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Electronic Supplementary Information

A multifunctional toolkit for target-directed cancer therapy

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General experimental methods

Common chemicals and solvents in addition to 2-cyanoethyl diisopropyl-phosphoramidochloridite were purchased from commercial sources and used without further purification. Anhydrous solvents and deuterated solvents ($CDCl_3$) were obtained from reputable sources and used as received.

Reagents for oligonucleotide synthesis including 2'-*O*-TBDMS-protected phosphoramidite monomers of A^{Bz}, C^{Ac}, G^{dmf} and U, the 5'-deblocking solution (3% TCA in CH_2Cl_2), activator solution (0.3 M 5-benzylthio-1-H-tetrazole in CH_3CN), CAP A solution (acetic anhydride/pyridine/THF), CAP B solution (THF/*N*-methylimidazole 84/16) and oxidizing solution (0.02 M iodine in tetrahydro-furan/pyridine/water (7:2:1)) where obtained from commercial sources.

For the preparation of modified linear siRNAS, 20 µM unmodified, 5'-FAM or 5'-Cy5-bearing sense strands (see Fig. S5 for details) were incubated in siRNA buffer (100 mM KOAc, 30 mM HEPES-KOH at pH 7.4, 2 mM $MgCl_2$) for 1 min at 90 ºC followed by 1 h at 37 ºC. 5'-FAM- and 5'-Cy5-labelled sense strands were purchased from Sigma Aldrich.

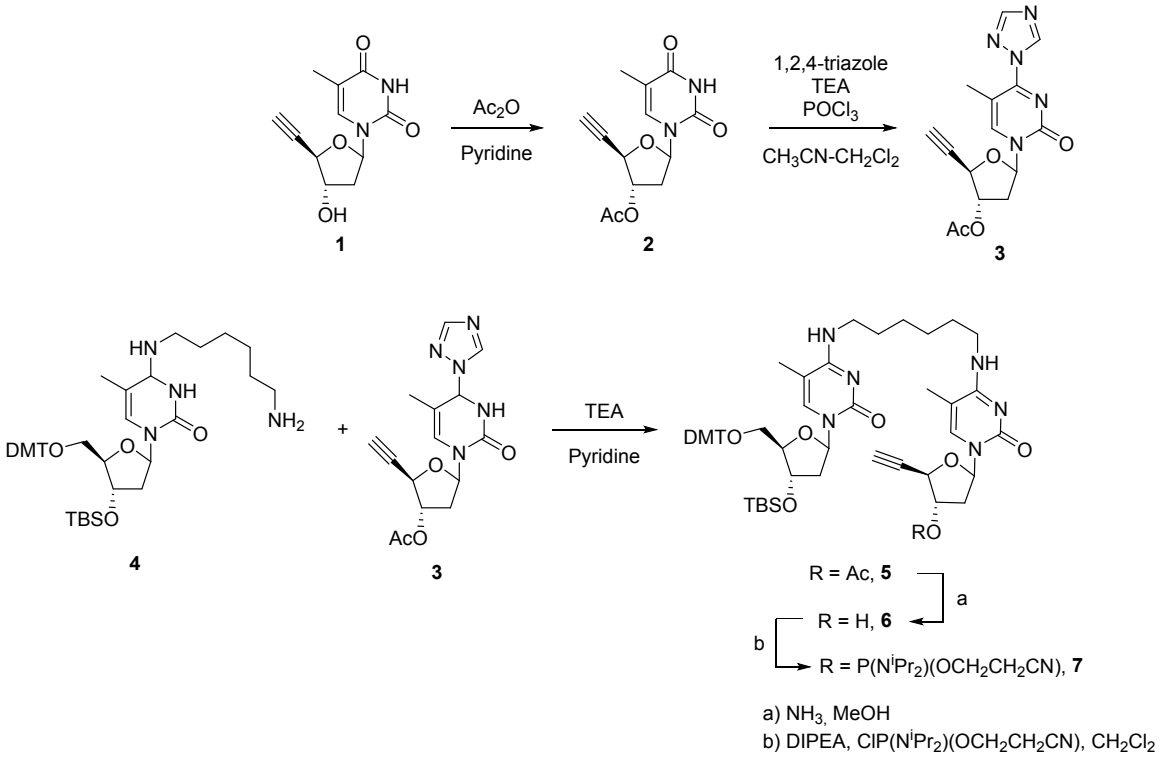
All reactions were carried out under argon atmosphere in oven-dried glassware. Thin-layer chromatography was carried out on aluminium-backed Silica-Gel 60 F_{254} plates. Column chromatography was performed using Silica Gel (60 Å , 230 x 400 mesh). NMR spectra were measured on a Varian Mercury-400 instrument. Chemical shifts are given in parts per million (ppm); *J* values are given in hertz (Hz). All spectra were internally referenced to the appropriate residual undeuterated solvent.

HRMS spectra were performed on a LC/MSD-TOF (Agilent Technologies) mass spectrometer.

MALDI-TOF spectra were performed using a Perspective Voyager DETMRP mass spectrometer, equipped with nitrogen laser at 337 nm using a 3ns pulse. The matrix used contained 2,4,6-trihydroxyacetophenone (THAP, 10 mg/mL in CH_3CN/water 1:1) and ammonium citrate (50 mg/mL in water).

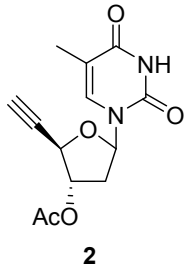
Oligonucleotide sequences that did not contain modified nucleotides were purchased from Sigma Aldrich.

Synthesis of ethynyl-bearing N-hexyl-N dimeric nucleoside



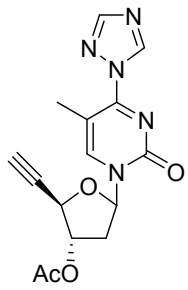
Alkyne **1** was synthesized as previously described.[1]

1-(3-*O*-Acetyl-2,5,6-trideoxy-β-D-erythro-hex-5-ynofuranosyl)-5-methyluracil (2)



DMAP (0.008 g, 0.06 mmol) and acetic anhydride (0.15 mL, 1.6 mmol) were added to a solution of alkyne **1** (0.150 g, 0.64 mmol) in anhydrous pyridine (4 mL). The reaction was stirred overnight at room temperature. The concentrated crude was purified by flash chromatography on silica gel (5% MeOH in CH_2Cl_2) to obtain a yellow foam (**2**, 178 mg, quantitative yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.93 (br, 1H), 7.54 (d, *J* = 1.2 Hz, 1H), 6.44 (dd, *J* = 8.6, 5.8 Hz, 1H), 5.38 (d, *J* = 4.7 Hz, 1H), 4.78 (m, 1H), 2.83 (d, *J* = 2.2 Hz, 1H), 2.62 (dd, *J* = 14.5, 5.8 Hz, 1H), 2.37 – 2.26 (m, 1H), 2.12 (s, 3H), 1.94 (d, *J* = 1.1 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.1, 164.1, 150.8, 135.1, 111.7, 86.5, 79.4, 78.3, 77.7, 74.1, 37.1, 20.9, 12.8. **HRMS** (ESI+) *m/z* calcd. for $C_{13}H_{15}N_2O_5$ $(M + H)^+$ 279.0975, found 279.0972.

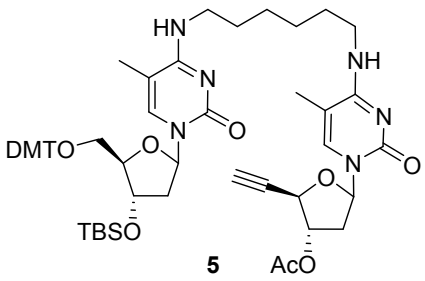
1-(3-*O*-Acetyl-2,5,6-trideoxy-β-D-erythro-hex-5-ynofuranosyl)-5-methyl-4-(*N*-1-triazolyl)uracil (3)



3

1,2,4-Triazole (0.476 g, 6.9 mmol) and triethylamine (1.37 mL, 9.9 mmol) were dissolved in anhydrous acetonitrile-CH_2Cl_2 (1:1, 6 mL) at 0 ºC for 5 min, followed by the addition of $POCl_3$ (0.1 mL, 1.08 mmol) at the same temperature. After stirring at 0 ºC for 30 min, a solution of alkyne **2** (0.120 g, 0.431 mmol) in acetonitrile-CH_2Cl_2 (2 mL) was cannulated to the mixture and the reaction mixture was stirred at room temperature for 1 hour. The reaction was monitored by TLC (5% MeOH in CH_2Cl_2). 5% $NaHCO_3$ in water was added to the mixture and extracted with CH_2Cl_2 three times. The organic phase was washed with brine, dried with $MgSO_4$, filtered and concentrated in vacuum to give **3** as a yellow foam in quantitative yield, which was used without further purification. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.29 (s, 1H), 8.28 (m, 1H), 8.12 (s, 1H), 6.36 (dd, *J* = 7.6, 6.0 Hz, 1H), 5.41 (d, *J* = 4.7 Hz, 1H), 4.95 – 4.93 (m, 1H), 3.09 (m, 1H), 2.80 (d, *J* = 2.2 Hz, 1H), 2.49 (d, *J* = 0.7 Hz, 3H), 2.33 (ddd, *J* = 14.8, 7.7, 4.9 Hz, 1H), 2.13 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.0, 158.4, 153.9, 153.6, 146.3, 145.1, 105.8, 89.6, 78.9, 78.3, 78.1, 75.1, 38.9, 20.9, 17.4. **HRMS** (ESI+) *m/z* calcd. for $C_{15}H_{16}N_5O_4$ $(M+H)^+$ 330.1197, found 330.1191.

1-{N^4-[3'-*O*-*tert*-Butyldimethylsilyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxy-5-methylcytidylyl]}-6-{N^4-[1-(3-*O*-Acetyl-2,5,6-trideoxy-β-D-erythro-hex-5-ynofuranosyl)]-5-methylcytosinyl}hexane (5)

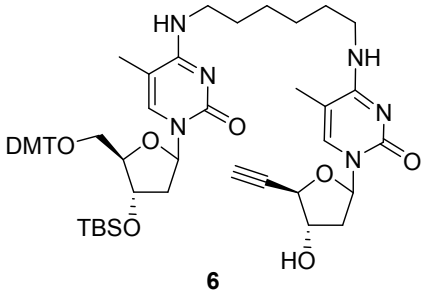


5

Aminonucleoside **4**[2] (0.303 g, 0.40 mmol) and triazolyl derivative **3** (0.120 g, 0.36 mmol) were dissolved in anhydrous pyridine (9.3 mL) at room temperature under argon. Triethylamine (0.43 mL, 3.1 mmol) was added to the mixture and the reaction was stirred overnight at room temperature. The solvent was removed to dryness and the crude was purified by flash chromatography on silica gel (from 1% MeOH in CH_2Cl_2 to 5% MeOH in CH_2Cl_2) to provide **5** as a yellow foam (300 mg, 81%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.64 (d, *J* = 0.9 Hz, 1H), 7.49 (d, *J* = 1.0 Hz, 1H), 7.44 – 7.40 (m, 2H), 7.33 – 7.19 (m, 7H), 6.82 (dd, *J* = 9.0, 1.2 Hz, 1H), 6.52 (dd, *J* = 8.5, 5.7 Hz, 1H), 6.36 (t, *J* = 6.1 Hz, 1H), 6.25 (NH, br, 1H), 5.93 (NH, br, 1H), 5.37 (d, *J* = 4.3 Hz, 1H), 4.78 (d, *J* = 2.1 Hz, 1H), 4.47 (dt, *J* = 6.3, 4.7 Hz, 1H), 3.93 (dt, *J* = 5.6, 2.9 Hz, 1H), 3.78 (s, 6H), 3.49 (dd, *J* = 10.5, 2.7 Hz, 1H), 3.45 – 3.35 (m, 4H), 3.24 (dd, *J* = 10.6, 3.1 Hz, 1H), 2.74 (s, *J* = 2.2 Hz, 1H), 2.75 – 2.69 (m, 1H), 2.42 – 2.35 (m, 1H), 2.24 – 2.13 (m, 2H), 2.11 (s, 3H), 2.10 (d, *J* = 0.7 Hz, 3H), 1.65 (s, 3H), 1.37 (m, 4H), 1.14 (m, 4H), 0.80 (s, 9H), -0.02 (s, 3H), -0.08 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.1, 163.5, 163. 5, 158.7, 157.1, 157.0, 144.6,

135.7, 135.6, 135.6, 135.3, 130.2, 130.1, 128.2, 128.0, 127.0, 113.3, 113.2, 105.5, 104.7, 87.4, 86.6, 86.2, 85.5, 79.9, 78.6, 77.3, 73.9, 71.7, 62.9, 55.3, 42.1, 42.0, 37.8, 28.6, 28.4, 27.0, 25.8, 21.0, 18.0, 14.0, 13.3, -4.6, -4.8. **HRMS** (ESI+) *m/z* calcd. for $C_{56}H_{73}N_6O_{10}Si$ $(M+H)^+$ 1017.5152, found 1017.5146.

