Transcriptome-wide profiling of multiple RNA modifications simultaneously at single-base resolution

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The breadth and importance of RNA modifications are growing rapidly as modified ribonucleotides can impact the sequence, structure, function, stability, and fate of RNAs and their interactions with other molecules. Therefore, knowing cellular RNA modifications at single-base resolution could provide important information regarding cell status and fate. A current major limitation is the lack of methods that allow the reproducible profiling of multiple modifications simultaneously, transcriptome-wide and at single-base resolution. Here we developed RBS-Seq, a modification of RNA bisulfite sequencing that enables the sensitive and simultaneous detection of mC, ψ, and mA at single-base resolution transcriptome-wide. With RBS-Seq, mC and mA are accurately detected based on known signature base mismatches and are detected here simultaneously along with ψ sites that show a 1–2 base deletion. Structural analyses revealed the mechanism underlying the deletion signature, which involves ψ-monobisulfite adduction, heat-induced ribose ring opening, and Mg2+-assisted reorientation, causing base-skipping during cDNA synthesis. Detection of each of these modifications through a unique chemistry allows high-precision mapping of all three modifications within the same RNA molecule, enabling covariation studies. Application of RBS-Seq on HeLa RNA revealed almost all known mC, mA, and ψ sites in tRNAs and rRNAs and provided hundreds of new mC and ψ sites in noncoding RNAs and mRNAs. However, our results diverge greatly from earlier work, suggesting ~10-fold fewer mC sites in noncoding and coding RNAs and the absence of substantial mA in mRNAs. Taken together, the approaches and refined datasets in this work will greatly enable future epitranscriptome studies.

RNA modification | pseudouridine | RNA methylation | mA | methyl adenosine

Covalent modifications of RNA are numerous (1), and transcriptome-wide profiling enables broad and systematic analyses (2–4). Thus far, transcriptome-wide profiling has been reported for a limited number of modifications including N6-methyladenosine (mA), 5-methylcytosine (mC), pseudouridine (ψ), and N4-methyladenosine (mA) (5–14). However, profiling methods that provide sensitive and true single-base resolution are currently available only for mC (9, 14, 15) and mA (16); three of these (mA, mA, and ψ) have involved initial enrichment or detection via antibodies (for mA or mA) (5, 6, 8, 10) or by techniques involving polymerase pausing/termination during reverse transcription (for mA and ψ) (7, 11–13, 17, 18). Recent single-base techniques for ψ (19) rely on a bulky adduct formation before detection. Furthermore, although the current methods for ψ profiling are useful, most lack the sensitivity, resolution, and technical ease needed for widespread adoption or straightforward candidate site validation (7, 11–13, 20). To provide simultaneous detection of mC, mA, and ψ at single-base resolution transcriptome-wide from the same sample, we developed a molecular approach and analysis pipelines for ψ and improved sequencing-based methods for mC and mA.

First, we provide the conceptual basis for sequencing/mismatch-based detection of all three modifications (Fig. 1A) and an example tRNA (glycine) that illustrates modification clarity within our HeLa cell dataset (Fig. 1 B and C, with multiple additional examples in SI Appendix, Figs. S1–S3).

Detection of mC in RNA (and DNA) relies on differential sensitivity to bisulfite: unmethylated cytosine is efficiently denatured by bisulfite ions converting cytosine to uridine, which is subsequently read as thymidine following desulfonation, RT-PCR, and sequencing. In contrast, mC resists bisulfite and remains cytosine after sequencing (15, 21) (Fig. 1A). We improved prior mC profiling methods by combining heat and the strong chemical denaturant formamide, which improves RNA denaturation and bisulfite treatment (which preferentially modifies single-stranded RNA), providing a global C → T conversion.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE90963). All custom computer scripts reported in this paper have been deposited in GitHub, https://github.com/HuntsmanCancerInstitute/RBSSeqTools.

