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Stability studies on the newly discovered cyclic form of tRNA N^6-threonylcarbamoyladenosine (ct^{6}A)

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ABSTRACT

A cyclic form of N^6-threonylcarbamoyladenosine bearing an oxazolone moiety (ct^{6}A) was discovered very recently at the position 37 in several tRNA sequences. Our study on the synthesized 5′,3′,2′-O-acetylated derivative of ct^{6}A confirmed high stability of the modified nucleoside under physiological conditions (PBS buffer, pH 7.4) and revealed remarkable stability of the oxazolone ring in acidic (100 mM HCl, pH 1) and basic (0.1 mM NaOH, pH 10) conditions. This feature may allow for the post-synthetic conversion of t^{6}A into ct^{6}A in assembled oligoribonucleotides.

N^6-threonylcarbamoyladenosine (t^{6}A, Fig. 1), a highly conserved hypermodified purine nucleoside found in transfer RNAs (tRNAs) in all three domains of life, is known for more than forty years.[1] This modified unit, located at the position 37 of several tRNAs (a position 3′ adjacent to the anticodon), is very important for fidelity of the decoding process and for protein biosynthesis in cells.[2–7] The t^{6}A nucleoside is required for efficient aminoacylation of tRNA,[2] tRNA binding to the A-site codon,[3] efficient translocation,[4] and reading frame maintenance, preventing leaky scanning of initiation codons and read-through of stop codons.[5] The bulky structure of t^{6}A prevents U33-A37 base pairing[6] and helps an elongator tRNA molecule to form the canonical U-turn structure of the anticodon loop,[7] while in initiator tRNA of *Schizosaccharomyces pombe* t^{6}A modification provides additional hydrogen bonds that causes formation of a unique U-turn motif.[8]

Although the presence of t^{6}A in cellular tRNAs has been well documented, it was very recently found by Suzuki and coworkers that at least in some populations of bacteria, fungi and plants the native structure of t^{6}A is a nucleoside with the threonine residue cyclized to the structure of the oxazolone ring (cyclic-t^{6}A or ct^{6}A, Fig. 1).[9] The authors provided strong evidence that t^{6}A is an artifact nucleoside resulting from hydrolysis of the native ct^{6}A due to conditions used in conventional methods of nucleoside isolation.[10] A new isolation methodology applied by Suzuki and coworkers (pH 7, a short time of tRNA digestion) furnished the intact ct^{6}A nucleoside.[9] Additionally, ct^{6}A obtained from a small sample of t^{6}A (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was used for oxazolone ring formation), allowed to confirm the structure of the new nucleoside by a high resolution NMR analysis. Moreover, the authors identified (in *Escherichia coli* and yeast) an ATP-dependent dehydratase for the conversion of t^{6}A to ct^{6}A, and estimated the decoding efficiency conferred by ct^{6}A. Their structural model studies clearly suggest that ct^{6}A is more effective than t^{6}A at enhancing the decoding efficiency of tRNA^{Lys}.[9]

The discovery of cyclic-t^{6}A indicates that many previous reports regarding t^{6}A functions in vitro and in vivo should be reconsidered. The structure–function studies were based mainly on the use of t^{6}A containing synthetic oligoribonucleosides,[11–15] and should be repeated with oligonucleotides containing the discovered ct^{6}A molecule. To start investigations on the introduction of ct^{6}A into an oligonucleotide chain, one has to know stability properties of the ct^{6}A nucleoside. In this communication we discuss stability of ct^{6}A under different hydrolytic conditions. For the tests we used 5′,3′,2′-tri-O-acetylated ct^{6}A (abbreviated as Ac$_3$ct^{6}A) as a model compound. We believed that the acetyl protection would allow for more efficient synthesis and easier purification of the ct^{6}A derivative (a less polar compound), while should not affect the reactivity of the oxazolone modification.

The Ac$_3$t^{6}A substrate was obtained in good yield according to the well known procedure introduced by Chheda and coworkers[16–18] which involves a displacement of the ethoxy group in

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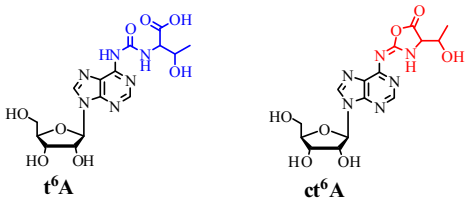
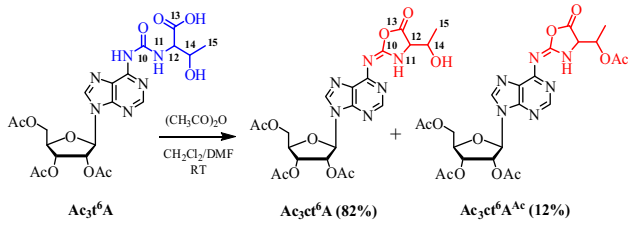


Figure 1. Structures of t^6A and ct^6A.

