Synthetic Studies Towards Oligonucleotide Derivatives and Conjugates

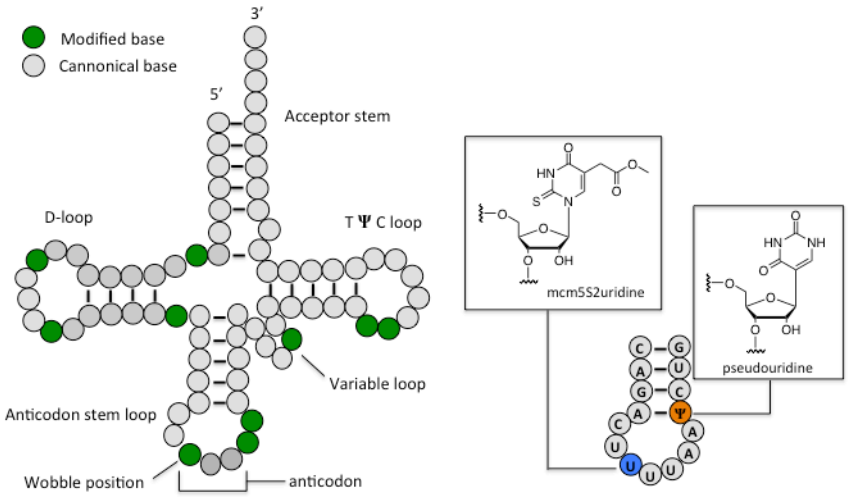
Delft, P. van

4 Modified (oligo)nucleotides as haptens conjugated to BSA as the protein carrier

3.1 Introduction

Nucleic acid modification is a widespread phenomenon in eukaryotes. Both DNA and RNA are subject to post-transcriptional modification.[1] For instance, an important feature in regulating gene translation entails the methylation of 2-deoxy-cytosine to 5-methyl-dC residues in DNA CpG dinucleotides.[2,3] Although recently several new modifications have been found on DNA as epigenetic markers[1], the occurrence of modified residues in RNA has already been known for over five decades and now exceeds well over a hundred identified modifications.[4] These modifications range from small changes such as thiolation and methylation of nucleobases to larger glycosylated and amino acid containing adducts. tRNA for example is known to contain a large variety and amount of these post-transcriptional modifications. Figure 4.1 depicts structural features of tRNA's and their modifications.

Figure 4.1: Schematic representation of tRNA and their post-transcriptional modifications. On the left: full length tRNA for alanine and sites of modification. On the right: anticodon loop of the tRNA for lysine ($tRNA^{lys}$).



Besides modified nucleosides in tRNA that contribute to the folding and provide stability, modifications in the anti-codon of tRNA allow the recognition of multiple codons by a single tRNA molecule. This phenomenon is called Wobble base pairing and the 5' base of the anticodon is therefore called the Wobble position and accounts for the smaller number of tRNA's compared to the number of different codons.[5,6] The anticodon loop of $tRNA^{lys}$ (figure 4.1, right) holds two modifications, pseudouridine (ψ) and at the wobble position mcm^5S^2U. Here the mcm^5S^2U nucleotide is important for recognition of both the AAA and the AAG codon, whereas ψ is important for the loop's stability increasing the T^m by 5 ^{0}C.

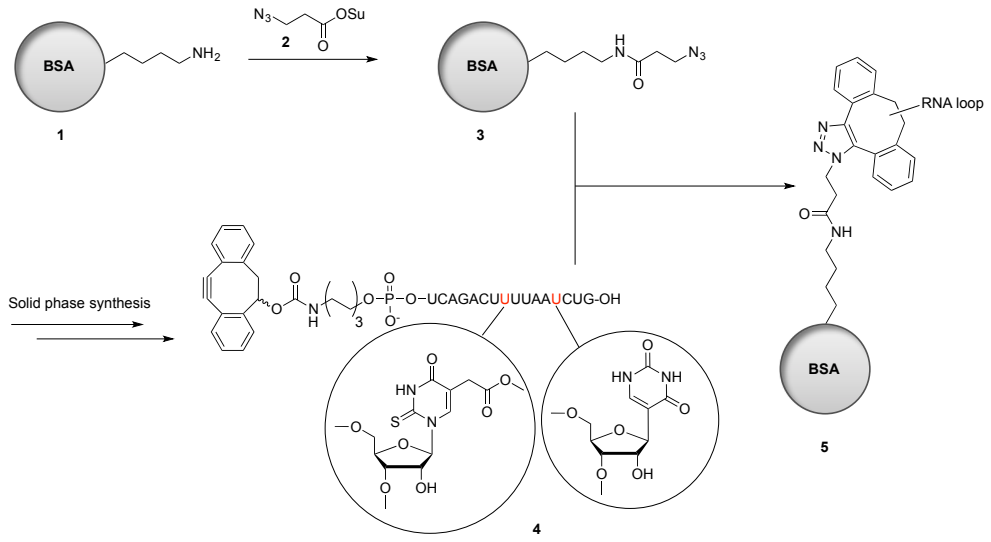
Although, in eukaryotes RNA modifications are mostly present in tRNA and rRNA where they aid formation of distinct tertiary structures and fine-tune their function, modifications on for example mRNA have also been found.[7] Additionally, modifications have been found in small functional non-coding RNA such as small nucleolar RNA(snoRNA), small nuclear RNA(snRNA), microRNA(miRNA)[8], and piwi interacting RNA(piRNA).[9] Insights in the presence of non-canonical bases on these RNA's might be of great value in the understanding of their function.

Whilst many RNA modifications are not essential for the survival of organisms, alterations in the modification profile of RNA's may have serious effect on its proper functioning.[10] For instance, some changes in RNA modification have been correlated with diseases such as cancer.[11,12] The postulate, that RNA modification is a dynamic system acting on cellular signals, makes the mapping of the events in RNA modification an interesting field of research.[13] The pathways and proteins involved are of complex nature and are often not fully understood or known.

The research on modifications throughout the different classes of RNA will benefit from the availability of suitable tools. For instance, the availability of a method for detecting the presence or absence of a specific modification in the different classes of RNA will provide information under which cellular conditions and/or presence of certain proteins the event of modification occurs. Antibodies are widely used for the detection of cellular components and have also been applied to (modified) nucleosides.[14] Antibodies against several DNA modifications are now commercially available but those have been raised against small molecular haptens rather than against the modified oligonucleotide fragment as a whole. This chapter describes the construction of protein-(oligo) nucleic acid conjugates of mcm^5U, mcm^5S^2U and the full length anti-codon stem loop of $tRNA^{lys}$, as haptens to raise such antibodies.

The conjugation chemistry commonly used for mononucleotides is unsuitable for a RNA oligomer with distinct structural motifs such as the mcm^5-ester substituent and the s^2-thiocarbonyl. For instance the commonly applied reagents and conditions making use of $NaIO_4$ and $NaBH_4$ might reduce the ester or oxidize the thiocarbonyl.[15] The copper-free click chemistry using the strained cyclooctyne phosphoramidite, described in the previous chapters, is selected as a valuable alternative conjugation procedure. To this end, bovine serum albumin (BSA) is provided with azide functions, while the mcm^5U and mcm^5S^2U nucleosides and the hypermodified RNA oligomer are functionalized at the 5'-end with the dibenzocylcooctyne moiety to install the linkage between the hapten and antigenic protein under mild conditions without potentially harmful additives. Scheme 4.2 gives an outline of this approach for the

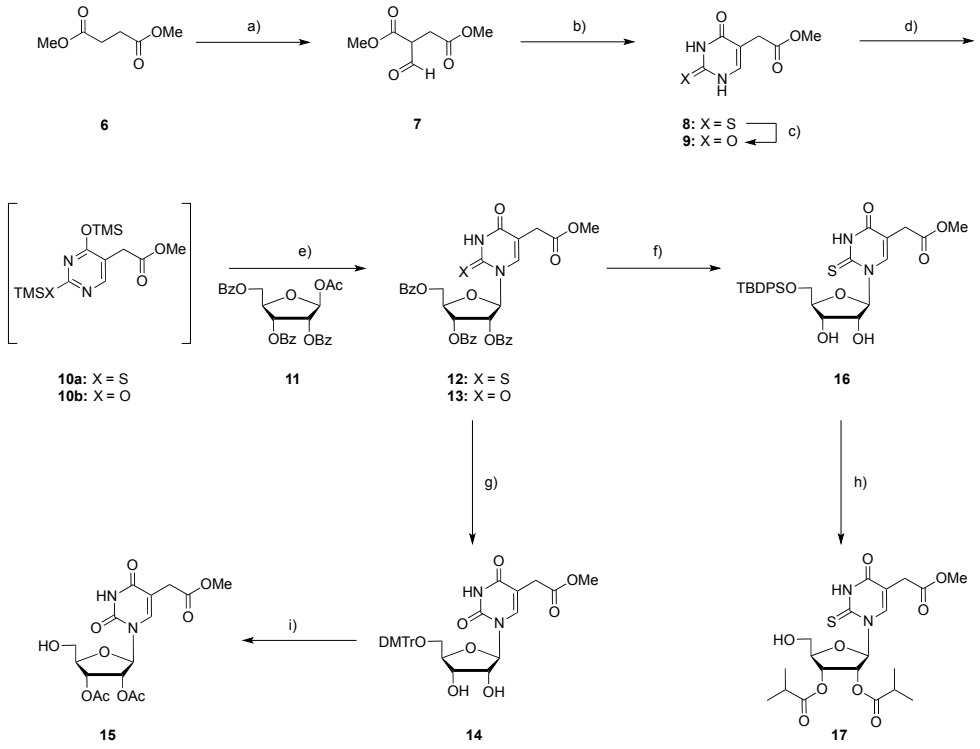
Scheme 4.2: Approach towards full length tRNA anti-codon stemloop BSA conjugate based on Cu-free click.



