Electrophilic RNA for peptidyl-RNA synthesis and site-specific cross-linking with tRNA-binding enzymes

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Supporting Informations

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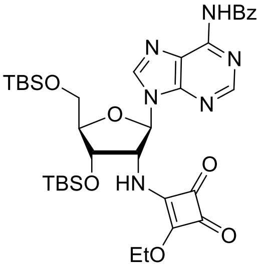
1- General information

Solvents were dried using standard methods and distilled before use. Unless otherwise specified, materials were purchased from commercial suppliers and used without further purification. Flash chromatography: silica gel, 180-240 mesh (Merck). Spectra were recorded on Bruker AVANCE 400 spectrometer for ^{1}H (250 MHz), ^{13}C (63 MHz), in Acetone, or DMSO, as indicated. Chemical shifts (δ) are expressed in ppm relative to residual $(CH_3)_2CO$ (δ=2.05) or $(CH_3)_2SO$ (δ=2.50) for ^{1}H and $(CH_3)_2CO$ (δ=29.84) for ^{13}C, as internal references. Signals were attributed based on DEPT 135, COSY, HSQC and HMBC. High-resolution mass spectroscopy (HRMS) spectra were carried out on a Bruker microTOF spectrometer.

2- Organic synthesis

Compound 2 :



N-(9-((2R,3R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)-3-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)tetrahydrofuran-2-yl)-9H-purin-6-yl)benzamide (2)

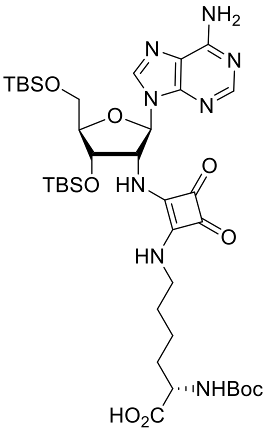
To a solution of **1**[1] (150 mg, 0.25 mmol) in EtOH (1.5 mL) were added DIPEA (22 µL, 0.13 mmol) and diethylsquarate (42 µL, 0.37 mmol). The reaction mixture was stirred at RT for 45 min then concentrated. Flash chromatography (Cyclohexane/AcOEt 4/6) of the residue yielded squaramate **2** (145 mg, 80 %) as a white foam.

^{1}H NMR (250 MHz, Acetone) δ 8.68 (s, 1H, H2 or H8), 8.53 (s, 1H, H2 or H8), 8.17 (d, *J* = 7.1 Hz, 2H, Bz), 7.72 – 7.35 (m, 3H, Bz), 6.53 (d, *J* = 7.5 Hz, 1H, H1'), 4.83 (d, *J* = 3.7 Hz, 1H, H3'), 4.59 (s, 2H, CH_2), 4.33 (s, 1H, H4'), 4.10 (m, 1H, H5'), 3.98 (dd, *J* = 11.3, 3.5 Hz, 1H, H5'), 3.04 (s, 3H, CH_3), 1.02 (s, 9H, tBu^{TBS}), 0.99 (s, 9H, tBu^{TBS}), 0.26 (s, 3H, Me^{TBS}), 0.23 (s, 3H, Me^{TBS}), 0.19 (s, 6H, 2 Me^{TBS}).

^{13}C NMR (63 MHz, Acetone) δ 173.4, 164.8, 152.7, 151.3, 143.2, 134.8, 133.1, 129.3, 129.1, 125.8, 87.6, 73.8, 70.0, 63.8, 60.4, 26.1, 26.4, 19.0, 18.7, 16.0, -4.4, -4.6, -5.2.

HRMS: *m/z* calcd. for $C_{35}H_{51}N_6O_7Si_2$: 723.3358 [*M*+H]; Found: 723.3325.

Compound 3 :

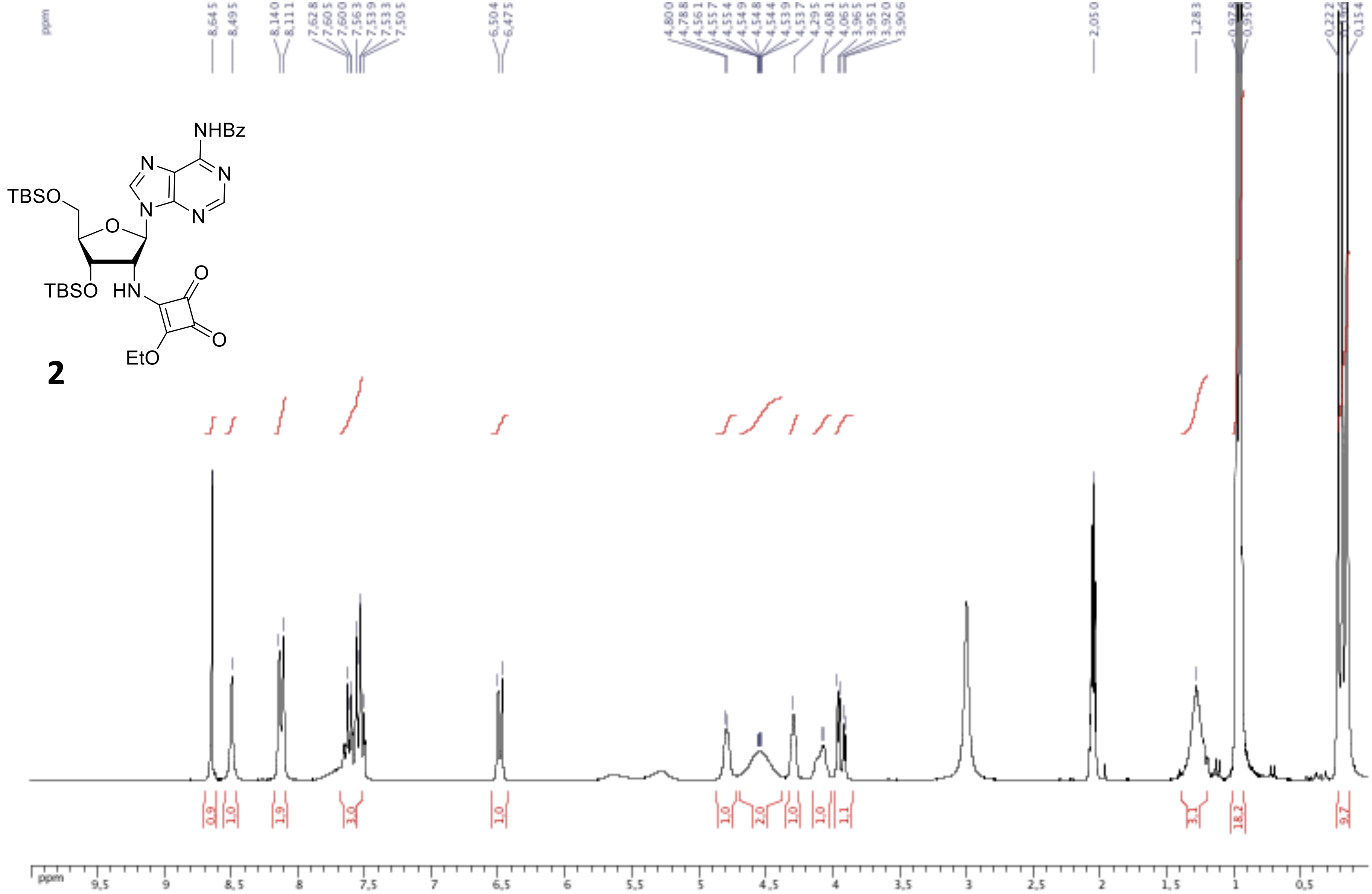


6-((2-(((2R,3R,4S,5R)-2-(6-amino-9H-purin-9-yl)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-3-yl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)-2-((tert-butoxycarbonyl)amino)hexanoic acid (3)

