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5-Benzylidenethiazolidin-4-ones as Multitarget Inhibitors of Bacterial Mur Ligases

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Mur ligases participate in the intracellular path of bacterial peptidoglycan biosynthesis and constitute attractive, although so far underexploited, targets for antibacterial drug discovery. A series of hydroxy-substituted 5-benzylidenethiazolidin-4-ones were synthesized and tested as inhibitors of Mur ligases. The most potent compound **5a** was active against MurD–F with IC_{50} values between 2 and 6 μM, making it a promising multitarget inhibitor of Mur ligases. Antibacterial activity against different strains, inhibitory activity against protein kinases, mutagenicity and genotoxicity of **5a** were also investigated, and kinetic and NMR studies were conducted.

Introduction

The search for new antibacterial agents with novel mechanisms of action is one of the key strategies emerging for combating drug-resistant bacteria.[1] The biochemical machinery involved in peptidoglycan biosynthesis remains a viable source of novel, previously unexploited targets for antibacterial drugs.[2] A large number of important antibiotic classes act by inhibiting extracellular steps of peptidoglycan biosynthesis. Peptidoglycan is an essential cell-wall polymer unique to prokaryotic cells that provides the rigidity, flexibility and strength required for bacterial cells to grow and divide, while withstanding high internal osmotic pressure.[3] Recently, there has been an increased interest in exploiting the enzymes involved in the early intracellular steps of cytoplasmic peptidoglycan precursor biosynthesis for antibacterial drug discovery. Among them are the ATP-dependent Mur ligases (MurC–F) that catalyze a series of reactions leading to UDP-Mur*N*Ac-pentapeptide (Park's nucleotide) by sequentially adding L-Ala (MurC), D-Glu (MurD), L-Lys or *meso*-diaminopimelic acid (MurE) and D-Ala–D-Ala dipeptide (MurF) to the starting MurC substrate UDP-Mur*N*Ac. The fact that Mur enzymes are vital for the survival of bacteria makes them promising targets for antibacterial drug discovery.[4]

Mur ligases catalyze the formation of an amide or peptide bond between the UDP substrate and the condensing amino acid. Initially, the terminal carboxyl group of the UDP substrate is activated by ATP phosphorylation, resulting in the formation of an acylphosphate intermediate that is subsequently attacked by the amino group of the incoming amino acid or dipeptide. The tetrahedral high-energy intermediate formed collapses with elimination of inorganic phosphate and concomitant formation of the amide or peptide bond (Figure 1).[3,5,6] Moreover, based on 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 finally the condensing amino acid or dipeptide.[7] All the Mur ligases share the same three-domain topology, with the N-terminal and central domains binding UDP precursor and ATP respectively, while the C-terminal domain binds the condensing amino acid or dipeptide residue.[3,8]

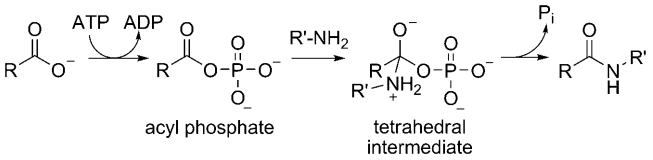

Figure 1. Catalytic mechanism of Mur ligases.

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There have been several attempts to design inhibitors of the Mur ligases by mimicking substrates, products or tetrahedral intermediates.[4,9] The crystal structures of apoenzymes and enzymes in complex with their substrates, products or inhibitors,[5,10] have enabled structure-based design of inhibitors and recently led to the discovery of diverse Mur ligase inhibitors by virtual screening of compound libraries.[11]

It is now widely recognized that compounds designed to bind to more than one target (designed multitarget ligands) can be more therapeutically beneficial than highly target-specific ligands. Designing multitarget ligands is usually a demanding challenge, with the need to appropriately balance affinities for different targets while preserving their drug-like properties. Nevertheless, designed multitarget ligands have rapidly become a paradigm in drug discovery in different therapeutic areas, such as cancer, hypertension, allergic, psychiatric and metabolic diseases.[12a] Using network models of antimicrobial drugs, Csermely et al.[12b] showed that multitarget attacks perturb complex systems more effectively than focused attacks, even if the number of targeted interactions is the same. Consequently, we believe that inhibition of multiple Mur ligases would result in potent antibacterial activity. Moreover, it should be beneficial in combating the proliferation of bacterial resistance caused by mutation,[2] a major advantage for multitarget inhibition in bacteria. Since Mur ligases share the same catalytic mechanism and possess several conserved residues in their active sites, particularly in the ATP-binding site,[13] it should be possible to inhibit all Mur ligases (MurC–F) with a single molecular entity.[4b] Indeed, Mansour et al. recently reported naphthyl tetronic acids as multitarget inhibitors of bacterial peptidoglycan biosynthesis that inhibit MurB–F from *Staphylococcus aureus* and *Escherichia coli*, and MurA from *E. coli*.[12c] There are at least three possible ways by which inhibition of multiple Mur ligases could be achieved: 1) by ATP-competitive inhibition, 2) by an inhibitor mimicking the UDP-Mur*N*Ac moiety of the UDP substrate and 3) by binding at an allosteric site common to all four Mur ligases.

As part of our efforts to discover new small-molecule inhibitors of the intracellular steps of peptidoglycan biosynthesis, we recently reported glutamic-acid-based selective MurD inhibitors containing a rhodanine moiety that act as MurD product mimics.[14] Since compounds bearing a rhodanine ring often exhibit antibacterial activity due to inhibition of bacterial enzymes,[15] we have further explored rhodanine-based inhibitors of Mur ligases. The rhodanine ring has already been employed as a diphosphate surrogate or phosphate mimetic,[16] which makes it a convenient scaffold for the design of Mur ligase inhibitors. Since the ATP-binding and UDP-binding pockets of MurC–F active sites are the most highly conserved, and since visual inspection of the crystal structures suggests several possible hydrogen-bond interactions with the substituted rhodanine scaffold, compounds based on the 5-benzylidenerhodanine scaffold could bind to either the ATP- or UDP-binding pocket of the Mur enzymes, which is expected to result in multiple Mur ligase inhibition. Attachment of hydroxy groups to the benzylidene moiety would offer potential hydrogen-bond formation and should improve inhibitory activity against Mur ligases. Indeed, some *N*-acylhydrazones incorporating the 2,3,4-trihydroxyphenyl group have already shown inhibition of MurC and MurD.[17] For these reasons, we synthesized a series of hydroxy-substituted 5-benzylidenethiazolidin-4-one derivatives and assayed them as inhibitors of the MurC–F enzymes.

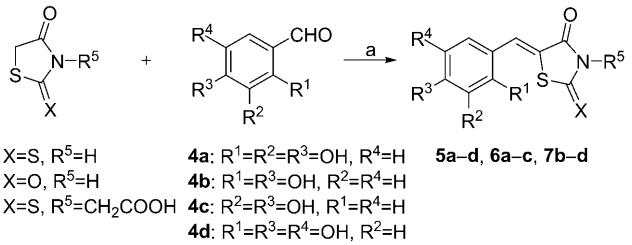
Results and Discussion

Chemistry

The thiazolidine-4-one-based compounds **5a–d**, **6a–c** and **7b–d** (Table 1) were synthesized via a Knoevenagel condensation between the 2-thioxothiazolidin-4-one (rhodanine, **1**), thiazolidine-2,4-dione (**2**) or 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid (rhodanine-*N*-acetic acid, **3**) and the corresponding benzaldehyde **4a–d** (Scheme 1). The target compounds were prepared under a variety of reaction conditions under microwave irradiation, using piperidine and glacial acetic acid as catalysts. Compounds **5a–d** and **6a–c** were obtained in good yields by heating the reaction mixtures at 140 °C for 30 min. In contrast, the use of the same conditions for the synthesis of **7b–d** did not give the target compounds. Nevertheless, by varying the temperature and reaction time, **7b–d** could be synthesized by heating the reactants at 110 °C for 40 min, but pure reaction products could only be isolated in low yields. In theory, there are two possible geometrical isomers (*E/Z*) for 5-benzylidenethiazolidin-4-ones; ^{1}H NMR spectra of compounds **5a–d**, **6a–c** and **7b–d** show only one signal for the methyne proton in the

Table 1. Structures of compounds **5a–d**, **6a–c** and **7b–d**.

