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Esterification of Cyclic N^6-Threonylcarbamoyladenosine During RNA Sample Preparation

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The continuous deciphering of crucial biological roles of RNA modifications and their involvement in various pathological conditions, together with their key roles in the use of RNA-based therapeutics, has reignited interest in studying the occurrence and identity of non-canonical ribonucleoside structures during the past years. Discovery and structural elucidation of new modified structures is usually achieved by combination of liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) at the nucleoside level and stable isotope labeling experiments. This approach, however, has its pitfalls as demonstrated in the course of the present study: we structurally elucidated a new nucleoside structure that showed significant similarities to the family of (c)t^{6}A modifications and was initially considered a genuine modification, but subsequently turned out to be an *in vitro* formed glycerol ester of t^{6}A. This artifact is generated from ct^{6}A during RNA hydrolysis upon addition of enzymes stored in glycerol containing buffers in a mildly alkaline milieu, and was moreover shown to undergo an intramolecular transesterification reaction. Our results demand for extra caution, not only in the discovery of new RNA modifications, but also with regard to the quantification of known modified structures, in particular chemically labile modifications, such as ct^{6}A, that might suffer from exposure to putatively harmless reagents during the diverse steps of sample preparation.

Introduction

RNA is a ubiquitous biopolymer whose functions and functionality are greatly enhanced by implementation of a complex network of chemically modified nucleoside structures with a structural versatility that is generally underappreciated.[1] Driven by advances in technology and concomitantly lowered detection limits, the number of new modified ribonucleosides is ever-growing.[2–5] Of particular interest in the discovery of new modifications is transfer RNA (tRNA), not only with its outstanding level of modifications per molecule, but also with their diversity and complexity, including so-called hypermodifications that are introduced in multi-step enzymatic processes.[6]

LC–MS/MS analysis of RNA samples on nucleoside level has established itself as the gold standard for both the discovery and the quantification of RNA modifications.[7] Besides detection of genuine RNA modifications with a biological function, several recent studies highlight the existence of RNA modification damages which are accompanied by a loss of function and demand more critical scrutiny.[2,8] Even more challenging for LC–MS/MS-based analysis of the so-called epitranscriptome are artifacts that are generated *in vitro* during sample preparation and storage.[9,10] Such artifacts can share the structure of naturally occurring modifications, thus leading to an adulteration of modification levels. Prominent examples include deamination of adenosine to inosine, Dimroth rearrangement of 1-methyladenosine resulting in N^6-methyladenosine, or cyclic N^6-threonylcarbamoyladenosine (ct^{6}A) which remained concealed for decades due to its fast hydrolysis to N^6-threonylcarbamoyladenosine (t^{6}A).[11–14] In addition, the formation of non-natural artifacts from chemically labile RNA modifications holds the risk of their misidentification as new RNA modifications and underlines the importance of a careful choice of sample preparation procedures. Among such structures are amino/imino ribonucleoside artifacts that are generated from carbon-othiolated nucleosides during RNA hydrolysis and artifacts resulting from the nucleophilic ring-opening of ct^{6}A with primary amines as for example tris(hydroxymethyl)amine, glycine and ethanolamine to yield corresponding amides under alkaline conditions.[14–17]

Reaching for hitherto unknown chemically modified nucleosides in tRNA from *E. coli*, we generated an LC–MS/MS data set that indicated the existence of further potentially new RNA modifications, yet to be elucidated.[5,18] In the present study we scrutinized one of these modification candidates which was

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considered as RNA modification in the beginning, but was unraveled to be a glycerol ester of t^6A, formed during RNA sample preparation. These findings did not only emphasize difficulties in the use of stable isotope labeling for structural elucidation purposes with regard to artifacts but also suggest to extend the previously perceived vulnerability of ct^6A towards primary amines to chemicals containing free hydroxyl groups.

