**Amino-acyl tXNA as inhibitors or amino acid donors in peptide synthesis.**

Lauriane Rietmeyer†[a], Inès Li De La Sierra Gallay†[b], Guy Schepers[c], Delphine Dorchène[a], Laura Iannazzo[d], Delphine Patin[b], Thierry Touzé[b], Herman van Tilburgh[b], Piet Herdewijn[c], Mélanie Ethève-Quelquejeu[d,*, and Matthieu Fonvielle[a,]****

[a] Lauriane Rietmeyer, Delphine Dorchène, Dr. Matthieu Fonvielle*, INSERM, Université Paris Cité, Sorbonne Université, Centre de Recherche des Cordeliers, F-75006 Paris, France
[b] Dr. Inès Li De La Sierra Gallay, Delphine Patin, Dr. Thierry Touzé, Dr. Herman van Tilburgh Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Saclay, 91198 Gif-sur-Yvette, France
[c] Guy Schepers, Dr. Piet Herdewijn, Laboratory of Medicinal Chemistry, Rega Institute for Biomedical Research, KU Leuven, Herestraat 49, Box 1041, 3000 Leuven, Belgium
[d] Dr. Laura Iannazzo, Dr. Mélanie Ethève-Quelquejeu, Université Paris Cité, CNRS, Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologiques, F-75006 Paris, France

**Supplementary information**

1. General information ......................................................................................................................................................... 1
2. Peptidoglycan (PG) synthesis in Gram-positive bacteria ..................................................................................................... 2
3. Mass spectrometry analysis of oligonucleotide-containing compounds ............................................................................. 3
4. Enzyme production and purification ....................................................................................................................................... 3
5. tRNA production and purification .......................................................................................................................................... 3
6. Purification of nucleotide precursor from bacterial extracts .................................................................................................. 3
7. Semi-synthesis. ........................................................................................................................................................................... 4
8. Enzymatic assays ....................................................................................................................................................................... 7
9. FemX crystallization ................................................................................................................................................................. 8
10. Crystal structure determination, and 3D supplementary figures ............................................................................................. 8
11. Mass spectrometry analyses of synthesized compounds .................................................................................................. 12
12. rpHPLC analysis of synthesized compounds ........................................................................................................................ 13
13. References ............................................................................................................................................................................. 22

**1. General information**

Solvents were dried using standard methods and distilled before use. Unless otherwise specified, materials were purchased from commercial suppliers and used without further purification.
2. Peptidoglycan (PG) synthesis in Gram-positive bacteria

PG is a giant mesh-like polymer consisting of glycan strands made of alternating β-1,4-linked N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues surrounding the bacteria (right inset in Supplementary Scheme S1). Glycan strands are cross-linked by short-branched peptides of various side chain composition (left inset in Supplementary Scheme S1) synthesized by Fem transferases. Synthesis of the branched peptide can be performed either from the soluble precursor UDP-MurNAc pentapeptide (Park’s nucleotide, Class I Fem, Supplementary Scheme S1) or the peptidy-undecaprenyl-lipid carrier bound to the cytoplasmic membrane (Lipid II, Class II Fem, Supplementary Scheme S1), depending on the bacterial species. In W. viridescens, FemX incorporates the first L-alanyl residue into Park’s nucleotide (Supplementary Scheme S1), whereas in S. aureus or Streptomyces pneumoniae, the Fem transferases incorporate the side chain residues onto the lipid II precursor (1). Completion of the branched-peptide synthesized by Fem transferases is paramount to the structural integrity of the cell wall and has an impact on susceptibility to antibiotics targeting cell-wall synthesis (2-5). Fem transferases are considered to be attractive targets for the development of novel antibiotics against multi-resistant bacteria (6-11), as these enzymes have a unique catalytic mechanism (12) and are essential for the synthesis of the appropriate substrate for D,D-transpeptidases, including low-affinity PBPs responsible for resistance to β-lactam antibiotics (13).

Scheme. S1. Main steps of peptidoglycan synthesis in Gram-positive bacteria using Park’s nucleotide soluble substrate. The assembly of the subunit starts in the cytoplasm by the synthesis of UDP-MurNAc. The following steps are catalyzed by Mur ligases sequentially adding L-Ala (MurC), D-iGlu (MurD), L-Lys (MurE), and the dipeptide D-Ala-D-Ala (MurF) to form the Park nucleotide (steps not shown in this scheme). In W. viridescens, the Fem aminoacyl transferases add aminoacyl residues of the side chain onto the Park nucleotide (Class I Fem), whereas this enzymatic step performs the same addition onto the Lipid II intermediate in S. aureus (class II Fem). Transfer of the phospho-MurNAc-pentapeptide moiety of Park’s nucleotide to the C55 lipid carrier undecaprenyl phosphate by MraY leads to lipid intermediate I (lipid I). The addition of GlcNAc by MurG transferase to lipid I leads to lipid intermediate II (lipid II). The resulting mature PG precursor is externalized and inserted into preexisting PG by penicillin-binding proteins (PBPs). The inset shows the composition of the side chains among Gram-positive bacteria (14).
3. Mass spectrometry analysis of oligonucleotide-containing compounds

Mass spectroscopy (MS) spectra were carried out on a LCQ-Deca XP-Max spectrometer in positive or negative ionization mode. Mass spectrometry analyses of oligonucleotides, azido-oligonucleotides, and lipid-carbohydrate-peptidyl-oligonucleotide conjugates were performed by LC-MS or by MALDI-TOF. MALDI-TOF was performed on a MALDI TOF-TOF UltraflexTreme (Bruker daltonic). Liquid chromatography (LC) was performed by anionic rpHPLC using a DNAPac RP column (4 µm; 2.1 x 50 mm; Thermo Fisher Scientific) at a flow rate of 300 µl min⁻¹ with a 0 to 50% MeOH linear gradient applied from 2 to 10 min in Solution A [Solution A, triethylamine (15 mM) and 1,1,1,3,3,3-hexafluoro-2-propanol (400 mM)]. For mass spectrometry (MS), liquid chromatography was coupled to a LCQ Deca XP-Max mass spectrometer operating in the negative mode.

