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Synthesis and biological evaluation of new glutamic acid-based inhibitors of MurD ligase

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ABSTRACT

Mur ligases catalyze the biosynthesis of the UDP-MurNAc-pentapeptide precursor of peptidoglycan, an essential polymer of bacterial cell-wall. They constitute attractive targets for the development of novel antibacterial agents. Here we report on the synthesis of a series of 2,4-diaminoquinazolines, quinazoline-2,4(1*H*,3*H*)-diones, 5-benzylidenerhodanines and 5-benzylidenethiazolidine-2,4-diones and their inhibitory activities against MurD from *Escherichia coli*. Compounds (***R***)-**27** and (***S***)-**27** showed inhibitory activity against MurD with IC_{50} values of 174 and 206 μM, respectively, which makes them promising starting points for optimization.

Bacterial resistance to currently available antibiotics has created an urgent need to discover novel effective antibacterial agents directed towards previously unexploited targets.[1,2] One of the best known and most validated targets for antibacterial therapy is the enzyme system responsible for peptidoglycan biosynthesis.[3] Peptidoglycan is an essential cell-wall polymer whose main function is to provide the rigidity, flexibility and strength that are necessary for bacterial cells to grow and divide while withstanding high internal osmotic pressure.[4,5] As it is unique to prokaryotic cells, it represents an optimal target with respect to selective toxicity. A large number of antibiotics currently used in therapy act by inhibiting late extracellular steps of peptidoglycan biosynthesis. In contrast, the early intracellular steps of biosynthesis of cytoplasmic peptidoglycan precursor have been less exploited to date.[3,4]

The biosynthesis of peptidoglycan is a complex two-stage process involving intracellular assembly of the UDP-MurNAc-pentapeptide which is subsequently translocated through the cytoplasmic membrane to its outer side, where the polymerization reactions take place.[6–8] Enzymes of the Mur ligase family, MurC to MurF, catalyze a series of reactions leading to UDP-MurNAc-pentapeptide by stepwise addition of L-Ala (MurC), D-Glu (MurD), *meso*-diaminopimelic acid or L-Lys (MurE) and D-Ala-D-Ala (MurF) to the starting MurC substrate UDP-MurNAc.[3,6]

The crystal structures of Mur ligases from different bacterial species are known and all reveal the same three-domain topology, with N-terminal and central domains responsible for binding of the UDP-precursor and ATP, respectively, while the C-terminal domain binds the condensing amino acid or dipeptide residue.[9–16] Surprisingly, sequence alignment of Mur ligase orthologues and paralogues revealed relatively low overall homologies, but importantly, quite high homology of residues present in the active sites. The ATP binding pocket in particular seems to be well conserved throughout the family.[17–20]

The catalytic mechanism of Mur ligases has been studied in detail and they most probably operate by a similar chemical mechanism. Initially, the terminal carboxyl group of the UDP-substrate is activated by phosphorylation, resulting in formation of an acyl-phosphate intermediate which is subsequently attacked by the amino group of the condensing amino acid residue or dipeptide. The tetrahedral high-energy intermediate formed collapses with elimination of inorganic phosphate and concomitant peptide bond formation.[4,21] Moreover, based on the results of biochemical studies of MurC and MurF, Mur ligases exhibit an ordered kinetic mechanism in which ATP binds first to the free enzyme, followed by the corresponding UDP-substrate and condensing amino acid or dipeptide last.[22,23] X-Ray experiments revealed two distinct 'open' conformations and one 'closed' conformation of MurD from *Escherichia coli*, in which the binding of the substrates is accompanied by conformational closure of the C-terminal domain.[12,24]

There have been several attempts to design MurC to MurF inhibitors by using substrate or transition-state analogues and structure-based design, reviewed by El Zoeiby et al.[3] and more recently by Barreteau et al.[6] and Štefanič Anderluh et al.[25] Recently,

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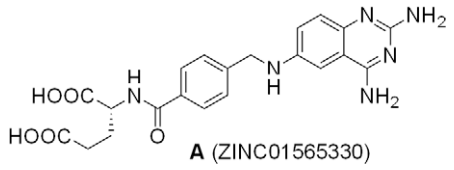