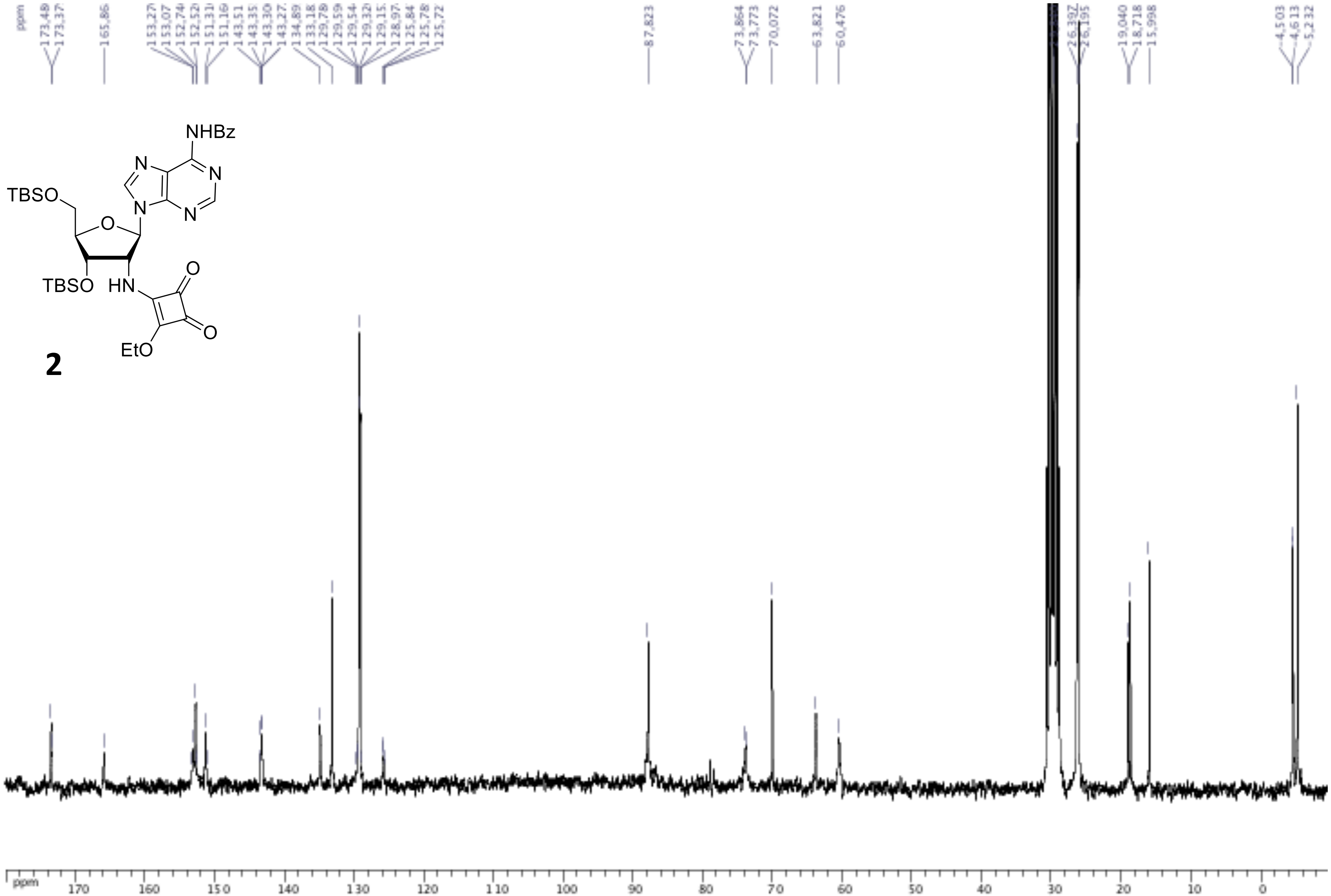
To a solution of **2** (40 mg, 0.055 mmol) in EtOH (1 mL) were added DIPEA (5 µL, 0.029 mmol, 0.5 Eq) and Boc-Lys-OH (14 mg, 0.055 mmol). The reaction mixture was stirred at 40°C for 24 h then concentrated. Flash chromatography (DCM/MeOH 96/4 to 90/10) of the residue yielded squaramide **3** (33 mg, 73 %) as a white foam.

^{1}H NMR (250 MHz, DMSO) δ 8.32 (s, 1H, H2 or H8), 8.15 (s, 1H, H2 or H8), 7.35 (s, 1H, NH), 6.15 (m, 1H, H1'), 5.41 (s, 1H), 4.58 (s, 1H), 4.07 (s, 1H), 3.92-3.86 (m, 1H), 3.74-3.71 (m, 2H), 3.41 (s, 8H, CH_2), 1.32 (s, 9H, *t*Bu^{Boc}), 0.87 (s, 9H, *t*Bu^{TBS}), 0.85 (s, 9H, *t*Bu^{TBS}), 0.08 (m, 6H, Me^{TBS}), 0.005 (m, 6H, Me^{TBS}).

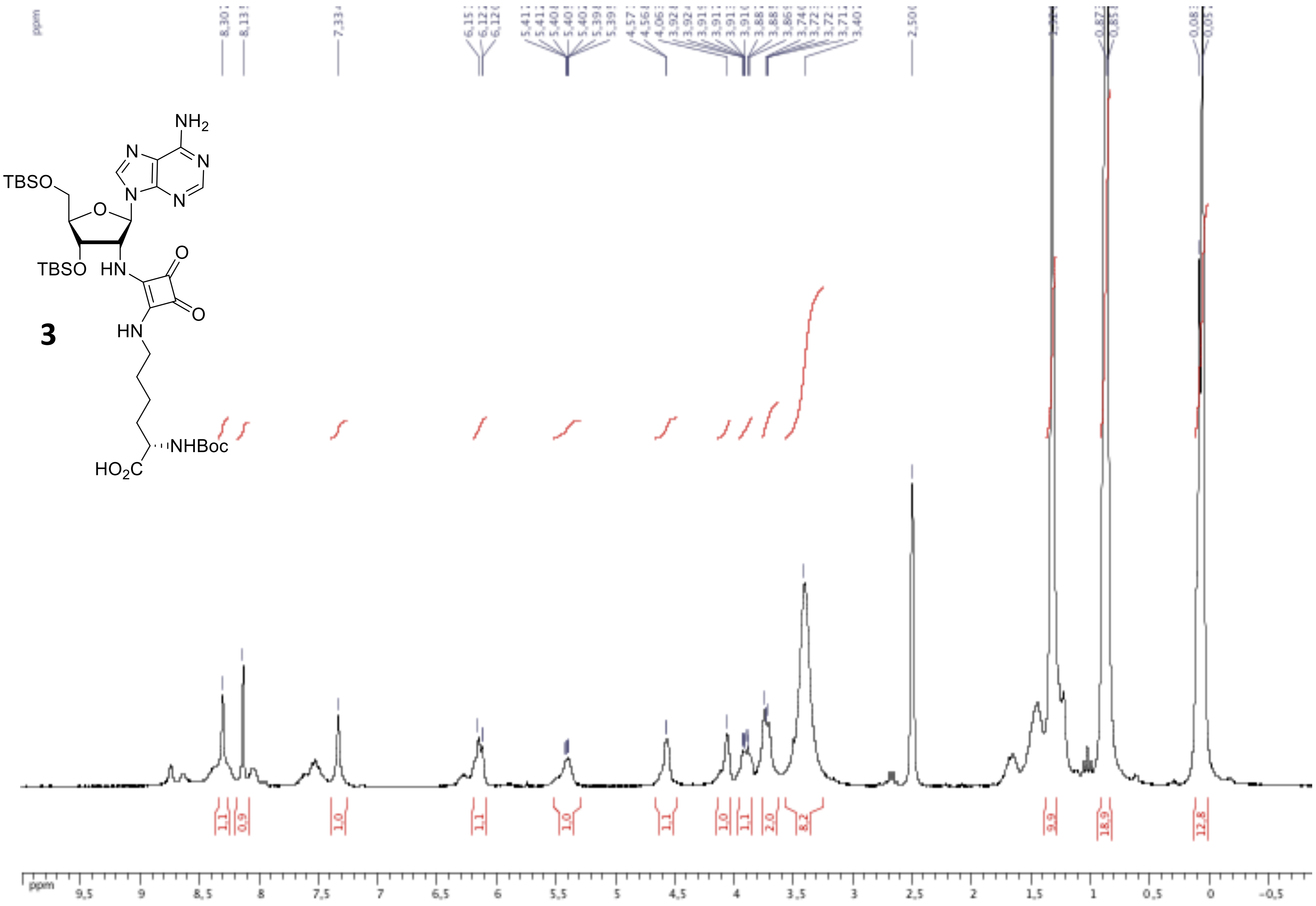
HRMS: *m/z* calcd. for $C_{37}H_{63}N_8O_9Si_2$: 819.4257 [*M*+H]; Found: 819.4313.



