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Synthesis of 3′-Fluoro-tRNA Analogues for Exploring Non-ribosomal Peptide Synthesis in Bacteria

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Aminoacyl-tRNAs (aa-tRNAs) participate in a vast repertoire of metabolic pathways, including the synthesis of the peptidoglycan network in the cell walls of bacterial pathogens. Synthesis of aminoacyl-tRNA analogues is critical for further understanding the mechanisms of these reactions. Here we report the semi-synthesis of 3′-fluoro analogues of Ala-tRNA^{Ala}. The presence of fluorine in the 3′-position blocks Ala at the 2′-position by preventing spontaneous migration of the residue between positions 2′ and 3′. NMR analyses showed that substitution of the 3′-hydroxy group by fluorine in the *ribo* configuration favours the S-type conformation of the furanose ring of terminal adenosine A76. In contrast, the N-type conformation is favoured by the presence of fluorine in the *xylo* configuration. Thus, introduction of fluorine in the *ribo* and *xylo* configurations affects the conformation of the furanose ring in reciprocal ways. These compounds should provide insight into substrate recognition by Fem transferases and the Ala-tRNA synthetases.

Introduction

Aminoacyl-tRNAs (aa-tRNAs) are a source of ester-activated amino acids. In addition to their role in protein synthesis by the ribosome, they participate in various metabolic pathways.[1] The vicinal hydroxy groups in positions 2′ and 3′ of the terminal nucleotide in a tRNA (A76) have pivotal roles. The tRNAs are esterified by aminoacyl-tRNA synthetases,[2] which catalyse the transfer of a specific aminoacyl residue from an adenylate to the 2′- or the 3′-hydroxy group of A76 (Scheme 1 A). Isomerisation of the ester between positions 2′ and 3′ occurs in the absence of any enzyme with a rate and a thermodynamic equilibrium of the order of 5 s^{-1} and 1, respectively.[3] The A site of the ribosome is specific for the 3′-*O*-aminoacyl isomer, and the 3′-linkage is conserved in the product of the peptidyl transfer reaction[4] (Scheme 1 A). The 2′-hydroxy group of peptidyl-tRNA at the P site of the ribosome was reported to be essential, with a 10^6–10^7-fold reduction in the rate of peptide bond formation being reported for a 2′-deoxy analogue.[5] It was proposed that the critical role of the 2′-OH group reflected substrate-assisted catalysis in the peptidyltransferase centre of the ribosome.[5a]

Among the tRNA-dependent aminoacyl transferases, Fem transferases participate in peptidoglycan synthesis in Gram-positive bacteria (Scheme 1 A). Because these enzymes are essential for resistance to β-lactam antibiotics in major pathogens, including *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis*, they are considered attractive targets for the development of new antibiotics active against multi-resistant bacteria.[6] FemX$_{Wv}$ of *Weissella viridescens*, the model enzyme of the Fem family, transfers L-Ala from Ala-tRNA to the peptidoglycan precursor (Scheme 1 A). We have previously determined the regiospecificity of FemX$_{Wv}$ and shown that transfer of Ala occurs from the 2′-position.[7] We have also shown that the 3′-OH group is not essential for catalysis, although a 240-fold reduction in the turnover number was observed for the 3′-deoxy analogue. We proposed that the 3′-OH group participates in proton shuttling in the substrate-assisted catalytic mechanism (Scheme 1 B).[7,8]

Chemical modification of the terminal nucleotide of tRNAs (A76) is critical for better understanding of the pivotal roles of the vicinal 2′- or 3′-hydroxy groups in protein and peptidoglycan synthesis. Two analogues—the deoxy or the fluoro analogues, in which one of the hydroxy groups has been replaced by hydrogen[5a,9] or fluorine,[5a,10] respectively—are mainly used in this context. Both substitutions freeze the amino acid residue at the 2′- or 3′-position. The deoxy analogues lack both the hydrogen bond donor and acceptor characters of the hydroxy group. The fluoro analogues have distinct characteristics because fluorine mimics the polarity of the hydroxy group and is a weak hydrogen bond acceptor.[11] In addition, the electron-withdrawing nature of fluorine strongly affects sugar puckering.[12]

Previous studies have reported on the introduction of 2′-deoxy-2′-fluoroadenosine at the extremities of peptidyl-tRNAs

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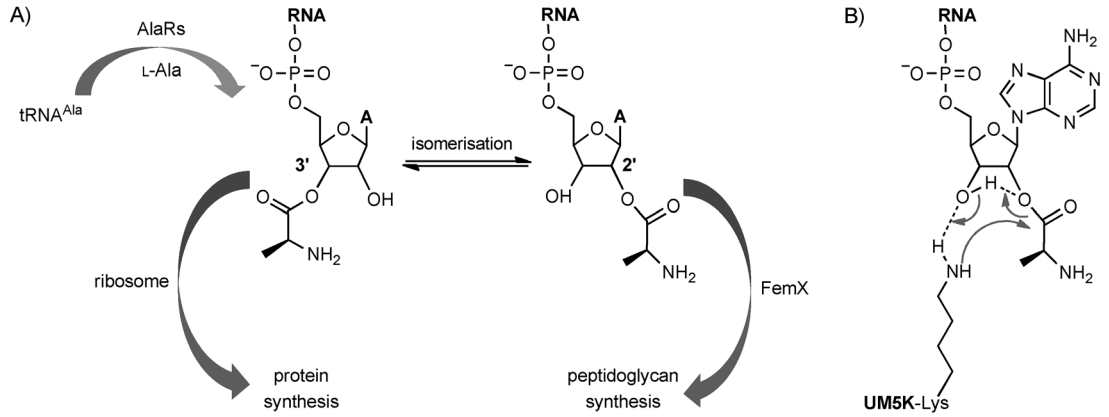
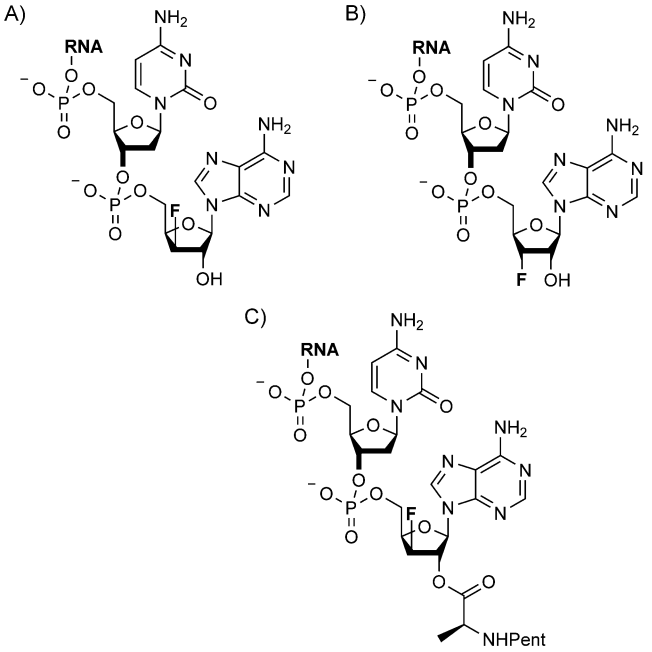


Scheme 1. A) Schematic representation of Ala-tRNA^{Ala} regioisomers in protein and peptidoglycan synthesis. B) FemX transfers an alanyl residue from Ala-tRNA^{Ala} to the side chain of L-Lys in the peptidoglycan precursor UDP-*N*-acetyl-muramyl-pentapeptide (UM5K). The 3′-OH group may be involved in proton shuttling during catalysis.

and aminoacyl-tRNAs by a two-step enzymatic process.[5a,10a] Firstly, the terminal CA nucleotides were removed from the 3′-end of natural tRNA^{Lys} by two rounds of periodate/aniline/polynucleotide kinase reactions.[13] Secondly, the CCA-adding enzyme was used to restore the full tRNA sequence with cytosine triphosphate (for position 75) and 2′-deoxy-2′-fluoroadenosine triphosphate (for position 76) as the substrates.[5a] We report here an alternative to this enzymatic route. Our strategy relies on chemical synthesis of a fluorinated dinucleotide (pdCpA-F), followed by ligation to incomplete tRNAs lacking the terminal pCpA dinucleotide.[14] The ligation reaction, catalysed by T4 RNA ligase, restores the complete tRNA sequence. We report the application of this strategy to the synthesis of 3′-fluoro-tRNA^{Ala} analogues with the fluorine in the *xylo* and *ribo* configurations and the acylated derivative of the former analogue (Scheme 2). In addition, we have explored the conformational preference of the corresponding dinucleotide by NMR spectroscopy.



Scheme 2. A) and B) Fluoro analogues of tRNA^{Ala} in the *xylo* and *ribo* configurations, respectively. C) Fluoro analogue of Ala-tRNA^{Ala} in the *xylo* configuration.

Results and Discussion

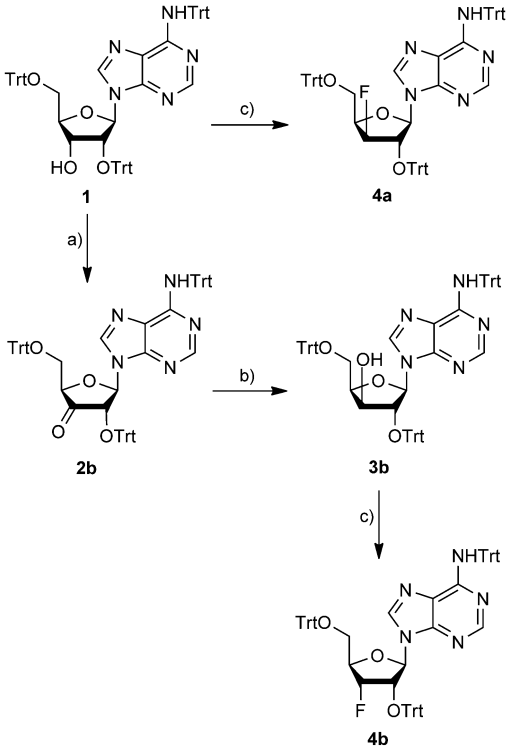
Synthesis

Syntheses of 2′-deoxy-2′-fluoroadenosine have been widely described,[15] whereas those of 3′-deoxy-3′-fluoroadenosine have been less studied.[16] Fluorinated nucleosides can be synthesised either by fluorination of preformed nucleosides or by condensation of fluorine-substituted furanose moieties with suitable heterocyclic bases. In this study we followed the first strategy, starting from the known tritylated compound **1**[16a,17] (Scheme 3).