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frequency of 99.7% in HeLa RNA (SI Appendix, Figs. S4–S8). Optimization was quantified via synthetic RNA oligomers, with mC bases placed within and/or outside of regions of secondary structure (SI Appendix, Fig. S5). We applied RBS-Seq to HeLa RNA, using three types of input RNA species [polyA-selected (~85 M reads), rRNA-depleted (~200 M reads), and small RNA (~92 M reads)] (22), and our analysis pipelines (SI Appendix, Fig. S9) (23) compared datasets derived from bisulfite-treated (BS) and non–bisulfite-treated (NBS; untreated) RNAs of the same sample, a regimen which reduces false positives generated by incorrect alignments resulting from reduced nucleotide complexity. We then aggregated results from all three input types and filtered out additional false positives via computational and visual inspection for C/G tracts and strong secondary structure and imposed thresholds for nonconversion [≥20% (FDR < 0.05)] (SI Appendix, Fig. S9). This combination of approaches and thresholds yielded a list of high-threshold candidate sites in the following RNA categories: 486 total mC sites, representing 297 unique sites in abundant noncoding RNAs (tRNAs and rRNA, together), 143 sites in mRNAs (e.g., PTEN and HDGF; Figs. 2A and B and 3A, SI Appendix, Fig. S24, and Datasets S1 and S2), 14 pseudogenes, and 32 other noncoding RNAs. New sites within prominent mRNAs include PTEN, XRCC3, FANCA, RXRB, FGFR4, and EIF3B (Dataset S1). Importantly, examination of known/validated sites in tRNAs demonstrated that RBS-Seq has a dynamic range for mC approaching 100% at single cytosines (e.g., C49 in Fig. 1C). Although our read numbers exceeded prior studies, our yield of 486 high-threshold candidate mC sites in mRNA was far lower than the 10,275 sites reported previously (14), largely due to our more effective denaturation and deamination/conversion, lowering false positives (SI Appendix, Figs. S10 and S11, and Dataset S1). In keeping, more recent mC profiling in mouse ESCs that applies additional statistical and analytical parameters to remove false-positives reports 266 sites in mRNA passing thresholds (24).

Unlike mA, mA compromises A/T Watson–Crick base pairing, which pauses reverse transcriptase and elicits frequent nucleotide misincorporation, generating a single-base mismatch signature useful for mA identification (8, 17, 25). As expected, in our NBS datasets from RBS-Seq, we indeed detected significant (FDR < 0.01) mA-related mismatches at well-known mA sites in noncoding RNAs [e.g., mA-1322 in 28S rRNA (Fig. 2C and SI Appendix, Fig. S2B) and mA-58 in all tRNAs (Fig. 2D and Dataset S3)]. Unexpectedly, these mismatches were wholly absent or greatly diminished in our BS datasets (SI Appendix, Table S3), with tRNAs displaying the remarkable dynamic range of our method (~90% for tRNA\textsuperscript{18S} and tRNA\textsuperscript{16S}; Fig. 2D). Regarding the basis, conversion of mA to mU (involving transfer of the methyl group from N\textsuperscript{6} to N\textsuperscript{2}), which faithfully templates thymine (cDNA remains adenine) in the BS sample. Thus, comparison of NBS and BS samples identifies mA sites. For \(\Psi\) and \(\Psi\) nucleotides upon bisulfite treatment form a stable monobisulfite adduct (Fig. 4) causing frequent bypass with reverse transcriptase, leaving a deletion signature at the exact modified sites, evident only in the BS sequence. For mA, cDNA synthesis at sites with mA often confers misincorporation/mismatch in the BS sample. In contrast, during treatment, m\textsuperscript{A} becomes converted to m\textsuperscript{A} via Dimroth rearrangement (methyl passes from N\textsuperscript{2} to N\textsuperscript{6}), which faithfully templates thymine (cDNA remains adenine) in the BS sample. Thus, comparison of NBS and BS samples identifies mA sites. For \(\Psi\) and \(\Psi\) modified sites. (C) Bar graph summarizing the actual RBS-Seq results from HeLa cell line for a tRNA\textsuperscript{A\textsubscript{\textsuperscript{\textgamma}}} locus indicating the exact locations of the modified nucleotides and their levels. The low levels of mC sites shown at positions 40 and 60–66 have been shown previously for a subset of tRNA types (31, 32).
Sites in prominent mRNAs include SMC2, EIF3D, POLE4, LM07, CCDC22, ATP5F1, and TRIM8 (Dataset S5).