2′,3′,5′-tri-*O*-acetyl-adenosine N^6-carbamate with an L-threonine residue (details are given in Supplementary data). The structure of Ac_3t^6A was unambiguously confirmed by ^1H NMR, ^{13}C NMR, FAB MS, UV, IR spectra and HPLC data (Supplementary data, Figs. S1–S8) and obtained results conform with earlier published data.[16–19] Dehydration of Ac_3t^6A by the use of 5-fold molar excess of acetic anhydride (a convenient procedure for formation of amino acid oxazolones[20]) furnished the cyclic form of threonine residue. The reaction was performed at room temperature in an 85/15 (v/v) mixture of anhydrous methylene chloride and dimethylformamide (Scheme 1).

After 3.5 h, TLC analysis ($CHCl_3$/MeOH 9:1, v/v) revealed almost complete disappearance of the nucleoside substrate and formation of two products, the major one with $R_f = 0.40$ and the minor with $R_f = 0.56$, which were isolated and subjected to ^1H NMR analysis in anhydrous DMSO-d_6. The spectrum of the major product (isolated in 82% yield) clearly confirmed the structure of the desired Ac_3ct^6A nucleoside (Fig. S9). The signal for the diagnostic H11 atom (NH) at δ 8.74 ppm is located upfield to that at 9.85 ppm for the same proton in opened Ac_3t^6A (compare the spectra in DMSO-d_6 on Fig. S1 and Fig. S9), and the observed relation is the same as that



Scheme 1. Synthesis of the cyclic form of 2′,3′,5′-tri-*O*-acetyl-threonylcarbamoyl-adenosine (Ac_3ct^6A).

published for ct^6A and t^6A molecules.[9] Additionally, Ac_3ct^6A was further characterized by ^1H NMR (D_2O), ^{13}C NMR, FAB MS, UV, and IR spectra (Figs. S10–S15), and its purity was confirmed by HPLC (Fig. S16). The minor product (yield 12%) was identified on the basis of ^1H NMR spectrum in DMSO-d_6 (Fig. S17) as a derivative of Ac_3ct^6A additionally acetylated at the β-hydroxyl function of the threonine residue (abbreviated as $Ac_3ct^6A^{Ac}$). This compound was the only product when the dehydration reaction was performed in neat acetic anhydride (Supplementary data, p. 24). It is noteworthy that in the case of synthesis of Ac_3ct^6A, the formation of the threonine cyclic ester under acetic anhydride treatment in methylene chloride appeared to be much more convenient than the procedure employing soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)),[21] as the latter methodology provides difficult to separate urea side-products (unpublished data).

To study the susceptibility of the oxazolone type Ac_3ct^6A cyclic ester to hydrolysis under different conditions ^1H NMR technique was used, as the spectra (in D_2O) of cyclic Ac_3ct^6A and opened Ac_3t^6A are sufficiently different (Fig. 2 and Fig. S24). The signals of heterobase protons H8 and H2 are diagnostic for quantification of the product formation.

Cyclic Ac_3ct^6A was subjected to hydrolysis in a 0.2 M PBS/D_2O buffer (pD 7.4). The reactions were performed at 23 and 37 °C in NMR tubes, and the time course of the process was monitored (Fig. 3, panel Ia and Ib, respectively, and Fig. S26 and Fig. S27).

The hydrolyses carried out at 23 and 37 °C required ca. 230 and 70 h, respectively, to reach the mid-point. The reactions followed the kinetics of a pseudo-first order process (Fig. S28 and Fig. S29) with the rate constant values of 66.7×10^{-4} min^{-1} and 2.17×10^{-4} min^{-1}, respectively.

