Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Chemo-enzymatic Approach to specifically click-modified RNA molecules

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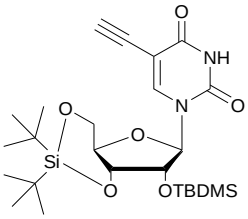
I. Experimental Procedures for the Synthesis of modified Nucleosides

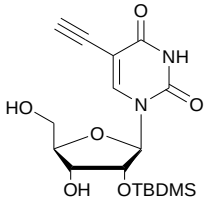
General information: All reagents were purchased from Sigma–Aldrich or Acros and were used as received. Dry solvents were taken from a *solvent purification system SPS 800* von MBraun or purchased and stored over molecular sieves. Whenever required, reactions were performed under an argon atmosphere using standard Schlenk-technique. For reaction monitoring thin-layer chromatography aluminium sheets from Merck (Kieselgel 60 F_{254}) were used. Flash chromatography was performed on silica gel 60 from Merck or octadecyl-modified silica gel from Macherey-Nagel. ^{1}H- and ^{13}C-NMR spectra were recorded with Avance-II-300- or Avance-II-500-NMR-spectrometer. Chemical shifts are reported in parts per million relative to the residual solvent signal.[1] High-resolution mass spectra (HRMS) were obtained by electrospray ionization (ESI) with a LTQ FT Ultra™ spectrometer. Compound **2**,[2] THPTA,[3] FAM and Dabcyl azide,[4] teab buffer[5] as well as the used phosphoramidites[6, 7] were synthesized and produced according to literature, teaa buffer was prepared by dissolving 5.6 ml glacial acetic acid in 950 ml water. Analytical HPLC-UV-MS was performed with an *Agilent 1100* series system equipped with a VL-Quadrupol mass spectrometer, a UV-detector (λ = 260 nm) and a Polaris-C18-A column from Varian (5 µm, tempered to 55 °C), the flow rate was set to 0.3 ml min^{-1}.

2'-*O*-*tert*-Butyldimethylsilyl-3',5'-*O*-bis(*tert*-butyl)silyl-5-ethinyl uridine 3



Di-*tert*-butylsilybis(trifluoromethanesulfonate (217 mg, 0.49 mmol) was added dropwise to a cooled solution of 5-ethinyl uridine **2** (120 mg, 0.45 mmol) in 0.9 ml dmf. After stirring for 60 min at 0 °C, imidazole (152 mg, 2.25 mmol) was added, the ice-bath was removed and the solution was stirred for further 25 min. Upon addition of *tert*-butyldimethylsilyl chloride (73.8 mg, 0.49 mmol) the solution was heated to 60 °C for 2.5 h, cooled to room temperature within 30 min and finally quenched with 20 ml 0.1 M aq. HCl solution. The aqueous phase was extracted with 3 × 100 ml CH_2Cl_2. The combined organic layers were washed with 100 ml saturated $NaHCO_3$ solution, dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by flash chromatography (0-2.5% meoh in CH_2Cl_2) to yield **3** as a colorless solid (162 mg, 0.31 mmol, 69 %). | $C_{25}H_{42}N_2O_6Si_2$ | R_f = 0.43, 5 % MeOH in CH_2Cl_2 | ^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 0.12 (s, 3H, Si-CH_3), 0.17 (s, 3H, Si-CH_3), 0.91 (s, 9H, ^{t}BuSi-CH_3), 1.01 (s, 9H, ^{t}BuSi-CH_3), 1.04 (s, 9H, ^{t}BuSi-CH_3), 3.19 (s, 1H, ≡CH), 3.83 (dd, J = 9.7, 4.7 Hz, 1H, H5'), 3.96 – 4.02 (m, 1H, H3'), 4.17 (td, J = 10.2, 5.2 Hz, 1H, H4'), 4.27 (d, J = 4.6 Hz, 1H, H2'), 4.51 (dd, J = 9.3, 5.1 Hz, 1H, H5'), 5.64 (s, 1H, H1'), 7.54 (s, 1H, H6), 9.54 (s, 1H, 3NH) ppm | ^{13}C-NMR ($CDCl_3$, 126 MHz): δ = -5.1 (Si-CH_3), -4.3 (Si-CH_3), 18.1 (^{t}BuSi-C_q), 20.3 (^{t}BuSi-C_q), 22.7 (^{t}BuSi-C_q), 3 × 25.8 (^{t}BuSi-CH_3), 3 × 26.9 (^{t}BuSi-CH_3), 3 × 27.4 (^{t}BuSi-CH_3), 67.5 (C5'), 74.4 (≡CH), 74.6 (C2'), 75.3 (C3'), 75.9 (C4'), 82.2 (≡C_q), 93.9 (C1'), 99.1 (C5), 142.8 (C6), 148.7 (C2) 161.3 (C4) ppm | ESI-HRMS m/z calc. for $[C_{25}H_{41}N_2O_6Si_2]^-$: 521.2503, found 521.2508

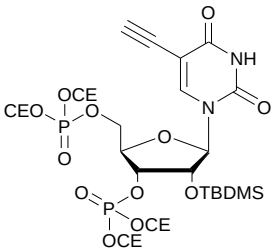
2'-*O*-tert-Butyldimethylsilyl-5-ethinyl uridine



HF × pyridine (28.3 µl, 70 % HF) was diluted with 400 µl dry pyridine and added slowly to an ice-cold solution of **3** (160 mg, 0.31 mmol) in 1.5 ml dry thf. Within 40 min the solution was warmed to room temperature, stopped by addition of 540 µl pyridine and diluted with 20 ml CH_2Cl_2 and 15 ml water. The aqueous phase was extracted with 3 × 30 ml CH_2Cl_2 and the combined extracts were washed with 20 ml saturated $NaHCO_3$ solution. The $NaHCO_3$ layer was extracted with 30 ml CH_2Cl_2 and the combined organic layers were dried over Na_2SO_4 and evaporated to dryness to afford 2'-*O*-tert-Butyldimethylsilyl-5-ethinyl uridine as a colorless solid (130 mg, 0.34 mmol, > 99 %). | $C_{17}H_{26}N_2O_6Si$

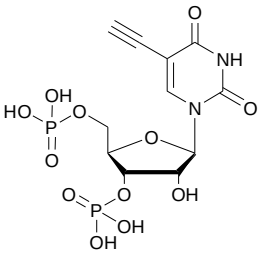
| R_f = 0.27, 2 % meoh in CH_2Cl_2 | ^{1}H-NMR (CD_3OD, 500 MHz): δ = 0.11 (s, 3H, Si-CH_3), 0.12 (s, 3H, Si-CH_3), 0.92 (s, 9H, ^{t}BuSi-CH_3), 3.76 (dd, J = 12.2, 2.4 Hz, 1H, H5'), 3.90 (dd, J = 12.2, 2.4 Hz, 1H, H5'), 4.03 – 4.06 (m, 1H, H3'), 4.12 (t, J = 5.0 Hz, 1H, H4'), 4.30 (t, J = 4.5 Hz, 1H, H2'), 5.88 (d, J = 4.2 Hz, 1H, H1'), 8.53 (s, 1H, H6) ppm | APT (CD_3OD, 126 MHz): δ = -5.2 (Si-CH_3), -5.0 (Si-CH_3), 18.7 (^{t}BuSi-C_q), 3 × 25.9 (^{t}BuSi-CH_3), 61.3 (C5'), 70.7 (C2'), 75.5 (≡CH), 77.4 (C3'), 82.5 (≡C_q), 86.0 (C4'), 90.5 (C1'), 99.4 (C5), 145.9 (C6), 150.9 (C2), 163.9 (C4) ppm | HPLC-MS m/z calc. for $[C_{17}H_{27}N_2O_6Si]^+$ 383.5, found 383.2

2'-*O*-tert-Butyldimethylsilyl-3',5'-*O,O*-bis[di(2-cyanoethyl)]phosphoryl-5-ethinyl uridine 4



Phosphoramidite $P(OCE)_2(NiPr_2)$ (193 mg, 0.71 mmol) and a tetrazole solution (1.59 ml, 0.71 mmol, 0.45 M in acetonitrile) were added dropwise to an ice-cooled suspension of 2'-O-tert-Butyldimethylsilyl-5-ethinyl uridine (90.8 mg, 0.24 mmol) in 0.6 ml dry CH_2Cl_2. After stirring for 2 h at 0 °C a solution of tert-Butyl hydroperoxide (0.26 ml, 1.43 mmol, 5-6 M in decan) was added. The solution was allowed to stir for 10 min at 0 °C and further 45 min at room temperature. The organic solvents were removed under reduced pressure (max 35 °C) and the resulting residue was purified by flash chromatography (0-5% meoh in CH_2Cl_2) to yield the phosphorylated compound **4** as a colorless solid (124 mg, 0.17 mmol, 69 %). | $C_{29}H_{40}N_6O_{12}P_2Si$ | R_f = 0.42, 5 % meOH in CH_2Cl_2 | ^{1}H-NMR (CD_3CN, 500 MHz): δ = 0.11 (s, 3H, Si-CH_3), 0.13 (s, 3H, Si-CH_3), 0.89 (s, 9H, ^{t}BuSi-CH_3), 2.82 – 2.86 (m, 8H, CH_2), 3.47 (s, 1H, ≡CH), 4.26 – 4.32 (m, 8H, O-CH_2), 4.38 – 4.43 (m, 1H, H5'), 4.43 – 4.50 (m, 3H, H3', H4', H5'), 4.80 (dt, J = 8.2, 5.0 Hz, 1H, H2'), 5.79 (d, J = 4.4 Hz, 1H, H1'), 7.90 (s, 1H, H6), 8.95 (s, 1H, 3NH) ppm | ^{13}C-NMR (CD_3CN, 126 MHz): δ = – 4.8 (Si-CH_3), – 4.7 (Si-CH_3), 18.6 (^{t}BuSi-C_q), 2 × 20.2 (d, J = 3.8 Hz, CH_2), 2 × 20.3 (d, J = 3.8 Hz, CH_2), 3 × 25.9 (^{t}BuSi-CH_3), 2 × 63.9 (d, J = 4.3 Hz, O-CH_2), 64.1 (d, J = 4.3 Hz, O-CH_2), 64.2 (d, J = 4.3 Hz, O-CH_2), 66.8 (d, J = 4.9 Hz, C5'), 74.7 (d, J = 4.9 Hz, C3'), 75.2 (d, J = 4.9 Hz, C2'), 76.0 (≡CH), 81.3 (dd, J = 8.2, 4.1 Hz, C4'), 83.1 (≡C_q), 90.3 (C1'), 99.7 (C5), 2 × 118.4 (CN), 2 × 118.4 (CN), 144.6 (C6), 150.3 (C2), 162.2 (C4) ppm | ^{31}P-NMR (CD_3CN, 162 MHz): δ = – 0.8, – 1.2 ppm | ESI-HRMS m/z calc. for $[C_{29}H_{40}N_6O_{12}P_2SiNa]^+$ 777.1846, found 777.1835

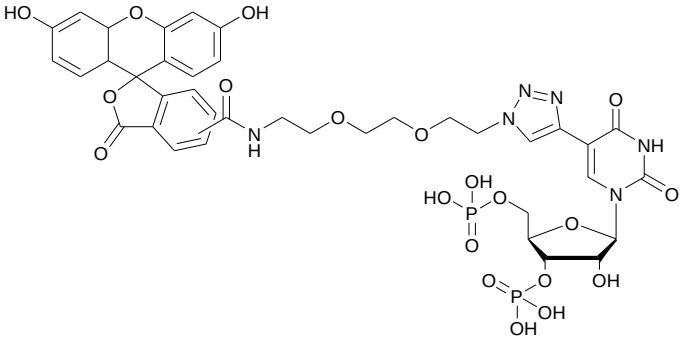
3',5'-*O,O*-Bisphosphate-5-ethinyl uridine 5 pUp^{Alk}



Substance **4** (101 mg, 0.13 mmol) was dried by co-evaporation with 2 × 5 ml dry pyridine and dissolved in 13 ml dry pyridine. To this solution 1.25 ml N,O-Bis(trimethyl silyl)acetamide and 120 µl DBU were added and the resulting solution was stirred for 5.5 h. The reaction was quenched with 25 ml water and diluted with 25 ml diethyl ether. The organic layer was extracted with 2 × 10 ml water and the combined aqueous phases were evaporated to dryness. The residue was treated with 3 × 5 ml toluene, 2 × 5 ml pyridine and dried in fine vacuum. In an argon atmosphere the brown oil was dissolved in 1 stirred for 20 h at room temperature. The reaction was stopped by slow addition of teab buffer (0.5 M, pH 7.5, vigorous foaming!). After 45 min stirring at room temperature the solvent was removed (< 35 °C). The final compound was isolated after purification by column chromatography on DEAE-Sephadex® (∅1.5 cm × 12 cm, conditioning in 0.3 M teab buffer, equilibrating in 0.3 M teab buffer, stepwise gradient 0.3-0.4-0.5…1.0 M). The product containing fractions were pooled, concentrated in vacuo and lyophilized to yield **pUp^{Alk}** as a colorless solid (101 mg, 0.11 mmol with 49 m% $NHEt_3^+$, 85 %). | $C_{11}H_{14}N_2O_{12}P_2$ | R_f = 0.5, 60 % ^{i}PrOH in water with

0.1 % 1 M aq. HCl-solution | ^{1}H-NMR (D_2O, 500 MHz): δ = 1.27 (t, J = 7.3 Hz, 29H, $NHEt_3^+$), 3.20 (q, J = 7.3 Hz, 15H, $NHEt_3^+$), 3.60 (s, 1H, ≡CH), 4.09 – 4.21 (m, 2H, H5'), 4.45 (d, J = 4.7 Hz, 2H, H3', H4'), 4.62 – 4.68 (m, 1H, H2'), 6.00 (d, J = 5.3 Hz, 1H, H1'), 8.24 (s, 1H, H6) ppm | 13C-NMR (D_2O, 126 MHz): δ = 8.2 ($NHEt_3^+$), 46.6 ($NHEt_3^+$), 63.8 (d, J = 4.2 Hz, C2'), 72.3 (d, J = 3.8 Hz, C5'), 73.5 (d, J = 2.6 Hz, C3'), 74.3 (≡CH), 83.0 (dd, J = 8.0, 5.9 Hz, C4'), 83.5 (≡C_q), 88.8 (C1'), 99.0 (C5), 145.4 (C6), 150.7 (C2), 164.6 (C4) ppm | ^{31}P-NMR (D_2O, 202 MHz): δ = 0.6, 1.0 ppm | ESI-HRMS m/z calc. for $[C_{11}H_{13}N_2O_{12}P_2]^-$ 426.9944, found 426.9948.

