Discovery of a fragment hit compound targeting D-Ala:D-Ala ligase of bacterial peptidoglycan biosynthesis

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ABSTRACT
Bacterial resistance is an increasing threat to healthcare systems, highlighting the need for discovering new antibacterial agents. An established technique, fragment-based drug discovery, was used to target a bacterial enzyme Ddl involved in the biosynthesis of peptidoglycan. We assembled general and focused fragment libraries that were screened in a biochemical inhibition assay. Screening revealed a new fragment-hit inhibitor of DdlB with a Ki value of 20.7 ± 4.5 μM. Binding to the enzyme was confirmed by an orthogonal biophysical method, surface plasmon resonance, making the hit a promising starting point for fragment development.

GRAPHICAL ABSTRACT

Introduction
Infections caused by antibiotic-resistant bacteria pose a serious challenge to healthcare systems worldwide. The increasing resistance of gram-positive and gram-negative pathogens causing infections in hospitals and in the general population and the worldwide spread of antibiotic-resistant 'superbugs' represent a major global health problem. Reportedly, 25,000 Europeans die each year as a direct result of infections with multidrug-resistant strains of pathogenic bacteria, with an estimated economic impact of 1.56 billion per year. However, the pipeline for new drugs is small because major pharmaceutical companies have largely abandoned antibiotic research, despite the urgent need for new drugs. The fact that few new antimicrobials are available and multidrug resistance is becoming more common means that we need to increase our efforts in finding new antimicrobials. It is imperative that we continue to search for new antibacterial agents by using innovative screening methods for carefully selected protein targets and by conducting rational drug design using the advances offered by protein crystal structures.

Peptidoglycan is a macromolecule essential for bacterial survival and is found only in the bacterial cell wall. Therefore, enzymes involved in the peptidoglycan biosynthetic pathway represent potential targets for the discovery of new antimicrobial agents. Among the intracellular enzymes involved in peptidoglycan biosynthesis, only two enzymes have been validated as antibacterial targets by inhibitors that are in clinical use: UDP-N-acetylglucosamine-enolpyruvyl transferase (MurA, EC 2.5.1.7) is validated by fosfomycin, which is used to treat urinary tract infections, and D-alanine:D-alanine ligase (Ddl, EC 6.3.2.4) is validated by cycloserine, which is a second-line drug for the treatment of tuberculosis.

Ddl is an ATP-dependent bacterial enzyme that catalyses the ligation of two D-alanines to a D-alanyl-D-alanine product, which in the next step is incorporated into the final intracellular peptidoglycan precursor UDP-N-acetylmuramoyl pentapeptide. Subsequently, this D-alanyl-D-alanine terminus is involved in transpeptidation, the cross-linking of the growing peptidoglycan chains. In Escherichia coli, there are two isozymes of Ddl, DdlA and DdlB.
and DdlB, which have similar catalytic efficiencies and substrate recognition properties, as well as similar sensitivity to inhibitors. We focussed on the search for inhibitors of DdlB because it is the best studied and the crystal structures are available. Although many Ddl inhibitors have been discovered in the past two decades, mainly by various screening campaigns or by classical medicinal chemistry approaches (for a comprehensive review, see ref.10), these inhibitors usually had only weak or no antibacterial activity. The most promising recent inhibitors appear to be thiosalicylic acid 1 and thiosemicarbazone 2 (Figure 1(B)), which had promising antibacterial activity and have been shown to target Ddl in bacteria.

One of the innovative and underutilised approaches to target Ddl is fragment-based drug discovery (FBDD). This approach is increasingly being used in the pharmaceutical industry and academia to reduce attrition and provide leads for previously inaccessible biological targets. FBDD identifies low molecular weight ligands (∼150 Da) that bind to biologically important macromolecules. The three-dimensional experimental-binding mode of these fragments is determined by X-ray crystallography or NMR spectroscopy and is used to facilitate their optimisation into potent lead compounds with drug-like properties by known methods such as fragment growing and fragment linking. Compared with high-throughput screening, the fragment-based approach requires a smaller number of compounds to be screened and provides more efficient and fruitful optimisation campaigns. In this manuscript, we describe the construction of a new library of fragments and its screening on DdlB from E. coli to discover new fragment-hit inhibitors.

Results and discussion

The definition of fragments in this study was based on physicochemical properties, the most important criterion being a molecular weight not exceeding 300 Da. Half of the fragment library was designed for a general purpose and will be screened on other targets as well. It contained diverse fragments (purchased or available in-house) without reactive functional groups. The second half consisted of subsets specifically designed to target DdlB. Since the enzyme is a dinuclear Mg$_{2+}$-dependent metalloenzyme, fragments capable of chelating metal ions could potentially bind to the active site via coordination interactions with Mg$_{2+}$ ions. A subset of chelating fragments was designed using substructure filters for chelating groups (Supplementary Table S2). DdlB is also ATP-dependent, thus we designed a subset of fragments containing phosphate bioisosteres (Supplementary Table S3) aiming to bind into the phosphate-binding site pockets normally occupied by ATP. Next, we designed a subset of cycloserine analogues that have similar substructures to an approved drug cycloserine and could potentially bind to the D-Ala-binding site (Supplementary Table S4). The final subset was designed by selecting the highest scoring hits obtained by docking a virtual library of available fragments to ATP- and D-Ala-binding sites. In total, the fragment library contained 943 compounds (Table 1) distributed across a range of physicochemical properties (Supplementary Figure S1).

