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RNA-methyltransferase TrmA is a dual-specific enzyme responsible for C^5-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 $m^5U\psi C$. In tRNAs of gram-negative bacteria, formation of m^5U is catalyzed by the SAM-dependent methyltransferase TrmA, while formation of m^5U at two different positions in ribosomal RNA is catalyzed by distinct site-specific methyltransferases RlmC and RlmD. Here we show that m^5U formation

in tmRNAs is exclusively due to TrmA and should be considered as a dual-specific enzyme. The evidence comes from the lack of m^5U in purified tmRNA or TLD variants recovered from an *Escherichia coli* mutant strain deleted of the *trmA* gene. Detection of m^5U 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 ($G_{18}.\Psi_{55}$ and $G_{19}.C_{56}$). 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 TψC.[6] Because of obvious structural similarity between the T-arms of tmRNA and tRNA, it has been thought that formation of ribo-T (m^5U), 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 m^5U-RNA methyltransferases exist: TrmA (formerly called RumT) catalyzing the formation of m^5U at position 54 of tRNA, RlmD catalyzing the formation of m^5U at position 1939 of 23S rRNA and RlmC that methylates U_{747} in 23S rRNA.[12,13]

Here we aimed at identifying which of the three rRNA-m^5U 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 NMR[8]. 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 (*ec*tmRNA, 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 m^5U 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 *ec*tmRNA 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 m^5U in the naturally occurring full-length *E. coli* tmRNA is 341.

The wild-type and the mutant *E. coli* strains lacking either tRNA:m^5U methyltransferase *trmA, or trmA* and both *rRNA:m^5U 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:m^5U-methyltransferase(s). We were able to recover enough material (*i.e.* between 1 to 5 mg) from the wild-type and *Δtrm*A 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 Δ*trmA* and Δ*trmA-rlmC-rlmD* mutant strains respectively by "Δ1-" and "Δ3-".

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 m^5U_{38} (or T_{38}), 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 Ψ39, 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 Δ1-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 Δ1-TLDaa (Figure 2, right panel) and Δ3-TLDaa (not shown).

The NOESY spectrum of the full-length wt-*ec*tmRNA 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-*ec*tmRNA 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 G13 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 Δ*trmA* and Δ*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 m^5U in both tRNA and tmRNA, while neither rRNA methyltransferases RlmD nor RlmC was able to catalyze even trace amounts of m^5U 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 m^5U_{97}. 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 Δ*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 m^5U 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*, m^5U 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 m^5U-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, ΔlacZ4787*(::rrnB-3), λ^-, *rph-1, Δ(rhaD-rhaB)568, hsdR514*] and were obtained from the Coli Genetic Stock Center. The BW 25113 single *ΔtrmA* and triple *ΔtrmA/ΔrlmC/ΔrlmD* 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 μg/ml).[23]

Preparation and purification of the RNAs

The genes of TLDaa, TLD-Haa and *ec*tmRNA 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 μg/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 *ec*tmRNA 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 Δ*trmA* and Δ*trmA*/Δ*rlmC*/Δ*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% H_2O-5% D_2O. 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 μM. 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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FIGURES LEGENDS

Figure 1. Secondary structure models of (A) TLDaa, (B) TLD-Haa and (C) full-length *ec*tmRNA. The TLD parts of the *A. aeolicus* and *E. coli* models are very similar in sequence. An arrow stresses the position of the ribothymidine m^5U (labeled T for simplification) (*i.e.* 38 in TLDaa, 121 in TLD-Haa and 341 in *ec*tmRNA). The second methylated uridine observed in TLD-Haa, as explained in text, is most probably U_{97}.

Figure 2. Fingerprint regions of two NOESY spectra recorded at 150 ms mixing times and 288 K of the wt-TLDaa (left) and Δ1-TLDaa (right). The spectra are almost identical, except for the boxed cross-peaks arising from m^5U_{38} 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-*ec*tmRNA 0.2 mM (upper spectrum) and in Δ1-*ec*tmRNA 50 μM (lower spectrum). Upper: a strong cross-peak connects a methyl group and an H6 proton. T_{341}H3 connectivity pathway to G_{13} is similar to that linking wt-TLDaa T_{38}H3 to G_{12}H3. 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 m^5U_{121} and the other probably from m^5U_{97}.