4. Enzyme production and purification

FemX was produced in Escherichia coli TOP10 harboring derivatives of vector pTrc-His60. Protein was purified by nickel-affinity, anion-exchange, and size-exclusion chromatographies (15). Protein was concentrated to a final concentration of 10 mg mL⁻¹ by ultrafiltration and stored at -20 °C in 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl supplemented with 50% glycerol. The alanyl-tRNA synthetase of E. faecalis strain JH2-2 and the T4 RNA ligases were produced as previously described (16). MurC, MurD, MurE, and MurF were produced and purified as previously described (17). Concentration of proteins were determined by the Bradford assay (Biorad) using BSA as a standard.

5. tRNA production and purification

tRNA^{Ala} (5'-GGGGCCUAGCUCAGCGAGACGUCCGCUUUGCACG CAGGAGGUCAGCGGCUUCGAUCCGUAGGCCUCCACCA-3') corresponding to 76-nucleotides (76-nt) tRNA^{Ala} of E. faecalis strain V583 was obtained by in vitro transcription using T7 RNA polymerase as previously described (16).

6. Purification of nucleotide precursor from bacterial extracts

Park's nucleotide 5 was purified from vancomycin-susceptible S. aureus RN4220 strain. Bacteria were grown at 37°C in 2 L of brain heart infusion (BHI) to an optical density of 1.0 unit absorbance. Bacteria were further incubated in the presence of vancomycin (25 mg L⁻¹) for 2 hours at 37 °C. Bacteria were collected by centrifugation and transfer into boiling water (300 mL) and kept at 95°C for 30 min under agitation. The mixture was then cold to 0°C on an iced bath and trichloroacetic acid (TCA) was added to precipitate bacteria fragments (5% w/v). The mixture was kept for 30 min at 0°C and centrifuged at
4,000 rpm for 30 min. The supernatant was extracted three times with diethyl-ether (100 mL) to eliminate TCA. The aqueous phase containing the peptidoglycan precursor was desalted on a G-25 column, and lyophilized. Peptidoglycan precursor was solubilized in 0.1% TFA and purified by rpHPLC with a preparative C18 column (5 µm; 22 x 250 mm; Nucleosil, Macherey-Nagel) equilibrated with 0.1% TFA at a flow rate of 10 mL min⁻¹. A linear gradient of acetonitrile was applied from 0 to 20 % between 10 and 40 min. Fractions containing 5 were identified by mass spectrometry and lyophilized. The precursors were dissolved in water and quantified by UV absorption at 260 nm ($\epsilon_{260nm} = 10,000 \text{ M}^{-1} \text{ cm}^{-1}$). Yield: 45 µmol per litre of bacterial culture.

7. Semi-synthesis.

Synthesis of alkyne-containing UDP-MurNAc-peptide analogue. Semi-synthesis of the UDP-MurNAc-peptide analogue 4 was performed as previously described (Scheme S2) (17). Briefly, (i) Sequential addition of L-Ala, d-iGlu, and meso-cystine to uridine di-phospho-N-acetyl-muramic acid (UDP-MurNAc) by synthetases MurC, MurD, and MurE afford compound 1. (ii) Addition of d-Ala-d-Ala to UDP-MurNAc-tripeptide 1 by synthetase MurF lead to compound 2. (iii) One-pot reduction of disulfure bond and oxidation-elimination of the sulfhydryl group of the L-Cys of compound 2 as previously described (17). Briefly, the mixture comprised meso-cystine-containing precursor 2 (5 mM), TCEP (20 mM), tris-(2,5-dibromohexanediamide) (100 mM), potassium phosphate (100 mM, pH 8.0), and 20% DMF. The solution of tris-(2,5-dibromohexanediamide) (DBHD) was freshly prepared in DMF. Incubation was performed for 1 h at 37°C. The dehydroalanine containing precursor 3 was purified by size-exclusion chromatography (SuperDexpeptide 10/300 GL, general Electrics) in water (260 nm, 1 mL min⁻¹; RT = 8.1 min) followed by rpHPLC (EC 250/22 Nucleosil 100-5 C18; Macherey Nagel) with a linear gradient of acetonitrile (0 to 15%) in ammonium acetate (50 mM; pH 5.0) applied between 10 and 40 min (260 nm, 10 mL min⁻¹). RTs = 16.8 min for 3. Yields: 30%. (iv) Addition of 3-butyne-1-thiol (50 mM) to 3 (0.5 mM) was performed in DMF-phosphate buffer (100 mM; pH 8.0) (1/19, v/v) for 2.5 h at 37°C. After the addition of 1 volume of buffer A (50 mM ammonium acetate, pH 5.0), the two resulting diastereoisomer were separated by rpHPLC (EC 250/4.6 Nucleosil 100-5 C18; Macherey Nagel) with a linear gradient of acetonitrile (0 to 5%) in ammonium acetate (50 mM; pH 5.0) applied between 10 and 40 min at a flow rate of 1 mL min⁻¹. The final product 4 was lyophilized and dissolved in RNAse free water (Sigma-Aldrich). Purity of the two diastereoisomers was assessed by mass spectrometry and analytical rpHPLC. Concentrations were determined by spectrophotometry ($\epsilon_{260nm} = 10,000 \text{ M}^{-1} \text{ cm}^{-1}$). Yields: 19.3%.
Scheme S2. (i) L-Ala, MurC; 2. D-Glu, MurD; 3. meso-cystine, MurE; (ii) D-Ala-D-Ala, MurF; (iii) TCEP, DBHD, Phosphate buffer/DMF (4/1 v/v), 1 h, 37°C; (v) 3-butyne-1-thiol, DMF, 2 h, 37°C.