Results and Discussion

We previously described the combination of an untargeted LC–MS/MS approach and stable isotope labeling as a powerful tool to discover new RNA modifications using triple quadrupole (QQQ) mass spectrometry.[5,18] This approach relies on the characteristic fragmentation of nucleoside species at the *N*-glycosidic bond, along with the neutral loss of the ribose moiety (−132). The initial screening for potentially new RNA modifications was followed by several filtering steps, resulting in a set of so-called candidates, which were named after their mass-to-charge ratio and subjected to further experiments for structural elucidation.[5] One of these modification candidates eluted after 25.6 min from the reversed-phase column and was characterized by an *m/z* of 487. The analysis of stable isotope labeled tRNA suggested an elemental composition of 15 carbon atoms, 6 nitrogen atoms and no sulfur atoms as indicated by respective shifts in the mass spectrum (Figure 1a). In order to determine the main nucleoside prior to modification we applied a differential labeling approach, i.e. we supplied a ^{15}N-labeled culture with the unlabeled (^{14}N) nucleobases. Upon addition of ^{14}N-adenine, we detected a compound with *m/z* 488 as the most abundant species, which did not only identify adenosine as the parent species in case of candidate 487, but also implied the incorporation of one ^{15}N from nitrogen containing metabolites, e.g. amino acids, generated by *E. coli* in the labeled medium, which was in line with the previously determined total amount of six nitrogen atoms for candidate 487 (Supplementary Figure S1). Importantly, although we observed equal labeling results regarding both the elemental composition and the underlying main nucleoside for the other substances with *m/z* 487 displayed in the EIC in Figure 1a (Supplementary Figure S1), the focus of this study was on the substance eluting after 25.6 min because this was the most abundant species. However, we considered it a plausible possibility that the other candidates might be structurally related to candidate 487. For convenient discussion we assigned letters from A to D to the peaks with *m/z* 487 in order of their elution time, with peak C representing candidate 487. It should also be noted that exact retention times and chromatographic performance varied in different experiments as a consequence of changes to the instrument configuration, reversed-phase column or applied gradients.

In a next step we assessed the fragmentation behavior of candidate 487 in a so-called pseudo-MS^3 scan. Here, the application of high fragmentor voltages induces cleavage already in the ion source, enabling filtering for the nucleobase ions of interest in the first mass filter, their fragmentation in the collision cell and detection of resulting product ions in the second mass filter. The obtained fragmentation patterns are very characteristic and can provide important information not only on functional groups within the molecule, but also on structural similarities to other modified nucleosides and can become an integral part within structural elucidation of unknown structures, as convincingly demonstrated by the Limbach group who generated a mass spectral network and thereby identified s^2Cm among others.[3] Comparing the fragmentation pattern of candidate 487 to other modified adenosine structures, we noticed significant similarities with the fragmentation patterns of ct^6A and t^6A (Figure 1b). To examine if there was a structural or metabolic relation between candidate 487 and these known modifications, we aimed to analyze tRNA isolated from *E. coli* mutant cells, defective in enzymes that are required for the biosynthesis of t^6A or ct^6A, respectively. Via the Keio collection, a collection of single gene knockout (KO) mutants from *E. coli*, we had access to the KO strain for TcdA which catalyzes the dehydration of t^6A to yield ct^6A, however, it was not possible to directly address a potential relation with t^6A, as KO mutants for the enzymes involved in its synthesis were presumably not viable.[19] Importantly, candidate 487 was completely absent in the analysis of hydrolyzed tRNA from the Δ*tcdA* strain (Figure 1c), indicating a direct relation to the ct^6A modification. At this point, previous reports on the lability of ct^6A towards reaction with nucleophiles under mildly alkaline conditions (*ex vivo*) gave rise to question the authenticity of the labeling results and the composition of candidate 487 derived thereof.[11,14,17] Especially intriguing was the fact that, according to the labeling results, the potentially new modification was supposed to consist of the same amount of carbon and nitrogen atoms as ct^6A, but the candidate's *m/z* was significantly higher. To address this issue, we intended to determine the elemental composition independent of uniform stable isotope labeling and moved to high-resolution mass spectrometry (HRMS) which allows calculation of potential sum formulas based on the detected exact mass. In case of candidate 487 we found an exact mass of 487.1777 well in line with an ion formula of $C_{18}H_{27}N_6O_{10}^+$, displaying a distinct discrepancy between the labeling results and the actual composition of candidate 487 (Figure 1d). The most plausible explanation for this observation was that one part of the candidate's structure was introduced subsequent to the isolation of the labeled RNA, i.e. during work-up or sample preparation, and was therefore unrecognizable in stable isotope labeling experiments, i.e. did not lead to *m/z* shifts compared to the unlabeled sample (Figure 2a). In our particular case, the compositional imbalance of $C_3H_8O_3$ between ct^6A ($C_{15}H_{18}N_6O_7$) and candidate 487 ($C_{18}H_{26}N_6O_{10}$) as well as a consideration of reagents that RNA samples are exposed to during work-up and sample preparation, was strongly suggestive of glycerol being involved in the *ex vivo* formation of candidate 487. The latter was consequently proposed to be a glycerol ester of t^6A. Figure 2a shows one possible structure proposal resulting from a nucleophilic attack of the primary alcohol group of glycerol, however, a nucleophilic attack of the secondary alcohol group was also plausible. In order to examine if candidate 487 was

