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Chemical, biochemical and microbiological properties of a brominated nitrovinylfuran with broad-spectrum antibacterial activity

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ABSTRACT

A di-bromo substituted nitrovinylfuran with reported broad-spectrum antibacterial activity was found to be a potent inhibitor of MurA, a key enzyme in peptidoglycan biosynthesis. Further characterization of the compound was carried out to assess its reactivity towards thiol nucleophiles, its stability and degradation under aqueous conditions, inhibitory potential at other enzymes, and antibacterial and cytotoxic activity. Our results indicate that the nitrovinylfuran derivative is reactive towards cysteine residues in proteins, as demonstrated by the irreversible inhibition of MurA and bacterial methionine aminopeptidase. Experiments with proteins and model thiols indicate that the compound forms covalent adducts with SH groups and induces intermolecular disulfide bonds, with the intermediate formation of a monobromide derivative. The parent molecule as well as most of its breakdown products are potent antibiotics with MIC values below 4 μg/mL and are active against multiresistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA). Further development of the bromonitrovinyl scaffold towards antibiotics with clinical relevance, however, requires optimization of the antibiotic–cytotoxic selectivity profile.

1. Introduction

Resistance of bacteria against antibiotics is an increasingly problematic clinical issue. The development of novel selective antibacterial agents as well as the discovery of novel antibacterial targets is therefore urgently and constantly required.[1,2] The cell wall is an essential and exclusive structural element of prokaryotes and therefore represents an ideal target for the discovery and development of novel antibiotics. Peptidoglycan—the macromolecular component of the bacterial cell wall—consists of linear glycan chains that are cross-linked by short peptides.[3] Penicillins and glycopeptides interfere with the final steps of cell wall synthesis. The earlier steps of the peptidoglycan biosynthetic pathway, however, provide further attractive targets for the development of antibacterial compounds. The cytoplasmic steps in peptidoglycan synthesis are catalyzed by a series of enzymes that form, in a sequence of reactions, the monomeric precursor UDP-*N*-acetylmuramic acid pentapeptide (Scheme 1).

Because all enzymes involved in this pathway are essential to the vital function of the prokaryotic cell, the inhibition of one or more of the 'Mur' enzymes has a lethal effect on bacteria. Approaches in targeting this multi-step pathway have been compiled by El Zoeiby et al.[4]

One inhibitor of the Mur-enzyme biosynthetic pathway has acquired clinical importance: The epoxide fosfomycin is a covalent and irreversible inhibitor of MurA, the enzyme that catalyzes the first biosynthetic step. Fosfomycin irreversibly alkylates a cysteine residue (Cys115 in *Escherichia coli* MurA) in the active site of the enzyme. This cysteine residue is of critical relevance for the catalytic activity of MurA.[5–8] Our own efforts in this field led to the identification of the sesquiterpene lactone cnicin as potent, non-covalent suicide inhibitor of MurA.[9] X-ray structure analysis revealed the formation of a covalent adduct between the substrate UNAG and cnicin. A similar binding mode may be relevant for some natural products from tulips that are structurally related to the cnicin side chain.[10]

Nitrovinylfuran derivatives have been known as antimicrobials for a long time.[11,12] Some of them were evaluated for clinical and industrial applications.[13–18] Nitrovinylfurans with exocyclic nitro function have been demonstrated to be non-mutagenic in contrast

Abbreviations: BSA, bovine serum albumine; EP-UNAG, enolpyruvyl-UDP-*N*-acetylglucosamine; GSH, glutathione; GSSG, oxidized glutathione; MH medium, Müller-Hinton medium; PEP, phosphoenolpyruvate; UNAG, UDP-*N*-acetyl glucosamine.

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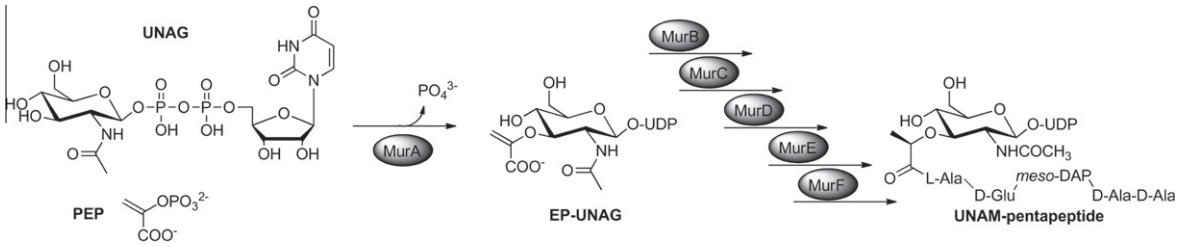
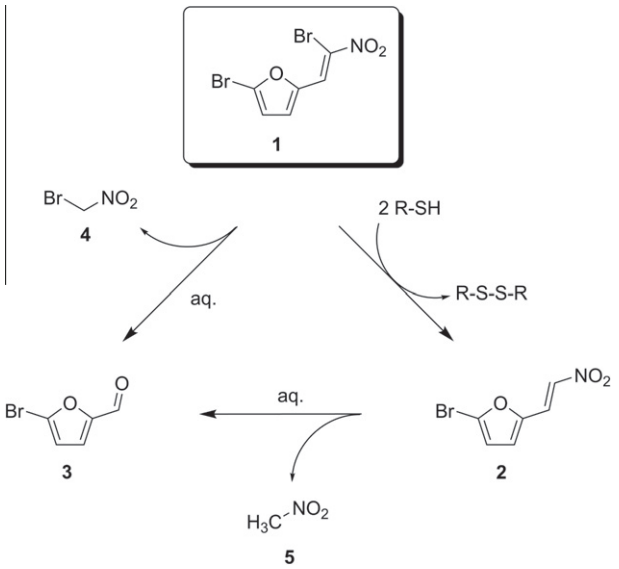


Scheme 1. The cytoplasmic steps in peptidoglycan biosynthesis are catalyzed by the enzymes MurA–F (Scheme for gram-negative bacteria).

to classical 5-nitrofurans.[11,19–29] Our interest in this class of compounds (Scheme 2) was spawned by the published reports on their broad-spectrum antibacterial and antifungal properties.[11,13,14,16,30–33] Related β-nitrostyrene derivatives are active against Gram-positive and Gram-negative bacteria.[34] The activity of β-nitrostyrenes against bacteria and fungi, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE), is currently still under investigation.[33,35,36]

