Posttranscriptional Site-Directed Spin Labeling of Large RNAs with an Unnatural Base Pair System Under Non-Denaturing Conditions

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Submitted date: 01/04/2020 • Posted date: 06/04/2020
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Citation information: Wang, Yan; Kathiresan, Venkatesan; Chen, Yaoyi; Hu, Yanping; Jiang, Wei; Bai, Guangcan; et al. (2020): Posttranscriptional Site-Directed Spin Labeling of Large RNAs with an Unnatural Base Pair System Under Non-Denaturing Conditions. ChemRxiv. Preprint.
https://doi.org/10.26434/chemrxiv.12058914.v1

Site-directed spin labeling (SDSL) of large RNAs for electron paramagnetic resonance (EPR) spectroscopy remains challenging up-to-date. We here demonstrate an efficient and generally applicable posttranscriptional SDSL method for large RNAs under non-denaturing conditions using an expanded genetic alphabet containing the NaM-TPT3 unnatural base pair (UBP). An alkyne-modified TPT3 ribonucleotide triphosphate (rTPT3\textsuperscript{CO-TP}) is synthesized and site-specifically incorporated into large RNAs by in vitro transcription, which allows attachment of the azide-containing nitrooxide through click chemistry. We validate this strategy using a 419-nucleotide Ribonuclease P (RNase P) RNA from Bacillus stearothermophilus. The effects of site-directed UBP incorporation and subsequent spin labeling to global structure and function of RNase P are marginal as evaluated by Circular Dichroism spectroscopy, Small Angle X-ray Scattering, and enzymatic assay. Continuous-wave EPR analyses reveal that the labeling reaction is efficient and specific, and Pulsed Electron-Electron Double Resonance measurements yield an inter-spin distance distribution that agrees well with the crystal structure. Thus, the labeling strategy as presented overcomes the size constraint of RNA labeling, opening new possibilities for application of EPR spectroscopy in investigating structure and dynamics of large RNA.

File list (2)

- UBP-EPR-ChemRxiv-0403.pdf (0.98 MiB)
- SI-UBP-EPR-ChemRxiv-0403.pdf (1.97 MiB)
Supporting Information

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

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Table of Content

Synthetic procedures and characterizations of rTPT3^COTP ..........................................................3

Supporting Figures

Selection of labeling sites .......................................................................................................................10
   Figure S1
Native purification of TPT3CO-modified and spin-labeled RNase P RNAs .................................11
   Figure S2, S3
Spin counting and assessment of nitroxide labeling efficiency ......................................................12
   Figure S4
Assessment of the specificity of nitroxide labeling ........................................................................13
   Figure S5
Additonal EPR data ..........................................................................................................................14
   Figure S6
   Figure S7

Supporting Tables

The primary sequences of plasmid DNAs, native and unnatural primers ........................................16
   Table S1, S2
Reaction conditions for PCR and in vitro transcription .................................................................18
   Table S3, S4
SAXS-related parameters .................................................................................................................19
   Table S5, S6
Data for determination of calibration curve and spin labeling efficiencies ....................................20
   Table S7
Synthetic procedures and characterizations of rTPT3-COTP

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 [\textsuperscript{1}H-NMR: CDCl\textsubscript{3} (7.26 ppm), DMSO-
\textsubscript{d6} (2.50 ppm); \textsuperscript{13}C-NMR: CDCl\textsubscript{3} (77.16 ppm), DMSO-
\textsubscript{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 mixture was quenched by addition Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} (500 mL) at 20 °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\textsubscript{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

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

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, \( R_f = 0.24 \)

**\(^1H\) NMR:** (400 MHz DMSO)

\( \delta \) 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\(_3\))
\(\delta\) 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)

$\delta$ 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

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 NH₄HCO₃)-ACN]; B%: 0%-20%, 21 min. Compound 7 (1.1 g, 1.86 mmol, 60.0% yield, 95.0% purity) was obtained as a yellow solid.

**¹H NMR:** (400 MHz D₂O)

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)

**³¹P NMR:** (400 MHz D₂O)

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 rTPT3COTP

A mixture of compound 7 (0.8 g, 1.4 mmol, 1.00 eq) in H₂O (10 mL), then through Li⁺ resin at 25°C. The target compound showed spot at TLC, when the spot disappears, stop added water.
Then lyophilized the water, rTPT3\textsuperscript{CO}TP (0.4, 642.8 umol, 45.2% yield, 95.0% purity, 4Li\textsuperscript{+}) was obtained as a yellow solid.

