Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Posttranscriptional spin labeling of RNA by tetrazine-based cycloaddition

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Supporting Information

Contents

General Methods

MS

High resolution (HR) $ESI^{+/-}$ mass spectra were recorded on a *micrOTOF-Q* mass spectrometer from *Bruker Daltonik* or on an *Orbitrap XL* from *Thermo Fisher Scientific*. LC-MS measurements were performed on an *HTC esquire* from *Bruker Daltonik* in combination with an *Agilent 1100* Series HPLC system (*Agilent Technologies*) using a *Zorbax Narrow Bore SB C18* (2.1×50 mm, 5 µm) column (*Agilent Technologies*). 10 mM triethylamine/100 mM hexafluoroisopropanol was used as solvent A for the analysis of oligonucleotides with a gradient of 3 → 20% B (MeCN, flow rate 0.4 mL min^{-1}) in 20 min. MALDI-TOF mass spectrometry was performed on a *Voyager DE*, *PerSeptive Biosystems* by *Ella Biotech*, Germany

HPLC

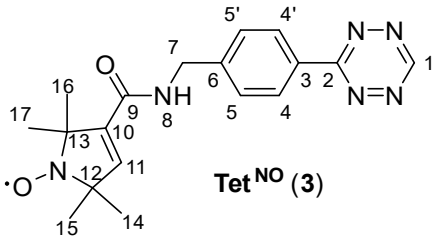
HPLC purification/analysis was performed on an *Agilent 1100* or on an *Agilent 1260 Infinity II* Series HPLC system (*Agilent Technologies*) with an *EC 150/4.6 Nucleodur 100-5 C_{18} ec* column (*Macherey-Nagel*) with H_2O as solvent A and acetonitrile as solvent B at a flow rate of 1 mL min^{-1} (small molecules) or 0-15 % MeCN (B)/0.1 M ammonium acetate (A) in 15 min, flow rate: 1 mL min^{-1} (RNA).

NMR

NMR spectra were recorded using an *Avance I 500 MHz* or an *Avance III HD 700 MHz Cryo*, both from *Bruker*. Chemical shifts (*δ*) are given in ppm and spectra were calibrated to the respective deuterated solvent residue signal of DMSO according to literature values (2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C spectra).[1] Residual peaks in the spectra of nitroxyl-containing compounds correspond to phenylhydrazine.

Synthesis of 1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-3-carboxylic acid 4-(1,2,4,5) tetrazin-3-yl-benzylamide (3, Tet^{NO})

3-(*p*-Benzylamino)-1,2,4,5-tetrazine (**1**, 1.0 eq., 22.4 µmol, 5.00 mg, *Jena Bioscience*) and 1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylate *N*-hydroxysuccinimide ester (TEMPYO-NHS, **2**, 1.5 eq., 33.5 µmol, 9.43 mg, *Alfa Aesar*) were dissolved in a solution of NEt_3 (anhydr., 1.5 eq, 33.5 mmol, 4.60 µL) and DMSO (anhydr., 500 µL) under an atmosphere of argon and exclusion of light. After 1 h the reaction mixture was diluted with water (5 mL) and rapidly frozen in liquid nitrogen. After freeze-drying the crude product was purified via HPLC (0 → 75% B in 20 min; A: ddH_2O; B: MeCN) and **3** was isolated (21.7 µmol, 7.67 mg, 97%) as pink solid.



Chemical formula: $C_{18}H_{21}N_6O_2^{\cdot}$; **MW**: 353.4.

^{1}H-NMR (DMSO-d_6, 500 MHz, r.t.): δ [ppm] = 10.59 (s, 1H, H-1), 8.49 (s, 2H, H-4/4'), 7.61 (s, 2H, H-5/5'), 4.71(s, 2H, H-7).

The signals of the methyl groups and of the olefinic proton (H-11) are not seen in the spectrum (Fig. S13). The signals reappear upon in situ reduction by phenylhydrazine (reduced form: d (ppm) = 1.16 (CH_3-14/15), 1.25 (CH_3-16/17), 6.35 (H-11)); see Figures S14 - 16.

HR MS (ESI$^+$): calcd. for $[M]^{+\cdot}$: 353.1721; found: *m/z* = 353.1709.

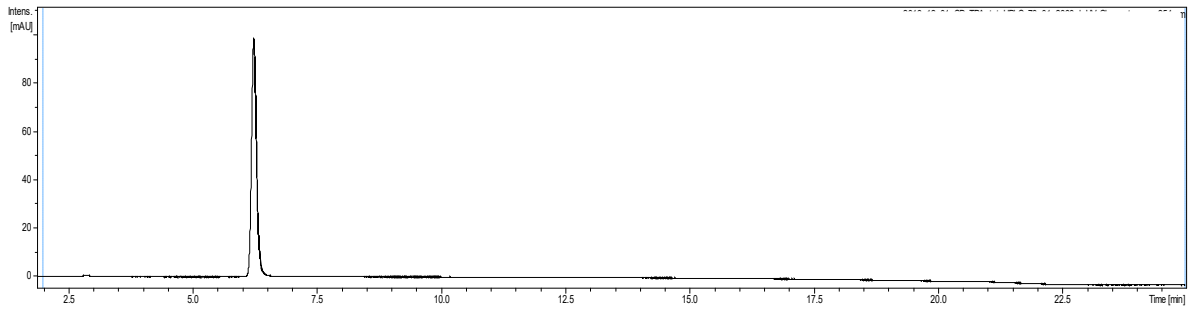


Figure S1. HPL-chromatogram of purified **Tet^{NO} 3**.

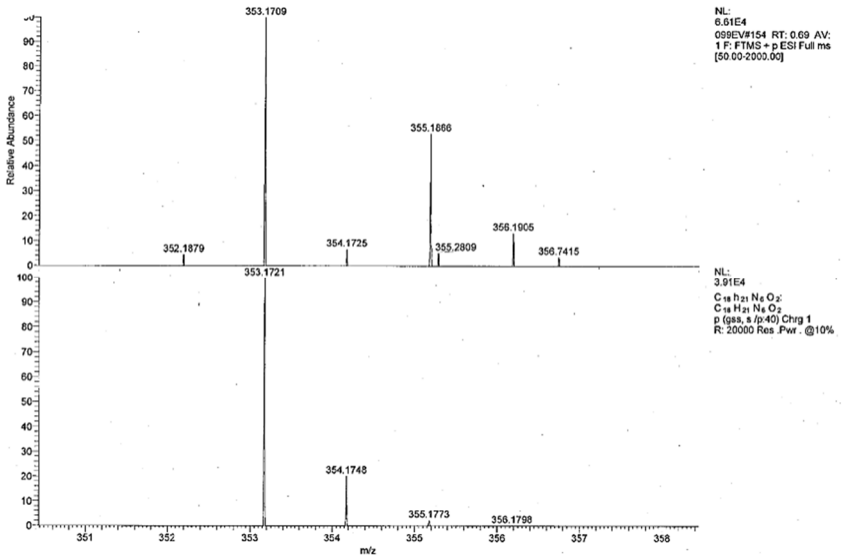


Figure S2. HR-MS (ESI$^+$) of **Tet^{NO} 3** (calcd. for $[M]^{+\cdot}$: 353.1721 (bottom panel: simulated spectrum); found: *m/z* = 353.1709 (top panel: measured spectrum).

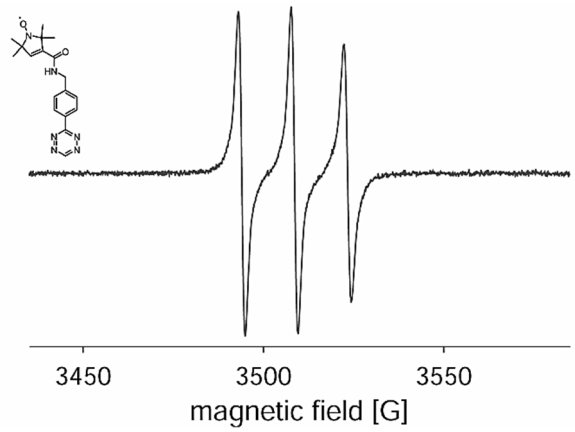


Figure S3. *Cw*-X-band EPR spectrum of **Tet^{NO} 3** (in MeCN).

