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Sesquiterpene lactones are potent and irreversible inhibitors of the antibacterial target enzyme MurA

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Abstract—We report the identification of the sesquiterpene lactones cnicin and cynaropicrin as potent, irreversible inhibitors of the bacterial enzyme MurA. They covalently bind to the thiol group of Cys115. Judging from the structure–activity relationships, we conclude that the unsaturated ester side chain of cynaropicrin and cnicin is of particular importance for the inhibition of MurA. These results provide evidence that MurA is a target protein of SLs with a probably high relevance for their known antibacterial effect.

The bacterial enzyme MurA (EC 2.5.1.7) is responsible for the first step in the cytoplasmatic biosynthesis of peptidoglycan precursor molecules. It catalyzes the transfer of phosphoenolpyruvate (PEP) to the 3′ hydroxyl group of UDP-*N*-acetylglucosamine (UNAG) yielding enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UNAG) and inorganic phosphate (P_i). The enzyme consists of two globular domains with a flexible, superficial loop of ten amino acids from Pro112 to Pro121 (numbering is for the *Escherichia coli* enzyme) that hosts a cysteine residue (Cys115 in *E. coli*, Cys117 in *Pseudomonas aeruginosa* MurA) which is essential for catalysis.[1] The broad-spectrum antibiotic fosfomycin, an epoxide, acts as an analogue of the substrate PEP and irreversibly alkylates the thiol group of Cys115.[2] The MurA-dependent metabolites are of vital importance for bacteria, and the enzyme is therefore in the focus of several drug development projects in academic and industrial groups (Fig. 1).[3–5]

Our own efforts in this field have been in the area of rational drug design (Klein and Bachelier, accepted), assay development and inhibitor screening. We performed a screening project on synthetic compounds and natural products which were pre-selected under the aspects of similarity to the MurA substrates and the presence of potentially reactive functional groups. A few sesquiterpene lactones (SLs) of natural origin (Fig. 2) were included in the screening because this class of compounds can potentially alkylate thiol groups (Cys115 side chain of MurA).[6] We observed a pronounced inhibitory activity for the compound cnicin, a SL isolated from the plant *Cnicus benedictus* L. This observation prompted us to evaluate other SLs with diverse structural elements in order to elucidate the structural requirements for MurA inhibition (cf. Supplementary data). The antibiotic activity of SLs is a well-known effect and has been shown for various SLs and numerous bacterial strains.[7–9] SL-containing plants are, for example, used in traditional African medicine as chewing sticks for oral hygiene due to their antimicrobial properties.[10] Since many SLs contain electrophilic functional groups, it appears likely that they react with cellular nucleophiles in DNA or enzymes. Up to now, however, no definite target structure relevant for the antibiotic activity of SLs has been reported.

The MurA enzymes of *E. coli* K12 and *P. aeruginosa* PAO1293 were overexpressed as His-tag fusion proteins with a thrombin cleavage site.[11,20] Site-directed mutagenesis was performed on the *E. coli murA* gene using the megaprimer PCR mutagenesis procedure.[12] The mutagenic oligonucleotide contained the base substitution ‘tgt‘$_{cys115}$ → ‘gat‘$_{asp115}$ at position 343–345.[13]

Keywords: Sesquiterpene lactones; Antibiotic; Peptidoglycan biosynthesis; MurA; Structure–activity relationships.
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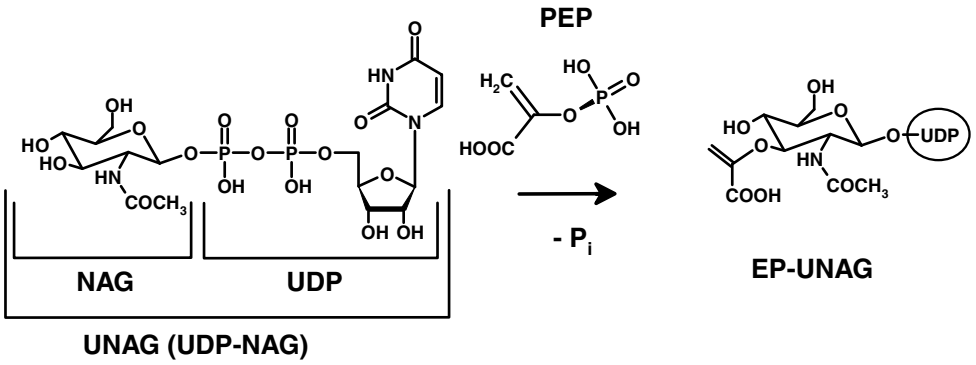

Figure 1. MurA catalyzes the formation of enolpyruvyl-UNAG (EP-UNAG) from phosphoenolpyruvate (PEP) and UDP-*N*-acetylglucosamine (UNAG).

