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Synthesis of Stable Aminoacyl-tRNA Analogues Containing Triazole as a Bioisoster of Esters

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Abstract: Aminoacyl-tRNAs have important roles in a variety of biological processes, including protein synthesis by ribosomes, targeting of proteins for degradation by the proteasome, and bacterial cell wall synthesis. Here we describe the synthesis of stable aminoacyl-tRNA analogues containing 1,4- and 1,5-substituted 1,2,3-triazole rings. The procedure involves i) Cu- and Ru-catalysed cycloadditions of 3′-azidoadenosine and alkynes, which produced the 1,4 and 1,5 regioisomers of the triazoles, respectively, ii) coupling between the resulting triazole-deoxyadenosine derivatives and a deoxycytidine phosphoramidite, and iii) the enzymatic ligation of the substituted dinucleotides with a 22 nt RNA microhelix that mimics the acceptor arm of tRNA. Nucleoside and nucleotide compounds were characterized by MS spectrometry and ^{1}H, ^{31}P and ^{13}C NMR spectroscopy and were assayed for inhibition of $FemX_{Wv}$, an alanyltransferase essential for the formation of the peptidoglycan network of Gram-positive bacterial pathogens. The low IC_{50} values obtained (2 to 4 μM) indicate that the five-membered triazole rings acted as bioisosters of esters and can be used for the design of stable aminoacyl-tRNA analogues.

Keywords: aminoacyl-tRNA · click chemistry · dinucleotides · inhibitors · triazoles

Introduction

Peptidoglycan is an essential component of the bacterial cell envelope because it provides mechanical protection against the osmotic pressure of the cytoplasm. The peptidoglycan subunit (Figure 1) consists of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) substituted with a conserved stem pentapeptide.[1] In most Gram-positive bacteria the subunit contains an additional side chain linked to the third position of the stem pentapeptide. The side chains display considerable interspecies sequence diversity, consisting, for example, of five glycines in *Staphylococcus aureus* and of the sequence L-Ser-L-Ala or L-Ala-L-Ala in *Streptococcus pneumoniae*.[2] The side chains are assembled by transferases of the Fem family that have the particularity of using aminoacyl-tRNAs as substrates.[3] These enzymes have a pivotal role in peptidoglycan synthesis because the side chains supply the branching points to cross-link peptides from adjacent glycan chains, an essential reaction catalysed by the D,D-transpeptidase catalytic domain of penicillin-binding proteins (PBPs).[4] For this reason, Fem transferases are considered attractive targets for the development of novel antibiotics active against multiply resistant bacteria.[5]

Recently, we have developed the synthesis of novel aminoacyl-tRNA analogues for inhibition of $FemX_{Wv}$,[6] the prototypic enzyme of the Fem family. The inhibitor **A** (Scheme 1) contains a 1,2,4-oxadiazole ring as a mimic of the natural 3′-aminoacyl ester **B** (Figure 2). Oxadiazoles, including 1,2,4-oxadiazoles, are stable analogues of esters,[7] due to their geometries and electronic properties.[8] The oxadiazole-containing $FemX_{Wv}$ inhibitor differs from previously

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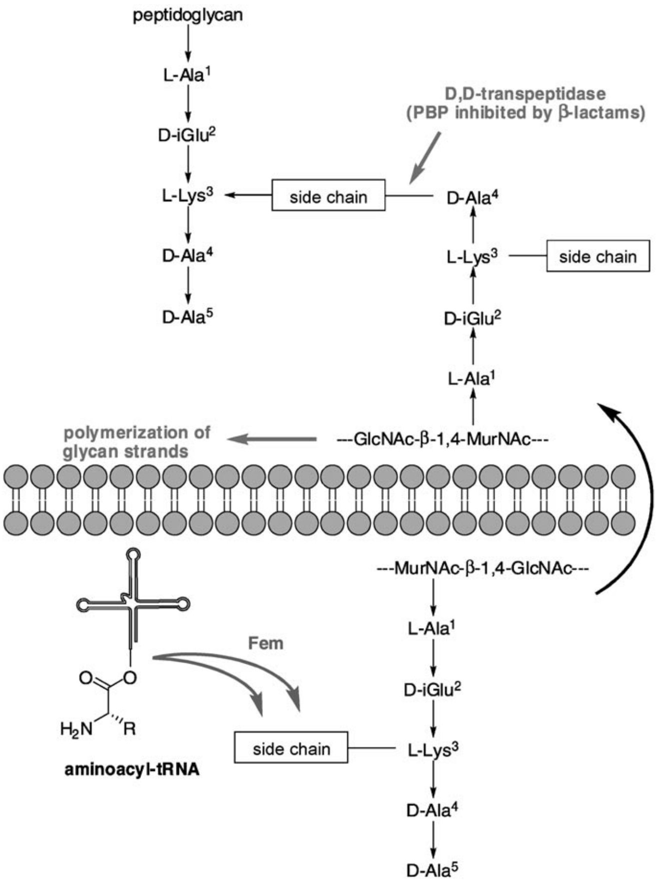


Figure 1. Synthesis of bacterial cell wall peptidoglycan. Arrows show peptide bond orientation CO→NH. GlcNAc: *N*-acetylglucosamine. MurNAc: *N*-acetylmuramic acid.

reported aminoacyl-tRNA analogues used in the study of aminoacyl-tRNA synthetases and of the peptidyl-transferase centre of the ribosome.[9] In those studies, the ester linkages at the 3′-end of the aminoacyl- or peptidyl-tRNAs have been typically replaced by amide linkages (puromycin analogues) or by phosphate or phosphoramidate groups that mimic the tetrahedral transition state formed during the aminoacyl transfer reactions. A deoxyadenosine 3′-phosphonate analogue has also been reported to inhibit MurM, a transferase of the Fem family ($IC_{50} = 100\ \mu M$).[10]

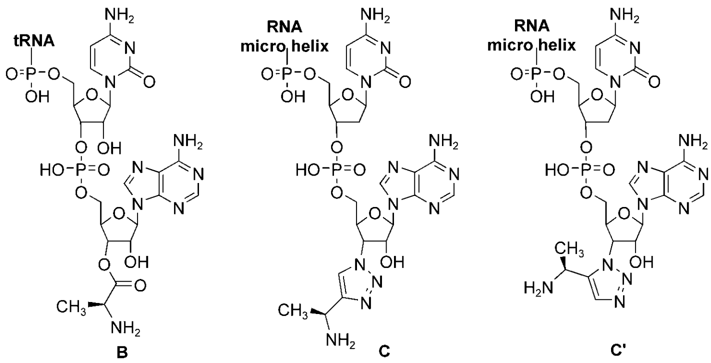


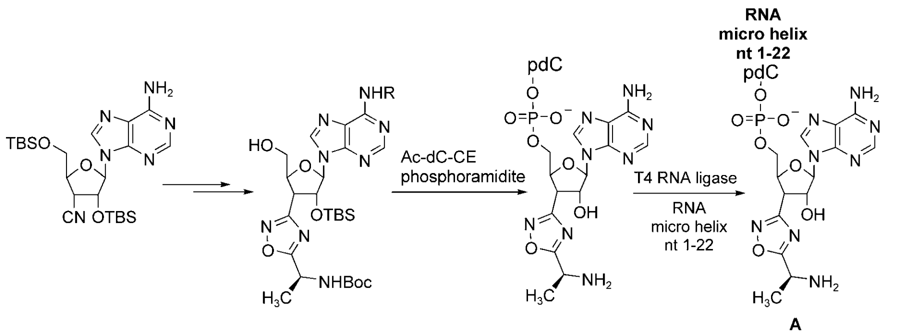
Figure 2. Ala-tRNA^{Ala} substrate of $FemX_{Wv}$ (**B**). Stable analogues (**C**, **C′**) of tRNA^{Ala} containing triazole as ester isoster.

The synthesis of the $FemX_{Wv}$ inhibitor[6] (**A** in Scheme 1) starts from the 3′-cyanodeoxyadenosine, which is converted into the oxadiazole derivative by treatment with hydroxylamine in methanol and condensation with activated Boc-L-alanine. The oxadiazole nucleoside is then coupled with the deoxycytidine in the classical phosphoramidite approach. The resulting dinucleotide is phosphorylated and ligated with an RNA microhelix by T4 RNA ligase to afford compound **A**. This oxadiazole-tRNA was shown to inhibit $FemX_{Wv}$ with an IC_{50} value of 1.4 μM, indicating that the five-membered heterocycle ring was acting as an ester surrogate.

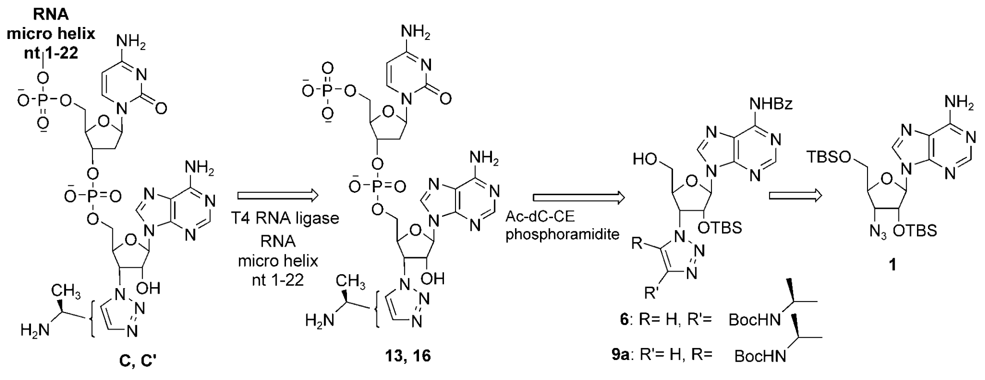
For this report we have developed novel aminoacyl-tRNA analogues each containing a triazole ring instead of an oxadiazole ring (**C** and **C′** in Figure 2). These units are heterocyclic structural motifs with considerable medicinal potential, because they are more than just passive linkers, due to their high potential for association with biological targets through hydrogen bonding and dipole interactions.[11] They can be obtained by azide–alkyne cycloaddition (Huisgen's 1,3-dipolar cycloaddition).[12] A remarkable development of this approach, based on the Cu^{I}-catalysed reaction, afforded regioselective formation of the 1,4-substituted 1,2,3-triazole, reported as the first "click chemistry" reaction.[13] This has led to growing use of triazoles for drug discovery. In the field of nucleoside and nucleotide chemistry, Huisgen–Sharpless cycloaddition has been used as powerful linking reaction to obtain a variety of bioconjugates,[14] modified bases,[15] sugar residues[16] and altered phosphodiester backbones.[17] Here we have applied this approach to the synthesis of aminoacyl-tRNA analogues **C** and **C′** and have shown that these compounds inhibit the $FemX_{Wv}$ cell wall target.

