Electronic Supplementary Material (ESI) for Chemical Communications

Electronic Supporting Information

Inverse electron-demand Diels-Alder reaction for the selective and efficient labeling of RNA

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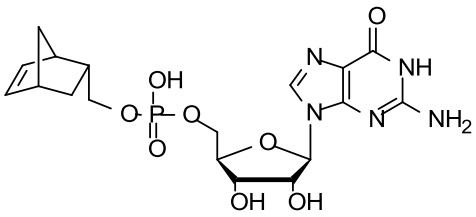
List of abbreviations. TBAF = tetrabutylammoniumfluoride, TBDMS = *tert*-butyl-dimethylsilane, ACN = acetonitrile, min = minutes, h = hours, DA = Diels-Alder, DAinv = Inverse electron-demand Diels-Alder reaction.

General materials and methods

All reagents were purchased from Sigma-Aldrich and used without further purification. Reversed-phase HPLC purification was performed on an Agilent 1100 Series HPLC system equipped with a diode-array detector using a semi-preparative Phenomenex Luna C18 column (5 µm, 15.0 × 250 mm) at a flow rate of 5 ml/min and eluted with a gradient of 100 mM triethylammonium acetate *p*H 7.0 (buffer A) and 100 mM triethylammonium acetate in 80% acetonitrile (buffer B). LC-MS experiments were performed on a Bruker microTOFQ-II ESI mass spectrometer connected to an Agilent 1200 Series HPLC system equipped with a multi-wavelength detector. A Phemonenex Kinetex C18 column (2.6 µm, 2.1 × 100 mm) was used and eluted at a flow rate of 0.2 ml/min using a gradient of 100 mM hexafluoroisopropanol/ 8.6 mM triethylamine *p*H 8.3 and methanol (LC-MS grade). Analysis of the LC-MS measurements was carried out using Hyphenation Star PP (Version 3.2.44.0) and DataAnalysis (Version 4.0, SP 4) software (Bruker Daltonics). MS-spectra were deconvoluted using Maximum Entropy deconvolution. For high-resolution mass spectra, internal calibration was performed (enhanced quadratic mode) using ESI Tunemix (Fluka) as calibrant. Calculated molecular weights refer to the m/z values given by the DataAnalysis software. NMR spectra were recorded on a Varian Mercury Plus 500 MHz spectrometer. The assignment of proton and carbon resonances is based on two-dimensional correlation experiments (COSY, GHSQC, GHMBC). Flash purification was done on a Varian IntelliFlash 310 Discovery Scale Flash Purification System. Oligonucleotide synthesis was performed on an Expedite™ 8909 automated synthesizer using standard reagents from Sigma Aldrich Proligo. Fluorescence scans of polyacrylamide gels were done using AlphaImager™ 2200. Non-radioactive denaturing polyacrylamide gels were stained with SYBR Gold (Invitrogen). Both non-radioactive and radioactive gels were scanned on a Typhoon 9400 Imager (Amersham Biosciences). Analysis and calculation of conversions were carried out with Image Quant software (Molecular Dynamics, Version 5.2).

In Diels-Alder reactions of this type, different product isomers can be formed.[1] Depending on the gradient used in HPLC analysis, separation of the different product isomers of tetrazine and norbornene-oligonucleotide is possible.[2] In the current study, however, no attempts were made to separate these isomers as this was not required for product analysis.

Synthesis of initiator nucleotide



Initiator nucleotide

5'-*O*-(*endo*-Bicyclo[2.2.1]hept-2-en-2-ylmethyl)-guanosine (initiator nucleotide).

5′-*O*-(2-cyanoethyl-N,N′-diisopropylaminophosphino)-2',3'-di-*O*-(*tert*butyldimethylsilyl) guanosine[3, 4] (684 mg, 962 mmol, 1.2 eq) was dissolved in abs. THF (11 ml) and added to e*ndo*-bicyclo[2.2.1]hept-2-en-2-ylmethanol[5] (100 mg, 806 mmol). Benzylthiotetrazole (155 mg, 806 mmol) was added and the solution stirred for 1h 45 min at RT. 1.08 ml aqueous *tert*BuOOH was added and stirring continued for 30 min. The solvent was evaporated and then TBAF (1M in THF, 4.85 ml) was added. After stirring overnight at room temperature, the solvent was evaporated and the crude product was purified by flash chromatography (C18 column, 5 min 99% H_2O/ 1% ACN, increase to 50% ACN over 16 min, R_t ((initiator nucleotide) ~15 min). Fractions containing the initiator nucleotide were lyophilized and one round of ion-exchange was performed (Na^+ cycle). After lyophilization, **initiator nucleotide** (268.4 mg, 547 µmol, 68%) was obtained as a white solid (mixture of two isomers).

