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Efficient access to 3′-*O*-phosphoramidite derivatives of tRNA related *N*⁶-threonylcarbamoyladenosine (t⁶A) and 2-methylthio-*N*⁶-threonylcarbamoyladenosine (ms²t⁶A)†

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An efficient method of ureido linkage formation during epimerization-free one-pot synthesis of protected hypermodified *N*⁶-threonylcarbamoyladenosine (**t⁶A**) and its 2-SMe analog (**ms²t⁶A**) was developed. The method is based on a Tf₂O-mediated direct conversion of the *N*-Boc-protecting group of *N*-Boc-threonine into the isocyanate derivative, followed by reaction with the *N*⁶ *exo*-amine function of the sugar protected nucleoside (yield 86–94%). Starting from 2′,3′,5′-tri-*O*-acetyl protected adenosine or 2-methylthioadenosine, the corresponding 3′-*O*-phosphoramidite monomers were obtained in 48% and 42% overall yield (5 step synthesis). In an analogous synthesis, using the 2′-*O*-(*tert*-butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilylene) protection system at the adenosine ribose moiety, the **t⁶A**-phosphoramidite monomer was obtained in a less laborious manner and in a remarkably better yield of 74%.

Introduction

Transfer RNAs (tRNAs) are known for having a substantial content of modified nucleoside units.[1,2] To date, in tRNAs from all domains of life, more than 130 modified units have been identified, which differ in chemical structure,[3–6] distribution within the tRNA molecules,[7] and their biological activity.[8–13]

The majority of modified units are present in the anticodon loop and stem domain of tRNAs, particularly at position 34 (the wobble position) and at position 37, *i.e.* adjacent to the anticodon at its 3′-side.[3,7,14,15] Considering the latter modifications, special interest has been paid to several *N*⁶-threonylcarbamoyladenosines (depicted in Fig. 1), which are widely involved in the decoding of the A-starting codons (ANN).[15,16] Among them, the most abundant *N*⁶-threonylcarbamoyladenosine (**t⁶A**)[17] and its analogs containing either the –SMe group at the purine C2 atom (**ms²t⁶A**),[18] or the methyl substituent at the *N*⁶-atom (**m⁶t⁶A**)[19] have been known for many years and their diverse functions during the protein biosynthesis were intensively studied.[6,9,15,16] Recently, next members of the **t⁶A** family have been identified in the tRNA anticodon loops, *i.e.* cyclic *N*⁶-threonylcarbamoyladenosine (**ct⁶A**)[20,21] cyclic 2-methylthio-*N*⁶-threonylcarbamoyladenosine (**ms²ct⁶A**),[22] and a **t⁶A** derivative

having the threonine methyl group converted into a hydroxymetyl one (hydroxy-*N*⁶-threonylcarbamoyl-adenosine, **ht⁶A**).[23]

Recognition of the structural aspects and biological functions of the **t⁶A** nucleoside family is highly dependent on the synthetic availability of these nucleosides, as well as their 3′-*O*-phosphoramidite derivatives, which are essential for fast and efficient synthesis of model oligonucleotides with the sequence of the appropriate tRNA anticodon stems and loops (ASL of tRNAs). To date, several procedures have been developed to modify adenosine or 2-methylthioadenosine (**ms²A**) at the *N*⁶ position with a threonylcarbamoyl chain (a ureido system is formed) using either a carbamate or isocyanate approach (Scheme 1, paths A and B, respectively).

Adenosine *N*⁶-ethyl carbamate was used in the first syntheses of "free" **t⁶A** nucleoside,[24–27] as well as in the preparation of its stereoisomers containing L, D, L-*allo*, and D-*allo*-Thr.[28]
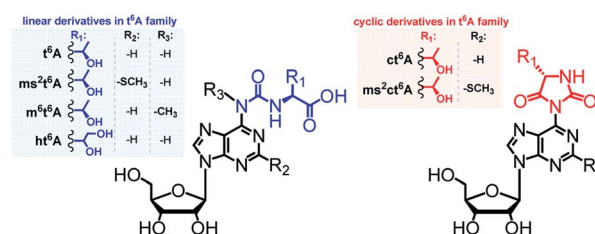
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† Electronic supplementary information (ESI) available: Experimental details for synthesis **6a/6b** from **4a/4b** and spectroscopic data (NMR, IR, HRMS). See DOI: 10.1039/d0ra09803e
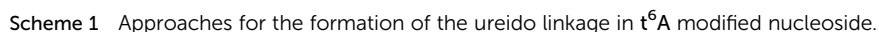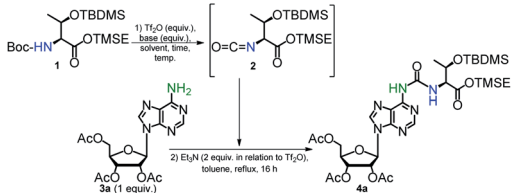

Fig. 1 Abbreviations and structures of L-threonylcarbamoyl modified adenosines (the **t⁶A**₃₇ family) located in tRNAs at the position 37.

Scheme 1 Approaches for the formation of the ureido linkage in t⁶A modified nucleoside.

Analogously, **t⁶A** derivatives suitable for synthesis of the corresponding 3′-O-phosphoramidite derivative (protected with *tert*-butyldimethylsilyl (TBDMS) on the OH and trimethylsilylethyl ester (TMSE) at the COOH of threonine residue) can be prepared.[29] The carbamate method has been significantly improved by the use of more active phenyl carbamate derivatives of adenosine/2-methylthio-adenosine.[30–40] This was possible with phenoxycarbonyl tetrazole[31] or 1-N-methyl-3-phenoxycarbonyl-imidazolium chloride[41] used as effective reagents introducing the carbamate functionality onto the weakly nucleophilic N⁶-amine function of **A/ms²A** nucleosides.

