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2-Aminotetralones: Novel inhibitors of MurA and MurZ

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Abstract—Several 2-aminotetralones were identified as novel inhibitors of the bacterial enzymes MurA and MurZ. A number of these inhibitors demonstrated antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* with MICs in the range 8–128 μg/ml. Based on structure–activity relationships we propose that the α-aminoketone functionality is responsible for the inhibitory activity and evidence is provided to support a covalent mode of action involving the C115 thiol group of MurA/MurZ.

Peptidoglycan is an essential component of the cell walls of Gram-positive and Gram-negative bacteria, providing protection of the bacterial cell from destruction by osmotic pressure and conferring cellular shape.[1] Interference with bacterial cell wall biosynthesis is firmly established as an excellent basis for the development of potent antibacterials; for example the penicillins and cephalosporins specifically inhibit enzymes involved at the crosslinking stages of cell wall synthesis.[2]

The bacterial enzyme MurA (UDP-GlcNAc enolpyruvyl transferase), in the first committed step of peptidoglycan biosynthesis, catalyses the transfer of enolpyruvate from phosphoenolpyruvate (PEP) to the 3′-hydroxyl group of UDP-*N*-acetylglucosamine (UDP-GlcNAc) yielding enolpyruvyl UDP-*N*-acetylglucosamine (EP-UDP-GlcNAc) and inorganic phosphate (Fig. 1).[3] Low-GC Gram-positive bacteria contain two copies of the *murA* gene (*murA* and *murZ*) which encode, respectively, the enolpyruvyl transferase enzymes, MurA and MurZ.[4]

As MurA is conserved across both Gram-positive and Gram-negative bacterial species and is an essential enzyme with no mammalian counterpart, it is an attractive target for the development of new antibacterials, which are urgently required as resistance to established antibiotics continues to escalate.[5,6]

Inhibition of MurA by the naturally occurring epoxide-based antibiotic fosfomycin has been extensively described.[7–9] This drug acts as a PEP surrogate and forms a covalent adduct with C115 (*Escherichia coli* numbering) via epoxide ring-opening. Fosfomycin also inhibits MurZ from *Staphylococcus aureus* by the same mechanism.

Here, we report the identification of 2-aminotetralones as a new class of MurA inhibitors and propose a mode of action involving formation of a covalent adduct.

High throughput screening of the Novartis compound collection identified compounds **3** and **4** as good inhibitors of *E. coli*[10] MurA with IC_{50} values of 3.1 and 8.5 μM, respectively (Table 1).[11] Compounds **3** and **4** were also identified as inhibitors of MurA and MurZ from *S. aureus*[12,13] with IC_{50}'s in the range 12–23 μM

Keywords: 2-Aminotetralone; MurA inhibitor; MurZ inhibitor; Antibacterial activity.
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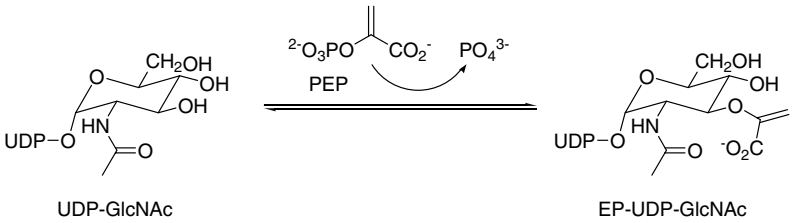
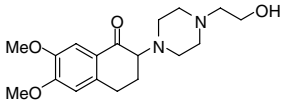
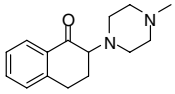
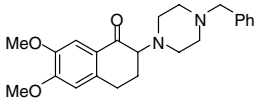
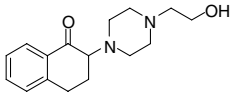
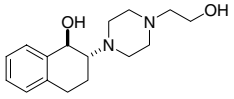
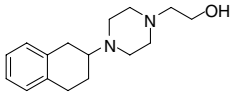
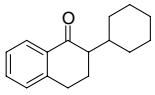
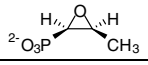


Figure 1. MurA catalyses the formation of enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UDP-GlcNAc) from phosphoenolpyruvate (PEP) and UDP-*N*-acetylglucosamine (UDP-GlcNAc).