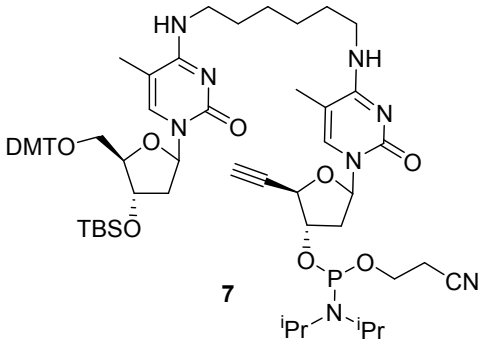
1-{*N*^{4}-[3'-*O*-*tert*-Butyldimethylsilyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxy-5-methylcytidylyl]}-6-{*N*^{4}-[1-(2,5,6-trideoxy-β-D-erythro-hex-5-ynofuranosyl)]-5-methylcytosinyl}hexane (6)



6

Ammonium hydroxide (0.37 mL, 28.0-30% in water) was added to a solution of **5** (0.070 g, 0.069 mmol) in methanol (2.15 mL) and the reaction was allowed to stir for 15 h at room temperature. Solvent was removed under reduced pressure to give **6** in quantitative yield as a white foam. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.67 (s, 1H), 7.53 (s, 1H), 7.42 (m, 2H), 7.33 – 7.15 (m, 7H), 6.82 (m, 2H), 6.49 (dd, *J* = 7.4, 5.9 Hz, 1H), 6.36 (t, *J* = 6.1 Hz, 1H), 5.80 (br, 1H), 5.57 (br, 1H), 4.71 (s, 1H), 4.57 (m, 1H), 4.47 (m, 1H), 3.93 (m, 1H), 3.79 (s, 6H), 3.46 (m, 5H), 3.23 (dd, *J* = 10.5, 3.0 Hz, 1H), 2.69 (d, *J* = 2.2 Hz, 1H), 2.67 (m, 1H), 2.44 – 2.37 (m, 1H), 2.19 (m, 2H), 2.03 (s, 3H), 1.48 (m, 4H), 1.26 (s, 3H), 1.26 (m, 4H), 0.80 (s, 9H), -0.01 (s, 3H), -0.08 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 163.5, 163.4, 158.7, 157.1, 156. 9, 144. 6, 136.3, 136.1, 135.7, 135.6, 130.2, 130.2, 129.1, 128.3, 128.2, 128.0, 127.1, 125.4, 113.3, 113.3, 104.3, 103.8, 87.8, 86.7, 86.2, 85.6, 81.3, 77.0, 76.7, 76.5, 71.6, 62.8, 55.3, 50.6, 42.1, 41.6, 41.6, 40.8, 29.8, 28.8, 28.7, 26.7, 25.8, 21.5, 18.0, 13.8, 13.1, -4.6, -4.8. **HRMS** (ESI+) *m/z* calcd. for $C_{54}H_{71}N_6O_9Si$ $(M+H)^+$ 975.5046, found 975.5035.

1-{*N*^{4}-[3'-*O*-*tert*-Butyldimethylsilyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxy-5-methylcytidylyl]}-6-{*N*^{4}-[1-(*O*-(β-cyanoethyl-*N*,*N*'-diisopropyl)phosphoramidite-2,5,6-trideoxy-β-D-erythro-hex-5-ynofuranosyl)]-5-methylcytosinyl]}hexane (7)



7

Diisopropylethylamine (DIPEA, 42 μL, 0.24 mmol) was added to a solution of **6** (0.067 g, 0.069 mmol) in anhydrous dichloromethane (3.3 mL) at 0 ºC under argon atmosphere. After 15 min, 2-cyanoethyl-diisopropylphosphoramidochloridite (37 μL, 0.17 mmol) was added and the mixture was stirred for 1 h at room temperature. Once the TLC (5% MeOH in CH_2Cl_2)

showed total conversion of the starting material, the mixture was poured into 5% $NaHCO_3$ (aqueous) and was extracted three times with CH_2Cl_2. The organic phase was dried over $MgSO_4$, filtered and concentrated. The crude phosphoramidite was used without further purification. **^{31}P NMR** ($CDCl_3$, 162 MHz) δ 149.1, 148.7.

RNA synthesis

All sequences were synthesized at the 1 μmol scale via solid phase synthesis using standard phosphoramidite methods.[3] For the synthesis of RNA strands containing BC6 loops and internal bulge, commercially available 5'-*O*-DMT-A^{Bz}-3'-succinyl-LCAA-CPG, 5'-*O*-DMT-C^{Ac}-3'-succinyl-LCAA-CPG, 5'-*O*-DMT-G^{dmf}-3'-succinyl-LCAA-CPG and 5'-*O*-DMT-U-3'-succinyl-LCAA-CPG were used as the solid supports.

Activated BC6 dimer was incorporated into the BC6-loop or the BC6-bulge internal position of a set of hairpins or single-stranded RNAs composing the central building blocks (**An**, **Bn** and $\mathbf{C_Tn}$, respectively) by using an automated DNA/RNA synthesizer and 2'-*O*-TBDMS-protected phosphoramidites of natural ribonucleotides. The coupling reaction was performed for 15 min leading to natural and modified phosphoramidites with a yield around 95%. Incorporation of the dimeric nucleoside modification did not have a negative effect in the yield. All oligonucleotides were synthesized in DMT-ON mode. After the solid-phase synthesis, the solid support was transferred to a screw-cap vial and incubated at 55 ºC for 2 h with 1.5 mL of NH_3 solution (33%) and 0.5 mL of ethanol. The vial was then cooled on ice and the supernatant was transferred into a 2 mL Eppendorf tube. The solid support and vial were rinsed with 50% ethanol (2 x 0.25 mL). The combined solutions were evaporated to dryness using an evaporating centrifuge. The residue that was obtained was dissolved in DMSO (115 μL). After addition of 60 μL of triethylamine and 75 μL of triethylamine trihydrofloride, the resulting solution was incubated at 65 ºC for 2.5 h. Then, the oligonucleotides were purified using Glen-Pack Cartridges (Glen Research) according to manufacturer's instructions. The oligonucleotides were then purified by 20% polyacrylamide gel electrophoresis (DMT-OFF). After purification, the RNAs were isolated by the crush and soak method, dialyzed, quantified by absorption at 260 nm and confirmed by MALDI mass spectrometry (see Table S1).

Construction of branched RNAs

For the construction of branched nanostructures (100 µL of a 20 µM solution), equimolar amounts of **An**, **Bn**, **C_Tn** and **C_Bn** subunits (2 nmol) were mixed together in an eppendorf tube. The resulting solution was evaported to dryness. Then, the pellet that was obtained was dissolved in 100 µL of siRNA buffer (100 mM KOAc, 30 mM HEPES-KOH at pH 7.4, 2 mM $MgCl_2$) and the resulting solution was heated for 1 min at 95 ºC followed by 1 h at 37 ºC. For annealing of linear siRNAs, 20 µM single strands were incubated in siRNA buffer (100 mM KOAc, 30 mM HEPES-KOH at pH 7.4, 2 mM $MgCl_2$) for 1 min at 90 ºC followed by 1 h at 37 ºC.

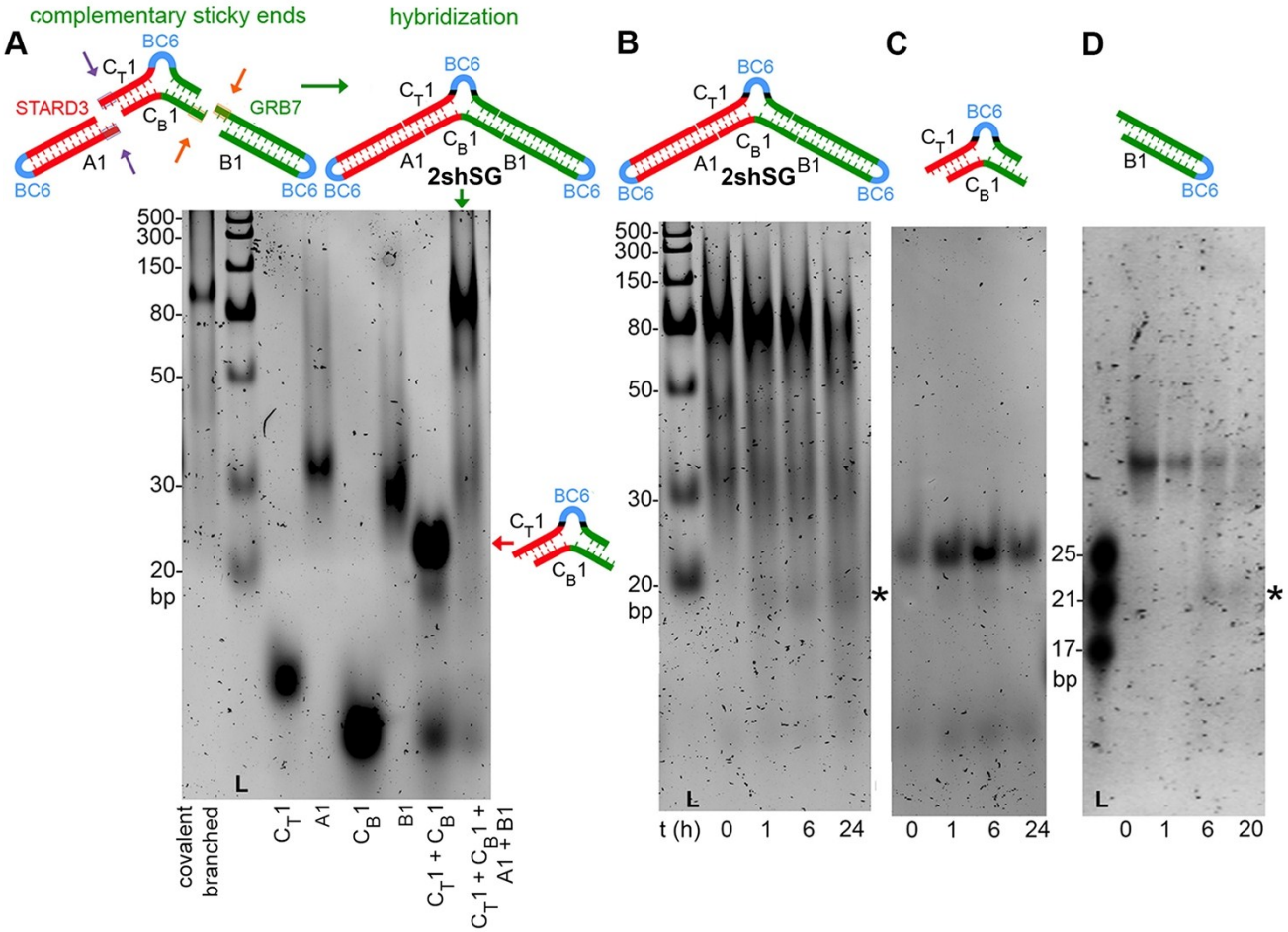


Figure S1. Construction of **2shSG** and recognition by Dicer. (A) Native PAGE analysis revealing the formation of **2shSG** by combination of **C_T1**, **A1**, **C_B1** and **B1** components. (B-D) Dicer cleavage of **2shSG** (B), central part **C_T1:C_B1** (C) and hairpin **B1** (D).

