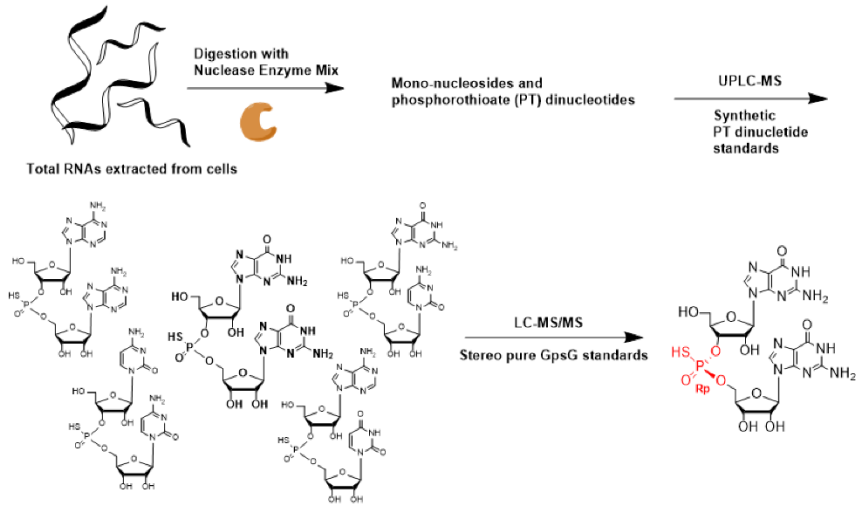
Digestion with
Nuclease Enzyme Mix
Mono-nucleosides and
phosphorothioate (PT) dinucleotides
UPLC-MS
Synthetic
PT dinucletide
standards
Total RNAs extracted from cells
LC-MS/MS
Stereo pure GpsG standards
Rp

TOC graph

RNA Phosphorothioate Modification in Prokaryotes and Eukaryotes

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Supporting Information Placeholder

ABSTRACT: RNA modifications play important roles in RNA structures and regulation of gene expression and translation. We report the first RNA modification on the phosphate, the RNA phosphorothioate (PT) modification, discovered in both prokaryotes and eukaryotes. The PT modification is also first reported on nucleic acids of eukaryotes. The GpsG modification exists in the Rp configuration and was quantified with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). By knocking out the *Dnd*A gene in *E*. coli, we show the *Dnd* clusters that regulate DNA PT modification may also play roles in RNA PT modification.

Post-transcriptional modifications of cellular RNAs play important roles in both normal and disease cells.[1,2] Over 150 distinct post-transcriptional RNA modifications have been identified. Beyond simply fine-tuning the structure and function of RNA, emerging studies have linked RNA modifications to various disease syndromes such as cancer and neurological disorder.[2] However, all the RNA modifications known to date are either on the ribose or on nucleobases. To the best of our knowledge, no natural modification has been reported on the phosphate backbone of RNA thus far.

The phosphorothioate (PT) modification on DNA has been discovered in bacteria,[3-6] and more recently in archaea[7]. In a phosphorothioate linkage, a sulfur atom replaces one of the non-bridging oxygen atoms and renders the stereogenic phosphorus center. The DNA phosphorothioate modifications in bacteria and archaea are known to be in the Rp configuration, with an occurrence of 4-31 PT modifications per 10^4 deoxynucleotides.[3,5] The DNA phosphorothioation is the product of the *Dnd* gene clusters *Dnd*A-E. Together with the DNA PT modification-based restriction enzymes by *Dnd*FGH, the *Dnd* genes constitute a restriction-modification system that protects bacteria from invading foreign DNA. The cellular functions of DNA PT modifications in bacteria and archaea also include the maintenance of cellular redox homeostasis,[3,8] cellular stress response,[4] and the epigenetic control of gene expression.[3] However, there is no report on PT modifications in eukaryote to date.

In the rapidly growing molecular-based gene therapy field, phosphorothioate modified oligonucleotides are of central importance for therapeutic strategies that employ microRNA, small interfering RNA, and antisense oligonucleotides.[9] Most of the aforementioned therapeutic oligonucleotides approved by the U.S. Food and Drug Administration (FDA) or currently under clinical trials utilize phosphorothioate modifications to improve the metabolic stability and cellular uptake.[10,11] The traditional synthesis of phosphorothioate oligonucleotides based on phosphoramidites affords a mixture of stereoisomers. Recently, the stereogenic synthesis of phosphorothioate oligonucleotides have been significantly improved,[12-16] which will have huge impacts on the future therapeutic developments.

