Acylated sulfonamide adenosines as potent inhibitors of the adenylate-forming enzyme superfamily

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ABSTRACT

The superfamily of adenylate-forming enzymes all share a common chemistry. They activate a carboxylate group, on a specific substrate, by catalyzing the formation of a high energy mixed phosphoanhydride-linked nucleoside intermediate. Members of this diverse enzymatic family play key roles in a variety of metabolic pathways and therefore many have been regarded as drug targets. A generic approach to inhibit such enzymes is the use of non-hydrolysable sulfur-based bioisosteres of the adenylate intermediate. Here we compare the activity of compounds containing a sulfamoyl and sulfonamide linker respectively. An improved synthetic strategy was developed to generate inhibitors containing the latter that target isoleucyl- (IleRS) and seryl-tRNA synthetase (SerRS), two structurally distinct representatives of class I and II aminoacyl-tRNA synthetases (aaRSs). These enzymes attach their respective amino acid to its cognate tRNA and are indispensable for protein translation. Evaluation of the ability of the two similar isosteres to inhibit serRS revealed a remarkable difference, with an almost complete loss of activity for seryl-sulfonamide **15** (SerSoHA) compared to its sulfamoyl analogue (SerSA), while inhibition of IleRS was unaffected. To explain these observations, we have determined a 2.1 Å crystal structure of *Klebsiella pneumoniae* SerRS in complex with SerSA. Using this structure as a template, modelling of **15** in the active site predicts an unfavorable eclipsed conformation. We extended the same modelling strategy to representative members of the whole adenylate-forming enzyme superfamily, and were able to disclose a new classification system for adenylating enzymes, based on their protein fold. The results suggest that, other than for the structural and functional orthologues of the class II aaRSs, the O to C substitution within the sulfur-sugar link should generally preserve the inhibitory potency.

Keywords:

Adenylating enzymes
Aminoacyl-tRNA synthetases
Hydrolytically stable inhibitors
Eclipsed interactions
Homo-adenosine derivatives

1. Introduction

Adenylate-forming enzymes all catalyse the same reaction: the condensation between ATP and a weakly nucleophilic carboxylate group of a substrate molecule, with the concomitant release of a pyrophosphate byproduct. The resulting unstable acyl-AMP intermediate (Fig. 1A) can be converted into a carboxylic acid derivative as it readily reacts with common nucleophiles such as alcohols, thiols or amines, giving rise to esters, thioesters and amides respectively. The large superfamily of enzymes, functionally linked by this chemistry, are involved in a variety of essential cellular processes, that include but are not limited to nucleotide and enzyme co-factor biosynthesis, lipid catabolism, and the modification and aminoacylation of tRNAs

necessary for protein translation [1]. The critical roles of these enzymes have stimulated considerable interest in targeting them for the development of new antimicrobials [2,3].

Despite the common chemistry these enzymes share, they can be divided into five structurally distinct classes (Fig. 2). While a previously reported classification system employed a mixture of structural and functional similarity to make such divisions [1], we have updated this to account for newly available X-ray crystallographic structures and separated the clusters purely based on their protein fold. Class I members inherit their grouping as a result of older nomenclature in the literature for the main representatives of this group, the HIGH-motif containing Class I aminoacyl-tRNA synthetases (aaRS) [4,5]. Furthermore, this class also includes enzymes belonging to the N-type ATP pyrophosphatase family, such as GMP and NAD synthetase [6]. Both enzyme families are part of a larger functionally diverse group collectively known as the HUP family (CATH 3.40.50.620 [7]). Similar to the first class, the previously annotated Class II aminoacyl-tRNA synthetases designate the numbering of the

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Fig. 1. (190mm width; for convenience, all figures and schemes are provided separately as well): Chemical structures of the acyl-adenylate intermediate and different non-hydrolysable alternatives. [A] The natural acyl-adenylate; a variety of R-groups are possible as shown in Fig. 2. [B] The high affinity acyl-SA, mimicking the reaction intermediate, is prone to cyclative degradation into *N*-cycloadenosine (blue arrows). [C] Alternative sulfur-based intermediate analogues. Left: 5'-deoxyadenosine (SoA); Middle: 3-deazadenosine (S3DA) and the reported sulfonamide (SoHA) that target aminoacyl-tRNA synthetases, where the R-group corresponds to the side chain of a proteinogenic amino acid (aa); Right: Stable sulfamide based analogue targeting biotin ligase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. (140mm width): Proposed structure-based classification system of the adenylate-forming enzyme superfamily. The tertiary structure of an example member for each class is shown as a cartoon representation, with β -strands coloured magenta and α -helices in cyan. The molecular structure of the corresponding acyl group is shown for each. Class I, tyrosyl-tRNA synthetase (PDB ID 1N3L); Class II, alanyl-tRNA synthetase (PDB ID 4XEM); Class III, firefly luciferase (PDB ID 2D1S); Class IV, achromobactin synthetase protein D (PDB ID 2X3J); Class V, L-threonylcarbamoyladenylate synthase (SUA5, PDB ID 4E1B). For clarity only a monomeric subunit is shown for the representatives that form higher-order oligomers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

second structural cluster (CATH 3.30.930.10 [4,5], Fig. 2). This family also includes biotin and lipoate ligases that attach their respective substrate to protein targets [8]. Members of the Class III cluster (originally termed Class I in the older classification scheme [1]) are part of the functionally diverse ANL family (CATH 3.40.50.980 [9]). This family includes acetyl-CoA synthetase, fatty acid ligase and the non-ribosomal peptide synthetases (NRPS). Class IV adenylate-forming enzymes encompass the NRPS-independent peptide (NIS) synthetases involved in siderophore biosynthesis [10]. Finally, the Class

V group contains enzymes that transfer an activated carbamoyl moiety, which is necessary in some antibiotic biosynthetic pathways as well as for the formation of the universal N^6 -threonylcarbamoyl adenosine tRNA modification [11].

In general, mimicking the acyl-AMP intermediates by hydrolytically stable competitive inhibitors has proven a potent way of targeting the adenylate-forming enzymes from the different structural classes [12–14]. Focusing on aaRSs, enzymes which catalyse the activation and esterification of amino acids to their cognate tRNA, there

have been many successful approaches to enhance the stability of the aminoacyl-adenylate intermediate by substitution of the mixed phosphoanhydride linker with a sulfur based isoster [15–18]. Amongst these the aminoacyl-sulfamate isosteres (Fig. 1B, where R is a decarboxylated amino acid) have been established as the most potent aaRS inhibitors during the last two decades, but further elaboration of this scaffold to improve uptake has been limited as the sulfamate linker is prone to *N*-cycloadenosine formation (Fig. 1B) [12]. Numerous attempts have been made to improve the stability of these isosteres. Our group deleted the 5'-oxygen atom resulting in aaSoA analogues (Fig. 1C, aaSoA) [19]. This leads to a less electrophilic 5'-carbon atom because the resulting sulfonamide moiety is a poorer leaving group compared to the sulfamoyl group. Unfortunately, the majority of the resulting compounds no longer efficiently inhibited their target aaRS. More recently, we applied another strategy involving the synthesis of 3-deazaadenosine analogues (Fig. 1C, aaS3DA). These compounds lack the N³ atom of the adenine base, which is responsible for the nucleophilic attack on the 5'-carbon atom. An evaluation of six different analogues highlighted a specific class dependent inhibitory activity [20].

Alternative stabilization strategies have been applied to sulfamate based acyl-AMP bioisosteres targeting other adenylate-forming enzymes. In an attempt to limit disproportionation of biotinoyl-sulfamoyl-adenosine, a potent inhibitor of the Class II member biotin ligase, modifications of the adenine base [12] or addition of a methyl group to the C5'-position of the ribose [13] resulted in stabilization of the compounds. This was rationalized as likely the result of steric hindrance, preventing the *syn*-conformation required for the adenine N3 attack on the C5' atom. In both cases the modifications had a minimal effect on binding affinity, but the base modifications dramatically reduced the activity on whole cells [12]. In addition to these

scaffold elaborations, attempts have been made to substitute the 5'-oxygen with alternative atoms [12,21]. Substitution with an amine, yielding biotinoyl-AMS resulted in a more stable compound that is a potent and selective antitubercular [12]. Although chemically more difficult, replacement of the same atom with a methylene group also resulted in a potent inhibitor that was resistant to chemical and biochemical induced degradation [12,13].

