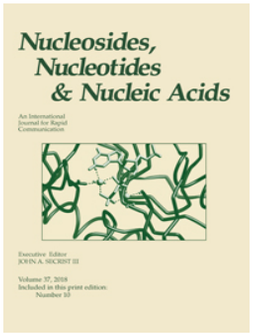

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Fluorescent labeling of s^2T-incorporated DNA and m^5s^2U-modified RNA

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ABSTRACT

We report herein comprehensive investigations of alkylation/sulfur exchange reactions of sulfur-containing substrates including nucleosides such as s^2U, m^5s^2U, s^4U, s^2A and s^2T-incorporated DNA enable by comprehensive screenings of the reagents (**2a–2h**). It has been proven that iodoacetamide (**2a**) displays the most promising feasibility toward sulfur-containing substrates including s^2T, s^2U, m^5s^2U, s^4U and s^2A. In sharp contrast, the alkylation process with S-benzyl methanethiosulfonate (BMTS, **2h**) displays the best application potential only for s^4U. Based on these results, the fluorescent labeling of s^2T-incorporated DNA and m^5s^2U-modified RNA has been achieved.

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Sulfur modification; s2T; m5s2U; alkylation; sulfur exchange

1. Introduction

Nucleic acids play fundamental roles in living organism. In addition to the four canonical nucleosides (adenosine, guanosine, cytosine and uridine), nucleic acids use chemical modifications to diversify their structures and functions.[1] Among the reported over 150 chemical modifications in a wide range of RNA species, it has been revealed that thio-modifications exist broadly in various cells (Fig. 1).[2] For instance, Liu et al. recently disclosed that ges^2U, mnm^5ges^2U and $cmnm^5ges^2U$ are identified in living organisms including *Escherichia coli, Enterobacter aerogenes, Pseudomonas aeruginosa* and *Salmonella enterico var. Typhimurium*.[3,4] Considering the roles of the sulfur-containing nucleotides of varied RNA species in regulation and enhanced translation efficiency and fidelity, the transcriptomics-based deep analysis of thio-modifications in nucleic acids show a great potential for illustration of its molecular mechanism although it remains elusive.[5,6] To that end, development of tools for selective labeling of thio-modified nucleic acids is of highly urgent.

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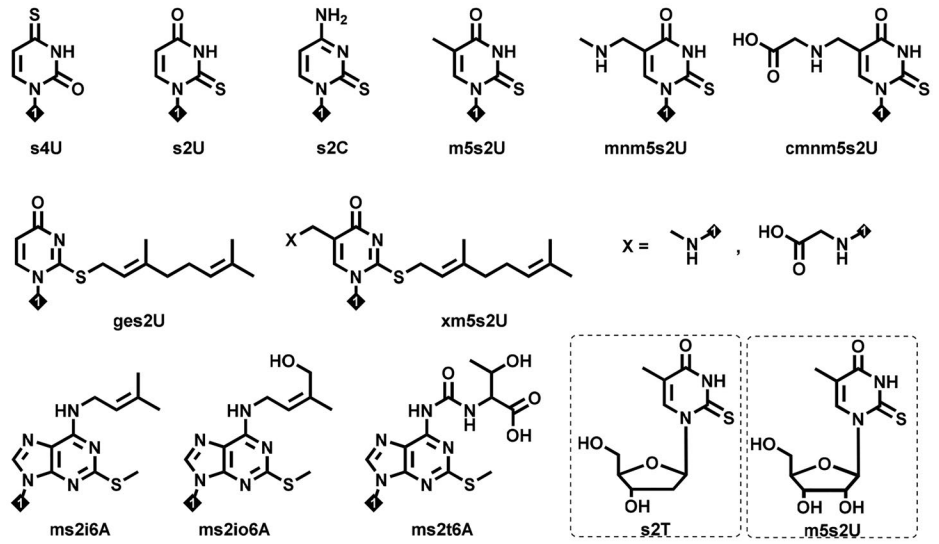

Figure 1. Overall illustration of sulfur-containing nucleosides. Nucleosides (s^2T and m^5s^2U) in black dotted are presented as model substrates.