Among the nitrovinylfurans, 2-bromo-5-(2-bromo-2-nitrovinyl)furan (compound **1**) has gained special attention. The biological properties of **1** have been described before.[13,16,30,31] The patent specification reports biological activity in vitro and in vivo, including broad-spectrum antibacterial and antifungal properties, and a phase 3 study of a topical preparation. Studies on the stability have also been documented.[37] Compound **1** has been described to be less genotoxic than 5-nitrofuran derivatives[21,25,26,38], though Ramos et al. report mutagenic effects.[39] The indication spectrum of nitrovinylfuran derivatives was extended to include leishmaniasis and trypanosomiasis.[40]

The antibacterial mechanism of action of **1** remains unclear. The inhibition of bioenergetic processes, such as glycolysis, due to chemical modification of functional thiol groups in enzymes has been proposed as the basis of the antimicrobial and cytotoxic activity of vinylfurans.[11,41–45] Inhibition of glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, malate dehydrogenase and glutathione reductase has been shown in vitro and the modification of enzyme thiol groups was shown with purified mammalian glyceraldehyde-3-phosphate dehydrogenase.[44,45] The addition of thiols to the exocyclic double bond of vinylfurans was demonstrated with model thiols.[41,45–48]



Scheme 2. The nitrovinylfuran **1**, its degradation pathways and breakdown products.

Given the sensitivity of MurA to alkylation of the catalytically relevant Cys115 residue, along with the antibacterial activity of the nitrovinylfurans and their propensity to bind to thiol groups, we decided to determine whether MurA could be a target of this compound class. In the course of these investigations, we also studied the stability of **1** in aqueous media, its reactivity towards model nucleophiles such as glutathione, and the corresponding degradation and reaction products.

2. Results and discussion

2.1. Bioactivity

2.1.1. Enzyme inhibition

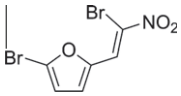
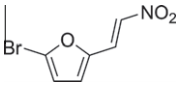
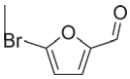
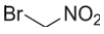
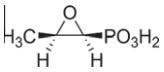
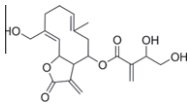
Inhibitory effects of **1** and its degradation products were evaluated for several MurA species, including *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (isoform 2). Table 1 comprises enzyme inhibition data, as well as the in vitro antibacterial data, expressed as minimum inhibitory concentrations (MIC). IC_{50} values of cytotoxicity against various cell lines complement the profiling of the compounds. Data related to the known *E. coli* MurA inhibitory agents fosfomycin and cnicin are given as references. Compound **1** is a potent inhibitor of the MurA enzymes, with an IC_{50} of 2.8 (±0.2) μM for the *E. coli* MurA enzyme (Fig. 1). Compound **1** is also a potent inhibitor of *E. coli* MetAP (methionine aminopeptidase), but not of the human MetAPs and the two serine proteases (thrombin, Dengue NS2B-NS3). The nitrovinyl moiety in **1** and several congeners could be responsible for a Michael-addition to nucleophilic structures in the target proteins. In *E. coli* MurA, the Cys115 residue is solvent-exposed in the unliganded state and is attacked by the epoxide electrophile of the antibiotic fosfomycin.[49] In *E. coli* MetAP, the active site also contains exposed cysteine residues.[50,51]

To study the reactivity of the compounds towards Cys115 in MurA, we compared the inhibitory effects on the C115D MurA mutant. This mutant is catalytically active but resistant to the antibacterial agent fosfomycin as it lacks the Cys115 nucleophile and is therefore not reactive towards electrophilic attack at this site.[7] The results (cf. Fig. 1) indicate a somewhat reduced but still significant inhibition of the mutant enzyme with an IC_{50} of 18.7 (±4.8) μM for **1**. Cys115 is therefore not the only target site. The different steepness of the dose–response curves of **1** for native and C115D MurA indicates a particular involvement of the geminal bromonitrovinyl moiety and Cys115 in the binding mode (Fig. 1). This effect is under further investigation.

The de-bromo derivative **2** was significantly less potent against all tested enzymes. Interestingly, the IC_{50} values of **2** at the various MurA enzymes, including the C115D mutant, are nearly identical. In contrast, inhibition by the parent compound **1** was less pronounced for the mutant lacking the active site thiolate.

The aldehyde **3** is inactive against the enzymes and has neither antibacterial nor cytotoxic activity, whereas bromonitromethane **4** shows significant inhibition of the MurA enzymes and antibacterial activity. The antibiotic effect of **4** is known[34,53–55] and its use as

Table 1
Enzymatic, antibacterial and cytotoxicity profile of compound **1** and its breakdown products

Compound	1 	2 	3 	4 	5 	Fosfomycin 	Cnicin 
IC_{50} (μM) on MurA species							
E. coli MurA	2.8 (±0.2)	27.3 (±4.4)	n.i.[a]	4.6 (±2.8)	n.i.	0.1[e] (±0.01)	16.7[e] (±0.7)
E. coli MurA C115D	18.7 (±4.8)	23.8 (±7.2)	n.i.	7.8 (±3.3)	n.i.	n.i.[e]	n.i. [e]
Ps. aerug. MurA	2.8 (±0.3)	20.3 (±1.9)	n.i.	4.0 (±0.5)	n.i.	10.5[e] (±0.5)	10.3[e] (±1.5)
S. aureus MurA2	3.9 (±0.3)	20.7 (±1.6)	n.i.	6.2 (±0.1)	n.i.	1.1 (±0.1)	20.4 (±1.1)
Other enzymes (% inh)[c]							
E. coli MetAP	85 (±3)	30 (±9)	n.i.	85 (±2)	n.i.	n.i.	n.i.
Hs MetAP 1	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Hs MetAP 2	13 (±4)	7 (±2)	n.i.	22 (±1)	n.i.	n.i.	9 (±1)
Thrombin	8 (±3)	19 (±5)	n.i.	n.i.	n.i.	n.i.	n.i.
Dengue NS2B-NS3	12 (±2)	26 (±3)	n.i.	n.i.	n.i.	n.i.	n.i.
MIC (μg/mL)							
Gram-positive							
MSSA (ATCC 25923)	4	2	>32	2	8	4	>32
MRSA (NCTC 10442)	4	2	>32	2	>32	4	>32
Gram-negative							
E. coli (ATCC 25922)	4	2	>32	1	32	2	>32
Ps. aerug. (ATCC 27853)	2	4	>32	0.5	8	1	>32
Cytotoxicity IC_{50} (μM)							
Non cancer cell lines							
HaCaT	33	15	⩾100	25	⩾100	⩾100	80
Flow	9	6	⩾100	14	⩾100	⩾100	82
Cancer cell lines							
A-431	24	12	⩾100	38	⩾100	⩾100	27
MeWo	43	21	⩾100	40	⩾100	⩾100	⩾100
Calu-6	10	5	⩾100	23	⩾100	⩾100	44
CaSki	50	18	⩾100	99	⩾100	⩾100	⩾100
Capan-1	36	19	⩾100	69	⩾100	⩾100	93
SW-707	47	22	⩾100	73	⩾100	⩾100	⩾100
$t_{1/2}$ (h)[d]							
Tris buffer, pH 7.8	0.5	58	n.d.[b]	n.d.	n.d.	n.d.	n.d.
Citrate buffer, pH 6	6	116	n.d.	n.d.	n.d.	n.d.	n.d.
GSH-reactivity							
GSH-adduct formation (addition)	+	+	–	–	n.d.	n.d.	n.d.
Oxidation to GSSG	+	–	–	+	n.d.	n.d.	n.d.