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 CH_3 methyl resonance at 1.2 ppm is absent when TLDaa is overproduced in the Δ*trmA"* strain. The other peaks around 1 ppm arise from a buffer contaminant (stars).

Figure S2. Selected regions of the Δ1TLD-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

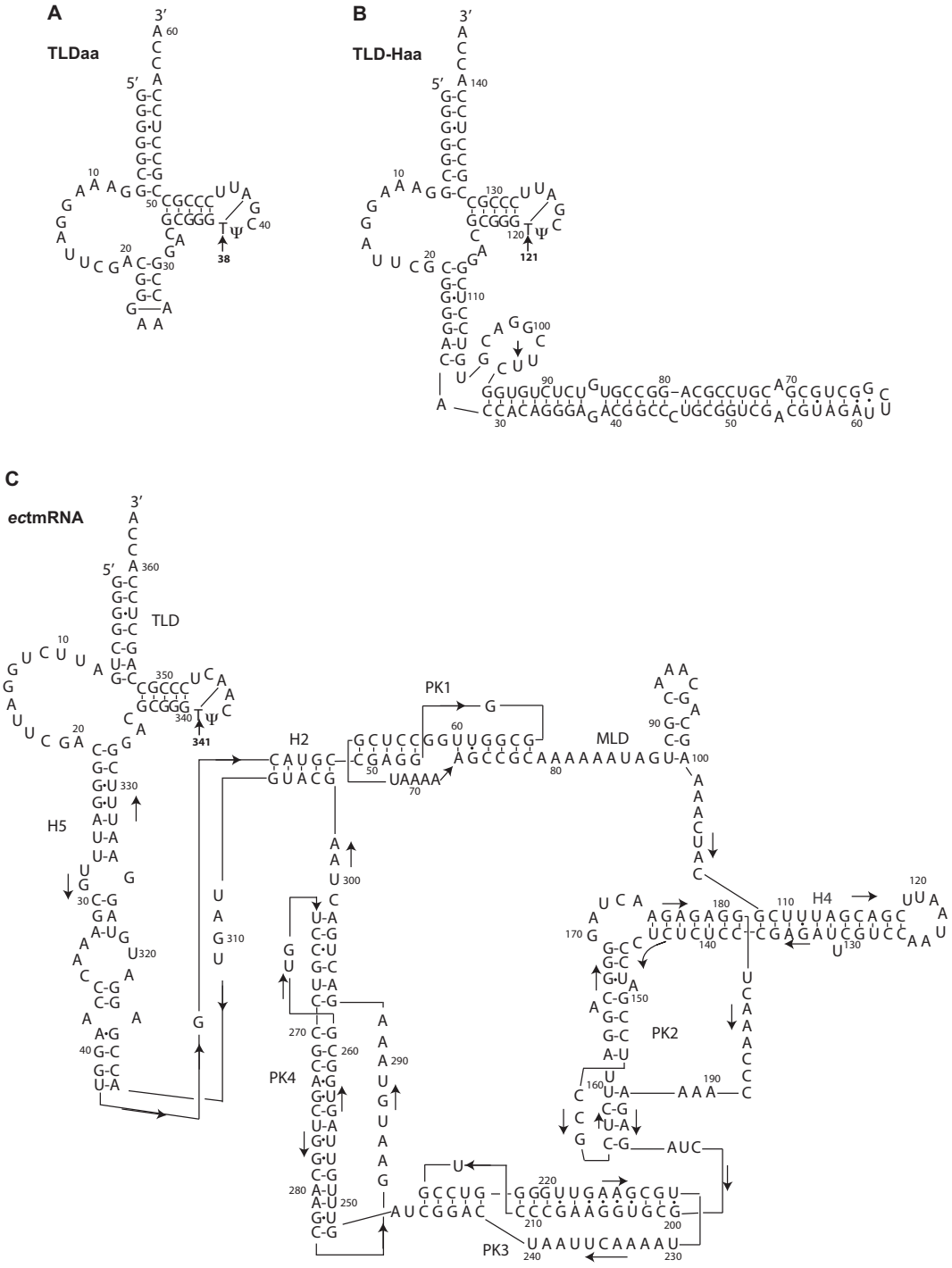


FIGURE 2

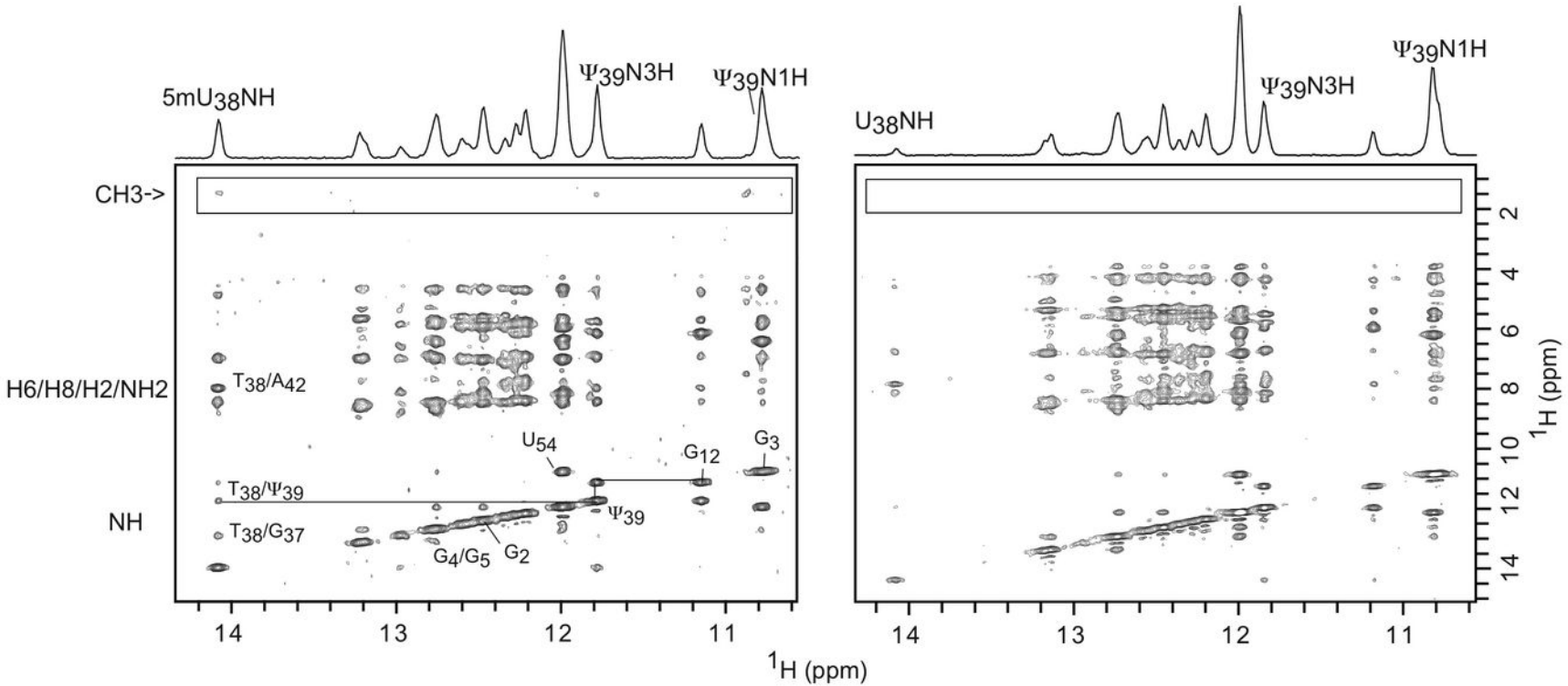

5mU38NH
Ψ39N3H
Ψ39N1H
U38NH
Ψ39N3H
Ψ39N1H
CH3->
H6/H8/H2/NH2
NH
T38/A42
U54
G12
G3
T38/Ψ39
Ψ39
T38/G37
G4/G5
G2
14
13
12
11
1H (ppm)
2
4
6
8
10
12
14
1H (ppm)

