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Supplemental Material to:

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Supplementary material

Preparation of modified long-mer RNAs and analysis of FMN binding to the *ypaA* aptamer from *B. subtilis*

Jennifer Frommer, Robert Hieronymus, Tamil Selvi Arunachalam, Sabine Heeren, Maria Jenckel, Anne Strahl, Bettina Appel, Sabine Müller*

Preparation of *el-WT* by enzymatic ligation of chemically synthesized fragments

More than ten different enzymatic ligation systems were tested (Fig. S1). All donor RNA fragments were freshly phosphorylated before use. Initially, T4 RNA ligase was used for ligation of three fragments, but this strategy failed. As analysed by RNA folding algorithms, one of the three fragments (the 38 nt fragment in three fragment strategy, Fig. S1) folds in a stable secondary structure preventing it from association with the other parts of the aptamer RNA, and consequently from ligation. As an alternative, we tried to join the fragments by T4 DNA ligase in presence of a full length DNA splint, according to the procedure used by Helm and co-workers for preparation of t-RNA molecules.[1] However, also this protocol was not successful. We screened a variety of reaction conditions, focussing on the hybridisation protocol, ligation temperature and ligase concentration. In all cases, ligation yields were below 10%. Presumably, the strong secondary structure of the 38 nt fragment mentioned above (see Fig. S1) could not be resolved, in spite of using a full-length DNA splint. In order to overcome the secondary structure problem, the strategy was changed, and the aptamer was divided into four fragments of 22 to 45 nt lengths (Fig. S1). Ligation was tried with the full lengths DNA splint as before but without success. Again, we have carried out an extensive optimization study looking at ligases from different suppliers, different concentration relations of ligase, ligation fragments and DNA splint, different hybridization protocols and sample preparation, varying ligation temperatures, buffer compositions and added stabilizing agents. It should be mentioned that the full length ligation product was formed only at higher DMSO concentrations (>16%) (for more detailed information see Fig. S2), although with very low yield. In the aggregate, also with the four fragment strategy we were not successful enough, and it was surely not worthwhile to synthesize fragments consuming the precious 2-AP phosphoramidite and to use them for a low yield ligation procedure.

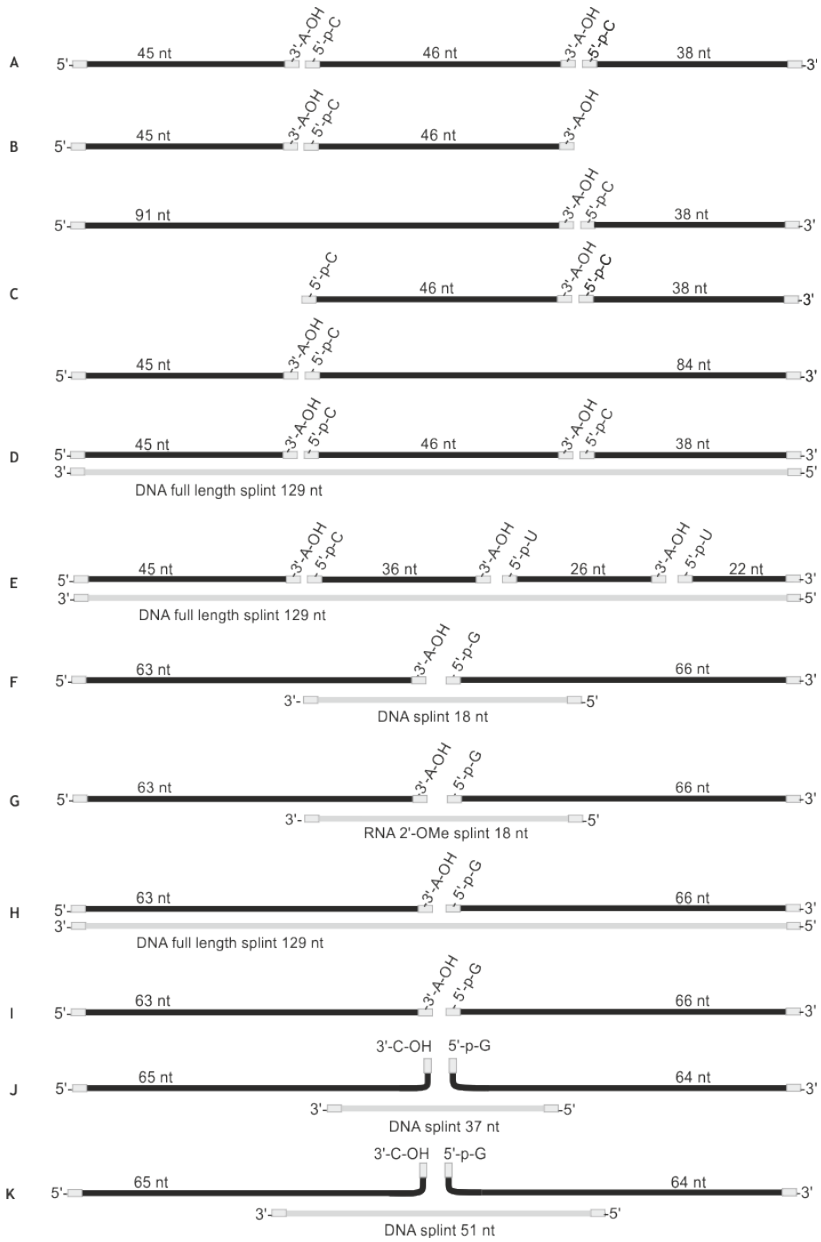

Figure S1: Schematic presentation of strategies for splint ligation of aptamer fragments with T4 DNA ligase and T4 RNA ligase. System A to C were ligated with T4 RNA ligase from Fermentas, D to H with T4 DNA ligase, I with T4 RNA ligase from Fermentas and T4 RNA ligase I from NEB, the systems J and K were ligated with the T4 RNA ligase I from NEB. The yield of the full length aptamer domain product *el-WT* (129 nt) in all systems from A to I were below 15%. With the strategies J and K yields between 7 and 42% could be achieved (see Table S1 and additional information to Figure 3 below for details on strategy J and K).