Synthesis of oligonucleotides by solid-phase synthesis. The oligonucleotide helices comprising RNA, 2'-F-RNA, HNA, 2'F-ANA and DNA nucleotides used in this study have the sequence corresponding to the acceptor arm of tRNA<sup>Ala</sup>. Two types of oligonucleotides were obtained by solid phase synthesis. First, 22-nt helices 6a to 11a (5'-GGGGCCUUAGCUCAGGCUCCAC-3') mimicking the acceptor arm of tRNA<sup>Ala</sup>. Second, 8-nt micro-helices 22 to 25 constituted of a 2-nt (5'-GG-3') covalently bound to a 6-nt (5'-CCACCA-3') by a hexapolyethylene glycol linker (Glen Research) mimicking the terminal part of the acceptor arm of tRNA<sup>Ala</sup>. The ribose at the 3'-terminus of the 8-nt contained a 2'-azido moiety. Production of the resin substituted by the 2'-azido nucleotide were performed as previously described (15).

Oligonucleotide assembly was performed on an Expedite® 8909 DNA synthesizer (applied Biosystems) by using the phosphoroamidite approach. The standard DNA assembly protocols were adjusted for HNA 420 sec and RNA 600 sec coupling time. Oligomers were deprotected and cleaved from the solid support by treatment with AMA solution, half part composed of ammonia 33% and half of methylamine 40% ) for 2h at 40°C. After gel filtration on NAP-25® column (Sephadex G25-DNA grade; Pharmacia) with ethanol 10%, the crude mixture was lyophilized. The desililation procedure was been achieved by treating the crude with a solution made by N-methylpyrrolidone, triethylamine, hydrogen fluoride [NMP/TEA/TEA.3HF (6/3/4)] and then neutralized by treating with a solution 1.5 M of NH<sub>4</sub>OAc. After gel filtration on NAP-25® column (Sephadex G25-DNA grade; Pharmacia) with ethanol 10%, the crude mixture was analyzed on a Mono Q TM 10/100 column (Pharmacia) ion exchange column at gradient (pH=7.4) which purification was achieved with the following gradient system (A= 10 mM NaClO<sub>4</sub> (pH=7.4), 20 mM Tris-HCl, 0.1 mM EDTA and 15% CH<sub>3</sub>CN); B= 600 mM NaClO<sub>4</sub> (pH= 7.4), 20 mM Tris-HCl, 0.1 mM EDTA and 15% CH<sub>3</sub>CN). The high-pressure liquid chromatography system consisted of a Primaide-Hitachi 1110 pump, a Mono Q TM 10/100 column (Pharmacia) a Uvicord SII 1410 UV detector (Pharmacia-LKB) and a recorder. The product-containing fraction was desalted on a NAP-25® column and lyophilized, ready for mass analysis to test purity. Spectra were acquire on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were
infused at 3 μL min\(^{-1}\) and spectra were obtained in positive (or negative) ionization mode with a resolution of 15,000 (FWHM) using leucine enkephalin as lock mass.

**Ligation of azido-dinucleotide to oligonucleotides helices.** pdCpA-2'-azido was ligated to helices 7a to 11a with purified T4 RNA ligase as previously described (18,19). The azido containing helices 12 to 16 were purified by anion exchange chromatography (DNA Pac-100, Dionex) with a two-step gradient of ammonium acetate pH 8.0 containing 0.5 % acetonitrile (step 1 from 25 to 750 mM between 2 and 3 min and step 2 from 750 to 2,500 mM applied between 3 and 22 min). Fractions containing ligation products were lyophilized, resuspended in RNAse free water (Sigma), and stored at -20°C. The concentrations of the azido containing helices 12 to 16 were determined spectrophotometrically (\(\varepsilon_{260\text{nm}} = 226,000 \text{ M}^{-1} \text{cm}^{-1}\)) and the purity of the molecules was assessed by denaturing urea-bisacrylamide gel (Figure S1), mass spectroscopy (Supplementary section 11) and analytical HPLC (Supplementary section 12).