^{1}H, Acetone, 250 MHz

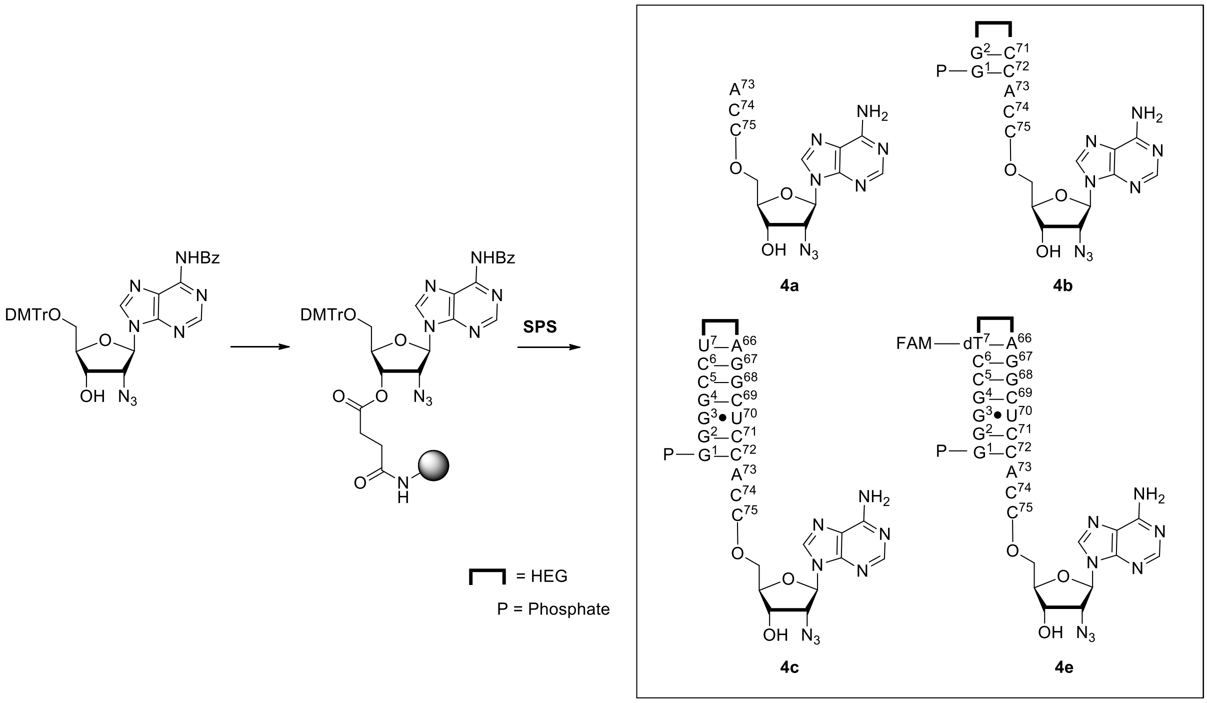


^{13}C, Acetone, 63 MHz



^{1}H, DMSO, 250 MHz

3- Solid-phase synthesis



a- Preparation of resin

Amino-SynBase resin 1000/100 (Link Technologies, Glasgow, UK) (1000 Å pore size, loading 59 micromole/g, 1.5 g) was treated with 3% trichloroacetic acid (TCA) in dichloromethane (CH_2Cl_2) for 4 h in a stoppered glass vessel. The solvents were removed by filtration and the resin was washed with triethylamine:di-isopropylethylamine (9:1), CH_2Cl_2 and diethyl ether, dried under vacuum, and soaked in dry pyridine for 10 min. Succinic anhydride (7.5 mmol) and DMAP (0.62 mmol) in dry pyridine (15 mL) were added and the vessel was rotated for 20 h. The resin was washed with pyridine, CH_2Cl_2 and diethyl ether, dried, and soaked in pyridine for 10 min. Ethyldimethylaminopropylcarbodiimide hydrochloride (1.5 mmol), DMAP (0.098 mmol), and triethylamine (60 µL) in dry pyridine (5 mL) were added to the resin followed by 2'-azido-5'-dimethoxytrityl-benzoyl adenosine (0.072 mmol) in dry pyridine (2 mL). The reaction was rotated for 20 h at room temperature, pentachlorophenol (0.13 mmole) was added, and the vessel was rotated for a further 3 h. The resin was filtered and washed with pyridine, CH_2Cl_2, and diethyl ether. Piperidine (10% in DMF, 10 mL) was added and the resin was washed after 1 min at room temperature with CH_2Cl_2 and diethyl ether. Capping reagent (oligonucleotide synthesis grade, acetic anhydride/pyridine/tetrahydrofuran: *N*-methyl imidazole in tetrahydrofuran, 1:1, 10 mL, Applied Biosystems) was added and the vessel was rotated for 1 h. The resin was washed with pyridine, CH_2Cl_2, diethyl ether, and dried under vacuum overnight. The loading of 2'-azido-5'-dimethoxytrityl-benzoyl adenosine on the resin was 24 micromol/g as determined from the cleaved DMT group.

b- Synthesis

The building blocks for the RNA analogues were prepared using 2'-TBS protected RNA phosphoramidite monomers with t-butylphenoxyacetyl protection of the A, G and C nucleobases and unprotected U (Sigma-Aldrich). Hexaethylene glycol phosphoramidite monomer were purchased from Link Technologies Ltd (Glasgow). A solution of 0.3 M benzylthiotetrazole in acetonitrile (Link Technologies) was used as the coupling agent, t-butylphenoxyacetic anhydride as the capping agent and 0.1 M iodine as the oxidizing agent (Sigma-Aldrich). All phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use, and the coupling time for all monomers was 6 min. Stepwise coupling efficiencies were determined by automated trityl cation conductivity monitoring and in all cases were >97%. Cleavage of oligonucleotides from the solid support and deprotection were achieved by exposure to concentrated 33% aqueous ammonia/ethanol (3/1 v/v) for 2 hr at room temperature followed by heating in a sealed tube for 45 min at 55°C.