Compd	X	R^1	R^2	R^3	R^4	R^5
5a	S	OH	OH	OH	H	H
5b	S	OH	H	OH	H	H
5c	S	H	OH	OH	H	H
5d	S	OH	H	OH	OH	H
6a	O	OH	OH	OH	H	H
6b	O	OH	H	OH	H	H
6c	O	H	OH	OH	H	H
7b	S	OH	H	OH	H	CH_2COOH
7c	S	H	OH	OH	H	CH_2COOH
7d	S	OH	H	OH	OH	CH_2COOH



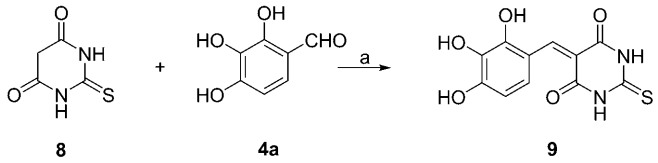
1: X=S, R^5=H
2: X=O, R^5=H
3: X=S, R^5=CH_2COOH

4a: R^1=R^2=R^3=OH, R^4=H
4b: R^1=R^3=OH, R^2=R^4=H
4c: R^2=R^3=OH, R^1=R^4=H
4d: R^1=R^3=R^4=OH, R^2=H

5a–d, **6a–c**, **7b–d**

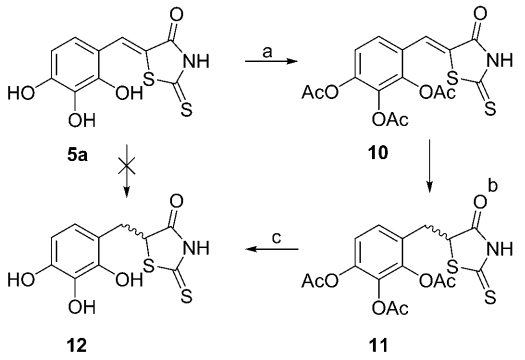
Scheme 1. *Reagents and conditions*: a) piperidine, AcOH, EtOH, 30 W, 18 bar, 140 °C, 30 min for **5a–d** and **6a–c** or 110 °C, 40 min for **7b–d**.

range 7.47 to 8.01 ppm, at lower field values than those expected for the *E* isomers, which strongly indicates that the compounds have the *Z* configuration. The latter has been reported as thermodynamically more stable than the *E* configuration.[18a,b] The *Z* configuration of compounds **5a**–**d**, **6a**–**c** and **7b**–**d** can also be inferred from the X-ray crystal structures[18c] and from the splitting pattern and coupling constants of the proton signal in ^{1}H-coupled ^{13}C NMR spectrum of similar compounds arising due to interaction with the C=O group of the rhodanine system.[18d] 2-Thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (**9**), a thiobarbituric acid derivative, was prepared by refluxing thiobarbituric acid (**8**) and 2,3,4-trihydroxybenzaldehyde (**4a**) in water overnight (Scheme 2), since application of the procedure described above proved unsuccessful.



Scheme 2. *Reagents and conditions*: a) H_2O, reflux, o/n.

Only a few methods have been reported for reducing the exocyclic double bond in 5-benzylidenethiazolidin-4-ones. Lithium borohydride can be used as a reducing agent, however, long reaction times and poor conversion have been reported for phenol-containing compounds, because the phenolates generated under the reaction conditions were insoluble in the reaction media.[19] We therefore considered reduction using diethyl 2,6-dimethyl-1,4-dihydro-3,5-pyridinedicarboxylate (Hantzsch ester)[20] and activated silica gel.[21] Our first attempts to reduce the exocyclic double bond of **5a** were unsuccessful due to the insolubility of **5a** in toluene, even at elevated temperatures. To increase its solubility, different solvents (THF, DMF and EtOAc) were used instead of toluene but these also failed to yield the desired product. Therefore, we decided to acetylate the hydroxy groups of **5a** (compound **10**, Scheme 3) in order to 1) increase the solubility of **5a** in toluene and then use the Hantzsch ester method, or 2) prevent phenolate generation and use lithium borohydride reduction. The treatment of **5a** with acetic anhydride in the presence of potassium carbonate in diethyl ether gave compound **10**, which was successfully reduced to compound **11** in moderate yield using the Hantzsch ester method. Finally, hydrazinolysis[22] of the ester groups of **11** gave the target compound **12**.



Scheme 3. *Reagents and conditions*: a) Ac_2O, K_2CO_3, Et_2O, 0 °C→RT, 15 h; b) diethyl 2,6-dimethyl-1,4-dihydro-3,5-pyridinedicarboxylate, silica gel 60, toluene, 100 °C, 24 h; c) $NH_2NH_2{\cdot}H_2O$, CH_3CN, RT, 20 min.

Biology

Following our idea to combine rhodanine with a hydroxyphenyl moiety, we first synthesized compound **5a** and tested its ability to inhibit Mur ligases (MurC, MurD and MurF from *E. coli* and MurE from *S. aureus*) using the malachite green assay for detecting orthophosphate generated during the enzymatic reaction.[23] To exclude possible nonspecific (promiscuous) inhibition, the compounds were tested in the presence of detergent (Triton X-114, 0.005%).[24] Compound **5a** inhibited MurD and MurF with an IC_{50} value of 2 μM, and MurE with IC_{50} of 6 μM. It thus possessed well-balanced inhibitory activity against enzymes MurD–F. Inspired by this promising result, we synthesized a series of analogues by varying the number and position of hydroxy groups on the benzylidene ring (compounds **5b**–**d**) in order to obtain some insight into the structure–activity relationships (SAR). The similarity of thiazolidine-2,4-dione (**2**) to rhodanine (**1**) prompted us to synthesize analogues **6a**–**c.** The SAR were further exploited by the synthesis of compounds bearing the N-substituted rhodanine ring (**7b**–**d**) or thiobarbituric acid moiety (compound **9**).

Compounds **5b**–**d**, **6a**–**c** and **7b**–**d** were first tested for inhibition of MurE ligase; those compounds inhibiting MurE were further tested on the other Mur ligases. The results are presented in Table 2 as percent inhibition of the enzymes in the presence of 100 or 500 μM of the tested compound, or IC_{50} values for the most active compounds.

Some SAR can be deduced from structural analysis of the tested compounds and their inhibitory activities: 1) The most potent multitarget MurD–F inhibitors **5a** and **5d** bear a trihy-

Table 2. Inhibitory activities of compounds against Mur ligases.

Compd	Inhibition[a] [%] or IC_{50} [μM]			
	MurC	MurD	MurE	MurF
5a	52%	2 μM	6 μM	2 μM
5b	nt	nt	14%	nt
5c	nt	nt	11%	nt
5d	41%	8 μM	9 μM	4 μM
6a	56%	59%	3 μM	3 μM
6b	nt	nt	15%	nt
6c	nt	nt	14%	nt
7b	32%	21%	39 μM	14 μM
7c	nt	nt	0%	nt
7d	42%	46%	19 μM	6 μM
9	nt	12%	nt	20%
11	nt	10%	24%[b]	11%
12	nt	40%	33%[b]	38%

[a] Enzyme inhibition (%) in the presence of 100 μM concentration of test compound. nt, not tested. [b] Compound tested at 500 μM concentration.

droxyphenyl-substituted rhodanine moiety. The replacement of the rhodanine ring by the thiazolidine-2,4-dione (**6a**) or rhodanine-*N*-acetic acid (**7d**) moiety led to decreased inhibition of MurD, while MurE and MurF inhibition remained in the low micromolar range (IC_{50} = 3–19 μM). 2) In the case of MurE, compounds bearing the dihydroxyphenyl-substituted rhodanine (**5b**, **5c**) or thiazolidine-2,4-dione moiety (**6b**, **6c**) were devoid of inhibitory activity, while 2,4-dihydroxybenzylidenerhodanine-*N*-acetic acid (**7b**) inhibited MurE with an IC_{50} value of 39 μM.