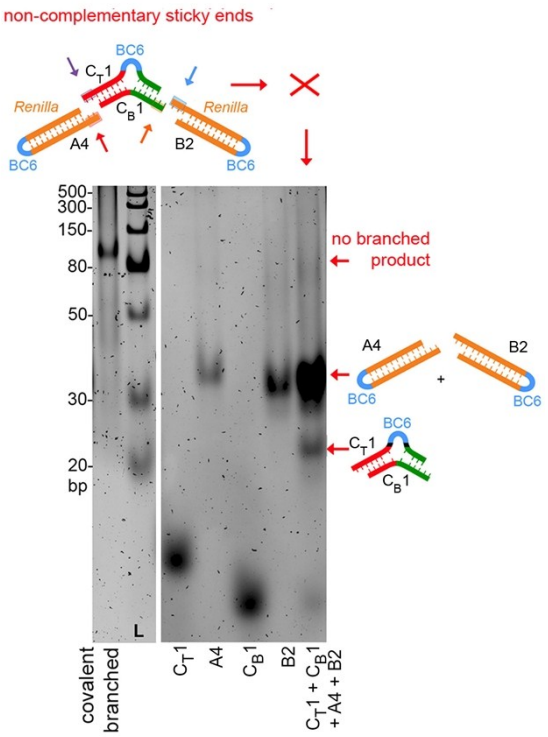


Figure S2. Native PAGE analysis confirming the specificity of our approach: combination of four RNA building blocks possessing non-complementary sticky ends [**A4** and **B2**, corresponding to the **2shRR** group and $\mathbf{C_T1}$ and $\mathbf{C_B1}$, corresponding to the **2shHG** group (Fig. 1D)] produced only the separated hairpin (**A4** and **B2**; of about 30 bp) and central part (double-stranded RNA formed by hybridization of $\mathbf{C_T1}$ and $\mathbf{C_B1}$; of about 20 bp) components.

Formation of the "covalent" version of branched RNA 2shSG

A solution of RNAs **A1** (1 µM, 200 pmol), **B1** (1 µM, 200 pmol), $\mathbf{C_T1}$ (1 µM, 200 pmol) and $\mathbf{C_B1}$ (1 µM, 200 pmol) in 500 mM Tris·HCl, 100 mM $MgCl_2$, 10 mM ATP, 100 mM DTT, pH 7.5 buffer (total volume 200 µL) was treated with T4 Polynucleotide Kinase (100 U) at 37 ºC for 30 minutes. Then, the enzyme was heat inactivated at 65 ºC for 20 min. After 5'-phosphorylated RNAs had been annealed, T4 RNA ligase 2 (30 U; New Englands BioLabs) was added and incubated at 37 ºC overnight. Ligation reaction was analyzed by 10% native PAGE (running conditions: TBM buffer, containing 89 mM Tris, 89 mM boric acid, 1 mM $MgCl_2$; 80 V, 4 ºC, 24 h). Bands were visualized by staining with SYBR Gold. The ligated covalent branched was purified by native PAGE (under identical conditions). In this case, bands were visualized by UV shadowing, and crushed and extracted with 0.1 M NaCl. The eluate was desalted by using Slide-A-Lyzer dyalisis columns (ThermoFisher Scientific). The ligated structure was confirmed by heating at 95 ºC followed by denaturing PAGE analysis.

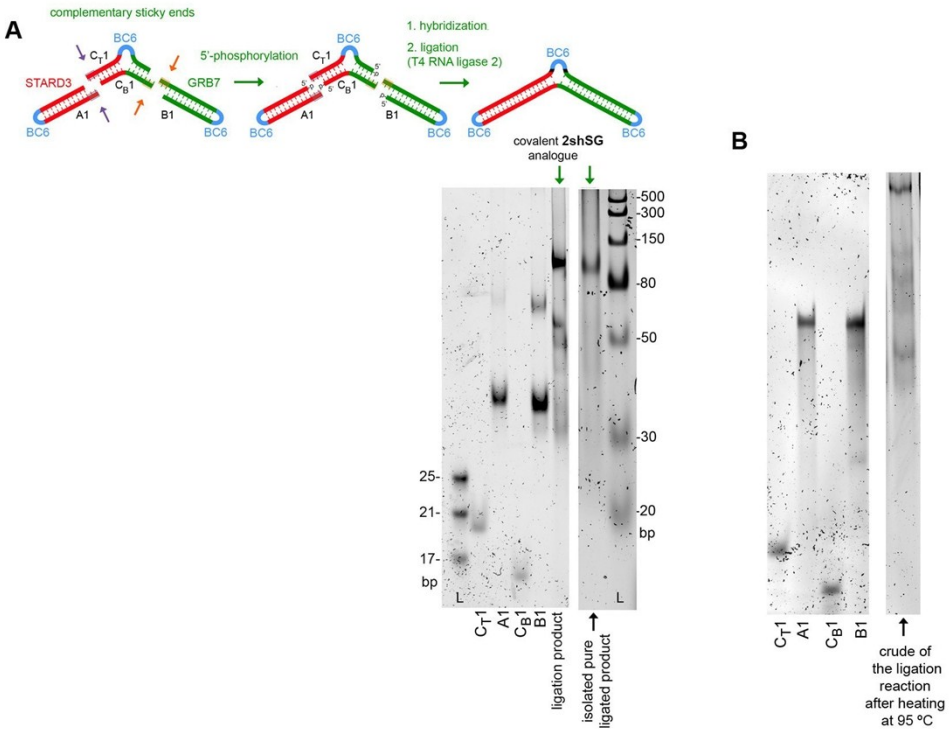


Figure S3. (A) Construction of the "covalent" version of branched **2shSG**, by 5'-phosphorylation of **A1**, **B1**, $\mathbf{C_T1}$ and $\mathbf{C_T1}$ components (which have complementary sticky ends), followed by annealing and treatment with RNA ligase 2. Analytic gel of each of the four components, as well as the crude of the ligation reaction and the pure isolated ligated product (conditions: 15% PAGE; TBM running buffer containing 89 mM Tris, 89 mM boric acid, 1 mM $MgCl_2$; loading buffer: 9M urea; 4 ºC, 80 V, 24 h). (B) 20% Denaturing PAGE (containing 8.3 M urea) of the crude of the ligation reaction after heating at 95

ºC for 5 min, as well as of the four independent **A1**, **B1**, **C_T1** and **C_T1** components, treated also at 95 ºC before loading them onto the gel.

Dicer cleavage reaction of RNAs

RNAs (0.91 μM) were mixed with Dicer enzyme (0.091 units/μL; Recombinant Human Turbo Dicer Enzyme Kit from Genlantis, USA) in the buffer system supplied. The mixtures were incubated at 37 °C and aliquots (2.2 μL) were taken from the mixture after 0, 1, 6 and 20 h. They were analyzed by 15% non-denaturing PAGE. The gels were visualized with SYBR Gold.

Synthesis of conjugates C_T1-AHNP, A2-Cy and B1-FAM

Stock solutions of $CuSO_4$ (150 mM) and sodium ascorbate (150 mM), dry peptide and fluorophores, alkynyl-modified RNA and 0.1 mM Tris·HCl buffer (pH 7.5) were flushed with argon for 15 min prior any further treatment. To an argon-flushed vial containing 0.3 μmol of azido-fluorescein/Cy5/Tat-AHNP, 120 μL of a freshly prepared $CuSO_4$/sodium ascorbate solution (prepared by addition of 15 μL of 150 mM $CuSO_4$ in 0.1 mM Tris·HCl pH 7.5/CH_3CN 8:2 and 15 μL of 150 mM sodium ascorbate in 0.1 mM Tris·HCl pH 7.5/CH_3CN 8:2 to a vial containing 90 μL of 0.1 mM Tris·HCl pH 7.5/CH_3CN 8:2) were added. The resulting pale-blue solution was immediately added to an argon-flushed vial containing 0.15 μmol of dry alkynyl-bearing RNA (**C_T1'**, **A2'** or **B1'**). The vial containing the corresponding azide (Tat-AHNP-azide, Cy5-azide or FAM-azide, respectively) was rinsed with 30 μL of 0.1 mM Tris·HCl pH 7.5/CH_3CN 8:2 and the resulting solution was added to the RNA-peptide mixture. The resulting yellow-coloured solution was throroughly shaken for 30 seconds and allowed to run at r.t. for 90 min. The reaction was subsequently diluted with Milli-Q water and then purified by 20% polyacrylamide gel electrophoresis (DMT-OFF). After purification, the RNA was isolated by the crush and soak method, dialyzed and quantified by absorption at 260 nm.

Analysis of the reaction of formation of C_T1-AHNP, B1-FAM and **A2-Cy and conjugates**

Figure S4B shows the PAGE analysis of ethynyl-bearing RNA **C_T1'** (lane 1), the purified Tat-AHNP-bearing product (**C_T1-FAM**) (lane 3) and a mixture of both (lane 2), visualized with SYBR Gold.

Figure S4D shows the PAGE analysis of ethynyl-bearing RNA **B1'** (lane 1), the purified FAM-labeled product (**B1-FAM**) (lane 3) and a mixture of both (lane 2), visualized in the absence of staining agent (SYBR Gold) with a Typhoon imager (at an excitation wavelength of 495 nm using a 510lP filter). Whereas the band corresponding to the alkynyl-RNA remains undetectable, an intense band is observed in the case of the product of the click reaction (lane 3), confirming efficient FAM-labeling. Staining of this gel with SYBR Gold (Figure S4E) allowed us to detect also the band corresponding to the non-labeled ethynyl-bearing RNA **B1'** (lane 1), of very similar mobility to that of the labeled product (**B1-FAM**; lane 3).

Figures S4G and S1H show the PAGE analysis of ethynyl-bearing RNA **A2'** (lane 1) and the purified Cy5-bearing product (**A2-Cy**) (lane 2), visualized in the absence (Fig. S4G) and in the presence (Fig. S4H) of SYBR Gold . The Cy5-labeled RNA **A2'-Cy** quenches SYBR fluorescence, resulting in low signal (Fig S4H, lane 2).

