Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Reactive oxygen species - responsive RNA interference

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1 Synthesis

1.1 Chemicals

If not stated otherwise, all chemicals were acquired commercially from Sigma-Aldrich (Germany) in at least 98 percent purity. The reagents were used without further purification. Anhydrous solvents and NMR-solvents were stored under nitrogen or argon atmosphere and, if possible, with use of molecular sieve. As solid phase for DNA synthesis dC(bz) CPG (1000 Å, 28 µmol/g, Sigma Aldrich) and for RNA synthesis dT CPG (1000 Å, 25 – 35 µmol/g, Sigma Aldrich) were used. The strands were synthesised with DMT-dT and DMT-2´-O-TC (rA(bz), rC(ac), rU, rG(ib), Sigma Aldrich) phosphoramidites. Details of synthesis of chemically modified siRNAs used for in vivo studies are provided in section 4.1. Gibco™ Opti-MEM™ Reduced Serum Media (Opti-MEM), Lipofectamine® RNAiMAX, random hexamer primer and dNTPs, the reverse transcriptase and its 5x buffer were all obtained from Thermo Fisher Scientific (Germany. Zymo-Spin IC Columns were purchased from Zymo Research Corporation (USA). LightCycler® 480 SYBR® Green Master from Roche Diagnostics GmbH (Germany) was used for the qPCR analysis. For the microscopy 35 mm imaging dishes (µ-Dish 35 mm, high, ibidi GmbH, Germany) were used.

1.2 Instruments

Automated oligonucleotide synthesis was performed on a H-8 synthesizer from the company K&A Laborgeräte GbR (Germany). Purification of oligonucleotide conjugates was done on HPLCs of the company Shimadzu, equipped either with only a UV-vis detector or additionally with a fluorescence detector. The chromatographic separations were performed at 22°C. If not otherwise stated, a reversed phase Nucleosil C_{18} column (250 mm x 4.6 mm) from Macherey-Nagel was used. HPLC fractions were analyzed on a UltrafleXtreame MALDI-TOF mass spectrometer from Bruker. Samples were prepared by dried droplet method on a MTP 384 polished steel plate from Bruker. Concentrations of modified and non-modified RNAs and DNAs were determined with a Nanodrop UV-visible spectrophotometer from the company Thermo Fisher Scientific (Germany). The reverse transcription was performed with a Dual Block Gradient PCR Thermal Cycler GE4852T™ from Biogen (US). For the qPCR experiments the LightCycler® 480 from Roche Molecular Systems, Inc. (US) was used. The confocal microscopy images were taken with a Zeiss Spinning Disk Axio Observer Z1 with Plan-Apochromat 63x/1.40 oil objective in spinning disc confocal mode using a EVOLVE 512 EMCCD camera from ZEISS (Germany).

1.3 Synthesis protocols (Scheme S1)

Glassware was dried by heating with a hot air gun under applied vacuum. After cooling to 22°C, the flask was flushed with nitrogen or argon. For thin layer chromatography (TLC) silica or allox plates with a fluorescent dye of the company Macherey-Nagel (Düren, Germany) were used (Silica: SIL G/UV 0.2 mm; aluminum oxide: ALOX N/UV 0.2 mm). Column chromatography was performed on neutral silica or aluminum oxide (allox) of the company Macherey-Nagel. The bought allox was brought to activity 3 by addition of water (10% v/m).

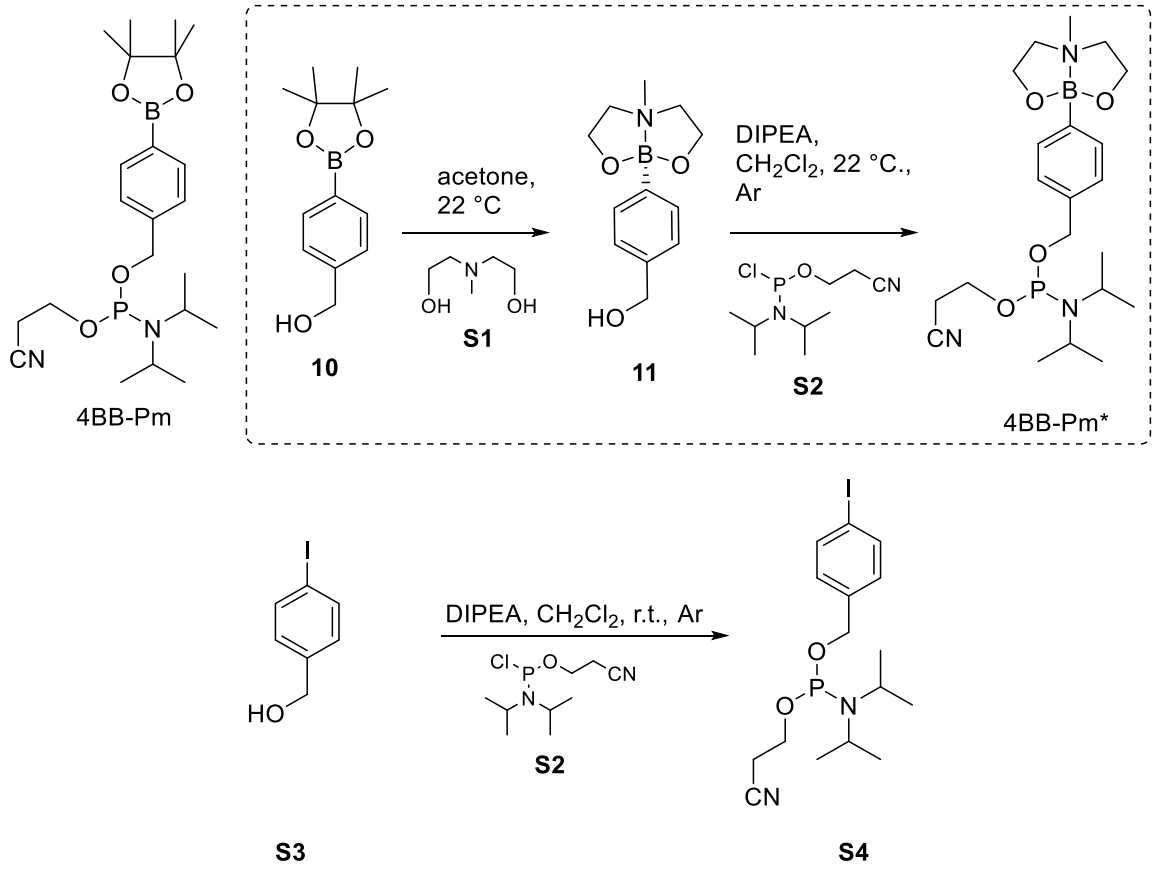


Figure **S1**: Structure of known 4BB-Pm and synthesis of phosphoramidites 4BB-PM* and **S4**.

Synthesis of compound 11

4-(Hydroxymethyl)phenylboronic acid pinacol ester (**10**) (502 mg, 2.14 mmol) was suspended in acetone (18 mL). Under stirring bis-(2-hydroxyethyl)methylamine (**S1**) (300 µL, 2.62 mmol, 1.2 eqv.) was added dropwise. The colorless solution was stirred overnight at 22°C. Formation of precipitate could already be observed after few hours. To fully precipitate the product *iso*-hexane was added to the total volume of 80 mL. The mixture obtained was cooled for at least 3 hours at -20°C. Afterwards, the supernatant was removed, the precipitate was washed with *iso*-hexane and then dried *in vacuo* to give compound **11** as colorless crystals with a yield of

77% (390 mg, 1.66 mmol). If traces of the starting material were left, addition of a small amount of acetone and ultra-sonification removed all impurities.

^{1}H-NMR spectrum (300 MHz, $CDCl_3$) δ (ppm) = 7.61 (d, *J* = 5.8 Hz, 2H), 7.29 (d, *J* = 6.8 Hz, 2H), 4.66 (d, *J* = 5.7 Hz, 2H), 4.27 – 4.05 (m, 4H), 3.18 (dt, *J* = 9.5, 4.0 Hz, 2H), 2.99 (dt, *J* = 12.0, 7.0 Hz, 2H), 2.31 (d, *J* = 2.1 Hz, 3H), 1.92 – 1.64 (m, 1H).

^{13}C-NMR spectrum (76 MHz, $CDCl_3$) δ (ppm) = 140.25, 133.64, 126.34, 65.83, 62.47, 60.75, 47.93.

Atmospheric pressure photoionization (APPI) mass spectrum (positive mode): calculated for $C_{12}H_{19}BNO_3$ 236.1453 m/z $[M+H]^+$; found: 236.1461 m/z.

C, H, N analysis: calculated for $C_{12}H_{18}BNO_3$: *C* 61.31, *H* 7.72, *N* 5.96; found: *C* 61.43, *H* 7.60, *N* 5.92.

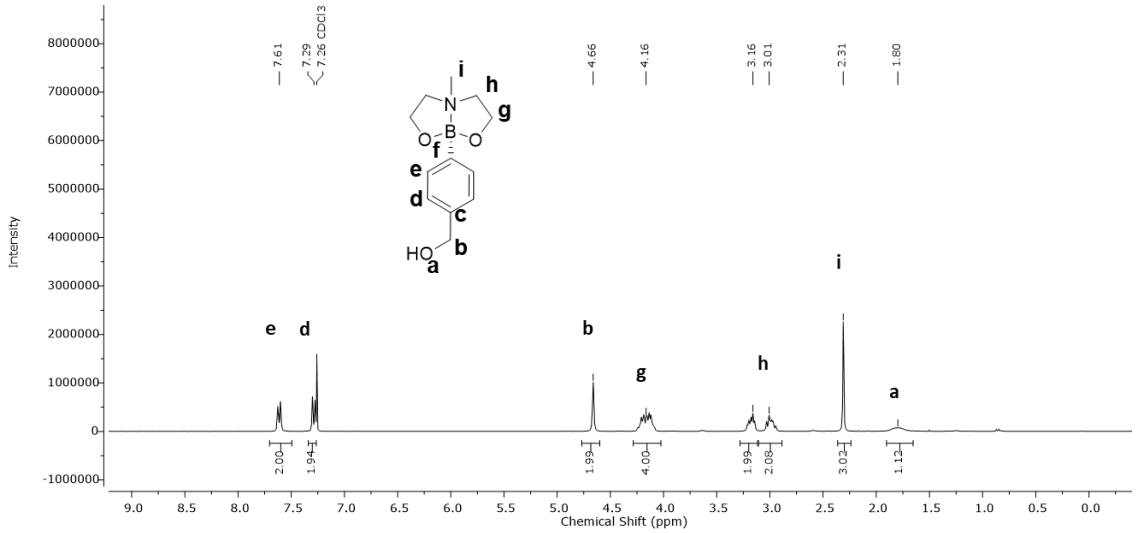


Figure **S2** :^{1}H-NMR spectrum of compound **11**.

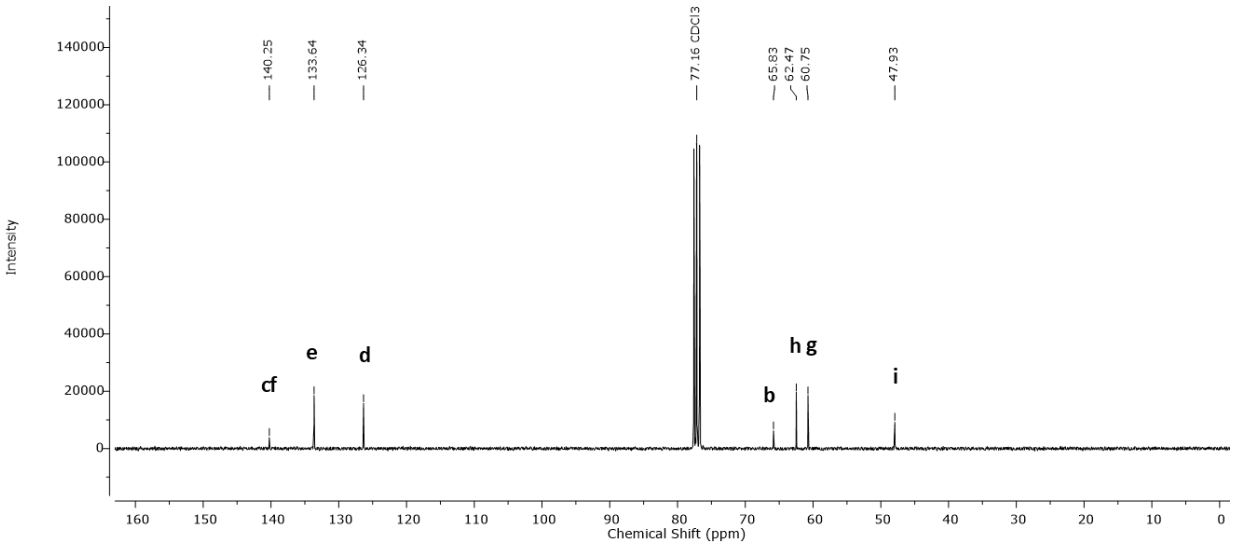


Figure **S3**:^{13}C-NMR spectrum of compound **11**.

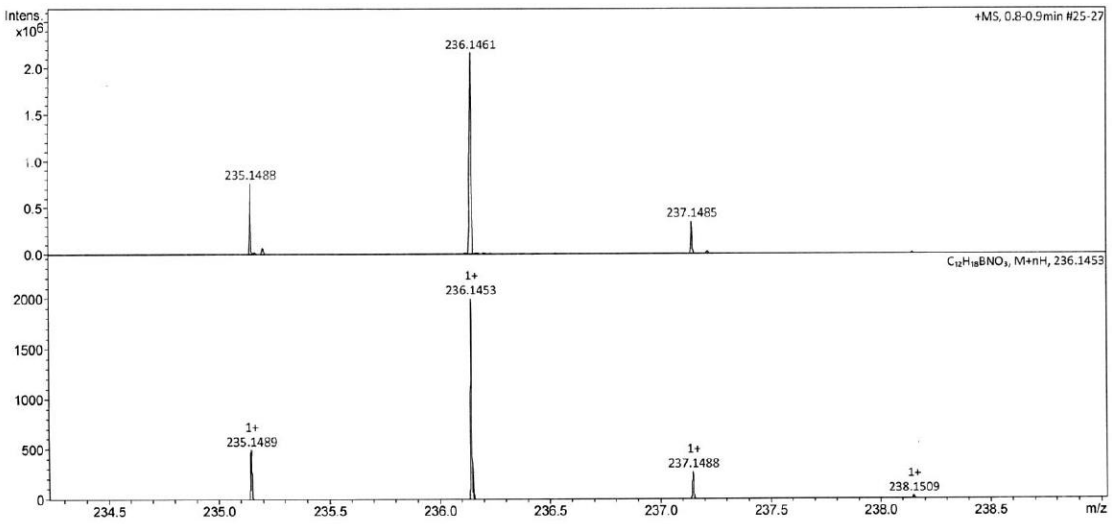


Figure **S4**: APPI mass spectrum of compound **11.**

Synthesis of compound 4BB-Pm*

In a vacuum dried flask compound **11** (200 mg, 0.851 mmol) and N,N-diisopropylethylamine (DIPEA) (0.44 mL, 2.55 mmol, 3 eqv.) were dissolved under argon in anhydrous dichloromethane (DCM) (10 mL). Then 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (**S2**) (0.21 mL, 0.940 mmol, 1.1 eqv.) was added dropwise while stirring. The resulting reaction mixture was stirred for 1 – 2 h at 22 °C. Then, the DCM was evaporated. The resulting gel was directly applied to column chromatography on aluminum oxide. First, 1:1 isohexane/DCM (v/v) then pure DCM were used as eluents. If the product is not eluted fast enough, a mixture of DCM/Acetone 1:1 (v/v) can be used as no other impurity was eluted with this mixture. Compound 4BB-Pm* could be obtained as a colorless gel with a yield of 57% (188 mg, 0.432 mmol).

