Supporting Information

Posttranscriptional site-directed spin labeling of large RNAs with an unnatural base pair system under non-denaturing conditions

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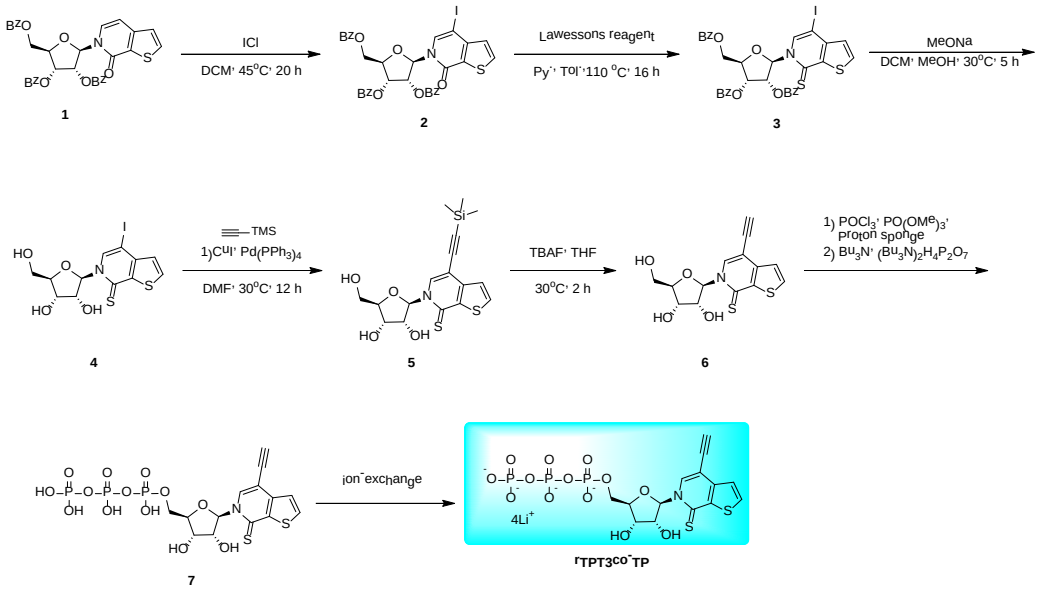
S1: Synthetic procedures and characterizations of rTPT3^{CO}TP

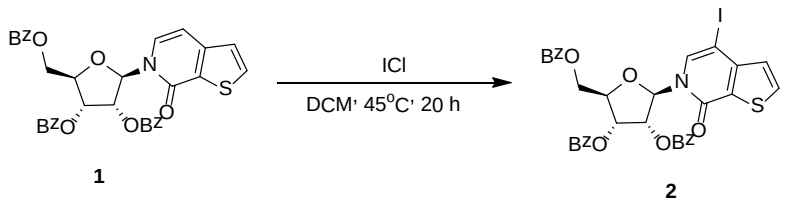
General

All solvents and reagents were purchased commercially and used without further purification. For synthetic procedures all reactions were carried out in oven-dried glassware under an inert atmosphere. Solvents were distilled and/or dried over 4 Å molecular sieves. NMR spectra were recorded on an AVANCE III 1 BAY 400 MHz Bruker NMR spectrometer and the chemical shifts were reported relative to the deuterated NMR solvent used [^{1}H-NMR: CDCl3 (7.26 ppm), DMSO-d6 (2.50 ppm); ^{13}C-NMR: CDCl3 (77.16 ppm), DMSO-d6 (39.52 ppm)]. Mass spectra were recorded on an Agilent 1200 + G6110A.

Synthetic schemes and procedures

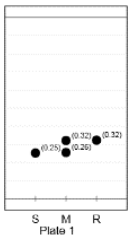


General procedure for preparation of compound 2

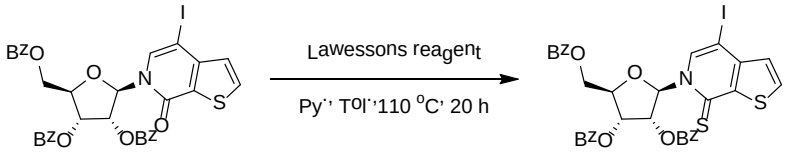


To a solution of compound **1** (50.0 g, 83.9 mmol, 1.00 *eq*) in DCM (300 mL) was added ICl (27.3 g, 167.9 mmol, 8.6 mL, 2.00 *eq*) at 0 °C. The mixture was stirred at 45 °C for 20 hrs under exclusion of light. TLC (petroleum ether/ethyl acetate = 3/1, R_f = 0.32) indicated compound **1** was consumed completely and one new spot formed. The reaction was clean according to TLC. The reaction mixture was quenched by addition $Na_2S_2O_3$ (500 mL) at 2**0** °C, and then diluted with DCM (500 mL x 2). The organic layers were concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether/ethyl acetate = 10/1 to 5/1). Compound **2** (28.0 g, 34.9 mmol, 41.6% yield, 90.0% purity) was obtained as a yellow solid.

TLC: petroleum ether/ethyl acetate = 3/1, R_f = 0.32



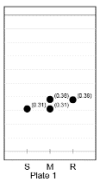
General procedure for preparation of compound 3



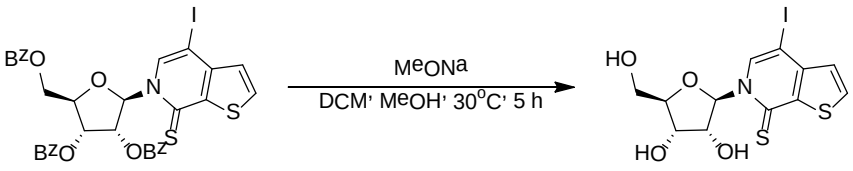
2 **3**

To a solution of compound **2** (43.0 g, 59.6 mmol, 1.00 *eq*) in toluene (300 mL) was added LAWESSON'S REAGENT (36.2 g, 89.4 mmol, 1.50 *eq*) and Py (4.7 g, 59.6 mmol, 4.8 mL, 1.00 *eq*). The mixture was stirred at 110 °C for 20 h. TLC (petroleum ether/ethyl acetate = 3/1, R_f = 0.38) indicated compound **2** was consumed completely and one new spot formed. The reaction was clean according to TLC. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether/ethyl acetate = 10/1 to 3/1). Compound **3** (27.0 g, 32.9 mmol, 55.3% yield, 90.0% purity) was obtained as a brown solid.

