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Inhibition of tRNA-dependent ligase MurM from *Streptococcus pneumoniae* by phosphonate and sulfonamide inhibitors

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ABSTRACT

Ligase MurM catalyses the addition of Ala from alanyl-tRNA^{Ala}, or Ser from seryl-tRNA^{Ser}, to lipid intermediate II in peptidoglycan biosynthesis in *Streptococcus pneumoniae*, and is a determinant of high-level penicillin resistance. Phosphorus-based transition state analogues were designed as inhibitors of the MurM-catalysed reaction. Phosphonamide analogues mimicking the attack of a lysine nucleophile upon Ala-tRNA^{Ala} showed no inhibition of MurM, but adenosine 3′-phosphonate analogues showed inhibition of MurM, the most active being a 2′-deoxyadenosine analogue (IC_{50} 100 μM). Structure/function studies upon this analogue established that modification of the amino group of the aminoalkylphosphonate resulted in loss of potency, and modification of the adenosine 5′-hydroxyl group with either a *t*-butyl dimethyl silyl or a carbamate functional group resulted in loss of activity. A library of 48 aryl sulfonamides was also screened against MurM using a radiochemical assay, and two compounds showed submillimolar inhibition. These compounds are the first small molecule inhibitors of the Fem ligase family of peptidyltransferases found in Gram-positive bacteria.

1. Introduction

Infections caused by *Streptococcus pneumoniae* are responsible for approximately 3 million deaths each year worldwide due to pneumonia, meningitis and sepsis, and cause serious upper airway infections such as sinusitis and otitis media.[1] Penicillin resistance in *S. pneumoniae* is a significant clinical problem in a number of countries worldwide, with up to 39% of *S. pneumoniae* isolates resistant to penicillin in some European countries in 2005.[2] Given the steady worldwide increase in resistance rates, and the lack of effective new antibacterial agents appearing from pharmaceutical development,[3] there is a need to identify new targets for antibacterial action and strategies to combat antibiotic resistance.

The peptidoglycan layer of many highly penicillin resistant strains of *S. pneumoniae* contain additional -Ala-Ala- and -Ser-Ala- inter-strand cross-links,[4] as shown in Figure 1. The presence of these cross-links is associated with particular sequences of the *murM* and *murN* genes,[5] in the same gene family as the *femABX* genes responsible for formation of the $(Gly)_5$ cross-link in *Staphylococcus aureus*.[6] The encoded MurMN proteins catalyse the addition of Ala(Ser) and Ala, respectively, to lipid intermediate II in peptidoglycan biosynthesis,[7] using Ala-tRNA^{Ala} or Ser-tRNA^{Ser}, respectively (see Fig. 1C), analogous to the addition of five glycine

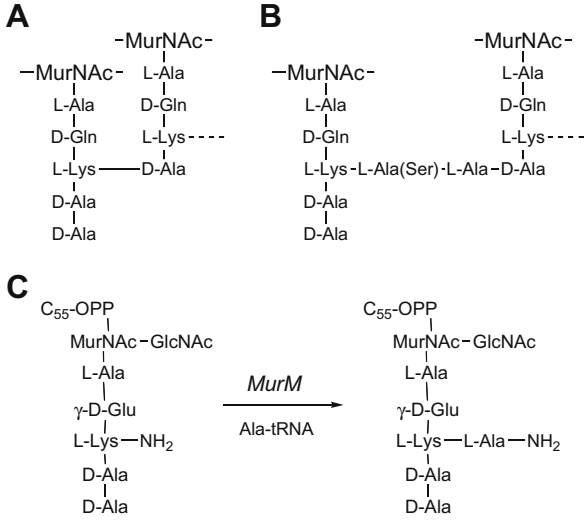

Figure 1. Peptidoglycan cross-links found in the cell walls of (A) penicillin-sensitive and (B) highly penicillin-resistant *Streptococcus pneumoniae*. (C) Reaction catalysed by *S. pneumoniae* ligase MurM. C_{55}-OPP, undecaprenyl diphosphate.

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units from Gly-tRNA^{Gly} by FemABX in *S. aureus*.[8] Deletion of the *murM* gene in *S. pneumoniae* leads to loss of the high-level resistance phenotype,[5] therefore MurM is a potential target for anti-resistance agents that could be applied synergistically with penicillin, against antibiotic-resistant bacteria.

We have recently reported the in vitro reconstitution of the MurM-catalysed reaction for penicillin-resistant and -susceptible MurM variants of *S. pneumoniae*,[9] allowing the study of inhibitors of MurM. The catalytic mechanism of the MurM-catalysed reaction is likely to proceed via a tetrahedral transition state, shown in Figure 2. This transition state could be effectively mimicked by a phosphonate or phosphonamide group. We have previously reported in a communication the identification of the first synthetic inhibitor of MurM, an adenosine phosphonate transition state analogue.[10] In this paper we report the synthesis and evaluation of two series of phosphorus-based transition state analogues for the MurM-catalysed reaction, and the screening of a library of aryl sulfonamides against MurM. The first series of phosphonamides mimic the attack of a lysine nucleophile upon Ala-tRNA^{Ala}; the second series of adenosine phosphonates incorporate the adenosine base found at the 3′-terminus of tRNA, as shown in Figure 2.

2. Results

2.1. Synthesis and assay of phosphonamide transition state analogues

The first series of phosphonamide transition state analogues (see Fig. 2) was synthesised using the synthetic route illustrated in Figure 3. Cbz-protected aminoalkylphosphonate monoesters **1a–e** were prepared in a one-pot condensation of benzylcarbamate, an aldehyde and PCl_3, as reported by Yuan et al.[11] This reaction proceeded in 25–37% yield, to give the phosphonate monoester as the major product, though with aliphatic aldehydes the phosphonate diester and diacid were also observed, which were separated by chromatography. Three different alcohols were used for monoester synthesis: methanol,

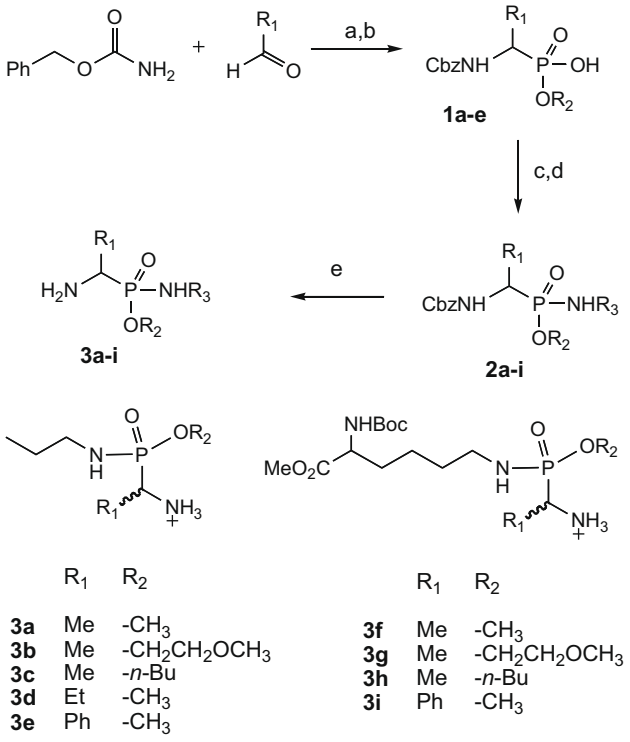


Figure 3. Synthetic route for phosphonamide series. Reagents: (a) RCHO, PCl_3, CH_2Cl_2; (b) R_1OH, CH_2Cl_2; (c) $(COCl)_2$, DMF; (d) R_2NH_2, Et_3N, CH_2Cl_2; (e) H_2, Pd/C, MeOH.

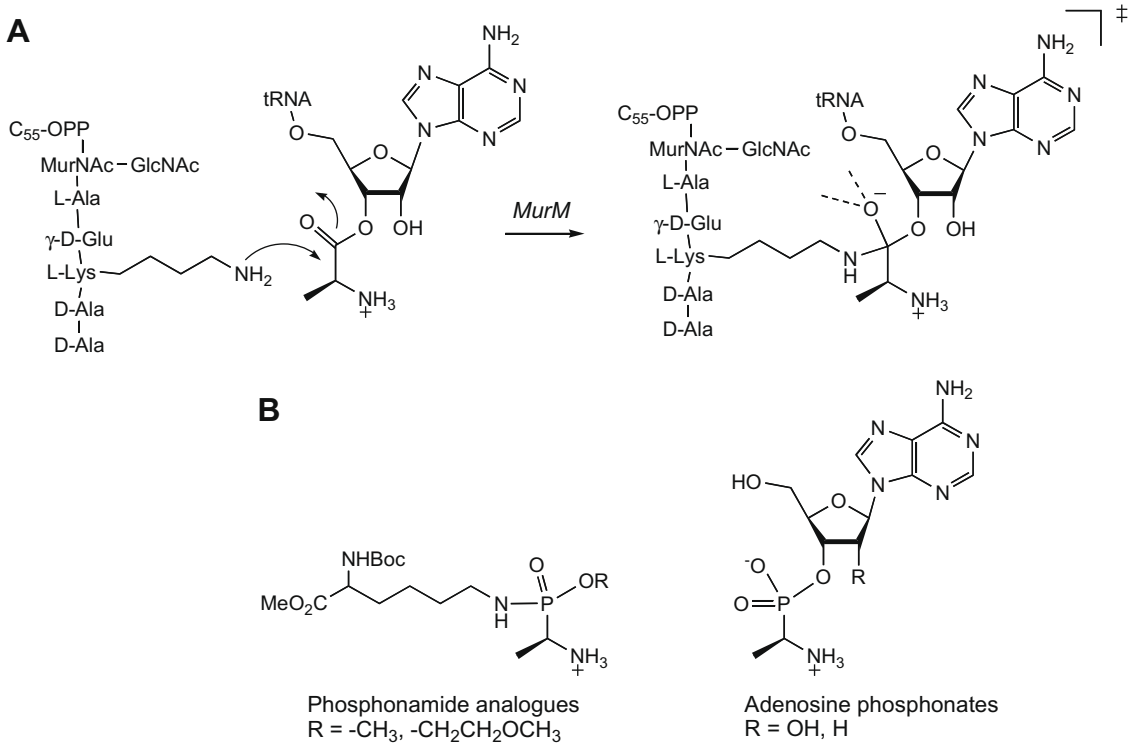


Figure 2. Transition state analogues designed for MurM. (A) The presumed oxyanion intermediate for the MurM-catalysed reaction. (B) Phosphonamide and adenosine phosphonate transition state analogues.

2-methoxyethanol (mimicking the 2′-hydroxyl group of the adenosine nucleotide) and 1-butanol.

Phosphonate monoesters **1a–e** were then converted to the corresponding phosphonochloridate using oxalyl chloride, and then coupled with either 1-propylamine or N^{α}-Boc-lysine methyl ester to give protected phosphonamides **2a–i**, typically in 40–70% yield. Deprotection of the N-Cbz group was achieved by hydrogenation, typically in 80–100% yield.

Each phosphonamide was tested as an inhibitor of recombinant MurM from the highly penicillin-resistant strain 159 of *S. pneumoniae*, using a radiochemical assay, but no inhibition was observed at 1 mM concentration for any of the compounds. The stability of the phosphonamides under the assay conditions (pH 6.8, 37 °C) was verified, therefore this series of compounds do not act as inhibitors for MurM.

2.2. Synthesis and assay of adenosine phosphonate transition state analogues

The synthetic route employed for the adenosine 3′-phosphonate analogue is illustrated in Figure 4. Earlier attempts to couple either a phosphonate monoester **1a** or the corresponding phosphonate diacid **4** to a 2′,5′-TBDMS-protected adenosine derivative were unsuccessful using a range of peptide coupling agents (data not shown), indicating that the 3′-position of this nucleoside is sterically hindered. Zemlicka et al. have previously reported the coupling of phosphonate diacid **4** to a similar protected adenosine derivative, to give a mixture of 2′- and 3′-substituted phosphonate esters, using DCC as coupling agent.[12] In our hands, phosphonate diacid **4** was coupled using EDC to the known adenosine derivative **5**,[13] to give a 3:1 mixture of 3′- and 2′-substituted phosphonate esters **6a** and **6b** in 60% overall yield. Deprotection of the N^6-benzoyl group of adenosine with ammonia/methanol gave **7a/7b** in 97% yield; desilylation with $Et_3N{\cdot}HF$ gave **8a/8b** in 99% yield; and removal of the Cbz group was achieved by hydrogenation in 87% yield, to give **9a/9b** as a 3:1 mixture of regioisomers.

Attempts were made to separate **9a** and **9b**, and, although challenging to separate, a small sample of a single regioisomer was obtained after MonoQ anion exchange chromatography, which was found to be the 2′-regioisomer. Analysis by C_{18} reverse phase HPLC confirmed that the two regioisomers **9a** and **9b** had retention times of 15.5 and 16.1 min, and the purified sample of **9b** showed a single peak at 16.06 min.

The corresponding derivative of 2′-deoxyadenosine was synthesised using a similar synthetic sequence, illustrated in Figure 5. The N^6-benzoyl,5′-OTBDMS derivative **10** was prepared using literature methods.[13] Coupling to phosphonate diester **4** was found to proceed in low yield using EDC, but coupling with phosphonate monoester **1a** was successful using DCC as coupling agent, giving the demethylated product **11** in 54% yield after 6 days reaction time. **11** was deprotected by treatment with ammonia/methanol to give debenzoylated **12** in 85% yield, followed by desilylation with $Et_3N{\cdot}HF$ to give **13** in 99% yield, and hydrogenation to give the target phosphonate **14** in 62% yield.