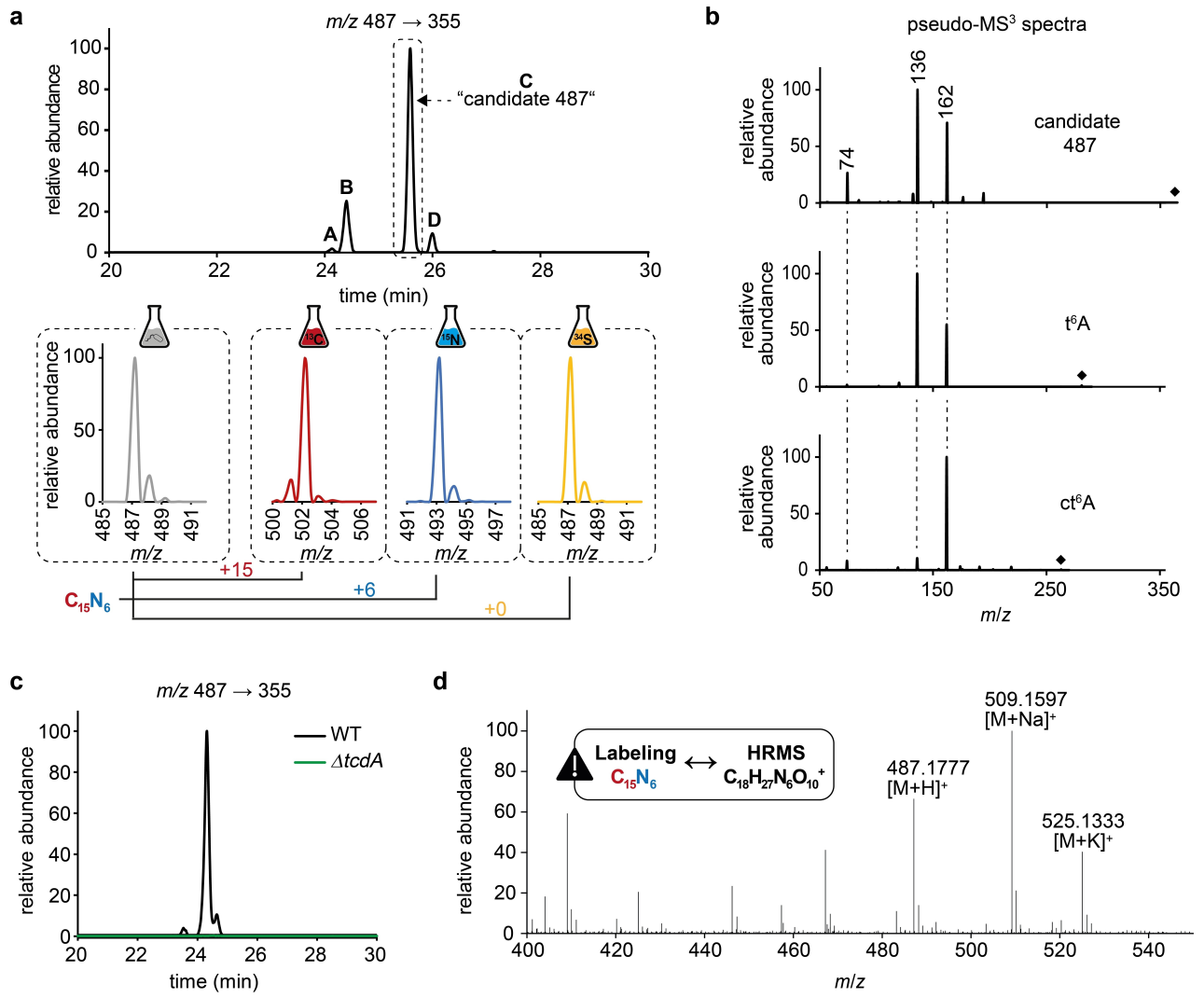


Figure 1. Structural characterization of candidate 487 by LC–MS/MS. Abundances were set in relation to the highest peak or signal in the respective extracted ion chromatogram (EIC) or mass spectrum (relative abundance). a) Upper panel: EIC at *m/z* 487 from a neutral loss (−132) measurement of an *E. coli* tRNA hydrolysate showing peaks A, B, C and D with peak C corresponding to candidate 487 which is highlighted in a dotted box. Lower panel: QQQ mass spectra of candidate 487, recorded from an NLS of hydrolyzed unlabeled (gray) or labeled (^{13}C = red, ^{15}N = blue, ^{34}S = yellow) *E. coli* tRNA, indicating the elemental composition by shifts in *m/z*. b) QQQ fragmentation mass spectra for candidate 487 (precursor *m/z* 355), t^{6}A (precursor ion *m/z* 281) and ct^{6}A (precursor ion *m/z* 263), resulting from the respective compounds after in-source fragmentation (pseudo-MS^3 scan) with similarities in the occurring fragmentation patterns indicated by dotted lines and the *m/z* of the precursor ions marked with a square. The spectrum for candidate 487 was recorded from an *E. coli* tRNA hydrolysate enriched for candidate 487 while synthetic standards were used for t^{6}A and ct^{6}A. c) Merged EICs illustrating the occurrence of candidate 487 in a QQQ measurement of tRNA isolated from the Keio wild type (WT) strain (black) and the ct^{6}A-related knockout strain Δ*tcdA* (green). d) Mass spectrum and calculated ion formula (protonated species) for candidate 487 from the exact mass detected by HRMS analysis of digested tRNA enriched for candidate 487 which do not match the previous results from the labeling experiment in a).

indeed formed during our RNA hydrolysis procedure, which included enzymes stored in glycerol-containing buffers, we treated ct^{6}A nucleoside according to our hydrolysis protocol and subjected this sample to LC–MS/MS analysis, together with an untreated control sample. While we did not detect any candidate 487 in the untreated control sample, after exposure to the hydrolysis mixture we clearly detected candidate 487 (Figure 2b), supporting our previous assumption of an *ex vivo* formation of candidate 487. Interestingly, this did not only apply to the peak with the highest intensity, which we refer to as candidate 487, but also to the smaller peaks which had initially shown the same labeling results, emphasizing that these substances might be structurally related to candidate 487.

Although a formation of the proposed glycerol ester via the more reactive cyclic active ester ct^{6}A seems plausible and was also supported by absence of candidate 487 in tRNA from the Δ*tcdA* cells, a formation of the ester from t^{6}A was also conceivable and hence assessed by similar treatment with the hydrolysis mixture and subsequent analysis by LC–MS/MS. In contrast to the analysis of ct^{6}A, the treatment of t^{6}A only led to the formation of an almost negligibly low amount of candidate 487 (Supplementary Figure S2), indicating that the favored route for formation of the presumed ester starts from ct^{6}A.