Results and Discussion

Synthesis: The general strategy (Scheme 2) for the preparation of the target compounds **C** and



Scheme 1. Stable analogue of tRNA^{Ala} (**A**).



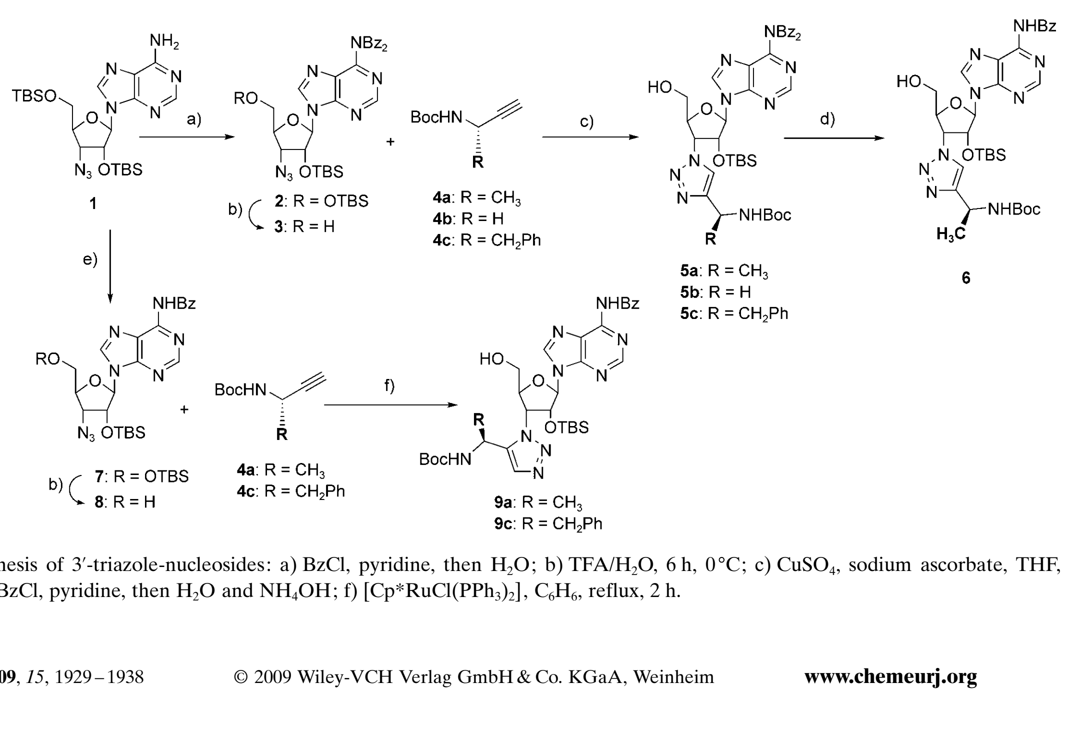
Scheme 2. General strategy for the preparation of Ala-tRNA^{Ala} analogues **C** and **C′**.

C′ was based on enzymatic ligation of **13** or **16** with a 22 nt RNA microhelix that mimics the acceptor arm of tRNA. This approach, initially developed for the introduction of non-natural amino acids in proteins,[18] involves the chemical synthesis of an aminoacylated dinucleotide and its enzymatic coupling to an incomplete RNA lacking the terminal dinucleotide unit. We planned to develop the synthesis of dinucleotides **13** or **16** by coupling between Ac-dC-CE phosphoramidite and nucleosides **6** or **9a**. The key step to obtaining **6** and **9a** was to be a cycloaddition between a 3′-azido-adenosine derivatives and alkynes.

Cycloaddition: Cycloaddition of organic azides and alkynes is the most direct route to 1,2,3-triazoles. In this study, we used two different catalysts to achieve this reaction: the Cu^I catalyst, which has the advantage of exclusively providing the 1,4-disubstituted 1,2,3-triazole regioisomers,[13] and the $[Cp^*RuCl(PPh_3)_2]$ catalyst, which has recently been described for regioselective synthesis of 1,5-disubstituted 1,2,3-triazole systems.[19] The synthesis (Scheme 3) started from the known 3′-azido-2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyadenosine (**1**),[20] which was protected with benzoyl chloride (BzCl) to afford **2** in 89% yield. The selective removal of the silyl group by treatment with trifluoroacetic acid gave **3** in 84% yield. Nucleoside **3** was converted into the 1,4-disubstituted 1,2,3-triazoles **5a–c** through Huisgen–Sharpless 1,3-dipolar cycloadditions with alkynes **4a–c**,[21] in the presence of copper(II) sulfate and sodium ascorbate.

The use of alkynes **4a–c** provides mimics of Ala, Gly and Phe and shows the potential for application to various amino acid residues in the field of modified nucleoside synthesis. The 1,4-disubstituted 1,2,3-triazoles **5a–c** were obtained in 99, 89, and 81% yields respectively, from **3**. Very high yields for the formation of triazole-nucleosides were also obtained under the same conditions from nucleosides **1** or **8**, which differ from **3** in their protecting groups (results not shown).

To obtain the regioisomer 1,5-disubstituted 1,2,3-triazoles, we started from the dibenzoyl-nucleoside **3** in the presence of alkynes **4a–c** and $[Cp^*RuCl(PPh_3)_2]$. From these starting materials we variously observed a low yield (9% from **4a**) or no pure products from the two other derivatives **4b** and **4c**. These poor results were partly due to the removal of



Scheme 3. Synthesis of 3′-triazole-nucleosides: a) BzCl, pyridine, then H_2O; b) TFA/H_2O, 6 h, 0°C; c) $CuSO_4$, sodium ascorbate, THF, H_2O, RT, 24 h; d) NH_4OH; e) BzCl, pyridine, then H_2O and NH_4OH; f) $[Cp^*RuCl(PPh_3)_2]$, C_6H_6, reflux, 2 h.

one of the benzoyl groups in compound **3**. To bypass this problem, we synthesized **8** (Scheme 3) from **1** through a protection step with benzoyl chloride and a selective deprotection of the 5′-hydroxy group with trifluoroacetic acid (45% yield over the two steps) and used it under the same conditions to afford the 1,5-disubstituted triazoles **9a** and **9c** in 37 and 39% yields. These results show that the efficiencies of the cycloadditions in providing triazole-nucleosides is dependent on the natures of alkynes and azido derivatives in the ruthenium-catalysed reactions. In contrast, the copper-catalysed reaction (a "click reaction") provides high yields with various compounds.

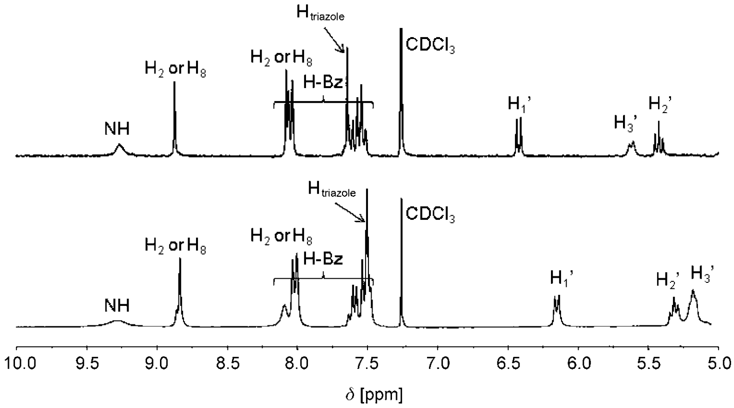
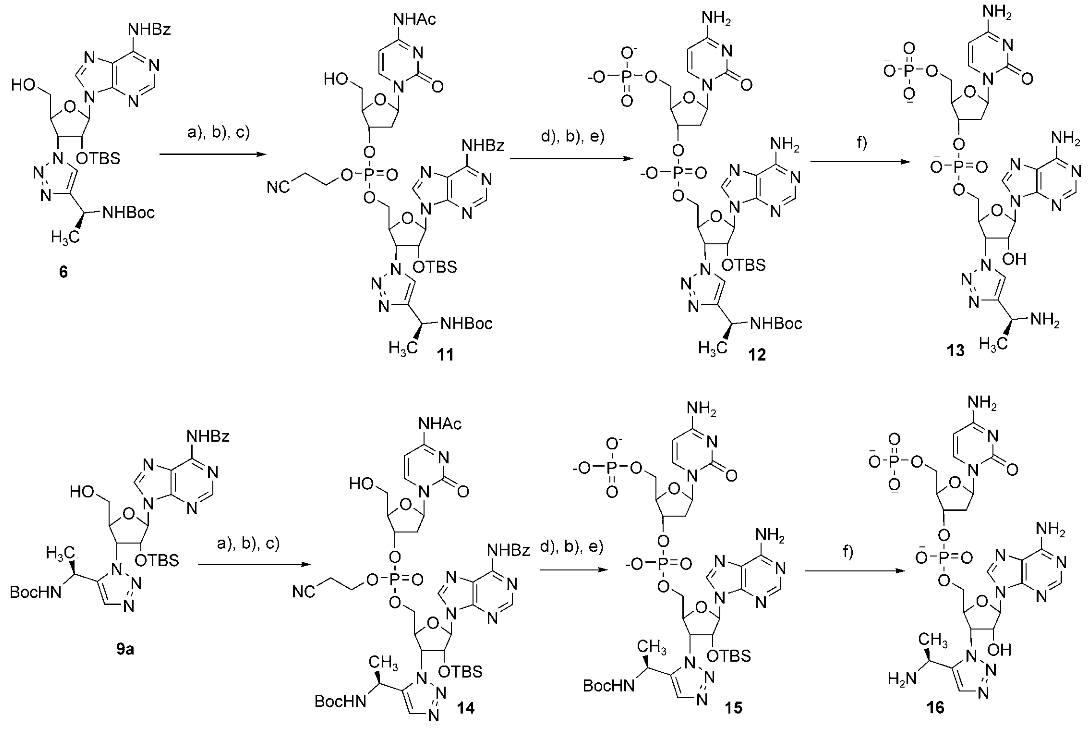


Figure 3. Comparison of the downfield regions of the ^{1}H NMR spectra of **9a** (top) and **6** (bottom) in $CDCl_3$.

