signals were monitored at 530 nm and collected with a magic angle. The instrument response function (IRF) was collected with an aqueous Ludox solution used to scatter the excitation light. Unless otherwise stated, data were accumulated until the number of counts in the channel with highest intensity reached 10 000. Our full-width half maxima (FWHM) of TCSPC apparatus was estimated about ~200 ps and all the picosecond fluorescence kinetics were reported after deconvolution using a Gaussian-shaped instrument response function (IRF). The emission decays were fit using the software (Fluoracle®) from Edinburgh Instruments assuming a multiexponential decay function, $I(t) = \sum_{i=1}^{n} \alpha_i exp(-t/\tau_i)$, where α_i is the amplitude and τ_i is the fluorescence lifetime of the i-th decay component. The maximum number of exponentials allowed by this software is four. For all measured decay traces, no more than three exponentials were needed to reasonably fit the data. The number of exponentials required for each trace was determined by the quality of the fit, judged on the basis of the reduced chi-square χ^2 and the randomness of residuals. The mean lifetime was calculated by $\langle\tau\rangle = \sum\alpha_i \tau_i$, using the lifetimes τ_i associated with the α_i amplitudes.

3. Figures and Tables

Table S 1. Mass data on DNA probes with the AP-C3 dT modification labelled with dyes and appropriate targets. In the table TO stands for TO-N_3 attached *via* CuAAC and ROX stands for ROX-NHS ester attached *via* amide bond formation. A = alkyne.

Sequence	X =	Probe	Mass calculated	Mass found
ODN1 5′-CGCTTC**X**GTATCTATATTCATC	AP-C3	d1-Y/A	6761	6761
	AP-C3-(TO)	d1-TO/A	7135	7133
	AP-C3-(6-ROX)	d1-Y/RX	7277	7276
	AP-C3-(TO/ROX)	d1-TO/RX	7651	7651
ODN2 5′-CGCTTC**X**GTATCTA**X**ATTCATC	AP-C3	d2-Y/A	6895	6895
	AP-C3-(TO)	d2-TO/A	7643	7642
	AP-C3-(ROX)	d2-Y/RX	7927	7929
	AP-C3-(TO/ROX)	d2-TO/RX	8675	8675
ODN3 5′-CGCT**X**CTGTA**X**CTATA**X**TCATC	AP-C3	d3-Y/A	7029	7029
	AP-C3-(TO)	d3-TO/A	8151	8150
	AP-C3-(ROX)	d3-Y/RX	8577	8579
	AP-C3-(TO/ROX)	d3-TO/RX	9699	9698
5′-dCTATGATGAATATAGATACAGAAGCGTCAT	**DNA Target**	-	9262	9263
5′-rGAUGAAUAUAGAUACAGAAGCG	**RNA Target**	-	7136	7136
5′-dGATGAATATAGATACGGAAGCG	G:T mismatch	-	6856	6858

	DNA target			
5′-dGATGAATATAAATACAGAAGCG	A:C mismatch DNA target	-	6824	6826
5′-rGAUGAAUAUAGAUACGGAAGCG	G:T mismatch RNA target	-	7151	7152
5′-rGAUGAAUAUAAAUACAGAAGCG	A:C mismatch RNA target	-	7119	7121

Note: in probe names, d = DNA oligo, r = RNA oligo, number = probe number, A = unlabelled amine, Y = unlabelled alkyne, TO = labelled with thiazole orange, RX = labelled with ROX, TO/RX = labelled with thiazole orange and ROX.

Table S 2. Mass data on 2′-OMe RNA probeswith the AP-C3 dT modification labelled with dyes and appropriate targets. In the table TO stands for TO-N_3 attached *via* CuAAC and ROX strand for ROX-NHS ester attached *via* amide bond formation.

Sequence (all 2′-OMe but X)	X =	Probe	Mass calculated	Mass found
5′-rCUUCUGUAUCUAUA**X**UC	AP-C3	r1-Y/A	5602	5605
	AP-C3-(TO)	r1-TO/A	5976	5977
	AP-C3-(ROX)	r1-Y/RX	6119	6121
	AP-C3-(TO/ROX)	r1-TO/RX	6493	6495
5′-rCU**X**CUGUAUCUAUA**X**UC	AP-C3	r2-Y/A	5720	5723
	AP-C3-(TO)	r2-TO/A	6468	6468
	AP-C3-(ROX)	r2-Y/RX	6754	6756
	AP-C3-(TO/ROX)	r2-TO/RX	7502	7502
5′-rCU**X**CUGUA**X**CUAUA**X**UC	AP-C3	r3-Y/A	5837	5841
	AP-C3-(TO)	r3-TO/A	6959	6959
	AP-C3-(ROX)	r3-Y/RX	7388	7390
	AP-C3-(TO/ROX)	r3-TO/RX	8510	8509
5′-dGAATATAGATACAGAAG	**DNA Target**	-	5275	5276
5′-rGAAUAUAGAUACAGAAG	**RNA Target**	-	5504	5505
5′-dGAGTATAGATACAGAAG	G:T mismatch DNA target	-	5291	5293
5′-dGAATATAAATACAGAAG	A:C mismatch DNA target	-	5259	5261
5′-rGAGUAUAGATUCAGAAG	G:T mismatch RNA target	-	5521	5521
5′-rGAAUAUAAAUACAGAAG	A:C mismatch RNA target	-	5488	5489

Note: In probe names, d = DNA oligo, r = RNA oligo, number = probe number, A = unlabelled amine, Y = unlabelled alkyne, TO = labelled with thiazole orange, RX = labelled with ROX, TO/RX = labelled with thiazole orange and ROX.

Table S 3. Sequence information and mass data on oligonucleotide probe d2(PCR)-TO/RX and primers used in PCR. In the table TO stands for TO-N_3 attached *via* CuAAC and ROX strand for ROX-NHS ester attached *via* amide bond formation. The analyte is the PCR product made from a region of a 2665mer RNA template made by in vitro transcription from plasmid pcDNA3.1-hAsCpf1 (Addgene, plasmid number #69982), kindly gifted by Dr. Feng Zhang).

Sequence	X =	Probe	Mass calculated	Mass found
5′-dA**X**GGCGCCATCCTGT**X**TGTGAAGA-propanol	AP-C3-(TO/ROX)	d2(PCR)-TO/RX	9570	9571
5′-dGTCACTGGTCACAGATAGTAC;	-	Forward primer	6430	6431
5′-dCGTAGGTCAGACGTGAATAAG;	-	Reverse primer	6519	6520
5′-	-	PCR template	-	-

dCGTAGGTCAGACGTGAATAAGGAGAAGAACAA TGGCGCCATCCTGTTTGTGAAGAACGGCCTGTACT ATCTG;				

Note: in probe names, d = DNA oligo, number = probe number, TO = labelled with thiazole orange, RX = labelled with ROX, TO/RX = labelled with thiazole orange and ROX.

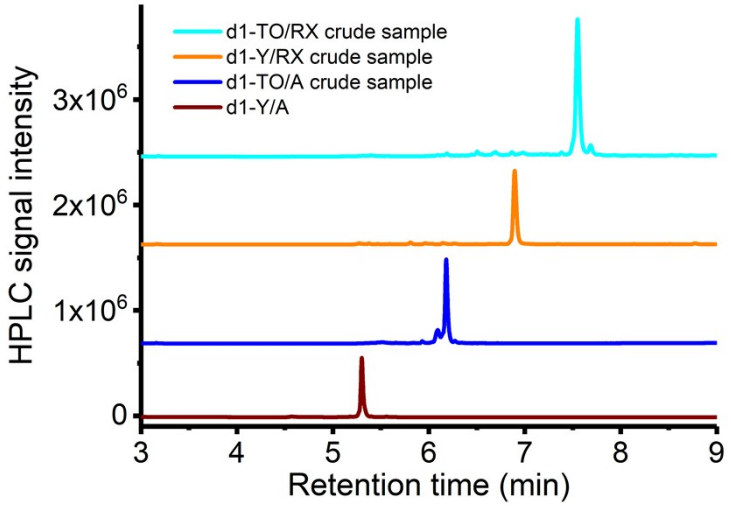


Figure S 1. HPLC chromatograms of d1-Y/A before (brown trace) and after reaction with TO azide (blue trace), ROX NHS ester (orange trace), and both ROX NHS ester and TO azide (cyan trace).

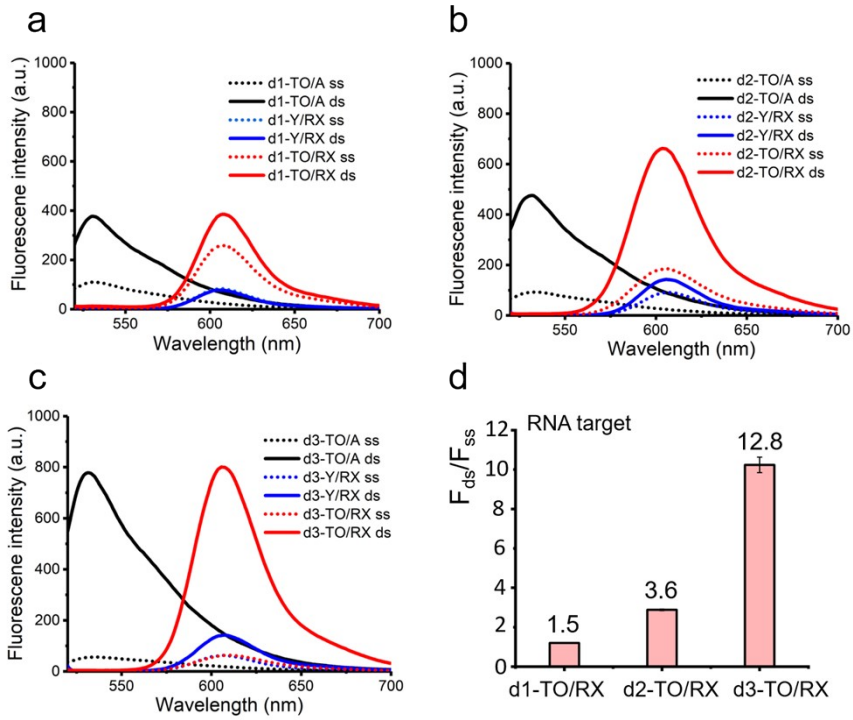


Figure S 2. Fluorescence spectra of TO, RX or TO/RX-functionalised DNA probes to RNA target when excitation wavelength was 510 nm. (a) Fluorescence spectra of 0.25 μM single TO, RX or TO/RX-functionalised DNA probes to 0.275 μM RNA target. (b) Fluorescence spectra of 0.25 μM double TO, RX or TO/RX-functionalised DNA probes to 0.275 μM RNA target. (c) Fluorescence spectra of 0.25 μM triple TO, RX or TO/RX-functionalised DNA probes to 0.275 μM RNA target. (d) Ratios of fluorescence intensity of TO/RX-functionalised DNA probes before and after hybridization with RNA target. Conditions: 10 mM Na-phosphate buffer, 200 mM NaCl, pH 7.0. Data points were measured in triplicate.

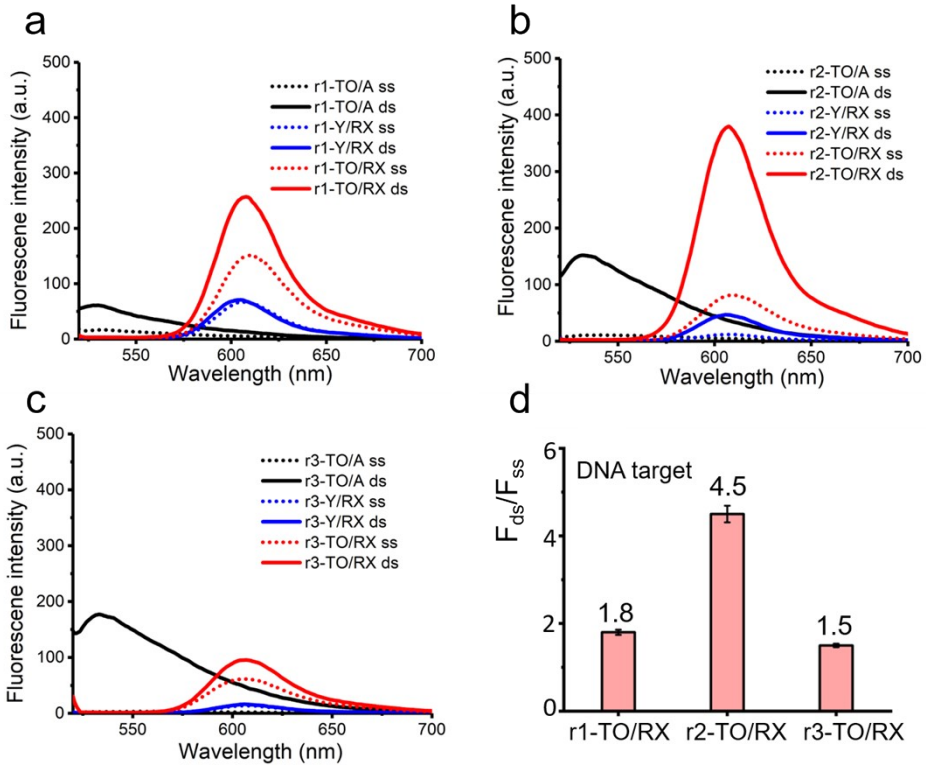


Figure S 3. Fluorescence spectra of TO, RX or TO/RX-functionalised 2'-OMe RNA probes to DNA target when excitation wavelength was 510 nm. (a) Fluorescence spectra of 0.25 μM single TO, RX or TO/RX-functionalised 2'-OMe RNA probes to 0.275 μM DNA target. (b) Fluorescence spectra of 0.25 μM double TO, RX or TO/RX-functionalised 2'-OMe RNA probes to 0.275 μM DNA target. (c) Fluorescence spectra of 0.25 μM triple TO, RX or TO/RX-functionalised 2'-OMe RNA probes to 0.275 μM DNA target. (d) Ratios of fluorescence intensity of TO/RX-functionalised 2'-OMe RNA probes before and after hybridization with DNA target. Conditions: 10 mM Na-phosphate buffer, 200 mM NaCl, pH 7.0. Data points were measured in triplicate.