FIGURE 3

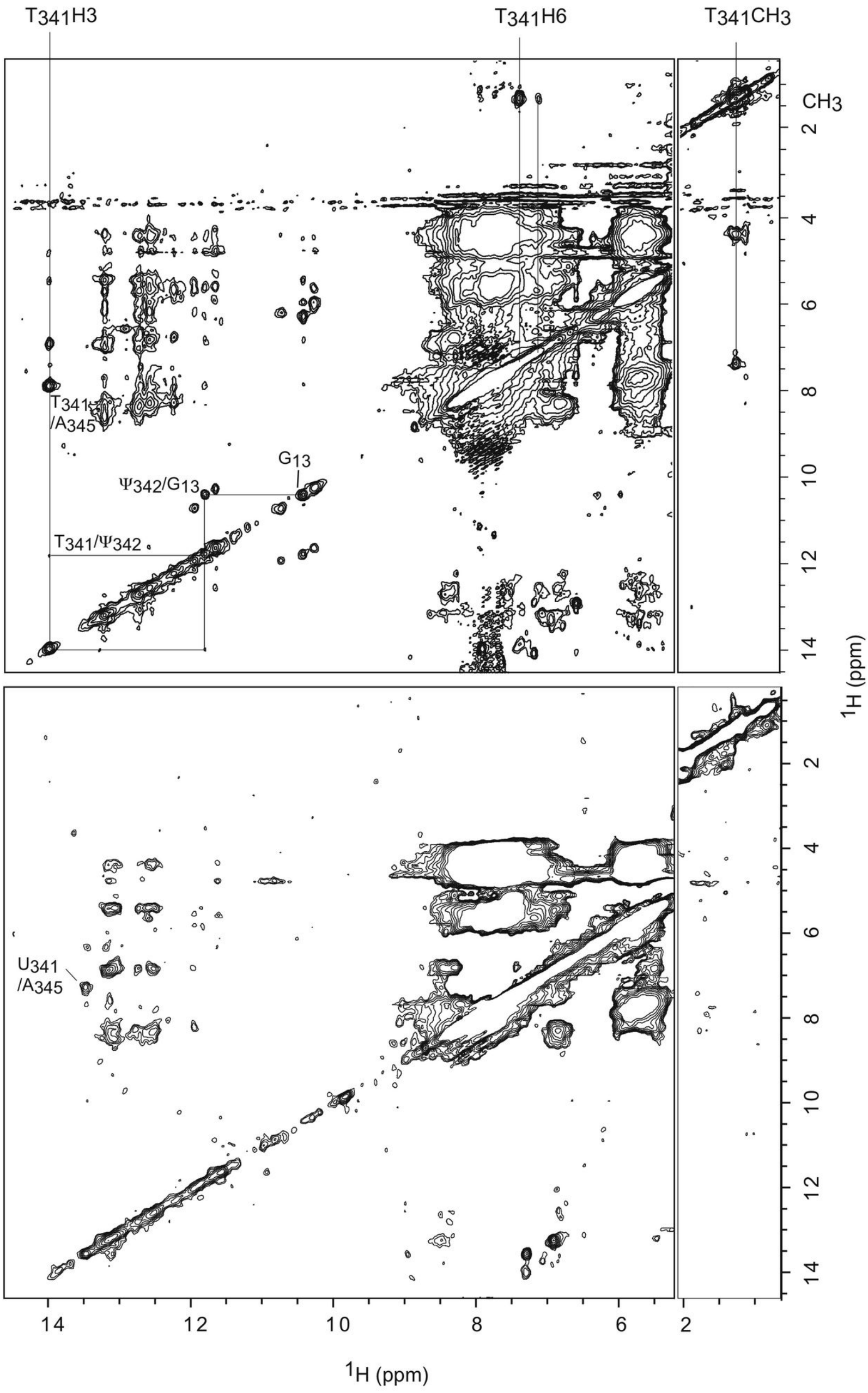

T341H3
T341H6
T341CH3
CH3
T341
/A345
G13
Ψ342/G13
T341/Ψ342
U341
/A345
14
12
10
8
6
2
1H (ppm)