The reported stability and effectiveness of the adenosine sulfonamide scaffold raises an open question as to whether it can be applied to other adenylate-forming enzymes. We developed an improved synthetic strategy to generate two aminoacyl analogues (Fig. 1C, aaSoHA) targeting the Class I isoleucyl-tRNA synthetase (IleRS) and the Class II seryl-tRNA synthetase (SerRS). Following synthesis of IleSoHA **14** and SerSoHA **15** (Scheme 1), the activity against the corresponding enzymes was evaluated. Combined with X-ray crystallographic studies and modelling we extended this characterization to representatives of each class of the adenylate-forming enzyme superfamily.

2. Results

2.1. Synthesis of aminoacylated sulfonamide homo-adenosines (aaSoHAs)

Reagents and conditions: [A] (a) DMP, PTSA·H₂O, acetone, rt, overnight; (b) TBDMS-Cl, imidazole, anh. DMF, rt, 3 d; (c) Boc₂O, DMAP, TEA, anh. DMF, 0 °C - rt, 3 d; (d) TBAF·3H₂O, anh. THF, rt, overnight; (e) Dess-Martin periodinane, anh. DCM, rt, 1.5 h; (f) (1) **9**, LiHMDS, anh. DMF/THF, -78 °C, 2.5 h; (2) **6**, anh. THF, -78 °C - rt, 2 d; (g) H₂, Pd/C, anh. MeOH, rt, 3 d; (h) TFA, anh. DCM, 0 °C, 2 h; (i) Boc-aa-(Bn)-OSu, Cs₂CO₃, anh. DMF, 0 °C - rt, 2 d - 4 d; (j) H₂,

Scheme 1. (190 mm width): [A] Successful reaction sequence leading to the desired sulfonamide nucleosides IleSoHA **14** and SerSoHA **15**; [B] Synthesis of Wittig-Horner reagent **9**.

Pd/C, anh. MeOH, rt, 2 d; (k) TFA/H₂O, 0 °C - rt, 3 h; [B] (a) Boc₂O, DMAP, TEA, anh. DCM, 0 °C - rt, overnight; (b) (1) (*i*Pr)₂NH, *n*-BuLi, anh. THF, -78 °C - 0 °C, 1 min; (2) **8**, anh. THF, -78 °C, 10 min; (3) Ph₂P(O)Cl, -78 °C, 1.5 h.

The synthetic scheme for the desired compounds **14** and **15** (Scheme 1A) started with selective introduction of the isopropylidene moiety. TBDMS protection of the remaining primary alcohol and subsequent di-Boc protection of the heterocyclic amine gave compound **4**. Selective deprotection of the silyl moiety resulted in protected nucleoside **5**. In analogy with the work of Shi et al., **5** was used in a Wittig-Horner reaction which, in our hands, afforded only **10** with a yield of 23% [12]. In spite of several attempts, we could not reproduce the previously published results whereby only **11** was obtained with a better yield than what we achieved for **10**. The employed Wittig-Horner reagent **9** was synthesized, utilizing methanesulfonamide **7** as the starting material in which the amino group was first Boc protected (Scheme 1B). The di-anion of **8** was then prepared with freshly made LDA, whereafter it was treated with diphenylphosphinic chloride in a substitution reaction (Scheme 1B). An important issue in this Wittig-Horner reaction was the instability of aldehyde **6** (Scheme 1A). This compound was synthesized in a high (isolated) yield (88%) utilizing Dess-Martin periodinane, but could not be stored overnight. Degradation of **6** was confirmed by NMR analysis and as a consequence, the crude aldehyde had to be used immediately upon production in order to obtain **10**. In addition, we observed that the selective (*E*)-alkene forming reaction limited the amount of starting material **5** which can be used, because attempts to scale-up (0.5 g) in our hands no longer afforded the desired product **10**. Quantitative reduction of the double bond using hydrogen gas resulted in com-

pound **12**, which was confirmed by mass analysis. Hence, **12** was immediately deprotected under anhydrous acidic conditions. Unfortunately, the desired compound **13** in our hands decomposed during work-up, in contrast to previously reported literature [12].

To avoid the use of a Boc protecting group on the sulfonamide moiety, we chose to prepare Wadsworth-Horner-Emmons and Wittig-Horner compounds **16** and **18** respectively (Scheme 2) in a similar reaction sequence as described for Boc protected Wittig-Horner product **9** (Scheme 1B). Both benzhydryl (**16**) and Cbz (**18**) protecting groups can be removed upon hydrogenolysis, thus avoiding the use of acid. Compounds **16** and **18** were both utilized for the C—C coupling reaction with crude aldehyde **6**, but both reactions failed to give the desired products **17** or **19** respectively (Scheme 2). The reaction of **6** with **16** resulted in the formation of two unknown (and incorrect) compounds, while reaction of **6** with **18** unexpectedly caused a Boc transfer from the adenine base to the sugar part of **5**, resulting in protected 5'-*O*-Boc-adenosine.

Reagents and conditions: (a) (1) DMAP, SOCl₂, anh. MeCN, 0 °C - rt, 4 h; (2) anh. MeCN, rt, overnight; (b) Boc₂O, DMAP, TEA, anh. MeCN, 0 °C - rt, 3 d; (c) (1) (*i*Pr)₂NH, *n*-BuLi, anh. THF, -78 °C - 0 °C, 1 min; (2) *N*-benzhydrylmethanesulfonamide, anh. DMF, -78 °C, 1 h; (3) **21**, anh. DMF, -78 °C, 1 h; (4) anh. DMF/THF, -78 °C - rt, 3 d.

A different approach for these modified Wittig reactions had to be found for obtaining the C—C linked molecules. Therefore, a nucleophilic substitution was envisioned in order to extend the 5'-carbon side chain (Scheme 3). Hereto, the remaining alcohol group of **2** was substituted by a chlorine atom, using thionyl chloride as the halogen donor. Chlorine would act as a stable, but still good leaving group

Scheme 2. (90 mm width): Two unsuccessful C—C coupling reactions (similar as in Scheme 1A, steps e and f) using either synthesized Wadsworth-Horner-Emmons reagent **16** or Wittig-Horner compound **18** (similar as in Scheme 1B).

Scheme 3. (190 mm width): Unsuccessful nucleophilic substitution approach which would have resulted in the desired sulfonamide **22**.

(installing better leaving groups immediately results in the formation of *N*-cycloadenosine (Fig. 1A), as seen with the sulfamate analogues of **14** and **15**). The adenine base was then di-Boc protected to obtain **21** devoid of acidic protons. This compound was subsequently reacted with the di-anion of *N*-benzhydrylmethanesulfonamide, which should lead to sulfonamide **22**. Unfortunately, no reaction occurred, even at room temperature, while at elevated temperatures, decomposition of the nucleoside was observed.

In view of decomposition of nucleosides at elevated temperatures in acidic medium, prior to work-up, the mixture, as obtained in step h of Scheme 1, was neutralized immediately following reaction with an excess of bicarbonate. Following conventional purification, **13** was isolated as its TFA salt in moderate yield. Amino acids isoleucine and serine were then coupled to the sulfonamide moiety using Cs_2CO_3 . Following removal of all remaining protecting groups, crude products **14** and **15** were purified by preparative reversed-phase HPLC, resulting in the desired analogues IleSoHA **14** and SerSoHA **15**.

2.2. Inhibitory activity

The ability of **14** and **15** to inhibit the respective aaRS was evaluated using an *in vitro* aminoacylation assay, monitoring their effect on the transfer of the appropriate C^{14} radiolabelled amino acid to tRNA (Fig. 3). In both cases the purified recombinant *E. coli* protein was utilized with a total tRNA pool from the same organism. Compound **14** showed potent inhibitory potential for the target *E. coli* IleRS. The inhibition profile, including the steep increase in aaRS activity between 50 and 10 nM of the compound, is in line with previous studies of the sulfamoyl analogue [20]. This observation is also reflected in an even slightly improved apparent K_i value of 1.2 nM for **14** (Supplemental Fig. S1) compared to its sulfamoyl analogue ($K_{i,\text{app}} = 1.9 \text{ nM}$ [20]). Surprisingly, **15** demonstrated little inhibitory activity. At a final concentration of 2 μM , it only reduced the ability of *E. coli* SerRS to aminoacylate tRNA by 30%. This is more than a 200-fold reduction in inhibitory capacity when compared to **14**, and a marked reduction to the previously reported activity of the analogous sulfamoyl, which showed an apparent K_i of 0.18 nM [20].

Fig. 3. (90mm width): Aminoacylation activity of *E. coli* ileRS and serRS in the presence of the respective sulfonamide linked intermediate analogue. Values were normalized based on the measured activity of each enzyme in the absence of inhibitor. Experiments were performed in triplicate, the mean and standard error are presented.

