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Synthesis and biological evaluation of non-isomerizable analogues of Ala-tRNA^{Ala}

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Aminoacyl-tRNAs serve as amino acid donors in many reactions in addition to protein synthesis by the ribosome, including synthesis of the peptidoglycan network in the cell wall of bacterial pathogens. Synthesis of analogs of aminoacylated tRNAs is critical to further improve the mechanism of these reactions. Here we have described the synthesis of two non-isomerizable analogues of Ala-tRNA^{Ala} containing an amide bond instead of the isomerizable ester that connects the amino acid with the terminal adenosine in the natural substrate. The non-isomerizable 2′ and 3′ regioisomers were not used as substrates by FemX$_{Wv}$, an alanyl-transferase essential for peptidoglycan synthesis, but inhibited this enzyme with IC$_{50}$ of 5.8 and 5.5 μM, respectively.

Introduction

Aminoacyl-tRNAs (aa-tRNAs) participate in a vast repertoire of reactions, including synthesis of peptidoglycan precursors and tetrapyrrole, modification of bacterial membrane lipids, and N-terminal labeling of proteins targeted to proteolysis.[1] Aminoacyl-tRNAs also participate in secondary metabolism including synthesis of antibiotics[2] and cyclic dipeptides.[3] The corresponding tRNA-dependent aminoacyl-transferases have only recently been investigated revealing a high diversity in the catalytic mechanisms and protein structures. Recognition of aminoacyl-tRNAs by the transferases and by components of the translation machinery potentially involves different mechanisms relevant to enzyme specificity and competition between different pathways for the same aminoacyl-tRNA pools. This has stimulated interest in the chemical mutagenesis[4] of aminoacyl-tRNA to assess the role of specific groups in enzyme efficacy and specificity.

One important aspect of tRNA recognition by aminoacyl-transferases is the regiospecificity for the 2′ or 3′ position of the terminal adenine. In natural aminoacyl-tRNAs, the aminoacyl residue is linked by an ester bond to the 2′ or 3′ hydroxyls of the terminal adenosine of the tRNA (Fig. 1). An uncatalyzed transesterification reaction leads to isomerization between the O2′ and O3′ positions in aqueous solution with a thermodynamic equilibrium and a rate of 1 and 5 s^{-1}, respectively,[5] as evaluated by NMR analysis of acylated mononucleotides. Aminoacyl-tRNA synthetases, which charge the 20 natural amino acids, fall into two classes with respect to the hydroxyl used for aminoacylation.[6] The ribosome A site is specific for the 3′ isomer and the position of the acyl-group is conserved during peptide bond formation and translocation to the P site.[7] This implies that the aminoacyl residue migrates from the 2′ to the 3′ position in the case of aminoacyl-tRNA

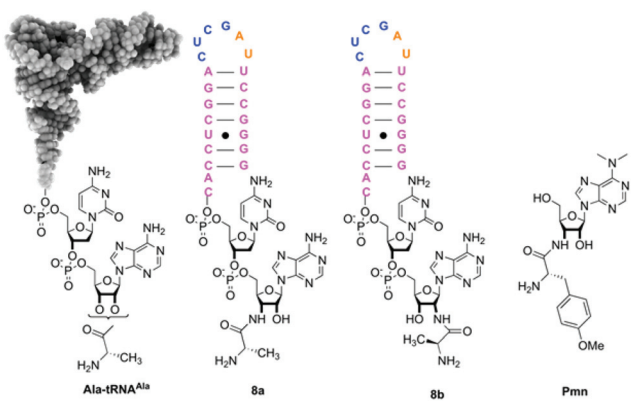

Fig. 1 Ala-tRNA^{ALa}: the natural substrate of FemXwv; **8a** and **8b**: Ala-tRNA^{Ala} analogues; Pmn: Puromycin.

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synthetases that acylate the 2′ OH. The presumably uncatalyzed trans-esterification reaction is thought to be kinetically limiting for protein synthesis despite the elevated rate reported for model molecules.[5] We have recently shown that trans-esterification is also required in the case of the aminoacyl transferase $FemX_{Wv}$, which adds an alanyl residue to the side chain of peptidoglycan precursors in the Gram-positive bacterium *Weissella viridescens*.[8] In this pathway, alanyl-tRNA synthetase acylates the 3′ position of $tRNA^{Ala}$ but $FemX_{Wv}$ transfers Ala only from the 2′ position.[8] Since $FemX_{Wv}$ displays similar affinities for Ala-$tRNA^{Ala}$ analogues in which Ala has been blocked at the 2′ or 3′ position by removing the adjacent hydroxyl, we have proposed that the enzyme binds the 3′ regioisomer formed by alanyl-tRNA synthetase. This implies that trans-esterification occurs in the catalytic site of $FemX_{Wv}$ to produce the 2′ isomer used in the aminoacyl transfer reaction. This would enable $FemX_{Wv}$ to efficiently compete with the ribosome for the 3′ isomer formed by alanyl-tRNA synthetase.

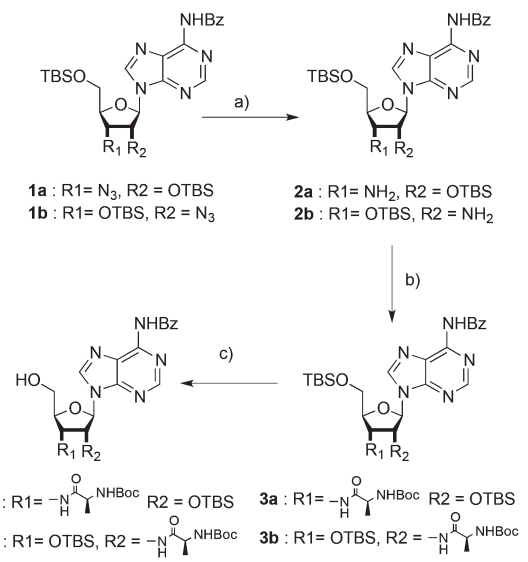
Two types of chemical modifications have been introduced into aa-tRNAs to block trans-esterification between the 2′ and 3′ positions. In a first approach, the adjacent hydroxyl is removed or replaced by a methoxy group.[9] This leads to the desired stable isomers but also introduces potentially confounding factors, due to modification of the furanose conformation of the terminal nucleotide. In addition, the vicinal hydroxyl may have a crucial role in substrate-assisted catalysis by participating in proton shuttle as proposed for peptide bond formation by the ribosome and by $FemX_{Wv}$.[8] In a second approach, the ester bond connecting the amino acid residue with the tRNA is substituted by a stable bond that prevents isomerization. Phosphate, phosphoramidate, triazole, and oxadiazole provide such stable analogues that retain an adjacent hydroxyl.[9,10] Phosphate and phosphoramidate have the potential advantage to mimic the tetrahedral transition state of aminoacyl-transfer reactions. Triazole or oxadiazole rings act as stable isosters of esters, both with respect to geometries and stereoelectronic properties.[10] However, these five-membered rings increase the distance between the ribose and the amino acid since they harbor an additional carbon in comparison to the ester of natural aa-tRNAs. Aminoacyl-tRNA analogues were also generated by replacement of the ester bond by an amide linkage[11] (Fig. 1). Puromycin has been the first analogue of aa-tRNA shown to act as a translation inhibitor by entering the ribosomal A site and participating in peptide bond formation with the nascent peptidyl chain, thereby blocking further polypeptide elongation.[12] Puromycin analogues have been developed in which the methyl groups of the nucleobase have been removed and the amino acid moiety has been modified.[13] Puromycin and its analogues have been broadly used to solve the X-ray structure of nucleoprotein complexes. This structural approach has been instrumental to mechanistic studies of the peptidyl transfer center of the ribosome,[14,9c] the editing domain of aa-tRNA synthetases (ThrRS,[15] LeuRS,[16] and IleRS),[17] and the catalytic cavity of L/F transferase.[18] Puromycin analogues have also been used to assess stereo- and regio-chemical constraints on peptide bond formation in the ribosome[19] and in the active site of GatCAB amido transferase.[20]