TLC: petroleum ether/ethyl acetate = 3/1, R_f = 0.38



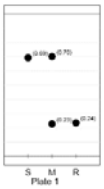
General procedure for preparation of compound 4



3 **4**

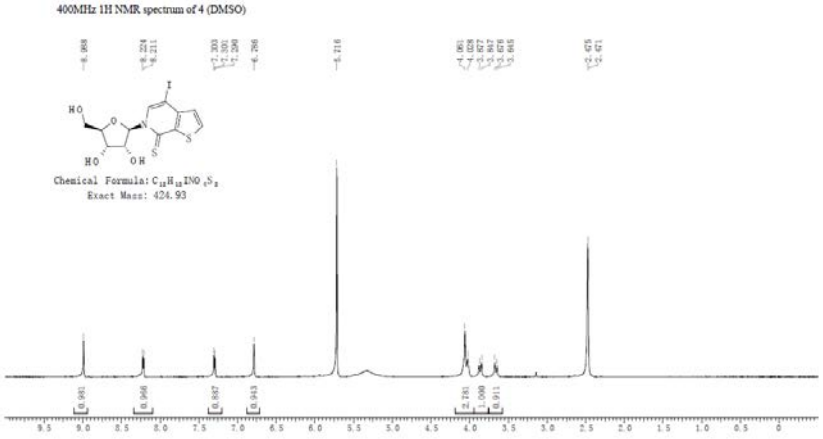
To a solution of compound **3** (27.0 g, 36.6 mmol, 1.00 *eq*) in MeOH (200 mL) and DCM (200 mL) was added NaOMe (988.8 mg, 18.30 mmol, 0.50 *eq*). The mixture was stirred at 30 °C for 5 h. TLC (dichloromethane/methanol = 10/1, R_f = 0.24) indicated compound **3** was consumed completely and one new spot formed. The reaction was clean according to TLC. The reaction mixture was concentrated under reduced pressure to give a residue. The crude product was triturated with DCM at 20 °C for 20 min, filtered and get the filter cake. Compound **4** (10 g, 21.16 mmol, 57.81% yield, 90.0% purity) was obtained as a yellow solid.

TLC: dichloromethane/methanol = 10/1, R_f = 0.24

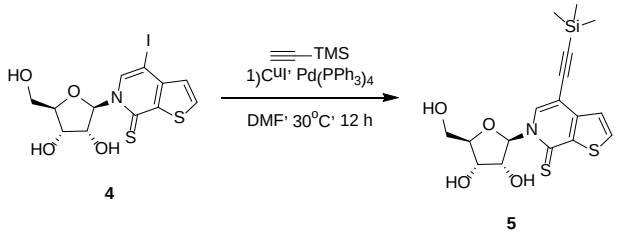


^{1}H NMR: (400 MHz DMSO)

δ ppm 8.99 (s, 1H), 8.22 (d, *J* = 5.2 Hz, 1H), 7.21-7.39 (m, 1H), 6.79 (s, 1H), 3.97-4.17 (m, 3H), 3.86 (d, *J* = 11.8 Hz, 1H), 3.66 (d, *J* = 12.2 Hz, 1H).



General procedure for preparation of compound 5

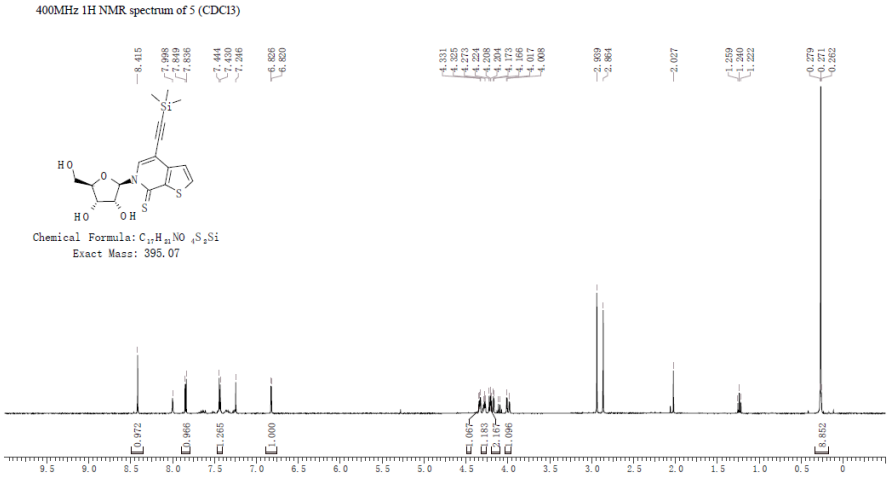


To a solution of compound **4** (6.4 g, 15.1 mmol, 1.00 *eq*) in DMF (50 mL) was added $Pd(PPh_3)_4$ (1.7 g, 1.50 mmol, 0.10 *eq*) and CuI (573.2 mg, 3.0 mmol, 0.20 *eq*) and TEA (2.3 g, 22.6 mmol, 3.1 mL, 1.50 *eq*), then added ethynyl (trimethyl) silane (2.2 g, 22.6 mmol, 3.1 mL, 1.50 *eq*). The mixture was stirred at 30 °C for 12 h. TLC (dichloromethane/methanol = 10/1, R_f = 0.46) indicated compound **4** was consumed completely and one new spot formed. The reaction was clean according to TLC. The reaction mixture was concentrated under reduced pressure to give a residue. The crude product black oil compound **5** (5.5 g, 11.82 mmol, 78.5% yield, 85.0% purity) was used into the next step without further purification.

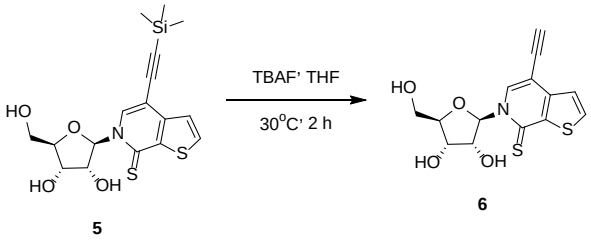
TLC: dichloromethane/methanol = 10/1, R_f = 0.46

^{1}H NMR: (400 MHz $CDCl_3$)

δ ppm 8.42 (s, 1H), 7.84 (d, *J* = 5.2 Hz, 1H), 7.44 (d, *J* = 5.8 Hz, 1H), 6.82 (d, *J* = 2.2 Hz, 1H), 4.34 (dd, *J* = 6.0, 2.4 Hz, 1H), 4.25-4.31 (m, 1H), 4.15-4.24 (m, 2H), 4.00 (dd, *J* = 12.2, 3.6 Hz, 1H), 0.27 (s, 9H).