Enzyme concentrations were 12 nM for the native MurA and C115D mutant and *S. aureus* MurA2. *Ps. aeruginosa* MurA was assessed at 150 nM. Other enzyme concentrations were: MetAP enzymes 500 nM, Thrombin 10 nM, Dengue NS2B-NS3 100 nM. Compound concentration for determining percentage inhibition of investigated enzymes were as follows: MetAP assay 10 μM, Thrombin assay 25 μM and DV NS2B-NS3 assay 50 μM.

[a] No inhibition
[b] Not determined
[c] Percentage inhibition
[d] Half-life
[e] Bachelier et al. (2006).[52]

biocide has been patented,[13,56] but a mechanism of action has not been proposed yet. Considering the structure–activity relationships presented here, it appears that the bromonitromethyl moiety, either isolated or in conjunction with a vinylfuran structure, is essential for the antibacterial and MurA inhibition properties of this compound class.

In order to obtain information on the target-selectivity of these compounds, we screened their activity against other enzymes. No compound was active against the serine proteases thrombin and Dengue virus NS2B-NS3 and the two human methionine aminopeptidases MetAP I and II. In contrast, the methionine aminopeptidase from *E. coli* is highly sensitive towards **1** and **4**. Bacterial MetAPs are potential targets for antibacterial drug discovery. The bromonitromethyl moiety may therefore be considered as a fragment in the design of inhibitors of bacterial MetAPs.

2.1.1.1. Time-dependent enzyme inhibition. Reversible and irreversible binding modes can be distinguished by varying the pre-incubation period of inhibitor and enzyme. For a reversible inhibitor, the inhibitory potency is independent of the pre-incubation time, whereas irreversible inhibitors appear as increasingly potent if the incubation time is prolonged.

For compound **1** the enzyme inhibition increased with preincubation time, in analogy to the irreversible MurA inhibitor fosfomycin (Fig. 2), indicating an irreversible mode of action.

2.1.1.2. UNAG dependency. Inhibitor binding to MurA often depends on the presence of the substrate UNAG.[52,57] UNAG triggers a conformational change towards a 'closed' conformation of MurA.[58] Thus, augmented inhibitory potency in the presence of UNAG indicates that the inhibitor binds to the 'closed'

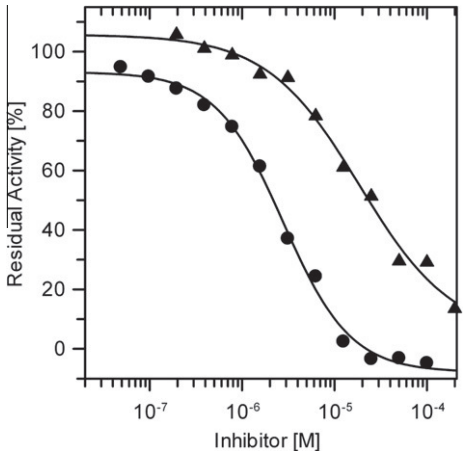


Figure 1. Dose–response curves for native *E. coli* MurA (circle) and C115D mutant (triangle) upon incubation with **1**.

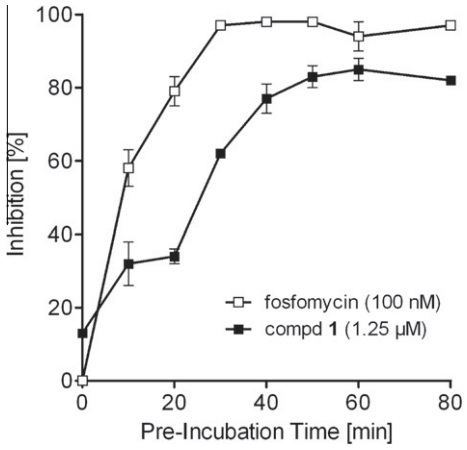


Figure 2. Time-dependent inhibition of *E. coli* MurA by **1** and fosfomycin as reference inhibitor.

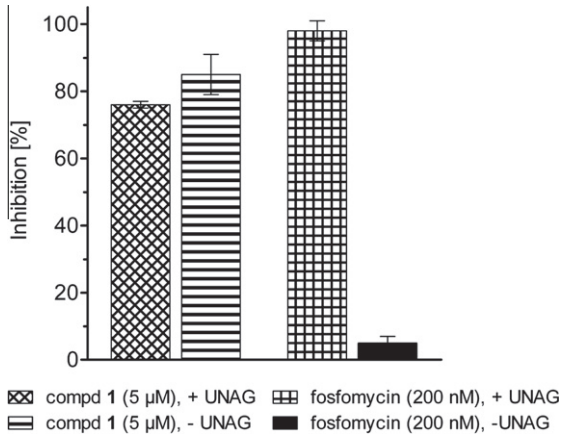


Figure 3. The inhibitory effect of fosfomycin, but not of compound **1** depends on the presence of UNAG.

conformation. Additionally, MurA isolated from *E. coli* contains PEP bound covalently to Cys115,[59] which can be liberated from the enzyme by addition of UNAG, leading to the formation of the reaction product EP-UNAG. Thus, if the binding site of an inhibitor overlaps with the PEP binding site, the effect of UNAG would be to liberate PEP, thereby opening access for the inhibitory compound.

The binding of fosfomycin is clearly, and absolutely, dependent on the presence of UNAG. This effect is not present for **1**. Figure 3 shows the results of an experiment where fosfomycin and **1** were pre-incubated with MurA in the absence and presence of UNAG. Whereas fosfomycin remains inactive in absence of substrate UNAG, compound **1** binds in a way that is independent of UNAG. These results show that **1** does not require the UNAG-triggered closure of the MurA active site for binding.