The fragment library was assayed in a DdlB inhibition assay, where fragments were tested for their ability to inhibit the D-Ala-adding activity of DdlB ligase. The orthophosphate formed during the enzymatic reaction was measured spectrophotometrically using malachite green reagent. The tested fragment concentrations varied from 1.5 to 5 mM. The criteria for classifying a fragment as a hit were: <50% residual DdlB activity and solubility (low background absorbance of the compound at 650 nm). The results are provided in a Supplementary excel file. We re-purchased six hit compounds that matched the criteria to determine the inhibition of DdlB from freshly prepared compound solutions (Table 2). However, only two hits showed sufficient inhibition for IC$_{50}$ determination (3 and 4).

To pursue only compounds with tractable mechanism of action, extensive profiling of fragment hits was performed. First, deselection was performed with a MurA inhibition assay using the same assay technology to find compounds that interfered with the malachite green assay system. Second, the fragment hits were tested in a phosphate-binding assay that omitted the enzyme and again used the same assay technology. Compounds 4 and 5 were found to interfere with the assay by inhibiting MurA, DdlB, and binding phosphate (Table 2). Redox activity assays were performed to find compounds that interfered with the assay or had undesirable mechanisms of bioreactivity leading to false positive inhibitions. The horseradish peroxidase–phenol red (HRP-PR) assay was used to detect H$_2$O$_2$ generated by redox cycling compounds in the presence or absence of DTT. A fluorescent probe, 2,7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA), was used to detect compounds generating reactive oxygen species in the presence or absence of a reducing agent TCEP. The resazurin assay was used to detect redox active compounds that catalyse the conversion of resazurin to resorufin by the formation of free radicals in the presence of the reducing agent DTT. Compounds 4–6 and 8 were found to be active in at least one of the redox activity assays (Table 2, Table S5). Because our goal was to discover non-covalently binding inhibitors of DdlB, the fragment hits were assayed for their potential reactivity with a nucleophilic thiol surrogate, reduced 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB). Fragment 4 was found to be reactive in this assay (Table 2 and Supplementary Figure S2).

Table 1. Fragment library subsets and number of fragments used for screening on DdlB.

| Fragment library subsets                  | Number of fragments |
|-------------------------------------------|---------------------|
| In-house diverse fragments                | 195                 |
| Purchased diverse fragments               | 283                 |
| Chelating fragments                       | 214                 |
| Phosphate bioisostere fragments           | 159                 |
| Cycloserine analogues                     | 31                  |
| Docked fragments to DdlB                  | 61                  |
| Total                                      | 943                 |

Figure 1. (A) Reaction mechanism and (B) inhibitors of Ddl.
Based on the fragment hit profiling results, compounds with at least two red flags were excluded. For further evaluation, we selected fragment hit 3 without red flags and with an IC_{50} value of 168 µM (Figure 2(A) and Table 2), which is in the same range as that of the approved drug cycloserine (IC_{50} = 108 µM). Compound 3 was part of a Docked fragments to DdlB subset of the fragment library. In particular, docking to the ATP-binding site was used in the selection of this compound as part of the fragment library design. Structurally, 3 is a pyrazolopyrimidinocarboxylic acid with a molecular weight of 253 Da.

### Table 2. Profiling of repurchased fragment hits from the screening.

| Structure | Label | Fragment library subset | DdlB inhibition | MurA inhibition | Phosphate binding | HRP-PR assay | H_2DCFDA assay | Resazurin assay | Thiol reactivity |
|-----------|-------|--------------------------|----------------|----------------|------------------|-------------|--------------|----------------|----------------|
| 3         | Docked fragments to DdlB | IC_{50} = 168 µM | 64% RA at 5 mM | 111% free phosphate at 5 mM | Not active | Not active | Not active | Not reactive |
| 4         | Purchased diverse fragments | IC_{50} = 1790 µM | 39% RA at 1.5 mM | 29% free phosphate at 5 mM | Not active | Active | Active | Reactive |
| 5         | Purchased diverse fragments | 28% RA at 2.5 mM | 6% RA at 5 mM | 10% free phosphate at 5 mM | Not active | Active | Not active | Not reactive |
| 6         | Purchased diverse fragments | 25% RA at 5 mM | 10% RA at 5 mM | 23% free phosphate at 5 mM | Not active | Not active | Active | Not reactive |
| 7         | Docked fragments to DdlB | 47% RA at 5 mM | 81% RA at 5 mM | 89% free phosphate at 5 mM | Not active | Not active | Not active | Not reactive |
| 8         | Purchased diverse fragments | 95% RA at 5 mM | 100% RA at 5 mM | 85% free phosphate at 5 mM | Not active | Active | Not active | Not reactive |

RA: Residual Activity.
In an attempt to investigate the structure-activity relationship, 15 analogues of compound 3 were purchased (analogue-by-catalogue) (Table 3). Compounds 9–14 differ by the substituents on the phenyl ring, while other heterocycles were explored with compounds 15–18. The carboxylic group was switched from position 3 to position 2 for compounds 18 and 19. Compounds 20–23 contain isosteric replacements for the carboxylic group. None of the assayed analogues inhibited DdlB in an inhibition assay. However, because they are fragment-sized compounds (molecular weight between 234 and 322 Da), even small changes may contribute to loss of inhibitory potency.