4.2 Results and Discussion

The study towards the applicability of strain induced alkyne azide cyclo-addition in the construction of protein nucleic acid conjugates started with the synthesis and functionalization of both the mcm^5U and mcm^5S^2U nucleosides. The synthesis of the mcm^5U and mcm^5S^2U nucleobases was performed as described[16]; formylation of dimethyl succinate followed by cyclisation using thiourea, to obtain 2-thio-5-methylcarboxymethyluracil (**8**). Desulfurization using 2-chloroacetic acid and subsequent Fisher esterification yielded 5-methylcarboxymethyluracil (**9**) from the sulfurized precursor. Adaptation of a method, described by Vorbrüggen[17], led to the coupling of the nucleobases to the ribose sugar. Silylation of the nucleobases using TMS-Cl and HMDS under reflux was followed by addition of β-1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribose (**11**) and $SnCl_4$ as a Lewis acid at 0 °C, to afford nucleosides **12** and **13** in good to excellent yields. Saponification of the benzoyl esters followed by selective protection of the 5' hydroxyl with either the TBDPS or the DMT protective group allowed orthogonal protection of 2' and 3' hydroxyls with acyl protective groups. The mcm^5U building block **13** was acetylated using Ac_2O, for solubility reasons the mcm^5S^2U nucleoside **12** was treated with iso-butyric acid anhydride. Removal of the 5' protective groups under acidic conditions or TBAF gave the suitably protected nucleosides **15** and **17** respectively.

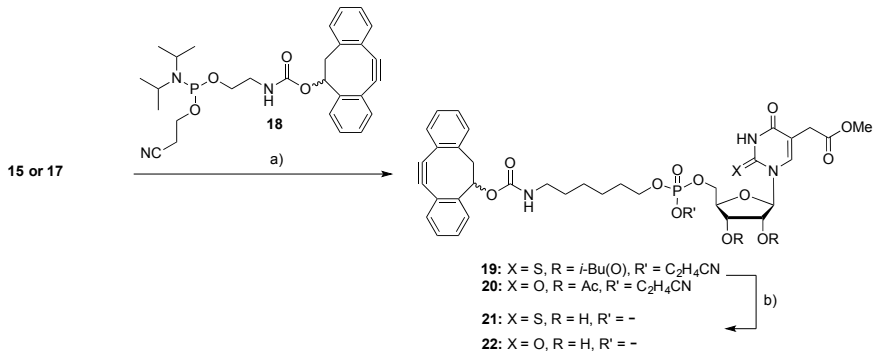
Scheme 4.3: Synthesis of suitably protected mcm^{5}U and mcm^{5}S^{2}U nucleosides.



Reagents and conditions: a) Methyl formate, NaOMe, Et_2O, 36%; b) Na(s), thiourea, MeOH, 56% c) chloroacetic acid, aqueous HCl, reflux then MeOH, H_2SO_4, reflux, 75% over two steps; d) TMS-Cl, HMDS, MeCN, reflux; e) $SnCl_4$, MeCN, 0 °C to r.t. **12** : 57%, **13** : 94%; f) NaOMe, MeOH, then TBDPS-Cl, pyridine, 70% over two steps; g) NaOMe, MeOH then DMT-Cl, pyridine, 80% over two steps; h) *iso*-butyric anhydride, pyridine then TBAF, THF, 45% over two steps; i) Ac_2O, pyridine then pTosOH, $CHCl_3$/MeOH (9 : 1, v/v), 67% over two steps.

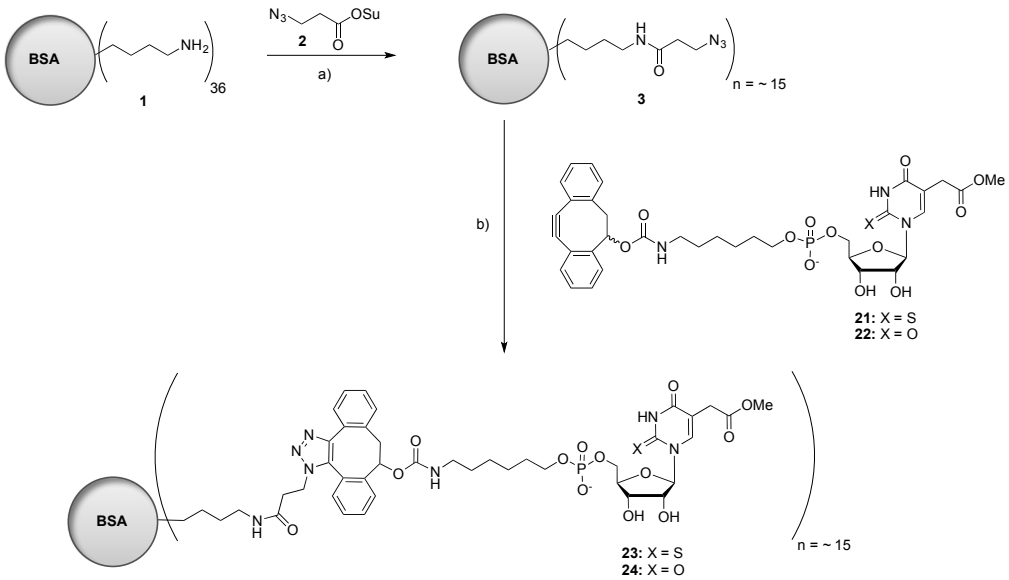
To functionalize the protected nucleosides **15** and **17** dibenzocylcooctyne phosphoramidite **18** and 4,5-dicyano-1-*H*-imidazole as the activator were used (scheme 4.4). Upon disappearance of starting material and formation of the phosphite intermediate (^{31}P-NMR of an aliquot), *t*-BuOOH was added as the oxidizing agent forming the desired functionalized nucleotide phosphotriesters **19** and **20**. This sequence of reactions proceeded smoothly for the mcm^{5}U building block (**20**). Contrary, nucleoside **19** was isolated in slightly disappointing yield of 54% probably because of the poor solubility and the associated problems with the detection of the starting material by TLC analysis. Next the protecting groups in the functionalized nucleotides **19** and **20** were removed by treating both phosphotriesters with DBU in anhydrous MeOH. The deprotection reaction was monitored by LC/MS analysis and after full conversion, the reaction mixture was quenched and purified by RP-HPLC to furnish target compounds **21** and **22** in good yield.

Scheme 4.4: The functionalization of modified nucleosides with a cyclooctyne conjugation handle.



Reagents and conditions: a) **18**, DCI, MeCN then *t*-BuOOH, **19** : 54%, **20** : quant.; b) DBU, MeOH followed by HPLC purification, **21** : 33%, **22** : 25%.

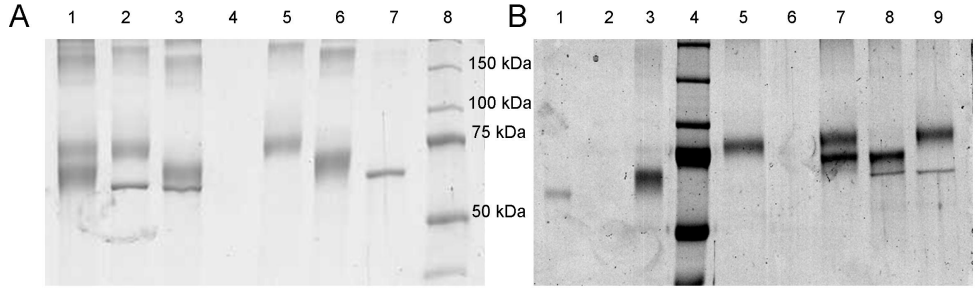
Scheme 4.5: Synthesis of azido-BSA and conjugation reaction thereof with mcm^5U and mcm^5S^2U haptens.



Reagents and conditions: a) PBS (0.2 M, pH 7.3) / MeCN (1 : 1); b) PBS (0.2 M, pH 7.3) / MeOH (5 : 1).

For the functionalization of bovine serum albumin with azide moieties 3-azidopropionic succinyl ester **2** was prepared according to a reported procedure.[18] The OSu ester of propionic acid was chosen instead of azido-acetic acid due to the lability of the azido acetic acid OSu ester. The activated ester **2** was added to a solution of BSA in phosphate buffer saline at pH 7.3 in 3 portions over a six hour period of time. The protein solution was then dialyzed against water to remove any unreacted azido propionic acid material and subsequently lyophilized. SDS-PAGE (figure 4.3) analysis of the isolated material revealed a shift of the azido-BSA (**3**) compared to a sample of the native protein (A, lanes 6 and 7; B, lanes 1 and 3) corresponding to an estimated coupling of 15 azido propionic acid residues.

Figure 4.3: SDS-PAGE analyis of BSA-nucleoside conjugates **23** and **24**.