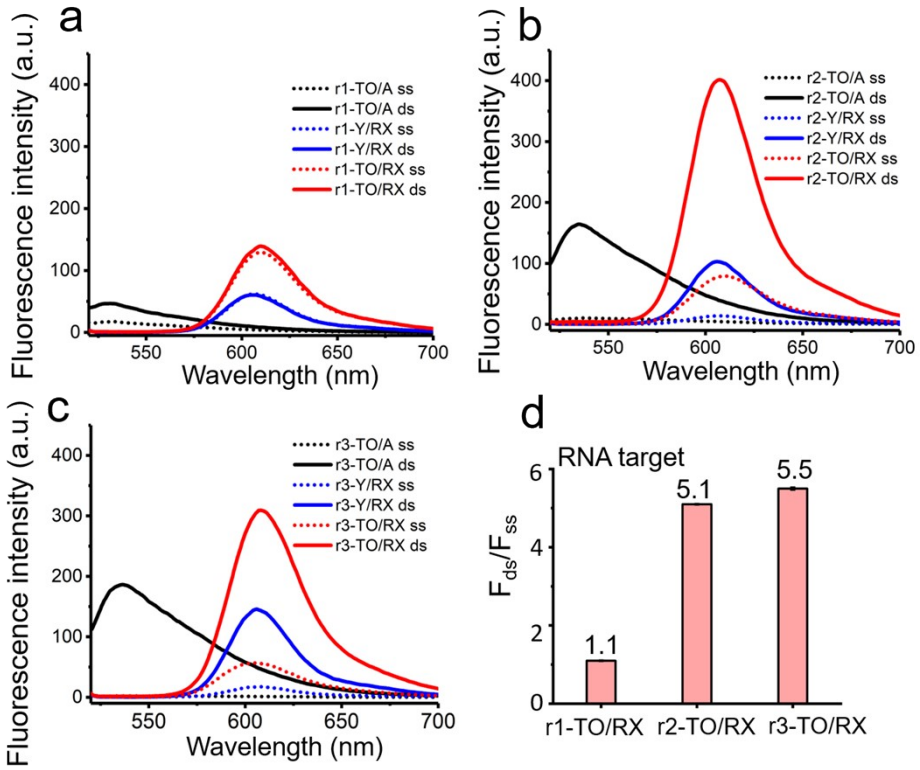


Figure S 4. Fluorescence spectra of TO, RX or TO/RX-functionalised 2'-OMe RNA probes to RNA target when excitation wavelength was 510 nm. (a) Fluorescence spectra of 0.25 μM single TO, RX or TO/RX-functionalised 2'-OMe RNA probes to 0.275 μM RNA target. (b) Fluorescence spectra of 0.25 μM double TO, RX or TO/RX-functionalised 2'-OMe RNA probes to 0.275 μM RNA target. (c) Fluorescence spectra of 0.25 μM triple TO, RX or TO/RX-functionalised 2'-OMe RNA probes to 0.275 μM RNA target. (d) Ratios of fluorescence intensity of TO/RX-functionalised 2'-OMe RNA probes before and after hybridization with RNA target. Conditions: 10 mM Na-phosphate buffer, 200 mM NaCl, pH 7.0. Data points were measured in triplicate.

Table S 4. Fluorescence emission data for DNA probes against DNA target. F_{ds}/F_{ss} – ratio of fluorescence emission at $\lambda_{em,\ max}$ of oligonucleotide duplexes to single stranded, $I_{0,\ max}$– fluorescence emission intensity at $\lambda_{em,\ max}$ of single stranded oligonucleotides probes, I_{max} – fluorescence emission intensity at $\lambda_{em,\ max}$ of probe-target duplexes. [a] – saturation of detector. Conditions: Probe concentration 0.25 µM, target concentration 0.275 µM in 10 mM Na-phosphate buffer, 200 mM NaCl, pH 7.0. Data points were measured in triplicate.

λ_{ex}	Probe	F_{ds}/F_{ss} at $\lambda_{em,\ max}$	$I_{0,\ max}$	I_0 error	I_{max}	I_{max} error	$\lambda_{em,\ max}$ probe-target
484 nm	d1-TO/A	2.4	73.7	0.2	178.1	0.9	528
	d1-Y/RX	0.9	36.6	0.6	32.8	0.4	607
	d1-TO/RX	1.4	174.4	2.5	236.0	1.7	606
	d2-TO/A	3.5	86.8	1.6	307.9	0.1	528
	d2-Y/RX	1.5	37.8	1.6	58.2	0.9	607
	d2-TO/RX	3.2	178.6	0.7	567.7	0.7	607
	d3-TO/A	10.0	43.0	0.1	428.7	3.9	531
	d3-Y/RX	2.5	31.4	0.0	78.5	1.6	608
	d3-TO/RX	7.4	104.2	0.4	772.9	3.6	606
510 nm	d1-TO/A	2.4	103.3	0.6	249.8	0.1	527
	d1-Y/RX	0.9	74.5	0.4	68.9	0.8	607
	d1-TO/RX	1.5	240.9	2.5	353.4	8.5	605
	d2-TO/A	4.6	93.0	0.8	424.8	1.8	528
	d2-Y/RX	1.5	75.9	1.5	116.5	2.2	607
	d2-TO/RX	5.9	135.9	2.4	804.3	4.1	606
	d3-TO/A	13.5	42.4	0.1	571.9	8.1	530
	d3-Y/RX	2.5	62.5	1.7	153.2	5.8	606
	d3-TO/RX	>16.7	59.8	0.5	>1000.0[a]	N/A	N/A
570 nm	d1-TO/A	0.6	4.4	0.9	2.5	0.0	578
	d1-Y/RX	0.9	347.8	3.0	322.2	0.5	605
	d1-TO/RX	1.8	136.6	0.1	249.1	0.9	605
	d2-TO/A	1.3	2.7	0.3	3.5	0.8	578
	d2-Y/RX	1.5	343.1	5.2	525.8	8.2	606
	d2-TO/RX	5.5	103.5	1.1	564.3	1.8	606
	d3-TO/A	3.0	2.7	0.4	8.1	1.6	578
	d3-Y/RX	2.4	277.5	5.5	668.4	11.2	607
	d3-TO/RX	13.2	58.3	0.7	772.3	6.1	605

Table S 5. Fluorescence emission data for DNA probes against RNA target. F_{ds}/F_{ss} – ratio of fluorescence emission at $\lambda_{em,\ max}$ of oligonucleotide duplexes to single stranded, $I_{0,\ max}$– fluorescence emission intensity at $\lambda_{em,\ max}$ of single stranded oligonucleotides probes, I_{max} – fluorescence emission intensity at $\lambda_{em,\ max}$ of probe-target duplexes. Conditions: Probe concentration 0.25 µM, target concentration 0.275 µM in 10 mM Na-phosphate buffer, 200 mM NaCl, pH 7.0. Data points were measured in triplicate.

λ_{ex}	Probe	F_{ds}/F_{ss} at $\lambda_{em,\ max}$	$I_{0,\ max}$	I_0 error	I_{max}	I_{max} error	$\lambda_{em,\ max}$ probe-target
484 nm	d1-TO/A	3.1	80.8	0.4	251.5	6.3	531
	d1-Y/RX	0.9	42.1	1.4	37.8	1.6	607
	d1-TO/RX	1.3	188.9	1.4	248.4	0.2	608
	d2-TO/A	4.1	84.0	1.6	347.4	8.8	531
	d2-Y/RX	1.9	36.7	2.7	70.6	1.5	607
	d2-TO/RX	2.1	215.7	0.3	449.4	4.8	605
	d3-TO/A	11.5	48.1	0.2	551.6	0.6	532
	d3-Y/RX	2.2	32.2	2.9	72.2	1.2	607
	d3-TO/RX	6.2	92.0	0.1	569.0	10.0	607
510 nm	d1-TO/A	3.4	111.1	0.9	372.5	5.1	531
	d1-Y/RX	0.9	83.3	2.3	77.9	1.8	605
	d1-TO/RX	1.5	260.1	2.0	386.6	1.3	609
	d2-TO/A	5.1	94.2	2.3	484.5	8.9	532
	d2-Y/RX	1.6	91.2	2.0	142.9	3.3	606
	d2-TO/RX	3.6	180.2	3.3	656.5	6.2	604
	d3-TO/A	13.6	56.9	1.8	776.1	2.4	532
	d3-Y/RX	2.3	63.2	1.6	142.4	3.7	608
	d3-TO/RX	12.8	62.6	2.1	801.2	6.4	606
570 nm	d1-TO/A	1.0	3.1	0.3	3.1	0.6	578
	d1-Y/RX	0.9	387.2	6.3	358.7	2.0	607
	d1-TO/RX	1.3	156.3	0.4	204.1	0.4	607
	d2-TO/A	1.6	3.4	0.1	5.5	0.0	578
	d2-Y/RX	1.6	406.8	8.1	646.6	16.3	606
	d2-TO/RX	2.7	166.2	6.3	445.5	4.4	604
	d3-TO/A	2.1	4.3	0.4	9.0	0.5	578
	d3-Y/RX	2.3	266.1	7.0	601.3	16.2	607
	d3-TO/RX	9.4	59.8	5.3	564.6	3.1	606

Table S 6. Fluorescence emission data for 2'-OMe RNA probes against DNA target. F_{ds}/F_{ss} – ratio of fluorescence emission at $\lambda_{em, max}$ of oligonucleotide duplexes to single stranded, $I_{0, max}$– fluorescence emission intensity at $\lambda_{em, max}$ of single stranded oligonucleotides probes, I_{max} – fluorescence emission intensity at $\lambda_{em, max}$ of probe-target duplexes. Conditions: Probe concentration 0.25 µM, target concentration 0.275 µM in 10 mM Na-phosphate buffer, 200 mM NaCl, pH 7.0. Data points were measured in triplicate.

λ_{ex}	Probe	F_{ds}/F_{ss} at $\lambda_{em, max}$	$I_{0, max}$	I_0 error	I_{max}	I_{max} error	$\lambda_{em, max}$ probe-target
484 nm	r1-TO/A	3.4	12.5	0.2	42.5	0.1	529
	r1-Y/RX	1.1	32.2	0.1	34.6	0.3	603.5
	r1-TO/RX	1.6	116.8	3.7	181.7	2.8	608
	r2-TO/A	9.0	12.1	0.5	109.4	2.4	533
	r2-Y/RX	3.6	6.0	0.5	21.9	0.2	605.5
	r2-TO/RX	2.4	116.3	2.0	280.4	0.7	608
	r3-TO/A	36.0	3.5	0.0	127.4	2.1	531.5
	r3-Y/RX	1.2	7.3	0.4	8.3	0.4	608
	r3-TO/RX	1.4	57.1	3.3	82.7	0.8	611
510 nm	r1-TO/A	3.9	15.5	0.7	59.9	1.1	527.5
	r1-Y/RX	1.1	67.5	0.5	71.1	0.2	605
	r1-TO/RX	1.8	144.8	5.6	253.6	3.4	608
	r2-TO/A	14.2	10.9	0.3	154.3	2.2	531.5
	r2-Y/RX	3.9	11.9	0.0	46.9	0.0	605.5
	r2-TO/RX	4.5	82.7	1.3	374.2	5.5	607.5
	r3-TO/A	64.4	2.8	0.1	180.4	3.4	533.5
	r3-Y/RX	1.2	14.6	0.9	17.0	0.8	606.5
	r3-TO/RX	1.5	59.8	1.7	92.4	3.1	606.5
570 nm	r1-TO/A	0.9	5.1	1.4	4.8	1.4	578
	r1-Y/RX	1.1	318.4	5.4	341.8	4.1	604
	r1-TO/RX	1.7	79.1	2.3	136.1	0.5	606
	r2-TO/A	1.4	2.8	0.6	3.7	0.8	578
	r2-Y/RX	3.5	62.3	0.1	219.6	8.0	605.5
	r2-TO/RX	3.1	71.1	1.1	223.8	3.7	604.5
	r3-TO/A	2.9	11.7	1.7	33.4	10.1	578
	r3-Y/RX	1.2	65.2	4.2	75.6	4.8	607
	r3-TO/RX	1.1	208.9	0.7	228.0	2.3	601.5

Table S 7. Fluorescence emission data for 2'-OMe RNA probes against RNA target. F_{ds}/F_{ss} – ratio of fluorescence emission at $\lambda_{em, max}$ of oligonucleotide duplexes to single stranded, $I_{0, max}$– fluorescence emission intensity at $\lambda_{em, max}$ of single stranded oligonucleotides probes, I_{max} – fluorescence emission intensity at $\lambda_{em, max}$ of probe-target duplexes. Conditions: Probe concentration 0.25 µM, target concentration 0.275 µM in 10 mM Na-phosphate buffer, 200 mM NaCl, pH 7.0. Data points were measured in triplicate.