Two strategies for synthesis of RNA adducts containing an amide linkage at the 3′-terminus have been reported. The amide bond could be introduced directly into the full RNA or into the nucleoside, which is then linked to a solid support and provides the precursor for solid phase synthesis. For the first strategy, direct coupling of an activated amino acid to an 18-nt 3′-amino-RNA has been described in a single report.[11b] Efficient coupling was only obtained for formyl-methionine. For the second strategy, classical peptidic[21] or Staudinger coupling[21c] has been used to link activated amino acids to amino- or azido-deoxyadenosine, respectively. Here we report an alternative to solid phase synthesis that relies on enzymatic ligation. The amide linkage is introduced into the protected adenosine, followed by nucleotidic coupling to obtain the corresponding fully deprotected dinucleotide, which is enzymatically ligated to unprotected RNA. Two analogues of Ala-$tRNA^{Ala}$ **8** containing a 2′- or 3′-amide linkage were generated by this approach and evaluated as inhibitors or substrates of $FemX_{Wv}$, a model aminoacyl transferase that catalyzes an essential step of peptidoglycan synthesis (Fig. 1).

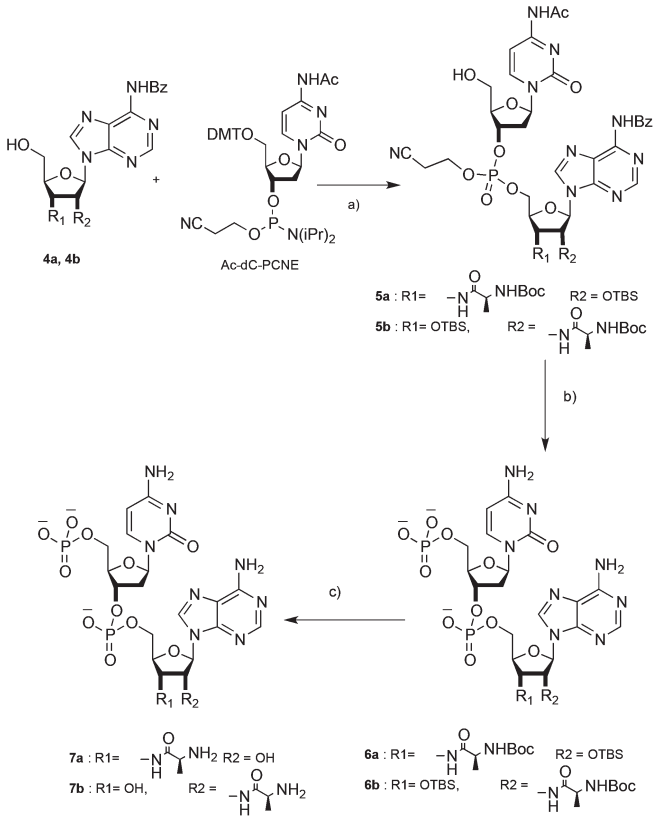
Results and discussion

Synthesis of nucleosides and dinucleotides

The synthesis (Scheme 1) started with the hydrogenolysis of 6-*N*-benzoyl-3′-azido-2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyadenosine **1a**[8] and 6-*N*-benzoyl-2′-azido-3′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyadenosine **1b**[8] to afford compounds **2a** and **2b** in quantitative yield. N-Boc-L-alanine was used as this protecting group is stable during phosphoramidite coupling and easily removed in the last step of dinucleotide synthesis. Coupling of N-Boc-L-Ala to protected amino-nucleosides **2a**



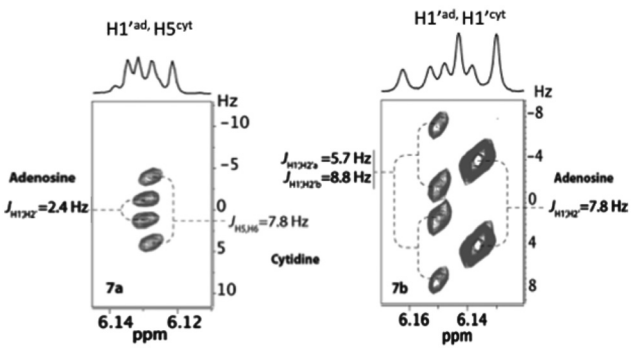
Scheme 1 (a) H_2, Lindlar's cat., MeOH, RT, 12 h; (b) L-AlaNHBoc, EDCI, DMAP, CH_2Cl_2, 0 °C to RT, 18 h; (c) TFA, THF–H_2O, 0 °C, 12 h.



Scheme 2 (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; (b) (i) bis(2-cyanoethyl)diisopropylphosphoramidite, tetrazole, CH_2Cl_2, RT, 1 h; (ii) I_2, H_2O/Pyr/THF, RT, 30 min; (iii) CH_3CN, EtOH/H_2O, RT, 24 h; (c) TBAF, THF then TFA/H_2O, RT, 15 min.

and **2b** was done by EDCI/DMAP-mediated amide bond formation in dichloromethane overnight at room temperature. The 3′-L-Ala-amido-3′-deoxyadenosine **3a** and the 2′-L-Ala-amido-2′-deoxyadenosine **3b** were obtained in 62% and 74% yields, respectively. Selective deprotection of the *tert*-butyldimethylsilyl group at position 5′ was performed in the presence of TFA to afford **4a** and **4b** in 64% and 78% yields, respectively.

Dinucleotides **7a** and **7b** were obtained by the phosphoramidite approach (Scheme 2). Nucleosides **4a** and **4b** were coupled to commercial deoxycytidine phosphoramidite (Ac-dC-CE-phosphoramidite) in the presence of tetrazole. The crude products were oxidized with iodine and treated with trichloroacetic acid to afford compounds **5a** and **5b** in 54% and 72% yields, respectively. Phosphorylation of **5a** and **5b** with bis(2-cyanoethyl)diisopropylphosphoramidite in tetrazole, followed by deprotection of cyanoethyl, acetyl, and benzoyl groups with methylamine afforded **6a** and **6b**, respectively. The N-Boc and TBS protecting groups were removed in **6a** and **6b** by treating with a solution of TBAF and then with a solution of TFA/H_2O for 15 min, producing **7a** and **7b**. The two deprotection steps were performed without purification of compounds **6a** and **6b**. Compounds **7a** and **7b** were obtained from **5a** and **5b** in 40% and 42% yields, respectively. COSY NMR experiments were performed to assign all protons of dinucleotides **7a** and **7b**. Carbons were assigned by HSQC and HMBC experiments.



B Compounds	$J^{1'-2'}$/Hz	S(%)
7a	2.4	24
7b	7.8	78
Pmn[23]	1.8	18

Fig. 2 (A) Magnification of J_{RES}-NMR spectra of **7a** and **7b**. (B) Coupling constants for adenosine H1′ and *S*% values in D_2O of **7a** and **7b**.