Compounds **5a**, **6a** and **7d** were tested for their antibacterial activity against two Gram-negative (*E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two Gram-positive (*S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212) bacterial strains using the macrodilution method (Table 3) and

Table 3. MIC and MBC values of selected compounds against selected bacterial strains.[a]

Compd	MIC [μg mL^{-1}]			
	E. coli	*P. aeruginosa*	*E. faecalis*	*S. aureus*
5a	>128	128[b]	>128	>128
6a	>128	>128	>128	128
7d	>128	>128	128	128

[a] *E. coli* ATCC 25922; *P. aeruginosa* ATCC 27853; *E. faecalis* ATCC 29212; *S. aureus* ATCC 29213. [b] Minimal bactericidal concentration (MBC) for compound **5a** against *P. aeruginosa* ATCC 27853 was 128 μg mL^{-1}.

were found to be weak inhibitors of bacterial growth in vitro. According to the bacterial reverse mutation assay with *Salmonella typhimurium* (TA98 and TA100), compound **5a** can be considered as a bacterial nonmutagen (see the Experimental Section and table S1 in the Supporting Information for details). Compound **5a** was also not genotoxic in human hepatoma HepG2 cells at noncytotoxic concentrations (see the Experimental Section and table S2 in the Supporting Information for details).

The inhibition of multiple Mur ligases by thiazolidin-4-ones **5a**, **5d**, **6a**, **7b** and **7d**, together with their, albeit weak, antibacterial activity, stimulated us to investigate the mode-of-action and selectivity of these Mur inhibitors. Following our hypothesis of putative ATP-competitive binding of these Mur ligase inhibitors, we evaluated the selectivity of compounds **5a** and **6a** against a diverse panel of 76 protein kinases using the gold standard radioactive (^{33}P-ATP) filter-binding assay.[25] This revealed that both compounds are also inhibitors of some protein kinases (see table S3 in the Supporting Information for details).

Compounds **5a** and **6a** were also tested against D-alanine:D-alanine ligase (Ddl from *E. coli*), another ATP-dependent enzyme responsible for supplying MurF ligase with the substrate D-alanyl-D-alanine.[4] They were found to be inactive at 100 μM (inhibition = 19 and 13%, respectively). Different activities against various ATP-dependent enzymes, inactivity against Ddl and some protein kinases, and inhibition of MurD–F and several protein kinases, raised questions regarding the mechanism-of-action of these two compounds.

A literature survey suggested that 5-benzylidenerhodanines could act as substrates for reversible Michael-type 1,4-conjugative addition of nucleophilic cysteine residues of proteins.[15,26] However, such a mechanism is contradicted by the fact that dihydroxybenzylidene-substituted compounds **5b** and **5c**, analogous to trihydroxybenzylidene compound **5a**, were found to be inactive. Moreover, thiobarbituric acid derivative **9**, which could also act as Michael acceptor, was only very weakly active against MurD and MurF, with 12% and 20% inhibition at 100 μM. If these compounds act via the above mechanism, the inhibition of *E. coli* MurD, which was shown to possess a reactive cysteine in its active site,[27] would be stronger. In order to obtain further insight into the role of the exocyclic double bond, **5a** was reduced to give the racemic compound **12** (Scheme 3) that displayed some inhibition of MurD and MurF (inhibition = 38–40% at 100 μM), while inhibition of MurE was practically lost, even at higher concentrations of compound **12** (inhibition = 33% at 500 μM). We believe that the observed decrease in inhibitory potency of **12** as compared to that of **5a** is exclusively due to the flexibility of the molecule and to the loss of conjugation between the rhodanine and the phenyl ring.

2,3,4-Trihydroxyphenyl and 2,4,5-trihydroxyphenyl moieties have recently been recognized as functional groups that are not correlated with false-positive activity in high-throughput screening of chemical libraries against β-lactamase.[28] To check whether the presence of these functionalities might be the sole reason for the inhibition of Mur enzymes, we tested the susceptibility of MurD to pyrogallol (benzene-1,2,3-triol) and found that it was inactive at 100 μM concentration. The absence of potent inhibition of Mur enzymes by compounds **9** and **12**, which also possess the trihydroxyphenyl motif, further excludes inhibition due to the presence of the trihydroxyphenyl moiety alone. However, a weaker inhibition by **11** as compared to **12** lends support to the role of the phenol groups. Furthermore, the inactivity of (*Z*)-5-(ethoxymethylidene)rhodanine in the MurD inhibition assay (inhibition = 1% at 100 μM) also indicates that the rhodanine moiety alone cannot be responsible for inhibiting Mur enzymes.

Next we investigated the mode-of-action of compound **5a** with a steady-state kinetic study on MurD ligase. Kinetic analysis revealed that compound **5a** acts as a noncompetitive MurD inhibitor with respect to all three substrates, namely ATP, D-Glu and UDP-Mur*N*Ac-L-Ala (UMA), with K_i values of 2.2 ± 0.12 μM, 2.2 ± 0.10 μM and 1.8 ± 0.12 μM, respectively (Figure 2). Due to known promiscuity of the rhodanine scaffold,[30] to support the specific action of the inhibitors by structural data, an NMR study of the interactions of **5a** with MurD was performed. This study showed that **5a** mainly interacts with the residues flanking the UMA-binding site, while binding to the ATP-binding site or other parts of the protein was not observed. These conclusions are based on monitoring the ^{1}H/^{13}C chemical shift changes of MurD selectively labeled with ^{13}C at the methyl groups of Ile, Val, and Leu[31] upon binding of **5a**, UMA, adenylyl 5′-(β,γ-methylene)diphosphonate (AMPPCP), and 6-butoxy-