λ_{ex}	Probe	F_{ds}/F_{ss} at $\lambda_{em, max}$	$I_{0, max}$	I_0 error	I_{max}	I_{max} error	$\lambda_{em, max}$ probe-target
484 nm	r1-TO/A	2.6	13.2	0.0	33.6	0.2	530
	r1-Y/RX	1.0	29.7	0.3	30.3	0.5	607
	r1-TO/RX	1.0	105.6	0.6	107.7	0.2	612.5
	r2-TO/A	9.6	11.3	0.5	108.8	0.2	534.5
	r2-Y/RX	7.1	6.8	0.3	48.3	3.1	607
	r2-TO/RX	2.5	107.9	3.0	269.5	7.4	607
	r3-TO/A	45.0	2.9	0.3	129.3	1.1	537.5
	r3-Y/RX	8.2	8.6	0.1	70.4	0.1	607.5
	r3-TO/RX	4.4	52.8	1.2	232.3	2.5	610
510 nm	r1-TO/A	2.7	17.2	0.1	47.2	0.4	531.5
	r1-Y/RX	1.0	63.4	1.5	61.8	1.3	606.5
	r1-TO/RX	1.1	130.4	1.1	140.4	1.0	610
	r2-TO/A	15.6	10.4	0.1	162.4	2.0	535
	r2-Y/RX	7.9	13.6	0.2	108.0	4.9	606
	r2-TO/RX	5.1	75.6	3.0	387.0	14.5	607.5
	r3-TO/A	79.3	2.3	0.1	186.3	0.2	536.5
	r3-Y/RX	8.5	17.1	0.1	144.9	0.9	606
	r3-TO/RX	5.5	56.3	0.4	309.9	0.7	608
570 nm	r1-TO/A	1.6	3.1	0.4	4.8	0.4	578
	r1-Y/RX	1.0	300.8	5.8	296.8	5.6	606
	r1-TO/RX	0.9	71.0	0.9	63.9	0.6	605.5
	r2-TO/A	2.3	2.5	0.5	5.8	0.1	578
	r2-Y/RX	8.2	62.8	0.2	517.4	13.0	606.5
	r2-TO/RX	3.5	66.9	2.5	232.4	9.9	607
	r3-TO/A	1.1	10.0	3.4	10.8	3.2	578
	r3-Y/RX	7.5	78.3	0.9	588.0	8.2	607.5
	r3-TO/RX	1.7	202.8	4.5	340.4	3.0	603.5

Table S 8. Fluorescence and UV-derived melting temperatures of DNA (left) and 2'-OMe RNA probes (right) hybridised to complementary targets. Samples were made up to 0.4 µM oligonucleotide probes against 0.44 µM targets in buffer (NaH_2PO_4, 10 mM; NaCl 200 mM) at pH 7.0. The samples were initially denatured by heating to 85 °C at 1 °C min^{-1} then cooled to 25 °C at 1 °C min^{-1} and fluorescence was recorded every 0.1 °C, where FL=fluorescence melting and UV= UV melting. Oligonucleotide sequences: d1: 5'-CGCTTCXGTATCTATATTCATC; d2: 5'-CGCTTCXGTATCTAXATTCATC; d3: 5'-CGCTXCTGTAXCTATAXTCATC, where X=labelled dT. Data points were measured in triplicate.

In several cases melting temperatures were measured by both UV and fluorescence melting with reasonable agreement. UV melting temperatures are slightly higher because of the increased oligonucleotide concentration required.

Probe	Tm/°C (DNA target)	Tm/°C (RNA target)	Probe	Tm/°C (DNA target)	Tm/°C (RNA target)
d1-Y/A	63.7(UV)	61.9(UV)	r1-Y/A	45.5(UV)	63.5(UV)
d2-Y/A	64.7(UV)	63.5(UV)	r2-Y/A	46.8(UV)	63.1(UV)
d3-Y/A	65.6(UV)	63.8(UV)	r3-Y/A	48.7(UV)	63.4(UV)
d1-TO/A	64.5(FL)	64.0(FL)	r1-TO/A	49.0(FL)	66.0(FL)
d2-TO/A	69.5(FL)/71.2(UV)	65.5(FL)	r2-TO/A	53(FL)/53.8(UV)	67(FL)/68.3(UV)
d3-TO/A	74.5(FL)	69.0(FL)	r3-TO/A	51.0(FL)	62.0(FL)
d1-TO/RX	60.5(FL)	59.5(FL)	r1-TO/RX	48.0(FL)	65.0(FL)
d2-TO/RX	56.0(FL)	54.0(FL)	r2-TO/RX	45.0(FL)	63.0(FL)
d3-TO/RX	51.5(FL)	51.5(FL)	r3-TO/RX	53.0(FL)	50.0(FL)
d1-Y/RX	57.0(FL)	57.5(FL)	r1-Y/RX	47.5(FL)/48.7(UV)	65.5(FL)/66.3(UV)
d2-Y/RX	52.0(FL)	54.0(FL)	r2-Y/RX	36.5(FL)	60.5(FL)
d3-Y/RX	48.0(FL)	52.0(FL)	r3-Y/RX	24.0(FL)/25.9(UV)	51.0(FL)

Table S 9. Fluorescence melting temperatures of single TO and TO/ROX labelled probes hybridised to fully complementary and specific single mismatched targets. Measurement was performed with probe concentration of 0.4 µM and target concentration of 0.44 µM in 10 mM Na-phosphate buffer, 200 mM NaCl, pH 7.0. Samples were initially denatured by heating to 85 °C at 1 °C min^{-1} then cooled to 25 °C at 1 °C min^{-1}. Fluorescence emission was recorded every 0.1 °C in TET channel (Excitation wavelength: 515 nm-535 nm; Emission wavelength: 560 nm- 580 nm) and ROX channel (Excitation wavelength: 560 nm-590 nm; Emission wavelength: 610 nm- 650 nm). Oligonucleotides sequence was shown in Table S1 and S2. Data points were measured in triplicate.

Probe	Tm/°C (DNA target)	T_m/°C (G:T mismatch DNA target)	T_m/°C (A:C mismatch DNA target)	T_m/°C (RNA target)	T_m/°C (G:T mismatch RNA target)	T_m/°C (A:C mismatch RNA target)
d1-TO/A	64.5	54.0	48.0	64.0	57.5	53.5
d1-TO/RX	60.5	55.0	48.5	59.5	53.5	48.0
r1-TO/A	49.0	43.0	34.0	66.0	61.0	53.0
r1-TO/RX	48.0	44.0	31.5	65.0	63.5	55.0

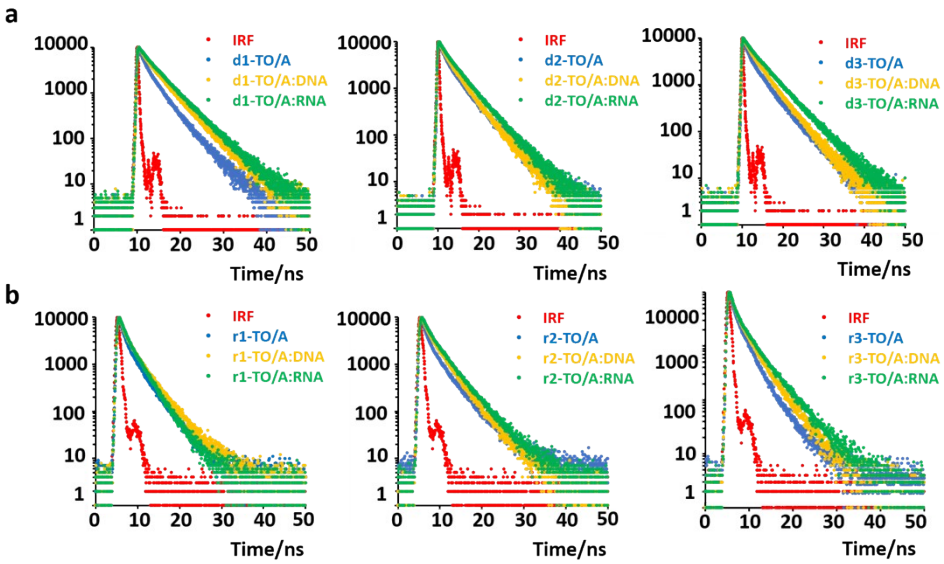


Figure S 5. Fluorescence decay of TO-labelled AP-C3 dT probes. a. d1-TO/A, d2-TO/A, d3-TO/A and b. r1-TO/A, r2-TO/A, r3-TO/A. 1 µM of probe was hybridised to 1.1 uM of complementary DNA and RNA target. Samples were excited at 475 nm and fluorescence decays were measured at 530 nm. Buffer is 10 mM Na-phosphate, 200 mM NaCl, pH = 7.0. Data points were measured in triplicate.

Table S 10. Time-resolved fluorescence data for d1- to d3-TO/A, r1- to r3-TO/A and r1 to r3-TO/RX probes against DNA and RNA targets. Excitation wavelength was 475 nm. Emission wavelength is 530 nm for d/r1- to d/r3-TO/A probes and is 610 nm for r1- to r3-TO/RX probes. Samples were made up to 1 µM of probe with 1.1 µM of DNA and RNA targets in buffer (10 mM Na-phosphate, 200 mM NaCl, pH = 7.0). a_1 = fraction of τ_1, a_2 = fraction of τ_2.

Probe:Target	τ_1 (ns)	a_1	τ_2 (ns)	a_2	$\langle\tau\rangle$ (ns)	$\langle\tau\rangle_{ds/ss}$
d1-TO/A	1.10	0.67	3.73	0.33	1.97	
d1-TO/A:DNA	1.49	0.50	4.37	0.50	2.93	1.48
d1-TO/A:RNA	1.50	0.33	4.56	0.67	3.54	1.79
d2-TO/A	1.11	0.67	4.29	0.33	2.17	
d2-TO/A:DNA	1.23	0.50	3.90	0.50	2.56	1.18
d2-TO/A /A:RNA	1.27	0.33	4.27	0.67	3.27	1.50
d3-TO/A	0.88	0.67	4.04	0.33	1.93	
d3-TO/A:DNA	1.10	0.43	3.88	0.57	2.69	1.39
d3-TO/A:RNA	1.09	0.33	4.55	0.67	3.39	1.75
r1-TO/A	0.18	0.26	2.73	0.74	2.08	
r1-TO/A:DNA	0.62	0.33	3.50	0.67	2.56	1.23
r1-TO/A:RNA	0.24	0.16	2.61	0.84	2.24	1.08
r2-TO/A	0.19	0.24	3.14	0.76	2.44	
r2-TO/A:DNA	0.25	0.13	3.15	0.87	2.76	1.13
r2-TO/A:RNA	0.23	0.10	3.44	0.90	3.11	1.27
r3-TO/A	0.15	0.28	2.07	0.72	1.53	
r3-TO/A:DNA	0.22	0.19	2.92	0.81	2.40	1.57
r3-TO/A:RNA	0.22	0.18	3.39	0.82	2.81	1.83

4. Mass spectra of all synthesized oligonucleotides

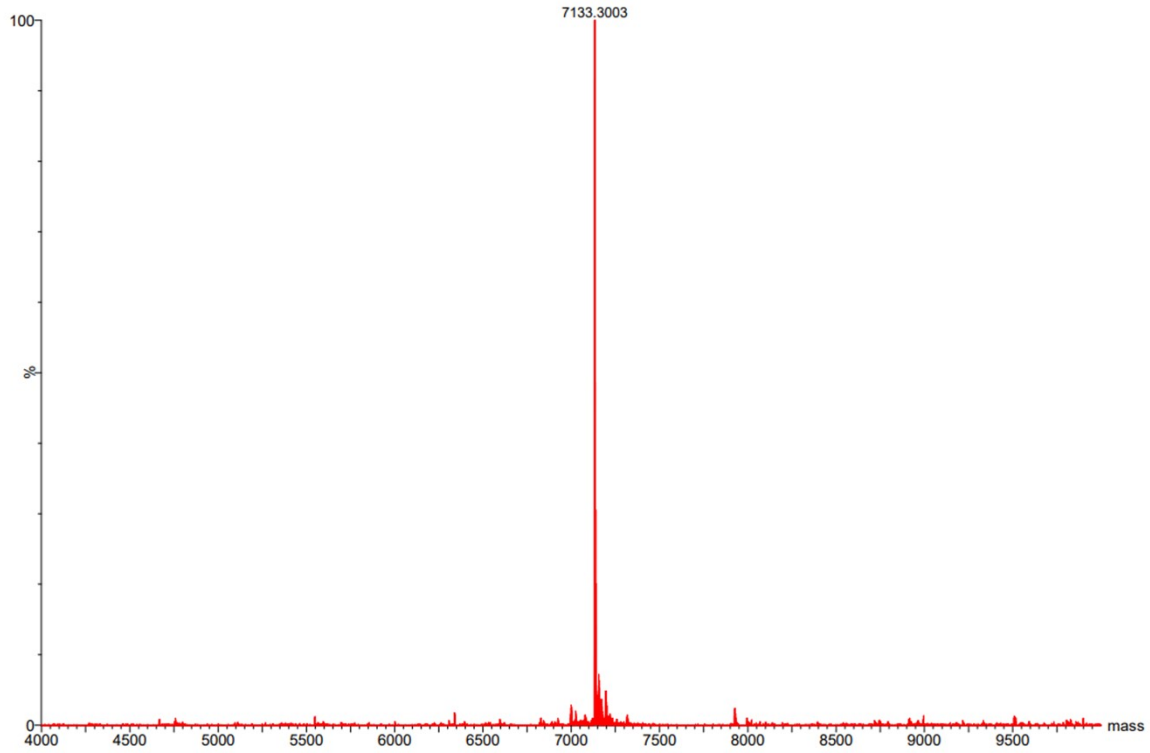


Figure S 6. Mass spectrum of d1-TO/A. Mass calculated: 7135; mass found: 7133.3.

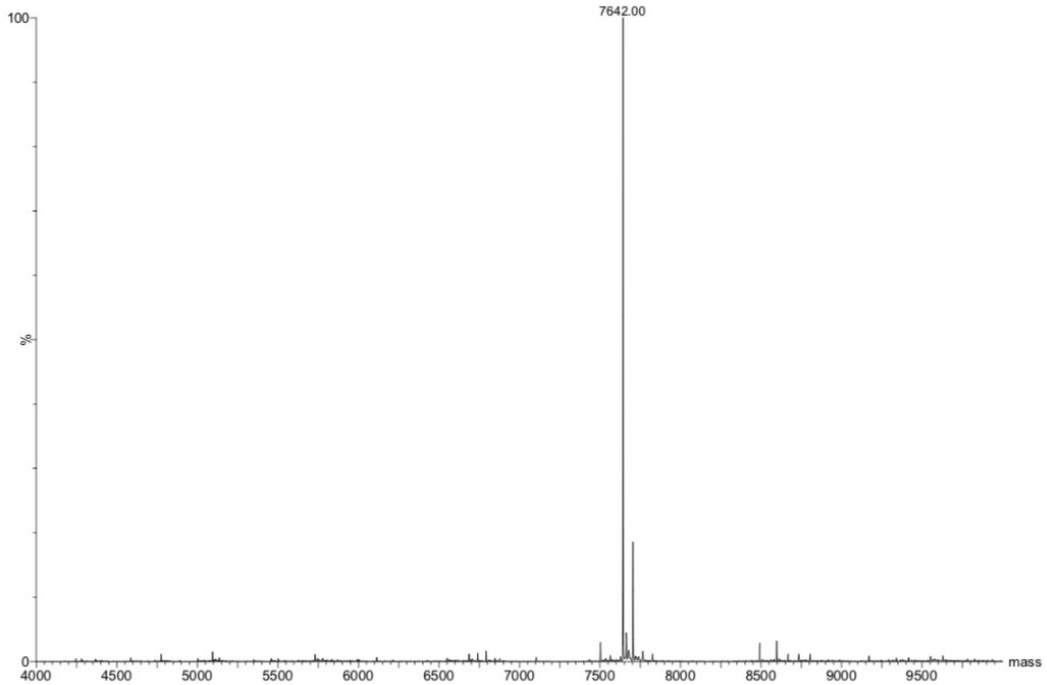


Figure S 7. Mass spectrum of d2-TO/A. Mass calculated: 7643; mass found: 7642, 7704(+62, Cu^{2+}).