Binding of compound 3 to DdlB was confirmed by an orthogonal biophysical assay, surface plasmon resonance (SPR). The protein was covalently attached to the surface of the CM5 chip (~7700 response units), and the ligand was titrated across the surface (Figure 2(B)). The apparent equilibrium dissociation constant ($K_D = 3.2 \text{ mM}$) was estimated from the steady-state binding levels (Figure 2(C)). Steady-state kinetics were performed to gain mechanistic insight into the inhibition of compound 3 (Supplementary Figure S3 and Table S6). The resulting best model showed that 3 is a competitive inhibitor with respect to ATP with a $K_i$ value of $20.7 \pm 4.5 \text{ mM}$. These data are in agreement with the docking results obtained during the design of fragment library, where 3 was identified as a potential ATP competitive inhibitor.

Compound 3 was then additionally docked using extra-precision Glide (Glide XP) and also redocked using QM-Polarized Ligand Docking (QPLD) protocol that generates partial charges on the ligand atoms by quantum mechanical calculations on the ligand in the field of the receptor. This accounts for the polarisation of the charges on the ligand by the receptor environment, and redocking with these new charges can lead to improved docking accuracy. The QPLD pose showed a difference of 2.54 Å from the original pose, mainly due to the $m$-tolyl ring flip (Figure 2(E)). Based on the putative binding mode, the carboxylic acid mimics the phosphate groups of ATP that coordinate Mg$^{2+}$ ions (Figure 2(D,E)). As shown in Table 4, the electrostatic term (XP Electro) from Coulombic and metal interactions contributes the most, followed by XP Zpotr, which denotes a reward for ligand atoms placed in a favourable electrostatic environment of the protein, and XP PhobEn, a reward for hydrophobic enclosure. Thus, the appropriately positioned carboxylate group is mainly responsible for successful binding.

**Table 3.** Analogues of compound 3 that were evaluated in the DdlB inhibition assay.

| Structure | Label | DdlB inhibition |
|-----------|-------|-----------------|
| ![Structure 3](image) | | $IC_{50} = 168 \mu M$ $Ki = 20.7 \mu M$ |
| ![Structure 9](image) | 9 | 90% RA at 5 mM |
| ![Structure 10](image) | 10 | 101% RA at 0.5 mM |
| ![Structure 11](image) | 11 | 100% RA at 0.5 mM |
| ![Structure 12](image) | 12 | 0% RA at 5 mM 90% RA at 1 mM |
| ![Structure 13](image) | 13 | 89% RA at 0.5 mM |
| ![Structure 14](image) | 14 | 100% RA at 0.5 mM |
| ![Structure 15](image) | 15 | 96% RA at 1 mM |
| ![Structure 16](image) | 16 | 89% RA at 0.5 mM |
| ![Structure 17](image) | 17 | 26% RA at 5 mM Nonsigmoidal curve |
| ![Structure 18](image) | 18 | 69% RA at 5 mM 87% RA at 1.5 mM |
| ![Structure 19](image) | 19 | 50% RA at 5 mM 90% RA at 1.5 mM |
| ![Structure 20](image) | 20 | 100% RA at 0.5 mM |
| ![Structure 21](image) | 21 | 96% RA at 0.5 mM |
| ![Structure 22](image) | 22 | 100% RA at 0.5 mM |
| ![Structure 23](image) | 23 | 94% RA at 0.5 mM |
| ![Structure D-Cyclo-serine](image) | D-Cyclo-serine | $IC_{50} = 108 \mu M$ |

(continued)
To confirm the relevance of the predicted docking pose, a 100 ns molecular dynamics (MD) simulation on the 3 binding pose was run. The pose was fairly stable during the simulation time (Supplementary Figure S4), with the largest fluctuations resulting from the flipping of the m-tolyl ring. The metal coordinating and ionic interactions with both of the active site Mg$^{2+}$ ions through the carboxylate moiety and one pyrimidino nitrogen anchored 3 in the active site (Supplementary Figure S5). Additionally, a cation-p interaction of the m-tolyl ring with Lys144 occurred quite frequently. Overall, in the absence of an experimental crystal structure, these results provide strong evidence for the proposed binding mode of compound 3 in the ATP-binding site.

The antibacterial activities of 3 were determined according to the European Committee for Antibacterial Susceptibility Testing recommendations and Clinical Laboratory Standards Institute protocol23. Compound was tested against reference strains of E. coli and Staphylococcus aureus and against two mutant E. coli strains. E. coli D22 bears a mutation in the LpxC gene that increases membrane permeability, whereas E. coli N43 is an AcrA knockout strain (knockout of the cell membrane pump). The 3 showed no antibacterial activity against both E. coli and S. aureus (Table S7). The most plausible reason is the low on target activity of the hit fragment, however, also the poor penetration into the bacterial cell or efflux pumps effect cannot be ruled out. Although the lack of antibacterial activity is a limitation, hit compound 3 represents a fragment-sized starting point for the development of new compounds with increased DdlB inhibitory potency as well as antibacterial activity.

### Conclusions

Here, we describe the construction of a fragment library containing 943 fragments to discover novel inhibitors of a bacterial enzyme DdIB involved in intracellular steps of peptidoglycan biosynthesis. FBDD is an innovative and underutilised approach to target DdIB. The library contained diverse fragments suitable for screening on different targets, as well as fragments specifically designed to target DdIB. Library screening using a biochemical assay was followed by profiling of the fragment hits. Compounds that interfered with the assay, redox active compounds, and reactive compounds were flagged. The fragment hit 3 without flags and with an IC$_{50}$ value of 168 µM was selected for further studies. We confirmed binding of 3 to DdIB with SPR analysis. Furthermore, steady-state kinetics showed 3 to be a competitive inhibitor with respect to ATP with a Ki value of 20.7 ± 4.5 µM. An analogue-by-catalogue campaign did not reveal more potent compounds, making fragment hit 3 a starting point for further synthetic optimisation to develop new antibacterial agents.