5(6)FAM-labeled 3',5'-*O,O*-Bisphosphat uridine pUp^{FAM}



In a 15 ml flask 3',5'-O,O-Bisphosphat-5-ethinyl uridine (30 mg with 43 m% $NHEt_3^+$, 0.04 mmol) was dissolved in 0.5 ml water and diluted with 6 ml of a dmso-water mixture (1:1, v/v). Azido labeled 5(6)-FAM **10** was added (103 µl, 82.4 µmol, 0.8 M in dmso) and the solution was heated to 40 °C. Then a portion of THPTA (208 mg, 0.48 mmol) was added. The reaction mixture was degassed for 30 sec with argon and sodium ascorbate (181 mg, 0.96 mmol) as well as copper sulfate pentahydrate (76.6 mg, 0.30 mmol) were added. The resulting solution was stirred for 2 d at 40 °C. Despite incomplete conversion of **5** the reaction was stopped by evaporation of the solvents to a minimum and impurities were removed by column chromatography on RP_{18}-modified silica gel (∅ 1 cm × 3 cm, conditioning in acetonitrile, equilibrating in 3 × 6 ml water with 0.1 % 1 M aqueous HCl, 0.1 % acetonitrile, stepwise gradient with increasing amounts of acetonitrile from 0.1-3-5-7.5 %). Pooling the product containing fractions yielded a yellow solid (~ 5 mg, 5.1 µmol, 13 %). | $C_{38}H_{38}N_6O_{20}P_2$ | R_f = 0.90, 0.1 % 1 M aqueous HCl 70 % ^{i}PrOH in water | ^{31}P-NMR (DMSO-d_6, 121 MHz): δ = – 0.1, – 0.2 ppm | ESI-HRMS m/z calc. for $[C_{38}H_{37}N_6O_{20}P_2]^-$ 959.1538, found 959.1549 | HPLC/UV/MS 13.92 and 14.90 min, Gradient 0-50 % B (98.9 % acetonitrile, 1 % water, 0.1 %) in A (98.9 % water, 1 % acetonitrile, 0.1 % HCOOH) within 20 min

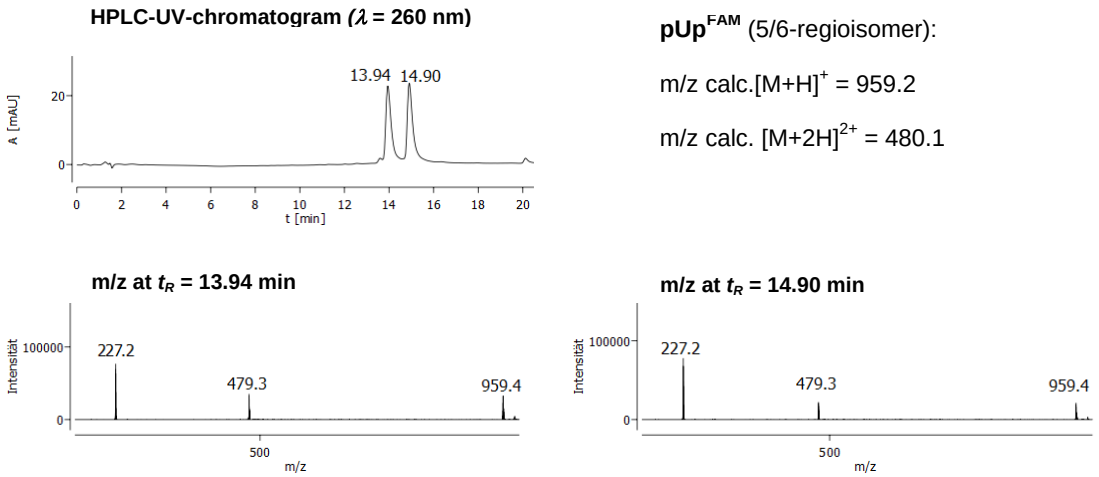
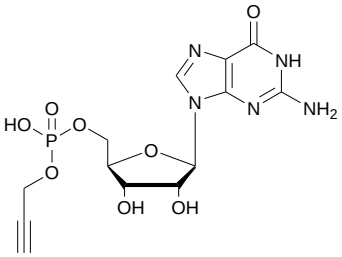


Fig. S1 HPLC-UV-chromatogram (λ = 260 nm) and m/z at retention times as specified

***O*-(5'-guanosine)-*O*-propargyl monophosphate 9 GMP^{Prg}**



To a suspension of protected guanosine **7** (760 mg, 2.01 mmol) in 5 ml dry CH_2Cl_2 phosphoramidite $P(OCE)(NiPr_2)_2$ (757 mg, 2.51 mmol) and tetrazole solution (5.58 ml, 2.51 mmol, 0.45 M in acetonitrile) were added at 0 °C. The reaction was allowed to warm to room temperature within 60 min and cooled again to 0 °C followed by the addition of 3-(Trimethylsilyl)propargyl alcohol (513 mg, 4.02 mmol) and a further portion of tetrazole solution (5.58 ml, 2.51 mmol, 0.45 M in acetonitrile). After stirring at room temperature for 60 min oxidation was initiated by addition of tert-Butyl hydroperoxide (1.10 ml, 6.03 mmol, 5 - 6 M in decan) at 0 °C. After stirring for 30 min at room temperature, the solution was diluted with 50 ml chloroform, consecutively washed with 0.3 % $NaHSO_3$, brine and water (each with 20 ml). The organic layer was separated, dried over Na_2SO_4 and concentrated in vacuo to a yellowish oil. The purification was effected by column chromatography on silica gel with a stepwise gradient 0-1.5-3 % meoh in CH_2Cl_2 and enabled the isolation of the fully protected intermediate **8** as a colorless solid (R_f = 0.24, 5 % meoh in CH_2Cl_2, 706 mg, 1.14 mmol, 57 %). For removal of the protecting groups **8** was dissolved in 10 ml 7 N ammonia solution in meoh and heated to 50 °C for 2 h without venting. Then the solution was allowed to cool to room temperature, stirred for further 20 h, evaporated to dryness and kept in fine vacuum overnight. The colorless solid was washed with 2 × 10 ml ethyl acetate and again dried in in fine vacuum for 12 h. The residue was suspended in 1.5 ml water, cooled to 0 °C and diluted with 1.5 ml trifluoracetic acid. After stirring the solution for 5 h at room temperature, all volatiles were removed and the crude product was dried in in fine vacuum overnight. A column chromatography on DEAE-Cellulose (∅ 1.5 cm × 7 cm, conditioning and equilibrating in 0.1 M teab buffer pH 7.7, stepwise gradient 0.1-0.2 M) ended in the collection of two product containing fractions which were concentrated in vacuo (< 35 °C) separately. Apart from varying amounts of triethylamine salt both were identical in accordance to NMR. The transcription starter **9** was isolated a yellowish solid (556 mg, 0.32 mmol with 77m% NEt_3, 16 % and 148 mg, 0.28 mmol, 25m% NEt_3, 14 %). | $C_{13}H_{16}N_5O_8P$ | R_f =0.71, 15 % water in ^{i}PrOH | ^{1}H-NMR (D_2O, 500 MHz): δ = 1.19 (t, J = 7.4 Hz, 119H, NEt_3), 2.83 (t, J = 2.4 Hz, 1H, ≡CH), 3.11 (q, J = 7.3 Hz, 79H, NEt_3), 4.01 – 4.11 (m, 2H, H5'), 4.25 (dt, J = 5.3, 2.8 Hz 1H, H4'), 4.37 (dd, J = 9.4, 2.4 Hz, 2H, O-CH_2), 4.39 – 4.42 (t, J = 4.8 Hz, 1H, H3'), 4.79 (m, 1H, H2'), 5.85 (d, J = 5.2 Hz, 1H, H1'), 8.26 (s, 1H, H8) ppm | ^{13}C-NMR (D_2O, 126 MHz): δ = 8.2 (NEt_3), 46.7 (NEt_3), 53.5 (d, J = 3.3 Hz, O-CH_2), 65.0 (d, J = 4.9 Hz, C5'), 70.4 (br, C3'), 73.6 (br, C2'), 75.6 (br, ≡CH), 79.1 (d, J = 7.6 Hz, ≡C_q), 83.6 (dd, J = 8.1, 2.1 Hz, C4'), 87.1 (C1'), 116.2 (C5), 137.4 (C8), 151.6 (C4), 153.8 (C2/6), 158.7 (C2/6) ppm | ^{31}P (D_2O, 202 MHz) δ = 0.5 ppm | ESI-HRMS m/z calc. for $[C_{13}H_{15}N_5O_8P]^-$ 400.0658, found 400.0662

II. Secondary Structures of pre-miRNAs

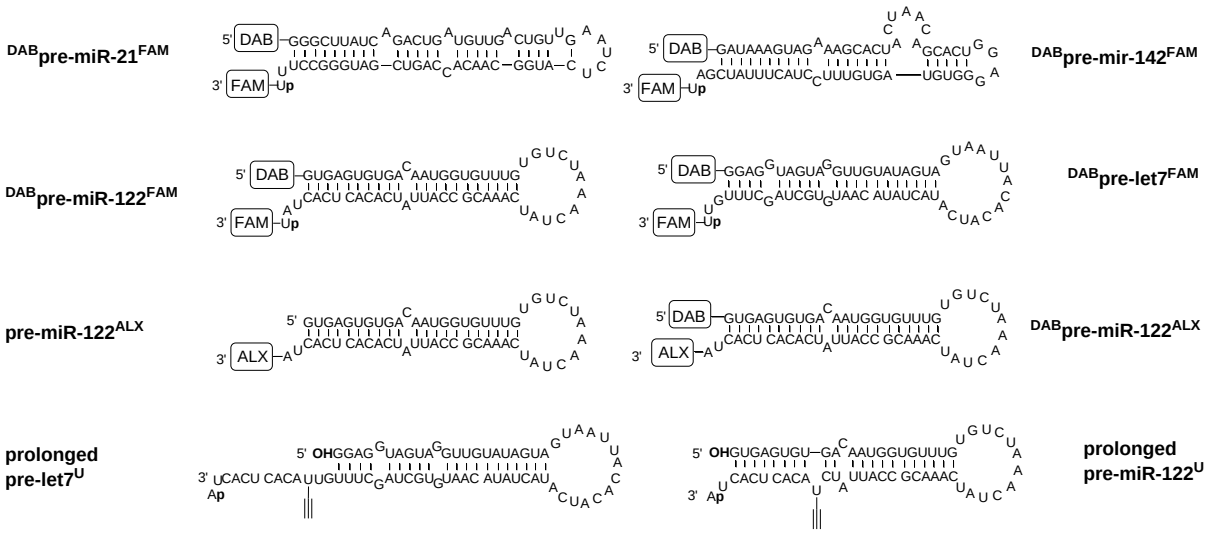


Fig. S3 Sequences of modified pre-miRNAs: Secondary Structures were modeled with the *Mfold* Web Server.[8] Original sequences were taken from miRBase.[9]

III. Experimental Procedures for Labeling of RNA

Plastic materials for RNA experiments were certified pyrogen/DNase/RNase free whenever possible. Deionized water was obtained from a membraPure Astacus purification system. All buffers were additionally sterile filtered through 0.22 µM filters. Filters, enzymes and reagents were purchased from the following providers: Amicon Ultra-0.5 ml centrifugal filters 3/10K (Merck Millipore), SYBR® Gold (Invitrogen), recombinant human Dicer (Genlantis), RiboLock® (Fermentas), Calf Intestinal Phosphatase/miRNA marker/low range ssRNA ladder/T4 RNA Ligase 1 (New England Biolabs), RiboMAX™ Large Scale RNA Production System-T7 (Promega). RNA analyses in terms of identity and concentration were based on ε_{260} and mass values which were calculated with the Oligo Analyzer 3.1, that is available online.[10] Mass detection was performed on an Agilent UHPLC system equipped with a BEH C18 column from waters (1.7 µm, tempered to 70 °C) and connected with an ESI-Q-TOF-mass spectrometer. A gradient from 0 to 40% meoh in aq. triethylamine with hexafluor isopropanol was used as eluent and the flow rate was set to 0.3 ml min^{-1}. Additionally denaturing urea (8M) PAGE-gels were made to verify the formation of the full-length product. Pre-miRNAs were purified on a Gilson *1105 HPLC System* with detection at λ = (260/280) nm, (260/495) nm or (260/633) nm using a *XBridge* C18 column from Waters (5 µm, tempered to 70 °C), the gradient 0-40% acetonitrile in 0.1 M teaa buffer pH 7.7 in 40 min with a flow rate set to 1 ml min^{-1}.