It has been proven that most of the thio group in nucleosides (s^2U, s^4U and s^2A derivatives, etc.) have very close reactivity's with cysteine in proteins. It is known that sulfur-containing derivatives of nucleic acids are present in the tautomeric form thiol. Thio-modified nucleosides display in two or more tautomeric forms which can further undergo nucleophilic substitution reactions. To the regard, alkylation of thiolated nucleic acids with various nucleophiles have been demonstrated successfully.[7]

In 1973, Söll and coworkers have reported that 4-thiouridine reacted with the fluorescent reagent 4-bromomethyl-7-methoxy-2-oxo-2H-benzopyran under physiological condition, the selective labeling of the 4-thiouridine in *Bacillus* tRNA has been achieved.[8]

Later, s^4U has been demonstrated successfully in metabolic labeling studies. In more details, s^4U incorporated RNA were selectively enriched by use of streptavidin matrix HPDP-biotin (2-pyridylthio-activated biotin disulfide) via sulfur exchange reaction, which have been served as a practical method for biotinylation of s^4U-RNA.[9]

Duffy and coworkers have reported a chemically selective approach to label and enrich s^4U-modified RNA.[7] It has demonstrated that methanethiolsulfonate (MTS) reagents reacted with s^4U efficiently, leading to higher yields and less biased enrichment than conventional HPDP-biotin. However, although this method is useful, the application scope of other sulfur-containing nucleosides such as s^2T, s^2U, m^5s^2U and s^2A have not been clarified. Other method that can be applied for the studies of highly selective labeling or enrichment of sulfur-containing RNA/DNA molecules remain unknown.

Notably, mercury has also been used for labeling of thiolated RNA.[10–15] It is noted that organomercury functionalized agarose and mercury containing cellulose has been employed for the purification of thiolated RNA since 1977. Interestingly, it has been showed that the degree of retardation depends on the type of RNA modification with 4-thiouridine being more retarded than 2-thiouridine or 5-methylamino-methyl-2-thiouridine (mnm^5s^2U). Organomercury gels are now commonly used to study the position 34 thiolated uridines in tRNAs.[16,17]

In general, thiols are strong nucleophiles that could react with a wide range of electrophiles. For instance, iodoacetamides and bromoacetamides[18,19] have been employed to label s^2U- and s^4U-incorporated nucleic acids. However, reagents such as bromomethyl-coumarin[8] was reported to react with s^4U, the selectivity toward other thiolated nucleosides like s^2U, s^2A or s^2C remains unknown.

Therefore, intensive illustration of the intrinsic mechanism of thio-modified nucleosides is of highly urgent and necessary. We report here comprehensive investigations of nucleophiles used for highly selective labeling and enrichment reagent for studies of thio-modified nucleosides such as s^2T, s^2U, m^5s^2U, s^2A, s^4U and so forth. Based on preliminary screenings, we demonstrate successfully the fluorescent labeling of s^2T-incorporated DNA and m^5s^2U-containing RNA.

2. Results and discussion

To evaluate reactivities of reagents used in the studies of sulfur-containing nucleosides and further DNA and RNA molecules, we first chose thiouracil as a model substrate. As depicted in Scheme 1, a series of alkylation reagents including iodoacetamide (for **2a** derivative, see Supporting Information Figs. S1–S3), propargyl bromide (**2b**), prenyl bromide (**2c**), geranyl bromide (**2d**), 4-methylcumarin bromide (**2e**), acrylonitrile (**2f**, Supporting Information Figs. S4–S7),[20] vinyl sulfonate (**2g**, Supporting Information Figs. S8–S11) [21] and S-benzyl methanethiosulfonate (BMTS, **2h**, Supporting Information Figs. S12–S16) were gingerly investigated. It was not surprising that nucleophiles such as reagents **2a** and **2h** displayed unique dynamic properties. Comparing with allylic bromide (**2c–2e**, Supporting Information Figs. S19–S21, Charts S5–S10) or alkynyl analogue (**2b**, Supporting Information Fig. S18, Charts S3–S4), iodoacetamide (**2a**, Supporting Information Fig. S17, Charts S1 and S2) and derivative displayed higher conversion rate in sulfur alkylation process. Unsaturated alkenyl substrates such as acrylonitrile (**2f**, Supporting Information Figs. S22, Chart S11), vinyl sulfonate (**2g**, Supporting Information Fig. S23, Chart S12) had a very slow conversion rate toward those thiouracil. The