The isocyanate approach to the synthesis of **t⁶A/ms²t⁶A** (Scheme 1, path B1,2) was shown to have limited applicability in the preparation of "free" nucleosides.[24] Because this method required a threonine derivative protected on the OH and COOH functions, it was considered inferior to the carbamate approach in which unprotected amino acid can be used.[24–26] However, the isocyanate route was recently postulated by the Carell's group as a possible pathway for the formation of **t⁶A** under prebiotic conditions.[42]

In the synthesis of threonine protected **t⁶A/ms²t⁶A** derivatives for the subsequent preparation of the corresponding 3′-O-phosphoramidites, the isocyanate approach[39,43] is much less explored than the carbamate procedures.[29,34–40] Initially, the isocyanate derivative was generated from the N⁶-amine function of sugar protected adenosine (Scheme 1, path B1), but its condensation with the free amine function of L-threonine was ineffective and the ureido-nucleoside product was obtained in a low 19% yield.[43] Noticeably better results were obtained in our recently published method (Scheme 1, path B2), based on the reaction of isocyanate derivative of the amino acid substrate (prepared by removing of Boc-protection and phosgene treatment of the free amine function of L-threonine appropriately blocked on the OH and COOH functions) with the sugar protected nucleoside (overall yield of this three steps procedure ~55%).[39] This result of isocyanate procedure turned our attention to the methods of synthesis of unsymmetrical ureas involving the formation of the isocyanate functionality directly from the carbamate type protecting groups of amino acids (*e.g.* N-Boc protecting group).[44–53] Most likely, such variant of the isocyanate method (Scheme 1, path B3) applied in the synthesis of the 3′-O-phosphoramidite derivatives of **t⁶A/ms²t⁶A** would be

greatly advantageous in comparison to our previous isocyanate route (Scheme 1, path B2) owing to a smaller number of synthesis steps in the preparation of threonine derivative (the removal of N-Boc protection is unnecessary) and escaping the use of toxic phosgene.

Here we report a new one-pot procedure for the introduction of an ureido linkage into **t⁶A/ms²t⁶A** using a Tf₂O-mediated generation of the isocyanate derivative directly from N-Boc-protecting group of L-threonine, followed by its straight reaction with the N⁶ *exo*-amine function of the sugar protected nucleoside. We have also showed that this approach is compatible with the use of the recently introduced 2′-O-(*tert*-butyldimethylsilyl)-3′,5′-O-(di-*tert*-butylsilylene) ribonucleoside sugar protection system,[54–56] that allows to prepare the 3′-O-phosphoramidite monomeric unit more effectively and in a less laborious manner.

Results and discussion

Search for the best conditions leading to the formation of the ureido compound **4a** was performed using trimethylsilylethyl (TMSE) ester of N-Boc-O-*tert*-butyldimethylsilyl (TBDMS) protected L-threonine[39] (**1**) and 2′,3′,5′-tri-O-acetyladenosine (**3a**) (Table 1, see ESI for spectroscopic data of **1**, **2**, **3a**, Fig. S1–S7†). In all cases, the final condensation of isocyanate **2** with **3a** was performed in the presence of Et₃N (2-fold molar excess over the Tf₂O activator used for isocyanate formation) in boiling toluene for 16 h. It was reported that addition of Et₃N, which is an effective scavenger of trifluoromethanesulfonic acid (generated in the step of isocyanate formation), helps to maintain a concentration of the unprotonated amine component sufficient for effective nucleophilic attack on the isocyanate moiety.[48,50]

To optimize the triflic anhydride (Tf₂O) mediated conversion of N-Boc-protected threonine **1** (a dichloromethane solution) into the isocyanate derivative **2** (the first step of the one-pot synthesis of **4a**) we were changing the amount of Tf₂O activator, basicity of amine, temperature and reaction time (entries 1–7). When the amount of **2** reached the plateau (TLC monitoring) the reaction mixture was concentrated, the residue was dissolved in toluene and Et₃N and the nucleoside substrate **3a** was added. The reaction **1** → **2** for 15 min at room temp. (entry

Table 1 Optimization of the reaction conditions for the synthesis of t^6A from Boc-Thr derivative **1** and the sugar-protected adenosine **3a**[a]

Entry	Boc-Thr **1**[b] (equiv.)	Tf$_2$O/base[c] (equiv.)	Solvent, time, temp. (°C)	Yield of **4a**[d] (%)
1	1.0	Tf$_2$O (1.5)/2-Cl-Py (3.0)	CH$_2$Cl$_2$, rt, 15 min	19%
2	1.0	Tf$_2$O (1.5)/2-Cl-Py (3.0)	CH$_2$Cl$_2$, 0 °C, 5 min	46%
3	1.0	Tf$_2$O (2.0)/2-Cl-Py (4.0)	CH$_2$Cl$_2$, 0 °C, 5 min	42%
4	1.0	Tf$_2$O (1.5)/Py (3.0)	CH$_2$Cl$_2$, rt[e], 3 h	—
5	1.0	Tf$_2$O (1.5)/DMAP (3.0)	CH$_2$Cl$_2$, rt[e], 3 h	—
6	1.0	Tf$_2$O (1.5)/Et$_3$N (3.0)	CH$_2$Cl$_2$, rt[e], 3 h	—
7	1.0	Tf$_2$O (1.5)/2, 6-lutidine (3.0)	CH$_2$Cl$_2$, rt[e], 30 min	16%
8	1.5	Tf$_2$O (2.25)/2-Cl-Py (4.5)	CH$_2$Cl$_2$, 0 °C, 5 min	71%
9	2.0	Tf$_2$O (3.0)/2-Cl-Py (6.0)	CH$_2$Cl$_2$, 0 °C, 5 min	80%
10	2.5	Tf$_2$O (3.75)/2-Cl-Py (7.5)	CH$_2$Cl$_2$, 0 °C, 5 min	92%
11	2.5	Tf$_2$O (3.75)/2-Cl-Py (7.5)	Toluene, rt, 15 min	92%

[a] All reactions were performed in a 0.2 mmol scale in 6 mL of the corresponding solvent. [b] The number of equivalents was calculated in respect to the nucleoside reagent **3a**. [c] The ratios of **1**/Tf$_2$O = 1.5 and Tf$_2$O/base = 2 were applied. [d] Isolated yield after column chromatography. [e] The reaction was carried out also in 0 °C and after stirring for 3 h no consumption of **1** was observed according to TLC analysis.