Having established that candidate 487 was formed during RNA sample preparation, we set out to confirm the proposed structure and investigate whether the nucleophilic attack

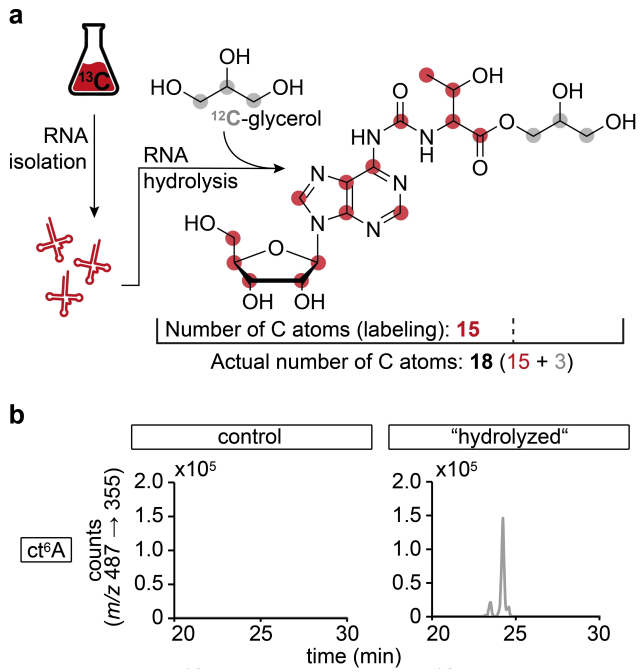
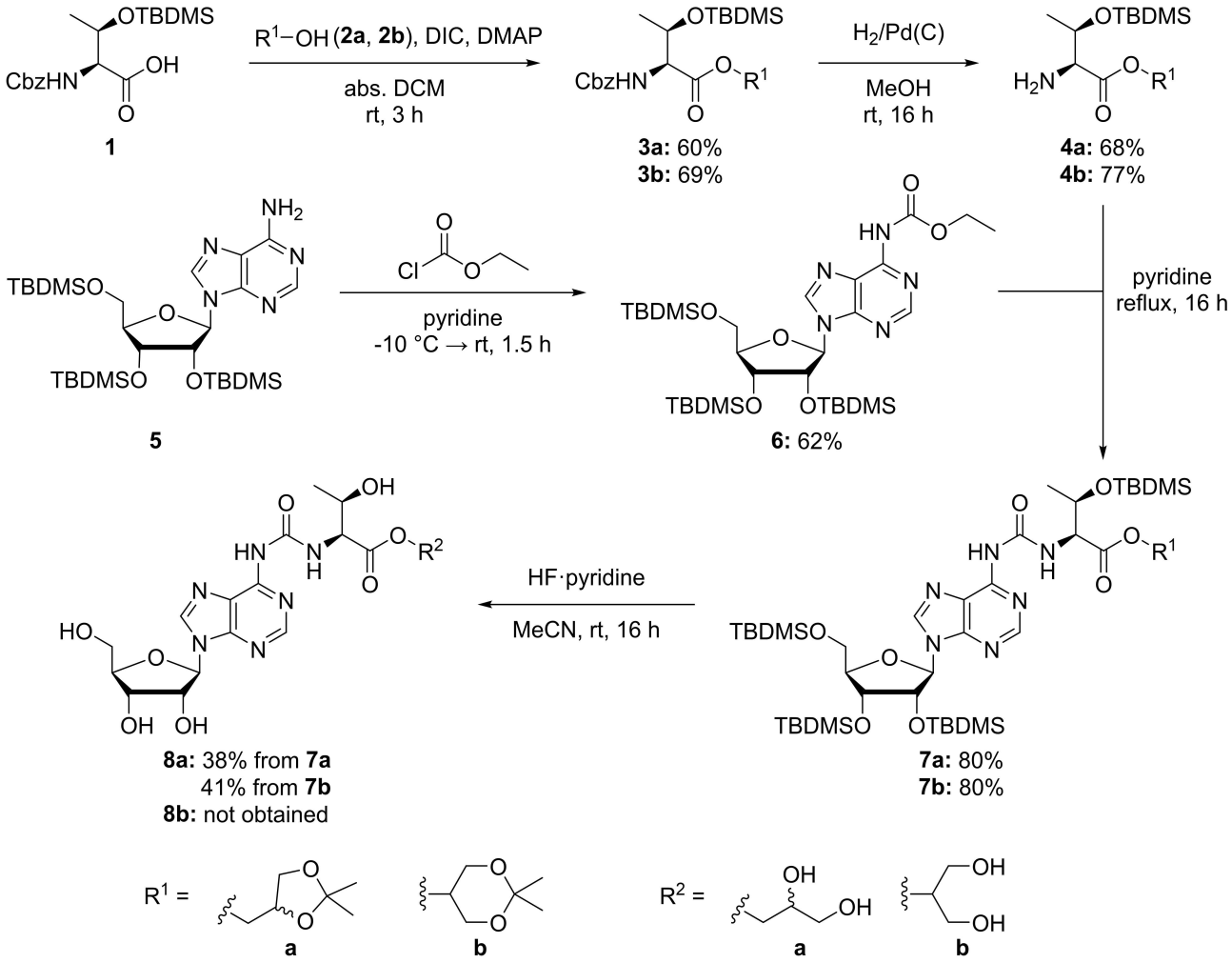


