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Structure–activity relationships of tulipalines, tuliposides, and related compounds as inhibitors of MurA

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ABSTRACT

The enzyme MurA performs an essential step in peptidoglycan biosynthesis and is therefore a target for the discovery of novel antibacterial compounds. We report here the inhibition of MurA by natural products from tulips (tulipalines and tuliposides), and the structure–activity relationships of various derivatives. The inhibition of MurA can be related to antibacterial activity, and MurA is probably one of the relevant molecular targets of the tulipaline derivatives. MurA inhibition by this class of compounds depends on the presence of the substrate UNAG, which indicates non-covalent suicide inhibition as observed previously for cnicin. With respect to selectivity, however, the reactivity against arbitrary sulfhydryl groups, such as in glutathione, could not yet be sufficiently separated from MurA inhibition in the present dataset.

Bacteria are surrounded by the peptidoglycan polymer, which is responsible for cell integrity. The first step in the biosynthesis of peptidoglycan is catalyzed by the enzyme UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (MurA). This step includes a nucleophilic attack of the 3′-hydroxyl group of UDP-*N*-acetylglucosamine (UNAG) to the C2 position of phosphoenolpyruvate (PEP) to form enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UNAG) under release of phosphate (Fig. 1).[1,2]

The inhibition of an enzyme from the biosynthetic pathway of the bacterial cell wall induces cell lysis and is therefore a relevant approach for antibacterial drug discovery. In case of MurA the only inhibitor in clinical use is the epoxide fosfomycin, which covalently binds to Cys115 of MurA.[3]

Recently we reported a couple of potent inhibitors of MurA: These are natural products of the sesquiterpene lactone type with cnicin as its most potent representative.[4] We initially expected a covalent binding of the α,β-unsaturated carbonyl function to Cys115 of MurA, which would have been analogous to fosfomycin's binding mode. However, an irreversible binding mode could not be confirmed by bio-analytical methods such as mass spectrometry of proteolytic digests. Eventually, an X-ray structure of the cnicin–UNAG–MurA complex indicated an unexpected binding mode: The side chain of cnicin—closely resembling PEP, with the Michael acceptor and the diol group—is converted by MurA to a 'wrong' product, which remains bound to the active site.[5] This mode of action represents an auto-catalytic, non-covalent suicide inhibition. Attempts to generate larger amounts of the product or to detect it by mass spectrometry, remained ambiguous. In order to gain a further understanding of the underlying SAR and to increase the potency of the compounds, we decided to synthesize and evaluate a series of acrylic acid derivatives as mimics of the cnicin side chain.

Considering the preparation and modification of the side chain, we identified various constituents of tulips, tulipaline A and B, and their corresponding glycosides 1-tuliposide A and B (cf. Table 1).[6] The antibiotic activity of tulips was first described in 1943. It was also observed that certain fungi do not affect the pistil of tulips. The tulipalines and tuliposides, which are present in high concentrations in this part of the plants, are believed to be responsible for this effect.[6–10] While the present work was in progress, Ubukata and co-workers[11] described the antibiotic activity of tulipalines against a variety of pathogenic organisms and hypothesized that MurA was the responsible target protein.

In the first part of this study we investigated the inhibition of MurA by the natural products from tulips and their derivatives. **1-tuliposide A** and **1-tuliposide B** were extracted from freeze-dried tulips. The commercially available α-methylene-γ-butyrolactone **tulipaline A** was converted into **(±)-tulipaline B** with selenium dioxide. Ring opening was achieved with potassium hydroxide in methanol to obtain **1** and **2** in good yields (Scheme 1).

The IC_{50} values at the native *Escherichia coli* MurA and the mutant (C115D) enzyme were determined as described previously (Table 1).[4,12,13]Whereas **tulipaline A** has no inhibitory effect, its

Abbreviations: UNAG, UDP-*N* acetylglucosamine; EP-UNAG, enolpyruvyl-UDP-*N*-acetylglucosamine; DMP, Dess–Martin-periodinane; PEP, phosphoenolpyruvate; GSH, glutathione.