1), followed by reaction with **3a**, afforded the final product **4a** in a low 19% yield and several by-products were detected by TLC analysis. An experiment conducted at lower temperature (0 °C)

for much shorter time (5 min) (entry 2) was more productive (46% yield) but the yield did not further increase when higher concentration of Tf$_2$O (2 equiv.) was used (entry 3). Compound **1** did not react when more common bases such as pyridine, 4-dimethylaminopyridine or triethylamine were used (entries 4–6). In the case of 2,6-lutidine, some isocyanate **2** was generated after 30 min at rt, but the final product **4a** was formed in only 16% yield (entry 7). Neither acetic anhydride nor trifluoroacetic anhydride were able to promote the formation of isocyanate **2** regardless of the temperature applied.

In so far reported procedures for the one-pot syntheses of ureas from carbamates, the use of an excess of amine substrate, usually up to 3 equivalents (or more for less nucleophilic amines) is recommended to obtain the higher efficiency of the process.[48,50,51] However, in the case of t^6A/ms^2t^6A synthesis, the amine nucleoside substrate, especially non-native 2-methyl-thioadenosine (**ms^{2}A**) is a very costly reagent. Therefore, in the second step of optimizations we examined an excess of N-Boc protected L-threonine derivative **1** to nucleoside **3a** in a range 1.5–2.5 (entries 8–10), yet the concentrations of Tf$_2$O and 2-Cl-Py against **1** were kept as determined previously (entry 2). We were glad to see that 1.5 molar excess of **1** to **3a** led to a significantly better yield of **4a** (71%, entry 8), while very high conversion of **3a** to **4a** was observed when 2.5 equivalents of **1** was applied (92%, entry 10). Unfortunately, further increase in the excess of **1** (3 equiv. or more) did not lead to a higher isolated yield of product **4a**. Finally, the use of toluene instead of dichloromethane for the formation of **2** allowed us to carry out the whole process in the same solvent (92% yield, entry 11) which facilitate the preparative procedure for the one-pot synthesis of t^6A derivative **4a**.

The optimized method described above was used in synthesis of the phosphoramidite derivatives of t^6A and ms^2t^6A (**6a**, and **6b**, respectively; Scheme 2). Starting from 2.5 mmol of appropriately protected Boc-L-threonine **1**[39] and 1 mmol of adenosine derivative **3a**[57] or **3b**,[39] the modified nucleosides **4a** and **4b** were obtained in 92% and 86% yield, respectively. Next, the acetyl groups in **4a**/**4b** were removed under conditions safe for the installed N^6-threonylcarbamoyl chain (Et$_3$N/MeOH, rt, 24 h) and the resultant **5a**/**5b** were appropriately protected and
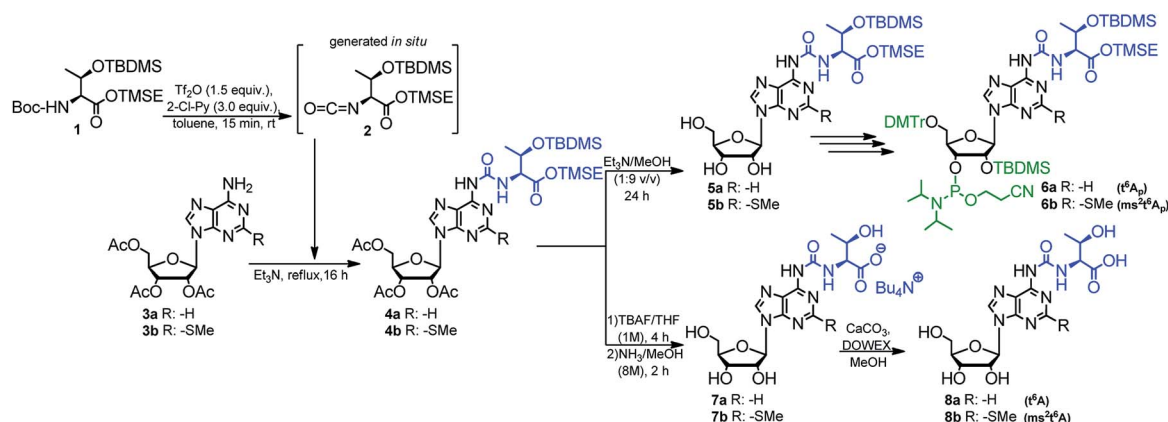
Scheme 2 Preparation of t^6A and ms^2t^6A 3′-O-phosphoramidites and samples of modified nucleosides t^6A and ms^2t^6A.

phosphitylated according to the previously reported procedures[39] to give **t⁶A/ms²t⁶A**-phosphoramidites (**6a/6b**) in 48% and 42% overall yield, respectively (see ESI† for details).