**Synthesis of peptidyl-oligonucleotide conjugates (17-21).** Each 2'-azido-oligonucleotide helices 12 to 16 and micro-helices 22 to 25 were coupled to UDP-MurNAc-L-Ala-\(\Delta\)-Glu-L-Cys(S-but-3-ynyl)-\(\Delta\)-Ala-\(\Delta\)-Ala 4 by Cu-AAC reaction in the presence of tris[(1-hydroxy-propyl-1H-1,2,3-triazole-4-yl)methyl]amine (THPTA) to stabilize Cu\(^{I}\) and avoid RNA degradation (17). Reactions were performed at 37 °C for 2 h in 20 μL of phosphate buffer (100 mM, pH 8.0) containing 2'-azido-oligonucleotides (12 to 16 and 22 to 25) (50 nmol, 2.5 mM), alkyne peptide 4 (50 nmol, 2.5 mM), CuSO\(_4\) (0.5 mM), Na-ascorbate (5 mM), and THPTA (3.5 mM). Peptidyl-oligonucleotide products of the reactions (17 to 21 and 26 to 29) were purified by anion-exchange chromatography (DNA Pac PA-100, Dionex) at a flow rate of 2 mL min\(^{-1}\) in 25 mM of NH\(_4\)OAc (pH 8.0) containing 0.5% CH\(_3\)CN with a linear gradient (25 to 1,250 mM NH\(_4\)OAc) applied between 5 and 20 min. Fractions containing peptidyl-oligonucleotides were

---

**Figure S1.** Analysis of ligation by acrylamide-urea denaturing gel electrophoresis. 500pmol of each product were loaded onto the gel. 20 cm high, 0.1cm width, 7M urea, 4% acrylamide gel stacking (5cm high) and 12% acrylamide gel separating (15 cm high). 1h at 600 V followed by BET staining.
lyophilized and dissolved in RNA-free water (Sigma). The concentration of peptidyl-oligonucleotide adducts were determined by UV spectrophotometry at 260 nm (ε<sub>260 nm</sub> = 236,000 and 85,500 M<sup>-1</sup> cm<sup>-1</sup> for 24- and 8-nt peptidyl-oligonucleotides, respectively), analyzed by mass spectroscopy (Supplementary section 11), and by analytical HPLC (Supplementary section 12).

**Ligation of N-pentenoyl-L-alanyl-dinucleotide (pdCpA-2'-L-Ala-pentenoyl) to 22-nt helices.** Modified dinucleotide pdCpA-L-alanyl-pentenoyl was ligated to helices 6a to 11a with purified T4 RNA ligase as previously described (18,19). Briefly, 1 nmol (40 µM) of each helices were dissolved in 25 µL of a solution containing 50 mM sodium HEPES buffer (pH 7.5), MgCl<sub>2</sub> (15 mM), ATP (1 mM), DMSO (10% v/v), pdCpA-L-alanyl-pentenoyl (400 µM), and T4 RNA ligase (0.6 mg mL<sup>-1</sup>). After 2h of incubation at 37°C, 250 µL of cold ethanol were added and the mixtures were kept 1h at -65°C. After 30 min of centrifugation at 16,400 rpm and elimination of supernatants, products were washed by 500 µL of a cold 70% ethanol solution, centrifuged for 10 min and supernatants discarded. Ligation products were dry 5 min under reduced pressure (SpeedVac) and were kept at -20°C until removal of the pentenoyl protecting group.

**Iodine mediated L-alanyl deprotection.** The deprotection procedure was performed with iodine extemporaneously before enzyme assay as previously described (20). Briefly, each ligation product was dissolved in free RNAse water (40 µL, Sigma-Aldrich) and 10 µL of a solution of iodine (25mM) dissolved in a THF/H<sub>2</sub>O solution (1/1; v/v) were added. After 10 min of incubation at room temperature, the deprotected L-alanyl-helices were precipitated by the addition of cold ethanol (1 mL). Each product 6b to 11b was kept at -65°C for 30 min, centrifuged (30 min; 16,400 rpm; 4°C), and washed two times with a cold 70% ethanol solution (500 µL). The supernatants were discarded and the L-alanyl-helice pellet was dry for 5 min under reduced pressure (SpeedVac) and used immediately for kinetic studies.

**8. Enzymatic assays**

**Determination of the efficacy of FemX with aminoacylated helices.** FemX activity was determined at 21°C in 20 µl of 50 mM ammonium acetate (pH 7.2) containing, enzyme (50 nM), Park’s nucleotide 5 (50 µM), and amino-acylated helices 6b to 11b (50 µM if quantitative ligation). After 15, 30, 60, 120, 300, and 1,100 min, 2.5 µL of the reaction was quenched by addition of 2.5 µL of a solution of water/acetonitrile/formic acid (50/50/0.1) and 1.25 µL of a solution of <sup>13</sup>C and <sup>15</sup>N labelled UDP-MurNAc-hexapeptide ([<sup>13</sup>C-<sup>15</sup>N]-L-Ala)-UDP-MurNAc-pentapeptide (67 µM; 84 pmol) as an internal standard. 5 µL of the resulting solution was analyzed by MS on positive mode. Concentrations of UDP-MurNAc-hexapeptide synthetized during incubations times were determined by comparing the sum of the intensity of peaks corresponding to the [M+H]<sup>+</sup>, [M+Na]<sup>+</sup> of the product with the corresponding peaks of the [<sup>13</sup>C-<sup>15</sup>N]-hexapeptide internal standard. Velocities of the L-Ala transfer from various helices to UDP-MurNAc-pentapeptide were calculated using the linear part of the curves (0, 15, and 30 min). To avoid biases caused by differences in concentrations of acylated amino helices resulting from ligation,
precipitation, and deprotection steps during synthesis, transfer efficacies were calculated by dividing the velocity of each reaction by the final product concentration determined at 1,100 min.

For L-analy transfer from 2′F-ANA and DNA aminoacylated helices, signal/noise ratio were too small to detect transfer reaction. To be sure that ligation and deprotection steps were correctly done, a sample of each reaction mixture (2.5 μL) were taken and a large excess of FemX (2 μM) was added. After 15 min of incubation, the internal standard was added. For each sample, the reaction product was observed indicating that ligation, deprotection and precipitations steps were correctly performed.