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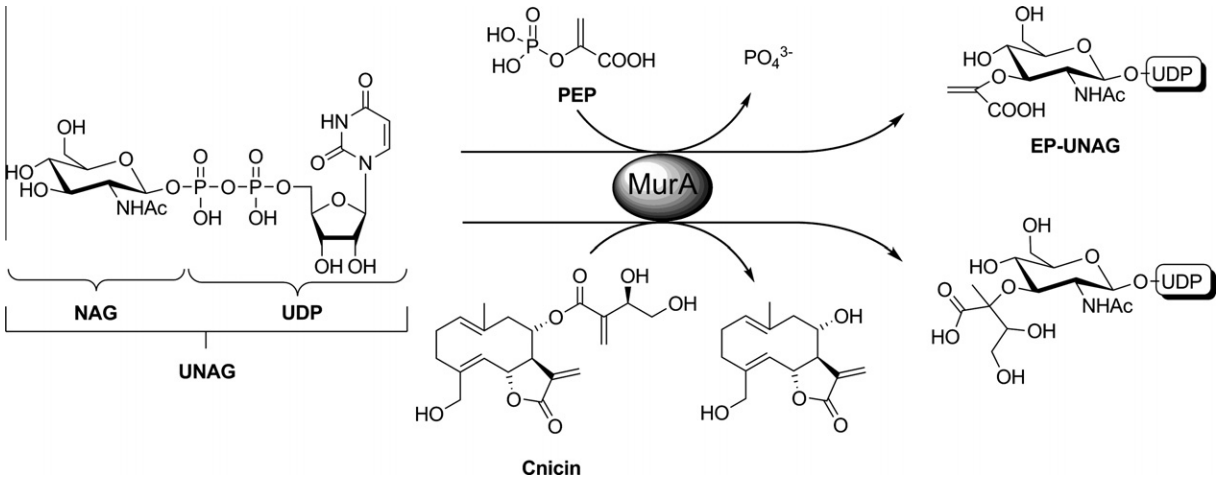


Figure 1. MurA catalyzes the formation of enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UNAG) from UDP-*N*-acetylglucosamine (UNAG) and phosphoenolpyruvate (PEP). Cnicin acts as a substrate mimic for PEP, which leads to the formation of an UNAG–cnicin adduct that remains bound in the active site of MurA.

hydroxylated analog **(±)-tulipaline B**, which represents a lactonized form of the cnicin side chain, is a potent inhibitor of the native *E. coli* MurA. The glycoside **1-tuliposide B** with an IC_{50} value of 5 μM is highly active, whereas **1-tuliposide A** shows no inhibition of MurA. Since the only difference between the A- and B-series is the hydroxyl group, this moiety seems to be crucial. Both representatives of the B-series inhibit MurA in a time-dependent fashion, indicating either formation of a covalent enzyme-inhibitor complex or non-covalent suicide inhibition (cf. Fig. 2).

Stimulated by these encouraging results we synthesized various tulipaline derivatives to further modulate the steric and electronic properties of the compound series. Derivatives with shorter chain-length were synthesized according to Scheme 2. The ester **3** was formed by a Baylis–Hillman reaction, where the reaction time could be shortened from 3 days to 6 h by use of ultrasonic irradiation.[14,15] Subsequent hydrolysis afforded the carboxylic acid **4**.

Derivatives with longer chain-lengths were synthesized according to Scheme 3. Starting with DL-1,2-isopropylideneglycerol, the first step was an oxidation with Dess–Martin-periodinane (DMP) followed by a Baylis–Hillman reaction to form **5**.[16,17] Acetylation and deprotection of the dimethylacetal with *p*-TsOH lead to **6**. Deprotection under variable conditions afforded **7** and **8**.[6]

The **(±)-tulipaline B** analog **9** (Scheme 4) was synthesized from **5** using TFA/H_2O for deprotection and ring closure.[17] The following selective halogenation of the primary alcohol was problematic due to different side reactions of the double bond and the secondary alcohol. Finally, an Appel reaction with CBr_4 and PPh_3 furnished the bromo derivative **10**.

To form the dehydroxy-derivative of **9** a two step procedure was used. Starting with a Reformatzky reaction of bromomethylacrylate **11** and acetylethanal using activated zinc furnished intermediate **12**, which was deacetylated with sodium methanolate to give compound **13** (Scheme 5).[18]

Compound **15** was designed to mimic PEP more closely (cf. Scheme 6). Intermediate **11** was obtained by halogenation of **3** and converted into the phosphonic acid ester **14** using a Perkov reaction with triethyl phosphite.[19] Deprotection with concd HCl afforded **15** in quantitative yield.