Structural assignment: The use of the ruthenium catalyst $[Cp^*RuCl(PPh_3)_2]$ has been reported mainly to provide 1,5-disubstituted 1,2,3-triazoles, although the nature of the azide component can also affect the regioselectivity, leading to mixtures of 1,5- and 1,4-disubstituted triazoles.[19b] The structure of triazole **9a** was thus confirmed by comparison with compound **6**. Their ^{1}H and ^{13}C NMR spectra were very similar, except for H3′, H1′ and $H^{triazole}$ in the ^{1}H NMR (Figure 3) and for the C3′ and $CH^{triazole}$ in the ^{13}C NMR (see Experimental Section). The ^{1}H NMR signals for H3′, H1′ and $H^{triazole}$ for compound **6** appeared at 5.18, 6.15 and 7.51 ppm, respectively, whereas in compound **9a** the signals were shifted downfield (5.62, 6.42 and 7.64 ppm, respectively). In their ^{13}C NMR spectra, $CH^{triazole}$ and C3′ of the nucleoside were observed at 122.8 and 63.1 ppm, respectively, for compound **6** and at 129.5 and 60.4 ppm, respectively, for **9a**.

Phosphoramidite coupling: The dinucleotides **13** and **16** were obtained by the phosphoramidite approach (Scheme 4). Nucleosides **6** and **9a** were coupled in the presence of tetrazole with the commercially available deoxycytidine phosphoramidite (Ac-dC-CE-phosphoramidite). Re-



Scheme 4. a) Ac-dC-CE phosphoramidite, tetrazole, CH_2Cl_2, RT, 1 h; b) I_2, 30 min, RT; c) TCA, CH_2Cl_2, 30 min, RT; d) bis(2-cyanoethyl)diisopropylphosphoramidite, tetrazole, CH_2Cl_2, 1 h, RT; e) CH_3NH_2, 24 h, RT; f) HCl (6 N)/THF/CH_3OH, 24 h.

placement of cytidine with deoxycytidine simplified the synthesis because it eliminated the 2′-OH reactive group without affecting biological activity.[22] After the coupling step, the crude products were oxidized with iodine and treated with trichloroacetic acid to afford compounds **11** and **14** in 69% and 70% yields, respectively. Phosphorylation of **11** and **14** with bis(2-cyanoethyl)diisopropylphosphoramidite in tetrazole, followed by removal of cyanoethyl, acetyl and benzoyl groups with methylamine, afforded **12** and **15** in 54 and 64% yields. The *N*-Boc and TBS protecting groups were removed by stirring with HCl for 24 h, producing **13** and **16** in 44 and 58% yields.

Enzymatic ligation: Aminoacyl-tRNAs are obtained through the coupling of protected aminoacylated dinucleotides with tRNAs lacking the 3′ terminal pdCpA moiety by use of T4 RNA ligase.[18] Stable aminoacyl-pdCpA analogues are also substrates of T4 RNA ligase,[6] and **13** and **16** were ligated to the 3′-end of an RNA microhelix with this enzyme (Scheme 5). In this study we chose a 22 nt RNA microhelix to mimic the acceptor arm of tRNA, because oxadiazole-containing analogues with full-length tRNA (76 nt) or microhelix RNA (24 nt) had previously been shown to inhibit $FemX_{Wv}$ with similar efficiencies (IC_{50} = 0.17 and 1.4 μM, respectively).[6] The ligation was performed in HEPES buffer containing the RNA microhelix (22 nt), compound **13** or **16**, T4 RNA ligase, ATP and $MgCl_2$. After two hours, compounds **C** and **C′** had been obtained and were purified by anion-exchange chromatography. Fractions containing the ligation product were analysed by denaturing polyacrylamide gel electrophoresis (Figure 4), which revealed that the microhelix (22 nt) had been quantitatively converted into the 24 nt triazole-containing oligonucleotides.

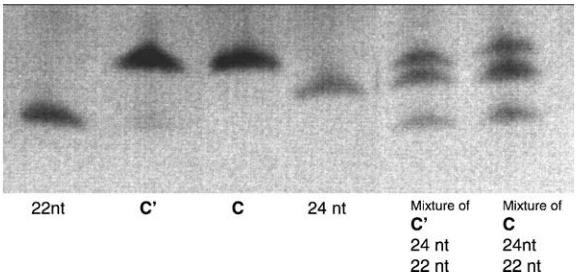
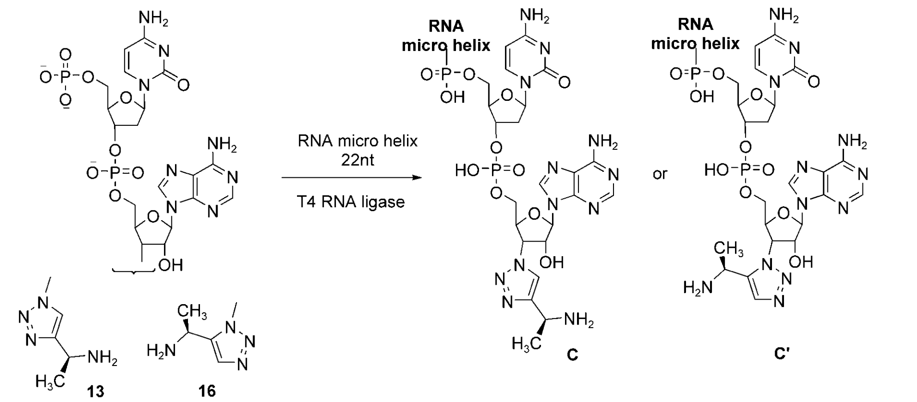


Figure 4. Analysis of **C** and **C′** by polyacrylamide gel electrophoresis. **C** = 5′-GGGGCCUUAGCUCAGGCUCCACdCA-(1,4-disubstituted 1,2,3-triazole)-ethanamine-3′; **C′** = 5′-GGGGCCUUAGCUCAGGCUCCACdCA-(1,5-disubstituted 1,2,3-triazole)ethanamine-3′; 22 nt = microhelix 5′-GGGGCCUUAGCUCAGGCUCCAC-3′; 24 nt = microhelix 5′-GGGGCCUUAGCUCAGGCUCCACCA-3′.



Scheme 5. Reaction was performed at 37°C for 120 min in HEPES buffer (50 mM, 500 μL) containing the 22 nt RNA microhelix (20 nmol), compound **13** or **16** (200 nmol), T4 RNA ligase (3.1 mg), DMSO (10%), ATP (1 mM), $MgCl_2$ (15 mM).

In vitro inhibition assays: $FemX_{Wv}$ from *Weissella viridescens* has been used as a model enzyme for kinetics and structural analyses of transferases of the Fem family.[23] The enzyme catalyses the transfer of L-Ala from Ala-$tRNA^{Ala}$ to the peptidoglycan precursor UDP-MurNAc-pentapeptide in order to introduce the first residue of an L-Ala-L-Ser-L-Ala side chain. Inhibition of $FemX_{Wv}$ was tested in a radioactive coupled assay as previously described.[6] Inhibition of the transfer of L-Ala by $FemX_{Wv}$ revealed IC_{50} values of 2.4 ± 0.4 μM for **C** and of 4.1 ± 0.4 μM for **C′** (Figure 5). The low IC_{50} values indicate that the triazole unit can be used as an ester bioisoster.

Compound **C′** was only slightly less active than **C** (4.1 versus 2.4 μM). This result suggests that the ethylamine substituent of the triazole, which has different orientations in the two compounds, has little effect on the interaction of the inhibitors with the enzyme. The 1-aminoethyl group was introduced in **C** and **C′** in order to mimic the amine and the methyl side chain of L-Ala. $FemX_{Wv}$ had previously been shown to catalyse aminoacyl transfer from Gly-$tRNA^{Gly}$, indicating that the methyl group of L-Ala is not essential for activity.[24] This observation could account for the similar behaviour of **C** and **C′** as inhibitors.

Conclusions

We have described an efficient method for the synthesis of a novel class of stable analogues of aminoacyl-tRNAs. In contrast with native aminoacyl-tRNAs, which bear readily hydrolysable ester linkages, the analogues each contained a stable five-membered triazole ring. The use of "click chemistry" techniques afforded 3′deoxy-3′-triazole-nucleosides in very high yields, and cycloadditions catalysed by copper or ruthenium allowed the 1,4- and 1,5-regioisomers of the triazoles to be obtain from the same starting materi-

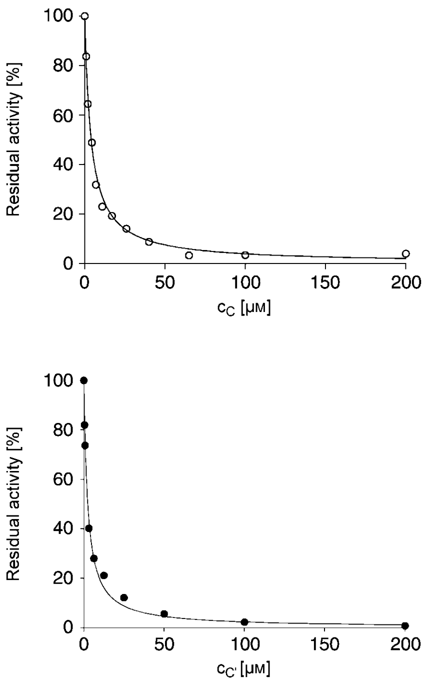


Figure 5. Inhibition of $FemX_{Wv}$ by compounds **C** and **C′**. Curves represent nonlinear regressions (four-parameter logistic curve, sigma Plot 9.0) of experimentally determined values. ○) $IC_{50}=2.4\pm0.4$ μM for **C** Hillslope 1.03 ± 0.05; $R=0.9998$, and ●) $IC_{50}=4.1\pm0.4$ μM for **C′** Hillslope 1.0 ± 0.1; $R=0.9986$.

als. The IC_{50} values of **C** and **C′** for $FemX_{Wv}$ indicate that their triazole rings are good ester bioisosters. The strategy is of broad interest for the design of inhibitors of various types of enzymes that use aminoacyl-tRNA as substrates.