2.1.2. Antimicrobial activity

Encouraged by the enzyme inhibition data, the antibacterial properties of the compounds against selected strains of Gram-positive and Gram-negative bacteria were determined (Table 1). Compounds that exerted inhibitory activity on the isolated MurA enzymes, for example, **1**, **2** and **4** had antibacterial MIC (minimal inhibitory concentration) values of 4 μg/mL or less. Interestingly, a clinical isolate with highly problematic resistance profile (methicillin-resistant *Staphylococcus aureus*, MRSA) was equally sensitive towards these agents, indicating that the compounds were not inactivated by the resistance mechanisms of that strain. Antibacterial efficacy of **1** against resistant strains was already noted and hence an orthogonal mechanism of action was presumed, although no detailed analysis of the phenomenon was made.[31] Antibacterial potency was also striking for **2**, although it was considerably less potent than **1** as MurA inhibitor. This clearly indicates that MurA is not the only target of the nitrovinylfuran derivatives. Results of our examination are in good accordance with the antibacterial susceptibilities already reported elsewhere.[16,31] The antibacterial activity of the decomposition products **3** and **5** was not significant. Thus, the bromonitromethyl element is essential for antibacterial activity and enzyme inhibition.

2.1.3. Cytotoxicity

Profiling of the compounds under study was completed by cytotoxicity data on several cancer and non-cancer cell lines. All compounds which were active on the enzymatic and bacterial level also displayed cytotoxic effects with no significant selectivity towards tumorigenic cell lines (Table 1). The cytotoxic effects of **1** and **2** was more pronounced than that of the halo-nitro compound **4**. For **3** and **5** no cytotoxic properties were observed, in analogy to the lack of activity in enzymatic or bacterial tests. Interestingly, the cytotoxic action of the de-bromo compound **2** was greater than for the parent molecule **1** on all investigated cell lines. Cytotoxic effects of **2** on cultured human lymphocytes have been reported at 15 μg/mL and the reduction of cytotoxic events due to exogenous metabolic inactivation by S9 fraction proteins was demonstrated.[23] In a study using a human lymphoblastoid cell line viability was >70% at the highest tested dose which was 1 μg/mL for **1** (3.4 μM) and 10 μg/mL for **2** (46 μM).[27] Recent investigations demonstrate a low cytotoxicity for **1** in mouse spleen macrophages (cytotoxicity 27% at 10 μg/mL resp. 34 μM).[60] In a comparative study, the nitrostyrene scaffold was demonstrated to be twofold more cytotoxic than unsubstituted nitrovinylfuran.[61] In contrast, the reference compounds fosfomycin and cnicin did not display cytotoxic effects at the highest concentration tested. Nitrofurantoin, as classical nitrofuran derivative with the nitro group attached to the furan ring, did not exhibit cytotoxic effects on the investigated cell lines at the highest dose (data not shown) in accordance with the documented need for metabolic activation.[62–64] The therapeutic window between antibiotic and cytotoxic effects is limited for all tested compounds. There is no selectivity between cancerous and non-cancerous cell lines.

2.2. Compound stability and reactivity

2.2.1. Nitrovinylfuran derivatives decompose to aldehydes in aqueous media

The instability of **1** in aqueous media has been reported before. The decomposition was studied by TLC and UV spectroscopy and the monobromide derivative 2-bromo-5-(2-nitrovinyl)furan **2** and 5-bromofurfural **3** were the reported degradation products.[30,37]

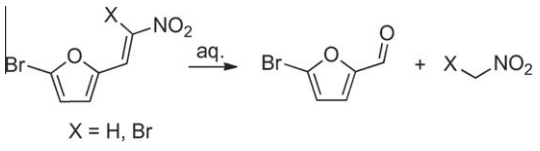
We further investigated the stability of parent molecule **1** in aqueous solution by HPLC and calculated half-lives of the compounds (Table 1). This confirmed the formation of **3** out of **1** with concomitant release of bromonitromethane **4**, but the formation of **2** could not be verified. Reference chromatograms of commercially available furan-aldehydes showed that all studied nitrovinyl derivatives decompose to the corresponding aldehyde under aqueous conditions (Scheme 3).

In all cases, the decrease of the starting material follows a first order reaction rate law with logarithm of concentration plotted versus time resulting in a linear slope. The half-lives can be calculated from this slope. The geminal bromonitro derivative show much shorter half-lives than the corresponding nitrovinyl compounds, for example, 29 min for **1**, but 58 h for **2**.

For compounds **1** and **2**, the degradation was also studied at pH 6 and in Müller-Hinton (MH) medium, pH 7.2 (data not shown). At pH 6, all compounds show longer half-lives compared to pH 7.8 (e.g., 6 h for **1**). In MH medium, we observed a time-dependent disappearance of the starting materials, but were unable to detect defined degradation products. This is probably due to a reaction of either the starting materials or the released derivatives with macromolecular constituents of the MH medium. Compared to Tris buffer at pH 7.8, the disappearance of the starting material in MH medium is much faster (e.g., 8 min for **1**).

The half-lives for **1** lie within the incubation periods of the performed bioassays. This means—apart from any possible influence of the enzyme—that a large quantity of the molecules decomposes within the assay time span to give the respective furaldehyde. However, this decomposition product has been shown to be inactive against the isolated enzyme and to have insignificant antibiotic efficacy. The presumed co-product of this degradation route, however, would be bromonitromethane **4**, for which potent inhibitory efficacy was demonstrated (Table 1). In any case, the release of **4** as active component can not be the only mechanism of enzyme inhibition, because the nitrovinyl derivative **2**—which releases inactive nitromethane **5**—also shows significant MurA inhibition.

Because a shift of pH to slightly acidic conditions provokes a clear increase in compound stability, the relevance of these findings was monitored for the determination of bacterial susceptibility. MIC determination was repeated using MH medium which had been previously adjusted to pH 5.8. At lower pH (change from 7.2 to 5.8) the half-life of **1** increases from 8 min to 60 min. However, the MICs were not affected by the pH-change of the medium. While the compound is more stable under acidic conditions, cellular uptake or binding to the target site might also be hindered at lower pH.



Scheme 3. Degradation of nitrovinylfuran derivatives to aldehydes. This decomposition route was demonstrated for aqueous conditions and for the following buffer systems: Tris pH 7.8; citrate pH 6.

2.2.2. Reactivity of compound 1 and derivatives towards glutathione

To investigate the reactivity of nitrovinylfuran **1** and its breakdown products towards thiols, we used glutathione (GSH) as a model sulphur nucleophile to simulate the SH groups of target proteins. The reactivity of **1** towards thiols has not been studied before. We studied the reaction between **1** and GSH in equal concentrations at pH 6. The samples were analyzed by HPLC-UV and HPLC-ESI-MS. Reactivity towards thiol-nucleophiles has been analyzed for β-nitrostyrenes and several vinylfurans and the thioether conjugate has been the reported product.[41,45–47,65]

Surprisingly, **1** turned out to react with GSH in a different manner. The major reaction product is the de-bromo derivative **2**, and not the expected GSH-adduct. The product was verified by isolation and ^{1}H NMR and APCI-MS-analysis of **2** from the reaction of **1** and GSH in citrate buffer (data not shown). In contrast to the degradation of **1** in citrate buffer, the incubation with GSH in the same buffer does not lead directly to the formation of **3**. HPLC-UV-ESI-MS analysis shows, besides **2**, the formation of the GSH-adducts of **1** and the released **2** (Fig. 4).