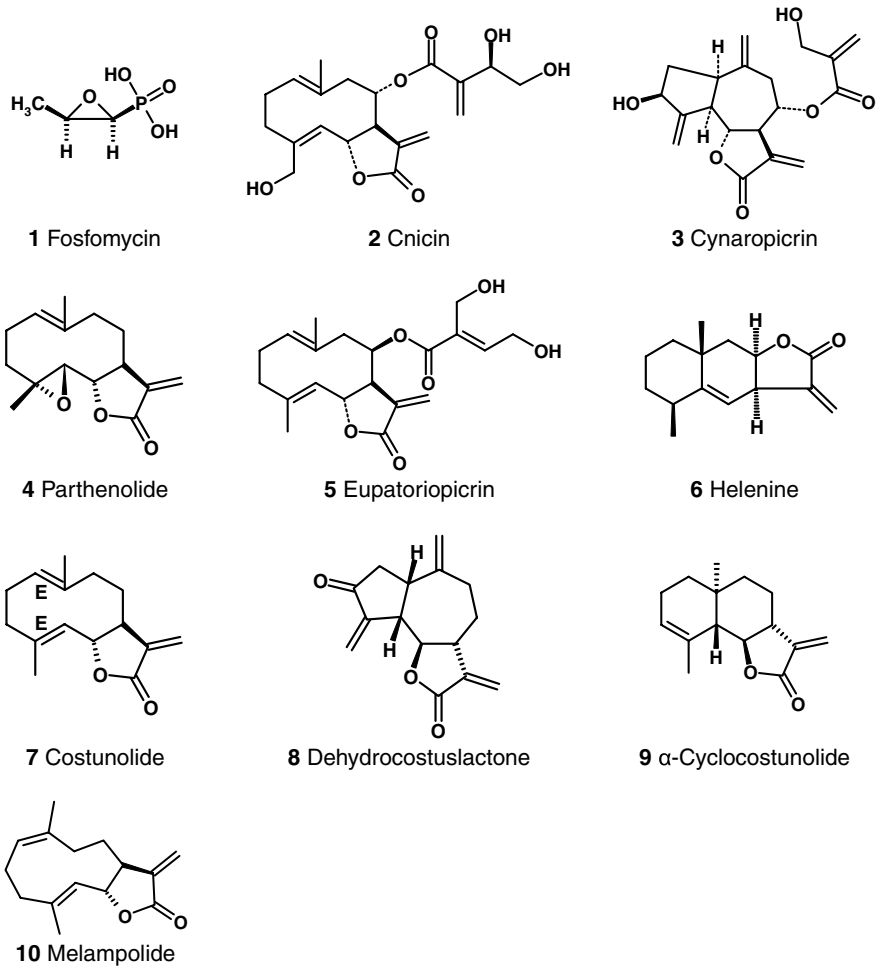

Figure 2. Structural formulas of the tested SLs and fosfomycin.

Assays were performed in 96-well plates using the method described by Lanzetta.[14,21]

SLs from different skeleton classes (germacranolide, guaianolide, eudesmanolide and melampolide) were tested for their activity against *P. aeruginosa* and *E. coli* MurA. The enzyme derived from *E. coli* has a significantly higher turn-over number and was used in the biological assay at 12 nM compared to 150 nM for *P. aeruginosa* MurA.

For *P. aeruginosa* MurA, we found that cnicin and cynaropicrin displayed IC_{50} values of 10.3 and 12.1 μM, respectively, compared to 10.5 μM for the known MurA inhibitor fosfomycin. In contrast, the germacranolide eupatoriopicrin, which possesses an ester side chain at the C8-position, but no further α,β-unsaturated carbonyl function in the side chain, exhibited a lower inhibitory effect on *P. aeruginosa* MurA (19.8 μM). Interestingly, the SLs parthenolide, helenine, costunolide, α-cyclocostunolide, dehydrocostuslactone and melampolide

which lack the ester function in the molecule showed marginal inhibition. At the *E. coli* MurA, cnicin (16.7 μM) and cynaropicrin (19.5 μM) were also more potent than the other SLs.

Two assays were employed to characterize the binding mode of SLs to MurA. First, we performed a standard MurA assay with variable pre-incubation periods. As shown in Figure 3, the inhibitory activity of the tested SLs (and fosfomycin as a control) is time-dependent which is characteristic for an irreversible binding mode.