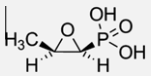
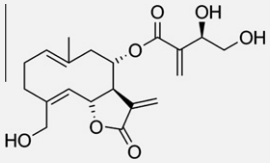
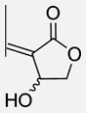
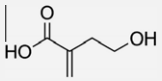
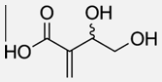
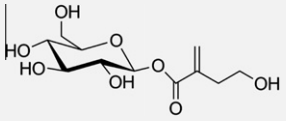
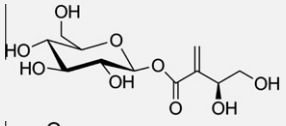
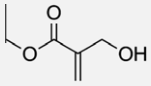
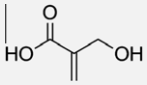
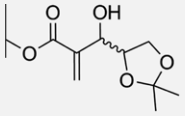
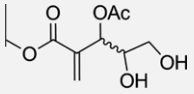
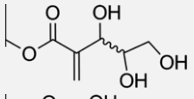
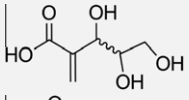
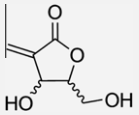
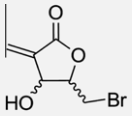
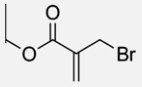
The results of the enzymatic and antibacterial assays are given in Table 1 with fosfomycin and cnicin as reference compounds. From the top of Table 1, it becomes immediately clear that the A-series of tulipalines and related compounds is practically inactive, whereas the B-series is highly active in the enzymatic assay. As recently reported,[11] the side chain of tuliposide B is very important for a strong antibacterial activity and a small change in the structure leads to complete loss of activity. These results are consistent with our observations presented here, indicating that MurA is one of the major molecular targets of tulipalines and tuliposides. Compound **2**, the open-ring analog of **(±)-tulipaline B**, is the single carboxylic acid in the dataset that shows activity (cf. the inactive compds **1**, **4**, **8**, and **15**). It appears that the gem-diol moiety adjacent to the methylene group, as realized in compd **2** (and, in masked form, in **(±)-tulipaline B**) represents the optimum structure: The extension with a third hydroxy group (compds **6**, **7**, **8**) reduces activity, and 'contraction' (compds **3** and **4**) results in completely inactive compounds. The inactivity of compds **3** and **4** indicates that the relevant factor is not the inductive effect of the adjacent hydroxy function on the methylene group, but—in line with the observed binding mode of the UNAG–cnicin-adduct[5]—the bioisosteric similarity of the gem-diol moiety to PEP's phosphate group. This SAR can be extended to the lactonized compounds, which have a similar activity as the corresponding ring-opened methyl esters (**7** ↔ **9**). The essentiality of a hydroxy group adjacent to the methylene moiety becomes evident in the comparison of compounds **9** and **13**.

All active analogs except compound **2** are either esters or lactones. Apart from compound **2**, the carboxylic acids are inactive. It has been hypothesized by others[11] that lactonization is a prerequisite for antibacterial activity. Our data indicates that lactones and esters are both active in the MurA assay and the antibacterial screen. We therefore conclude that the relevant effect is not a steric (cyclic vs linear ester) but an electronic one (acid/anion vs neutral compound). The outlier, compound **2**, may either have a particularly strong propensity to form the lactone in situ, or the other structural features of the compound (which otherwise represents the optimum structure as discussed above) offset the detrimental electronic effect of the carboxylic acid group.

The bromo-analogs **10** and **11** were synthesized in an attempt to generate covalent UNAG-inhibitor adducts with increased electron density for protein-inhibitor co-crystallization experiments. Unfortunately, we were unable to obtain co-crystals of these compounds that were of sufficient quality for structure determination by X-ray diffraction. Of the phosphonic acid derivatives **14** and **15**, only the ester analog **14** showed some residual activity against the native MurA.