Also, the nucleosides **4a/4b** were deprotected to yield **8a/8b** (Scheme 2), to be used as standards in analysis of enzymic hydrolysates of **t⁶A**- or **ms²t⁶A**-containing oligomers. The silyl protecting groups (TBDMS, TMSE) were removed with excess 1 M tetrabutylammonium fluoride (TBAF) in THF (4 h, rt), and the acetyl groups were cleaved off with NH₃/MeOH (2 h, rt) (see experimental details in ESI†). The reactions were virtually quantitative and the HPLC profiles recorded for the reaction mixtures (Fig. 2) contained single, slightly tailing peaks (profiles in panels (A), part I for **t⁶A** and part II for **ms²t⁶A**). The tailing was not observed, when the highly lipophilic tetrabutylammonium cations were replaced with H⁺ ions using DOWEX, H⁺/CaCO₃ treatment.[58] The resultant acidic forms **8a/8b** had the same HPLC mobility as genuine ʟ-**t⁶A**/ʟ-**ms²t⁶A** standards[21,22,38,39] (compare profiles in panels (B) and (C)). The profiles recorded for **8a** co-injected with ᴅ-*allo*-**t⁶A**[21,38] and for **8b** co-injected with ᴅ-*allo*-**ms²t⁶A**[22,39] (panels (E)) indicate that the new procedure for ureido linkage formation is safe in terms of the stereochemistry at the Cα of the amino acid component. The profiles for the ᴅ-*allo* nucleoside standards are shown in panels (D).
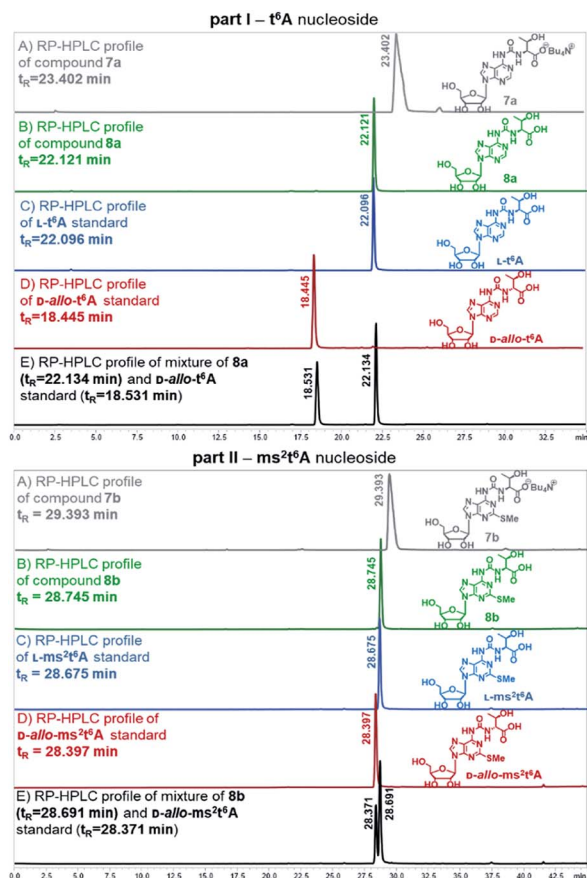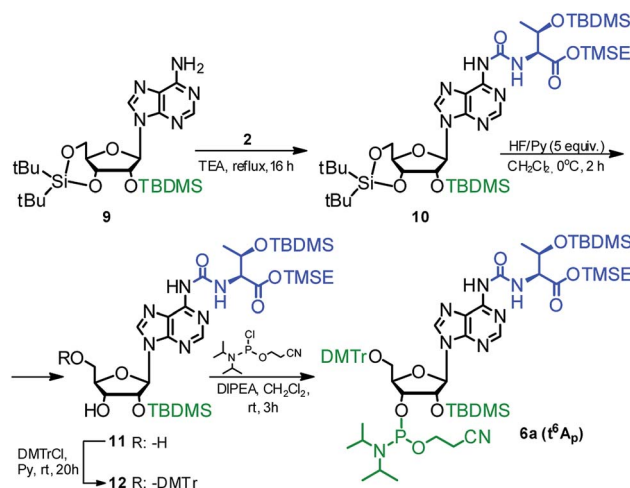
Scheme 3 Synthesis of **t⁶A** phosphoramidite (**6a**) using 2′-*O*-(*tert*-butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilylene)adenosine as the nucleoside substrate.

The phosphoramidite derivative of **t⁶A** (**6a**) was also synthesized using 2′-*O*-(*tert*-butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilylene) protected adenosine **9**[54,59] as the nucleoside substrate (Scheme 3). The one-pot conversion **9 → 10** proceeded in 94% yield. Subsequent selective removal of the cyclic silyl protecting group (HF in pyridine, 0 °C) furnished compound **11** (96% yield), further converted into the 5′-*O*-DMTr derivative **12** (90% yield). The reaction of **12** with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite gave finally the target **t⁶A**-phosphoramidite in 91% yield (combined yield 74% for **9 → 6a**).

Conclusions

The presented here modification of the isocyanate method of formation of the ureido linkage between adenosine and threonine greatly facilitates synthesis of fully protected ʟ-threonylcarbamoyl modified adenosines **4a,b** rendering subsequent preparation of the **t⁶A/ms²t⁶A** phosphoramidite monomers **6a,b** much more efficient. The developed one-pot procedure for **4a,b** synthesis, consisting in the epimerization-free formation of ʟ-threonine isocyanate directly from the *N*-Boc-Thr upon activation with Tf₂O in the presence of 2-Cl-Py, followed by its straight reaction with the *N⁶* *exo*-amine function of the sugar protected nucleoside, eliminates the use of toxic phosgene and provides a shorter protocol for the preparation of the protected **t⁶A/ms²t⁶A** derivatives compared to the previously reported isocyanate and carbamate routes. In addition, the protected nucleosides **4a,b** were efficiently deprotected yielding free nucleosides **8a,b** to be used as the standards, *e.g.* in HPLC analysis of enzymatically digested oligomers bearing **t⁶A/ms²t⁶A** units. Moreover, the *in situ* formed threonine isocyanate reacted efficiently with 2′-*O*-(*tert*-butyldimethyl-silyl)-3′,5′-*O*-(di-*tert*-butylsilylene)adenosine and the resultant conjugate was conveniently transformed into the **t⁶A**-phosphoramidite in a very good overall yield 74%. Developed procedures for the synthesis of **t⁶A/ms²t⁶A** 3′-*O*-phosphoramidities will significantly facilitate the