We finally decided to try to generate the aptamer domain by ligation of only two oligonucleotides. A two fragment strategy requires chemical synthesis of fragments with the lengths of around 64 nt. The chemical preparation of long oligoribonucleotides is less efficient; however, it has the advantage that only one ligation is required. The ligation site was positioned between A63 and G64 in the single stranded loop region to allow ligation of a ssRNA site with T4 RNA ligase or alternatively, ligation with T4 DNA ligase in the presence of a splint. The 63 nt acceptor strand and the 66 nt donor strand were chemically synthesized and joined by either of the two ligases; in case of T4 DNA ligase in the presence of a full length DNA splint, a 18-mer DNA or a 2'-*O*-methyl-RNA splint. Ligation with either of the two 18-mer splints was not successful, presumably due to secondary structure problems as discussed above. However, in the presence of the full length DNA splint, the ligation product was well detectable in analytical experiments. Single strand ligation with T4 RNA ligase also proceeded with product formation, although less efficient.

In each row of experiments different ratios between acceptor : splint : donor and concentrations were tested. Reaction temperature and ligase concentration were varied as well the concentration of DMSO as a ligation mediator. The influence of DMSO concentration on T4 DNA ligase mediated ligation was exemplary analysed in the four fragment system (E in Fig. S1). The higher the concentration of DMSO, the higher the concentration of the aptamer domain (129 nt) as shown in Figure S2.

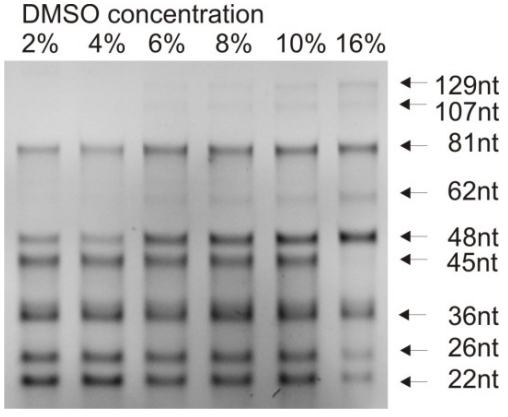

Figure S2: Denaturing PAA gel for the analysis of the influence of DMSO concentration on T4 DNA ligase mediated splint ligation of four fragments. Bands were visualized by ethidium bromide staining.

The lower four bands in all lanes represent the individual ligation fragments. With increasing DMSO concentration, the band corresponding to the 48 nt single site ligation product of fragments with the length of 26 and 22 nt becomes more pronounced, as do the bands corresponding to the 62 nt single site ligation product of the fragments with the length 36 and 26 nt, and to the single site ligation product of fragments with the length 45 and 36 nt. Only at higher DMSO concentrations, also bands corresponding to the ligation products of three fragments (107 nt) and to the full length product obtained from ligation of four fragments (129 nt) are visible. This result demonstrates that DMSO positively influences ligation yields, and that ligation as such is working.

Additional information to Figure 3 from main text

Ligation reactions of two fragments corresponding to systems J and K in Figure S1 and shown in Figure 3 of the main text were conducted in a total volume of 20 µl with short (37 nt) or long (51 nt) DNA splint. Reaction conditions and the final yield of the full-length *ypaA* aptamer *el-WT* are given in table S1. The best result (42% yield) was achieved with the long splint and an acceptor : splint : donor ratio of 1 : 1.5 : 2.

In lane 5 and 7 of the gel shown in Figure 3 (main text) an additional band is seen corresponding to the lengths of 194 nt. We hypothesized that this is the double-ligation product where the donor fragment (64 nt length) is ligated to it-self and in addition to the 65nt acceptor (Fig. S3 A). Analysis of the ligation product by polyacrylamide gel electrophoresis (Fig. S3 B) supports this suggestion.

Table S1. Various conditions for ligation systems J and K

	system	acceptor concentration	acceptor : splint : donor	yield %
lane 2	J	5 µM	1:1.5:2	15.10
lane 3	K	5 µM	1:1.5:2	31.80
lane 4	J	10 µM	1:0.5:1	6.72
lane 5	K	10 µM	1:0.5:1	12.27
lane 6	J	10 µM	1:1.5:2	7.35
lane 7	K	10 µM	1:1.5:2	41.64