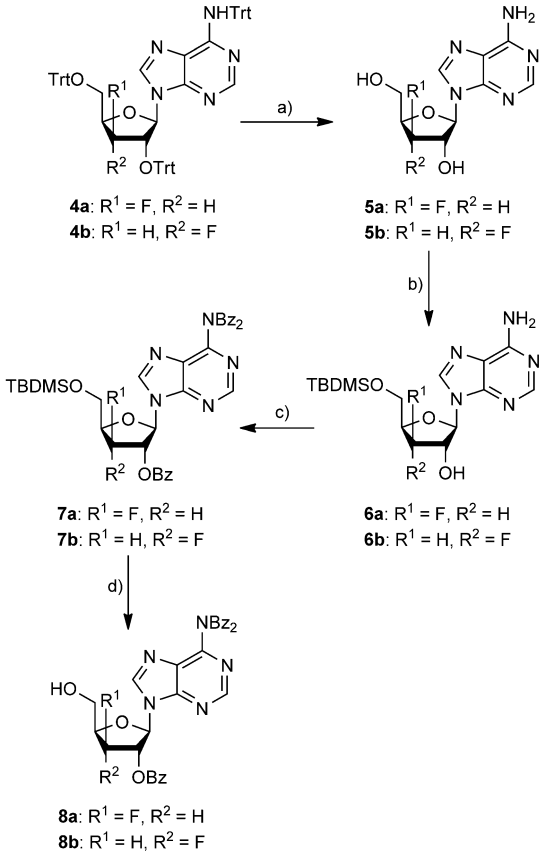
The 3′-*xylo*-Fluoro analogue **4a** was directly obtained by using diethylaminosulfur trifluoride (DAST) as the fluorinating agent (50% yield). For the synthesis of 3′-*ribo*-fluoro analogue **4b** (Scheme 3), compound **1** was sequentially oxidised to ketone **2b** with Dess–Martin periodinane and reduced to **3b** in an overall yield of 47%. Fluorination of **3b** was achieved with DAST (67%).

For compatibility with the dinucleotide synthesis, protecting groups were changed in two steps (Scheme 4). Firstly, **4a** and **4b** were fully deprotected (TFA), to afford **5a** and **5b** in 94 and 76% yields, respectively. Secondly, silyl ether derivatives of **5a** and **5b** were obtained by treatment with TBDMSCl to give **6a** and **6b.** These were treated with benzoyl chloride to afford **7a** and **7b** in 90 and 100% yields, respectively. The 5′-silyl ether groups were removed with the aid of a 1:1 TFA/H_2O mixture to provide **8a** and **8b** in 90 and 81% yields, respectively. Compounds were purified by column chromatography and characterised by ^{1}H, ^{13}C and ^{19}F NMR spectroscopy and mass spectrometry analysis.

Dinucleotides **11a** and **11b** were obtained by the phosphoramidite approach (Scheme 5). Phosphodiesters **9a** and **9b**



Scheme 3. a) Dess–Martin periodinane, CH_2Cl_2, RT, 4 h, **2b** (95%); b) $NaBH_4$, $EtOH/H_2O$, 0 °C then RT, 2 h, **3b** (50%); c) DAST, pyridine, $CHCl_3$, RT, 24 h, **4a** (50%), **4b** (67%).



Scheme 4. a) TFA, CH_2Cl_2, RT, 1 h, **5a** (94%), **5b** (76%); b) TBDMSCl, DMAP, pyridine, RT, overnight, **6a** (36%), **6b** (22%), c) BzCl, pyridine, RT, overnight, **7a** (90%), **7b** (quant.); d) TFA/H_2O 1:1, THF, RT, 1 h, **8a** (90%), **8b** (81%).

were prepared from tetrazole-activated deoxycytidine phosphoramidite Ac-dC-PCNE (Scheme 5) and nucleosides **8a** and **8b**, respectively. After oxidation of the phosphite intermediate and removal of the dimethoxytrityl group by treatment with trichloroacetic acid, **9a** and **9b** were obtained in 72 and 70% yields, respectively. Phosphorylation of **9a** and **9b** with bis-(2-cyanoethyl)diisopropylphosphoramidite gave **10a** and **10b** in 80 and 87% yields, respectively. The final removal of acetyl, benzoyl (Bz) and cyanoethyl groups was performed with methylamine, to afford **11a** and **11b** in 34 and 46% yields, respectively. Dinucleotides **11a** and **11b** were purified by RP-HPLC and fully characterised by ^1H, ^{13}C, ^{19}F and ^{31}P NMR spectroscopy and mass spectrometry analysis.

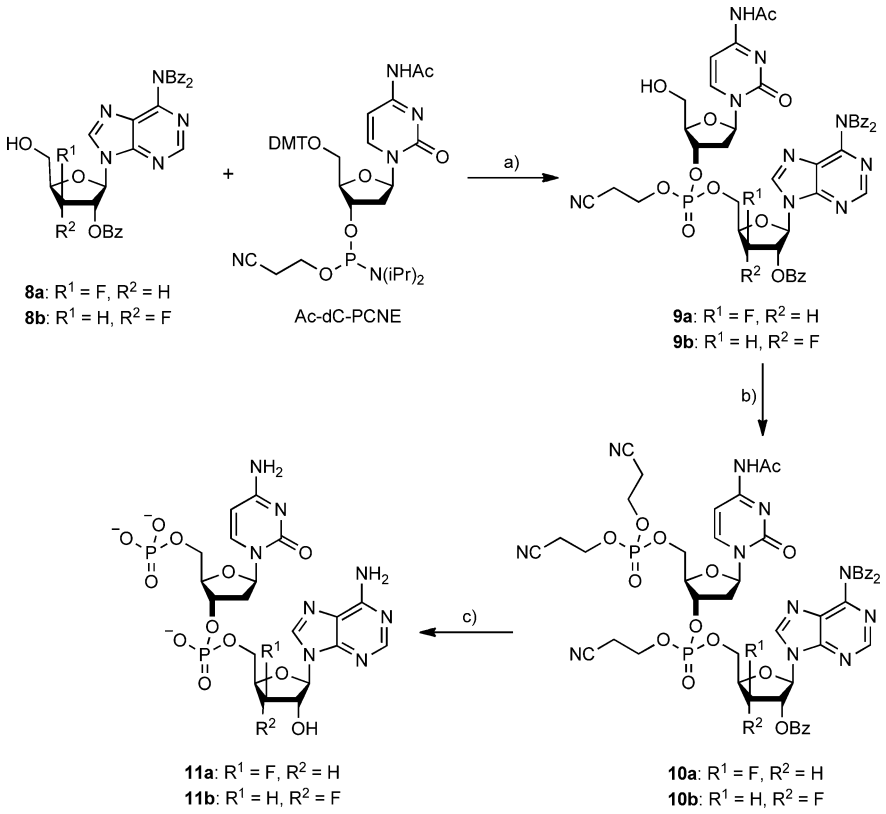
Conformation study

Nucleosides and their analogues are known to exist in equilibrium between S- and N-type conformations, based on furanose ring puckering. Factors that influence the preferred conformations of furanose rings in nucleosides and nucleotides are the anomeric effect (AE, O4′-C1-N1/9 stereoelectronic effect), the *gauche* effect (GE) and the steric effects of the bases.[18] In 3′-substituted 3′-deoxyadenosine systems, it has been shown that the proportions of S- or N-type conformers are mostly determined by the *gauche* effect of (X3′-C3′-C4′-04′), provided that X3′ is an electron-withdrawing group, as is the case for our fluorinated compounds (Scheme 6). In the 3′-*xylo* configuration (6A), the *gauche* and anomeric effects act in cooperation to favour the N-type conformer. For the 3′-*ribo* configuration (6B), the *gauche* effect prevails over the anomeric effect, and the sugar preferentially adopts the S-type conformation[18,19] (Scheme 6).

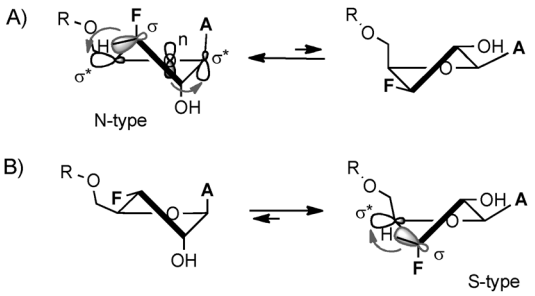
Several equations have been proposed in efforts to deduce the relative abundances of S- and N-type conformations from ^1H NMR spectra.[12c] Here we have used the "10-Hertz rule-of-thumb" (% S-type = 10 $J_{1',2'}$) applied to the $J_{1',2'}$ constant in ^1H NMR spectra collected in CD_3OD (Table 1). The NMR spectra show, as expected, that 3′-deoxy-3′-fluoroadenosine with fluorine in the *xylo* configuration (compound **5a**) has a preference for the N type conformation, whereas its counterpart with fluorine in the *ribo* configuration (compound **5b**, Table 1) prefers the S type. More precisely, we observed 20 and 65% of S-type conformer for nucleosides **5a** and **5b**, respectively, and 22 and

Table 1. $J_{1',2'}$ coupling constants and *S* values (in CD_3OD) of 3′-deoxy-3′-fluoroadenosine derivatives.