^{1}H-NMR (500 MHz, D_2O, 25°C): δ = 0.22 (2ddd, *J* = 11.78, 4.52, 2.62 Hz, 1H), 1.10-1.24 (m, 1H), 1.27 (ddd, *J* = 8.23, 3.84, 1.90 Hz, 1H), 1.64 (dddd, *J* = 15.30, 11.78, 9.28, 3.77 Hz, 1H), 2.14-2.25 (m, 1H), 2.66-2.75 (m, 2H), 3.09-3.45 (m, 2H), 4.03-4.09 (m, 2H), 4.29-4.35 (m, 1H), 4.56 (ddd, *J* = 5.42, 4.01, 2.95 Hz, 1H), 4.80-4.91 (m, 1H), 5.78 (2dd, *J* = 5.76, 2.86 Hz, 1H), 5.92 (dd, J = 5.60, 2.42 Hz, 1H), 6.02 (dd, *J* = 5.76, 2.95 Hz, 1H), 8.06, 8.07 (2s, 1H), 8.11 (s, NH, 1H). ^{13}C {^{1}H} NMR (75 MHz, D_2O, 25°C, TMS): δ = 27.8, 38.7, 41.8, 43.2, 48.6, 64.9, 69.4, 70.5, 73.0, 83.7, 87.0, 116.2, 132.0, 137.4, 137.5, 151.8, 153.8, 158.8. ^{31}P-NMR (121 MHz, D_2O, 25°C, H_3PO_4): δ = -0.03, 0.08. MS (HR-ESI): m/z 468.1290 (calculated for $[C_{18}H_{24}N_5O_8P_1\text{-}H]^-$ 468.1290) , ε (254 nm) = (11814 ± 255) $lmol^{-1}cm^{-1}$.

The mass spectrum of the **initiator nucleotide** is shown in Figure S1.

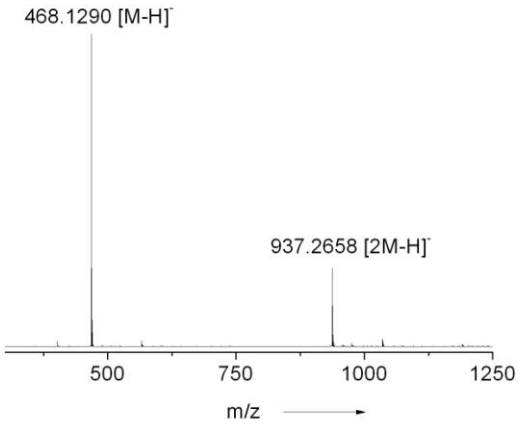


Figure S1. Mass spectrum (HR-ESI⁻) of the **initiator nucleotide**.

Tetrazines

Tetrazines used in this study are shown in Figure S2.

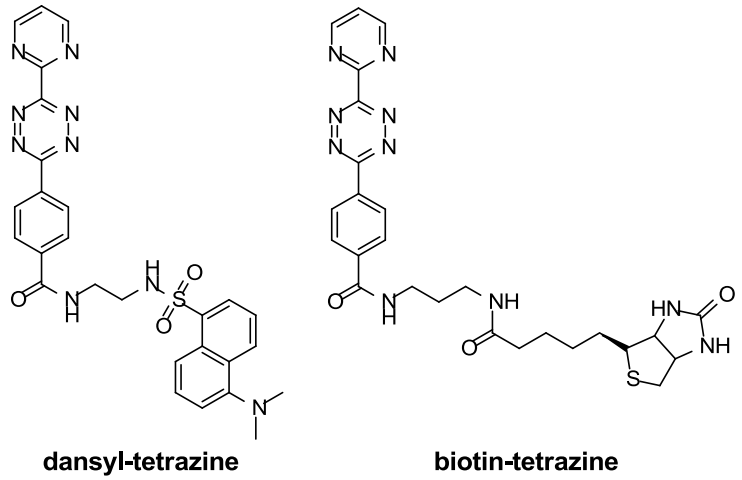


Figure S2. Tetrazines used as dienes for inverse Diels-Alder reactions in this study.