General procedure for preparation of compound 6

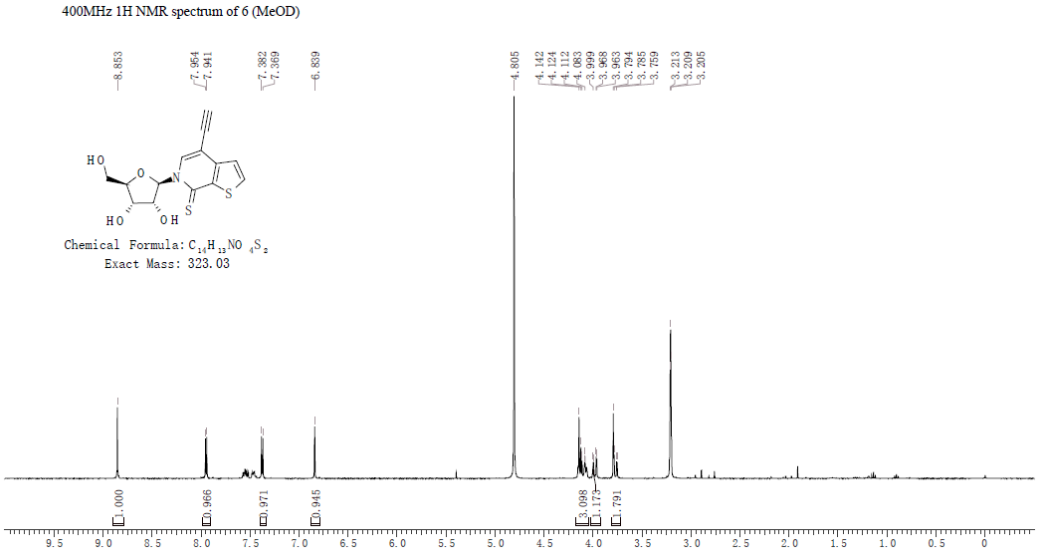


To a solution of compound **5** (6.5 g, 16.4 mmol, 1.00 *eq*) in THF (60 mL) was added TBAF (8.6 g, 32.9 mmol, 2.00 *eq*). The mixture was stirred at 30 °C for 2 h. TLC (petroleum ether/ethyl acetate = 3/1, R_f = 0.44) indicated compound **5** was consumed completely and one new spot formed. The reaction was clean according to TLC. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether/ethyl acetate = 3/1 to DCM/MeOH = 20/1). Compound **6** (5.0 g, 13.9 mmol, 84.7% yield, 90.0% purity) was obtained as a yellow solid.

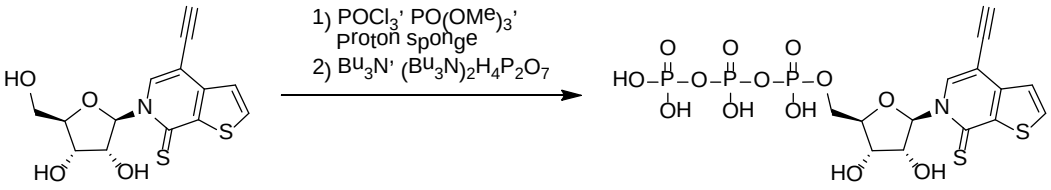
TLC: petroleum ether/ethyl acetate = 3/1, R_f = 0.44

^{1}H NMR: (400 MHz MeOD)

δ ppm 8.85 (s, 1H), 7.95 (d, *J* = 5.4 Hz, 1H), 7.38 (d, *J* = 5.4 Hz, 1H), 6.84 (s, 1H), 4.04-4.20 (m, 3H), 3.98 (dd, *J* = 12.4, 2.0 Hz, 1H), 3.69-3.83 (m, 2H).



General procedure for preparation of compound 7



6 **7**

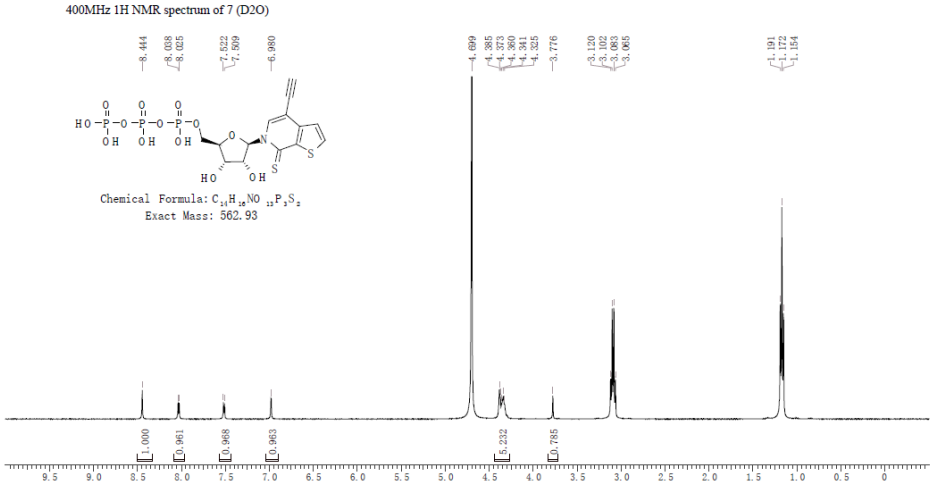
To a solution of compound **6** (1.0 g, 3.1 mmol, 1.00 *eq*) and proton sponge (662.7 mg, 3.1 mmol, 1.00 *eq*) in $PO(OMe)_3$ (10 mL) was added $POCl_3$ (616.4 mg, 4.02 mmol, 373.6 uL, 1.30 *eq*) at 0 °C. The mixture was stirred at 25 °C for 1 hr. LC-MS (product: RT = 1.045 min) showed compound **6** was consumed completely and one main peak with desired m/z was detected. The crude product monophosphate (1.36 g, crude) with yellow colour was used into the next step without further purification.

To a solution of monophosphate (1.36 g, 3.09 mmol, 1.00 *eq*) in $PO(OMe)_3$ (10 mL) was added N, N- dibutylbutan-1-amine; phosphono dihydrogen phosphate (8.5 g, 15.4 mmol, 5.00 *eq*) and N, N-dibutylbutan-1-amine (3.4 g, 18.5 mmol, 4.4 mL, 6.00 *eq*). The mixture was stirred at 0 °C for 1 hr. LC-MS (product: RT = 0.791 min) showed monophosphate was consumed completely and one peak with desired m/z was detected. Added 1M TEAB adjust pH 7, the crude product was combined together for purification. The residue was purified by prep-HPLC (neutral condition;

column: Agela DuraShell C18 250 x 50 mm x 10 μm; mobile phase: [water (10 mM NH_4HCO_3)-ACN]; B%: 0%-20%, 21min). Compound **7** (1.1 g, 1.86 mmol, 60.0% yield, 95.0% purity) was obtained as a yellow solid.

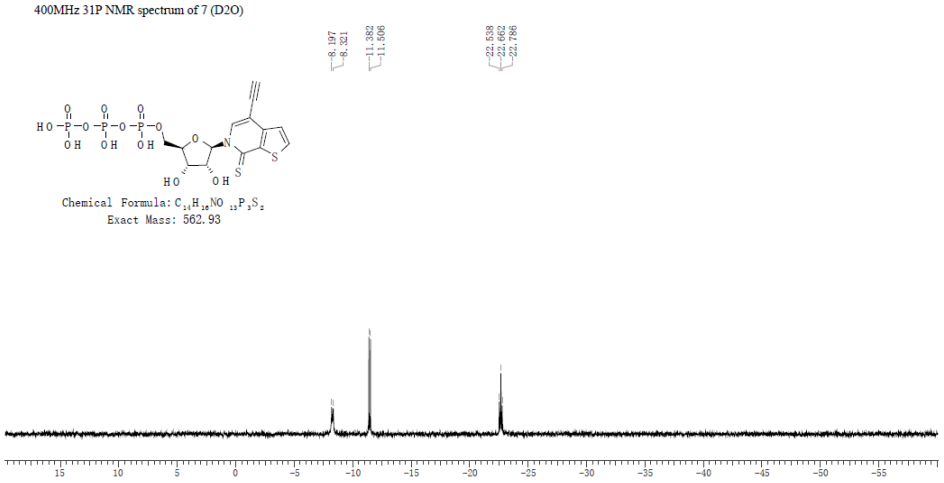
^{1}H NMR: (400 MHz D_2O)

δ ppm 8.44 (s, 1H), 8.03 (d, *J* = 5.2 Hz, 1H), 7.52 (d, *J* = 5.4 Hz, 1H), 6.98 (s, 1H), 4.25-4.45 (m, 5H), 3.78 (s, 1H)