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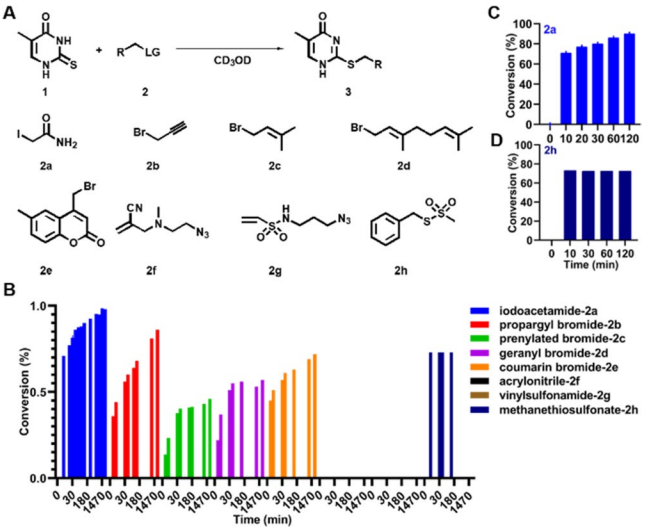

Scheme 1. Model reaction investigations of thiouracil with various reagents. (A) Overall reaction illustration using various reagents (**2a–2h**). Thiouracil (1.0 eq.) and reagent (2.0 eq.) in CD_3OD (0.6 mL) were used for all experiments otherwise stated. (B) Dynamic results of varied regents (**2a–2h**) with thiouracil (**1**). It was demonstrated that **2a** (blue bar) and **2h** (black bar) showed the best results. (C and D) Dynamic details of reagents **2a** or **2h** with thiouracil (**1**). For reagent **2a**, the reaction proceeded rapidly in 70% conversion within 10 minutes, with the extension of the reaction time, the conversion reached to 85% in 2 hours. For reagent **2h**, the reaction proceeded rapidly in 70% conversion in 10 minutes, with the extension of the reaction time, the conversion did not increase.

neutral reaction condition may be accounted for the low nucleophilic substituent reactivity. To our delight, the S-benzyl methanethiosulfonate (BMTS, **2h**, Supporting Information Figs. S24 and S25, Charts S13 and S14) as well as allylic substrates (**2c–2e**) showed moderate to good conversion rate, which was equal to iodoacetamide (**2a**, Scheme 1 and Supporting Information Fig. S17, Charts S1 and S2). We next paid our attentions toward these two reagents in the labeling studies of thio-containing DNA and RNA. Monitoring of the reaction of thiouracil with either iodoacetamide (**2a**) or S-benzyl methanethiosulfonate (BMTS, **2h**, Supporting Information Figs. S24 and S25) *in situ* showed different dynamic. For instance, using of iodoacetamide (**2a**) resulted in a pseudo-first order kinetics with a half-time of approximate 50.8 minutes (Scheme 1B,C, and Supporting Information Chart S1 and S2), while use of S-benzyl methanethiosulfonate (BMTS, **2h**) yielded a very new result (Scheme 1B,D, and Supporting Information Chart S24 and S25), the conversion stayed at a constant conversion of about 70% in 10 minutes, probably because this process was partial reversible.