^{1}H-NMR spectrum (300 MHz, acetone-d_6) δ (ppm) = 7.57 (d, *J* = 8.0 Hz, 2H), 7.24 (d, *J* = 8.0 Hz, 2H), 4.71 (qd, *J* = 12.3, 8.4 Hz, 2H), 4.11 – 3.93 (m, 4H), 3.91 – 3.81 (m, 2H), 3.74 – 3.62 (m, 2H), 3.30 (dt, *J* = 11.5, 4.6 Hz, 2H), 3.04 (ddd, *J* = 11.6, 8.4, 6.7 Hz, 2H), 2.75 (t, *J* = 6.0 Hz, 2H), 2.60 (s, 2H), 2.30 (s, 3H), 1.23 – 1.16 (m, 18H).

^{31}P-NMR spectrum (122 MHz, acetone-d_6) δ (ppm) = 147.93.

APPI mass spectrum (positive mode): calculated for $C_{21}H_{35}BN_3PO_4$ 435.2457 m/z $[M\text{-}e^-]^+$, found 435.2469 m/z.

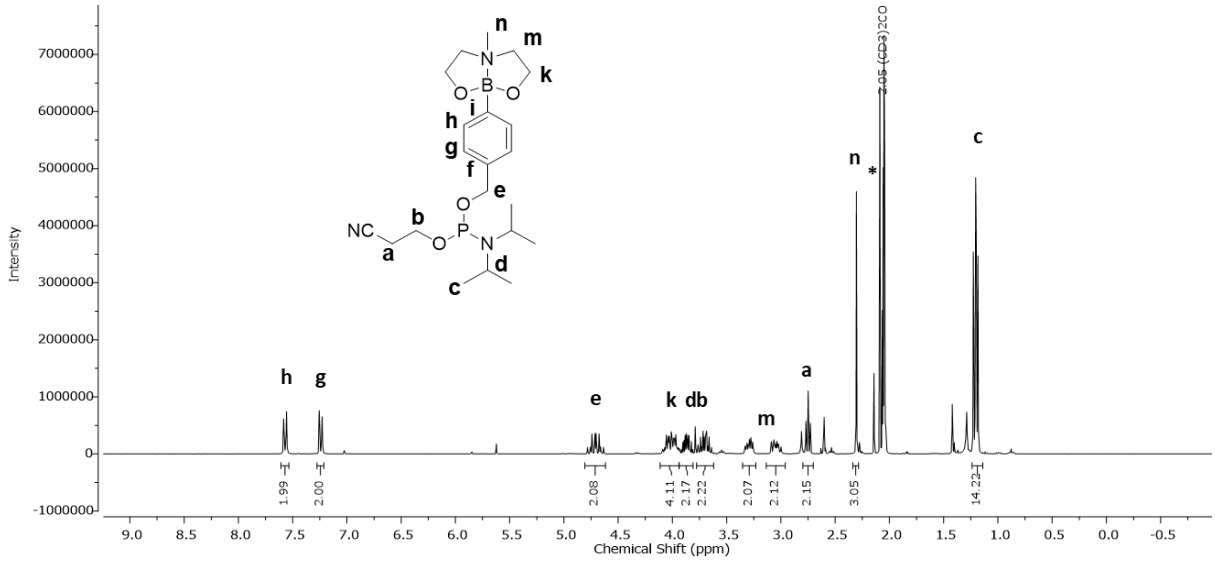


Figure **S5**: ^{1}H-NMR of compound 4BB-Pm*. Impurity (*) results from residues of acetone.

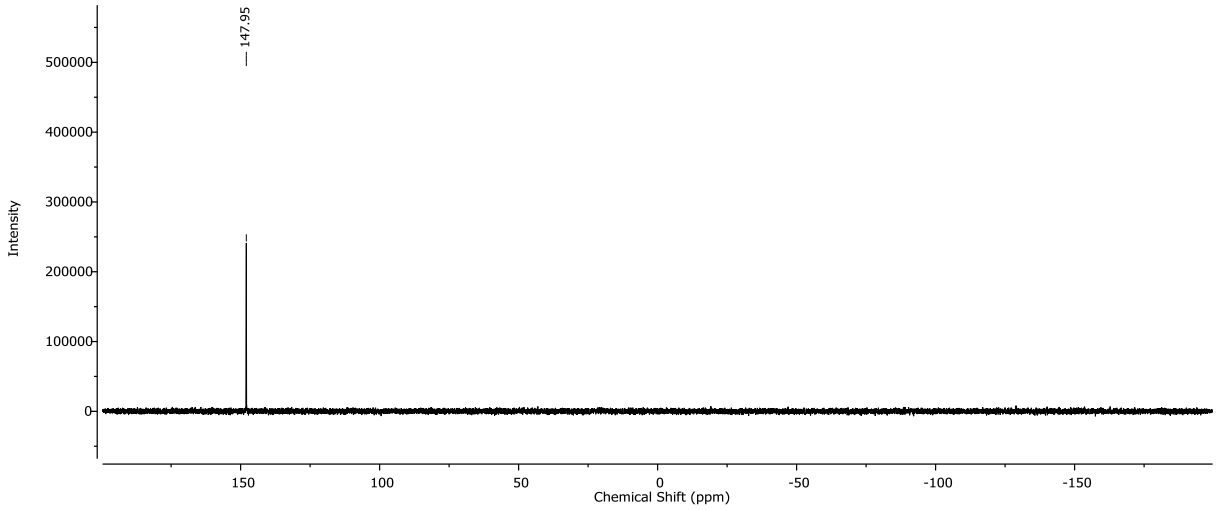


Figure **S6**: ^{31}P-NMR spectrum of compound 4BB-Pm*.

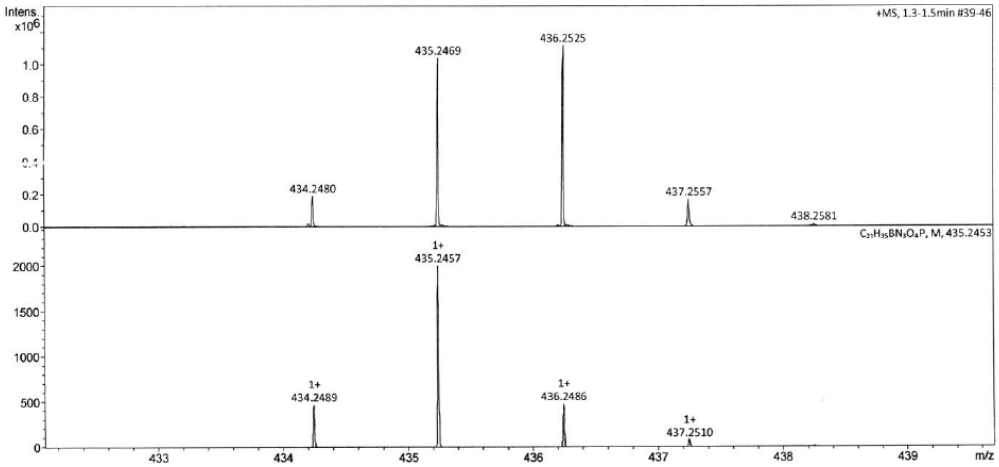


Figure **S7**: APPI mass spectrum of compound 4BB-Pm*.

Synthesis of compound S4

In a vacuum dried flask 4-iodobenzyl alcohol (**S3**) (150 mg, 0.601 mmol) and DIPEA (0.40 mL, 2.30 mmol, 3.8 eqv.) were dissolved under argon in anhydrous DCM (8 mL). Then 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (**S2**) (0.21 mL, 0.940 mmol, 1.56 eqv.) was added dropwise while stirring. The resulting reaction mixture was stirred for 2 h at 22°C. Then the reaction mixture was directly applied to column chromatography on allox with pure dichloromethane as eluent to give compound **S4** as a colorless gel with a yield of 99% (274 mg, 0.631 mmol).

^{1}H-NMR spectrum (300 MHz, acetone-d_6) δ (ppm) = 7.73 (d, *J* = 8.4 Hz, 2H), 7.23 (d, *J* = 8.5 Hz, 2H), 4.84 – 4.60 (m, 2H), 3.95 – 3.80 (m, 2H), 3.78 – 3.61 (m, 2H), 2.77 (t, *J* = 6.0 Hz, 2H), 1.20 (t, *J* = 6.6 Hz, 13H).

^{31}P-NMR spectrum (122 MHz, acetone-d_6) δ (ppm) = 148.27.

^{13}C-NMR spectrum (76 MHz, acetone-d_6) δ (ppm) = 140.55, 140.45, 138.19, 129.99, 118.96, 92.91, 65.49, 65.25, 59.74, 59.49, 43.95, 43.79, 24.94, 24.84, 20.80, 20.71.

ESI mass spectrum (positive mode): calculated for $C_{16}H_{25}IN_2PO_2$ 435.0693 m/z $[M+H]^+$, found 435.0704 m/z.

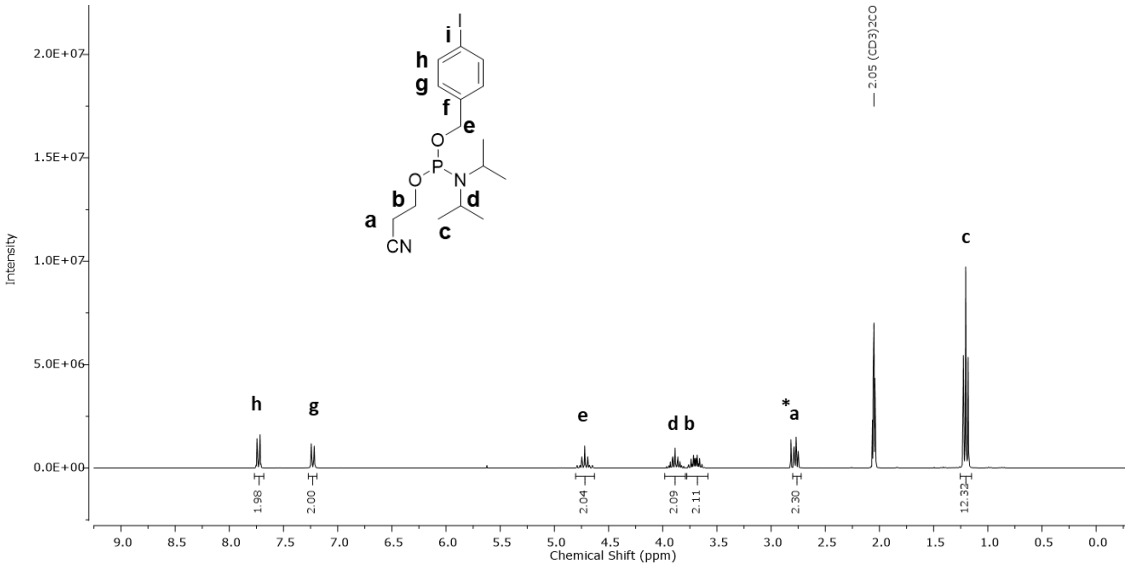


Figure **S8**: ^{1}H-NMR spectrum of compound **S4.** Impurity * results from residues of water in the deuterated solvent.

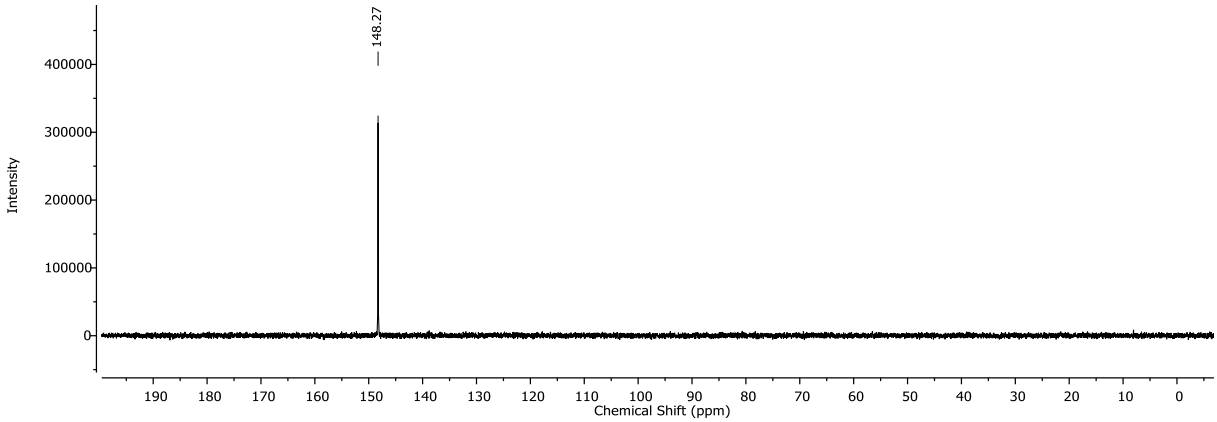


Figure **S9**: ^{31}P-NMR spectrum of compound **S4.**

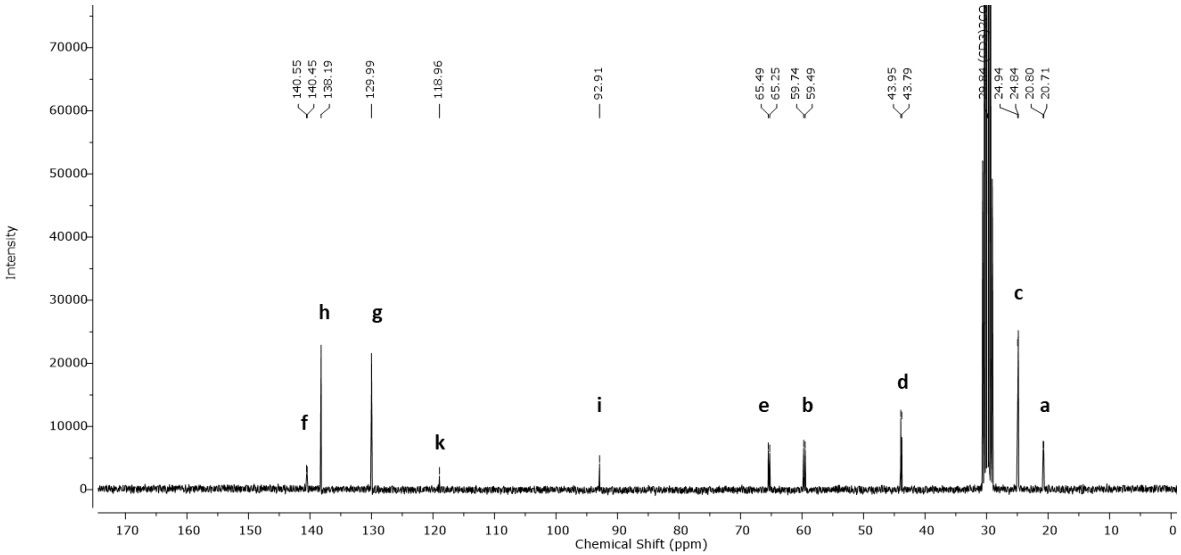


Figure **S10**: ^{13}C-NMR spectrum of compound **S4.**

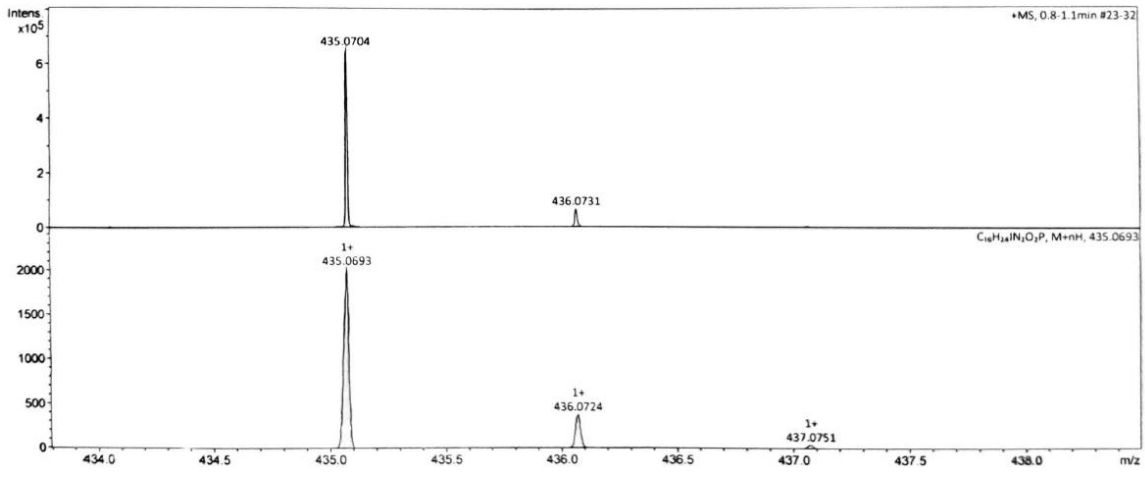


Figure **S11**: ESI mass spectrum of compound **S4**.