Four groups have previously conducted transcriptome-wide Ψ profiling reports under different names: Pseudoseq (7) and Ψ-seq (13) (using yeast and mammalian cells), PSI-seq (12) (yeast), and CeU-Seq (11) (mammalian cells). All four methods share the same principle: treatment of RNA with the chemical N-cyclohexyl-N′-(2-morpholinoethyl)-carbodiimide-metho-p-toluenesulfonate (CMC), which leaves a bulky group on pseudouridines, stopping reverse transcriptase during cDNA synthesis, thus indicating sites of pseudouridylation globally via RNASeq. These CMC-based techniques have proven useful for identifying Ψ sites, especially in high-abundance RNAs, and for identifying candidate Ψ sites in mRNAs. However, despite utilizing similar methods and identical yeast strains, overlap between candidate sites was extremely low (typically <4%), and was equivalently low when we compared published CMC-based results in mammalian cell types (typically HeLa or HEK293) (20) (SI Appendix, Fig. S20, and Dataset S7). Interestingly, candidate Ψ sites from RBS-Seq using HeLa cells overlapped better with prior CMC-based studies (either HeLa or HEK293) than did the prior studies with themselves (SI Appendix, Fig. S21, and Dataset S7), consistent with RBS-Seq revealing a higher proportion of positives.

To better understand these differences, we turned to validation approaches. CMC-based methods that rely on cDNA chain termination for mapping Ψ sites present challenges for validation, requiring quantities of pure target RNA beyond feasibility for most mRNAs; thus, most prior studies either lacked validation, or verified only very low (fewer than five) highly abundant candidate sites (11, 12). RBS-Seq, in contrast, provides a straightforward high-throughput validation protocol easily adapted to mRNA because the Ψ-dependent deletion signatures that appear within the corresponding CDNA are easily scaled up through gene-specific PCR amplification and quantified by high-throughput sequencing of barcoded amplicons. Thus, for validation comparisons to prior studies, we tested 60 candidate sites, which we partitioned into four groups: group I, sites uniquely identified by RBS-Seq (12 sites); group II, sites shared between RBS-Seq and at least one CMC-based method (25 sites); group III, sites detected in at least one of the CMC-based methods but not identified by RBS-Seq due to falling below our read coverage thresholds (14 sites); and group IV, sites from one or more CMC-based methods but not RBS-Seq, despite sufficient coverage in RBS-Seq (nine sites) (Dataset S8). For validation tests, we treated HeLa or HEK293 total RNA to a streamlined bisulfite + heat + MgCl2 protocol via chemistry described below followed by RT-PCR involving barcoded ~125-bp amplicons (on average), sequencing (termed RBS-MiSeq), and our deletion signature analysis pipeline (SI Appendix, Fig. S22A). Notably, the vast majority (34 of 37) of group I and II sites validated, yielding a clear deletion signature involving tens of thousands of sequenced reads with HeLa and/or HEK293 datasets, providing confidence in sites identified by RBS-Seq. For group III, 10 of 14 validated, suggesting that increasing the sequencing depth and/or applying our focused validation approach can resolve the rare false negatives generated by RBS-Seq. Finally, none of the candidates in group IV (0 of 9) validated, strongly suggesting a much higher false positive rate with any of the CMC-based techniques alone compared with RBS-Seq, consistent with their low overlap in prior studies. Examples of each group are provided in SI Appendix, Fig. S22B, and complete results are provided in Dataset S8.
To independently test our Ψ profiling approaches and results, we examined Dyskerin (DKC1), the most disease-relevant human Ψ synthase. Mutation of DKC1 causes dyskeratosis congenita, characterized by short telomeres and bone marrow failure (30, 31). DKC1 utilizes H/ACA box snoRNAs to guide Ψ targeting to rRNAs via base pairing between the snoRNA and the target tRNA (32), and DKC1 also interacts with telomerase noncoding RNA (TERC) (33, 34), but whether TERC receives substantial Ψ is uncertain (35). To resolve this issue, high-throughput RBS-Seq followed by deletion signature analysis was performed on both total and polyA-selected RNAs isolated from DKC1-depleted HeLa cell via siRNAs, yielding ~84% reduction in DKC1 transcript levels (SI Appendix, Fig. S22 C and D). Comparison of the DKC1-siRNA with control-siRNA data-sets revealed significant reduction (>25% reduction, FDR < 0.01) of the deletion signature levels in 227 sites; most reside within rRNAs, although 18 sites were observed within mRNAs (SI Appendix, Fig. S22 E and F, and Dataset S9). Curiously, the 58 DKC1-dependent sites in HEK293 mRNAs reported by Ψ-seq show no overlap with the 18 sites found in HeLa mRNAs by RBS-Seq. Moreover, because Ψ-seq but not RBS-Seq reported two DKC1-dependent Ψ sites within TERC in HEK293 cells (13), we specifically tested TERC at both sites with our streamlined RBS-MiSeq validation procedure in both HeLa and HEK293 cells. Notably, despite over 30 K reads overlapping both sites in both cell types, no significant deletion was observed (Dataset S8), suggesting that TERC is an interacting partner of DKC1 but not a direct pseudouridylation substrate in these cell types under the conditions tested.