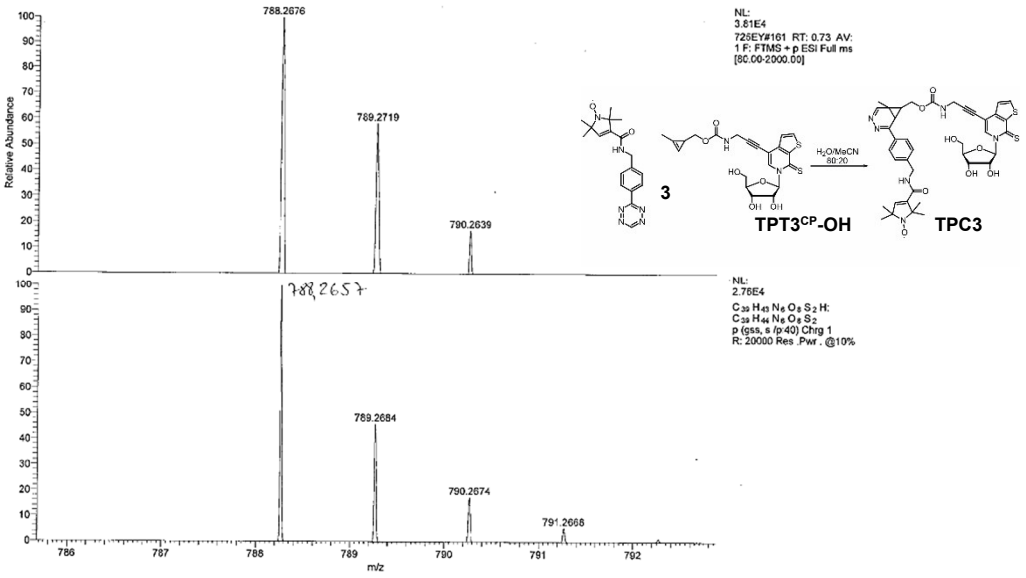


Figure S4. HR-MS (ESI$^+$) of click product **TPC3** resulting from the reaction of **TPT3^{CP}-OH** and **Tet^{NO} 3** (calcd. for $[M]^{+\cdot}$: 788.2657 (bottom panel: simulated spectrum); found: *m/z* = 788.2676 (top panel: measured spectrum).

List of DNA and RNA oligonucleotide sequences

d**NaM** cyanoethyl phosphoramidite was purchased from *Berry & Associates Inc.*, USA. Solid phase syntheses and purification of d**NaM**-modified oligonucleotides were performed in 200 nmol scale by *Ella Biotech*, Germany. DNA primer and unmodified RNA oligonucleotides were synthesized and purified by *Biomers.net*, Germany.

A. *Primer for T7 in vitro transcription*

DNA^{T7} 5'-ATA ATA CGA CTC ACT ATA GG-3'

B. *Template strand containing **X** = d**NaM***

DNA^{NaM} 5'-GG**X** TCT GAT ATC AGA TCC TA TAG TGA GTC GTA TTA T-3'

*C. RNA transcript containing **Y** = **TPT3^{CP}***

RNA^{CP} 5'-GGA UCU GAU AUC AGA **Y**CC-3'

*RNA transcript containing **Z** = **TPT3^{CP}** after iEDDA reaction with **Tet^{NO}***

RNA^{NO} 5'-GGA UCU GAU AUC AGA **Z**CC-3'

D. Solid-phase synthesized RNA

RNA^{C} 5'-GGA UCU GAU AUC AGA UCC-3'

Polyacrylamide gel electrophoresis

For analytical (20%) and preparative (20%) denaturing PAGE separation, a solution of formamide/8.3 M urea (95/5, *v*/*v*) supplemented with 20 mM ethylenediaminetetraacetic acid (EDTA) was used as loading buffer in equal ratio with the sample volume. Samples were heated to 95 °C for 2 min prior to gel loading. Analytical gels were run at 300 V for 1 h, preparative gels were run at 400 V for 4 h. 1×Tris-borate-EDTA buffer (1×TBE) was employed as running buffer.

Analytical polyacrylamide gels were stained with *SYBR® Safe* (*Life Technologies)* and visualized by UV illumination using a *Genoplex* gel documentation system (*VWR*).

***In vitro* transcription**

In vitro transcription reactions were prepared in 100 µL scale with final concentrations of 40 mM Tris·HCl pH 7.9, 25 mM $MgCl_2$, 5 mM DTT, 2.5 mM each canonical triphosphate, 1 mM unnatural triphosphate (**TPT3^{CP}** TP[2]), 3 µM template DNA and primer, 0.5 U µL^{-1} RNasin (*Promega*), 3 ng µL^{-1} iPP (*Roche*), and 5 U µL^{-1} T7 RNA polymerase (*self-made*, AA sequence conforms with GenBank[3]: AY264774.1), which was added to the mixture endmost. Transcriptions were run at 37 °C for 4 h.

Crude reactions were DNase digested by subsequent addition of 10.0 µL 10xDNase I reaction buffer (100 mM Tris·HCl pH 7.6, 25 mM $MgCl_2$, 5 mM $CaCl_2$, *New England Biolabs*) and RNase-free DNase I (*New England Biolabs*) to a final concentration of $4 \cdot 10^{-2}$ U μL^{-1}. Samples were incubated at 37 °C for 30 min followed by enzyme inactivation at 95 °C for 2 min.

PAGE Purification transcribed RNA^{CP}

Crude, DNase-digested transcription reactions of **RNA^{CP}** were purified via preparative denaturing PAGE. After visualization by UV-shadowing and excision of the RNA, electroelution was performed into 170 µL 8 M NH_4OAc using an electroelution chamber (*self-made*, 150 V, 45 min) and precipitated from $EtOH_{abs.}$.

Posttrancriptional labeling of RNA^{CP} with tetrazine spin label Tet^{NO}

Purified **RNA^{CP}** was incubated with an excess of tetrazine conjugate **3** (100-fold) at room temperature for 1 h in aqueous solution. Prior to HPLC purification, the RNA sample (**RNA^{NO}**) was purified by Microspin™ G-25 Columns (*GE Healthcare*).

RNA concentration determination

The absorption at 260 nm (A_{260}) was determined using a *Nanodrop* UV-spectrometer *2000c* (*Thermo Fisher Scientific*). Concentrations were obtained from the A_{260} value and software-assisted calculation (*http://biotools.nubic.northwestern.edu/ OligoCalc.html*).

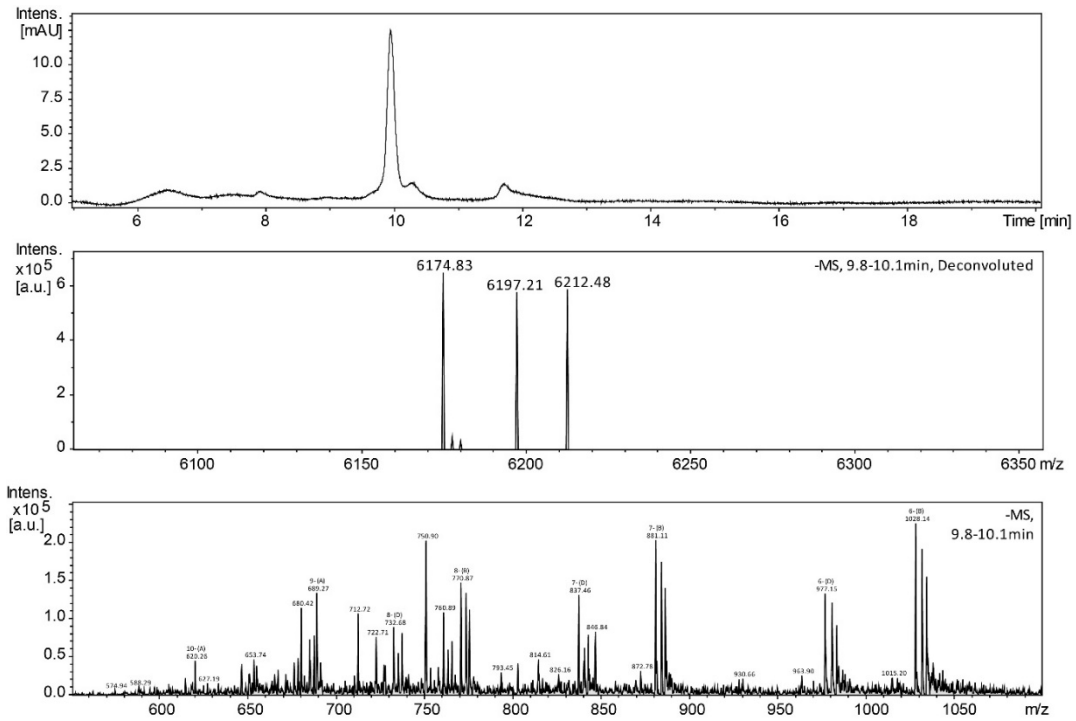


Figure S5. LC-MS analysis showing the UV-trace at 260 nm (panel above), deconvoluted ESI^- spectrum (central panel) and raw ESI^- data (panel below) of *in vitro* transcribed **RNA^{CP}** ($M_{calcd.}$ for **RNA^{CP}**: 6174.4 (5'-PPP), found: *m*/*z* = 6174.8, $M_{calcd.}$ + Na^+: 6197.4, found: *m*/*z* = 6197.2, $M_{calcd.}$ + K^+: 6212.4, found : *m*/*z* = 6212.5).

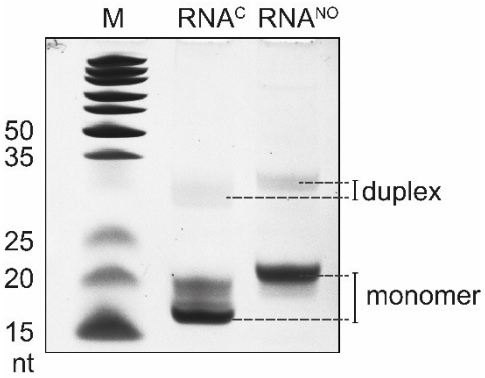


Figure S6. 20% denaturing PAGE analysis of 18mer **RNA^{NO}** used in PELDOR experiments and an unmodified synthetic control **RNA^{C}**. Despite the strong denaturing conditions of the PAGE experiment, duplex formation is observed for both self-complementary RNA sequences.