2.2.3. Compound 1 promotes glutathione oxidation

As the conversion of **1**–**2** represents a reduction reaction, we examined whether oxidized glutathione (GSSG) is formed during the incubation of **1** and GSH. As shown in Figure 5, formation of GSSG occurs.

Scheme 4 shows the proposed mechanism for the reaction between **1** and GSH. Oxidation of protein thiol groups could be another possible mechanism of action for **1** at the studied enzymes.

Formation of intramolecular, non-native disulfide bonds of active site cysteines, resulting in conformational changes as enzyme inactivating mechanism has already been suggested for the ageing of *E. coli* MetAP and could account for the observed inhibitory effects of **1** and **4** on this enzyme.[66] However, other enzymes that were investigated in the course of this study were resistant to **1** and **4** because they do not harbour sensitive cysteine residues.

Table 1 includes an overview of the observed reaction products of investigated substances with GSH. Oxidation of GSH was also demonstrated for the decomposition product **4**. In contrast, **2** forms the GSH-conjugate without concomitant oxidation product. These results indicate that the geminal bromonitro feature is responsible for the observed oxidative processes. In accordance to the results in the enzymatic assays, compounds **3** and **5** were inactive towards GSH.

The interference of compounds **1**, **2** and **4** with thiol nucleophiles in proteins can have severe consequences for the vitality of prokaryotic organisms. Similar observations have been made previously for antibiotic compounds that contain related structural elements: Oxidative modification of cysteine residues has been described as the mechanism of action of the broad-spectrum antibiotic nitrofurantoin.[67,68] Depletion of GSH pool levels as consequence of the oxidative stress has been linked to eukaryotic toxicity of nitrofurantoin.[69–71] Thiol protein oxidation has also been observed for the preservative 2-bromo-2-nitropropane-1,3-diol, which is a close structural analog of the compounds described here.[72] We therefore conclude that the thiol-reactivity of compounds **1** and **4** could provoke uncontrolled oxidative processes comprising formation of disulfide bonds within and between vital enzymes, thereby leading to cell damage.

2.2.4. Compound 1 interacts with MurA cysteines

To verify the proposed interaction between **1** and cysteine residues of MurA, 2,2′-dithiodipyridine (2-DPS) was used to quantify free thiol groups of the enzyme. 2-DPS reacts with thiols in a thiol-disulfide exchange and releases a stoichiometric amount of the chromogenic 2-thiopyridone (2-TP), which can easily be

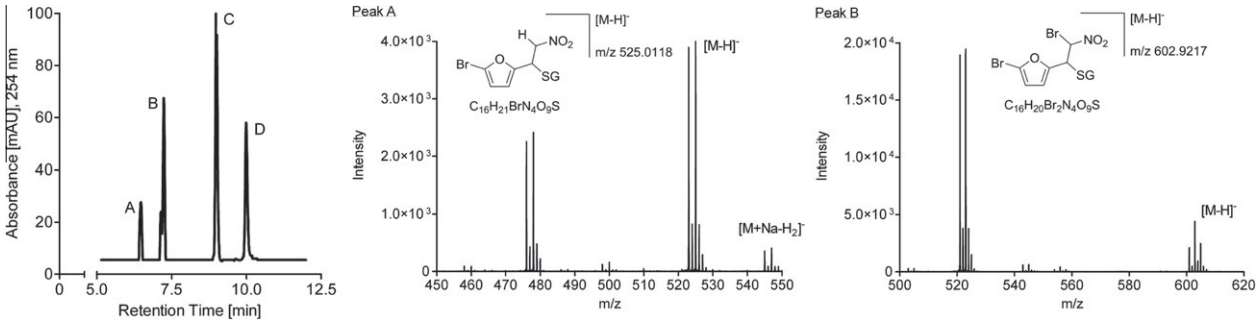


Figure 4. Chromatogram of **1** after 60 s incubation with GSH. Peak labelling: (A) GSH-adduct of **2**, (B) GSH-adduct of **1**, (C) compd **2**, (D) compd **1**. Corresponding ESI mass spectra of peaks A and B. (A) GSH-adduct of **2** showing the characteristic Br_1-substitution pattern and fragment molecules through HNO_2-loss (m/z 478.0096, $\Delta m/z$ 47.0022) and sodium adduct (m/z 546.9902). Mass accuracy −0.4 ppm with respect to the calculated mass m/z 525.0120. (B) GSH-adduct of **1** showing the characteristic Br_2-substitution pattern and fragment molecule through HBr-loss (m/z 522.9960, $\Delta m/z$ 79.9254). Mass accuracy −1.3 ppm with respect to the calculated mass m/z 602.9225.

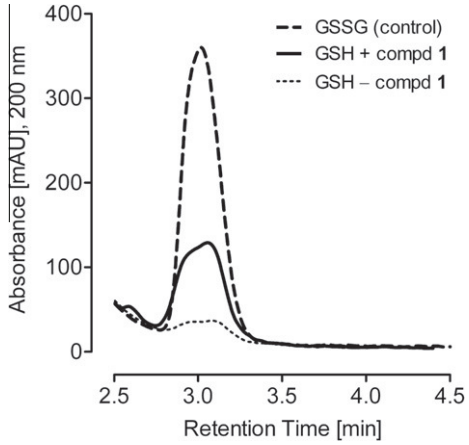
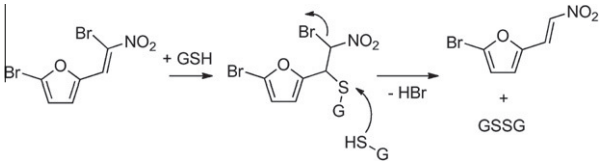


Figure 5. Compound **1** promotes GSSG formation.



Scheme 4. Proposed mechanism for the redox reaction between **1** and GSH.

detected by HPLC. MurA was incubated with an equimolar concentration of **1** for 30 min before adding 2,2′-dithiodipyridine. The amount of 2-TP released from an excess of 2-DPS was compared to a control containing only MurA.