Oligonucleotides were purchased from IBA (Göttingen, Germany), Biotez (Berlin, Germany) or Proligo (Boulder, USA):

>>DNA-T7-promotor | Proligo | 20 nt | MW = 6126 g mol^{-1} | ε_{260} = 203900 l $(mol\ cm)^{-1}$ | 5'- GGT AAT ACG ACT CAC TAT AG -3'
>>DNA-template pre-let-7 | IBA | 77 nt | MW = 23705 g mol^{-1} | ε_{260} = 769400 l $(mol\ cm)^{-1}$ | 5'- $A_{OMe}C_{OMe}$A AAG CTA GCA CAT TGT ATA GTA TGA TGT GTA ATT ACT ACT ATA CAA CCT ACT ACC TCC TAT AGT GAG TCG TAT TA -3'
>>DNA-template pre-miRNA-21 | IBA | 76 nt | MW = 23512 g mol^{-1} | ε_{260} = 748100 l $(mol\ cm)^{-1}$ | 5'- $A_{OMe}A_{OMe}$G GCC CAT CGA CTG GTG TTG CCA TGA GAT TCA ACA GTC AAC ATC AGT CTG ATA AGC CCT ATA GTG AGT CGT ATT A -3'

>>DNA-template pre-miRNA-142 | Biotez | 76 nt | MW = 23371 g mol^{-1} | ε_{260} = 741700 l $(mol\ cm)^{-1}$ | 5'- TCG ATA AAG TAG GAA ACA CTA CAC CCT CCA GTG CTG TTA GTA GTG CTT TCT ACT TTA TCT ATA GTG AGT CGT ATT A -3'
>>DNA-template pre-miRNA-122 | IBA | 75 nt | MW = 23240 g mol^{-1} | ε_{260} = 749900 l $(mol\ cm)^{-1}$ | 5'- $U_{OMe}A_{OMe}$G TGA GTG TGA TAA TGG CGT TTG ATA
GTT TAG ACA CAA ACA CCA TTG TCA CAC TCA CTA TAG TGA GTC GTA TTA -3'
>>DNA-template pre-miRNA-122^{A} | Biotez | 65 nt | MW = 20048 g mol^{-1} | ε_{260} = 648700 l $(mol\ cm)^{-1}$ | 5'- $G_{OMe}A_{OMe}$T AAT GGC GTT TGA TAG TTT AGA CAC AAA CAC CAT TGT CAC ACT CAC TAT AGT GAG TCG TAT TA -3'
>>RNA-Oligomer pre-miRNA-122-B^{ALX} | IBA | 10 nt | MW = 4285 g mol^{-1} | ε_{260} = 99800 l $(mol\ cm)^{-1}$ | $\varepsilon_{632,ALX}$ = 159000 l $(mol\ cm)^{-1}$ | 5'- pACA CUC ACU A^{ALX} -3'
>>RNA-Oligomer pre-miRNA -122-C | IBA | 10 nt | MW = 3088 g mol^{-1} | ε_{260} = 99800 l $(mol\ cm)^{-1}$ | 5'- pACA CUC ACU Ap -3'

Fluorescence-based detection of Dicer-cleavage:[11, 12] The rate of Dicer cleavage was measured in 384-well plates and a final volume of 40 µL was used. A 40 µL reaction which contained 20 nM beacon (renaturated prior use in dicer buffer: 3 min at 95 °C, cooled to room temperature within 30 min) and Dicer buffer (20 mM Tris–HCl, pH 6.8, 12.5 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT) was placed on ice. Then 0.05 U Dicer was added and the fluorescence increase measured every minute for 3 h at 37 °C. All Fluorescence measurements were done in triplicate on a BMG Labtech Fluorostar Optima plate reader with $\lambda_{ex/em}$ = (485/520±5) nm and the average values are given. For the Alexa-labeled probe (90 nM in 50 µl final volume, 0.5 U Dicer, 37 °C) the fluorescence increase was measured every 60 min in a Varian Cary Eclipse fluorescence spectrophotometer with λ_{ex} = (633±5) nm, λ_{em} = 645±5 - 750±5 nm using 3 × 3 mm ultra-micro-cuvettes.

In-vitro-transcription of pre-miRNAs: Transcriptions of pre-miRNAs were performed using a RiboMAX™ Large Scale RNA Production System-T7 kit with DNA template and T7 promotor according to manufacturer's protocol but with minor modifications. To generate the promotor double strand, water, T7-promotor and DNA-template were heated to 95 °C for 2 min. After 10 min at room temperature, the tabulated volumes of buffer, $MgCl_2$, rNTPs and GMP^{Prg} were added in the order specified (Tab. 1). The reaction was warmed to 37 °C and a further equivalent of GTP was added every 15 min (8 ×). Afterwards the transcription was continued for 30 min. Then the reaction was stopped by addition of DNase. RNA was isolated by PCI-extraction, ethanol precipitation and used for CuAAC without further purification.

Tab. 1 Pipetting scheme for in-vitro-transcription

Compound	c_{Stock}	c_{Final}	V [µl]
water	-	-	45
T7-Promotor	310 µM	7.5 µM	7.2
DNA-template	100 µM	3.7 µM	11.2
T7-buffer	5 ×	1 ×	60
$MgCl_2$	1 M	10 mM	3.0
rNTPs	25 mM ACU, 2.5 mM G	7.5 mM, 0.75 mM	90
GMP^{Prg}	37.8 mM	6.75 mM	53.6
Enzyme mixture	10 ×	1 ×	30
sum			**300**
DNase	1 U μl^{-1}		45

CuAAC: RNA transcript was dissolved in water and diluted with PUS-buffer (1 ×: 1 M Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 M NH_4OAc). Stock solutions of sodium ascorbate (freshly prepared prior use), copper sulfate, THPTA and azide were degassed with Ar through an Eppendorf tip (10 sec) and added in the amounts and order outlined in Tab. 2 . The resulting reaction volume was degassed again and shaken for 2 h at 25 °C followed by PCI extraction (containing additional 10 mM EDTA to remove copper). The aqueous layer was purified and concentrated with Amicon Ultra-0.5 ml centrifugal filters 10 K to separate non-incorporated rNTPs from transcription and other small molecules. The labeled RNA was separated by HPLC (0-40 % acetonitrile in 0.1 teaa buffer, 40 min). The product containing fractions (t_R ~ 34 min in case of labeling with Dabcyl) as well as the GTP-primed fractions (t_R ~ 22 min) were pooled, desalted and concentrated by Amicon Ultra-0.5 ml centrifugal filters 10 K. In case of small scale CuAAC (e. g. internal labeling with known conc. of RNA) the concentrations of reagents were changed slightly (Tab.2, right).

Tab. 2 Pipetting scheme for CuAAC

Compound	c_{Stock}	c_{Final}	V [µl]	Compound	c_{Stock}	c_{Final}	V [µl]
water	-	-	64.5	water	-	-	34.7
PUS-buffer	10 ×	1 ×	30	PUS-buffer	10 ×	1 ×	10
pre-miR-transcript	~50 µM	~ 25µM	150	pre-miR-transcript	7.5 µM	2.5 µM	35
DAB-N_3[4] in dmso	10 mM	500 µM	15	**FAM-N_3**[4] in dmso	1 mM	100	10
THPTA	50 mM	3.75 mM	22.5	THPTA	50 mM	5 mM	5
Sodium ascorbate	150 mM	7.5 mM	15	Sodium ascorbate	150	2.5	3.3
$CuSO_4$	75 mM	0.75 mM	3	$CuSO_4$	25 mM	0.5	2
sum			**300**	**sum**			**100**

^{DAB}**pre-let7** | 60 nt | ε_{260} = 642000 l (mol cm)$^{-1}$ | n = 0.89 nmol | 5'- DAB-GGA GGU AGU AGG UUG UAU AGU AGU AAU UAC ACA UCA UAC UAU ACA AUG UGC UAG CUU UGU -3'
pre-let7 | 60 nt | ε_{260} = 630900 l (mol cm)$^{-1}$ | n = 1.57 nmol | 5'-pppGGA GGU AGU AGG UUG UAU AGU AGU AAU UAC ACA UCA UAC UAU ACA AUG UGC UAG CUU UGU -3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 19502.4, found 19502.4
isolated yield n_{total} = 2.46 nmol (36 % ^{DAB}**pre-let7**, 64 % **pre-let7**)

^{DAB}**pre-miR-21** | 59 nt | ε_{260} = 589000 l (mol cm)$^{-1}$ | n = 0.65 nmol | 5'- DAB-GGG CUU AUC AGA CUG AUG UUG ACU GUU GAA UCU CAU GGC AAC ACC AGU CGA UGG GCC UU -3'
pre-miR-21 | 59 nt | ε_{260} = 577900 l (mol cm)$^{-1}$ | n = 1.84 nmol | 5'- pppGGG CUU AUC AGA CUG AUG UUG ACU GUU GAA UCU CAU GGC AAC ACC AGU CGA UGG GCC UU -3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 19153.2, found 19153.3
isolated yield n_{total} = 2.49 nmol (26 % ^{DAB}**pre-miR-21**, 74 % **pre-miR-21**)

^{DAB}**pre-mir-142** | 59 nt | ε_{260} = 621400 l (mol cm)$^{-1}$ | n = 0.41 nmol | 5'- DAB-GAU AAA GUA GAA AGC ACU ACU AAC AGC ACU GGA GGG UGU AGU GUU UCC UAC UUU AUC GA -3' | ESI-MS (dekonvuliert) aus UHPLC/UV-Vis/MS ber. 19519.6, found 19519.8
pre-miR-142 | 59 nt | ε_{260} = 610300 l (mol cm)$^{-1}$ | n = 0.68 nmol | 5'- pppGAU AAA GUA GAA AGC ACU ACU AAC AGC ACU GGA GGG UGU AGU GUU UCC UAC UUU AUC GA -3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 19216.3, found 19216.3
isolated yield n_{total} = 1.09 nmol (38 % ^{DAB}**pre-mir-142**, 62 % **pre-miR-142**) starting from 100 µl scale in-vitro-transcription

^{DAB}Pre-miR-122 | 58 nt | ε_{260} = 595700 l (mol cm)$^{-1}$ | n = 0.56 nmol | 5'- DAB-GUG AGU GUG ACA AUG GUG UUU GUG UCU AAA CUA UCA AAC GCC AUU AUC ACA CUC ACU A -3'
pre-miR-122 | 58 nt | ε_{260} = 584600 l (mol cm)$^{-1}$ | n = 2.05 nmol | 5'- pppGUG AGU GUG ACA AUG GUG UUU GUG UCU AAA CUA UCA AAC GCC AUU AUC ACA CUC ACU A -3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 18745.0, found 18745.2
isolated yield n_{total} = 2.61 nmol (21 % **^{DAB}Pre-miR-122**, 79 % **pre-miR-122**)

^{DAB}Pre-miR-122-A | 48 nt | ε_{260} = 497500 l (mol cm)$^{-1}$ | n = 0.73 nmol | 5'- DAB-GUG AGU GUG ACA AUG GUG UUU GUG UCU AAA CUA UCA AAC GCC AUU AUC -3'
pre-miR-122-A | 48 nt | ε_{260} = 486400 l (mol cm)$^{-1}$ | n = 1.67 nmol | 5'- pppGUG AGU GUG ACA AUG GUG UUU GUG UCU AAA CUA UCA AAC GCC AUU AUC -3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 15595.1, found 15595.2
isolated yield n_{total} = 2.40 nmol (30 % **^{DAB}Pre-miR-122-A**, 70 % **pre-miR-122-A**)

Ligation of pUp$^{Alk/FAM}$: *T4 RNA Ligase 1* was used for ligation of **pUp^{Alk}** or **pUp^{FAM}** to the 3'-end of RNA strands. Compound **pUp^{FAM}** was dissolved in a mixture of dmso/water (3:2, v/v), **pUp^{Alk}** was dissolved in water. Both stock solutions were stored at – 20 °C. For renaturation a mixture of buffer, water, RNA (final conc.: 1.75 µM) and dmso (final conc.: 20 %) were vortexed, heated to 95 °C for 3 min and placed on ice for 5 min. Afterwards modified uridine (final conc.: 0.65 mM) as well as ATP (final conc.: 1 mM) were added. Again the reaction was allowed to cool on ice for 5 min. After addition of Ribolock® and Ligase (both final conc.: 0.70 U µl^{-1}) the vial was kept at 16 °C for 20 h. RNA was purified with PCI extraction and HPLC (0-40 % acetonitrile in 0.1 teaa buffer, 40 min). The product containing fractions (t_R ~ 36 min in case of labeling with FAM/Dabcyl) were pooled, desalted and concentrated by Amicon Ultra-0.5 ml centrifugal filters 3 K. In case of **pUp^{Alk}** ligation RNA was purified with PCI extraction, desalted and concentrated by Amicon Ultra-0.5 ml centrifugal filters 3 K.