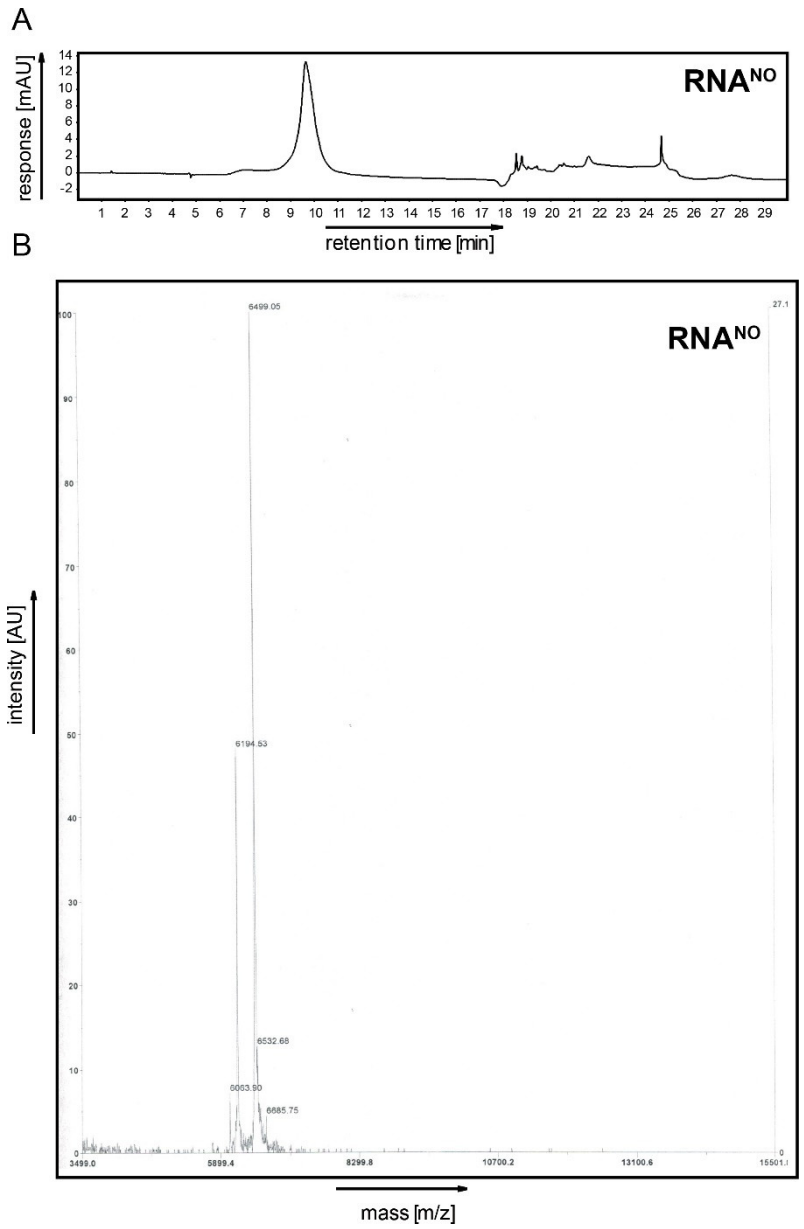


Figure S7. HPLC and MALDI-TOF MS analysis of **RNA^{NO}**. A. HPL chromatogram at 260 nm of purified **RNA^{NO}**. B. MALDI-TOF MS spectrum of **RNA^{NO}** (eluted sample of the HPLC trace shown in A). MALDI-TOF mass spectrometry was performed on a *Voyager DE*, *PerSeptive Biosystems* by *Ella Biotech*, Germany ($M_{calcd.}$ for **RNA^{NO}**: 6499.7 (5'-PPP), found: *m*/*z* = 6499.1, $M_{calcd.}$ + K^+: 6537.6, found: *m*/*z* = 6532.7; unreacted **RNA^{CP}** + Na^+ $M_{calcd.}$: 6196.4, found: *m*/*z* = 6194.5).

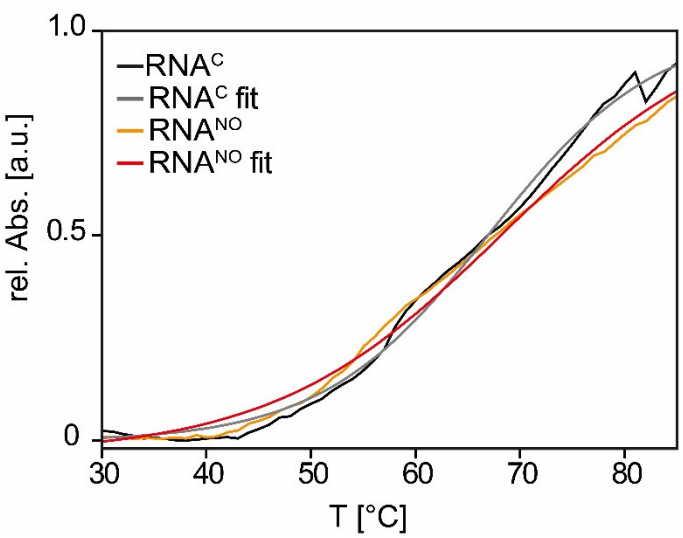


Figure S8. UV melting curves (average of four measurements) of **RNA^{NO}** duplex and unmodified duplex **RNA^{C}** overlaid with fitted data. Thermal denaturation experiments of modified and unmodified oligonucleotides were carried out on a *Cary 100 UV-Vis* spectrophotometer (*Agilent Technologies*). 1 µM samples were prepared in 100 µL phosphate buffer (145 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.0) containing 10 mM MgCl2, annealed 5 min at 70 °C followed by cooling to 18 °C with 2 °C min^{-1}, and analyzed in micro-cuvettes (*Hellma Analytics*). The temperature range for melting curve measurements was set from 20 °C to 95 °C with a rate of 1.0 °C min^{-1}. Melting points were determined from four measurements (**RNA^{NO}** duplex: 68.4 ± 0.5; **RNA^{C}** duplex: 67.1 ± 0.3).

EPR spectroscopy

Sample preparation for EPR measurements

Purified **RNA^{NO}** was dissolved in 80 µL phosphate buffer containing 145 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.0 to a final concentration of 20 µM, and hybridized by heating to 70 °C for 5 min, followed by a gradient 70 → 18 °C with a cooling rate of 2 °C min^{-1}.

Continuous-wave-(*cw*)-X-band electron paramagnetic resonance spectroscopy

10 µL samples of spin labeled **RNA^{NO}** was used to record room temperature *cw*-X-band EPR spectra on an *EMXnano* spectrometer from *Bruker*. The samples were measured at room temperature with a microwave power of 0.32 mW, a modulation amplitude of 1 G, a time constant of 20.48 ms, a conversion time of 20.10 ms.

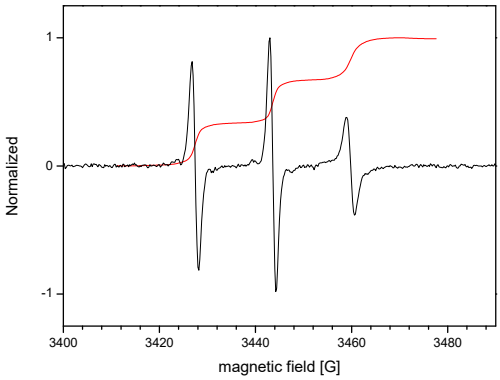


Figure S9. Spin counting of **RNA^{NO}**: [spins] = 98 µM spins, [A_{260}] = 104 µM, 94% (the EMXnano spectrometer (*Bruker)* allowed reference free concentration determination using an internal standard, the absolute number of spins was determined using the software *SpinCount*: Estimated error: 10%).