Fig. 2 HPLC profiles recorded for ʟ-**t⁶A** and ʟ-**ms²t⁶A** nucleosides and the corresponding ᴅ-*allo* nucleoside standards (ᴅ-*allo*-**t⁶A**, ᴅ-*allo*-**ms²t⁶A**).

availability of monomeric units for the chemical synthesis of various model tRNA fragments suitable for the structure–activity-relationship and biological studies of the **t⁶A** family nucleosides.

Experimental

General remarks

Commercial reagents and analytical grade solvents were used without additional purification unless otherwise stated. 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 60 (mesh 230–400, Fluka) column eluted with the indicated solvent mixtures. NMR spectra were recorded using a 700 MHz (for ^1H) instrument, 176 MHz for ^{13}C and 283 MHz for ^{31}P. Chemical shifts (δ) are reported in ppm relative to residual solvent signals CDCl₃: 7.26 ppm for ^1H NMR, 77.16 ppm for ^{13}C NMR; DMSO-d_6: 2.50 ppm for ^1H NMR, 39.52 ppm for ^{13}C NMR. The signal multiplicities are described as s (singlet), d (doublet), dd (doublet of doublets), ddd (doublets of doublets of doublets), dq (dublet of quartets), t (triplet), td (triplet of doublets), q (quartet), qd (quartet of doublets), m (multiplet), and br s (broad singlet). High-resolution mass spectra were recorded on Synapt G2Si mass spectrometer (Waters) equipped with an ESI source and quadrupole-time-of-flight mass analyzer. HPLC analysis of nucleosides was 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). Analyses were run at 30 °C and the elution profiles were UV monitored at $\lambda = 254$ nm.

General procedure for the one-pot synthesis of 4a, 4b and 10 from Boc-L-threonine 1

To a stirred solution of Boc-L-threonine **1** (1.08 g, 2.5 mmol) in dry toluene (30 mL) 2-chloropyridine (2-Cl-Py, 0.7 mL, 7.5 mmol) was added, followed by trifluoromethanesulfonic anhydride (Tf₂O, 0.64 mL, 3.75 mmol) and after stirring for 15 min at room temperature triethylamine (Et₃N, 1.04 mL, 7.5 mmol) and sugar-protected adenosine (**3a**, **3b** or **9**, 1.0 mmol) were added. The reaction mixture was stirred under reflux for 16 h. Then the solvent was evaporated under reduced pressure and **4a,b** or **10** were isolated by silica gel column chromatography.

One-pot synthesis of 4a from Boc-L-threonine 1 and adenosine derivatives 3a. Starting with 2′,3′,5′-tri-O-acetyladenosine **3a** (0.39 g, 1.0 mmol) **4a** was obtained as a white solid in 92% yield (0.69 g, 0.92 mmol) after purification by silica gel column chromatography (0–2% MeOH in CHCl₃). TLC: R_f = 0.53 (CHCl₃/MeOH, 95 : 5 v/v).

^1H NMR (700 MHz, DMSO-d_6) δ: 9.98 (s, 1H, NH-6), 9.86 (d, 1H, ^3J = 9.2 Hz, NH Thr), 8.65 (s, 1H, H-8), 8.42 (s, 1H, H-2), 6.30 (d, 1H, ^3J = 5.4 Hz, H-1′), 6.07–5.99 (m, 1H, H-2′), 5.64 (dd, 1H, ^3J = 5.9 Hz, ^3J = 4.5 Hz, H-3′), 4.49 (qd, 1H, ^3J = 6.2 Hz, ^3J = 1.8 Hz, CH-β Thr), 4.44–4.37 (m, 3H, CH-α Thr, H-4′, H-5′), 4.30–4.24 (m, 1H, H-5″), 4.18 (ddd, 1H, ^2J = 11.0 Hz, ^3J = 10.0 Hz, ^3J =

6.8 Hz, O–CH TMSE), 4.12 (ddd, 1H, ^3J = 10.9 Hz, ^3J = 10.0 Hz, ^3J = 6.5 Hz, O–CH TMSE), 2.13 (s, 3H, CH₃–CO Ac), 2.04 (s, 3H, CH₃–CO Ac), 2.01 (s, 3H, CH₃–CO Ac), 1.19 (d, ^3J = 6.3 Hz, 3H, CH₃ Thr), 1.01–0.92 (m, 2H, Si–CH₂ TMSE), 0.89 (s, 9H, Si–C(CH₃)₃ TBDMS), 0.08 (s, 3H, Si–CH₃ TBDMS), 0.01 (s, 3H, Si–CH₃ TBDMS), −0.00 (s, 9H, Si(CH₃)₃ TMSE); HRMS (ESI-TOF) m/z: [M + H]⁺ calcd for C₃₂H₅₃N₆O₁₁Si₂ 753.3311; found 753.3307 (see Fig. S8 and S26 in the ESI†).

One-pot synthesis of 4b from Boc-L-threonine 1 and adenosine derivatives 3b. Starting with 2′,3′,5′-tri-O-acetyl-2-methylthioadenosine **3b** (0.44 g, 1.0 mmol) **4b** was obtained as white solid in 86% yield (0.68 g, 0.86 mmol) after purification by silica gel column chromatography (0–1% MeOH in CHCl₃). TLC: R_f = 0.52 (CHCl₃/MeOH, 95 : 5 v/v).

