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Efficient Access to Peptidyl-RNA Conjugates for Picomolar Inhibition of Non-ribosomal $FemX_{Wv}$ Aminoacyl Transferase

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Dedicated to Professor Michel Therisod on the occasion of his 65th birthday

Abstract: Peptidyl–RNA conjugates have various applications in studying the ribosome and enzymes participating in tRNA-dependent pathways such as Fem transferases in peptidoglycan synthesis. Herein a convergent synthesis of peptidyl–RNAs based on Huisgen–Sharpless cycloaddition for the final ligation step is developed. Azides and alkynes are introduced into tRNA and UDP-MurNAc-pentapeptide, respectively. Synthesis of 2′-azido RNA helix starts from 2′-azido-2′-deoxyadenosine that is coupled to deoxycytidine by phosphoramidite chemistry. The resulting dinucleotide is deprotected and ligated to a 22-nt RNA helix mimicking the acceptor arm of Ala-$tRNA^{Ala}$ by T4 RNA ligase. For alkyne UDP-MurNAc-pentapeptide, *meso*-cystine is enzymatically incorporated into the peptidoglycan precursor and reduced, and L-Cys is converted to dehydroalanine with *O*-(mesitylenesulfonyl)hydroxylamine. Reaction of but-3-yne-1-thiol with dehydroalanine affords the alkyne-containing UDP-MurNAc-pentapeptide. The Cu^{I}-catalyzed azide alkyne cycloaddition reaction in the presence of tris[(1-hydroxypropyl-1*H*-1,2,3-triazol-4-yl)methyl]amine provided the peptidyl-RNA conjugate, which was tested as an inhibitor of non-ribosomal $FemX_{Wv}$ aminoacyl transferase. The bi-substrate analogue was found to inhibit $FemX_{Wv}$ with an IC_{50} of (89 ± 9) pM, as both moieties of the peptidyl–RNA conjugate contribute to high-affinity binding.

Keywords: click chemistry · inhibitors · peptidyl–RNA conjugates · RNA · transferases

Introduction

Peptidyl–RNA conjugates have various applications in functional and structural studies of the ribosome and for improving stability and cell permeability of short interfering RNAs.[1] Two strategies are currently explored for synthesis of peptidyl–RNA conjugates. In the divergent method, the peptide and oligonucleotide fragments are sequentially assembled by solid-phase synthesis on the same support. In the convergent method, the peptide and oligonucleotide fragments are independently synthesized prior to ligation. The first method is limited by the lack of a suitable solid support to assemble the peptide and oligonucleotide moieties and the lack of a uniform protection strategy suitable for any peptide sequence. A promising approach relies on polymerization of 3′-aminoacylamino-3′-deoxyadenosine linked to the solid support at the 2′ position.[2] The modified adenosine provides the C-terminal extremity of the peptide chain and the 3′ terminus of the oligonucleotides. Automated synthesis proceeds by Fmoc solid-phase synthesis followed by standard automated DMT/phosphoramidite synthesis. Successful protection of amino acid side chains is currently limited to Ser, Thr, Tyr, Asp and Glu.[3] To incorporate Arg in peptidyl–RNA conjugates, a convergent approach was developed which relies on a final native chemical ligation step.[4] This requires synthesis of a cysteinyl 3′-adenosine analogue resulting in the obligate presence of a cysteinyl residue at the C terminus of the peptide moiety of the conjugate. Here we develop a convergent synthetic route to peptidyl–RNA conjugates based on the Huisgen–Sharpless Cu^{I}-

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catalyzed azide–alkyne cycloaddition reaction (Cu-AAC) and apply the method to the synthesis of a picomolar inhibitor of non-ribosomal $FemX_{Wv}$ aminoacyl transferase.

Aminoacyl–tRNAs participate in various biosynthetic pathways as donors of activated aminoacyl residues in addition to their role in protein synthesis by the ribosome.[5] Among non-ribosomal aminoacyl transferases, $FemX_{Wv}$ from *Weissella viridescens*[6] catalyzes transfer of Ala from Ala-$tRNA^{Ala}$ to the amino group of L-Lys at the 3-position of peptidoglycan precursors[7] (Figure 1 A). Enzymes of the Fem family are attractive targets for the development of antibiotics active against resistant bacteria, since the residues incorporated by the enzymes are essential for the last cross-linking step of peptidoglycan polymerization in several important β-lactam-resistant pathogens such as staphylococci, pneumococci and streptococci.[8] Blocking Fem activity would therefore result in production of incomplete precursors acting as chain terminators that block formation of the essential stress-bearing peptidoglycan layer of the bacterial cell wall.

The alanyl-tRNA substrate of $FemX_{Wv}$ is produced by the alanyl-tRNA synthetase that aminoacylates the 3′-position of the terminal adenosine residue (A76) of $tRNA^{Ala}$ (Figure 1 A).[7] The resulting 3′-acylated tRNA binds to the acceptor site and delivers Ala to the ribosome. The same 3′ Ala-$tRNA^{Ala}$ regioisomer binds to $FemX_{Wv}$ with high affinity, although the enzyme catalyzes aminoacyl transfer only from the 2′-position following trans-esterification within the active site.[9] This enables $FemX_{Wv}$ to efficiently compete with the translation machinery for a common pool of Ala-$tRNA^{Ala}$.[9] The aminoacyl-transfer reaction catalyzed by $FemX_{Wv}$ may proceed by a substrate-assisted catalytic mechanism involving direct attack of the amino group of the peptidoglycan precursor on the carbonyl group of Ala attached to the 2′ position of the terminal ribose.[9] Our target (Figure 1 B) is a bi-substrate composed of a peptidoglycan precursor analogue covalently linked to an RNA helix mimicking the $tRNA^{Ala}$ acceptor arm. The convergent synthesis of the bi-substrate (Scheme 1) is based on a Cu-AAC reaction between alkyne UDP-MurNAc-pentapeptide **9** and 2′-azido RNA helix **11**.

Results and Discussion

For synthesis of 2′-azido RNA helix **11**, we started from the 2′-azido-2′-deoxyadenosine **1** (Scheme 1).[9,10] Coupling of **1** with commercially available Ac-dC-PCNE afforded dinucleotide **2**, which was phosphorylated and deprotected to obtain **3**. An additional deprotection step afforded dinucleotide **4**, which was ligated to 22-nt RNA helix **10** by T4 RNA ligase to obtain 24-nt azido RNA helix **11**.