Conformational study

Nucleosides and their analogues are known to exist in equilibrium between S- and N-type conformations based on their furanose-ring puckering. Conformational modifications of the furanose ring produce major structural changes in natural nucleic acids and have important consequences for their biological functions. Altona and co-workers reported that ^1H–^1H coupling constants $J^{1'2'}$, $J^{2'3'}$and $J^{3'4'}$ vary with ribose conformation and several equations have been proposed to predict the relative abundance of S- and N-type conformations.[22] Here, we used the "10-Hertz rule-of-thumb" (% S-type = $10J^{1'-2'}$) to determine the pucker preference of compounds **7a** and **7b**. ^1H NMR was not sufficient to determine $J^{1'-2'}$ since the signal of the anomeric proton of adenosine (H1′) overlapped with other signals (H5 of the cytidine base for **7a** and H1′ of the cytosine for **7b**). The $J^{1'-2'}$ constant was successfully determined by 2D ^1H *J*-resolved (J_{RES}) NMR spectroscopy, which provided well-resolved signals for adenosine H1′ (Fig. 2). The deduced *S*% values in D_2O were 25 and 78 for **7a** and **7b**, respectively. The N-type conformation is also preponderant for puromycin, which is 3′-substituted (Fig. 3).[23]

Enzymatic ligation

Ala-tRNA^{Ala} analogues **8a** and **8b** were obtained by ligation of dinucleotides **7a** and **7b** to a 22-nt RNA helix, which mimics the acceptor arm of tRNA^{Ala} (helix^{Ala}: 5′-GGG GCC UUA GCU CAG GCU CCA C-3′) (Fig. 4). The ligation reaction was performed with T4 RNA ligase, as previously described.[24] Compounds **8a** and **8b** were purified by anion exchange chromatography and analyzed by denaturing PAGE.

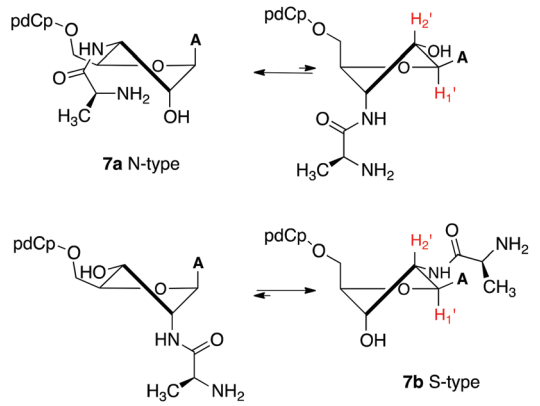


Fig. 3 Conformers of **7a** and **7b**.

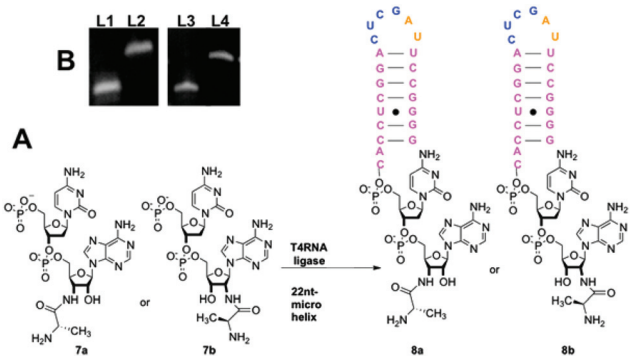


Fig. 4 (A) Ligation of dinucleotides to the RNA helix and (B) PAGE analysis. Lanes 1 and 3, helix^{Ala}; lane 2, **8a**; lane 4, **8b**.

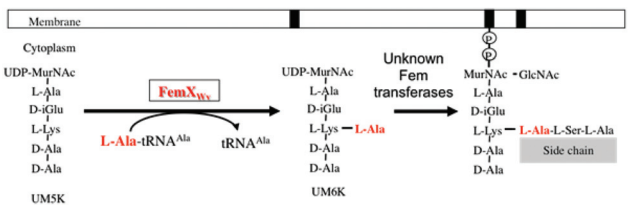


Fig. 5 Synthesis of peptidoglycan precursors in *W. viridescens*. GlcNAc: *N*-acetylglucosamine; MurNAc: *N*-acetylmuramic acid. The black box represents the undecaprenyl lipid carrier linked to the peptidoglycan precursor by a pyrophosphate.

Biological tests

Compounds **8a** and **8b** were evaluated as inhibitors and substrates of transferase FemX$_{Wv}$ from *W. viridescens*, which has been broadly used as a model enzyme for kinetic and structural analyses of transferases of the Fem family.[25] The enzyme catalyzes the transfer of L-Ala from Ala-tRNA^{Ala} to UDP-MurNAc-pentapeptide, thereby introducing the first residue into the L-Ala-L-Ser-L-Ala side-chain of peptidoglycan precursors (Fig. 5).

Compounds **8a** and **8b** inhibited FemX$_{Wv}$ with IC$_{50}$ of 5.8 ± 0.4 and 5.5 ± 0.6 μM, respectively (Fig. 6A), but these

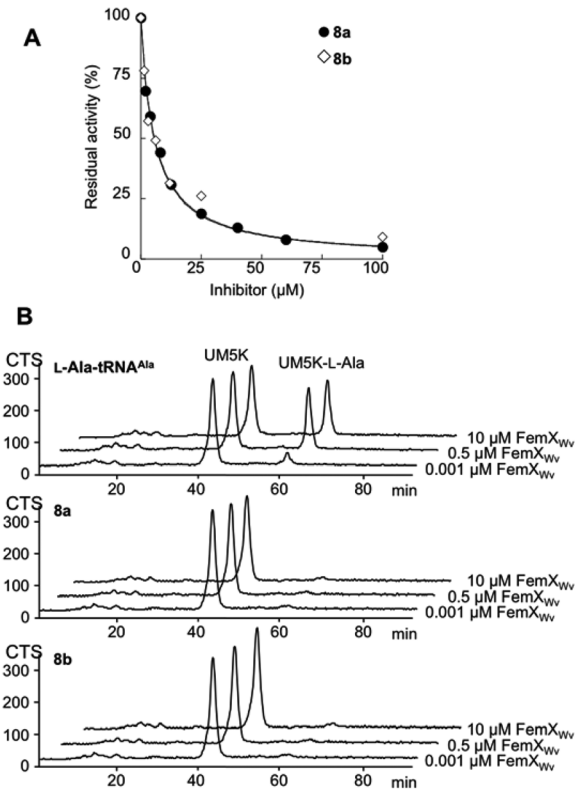


Fig. 6 (A) Inhibition of FemX$_{Wv}$ by **8a** and **8b**. The inhibition of the transfer of [^{14}C]L-Ala from [^{14}C]L-Ala-tRNA^{Ala} to UDP-MurNAc-pentapeptide was determined as previously described.[26] (B) Assay of **8a** and **8b** as substrates of FemX$_{Wv}$. The transfer of L-Ala from Ala-tRNA^{Ala} (1), **8a** (2), and **8b** (3) to [^{14}C]UDP-MurNAc-pentapeptide (UM5 K) was determined by rp-HPLC as previously described.[27]

compounds (up to 20 μM) were not used as substrates of FemX$_{Wv}$ (10 μM) (Fig. 6B). Under the same conditions, transfer of L-Ala from L-Ala-tRNA^{Ala} to UDP-MurNAc-pentapeptide was observed at lower substrate and enzyme concentrations (10 and 0.001 μM, respectively).

