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Discovery of new inhibitors of the bacterial peptidoglycan biosynthesis enzymes MurD and MurF by structure-based virtual screening

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Dedicated to Professor Slavko Pečar, on the occasion of his 60th birthday

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ABSTRACT

The ATP-dependent Mur ligases (MurC, MurD, MurE and MurF) successively add L-Ala, D-Glu, *meso*-A_2pm or L-Lys and D-Ala-D-Ala to the nucleotide precursor UDP-Mur*N*Ac, and they represent promising targets for antibacterial drug discovery. We have used the molecular docking programme eHiTS for the virtual screening of 1990 compounds from the National Cancer Institute 'Diversity Set' on MurD and MurF. The 50 top-scoring compounds from screening on each enzyme were selected for experimental biochemical evaluation. Our approach of virtual screening and subsequent in vitro biochemical evaluation of the best ranked compounds has provided four novel MurD inhibitors (best IC_{50} = 10 μM) and one novel MurF inhibitor (IC_{50} = 63 μM).

1. Introduction

The emergence of drug-resistant bacterial strains represents a global health threat and has emphasized the need to develop new antibacterial agents.[1] The biochemical machinery involved in bacterial peptidoglycan biosynthesis is an important target of many known antibacterial agents. Peptidoglycan is an essential component of the bacterial cell wall that provides structural integrity and protection against osmotic pressure.[2] Many important antibacterial drug classes, such as the β-lactams, cephalosporins and glycopeptides, have antibacterial activities due to inhibition of the formation of peptidoglycan. Most of these target the extracellular steps of peptidoglycan biosynthesis. The intracellular steps, however, still remain mostly underexploited. Among the intracellular stages of bacterial peptidoglycan biosynthesis, the ATP-dependent Mur ligases (MurC, MurD, MurE and MurF) deserve particular attention. These enzymes successively add L-Ala, D-Glu, *meso*-A_2pm or L-Lys, and D-Ala-D-Ala to the nucleotide precursor UDP-Mur*N*Ac, and they represent promising targets for antibacterial drug discovery.[3–5] In the present study, we have focussed our attention on MurD and MurF, which catalyze the addition of a D-amino acid and a D,D-dipeptide, respectively. As there are no human counterparts to the peptidoglycan-synthesizing enzymes, MurD and MurF inhibitors should be highly selective for the bacterial enzymes.

MurD catalyses the formation of the peptide bond between UDP-Mur*N*Ac-L-Ala and D-Glu. The reaction starts by phosphorylation of UDP-Mur*N*Ac-L-Ala to form an acylphosphate, followed by nucleophilic attack by the amino group of the incoming D-Glu. A high energy tetrahedral intermediate is formed, which eventually collapses to yield UDP-Mur*N*Ac-L-Ala-D-Glu, ADP and inorganic phosphate.[6,7] Many inhibitors of MurD have been designed as phosphinic[8–13] and sulfonamide[14] tetrahedral transition-state analogues, with those incorporating a D-Glu fragment having better affinities.

MurF catalyses the formation of the peptide bond between UDP-Mur*N*Ac-tripeptide and D-Ala-D-Ala, to form UDP-Mur*N*Ac-pentapeptide. Its reaction mechanism is similar to that described above for MurD.[15] There are only a few known inhibitors of MurF. The first inhibitors were pseudo-tripeptide and pseudo-tetrapeptide aminoalkylphosphinic acids, which function as simplified transition-state analogues.[16] Abbott Laboratories have developed two small molecule lead compounds, which have been co-crystallized with MurF.[17] Based on these data, a series of potent structurally related inhibitors was obtained; however, none of these showed significant antibacterial activity.[18]

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Computational methods have become increasingly popular in hit discovery and lead compound optimization. The purpose of the present study was to use virtual high-throughput screening (VHTS) to find novel inhibitors of these target bacterial enzymes, MurD and MurF. Virtual screening is an emerging drug discovery technology that involves the analysis of large collections of compounds, and which can lead to the definition of smaller subsets of hits for biological testing. Structure-based virtual screening requires computational fitting of compounds into the active site of a receptor (docking), followed by the scoring and ranking of the compounds to identify potential hits. We report here the results of structure-based virtual screening using the National Cancer Institute (NCI) 'Diversity Set'[19] with the new eHiTS software,[20] which has identified some chemically diverse compounds as novel MurD and MurF inhibitors. In addition, the importance of using a suitable rapid primary in vitro biochemical assay in combination with a more accurate secondary assay is demonstrated.

