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RNA-methyltransferase TrmA is a dual-specific enzyme responsible for C⁵-methylation of uridine in both tmRNA and tRNA.

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RUNNING HEAD: tmRNA-TLD is methylated by TrmA in Gram- bacteria

Keywords: methylation, methyltransferase, TrmA, NMR, post-transcriptional modification, t-RNA like domain (TLD), tmRNA.

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ABSTRACT

In bacteria, trans-translation rescues stalled ribosomes by the combined action of tmRNA (transfer-messenger RNA) and its associated protein SmpB. The tmRNA 5' and 3' ends fold into a tRNA-like domain (TLD), that shares structural and functional similarities with tRNAs. As in tRNAs, the UUC sequence of the T-arm of the TLD is post-transcriptionally modified to m⁵UψC. In tRNAs of gram-negative bacteria, formation of m⁵U is catalyzed by the SAM-dependent methyltransferase TrmA, while formation of m⁵U at two different positions in ribosomal RNA is catalyzed by distinct site-specific methyltransferases RImC and RImD. Here we show that m⁵U formation

in tmRNAs is exclusively due to TrmA and should be considered as a dual-specific enzyme. The evidence comes from the lack of m⁵U in purified tmRNA or TLD variants recovered from an *Escherichia coli* mutant strain deleted of the *trmA* gene. Detection of m⁵U in RNA was performed by NMR analysis.

INTRODUCTION

Defective mRNAs can cause stalling of translating ribosomes. In bacteria, ribosomes are mainly rescued by the trans-translation process, the essential components of which are tmRNA (transfer-messenger RNA, formerly called 10Sa RNA), a large ubiquitous RNA of about 360 nt with both mRNA and tRNA activities, and a partner protein SmpB. tmRNA is alanylated prior to entering the ribosome (for recent reviews see refs. 1, 2). Alanine is transferred to the nascent peptide whose synthesis has been stopped. Translation then resumes on the open reading frame internal to tmRNA (mRNA-like domain or MLD) and ends normally at an internal stop codon, allowing ribosome recovery. The tmRNA-encoded carboxy-terminal fusion peptide targets the incomplete protein for degradation.

The biosynthesis of tmRNA shares many features with that of tRNAs. The precursor tmRNA is processed at the 5' end by RNase P and at the 3' end by several enzymes including RNase PH and RNase T. Both ends then fold into a tRNA-like domain (TLD) containing an amino acid acceptor stem and a T-arm closed by a TΨC-loop.³⁻⁶ The 3'-CCA sequence of the TLD is either encoded in the gene or added post-transcriptionally and is charged *in vivo* by the Ala-RNA synthetase (AlaRS).^{3,7} There is no D-stem in tmRNAs. However their D-loop contains the consensus GG doublet found in all canonical tRNAs, which is known to form two base pairs with T-loop residues (G₁₈.Ψ₅₅ and G₁₉.C₅₆). An NMR study of the D/T loop interactions stabilizing the *Escherichia coli* and *Aquifex aeolicus* TLDs and two crystallographic structures of the TLDs of *A. aeolicus* and *Thermus thermophilus* in complex with the partner protein SmpB, confirm that the fold of the TΨC-loop and the existence of the two base pairs between doublets of the T-loop are very similar to what is observed in all cytoplasmic tRNAs.⁸⁻¹⁰

Methylation occurs in most non-coding RNAs and accounts for more than half of the post-transcriptional modifications.¹¹ As in regular tRNAs of all three phylogenetic domains, the UUC sequence of the tmRNA T-arm loop is usually post-

transcriptionally modified into T ψ C.⁶ Because of obvious structural similarity between the T-arms of tmRNA and tRNA, it has been thought that formation of ribo-T (m⁵U), as well as ψ in both types of nucleic acids, would be catalyzed by the same set of modification enzymes. However, in the case of tmRNA, no experimental evidence has yet been provided.

In *E. coli*, three m⁵U-RNA methyltransferases exist: TrmA (formerly called RumT) catalyzing the formation of m⁵U at position 54 of tRNA, RImD catalyzing the formation of m⁵U at position 1939 of 23S rRNA and RImC that methylates U₇₄₇ in 23S rRNA.^{12,13}

Here we aimed at identifying which of the three rRNA-m⁵U methyltransferase paralogs in *E. coli* is responsible for the methylation of uridine of the T-arm of tmRNA. For this purpose, we used three RNAs recapitulating the functional 5' and 3' tmRNA ends (Figure 1). One of them is the TLD fragment of *A. aeolicus* (TLDaa, 60 nt in length) whose structure was formerly investigated by NMR⁸. The other two RNAs are longer: an *A. aeolicus* TLD-H2 derivative of 143 nucleotides (TLD-Haa) and the full-length processed *E. coli* tmRNA itself (ectmRNA, 363 nt). All RNAs were overproduced in three *E. coli* strains, wild-type or deleted either of the *trmA* gene alone or of the set of the three *trmA*, *rumB* and *rumC* genes. The triple deletion was used as negative control. After recovery and purification of the expressed RNAs, the presence or the absence of methylation of the first uridine of the T-loop UUC sequence, was investigated by NMR. One goal of our work was also to expand the application of the NMR technique to test the presence or absence of a naturally occurring methyl group in a long cellular RNA molecule. At the same time, this approach gives conformational and dynamic information around the site of methylation, which would not be easily obtained by other techniques, such as *in vitro* tests with recombinant enzymes. While tedious for sample preparation, this technique is nevertheless particularly suited to analyze the TLD portion of tmRNA because it can rapidly and unambiguously search for the presence or absence of methylation, determining the position of the methylated nucleotide.