The synthesis of these dienes was carried out by activation and coupling of an acid precursor. This tetrazine-precursor can be synthesized according to literature[6] in two steps (Figure S3). The product was further purified on HPLC (using water/ ACN as eluent system).

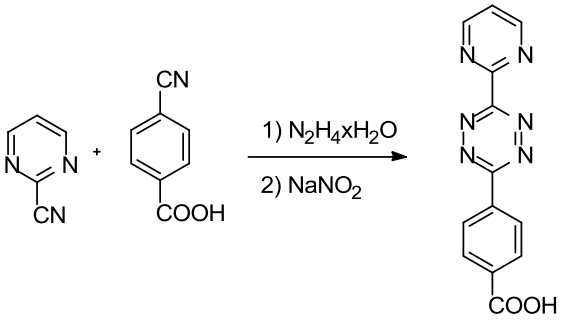
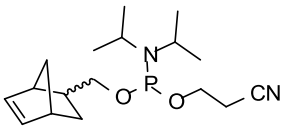


Figure S3. Two step synthesis of tetrazine-acid used as precursor for dansyl- and biotin-tetrazine synthesis.

Solid-phase oligonucleotide synthesis

For introducing a norbornene moiety at the 5'-end of RNA using solid-phase oligonucleotide synthesis, the following phosphoramidite (Fig. S4) was prepared according to our previous report.[2]



Phosphoramidite

Figure S4. Phosphoramidite for chemical incorporation of norbornene on the 5'-end of RNA.

Oligonucleotide synthesis was performed at 1 µmol scale using standard reagents and standard protocols for solid-phase synthesis of RNA. For introducing the norbornene modification, norbornene phosphoramidite was coupled at the 5'-end. After synthesis, the oligonucleotide was cleaved from the solid support using aqueous ammonia (28-30%). The cleaved product was TBDMS-deprotected using TBAF (1M in THF) and isopropanol-precipitated. The oligonucleotide was then purified by semi-preparative HPLC and analyzed by reversed-phase LC-MS (HR-ESI$^-$) (Fig. S5).

Gradients used for LC purification and LC-MS analysis:

Semi-preparative HPLC: Increase from 20% buffer B to 55% buffer B over 35 minutes.

LC-MS: Increase from 5% methanol to 35% methanol over 30 minutes.

Table S1. LC-MS (HR-ESI$^-$) analysis of 5'-norbornene modified RNA

Sequence	Retention time [min]	[M] calculated	[M] observed	Deviation [ppm]
5'-**norbornene**-GGAGCUCAGCCUUCACUGC-3'	19.7	6197.8791	6197.8628	2.6

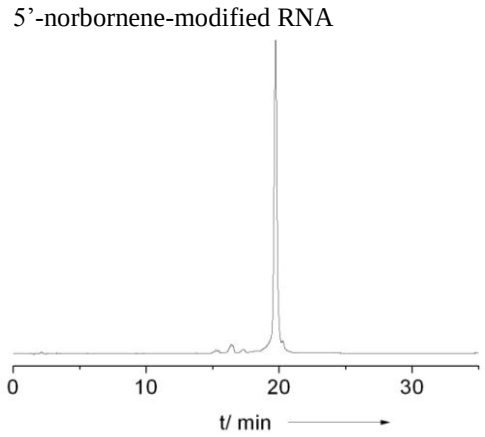


Figure S5. HPLC trace of 5'-norbornene-modified 19 mer RNA.

DAinv on the chemically synthesized RNA oligonucleotide

Procedure. An aqueous solution of norbornene-modified RNA oligonucleotide (c = 10 µM) was either mixed with a solution of dansyl-tetrazine (c = 50 µM) or biotin-tetrazine (c = 50 µM) and incubated for different periods of time at room temperature.

Analysis by LC-MS (HR-ESI⁻). The DAinv reaction mixture with dansyl-tetrazine was incubated for 90 minutes at room temperature and afterwards directly injected into LC-MS system (volume = 45 µl). Gradient used for analysis: 5 min 15% methanol, increase to 100% methanol over 5 min, decrease to 15% methanol over 10 min.

Analysis by PAGE gel electrophoresis. The DAinv reaction mixture with dansyl-tetrazine or biotin-tetrazine was incubated for 50 minutes at room temperature. For DAinv with biotin-tetrazine, 1 eq. of streptavidin (New England Biolabs) was added and analyzed on a 15% denaturing polyacrylamide gel (1h 30 min at 17W). For DAinv with danysl-tetrazine as well as biotin-tetrazine, the DA product could be detected with a conversion higher than 50% after SYBR Gold staining (Fig. S6).