c- 2'-TBS deprotection of oligoribonucleotides

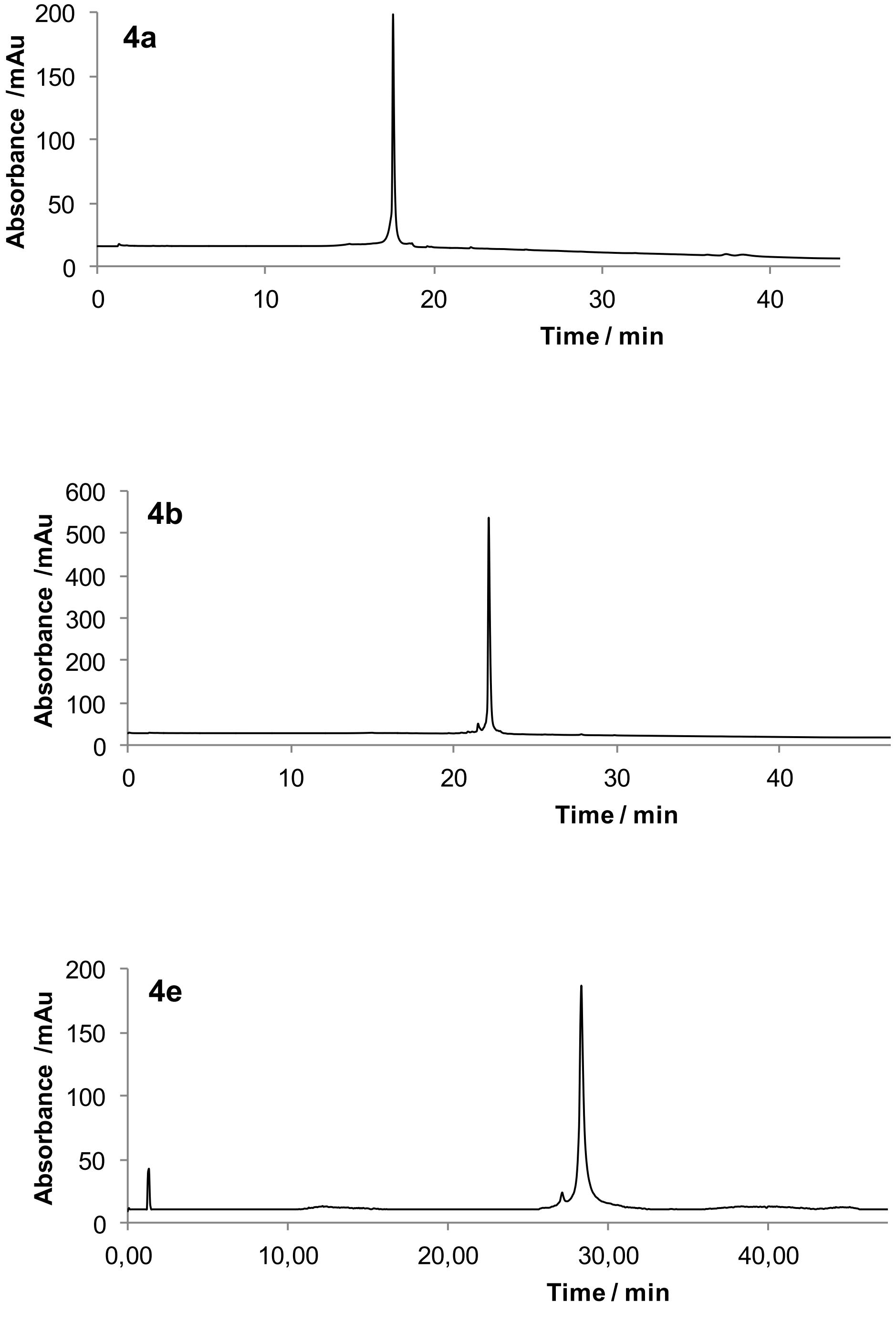
After cleavage from the solid support and deprotection of the nucleobases and phosphodiesters backbone, oligonucleotides were concentrated to a small volume in vacuo, transferred to 15 mL plastic tubes and freeze dried. The residue was dissolved in DMSO (300 μL) and triethylamine trihydrofluoride (300 μL) was added after which the reaction mixtures were kept at 65°C for 2.5 hr. Sodium acetate (3 M, 50 μL) and butanol (3 mL) were added with vortexing and the samples were kept at -80 °C for 30 min then centrifuged at 4 °C at 13,000 rpm for 10 min. The supernatant was decanted and the precipitate was washed twice with ethanol (0.75 mL) and then dried under vacuum.

d- Purification of oligonucleotides

The fully deprotected oligonucleotides **4a-c** and **4e** were purified by reversed-phase HPLC on a Gilson system using a Luna 10µ C8 100 Å pore Phenomenex column (10 x 250 mm) with a gradient of acetonitrile in triethylammonium acetate (0% to 50% buffer B over 20 min, flow rate 4 mL/min), (buffer A: 0.1 M triethylammonium acetate, pH 7.0, buffer B: 0.1 M triethylammonium acetate, pH 7.0, with 50% acetonitrile). Elution was monitored by UV absorption at 295 nm. After HPLC purification, oligonucleotides **4a-c** and **4e** were desalted using NAP-25 then NAP-10 columns (GE Healthcare). Oligonucleotides **5a-c et 5e**, **6a-e** and **7a-c** were purified by anion exchange-HPLC on an Akta purifier 10 (General Electric) system using a DNAPac PA100 column (9 x 250 mm, ThermoFisher) with variable gradient of ammonium acetate at 3 mL/min^{-1} (buffer A: 25 mM ammonium acetate pH 8.0, 0.5% acetonitrile; buffer B: 2500 mM ammonium acetate pH 8.0, 0.5% acetonitrile). Elution was monitored by UV absorption at 260 and 280 nm. Oligonucleotides **5a-c et 5e**, **6a-e** and **7a-c** were lyophilized and taken in RNAse-free water (Sigma). Concentrations of oligonucleotides were determined spectrophotometrically at 260 nm. All oligonucleotides were characterized by mass spectrometry using an electrospray time-of-flight mass spectrometer operating in negative mode (Qstar Pulsar I, Applied biosystem, Courtaboeuf, France or LCQ-Deca XP Max, Thermofisher scientific) and analytical anion exchange-HPLC on an Hitachi LACHROM system using a DNAPac PA100 column (4 x 250 mm; ThermoFisher) with a gradient of ammonium acetate (0% buffer B for 10 min then 0% to 100% buffer B in 30 min at 1 mL/min). Elution was monitored by UV absorption at 260 nm.

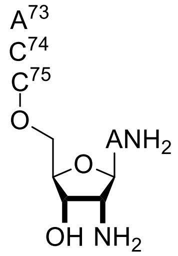
4- 2’-azido-RNAs 4a, 4b and 4e

Analytic anion exchange-HPLC chromatograms for compound **4a, 4b** and **4e**



5- Synthesis of 2’-amino-RNAs 5a, 5b, 5c and 5e

ACCA-2’-NH$_2$ (5a)

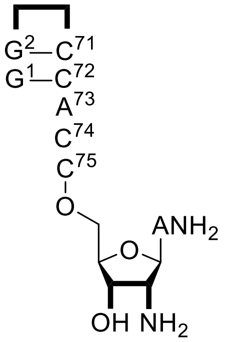


To an aqueous solution of TCEP (500 µM) in Tris.HCl buffer pH 8.0 (100 mM) was added 2’-azido-RNA **4a** (63 nmol, 20 µM). The reaction mixture was kept at -20 °C for 12 h and ACCA-2’-amino oligonucleotide **5a** was purified with the semi-preparative DNAPac PA100 column (ThermoFisher) (0% for 8.5 min then 0% to 45% buffer B in 37 min at 3 mL.min^{-1}; RT= 17.4 min. The recovered product was lyophilized, dissolved in 60 µL of free RNAse water. Final concentration was determined spectrophotometrically at 260 nm (ε^{260nm} = 37 200 M^{-1}.cm^{-1}). Yield 51% (32 nmol).

Analytical DNAPac-HPLC: RT= 17.02 min

HRMS: *m/z* calcd. for $C_{38}H_{50}N_{17}O_{23}P_3$: 1205.2478 [*M*]; found: 1205.2230

GGHHCCACCA-2’-NH$_2$ (5b)



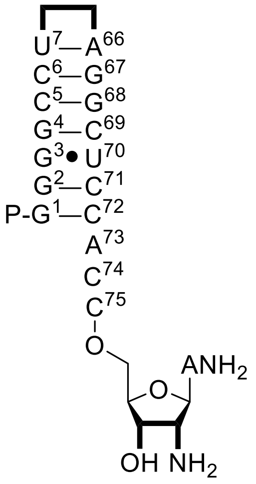
To an aqueous solution of TCEP (500 µM) in Tris.HCl buffer pH 8.0 (100 mM) was added 3’-azido-RNA **4b** (63 nmol, 20 µM). The reaction mixture was kept at -20 °C for 12 h and the product was purified with the semi-preparative DNAPac PA100 column (ThermoFisher) (0% for 14 min then 0% to 15% buffer B in 3 min and 15% to 40% B in 37 min at 3 mL/min; RT = 26.8 min). The recovered 2’-amino-oligonucleotide **5b** was lyophilized dissolved in 60 µL of free RNAse water. Final concentration was determined spectrophotometrically at 260 nm (ε^{260nm} = 85 500 M^{-1}.cm^{-1}). Yield 44% (28 nmol).

