Efficient access to 3′-O-phosphoramidite derivatives of tRNA related \(N^6\)-threonylcarbamoyladenosine (t\(\text{A}\)) and 2-methylthio-\(N^6\)-threonylcarbamoyladenosine (ms\(^2\)t\(\text{A}\))

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An efficient method of ureido linkage formation during epimerization-free one-pot synthesis of protected hypermodified \(N^6\)-threonylcarbamoyladenosine (t\(\text{A}\)) and its 2-SMe analog (ms\(^2\)t\(\text{A}\)) was developed. The method is based on a Tf\(2\text{O}\)-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 t\(\text{A}\)-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,\(^3,4\) distribution within the tRNA molecules,\(^5,6\) and their biological activity.\(^7\)\(^–\)\(^11\)

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.\(^12,13,14\) 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).

An extensive access to \(N^6\)-threonylcarbamoyladenosine (t\(\text{A}\)) and its analogs containing either the threonylcarbamoyl chain (a ureido system is formed) using either a carbamate or isocyanate approach (Scheme 1, paths A and B, respectively).

Fig. 1. Abbreviations and structures of \(L\)-threonylcarbamoyl modified adenosines (the t\(\text{A}_{37}\) family) located in tRNAs at the position 37.
Analogously, $t^6A$ derivatives suitable for synthesis of the corresponding $3'$-O-phosphoramidite derivative (protected with tert-butylmethyldimethylsilylester (TBDMS) on the OH and trimethylsilyl ester (TMSE) at the COOH of threonine residue) can be prepared. The carbamate method has been significantly improved by the use of more active phenyl carbamate derivatives of adenosine/2'-methylthio-adenosine. This was possible with phenoxycarbonyl tetrazole or 1-N-methyl-3-phenoxycarbonyl-imidazolium chloride used as effective reagents introducing the carbamate functionality onto the weakly nucleophilic $N^6$-amine function of $A_{ms^5}A$ nucleosides.

The isocyanate approach to the synthesis of $t^6A_{ms^5}t^6A$ (Scheme 1, path B1,2) was shown to have limited applicability in the preparation of “free” nucleosides. 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. However, the isocyanate route was recently postulated by the Carell’s group as a possible pathway for the formation of $t^6A$ under prebiotic conditions.

In the synthesis of threonine protected $t^6A_{ms^5}t^6A$ derivatives for the subsequent preparation of the corresponding $3'$-O-phosphoramidites, the isocyanate approach is much less explored than the carbamate procedures. Initially, the isocyanate derivative was generated from the $N^6$-amine function of sugar protected adenosine (Scheme 1, path B1), but its condensation with the free amine function of $\lambda$-threonine was ineffective and the ureido-nucleoside product was obtained in a low 19% yield. 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 $\lambda$-threonine appropriately blocked on the OH and COOH functions) with the sugar protected nucleoside (overall yield of this three steps procedure ~55%). 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). Most likely, such variant of the isocyanate method (Scheme 1, path B3) applied in the synthesis of the $3'$-O-phosphoramidite derivatives of $t^6A_{ms^5}t^6A$ 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 $t^6A_{ms^5}t^6A$ using a TF$_2$O-mediated generation of the isocyanate derivative directly from N-Boc-protecting group of $\lambda$-threonine, followed by its straight reaction with the $N^6$-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-butylylene) ribonucleoside sugar protection system, 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 trimethylsilyl (TMSE) ester of N-Boc-O-tert-butyldimethylsilyl (TBDMS) protected $\lambda$-threonine$^{29}$ (1) and 2',3',5'-O-acetyladenosine (3a) (Table 1, see ESI for spectroscopic data of 1, 2, 3a). In all cases, the final condensation of isocyanate 2 with 3a was performed in the presence of Et$_3$N (2-fold molar excess over the TF$_2$O activator used for isocyanate formation) in boiling toluene for 16 h. It was reported that addition of Et$_3$N, 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.