To determine feasibility of this method, alkylation of 5-methyl-2-thiouridine (m^5s^2U) following this protocol was carried out. It was observed

that the alkylation using **2a** or **2h** was not so efficient. We tried to optimize the reaction conditions to enhance its feasibility of implementation (Supporting Information Figs. S26–S30). When reagent **2h** was applied, temperatures (40 or 60 °C) or concentrations (Supporting Information Chart S14) trials has been screened, but this influence was minimal (Supporting Information Fig. S26). Screenings of the reaction of m^5s^2U with reagents **2h** at different pH values and temperature failed, significantly low conversion was observed. Interestingly, substrate s^4U proceeded well with reagent **2h** (Supporting Information Figs. S27–S29) and **2a** (Supporting Information Figs. S30 and S31) in almost quantitative yields. In sharp contrast, the reaction of m^5s^2U with **2a** run well to produce alkylated product in approximate 65% conversion at 40 °C in 12 hours (Supporting Information Fig. S32). These results indicated the methyl has a crucial influence on the reactivity toward sulfur-containing nucleosides. Multiple parameters (pH values and temperatures) have also been evaluated (Supporting Information Figs. S32–S35). It was anticipated that basic condition would promote formation of sulfur anion, thus, accelerated the nucleophile attachment process of sulfur-containing substrate with various alkylation reagents, which indeed was observed in these experiments (Supporting Information Figs. S34 and S35). In addition, the reaction of the substrates s^2U with **2a** proceeded faster (Supporting Information Fig. S37) than **2h** (Supporting Information Fig. S36), and s^2A (Supporting Information Figs. S38–S40) displayed low activities toward reagents **2a** or **2h**. Moreover, the reactions of 2-thiouracil with reagent **2h** under varied conditions have also explored (Supporting Information Figs. S41 and S42). It has demonstrated that m^5s^2U and 2-thiouracil had a similar reactivities when reagent **2a** was employed (Supporting Information Figs. S32–S35 versus S17), but the difference was obvious when using of **2h** (Supporting Information Figs. S26 versus S24). Thus, it has demonstrated that sulfur-modified sites have a significant influence on the reactivity toward defined reagents such as iodoacetamide (**2a**) or S-benzyl methanethiosulfonate (**2h**). 2-Thio nucleosides have no activities in the absence or presence of 5-methyl substituent (Supporting Information Figs. S36, S26 and S38) when the S-benzyl methanethiosulfonate (**2h**) was used.

Next, we aimed to investigate whether our approach could be utilized into thio-modified oligos (Supporting Information Scheme 2 and Supporting Information Figs. S43–S54). [22–24] The thio-modified base skeleton (**3d**) was constructed[25] in 3 linear steps (23% overall yields, Scheme 2) from aldehyde (**3a**). Subsequently, the Vorbrüggen nucleoside synthesis was applied, the key intermediate **3e** was obtained in relative low yield with two isomers which was proven to be inseparable by flash chromatography or high-performance liquid chromatography. Nevertheless, the deprotection