It has been previously shown that FemX$_{Wv}$ transfers L-Ala from the 2′-position of tRNA^{Ala}.[8] In this study, FemX$_{Wv}$ did not catalyze the transfer of L-Ala from 2′-modified **8b**, despite the relatively high affinity of the enzyme for this compound (IC$_{50}$ of 5.5 ± 0.6 μM). This result is consistent with the fact that puromycin and its analogues, which also contain an amide instead of an ester bond, block polypeptide elongation by the ribosome. Although amide bonds are considered chemically stable, several peptide-bond forming enzymes catalyze aminoacyl transfer both from amide- and ester-containing substrates. In peptidoglycan synthesis, this is the case of D,D-transpeptidases that cross-link peptidoglycan precursors ending in D-Ala and D-lactate in vancomycin-resistant enterococci.[26]

Both the 2′-modified **8b** and the 3′-modified **8a** inhibited FemX$_{Wv}$ with IC$_{50}$ that were similar to each other (5.5 ± 0.6 and 5.8 ± 0.4 μM, respectively) and also similar to the IC$_{50}$ previously reported for 3′- and 2′-triazole-substituted RNA analogues (2.4 ± 0.4 and 2.3 ± 0.1 μM, respectively).[8] These observations confirmed that FemX$_{Wv}$ does not interact with

the Ala moiety of the Ala-tRNA^{Ala} substrate allowing trans-acylation within the enzyme active site, as previously discussed.[27] The amide bond and the triazole linker had similar positive effects on recognition by FemX$_{Wv}$ in comparison to free tRNA^{Ala} (IC_{50} of 89 ± 4 μM[8]).

Conclusions

We have described the synthesis of two Ala-tRNA^{Ala} analogues which contained a stable amide bond instead of the hydrolysable and isomerizable ester linkage present in the natural substrate. The compounds were inhibitors but not substrates of aminoacyl-transferase FemX$_{Wv}$. H^1 NMR spectroscopy, performed on the dinucleotides **7a** and **7b**, indicated that the 3′- and 2′-substituted analogues show a preference for N-type and S-type conformations, respectively. The 3′-substituted ribose of **7a** and puromycin display similar preference for N-type conformation.[24] The semi-synthetic route reported here is expected to be applicable to various combinations of amino acid and RNA due to the promiscuous activity of T4 RNA ligase.[28]

Experimental part

General reagents and materials

Solvents were dried using 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 10% H_2SO_4 in ethanol followed by heating. Flash chromatography: silica gel 60 Å, 180–240 mesh from Merck. Spectra were recorded using Bruker spectrometers ARX 250 for ^1H (250.13 MHz) and ^{13}C (62.89 MHz), AC 400 for ^{31}P (161.97 MHz), Bruker Avance II 600 MHz for ^1H and ^{13}C in D_2O as indicated below. Chemicals shifts (δ) are expressed in ppm relative to residual $CHCl_3$ (δ 7.26) or CHD_2OD (δ 3.31) or HDO (δ 4.79) for ^1H, $CDCl_3$ (δ 77.16) or CD_3OD (δ 49.00) for ^{13}C as internal references and H_3PO_4 (δ 0) for ^{31}P as external references. Signals were assigned based on COSY and DEPT 135 (^{13}C). High-resolution mass spectroscopy (HRMS) was recorded using a Bruker micrOTOF spectrometer. High-performance liquid chromatography (HPLC) was performed on a HPLC system with a reverse phase C-18 column (250 mm × 21.2 mm; HYPERSIL HSC18; Thermoelectron Corporation) using a solvent system consisting of 50 mM aqueous NH_4OAc–CH_3CN (linear gradient from 100 : 0 to 50 : 50 in 50 min) at a flow rate of 17 mL min^{-1} and UV detection at 260 nm. Fast protein liquid chromatography (FPLC) was performed using an AKTA purifier (Amersham Pharmacia Biotech). Optical rotations were carried out using a PerkinElmer Model 341 Polarimeter.

General procedure for reduction of azido-nucleosides

A mixture of **1** (1.0 eq., 80 μmol) in methanol (4.2 mL) was subjected to hydrogenation (1 atm) in the presence of Lindlar palladium catalyst (10 mg) at room temperature for 12 hours. The reaction mixture was filtered through celite and the solvent was removed under vacuum.

3′-Amino-*N*^{6}-benzoyl-2′,5′-bis-*O*-(*tert*-butyl-dimethylsilyl)-3′-deoxyadenosine (2a). Compound **1a** (50 mg, 80 μmol) and Lindlar palladium catalyst (10 mg) were reacted under a hydrogen atmosphere according to the general procedure to give crude product **2a** (48 mg, quantitative yield) as a white foam which was used in the next step without further purification. Physical data were in agreement with published data.[29]

2′-Amino-*N*^{6}-benzoyl-3′,5′-bis-*O*-(*tert*-butyl-dimethylsilyl)-2′-deoxyadenosine (2b). Compound **1b** (50 mg, 80 μmol) and Lindlar palladium catalyst (10 mg) were reacted under a hydrogen atmosphere according to the general procedure to give crude product **2b** (46 mg, 96%) as a white foam which was used in the next step without further purification. R_f = 0.36 (AcOEt–Et_3N, 99 : 1); $[\alpha]_D^{25}$ = −54 (c = 1 in CH_2Cl_2); ^1H NMR (250 MHz, $CDCl_3$) δ = 8.73 (s, 1H, H2 or H8), 8.26 (s, 1H, H2 or H8), 7.99 (d, ^3J(H–H) = 5 Hz, 2H, H–Bz), 7.44 (m, 3H, H–Bz), 5.93 (d, ^3J(H–H) = 7.5 Hz, 1H, H1′), 4.30 (d, ^3J(H–H) = 7.5 Hz, 1H, H3′), 4.13 (m, 1H, H4′), 3.94 (m, 1H, H2′), 3.81 (dq, ^3J(H–H) = 2.5 Hz–15 Hz, 2H, H5′), 0.94 (s, 9H, tBu^{TBS}), 0.89 (s, 9H, tBu^{TBS}), 0.12 (2 s, 6H, 2 × Me^{TBS}), 0.01 (2 s, 6H, 2 × Me^{TBS}). ^{13}C NMR (63 MHz, $CDCl_3$) δ = 165.8 (2 × C═O–Bz), 152.74 (C2 or C8), 152.37 (Cq-Ad), 150.29 (Cq-Ad), 134.18 (Cq-Bz), 124.03 (Cq-Bz), 132.80, 128.39, 128.02 (C–Bz), 89.93 (C1′), 87.19 (C4′), 74.12 (C3′), 63.46 (C5′), 59.45 (C2′), 26.30 (C-tBu^{TBS}), 18.53 (Cq-tBu^{TBS}), −4.17, −4.37, −5.05, −5.15 (4 × Me^{TBS}).

General procedure for the coupling step

4-Dimethylaminopyridine (1.2 eq., 0.260 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.2 eq., 0.260 mmol) were added to a mixture of Boc-L-Ala (1.2 eq., 0.260 mmol) in anhydrous dichloromethane (1.0 mL) at 0 °C. After being stirred at 0 °C for 15 min., a solution of **2a–b** diluted in anhydrous dichloromethane (1.5 mL) was added to the mixture. After being stirred at room temperature for 18 hours, the solvent was removed under vacuum. The crude product was subjected to column chromatography and eluted with EtOAc–cyclohexane (5 : 5).