Alkyne UDP-MurNAc-pentapeptides **9a** and **9b** were obtained by semisynthesis (Scheme 1). Briefly, UDP-MurNAc-pentapeptide analogue **6**, which contained *meso*-cystine instead of L-Lys, was obtained enzymatically by sequential addition of L-Ala, D-Glu, *meso*-cystine and D-Ala-D-Ala to UDP-MurNAc by Mur synthetases MurC, D, E and F from *Escherichia coli*, respectively.[11] The natural substrate of *E. coli* MurE, *meso*-diaminopimelic acid (*meso*-DAP), differs from *meso*-cystine only by replacement of a methylene by a disulfide group. This account for successful incorporation of *meso*-cystine instead of *meso*-DAP by MurE, albeit at the cost of 28-fold reduction in enzyme catalytic efficiency (data not shown). The *meso*-cystine-containing UDP-MurNAc-pentapeptide **6** was reduced by dithiothreitol to afford L-cysteine-containing UDP-MurNAc-pentapeptide **7**. Transformation of L-Cys of **7** into dehydroalanine of **8** was achieved with *O*-(mesitylenesulfonyl)hydroxylamine (MSH) by using procedures developed for proteins,[12] which proved compatible with the peptidoglycan precursor. In the last step, reaction of but-3-yne-1-thiol with dehy-

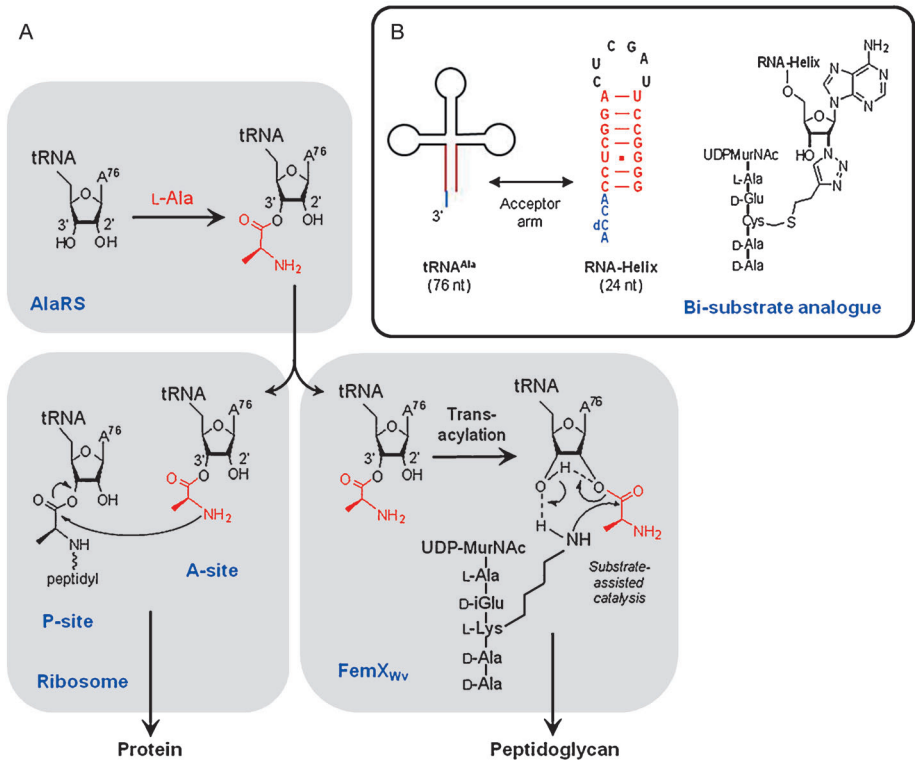
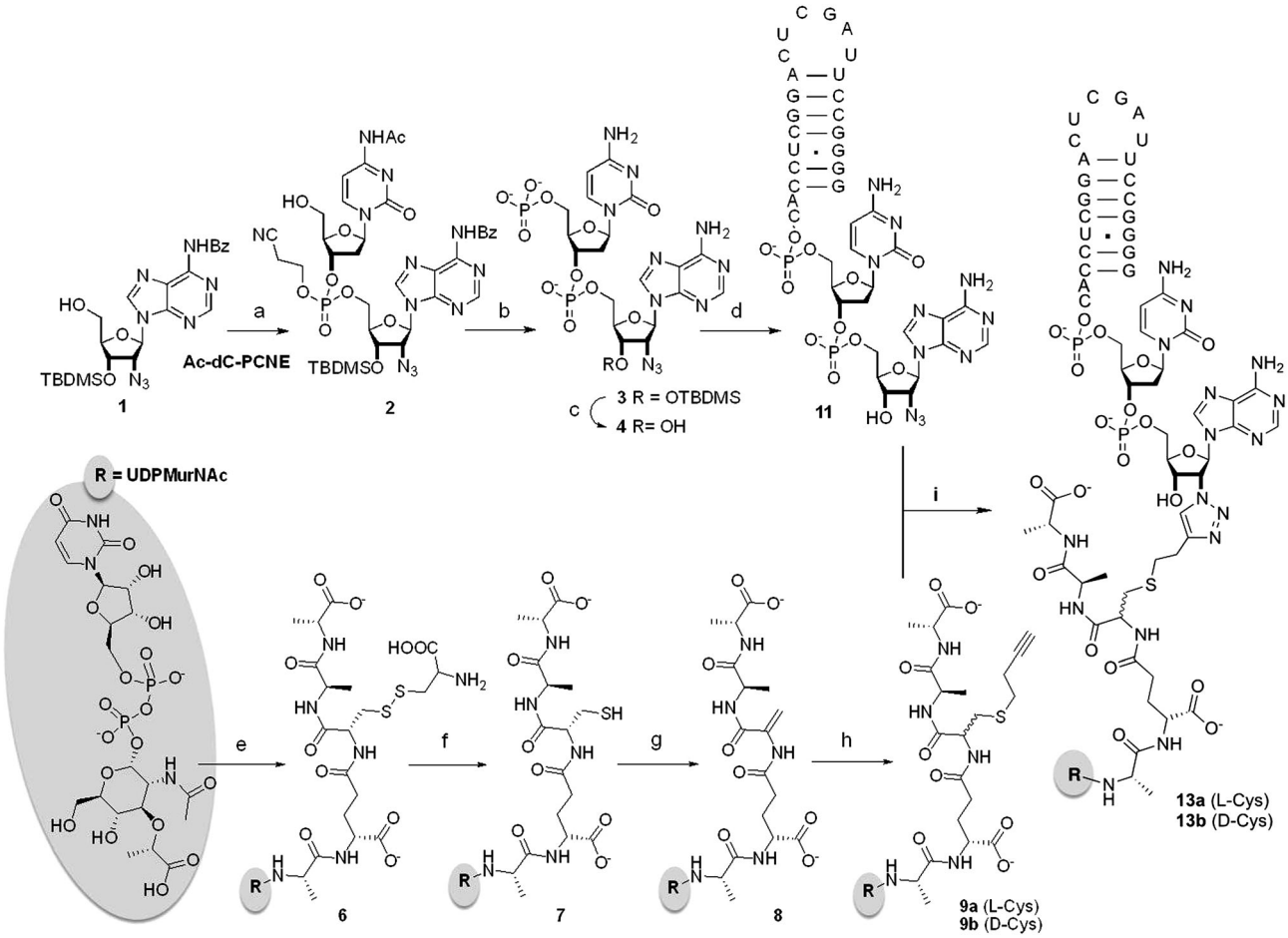