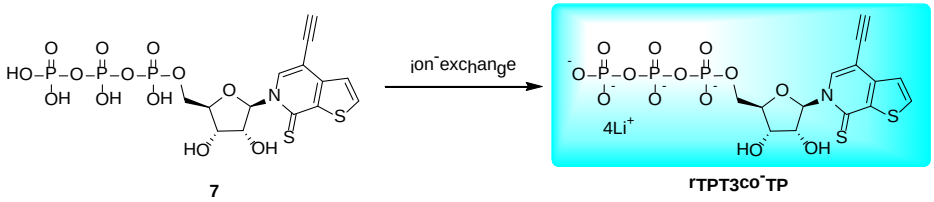


^{31}P NMR: (400 MHz D_2O)

δ ppm -8.26 (d, *J* = 20.0 Hz, 1P), -11.44 (d, *J* = 20.0 Hz, 1P), -22.66 (t, *J* = 20.0 Hz, 1P)



General procedure for preparation of rTPT3^{CO}TP

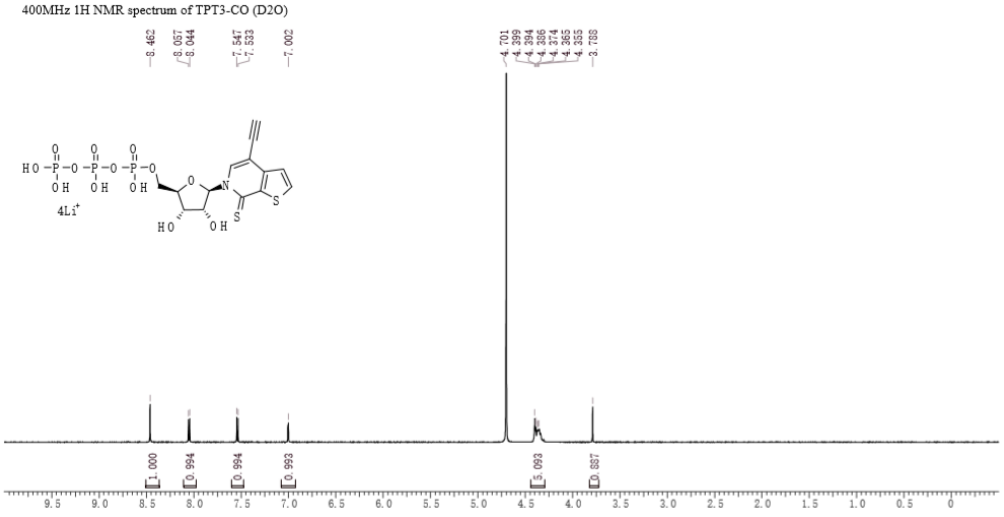


A mixture of compound **7** (0.8 g, 1.4 mmol, 1.00 *eq*) in H_2O (10 mL), then through Li^+ resin at 25°C. The target compound was showed spot at TLC, when the spot disappears, stop added water.

Then lyophilized the water, **rTPT3^{CO}TP** (0.4, 642.8 umol, 45.2% yield, 95.0% purity, 4Li^{+}) was obtained as a yellow solid.

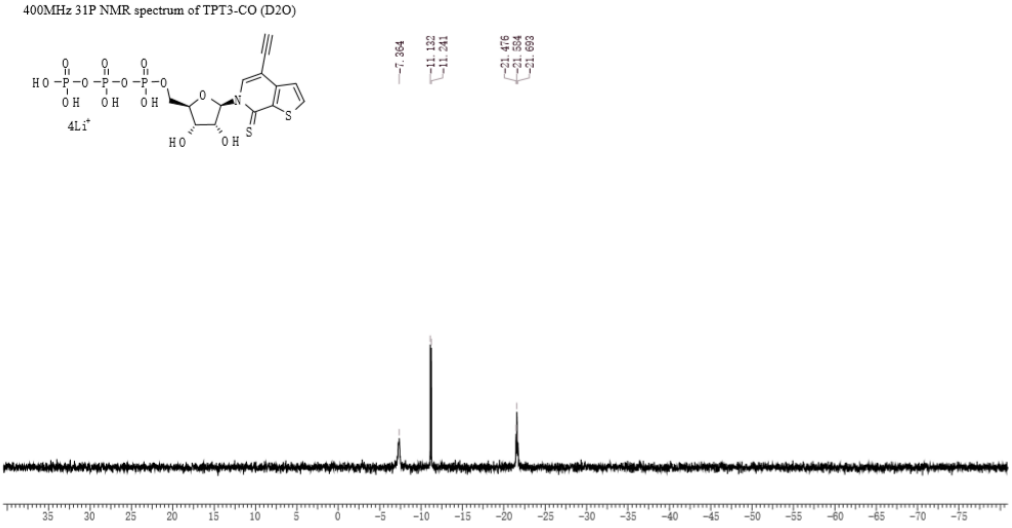
^{1}H NMR: (400 MHz D_2O)

δ ppm 8.46 (s, 1H), 8.05 (d, *J* = 5.4 Hz, 1H), 7.54 (d, *J* = 5.4 Hz, 1H), 7.00 (d, *J* = 1.50 Hz, 1H), 4.22-4.47 (m, 5H), 3.79 (s, 1H).



^{31}P NMR: (400 MHz D_2O)

δ ppm -7.36 (m, 1P), -11.19 (d, *J* = 17.6 Hz, 1P), -22.42--21.04 (m, 1P)



S2: Selection of labeling sites

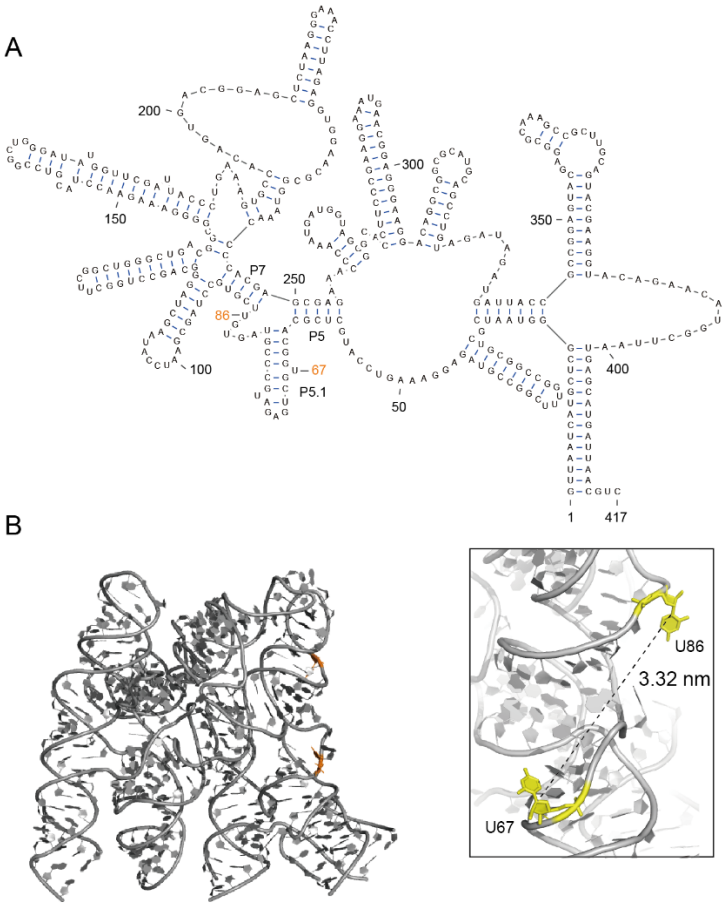


Fig. S1. Structure of the RNase P RNA from *Bacillus stearothermophilus*. (**A**) The spin labeling sites of U67 and U86 are indicated on secondary structure of RNase P. (**B**) The spin labeling sites of U67 and U86 are indicated on crystal structure of RNase P (left), the distance between the N1 atoms is measured as 3.32 nm (right).

