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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 4654–4656

Adenosine phosphonate inhibitors of lipid II: Alanyl tRNA ligase MurM from *Streptococcus pneumoniae*

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Received 7 May 2007; revised 20 May 2007; accepted 22 May 2007
Available online 25 may 2007

Abstract—Adenosine and 2′-deoxyadenosine phosphonate transition state analogues act as the first inhibitors for the MurMN/FemABX family of tRNA-dependent ligases implicated in high-level penicillin resistance in Gram-positive bacteria.

High-level penicillin resistance in *Streptococcus pneumoniae* is associated with the presence of additional -Ala-Ala- and -Ser-Ala- cross-links in the peptidoglycan structure,[1] as shown in Figure 1. The presence of these cross-links is associated with the presence of particular sequences of the *murM* and *murN* genes,[2] related to the *femABX* genes responsible for formation of the $(Gly)_5$ cross-link in *Staphylococcus aureus*.[3] The encoded MurMN proteins catalyse the addition of Ala(Ser) and Ala, respectively, to lipid intermediate II in peptidoglycan biosynthesis,[4] using Ala-tRNA and Ser-tRNA, respectively, analogous to the addition of Gly-tRNA by FemABX in *S. aureus*.[5] Deletion of the *murM* gene in *S. pneumoniae* leads to loss of the high-level resistance phenotype,[2] therefore MurM is a potential target for anti-resistance agents that could be applied synergistically with penicillin, against antibiotic-resistant bacteria.

The catalytic mechanism of the MurM-catalysed reaction is likely to proceed via a tetrahedral transition state, shown in Figure 2. Since adenosine is found at the 3′-terminus of tRNA nucleic acids, a possible transition state mimic for this family of enzymes is an adenosine 3′-phosphonate, shown in Figure 2. This paper describes the synthesis and in vitro assay of a series of adenosine and 2′-deoxy-adenosine phosphonates as MurM inhibitors.

In the 2′-deoxy series, a protected 2′-deoxyadenosine derivative (**1**)[6] was coupled with aminoethylphosphonic monomethyl ester **2**[7] using DCC, in 54% yield. Deprotection was achieved in 52% yield, to give phosphonate **3**, as shown in Figure 3.

In the ribo-series, attempts to couple **2** to a 2′,5′-TBDMS-protected adenosine to obtain selectively the 3′ phosphonate (**6a**) were unsuccessful, using a range of coupling agents. Therefore, an improved version of the procedure reported by Zemlicka[8] to obtain a mixture of 2′ and 3′ regioisomers was followed. A 5′-TBDMS-protected adenosine (**4**)[6] was coupled with aminoethylphosphonic acid **5**[7] to give a mixture of 2′- and 3′-phosphonates **6a** and **6b**, in a 3:1 ratio,

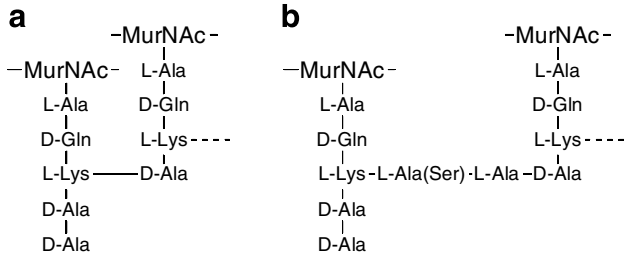

Figure 1. (a) Direct peptidoglycan cross-link found in normal *S. pneumoniae*; (b) indirect peptidoglycan cross-link found in penicillin-resistant *S. pneumoniae*.

Keywords: MurM; Inhibitor; Peptidoglycan biosynthesis; Antibacterial; *Streptococcus pneumoniae*.
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doi:10.1016/j.bmcl.2007.05.071