2. Results and discussion

2.1. Docking of the NCI diversity set to MurD and MurF, and selection of the top scoring compounds for in vitro testing

One of the most widely used methods for VHTS is the docking of small molecules into the active site of a protein target, and the subsequent scoring.[21] A wide range of different docking programmes are available, most of which use semi-rigid docking, where the ligands are treated as flexible and the receptors as rigid. The treatment for ligand flexibility can be divided into three basic categories: systematic methods, random or stochastic methods and simulation methods.[22] After successful docking, each programme evaluates and ranks the predicted conformations. Therefore, the design of the scoring function is of fundamental importance. Different scoring functions can also be divided into three different classes: force field based, empirical, and knowledge based.[22]

For our docking, we used the eHiTS 6.0 software. eHiTS is a programme for flexible ligand docking that applies a systematic algorithm to sample all of the possible degrees of freedom of the ligand molecules. These are first divided into rigid fragments and flexible chains, and each fragment is then docked independently into the active site. The programme applies a fast graph-matching algorithm to reconstruct the original molecule from the dockings of the small rigid fragments. These fragments are then reconnected with flexible chains. Local energy optimization within the receptor is then performed, followed by the scoring. This latter is based on the eHiTS_Score, which is a statistically derived empirical scoring function.[23,24]

Using eHiTS for the virtual screening of the NCI Diversity Set on both MurD and MurF, the 50 top scoring compounds for each enzyme were selected for experimental biochemical evaluation. We carefully inspected the docking poses and possible interactions with the active site for all of the active compounds.

A characteristic structural feature of all of the Mur ligases is that they consist of three domains. The N-terminal domain is involved in the binding of the UDP-precursor, the central domain in the binding of ATP, and the C-terminal domain in the binding of the incoming amino acid (MurC-E) or dipeptide (MurF).[3] Mur ligases also have 'closed' and 'open' conformations, and the closure of the domains is believed to be caused by ligand binding.[4] From the crystal structure of MurD in complex with the reaction product UDP-Mur*N*Ac-L-Ala-D-Glu (PDB entry 4UAG; Fig. 1), we can see that the ligand is bound by Leu-15, Thr-16, Thr-36 and Gly-73 of the N-terminal domain, Asn-138 and Gln-162 of the central domain, and Lys-348, Thr-321, Ser-415 and Phe-422 of the C-terminal domain.

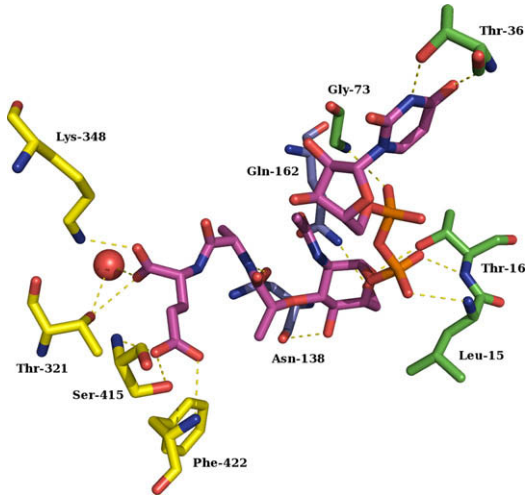


Figure 1. The active site of MurD co-crystallized with the product UDP-MurNAc-L-Ala-D-Glu (magenta). Only the relevant amino-acid residues are shown: N-terminal domain—green, central—blue, C-terminal—yellow. Yellow dashes represent H-bonds.