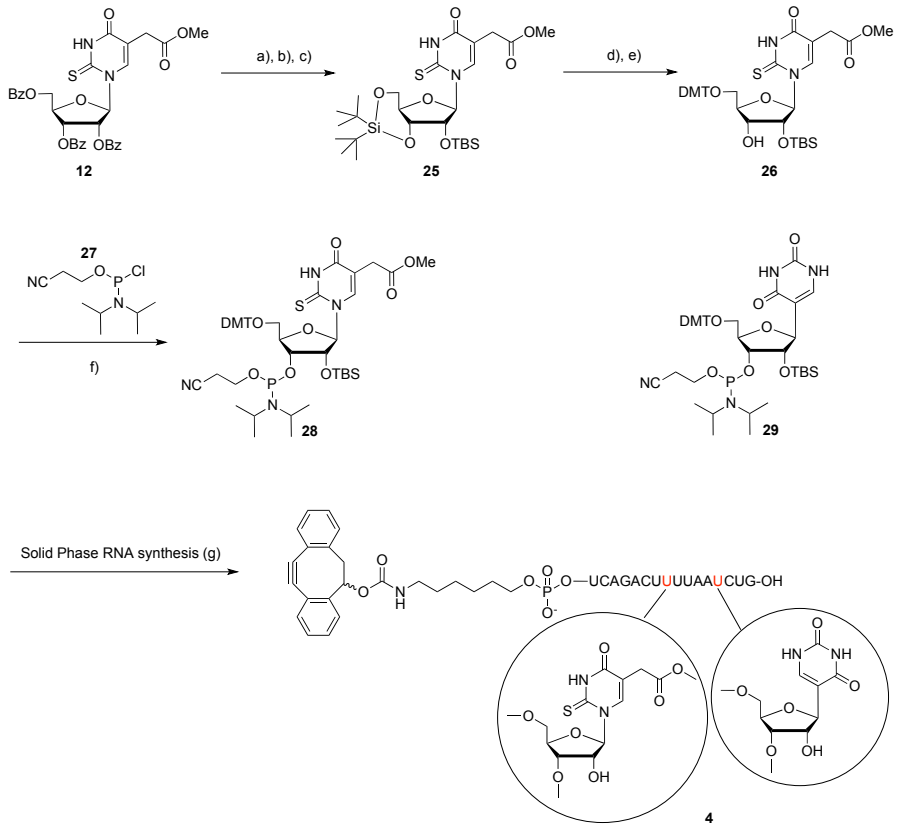
A) 7.5% AA, coumassie stain**:** 1) **3** and **24**; 2) **1** and **24**; 3) **1** and **3**; 4) blank; 5) **24**; 6) **3**; 7) **1**; 8) DC marker. **B)** 7.5% AA, coumassie stain**:** 1) **1**; 2) blank; 3) **3**; 4) DC marker; 5) **23**; 6) blank; 7) **3** and **23**; 8) **1** and **3**; 9) **1** and **23**.

To obtain the target conjugates of the modified nucleosides a solution of azido-BSA in PBS was added a solution of cyclooctyne functionalized nucleotide haptens **21** and **22** in MeOH, respectively. The reaction mixtures were shaken 8 hours prior to dialysis over a 10 kDa molecular weight cut-off membrane yielding target conjugates **23** and **24** as identified by SDS-PAGE analysis and coumassie staining. (figure 4.3). Lanes A5 and B5 illustrate bands shifted from the starting azido BSA indicating complete coupling.

Guided by the successful construction of the mono-nucleotide BSA conjugates **23** and **24** the preparation of the proposed full-length anti-codon stem loop RNA protein conjugate (scheme 4.1, **5**) was explored. To this end the modified mcm^5S^2U phosphoramidite building block required for the solid phase RNA synthesis was prepared (scheme 4.6).

Starting from acyl protected **12**; literature procedures[19] describe the saponification of the benzoyl esters, subsequent protection of the primary alcohol using DMT-Cl followed by 2'-*O*-TBDMS protection and finally phosphitylation. However, in this sequence of reactions, the protection of the 2'-hydroxyl in presence of the free 3'-hydroxyl results in an regio isomeric mixture of 2'- and 3'-silyl ethers of which the separation can be cumbersome. Therefore the method described by Serebryany and Beigelman[20] was adopted (scheme 4.6). In a one pot procedure the benzoyl esters in **12** were removed, the cyclic di-*tert*-butyl-silandiyl protective group on the 5'- and 3'-OH and the TBDMS on the 2'-OH protection were introduced to give **25** in good yield. Next the cyclic di-*tert*-butyl-silandiyl group in nucleoside **25** was selectively removed using HF in pyridine at 0 ^oC followed by DMT protection to give **26** which was phosphitylated giving target building block **28**.

Scheme 4.6: Synthesis of mcm^{5}S^{2}U phosphoramidite building block and subsequent incorporation.



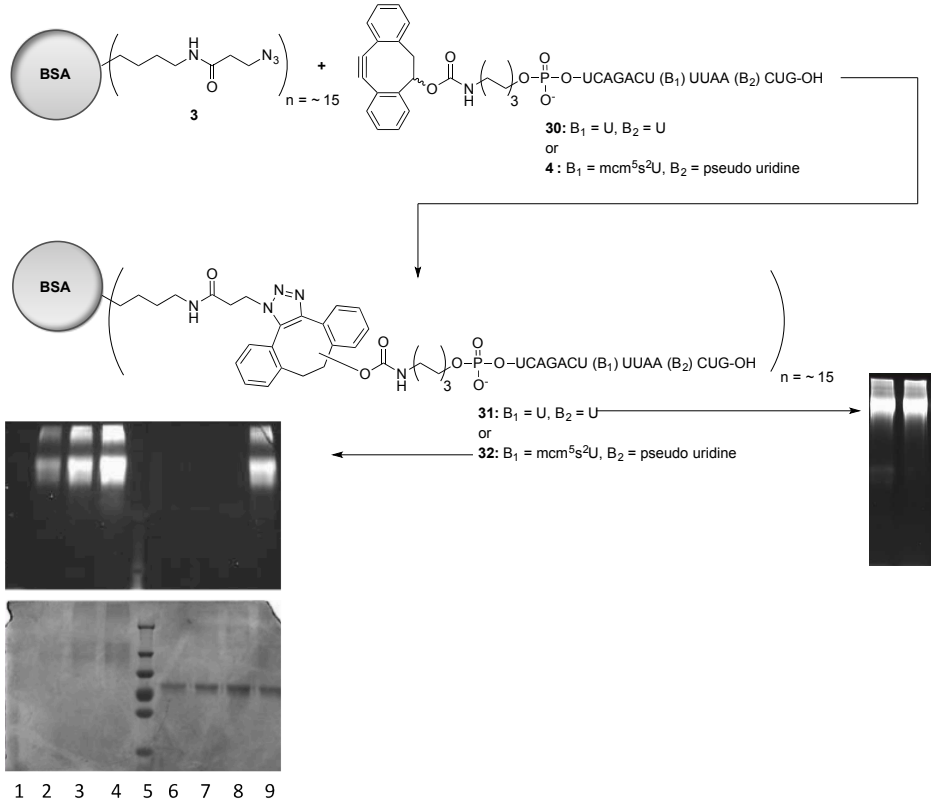
Reagents and conditions: a) NaOMe, MeOH; b) di-*tert*-butyl-silyl-bis-(trifluoromethanesulfonate), DMF; c) TBDMS-Cl, imidazole, 74% over three steps. d) pyridine·HF, pyridine, 0 °C; e) DMT-Cl, pyridine, 0 °C, 60% over two steps. f) DIPEA, DCM, 63%.

The automated synthesis of RNA 16-mer **4** was accomplished using polystyrene support preloaded with guanosine and a standard *tert*-butyl-phenoxy-acetyl (TAC) based RNA solid phase synthesis protocol similar to a previously reported synthesis.[19] The applied nucleoside amidites including pseudouridine building block **29** (scheme 4.6) were commercially obtained. When the repeated elongation cycle reached the incorporation of the mcm^{5}S^{2}U via building block **28**, the oxidation procedure was changed from I_2/H_2O/pyridine to *t*-BuOOH in MeCN to avoid desulfurization. In the final coupling cycle dibenzocylcooctyne phosphoramidite **18** was used to obtain the protected 5' functionalized immobilized target oligonucleotide. Cleavage from the solid support and removal of the protecting groups were realized by treatment with DBU in anhydrous MeOH in order to prevent hydrolysis or ammonolysis of the methyl ester. Treatment with neat triethylammonium fluoride followed by purification led to the isolation of RNA 16-mer **4**.

Prior to the conjugation of hyper modified anti-codon RNA loop **4** with azido-BSA, previously described (chapter 2) unmodified RNA-16 mer **30** was coupled to azido-BSA to

test the conjugation conditions simultaneously obtaining a good reference conjugate. SDS-PAGE analysis of the acquired RNA-BSA conjugate (scheme 4.7, **31**) showed a gel shift towards 150 kDa when ethidium bromide staining and UV-trans luminescence was applied. Using the same procedure azido-BSA was incubated with the hyper modified anti-codon RNA loop (**4**) to reveal, after dialysis, the same pattern on SDS-PAGE gel. Target conjugate **32** was also visualized by UV transluminesence after EtBr staining and subsequent Coumassie Brilliant blue staining of the same gel slab revealed faint bands for the negatively charged conjugate (lanes 2-4), the molecular weight marker (lane 5) and the azido-BSA **3** (lanes 6-9) protein. The slot containing both conjugate **32** and **3** colored as expected (150 kDa EtBr, 70 KDa Coumassie BB).

Scheme 4.7: Conjugation of modified and unmodified RNA 16-mer stem loops to BSA.



Reagents and conditions: Conjugation: PBS (0.1 M, pH 7.3), dialysis. SDS PAGE conjugate **32** : 7.5% AA, EtBr (top) and subsequent Coumassie (bottom) staining. lanes 1) blank, 2, 3, 4) 400 ng, 600 ng, 800 ng **32**, 5) dual color marker, 6, 7, 8) 400 ng, 600 ng, 800 ng **3**, 9) 400 ng **3** + 400 ng **32**. SDS PAGE conjugate **31** : 7.5% AA, EtBr staining, 400 ng **31**.

Conclusion

In summary a fast, mild and clean method for the conjugation of (oligo) ribonucleic acids to a bovine serum albumin protein carrier was developed. This method makes use of strain induced alkyne azide [3+2] cycloaddition and requires no reagents other than an alkyne functionalized (oligo)nucleotide acid and an azides containing protein. The success of this strategy was demonstrated by the coupling of two modified nucleotides and a full length (modified) RNA anti-codon stem loop to BSA. These conjugates are presently evaluated on their ability to generate antibodies against these motifs.