^1H NMR (700 MHz, DMSO-d_6) δ: 9.98 (s, 1H, NH-6), 9.24 (d, 1H, ^3J = 8.6 Hz, NH Thr), 8.45 (s, 1H, H-8), 6.24 (d, 1H, ^3J = 4.3 Hz, H-1′), 6.07 (dd, 1H, ^3J = 6.0 Hz, ^3J = 4.2 Hz, H-2′), 5.69 (t, 1H, ^3J = 6.1 Hz, H-3′), 4.46–4.44 (m, 2H, CH-α Thr, CH-β Thr), 4.42 (dd, 1H, ^2J = 12.0 Hz, ^3J = 3.7 Hz, H-5′), 4.40–4.35 (m, 1H, H-4′), 4.24–4.16 (m, 2H, 2H, H-5″, O–CH TMSE), 4.11 (td, 1H, ^2J = 10.7 Hz, ^3J = 6.2 Hz, O–CH TMSE), 2.58 (s, 3H, S–CH₃), 2.11 (s, 3H, CH₃–CO Ac), 2.07 (s, 3H, CH₃–CO Ac), 1.95 (s, 3H, CH₃–CO Ac), 1.19 (d, 3H, ^3J = 6.3 Hz, CH₃ Thr), 1.04–0.93 (m, 2H, Si–CH₂ TMSE), 0.85 (s, 9H, Si–C(CH₃)₃ TBDMS), 0.08 (s, 3H, Si–CH₃ TBDMS), 0.03 (s, 3H, Si–CH₃ TBDMS), 0.01 (s, 9H, Si(CH₃)₃ TMSE); HRMS (ESI-TOF) m/z: [M + H]⁺ calcd for C₃₃H₅₅N₆O₁₁-SSi₂ 799.3188; found 799.3177 (see Fig. S14 and S27 in the ESI†).

Preparation of nucleoside standards 8a and 8b

Fully-protected adenosine **4a** or **4b** (0.02 g, 0.03 mmol) was dissolved in 1 M solution of TBAF in THF (0.4 mL, 0.40 mmol) and the reaction mixture was stirred for 4 h at room temperature. After this time NH₃ in dry MeOH (8 M solution, 0.2 mL) was added for deprotection of all acetyl groups from ribose moiety. The reaction was carried out for 2 h and then NH₃ was removed under reduced pressure to obtain tetrabutylammonium salts **7a/7b**. To exchange Bu₄N⁺ counterion to H⁺, CaCO₃ (0.28 g), dry DOWEX 50WX8 H⁺ form (0.84 g) and distilled methanol (0.6 mL) were added and the reaction mixture was stirred for 1 h at room temperature.[58] After this time the resulting mixture was filtered through Celite plug and washed with MeOH. The filtrate was analysed by HPLC and the presence of fully-deprotected only one isomer of **8a/8b** with natural L-threonine residue was confirmed (for **8a** R_t = 22.121 min, for **8b** R_t = 28.745 min, see Fig. 2 panel (B)).

RP-HPLC conditions for analysis of **t⁶A** derivatives: C18 column with linear gradient of buffer A (0.1% AcOH in H₂O) and buffer B (ACN) with a flow of 1 mL min⁻¹ as follows: 0–15 min from 2% to 8% B, 15–30 min from 8% to 25% B, 30–35 min 2% B. RP-HPLC conditions for analysis of **ms²t⁶A** derivatives: C18 column with linear gradient of buffer A (0.1% AcOH in H₂O) and buffer B (ACN) with a flow of 1 mL min⁻¹ as follows: 0–30 min from 2% B to 15% B, 30–40 min from 15% B to 30% B, 40–45 min 2% B.

Synthesis of t⁶A 3′-O-phoshoramidite 6a from 9

One-pot synthesis of 10 from Boc-L-threonine 1 and adenosine derivatives 9. Compound **10** was prepared using 2′-O-(tert-butyldimethylsilyl)-3′,5′-O-(di-tert-butylsilylene)-adenosine **9** (0.52 g, 1.0 mmol) according to general one-pot procedure. The crude product **10** was purified by silica gel column chromatography (0–1% MeOH in CHCl₃) to obtain pure **10** as white solid with 94% yield (0.83 g, 0.94 mmol). TLC: R_f = 0.68 (CHCl₃/MeOH, 95 : 5 v/v).