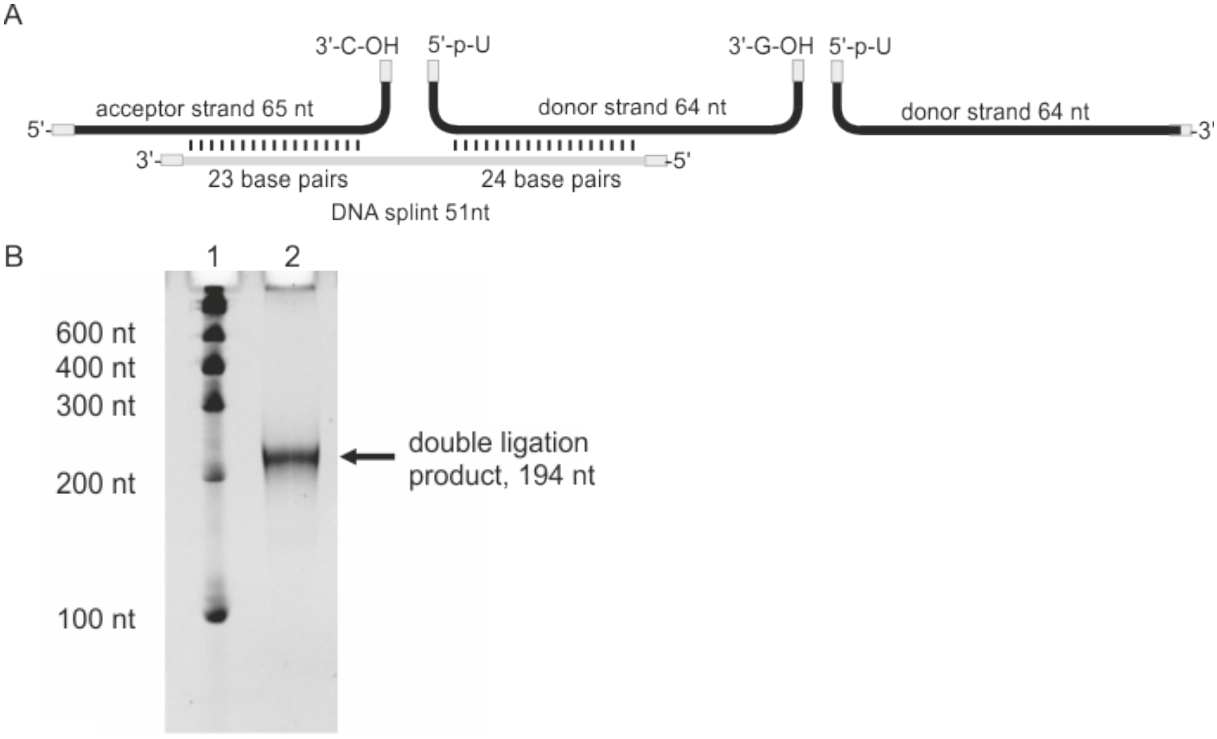

Figure S3: Double ligation. (A) Ligation reaction that may lead to a 194mer; (B) Denaturing polyacrylamide gel, bands were visualized by ethidiumbromid staining: lane 1: RNA low range ladder; lane 2: isolated upper band from lane 5 of Figure 3 (main text).

In lanes 4 to 7 (Figure 3, main text) a band corresponding to about 64 nt is visible. We assumed that this band is a circular version of the fragment with the length of 64 nt, formed by circular self-ligation. The migration behaviour of circular RNA differs from that of linear RNA. Therefore it is possible to observe circular RNA with the help of 2D-polyacrylamide gel electrophoresis. Linear RNAs form a diagonal in the second dimension, whereas circular RNA deviates from this diagonal.[2,3] Without splint, the formation of a circular product is favorable over intermolecular ligation to the 129mer. We performed another ligation reaction with and without splint (Fig. S4 A). The entire lane 2 was cut out and used for electrophoresis in the second dimension (Fig. S4 B).

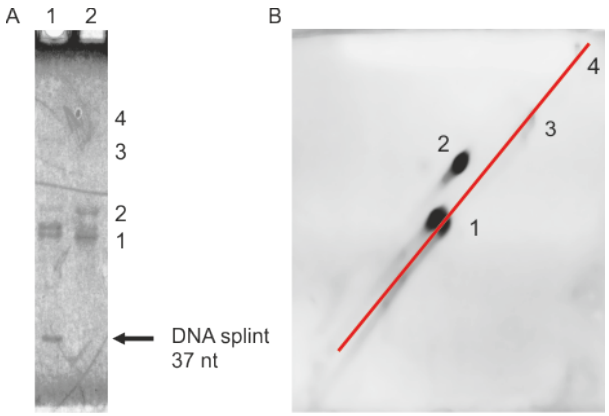

Figure S4: Analysis of the circular RNA c64 by 2D polyacrylamide gel electrophoresis: (A) denaturing polyacrylamide gel (10%) of ligation reaction with (lane 1) or without (lane 2) splint (37 nt) according to system K, (lane 7, Fig. 3, main text). Bands were visualized by UV shadowing; (B) denaturing polyacrylamide gel (15%) showing second dimension electrophoresis of cut out lane 2 from the gel shown in (A). Bands were visualized by ethidiumbromide staining; 1: 64 and 65mer, 2: circular 64mer, 3: full length ligation product (129 nt), 4: double ligation product (194 nt).