S3: Non-denaturing purification of TPT3^{CO}-modified and spin-labeled RNase P RNAs

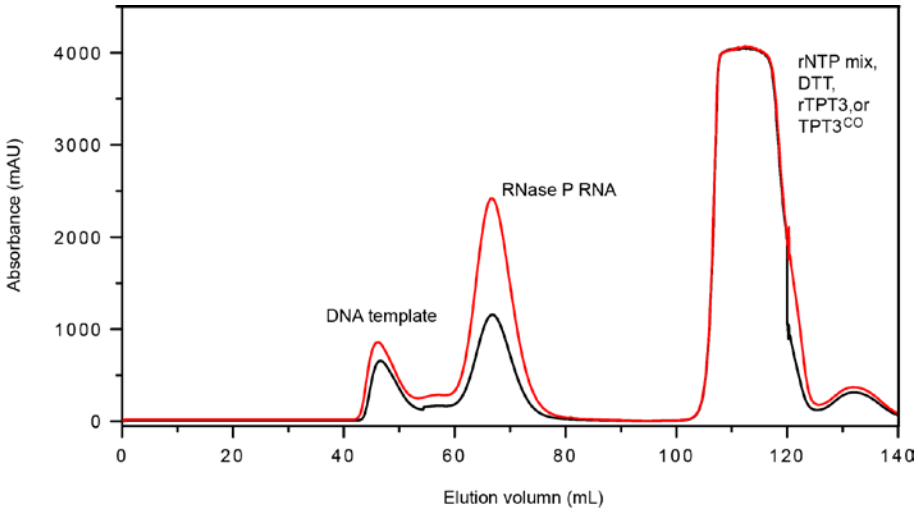


Fig. S2. Non-denaturing purification of the RNA transcripts of the doubly TPT3^{CO}-modified RNase P RNA by Size-Exclusion Chromatography. The DNA template, RNase P RNA as well as excess rNTPs, rTPT3 or rTPT3^{CO}, and residual DTT are well separated. Note that while the experimental approach reported in this work allows successful non-denaturing purification of functional RNase P RNA free from interference of "aborts" or "miss folding" transcripts as well as the DNA template, for other RNAs a suitable non-denaturing purification approach needs to be explored case-by case. As demonstrated in X-ray crystallography and NMR studies, the RNA constructs should be optimized (such as deletion or addition of sequences, alteration of 5'- and/or 3'-terminus) to overcome the aborts or misfolding.

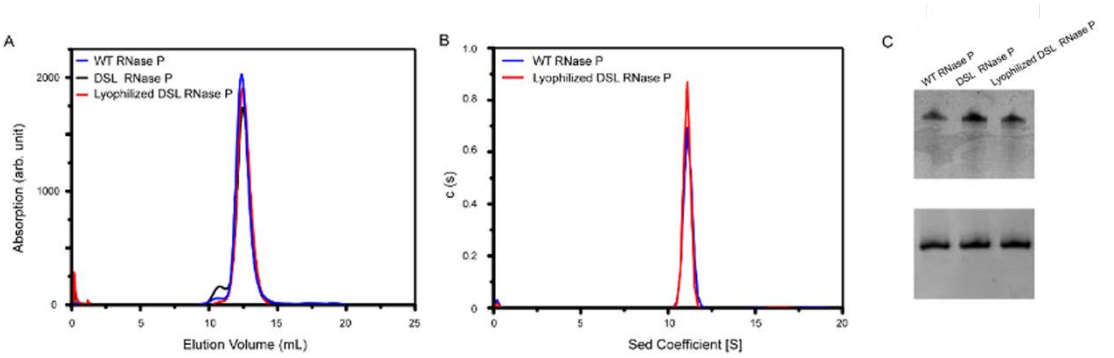


Fig. S3. Assessing impacts of lyophilization on RNase P samples. (A) Elution profiles of wild type, double spin-labeled (DSL) and lyophilized DSL RNase P RNAs through the Superose 6 Increase 10/300 GL column. (B) SV-AUC profiles of wild type and lyophilized DSL RNase P samples. Each sample gives a profile with a sharp peak with Stokes radius around 4.9 nm. (C) Denaturing (top) and native (bottom) PAGE gels of purified wild type and lyophilized DSL RNase P samples, both show no obvious differences in RNA migration. Collectively, these biochemical and biophysical characterization suggest lyophilization minimally effects the global folding of DSL RNase P RNA in this work.

S4: Additional CD data

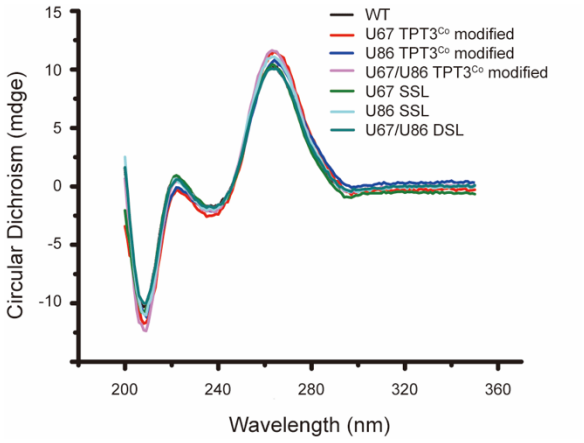


Figure S4. CD spectra of wild type, single- (U67, U86) and double- (U67/86) TPT3^{CO} modified, and single- or double- spin labeled RNase P RNAs, which exhibit very similar spectral features.

S5: Spin counting and assessment of nitroxide labeling efficiency

The nitroxide labeling effeciency is the ratio of the nitroxide concentration to that of the RNA for a given sample. To determine nitroxide concentration, CW-EPR spectra of Az-TMIO with varying concentrations were measured. The 2nd integral values [I_{2nd} (NOX)] of these spectra were computed, and were plotted against the Az-TMIO concentrations to generate a standard calibration curve (Fig. S5, Table S5†). From this plot, a linear fit yielded:

$$I_{2nd}\,(NOX) = 901187 \times [\text{Az-TMIO}] + 3 \times 10^7 \qquad \text{(eq. S1)}$$

In parallel, CW-EPR spectra were obtained with each nitroxide-labeled RNA under the same experimental setting as that of the Az-TMIO, and the corresponding 2nd integral values of the spectrum without normalization, I_{2nd}(sample), were computed. The nitroxide concentration in each sample, [NOX], was computed according to:

$$[NOX] = [I_{2nd}(\text{sample}) - 3 \times 10^7] / 901187 \qquad \text{(eq. S2)}$$