Figure 6 shows the experiment of SH group detection for **1** and the reference compound fosfomycin upon incubation of equimolar and excess substance/inhibitor ratios. The results are compared to an experiment with the C115D mutant enzyme. For the reference substance fosfomycin, the amount of detected thiol groups is not further decreased under compound excess, correlating with the C115-selectivity of this compound in a 1:1 stoichiometry. Upon incubation with the C115D mutant, the amount of detected thiol groups remains unchanged for fosfomycin compared to the control without inhibitor, again correlating with the reported specificity of fosfomycin for the active site nucleophile C115 and the resistance of the C115D mutant towards fosfomycin. For **1** the amount of free SH groups is reduced to a much larger extent when incubated in equimolar ratio and decreases even more upon compound excess. This decrease in detected SH groups is obvious for both wild-type and mutant enzyme. This experiment provides another indication that **1** interacts with cysteine residues in MurA besides the catalytic C115. On the other hand, this experiment clearly points out that enzyme inhibition is not promoted through 1:1 stoichiometry, but involves several accessible cysteine residues.

2.2.5. MurA induces the debromination of compound 1

The reaction of **1** with GSH leads to formation of the de-bromo derivative **2** and oxidized GSH. We studied the conversion of **1** in the presence of MurA to examine whether cysteine oxidation might play a role in enzyme inhibition. In order to rule out non-specific transformation of **1** by the assay additive BSA (bovine serum albumine) we conducted appropriate controls of compound incubation in presence and absence of 0.1% BSA. Reaction progress was followed by HPLC-UV. The presence of BSA has only minor effects on the conversion of **1**. We also showed that the enzyme substrates UNAG or PEP have no significant effects on the conversion of **1** (data not shown). The main product of the degradation in absence of MurA is **3**, while the presence of **2** is near detection limit (Fig. 7). In contrast, in the presence of MurA the formation of the de-bromo derivative **2**, which must occur via redox reaction, can be detected, while only a small amount of **3** is formed (Fig. 7). Additionally, the decomposition of **1** in solution proceeds much faster in presence of the enzyme. The concentration of **1** is greatly diminished even at time point zero. The rapid disappearance of **1** in solution indicates either a covalent binding to the enzyme or an enzyme-catalysed transformation. The decrease of **2** can be attributed to a binding process to the enzyme, because **2** is decomposing very slowly under assay conditions (half-life 58 h) and there is no further rise in concentration of furaldehyde **3**.

2.2.6. Compound 1 induces disulfide bond formation between MurA and a fluorescent thiol

To determine whether the formation of disulfide bonds mediated through **1** also occurs with the MurA enzyme (analogous to the reaction with GSH), MurA was incubated with the fluorescent coumarin derivative **6** in the absence and presence of **1**. The samples were analyzed by SDS–PAGE and the fluorescent dye was visualized under UV.

Figure 8 shows the formation of a fluorescent adduct with the molecular weight of MurA in the sample containing **1** and **6** (lanes 2 and 8) according to the proposed reaction scheme (Scheme 5). A minor band of this size can also be seen in lane 3, where **1** was omitted. Addition of the thiol nucleophile DTT in reduced Laemmli buffer (lane 4) reverses disulfide bond formation, coinciding with a disappearance of this distinct band. This confirms that a disulfide bond was initially formed between enzyme and dye.

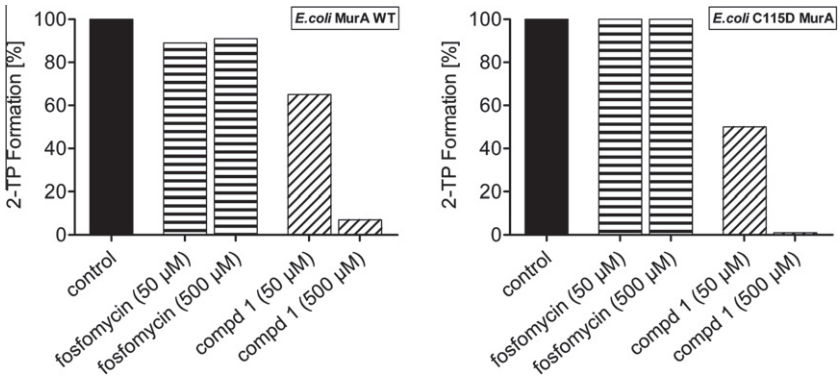


Figure 6. Quantification of free cysteines after treatment of MurA with fosfomycin and **1**. HPLC-analysis of 2-TP released from native and C115D MurA.

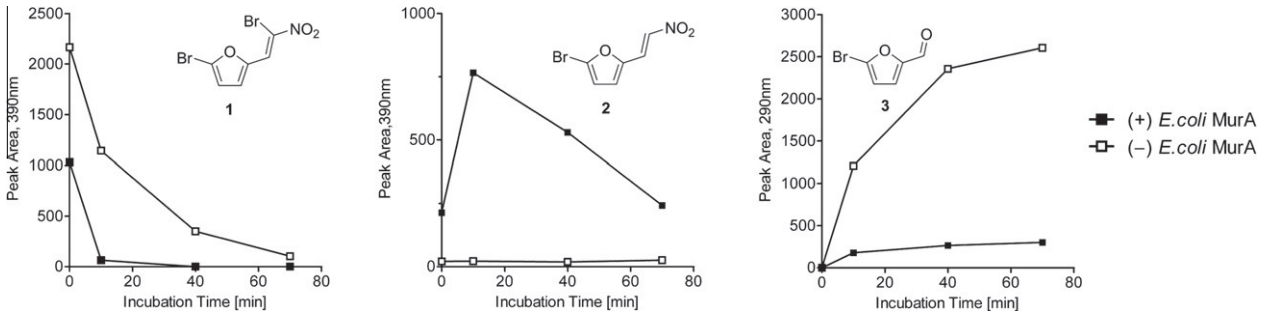
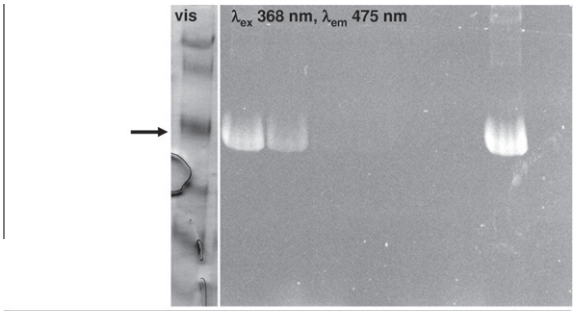


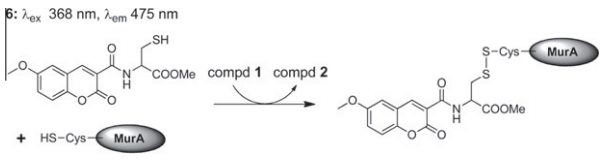
Figure 7. Time course of compound **1** conversion into its degradation products **2** and **3** in presence and absence of *E. coli* MurA (100 μM).