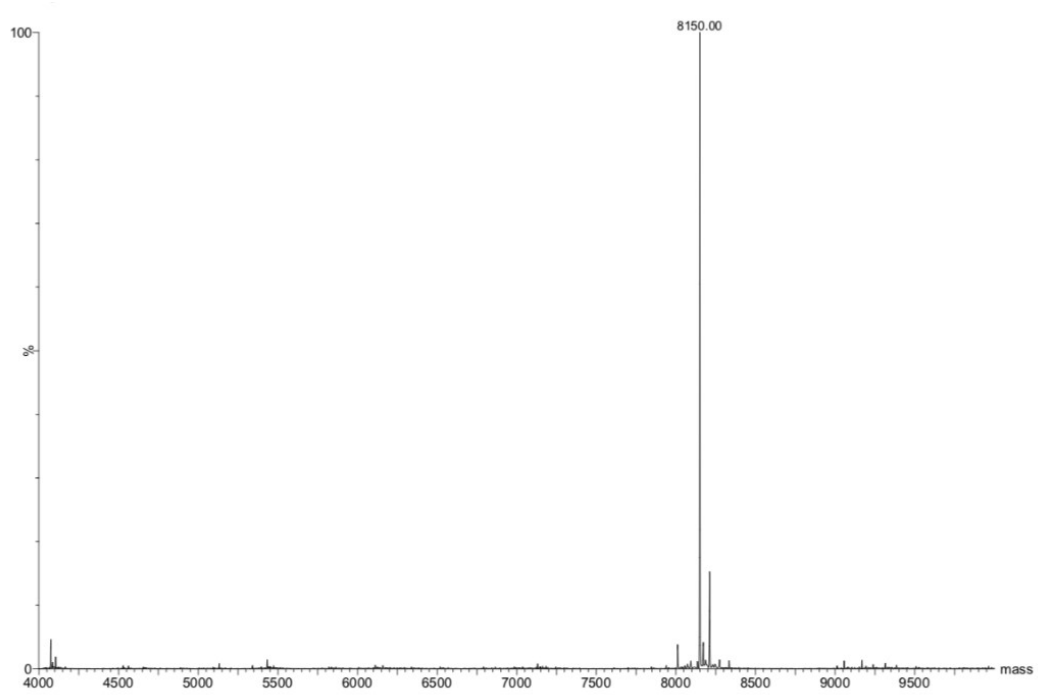


Figure S 8. Mass spectrum of d3-TO/A. Mass calculated: 8151; mass found: 8150.

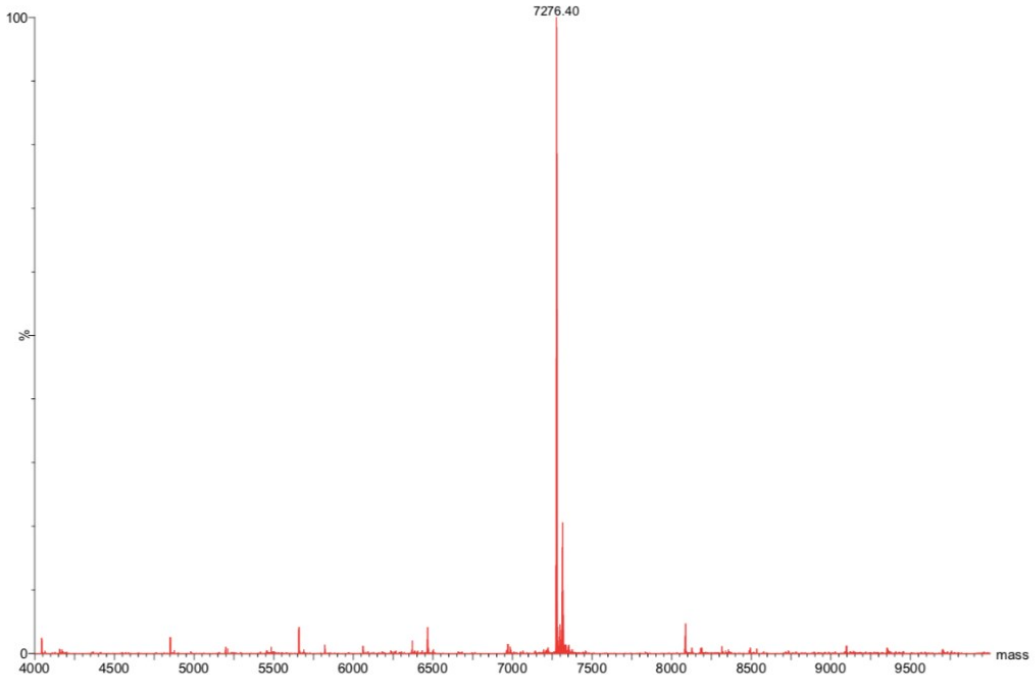


Figure S 9. Mass spectrum of d1-Y/RX. Mass calculated: 7277; mass found: 7276.4, 7338.2(+62, Cu^{2+}).

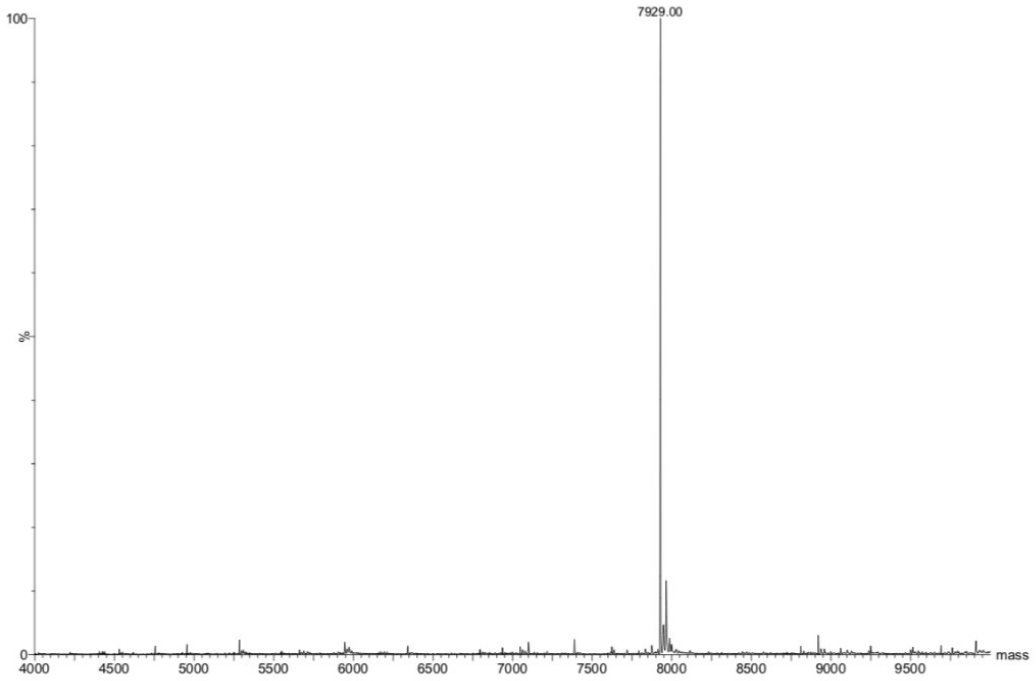


Figure S 10. Mass spectrum of d2-Y/RX. Mass calculated: 7927; mass found: 7929.

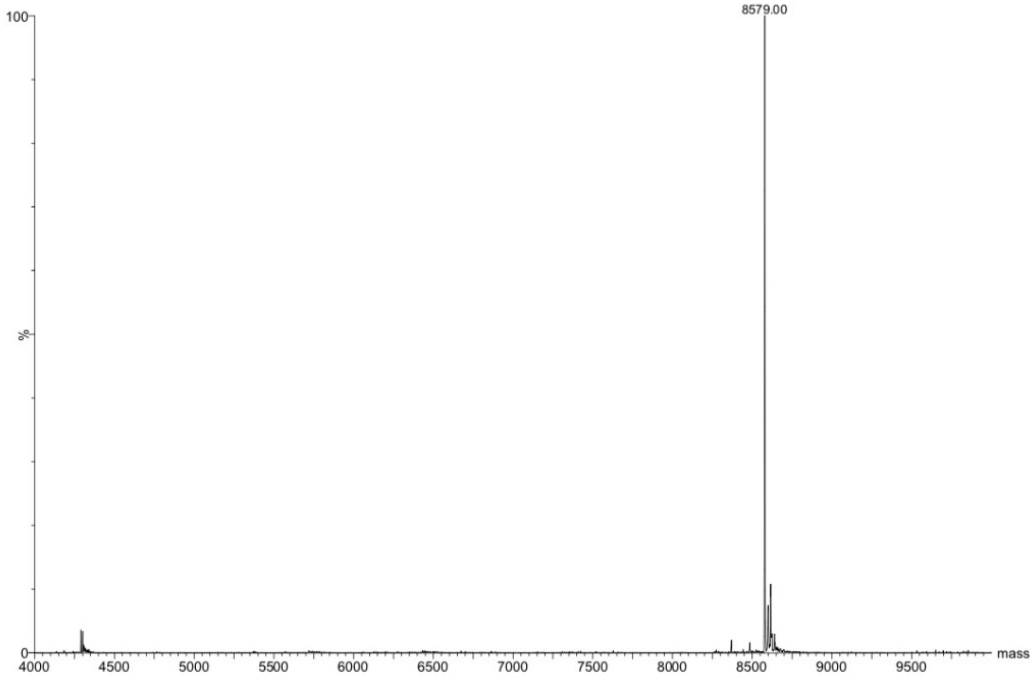


Figure S 11. Mass spectrum of d3-Y/RX. Mass calculated: 8577; mass found: 8579.

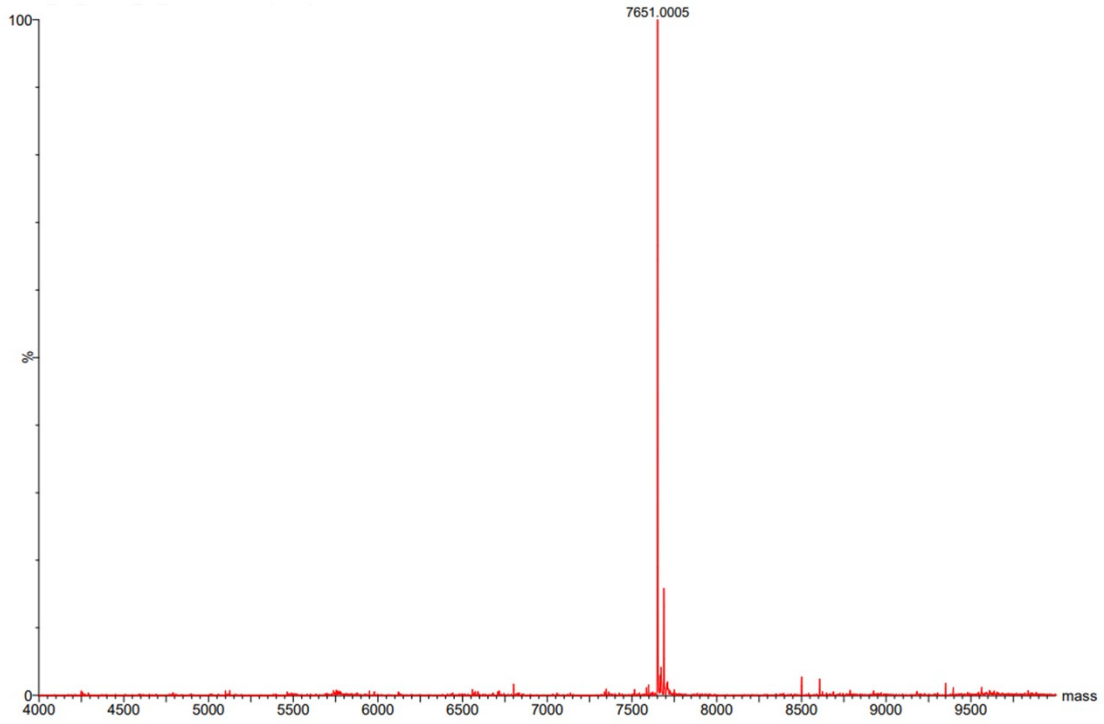


Figure S 12. Mass spectrum of d1-TO/RX. Mass calculated: 7651; mass found: 7651, 7713(+62, Cu^{2+}).

Figure S 13. Mass spectrum of d2-TO/RX. Mass calculated: 8675; mass found: 8675.