PELDOR spectroscopy

For PELDOR spectroscopy, the spin labeled **RNA^{NO}** duplex was dissolved in 80 µL D_2O buffer (145 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.0 supplemented with 20% deuterated ethylene glycol). The sample was transferred to a 3 mm quartz Q-band EPR tube and flash-cooled in liquid nitrogen. The PELDOR time traces were recorded on an *ELEXSYS E580* pulsed Q-band EPR spectrometer (*Bruker*), with an *ER 5106QT-2* Q-band resonator. The instrument was equipped with a continuous flow helium cryostat (*CF935*) and temperature control system (*ITC 502*), both from *Oxford Instruments.* The second microwave frequency was coupled into the microwave bridge using a commercially available setup from

Bruker. All pulses were amplified via a 150 W pulsed traveling wave tube amplifier. PELDOR experiments were performed with the pulse sequence p/2(nA)-t1-p(nA) (t1 þ t) p(nB)-(t2 t)-p(nA)-t2-echo. The detection pulses (nA) were set to 12 ns for the p/2 and 24 ns for the p-pulses and applied at a frequency 80 MHz lower than the resonance frequency of the resonator. The pulse amplitudes were chosen to optimize the refocused echo. The p/2-pulse was phase-cycled to eliminate receiver offsets. The pump pulse (nB) was set at the resonance frequency of the resonator and its optimal length (typically 16 ns) was determined using a transient nutation experiment for each sample. The field was adjusted such that the pump pulse was applied to the maximum of the nitroxide spectrum. The pulse amplitude was optimized to maximize the inversion of a Hahn-echo at the pump frequency. All PELDOR spectra were recorded at 50 K with an experiment repetition time of 1 ms, a video amplifier bandwidth of 20 MHz, and an amplifier gain of 42 dB. The parameter t1 was set to 260 ns and the maximum of t2 was set to values ranging from 10 µs. Deuterium modulation was suppressed by addition of 8 spectra of variable t1 with a dt1 of 16 ns. The obtained time traces were divided by a mono exponential decay to eliminate intermolecular contributions and renormalized. Distance distributions were obtained from the background-corrected data by using the program *DEER Analysis 2016* (*http://www.epr.ethz.ch/software.html*) developed by Jeschke *et al.*[4].

MD Simulation

The self-complementary 18mer **RNA^{NO}** duplex was constructed based on A-form RNA in HyperChem (Release 7.01, *Hypercube, Inc.*) with one **TPC3** residue per oligonucleotide. The structure and internal atom numbering of the click-spin-labeled nucleotide **TPC3** is shown in Fig. S9. The obtained duplex geometry was converted to Gromacs 2018[5] format using new parameters for **TPC3** (see below) within the CHARMM 36 all atom force field (version January 2018)[6]. The structure was placed in a cubic box (7.879 nm^3) solvated with 15486 TIP3P water molecules and 34 Na^+ counterions.

The system was energy minimized 6 times switching alternatively between runs using steepest descent gradients or Polak-Ribiere conjugate gradients until convergence to machine precision. Subsequently, 480ps MD calculations at constant temperature (300 K, NVT) followed by 480 ps MD calculations at constant pressure (1 bar, NPT) equilibrate solvent and ions. Finally, a MD trajectory was calculated without restrains or constrains (except bond lengths) over 1200 ns at 300 K. The time span between 200 ns and 1200 ns has been used for the characterization of the system.

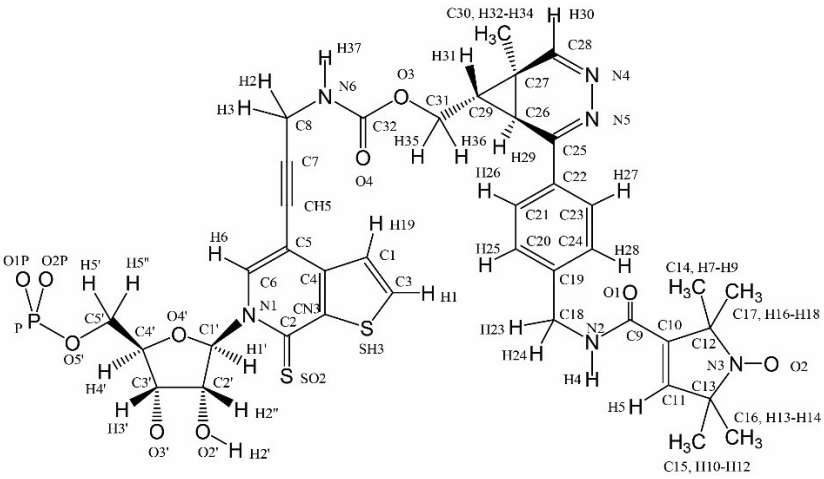


Figure S10. Structure of the spin label **TPC3** with atom definitions of the residue "**TPC3**" used in the Gromacs – Charmm36 force field. The indicated C26[S]-C27[S]-C29[S] isomer was used for the MD-calculations.

Parametrization of TPC3

The **TPC3** residue was parametrized in CHARMM using CGenFF[7]. 6-31G* *ab initio* calculations were used on selected groups to adjust the charges, bond lengths, angles and dihedral angles suggested by CGenFF being expected to be unreliable. This was particularly true for the nitroxide label and the diazine ring. All charges and parameters together with the atom numbering (Figure S9) are listed in Gromacs format in Table S1. The final charge distribution in CHARMM at the nitroxide label (N8, -0.09; O9, -0.36; N-O bond length 0.139 nm) compares well to the somewhat higher polarity used in an Amber parametrization (atoms analogue to N8, +0.18, O9, -0.403; N-O bond length 0.125 nm).[7c]

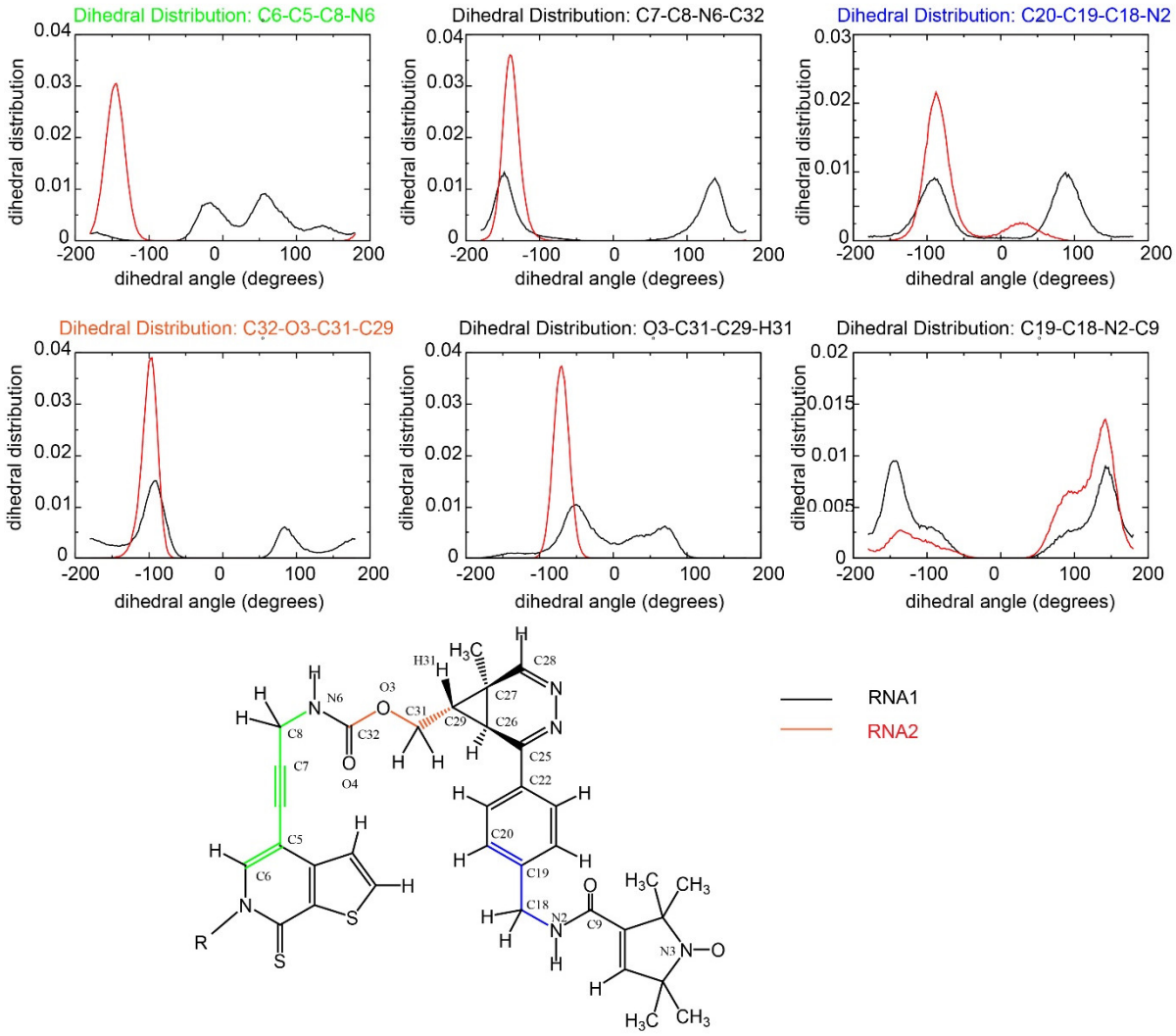


Figure S11. Rotamer distribution of selected "dihedral" angles of the two **TPC3** units in the duplex formed by **RNA^{NO}**. Red lines belong to RNA2 with the **TPC3** residue in the more rigid 3' end (upper position in Figure 2D and E), black lines to RNA1 with the **TPC3** residue in the more flexible 3' end (lower position in Figure 2D and E).