Lane	1	2	3	4[a]	5	6	7	8	9
E. coli MurA [μM]	-	100	100	100	100	-	-	100	100
compound **1** [μM]	-	200	-	200	200	-	200	300	-
compound **6** [μM]	-	200	200	200	-	200	200	300	-

[a] with reducing Laemmli buffer

Figure 8. SDS-PAGE of fluorescently labelled MurA. Excitation at 365 nm, emission at 475 nm. Lane 1: Molecular weight marker (at visible light); the arrow indicates the 47 kDa band corresponding to *E. coli* MurA (47.4 kDa). Intermolecular disulfide bond formation of *E. coli* MurA and **6** is markedly increased in presence of **1**.



Scheme 5. Fluorescent labelling of *E. coli* MurA with the coumarine derivative **6**, induced by redox reaction with **1**.

3. Conclusion

Nitrovinylfuran and bromonitromethane derivatives possess a significant antibacterial activity. We started to explore the efficacy of these compounds as inhibitors of enzymes within the early peptidoglycan biosynthetic pathway. Our data identify the nitrovinylfuran and bromonitromethane scaffolds as inhibitors of MurA from various species and *E. coli* MetAP. In particular, compounds **1** and **4** specifically target bacterial and not human MetAPs. The observed activity on the enzymatic and cellular level is induced by a complex interplay of **1** and its breakdown products. Depending on environmental conditions, the transformation of **1** to the aldehyde proceeds either directly in aqueous media or through intermediate formation of the de-bromo derivative **2** in presence of thiol nucleophiles (Scheme 2). Reactivity of **1** and its descendants involves thiol functionalities through addition reactions or oxidative processes, including the formation of non-native disulfide bonds, thereby impairing stability and catalytic properties of bacterial proteins. Enzymatic, antibacterial, cytotoxic and GSH-reactivity data presented here indicate that neither MurA, nor the other targets which were previously identified, are exclusively responsible for the antibacterial effect of **1**, but that this molecule exerts pleiotropic effects on numerous proteins by interference with cysteine residues.

The cytotoxic activity of the compounds against human cell lines is significant and does not encourage the further development of the compounds as antibacterial drugs. However, the bromonitrovinyl scaffold may be used for the design of inhibitors that specifically interact with cysteine residues in target proteins. This will require a significant selectivity, which must be obtained by other, non-covalent recognition elements.

4. Experimental

4.1. Materials

Chemicals and solvents for HPLC were from Sigma–Aldrich (Schnelldorf, Germany). 96-well plates, U-bottom (polystyrene), and 96-well cell culture plates (Cellstar®, sterile, F-bottom) were purchased from Greiner Bio-One (Frickenhausen, Germany).

Compounds **1** and **2** were synthezised according to the procedures specified in the supporting information.

Compound stock solutions were 10 mM in DMSO. Fosfomycin stock solution was prepared freshly in water. All investigated enzymes were expressed in *E. coli* BL21 (λ DE3) and purified by chromatographic techniques according to procedures described previously,[52,73,74] except for thrombin which was obtained from Sigma–Aldrich.

4.2. Enzyme inhibition studies

Experiments were performed in triplicate with standard deviations of average values generally $\leqslant$10%.

4.2.1. IC_{50} determination

Dose–response curves were established by measuring enzyme activity in triplicate determinations of at least eight different compound concentrations. Assays were performed as described above. The resulting data were plotted using GraFit version 5.0.12 (Erithacus Software Ltd, Horley, Surrey, United Kingdom) and IC_{50} values, representing the concentration of compound at which the residual activities were 50%, were determined from a four-parameter fit.

4.2.2. MurA assay

In a standard assay, MurA enzyme was pre-incubated with the substrate UNAG and inhibitor for 10 min at 37 °C.[52] To determine the influence of the substrate UNAG on the binding process, experiments were performed in absence of UNAG during pre-incubation. The reaction was started by the addition of the second substrate PEP, resulting in a total volume of 100 μL with the following concentrations: *E. coli* wild-type, C115D mutant MurA or *S. aureus* MurA2 12 nM, *Ps. aeruginosa* MurA 150 nM, BSA 0.1% (v/v), UNAG 250 μM, PEP 125 μM, Tris 25 mM pH 7.8, DMSO 1% (v/v). The reaction was stopped after 60 min at 37 °C by adding 100 μL of Lanzetta reagent, composed of malachite green solution (0.045% (w/v)) and ammonium heptamolybdate (8.4% (w/v) in 8 N HCl) in a ratio of 4:1, and 0.03% (w/v) Tergitol NP-40 as dye-stabilizing detergent. After 10 min the absorbance at 620 nm was measured using a BMG Labtech Fluostar Galaxy (Ortenberg, Germany) multiplate reader to quantify the released inorganic phosphate.

4.2.3. Other enzymes

Inhibitor screening on the Dengue virus NS2B-NS3 protease and thrombin were conducted as reported previously.[74,75] Activity on the MetAP enzymes was assessed as described elsewhere.[75]

4.3. Microbiological studies—determination of MIC in pathogenic bacteria

Antimicrobial activities were determined against *S. aureus* (MSSA) ATCC 25923, methicillin-resistant *S. aureus* (MRSA) NCTC 10442, *E. coli* ATCC 25922 and *Ps. aeruginosa* ATCC 27853 strains. The strains were sub-cultured on Columbia agar containing 5% sheep blood (Becton Dickinson, Germany) 24 h prior to any antimicrobial test. Broth microdilution assays were performed according to CLSI (Clinical and Laboratory Standards Institute, 2011) in Müller-Hinton broth. Briefly, the samples were pipetted to 96-well microtiter plates in an appropriate medium followed by a twofold serial dilution. Hundred microliters of microbial suspension was added to give a final concentration of 5×10^5 cfu/mL. After incubation at 36 °C for 20–24 h, minimal inhibitory concentration (MIC) was determined as the lowest concentration at which no growth occurred.