Experimental Section

General reagents and materials: Solvents were dried by standard methods and were distilled before use. Unless otherwise specified, materials were purchased from commercial suppliers and were used without further purification. TLC: precoated thin-layer silica gel 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, Merck). Spectra were recorded on Bruker spectrometers: ARX 250 for ^{1}H NMR (250.13 MHz) and ^{13}C NMR (62.89 MHz), AC 400 for ^{31}P NMR (161.97 MHz), Avance III 500 for ^{1}H NMR (500.11 MHz) and ^{13}C NMR (125.75 MHz), and DRX 500 for ^{31}P NMR (202.31 MHz), in $CDCl_3$, [D_6]DMSO, CD_3OD or D_2O as indicated below. Chemical shifts (δ) are expressed in ppm relative to residual $CHCl_3$ ($\delta=7.26$ ppm), CHD_2OD ($\delta=3.31$ ppm), CHD_2SOCD_3 ($\delta=2.50$ ppm) or HDO ($\delta=4.79$ ppm) for ^{1}H, and to $CDCl_3$ ($\delta=77.16$ ppm), CD_3OD ($\delta=49.00$ ppm) or CD_3SOCD_3 ($\delta=39.52$ ppm) for ^{13}C as internal references, and to H_3PO_4 ($\delta=0$ ppm) for ^{31}P as external reference. Signals were attributed on the basis of COSY and DEPT 135 (^{13}C). High-resolution mass spectrometry (HRMS) was carried out with a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc.) by electrospray ionization (ESI^+ or ESI^-) at the Mass Spectrometry Centre of the University Pierre & Marie Curie (Paris). High-performance liquid chromatography (HPLC) was performed on a HPLC system with a reversed-phase C-18 column (250 mm×21.2 mm, HYPERSIL HSC18, Thermoelectron Corporation) with a solvent system consisting of aqueous NH_4OAc (50 mM)/CH_3CN (linear gradient from 100:0 to 50:50 over 50 min) at a flow rate of 17 mL min^{-1} and UV detection at 260 nm. Fast protein liquid chromatography (FPLC) was performed with an AKTA purifier (Amersham Pharmacia Biotech). Optical rotations were carried out on a Perkin–Elmer Model 341 polarimeter.

3′-Azido-6-*N*,*N*-dibenzoyl-2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyadenosine (2): Benzoyl chloride (975 μL, 8.40 mmol) was added dropwise at 0 °C to a solution of **1** (875 mg, 1.68 mmol) in anhydrous pyridine (50 mL). The mixture was stirred at room temperature for 4 h, and the solution was concentrated to dryness. The residue was dissolved in CH_2Cl_2 and washed with H_2O, saturated $NaHCO_3$ and brine. The combined organic layers were dried over anhydrous Na_2SO_4. After removal of the solvents, the crude product was purified on a silica gel column with elution with EtOAc/cyclohexane 2:8 to give **2** (1.09 g, 89%) as a white foam. $R_f=0.6$ (EtOAc/cyclohexane 2:8); $[\alpha]_D^{25}=-20.2$ ($c=0.5$ in $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=8.65$ (s, 1H; H2 or H8), 8.37 (s, 1H; H2 or H8), 7.93–7.78 (m, 4H; H-Bz), 7.53–7.29 (m, 6H; H-Bz), 6.08 (d, $^3J_{H,H}=4.5$ Hz, 1H; H1′), 4.88 (t, $^3J_{H,H}=4.7$ Hz, 1H; H2′), 4.22 (dd, $^3J_{H,H}=2.7$, 5.2 Hz, 1H; H4′), 4.10–3.99 (m, 2H; H3′/H5′a), 3.83 (dd, $^3J_{H,H}=2.8$, $^2J_{H,H}=11.7$ Hz, 1H; H5′b), 0.93 (s, 9H; H-*t*Bu^{TBS}), 0.83 (s, 9H; H-*t*Bu^{TBS}), 0.12 (s, 6H; H-Me^{TBS}), 0.04 (s, 3H; H-Me^{TBS}), −0.02 ppm (s, 3H; H-Me^{TBS}); ^{13}C NMR (63 MHz, $CDCl_3$): $\delta=172.3$ (C=O−Bz), 152.8 (Cq-Ad), 152.4 (C2 or C8), 151.9 (Cq-Ad), 143.4 (C2 or C8), 134.2 (Cq-Bz), 133.0 (C-Bz), 129.6 (C-Bz), 128.8 (C-Bz), 88.9 (C1′), 82.6 (C4′), 77.1 (C2′), 62.7 (C5′), 61.3 (C3′), 26.1 (C-*t*Bu^{TBS}), 25.6 (C-*t*Bu^{TBS}), 18.6 (Cq-*t*Bu^{TBS}), 18.0 (Cq-*t*Bu^{TBS}), −4.9 (C-Me^{TBS}), −5.1 (C-Me^{TBS}), −5.2 (C-Me^{TBS}), −5.3 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{36}H_{49}N_8O_5Si_2$: 729.3359 $[M+H]^+$; found: 729.3359.

3′-Azido-6-*N*,*N*-dibenzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyadenosine (3): Aqueous TFA (5 mL, 1:1) was added at 0 °C to a stirred solution of **2** (459 mg, 0.630 mmol) in THF (7 mL). After having been stirred for 6 h at 0 °C, the reaction mixture was neutralized with saturated aqueous $NaHCO_3$ and diluted with EtOAc. After separation, the organic phase was washed with H_2O and brine, dried over anhydrous Na_2SO_4 and concentrated at reduced pressure. The residue was subjected to flash chromatography with elution with EtOAc/cyclohexane (:7 to provide **3** (324 mg, 84%) as a white foam. $R_f=0.24$ (EtOAc/cyclohexane 3:7); $[\alpha]_D^{25}=-36.1$ ($c=1$ in $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=8.65$ (s, 1H; H2 or H8), 8.09 (s, 1H; H2 or H8), 7.92–7.80 (m, 4H; H-Bz), 7.50 (m, 2H; H-Bz), 7.35 (m, 4H; H-Bz), 5.82 (d, $^3J_{H,H}=7.5$ Hz, 1H; H1′), 5.70 (d, $^3J_{H,H}=10.2$ Hz, 1H; OH), 5.28 (dd, $^3J_{H,H}=5.5$, 7.5 Hz, 1H; H2′), 4.25 (d, $^3J_{H,H}=5.4$ Hz, 1H; H3′), 4.16 (s, 1H; H4′), 3.83 (dd, $^3J_{H,H}=2.8$, $^2J_{H,H}=11.6$ Hz, 2H; H5′a/H5′b), 0.78 (s, 9H; H-*t*Bu^{TBS}), −0.12 (s, 3H; H-Me^{TBS}), −0.53 ppm (s, 3H; H-Me^{TBS}); ^{13}C NMR (63 MHz, $CDCl_3$): $\delta=172.1$ (C=O−Bz), 153.1 (Cq-Ad), 151.8 (C2 or C8), 145.0 (C2 or C8), 133.9 (Cq-Bz), 133.3 (C-Bz), 129.6 (C-Bz), 128.9 (C-Bz), 90.8 (C1′), 85.4 (C4′), 74.9 (C2′), 63.6 (C3′), 63.4 (C5′), 25.7 (C-*t*Bu^{TBS}), 17.9 (Cq-*t*Bu^{TBS}), −5.1 (C-Me^{TBS}), −5.8 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{30}H_{34}N_8O_5SiNa$: 637.2308 $[M+Na]^+$; found: 637.2309.

General procedure for Cu-catalysed cycloadditions: A solution of aq sodium ascorbate (1 M, 0.2 equiv, 16 μL, 16 μmol) and aq $CuSO_4$ (7.5%, 0.1 equiv, 81 μL, 8 μmol) was added at 0 °C to a mixture of **3** (1.0 equiv, 81 μmol) and one of the compounds **4a–c** (1.1 equiv, 89 μmol) in H_2O/THF (3 mL, 1:1). The heterogeneous mixture was stirred vigorously at room temperature until complete consumption of the reactants was indicated by TLC analyses. After removal of THF under reduced pressure, water (3 mL) was added, and the product was extracted with EtOAc (3× 10 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude product was subjected to column chromatography with elution with EtOAc/cyclohexane 5:5.