***N*^{6}-Benzoyl-2′,5′-bis-*O*-(*tert*-butyl-dimethylsilyl)-3′-(*S*)-methylcarbamate 3′-deoxyadenosine (3a).** Compound **2a** (0.13 g, 0.217 mmol) and Boc-L-Ala (49 mg, 0.260 mmol) were reacted according to the general procedure to give compound **3a** (104 mg, 62%) as a yellow oil. R_f = 0.39 (cyclohexane–EtOAc: 5/5); $[\alpha]_D^{20}$ = −35.5 (c = 1 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ = 9.33 (bs, 1H, NH-Ad), 8.77 (s, 1H, H2 or H8), 8.55 (s, 1H, H2 or H8), 8.01 (d, ^3J(H–H) = 9.0 Hz, 2H, H–Bz), 7.45–7.56 (m, 3H, H–Bz), 6.55–6.58 (bs, 1H, NH-amide), 6.12 (d, ^3J(H–H) = 4.0 Hz, 1H, H1′), 4.88 (d, ^3J(H–H) = 12 Hz, 1H, NH-Boc), 4.58–4.64 (m, 2H, H2′ et H4′), 3.84–4.15 (m, 4H, H3′, H5′ and CHα), 1.43 (s, 9H, tBu-Boc), 1.34–1.37 (d, ^3J(H–H) = 9.0 Hz, 3H, CH3α), 0.89–0.94 (m, 18H, H-tBu^{TBS}), 0.03–0.13 (m, 12H, H-Me^{TBS}); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 172.8 (C═O–Bz), 164.9 (C═O-amide), 155.2 (Cq-Ad), 152.8 (C2 or C8), 151.3

(C═O-Boc), 149.6 (Cq-Ad), 141.2 (C2 or C8), 133.9 (Cq-Bz), 132.7 (C–Bz), 128.9 (C–Bz), 128.0 (C–Bz), 123.0 (Cq), 89.6 (C1′), 84.5 (C3′), 80.5 (Cq-*t*Bu^{Boc}), 76.5 (C3′), 62.5 (C5′), 50.3 (C2′), 28.3 (C-*t*Bu^{Boc}), 26.1 (C-*t*Bu^{TBS}), 25.8 (C-*t*Bu^{TBS}), 18.7 (CH3), 18.6 (Cq-*t*Bu^{TBS}), 18.0 (Cq-*t*Bu^{TBS}), −4.7 (C-Me^{TBS}), −5.0 (C-Me^{TBS}), −5.3 (C-Me^{TBS}), −5.5 (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{37}H_{59}N_7O_7Si_2Na_1$: 792.3907, found: 792.3908 [M + Na$^+$].

***N*^{6}-Benzoyl-3′,5′-bis-*O*-(*tert*-butyl-dimethylsilyl)-2′-(*S*)-methylcarbamate 2′-deoxyadenosine (3b).** Compound **2b** (0.13 g, 0.217 mmol) and Boc-L-Ala (49 mg, 0.260 mmol) were reacted according to the general procedure to give compound **3b** (144 mg, 74%) as a white powder. R_f = 0.32 (cyclohexane–EtOAc: 7/3) $[\alpha]_D^{20}$ = −69 (*c* = 1 in CH_2Cl_2); ^{1}H NMR (250 MHz, $CDCl_3$) δ = 9.12 (ls, 1H, NHBz), 8.79 (s, 1H, H2 or H8), 8.02 (d, ^3J(H–H) = 7.5 Hz, 2H, H–Bz), 7.51 (m, 3H, H–Bz), 6.99 (m, 1H, NHCO), 6.14 (d, ^3J(H–H) = 7.5 Hz, 1H, H1′), 5.02 (m, 1H, H2′), 4.75 (m, 1H, NHBoc), 4.49 (d, ^3J(H–H) = 2.5 Hz, 1H, H3′), 4.18 (m, 1H, H4′), 4.10 (m, 1H, H^{Ala}), 3.85 (dq, ^3J(H–H) = 5 Hz–7.5 Hz, 2H, H5′), 1.42 (s, 9H, *t*Bu^{Boc}), 1.15 (d, ^3J(H–H) = 7.5 Hz, 3H, CH_3^{Ala}), 0.99, 0.92, (2 s, 18H, 2 × *t*Bu^{TBS}), 0.17 (2 s, 6H, 2 × Me^{TBS}), 0.10 (2 s, 6H, 2 × Me^{TBS}). ^{13}C NMR (63 MHz, $CDCl_3$) δ = 173.0 (C═O–Bz), 153.0 (C2 or C8), 141.86 (C2 or C8) 151.5, 149.9 (2 × Cq-Ad), 134.1 (Cq-Bz), 133.0 (C–Bz), 128.8 (C–Bz), 128.2 (C–Bz), 123.2 (Cq^{Ad}), 87.6 (C4′), 87.0 (C1′), 73.2 (C3′), 63.5 (C5′), 56.4 (C2′), 28.6 (*t*Bu^{Boc}), 26.3, 26.2 (2 × C-*t*Bu^{TBS}), 18.8, 18.4 (Cq-*t*Bu^{TBS}), 14.26 (C-CH_3^{Ala}) −4.1, −4.2, −5.0, −5.1 (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{37}H_{59}N_7O_7Si_2Na$: 792.3907; found: 792.7258 [M + Na$^+$].

General procedure for selective desilylation

To a stirred solution of **3** (1.0 eq., 0.126 mmol) in THF (2 mL) was added aqueous TFA (2 mL, TFA–H_2O: 1/1) at 0 °C. After stirring for 12 hours at 0 °C, the reaction mixture was neutralized with saturated aqueous $NaHCO_3$ and diluted with ethyl acetate (10 mL). After separation, the organic phase was washed with H_2O (10 mL) and brine (10 mL), dried over anhydrous Na_2SO_4 and evaporated at reduced pressure. The crude product was subjected to column chromatography and eluted with EtOAc–cyclohexane (5 : 5).

***N*^{6}-Benzoyl-2′-*O*-(*tert*-butyl-dimethylsilyl)-3′-(*N*-*tert*-butyloxycarbonyl-L-alanine)amido-3′-deoxyadenosine (4a).** Compound **3a** (0.10 g, 0.129 mmol) was reacted according to the general procedure to give compound **4a** (54 mg, 64%) as a white foam. R_f = 0.20 (EtOAc); $[\alpha]_D^{25}$ = −40.2 (*c* = 1 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.31 (bs, 1H, NH-Ad), 8.78 (s, 1H, H2 or H8), 8.38 (s, 1H, H2 or H8), 8.03 (d, ^3J(H–H) = 9.0 Hz, 2H, H–Bz), 7.49–7.52 (m, 3H, H–Bz), 6.80–6.81 (bs, 1H, NH-amide), 5.95 (d, ^3J(H–H) = 4.0 Hz, 1H, H1′), 4.84–4.87 (m, 3H, NH-Boc, H2′ and OH), 4.50 (m, 1H, H3′), 4.16–4.26 (m, 2H, H4′ and CHα), 3.85–4.03 (m, 2H, H5′), 1.48 (s, 9H, *t*Bu-Boc), 1.36 (d, ^3J(H–H) = 8.0 Hz, 3H, CH3α), 0.88 (m, 9H, H-*t*Bu^{TBS}), 0.01–0.04 (m, 6H, H-Me^{TBS}); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 173.9 (C═O–Bz), 164.8 (C═O-amide), 155.4 (Cq-Ad), 152.7 (C2 or C8), 150.8 (C═O-Boc), 150.1 (Cq-Ad), 142.2 (C2 or C8), 133.7 (Cq-Bz), 132.9 (C–Bz), 128.9 (C–Bz), 128.0 (C–Bz), 123.9 (Cq), 91.2 (C1′), 85.4 (C4′), 80.5 (Cq-*t*Bu^{Boc}), 74.9 (C2′), 61.9 (C5′), 51.0 (C3′ and CHα), 28.4 (C-*t*Bu^{Boc}), 25.8 (C-*t*Bu^{TBS}), 18.3 (CH3^{Ala}), 18.0 (Cq-*t*Bu^{TBS}), −4.8 (C-Me^{TBS}), −5.0 (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{31}H_{45}N_7O_7SiNa_1$: 678.3042, found: 678.3100 [M + Na$^+$].