RESULTS AND DISCUSSION

Investigations were performed exclusively with *E. coli*, a Gram-negative bacterium. Three RNAs were used as templates to probe methylation of the uridines

in vivo (Figure 1). The shorter fragment (TLDaa) recapitulates the TLD part of the *A. aeolicus* tmRNA and is closed by an artificial GAAA loop. The m⁵U was expected at position 38. The second RNA was originally designed so as to extend the natural part of the TLDaa by an extended bulged helical segment mimicking the tmRNA H2 connecting stem. Unfortunately, this construct proved to be toxic for the bacteria. However, the bacteria evolved a mutation, in which one adenine in the D-loop is deleted and that bears an extra nine-residue bulge whose sequence (CUUCGGACG) resembles that of the TLDaa T-loop. The corresponding RNA is called hereafter TLD-Haa. According to MFOLD, a secondary structure prediction program, deletion of the adenine does not break the L-shaped fold of the TLD part.¹⁴ This prediction is confirmed by the overproduction of an intact RNA, that attests the formation of a protecting tRNA-like scaffold. In this TLD-Haa, uridine methylation was expected at position 121. The third RNA is the full-length ectmRNA itself. The T-arms of the three RNAs are identical. The T-loops only differ by the fourth and sixth residues, which however remain in all cases respectively a purine and a pyrimidine. The position of the m⁵U in the naturally occurring full-length *E. coli* tmRNA is 341.

The wild-type and the mutant *E. coli* strains lacking either tRNA:m⁵U methyltransferase *trmA*, or *trmA* and both rRNA:m⁵U methyltransferases *rlmC* and *rlmD* were transformed with each of the three plasmids. Bacterial growth of the mutant strains was not totally abolished by the deletion of one, or all three RNA:m⁵U-methyltransferase(s). We were able to recover enough material (*i.e.* between 1 to 5 mg) from the wild-type and $\Delta trmA$ strains to perform NMR studies. For simplification, the names of the RNAs produced in the wild-type strain will be preceded by “wt-”, and those produced from the $\Delta trmA$ and $\Delta trmA-rlmC-rlmD$ mutant strains respectively by “ $\Delta 1$ -” and “ $\Delta 3$ -”.

For each sample, the 1D and NOESY spectra disclose the characteristic resonance(s) of a methyl proton (sharp peak between 1.0 and 1.5 ppm) when the RNAs are overproduced in the wt-BW25113 strain, but not when produced in the mutant strains (Figures 2-4).

In a previous study, we investigated the TLDaa by NMR and proved that it adopts a tRNA-like tri-dimensional fold. In this former study, the TLDaa was overproduced in JM101 strains and we were able to unambiguously assign the resonances of both the imino and the methyl protons of m⁵U₃₈ (or T₃₈), the equivalent

position of m^5U_{55} in tRNAs.⁸ The spectra recorded here for the TLDaa produced in BW25113 *E. coli* cells are identical to our previous spectra, confirming: i) that the RNA is correctly folded, and ii) that the position of methylation is independent of the nature of the *E. coli* strains. TLDaa 1D and NOESY spectra recorded in both strains also display one set of the two imino proton resonances of the Ψ_{39} , emphasizing the quality of our RNAs. The 1D spectrum of wt-TLDaa exhibits an extra sharp resonance at 1.19 ppm compared to that of $\Delta 1$ -TLDaa (Figure S1). As expected, the NOESY spectrum of the wt-TLDaa discloses a cross-peak pattern similar to that of our previously studied TLD, which allowed the resonance at 1.19 ppm to be assigned to m^5U_{38} (T_{38}) methyl protons (Figure 2, left panel) and therefore confirming the site of methylation.⁸ This set of cross-peaks is totally absent from the equivalent spectra recorded for $\Delta 1$ -TLDaa (Figure 2, right panel) and $\Delta 3$ -TLDaa (not shown).

The NOESY spectrum of the full-length wt-ectmRNA presents very broad resonances, which is not surprising for a RNA of 117.4 kDa (Figure 3). Contrary to the cases of the shorter RNA derivatives, the spectral resolution is thus much too low to allow direct identification of the methylation position. However, the wt-ectmRNA spectrum discloses two well-resolved cross-peaks, between a methyl group and two aromatic protons demonstrating the presence of a ribothymidine (m^5U). The stronger cross-peak arises from the intra-residual CH3-H6 NOE. It is connected to the resonance at 14.0 ppm whose connection pathway to G_{13} is reminiscent of that of wt-TLDaa $T_{38}H3$ to G_{12} (Figure 2) and that of $T_{39}H3$ to G_{13} in the spectrum we recorded for the RNA recapitulating the *E. coli* tmRNA TLD.⁸ As only one uridine is post-transcriptionally methylated, we could infer that it indeed stems from m^5U_{341} in the T-loop and that the imino proton resonance that is at 14.0 ppm is that of $m^5U_{341}H3$ (or T_{341}).⁶ As expected, this resonance is also connected to the N3H imino resonance of the neighboring Ψ_{342} at 11.85 ppm, which is in turn connected to G_{13} N3H at 10.2 ppm.

The overproduction of each of the three RNAs was a little less efficient in the deleted strains than in the wild-type strain (not shown). This is not surprising since the lack of m^5U_{54} in tRNA was reported to only marginally reduce (4%) the growth rate (reviewed in ref. 15). Consequently, we can rule out any saturation of the methylation enzymes to explain the absence of observable methylation on each of the RNAs produced in the $\Delta trmA$ and $\Delta trmA-rlmC-rlmD$ strains. Our data thus

definitively prove that under *in vivo* experimental conditions, only tRNA methyltransferase TrmA of *E. coli* is able to catalyze the formation of m⁵U in both tRNA and tmRNA, while neither rRNA methyltransferases RlmD nor RlmC was able to catalyze even trace amounts of m⁵U in any of the tmRNA-TLD derivatives tested.