2.3. Structural studies

To understand why SerSoHA **15** showed a dramatically reduced activity relative to **14**, we solved the X-ray crystallographic structure of *Klebsiella pneumoniae* SerRS (KpSerRS) in complex with the canonical SerSA analogue (Fig. 4A and Supplemental Table 1). Examination of datasets of similar crystals soaked with high concentrations of **15** failed to reveal any suitable electron density corresponding to the compound (data not shown). KpSerRS shares 93.7% sequence identity (97.4% similarity) with the *E. coli* homologue, therefore interpretation of the ligand bound structure can be compared to the *in vitro* studies with the latter enzyme. The asymmetric unit of the crystal contains one protomer of the dimeric enzyme (Fig. 4A), the canonical homodimer formed by a crystallographic 2-fold rotation axis. The KpSerRS structure shows high similarity to the homologous enzyme from other bacterial species [22,23], with an N-terminal antiparallel coiled-coil arm, involved in tRNA binding, and a mixed α/β catalytic domain (Fig. 4A and Supplemental Fig. S2). The 2.1 Å crystal structure, currently the highest resolution for a bacterial SerRS orthologue in the PDB, unambiguously reveals the conformation of the intermediate analogue as evidenced by the polder omit map (Fig. 4A, insert). The compound makes numerous interactions with the protein, showing high complementarity with the large active site. Careful examination of the 5'-oxygen atom suggests it only makes weak Van der Waals interactions with the sidechain NH1 and NH2 atoms of Arg268. This residue, which is part of the catalytic core, is found in all Class II aaRSs where it anchors the N-terminal side of a conserved sequence motif [5,20]. In the ligand bound structure Arg268 makes H-bond and salt bridge interactions with the sulfamoyl and carbonyl group of the intermediate analogue (Fig. 4B). The latter is mediated by a strong ionic interaction as the tautomer of conjugated peptide bond, with an estimated pKa of 3.1 using Schrodinger Epik software [24], will be deprotonated at physiological pH. This calculated value is comparable to what was determined in previous literature for equivalent molecules [25]. In the case of **15**, calculations suggest a slight increase in the pKa of this group to 4.1, therefore it is also predicted to be deprotonated under the native conditions that were evaluated and therefore is likely not the cause of the observed loss of activity.

Using the empirically determined structure, SerSoHA **15** was modelled in the enzyme active site maintaining the same conformation of SerSA. The substitution of the O5' atom with a methylene group is readily accommodated, with no inter- or intramolecular clashes. Maintaining a torsional angle of 127.8° between the C4' atom and the sulfone group, the minimised model shows that the additional protons introduced by the methylene substitution are found in an energetically unfavorable (nearly) eclipsed position (Fig. 4C). To start with, a torsional angle of 13° is observed between the C5' and C6' protons (Fig. 4C, insert). Secondly, the other C6' proton is offset from the C4'-C5' bond by a 16° torsional angle. Finally, a similar torsional angle of 19° is seen between the second C5' proton and the C6'—S bond, a near overlap of atoms that is also observed in the experimental SerSA bound structure between the C5' proton and the O5'—S bond. Hence this structural pose of SerSoHA **15** is energetically unfavorable.

2.4. Comparative modelling studies

As IleSoHA **14** demonstrated high affinity for its target aaRS (Fig. 3), we wanted to examine whether the conformation of the atoms around the C5'-C6' bond of this compound was similar to that ob-

Fig. 4. (90mm width): (A) The crystal structure of *K. pneumoniae* SerRS bound to the intermediate analogue SerSA (PDB ID 6H9X). The protein is depicted as a cartoon representation and the SerSA shown as spheres. The insert shows the phenix.polder omit map for the bound SerSA contoured at 6σ. (B) Complementarity of SerSA with the enzyme active site. The protein surface has been calculated using a 1.4 Å probe. The side chains of interacting residues are shown as stick representations, the carbon atoms are coloured based on the secondary structure in panel A. R268 interacts with the carbonyl and sulfamate oxygen atoms of SerSA (yellow dashed lines). (C) Model

of SerSoHA **15** based on the pose of SerSA, showing the 3'-endo ribose conformation and the eclipsing protons and bonds. The insert shows the torsional angle between the eclipsed C5' and C6' protons when looking down the C6'—C5' bond. The predicted protonation state at physiological pH is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

served for SerSoHA **15** bound to SerRS. As there are no high resolution (sub 2.5 Å) crystal structures of IleRS in complex with an aminoacyl-AMP intermediate, or analogues thereof, we used the 1.85 Å resolution structure of the related leucyl-tRNA synthetase (LeuRS) in complex with LeuSA for comparative analysis (Fig. 5A; PDB ID 2V0C). Using a re-refined structure of this protein-ligand complex LeuSoHA was modelled in the active site, by substituting the O5' atom of LeuSA with a methylene group whilst maintaining the same bound conformation (Fig. 5B). In the original LeuSA structure one of the oxygen atoms of the sulfamoyl group makes a H-bond with a backbone atom which is maintained in the LeuSoHA model. Further examination of the model shows no clashing atoms between the compound and the protein suggesting this pose is a viable conformation. Focusing on the intramolecular contacts of LeuSoHA a staggered relationship for the C5' and C6' protons (torsional angle of 42°) was observed (Fig. 5B, insert). Similar values were also observed between the other C5' proton and the C6'—S bond (torsional angle of 51°). The second C6' proton and the C4'—C5' bond exhibited a torsional angle of 35°.

In order to investigate if we can extrapolate the above analyses of LeuSoHA and SerSoHA **15** to all other Class I and Class II aaRSs respectively, we examined the available intermediate-bound or non-hydrolysable analogue-bound crystal structures of the different aaRS orthologues (Fig. 5C and D). The ligands in the Class I representatives were superposed using the C1', C4' and O4' atoms of the ribose, which all demonstrated the same 2'-endo pucker (Fig. 5C). It is clear from this overlap that the position of the sulfamate group and the adenine ring is not completely fixed between enzymes. Rotating around the base N9 atom, the angle between the most displaced adenine rings is 16°, resulting in a maximum distance of 1.98 Å between their distal ends. Similarly, with a mean value of 161.6° but a standard deviation of 13.7°, the torsion angle between the C4'—C5' and the C6'—S bonds also demonstrate some variability. In all cases though the staggered conformation of the atoms circling the C5'—C6' bond is maintained, suggesting that the O5' to C substitution will not result in a detrimental effect on binding of the inhibitor to any Class I aaRS. The same ring based superposition strategy was also applied to all available Class II bound ligands, each showing the same 3'-endo ribose pucker (Fig. 5D). The overall conformation of the Class II inhibitors is more strictly fixed, with an all non-H atom RMSD of 0.417 Å. In particular the torsion angle between the C4'—C5' and the C6'—S bonds, with a mean value of 127.4°, has a standard deviation of 7.5°—just over half that was observed for the Class I intermediate analogues. This strongly suggests that the energetically unfavorable conformation observed for the SerSoHA model would be replicated with an O5' to C substitution in each of the examined structures, likely negatively affecting the binding affinity of all Class II aaRS targeting sulfonamide-linked intermediate analogues.

As the two aaRS classes are members of a larger superfamily of adenylate-forming enzymes (Fig. 2), we extended our structural analyses to other representatives, to evaluate whether the acyl-sulfonamide linker strategy can be employed to this structurally and functionally diverse group of enzymes (Fig. 6). A search for suitable ligand bound crystal structures, specifically of enzymes that have been previously considered as druggable targets, was performed. In all cases the O5' of the acyl-SA intermediate analogue was replaced by a methylene group in the minimised structure. For the Class I

Fig. 5. (90mm width): [A] Structure of re-refined *T. thermophilus* LeuRS, shown as a cartoon representation, bound to leucyl-sulfamoyl-adenosine (LeuSA - green spheres); [B] Stick representation of the bound pose of LeuSA. The insert in the lower panel shows the torsional angle between the staggered C5' and C6' protons when looking

down the C6'—C5' bond. The predicted protonation state at physiological pH is shown; [C] Superposition of class I aaRS intermediate analogues, making use of the available ligand bound structures for IleRS (PDB ID 1JZQ - red), TyrRS (PDB ID 6I5Y - yellow), LeuRS (PDB ID 2V0C - green), MetRS (PDB ID 1PFY - blue), GlnRS (PDB ID 1QTQ - white), GluRS (PDB ID 3AFH - grey) and TrpRS (PDB ID 1I6K - black); [D] Overlap of the bound intermediate analogues of class II aaRS representatives LysRS (PDB ID 3E9H - red), SerRS (PDB ID 6H9X - green), ProRS (PDB ID 2J3L - magenta), ThrRS (PDB ID 1NYQ - cyan), GlyRS (PDB ID 5F5W - blue), AsnRS (PDB ID 1X55 - white), AspRS (PDB ID 1G51 - grey) and AlaRS (PDB ID 3HXZ - orange). The stated colors correspond to the carbon atoms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

NAD⁺ synthetase, a member of the N-type ATP pyrophosphatase family, the conformation of the acyl-adenylate analogue is distinct to that observed for the Class I aaRS (1KQP, Fig. 6A). These variances include the pucker of the ribose and the position of the adenine relative to the sugar, and reflect differences in the binding mode between these two class members. Despite this, the sulfonamide based analogue of the NAD⁺-adenylate intermediate displays a staggered conformation with a 164° torsion angle between the C4'-C5' and C6'—S bonds, close to the mean value seen for the Class I tRNA synthetases. This positioning of the sulfonamide group within the active site, relative to the ribose, results in a 50° offset between the C6' and C5' H-atoms (Fig. 6A).