6-*N*,*N*-Dibenzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-(*S*)-*tert*-butyl-1-(1*H*-1,2,3-triazol-4-yl)ethylcarbamate-3′-deoxyadenosine (5a): Compound **3** (50 mg, 81 μmol) and alkyne **4a** (15 mg, 89 μmol) were treated as described in the general procedure to give compound **5a** (55 mg, 99%) as a white solid. $R_f=0.71$ (EtOAc/cyclohexane 8:2); $[\alpha]_D^{25}=-67.2$ ($c=1$ in $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=8.65$ (s, 1H; H2 or H8), 8.16 (s, 1H; H2 or H8), 7.82 (d, $^3J_{H,H}=7.4$ Hz, 4H; H-Bz), 7.51 (s, 1H; H^{triazole}), 7.51–7.41 (m, 2H; H-Bz), 7.32 (t, $^3J_{H,H}=7.6$ Hz, 4H; H-Bz), 6.14 (d,

$^3J_{H,H}$=6.9 Hz, 1H; H1′), 5.77 (d, $^3J_{H,H}$=10.3 Hz, 1H; OH), 5.30–5.10 (m, 3H,H2′/H4′/NH), 4.93 (m, 2H; H3′/CH), 4.12–3.96 (m, 1H; H5′a), 3.82–3.66 (m, 1H; H5′b), 1.52 (d, $^3J_{H,H}$=6.9 Hz, 1H; CH_3), 1.41 (s, 9H; *t*Bu-Boc), 0.55 (s, 9H; H-*t*Bu^{TBS}), −0.23 (s, 3H; H-Me^{TBS}), −0.61 ppm (s, 3H; H-Me^{TBS}); ^{13}C NMR (63 MHz, $CDCl_3$): δ=172.0 (C=O−Bz), 155.2 (Cq-Ad), 153.0 (C2 or C8), 151.8 (C=O−Boc), 149.59 (Cq-Ad), 145.0 (C2 or C8), 133.8 (Cq-Bz), 133.2 (C-Bz), 129.5 (C-Bz), 128.8 (C-Bz), 122.7 (C-triazole), 91.3 (C1′), 84.7 (C4′), 79.6 (Cq-*t*Bu^{Boc}), 73.6 (C2′), 63.3 (C5′), 62.8 (C3′), 42.6 (CH), 28.4 (C-*t*Bu^{Boc}), 25.2 (C-*t*Bu^{TBS}), 21.2 (CH_3), 17.4 (Cq-*t*Bu^{TBS}), −5.1 (C-Me^{TBS}), −5.9 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{39}H_{49}N_9O_7SiNa$: 806.3422 [*M*+Na]$^+$; found: 806.3416.

6-*N*,*N*-Dibenzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-(*S*)-*tert*-butyl-1-(1*H*-1,2,3-triazol-4-yl)methylcarbamate-3′-deoxyadenosine (5b): Compound **3** (50 mg, 81 µmol) and alkyne **4b** (14 mg, 89 µmol) were treated as described in the General Procedure to give compound **5b** (56 mg, 89%) as a white solid. R_f=0.59 (EtOAc/cyclohexane 8:2); $[\alpha]_D^{25}$=−75.7 (*c*=1 in $CHCl_3$); ^{1}H NMR (250 MHz, CD_3OD): δ=8.84 (s, 1H; H2 or H8), 8.67 (s, 1H; H2 or H8), 7.97 (s, 1H; H^{triazole}), 7.84 (d, $^3J_{H,H}$=7.2 Hz, 4H; H-Bz), 7.54 (t, $^3J_{H,H}$=7.4 Hz, 2H; H-Bz), 7.39 (t, $^3J_{H,H}$=7.6 Hz, 4H; H-Bz), 6.38 (d, $^3J_{H,H}$=5.5 Hz, 1H; H1′), 5.49 (m, 1H; H3′), 5.19 (t, $^3J_{H,H}$=6.0 Hz, 1H; H2′), 4.92 (m, 1H; H4′), 4.33 (s, 2H; CH_2), 3.91 (dd, $^2J_{H,H}$=10.0 Hz, 2H; H5′a/H5′b), 1.44 (s, 9H; H-*t*Bu^{Boc}), 0.60 (s, 9H; H-*t*Bu^{TBS}), −0.19 (s, 3H; H-Me^{TBS}), −0.39 ppm (s, 3H; H-Me^{TBS}); ^{13}C NMR (63 MHz, CD_3OD): δ=173.6 (C=O−Bz), 158.2 (Cq-Ad), 154.02 (Cq-Ad), 153.2 (C2 or C8), 147.1 (Cq-Ad), 146.5 (C2 or C8), 135.30 (Cq-Bz), 134.4 (C-Bz), 130.5 (C-Bz), 129.9 (C-Bz), 129.4 (Cq-triazole), 125.4 (C-triazole), 91.2 (C1′), 84.7 (C4′), 80.4 (Cq-*t*Bu^{Boc}), 76.6 (C2′), 63.2 (C3′), 62.7 (C5′), 36.5 (CH_2), 28.8 (C-*t*Bu^{Boc}), 25.8 (C-*t*Bu^{TBS}), 18.4 (Cq-*t*Bu^{TBS}), −5.01 (C-Me^{TBS}), −5.31 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{38}H_{47}N_9O_7SiNa$: 792.3265 [*M*+Na]$^+$; found: 792.3260.

6-*N*,*N*-Dibenzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-(*S*)-*tert*-butyl-1-(1*H*-1,2,3-triazol-4-yl)-2-phenylethylcarbamate-3′-deoxyadenosine (5c): Compound **3** (50 mg, 81 µmol) and alkyne **4c** (22 mg, 89 µmol) were treated as described in the General Procedure to give compound **5c** (57 mg, 81%) as a white solid. R_f=0.71 (EtOAc/cyclohexane 8:2); $[\alpha]_D^{25}$=−72.1 (*c*=1 in $CHCl_3$); ^{1}H NMR (250 MHz, CD_3OD): δ=8.84 (s, 1H; H2 or H8), 8.67 (s, 1H; H2 or H8), 7.91 (s, 1H; H^{triazole}), 7.89–7.79 (m, 4H; H-Bz), 7.54 (m, 2H; H-Bz), 7.39 (m, 4H; H-Bz), 7.24 (m, 5H; H-Bz), 6.36 (d, $^3J_{H,H}$=5.3 Hz, 1H; H1′), 5.49 (m, 1H; H3′), 5.19 (m, 1H; H2′), 5.08 (m, 1H; CH), 4.88 (m, 1H; H4′), 4.03–3.74 (m, 2H; H5′a/H5′b), 3.29–2.94 (m, 2H; CH_2), 1.34 (s, 9H; H-*t*Bu^{Boc}), 0.59 (s, 9H; H-*t*Bu^{TBS}), −0.21 (s, 3H; H-Me^{TBS}), −0.39 ppm (s, 3H; H-Me^{TBS}); ^{13}C NMR (63 MHz, CD_3OD): δ=173.6 (C=O−Bz), 157.4 (Cq), 154.0 (Cq), 153.2 (C2 or C8), 150.4 (Cq), 146.5 (C2 or C8), 139.2 (Cq), 135.3 (Cq-Ar), 134.3 (C-Ar), 130.5 (C-Ar), 129.9 (C-Ar), 129.4 (C-Ar), 127.5 (Cq-triazole), 124.6 (C-triazole), 91.1 (C1′), 84.7 (C4′), 80.2 (Cq-*t*Bu^{Boc}), 76.6 (C2′), 63.2 (C3′), 62.6 (C5′), 50.0 (CH), 42.5 (CH_2), 28.7 (C-*t*Bu^{Boc}), 25.9 (C-*t*Bu^{TBS}), 18.5(Cq-*t*Bu^{TBS}), −5.00 (C-Me^{TBS}), −5.28 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{45}H_{53}N_9O_7SiNa$: 882.3735 [*M*+Na$^+$]; found: 882.3729.

6-*N*-Benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-(*S*)-*tert*-butyl-1-(1*H*-1,2,3-triazol-4-yl)ethylcarbamate-3′-deoxyadenosine (6): Compound **5a** (97 mg, 12.4 mmol) was stirred in CH_2Cl_2/NH_4OH (8 mL, 1:1) for 4 h at room temperature. The reaction mixture was washed with aqueous satured NH_4Cl solution, water and brine. The organic phase was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the crude residue was purified by flash chromatography (EtOAc/cyclohexane 8:2) to give **6** (77 mg, 92%) as a white solid. R_f=0.49 (EtOAc); $[\alpha]_D^{25}$=−51.0 (*c*=0.8 in MeOH); ^{1}H NMR (250 MHz, $CDCl_3$): δ=9.30 (brs, 1H; NH), 8.86 (s, 1H; H2 or H8), 8.09 (s, 1H; H2 or H8), 8.03–8.00 (m, 2H; H-Bz), 7.60–7.50 (m, 3H; H-Bz), 7.48 (s, 1H; H^{triazole}), 6.14 (d, $^3J_{H,H}$=7.5 Hz, 1H; H1′), 5.32 (m, 1H; H2′), 5.18 (m, 2H; H3′/NH), 4.96 (m, 2H; CH/H4′), 4.11–3.75 (dd, $^2J_{H,H}$=12.5 Hz, 2H; H5′a/H5′b), 1.56 (d, $^3J_{H,H}$=7.5 Hz, 3H; CH_3), 1.42 (s, 9H; H-*t*Bu^{Boc}), 0.55 (s, 9H; H-*t*Bu^{TBS}), −0.20 (s, 3H; H-Me^{TBS}), −0.56 ppm (s, 3H; H-Me^{TBS}); ^{13}C NMR (63 MHz, $CDCl_3$): δ=164.8 (C=O−Bz), 155.3 (Cq), 152.6 (C2 or C8), 150.5 (Cq), 143.3 (C2 or C8), 133.2 (Cq-Bz), 129.0 (C-Bz), 128.0 (C-Bz), 124.2 (Cq-Bz), 122.8 (C-triazole), 91.6 (C1′), 84.9 (C4′), 79.7 (Cq-*t*Bu^{Boc}), 73.7 (C2′), 63.5 (C5′), 63.1 (C3′), 42.7 (CH), 28.5 (C-*t*Bu^{Boc}), 25.2 (C-*t*Bu^{TBS}), 21.3 (CH_3), 17.5(Cq-*t*Bu^{TBS}), −5.00 (C-Me^{TBS}), −5.72 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{32}H_{45}N_9O_6SiNa$: 702.3160 [*M*+Na]$^+$; found: 702.3154.