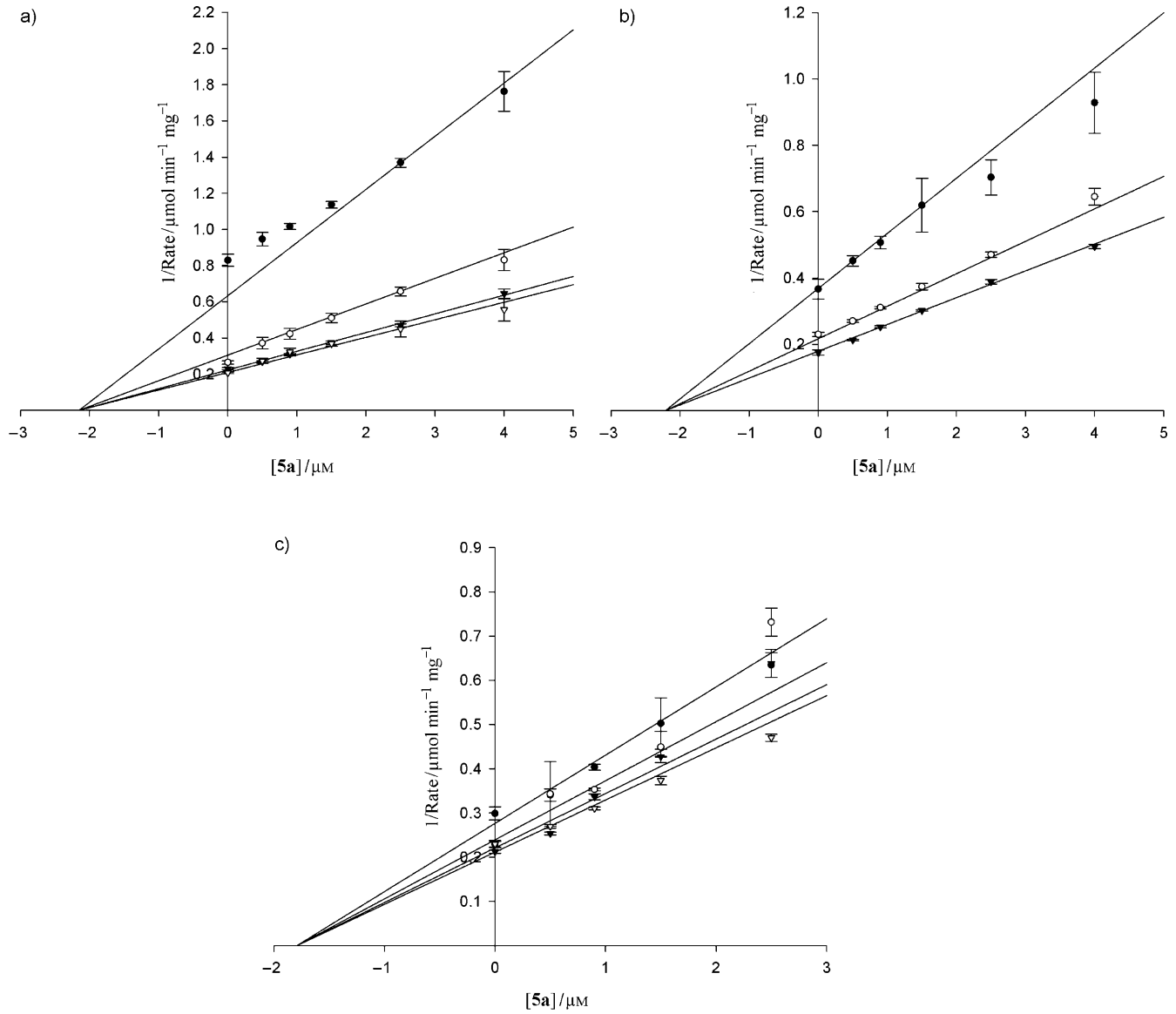


Figure 2. Kinetic analysis of MurD (*E. coli*) inhibition by compound **5a**. The reciprocal of the initial velocity was plotted according to Dixon vs the inhibitor concentration (compound **5a**) at different concentrations of one substrate and fixed concentrations of the other two: a) differing ATP (25 (●), 100 (○), 400 (▼), 800 μM (▽) ATP; 80 μM UMA; 100 μM D-Glu); b) differing D-Glu (25 (●), 100 (○), 400 μM (▼) D-Glu; 80 μM UMA, 400 μM ATP); c) differing UMA (10 (●), 20 (○), 40 (▼), 80 μM (▽) UMA; 100 μM D-Glu, 400 μM ATP). Concentrations of **5a** were 0, 0.5, 0.9, 1.5, 2.5 and 4.0 μM. Data were fitted to competitive, noncompetitive and uncompetitive inhibition models using SigmaPlot 11.0 software[29] and K_i values for the best fitted model were calculated.

naphthalene-*N*-sulfonyl-D-glutamic acid. The binding mode of several naphthalene-*N*-sulfonyl-D-Glu derivatives as novel inhibitors of MurD has recently been determined by X-ray and NMR.[10f–g,32] The binding interactions of ADP and UMA with MurD are known from the X-ray co-crystal structures.[5] Several pronounced effects on the MurD methyl chemical shifts are observed in $^{1}H/^{13}C$ HSQC spectra upon binding of these ligands (see figure S1 in the Supporting Information). The UMA binding has the most pronounced influence on MurD resonances. The effect of **5a** is more local and limited to the signals, which are also influenced by UMA binding. Apparently **5a** interacts with part of the UMA-binding site in a way that does not prevent the binding of UMA. Several resonances with pronounced chemical-shift perturbations induced by binding of **5a** are strongly influenced also by UMA binding, while the effect of 6-butoxy-naphtalene-*N*-sulfonyl-D-Glu is strikingly lower. This observation indicates that **5a** extends towards the uracil-binding pocket, which is occupied by the C6 substituents of naphthalene-*N*-sulfonyl-D-Glu derivatives.[10f,g,32] Due to highly conserved active sites of MurD–F, and similar structures of inhibitors **5a**, **5d**, **6a**, **7b** and **7d**, we hypothesize that these compounds bind to the same regions of MurD–F.

Conclusions

To conclude, the 5-(trihydroxybenzylidene)rhodanines **5a** and **5d** were shown to be inhibitors of multiple MurD–F ligases with well-balanced IC_{50} values in the low micromolar range.

The most potent compound **5a** inhibited MurD and MurF with IC_{50} values of 2 μM, and MurE with an IC_{50} value of 6 μM. In contrast, the N-substituted (*Z*)-5-(di-/tri-hydroxybenzylidene)rhodanines **7b** and **7d** strongly inhibited only MurE and MurF ligases. The molecular interactions of (*Z*)-5-(hydroxybenzylidene)rhodanines and (*Z*)-5-(hydroxybenzylidene)thiazolidine-2,4-diones with the MurD–F enzymes are currently the subject of further investigation using NMR and X-ray crystallography to obtain further insight into their inhibition mechanism. The observed inhibition of different Mur enzymes, involved in the biosynthesis of bacterial peptidoglycan, makes these compounds interesting leads in the search for multitarget antibacterial agents.

Experimental Section

Chemistry

General Methods: Chemicals were obtained from Acros, Sigma–Aldrich and Fluka and used without further purification. Analytical thin-layer chromatography (TLC) was performed using silica gel 60 F_{254} precoated plates (0.25 mm) from Merck (Germany). Flash column chromatography was carried out on silica gel 60 (particle size 0.040–0.063 mm; Merck, Germany). Melting points were determined on a Reichert hot-stage microscope and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE DPX_{300} spectrometer at 300 MHz and 75 MHz, respectively. Samples were prepared in $CDCl_3$ or [D_6]DMSO solution with TMS as the internal standard. IR spectra were recorded on a Perkin–Elmer 1600 FTIR spectrometer. Elemental analyses were performed on a Perkin–Elmer C, H, N analyzer 240 C and were within ±0.4% of the theoretical values. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. (*Z*)-5-(Ethoxymethylidene)rhodanine was synthesized as described previously.[33]

Microwave-assisted reactions were performed using a focused microwave reactor (Discover™, CEM Corporation, Matthews, USA). Reactions were performed in septum-sealed glass vials (10 mL) which enable high-pressure reaction conditions (20 bar). The temperature of the reaction mixture was monitored using a calibrated infrared temperature controller mounted under the reaction vessel. The maximum power used was 30 W and the pressure limit was set at 18 bar, unless otherwise stated.

General procedure for microwave-assisted synthesis of 5-benzylidenerhodanines (5a–d): A suspension of rhodanine (0.200 g, 1.50 mmol, 1.0 equiv) in dry EtOH (5 mL) was treated with aldehyde (1.50 mmol, 1.0 equiv), piperidine (0.150 mmol, 0.1 equiv) and glacial AcOH (0.150 mmol, 0.1 equiv). The reaction mixture was heated by microwave irradiation to 140 °C and the temperature maintained for 30 min. The reaction vessel was cooled in an ice bath; the precipitate was filtered off, washed with ice-cold EtOH and dried in vacuo.

(*Z*)-5-(2,3,4-Trihydroxybenzylidene)-2-thioxothiazolidin-4-one (5a): Recrystallization from MeOH gave **5a** as a brown crystalline solid (0.285 g, 71%): R_f = 0.31 (CH_2Cl_2/MeOH/CH_3COOH; 9:1:0.1); mp: > 300 °C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 13.54 (s, 1H, N*H*), 10.16 (s, 1H, O*H*), 9.57 (s, 1H, O*H*), 8.77 (s, 1H, O*H*), 7.84 (s, 1H, C*H*), 6.71 (d, *J* = 8.7 Hz, 1H, Ar$H_{(6)}$), 6.51 ppm (d, *J* = 8.7 Hz, 1H, Ar$H_{(5)}$); ^{13}C NMR (75 MHz, [D_6]DMSO): δ = 195.6, 169.4, 150.0, 147.8, 132.9, 128.4, 120.4, 119.1, 112.7, 108.3 ppm; IR (KBr): $\tilde{\nu}$ = 3552, 3409, 3088, 2841, 1695, 1621, 1570, 1509, 1445, 1391, 1326, 1301, 1257, 1196, 1070, 1045, 1014, 967, 776, 722, 691, 636, 562, 541, 477 cm^{-1}; MS (ESI+): *m/z* (%): 270 (70) [*M*+H]$^+$, 214 (100); Anal. calcd for $C_{10}H_7NO_4S_2$: C, 44.60; H, 2.62; N, 5.20; found: C, 44.70; H, 2.85; N, 5.10.