Evidence for the formation of a covalent adduct was also obtained from the dilution assay. After a pre-incubation period of 2 h at RT containing *P. aeruginosa* MurA (50 μM), cnicin (900 μM) and UNAG (400 μM), the enzyme–inhibitor complex was diluted in a ratio of 1:500 resulting in a nanomolar concentration of cnicin. Assuming a reversible binding mode, one could not expect to see a significant inhibition of MurA in this concentration range. However, nearly 64% inhibition was observed after the dilution indicating that a covalent adduct of cnicin and *P. aeruginosa* MurA had been formed during the pre-incubation period.

The IC_{50} values given in Table 1 were determined in the presence of the substrate UNAG during the pre-incubation period. As shown in Figure 4, the presence of the substrate UNAG facilitates the binding of fosfomycin and the SL cnicin to MurA.[15]

In order to determine the role of Cys115 in the inhibition of MurA by SLs, we cloned the Cys115Asp mutant protein of *E. coli*. The Cys115Asp substitution occurs naturally in *Mycobacteria*, *Chlamydia* and *Nocardia* species.[16–18] While catalytic activity is maintained, the Cys115Asp mutant is not reactive towards electrophilic attack by epoxides (fosfomycin). None of the tested SLs displayed any inhibitory effect on the Cys115Asp mutant of *E. coli* MurA at the highest tested concentration (50 μM) (see Table 1). Therefore, it is very likely that SLs with an α,β-unsaturated carbonyl function act through Michael addition at the thiol group of Cys115.

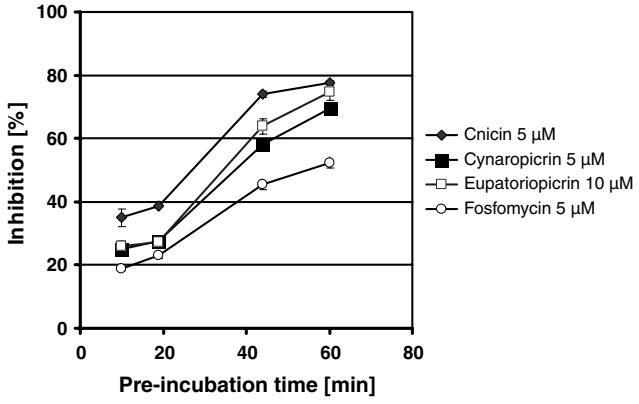

Figure 3. Time-dependent and irreversible inhibition of MurA by SLs. The inhibitory effect of SLs increases with pre-incubation period.

Table 1. Inhibition of MurA by fosfomycin and SLs in the presence of UNAG

Compound	IC_{50} (μM) *E. coli* MurA, 12 nM	IC_{50} (μM) *P. aeruginosa* MurA, 150 nM	Inhibition at 50 μM *E. coli*-MurA C115D, 12 nM
1	0.118	10.5	n.i.[a]
2	16.7	10.3	n.i.
3	19.5	12.1	n.i.
4	>50	27.8	n.i.
5	32.7	19.8	n.i.
6	>50	>50	n.i.
7	>50	>50	n.i.
8	>50	>50	n.i.
9	>50	>50	n.i.
10	>50	>50	n.i.

The pre-incubation time of the inhibitors with the enzyme and UNAG was 10 min.
[a] No inhibition.

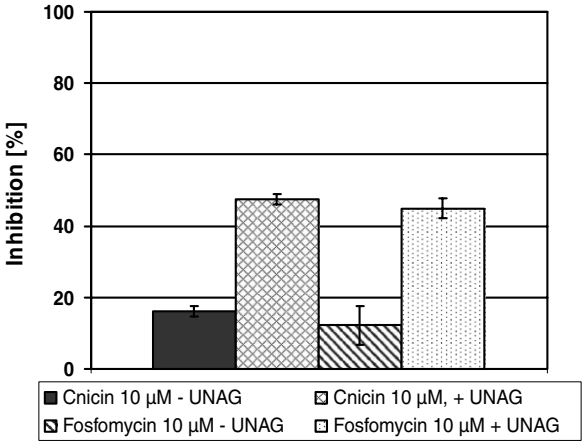

Figure 4. MurA inhibition by cnicin and fosfomycin depends on the presence of UNAG. Shown are the inhibition values of cnicin and fosfomycin (10 μM) with and without the substrate UNAG during the pre-incubation period.