Preparation of 3'-Azido-adenosine loaded solid support

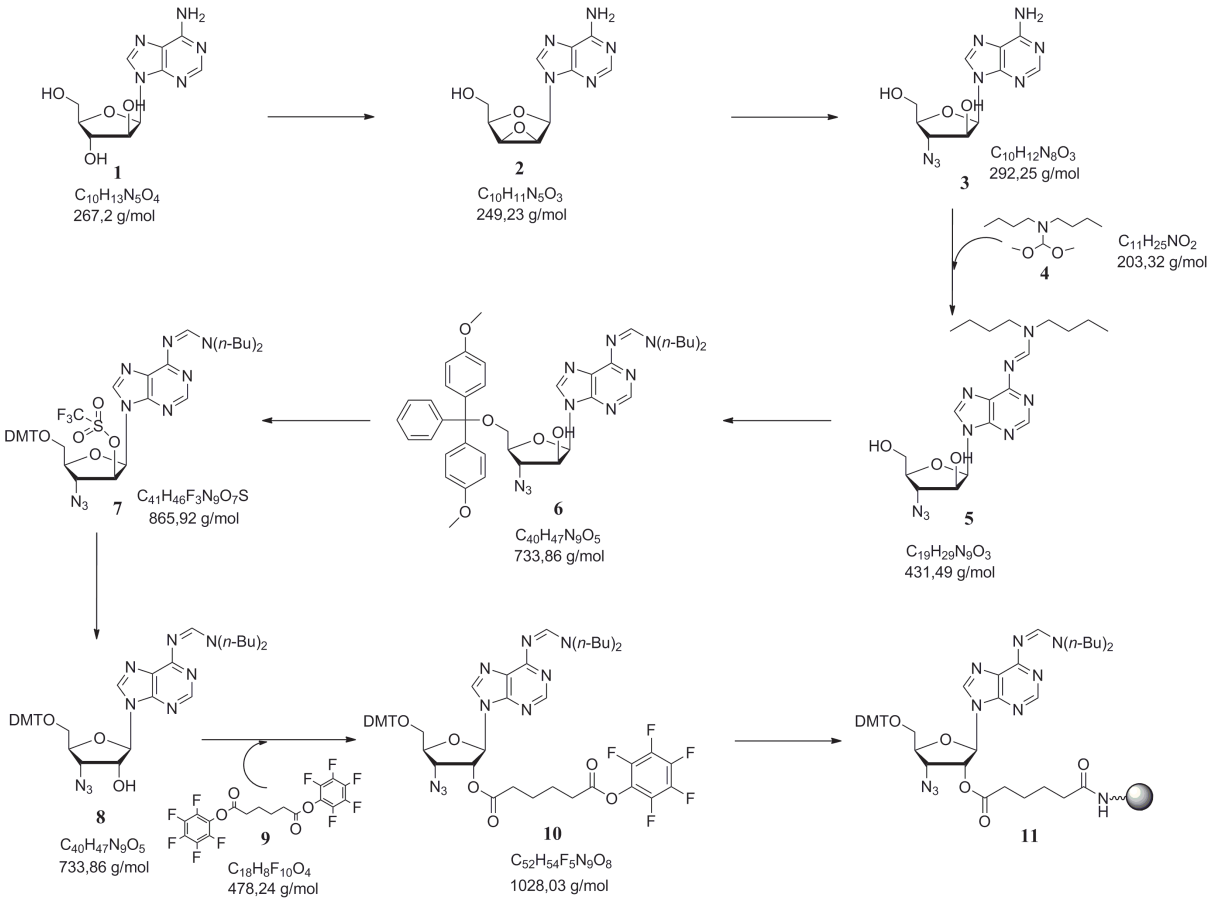

Figure S5: Synthesis scheme for the preparation of 3'-azido-modified adenosine linked to a solid support for RNA solid phase synthesis.

*2,3-Anhydro-ß-lyxofuranosyladenine (***2***)*

Arabinofuranosyladenosine **1 (**1.44 g, 5.4 mmol) and triphenylphosphane (2.17 g, 8.3 mmol) were suspended in 40 ml dry dimethylformamide and 40 ml 1,4-dioxane under argon atmosphere. After heating at 70 °C for 20 min diethyl azodicarboxylate (1.3 ml, 8.3 mmol) was added to the suspension. After stirring for 50 min at 70 °C the solvents were distilled under *vacuo*. The epoxid **2** was isolated via column chromatography (ethyl acetate/ methanol, 95:5) with a yield of 95%. Preparation according to Ref. 4.

Lithium azide

Dry methanol (55 ml) was added dropwise to a mixture of sodium azide (37.2 g, 572 mmol) and lithium chloride (22 g, 517 mmol). The reaction mixture was stirred for 30 hours at 60 °C. The side product sodium chloride crystallized and was filtered off under reduced pressure. The product was isolated by removing the solvent under reduced pressure with a yield of 57%. No further purification was carried out.

3'-Azido -arabinofuranosyl-adenine (**3**)

The epoxide **2** (1.1 g, 4.38 mmol) and lithium azide (1.03 g, 22 mmol), were suspended in 54 ml dry dimethylformamide and heated at 80 °C for 2 hours under argon atmosphere. Subsequently the solvent was removed under reduced pressure, the residue was resolved in 134 ml boiling water and the solution was slowly cooled down to room temperature. The isolated slightly yellow needles were dried over phosphorus pentoxide *in vacuo*. A yield of 79% was achieved. Preparation according to Ref. 4.

N,N-di-n-butylformamide-dimethyl-acetal (**4**)

Di-*n*-butylamine (16.8 ml, 100 mmol) and *N,N*-dimethylformamide-dimethyl-acetal (14.6 ml, 110 mmol) were heated at 100 °C under reflux and dry conditions for 3 days. The product was isolated by fractionated distillation using a vigreux column at 110 °C and 0.75 mbar. The product was isolated at 70 °C in a yield of 34%. Preparation according to Ref. 5.