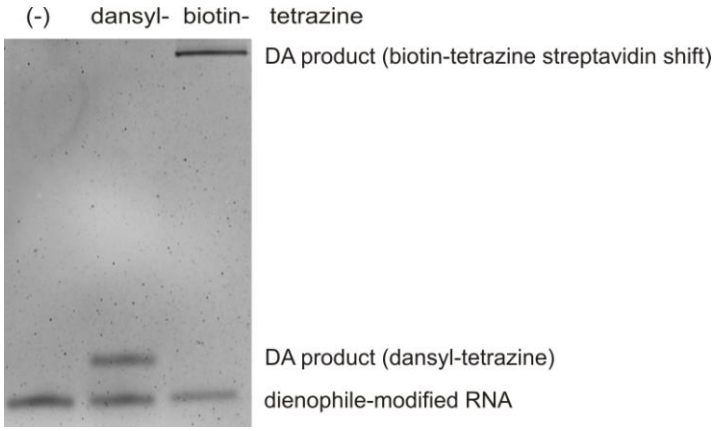


Figure S6. 15% denaturing PAGE gel of DA reactions using dansyl-tetrazine and biotin-tetrazine as dienes. Lane 1: Norbornene-modified RNA, lane 2: DA reaction mixture using dansyl-tetrazine as diene after incubation for 50 min at RT, lane 3: DA reaction mixture using biotin-tetrazine as diene after incubation for 50 min at RT and addition of 1 eq. of streptavidin.

To monitor the reaction kinetics, DA reaction mixtures were incubated with dansyl-tetrazine for different periods of time and then subjected to gel electrophoresis (15% denaturing PAGE, 1h 30 min at 17W). As fluorescence of dansyl-tetrazine is turned on after Diels-Alder reaction, bands corresponding to the DA product are visible in the fluorescent scan with increasing intensity. After SYBR Gold staining, the fluorescent bands are also visible in the SYBR gold scan. Therefore these bands correspond to the DA product as they carry both, the fluorophore and the RNA oligonucleotide (Fig. S7).

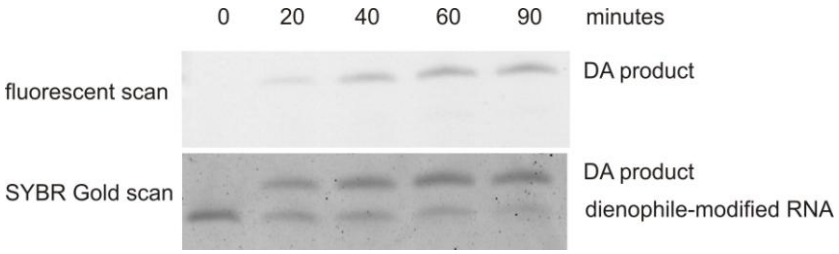


Figure S7. 15% denaturing PAGE gel of DA reactions using dansyl-tetrazine as diene and incubation for different periods of time. Upper panel: Fluorescent scan, excitation at 302 nm, Lower panel: SYBR Gold scan. Lane 1: 0 min, lane 2: 20 min, lane 3: 40 min, lane 4: 60 min, lane 5: 90 min incubation time.

In vitro transcription

General procedure. For *in vitro* transcription, 0.2 µM of dsDNA template in transcription buffer (40 mM Tris *p*H 8.1, 1 mM spermidine, 22 mM $MgCl_2$, 0.01% Triton-X-100) along with 10 mM DTT, 0.01 mg/mL Bovine Serum Albumin, 0.4 mM GTP, and 4 mM of each ATP, CTP & UTP was mixed in an Eppendorf tube. The transcription reaction was doped with 30 µCi of radioactive CTP (^{32}P α-CTP, 10 µCi/µL, Hartmann Analytics, Germany). Then 10 U/µL of T7 RNA polymerase was added (lab prepared stock) and the transcription mixture was incubated at 37°C for 4 h. The reaction was stopped by addition of gel loading

buffer (10% TBE in formamide containing xylene cyanol and bromophenol blue) and purified over a 8% denaturing polyacrylamide gel using standard electrophoresis conditions (1X TBE buffer, run at 25 W for 1 h). Transcript bands were excised and eluted in 0.3 M Na-Acetate *p*H 5.5 overnight at room temperature. The eluted solution was ethanol precipitated and then dissolved in neutral water (Millipore water, MilliQ) for inverse Diels-Alder reaction.