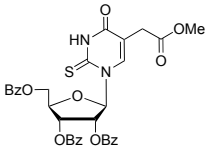
Experimental Section

General methods and materials

Chemicals were purchased from Acros Organics, Sigma Aldrich and Proligo and used as received. Dichloromethane was distilled over CaH_2 and stored on 4 Å molecular sieves. DIPEA was distilled and stored on KOH pellets. Compounds used in reactions requiring anhydrous conditions were co-evaporated with 1,4-dioxane, pyridine or toluene three times. All reactions were performed at ambient temperature under an argon atmosphere unless stated otherwise. Oligonucleotides were synthesized on an ÄKTA Oligopilot Plus oligonucleotide synthesizer (GE Healthcare Life Sciences). Reactions were monitored by TLC on Kieselgel 60 F254 (Merck). Compounds were visualized by using UV light (254 nm) or applying a solution of $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ 25 g/L, $(NH_4)_4Ce(SO_4)_4\cdot 2\ H_2O$ 10 g/L, 10% H_2SO_4 in H_2O followed by charring (+/- 150 ºC). LC/MS analysis was performed on a Jasco HPLC system (UV detection simultaneously at 214 and 254 nm) coupled to a PE/SCIEX API 165 single quadruple mass spectrometer (Perkin-Elmer). An analytical Gemini C_{18} column (Phenomex, 50 x 4.60 mm, 3 micron) was used in combination with eluents A: H_2O; B: MeCN and C 0.1 M aq. NH_4OAc as the solvent system. Analytical anion-exchange was performed on a GE ÄKTAexplorer 10 using a Dionex DNA-PAC PA-200 4x250 mm column with eluents A: 500 mM NaOAc and 50 mM $NaClO_4$ and B: 500 mM NaOAc and 500 mM $NaClO_4$ using a linear gradient (0 - 20%). Anion exchange purification was performed on a GE ÄKTAexplorer 10 using a GE Q-Sepharose HR 26 x 10 column with eluents A: 500 mM NaOAc and 50 mM $NaClO_4$ and B: 500 mM NaOAc and 500 mM $NaClO_4$ followed by a desalting procedure using a Sephadex G25 column with 150 mM NH_4OAc as the solvent system. Preperative RP HPLC was performed on a Gilson GX-281 HPLC system. A semipreparative Altima C_{18} column (Phenomex, 250 x 10 mm, 5 micron) was used in combination with eluents A 50 mM aq. Et_3HN OAc and B: MeCN as the solvent system. ^1H, ^{13}C and ^{31}P NMR were recorded on a Bruker AV-400 instrument. Chemical shifts (δ) of ^1H and ^{13}C spectra are relative to tetramethylsilane. ^{31}P chemical shifts are relative to phosphoric acid, reaction mixture aliquots measured by means of an acetone-d6 capillary. $CDCl_3$ was neutralized by filtration over neutral Al_2O_3 (Merck). HRMS spectra were recorded by direct injection (2 μL of a μM solution in H_2O or MeCN and 0.1% formic acid) on a Thermo Finnigan LTQ Orbitrap equipped with an electro spray ion source in positive mode. IR spectra were recorded on a Shimadzu FT-IR 8300 and are reported in cm^{-1}. The yields of the oligonucleotides were determined specrophotometrically using Optical Density measurements on a Varian Cary 50 Bio UV-VIS Spectrophotometer at 260 nm.

Protein purification and SDS-PAGE analysis

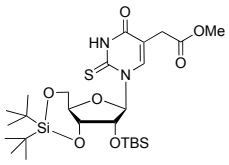
Slide-A-Lyzer (0.5 - 3 mL, 10K MWCO) dialysis cassettes were purchased from Thermo Scientific. SDS-PAGE analysis was performed using 0.75 mm 7.5% acrylamide gels. Stacking was done at 80 V for 15 mins prior to a 120 V 2 hour run. Dual color (1 µL) (Thermo Scientific) marker was used. Coumassie Brilliant Blue staining was performed using standard protocols and visualized on a Biorad GS-800 densitometer. Ethidium bromide staining was performed by treating the gel 15 minutes with a solution of ethidium bromide (1 µL of a 10% solution in H_2O was diluted with 20 mL H_2O). Destaining was performed (2 x 15 minutes, 20 mL H_2O) prior to visualization on a Biorad ChemiDoc MP using UV-trans luminescence settings.



2',3',5'-tri-*O*-Benzoyl-5-methylcarboymethyl-2-thio-Uridine (12)

A suspension of 5-methylcarboxymethyl-2-thio-Uracil (4845 mg, 20 mmol), HMDS (3.4 mL, 16 mmol), TMS-Cl (2.05 mL, 16 mmol) and 1-*O*-Acetyl-2,3,5-tri-*O*-Benzoyl-α-D-ribose (10.09 g, 20 mmol) in MeCN (200 mL) was refluxed until a clear solution was obtained. The solution was cooled to 0 °C and added dropwise to a pre-cooled (0 °C) solution of $SnCl_4$ (2.82 mL, 24 mmol, 1.2 eq) in MeCN (50 mL). The mixture was stirred for 17 hours under gradual warming to r.t. The mixture was filtered over celite and concentrated. The resulting syrup was taken up in DCM (200 mL), filtered over celite and washed with sat. aq. $NaHCO_3$ (100 mL) and H_2O (4 x 100 mL). The sodium bicarbonate fraction was diluted with H_2O (100 mL) and extracted with DCM (100 mL). The organic phases were combined, dried (Na_2SO_4) and concentrated under reduced pressure. Silica gel column chromatography (EtOAc : Toluene, 1 : 9 → 15 : 85) yielded the title nucleoside (**12**) as a white foam (7.4 g, 11.5 mmol, 57%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.34 (s, 1H), 8.09 (d, *J* = 7.7 Hz, 2H), 8.01 (d, *J* = 7.8 Hz, 2H), 7.94 (d, *J* = 7.8 Hz, 2H), 7.71 (s, 1H), 7.64 – 7.21 (m, 9H), 7.26 (s, 1H), 5.89 (t, *J* = 4.8 Hz, 1H), 5.81 (t, *J* = 5.6 Hz, 1H), 4.89 (d, *J* = 12.1 Hz, 1H), 4.81 – 4.76 (m, 1H), 4.73 – 4.65 (m, 1H), 3.57 (s, 3H), 3.01 (d, *J* = 17.4 Hz, 1H), 2.82 (d, *J* = 17.5 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 175.5, 169.6, 165.8, 165.2, 165.0, 159.1, 137.4, 133.6, 133.6, 129.9, 129.7, 129.4, 129.1, 128.8, 128.4, 128.3, 113.7, 90.0, 80.8, 74.4, 70.7, 63.5, 52.1, 31.2. IR (neat) cm^{-1} : 1724.4; 1695.5; 1480.4; 1450.5; 1259.6; 1251.9; 1090.8. HRMS (ESI): calculated for $[C_{33}H_{28}N_2O_{10} + H]^+$: 645.15374 found : 645.15433 $[M + H]^+$.

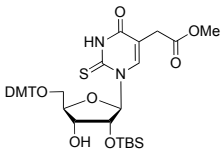


2'-*O*-dimethyl-*tert*-butylsilyl-3',5'-*O*-di-*tert*-butylsilandiyl-5-methylcarboxymethyl-2-thio-Uridine (25)

Acyl protected nucleoside **12** (7.6 g, 11.8 mmol) was suspended in MeOH (200 mL). A catalytic amount of NaOMe (432 mg, 8.0 mmol) was added and the reaction mixture was stirred for 2 days. The clear and basic solution was treated with amberlite IR-120 H^+ resin until neutral. The reaction mixture was concentrated and co-evaporated three times with 1,4-dioxane, taken up in DMF (30 mL) and cooled (0 °C). Di-*tert*-butylsilyl-bis-trifluoromethylsulfonate (3.4 mL, 10.6 mmol) was added dropwise over 15

minutes and the reaction mixture was stirred for 30 minutes. Imidazole (3.2g, 48.2 mmol) was added and the mixture was allowed to warm to r.t. TBDMS-Cl (1743 mg, 11.57 mmol) was added and the reaction mixture was stirred for 48 hrs prior to concentration. The concentrate was taken up in EtOAc (75 mL), washed with sat. aq. $NaHCO_3$ (3 x 25 mL), brine (25 mL), dried ($MgSO_4$) and concentrated under reduced pressure. Silica gel column chromatography (EtOAc : Pentane, 1 : 9 → 14 : 86) yielded silylated nucleoside (**25**) as a white foam (5.1 g, 8.7 mmol, 74% over three steps).

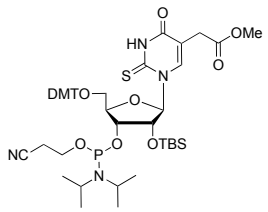
^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.75 (s, 1H), 7.66 (s, 1H), 6.46 (s, 1H), 4.55 (dd, J = 9.2, 5.2 Hz, 1H), 4.36 (d, J = 4.3 Hz, 1H), 4.28 (td, J = 10.2, 5.1 Hz, 1H), 3.89 (dd, J = 9.7, 4.3 Hz, 1H), 4.20 – 4.08 (m, 1H), 3.72 (s, 3H), 3.52 (d, J = 18.1 Hz, 1H), 3.30 (d, J = 18.2 Hz, 1H), 1.06 (s, 9H), 1.03 (s, 9H), 0.96 (s, 9H), 0.24 (s, 3H), 0.17 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0, 170.3, 159.3, 137.8, 112.1, 96.5, 77.3, 77.0, 76.7, 75.8, 75.7, 75.1, 67.4, 52.3, 30.6, 27.5, 27.2, 26.9, 25.9, 22.7, 20.3, 18.2, -3.5, -4.5. IR (neat) cm^{-1} : 2931.8, 2856.6, 1724.4, 1672.3, 1483.3, 1275.0, 1163.0, 1055.1. HRMS (ESI): calculated for $[C_{26}H_{46}N_2O_7SSi_2 + H]^+$: 587.26409 found : 587.26370 $[M + H]^+$.