A biotinoyl-SoHA intermediate analogue, targeting the alternative Class II member biotin-protein ligase BirA (Fig. 6B), has previously been described as a potent inhibitor of this enzyme with demonstrable antitubercular activity [12,13]. This contrasts with our results for SerRS and what is predicted for the other Class II aaRS. However, while BirA shares the core six-stranded antiparallel β -sheet flanked by structurally conserved α -helices seen in SerRS and other class II aaRS, the orientation of the loop region involved in nucleoside recognition is significantly different (Supplemental Fig. S2). This difference leads to an alternative conformation of the intermediate whereby the S—C6'-C5'-C4' torsion angle of biotinoyl-SoHA is 201.8°, resulting in no eclipsing interactions around the C6'-C5' bond (Fig. 6B). Comparatively, the conformation of the biostere linker in biotinoyl-SoHA resembles more that of the intermediate analogues bound to the structurally distinct members of the Class III adenylyate-forming enzymes (Fig. 6C). Both share the same 3'-endo conformation of the sugar ring, and a similar C5'-C6' bond position relative to the ribose. In the case of the inhibitor conformation bound to the *Mycobacterium tuberculosis* long-chain-fatty-acid-AMP ligase the sulfone group is positioned in an anti configuration, relative to the C4' atom, with a S—C6'-C5'-C4' torsion angle of 179.2°. This pose results in a staggered relation of the C5' and C6' protons similar to that seen for biotinoyl-SoHA (Fig. 6B and C).

The Class IV NIS members of the adenylyate-forming family, are all involved in siderophore biosynthesis [10]. These chelating molecules sequester iron, in nutrient-poor environments and are important virulence factors in pathogenic bacteria [10]. Despite the recognition that this family are suitable targets for antimicrobial development, no structures are available of a representative enzyme bound to the reaction intermediate, or an analogue thereof. Therefore *N*-citryl-ethyl-enediamine-SoHA was modelled and minimised, by linking the natural substrates observed in the crystal structure of achromobactin synthetase protein D (PDB ID 2×3J, Fig. 6D). This intermediate seems to be an outlier compared to all other adenylyate classes in that the adenine base adopts a syn conformation. The sugar ring has a 2'-endo conformation, observed in the intermediate of the Class I aaRSs (Fig. 5B and C), but because of the position of the adenine base, the C6'-C5' bond is rotated away from the nucleoside scaffold. Despite this difference, the S—C6'-C5'-C4' torsion angle is 172.0°, similar to that observed for LeuSoHA, and the angular offset of the

Fig. 6. (90mm width): Conformation analysis of sulfonamide-linked biosters for the different intermediates of class representative adenylate-forming enzymes. (A) Left: C-5'-(nicotinamide-adenine-dinucleotidyl)-adenosyl-methansulfonamide (R_1 = -ribose-ADP), Right: Class I crystal structure of *Bacillus subtilis* NH_3 -dependent NAD $^+$ synthetase (PDB ID 1KQP); (B) Left: C-5'-(biotinoyl)-adenosyl-methansulfonamide, Right: Class II crystal structure of *Mycobacterium tuberculosis* biotin-protein ligase (PDB ID 3RUX); (C) Left: C-5'-(11-phenoxyundecanoyl)-adenosyl-methansulfonamide (R_2 = $-(\text{CH}_2)_5-\text{O-Ph}$), Right: Class III crystal structure of *Mycobacterium tuberculosis* long-chain-fatty-acid-AMP ligase (PDB ID 5HM3); (D) Left: C-5'-(citryl)-adenosyl-methansulfonamide, Right: Class IV crystal structure of *Pectobacterium chrysanthemi* achromobactin synthetase protein D (PDB ID 2x3J); (E) Left: C-5'-(threonylcarbamoyl)-adenosyl-methansulfonamide, Right: Class V crystal structure of *Sulfolobus tokodaii* L-threonylcarbamoyl adenine synthase (PDB ID 4E1B).

thetase (PDB ID 1KQP); (B) Left: C-5'-(biotinoyl)-adenosyl-methansulfonamide, Right: Class II crystal structure of *Mycobacterium tuberculosis* biotin-protein ligase (PDB ID 3RUX); (C) Left: C-5'-(11-phenoxyundecanoyl)-adenosyl-methansulfonamide (R_2 = $-(\text{CH}_2)_5-\text{O-Ph}$), Right: Class III crystal structure of *Mycobacterium tuberculosis* long-chain-fatty-acid-AMP ligase (PDB ID 5HM3); (D) Left: C-5'-(citryl)-adenosyl-methansulfonamide, Right: Class IV crystal structure of *Pectobacterium chrysanthemi* achromobactin synthetase protein D (PDB ID 2x3J); (E) Left: C-5'-(threonylcarbamoyl)-adenosyl-methansulfonamide, Right: Class V crystal structure of *Sulfolobus tokodaii* L-threonylcarbamoyl adenine synthase (PDB ID 4E1B).

C5' and C6' protons is 55° reflecting a staggered conformation (Fig. 6D). Finally, the threonylcarbamoyl-adenylate intermediate in the Class V representative *L*-threonylcarbamoyl-adenylate synthase (4E1B, Fig. 6E) has a similar nucleoside binding pose as the Class III inhibitor. Uniquely though, amongst the adenylate-forming superfamily, the sulfone group is positioned at an acute angle to the C4' atom with a torsion angle of 76.9°. However, the C5' and C6' protons demonstrate a staggered offset with a torsion angle of 39° for the closest proton pair (Fig. 6E).

Combined, this family-wide analysis indicates that the O5' to C substitution in the sulfonamide bioisosteres or the acyl-adenylate would likely be tolerated by all classes of the adenylate-forming enzyme superfamily except for the Class II aaRSs.

3. Discussion and conclusion

Non-hydrolysable acyl-adenylate analogues of the enzymatic intermediate of a variety of adenylate-forming enzymes have been consistently shown to have inhibitory activity [2,3]. Functioning as bi-substrate competitive inhibitors, the potency of these compounds emanates from the entropic favorability of binding over the unlinked substrates. Sulfur-based bioisosteres of the labile phosphoanhydrides have been typically employed, of which the sulfamate containing compounds have proven to be particularly effective [26,27]. In the case of targeting aminoacyl-tRNA synthetases subnanomolar binding has been observed *in vitro*, but unfortunately this has not translated to *in vivo* activity due to the high polarity of the compounds. Attempts to modify this scaffold, for prodrug strategies [28,29], has been limited due to the chemical instability of the compound [12]. In this work, we synthesized aminoacylated sulfonamide adenosines IleSoHA **14** and SerSoHA **15** (Scheme 1), aimed to inhibit their corresponding Class I and II aminoacyl-tRNA synthetase. The rationale for this oxygen-carbon substitution was to make hydrolytically stable aaSA analogues that are resistant to degradation at the 5'-position due to the less electrophilic 5'-carbon atom (Fig. 1A).

Inspired by the work of Shi et al. [12], we first attempted a Boc-Wittig-Horner procedure in order to obtain the C—C linked sulfonamide nucleosides. Unfortunately, compound **13** in our hands decomposed during work-up, in contrast to the previous report. Therefore, we explored two related reaction routes, using compounds **16** and **18**, but both failed to provide the desired products (Scheme 2). A nucleophilic substitution approach was subsequently tried in vain in order to extend the amino acid side chain (Scheme 3). With the intention of solving the degradation problem, we slightly modified the Boc-Wittig-Horner reaction procedure avoiding acidic circumstances. This alteration in the reported reaction procedure enabled us to obtain the desired products **14** and **15** (Scheme 1) in a reproducible way.