The sample of pure 2′-phosphonate **9b** showed weak inhibition of MurM (IC_{50} 780 μM), whereas the 3:1 mixture of **9a/9b** showed ~20% inhibition of MurM at 1 mM concentration, therefore indicating that the 2′-regioisomer binds more tightly to MurM than the 3′-regioisomer (see Section 3). 2′-Deoxyadenosine phospho-

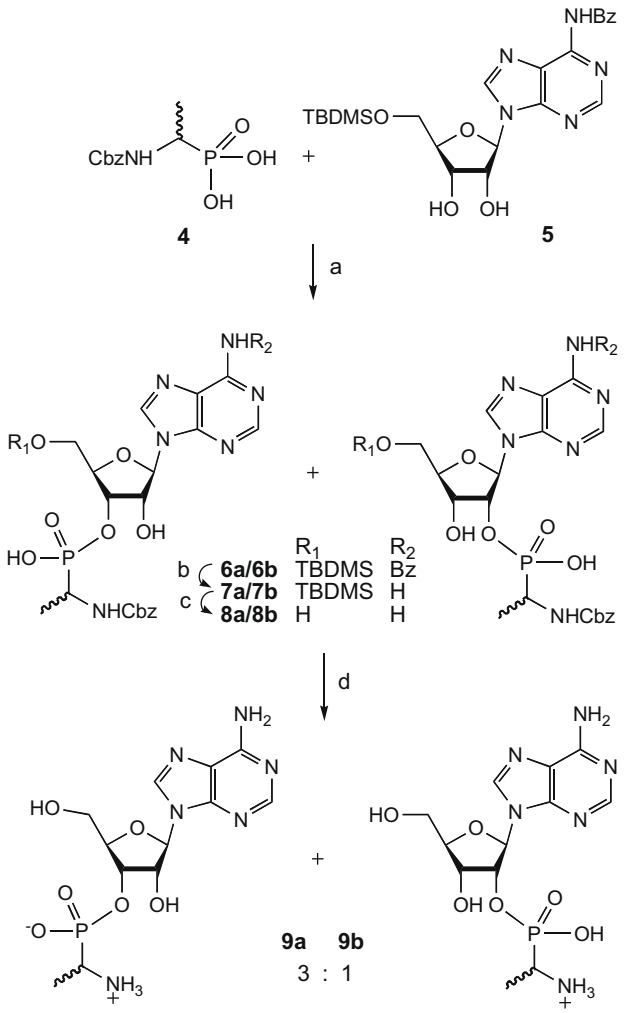


Figure 4. Synthetic route for adenosine phosphonates. Reagents and conditions: (a) EDC, Dowex (pyr^+), pyridine, 5–6 days; (b) NH_3, MeOH; (c) $Et_3N{\cdot}HF$, THF; (d) H_2, Pd/C, $MeOH/H_2O$.

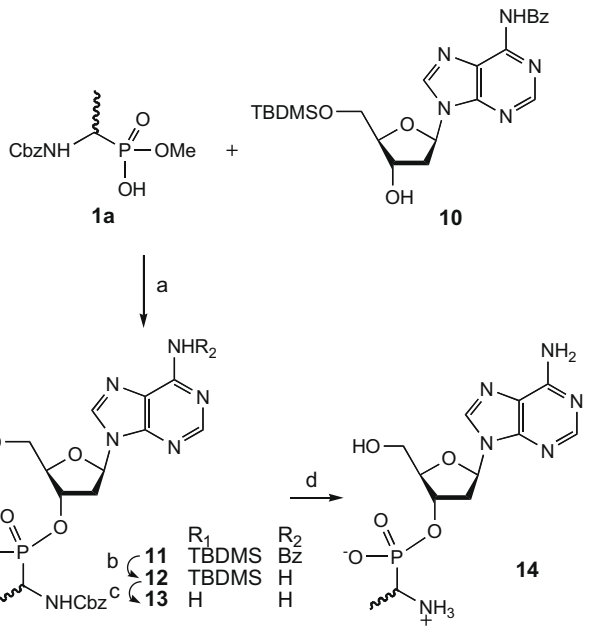


Figure 5. Synthetic route for 2′-deoxyadenosine phosphonate. Reagents and conditions: (a) DCC, Dowex (pyr^+), pyridine, 5–6 days; (b) NH_3, MeOH; (c) $Et_3N{\cdot}HF$, THF; (d) H_2, Pd/C, $MeOH/H_2O$.

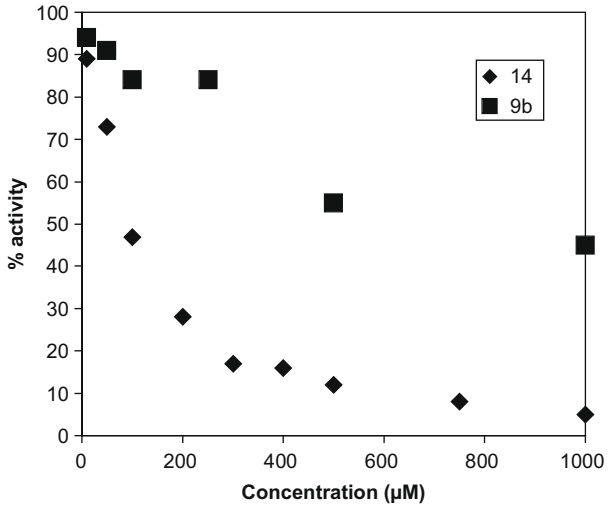


Figure 6. Inhibition of MurM by adenosine phosphonates **14** and **9b**.

nate **14** showed much higher levels of inhibition, as shown in Figure 6, giving an IC_{50} value of 100 μM.

2′-Deoxyadenosine phosphonate **14** was tested for antibacterial activity against *S. pneumoniae* strains pn16 (penicillin-sensitive) and 159 (penicillin-resistant, MIC 16 μg/ml). No growth inhibition was observed at 1 mM concentration (380 μg/ml), and no effect on penicillin MIC was observed for strain 159. Compound **14** was also tested against other strains containing *femABX* genes, namely *S. aureus*, *Enterococcus faecalis* and *Micrococcus flavus*, but no antimicrobial activity was observed in each case.

2.3. Structure/activity studies on adenosine phosphonate inhibitors

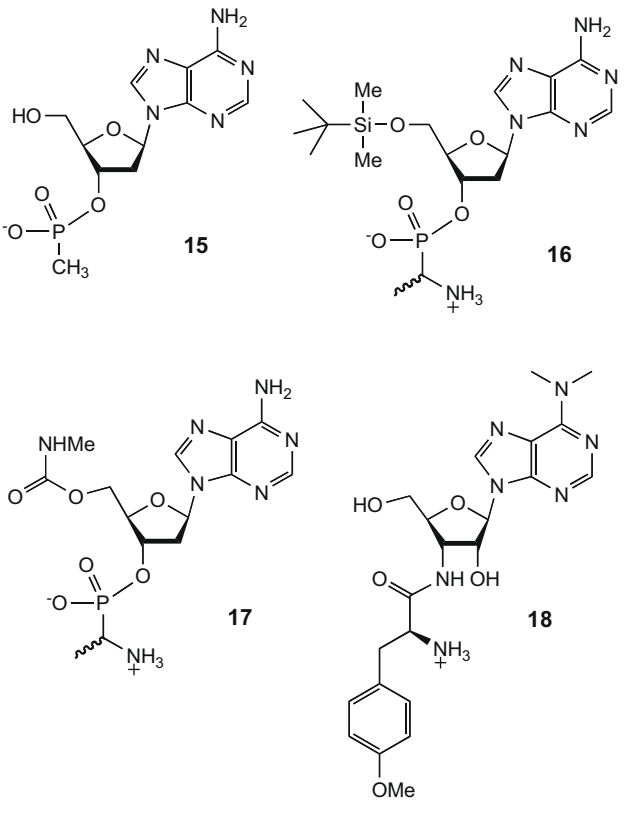
In order to assess the role of different functional groups in the inhibitor structure, several protected compounds were also tested against MurM, as listed in Table 1. In the ribo-phosphonate series, the N-Cbz protected derivative **8** showed 15% inhibition of MurM at 1 mM concentration, similar to the deprotected compound, whereas the fully protected compound **6** and the N-Cbz,5′-OTBDMS protected derivative **7** showed no enzyme inhibition. In the 2′-deoxyphosphonate series, the N-Cbz protected derivative **13** showed no enzyme inhibition, neither did the protected derivatives **11** and **12**.

Table 1
Structure/activitiy data for inhibition of *S. pneumoniae* 159 MurM by adenosine derivatives

Compound	Description	% Inhibition at 1 mM concentration
6a/**6b**	5′-OTBDMS, N-Cbz, Bz-Ad ribofuranose phosphonate	0
7a/**7b**	5′-OTBDMS, N-Cbz ribofuranose phosphonate	0
8a/**8b**	N-Cbz ribofuranose phosphonate (3:1 mixture of 2′,3′ isomers)	15
9a/**9b**	Ribofuranose phosphonate (3:1 mixture of 2′,3′ isomers)	19
9b	Ribofuranose 2′-phosphonate	55
—	2′-Deoxyadenosine	13
—	2′-Deoxyadenosine 3′-phosphate	15
11	5′-OTBDMS, N-Cbz, Bz-Ad deoxyribofuranose phosphonate	0
12	5′-OTBDMS, N-Cbz deoxyribofuranose phosphonate	0
13	N-Cbz deoxyribofuranose phosphonate	0
14	Deoxyribofuranose phosphonate	95
15	Deoxyribofuranose 3′-methylphosphonate	7
16	5′-OTBDMS deoxyribofuranose phosphonate	0
17	5′-Methylcarbamate deoxyribofuranose phosphonate	3
18	Puromycin	0

2′-Deoxyadenosine showed only 13% inhibition of MurM at 1 mM concentration, indicating the importance of the phosphonate group. 2′-Deoxyadenosine 3′-phosphate was tested against MurM, and showed 20% inhibition at 1 mM concentration, indicating that the aminoalkyl substituent is required for efficient binding. A methylphosphonate derivative **15** lacking the amino-substituent was synthesised by coupling of commercially available methylphosphonic acid to **10** using DCC, in 47% yield, followed by deprotection as before. Phosphonate **15** was assayed against MurM, but showed only 7% inhibition at 1 mM concentration, less active than the 3′-phosphate derivative. Therefore, the amino-substituent does appear to be required for efficient binding to the MurM active site.

In the Ala-tRNA substrate for MurM, the adenosine nucleotide is at the 3′-terminus of a large tRNA molecule, therefore, synthetic modification at the 5′ position of **14** seemed a logical approach to higher affinity MurM inhibitors. The 5′-OTBDMS derivative **16** was prepared by hydrogenation of **11**, in 99% yield. Compound **16** was assayed against MurM, but showed no enzyme inhibition at 1 mM, indicating that a bulky substituent is not tolerated in this position. As a neutral mimic of the phosphodiester linkage present in alanyl $tRNA^{Ala}$, the 5′-carbamate derivative **17** was synthesised. Using the method of Gotor et al.,[14] 2′-deoxyadenosine was reacted with acetone *O*-(phenyloxycarbonyl) oxime in the presence of *Candida antarctica* lipase B, yielding the 5′-phenyl carbonate derivative in 73% yield, which was then reacted with methylamine, followed by deprotection, to give **17**. Assays versus MurM showed only 3% inhibition by **17** at 1 mM concentration, therefore, modification to the 5′ carbamate has reduced activity significantly, which is surprising, given that a phosphodiester linkage is found at this position in alanyl tRNA.



Puromycin (**18**) is a 3′-acylamino derivative of adenosine, which inhibits the action of the bacterial ribosome by mimicking tyrosyl tRNA.[15] Puromycin was tested as an inhibitor of MurM, but showed no inhibition at 1 mM concentration.

In view of the similarity of phosphonate **14** to the transition state for the formation of acyl-tRNA by aminoacyl tRNA synthetase enzymes, which are known antibacterial targets,[16] **14** was also assayed as an inhibitor of *S. pneumoniae* alanyl tRNA synthetase (which is used here for reconstitution of MurM[9]), and seryl-tRNA synthetase, as described in Section 4. At 1 mM concentration, phosphonate **14** showed 22% inhibition of *S. pneumoniae* alanyl-tRNA synthetase, and 28% inhibition of *S. pneumoniae* seryl tRNA synthetase; no inhibition was observed at 100 μM concentration.

2.4. Screening of library of aryl sulfonamides

Sulfonamides represent an alternative transition state mimic to phosphonates, and, being less polar and uncharged, offer improved pharmacokinetic properties. Caddick et al. have recently reported the use of microwave-assisted synthesis to prepare libraries of aryl sulfonamides.[17] Using this methodology, a library of 48 aryl sulfonamides having the general structure shown in Figure 6 was prepared, and was screened for inhibition of MurM at 1 mM concentration, using the radiochemical enzyme assay. Two compounds were found to show inhibition of MurM, whose structures are shown in Figure 7. They showed 50% (R = H) and 34% (R = Br) inhibition of MurM at 1 mM concentration of inhibitor. These structures represent possible lead structures for development of non-nucleoside inhibitors of the MurMN/FemABX ligase family.

3. Discussion

The MurMN/FemABX family of aminoacyl-tRNA ligases represent an possible target for chemotherapeutic intervention against a number of pathogenic Gram-positive bacteria that contain inter-strand cross-links in their peptidoglycan structures, including *S. aureus*, *E. faecalis* and penicillin-resistant *S. pneumoniae*. Many Gram-positive bacteria contain inter-strand cross-links, and considerable diversity in the nature of these cross-links exists,[18] therefore it could be possible to target certain types of bacteria, if a selective agent could be developed. One would expect that such an agent would increase the susceptibility towards penicillin, and might be bacteriocidal, since it is known for example that the *femX* gene in *S. aureus* is essential.[6]

In this work, we have shown that *S. pneumoniae* MurM is inhibited in vitro by a 2′-deoxyadenosine 3′-phosphonate analogue **14**, that functions as a transition state mimic, but no inhibition was observed using phosphonamide dipeptide analogues **3a–i**, and only weak inhibition was observed using the corresponding ribo-phosphonate analogue **9b**. The lack of activity of the phosphonamide series could be due to a requirement for the adenosine nucleoside structure for binding, or might indicate that a negatively charged phosphonate group is needed, in order to mimic the transition state structure.

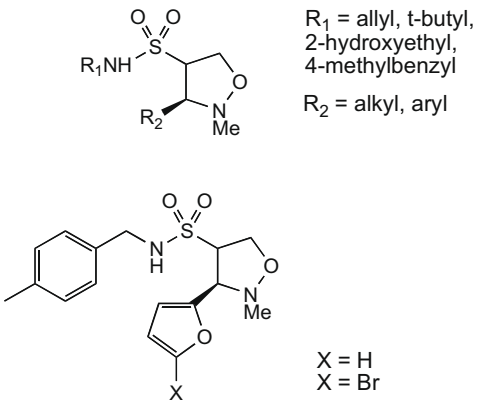


Figure 7. Structures of aryl sulfonamide library, and the two compounds found to inhibit MurM.