2'-*O*-dimethyl-*tert*-butylsilyl-5'-*O*-(4,4'-dimethoxy)trityl-5-methylcarboxymethyl-2-thio-Uridine (26)

Pyridine·HF (0.88 mL, 30.8 mmol, 3.85 eq) was carfully diluted at 0 °C with pyridine (5.5 mL). The cooled HF solution was added dropwise to a pre-cooled (0 °C) solution of silyl protected **25** (4.69 g, 8 mmol) in DCM (40 mL). The reaction mixture was stirred at 0 °C for 3 hours. The reaction was quenched by the addition of H_2O (20 mL), diluted with DCM (20 mL), washed with sat. aq. $NaHCO_3$ (20 mL) and dried ($MgSO_4$). Toluene was added (20 mL) and the reaction mixture was concentrated under reduced pressure (30 °C). to approximately 5 mL followed by a second addition of toluene (20 mL) and consecutive final concentration. The resulting crude product was taken up in pyridine (14 mL) and cooled (0 °C). DMT-Cl (3.5 g, 8 mmol, 1.14 eq) was added and the reaction mixture was stirred overnight. The reaction was quenched by the addition of dry MeOH (1 mL) followed by concentration to approximately 15 mL (reduced pressure, 30 °C) and dilution with DCM (200 mL). The mixture was washed with H_2O (30 mL), sat. aq. $NaHCO_3$ (2 x 30 mL), brine (50 mL) and dried ($MgSO_4$). Concentration under reduced pressure was performed as described above (30 °C, 2x 100 mL toluene). Silica gel column chromatography (EtOAc : DCM, 1 : 9) yielded the title nucleotide **26** as a white foam (3.58 g, 4.8 mmol, 60% over 2 steps).

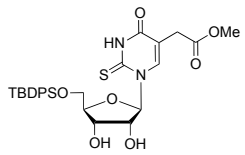
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (s, 1H), 7.39 – 7.18 (m, 10H), 6.92 – 6.78 (m, 5H), 4.55 (t, J = 5.1 Hz, 1H), 4.35 – 4.30 (m, 1H), 4.26 – 4.21 (m, 1H), 3.80 (s, 6H), 3.58 (s, 4H), 3.52 – 3.37 (m, 2H), 2.45 (d, J = 17.1 Hz, 1H), 2.32 (d, J = 17.1 Hz, 1H), 0.95 (s, 9H), 0.19 (d, J = 7.7 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.9, 169.7, 159.1, 158.8, 143.8, 138.8, 134.9, 134.8, 130.1, 130.1, 128.2, 128.1, 127.4, 114.0, 113.4, 113.4, 92.4, 87.3, 84.3, 77.3, 77.0, 76.7, 76.6, 71.4, 63.1, 55.2, 52.0, 31.4, 25.7, 18.1, -4.4, -5.1. IR (neat) cm^{-1} : 2952.2, 1738.9, 1695.5, 1607.7, 1441.9, 1249.9, 1173.7, 1033.9.



2'-*O*-dimethyl-*tert*-butylsilyl-5'-*O*-(4,4'-dimethoxy)trityl-3'-*O*-([2-cyanoethyl]-*N,N*-diisopropylamino phosphoramidite)-5-methylcarboxymethyl-2-thio-Uridine (28)

Partially protected mcm^{5}S^{2}U **26** (0.75 g, 1 mmol) was taken up in DCM (10 mL), Dipea (0.49 mL, 3 mmol, 3 eq) and 2-*O*-cyanoethyl-*N,N,*-diisopropylamino chlorophosphine (0.45 mL, 2 mmol, 2 eq) was added. The reaction mixture was stirred overnight. (product and starting material run at the same height on TLC hence a sample was treated with DCI to hydrolyse the amidite product and visualize any remaining starting material) An aliquot was analysed by ^{31}P-NMR (acetone-d6 capillary) for formation of the amidite product. The reaction mixture was quenched by the addition of sat. aq. $NaHCO_3$ (20 mL), diluted with EtOAc (40 mL, containing 2% TEA), washed with sat. aq. $NaHCO_3$ (2 x 20 mL), brine (20 mL), dried ($NaSO_4$) and concentrated under reduced pressure. The crude phosphoramidite was taken up in THF : TEA (97 : 3, 2 mL) and eluted over a sephadex column (THF : TEA, 97 : 3). Concentration followed by silicagel column chromatography (EtOAc : DCM : TEA, 5 : 93 : 2) yielded the title nucleotide as a amorphous solid (600 mg, 0.63 mmol, 63%).

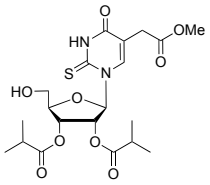
^{1}H NMR (400 MHz, CD_3CN) δ 7.87 – 7.72 (m, 2H), 7.46 – 7.33 (m, 4H), 7.33 – 7.18 (m, 15H), 6.99 (d, *J* = 6.9 Hz, 1H), 6.90 – 6.76 (m, 9H), 4.58 (dd, *J* = 6.8, 4.7 Hz, 1H), 4.54 (t, *J* = 5.1 Hz, 1H), 4.40 – 4.18 (m, 4H), 3.97 – 3.79 (m, 2H), 3.73 (s, 12H), 3.69 – 3.49 (m, 11H), 3.44 – 3.27 (m, 5H), 2.76 – 2.58 (m, 4H), 2.48 – 2.38 (m, 4H), 1.16 – 1.09 (m, 18H), 0.98 (d, J = 6.8 Hz, 6H), 0.92 – 0.86 (m, 18H), 0.17 – 0.08 (m, 12H). ^{13}C NMR (101 MHz, CD_3CN) δ 177.9, 177.5, 170.8, 170.7, 160.2, 159.8, 159.8, 145.3, 145.2, 139.1, 136.3, 136.1, 135.8, 131.0, 131.0, 130.9, 130.9, 129.1, 129.0, 128.8, 128.2, 128.1, 119.4, 15.2, 114.9, 114.2, 114.1, 93.0, 91.9, 88.1, 87.9, 84.9, 84.6, 77.1, 76.4, 74.1, 73.8, 73.6, 64.5, 64.2, 60.1, 59.9, 58.8, 58.6, 55.9, 52.6, 52.5, 44.1, 44.0, 43.7, 43.6, 32.5, 26.3, 26.2, 25.0, 24.9, 24.8, 21.1, 21.1, 20.9, 20.8, 18.8, -4.3. ^{31}P NMR (162 MHz, CD_3CN) δ 151.3, 150.3. IR (neat) cm^{-1} : 2962.7, 2927.9, 1739.8, 1697.4, 1508.3, 1442.8, 1247.9, 1174.7, 1151.5. HRMS (ESI): calculated for $[C_{48}H_{65}N_4O_{10}PSSi + H]^+$: 949.40010 found : 949.40143 $[M + H]^+$.



5'-*O*-diphenyl-*tert*-butylsilyl-5-methylcarboxymethyl-2-thio-Uridine (16)

2',3',5'-tri-*O*-Benzoyl protected nucleotide **12** (2.21 g, 3.43 mmol) was taken up in anhydrous MeOH (100 mL) and NaOMe (113 mg, 2.1 mmol) was added. The reaction mixture was stirred for 48 hours. Amberlite resin (IR-120 H^+) was added until the solution was slightly acidic (pH 5). The solution was filtered, pyridine (2 mL) was added and the reaction mixture was concentrated under reduced pressure followed by co-evaporation with pyridine (3 x 5 mL). The reaction crude was dissolved in pyridine (15 mL) and TBDPS-Cl (1.07 mL, 4.12 mmol, 1.2 eq) was added. The reaction mixture was stirred for 48 hours, quenched with MeOH (0.5 mL), concentrated, diluted with EtOAc (60 mL), washed with sat. aq. $NaHCO_3$ (5 x 20 mL), brine (20 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Silica gel column chromatography (EtOAc : petroleum ether, 2 : 8 → 6 : 4) yielded silylated nucleoside **16** as a white foam (1350 mg, 2.36 mmol, 70% over two steps).

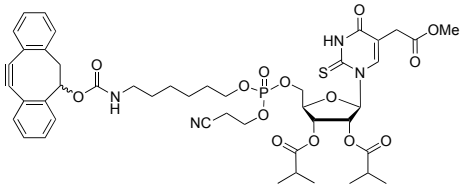
^{1}H NMR (400 MHz, $CDCl_3$) δ 10.04 (s, 1H), 7.83 (s, 1H), 7.67 – 7.61 (m, 4H), 7.47 – 7.36 (m, 6H), 6.51 (d, J = 3.2 Hz, 1H), 4.44 – 4.30 (m, 2H), 4.23 – 4.14 (m, 2H), 3.96 (dd, J = 11.8, 2.2 Hz, 1H), 3.59 (s, 3H), 2.84 (d, J = 17.3 Hz, 1H), 2.73 (d, J = 17.3 Hz, 1H), 1.08 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.0, 169.9, 159.4, 138.1, 135.4, 135.1, 133.1, 132.2, 130.2, 130.1, 128.1, 128.1, 113.6, 94.1, 85.3, 76.1, 69.5, 62.6, 52.2, 31.6, 27.0, 19.4. IR (neat) cm^{-1} : 3433.4, 2390.0, 1738.9, 1675.3, 1484.3, 1427.4, 1264.4, 1105.3, 1048.4. HRMS (ESI): calculated for $[C_{28}H_{34}N_2O_7SSi + H]^+$: 571.19288 found : 571.19285 $[M + H]^+$.