To optimize the triflic anhydride (TF$_2$O) 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 TF$_2$O 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 Et$_3$N and the nucleoside substrate 3a was added. The reaction $1 \rightarrow 2$ for 15 min at room temp. (entry...
Table 1  Optimization of the reaction conditions for the synthesis of t⁶A from Boc-Thr derivative 1 and the sugar-protected adenosine 3a

| Entry | Boc-Thr ¹⁰ (equiv.) | Tf₂O/base ¹⁰ (equiv.) | Solvent, time, temp. (°C) | Yield of 4a ¹⁰ (%) |
|-------|---------------------|-----------------------|--------------------------|-------------------|
| 1     | 1.0                 | Tf₂O (1.5)/2-Cl-Py (3.0) | CH₂Cl₂, rt, 15 min       | 19%               |
| 2     | 1.0                 | Tf₂O (1.5)/2-Cl-Py (3.0) | CH₂Cl₂, 0 °C, 5 min      | 46%               |
| 3     | 1.0                 | Tf₂O (2.0)/2-Cl-Py (4.0) | CH₂Cl₂, 0 °C, 5 min      | 42%               |
| 4     | 1.0                 | Tf₂O (1.5)/Py (3.0)     | CH₂Cl₂, rt, 3 h          | —                 |
| 5     | 1.0                 | Tf₂O (1.5)/DMAP (3.0)   | CH₂Cl₂, rt, 3 h          | —                 |
| 6     | 1.0                 | Tf₂O (1.5)/Et₃N (3.0)   | CH₂Cl₂, rt, 3 h          | —                 |
| 7     | 1.0                 | Tf₂O (1.5)/2, 6-lutidine (3.0) | CH₂Cl₂, rt, 3 h | 16%               |
| 8     | 1.5                 | Tf₂O (2.23)/2-Cl-Py (4.5) | CH₂Cl₂, 0 °C, 5 min | 71%               |
| 9     | 2.0                 | Tf₂O (3.0)/2-Cl-Py (6.0) | CH₂Cl₂, 0 °C, 5 min      | 80%               |
| 10    | 2.5                 | Tf₂O (3.75)/2-Cl-Py (7.5) | CH₂Cl₂, 0 °C, 5 min      | 92%               |
| 11    | 2.5                 | Tf₂O (3.75)/2-Cl-Py (7.5) | Toluene, rt, 15 min     | 92%               |

All reactions were performed in a 0.2 mmol scale in 6 mL of the corresponding solvent. The number of equivalents was calculated in respect to the nucleoside reagent 3a. The ratios of 1/Tf₂O = 1.5 and Tf₂O/base = 2 were applied. Isolated yield after column chromatography. 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 Tf₂O (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 ura 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. However, in the case of t⁶A/ms₂t⁶A synthesis, the amine nucleoside substrate, especially non-native 2-methylthioadenosine (ms₂A) is a very costly reagent. Therefore, in the second step of optimizations we examined an excess of N-Boc protected α-threonine derivative 1 to nucleoside 3a in a range 1.5–2.5 (entries 8–10), yet the concentrations of Tf₂O 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 t⁶A derivative 4a.

The optimized method described above was used in synthesis of the phosphoramidite derivatives of t⁶A and ms₂t⁶A (6a, and 6b, respectively; Scheme 2). Starting from 2.5 mmol of appropriately protected Boc-α-threonine 1 ¹⁰ and 1 mmol of adenosine derivative 3a ²⁷ and 3b ²⁸ 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 N⁶-threonylcarbamoyl chain (Et₃N/MeOH, rt, 24 h) and the resultant 5a/5b were appropriately protected and