Unexpectedly, the spectrum of TLD-Haa also discloses cross-peaks connected to a second methyl group (Figure 4). The presence of this second spin system connected to a methyl group might result from the formation of two alternative conformations in slow equilibrium on the NMR time scale or from two methylation sites within the same conformation. We ruled out the first possible explanation because if two conformations of the TLD were in equilibrium, then two sets of proton resonances, one for each conformation should be visible. This is not the case. The imino spectrum, assigned by analogy with the wt-TLDaa equivalent spectrum, discloses only one characteristic imino pathway (Figure 4). Moreover, the intensities of the two methyl resonances remain identical whatever the experimental conditions (concentration, temperature, thermal denaturation of the sample followed by fast freezing). Therefore, the observed second methyl resonance results from the existence of a second cryptic methylation site in the molecule, identified as m⁵U₉₇. Indeed, the additional bulge whose sequence resembles that of the TLD T-loop and fits the consensus descriptors, *i.e.* the triplet UUC next to a stack of purines, makes its first uridine a target for TrmA.¹⁶ This second methylation is absent when TLD-Haa is produced in the $\Delta trmA$ strain attesting that TrmA also catalyzes the methylation of this second uridine target (Figure S2). Nevertheless beyond the scope of this report, it would be interesting to assess the methylation by TrmA of an oligonucleotide recapitulating this bulge.

The tRNA mimicry of the 5' and 3' ends of bacterial tmRNAs was postulated before the discovery of its structure and the exact chemical nature of the post-transcriptional modifications. We have now demonstrated that the *E. coli* SAM-dependent U-methyltransferase acting on the T-loop of tRNA, also acts on the T-loop of the tRNA-like domain of tmRNA and should therefore be considered as a dual-specific enzyme, as the pseudouridine synthase RluA acting on 23S rRNA (position 746) and tRNA (position 32) or the SAM-dependent methyltransferase RlmN acting on 23S rRNA (position 2503) and tRNA (position 37).^{17,18} In the same line, we expect TruB, the Psi-synthase catalyzing formation of Ψ_{55} within the same T-loop in tRNAs

in *E. coli*, to also be a dual-specific enzyme responsible for pseudouridylation of the T-loop in both the tmRNA and tRNA.

In the Gram-positive *B. subtilis*, only one enzyme (RlmCD) catalyzes the formation of m⁵U at both positions 747 and 1939 in 23S RNA, instead of two enzymes (RlmC and RlmD) in the Gram-negative *E. coli*. Moreover, in *B. subtilis*, m⁵U at position 54 in tRNA is catalyzed by TrmFO, a methylene-tetrahydrofolate-dependent enzyme, while in *E. coli* it is the SAM-dependent TrmA enzyme that catalyzes the formation of the same m⁵U-54 in tRNA.¹⁹⁻²¹ Very recently, Yamagami and collaborators have demonstrated that TrmFO, like TrmA, recognizes the T-arm structure of *T. thermophilus* tRNAs.²² We can thus infer from this and our results that TrmFO most probably methylates the TLD T-loop in tmRNAs of Gram-positive bacteria (another dual-specific enzyme).

MATERIALS AND METHODS

E. coli strains and media

E. coli simple mutant strains are derivatives of BW 25113 [F⁻, Δ (*araD-araB*)567, Δ *lacZ*4787(::*rrmB*-3), λ^- , *rph*-1, Δ (*rhaD-rhaB*)568, *hsdR*514] and were obtained from the Coli Genetic Stock Center. The BW 25113 single Δ *trmA* and triple Δ *trmA*/ Δ *rlmC*/ Δ *rlmD* strains were initially constructed by Damien Brégeon (University of Orsay) and previously described in Desmolaize et al.¹⁹ Electrocompetent cells were transformed with the pBSTNAV vectors and selected on LB plates containing ampicillin (100 μ g/ml).²³

Preparation and purification of the RNAs

The genes of TLDaa, TLD-Haa and *ectmRNA* were cloned in the pBSTNAV vector (AmpR) between *EcoRI* and *PstI* according to the procedure described by Ponchon et al.^{24,25} The expression of the recombinant RNAs was monitored by carrying out extractions of total soluble RNAs from small-scale overnight cultures in 2XTY growth medium. Large-scale expression was carried out overnight at 37°C in 2 litres of 2XTY medium containing 100 μ g/ml ampicillin. The RNAs were recovered and purified as described in Gaudin et al. and in Ponchon et al.^{8,25} Pure fractions of monomeric *ectmRNA* were separated from aggregates on a Superdex-200 prep grade gel filtration column (GE-Healthcare) equilibrated with 50 mM NaCl, 20 mM potassium phosphate pH 6.5 and 1 mM EDTA water solution. The purity of all

samples was checked by 12% polyacrylamide gel electrophoresis containing 7 M urea. The gels were visualized by UV shadowing. The final yields were about 10 mg/L for the production in wild-type strains and 2 mg/L in $\Delta trmA$ and $\Delta trmA/\Delta rlmC/\Delta rlmD$ strains.

NMR sample preparation and analysis

The purified samples were deacylated in Tris buffer pH 8 at 37°C for 1 h and dialyzed several times against 1 mM EDTA water solutions and finally extensively against water. They were then freeze-dried before re-suspension in 95% H₂O-5% D₂O. The pH was adjusted to 6.5 by addition of small aliquots of NaOH 0.1 N under stirring. The sample strand concentrations were between 0.3 mM and 50 μ M. TLDaa and TLD-Haa samples were heated at 95°C and snap-cooled in ice water to ensure monomeric species before NMR experiments. The spectra were recorded in 5 mm Shigemi tubes at 600 MHz on a Bruker Avance DRX600 spectrometer equipped with a cryoprobe and Z-axis gradient. 1D and 2D NOESY experiments were recorded at 288 K and 293 K using the Jump and Return procedure (jrNOESY) to avoid excitation of the water resonance, or the Watergate pulse sequence (wgNOESY) for solvent suppression.^{26,27} The data were processed using TOPSIN 3.1 (Bruker).

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References

1. Barends S, Kraal B, van Wezel GP. The tmRNA-tagging mechanism and the control of gene expression: a review. *Wiley Interdiscip Rev RNA*. 2011; 2:233-46.
2. Felden B, Gillet R. SmpB as the handyman of tmRNA during trans-translation. *RNA Biol* 2011; 8:440-9.