### Experimental section

#### Fragment library preparation

The in silico part of the fragment library preparation was performed using the KNIME analytics platform24. SMARTSview server was used to visualise the SMARTS patterns25. For fragment library preparation, we selected compounds from more than 4500 in-house chemicals and more than 1.57 million compounds available for cherry-picking from ChemDiv (the database was downloaded on 05 October 2020). First, the RDKit Descriptor Calculation node26 was used to calculate the molecular descriptors for each compound in the library. Then, the compounds were filtered using the following criteria to retain only fragments: molecular weight between 80 and 300, no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, and estimated logP value no greater than 4. The Speedy SMILES Organic Subset Molecules Splitter node was used to remove compounds with non-organic atoms other than C, N, O, S, P, B, F, Cl, Br, I, and H. To remove reactive and unwanted functional groups, a set of SMARTS filters (Supplementary Table S1) were applied using the SMARTS Query node27. Finally, RDKit Molecule Catalogue Filter node was used to remove PAINS compounds known to cause interference in assay systems28. The resulting virtual fragment library of 1087 in-house and 91,379 commercially available compounds was then used to design six subsets.

The selected commercially available fragments were obtained at 1.0–1.2 mg on 96-well polypropylene plates. To each well, 100 µL of dimethyl sulfoxide (DMSO) was added, and the plates were covered with an aluminum seal, and sonicated to prepare 26–125 mM stock solutions, which were used for screening in a DdIB inhibition assay.

#### In-house diverse fragments

Additional physicochemical filters were applied to the in-house available fragments, namely no more than 5 hydrogen bond acceptors, no more than 3 rotatable bonds, and a polar surface area greater than 0 and no greater than 80, resulting in a library of 887 in-house available fragments. Then, 400 diverse fragments were selected using the RDKit Diversity Picker node. The node uses a fast MaxMin algorithm to select diverse compounds based on the Tanimoto distance between the Daylight-like topological fingerprints. The diverse fragments were then manually inspected and a set of 195 fragments was selected for the physical fragment library. The fragments were prepared as 50 mM DMSO stock solutions and transferred to 96-well polypropylene plates.

#### Purchased diverse fragments

From the 91,379 commercially available fragments, 2000 diverse fragments were selected using the RDKit Diversity Picker node. Subsequently, k-means clustering into 500 clusters was applied to the calculated distance matrix for Daylight-like topological fingerprints using the k-Medoids node. Next, we manually inspected 500 medoids and selected a set of 283 to be included in the physical fragment library.

#### Chelating fragments

The Brønsted filter (Supplementary Table S1) without filters for phosphor and catechol was used in the previous step to prepare the subset of chelating fragments. Based on recent reviews17,18, we designed SMARTS patterns (Supplementary Table S2) to find fragments capable of metal ions chelation. The RDKit Substructure Filter was used in the KNIME analytics platform. When filtering with a particular SMARTS pattern resulted in more than 100

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### Table 4. The breakdown of GlideScore scores into individual contributions, only non-zero terms are shown.

|                  | XP GScore | XP Hbond | XP PhobEn | XP LowMW | XP LipophilicEvdW | XP Electro | XP Zpotr |
|------------------|-----------|----------|-----------|----------|-------------------|-----------|----------|
| Glide XP (original) pose | −12.549 | −0.76 | −2.00 | −0.5 | −1.664 | −5.125 | −2.5 |
| QPLD pose | −12.009 | −0.57 | −2.05 | −0.5 | −1.261 | −5.125 | −2.5 |
fragment chelators, the RDKit Diversity Picker node was used to select 100 diverse fragment chelators. A total of 214 fragments were then manually selected.

**Phosphate bioisostere fragments**

The Brenk filter (Supplementary Table S1) without filters for Michael acceptors was used in the previous step to prepare the subset of phosphate bioisosters. SMARTS patterns for phosphate bioisosters were designed based on a review. The RDKit Substructure Filter was used in the KNIME analytics platform. When filtering with a particular SMARTS pattern resulted in more than 100 phosphate bioisostere fragments, the RDKit Diversity Picker node was used to select 100 diverse fragment chelators. A total of 159 fragments were then manually selected.

**Cycloserine analogues**

We applied several SMARTS patterns (Supplementary Table S4) to find fragments containing similar substructures to cycloserine. The RDKit Substructure Filter was used in the KNIME analytics platform. A total of 31 cycloserine analogues were manually selected.