Scheme 2  Preparation of t⁶A and ms₂t⁶A 3′-O-phosphoramidites and samples of modified nucleosides t⁶A and ms₂t⁶A.
phosphitylated according to the previously reported procedures to give \( \text{t}^\theta \text{A}/\text{ms}^2\text{t}^\theta \text{A}-\text{phosphoramidites} (6a/b) \) 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 \( \text{t}^\theta \text{A} \)- or \( \text{ms}^2\text{t}^\theta \text{A} \)-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 \( \text{NH}_3/\text{MeOH} \) (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 \( \text{t}^\theta \text{A} \) and part II for \( \text{ms}^2\text{t}^\theta \text{A} \)). The tailing was not observed, when the highly lipophilic tetrabutylammonium cations were replaced with \( \text{H}^+ \) ions using DOWEX, \( \text{H}^+/\text{DOWEX} \) (compare profiles in panels (B) and (C)). The profiles recorded for 8a co-injected with \( \text{d}-\text{allo}-\text{t}^\theta \text{A} \) and for 8b co-injected with \( \text{d}-\text{allo}-\text{ms}^2\text{t}^\theta \text{A} \) (panels (E)) indicate that the new procedure for ureido linkage formation is safe in terms of the stereochemistry at the \( \text{C}_\alpha \) of the amino acid component. The profiles for the \( \text{d}-\text{allo} \) nucleoside standards are shown in panels (D).

![HPLC profiles](image)

**Fig. 2** HPLC profiles recorded for \( \text{l}-\text{t}^\theta \text{A} \) and \( \text{d}-\text{ms}^2\text{t}^\theta \text{A} \) nucleosides and the corresponding \( \text{d}-\text{allo} \) nucleoside standards (\( \text{d}-\text{allo}-\text{t}^\theta \text{A} \), \( \text{d}-\text{allo}-\text{ms}^2\text{t}^\theta \text{A} \)).

The phosphoramidite derivative of \( \text{t}^\theta \text{A} \) (6a) was also synthesized using 2’-O-(tert-butyldimethylsilyl)-3’,5’-O-(di-tert-butylsilylene) protected adenosine to give nucleoside substrate (Scheme 3). The one-pot conversion 9 \( \rightarrow \) 10 proceeded in 94% yield. Subsequent selective removal of the cyclic silyl protecting group (HF in pyridine, \( 0^\circ \text{C} \)) furnished compound 11 (96% yield), further converted into the 5’-O-DMT derivative 12 (90% yield). The reaction of 12 with 2-cyanoethyl \( \text{N,N}-\text{disopropylchloro-phosphoramidite} \) gave finally the target \( \text{t}^\theta \text{A}-\text{phosphoramidite} \) in 91% yield (combined yield 74% for 9 \( \rightarrow \) 6a).

**Scheme 3** Synthesis of \( \text{t}^\theta \text{A} \) phosphoramidite (6a) using 2’-O-(tert-butyldimethylsilyl)-3’,5’-O-(di-tert-butylsilylene) protected adenosine as the nucleoside substrate.

**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 \( \text{l}-\text{threonylcarbamoyl modified adenosines 4a,b} \) rendering subsequent preparation of the \( \text{t}^\theta \text{A}/\text{ms}^2\text{t}^\theta \text{A} \) phosphoramidite monomers 6a,b much more efficient. The developed one-pot procedure for 4a,b synthesis, consisting in the epimerization-free formation of \( \text{l}-\text{threonine isocyanate} \) directly from the \( \text{N}^\theta-\text{Boc-Thr} \) upon activation with \( \text{Tf}_2\text{O} \) in the presence of 2-Cl-Py, followed by its straight reaction with the \( \text{N}^\theta-\text{exo-amine function} \) of the sugar protected nucleoside, eliminates the use of toxic phosgene and provides a shorter protocol for the preparation of the protected \( \text{t}^\theta \text{A}/\text{ms}^2\text{t}^\theta \text{A} \) 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 \( \text{t}^\theta \text{A}/\text{ms}^2\text{t}^\theta \text{A} \) units. Moreover, the \( \text{in situ} \) 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 \( \text{t}^\theta \text{A}-\text{phosphoramidite} \) in a very good overall yield 74%. Developed procedures for the synthesis of \( \text{t}^\theta \text{A}/\text{ms}^2\text{t}^\theta \text{A} \) 3’-O-phosphoramidities will significantly facilitate the
availability of monomeric units for the chemical synthesis of various model tRNA fragments suitable for the structure-activity-relationship and biological studies of the t^A 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 13C and 283 MHz for 31P. Chemical shifts (δ) are reported in ppm relative to residual solvent signals CDCl3: 7.26 ppm for 1H NMR, 77.16 ppm for 13C NMR; DMSO-d6: 1H, 3.3a, 3.5 Hz, 3b, 4.44 ppm (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 right 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 λ = 254 nm.