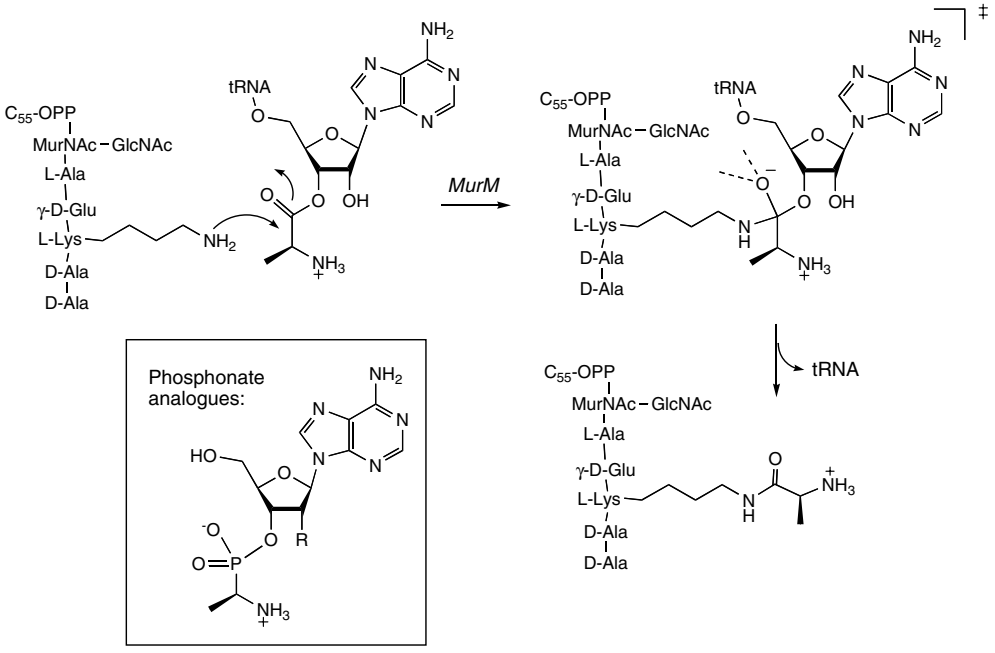

Figure 2. The MurM-catalysed ligation of lipid intermediate II with alanyl-tRNA, showing the likely tetrahedral oxyanion intermediate, and the structures of the phosphonate analogues. R = OH or H; C_{55}, undecaprenyl.

which were not separable. Deprotection gave a 3:1 mixture of 2′- and 3′-phosphonates **7a** and **7b** (see Fig. 4). Anion exchange FPLC of the Cbz-protected intermediate yielded a small amount of pure **7b** for enzyme assays.

Phosphonates **3**, **7b**, and the 3:1 mixture of **7a**/**7b** were assayed as inhibitors of MurM from *S. pneumoniae* strain 159, whose expression and purification is described elsewhere.[9] Lipid intermediate II was prepared enzymatically, using the method of Breukink et al.[10] [^{3}H]-Ala-tRNA was prepared enzymatically, using *S. pneumoniae* alanyl tRNA synthetase, and tRNA isolated from *Micrococcus flavus*.[9] The MurM reaction

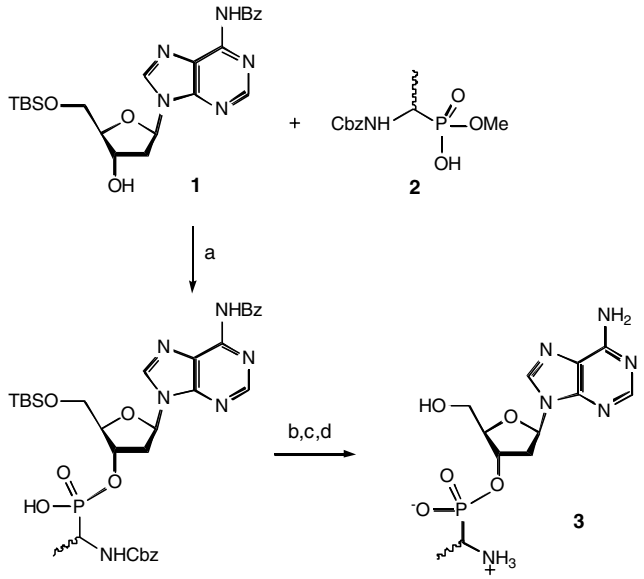

Figure 3. Synthesis of 2′-deoxyadenosine phosphonate **3**. Reagents and yields: (a) DCC, pyridine, Dowex (pyr$^+$), 54%; (b) NH_3, MeOH, 85%; (c) $Et_3N{\cdot}HF$, THF, 99%; (d) H_2/Pd/C, MeOH, 62%.