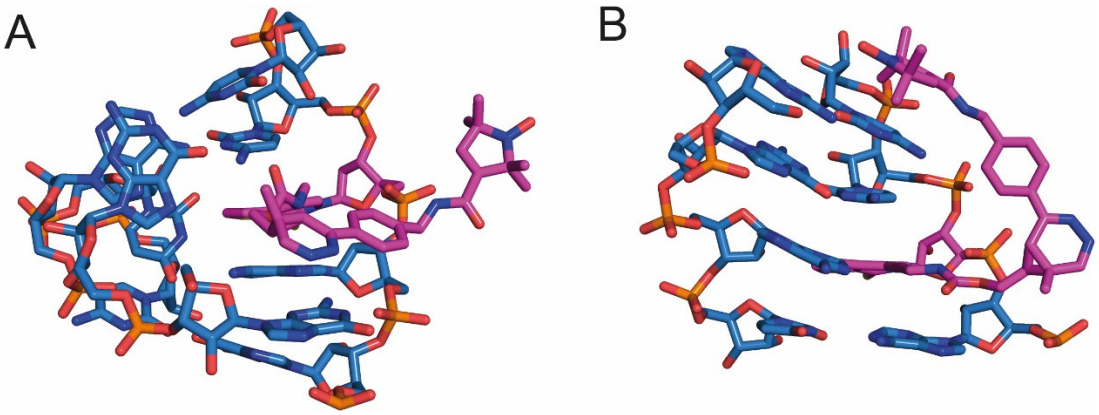


Figure S12. Typical conformations of the rigid (B) and of the more flexible (A) end of the duplex formed by **RNA^{NO}** (the **TPC3** residue is colored in pink). The structures belong to a frame at 537564 ps which has been selected as the center of a cluster by its smallest rmsd deviation from all other structures (program cluster in gmx).

Discussion of MD results

The distance distribution of the nitroxide labels over 1000 ns fits well to the distribution obtained by the PELDOR experiments (Figure 2C). However, the distribution of the *N-N* distances of the RNA base pairs indicates two different conformational regions at the two ends of the duplex (Fig. 2D), Obviously, the sampling over 1000 ns at 300 K is not sufficient to produce identical distributions of conformational states at the two ends of the duplex being chemically identical. A more detailed analysis of the involved conformations is depicted in Figure S12 showing a flexible and a rigid linker conformation in the duplex.

Table S1. Force field parameters[5] for **TPC3** (spin labeled **TPT3^{CP}** nucleoside):

A. New residue topology entry “TPC3” in “merged.rtp” of the Gromacs – CHARMM 36 force field**.** Partial charges were suggested by CGenFF[10] (interface 1.0.0, force field 3.0.1) and adjusted using AM1 and 6-31G* QM calculations

```
 [ TPC3 ]
   [ atoms ]
;  atom   atomtype  charge
    P       P       1.500  0
    O1P     ON3    -0.780  1
    O2P     ON3    -0.780  2
    N2      NG2S1  -0.430  3
    C9      CG2O1   0.550  4
    C10     CG2R51 -0.100  5
    C11     CG2R51 -0.080  6
    C12     CG3C50  0.200  7
    N3      NG3C51 -0.090  8
    C13     CG3C50  0.200  9
    C14     CG331  -0.240 10
    C15     CG331  -0.240 11
    C16     CG331  -0.240 12
    C17     CG331  -0.240 13
    O1      OG2D1  -0.450 14
    H4      HGP1    0.311 15
    H5      HGR51   0.150 16
    O2      OG312  -0.360 17
    H7      HGA3    0.090 18
    H8      HGA3    0.090 19
    H9      HGA3    0.090 20
    H10     HGA3    0.090 21
    H11     HGA3    0.090 22
    H12     HGA3    0.090 23
    H13     HGA3    0.090 24
    H14     HGA3    0.090 25
    H15     HGA3    0.090 26
    H16     HGA3    0.090 27
    H17     HGA3    0.090 28
    H18     HGA3    0.090 29
    C18     CG321   0.003 30
    C19     CG2R61 -0.003 31
    C20     CG2R61 -0.117 32
    C21     CG2R61 -0.110 33
    C22     CG2R61  0.249 34
    C23     CG2R61 -0.110 35
    C24     CG2R61 -0.117 36
    C25     CG2DC1  0.040 37
    C26     CG3RC1 -0.030 38
    C27     CG3RC1 -0,010 39
    C28     CG2DC1  0,015 40
    N4      NG2D1  -0,230 41
    N5      NG2D1  -0,235 42
    C29     CG3C31 -0,072 43
    C30     CG331  -0,268 44
    C31     CG321   0,136 45
    O3      OG302  -0,299 46
    C32     CG2O6   0,164 47
    O4      OG2D1  -0,390 48
    N6      NG2S1  -0,430 49
    O5'     ON2    -0.570 50
    C5'     CN8B   -0.080 51
    H5'     HN8     0.090 52
    H5''    HN8     0.090 53
    C4'     CN7     0.160 54
    H4'     HN7     0.090 55
    O4'     ON6B   -0.500 56
    C1'     CN7B    0.160 57
    H1'     HN7     0.090 58
    N1      NN2B   -0.300 59
    C6      CG2R61  0.025 60
    H6      HGR62   0.190 61
    C5      CG2R61  0.105 62
   CH5      CG1T1  -0.005 63
    C4      CG2RC0 -0.095 64
   CN3      CG2RC0  0.060 65
   SH3      SG2R50 -0.050 66
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```
   C2     CG2R63  0.130 67
  SO2     SG2D1  -0.206 68
   C2'    CN7B    0.140 69
  H2''    HN7     0.090 70
   O2'    ON5    -0.660 71
   H2'    HN5     0.430 72
   C3'    CN7     0.010 73
   H3'    HN7     0.090 74
   O3'    ON2    -0.570 75
   C1     CG2R51 -0.250 76
   C3     CG2R51 -0.069 77
   C7     CG1T1  -0.110 78
   C8     CG321  -0.020 79
   H1     HGR52   0.185 80
   H2     HGA2    0.090 81
   H3     HGA2    0.090 82
   H19    HGR51   0.209 83
   H23    HGA2    0.090 84
   H24    HGA2    0.090 85
   H25    HGR61   0.115 86
   H26    HGR61   0.115 87
   H27    HGR61   0.115 88
   H28    HGR61   0.115 89
   H29    HGA1    0.110 90
   H30    HGA4    0.175 91
   H31    HGA1    0.110 92
   H32    HGA3    0.090 93
   H33    HGA3    0.090 94
   H34    HGA3    0.090 95
   H35    HGA2    0.090 96
   H36    HGA2    0.090 97
   H37    HGP1    0.329 98

[ bonds ]
     N2   C9
     C9   C10
     C10  C11
     C10  C12
     C12  N3
     C11  C13
     N3   C13
     C12  C14
     C12  C15
     C13  C16
     C13  C17
     C9   O1
     N2   H4
     C11  H5
     N3   O2
     C14  H7
     C14  H8
     C14  H9
     C15  H10
     C15  H11
     C15  H12
     C16  H13
     C16  H14
     C16  H15
     C17  H16
     C17  H17
     C17  H18
     N2   C18
     C18  C19
     C19  C20
     C20  C21
     C21  C22
     C22  C23
     C19  C24
     C23  C24
     C22  C25
     C25  C26
     C26  C27
     C27  C28
     C28  N4
     C25  N5
     N4   N5
     C26  C29
     C27  C29
```

```
      C27  C30
      C29  C31
      C31  O3
      O3   C32
      C32  O4
      C32  N6
      P   O1P
      P   O2P
      P   O5'
      O5'  C5'
      C5'  H5'
      C5'  H5''
      C5'  C4'
      C4'  H4'
      C4'  O4'
      O4'  C1'
      C1'  H1'
      C1'  N1
      N1   C6
      C6   H6
      C6   C5
      C5   CH5
      C5   C4
      C4   CN3
      CN3  SH3
      N1   C2
      CN3  C2
      C2   SO2
      C1'  C2'
      C2'  H2''
      C2'  O2'
      O2'  H2'
      C4'  C3'
      C2'  C3'
      C3'  H3'
      C3'  O3'
      C4   C1
      SH3  C3
      C1   C3
      CH5  C7
      N6   C8
      C7   C8
      C3   H1
      C8   H2
      C8   H3
      C1   H19
      O3'   +P
      C18  H23
      C18  H24
      C20  H25
      C21  H26
      C23  H27
      C24  H28
      C26  H29
      C28  H30
      C29  H31
      C30  H32
      C30  H33
      C30  H34
      C31  H35
      C31  H36
      N6   H37

[ impropers ]
     C9     C10    N2     O1
     C25    C22    C26    N5
     C28    C27    N4     H30
     C32    N6     O4     O3
     C2     CN3    N1     SO2
```