Figure 1. The highest ranked compound in virtual screening.

the first crystal structures of MurD- and MurF-small molecule inhibitor complexes were published, providing a good starting point for the design of inhibitors with improved properties.[26,27]

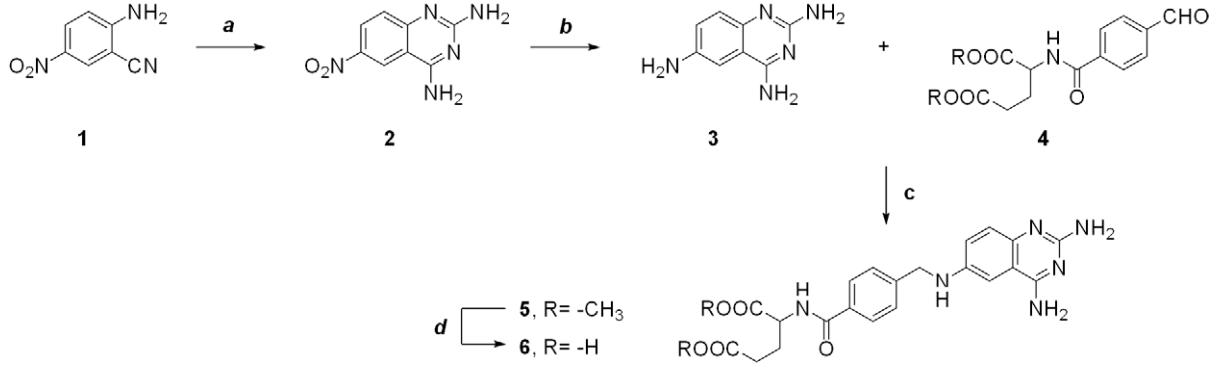
As a part of our efforts toward the discovery of new inhibitors of MurD, we performed a virtual screening[28] using FlexX[29] docking program. Commercially available quinazolines from ZINC database[30] were docked into the crystal structure 4UAG.pdb of MurD from *E. coli*.[11] Compound **A** (Fig. 1) with the highest score of FlexX scoring function was identified as a potential inhibitor of MurD ligase.

MurD is highly specific for D-Glu as a substrate,[31,32] which is also present in the structure of **A**, and was thus suggested to be an essential fragment of a potent inhibitor.[33] Therefore, we decided to synthesize **A** and its L-Glu containing analogue to further explore the difference in potencies of L-Glu- and D-Glu-based inhibitors. As a matter of fact, it has recently been found that an *N*-sulfonyl-L-Glu derivative was not devoid of affinity, being only 4-fold less potent than its D-Glu counterpart.[26]