Figure 2. Pitfalls in determination of elemental compositions by stable isotope labeling and identification of candidate 487 as an artifact. a) Behavior of artifacts resulting from the handling or sample preparation in labeling experiments highlighting the discrepancy in the number of certain atoms (here: number of carbon atoms, displayed in red) according to labeling (here: ^{13}C labeling) versus the actual number of the respective atoms as a consequence of the incorporation of unlabeled species (e.g. ^{12}C-glycerol, displayed in gray) into the molecule as illustrated within one of the structure proposals for candidate 487. b) EICs of candidate 487 (*m/z* 487→ 355) from a neutral loss measurement of untreated synthetic ct^{6}A (control) as well as the corresponding synthetic ct^{6}A incubated with the enzyme mixture usually used for tRNA hydrolysis prior to LC–MS/MS analysis ("hydrolyzed").

involved the primary or the secondary alcohol group of the glycerol. Therefore, we intended to synthesize both variants of the artifact (Scheme 1). For the synthesis of t^{6}A glycerol esters **8a** and **8b**, we first prepared Cbz- and TBDMS-protected threonine **1** according to literature procedures.[20] Then, **1** was reacted in a Steglich esterification with solketal (**2a**) and 2,2-dimethyl-1,3-dioxan-5-ol (**2b**) by using DIC as the coupling reagent to afford glycerol esters **3a** and **3b**, which were deprotected at the *N*-terminus under hydrogenative conditions to give **4a** and **4b**.[21] Additionally, TBDMS-protected adenosine **5** was prepared as reported in the literature and reacted with ethyl chloroformate to carbamate **6**, which readily reacted with **4a** and **4b** in pyridine under reflux to give **7a** and **7b**, respectively.[22] In the last synthesis step, HF·pyridine was employed for the cleavage of the acetonide moiety in **7a** and **7b** and the removal of the TBDMS-protecting groups to afford t^{6}A glycerol ester **8a** as the only product in both cases, as identified by NMR. These findings suggest that **7b** might undergo an intramolecular transesterification under the deprotection conditions, leading to the formation of **8a**.

In order to confirm the proposed structures, we analyzed the synthesized compounds **8a** and, putatively, **8b** as well as hydrolyzed tRNA from *E. coli* and compared not only their chromatographic but also their fragmentation behavior. In both synthetic samples, we observed the occurrence of candidate 487 (peak C) together with peak D at the same retention times, in equal quantities compared to the occurrence of candidate 487 in the hydrolyzed tRNA sample (Figure 3a) and with the congruent fragmentation patterns (Supplementary Figure S3). On the one hand this confirmed our structure proposal of a glycerol adduct of t^{6}A, but on the other hand also seemed to indicate that the synthetic samples consisted of isomeric structures, providing further evidence for the suggested transesterification reaction.[23] Although **8a** was suggested as the only product via NMR spectrum (Supplementary Figure S31), the two peaks occurring in the chromatogram of **8a** implied the presence of two distinct substances with only slight differences in their molecular structure. To take a more detailed look at the presumed transesterification, we optimized our HPLC method in terms of separation of the two peaks detected in the synthetic samples, which consequently allowed us to separately collect both peaks, based on their UV signal (Figure 3b). After lyophilization, the collected samples were dissolved in water and one sample of each collected peak was directly subjected to renewed LC–MS analysis. Here, we observed that reinjection of one individual peak resulted in the renewed occurrence of both peaks, respectively, indicating an interconvertibility of these two substances which would be consistent with the previously mentioned transesterification reaction (Figure 3d). Since previous chromatographic results from samples with different backgrounds (Figures 1a, 2b and 3a) gave the impression of an equal ratio between candidate 487 and the later eluting substance (peak D), we wondered if the influence of time and temperature might result in a chemical equilibrium. Therefore, we reinjected samples of the separately collected peaks after storage at different temperatures overnight. For all samples the analysis showed a similar ratio of the later eluting peak D to the peak of candidate 487 (peak C) (Figure 3c), consistently demonstrating a chemical equilibrium which is strongly shifted towards candidate 487 (Figure 3d) and which can only be observed by direct analysis of the collected peaks (Figure 3b,c). These findings support the presumed transesterification of **8a** and **8b**, also under analysis conditions, and, with the NMR spectroscopy matching compound **8a**, it is likely that the more abundant species can be assigned to the sterically less hindered primary ester **8a** which would then be the structure of the substance corresponding to the original peak of candidate 487.

