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Chemical synthesis of oligoribonucleotide (ASL of $tRNA^{Lys}$ *T. brucei*) containing a recently discovered cyclic form of 2-methylthio-N^6-threonylcarbamoyladenosine (ms^2ct^6A)

Katarzyna Debiec,[a] Michal Matuszewski,[a] Karolina Podskoczyj,[a] Grazyna Leszczynska, [a] and Elzbieta Sochacka *[a]

Abstract:

The synthesis of the protected form of 2-methylthio-N^6-threonylcarbamoyl adenosine (**ms^2t^6A**) was developed starting from adenosine or guanosine using the optimized carbamate method and, for the first time, the isocyanate route. The hypermodified nucleoside was subsequently transformed to the protected **ms^2t^6A**–phosphoramidite monomer and used in a large scale synthesis of the precursor 17nt **ms^2t^6A**-oligonucleotide (the anticodon stem and loop fragment of $tRNA^{Lys}$ from *T. brucei*). Finally, stereochemically secure **ms^2t^6A→ms^2ct^6A** cyclization at the oligonucleotide level afforded efficiently tRNA fragment bearing the **ms^2ct^6A** unit. Applied post-synthetic approach provides two sequentially homologous **ms^2t^6A**- and **ms^2ct^6A**-oligonucleotides suitable for further comparative structure-activity relationship studies.

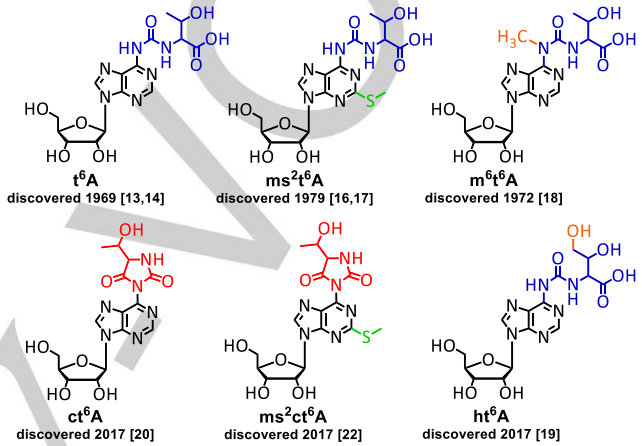

Figure 1. Structures of L-threonylcarbamoyl modified adenosines located at position 37 of tRNAs

Introduction

Transfer RNAs (tRNAs) constitute a unique subset of natural RNA, because in all domains of life these relatively short biopolymers utilize, in total, more than 130 structurally diverse analogs of canonical nucleosides.[1-3] Their impact on the biological processes (mostly related to the decoding of the genetic code) is a matter of intense studies.[4-10] Interestingly, the modifications are not distributed evenly along the tRNA molecules[11] and predominantly occupy positions 34 (the wobble position) and 37 (3′ side downstream from the anticodon) in the anticodon loop and stem domains.[1,5-7,11,12]

Among the modified nucleosides found at the position 37, special interest is paid to N^6-threonylcarbamoyladenosine (**t^6A_{37}**, Figure 1) and its derivatives (**ms^2t^6A** and **m^6t^6A**, *vide infra*), present in almost all known tRNAs decoding the A-starting codons (ANN).[1,11] The most abundant, namely **t^6A_{37}** has been intensively studied (for already 50 years[13,14]) because of diverse functions it plays during protein biosynthesis.[15] In some tRNAs, **t^6A_{37}** is replaced with an analog containing either an -SMe group attached to the purine C2 atom (**ms^2t^6A**)[16,17] or a methyl substituent at the N^6-atom (**m^6t^6A**).[18]

Recently, due to a remarkable development of biochemical and analytical methods, other analogs of **t^6A** have been identified in the tRNA anticodon loops, *i.e.* a **t^6A** "linear" derivative having a hydroxylated threonine methyl group[19] (**ht^6A**, Figure 1) and two "cyclic" species bearing a hydantoin ring - N^6-threonylcarbamoyl-adenosine (**ct^6A**)[20,21] and 2-methylthio-N^6-threonylcarbamoyl adenosine (**ms^2ct^6A**).[22] It was documented that **ct^6A** and **ms^2ct^6A** are formed intracellularly upon enzymatic dehydration of "linear" **t^6A** and **ms^2t^6A** units.[20,22] Importantly, the hydantoin derivatives were identified in several tRNAs in which the "linear" **t^6A** and **ms^2t^6A** were previously detected.[20-22] This initial misidentification resulted from high susceptibility of **ct^6A** and **ms^2ct^6A** to hydrolysis,[20-23] (leading to **t^6A** and **ms^2t^6A** units, respectively) under alkaline conditions commonly used in the standard protocol of tRNA isolation.[24] Thus, the reported existence of "linear" **t^6A/ms^2t^6A** in tRNA molecules must be verified and the results may lead to new ideas regarding the biological functions of tRNAs bearing "cyclic" analogs.

Structural studies revealed that the N^6-threonylcarbamoyl chain in **t^6A** adopts a planar alignment in respect to the adenine ring.[25,26] The same phenomenon was observed in the **t^6A**-containing RNA oligomers[27-31] and that planarity is claimed to be a crucial factor for proper strength of the codon-anticodon interactions[27-32] However, X-ray studies of the **ct^6A** nucleoside revealed a twisted arrangement of the hydantoin ring and the adenine moiety, most likely enforced by strong electrostatic repulsion between the carbonyl oxygen atoms of hydantoin and the N1 and N7 nitrogen atoms of adenine.[20] It is highly possible that the hydantoin and adenine rings are also not coplanar in biological milieu, with still unknown consequences for the translation process. Thus, to investigate structural aspects and biological functions of the tRNA containing **ct^6A** or **ms^2ct^6A** units, a reliable chemical method for the synthesis of suitable model oligonucleotides is highly desired.

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Recently, we have reported a successful transformation of **t^{6}A** into **ct^{6}A** and a method suitable for that cyclization at the oligonucleotide level.[33] Here we present the first introduction of an **ms^{2}ct^{6}A** unit into a 17-mer with the sequence of anticodon stem and loop (ASL) fragment of tRNA^{Lys} from *T. brucei*, where **ms^{2}ct^{6}A** was found as a native modification.[22]

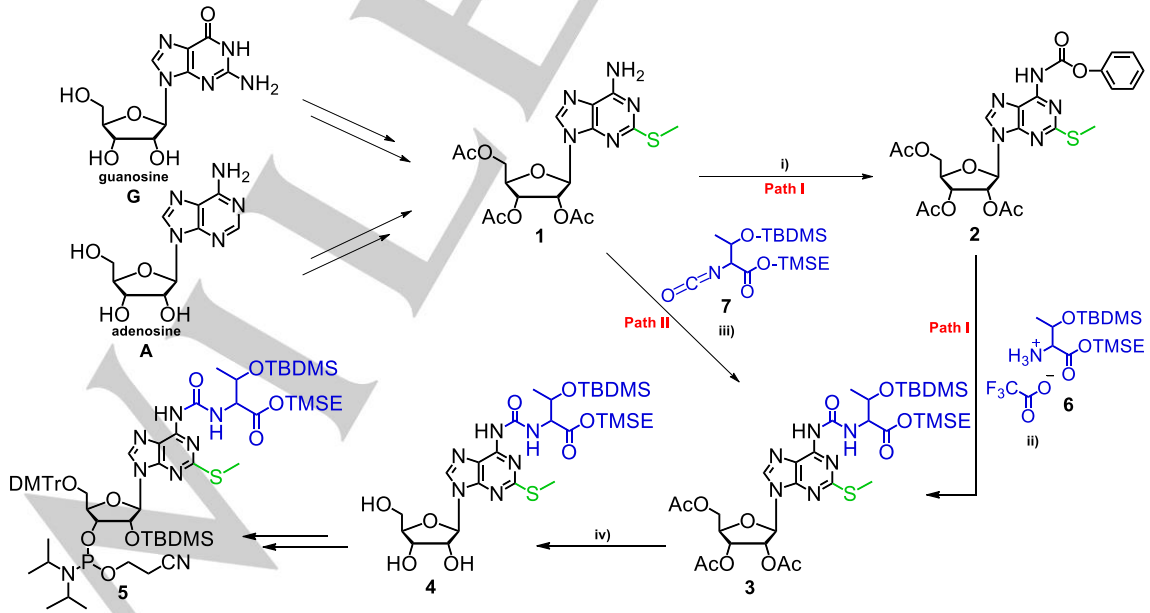
Results and Discussion

Although chemical synthesis of RNA oligomers containing the **t^{6}A** or **ms^{2}t^{6}A** units by the phosphoramidite approach[34-36] using the ultra-mild protected monomers is feasible,[37-43] this approach is inappropriate for synthesis of RNAs bearing **ms^{2}ct^{6}A** due to the aforementioned instability of the hydantoin ring in even slightly alkaline media. Therefore, we decided to explore an alternative post-synthetic methodology in which the **ms^{2}t^{6}A→ms^{2}ct^{6}A** cyclization is performed in the already assembled oligoribonucleotide containing the **ms^{2}t^{6}A** unit. First, we optimized the synthesis of the 5′-*O*-DMTr-2′-*O*-TBDMS protected "linear" **ms^{2}t^{6}A** nucleoside (protected at the threonine –OH and –COOH functions) and made its 3′-*O*-phosphoramidite derivative (**5**, Scheme 1). The monomer was then used in the synthesis of an **ms^{2}t^{6}A**-modified oligonucleotide (Figure 2). Finally, we performed an effective and stereochemically safe conversion of **ms^{2}t^{6}A→ms^{2}ct^{6}A** at the oligonucleotide level (Figure 2 and 3).