In addition, the RNA concentration [RNA] for each sample was determined from UV-Vis measurements, and the labeling efficiency for singly labeled RNA was computed as:

$$\%\text{label} = [NOX] / [RNA] \qquad \text{(eq. S3)}$$

For the doubly labeled RNA, the labeling efficiency was computed as:

$$\%\text{label} = ([NOX]/2)/[RNA] \qquad \text{(eq. S4)}$$

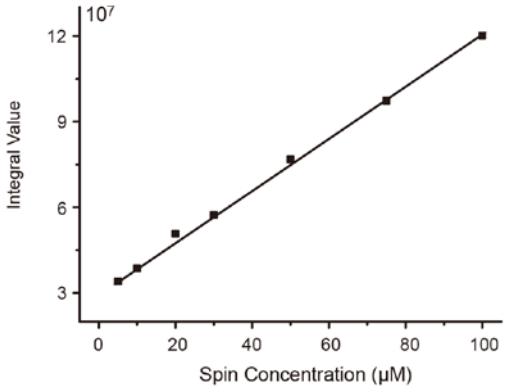


Fig. S5. Calibration curve for spin counting.

S6: Assessment of the specificity of nitroxide labeling

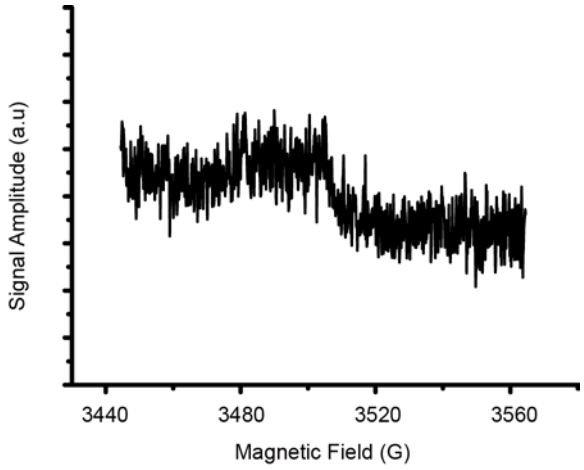


Fig. S6. A representative CW-EPR spectrum measured on a wild type RNase P RNA subjected to the nitroxide labeling and purification procedure. The RNA does not contain the $TPT3^{CO}$ modification, and no nitroxide signal was detected. This indicates that the reported labeling procedure specifically directs the nitroxide to the site of $TPT3^{CO}$ incorporation.

S7: Additional EPR data

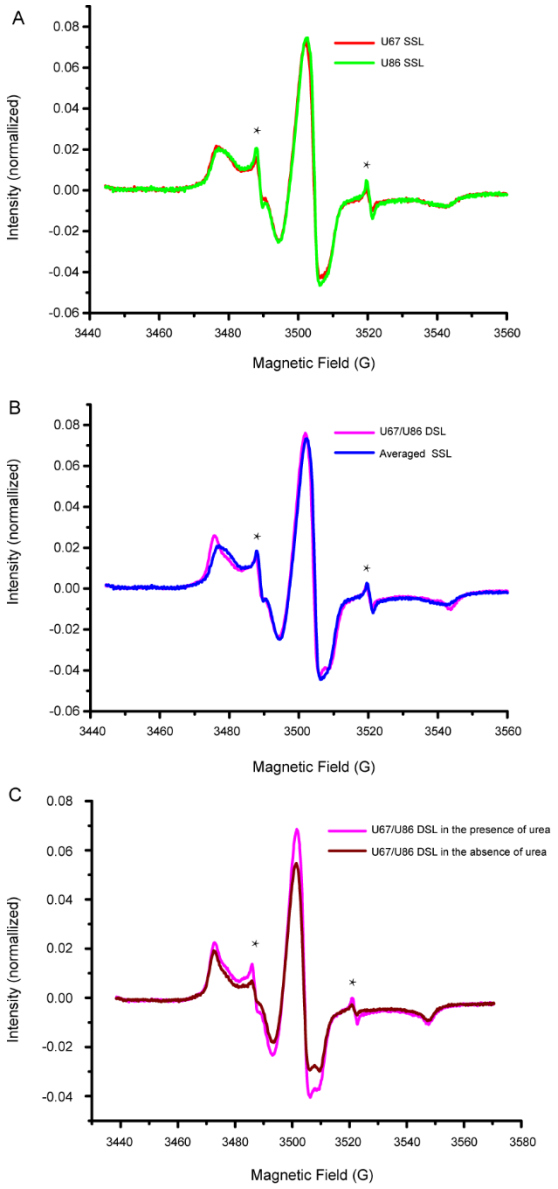


Fig. S7. (A) An overlay of the normalized, uncorrected CW-EPR spectra of the U67 singly spin-labeled (SSL) (red) and the U86 SSL (green) RNase P RNAs. (B) An overlay of the normalized, uncorrected CW-EPR spectrum of the U67/U86 doubly spin-labeled (DSL) (magenta) on that of the averaged spectrum (blue) of the U67- and U86- SSL RNase P RNAs. (C) Comparison of the original CW-EPR spectra of U67/U86 DSL RNase P RNAs labeled via click reaction in the presence (magenta) or absence (wine) of urea (0.5 M). As shown the two spectra were obtained using the same spectral acquisition parameters but not normalized to their respective number of spins. While the lineshapes are very similar, labeling in the absence of 0.5M urea gave a labeling efficiency of 70%, which is 15% lower than that obtained from labeling in the presence of 0.5 M urea (Table S5). In all three panels, "*" signals arise from trace amount of free nitroxide.