3'-Azido-6-N-(di-butylamino)methylene-arabinofuranosyl-adenine (**5**)

The azide **3** (0.59 g, 2.0 mmol) and *N,N-di-n-butylformamide-dimethyl-acetal* **4** (1.3 ml, 6.4 mmol) were solved in 7 ml dry dimethylformamide and stirred for 2 hours at 80 °C at dry conditions. After removing the solvent under *vacuo* the residue was resolved in dichloromethane. After aqueous workup dichloromethane was removed under reduce

pressure. Purification with column chromatography (dichloromethane/methanol, 98:2) gives the product in 71%. Preparation was carried out according to the literature with slightly changes during the work-up.[6]

3'-Azido-6-N-(di-n-butylamino)methylene-5'-O-(4,4'-dimethoxytrityl)-arabinofuranosyl-adenine (**6**)

Compound **5** (0.4 g, 0.9 mmol) was solved in 3 ml dry pyridine. The reaction mixture was stirred at 0 °C for 10 min. Subsequently dimethoxytritylchloride (0.37 g, 1.08 mmol) was added at 0 °C, the suspension stirred for 10 min. After removing the ice bath, the reaction mixture was stirred over night at room temperature. Pyridine was removed under reduced pressure and the residue was coevaporated three times with toluene. The residue was resolved in dichloromethane and the resulting solution was washed with saturated sodium bicarbonate solution. Subsequently the combined phases were washed with brine and dried with sodium sulfate. The solvent was removed *in vacuo*. The isolation of the product could be achieved via column chromatography (dichloromethane/methanol, 99:1) with a yield of 51%.

3'-Azido-6-N-(di-butylamino)methylene-5'-O-(4,4'-dimethoxytrityl)-2'-O-(trifluoromethanesulfonyl)-arabinofuranosyl-adenine (**7**)

The DMT protected azide **6** (0.8 g, 1.1 mmol) and 4-dimethylaminopyridine (0.2 g, 1.6 mmol) were solved in 20 ml dry dichloromethane and stirred at 30 °C under argon atmosphere. After freshly distilled *N,N*-diisopropylethylamine (480 µl) was slowly added, the mixture was cooled to 0 °C. Trifluoromethanesulfonic chloride (250 µl) was added dropwise and the reaction mixture was slowly warmed up to 30 °C and stirred for 20 min. The reaction was stopped by adding 20 ml dichloromethane. Subsequently the solution was neutralized with saturated sodium hydrogen carbonate solution. After aqueous workup with brine the solvent

was remove in *vacuo*. The crude product was used for the next step without purification. Preparation according to Ref. 6.

3'-Azido-6-N-(di-n-butylamino)methylene-5'-O-(4,4'-dimethoxytrityl)-ß-D-adenosine (**8**)

The crude methanesulfonate **7** (1.67 g, 1.93 mmol) was solved in 30 ml dry toluene at dry conditions. 18-crown-6-ether (0.87 g, 3.28 mmol) and potassium trifluoroacetate (1.34 g, 8.78 mmol) were added. Freshly distilled *N,N*-diisopropylethylamine (420 µl, 2.36 mmol) was added dropwise. The reaction mixture was stirred at 80 °C for 2.5 hours, wherein the flask was wrapped into alumina foil to ensure a complete light exclusion. The toluene was removed under reduced pressure and the residue was resolved in dichloromethane. The solution was washed with saturated sodium bicarbonate solution and brine. After drying with sodium sulfate the solvent was removed *in vacuo*. The crude product was purified by column chromatography, wherein the column was wrapped into alumina foil, with dichloromethane/methanol/triethylamine (triethylamine was used to avoid azide decomposition) in a ratio of 98:1:1 and ethyl acetate/hexane in a ratio of 1:1 → 2:1. A yield of 35% over two steps could be achieved. Preparation was carried out according to the literature with precautions regarding azide decomposition.[6]

Adipinic-acid-dipentafluorophenylester (**9**)

Adipic acid (1.2 g, 8.2 mmol) and pentafluorophenol (4 g, 22 mmol) were solved in 16 ml dry dimethylformamide at dry conditions. After cooling to 0 °C 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (3.14 g, 16.4 mmol) was added. After stirring for 2.5 h at room temperature the solvent was removed at reduced pressure. The product was isolated by column chromatography with pure dichloromethane. Yield: 68%. Preparation according to Ref. 7.

3'-Azido-6-N-(di-n-butylamino)methylene-5'-O-(4,4'-dimethoxytrityl)-2'-O-[1,6-dioxo-6-(pentafluorophenyloxy)hexyl]-ß-D-adenosine (**10**)

The azide **8** (280 mg, 0.38 mmol) was solved in 3.4 ml dry dimethylformamide and 3.4 ml dry pyridine at dry conditions and with light exclusion. 4-dimethylaminopyridine (50 mg, 0.38 mmol) and active ester **9** (0.86 g, 1.8 mmol) were added and the reaction mixture was stirred 1 h at room temperature. The solvents were removed under reduced pressure and the residue was coevaporated twice with toluene and once with dichloromethane. The product was purified by column chromatography (dichloromethane/acetone/triethylamine, 96.5:3:0.5). The column was wrapped into aluminum foil to avoid light induced degradation of the azide. A yield of 28% could be achieved. Preparation was carried out according to the literature with precautions regarding azide decomposition.[7]