Figure 1. Participation of Ala-$tRNA^{Ala}$ in protein and peptidoglycan synthesis (A). Target peptidyl-RNA conjugate (B). The RNA helix mimics the acceptor arm (red) and ACCA extremity (blue) or $tRNA^{Ala}$.



Scheme 1. Convergent synthesis of peptidyl–RNA conjugates **13a** and **13b**. a) 1. Tetrazole, CH_2Cl_2, RT, 1 h; 2. I_2, 30 min, RT; 3. TCA, CH_2Cl_2, RT, 30 min; b) 1. bis(2-cyanoethyl)diisopropylphosphoramidite, tetrazole, CH_2Cl_2, RT, 1 h; 2. I_2, RT, 30 min; 3. CH_3NH_2, RT, 24 h; c) HCl (6 M)/THF/CH_3OH, 24 h; d) 22 nt RNA helix^{Ala}, T4 RNA ligase; e) 1. L-Ala, MurC; D-Glu, MurD; 2. *meso*-Cystine, MurE; 3. D-Ala-D-Ala, MurF; f) DTT, Tris-HCl buffer, pH 8.0, 2 h, RT; g) MSH, DMF, 2 h, RT; h) (*S*)-But-3-ynyl ethanethioate, DMF, 2 h, 37 °C; i) $CuSO_4$, THPTA, Na ascorbate, 4 °C, 24 h.

droalanine afforded UDP-MurNAc-pentapeptide analogues **9a** and **9b**, which contained the desired alkyne. Diastereoisomers **9a** and **9b**, which resulted from non-stereoselective thiol conjugate addition[13] to dehydroalanine **8**, were purified by HPLC.

Optimization of the cycloaddition reaction[14] was performed with 24-nt azido RNA helix **11** and commercially available 1,8-nonadiyne, which afforded compound **12** (Figure 2A). The reaction conditions for this critical step were azido-RNA helix **11** (0.1 mM), nonadiyne (5 mM), copper sulfate (0.5 mM), sodium ascorbate (5 mM) and the tris[(1-hydroxypropyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (THPTA) ligand (3.5 mM) in water for 24 h at 7 °C. The THPTA ligand[15] was used to stabilize Cu^I in aqueous buffer and avoid RNA degradation. These conditions provided the expected triazole analogue **12** in 70% yield, as detected by gel electrophoresis (Figure 2A) and mass spectrometry (calculated and observed masses of 7771.8 and 7772.1, respectively). The same approach was used to connect alkyne UDP-MurNAc-pentapeptides **9a** and **9b** (100 μM) to azido RNA helix **11** (50 μM; Scheme 1). The resulting bi-substrates **13a** and **13b** were purified by denaturing polyacrylamide gel electrophoresis (Figure 2A). These initial conditions were optimized by increasing the concentrations of UDP-MurNAc-pentapeptide **9a** (20 mM) and RNA helix **11** (1 mM), which increased the yield from 4 to 36%.

Interaction of **13a** with $FemX_{Wv}$ was analyzed by size-exclusion chromatography (Figure 3A). In the presence of a threefold molar excess of $FemX_{Wv}$, **13a** was quantitatively eluted as a complex. Highly purified bi-substrate **13a** was recovered from the corresponding fractions following phenol extraction to remove the protein (Figure 3B) and analyzed by mass spectrometry (calculated and observed masses of 8828.6 and 8828.4, respectively).

The RNA helices were tested as inhibitors of $FemX_{Wv}$ by using a coupled assay[16] that relies on aminoacylation of $tRNA^{Ala}$ by aminoacyl-tRNA synthetase AlaRS and transfer of Ala from the resulting Ala-$tRNA^{Ala}$ to UDP-MurNAc-pentapeptide by $FemX_{Wv}$ (Figure 2B). RNA helix **10** inhibited $FemX_{Wv}$ with an IC_{50} of (89 ± 4) μM.[9] Low affinity is accounted for by the presence of vicinal hydroxyl groups in the terminal adenosine, which are also present in the