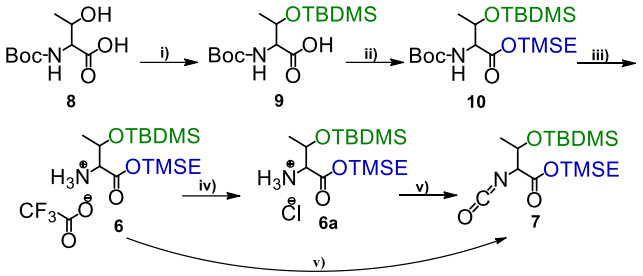
Optimization of ms^{2}t^{6}A phosphoramidite synthesis

In our preparation of the **ms^{2}t^{6}A** monomer **5**, 2′,3′,5′-tri-*O*-acetyl derivative of **ms^{2}A** (**Ac$_3$ms^{2}A**, **1**, Scheme 1) was a key intermediate. This nucleoside was previously synthesized by the D. R. Davis group with the use of the silyl method of *N*-glycosidic bond formation between 2-methylthioadenine (not commercially available, obtained with 4 steps synthesis from thiourea and malonitrile) and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, followed by the conversion into 2′,3′,5′-tri-*O*-acetyl derivative.[41] In our approach we used adenosine or guanosine as the starting material, and successfully combined synthetic methods already reported in several sources.[44-49] In brief, adenosine was converted into adenosine N-oxide,[44] which was subjected to the opening of the 6-membered ring (5 M NaOH) with the formation of 5-amino-1-(β-D-ribofuranosyl)imidazole-4-carboxamidoxime, and later to the ring closure/thiolation (a reaction with CS_2), followed by simple *S*-methylation with MeI.[44-46] Final acetylation of the ribose hydroxyl groups yielded **1** in 31 % overall yield (for experimental details see Scheme S1, Supporting Information). The same protected nucleoside **1** was also obtained in a four-step synthesis from guanosine (in 36 % overall yield), involving acetylation of the ribose hydroxyl groups of nucleoside, followed by a C^6=O→C^6-Cl conversion with $POCl_3$,[47] incorporation of the -SMe group at the C^2 atom and the final C^6-Cl→C^6-NH$_2$ conversion[48,49] (Scheme S2, Supporting Information).

To modify the nucleobase in **Ac$_3$ms^{2}A** with a threonylcarbamoyl residue at the N^6 position we applied a carbamate or an isocyanate route (Scheme 1, path I and II, respectively). The former is advantageous when the attachment of an unprotected L-threonine residue (in a ureido fashion) is the synthetic goal,[50-52] but in the synthesis of **5**, the phosphitylation reaction must be preceded by an appropriate protection of the hydroxyl and carboxylic groups. In the reported methods, the *tert*-butyldimethylsilyl group (TBDMS) was used to protect the hydroxyl function, and the carboxylic group was converted into a base-labile 2-(4-nitrophenyl)ethyl ester (pNPE)[38,41] or a fluoride-sensitive trimethylsilylethyl ester (TMSE).[37,39,40,42] Within the TBDMS/pNPE protection scheme, *O-tert*-butyldimethylsilyl-L-threonine 2-(4-nitrophenyl) ethyl ester was reacted with **ms^{2}A** carbamate,[38] while the TBDMS/TMSE protection was carried out in the already threonylated **ms^{2}t^{6}A** nucleoside by the reaction with TBDMS triflate, followed by esterification with 2-(trimethylsilyl)ethanol in the presence of DCC.[40,42]



Scheme 1. Synthesis of **ms^{2}t^{6}A** phosphoramidite i) phenoxycarbonyl tetrazole (3.0 equiv), 1,4-dioxane, 40 °C, 20 h, 78 % yield; ii) pyridine, 40 °C, 24 h, 75 % yield; iii) toluene, reflux, 16 h, 83 % yield; iv) triethylamine in methanol (10 % solution), rt, 24 h, 86 % yield; (synthetic steps from G to **1** and from A to **1** as well as the procedures for the nucleoside **4** protection with DMTr and TBDMS groups and phosphitylation are described in details in Supporting Information).



Scheme 2. Synthesis of L-threonine component i) *tert*-butyldimethylsilyl chloride (2.2 equiv), imidazole (2.2 equiv), pyridine, 24 h, 98 % yield; ii) 2-(trimethylsilyl)ethanol (1.1 equiv), DCC (1.6 equiv), DMAP (0.1 equiv) dichloromethane, 0 °C, 3 h, 98 % yield; iii) trifluoroacetic acid in dichloromethane (60 % solution), dichloromethane, rt, 5 min, 64 % yield; iv) 1,4-dioxane saturated with hydrogen chloride (4 M), dichloromethane, 5 min, 100 % yield; v) phosgene in toluene (20 % solution), pyridine, dichloromethane, 0 °C, 2 h, (100 % conversion observed for crude **7** from **6a**)

We explored the carbamate route (Scheme 1, path I) using synthesized for the first time TBDMS/TMSE protected L-threonine (**6**), which was obtained starting from commercially available *N*-Boc-L-threonine (**8**, Scheme 2 and Experimental Section). Compound **8** was almost quantitatively converted into an *O*-TBDMS derivative **9**, then esterified with 2-(trimethylsilyl)ethanol in the presence of DCC, and, finally, *N*-deprotected with trifluoroacetic acid to furnish the corresponding salt **6** in 61 % overall yield. It was further coupled with an active carbamoyl derivative **2** (Scheme 1), which was prepared in the reaction of 2′,3′,5′-tri-*O*-acetyl-2-methylthioadenosine (**1**) with phenoxycarbonyl tetrazole according to the reported procedure.[41,42] The condensation gave fully protected **ms^{2}t^{6}A** (**3**) in 75 % yield. The same intermediate **3** was obtained on the isocyanate path II (Scheme 1). To this goal, compound **6** (or **6a**, Scheme 2) was treated with a commercially available solution of phosgene in toluene at 0 °C, according to the racemization-free Nowick's procedure.[53] After short aqueous workup (to avoid hydrolysis) an isocyanate derivative **7** was obtained and reacted (without purification) with 2′,3′,5′-tri-*O*-acetyl-2-methylthioadenosine (**1**) to give nucleoside **3** with a good 68 % yield (Scheme1). The reaction was remarkably more efficient (83 % yield) when **7** was obtained from the more stable hydrochloride salt of L-threonine **6a**. (Note: in a similar approach, used by Chheda and Hong for synthesis of **t^{6}A**, the "isocyanated" *O*-benzyl-L-threonine benzyl ester was reacted with 2′,3′,5′-tri-*O*-acetyl-adenosine and the product was obtained with a moderate 48 % yield. [50] In a "mirror" reaction of the "isocyanated" adenosine with *O-tert*-butyldimethylsilyl-L-threonine *p*-nitrophenylethyl ester the urethane nucleoside product was obtained in only 19 % yield[38]). The acetyl groups in **3** were safely removed with 10 % TEA in methanol to give the conjugate **4** in 86 %, so the three step synthesis (path I, **1**→**2**→**3**→**4**, Scheme 1) afforded the TBDMS/TMSE derivative of **ms^{2}t^{6}A** in 50 % overall yield, compared to 40 % reported for the use of *O-tert*-butyldimethylsilyl-L-threonine-(2-(4-nitrophenyl)ethyl) ester intermediate.[41] For the alternative two step synthesis (path II, **1**→**3**→**4**), the overall 60-70 % efficiency was observed, depending on the way of preparation of **7**, so in terms of chemical efficiency this newly developed isocyanate route is advantageous over the path I.

The final transformation **4**→**5** (Scheme 1) was accomplished using the chemistry reported for previous syntheses of **t^{6}A** / **ms^{2}t^{6}A** phosphoramidite building blocks.[38-43] The details are given in Supporting Information (Scheme S3).