B. New force field parameters used within the CHARMM-36- Gromacs parameter file "ffbonded.itp". The data were suggested by CGenFF[5] and adjusted using 6-31G* QM calculations (indicated).

```
[ bondtypes ]
;      i        j  func           b0           kb
   CG1T1   CG2R61     1   0.14350000    288696.00
   CG1T1    CG321     1   0.14650000    343088.00
   CG2O1   CG2R51     1   0.14950000    251040.00 ;631Gs
  CG2R51   CG3C50     1   0.15100000    292880.00
   CG331   CG3C50     1   0.15280000    186188.00
  CG3C50   NG3C51     1   0.14780000    334720.00
  NG3C51    OG312     1   0.13900000    259408.00 ;631Gs
  CG2R61     NN2B     1   0.13790000    317984.00
  CG2R63     NN2B     1   0.13830000    284512.00
  CG2DC1   CG3RC1     1   0.14850000    305432.00 ;631Gs
   NG2D1    NG2D1     1   0.13660000    267776.00 ;631Gs

[ angletypes ]
;      i        j        k  func       theta0       ktheta          ub0          kub
   CG1T1    CG1T1   CG2R61     5   180.000000   334.720000   0.00000000         0.00
   CG1T1    CG1T1    CG321     5   180.000000   158.992000   0.00000000         0.00
  CG2R51   CG2O1    NG2S1     5   116.500000   669.440000   0.00000000         0.00
  CG2R51   CG2O1    OG2D1     5   121.000000   251.040000   0.00000000         0.00
   CG2O1   CG2R51   CG2R51     5   120.000000   383.254400   0.00000000         0.00 ;631Gs
   CG2O1   CG2R51   CG3C50     5   129.700000   543.920000   0.00000000         0.00 ;631Gs
  CG2R51   CG2R51   CG3C50     5   109.000000   962.320000   0.00000000         0.00
  CG3C50   CG2R51    HGR51     5   124.600000   242.672000   0.00000000         0.00
   CG1T1   CG2R61   CG2R61     5   120.000000   292.880000   0.00000000         0.00
   CG1T1   CG2R61   CG2RC0     5   120.000000   292.880000   0.00000000         0.00
  CG2RC0   CG2R63    SG2D1     5   125.100000   317.984000   0.00000000         0.00
  CG2R63   CG2RC0   SG2R50     5   123.700000   376.560000   0.00000000         0.00
   CG1T1    CG321    NG2S1     5   115.200000   443.504000   0.00000000         0.00
   CG1T1    CG321     HGA2     5   111.500000   393.296000   0.00000000         0.00
  CG3C50    CG331     HGA3     5   110.100000   289.532800   0.21790000     18853.10
  CG2R51   CG3C50    CG331     5   112.000000   518.816000   0.00000000         0.00 ;631Gs
  CG2R51   CG3C50   NG3C51     5   100.100000   585.760000   0.00000000         0.00 ;631Gs
   CG331   CG3C50    CG331     5   113.500000   488.272800   0.25610000      9338.69
   CG331   CG3C50   NG3C51     5   111.500000   376.560000   0.00000000         0.00
  CG2RC0   CG2R63     NN2B     5   107.800000   585.760000   0.00000000         0.00
    NN2B   CG2R61    HGR62     5   115.000000   276.144000   0.00000000         0.00
  CG2R61   CG2R61     NN2B     5   120.000000  1046.000000   0.00000000         0.00
    CN7B     NN2B   CG2R61     5   119.600000   376.560000   0.00000000         0.00
    CN7B     NN2B   CG2R63     5   118.400000   376.560000   0.00000000         0.00
  CG2R61     NN2B   CG2R63     5   123.900000    83.680000   0.00000000         0.00
  CG3C50   NG3C51   CG3C50     5   110.000000   500.520000   0.00000000         0.00
  CG3C50   NG3C51    OG312     5   121.000000   393.296000   0.00000000         0.00
    NN2B   CG2R63    SG2D1     5   123.000000   468.608000   0.00000000         0.00
  CG2R61   CG2DC1   CG3RC1     5   120.300000   401.664000   0.00000000         0.00 ;631Gs-
Cgenff
  CG3RC1   CG2DC1    NG2D1     5   122.800000   401.664000   0.00000000         0.00 ;631Gs-
Cgenff
  CG3RC1   CG2DC1     HGA4     5   118.400000   334.720000   0.00000000         0.00 ;631Gs-
Cgenff
  CG3C31    CG321    OG302     5   110.100000   633.457600   0.00000000         0.00 ;Cgenff
   CG321   CG3C31   CG3RC1     5   120.800000   563.584800   0.00000000         0.00 ;631Gs-
Cgenff
  CG3RC1   CG3C31     HGA1     5   115.000000   288.696000   0.21790000     18853.10 ;631Gs-
Cgenff
  CG2DC1   CG3RC1    CG331     5   114.500000   488.270000   0.25610000      9338.69 ;631Gs-
Cgenff
  CG2DC1   CG3RC1   CG3C31     5   120.000000   585.760000   0.00000000         0.00 ;631Gs-
Cgenff
  CG2DC1   CG3RC1   CG3RC1     5   115.800000   585.760000   0.00000000         0.00 ;631Gs-
Cgenff
  CG2DC1   CG3RC1     HGA1     5   116.000000   418.400000   0.00000000         0.00 ;631Gs-
Cgenff
   CG331   CG3RC1   CG3C31     5   122.100000   447.699000   0.25610000      6694.40 ;631Gs-
Cgenff
  CG2DC1    NG2D1    NG2D1     5   121.300000   585.760000   0.00000000         0.00 ;631Gs-
Cgenff
```

```
[ dihedraltypes ]
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   NG2S1    CG2O1   CG2R51   CG3C50     9   180.000000     4.184000     2
   OG2D1    CG2O1   CG2R51   CG2R51     9   180.000000     4.184000     2
   OG2D1    CG2O1   CG2R51   CG3C50     9   180.000000     4.184000     2
  CG2R51    CG2O1    NG2S1    CG321     9     0.000000     6.694400     1
  CG2R51    CG2O1    NG2S1    CG321     9   180.000000    16.736000     2
  CG2R51    CG2O1    NG2S1     HGP1     9   180.000000    10.460000     2
   CG2O1   CG2R51   CG2R51   CG3C50     9   180.000000    16.736000     2
   CG2O1   CG2R51   CG2R51    HGR51     9   180.000000     4.184000     2
  CG3C50   CG2R51   CG2R51   CG3C50     9   180.000000    50.208000     2
  CG3C50   CG2R51   CG2R51    HGR51     9   180.000000    12.133600     2
   CG2O1   CG2R51   CG3C50    CG331     9   180.000000     0.209200     3
   CG2O1   CG2R51   CG3C50   NG3C51     9   180.000000     2.928800     3
  CG2R51   CG2R51   CG3C50    CG331     9   180.000000     0.209200     3
  CG2R51   CG2R51   CG3C50   NG3C51     9   180.000000     2.928800     3
   HGR51   CG2R51   CG3C50    CG331     9   180.000000     8.368000     2
   HGR51   CG2R51   CG3C50   NG3C51     9   180.000000    12.970400     2
   CG1T1   CG2R61   CG2R61     NN2B     9   180.000000     4.184000     2
   CG1T1   CG2R61   CG2R61    HGR62     9   180.000000    10.041600     2
  CG2RC0   CG2R61   CG2R61     NN2B     9   180.000000    15.480800     2
   CG1T1   CG2R61   CG2RC0   CG2R51     9   180.000000    16.736000     2
   CG1T1   CG2R61   CG2RC0   CG2RC0     9   180.000000    12.970400     2
  CG2R61   CG2R61     NN2B     CN7B     9   180.000000    46.024000     2
   HGR62   CG2R61     NN2B     CN7B     9   180.000000     1.255200     2
    NN2B   CG2R63   CG2RC0   SG2R50     9   180.000000     0.000000     2
   SG2D1   CG2R63   CG2RC0   CG2RC0     9   180.000000    18.828000     2
   SG2D1   CG2R63   CG2RC0   SG2R50     9   180.000000     0.000000     2
  CG2RC0   CG2R63     NN2B   CG2R61     9   180.000000     0.836800     2
  CG2RC0   CG2R63     NN2B     CN7B     9   180.000000    46.024000     2
   SG2D1   CG2R63     NN2B     CN7B     9   180.000000     6.694400     2
  CG2R51   CG2RC0   CG2RC0   CG2R63     9   180.000000     6.276000     2
  CG2R61   CG2RC0   CG2RC0   CG2R63     9   180.000000    12.552000     2
  CG2R63   CG2RC0   SG2R50   CG2R51     9   180.000000    35.564000     2
   CG1T1    CG321    NG2S1    CG2O1     9     0.000000     0.062760     1
   CG1T1    CG321    NG2S1    CG2O1     9     0.000000     1.246832     2
   CG1T1    CG321    NG2S1    CG2O1     9   180.000000     0.317984     3
   CG1T1    CG321    NG2S1    CG2O1     9     0.000000     2.589896     4
   CG1T1    CG321    NG2S1    CG2O1     9     0.000000     0.619232     6
   CG1T1    CG321    NG2S1     HGP1     9     0.000000     0.000000     1
    HGA3    CG331   CG3C50   CG2R51     9     0.000000     0.669440     3
    HGA3    CG331   CG3C50    CG331     9     0.000000     0.669440     3
    HGA3    CG331   CG3C50   NG3C51     9     0.000000     0.669440     3
  CG2R51   CG3C50   NG3C51   CG3C50     9   180.000000     8.577200     3
  CG2R51   CG3C50   NG3C51    OG312     9   180.000000     8.577200     3
   CG331   CG3C50   NG3C51   CG3C50     9     0.000000     0.753120     3
   CG331   CG3C50   NG3C51    OG312     9     0.000000     3.765600     3
    ON6B     CN7B     NN2B   CG2R63     9     0.000000     2.928800     3
    ON6B     CN7B     NN2B   CG2R63     9   180.000000     3.347200     1
    ON6B     CN7B     NN2B   CG2R61     9     0.000000     3.765600     1
     HN7     CN7B     NN2B   CG2R63     9     0.000000     0.000000     3
     HN7     CN7B     NN2B   CG2R61     9     0.000000     0.815880     3
    CN7B     CN7B     NN2B   CG2R63     9   180.000000     0.836800     3
    CN7B     CN7B     NN2B   CG2R61     9   180.000000     0.000000     3
  CG2R61   CG2R61     NN2B   CG2R63     9   180.000000     2.092000     2
   HGR62   CG2R61     NN2B   CG2R63     9   180.000000    19.246400     2
    NN2B   CG2R63   CG2RC0   CG2RC0     9   180.000000     0.836800     2
   SG2D1   CG2R63     NN2B   CG2R61     9   180.000000     6.694400     2
  CG3RC1   CG2DC1   CG2R61   CG2R61     9   180.000000     6.694400     2
  CG2R61   CG2DC1   CG3RC1   CG3C31     9     0.000000     1.255200     3
  CG2R61   CG2DC1   CG3RC1   CG3RC1     9     0.000000     1.255200     3
  CG2R61   CG2DC1   CG3RC1     HGA1     9   180.000000     1.255200     3
   NG2D1   CG2DC1   CG3RC1    CG331     9     0.000000     2.092000     2
   NG2D1   CG2DC1   CG3RC1    CG331     9     0.000000     1.255200     3
   NG2D1   CG2DC1   CG3RC1   CG3C31     9     0.000000     2.092000     2
   NG2D1   CG2DC1   CG3RC1   CG3C31     9     0.000000     1.255200     3
   NG2D1   CG2DC1   CG3RC1   CG3RC1     9     0.000000     2.092000     2
   NG2D1   CG2DC1   CG3RC1   CG3RC1     9     0.000000     1.255200     3
   NG2D1   CG2DC1   CG3RC1     HGA1     9     0.000000     0.125520     3
    HGA4   CG2DC1   CG3RC1    CG331     9     0.000000     0.836800     3
    HGA4   CG2DC1   CG3RC1   CG3C31     9     0.000000     0.836800     3
    HGA4   CG2DC1   CG3RC1   CG3RC1     9     0.000000     0.836800     3
  CG2R61   CG2DC1    NG2D1    NG2D1     9   180.000000    50.208000     2
  CG3RC1   CG2DC1    NG2D1    NG2D1     9   180.000000    50.208000     2
    HGA4   CG2DC1    NG2D1    NG2D1     9   180.000000    16.736000     2
   OG302    CG321   CG3C31   CG3RC1     9     0.000000     0.815880     3
   OG302    CG321   CG3C31     HGA1     9     0.000000     0.836800     3
```

```
    HGA2    CG321   CG3C31   CG3RC1     9     0.000000     0.041840     1
    HGA2    CG321   CG3C31   CG3RC1     9     0.000000     0.083680     3
  CG3C31    CG321    OG302    CG2O6     9   180.000000     0.418400     1
  CG3C31    CG321    OG302    CG2O6     9     0.000000     2.510400     2
  CG3C31    CG321    OG302    CG2O6     9   180.000000     0.418400     3
    HGA3    CG331   CG3C50   CG2R51     9     0.000000     0.669440     3
    HGA3    CG331   CG3C50    CG331     9     0.000000     0.669440     3
    HGA3    CG331   CG3C50   NG3C51     9     0.000000     0.669440     3
    HGA3    CG331   CG3RC1   CG2DC1     9     0.000000     0.669440     3
    HGA3    CG331   CG3RC1   CG3C31     9     0.000000     0.815880     3
   CG321   CG3C31   CG3RC1   CG2DC1     9     0.000000     2.928800     3
   CG321   CG3C31   CG3RC1    CG331     9     0.000000     0.627600     3
   CG321   CG3C31   CG3RC1   CG3RC1     9     0.000000     0.627600     3
   CG321   CG3C31   CG3RC1     HGA1     9     0.000000     0.627600     3
  CG3RC1   CG3C31   CG3RC1   CG2DC1     9     0.000000     2.928800     3
  CG3RC1   CG3C31   CG3RC1    CG331     9     0.000000     0.627600     3
    HGA1   CG3C31   CG3RC1   CG2DC1     9     0.000000     0.627600     3
    HGA1   CG3C31   CG3RC1    CG331     9     0.000000     0.815880     3
    HGA1   CG3C31   CG3RC1   CG3RC1     9     0.000000     0.627600     3
    HGA1   CG3C31   CG3RC1     HGA1     9     0.000000     0.627600     3
  CG2DC1   CG3RC1   CG3RC1   CG2DC1     9     0.000000     0.627600     3
  CG2DC1   CG3RC1   CG3RC1    CG331     9     0.000000     0.209200     3
  CG2DC1   CG3RC1   CG3RC1   CG3C31     9     0.000000     0.627600     3
  CG2DC1   CG3RC1   CG3RC1     HGA1     9     0.000000     0.627600     3
   CG331   CG3RC1   CG3RC1   CG3C31     9     0.000000     0.627600     3
  CG2DC1    NG2D1    NG2D1   CG2DC1     9   180.000000    50.208000     2
  CG3C50   NG3C51    OG311     HGP1     9     0.000000     1.158968     3
; CG3C31    CG321    OG302    CG2O6     9   180.000000     0.418400     3
   CG1T1    CG321    NG2S1    CG2O6     9     0.000000     0.062760     1
   CG1T1    CG321    NG2S1    CG2O6     9     0.000000     1.246832     2
   CG1T1    CG321    NG2S1    CG2O6     9   180.000000     0.317984     3
   CG1T1    CG321    NG2S1    CG2O6     9     0.000000     2.589896     4
   CG1T1    CG321    NG2S1    CG2O6     9     0.000000     0.619232     6

[ dihedraltypes ]
; 'improper' dihedrals
   CG2O1   CG2R51    NG2S1    OG2D1     2     0.000000  1004.160000
  CG2R63   CG2RC0     NN2B    SG2D1     2     0.000000   259.408000
  CG2DC1   CG2R61   CG3RC1    NG2D1     2     0.000000  1004.160000
  CG2DC1   CG3RC1    NG2D1     HGA4     2     0.000000   251.040000
```