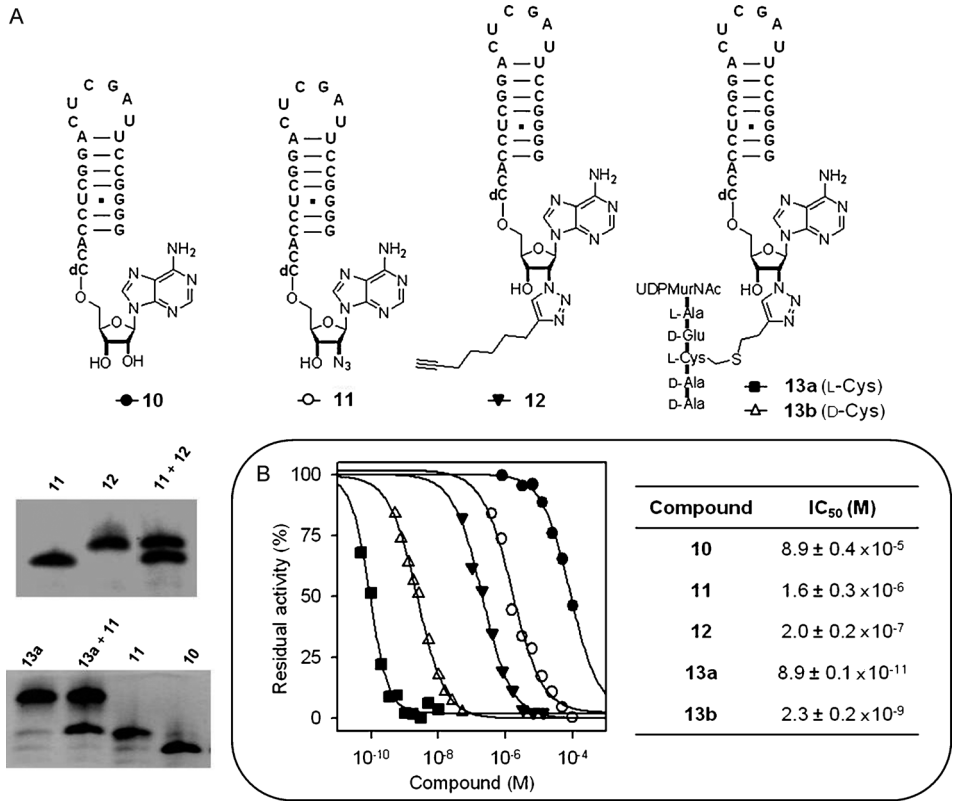


Figure 2. Inhibition of $FemX_{Wv}$. Structure of tRNA analogues and analysis by denaturing PAGE (A). IC_{50} of the tRNA analogues (B).

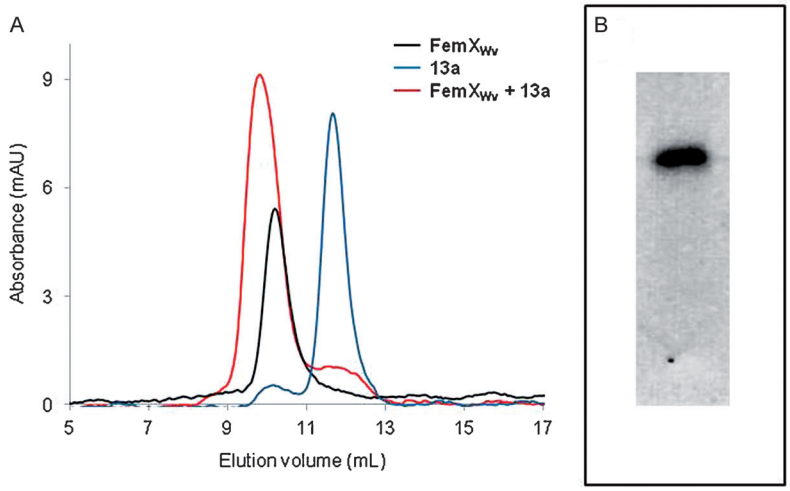


Figure 3. Isolation of the complex containing **13a** and $FemX_{Wv}$. Size-exclusion chromatography of the complex (A) and denaturing PAGE analysis of purified **13a** (B).

$tRNA^{Ala}$ product of the $FemX_{Wv}$-catalyzed reaction.[9] In agreement, substitution of 2′-hydroxyl by an azido group (helix **11**) led to a 56-fold decrease in the IC_{50} ((1.6 ± 0.3) μM). Addition of nonadiyne to **11** by the Cu-AAC reaction (compound **12**) led to a further eightfold decrease in the IC_{50} ((0.20 ± 0.02) μM). Thus, the nonadiyne carbon chain may mimic the L-Lys side chain of UDP-MurNAc-pentapeptide. Bi-substrate **13a** inhibited $FemX_{Wv}$ with an IC_{50} of 89 ± 9 pM, whereas diastereoisomer **13b** inhibited $FemX_{Wv}$ with an IC_{50} of 2.3 ± 0.2 nM. The L and D configurations of Cys were assigned to diastereoisomers **13a** and **13b**, respectively, based on the higher affinity of $FemX_{Wv}$ for **13a** and the presence of L-Lys at the analogous position of the natural substrate. Together, these results indicate that the RNA helix and UDP-MurNAc-peptide moieties of bi-substrate **13a** both contributed to high-affinity binding to $FemX_{Wv}$.

Conclusion

We have developed a convergent method to synthesize peptidyl–RNA conjugates based on Huisgen–Sharpless cycloaddition for the final ligation step. The reaction conditions reported here are fully compatible with two types of biomolecules, RNA and the peptidoglycan precursor. We also show that introduction of dehydroalanine as electrophilic site by using the MSH reagent is compatible with the peptidoglycan precursor containing nucleotide (UDP), sugar (MurNAc) and peptide (L-Ala-γ-D-Glu-L-Cys-D-Ala-D-Ala) moieties. Finally, association of both partners of the enzymatic acyl-transfer reaction in peptidyl-RNA **13a** induces an 18000-fold increase in $FemX_{Wv}$ inhibition efficiency and paves the way for the design of antibacterial agents acting on this new target.

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). Flash chromatography: silica gel, 180–240 mesh (Merck). Optical rotations were measured on a PerkinElmer 341 digital polarimeter. Spectra were

recorded on Bruker AVANCE 400 spectrometer for ^{1}H (400 MHz), ^{13}C (101 MHz) and ^{31}P (122 or 162 MHz) in $CDCl_3$, D_2O or DMSO. Chemicals shifts are expressed in parts per million (ppm) relative to residual $CHCl_3$ ($\delta = 7.26$) or HDO ($\delta = 4.79$) for ^{1}H and $CDCl_3$ ($\delta = 77.16$) for ^{13}C as internal references. Signals were assigned on the basis of DEPT 135, COSY, HSQC and HMBC. High-resolution mass spectra were carried recorded on a Bruker micrOTOF spectrometer. Unless otherwise specified, HPLC was performed on a HPLC system with C18 reverse-phase columns (analytical column, 250×4.6 mm, HYPERSIL-100 C18; semipreparative column, 250×21.2 mm, HYPERSIL HS C18; Thermoelectron Corporation). The solvent system consisted of 50 mM aqueous NH_4OAc:CH_3CN with a linear gradient (100:0 to 67:33) applied from 5 to 50 min at a flow rate of 1 (analytical) or 10 mL min^{-1} (semipreparative) and UV detection at 260 nm. For separation of UDP-MurNAc-peptides, fast protein liquid chromatography (FPLC) was performed on an Äkta purifier system (Amersham Pharmacia Biotech) with an analytical C18 reverse-phase column (Nucleosil 100-3 C18, 250×4.6 mm, Macherey-Nagel). The solvent system consisted of 50 mM aqueous NH_4OAc/CH_3CN with a linear gradient (100:0 to 95:5) applied from 7 to 37 min at a flow rate of 1 mL min^{-1} and UV detection at 260 nm. Analytical denaturing polyacrylamide gel electrophoresis (PAGE) was performed in gels (20×20×0.1 cm) containing acrylamide (13%, w/v), bis-acrylamide (4.4%, w/v), urea (8M), tetramethylethylenediamine (TEMED, 0.04% v/v), ammonium persulfate (0.08% w/v) and 1× TBE buffer (pH 8.2) [Tris (89 mM), borate (89 mM), EDTA (2 mM)]. Gels were loaded (lane width 6 mm) with RNA helices (100 pmol) in a volume of 10 μL containing bromophenol blue (0.01% w/v) and glycerol (25% v/v). Electrophoresis was performed for 120 min at 600 V, and gels stained with ethidium bromide (0.5 μg mL^{-1}) were imaged with a Herolab E.A.S.Y 429K camera. Preparative denaturing PAGE was performed under the same conditions, except that 1 nmol of RNA helices was loaded per lane and the concentration of ethidium bromide used for staining was reduced to 0.25 μg mL^{-1}. RNA bands were cut off from gels with a sterile scalpel and electroeluted for 2 h at 100 V in dialysis bags (cut-off 1 kDa; Spectra/Por 7 Dialysis Membrane, Spectrum Labs). RNA helices were concentrated to a final volume of 250 μL by ultrafiltration (Microcon, cut-off 3 kDa, Millipore), purified by gel filtration (Sephadex G-75 column 10/300 GL, GE Healthcare) equilibrated in Tris-HCl buffer (25 mM, pH 7.5) containing NaCl (100 mM) and $MgCl_2$ (5 mM).