Incorporation of initiator nucleotide using a 38 mer template. DNA strands: 5'-TCTAATACGACTCACTATA-3',5'-GGAGTGAAGGCTGATGACCTATAGTGAGTCGTATTAGA-3'. Underlined nucleotides carry a 2'-O-methyl modification to reduce the non-templated addition of nucleotides at the 3'-end of RNA transcripts by T7 RNA polymerase.[7] The two DNA strands (25 µM each) were hybridized to each other in TE buffer (10 mM Tris-HCl *p*H 7.5, 80 mM NaCl) to form the dsDNA template for *in vitro* transcription. Hybridization was done by heating at 85°C for 3 minutes and then annealing at room temperature for 10 minutes.

To find optimal incorporation conditions for norbornene-modified guanosine (initiator nucleotide), different ratios of initiator nucleotide *vs* GTP were investigated. In this experiment at a constant concentration of initiator nucleotide (4 mM), RNA was *in vitro* transcribed with different concentrations of GTP (4 mM, 0.8 mM & 0.4 mM) using the 38 mer DNA template. All other conditions of *in vitro* transcription are as described above in the general procedure. Transcription yielded a 5'-initiated 19 nucleotide long RNA. The *in vitro* transcribed RNA was resolved on a 15% denaturing polyacrylamide sequencing gel under standard electrophoresis conditions (1X TBE buffer, run at 40 W for 4 h 50 min, Fig. S8).

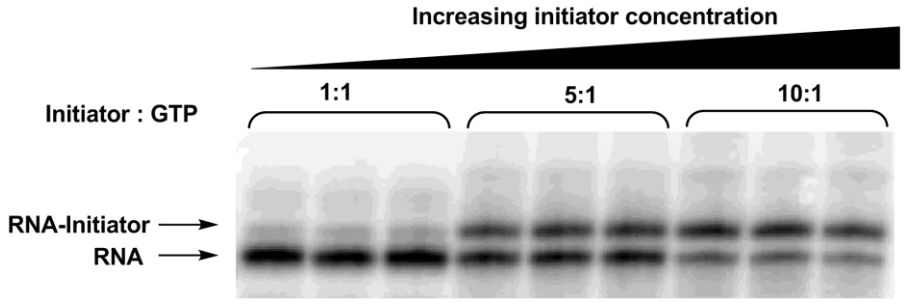


Figure S8. *In vitro* transcription using different ratios of initiator nucleotide and GTP. Gel lanes: 1:1, 5:1 &10:1 are RNA initiation with 1, 5 &10 fold excess of initiator (each ratio was done in triplicates to calculate the amount of RNA-initiated with 5'-norbornene guanosine). Gel labels: RNA-Initiator: 5'-initiated RNA (with norbornene-modified guanosine), RNA: non-initiated RNA.

DAinv on the enzymatically transcribed RNA oligonucleotide

DAinv varying the incubation time. To investigate the effect of the reaction time, RNA (10 µM) was incubated with dansyl-tetrazine (50 µM, 5-fold excess) in neutral water at RT. After appropriate intervals, reaction aliquots were mixed with gel loading buffer. The reacted RNA-initiator DA product was resolved on a 15% denaturing polyacrylamide sequencing gel under standard electrophoresis conditions (1X TBE buffer, run at 40 W for 5 h, Fig. S9).

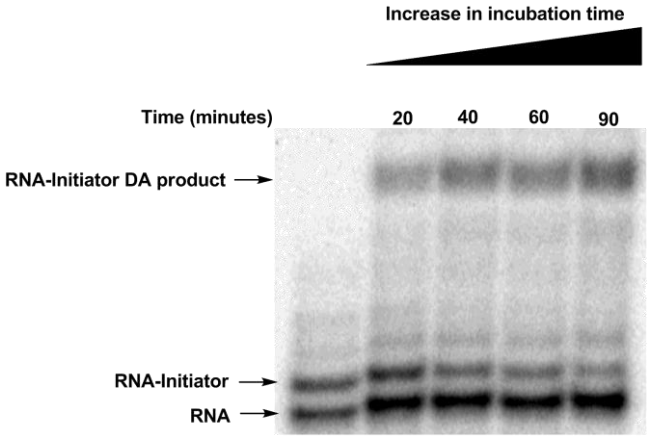


Figure S9. 15% denaturing PAGE of DA reactions using *in vitro* transcribed RNA, incubated for different periods of time. Lane 1: 0 min, lane 2: 20 min, lane 3: 40 min, lane 4: 60 min, lane 5: 90 min incubation time. Gel labels: RNA-Initiator DA product: Diels-Alder product formed between 5'-norbornene initiated RNA and dansyl-tetrazine.