Synthesis of an ms^{2}t^{6}A-containing RNA oligonucleotide by phosphoramidite chemistry

Manual synthesis of a 17nt oligoribonucleotide of the sequence 5'-CACGGCUUUU**ms^{2}t^{6}A**ACCGUG-3' (**ms^{2}t^{6}A-ASL,** Figure 2) was performed on a CPG-support at a 2.5 µmol scale using the modified Sproat's protocol.[35] The phosphoramidite monomers other than **5**, as well as guanosine attached to the solid support, were *N*-protected with a 4-*tert*-butylphenoxyacetyl (tac) groups to make the final deprotection safe for the relatively base labile carbamoyl linkage in **ms^{2}t^{6}A** nucleoside. Also, the oxidation step was modified to protect the 2-methylthio group in the **ms^{2}t^{6}A** unit. Whereas in the previous synthesis of an **ms^{2}t^{6}A**-oligonucleotide 10 % *t*BuOOH in acetonitrile was applied as the oxidizing agent,[41,42] we used a diluted 0.02 M iodine solution in THF/H_2O/pyridine, which we effectively and safely used in synthesis of oligonucleotides containing the highly oxidation sensitive 2-thiouridines.[54-56] In the coupling steps (lasting for 8 min), 0.1 M solutions of canonical phosphoramidites in dry acetonitrile and 5-(3,5-bis(trifluoromethyl)phenyl)-1*H*-tetrazole (0.25 M solution in ACN) as an activator were used. To increase the efficiency of incorporation of the **ms^{2}t^{6}A** unit, double coupling of **5** was performed, each lasting for 25 min. Next, the protected **ms^{2}t^{6}A-ASL** (still CPG-bound) was treated with TEA–ACN (1:1, v/v) to remove the 2-cyanoethyl groups, followed by 8 M ethanolic ammonia to remove the *N*-protecting groups and to release the oligoribonucleotide from the support. The resultant solution was concentrated *in vacuo* and treated with 1 M TBAF in THF to remove the TBDMS and TMSE groups. The deprotected RNA was isolated by IE-HPLC (for details see Experimental Section and Supporting Information, Figure S2). The eluate was concentrated to a small volume, desalted and lyophilized to yield 94 OD of **ms^{2}t^{6}A-ASL**, the structure of which was confirmed by MALDI-TOF MS (Figure S3).

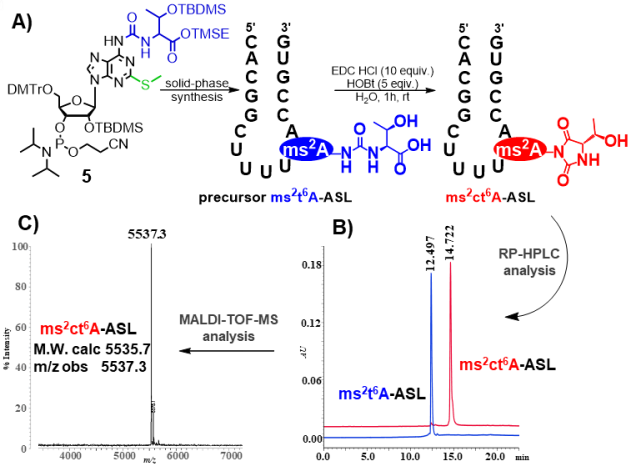


Figure 2. A) Synthesis of **ms^{2}ct^{6}A**-modified oligoribonucleotide with the sequence corresponding to the anticodon stem and loop of tRNA^{Lys} *T. brucei* (**ms^{2}ct^{6}A-ASL**); **B**) comparison of RP-HPLC profiles of precursor **ms^{2}t^{6}A-ASL** and cyclic **ms^{2}ct^{6}A-ASL**; **C**) MALDI-TOF MS for **ms^{2}ct^{6}A-ASL**

Conversion of ms²t⁶A → ms²ct⁶A at oligonucleotide level

To convert the *N*⁶-threonylcarbamoyl residue in **ms²t⁶A-ASL** into a hydantoin ring, we applied the conditions developed recently for the post-synthetic **t⁶A→ct⁶A** conversion in **t⁶A**-containing oligomers.[33] In a small scale dehydration/cyclization of **ms²t⁶A-ASL** (0.5 OD), 5 equivalents of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and 5 equivalents of 1-hydroxy-1*H*-benzotriazole (HOBt) were used. After 1 hour, an RP-HPLC analysis revealed ca. 50 % conversion. Longer reaction time did not promote the further conversion. When the excess of EDC·HCl/HOBt was doubled, 1-hour reaction time was sufficient for almost quantitative **ms²t⁶A-ASL→ms²ct⁶A-ASL** transformation (Supporting Information, Figure S4). This 10-fold excess of dehydrating reagents was maintained in a large scale (50 OD) transformation, which after ultrafiltration of the reaction mixture (Amicon® Ultra 3K; 3,000 MWCO) furnished 45 OD (90 % conversion) of **ms²ct⁶A-ASL** (for details see Experimental Section and Supporting Information, Figure S5). The structure of **ms²ct⁶A-ASL** was confirmed by MALDI-TOF MS (Figure 2, Figure S6) and determination of the nucleoside content (Figure 3 and Figure S7). In the latter, the isolated product was hydrolyzed with nuclease P1 and the resultant nucleoside 5'-*O*-phosphates were dephosphorylated with alkaline phosphatase in 20 mM TEA·HCl (pH 7).[21] RP-HPLC analysis (monitored at 254 nm) of the mixture showed the presence of four canonical and one modified nucleoside product (Figure 3, part C).

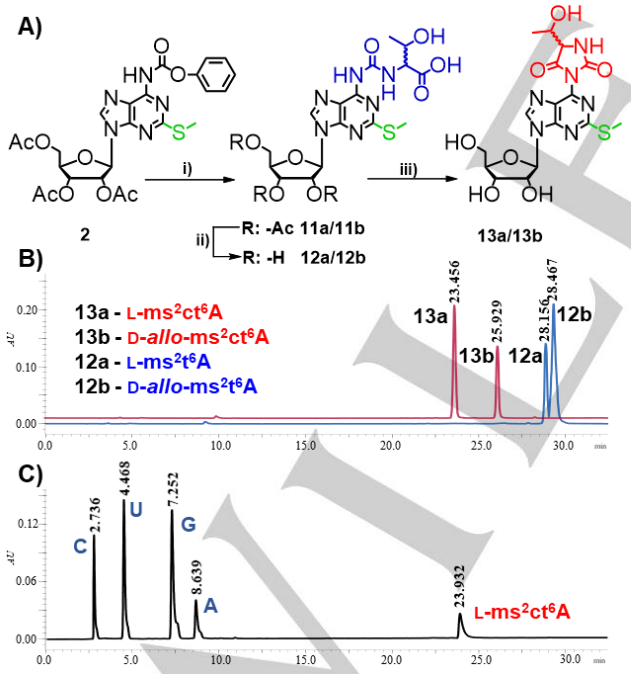


Figure 3. A) Synthesis of **L-ms²ct⁶A** and **D-*allo*-ms²ct⁶A** i) L-threonine (a) or D-*allo*-threonine (b) (3.0 equiv), pyridine, 40 °C, 10 h, 88 % yield for **11a**, 82 % yield for **11b**; ii) NH_3 in methanol (10 M), rt, 5 h, 90 % yield for **12a**; 87 % yield for **12b**; iii) EDC-P (5 equiv), HOBt (5 equiv), water, rt, 1 h, 90 % yield for **13a**, 92 % yield for 13b; **B**) RP-HPLC profile of a mixture of **L-ms²t⁶A** / **D-*allo*-ms²t⁶A** and **L-ms²ct⁶A** / **D-*allo*-ms²ct⁶A** nucleosides **C**) The nucleoside composition analysis of **ms²ct⁶A-ASL** after enzymatic digestion.

Taking into account that the conditions applied in the synthesis and purification of **ms²ct⁶A-ASL** might lead to the hydrolysis of the hydantoin ring in **L-ms²ct⁶A** and/or the epimerization at the C-α atom of the amino acid residue, four HPLC-standards were prepared: "linear" **L-ms²t⁶A** (**12a**) and **D-*allo*-ms²t⁶A** (**12b**) and "cyclic" **L-ms²ct⁶A** (**13a**)/ **D-*allo*-ms²ct⁶A** (**13b**) (Figure 3, part A, and Supporting Information Scheme S4 and Figures S8, S9). Intermediates **11a** and **11b** were synthesized in the reactions of phenoxycarbamate **2** with L-threonine or its C-α epimer (D-*allo*-threonine), respectively. Their treatment with 10 M methanolic solution of ammonia afforded the corresponding ammonium salts of **12a** or **12b**, which were further converted into free acids by ion exchange chromatography and treated with the polymer-bound 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC-P, 5 equiv) and hydroxybenzotriazole (HOBt, 5 equiv). After 1h reaction at rt, the polymer beads were filtered off and "cyclic" **13a** or **13b** were isolated (in 90 % and 92 %, respectively) by silica gel column chromatography. An RP-HPLC analysis revealed that the peaks corresponding to **12a**, **12b**, **13a**, and **13b** were well separated (Figure 3, part B). Using these standards in analysis of the aforementioned mixture of nucleosides obtained after enzymatic hydrolysis of **ms²ct⁶A-ASL** we confirmed the presence of the **ms²ct⁶A** unit with a natural L-threonine residue (Figure 3, part B and C).

Conclusions

The optimization of the carbamate route (Scheme 1, path I) as well as the first synthesis of the protected **ms²t⁶A** derivative **4** on the isocyanate route (path II), significantly improved the availability of this synthetically demanding nucleoside and made the preparation of the **ms²t⁶A**-phosphoramidite monomer **5** more efficient. Compound **5** was used in a manual synthesis of the 17nt **ms²t⁶A**-oligonucleotide (the anticodon stem and loop fragment of $tRNA^{Lys}$ from *T. brucei*), which was post-synthetically subjected to the epimerization-free **ms²t⁶A→ms²ct⁶A** cyclization. Since this approach furnishes the "linear" **ms²t⁶A**-oligonucleotide and its "cyclic" **ms²ct⁶A**-containing derivative, the comparative structure-activity relationship studies may commence, which should result in a better understanding of the role of **ms²t⁶A** cyclization in the tuning of decoding process in protein biosynthesis.