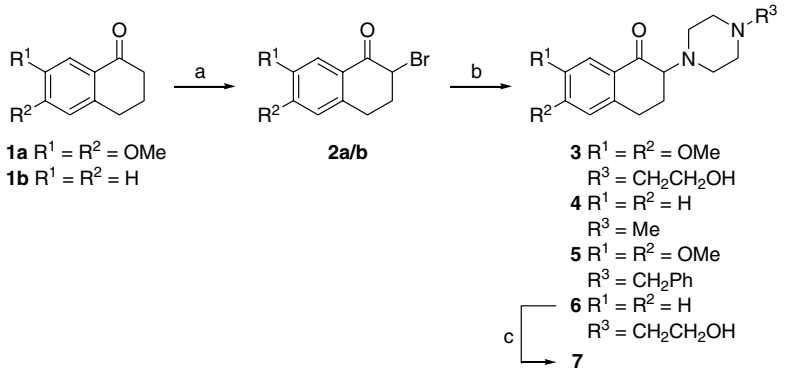
Table 1. Some 2-aminotetralone-derivatives that inhibit isolated MurA and MurZ enzymes and have antibacterial activities against *E. coli* and *S. aureus*

Compound	Structure[a]	IC_{50} (*E. coli MurA*)[b] (μM)	IC_{50} (*S. aureus* MurA)[b] (μM)	IC_{50} (*S. aureus* MurZ)[c] (μM)	MIC (*E. coli*)[d] (μg/ml)	MIC (*S. aureus*)[e] (μg/ml)
3		3.13 (±1.51)	17.35 (±3.30)	18.83 (±2.37)	256	128
4		8.53 (±2.39)	12.04 (±0.40)	22.91 (±0.80)	128	64
5		22.56 (±4.48)	29.00 (±0.16)	28.93 (±1.02)	>256	64
6		22.49 (±0.74)	12.52 (±1.82)	16.09 (±3.15)	128	8
7[f]		>120	>120	>120	>256	>256
10		>120	>120	>120	>256	>256
12		>120	>120	>120	>256	>256
Fosfomycin		0.46 (±0.06)	1.36 (±0.06)	1.37 (±0.02)	8	16

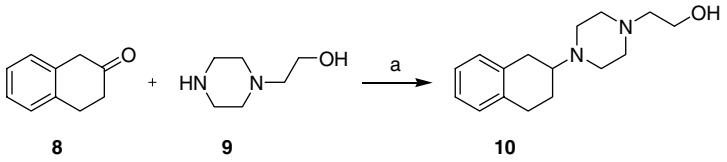
[a] Compounds **3**, **4**, **6**, **7**, and **10** were assayed as their dihydrochloride salts.
[b] Values are means of four experiments, standard deviation is given in parentheses.
[c] Values are means of three experiments, standard deviation is given in parentheses.
[d] Strain JM109.[10]
[e] Strain SH1000.[12]
[f] Single diastereomer isolated after purification. Confirmed as *trans* (*J* C(1)H-C(2)H coupling constant = 9.5 Hz).

(Table 1). These are the first reported synthetic inhibitors of both forms of UDP-GlcNAc enolpyruvyl transferase from *S. aureus*.

To probe the key structural features responsible for inhibition, analogues were synthesized by reaction of 2-bromotetralones **2a/b** (obtained from bromination of commercially available 6,7-dimethoxy-1-tetralone **1a** and α-tetralone **1b**, respectively) with the appropriate substituted piperazine (Scheme 1).[14] Inhibitory activity was maintained following removal of the aryl methoxy groups (compound **6**) or incorporation of 4-benzylpiperazine (compound **5**) (Table 1). These findings led us to investigate the importance of the ketone moiety to the mode of action of these inhibitors. Hence, we synthesized the des-carbonyl compound **10**, from β-tetralone **8** and 1-(2-hydroxylethyl)piperazine **9** as shown in Scheme 2. Compound **10** failed to inhibit MurA and MurZ (Table 1) suggesting the ketone is crucial for inhibitory activity. Next, we investigated whether an amine alpha to the ke-



Scheme 1. Synthesis of 2-aminotetralone derivatives **3**–**7**. Reagents and conditions: (a) *N*-bromosuccinimide, amberlyst-15, EtOAc, rt, 24 h; (b) 4-R^3-piperazine, K_2CO_3, DMF, rt, 24 h; (c) $NaBH_4$, THF, rt, 24 h.