Despite the wide investigations on DNA phosphorothioate modifications and synthetic phosphorothioate oligonucleotides, the existence of RNA phosphorothioate modification remains unknown. Since RNAs and RNA epitranscriptomics play central roles in numerous cellular processes,[17] research on RNA PT modification and the related biological functions will also benefit the drug development in gene therapeutics, in addition to expanding our understandings of RNA biochemistry and cell biology. For instance, if the phosphorothioate modification occurs on RNA, the biological functions of RNA PT modification might impact the pharmacology studies of phosphorothioate oligonucleotide-based gene therapies.[18] Encouraged by the significance of RNA phosphorothioate modification in both fundamental and health-related science, we set out to search the RNA PT modification with mass spectrometry.

In the present work, we discovered the RNA PT modifications in both prokaryotes and eukaryotes. We first digested synthetic RNA oligonucleotides containing PT modification on certain locations with a mixture of nuclease enzymes to study the kinetics of the RNA phosphorothioate hydrolysis (Supplementary Figure S7). After identifying the optimal reaction conditions in which the normal phosphodiester bonds are hydrolyzed while the phosphorothioate bonds are intact, we then digested the total RNAs extracted from various types of cells including bacteria E.coli and L.lactis, model systems such as Drosophila melanogaster S2 cells, and mouse liver tissues, as well as Hela cells and HCT116 cells. After digesting the total RNA with a nuclease enzyme mixture, the resulting mono-nucleosides and anticipated phosphorothioate dinucleotides, together with 16 synthetic RNA dinucleotide standards linked by phosphorothioate bond, are subjected to ultra-performance liquid chromatography coupled with mass spectrometry(UPLC-MS) for identifying the RNA PT modifications. As a result, we found the RNA PT modifications, just like the DNA counterpart, occur in a sequence-specific manner. For instance, CpsA, GpsC and GpsG are all found in the total RNA of L. lactis (Supplementary Chart S1).

Next, we chose to further study the GpsG modification (shown in Figure 1) as this dinucleotide occurs at the highest frequencies and exists across prokaryotes and eukaryotes (Supplementary Chart S1). The stereo pure GpsG dinucleotides were prepared by HPLC separation after phosphoramidite-based synthesis with 3-((N,N-dimethyl-aminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (DDTT) as the sulfurizing reagent on an automated solid-phase oligonucleotide synthesizer. The stereo configurations of GpsG-Rp and GpsG-Sp were assigned with ^{31}P-NMR (Supplementary Figures S3 and S4). The extinction coefficients at 254 nm of the GpsG-Rp and GpsG-Sp were determined by UV spectroscopy to be 6460.3 (AU*cm^{-1}*M^{-1}) and 5705.4 (AU*cm^{-1}*M^{-1}), respectively. LC-MS/MS has been widely used as a sensitive technique for the detection and quantification of nucleic acid modifications.[19-24] Synthetic GpsG-Rp was used to develop the LC-MS/MS method. As being expected, the LC retention time, ion fragments and ratios in the digested total RNA samples match with the data from our synthetic GpsG-Rp 643.084>227.056, 643.084>150.091, 643.084>133.125 in a negative electrospray ionization (ESI-) mode. (Supplementary Chart S2, Figures S8 and S9)

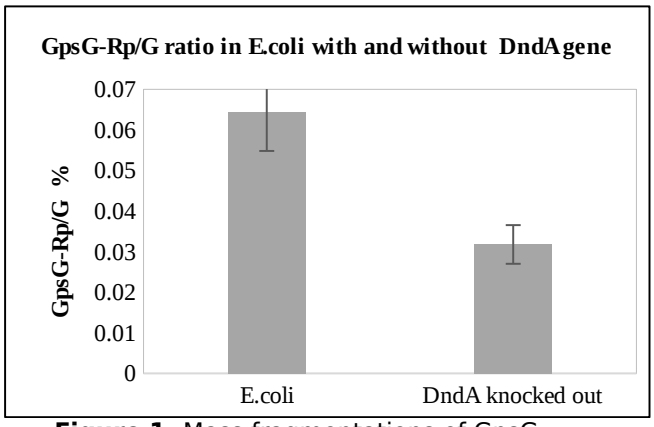
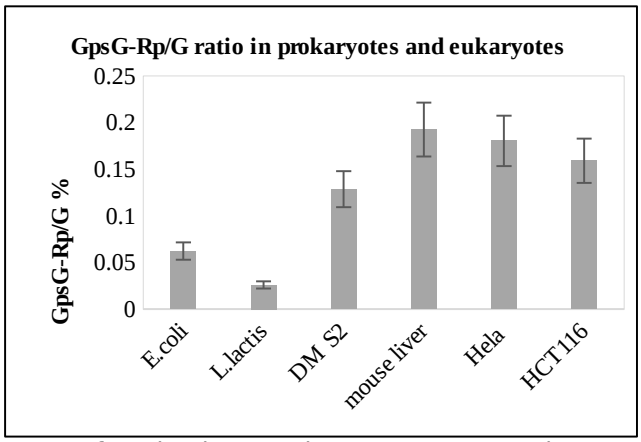


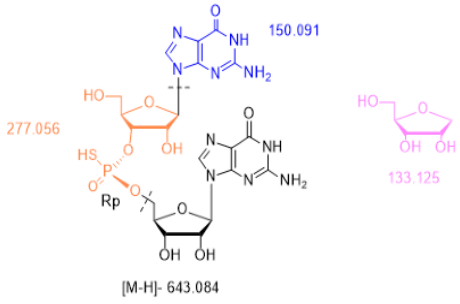
Figure 1. Mass fragmentations of GpsG.