**Ki determination.** 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), MgCl₂ (12.5 mM), [¹⁴C]-L-Ala (50 μM; 3,700 Bq nmol⁻¹, Perkin Elmer), tRNAAla (0.4 μM), Park’s nucleotide (50 μM), FemX (2.5 nM), and various concentrations of compounds 12 to 16 and 26 to 29. Reactions were performed at 37°C for 120 min and stopped by incubation at 95°C for 10 min. [¹⁴C]-L-Ala and [¹⁴C]-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. Ki were determined by plotting residual activity versus the inhibitor concentration. Curves represent fits of the Morisson equation function of the experimental values (Sigma Plot 12.0).

9. FemX crystallization

FemX (255 μM) and peptidyl-RNA 26 to 29 (306 μM) were co-crystallized by the sitting-drop vapor diffusion method at 295 K using the MOSQUITO robot as previously described with minor modifications (15). Briefly, the droplet (0.2 μL) consisted of an equal volume of protein, peptidyl-nucleic acids, and reservoir solutions [0.1 M Tris-sodium citrate buffer (pH 5.6), 0.2 M ammonium acetate, and 33% polyethylene glycol 4K]. Crystals grew in one day and were used for seeding 1.5 μl drops containing the same solution, except for the polyethylene glycol 4K concentration (30 to 35%). The next day, the freshly obtained crystals were mounted in cryo-loops, successively transferred into reservoir solution containing increasing glycerol concentrations (7, 15, and 20%), and snap-frozen in liquid nitrogen.

10. Crystal structure determination, and 3D supplementary figures

Diffraction data collection, phasing and refinement statistics are given in Table S1. Diffraction data were collected at 100 K on the PROXIMA-2A and ID23-2 beamlines at the SOLEIL Synchrotron (Saint-Aubin, France) and ESRF Synchrotron (Grenoble, France), respectively. Data were processed with XDS (21) through XDSME (https://github.com/legrandp/xdsme) (22). The crystal structures of the complexes were solved by molecular replacement (MR) with the MOLREP program (23) using the X-ray structure of FemX as search model (PDB ID 4II9). The initial models were then manually corrected and completed using COOT (24). Significant extra electron density allowed the manual building of the peptidyl-RNA
molecules. The structures were iteratively improved by manual building steps followed by refinement cycles. The model refinement was conducted with Phenix program (25).

|                  | 2’F-RNA (26) | HNA (27) | 2’F-ANA (28) | DNA (29) |
|------------------|--------------|----------|--------------|----------|
| Wavelength (Å)   | 0.9801       | 0.8731   | 0.9801       | 0.9801   |
| Unit-cell (Å, °) | 41.9, 100.88, 44.29, 90.0, 110.65, 90.0 | 42.16, 101.96, 46.19, 90.0, 102.78, 90.0 | 42.22, 101.13, 46.05, 90.0, 102.57, 90.0 | 42.19, 101.49, 46.25, 90.0, 102.51, 90.0 |
| Space group      | P21          | P21      | P21          | P21      |
| Resolution limits (Å) | 41.44-1.66(1.76-1.66) | 41.2-1.71(1.82-1.71) | 44.95-1.6(1.69-1.6) | 45.16-1.49 (1.58-1.49) |
| Total reflections | 276925       | 233105   | 339367       | 418872   |
| Unique reflections | 40415       | 38530    | 49872        | 60734    |
| R-meas (%)       | 23.4(149.0)  | 26.7(292.5) | 7.5(190.2)   | 7.9(222.1) |
| Completeness (%) | 99(95.4)     | 94.0(69.9) | 99.3(97.3)   | 98.3(93.4) |
| Mean I/σ (I)     | 8.93(1.25)   | 5.22(0.77) | 13.3(0.74)   | 12.91(0.82) |
| CC (1/2)         | 0.99(0.68)   | 0.99(0.31) | 0.99(0.68)   | 0.99(0.53) |
| Wilson B factor (Å2) | 23.0        | 27.4     | 30.0         | 24.1     |
| Number of non-hydrogen atoms (protein/ligand/solvent) | 2701/257/134 | 2691/165/140 | 2691/171/269 | 2702/167/272 |
| R/Rfree (%)      | 19.8/22.9    | 21.2/24.2 | 19.7/22.5    | 19.5/22.4 |
| R.M.S.D. Bonds (Å)/angles (°) | 0.009/1.26   | 0.008/1.002 | 0.007/1.027 | 0.006/0.912 |
| Average temperature factors (protein/ligand/solvent) | 28.22/49.9/32.15 | 29.72/42.81/32.94 | 30.8/42.6/37.8 | 29.98/32.89/36.16 |
| Ramachandran plot (%) (Favored/Outliers) | 99/0         | 99/1     | 99 /0        | 99/0     |
| PDB              | 7Z6A         | 7Z5Y     | 7Z6K         | 7Z5Z     |

**Table S1.** Data collection and refinement statistics. Statistics for the highest-resolution shell are shown in parentheses.
Fig. S2. Superposition of models of FemX in complex with RNA-bi-substrate (PDB 4II9) and compounds 26, 27, 28, and 29. In this figure, only the protein is represented. The inset shows the only minor variation observed for loop Met260-Thr264. Color code: FemX in complex with RNA, HNA, 2’F-RNA, 2’F-ANA, and DNA are represented in gray, blue, pink, orange, and green, respectively.