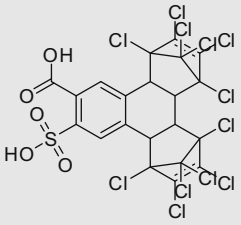
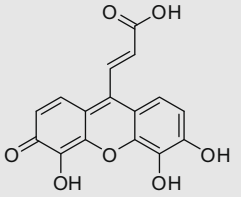
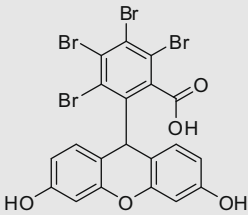
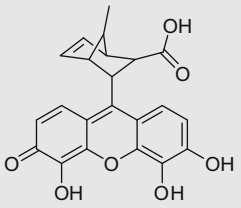
As described in Section 4, we modified the co-crystal structure of MurD prior to docking, to reduce the size of its active site. We deleted the uridine monophosphate moiety of UDP-Mur*N*Ac-L-Ala-D-Glu, so that the active site detected would be smaller, and eHiTS would dock compounds preferably to the phospho-Mur*N*Ac-L-Ala-D-Glu-binding site. This decision was based on three important findings: (i) Gegnas et al.[11] described 1-phospho-Mur*N*Ac-containing phosphinates as very potent inhibitors of MurD; (ii) 1-phospho-Mur*N*Ac-L-Ala is a substrate of MurD, indicating the relatively good affinity of the enzyme for this part of the nucleotide substrate[25] and (iii) the majority of the sulfonamide MurD inhibitors that we have synthesized recently have either D-Glu or L-Glu moieties and bind to the phospho-Mur*N*Ac-L-Ala-D-Glu binding site that is formed by amino-acid residues Leu-15, Thr-16, Asn-138, Gln-162 Lys-348, Thr-321, Ser-415 and Phe-422.[14] The uridine monophosphate moiety of UDP-Mur*N*Ac-L-Ala-D-Glu interacts only with Thr-36 and Gly-73, which we considered to be less important for inhibitor binding, and thus also for our virtual screening.

For the virtual screening with MurF, the crystal structure of MurF from *Streptococcus pneumoniae* (PDB entry 2AM1) was used, as this is the only MurF enzyme that has been co-crystallized with a small molecule inhibitor.[17]

2.2. The MurD inhibitors

Out of the 50 compounds from the eHiTS virtual screening that were tested in the phosphate release assay, four (NSC 22225, NSC 270718, NSC 119911 and NSC 119889) showed inhibitory activities (residual activities at 250 μM below 50%). To confirm these results, we used a secondary assay that was based on the use of radiolabelled D-Glu. Here, the inhibitory activities were confirmed for NSC 270718, NSC 119911 and NSC 119889, while NSC 22225 was inactive. As NSC 119911 and NSC 119889 share a similar xanthene scaffold (Table 1), we used the Open Babel software to search for related compounds in the NCI Diversity Set. This identified NSC 119913, which also turned out to be active in both the primary and secondary assays. The IC_{50} values were determined for all of these active compounds, and in all cases they were in the low micromolar range. The most active compound, NSC 119889, has an IC_{50} of 10 μM. The three active compounds arising from the docking experiments were among the first 15 best ranked compounds identified by eHiTS (Table 1).

Table 1
The MurD inhibitors

NSC#	eHiTS score	Rank	IC_{50} (μM)	Formula
270718	−5.299	1	34	
119911	−4.096	15	47	
119889	−4.727	14	10	
119913	[a]	[a]	43	

[a] Not applicable.

Our docking experiments predicted binding to the D-Glu-binding site (Lys-348, Thr-321, Ser-415 and Phe-422) for all of these inhibitors that were active in the in vitro assays. Figure 2 shows the predicted binding conformation for the most active compound, NSC 119889, with the possible H-bond interactions with residues Lys-348, Thr-321, Ser-415 and Phe-422 shown. NSC 270718 and NSC 119911 also formed similar interactions with these residues.

As noted above, NSC 119889, NSC 119911 and NSC 119913 share similar scaffolds: they all have a hydroxylated condensed three-cyclic xanthene system and a carboxylate group separated by a two-carbon spacer. These hits should be considered as promising starting points for the synthesis of analogues with improved inhibitory activities and for scaffold hopping.