(*Z*)-5-(2,4-Dihydroxybenzylidene)-2-thioxothiazolidin-4-one (5b): Purification by column chromatography (CH_2Cl_2/CH_3OH; 20:1) gave compound **5b** as a brown crystalline solid (0.209 g, 55%): R_f = 0.38 (CH_2Cl_2/MeOH/CH_3COOH; 9:1:0.1); mp: > 300 °C (literature value,[34] 162–163 °C); ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 13.52 (br s, 1H, N*H*), 10.58 (s, 1H, O*H*), 10.27 (s, 1H, O*H*), 7.79 (s, 1H, C*H*), 7.15 (d, *J* = 9.3 Hz, 1H, Ar$H_{(6)}$), 6.43–6.40 ppm (m, 2H, Ar$H_{(3,5)}$); ^{13}C NMR (75 MHz, [D_6]DMSO): δ = 195.8, 170.0, 162.3, 159.8, 131.0, 127.8, 119.0, 111.9, 108.8, 102.5 ppm; IR (KBr): $\tilde{\nu}$ = 3413, 3067, 2845, 2364, 1690, 1637, 1617, 1560, 1516, 1466, 1335, 1272, 1187, 1110, 974, 837, 791, 754, 693, 623, 563, 513, 478 cm^{-1}; MS (EI): *m/z* (%): 253 (65) [*M*]$^+$, 166 (100); HRMS (ESI−): *m/z* [*M*−H]$^-$ calcd for $C_{10}H_6NO_3S_2$: 251.9789, found: 251.9792.

(*Z*)-5-(3,4-Dihydroxybenzylidene)-2-thioxothiazolidin-4-one (5c): Recrystallization from MeOH gave **5c** as a brown crystalline solid (0.229 g, 60%): R_f = 0.45 (CH_2Cl_2/MeOH/CH_3COOH; 9:1:0.1); mp: > 300 °C (literature value,[35] 270–280 °C); ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 13.66 (s, 1H, N*H*), 9.93 (s, 1H, O*H*), 9.51 (s, 1H, O*H*), 7.47 (s, 1H, C*H*), 7.02–6.98 (m, 2H, Ar$H_{(2,6)}$), 6.88 ppm (d, 1H, Ar$H_{(5)}$); ^{13}C NMR (75 MHz, [D_6]DMSO): δ = 195.4, 169.4, 149.1, 146.0, 132.8, 124.8, 124.3, 120.6, 116.6, 116.4 ppm; IR (KBr): $\tilde{\nu}$ = 3445, 2054, 1677, 1640, 1611, 1584, 1527, 1435, 1288, 1259, 1166, 1117, 968, 906, 844, 758, 615, 556, 510, 484 cm^{-1}; MS (ESI+): *m/z* (%): 254 (100) [*M*+H]$^+$; HRMS (ESI−): *m/z* [*M*−H]$^-$ calcd for $C_{10}H_6NO_3S_2$: 251.9789, found: 251.9796.

(*Z*)-5-(2,4,5-Trihydroxybenzylidene)-2-thioxothiazolidin-4-one (5d): Recrystallization from MeOH gave **5d** as a red crystalline solid (0.080 g, 20%): R_f = 0.23 (CH_2Cl_2/CH_3OH; 9:1); mp: 270–272 °C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 13.45 (s, 1H, N*H*), 10.01 (d, 2H, *J* = 5.1 Hz, O*H*), 8.93 (s, 1H, O*H*), 7.80 (s, 1H, C*H*), 6.72 (s, 1H, Ar$H_{(6)}$), 6.43 ppm (s, 1H, Ar$H_{(3)}$); ^{13}C NMR (75 MHz, [D_6]DMSO): δ = 194.8, 169.0, 152.8, 151.0, 138.9, 127.3, 117.1, 113.2, 109.9, 102.8 ppm; IR (KBr): $\tilde{\nu}$ = 3451, 3206, 1684, 1578, 1533, 1465, 1441, 1400, 1360, 1326, 1294, 1242, 1169, 1132, 1065, 930, 902, 853, 747, 684, 630, 557, 511 cm^{-1}; MS (ESI−): *m/z* (%): 268 (83) [*M*−H]$^-$, 209 (100); HRMS (ESI−): *m/z* [*M*−H]$^-$ calcd for $C_{10}H_6NO_4S_2$: 267.9738, found: 267.9737.

General procedure for microwave-assisted synthesis of 5-benzylidenethiazolidine-2,4-diones (6a–c): A suspension of thiazolidine-2,4-dione (0.200 g, 1.71 mmol, 1.0 equiv) in dry EtOH (5 mL) was treated with aldehyde (1.71 mmol, 1.0 equiv), piperidine (0.171 mmol, 0.1 equiv) and glacial AcOH (0.171 mmol, 0.1 equiv). The reaction mixture was heated by microwave irradiation to 140 °C and the temperature maintained for 30 min. The reaction vessel was cooled in an ice bath; the precipitate was filtered off, washed with ice-cold EtOH and dried in vacuo.

(*Z*)-5-(2,3,4-Trihydroxybenzylidene)thiazolidine-2,4-dione (6a): Recrystallization from MeOH gave **6a** as a brown crystalline solid (0.344 g, 74%): R_f = 0.36 (CH_2Cl_2/MeOH/CH_3COOH; 9:1:0.1); mp: > 300 °C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 12.31 (s, 1H, N*H*), 10.03 (s, 1H, O*H*), 8.74 (s, 1H, O*H*), 8.40 (s, 1H, -O*H*), 8.01 (s, 1H, C*H*), 6.73 (d, *J* = 8.7 Hz, 1H, Ar$H_{(6)}$), 6.48 ppm (d, *J* = 8.7 Hz,1H, Ar$H_{(5)}$); ^{13}C NMR (75 MHz, [D_6]DMSO): δ = 168.3, 167.7, 149.1, 147.3, 132.8, 127.5, 119.0, 117.3, 112.6, 107.8 ppm; IR (KBr): $\tilde{\nu}$ = 3436, 3050, 2789, 2043, 1775, 1729, 1673, 1640, 1624, 1590, 1511, 1481, 1384, 1347, 1311, 1256, 1159, 1052, 1023, 965, 910, 790, 692, 637, 615, 549, 498, 481 cm^{-1}; MS (ESI+): *m/z* (%): 254 (100) [*M*+H]$^+$; HRMS

(ESI−): *m/z* $[M-H]^-$ calcd for $C_{10}H_6NO_5S$: 251.9967, found: 251.9964.

(*Z*)-5-(2,4-Dihydroxybenzylidene)thiazolidine-2,4-dione (6b): Purification by column chromatography (CH_2Cl_2/CH_3OH; 20:1) gave compound **6b** as a brown crystalline solid (0.280 g, 69%): $R_f = 0.50$ (CH_2Cl_2/MeOH/CH_3COOH; 7:1:0.1); mp: 276–278 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 12.30$ (s, 1H, N*H*), 10.42 (s, 1H, O*H*), 10.14 (s, 1H, O*H*), 7.98 (s, 1H, C*H*), 7.17 (d, $J = 8.1$ Hz, 1H, Ar$H_{(6)}$), 6.40 ppm (m, 2H, Ar$H_{(3,5)}$); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 167.9$, 167.2, 161.2, 158.9, 129.4, 127.0, 116.4, 111.2, 107.8, 102.1 ppm; IR (KBr): $\tilde{\nu} = 3418$, 2060, 1638, 1465, 1343, 1323, 1272, 1159, 1099, 1029, 844, 805, 697, 627, 482 cm^{-1}; MS (EI): *m/z* (%): 237 (70) $[M]^+$, 166 (100); HRMS (ESI−): *m/z* $[M-H]^-$ calcd for $C_{10}H_6NO_4S$: 236.0018, found: 236.0020.