NMR spectra

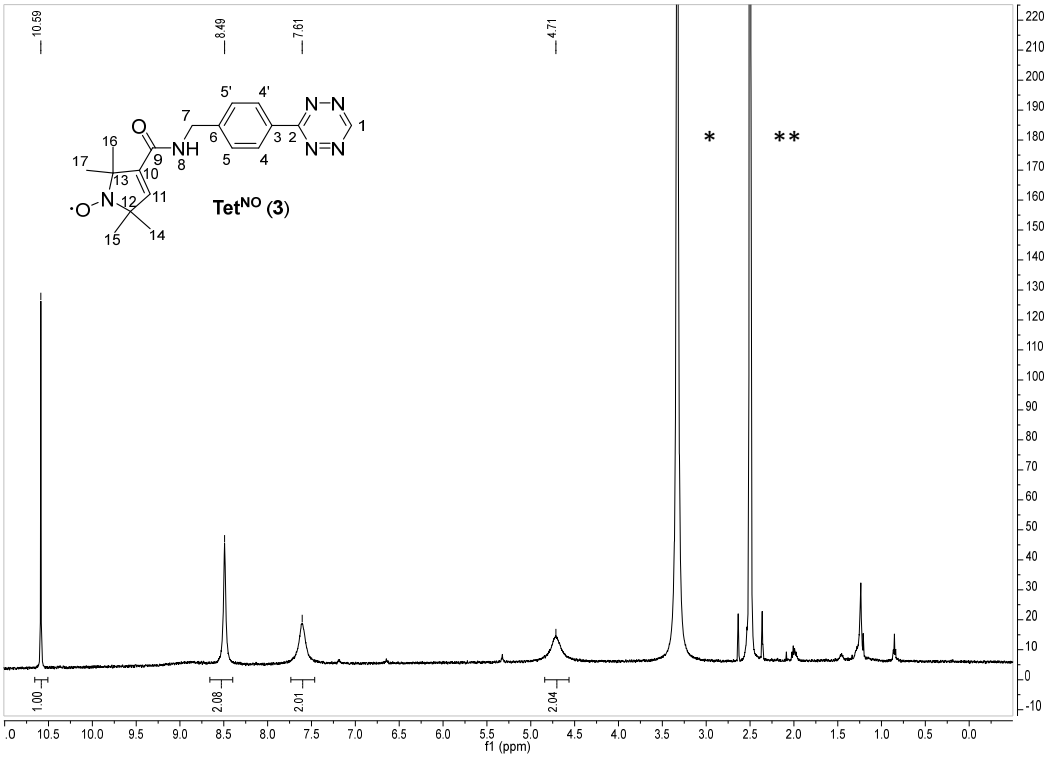

Figure S13. ^{1}H-NMR spectrum (DMSO-d_6, 500 MHz, r.t.) of **Tet^{NO}** (**3**); *H_2O, **DMSO residual signal.