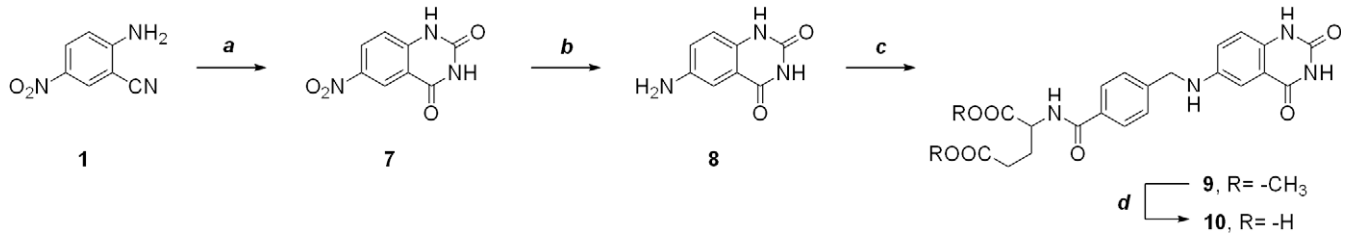
Compound **A** was synthesized for the first time in the series of isofolic acid analogues and was shown to be an inhibitor of the rat liver dihydrofolate reductase in vitro.[34,35] In the present work we prepared it using an alternative synthetic strategy (Scheme 1). First, we synthesized 2,4,6-triaminoquinazoline (**3**) according to the method of Davoll and Johnson by condensation of 2-amino-5-nitrobenzonitrile (**1**) and guanidine carbonate with subsequent reduction of the nitro group of 6-nitro-2,4-diaminoquinazoline (**2**).[36] Since **2** is usually prepared by refluxing the reaction mixture in ethanol overnight with yields around 70%, we tried to increase the yield and shorten the reaction time by using microwave-assisted synthesis.[37] Different solvents and various reaction conditions were tested. When ethanol as a solvent and temperatures above 75 °C were used, we observed the formation of a byproduct which decreased the final yield and purity of the desired product. Nevertheless, we succeeded in preparing **2** of high purity in yields above 85% by using methanol as a solvent and microwave irradiation at 100 °C for 15 min. In parallel, we synthesized aldehydes (***S***)-**4** and (***R***)-**4** from 4-carboxybenzaldehyde and enantiomerically pure dimethyl ester of L- or D-Glu, using EDC/HOBt promoted amide bond formation. Finally, reductive amination of 2,4,6-triaminoquinazoline (**3**) with (***S***)-**4** or (***R***)-**4** afforded derivatives (***S***)-**5** and (***R***)-**5**, containing dimethyl ester of L- and D-Glu, respectively. After saponification of (***S***)-**5** and (***R***)-**5** using 1 M aqueous NaOH in dioxane, free glutamic acid derivatives (***S***)-**6** and (***R***)-**6** were obtained.

By replacing 2,4,6-triaminoquinazoline scaffold (**3**) with 6-aminoquinazoline-2,4(1*H*,3*H*)-dione scaffold (**8**) we synthesized compounds (***S***)-**10** and (***R***)-**10**. 6-Nitroquinazoline-2,4(1*H*,3*H*)-dione (**7**) was prepared from 2-amino-5-nitrobenzonitrile (**1**) according to the known procedure (Scheme 2).[38] After reduction of the nitro group of **7**, reductive amination of the thus obtained amine **8** and aldehyde (***S***)-**4** or (***R***)-**4** afforded compounds (***S***)-**9** and (***R***)-**9**. Compounds (***S***)-**10** and (***R***)-**10** were prepared after hydrolysis of (***S***)-**9** and (***R***)-**9**, using 1 M aqueous NaOH in dioxane.

Target compounds (***S***)-**5**, (***R***)-**5**, (***S***)-**6**, (***R***)-**6**, (***S***)-**9**, (***R***)-**9**, (***S***)-**10** and (***R***)-**10** were tested for inhibitory activity on MurD from *E. coli* using the Malachite green assay for detecting orthophosphate generated during the reaction.[39] To exclude possible nonspecific (promiscuous) inhibition, all the compounds were tested in the presence of detergent (Triton X-114, 0.005%).[40] The results are presented as residual activities (RAs) of the enzyme in the presence of 500 μM concentration of each compound (Table 1). Based on these results we can conclude that the synthesized compounds are poor inhibitors, of MurD if at all, that need further optimization to obtain higher affinity towards the enzyme.



Scheme 1. Reagents and conditions: (a) guanidine carbonate, NaOMe/MeOH then MeOH, 5 W, 15 bar, 100 °C, 15 min or EtOH, reflux, 24 h; (b) H_2, Pd/C, DMF/AcOH, 5 bar, 4 h; (c) $NaCNBH_3$, DMF, rt, 24–72 h; (d) 1 M NaOH, dioxane/water, rt, 2-4 h.



Scheme 2. Reagents and conditions: (a) DBU, CO_2, DMF, rt, 24 h; (b) H_2, Pd/C, DMF, 5 bar, 12 h; (c) **4**, $NaCNBH_3$, DMF, rt, 48–72 h; (d) 1 M NaOH, dioxane/water, rt, 4–6 h.