(*Z*)-5-(3,4-Dihydroxybenzylidene)thiazolidine-2,4-dione (6c): Recrystallization from MeOH gave **6c** as a brown crystalline solid (0.265 g, 66%): $R_f = 0.57$ (CH_2Cl_2/MeOH/CH_3COOH; 7:1:0.1); mp: >330 °C (literature value,[36] 266–268 °C); ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 12.41$ (s, 1H, N*H*), 9.81 (s, 1H, O*H*), 9.43 (s, 1H, O*H*), 7.61 (s, 1H, C*H*), 7.00–6.95 (m, 2H, Ar$H_{(2,6)}$), 6.87 ppm (d, $J = 8.1$ Hz, 1H, Ar$H_{(5)}$); ^{13}C (75 MHz, [D$_6$]DMSO): $\delta = 167.8$, 167.2, 148.3, 145.5, 132.4, 124.0, 123.6, 118.4, 116.1, 116.0 ppm; IR (KBr): $\tilde{\nu} = 3491$, 3257, 3048, 2783, 1734, 1664, 1589, 1515, 1451, 1379, 1332, 1314, 1276, 1178, 1153, 1111, 1029, 963, 919, 859, 799, 778, 738, 695, 630, 611, 510 cm^{-1}; MS (ESI+): *m/z* (%): 238 (100) $[M+H]^+$; HRMS (ESI−): *m/z* $[M-H]^-$ calcd for $C_{10}H_6NO_4S$: 236.0018, found: 236.0016.

General procedure for microwave-assisted synthesis of 5-benzylidenerhodanine-3-acetic acids (7b–d): A suspension of rhodanine-3-acetic acid (0.200 g, 1.05 mmol, 1.0 equiv) in dry EtOH (5 mL) was treated with aldehyde (1.05 mmol, 1.0 equiv), piperidine (0.105 mmol, 0.1 equiv) and glacial AcOH (0.105 mmol, 0.1 equiv). The reaction mixture was heated by microwave irradiation to 110 °C and the temperature maintained for 40 min. The solvent was evaporated in vacuo and the residue purified by column chromatography (CH_2Cl_2/CH_3OH; 9:1).

(*Z*)-2-(5-(2,4-Dihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid (7b): Brown crystalline solid (0.027 g, 8%): $R_f = 0.10$ (CH_2Cl_2/MeOH/CH_3COOH; 7:1:0.1); mp: 215–217 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 13.29$ (s, 1H, COO*H*), 10.80 (s, 1H, O*H*), 10.46 (s, 1H, O*H*), 8.00 (s, 1H, C*H*), 7.24 (d, $J = 8.6$ Hz, 1H, Ar$H_{(6)}$), 6.45 (d, $J = 7.8$ Hz, 2H, Ar$H_{(3,5)}$), 4.71 ppm (s, 2H, CH_2); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 193.1$, 167.0, 166.3, 162.6, 159.7, 131.4, 129.9, 114.6, 111.5, 108.7, 102.1, 44.6 ppm; IR (KBr): $\tilde{\nu} = 3220$, 1724, 1679, 1614, 1579, 1514, 1467, 1438, 1398, 1322, 1286, 1252, 1191.95, 1136, 1094, 1055, 959, 853, 785, 748, 682, 636, 614, 550, 495 cm^{-1}; MS (ESI−): *m/z* (%): 310 (100) $[M-H]^-$; HRMS (ESI−): *m/z* $[M-H]^-$ calcd for $C_{12}H_8NO_5S_2$: 309.9844, found: 309.9852.

(*Z*)-2-(5-(3,4-Dihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid (7c): Pale yellow crystalline solid (0.037 g, 12%): $R_f = 0.26$ (CH_2Cl_2/MeOH/CH_3COOH; 9:1:0.1); mp: 217–219 °C (literature value,[37] 328–329 °C); ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 7.64$ (s, 1H, C*H*), 7.05–7.00 (m, 2H, Ar$H_{(2,6)}$), 6.89 (d, 1H, $J = 8.0$ Hz, Ar$H_{(5)}$), 4.41 ppm (s, 2H, CH_2)—O*H* and COO*H* signals were not seen; ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 192.7$, 166.9, 166.5, 149.4, 145.9, 133.5, 123.9, 117.1, 116.5, 116,1, 43.1, 21.9 ppm; IR (KBr): $\tilde{\nu} = 3430$, 3200, 2509, 1696, 1563, 1452, 1407, 1365, 1304, 1283, 1203, 1102, 1051, 922, 862, 798, 671, 632, 609, 526 cm^{-1}; MS (ESI−): *m/z* (%): 310 (45) $[M-H]^-$, 165 (100); HRMS (ESI−): *m/z* $[M-H]^-$ calcd for $C_{12}H_8NO_5S_2$: 309.9844, found: 309.9850.

(*Z*)-2-(5-(2,4,5-Trihydroxybenzylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid (7d): Orange crystalline solid (0.028 g, 8%): $R_f = 0.08$ (CH_2Cl_2/MeOH/CH_3COOH; 9:1:0.1); mp: 260–262 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 13.34$ (s, 1H, COO*H*), 10.16 (s, 1H, O*H*), 8.99 (s, 1H, O*H*), 7.99 (s, 1H, C*H*), 6.79 (s, 1H, Ar$H_{(6)}$), 6.46 (s, 1H, Ar$H_{(3)}$), 4.72 ppm (s, 2H, CH_2)—O*H* signal was not seen; ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 192.4$, 166.8, 165.9, 153.3, 151.7, 139.1, 129.4, 113.3, 113.1, 109.9, 102.8, 44.3 ppm; IR (KBr): $\tilde{\nu} = 3247$, 1719, 1657, 1608, 1570, 1531, 1459, 1396, 1342, 1307, 1244, 1190, 1106, 1090, 1065, 989, 890, 850, 743, 599, 515 cm^{-1}; MS (ESI−): *m/z* (%): 326 (38) $[M-H]^-$, 209 (100); HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{12}H_{10}NO_6S_2$: 327.9950, found: 327.9950.

2-Thioxo-5-(2,3,4-trihydroxybenzylidene)dihydropyrimidine-4,6(1*H*,5*H*)-dione (9): A solution of 2-thiobarbituric acid (0.100 g, 0.649 mmol) and 2,3,4-trihydroxybenzaldehyde (0.094 g, 0.649 mmol) in H_2O (15 mL) was heated at reflux overnight. The precipitate was filtered, washed with H_2O and Et_2O and dried (Na_2SO_4) to give **9** as a red-brown solid (0.110 g, 61%): $R_f = 0.10$ (CH_2Cl_2/MeOH/CH_3COOH; 9:1:0.1); mp: >300 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 12.18$–12.09 (m, 2H, N*H*), 10.77 (s, 1H, O*H*), 9.99 (s, 1H, O*H*), 8.84 (s, 1H, C*H*), 8.79 (s, 1H, O*H*), 8.42 (d, $J = 9.2$ Hz, 1H, Ar$H_{(6)}$), 6.43 ppm (d, $J = 9.2$ Hz, 1H, Ar$H_{(5)}$); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 208.7$, 204.1, 189.1, 174.6, 173.2, 164.9, 138.1, 132.6, 116.1, 112.1, 100.4 ppm;; IR (KBr): $\tilde{\nu} = 3359$, 1654, 1506, 1398, 1308, 1257, 1191, 1145, 1050, 967, 804, 788, 754, 718, 568, 517, 496, 471 cm^{-1}; MS (ESI−): *m/z* (%): 279 (83) $[M-H]^-$, 309 (100) $[M+K]^+$; Anal. calcd for $C_{11}H_8N_2O_5S{\cdot}H_2O$: C, 44.29; H, 3.38; N, 9.39; found: C, 44.48; H, 3.36; N, 9.39.