One possible rationalisation of the higher activity of the 2′-deoxy analogue **14**, compared with the ribofuranose analogue **9**, is that the conformation of the furanose ring is important for binding to MurM. Ribo-nucleoside derivatives are known to adopt preferentially 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, as shown in Figure 8.[19] The $^{1}H–^{1}H$ NMR coupling constant between H-1′ and H-2′ is a reliable reporter of furanose conformation.[19] In the case of the ribo-phosphonates **9a/9b**, $J_{1'2'}$ values of 6.6 Hz and 6.0 Hz were measured, respectively, which are similar to literatures values of 5.8 Hz for adenosine 3′-phosphate, and 5.9 Hz for adenosine 2′-phosphate,[20] consistent with the adoption of a 3′-*endo* conformation in solution. In the case of 2′-deoxyribophosphonate **14**, $J_{1'2'}$ values of 7.7 and 5.7 Hz were measured. The calculated $J_{1'2'}$ values for a 2′-*endo* conformation are 10.1 and 5.5 Hz, whereas the calculated values for a 3′-*endo* conformation are 7.4 and 0.1 Hz,[21] with one very small coupling constant, due to a 90° dihedral bond angle, that is not observed in **14**. Therefore, the NMR data for 2′-deoxy analogue **14** are consistent with a 2′-*endo* conformation, bearing an axial 3′-phosphonate group, whereas the 3′-phosphonate of ribo-analogue **9a** 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.

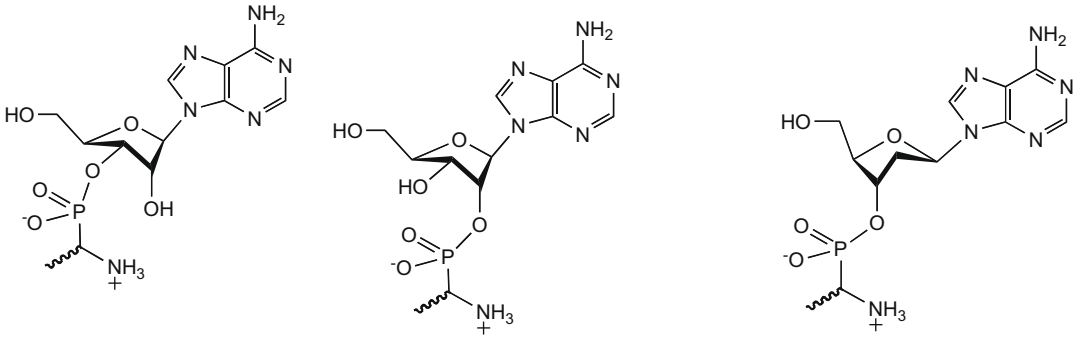
The lack of antibacterial activity for **14** against *S. pneumoniae* strain 159, and the lack of effect on penicillin MIC, suggests that **14** is not effectively transported across the cell membrane. A tRNA-based inhibitor for *Weissella viridescens* FemX has been reported, which also shows no antibacterial activity.[22] It would therefore be of interest to prepare neutral, less polar MurM inhibitors that would be more likely to be taken up into the cell. It is therefore of interest that two aryl sulfonamides obtained from library screening show weak inhibitory activity against MurM.

There is protein structural data available for *S. aureus* FemA[23] and *W. viridescens* FemX,[24] in the latter case co-crystallised with UDPMurNAc-pentapeptide.[24] At present there is no structure available containing the aminoacyl tRNA substrate, therefore the precise location of the aminoacyl-tRNA binding site is not known. Active site residue Arg-211, located close to the diphosphate linkage, has been implicated in substrate binding, using site-directed mutagenesis.[25] On the opposite side of the FemX active site, as shown in Figure 9, there is a cluster of conserved amino acid residues, comprising Tyr-72, Asp-108, and Gln-143, of which Asp-108 has been suggested to act as a catalytic base.[26]

These three amino acid residues are conserved in all MurM/FemABX homologues, and seem a possible site for acyl transfer. Situated close to this conserved triad is the sidechain of Lys-75, which might provide electrostatic stabilisation to the oxyanion transition state. An electrostatic interaction of this type might rationalise why enzyme inhibition is only observed using charged phosphonate analogues, whereas the phosphonamide series of analogues was uncharged at the phosphorus centre.

Phosphonate analogue **14** was also tested as an inhibitor of seryl and alanyl-tRNA synthetases from *S. pneumoniae*, since it also mimics the transition state for acyl tRNA formation. Only rather weak inhibition was observed, in the millimolar range,

Ribofuranoses - 3'-*endo* conformation: 2'-deoxyfuranose- 2'-*endo* conformation:



9a INACTIVE **9b** IC_{50} 780 μM **14** IC_{50} 100 μM

Figure 8. Expected lowest energy conformations for the ribofuranose ring of phosphonates **9a**, **9b** and **14**.

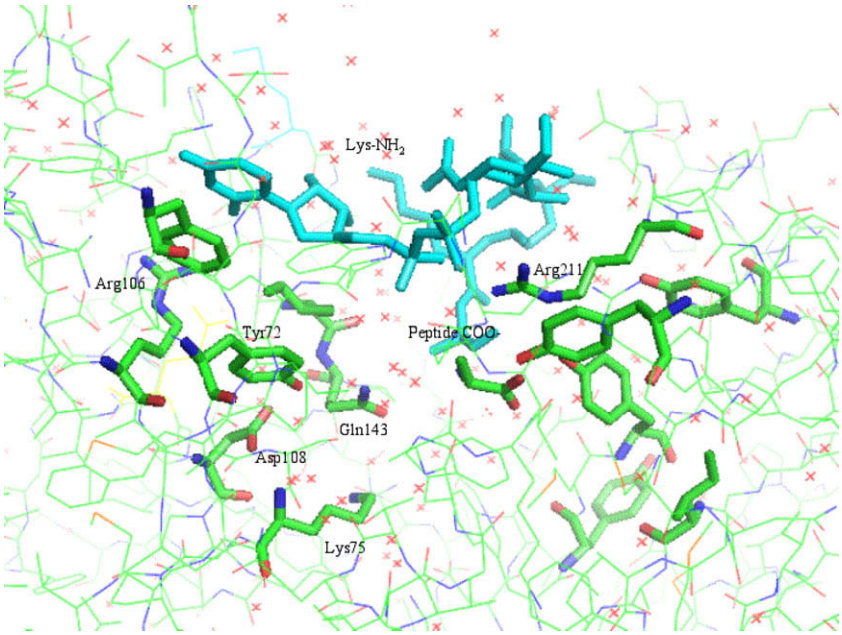


Figure 9. Active site of *Weissella viridescens* FemX, complexed with UDPMurNAc-pentapeptide (in cyan, Lys-NH_2 at position 3 and D-Ala-COO^- at position 5 are indicated), showing active site residues Arg-211, Tyr-72, Asp-108, Gln-143, Lys-75 and Arg-106. Figure prepared using PYMOL software, from PDB file 1FYZ.

whereas binding constants for tRNA substrates by these enzymes are typically in the sub micromolar range. Therefore, there is a prospect for development of a selective transition state analogue for the MurMN/FemABX family of enzymes, provided that transport across the cell membrane can be achieved.

4. Experimental

4.1. Materials

N^6-Benzoyl 2′-deoxyadenosine was prepared from 2′-deoxyadenosine, using the procedure of Ti et al.[13] N^6-Benzoyl 5′-(*tert*-butyldimethylsilyl) adenosine (**5**) and N^6-benzoyl 5′-(*tert*-butyldimethylsilyl) 2′-deoxyadenosine (**10**) were prepared by silylation of N^6-benzoyl adenosine and N^6-benzoyl 2′-deoxyadenosine, respectively, using the procedure of Ogilvie et al.[13] Acetone *O*-((phenyloxy)carbonyl) oxime was prepared by the method of Fernandez et al.[27] Puromycin (**18**) was purchased from Sigma Aldrich Chemical Co. Undecaprenyl phosphate was purchased from Larodan Fine Chemicals AB. UDP-Mur*N*Ac-pentapeptide (AE-KAA), *M. flavus* membranes, lipid intermediate II, recombinant *S. pneumoniae* Pn16 alanyl-tRNA synthetase (AlaRS) and *S. pneumoniae* MurM, and *M. flavus* tRNA were prepared as previously described.[9]

4.2. General procedure for monoalkyl 1-(benzyloxycarbonylamino) phosphonates (1a–e) (based upon Ref. 11)

Benzylcarbamate (2 g, 13.2 mmol) was suspended in dry CH_2Cl_2 (50 mL) under N_2 and cooled to approximately −15 °C in a ice/methanol bath. The appropriate aldehyde (15.8 mmol) was added to the suspension followed by phosphorus trichloride (1.38 mL, 15.8 mmol). The mixture was brought to 0 °C in an ice bath and left stirring for 1 h 30 min. After this time, the solution was warmed to rt and flushed with N_2 to eliminate the residue aldehyde. Then dry

alcohol (25.0 mmol) was added dropwise and the mixture was stirred for 4 h. After this time, the solvent was removed under reduced pressure and the remaining yellow oil was dissolved in ethyl acetate (50 mL) and extracted with 5% $NaHCO_3$ (3 × 100 mL). The combined water phases were acidified to pH 1 with concentrated HCl and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic phases were dried over $MgSO_4$ and the solvent removed under reduced pressure. The residue was purified by flash chromatography on silica gel (eluent CH_2Cl_2/MeOH from 100% CH_2Cl_2 to 98:2 at 0.5% gradient of methanol).

4.2.1. Methyl 1-(benzyloxycarbonylamino) ethyl phosphonate (1a)

Yield: 33% (white solid). Mp: 120–123 °C (lit.[11] 119–120 °C). R_f (CH_2Cl_2/MeOH 8:2) = 0.1. ^{1}H NMR (300 MHz; $CDCl_3/CD_3OD$): δ_H 7.30 (5H, m, Ph), 5.06 (2H, s, CH_2-Ph), 4.03 (1H, m, CH–P), 3.67 (3H, d, J = 10.2, OCH_3), 1.28 (3H, dd, J_{PH} = 16.4, J_3 = 7.3, C*H*$_3$–CH–P). ^{13}C NMR (75.5 MHz; $CDCl_3/CD_3OD$): δ_C 155.5, 135.8, 128.3, 128.0, 127.9, 67.0, 52.5, 43.8, 41.7 (d, J_{CP} = 159), 15.4. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 22.9. ES-MS: *m/z* 318.1 (M+2Na$^+$). HR MS (FAB): 274.0845 Da (calcd for $C_{11}H_{17}NO_5P$ (M+H$^+$): 274.0844). IR (neat): ν (cm^{-1}) 3304, 2956, 1694, 1531, 1454.

4.2.2. 2-(Methoxy)ethyl 1-(benzyloxycarbonylamino) ethyl phosphonate (1b)

Yield: 36% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.15. ^{1}H NMR (300 MHz; $CDCl_3/CD_3OD$): δ_H 7.32 (5H, m, Ph), 5.10 (2H, m, CH_2-Ph), 4.10 (3H, m, CH–P, CH_2–O–P), 3.55 (2H, m, C*H*$_2$–O–CH_3), 3.31 (3H, m, OCH_3 and CD_3OD), 1.36 (3H, dd, J_3 = 7.3, J_{PH} = 16.5, C*H*$_3$–CH–P). ^{13}C NMR (125 MHz; $CDCl_3$): δ_C 156.4, 136.7, 128.9, 128.4, 74.0, 71.8, 67.5, 65.8, 62.0, 44.7, 43.4 (d, J_{CP} = 161), 16.2. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 28.16. ES-MS: *m/z* 362.3 (M+2Na$^+$). IR (neat): ν (cm^{-1}) 3295, 2941, 1698, 1530, 1453.

4.2.3. *n*-Butyl 1-(benzyloxycarbonylamino) ethyl phosphonate (1c)

Yield: 31% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.15. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 11.6 (1H, br, OH), 7.33 (5H, m, Ph), 5.57 (1H, br, NH), 5.10 (2H, s, CH_2-Ph), 4.16 (1H, m, CH–P), 4.02 (2H, dd, J_{PH} = 13.4, J_3 = 7.2, CH_2O but), 1.60 (2H, qui, J_3 = 7.2, CH_2 but), 1.35 (5H, m, CH_2 but, C*H*$_3$–CH–P), 0.89 (3H, t, J_3 = 7.2, CH_3 but). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 156.2, 136.6, 128.9, 128.6, 128.5, 67.5, 66.6, 66.5 (t, J_{C-P} = 6), 44.7, 42.6 (d, J_{C-P} = 160), 32.7, 19.0, 16.1, 13.9. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 28.3. LSI MS: 316.09 (M+H$^+$), 338.07 (M+Na$^+$). HR MS: 316.1318 Da (calcd for $C_{14}H_{23}NO_5P$ (M+H$^+$): 316.1313). IR (neat): ν (cm^{-1}) 3285, 3063, 2958, 2873, 1688, 1541, 1453.

4.2.4. Methyl 1-(benzyloxycarbonylamino) propyl phosphonate (1d)

Yield: 25% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.1. ^{1}H NMR (300 MHz; DMSO-d_6): δ_H 8.31 (1H, s, OH), 7.35 (5H, s, Ph), 5.05 (2H, s, CH_2-Ph), 3.58 (4H, m, CH–P, OCH_3), 1.75–1.43 (2H, m, C*H*$_2$–CH–P), 0.85 (3H, m, CH_2–C*H*$_3$). ^{13}C NMR (75.5 MHz; DMSO-d_6): δ_C 156.5, 137.5, 128.6, 128.0, 128.7, 65.7, 55.2, 50.6, 48.6 (d, J_{CP} = 154), 22.5, 11.3. ^{31}P NMR (121.5 MHz; DMSO-d_6): δ_P 24.7. LSI MS: *m/z* 288.10 (M+H$^+$), 310.13 (M+Na$^+$). IR (neat): ν (cm^{-1}) 3304, 2956, 1694, 1531, 1454.

4.2.5. Methyl 1-(benzyloxycarbonylamino) benzyl phosphonate (1e)

Yield: 37% (white solid). Mp: 178–180 °C (lit.[11] 175 °C). ^{1}H NMR (300 MHz; DMSO-d_6): δ_H 8.27 (1H, d, J = 8.8, NH), 7.32 (10H, m, Ph), 5.02 (3H, m, CH–P, CH_2-Ph), 3.50 (3H, d, J_{PH} = 10.3, OCH_3). ^{13}C NMR (75.5 MHz; DMSO-d_6): δ_C 155.3, 137.2, 128.6, 128.4, 128.3, 128.2, 128.0, 127.5, 66.1, 52.8, 53.8, 51.8 (d, J_{CP} = 150). ^{31}P NMR (121.5 MHz; DMSO-d_6): δ_P 20.5. ES-MS: *m/z* 336.3 (M+H$^+$), 358.6 (M+Na$^+$). IR (neat): ν (cm^{-1}) 3294, 2936, 1714, 1543.