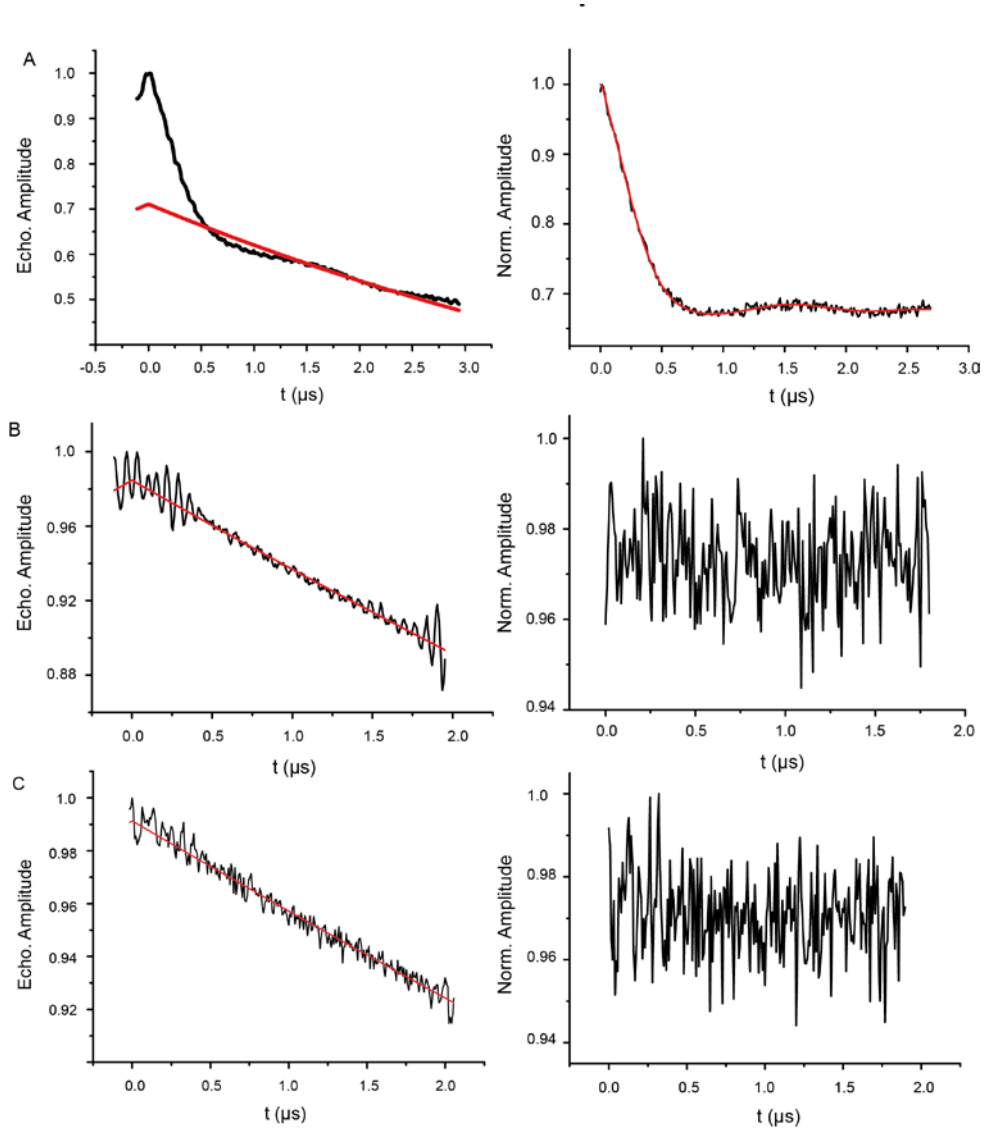


Fig. S8. Additional PELDOR data. (A) PELDOR data for the U67/86 double-labeled RNase P RNA. Shown on the left is the original dipolar evolution trace (black) overlaid with the simulated background decay (red). Shown on the right the measured background-corrected dipolar evolution curve (black) overlaid with the fit trace. (B) PELDOR data for the U67 single-labeled RNA. Shown on the left is the original dipolar evolution trace (black) overlaid with the simulated background decay (red). Shown on the right is the background-corrected dipolar evolution trace. No decay was observed, indicating a complete lack of dipolar coupling between the spin labels. (C) PELDOR data for the U86 single-labeled RNA. Shown on the left is the original dipolar evolution trace (black) overlaid with the simulated background decay (red). Shown on the right is the background-corrected dipolar evolution trace. No decay was observed, indicating a complete lack of dipolar coupling between the spin labels.

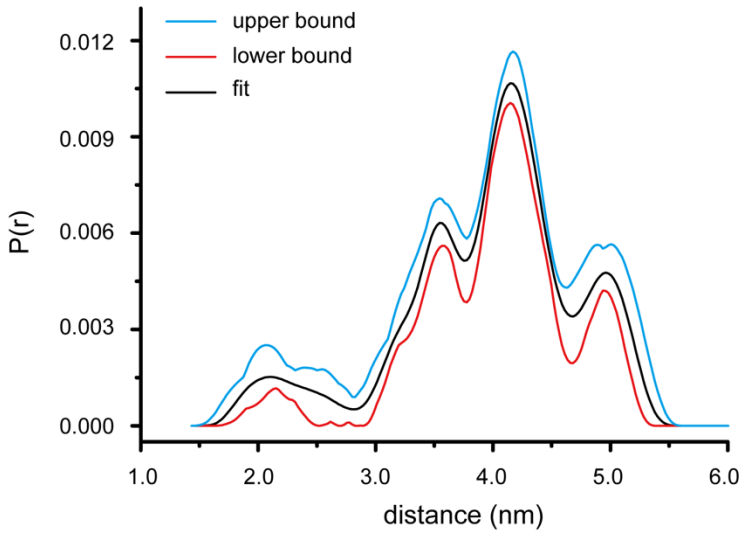


Fig. S9: Uncertainty in the distance distribution of the U67/86 double-labeled RNase P RNA. As shown data were obtained with the data set of 3-µs using DeerAnalysis2013.

S8: The primary sequences of plasmid DNAs, native and unnatural primers

Table S1. The DNA sequences of the total gene synthesized plasmids coding for full-length RNase P RNA from *Bacillus stearothermophilus* and pre-tRNA^{Phe} from yeast.

Plasmids	Primary DNA sequence
RNase P	AAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAA CCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTTGTAAAACGA CGGCCAGTCGAACCACGCAATGCGTCTCGATCCGCAGTGTCTTGCGTCTCTGGTAATACGACTCA CTATAggGTTAATCATGCTCGGGTAATCGCTGCGGCCGGTTTCGGCCGTAGAGGAAAGTCCATGCT CGCACGGTGCTGAGATGCCCGTAGTGTTCGTGCCTAGCGAATCCATAAGCTAGGGCAGCCTGGCT TCGGCTGGGCTGACGGCGGGGAAAGAACCTACGTCCGGCTGGGATATGGTTCGATTACCCTGAA AGTGCCACAGTGACGGAGCTCTAAGGGAAACCTTAGAGGTGGAACGCGGTAAACCCCACGAGC GAGAAACCCAAATGATGGTAGGGGCACCTTCCCGAAGGAAATGAACGGAGGGAAGGACAGGCG GCGCATGCAGCCTGTAGATAGATGATTACCGCCGGAGTACGAGGCGCAAAGCCGCTTGCAGTAC GAAGGTACAGAACATGGCTTATAGAGCATGATTAACGTCAGAGACGGAGTCACTGCCAACCGAG ACGGTCATAGCTGTTTCCTGTGTGCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGG CTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTACCCACAGAATCAGGGGATA ACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG TTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCA GAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTG CGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGT GGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGG GCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAG TCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAG CGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAG AACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTT GATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCG CAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAC GAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTA AATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAA TGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTCTTTCGTTCATCCATAGTTGCCTGACTC CCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATAATACC GCGGGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAG CGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAG AGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATCGCTACAGGCATCGTGGTATC ACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGAT CCCCCATGTTGCGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG GCCGCCGTGTTATCACTCATGGTTATGGCAGCACTACATAATTCTCTTACTGTCATGCCATCCGTAA GATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCG AGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCT CATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTT CGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGT GAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGA ATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATA CATATTTGAATGTATTTAG
Yeast pre-tRNA^{Phe}	AAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAA CCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTTGTAAAACGA CGGCCAGTCCGTCTCTATCCGGTCTCGATCCGCAGTCTCTTGGCACAGGTAATACGACTCACTATA GGAATAGAAGCGGATTTAGCTCGATTGGGAGAGCGCCAGACTGAAGATCTGGAGGTCCTGTGTT CGATCCACAGAATTCGCTTTCATTTTAACCGTGCGAGTTACTGCCAACCGAGACCCAACCGAGAC GGGTCATAGCTGTTTCCAGTGTGCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGC TGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTACCCACAGAATCAGGGGATAA CGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGT TGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAG AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGC GCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTG GCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGG CTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGT CCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGC GAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGG ACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTG ATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGC AGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACG AAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAA ATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAAT GCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCC CCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATAATACCG CGGGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGC GCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGA GTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATCGCTACAGGCATCGTGGTGTC

	ACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGAT CCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG GCCGCCGTGTTATCACTCATGGTTATGGCAGCACTACATAATTCTCTTACTGTCATGCCATCCGTAA GATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCG AGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCT CATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTT CGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGT GAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGA ATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATA CATATTTGAATGTATTTAG

[a]: The DNA sequences coding for RNase P (419 nts) and yeast pre tRNA^{Phe} (91 nts) RNAs are colored in red;
[b]: The T7 promoter is colored in green;
[c]: The common upstream sequence targeted by pMVF primer is colored in blue.