(*Z*)-4-((4-Oxo-2-thioxothiazolidin-5-ylidene)methyl)benzene-1,2,3-triyl triacetate (10): A stirred suspension of compound **5a** (1.60 g, 5.96 mmol) and K_2CO_3 (3.29 g, 23.8 mmol) in Et_2O (60 mL) was cooled to 0 °C and treated dropwise with Ac_2O (8.45 mL, 89.3 mmol). After stirring at RT for 15 h, EtOAc (50 mL) and H_2O (50 mL) were added to the reaction mixture. The combined organic extracts were washed successively by H_2O (2×40 mL), saturated aq $NaHCO_3$ (2×20 mL) and brine (2×20 mL), then dried (Na_2SO_4), filtered and concentrated in vacuo to give compound **10** as a yellow solid (2.11 g, 89.6%): $R_f = 0.39$ (CH_2Cl_2/CH_3OH; 20:1); mp: >300 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 13.92$ (br s, 1H, N*H*), 7.50 (d, $J = 8.7$ Hz, 1H, Ar$H_{(6)}$), 7.44 (d, 1H, $J = 8.7$ Hz, Ar$H_{(5)}$), 7.39 (s, 1H, C*H*),2.41 (s, 3H, CH_3), 2.34 (s, 3H, CH_3), 2.31 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 192.2$, 167.9, 167.4, 167.3, 166.5, 145.3, 143.6, 135.8, 128.0, 125.6, 125.3, 125.1, 121.5, 20.7, 20.3, 20.1 ppm; IR (KBr): $\tilde{\nu} = 3418$, 3170, 3078, 2840, 2056, 1789, 1768, 1704, 1637, 1592, 1490, 1446, 1432, 1369, 1335, 1274, 1223, 1190, 1100, 1063, 1022, 897, 850, 798, 776, 678, 664, 644, 598, 585, 550, 521, 506, 458 cm^{-1}; MS (ESI−): *m/z* (%): 394 (100) $[M-H]^-$; HRMS (ESI−): *m/z* $[M-H]^-$ calcd for $C_{16}H_{12}NO_7S_2$: 394.0055, found: 394.0045.

4-((4-Oxo-2-thioxothiazolidin-5-yl)methyl)benzene-1,2,3-triyl triacetate (11): A stirred suspension of **10** (0.513 g, 1.30 mmol) in toluene (50 mL) was treated with diethyl 2,6-dimethyl-1,4-dihydro-3,5-pyridinedicarboxylate (0.427 g, 1.69 mmol) and silica gel 60 (1.3 g, 1 g mm^{-1}ol), previously activated by heating at 120 °C for 5 h. The mixture was heated to 100 °C for 24 h in the dark under Ar. The reaction mixture was cooled and filtered. The filter cake was rinsed with EtOAc. The combined filtrate and rinse were concentrated to dryness. The residue was redissolved in EtOAc (30 mL) and washed with aq HCl (1 M, 3×30 mL) and brine (30 mL). The organic phase was dried (Na_2SO_4), filtered and concentrated in vacuo. Purification by column chromatography (CH_2Cl_2/CH_3OH; 20:1) gave compound **11** as a yellow solid (0.286 g, 57.0%): $R_f = 0.41$ (CH_2Cl_2/CH_3OH;

20:1); mp: 145–146 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 13.25 (br s, 1H, N*H*), 7.28 (d, J = 8.6 Hz, 1H, Ar$H_{(6)}$), 7.21 (d, J = 8.6 Hz, 1H, Ar$H_{(5)}$), 4.96 (dd, J = 5.0 Hz, J = 9.2 Hz, 1H, SC*H*CO), 3.27–3.13 (m, 2H, CH_2CH), 2.34 (s, 3H, CH_3), 2.29 (s, 3H, CH_3), 2.26 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, CDCl$_3$): δ = 199.0, 175.6, 167.4, 167.1, 166.2, 142.5, 141.6, 134.8, 127.1, 126.6, 120.6, 54.2, 33.0, 20.1, 19.8, 19.6 ppm; IR (KBr): $\tilde{\nu}$ = 3430, 2935, 2867, 2081, 1779, 1759, 1637, 1496, 1449, 1372, 1284, 1194, 1106, 1080, 1039, 1015, 968, 923, 864, 815, 769, 724, 675, 659, 579, 535, 524 cm^{-1}; MS (ESI+): *m/z* (%): 398 (72) [*M*+H]$^+$, 420 (100) [*M*+Na]$^+$; Anal. calcd for $C_{16}H_{15}NO_7S_2$: C, 48.35; H, 3.80; N, 3.52; found: C, 48.20; H, 3.93; N, 3.53.

2-Thioxo-5-(2,3,4-trihydroxybenzyl)thiazolidin-4-one (12): A solution of **11** (0.118 g, 0.297 mmol) and N_2H_4·H_2O (0.028 g, 0.892 mmol) in CH_3CN (5 mL) was stirred at RT for 20 min. The reaction mixture was neutralized with glacial AcOH and the solvent evaporated in vacuo. The residue was dissolved in EtOAc and washed successively with H_2O (2×10 mL) and brine (10 mL). The organic phase was dried (Na_2SO_4), filtered and concentrated in vacuo. Purification by flash column chromatography (CH_2Cl_2/CH_3OH/AcOH; 90:10:1) gave compound **12** as a yellow solid (0.081 g, 73.2%): R_f = 0.33 (CH_2Cl_2/MeOH/CH_3COOH; 9:1:0.1); mp: 208–210 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 13.08 (br s, 1H, N*H*), 9.01 (s, 1H, O*H*), 8.46 (s, 1H, O*H*), 8.29 (s, 1H, O*H*), 6.36 (d, J = 8.2 Hz, 1H, Ar$H_{(6)}$), 6.22 (d, J = 8.2 Hz, 1H, Ar$H_{(5)}$), 4.89 (dd, J = 4.5 Hz, J = 10.3 Hz,1H, SC*H*CO), 3.41–3.33 ppm (m, 2H, CH_2CH, signal overlapped with residual H_2O); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 203.5, 177.9, 145.1, 144.2, 132.6, 119.3, 114.6, 106.0, 54.9, 31.9 ppm; IR (KBr): $\tilde{\nu}$ = 3460, 3103, 2903, 2849, 2094, 1719, 1636, 1540, 1514, 1449, 1388, 1310, 1293, 1227, 1189, 1153, 1107, 1080, 1035, 960, 905, 761, 728, 688, 621, 582, 503, 481, 458 cm^{-1}; MS (ESI+): *m/z* (%): 272 (60) [*M*+H]$^+$, 294 (65) [*M*+Na]$^+$, 252 (100); Anal. calcd for $C_{10}H_9NO_4S_2$: C, 44.27; H, 3.34; N, 5.16; found: C, 44.43; H, 3.59; N, 5.41.

Biology

Enzyme assays

The inhibition of Mur ligases and Ddl was determined using the malachite green assay, as previously reported.[11a,38] For compound **5a**, which showed inhibitory activity against MurD–F ligases, K_i values were determined against MurD from *E. coli*. K_i determinations were performed under similar conditions as described for the Mur ligases and Ddl inhibition assay: different concentrations of one substrate and fixed concentrations of the other two. First, the concentration of ATP (25, 100, 400 and 800 μM) was varied at fixed concentrations of UMA (80 μM) and D-Glu (100 μM), then the concentration of D-Glu (25, 100 and 400 μM) was modified at fixed concentrations of ATP (400 μM) and UMA (80 μM), and finally, the concentration of UMA (10, 20, 40 and 80 μM) was changed at fixed concentrations of ATP (400 μM) and D Glu (100 μM). The concentrations of **5a** used were 0, 0.5, 0.9, 1.5, 2.5 and 4.0 μM. After incubation for 15 min at 37 °C, the enzyme reaction was terminated by adding Biomol green™ reagent and the absorbance was read at 650 nm. Initial velocity data were fitted to competitive, noncompetitive and uncompetitive inhibition models using SigmaPlot 11.0 software[29] and K_i values were calculated for the best fitted model.