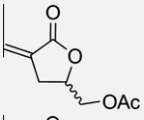
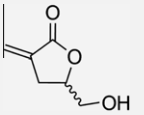
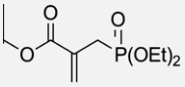
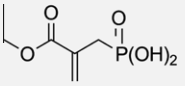
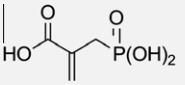
The C115D mutant MurA is practically resistant towards all tested compounds. Therefore, the cysteine residue must play a pivotal role in the formation of the UNAG-inhibitor adduct. It may be hypothesized that a covalent intermediate between inhibitor and the thiol group of Cys115 is generated before the formation of

Table 1
Inhibition of native and C115D *E. coli* MurA in the presence of UNAG, and growth inhibition of *E. coli* cells (GI) in full and minimal medium

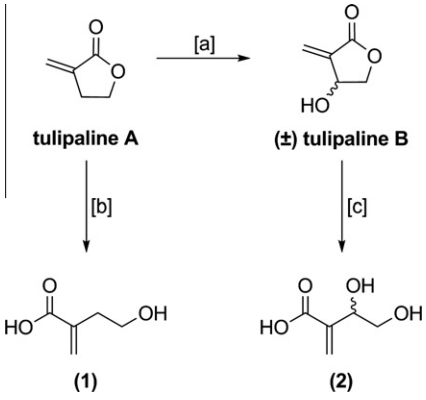
Compound	Structure	*E. coli* MurA [% inh.]	*E. coli* MurA IC_{50} [μM]	*E. coli* MurA C115D [% inh.]	GI [% inh.]	GI (min. medium) [% inh.]
Fosfomycin[4]		99 (±1)	0.1 (±0.01)	n.i.	100	96 (±6)
Cnicin[4]		97 (±2)	16.7 (±0.1)	n.i.	19 (±4)	92 (±5)
Tulipaline A		n.i.	n.d.	n.i.	5 (±3)	n.i.
(±)-Tulipaline B		97 (±3)	2.0 (±0.6)	n.i.	62 (±4)	93 (±9)
1		n.i.	n.d.	n.i.	n.d.	n.i.
2		87 (±4)	8.6 (±0.4)	n.i.	18 (±4)	100 (±4)
1-Tuliposide A		11 (±5)	n.d.	n.i.	n.i.	n.i
1-Tuliposide B		84 (±2)	5.0 (±0.8)	n.i.	64 (±5)	97 (±6)
3		n.i.	n.d.	n.i.	n.i.	n.i.
4		n.i.	n.d.	n.i.	n.i.	n.i.
5		n.i.	n.d.	n.i.	n.i.	n.i.
6		79 (±2)	4.5 (±1.1)	10 (±4)	30 (±4)	98 (±6)
7		77 (±3)	6.3 (±1.0)	10 (±5)	17 (±2)	n.d.
8		n.i.	n.d.	n.i.	n.i.	n.i.
9		74 (±2)	6.6 (±1.3)	n.i.	26 (±5)	102 (±4)
10		85 (±1)	10.4 (±2.0)	18 (±7)	84 (±12)	103 (±4)
11		55 (±6)	n.d.	n.i.	20 (±7)	101 (±6)

(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	*E. coli* MurA [% inh.]	*E. coli* MurA IC_{50} [μM]	*E. coli* MurA C115D [% inh.]	GI [% inh.]	GI (min. medium) [% inh.]
12		n.i.	n.d.	n.i.	n.i.	74 (±8)
13		17 (±4)	n.d.	n.i.	n.i.	47 (±9)
14		20 (±1)	n.d.	n.i.	n.i.	n.i.
15		n.i.	n.d.	n.i.	n.i.	n.i.
16		13 (±3)	n.d.	n.i.	n.i.	n.i.

Inhibitors were screened at a concentration of 25 μM in the enzyme assays and at 200 μM in the GI assay. IC_{50} values were determined for compounds with significant activity. Enzyme concentrations were 12 nM for the native MurA and 24 nM for the C115D mutant. n.i.: no inhibition; n.d.: not determined.



Scheme 1. Reagents and conditions: (a) SeO_2, 100 °C, 84%; (b) KOH, 70 °C, 85%; (c) KOH, 70 °C, 94%.