**Docked fragments to DdlB**

The final subset contained the highest scoring fragments from docking to DdlB. The DdlB protein was prepared from the X-ray structure in complex with ADP and D-cycloserine phosphate (PDB ID: 4C5A), using Protein Preparation Wizard (Schrödinger Suite 2020–2, Schrödinger, LLC, New York, NY, 2020). Briefly, hydrogen atoms were added, residues were protonated at pH 7.0, the hydrogen bonding network was refined, waters were removed, and restrained minimisation was performed. Next, Make Receptor 3.4.0.2 (OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com) was used to define grid boxes for each of the binding sites separately. The ATP binding site was defined with a box of 4214 Å³ (14.37 Å × 14.37 Å × 20.38 Å) and the outer contour of 739 Å³. The D-Ala binding site where cycloserine was defined with a box of 2155 Å³ (12.71 Å × 11.43 Å × 14.84 Å) and the outer contour of 308 Å³. The virtual fragment library containing 42,700 commercially available fragments was first processed using FixpKa (OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com) to obtain proper ionisation at pH 7.4. Subsequently, the stereoisomer and conformational model generator OMEGA 3.1.2.2 (OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com) was used to enumerate stereocenters and to generate up to 200 conformers per compound. The prepared fragment library was then docked to each binding site individually using FRED 3.4.0.2 (OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com). Validation of the docking protocol was performed by redocking the co-crystallised ADP and D-cycloserine phosphate yielding RMSD < 2.0 Å. The 200 highest scoring virtual screening hits for the ATP binding site were clustered into 50 clusters, and 36 diverse fragments were manually selected. The 400 highest scoring virtual screening hits for the ATP binding site were clustered into 60 clusters, and 25 diverse fragments were manually selected.

**DdlB inhibition assay**

A recombinant E. coli DdlB enzyme (D-alanine:D-alanine ligase) was expressed in E. coli. Inhibition of the enzyme was determined using an end-point malachite green assay by detecting the orthophosphate formed during the enzymatic reaction. The final mixture (50 µL) contained: 50 mM HEPES, pH 8.0, 0.005% Triton X-114, 5 mM MgCl₂, 6.5 mM (NH₄)₂SO₄, 10 mM KCl, 700 µM D-Ala, 100 µM ATP, purified DdlB (diluted in 50 mM HEPES, pH 8.0), and the test compound dissolved in DMSO. The final DMSO concentration was 5%. After 20 min of incubation at 37 °C, the reaction was terminated by adding Biomol reagent (100 µL) and after 5 min at room temperature the absorbance was measured at 650 nm using a microplate reader (Synergy H4, BioTek Instruments, Inc., USA). A parallel experiment without the enzyme was performed to detect insoluble compounds under the assay conditions and subtracted from each measurement. All experiments were performed in duplicates. RAs were calculated with respect to blank experiments without tested compounds and with 5% DMSO. The final concentrations for fragment screening were 1.3–6.2 µM for purchased fragments and 2.5 mM for in-house available fragments. IC₅₀ values were determined by measuring the residual activities in quadruplicate at seven different compound concentrations and calculated using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

For compound 3, Ki values were determined against DdlB from E. coli. Ki determinations were performed under similar conditions as described for DdlB inhibition assay, where different concentration of one substrate and fixed concentration of the other were used. The concentration of ATP (50, 100, 200, 300 and 500 µM) was varied at fixed concentration of D-Ala (700 µM). The concentrations of compound 3 were 0, 25, 50, 100, 250, 500, and 1000 µM. After 20 min of incubation, 100 µM of Biomol green reagent was added, and absorbance was read at 650 nm after 5 min. Initial data were fitted to competitive, (v/V = [S]/(Kₘ+[I]+[S])), non-competitive (v/V = [S]/(1+[I]/Kₘ+[S])), and uncompetitive (v/V = [S]/(Kₘ+[I]+[S]([1+[I]/Kₘ]+[S]))) inhibition models in which v is observed reaction rate, V is the maximum rate, Kₘ is the Michaelis constant, using SigmaPlot 12.0 software. The best fit was taken as the one having the highest R², the lowest standard error and the narrowest 95% confidence interval in each parameter (see Supplementary Table S6).

**Mura inhibition assay**

A recombinant E. coli MurA enzyme (UDP-N-acetylglucosamine enolpyruvyl transferase) was expressed in E. coli. Inhibition of the enzyme was determined using an end-point malachite green assay by detecting the orthophosphate formed during the enzymatic reaction. The final mixture (50 µL) contained: 50 mM HEPES, pH 8.0, 0.005% Triton X-114, 200 µM UDP-N-acetylglucosamine, 100 µM phosphoenolpyruvate, purified MurA (diluted in 50 mM HEPES, pH 8.0), and 5 mM of each test compound dissolved in DMSO. The final DMSO concentration was 5%. After 15 min of incubation at 37 °C, the reaction was terminated by adding Biomol reagent (100 µL), and after 5 min at room temperature the absorbance was measured at 650 nm using a microplate reader (Synergy H4, BioTek Instruments, Inc., USA). A parallel experiment without the enzyme was performed to detect insoluble compounds under the assay conditions and subtracted from each measurement. All experiments were performed in duplicates. RAs were calculated with respect to blank experiments without tested compounds and with 5% DMSO.

**Phosphate-binding assay**

Phosphate binding was determined by colorimetric measurement of phosphate with malachite green in the presence or absence of
the compound. To 46.9 µL of buffer (50 mM HEPES, pH 8.0), 0.625 µL of 800 µM phosphate standard, and 2.5 µL of 100 mM compound DMSO stock was added. The reaction mixture was incubated for 5 min at room temperature. Then, 100 µL of Biomol
reagent was added and after 5 min, the absorbance was measured at 650 nm using a microplate reader (Synergy H4, BioTek Instruments, Inc., USA). Background absorbance was determined in a parallel experiment without phosphate standard and subtracted from each measurement. To determine the blank value, the compound solution was replaced with pure DMSO and the background-subtracted measurements were divided by the blank value.