2.3. The MurF inhibitor

In the primary spectroscopic in vitro assay, 12 compounds (NSC 209931, NSC 659162, NSC 45576, NSC 7223, NSC 668394, NSC 37031, NSC 49789, NSC 62406, NSC 86372, NSC 117079, NSC 270156 and NSC 17383) showed significant inhibitory activity for MurF (residual activities at 250 μM below 50%). To confirm these results, we used a secondary assay that is based on the use of radiolabelled UDP-Mur*N*Ac-tripeptide. Only one of these 12 compounds, NSC 209931 (Fig. 3), demonstrated inhibitory activity (IC_{50} = 63 μM), thereby indicating that the other 11 were false positives in the primary assay. NSC 209931 was ranked as the fifth most active compound by eHiTS (score, −7.007).

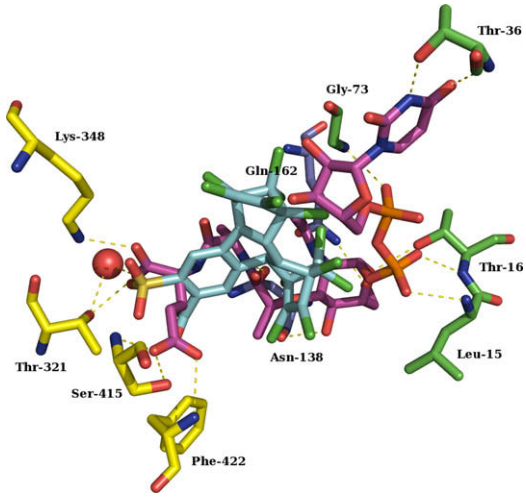


Figure 2. The MurD interactions with NSC 119889 (cyan) docked in the active site. UDP-Mur*N*Ac-L-Ala-D-Glu (magenta) is also shown.

Recently, Abbot laboratories published two small molecule inhibitors in complex with MurF.[18] For our virtual screening we selected the crystal structure 2AM1.pdb with sulphonamide inhibitor **1** (Fig. 3). In this crystal structure, the inhibitor **1** binds to the interface between the three domains, which are presented in different colours in Figure 4. The cyanothiophene ring is located between the N- and C-terminal domains, and the nitrile functionality forms an H-bond with Arg-49. The cyclohexyl ring forms hydrophobic interactions with the hydrophobic residues Pro-329, Leu-360 and Leu-367 from the C-terminal domain, and Phe-54 from the N-terminal domain. The dichlorophenyl and substituted sulfonamide moiety are located at the interface between all three of the domains, and they form hydrophobic interactions with Phe-31, Leu-45, Tyr-135 and Ile-139. These parts of the inhibitor **1** are also in contact with Asn-326, Asn-328 and Thr-330.

The biochemical assays with MurF were carried out using *Escherichia coli* because only the enzyme from this species was available. The crystal structure of *E. coli* MurF was solved only as the apo-enzyme, which is in an open form[26] and as such, is not suitable for docking experiments. However, the primary sequences of MurF from *E. coli* and *S. pneumoniae* share 26% identity, and the similarity of the individual domains from both of the structures is evident in structural alignments. Here, the overlap of the first two domains yields a RMSD value of 2.1 Å for 278 α-carbon atoms, while the C-terminal domains can be separately aligned with an RMSD value of 2.0 Å for 90 α-carbon atoms. Although the three domains show close structural similarities individually, the spatial arrangement of the domains differs substantially (open form of MurF from *E. coli* and closed form of MurF from *S. pneumoniae*).[17]

The lower hit rate as compared to the virtual screening for MurD might be attributed in part to our use of MurF from *S. pneu-*

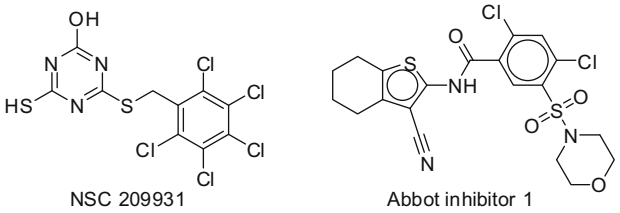


Figure 3. Structures of NSC 209931 and Abbot inhibitor **1**.