4.2.6. 1-(Benzyloxycarbonylamino) ethyl phosphonic acid (4)

The above procedure was followed, using acetaldehyde (1.09 mL, 19.5 mmol), except that the reaction was quenched with a chilled solution of NaOH (500 mg in 10 mL). The standard work-up yielded acid **4** (1.336 g 5.15 mmol, 40% yield) as a white solid. ^{1}H NMR (300 MHz; $CDCl_3/CD_3OD$): δ_H 7.32 (5H, m, Ph), 5.04 (2H, m, CH_2-Ph Cbz), 4.00 (1H, m, CH–P), 1.35 (3H, m). ^{13}C NMR (75.5 MHz; $CDCl_3/CD_3OD$): δ_C 158.6, 138.2, 130.0, 129.6, 129.5, 68.5, 47.1, 45.1 (d, J_{CP} = 159), 17.0. ^{31}P NMR (121.5 MHz; $CDCl_3/CD_3OD$): δ_P 27.8. LSI MS: *m/z* 260.04 (M+H$^+$), 282.01 (M+Na$^+$). HR MS (FAB): 260.0696 Da (calcd for $C_{10}H_{15}NO_5P$ (M+H$^+$): 260.0687).

4.3. General procedure for *O*-alkyl *N*-alkyl 1-(benzyloxycarbonylamino) alkyl(aryl) phosphonamides (2a–i)

Phosphonate monoester **1** (1.48 mmol) was suspended in dry CH_2Cl_2 (20 mL) and added dropwise to a solution of $(COCl)_2$ (258 μL, 2.96 mmol) and DMF (10 μL, 0.132 mmol) in dry CH_2Cl_2 (20 mL) and the solution was stirred at room temperature for 1 h under nitrogen. After this time, the volatile components were removed under reduced pressure and the residue re-dissolved in dry CH_2Cl_2 (30 mL) under N_2 and cooled to 0 °C. Triethylamine (412 μL, 2.38 mmol) is added dropwise followed by either *n*-propylamine (182 μL, 2.22 mmol) or a solution of Boc-Lys-OMe (613 mg, 2.35 mmol) in CH_2Cl_2. The solution was brought to room temperature and stirred for 1 day. After this time, the solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (50 mL) and extracted with 5% $NaHCO_3$ (3 × 50 mL) then with water (20 mL) and brine (20 mL). The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography on silica gel (eluent: CH_2Cl_2 100% to CH_2Cl_2/MeOH 98:2, 0.5% gradient of methanol).

4.3.1. *O*-Methyl *N*-propyl 1-(benzyloxycarbonylamino) ethyl phosphonamide (2a)

Yield: 79% (oil). R_f (AcOEt/MeOH 95:5): 0.5. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 7.25 (5H, m, Ph), 6.19 and 5.65 (1H, d, J = 9.8, NH Cbz, two diastereoisomers), 5.01 (2H, s, CH_2-Ph), 3.98 (1H, m, CH–P), 3.55, 3.53 (3H, 2 × d, J_{PH} = 10.5, OCH_3, two diastereoisomers), 3.05 (1H, m, NH–P), 2.74 (2H, m, C*H*$_2$–NH prop), 1.32 (5H, m, C*H*$_2$–CH_2–NH, C*H*$_3$–CH–P), 0.78 (3H, m, CH_3 prop). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 156.0, 155.6, 136.2, 128.2, 127.9, 127.8, 127.7, 66.5, 50.6, 50.5, 50.4, 45.2, 43.3 (d, J_{CP} = 144), 44.1, 41.8 (d, J_{CP} = 146), 42.6, 42.3, 25.0, 15.4, 15.1, 10.8. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 33.3, 32.0. ES-MS: *m/z* 337.2 (M+Na$^+$). Anal. Calcd for $C_{14}H_{23}N_2O_4P$: C, 53.50; H, 7.38; N, 8.91. Found: C, 52.99; H, 7.41; N, 8.66. IR (neat): ν (cm^{-1}) 3233, 3033, 2961, 2875, 1702, 1537, 1453.

4.3.2. O-(2-(Methoxy)ethyl) *N*-propyl 1-(benzyloxycarbonylamino) ethyl phosphonamide (2b)

Yield: 26% (oil). R_f (CH_2Cl_2/MeOH 97:3) = 0.5. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 7.21 (5H, m, Ph), 5.67 and 5.33 (1H, d, J = 9.7, NH Cbz, two diastereoisomers), 5.03 (2H, m, CH_2-Ph), 4.08–3.96 (3H, m, CH–P, CH_2–O–P), 3.45 (2H, m, C*H*$_2$–O–CH_3), 3.28 (3H, 2 × s, OCH_3, diastereoisomers), 2.87–2.68 (3H, m, C*H*$_2$–NH prop, NH–P), 1.41–1.26 (5H, m, C*H*$_2$–CH_2–NH, C*H*$_3$–CH–P), 0.78 (3H, t, CH_3 prop). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 156.4, 136.8, 128.8, 128.5, 128.4, 128.3, 72.1, 67.2, 63.7, 63.6, 63.5, 63.4, 59.3, 46.3, 44.4 (d, J_{CP} = 144), 45.3, 43.3 (d, J_{CP} = 146), 42.9, 25.6, 16.2, 15.8, 11.4. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 32.6, 30.6. ES-MS: *m/z* 358.1 (M$^+$), 381.2 (M+Na$^+$). LSI MS: *m/z* 359.10 (M+H$^+$), 381.07 (M+Na$^+$), 397.03 (M+K$^+$). HR MS (FAB): 359.1731 Da (calcd for $C_{16}H_{28}N_2O_5P$ (M+H$^+$): 359.1735). Anal. Calcd for $C_{16}H_{27}N_2O_5P$: C, 53.62; H, 7.59; N, 7.82. Found: C, 52.95; H, 7.56; N, 7.11. IR (neat): ν (cm^{-1}) 3323, 3179, 3067, 2953, 2872, 1691, 1528, 1452.

4.3.3. *O*-*n*-Butyl *N*-propyl 1-(benzyloxycarbonylamino) ethyl phosphonamide (2c)

Yield: 86% (white solid). Mp: 42–48 °C. R_f (CH_2Cl_2/MeOH 97:3): 0.4. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 7.33 (5H, m, Ph), 5.57 and 5.10 (1H, d, J = 9.8, NH Cbz, two diastereoisomers), 5.11 (2H, m, CH_2-Ph), 4.03 (2H, m, CH_2O), 3.90 (1H, m, CH–P), 2.92–2.58 (3H, m, C*H*$_2$–NH, NH–P), 1.60 (2H, m, CH_2 but), 1.51–1.28 (7H, m, C*H*$_3$–CH–P, C*H*$_2$–CH_2–NH, CH_2 but), 0.95–0.82 (6H, m, CH_3 but, CH_3 prop). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 156.5, 136.8, 128.9, 128.8, 128.6, 128.5, 128.4, 67.3, 64.4, 64.1, 46.1, 44.9, 44.2, 43.3, 43.0, 32.9, 32.8, 25.7, 25.6, 19.1, 16.2, 15.9, 14.0, 11.5. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 31.8, 30.6. LSI MS: *m/z* 357.12 (M+H$^+$), 379.09 (M+Na$^+$). HR MS (FAB): 357.1936 Da (calcd for $C_{17}H_{30}N_2O_4P$ (M+H$^+$): 357.1943). FT-IR (neat): ν (cm^{-1}) 3319, 3183, 3035, 2957, 2932, 2872, 1688, 1529.

4.3.4. *O*-Methyl *N*-propyl 1-(benzyloxycarbonylamino) propyl phosphonamide (2d)

Yield: 35% (oil). R_f (AcOEt) = 0.2. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 7.32 (5H, m, Ph), 6.03 and 5.46 (1H, d, J = 9.7, NH Cbz, two diastereoisomers), 5.11 (2H, m, CH_2-Ph), 3.89 (1H, m, CH–P), 3.62 (3H, m, OCH_3), 3.10–2.69 (3H, m, C*H*$_2$–NH, NH–P), 1.75–1.43 (2H, m, C*H*$_2$–CH–P, C*H*$_2$–CH_2–NH), 0.90 (6H, m, C*H*$_3$–CH_2–CH–P, CH_3 prop). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 157.1, 136.9, 128.8, 128.4, 128.3, 128.2, 67.2, 51.8, 51.0, 50.9, 49.9, 48.9, 43.1, 25.6, 22.2, 11.5, 10.9. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 32.6, 31.1. LSI MS: *m/z* 329.14 (M+H$^+$), 657.29 (2M+H$^+$). IR (neat): ν (cm^{-1}) 3312, 3200, 2961, 1686, 1535.

4.3.5. *O*-Methyl *N*-propyl 1-(benzyloxycarbonylamino) benzyl phosphonamide (2e)

Yield: 56% (white solid). Mp: 107–130 °C. R_f (AcOEt) = 0.4. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 7.35 (10H, m, Ph), 6.24 and 6.07 (1H, dd, J = 9.2, J = 5.5, NH Cbz, 2 × diastereoisomers), 5.08 (3H, m, CH–P, CH_2-Ph), 3.66 and 3.47 (3H, d, J_{PH} = 10.7, OCH_3, 2 × diastereoisomers), 2.83 (2H, m, C*H*$_2$–NH prop), 2.65 (1H, br m, NH–P), 1.42 and 1.29 (2H, 2 × qui, J = 7.3, C*H*$_2$–CH_2–NH, two diastereoisomers), 0.85 and 0.79 (3H, 2 × t, J = 7.3, CH_3 prop, two diastereoisomers). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 155.7, 136.1, 128.4, 128.3, 128.2, 127.8, 127.7, 67.0, 53.7, 51.9 (d, J_{CP} = 147), 51.3, 42.8, 25.1, 11.0. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 29.3, 28.5. ES-MS: *m/z* 399.1 (M+Na$^+$). LSI MS: *m/z* 377.10 (M+H$^+$). HR MS (FAB): 377.1634 Da (calcd for $C_{19}H_{26}N_2O_4P$ (M+H$^+$): 377.1630). Anal. Calcd for $C_{19}H_{25}N_2O_4P$: C, 60.63; H, 6.69; N, 7.44. Found: C, 60.34; H, 6.72; N, 7.29. IR (neat): ν (cm^{-1}) 3326, 3211, 2931, 2873, 1688, 1533, 1454.

4.3.6. *O*-Methyl *N*-(ε-amino N$^\alpha$-*tert*-butoxycarbonylamino lysyl methylester) 1-(benzyloxycarbonylamino) ethyl phosphonamide (2f)

Yield: 64% (oil). R_f (AcOEt/MeOH 95:5) = 0.5. ^{1}H NMR (400 MHz; $CDCl_3$): δ_H 7.32 (5H, m, Ph), 6.10–5.76 (1H, br m, NH Cbz), 5.37 (1H, br, NH Boc), 5.09 (2H, s, CH_2-Ph), 4.25 (1H, m, α-CH Lys), 4.04 (1H, m, CH–P), 3.76–3.60 (6H, m, $COOCH_3$, $POCH_3$), 3.21 (1H, br, NH–P), 2.88 (2H, m, ε-CH_2 Lys), 1.62–1.33 (18H, m). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 173.3, 156.1, 155.5, 136.4, 128.4, 128.0, 127.9, 79.7, 66.9, 53.4, 52.1, 50.9, 45.2, 44.2, 43.7, 43.5, 42.7, 40.5, 40.2, 40.1, 32.0, 31.9, 31.7, 31.4, 28.3, 22.3, 15.7, 15.3. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 33.5, 33.2, 32.2, 32.0. LSI MS: *m/z* 516.15 (M+H$^+$). HR MS (FAB): 516.2466 Da (calcd for $C_{23}H_{39}N_3O_8P$ (M+H$^+$): 516.2474). IR (neat): ν (cm^{-1}) 3259, 2949, 1698, 1526, 1454.

4.3.7. O-(2-(Methoxy)ethyl) N-(ε-amino N$^\alpha$-*tert*-butoxycarbonylamino lysyl methylester) 1-(benzyloxycarbonylamino) ethyl phosphonamide (2g)

Yield: 48% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.75. ^{1}H NMR (400 MHz; $CDCl_3$): δ_H 7.31 (5H, m, Ph), 5.64–5.05 (2H, br m, N*H*-Boc, NH Cbz), 5.02 (2H, s, CH_2-Ph), 4.23–4.00 (4H, m, α-CH Lys, CH_2–O–P, CH–P), 3.70 (3H, s, $COOCH_3$), 3.51 (2H, m, C*H*$_2$–O–CH_3), 3.33 (3H, s, OCH_3), 2.96–2.86 (3H, m, ε-CH_2 Lys, NH–P), 1.73–1.31 (18H, m). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 173.3, 155.4, 136.4, 128.4, 128.1, 128.0, 79.8, 71.7, 66.9, 63.3, 58.9, 53.3, 52.2, 44.3, 43.4, 40.4, 32.2, 31.6, 28.3, 22.2, 15.7. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 32.4, 32.3, 32.0, 30.9. LSI MS: *m/z* 560.19 (M+H$^+$), 582.19 (M+Na$^+$). HR MS (FAB): 560.2724 Da (calcd for $C_{25}H_{43}N_3O_9P$ (M+H$^+$): 560.2736). IR (neat): ν (cm^{-1}) 3269, 2935, 1703, 1525.