3′-Azido-6-*N*-benzoyl-2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyadenosine (7): Benzoyl chloride (953 µL, 8.2 mmol) was added dropwise at 0°C to a solution of **1** (0.855 g, 1.64 mmol) in anhydrous pyridine (12 mL). The mixture was stirred for 2 h at room temperature. Ice-water (10 mL) was added, and the mixture was allowed to stir for 30 min at 0°C. Aqueous NH_4OH solution (30% 10 mL) was then added, and the reaction was stirred for 1 hour at 0°C and for another 3 h at room temperature. The reaction mixture was washed with aqueous saturated NH_4Cl solution (2×50 mL), water (2×50 mL) and brine (2×50 mL). The organic layers were dried over Na_2SO_4. After removal of the solvents, the residue was purified on a flash column of silica gel with elution with EtOAc/cyclohexane 2:8 to give **7** (629 mg, 61%) as a white solid. R_f=0.70 (EtOAc/cyclohexane 5:5); $[\alpha]_D^{25}$=−14.7 (*c*=1 in $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): δ=9.39 (brs, 1H; NH), 8.78 (s, 1H; H2 or H8), 8.40 (s, 1H; H2 or H8), 8.01 (d, $^3J_{H,H}$=9.0 Hz, 2H; H-Bz), 7.50 (m, 3H; H-Bz), 6.11 (d, $^3J_{H,H}$=5.0 Hz, 1H; H1′), 4.86 (t, $^3J_{H,H}$=5.0 Hz, 1H; H2′), 4.26 (m, 1H; H4′), 4.07 (m, 2H,H3′/H5′a), 3.84 (dd, $^3J_{H,H}$=2.5, $^2J_{H,H}$=12.5 Hz, 1H; H5′b), 0.94 (s, 9H; H-*t*Bu^{TBS}), 0.86 (s, 9H; H-*t*Bu^{TBS}), 0.15 (s, 6H; H-Me^{TBS}), 0.06 (s, 3H; H-Me^{TBS}), −0.06 ppm (s, 3H; H-Me^{TBS}); ^{13}C NMR (63 MHz, $CDCl_3$): δ=165.0 (C=O−Bz), 152.4 (C2 or C8), 141.3 (C2 or C8), 151.5, 149.7 (Cq-Ad), 133.3 (Cq-Bz), 133.8 (C-Bz), 128.8 (C-Bz), 128.1 (C-Bz), 89.1 (C1′), 82.3 (C4′), 77.2 (C2′), 62.5 (C5′), 60.9 (C3′), 26.1 (C-*t*Bu^{TBS}), 25.7 (C-*t*Bu^{TBS}), 18.6 (Cq-*t*Bu^{TBS}), 18.0 (Cq-*t*Bu^{TBS}), −4.9 (C-Me^{TBS}), −5.0 (C-Me^{TBS}), −5.2 (C-Me^{TBS}), −5.4 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{29}H_{44}N_8O_4Si_2Na$: 647.2922 [*M*+Na]$^+$; found: 647.2916.

3′-Azido-6-*N*-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyadenosine (8): Aqueous TFA (6 mL, 1:1) was added at 0°C to a stirred solution of **7** (594 mg, 0.95 mmol) in THF (8 mL). After having been stirred for 8 h at 0°C, the reaction mixture was neutralized with aqueous saturated $NaHCO_3$ solution and diluted with EtOAc. After separation, the organic phase was washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to flash chromatography with elution with EtOAc/cyclohexane 2:8 to provide **8** (359 mg, 74%) as a white foam. R_f=0.62 (EtOAc); $[\alpha]_D^{25}$=+7.8 (*c*=1 in $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): δ=9.39 (brs, 1H; NH), 8.75 (s, 1H; H2 or H8), 8.05 (s, 1H; H2 or H8), 8.01 (d, $^3J_{H,H}$=9.0 Hz, 2H; H-Bz), 7.51 (m, 3H; H-Bz), 6.05 (brs, 1H; OH), 5.81 (d, *J*=7.5 Hz, 1H; H1′), 5.29 (dd, $^3J_{H,H}$=5.0, 7.5 Hz, 1H; H2′), 4.24 (d, $^3J_{H,H}$=7.5 Hz, 1H; H3′), 4.13 (s, 1H,H4′), 3.91 (m, 1H; H5′a), 3.70 (m,1H; H5′b), 0.75 (s, 9H; H-*t*Bu^{TBS}), −0.12 (s, 3H; H-Me^{TBS}), −0.49 ppm (s, 3H; H-Me^{TBS}); ^{13}C NMR (63 MHz, $CDCl_3$): δ=164.7 (C=O−Bz), 152.4 (C2 or C8), 150.7 (Cq-Ad), 143.1 (C2 or C8), 133.5 (Cq-Bz), 133.0 (C-Bz), 128.9 (C-Bz), 128.0 (C-Bz), 91.0 (C1′), 85.4 (C4′), 74.9 (C2′), 63.3 (C3′), 60.9 (C5′), 25.2 (C-*t*Bu^{TBS}), 15.2 (Cq-*t*Bu^{TBS}), −5.0 (C-Me^{TBS}), −5.8 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{23}H_{31}N_8O_4SiNa$: 533.2057 [*M*+Na]$^+$; found: 533.2052.

General procedure for Ru-catalysed cycloadditions: A mixture of azide **8** (1.0 equiv, 0.2 mmol), alkyne **4a** or **4c** (2.5 equiv) and [Cp*RuCl($PPh_3)_2$] (0.1 equiv, 0.02 mmol) in anhydrous benzene (1 mL) was heated at reflux at 80°°C for 24 h. The progress of the reaction was monitored by TLC. The mixture was then cooled and evaporated under reduced pressure. The product was purified by flash chromatography with elution with EtOAc/cyclohexane 6:4.

6-*N*-Benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-(*S*)-*tert*-butyl-1-(1*H*-1,2,3-triazol-5-yl)ethylcarbamate-3′-deoxyadenosine (9a): Azide **8** (100 mg, 0.2 mmol), alkyne **4a** (83 mg, 0.5 mmol) and [Cp*RuCl($PPh_3)_2$] (16 mg, 0.02 mmol) were treated as described in the General Procedure. The product **9a** (49 mg, 37%) was obtained as a yellow oil. R_f=0.68 (EtOAc); $[\alpha]_D^{25}$=−37.8 (*c*=1 in MeOH); ^{1}H NMR (250 MHz, $CDCl_3$): δ=9.32 (brs, 1H; NH), 8.85 (s, 1H; H2 or H8), 8.08 (s, 1H; H2 or H8), 8.03 (m, 2H; H-Bz), 7.83 (s, 1H; H^{triazole}), 7.56 (m, 3H; H-Bz), 6.41 (d, $^3J_{H,H}$=7.2 Hz, 1H; H1′), 6.14 (brs, 1H; NH), 5.60 (m, 1H; H3′), 5.41 (t, $^3J_{H,H}$=7.1 Hz, 1H; H2′), 4.96 (m, 1H; CH), 4.81 (s, 1H; H4′), 4.20–3.96 (m, 2H; H5′a/H5′b), 1.61 (d, $^3J_{H,H}$=5.0 Hz, 3H; CH_3), 1.45 (s, 9H; H-*t*Bu^{Boc}), 0.54 (s, 9H; H-*t*Bu^{TBS}), −0.12 (s, 3H; H-Me^{TBS}), −0.72 ppm (s, 3 H; H-Me^{TBS}); ^{13}C NMR (63 MHz, CDCl$_3$): δ=172.1 (C=O–Bz), 152.6 (C2 or C8), 143.4 (C2 or C8), 132.1 (C-Bz), 129.5 (C-triazole), 129.0 (C-Bz), 128.7 (C-Bz), 92.0 (C1′), 86.1 (C4′), 73.5 (C2′), 63.4 (C5′), 60.4 (C3′), 39.8 (CH), 28.4 (C-*t*Bu^{Boc}), 25.1 (C-*t*Bu^{TBS}), 20.2 (CH$_3$), −4.9 (C-Me^{TBS}), −5.9 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{32}H_{45}N_9O_6SiNa$: 702.3160 [*M*+Na]$^+$; found: 702.3154.

6-*N*,*N*-Dibenzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-3′-(*S*)-*tert*-butyl-1-(1*H*-1,2,3-triazol-5-yl)-2-phenylethylcarbamate-3′-deoxyadenosine (9c): Azide **8** (100 mg, 0.2 mmol), alkyne **4c** (120 mg, 0.5 mmol) and [Cp*RuCl(PPh$_3$)$_2$] (16 mg, 0.02 mmol) were treated as described in the General Procedure. The product **9c** (57 mg, 39%) was obtained as a yellow oil. R_f=0.72 (EtOAc); $[\alpha]_D^{25}$=−42.0 (*c*=1 in CHCl$_3$); ^{1}H NMR (250 MHz, CDCl$_3$): δ=9.36 (brs, 1 H; NH), 8.82 (s, 1 H; H2 or H8), 8.10 (s, 1 H; H2 or H8), 8.03 (m, 2 H; H-Bz), 7.61 (s, 1 H; H^{triazole}), 7.53–7.47 (m, 8 H; H-Bz/H-Ph), 6.49 (d, $^3J_{H,H}$=7.5 Hz, 1 H; H1′), 6.09 (brs, 1 H; NH), 5.39 (t, $^3J_{H,H}$=7.5 Hz, 1 H; H2′), 5.01 (m, 1 H; H4′), 4.24–3.87 (m, 4 H; CH/H3′/H5′a/H5′b), 3.53–3.05 (m, 2 H; CH$_2$ Ph), 1.21 (s, 9 H; H-*t*Bu^{Boc}), 0.55 (s, 9 H; H-*t*Bu^{TBS}), −0.25 (s, 3 H; H-Me^{TBS}), −0.67 ppm (s, 3 H; H-Me^{TBS}); ^{13}C NMR (63 MHz, CDCl$_3$): δ=164.6 (C=O–Bz), 152.6 (C2 or C8), 150.6 (Cq), 143.4 (C2 or C8), 132.0 (C-Bz or C-Ph), 132.8 (C-Bz or C-Ph), 132.2 (C-Bz or C-Ph), 131.0 (C-triazole), 127.3 (C-Bz or C-Ph), 92.1 (C1′), 86.2 (C4′), 73.5 (C2′), 63.2 (C5′), 60.3 (C3′), 43.5 (CH), 29.8 (C-CH$_2$Ph), 28.1 (C-*t*Bu^{Boc}), 25.4 (C-*t*Bu^{TBS}), −4.8 (C-Me^{TBS}), −5.7 ppm (C-Me^{TBS}); HRMS (ESI): *m*/*z*: calcd for $C_{38}H_{49}N_9O_6SiNa$: 778.3473; found: 778.3467 [*M*+Na]$^+$.