Table S2. The native and UBP-modified DNA primers used in this study.

Primers	Sequence	Application
pMVF	5'- CATAATTCTCTTACTGTCATGCCATC-3'	Common forward primer
RPR	5'- GACGTTAATCATGCTCTATAAGCC -3'	Reverse primer for RNase P
67/86F	5'- GTCCATGCTCGCACGG (dTPT3)GCTGAGA TGCCCGTAG (dTPT3) GTTCGTGCCTAG -3'	Forward primer for U_{67}/ U_{86} NaM modification
67/86R	5'-GATTCGCTAGGCACGAAC(dNaM) CTACGG GCATCTCAGC (dNaM) CC-3'	Reverse primer for U_{67}/ U_{86} NaM modification
67F	5'- GTCCATGCTCGCACGG (dTPT3) GCTGAGA TGCCCGTAGTG -3'	Forward primer for U_{67} NaM modification
67R	5'- GGCACGAACACTACGGGCATCTCAGC (dNaM) CCGTGCGAG-3'	Reverse primer for U_{67} NaM modification
86F	5'-GCTGAGATGCCCGTAG (dTPT3) GTTCGTGC CTAG-3'	Forward primer for U_{86} NaM modification
86R	5'-CTAGGCACGAAC (dNaM) CTACGGGCATCT CAGCACCGTG-3'	Reverse primer for U_{86} NaM modification
tRNAR	5'-TAAAATGAAAGCGAATTCTGTGGATCG-3'	Reverse primer for yeast pre-$tRNA^{Phe}$ R

S9: Reaction conditions for PCR and *in vitro* transcription

Table S3. Conditions for overlap extension PCR amplification of the UBP modified DNA templates.

Coding RNA	U_{67}-single labeled		U_{86}-single labeled		U_{67}/U_{86}-double labeled	
Fragments	[a]F1	[a]F2	[b]F1	[b]F2	[c]F1	[c]F2
2×PCR mix	25 μl					
Template	RNase P plasmid (4 ng/μL)					
Fprimer	pMVF(0.8M)	67F(0.8μM)	pMVF(0.8μM)	86R(0.8μM)	pMVF(1μM)	67/86F(1μM)
Rprimer	67R(0.8μM)	RPR(0.8μM)	86R(0.8μM)	RPR(0.8μM)	67/86R(1μM)	RPR(1μM)
dTPT3TP	1mM	/	1mM	/	1.4mM	/
dNaMTP	/	1 mM	/	1 mM	/	1.4 mM
ddH2O	To 50 μL					
2×PCR mix	25 μl					
Template	[a]F1+[a]F2 (1:1)		[b]F1+[b]F2 (1:1)		[c]F1+[c]F2 (1:1)	
Fprimer	pMVF(1μM)					
Rprimer	RPR(1μM)					
dTPT3TP	1.4 mM					
dNaMTP	1.4 mM					
ddH2O	To 50 μL					

[a]: DNA fragments for U_{67}-single labeled RNase P;
[b]: DNA fragments for U_{86}-single labeled RNase P;
[c]: DNA fragments for U_{67}/U_{86}-double labeled RNase P;
[d]: Conditions for the first step of overlap extension PCR are colored in light blue;
[e]: Conditions for the second step of overlap extension PCR are colored in light green;

Table S4. Typical conditions for *in vitro* transcription of UBP modified RNase P.

RNA	U_{67} modified	U_{86} modified	U_{67}/U_{86} modified
10 ×Transcription buffer	10 μL		
$MgCl_2$	30 mM		
T7 RNA polymerase	200 ng/μL		
UBP-modified DNA template	4 μM	4 μM	6 μM
rNTP mix	4 mM each		
rTPT3 or $rTPT3^{Co}$	0.8 mM		1 mM
DTT	10 mM		
DEPC-H_2O	To 50 μL		

S10: Data for determination of calibration curve and spin labeling efficiencies

Table S5. Data used for spin counting and determination of RNA labeling efficiency.

Sample	I_{2nd}(sample)	[NOX] (μM)	[RNA] (μM)	%label
Az-TMIO [(a)] (free nitroxide)	34072022	5	N.A	N.A
	38660181	10		
	50789134	20		
	57035610	30		
	76884429	50		
	97589025	75		
	120117208	100		
U67 SSL	117415139	97 [(b)]	116	84[(c,e)]
U86 SSL	77762911	53 [(b)]	65	82[(c,e)]
U67/86 DSL	114711578	94 [(b)]	55	85[(d,e)]
U67/86 DSL without urea	98874603	76 [(b)]	54	70[(d,f)]

(a) Data for generating the calibration curve shown in Fig. S5.
(b) Computed according to eq. S2.
(c) Efficiency for single-labeling computed as %label=[NOX]/[RNA] (i.e., eq. S3).
(d) Efficiency for double-labeling with the average efficiency at each labeling site computed as %label=([NOX]/2)/[RNA] (i.e., eq. S4).
(e) Labeling carried out in the presence of 0.5 M urea. These are higher that the 44% -- 76% values reported in the recent work of Dominck and co-workers (*Angew Chem Int Ed Engl*, 2020, **59**, 7891-7896).
(f) Labeling carried out in the absence of urea.

S11: SAXS-related parameters

Table S6. SAXS data collection parameters and software employed for data analysis.

Data Collection Parameters	
Facilities and parameters	Settings and values
Beam line	12ID-B (APS, ANL)
Wavelength (Å)	0.8857
Detector	Pilatus 1M (SAXS)
q range ($Å^{-1}$)	0.005-0.89
Exposure time (s)	30-60
Concentration range (mg/ml)	0.50-2
Temperature (K)	298
Software Employed	
Primary Data Processing	Matlab/PRIMUS
P(r) Function	GNOM

Table S7. Overall structural parameters for native and double spin-labeled (DSL) RNase P RNAs.

Sample	I_0[a]	Rg[a] (Å)	I_0[b]	R_g[b] (Å)	D_{max} (Å)	MW[c] (kDa)	MW[d] (kDa)	Oligomerization
RNase P	1.18±0.05	49.51±0.26	1.18±0.05	50.08±0.36	181	137.6	135.6	monomer
U67/86 DSL	1.14±0.04	49.15±0.18	1.15±0.06	50.13±0.44	180	138.2	137.8	monomer

[a]Derived from Guinier fitting; [b]derived from GNOM analysis; [c]MW: molecular weight predicted from sequences; [d]MW: molecular weight calculated based on the power law of volume of correlation.