4.3.8. *O*-*n*-Butyl N-(ε-amino N$^\alpha$-*tert*-butoxycarbonylamino lysyl methylester) 1-(benzyloxycarbonylamino) ethyl phosphonamide (2h)

Yield: 38% (oil). R_f (CH_2Cl_2, MeOH 95:5) = 0.5. ^{1}H NMR (400 MHz; $CDCl_3$): δ_H 7.28 (5H, m, Ph), 5.91–5.24 (2H, br, NH Cbz, NH-Boc), 5.08 (2H, s, CH_2-Ph), 4.20 (1H, m, α-CH Lys), 3.96 (2H, m, CH_2O but), 3.82 (1H, m, CH–P), 3.66 (3H, s, OCH_3), 3.09–2.96 (1H, m, NH–P), 2.83 (2H, m, ε-CH_2 Lys), 1.69–1.27 (22H, m), 0.85 (3H, m, CH_3 but). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 173.3, 155.9, 155.4, 136.4, 128.4, 128.0, 127.9, 79.7, 66.7, 64.0, 53.3, 52.1, 45.3, 44.4, 43.9, 42.9, 40.6, 40.4, 40.1, 32.5, 32.0, 31.7, 28.2, 22.3, 18.8, 15.6, 13.6. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 32.0, 31.6, 30.7, 30.6. LSI MS: *m/z* 558.23 (M+H$^+$), 580.2 (M+Na$^+$). HR MS (FAB): 558.2928 Da (calcd for $C_{26}H_{45}N_3O_8P$ (M+H$^+$): 558.2944). IR (neat): ν (cm^{-1}) 3266, 2956, 1703, 1524.

4.3.9. *O*-Methyl *N*-(ε-amino N$^\alpha$-*tert*-butoxycarbonylamino lysyl methylester) 1-(benzyloxycarbonylamino) benzyl phosphonamide (2i)

Yield: 67% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.4. ^{1}H NMR (400 MHz; $CDCl_3$): δ_H 7.33 (10H, m, Ph), 6.08 (1H, br, NH Cbz), 5.05 (4H, m, CH-Ph, CH_2-Ph, NH-Boc), 4.22 (1H, br, α-CH Lys), 3.72 and 3.71 (3H, 2 × s, $COOCH_3$, diastereoisomers), 3.67 and 3.46 (3H, 2 × d, J_{PH} = 10.9, $POCH_3$, diastereoisomers), 2.88 (1H, m, NH–P), 2.72–2.54 (2H, m, ε-CH_2 Lys), 1.68–1.18 (15H, m). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 173.2, 155.4, 136.1, 128.7, 128.6, 128.5, 128.1, 127.9, 79.9, 67.2, 53.3, 52.2, 51.5, 40.6, 31.9, 28.3, 22.1. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 29.2, 29.0, 28.9. ES-MS: *m/z* 600.3 (M+Na$^+$), 616.6 (M+K$^+$). LSI MS: *m/z* 578.21 (M+H$^+$). HR MS (FAB): 578.2628 Da (calcd for $C_{28}H_{41}N_3O_8P$ (M+H$^+$): 578.2631). IR (neat): ν (cm^{-1}) 3340, 2948, 1697, 1497.

4.4. General procedure for *O*-alkyl *N*-alkyl 1-amino alkyl(aryl) phosphonamides (3a–i)

Phosphonamide 2 (100 mg) was dissolved in methanol (10 mL) and Pd/C 10% (10 mg) was added. The solution was stirred under H_2 until the reaction went to completion (monitored by TLC). The catalyst was then filtered on Celite and the remaining solution was removed under reduced pressure. The crude product 3 required generally further purification which was performed by flash chromatography on silica gel (eluents: CH_2Cl_2 100% to CH_2Cl_2/MeOH 95:5 (1% gradient) or AcOEt 100% to AcOEt/MeOH 95:5 (1% gradient)).

4.4.1. *O*-Methyl *N*-propyl 1-amino ethyl phosphonamide (3a)

Yield: 77% (oil). R_f (AcOEt/MeOH 95:5) = 0.2. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 3.61 and 3.60 (3H, 2 × d, J_{PH} = 10.4, OCH_3, diastereoisomers), 3.07–2.80 (4H, m), 1.70 (2H, br s, N*H*$_2$), 1.47 and 1.46 (2H, sext, J = 7.2, C*H*$_2$–CH_2–NH, two diastereoisomers), 1.26 and 1.25 (3H, 2 × dd, J_{PH} = 16.9, J_3 = 7.2, two diastereoisomers), 0.87 (3H, t, J = 7.2, CH_3 prop). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 50.9, 50.7, 46.2, 45.7, 44.3 and 43.6 (2 × d, J_{CP} = 140, diastereoisomers), 43.3, 26.0, 17.7, 17.3, 11.5. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 36.6, 36.2. ES-MS: *m/z* 180.103 (M$^+$). HR MS (FAB): 180.1030 Da (calcd for $C_6H_{18}N_2O_2P$ (M$^+$): 180.1027). IR (neat): ν (cm^{-1}) 3218, 2961, 2874, 1595, 1456.

4.4.2. *O*-2-((Methoxy)ethyl) *N*-propyl 1-amino ethyl phosphonamide (3b)

Yield: 81% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.3. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 4.07 (2H, m, CH_2–O–P), 3.52 (2H, m, CH_2–O–CH_3), 3.32 (3H, s, OCH_3), 3.07 (1H, m, CH–P), 2.88 (2H, m, CH_2–NH), 2.77 (1H, m, NH–P), 1.75 (2H, br s, NH_2), 1.46 and 1.45 (2H, 2 × qui, J = 7.3, CH_2–CH_2–NH, two diastereoisomers), 1.26 and 1.25 (3H, 2 × dd, J_{C-H} = 7.2, J_{P-H} = 17.1, CH_3–CH, diastereoisomers), 0.86 (3H, t, J = 7.3, CH_3 prop). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 71.7, 62.5, 58.7, 45.9, 44.1 and 43.6 (2 × d, J_{CP} = 140, diastereoisomers), 42.7, 25.5, 17.0, 16.7, 10.9. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 35.4, 35.1. LSI MS: *m/z* 225.13 (M+H$^+$). HR MS (FAB): 225.1369 Da (calcd for $C_8H_{22}N_2O_3P$ (M+H$^+$): 225.1368). IR (neat): ν (cm^{-1}) 3255, 2962, 2934, 2876, 1658, 1453.

4.4.3. *O*-*n*-Butyl *N*-propyl 1-amino ethyl phosphonamide (3c)

Yield: 65% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.4. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 3.96 and 3.85 (2H, 2 × m, CH_2O, diastereoisomers), 3.02 (1H, m, CH–P), 2.90 (2H, m, CH_2–NH), 2.63 (1H, m, NH–P), 1.58 (4H, m, NH_2, CH_2 but), 1.46 and 1.45 (2H, 2 × sext, J = 7.2, CH_2–CH_2–NH, diastereoisomers), 1.33 (2H, sext, J = 7.2, CH_2 but), 1.25 and 1.24 (3H, 2 × dd, J_{C-H} = 7.3, J_{P-H} = 16.9, CH_3–CH, diastereoisomers), 0.86 (6H, m). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 62.5, 45.0, 44.5, 43.2 and 42.6 (2 × d, J_{CP} = 138, diastereoisomers), 42.0, 31.6, 24.7, 17.8, 16.4, 16.0, 12.6, 10.1. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 29.6, 29.4. LSI MS: *m/z* 223.15 (M+H$^+$). HR MS (FAB): 223.1586 Da (calcd for $C_9H_{24}N_2O_2P$ (M+H$^+$): 223.1575). IR (neat): ν (cm^{-1}) 3209, 2960, 2933, 2874, 1598.

4.4.4. *O*-Methyl *N*-propyl 1-amino propyl phosphonamide (3d)

Yield: 96% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.5. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 3.68 (3H, d, J_{PH} = 10.5, OCH_3), 3.03–2.76 (4H, m, CH–P, CH_2–NH, NH–P), 1.85 (3H, m, CH_AH$_B$–CH–P, NH_2), 1.52 (3H, m, CH$_A$$H_B$–CH–P, C$H_2$–$CH_2$–NH), 1.06 (3H, t, J = 7.3, CH_3–CH_2–CH–P), 0.93 (3H, m, CH_3 prop). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 51.9, 51.0, 50.5 and 49.6 (2 × d, J_{CP} = 139, diastereoisomers), 50.3, 42.9, 25.6, 24.4, 24.1 (t, J_{CP} = 36), 11.1, 10.9. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 36.1, 35.5. LSI MS: *m/z* 195.08 (M+H$^+$). HR MS (FAB): 194.1176 Da (calcd for $C_7H_{19}N_2O_2P$ (M$^+$): 194.1184). IR (neat): ν (cm^{-1}) 3220, 2960, 2874, 1457.

4.4.5. *O*-Methyl *N*-propyl 1-amino benzyl phosphonamide (3e)

Yield: 100% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.5. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 7.30 (5H, m, Ph), 4.25 and 4.22 (1H, 2 × d, J_{PH} = 15.5, CH–P, two diastereoisomers), 3.67 and 3.62 (3H, 2 × d, J_{PH} = 10.7, OCH_3), 2.80–2.69 (2.5H, m, CH_2–NH prop, NH–P diastereoisomer A), 2.50 (0.5H, m, NH–P, diastereoisomer B), 2.07 (2H, br s, NH_2), 1.40 (2H, m, CH_2–CH_2–NH), 0.84 (3H, m, CH_3 prop). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 138.8, 138.5, 128.8, 128.2, 128.1, 128.0, 127.9, 71.7, 56.2, 54.4 (d, J_{CP} = 137), 51.5, 43.5, 25.8, 11.5. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 32.0, 31.6. LSI MS: *m/z* 243.15 (M+H$^+$), 485.27 (2M+H$^+$), 727.35 (3M+H$^+$). HR MS (FAB): 243.1274 Da (calcd for $C_{11}H_{20}N_2O_2P$ (M+H$^+$): 243.1262). IR (neat): ν (cm^{-1}) 3215, 2958, 2873, 1668, 1602, 1554, 1494, 1452.

4.4.6. *O*-Methyl *N*-(ε-amino N$^{\alpha}$-*tert*-butoxycarbonylamino lysyl methylester) 1-amino ethyl phosphonamide (3f)

Yield: 100% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.3. ^{1}H NMR (400 MHz; $CDCl_3$): δ_H 5.28 (1H, br, NH-Boc), 4.20 (1H, m, α-CH Lys), 3.72 and 3.60 (3H, 2 × d, J_{PH} = 10, $POCH_3$, diastereoisomers), 3.66 (3H, s, $COOCH_3$), 3.11–2.97 (4H, m, ε-CH_2 Lys, CH–P, NH–P), 1.89–1.18 (20H, m). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 173.2, 155.4, 79.7, 53.2, 52.2, 50.5, 45.7, 45.2, 44.6, 44.3, 43.8, 43.3, 43.1, 40.7, 32.1, 31.7, 28.2, 22.3, 17.4, 17.3, 17.0. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 36.0, 35.6. LSI MS: *m/z* 382.22 (M+H$^+$), 404.21 (M+Na$^+$). HR MS (FAB): 382.2098 Da (calcd for $C_{15}H_{33}N_3O_6P$ (M+H$^+$): 382.2107). IR (neat): ν (cm^{-1}) 3258, 2948, 2867, 1742, 1704, 1525.

4.4.7. *O*-(2-(Methoxy)ethyl) *N*-(ε-amino N$^{\alpha}$-*tert*-butoxycarbonyl-amino lysyl methylester) 1-amino ethyl phosphonamide (3g)

Yield: 99% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.3. ^{1}H NMR (400 MHz; $CDCl_3$): δ_H 5.16 (1H, br, NH-Boc), 4.20 (1H, m, α-CH Lys), 4.06 (2H, m, CH_2–O–P), 3.66 (3H, s, $COOCH_3$), 3.52 (2H, m, CH_2–O–CH_3), 3.32 (3H, s, OCH_3), 3.08–2.77 (4H, m, ε-CH_2 Lys, CH–P, NH–P), 1.96 (2H, br s, NH_2), 1.72–1.22 (18H, m). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 173.3, 155.4, 79.8, 71.9, 66.9, 62.9, 58.9, 53.3, 52.2, 45.5, 44.1, 40.6, 32.2, 31.9, 28.3, 22.3, 17.0. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 35.4, 35.0. LSI MS: *m/z* 426.23 (M+H$^+$). HR MS (FAB): 426.2355 (calcd for $C_{17}H_{37}N_3O_7P$ (M+H$^+$): 426.2369). IR (neat): ν (cm^{-1}) 3272, 2932, 1741, 1705, 1526.

4.4.8. *O*-*n*-Butyl N-(ε-amino N$^{\alpha}$-*tert*-butoxycarbonylamino lysyl methylester) 1-amino ethyl phosphonamide (3h)

Yield: 80% (oil). R_f (AcOEt 100%): 0.4. ^{1}H NMR (400 MHz; $CDCl_3$): δ_H 5.29 (1H, br, NH-Boc), 4.27 (1H, m, α-CH Lys), 4.04 and 3.91 (2H, 2 × m, CH_2–O–P), 3.73 (3H, s, OCH_3), 3.09–2.93 (3H, m, CH–P, ε-CH_2 Lys), 2.76 (1H, br, NH–P), 1.79–1.37 (22H, m), 1.32 and 1.31 (3H, 2 × dd, J_{C-H} = 7.2, J_{P-H} = 16.7, CH–CH_3, diastereoisomers), 0.94 (3H, t, J = 7.2, CH_3 but). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 173.2, 155.4, 79.8, 63.6, 53.2, 52.2, 45.9, 45.3, 44.5, 43.9, 40.8, 32.6, 32.1, 31.9, 28.2, 22.3, 18.8, 17.5, 17.1, 13.6. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 34.5, 34.1. LSI MS: *m/z* 424.18 (M+H$^+$), 446.08 (M+Na$^+$). HR MS (FAB): 424.2562 Da (calcd for $C_{23}H_{41}N_3O_6P$ (M+H$^+$): 424.2576). IR (neat): ν (cm^{-1}) 3237, 2959, 2871, 1743, 1704, 1527.

4.4.9. *O*-Methyl *N*-propyl 1-amino benzyl phosphonamide (3i)

Yield: 100% (oil). R_f (CH_2Cl_2/MeOH 9:1) = 0.5. ^{1}H NMR (300 MHz; $CDCl_3$): δ_H 7.30 (5H, m, Ph), 4.25 and 4.22 (1H, 2 × d, J_{PH} = 15.5, CH–P, two diastereoisomers), 3.67 and 3.62 (3H, 2 × d, J_{PH} = 10.7, OCH_3), 2.80–2.69 (2.5H, m, CH_2–NH prop, NH–P diastereoisomer A), 2.50 (0.5H, m, NH–P, diastereoisomer B), 2.07 (2H, br s, NH_2), 1.40 (2H, m, CH_2–CH_2–NH), 0.84 (3H, m, CH_3 prop). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 138.8, 138.5, 128.8, 128.2, 128.1, 128.0, 127.9, 71.7, 56.2, 54.4 (d, J_{CP} = 137), 51.5, 43.5, 25.8, 11.5. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 32.0, 31.6. LSI MS: *m/z* 243.15 (M+H$^+$), 485.27 (2M+H$^+$), 727.35 (3M+H$^+$). HR MS (FAB): 243.1274 Da (calcd for $C_{11}H_{20}N_2O_2P$ (M+H$^+$): 243.1262). IR (neat): ν (cm^{-1}) 3215, 2958, 2873, 1668, 1602, 1554, 1494, 1452.