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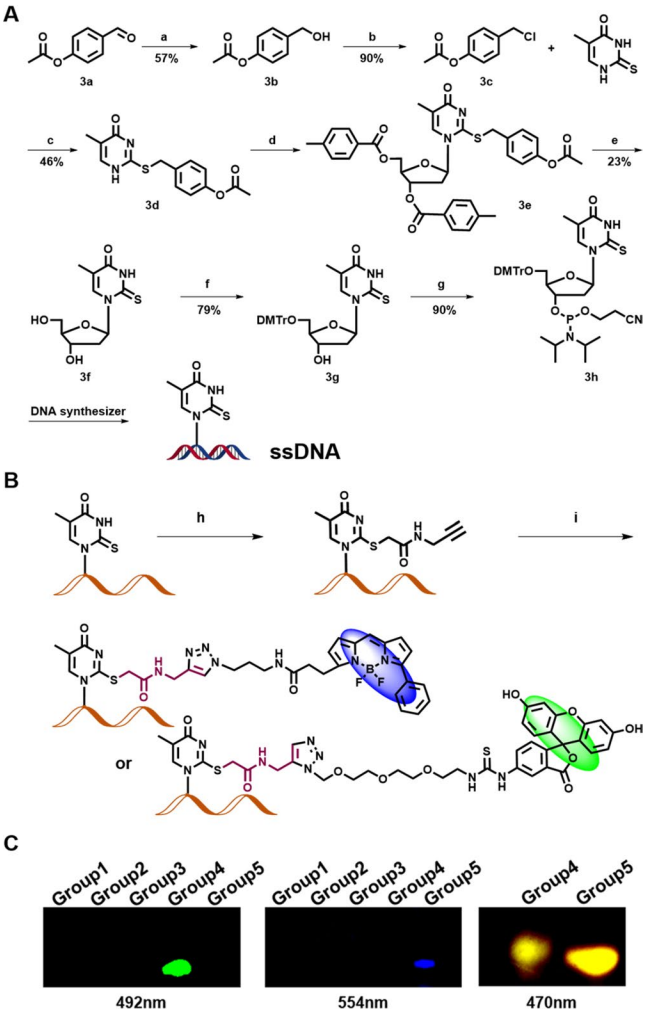

Scheme 2. Labeling studies of s^2T-DNA. (A) Synthesis details: (a) $NaBH_4$, THF, r.t., 12 hours, 57%; (b) $SOCl_2$, r.t., 90%; (c) K_2CO_3, acetone, refluxed, 46%; (d) *N, O*-bis(trimethylsilyl) acetamide, CH_2Cl_2; then, $SnCl_4$, used directly for next step; (e) NaOH (1.0 M), THF-MeOH, 23% yield for 2 steps; (f) DMTCl, pyridine, r.t., 79%; (g) 2-Cyanoethyl *N, N*-diisopropyl chlorophosphormidite, DIPEA, CH_2Cl_2, r.t., 2 hours, 90%. (B) Group1: native DNA; Group2: modified DNA; Group3: modified DNA with PIA; Group4: modified-DNA + PIA + azido-FITC; Group5: modified-DNA + PIA + azido-BODIPY. Method used here for the labeling of s^2T-modified DNA: (i) propargyl iodoacetamide (PIA), 90 °C, 5 minutes, NaOH (1.0 M, 1%); (ii) $CuSO_4$ (5.0 mM), TBTA (5.0 mM), sodium ascorbate (20.0 mM), tBuOH: H_2O (3: 1); Labeling approaches using azido-BODIPY (1.8 mM) or azido-FITC (1.8 mM) versus DNA (120 μM) via click reactions; (iii) Gel electrophoresis assays and subsequent imaging under blue-light or UV-light illumination were confirmed. 20% denature PAGE gel was utilized. Left: fluorescent image (Excitation wavelength are 554 nm for BODIPY and 492 nm for FITC). Right: GoldView dye was used.

of substrate (**3e**) proceeded well, leading to the deprotected nucleoside **3f** in 23% yield (Supporting Information Fig. S49). It was delighted to find that when DMT group was employed for protection of 5′-OH function in nucleoside **3f**, **3g** was obtained in 79% yield and the *α/β* isomers could

be isolated in over 95% purity based on the NMR data (Supporting Information Figs. S50 and S51). Eventually, the DMT-protected nucleoside **3g** reacted with 2-cyanoethyl *N, N*-diisopropylchlorophosphormidite (CEP-Cl) under the optimized condition to give s^2T-phosphormidite (**3h**, Supporting Information Figs. S52–S54) in 90% yield. The synthesis of 2-thiothymidine (s^2T)-containing oligo was carried out *via* solid-phase synthesis (Supporting Information Table S1). After ammonium cleavage/deprotection and HPLC purification, the full-length s^2T-DNA product was confirmed by HPLC and mass spectrum (Supporting Information Figs. S55 and S56).