¹H NMR (700 MHz, CDCl₃) δ: 10.08 (d, 1H, ^3J = 9.1 Hz, NH Thr), 8.49 (s, 1H, H-2), 8.43 (br s, 1H, NH-6), 8.14 (s, 1H, H-8), 5.97 (br s, 1H, H-1′), 4.61–4.56 (m, 3H, H-2′, CH-β Thr, CH-α Thr), 4.52–4.47 (m, 2H, H-3′, H-5′), 4.29–4.21 (m, 2H, H4′, O-CH TMSE), 4.21–4.15 (m, 1H, O-CH₂ TMSE), 4.06 (dd, ^2J = 10.5 Hz, ^3J = 9.3 Hz, 1H, H5″), 1.26 (d, 3H, ^3J = 6.3 Hz, CH₃ Thr), 1.09 (s, 9H, Si-C(CH₃)₃ tBu₂Si), 1.05 (s, 9H, Si-C(CH₃)₃ tBu₂Si), 1.02–0.99 (m, 2H, Si-CH₂ TMSE), 0.95 (s, 9H, Si-C(CH₃)₃ TBDMS), 0.94 (s, 9H, Si-C(CH₃)₃ TBDMS), 0.17 (s, 3H, Si-CH₃ TBDMS), 0.15 (s, 3H, Si-CH₃ TBDMS), 0.10 (s, 3H, Si-CH₃ TBDMS), 0.05 (s, 3H, Si-CH₃ TBDMS), 0.02 (s, 9H, Si(CH₃)₃ TMSE); ¹³C NMR (176 MHz, CDCl₃) δ: 171.16 (C=O Thr), 154.44 (NH-CO-NH), 151.48 (C-2), 150.61 (C-6), 149.82 (C-4), 141.31 (C-8), 121.25 (C-5), 92.45 (C-1′), 76.01 (C-3′), 75.74 (C-2′), 74.93 (C-4′), 68.97 (Cα Thr), 67.91 (C-5′), 63.81 (O-CH₂ TMSE), 59.75 (Cβ Thr), 27.64 (Si-C(CH₃)₃ tBu₂Si), 27.19 (Si-C(CH₃)₃ tBu₂Si), 26.05 (Si-C(CH₃)₃ TBDMS), 25.75 (Si-C(CH₃)₃ TBDMS), 22.88 (Si-C(CH₃)₃ tBu₂Si), 21.32 (CH₃ Thr), 20.51 (Si-C(CH₃)₃ tBu₂Si), 18.47 (Si-C(CH₃)₃ TBDMS), 18.03 (Si-C(CH₃)₃ TBDMS), 17.51 (Si-CH₂ TMSE), −1.41(Si(CH₃)₃ TMSE), −4.07 (Si-CH₃ TBDMS), −4.15 (Si-CH₃ TBDMS), −4.84 (Si-CH₃ TBDMS), −5.15 (Si-CH₃ TBDMS); HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for C₄₀H₇₇N₆O₈Si₄ 881.4880; found 881.4868 (see Fig. S20, S21 and S28 in the ESI†).

Preparation of 11 by removal of 3′,5′-O-di-tert-butyl silylether protection from 10. Fully-protected nucleoside **10** (0.72 g, 0.84 mmol) was dissolved in anhydrous CH₂Cl₂ (7.2 mL) and cooled to 0 °C. Then a mixture of 70% HF in pyridine (0.1 mL, 4.2 mmol) and anhydrous pyridine (0.66 mL) was cooled to 0 °C and added to the reaction mixture. After 2 h stirring at 0 °C the mixture was diluted with CH₂Cl₂ (15 mL) and extracted with saturated NaHCO₃ (3 × 15 mL). The organic layer was dried over anhydrous MgSO₄, filtered and evaporated under reduced pressure. The oily residue was co-evaporated with toluene (2 × 15 mL) and silica gel column chromatography (0–1% MeOH in CH₂Cl₂) furnished **11** in 96% yield (0.60 g, 0.81 mmol). TLC: R_f = 0.48 (CHCl₃/MeOH, 95 : 5 v/v).

¹H NMR (700 MHz, DMSO-d₆) δ: 9.92 (s, 1H, NH-6), 9.88 (d, 1H, ^3J = 9.0 Hz, NH Thr), 8.71 (s, 1H, H-8), 8.39 (s, 1H, H-2), 6.03 (d, 1H, ^3J = 5.6 Hz, H-1′), 5.18 (dd, 1H, ^3J = 6.3 Hz, ^3J = 5.1 Hz, 5′OH), 5.13 (d, 1H, ^3J = 5.3 Hz, 3′OH), 4.71 (dd, 1H, ^3J = 5.7 Hz, ^3J = 4.8 Hz, H2′), 4.48 (qd, 1H, ^3J = 6.2 Hz, ^3J = 1.9 Hz, CH-β Thr), 4.41 (dd, 1H, ^3J = 9.0 Hz, ^3J = 2.0 Hz, CH-α Thr), 4.20–4.15 (m, 2H, H3′, O-CH TMSE), 4.12 (ddd, 1H, ^2J = 11.0 Hz, ^3J = 9.9 Hz, ^3J = 6.6 Hz, O-CH TMSE), 4.02 (q, 1H, ^3J = 3.7 Hz, H4′), 3.74 (ddd, 1H, ^2J = 12.1 Hz, ^3J = 5.1 Hz, ^3J = 4.0 Hz, H5′), 3.74 (ddd, 1H, ^2J = 12.1 Hz, ^3J = 6.3 Hz, ^3J = 3.6 Hz, H5″), 1.19 (d, ^3J = 6.3 Hz, 3H, CH₃ Thr), 0.99–0.91 (m, 2H, Si-CH₂ TMSE), 0.88 (s,

9H, Si-C(CH₃)₃ TBDMS), 0.72 (s, 9H, Si-C(CH₃)₃ TBDMS, 0.07 (s, 3H, Si-CH₃ TBDMS), 0.00 (s, 3H, Si-CH₃ TBDMS), −0.02 (s, 9H, Si-(CH₃)₃ TMSE), −0.07 (s, 3H, Si-CH₃ TBDMS), −0.18 (s, 3H, Si-CH₃ TBDMS); ¹³C NMR (176 MHz, DMSO-d₆) δ: 170.62 (C=O Thr), 153.68 (NH-CO-NH), 150.34 (C-6), 150.13 (C-4), 150.10 (C-2), 142.27 (C-8), 120.55 (C-5), 87.90 (C-1′), 85.98 (C-4′), 75.66 (C-2′), 70.12 (C-3′), 68.36 (Cβ Thr), 62.85 (O-CH₂ TMSE), 61.06 (C-5′), 58.90 (Cα Thr), 25.47 (Si-C(CH₃)₃ TBDMS), 25.34 (Si-C(CH₃)₃ TBDMS), 20.86 (CH₃ Thr), 17.73 (Si-C(CH₃)₃ TBDMS), 17.47 (Si-C(CH₃)₃ TBDMS), 16.69 (Si-CH₂ TMSE), −1.62 (Si(CH₃)₃ TMSE), −4.35 (Si-CH₃ TBDMS), −4.94 (Si-CH₃ TBDMS), −5.42 (Si-CH₃ TBDMS), −5.59 (Si-CH₃ TBDMS); HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for C₃₂H₆₁N₆O₈Si₃ 741.3859; found 741.3860 (see Fig. S22, S23 and S29 in the ESI†).