Redox activity assays

HRP-PR assay

The HRP-PR assay was performed according to a previously optimised procedure. Briefly, to 58 µL of buffer (50 mM HEPES, 50 mM NaCl, pH 7.5), 10 µL of 2 mM compound DMSO stock solution, and 66 µL of buffer (redox-free) or 66 µL of 3 mM DTT in buffer were added. After 15 min of incubation at room temperature, 66 µL of HRP-PR detection reagent (300 µg/mL phenol red and 180 µg/mL HRP [150–250 units/mg solid] in buffer) was added and incubated for an additional 5 min at room temperature. The final concentrations were: 100 µM compound, 100 µg/mL (282 µM) phenol red, 60 µg/mL HRP, 1 mM DTT, 5% DMSO. The reaction was then quenched by adding 10 µL of 1 M NaOH solution and the absorbance was measured at 610 nm using a microplate reader (Synergy H4, BioTek Instruments, Inc., USA). To determine the blank value, the compound solution was replaced with pure DMSO. The measured absorbance for each compound was then divided by the blank value. 3-Methyltoxoflavin was used as a control compound. All reagent solutions were freshly prepared before performing the experiments.

H₂DCFDA assay

The H₂DCFDA assay was performed according to a previously optimised procedure. Briefly, the probe H₂DCFDA was prepared as a 5 mM stock in DMSO and diluted 10-fold to 500 µM with 0.01 M NaOH. The probe was freshly prepared for each plate and stored in the dark at room temperature for 30 min to hydrolyse the ester. To 52.5 µL of buffer (50 mM HEPES, 50 mM NaCl, pH 7.5), 7.5 µL of 2 mM compound DMSO stock solution, 75 µL of buffer (redox-free) or 75 µL of 200 µM TCEP in buffer were added, and 15 µL of 500 µM H₂DCFDA. The final concentrations were: 100 µM compound, 50 µM H₂DCFDA, 100 µM TCEP, and 5% DMSO. The microplate was covered with a lid and stored in the dark at room temperature for 30 min. Fluorescence intensity was then measured using 560 nm excitation and 590 nm emission filters (Synergy H4, BioTek Instruments, Inc., USA). To determine the blank value, the compound solution was replaced with pure DMSO. The measured fluorescence for each compound was then divided by the blank value. 3-Methyltoxoflavin was used as a control compound. All reagent solutions were freshly prepared before performing the experiments.

Resazurin assay

The resazurin assay was performed according to a previously optimised procedure. Briefly, to 100 µL of buffer (50 mM HEPES, 50 mM NaCl, pH 7.5), 2 µL of 1, 0.1, or 0.01 mM compound DMSO stock solution, and 100 µL of resazurin solution (10 µM resazurin and 200 µM DTT in buffer) were added. The final concentrations were: 0.1, 1, or 10 µM compound, 5 µM resazurin, 100 µM DTT, and 1% of DMSO. The microplate was covered with a lid and stored in the dark at room temperature for 30 min. Fluorescence intensity was then measured using 560 nm excitation and 590 nm emission filters (Synergy H4, BioTek Instruments, Inc., USA). To determine the blank value, the compound solution was replaced with pure DMSO. The measured fluorescence for each compound was then divided by the blank value. 3-Methyltoxoflavin was used as a control compound. All reagent solutions were freshly prepared before performing the experiments.

Thiol reactivity assay

The assay was performed as previously described. Briefly, 100 µM of compound was incubated at 37 °C with a mixture of 100 µM TCEP and 25 µM DTNB (generating 50 µM TNB in situ) in buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) with 5% final DMSO concentration. To monitor TNB depletion, absorbance at 412 nm was measured every 5 min for 12 h (Synergy H4, BioTek Instruments, Inc., USA). Compound background absorbance was subtracted from each measurement. 2-Chloro-N-(3-chlorophenyl)acetamide was used as a control compound. All reagent solutions were freshly prepared before performing the experiments.

Surface plasmon resonance

The SPR measurements were performed at the Infrastruktural Centre for Analysis of Molecular Interactions at the Department of Biology, University of Ljubljana on a Biacore T200 (GE Healthcare) at 25 °C. To assay the interaction of compound with the DdIB protein we immobilised approximately 7700 RU of the purified DdIB protein on the carboxymethyl dextran-coated gold surface of the series 5 sensor chip CMS (GE Healthcare) to which DdIB was immobilised via the free amino groups by an amine coupling procedure. For this, DdIB protein was in 10 mM sodium acetate buffer (pH 4). Compound was serially diluted in running buffer composed of 50 mM HEPES (pH 7.4), 140 mM NaCl, 0.005% P20. To assay the fragment–DdIB interaction, each fragment concentration tested (ranging from 78.125 to 5000 µM) for 40 s at a flow rate of 30 µL/min and dissociation was followed for 40 s. For the regeneration of the surface of the sensor chip, 2 mM NaOH was used. As the stock of the compound was prepared in 5% DMSO, we referenced the sensorgrams for the buffer response containing identical concentration of DMSO as the diluted sample of the compound. Furthermore, sensorgrams were referenced for the untreated surface flow-cell 1 response. The data were analysed with the Biacore T200 Evaluation Software and was determined by fitting the data to the steady state affinity model. The average Kd of standard deviations were determined from three titrations of 3.

