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COMMUNICATION

Synthesis and structure–activity relationships of *o*-sulfonamido-arylhydrazides as inhibitors of LL-diaminopimelate aminotransferase (LL-DAP-AT)†‡

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Recently, LL-diaminopimelate aminotransferase (LL-DAP-AT), a pyridoxal-5′-phosphate (PLP)-dependent enzyme, was reported to catalyze a key step in the biosynthesis of L-lysine in plants and *Chlamydia*. Previous screening of a 29 201-compound library against LL-DAP-AT identified an *o*-sulfonamidoarylhydrazide as a reversible inhibitor with $IC_{50} \sim 5$ μM. Structure–activity relationship (SAR) studies based on this lead compound identified key structural features essential for enzyme inhibition and led to slightly improved inhibitors. Preliminary studies on the mode of inhibition of LL-DAP-AT by this class of compounds are also reported.

Introduction

Lysine biosynthesis occurs in bacteria and plants *via* variants of the diaminopimelate (DAP) pathway.[1,2] However, formation of this essential amino acid is absent in mammals, and thus they must obtain it from their diet.[3] The direct precursor of lysine in this biosynthetic route, *meso*-DAP, is also involved in formation of cross-links between stem peptides of peptidoglycan in Gram-negative bacteria. Hence it has an important role in maintaining integrity of bacterial cell walls. Its metabolic product, L-lysine has an analogous function in many Gram-positive bacteria.[4,5]

The DAP pathway is a target for a new class of antibiotics or herbicides with low-toxicity to mammals. This has prompted study of both the structures of the enzymes involved in DAP biosynthesis, as well as design of inhibitors for them.[3,6–9] As lysine intake can be limited in human diets, complete understanding of lysine biosynthesis could also assist in engineering plants with greater lysine content.[10]

In most bacteria, lysine is biosynthesized by the route shown in Fig. 1a. The pathway starts with pyruvate and L-aspartate semialdehyde to form L-tetrahydrodipicolinate (L-THDP) in two enzymatic steps. Bacterial conversion of L-THDP to LL-diaminopimelate (LL-DAP) is generally achieved by three enzymatic steps, acylation, transamination, and deacylation.[11] At a later stage, LL-DAP is converted by DAP epimerase to *meso*-DAP, a crucial component of many Gram-negative bacterial cell walls.[12–14] Finally, L-lysine is formed upon decarboxylation of *meso*-DAP. Until recently this biosynthetic pathway was believed to be used by both plants and bacteria. However, a newly discovered aminotransferase (AT) enzyme was isolated which converts L-THDP directly to LL-DAP (Fig. 1b).[15] This represents a shorter route in the biosynthetic pathway, which normally requires three enzymes for catalysis, but here is achieved within a single step. This pyridoxal-5′-phosphate (PLP) dependent enzyme catalyzes a transamination reaction, and is therefore named LL-diaminopimelate aminotransferase (LL-DAP-AT).[16,17] It occurs in higher plants, some bacteria, archaea and algae, including cyanobacteria and chlamydia species.[18] Specifically, recent genetic analysis has shown that LL-DAP-AT occurs in *Cyanobacteria*, *Desulfuromonadales*, *Firmicutes*, *Bacteroidetes*, *Chlamydiae*, *Spirochaeta*, and *Chloroflexi* and two archaeal groups, *Methanobacteriaceae* and *Archaeoglobaceae*.[19,20]

In some cases, inhibitors of enzymes involved in the DAP pathway have already been shown to have antimicrobial activity.[8,21,22] Inhibitors that directly target LL-DAP-AT could potentially act as herbicides or as antibiotics specific to *Chlamydiae*. Substrate-based inhibitors could be very effective; however, they often suffer from poor uptake by the bacterial cells.[8] Compound library screening provides an alternative approach to overcome this problem.