3. Komine, Y, Kitabatake, M, Yokogawa, T, Nishikawa, K, Inokuchi, H. A tRNA-like structure is present in 10Sa RNA, a Small stable RNA from *Escherichia coli*. Proc Natl Acad Sci U S A 1994; 91:9223-27.
4. Li Z, Pandit S, Deutscher MP. 3' exoribonucleolytic trimming is a common feature of the maturation of small, stable RNAs in *Escherichia coli*. Proc Natl Acad Sci U S A 1998; 95:2856-61.
5. Felden B, Himeno H, Muto A, Mccutcheon JP, Atkins JF, Gesteland RF. Probing the structure of the *Escherichia coli* 10Sa RNA (tmRNA). RNA 1997; 3:89-103.
6. Felden B, Hanawa K, Atkins JF, Himeno H, Muto A, Gesteland RF et al. Presence and location of modified nucleotides in *Escherichia coli* tmRNA: structural mimicry with tRNA acceptor branches. EMBO J 1998; 17:3188-96.
7. Ushida C, Himeno H, Watanabe T, Muto A. tRNA-like structures in 10Sa RNAs of *Mycoplasma capricolum* and *Bacillus subtilis* Nucleic Acids Res 1994; 22:3392-96.
8. Gaudin C, Nonin-Lecomte S, Tisné C, Corvaisier S, Bordeau V, Dardel F et al. The tRNA-like domains of *E. Coli* and *A. aeolicus* transfer-messenger RNA: structural and functional studies. J Mol Biol 2003; 331:457-71.
9. Gutmann S, Haebel PW, Metzinger L, Sutter M, Felden B, Ban N. Crystal structure of the transfer-RNA domain of transfer-messenger RNA in complex with SmpB. Nature 2003; 424:699-703.
10. Bessho Y, Shibata R, Sekine S, Murayama K, Higashijima K, Hori-Takemoto C et al. Structural basis for functional mimicry of long-variable-arm tRNA by transfer-messenger RNA. Proc. Natl. Acad. Sci. U S A 2007; 104:8293-98.
11. Motorin Y, Helm M. RNA nucleotide methylation. Wiley Interdiscip. Rev RNA 2011; 2: 611-31.
12. Ny T, Björk GR. Cloning and restriction mapping of the trmA gene coding for transfer ribonucleic acid (5-methyluridine)-methyltransferase in *Escherichia coli* K-12. J Bacteriol 1980; 142:371-9.
13. Madsen CT, Mengel-Jørgensen J, Kirpekar F, Douthwaite S. Identifying the methyltransferases for m(5)U747 and m(5)U1939 in 23S rRNA using MALDI mass spectrometry. Nucleic Acids Res. 2003; 31:4738-46.
14. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003; 31:3406-15.
15. Björk GR. The role of modified nucleosides in tRNA interactions. In Hatfield DL, Lee BJ and Pirtle RM, ed. Transfer RNA in Protein Synthesis. CRC Press, Boca Raton, FL, 1992:23-85.
16. Alian A, Lee TT, Griner SL, Stroud RM, Finer-Moore J. Structure of a TrmA-RNA complex: a consensus RNA fold contributes to substrate selectivity and catalysis in

m5U methyltransferases. Proc Natl Acad Sci U S A 2008; 105:6876-81.

17. Wrzesinski J, Nurse K, Bakin A, Lane BG, Ofengand J. A dual-specificity pseudouridine synthase: an *Escherichia coli* synthase purified and cloned on the basis of its specificity for psi 746 in 23S RNA is also specific for psi 32 in tRNA(phe). RNA 1995; 1:437-48.

18. Benítez-Páez A, Villarroya M, Armengod ME. The *Escherichia coli* RlmN methyltransferase is a dual-specificity enzyme that modifies both rRNA and tRNA and controls translational accuracy. RNA 2012; 18:1783-95.

19. Desmolaize B, Fabret C, Brégeon D, Rose S, Grosjean H, Douthwaite S. A single methyltransferase YefA (RlmCD) catalyses both m5U747 and m5U1939 modifications in *Bacillus subtilis* 23S rRNA. Nucleic Acids Res 2011; 39:9368-9375.

20. Urbonavicius J, Skouloubris S, Myllykallio H, Grosjean H. Identification of a novel gene encoding a flavin-dependent tRNA:m5U methyltransferase in bacteria--evolutionary implications. Nucleic Acids Res 2005; 33:3955-64.

21. Yamagami R, Yamashita K, Nishimasu H, Tomikawa C, Ochi A, Iwashita C et al. The tRNA Recognition Mechanism of Folate/FAD-dependent tRNA Methyltransferase (TrmFO). J Biol Chem 2012; 287:42480-94.

22. Nishimasu H, Ishitani R, Yamashita K, Iwashita C, Hirata A, Hori H et al. Atomic structure of a folate/FAD-dependent tRNA T54 methyltransferase. Proc Natl Acad Sci U S A 2009; 106:8180-5.

23. Meinnel T, Mechulam Y, Fayat G. Fast purification of a functional elongator tRNA^{met} expressed from a synthetic gene *in vivo*. Nucl Acids Res 1988; 16:8095-96.

24. Ponchon L, Dardel F. Recombinant RNA technology: the tRNA scaffold Nature Methods 2007; 4:571-6.

25. Ponchon L, Beauvais G, Nonin-Lecomte S, Dardel F. A generic protocol for the expression and purification of recombinant RNA in *Escherichia coli* using a tRNA scaffold. Nature Protocols 2009; 4:947-9.

26. Guéron M, Plateau P., Descorps M. Solvent signal suppression in NMR. Progress in NMR Spectroscopy 1991; 23:135-209.

27. Piotto M, Saudek V, Sklenar V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. J Biomol NMR 1992; 2:661-5.

FIGURES LEGENDS

Figure 1. Secondary structure models of (A) TLDaa, (B) TLD-Haa and (C) full-length ectmRNA. The TLD parts of the *A. aeolicus* and *E. coli* models are very similar in sequence. An arrow stresses the position of the ribothymidine m⁵U (labeled T for simplification) (*i.e.* 38 in TLDaa, 121 in TLD-Haa and 341 in ectmRNA). The second methylated uridine observed in TLD-Haa, as explained in text, is most probably U₉₇.