Fig. S3. Alternative positioning of the peptide part of the 2’F-RNA bi-substrate in the catalytic pocket of FemX. (A) Protein-ligand interactions between FemX and the published RNA bi-substrate. (B) Protein-ligand interactions between FemX and the 2’F-RNA based bi-substrate 26. Black arrows represent the position of triazole moieties. The green ball represents a Mg$^{2+}$ ion in complex with carbonyls of His$^{139}$, Ala$^{140}$, and Ile$^{142}$ of the protein and the carbonyl and carboxylate moieties of D-iGlu$^2$ of the peptidyl part of the 2’F-RNA-bi-substrate 26.
Fig. S4. (A) G₁-C₇² and G²-C₇¹ Watson-crick interactions observed in the complex between FemX and the 2'F-RNA containing bi-substrat inhibitor (26). (B) FemX residues in the vicinity of the bases C₇¹ and C₇² implicated in the substrate specificity of FemX towards tRNAAla (16).

Fig. S5. Conformation of HNA A₇³, C₇⁴, and C₇⁵ in FemX catalytical site. (A) Visible 5'-A₇³C₇⁴C₇⁵A₇⁶-3' terminal portion of compound 27. (B) π-stacking interaction observed between C₇⁵ and C₇⁴ and between C₇⁴ and A₇³. Distances are given in Å. (C) independent representation of the three visible hexitol rings of A₇³, C₇⁴ and C₇⁵.
## 11. Mass spectrometry analyses of synthesized compounds

| Compound Description | N° | Formula | Calculated | Observed |
|----------------------|----|---------|------------|----------|
| UML-L-Ala-D-KAnu-cystine | 1 | C₉H₁₇NO₅P₂S²⁻ | [M⁺H]⁺ = 1,102.20 | 1,102.1 |
| UML-L-Ala-D-Glu-cystine-D-Ala-D-Ala | 2 | C₁₀H₁₉NO₅P₂S²⁻ | [M⁺H]⁺ = 1,244.26 | 1,244.3 |
| UML-L-Ala-D-Glu-dha-D-Ala-D-Ala | 3 | C₁₀H₁₉NO₅P₂S²⁻ | [M⁺H]⁺ = 1,089.27 | 1,089.3 |
| UML-L-Ala-D-2Klu-(R)-thio-butyryl-O-Ala-D-Ala | 4 | C₁₀H₁₉NO₅P₂S²⁻ | [M⁺H]⁺ = 1,175.33 | 1,175.3 |
| UML-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (Park's nucleotide) | 5 | C₁₀H₁₉NO₅P₂S²⁻ | [M⁺H]⁺ = 1,148.34 | 1,148.3 |
| UML-L-Ala-D-Glu-L-Lys-Ala-D-Ala | 6 | C₁₀H₁₉NO₅P₂S²⁻ | [M⁺H]⁺ = 1,221.40 | 1,221.3 |
| UML-L-Ala-D-2Klu-L-Lys[¹³C]° | 7 | C₁₀H₁₉NO₅P₂S²⁻ | [M⁺H]⁺ = 1,225.40 | 1,225.3 |
| 2F-RNA helix (5'-GGGGCCSUAGCAAGGCUCACAC-3') | 8a | C₉H₁₇NO₅P₂S²⁻ | 7,005.0 | 7,005.0 |
| 2F-RNA helix (5'-GGGCGCUAGCAAGGCUCACAC-3') | 7a | C₉H₁₇NO₅P₂S²⁻ | 7,032.9 | 7,032.9 |
| HNA helix (5'-GGGCGCUAGCAAGGCUCACAC-3') | 8a | C₉H₁₇NO₅P₂S²⁻ | 6,973.3 | 6,973.3 |
| HNA helix (5'-GGGCGCUAGCAAGGCUCACAC-3') | 8a | C₉H₁₇NO₅P₂S²⁻ | 6,965.4 | 6,965.4 |
| 2F-ANA helix (5'-GGGCGCUAGCAAGGCUCACAC-3') | 9a | C₉H₁₇NO₅P₂S²⁻ | 7,032.9 | 7,032.9 |
| 2F-ANA helix (5'-GGGCGCUAGCAAGGCUCACAC-3') | 9a | C₉H₁₇NO₅P₂S²⁻ | 6,609.1 | 6,609.0 |
| 2F-RNA helix-pd2PA-2N₈ (5'-GGGCGCUAGCAAGGCUCACAC-3') | 10a | C₉H₁₇NO₅P₂S²⁻ | 7,679.3 | 7,675 |
| HNA helix-pd2PA-2N₈ (5'-GGGCGCUAGCAAGGCUCACAC-3') | 13 | C₉H₁₇NO₅P₂S²⁻ | 7,624.0 | 7,621 |
| Full HNA helix-pd2PA-2N₈ (5'-GGGCGCUAGCAAGGCUCACAC-3') | 14 | C₉H₁₇NO₅P₂S²⁻ | 7,611.2 | 7,611 |
| 2F-ANA helix-pd2PA-2N₈ (5'-GGGCGCUAGCAAGGCUCACAC-3') | 15 | C₉H₁₇NO₅P₂S²⁻ | 7,679.5 | 7,679.7 |
| DNA helix-pd2PA-2N₈ (5'-GGGCGCUAGCAAGGCUCACAC-3') | 16 | C₉H₁₇NO₅P₂S²⁻ | 7,455.7 | 7,455.9 |
| 2F-RNA helix inhibitor (5'-GGGCGCUAGCAAGGCUCACAC-3') | 17 | C₉H₁₇NO₅P₂S²⁻ | 8,655.8 | 8,657.4 |
| HNA helix inhibitor (5'-GGGCGCUAGCAAGGCUCACAC-3') | 18 | C₉H₁₇NO₅P₂S²⁻ | 8,601.0 | 8,601.1 |
| Full HNA helix inhibitor (5'-GGGCGCUAGCAAGGCUCACAC-3') | 19 | C₉H₁₇NO₅P₂S²⁻ | 8,795.2 | 8,790 |
| 2F-ANA helix inhibitor (5'-GGGCGCUAGCAAGGCUCACAC-3') | 20 | C₉H₁₇NO₅P₂S²⁻ | 8,656.8 | 8,658.1 |
| DNA helix inhibitor (5'-GGGCGCUAGCAAGGCUCACAC-3') | 21 | C₉H₁₇NO₅P₂S²⁻ | 8,632.7 | 8,633.3 |
| 2F-RNA micro-helix-2N₈ (5'-GGHCCACCA-3') | 22 | C₉H₁₇NO₅P₂S²⁻ | 2,896.2 | 2,896.2 |
| HNA micro-helix-2N₈ (5'-GGHCCACCA-3') | 23 | C₉H₁₇NO₅P₂S²⁻ | 2,861.7 | 2,861.7 |
| 2F-ANA micro-helix-2N₈ (5'-GGHCCACCA-3') | 24 | C₉H₁₇NO₅P₂S²⁻ | 2,889.5 | 2,889.5 |
| DNA micro-helix-2N₈ (5'-GGHCCACCA-3') | 25 | C₉H₁₇NO₅P₂S²⁻ | 2,763.6 | 2,763.6 |
| 2F-RNA micro-helix-UMSK (5'-GGHCCACCA-3') | 26 | C₉H₁₇NO₅P₂S²⁻ | 4,067.2 | 4,068.2 |
| HNA micro-helix-UMSK (5'-GGHCCACCA-3') | 27 | C₉H₁₇NO₅P₂S²⁻ | 4,040.1 | 4,040.1 |
| 2F-ANA micro-helix-UMSK (5'-GGHCCACCA-3') | 28 | C₉H₁₇NO₅P₂S²⁻ | 4,067.8 | 4,067.9 |
| DNA micro-helix-UMSK (5'-GGHCCACCA-3') | 29 | C₉H₁₇NO₅P₂S²⁻ | 3,941.6 | 3,941.6 |