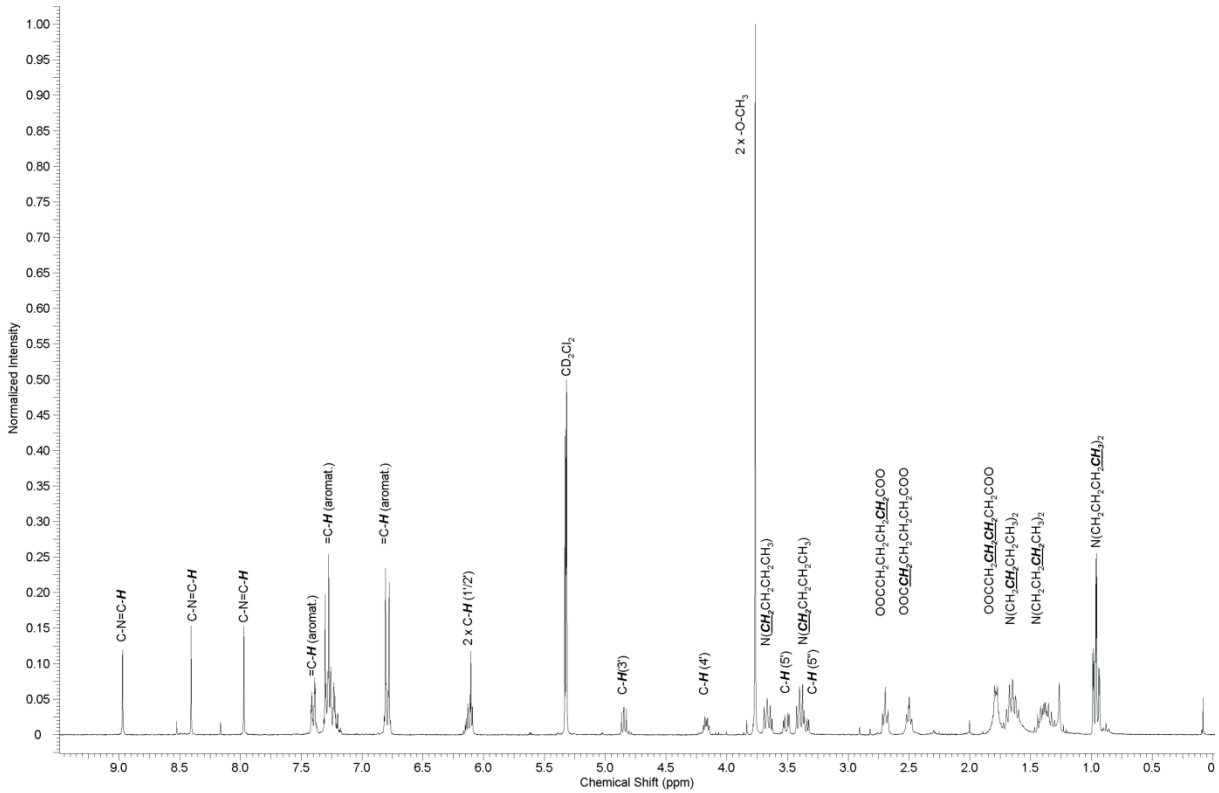

Figure S6: ^{1}H NMR (300 MHz) spectra of compound **10** in deuterated dichloromethane. The particular signals could clearly assigned.

Coupling to solid support **(11)**

As solid support Custom Primer SupportTM 200 Amino from GE Healthcare was used (polystyrene solid support). The solid support (300 mg) and the modified nucleoside **11 (**109 mg, 0.106 mmol) were solved in 1.7 ml dry dimethylformamide at dry conditions and light exclusion. Dry pyridine (17 µl, 0.212 mmol) was added. The flask was sealed with a stopper and parafilm. The reaction mixture was stirred at room temperature for 22 hours. The solvents were removed, and the remaining solid was washed with dry dimethylformamide, methanol and dichloromethane. Afterwards, capping of the remaining amino groups was carried out with typical reagents as used for RNA solid phase synthesis. 3 ml of capping A (4-dimethylaminopyridine/acetonitrile 0.5 M) and 3 ml of capping B (acetic anhydride/2,4,6-trimethylpyridine/acetonitrile in a ratio of 2:3:5) were added to the azide-loaded support. The aluminium foil wrapped flask was slightly shaken for 10 min at room temperature. After the solvents were removed, the solid was washed with dry acetonitrile, methanol, dichloromethane and acetonitrile again. The resulting solid was dried overnight. The loading density could be determined by light absorption of the dimethoxytritylcation at 498 nm. Dimethoxytritylchloride was weighed and solved with 3% dichloroacetic acid in dichloroethane. With a dilution series a specific absorption depending on the concentration of the cation in solution could be measured to obtain a calibration curve. 2 mg of the loaded support was solved in 3% dichloroacetic acid in dichloroethane. With the measured extinction the loading density could be easily calculated. The loading density of the support is 102 µmol/g. Preparation according to Ref. 7.