(2*R*,3*S*,5*R*)-5-[4-Acetamido-2-oxopyrimidin-1(2*H*)-yl]-2-(hydroxymethyl)tetrahydrofuran-3-yl[(2*R*,3*S*,4*R*,5*R*)-4-azido-5-(6-benzamido-9*H*-purin-9-yl)-3-(*tert*-butyldimethylsilyloxy)tetrahydrofuran-2-yl]methyl 2-cyanoethyl phosphate (2): A 0.45M solution of tetrazole in MeCN (2.9 mL, 1.29 mmol) and Ac-dC-PCNE (250 mg, 0.324 mmol) were added to a solution of **1**[9,10] (66 mg, 0.130 mmol) in CH_2Cl_2 (700 μL) at room temperature under argon atmosphere. After stirring for 1 h at room temperature, 0.1M iodine in THF:H_2O:pyridine (75:2:20, 3.3 mL) was added. After 30 min, the reaction mixture was diluted with EtOAc (10 mL), washed with water (10 mL), aqueous saturated $Na_2S_2O_3$ solution (10 mL) and brine (10 mL), dried over anhydrous $MgSO_4$, filtered and concentrated at reduced pressure. The residue was then stirred with 0.18M trichloroacetic acid solution in dichloromethane (7.2 mL) at room temperature for 30 min. The reaction mixture was diluted with CH_2Cl_2 (5 mL) and the organic phase was washed with water (5 mL), aqueous saturated $NaHCO_3$ solution at 0°C (5 mL) and brine (5 mL), dried over anhydrous $MgSO_4$, filtered and concentrated. The crude product was purified by preparative TLC with CH_2Cl_2:MeOH (90:10) as eluent to give **2** (two diastereoisomers, ratio 1:1, 95 mg, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 9.44 (br, 1H, NH^{Ac}), 8.75 (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 8.31 (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 8.16 (dd, $J = 7.5$, 1.9 Hz, 1H, $H6^{Cyt}$), 8.02 (d, $J = 1.3$ Hz, 2H, Bz), 7.58 (ddd, $J = 7.2$, 5.1, 1.6 Hz, 1H, Bz), 7.49 (ddd, $J = 8.3$, 6.9, 2.5 Hz, 2H, Bz), 7.31 (dd, $J = 7.5$, 2.8 Hz, 1H, $H5^{Cyt}$), 6.15–6.05 (m, 2H, $H1'^{Ad}$, $H1'^{Cyt}$), 5.07 (ddd, $J = 8.6$, 5.9, 2.6 Hz, 1H, $H3'^{Cyt}$), 4.97–4.93 (m, 1H, $H3'^{Ad}$), 4.80–4.75 (m, 1H, $H2'^{Ad}$), 4.27–4.15 (m, 5H, OCH_2, $H4'^{Ad}$, $H5'^{Cyt}$), 3.77 (dd, $J = 8.7$, 2.7 Hz, 2H, $H5'^{Ad}$), 3.47 (s, 3H, Me^{Ac}), 2.76–2.71 (m, 2H, CH_2CN), 2.68–2.57 (m, 1H, $H2'a^{Cyt}$), 2.36–2.29 (m, 1H, $H2'_b{}^{Cyt}$), 0.97 (s, 9H, tBu^{TBS}), 0.21 (s, 3H, Me^{TBS}), 0.20 ppm (s, 3H, Me^{TBS}); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 170.7$ ($C{=}O^{Bz}$), 162.6 ($C{=}O^{Bz}$), 155.5 ($C2^{Ad}$ or $C8^{Ad}$), 145.3 ($C6^{Cyt}$), 142.5, ($C2^{Ad}$ or $C8^{Ad}$), 133.0, 128.9, 128.3 (Bz), 116.5 (Cq), 96.8 ($C5^{Cyt}$), 87.8 ($C1'^{Ad}$), 87.3 ($C1'^{Cyt}$), 77.5 ($C3'^{Cyt}$), 71.9 ($C3'^{Ad}$), 62.6 ($C2'^{Ad}$), 61.5 ($C5'^{Cyt}$), 61.3 ($C5'^{Ad}$), 39.7 ($C2'^{Cyt}$), 25.8 (tBu^{TBS}), 25.0 ($CH_3{}^{Ac}$), 18.5 (CH_2CN), 18.1 (Cq^{TBS}), −4.5, −4.8 ppm (2 × Me^{TBS}); ^{31}P NMR (^{1}H-decoupled, 122 MHz, DMSO): $\delta = -2.60$, −2.66 (2s); ESI$^+$ MS: *m/z* calcd for $C_{37}H_{47}N_{12}O_{11}PNaSi^+$ $[M+Na]^+$: 917.2886; found: 917.3253.