Analytical DNAPac-HPLC: RT= 21.63 min

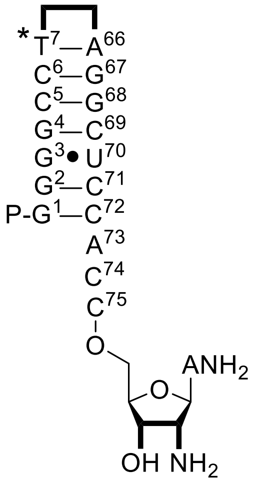
HRMS: *m/z* calcd. For $C_{88}H_{123}N_{33}O_{60}P_8$: 2850.9 [*M*]; found: 2850.8

5’P-GGGGCCUHHAGGCUCCACCA-2’-NH$_2$(5c) and

5’P GGGGCCT*HHAGGCUCCACCA-2’-NH$_2$ (5e)



5c



5e

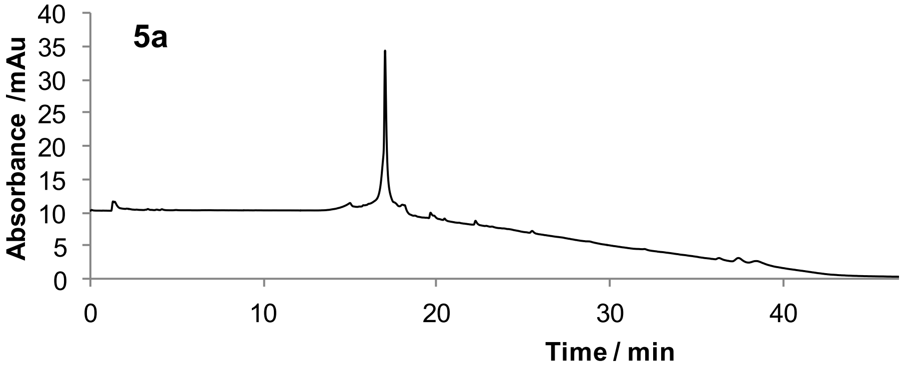
To an aqueous solution of TCEP (2.5 µmol, final concentration 500 µM) in Tris-HCl buffer pH 8.0 (100 mM) was added 3’-azido-RNA **4c** or **4e** (100 nmol, final concentration 20 µM) and the reaction mixture was kept at -20 °C for 12 h. Final product was purified by DNAPac-HPLC semi preparative DNAPac-HPLC (0% for 7 min then 0% to 10% buffer B in 3 min then 10% to 50% buffer B in 37 min at 3 mL.min^{-1}; RT = 30.7 min for **5c** 0% for 7min then 0% to 30% Buffer B in 3 min then 10% to 70% Buffer B in 30 min at 3 mL.min^{-1}; RT = 20.8 min for **5e**).The amino oligonucleotides **5c** and **5e** were lyophilized, taken in water and concentrations were determined spectrophotometrically at 260 nm (Ɛ^{260nm} = 170 000 M^{-1}.cm^{-1}). 47 and 22 nmol were obtained (yields = 47% and 22%, respectively).

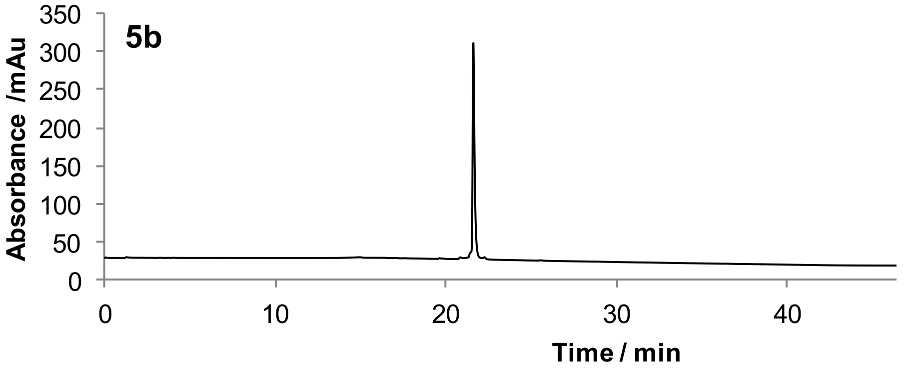
Analytical DNAPac-HPLC: RT(**5c**) = 25.20 min; RT(**5e**)= 27.84

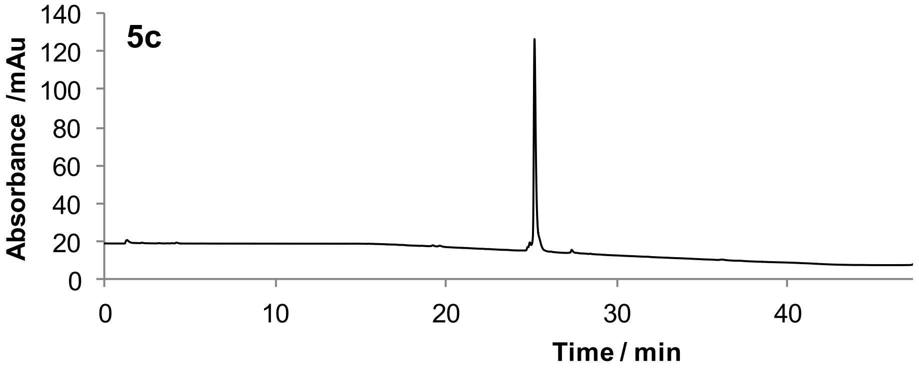
5c: HRMS: *m/z* calcd. for $C_{183}H_{242}N_{71}O_{134}P_{19}$: 6165.9319 [*M*]; found: 6165.8095.

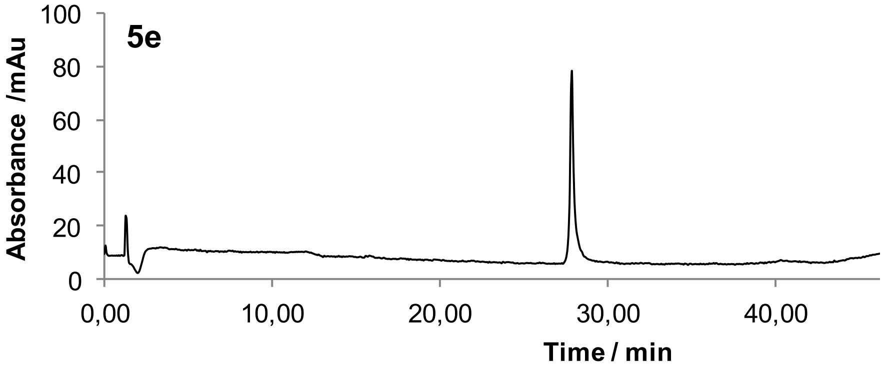
5e: MS: *m/z* calcd. for $C_{213}H_{268}N_{73}O_{140}P_{19}$: 6679.4 [*M*]; found: 6679.3.

Analytic anion exchange-HPLC chromatograms for compound **5a-c** and **5e**



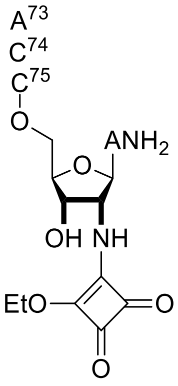



5c
Absorbance /mAu
0
20
40
60
80
100
120
140
0
10
20
30
40
Time / min


5e
Absorbance /mAu
0
20
40
60
80
100
0,00
10,00
20,00
30,00
40,00
Time / min

6- Synthesis of Ethyl or Methyl squaramate-RNA conjugates 6a-e

ACCA-2'-NH-Sq-OEt (6a)

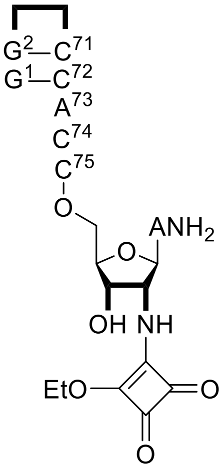


To an aqueous solution of oligo **5a** (67 nmol, final concentration 100 µM) in a 100 mM HEPES buffer pH 7.5, was added a 5 mM solution of diethylsquarate in DMF (final concentration 1 mM). The reaction mixture was kept at RT for 12 h and ACCA-2'-NH-Sq-OEt squaramate **6a** was purified using semi-preparative DNAPac PA100 column (ThermoFisher) (0% for 12.5 min then 0% to 30% buffer B in 37 min at 3 mL.min^{-1}; RT = 23.6 min). The recovered product was lyophilized, dissolved in 27 µL of free RNAse water and its final concentration was determined spectrophotometrically at 260 nm (ε^{260nm} = 37 200 M^{-1}.cm^{-1}). Yield: 79% (53 nmol).