4.4. Cytotoxicity studies

The cytotoxicity of the investigated compounds was determined using the following cell lines: HaCaT (human skin keratinocyte) and Flow 2002 (diploid human embryonic lung fibroblast) as nontumorigenic cell lines; A-431 (human skin epidermoid carcinoma), MeWo (human skin melanoma), Calu-6 (human lung adenocarcinoma), CaSki (human cervical carcinoma), Capan-1 (human pancreas adenocarcinoma) and SW-707 (human colon carcinoma) as cancerous representatives. The assay was performed in 96-well plates with an inoculum of 3×10^4 cells/well, which were seeded the day before treatment. Compound test solutions were prepared freshly by diluting the stock solution 1:10 with the respective strain medium. Total assay volume was 100 μL with a final DMSO concentration of 1% (v/v). Plates were incubated for 72 h at 37 °C, then deprived of medium and fixed with formalin solution 3% (v/v). For quantification of cytotoxicity cells were stained by adding 80 μL of crystal violet solution 0.5% (w/v) per well. After removal of excessive dye and desiccation of the plates, the optical density was measured at 590 nm using a BMG Labtech Fluostar Galaxy multiplate reader. IC_{50} values were calculated from eight different compound concentrations using GraFit version 5.0.12 (Erithacus Software Ltd, United Kingdom).

4.5. Chromatographic analysis (compound stability and reactivity)

4.5.1. Instrumentation and chromatographic conditions

Samples of degradation studies and for reactivity analysis with GSH or enzyme were analyzed by HPLC. The HPLC system consisted of an Agilent 1200 series device (consisting of G1322A degasser, G1311A quaternary pump, G1329A autosampler, G1316A column oven and G1315A diode array detector controlled by Chemstation Software, Agilent Technologies, Germany) equipped with a Waters XTerra® C18 column (2.1 × 50 mm, particle size 2.5 μm) unless otherwise noted. A linear gradient mobile phase was adequate for all analytical separations and accomplished using 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) with a flow rate of 0.3 mL/min and a column temperature of 37 °C. A typical gradient elution profile started with 10% solvent B for 1 min, was ramped linearly to 95% over 8 min and maintained at 95% for 2 min. Injection volume was 10 μL unless indicated otherwise. The column eluate was monitored by measuring the absorbance as indicated within the respective experimental setup. RP-LC/MS-analysis was performed using the same chromatographic setup coupled to a micrOTOF-Q II (Bruker Daltonik, Bremen, Germany) operating in electrospray negative ionization mode. Chromatographic conditions were employed as mentioned, except that the solvent additive TFA was replaced by formic acid 0.1% to allow for MS-compatibility. Reference masses of sodium formate clusters were used for external calibration of each chromatographic run.

4.5.2. Half-life of nitrovinylfuran derivatives

The decomposition of nitrovinylfuran derivatives into corresponding aldehydes was studied using HPLC-UV. Solutions of the compounds (100 μM) in water as well as different buffers (sodium citrate 50 mM pH 6, Tris 50 mM pH 7.8) and in Müller-Hinton

medium (pH 7.2) were prepared and aliquots of 10 μL of these samples were injected into the HPLC system at different time points. Disappearance of the nitrovinylfuran derivatives was monitored at 390 nm. Formation of corresponding aldehydes was observed at 290 nm. Microsoft® Office Excel 2003 was used to construct first order plots from the logarithm of peak area versus time and the half-lives were calculated using the equation $t_{1/2} = \ln 2/k_{obs}$.

4.5.3. Reactivity of nitrovinylfuran derivatives towards glutathione

In order to study the reactivity towards glutathione, 10 μL inhibitor stock solution (10 mM in DMSO) and 10 μL GSH stock solution (10 mM in H_2O) were dissolved in 980 μL sodium citrate buffer (50 mM pH 6.0), resulting in final concentrations of 100 μM. Aliquots (100 μL) of the reaction mixture were withdrawn at regular intervals (every 30 s over a period of 3 min, and at 4 and 5 min) and the reaction in these aliquots was stopped by quenching with 1 μL of orthophosphoric acid (85%). Samples were analyzed by HPLC.

The presence of glutathione-conjugates was shown by RP-LC/MS. To verify the formation of oxidized glutathione (GSSG), a Merck Chromolith® Performance RP-18e column (4.6 × 100 mm) was used. Elution started with 100% aqueous conditions for 3 min, followed by a linear gradient to 95% solvent B over 5 min at a flow rate of 1 mL/min. Formation of GSSG could be detected at 200 nm at a retention time of 3.0 min.

4.5.4. Detection of protein thiols using 2,2′-dithiodipyridine

Ten millilitres of *E. coli* MurA stock solution (500 μM) and 5 μL of compound **1** stock solution (1 mM for equimolar ratio and 10 mM for 10-fold excess experiments) were dissolved in 35 μL Tris buffer 50 mM pH 7.8 and incubated at room temperature. After 30 min, 50 μL of a 2,2′-dithiodipyridine solution (2 mM in sodium citrate buffer 0.5 M at pH 4, containing 6 M urea) were added. After subsequent 30 min incubation at room temperature, the reaction was quenched by the addition of 10 μL TFA (4%). The reaction product 2-thiopyridone was detected by HPLC at 342 nm. Injection volume was 20 μL. The resulting peak area was compared to a control in which **1** was omitted.

4.5.5. Reactivity of compound 1 towards MurA

To determine the effects of MurA on the degradation route of **1**, conditions were identical to those described for the standard enzyme activity assay, except for enzyme and compound concentrations which were 100 μM. For reactions in the absence of enzyme, identical reaction buffers were used without addition of the enzyme. For measurements in absence of the assay additive BSA, the respective volume was replaced with water. Reactions were stopped at different time points by addition of 1 μL orthophosphoric acid (85%). For determinations at time point zero the stopping reagent was added before all other components. Standards for the retention time determination of **1**, **2** and **3** were prepared at 100 μM in 0.1% formic acid in order to prevent unspecific degradation steps. Conversion of **1** in presence of enzyme was tracked at 290 and 390 nm according to the expected decomposition products.

4.6. Detection of disulfide bond formation using the fluorescent thiol 6

E. coli MurA (final concn 100 μM) and the fluorescent thiol derivative **6** (final concn 200 μM) were incubated in Tris buffer 50 mM pH 7.8 at room temperature in the absence or presence of **1** (final concn 200 μM). Appropriate controls were performed by incubation of enzyme and compound **1** alone and without any additive. After 1 h, a 10 μL aliquot was quenched with 10 μL of non-reducing Laemmli buffer. Additionally, one sample containing MurA and compounds **1** and **6** was quenched with reducing Laemmli buffer. After heating to 95 °C for 3 min, 10 μL were analyzed by SDS–PAGE (12%). Protein bands were analyzed using an Alpha-Imager (Alpha Innotec, Kasendorf, Germany) gel documentation system with an excitation wavelength of 365 nm to visualize protein-bound fluorescent **6**. Compound **6** was synthesized from 6-methoxy-coumarin-3-carboxylic acid and 4,4′-dimethoxytrityl protected cysteine methylester. Details are described in the Supplementary data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.018.

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