[(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-azido-3-(tert-butyldimethylsilyloxy)tetrahydrofuran-2-yl]methoxy}oxidophosphoryloxy)tetrahydrofuran-2-yl]methyl hydrogenphosphate (3): Bis(2-cyanoethyl)diisopropylphosphoramidite (88 mg, 320 μmol, 2.5 equiv) was added to phosphotriester **2** (115 mg, 130 μmol). Ultradry CH_2Cl_2 (400 μL) was added, followed by tetrazole in CH_3CN (2.8 mL, 1.3 mmol). The mixture was stirred at room temperature for 1 h, and I_2 in THF:H_2O:pyridine, (75:2:20, 3.2 mL, 320 μmol) was added. After being stirred at room temperature for 30 min, the mixture was diluted with EtOAc (5 mL) and washed successively with saturated aqueous $Na_2S_2O_3$ solution (5 mL) and brine (5 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated to dryness. The crude intermediate was dissolved in 15 mL of aqueous 5M $MeNH_2$. The reaction was stirred for 12 h at room temperature and concentrated under reduced pressure. The ammonium salt of dinucleotide **3** was purified by HPLC, lyophilized and recovered as a white solid (35 mg, 33%). $R_f = 0.2$ (CH_2Cl_2:MeOH 94:6); ^{1}H NMR (400 MHz, D_2O): $\delta = 8.47$ (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 8.30 (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 7.86 (d, $J = 7.7$ Hz, 2H, $H6^{Cyt}$), 6.19 (d, $J = 4.8$ Hz, 1H, $H5^{Cyt}$), 6.13–6.06 (m, 2H, $H1'^{Ad}$, $H1'^{Cyt}$), 4.97 (t, $J = 5.5$ Hz, 1H, $H3'^{Ad}$), 4.89–4.84 (m, 1H, $H3'^{Cyt}$), 4.34–4.28 (m, 2H, $H4'^{Ad}$, $H4'^{Cyt}$), 4.24–4.10 (m, 2H, $H5'^{Ad}$), 4.03 (s, 2H, $H5'^{Cyt}$), 2.42 (ddd, $J = 7.9$, 5.4, 2.7 Hz, 1H, $H2'^{Cyt}$), 1.93–1.85 (m, 1H, $H2'^{Cyt}$), 1.00 (s, 9H, tBu^{TBS}), 0.28 (s, 3H, Me^{TBS}), 0.27 ppm (s, 3H, Me^{TBS}); HPLC retention time: 24.7 min (MeCN:NH_4OAc 0:100 to 50:50); ESI$^-$ MS: *m/z* calcd for $C_{25}H_{37}N_{11}O_{12}P_2Si^-$ $[M-H]^-$: 774.1951; found: 774.1959.

[(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-azido-3-hydroxytetrahydrofuran-2-yl]methoxy}oxidophosphoryloxy)tetrahydrofuran-2-yl]methyl hydrogenphosphate (4): Partially protected dinucleotide **3** (34 mg, 44 μmol) was treated with aqueous 6M HCl:THF:MeOH (1:2:1, 2.8 mL) at room temperature for 24 h. The reaction mixture was concentrated in vacuo, diluted with water (3.5 mL) and washed with CH_2Cl_2 (3.5 mL). The aqueous layer was evaporated under reduced pressure, and the ammonium salt of dinucleotide **4** was purified by HPLC, lyophilized and recovered as a white solid (13.9 mg, 44%). ^{1}H NMR (400 MHz, D_2O): $\delta = 8.49$ (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 8.25 (s, 1H, $H2^{Ad}$ or $H8^{Ad}$), 7.81 (d, $J = 7.7$ Hz, 1H, $H6^{Ad}$), 6.13–6.10 (m, 2H, $H5^{Cyt}$, $H1'^{Ad}$), 6.08 (d, $J = 8.0$ Hz, 1H, $H1'^{Cyt}$), 4.87–4.84 (m, 2H, $H2'^{Ad}$, $H3'^{Cyt}$), 4.35 (br, 1H, $H3'^{Ad}$), 4.29 (br, 2H, $H4'^{Cyt}$, $H4'^{Ad}$), 4.22–4.12 (m, 2H, $H5'^{Ad}$), 4.02 (dd, $J = 4.6$, 2.6 Hz, 2H, $H5'^{Cyt}$), 2.40 (ddd, $J = 7.7$, 5.7, 1.9 Hz, 1H, $H2'_a{}^{Cyt}$), 1.92 −1.85 ppm (m, 1H, $H2'_b{}^{Cyt}$); ^{13}C NMR (101 MHz, D_2O): $\delta = 155.0$ ($C2^{Ad}$ or $C8^{Ad}$), 152.5 ($C2^{Ad}$ or $C8^{Ad}$), 141.8 ($C6^{Cyt}$), 139.7(Cq), 96.0 ($C1'^{Cyt}$), 85.7 ($C1'^{Ad}$), 85.5 ($C5^{Cyt}$), 84.7 ($C4'^{Cyt}$, $C4'^{Ad}$), 83.5 ($C3'^{Ad}$), 70.4 ($C2'^{Ad}$), 64.9 ($C3'^{Cyt}$), 64.5 ($C5'^{Cyt}$), 64.2 ($C5'^{Ad}$), 38.1 ppm ($C2'^{Cyt}$); ^{31}P NMR (^{1}H-decoupled, 162 MHz, D_2O): $\delta = 0.19$, −1.12 (2s); HPLC retention time: 19.2 min (MeCN:NH_4OAc 0:100 to 25:75 from 7 to 37 min); ESI$^-$ MS: *m/z* calcd for $C_{19}H_{24}N_{11}O_{12}P_2^-$ $[M-H]^-$: 660.1087; found: 660.1116.

UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-cystine (5): The reaction mixture (100 mL) contained Tris-HCl (100 mM, pH 8.6), $MgCl_2$ (40 mM), potassium phosphate (20 mM), ATP (5 mM), UDP-MurNAc-L-Ala-D-Glu (0.1 mM),[11] *meso*-cystine (0.5 mM) and His-tagged MurE from *E. coli* (8.7 mg).[17] After 18 h at 37°C under gentle stirring, the magnesium phosphate precipitate was removed by centrifugation and the supernatant was lyophilized. Product **5** was purified by gel filtration on a Sephadex G-25 column (115×2 cm) in water.[18] Yield, 8.4 μmol (80%). The reaction product was used without further purification, although analytical HPLC revealed the presence of UDP-MurNAc-L-Ala-D-Glu (ca. 10%) in addition to **5**.

UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-cystine-D-Ala-D-Ala (6): The reaction mixture (8 mL) contained Tris-HCl (100 mM, pH 8.6), $MgCl_2$ (30 mM), ATP (5 mM), UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-cystine **5** (1 mM), D-Ala-D-Ala (3 mM) and His-tagged MurF from *E. coli*

(90 μg).[19] After 3 h of incubation at 37 °C, the reaction mixture was lyophilized and product **6** was purified on a Sephadex G-25 column (115×2 cm) in water. Yield, 7 μmol (90 %). ESI MS: *m/z* calcd for $C_{40}H_{62}N_9O_{28}P_2S_2^-$ 1242.2626 $[M-H]^-$; found 1242.1827. The reaction product was used without further purification although analytical HPLC revealed the presence of UDP-MurNAc-L-Ala-D-Glu (ca. 10 %) in addition to **6**.