An evaluation of the *in vitro* inhibitory activity of **14** and **15** surprisingly demonstrated a class correlated difference, with at least a 10000-fold decrease in the IC_{50} for the SerSoHA **15** over that reported for the analogous sulfamoyl [20] (Fig. 3). Detailed examination of the modelled SerSoHA bound conformation, employing a 2.1 Å resolution crystal structure of *K. pneumoniae* SerRS soaked with SerSA suggests that an accumulation of torsional penalties, re-

sulting from the energetically unfavorable eclipsed positions of the C5' and C6' protons with regard to each other, and with the C6'—S and C4'-C5' bonds respectively, are in part likely to cause the observed loss of activity (Fig. 4C). By itself this appears surprising, as the relative energy between the staggered and eclipsed conformations of butane is only about 4 kcal/mol [30]. Examination of the other properties of the molecule suggest though that it is the major determinant.

An estimate of the pKa of the sulfonamide nitrogen predicts it to be deprotonated under physiological conditions in analogy with the sulfamoyl moiety. This same bioisosteric linker has been successfully employed in compounds targeting *M. tuberculosis* biotin ligase, an alternative Class II adenylate-forming enzyme (Fig. 6B and Supplemental Fig. S2). In this particular enzyme, the phosphate makes numerous charge-based interactions with basic residues in the protein active site including a conserved lysine that is essential for activity [21,31]. As the deprotonated sulfonamide effectively replicates these ionic interactions in this BirA active site, by extrapolation this should also apply in the case of the aminoacyl-SoHA analogues.

The switch from the S—O (1.577 Å) to a S—C (1.758 Å) bond results in a small shift in bond length ($\Delta=0.181$ Å) which may have an effect on the overall conformation of the resultant compound [32]. This value though is within one standard deviation of the observed S—C6'-C5'-C4' torsion angles from available Class II ligand bound crystal structures suggesting it can be accommodated. Ultimately, improved binding of the two bisubstrate-based competitive inhibitors over the amino acid and ATP substrates, which cumulatively make a similar number of interactions with the aaRS, is dictated by an entropic benefit [33]. It therefore appears that the torsion angle penalty caused by the O5'—C substitution in the bound state of **15** affects this profoundly.

A comprehensive examination of all Class II aaRSs shows that the conformation modelled for SerSoHA **15** bound to SerRS is not just restricted to this orthologue. Superposition of this scaffold on the intermediate observed in the available structures shows a highly restricted bent conformation, where the O5'—S bond (or O5'—P in the natural adenylate) is found always eclipsing the C6' protons (Fig. 5D). This torsion penalty, even for the regular intermediate, appears to be overcome by extensive interactions that are made between the aminoacyl-adenylate and the active sites of the different Class II aaRSs [34,35]. In contrast, examination of the intermediate conformation in all available Class I aaRSs shows no eclipsed conformation for the side-groups of the C5'-C6' (or C5'-O), as would be predicted based on the observed inhibitory activity results of IleSoHA **14** (Fig. 3). Together this suggests that the use of the sulfonamide linker in aminoacyl-adenylate intermediate analogues, is suitable for Class I aaRS but should be avoided for Class II enzymes performing the equivalent function.

To evaluate the further use of the sulfonamide-linked bioisosteres as pharmacologically relevant molecules we examined the binding conformation of the acyl-adenylate analogue to additional representatives of all five structurally distinct classes of the adenylate-forming enzyme superfamily (Figs. 2 and 6). In all cases we observed no steric clashes or torsion angle penalties with the introduction of this linker suggesting it could be a viable alternative for this diverse family. Interestingly, as stated above, a sulfonamide based intermediate analogue has been reported to be effective against biotin ligase (BirA), with demonstrable antitubercular activity [12]. Like SerRS, this particular enzyme is a member of the Class II adenylate-forming enzymes which all share a core conserved β -sheet structure (Figs. 2, 4 and 6). That said, the binding mode in BirA, particular of the nucleoside portion, is significantly different to that seen within the Class II

aaRSs (Supplemental Fig. S2). This ultimately results in a distinct conformation of the intermediate analogue that is more similar to that observed in the functionally diverse Class III family enzymes, where there are no observed issues with torsion angle overlaps.

As a final conclusion, we have observed that a theoretically innocuous atom substitution, from a sulfamate to a sulfonamide, in a known potent class II aaRS inhibitor, has a dramatic negative effect on the inhibitory activity of the resultant compound. Our comprehensive analysis points to a torsion angle penalty as being the likely cause of this loss. This problem is manifested due to conserved conformation of the aminoacyl-adenylate intermediate in all class II aaRS, which is ultimately dictated by the enzymatic spatial arrangement of the α -phosphate of ATP and the amino acid carboxylate group during catalysis [34,35]. A comprehensive examination of the acyl-adenylate bound to representative members of all adenylate-forming enzyme classes, shows considerable variation in the conformation of this reaction intermediate between the different groups. Our analysis shows that, with the exception of the class II aaRS, the sulfonamide-based phosphate bioisostere is likely a viable non-hydrolysable linker for developing family-wide bisubstrate inhibitors that work by mimicking the acyl-adenylate. The incorporation of the sulfonamide has been shown to improve chemical stability over other sulfur-based linkers [12], and was more recently shown to limit enzymatic-based disproportionation of the compound [13]. The latter is an important consideration in developing inhibitors that can avoid natural resistance mechanisms [36]. The optimized chemical synthesis strategy outlined here should permit elaboration of such compounds, increasing opportunities to develop much needed antimicrobials.

4. Experimental section

4.1. Reagents and analytical procedures

Reagents and solvents were purchased from commercial suppliers (Acros, Sigma-Aldrich) and used as provided, unless indicated otherwise. DMF, THF, DCM and MeOH were of analytical grade and were stored over 4 Å molecular sieves. All other solvents used for reactions were analytical grade and used as provided. Reactions were carried out in oven-dried glassware under a nitrogen atmosphere with stirring at room temperature, unless indicated otherwise. ^{14}C -radiolabeled amino acids and scintillation liquid were purchased from PerkinElmer.

^1H and ^{13}C NMR spectra of the compounds dissolved in CDCl_3 , CD_3OD , $\text{DMSO}-d_6$ or D_2O were recorded on a Bruker UltraShield Avance 300 MHz, 400 MHz or when needed on a 600 MHz spectrometer. The chemical shifts are expressed as δ values in parts per million (ppm), using the residual solvent peaks (CDCl_3 : ^1H , 7.26 ppm; ^{13}C , 77.16 ppm; DMSO : ^1H , 2.50 ppm; ^{13}C , 39.52 ppm; D_2O : ^1H , 4.79 ppm; CD_3OD : ^1H , 3.31 ppm; ^{13}C , 49.00 ppm) as a reference. Coupling constants are reported in Hertz (Hz). The peak patterns are indicated by the following abbreviations: br s=broad singlet, d=doublet, m=multiplet, q=quadruplet, s=singlet and t=triplet. High resolution mass spectra were recorded on an ion-mobility mass spectrometer (SYNAPT G2-Si HDMS, Waters, Milford, US) equipped with a standard ESI interface; samples were infused in 2-propanol/ H_2O (1:1) at 3 mL min $^{-1}$. For TLC, precoated aluminum sheets were used (Merck, Silica gel 60 F254). The spots were visualized by UV light at 254 nm. Chromatography was performed on ICN silica gel 60A 60–200. Final products (acylated sulfonamide nucleosides) were purified using a semi-prep PLRP-S 100 Å column (7.5 $\times$ 300 mm) connected to a Merck-Hitachi L6200A Intelligent pump. Eluent compositions are expressed as v/v. The characterization

of the final compounds by NMR and mass are provided in the supplementary file. Compounds **2**, **3**, **8** and **9** were prepared according to literature procedures (references [37–40], respectively) and proved identical.