Experimental Section

General remarks

Commercial reagents purchased from Aldrich and Acros companies were used without additional purification unless otherwise stated. All solid reagents were dried under high vacuum prior to use. Analytical thin layer chromatography (TLC) was done on silica gel coated plates (60 F254, Supelco) with UV light (254 nm) or the ninhydrin test (for amino acids) detection. The products were purified by chromatography on a silica gel column 60 (mesh 230 – 400, Fluka) eluted with the indicated solvent mixtures. NMR spectra were recorded using a 700 MHz (for ^{1}H) instrument (176 MHz for ^{13}C, 101 MHz for ^{31}P). Chemical shifts (δ) are

reported in ppm relative to TMS (an internal standard) for ^{1}H and ^{13}C, and 85 % phosphoric acid (an external standard) for ^{31}P. The signal multiplicities are described as s (singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplets), dq (doublet of quartets), t (triplet), td (triplet of doublets), q (quartet), qd (quartet of doublets), m (multiplet), and bs (broad singlet). MALDI-TOF MS spectra were recorded on Applied Biosystems Voyager-Elite mass spectrometer in a linear, negative-ion mode. IR data were recorded on an FT-IR ALPHA instrument (Bruker) equipped with a Platinum ATR QuickSnap™ module. Analytical HPLC of nucleosides/oligonucleotides were performed on a Shimadzu Prominence HPLC system equipped with an SPD-M20A spectral photodiode array detector using a Kinetex® column (RP, C18, 5 µm, 4.6 × 250 mm, 100 Å, Phenomenex) or an Ascentis® column (RP, C18, 5 µm, 4.6 × 250 mm, Supelco). Preparative IEX-HPLC of oligoribonucleotides were performed on a Waters 515 HPLC system equipped with a 996 spectral diode array detector, using a SOURCE™ column (IE, 15Q 4.6/100PE®, GE Healtcare). Analyses were run at 30 °C for RP-HPLC and at 38 °C for IEX-HPLC. The elution profiles were UV monitored at λ = 254 nm.

Synthesis of 2-methylthio-*N*^{6}-threonylcarbamoyladenosine protected at threonyl –OH and –COOH functions (4)

[9-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl)-2-methylthiopurin-6-yl] phenylcarbamate (**2**)

To a sample of 2',3',5'-tri-*O*-acetyl-2-methylthioadenosine (**1**) (0.60 g; 1.4 mmol) dissolved in anhydrous 1,4-dioxane (3 mL) phenoxycarbonyl tetrazole (0.80 g; 4.2 mmol) was added in one portion under argon atmosphere. The mixture was stirred at 40 °C for 20 h and the solvent was evaporated under reduced pressure. The product **2** was isolated by column chromatography (silica gel, 0-20 % AcOEt in CH_2Cl_2) as a white solid (0.62 g; 1,1 mmol; 78 % yield). TLC: R_f = 0.74 ($CHCl_3$/MeOH, 95/5, v/v); ^{1}H NMR: (DMSO-d6) δ: 11.19 (s, 1H, NH-6), 8.51 (s, 1H, H-8), 7.47-7.43 (m, 2H, H_{Ar}), 7.30-7.27 (m, 1H, H_{Ar}), 7.26-7.25 (m, 2H, H_{Ar}), 6.26 (d, 1H, ^{3}J = 4.4 Hz, H-1'), 6.07 (dd, 1H, ^{3}J = 6.2 Hz, ^{3}J = 4.4 Hz, H-2'), 5.70-5.68 (m, 1H, H-3'), 4.42 (dd, 1H, ^{2}J = 12.0 Hz, ^{3}J = 3.7 Hz, H-5'), 4.39-4.37 (m, 1H, H-4'), 4.22 (dd, 1H, ^{2}J = 12.0 Hz, ^{3}J = 5.4 Hz, H-5″), 2.59 (s, 3H, S-CH_3), 2.12 (s, 3H, CH_3-CO), 2.07 (s, 3H, CH_3-CO), 1.96 (s, 3H, CH_3-CO). (Spectrum S15, Supporting Information).

N-[[9-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl)-2-methylthiopurin-6-yl]carbamoyl]-*O*-*tert*-butyldimethylsilyl-*L*-threonine trimethylsilylethyl ester (**3**)

Path I: To a solution of **2** (1.12 g; 2.0 mmol) in dry pyridine (9 mL) threonine trifluoroacetate **6** (1.00 g; 2.2 mmol) was added. The mixture was stirred at 40 °C for 24 h and the solvent was evaporated under reduced pressure. The oily residue was co-evaporated with toluene (3 × 15 mL), diluted with CH_2Cl_2 and washed with 5 % aq. $NaHCO_3$ (2 × 40 mL) and H_2O (50 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The product **3** was isolated by column chromatography (silica gel, 0-1 % MeOH in CH_3Cl) as a light-yellow solid (1.13 g; 1.5 mmol; 75 % yield).

Path II: 2',3',5'-Tri-*O*-acetyl-2-methylthioadenosine (**1**, 0.31 g; 0.70 mmol) and threonine isocyanate (crude **7** obtained from **6a**, 0.76 g; 2.10 mmol) were dissolved in dry toluene (12 mL). The reaction mixture was stirred under reflux for 16 h and the solvent was evaporated under reduced pressure. The product **3** was isolated by column chromatography (silica gel, 0-1 % MeOH in $CHCl_3$) as a light-yellow solid (0.47 g; 0.58 mmol; 83 % yield).

TLC: R_f = 0.52 ($CHCl_3$/MeOH, 95/5, v/v); ^{1}H NMR: (DMSO-d6) δ: 9.98 (s,1H, NH-6), 9.25 (d, 1H, ^{3}J = 8.6 Hz, NH Thr), 8.45 (s, 1H, H-8), 6.25 (d, 1H, ^{3}J = 4.3 Hz, H-1'), 6.08 (dd, 1H, ^{3}J = 6.0 Hz, ^{3}J = 4.3 Hz, H-2'), 5.70-5.68 (m, 1H, H-3'), 4.46-4.44 (m, 2H, CH-α Thr, CH-β Thr), 4.42 (dd, 1H, ^{2}J = 12.0 Hz, ^{3}J = 3.7 Hz, H-5'), 4.38 (dt, 1H, ^{3}J = 5.8 Hz, ^{3}J = 3.8 Hz, H-4'), 4.22-4.18 (m, 2H, H-5″,O-CH_2 TMSE), 4.11 (dt, 1H, ^{2}J = 10.7 Hz, ^{3}J = 6.2 Hz, O-CH_2 TMSE), 2.58 (s, 3H, S-CH_3), 2.11 (s, 3H, CH_3-CO), 2.08 (s, 3H, CH_3-CO), 1.96 (s, 3H, CH_3-CO), 1.19 (d, 3H, ^{3}J = 6.2 Hz, CH_3 Thr), 1.03-0.94 (m, 2H, Si-CH_2 TMSE), 0.85 (s, 9H, Si-C(CH_3)$_3$ TBDMS), 0.07 (s, 3H, Si-CH_3 TBDMS), 0.03 (s, 3H, Si-CH_3 TBDMS), 0.01 (s, 9H, Si(CH_3)$_3$ TMSE); ^{13}C NMR: (DMSO-d6) δ: 170.25 (C=O Thr), 169.96 (CH_3-<u>C</u>O), 169.43 (CH_3-<u>C</u>O), 169.30 (CH_3-<u>C</u>O), 164.58 (C-2), 153.23 (NH-CO-NH), 150.68 (C-4), 149.90 (C-6), 141.81 (C-8), 118.03 (C-5), 86.50 (C-1'), 78.88 (C4'), 71.96 (C-2'), 69.60 (C-3'), 68.69 (CH-β Thr), 62.89 (O-CH_2 TMSE), 62.51 (C-5'), 59.04 (CH-α Thr), 25.71 (Si-C(<u>C</u>H$_3$)$_3$ TBDMS), 20.56 (CH_3 Thr), 20.35 (<u>C</u>H$_3$-CO), 20.33 (<u>C</u>H$_3$-CO), 20.26 (<u>C</u>H$_3$-CO), 17.71 (Si-<u>C</u>(CH_3)$_3$ TBDMS), 16.79 (Si-CH_2 TMSE), 14.12 (S-CH_3), -1.58 (Si(CH_3)$_3$ TMSE), -4.25 (Si-CH_3 TBDMS), -5.26 (Si-CH_3 TBDMS) (Spectra S16/S17, Supporting Information).