UDP-MurNAc-L-Ala-γ-D-Glu-L-Cys-D-Ala-D-Ala (7): Dithiothreitol (15.43 mg, 100 μmol) was added to **6** (3.3 mM, 3 μmol) in Tris-HCl buffer (50 mM, pH 8.0). After 2 h of incubation at room temperature, the product was purified by size-exclusion chromatography on a Sephadex G-25 column (30×1 cm) in water, lyophilized, dissolved in water and UV-quantified ($\varepsilon_{260\,nm}=10\,000\,M^{-1}cm^{-1}$, 2.7 μmol, 90 % yield). Purity was assessed by analytical FPLC on a C18 column and product **7** was used without further purification. Retention time: 22.0 min; ESI MS: *m/z* calcd for $C_{37}H_{57}N_8O_{26}P_2S^-$ $[M-H]^-$: 1123.2585; found 1123.1970.

UDP-MurNAc-L-Ala-γ-D-Glu-dehydroalanine-D-Ala-D-Ala (8): *O*-Mesitylenesulfonylhydroxylamine (MSH)[12] in DMF (7 mM, 7.04 μmol) was added to a solution of **7** (1.4 mM, 704 nmol) in phosphate buffer (50 mM, pH 8.0) at room temperature. Total conversion of Cys to dehydroalanine was typically obtained after 2 h of incubation at room temperature, as assessed by determining the sulfhydryl concentration with Ellman's reagent. Product **8** was purified by size-exclusion chromatography on a Sephadex G-25 column (30×1 cm) in water followed by FPLC on a C18 column. Product **8** was lyophilized, dissolved in water and UV-quantified ($\varepsilon_{260\,nm}=10\,000\,M^{-1}cm^{-1}$, 200 nmol, 28 % yield). Retention time: 20.2 min; ESI MS: *m/z* calcd for $C_{37}H_{55}N_8O_{26}P_2^-$ $[M-H]^-$: 1089.2708; found: 1089.2710.

UDP-MurNAc-L-Ala-γ-D-Glu-L-Cys(*S*-but-3-ynyl)-D-Ala-D-Ala (9a) and UDP-MurNAc-L-Ala-γ-D-Glu-D-Cys(*S*-but-3-ynyl)-D-Ala-D-Ala (9b): NaOH (1 M, 10 μmol) was added at room temperature to (*S*)-but-3-ynyl ethanethioate in DMF (1 M, 10 μmol). After 10 min phosphate buffer (80 μL, 250 mM, pH 8.0) and compound **8** in water (1 mM, 100 nmol) were sequentially added and the mixture was incubated for 2 h at 37 °C. The mixture of **9a** and **9b** was purified by size-exclusion chromatography on a Sephadex G-25 column (30×1 cm), lyophilized and dissolved in water. Diastereoisomers **9a** and **9b** were purified by FPLC on a C18 column, lyophilized and dissolved in RNAse-free water (Sigma) ($\varepsilon_{260\,nm}=10\,000\,M^{-1}cm^{-1}$; **9a**: 25 nmol, 25 %; **9b**:25 nmol, 25 %). Retention times: 36.5 and 33.9 min for **9a** and **9b**, respectively; ESI MS: *m/z* calcd for $C_{41}H_{61}N_8O_{26}P_2S^-$ 1175.2898 $[M-H]^-$; found: 1175.2820 and 1175.2893 for **9a** and **9b**, respectively.

2′-Azido-RNA helix (11): 2′-Azido-dinucleotide **4** was ligated to RNA-helix **10** (5′-GGGGCCUUAGCUCAGGCUCCAC-3′) mimicking the acceptor arm of the $tRNA^{Ala}$[6c] with purified T4 RNA ligase.[6c] RNA-helix **10** was synthesized by the phosphoramidite method and purified by polyacrylamide gel electrophoresis (Eurogentec). The ligation reaction was performed at 37 °C for 120 min in 500 μL of HEPES buffer (50 mM, pH 7.5) containing RNA helix **10** (40 nmol), 2′-azido-dinucleotide **4** (400 nmol), T4 RNA ligase (0.6 mg), DMSO (10 %), ATP (1 mM) and $MgCl_2$ (15 mM). The product of the ligation reaction, **11**, was purified by anion-exchange chromatography (DNAPac PA100, 250×4 mm; Dionex) with a linear ammonium acetate gradient (25 to 2500 mM, pH 8.0) containing 0.5 % acetonitrile, which was applied for 30 min at a flow rate of 1 mL min^{-1}.[6b] Fractions containing the ligation product were identified by PAGE, lyophilized, dissolved in RNAse-free water and stored at −20 °C ($\varepsilon_{260\,nm}=2.26\ 10^5\,M^{-1}cm^{-1}$; 15.4 nmol, 46 %); ESI MS: *m/z* calcd for $C_{227}H_{284}N_{93}O_{164}P_{23}$ [*M*]: 7651.6; found: 7651.5.

Nonadiyne derivative of 2′-azido-RNA-helix (12): 2′-Azido-RNA-helix **11** was coupled to 1,8-nonadiyne by Cu^I-catalyzed azido–alkyne cycloaddition (Cu-AAC) reaction. The mixture (50 μL in water) contained 2′-azido-RNA-helix **11** (2 nmol, 100 μM), 1,8-nonadiyne (5 mM), $CuSO_4$ (0.5 mM), Na ascorbate (5 mM) and the Cu^I-stabilizing ligand THPTA (3.5 mM).[15a] After 24 h of incubation at 4 °C, Cu–THPTA complex and Na ascorbate were removed by gel filtration on a Sephadex G-25 column (30×1 cm) in water. Product **12** was purified by denaturing PAGE followed by gel filtration. Concentration was determined spectrophotometrically ($\varepsilon_{260\,nm}=2.26\ 10^5\,M^{-1}cm^{-1}$; 1.4 nmol, 70 %). ESI MS: *m/z* calcd for $C_{236}H_{296}N_{93}O_{164}P_{23}$ [*M*]: 7771.8; found: 7772.1.