Finally, to elucidate the chemistry of the observed 1–2 base deletion signature at Ψ and to guide validation methodologies, we determined which step(s) of our RBS-Seq protocol elicited base deletion by utilizing a synthetic 70-mer oligonucleotide bearing two Ψ sites and quantifying base deletion frequencies. Strikingly, bisulfite treatment alone failed to induce any deletion signature, whereas heating (75 °C) the BS RNA in the presence of magnesium ions (20 mM) for 15 min was both necessary and sufficient for generating the penetrant deletion signature (Fig. 4 A–C).

![Fig. 4. Characterization of a pseudouridine-bisulfite adduct and heat/MgCl₂-induced rearrangement to elicit reverse transcriptase bypass.](#)

A. Sequence and intramolecular folding of pseudouridylated 70-mer RNA oligonucleotide used in the downstream experiments. The two Ψ sites (in red) are indicated by arrowheads.
B. Flowchart of oligo treatments, RT-PCR, TA cloning, and Sanger sequencing of individual colonies.
C. Summary of the deletion signatures obtained from oligonucleotide experiments with the reference sequence and the two Ψ sites at the bottom, showing the insufficiency of the bisulfite step, and requirement for the subsequent heat + MgCl₂ step to generate the deletion signatures. D. Sequence and calculated mass for 12-mer control (12-U) and pseudouridylated (12-Ψ) oligomers used in the downstream experiments. E. Reaction sequence and methods used for Ψ reactivity studies with 12-mers. F. Mass spectrum for 12-Ψ after bisulfite and subsequent heat + MgCl₂ treatments shows formation of a stable monobisulfite adduct. Mass spectrum for the 12-U and 12-Ψ with only bisulfite treatment is provided in SI Appendix, Fig. S24. G. A proposed model showing that during cDNA synthesis, ribose ring-opened Ψ-monobisulfite is oriented away from the polymerization site, reinforced by Mg²⁺, explaining base skipping.
and SI Appendix, Figs. S24–S27). Similar treatments with a synthetic 12-mer RNA oligonucleotide containing a single Ψ followed by MS analysis displayed a major peak consistent with a stable, monooxidant of bisulfite (Fig. 4 D–F and SI Appendix, Fig. S28). Next, the site of covalent attachment of the bisulfite group was determined by exposing the Ψ nucleoside itself to the reaction sequence followed by structural analysis (SI Appendix, Fig. S23A). Notably, the nucleoside reaction analyzed by LC-MS revealed two stable monobisulfite adducts (SI Appendix, Figs. S29 and S30). The UV-vis for these compounds displayed $\lambda_{\text{max}} \sim 260$ nm (SI Appendix, Fig. S31), supporting the aromaticity of the base remaining intact after the reaction. On the basis of $^{1}H$-NMR, the bisulfite attachment was determined to be on the $^1$C carbon, which involved an opening of the ribose ring to yield a pair of isomeric products, consistent with prior preliminary results (SI Appendix, Fig. S32) (36). We propose initial addition of bisulfite to the base, followed by a heat-induced migration of the bisulfite to the ribose, yielding recarboxylation and the formation of a stable ring-opened sugar adduct (SI Appendix, Fig. S23B). Notably, previous studies examining polymerase bypass of similar ring-opened template sites reported a strong tendency for deletion under the same conditions. Interestingly, although Mg$^{2+}$ was an absolute requirement for generation of the deletion signature assessed by cDNA sequencing (Fig. 4 C), it proved dispensable for generating the ring-opened sugar adduct in the nucleoside experiments, suggesting that Mg$^{2+}$ helps reorient the ribose ring-opened Ψ bisulfite adduct away from the polymerization site or stabilizes a preexisting configuration that eventually causes polymerase bypass (Fig. 4 G).