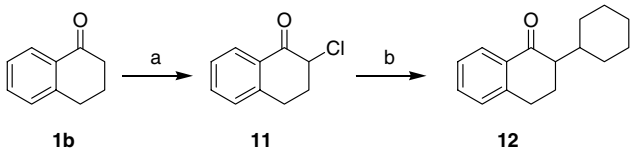


Scheme 2. Synthesis of **10**. Reagents and conditions: (a) $NaBH(OAc)_3$, AcOH, DCM, rt, 24 h.

tone was necessary for activity by synthesizing the cyclohexyl derivative **12** by a previously published route (Scheme 3).[15] When tested in the MurA and MurZ assays, **12** did not inhibit the enzymes (Table 1) suggesting that the α-aminoketone functionality is responsible for the activity of this class of inhibitors.

To determine whether the binding mode of the aminotetralone inhibitors was reversible, a dilution assay was performed. *E. coli* MurA was treated with **4**, UDP-GlcNAc and PEP followed by a 50-fold dilution of the enzyme–inhibitor complex. Assuming a reversible mode of action, there should be recovery of enzyme activity upon dilution of the reaction mix. However, as can be seen from Figure 2, the level of inhibition was maintained over the course of an hour in a manner similar to that of the covalent inhibitor, fosfomycin. Hence, these results indicate a mode of action of these inhibitors consistent with either covalent or very tight non-covalent binding.

However, attempts to detect a covalent adduct between MurA and **4** using electrospray mass spectrometry were unsuccessful, under conditions that did detect an adduct between MurA and fosfomycin (data not shown). If the covalent adduct did form in the case of **4**, it was not stable to the conditions of mass spectrometry.



Scheme 3. Synthesis of **12**.[15] Reagents and conditions: (a) *N*-chlorosuccinimide, amberlyst-15, EtOAc, rt, 24 h; (b) *c*-$C_6H_{11}MgCl$ + $ZnCl_2$, $Cu(acac)_2$, Et_2O, rt, 24 h.

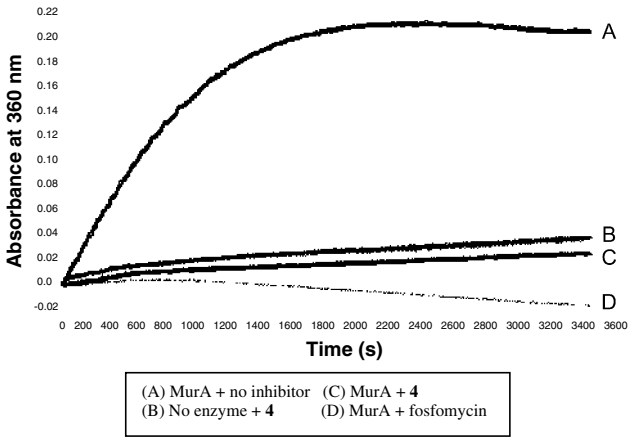


Figure 2. Test for reversible inhibition of *E. coli* MurA by **4**. No recovery of enzyme activity over time after diluting out inhibitor supports a tight-binding mode of inhibition possibly involving a covalent adduct.

Since the mode of action of fosfomycin involves attack by C115, we investigated if this residue was required for inhibition by the 2-aminotetralone inhibitors. We generated and purified the C115D MurA mutant protein[16] of *E. coli* and treated it separately with **3**, **4**, and fosfomycin. Although this mutant protein was found to be functional and, as expected, resistant to fosfomycin, no inhibition was observed with **3** and **4** at the highest concentration tested (120 μM) (data not shown). These data support the hypothesis that C115 is involved in the mechanism of these inhibitors, presumably through attack by the thiol group on the ketone moiety to generate a thiohemiketal adduct **13** (Fig. 3), which have been shown to be formed by some cysteine protease inhibitors.[17] The limited stability associated with this type of thiohemiketal may account for our failure to

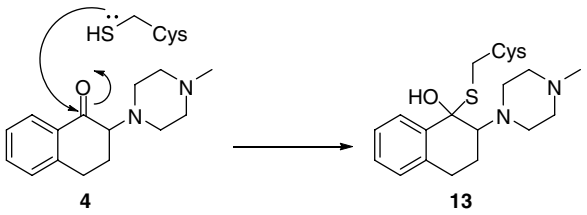


Figure 3. Proposed mechanism of inhibition for 2-aminotetralone inhibitors.

detect an adduct using MS. Indeed, rearrangement of such adducts has been previously proposed for certain cysteine protease inhibitors derived from halomethyl ketones.[18]

Using the docking program eHiTS[19] together with the X-ray crystal structure of *E. coli* MurA[9] (pdb code: 1UAE), **4** was docked into the enzyme active site (Fig. 4). As illustrated in Figure 4, **4** is predicted to bind close to the key C115 residue. Since the loop that contains C115 is known to exhibit a high degree of flexibility,[20] the cysteine residue should be capable of approaching the ketone prior to attack. Furthermore, the ketone is predicted to make a hydrogen-bond to the conserved active site residue R120, which would be expected to facilitate thiohemiketal formation.