We found the nuclease enzyme mixture, purchased from New England Biolab, in the experiments inhibits the hydrolysis of both GpsG-Rp and GpsG-Sp in synthetic oligonucleotides (Supplementary Figure S6). We observed GpsG-Rp modifications in E.coli, L.lactis, DM S2, mouse liver, Hela, and HCT116 cells. No GpsG-Sp modification was detected in these samples. Next, for the quantification of GpsG-Rp in cells, we digested the extracted total RNAs, subjected the samples for LC-MS/MS analysis, and obtained the concentrations of GpsG-Rp and guanosine respectively. The ratios of GpsG-Rp/G are shown in Figure 2.

Figure 2. GpsG-Rp modification in several types of cells.

Unlike the DNA PT modification, which is reported only in bacteria and archaea, we



discovered the RNA PT modification also exist in eukaryotes. This discovery expands the knowledge of the phosphorothioate modification in nucleic acids and attracted us to further investigate the enzymes responsible for the regulation of RNA PT modification. Since the PT modification GpsG also exists only in the Rp form and the abundance is similar to that of DNA PT modifications found in bacteria, it might be possible that this modification is regulated by the same set of enzymes. Due to these similarities, we set out to knock out the *Dnd*A gene, which is reported to provide the sulfur source in the biosynthesis of DNA PT bonds in bacteria.[3]

The lambda red homologous recombination method was chosen to manipulate the *Dnd*A gene in the E.coli BUN21/pML300 strain. The pML300 carries the genes encoding the exo, beta, and gam recombination functions of bacteriophage lambda, as well as RecA, to render the E. coli strain recombination proficient.[25] A bioinformatic search and DNA sequencing of the anticipated PCR products confirmed that BUN21/Pml300 strain harbors the *Dnd*A gene (GenBank accession number ZP 00714230)[6]. After recombineering,[26] the ampicillin-resistant gene replaces the *Dnd*A gene with 50-nucleotide homologous arms to delete *Dnd*A gene and serve as the selection marker. The *Dnd*A-knocked out colony was confirmed by DNA sequencing of the PCR amplified gene of interest. The BUN21/pML300 and DndA-knocked out strains were grown, the total RNAs were extracted and digested for GpsG quantification with LC-MS/MS.

The LC-MS/MS analysis of the GpsG (Figure 3) shows the modification level of the *Dnd*A-knocked out strain is less than that of the BUN21 strain by half. The result suggests *Dnd*A is also involved in the production of RNA PT modification. Previously, it was reported that DNA PT modification was also detected in bacteria strains lacking the DndA gene and this backbone thiolation was performed by other sulfur-providing enzymes such as cysteine desulphurase IscS.[27,28] Similarly in the RNA PT modification case, other enzymes in the *Dnd* gene cluster as well as other desulphurases will need to be investigated in both prokaryotes and eukaryotes, probably guided by the bioinformatic studies.

Figure 3. GpsG-Rp modification before and after *Dnd*A gene was knocked out in E.coli.

For future investigations, the distribution and abundance of this PT modification in mRNA, tRNA, and other non-coding RNAs in different cellular environments will shed light on its biological functions in both normal and diseased cells. Other closely related research topics, such as whether the RNA PT modification is permanent or reversible, its dynamic regulation mechanisms, its potential epigenetic functions, and the existence of PT modification combined with base or ribose modifications, will attract further investigations and the developments of other detection techniques and molecular tools[29] focusing on RNA PT modification. Meanwhile, the recently developed stereo-controlled synthesis of phosphorothioate oligonucleotides[16] could benefit the synthesis of longer sterically pure phosphorothioate standards to further investigate the PT modifications in forms of trinucleotides and beyond.

In summary, RNA phosphorothioate modification exists in both prokaryotes and eukaryotes. Our discovery expands the library of nucleic acid modifications and provides new opportunities to investigate the regulation of genetic information by the phosphorothioate modification. Because the phosphorothioate oligonucleotides are common in gene therapy, it also raises the significance of the cellular function of RNA PT modification for developing future gene therapeutics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Description of the synthesis and purification of oligonucleotide, phosphorothioate dinucleotide standards, extinction coefficient data, UPLC-MS and LC-MS/MS analysis, and DndA gene knock out with lambda-red homologous recombineering. (PDF) DNA sequencing result of knocking out the *Dnd*A gene. (AB1)

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Notes

The authors declare no competing financial interest.

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