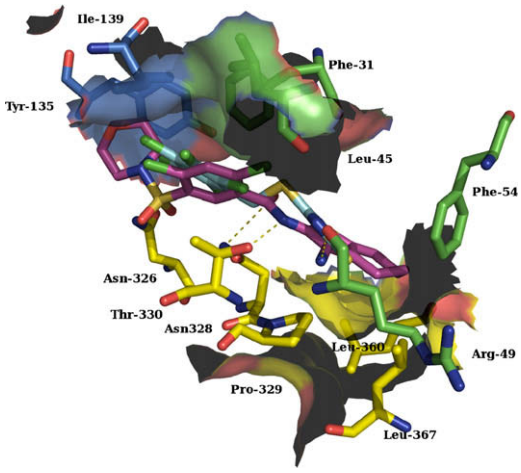


Figure 4. Crystal structure of MurF co-crystallized with the Abbot inhibitor **1** (magenta). The relevant amino-acid residues are shown: N-terminal domain—green, central domain—blue and C-terminal—yellow. Hydrophobic residues are shown as surfaces. NSC 209931 (cyan) docked into the MurF active site is also presented.

moniae for the docking experiments, and MurF from *E. coli* for the biochemical assays.

The experimental assays with MurF demonstrated that NSC 209931 has significant inhibitory activity. The eHiTS-predicted binding pose for NSC 209931 is shown in Figure 4. The lipophilic part of NSC 209931 interacts with the hydrophobic shelf formed by Phe-31 and Leu-45 of N-terminal domain and Tyr-135 and Ile-139 of the central domain. The hydrophilic part (3-thio-1,3,5-triazinol) of NSC 209931 could make H-bonds with Asn-328, Thr-330 or Arg-49. The predicted docking structure for NSC 209931 is also well aligned with inhibitor **1** co-crystallized with MurF (PDB entry 2AM1). Chlorinated phenyls from both inhibitor **1** and NSC 209931 are overlapping, and 3-thio-1,3,5-triazinol ring from NSC 209931 also overlaps with the cyanothiophene ring of the co-crystallized inhibitor **1**.

2.4. Primary and secondary assays

One of the serious problems associated with HTS, and also with biological evaluations after VHTS, is the occurrence of 'false-positives'. These are the molecules that are active in the biochemical assays that operate via mechanisms that are not directly relevant to the assay design, like interference with the assay signal, oxidation of, or other chemical reactions with, the target, or the forming of promiscuous aggregates that non-specifically inhibit the target.[27] One of the most often used methods for biochemical evaluation of inhibitors of the Mur enzymes is the Malachite green method, which involves the detection of the free phosphate that is liberated during the reaction through the formation of a complex with the Malachite dye that can be detected spectroscopically at 650 nm. This method is very sensitive, and it allows the rapid evaluation of large numbers of compounds on microtiter plates. However, one of its main drawbacks is that some compounds absorb at the same wavelength as the phosphomolybdate-Malachite green complex, or bind the free phosphate, or react directly with the Malachite green dye. To overcome this problem, and to verify all of the hits that were identified with the primary Malachite green assay, we used a more reliable secondary assay, which used a radioactively labelled substrate to directly detect the reaction products. In addition, all of the primary and secondary assays were performed in the presence of detergent (Triton X-114 or Tween 20), to exclude the formation of detergent-sensitive promiscuous aggregates.

2.5. Microbiological evaluation

The antibacterial activity of each hit compound was determined against *E. coli* 1411, *E. coli* SM1411, and *Staphylococcus aureus* 8325-4. *E. coli* SM1411 is an AcrAB$^-$ deficient derivative of *E. coli* 1411 that exhibits increased susceptibility to a range of antimicrobial agents.[28] None of the hit compounds demonstrated antibacterial activity against *E. coli* or *S. aureus* strains except for NSC 209931, that prevented growth of *S. aureus* 8325-4 at a high concentration (MIC 128 μg/mL). Lack of antibacterial activity could reflect failure of the inhibitors to enter the bacterial cell.

3. Conclusions

Structure based virtual screening is widely used for hit discovery and the development of new potential lead compounds. It allows the user to screen large databases of compounds in short periods of time, and at relatively low material consumption and cost. Ideally the result of such screening is an enriched subset of compounds that are highly ranked by a docking programme and then tested for in vitro inhibitory activity. Our approach employing eHiTS for virtual screening and the subsequent in vitro biochemical evaluation of the best ranked compounds provided four novel MurD inhibitors (best IC_{50} = 10 μM) and one novel MurF inhibitor (IC_{50} = 63 μM). It should be emphasized that docking scores rarely make reliable quantitative estimation of IC_{50} and most often result in 'enrichments' of only a few percent. In the present example 4 of 50 compounds (8%) had high inhibitory potential on MurD. In addition, in vitro assays can produce false-positive results, so a more reliable secondary assay needs to be available for verification of the results obtained in a primary assay. For the discovery of Mur ligase inhibitors, our combination of a colorimetric primary assay with a radioactivity secondary assay appears to be appropriate.