\textbf{\textsuperscript{1}H NMR:} (400 MHz D\textsubscript{2}O)

\[ \delta \text{ ppm } 8.46 \text{ (s, 1H), 8.05 (d, } J = 5.4 \text{ Hz, 1H), 7.54 (d, } J = 5.4 \text{ Hz, 1H), 7.00 (d, } J = 1.50 \text{ Hz, 1H), 4.22-4.47 (m, 5H), 3.79 (s, 1H).} \]

\textbf{\textsuperscript{31}P NMR:} (400 MHz D\textsubscript{2}O)

\[ \delta \text{ ppm } -7.36 \text{ (m, 1P), -11.19 (d, } J = 17.6 \text{ Hz, 1P), -22.42--21.04 (m, 1P) } \]
Supporting Figures

Selection of labeling sites

**Figure 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).
Native purification of TPT3CO-modified and spin-labeled RNase P RNAs

**Figure S2.** Native purification of the RNA transcripts of the doubly TPT3CO-modified RNase P RNA by Size-Exclusion Chromatography. The DNA template, RNase P RNA and excess rNTPs, rTPT3CO, residual DTT were well separated.

**Figure S3.** Native purification of spin-labeled RNase P RNA by Size-Exclusion Chromatography. **(A)** The elution profiles of wild type RNase P and double spin-labeled RNase P through the Superose 6 Increase 10/300 GL column. **(B)** Native PAGE of purified wild type RNase P and doubly spin-labeled RNase P, which has no obvious difference in migration.
Spin counting and assessment of nitroxide labeling efficiency

The nitroxide labeling efficiency 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 (Figure S4). From this plot, a linear fit yielded:

\[I_{2nd}(NOX) = 90118 \times [Az-TMIO] + 3 \times 10^7\]  \hfill (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, \(I_{2nd}(\text{sample})\), were computed. The nitroxide concentration in each sample, \([NOX]\), was computed according to:

\[[NOX] = [I_{2nd}(\text{sample}) - 3 \times 10^7] / 90118\]  \hfill (eq. S2)

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

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

**Figure S4.** The calibration curve for spin counting generated from CW-EPR spectra of standard nitroxide at different concentrations.
Assessment of the specificity of nitroxide labeling

Figure S5. A representative CW-EPR spectrum measured on a native RNase P subjected to the standard labeling procedure. The RNA does not contain the TPT3\(^{\text{CO}}\) modification, and no nitroxide signal was detected. This indicates that the reported labeling procedure specifically directs the nitroxide to the site of TPT3\(^{\text{CO}}\) incorporation.
Figure S6. (A) An overlay of the CW-EPR spectra of the U67 singly spin-labeled (red) and the U86 singly spin-labeled (green) RNase P RNAs. (B) An overlay of the CW-EPR spectrum of the U67/U86 doubly spin-labeled (magenta) on that of the averaged spectrum (blue) of the U67- and U86-singly spin-labeled RNase P RNAs.
Figure S7. (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.
Supporting Tables

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  | AAAAATAACAAATAGGCTCCGGCGACATTTCGGCGAAAAGTGCATACGTTAGGAAAAGGCTTTACCTAAAAGATAGGGCATTACCCAGCGCCGCTTCTGCTGTTTAAGAAAAGGCA GGGCCAGTCAAACGATGCTGCTGATTCCGTCCGGAAGGTGAATACGACACCGCTTCTGCTGACATTGCTAGTAAAAACGA CCGCCGCTGGTCGGAATTGGCAATGGAATAGGCGCTTCTGCTGACGATAGGTCACTTCTGCTGCTGTCTTGGTCGGCAGTCGAGCTGACGCGGTAGCTGATTCTGCTGCTGGTCAACTACTGTCGAGGCTGCCGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG}
Table S2. The native and UBP-modified DNA primers used in this study.