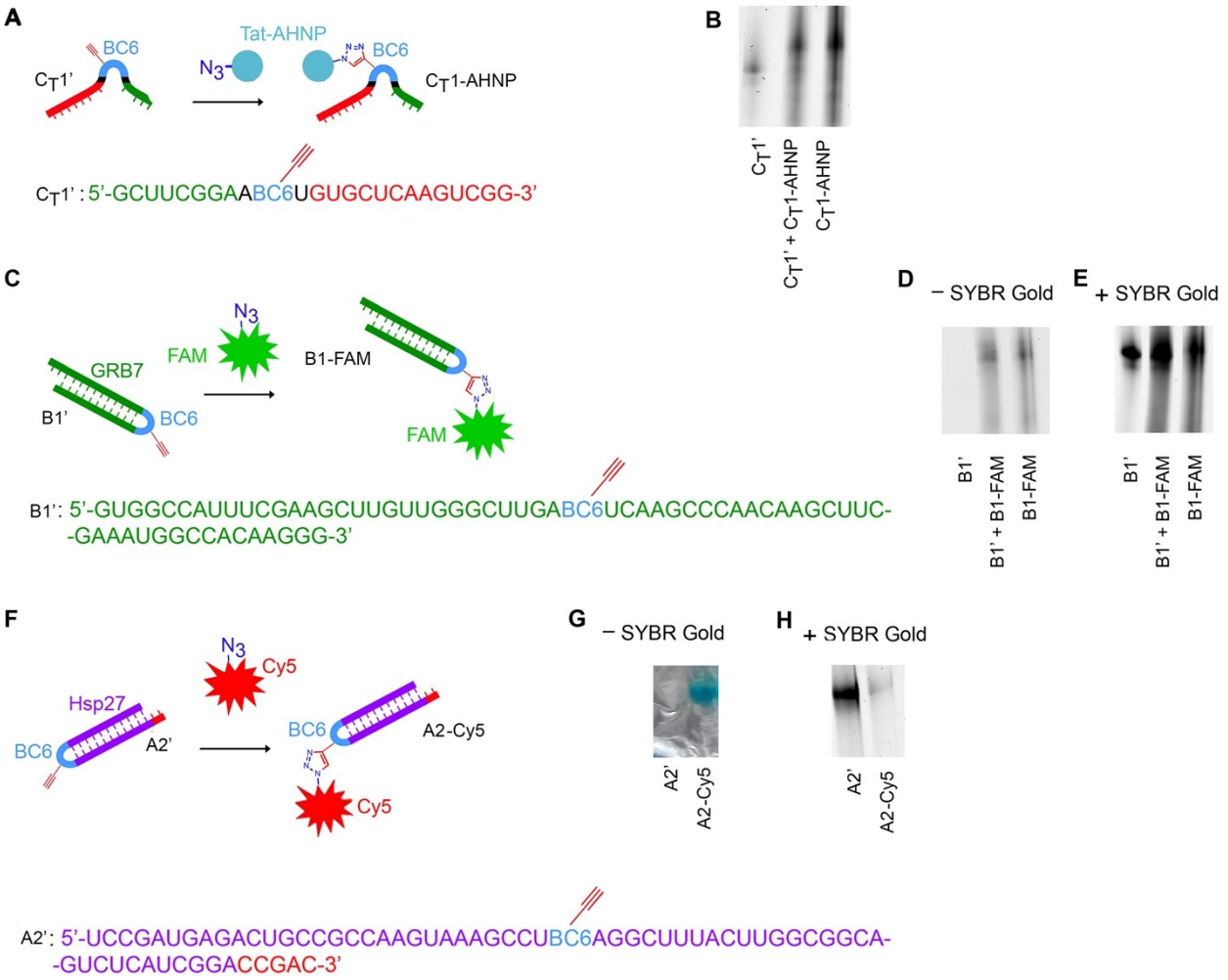


Figure S4. (A) Copper (I)-catalyzed lick reaction between **C_T1'** and azido-Tat-AHNP. (B) 20% Denaturing polyacrylamide gel of **C_T1'** and **C_T1-AHNP** visualized with SYBR Gold. (C) Copper (I)-catalyzed lick reaction between **B1'** and azido-FAM. (D,E) 20% Denaturing polyacrylamide gel of **B1'** and **B1-FAM** visualized in the presence (D) or in the absence (E) of SYBR Gold. (F) Copper (I)-catalyzed lick reaction between **A2'** and azido-Cy5. (G,H) 20% Denaturing polyacrylamide gel of **A2'** and **A2-Cy** visualized in the presence (H) or in the absence (G) of SYBR Gold.

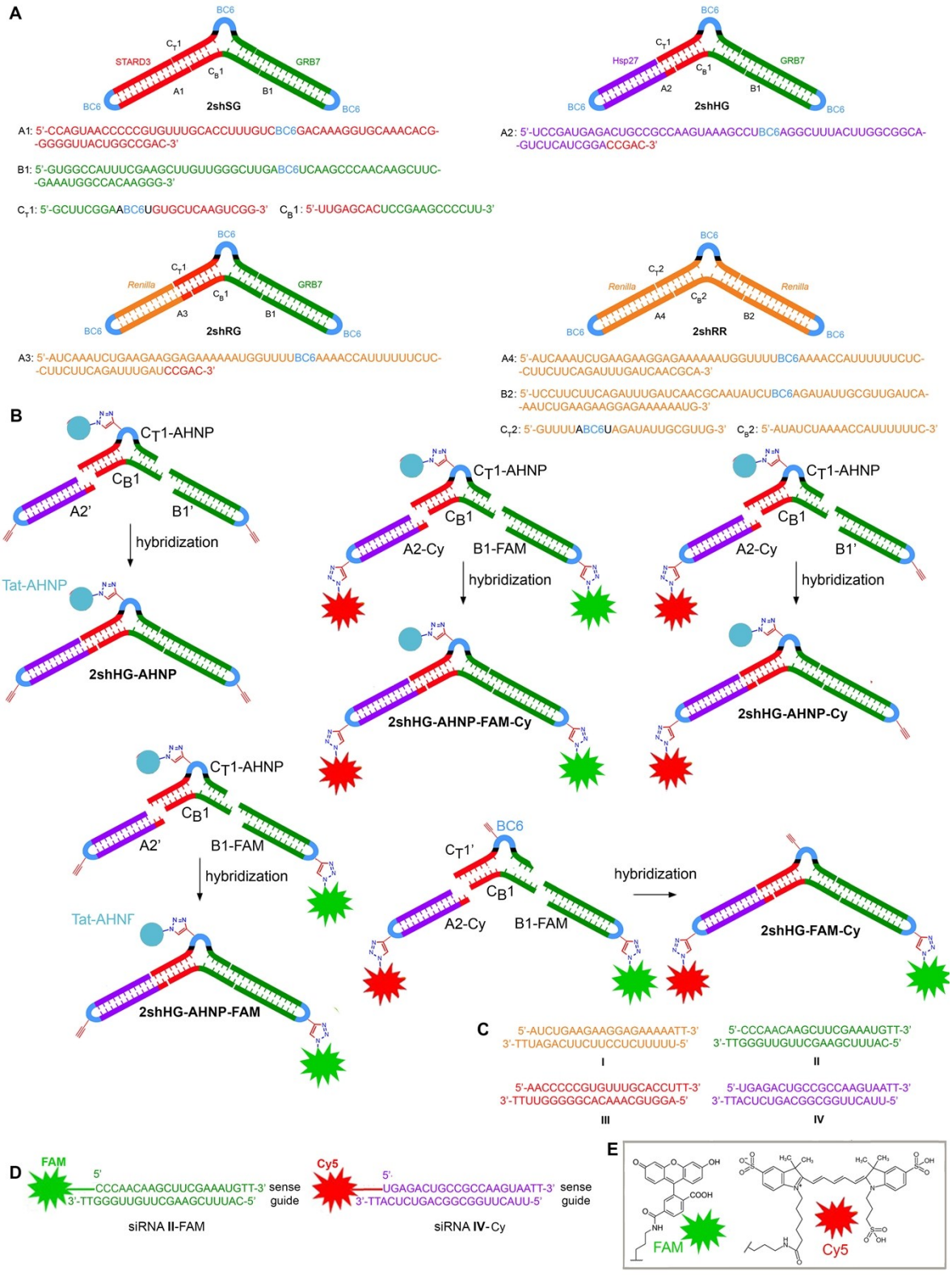


Figure S5. (A) "Naked" branched RNA structures and sequences used in this study. (B) Schematic representation of the formation of the 2shRNA conjugates used in this study. (C,D) Linear siRNA controls (**I**: *Renilla*; **II**: GRB7; **III**: STARD3; **IV**: Hsp27; C) and the corresponding siRNA analogues functionalized with FAM and Cy5; (E) Chemical structures of 6-carboxyfluorescein and Cy5 dyes.

Cell culture

The SKBR3, BT-4T4, UACC-732, HEK-293T and HeLa cell lines were obtained from the American Type Culture Collection. UACC-732 cells were grown on collagen-coated plates. All cell lines were mantained at 37 ºC in a humidified atmosphere with 5% CO_2. HeLa, BT-474, UACC-732 and HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F-12; GIBCO) supplemented with glutamine (2 mmol L^{-1}), fetal bovine serum (FBS, 10%), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). SKBR3 cells were cultured in McCoy's modified medium (GIBCO) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}).

Luciferase siRNA assays

HeLa cells were regularly passaged to mantain exponential growth. The cells were seeded one day prior to the experiment in a 24-well plate at a density of 150.000 cells/well in complete DMEM containing 10% FBS (500 μL per well). Following overnight culture, the cells were treated with luciferase plasmids and siRNAs. Two luciferase plasmids –*Renilla* luciferase (pRL-TK) and firefly luciferase (pGL3) from Promega– were used as a reporter and control. Cotransfection of plasmids and siRNAs was carried out with Lipofectamine 2000 (Life Technologies) as described by the manufacturer for adherent cell lines; pGL3-control (1.0 μg), pRL-TK (0.1 μg), and RNAs (siRNA duplex or branched RNA; 20 nM dose) formulated into liposomes were added to each well with a final volume of 600 μL. After a 5-h incubation period, cells were rinsed once with PBS and fed with 600 μL of fresh medium (DMEM) containing 10% FBS. After a total incubation period time of 22 h, the cells were harvested and lysed with passive lysis buffer (100 μL per well) according to the instructions of the Dual-Luciferase Reporter Assay System (Promega). The luciferase activities of the samples were measured with a MicroLuma*Plus* LB 96V (Berthold Technologies) with a delay time of 2 s and an integration time of 10 s. The following volumes were used: 20 μL of sample and 30 μL of each reagent (Luciferase Assay Reagent II and Stop and Glo Reagent). The inhibitory effects generated by siRNAs or branched RNAs were expressed as normalized ratios between the activities of the reporter (*Renilla* or Firefly) luciferase gene and the control (Firefly or *Renilla*, respectively) luciferase gene.

Analysis of GRB7, STARD3 and Hsp27 protein knockdown by Western blot

In the presence of Lipofectamine 2000

SKBR3 cells were seeded 24 h before transfection in 60 mm dishes at a density of 800.000 cells/dish in medium containing 10% FBS. Following overnight culture, a mixture of two siRNA duplexes (40 nM each) or a branched RNAs (40 nM per dish) formulated into liposomes were added to each dish with a final volume of 2 mL. Cotransfection of RNAs was carried out using Lipofectamine 2000. After a 5-h incubation period, the transfection medium was changed to complete medium containing 10% FBS. After a 48-h incubation time, the cells were harvested with PBS and lysed by incubation in RIPA buffer containing protease inhibitors (Roche) at 4 ºC for 1 h. Cell debris were removed by centrifugation at 8000 x g for 20 min at 4 ºC, and protein concentration was determined using the BCA assay (Pierce). 30 μg of protein were resolved by SDS electrophoresis and transferred to a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore). The membrane was blocked with 5% skim milk in TBS containing 0.1% Tween for 1 h at r.t. and subsequently probed with anti-GRB7 monoclonal rabbit antibody (Santa Cruz Biotechnology) (diluted 1:500 in blocking buffer), anti-STARD3 monoclonal rabbit antibody (diluted in 1:3500 in blocking buffer) or Hsp27 monoclonal rabbit antibody (diluted 1:500 in blocking buffer) overnight at 4 ºC. Anti-rabbit (goat) IgG HRP conjugated secondary antibody (Thermo Scientific, Rockford, IL) was incubated at 1:5000 dilution in the blocking solution for 1 h at r.t. β-Actin was selected as internal control and was detected by incubation with anti-β-actin HRP conjugated antibody (Abcam) (at a dilution of 1:20.000 in blocking buffer) for 1 h at r.t. The intensities of the bands were analyzed using ImageJ 1.45 software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011).