Antimicrobial activities were tested according to the Clinical Laboratory Standards Institute (CLSI) protocol.[21]

Of the compounds that showed in vitro activity against MurA and MurZ, **3**, **4**, **5**, and **6** had MICs of 128, 64, 64, and 8 μg/ml, respectively, against *S. aureus* (Table 1). In addition, **4** and **6** had an MIC of 128 μg/ml against *E. coli*.

Selectivity assays were performed using malate dehydrogenase (MDH) and chymotrypsin.[22] Those compounds that inhibited *E. coli* MurA (**3**–**6**) showed no activity against either MDH or chymotrypsin (data not shown),

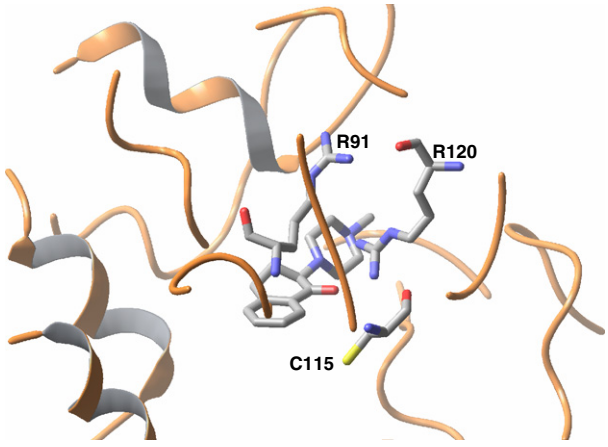


Figure 4. Inhibitor **4** docked into the active site of *E. coli* MurA. The keto-carbonyl group shown to be essential for inhibition lies in close proximity to the key C115 residue of the enzyme.

supporting the interpretation that the 2-aminotetralones are not 'promiscuous' inhibitors. These findings suggest that inhibition of MurA/MurZ is an important factor in the antimicrobial action of these inhibitors although further studies are required in order to establish whether this inhibition is the primary mode of antibacterial activity associated with these inhibitors.

In summary, we have discovered a new series of inhibitors of MurA based around a 2-aminotetralone motif. In addition, these inhibitors also displayed activity against MurA and MurZ from *S. aureus*, the first time these enzymes have been targeted by synthetic inhibitors. These results continue to provide insight into the design of new antibacterial agents.

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11. MurA activity was monitored by the detection of inorganic phosphate generated during the reaction based on the colourimetric malachite green method described by Marquadt, J.L.; Siegele, D.A.; Walsh, C.T. *J. Bacteriol.* **1992**, *174*, 5748. Inhibition of MurA was measured according to the method of Baum, E.Z.; Montenegro, D.A.; Licata, L.; Turchi, I.; Webb, G.C.; Foleno, B.D.; Bush, K. *Antimicrob. Agents Chemother.* **2001**, *45*, 3182. All assays were carried out at 25 °C. To measure MurA inhibition, a 2-fold dilution series of the inhibitor (15 μL) was added to 33.3 μg/mL MurA preincubated for 10 min. in 50 mM Tris–HCl buffer (pH 7.8) containing 187.5 μM UDP-*N*-acetylglucosamine (20 μL). The reaction was started with the addition of 15 μL of 66.7 μM phosphoenolpyruvate and the reaction mixture was incubated for 30 min. 50 μL malachite green-molybdate (0.045% w/v malachite green hydrochloride and 4.2% w/v ammonium molybdate in 4 N HCl mixed in a 3:1 ratio for 30 min and

then passed through a Millipore Steritop 0.22 mm bottle top filter unit) was added and incubated for 10 min. Absorbance at 660 nm was then measured with a Molecular Devices SpectraMax 250 plate reader. An accompanying series of inorganic phosphate standards was run for each plate. It was established that under these conditions, the production of phosphate versus time was linear.