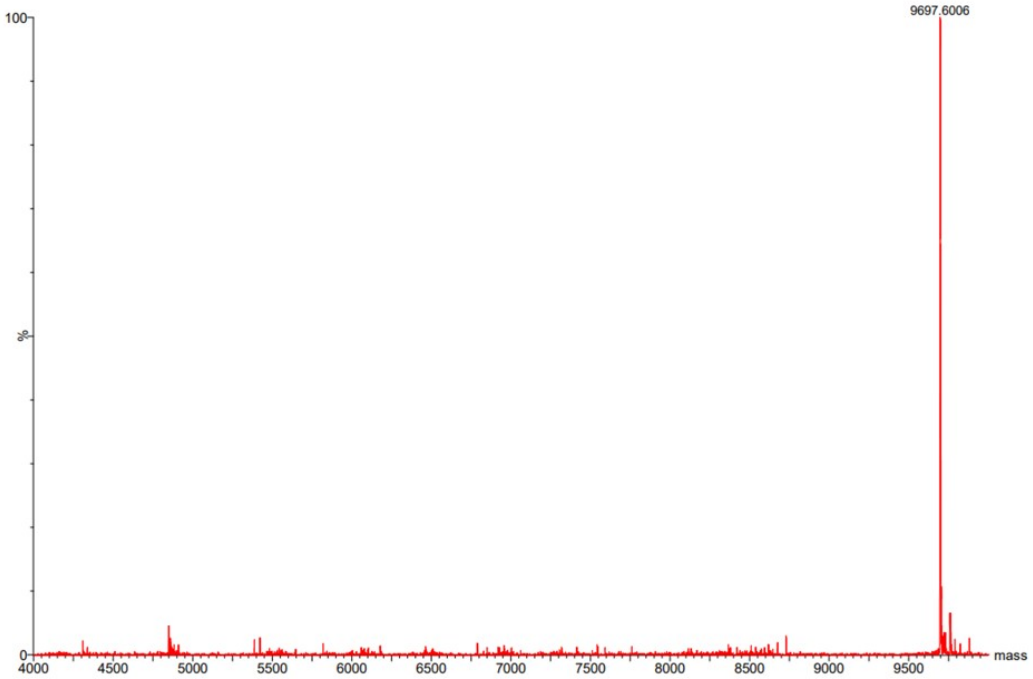


Figure S 14. Mass spectrum of d3-TO/RX. Mass calculated: 9699; mass found: 9697.6.

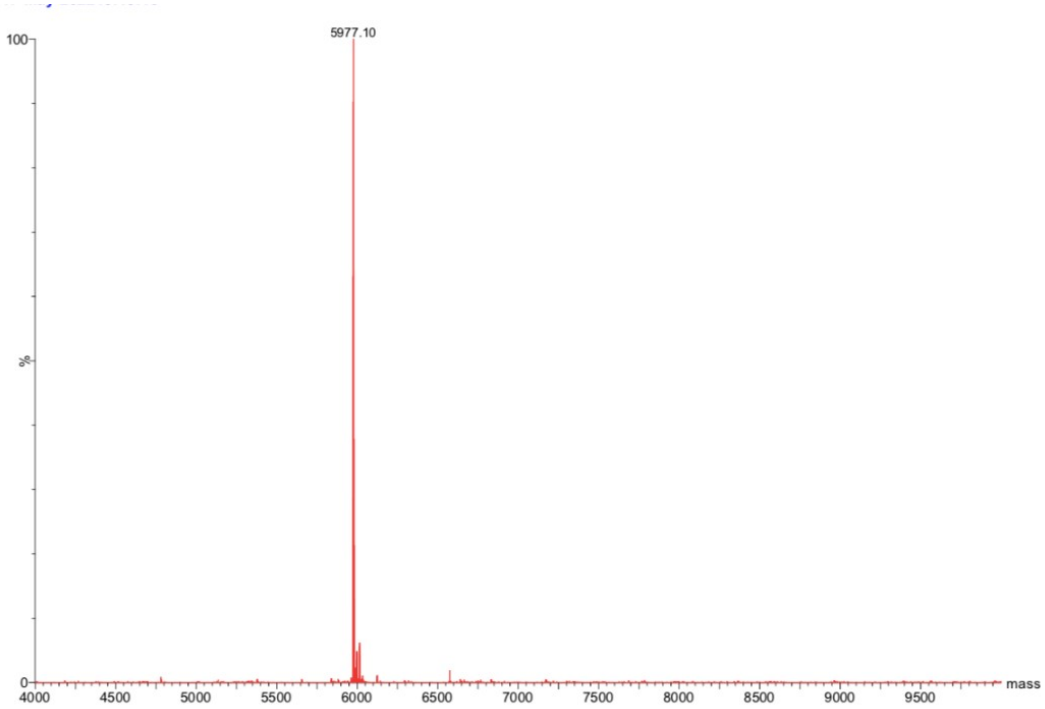


Figure S 15. Mass spectrum of r1-TO/A. Mass calculated: 5976; mass found: 5977.1.

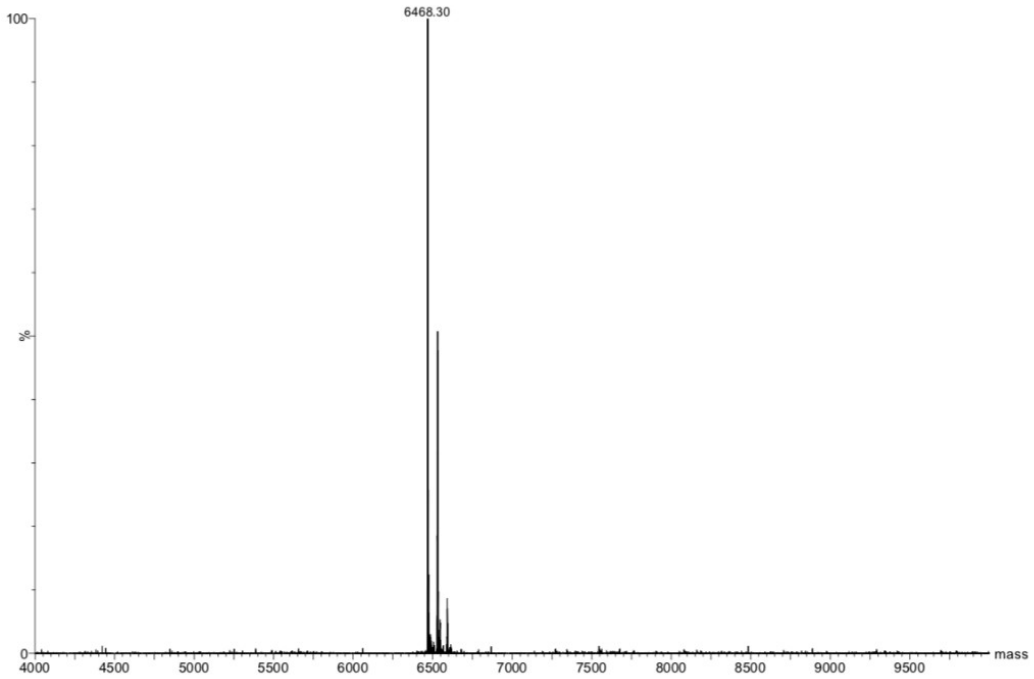


Figure S 16. Mass spectrum of r2-TO/A. Mass calculated: 6468; mass found: 6468.3, 6530.3(+62, Cu^{2+}).

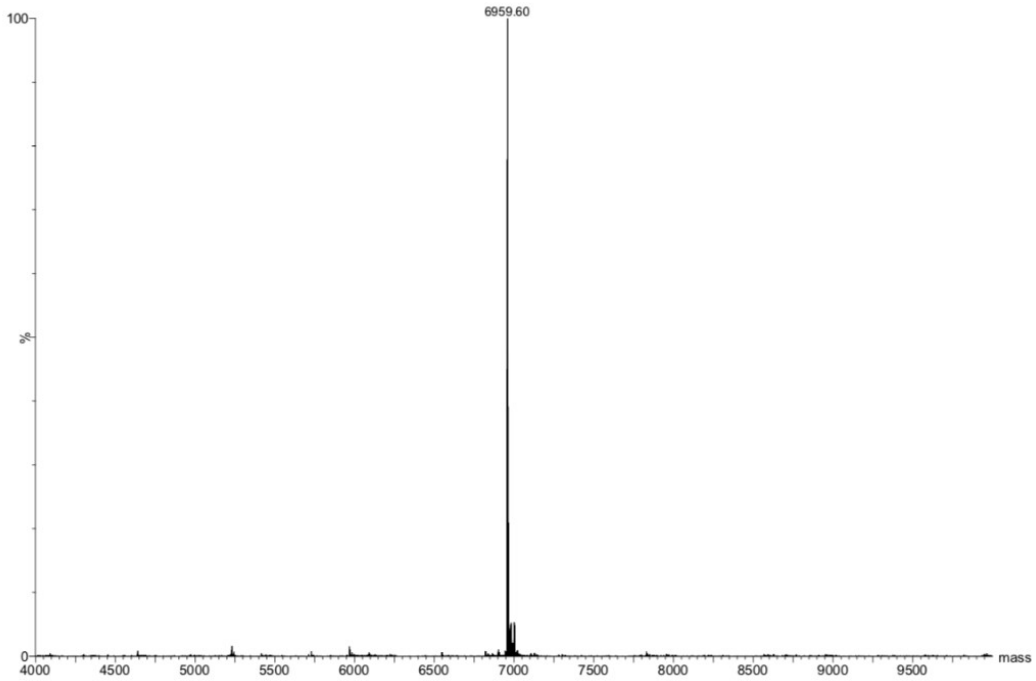


Figure S 17. Mass spectrum of r3-TO/A. Mass calculated: 6959; mass found: 6959.6.

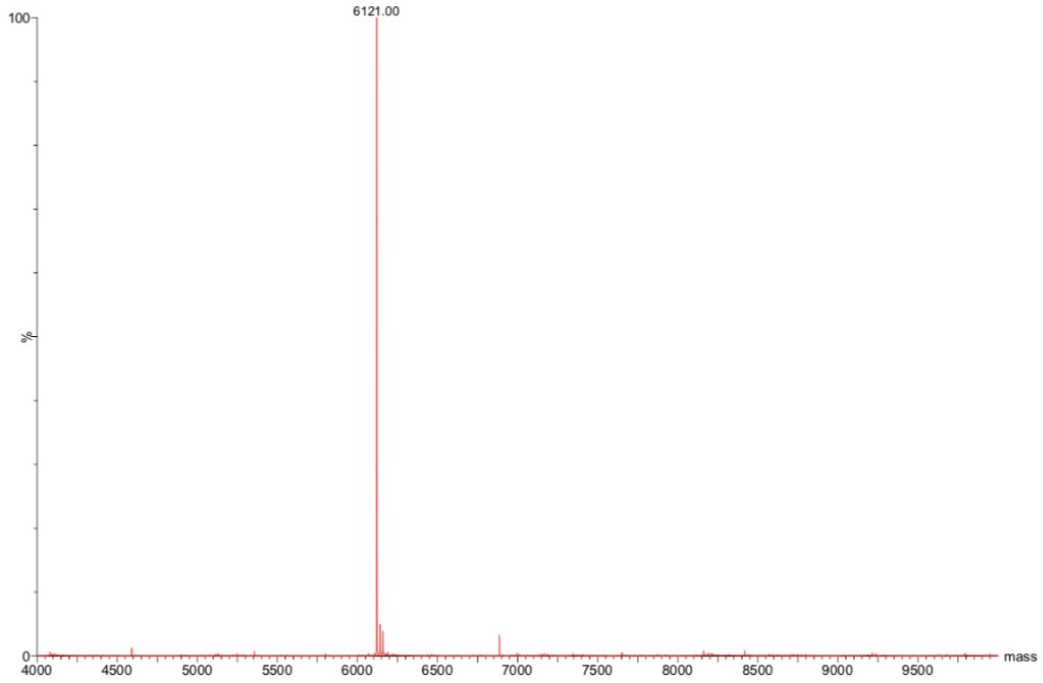


Figure S 18. Mass spectrum of r1-Y/RX. Mass calculated: 6119; mass found: 6121.

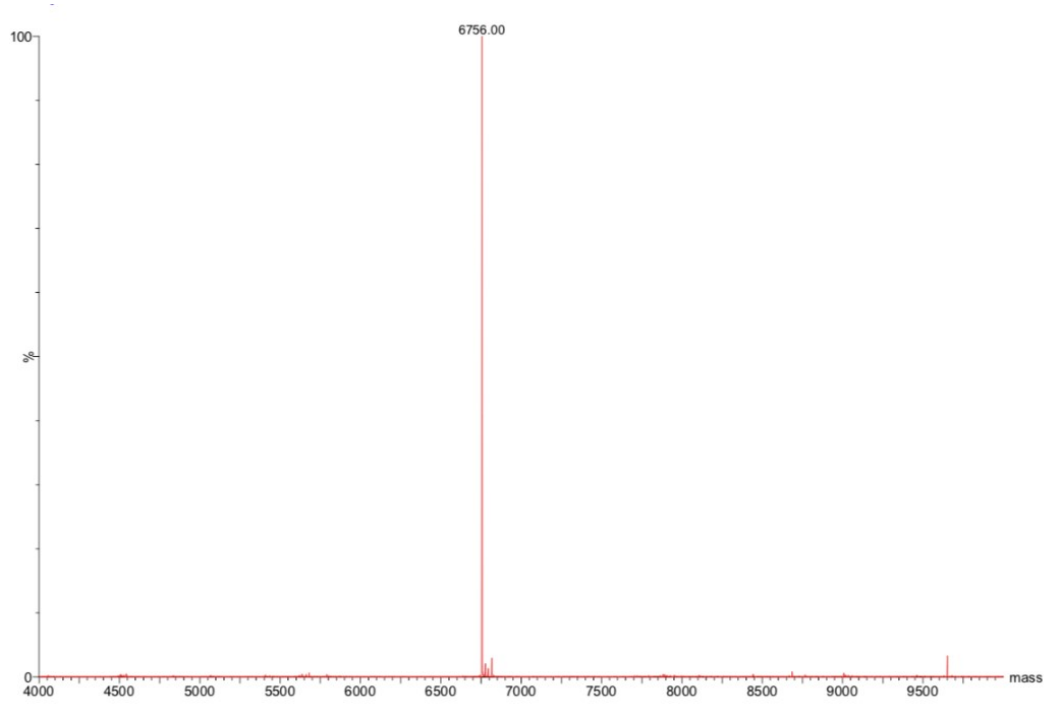


Figure S 19. Mass spectrum of r2-Y/RX. Mass calculated: 6754; mass found: 6756.