The circular dichroism (CD) spectra of double-strand of the native and s^2T-modified DNA were measured first (Supporting Information Fig. S57.[26] As expected, both oligomers presented a spectrum typical of B-DNA, which exhibited characteristic cross-over near 260 nm with a large negative ellipticity at 250 nm. In more details, in the U-A or s^2U-A duplexes spectrum (Supporting Information Fig. S57A) there was a strong positive peak around 275 nm, a relatively weak negative peak around 245 nm. Further *T*m calculations based on the CD spectra have also been evaluated, it was demonstrated the s^2T-incorporated DNA comparing with its native form resulted in a decrease temperature of 3.6 °C. The thio-modification on 2-position perturbed the T-A base-pairing, leading to small biases of the structure.

To evaluate the feasibility of this s^2T-involved labeling and enrichment of DNA as well as RNA molecules, we tried to further evaluate those iodoacetamide (**2a**) or S-benzyl methanethiosulfonate (**2h**). It was observed that the iodoacetamide (**2a**) derivative with propargyl handle proceeded successfully under the optimized condition (95 °C, 5 minutes), which was confirmed by HPLC (Supporting Information Figs. S58 and S59) and mass spectra (Supporting Information Fig. S60). Notably, direct fluorescent labeling of s^2T-DNA (Supporting Information Scheme 3) using iodoacetamide-FITC (**3**) followed the optimized condition has also been performed and further were confirmed by green fluorescence imaging (Supporting Information Fig. S61) and mass spectra result (Supporting Information Fig. S62 and Table S2).

Considering the propargyl handle installed on s^2T-DNA, we next turned to illustrate the possibility of labeling of s^2T-DNA *via* click reaction. As depicted in Supporting Information Scheme S3, the azido-incorporated BODIPY were successfully inserted into the s^2T-DNA which were further analyzed *via* fluorescent spectroscopy and polyacrylamide gel electrophoresis (Scheme 2B, see Supporting Information Scheme S3 for details). Nucleic acid staining (right, Scheme 2B) or fluorescent detection (left, Scheme 2B) of the gel revealed that selective labeling thio-modified DNA

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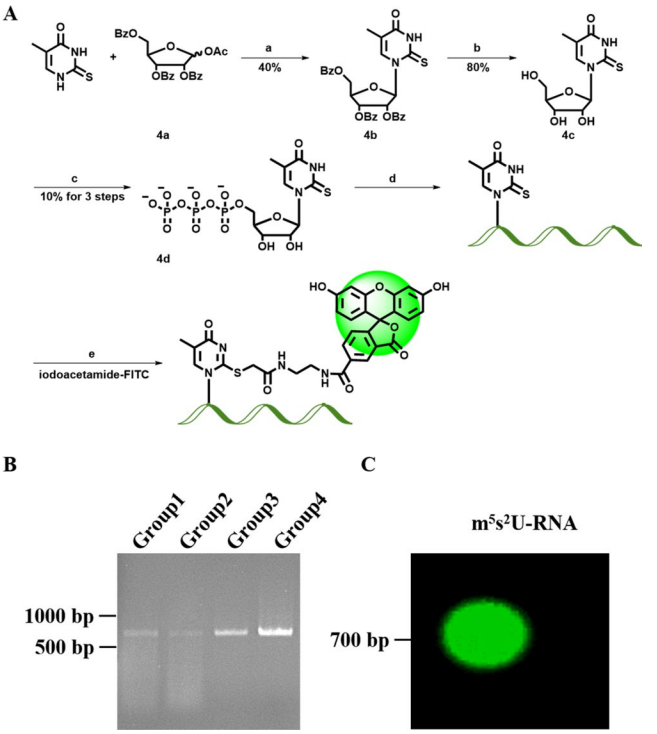