4. Experimental

4.1. Materials

The Diversity Set[19] consists of 1990 compounds in DMSO solution in 96-well microtiter plates and was obtained from the NCI.

The MurD (native form) and MurF (C-terminal His-tagged form) enzymes were overproduced in *E. coli* JM83 cells harbouring the pMLD58 and pMLD116 plasmids, respectively. These enzymes were purified by ion-exchange[29] and affinity[30] chromatography, respectively. The nucleotide substrates UDP-Mur*N*Ac-L-Ala and UDP-Mur*N*Ac-L-Ala-γ-D-Glu-*meso*-[^{14}C]A_2pm were prepared by chemo-enzymatic synthesis.[31]

4.2. Hardware and software

The virtual screening was carried out on a workstation with two Xeon 2.4 GHz processors, 2 GB RAM, a 120 GB hard drive and an Nvidia Geforce 6600 GT graphic card, running Red Hat Linux 9.0. eHiTS 6.0 from SimBioSys Inc.[20] was used for the active site detection and docking. Open Babel[32] was used for manipulating the ligands with various chemical formats. PyMol[33] from DeLano Scientific was used for visual inspection of the results and the graphical representations. CACTVS[34] tools was used for 2D structure visualization.

4.3. Structures

The 3D structures of the compounds from the NCI Diversity Set were obtained from the NCI webpage (http://dtp.nci.nih.gov/dw/testmasters/chem3d.html). The 1990 highly diverse compounds

of the NCI Diversity Set represented a broad chemical spectrum. However, no special preparation of the 3D structures was applied since eHiTS automatically evaluates all of the possible protonation states for ligands and enzymes.

For MurD, we used its crystal structure from *E. coli* as a complex with UDP-Mur*N*Ac-L-Ala-D-Glu, the product of the reaction (PDB entry 4UAG).[6] The structure was solved at 1.66 Å. eHiTS automatically detected the enzyme active site as the region of MurD around the ligand. To reduce the size of the active site before the docking, the 4uag.pdb file was manually edited, with the uridine monophosphate moiety deleted, as it does not make crucial interactions with MurD: only parts of the ligand (phospho-Mur*N*Ac-L-Ala-D-Glu) that are of greatest importance for substrate binding to MurD were thus retained.

The virtual screening with MurF was carried out on the crystal structure of MurF from *S. pneumoniae* in complex with its inhibitor 2,4-dichloro-*N*-(3-cyano-4,5,6,7-tetrahydro-benzothiophen-2yl)-5-(morpholine-4-sulfonyl)-benzamide (inhibitor **1**; PDB entry 2AM1).[17] This structure was solved at 2.50 Å and was used without any modifications.

4.4. Docking and scoring

Both MurD and MurF were initially prepared with eHiTS. Their active site detection was carried out using the '-complex' parameter with high accuracy. The programme automatically detected the ligand in the complex and selected the part of the enzyme within a 7-Å margin around the ligand as the active site. The NCI Diversity Set was then docked to the active site using the fastest mode of docking (accuracy set to 1). Two hundred of the best scoring compounds from the first run were then re-docked at higher accuracy (accuracy set to 6). The scoring was according to the eHiTS_Score, which is a statistically derived empirical scoring function that is included in the eHiTS software package.[23]