DAinv varying the amount of dansyl-tetrazine. To label 5'-initiated RNA efficiently with the dansyl-dye different excess of dansyl-tetrazine was titrated over a constant concentration of RNA (10 µM). RNA and dansyl-tetrazine were mixed in neutral water and incubated for 50 minutes. After incubation, gel loading buffer was added to the reaction and the mixture loaded on a gel. The DA product was resolved on a 15% denaturing polyacrylamide sequencing gel under standard electrophoresis conditions (1X TBE buffer, run at 40 W for 5 h, Fig. S10).

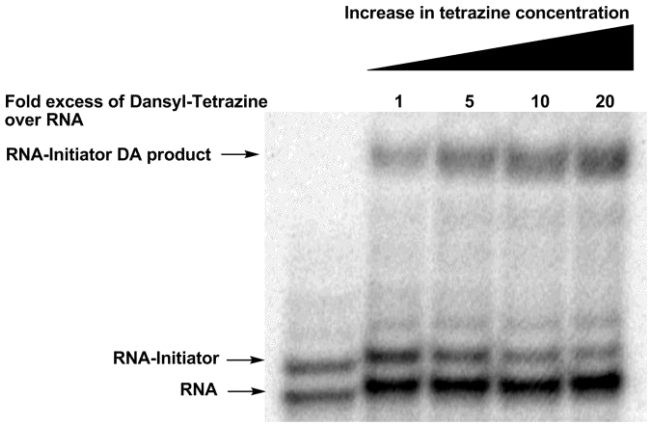


Figure S10. 15% denaturing PAGE of DA reactions using *in vitro* transcribed RNA and incubated with different amounts of dansyl-tetrazine. Gel lanes; 1, 5, 10, 20 represents 10 µM, 50 µM, 100 µM & 200 µM, respectively, of dansyl-tetrazine. Gel labels: RNA-Initiator DA product: Diels-Alder product formed between 5'-norbornene initiated RNA and dansyl-tetrazine.

Table S2. MS (HR-ESI$^-$) analysis of RNA-Initiator and RNA-Initiator DA product

ODN	Sequence	[M] calculated	[M] observed	Deviation [ppm]
RNA-Initiator	5'-**Initiator**-GUCAUCAGCCUUCACUCC-3'	6118.8519	6118.8239	4.6
RNA-Initiator DA product	5'-**DA product**-GUCAUCAGCCUUCACUCC-3'	6654.0713	6654.0584	1.9

Incorporation of initiator nucleotide and DAinv using a 252 mer DNA template. A dsDNA pool (252 nucleotide long) was used for *in vitro* transcription, having a constant 5'-end (5'-TCTAATACGACTCACTATAGGAGCTCAGCCTTCACTGC-3', underlined T7-promoter for T7 RNA polymerase binding), a constant 3'-end (5'-GGCACCACGGTCGGATCCAC-3'**)** and a randomized region in-between. The dsDNA template was *in vitro* transcribed following the general procedure using a 10 fold excess of initiator nucleotide over GTP. Transcription yielded a 5'-initiated 233 nucleotide long RNA. The incorporation of the initiator nucleotide was determined to be 70% using DNAzyme cleavage of 5'-end from this 233 mer RNA. The norbornene-modified RNA (10 µM) was incubated with a 5-fold excess of biotin-tetrazine in water for 90 min at RT. After incubation, a 2-fold excess of streptavidin (New England Biolabs) was added to facilitate the separation of reacted and non-reacted RNAs. Gel loading buffer was added to the reaction mixture and then loaded on a gel. The DA product was resolved on a 12% denaturing polyacrylamide gel under standard electrophoresis conditions (1X TBE buffer, run at 24 W for 2 h 40 min, Fig. S11).

Figure S11. 12% denaturing PAGE of DA reactions using *in vitro* transcribed RNA with subsequent incubation with biotin-tetrazine. Gel lanes; (**+**) represents the RNA reacted with biotin-tetrazine and shifted with streptavidin. (-) represents negative control RNA without incubation with biotin-tetrazine but with the addition of streptavidin. Gel labels: RNA-Initiator DA product: Diels-Alder product formed between 5'-norbornene initiated RNA and biotin-tetrazine.

References

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