Compounds	$J_{1',2'}$ [Hz]	*S* [%]
xylo-F-adenosine **5a**	2.0	20
xylo-F-pdCpA **11a**	2.2	22
xylo-F-Ala-pdCpA **12a**	2.4	24
ribo-F-adenosine **5b**	6.5	65
ribo-F-pdCpA **11b**	8.0	80



Scheme 5. a) i: Tetrazole, CH_2Cl_2, RT, 1 h; ii: I_2, H_2O/Pyr/THF, RT, 30 min; iii: TCA, CH_2Cl_2, RT, 30 min, **9a** (72%), **9b** (70%); b) i: bis(2-cyanoethyl)diisopropylphosphoramidite, tetrazole, CH_2Cl_2, RT, 1 h; ii: I_2, H_2O/Pyr/THF, RT, 30 min, **10a** (80%), **10b** (87%); c) CH_3NH_2, EtOH/H_2O, RT, 24 h, **11a** (34%), **11b** (46%).



Scheme 6. Conformation equilibrium of A) *xylo*-, and B) *ribo*-3′-fluoroadenosine. The arrows between C3′ and C4′ (A and B) show the *gauche* effect (GE), which involves interaction between the best donor ($\sigma_{C3'-H3'}$) and the best acceptor ($\sigma^*_{C4'-O4'}$) orbitals. The arrow between O and C1′ (A) indicates the interaction between the $\sigma^*_{C1'-N9}$ orbital and the lone electron pair of O4′, which contributes to the anomeric effect (AE). The N-type conformer is stabilised by the GE and by the AE in (A). The S-type conformer is stabilised by the GE in (B).

80% of S-type for the corresponding dinucleotides **11a** and **11b**, respectively. These values are similar to those reported in the literature for 3′-fluoro analogues of adenosine.[12c, 19a, b] These results show that the proportions of the conformers were not affected by conversion of nucleosides into the corresponding dinucleotides.

The first set of NMR spectra was acquired in CD_3OD (Table 1). Because aqueous solutions are relevant to biological activities, we have also evaluated the influence of the solvent on the conformation equilibrium.

^{1}H NMR spectra of dinucleotide **11a** were acquired in three solvents (Figure 1): $J_{1',2'}$ values of 2.7, 2.5 and 2.2 Hz were found in water, DMSO and methanol, respectively. Thus, a decrease in the polarity of the solvent resulted in a marginal decrease in the $J_{1',2'}$ coupling constant. This result confirms that the *gauche* effect is the main factor that drives the equilibrium, because this effect is not influenced by the polarity of the solvent, unlike the anomeric effect, which involves dipole–dipole interaction in addition to orbital interaction. This result also suggests that hydrogen bonding is not involved in the stabilisation of the N-type conformer.[18]

Aminoacylation

Chemical aminoacylation of the fluorinated dinucleotide **11a** with L-Ala was performed as previously described.[14] Before the coupling reaction, the ammonium salt **11a** was converted into the tetrabutylammonium salt to enhance its solubility. The tetrabutylammonium salt of **11a**, dissolved in DMF, reacted with the activated pentenoyl-cyanomethyl ester of L-alanine to give **12a** in 10% yield (Scheme 7). The pentenoyl group is easily cleavable under conditions that did not affect the tRNA

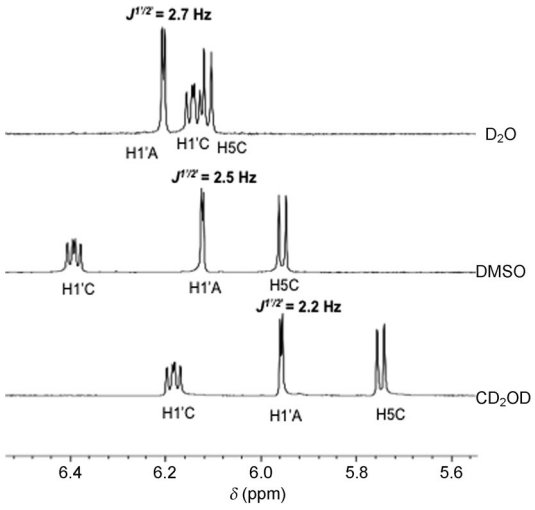
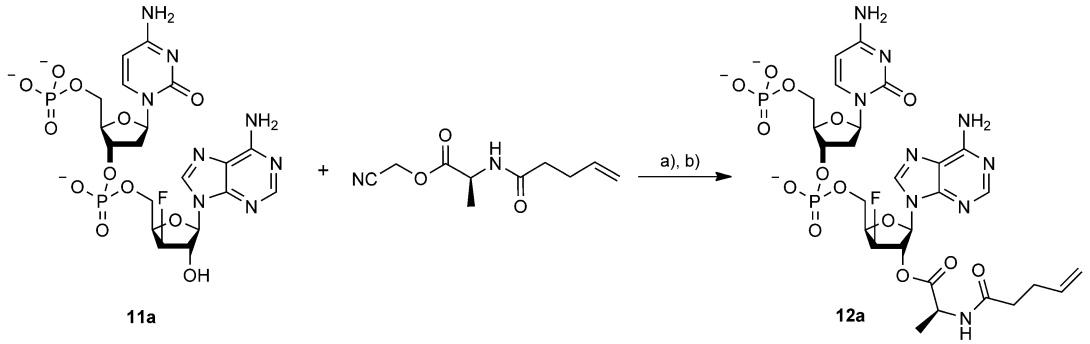


Figure 1. Selected areas of the ^{1}H NMR spectra of compound **11a** in D_2O, DMSO and CD_3OD.



Scheme 7. a) DOWEX NBu_4^+; b) DMF, RT, 24 h, **12a** (10% over two steps).

moiety. The fluoro-Ala-dinucleotide analogue was characterised by ^1H, ^{19}F and ^{31}P NMR spectroscopy and by mass spectrometry. The coupling constant $J_{1',2'}$ determined by ^1H NMR was 2.4 Hz (Table 1), thus indicating that acylation at 2′-OH hardly affected the ribose conformation.

Enzymatic ligation

The fluoro-tRNA^{Ala} analogues were obtained by enzymatic ligation of dinucleotides **11a**, **11b** and **12a** with a 22-nt RNA molecule that mimics the acceptor arm of the tRNA^{Ala} (Figure 2). The ligation was performed in HEPES buffer containing the 22-nt RNA, compound **11a**, **11b** or **12a**, T4 RNA ligase, ATP and $MgCl_2$. After ligation, the Ala-tRNA^{Ala} analogues were purified by anion-exchange chromatography and analysed by denaturing PAGE (Figure 2). The ligation reaction was efficiently catalysed by T4 RNA ligase, thus indicating that this enzyme tolerates fluorination of the terminal adenosine moiety.

Conclusion

We report synthetic routes to 3′-fluorinated dinucleotides with the *xylo* and *ribo* configurations, as well as to the acylated form of the *xylo* isomer. These dinucleotides were efficiently ligated to synthetic RNA molecules, thus providing access to the corresponding fluoro analogues of tRNA^{Ala} and Ala-tRNA^{Ala}. These molecules are non-isomerisable analogues of the substrates of alanyl-tRNA synthetases and FemX transferases, respectively. We also analysed the conformations of the 3′-fluorinated sugar components and showed that the *xylo* and *ribo* stereoisomers preferentially adopt the N and S conformations, respectively. This was observed for the nucleosides, the dinucleotides and the acylated *xylo*-configured dinucleotide. The conformations were not affected by the polarity of the solvent. Our results indicate that the populations of the N and S conformations are likely to be similar for 3′-fluorinated tRNA^{Ala} and Ala-tRNA^{Ala} analogues. These compounds should provide new insight into substrate recognition by Fem transferases and Ala-tRNA synthetases.

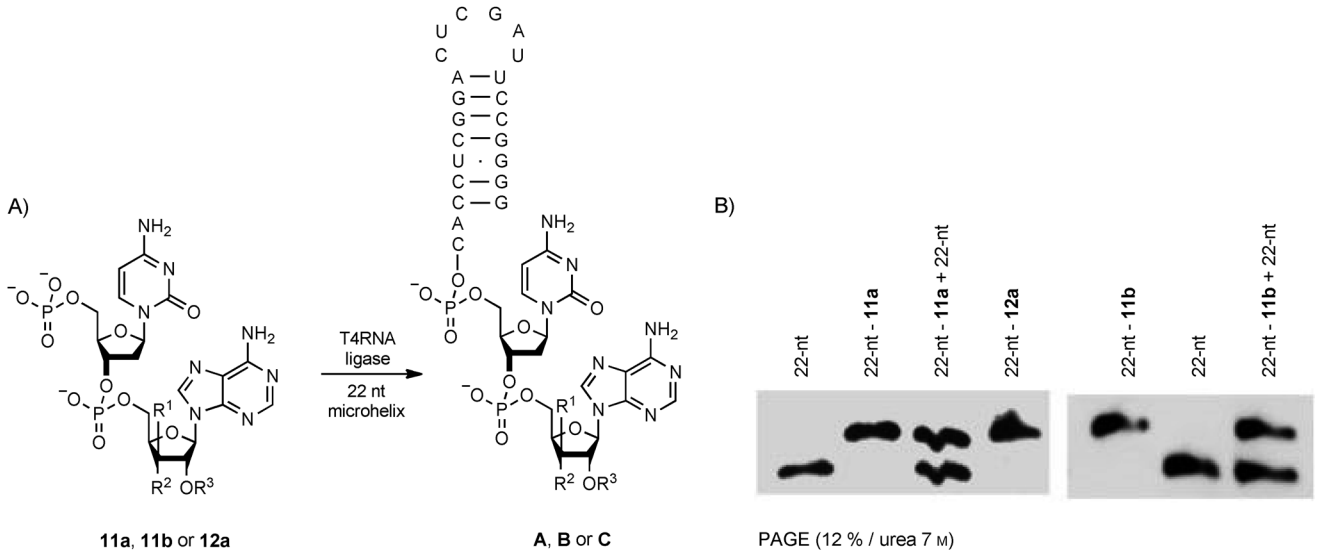


Figure 2. A) Ligation of **11a**, **11b** and **12a** to the 22-nt RNA by T4RNA ligase. B) PAGE analysis.