1.4 Synthesis of modified oligonucleotides

Automated oligonucleotide synthesis was performed on a 1 µmol-scale by using the standard (3′→5’) synthesis according to the recommendations of the manufacturer. Chemical modifications were introduced by manual coupling described in the synthetic section of the corresponding oligonucleotides.

The synthesized and chemically modified oligonucleotides were purified on reversed phase HPLC. Gradients of acetonitrile (solution B) in triethylammonium acetate buffer (0.1 M, pH 7.4) containing acetonitrile (5%, v/v, solution A) were used as eluents.

HPLC fractions of DNA and RNA samples were analysed by MALDI-TOF mass spectrometry. The samples were prepared by dried droplet method on a MTP 384 steel plate from Bruker. First, 1 µL of a prepared matrix was spotted on a designated spot on the MALDI plate. After the spot was dried, 0.8 µL of DNA or RNA solution was spotted on the top and dried. Under these conditions mass accuracy with external calibration was better than 0.05% of the peak mass, that is ± 2.1 at m/z 7.000 (RNA) or ± 1.0 at m/z 2.000 (DNA). For matrix preparation either 2',4',6'-trihydroxyacetophenone (THAP) (2.5 mg) or 3-hydroypicolinic acid (3-HPA) (5.0 mg) was dissolved in HPLC grade acetonitrile (0.5 mL) while ammonium citrate (10 mg) was dissolved in HPLC grade water containing 0.1% trifluoroacetic acid (0.5 mL). Both solutions were then mixed in a ratio of 1:1 (v/v).

Concentrations of modified and non-modified RNAs and DNAs were determined by measuring the absorption at 260 nm for a defined dilution of the stock solution of unknown concentration.

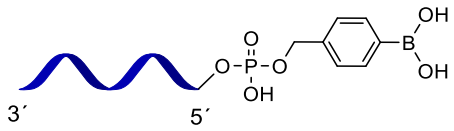
The unmodified antisense strand for KIF11 (AS strand, RNA 14) has the sequence 5´-dT r(AUA GAU GUU CUU GUA CGC) dTdT with ε = 209 400 l/(mol*cm), the sense strand (S strand, RNA 13) has the sequence 5´-r(GCG UAC AAG AAC AUC UAU A) dTdT with ε = 213 900 l/(mol*cm). Extinction coefficients were calculated with the Oligo Evaluator™ from Sigma Aldrich. For extinction coefficients of modified oligonucleotide see experimental section for the corresponding modified DNAs or RNAs.

Annealing: Annealing buffer: 100 mM Tris with pH 7.8, 500 mM NaCl and 10 mM EDTA

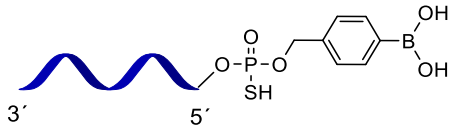
The (5´-modified) antisense strand and the complementary sense strand were mixed in annealing buffer with a concentration of 10 µM per strand. The strands were then annealed by heating the mixture to 90°C for 15 min and cooling it afterwards with 3°C per 2 min to 22°C. The annealed siRNA was then diluted 1:10 with water to a final concentration of 1 µM.

1.4.1 5´-modified DNAs

Conjugates S5 and DNA 14



S5



DNA 14

Synthesis of conjugate **S5** (unsuccessful):

Solid support dC (bz) 500Å (40µmol/g) was used. The DNA strand 5´-TTTTC was synthesized at standard conditions using a DNA synthesizer. Phosphoramidite 4BB-Pm* was coupled manually under argon atmosphere one time for 10 min. For that, the phosphoramidite in water free acetonitrile (0.1 mL, 0.1 M) together a solution of ETT activator in acetonitrile (0.1 mL, 0.5 M) were used. Oxidation was performed on the synthesizer with standard iodine solution but different time lengths: 0s, 5s, 10s and 30s. Also, a 1:1 or 1:5 dilution with iodine solution/acetonitrile for 25s were tested. After washing with acetonitrile, the solid support was cleaved and deprotected by the treatment with aqueous ammonia solution (25%, 0.15 mL) for 2 h at 55 °C to get conjugate **S5**. Then, the solid phase and the solution were separated, and the aqueous ammonia solution was diluted to a total volume of 1 mL with HPLC grade water and evaporated by lyophilization. The crude DNA mixture was dissolved with HPLC grade water. For each of the tested oxidation times MALDI-TOF mass spectra of the crude mixtures were measured (Figure S12). Selected mixtures (for 10 and 30 s oxidation times) were purified by reversed phase HPLC (example see Figure S13) using the gradient of solution B in solution A: from 0 to 0.1 min at 5% B, in 20 min to 20% B, then in 10 min to 30%. Additionally, the HPLC of the unmodified strand (5´-TTTTC) (expected mass of 1443.99 m/z) and the 5´-phosphorylated strand (expected mass 1523.97 m/z) were recorded. As shown in Figures S12 – 14 the main products of the manual coupling were the unmodified 5´-mer and the 5´-phosphorylated strand suggesting that coupling occurred but the boronic acid was cleaved under the described conditions.

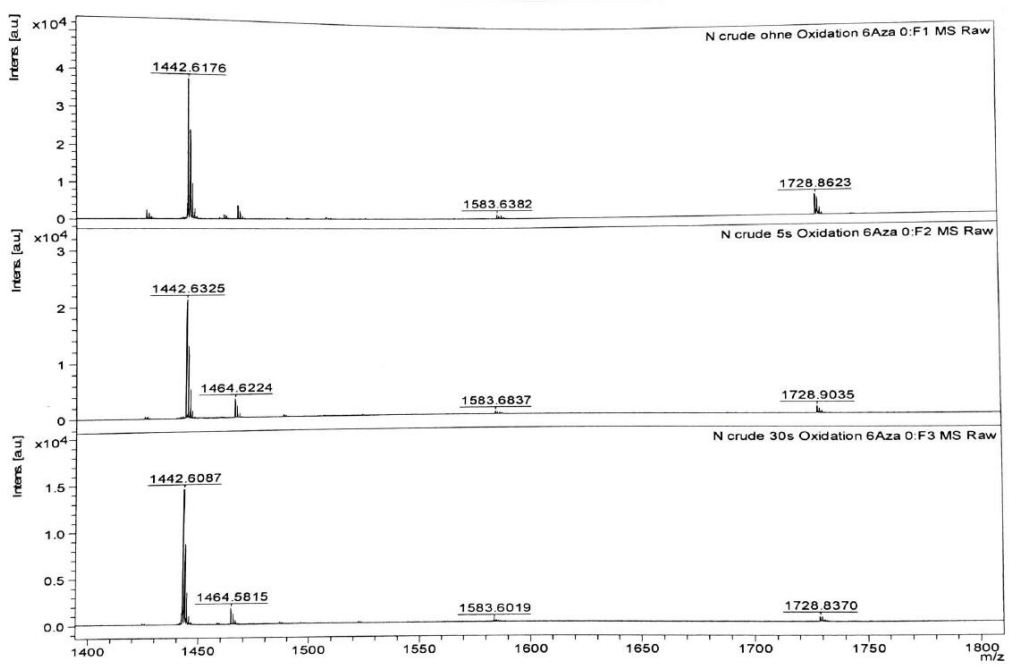


Figure **S122**: MALDI-TOF mass spectra of crude mixtures after cleavage from the solid supports obtained in synthesis of **S5**. Measured for three different tested conditions: upper - no oxidation, middle – oxidation for 5s, lower - oxidation for 30s.

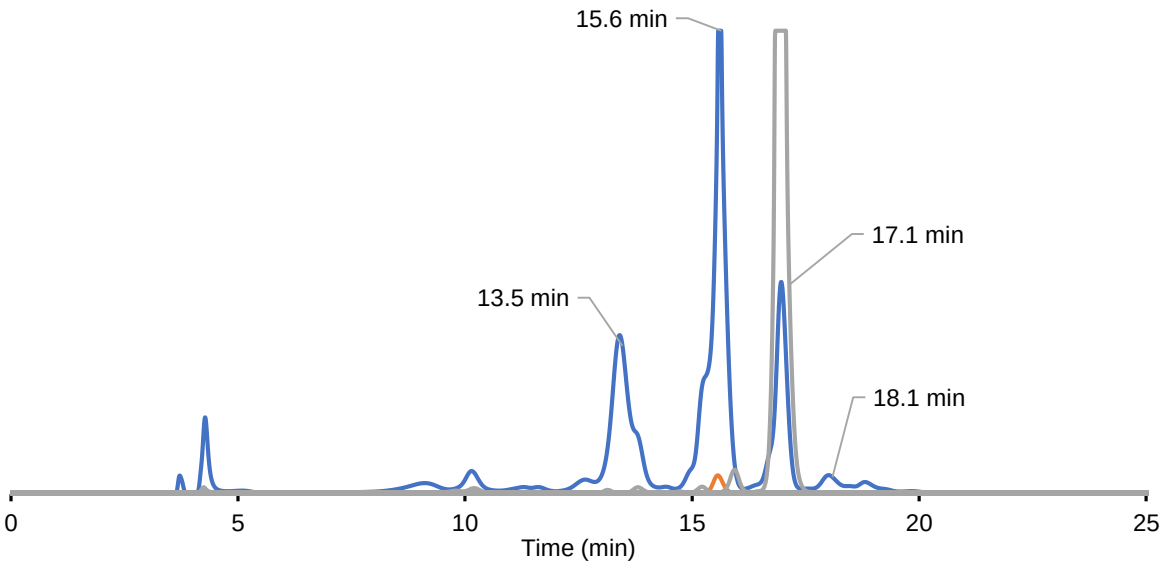


Figure **S133**: HPLC profile of the crude after cleavage from the solid supports obtained in synthesis of **S5** (blue) (oxidation time: 10s) using the gradient of solution B in solution A: from 0 to 0.1 min at 5 % B, in 20 min to 20 % B, then in 10 min to 30%. The HPLC traces obtained for reference conjugates TTTTC (grey) and 5´-phosphorylated-TTTTC (orange) under identical conditions are also included. No conjugate **S5** could be identified (Figure S15). All peaks at lower retention times show masses (*m/z*) lower than that expected for TTTTC.

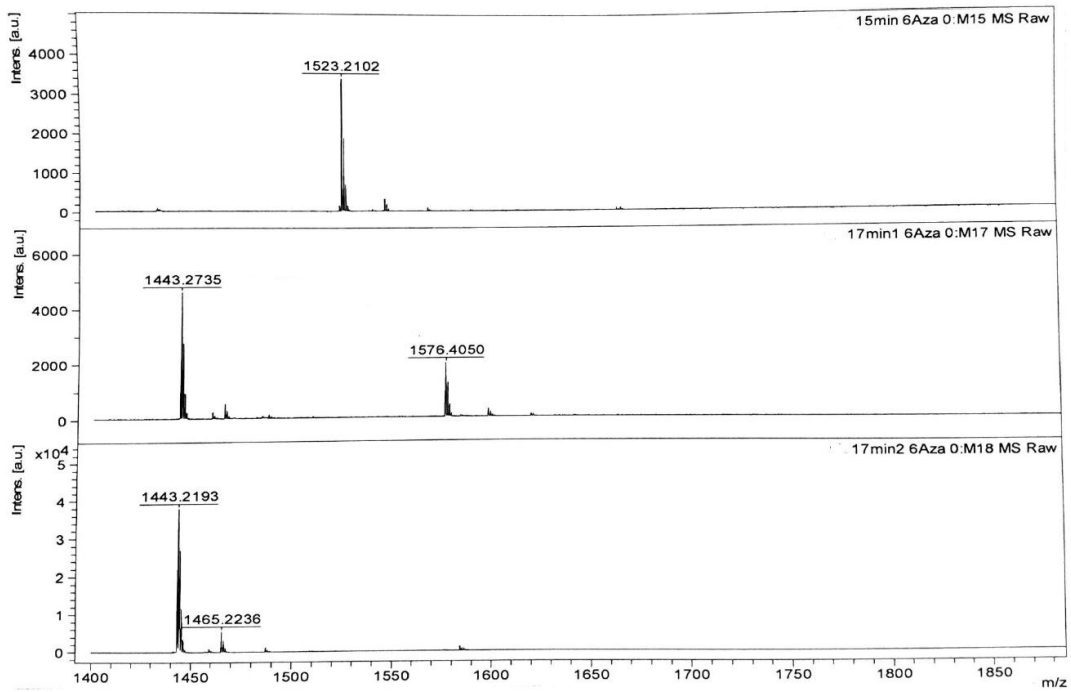


Figure **S144**: MALDI-TOF mass spectra of the selected HPLC fractions from the HPLC shown in Figure S14. The measured masses (*m/z*) could be assigned to unmodified strand (TTTTC, expected *m/z* 1443.99 Da) and 5´-phosphorylated TTTTC (expected *m/z* 1523.97 Da).

One possible explanation of why synthesis of conjugate **S5** failed could be the sensitivity of B-C bond in **S5** to the oxidative conditions used in solid phase DNA synthesis. To test whether this could be the case, we investigated the stability of model compound **11** having a B-C bond towards the oxidative conditions (Figure S15).