Another intriguing observation in the analysis of the synthetic samples (Figure 3a) was the lack of any peaks eluting before the candidate 487 peak while these peaks A and B, which exhibited similar labeling results as candidate 487 (*vide supra*), were detected in the hydrolyzed tRNA sample (Figure 1a and 3a) and the ct^{6}A standard that was incubated under hydrolysis conditions (Figure 2b). Given previous reports on the epimerization of ct^{6}A under mild alkaline conditions (pH 8.2–pH 9), we hypothesized that the glycerol adducts which are primarily formed from ct^{6}A might be prone to epimerization.[11] To assess this hypothesis, we treated the synthetic glycerol adduct according to our hydrolysis protocol



Scheme 1. Planned synthesis of t^{6}A glycerol esters **8a** and **8b** from Cbz- and TBDMS protected threonine **1** and TBDMS-protected adenosine **5**. See supplementary information for the synthesis of **1**, **2b**, and **5**.

(pH 7.5) and indeed observed the occurrence of the earlier eluting peaks A and B (Supplementary Figure S4), strongly supporting the assumption that the earlier eluting peaks A and B with *m/z* 487 in the hydrolyzed tRNA sample might correspond to different epimers of the glycerol adduct.

Conclusions

The new nucleoside structure elucidated here joins a series of artifacts already found in epitranscriptomic analyses and illustrates how important it is to consider a potential lability of RNA and its modifications under the conditions of RNA isolation and sample preparation for LC–MS analysis. Considerations range from susceptibility to oxidation, nucleophilic attacks, and rearrangement to reactions with solvents and chemicals used during the workflow.[9,12–15,24] In addition to the previously reported reaction of the cyclic active ester ct^{6}A with amines to form amides, the present study showed that esterification with hydroxyl groups, e.g. in glycerol, can also occur.[14,16,17] Although such derivatives have no biological function themselves, they would escape the quantification of their parent compounds, e.g. ct^{6}A, thereby leading to misinterpretation of quantitative results. Hence it is of importance to be aware of their existence and the circumstances leading to their formation, in order to reliably and robustly quantify chemically labile modifications within the epitranscriptome. In line with the efforts of some groups in this direction, including proposed strategies to avoid artifacts as well as a classification and characterization of different error sources, our results underline the need for special caution not only in the context of LC–MS/MS based analysis, but also in the application of other technologies to analyze both native and therapeutic RNA.[9,10]

Supporting Information

The authors have cited additional references within the Supporting Information.[25]