N-[[9-(β-D-ribofuranosyl)-2-methylthiopurin-6-yl]carbamoyl]-*O*-*tert*-butyldimethylsilyl-*L*-threonine trimethylsilylethyl ester (**4**)

To a solution of **3** (0.98 g; 1.3 mmol) in anhydrous MeOH (5 mL) a 10 % solution of TEA in MeOH (5 mL) was added and allowed to stir at room temperature. After 24 h reaction mixture was diluted with toluene (50 mL) and the solvents were removed under reduced pressure. The oily residue was extra co-evaporated with toluene (3 × 15 mL) and the product **4** was isolated by column chromatography (silica gel, 0-7 % MeOH in $CHCl_3$) as a white solid (0.75 g; 1.1 mmol; 86 % yield). TLC: R_f = 0.18 ($CHCl_3$/MeOH, 95/5, v/v); ^{1}H NMR: (DMSO-d6) δ: 9.90 (s, 1H, NH-6), 9.27 (d, 1H, ^{3}J = 8.5 Hz, NH Thr), 8.51 (s, 1H, H-8), 5.93 (d, 1H, ^{3}J = 5.7 Hz, H-1'), 5.49 (d, 1H, ^{3}J = 6.0 Hz, 2'-OH), 5.22 (d, 1H, ^{3}J = 5.0 Hz, 3'-OH), 4.99 (t, 1H, ^{3}J = 5.6 Hz, 5'-OH), 4.64-4.62 (m, 1H, H-2'), 4.47-4.43 (m, 2H, CH-α Thr, CH-β Thr), 4.22-4.16 (m, 2H, H-3', O-CH_2 TMSE), 4.11 (td, 1H, ^{2}J = 10.8 Hz, ^{3}J = 6.1 Hz, O-CH_2 TMSE), 3.95-3.94 (m, 1H, H-4'), 3.68-3.65 (m, 1H, H-5'), 3.60-3.53 (m, 1H, H-5″), 2.56 (s, 3H, S-CH_3), 1.19 (d, 3H, ^{3}J = 6.1 Hz, CH_3 Thr), 1.03-0.94 (m, 2H, Si-CH_2 TMSE), 0.85 (s, 9H, Si-C(CH_3)$_3$ TBDMS), 0.08 (s, 3H, Si-CH_3 TBDMS), 0.03 (s, 3H, Si-CH_3 TBDMS), 0.01 (s, 9H, Si(CH_3)$_3$ TMSE); ^{13}C NMR: (DMSO-d6) δ: 170.26 (C=O Thr), 163.95 (C-2), 153.30 (NH-CO-NH), 151.26 (C-4), 149.70 (C-6), 141.24 (C-8), 117.80 (C-5), 87.46 (C-1'), 85.54 (C4'), 73.53 (C-2'), 70.31 (C-3'), 68.72 (CH-β Thr), 62.87 (O-CH_2 TMSE), 61.34 (C-5'), 59.02 (CH-α Thr), 25.71 (Si-C(<u>C</u>H$_3$)$_3$ TBDMS), 20.54 (CH_3 Thr), 17.71 (Si-<u>C</u>(CH_3)$_3$ TBDMS), 16.79 (Si-CH_2 TMSE), 14.13 (S-CH_3), -1.59 (Si-(CH_3)$_3$ TMSE), -4.30 (Si-CH_3 TBDMS), -5.23 (Si-CH_3 TBDMS). (Spectra S18/S19, Supporting Information).

Synthesis of L-threonine components

N-(*tert*-butoxycarbonyl)-*O*-*tert*-butyldimethylsilyl-*L*-threonine (**9**)

N-*tert*-butoxycarbonyl-L-threonine **8** (5.00 g; 22.8 mmol) was co-evaporated with anhydrous pyridine (3 × 15 mL), dissolved in dry pyridine (40 mL) and treated with imidazole (3.42 g; 50 mmol) and TBDMS-Cl (7.54 g; 50 mmol). After stirring for 72 h at room temperature, H_2O (150 mL) was added and the stirring was continued for 0.5 h. The mixture was extracted with AcOEt (3 × 150 mL). The combined organic layers were washed with 1M aq. $KHSO_4$ (2 × 150 mL) and H_2O (150 mL), dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The oily residue was co-evaporated with toluene (3 × 150 mL) and the product **9** was isolated by column chromatography (silica gel, 0-10 % MeOH in CH_2Cl_2) as colorless oil (7.45 g; 22.3 mmol, 98 % yield). TLC: R_f = 0.33 (AcOEt/hexane/AcOH, 60/40/1, v/v); ^{1}H NMR: (DMSO-d_6) δ: 12.71 (s, 1H, COOH), 5.91 (d, 1H, ^{3}J = 9.8 Hz, NH), 4.30 (dq, 1H, ^{3}J = 6.2 Hz, ^{3}J = 2.8 Hz, CH-β), 3.97 (dd, 1H, ^{3}J = 9.1 Hz, ^{3}J = 2.1 Hz, CH-α), 1.39 (s, 9H, C(CH_3)$_3$ Boc), 1.11 (d, 3H, ^{3}J = 6.3 Hz, CH_3), 0.82 (s, 9H, Si-C(CH_3)$_3$ TBDMS), 0.03 (s, 3H, Si-CH_3 TBDMS), 0.00 (s, 3H, Si-CH_3 TBDMS); ^{13}C NMR: (DMSO-d6) δ: 172.09 (C=O), 156.56 (C=O Boc), 78.40 (<u>C</u>(CH_3)$_3$ Boc), 68.39 (CH-β), 59.13 (CH-α), 28.09 (C(<u>C</u>H$_3$)$_3$ Boc), 25.63 (Si-<u>C</u>(CH_3)$_3$ TBDMS), 20.55 (CH_3), 17.62 (Si-C(<u>C</u>H$_3$)$_3$ TBDMS), -4.54 (Si-CH_3 TBDMS), -5.25 (Si-CH_3 TBDMS). (Spectra S5/S6, Supporting Information).

N-(*tert*-butoxycarbonyl)-*O*-*tert*-butyldimethylsilyl-L-threonine trimethylsilylethyl ester (**10**)

To a solution of protected threonine **9** (3.00 g; 9.0 mmol) in anhydrous CH_2Cl_2 (40 mL) 2-(trimethylsilyl)ethanol (1.81 mL; 12 mmol) was added. The mixture was stirred for 10 minutes at 0 °C and DCC (3.10 g;

15 mmol) and DMAP (0.11 g, 0.9 mmol) were added. After stirring for 3 h, the DCU precipitate was filtered off and washed with CH_2Cl_2 (3 × 10 mL). The solvent was evaporated under reduced pressure and the product **10** was isolated by column chromatography (silica gel, hexane/$CHCl_3$, 1:1, v/v) as a white solid (3.82 g; 8.8 mmol; 98 % yield). TLC: R_f = 0.45 ($CHCl_3$); ^{1}H NMR: (DMSO-d6) δ: 6.19 (d, 1H, ^3J = 9.4 Hz, NH), 4.27 (dq, 1H, ^3J = 6.2 Hz, ^3J = 3.1 Hz, CH-β), 4.14 (dt, 1H, ^2J = 10.7 Hz, ^3J = 6.5 Hz, O-CH_2 TMSE), 4.09 (dt, 1H, ^2J = 10.7 Hz, ^3J = 6.2 Hz, O-CH_2 TMSE), 4.04 (dd, 1H, ^3J = 9.4 Hz, ^3J = 3.1 Hz, CH-α), 1.39 (s, 9H, C(CH_3)$_3$ Boc), 1.11 (d, 3H, ^3J = 6.2 Hz, CH_3), 0.99-0.90 (m, 2H, Si-CH_2 TMSE), 0.82 (s, 9H, Si-C(CH_3)$_3$ TBDMS), 0.03 (s, 12H, Si(CH_3)$_3$ TMSE; Si-CH_3 TBDMS), -0.01 (s, 3H, Si-CH_3 TBDMS); ^{13}C NMR: (DMSO-d6) δ: 170.62 (C=O), 155.52 (C=O, Boc), 78.52 (C(CH_3)$_3$ Boc), 68.35 (CH-β), 62.66 (O-CH_2 TMSE), 59.42 (CH-α), 28.07 (C(CH_3)$_3$ Boc), 25.53 (Si-C(CH_3)$_3$ TBDMS), 20.36 (CH_3), 17.55 (Si-C(CH_3)$_3$ TBDMS), 16.76 (Si-CH_2 TMSE), -1.58 (Si-(CH_3)$_3$ TMSE), -4.44 (Si-CH_3 TBDMS), -5.42 (Si-CH_3 TBDMS). (Spectra S7/S8, Supporting Information).