Microbiological evaluation

Minimum inhibitory concentrations (MICs) for 3 were determined by broth microdilution in Cation-adjusted Mueller-Hinton broth (MH) with TES, TREK Diagnostic Systems Ltd. against S. aureus ATCC 29213, E. coli ATCC 25922, E. coli N43 and E. coli D22. All of the experiments were performed in duplicate in 96-well plate format according to CLSI guidelines and European Committee for Antimicrobial Susceptibility Testing recommendations. The bacterial suspension of a specific bacterial strain was diluted with MH.
broth to obtain a final inoculum of 5 × 10^5 CFU/mL in the assay. Compound 3 dissolved in DMSO and inoculum were mixed together and incubated for 20 h at 35 °C. After incubation, the minimal inhibitory concentration (MIC) values were determined by visual inspection as the lowest dilution of compounds showing no turbidity. The MICs were determined against S. aureus (ATCC 29213), E. coli (ATCC 25922), E. coli N43, and E. coli D22 bacterial strains. D-Cycloserine was used as a positive control.

**High-precision docking and molecular dynamics**

The DdIB protein was prepared from the X-ray structure in complex with ADP and D-Ala-D-Ala (PDB ID: 4C5C) using Protein Preparation Wizard34 (Schrödinger Suite 2021–1, Schrödinger, LLC, New York, NY, 2021). Briefly, hydrogen atoms were added, residues were protonated at pH 7.0, the hydrogen bonding network was refined, waters were removed, and restrained minimisation was performed. Only chain A was retained. The receptor grid was generated using Receptor Grid Generation with van der Waals and Cl– ions were added to neutralise the system and produce the final 0.15 M concentration, and OPLS_2005 force field35, while the co-crystallised ADP defined the radii scaling by 1, partial charge cut-off 0.25 (default settings), and generated using Receptor Grid Generation with van der Waals. The system was prepared with Preparation Wizard29 (Schrödinger, LLC) with Waters Acquity UPLC® HSS C18 SB column (2.1 × 50 mm, 1.8 μm) thermostated at 40 °C, injection volume, 1 μL; flow rate, 0.3 mL/min; detector λ, 220 nm and 254 nm; mobile phase A: 0.1% TFA (v/v) in water; mobile phase B: MeCN. Method: 0–9 min, 5–95% B; 9–11 min, 95% B; 11–15.5 min, 95–5% B. Compounds are >95% pure by HPLC analysis, unless stated otherwise.

7-(m-Tolyl)pyrazolo[1,5-a]pyrimidine-3-carboxylic acid (3, Vitas-M laboratory, STL396729)

1H NMR (400 MHz, DMSO-d6) δ 13.36 (s, 1H, COOH), 8.70 (d, J = 4.3 Hz, 1H, C5-H), 7.92 (dt, J = 7.7, 1.7 Hz, 1H, C6'-H), 7.89 – 7.84 (m, 1H, C2'-H), 7.52 (t, J = 7.6 Hz, 1H, C5'-H), 7.49 – 7.43 (m, 1H, C4'-H), 7.35 (t, J = 4.3 Hz, 1H, C6-H), 7.21 (s, 1H, C2-H), 2.43 (s, 3H, CH3). 13C NMR (101 MHz, DMSO-d6) δ 163.95 (COOH), 151.16 (C5), 150.09 (C3), 148.02 (C1'), 146.70 (C7), 138.39 (C3'), 132.66 (C4'), 130.67 (C3a), 130.23 (C2'), 128.93 (C5'), 127.12 (C6), 110.15 (C6), 99.38 (C2), 21.50 (CH3). HRMS (ESI+) m/z [M + H]+, calcd. for C14H12N2O2: 254.09240, found: 254.09159. Purity by HPLC: 95.6%. 2D NMR spectra are shown in Supplementary Figures S6–S12.

(2-Ethoxy-7,8-dihydro-1,6-naphthyridin-6(5H)-yl)(pyridin-2-yl) methane (6, ChemDiv, S422-0111)

1H NMR (400 MHz, DMSO-d6) δ 8.63 (ddd, J = 8.1, 4.4, 2.6 Hz, 1H), 8.01 – 7.90 (m, 1H), 7.68 – 7.58 (m, 2H), 7.57 – 7.47 (m, 1H), 6.67 (d, J = 8.4 Hz, 1H), 4.74 (s, 1H), 4.57 (s, 1H), 4.26 (q, J = 7.0 Hz, 2H), 3.96 (t, J = 6.0 Hz, 1H), 3.68 (t, J = 5.9 Hz, 1H), 2.97 – 2.80 (m, 2H), 1.30 (td, J = 7.0, 2.0 Hz, 3H). Purity by HPLC: 92.6%.

6-Amino-4-hydroxyquinoline-3-carboxylic acid (7, ChemBridge, 9220711)

1H NMR (400 MHz, DMSO-d6) δ 8.61 (s, 1H), 7.55 (d, J = 8.9 Hz, 1H), 7.31 (d, J = 2.6 Hz, 1H), 7.18 (dd, J = 8.9, 2.6 Hz, 1H), 5.81 (s, 2H). Purity by HPLC: 98.5%.
7-Phenylpyrazolo[1,5-a]pyrimidine-3-carboxylic acid (9, Enamine, EN300-39113)

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.46 (s, 1H), 8.86 (d, $J = 4.5$ Hz, 1H), 8.63 (s, 1H), 8.15 – 8.06 (m, 2H), 7.70 – 7.57 (m, 3H), 7.46 (d, $J = 4.4$ Hz, 1H). Purity by HPLC: 98.8%.

7-(p-Tolyl)pyrazolo[1,5-a]pyrimidine-3-carboxylic acid (10, ChemBridge, 8928448)

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.82 (d, $J = 4.5$ Hz, 1H), 8.62 (s, 1H), 8.06 – 7.99 (m, 2H), 7.47 – 7.40 (m, 3H), 2.43 (s, 3H). Purity by HPLC: 98.0%.