Considering the inhibition data at the native and mutant MurA enzymes, we conclude that the reactive ester side chain in the C8-position of cynaropicrin and cnicin is essential for the inhibition of MurA. In contrast, the α-methylene butyrolactone moiety and the exocyclic methylene groups in the macrocyclic part of the molecules are less relevant. Concerning the binding mode, the side chains of cnicin and cynaropicrin—and, to a lesser extent, that of eupatoriopicrin—mimic the substrate PEP, whereas the macrocyclic part of the molecules is of minor importance. It may, however, be involved in 'ancillary' processes that precede the eventual irreversible binding to Cys115, for example, the closure of the Cys115 surface loop. The macrocyclic part of eupatoriopicrin is nearly identical to that of cnicin. However, the compound is considerably less potent against MurA. This is very probably due to the fact that the reactive methylene carbon in the ester side chain is less exposed and therefore not as accessible for nucleophilic attack than in cnicin and cynaropicrin (Fig. 5).

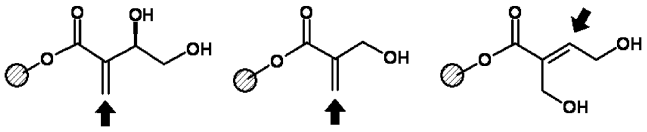

Figure 5. Electrophilic sites of the cnicin, cynaropicrin and eupatoriopicrin side chains. The reactive site in the eupatoriopicrin side chain (right) is less exposed and thus less accessible towards nucleophilic attack by Cys115.

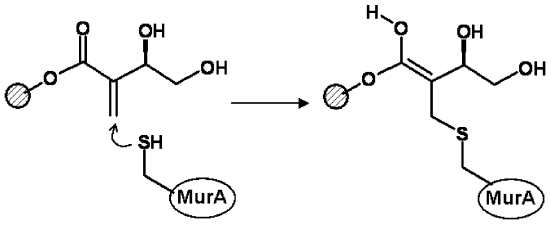

Figure 6. Reaction of the Cys115 side chain of MurA with sesquiterpene lactones.

The proposed Michael addition of SLs with the thiol group of Cys115 results in a stable binding and the alkylation of this important residue (Fig. 6). The crystal structure of the *E. coli* MurA complexed with the substrate UNAG and the inhibitor fosfomycin (PDB code 1UAE) shows that, upon the alkylating of Cys115 by fosfomycin, the loop from Pro112 to121 closes the active site completely.[2] In case of the inhibition of MurA by the SLs, it is likely that the macrocyclic isoprenoid skeleton prevents the closure of the active site.

Parthenolide contains an epoxide functionality at the macrocylic part of the molecule and could therefore, in theory, bind to MurA in a way that would resemble the binding of fosfomycin. However, the inhibitory potency of parthenolide is relatively low and we therefore conclude that it is the steric location, and not the chemical functionality of the reactive group, which is of prime relevance for MurA inhibition.

We cannot assume that the antibacterial effect of all SLs is exclusively due to inhibition of MurA, because there are numerous other nucleophilic binding sites within the cell that may be attacked by the exocyclic methylene groups on the macrocycle or the side chain electrophiles. However, the data presented here provide clear evidence that SLs are potent and irreversible inhibitors of a metabolic process that is of vital importance for bacterial cells. It offers the first explanation of the antibacterial mode of action of SLs on a molecular basis.

The α,β-unsaturated carbonyl function of the presented SLs has a similar reactivity towards biological nucleophiles as the epoxides such as fosfomycin, and it is questionable whether the other structural features of cnicin or cynaropicrin are able to impart a sufficient selectivity for the desired target MurA. In fact, many SLs have a known allergenic potential because of their unselective binding to various (unknown) biological macromolecules.[19] It is therefore not sensible to promote the SLs per se as antibacterial drugs, but rather to employ the structural scaffolds of the ester side chains as reactive core structures for the design of compounds that have an increased selectivity for MurA. To this end, it may be necessary to modulate the reactivity of the α,β-unsaturated carbonyl function by introducing electron-donating substituents. One aspect, however, will—as in the case of fosfomycin—remain critical for this class of compounds: they will require the presence of the nucleophilic Cys115 in the target enzyme, which is a priori not present in some species and can be substituted by aspartate, thus yielding a resistant enzyme.

In summary, these results shed light on the 'antibiotic' mechanism of action of the sesquiterpene lactones and present SLs as in vitro potent MurA inhibitors. The structure–activity relationships presented here may have relevance for the design of more potent and selective MurA inhibitors.

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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.2006.08.021.

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21. In a typical assay, MurA (15 nM WT and mutant *E. coli* MurA, 187.5 nM *P. aeruginosa* MurA) was pre-incubated with 31.25 μM 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 of SLs, 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 (*P. aeruginosa* MurA 150 nM), BSA 0.1%, UNAG 250 μM, PEP 125 μM, Tris 25 mM, pH 7.8, and 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 Wallac Victor2 multiplate reader to quantify the released inorganic phosphate. KH_2PO_4 was utilized as a standard.