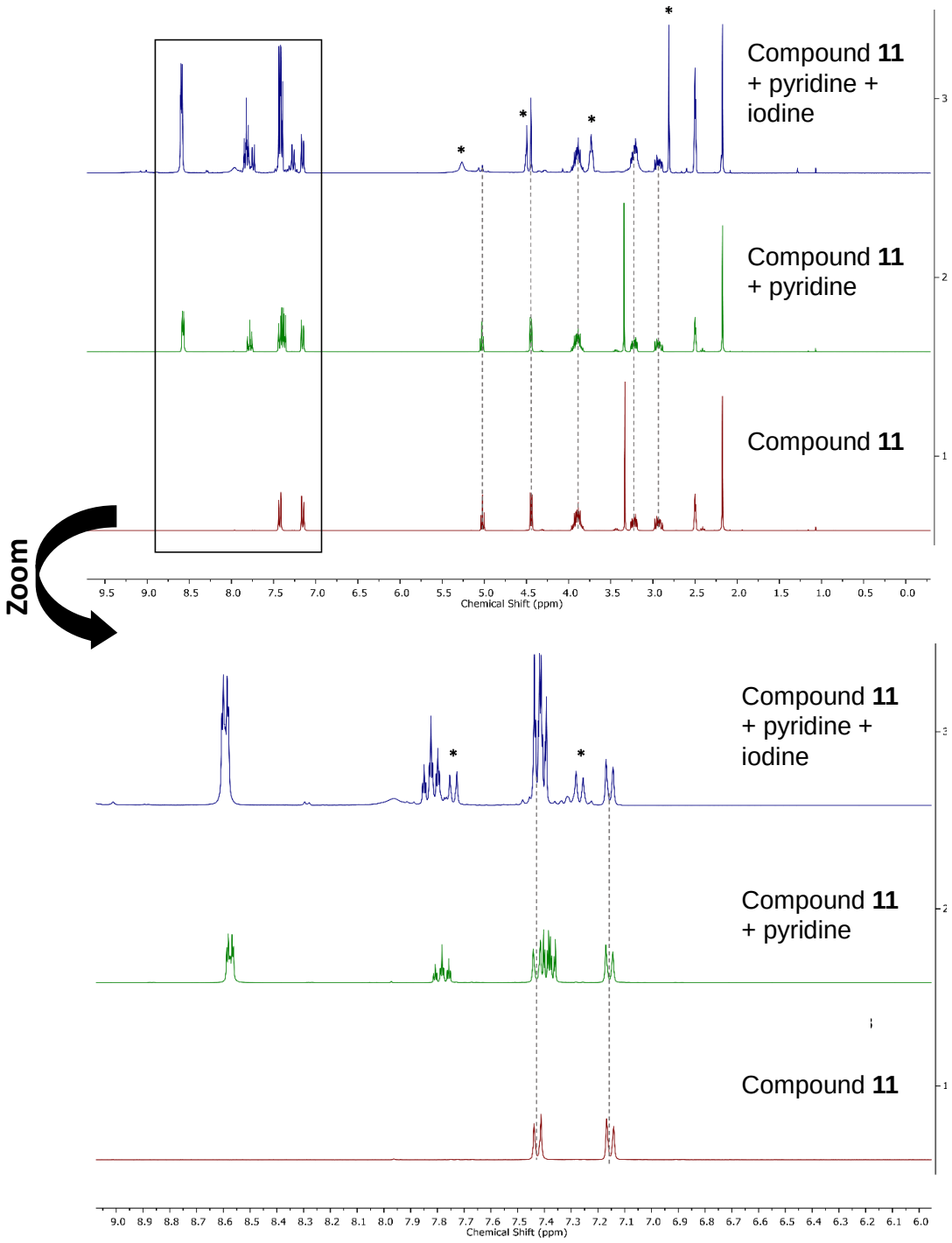


Figure **S155**: Monitoring stability of **11** (56 mM) under different conditions using ^{1}H NMR spectroscopy. Solvent: DMSO-d^{6} (300 MHz). Measurements were performed at 22°C. Upper diagram: Full spectra, lower diagram: zoom in of the aromatic region. Peaks marked with a (*) appear during treatment of **11** with the oxidant.

A protocol of the experiment (Figure S15): compound **11** (8 mg, 34 µmol) was dissolved in DMSO-d^{6} (600 µL). After recording of ^{1}H NMR spectrum, pyridine (4 µL) was added, and the spectrum was measured again. Then, iodine (8.6 mg, 34 µmol) was added, the NMR tube was ultrasonicated for 20s and the spectrum was measured. All NMRs were recorded at 22°C. Oxidation of **11** with iodine causes changes in the area of aromatic signals. Furthermore, few new peaks appear in the aliphatic region (Figure S15, marked with *). As a new set of signals

appear, formation of a new species can be assumed. However, unambiguous identification of these species was not possible.

Synthesis of DNA **14**:

We first tested whether a B-C bond is stable towards 3-ethoxy-1,2,4-thiazoline-5-one (EDITH) to evaluate whether the synthesis of DNA **14** is possible in principle. For this test, we used model compound **11**. In particular, compound **11** (8 mg, 34 µmol) was mixed with EDITH (9.1 mg, 34 µmol) in DMSO-d^6 (600 µL) and stability of **11** was monitored by ^{1}H NMR spectroscopy (Figure S16). We observed that compound **11** is stable in the presence of EDITH. This result suggested that EDITH is better suited than iodine as oxidizing agent for solid phase DNA synthesis.

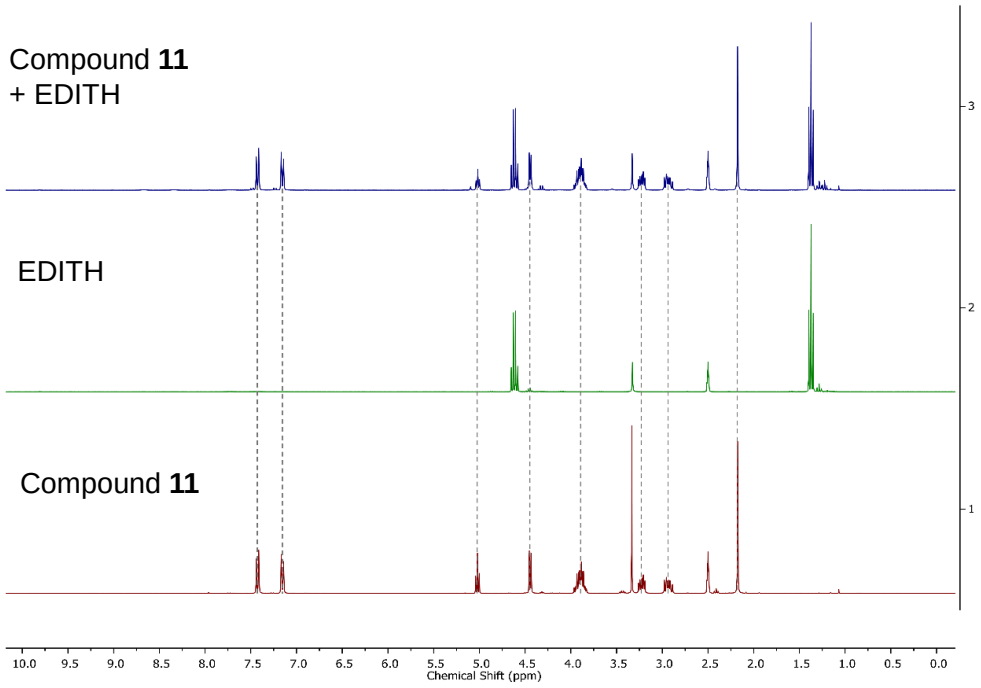


Figure **S166**: Monitoring stability of **11** (56 mM) under different conditions using ^{1}H NMR spectroscopy. Solvent: DMSO-d^6 (300 MHz). Measurements were performed at 22°C.

Synthesis of the DNA part for DNA **14** as well as the final manual coupling were performed analogously to the protocol described above for conjugate **S5**. For the oxidation, a solution of EDITH in acetonitrile (0.05 M) was used. The oxidation was performed at the DNA synthesizer for 30s. After washing with acetonitrile, the solid support was cleaved and deprotected by the treatment with aqueous ammonia solution (25 %, 0.15 mL) for 2 h at 55 °C. Then, the solid phase and the solution were separated, the aqueous ammonia solution was diluted with HPLC grade water (0.7 mL) and evaporated by lyophilization. The crude DNA mixture was dissolved in water and purified by reversed phase HPLC using the gradient of solution B in solution A: from 0 to 0.1 min at 5 % B, in 20 min to 20 % B, then in 10 min to 30%.

Yield: 4.25 %.

Extinction coefficient at 260 nm (ε) = 47 400 L/(mol*cm)

MALDI-TOF mass spectrum (negative mode): calculated for $C_{56}H_{73}BN_{11}O_{26}P_5S$ $[M\text{-}H^+]^-$: 1829.2786 *m/z*, found (using THAP-based matrix): 1829.3768 *m/z* (elution at ~17 min) and 1829.3915 *m/z* (elution at ~18 min) (Figures S17, S18).

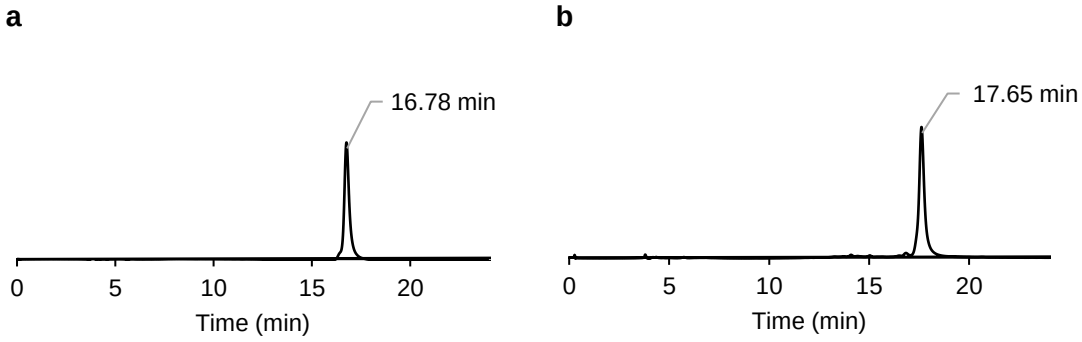


Figure **S17**: Analytical HPLCs of isomers of conjugate **DNA 14**. Gradient of solution B in solution A: 0 - 30 min to 30 % B. Two peaks were collected having the same mass (*m/z*) (Figure S19) suggesting that both possible isomers could be isolated.

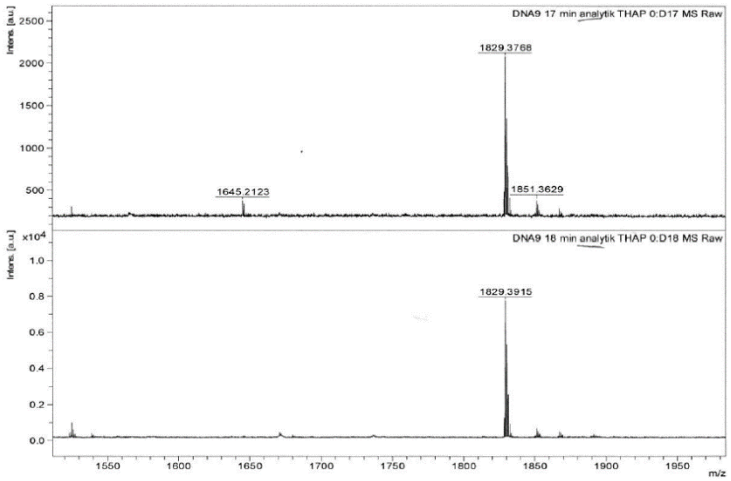
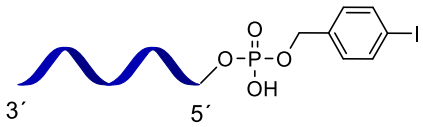


Figure **S18**: MALDI-TOF mass spectra of isomers of conjugate **DNA 14.** Upper diagram: HPLC peak eluted at ~17 min, lower diagram: (b) HPLC peak eluted at ~18 min.

Synthesis of **DNA 15**



DNA 15

Solid support dC (bz) (20 mg) was used. The DNA strand was synthesized at standard conditions using a DNA synthesizer. The phosphoramidite **S4** was coupled manually over 10 min using a solution of phosphoramidite **S4** in dry acetonitrile (0.1 mL, 0.1 M) and a solution of ETT activator in acetonitrile (0.1 mL, 0.5 M). Afterwards the solution mixture was removed, the solid support washed with dry acetonitrile and the coupling was repeated. Oxidation was performed on the synthesizer. Cleavage from the solid support and deprotection were performed by the treatment with aqueous ammonia solution (25%, 0.15 mL) for 2 h at 55 °C. After separation of the solid phase and the solution, the aqueous ammonia solution was diluted with HPLC grade water (0.7 mL) and removed via lyophilization. The crude DNA mixture was dissolved with water and purified by HPLC using the gradient of solution B in solution A: 0 – 30 min to 30 % B.

Yield: 8.47 %.

Extinction coefficient at 260 nm (ε) = 47 500 L/(mol*cm)

MALDI-TOF mass spectrum (negative mode): calculated for $C_{56}H_{71}IN_{11}O_{35}P_5$ $[M]^-$: 1739.1847 *m/z*, found (matrix based on THAP): 1739.2000 *m/z* (Figures S19, S20).

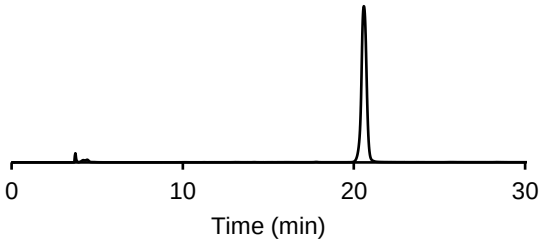


Figure **S19**: Analytical HPLC profile of conjugate **DNA 15**. Gradient of solution B in solution A: 0 – 30 min to 30% B.

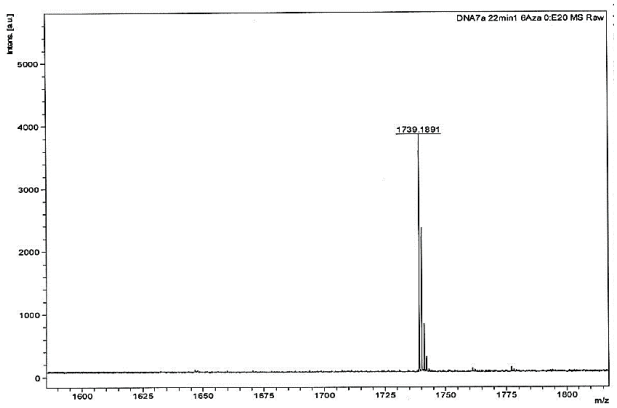
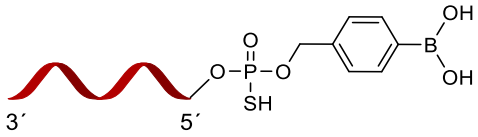


Figure **S20**: MALDI-TOS mass spectrum of conjugate **DNA 15**.