Table 1
Inhibitory activities against MurD of quinazoline and folic acid derivatives.

Compound	RA[a],%
(*S*)-5	81
(*R*)-5	87
(*S*)-6	80
(*R*)-6 or **A**	82
(*S*)-9	92
(*R*)-9	91
(*S*)-10	81
(*R*)-10	77
(*S*)-11	96
(*S*)-12	95

[a] Residual activity of the enzyme in the presence of the tested compound at 500 μM.

The similarity of compound **A** with dihydrofolate, the substrate of dihydrofolate reductase, led us to assume that the latter compound might inhibit MurD. Interestingly, dihydrofolate was shown to inhibit glutathione synthetase, which is also an amide-forming enzyme.[41] Therefore, commercially available folic acid ((***S***)-**12**, Fig. 2) and its dimethyl ester ((***S***)-**11**) were tested for inhibition of MurD ligase but were also found to be inactive (Table 1).

In spite of their weak inhibitory activity, we docked compounds **6** and **10** into the MurD active site using Gold v3.2 program.[42] The docked poses revealed that the pyrimidine ring of the quinazoline nucleus was located in the same position as the diphosphate group of UDP. This prompted us to replace the quinazoline ring by a phosphate isostere,[43] for example, a 2-thioxo-1,3-thiazolidin-4-one (rhodanine (**13**)) moiety.

Compounds bearing the rhodanine ring have shown a wide range of pharmacological activities, which also include antibacterial[44] and antifungal[45] effects. Additionally, rhodanines have been designed as inhibitors of various enzymes such as bacterial β-lactamase[46] and Mur ligases.[47–49] Thus, we decided to replace the quinazoline ring of compounds **6** and **10** with 5-benzylidenerhodanine or 5-benzylidenethiazolidine-2,4-dione moiety to obtain compounds **23, 24, 27** and **28**.

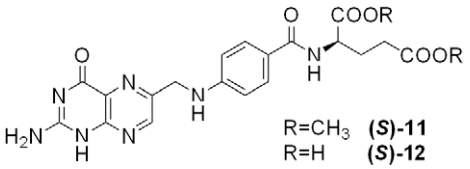


Figure 2. Folic acid (***S***)-**12** and its dimethyl ester (***S***)-**11**.

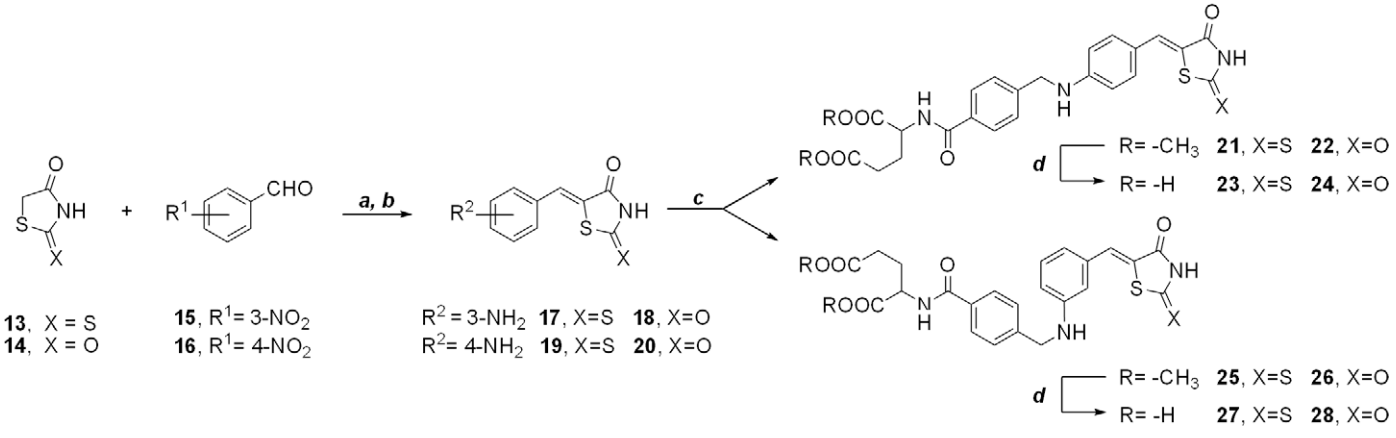
Rhodanine-based compounds were synthesized according to Scheme 3. The 5-benzylidenerhodanines **17** and **19** were prepared in good yields via a Knoevenagel condensation between the requisite rhodanine (**13**) and 3-nitrobenzaldehyde (**15**) or 4-nitrobenzaldehyde (**16**) using microwave irradiation, followed by reduction of the nitro group to the corresponding amine with tin(II) chloride. According to the literature,[50,51] only thermodynamically stable *Z*-isomers of 5-benzylidenerhodanines are formed in the reaction of rhodanine with aromatic aldehydes. An investigation of ^{1}H-NMR spectra of our compounds **17**–**20** demonstrated that only one isomer was formed, since only one signal for the methyne proton was observed, thus confirming the *Z*-configuration of 5-benzylidenerhodanines **17**-**20**. In the next step, compounds **17** and **19** were reacted with (***S***)-**4** or (***R***)-**4** to give (***S***)-**21**, (***R***)-**21**, (***S***)-**25** and (***R***)-**25**. After hydrolysis using 2,2 M LiOH solution we obtained target compounds (***S***)-**23**, (***R***)-**23**, (***S***)-**27** and (***R***)-**27**. Compounds (***S***)-**24**, (***R***)-**24**, (***S***)-**28** and (***R***)-**28** were synthesized from thiazolidine-2,4-dione (**14**) in an almost identical fashion to their rhodanine counterparts (Scheme 3).