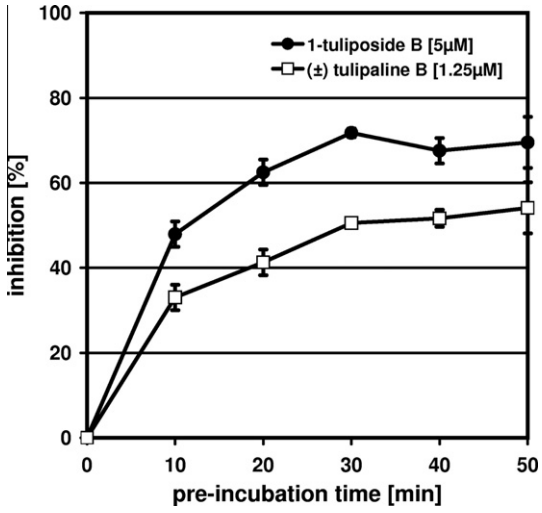
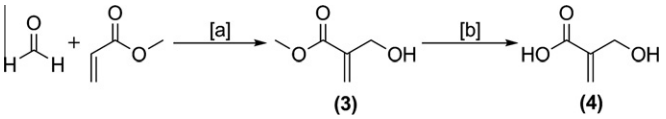


Figure 2. Time dependent inhibition of MurA by **(±)-tulipaline B** and **1-tuliposide B**.

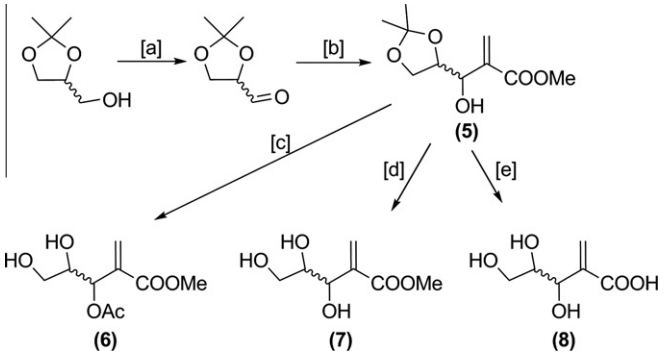


Scheme 2. Reagents and conditions: (a) DABCO, ultrasound, 78%; (b) KOH, 85%.

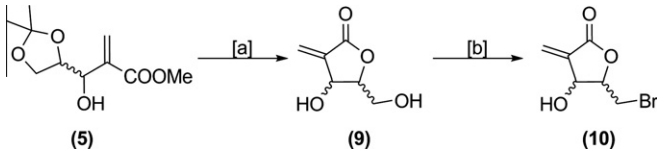
the UNAG-inhibitor adduct. Alternatively, a proton transfer from Cys115 may be essential in the reaction, and the higher acidity of the aspartate residue may not be favorable for this specific reaction.

The binding of UNAG to MurA triggers a conformational change in the highly flexible, superficial loop that contains Cys115. This precedes the transfer of the enolpyruvyl unit from PEP to UNAG. The Cys115 residue is solvent-exposed in the un-liganded state of MurA. Therefore, Cys115 should be highly reactive towards non-selective electrophiles if UNAG is not present. In the case of fosfomycin and cnicin, it has been shown previously that UNAG is necessary for the formation of the covalent adducts between inhibitor and enzyme (fosfomycin) or UNAG (cnicin). As demonstrated in Figure 3, the binding of the inhibitors presented here also depends on the presence of UNAG, albeit to varying degrees. The dependency is most pronounced for fosfomycin, compound **7** and **(±)-tulipaline B**, whereas the binding of compound **10** is least dependent on UNAG. This indicates that the former substances rely on the formation of a ternary complex between UNAG, inhibitor, and MurA that provides the necessary electronic and steric interactions for the auto-catalytic suicide inhibition (cnicin etc.) or the activation of the epoxide for the nucleophilic attack of Cys115 (fosfomycin).

The antibacterial activity was assayed using *E. coli* cells in a rich medium (LB broth) and in a minimal medium.[20] The correlation between enzyme inhibition and antibacterial activity is evident, which indicates that MurA may be a relevant target for this class of compounds. It is also obvious that only few compounds retain



Scheme 3. Reagents and conditions: (a) DMP, 60%; (b) DABCO, ultrasound, 80%; (c) Ac_2O, pyridine; TFA/H_2O, 0 °C, 28%, (d) *p*-TsOH, 56%; (e) *p*-TsOH, KOH, 32%.



Scheme 4. Reagents and conditions: (a) TFA/H_2O, 0 °C, 56%; (b) CBr_4, PPh_3, 28%.

their activity in 'full' medium—or, to state it differently, most compounds that are active in the enzymatic assay are captured and deactivated by nucleophilic compounds in the LB broth. A few compounds appear less prone to inactivation: These are **(±)-tulipaline B**, **1-tuliposide B**, and the bromo analog **10**.