Figure 2. Fingerprint regions of two NOESY spectra recorded at 150 ms mixing times and 288 K of the wt-TLDaa (left) and Δ 1-TLDaa (right). The spectra are almost identical, except for the boxed cross-peaks arising from m⁵U₃₈ that are absent when the RNA is overproduced in the Δ trmA strain.

Figure 3. wgNOESY spectra recorded at 150 ms mixing times and 293 K of wt-ectmRNA 0.2 mM (upper spectrum) and in Δ 1-ectmRNA 50 μ M (lower spectrum). Upper: a strong cross-peak connects a methyl group and an H6 proton. T₃₄₁H3 connectivity pathway to G₁₃ is similar to that linking wt-TLDaa T₃₈H3 to G₁₂H3. Lower: the methyl resonance and the linked cross-peaks are absent.

Figure 4. Superposition of TLD-Haa jrNOESY spectra recorded at 298 K at 100 and 150 ms mixing times: imino proton region (left) and methyl proton region (right). The pattern of the TLDaa cross-peaks and its assignment is transposable to these spectra, showing that the TLD parts of both RNAs fold similarly. The spectra disclose two methyl proton resonances (boxed cross-peaks), one arising from the expected m⁵U₁₂₁ and the other probably from m⁵U₉₇.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Stack plot of the 1D-watergate NMR spectra recorded at 288 K and pH 6.5. Selected regions: imino and aromatic protons (right); methyl protons (left). Lower spectrum: TLDaa from the wild-type *E. coli* strain. Upper spectrum: TLDaa from the Δ trmA *E. coli* strain. The spectra are very similar and attest that folding is identical.

The CH₃ methyl resonance at 1.2 ppm is absent when TLDaa is overproduced in the $\Delta trmA$ strain. The other peaks around 1 ppm arise from a buffer contaminant (stars).

Figure S2. Selected regions of the $\Delta 1TLD$ -Haa jrNOESY spectrum recorded at 298 K with a 100 ms mixing time. The box in the right panel stresses the lack of methyl-H6 cross-peaks.