In the absence of Lipofectamine 2000

SK-BR-3 cells were seeded in 48-well plates at a density of 75.000 cells/well. Following overnight culture, branched RNA (100 nM per dish) was added to each well (in the absence of Lipofectamine 2000) with a final volume of 190 μL. After a 5-day incubation time, the cells lysates were analyzed by Western blot following the same procedure used for cells treated with RNAs in the presence of Lipofectamine 2000.

Cell viability assay

In the presence of Lipofectamine 2000

Interference with *in vitro* growth rate of SKBR3, BT-474 and UACC-732 cells by natural siRNAs and branched RNAs in the presence or in the absence of Lapatinib was measured using crystal violet. 150.000 SKBR3, BT-474 and UACC-732 cells were plated in 24-well plates. Twenty-four hours after plating (0 hrs) cells were transfected with the corresponding RNAs [in the case of branched RNAs, a 40 nM dose was used; in the case of mixtures of siRNAs or shRNAs, a 1:1 molar mixture was used (40 nM of each of the siRNAs/shRNAs composing the mixture)] using Lipofectamine 2000 (final volume: 500 μL). 20 hours after transfection, 1.2 μL Lapatinib stock solution in DMSO (430 nM, 43 nM or 4.3 mM in the case of SK-BR-3, BT-474 and UACC-732 cells, respectively) or 1.2 μL of DMSO (vehicle) were added. 72 hours later, cells were fixed with 4% formalin for 10 minutes, then washed twice with distilled water and stained with 0.1% freshly prepared crystal violet for 30 minutes. After washing, the stain was dissolved with 10% acetic acid and subsequently quantified by absorbance at 570 nM.

Viability of HEK-293 cells after treatment with branched RNAs and siRNAs was performed using the same protocol, with the exception of the step involving treatment with DMSO or Lapatinib. Cell viability was assessed by crystal violet assay 72 hours transfection with RNAs.

In the absence of Lipofectamine 2000

Interference with *in vitro* growth rate of SKBR3 by branched 2shRNA conjugates, unmodified 2shRNA and mixtures of siRNAs was measured using crystal violet. 35.000 SKBR3 cells were plated in 48-well plates. Twenty-four hours after plating (0 hrs) cells were treated with the corresponding RNAs [in the case of branched RNAs, a 100 nM dose was used; in the case of mixtures of siRNAs, a 1:1 molar mixture was used (100 nM of each of the siRNAs composing the mixture)] in the absence of transfecting agent (final volume: 190 μL). 5 days later, cells were fixed with 4% formalin for 10 minutes, then washed twice with distilled water and stained with 0.1% freshly prepared crystal violet for 30 minutes. After washing, the stain was dissolved with 10% acetic acid and subsequently quantified by absorbance at 570 nM.

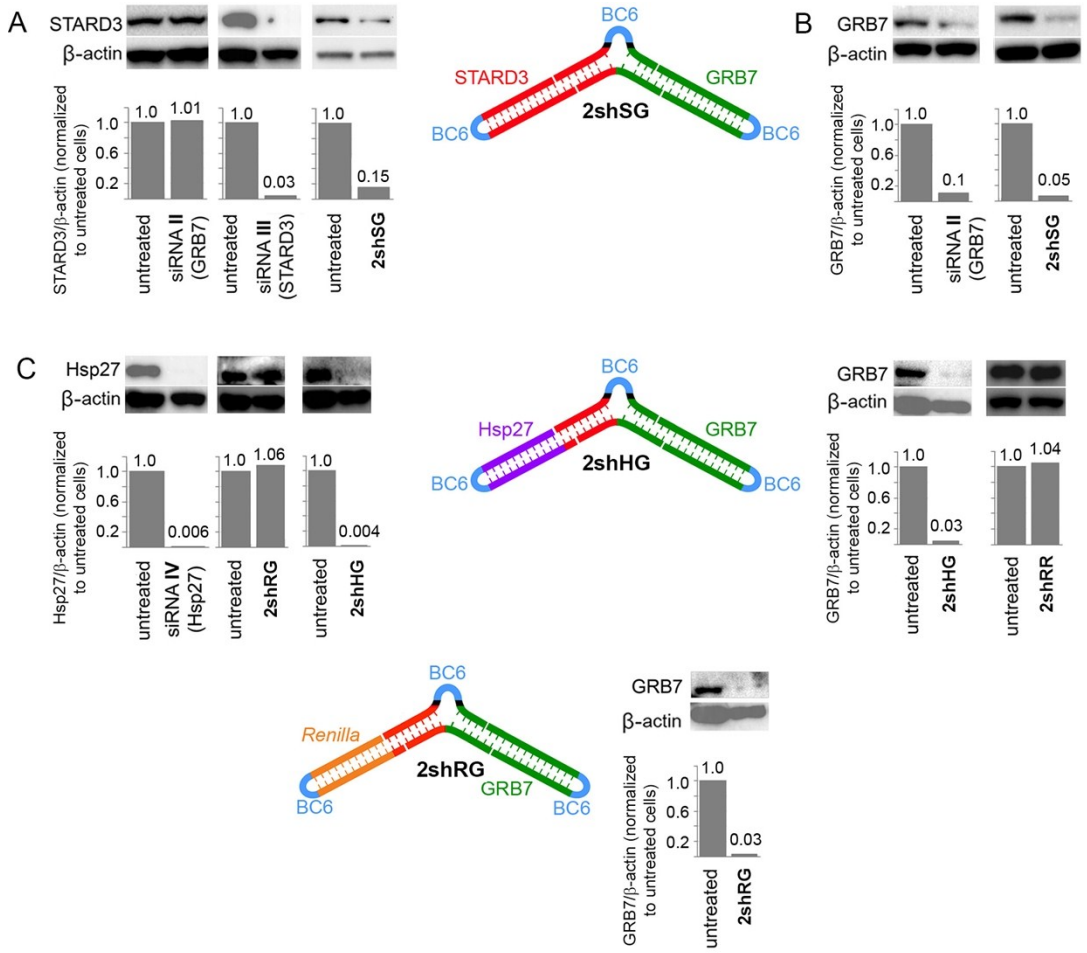


Figure S6. Analysis of STARD3, GRB7 and Hsp27 protein levels in lysates from SK-BR-3 cells after transfection with 2shRNAs or control siRNAs in the presence of Lipofectamine. Results of Western blots are shown as ratios of amounts of STARD3, GRB7 or Hsp27 and β-actin (internal control). Results were normalized to corresponding ratios in untreated control cells.

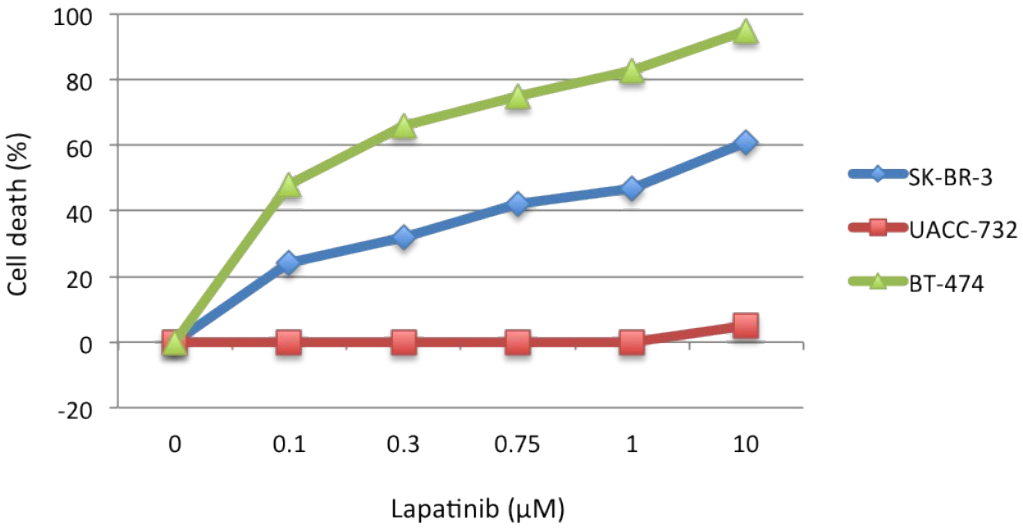


Figure S7. SK-BR-3, BT-474 and UAC-732CC cells were treated with Lapatinib. Lapatinib-specific cell death was determined using crystal violet assay and plotted as a percentage of cell death relative to untreated cells. Amount of the drug necessary to induce 50%-60% of cell death: 1 μM in the case of the SK-BR-3 cell line and 100 nM in the case of the BT-474 cell line (Lapatinib doses used in the cell viability studies depicted in Figures 2D and 2E).

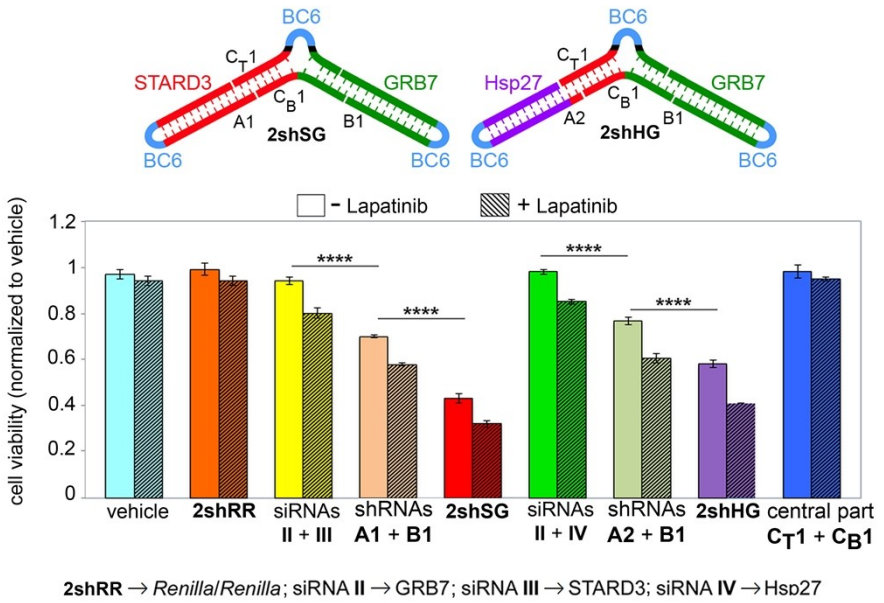


Figure S8. Viability of UACC-732 cells treated with 2shRNAs, mixtures of the corresponding shRNA components or mixtures of the corresponding siRNA analogues in the presence of Lipofectamine (72 h of incubation) and in the absence/presence of Lapatinib. The growth of the cells was plotted as a percentage of proliferation relative to the vehicle control cells. Vehicle: cells treated with Lipofectamine 2000 and DMSO alone. **** ($P < 0.0001$) versus indicated samples.

Statistical analysis

Data were analyzed by using the GraphPad Prism 5 program (GraphPad Software). Where appropriate, the results are expressed as mean ± standard deviation (SD). *P*-values of 0.05 or less were accepted as indicators of statistically significant data. Significant differences were assessed by Student's *t*-tests. Each experiment was performed in triplicate.