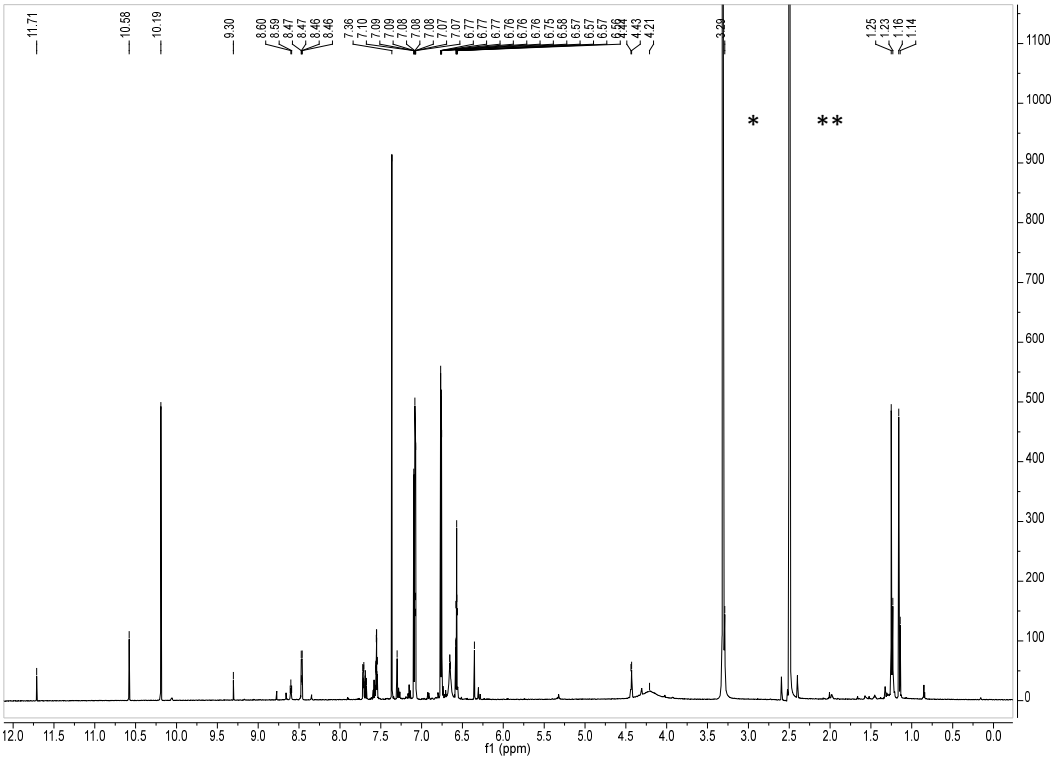

Figure S14. ^{1}H-NMR spectrum (DMSO-d_6, 700 MHz, r.t.) of **Tet^{NO}** (**3**), in situ reduced by phenyl hydrazine; *H_2O, **DMSO residual signal.

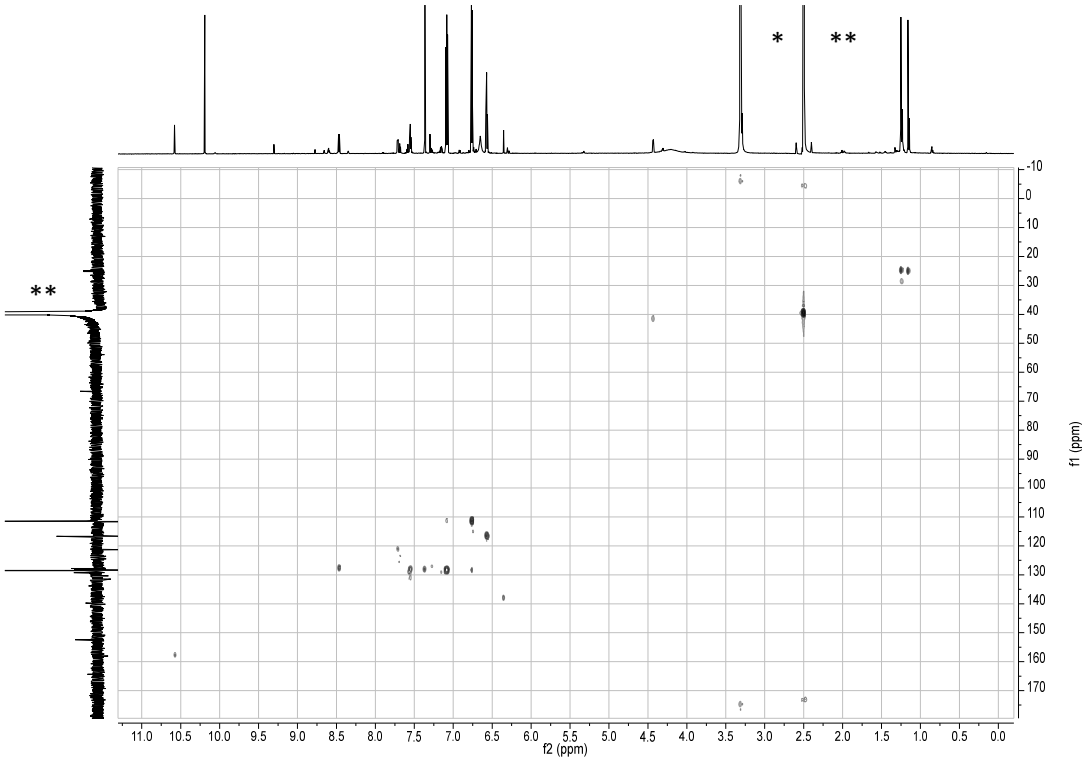

Figure S15. HSQC spectrum (DMSO-d_6, 700/176 MHz, r.t.) of **Tet^{NO}** (**3**), in situ reduced by phenyl hydrazine; *H_2O, **DMSO residual signal.

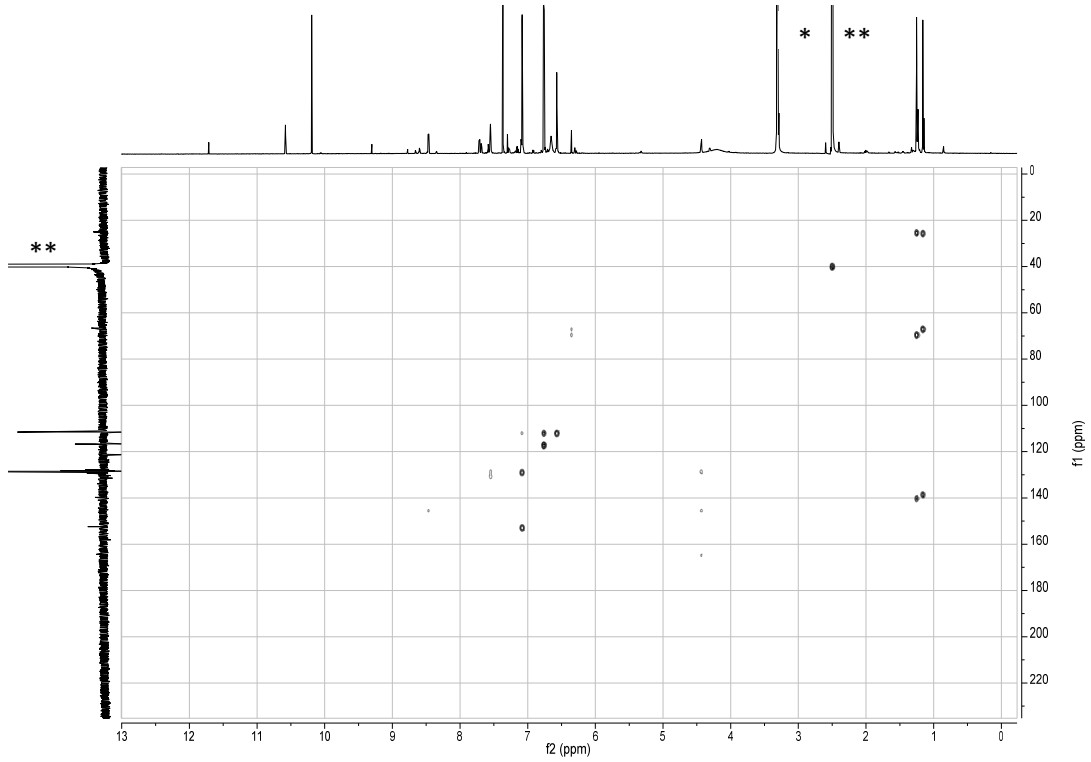

Figure S16. HMBC spectrum (DMSO-d_6, 700/176 MHz, r.t.) of **Tet^{NO}** (**3**), in situ reduced by phenyl hydrazine; *H_2O, **DMSO residual signal.

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a) H. J. C. Berendsen, D. van der Spoel, R. van Drunen, *Computer Physics Commun.,* **1995**, *91*, 43-56.

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