4.4.10. *N*^{6}-Benzoyl 5′-(*tert*-butyldimethylsilyl) 3′-(1-(benzyloxycarbonylamino) ethyl phosphonyl) adenosine (6a) and *N*^{6}-benzoyl 5′-(*tert*-butyldimethylsilyl) 2′-(1-(benzyloxycarbonylamino) ethyl phosphonyl) adenosine (6b)

Phosphonic acid **4** (163 mg, 0.61 mmol) was coevaporated three times with dry pyridine, then dissolved in the same solvent (2 mL). *N*^{6}-Benzoyl 5′-(*tert*-butyldimethylsilyl) adenosine (**5**) (244 mg, 0.50 mmol) was coevaporated three times with dry pyridine, dissolved in the same solvent (5 mL) and added to the stirring suspension of **62**. Then Dowex 50W 8X resin (pyr$^+$) (0.2 g), and EDC (477 mg, 2.5 mmol) were added, and the solution was stirred at 45 °C for 2 days. Water (10 mL) was added, and the solution was stirred for 10 min filtered, and removed under reduced pressure. The residue was dissolved in $CHCl_3$ (20 mL) and extracted with water (2 × 20 mL) and brine (20 mL). The organic phase was dried over $MgSO_4$, and removed under reduced pressure. The residue was purified by column chromatography on silica gel (eluent $CHCl_3$/MeOH/Et_3N: gradient from 68:2:0.1 to 5:2:0.1) to yield a 3:1 mixture of **6a** and **6b** as triethylammonium salts (219 mg, 0.35 mmol, 70%

yield). R_f ($CHCl_3$/MeOH/Et_3N 6:1:0.1): 0.3. ^1H NMR (400 MHz; $CDCl_3$): δ_H 11.90 (1H, br s, OH), 9.28 (1H, br s, Ad NH), 8.68 (1H, s, CH Ad), 8.30 and 8.27 (1H, 2 × s, CH Ad, 2 isomers), 7.96 (2H, d, J = 7.3, 2,6 CH Bz), 7.51 (1H, m, Bz), 7.42 (2H, m, Bz), 7.27–7.08 (5H, m, Cbz), 6.21 and 6.18 (0.2H, 2 × d, J = 3.6, 1′ CH (**6b**), two diastereoisomers), 6.13 and 6.11 (0.8H, 2 × d, J = 5.5, 1′ CH (**6a**), two diastereoisomers), 5.69 and 5.62 (0.2H, 2 × d, J = 8.5, NH Cbz (**6b**), two diastereoisomers), 5.44 and 5.33 (0.8H, 2 × d, J = 7.0 and 9.2, NH Cbz (**6a**), two diastereoisomers), 5.07–4.93 (2.2H, m, CH_2 Cbz and 2′ CH (**6b**)), 4.83 and 4.74 (0.8H, 2 × m, 3′ CH (**6a**), two diastereoisomers), 4.61 and 4.55 (0.8H, 2 × m, 2′ CH_2 (**6a**), two diastereoisomers), 4.45 (0.2H, m, 3′ CH (**6b**)), 4.25–4.05 (1H, m, 4′ CH), 3.96 (1H, m, CH–P), 3.84–3.64 (2H, m, 5′ CH_2), 2.97 (6H, q, J = 7.2, $(CH_3CH_2)_3NH^+$ salt), 1.34 (3H, m, CH_3–CH–P), 1.22 (9H, t, J = 7.2, $(CH_3CH_2)_3NH^+$ salt), 1.19 and 0.85 and 0.82 (9H, 3 × s, $(CH_3)_3C$–Si, isomers), 0.04 and 0.00 and −0.02 (6H, 3 × s, CH_3–Si, isomers). ^{13}C NMR (75.5 MHz; $CDCl_3$): δ_C 165.4, 156.6, 156.4, 156.3, 152.9, 152.7, 152.6, 152.0, 149.9, 141.8, 141.7, 137.1, 136.9, 134.0, 133.0, 129.3, 129.0, 128.8, 128.6, 128.4, 128.2, 123.6, 123.5, 87.8, 87.6, 86.1, 85.7, 85.9, 76.0, 75.6, 67.0, 66.9, 63.5, 46.4, 44.3 (d, J_{CP} = 153, isomers), 45.9, 44.6, 42.6 (d, J_{CP} = 150, isomers), 26.4, 26.3, 18.7, 17.5, 16.9 (q, J_{CP} = 48), 8.8, −5.0, −5.1. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 24.9, 22.4, 21.8. ES-MS (−ve ion): m/z 725.4 ($M-H^+$), (+ve ion): m/z 727.1 ($M+H^+$), 749.2 ($M+Na^+$). LSI MS: m/z 727.26 ($M+H^+$), HR MS (FAB): 727.2683 Da (calcd for $C_{33}H_{44}N_6O_9PSi$ ($M+H^+$): 727.2676). IR (neat): ν (cm^{-1}) 3255, 2931, 2857, 1697, 1609, 1578.

4.4.11. 5′-(*tert*-Butyldimethylsilyl) 3′-(1-(benzyloxycarbonylamino) ethyl phosphonyl) adenosine (7a) and 5′-(*tert*-butyl dimethyl silyl) 2′-(1-(benzyloxycarbonylamino) ethyl phosphonyl) adenosine (7b)

Compound **6a**/**6b** (250 mg, 0.30 mmol) was dissolved in methanol (5 mL), then NH_3 aq 33% (15 mL) was added, and the mixture was stirred at room temperature overnight. Then, the solvents were removed under reduced pressure and the residue was purified by column chromatography on silica gel (eluent $CHCl_3$/MeOH/Et_3N: gradient from 65:5:0.1 to 5:2:0.1) to yield a 3:1 mixture of **7a** and **7b** as triethylammonium salts (210 mg, 0.29 mmol, 97%). R_f ($CHCl_3$/MeOH/Et_3N 6:1:0.1): 0.3. ^1H NMR (400 MHz; $CDCl_3$): δ_H 11.7 (1H, br s, OH), 8.23 and 8.22 (1H, 2 × s, CH Ad, two isomers), 8.12 and 8.09 (1H, 2 × s, CH Ad, two isomers), 7.29 (5H, m, Cbz), 6.87 (2H, br s, Ad NH_2), 6.46 and 6.39 (1H, 2 × d, J = 7.7, J = 9.0, NH Cbz), 6.15 (0.2H, m, 1′ CH (**7b**)), 6.06 (0.8H, m, 1′ CH (**7a**)), 5.10–4.98 (2.2H, m, CH_2 Cbz and 2′ CH (**7b**)), 4.88 and 4.79 (0.8H, 2 × m, 3′ CH (**7a**), two diastereoisomers), 4.66 and 4.50 (0.8H, 2 × m, 2′ CH_2 (**7a**), two diastereoisomers), 4.58 (0.2H, m, 3′ CH (**7b**)), 4.27–3.92 (2H, m, 4′ CH, CH–P), 3.84–3.68 (2H, m, 5′ CH_2), 2.95 (6H, q, J = 7.3, $(CH_3CH_2)_3NH^+$ salt), 1.37 (3H, m, CH_3–CH–P), 1.22 (9H, t, J = 7.3, $(CH_3CH_2)_3NH^+$ salt), 0.87 and 0.83 (9H, 3 × s, $(CH_3)_3C$–Si, isomers), 0.06 and 0.00, (6H, 2 × s, CH_3–Si). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 156.1, 156.0, 155.6, 152.6, 150.2, 150.0, 138.7 139.6, 136.9, 136.7, 128.9, 128.4, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7, 119.5, 119.4, 87.3, 87.0, 85.6, 85.5, 85.2, 85.1, 75.5, 75.2, 74.8, 66.4, 66.3, 63.2, 45.7, 44.0 (d, J_{CP} = 174, isomers), 45.7, 44.2, 42.5 (d, J_{CP} = 171, isomers), 25.9, 25.8, 18.4, 17.8, 16.4 (q, J_{CP} = 137), 8.4, −5.4, −5.5. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 24.3, 22.2, 21.8, 21.5. ES-MS (−ve ion): m/z 621.3 ($M-H^+$), (+ve ion): m/z 623.2 ($M+H^+$), 645.2 ($M+Na^+$). LSI MS: m/z 623.30 ($M+H^+$), HR MS (FAB): 623.2427 Da (calcd for $C_{26}H_{40}N_6O_8SiP$ ($M+H^+$): 623.2414). IR (neat): ν (cm^{-1}) 3182, 2930, 2858, 1710, 1642, 1600, 1573.

4.4.12. 3′-(1-(Benzyloxycarbonylamino) ethyl phosphonyl) adenosine (8a) and 2′-(1-(benzyloxycarbonylamino) ethyl phosphonyl) adenosine (8b)

Compound **7a/7b** (140 mg, 0.193 mmol) was dissolved in THF (5 mL), then $Et_3N{\cdot}3HF$ (155 μL, 0.965 mmol) was added and the mixture was stirred at room temperature overnight. The solvent was then removed under reduced pressure, and the residue dissolved in ethyl acetate (10 mL) and extracted with water (3 × 20 mL). The combined aqueous phases were evaporated under reduced pressure and the residue was purified by column chromatography on silica gel (eluent $CHCl_3$/MeOH/NH_3: gradient from 6:1:0.1 to 5:2:0.1) to yield a 3:1 mixture of **8a** and **8b** as ammonium salts (100 mg, 0.19 mmol, 99%). R_f ($CHCl_3$/MeOH/NH_3 6:1:0.1): 0.2. ^1H NMR (400 MHz; CD_3OD): δ_H 8.27 and 8.23 (1H, 2 × s, CH Ad, two isomers), 8.16 and 8.15 (1H, 2 × s, CH Ad, two isomers), 7.35–7.23 (5H, m, Cbz), 6.12 and 6.09 (0.2H, 2 × d, J = 5.7, 1′ CH (**8b**), two diastereoisomers), 5.98 and 5.95 (0.8H, 2 × d, J = 7.0 and 6.5, 1′ CH (**8a**), two diastereoisomers), 5.31 (0.2H, m, 2′ CH (**8b**)), 5.12–4.77 (3.6H, m, CH_2 Cbz, 2′ and 3′ CH (**8a**)), 4.56 and 4.52 (0.2H, 2 × dd, J = 3.2 and 5.0, 3′ CH (**8b**), two diastereoisomers), 4.33 and 4.29 (0.8H, 2 × m, 4′ CH (**8a**), two diastereoisomers), 4.18 (0.2H, m, 4′ CH (**8b**)), 3.95 (1H, m, CH–P), 3.84–3.73 (2H, m, 5′ CH_2), 1.39 and 1.38 (2.4H, 2 × dd, J_{C-H} = 7.2, J_{P-H} = 15.0, CH_3–CH–P (**8a**), two diastereoisomers), 1.22 (0.6H, m, CH_3–CH–P (**8b**)). ^{13}C NMR (100.5 MHz; CD_3OD): δ_C 157.5, 153.5, 150.0, 142.0, 138.3, 129.5, 129.0, 128.8, 128.7, 121.0, 91.0, 90.9, 87.9, 87.8, 87.6, 87.5, 78.1, 78.0, 76.3, 75.1, 72.4, 67.7, 67.6, 63.2, 63.0, 47.1, 45.6 (d, J_{CP} = 154, isomers), 46.7, 45.2 (d, J_{CP} = 152, isomers), 17.0. ^{31}P NMR (121.5 MHz; CD_3OD): δ_P 22.7, 22.2, 22.0, 21.9. ES-MS (−ve ion): m/z 507.1 ($M-H^+$), (+ve ion): m/z 509.1 ($M+H^+$), 531.1 ($M+Na^+$). LSI MS: m/z 509.15 ($M+H^+$), HR MS (FAB): 509.1535 Da (calcd for $C_{20}H_{26}N_6O_8P$ ($M+H^+$) 509.1549). IR (neat): ν (cm^{-1}) 3180, 2935, 1693, 1645, 1577.

4.4.13. 3′-(1-Amino ethyl phosphonyl) adenosine (9a) and 2′-(1-amino ethyl phosphonyl) adenosine (9b)

Compound **8a/8b** (229 mg, 0.35 mmol), was dissolved in MeOH (5 mL), then water (5 mL) and Pd/C 5% Degussa type (5 mg) were added to the solution. The mixture was stirred under H_2 atmosphere for 18 h, then the solution was diluted with water (10 mL) filtered on paper in a Hirsch funnel. The catalyst was repeatedly washed with water (20 mL), then the filtrate was evaporated under reduced pressure and coevaporated several times with ammonia to yield a 3:1 mixture of **9a** and **9b** in as ammonium salts (121 mg, 0.31 mmol, 86%).

^1H NMR (500 MHz; D_2O): δ_H 8.42 and 8.41 (1H, 2 × s, CH Ad, isomers), 8.31 and 8.29 (1H, 2 × s, CH Ad, isomers), 6.30 and 6.29 (0.25H, 2 × d, J = 5.8, 1′ CH (**9b**)), 6.21 and 6.20 (0.75H, 2 × d, J = 6.6, 1′ CH (**9a**)), 5.36 (0.25H, m, 2′ CH (**9b**)), 5.00 (1.5H, m, 2′ and 3′ CH (**9a**)), 4.66 (0.25H, m, 3′ CH (**9b**)), 4.55 (0.75H, m, 4′ CH (**9a**)), 4.40 (0.25H, m, 4′ CH (**9b**)), 4.04–3.90 (2H, m, 5′ CH_2), 3.60 (0.75H, m, CH–P (**9a**)), 3.20 (0.25H, m, CH–P (**9b**)), 1.57 (2.25H, dd, J_{CH} = 7.4, J_{PH} = 16.8, CH_3–CH–P (**9a**)), 1.41 and 1.36 (0.75H, 2 × dd, J_{CH} = 7.3, J_{PH} = 16.7, CH_3–CH–P (**9b**), two diastereoisomers). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 154.9, 154.7, 152.0, 151.9, 147.8, 147.7, 140.3, 118.3, 87.8, 87.6, 87.5, 85.9, 85.8, 85.4, 76.2, 76.1, 74.9, 74.5, 72.8, 70.0, 69.9, 61.3, 61.2, 61.0, 45.2, 44.6, 43.7, 43.1, 13.3, 13.1. ^{31}P NMR (121.5 MHz; D_2O): δ_P 17.8, 17.1, 16.9, 16.6. ES-MS (−ve ion): m/z 373.1 ($M-H^+$), 747.2 (2M−H+), 769.2 ($2M-H^++Na^+$); (+ve ion): m/z 375.1 ($M+H^+$), 397.1 ($M+Na^+$), 749.2 ($2M+H^+$), 771.2 ($2M+Na^+$). LSI MS: m/z 375.12 ($M+H^+$). HR MS (FAB): 375.1184 Da (calcd for $C_{12}H_{20}N_6O_6P$ ($M+H^+$) 375.1181). RP-HPLC: *t*R, **9a**, 15.50 min; **9b**, 16.10 min.