**General procedure for the one-pot synthesis of 4a, 4b and 10 from Boc-\(\text{t}^{\text{A}}\)-threonine 1**

To a stirred solution of Boc-\(\text{t}^{\text{A}}\)-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 a fluoromethanesulfonic anhydride (Tf2O, 0.64 mL, 3.75 mmol). The reaction mixture was stirred for 16 h. Then the solvent was evaporated under reduced pressure to obtain tetrabutylammonium salts. The residue was dissolved in a mixture of 0–2% MeOH in CHCl3. TLC: \(R_f = 0.53\) (CHCl3:MeOH, 95 : 5 v/v).

1H NMR (700 MHz, DMSO-d6) δ: 9.98 (s, 1H, NH-6), 9.24 (d, 1H, \(\ddagger J = 6.3\) Hz, H-8), 8.45 (s, 1H, H-7), 2.50 ppm (for 1H NMR, 31P NMR; DMSO-d6: 39.52 ppm for 13C NMR. The signal multiplicities are described as s (singlet), d (doublet), dd (doublet of doublets), ddd (doublets of doublets of doublets), dq (double 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 Prominance 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 λ = 254 nm.

**One-pot synthesis of 4b from Boc-\(\text{t}^{\text{A}}\)-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-d6) δ: 9.98 (s, 1H, NH-6), 9.24 (d, 1H, \(\ddagger J = 8.6\) Hz, NH Thr), 8.45 (s, 1H, H-7), 6.24 (d, 1H, \(\ddagger J = 4.3\) Hz, H-1'), 6.07 (dd, 1H, \(\ddagger J = 6.0\) Hz, \(\ddagger J = 4.2\) Hz, H-2'), 5.69 (t, 1H, \(\ddagger J = 6.1\) Hz, H-3'), 4.46–4.44 (m, 2H, CH2 Thr, CHβ Thr), 4.42 (dd, 1H, \(\ddagger J = 12.0\) Hz, \(\ddagger J = 3.7\) Hz, H-5'), 4.40–4.35 (m, 1H, H-4'), 4.24–4.16 (m, 2H, 2H, H-5', O-CH2 TMSE), 4.11 (td, 1H, \(\ddagger J = 10.7\) Hz, \(\ddagger J = 6.2\) Hz, O-CH TMSE), 2.58 (s, 3H, S-CH3), 2.11 (s, 3H, CHβ-CH3 Ac), 2.07 (s, 3H, CH2-CH3 Ac), 1.95 (s, 3H, CH3-CH3 Ac), 1.19 (d, 3H, \(\ddagger J = 6.3\) Hz, CH3 Thr), 1.04–0.93 (m, 2H, 2H, CH2(O), 0.85 (s, 9H, Si(CH3)3 TMSE), 0.80 (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]+ caled for C32H53N6O11Si2 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. 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 \(\text{t}^{\text{A}}\)-threonine residue was confirmed (for 8a \(R_f = 22.121\) min, for 8b \(R_f = 28.745\) min, see Fig. 2 panel (B)).