In earlier work[23] we screened nearly 30 000 compounds against LL-DAP-AT from *Arabidopsis thaliana*, and found 46 compounds displaying inhibition greater than 13% at 10 μM by both robotic and manual testing. Three pharmacophores (barbiturate, thiobarbiturate, and rhodanine) were identified based on their high occurrence in the primary hits (17%, 17%, and 28%, respectively). From the screening, the best inhibitor (**1**, Table 1) contains a hydrazide functionality, which is a potentially reactive group. Two analogues of **1** tested earlier suggested that an unsubstituted hydrazide moiety appears to be necessary for inhibition.[23]

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‡Electronic supplementary information (ESI) available: Detailed enzyme assay, general synthetic procedure, and characterization data of analogues. See DOI: 10.1039/c2ob00040g

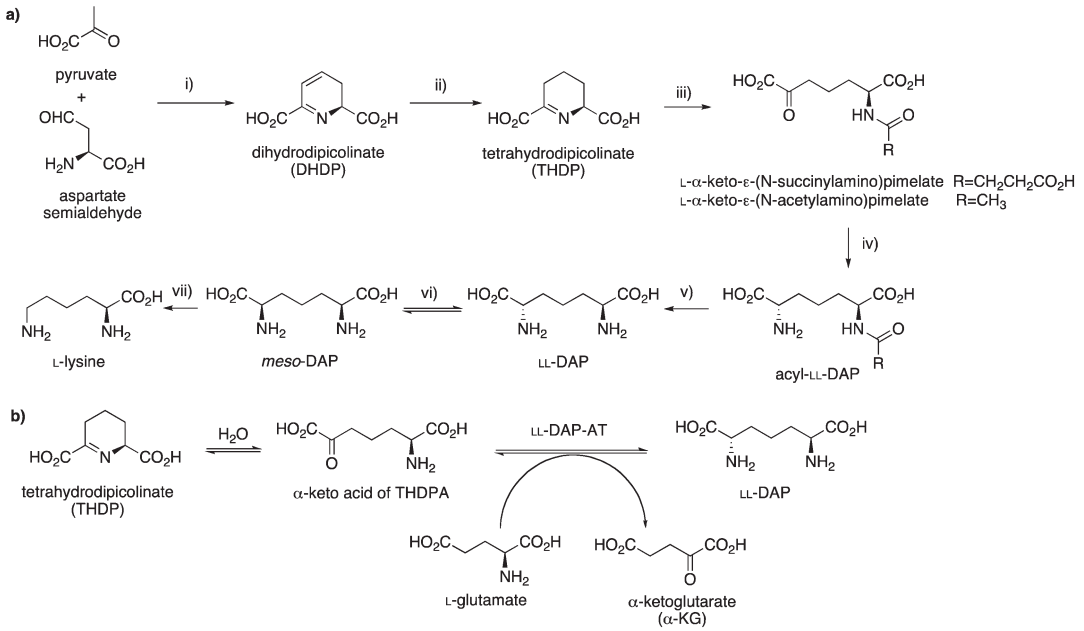


Fig. 1 (a) L-Lysine biosynthesis *via* the DAP pathway in bacteria. Enzymes involved: (i) DHDP synthase; (ii) DHDP reductase; (iii) THDP acyltransferase; (iv) *N*-acyl-DAP aminotransferase; (v) *N*-acyl-DAP deacylase; (vi) DAP epimerase; (vii) DAP decarboxylase. (b) The shortcut of the DAP pathway in higher plants and *Chlamydiae*.

In the current study, additional analogues based on **1** were synthesized and tested against LL-DAP-AT from *A. thaliana*. The assay used in this work is shown in Fig. 2.[15] Briefly, the assay involves monitoring the reverse of the transamination process, with α-ketoglutarate being used as an amino acceptor. Introduction of *o*-aminobenzaldehyde to the assay allows for the product (L-THDP) to be converted into a conveniently monitored chromophore. Furthermore, to test whether the hydrazide functional group reacts with PLP or not, NMR spectroscopy and mass spectrometry experiments were done. Isoniazid (**18**) was tested with the enzyme to examine whether this important anti-tuberculosis drug can inhibit DAP-AT. An adduct between one of the analogues and PLP was also tested against the enzyme.