1.4.2 5´-modified guide RNA strands for KIF11-siRNAs

Synthesis of **RNA 7a**



RNA 7a

Solid support dT(bz) 1000Å (25 – 30 µmol/g) was used. The RNA strand was synthesized at standard conditions using a DNA synthesizer. The phosphoramidite 4BB-Pm* was coupled manually over 10 min using a solution of phosphoramidite 4BB-Pm* in dry acetonitrile (0.1 mL, 0.1 M) and a solution of ETT activator in acetonitrile (0.15 mL, 0.5 M). Afterwards the solution mixture was removed, and oxidation was performed with a solution of EDITH in acetonitrile (0.05 M) for 30s. Manual coupling and oxidations were repeated two times. The solid support was dried under N_2 flow for 5 min. Then, ethylenediamine was added to the column under N_2. The closed column was incubated for 2 h. Afterwards, the ethylenediamine was removed and the column was washed with water free acetonitrile. After short drying by N_2 – flushing, the RNA was removed from the column by pressing 2 mL 1x PBS solution through. The collected solution was lyophilized, and the gained solid was dissolved in HPLC grade water. The crude RNA mixture was then purified by HPLC using the gradient of solution B in solution A: from 0 to 1 min 1% B, 1 - 10 min to 10% B, 10 min at 10% B, in 10 min to 20% B, 5 min at 20% B (Figure S21).

Yield: 2.85 %

Extinction coefficient at 260 nm (ε) = 216 300 L/(mol*cm)

MALDI-TOF mass spectrometry (negative mode): calculated for $C_{213}H_{260}BN_{69}O_{157}P_{21}S$ $[M+citrate]^-$: 6992.66 *m/z*, found (matrix based on 3-HPA): 6991.84 *m/z* (Figure S22).

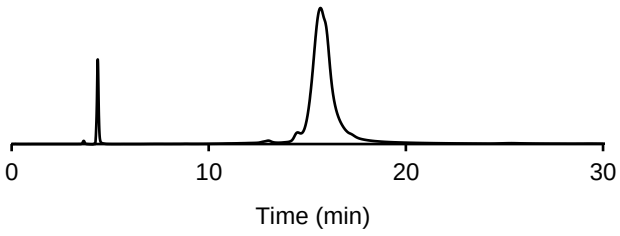


Figure **S21**: Analytical HPLC profile of conjugate **RNA 7a**.

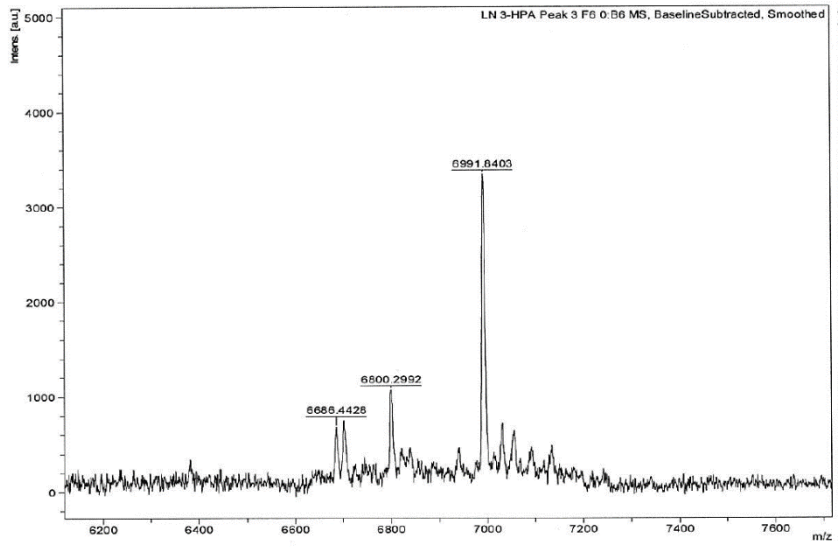
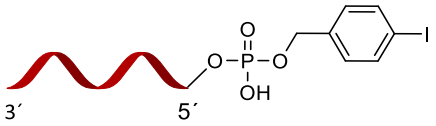
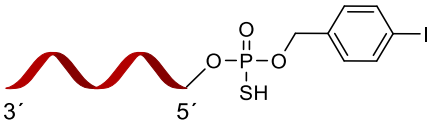


Figure **S22**: MALDI-TOF mass of conjugate **RNA 7a**.

Synthesis of **RNA 16** and **RNA 17**



RNA16



RNA17

Solid support dT(bz) 1000Å (25 – 35 µmol/g) was used. The RNA strand was synthesized at standard conditions using a DNA synthesizer. The phosphoramidite **S4** was coupled manually for 30 min using a solution of phosphoramidite **S4** in dry acetonitrile (0.1 mL, 0.1 M) and a solution of ETT activator in acetonitrile (0.15 mL, 0.5 M). Afterwards the solution mixture was removed, and oxidation was performed. For **RNA 16** standard iodine solution was used for oxidation with 10s oxidation time, while a solution of EDITH in acetonitrile (0.05 M) was used for **RNA 17** with 20s oxidation time. The manual coupling and oxidation were repeated two times. Further steps were analogous to those used for synthesis of **RNA 7a**. The crude RNA mixture was purified by HPLC using the gradient of solution B in solution A. For conjugate **RNA 16**: 0 to 1 min to 5 % B, in 24 min - to 25 % B; for conjugate **RNA 17**: from 0 to 1 min at 5% B, in 29 min – to 25% B (Figure S23).

Yields: RNA 16 (2.07 %), **RNA 17** (1.1 %)

Extinction coefficient at 260 nm (ε) = 216 400 L/(mol*cm)

MALDI-TOF mass spectrum (negative mode): **RNA 16** calculated for $C_{207}H_{255}IN_{69}O_{151}P_{21}$ $[M]^-$: 6903.00 *m/z*, found (matrix based on 3-HPA): 6902.46 *m/z*. **RNA 17** calculated for $C_{207}H_{255}IN_{69}O_{150}P_{21}S$ $[M]^-$: 6919.06 *m/z*, found (matrix based on 3-HPA): 6918.51 *m/z* (Figure S24).

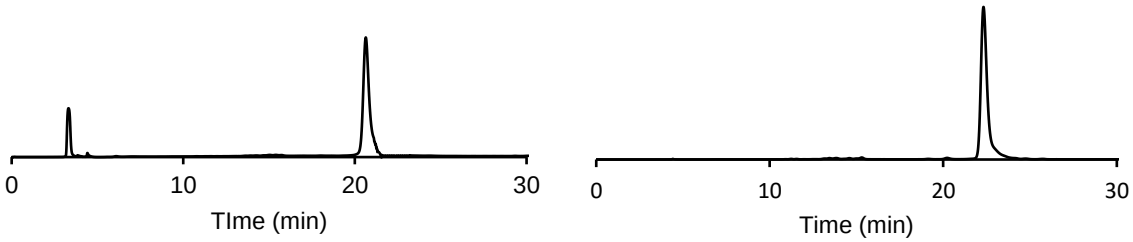


Figure **S23**: Analytical HPLC profiles of conjugates **RNA 16** and **RNA 17**.

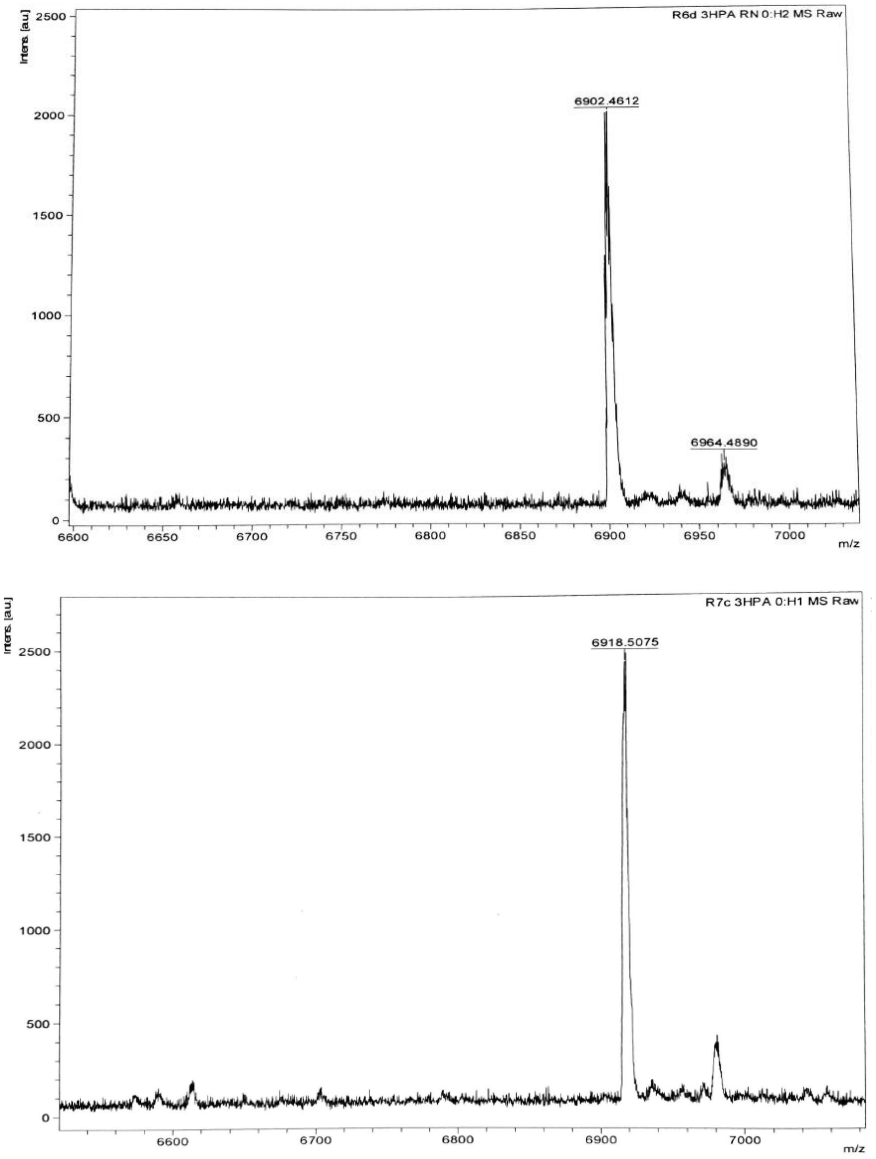


Figure **S24**: MALDI-TOF mass spectra of **RNA 16** (upper) and **RNA 17** (lower).

2 Reactions of ROS-responsive DNAs and RNAs as well as controls with H_2O_2 in cell free settings

DNA and RNA conjugates were dissolved in phosphate buffered saline (PBS) (10 mM, pH 7.4) containing NaCl (150 mM) in low µM range. H_2O_2 was added to these solutions to final concentrations of either 10 or 200 mM. After mixing, reaction vials were shaken at 35°C for 1 - 4 hours. Cleavage of DNAs and RNAs was monitored by HPLC. The HPLC fractions were collected and analyzed by MALDI-TOF mass spectrometry.

2.1 Experiments with conjugates carrying a 5'-4BB group

DNA **14** (Figures S25-S27):

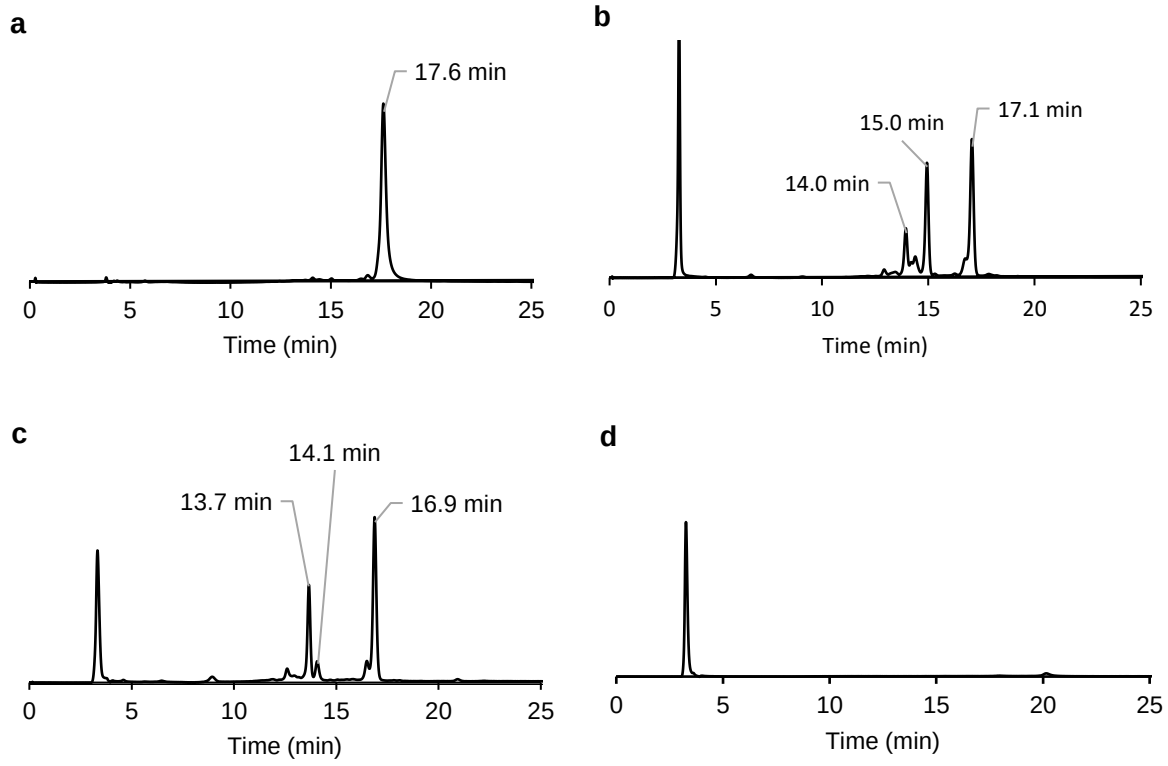


Figure **S25**: Monitoring cleavage of DNA **14** (130 µM, elution time 17.6 min, HPLC of the pure compound is shown in trace a) in the presence of H_2O_2 (200 mM) in water (trace b) and PBS buffer (trace c). Incubation time - 1h. The experiment was conducted at 35 °C. HPLC of H_2O_2 (200 mM) is shown in trace d.