- dha, dehydroalanine; UM, UDP-MurNAc; UMSK, UDP-MurNAc-pentapeptide; H, PEG₁₈
12. *rpHPLC* analysis of synthesized compounds

**22- and 24-nt oligonucleotides containing compounds**

![Graph](image-url)

Anionic *rpHPLC* on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min\(^{-1}\) with a two-step gradient applied between 2 and 3 min from 25 mM to 750 mM and between 3 and 22 min from 750 mM to 1,750 mM of ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).
Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min\(^{-1}\) with a two-step gradient applied between 2 and 3 min from 25 mM to 750 mM and between 3 and 22 min from 750 mM to 1,750 mM of ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).

Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min\(^{-1}\) with a 15 min gradient applied between 5 and 20 min from 25 mM to 1,250 mM ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).
Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min$^{-1}$ with a 15 min gradient applied between 5 and 20 min from 25 mM to 1,250 mM ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).
Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min\(^{-1}\) with a two-step gradient applied between 2 and 3 min from 25 mM to 750 mM and between 3 and 19 min from 750 mM to 1,750 mM of ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).

Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min\(^{-1}\) with a two-step gradient applied between 2 and 3 min from 25 mM to 750 mM and between 3 and 19 min from 750 mM to 1,750 mM of ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).
Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min$^{-1}$ with a 15 min gradient applied between 5 and 20 min from 25 mM to 1,250 mM ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).

Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min$^{-1}$ with a twostep gradient applied between 5 and 20 min from 25 mM to 1,250 mM and between 20 and 22.5 min from 1,250 mM to 2,500 mM of ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).
Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min⁻¹ with a two-step gradient applied between 2 and 3 min from 25 mM to 750 mM and between 3 and 22 min from 750 mM to 2,500 mM of ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).
Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min\(^{-1}\) with a two-step gradient applied between 2 and 3 min from 25 mM to 750 mM and between 3 and 22 min from 750 mM to 2,500 mM of ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).

8-nt oligonucleotides containing compounds

Anionic rpHPLC on analytical DNAPac PA-100 (Thermofisher Scientific, 4.6 x 250 mm) at 2 mL min\(^{-1}\) with a 15 min gradient applied between 5 and 20 min from 25 mM to 1,250 mM ammonium acetate pH 8.0 containing 0.5% ACN (absorbance measured at 260 nm).
13. References

1. Fiser, A., Filipe, S. R. and Tomasz, A. (2003) Cell wall branches, penicillin resistance and the secrets of the MurM protein. Trends Microbiol., 11, 547–553.

2. Rohrer, S. and Berger-Bachi, B. (2003) FemABX Peptidyl Transferases: a Link between Branched-Chain Cell Wall Peptide Formation and β-Lactam Resistance in Gram-Positive Cocci. Antimicrob. Agents Chemother., 47, 10.
3. Rohrer, S., Ehlert, K., Tschierske, M., Labischinski, H. and Berger-Bachi, B. (1999) The essential Staphylococcus aureus gene fmhB is involved in the first step of peptidoglycan pentaglycine interpeptide formation. Proc. Natl. Acad. Sci. USA, 96, 9351–9356.