Detection of cell internalization by confocal microscopy

Cellular uptake of 2shRNA constructs was examined by confocal laser scanning microscopy. SK-BR-3 or MCF7 cells were seeded at a density of 33.000 cells/well on cover glasses in 48-well plates. 24 hours later, cells were treated with the corresponding RNA (100 nM dose per well). After incubation for 24 hours, the medium was aspirated, and the cells were washed twice with PBS. Next, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min, followed by permeabilization with 0.5% Triton for 5 min and nuclei staining with Hoechst (1μg mL^{-1}) for 10 min at room temperature. Cells were then washed 3x with PBS. The cover glasses were carefully isolated from the wells and mounted on slide glasses. The fluorescence of the conjugates was observed using a confocal microscope (SPE, Leica) at excitation wavelengths of 635 (Cy5) and 488 nm (FAM), and at emission wavelengths of 640-700 nm (Cy5) and 500-550 nm (FAM). The images were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

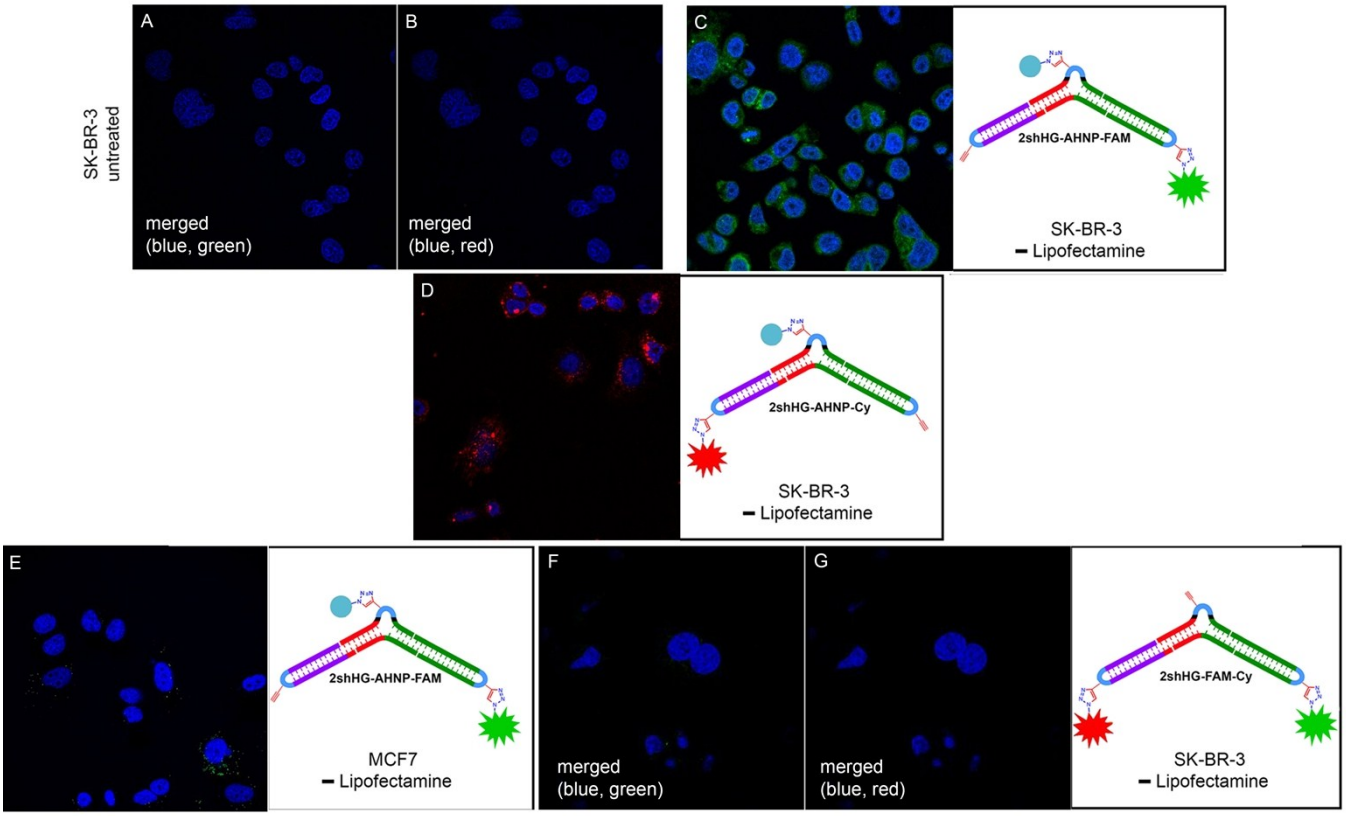


Figure S9. Confocal microscopy images. (A, B) Merged images [blue and green channels (A), and blue and red channels (B)] of untreated SK-BR-3 cells. (C) Merged image (blue and green channels) of SK-BR-3 cells incubated with **2shHG-AHNP-FAM** (100 nM; 24 hours) in the absence of Lipofectamine. (D) Merged image (blue and red channels) of SK-BR-3 cells incubated with **2shHG-AHNP-Cy** (100 nM; 24 hours) in the absence of Lipofectamine. (E) Merged image (blue and green channels) of MCF7 cells incubated with **2shHG-AHNP-FAM** (100 nM; 24 hours) in the absence of Lipofectamine. (F, G) Merged images [blue and green channels (F), and blue and red channels (G)] of SK-BR-3 cells incubated with **2shHG-FAM-Cy** (100 nM; 24 hours) in the absence of Lipofectamine.

Molecular dynamics simulations

The branched **2shHG** RNA was simulated as follows: the system was divided in 3 fragments [fragments A' (left arm; 28 bp), B' (right arm; 29 bp) and C' (central part; 35 bp)] and 200 ns molecular dynamics simulations were performed for each of them. The approach was similar to that used in our previous study.[2] Simulations were done using AMBER14 package[4] using the parmbsc0-OL3[5,6] force field. RNA structures were created using the make-na module (structure.usc.edu). The internal bulge was created by constructing a 5-nt internal bulge of natural ribonucleotides and replacing the three central nucleotides with the BC6 dimer, using the rnacomposer module (rnacomposer.cs.put.poznan.pl).[7] The structures were solvated with TIP3P water molecules[8] and neutralized with K^+ ions.[9] Systems were minimized, annealed and equilibrated following the standard AMBER simulation protocol followed by a 10 ns of post equilibration time prior to the 200 ns production runs.

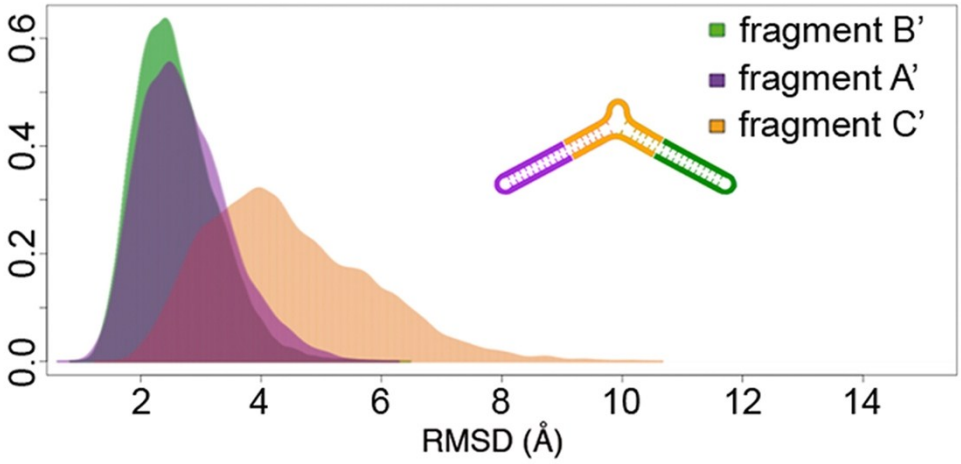
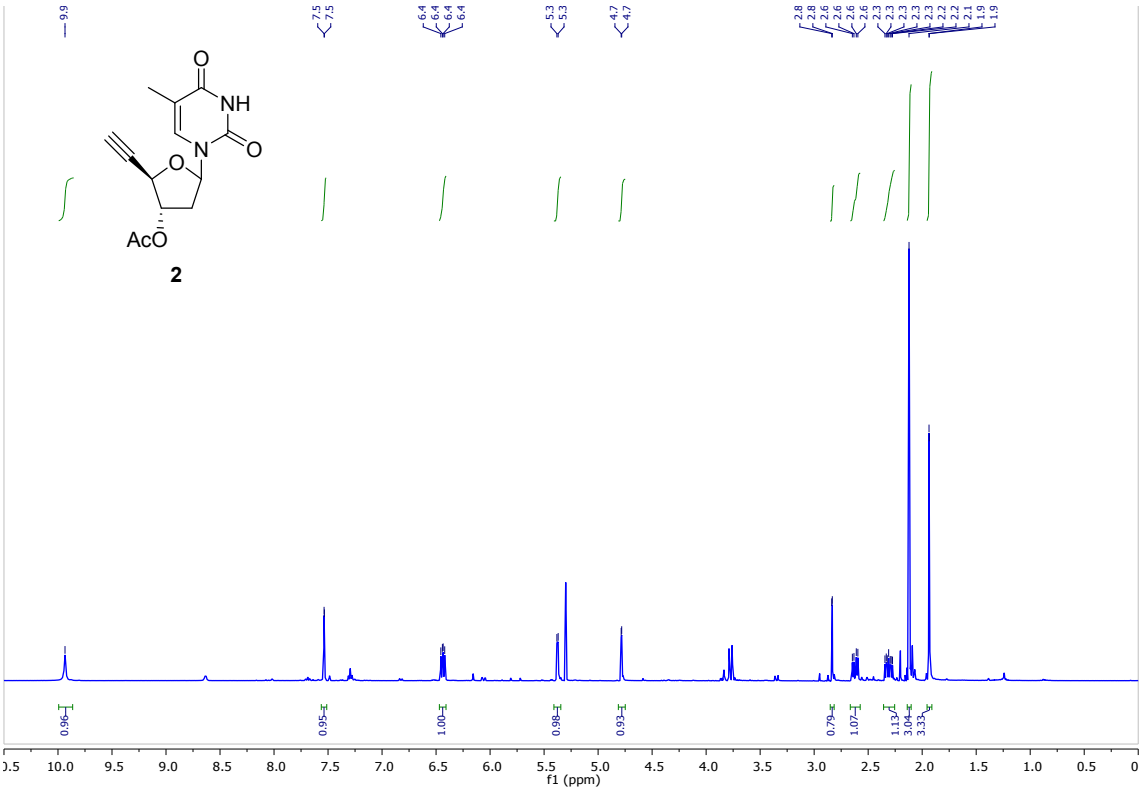


Figure S10. RMSD plots of fragments A', B' and C' with sequences 5'-UCCGAUGAGACUGCCGCCAAGUAAAGCCU**BC6**AGGCUUUACUUGGCGGCAGUCUCAUCG GA-3' (fragment A'; purple), 5'-GGCCAUUUCGAAGCUUGUUGGGCUUGA**BC6**UCAAGCCCAA-CAAGCUUCGAAAUGGCC-3' (fragment B'; green) and 5'-GGCCACAAGGGGCUUCGGAA**BC6**-UAGAAGCUGCAAAAUCC-3': 5'-GGAUUUUGCAGCUUCUUCCGAAGCCCCUUGUGGCC-3' (fragment C'; orange) respect to the average structures along the MD simulations