Scheme 3. Synthesis of m^5s^2U-RNA and its fluorescent labeling study. (A) Synthesis of m^5s^2U-incorporated RNA by use of transcriptional method. Synthesis: (a) *N*, O-bis(trimethylsilyl)acetamide, acetonitrile, 80 °C, 1 hour; trimethyl silyl triflate, 50 °C, 4 hours, 40%; (b) NaOMe, MeOH, 2 hours, 80%; (c) Proton sponge, trimethyl phosphate, 0 °C, 10 minutes; phosphorus oxychloride, 2 hours; tributylamine, bis-(tributylammonium) pyrophosphate, 10% for 3 steps. (d) Transcription protocol: EGFP DNA template (1.0 µg), T7 RNAp (1.0 µL), buffer (2.0 µL) and rNTP mix: Group1 (A/C/G, 0.5 µL, 100 mM, m^5s^2UTP, 2 µL, 100 mM), Group2 (A/C/G/U, 0.5 µL, 100 mM and m^5s^2UTP, 2 µL, 100 mM), Group3 (A/C/G/U, 0.5 µL, 100 mM and m^5s^2UTP, 4 µL, 100 mM), Group4 (A/C/G/U, 0.5 µL, 100 mM and m^5s^2UTP, 8 µL, 100 mM). Final volume was 20 µL (DEPC). (B) Transcription assays and analysis by gel electrolysis using GoldView dye. (C) Fluorescent labeling by use of iodoacetamide-FITC (**3**, 10.0 µM), 90 °C, 3 minutes. Fluorescent imaging was recorded via Cy3 tunnel (Ex.470 nm).

using our method have been achieved. Analysis of the mass spectroscopy of the BODIPY-labeled product have successfully demonstrated the feasibility of this method (Supporting Information Fig. S62 and Table S2). Thus, through the optimized alkylation process directly with iodoacetamide-FITC (**3**), or with propargyl iodoacetamide along with the click reaction, the fluorescent labeling of s^2T-incorporated DNA has been achieved.

In order to enhance feasibility of labeling of thio-modified nucleic acid following this protocol, i.e., m^5s^2U-RNA, the m^5s^2U triphosphate was synthesized in three steps (Scheme 3A and Supporting Information Figs. S63–S68). Nucleoside **4b** was synthesized first via the Vorbrüggen reaction

with two isomers which was inseparable by silica gel flash chromatography (Supporting Information Fig. S63). Deprotection of **4b** gave nucleoside **4c** in 80% yield, which was separated by HPLC and confirmed by proton NMR (Supporting Information Figs. S64–S66). The one-pot m^5s^2U triphosphate synthesis from unprotected **4c** was carried out, m^5s^2U triphosphate was obtained in 10% yield (Supporting Information Figs. S67 and S68). With this triphosphate in hand, we turned our attentions to corresponding transcription assays to introduce m^5s^2U into RNA. We constructed a EGFP gene first (Supporting Information Fig. S69). After concentration titration and optimization (Supporting Information Table S3), it was revealed that when the triphosphate **4d** reached to a concentration of 20 mM, EGFP is efficiently transcribed to produce RNA with m^5s^2U-modifications and band of this transcribed RNA on gel was obvious (Scheme 3B, left, Group3, Supporting Information Fig. S70). Subsequently, labeling of this m^5s^2U-modified transcript was performed using the optimized condition in the absence of base. After installation of a propargyl functionality into m^5s^2U-modified transcript with propargyl iodoacetamide described above (Supporting Information Scheme S2), click reaction was utilized with iodoacetamide-FITC reagents (**3**) in order to introduce a FITC fluorophore. It seemed that when the transcript concentration reached to 100 μM, and the concentration of iodoacetamide-FITC (**3**) (Supporting Information Fig. S3) was 10 μM, the fluorescent labeling of m^5s^2U-modified RNA was effective (Scheme 3B, right and Supporting Information Fig. S71). In a word, fluorescent labeling of thio-modified (m^5s^2U) newly transcribed RNA has been achieved via alkylation by direct use of iodoacetamide FITC (**3**).