General procedure for dinucleotide synthesis: Coupling was directly carried out under argon in a commercial Ac-dC-PCNE phosphoramidite vial (250 mg, 324 μmol) fitted with a flat magnetic stirrer. The adenosine derivative (130 μmol) was added first, followed by anhydrous CH$_2$Cl$_2$ (350 μL). A solution of tetrazole in CH$_3$CN (0.45 M, 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 solution of I$_2$ (0.1 M, 3.3 mL) was added. The reaction mixture was stirred at room temperature for 30 min, diluted with EtOAc and washed successively with aqueous saturated Na$_2$S$_2$O$_3$ solution and brine. The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated to dryness. A solution of trichloroacetic acid (0.18 M, 7.2 mL) was finally added to the resulting residue, and the mixture was stirred at room temperature for 30 min, diluted with CH$_2$Cl$_2$ and washed successively with aqueous ice-saturated NaHCO$_3$ solution and brine. The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated under vacuum. The crude product was purified by preparative TLC (CH$_2$Cl$_2$/MeOH 9:1) to afford the desired compound as two diastereomers.

Compound 11: Adenosine derivative **6** (88 mg, 129 μmol) was coupled to Ac-dC-PCNE-phosphoramide as described in the General Procedure to give dinucleotide **11** (95 mg, 69%) as two diastereomers. ^{1}H NMR (250 MHz, CDCl$_3$): δ=9.73 (s, 2 H; NHAc/NHBz), 8.76 (s, 1 H; H2^{Ad} or H8^{Ad}), 8.39 (s, 1 H; H2^{Ad} or H8^{Ad}), 8.16 (brs, 1 H; H6^{Cyt}), 8.02 (d, $^3J_{H,H}$=7.8 Hz, 2 H; H-Bz), 7.50–7.30 (m, 6 H; H-Bz/H^{triazole}), 7.31 (d, $^3J_{H,H}$=7.2 Hz, 1 H; H5^{Cyt}), 6.17 (s, 1 H; H1′^{Ad}), 6.07 (s, 1 H; H1′^{Cyt}), 5.68 (m, 1 H; H3′^{Ad}), 5.27 (m, 1 H; H2′^{Ad}), 5.12 (m, 2 H; H3′^{Cyt}/H4′^{Ad}), 4.96 (m, 1 H; CH), 4.55 (m, 2 H; H5′^{Cyt}), 4.19 (m, 5 H; H4′^{Cyt}/H5′^{Cyt}/CH$_2$O), 3.75 (m, 2 H; H5′^{Ad}), 2.73 (m, 4 H; H2′^{Ad}/H2′$_a^{Cyt}$/CH$_2$CN), 2.16 (s, 3 H; H-Me^{Ac}), 1.52 (d, $^3J_{H,H}$=6.8 Hz, 3 H; CH$_3$), 1.41 (s, 9 H; H-*t*Bu^{Boc}), 0.68 (s, 9 H; H-*t*Bu^{TBS}), −0.15 (s, 3 H; H-Me^{TBS}), −0.22 ppm (s, 3 H; H-Me^{TBS}); ^{13}C NMR (63 MHz, CDCl$_3$): δ=184.7 (C=O^{Boc}), 171.2 (C=O–Ac), 162.8 (C=O^{Cyt}), 155.4 (C=O–Bz), 152.7 (C2^{Ad} or C8^{Ad}), 151.4 (Cq), 150.1 (Cq), 145.2 (C6^{Cyt}), 142.1 (C2^{Ad} or C8^{Ad}), 132.9 (C-Bz), 128.8 (C-Bz), 128.2 (C-Bz), 124.0 (Cq-triazole), 122.0 (CH^{triazole}), 96.8 (C5^{Cyt}), 91.0 (C1′^{Ad}), 87.6 (C1′^{Cyt}), 86.4 (C4′^{Cyt}), 79.4 (Cq-*t*Bu^{Boc}), 79.2 (C4′^{Ad}/C3′^{Cyt}), 75.0 (C2′^{Ad}), 66.3 (C5′^{Cyt}), 62.8 (CH$_2$O), 61.2 (C5′^{Ad}), 60.4, 55.9 (CH), 42.9 (C3′^{Ad}), 39.7 (C2′^{Cyt}), 29.4 (Cq-*t*Bu^{TBS}), 28.4 (C-*t*Bu^{Boc}), 25.4 (C-*t*Bu^{TBS}), 24.8 (C-Me^{Ac}), 21.5 (CH$_3$), 19.7 (CH$_2$CN), −5.0(C-Me^{TBS}), −5.4 ppm (C-Me^{TBS}); ^{31}P NMR (^{1}H decoupled, 162 MHz, CDCl$_3$): δ=−0.68, 1.45 ppm (2×s, diast); HRMS (ESI): *m*/*z*: calcd for $C_{46}H_{63}N_{13}O_{13}PSi$: 1064.4175 [*M*+H]$^+$; found: 1064.4170.

Compound 14: Adenosine derivative **9a** (88 mg, 129 μmol) was coupled to Ac-dC-PCNE-phosphoramide as described in the General Procedure to give dinucleotide **14** (97 mg, 70%) as two diastereomers. ^{1}H NMR (250 MHz, CDCl$_3$): δ=9.60 (m, 2 H; NHAc/NHBz), 8.73 (s, 1 H; H2^{Ad} or H8^{Ad}), 8.41 (d, *J*=7.0, 1 H; H6^{Cyt}), 7.83 (d, $^3J_{H,H}$=7.3, 3 H; H2^{Ad} or H8^{Ad}/H-Bz), 7.41 (m, 5 H; H5^{Cyt}/H-Bz/H^{triazole}), 6.34 (s, 1 H; H1′^{Ad}), 6.09 (m, 2 H; H1′^{Cyt}/NH), 5.51 (m, 1 H; H3′^{Ad}), 5.03 (m, 3 H; H2′^{Ad}/H4′^{Ad}/H3′^{Cyt}), 4.50 (m, 1 H; CH), 4.22 (m, 5 H; H4′^{Cyt}/H5′^{Cyt}/CH$_2$O), 3.72 (m, 2 H; H5′^{Ad}), 2.68 (m, 4 H; H2′^{Ad}/H2′$_a^{Cyt}$/CH$_2$CN), 2.34 (m, 1 H; H2′$_b^{Cyt}$), 2.20 (s, 3 H; H-Me^{Ac}), 1.60 (m, 3 H; CH$_3$), 1.40 (s, 9 H; H-*t*Bu^{Boc}), 0.07 (2 s, 9 H; H-*t*Bu^{TBS}), −0.20 (2×s, 3 H; H-Me^{TBS}), −0.43 ppm (2×s, 3 H; H-Me^{TBS}); ^{31}P NMR (^{1}H decoupled, 162 MHz, CDCl$_3$): δ=−0.15, 1.98 ppm (2×s, diast). HRMS (ESI): *m*/*z*: calcd for $C_{46}H_{63}N_{13}O_{13}P_1SiNa$: 1086.3995 [*M*+Na]$^+$; found: 1086.3989.

General procedure for dinucleotide phosphorylation: Bis(2-cyanoethyl)-diisopropylphosphoramidite (5 equiv) was added neat to the flask containing the dinucleotide (1 equiv). Anhydrous CH$_2$Cl$_2$ (3.5 μL μmol^{-1}) was then added, followed by a solution of tetrazole in CH$_3$CN (0.45 M, 20 equiv). The mixture was stirred at room temperature for 1 hour, and a solution of I$_2$ (0.1 M, 5 equiv) was added. After having been stirred at room temperature for 30 min, the mixture was diluted with EtOAc and washed successively with aqueous saturated Na$_2$S$_2$O$_3$ solution and brine. The organic layer was dried over anhydrous Na$_2$SO$_4$, concentrated to dryness and then dissolved in a solution of MeNH$_2$ (5 M, large excess). The reaction mixture was stirred for 12 h at room temperature and concentrated under reduced pressure. The residue was purified by HPLC. After the appropriate fractions had been collected and lyophilized, the phosphorylated product was obtained as an NH$_4^+$ salt.

Compound 12: Dinucleotide **11** (56 mg, 52.6 μmol) was treated with bis(2-cyanoethyl)diisopropylphosphoramidite (71 mg, 263 μmol) in the presence of tetrazole (2.76 mL, 1.05 mmol) as described in the General Procedure and was then oxidized with I$_2$ (3 mL, 263 μmol) to give the NH$_4^+$ salt of the phosphorylated product **12** (27 mg, 54%) as a white solid. HPLC retention time: 37 min; ^{1}H NMR (250 MHz, CD$_3$OD): δ=8.69 (s, 1 H; H2^{Ad} or H8^{Ad}), 8.21 (s, 1 H; H2^{Ad} or H8^{Ad}), 8.09 (d, $^3J_{H,H}$=7.6, 1 H; H6^{Cyt}), 8.00 (s, 1 H; H^{triazole}), 6.78 (d, $^3J_{H,H}$=5.7 Hz, 1 H; H1′^{Ad}), 6.25 (m, 1 H; H1′^{Cyt}), 5.95 (d, $^3J_{H,H}$=7.6 Hz, 1 H; H5^{Cyt}), 5.57 (m, 1 H; H3′^{Ad}), 5.21 (m, 1 H; H2′^{Ad}), 4.92 (m, 3 H; H3′^{Cyt}/H4′^{Ad}/CH), 4.34 (s, 1 H; H4′^{Cyt}), 4.30–4.03 (m, 4 H; H5′^{Ad}/H5′^{Cyt}), 2.50 (m, 1 H; H2′$_a^{Cyt}$), 2.16 (m, 1 H; H2′$_b^{Cyt}$), 1.50 (d, $^3J_{H,H}$=7.0, 3 H; CH$_3$), 1.44 (s, 9 H; H-*t*Bu^{Boc}), 0.59 (s, 9 H; H-*t*Bu^{TBS}), −0.12 (s, 3 H; H-Me^{TBS}), −0.29 ppm (s, 3 H; H-Me^{TBS}); ^{13}C NMR (63 MHz, CD$_3$OD): δ=175.5 (C=O–Boc), 165.7 (C=O^{Cyt}), 157.2 (Cq), 154.0 (Cq), 151.7 (C2^{Ad} or C8^{Ad}), 151.0 (Cq), 143.6 (C2^{Ad} or C8^{Ad}), 141.2 (C6^{Cyt}), 130.4 (Cq), 124.4 (CH^{triazole}), 119.9 (Cq), 116.2 (Cq), 96.2 (C5^{Cyt}), 89.7 (C1′^{Ad}), 87.6 (C1′^{Cyt}), 87.1 (C4′^{Cyt}), 82.8 (C4′^{Ad}), 80.3 (Cq-*t*Bu^{Boc}), 77.7 (C3′^{Cyt}), 76.9 (C2′^{Ad}), 66.3 (C5′^{Cyt}), 66.0 (C5′^{Ad}), 63.4 (C3′^{Ad}), 44.2 (CH), 40.8 (C2′^{Cyt}), 28.8 (C-*t*Bu^{Boc}), 25.9 (C-*t*Bu^{TBS}), 20.9 (CH$_3$), 18.4 (Cq-*t*Bu^{TBS}), −3.7 (C-Me^{TBS}), −4.1 ppm (C-Me^{TBS}); ^{31}P NMR (^{1}H decoupled, 162 MHz, CDCl$_3$): δ=1.98, 0.17 ppm (2Σs).