Our next studies confirmed that the cyclic form easily hydrolyzes under the conditions of the conventional isolation of modified tRNA nucleosides.[10] Treatment of Ac_3ct^6A with 0.1 M ammonium acetate (pH 5.3) at 45 °C for 2 h, followed by 1 M ammonium bicarbonate (pH 8.2) at 37 °C for additional 2 h, gave almost exclusively the open form (Fig. 3, panel IIa). Most likely the reaction mixture contained the carboxylic acid and amide derivatives of Ac_3t^6A, as in the ^1H NMR region of the open nucleoside the signals of H2 and H8 protons were splitted. The presence of the amide is possible since treatment of the cyclic nucleoside only with 1 M ammonium bicarbonate (pH 8.2) afforded the amide derivative of Ac_3t^6A in good yield (Supplementary data, p. 26, Figs. S18–S23 and Fig. S25). The new methodology for isolation

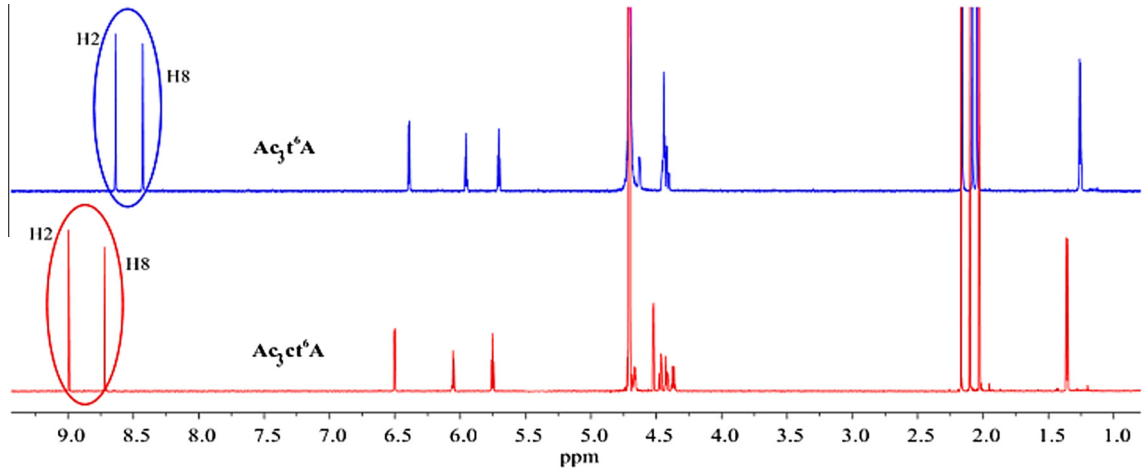
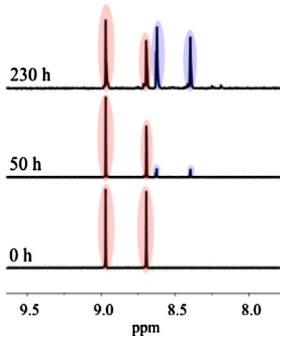
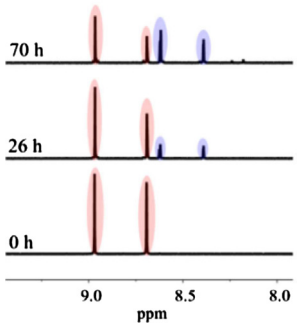


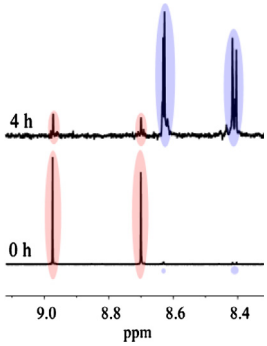
Figure 2. Comparison of ^1H NMR spectra in D_2O of Ac_3t^6A (an upper panel) and Ac_3ct^6A (a lower panel); the protons H2 and H8 diagnostic for Ac_3ct^6A stability studies are marked with ovals.



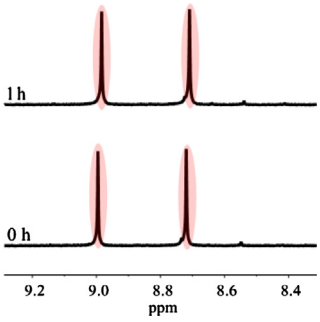
Ia. 0.2 M PBS buffer, pH 7.4, 23°C



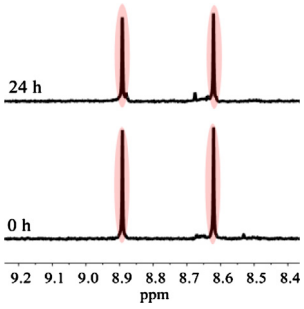
Ib. 0.2 M PBS buffer, pH 7.4, 37°C



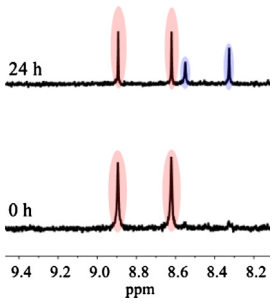
IIa. Condition of old isolation method[10] (0.1 M ammonium acetate, 45°C, 2h 1M and ammonium bicarbonate, 37°C, 2h)