Ligand for Click reaction

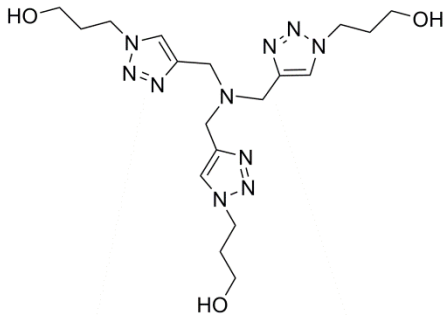

Figure S7: THPTA – Tris-(3-hydroxypropyltriazolylmethyl)amine, ligand for the Click reaction

Additional information to Figure 6 B from main text

The polyacrylamide gel shown in Figure 6B in the main part (and Fig. S8 A) shows results of Click reaction of two fragments in lanes 4 to 7.

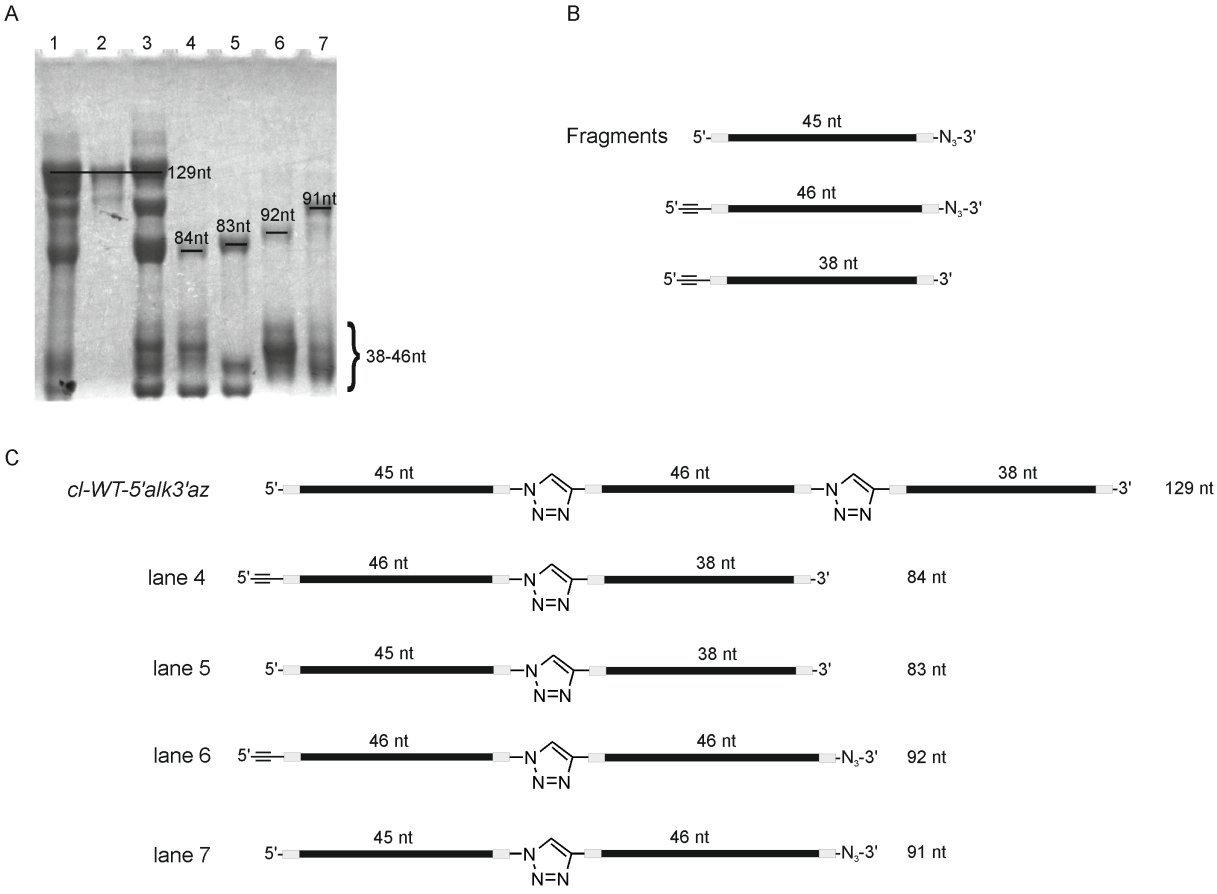

Figure S8: Click ligation with strategy II: (A) Polyacrylamide gel shown also in Figure 6B of main text; lane 1: ligation reaction to *cl-WT-5'az3'alk* with full length splint, lane 2: *wt-tr*, lane 3: ligation reaction to *cl-WT-5'az3'alk* without splint, lane 4: ligation of the two fragments with the length of 46 and 38 nt, lane 5: ligation of the two fragments with the length of 45 and 38 nt, lane 6: ligation of the two fragments with the length of 46 and 46 nt, lane 7: ligation of the two fragments with the length of 45 nt and 46 nt

Quenching curves of FMN derivatives

With the binding assay, we also wanted to address the question of the contribution of individual parts of FMN to binding. For this purpose we studied the binding of FMN analogues shown in Figure S9 to the aptamer *tr-WT*.