***N*^{6}-Benzoyl-3′-*O*-(*tert*-butyl-dimethylsilyl)-2′-(*N*-*tert*-butyloxycarbonyl-L-alanine)amido-3′-deoxyadenosine (4b).** Compound **3b** (0.20 g, 0.260 mmol) was reacted according to the general procedure to give compound **4b** (133 mg, 78%) as a white foam. R_f = 0.22 (EtOAc); $[\alpha]_D^{20}$ = −83 (*c* = 1 in $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$) δ = 9.33 (ls, 1H, NHBz), 8.74 (s, 1H, H2 or H8), 8.01 (H2 or H8), 7.97 (d, ^3J(H–H) = 7.5 Hz, 2H, H–Bz), 7.49 (m, 3H, H–Bz), 6.78 (m, 1H, NHCO), 6.05 (m, 1H, OH), 5.84 (d, ^3J(H–H) = 7.5 Hz, 1H, H1′), 5.20 (m, 1H, H2′), 5.01 (m, 1H, NHBoc), 4.58 (d, ^3J(H–H) = 5 Hz, 1H, H3′), 4.13 (m, 1H, H4′), 4.05 (m, 1H, H^{Ala}), 3.85 (m, 2H, H5′), 1.42 (s, 9H, *t*Bu-Boc), 1.15 (d, ^3J(H–H) = 7.5 Hz, 3H, CH_3^{Ala}), 0.96 (s, 9H, *t*Bu^{TBS}), 0.15, 0.14 (2 s, 6H, 2 × Me^{TBS}); ^{13}C NMR (63 MHz, $CDCl_3$) δ = 165.8 (2 × C═O–Bz), 152.7 (C2 or C8), 152.3, 150.2 (2 × Cq^{Ad}), 134.1 (Cq^{Bz}), 132.8 (C^{Bz}), 128.3, 128.0 (C^{Bz}), 124.0 (Cq^{Bz}), 89.9 (C1′), 87.1 (C4′), 74.1 (C3′), 63.4 (C5′), 59.4 (C2′), 26.3 (*t*Bu^{TBS}),18.8 (Cq *t*Bu^{TBS}), 18.45 (CH_3^{Ala}), −4.17, −4,37 (2 × Me^{TBS}). HRMS (ESI$^+$): *m*/*z*: calcd for $C_{31}H_{45}N_7O_7SiNa$: 678.3042; found 678.3041 [M + Na$^+$].

General procedure for dinucleotides synthesis

Coupling reaction was directly carried out in a commercial Ac-dC-CE phosphoramidite vial (250 mg, 324 μmol) equipped with a flat magnetic stirrer under an argon atmosphere. The adenosine derivative (130 μmol) was added first followed by anhydrous CH_2Cl_2 (350 μL). A 0.45 M solution of tetrazole in CH_3CN (2.9 mL) was then added slowly to start the reaction. The mixture was stirred at room temperature for 1 hour (TLC monitoring) and a 0.1 M solution of I_2 (3.3 mL, H_2O/Pyr/THF 2 : 20 : 75) was added. The reaction was stirred at room temperature for 30 minutes, diluted with EtOAc and washed successively with aqueous saturated $Na_2S_2O_3$ solution and brine. The organic layer was dried over anhydrous Na_2SO_4, concentrated to dryness. A 0.18 M solution of trichloroacetic acid (7.2 mL) was finally added to the resulting residue and the mixture was stirred at room temperature for 30 minutes, diluted with CH_2Cl_2 and washed successively with aqueous ice-saturated $NaHCO_3$ solution and brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under vacuum. The crude product was purified by preparative TLC (CH_2Cl_2–MeOH 9 : 1) affording the desired compound as two diastereomers.

***tert*-Butyl (2*R*)-1-((2*S*,3*R*,4*R*,5*R*)-2-((((2*R*,3*S*,5*R*)-5-(4-acetamido-2-oxopyrimidin-1(2*H*)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yloxy)(2-cyanoethoxy)phosphoryloxy)methyl)-5-(6-benzamido-9*H*-purin-9-yl)-4-(*tert*-butyldimethylsilyloxy)tetrahydrofuran-3-ylamino)-1-oxopropan-2-ylcarbamate (5a).** According to the general procedure, adenosine derivative **4a** (84 mg, 129 μmol) was coupled to Ac-dC-PCNE-phosphoramide to give dinucleotide **5a** (74 mg, 54%) as two diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.63 (bs, 1H, NHBz), 8.74 (s, 1H, H2 or H8), 8.46 (s, 1H, H2 or H8), 8.26 (m, 1H, H6^{Cyt}), 8.08, 7.46 (m, 5H, H–Bz)

7.31 (m, 1H, $H5^{Cyt}$), 6.17 (m, 1H, $H1'^{Cyt}$), 6.07 (m, 1H, $H1'^{Ad}$) 5.20 (m, 1H, NHBoc), 4.83 (m, 2H, $H2'^{Ad}/H4'^{Ad}$), 4.63 (m, 1H, $H3'^{Ad}$), 4.16–4.37 (m, 6H, $H4'^{Cyt}/H\alpha^{Ala}/CH_2O/H5'^{Cyt}$), 3.63–3.83 (m, 3H, $H5'^{Ad}/H3'^{Cyt}$), 2.69–2.78 (m, 3H, $CH_2CN/H2'^{Cyt}$), 2.41 (m, 1H, $H2'^{Cyt}$), 2.10 (m, 3H, CH_3^{NHAc}), 1.33 (s, 9H, $t\mathrm{Bu}^{Boc}$), 1.24 (m, 3H, CH_3^{Ala}), 0.87 (s, 9H, $t\mathrm{Bu}^{TBS}$) 0.06–0.20 (2 s, 6H, $2 \times Me^{TBS}$); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 173.9, 173.7 ($C{=}O^{Boc}$), 171.3, 171.0 ($C{=}O^{Ac}$), 162.7 ($C{=}O^{Cyt}$), 155.7 ($C{=}O^{Bz}$), 152.8 (C2 or C8), 151.3 (Cq), 149.9 (Cq), 145.2 (C2 or C8), 133.1 (Cq^{Bz}), 132.9, 129.0, 128.9, 128.4, 128.3 (CH^{Bz}), 123.7 (Cq^{Ad}), 116.9 (CN), 96.9 ($C5^{Cyt}$), 87.7 ($C1'^{Ad}$), 86.6 ($C1'^{Cyt}$), 80.6 ($C4'^{Ad}$), 77.4 ($C4'^{Cyt}$), 75.5 ($C3'^{Ad}$), 66.3 ($C5'^{Cyt}$), 62.8 (CH_2O), 61.4 ($C5'^{Ad}$), 50.8 ($C2'^{Ad}$), 49.9 (CHα), 40.0 ($C2'^{Cyt}$), 28.5 ($t\mathrm{Bu}^{Boc}$), 25.9 ($t\mathrm{Bu}^{TBS}$), 25.0 (CH_3^{Ac}), 18.9 (CH_2^{CH2CN}), 18.5 (Cq $t\mathrm{Bu}^{TBS}$), 18.3 (CH_3^{Ala}), −4.5, −4.8 ($2 \times Me^{TBS}$); ^{31}P NMR (162 MHz, $CDCl_3$) −2.9; HRMS (ESI): *m*/*z*: calcd for $C_{45}H_{62}N_{11}NaO_{14}PSi$: 1062.3883, found: 1062.3516 [M + Na^+].