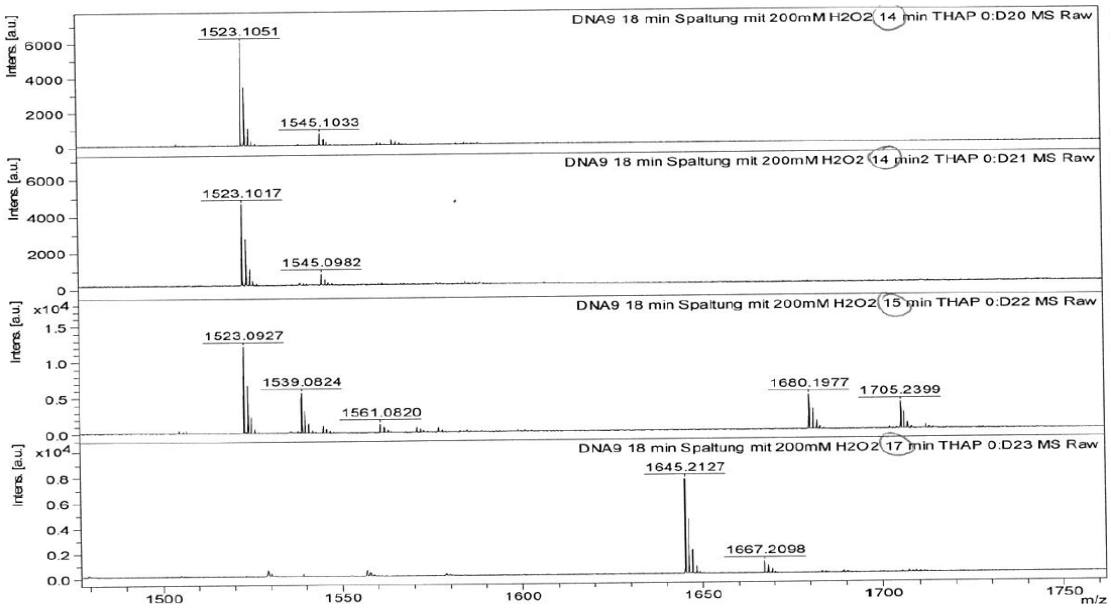
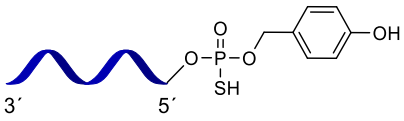
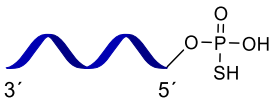


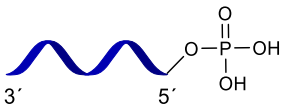
Figure **S26**: MALDI-TOF mass spectra of fractions from HPLC trace b (Figure S25). Upper two spectra: two fractions at ~14 min elution time. The second lowest spectrum: a fraction at 15 min elution time. The lowest spectrum: a fraction at 17.0 min elution time. Found and calculated *m/z* values of the identified products are shown in Figure S28.



found: 1645.21 m/z
calculated: 1645.26 m/z
for $C_{56}H_{72}N_{11}O_{35}P_5S$ $[M]^-$



found: 1539.21 m/z
calculated: 1539.08 m/z
for $C_{49}H_{66}N_{11}O_{34}P_5S$ $[M]^-$



found: 1523.10 m/z
calculated: 1523.24 m/z
for $C_{49}H_{66}N_{11}O_{35}P_5$ $[M]^-$

Figure **S27**: Found and calculated *m/z* values of the identified products in MALDI-TOF mass spectra shown in Figure S27.

RNA **7a** (Figures S28-S30):

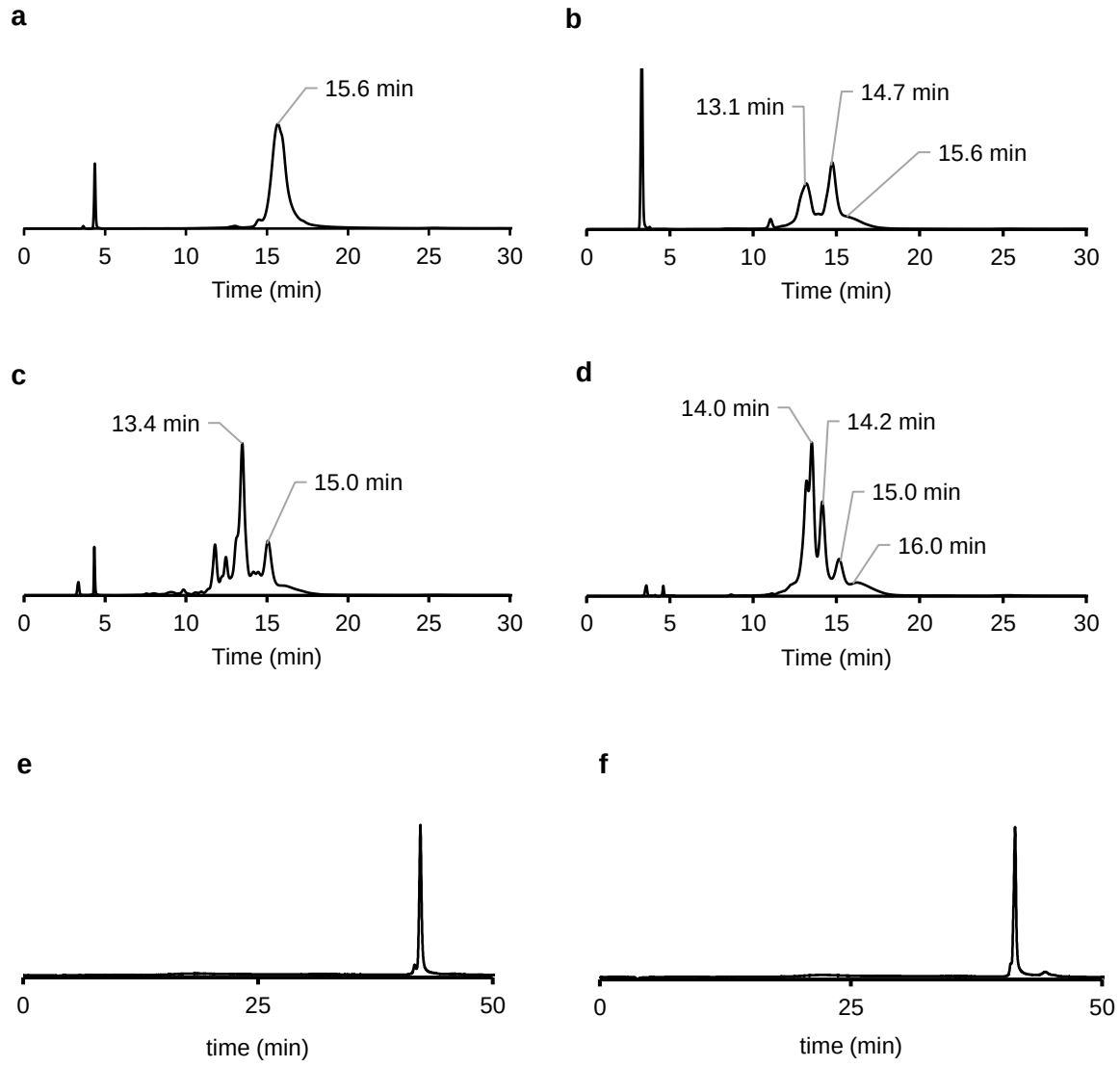


Figure **S28**: (a)-(d) Monitoring cleavage of RNA **7a** (65 µM, elution time 15.6 min, HPLC of the pure compound is shown in trace a) in PBS buffer (35 °C) at different conditions: trace b - in the presence of H_2O_2 (200 mM), 1 h incubation, trace c - in the presence of H_2O_2 (10 mM), 1 h incubation, trace d - in the presence of H_2O_2 (10 mM), 4 h incubation. (e), (f) Monitoring stability of RNA **7a** (2.7 µM) dissolved in PBS buffer (10 mM, pH 7.4) containing NaCl (150 mM). After mixing, the reaction vial was incubated at 22 °C for 0 h (e) and 24 h (f), after which aliquots (100 µL) were subjected to HPLC using a gradient of solution B in solution A: from 0 to 50 min from 0% to 20%.

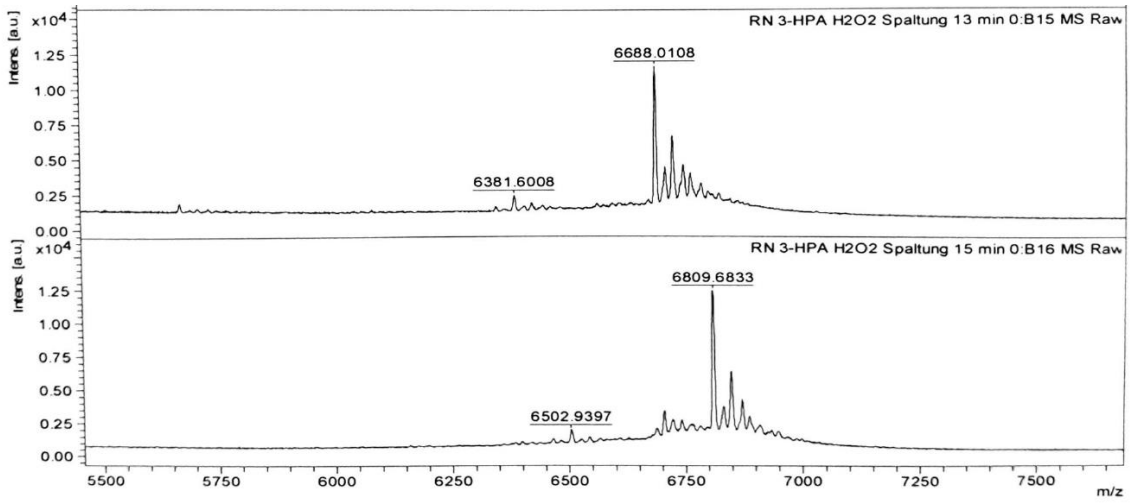


Figure **S29**: MALDI-TOF mass spectra of fractions from HPLC trace b (Figure S28). Upper spectrum: a fraction at ~13-14 min elution time. Lower spectrum: a fraction at 15 min elution time. Found and calculated *m/z* values of the identified products are shown in Figure S31.

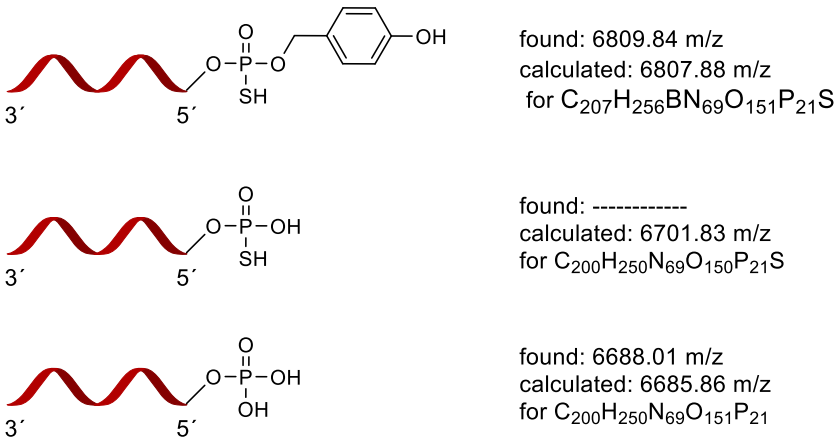


Figure **S29**: Found and calculated *m/z* values of the identified products in MALDI-TOF mass spectra shown in Figure S30.

2.2 Experiments with conjugates carrying a ROS resistant 5'-cap

DNA **15** (Figure S31):

First test cleavages were performed with the **DNA 15**. The cleavage was performed with 200 mM H_2O_2 in PBS at 35°C for 1 hour (Figure S31).

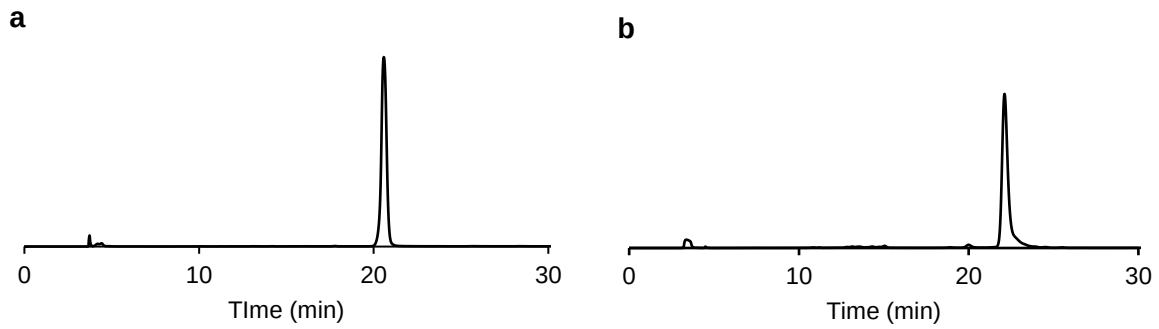


Figure **S30**: Monitoring cleavage of DNA **15** (130 μM, elution time ~21 min, HPLC of the pure compound is shown in trace a) in the presence of H_2O_2 (200 mM) in PBS buffer, incubation time - 1 h, at 35 °C.

RNA **16** and RNA **17** (Figure S32)

Cleavage of conjugate **RNA 16** (a and b) and **RNA 17** (c and d) were performed for 1 hour with either 10 mM or 200 mM H_2O_2 (Figure S32). No cleavage could be observed. For conjugate **RNA 17** a small portion of the thiophosphate bond was converted to phosphate.

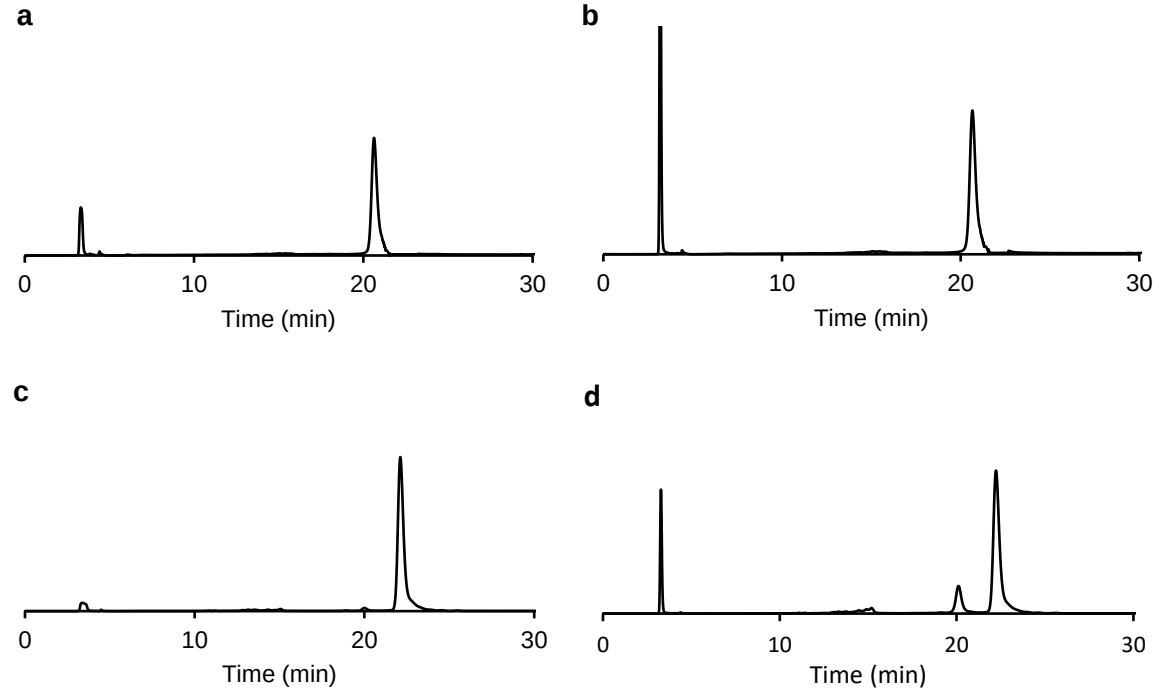


Figure **S31**: HPLC-monitoring cleavage of RNA **16** (8 μM, elution time ~20-21 min) in PBS buffer (pH 7.4) in the presence of H_2O_2 (10 mM – trace a or 200 mM – trace b), incubation time - 1 h, at 35 °C. Related data for RNA **17** (11 μM, elution time ~22 min) are shown in traces c (H_2O_2, 10 mM) and d (H_2O_2, 200 mM). H_2O_2 at 200 mM (trace d) causes partial conversion of RNA **17** (elution time ~22 min) to RNA 10 (elution time ~20-21 min).