Results and discussion

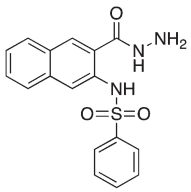
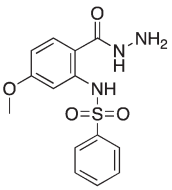
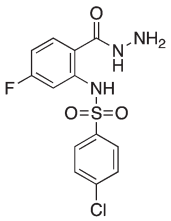
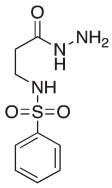
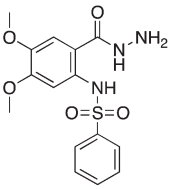
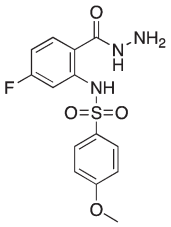
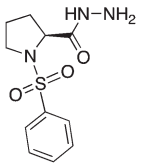
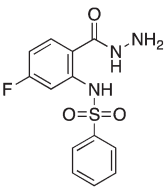
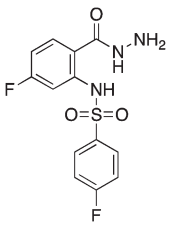
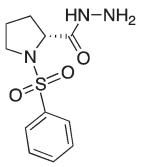
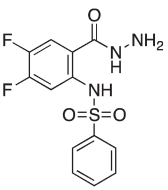
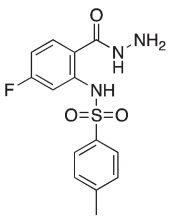
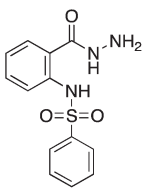
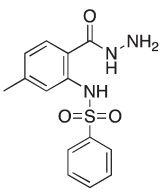
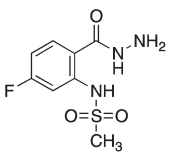
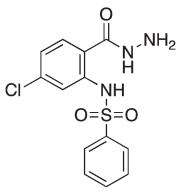
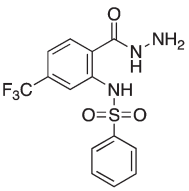
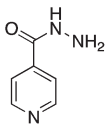
Compound **1** has an IC_{50} value of 5 μM[23] and was chosen as a lead for our structure–activity relationship (SAR) analysis of LL-DAP-AT. The simple synthetic route employed is shown in Scheme 1. Various amino substituted carboxylic acids were first reacted with sulfonyl chlorides under basic conditions to afford a sulfonamide (**2a–17a**). Then the sulfonamide carboxylic acid was coupled with hydrazine using carbonyl diimidazole as a coupling reagent to form the product (**2–17**).

It has been shown that the terminally unsubstituted NH_2 of the hydrazide group is necessary for inhibition;[23] thus, no further modifications of this group were done. The first set of modifications focused on the naphthalene ring moiety. Compound **2**, lacking the aromatic system, was totally devoid of inhibitory activity against the enzyme. Furthermore, L- and D-proline derivatives (**3** and **4**) were synthesized and tested against the LL-DAP-AT and both compounds showed no inhibition at 200 μM. These results suggest that a β-amino acid derived aromatic system is necessary for inhibition.