***tert*-Butyl (2*R*)-1-((2*R*,3*R*,4*S*,5*R*)-5-((((2*R*,3*S*,5*R*)-5-(4-acetamido-2-oxopyrimidin-1(2*H*)-yl)-2-(hydroxymethyl)tetrahydrofuran-3-yloxy)(2-cyanoethoxy)phosphoryloxy)methyl)-2-(6-benzamido-9*H*-purin-9-yl)-4-(*tert*-butyldimethylsilyloxy)tetrahydrofuran-3-ylamino)-1-oxopropan-2-ylcarbamate (5b).** According to the general procedure, adenosine derivative **4b** (84 mg, 129 μmol) was coupled to Ac-dC-PCNE-phosphoramide to give dinucleotide **5b** (92 mg, 72%) as two diastereomers. ^1H NMR (250 MHz, $CDCl_3$) δ = 8.62 (1 s, 1H, H2 or H8), 8.27 (1 s, 1H, H2 or H8), 8.15 (m, 1H, $H6^{Cyt}$), 7.91, 7.41 (m, 5H, H–Bz) 7.29 (m, 1H, $H5^{Cyt}$), 6.09 (m, 1H, $H1'^{Cyt}$), 6.03 (m, 1H, $H1'^{Ad}$) 5.86 (bs, 1H, OH), 4.94 (m, NHBoc), 4.83 (m, 1H, $H2'^{Ad}$), 4.63–4.61 (m, 1H, $H3'^{Ad}$), 4.28–4.26 (m, 2H, $H4'^{Ad}/H\alpha^{Ala}$) 4.16, 4.14 (m, 3H, $CH_2O/H3'^{Cyt}$), 4.01 (m, 2H, $H5'^{Cyt}$), 3.63 (m, 1H, $H4'^{Cyt}$), 3.51, 3.22 (m, 2H, $H5'^{Ad}$), 2.68 (m, 2H, CH_2CN), 2.66 (m, 1H, $H_2'^{Cyt}$), 2.15 (m, 1H, $H2'^{Cyt}$), 2.03 (s, 3H, CH_3^{NHAc}), 1.26 (s, 9H, $t\mathrm{Bu}^{Boc}$), 1.04 (d, ^3J(H–H) = 7.5 Hz, CH_3^{Ala}), 0.84 (s, 9H, $t\mathrm{Bu}^{TBS}$) 0.05, 0.03, (2 s, 6H, $2 \times Me^{TBS}$). ^{13}C NMR (63 MHz, $CDCl_3$) δ = 173.7 ($C{=}O^{Boc}$), 171.2 ($C{=}O^{Ac}$), 162.5 ($C{=}O^{Cyt}$), 155.9 ($C{=}O^{Bz}$)152.2 (C2 or C8), 151.4 (Cq), 149.8 (Cq), 144.7 (C2 or C8) 132.7 (Cq^{Bz}), 132.6 (C^{Bz}), 128.5 (C^{Bz}), 128.0 (C^{Bz}), 122.8 (Cq^{Ad}), 116.3 (CN), 97.0 ($C5^{Cyt}$), 87.1 ($C1'^{Ad}$), 86.8 ($C1'^{Cyt}$), 80.0 ($C4'^{Ad}$), 79.0 ($C4'^{Cyt}$), 71.2 ($C3'^{Ad}$), 67.3 ($C5'^{Cyt}$), 62.7 (CH_2O), 60.8 ($C5'^{Ad}$), 49.7 ($C2'^{Ad}$), 39.8 ($C2'^{Cyt}$), 27.9 ($t\mathrm{Bu}^{Boc}$), 24.2 ($t\mathrm{Bu}^{TBS}$), 24.0 (CH_3^{Ac}), 24.2 (CHα), 19.2 (CH_2^{CH2CN}), 17.7 (CH_3^{Ala}), −5.0 ($2 \times Me^{TBS}$). HRMS (ESI): *m*/*z*: calcd for $C_{45}H_{62}N_{11}NaO_{14}PSi$: 1062.3883, found: 1062.3646 [M + Na^+].

General procedure for dinucleotides phosphorylation

Bis(2-cyanoethyl)diisopropylphosphoramidite (5.0 eq.) was added neat into the flask containing the dinucleotide (1.0 eq.). Anhydrous CH_2Cl_2 (3.5 μL μmol^{-1}) was then added, followed by the 0.45 M solution of tetrazole in CH_3CN (20 eq.). The mixture was stirred at room temperature for 1 hour and a 0.1 M solution of I_2 (5.0 eq.) was added. After being stirred at room temperature for 30 minutes, the mixture was diluted with EtOAc and washed successively with aqueous saturated $Na_2S_2O_3$ solution and brine. The organic layer was dried over anhydrous Na_2SO_4, concentrated to dryness and then dissolved in a 5 M solution of $MeNH_2$ (large excess EtOH/H_2O 1 : 1). The reaction was stirred for 12 hours at room temperature and concentrated under reduced pressure. The residue was purified by HPLC. After the appropriate fractions were collected and lyophilized, the phosphorylated product was obtained as a NH_4^+ salt.

***tert*-Butyl (2*S*)-1-((2*S*,3*R*,4*R*,5*R*)-2-((((2*R*,3*S*,5*R*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-(phosphonooxymethyl)tetrahydrofuran-3-yloxy)(hydroxy)phosphoryloxy)methyl)-5-(6-amino-9*H*-purin-9-yl)-4-(*tert*-butyldimethylsilyloxy)tetrahydrofuran-3-ylamino)-1-oxopropan-2-ylcarbamate (6a).** According to the general procedure, dinucleotide **5a** (72 mg, 69.2 μmol) was reacted with bis(2-cyanoethyl)diisopropylphosphoramidite (94 mg, 346 μmol) in the presence of tetrazole (3.08 mL, 1.38 mmol) and then oxidized with I_2 (3.46 mL, 346 μmol) and finally a 5 M solution of $MeNH_2$ (20 mL) to give after lyophilization the NH_4^+ salt phosphorylated product **6a** (64 mg) as a yellow solid. ^1H NMR (250 MHz, MeOD) δ = 8.53 (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 8.21 (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 7.90 (d, ^3J(H–H) = 7.5 Hz, 1H, $H6^{Cyt}$), 6.22 (m, 1H, $H1'^{Cyt}$), 6.07 (m, 1H, $H1'^{Ad}$), 5.90 (d, ^3J(H–H) = 7.5 Hz, 1H, $H5^{Cyt}$), 4.81 (m, 1H, $H2'^{Ad}$), 4.65 (m, 1H, $H3'^{Ad}$), 4.11–4.26 (m, 6H, $H3'^{Cyt}/H4'^{Cyt}/CH\alpha/H4'^{Cyt}/H5'^{Ad}$), 3.72 (m, 2H, $H5'^{Cyt}$), 2.50 (m, 1H, $H2'^{Cyt}$), 2.16 (m, 1H, $H2'^{Cyt}$), 1.44 (s, 9H, H-$t\mathrm{Bu}^{Boc}$), 1.33 (d, ^3J(H–H) = 7.5 Hz, CH_3), −0.04 (s, 3H, H-Me^{TBS}), −0.03 (s, 3H, H-Me^{TBS}); HPLC retention time: 7.87 min.

General procedure for N-Boc and O-TBS deprotection

The partially protected dinucleotides **6a–b** were treated first with a 1 M solution of TBAF in THF (0.45 eq., 0.152 mmol) and then with a solution of TFA (50% aq.) at room temperature for 15 min. The reaction mixture was then concentrated under vacuum, diluted with water and washed with CH_2Cl_2. The aqueous layer was evaporated under reduced pressure and the residue was purified by HPLC. After the appropriate fractions were collected and lyophilized, the final dinucleotide was obtained as a NH_4^+ salt.