Preparation of 12 by 5′-O-dimethoxytritylation of 11. To a stirred solution of nucleoside **11** (0.55 g, 0.74 mmol) in dry pyridine (6.0 mL) DMTrCl (0.36 g, 0.96 mmol) was added. The reaction was stirred for 20 h at room temperature. The reaction mixture was cooled to 0 °C in an ice bath and quenched with H₂O (10 mL) and stirred at 0 °C for 15 min. The mixture was extracted with CH₂Cl₂ (3 × 15 mL) and the organic layer was dried over anhydrous MgSO₄, filtered and the solvent was evaporated. The oily residue was co-evaporated with toluene (2 × 10 mL). Silica gel column chromatography (0–1% MeOH in CH₂Cl₂) furnished **12** as a white solid in 90% yield (0.69 g, 0.67 mmol). TLC: R_f = 0.38 (CHCl₃/MeOH, 98 : 2 v/v).

¹H NMR (700 MHz, DMSO-d₆) δ: 9.92–9.91 (m, 2H, NH-6, NH Thr), 8.59 (s, 1H, H-8), 8.28 (s, 1H, H-2), 7.42–7.38 (m, 2H, H_Ar DMTr), 7.29–7.22 (m, 6H, H_Ar DMTr), 7.22–7.16 (m, 1H, H_Ar DMTr), 6.84–6.79 (m, 4H, H_Ar DMTr), 6.02 (d, 1H, ^3J = 5.3 Hz, H-1′), 5.17 (d, 1H, ^3J = 5.7 Hz, 3′OH), 5.01 (t, 1H, ^3J = 5.2 Hz, H-2′), 4.48 (qd, 1H, ^3J = 6.3 Hz, ^3J = 2.0 Hz, CH-β Thr), 4.41 (dd, 1H, ^3J = 9.0 Hz, ^3J = 1.9 Hz, CH-α Thr), 4.29–4.23 (m, 1H, H-3′), 4.18–4.15 (m, 1H, O-CH TMSE), 4.15–4.08 (m, 2H, H-4′, O-CH TMSE), 3.71 (s, 6H, 2× O-CH₃ DMTr), 3.32 (dd, 1H, ^2J = 10.6 Hz, ^3J = 3.9 Hz, H-5′), 3.25 (dd, 1H, ^2J = 10.5 Hz, ^3J = 5.1 Hz, H-5″), 1.19 (d, 3H, ^3J = 6.2 Hz, CH₃ Thr), 1.00–0.89 (m, 2H, Si-CH₂ TMSE), 0.85 (s, 9H, Si-C(CH₃)₃ TBDMS), 0.73 (s, 9H, Si-C(CH₃)₃ TBDMS), 0.06 (s, 3H, Si-CH₃ TBDMS), 0.00 (s, 3H, Si-CH₃ TBDMS), −0.02 (s, 9H, Si(CH₃)₃ TMSE), −0.05 (s, 3H, Si-CH₃ TBDMS), −0.16 (s, 3H, Si-CH₃ TBDMS); ¹³C NMR (176 MHz, DMSO-d₆) δ: 171.17 (C=O), 158.51 (C_Ar DMTr), 154.23 (NH-CO-NH), 150.86 (C-6), 150.59 (C-4), 150.47 (C-2), 145.35 (C_Ar DMTr), 143.44 (C-8), 135.95 (C_Ar DMTr), 130.18 (C_Ar DMTr), 128.17 (C_Ar DMTr), 128.15 (C_Ar DMTr), 127.05 (C_Ar DMTr), 121.26 (C-5), 113.52 (C_Ar DMTr), 88.89 (C-1′), 86.02 (⟩C⟨ DMTr), 84.38 (C-4′), 74.84 (C-2′), 70.69 (C-3′), 68.86 (C-β), 63.84 (C-5′), 63.36 (O-CH₂ TMSE), 59.40 (C-α), 55.44 (O-CH₃ DMTr), 25.99 (C-Si-(CH₃)₃ TBDMS), 25.79 (C-Si-(CH₃)₃ TBDMS), 21.35 (CH₃), 18.25 (C-Si-(CH₃)₃ TBDMS), 17.93 (C-Si-(CH₃)₃ TBDMS), 17.16 (Si-CH₂ TMSE), −1.11 (Si-(CH₃)₃ TMSE), −3.90 (Si-CH₃ TBDMS), −4.36 (Si-CH₃ TBDMS), −4.86 (Si-CH₃ TBDMS), −5.10 (Si-CH₃ TBDMS); HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for C₅₃H₇₉N₆O₁₀Si₃ 1043.5165; found 1043.5170 (see Fig. S24, S25 and S30 in the ESI†).

Preparation of 6a by 3′-O-phosphitylation of 12. 3′-O-Phoshoramidite **6a** was prepared according to the literature

procedure,[41] using compound **12** (0.60 g, 0.56 mmol), 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.24 mL, 1.12 mmol), DIPEA (0.4 mL, 2.24 mmol) and freshly distilled CH_2Cl_2 (3.2 mL). The crude product was purified by the flash chromatography (silica gel, petroleum ether/acetone, 2 : 1 v/v) to obtain pure product **6a** in 92% yield (0.64 g, 0.52 mmol). TLC: $R_f = 0.52$ ($CHCl_3$/acetone, 95 : 5 v/v).

^{31}P NMR: (283 MHz, C_6H_6) δ: 149.89, 148.04 (see Fig. S12 in the ESI†).

Conflicts of interest

There are no conflicts to declare.

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Notes and references

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