4.2. Chemical synthesis of intermediates and final compounds

4.2.1. *N*⁶,*N*⁶-bis-Boc-5'—*O*-tertbutyldimethylsilyl-2'-3'—*O*-isopropylideneadenosine (**4**)

Compound **3** (3.46 g, 8.22 mmol, 1.0 eq.), 4-dimethylaminopyridine (0.201 g, 1.64 mmol, 0.2 eq.) and triethylamine (2.41 mL, 17.25 mmol, 2.1 eq.) were combined and put under an atmosphere of nitrogen, whereafter dissolving the reactants in a 2.4:1 mixture of anhydrous acetonitrile:DMF (48:20 mL, 0.12 M). Di-*t*-butyl dicarbonate (6.28 g, 28.8 mmol, 3.5 eq.) was dissolved in DMF (6.0 mL) and added dropwise to the solution. The mixture was stirred for 3 days at room temperature under nitrogen. The solvents were subsequently removed under reduced pressure. The residue was purified by silica gel chromatography (heptane:ethyl acetate 50:50) to give compound **4** (4.611 g, 90%) as a light-yellow foam with *R*_f=0.68 (heptane:ethyl acetate 50:50). ¹H NMR (300 MHz, CDCl₃): δ=8.86 (s, 1H), 8.32 (s, 1H), 6.23 (d, *J*=2.6 Hz, 1H), 5.19 (dd, *J*=6.1, 2.6 Hz, 1H), 4.93 (dd, *J*=6.1, 2.4 Hz, 1H), 4.43 (dd, *J*=6.2, 3.6 Hz, 1H), 3.82 (ddd, *J*=33.3, 11.3, 3.8 Hz, 2H), 1.63 (s, 3H), 1.41 (s, 18H), 1.39 (s, 3H), 0.83 (s, 9H), 0.00 (s, 6H) ppm.

4.2.2. *N*⁶,*N*⁶-bis-Boc-2'-3'—*O*-isopropylideneadenosine (**5**)

Compound **4** (4.61 g, 7.42 mmol, 1.0 eq.) was put under an atmosphere of nitrogen and was dissolved in anhydrous tetrahydrofuran (THF, 42.4 mL, 0.18 M). Tetrabutylammonium fluoride trihydrate (4.68 g, 14.83 mmol, 2.0 eq.) was dissolved in anhydrous THF (14.83 mL) and added dropwise to the solution. The mixture was stirred overnight at room temperature under nitrogen. The solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (heptane:ethyl acetate 20:80) to give compound **5** (3.61 g, 96%) as a white solid with *R*_f=0.61 (heptane:ethyl acetate 20:80). ¹H NMR (300 MHz, DMSO): δ=8.88 (s, 1H), 8.82 (s, 1H), 6.29 (d, *J*=2.6 Hz, 1H), 5.45 (dd, *J*=6.1, 2.6 Hz, 1H), 5.13 (t, 1H), 5.00 (dd, *J*=6.2, 2.4 Hz, 1H), 4.29 (td, *J*=2.5 Hz, 1H), 3.55–3.50 (m, 2H), 1.56 (s, 3H), 1.39 (s, 18H), 1.34 (s, 3H) ppm.

4.2.3. *N*⁶,*N*⁶-bis-Boc-5'-deoxy-2',3'—*O*-isopropylidene-5'-oxoadenosine (**6**)

Alcohol **5** (0.1 g, 0.197 mmol, 1.0 eq.) was dissolved in anhydrous dichloromethane (7.88 mL, 0.025 M) and Dess-Martin periodinane (0.125 g, 0.296 mmol, 1.5 eq.) was added in 1 portion. The mixture was put under an atmosphere of nitrogen and stirred for 1.5 h at room temperature. The reaction solution was cooled to 0 °C and quenched through adding 1.0 M aqueous sodium thiosulfate (4.0 mL) and aqueous saturated NaHCO₃ (5.0 mL). The mixture was then stirred for 1.5 h at room temperature under nitrogen after which the aqueous layer was extracted with dichloromethane (3×). The combined organic layers were washed with water and brine and dried over anhydrous MgSO₄. Following filtration, the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (heptane:ethyl acetate 20:80) to give compound **6** (0.088 g, 88%) with *R*_f=0.42 (heptane:ethyl acetate 20:80). ¹H NMR (300 MHz, DMSO): δ=9.29 (s, 1H), 8.78 (s, 1H), 8.77 (s, 1H), 6.59 (s, 1H), 5.55 (dd, *J*=6.1, 1.7 Hz, 1H), 5.51 (d, *J*=6.1 Hz, 1H), 4.85 (d, *J*=1.7 Hz, 1H), 1.55 (s, 3H), 1.39 (s, 18H), 1.38 (s, 3H) ppm.

4.2.4. 6-Amino-*N*⁶,*N*⁶-bis-Boc-9-[5,6-dideoxy-6-(ethoxysulfonyl)-2,3-*O*-isopropylidene-β-*D*-ribo-hex-5-enofuranosyl]-9H-purine (**11**) and 6-Amino-*N*⁶-Boc-9-[5,6-dideoxy-6-(ethoxysulfonyl)-2,3-*O*-isopropylidene-β-*D*-ribo-hex-5-enofuranosyl]-9H-purine (**10**)

Using alcohol **5** (0.250 g, 0.493 mmol, 1.0 eq.), crude aldehyde **6** was obtained as a yellow oil with *R*_f=0.42 (heptane:ethyl acetate 20:80) as described above.

Wittig-Horner reactant **9** (0.214 g, 0.542 mmol, 1.1 eq.) was put under nitrogen and dissolved in a 1:1 mixture of anhydrous THF:DMF (5.0 mL, 0.1 M). The solution was cooled to −78 °C and lithium bis(trimethylsilyl)amide (1.08 mL, 2.2 eq., 1.0 M in THF) was added dropwise. The mixture was stirred for 2.5 h at −78 °C. Crude aldehyde **6** was dissolved in anhydrous THF (4.0 mL) and was added to the reaction mixture. The solution was allowed to warm to room temperature and was stirred for 2 days at room temperature. The solvents were removed under reduced pressure and the residue was resuspended into water (20 mL). The aqueous layer was acidified with aqueous HCl (1.0 M) to pH=4 and was extracted with ethyl acetate (3×). The combined organic layers were washed with water and brine and dried over anhydrous MgSO₄. After filtration, the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (ethyl acetate) to give compounds **11** (0.080 g, 24%) with *R*_f=0.89 (ethyl acetate) as a yellow solid and **10** (0.086 g, 30%) with *R*_f=0.64 (ethyl acetate) as a yellow oil. ¹H NMR (400 MHz, CDCl₃) of **11**: δ=8.84 (s, 1H), 8.11 (s, 1H), 6.82 (dd, *J*=15.2, 3.6 Hz, 1H), 6.39 (dd, *J*=15.4, 1.8 Hz, 1H), 6.21 (s, 1H), 5.77 (d, *J*=5.9 Hz, 1H), 5.35 (dd, *J*=5.9, 2.1 Hz, 1H), 4.94 (d, *J*=2.2 Hz, 1H), 1.62 (s, 3H), 1.51 (s, 18H), 1.50 (s, 9H), 1.44 (s, 3H) ppm. ¹³C NMR (101 MHz, CDCl₃) of **11**: δ=152.49, 150.96, 150.78, 149.50, 144.21, 141.76, 129.14, 128.57, 114.58, 90.95, 85.33, 84.56, 84.40, 83.97, 83.37, 27.86, 27.82, 26.78, 25.18 ppm. MS (ESI): calcd. for C₂₉H₄₁N₆O₁₁S₁ [M−H][−] 681.3; found 681.2. ¹H NMR (400 MHz, CDCl₃) of **10**: δ=8.72 (s, 1H), 8.63 (s, 1H), 8.20 (s, 1H), 7.04 (dd, *J*=15.1, 4.2 Hz, 1H), 6.42 (d, *J*=13.6 Hz, 1H), 6.26 (s, 1H), 5.53 (d, *J*=6.2 Hz, 1H), 5.25 (dd, *J*=5.9, 3.5 Hz, 1H), 4.93 (s, 1H), 1.63 (s, 3H), 1.56 (s, 9H), 1.43 (s, 9H), 1.41 (s, 3H) ppm. ¹³C NMR (101 MHz, CDCl₃) of **10**: δ=153.05, 150.32, 150.27, 149.96, 149.92, 142.61, 142.24, 128.51, 121.90, 114.96, 90.67, 85.27, 84.02, 83.84, 83.78, 82.18, 28.10, 27.85, 26.95, 25.21 ppm. MS (ESI): calcd. for C₂₄H₃₃N₆O₉S₁ [M−H][−] 581.2; found 581.1.

4.2.5. *C*-(2',3'—*O*-isopropylidene-5'-adenosyl)methansulfonamide (**13**)

Palladium on carbon (10 wt% loading, 0.015 g, 0.143 mmol) was put under nitrogen. Wittig-Horner compound **10** (0.086 g, 0.147 mmol, 1.0 eq.) was dissolved in anhydrous methanol (15 mL, 0.01 M) and added to the catalyst. The atmosphere was exchanged for hydrogen gas and the reaction mixture was vigorously stirred for 3 days at room temperature. After completion of the reaction (ESMS), the mixture was filtered over Celite. The solvent of the filtrate was removed under reduced pressure, affording crude reduced compound **12** with *R*_f=0.10 (heptane:ethyl acetate 50:50) as a colourless oil. MS (ESI): calcd. for C₂₄H₃₅N₆O₉S₁ [M−H][−] 583.2; found 583.2.