Trifluoroacetate of *O-tert*-butyldimethylsilyl-*L*-threonine trimethylsilylethyl ester (**6**)

To a solution of *N*-Boc trimethylsilylethyl ester **10** (3.00 g; 6.9 mmol) in CH_2Cl_2 (3.3 mL) was treated with freshly prepared mixture of TFA in anhydrous CH_2Cl_2 (60 vol %, 16 mL). After 5 min stirring at room temperature, toluene (9 mL) was added and the solvents were evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ (70 mL) and washed with H_2O (70 mL), 4 % aq. $NaHCO_3$ (2 × 70 mL), and H_2O (70 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The product **6** was isolated by column chromatography (silica gel, 0-1 % MeOH in $CHCl_3$) as a white solid (1.95 g; 4.5 mmol; 64 % yield). TLC: R_f = 0.30 ($CHCl_3$/MeOH, 98/2, v/v); ^{1}H NMR: (DMSO-d6) δ: 4.16 (dq, 1H, ^3J = 6.3 Hz, ^3J = 2.9 Hz, CH-β), 4.14-4.11 (m, 1H, O-CH_2 TMSE), 4.07-4.04 (m, 1H, O-CH_2 TMSE), 3.17 (d, 1H, ^3J = 2.8 Hz, CH-α), 1.59 (bs, 3H, NH_3^+), 1.16 (d, 3H, ^3J = 6.3 Hz, CH_3), 0.99-0.92 (m, 2H, Si-CH_2 TMSE), 0.81 (s, 9H, Si-C(CH_3)$_3$ TBDMS), 0.03 (s, 9H, Si(CH_3)$_3$ TMSE), 0.02 (s, 3H, Si-CH_3 TBDMS), -0.03 (s, 3H, Si-CH_3 TBDMS); ^{13}C NMR: (DMSO-d6) δ: 174.19 (C=O), 69.67 (CH-β), 61.98 (O-CH_2 TMSE), 59.90 (CH-α), 25.53 (Si-C(CH_3)$_3$ TBDMS), 20.35 (CH_3), 17.57 (Si-C(CH_3)$_3$ TBDMS), 16.83 (Si-CH_2 TMSE), -1.57 (Si(CH_3)$_3$ TMSE), -4.36 (Si-CH_3 TBDMS), -5.35 (Si-CH_3 TBDMS). (Spectra S9/S10, Supporting Information).

Hydrochloride of *O-tert*-butyldimethylsilyl-*L*-threonine trimethylsilylethyl ester (**6a)**

To a solution of threonine trifluoroacetate **6** (1.14 g; 2.54 mmol) in anhydrous CH_2Cl_2 (10.8 mL), anhydrous 1,4-dioxane saturated with hydrogen chloride (4 M, 1.1 mL) was added dropwise. After 5 min stirring the solution was concentrated under reduced pressure and the oily residue was co-evaporated with anhydrous toluene (2 × 20 mL). The product **6a** was obtained as a white solid (0.94 g; 2.54 mmol; 100 % yield). TLC: R_f = 0.26 ($CHCl_3$/MeOH, 98/2, v/v); ^{1}H NMR: ($CDCl_3$) δ: 8.71 (bs, 3H, NH_3^+), 4.50 (dq, 1H , ^3J = 6.6 Hz, ^3J = 1.7 Hz, CH-β), 4.30-4.26 (m, 1H, O-CH_2 TMSE), 4.25-4.20 (m, 1H, O-CH_2 TMSE), 4.03 (d, 1H, ^3J = 1.7 Hz, CH-α), 1.49 (d, 3H, ^3J = 6.6 Hz, CH_3), 1.08-1.03 (m, 2H, Si-CH_2 TMSE), 0.85 (s, 9H, C-Si(CH_3)$_3$ TBDMS), 0.08 (s, 3H, Si-CH_3 TBDMS), 0.03 (s, 9H, Si(CH_3)$_3$ TMSE), 0.02 (s, 3H, Si-CH_3 TBDMS); ^{13}C NMR: (DMSO-d6) δ: 167.77 (C=O), 67.58 (CH-β), 65.34 (O-CH_2 TMSE), 58.86 (CH-α), 25.83 (Si-C(CH_3)$_3$ TBDMS), 21.56 (CH_3), 18.02 (Si-C(CH_3)$_3$ TBDMS), 17.44 (Si-CH_2 TMSE), -1.45 (Si(CH_3)$_3$ TMSE), -4.08 (Si-CH_3 TBDMS), -5.09 (Si-CH_3 TBDMS). (Spectra S11/S12, Supporting Information).

"Isocyanated" *O-tert*-butyldimethylsilyl-*L*-threonine trimethylsilylethyl ester (**7**)

A solution of **6a** (0.94 g, 2.54 mmol) in anhydrous CH_2Cl_2 (8.6 mL) and anhydrous pyridine (0.9 mL) was stirred under argon atmosphere in an ice bath for 15 min. Then, a 20 % solution of phosgene in toluene (2 mL) was added dropwise and the resultant yellow solution was stirred at 0 °C for 2 h (TLC analysis revealed 100 % conversion; R_f = 0.94 for **7** vs. R_f = 0.26 for **6a** in $CHCl_3$/MeOH, 98/2, v/v). The mixture was diluted with CH_2Cl_2 (24 mL) and poured into a mixture of 0.5 M aq. HCl (24 mL) and crushed ice (24 mL). The organic layer was collected and the above wash step was repeated. The combined aqueous layers were extracted with CH_2Cl_2 (40 mL). The organic layers were combined and washed with brine (30 mL), water (30 mL) and dried over anhydrous $MgSO_4$. The solvents were removed under reduced pressure to afford **7** as a light brown oil. The crude product, without further purification, was used in the reaction with **1**. TLC: R_f = 0.94 ($CHCl_3$/MeOH, 98/2, v/v); IR (film) 2245 cm^{-1} (N=C=O), Figure S1, Supporting Information ; ^{1}H NMR: ($CDCl_3$) δ: 4.36 (dq, 1H, ^3J = 6.2 Hz, ^3J = 1.9 Hz, CH-β), 4.29 (dt, 1H, ^2J = 10.9 Hz, ^3J = 6.8 Hz, O-CH_2 TMSE), 4.21 (dt, 1H, ^2J = 10.8 Hz, ^3J = 6.7 Hz, O-CH_2 TMSE), 3.62 (d, 1H, ^3J = 1.9 Hz, CH-α), 1.26 (d, 3H, ^3J = 6.3 Hz, CH_3), 1.08-1.00 (m, 2H, Si-CH_2 TMSE), 0.87 (s, 9H, Si-C(CH_3)$_3$ TBDMS), 0.07 (s, 3H, Si-CH_3 TBDMS), 0.05 (s, 9H, Si(CH_3)$_3$ TMSE), 0.00 (s, 3H, Si-CH_3 TBDMS); ^{13}C NMR: (DMSO-d6) δ: 170.06 (C=O), 128.98 (N=C=O), 69.34 (CH-β), 64.89 (O-CH_2 TMSE), 64.67 (CH-α), 25.62 (Si-C(CH_3)$_3$ TBDMS), 21.33 (CH_3), 17.95 (Si-C(CH_3)$_3$ TBDMS), 17.54 (Si-CH_2 TMSE), -1.46 (Si(CH_3)$_3$ TMSE), -4.07 (Si-CH_3 TBDMS), -5.16 (Si-CH_3 TBDMS). (Spectra S13/S14, Supporting Information).

Synthesis of the (ms^{2}t^{6}A-ASL) oligonucleotide.

The manual synthesis was performed (according to the protocol reported by Sproat[35]) at a 2.5 µmol scale using 71.4 mg of the rG-succinyl-CPG (Proligo) support (the loading of 35 µmol/g) The commercially available phosphoramidite derivatives of A, C, U and G, carrying standard 5'-*O*-DMTr and 2'-*O*-TBDMS protecting groups and 4-*tert*-butylphenoxyacetyl (tac) moiety on the exocyclic amine functions in A, C, and G, were used as 0.1 M solutions in anhydrous acetonitrile. The coupling reactions were promoted with a 0.25 M solution of 5-(3,5-bis(trifluoromethyl)phenyl)-1*H*-tetrazole (Activator 42) in ACN. The coupling steps with the canonical A, U, C and G amidities were performed once (for 8 min), while for the modified unit double coupling was executed, each for 25 minutes. The capping steps were performed with tac anhydride (Fast deprotection Cap A : Cap B 1:1.1 v/v) for 2 min. A 0.02 M solution of elemental iodine in THF-H_2O-pyridine (90.54:9.05:0.41 v/v/v) was used as an oxidizing agent (2 min contact) for each oxidation step, followed by an additional capping step (to remove traces of iodine) and washing with dry acetonitrile. After the last coupling, the DMTr group was removed and the support was washed with acetonitrile, dried with argon and transferred to a screw cap glass vial for deprotection steps. To remove the 2-cyanoethyl protecting groups the support carrying already detritylated oligomer was suspended in a TEA-ACN mixture (3.3 mL, 1:1 v/v) and the suspension was stirred for 20 min in a tightly closed vessel. The liquid phase was removed and the CPG sediment was washed with ACN (3 × 2.5 mL), dried *in vacuo* for 30 min and treated with 8 M ethanolic ammonia (4.5 mL) for 16 h at 37 °C to remove the base-labile protecting groups and cleave the **ms^{2}t^{6}A-RNA** oligonucleotide from the solid support. The supernatant was collected and the CPG-support was rinsed with ethanol (3 × 4 mL). The ethanolic solutions were combined and the solvent was evaporated using a Speed-Vac concentrator. The solid residue was dried under high vacuum for 2 h and treated with 1 M TBAF in THF (1.8 mL) for 24 h at room temperature. The reaction was quenched by addition of phosphate buffer (0.2 M, 5 mL, pH 7) and desalted on a Sephadex column (G-25) using 20 % $EtOH_{aq}$ as eluent. The fully deprotected oligomer was purified by high resolution anion exchange (AEX) HPLC (a Source 15Q 4.6/100PE column) using linear gradient at a flow rate of 1 mL min^{-1} (Figure S2). The column was eluted with a linear gradient 50 mM to 600 mM NaBr in 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.5) containing 50 µM EDTA and 10 % ACN. The **ms^{2}t^{6}A-ASL** oligomer was eluted at R_t = 31.84 min and partially evaporated on Speed-Vac concentrator to remove acetonitrile. The remaining solution was diluted with 100 mM AcONa (up to ~ 6 mL) and loaded slowly onto a Sep-Pak C18 cartridge (Waters) equilibrated with

100 mM AcONa (pH 6.5, 10 mL). The packing bed was drop-by-drop washed with H_2O (miliQ quality, 6 × 8 mL). Finally, the cartridge was flushed with the elution solution (ACN/H_2O, 9:1, v/v) and the collected fraction was evaporated to dryness and lyophilized to give 94 OD_{260} units of precursor **ms²t⁶A-ASL**. The obtained product was analyzed by MALDI-TOF mass spectrometry (Figure S3).