5-Benzylidenerhodanines **21**, **23**, **25**, **27** and 5-benzylidenethiazolidine-2,4-diones **22**, **24**, **26** and **28** were tested for inhibitory activity on MurD from *E. coli*.[39] Results are presented in Table 2 as residual activities of the enzyme in the presence of 250 μM concentration of the tested compound. For the more active compounds, IC_{50} values were also determined. Compounds containing a dimethyl ester of Glu ((***S***)-**21**, (***R***)-**21**, (***S***)-**22**, (***R***)-**22**, (***S***)-**25**, (***R***)-**25**, (***S***)-**26**, (***R***)-**26**) were inactive (RA = 86–98%), which can be well rationalized since the natural substrate of the enzyme is free (unesterified) D-Glu. On the other hand, replacement of the quinazoline moiety in glutamic acid-containing compounds **5**, **6**, **9** and **10** by 5-benzylidenerhodanine resulted in the most potent inhibitors of the series, compounds (***R***)-**27** and (***S***)-**27**, with IC_{50} values of 174

Table 2
Inhibitory activities of rhodanine and 2,4-thiazolidinedione derivatives.

Compound	X	Substitution	RA[a],% or IC_{50}, μM
(*S*)-23	S	4-	68
(*R*)-23	S	4-	74
(*S*)-24	O	4-	88
(*R*)-24	O	4-	70
(*S*)-27	S	3-	IC_{50} = 206 μM
(*R*)-27	S	3-	IC_{50} = 174 μM
(*S*)-28	O	3-	87
(*R*)-28	O	3-	85

[a] Residual activity of the enzyme in the presence of the tested compound at 250 μM.



Scheme 3. Reagents and conditions: (a) piperidine, AcOH, EtOH, 20 W, 20 bar, 150 °C, 20 min; (b) $SnCl_2$, EtOH, reflux, 2 h; (c) **4**, $NaCNBH_3$, DMF, rt, 48 h; (d) 2.2 M LiOH, EtOH/water, rt, 24 h.

Table 3
Inhibitory activity of MurD inhibitors (***R***)-**27** and (***S***)-**27** against other Mur ligases.

Compound	RA[a],%		
	MurC	MurE	MurF
(*R*)-27	78	96	79
(*S*)-27	76	91	77

[a] Residual activity of the enzyme in the presence of the tested compound at 250 μM.