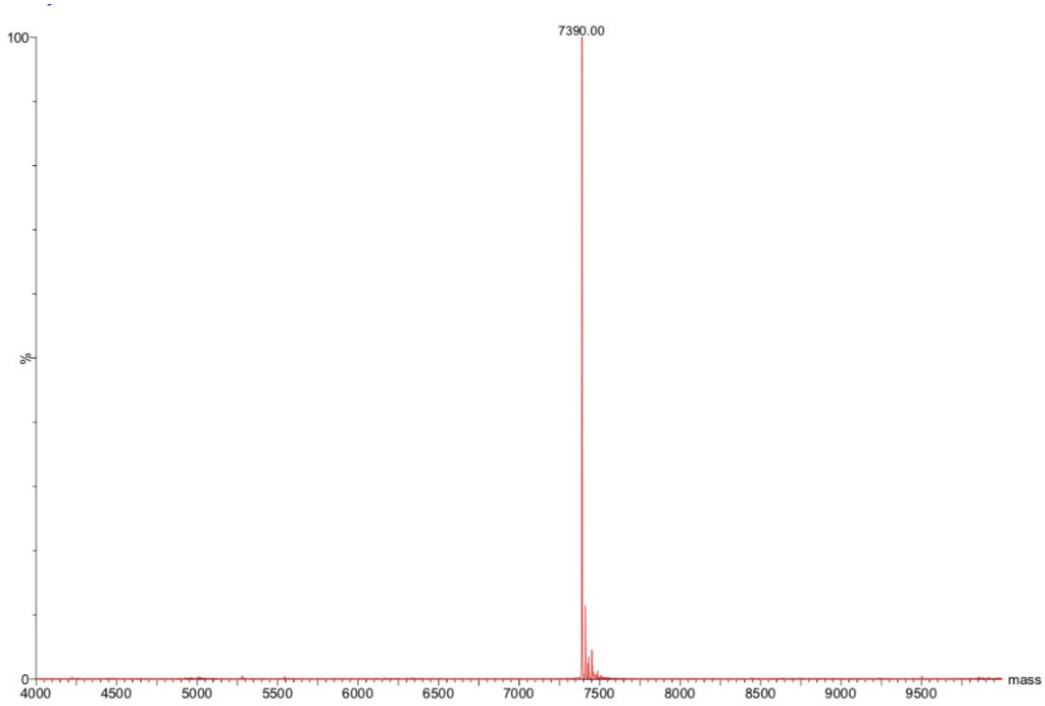


Figure S 20. Mass spectrum of r3-Y/RX. Mass calculated: 7388; mass found: 7390.

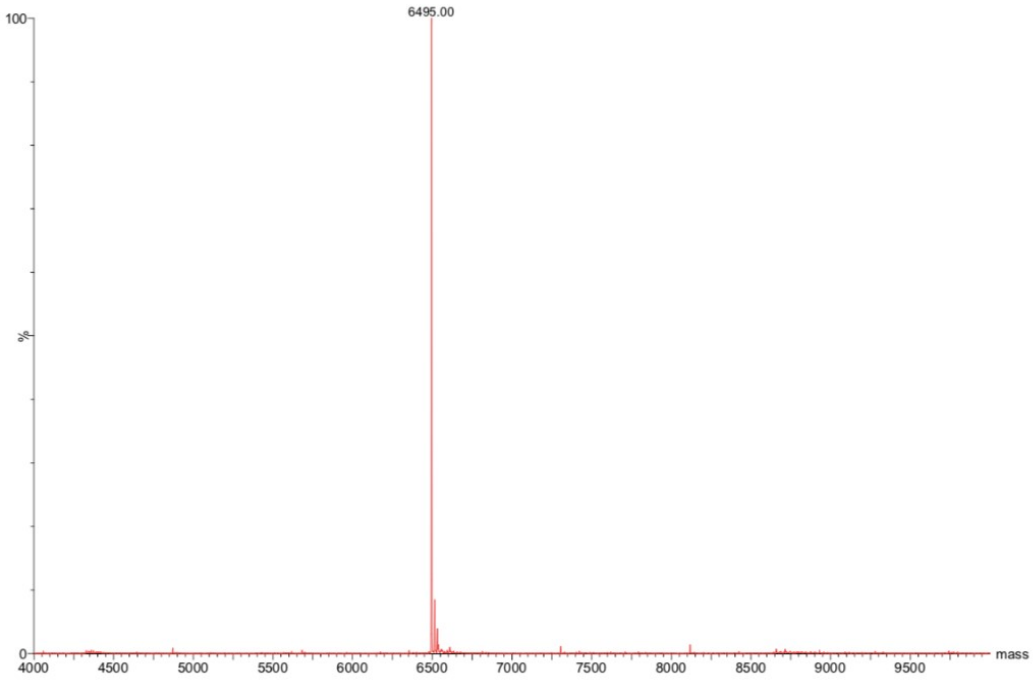


Figure S 21. Mass spectrum of r1-TO/RX. Mass calculated: 6493; mass found: 6495.

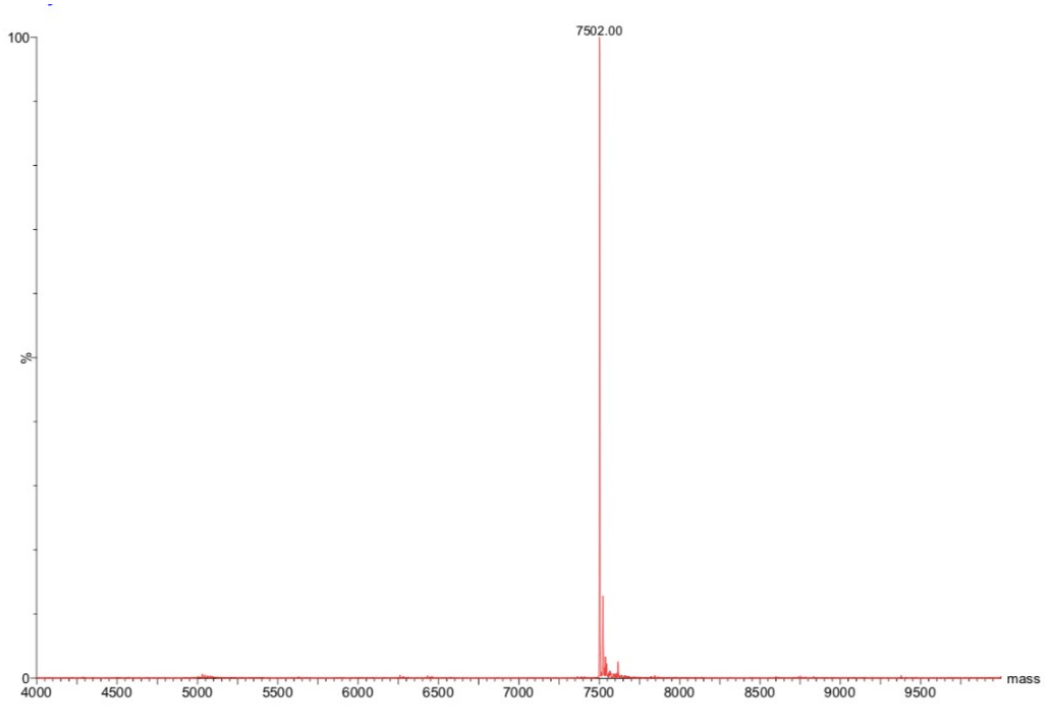


Figure S 22. Mass spectrum of r2-TO/RX. Mass calculated: 7502; mass found: 7502.

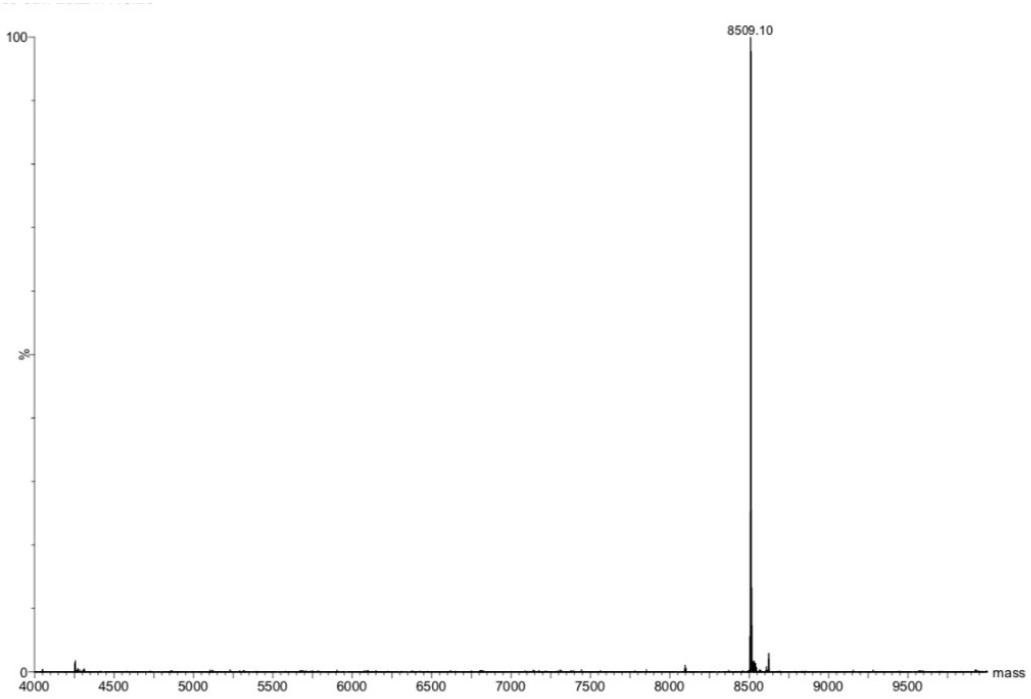


Figure S 23. Mass spectrum of r3-TO/RX. Mass calculated: 8510; mass found: 8509.1.

Figure S 24. Mass spectrum of d2(PCR)-TO/RX. Mass calculated: 9570; mass found: 9571.

5. NMR spectra

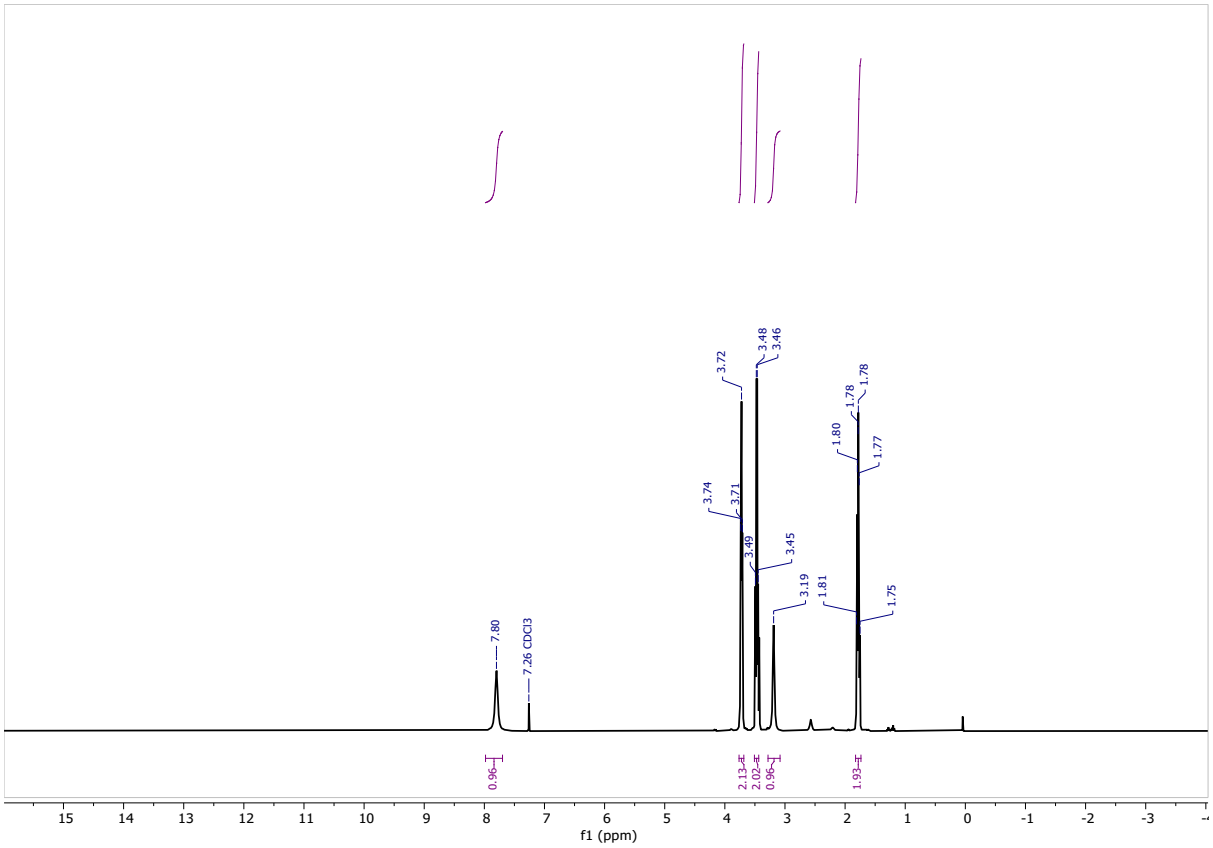


Figure S 25. ^{1}H NMR spectrum of compound 1 in $CDCl_3$ at 400 MHz.