Analytical DNAPac-HPLC: RT= 17.12 min

HRMS: *m/z* calcd. for $C_{44}H_{54}N_{17}O_{26}P_3$: 1329.2639 [*M*]; found: 1329.2406

GGCCACCA-2’-NH-Sq-OEt (6b)



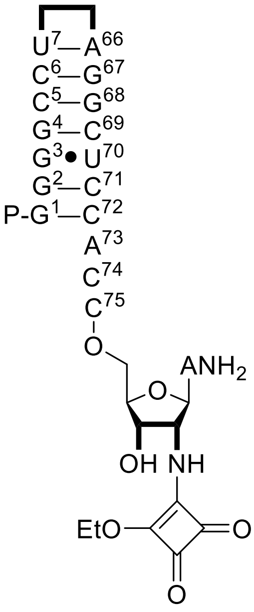
To an aqueous solution of oligo **5b** (20 nmol, final concentration 100 µM) in a 100 mM HEPES buffer pH 7.5, was added a 5 mM solution of diethylsquarate in DMF (final concentration 1 mM). The reaction mixture was kept at RT for 12 h and the product was purified using semi-preparative DNAPac PA100 column (ThermoFisher) (0% for 14 min then 0% to 30% buffer B in 37 min at 3 mL.min^{-1}; RT = 42.6 min). The recovered oligonucleotide **6b** was lyophilized, dissolved in 27 µL of free RNAse water and its final concentration was determined spectrophotometrically at 260 nm ($\mathcal{E}^{260nm}$ = 85 500 M^{-1}.cm^{-1}). Quantitative Yield (20 nmol).

Analytical DNAPac-HPLC: RT= 21.85 min

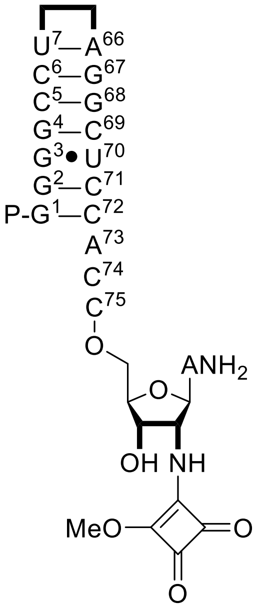
HRMS: *m/z* calcd. for $C_{94}H_{127}N_{33}O_{63}P_8$: 2973.5649 [*M*]; found: 2973.6433

5’P-GGGGCCUHHAGGCUCCACCA -2’-NH-Sq-OR (R=OEt : 6c – R=OMe : 6d;

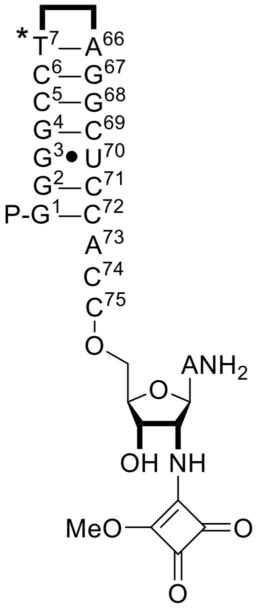
5’P-GGGGCC*THHAGGCUCCACCA -2’-NH-Sq-OCH_3, 6e)



6c



6d



6e

To an aqueous solution of oligo **5c** or **5e** (20 nmol for **5c** and 35 nmol for **5e**, final concentration 100 µM) in a 100 mM HEPES buffer pH 7.5, was added a 50 mM solution of diethyl- or dimethyl-squarate in DMF (80 µL, 4 µmol, final concentration 10 mM). The reaction mixture was kept at 37 °C for 12 h. Squaramide RNAs were purified by semi preparative DNAPac-HPLC (0% for 14 min then 0% to 10% buffer B in 3 min then 10% to 50% buffer B in 37 min at 3 $mL.min^{-1}$; RT = 40.0 min for OEt squaramide **6c** and 35.0 min for OMe squaramide **6d;** 0% for 12min then 0% to 10% buffer B in 3 min then 10% to 43% buffer B in 30 min then 43% to 61% buffer B in 7min at 3 $mL.min^{-1}$; RT = 52.1 min for **6e**). Oligonucleotide-squaramides **6c, 6d** and **6e** were lyophilized, dissolved in water and concentrations were determined spectrophotometrically at 260 nm (ε^{260nm} = 170 000 $M^{-1}.cm^{-1}$). 14.7 nmol were obtained for diethylsquarate **6c** (yield = 74%), 15.8 nmol for dimethylsquarate **6d** (yield=79%) and 13.4 nmol for **6e** (yield=38%).

Analytical DNAPac-HPLC (**6c**): RT= 25.28 min

Analytical DNAPac-HPLC (**6d**): RT= 25.61 min

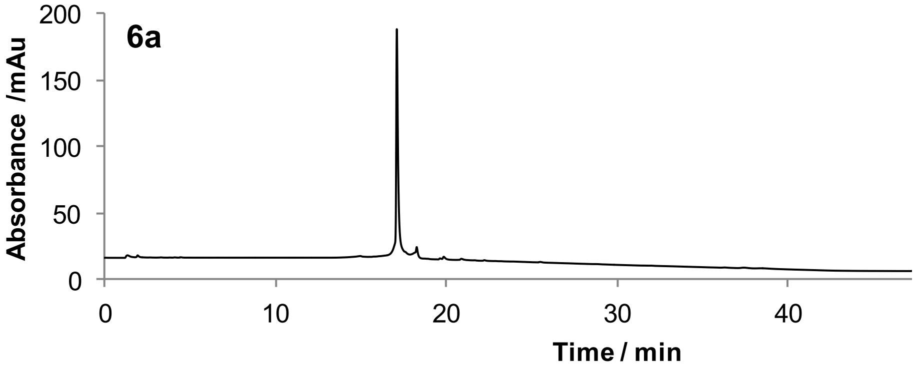
Analytical DNAPac-HPLC (**6e**): RT= 26.87 min

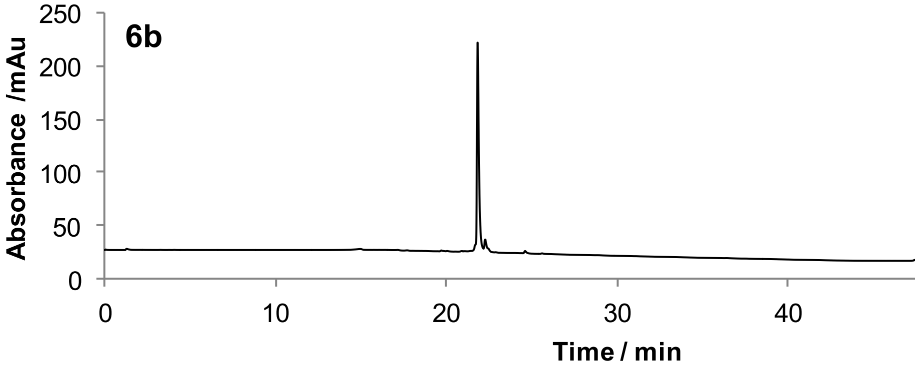
6c HRMS: *m/z* calcd. for $C_{189}H_{246}N_{71}O_{137}P_{19}$: 6289.9480 [*M*]; found: 6291.0191

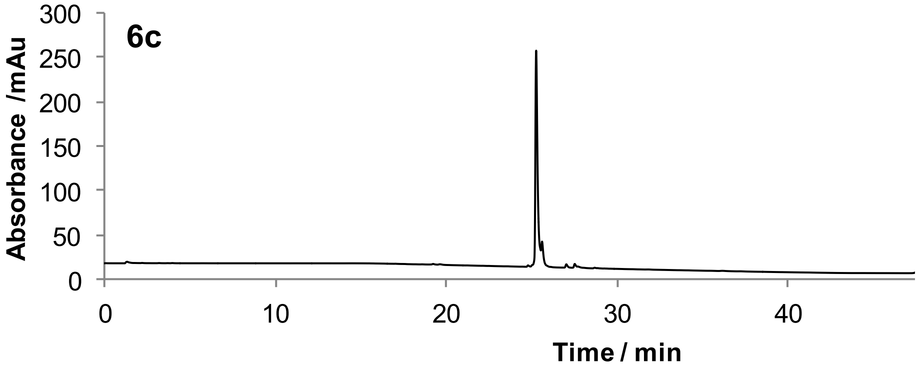
6d HRMS: *m/z* calcd. for $C_{188}H_{244}N_{71}O_{137}P_{19}$: 6275.9323 [*M*]; found: 6275.9852

6e MS: *m/z* calcd. for $C_{218}H_{270}N_{73}O_{143}P_{19}$: 6789.4 [*M*]; found: 6789.1