**Discussion**

This work provides five advances in RNA modification profiling: (i) improved methods for profiling m$^4$C and m$^1$A; (ii) quantitative methods for profiling Ψ sites at true base resolution transcriptome-wide; (iii) a chemical understanding of the Ψ-dependent deletion signature; (iv) a coupling of these methods for the simultaneous detection of all three modifications in the same sample, which has provided hundreds of candidate sites of modification; and (v) a streamlined Ψ candidate site validation procedure for bulk verification of dozens of candidate sites in the same sample. Together, the combinatorial ability and relative ease of execution provided by this procedure should greatly forward epitranscriptome studies involving these three very common RNA modifications, and the refined lists of high-threshold mapped sites in HeLa cells should enable better-focused downstream functional studies. Furthermore, because RBS-Seq also provides transcript abundance (like a typical RNA-seq), the combined outputs present a multidimensional (4D) dataset that may prove useful both for basic investigations and diagnostic settings.

**Methods**

Detailed methods are provided in SI Appendix, SI Methods.

**Cell Culture (Including siRNA Treatment).** HeLa cells were seeded in 100-mm plates at 2 $\times$ 10$^5$ per plate in DMEM (Gibco) containing 4.5 g/L d-glucose, l-glutamine, and 110 mg/L sodium pyruvate and supplemented with 10% FBS. At ~75% confluency, cells were harvested via TrypLE Express (Gibco) and washed once with 1X PBS, pH 7.4 (Gibco).

For the DKC1 depletion experiment, siRNA treatment was performed in two sequential transfections per sample, 72 h apart. HeLa cells were seeded at 3 $\times$ 10$^6$ per well and transfected with Lipofectamine RNAiMAX (Invitrogen) and 60 pmol of siRNA (either Dharmacon’s siGENOME Human DKC1 siRNA for the DKC1 knockdown or Dharmacon’s siGENOME non-targeting siRNA pool no. 1 for the control sample). Cells were split and reseeded 48 h after the first transfection and harvested 72 h after the second transfection.

**RNA Isolation and Preparation.** Total RNA from the above samples was isolated using TRIzol Reagent (Invitrogen) and split. RNA depleted samples were obtained via RiboMinus Transcriptome Isolation Kit for human/mouse (Ambion). The polyA-selected sample was isolated from total RNA using polyA Spin mRNA Isolation Kit (New England Biolabs). Small RNA (enriching transcripts <200 bp) was isolated using mirVana miRNA Isolation. See SI Appendix, SI Methods, for RNA fragmentation.

**Bisulfite Treatment.** Processed RNA was denatured by incubating 45 μL RNA (5 μg) in 240 μL denoised formamide at 95 °C for 5 min before chilling on ice. For the sulfonation step, 312 μL of freshly prepared 5 M sodium bisulfite (pH 5) with 3 μL 100 mM hydroquinone was added to each denatured sample and incubated at 50 °C. To desulfonate, each sample was purified on Illustra NAP-10 columns (GE) and incubated in 2 M Tris buffer, pH 9.0 (Trizma Preset crystals, pH 9.0: Sigma-Aldrich), for 2 h at 37 °C. The RNA was recovered by ethanol precipitation.

**Library Preparation and Sequencing.** For the transcriptome-wide study and for the DKC1 depletion experiment, we used the Illumina Tru-Seq Small RNA kit to generate paired-end libraries. The resulting libraries were sequenced in a 101-cycle paired-end format on an illumina HiSeq 2000 instrument.

**Validation of Candidate Ψ Sites by RBS-MSiSeq.** PCR amplification yielded ~300 bp regions surrounding each of 60 candidate sites via a primer design compatible with bisulfite, in which all unmethylated Cs have been converted to Ts. In addition, TruSeq-set-dependent primer sets, which were then pooled and used for MSiSeq DNA library preparation using different barcodes for HeLa or HEK293 sets according the illumina protocols. Libraries were sequenced on a MSiSeq instrument.