Experimental Section

General reagents and materials: Solvents were dried by standard methods and distilled before use. Unless otherwise specified, materials were purchased from commercial suppliers and used without further purification. TLC: precoated silica gel thin-layer sheets (60 F_{254}, Merck) and detection by charring with H_2SO_4 in ethanol (10%) followed by heating. Flash chromatography: silica gel 60 Å, 180–240 mesh from Merck. Spectra were recording with Bruker spectrometers (AM 250 or Advance II 500). Chemical shifts (δ) are expressed in ppm relative to residual $CHCl_3$ ($\delta = 7.26$ ppm) or CD_3OD ($\delta = 3.31$ ppm) for ^1H or relative to $CDCl_3$ ($\delta = 77.16$ ppm) or CD_3OD ($\delta = 049.00$ ppm) for ^{13}C as internal references. Signals were assigned by COSY and HSQC (^{13}C). HRMS was carried out with a Bruker micrOTOF spectrometer. HPLC was performed with a HPLC system including a reversed-phase C-18 column (250×21.2 mm) and use of a solvent system consisting of 50 mM aqueous CH_3CN/NH_4OAc (linear gradient from 0:100 to 63:37 in 40 min) at a flow rate of 15 mL min^{-1} and UV detection at 254 nm.

Compound 1: A mixture of adenosine (2.67 g, 10.0 mmol), DMAP (1.00 g, 8.0 mmol) and TrCl (13.9 g, 50.0 mmol) in pyridine (125 mL) was heated at 80 °C for three days. The reaction mixture was quenched with EtOH, concentrated in vacuo and coevaporated with toluene. CH_2Cl_2 was added to the resulting orange foam, the mixture was washed with H_2O, and the combined aqueous layers were then re-extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo, and the crude residue was purified by chromatography with cyclohexane/EtOAc (9:1 then 8:2) as the eluent to yield **1** as a white foam (4.63 g, 46%). ^1H NMR (250 MHz, $CDCl_3$): $\delta = 2.86$ (d, $J = 4.3$ Hz, 1H; H3′), 3.00 (dd, $J = 10.5$, 3.2 Hz, 1H; H5′a), 3.26 (dd, $J = 10.5$, 3.5 Hz, 1H; H5′b), 4.03 (br s, 1H; H4′), 5.16 (dd, $J = 7.5$, 4.7 Hz, 1H; H2′), 6.32 (d, $J = 7.5$ Hz, 1H; H1′), 7.00–7.42 (m, 45H; H^{Trt}), 7.86 (s, 1H; H2 or H8), 7.89 ppm (s, 1H; H2 or H8); ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 63.9$ (C5′), 70.7 (C^{Trt}), 71.4 (C3′), 76.9 (C2′), 84.3 (C4′), 86.5 (C^{Trt}), 87.1 (C1′), 87.8 (C^{Trt}), 121.3 (C^{Ad}), 126.9 (C^{Trt}), 127.1 (C^{Trt}), 127.6 (C^{Trt}), 127.8 (C^{Trt}), 127.9 (C^{Trt}), 128.1 (C^{Trt}), 128.3 (C^{Trt}), 128.7 (C^{Trt}), 129.0 (C^{Trt}), 139.5 (C2 or C8), 143.2 (C^{Trt}), 143.5 (C^{Trt}), 145.1 (C^{Trt}), 149.3 (C^{Ad}), 152.3 (C^{Ad}), 154.1 ppm (C2 or C8); HRMS: *m/z* calcd for $C_{67}H_{56}N_5O_4$: 994.4332 $[M+H]^+$; found: 994.4306.

Compound 4a: Compound **1** (2.00 g, 2.0 mmol) was dissolved in $CHCl_3$ (100 mL), and pyridine (4 mL) was added to the solution. DAST (1.32 mL, 10.0 mmol) was then added, and the solution was stirred at room temperature for 24 h. The reaction mixture was quenched with aqueous $NaHCO_3$, and the two layers were separated. The organic layer was then washed with water, and the combined aqueous layers were re-extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, concentrated in vacuo and purified by chromatography with cyclohexane/EtOAc (9:1) as the eluent to afford **4a** as a white foam (1.00 g, 50%). ^1H NMR (250 MHz, $CDCl_3$): $\delta = 3.24$–3.31 (m, 1H; H5′a), 3.43–3.50 (m, 1H; H5′b), 3.77 (d, $J = 51.7$ Hz, 1H; H3′), 4.20 (dt, $J = 29.7$, 6.25 Hz, 1H; H4′), 4.38 (d, $J = 14.5$ Hz, 1H; H2′), 6.45 (s, 1H; H1′), 6.90 (s, 1H; NH), 7.15–7.35 (m, 45H; H^{Trt}), 7.62 (s, 1H; H2 or H8), 8.10 ppm (s, 1H; H2 or H8); ^{19}F NMR (471 MHz, $CDCl_3$): $\delta = -199.60$ ppm (s, 1F; F3′); HRMS: *m/z* calcd for $C_{67}H_{55}FN_5O_3$: 996.4278 $[M+H]^+$; found: 996.4258.

Compound 5a: Trifluoroacetic acid (645 μL, 8.45 mmol) was added at 0 °C to a solution of **4a** (64 mg, 0.65 mmol) in CH_2Cl_2 (6 mL). The reaction mixture was allowed to warm to RT and further stirred for 1 h. Water and CH_2Cl_2 were then added to the resulting solution. After filtration, the aqueous layer was lyophilised and purified by chromatography with CH_2Cl_2/MeOH (9:1) to afford **5a** as a white foam (165 mg, 94%). ^1H NMR (500 MHz, CD_3OD): $\delta = 3.90$–3.99 (m, 2H; H5′), 4.47 (m, 1H; H4′), 4.71 (dt, $J = 14.0$, 3.5 Hz, 1H; H2′), 5.10 (ddd, $J = 52.0$, 3.6, 3.0 Hz, 1H; H3′), 6.09 (d, $J = 2.0$ Hz, 1H; H1′), 8.15 (s, 1H; H2 or H8), 8.22 ppm (s, 1H; H2 or H8); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 60.0$ (d, $J = 10.7$ Hz; C5′), 79.7 (d, $J = 27.1$ Hz; C2′), 83.9 (d, $J = 19.8$ Hz; C4′), 91.4 (C1′), 96.7 (d, $J = 183.2$ Hz; C3′), 140.2 (C2 or C8), 140.3 (C^{Ad}), 150.2 (C^{Ad}), 153.8 (C2 or C8), 157.3 ppm (C^{Ad}); HRMS: *m/z* calcd for $C_{10}H_{13}FN_5O_3$: 270.1002 $[M+H]^+$; found: 270.0992.

Compound 6a: DMAP (40 mg, 0.33 mmol) and TBDMSCl (273 mg, 1.8 mmol) were successively added at 0 °C to a solution of **5a** (444 mg, 1.65 mmol) in pyridine (10 mL). The reaction mixture was stirred at room temperature overnight and coevaporated with toluene in vacuo. EtOAc was added, and the aqueous layer was extracted. The organic extract was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by chromatography with CH_2Cl_2/MeOH (96:4) as the eluent, yielding **6a** as a white foam (228 mg, 36%). ^1H NMR (250 MHz, CD_3OD): $\delta = 0.10$ (s, 6H; Me^{TBS}), 0.90 (s, 9H; tBu^{TBS}), 4.00–4.36 (m, 2H; H5′), 4.45 (m, 1H; H4′), 4.66 (d, $J = 13.5$ Hz, 1H; H2′), 5.05 (dq, $J = 51.2$, 1.2 Hz, 1H; H3′), 6.11 (d, $J = 1.2$ Hz, 1H; H1′), 8.11 (s, 1H; H2 or H8), 8.21 ppm (s, 1H; H2 or H8); ^{13}C NMR (63 MHz, CD_3OD): $\delta = -5.4$ (Me^{TBS}), −5.2 (Me^{TBS}), 19.1 (Cq^{TBS}), 26.3 (tBu^{TBS}), 61.1 (d, $J = 10.1$ Hz; C5′), 79.8 (d, $J = 27.0$ Hz; C2′), 84.0 (d, $J = 18.7$ Hz; C4′), 91.5 (C1′), 96.5 (d, $J = 184.2$ Hz; C3′), 120.0 (C^{Ad}), 140.2 (C2 or C8), 150.2 (C^{Ad}), 154.0 (C2 or C8), 157.2 ppm (C^{Ad}); ^{19}F NMR (471 MHz, CD_3OD): $\delta = -203.3$ ppm (s, 1F; F3′); HRMS: *m/z* calcd for $C_{16}H_{27}FN_5O_3Si$: 384.1867 $[M+H]^+$; found: 384.1852.

Compound 7a: BzCl (392 μL, 3.3 mmol) was added at 0 °C to a solution of **6a** (185 mg, 0.48 mmol) in pyridine (5 mL), and the reaction mixture was stirred at room temperature overnight. The resulting solution was coevaporated with toluene in vacuo. The residue was suspended in EtOAc, washed with brine and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the product was purified by chromatography with cyclohexane/EtOAc (8:2) as the eluent to give **7a** as a white foam (300 mg, 90%). ^1H NMR (250 MHz, $CDCl_3$): $\delta = 0.11$ (s, 6H; Me^{TBS}), 0.91 (s, 9H; tBu^{TBS}), 4.04 (d, $J = 6.7$ Hz, 2H; H5′), 4.53 (ddt, $J = 29.2$, 6.5, 2.5 Hz, 1H; H4′), 5.32 (dd, $J = 50.0$, 2.0 Hz, 1H; H3′), 5.87 (d, $J = 12.7$ Hz, 1H; H2′), 6.56 (s, 1H; H1′), 7.33–7.64 (m, 9H; H^{Bz}), 7.86 (d, $J = 7.2$ Hz, 4H; H^{Bz}), 8.05 (t, $J = 6.7$ Hz, 2H; H^{Bz}), 8.38 (s, 1H; H2 or H8), 8.65 ppm (s, 1H; H2 or H8); ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = -5.4$ (Me^{TBS}), −5.3 (Me^{TBS}), 18.4 (Cq^{TBS}), 25.9 (tBu^{TBS}), 59.6 (d, $J = 9.1$ Hz; C5′), 79.8 (d, $J = 31.6$ Hz; C2′), 83.3 (d, $J = 19.7$ Hz; C4′), 87.5 (C1′), 92.7 (d, $J = 185.4$ Hz; C3′), 127.1 (C^{Ar}), 128.2 (C^{Ar}), 128.5 (C^{Ar}), 128.8 (C^{Ar}), 129.6 (C^{Ar}), 130.1 (C^{Ar}), 130.2 (C^{Ar}), 133.1 (C^{Ar}), 133.6 (C^{Ar}), 134.1 (C^{Ar}), 134.3 (C^{Ar}), 143.0 (C2 or C8), 152.0 (C^{Ad}), 152.6 (C2 or C8), 152.7 (C^{Ar}), 164.7 (C^{Ar}), 170.9 (C=O), 172.3 ppm (C=O); ^{19}F NMR (471 MHz, $CDCl_3$): $\delta = -202.65$ ppm (s, 1F; F3′); HRMS: *m/z* calcd for $C_{37}H_{39}FN_5O_6Si$: 696.2653 $[M+H]^+$; found: 696.2630.