A sample of **8b** (40 mg) was purified by anion exchange chromatography using a MonoQ FPLC column, eluting with 0.25 M NH_4HCO_3. The fractions were monitored at 260 nm and the first two peaks eluted were collected and freeze dried. The residue was then dissolved in MeOH (2 mL), then water (2 mL) and Pd/C 5% Degussa type (2 mg) was added to the solution. The mixture was stirred under H_2 atmosphere for 3 days, then it was diluted with water (5 mL) and filtered. The catalyst was repeatedly washed

with water (5 mL), then the filtrate was freeze-dried to yield a sample of single isomer **9b** (3.0 mg) in >95% purity. ^{1}H NMR (400 MHz; D_2O): δ_H 8.42 (1H, s, CH Ad), 8.32 (1H, s, CH Ad), 6.30 and 6.29 (1H, 2 × d, J = 6.0, 1′ CH, two diastereoisomers), 5.36 (1H, m, 2′ CH), 4.65 (1H, m, 3′ CH), 4.40 (1H, m, 4′ CH), 3.00 (1H, dd, J = 2.7,13.0, 5′ CH_AH$_B$), 3.92 (1H, dd, J_3 = 2.7,13.0, 5′ CH$_A$$H_B$), 3.23 (1H, m, CH–P), 1.32 (3H, m, CH_3–CH–P). ^{31}P NMR (121.5 MHz; D_2O): δ_P 17.2, 16.8. ES-MS (+ve ion): m/z 375.1174 (M+H$^+$); (−ve ion): m/z 373.1. (M−H$^+$). HR MS (TOF): 375.1176 Da (calcd for $C_{12}H_{20}N_6O_6P$ (M+H$^+$) 375.1181). RP-HPLC: tR, 16.06 min.

4.4.14. N^6-Benzoyl 5′-(*tert*-butyl dimethyl silyl) 3′-(1-(benzyloxycarbonylamino) ethyl phosphonyl) 2′-deoxyadenosine (11)

Phosphonic acid **1a** (528 mg, 1.93 mmol) was coevaporated three times with dry pyridine, then dissolved in the same solvent (2 mL). N^6-Benzoyl 5′-(*tert*-butyldimethylsilyl) 2′-deoxyadenosine (**10**) (755 mg, 1.60 mmol) was coevaporated three times with dry pyridine, dissolved in the same solvent (5 mL) and added to the stirred solution of **1a**. Dowex 50W 8X resin (pyr$^+$) (0.2 g), and dicyclohexylcarbodiimide (1.648 g, 8 mmol) were added, and the solution was stirred at room temperature for 6 days. Water (10 mL) was added, and the solution was stirred for 30 min. After this time, the suspension was filtered on paper in a Hirsch funnel and the solid was washed repeatedly with chloroform. The filtrate was then evaporated under reduced pressure, the residue was dissolved in $CHCl_3$ (20 mL) and extracted twice with water (20 mL). The organic phase was dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (eluent $CHCl_3$/MeOH/Et_3N: gradient from 68:2:0.1 to 6:1:0.1) to yield compound **11** as the triethylammonium salt (702 mg, 0.86 mmol, 54% yield). R_f: ($CHCl_3$/MeOH/Et_3N 6:1:0.1): 0.4. ^{1}H NMR (400 MHz; $CDCl_3$): δ_H 12.36 (1H, br s, OH), 9.72 (1H, br s, NH Ad), 8.73 (1H, s, CH Ad), 8.35 and 8.32 (1H, 2 × s, CH Ad, two diastereoisomers), 8.04 (2H, d, J = 7.5, 2,6 CH Bz), 7.54 (1H, m, Bz) 7.45 (2H, m, Bz), 7.27 (5H, m, Cbz), 6.53 (1H, m, 1′ CH), 5.78 (1H, br m, NH Cbz), 5.11–4.99 (3H, m, CH_2 Cbz, 3′ CH), 4.31 and 4.20 (1H, 2 × s, 4′ CH, two diastereoisomers), 3.98 (1H, m, CH–P), 3.79 (2H, m, 5′ CH$_2$), 2.96 (6H, q, J = 7.0, (CH$_3$–CH_2)$_3$NH$^+$), 2.50–2.37 (2H, m, 2′ CH$_2$), 1.36 (3H, dd, J_3 = 7.0, J_{PH} = 14.8, CH_3–CH–P), 1.35 (9H, t, J = 7.0, (CH_3–CH$_2$)$_3$NH$^+$), 0.85 (9H, s, (CH_3)$_3$C–Si), 0.07 and 0.05 (6H, 2 × s, CH$_3$–Si). ^{13}C NMR (100.5 MHz; $CDCl_3$): δ_C 165.0, 156.1, 156.0, 152.3, 151.6, 141.3, 137.8, 136.7, 133.7, 132.5, 128.6, 128.3, 128.1, 128.0, 127.9, 124.4, 87.5, 84.5, 75.9, 75.7, 66.5, 63.6, 45.4, 45.2, 43.7 (d, J_{CP} = 149), 45.0, 43.5 (d, J_{CP} = 150), 41.2, 40.8, 26.0, 18.3, 16.9, 8.5, −5.4. ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 20.8. ES-MS (−ve ion): m/z 709.3 (M−H$^+$). LSI MS: m/z 711.56 (M+H$^+$), 733.45 (M+Na$^+$), 749.53 (M+K$^+$). HR MS: 711.2722 Da (calcd for $C_{33}H_{44}N_6O_8SiP$ (M+H$^+$) 711.2727). IR (neat): ν (cm^{-1}) 3243, 2931, 2856, 1697, 1577.

4.4.15. 5′-(*tert*-Butyl dimethyl silyl) 3′-(1-(benzyloxycarbonylamino) ethyl phosphonyl) 2′-deoxyadenosine (12)

Compound **11** (485 mg, 0.598 mmol) was dissolved in methanol (5 mL) then aq NH_3 33% (15 mL) was added and the mixture was stirred at room temperature overnight. Then, the solvents were removed under reduced pressure and the residue was purified by column chromatography on silica gel (eluent $CHCl_3$/MeOH/Et_3N: gradient from 65:5:0.1 to 6:1:0.1) to yield compound **12** as the triethylammonium salt (362 mg, 0.512 mmol, 85%). R_f ($CHCl_3$/MeOH/Et_3N 6:1:0.1): 0.3.

^{1}H NMR (400 MHz; CD_3OD): δ_H 8.38 (1H, s, CH Ad), 8.26 (1H, s, CH Ad), 7.16 (5H, m, Cbz), 6.41 (1H, m, 1′ CH), 5.12 (1H, m, 3′ CH), 4.99 (2H, m, CH_2 Cbz), 4.27 and 4.18 (1H, 2 × s, 4′ CH, two diastereoisomers), 3.98–3.15 (3H, m, CH–P, 5′ CH$_2$), 3.17 (6H, q, J = 7.0, (CH$_3$–CH_2)$_3$NH$^+$), 2.67–2.55 (2H, m, 2′ CH$_2$), 1.37 (3H, dd, J_{CH} = 7.3, J_{PH} = 15.7, CH_3–CH–P), 1.29 (9H, t, J = 7.0, (CH_3–CH$_2$)$_3$NH$^+$), 0.85 (9H, s, (CH_3)$_3$C–Si), 0.05 (6H, s, CH$_3$–Si). ^{13}C NMR (100.5 MHz; CD_3OD): δ_C 158.1, 154.9, 150.2, 150.0, 141.7, 138.4, 129.9, 129.4, 129.0, 127.8, 127.9, 120.2, 88.9, 86.4, 76.7, 76.5, 67.6, 64.7, 47.7, 46.5, 45.0 (d, J_{CP} = 149), 41.8, 41.6, 26.5, 19.2, 16.8, 9.2, −5.2. ^{31}P NMR (121.5 MHz; CD_3OD): δ_P 21.5. ES-MS (−ve ion): m/z 605.3 (M−H$^+$). LSI MS: m/z 607.24 (M+H$^+$). HR MS (FAB): 607.2460 Da (calcd for $C_{26}H_{40}N_6OSiP$ (M+H$^+$): 607.2465). IR (neat): ν (cm^{-1}) 3205, 3034, 2953, 2930, 2857, 1709, 1644, 1601, 1573, 1535.

4.4.16. 3′-(1-(Benzyloxycarbonylamino) ethyl phosphonyl) 2′-deoxyadenosine (13)

Compound **12** (120 mg, 0.13 mmol) was dissolved in THF (5 mL), then $Et_3N{\cdot}3HF$ (67 μL, 0.418 mmol) was added, and the mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the residue dissolved in ethyl acetate and extracted three times with water. The combined water phases were evaporated under reduced pressure and the residue was purified by column chromatography on silica gel (eluent $CHCl_3$/MeOH/NH_3: gradient from 65:5:0.1 to 6:1:0.1) to yield compound **13** as the ammonium salt (66 mg, 0.13 mmol, 99%). R_f ($CHCl_3$/MeOH/NH_3 6:1:0.1): 0.2. ^{1}H NMR (400 MHz; CD_3OD): δ_H 8.26 and 8.24 (1H, 2 × s, CH Ad, two diastereoisomers), 8.17 (1H, s, CH Ad), 7.35–7.03 (5H, m, Cbz), 6.65 (1H, d, J = 8.3, NH), 6.41 (1H, m, 1′ CH), 5.15–4.91 (3H, m, 3′ CH, CH_2 Cbz), 4.23 and 4.14 (1H, 2 × m, 4′ CH, two diastereoisomers), 3.89 (1H, m, CH–P), 3.77 (2H, m, 5′ CH$_2$), 2.70 (1H, m, 2′ CH_AH$_B$), 2.36 (1H, m, 2′ CH$_A$$H_B$), 1.35 (3H, dd, J_{CH} = 7.3, J_{PH} = 15.6, CH_3–CH–P). ^{13}C NMR (100.5 MHz; CD_3OD): δ_C 165.7, 158.1, 157.7, 153.5, 150.3, 142.1, 138.7, 129.8, 129.7, 129.4, 129.3, 121.3, 89.7, 87.7, 77.5, 77.3, 67.8, 64.7, 41.2, 17.1. ^{31}P NMR (121.5 MHz; CD_3OD): δ_P 21.7. ES-MS (−ve ion): m/z 491.1 (M−H$^+$). LSI MS: m/z 493.15 (M+H$^+$). HR MS (FAB): 493.1578 Da (calcd for $C_{20}H_{26}N_6O_7P$ (M+H$^+$): 493.1600). IR (neat): ν (cm^{-1}) 3205, 2933, 1694, 1646, 1618, 1577.

4.4.17. 3′-(1-Amino ethyl phosphonyl) 2′-deoxyadenosine (14)

Compound **13** (66 mg, 0.13 mmol), was dissolved in methanol (5 mL), then water (10 mL) and Pd/C 5% Degussa type (5 mg) were added to the solution. The mixture was stirred under H_2 atmosphere for 2 days, then diluted with water (10 mL) filtered on paper in a Hirsch funnel. The catalyst was repeatedly washed with water (20 mL), then the filtrate was evaporated under reduced pressure to yield compound **14** (30 mg, 0.08 mmol, 62%) as a glassy solid.

^{1}H NMR (400 MHz; D_2O): δ_H 8.44 (1H, s, CH Ad), 8.35 (1H, s, CH Ad), 6.62 (1H, dd, J = 5.7, 7.7, 1′ CH), 5.17 (1H, m, 3′ CH), 4.46 (1H, m, 4′ CH), 3.92 (2H, m, 5′ CH$_2$), 3.54 (1H, m, CH–P), 3.00 (1H, m, 2′ CH_AH$_B$), 2.82 (1H, m, 2′ CH$_A$$H_B$), 1.56 (3H, dd, J_{CH} = 7.3, J_{PH} = 15.8, CH_3–CH–P). ^{13}C NMR (100.5 MHz; D_2O): δ_C 154.9, 151.9, 147.9, 140.1, 118.6, 86.8, 84.6, 75.0, 61.5, 44.8, 43.4 (d, J_{CP} = 146), 38.7, 13.4. ^{31}P NMR (121.5 MHz; D_2O): δ_P 17.1. ES-MS (−ve ion): m/z 357.5 (M−H$^+$). LSI MS: m/z 359.12 (M+H$^+$), 371.22 (M+Na$^+$). HR MS (FAB): 359.1223 Da (calcd for $C_{12}H_{20}N_6O_5P$ (M+H$^+$): 359.1232). IR (neat): ν (cm^{-1}) 3103, 2922, 2590, 1695, 1645, 1606, 1573. RP-HPLC: t_R, 15.0 min.