3 Cell tests

3.1 Cells and Cell Culture

Human ovarian cancer cell line A2780 was purchased from Sigma-Aldrich and cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with foetal bovine serum (FBS, 10%), penicillin/streptomycin (1%) and L-Glutamine (1%). Murine liver cell lines - normal AML12 (ATCC number: CRL-2254) and hepatoma Hepa 1-6 (Provided by Prof. O. Dontsova, Moscow State University, Russia) were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Thermo Scientific Gibco), supplemented with 10% fetal bovine serum (Thermo Scientific Gibco) and 1% penicillin-streptomycin (Thermo Scientific Gibco) at 37 °C and 5% CO_2. Cells were cultivated to around 80% confluence.

3.2 Transfection

A2780 were seeded in 6-well plates (200 cells/µL, 2 mL cultivation medium containing FBS (5%), penicillin/streptomycin (1%) and L-Glutamine (1%) per well). For the transfection, 10 pmol (for 5 nM end concentration) or 1 pmol (for 0.5 nM end concentration) siRNA in biograde water (10 µL) were diluted with Gibco™ Opti-MEM™ Reduced Serum Media (Opti-MEM) (40 µL). In addition, Lipofectamine™ RNAiMAX (1.5 µL) (Thermo Fisher Scientific™, Waltham, MA, USA) was diluted in Opti-MEM (48.5 µL) and incubated for 5 min at 22 °C before both solutions were combined. The transfection solution was then incubated for 20 min at 22 °C, while recurring shaking every 5 min. The cells were washed twice with PBS (2.5 mL per well) and a fresh portion of RPMI 1640 medium containing FBS (5%), penicillin/streptomycin (1%) and L-Glutamine (1%, 1.9 mL per well) was added. Finally, the 100 µL transfection solution was added to the cells. The samples were incubated for 38 h.

For dose dependence test AML12 and Hepa1-6 cells were seeded one day before the transfection in 24-well plates plates (70000 cells/µL, 0,5 mL cultivation medium containing FBS (5%), penicillin/streptomycin (1%) and L-Glutamine (1%) per well). siRNAs **23/22** and **7b/22** were pre-mixed with Lipofectmine RNAi (ThermoFisher Scientific), respectively, and opti-MEM reduced serum medium (ThermoFisher Scientific) according to manufacturer's protocol. To perform cell transfection premix was added to adherent cells in the final concentration of siRNAs 2; 0.2; 0.02; 0.002 nM. Cells were incubated for 1 day to measure siRNA efficacy by RT-qPCR.

3.3 Relative gene expression quantification via RT-qPCR

To isolate total RNA from cells, the cultivation medium was removed, the cells washed with DPBS and TRI reagent® (1 mL/well) was added. After 5 min incubation at 22 °C, the cell lysate was transferred into 1.5 mL Eppendorf tubes and the aqueous phase of the samples was extracted by adding chloroform (200 µL) followed by thorough mixing and centrifugation (12,000x g, 4 °C, 15 min). The aqueous phase was mixed with ice-cold ethanol (400 µL) and transferred onto Zymo-Spin IC Columns. The samples were subsequently centrifuged (1 min, 8,000x g, 4 °C). The eluate was discarded. The RNA on the column was washed with first sodium acetate buffer (3 M, 500 µL, pH=5.2) and secondly with ethanol (500 µL, 75 %, v/v, in water), by solvent addition, subsequent centrifugation as above and eluate removal. Finally, pure ethanol (500 µL) was added and the samples centrifuged (3 min, 10,000x g, 4 °C) to dry the column. The RNA was eluted by adding RNase-free water (25 µL), incubation for 5 min at 22°C and subsequent centrifugation (2 min, 8,000x g, 4 °C). To transcribe the isolated RNA into cDNA, RNA (1 µg in 12.5 µL RNase-free water) was incubated (65 °C for 5 min) with Random Hexamer Primer (1 µL). Subsequently, reverse transcriptase (0.5 µL), RT 5x buffer (4 µL) and dNTPs (2 µL) were added per sample. Finally, the reverse transcription protocol (10 min at 25 °C; 60 min at 42 °C; 10 min at 70 °C; forever at 4 °C) was run at the Dual Block Gradient PCR Thermal Cycler. For the relative quantification of the genes of interest, LightCycler® 480 SYBR® Green Master (5 µL) was mixed with a primer pair (1 µL) and cDNA (4 µL, 1:10 diluted) and subjected to the LightCycler® 480. The applied primer sequences were CAGCTGAAAAGGAAACAGCC; ATGAACAATCCACACCAGCA for KIF11 and TGGGCAAGAACACGATGATG; TGAGGTCCTCCTTGGTGAACA for the housekeeping gene RPLP0. The reaction mixtures were initially heated at 95 °C for 5 min, followed by 45 cycles, which consisted of 10 s at 95 °C, 30°s 60 °C, and 10 s at 72 °C. The data were normalized to the Ct value of the RPLP0 cDNA in the same sample. For the evaluation of the fold change, the $2^{-\Delta Ct}$ value was calculated. The $2^{-\Delta Ct}$ value of untreated cells was set to 100. Three independent experiments were performed and the mean ± SD calculated (Figure 3, main text).

3.4 Evaluation of siRNA activity on cell cycle arrest in A2780 cells

Cells depleted of KIF11 fail to establish a functioning spindle apparatus, resulting in a cell cycle arrest in the G2 phase. To evaluate the activity of the siRNAs on a protein level, the proportion of cells in the different phases of the cell cycle was determined. 38 h after transfection, the cells were washed twice with PBS, detached from the microtiter plate with the trypsin/EDTA solution (0.25% trypsin, v/v, 0.5 mL)), re-suspended in growth medium (5% FBS, 1 mL), and transferred into 1.5 mL Eppendorf tubes. The cells were then centrifuged for 3 min at 1000 rpm

and the cell pellet was washed with DPBS (0.5 mL). After centrifuging once again, the cells were re-suspended in cold PBS (4 °C, 0.4 mL). Subsequently, ice-cold ethanol (0.8 mL) was added to each sample dropwise over 1 min during gentle vortexing. For fixation and membrane permeabilization, the samples were incubated for 4 h at 4 °C. The cells were once again pelleted and washed with DPBS (0.5 mL), before resuspension in PI staining solution (10 µg/mL RNase A, 5 µg/mL propidium iodide (PI) in DPBS; 0.2 mL) and incubation for 30 min at 37 °C. The fluorescence of the DNA-intercalating PI was subsequently measured by flow cytometry (λ_{ex} = 488 nm) with the yellow filter (λ_{em} = 583/26 nm). For data evaluation, the inCyte™ software package from Merck Millipore and the ModFit LT™ 5.0 software from Verity Software House was used. The ratio of the cells in the G2 and the G1G0 phase was determined, and the means and the standard deviations of three independent experiments, which all consist of duplicates, were calculated (Figure 3, main text).

3.5 Evaluation of siRNA activity by microscopy

As previously described [DOI: 10.1101/gr.215103.116], the depletion of KIF11 results in a distinct prometaphase arrest phenotype, which can be detected by Hoechst33342 staining. A2780 cells (80 cells/µL in 500 µL cultivation medium) were seeded in 35 mm imaging dishes (µ- Dish 35 mm, high, ibidi GmbH, Germany). The next day, the cells were transfected as described above with 0.5 nM of either unmodified KIF11 siRNA **19**/**18** (unmod.) or siRNA **7a**/**18** or siRNA **17**/**18**. 38 h after transfection, the cells were washed twice with PBS (2.5 mL) and stained with Hoechst33342 (1 µg/mL, in 2 mLcultivation medium (5% FBS)) for 20 min at 37 °C, 5% CO_2. Subsequently, the cells were washed again twice with PBS (2.5 mL). Confocal fluorescent images were taken with a Zeiss Spinning Disk Axio Observer Z1 with Plan-Apochromat 63x/1.40 oil objective in spinning disc confocal mode using an Axiocam 506 mono from ZEISS (Germany) (λ_{ex} = 405 nm; λ_{em} = 450/50 nm). Representative images are shown in Figure 3, main text.

3.6 Measurement of ROS level in cells

AML12 and Hepa 1-6 cells were plated on 12-well plates at 90,000 cells/well for Hepa 1-6 and 80,000 cells/well for AML-12. 72h later, cells were harvested with Tripsin 0,25%. 1 mL of DMEM 10% FBS was added to each well, and transferred to a 5 mL Facs tube. After that cells were centrifuged at 300g for 7 min and left in 100 µL of media. 400 µL of 50 µg/ml DCFDA in HBSS was added to each tube, and incubated 40 min at 37 ^{o}C. The cells were washed with PBS and analyzed by flow cytometry on a BD Fortessa.

4 In vivo studies

4.1 siRNA synthesis, purification and LC-MS analysis

Previously we have designed and validated chemically modified siRNAs in vitro and in vivo that target murine eEF2 mRNA [https://doi.org/10.1038/s41598-020-72399-4]. Chemical modifications of siRNA increase stability against nucleases and reduce immune response that is crucial for in vivo studies [doi: 10.1038/nmeth911, doi: 10.1261/rna.30706]. We used eEF2 siRNA **23**/**22** as a positive control, while siRNA that targets the Firefly Luciferase gene as a negative control (Table S1).

Oligonucleotides were prepared on an MM-12 synthesizer (Bioautomation) with the phosphoramidite method, according to the manufacturer's recommendations at 1 µmol scale.

Protected thymidine 3'-phosphoramidite, 2'-OMe nucleoside 3'-phosphoramidites, 2'-TBDMS nucleoside 3'-phosphoramidites, Unylinker-CPG (500Å), DDTT and S-ethylthio-1H-tetrazole were purchased from ChemGenes. Coupling of phenylboronic phosphoramidite **4** (0.1 M) was performed during 10 min, average conversion exceeded 85-90%. Oligonucleotides were cleaved from the support and deprotected using AMA – 1:1 (v/v) concentrated aqueous ammonia and 40% aq. methylamine (500 µL) for 3 h at 65 °C. Next, the solid support was filtered and washed with DMSO (3×200 µl). Then the combined solution was evaporated to dryness, the gum was dissolved in the mixture of trimethylamine trihydrofluoride (150 µl) and DMSO (100 µl) and heated at 60°C for 3.5 h. Crude RNAs were precipitated by the addition of 20 µL of 3 M sodium acetate followed by 1 mL of butanol-1. Solids were separated by centrifugation at 10,000 g and washed thoroughly with ethanol (3×1 ml).

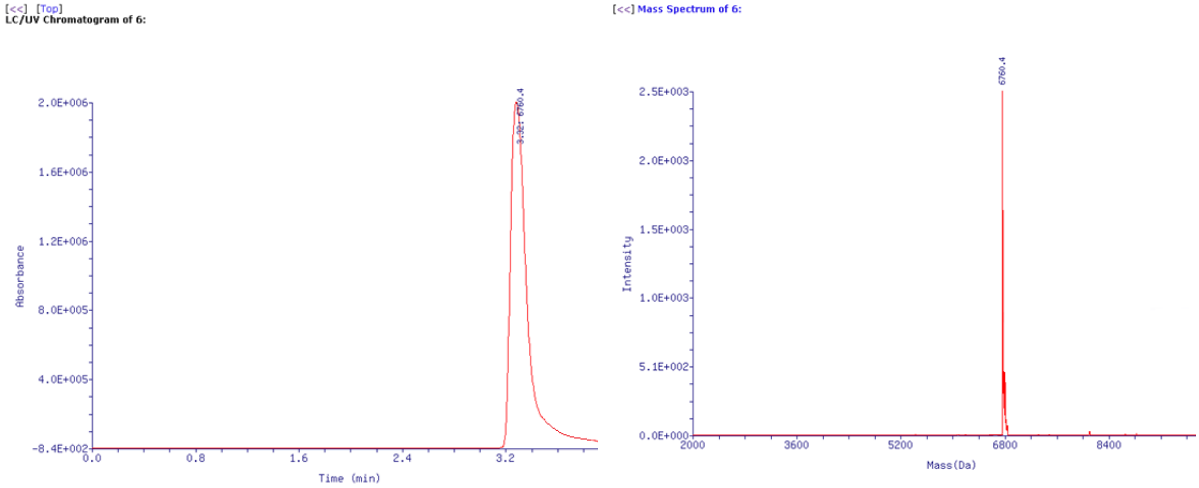
Oligonucleotides were purified by double HPLC. The IE-HPLC purification of oligonucleotides was carried out on a 7.7×75 mm TSKgel SuperQ-5PW column (13 µm, Tosoh); buffer A: 20 mM Tris-HCl (pH 7.5), 10% MeCN; buffer B: 20 mM Tris-HCl (pH 7.5), 600 mM sodium perchlorate, 10% MeCN; a gradient of B: 0-5% (1 CV), 5-25% (1 CV), 25-55% (10 CV); a flow rate of 1.1 mL/min; temperature 45°C. Fractions were analyzed by LC-MS (Thermo Scientific Ultimate 3000-LCQ system). Fractions with the full-length oligonucleotide (with purity more than 95%) were pooled and partially evaporated (~10X). Oligonucleotides were precipitated by excess of acetone (5X) and solids were separated by centrifugation at 10,000 g. The RP-HPLC purification of the oligonucleotides was carried out on a 4.6×250 mm Jupiter C18 column (5 µm, Phenomenex); buffer A: 0.05 M ammonium acetate (pH 7); buffer B: 0.03 M ammonium acetate (pH 7), 80% MeCN; a linear gradient of B: 0-35% (8 CV); a flow rate of 1 mL/min; temperature 45°C. Fractions were analyzed by LC-MS (Thermo Scientific Ultimate 3000-LCQ system). Fractions with the full-length oligonucleotide (with purity more than 95%) were pooled and evaporated. Oligonucleotides were dissolved in DNAse/RNAse-free water and the amount of material was quantified by UV-absorbance at 260 nm.

LC-MS analysis for the oligonucleotides was performed using Thermo Scientific LCQ Fleet with Dionex Ultimate 3000 HPLC system. The HPLC instrument was equipped with the 2.1×50 mm Jupiter C18 column (5 µm, Phenomenex); buffer A: 10 mM diisopropylamine, 15 mM 1,1,1,3,3,3-hexafluoroisopropanol; buffer B: 10 mM diisopropylamine, 15 mM 1,1,1,3,3,3-hexafluoroisopropanol, 80% MeCN. Elution was done in a step mode - salts were washed out with buffer A (4 CV) followed by a step of 100% buffer B (2 CV) with a flow rate of 0.3 mL/min; temperature 45 °C. The MS analysis of the oligonucleotides was carried out in negative mode (capillary voltage 3500 V, dry temp 160 °C), and raw spectra were deconvoluted using ProMass software (ENovatia, USA).

Table **S1:** List of eEF2 siRNAs used in the study including analytical LC-MS data (Upper case – ribonucleotide, lower case – 2'-O-methylribonucleotide, s –phosphorothioate, T – thymidine).