Crude compound **12** was put under nitrogen and was dissolved in anhydrous dichloromethane (5.66 mL, 0.026 M) and the mixture was cooled to 0 °C. Trifluoroacetic acid (TFA, 0.82 mL, 11.03 mmol, 75 eq.) was added dropwise after which the mixture was stirred for 2 h at 0 °C. The mixture was then poured in cooled (0 °C) aqueous saturated NaHCO₃, whereafter the aqueous phase was washed with dichloromethane (3×). The aqueous layer was subsequently extracted with ethyl acetate (3×). The combined organic layers were washed

with water and brine and dried over anhydrous MgSO_4 . After filtration, the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (ethyl acetate:methanol 100:0 $\rightarrow$ 90:10) to give the TFA salt of compound **13** (0.062 g, 69%) with $R_f=0.20$ (ethyl acetate) as a colourless oil. ^1H NMR (400 MHz, CD_3OD): $\delta=8.28$ (s, 1H), 8.25 (s, 1H), 6.18 (s, 1H), 5.52 (s, 1H), 5.00 (s, 1H), 4.31 (s, 1H), 3.13 (s, 2H), 2.25 (s, 2H), 1.61 (s, 3H), 1.40 (s, 3H) ppm. ^{13}C NMR (101 MHz, CD_3OD): $\delta=155.96$, 152.61, 148.89, 140.48, 118.11, 115.21, 89.74, 84.67, 83.80, 83.78, 50.90, 27.66, 26.05, 24.18 ppm.

4.2.6. *N-L-isoleucyl-C-5'-adenosyl-methansulfonamide (14)*

Cesium carbonate (0.171 g, 0.52 mmol, 6.37 eq.) was put under nitrogen and cooled to 0°C . Compound **13** (0.050 g, 0.082 mmol, 1.0 eq.) and *t*-butyloxycarbonyl-*L*-isoleucine hydroxysuccinimide ester (0.086 g, 0.26 mmol, 3.18 eq.) were dissolved in anhydrous DMF (4.37 mL, 0.019 M) and were added to the base. The mixture was gradually warmed to room temperature and was stirred for 4 days at room temperature. The solvent was then removed under reduced pressure and the residue was resuspended in dichloromethane:methanol 90:10. The suspension was filtered over Celite, whereafter the solvents were removed under reduced pressure, giving the crude isoleucine coupled compound with $R_f=0.64$ (dichloromethane:methanol 90:10) as a yellow solid.

The crude isoleucine coupled compound was put under nitrogen and was cooled to 0°C . An 8:2 (v/v) mixture of trifluoroacetic acid:water (0.21 mL, 2.81 mmol, 34 eq.:0.05 mL, 2.90 mmol, 35 eq.) was added dropwise. The mixture was stirred for 3 h at room temperature after which water was added and the mixture was lyophilized, affording a yellow oil.

The crude product was purified by preparative reversed-phase high-performance liquid chromatography with a PLRP-S 100 Å column connected to a Merck-Hitachi L6200A intelligent pump at a flow rate of 8.0 mL/min for 40 min. Hereto, following eluent was used: Milli-Q purified water: 5 min acetonitrile:water 5:95 (v/v); followed by a linear gradient of acetonitrile in 30 min from 5% to 95% and re-equilibrating in 5 min from 95% to 5%. The retention time of the product was 18.0 min. The appropriate fractions were pooled and lyophilized to afford the final compound **14** (IleSoHA, 0.003 g, 5%) as a white powder. ^1H NMR (600 MHz, D_2O): $\delta=8.35$ (s, 1H), 8.32 (s, 1H), 6.00 (d, $J=5.1$ Hz, 1H), 4.78 (t, $J=5.2$ Hz, 1H), 4.26 (t, $J=4.9$ Hz, 1H), 4.16 (dd, $J=11.2$, 6.4 Hz, 1H), 3.61 (d, $J=4.2$ Hz, 1H), 3.31–3.25 (m, 2H), 2.14 (dd, $J=15.8$, 6.9 Hz, 2H), 1.94–1.87 (m, 1H), 1.39–1.31 (m, 1H), 1.15–1.08 (m, 1H), 0.89 (d, $J=7.0$ Hz, 3H), 0.80 (t, $J=7.4$ Hz, 3H) ppm. ^{13}C NMR (151 MHz, D_2O): $\delta=174.57$, 150.68, 148.32, 145.52, 142.66, 119.03, 88.55, 82.81, 73.45, 72.87, 59.85, 48.19, 36.29, 26.92, 23.96, 14.43, 10.86 ppm. MS (ESI): calcd. for $\text{C}_{17}\text{H}_{28}\text{N}_7\text{O}_6\text{S}_1$ $[\text{M} + \text{H}]^+$ 458.2; found 458.1.

4.2.7. *N-L-seryl-C-5'-adenosyl-methansulfonamide (15)*

Cesium carbonate (0.116 g, 0.36 mmol, 4.0 eq.) was put under an atmosphere of nitrogen and was cooled to 0°C . Compound **13** (0.043 g, 0.089 mmol, 1.0 eq.) and *t*-butyloxycarbonyl-*O*-benzyl-*L*-serine hydroxysuccinimide ester (0.070 g, 0.18 mmol, 2.0 eq.) were dissolved in anhydrous DMF (2.93 mL, 0.03 M) and were added to the base. The mixture was gradually warmed to room temperature and was stirred for 2 days at room temperature. The solvent was then removed under reduced pressure and the residue was resuspended in dichloromethane:methanol 90:10. The suspension was filtered over Celite, whereafter the solvents were removed under reduced pressure. The residue was purified by silica gel chromatography (ethyl acetate:methanol 95:5 $\rightarrow$ 90:10) to give serine coupled compound

(0.021 g, 31%) with $R_f=0.43$ (ethyl acetate:methanol 90:10) as a white solid. HRMS (ESI): calcd. for $\text{C}_{34}\text{H}_{47}\text{N}_7\text{O}_{11}\text{S}_1\text{Na}_1$ $[\text{M} + \text{Na}]^+$ 784.29465; found 784.3362.

Palladium on carbon (10 wt% loading, 0.003 g, 0.028 mmol) was placed under a nitrogen blanket, while the serine coupled compound (0.021 g, 0.028 mmol, 1.0 eq.) was dissolved in anhydrous methanol (2.69 mL, 0.01 M) and added to the catalyst. The atmosphere was exchanged for hydrogen gas and the reaction mixture was vigorously stirred for 2 days at room temperature. The mixture was then filtered over Celite. The filtrate was evaporated under reduced pressure, giving the crude debenzylated compound.

The crude debenzylated compound was put under nitrogen and a 8:2 (v/v) mixture of trifluoroacetic acid:water (0.04 mL, 0.59 mmol, 21 eq.:0.01 mL, 0.61 mmol, 22 eq.) was added. The mixture was then stirred for 3 h at room temperature. Excess water was then added and the reaction mixture was lyophilized, giving a transparent oil.

The crude product was purified by preparative RP-HPLC as described for **14**. The retention time of the product was 17.6 min. The appropriate fractions were pooled and lyophilized to afford the desired compound **15** (SerSoHA, 0.002 g, 17%) as a white powder. ^1H NMR (600 MHz, D_2O): $\delta=8.30$ (s, 1H), 8.16 (s, 1H), 8.10 (s, 1H), 5.91 (d, $J=5.3$ Hz, 1H), 4.16 (t, $J=4.9$ Hz, 1H), 4.10 (dt, $J=9.0$, 4.6 Hz, 1H), 3.84–3.77 (m, 2H), 3.68 (t, $J=4.5$ Hz, 1H), 3.23 (pd, $J=14.0$, 5.6 Hz, 2H), 2.14–1.98 (m, 2H) ppm. ^{13}C NMR (151 MHz, D_2O): $\delta=170.86$, 160.16, 155.46, 152.69, 148.72, 139.81, 118.72, 87.31, 82.36, 73.17, 72.74, 60.68, 56.88, 47.81, 27.08 ppm. HRMS (ESI): calcd. for $\text{C}_{14}\text{H}_{20}\text{N}_7\text{O}_7\text{S}_1$ $[\text{M} - \text{H}]^-$ 430.1150; found 430.1144.