((2*R*,3*S*,5*R*)-5-(4-Amino-2-oxopyrimidin-1(2*H*)-yl)-3-((((2*S*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3-((*R*)-2-aminopropanamido)-4-hydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryloxy)tetrahydrofuran-2-yl)methyl dihydrogen phosphate (7a). According to the general procedure, dinucleotide **6a** (64 mg, 9 μmol) was first reacted with a 1 M solution of TBAF in THF (152 μL) and then with a solution of TFA (50% aq., 3 mL) at room temperature for 15 minutes. After purification by HPLC, the final dinucleotide **7a** was obtained as a NH_4^+ salt (20 mg, 40% yield) as a white solid. ^1H NMR (600 MHz, D_2O) δ = 8.52 (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 8.33 (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 8.00 (d, ^3J(H–H) = 7.8 Hz, $H6^{Cyt}$), 6.13 (dd, ^3J(H1′–H2′) = 2.4 Hz, ^3J(H5–H6) = 7.8 Hz, 2H, $H1'^{Ad}/H5^{Cyt}$), 6.08 (m, 1H, $H1'^{Cyt}$), 4.70–4.80 (m, 3H, $H2'^{Ad}/H3'^{Ad}/H3'^{Cyt}$), 4.48 (m, 1H, $H4'^{Ad}$), 4.30 (m, 1H, $H4'^{Cyt}$), 4.22 (m, 2H, $H5'^{Ad}/H\alpha$), 3.98 (m, 3H, $H5'^{Cyt}/H5'^{Ad}$), 2.45 (m, 1H, $H2'^{Cyt}$), 2.06 (m, 1H, $H2'^{Cyt}$), 1.55 (d, ^3J(H–H) = 7.2 Hz, 3H, CH_3^{Ala}); ^{13}C NMR (151 MHz, $CDCl_3$) δ = 171.2 (C═O), 163.3 ($C{=}O^{Cyt}$), 160.1, 152.9, 152.7, 149.4, 149.1, (Cq), 148.4 (C2 or C8), 143.6 ($C6^{Cyt}$), 141.1 (C2 or C8), 95.3 ($C5^{Cyt}$), 89.4 ($C1'^{Ad}$), 85.2 ($C1'^{Cyt}$), 85.1 ($C4'^{Cyt}$), 80.1 ($C4'^{Ad}$), 76.1 ($C3'^{Cyt}$), 73.3

(C3′^{Ad}), 64.6 (C5′^{Ad}), 64.1 (C5′^{Cyt}), 50.8 (C2′^{Ad}), 49.1 (CHα), 38.6 (C2′^{Cyt}), 16.7 (CH_3^{Ala}); ^{31}P NMR (1H decoupled, 242 MHz, D_2O) −0.57, −0.51; HRMS (ESI): *m*/*z*: calcd for $C_{22}H_{31}N_{10}O_{13}P_2^-$: 705.1553, found: 705.1111 [M − H$^+$]; HPLC retention time: 9.26 min.

((2*R*,3*S*,5*R*)-5-(4-Amino-2-oxopyrimidin-1(2*H*)-yl)-3-((((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-4-((*R*)-2-aminopropanamido)-3-hydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryloxy)tetrahydrofuran-2-yl)methyldihydrogen phosphate (7b). According to the general procedure, dinucleotide **6b** (82 mg, 108.1 μmol) was first reacted with a 1 M solution of TBAF in THF (152 μL, 0.152 mmol) and then with a solution of TFA–H_2O (50% aq., 3 mL) at room temperature for 15 minutes. After purification by HPLC, the final dinucleotide **7b** was obtained as a NH_4^+ salt (34.4 mg, 42% yield) as a white solid. HPLC retention time: 15 min. ^{1}H NMR (600 MHz, D_2O) δ = 8.51 (s, 1H, H2^{Ad} or H8^{Ad}), 8.20 (s, 1H, H2^{Ad} or H8^{Ad}), 8.00 (s, 1H, NH), 7.74 (d, ^3J(H–H) = 3.3 Hz, 1H, H6^{Cyt}), 6.15 (dd, ^3J(H1′^{cyt}–H2′^{cyt} a) = 8.8 Hz, ^3J(H1′^{cyt}–H2′^{cyt} b) = 5.7 Hz, 1H, H1′^{Cyt}), 6.13 (d, ^3J(H1′^{Ad}–H2′^{Ad}) = 7,8 Hz, 1H, H1′^{Ad}), 6.02 (d, ^3J(H–H) = 7.2 Hz, 1H, H5^{Cyt}), 5.11 (m, 1H, H2′^{Ad}), 4.77–4.75 (m, 1H, H3′^{Cyt}), 4.65 (m, 1H, H3′^{Ad}), 4.41–4.38 (s, 1H, H4′^{Ad}), 4.28 (s, 1H, H4′^{Cyt}), 4.15–4.11 (m, 2H, H5′^{Ad}), 4.07–3.98 (m, 2H, Hα/H5′^{Cyt}), 2.36 (m, 1H, H2′^{Cyt}), 1.82 (m, 1H, H2′^{Cyt}), 1.53 (d, ^3J(H–H) = 6.6 Hz, 3H, CH_3^{Ala}). ^{13}C NMR (600 MHz, $CDCl_3$) δ = 180.4 (C═O), δ = 171 (C═O), 164.8 (C═O^{cyt}), 155.3 (C2 or C8), 152.8 (C2 or C8), 141.3 (C6^{Cyt}), 96.4 (C5^{Cyt}), 85.6 (C1′^{Cyt}), 85.1 (C1′^{Ad}), 84.8 (C4′^{Ad}), 84.7 (C4′^{Cyt}), 76.4 (C3′^{cyt}), 69.7 (C3′^{Ad}), 64.9 (C5′^{Ad}), 64.6 (C5′^{Cyt}), 55.5 (C2′^{Ad}), 48.9 (Cα), 38.0 (C2′^{Cyt}), 16.5 (CH_3^{Ala}). 31P NMR (1H decoupled, 162 MHz, $CDCl_3$): 1.26, 0.2 (2 s). ^{31}P NMR (1H decoupled, 162 MHz, D_2O) 1.24, 0.17 (2 s). HRMS (ESI): *m*/*z*: calcd for $C_{22}H_{33}N_{10}O_{13}P_2$: 706.1626, found: 705.1510 [M − H$^+$].

Ligation of modified dinucleotides to RNA helices

Modified dinucleotides **7a** and **7b** were ligated to RNA helices with purified T4 RNA ligase.[10a] Compounds **8a** and **8b** 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 0.5% acetonitrile. Fractions containing the ligation product were identified by denaturing polyacrylamide gel electrophoresis,[10a] lyophilized, resuspended in RNAse free water (Sigma), and stored at −20 °C. The concentration of the inhibitors was determined spectrophotometrically (ε = 2.26 × 10^5 M^{-1} cm^{-1} at 260 nm).

Inhibition of FemX$_{Wv}$ by 8a and 8b

The assay contained Tris–HCl (50 mM, pH 7.5), alanyl-tRNA synthetase of *E. faecalis* (800 nM), ATP (7.5 mM), $MgCl_2$ (12.5 mM), L-[^{14}C]Ala (50 μM, 3700 Bq nmol^{-1}; ICN, Orsay, France), FemX$_{Wv}$ (2 nM), UDP-MurNAc-pentapeptide (50 μM), tRNA^{Ala} (0.4 μM) and inhibitors (0 to 150 μM). The reaction was performed at 37 °C for 10 min with a preincubation of 2 min in the absence of FemX$_{Wv}$ for synthesis of Ala-tRNA^{Ala} by the auxiliary system. The reaction was stopped at 96 °C for 10 min and analysed by descending paper chromatography (Whatman 4MM, Elancourt) with isobutyric acid–ammonia, 1 M (5 : 3 per vol). Radioactive spots were identified by autoradiography, cut out, and counted by a liquid scintillation.

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