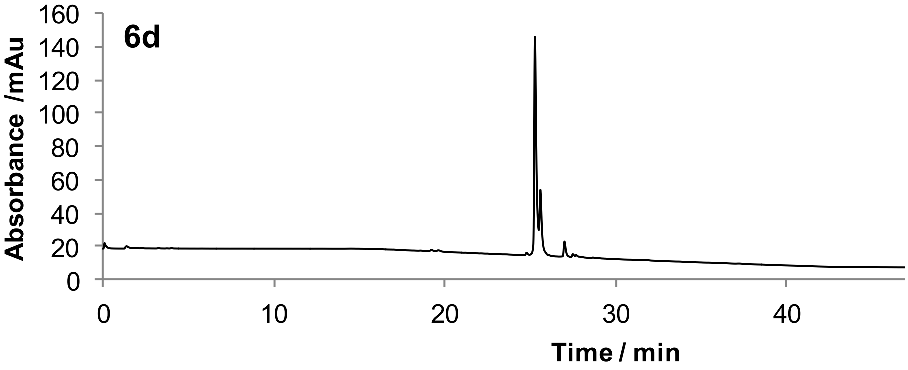
Analytic anion exchange-HPLC chromatograms for compound **6a-e**



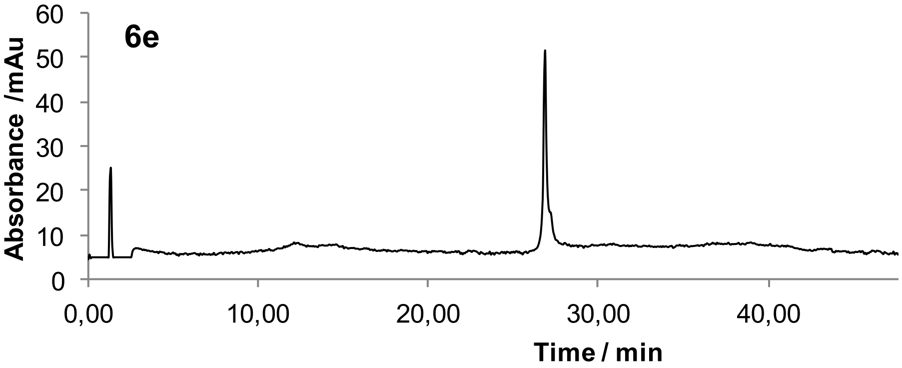




6d

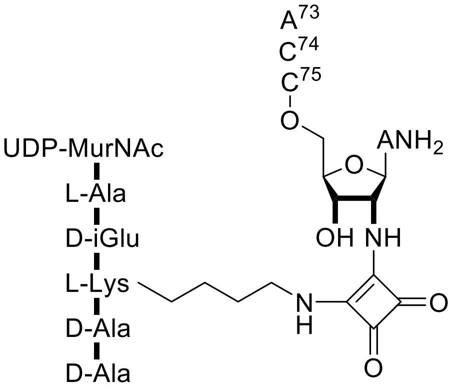

6d
Absorbance /mAu
160
140
120
100
80
60
40
20
0
0
10
20
30
40
Time / min

6e


6e
Absorbance /mAu
60
50
40
30
20
10
0
0,00
10,00
20,00
30,00
40,00
Time / min

7- Synthesis of peptidyl-squaramide-RNA conjugates 7a-7c

ACCA-2’-NH-Sq-NH-UM5K (7a)

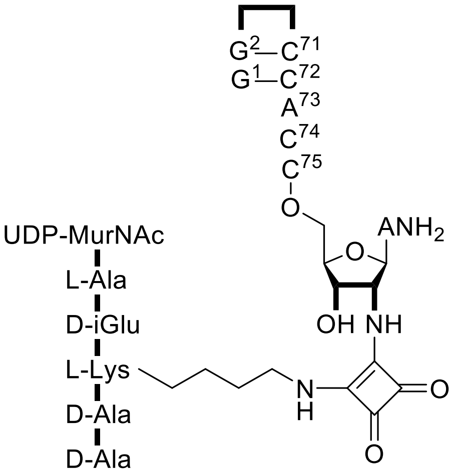


Oligo **6a** (20 nmol) and UDP-MurNAc-pentapeptide (200 nmol) were lyophilized and dissolved in 10 µL of a 500 mM borate buffer pH 9.2 containing 10% DMF. The reaction mixture was kept at 37°C for 72 h. ACCA-2’-bis-squaramide-UDP-MurNAc-pentapepide **7a** was purified using semi-preparative DNAPac PA100 column (ThermoFisher) (0% for 14 min then 0% to 30% buffer B in 37 min at 3 $mL.min^{-1}$; RT = 41.8 min). The recovered product was lyophilized, dissolved in 100 µL of free RNAse water and its final concentration was determined spectrophotometrically at 260 nm ($\mathcal{E}^{260nm} = 47\ 200\ M^{-1}.cm^{-1}$). Yield 22.4% (2.24 nmol).

Analytical DNAPac-HPLC: RT= 19.38 min

HRMS: *m/z* calcd. for $C_{82}H_{113}N_{26}O_{51}P_5$: 2432.5736 [*M*]; found: 2432.6277

GGCCACCA-2'-NH-Sq-NH-UM5K (7b)

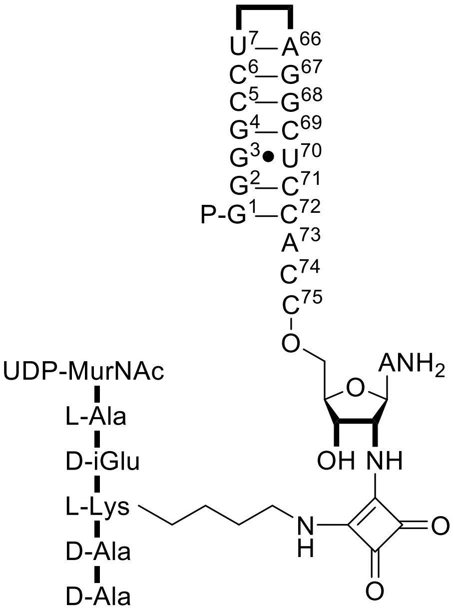


Oligo **6b** (20 nmol) and UDP-MurNAc-pentapeptide (200 nmol) were lyophilized and dissolved in 10 µL of a 500 mM borate buffer pH 9.2 containing 10% DMF. The reaction mixture was kept at 37°C for 72 h. Product was purified using semi-preparative DNAPac PA100 column (ThermoFisher) (0% for 5.5 min then 0% to 15% buffer B in 3 min then 15% to 50% buffer B in 37 min at 3 $mL.min^{-1}$; RT = 22.3 min). The recovered product **7b** was lyophilized, dissolved in 100 µL of free RNAse water and its final concentration was determined spectrophotometrically at 260 nm (ε^{260nm} = 95 500 $M^{-1}.cm^{-1}$). Yield 40% (7.93 nmol).

Analytical DNAPac-HPLC: RT= 21.67 min

HRMS: *m/z* calcd. for $C_{132}H_{186}N_{42}O_{88}P_{10}$: 4077.0070 [*M*]; found: 4076.8747

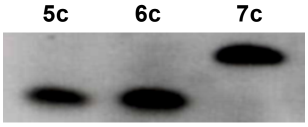
5’P-GGGGCCUHHAGGCUCCACCA -2’-NH-Sq -NH-UM5K (7c)



Oligo **6c** (11 nmol) and UDP-MurNAc-pentapeptide (110 nmol) were lyophilized and dissolved in 10 µL of a 500 mM borate buffer pH 9.2 containing 10% DMF. The reaction mixture was kept at 37 °C for 72 h. Compound **9c** was purified using semi-preparative DNAPac-HPLC (ThermoFisher) (0% for 14 min then 0% to 50% buffer B in 37 min at 3 mL.min^{-1} ; RT = 41.9 min). The recovered product was lyophilized, taken in 100 µL of free RNAse water and final concentration was determined spectrophotometrically at 260 nm (ε^{260nm} = 180 000 M^{-1}.cm^{-1}). Yield 45% (5 nmol). **7c** was characterized by denaturing PAGE (supplementary figure S1) and mass spectroscopy.