RNA-helix-UDP-MurNAc-pentapeptide bi-substrate (13a and 13b): 2′-Azido-RNA-helix **11** was coupled to **9a** or **9b** by Cu-AAC reaction. The mixture (200 μL in phosphate buffer, 25 mM, pH 8.0), which contained 2′-azido-RNA-helix **11** (5 nmol, 25 μM), **9a** or **9b** (20 nmol, 50 μM), $CuSO_4$ (0.5 mM), Na ascorbate (5 mM) and THPTA (3.5 mM), was incubated at 4 °C for 24 h. Compounds **13a** and **13b** were purified by denaturing PAGE and gel filtration as described above for compound **12** ($\varepsilon=2.36\times10^5\,M^{-1}cm^{-1}$ at 260 nm, 200 pmol; 4 %). The procedure was optimized by increasing the concentration of **9a** (20 mM, 200 nmol) and **11** (1 mM, 10 nmol) in the ligation step and by purifying **13a** by anion-exchange chromatography (DNAPac PA-100, Dionex) with a solvent system consisting of a 30 min linear gradient from 25 to 2500 mM of NH_4OAc (pH 8.0) containing 0.5 % CH_3CN at a flow rate of 1 mL min^{-1}. Compound **13a** was identified by denaturing PAGE and its concentration was determined spectrophotometrically ($\varepsilon_{260\,nm}=2.36\times10^5\,M^{-1}cm^{-1}$; 3.6 nmol, 36 %). ESI MS: *m/z* calcd for $C_{268}H_{346}N_{101}O_{190}P_{25}S$ [*M*]: 8828.6; found: 8828.4.

Analysis of the interaction of 13a with $FemX_{Wv}$ by size-exclusion chromatography: Bi-substrate **13a** (200 pmol) was incubated with $FemX_{Wv}$ (1200 pmol) for 10 min at 4 °C in 500 μL of buffer A (25 mM Tris-HCl, pH 7.5; 100 mM NaCl; 5 mM $MgCl_2$). The $FemX_{Wv}$-**13a** complex was loaded onto a Sephadex G-75 column (10/300 GL, GE Healthcare) equilibrated with buffer A and elution was performed at a flow rate of 0.8 mL min^{-1} at room temperature. Relevant fractions (5 mL) were pooled, concentrated to a final volume of 500 μL by ultrafiltration (3 kDa, Microcon, Millipore) and incubated for 10 min at 65 °C followed by 5 min at 0 °C. Compound **13a** was extracted twice with phenol (500 μL; phenol, water-saturated, stabilized, pH 4.1; Eurobio) and once with a phenol:chloroform solution (5:1 v:v, 500 μL, Sigma). The product was purified on a Sephadex 75 10/300 GL column in buffer A. Compound **13a** was concentrated to a final volume of 250 μL by ultrafiltration. The overall yield of the size-exclusion chromatography purification step was 43 % (85 pmol).

IC_{50} determination: *W. viridescens* $FemX_{Wv}$,[16] *E. faecalis* alanyl-tRNA synthetase (AlaRS)[7a] and T7 RNA polymerase[6c] were purified as previously described. Full-length $tRNA^{Ala}$ (5′-GGGGCCUUAGCUCAGCUGGGAGAGCGCCUGCUUUGCACGCAGGAGGUCAGCGGUUCGAUCCCGCUAGGCUCCACCA-3′) was obtained by in vitro transcription using T7 RNA polymerase.[6c] Inhibition of $FemX_{Wv}$ was tested in a radioactive coupled assay[8a] involving acylation of $tRNA^{Ala}$ by [^{14}C]Ala and transfer of [^{14}C]Ala from the resulting [^{14}C]Ala-$tRNA^{Ala}$ to UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) to form UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys(L-[^{14}C]Ala)-D-Ala-D-Ala ([^{14}C]UDP-MurNAc-hexapeptide). The standard assay mixture (10 μL) contained Tris-HCl (50 mM, pH 7.5), alanyl-tRNA synthetase (800 nM), ATP (7.5 mM), β-mercaptoethanol (2 mM), $MgCl_2$ (12.5 mM), L-[^{14}C]Ala (50 μM, 3700 Bq nmol^{-1}, ICN, Orsay, France), $tRNA^{Ala}$ (0.4 μM), UDP-MurNAc-pentapeptide (50 μM), $FemX_{Wv}$ (5 nM) and various concentrations of compounds **10**, **11** and **12**. Reactions were performed at 30 °C for 10 min and stopped at 95 °C for 10 min. Inhibition of $FemX_{Wv}$ by **13a** and **13b** was tested under the same conditions, except for the volume of the mixture (100 μL and 10 μL, respectively), $FemX_{Wv}$ concentration (0.05 μM and 0.5 μM, respectively) and incubation time (100 min for both). The reaction mixture was lyophilized and dissolved in water (10 μL). L-[^{14}C]Ala and [^{14}C]UDP-MurNAc-hexapeptide were separated by descending paper chromatography (Whatman 4MM, Elancourt, France) with isobutyric acid:1 M ammonium hydroxide (5:3). Radioactive spots were identified by autoradiography, cut out and counted by liquid scintillation. IC_{50} were determined by plotting residual activity versus inhibitor concentration. Curves represent fits of a four-parameter logistic function of experimental values (Sigma Plot 9.0).

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Peptidyl–RNA Conjugates

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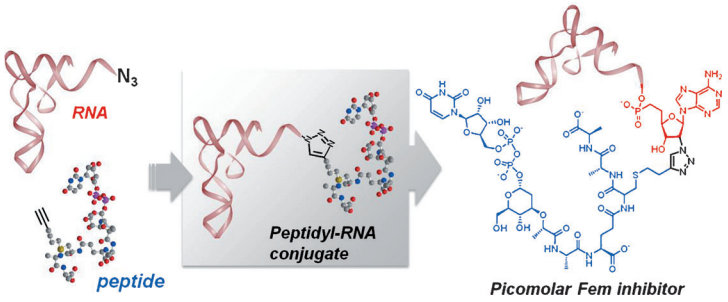
Efficient Access to Peptidyl-RNA Conjugates for Picomolar Inhibition of Non-ribosomal FemX$_{Wv}$ Aminoacyl Transferase



Conjugation of complex biomolecules: Peptidyl–RNA conjugates have various applications to study the ribosome and enzymes participating in tRNA-dependent pathways such as Fem transferases in peptidoglycan synthesis. Here, a convergent route for synthesis of peptidyl–RNAs based on Huisgen–Sharpless cycloaddition for the final ligation step is developed (see figure). Both moieties of the peptidyl–RNA conjugate contribute to high-affinity binding to FemX, providing a picomolar inhibitor.