RP-HPLC conditions for analysis of t^A 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 ms^t^A 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 \(^6\)A 3'-O-phosphoramidite 6a from 9

One-pot synthesis of 10 from Boc-\(\alpha\)-threonine 1 and adenosine derivatives 9. Compound 10 was prepared using 2'-O-(tert-butylidimethylsilyl)-3',5'-O-(di-tert-butylsilylene)-adenosine (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 CHCl\(_3\)) to obtain pure 10 as white solid with 94% yield (0.83 g, 0.94 mmol). TLC: \(R_f = 0.68\) (CHCl\(_3\)/MeOH, 95: 5 v/v).

1\(^1\)H NMR (700 MHz, CDCl\(_3\)) \(\delta = 10.08\) (d, 1H, \(J = 9.1\) 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-\(\beta\) Thr, CH-\(\alpha\) 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-CH\(_2\) TMSE), 4.06 (dd, \(J = 10.5\) Hz, \(J = 9.3\) Hz, 1H, H5'), 1.26 (d, 3H, \(J = 6.3\) Hz, CH3 Thr), 1.09 (s, 9H, Si-(CH3)\(_3\) TBDMS), 1.05 (s, 9H, Si-(CH3)\(_3\) TBDMS), 1.02–0.99 (m, 2H, Si-CH\(_2\) TMSE), 0.95 (s, 9H, Si-(CH3)\(_3\) TBDMS), 0.94 (s, 9H, Si-(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)\(_3\) TBDMS); \(^13\)C NMR (176 MHz, CDCl\(_3\)) \(\delta = 171.16\) (C-2'), 154.44 (NH-\(\alpha\)-NO), 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 (Cz Thr), 67.91 (C-5'), 63.81 (O-CH\(_2\) TMSE), 59.75 (Cz Thr), 27.64 (Si-(CH3)\(_3\) TBDMS), 27.19 (Si-(CH3)\(_3\) TBDMS), 26.05 (Si-(CH3)\(_3\) TBDMS), 25.75 (Si-(CH3)\(_3\) TBDMS), 22.88 (Si-(CH3)\(_3\) TBDMS), 21.32 (CH3 Thr), 20.51 (Si-(CH3)\(_3\) TBDMS), 18.47 (Si-(CH3)\(_3\) TBDMS), 18.03 (Si-(CH3)\(_3\) TBDMS), 17.51 (Si-CH\(_2\) TMSE), -1.41(Si(CH3)\(_3\) TBDMS), -4.07 (Si-CH3 TBDMS), -4.15 (Si(CH3)\(_3\) TBDMS), -5.15 (Si(CH3)\(_3\) TBDMS); HRMS (ESI-TOF) m/z: [M + H]+ calcd for C\(_{49}\)H\(_{32}\)N\(_6\)O\(_{14}\)Si\(_6\) 881.4880; found 881.4868 (see Fig. S20, S21 and S22 in the ESI†).

Preparation of 11 by removal of 3',5'-O-di-tert-butyldimethylsilyl protection from 10. Fully-protected nucleoside 10 (0.72 g, 0.84 mmol) was dissolved in anhydrous CH\(_2\)Cl\(_2\) (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 (0.06 mL) was cooled to 0°C and added to the reaction mixture. After 2 h stirring at 0°C the mixture was diluted with CH\(_2\)Cl\(_2\) (15 mL) and extracted with saturated NaHCO\(_3\) (3 × 15 mL). The organic layer was dried over anhydrous MgSO\(_4\), filtered and evaporated under reduced pressure. The oily residue was co-evaporated with toluene (2 × 10 mL) and silica gel column chromatography (0–1% MeOH in CHCl\(_3\)) furnished 11 as a white solid in 90% yield (0.69 g, 0.67 mmol). TLC: \(R_f = 0.38\) (CH\(_3\)Cl/MeOH, 98: 2 v/v).