Like fosfomycin, the tulipaline derivatives and cnicin have a bacteriostatic action at low concentrations and a bactericidal activity at higher concentrations. The MIC and MBC values of the tulipalines (cf. Supplementary data—Table 1) are well below those of fosfomycin in minimal medium. This may be due to decreased uptake of fosfomycin in minimal medium or other factors. The tulipaline derivatives are much less polar than fosfomycin and can be expected to cross the bacterial cell membrane(s) via passive diffusion, which does not depend on transport mechanisms and the expression of transmembrane transporters such as GlpT.

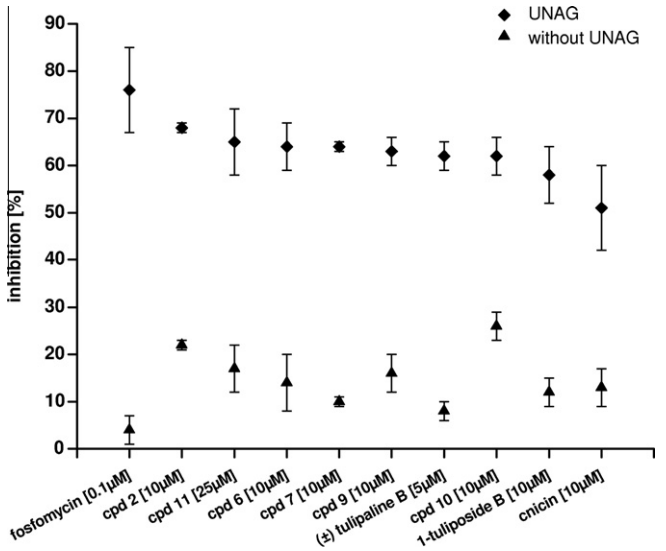


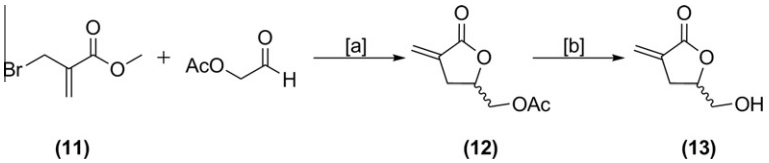
Figure 3. The inhibition of MurA by fosfomycin, cnicin, and the tulipaline derivatives depends on the presence of UNAG.

Table 2
Reactivity of selected compounds against glutathione (GSH), determined by NMR

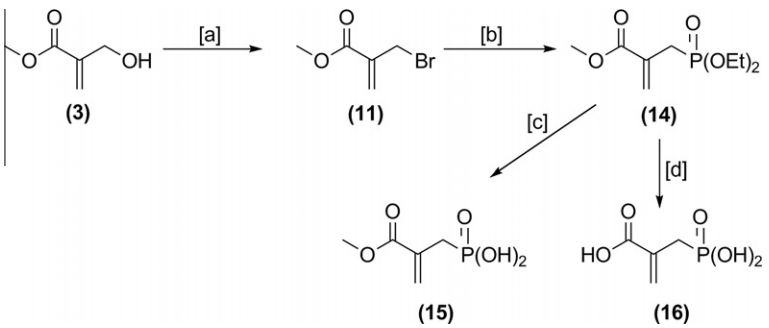
Compound	GSH reactivity $t_{1/2}$ [min]	IC_{50}[a] [µM]
Fosfomycin[4]	≫60	0.12 (±0.01)
Cnicin	<7[a]	17
Tulipaline A	~25	Inactive
(±)-Tulipaline B	<7	2 (±0.6)
2	<7	8.6 (±0.4)
3	>60	Inactive
4	>60	Inactive
9	<7	6.6 (±1.3)
13	~13	Inactive

Conditions: 25 °C; D_2O pH 7.8 (KH_2PO_4); 1 mM of GSH and test compound, respectively. Spectra were measured at 5-min intervals over a period of 60 min. Half-lives were calculated from the integration heights of suitable protons in the test compounds and products.

[a] Cnicin reacted with GSH preferably at the exocyclic methylene group of the sesquiterpene lactone macrocycle.



Scheme 5. Reagents and conditions: (a) Zn, 50 °C, 85%; (b) NaOMe, 42%.