Compound 8a: TFA/H_2O (1:1, 1.32 mL, 17.5 mmol) was added at 0 °C to a solution of **7a** (240 mg, 0.35 mmol) in THF (5 mL). The reaction mixture was stirred at RT for 1 h and quenched with aqueous $NaHCO_3$. EtOAc was added, and the two layers were separated. The organic layer was then washed with brine, and the combined aqueous layers were re-extracted with EtOAc. The combined organic layers were dried over Na_2SO_4, and concentrated in vacuo. Purification by chromatography with cyclohexane/EtOAc (4:6) as the eluent afforded **8a** as a white foam (180 mg, 90%). ^1H NMR (250 MHz, $CDCl_3$): $\delta = 3.98$ (d, $J = 5.5$ Hz, 2H; H5′), 4.49 (dq, $J = 27.5$, 4.4 Hz, 1H; H4′), 5.32 (d, $J = 52.0$ Hz, 1H; H3′), 5.90 (d, $J = 13.5$ Hz,

1H; H2′), 6.46 (d, J=1.7 Hz, 1H; H1′), 7.30–7.36 (m, 4H; H^{Bz}), 7.42–7.49 (m, 4H; H^{Bz}), 7.58–7.63 (m, 1H; H^{Bz}), 7.85 (d, J=7.2 Hz, 4H; H^{Bz}), 8.02 (d, J=7.5 Hz, 2H; H^{Bz}), 8.36 (s, 1H; H2 or H8), 8.63 ppm (s, 1H; H2 or H8); ^{13}C NMR (63 MHz, $CDCl_3$): δ=59.3 (d, J=9.1 Hz; C5′), 78.5 (d, J=30.6 Hz; C2′), 82.6 (d, J=19.7 Hz; C4′), 87.1 (C1′), 92.9 (d, J=187.4 Hz; C3′), 127.2 (C^{Ar}), 128.0 (C^{Ar}), 128.7 (C^{Ar}), 128.8 (C^{Ar}), 129.4 (C^{Ar}), 130.0 (C^{Ar}), 133.0 (C^{Ar}), 133.9 (C^{Ar}), 134.1 (C^{Ar}), 143.2 (C2 or C8), 151.8 (C^{Ar}), 152.4 (C2 or C8), 152.6 (C^{Ar}), 164.6 (C^{Ar}), 172.3 ppm (C=O); ^{19}F NMR (471 MHz, $CDCl_3$): δ=−202.46 ppm (s, 1F; F3′); HRMS: m/z calcd for $C_{31}H_{25}FN_5O_6$: 582.1789 $[M+H]^+$; found: 582.1771.

Compound 9a: A tetrazole solution in CH_3CN (0.45 M, 1.3 mmol, 2.8 mL) and adenosine derivative **8a** (0.13 mmol, 75 mg) in CH_2Cl_2 (350 μL) were added to a solution of phosphoramidite Ac-dC-PCNE (0.32 mmol, 250 mg) in CH_2Cl_2 (350 μL). After this system had been stirred at RT for 1 h, an iodine solution (0.1 M) in THF/H_2O/pyridine (75:2:20, 3.2 mL) was added. After 30 min, the reaction mixture was diluted with EtOAc, washed with water, saturated $Na_2S_2O_3$ solution and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was then stirred with a TCA solution (3%) in CH_2Cl_2 (7.1 mL) at RT for 30 min. The reaction mixture was diluted with CH_2Cl_2, and the organic phase was washed with a saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated. The crude product was purified by flash column chromatography with CH_2Cl_2/MeOH (96:4) as the eluent to yield **9a** as a white foam (90 mg, 72%). ^{1}H NMR (250 MHz, $CDCl_3$): δ=2.13–2.21 (m, 3H; Me^{Ac}), 2.26–2.35 (m, 1H; $H2'^{Cyt}$), 2.16 (s, 3H; CH_2CN, $H2'^{Cyt}$), 3.75 (br s, 2H; $H5'^{Ad\ or\ Cyt}$), 4.19–4.29 (m, 3H; CH_2O, $H4'^{Cyt}$), 4.50–4.54 (m, 2H; $H5'^{Ad\ or\ Cyt}$), 4.63–4.83 (m, 1H; $H4'^{Ad}$), 5.21–5.25 (m, 1H; $H3'^{Cyt}$), 5.43 (dd, J=49.9, 3.4 Hz, 1H; $H3'^{Ad}$), 5.94 (d, J=14.0 Hz, 1H; $H2'^{Ad}$), 6.13–6.19 (m, 1H; $H1'^{Cyt}$), 6.54 (d, J=1.7 Hz, 1H; $H1'^{Ad}$), 7.29–7.39 (m, 5H; H^{Bz}, $H5^{Cyt}$), 7.42–7.48 (m, 4H; H^{Bz}), 7.61–7.71 (m, 1H; H^{Bz}), 7.82 (d, J=7.6 Hz, 4H; H^{Bz}), 8.00 (d, J=8.1 Hz, 2H; H^{Bz}), 8.18–8.22 (m, 1H; $H6^{Cyt}$), 8.39 (s, 1H; $H2^{Ad}$ or $H8^{Ad}$), 8.61 (s, 1H; $H2^{Ad}$ or $H8^{Ad}$), 9.84 ppm (br s, 1H; NH^{Ac}); ^{13}C NMR (63 MHz, $CDCl_3$): δ=19.7 (CH_2CN), 24.8 (Me^{Ac}), 39.9 ($C2'^{Cyt}$), 61.4 ($C5'^{Ad\ or\ Cyt}$), 62.7 (CH_2O), 65.5 ($C5'^{Ad\ or\ Cyt}$), 79.2 ($C3'^{Cyt}$), 79.6 (d, J=19.3 Hz; $C4'^{Ad}$), 80.16 (d, J=30.7 Hz; $C2'^{Ad}$), 86.5 ($C4'^{Cyt}$), 87.3 ($C1'^{Ad\ or\ Cyt}$), 87.4 ($C1'^{Ad\ or\ Cyt}$), 93.1 (d, J=187.3 Hz; $C3'^{Ad}$), 97.0, 127.1, 127.9, 128.7, 128.8, 129.4, 130.0, 133.2, 133.9, 134.2, 143.1 (C2 or $C8^{Ad}$), 145.2 ($C6^{Cyt}$), 151.8 ($C2^{Ad}$ or $C8^{Ad}$), 152.6, 152.7, 152.8, 155.5 (C=O), 162.7 (C=O), 164.7 (C=N), 171.6 (C=O), 172.4 ppm (C=O); HRMS: m/z calcd for $C_{45}H_{42}FN_9O_{13}P$: 966.2623 $[M+H]^+$; found: 966.2596.

Compound 10a: Bis(2-cyanoethyl)diisopropylphosphoramidite (124 mg, 0.45 mmol) was added to a solution of dinucleotide **9a** (176 mg, 0.18 mmol) in CH_2Cl_2 (3 mL), followed by a solution of tetrazole in CH_3CN (0.45 M, 4 mL, 1.8 mmol). The reaction mixture was stirred at RT for 3 h, and an iodine solution (0.1 M, 4.5 mL, 0.45 mmol) was added. After having been stirred at RT for 1 h, the mixture was diluted in EtOAc and washed successively with $Na_2S_2O_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to dryness. Purification by flash column chromatography with CH_2Cl_2/MeOH (96:4) as the eluent afforded the desired phosphorylated dinucleotide **10a** as a white foam (166 mg, 80%). ^{1}H NMR (500 MHz, $CDCl_3$): δ=2.20 (s, 3H; CH_3), 2.30–2.34 (m, 1H; $H2'^{Cyt}$), 2.73–2.78 (m, 6H; 3×CH_2CN), 2.83–2.88 (m, 1H; $H2'^{Cyt}$), 4.26–4.58 (m, 11H; 3×CH_2–O, $H4'^{Cyt}$, $H5'^{Cyt}$, $H5'^{Ad}$), 4.74–4.81 (m, 1H; $H4'^{Ad}$), 5.16–5.17 (m, 1H; $H3'^{Cyt}$), 5.46 (d, J=51.0 Hz, 1H; $H3'^{Ad}$), 5.95–6.01 (m, 1H; $H2'^{Ad}$), 6.15–6.19 (m, 1H; $H1'^{Cyt}$), 6.57 (s, 1H; $H1'^{Ad}$),7.34–7.41 (m, 5H; H^{Ar}), 7.45–7.50 (m, 4H; H^{Ar}), 7.61–7.64 (m, 1H; H^{Ar}), 7.83–7.84 (m, 4H; H^{Ar}), 7.95 (d, J=7.5, 2.0 Hz; H^{Ar}), 8.03–8.06 (m, 2H; H^{Ar}), 8.44 (s, 1H; $H2^{Ad}$ or $H8^{Ad}$), 8.64 (s, 1H; $H2^{Ad}$ or $H8^{Ad}$, diast), 8.78 ppm (s, 1H; NH); ^{19}F NMR (471 MHz, $CDCl_3$): δ=−201.54 ppm (s, 1F; F3′); ^{31}P NMR (101 MHz, $CDCl_3$): δ=−3.06 (s, 1P), −2.54 ppm (s, 1P); HRMS: m/z calcd for $C_{51}H_{49}FN_{11}O_{16}P_2$: 1152.2818 $[M+H]^+$; found: 1152.2823.