2',3'-di-*O-iso*-butyryl-5-methylcarboxymethyl-2-thio-Uridine (17)

To a stirred and cooled (0 °C) solution of **16** (3750 mg, 6.56 mmol) in pyridine (30 mL) was added *iso*-butyric acid anhydride (2.62 mL, 15.7 mmol, 2.4 eq). The reaction mixture was stirred overnight under gradual warming to r.t. The reaction mixture was concentrated under reduced pressure, diluted with EtOAc (50 mL), washed with H_2O (4 x 10 mL), brine (10 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The crude nucleoside was taken up in THF (16 mL) and treated with TBAF (1 M in THF, 8.72 mL, 8.72 mmol, 1.33 eq). The reaction mixture was diluted with DCM (50 mL), washed with H_2O (3 x 10 mL), brine (10 mL), dried ($NaSO_4$) and concentrated under reduced pressure. Silica gel column chromatography (EtOAc : DCM, 5 : 95) yielded the title acylated nucleoside as a white solid (1.35 g, 2.9 mmol, 45% over two steps).

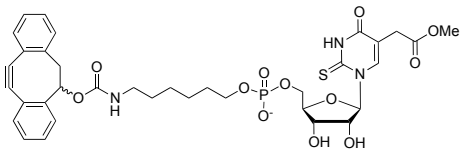
^{1}H NMR (400 MHz, DMSO-d6) δ 12.84 (s, 1H), 8.15 (s, 1H), 6.98 (d, *J* = 5.5 Hz, 1H), 5.53 (t, *J* = 4.9 Hz, 1H), 5.38 (t, *J* = 5.5 Hz, 1H), 5.35 – 5.29 (m, 1H), 4.23 (d, *J* = 3.4 Hz, 1H), 3.76 (ddd, *J* = 12.3, 5.0, 2.8 Hz, 1H), 3.71 – 3.63 (m, 1H), 3.62 (s, 3H), 3.34 (s, 2H), 2.66 – 2.56 (m, 1H), 2.56 – 2.51 (m, 1H), 1.15 – 1.10 (m, 6H), 1.08 (d, *J* = 2.6 Hz, 3H), 1.07 (d, *J* = 2.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d6) δ 175.9, 175.0, 174.7, 170.2, 159.6, 138.3, 113.7, 89.4, 83.3, 73.4, 70.2, 60.1, 52.0, 33.2, 33.2, 32.1, 18.8, 18.6, 18.6, 18.5. IR (neat) cm^{-1} : 3446.0, 2960.0, 1738.9, 1729.3, 1717.0, 1450.5, 1438.0, 1351.2, 1147.7. HRMS (ESI): calculated for $[C_{20}H_{28}N_2O_9S + H]^+$: 473.15883 found : 473.15866 $[M + H]^+$.



2',3'-di-*O-iso*-butyryl-5'-dibenzocyclooctyne functionalized mcm^5S^2U (19)

To a stirred solution of nucleoside **17** (157 mg, 0.33 mmol) in anhydrous 1,4-dioxane (3 mL) was added a solution of phosphoramidite **18** (225 mg, 0.4 mmol, 1.2 eq) in anhydrous MeCN (1 mL) and 4,5-dicyano imidazole (236 mg, 2 mmol, 5 eq). After 3 hours TLC analysis (Toluene : EtOAc, 1 : 1) showed near complete conversion of the starting material to a less polar compound. *tert*-Butyl hydroperoxide (5.5 M in nonane, 180 µL, 1 mmol, 3 eq) was added. The reaction mixture was stirred for an additional 3 hours prior to concentration. Silica gel column chromatography (EtOAc : DCM, 3 : 7 → 6 : 4) yielded the target compound as a thick oil (162 mg, 0.17 mmol, 52%).

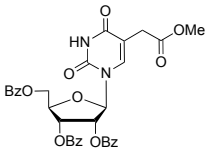
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.81 (s, 1H), 7.94 – 7.74 (m, 1H), 7.49 (d, J = 7.5 Hz, 1H), 7.45 – 7.21 (m, 9H), 7.04 – 6.85 (m, 1H), 5.47 (s, 1H), 5.37 (s, 2H), 5.21 – 5.02 (m, 1H), 4.49 – 4.20 (m, 5H), 4.20 – 4.04 (m, 3H), 3.77 – 3.64 (m, 3H), 3.56 – 3.36 (m, 2H), 3.25 – 3.11 (m, 3H), 2.94 – 2.68 (m, 3H), 2.69 – 2.51 (m, 2H), 1.76 – 1.66 (m, 2H), 1.61 – 1.51 (m, 2H), 1.47 – 1.34 (m, 4H), 1.23 – 1.12 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.6, 175.5, 175.4, 175.1, 170.5, 159.0, 158.9, 155.4, 152.2, 151.0, 138.0, 137.9, 129.9, 128.9, 128.0, 127.9, 127.0, 126.2, 125.9, 124.9, 123.8, 123.7, 121.3, 116.5, 114.1, 112.9, 109.9, 90.5, 90.4, 80.9, 80.8, 80.8, 73.5, 73.4, 69.0, 68.9, 68.9, 68.8, 65.9, 62.4, 62.3, 59.9, 52.3, 52.3, 46.2, 40.8, 33.8, 33.7, 32.0, 31.9, 30.1, 30.0, 29.8, 26.0, 24.9, 19.7, 19.7, 18.8, 18.8, 18.7, 18.7. ^{31}P NMR (162 MHz, $CDCl_3$) δ -1.19, -1.60. IR (neat) cm^{-1} : 3359.2, 2935.8, 1738.9, 1696.5, 1471.8, 1448.6, 1250.9, 1148.7, 1021.4. HRMS (ESI): calculated for $[C_{46}H_{55}N_4O_{14}PS + H]^+$: 951.32459 found : 951.32696 $[M + H]^+$, calculated for $[C_{46}H_{55}N_4O_{14}PS + NH_4]^+$: 968.35114 found : 968.35217 $[M + NH_4]^+$.



5'-dibenzocyclooctyne functionalized mcm^{5}S^{2}U (21)

Protected **19** (80 mg, 84 µmol) was taken up in anhydrous MeOH (2 mL) and treated with DBU (100 µL, 0.66 mmol, 7.85 eq) and the reaction mixture was stirred for 4.5 hours. LC/MS analysis revealed complete conversion of the starting material to the desired product. The reaction was quenched by the addition of AcOH (200 µL) followed by concentration. The reaction crude was taken up in magic (2 mL) and purified by RP-HPLC (MeCN : 50 mM NH_4OAc in H_2O, 2 : 8 → 8 : 2). The pooled fractions were co-evaporated with MeOH (3x) and lyophilized from H_2O yielding the target compound as an oil (21 mg, 27 µmol, 33%).

^{1}H NMR (400 MHz, CD_3OD) δ 8.24 (s, 1H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.44 – 7.26 (m, 7H), 6.63 (s, 1H), 5.41 (m, 1H), 4.31 – 4.19 (m, 3H), 4.19 – 4.06 (m, 2H), 3.88 (dd, *J* = 8.0 Hz, *J* = 12.0 Hz, 2H), 3.66 (s, 3H), 3.63 – 3.48 (m, 2H), 3.26 – 3.15 (m, 2H), 3.11 (t, J = 6.8 Hz, 2H), 2.89 – 2.76 (m, 1H), 1.70 – 1.60 (m, 2H), 1.57 – 1.47 (m, 2H), 1.46 – 1.35 (m, 4H). ^{13}C NMR (101 MHz, CD_3OD) δ 177.1, 173.0, 162.3, 158.0, 153.8, 152.5, 140.8, 131.0, 129.3, 129.2, 128.3, 128.2, 127.1, 126.9, 126.1, 125.0, 122.4, 114.4, 113.8, 111.0, 95.0, 84.4, 84.3, 77.8, 76.4, 69.9, 67.0, 66.9, 64.6, 52.6, 47.21, 41.78, 33.41, 31.74, 31.7, 30.9, 30.8, 27.5, 26.6. ^{31}P NMR (162 MHz, CD_3OD) δ 1.13. IR (neat) cm^{-1} : 3420.9, 2988.8, 1674.3, 1199.8, 1173.7, 1124.6. HRMS (ESI): calculated for $[C_{35}H_{40}N_3O_{12}PS + H]^+$: 758.21431 found : 758.21644 $[M + H]^+$.

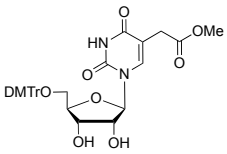


2',3',5'-tri-*O*-Benzoyl-5-methylcarboxymethyl-Uridine (13)

A suspension of 5-methylcarboxymethyl-Uracil (2.95 g, 16 mmol), HMDS (2.72 mL, 12.8 mmol), TMS-Cl (1.63 mL, 12.8 mmol) and 1-*O*-Acetyl-2,3,5-tri-*O*-Benzoyl-α-D-ribose (8.07 g, 16 mmol) in MeCN (160 mL) was refluxed until a clear solution was obtained. The solution was cooled to 0 ºC and added dropwise to a pre-cooled (0 ºC) solution of $SnCl_4$ (2.26 mL, 19.2 mmol, 1.2 eq) in MeCN (80 mL). The mixture was stirred for 17 hours under gradual warming to r.t. The mixture was filtered over celite and concentrated. The resulting syrup was taken up in DCM (200 mL), filtered over celite and washed with sat. aq. $NaHCO_3$ (100 mL) and H_2O (4 x 100 mL). The sodium bicarbonate fraction was diluted with

H_2O (100 mL) and extracted with DCM (100 mL). The organic phases were combined, dried (Na_2SO_4) and concentrated under reduced pressure. Silica gel column chromatography (EtOAc : Toluene, 1 : 9 → 2 : 8) yielded the title nucleoside (**13**) as a white foam (9.5 g, 15.1 mmol, 94%).