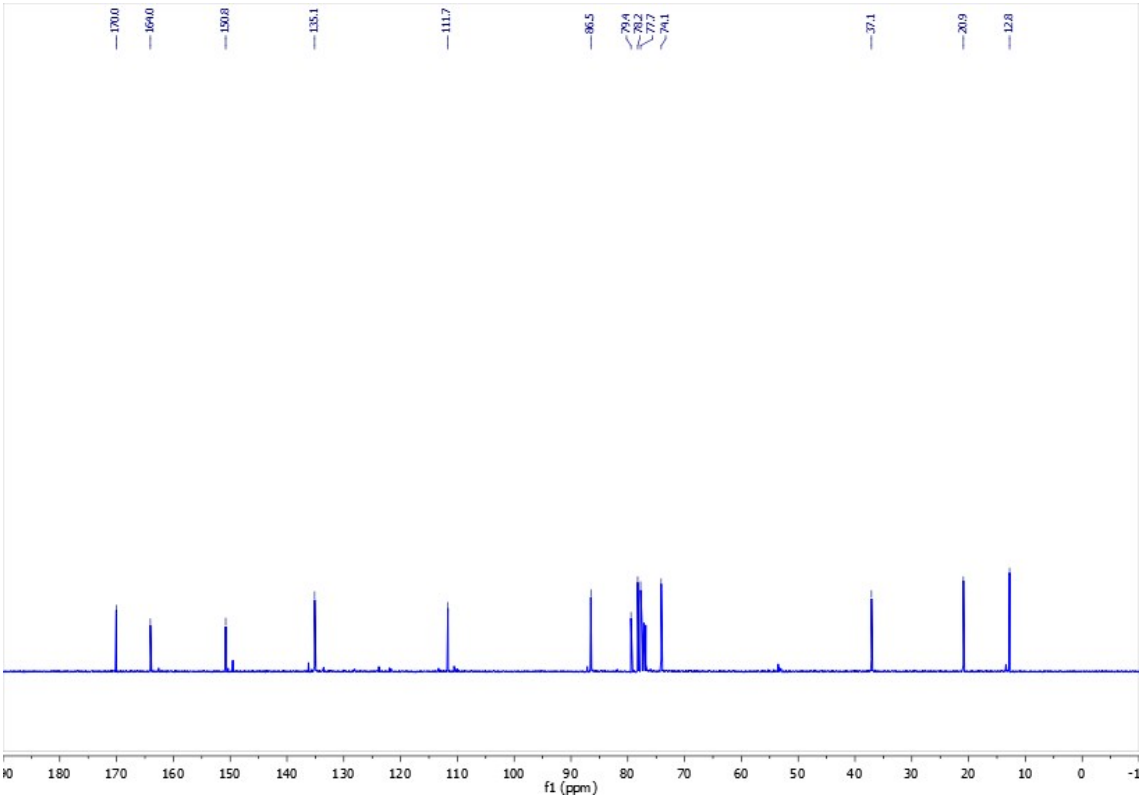
Table S1. Mass spectrometry analysis of synthesized oligonucleotides

RNA	Sequence	MW calcd.	MW found
A1	5'-CCA.GUA.ACC.CCC.GUG.UUU.GCA.CCU.UUG.UC.**BC6.**GAC.A-AAG.GUG.CAA.ACA.CGG.GGG.UUA.CUG.GCC.GAC-3'	20866.7 ($M+2Na^+$)	20870.4 ($M+2Na^+$)
B1	5'-GUG.GCC.AUU.UCG.AAG.CUU.GUU.GGG.CUU.GA.**BC6-**UCA.AGC.CCA.ACA.AGC.UUC.GAA.AUG.GCC.ACA.AGG.G-3'	20955.8 ($M+2Na^+$)	20967.9 ($M+2Na^+$)
C_T1	5'-GCU.UCG.GAA.**BC6.**UGU.GCU.CAA.GUC.GG-3'	8005.4	8007.3
C_B1	5'-UUG.AGC.ACU.CCG.AAG.CCC.CUU-3'	6607.3	6608.0
A2	5'-UCC.GAU.GAG.ACU.GCC.GCC.AAG.UAA.AGC.CU.**BC6-**A.GGC.UUU.ACU.UGG.CGG.CAG.UCU.CAU.CGG.ACC.GAC-3'	20820.7	20806.7
B2	5'-UCC.UUC.UUC.AGA.UUU.GAU.CAA.CGC.AAU.AUC.U-**BC6**.AGA.UAU.UGC.GUU.GAU.CAA.AUC.UGA.AGA.AGG.AGA.AAA.AAU.G-3'	23513.3 ($M+5Na^+$)	23516.9 ($M+5Na^+$)
C_T2	5'-GUU.UUA.**BC6**.UAG.AUA.UUG.CGU.UG-3'	6973.2	6972.3
C_B2	5'-AUA.UCU.AAA.ACC.AUU.UUU.UC-3'	6218.0	6216.1
A3	5'-AUC.AAA.UCU.GAA.GAA.GGA.GAA.AAA.AUG.GUU.UU-**BC6**.A.AAA.CCA.UUU.UUU.CUC.CUU.CUU.CAG.AUU.UGA.UCC.GAC-3'	22541.8	22556.1
A4	5'-AUC.AAA.UCU.GAA.GAA.GGA.GAA.AAA.AUG.GUU.UU.**BC6.-**AAA.ACC.AUU.UUU.UCU.CCU.UCU.UCA.GAU.UUG.AUC.AAC.GCA-3'	23349.0 ($M+3Na^+$)	23365.6 ($M+3Na^+$)
C_T1'	5'-GCU.UCG.GAA.**BC6^{ethynyl}**.UGU.GCU.CAA.GUC.GG-3'	7999.4	7993.6
A2'	5'-UCC.GAU.GAG.ACU.GCC.GCC.AAG.UAA.AGC.CU.**BC6^{ethynyl}-**A.GGC.UUU.ACU.UGG.CGG.CAG.UCU.CAU.CGG.ACC.GAC-3'	20830.7	20.846.5
B1'	5'-GUG.GCC.AUU.UCG.AAG.CUU.GUU.GGG.CUU.GA.**BC6^{ethynyl}-**UCA.AGC.CCA.ACA.AGC.UUC.GAA.AUG.GCC.ACA.AGG.G-3'	20903.7	20908.2

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



^{13}C NMR (101 MHz, $CDCl_3$) spectrum of 2



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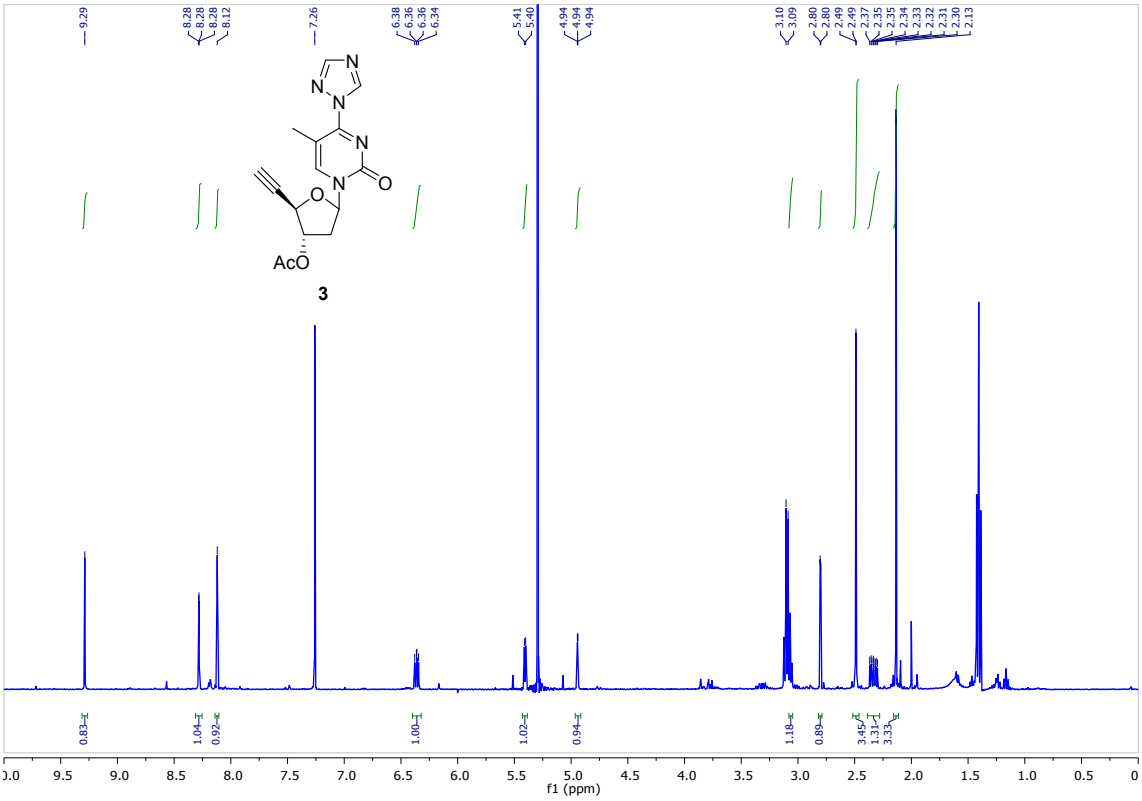