4.4.18. 3′-Methylphosphonyl 2′-deoxyadenosine (15)

Methyl phosphonic acid (122 mg, 1.27 mmol) was coevaporated three times with dry pyridine, then dissolved in the same solvent (5 mL). N^6-Benzoyl 5′-TBDMS 2′-deoxyadenosine **10** (500 mg, 1.06 mmol) was coevaporated three times with dry pyridine, dissolved in the same solvent (5 mL) and added. Then Dowex 50W 8X resin (pyr$^+$) (0.2 g), and dicyclohexylcarbodiimide (1.09 g, 5.3 mmol) were added, and the solution was stirred at room temperature for 2 days. Water (15 mL) was added and the solution was stirred for 10 min. The solvent was removed under reduced

pressure, and the residue was partitioned between $CHCl_3$ (20 mL) and water. The organic phase was then extracted with water (2×20 mL), dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (eluent $CHCl_3$/MeOH/Et_3N: gradient from 68:2:0.1 to 65:5:0.1) to yield N^6-benzoyl 5′-(*tert*-butyl dimethyl silyl) 3′-(methyl phosphonyl) 2′-deoxyadenosine as the triethylammonium salt (276 mg, 0.42 mmol, 42% yield). ^{1}H NMR (400 MHz; $CDCl_3$): δ_H 9.44 (1H, br, OH), 8.66 (1H, s, CH Ad), 8.30 (1H, s, CH Ad), 7.95 (2H, d, J = 7.2, 2,6 CH Bz), 7.48 (1H, d, J = 7.2, 4 CH Bz), 7.40 (1H, t, J = 7.2, 3,5 CH Bz), 6.51 (1H, dd, J = 5.7, J = 7.5, 1′ CH), 4.88 (1H, m, 3′ CH), 4.25 (1H, m, 4′ CH), 3.82 (2H, m, 5′ CH_2), 2.92 (6H, q, $(CH_3CH_2)NH^+$), 2.67 (1H, m, 2′ CH_AH_B), 2.57 (1H, m, 2′ CH_AH_B), 1.22 (13H, m, CH_3–P, $(CH_3CH_2)NH^+$), 0.80 (9H, s, $(CH_3)_3$C–Si), 0.00 (6H, s, CH_3–Si). ^{31}P NMR (121.5 MHz; $CDCl_3$): δ_P 21.7. ES-MS (−ve ion): *m/z* 546.1 ($M-H^+$); (+ve ion): *m/z* 548.0 ($M+H^+$); 570.1 ($M+Na^+$). HR MS (FAB): 548.2085 Da (calcd for $C_{24}H_{35}N_5O_6SiP$ ($M+H^+$) 548.2094).

The above compound (200 mg, 0.308 mmol) was dissolved in methanol (2 mL), then aq NH_3 33% (6 mL) were added and the mixture was stirred at room temperature overnight. The solvents were removed under reduced pressure and the residue was purified by column chromatography on silica gel (eluent $CHCl_3$/MeOH/Et_3N: gradient from 65:5:0.1 to 6:1:0.1) to yield the debenzylated compound as the triethylammonium salt (128 mg). This material was dissolved in THF (5 mL), then $Et_3N{\cdot}3HF$ (220 μL, 1.38 mmol) was added and the mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was partitioned between ethyl acetate (10 mL) and water (20 mL). The organic phase was further extracted with water (2×10 mL) and the combined water phases were evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel (eluent: $CHCl_3$/MeOH/NH_3: gradient from 6:1:0.1 to 5:2:0.1) to yield compound **15** as ammonium salt (74 mg, 70% overall). ^{1}H NMR (400 MHz; CD_3OD): δ_H 8.34 (1H, br s, CH Ad), 8.18 (1H, br s, CH Ad), 6.45 (1H, dd, J = 5.7,8.0, 1′ CH), 5.0 (1H, m, 3′ CH), 4.26 (1H, m, 4′ CH), 3.87 (1H, dd, J = 2.8,12.4, 5′ CH_AH_B), 3.83 (1H, dd, J = 3.1,12.4, 5′ CH_AH_B), 2.91 (1H, m, 2′ CH_ACH_B), 2.60 (1H, ddd, J = 2.6, 5.7,13.5, 2′ CH_ACH_B), 1.32 (3H, d, J_{PH} = 16.5, CH_3–P).^{13}C NMR (100.5 MHz; D_2O): δ_C 157.5, 153.5, 149.9, 141.6, 120.9, 89.1, 87.1, 75.5, 63.4, 40.7, 14.2, 12.8 (d, J_{CP} = 137).^{31}P NMR (121.5 MHz; D_2O): δ_P 24.4. ES-MS (−ve ion): *m/z* 328.0 ($M-H^+$), 679.1 ($2(M-H^+)+Na^+$); (+ve ion): *m/z* 330.1 ($M+H^+$), 352.1 ($M+Na^+$). HR MS (TOF): 328.0827 Da (calcd for $C_{11}H_{15}N_5O_5P$ ($M-H^+$) 328.0816). IR (neat): ν (cm^{-1}) 3042, 2861, 1652, 1606. RP-HPLC: t_R, 8.60 min.

4.5. 5′-(*tert*-Butyl dimethyl silyl) 3′-(1-amino ethyl phosphonyl) 2′-deoxyadenosine (16)

Compound **11** (100 mg, 0.123 mmol) was dissolved in MeOH/NH_3 aq 1:3 (15 mL). Pd/C 5% Degussa type (5 mg) was added and the reaction was stirred at room temperature under H_2 atmosphere for 18 h. After this time, the catalyst was filtered and the filtrate was evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (eluent $CHCl_3$/MeOH/NH_3: gradient from 6:1:0.1 to 5:2:0.1), affording compound **16** (60 mg, 0.123 mmol) in 99% yield. R_f ($CHCl_3$/MeOH/Et_3N 6:1:0.1): 0.1. ^{1}H NMR (400 MHz; $CDCl_3/CD_3OD/D_2O$ 2:3:1): δ_H 8.21 (1H, s, CH Ad), 8.11 (1H, s, CH Ad), 6.39 (1H, dd, J = 5.7, 8.2, 1′ CH), 4.97 (1H, m, 3′ CH), 4.26 (1H, m, 4′ CH), 3.82 (2H, m, 5′ CH_2), 3.19 (1H, m, CH–P), 3.00 (1H, m, 2′ CH_2), 2.67 (1H, ddd, J_3 = 2.0, 5.7, 13.8, 2′ CH_AH_B), 2.55 (1H, m, 2′ CH_AH_B), 1.37 (3H, dd, J_3 = 7.2, J_{PH} = 14.8, CH_3–CH–P), 0.79 (9H, s, $(CH_3)_3$C–Si), 0.00 (6H, s, CH_3–Si). ^{13}C NMR (100.5 MHz; $CDCl_3/CD_3OD/D_2O$ 2:3:1): δ_C 157.1, 153.9, 150.1, 140.4, 120.4, 88.6, 86.0, 76.8, 64.8, 46.7, 45.3 (d, J_{CP} = 149), 41.8, 27.0, 19.6, 15.2, −4.57. ^{31}P NMR (121.5 MHz; CD_3OD): δ_P 14.9. ES-MS (+ve ion): *m/z* 473.2 ($M+H^+$); (−ve ion): *m/z* 471.2 ($M-H^+$). HR MS (TOF): 473.2105 Da (calcd for $C_{18}H_{34}N_6O_5PSi$ ($M+H^+$): 473.2092). IR (neat): ν (cm^{-1}) 3182, 2929, 2852, 1615, 1574.

4.6. 5′-(Methylcarbamoyl) 2′-deoxyadenosine (17)

Following the method of Fernandez et al. (ref), acetone *O*-((phenyloxy)carbonyl) oxime (828 mg, 4.3 mmol) was added a solution of 2′-deoxyadenosine (270 mg, 1 mmol), 4 Å molecular sieves (0.2 g), and freshly dried *C. antarctica* (CAL-B, 0.2 g, >10,000 U/g) in anhydrous THF (20 mL) under N_2, and the mixture was shaken at 200 rpm, 37 °C in an incubator/shaker for 3 days. After this time, the suspension was filtered on a synthered glass filter, the filter was washed first with THF (10 mL) and then with methanol (20 mL) and the filtrate was evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel (eluent: gradient from AcOEt 100% to AcOEt/MeOH 9:1), affording the 5′-phenylcarbonate derivative (272 mg). ^{1}H NMR (400 MHz; CD_3OD): δ_H 8.27 (1H, s, CH Ad), 8.21 (1H, s, CH Ad), 7.35 (2H, m), 7.21 (1H, m), 7.10 (2H, m), 6.47 (1H, t, J = 6.5, 1′ CH), 4.67 (1H, m, 3′ CH), 4.53 (1H, dd, J = 11.8,3.9, 5′ CH_AH_B), 4.45 (1H, dd, J = 11.8, 4.7, 5′ CH_AH_B), 4.23 (1H, dd, J = 4.0, 8.5, 4′ CH), 2.83 (1H, m, 2′ CH_ACH_B), 2.52 (1H, ddd, J = 13.8, 6.5, 4.2, 2′ CH_ACH_B). HR MS (FAB): 372.1314 Da (calcd for $C_{17}H_{18}N_5O_5$ ($M+H^+$): 372.1307).

The above material was dissolved in anhydrous THF (5 mL) under N_2 and 2.0 M solution of methylamine in THF (336 μL, 0.673 mmol) was added. The solution was stirred at 50 °C until the starting material is consumed (18 h). After this time, the solvent was removed under reduced pressure and the result solid was triturated diethyl ether, and filtered. The solid was washed with enough Et_2O to remove phenol completely. The product **17** (226 mg, 72% overall) was afforded as a white solid in 99% yield. R_f (AcOEt/MeOH 9:1): 0.15. ^{1}H NMR (400 MHz; D_2O): δ_H 7.99 (1H, s, CH Ad), 7.90 (1H, s, CH Ad), 6.16 (1H, t, J = 6.0, 1′ CH), 4.53 (1H, m, 3′ CH), 4.27–3.99 (3H, m), 2.3 (1H, m, 2′ CH_ACH_B), 2.50 (1H, m, 2′ CH_ACH_B), 2.45 (3H, s, CH_3). ^{13}C NMR (100.5 MHz; D_2O): δ_C 158.2, 154.4, 152.3, 148.0, 139.0, 118.2, 84.6, 83.6, 70.5, 63.5, 38.6, 26.5. LSI MS: *m/z* 309.13 ($M+H^+$). HRMS (FAB): 309.1318 Da (calcd for $C_{12}H_{17}N_6O_4$ ($M+H^+$): 309.1311). FT-IR (neat): ν (cm^{-1}) 3325, 2946, 1697, 1613, 1574.

4.7. MurM Radiochemical enzyme assays

200 μL incubations containing 0.66 mg total *M. flavus* tRNA, 37.5 μM [^{3}H]-Ala (1.45 μCi, ~3,200,000 cpm, stock 0.5 mM, 500 cpm/pmol) or 65.7 μM [^{14}C]-Ala (1.45 μCi, ~3,200,000 cpm, 114 mCi/mmol, 0.1 mCi/mL), 4 units of inorganic pyrophosphatase (IPP), 0.16 mg Alanyl tRNA synthetase ($AlaRS_{Pn16}$), 10 mM DTT, in 30 mM HEPES pH 7.6, 15 mM $MgCl_2$, 25 mM KCl, 2 mM ATP were incubated at 37 °C for 2 h. Then, 20 μL of 3 M Na acetate pH 4.5 were added, followed by 205 μL of phenol equilibrated with 10 mM Tris HCl, pH 8.0, 1 mM EDTA. The emulsion was mixed and centrifuged for 3 min at 13,000 rpm. The aqueous phase was pooled and added to 205 μL of chloroform/isoamyl alcohol 24:1. After centrifugation, the aqueous phase was pooled and supplemented with 520 μL of absolute ethanol at −20 °C. The precipitated aminoacyl-tRNA and was collected by centrifugation at 13000 rpm. The pellet was washed with 1 mL of 70% ethanol, dried under reduced pressure in a desiccator and resuspended into 50 μL of 3 mM Na acetate pH 4.5. The specific activity for each incubation (Cpm/pmol) was estimated by spotting 0.5 μL of crude incubation mixture (before tRNA isolation) on filter paper, which was dried and placed directly into scintillation liquid for counting. The radiolabelled aminoacyl tRNA concentration was estimated from precipitation of radioactivity from a 1 μL

sample of purified tRNA onto filter paper disks in ice cold 10% (w/v) trichloroacetic acid for 15 min. This wash was repeated twice, and the papers disks were finally washed in ice-cold ethanol, dried and placed into scintillation liquid for counting.

To follow the generation of Lipid 2-[^{3}H]-Ala or Lipid 2-[^{14}C]-Ala in the presence of an inhibitor, an assay mix typically contained in a 40 μL total volume, 50 mM MOPS pH 6.8, 30 mM KCl, 10 mM $MgCl_2$, 1.5% CHAPS, 1 mM DTT, 1 mM L-alanine, 50 μM Lipid 2, 0.2 μM [^{3}H]-Ala-tRNA (5.5×10^{-3} μCi, 12,000 cpm total counts in each incubation) or 0.5 μM [^{14}C]-Ala-tRNA (5.5×10^{-3} μCi, 12,000 cpm total counts in each incubation), 50 nM (0.09 μg) $MurM_{159}$ and the inhibitor solution in water (1–0.1 mM). The incubations were kept at 37 °C for 5 min and then terminated with the addition of 40 μL 6 M pyridinium acetate pH 4.5 and 80 μL of *n*-butanol. The incubations were then mixed and centrifuged at 13,000 rpm for 3 min then the supernatant *n*-butanol phase was pooled and washed with 80 μL of water. The organic phase was then placed directly in vials containing scintillation liquid for counting.

Inhibitors were tested initially at 1 mM final concentration, and inhibitors **9** and **14** were subsequently tested as 20–1000 μM concentrations. Non water-soluble inhibitors were dissolved in DMSO. The DMSO amount in the incubation did not usually exceed 10% of the total volume (4 μL) and positive and negative controls containing the solvent (4 μL) were carried out. All the incubations were performed in duplicate and positive controls (containing no inhibitor) and negative controls (not containing either MurM or Lipid 2) were carried out for each set of assays.

4.8. Assay of *S. pneumoniae* alanyl tRNA synthetase and seryl tRNA synthetase

S. pneumoniae alanyl tRNA synthetase and seryl tRNA synthetase were expressed and purified as previously described.[9] Activity was monitored by trichloroacetic acid precipitation and subsequent quantitation of [^{3}H]-alanyl or seryl-tRNA formed by the enzymes in the presence of 37.5 μM [^{3}H]-amino acid (204 cpm $pmol^{-1}$), 2 mM ATP, 30 mM HEPES, 15 mM $MgCl_2$ 5 mM DTT, 25 mM KCl, 1 μM $tRNA^{Ala}$ or $tRNA^{Ser}$ and 0.17 μM alanyl or seryl tRNA synthetase. Enzyme inhibition assays were carried out in the presence of 100 μM and 1 mM inhibitor.

4.9. Antibacterial testing

Inhibitors were tested for growth inhibition of *S. pneumoniae* strains 159 and pn16, *S. aureus* (MRSA, MSSA), *E. faecalis*, *M. flavus* and *Escherichia coli* in liquid culture, using the NCCLS protocol, at 1–5 mM final concentration.

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