**Bioinformatics Methods.** Transcriptome-wide sequencing reads from BS, NBS, and the DKC1-1 experiments were aligned using Novoalign (Novocraft) to standard and bisulfite reference index of hg19 chromosome, scaffold and splice junction sequences, accommodating repeat reads, and trimming adaptor sequences. Reads mapping to certain small and repetitive RNA classes (RNA, rRNA, snRNA, and snoRNA) were extracted and realigned to a custom reference containing only unique representative sequences of the above. All alignment files were processed identically. The processed alignments were then analyzed using custom scripts (https://github.com/HuntsmanCancerInstitute/ RBSSeqTools) to generate tables of candidate sites for each individual modification based on the criteria as listed. For m$^4$C, we selected only those sites from all reference Ψ positions which had a read depth ≥10 in both BS and NBS datasets, a C→T nonconversion rate of ≥20% in the BS dataset, and an FDR ≤0.01 for nonconversion sites. Adjacent sites were further memoized in a custom Python script, which involved an opening of the ribose ring to yield a pair of isomeric products, consistent with prior preliminary results (SI Appendix, Fig. S32) (36). We propose initial addition of bisulfite helps reorient the ribose ring-opened Ψ bisulfite adduct away from the polymerization site or stabilizes a preexisting configuration that eventually causes polymerase bypass (Fig. 4 G).

**Investigating the Source of the Deletion Signature for Ψ.** A synthetic 12-mer RNA strand (10 nmols), 5’-GCU ACG TAC UAG-3’, was bisulfite treated (as described above) and dialyzed against ddH$_2$O at 36 h at 4 °C, and the water was changed every 8 h. After the 36-h dialysis, the sample was then dialyzed against ddH$_2$O containing 3 mM NH$_4$OAc for 36 h at 4 °C, and the NH$_4$OAc solution was changed every 8 h, then lyophilized to dryness and resuspended in 30 μL of 1 mM NH$_4$OAc and 30 μL of isopropanol. The adducted

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RNA sample was analyzed by ES−MS to yield an experimental mass (3880.8) consistent with a monobisulfite adducted RNA strand (calcld = 3880.3).

**Structural Analysis of the Monobisulfite Adduct to the Pseudouridine Nucleoside.**

All chemicals were obtained from commercial suppliers. The NaHSO$_3$ solution was freshly prepared before reaction as a 5 M stock solution (pH 5.0). The pseudouridine nucleoside (20 mM) was allowed to react with 3 M NaHSO$_3$ at pH 5.0 for 16 h at 50 °C to give a product yield of ~90%. The reaction was analyzed using a Hypercarb HPLC column running a mobile phase combination of A = 20 mM NaOAc (pH 7) and B = 1 M Li$_2$OAc (pH 7) and B = 1 M Li$_2$OAc with a flow rate = 1 mL/min while monitoring the elution profile at 220 and 260 nm. The method was held at 0% B for 5 min, after which B was changed to 95% B over 20 min via a linear gradient. The two product peaks were collected, dried, and submitted to mass spectrometric analysis in which they gave masses consistent with monoaducts of bisulfite to the nucleoside (calcld mass [M-H]− = 325.27 and the second isomer = 325.07). The purified samples were analyzed by UV-vis in d$_6$D$_2$O buffered with 20 mM NaP,

showing the first eluting peak named isomer 1 to have a λ$_{max}$ = 265 nm and the second eluting peak named isomer 2 to have a λ$_{max}$ = 266 nm. In a final experiment, the purified compounds were analyzed by $^1$H-NMR: isomer 1 (500 MHz, D$_2$O) δ 7.60 (s, 1 H), 4.40 (s, 0 Hz, 1 H), 4.33 (dd, 8.32 Hz, 1 H), 3.67 (m, 2.45 Hz, 1 H), 3.61 (dd, 2.17 Hz, 1 H), 3.45 (dd, 7.33, 6.85, and 4.40 Hz, 1 H), and 3.22 (dd, 4.89 and 2.93 Hz, 1 H) and isomer 2 (500 MHz, D$_2$O) δ 7.61 (s, 1 H), 4.39 (d, 6.85 Hz, 1 H), 3.98 (t, 6.85 and 6.35 Hz, 1 H), 3.75 (t, 5.87 Hz, 1 H), 3.65 (dd, 3.91 and 2.93 Hz, 1 H), 3.58 (dd, 3.42 and 2.94 Hz, 1 H), and 3.44 (dd, 6.85 and 4.89 Hz, 1 H).

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