FIGURE 4

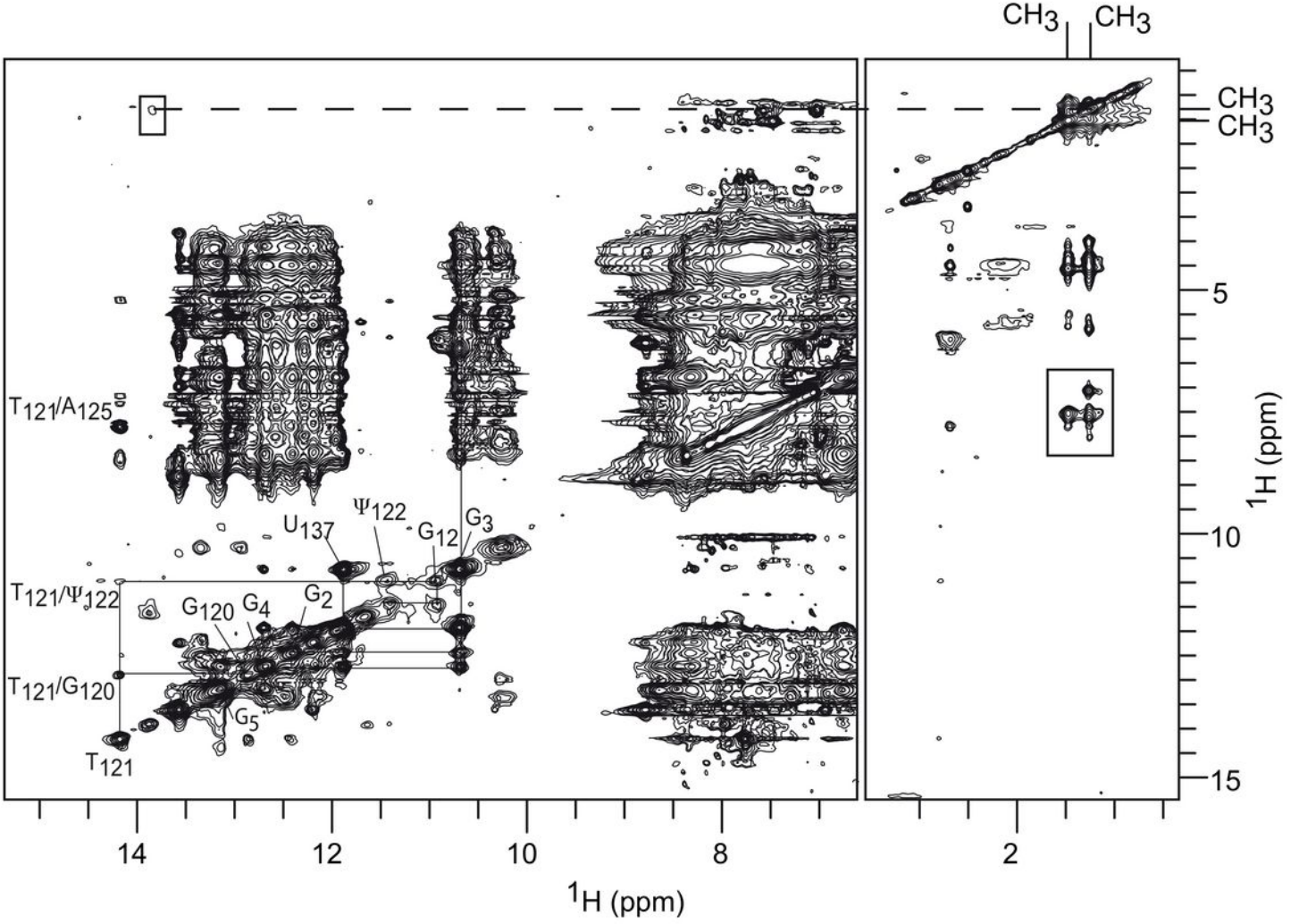

CH3
CH3
CH3
CH3
T121/A125
U137
Ψ122
G12
G3
T121/Ψ122
G120
G4
G2
T121/G120
G5
T121
14
12
10
8
2
5
10
15
1H (ppm)
1H (ppm)