Modification of the aromatic system was undertaken. Phenyl ring analogues were synthesized and tested against the enzyme (Table 1). The fact that compound **5** showed lower activity compared to the lead compound, implies that substitution is needed on the phenyl ring in order to retain activity. Electronic effects were also considered, and compounds bearing chlorine and methoxy groups *para* to the hydrazide moiety (compounds **6** and **7**) were synthesized. The results showed these two compounds had similar activity against the enzyme, which suggests that the electronic effects may not play a very significant role in the inhibition. Moreover, these two analogues showed slightly better activity than the lead compound, which supports the hypothesis mentioned above that substitution is needed on the phenyl ring. The dimethoxy-substituted analogue **8** showed worse inhibition than either the lead compound **1** or the mono-methoxy-substituted analogue **7**. The extra methoxy group *para* to the sulfonamide moiety may be hindering the enzyme–analogue interaction through steric effects. Since substitution is necessary on the phenyl ring and the size of the substituent cannot be too large, monofluoro- and difluoro-substituted analogues **9** and **10** were synthesized and evaluated. Difluoro-substituted analogue **10** showed slightly worse inhibition than the lead compound **1**, whereas the monofluoro-substituted analogue **9** showed two-fold improvement of activity relative to **1** and is the best inhibitor in the current study. In addition, *para*-methyl-hydrazide and *para*-trifluoromethyl-hydrazide analogues **11** and **12** were examined. The trifluoromethyl group enhances the activity compared to the methyl analogue, which indicates that an electron-withdrawing group on the benzene ring *para* to the hydrazide moiety can be beneficial for the inhibitory activity.

With the *para*-fluoro-hydrazide analogue as the best inhibitor in the series of aromatic ring modifications, we then decided to explore the sulfonamide moiety, based on the analogue **9**, Table 1 (compounds **13–17**). At first, the *para*-chloro and

Table 1 Inhibitor testing againt LL-DAP-AT

Entry	Structure	IC_{50}/μM	Entry	Structure	IC_{50}/μM	Entry	Structure	IC_{50}/μM
1[a]		5	**7**		4.5	**13**		5.3
2		No inhibition	**8**		20.7	**14**		3.4
3		No inhibition	**9**		2.5	**15**		7.5
4		No inhibition	**10**		5.9	**16**		5.6
5		13.2	**11**		7.9	**17**		No inhibition
6		4.1	**12**		3.8	**18**[b]		No inhibition

[a] Compound **1** purchased from ChemBridge Corporation. [b] Compound **18** purchased from Sigma-Aldrich.

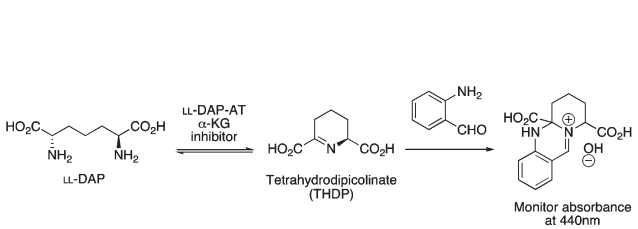
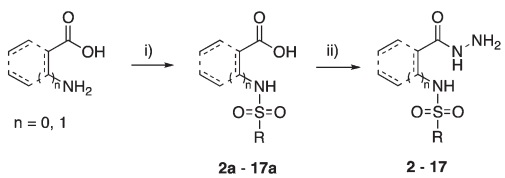
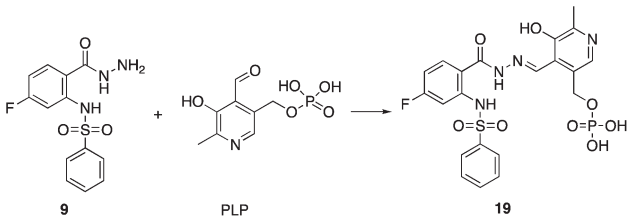


Fig. 2 Assay for inhibition against LL-DAP-AT.



Scheme 1 General synthetic route for analogues. Reagents: (i) R-SO_2Cl, Na_2CO_3, H_2O; (ii) carbonyl diimidazole, DMF; hydrazine monohydrate, DMF.