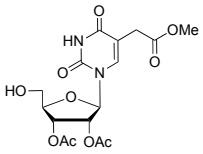
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.16 (s, 1H), 8.18 – 8.10 (m, 2H), 8.03 – 7.95 (m, 4H), 7.65 – 7.54 (m, 3H), 7.53 – 7.36 (m, 7H), 6.45 (d, J = 6.1 Hz, 1H), 5.95 (dd, J = 5.9, 3.9 Hz, 1H), 5.80 (t, J = 6.0 Hz, 1H), 4.89 (dd, J = 12.1, 2.5 Hz, 1H), 4.78 – 4.63 (m, 2H), 3.65 (s, 3H), 3.05 (d, J = 17.4 Hz, 1H, ab), 2.96 (d, J = 17.4 Hz, 1H, ab). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 166.0, 165.4, 165.3, 162.5, 150.1, 137.7, 133.8, 133.8, 133.7, 130.0, 129.9, 129.7, 129.3, 128.9, 128.7, 128.6, 128.6, 128.4, 109.4, 87.6, 80.8, 73.8, 71.5, 64.1, 52.2, 31.3. IR (neat) cm^{-1} : 3059.1, 1716.7, 1681.9, 1450.5, 1559.5. HRMS (ESI): calculated for $[C_{33}H_{28}N_2O_{11} + H]^+$: 629.17659 found : 629.17682 $[M + H]^+$.



5'-*O*-(4,4'-dimethoxy)-trityl-5-methylcarboxymethyl-uridine (14)

Acyl protected nucleoside **13** (0.81 g, 1.3 mmol) was dissolved in MeOH (55 mL) and NaOMe (118 mg, 2.18 mmol, 1.7 eq) was added. The reaction mixture was stirred and after 3 hours, TLC analysis showed complete conversion to the deprotected nucleotide. The reaction mixture was quenched by the addition of Amberlite resin (IR-120 H^+) until the solution was neutral. Filtration followed by concentration under reduced pressure yielded the crude nucleoside which was dissolved in pyridine (15 mL). DMT-Cl (525 mg, 1.55 mmol, 1.2 eq) was added and the reaction mixture was stirred for 3 days. The reaction was quenched by the addition of MeOH followed by concentration. The concentrate was diluted with DCM (50 mL), washed with 10% aq $KHSO_4$ (3 x 10 mL), sat aq $NaHCO_3$ (25 mL), H_2O (25 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Silica gel column chromatography (DCM : TEA : MeOH, 98 : 1 : 1 → 96 : 1: 3) yielded the tritylated nucleoside as a white foam (635 mg, 1 mmol, 80% over two steps).

^{1}H NMR (400 MHz, CD_3CN) δ 7.59 (s, 1H), 7.48 – 7.37 (m, 2H), 7.37 – 7.19 (m, 7H), 6.86 (m, 4H), 5.84 (d, J = 4.7 Hz, 1H), 4.34 (t, J = 5.0 Hz, 1H), 4.29 (t, J = 5.0 Hz, 1H), 4.02 (m, 1H), 3.76 (s, 6H), 3.56 (s, 3H), 3.36 (dd, J = 10.8, 3.3 Hz, 1H), 3.26 (dd, J = 10.8, 2.4 Hz, 1H), 2.58 (s, 2H). ^{13}C NMR (101 MHz, CD_3CN) δ 171.6, 163.8, 159.7, 151.5, 145.6, 139.1, 136.7, 136.4, 131.0, 130.9, 129.0, 129.0, 128.0, 114.1, 109.3, 89.7, 87.6, 84.3, 75.1, 71.3, 64.1, 55.9, 52.5, 32.3, 1.3. IR (neat) cm^{-1} : 3201.0, 2968.5, 2935.7, 1681.9, 1506.4, 1460.1, 1444.7, 1247.9, 1174.7. HRMS (ESI): calculated for $[C_{33}H_{34}N_2O_{10} + Na]^+$: 641.21057 found : 641.21077 $[M + Na]^+$.

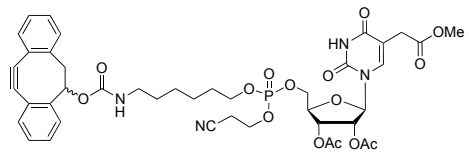


2',3'-di-*O*-Acetyl-5-methylcarboxymethyl uridine (15)

To a stirred solution of nucleoside **14** (0.93 g, 1.5 mmol) in DCE/pyridine (1 : 1, 9 mL) was added Ac_2O (0.56 mL, 6 mmol, 4 eq). The reaction mixture was stirred overnight and concentrated under reduced pressure. The crude acetylated compound was taken up in DCM (30 mL) and washed with H_2O (4 x 10 mL), brine (10 mL), dried (Na_2SO_4) and concentrated under reduced pressure and taken up in 5% (w/v) *p*TosOH in $CHCl_3$/MeOH (9 : 1, 25 mL) and stirred for 3 hrs. The reaction mixture was quenched by the addition of sat aq $NaHCO_3$ solution (10 mL). The aqueous layer was extracted with DCM (3 x 20

mL) and the combined organic phases were dried ($MgSO_4$). Silica gel column chromatography (DCM : MeOH, 1 : 0 → 98 : 2) yielded the title compound **15** (0.4 g, 1 mmol, 67% over two steps).

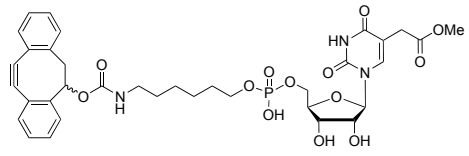
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.98 (s, 1H), 7.85 (s, 1H), 6.21 – 6.01 (m, 1H), 5.49 (d, *J* = 4.0 Hz, 2H), 4.21 (d, *J* = 2.1 Hz, 1H), 4.06 – 3.77 (m, 2H), 3.72 (s, 3H), 3.41 (d, J_{gem} = 17.5 Hz, 1H, ab), 3.35 (d, J_{gem} = 17.6 Hz, 1H, ab), 2.97 (t, *J* = 4.7 Hz, 1H), 2.14 (s, 3H), 2.09 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.3, 170.1, 169.8, 162.7, 150.3, 138.9, 108.8, 87.7, 83.7, 73.1, 71.3, 61.9, 52.4, 31.4, 20.8, 20.6. IR (neat) cm^{-1} : 3468.0, 2943.4, 1678.1, 1462.0, 1373.3, 1242.2, 1211.3, 1041.6. HRMS (ESI): calculated for $[C_{16}H_{20}N_2O_{10} + H]^+$: 401.11907 found : 401.11814 $[M + H]^+$.



2',3'-di-*O*-Acetyl-5'-dibenzocyclooctyne functionalized mcm^{5}U (20)

To a stirred solution of nucleoside **15** (100 mg, 0.25 mmol) and DCI (148 mg, 1.25 mmol, 5 eq) in MeCN (2 mL) was added a solution of phosphoramidite **18** (140 mg, 0.3 mmol, 1.2 eq). After 5 hours, TLC analysis showed complete conversion of the starting material to a less polar product. *t*-BuOOH (5.5 M in nonane, 110 µL, 0.6 mmol, 2 eq) was added and after 2 hours the reaction mixture was diluted with EtOAc (40 mL). The reaction mixture was washed with H_2O (2 x 10 mL), sat aq $NaHCO_3/H_2O$ (1 : 1, 2 x 10 mL), brine (10 mL), dried ($MgSO_4$) and concentrated under reduced pressure. Silica gel column chromatography (DCM : EtOAc, 3 : 7 → 0 : 1) yielded the title compound as an oil (260 mg, 0.25 mmol, quant).

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.28 (s, 1H), 7.67 – 7.42 (m, 2H), 7.42 – 7.10 (m, 7H), 6.26 – 5.98 (m, 1H), 5.56 – 5.38 (m, 2H), 5.39 – 5.12 (m, 1H), 4.43 – 4.20 (m, 4H), 4.20 – 4.02 (m, 2H), 3.70 (s, 3H), 3.39 (d, *J* = 5.1 Hz, 2H), 3.28 – 3.02 (m, 2H), 3.03 – 2.67 (m, 3H), 2.12 (s, 3H), 2.09 (s, 3H), 1.81 – 1.65 (m, 2H), 1.65 – 1.45 (m, 2H), 1.46 – 1.19 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.0, 169.8, 169.7, 169.7, 162.6, 155.6, 152.1, 151.1, 150.3, 138.0, 129.9, 128.1, 127.1, 126.3, 126.0, 123.8, 123.7, 121.3, 116.7, 112.9, 109.9, 109.6, 86.9, 86.7, 80.7, 76.7, 72.7, 70.1, 69.0, 68.2, 66.6, 62.4, 52.3, 46.2, 40.8, 38.7, 31.8, 30.0, 29.7, 26.1, 24.9, 20.6, 20.5, 19.8, 19.7. IR (neat) cm^{-1} : 2924.1, 2854.7, 1710.9, 1687.7, 1236.4, 1217.1. HRMS (ESI): calculated for $[C_{42}H_{47}N_4O_{15}P + NH_4]^+$: 896.31138 found : 896.31322 $[M + NH_4]^+$.



5'-dibenzocyclooctyne functionalized mcm^{5}U (22)

Protected **20** (170 mg, 200 µmol) was taken up in anhydrous MeOH (4 mL) and treated with DBU (200 µL, 1.3 mmol, 6.5 eq) and the reaction mixture was stirred for 5 hours. LC/MS analysis revealed complete conversion of the starting material to the desired product. The reaction was quenched by the addition of AcOH (4 mL) followed by concentration. The reaction crude was taken up in magic (2 mL) and purified by RP-HPLC (MeCN : 50 mM NH_4OAc in H_2O, 2 : 8 → 8 : 2). The pooled fractions were

concentrated and lyophilized from water (7 mL) yielding the target compound as an oily substance (37 mg, 50 μmol, 25%).