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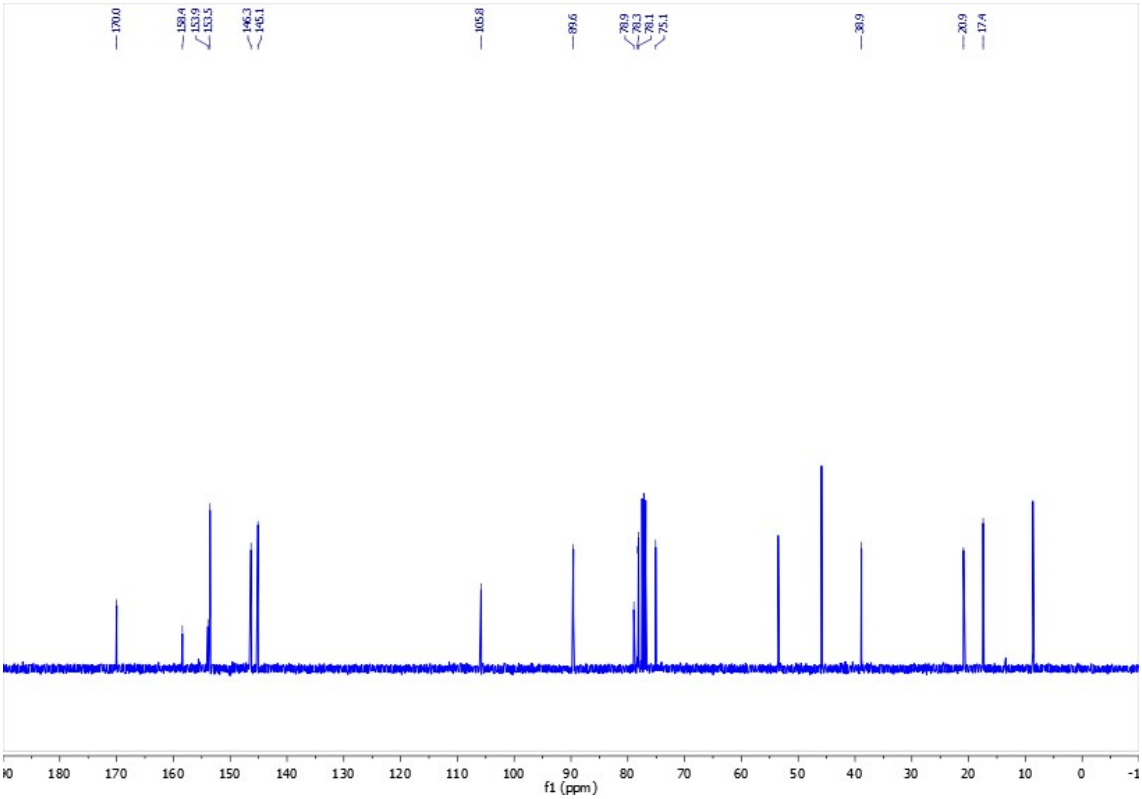
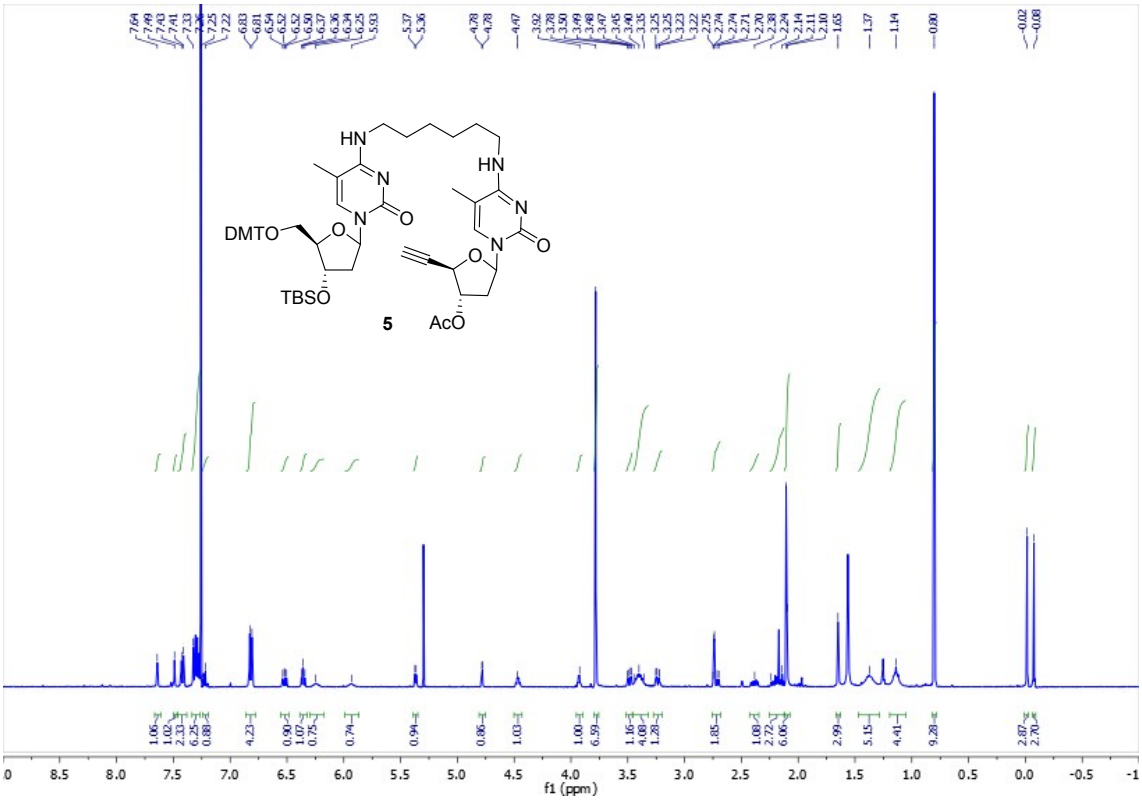
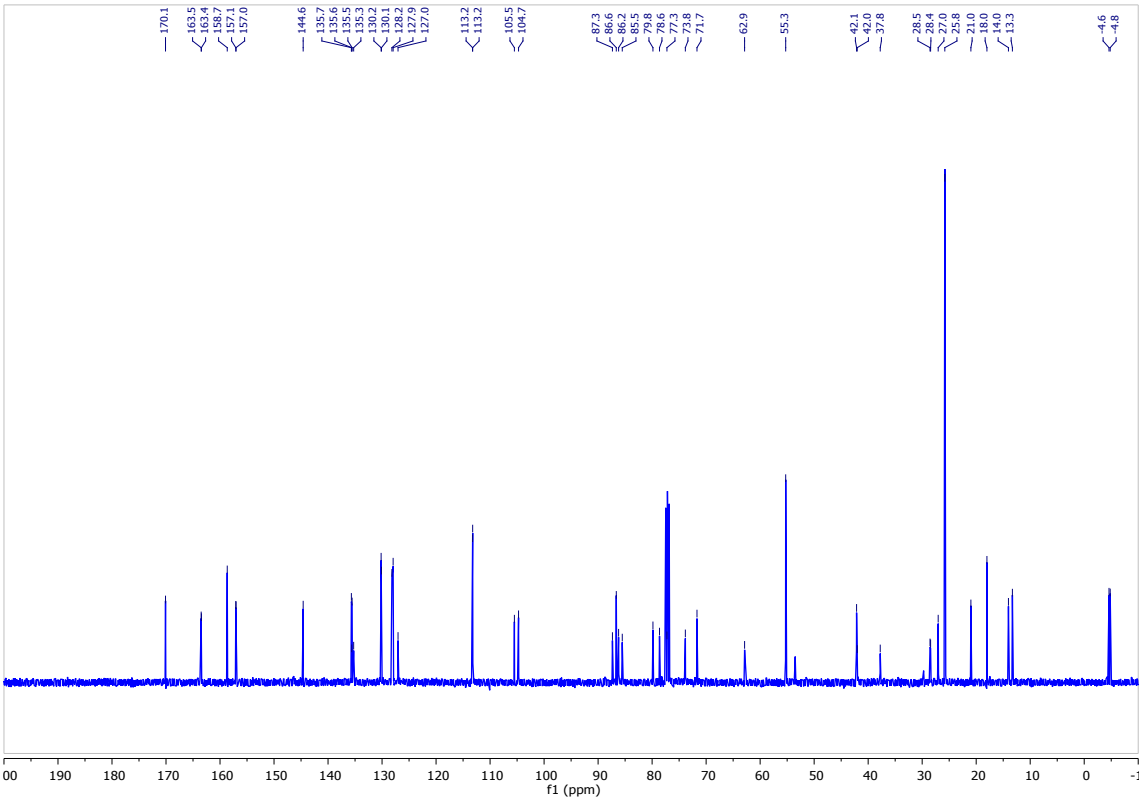


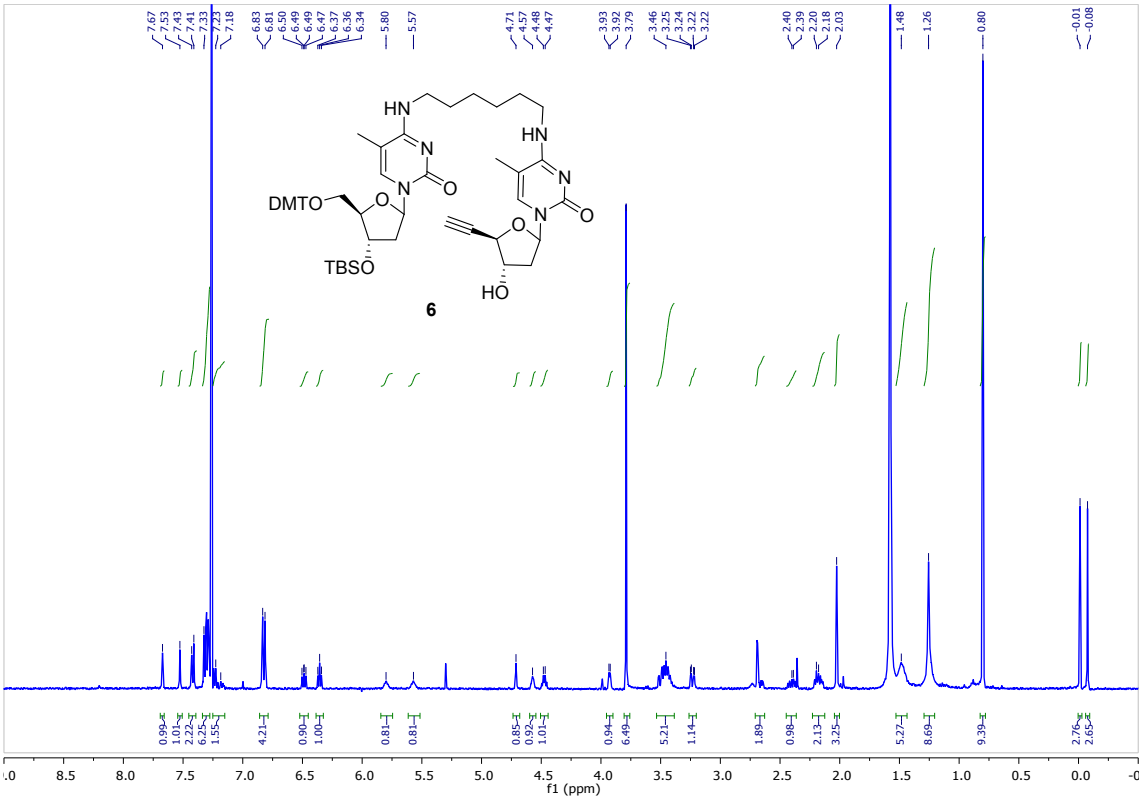
^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 5



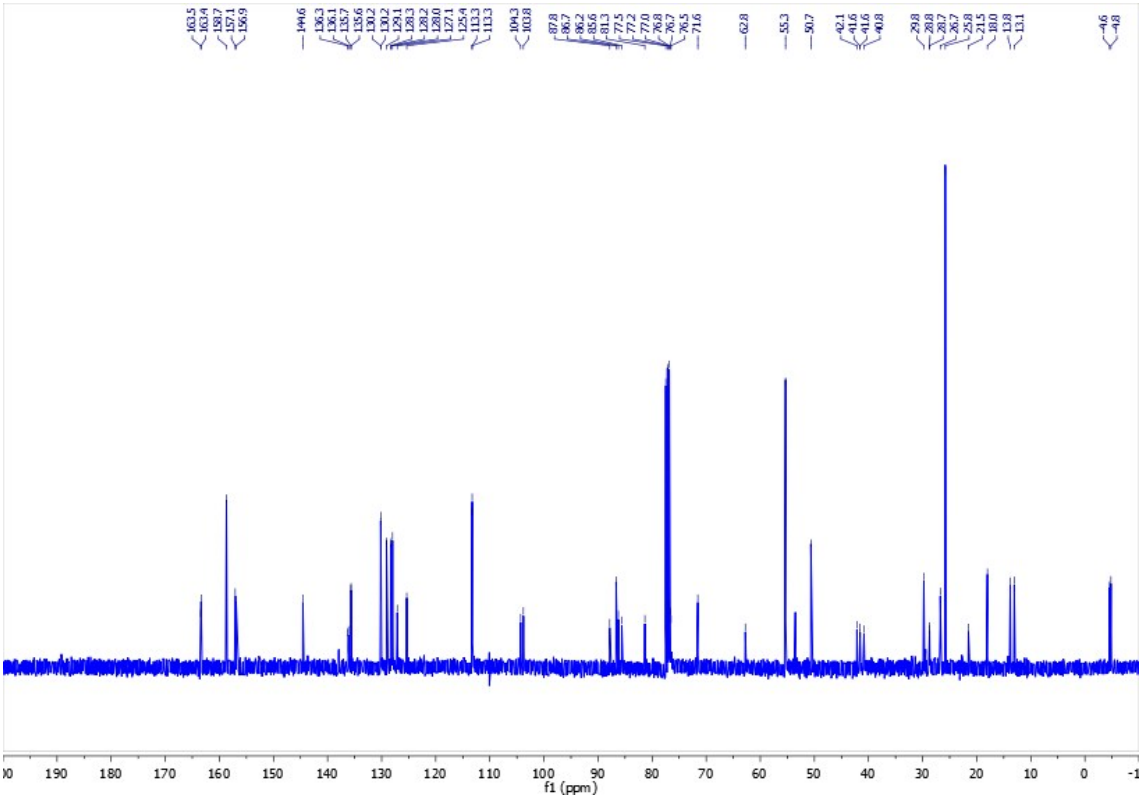
^{13}C NMR (101 MHz, $CDCl_3$) spectrum of 5



^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 6



^{13}C NMR (101 MHz, $CDCl_3$) spectrum of 6



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