Scheme 6. Reagents and conditions: (a) PBr_3, 62%; (b) $PO(Et)_3$, 80 °C, 76%; (c) TMS-Br, 0 °C, 72%; (d) HCl_{concd}, 110 °C, 95%.

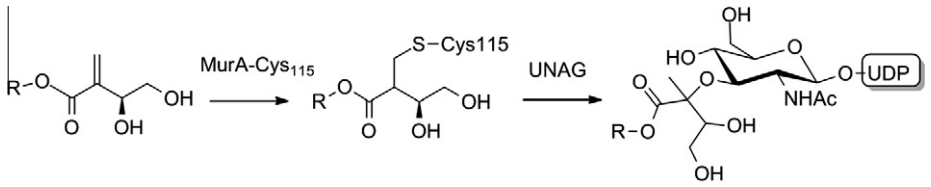


Figure 4. Formation of the adduct between tulipaline derivatives and UNAG via a covalent tulipaline–MurA intermediate.

Since the reactivity of the inhibitors towards sulfhydryl nucleophiles is probably the decisive factor for inactivation in the full medium and may also be related to MurA binding, we studied the reaction of a sub-group of compounds with glutathione (cf. Table 2). Fosfomycin is inert against glutathione. For the other compounds, there is a clear relationship between glutathione reactivity and inhibition of MurA. This indicates—along with the inactivity of the compounds against the C115D mutant—that the formation of a covalent intermediate between the cysteine sulfur and the inhibitors is probably required to form the non-covalent suicide inhibitor that was observed in the cnicin–UNAG–MurA crystal structure. This is illustrated in Figure 4.

To further elucidate whether MurA is the relevant molecular target of the tulipaline derivatives and cnicin, we studied the activity of the compounds against *E. coli* cells harboring the C115D mutant MurA. Details of these experiments are given in the Supplementary data. The experiments clearly show, however, that expression of the C115D mutant MurA makes *E. coli* resistant towards fosfomycin but not towards cnicin or the tulipaline derivatives. Therefore, MurA is not the only target addressed by cnicin and the tulipalines.

In conclusion, our data demonstrates that the natural products **1-tuliposide B** and the lactonized aglycon **(±)-tulipaline B** are potent inhibitors of MurA, which may partly explain the known antibacterial activity of these compounds. In the context of drug discovery, the 'desired' endpoints MurA inhibition and antibiotic activity can not be sufficiently separated from 'undesired' non-specific binding to sulfhydryl nucleophiles. Further steps in this direction must be focused on structural modifications that allow the separation of the desired and undesired endpoints, as realized in fosfomycin.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.139.

References and notes

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13. In a standard assay, MurA (15 nM) was pre-incubated with 31.25 mM Tris, pH 7.8, 312.5 μM UNAG, 0.125% BSA and inhibitor (10 μl, aqueous solution containing 10% DMSO) or without inhibitor (10 μl, water with 10% DMSO) for 10 min at 37 °C. To determine the influence of the substrate UNAG on the binding process, we also performed experiments in which UNAG was not present during pre-incubation. The reaction was started by the addition of the second substrate PEP (20 μl, 625 μM) resulting in a total volume of 100 μl with the following concentrations: *E. coli* WT and mutant MurA 12 nM, BSA 0.1%, UNAG 250 μM, PEP 125 μM, Tris 25 mM, pH 7.8, DMSO 1%. The reaction was stopped after 60 min at 37 °C by adding 100 μl of Lanzetta reagent. The absorbance at 620 nm was measured using a BMG Labtech Fluostar multiplate reader to quantify the released inorganic phosphate. KH_2PO_4 was utilized as a standard.
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20. Screening for antimicrobial activities was performed by monitoring the optical density (OD) at 590 nm using 96-well U-bottom polystyrene microtiter plates. Bacterial cells from an overnight culture of *E. coli* BL21(DE3) grown in LB- or minimal medium were diluted 50-fold with the respective medium, resulting in an OD (590 nm) of 0.03. 245 μl of the dilution were dispensed into the wells containing 5 μl of test solutions prepared in DMSO, giving a final concentration of the test compounds of 200 μM. Control wells were prepared with fosfomycin. Plates were incubated at 37 °C for 7 h. Bacterial growth was measured as increase in OD 590 nm. Antimicrobial activity (growth inhibition, GI) was calculated as the OD percentage relative to an uninhibited control.