1\(^1\)H NMR (700 MHz, DMSO-d\(_6\)) \(\delta = 9.92–9.91\) (m, 2H, NH-6, NH Thr), 8.59 (s, 1H, H-8), 8.28 (s, 1H, H-2), 7.42–7.38 (m, 2H, H2-C14, DMTr), 7.29–7.22 (m, 6H, H4-C18 DMTr), 7.22–7.16 (m, 1H, H3-C12 DMTr), 6.84–6.79 (m, 4H, H4, DMSO), 6.02 (d, 1H, \(J = 5.3\) Hz, H1'), 5.17 (d, 1H, \(J = 5.7\) Hz, 3'OH), 5.01 (t, 1H, \(J = 5.2\) Hz, H2'), 4.48 (qd, 1H, \(J = 6.3\) Hz, \(J = 2.0\) Hz, CH-\(\beta\) Thr), 4.41 (dd, 1H, \(J = 9.0\) Hz, \(J = 1.9\) Hz, CH-\(\alpha\) Thr), 4.29–4.23 (m, 1H, H3-C18 DMTr), 3.71 (s, 6H, 2\times O-CH3 DMTr), 3.32 (dd, 1H, \(J = 10.6\) Hz, \(J = 3.9\) Hz, H5'), 3.25 (dd, 1H, \(J = 10.5\) Hz, \(J = 5.1\) Hz, H5'), 1.19 (d, 3H, \(J = 6.2\) Hz, CH3 Thr), 1.00–0.89 (m, 2H, Si-CH3 TBDMS), 0.85 (s, 9H, Si-(CH3)\(_3\) TBDMS), 0.73 (s, 9H, Si-(CH3)\(_3\) TBDMS), 0.06 (s, 3H, Si-CH3 TBDMS), 0.00 (s, 3H, Si-CH3 TBDMS), -0.02 (s, 9H, Si(CH3)\(_3\) TBDMS), -0.05 (s, 3H, Si-CH3 TBDMS), -0.16 (s, 3H, Si-CH3 TBDMS); \(^13\)C NMR (176 MHz, DMSO-d\(_6\)) \(\delta = 171.17\) (C-2'), 158.51 (Cz DMTr), 154.23 (NH-\(\alpha\)-NO), 150.86 (C-6), 150.59 (C-4), 144.07 (C-2), 143.35 (Cz DMTr), 134.44 (C-8), 133.95 (Cz DMTr), 126.18 (Cz DMTr), 128.75 (Cz DMTr), 127.05 (Cz DMTr), 121.26 (Cz DMTr), 113.52 (Cz DMTr), 88.89 (C-1'), 86.02 (C-2'), 84.38 (C-4'), 74.84 (C-2'), 70.69 (C-3'), 68.68 (C-8), 63.84 (C-5'), 63.36 (O-CH\(_3\) TMSE), 59.40 (C-2'), 55.44 (O-CH3 DMTr), 25.79 (C-Si(CH3)2 TBDMS), 21.35 (CH3), 18.25 (C-Si(CH3)2 TBDMS), 17.93 (C-Si(CH3)2 TBDMS), 17.16 (Si-CH\(_2\) TMSE), -1.11 (Si(CH3)\(_3\) TMSE), -3.90 (Si(CH3)2 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 C\(_{52}\)H\(_{38}\)N\(_8\)O\(_{15}\)Si\(_6\) 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-Phosphoramidite 6a was prepared according to the literature
procedure,\textsuperscript{41} using compound 12 (0.60 g, 0.56 mmol), 2-cyanoethyl-\textit{N},\textit{N}-disopropylchlorophosphoramidite (0.24 mL, 1.12 mmol), DIPEA (0.4 mL, 2.24 mmol) and freshly distilled CH\textsubscript{2}Cl\textsubscript{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: \textit{R}\textsubscript{f} = 0.52 (CHCl\textsubscript{3}/acetone, 95 : 5 v/v).

\textsuperscript{13}P NMR: (283 MHz, C\textsubscript{6}D\textsubscript{6}) \delta: 149.89, 148.04 (see Fig. S12 in the ESI\textsuperscript{†}).

**Conflicts of interest**

There are no conflicts to declare.

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