In previous studies, we demonstrated that alkylation with iodoacetamide (**2a**) proceeded well for all substrates except s^2A, and it has been proven that s^4U displayed the fastest kinetics. When reagent S-benzyl methanethiosulfonate (**2h**) was utilized, s^4U exhibited great potential in the selective alkylation process. However, it has been demonstrated that 2-thio substrates exhibited low conversion rate toward those reagents (**2h**). To further expand feasibility, the bio-orthogonality have also been evaluated. It has demonstrated that reagent **2a** or **2h** were compatible for common amino acids including lysine (Supporting Information Figs. S72 and S74), tyrosine (Supporting Information Figs. S73 and S74) or cysteine analogue (Supporting Information Fig. S75. In addition, it has been reported that s^4U was easily transferred into uridine when treated with hydro peroxide (Supporting Information Fig. S79A. We try to explore whether this was also feasible for those 2-thio nucleoside substrates (Supporting Information Fig. S79B). Interestingly, all the thio-modified nucleosides were transformed to their native forms when treated with hydro peroxide, s^2U achieved the highest dynamics, while m^5s^2U

and s^2A displayed almost equal reactivities under the tested condition. Thus, our investigations have shed fundamental light on the difference between the reactivities of nucleosides including s^2T, s^2U, m^5s^2U, s^4U and s^2A.

3. Conclusions

We demonstrate herein comprehensive investigations of various sulfur-containing nucleosides such as s^2T, s^2U, m^5s^2U, s^4U, s^2A, s^2T-incorporated DNA and m^5s^2U-modified RNA. It has been proven that iodoacetamide (**2a**) display the most promising feasibility for sulfur-containing substrates such as s^2T, s^2U, m^5s^2U and s^4U. In sharp contrast, the alkylation with S-benzyl methanethiosulfonate (**2h**) exhibit the greatest potential in the studies of s^4U. Based on optimized condition, we have successfully demonstrated the fluorescent labeling of s^2T-incorporated DNA and m^5s^2U-modified RNA.

Activity comparisons between iodoacetamide (**2a**) or S-benzyl methanethiosulfonate (**2h**) with a wide range of sulfur-containing nucleosides have also been evaluated. It seemed that alkylation with iodoacetamide (**2a**) proceeded well for all substrates except s^2A, and it has been proven that s^4U displayed the fastest kinetics. When reagent S-benzyl methanethiosulfonate (**2h**) was utilized, s^4U exhibited great potential in the selective alkylation process. However, it has been demonstrated that 2-thio substrates exhibited low conversion rate toward those reagents (**2h**). In addition, we have also demonstrated that all the thio-modified nucleosides are able to be transformed to their native forms when treated with hydro peroxide, s^2U achieved the highest dynamics, while m^5s^2U and s^2A have almost equal reactivities under the tested condition. Epitranscriptome analysis based on this result is ongoing in my lab and will be reported in due course.

4. Experimental

4.1. General methods

Anhydrous solvents were used and redistilled using standard procedures. All solid reagents were dried under a high vacuum line prior to use. Air sensitive reactions were carried out under argon. Analytical TLC plates pre-coated with silica gel F254 (Yantai Chemical Industry Research Institute) were used for monitoring reactions and visualized by UV light and/or PMA or $KMnO_4$. Flash column chromatography was performed using silica gel (32–63 μm, Qingdao Marine Chemical Inc., China), or high-resolution liquid chromatography. All ^1H- and ^{13}C-NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz), and the ^1H and ^{13}C NMR chemical shifts were referenced to the solvent or solvent impurity peaks

for $CDCl_3$ at δ^1H 7.24 and δ^{13}C 77.23. High resolution mass spectra were recorded on a Bruker microTOF II spectrometer using electrospray ionization (ESI) achieving at Huazhong University of Science and Technology. The Supporting Information is available free of charge at https://www.tandfonline.com/

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Disclosure statement

No potential conflict of interest was reported by the authors.

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