Compound 11a: Dinucleotide **10a** (166 mg, 0.14 mmol) was dissolved in a solution of $MeNH_2$ (5 M, EtOH/H_2O 1:1; 14 mL), and the reaction mixture was stirred at RT for 24 h. After concentration under reduced pressure, the residue was purified by HPLC. The appropriate fractions were collected and lyophilised, to give the phosphorylated ammonium salt **11a** as a white foam (34 mg, 34%). t_R=15 min; ^{1}H NMR (500 MHz, CD_3OD): δ=2.17–2.23 (m, 1H; $H2'^{Cyt}$), 2.51–2.54 (m, 1H; $H2'^{Cyt}$), 4.03 (m, 1H; $H5'^{Ad}$), 4.09–4.13 (m, 1H; $H5'^{Ad}$), 4.24–4.29 (m, 2H; $H5'^{Cyt}$), 4.34 (s, 1H; $H4'^{Cyt}$), 4.66 (m, 1H; $H2'^{Ad}$), 4.74 (d, J=13.7 Hz, 1H; $H4'^{Ad}$), $H3'^{Cyt}$ masked in residual H_2O peak of CD_3OD, 5.20 (dt, J=51.5, 1.3 Hz, 1H; $H3'^{Ad}$), 5.95 (d, J=7.9 Hz, 1H; $H5^{Cyt}$), 6.12 (s, 1H; $H1'^{Ad}$), 6.35–6.38 (m, 1H; $H1'^{Cyt}$), 8.05 (d, J=7.8 Hz, 1H; $H6^{Cyt}$), 8.21 (s, 1H; $H2^{Ad}$ or $H8^{Ad}$), 8.24 ppm (s, 1H; $H2^{Ad}$ or $H8^{Ad}$); ^{13}C NMR (125 MHz, CD_3OD): δ=40.7 ($C2'^{Cyt}$), 63.4 ($C5'^{Cyt}$), 66.0 ($C5'^{Cyt}$), 77.6 ($C3'^{Cyt}$), 79.7 (d, J=27.1 Hz; $C4'^{Ad}$), 82.4 (d, J=30.3 Hz; $C2'^{Ad}$), 86.7 ($C4'^{Cyt}$), 87.3 ($C1'^{Cyt}$), 91.2 ($C1'^{Ad}$), 96.5 ($C5^{Cyt}$), 96.6 (d, J=182.2 Hz; $C3'^{Ad}$), 120.0, 140.2 (C2 or $C8^{Ad}$), 142.9 ($C6^{Cyt}$), 150.4, 153.9 (C2 or $C8^{Ad}$), 157.3, 158.2, 167.6, 178.9 ppm ($C=O^{Cyt}$); ^{19}F NMR (471 MHz, CD_3OD): δ=−203.16 ppm (s, 1F; F3′); ^{31}P NMR (203 MHz, CD_3OD): δ=−0.94 (s, 1P), 1.28 ppm (s, 1P); HRMS: m/z calcd for $C_{19}H_{26}FN_8O_{12}P_2$: 639.1151 $[M+H]^+$; found: 639.1125.

Compound 12a: Dowex ion-exchange beads in tetrabutylammonium form were added to a solution of pdCpdA·3NH_4^+ salt **11a** in Millipore water (4 mL), and the resulting mixture was stirred at RT for 30 min. The mixture was filtered, and the aqueous solution was lyophilised to afford pdCpdA as a tetrabutylammonium salt. The residue was added to a solution of *N*-pent-4-enoyl-L-alanine cyanomethyl ester (24 mg, 0.11 mmol) in DMF (250 μL). The reaction mixture was stirred for 24 h at RT, diluted to a total volume of 900 μL with a 1:2 CH_3CN/NH_4OAc (50 mM) solution and purified on a semi-preparative C-18 reversed-phase column. After lyophilisation of the appropriate fractions, dinucleotide **12a** was obtained as a white foam (4 mg, 10%). t_R=18 min (CH_3CN/NH_4OAc 0:100→63:37 over 40 min); ^{1}H NMR (250 MHz, CD_3OD): δ=1.42 (d, J=7.4 Hz, 3H; Me), 2.15–2.24 (m, 1H; $H2'^{Cyt}$), 2.31 (s, 4H; 2×CH_2), 2.47–2.59 (m, 1H; $H2'^{Cyt}$), 4.06–4.12 (m, 2H; $H5'^{Ad\ or\ Cyt}$), 4.24–4.29 (m, 2H; $H5'^{Ad\ or\ Cyt}$), 4.34 (s, 1H; $H4'^{Cyt}$), 4.43–4.69 (m, 3H; $H2'^{Ad}$, $H4'^{Ad}$, CH_α), $H3'^{Cyt}$ and CH= masked in residual H_2O peak of CD_3OD, 5.40 (d, J=50.5 Hz, 1H; $H3'^{Ad}$), 5.77–5.85 (m, 2H; CH=), 5.96 (d, J=7.6 Hz, 1H; $H5^{Cyt}$), 6.27 (d, J=2.4 Hz, 1H; $H1'^{Ad}$), 6.36–6.41 (m, 1H; $H1'^{Cyt}$), 8.09 (d, J=7.5 Hz, 1H; $H6^{Cyt}$), 8.19 (s, 1H; $H2^{Ad}$ or $H8^{Ad}$), 8.30 ppm (s, 1H; $H2^{Ad}$ or $H8^{Ad}$); ^{19}F NMR (471 MHz, CD_3OD): δ=−203.18 ppm (s, 1F; F3′); ^{31}P NMR (203 MHz, CD_3OD): δ=−0.93 (s, 1P), 0.62 ppm (s, 1P); HRMS: m/z calcd for $C_{27}H_{37}FN_9O_{14}P_2$: 792.1941 $[M+H]^+$; found: 792.2001.

Compound 2b: A solution of Dess–Martin periodinane (4.86 g, 11.4 mmol) in CH_2Cl_2 (40 mL) was added at 0 °C to a solution of **1** (3.77 g, 3.8 mmol) in CH_2Cl_2 (40 mL), and the reaction mixture was stirred at room temperature for 4 h. The reaction mixture was then cooled to 0 °C and quenched with an aqueous mixture of $NaHCO_3$ and $Na_2S_2O_3$. The two layers were separated, and the organic layer was washed with aqueous $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated in vacuo. Purification by flash column chromatography with cyclohexane/EtOAc (8:2) as the eluent afforded **2b** as a white foam (3.60 g, 95%).^{1}H NMR (250 MHz, $CDCl_3$): δ=3.49–3.66 (m, 2H; H5′), 4.66 (br s, 1H; H4′),

5.71 (d, J=6.1 Hz, 1H; H2′), 6.12 (d, J=6.1 Hz, 1H; H1′), 7.31–7.34 (m, 8H; H^{Trt}), 7.40–7.44 (m, 8H; H^{Trt}), 7.49–7.57 (m, 15H; H^{Trt}), 7.61–7.65 (m, 15H; H^{Trt}), 7.99 ppm (s, 1H; H2 or H8); ^{13}C NMR (63 MHz, $CDCl_3$): δ=64.01 (C5′), 71.4 (C2′), 80.8 (C4′), 85.6 (C1′), 87.2 (C^{Trt}), 89.4 (C^{Trt}), 121.1, 127.0, 127.2, 127.3, 127.5, 127.9, 128.0, 128.6, 128.7, 128.8, 129.1, 138.9, 143.3, 145.1, 148.6, 152.0, 153.8, 208.1 ppm (C=O); HRMS: m/z calcd for $C_{67}H_{54}N_5O_4$: 992.4176 $[M+H]^+$; found: 992.4145.

Compound 3b: Compound **2b** (1.68 g, 1.69 mmol) was suspended in EtOH/H_2O (15:1, 70 mL), and the suspension was cooled to 0 °C. $NaBH_4$ (73.4 mg, 1.94 mmol) was added, and the mixture was allowed to warm to room temperature and stirred for 1 h 30. $NaHCO_3$ was then added, and the mixture was extracted with $CHCl_3$. The organic layer was washed with water, dried over Na_2SO_4 and concentrated in vacuo. Purification by flash column chromatography with cyclohexane/EtOAc (8:2) as the eluent gave **3b** as a white foam (837 mg, 50%). ^{1}H NMR (500 MHz, $CDCl_3$): δ=3.44–3.47 (m, 1H; H5′a), 3.53–3.57 (m, 1H; H5′b), 3.97 (dd, J=11.0, 3.4 Hz, 1H; H3′), 4.31 (quint, J=3.5 Hz, 1H; H4′), 4.57 (s, 1H; H2′), 5.45 (s, 1H; H1′), 6.97 (d, J=13.6 Hz, 2H; H^{Trt}), 7.12–7.14 (m, 4H; H^{Trt}), 7.16–7.17 (m, 4H; H^{Trt}), 7.20–7.24 (m, 13H; H^{Trt}), 7.26–7.27 (m, 5H; H^{Trt}), 7.31–7.33 (m, 6H; H^{Trt}), 7.35–7.37 (m, 6H; H^{Trt}), 7.39–7.41 (m, 6H; H^{Trt}), 7.76 ppm (s, 1H; H2 or H8); ^{13}C NMR (125 MHz, $CDCl_3$): δ=62.6 (C5′), 77.0 (C3′), 82.6 (C2′), 84.6 (C4′), 87.1 (C^{Trt}), 88.7 (C^{Trt}), 92.39 (C1′), 121.5, 126.9, 127.1, 127.7, 127.8, 128.0, 128.2, 128.8, 128.9, 129.0, 140.7, 143.8, 143.9, 144.8, 146.4, 151.0, 154.3 ppm; HRMS: m/z calcd for $C_{67}H_{56}N_5O_4$: 994.4332 $[M+H]^+$; found: 994.4306.