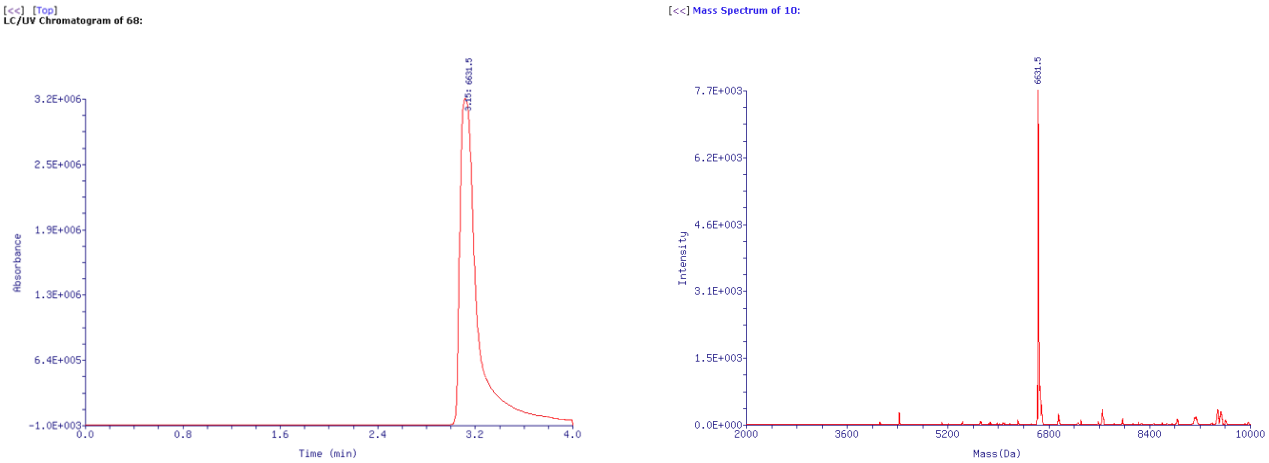
RNA **22** (eEF2 sense) cAGCcAAGCUGAUCGAGAATsT

Molecular mass (calculated/found) 6759.3/6760.4



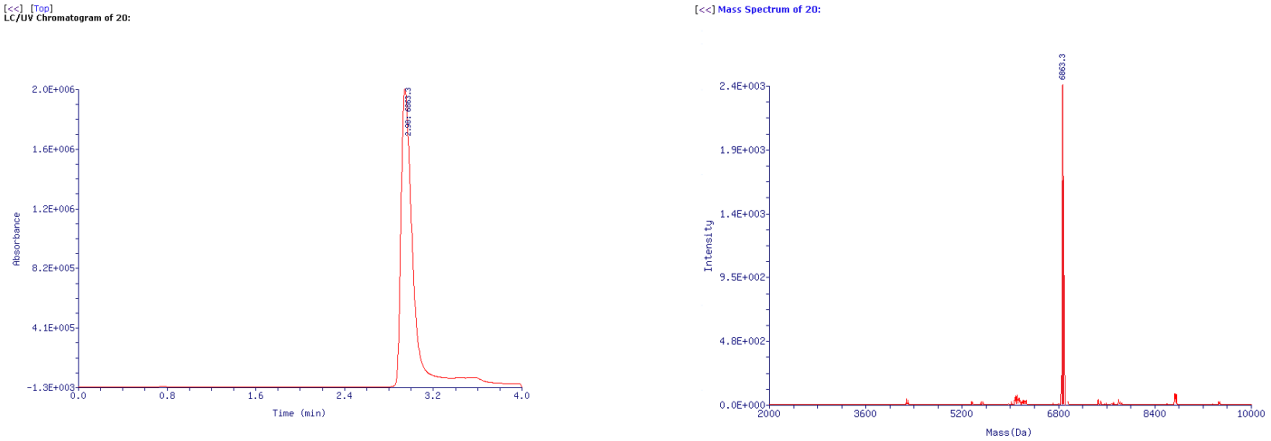
RNA **23** (eEF2 antisense) UUCUCGAUcAGCUUGGCUGTsT

Molecular mass (calculated/found) 6630.1/6631.5



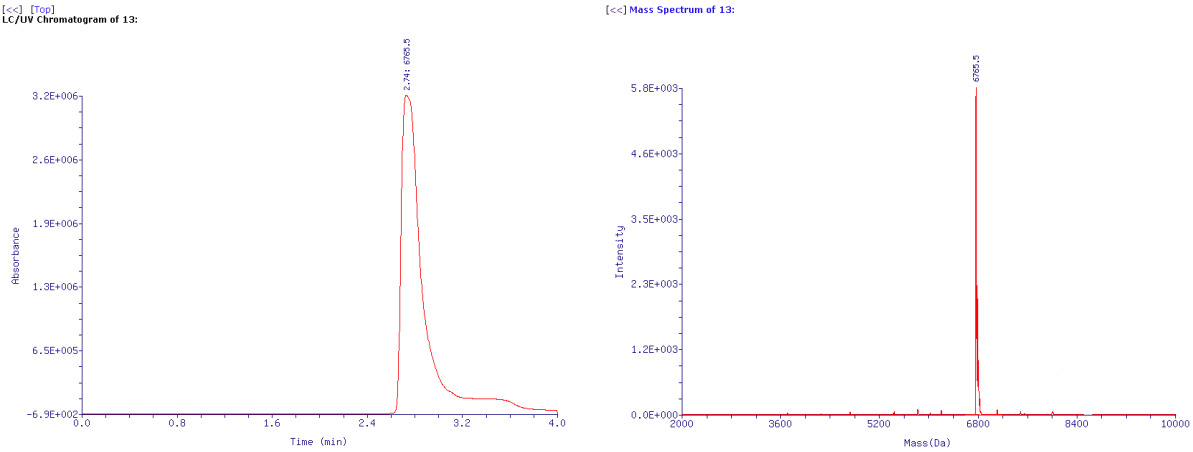
RNA **7b** (eEF2-M sense) 4BB-P-UUCUCGAUcAGCUUGGCUGTsT

Molecular mass (calculated/found) 6882.3/6883.3



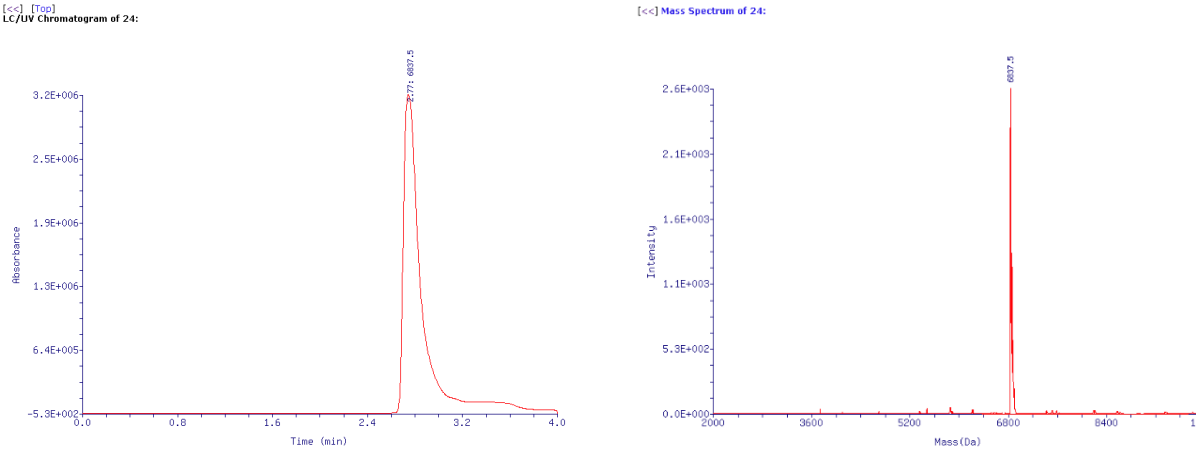
RNA **21** (Luc sense) cuUaCgCuGaGuAcUuCgATsT

Molecular mass (calculated/found) 6763.3/6765.5



RNA **20** (Luc antisense) UCgAaGuAcUcAgCgUaAgTsT

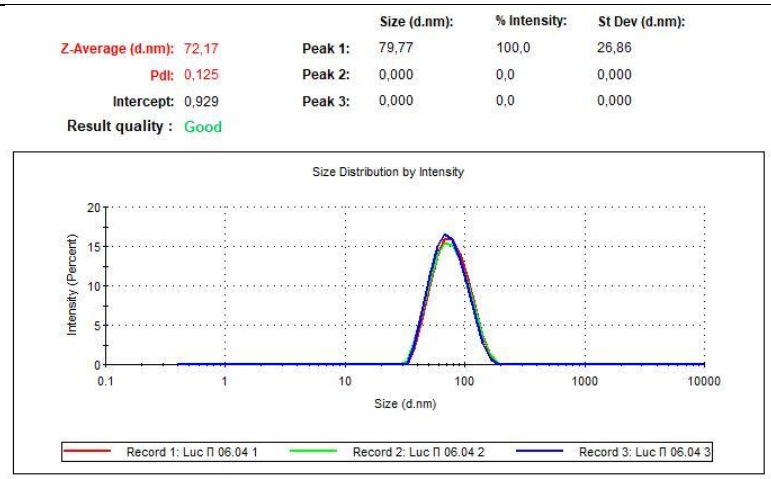
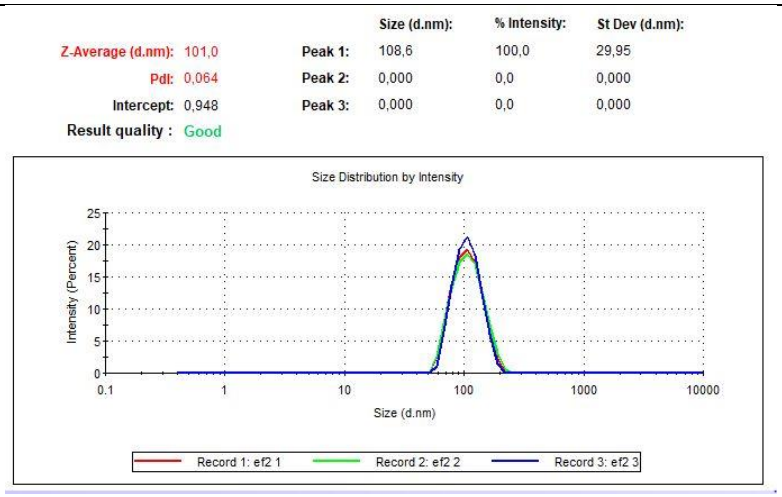
Molecular mass (calculated/found) 6835.4/6837.5

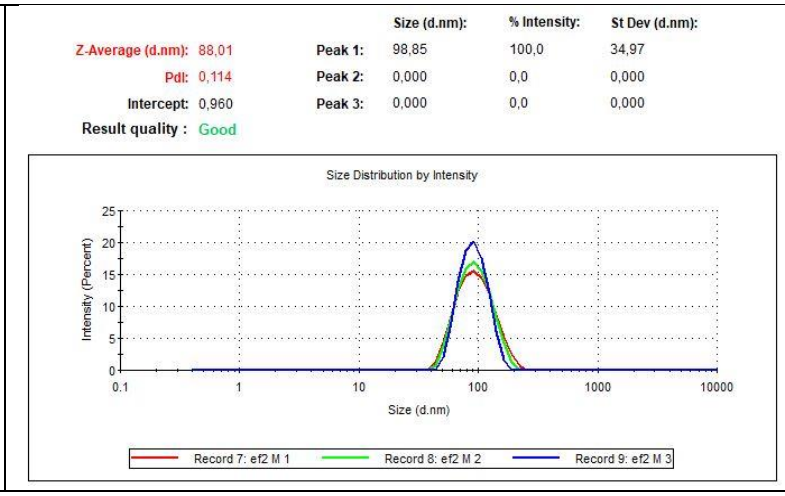


4.2 siRNA formulation in LNP

The eEF2 siRNA **23/22**, modified eEF2 siRNAs **7b/22** (#5 and #7) and the negative control siRNA were formulated in lipid nanoparticles (LNP) as previously described [doi: 10.1002/anie.201203263, doi: 10.1007/978-1-4939-0363-4_6]. LNP's were dialyzed against PBS at pH7.4 in 20'000 MW cut off dialysis cassettes overnight and filtered through a PES syringe filter (0.2 µm pores). Particle size analysis was carried out using a Zetasizer Nano ZSP (Malvern Panalytical, USA) according to the manufacturer's protocol. siRNA entrapment efficiency was determined (Table S2) using the Quant-iT™ RiboGreen® reagent (Thermo Fisher Scientific R11491) as described earlier [doi: 10.3390/pharmaceutics12111095].

Table **S2:** Characterization of LNP-siRNA used in the study.

	LNP size	siRNA conc., ng/µl	siRNA entrapment efficiency
LNP-control		137	63%
LNP-eEF2 siRNA **23/22**		111	51%

LNP-eEF2 siRNA **7b/22**		125	57%

4.3 RNA isolation and RT-qPCR

Total RNA was isolated from the cell and liver tissue samples using TRIzol (ThermoFisher Scientific, USA) according to the manufacturer's instructions. ~0.5-1 μg of RNA was further treated with DNase I (ThermoFisher Scientific, USA), supplied with RiboLock RNase Inhibitor (40 U/μL) to the final concentration 0.4 U/μL. For RT-qPCR, treated total RNA was used to synthesize cDNA using Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA), followed by qPCR using PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, USA). PCRs were performed using the primers listed in table S3. β-actin mRNA was used as a control for the analysis of total RNA

Table **S3:** List of primers used in the study.

Name	Forward, 5'→3'	Reverse, 5'→3'
β-actin	CTC TGG CTC CTA GCA CCA TGA AG	GTA AAA CGC AGC TCA GTA ACA GTC CG
eEF2	CCT GTA TGC CAG TGT GCT GA	CCA CCT GGG ACT CCT CAA AC

Upper case – 2'-deoxynucleotide.

4.4 Study of knockdown of eEF2 gene in healthy mice

All animal care and procedures were carried out according to the relevant National Institutes of Health guidelines and were approved by the Ethical Committee of the Pirogov Russian National Research Medical University (Moscow, Russia). Fvb mice (age - 6–8 weeks, males) were purchased from Stolbovaya Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency, Russia. Mice were housed at 22°C using a 12-h-light to 12-h-dark cycle, fed ad libitum with regular rodent chow. Lipid nanoparticles with siRNAs were diluted in sterile saline at different doses (0,01; 0,02; 0,05; 0,1 mg/kg) and injected

intravenously via tail vein at doses and regimes specified in the text (5 mice per group). Mice were sacrificed after 48 h and liver samples were collected for analysis. Liver samples were snap frozen, grounded and potions of homogenized liver were used for further mRNA analysis.

4.5 Animal Care and Treatments

All animal care and procedures were carried out according to the relevant National Institute of Health guidelines and were approved by the Bioethics Committee of Pirogov Russian National Research Medical University (Moscow, Russia), where the animal study was performed. Mice were housed at 22°C using a 12-h-light to 12-h-dark cycle, fed ad libitum with regular rodent chow. To study ROS-dependent activation of modified siRNA we performed partial hepatectomy as described previously with minor modifications [doi: 10.1038/nprot.2008.80].

We injected siRNA-lipid nanoparticles to FvB male mice (6-8 week old) at the dose 0,02 mg/kg. In 24h after injection we performed partial hepatectomy to induce ROS and activation of the 5'-modified siRNA **7b**/**22**. Briefly, mice were anesthetized (2% isoflurane), shaved and sanitized with 70% ethanol. Incision was made along the midline of abdomen, median lobe of liver was exposed and ligated with 4-0 silk suture and removed with scissors. After that peritoneum was closed by 4-0 suture needle and the skin was fixed by 7 mm wound clips. In 24 h after hepatectomy (48 after injection of siRNAs) mice were sacrificed, livers were harvested, frozen in liquid nitrogen and stored at -80°C for further mRNA analysis.

4.6 Statistical analysis of the experimental data

All diagrams are based on at least three independent experiments. Statistical data processing was performed using the GraphPad Prism software (version 8.3) with a t-test analysis. The data were considered statistically significant at $p < 0.05$.