Pre-let7^{U} | 61 nt | ε_{260} = 640600 l (mol cm)$^{-1}$ | isolated yield n = 0.43 nmol (87 %) | 5'- ppp-GGA GGU AGU AGG UUG UAU AGU AGU AAU UAC ACA UCA UAC UAU ACA AUG UGC UAG CUU UGUp-Ethinyl-3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 19912.6, found 19912.3

^{DAB}Pre-let7^{FAM} | 61 nt | ε_{260} = 672600 l (mol cm)$^{-1}$ | isolated yield n = 0.60 nmol (56 %)|* 5'- DAB-GGA GGU AGU AGG UUG UAU AGU AGU AAU UAC ACA UCA UAC UAU ACA AUG UGC UAG CUU UGUp-FAM -3' | ESI-MS (deconvouted) from UHPLC/UV-Vis/MS calc. 20748.0, found 20748.6

^{DAB}Pre-miR-21^{FAM} | 60 nt | ε_{260} = 619600 l (mol cm)$^{-1}$ | isolated yield n = 0.26 nmol (44 %) |* 5'- DAB-GGG CUU AUC AGA CUG AUG UUG ACU GUU GAA UCU CAU GGC AAC ACC AGU CGA UGG GCC UUUp-FAM -3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 20398.8, found 20399.3

^{DAB}Pre-miR-142^{FAM} | 60 nt | ε_{260} = 650900 l (mol cm)$^{-1}$ | isolated yield n = 0.10 nmol (39 %) |* 5'- DAB-GAU AAA GUA GAA AGC ACU ACU AAC AGC ACU GGA GGG UGU AGU GUU UCC UAC UUU AUC GAUp-FAM -3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 20461.9, found 20462.4

* isolated yield after HPLC

^{DAB}**Pre-miR-122**^{FAM} | 59 nt | ε_{260} = 625200 l (mol cm)$^{-1}$ | isolated yield n = 0.40 nmol (59 %) |* 5'-DAB-GUG AGU GUG ACA AUG GUG UUU GUG UCU AAA CUA UCA AAC GCC AUU AUC ACA CUC ACU AUp-FAM -3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 19990.5, found 19991.2

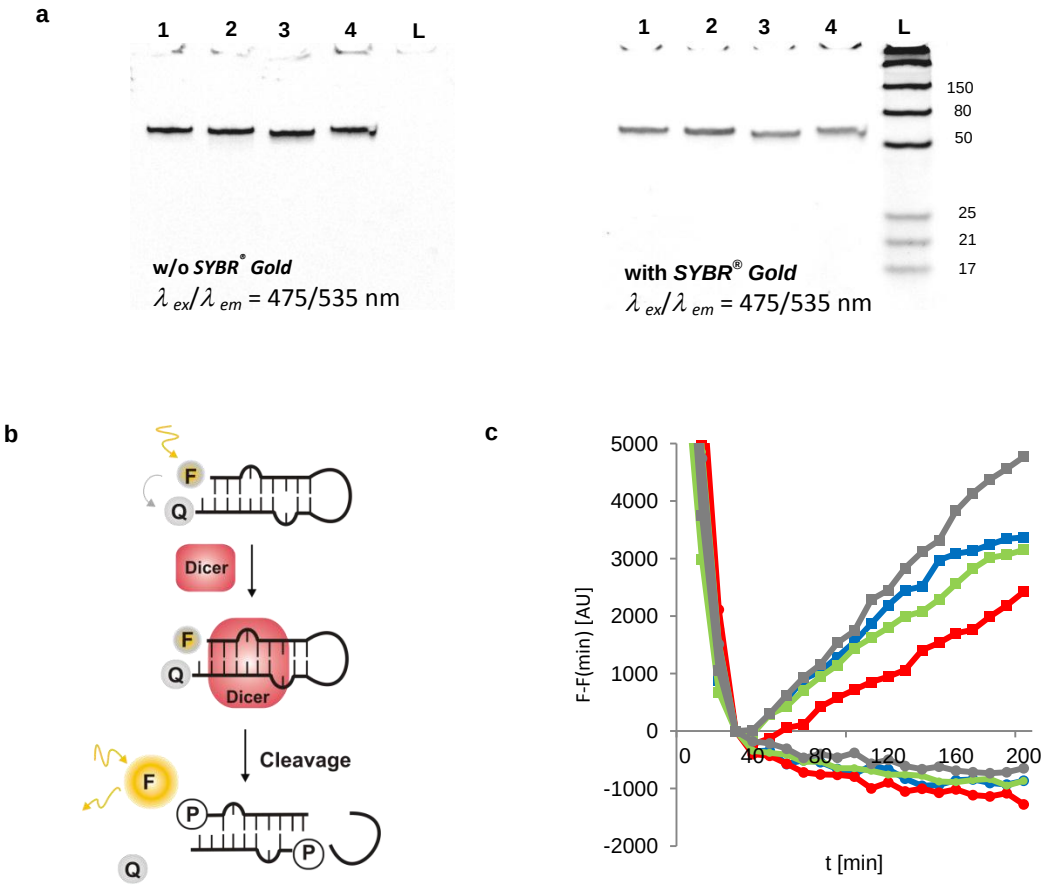


Fig. S4 **a** 20% denat. PAGE-Gel of pre-miRNA probes, legend: 1 =^{DAB}pre-let7^{FAM}, 2 = ^{DAB}pre-miR-21^{FAM}, 3 = ^{DAB}pre-miR-122^{FAM}, 4 = ^{DAB}pre-miR-142^{FAM}, L = RNA-ladder **b** schematic illustration of the concept of ^{DAB}pre-miRNA^{FAM} probes, **c** Fluorescence intensity (F-F_{min}) per min after incubation of ^{DAB}pre-miRNA^{FAM} (20 nM) at 37 °C with denat./nat. Dicer (0.05 U_{abs}): pre-miR-142 (●/■), pre-let7 (●/■), pre-miR-21 (●/■), pre-miR-122 (●/■), $\lambda_{ex/em}$ = (485/520±5) nm, all measurements were performed in triplex, mean values are given

Dephosphorylation: Calf Intestinal Phosphatase was used according to manufacturer's protocol with minor modifications: RNA was diluted to 3 µM and 4 U CIP per 0.1 nmol RNA was used. After incubation for 60 min at 37 °C the enzyme was removed by PCI-extraction. Finally the dephosphorylated RNA was purified and concentrated with Amicon Ultra-0.5 ml centrifugal filters 3K.

Ligation of RNA-oligonucleotides: *T4 RNA Ligase 1* was used for ligation of RNA strands. For renaturation a mixture of buffer, water, both RNA oligomers (final conc.: ^{DAB}pre-miR-122-A/pre-let7 3 µM, pre-miR-122-B^{ALX} 9 µM, pre-miR-122-C 30 µM) and dmso (final conc.: 20 %) were vortexed, heated to 95 °C for 3 min and placed on ice for 5 min. Afterwards dmso (final conc.: 10 %) as well as ATP (final conc.: 1 mM) were added. Again the reaction was allowed to cool on ice for 5 min. After addition of Ribolock® (final conc.: 0.75 U µl^{-1}) and Ligase (final conc.: 1 U µl^{-1}) the vial was kept at 16 °C for 20 h. RNA was purified with PCI extraction and HPLC (0-40 % acetonitrile in 0.1 teaa

buffer, 45 min). The product containing fractions (t_R ~ 40 min for ^{DAB}pre-miR-122^{ALX}) were pooled, desalted and concentrated with Amicon Ultra-0.5 ml centrifugal filters 3 K. In case of prolonged pre-miR-122^{U} no purifications steps were performed.

^{DAB}Pre-miR-122^{ALX} | 58 nt | ε_{260} = 595700 l (mol cm)$^{-1}$ | n = 0.20 nmol (29 %) |* 5'- DAB-GUG AGU GUG ACA AUG GUG UUU GUG UCU AAA CUA UCA AAC GCC AUU AUC -3' | ESI-MS (deconvoluted) from UHPLC/UV-Vis/MS calc. 20165.8, found 20166.3

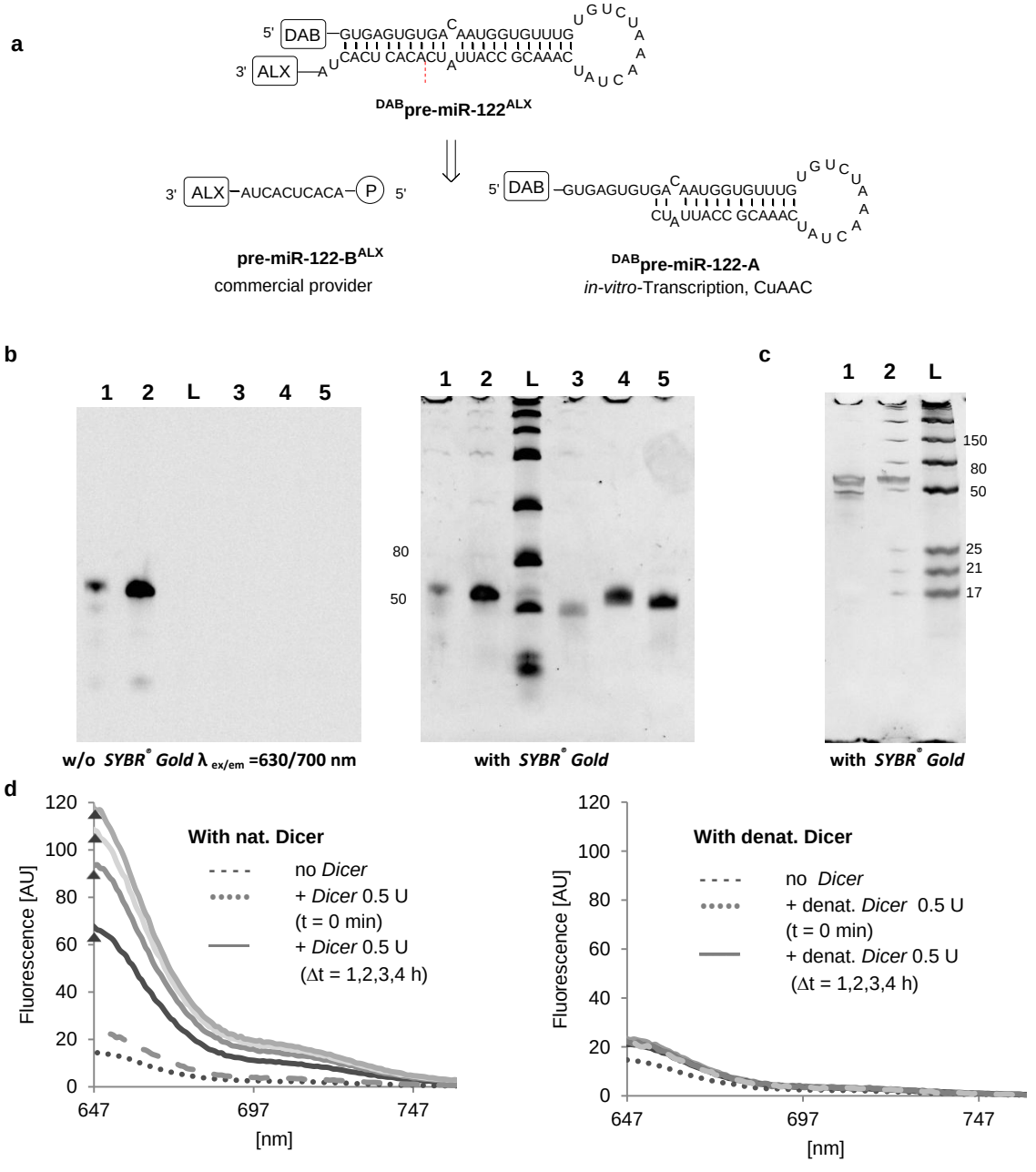


Fig. S5 **a** retrosynthetic analysis of ^{DAB}pre-miRNA-122^{ALX} **b** 10 % denat. PAGE gel, legend: 1 = ^{DAB}pre-miRNA-122^{ALX}, 2 = pre-miRNA-122^{ALX}, 3 = ^{DAB}pre-miRNA-122-A, 4 = ^{DAB}pre-miRNA-122^{FAM} 5 = pre-miRNA-122, L = RNA ladder **c** 20 % denat. PAGE gel, legend: 1 = ^{DAB}pre-miR-122^{ALX} before HPLC purification, 2 = ^{DAB}pre-miR-122^{ALX} after HPLC purification contaminated with L = RNA ladder **d** fluorescence intensity after incubation with nat./denat. Dicer (0.5 U), measurements were taken every 60 min, λ_{em} = (633±5) nm, λ_{em} = (645-750±5) nm, ^{DAB}pre-miRNA-122^{ALX} (90 nM), in Dicer buffer, 37 °C.