Cyclization of ms²t⁶A to ms²ct⁶A at the oligonucleotide level

To the lyophilized 5'-CACGGCUUUU**ms²t⁶A**ACCGUG-3' (50 OD, n = $3.6 \cdot 10^{-4}$ mmol, m = 2.0 mg) the 100 µL aliquots of the freshly prepared solutions of 7.0 mg of EDC·HCl in 1 mL H_2O (10 equiv, n = $3.6 \cdot 10^{-3}$ mmol, m = 0.70 mg) and 4.8 mg HOBt in 1mL DMF (10 equiv, n = $3.6 \cdot 10^{-3}$ mmol, m = 0.48 mg) were added. An HPLC analysis performed after 1 hour incubation at 25 °C showed one main product (R_t = 14.73 min, Supporting Information, Figure S5). The oligomer was isolated by ultrafiltration using an Amicon® Ultra-4 Centrifugal Filter 3.000 MWCO (for volumes up to 4 mL). The crude sample was placed in the filter device and diluted to 4 mL with 200 mM NaCl. The filter was put in a fixed-angle rotor and a 7,500×g acceleration was applied for 40 min. The filtrate was removed and the filtration was repeated three times using miliQ quality H_2O. Finally, the oligonucleotide was transferred from device to an Eppendorf tube and evaporated to dryness affording 45 OD_{260} units (90 % yield). The obtained **ms²ct⁶A-ASL** oligonucleotide was analyzed by MALDI-TOF mass spectrometry (*m/z* calc. 5535, found 5537) and its homogeneity was confirmed by RP-HPLC (Figures S5, S6, Supporting Information).

Synthesis of L-ms²t⁶A (12a) / D-*allo*-ms²t⁶A (12b) and L-ms²ct⁶A (13a) / D-*allo*-ms²ct⁶A (13b) nucleosides

2′,3′,5′-Tri-*O*-acetyl-2-thiomethyl-*L*-N^6-threonylocarbamoyladenosine (**11a**) and 2′,3′,5′-tri-*O*-acetyl-2-thiomethyl-*D*-*allo*-N^6-threonylocarbamoyl-adenosine (**11b**)

To the solution of **2** (0.67 g; 1.2 mmol) in anhydrous pyridine (5 mL) L-threonine (0.43 g, 3.6 mmol) was added. The mixture was stirred at 40 °C for 10 h and excess of amino acid was filtered off and washed with pyridine. The filtrates were combined and the solvent was evaporated under reduced pressure. The residue was co-evaporated with toluene (3 x 5 mL) and the product **11a** was isolated by column chromatography (silica gel, CH_2Cl_2/MeOH, 7:3, v/v) as a white solid (0.59 g; 1.0 mmol; 88 % yield). R_f = 0.67 (*n*-BuOH/H_2O/AcOH, 5:3:2, v/v/v); ^{1}H NMR: (DMSO-d6) δ: 9.84 (bs, 1H, NH-6), 9.32 (d, 1H, ^3J = 8.4 Hz, NH Thr), 8.46 (s, 1H, H-8), 6.23 (d, 1H, ^3J = 4.9 Hz, H-1'), 6.02 (dd, 1H, ^3J = 5.6 Hz, ^3J = 4.9 Hz, H-2'), 5.67-5.65 (m, 1H, H-3'), 4,42-4,37 (m, 2H, H-4', H-5'), 4.28 (dd, 1H, ^3J = 8.4 Hz, ^3J = 2.1 Hz, CH-α Thr) 4.25-4.22 (m, 2H, H-5″, CH-β Thr), 2.59 (s, 3H, S-CH_3), 2.12 (s, 3H, CH_3-CO), 2.06 (s, 3H, CH_3-CO), 1.98 (s, 3H, CH_3-CO), 1.13 (d, 3H, ^3J = 6.3 Hz, CH_3 Thr); ^{13}C NMR (DMSO-d_6) δ: 172.19 (C=O Thr), 169.97 (CH_3-<u>C</u>O), 169.39 (CH_3-<u>C</u>O), 169.25 (CH_3-<u>C</u>O), 164.62 (C-2), 153.24 (NH-CO-NH), 150.74 (C-4), 150.00 (C-6), 141.59 (C-8), 117.96 (C-5), 86.05 (C-1'), 79.15 (C-4'), 71.98 (C-2'), 69.75 (C-3'), 66.22 (CH-β Thr), 62.61 (C-5'), 58.53 (CH-α Thr), 20.65 (CH_3 Thr), 20.40 (<u>C</u>H_3-CO), 20.32 (<u>C</u>H_3-CO), 20.21 (<u>C</u>H_3-CO), 14.04 (S-CH_3). (Spectra S25/S26, Supporting Information).

Compound **11b** was prepared analogously as **11a** starting from **2** and D-*allo*-threonine and it was obtained as a white solid (0.56 g; 0.95 mmol; 82 % yield). R_f = 0.65 (*n*-BuOH/H_2O/AcOH, 5:3:2 v/v/v); ^{1}H NMR: (DMSO-d6) δ: 9.89 (bs, 1H, NH-6), 9.42 (d, 1H, ^3J = 7.7 Hz, NH Thr), 8.45 (s, 1H, H-8), 6.24 (d, 1H, ^3J = 4.2 Hz, H-1'), 6.06-6.05 (m, 1H, H-2'), 5.69-5.68 (m, 1H, H-3'), 4.42 (dd, 1H, ^2J = 12.6 Hz, ^3J = 4.2 Hz, H-5'), 4.39-4.35 (m, 2H, H-4', CH-α Thr), 4.22 (dd, 1H, ^2J = 11.9 Hz, ^3J = 5.6 Hz, H-5″), 4.06-4.03 (m, 1H, CH-β Thr), 2.60 (s, 3H, S-CH_3), 2.11 (s, 3H, CH_3-CO), 2.07 (s, 3H, CH_3-CO), 1.97 (s, 3H, CH_3-CO), 1.18 (d, 3H, ^3J = 7.0 Hz, CH_3 Thr); ^{13}C NMR (DMSO-d_6) δ: 171.85 (C=O Thr), 169.96 (CH_3-<u>C</u>O), 169.41 (CH_3-<u>C</u>O), 169.29 (CH_3-<u>C</u>O), 164.36 (C-2), 153.02 (NH-CO-NH), 150.66 (C-4), 149.98 (C-6), 141.71 (C-8), 117.97 (C-5), 86.38 (C-1'), 78.96 (C-4'), 71.96 (C-2'), 69.64 (C-3'), 66.57 (CH-β Thr), 62.49 (C-5'), 59.19 (CH-α Thr), 20.37 (<u>C</u>H_3-CO), 20.31 (<u>C</u>H_3-CO), 20.22 (<u>C</u>H_3-CO), 19.02 (CH_3 Thr), 13.96 (S-CH_3). (Spectra S27/S28, Supporting Information).

2-Thiomethyl-*L*-N^6-threonylcarbamoyladenosine (**12a**) and 2-thiomethyl-*D*-*allo*-N^6-threonylcarbamoyladenosine (**12b**)

Compound **11a** (0.21 g; 0.36 mmol) was dissolved in anhydrous MeOH saturated with NH_3 (10 M, 5 mL) and the mixture was stirred at room temperature for 5 h. The solvent was evaporated and the residue was dissolved in water and lyophilized. The resultant ammonium salt was then converted into an acidic form by ion exchange chromatography on Amberlite™ IR120 (H^+ form). The column was eluted with water and the eluate was concentrated down to 5 mL and lyophilized to yield **12a** as a white solid (0.15 g; 0.33 mmol; 90 % yield). R_f = 0.44 (*n*-BuOH/H_2O/AcOH, 5:3:2 v/v/v); ^{1}H NMR: (DMSO-d6) δ: 9.74 (bs, 1H, NH-6), 9.35 (d, 1H, ^3J = 8.4 Hz, NH Thr), 8.51 (s, 1H, H-8), 5.91 (d, 1H, ^3J = 6.3 Hz, H-1'), 5.49 (bs, 1H, 2'-OH), 5.21 (bs, 1H, 3'-OH), 5.01 (bs, 1H, 5'-OH), 4.58-4.59 (m, 1H, H-2'), 4.30 (dd, 1H, ^3J = 9.1 Hz, ^3J = 2.8 Hz, CH-α Thr), 4.26-4.23 (m, 1H, CH-β Thr), 4.17-4.16 (m, 1H, H-3'), 3.94 (dd, 1H, ^3J = 8.4 Hz, ^3J = 4.2 Hz, H-4'), 3.67-3.65 (m, 1H, H-5'), 3.57-3.55 (m, 1H, H-5″), 2.58 (s, 3H, S-CH_3), 1.13 (d, 3H, ^3J = 6.3 Hz, CH_3 Thr); ^{13}C NMR (DMSO-d_6) δ: 172.25 (C=O Thr), 164.06 (C-2), 153.33 (NH-CO-NH), 151.24 (C-4), 149.84 (C-6), 141.17 (C-8), 117.81 (C-5), 87.32 (C-1'), 85.57 (C-4'), 73.55 (C-2'), 70.30 (C-3'), 66.26 (CH-β Thr), 61.31 (C-5'), 58.51 (CH-α Thr), 20.67 (CH_3 Thr), 14.06 (S-CH_3). (Spectra S29/S30, Supporting Information).