| Primers | Sequence | Application |
|---------|----------|-------------|
| pMVF    | 5' - CATAATTCTTCTTACGTACATGCATCCATC-3' | Common forward primer |
| RPR     | 5' - GACGTTAATCTGGATATGCC -3' | Reverse primer for RNase P |
| 67/86F  | 5' - GTCCATGTCAGCGACG (dTPT3) GCTGAGA TGCCCGTAG (dTPT3) GTTCG -3' | Forward primer for U67/ U86 NaM modification |
| 67/86R  | 5' - GATCTCAGTAGGAACGCACGAGC (dNaM) CTACGG GCATCTCAGC (dNaM) CC -3' | Reverse primer for U67/ U86 NaM modification |
| 67F     | 5' - GTCCATGTCAGCGACG (dTPT3) GCTGAGA TGCCCGTAG -3' | Forward primer for U67 NaM modification |
| 67R     | 5' - GCCACGAACACTACGGGCGACGCGACG (dNaM) CCCTGCGAG -3' | Reverse primer for U67 NaM modification |
| 86F     | 5' - GCTGAGATGCCGGTAGGAG (dTPT3) GTTCGTCGC ATG-3' | Forward primer for U86 NaM modification |
| 86R     | 5' - CTAGGCACGGAC (dNaM) CTACGGGCATCT CAGACCGTG -3' | Reverse primer for U86 NaM modification |
| tRNAR   | 5' - TAAATGAAAGCGAATCTGTGGAGATCG -3' | Reverse primer for yeast pre-tRNA_Phe R |

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.
**Reaction conditions for PCR and *in vitro* transcription**

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

| Coding RNA | U67-single labeled | U86-single labeled | U67/U86-double labeled |
|------------|--------------------|--------------------|------------------------|
| 2× PCR mix |                    |                    |                        |
| Template   | RNase P plasmid (4 ng/μL) |                    |                        |
| Fprimer    | pMVF(0.8 μM)       | pMVF(0.8 μM)       | pMVF(1 μM)             |
|           | 67F(0.8 μM)        | 86R(0.8 μM)        | 67/86F(1 μM)           |
| Rprimer    | 67R(0.8 μM)        | RPR(0.8 μM)        | RPR(1 μM)              |
| dTPP3TP    | 1 mM               | 1.4 mM             |                        |
| dNaMTP     | /                  | 1 mM               | 1.4 mM                 |
| ddH2O      | /                  | /                  | 1.4 mM                 |
|            | 25 μL              |                    |                        |
| a:         | DNA fragments for U67-single labeled RNase P; |
| b:         | DNA fragments for U86-single labeled RNase P; |
| c:         | DNA fragments for U67/U86-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                  | U67 modified | U86 modified | U67/U86 modified |
|----------------------|--------------|--------------|-----------------|
| 10× Transcription buffer | 10 μL        |              |                 |
| T7 RNA polymerase    | 200 ng/μL    |              |                 |
| UBP-modified DNA template | 4 μM        | 4 μM         | 6 μM            |
| rTTP3 or rTTP3'α     | 0.8 mM       | 1 mM         |                 |
| DTT                  | 10 mM        |              |                 |
| DEPC-H2O             | To 50 μL     |              |                 |
SAXS-related parameters

Table S5. 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 S6. Overall structural parameters for native and spin-labeled RNase P RNAs.

| Sample          | l₀ᵃ | Rₐᵃ | lᵇ  | R₉ᵇ  | Dmax | MWᵃ (kDa) | MWᵇ (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 spin-labeled | 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
Data for determination of calibration curve and spin labeling efficiencies

Table S7. The respective 2nd integral values for CW-EPR spectra of Az-TMIO standard at various concentrations and spin-labeling efficiencies of RNase P RNAs.

| Sample                  | I_{2nd(sample)} | [NOX] (μM) | [RNA] (μM) | %label |
|-------------------------|-----------------|------------|------------|--------|
| Az-TMIO (free nitroxide)| 34072022        | 5          | N.A        | N.A    |
|                         | 38660181        | 10         |            |        |
|                         | 50789134        | 20         |            |        |
|                         | 57035610        | 30         |            |        |
|                         | 76884429        | 50         |            |        |
|                         | 97589025        | 75         |            |        |
|                         | 120117208       | 100        |            |        |
| U67 single-labeled      | 117415139       | 97         | 116        | 83.5   |
| U86 single-labeled      | 77762911        | 53         | 65         | 81.5   |
| U67/86 double-labeled   | 114711578       | 94         | 55         | 72.6   |