FIGURE 1

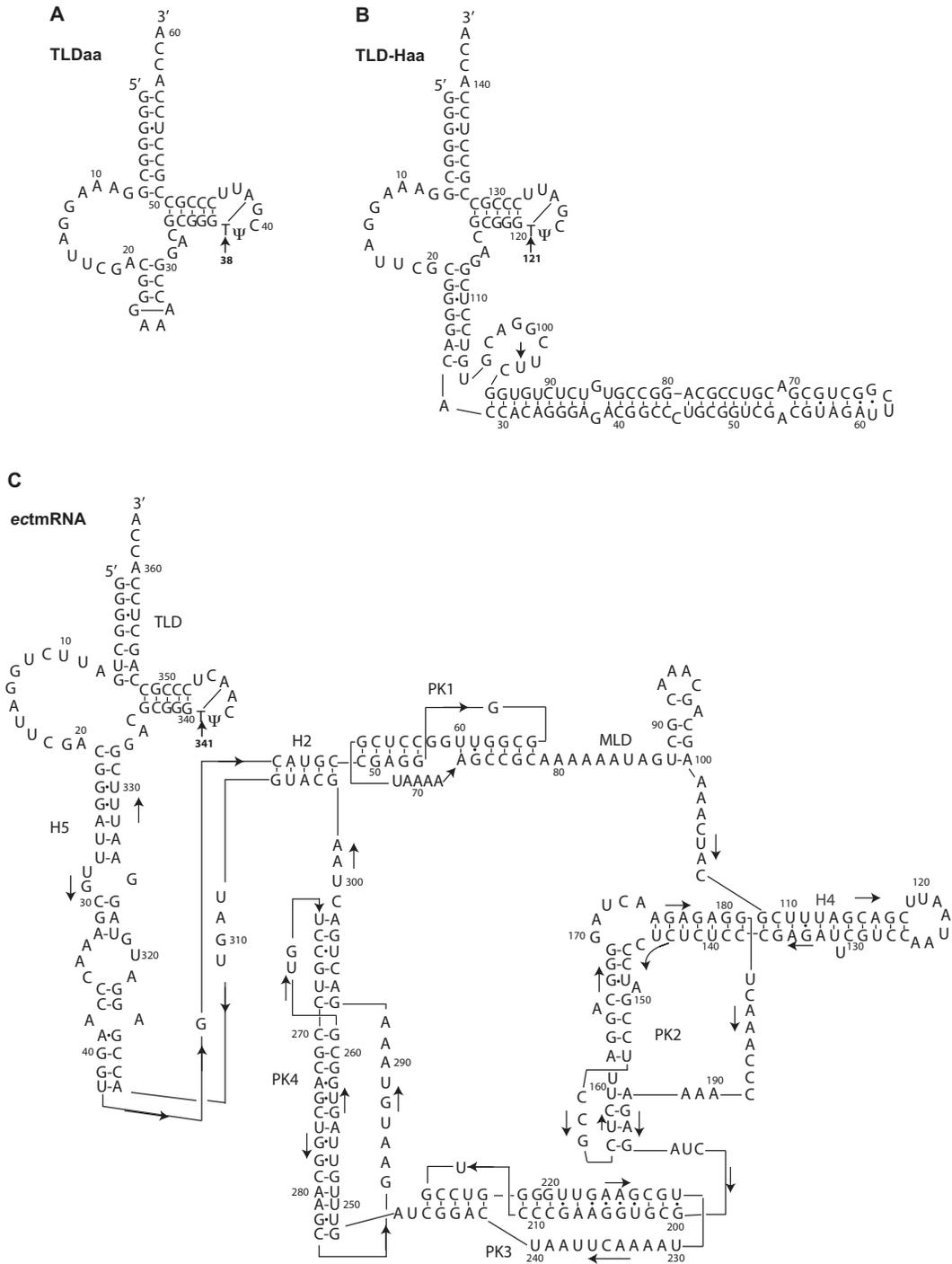


FIGURE 2

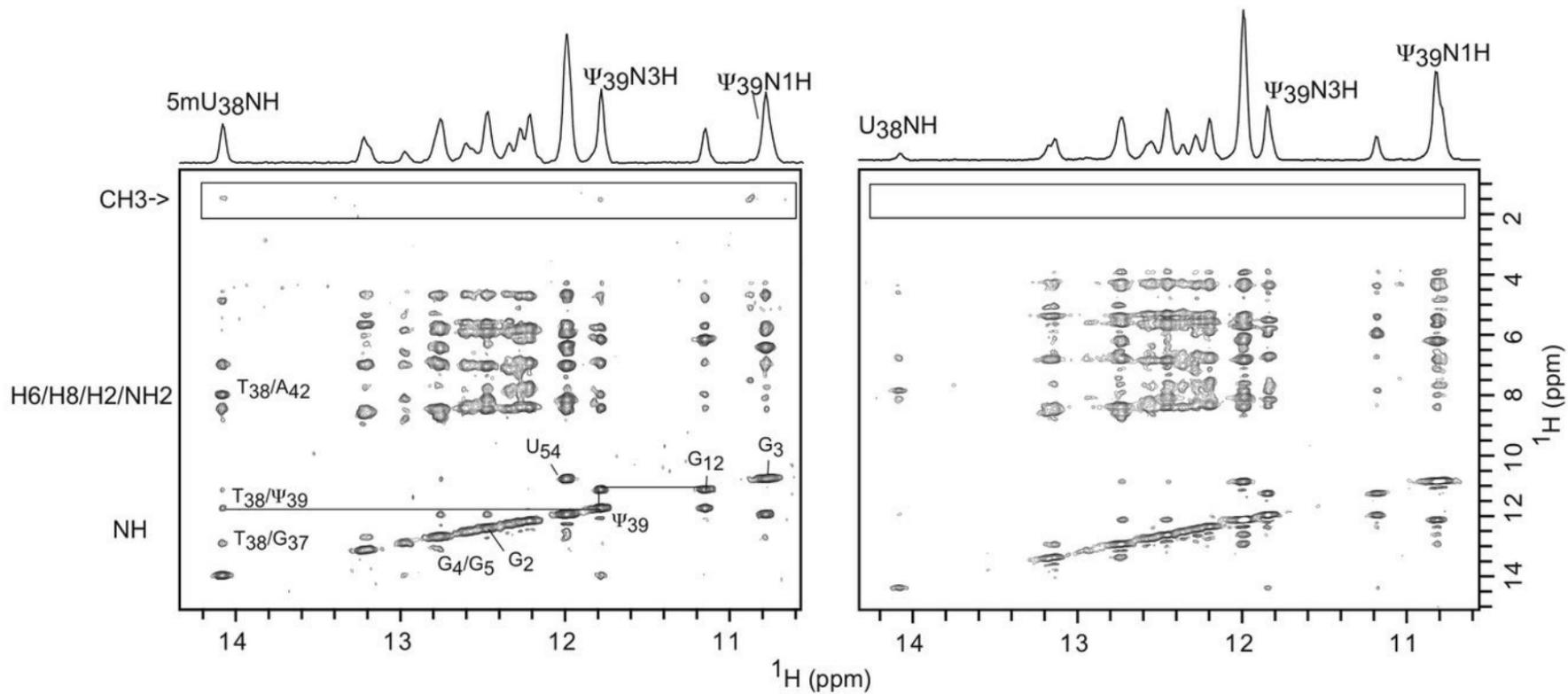