Compound 15: Dinucleotide **14** (7 mg, 6.6 μmol) was treated with bis(2-cyanoethyl)diisopropylphosphoramidite (9 mg, 33 μmol) in the presence of tetrazole (0.345 mL, 132 μmol) as described in the General Procedure and was then oxidized with I$_2$ (0.75 mL, 33 μmol) to give the NH$_4^+$ salt of the phosphorylated product **15** (4 mg, 64%) as a white solid. HPLC retention time: 35 min; ^{31}P NMR (^{1}H decoupled, 162 MHz, CDCl$_3$): δ=1.52, 0.55 ppm (2×s).

General procedure for *N*-Boc and *O*-TBS removal: The partially protected dinucleotide **12** or **15** was treated with a mixture of aqueous HCl (6 N)/THF/MeOH 1:2:1 at room temperature for 24 h. The reaction mixture was then concentrated under vacuum, diluted with water and washed with CH$_2$Cl$_2$. The aqueous layer was evaporated under reduced pressure, and the residue was purified by HPLC. After the appropriate fractions had been collected and lyophilized, the final dinucleotide was obtained as an NH$_4^+$ salt.

Compound 13: The protected dinucleotide **12** (23 mg, 24.3 μmol) was treated with a mixture of aqueous HCl (6 N)/THF/MeOH (2 mL) as described in the General Procedure to give the NH$_4^+$ salt of the deprotected dinucleotide **13** (8 mg, 44%) as a white solid. HPLC retention time: 13 min; $[\alpha]_D^{25}$=−8.0 (*c*=0.27 in H$_2$O); ^{1}H NMR (500 MHz, D$_2$O): δ=8.57 (s, 1 H; H2^{Ad} or H8^{Ad}), 8.32 (s, 1 H; H^{triazole}), 8.18 (s, 1 H; H2^{Ad} or H8^{Ad}), 7.74 (d, $^3J_{H,H}$=7.6 Hz, 1H; H6^{Cyt}), 6.25 (d, $^3J_{H,H}$=6.4 Hz, 1H; H1$'^{Ad}$), 6.11 (t, J=6.5 Hz, 1H; H1$'^{Cyt}$), 5.81 (d, $^3J_{H,H}$=7.6 Hz, 1H; H5^{Cyt}), 5.75–5.61 (m, 1H; H3$'^{Ad}$/H4$'^{Cyt}$), 5.15 (m, 2H; H2$'^{Ad}$/H4$'^{Ad}$), 4.91–4.69 (m, 2H; CH/H3$'^{Cyt}$), 4.23 (m, 2H; H5$'_b{}^{Ad}$), 4.17 (d, $^2J_{H,H}$=15.0 Hz, 1H; H5$'_b{}^{Ad}$), 4.01 (sl, 2H; H5$'^{Cyt}$), 2.40 (m, 1H; H2$'_a{}^{Cyt}$), 1.97 (m, 1H; H2$'_b{}^{Cyt}$), 1.72 ppm (d, $^3J_{H,H}$=7.0 Hz, 3H; H-CH$_3$); ^{13}C NMR (126 MHz, D$_2$O): δ=168.0 (C=O^{Cyt}), 159.6 (Cq), 157.9 (Cq), 155.6 (C2^{Ad} or C8^{Ad}), 151.8 (Cq), 147.5 (Cq), 143.8 (C6^{Cyt}), 142.0 (C2^{Ad} or C8^{Ad}), 128.5 (CH^{triazole}), 121.0 (Cq), 98.8 (C5^{Cyt}), 89.9 (C1$'^{Ad}$), 88.2 (C1$'^{Cyt}$), 86.7 (C4$'^{Cyt}$), 83.8 (C4$'^{Ad}$), 78.5 (C3$'^{Cyt}$), 77.5 (C2$'^{Ad}$), 66.7 (C5$'^{Cyt}$), 67.7 (C5$'^{Ad}$), 64.8 (C3$'^{Ad}$), 45.8 (CH), 41.2 (C2$'^{Cyt}$), 20.4 ppm (CH$_3$); ^{31}P NMR (^{1}H decoupled, 202 MHz, D$_2$O): δ=1.98, 0.30 ppm (2×s); HRMS (ESI): m/z: calcd for $C_{23}H_{31}O_{12}N_{12}P_2$: 729.1665 $[M-H]^-$; found: 729.1665.

Compound 16: The protected dinucleotide **15** (4 mg, 4.23 μmol) was treated with a mixture of aqueous HCl (6N)/THF/MeOH (large excess) as described in the General Procedure to give the NH_4^+ salt of the deprotected dinucleotide **16** (1.8 mg, 58%) as a white solid. $[\alpha]_D^{25}=-7.0$ (c=0.03 in H$_2$O); ^{1}H NMR (500 MHz, D$_2$O): δ=8.56 (s, 1H; H2^{Ad} or H8^{Ad}), 8.20 (s, 1H; H2^{Ad} or H8^{Ad}), 8.09 (s, 1H; H^{triazole}), 7.73 (d, $^3J_{H,H}$=7.6 Hz, 1H; H6^{Cyt}), 6.14 (m, 2H; H1$'^{Ad}$/H1$'^{Cyt}$), 5.91 (d, $^3J_{H,H}$=7.8 Hz, 1H; H5$'^{Cyt}$), 5.66 (dd, $^3J_{H,H}$=2.8, 6.7 Hz, 1H; H3$'^{Ad}$), 5.34 (s, 1H; H4$'^{Ad}$), 5.30 (m, 1H; H2$'^{Ad}$), 4.99 (dd, J=6.7, 13.5 Hz, 1H; CH), 4.79 (m, 1H; H3$'^{Cyt}$), 4.23 (m, 3H; H4$'^{Cyt}$/H5$'^{Ad}$), 4.00 (dd, $^3J_{H,H}$=4.5, 7.5 Hz, 2H; H5$'^{Cyt}$), 2.40 (m, 1H; H2$'_a{}^{Cyt}$), 1.90 (m, 1H; H2$'_b{}^{Cyt}$), 1.72 ppm (d, $^3J_{H,H}$=6.9, 3H; CH$_3$); ^{13}C NMR (126 MHz, D$_2$O): δ=155.4 (C2 or C8^{Ad}), 143.4 (C6^{Cyt}), 142.1 (C2 or C8^{Ad}), 135.1 (CH^{triazole}), 98.8 (C5^{Cyt}), 88.3 (C1$'^{Cyt}$/C1$'^{Ad}$), 86.7 (C4$'^{Cyt}$), 83.1 (C4$'^{Ad}$), 77.9 (C3$'^{Cyt}$), 75.3 (C2$'^{Ad}$), 67.6 (C5$'^{Ad}$), 66.4 (C5$'^{Cyt}$), 62.8 (C3$'^{Ad}$), 42.5 (CH), 40.3 (C2$'^{Cyt}$), 21.5 ppm (CH$_3$); ^{31}P NMR (^{1}H decoupled, 202 MHz, D$_2$O): δ=2.73, 1.61 ppm (2×s); HRMS (ESI): m/z: calcd for $C_{23}H_{31}O_{12}N_{12}P_2$: 729.1665; found: 729.1647 $[M-H]^-$; HPLC retention time: 12 min.

Ligation of dinucleotides 13 or 16 with the microhelix and purification of product C or C′: The ligation reaction was performed at 37°C over 120 min in Hepes buffer (500 μL, 50 mM) containing the RNA microhelix (22 nt, 20 nmol), compound **13** or **16** (200 nmol), T4 RNA ligase (3.1 mg), DMSO (10%), ATP (1 mM) and MgCl$_2$ (15 mM). Compound **C** was purified by FPLC (Superdex 75 HR 10/30 column, Amersham Pharmacia Biotech) in Tris-HCl (pH 7.5, 25 mM), NaCl (100 mM), MgCl$_2$ (5 mM) (retention volume=12.40 mL, corresponding to a 21 kDa protein). After desalting, the product was lyophilised, dissolved in H$_2$O (100 μL, RNAse-free, Sigma) and quantified by UV absorption at $\lambda_{max}(\varepsilon)$=260 nm (2.3×10^5). Analysis of **C** or **C′** by denaturing polyacrylamide gel electrophoresis was carried out in an acrylamide gel (20 cm, 13%) containing urea (8M) over 120 min at 600 V/300 mA/50 W.

Radioactive coupled FemX$_{Wv}$ assay: The standard assay used Tris-HCl (50 mM, pH 7.5), alanyl-tRNA synthetase of *E. faecalis* (800 nM), ATP (7.5 mM), MgCl$_2$ (12.5 mM), [^{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 inhibitor **C** or **C′** (0 to 500 μM). The reaction was performed at 30°C for 10 min with a preincubation time of 10 min in the absence of FemX$_{Wv}$ for synthesis of Ala-tRNA^{Ala} by the auxiliary system. The reaction was stopped at 95°C for 10 min, and analysis was performed by descending paper chromatography (Whatman 4MM, Elancourt, France) with isobutyric acid/1M ammonia 5:3 v/v. Radioactive spots were identified by autoradiography, cut out and counted by liquid scintillation.

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