IIb. Condition of new isolation method[9] (20 mM TMA·HCl, pH 7, 1h, 37°C)



IIIa. 100 mM DCl, pH 1, 22°C



IIIb. 0.1 mM NaOD, pH 10, 22°C

Figure 3. Stability of Ac_3ct^6A under different hydrolysis conditions. Signals of the H2 and H8 protons of the cyclic Ac_3ct^6A are marked in red and of the opened Ac_3t^6A in blue.

of modified nucleosides (the use of 20 mM trimethylamine × HCl solution, pH 7.0, 1 h) is completely safe for the cyclic nucleoside (Fig. 3, panel IIb).

Interestingly, the oxazolone ring in Ac_3ct^6A is stable in an acidic medium. Treatment of the nucleoside with 100 mM DCl solution (pD 1) for 24 h at room temperature did not produce any products of degradation (Fig. 3, panel IIIa and Fig. S30). Moreover, Ac_3t^6A appeared to be relatively stable under basic conditions (0.1 mM NaOD in D_2O, pH 10) as after 24 h the ^1H NMR analysis revealed only ca. 30% of the open product (Fig. 3, panel IIIb and Fig. S31), so the NaOH mediated hydrolysis of the threonine cyclic ester proceeds substantially slower than the reactions with ammonia and primary amines leading to corresponding amides.[10]

The observed high reactivity of the oxazolone ring towards nitrogen nucleophiles practically excludes the introduction of ct^6A into an oligonucleotide chain by typical procedures of solid phase synthesis as ammonium or amines are usually applied in the deprotection steps or for cleavage of oligonucleotides from the support.[22] On the other hand, our stability studies and the Suzuki's successful attempt at conversion of t^6A into ct^6A using EDC activation in an aqueous medium[9] offer some chances for the incorporation of ct^6A by a post-synthetic modification of the t^6A containing oligomers. Additionally, previous studies on the formation of different t^6A amides[23] clearly demonstrated that the four common nucleosides, as well as phosphodiester internucleotide bonds, remained unaffected by EDC treatment at a pH range needed for the oxazolone ring formation.

The presented efficient synthesis of the acetylated derivative of cyclic N^6-threonylcarbamoyladenosine (Ac_3ct^6A) allowed to assess the susceptibility of ct^6A to hydrolysis under different conditions. Our results indicate that rather unusual oxazolone ring of ct^6A is much more stable than typical oxazolones derived from the *N*-acyl or *N*-alkoxycarbonyl amino acid derivatives.[20,24,25] Additionally, the spectrum recorded for Ac_3ct^6A obtained from DL-threonine (in D_2O) contained the splitted signals for the H2′ and H3′ sugar moiety protons and for the α (H12 according to the numbering in ct^6A) and β (H14) protons of threonine residue, while that recorded in DMSO-d_6 showed splitted resonances for the purine

H2 and threonine β-OH (C14-OH) protons (Supplementary material, Fig. S32–S35). On the other hand, the corresponding signals in all ^{1}H NMR spectra taken in our study were non-splitted, so in the ct^{6}A oxazolone structure the configuration at the α-carbon of L-threonine residue has been retained.

In conclusion, slow hydrolysis of Ac$_3$ct^{6}A under aqueous physiological conditions (PBS buffer, pH 7.4) and remarkable stability under acidic and basic conditions (100 mM HCl, pH 1, and 0.1 mM NaOH, pH 10, respectively) gives the possibility for synthesis of ct^{6}A-containing oligonucleotides by post-synthetic transformation of oligomers bearing the opened t^{6}A unit. The availability of suitable model oligonucleotides will allow for future structure–function studies to compare biological roles of ct^{6}A and t^{6}A hypermodifications.

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Supplementary data

Supplementary data (general information, experimental procedures, spectral data for the compounds and characterization information of the hydrolysis reactions) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.048.

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