12. SH1000 strain: Horsburgh, M. J.; Aish, J. L.; White, I. J.; Shaw, L.; Lithgow, J. K.; Foster, S. J. *J. Bacteriol.* **2002**, *184*, 5457.
13. The *murA* and *murZ* genes from *Staphylococcus aureus* SH1000 were cloned into pET-26b for expression with a C-terminal His Tag. Constructs were used to transform *E. coli* BL21 Star (DE3) cells for protein expression. Expression of *murA* was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) over 24 h with shaking at 18 °C. The expression of *murZ* was achieved using the autoinduction media described by Studier, F.W. *Protein Expression & Purification* **2005** *41*, 207. The poly(His)-tagged MurA and MurZ were purified from the soluble protein extract by affinity chromatography to Co^{2+} resin (Talon resin, Clontech). The Co^{2+} resin was equilibrated with buffer containing 50 mM sodium phosphate, 300 mM NaCl and 0.5 mM THP. The resin and soluble protein were incubated on ice with gentle shaking for 3 h, then applied to 25 ml Cell Thru Disposable Columns (BD Biosciences). The resin was washed with 200 ml of 50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole. Protein was eluted using 300 mM imidazole. Eluted protein was then incubated with 1 mM UDP-*N*-acetylglucosamine (UDP-GlcNAc) for 20 min at 4 °C with shaking. Imidazole and UDP-GlcNAc were removed by dialysis into size exclusion buffer containing 50 mM HEPES (pH 7.5), 500 mM KCl, 3 mM dithiothreitol (DTT). MurA and MurZ were further purified using a HiLoad 16/60 Superdex 200 preparation grade gel filtration column. Pooled enzymes were dialysed into storage buffer containing 50 mM HEPES (pH 7.5), 500 mM KCl, 3 mM DTT and 50% glycerol and stored at −20 °C.
14. Typical procedure: To a solution of 6,7-dimethoxy-1-tetralone (2.4 mmol) **1a** (Scheme 1) in ethyl acetate (25 ml) was added *N*-bromosuccinimide (2.5 mmol) followed by Amberlyst-15® (0.75 g) (Ref.: Hershram, H.M.; Reddy, P.N.; Sadashiv, K.; Yadav, J.S. *Tetrahedron Lett.* **2005**, *46*, 623). The reaction was stirred at room temperature for 24 h, then filtered and the catalyst washed with ethyl acetate. The filtrate and washings were combined and reduced in vacuo to give the crude product which was purified by flash chromatography on silica gel using petroleum ether (40–60 °C)/EtOAc (4:1) as eluent. 2-Bromo-6,7-dimethoxy-1-tetralone (2.10 mmol) **2a** (Scheme 1) was dissolved in anhydrous DMF (20 ml) and anhydrous potassium carbonate (2.10 mmol) added. A solution of 1-(2-hydroxyethyl)piperazine (2.10 mmol) **9** in anhydrous DMF (10 ml) was then added dropwise with stirring. The reaction was stirred at room temperature under dry N_2 for 24 h. The mixture was poured into ice water (100 ml) and extracted with DCM (3 × 75 ml). The organic phase was exhaustively washed with water, dried ($MgSO_4$), and reduced in vacuo to give the crude product which was purified using flash chromatography on silica gel using DCM/MeOH/NH_3 (90:9:1) as eluent. The purified product was dissolved in DCM (10 ml), cooled to 0 °C, and treated dropwise with ethereal HCl (1 M, 1.5 ml). The solvent was removed in vacuo and the salt recrystallised from ethyl acetate/MeOH to afford pure **3**. mp = 172–173 °C; ^{1}H NMR (300 MHz, D_2O) δ 7.11 (s, 1H), 6.59 (s, 1H), 4.22 (m, 1H), 3.66 (m, 3H), 3.58 (m, 3H), 3.53 (m, 6H), 3.42 (m, 4H), 3.17 (m, 2H), 2.84 (m, 2H), 2.29 (m, 1H), 2.01 (m, 1H); ^{13}C NMR (75 MHz, D_2O) δ 191.1, 155.0, 147.8, 140.8, 123.6, 111.1, 108.9, 69.2, 58.5, 56.4, 56.1, 55.2, 49.7, 46.5, 27.5, 23.7; HRMS (ES+) *m/z* 335.1959 (MH^+); $C_{18}H_{26}N_2O_4H^+$ requires 335.1965; LC-MS (20–95% MeCN) t_R 2.88 min (*m/z* 335.1 (MH^+), 100%).
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