FIGURE 3

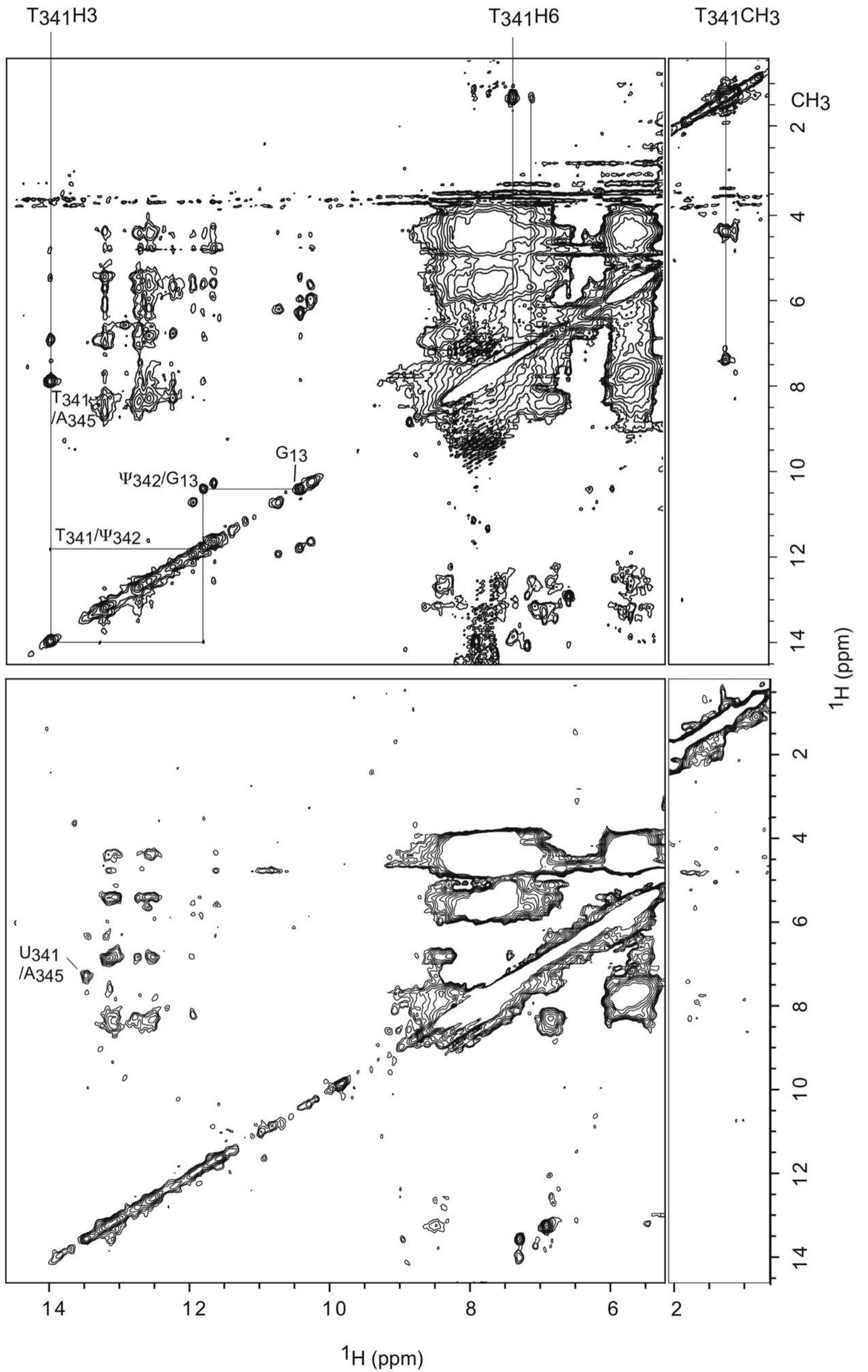


FIGURE 4

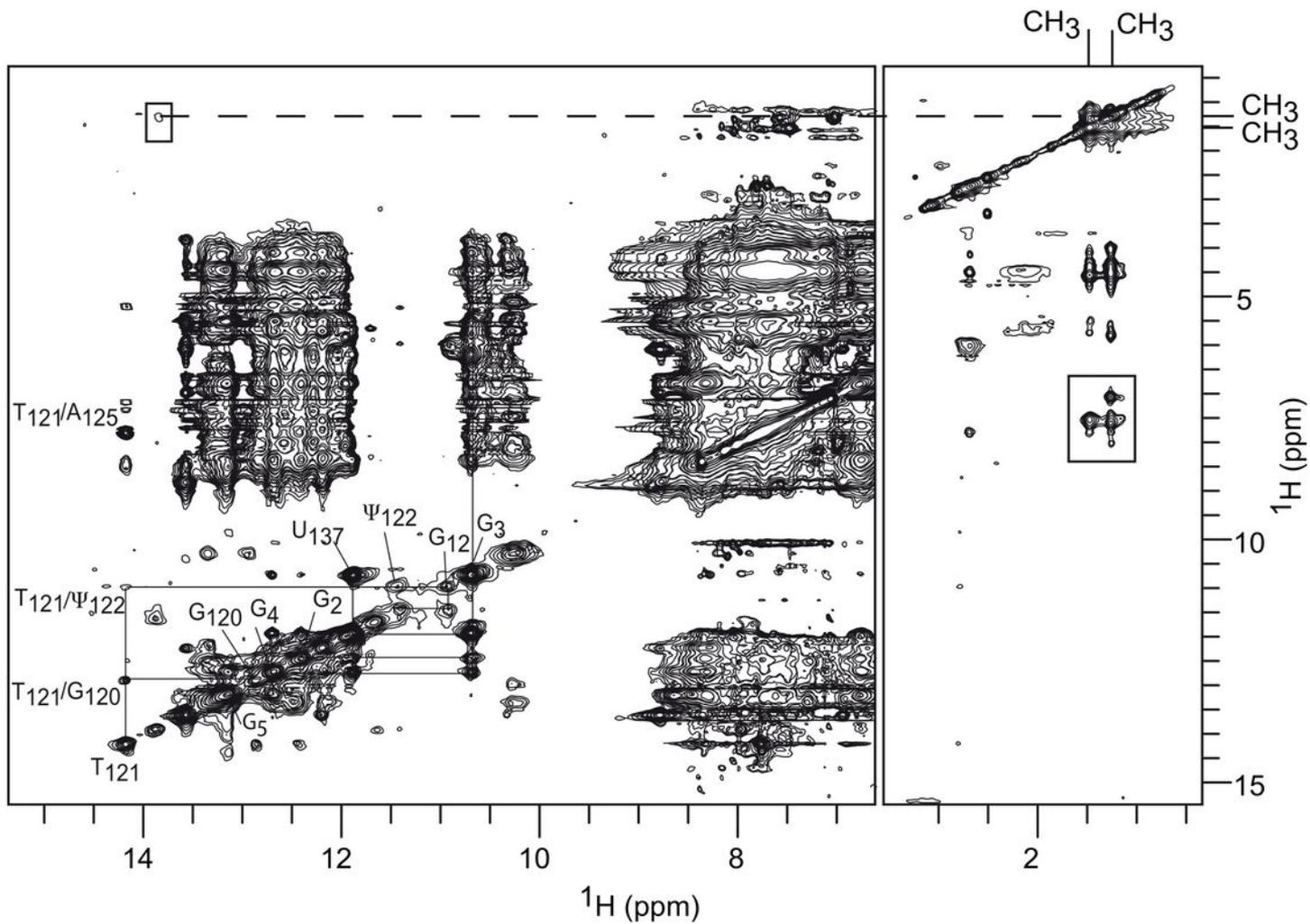