^{1}H NMR (400 MHz, CD_3OD) δ 7.99 (s, 1H), 7.52 (d, *J* = 7.7 Hz, 1H), 7.42 – 7.33 (m, 2H), 7.33 – 7.21 (m, 5H), 5.95 (d, *J* = 4.0 Hz, 1H), 5.37 (s, 1H), 4.26 – 4.13 (m, 2H), 4.10 – 3.93 (m, 3H), 3.81 (q, *J* = 6.4 Hz, 2H), 3.62 (s, 3H), 3.44 (s, 2H), 3.18 (dd, *J* = 15.0, 2.1 Hz, 1H), 3.06 (t, *J* = 6.9 Hz, 2H), 2.99 (q, *J* = 7.3 Hz, 6H), 2.78 (dd, *J* = 15.0, 3.9 Hz, 1H), 1.64 – 1.54 (m, 2H), 1.52 – 1.42 (m, 2H), 1.42 – 1.27 (m, 4H), 1.20 (t, *J* = 7.3 Hz, 9H). ^{13}C NMR (101 MHz, CD_3OD) δ 173.4, 165.5, 158.0, 153.8, 152.6, 152.5, 140.5, 131.1, 129.3, 128.2, 127.1, 126.9, 125.0, 122.4, 113.8, 111.0, 110.0, 89.9, 85.1, 85.0, 77.8, 75.6, 71.5, 66.6, 66.6, 65.6, 52.5, 49.0, 47.6, 47.2, 41.8, 33.2, 31.7, 30.8, 27.6, 26.6, 9.7. IR (neat) cm^{-1} : 2925.2, 1668.5, 1662.7, 1464.0, 1450.1, 1207.0, 1015.6. HRMS (ESI): calculated for $[C_{35}H_{40}N_3O_{13}P + H]^+$: 742.23715 found : 742.23821 $[M + H]^+$. calculated for $[C_{35}H_{40}N_3O_{13}P + NH_4]^+$: 759.26370 found : 759.26369 $[M + NH_4]^+$.

5'-Dibenzocyclooctyne-rUCAGACU(mcm^{5}S^{2}U)UUAAψCUG-3' (4)

The title oligonucleic acid was synthesized using the commercially available 2'-*O*-TBDMS building blocks of G(TAC), C(Ac), U, A(Bz) and pseudouridine (Berry & Assoc.) on a polystyrene solid support (2nd generation, GE). Standard RNA synthesis was performed as described in chapter 1 up until the incorporation of the mcm^{5}S^{2}U building block. From this point on tBuOOH (10%, w/v in MeCN) was used as the oxidizing agent. Cleavage and deprotection from the solid support was performed using DBU (10%, w/v in MeOH) followed by lyophilization and treatment with TEA·HF (1 mL, 12 hours). The reaction was quenched (H_2O) and the crude RNA was precipitated (*i*-propanol). The RNA pellet was redissolved in H_2O and purified as previously described.

LC/MS: (MeCN :10 mM aq NH_4OAc, 00 → 20 v/v) 7.70 min; ESI-MS: m/z 1950.5 $[M + 5H]^{5+}$; 1463.3 $[M + 4H]^{4+}$. HRMS (ESI): calculated for $[C_{186}H_{226}N_{59}O_{125}P_{17}S + 3H]^{3+}$: 1949.62290 found : 1949.62483 $[M + 3H]^{3+}$.

3-Azidopropionyl functionalized Bovine Serum Albumin (3)

Bovine serum albumin (5 mg, 80 nmol) was dissolved in PBS (0.2 M, pH 7.3, 340 μL) and treated with a solution of 3-azidopropionic acid *O*-succinyl ester (**2**) (0.5 mg, 2.4 μmol, 30 eq) in MeCN (100 μL). The reaction mixture was shaken and the addition of activated ester **2** was repeated twice at 2 hour intervals. The protein solution was transferred to a dialysis cassette (final volume 3 mL) and dialyzed against H_2O (2 L, 3 x 6 hours) followed by lyophilization yielded the azide functionalized protein **3**. SDS-PAGE gel analysis was performed as described in the general experimental section using coumassie brilliant blue staining.

mcm^{5}U bovine serum albumin conjugate (24)

To a solution of hapten **22** (0.55 mg, 650 nmol, ≈ 3 eq) in MeOH (84 μL) was added a solution of albumin **3** (1 mg, 14 nmol) in PBS (0.2 M, pH 7.3, 5 mL). The mixture was shaken for 4 hours and transferred to a dialysis cassette (final

volume 5.5 mL) and dialyzed against H_2O (2 L, 3 x 8 hours) followed by lyophilization yielding the title conjugate **24**. SDS-PAGE gel analysis was performed as described in the general experimental section using coumassie brilliant blue staining.

mcm^{5}S^{2}U bovine serum albumin conjugate (23)

To a solution of hapten **21** (0.61 mg, 800 nmol, ≈ 3 eq) in MeOH (100 µL) was added a solution of albumin **3** (1.2 mg, 17.5 nmol) in PBS (0.2 M, pH 7.3, 6 mL). The mixture was shaken for 4 hours and transferred to a dialysis cassette (final volume 7 mL) and dialyzed against H_2O (2 L, 3 x 8 hours) followed by lyophilization yielding the title conjugate **23**. SDS-PAGE gel analysis was performed as described in the general experimental section using coumassie brilliant blue staining.

Bovine serum albumin-5'-rUCAGACUUUUAAUCUG-3' conjugate (31)

To a solution of previously described oligo ribonucleic acid **30** (0.1 µmol) in PBS (0.2 M, pH 7.3, 21 µL) was added a solution of azido functionalized BSA **3** (126 µg, 1.85 nmol) in PBS (0.2 M, pH 7.3, 63 µL). The solution was shaken for 6 hours followed by dialysis against H_2O (2 L, 3 x 8 hours) followed by lyophilization yielding the title oligo ribonucleic acid-protein conjugate. SDS-PAGE gel analysis was performed as described in the general experimental section using consecutive staining with ethidium bromide and coumassie brilliant blue.

Bovine serum albumin-5'-rUCAGACU(mcm^{5}S^{2}U)UUAAψCUG-3' conjugate (32)

To a solution of previously described oligo ribonucleic acid **4** (57 nmol) in PBS (0.1 M, pH 7.3, 24 µL) was added a solution of azido functionalized BSA **3** (70 µg, 1.05 nmol) in PBS (0.2 M, pH 7.3, 24 µL). The solution was shaken for 6 hours followed by dialysis against H_2O (2 L, 3 x 8 hours) followed by lyophilization yielding the title oligo ribonucleic acid-protein conjugate. SDS-PAGE gel analysis was performed as described in the general experimental section using consecutive staining with ethidium bromide and coumassie brilliant blue.

References and notes:

(1) Carell, T.; Brandmayr, C.; Hienzsch, A.; Müller, M.; Pearson, D.; Reiter, V.; Thoma, I.; Thumbs, P.; Wagner, M. *Angew. Chem. Int. Ed.* **2012**, *51*, 7110–7131.

(2) Bergman, Y.; Cedar, H. *Nat. Struct. & Mol. Biol.* **2013**, *20*, 274–281.

(3) Jones, P. H. *Nature Rev. Genet.* **2012**, *13*, 484–92.

(4) Note: An up to date overview of all reported ribonucleoside modifications can be found online in the RNA modification database at : http://rna-mdb.cas.albany.edu/RNAmods or at the MODOMICS website at http://modomics.genesilico.pl

(5) Agris, P. F.; Vendeix, F. A. P.; Graham, W. D. *J. Mol. Biol.* **2007**, *366*, 1–13.

(6) Agris, P. F. *Nucleic Acids Res.* **2004**, *32*, 223–38.

(7) Limbach, P. A.; Crain, P. F.; McCloskey, J. a *Nucleic Acids Res.* **1994**, *22*, 2183–96.

(8) Yang, W.; Chendrimada, T. P.; Wang, Q.; Higuchi, M.; Seeburg, P. H.; Shiekhattar, R.; Nishikura, K. *Nat. struc. mol. biol.* **2006**, *13*, 13–21.

(9) Kim, V. N.; Han, J.; Siomi, M. C. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 126–39.

(10) Phizicky, E. M.; Hopper, A. K. *Genes & development* **2010**, *24*, 1832–60.

(11) Vinayak, M.; Pathak, C. *Biosci. Rep.* **2009**, *30*, 135–148.

(12) Langgut, W.; Reisser, T.; Nishimura, S.; Kersten, H. *FEBS lett.* **1993**, *336*, 137–42.

(13) Yi, C.; Pan, T. *Acc. Chem. Res.* **2011**, *44*, 1380–8.

(14) Inoue, A.; Shen, L.; Dai, Q.; He, C.; Zhang, Y. *Cell Res.* **2011**, *21*, 1670–6.

(15) Erlanger, F. F.; Beiser, S. M. *Proc. Nat. Ac. Sci. USA* **1964**, *52*, 68–74.

(16) Fissekis, J. D.; Sweet, F. *Biochemistry* **1970**, *9*, 3136–3142.

(17) Vorbrüggen, H. *Chem. Ber.* **1973**, *106*, 3039–3061.

(18) Grandjean, C.; Boutonnier, A.; Guerreiro, C.; Fournier, J.-M.; Mulard, L. A. *J. Org. Chem.* **2005**, *70*, 7123–32.

(19) Bajji, a C.; Davis, D. R. *Org. Lett.* **2000**, *2*, 3865–8.

(20) Serebryany, V.; Beigelman, L. *Tetrahedron Lett.* **2002**, *43*, 1983–1985.