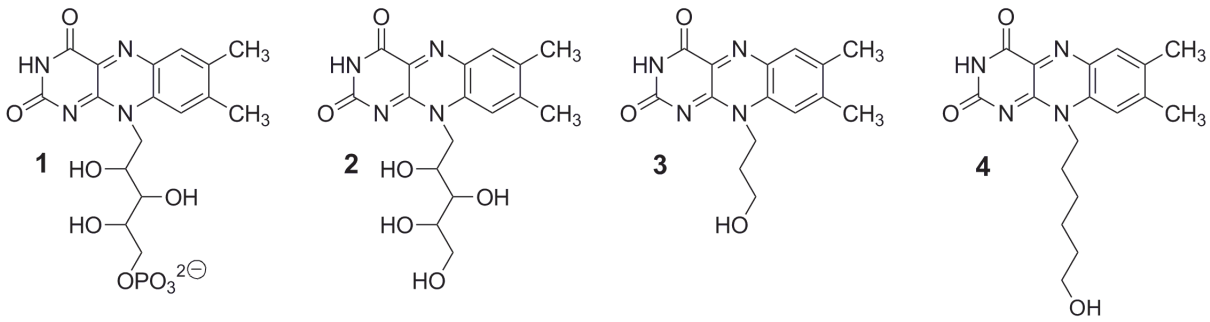

Figure S9: FMN and derivatives which are used for binding studies: FMN (**1**), riboflavin (**2**), 3-Hydroxypropylflavin (**3**) and 6-Hydroxyhexylflavin (**4**)

While fluorescence quenching was observed for the natural ligand FMN, neither of the analogues showed quenching upon addition of the RNA, indicating that the analogues do not bind to the aptamer (**Fig. S10**). This allows for the conclusion that the phosphate is strongly required for binding.

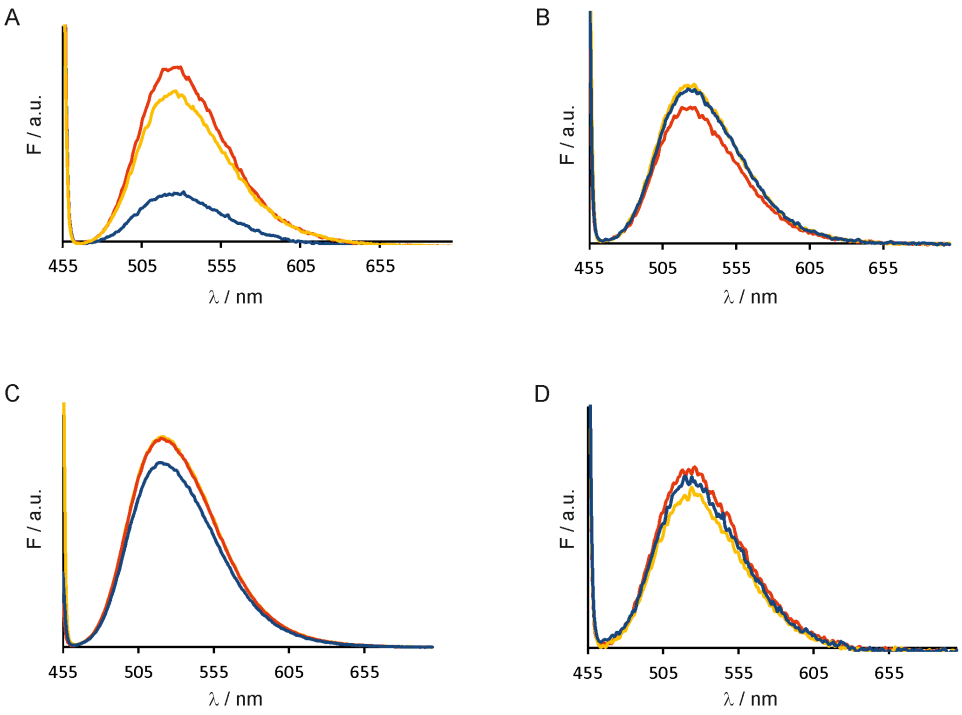

Figure S10: Fluorescence recordings of FMN (A) and analogues riboflavin (B), 3-Hydroxypropylflavin (C) and 6-Hydroxyhexylflavin (D), upon incubation with the *ypaA* aptamer. Fluorescence units are arbitrarily chosen. Blue curves are *tr-WT* with FMN, red and orange curves are free FMN.

Literature

1. Kurschat WC, Muller J, Wombacher R, Helm M. Optimizing splinted ligation of highly structured small RNAs. RNA 2005; 11:1909-14.
2. Umekage S, Kikuchi Y. In vitro and in vivo production and purification of circular RNA aptamer. J Biotechnol 2009; 139:265-72.
3. Petkovic S, Muller S. RNA self-processing: formation of cyclic species and concatemers from a small engineered RNA. FEBS Lett 2013; 587:2435-40.

4. Chen YCJ, Hansske F, Janda KD, Robins MJ. Nucleic-Acid Related-Compounds .64. Synthesis of 2',3'-Diazido-2',3'-Dideoxyadenosine and 2',3'-Diamino-2',3'-Dideoxyadenosine from 9-(Beta-D-Arabinofuranosyl)Adenine. J Org Chem 1991; 56:3410-3.

5. Froehler BC, Matteucci MD. Dialkylformamidines: depurination resistant N6-protecting group for deoxyadenosine. Nucleic Acids Res 1983; 11:8031-6.

6. Moroder H, Steger J, Graber D, Fauster K, Trappl K, Marquez V, et al. Non-hydrolyzable RNA-peptide conjugates: a powerful advance in the synthesis of mimics for 3'-peptidyl tRNA termini. Angew Chem Int Ed Engl 2009; 48:4056-60.

7. Steger J, Graber D, Moroder H, Geiermann AS, Aigner M, Micura R. Efficient access to nonhydrolyzable initiator tRNA based on the synthesis of 3'-azido-3'-deoxyadenosine RNA. Angew Chem Int Ed Engl 2010; 49:7470-2.