Compound **12b** was prepared analogously as **12a** starting from **11b** and was obtained as a white solid (0.14 g; 0.31 mmol; 87% yield). R_f = 0.44 (*n*-BuOH/H_2O/AcOH, 5:3:2 v/v/v); ^{1}H NMR: (DMSO-d6) δ: 9.81 (bs, 1H, NH-6), 9.45 (d, 1H, ^3J = 8.4 Hz, NH Thr), 8.52 (s, 1H, H-8), 5.93 (d, 1H, ^3J = 6.3 Hz, H-1'), 5.51 (bs, 1H, 2'-OH), 5.23 (bs, 1H, 3'-OH), 5.00 (bs, 1H, 5'-OH), 4.62-4.61 (m, 1H, H-2'), 4.38 (dd, 1H, ^3J = 8.4 Hz, ^3J = 4.9 Hz, CH-α Thr), 4.18-4.17 (m, 1H, H-3'), 4.06-4.05 (m, 1H, CH-β Thr), 3.69-3.95 (m, 1H, H-4'), 3.68-3.65 (m, 1H, H-5'), 3.56-3.55 (m, 1H, H-5″), 2.59 (s, 3H, S-CH_3), 1.19 (d, 3H, ^3J = 6.3 Hz, CH_3 Thr); ^{13}C NMR (DMSO-d_6) δ: 171.82 (C=O Thr), 163.80 (C-2), 153.11 (NH-CO-NH), 151.27 (C-4), 149.82 (C-6), 141.19 (C-8), 117.76 (C-5), 87.39 (C-1'), 85.57 (C-4'), 73.58 (C-2'), 70.33 (C-3'), 66.62 (CH-β Thr), 61.35 (C-5'), 59.15 (CH-α Thr), 19.13 (CH_3 Thr), 13.94 (S-CH_3). (Spectra S31/S32, Supporting Information).

Cyclic 2-thiomethyl-*L*-N^6-threonylcarbamoyladenosine (**13a**) and 2-thiomethyl-*D*-*allo*-N^6-threonylcarbamoyladenosine (**13b**)

To the solution of **12a** (50 mg; 0.11 mmol) in Milli-Q® water (5 mL) the polymer-bound EDC was added (390 mg; 0.55 mmol). The suspension was stirred at room temperature for 5 min and HOBt (74 mg; 0.55 mmol) was added. After 1 h stirring, the resin was filtered off and washed with H_2O (3 x 5 mL), MeOH (2 x 5 mL) and the collected solvents were removed under reduced pressure. The product **13a** was isolated by column chromatography (silica gel, 0-2 % H_2O in *n*-BuOH) as a white solid (43 mg; 0.10 mmol; 90% yield). R_f= 0,.48 (*n*-BuOH/H_2O, 85:15, v/v); ^{1}H NMR: (DMSO-d_6) δ: 8.72 (s, 1H, H-8), 8.71 (s, 1H, NH Thr), 6.00 (d, ^3J = 5.7 Hz, H-1'), 5.57 (d, 1H, ^3J = 5.9 Hz, 2'-OH), 5.24 (d, 1H, ^3J = 4.9 Hz, 3'-OH), 5.20 (d, 1H, ^3J = 5.9 Hz, -OH Thr), 5.01 (t, 1H, ^3J = 5.6 Hz, 5'-OH), 4.71-4.69 (m, 1H, H-2'), 4.22 (dd, 1H, ^3J = 2.7 Hz, ^3J = 1.2 Hz, CH-α Thr), 4.22-4.20 (m, 1H, H-3'), 4.08-4.03 (m, 1H, CH-β Thr), 3.97-3.96 (m, 1H, H-4'), 3.68-3.65 (m, 1H, H-5'), 3.59-3.55 (m, 1H, H-5″), 2.61 (s, 1H, S-CH_3), 1.24 (d, 3H, ^3J = 6.6 Hz, CH_3 Thr); ^{13}C NMR: (DMSO-d_6) δ: 170.99 (C=O Thr), 165.02 (C-2), 154.45 (NH-CO-NH), 154.30 (C-4), 145.00 (C-8), 143.93 (C-6), 127.29 (C-5), 87.70 (C-1'), 85.73 (C-4'), 73.35 (C-2'), 70.31 (C-3'), 65.41 (CH-β Thr), 63.26 (CH-α Thr), 61.25 (C-5'), 20.18 (CH_3 Thr), 14.12 (SCH_3); ESI-HRMS calcd for [$C_{16}H_{20}N_6O_7SNa$]: 463.1012, found: 463.1010. (Spectra S33/S34 and Figure S8, Supporting Information).

Compound **13b** was prepared analogously as **13a** starting from **12b** and was obtained as a white solid (45 mg; 0.10 mmol; 92 % yield). R_f = 0.50 (*n*-BuOH/H_2O, 85:15, v/v); ^{1}H NMR: (DMSO-d6) δ: 8.73 (s, 1H, H-8), 8.68 (bs, 1H, NH Thr), 6.00 (d, 1H, ^3J = 5.6 Hz, H-1'), 5.57 (d, 1H, ^3J = 6.3 Hz, 2'-OH), 5.33 (d, 1H, ^3J = 4.2 Hz, 3'-OH), 5.23 (d, 1H, ^3J = 4.9 Hz, -OH Thr), 5.00-4.99 (m,1H, 5'-OH), 4.70 (dd, 1H, ^3J = 11.2 Hz, ^3J = 5.6 Hz, H-2'), 4.42 (dd, 1H, ^3J = 2.8 Hz, ^3J = 1.4 Hz, CH-α Thr), 4.21 (dd, 1H, ^3J = 9.1 Hz, ^3J = 4.9 Hz, H-3'), 4.07-4.03 (m, 1H, CH-β Thr), 3.69 (dd, 1H, ^3J = 7.7 Hz, ^3J = 4.2 Hz, H-4'), 3.68-3.66 (m, 1H, H-5'), 3.58-3.55 (m, 1H, H-5″), 2.60 (s, 3H, S-CH_3), 1.21 (d, 3H, ^3J = 6.3 Hz, CH_3 Thr); ^{13}C NMR (DMSO-d_6) δ: 170.18 (C=O Thr), 165.10 (C-2), 154.30 (NH-CO-NH), 154.27 (C-4), 145.18 (C-6), 143.78 (C-8), 127.43 (C-5), 87.72 (C-1'), 85.73 (C-4'), 73.35 (C-2'), 70.31 (C-3'), 66.36 (CH-β Thr), 62.83 (CH-α Thr), 61.24 (C-5'), 17.27 (CH_3 Thr), 14.12 (S-CH_3). (Spectra S35/S36, Supporting Information).

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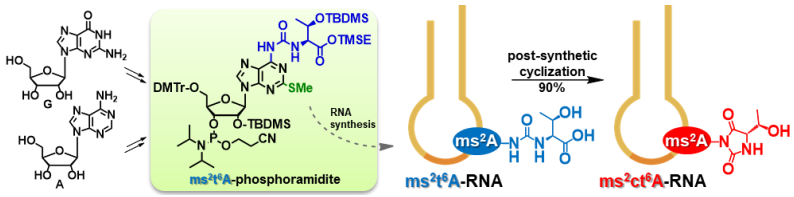

Hydantoin modified **ms^{2}ct^{6}A** nucleoside was successfully introduced into the RNA chain by post-synthetic **ms^{2}t^{6}A→ms^{2}ct^{6}A** cyclization at the oligonucleotide level. The precursor **ms^{2}t^{6}A**-RNA was prepared using **ms^{2}t^{6}A**-phosphoramidite monomeric unit obtained efficiently from adenosine or guanosine by the optimized carbamate and, for the first time, the isocyanate chemistry.

*Katarzyna Debiec, Michal Matuszewski, Karolina Podskoczyj, Grazyna Leszczynska, Elzbieta Sochacka**

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Chemical synthesis of oligoribonucleotide (ASL of tRNA^{Lys} *T. brucei*) containing a recently discovered cyclic form of 2-methylthio-*N*^{6}-threonylcarbamoyladenosine (ms^{2}ct^{6}A)