Compound 4b: Compound **3b** (1.37 g, 1.37 mmol) was dissolved in $CHCl_3$ (20 mL), and pyridine (33 mmol, 2.7 mL) was added to the solution. DAST (837 μL, 6.3 mmol) was then added at 0 °C, and the solution was stirred at room temperature for 24 h. The reaction mixture was quenched with aqueous $NaHCO_3$, and the two layers were separated. The organic layer was then washed with water, and the combined aqueous layers were re-extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, concentrated in vacuo and purified by chromatography with cyclohexane/EtOAc (8:2) as the eluent to afford **4b** as a white foam (927 mg, 67%). ^{1}H NMR (500 MHz, $CDCl_3$): δ=3.01–3.03 (m, 1H; H5′a), 3.29–3.32 (m, 1H; H5′b), 3.58 (dd, J=52.7, 3.7 Hz, 1H; H3′), 4.96 (dt, J=27.4, 4.0 Hz, 1H; H4′), 5.25 (dq, J=22.1, 4.0 Hz, 1H; H2′), 6.22 (d, J=8.1 Hz, 1H; H1′), 6.98–7.03 (m, 7H; H^{Trt}), 7.07–7.12 (m, 12H; H^{Trt}), 7.15–7.18 (m, 4H; H^{Trt}), 7.21–7.24 (m, 6H; H^{Trt}), 7.27–7.31 (m, 10H; H^{Trt}), 7.40–7.41 (m, 6H; H^{Trt}), 7.76 (s, 1H; H2 or H8), 7.81 ppm (s, 1H; H2 or H8); ^{13}C NMR (125 MHz, $CDCl_3$): δ=62.9 (d, J=10.8 Hz; C5′), 74.7 (d, J=16.2 Hz; C2′), 82.6 (d, J=23.5 Hz; C4′), 86.7 (C1′), 87.1 (C^{Trt}), 87.5 (C^{Trt}), 90.6 (d, J=184.1 Hz; C3′), 121.6, 126.9, 127.1, 127.3, 127.7, 127.8, 128.6, 129.0, 139.7, 143.3, 145.0, 149.0, 152.1, 153.9 ppm; ^{19}F NMR (471 MHz, $CDCl_3$): δ=−192.34 (s, 1F; F3′); HRMS: m/z calcd for $C_{67}H_{55}FN_5O_3$: 996.4289 $[M+H]^+$; found: 996.4262.

Compound 5b: Trifluoroacetic acid (1.15 mL, 14.9 mmol) was added at 0 °C to a solution of **4b** (1.15 g, 1.15 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was allowed to warm to RT and stirred for 1 h. Water and CH_2Cl_2 were then added to the resulting solution, the mixture was filtered, and the combined aqueous layers were lyophilised and purified by chromatography with CH_2Cl_2/MeOH (9:1) to afford **5b** as a white foam (235 mg, 76%). ^{1}H NMR (500 MHz, CD_3OD): δ=3.87–3.78 (m, 2H; H5′), 4.43 (d, J=2.81 Hz, 1H; H4′), H2′ masked in residual H_2O peak of MeOD, 5.13 (dd, J=55.7, 3.5 Hz, 1H; H3′), 6.01 (d, J=6.5 Hz, 1H; H1′), 8.17 (s, 1H; H2 or H8), 8.28 ppm (s, 1H; H2 or H8); ^{13}C NMR (125 MHz, CD_3OD): δ=63.0 (d, J=11.8 Hz; C5′), 74.5 (d, J=15.8 Hz; C4′), 86.3 (d, J=21.7 Hz; C2′), 90.0 (C1′), 94.5 (d, J=182.9 Hz; C3′), 142.1 (C2 or C8), 150.0 (C^{Ad}), 153.4 (C2 or C8), 157.6 (C^{Ad}), 163.4 ppm (C^{Ad}); ^{19}F NMR (471 MHz, CD_3OD): δ=−199.90 ppm (s, 1F; F3′); HRMS: m/z calcd for $C_{10}H_{13}FN_5O_3$: 270.1002 $[M+H]^+$; found: 270.0992.

Compound 6b: Compound **5b** (227 mg, 0.84 mmol) in pyridine (5 mL) was cooled to 0 °C, and DMAP (20 mg, 0.17 mmol) and TBDMSCl (140 mg, 0.92 mmol) were successively added. The reaction mixture was stirred overnight at RT and then concentrated in vacuo and coevaporated with toluene. EtOAc was added, and the aqueous layer was extracted. The combined organic extracts were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by chromatography with CH_2Cl_2/MeOH (96:4) as the eluent, yielding **6b** as a white foam (71 mg, 22%). ^{1}H NMR (250 MHz, CD_3OD): δ=0.12 (s, 6H; Me^{TBS}), 0.93 (s, 9H; tBu^{TBS}), 3.92 (d, J=3.4 Hz, 2H; H5′), 4.41 (dq, J=25.2 Hz, 2.1 Hz, 1H; H4′), 4.91–4.95 (m, 1H; H2′), 5.10 (dd, J=54.3 Hz, 4.3 Hz, 1H; H3′), 6.10 (d, J=7.2 Hz, 1H; H1′), 8.24 (s, 1H; H2 or H8), 8.31 ppm (s, 1H; H2 or H8); ^{13}C NMR (63 MHz, CD_3OD): δ=−5.4 (Me^{TBS}), 19.2 (Cq^{TBS}), 26.3 (tBu^{TBS}), 64.0 (d, J=11.7 Hz; C5′), 75.3 (d, J=19.5 Hz; C2′), 85.2 (d, J=23.3 Hz; C4′), 88.3 (C1′), 93.6 (d, J=181.8 Hz; C3′), 120.3 (Cq), 141.0 (C2 or C8), 150.9 (Cq), 152.5 (C2 or C8), 156.4 ppm (Cq); HRMS: m/z calcd for $C_{16}H_{27}FN_5O_3Si$: 384.1867 $[M+H]^+$; found: 384.1848.

Compound 7b: BzCl (150 μL, 1.29 mmol) was added at 0 °C to a solution of **6b** (71 mg, 0.18 mmol) in pyridine (2 mL), and the reaction mixture was stirred at room temperature overnight. The resulting solution was coevaporated with toluene in vacuo. The residue was suspended in EtOAc, washed with brine and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the product was purified by chromatography with cyclohexane/EtOAc (8:2) as the eluent to give **7b** (129 mg, quant.). ^{1}H NMR (500 MHz, $CDCl_3$): δ=0.15 (s, 3H; Me^{TBS}), 0.16 (s, 3H; Me^{TBS}), 0.95 (s, 9H; tBu^{TBS}), 3.92–4.01 (m, 2H; H5′), 4.57 (d, J=25.7 Hz, 1H; H4′), 5.50 (dd, J=54.1, 4.8 Hz, 1H; H3′), 5.90 (dq, J=21.0, 4.1 Hz, 1H; H2′), 6.66 (d, J=7.7 Hz, 1H; H1′), 7.26–7.34 (m, 4H; H^{Bz}), 7.40–7.47 (m, 4H; H^{Bz}), 7.53–7.57 (m, 1H; H^{Bz}), 7.85–7.87 (m, 4H; H^{Bz}), 8.02–8.04 (m, 2H; H^{Bz}), 8.48 (s, 1H; H2 or H8), 8.67 ppm (s, 1H; H2 or H8); ^{13}C NMR (125 MHz, $CDCl_3$): δ=−5.4 (Me^{TBS}), −5.3 (Me^{TBS}), 18.4 (Cq^{TBS}), 26.0 (tBu^{TBS}), 62.9 (d, J=10.8 Hz; C5′), 75.2 (d, J=16.0 Hz; C2′), 84.7 (d, J=23.5 Hz; C4′), 85.0 (C1′), 90.53 (d, J=188.8 Hz; C3′), 128.4, 128.5, 128.57, 129.5, 130.1, 133.0, 133.8, 134.1, 143.0, 151.9, 152.5, 152.2, 165.3, 170.8, 172.2 ppm; HRMS: m/z calcd for $C_{37}H_{39}FN_5O_6Si$: 696.2653 $[M+H]^+$; found: 696.2633.

Compound 8b: Compound **7b** (129 mg, 0.18 mmol) in THF (5 mL) was cooled to 0 °C, and TFA/H_2O (1:1, 709 μL, 9.25 mmol) was added. The reaction mixture was stirred at RT for 1 h, and then the reaction was quenched with aqueous $NaHCO_3$. EtOAc was added, and the two layers were separated. The organic layer was then washed with brine, and the combined aqueous layers were re-extracted with EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. Purification by chromatography with cyclohexane/EtOAc (4:6) as the eluent afforded **8b** as a white foam (86 mg, 81%). ^{1}H NMR (250 MHz, $CDCl_3$): δ=3.80–3.89 (m, 1H; H5′a), 3.98–4.03 (m, 1H; H5′b), 4.62 (d, J=30.5 Hz, 1H; H4′), 5.57 (dd, J=54.4, 4.5 Hz, 1H; H3′), 5.68 (br s, 1H; OH), 6.08 (dq, J=23.1, 4.3 Hz, 1H; H2′), 6.33 (d, J=7.8 Hz, 1H; H1′), 7.32–7.40 (m, 4H; H^{Bz}), 7.43–7.51 (m, 4H; H^{Bz}), 7.53–7.62 (m, 1H; H^{Bz}), 7.83–7.86 (m, 4H; H^{Bz}), 7.96–8.00 (m, 2H; H^{Bz}), 8.20 (s, 1H; H2 or H8), 8.65 ppm (s, 1H; H2 or H8); ^{13}C NMR (63 MHz, $CDCl_3$): δ=62.3 (d, J=11.2 Hz; C5′), 74.1 (d, J=14.9 Hz; C2′), 86.5 (d, J=21.9 Hz; C4′), 89.9 (C1′), 91.4 (d, J=187.9 Hz; C3′), 128.3, 128.6, 128.8, 128.9,

129.5, 129.9, 133.2, 133.8, 133.9, 144.4 (C2 or C8), 151.8, 152.0, 152.9 (C2 or C8), 165.0 (C=O^{Bz}), 172.1 ppm (C=O^{Bz}); ^{19}F NMR (471 MHz, $CDCl_3$): $\delta = -197.60$ ppm (s, 1F; F3′); HRMS: *m/z* calcd for $C_{31}H_{25}FN_5O_6$: 582.1789 $[M+H]^+$; found: 582.1770.