4. De Pascale, G., Lloyd, A.J., Schouten, J.A., Gilbey, A.M., Roper, D.I., Dowson, C.G. and Bugg, T.D.H. (2008) Kinetic Characterization of Lipid II-Ala:Alanyl-tRNA Ligase (MurN) from Streptococcus pneumoniae using Semisynthetic Aminoacyl-lipid II Substrates. Journal of Biological Chemistry, 283, 34571–34579.

5. Schneider, T., Senn, M.M., Berger-Bächli, B., Tossi, A., Sahl, H.-G., Wiedemann, I. (2004) In vitro assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly5) of Staphylococcus aureus: S. aureus in vitro interpeptide bridge synthesis. Mol. Microbiol., 53, 675–685.

6. Bugg, T.D.H., Braddick, D., Dowson, C.G., Roper, D.I. (2011) Bacterial cell wall assembly: still an attractive antibacterial target. Trends Biotechno., 29, 167–173.

7. Shepherd, J., Ibba, M. (2013) Direction of aminoacylated transfer RNAs into antibiotic synthesis and peptidoglycan-mediated antibiotic resistance. FEBS Letters, 587, 2895–2904.

8. Monteiro, J.M., Covas, G., Rausch, D., Filipe, S.R., Schneider, T., Sahl, H.-G., Pinho, M.G. (2019) The pentaglycine bridges of Staphylococcus aureus peptidoglycan are essential for cell integrity. Sci Rep, 9, 5010.

9. Filipe, S.R., Severina, E., Tomasz, A. (2002) The murMN operon: A functional link between antibiotic resistance and antibiotic tolerance in Streptococcus pneumoniae. Proc. Natl. Acad. Sci. USA, 99, 1550–1555.

10. Chopra, S., Reader, J. (2014) tRNAs as Antibiotic Targets. Int. J. Mol. Sci., 16, 321–349.

11. Strandén, A.M., Ehlert, K., Labischinski, H., Berger-Bächli, B. (1997) Cell wall monoglycine cross-bridges and methicillin hypersusceptibility in a femAB null mutant of methicillin-resistant Staphylococcus aureus. J. Bacteriol., 179, 9–16.

12. Fonvielle, M., Chemama, M., Lecerf, M., Villet, R., Busca, P., Bouhss, A., Ethève-Quelquejeu, M., Arthur, M. (2010) Decoding the Logic of the tRNA Regiospecificity of Nonribosomal FemXw Aminoacyl Transferase. Angew. Chem. Int. Ed. Engl., 49, 5115–5119.

13. Srisuknimit, V., Qiao, Y., Schaefer, K., Kahne, D., Walker, S. (2017) Peptidoglycan Cross-Linking Preferences of Staphylococcus aureus Penicillin-Binding Proteins Have Implications for Treating MRSA Infections. J. Am. Chem. Soc., 139, 9791–9794.

14. Dare, K., Ibba, M. (2012) Roles of tRNA in cell wall biosynthesis. Wiley Interdiscip. Rev. RNA, 3, 247–264.

15. Fonvielle, M., Li de La Sierra-Gallay, I., El-Sagheer, A.H., Lecerf, M., Patin, D., Mellal, D., Mayer, C., Blanot, D., Gale, N., Brown, T., et al. (2013) The Structure of FemXw in Complex with a Peptidyl-RNA Conjugate: Mechanism of Aminoacyl Transfer from Ala-tRNAAla to Peptidoglycan Precursors. Angew. Chem. Int. Ed. Engl., 52, 7278–7281.

16. Villet, R., Fonvielle, M., Busca, P., Chemama, M., Maillard, A.P., Hugonnet, J.-E., Dubost, L., Marie, A., Josseaume, N., Mesnago, S., et al. (2007) Idiosyncratic features in tRNAs participating in bacterial cell wall synthesis. Nucleic Acids Res., 35, 6870–6883.
17. Fonvielle, M.; Mellal, D.; Patin, D.; Lecerf, M.; Blanot, D.; Bouhss, A.; Santarem, M.; Mengin-Lecreulx, D.; Sollogoub, M.; Arthur, M.; Ethève-Quelquejeu, M. (2013) *Chem. Eur. J.*, 19 (4), 1357–1363.

18. Chemama, M., Fonvielle, M., Villet, R., Arthur, M., Valéry, J.-M., Ethève-Quelquejeu, M. (2007) Stable Analogues of Aminoacyl-tRNA for Inhibition of an Essential Step of Bacterial Cell-Wall Synthesis. *J. Am. Chem. Soc.*, 129, 12642–12643.

20. Lodder, M., Wang, B., Hecht, S.M. (2005) The N-pentenoyl protecting group for aminoacyl-tRNAs. *Methods*, 36, 245–251.

21. Kabsch, W., (2010) Integration, scaling, space-group assignment and post-refinement *Acta Crystallogr. D*66(2), 125-32.

22. Legrand, P., (2017) *GitHub repository*.

23. Vagin, A., Teplyakov, A., (2010) Molecular replacement with MOLREP *Acta Crystallogr. D*66(1), 22-5.

24. Emsley, P., Lohkamp, B., Scott, W.G., Cowtan, K., (2010) Features and development of Coot, *Acta Crystallogr. D*66(4), 486-501.

25. Afonine, P.V., Grosse-Kunstleve, R.W., Echols, N., Headd, J.J., Noriarty, N.W., Mustyakimov, M., Terwilliger, T.C., Urzhumtsev, A., Zwart, P.H., Adams, P.D., (2012) Towards automated crystallographic structure refinement with phenix.refine, *Acta Crystallogr. D*68, 352-67.