* isolated yield after HPLC

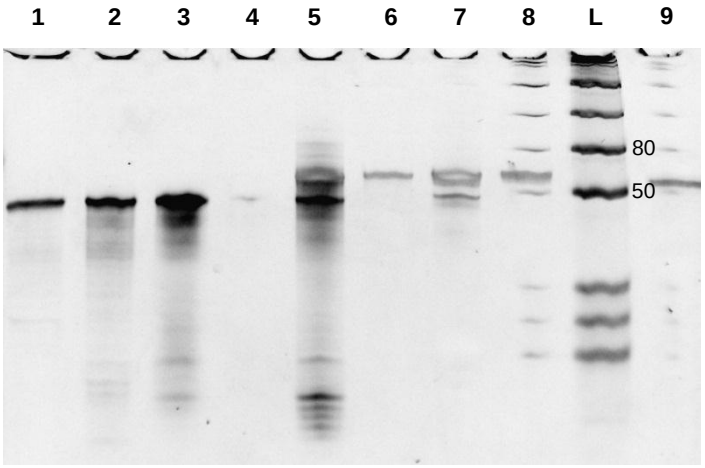
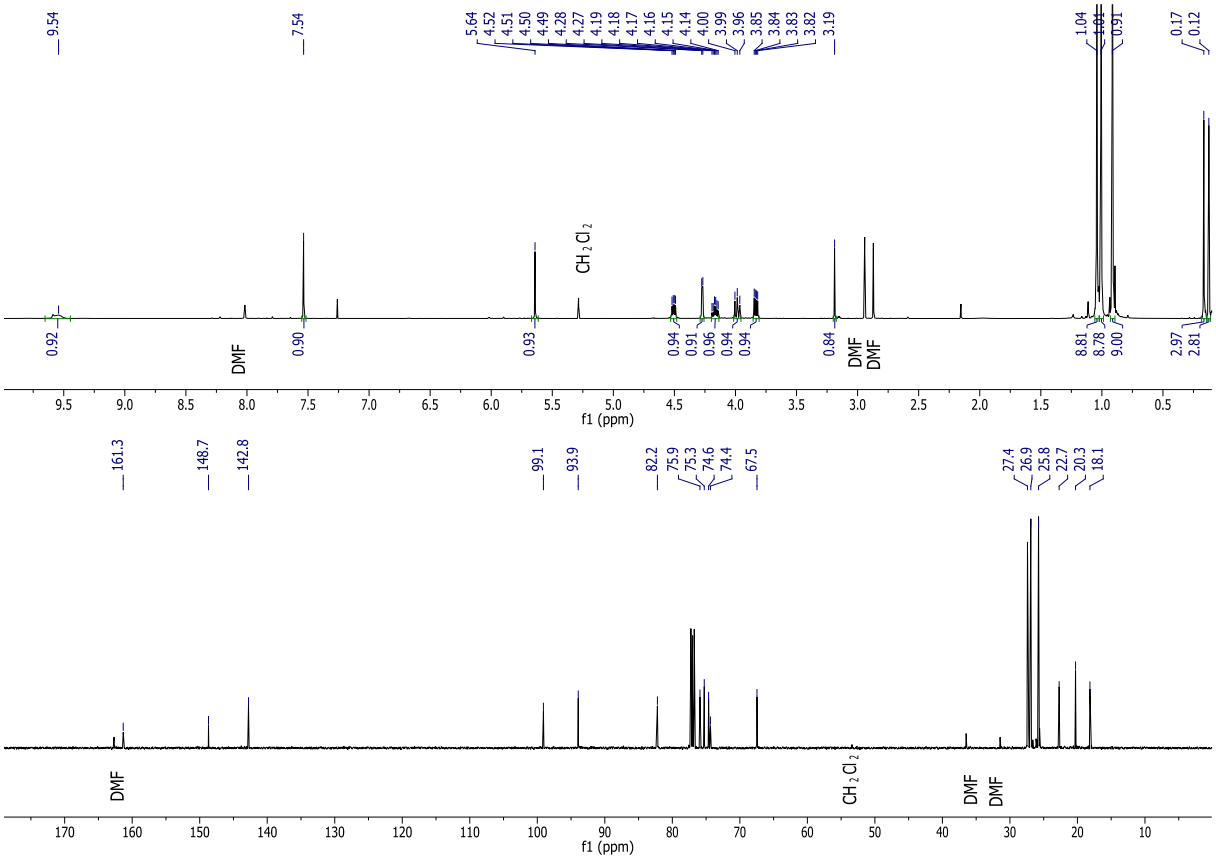


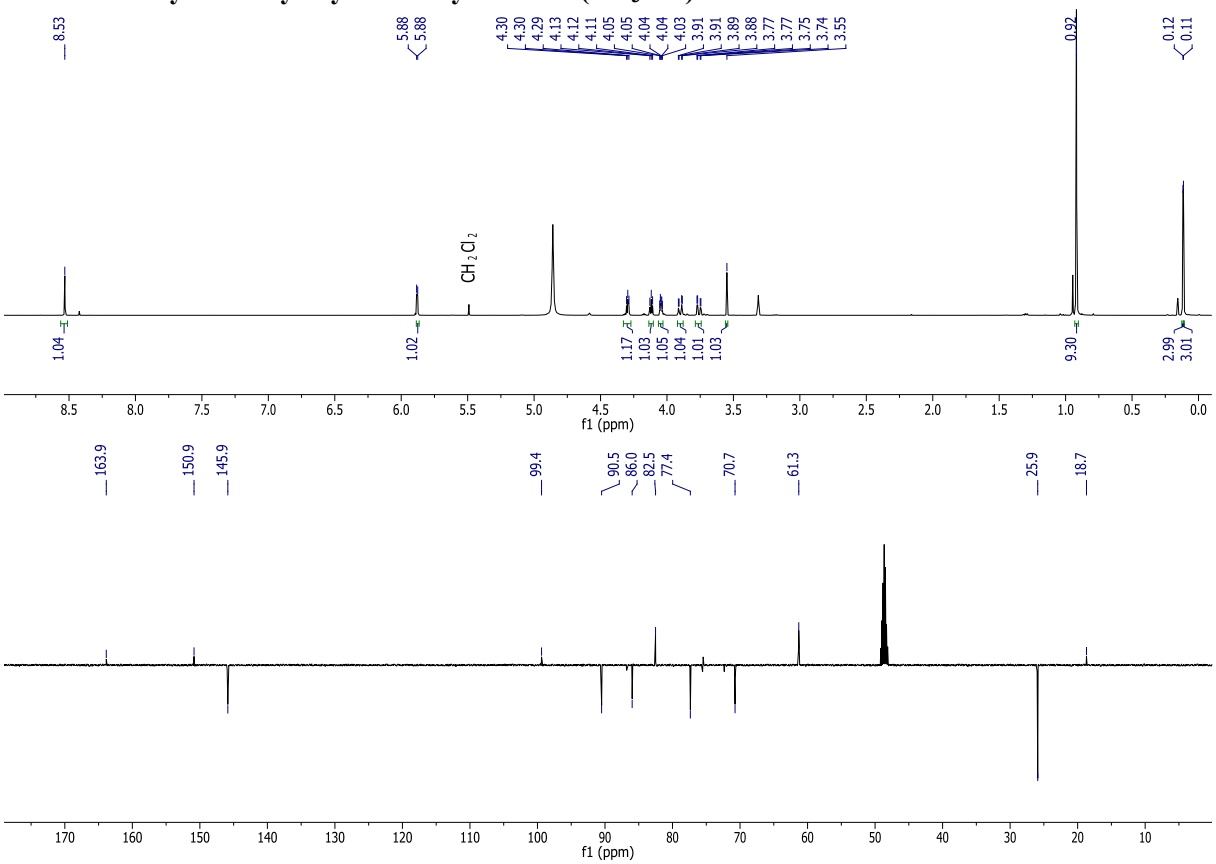
Fig. S6 PAGE analysis of internal labeling experiments, 20 % denat. PAGE gel after ligation, legend: 1 ^{DAB}Pre-miR-122, 2 und 3 Pre-miR-122-A in different concentrations, **4** Pre-miR-122-B**, 5** Pre-miR-122^{ALX} before HPLC, **6** Pre-miR-122^{ALX} after HPLC, **7** ^{DAB}Pre-miR-122^{ALX} before HPLC, **8** ^{DAB}Pre-miR-122^{ALX} after HPLC, **9** pre-miR-122, lane **8** and **9** are contaminated with RNA ladder

IV. ^{1}H-, ^{31}P- and ^{13}C-NMR Spectra

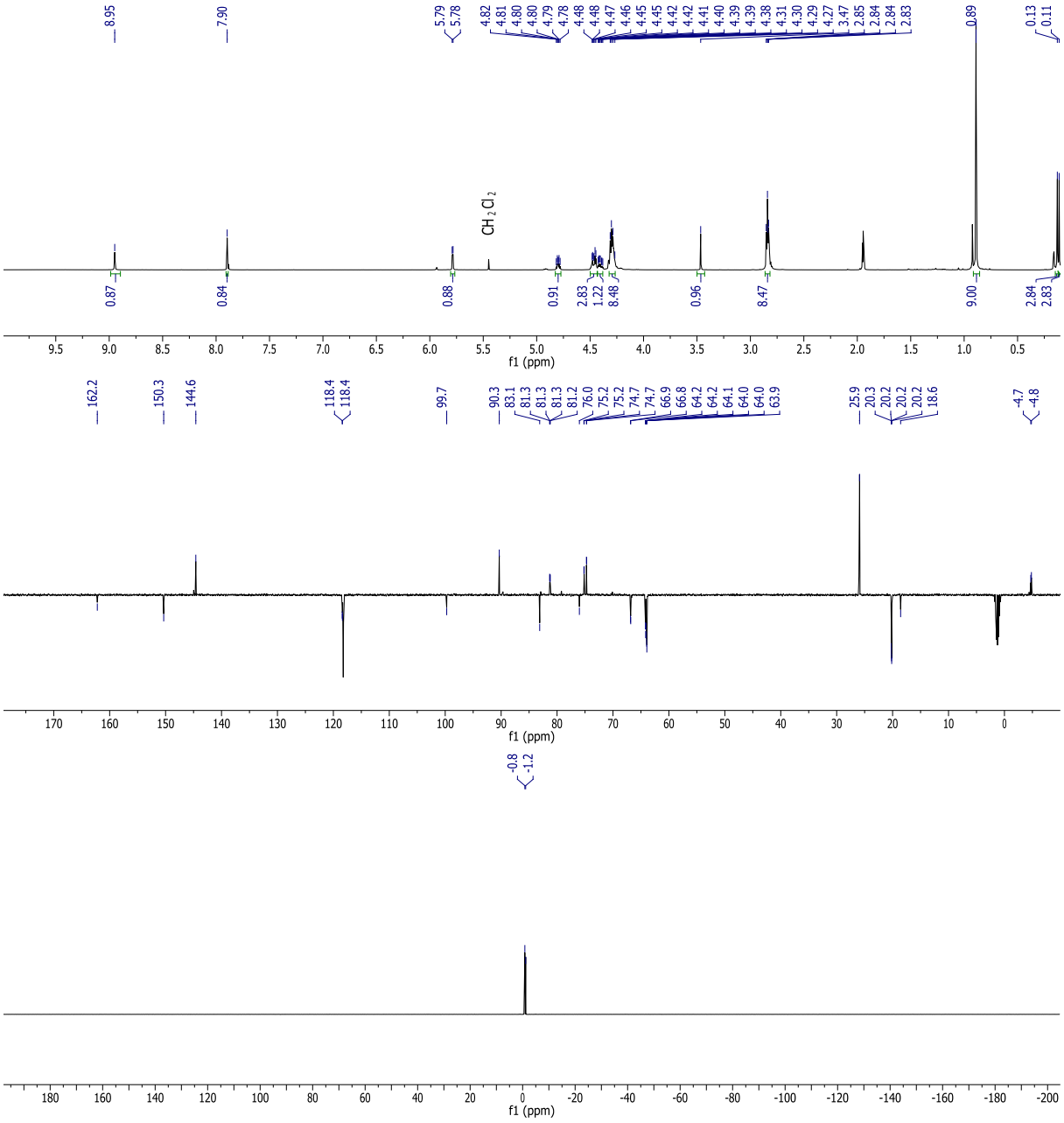
2'-*O*-*tert*-Butyldimethylsilyl-3',5'-*O*-bis(*tert*-butyl)silyl-5-ethinyl uridine 3 ($CDCl_3$)



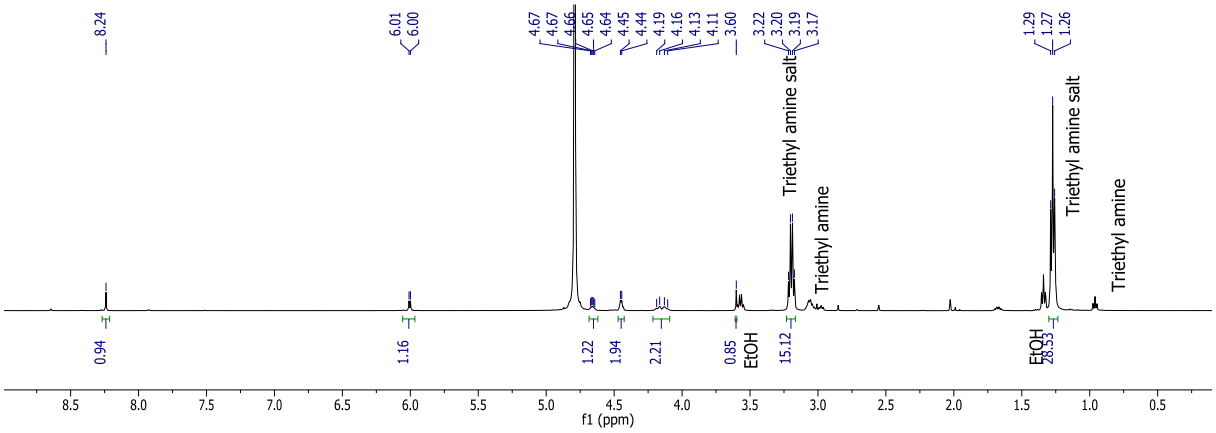
2'-*O*-tert-Butyldimethylsilyl-5-ethinyl uridine (CD_3OD)