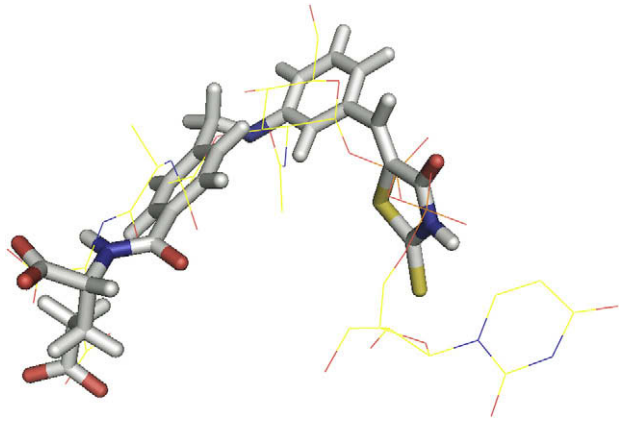


Figure 3. Superposition of the highest ranked solution of inhibitor (***R***)-**27** (in sticks), as calculated by the GOLD v3.2 program, and UDP-MurNAc-L-Ala-D-Glu (in lines), from the X-ray structure of MurD (the picture was prepared by Pymol[53]).

and 206 μM, respectively. In contrast, introduction of 5-benzylidenethiazolidine-2,4-dione was detrimental to the activity ((***S***)-**28**, (***R***)-**28**). The difference between the activities of rhodanines and the thiazolidine-2,4-diones tested is in agreement with our literature survey. There have been several reports that various rhodanines possess antimicrobial activity and inhibit bacterial enzymes, while their thiazolidine-2,4-dione counterparts usually displayed weaker activity or were inactive.[46,52] Moreover, the inhibitory activity was also dependent on the substitution of the benzylidene ring. While 4-substituted 5-benzylidenerhodanines ((***S***)-**23**, (***R***)-**23** and (***R***)-**24**) were weak inhibitors of MurD ligase, with RA values between 68% and 74% at 250 μM, their 3-substituted counterparts ((***R***)-**27** and (***S***)-**27**) were the most potent MurD inhibitors among the synthesized compounds. Furthermore, the inhibition appeared not to be strictly dependent on the stereochemistry of Glu, since compound (***S***)-**27**, containing L-Glu, was almost as potent as (***R***)-**27**, containing D-Glu. The difference in potencies of (***R***)-**27** and (***S***)-**27** was significantly smaller than in the previously reported *N*-sulfonyl-L- and D-Glu-based inhibitors.[26] To test the specificity of compounds (***R***)-**27** and (***S***)-**27** for MurD ligase they were also tested for inhibition of MurC and MurF from E. coli and MurE from *S. aureus* but were found to be practically inactive with RA values between 76% and 96% at 250 μM concentration (Table 3).

In order to investigate the possible binding mode of our inhibitors, compound (***R***)-**27** was docked into the MurD active site (pdb code 4UAG) using Gold v3.2 program.[42] This indicated that D-Glu of (***R***)-**27** binds in the D-Glu pocket of the enzyme, while the rhodanine ring occupies the place of the diphosphate of UDP (Fig. 3). The predicted binding mode is reasonable, since rhodanines and 4-thiazolidinones have already been used as diphosphate surrogates in inhibitors of the enzymes that act on UDP-containing substrates.[47,49,54]

To conclude, we reported on the synthesis and inhibitory activity of a series of enantiomerically pure L- or D-glutamic acid-containing compounds as inhibitors of MurD ligase from *E. coli*. Although our highest ranked virtual screening compound (***R***)-**6** was practically inactive, starting from it, we managed to obtain inhibitors of MurD. Compounds (***R***)-**27** and (***S***)-**27**, bearing the 5-benzylidenerhodanine moiety, inhibited MurD with IC_{50} values of 174 and 206 μM, respectively, and were also found to be more specific for MurD than other Mur ligases. They thus constitute a promising starting point for the development of novel, more potent inhibitors of this enzyme by using structure-based drug design.

Acknowledgments

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