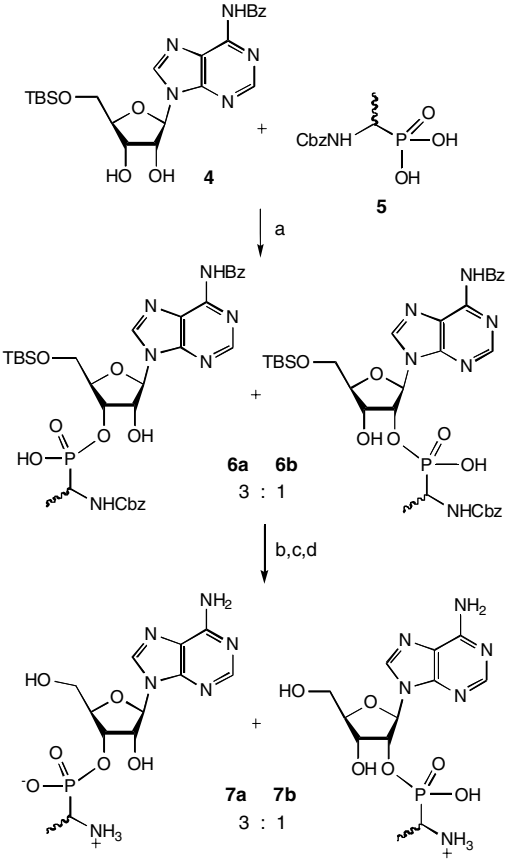

Figure 4. Synthesis of ribo-phosphonates **7a** and **7b**. Reaction conditions: (a) EDC, pyridine, Dowex (pyr$^+$), 70%; (b) NH_3, MeOH, 97%; (c) $Et_3N{\cdot}HF$, THF, 99%; (d) H_2/Pd/C, MeOH, 86%.

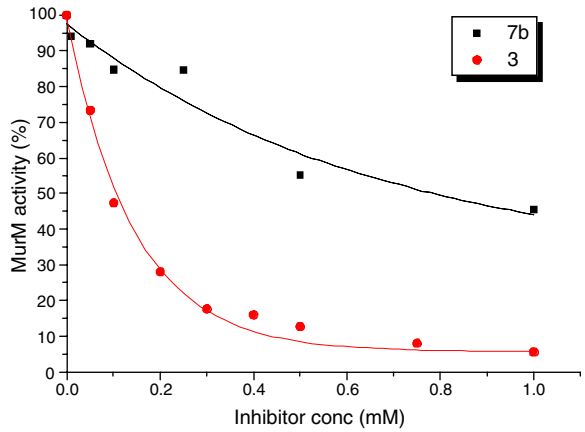

Figure 5. Inhibition of *S. pneumoniae* 159 MurM by **3** and **7b**.

was then monitored radiochemically, by extraction of the lipid II-[^{3}H]-Ala product into *n*-butanol, followed by scintillation counting.[†]

The 2′-ribo-phosphonate **7b** showed moderate levels of inhibition, yielding an IC_{50} value of 780 μM, whereas the 3:1 mixture of **7a**/**7b** showed no inhibition of MurM at 1 mM concentration. The 2′-deoxyadenosine phosphonate **3** showed much higher levels of inhibition, as shown in Figure 5, giving an IC_{50} value of 100 μM.

Assay of synthetic precursors containing either an *N*-Cbz protecting group or 5′-*O*-TBDMS-protecting group showed no enzyme inhibition at 1 mM concentration. Puromycin, an adenosine analogue containing a 3′-amino-tyrosyl substituent,[11] also showed no inhibition of MurM at 1 mM concentration, indicating the importance of the phosphonate group for enzyme inhibition.

One possible rationalisation of the observed inhibition data is that the potency of inhibition depends upon the conformation of the furanose ring. Ribo-nucleoside derivatives are known to prefer the 3′-*endo* conformation, in which the 2′-substituent is axial and 3′-substituent equatorial, whereas 2′-deoxy derivatives prefer the 2′-*endo* conformation, in which the 3′-substituent is axial.[12] ^{1}H–^{1}H NMR coupling constants from the NMR spectrum of 2′-deoxy analogue **3** are consistent with a 2′-*endo* conformation, bearing an axial 3′-phosphonate group, whereas the 3′-phosphonate of ribo-analogue **7a** is equatorial, implying that an axial phosphonate group is required for efficient binding to MurM. This is supported by the observation that 2′-phosphonate **7b**, which contains an axial phosphonate at C-2′, still shows modest enzyme inhibition.

Phosphonate **3**, the first in vitro inhibitor of the MurMN/FemABX ligase family, was tested for antibacterial activity against penicillin-resistant *S. pneumoniae* strain 159, but showed no growth inhibition at 1 mM concentration (380 μg/ml), and no effect upon penicillin MIC, perhaps due to lack of transport across the cell membrane. Further derivatives of **3** are being synthesised for in vitro and antibacterial testing.

Acknowledgment

This work was supported by the European Commission COBRA Network (Contract LSHM-CT-2003-503335), and a BBSRC studentship (to G.P.).

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[†] Assays contained 50 μM lipid intermediate II, 0.2 μM [^{3}H]-Ala-tRNA, 0.01–1 mM inhibitor and 25 nM *S. pneumoniae* 159 MurM in 50 mM MOPS buffer, pH 6.8, containing 30 mM KCl, 10 mM $MgCl_2$, 1.5% CHAPS, 1 mM L-Ala and 2 mM DTT.