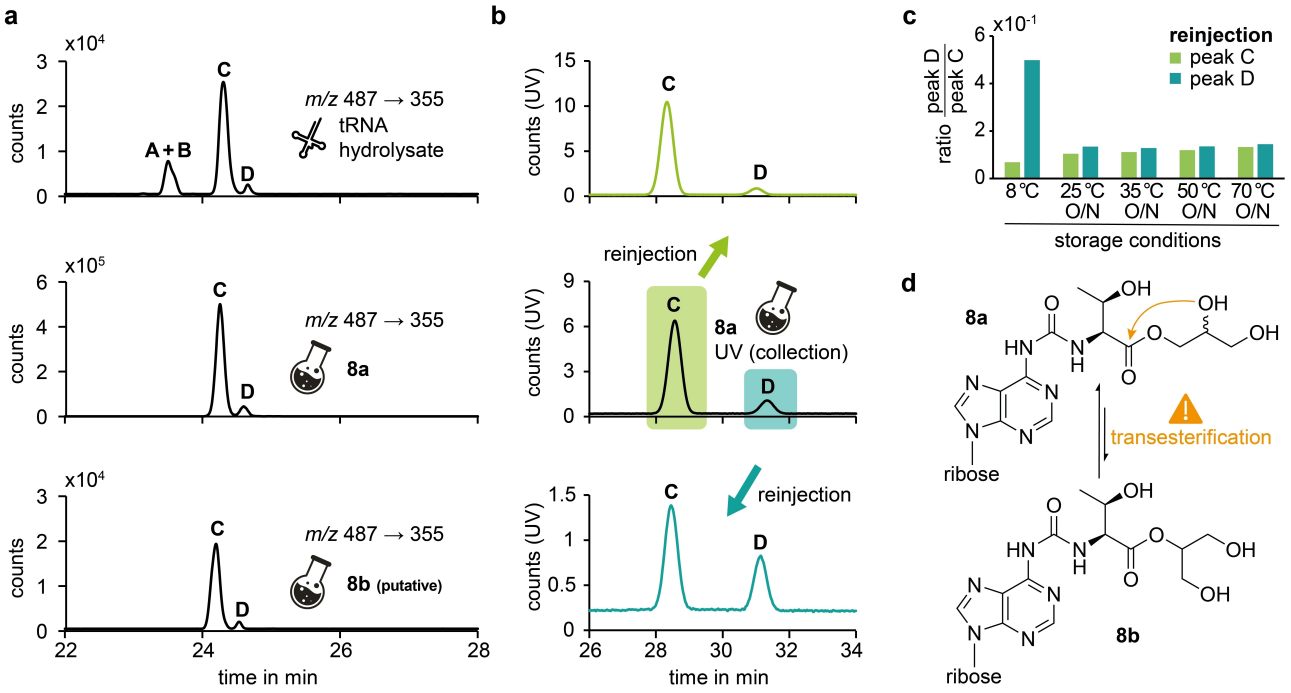


Figure 3. LC–MS/MS analysis of synthesized glycerol esters of t^{6}A. a) Each chromatogram shows the EIC for the mass transition *m/z* 487→355 with peak C corresponding to candidate 487, which were recorded from the QQQ LC–MS/MS analysis of a hydrolyzed tRNA sample as reference for the retention time (upper panel), the synthesized compound **8a** (middle panel) and, putatively, the synthesized compound **8b** (lower panel). b) UV chromatogram of the synthesized compound **8a** using an optimized method that was developed to separately collect both peaks occurring in the synthetic samples (middle panel), i.e. the peak C corresponding to candidate 487 (green) and peak D with a little longer retention on the column (blue). After collection, the eluates were lyophilized, resuspended in water and again subjected to LC–MS analysis (storage until injection in the autosampler at 8 °C), illustrating that both individual peaks resulted in the renewed occurrence of both peaks upon reinjection in the respective UV chromatograms which are displayed in green (reinjection of peak C, upper panel) and blue (reinjection of peak D, lower panel). c) Ratio of peak D to peak C (corresponding to candidate 487) calculated from the UV abundances in the LC–MS analysis of the collected samples (details see b)) after exposure to different temperatures overnight (O/N). d) Suggested transesterification reaction of glycerol adduct regioisomers **8a** and **8b** under deprotection and analysis conditions by intramolecular attack of one of the free hydroxyl groups with the chemical equilibrium presumably shifted to the side of the primary ester **8a**.

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Conflict of Interests

Mark Helm is a consultant for Moderna Inc.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: epitranscriptome · hydrolysis artifact · tRNA · nucleoside analysis · mass spectrometry

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