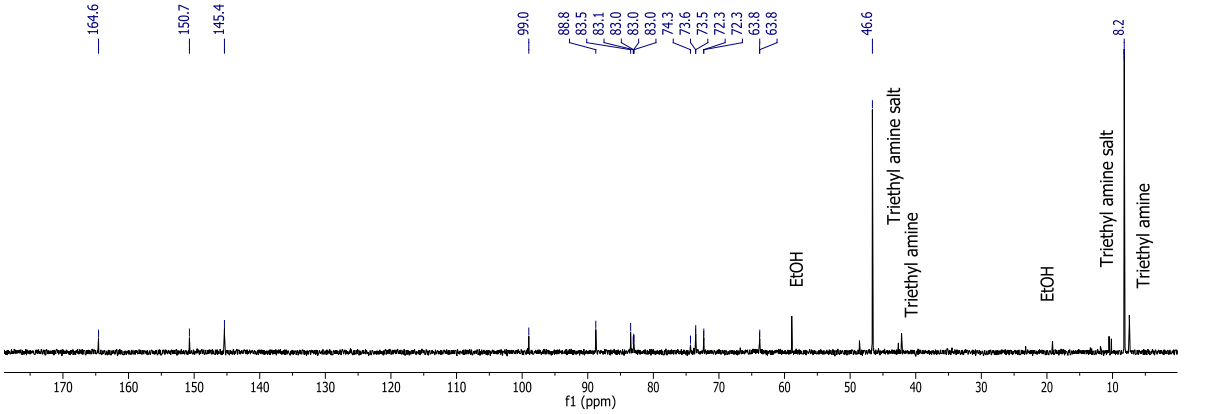


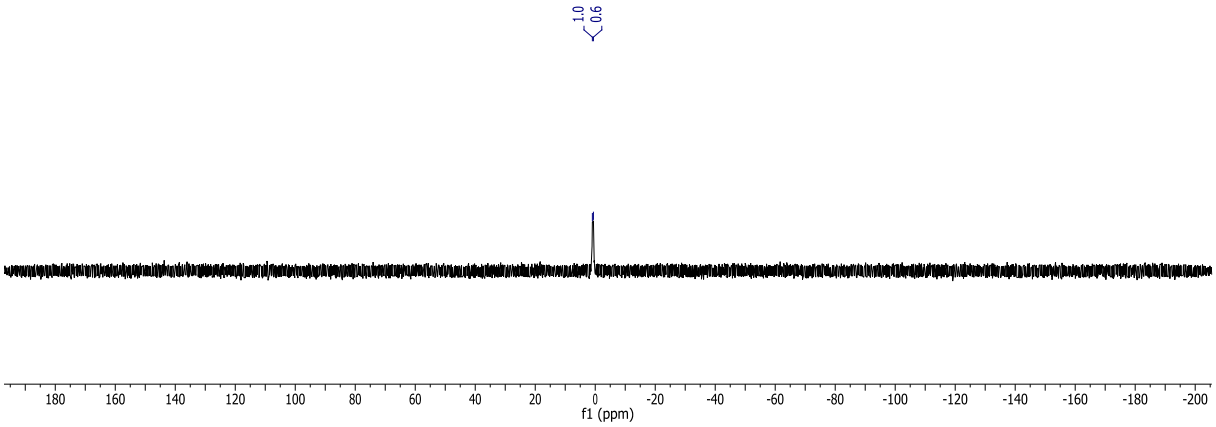
2'-*O*-tert-Butyldimethylsilyl-3',5'-*O*,*O*-bis[di(2-cyanoethyl)]phosphoryl-5-ethinyl uridine 4 (CD_3CN)



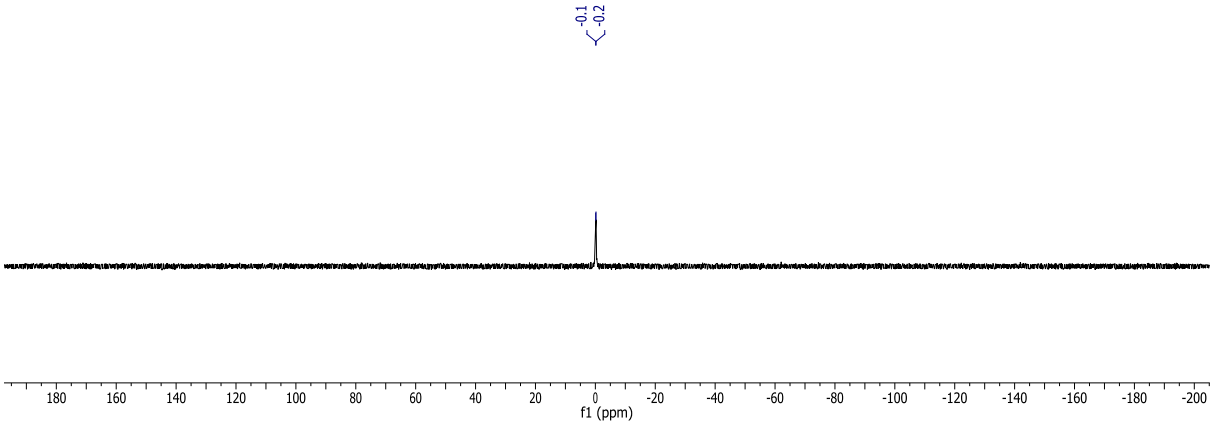
3',5'-*O,O*-Bisphosphate-5-ethinyl uridine 5 pUp^{Alk} (D_2O)



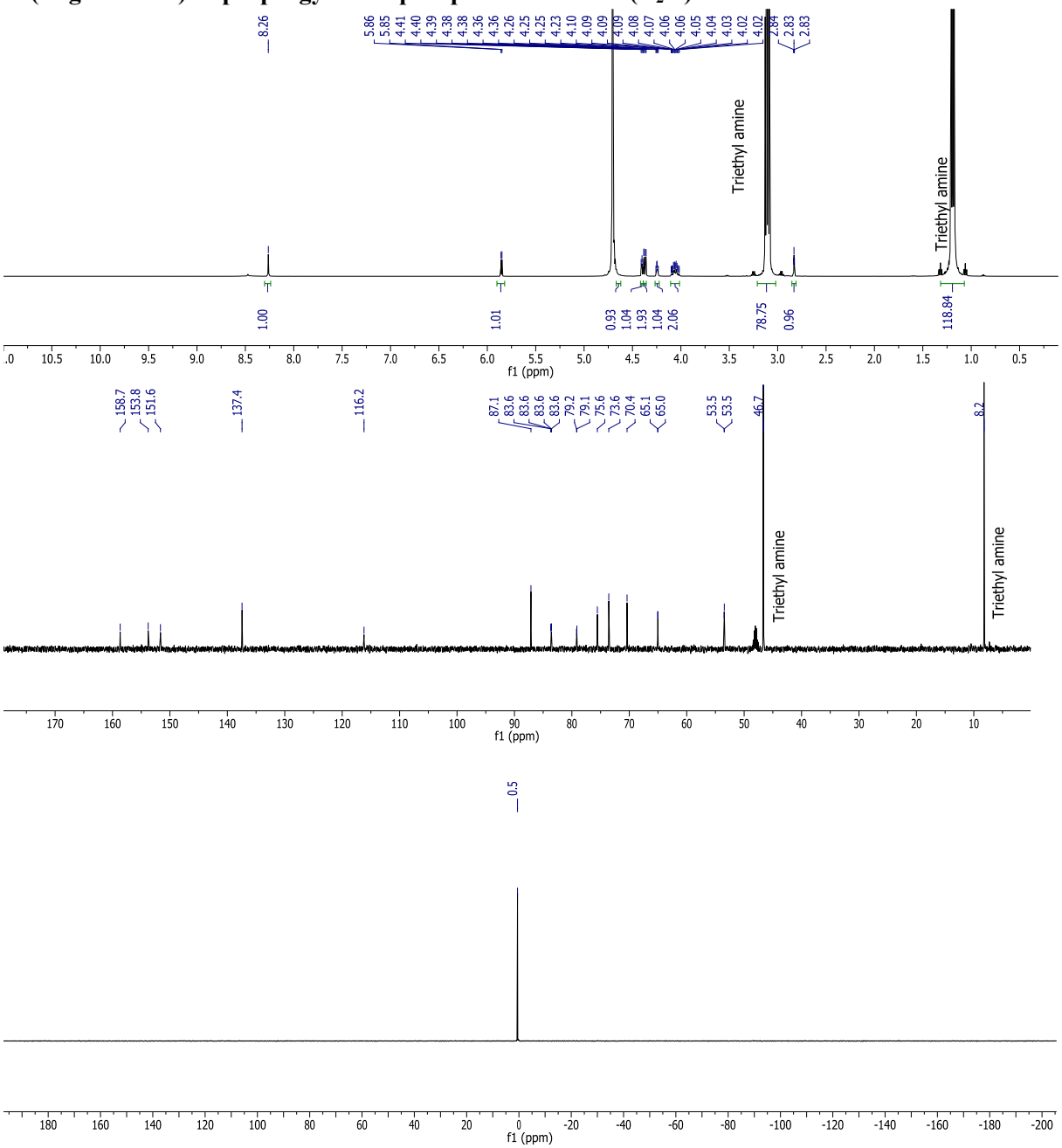




5(6)FAM-labeled 3',5'-*O,O*-Bisphosphat uridine pUp^{FAM} (D_2O)

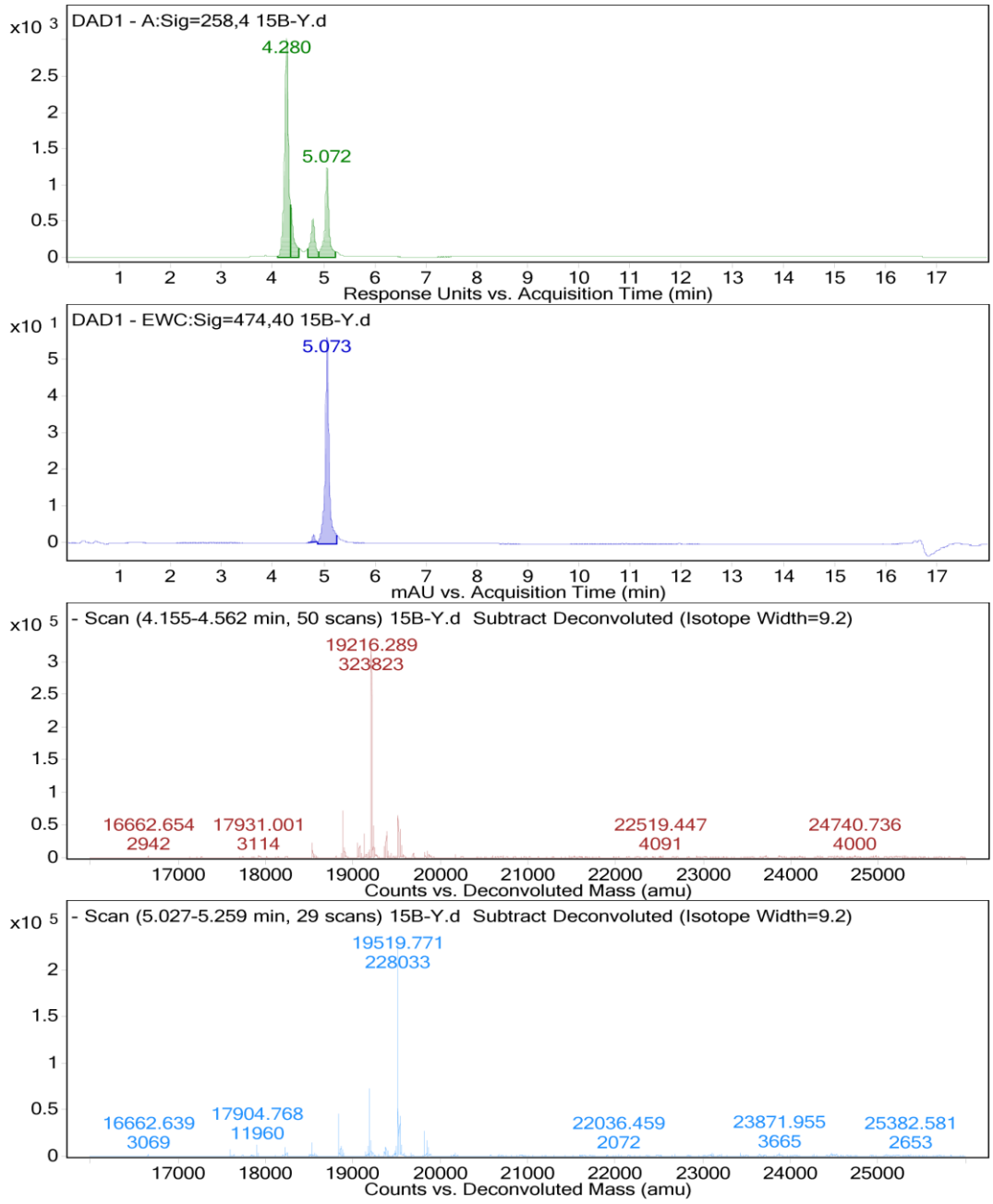


_O_-(5'-guanosine)-_O_-propargyl monophosphate 9 GMP^{Prg} (D_2O)