Antibacterial activity

The susceptibilities of four standard strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212) to compounds **5a**, **6a**, **6c**, **7d** and **9** were tested, using the macrodilution method. For the compounds that showed activity against *S. aureus*, susceptibility of a standard strain ATCC 43300 of methicillin-resistant *S. aureus* (MRSA) was additionally tested using the same method. The test compound (10 mg) was dissolved in 5 mL of DMSO to give a stock solution of 2 mg mL^{-1}. Working solutions were made by serially diluting stock solution in cation-adjusted Mueller–Hinton broth (CAMHB) as described by Amsterdam and Barry.[39,40] CAMHB (34 mL) was added to a 5 mL stock solution to give 256 μg mL^{-1} concentration and then passed through a sterilized filter. The compound was further serially diluted to give 14 dilutions down to the lowest concentration of 0.031 μg mL^{-1} and stored frozen for a maximum of two weeks.[39] Just prior to bacterial inoculation, 0.5 mL dilutions in the range of the compounds were pipetted into 13×100 mm screw-cap tubes.

The inoculum was prepared in such a way that four colonies of a fresh overnight culture on a nonselective agar plate were inoculated into saline. The turbidity was adjusted to match that of 0.5 McFarland standard (~10^8 CFU mL^{-1}). A portion of a standardized suspension was diluted (~1:1000; 10^5 CFU mL^{-1}), and a 0.5 mL aliquot of this dilution was then added to each tube containing 0.5 mL of the tested compound diluted in CAMHB within 30 min and incubated at 35 °C for 18–24 h. After inoculum was added, dilutions 0.016 to 128 μg mL^{-1} of the compound were achieved. Broth not containing any compound was inoculated as a growth control. If the compound inhibited bacterial growth, it was considered a potential antimicrobial agent. The lowest concentration of antimicrobial agent that resulted in complete inhibition of visible growth was the minimal inhibitory concentration (MIC). A sample from each tube that displayed no visible growth portion was plated to blood agar to determine the minimal bactericidal concentration (MBC). Quality control of the methods was done by testing *S. aureus* ATCC 29213 and gentamycin. Dilutions of antibiotic were made in the same way as for tested compounds and the MIC values obtained were in the range proposed by the Clinical Laboratory Standards Institute.[41]

Mutagenicity and genotoxicity of compound 5a

Bacterial strains and human hepatoma HepG2 cells: *Salmonella typhimurium* strain TA98, which detects frame-shift mutations, and TA100, which detects base-pair substitution mutations, were obtained from Professor B. N. Ames (University of California, Berkeley, USA). The strains were kept at −80 °C and were checked for their histidine/biotin dependence, rfa marker (crystal violet), uvrB deletion (UV sensitivity), and the presence of the plasmid pKM101 (ampicillin resistance). The HepG2 cell line was obtained from the ECACC (European Collection of Cell Culture, UK). The cells were grown in monolayer culture in EMEM medium supplemented with 4 mM L-glutamine, 1% nonessential amino acids and 15% FBS in a humidified atmosphere of 5% CO_2 at 37 °C.

Ames test

Mutagenicity of **5a** was tested using the *S. typhimurium* reverse mutation assay (Ames test) with strains TA98 and TA100, with and without metabolic activation as described by Maron and Ames.[42] 0.1 mL of diluted **5a** solution (in DMSO, vehicle or positive control), 0.1 mL of overnight grown bacterial culture and 0.5 mL of S9 mix (containing 4% S9—Arachlor-induced rat liver microsomal fraction; Moltox, USA) or phosphate-buffer solution (for treatment without activation) were added to 2 mL of molten top agar containing a limited amount of histidine/biotin at 45 °C, gently mixed and

poured onto Vogel–Bonner minimal glucose-agar plates. His^+ revertants were counted after 48 h incubation at 37 °C. The background lawn was inspected for signs of toxicity or compound precipitation. All experiments were carried out in triplicate using three concentrations (0.019, 0.095 and 0.476 mM per plate) of **5a**. The highest tested concentration was selected based on previously determined MIC values against *P. aeruginosa* and *S. aureus*. Benzo[*a*]pyrene (B[*a*]P, 10 μg per plate) and 4-nitroquinoline oxide (4-NQNO, 0.5 μg per plate) were used as positive controls in tests with and without metabolic activation, respectively.

Cytotoxicity assay

The cytotoxicity of compound **5a** was measured using the MTT reduction assay.[43] The HepG2 cells were seeded onto 96-well microplates at a density of 10^4 cells well^{-1} and incubated for 24 h at 37 °C to attach. The medium was then replaced with fresh complete medium containing graded concentrations of (0.000128–0.4 mM) **5a** and incubated for 24 h. MTT (final concentration = 0.5 mg mL^{-1}) was then added and the plates were incubated for an additional 3 h. At the end of the incubation with MTT, the medium was removed and the formazan crystals dissolved in DMSO. Optical density (OD) was measured at 570 nm (reference filter 690 nm) using a microplate reading spectrofluorometer (GENios™, Tecan, Männedorf, Switzerland). Viability was determined by comparing the OD of the wells containing the cells treated with **5a** with the vehicle-treated cells (DMSO, 0.1% *v/v*). The Student's t test was used to evaluate the statistical significance between exposed and control cells; $P < 0.05$ was considered significant.

Comet assay

The genotoxicity of compound **5a** was measured using single-cell gel electrophoresis (the comet assay), which is a very sensitive method for detecting single- and double-strand breaks, alkali-labile sites, DNA–DNA/DNA–protein crosslinks, and single-strand breaks associated with incomplete excision repair at the level of single cells.[44] HepG2 cells were seeded into 12-well tissue-culture-treated plates (Corning Costar Corporation, New York, USA) and left overnight at 37 °C in 5% CO_2 to attach. The medium was then replaced with fresh medium containing 0.00064, 0.0125 and 0.016 mM of **5a**, and 50 μM B[*a*]P as the positive control. Medium with DMSO (0.1% *v/v*) served as the vehicle control. DNA damage was determined with the comet assay after 24 h exposure to **5a** or B[*a*]P.

The comet assay was performed as described by Singh et al.[45] with minor modifications.[46] The slides were stained with ethidium bromide (5 mg mL^{-1}) and analyzed using a fluorescence microscope (Nikon, Eclipse 800) and image analysis software (Comet IV, Perceptive Instruments). Fifty nuclei were analyzed per experimental point in each of the three independent experiments. The statistical differences between treatments within each experiment were analyzed with one-way analysis of variance (Kruskal–Wallis test) followed by Dunn's post test, while a Student's t test was used to compare median values of the percentage of tail DNA and tail length in three independent experiments; $P < 0.05$ was considered significant.

NMR spectroscopy

The $^1H/^{13}C$ HSQC[47] spectra were recorded at 25 °C on a Varian DirectDrive 800 MHz spectrometer equipped with a Cryoprobe. The pulse sequence provided in the Varian BioPack library of pulse programs was used. NMR samples were prepared in a $D_2O/[D_6]$DMSO mixture (9:1, *v/v*) containing HEPES buffer (20 mM, pH 7.2), $(NH_4)_2SO_4$ (7 mM), $MgCl_2$ (3.5 mM), DTT (0.3 mM) and ATP (0.4 mM). The concentration of MurD selectively labeled with ^{13}C at the methyl groups of Ile (δ1 only), Val, and Leu was 0.07 mM. The protein was titrated by the ligands in MurD:ligand molar ratios of 0.5, 1, 2, 5, and 10. Spectra were acquired with 1024 data points in t_2, 32 scans, 64 complex points in t_1, and a relaxation delay of 1 s. The ^1H and ^{13}C sweep widths were 9470 and 3340 Hz, respectively. Spectra were processed and analyzed with Sparky software.[48] Spectra were zero-filled twice and apodized with a squared sine bell function shifted by $\pi/2$ in both dimensions, using linear prediction of the data in the incremented dimension. The combined chemical-shift perturbations $\Delta\delta$ were calculated from ^1H and ^{13}C chemical-shift changes using the equation: $\Delta\delta = (\Delta^1H + (0.252 \times \Delta^{13}C)^2)^{\frac{1}{2}}$.[49]

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