Compound 9b: A tetrazole solution in CH_3CN (0.45 M, 1 mmol, 2.3 mL) and adenosine derivative **8b** (0.1 mmol, 60 mg) in CH_2Cl_2 (350 μL) were added to a solution of phosphoramidite Ac-dC-PCNE (0.32 mmol, 250 mg) in CH_2Cl_2 (350 μL). After the system had been stirred at RT for 1 h, an iodine solution (0.1 M) in THF/H_2O/pyridine (75:2:20, 2.6 mL) was added. After 30 min, the reaction mixture was diluted with EtOAc, washed with water, saturated $Na_2S_2O_3$ solution and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was then stirred with a solution of TCA (3%) in CH_2Cl_2 (5.7 mL) at RT for 30 min. The reaction mixture was diluted with CH_2Cl_2, and the organic phase was washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated. The crude product was purified by flash column chromatography with CH_2Cl_2/MeOH (96:4) as the eluent to yield **9b** as a white foam (70 mg, 70%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 2.16$ (s, 3H; CH_3), 2.29–2.41 (m, 1H; H2′^{Cyt}), 2.69–2.76 (m, 3H; CH_2CN, H2′^{Cyt}), 3.75 (s, 2H; H5′^{Cyt}), 4.19–4.27 (m, 3H; CH_2O, H4′^{Cyt}), 4.48 (br s, 2H; H5′^{Ad}), 4.68 (m, 1H; H4′^{Ad}), 5.15–5.19 (m, 2H; H3′^{Cyt}), 5.67 (m, 1H; H3′^{Ad}), 6.01–6.21 (m, 2H; H1′^{Cyt}, H2′^{Ad}), 6.53, 6.56 (d, $J = 7.2$ Hz, 1H; H1′^{Ad}, diast), 7.28–7.43 (m, 9H; H^{Ar}), 7.52–7.58 (m, 1H; H^{Ar}), 7.79 (d, $J = 7.4$ Hz, 4H; H^{Ar}), 7.94–7.97 (m, 2H; H^{Ar}), 8.17–8.22 (m, 1H; H^{Ar}), 8.52, 8.53 (s, 1H; H2^{Ad} or H8^{Ad}, diast), 8.64, 8.65 (s, 1H; H2^{Ad} or H8^{Ad}, diast), 8.82 ppm (s, 1H; NH); ^{19}F NMR (471 MHz, $CDCl_3$): $\delta = -200.69$ ppm (s, 1F; F3′); ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -2.94$ ppm (s, 1P); HRMS: *m/z* calcd for $C_{45}H_{42}FN_9O_{13}P$: 966.2623 $[M+H]^+$; found: 966.2615.

Compound 10b: Bis(2-cyanoethyl)diisopropylphosphoramidite (50 mg, 0.18 mmol) was added to a solution of dinucleotide **9b** (70 mg, 0.07 mmol) in CH_2Cl_2 (2 mL), followed by a solution of tetrazole in CH_3CN (0.45 M, 1.6 mL, 0.70 mmol). The reaction mixture was stirred at RT for 3 h, and an iodine solution (0.1 M) in THF/H_2O/pyridine (75:2:20, 1.8 mL) was added. After having been stirred at RT for 1 h, the mixture was diluted with EtOAc and washed successively with $Na_2S_2O_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to dryness. Purification by flash column chromatography with CH_2Cl_2/MeOH (96:4) as the eluent afforded the desired phosphorylated dinucleotide **10b** as a white foam (72 mg, 87%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 2.17$ (s, 3H; CH_3), 2.27–2.39 (m, 1H; H2′^{Cyt}), 2.71–2.75 (m, 7H; 3×CH_2CN, H2′^{Cyt}), 4.25–4.50 (m, 11H; 3×CH_2O, H4′^{Cyt}, H5′^{Cyt}, H5′^{Ad}), 4.70 (m, 1H; H4′^{Ad}), 5.16 (br s, 1H; H3′^{Cyt}), 5.70 (m, 1H; H3′^{Ad}), 6.03–6.21 (m, 2H; H1′^{Cyt}, H2′^{Ad}), 6.52–6.57 (m, 1H; H1′^{Ad}), 7.29–7.48 (m, 9H; H^{Ar}), 7.54–7.59 (m, 1H; H^{Ar}), 7.80 (d, $J = 7.3$ Hz, 4H; H^{Ar}), 7.93–8.00 (m, 3H; H^{Ar}), 8.51, 8.53 (s, 1H; H2^{Ad} or H8^{Ad}, diast), 8.65 (s, 1H; NH or H2^{Ad} or H8^{Ad}), 8.83 ppm (s, 1H; NH or H2^{Ad} or H8^{Ad}); ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -2.91$ (s, 1P), -2.51 ppm (s, 1P); HRMS: *m/z* calcd for $C_{51}H_{49}FN_{11}O_{16}P_2$: 1152.2818 $[M+H]^+$; found: 1152.2889.

Compound 11b: Dinucleotide **10b** was dissolved in a solution (5 M) of $MeNH_2$ (EtOH/H_2O 1:1; 3 mL), and the reaction mixture was stirred at RT for 24 h. After concentration under reduced pressure, the residue was purified by HPLC. The appropriate fractions were collected and lyophilised, to give the phosphorylated ammonium salt **11b** as a white foam (20 mg, 46%). $t_R = 15$ min (CH_3CN/NH_4OAc 0:100→63:37 over 40 min); ^{1}H NMR (250 MHz, CD_3OD): $\delta = 2.04$–2.08 (m, 1H; H2′^{Cyt}), 2.33–2.41 (m, 1H; H2′^{Cyt}), 4.06–4.12 (m, 4H; H5′^{Ad}, H5′^{Cyt}), 4.29 (s, 1H; H4′^{Cyt}), 4.52 (m, 1H; H2′^{Ad}), H4′^{Ad}, H3′^{Cyt} and H3′^{Ad} masked in residual H_2O peak of CD_3OD, 5.94 (d, $J = 7.5$ Hz, 1H; H5^{Cyt}), 6.15 (d, $J = 8.0$ Hz, 1H; H1′^{Ad}), 6.29–6.35 (m, 1H; H1′^{Cyt}), 7.97 (d, $J = 7.8$ Hz, 1H; H6^{Cyt}), 8.20 (s, 1H; H2^{Ad} or H8^{Ad}), 8.61 ppm (s, 1H; H2^{Ad} or H8^{Ad}); ^{13}C NMR (63 MHz, CD_3OD): $\delta = 40.5$ (C2′^{Cyt}), 66.0 (C5′^{Cyt} or C5′^{Ad}), 66.1 (C5′^{Cyt} or C5′^{Ad}), 74.9 (d, $J = 15.7$ Hz; C4′^{Ad}), 77.8 (C3′^{Cyt}), 84.2 (d, $J = 21.8$ Hz; C2′^{Ad}), 86.8 (C4′^{Cyt}), 87.2 (C1′^{Cyt}), 87.4 (C1′^{Ad}), 94.3 (d, $J = 176.6$ Hz; C3′^{Ad}), 96.6 (C5^{Cyt}), 119.8, 140.8 (C2^{Ad} or C8^{Ad}), 142.8 (C6^{Cyt}), 151.3 (C2^{Ad} or C8^{Ad}), 154.1, 158.1, 167.4, 178.2 ppm (C=O); ^{19}F NMR (471 MHz, CD_3OD): $\delta = -199.30$ ppm (s, 1F; F3′); ^{31}P NMR (101 MHz, CD_3OD): $\delta = -1.13$ (s, 1P), 0.94 ppm (s, 1P); HRMS: *m/z* calcd for $C_{19}H_{24}FN_8O_{12}P_2$: 637.0973 $[M-H]^+$; found: 637.0833.

Ligation of fluorinated dinucleotides to RNA helices: Dinucleotides **11a**, **11b** and **12a** were ligated to RNA^{helix} (5′-GGGGC CUUAG CUCAG GCUCC AC-3′), thus mimicking the acceptor arm of the tRNA^{Ala} with purified T4 RNA ligase.[7] RNA^{helix} was synthesised by the phosphoramidite method and purified by polyacrylamide gel electrophoresis (Eurogentec). The ligation reaction was performed at 16 °C for 12 h in HEPES buffer (50 mM, pH 7.5) containing RNA^{helix} (40 μM), dinucleotide (400 μM), T4 RNA ligase (0.6 mg mL^{-1}), DMSO (10%), ATP (1 mM) and $MgCl_2$ (15 mM). Compounds **A**, **B** and **C** were purified by anion-exchange chromatography (DEAE column, DNAPac-100, Dionex) with a linear gradient of ammonium acetate (pH 8.0, 25–2500 mM) containing acetonitrile (0.5%). Fractions containing the ligation product were identified by denaturing polyacrylamide gel electrophoresis, lyophilised, resuspended in RNAse-free water (Sigma) and stored at −20 °C.

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Keywords: aminoacyl-tRNAs · dinucleotides · fluoronucleosides · peptidoglycan · transferases · tRNA

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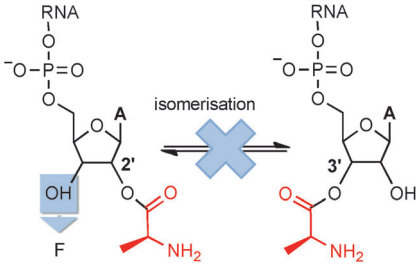
Fluorinated aminoacyl-tRNA analogues: Semisynthetic routes to fluoro analogues of $tRNA^{Ala}$ and Ala-$tRNA^{Ala}$ have been developed. These molecules are non-isomerisable analogues of the substrates of alanyl-tRNA synthetases and FemX transferases and should provide new insight into substrate recognition by these enzymes.



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Synthesis of 3′-Fluoro-tRNA Analogues for Exploring Non-ribosomal Peptide Synthesis in Bacteria