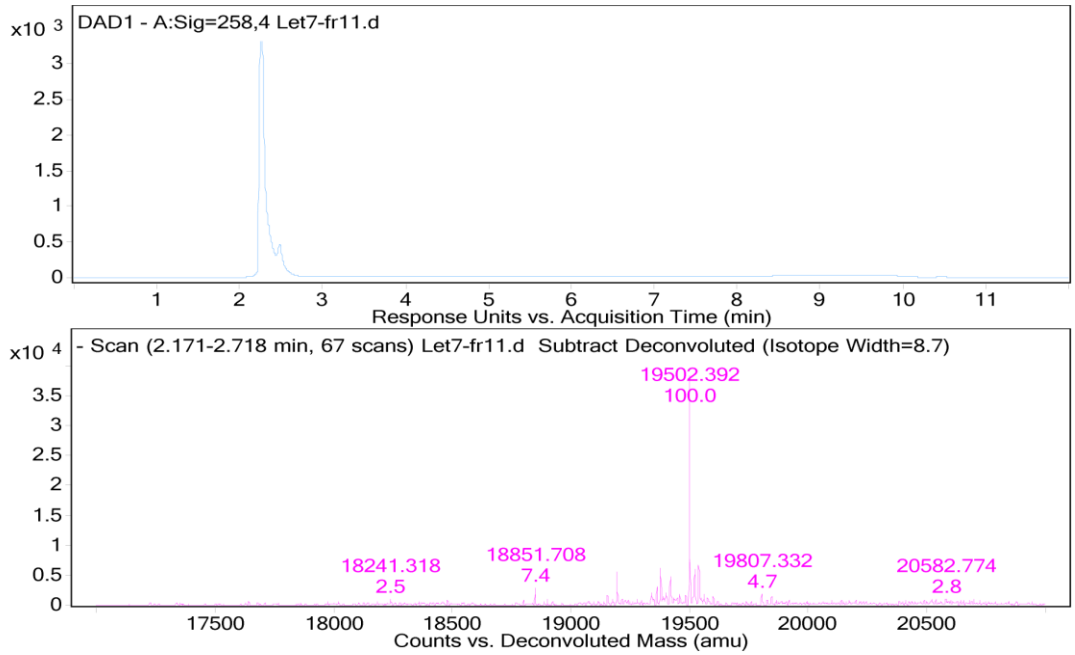
V. UPLC/UV-Vis/MS-chromatograms of selected pre-miRNAs

pre-miR-142 /^{DAB}pre-miR-142 before HPLC purification

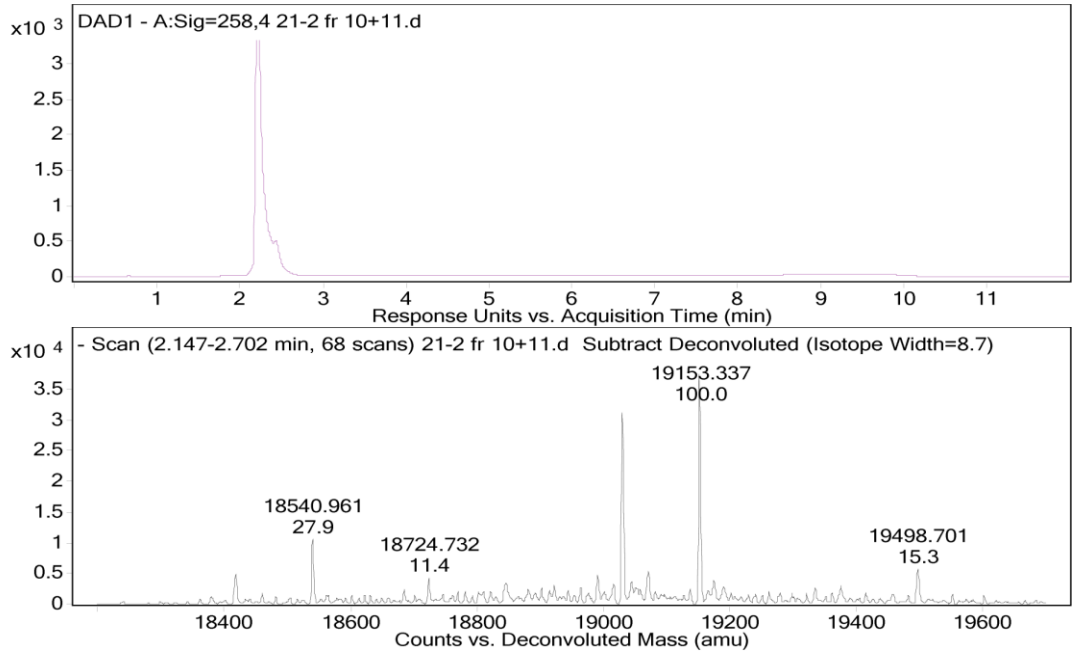


UPLC/UV-Vis/MS-chromatograms of isolated oligomers after HPLC with t_R ~ 22 min

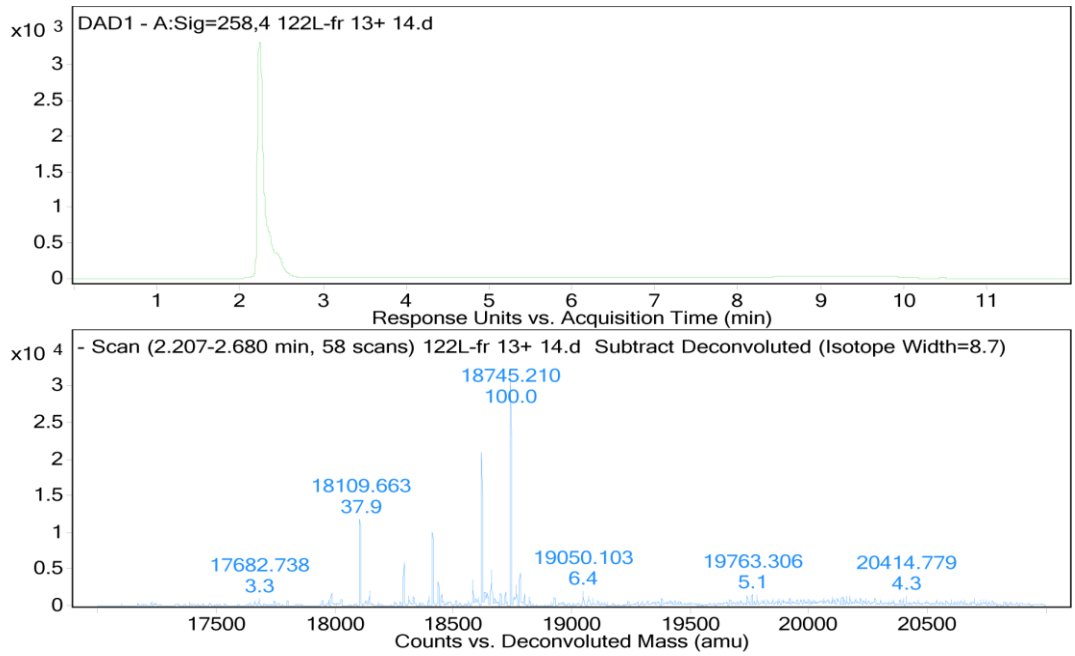
pre-miR-let7 after HPLC purification



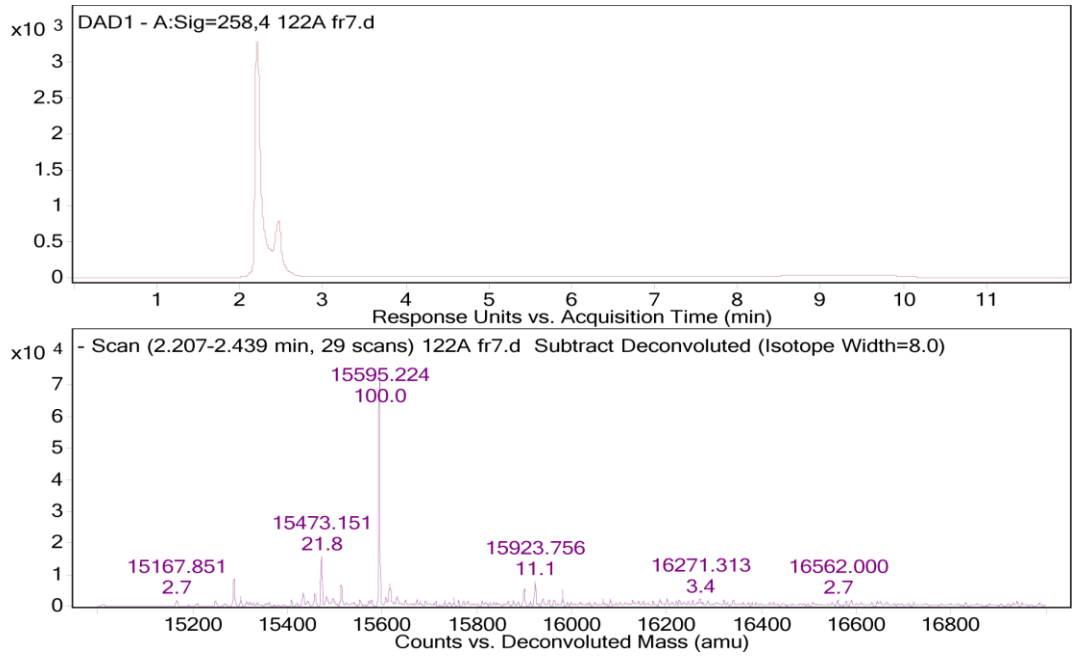
pre-miR-21 after HPLC purification



pre-miR-122 after HPLC purification

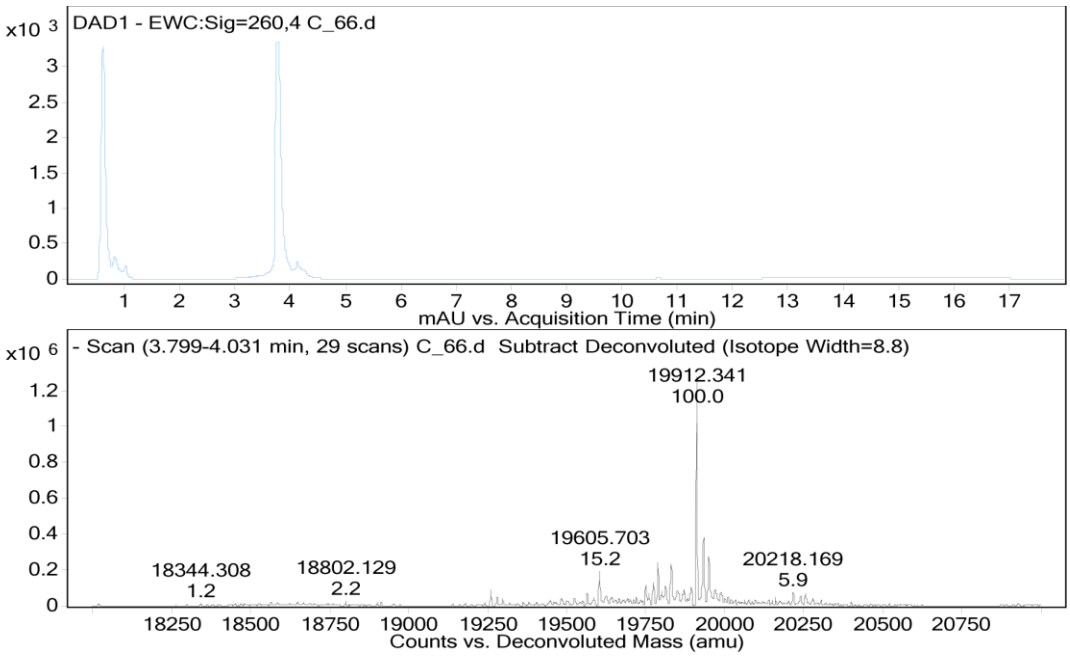


pre-miR-122-A after HPLC purification



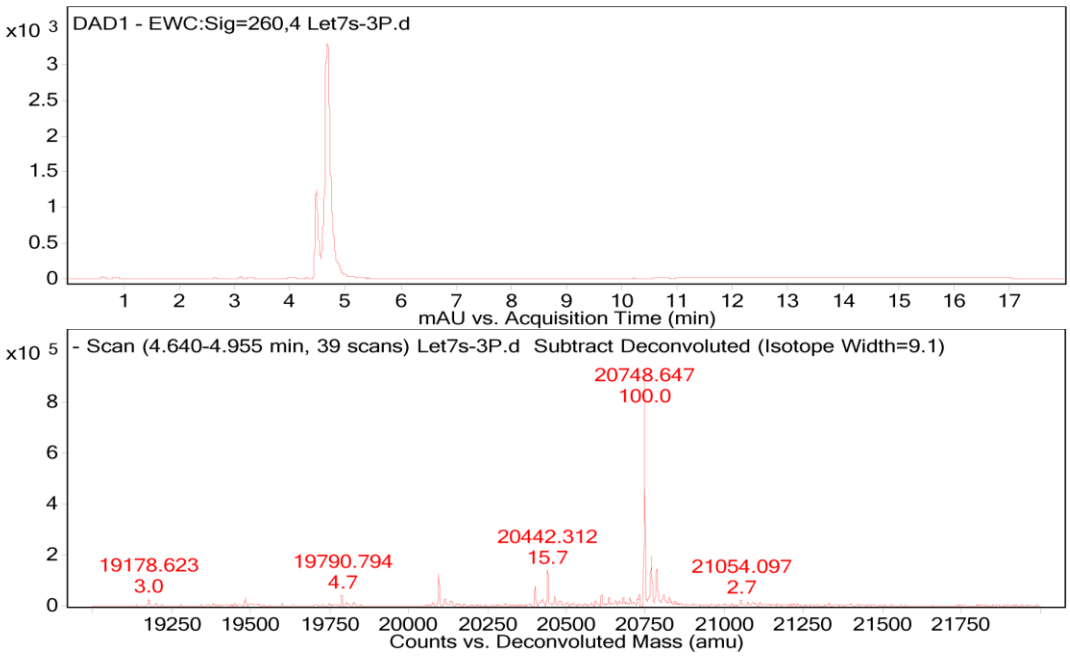
UPLC/UV-Vis/MS-chromatograms of pre-let7^{U} after labeling with pUp^{Alk} without any HPLC but with filtration with centrifugal filters