FIGURE S1

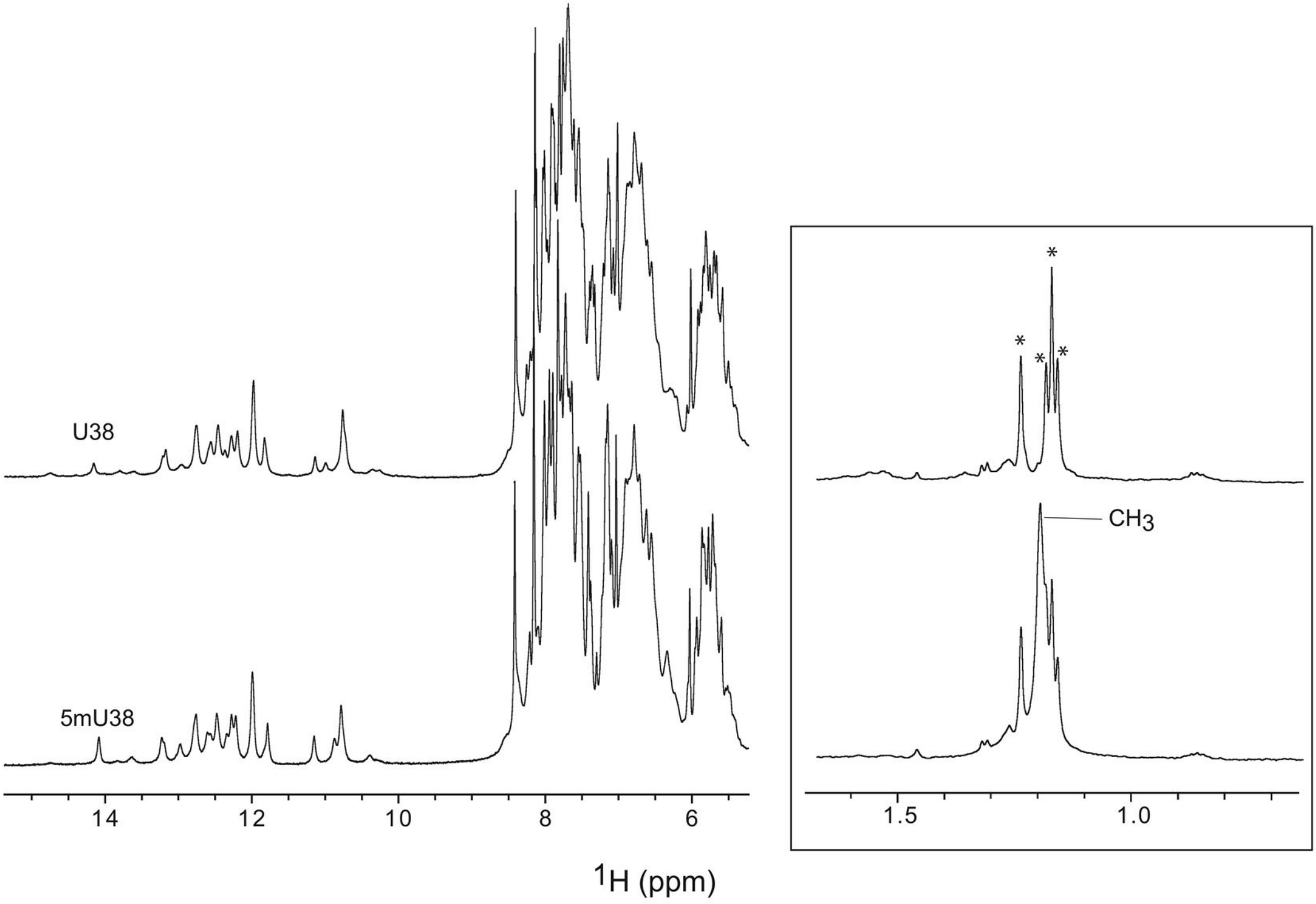
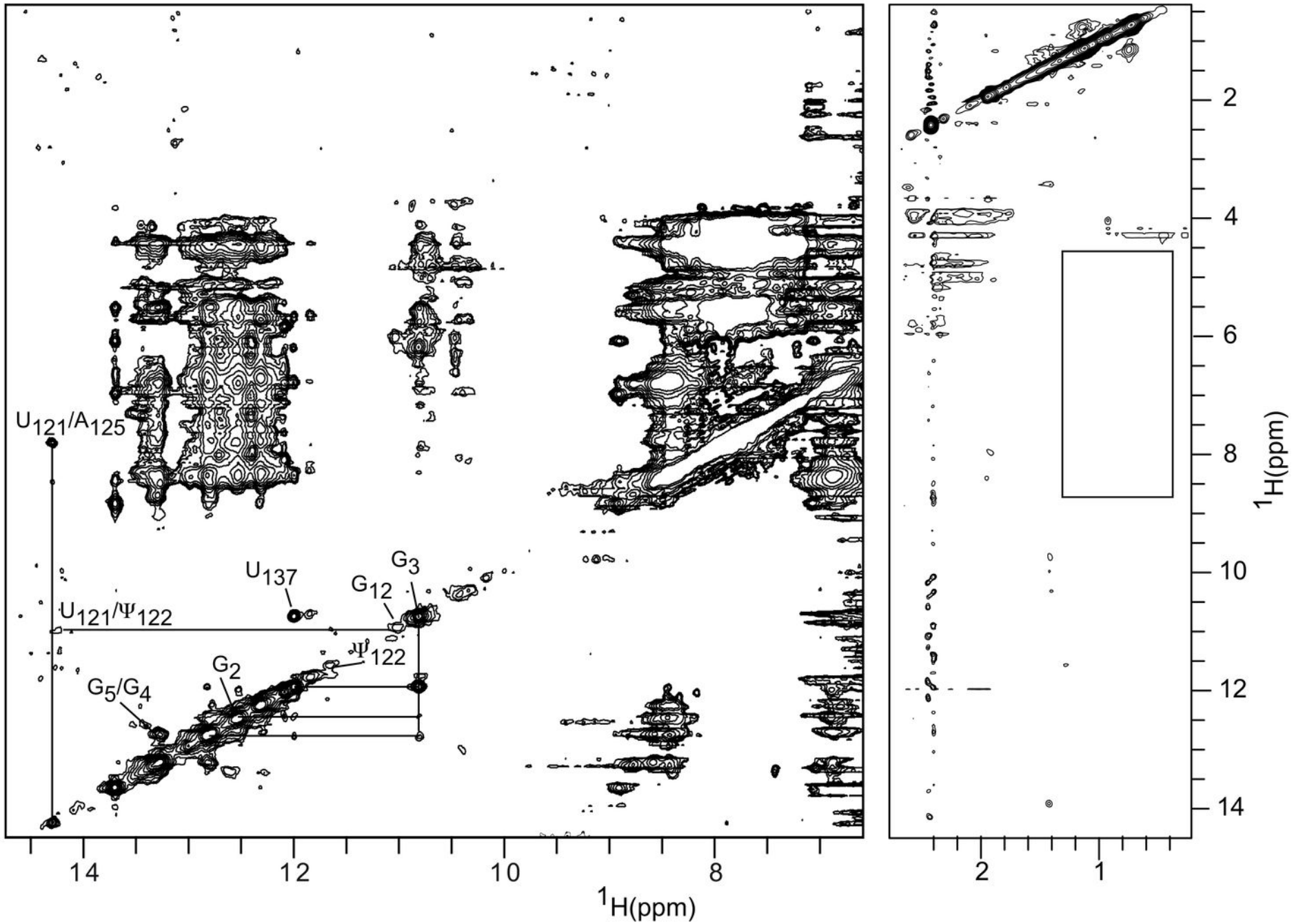


FIGURE S2


U121/A125
U137
G3
G12
U121/Ψ122
Ψ122
G2
G5/G4
14
12
10
8
1H(ppm)
2
1
2
4
6
8
10
12
14
1H(ppm)