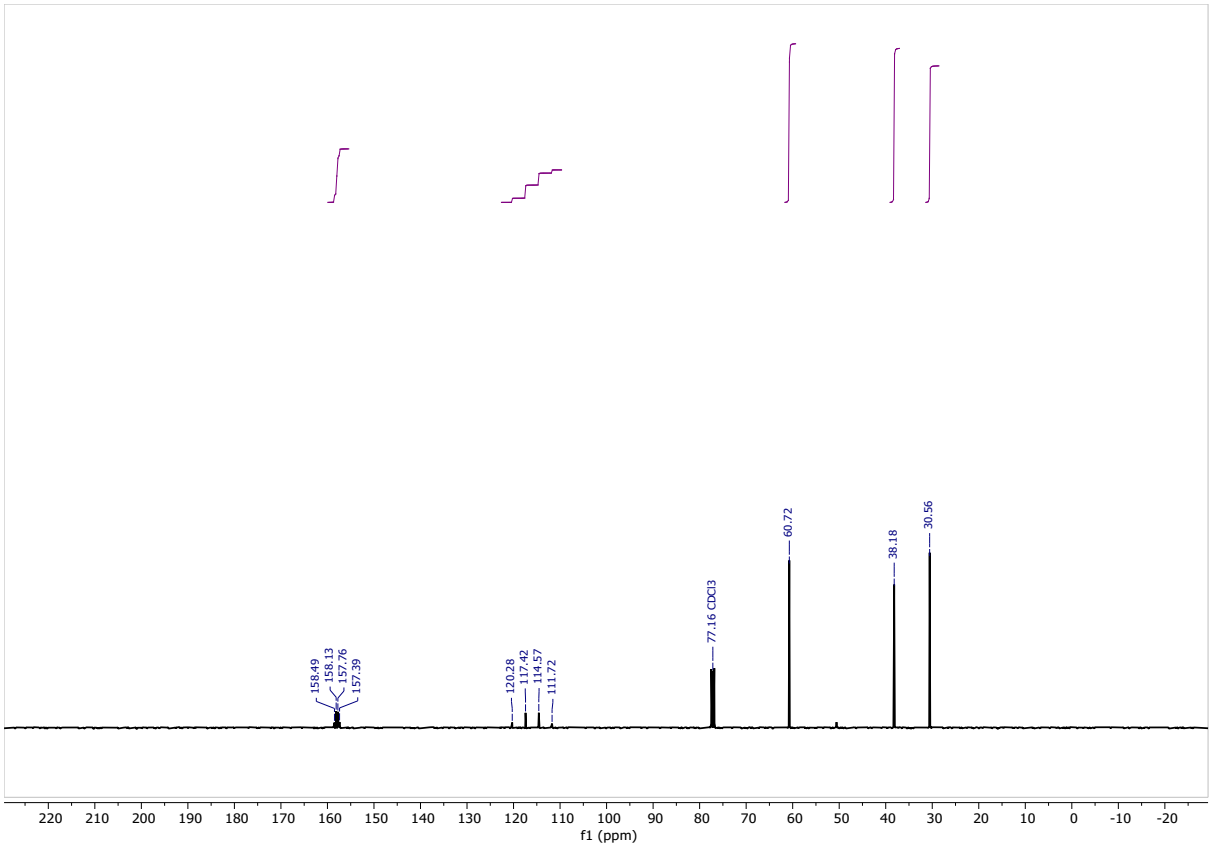


Figure S 26. ^{13}C NMR spectrum of compound 1 in $CDCl_3$ at 101 MHz.

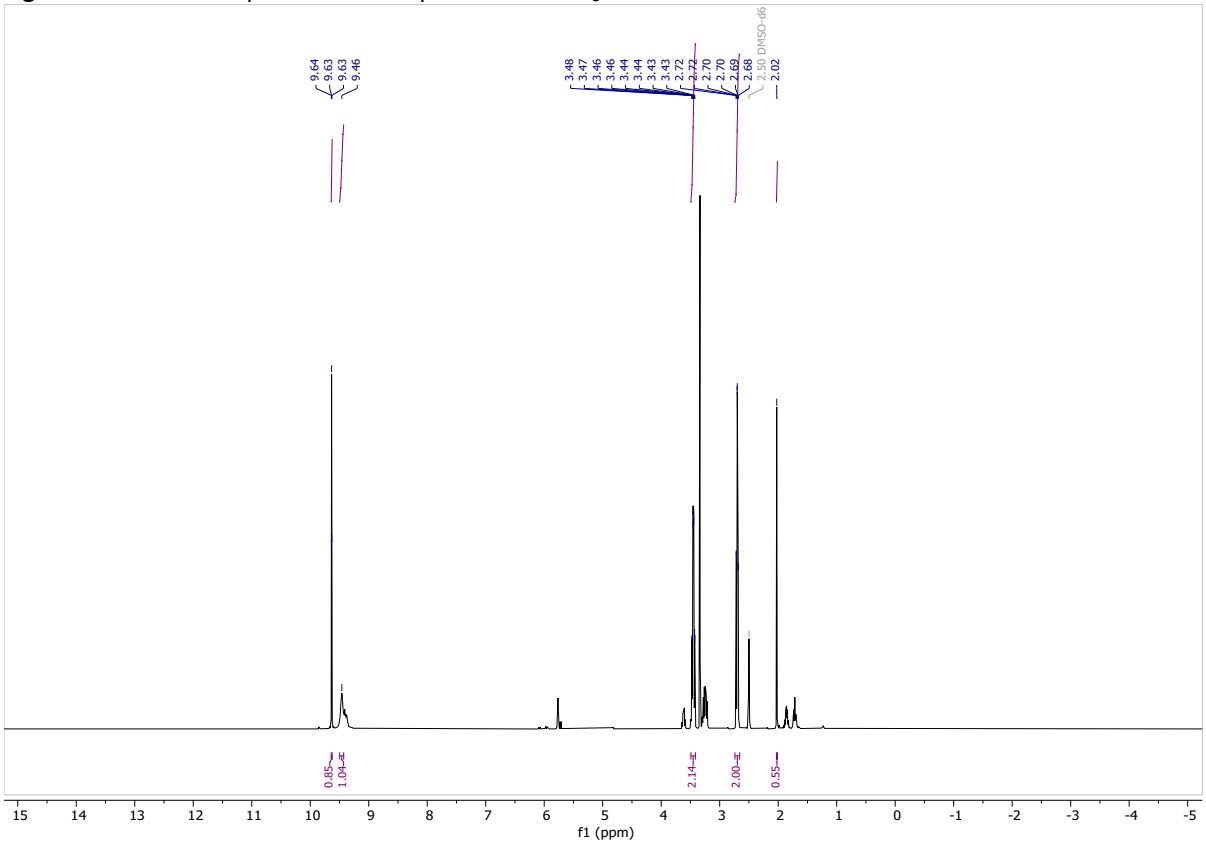


Figure S 27. ^{1}H NMR spectrum of compound 2 in $CDCl_3$ at 400 MHz.

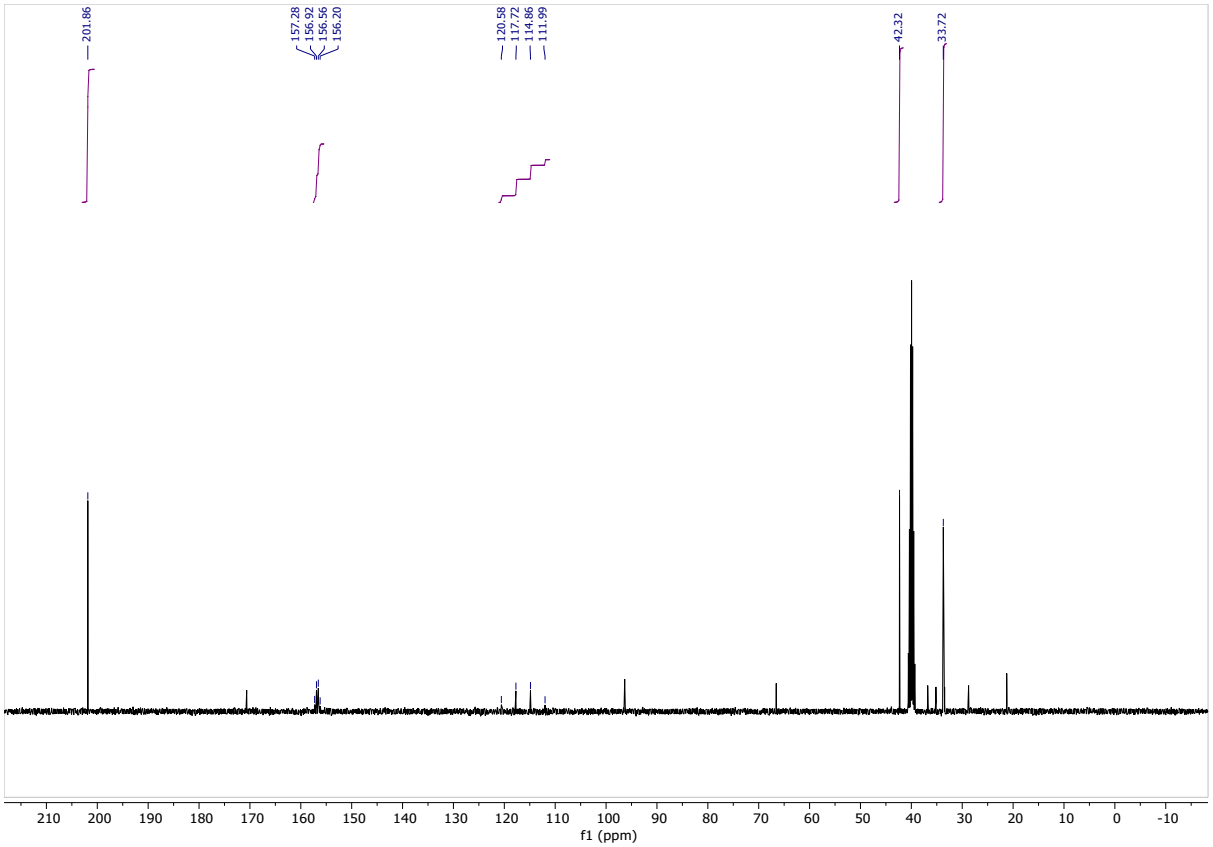


Figure S 28. ^{13}C NMR spectrum of compound 2 in $CDCl_3$ at 101 MHz.

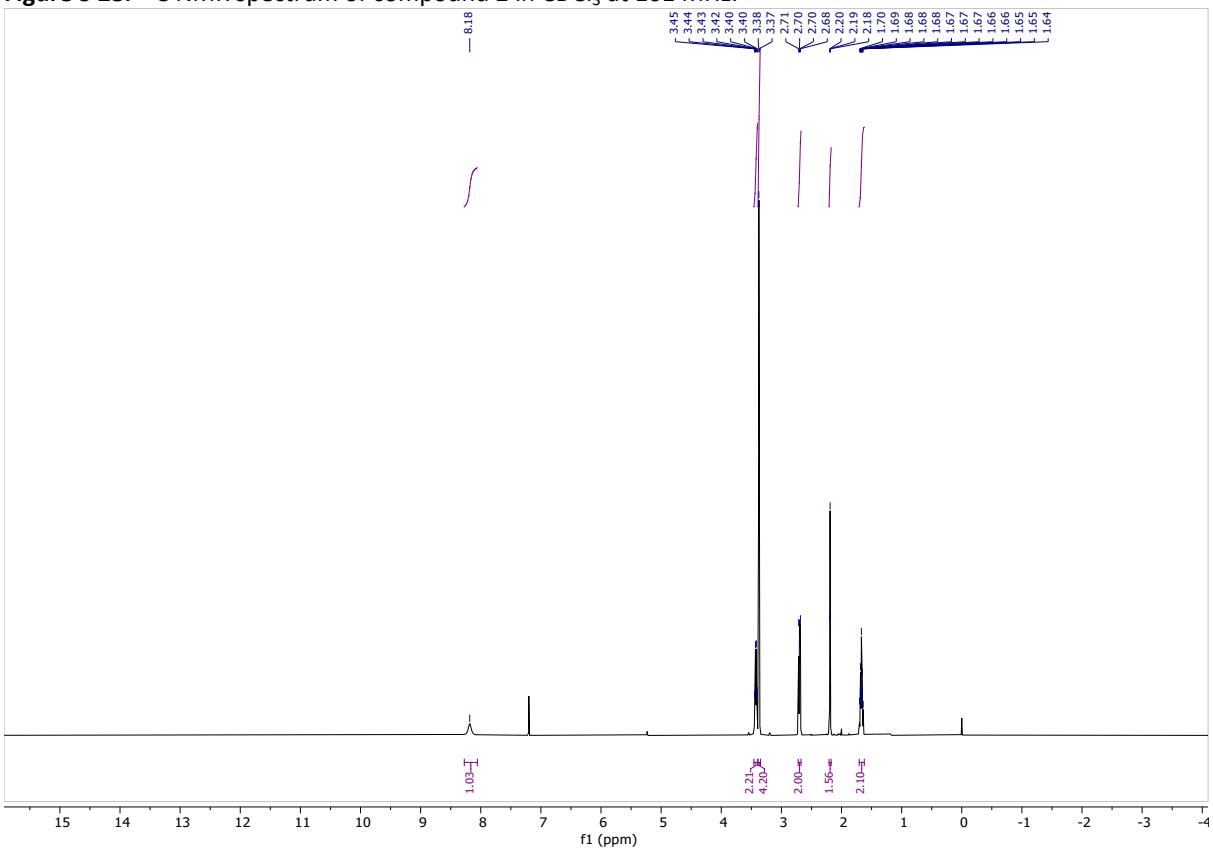


Figure S 29. ^{1}H NMR spectrum of compound 3 in $CDCl_3$ at 400 MHz.

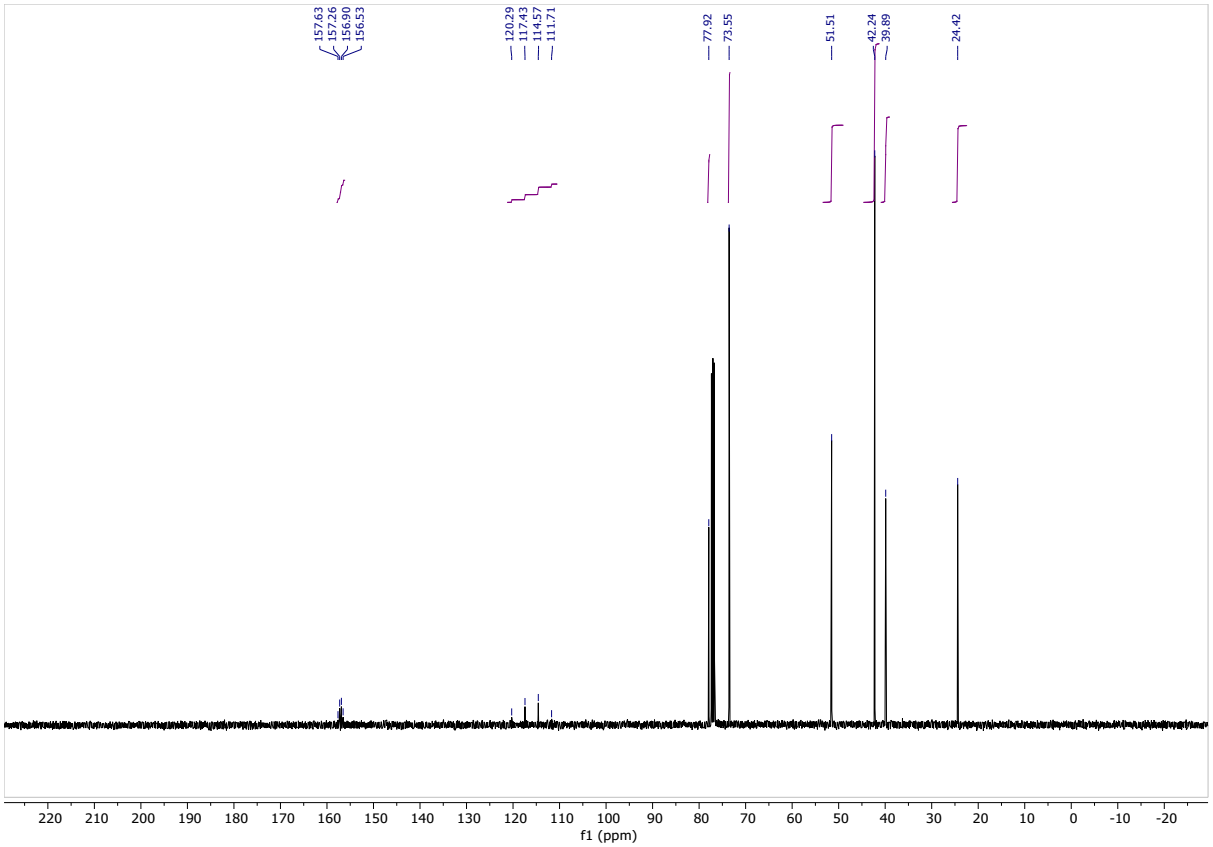


Figure S 30. ^{13}C NMR spectrum of compound 3 in $CDCl_3$ at 101 MHz.