pre-let7^{U}

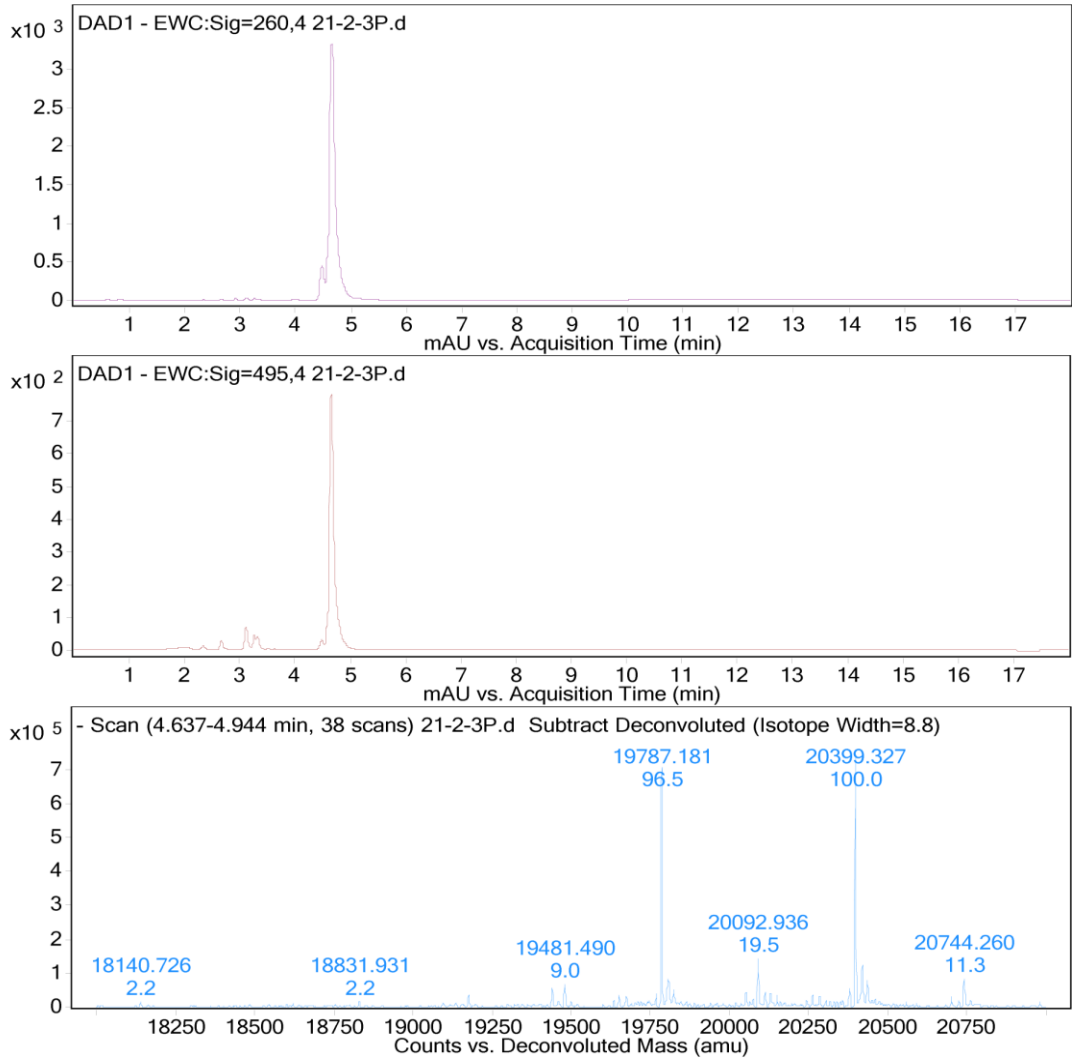


UPLC/UV-Vis/MS-chromatograms of fully labeled oligomers after HPLC with t_R ~ 36 min

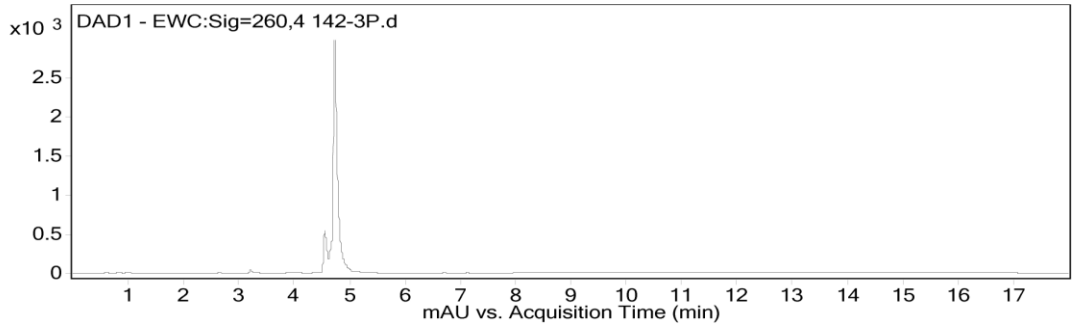
^{DAB}pre-let7^{UFAM}

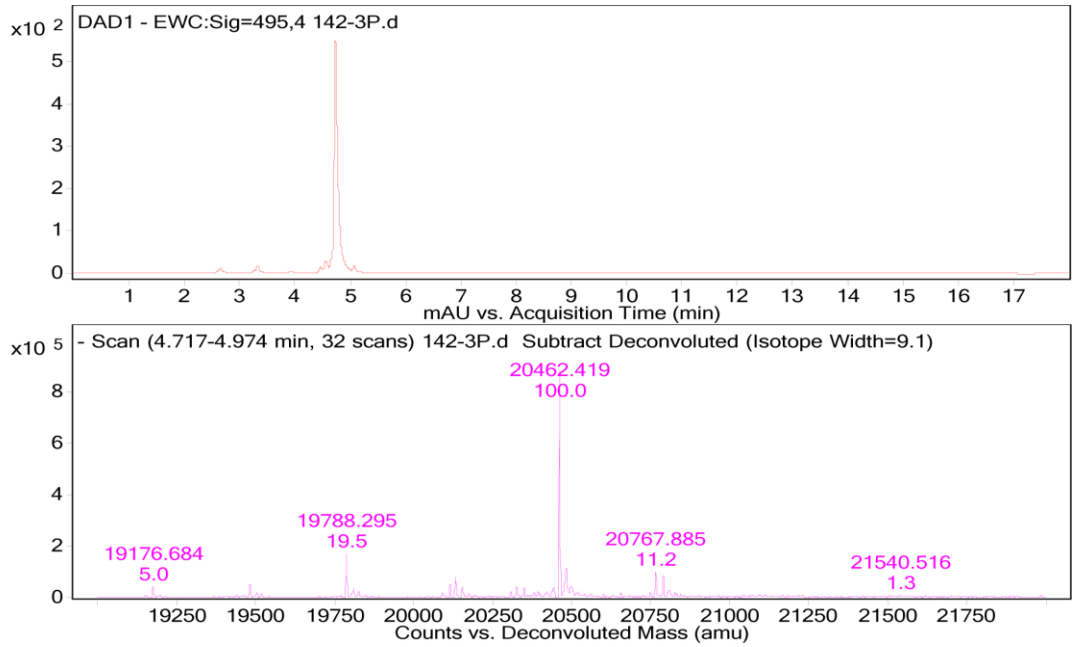


^{DAB}pre-miR-21^{FAM}

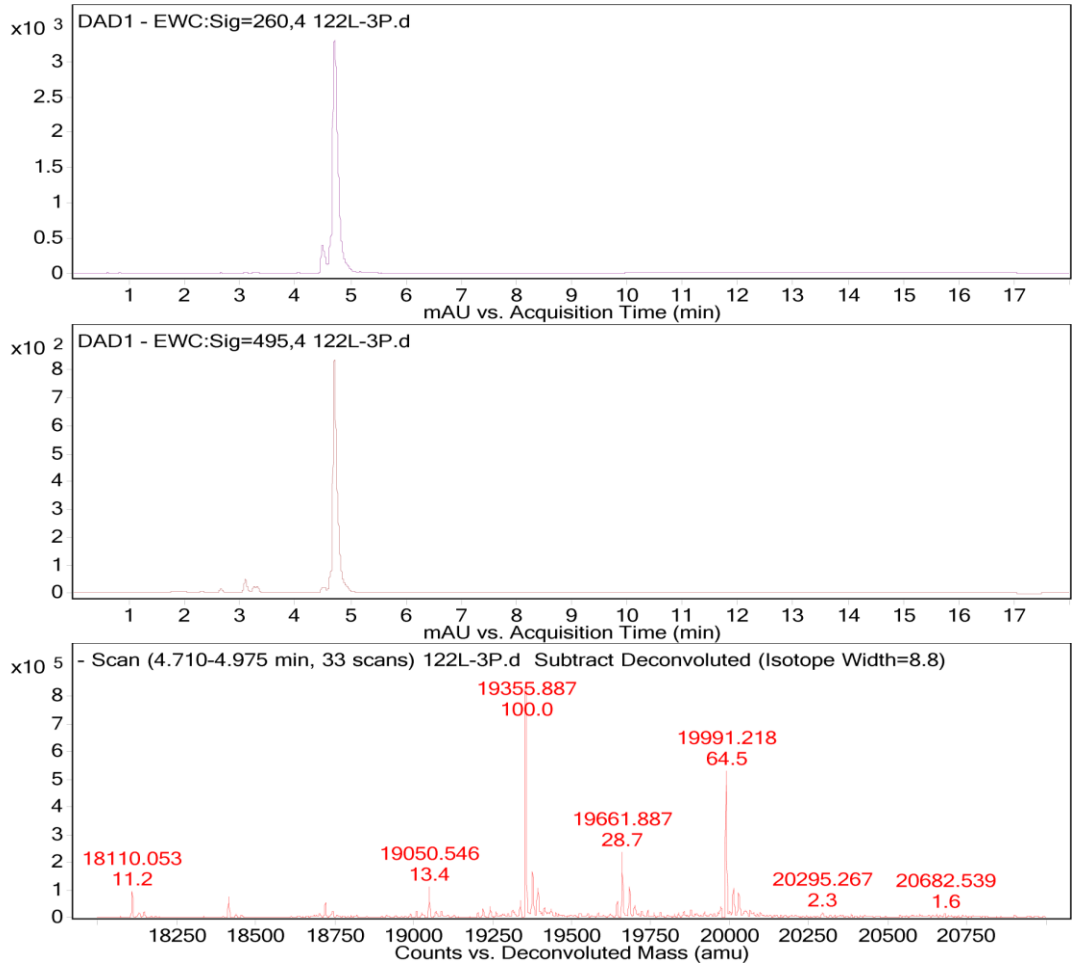


^{DAB}pre-miR-142 ^{FAM}

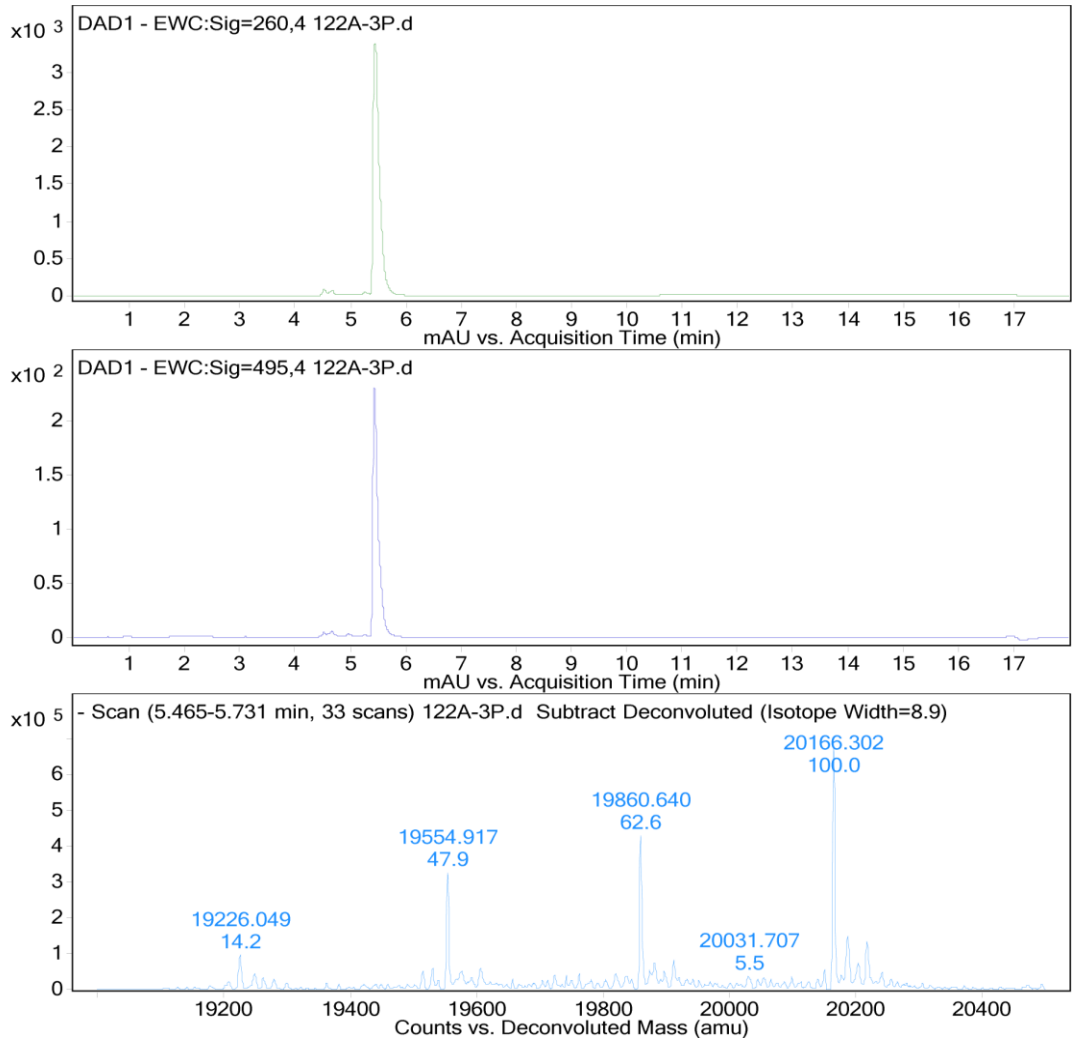




^{DAB}pre-miR-122^{FAM}



^{DAB}pre-miR-122^{ALX}(t_R ~ 40 min)



VI. References

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Electronic Supplementary Material (ESI) for Chemical Communications