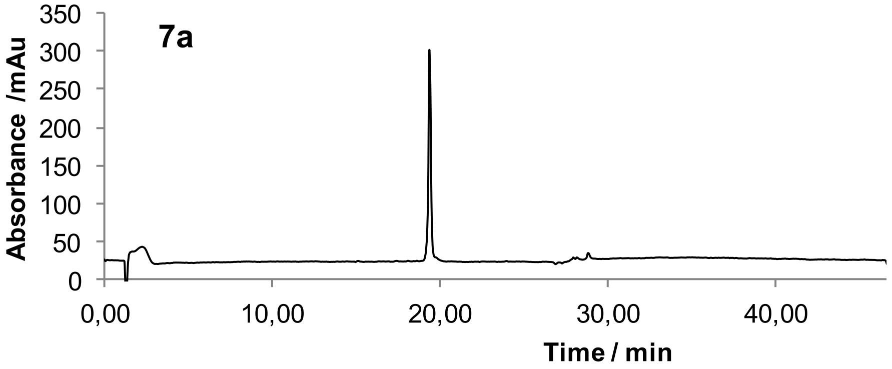
Analytical DNAPac-HPLC: RT= 23.74 min

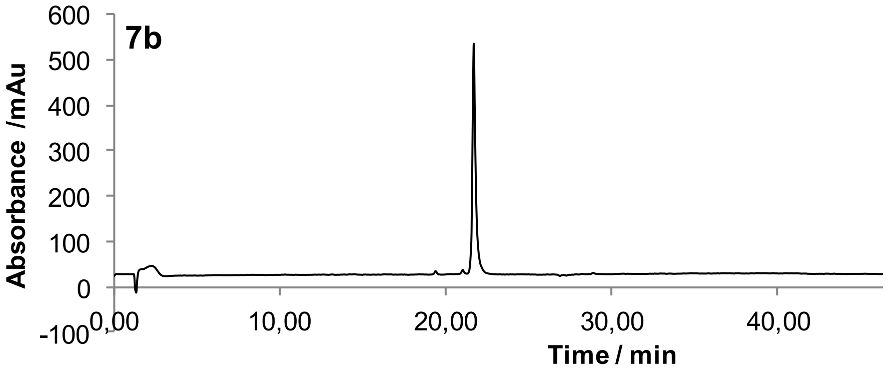
MS: *m/z* calcd. for $C_{227}H_{305}N_{80}O_{162}P_{21}$: 7396.8 [*M*]; found: 7396.6

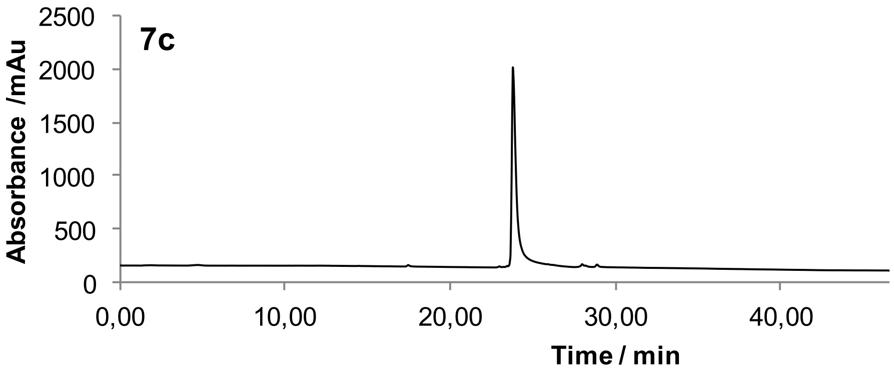


Supplementary Figure S1. Denaturing PAGE of peptidyl-squaramide-RNA **7c**. Analytical denaturing PAGE was performed in gels (20 x 20 x 0.1 cm) containing acrylamide (13%, w/v), bis-acrylamide (4.4 %, w/v), urea (8 M), TEMED (0.04%, v/v), ammonium persulfate (0.08% w/v), and 1xTBE buffer (pH 8.2) [Tris (89 mM), borate (89 mM), EDTA (2 mM)]. Gels were loaded (lane width, 6 mm) with 200 pmol of **5c**, **6c** and **7c** in a volume of 10 µL containing bromophenol blue (0.01%, w/v), and glycerol (25%, v/v). Electrophoresis was performed for 90 min at 600 V and gel stained with ethidium bromide (0.5 µg.mL^{-1}) was imaged with a Herolab E.A.S.Y 429K camera.

Analytic anion exchange-HPLC chromatograms for compound **7a-c**







8- Protein purification and determination of aminoacyl-transferase activity

Wild-type FemX_{Wv}, FmhB and mutant of FemX_{Wv} mutants proteins were produced in *Escherichia coli* TOP10 harboring derivatives of vector p*Trc*-His60. Proteins were purified by nickel-affinity, anion-exchange, and size-exclusion chromatographies.[2] Protein concentration was determined using the Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as a standard. FemX_{Wv} and mutant derivative proteins were concentrated to a final concentration of 10 g.L^{-1} by ultrafiltration and stored at -20°C in 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl supplemented with 50% glycerol. FmhB was concentrated to a final concentration of 10 g.L^{-1} by ultrafiltration and stored at -20°C in 100 mM phosphate buffer pH 7.5 containing 500 mM KCl supplemented with 50% glycerol. FemX_{Wv} aminoacyl-transferase activity was determined in a coupled assay containing an auxiliary system to generate aminoacyl-tRNAs as previously described.[2] Briefly, the assay contained 50 mM Tris-HCl (pH 7.5), 12.5 mM MgCl_2, 2 mM β-mercaptoethanol, 7.5 mM ATP, 800 nM aminoacyl-tRNA synthetase, 50 µM UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (UDP-MurNAc-pentapeptide), 50 µM of $[^{14}\text{C}]$L-Ala (3,700 Bq.nmol-1, ICN), and 0.4 µM tRNA^{Ala}. Reactions were performed at 30°C with a preincubation of 10 min prior to the addition of FemX_{Wv} or mutant proteins for synthesis of the L-Ala-tRNA^{Ala} by the auxiliary system. The concentration of FemX_{Wv} and mutants (0.75 to 800 nM) were adjusted to obtain initial velocities in conditions where transferase activity was rate limiting. Reactions were stopped at 95 °C for 10 min. [14C]Ala and UDP-MurNAc-pentapeptide($[^{14}\text{C}]$Ala) were separated by descending paper chromatography (Whatman 4MM) with isobutyric acid:ammonia 1M (5:3 per vol). Radioactive spots were identified by autoradiography, cut out, and counted by liquid scintillation. Inhibition of aminoacyl-transferase activity by peptidyl-squaramide-RNAs was determined as previously described.[3] Curves (Figure 2B) represent fits of a four-parameter logistic function of triplicates of experimental values (Sigma Plot 12.0).

9- Protein-squaramide ester RNA cross linking

Compounds **6c**, **6d** or **6e** (125 pmol, 25 µM final) and proteins (5 µg, 250 pmol, 50 µM final) were dissolved in 500 mM borate buffer pH 9.2. After 5 hours of incubation at 37°C, 5 µL of a solution of 50% glycerol in water containing 0.25% xylene cyanol and 0.25% bromophenol were added for each experiment. Analysis of cross linking formation was performed by denaturing polyacrylamide gel electrophoresis in a 12% acrylamide gel containing 8 M urea and 0.005% sodium dodecyl sulfate for 40 min at 200V. Formation of protein-RNA conjugates was assessed by co-localization of RNA and protein by fluorescence emission of fluorescein containing RNA by UV excitation at 312 nm (Herolab E.A.S.Y 429K camera), Coomassie blue staining and immuno-detection for protein identification. Immuno detection of FemX$_{Wv}$ proteins and variants was performed by classical chemoluminescence Western-blot techniques using rabbit anti-FemX$_{Ww}$ primary antibodies and goat anti-rabbit secondary antibodies conjugated to horse radish peroxidase (HRP) from ThermoScientific.

10- Bibliography

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[2] A. P. Maillard, S. Biarrotte-Sorin, R. Villet, S. Mesnage, A. Bouhss, W. Sougakoff, C. Mayer, M. Arthur, *J. Bacteriol.* **2005**, *187*, 3833-3838.

[3] M. Fonvielle, D. Mellal, D. Patin, M. Lecerf, D. Blanot, A. Bouhss, M. Santarem, D. Mengin-Lecreulx, M. Sollogoub, M. Arthur, M. Etheve-Quelquejeu, *Chemistry* **2013**, *19*, 1357-1363.