7-(3,4-Dimethylphenyl)pyrazolo[1,5-a]pyrimidine-3-carboxylic acid (11, Vitas-M laboratory, STK902058)

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.40 (s, 1H), 8.81 (d, $J = 4.5$ Hz, 1H), 8.63 (s, 1H), 7.92 – 7.82 (m, 2H), 7.42 (d, $J = 4.5$ Hz, 1H), 7.38 (d, $J = 8.0$ Hz, 1H), 2.34 (s, 3H), 2.33 (s, 3H). Purity by HPLC: 93.0%.

7-(4-Chlorophenyl)pyrazolo[1,5-a]pyrimidine-3-carboxylic acid (12, ChemDiv, C201-1701)

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.46 (s, 1H), 8.86 (s, 1H), 8.63 (d, $J = 3.9$ Hz, 1H), 8.17 – 8.10 (m, 2H), 7.74 – 7.67 (m, 2H), 7.49 (s, 1H). Purity by HPLC: 98.6%.

7-(3-Nitrophenyl)pyrazolo[1,5-a]pyrimidine-3-carboxylic acid (13, Vitas-M laboratory, STK349882)

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.50 (s, 1H), 8.98 (t, $J = 2.0$ Hz, 1H), 8.91 (d, $J = 4.4$ Hz, 1H), 8.67 (s, 1H), 8.53 – 8.45 (m, 2H), 7.93 (t, $J = 8.1$ Hz, 1H), 7.62 (d, $J = 4.4$ Hz, 1H). Purity by HPLC: 98.1%.

7-(Benzo[d][1,3]dioxol-5-yl)pyrazolo[1,5-a]pyrimidine-3-carboxylic acid (14, Vitas-M laboratory, STK902056)

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.76 (d, $J = 4.5$ Hz, 1H), 8.56 (s, 1H), 7.75 – 7.68 (m, 2H), 7.38 (d, $J = 4.5$ Hz, 1H), 7.19 – 7.13 (m, 1H), 6.17 (s, 2H). Purity by HPLC: 98.6%.

7-(3,4-Dimethylphenyl)pyrazolo[1,5-a]pyrimidine-2-carboxylic acid (18, Vitas-M laboratory, STK349529)

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 13.45 (s, 1H), 8.69 (d, $J = 4.7$ Hz, 1H), 8.58 (dd, $J = 3.9$, 1.2 Hz, 1H), 8.14 (dd, $J = 5.0$, 1.2 Hz, 1H), 7.88 (d, $J = 4.7$ Hz, 1H), 7.41 (dd, $J = 5.1$, 3.9 Hz, 1H), 7.23 (s, 1H). Purity by HPLC: 96.6%.
7-Phenylpyrazolo[1,5-a]pyrimidine-2-carboxylic acid (19, Vitas-M laboratory, STK350440)

![Structure](image1)

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 13.37 (s, 1H), 8.71 (d, \(J\) = 4.3 Hz, 1H), 8.15 – 8.05 (m, 2H), 7.70 – 7.59 (m, 3H), 7.38 (d, \(J\) = 4.3 Hz, 1H), 7.22 (s, 1H). Purity by HPLC: 96.9%.

Ethyl 7-(3,4-dimethylphenyl)pyrazolo[1,5-a]pyrimidine-3-carboxylate (20, Vitas-M laboratory, STK902137)

![Structure](image2)

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 8.85 (d, \(J\) = 4.5 Hz, 1H), 8.67 (s, 1H), 7.91 – 7.82 (m, 2H), 7.45 (d, \(J\) = 4.5 Hz, 1H), 7.38 (d, \(J\) = 8.0 Hz, 1H), 4.32 (q, \(J\) = 7.1 Hz, 2H), 2.34 (s, 3H), 2.33 (s, 3H), 1.33 (t, \(J\) = 7.1 Hz, 3H). Purity by HPLC: 94.0%.

7-Phenylpyrazolo[1,5-a]pyrimidine-3-carboxamide (21, Vitas-M laboratory, STK649786)

![Structure](image3)

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 8.17 (m, 2H), 7.82 (m, 2H), 7.45 (d, \(J\) = 7.1 Hz, 2H), 2.34 (s, 3H), 2.33 (s, 3H), 1.33 (t, \(J\) = 7.1 Hz, 3H). Purity by HPLC: 99.0%.

7-(m-Tolyl)pyrazolo[1,5-a]pyrimidine-3-carbonitrile (22, ChemBridge, 8909699)

![Structure](image4)

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 8.92 – 8.84 (m, 2H), 7.93 – 7.85 (m, 2H), 7.59 – 7.44 (m, 3H), 2.42 (s, 3H). Purity by HPLC: 94.0%.

(Z)-N’-Hydroxy-7-(3-(trifluoromethyl)phenyl)pyrazolo[1,5-a]pyrimidine-3-carboximidamide (23, Maybridge, SEW04848)

![Structure](image5)

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 9.43 (d, \(J\) = 1.1 Hz, 1H), 8.72 (dd, \(J\) = 4.4, 1.1 Hz, 1H), 8.54 (m, 1H), 8.45 (d, \(J\) = 1.1 Hz, 1H), 8.38 (d, \(J\) = 8.1 Hz, 1H), 8.01 (d, \(J\) = 7.9 Hz, 1H), 7.87 (t, \(J\) = 7.9 Hz, 1H), 7.43 (dd, \(J\) = 4.4, 1.1 Hz, 1H), 6.03 (s, 2H). Purity by HPLC: 98.5%.

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