Scheme 2 Imine formation.

para-methoxy benzenesulfonamide were employed to examine the electronic effect. Both analogues **13** and **14** showed worse inhibition than compound **9**. However, an electron-donating group appears to be better on the *para* position of the benzenesulfonamide compared to an electron-withdrawing group. Furthermore, *para*-fluorobenzenesulfonamide and *para*-toluenesulfonamide were used to construct the other two analogues **15** and **16**. Ultimately, both of the analogues were reasonable inhibitors, but not as effective as unsubstituted phenyl ring without any substituents. Finally, the methanesulfonamide analogue **17** was synthesized and tested. Surprisingly, the analogue did not show any inhibition up to 100 μM against the enzyme, indicating that an aromatic system connected to the sulfonyl group is necessary for the inhibition.

If the NH_2 of the hydrazide moiety is capped by a ring or acetyl group, the analogues show no activity against the enzyme, which indicates that a free hydrazide amino group is essential for the inhibition.[23] Although **1** displays completely reversible time-independent inhibition,[23] the free hydrazide moiety is reasonably nucleophilic and could react with the enzyme cofactor, pyridoxal-5′-phosphate (PLP). To determine whether the hydrazide analogues react with PLP or not, compound **9** was mixed with one molar equivalent of PLP, in a 1 : 1 mixture of deuterated water and deuterated methanol solution at room temperature. The ^{1}H- and ^{13}C-NMR results matched with the proposed imine structure **19** shown in Scheme 2. Isolation of the imine compound and high resolution mass-spectrometry (HR-MS) confirmed its identity. These data indicate that, as expected, the hydrazide moiety can react with PLP to form an imine (hydrazone) adduct.

Despite this reactivity, removal of PLP from the enzyme by the free amino terminus of the hydrazide moiety cannot be the sole factor for inhibition. Compounds **2–4**, **17** and isoniazid (**18**) display no detectable inhibition of DAP-AT at concentrations up to 200 μM. Some of these compounds, including the clinically used anti-tuberculosis drug, isoniazid (**18**),[24] are sterically much less demanding than lead compound **1** or the best inhibitor **9**. Since the isoniazid has a hydrazide moiety with a free terminus, the hydrazone adduct is readily formed between isoniazid and PLP in the absence of enzyme. If the formation of the PLP-hydrazone adduct or sequestration of the cofactor were to be the main requirement for enzyme inhibition, then isoniazid should show activity against LL-DAP-AT, but this is not observed. Hydrazone adduct formation may occur after the active analogues enter the enzyme active site that contains PLP bound as an imine to lysine-270.[16] However, hydrazone formation cannot be the only factor required to inhibit to the enzyme. Additional interactions between the inhibitor and enzyme active site are required. Furthermore, since the hydrazone adduct formation is a reversible process whose rate can be catalyzed by other nucleophilic groups,[25,26] active compounds such as **1** are completely reversible inhibitors.

Finally, in a preliminary experiment, the preformed adduct between compound **9** and PLP, namely **19**, was also tested against the enzyme. However, the adduct **19** only showed 40% inhibition at 100 μM. In contrast, the free hydrazide **9** displays ~98% inhibition at 100 μM. The adduct showed much worse inhibition. Although it is not possible to fully stop hydrolysis of **19** to **9** in the enzyme assay, this result suggests that the cofactor exchange is relatively slow.[25,26] The mode of action of the inhibitors to LL-DAP-AT will be studied further by X-ray crystallographic analysis and detailed enzyme kinetics.

Conclusions

In this SAR study, 16 hydrazide analogues were synthesized based on a lead compound **1** identified from previous library screening. The analogues were tested as inhibitors against LL-DAP-AT from *A. thaliana*, and the best inhibitor was found to be an *o*-sulfonamido-*p*-fluorophenylhydrazide **9**, with an IC_{50} value of 2.5 μM. This compound was also found to react with PLP readily, but the hydrazone adduct **19** was not as effective an inhibitor of the enzyme. Testing of isoniazid (**18**), a hydrazide-containing drug, showed no inhibition against LL-DAP-AT, which indicates that the reaction between analogues and PLP may play a role in the inhibition, but is not the key factor.

Acknowledgements

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