FIGURE S1

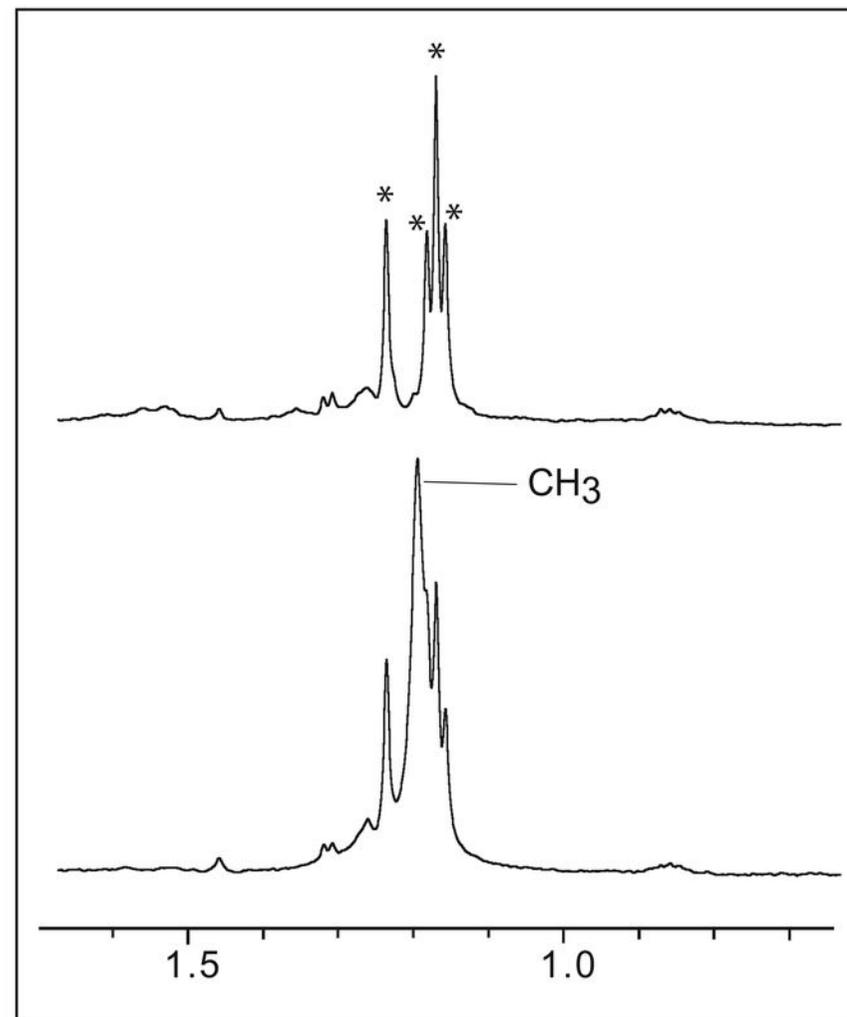
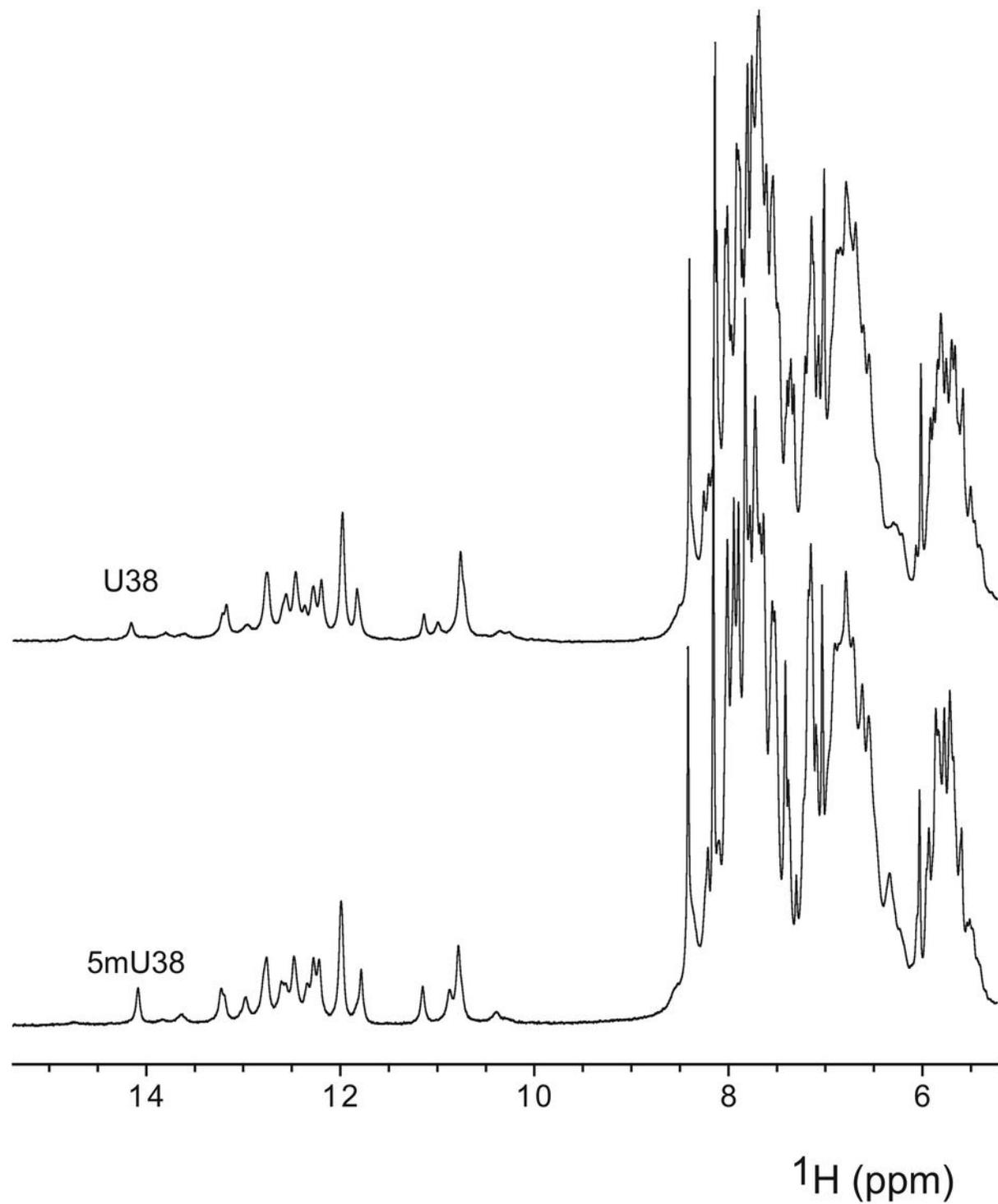


FIGURE S2