4.3. Cloning, expression and purification of *E. coli* SerRS and IleRS and *K. pneumoniae* SerRS

The *E. coli* SerRS and IleRS were recombinantly expressed and purified using the previously described procedures [20]. In the case of the *Klebsiella pneumoniae* SerRS the encoding sequence was amplified by PCR from a genomic DNA preparation from the strain *K. pneumoniae* BAA 1705. The reaction was performed using Phusion polymerase (New England Biolabs) and the primers 5'-gcgacagattggtggtATGCTCGATCCCAATCTGCTG-3' and 5'-tgttagcagaagcttaTTAGCCGATATATTCGAGGCCG-3', where the capitalized letters correspond to the annealing sequence. The purified PCR product was cloned into the linearized pETRUK plasmid, an in-house derivative of the SUMO fusion vector pETHSUL [41] containing a modified sequence for a pI enhanced SUMO tag. The integrity of the pETRUK-KpSerRS construct was confirmed by sequencing. This construct was transformed into Rosetta 2 (DE3) pLysS strain (EMD biosciences). A single clone was grown in 2 L of ZYP-5052 auto-induction medium [42] supplemented with chloramphenicol (10 $\mu\text{g}/\text{mL}$), ampicillin (100 $\mu\text{g}/\text{mL}$) and 1 mL of antifoam SE-15 (Sigma Aldrich). The culture was grown at 24°C overnight until it reached an $\text{OD}_{600\text{nm}}$ of 4. At this point, the temperature was decreased to 18°C . After a further 24 h incubation, cells were harvested by centrifugation at 6000g at 4°C . The cell pellet was resuspended in approximately 4 times w/v of cation exchange buffer A (CEXA; 25 mM Hepes pH 8, 200 mM NaCl, 5 mM β -mercaptoethanol) and then stored at -20°C . The frozen cell resuspension was quickly thawed and diluted a further two-fold in CEXA buffer. 100 U caryonase (Takara) and 10 mM (final concentration) MgCl_2 were added to the slurry and the cells were lysed by sonication on ice. The resulting lysate was clarified by centrifugation at 18,000g at 4°C for 30 min and the supernatant was loaded onto a HiTrap SP HP column (GE Healthcare Life Sciences). The tagged KpSerRS was eluted from the column using a linear gradient of cation exchange buffer B (CEXB; 25 mM Hepes pH 8,

1000 mM NaCl, 5 mM β -ME). Protein fractions were combined and the SUMO tag removed by addition of recombinant SUMO hydrolase in a 1:250 (hydrolase: fusion protein) mass ratio. The cleavage reaction was immediately placed in Spectra/Por dialysis tubing (standard regenerated cellulose tubing with size 12–14 kDa) and the sample was dialyzed against 1 L of anion exchange buffer A (AEXA; 20 mM Tris pH 7, 10% w/v Glycerol and 5 mM DTT) overnight at 4°C. The dialysis bag was placed in a fresh 1 L of AEXA buffer for an additional 2 h at 4°C to remove the remaining salt, then the retentate was applied to the same HiTrap SP HP column to subtract SUMO tag and initial contaminating proteins. Further purification was carried out by anion exchange chromatography using a HiTrap Q HP column (GE Healthcare Life Sciences). Fractions corresponding to the KpSerRS were pooled and the protein precipitated by addition of 2.8 M ammonium sulfate and 1 mM EDTA. This sample was stored at 4°C until required.

4.4. Total tRNA purification and aminoacylation assay

The total tRNA pool was extracted and purified from BL21 (DE3) using the previously described procedure with a minor modification [20]. Following resuspension of the final isopropanol precipitated tRNA in assay buffer (20 mM Tris pH 7.5, 10 mM MgCl₂, 100 mM KCl, 2.5 mM β -ME) the sample was incubated at 85°C for 5 min and then allowed to cool down to room temperature on the bench before performing the aminoacylation assay.

The inhibitory activity of SerSoHA **15** and IleSoHA **14** (and the SA analogues) were assessed using the previously described radiolabel transfer assay [20]. Briefly, purified 10 nM *E. coli* IleRS or 2 nM *E. coli* SerRS in assay buffer as mentioned before was pre-incubated with the corresponding compound, at different concentrations, at 37°C in the presence of 50 μ M of the appropriate ¹⁴C-labeled amino acid, 2 mg/mL tRNA and 0.5 mg/mL inorganic pyrophosphatase. After 10 min, the reaction was initiated by adding pre-warmed ATP at a final concentration of 500 μ M. Upon reaching 6 min, 4 μ L quenching buffer (0.2 M sodium acetate pH 4, 0.1% *N*-lauroylsarcosine and 5 mM unlabeled amino acid) was added to the mixture. 20 μ L was spotted on 3 MM Whatmann paper, precipitated using cold 10% TCA, washed twice with 10% TCA and once with acetone and dried. Addition of 12 mL scintillation liquid was followed by measurement of the radioactivity using scintillation counter.

4.5. Crystallization and structure determination

Prior to crystallization, the ammonium sulphate precipitated KpSerRS was pelleted and resuspended in size exclusion chromatography (SEC) buffer (10 mM Tris pH 7, 100 mM NaCl, 5 mM DTT). The sample was loaded onto a Superdex 200 10/300 GL column (GE Healthcare Life Sciences) equilibrated in the same buffer. Fractions containing the eluted protein were concentrated using a microspin centrifugal filter device with PES 10 kDa (VWR international) to 10 mg/mL. Initial crystallization screens were performed using mosquito liquid transfer robot and sitting drop vapor diffusion method. Single crystals were produced by hanging drop vapor diffusion mixing 1 μ L KpSerRS (10 mg/mL) with 1 μ L reservoir solution (100 mM MES/imidazole buffer system pH 6.5, 50 mM CaCl₂, 7.5% w/v PEG 8000 and 20% v/v ethylene glycol) at 20°C. These crystals were soaked with 1 mM 5'-*O*-(*N*-(*L*-seryl)-sulfamoyl)adenosine [20] for 1 h in similar solution contain 10% w/v PEG 8000. The soaked crystals were transferred to cryoloops and flash frozen in liquid nitrogen. Diffraction data was collected on ESRF beamline MASSIF-3 (Grenoble, France). The data were processed using auto-

PROC [43]. The initial structure, containing a single protomer in the asymmetric unit, was solved by molecular replacement with Phaser using *Aquifex aeolicus* SerRS structure 2DQ3 as a model [44]. Further iterative improvement of the model was performed by manual building in coot followed by refinement using Buster 2.10.3 [45].

4.6. Computational analysis

As the bound LeuSA in the available structure of *Thermus thermophilus* LeuRS (PDB ID 2V0C) is split into two chemical moieties (the amino acid and 5'-sulfamoyl modified nucleoside) the structure was re-refined with a new geometrical restraints file encapsulating the full chemical entity. A suitable crystallographic information file for LeuSA, corresponding to the ligand ID LSS in the PDB, was generated using the grade webserver (<http://grade.globalphasing.org>). Using the deposited model and structure factor data, the full LeuSA was placed into the calculated electron density map. This model was then refined using Buster [45] applying a single TLS group to the whole protein chain. The resulting R and R_{free} values of 0.1730 and 0.1927, respectively, were 0.7% better than those reported for the original model [46].

Modelling of the O—C of substitution of the various acyl-adenylate analogues was carried out using the Schrodinger Maestro software. The software was first used place or replace H-atoms in the structures of *K. pneumoniae* SerRS, the re-refined *T. thermophilus* LeuRS-LeuSA complex, *Bacillus subtilis* NH₃-dependent NAD⁺ synthetase (PDB ID 1KQP [47]; *Mycobacterium tuberculosis* biotin-protein ligase (PDB ID 3RUX, [21]), *M. tuberculosis* long-chain-fatty-acid-AMP ligase (PDB ID 5HM3 [48]); *Pectobacterium chrysanthemi* achromobactin synthetase protein D (PDB ID 2×3J [49]) and the *Sulfolobus tokodaii* L-threonylcarbamoyl adenine synthase (PDB ID 4E1B, [11]). The H-bonding was then optimized before performing a restrained minimization of the protein and ligand employing the OPLS3e force field [50]. The resultant structure was then used to perform the appropriate atom substitutions. In the case of the *P. chrysanthemi* achromobactin synthetase protein D, the original structure contained ATP and (3' S)—*N*-citryl-ethylenediamine [49], therefore the ethylenediamine atoms were removed and the citrate molecule linked to the α -phosphate, keeping the resultant pyrophosphate. This structure then went under a second round of restrained energy minimization prior to atom substitution.

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Conflicts of interest

No conflicts of interest are declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online.

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