4.5. Biochemical assays

4.5.1. Primary assay (Malachite green assay)

The selected compounds were tested for their inhibition of the addition of D-Glu (D-Ala-D-Ala) to UDP-Mur*N*Ac-L-Ala (UDP-Mur*N*Ac-L-Ala-D-Glu-*meso*-A_2pm) catalyzed by MurD (MurF) from *E. coli*. The detection of the orthophosphate generated during the reaction was based on the colorimetric, Malachite green method, as described elsewhere,[35] with slight modifications, using a mixture (final volume, 50 μL) containing 50 mM Hepes, pH 8.0, 5 mM $MgCl_2$ (50 mM $MgCl_2$), 80 μM UDP-Mur*N*Ac-L-Ala (100 μM UDP-Mur*N*Ac-L-Ala-γ-D-Glu-*meso*-A_2pm), 100 μM D-Glu (600 μM D-Ala-D-Ala), 400 μM ATP (500 μM ATP), 0.005% Triton X-114, purified MurD (MurF) from *E. coli* and 250 μM of each test compound dissolved in DMSO. The final concentration of DMSO was 5% (v/v). The mixture was incubated at 37 °C for 15 min (20 min), and then quenched with 100 μL Biomol® reagent. The absorbance was measured at 650 nm after 5 min. All of the experiments were run in duplicate. The residual activity was calculated with respect to a similar assay without the test compounds. The IC_{50} values were determined by measuring the residual activity at seven different test compound concentrations, and they represent the concentrations of test compounds for which the residual activity was 50%.

4.5.2. Secondary assay (radioactivity assay)

Selected compounds were also tested for inhibition of the addition of the radiolabelled amino-acid substrate D-[^{14}C]Glu to UDP-Mur*N*Ac-L-Ala catalyzed by MurD, using a mixture (final volume, 50 μL) containing 0.1 M Tris–HCl, pH 8.6, 5 mM $MgCl_2$, 5 mM ATP, 25 μM UDP-Mur*N*Ac-L-Ala, 25 μM D-[^{14}C]Glu (50,000 cpm), 5% (v/v) DMSO, 30 μM Tween 20, purified MurD (diluted in 20 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol, 1 mg/mL BSA), and 250 μM of each test compound. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by adding 10 μL glacial acetic acid. The resulting mixture was lyophilized and taken up in water (ca. 10 μL). The radioactive substrate and product were separated by TLC on LK6D silica gel plates (Whatman), which were developed in 1-propanol/ammonium hydroxide/water 6:3:1 (v/v), and quantified with a radioactivity scanner (model Multi-Tracemaster LB285, Berthold-France, Thoiry, France).

The MurF assay was similar, except the radiolabel was on the nucleotide substrate. The assay mixtures (final volume, 50 μL) contained 0.1 M Tris–HCl, pH 8.6, 100 mM $MgCl_2$, 5 mM ATP, 85 μM UDP-Mur*N*Ac-L-Ala-γ-D-Glu-*meso*-[^{14}C]A_2pm (50,000 cpm), 75 μM D-Ala-D-Ala, 5% (v/v) DMSO, 30 μM Tween 20, purified MurF (diluted in 20 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol, 1 mg/mL BSA), and 250 μM of each test compound. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by adding 10 μL glacial acetic acid. The resulting mixture was lyophilized and taken up in the HPLC elution buffer. The radioactive substrate and product were separated by reverse-phase HPLC on a Nucleosil $5C_{18}$ column (150 × 4.6 mm) as stationary phase, and with isocratic elution at a flow rate of 0.6 mL/min with 50 mM ammonium formate, pH 4.7. The compounds were detected and quantified with an LB 506 C-1 HPLC radioactivity monitor (Berthold-France) using Quickszint Flow 2 scintillator (Zinsser Analytic, Maidenhead, UK) at 0.6 mL/min.

4.6. Microbiological evaluation

Minimal inhibitory concentrations (MICs) of compounds were determined by broth microdilution in IsoSensitest broth (Oxoid) using an inoculum of 10^4 cells per mL for *E. coli* or 10^6 cells per mL for *S. aureus*. The potential antimicrobial agents were prepared in a twofold dilution series in 50% dimethyl sulfoxide (Sigma–Aldrich). Microwell plates with 96 wells (Nunc, Fisher Scientific), each containing a potential antimicrobial agent and a bacterial suspension, were incubated for 16 h at 37 °C in a Spectramax 384 plus microwell plate reader (Molecular Devices) running the SOFTmax PRO 3.1.1 software. Readings of optical density at 600 nm were made at 10 min intervals. Plates were shaken for 60 s before each reading. The MIC was taken as the lowest concentration of potential antimicrobial agent that prevented the growth of bacteria in a well.

Acknowledgements

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