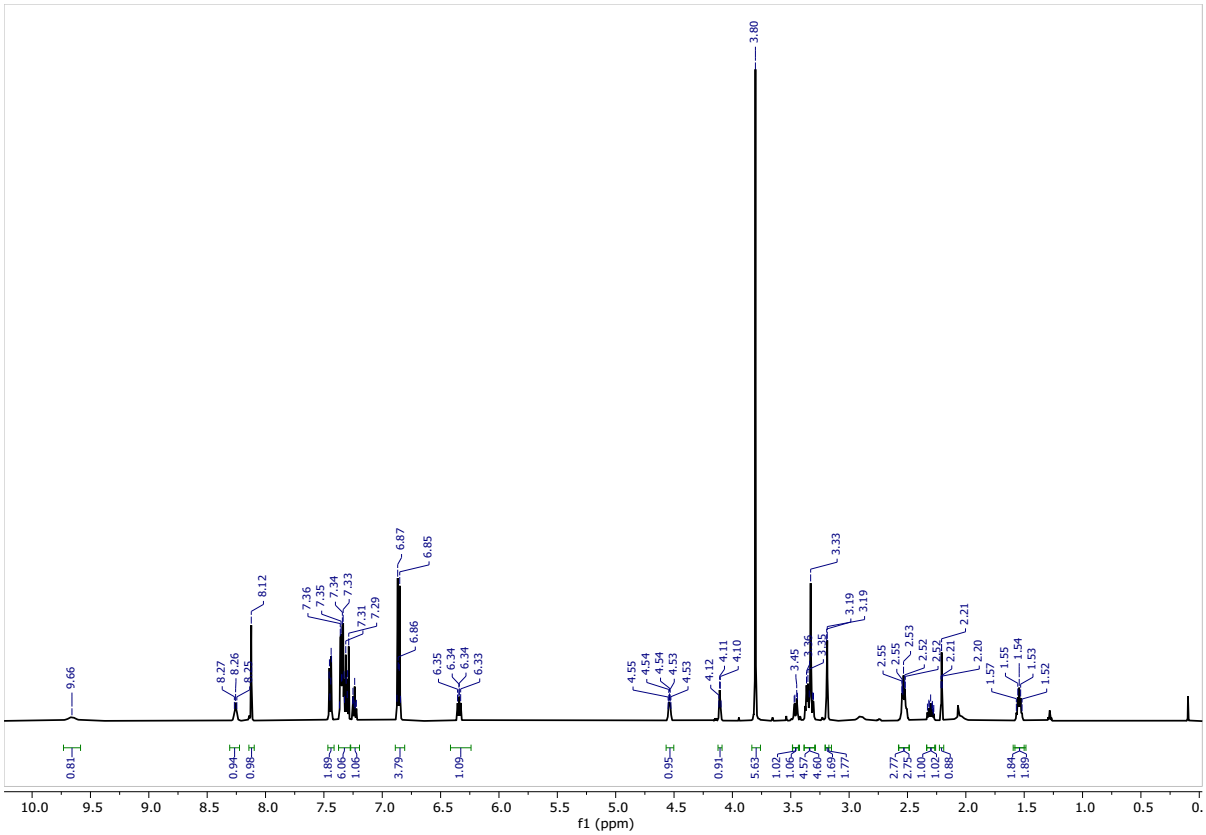


Figure S 31. ^{1}H NMR spectrum of compound 4 in $CDCl_3$ at 500 MHz.

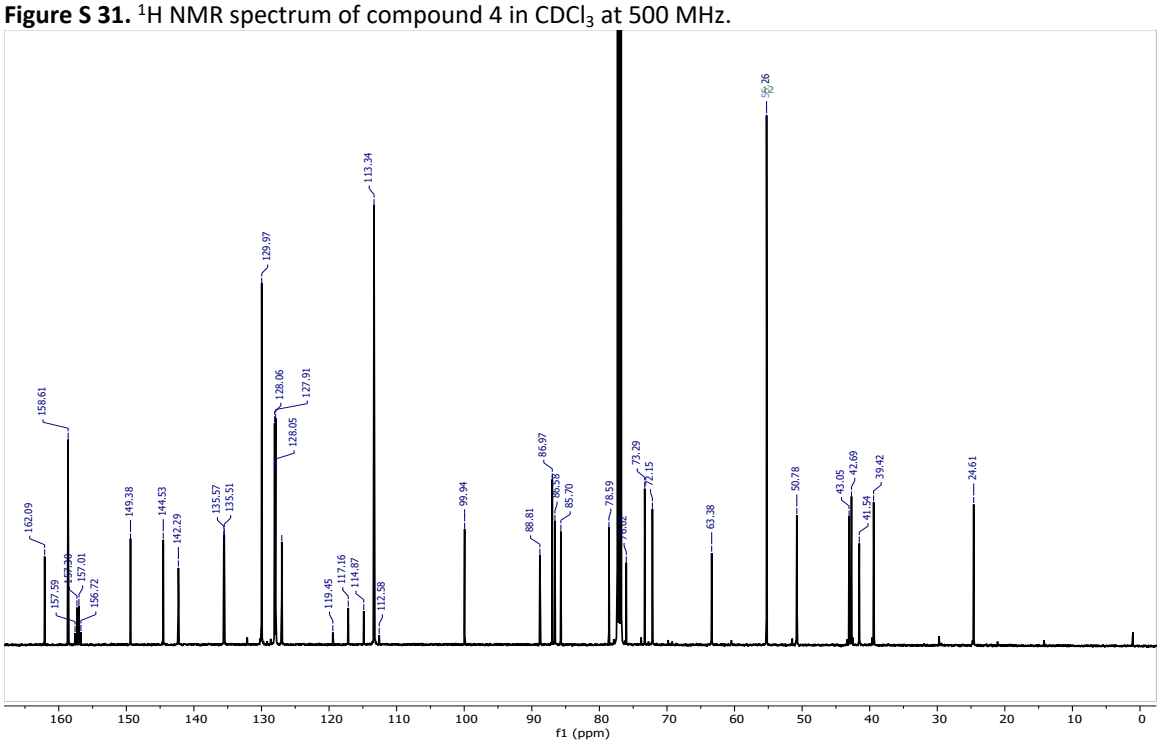


Figure S 32. ^{13}C NMR spectrum of compound 4 in $CDCl_3$ at 126 MHz.

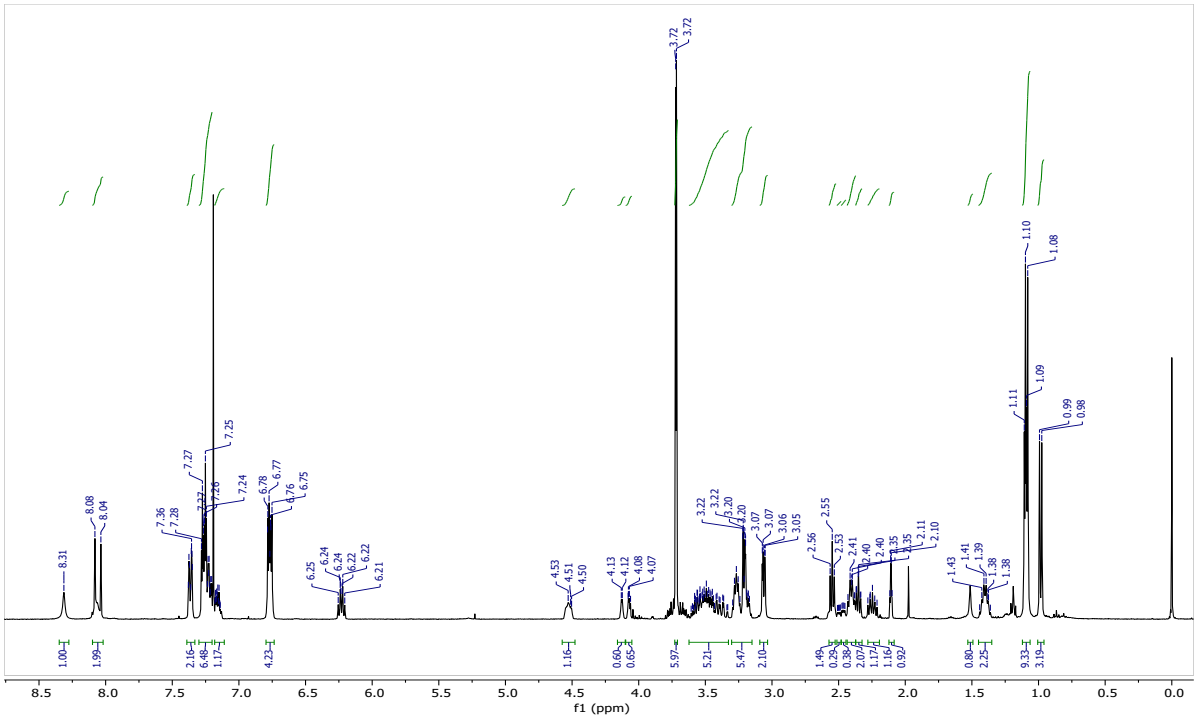


Figure S 33. ^{1}H NMR spectrum of compound 5 in $CDCl_3$ at 400 MHz.

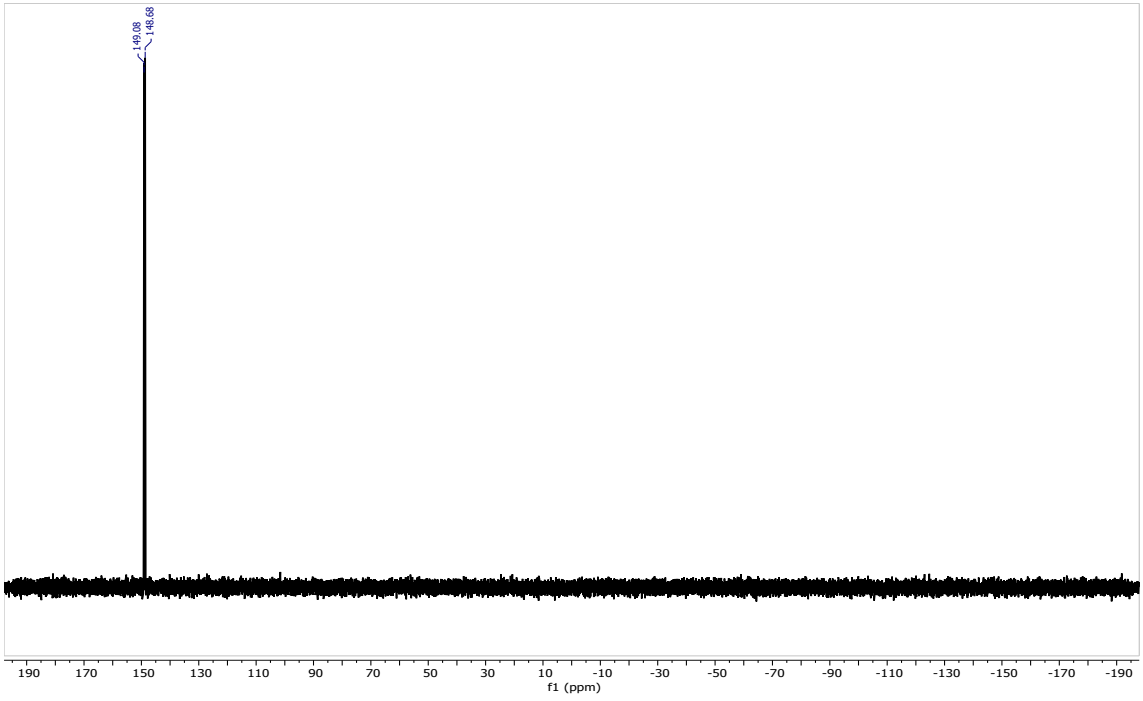


Figure S 34. ^{31}P NMR spectrum of compound 5 in $CDCl_3$ at 162 MHz.

6. References

1. Milelli, A.; Marchetti, C.; Greco, M. L.; Moraca, F.; Costa, G.; Turrini, E.; Catanzaro, E.; Betari, N.; Calcabrini, C.; Sissi, C.; Alcaro, S.; Fimognari, C.; Tumiatti, V.; Minarini, A., Naphthalene diimide-polyamine hybrids as antiproliferative agents: Focus on the architecture of the polyamine chains. *Eur. J. Med. Chem.* **2017,** *128*, 107-122.

2. Franzini, R. M.; Kool, E. T., Efficient Nucleic Acid Detection by Templated Reductive Quencher Release. *J. Am. Chem. Soc.* **2009,** *131* (44), 16021-16023.