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Design, synthesis and structure–activity relationships of new phosphinate inhibitors of MurD

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Abstract—A series of new phosphinate compounds were designed and synthesized as inhibitors of the D-glutamic acid-adding enzyme (MurD) involved in peptidoglycan biosynthesis. They were tested against the MurD enzyme from *Escherichia coli*, allowing initial structure–activity relationships to be deduced. Two compounds had IC_{50} values near 100 μM and constitute a promising starting point for further development.

The increasing emergence of pathogenic bacterial strains with high resistance to antibiotic therapy constitutes a serious public threat.[1] This has created an urgent need for the development of new antibacterial agents directed towards novel targets. One of the best known and most validated targets for antibacterial therapy is the machinery for peptidoglycan biosynthesis.[2,3] However, the early biosynthetic steps have received relatively little attention as potential drug targets, even though this part of the biosynthetic pathway utilizes essential enzymes that have no mammalian counterparts.

Peptidoglycan is an essential macromolecular component of the cell wall of both Gram-positive and Gram-negative bacteria. Its main function is to preserve cell integrity by withstanding the internal osmotic pressure. The glycan chains are composed of alternating units of *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac). The carboxyl group of Mur*N*Ac residues is substituted in most bacteria by a peptide unit, L-alanyl-γ-D-glutamyl-*meso*-diaminopimeloyl(or L-lysyl)-D-alanine.[3] The enzyme MurD (UDP-Mur*N*Ac-L-alanine: D-glutamate ligase), which catalyses the addition of D-glutamate to the cytoplasmic peptidoglycan precursor UDP-Mur*N*Ac-L-Ala, is present in all bacteria and is highly specific for D-glutamate.[4] High specificity, ubiquity among bacteria and absence in mammals make MurD a promising target for antibacterial therapy.[5]

There have been several attempts to target MurD with novel inhibitors.[6–13] The most potent compounds were designed as phosphinate transition state analogues.[6,7,12] In all of them the phosphinodipeptide part, Ala-ψ(PO_2-CH_2)-Glu, was conserved. They differ in the moiety mimicking the UDP-Mur*N*Ac part, for which a QSAR study has recently been made.[14] On the other hand, phosphinate transition state analogues for MurD with modifications in the peptide part have not been reported.

We were interested in the design, synthesis and biological evaluation of simplified phosphinate inhibitors of MurD. For this purpose, we selected compound **2**[7] as the starting point for an exploratory structure–activity relationship (SAR) study (Fig. 1). This compound, although lacking the UMP moiety, still possesses good inhibitory potency (IC_{50} = 20 nM). As a first step we sought a simple substitute for the phospho-sugar residue as well as an appropriate replacement of the D-lactoyl residue ('linker'). Following this we introduced modifications into the phosphinodipeptide part of the inhibitors. In lieu of phosphinoalanine, certain other phosphino amino acids were incorporated and, in addition, the glutamic acid mimetic, 2-methylenepentane-1,5-dioic acid, was truncated (Fig. 1).

The synthesis of phosphinate inhibitors **8a**, **12a**–**g** and **13a**–**j** is presented in Scheme 1. The key intermediate, the trimethyl ester of (L,D)-Ala-ψ(PO_2-CH_2)(L,D)-Glu (**9**), was prepared according to a known procedure.[6] Acetaldehyde, diphenylmethylamine hydrochloride and

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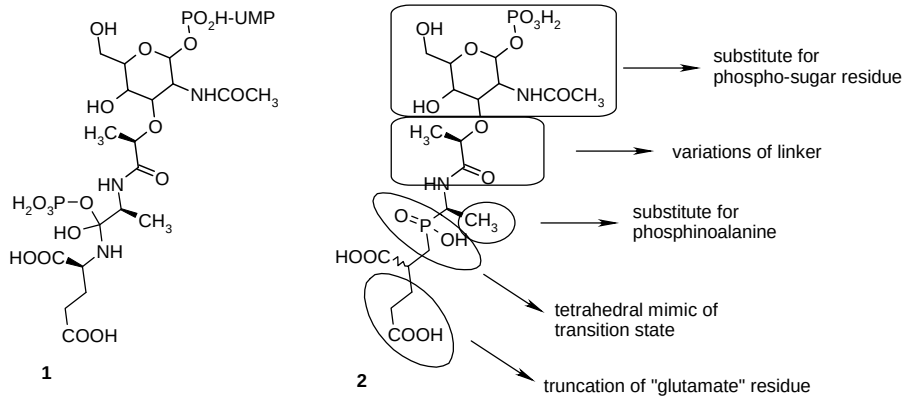
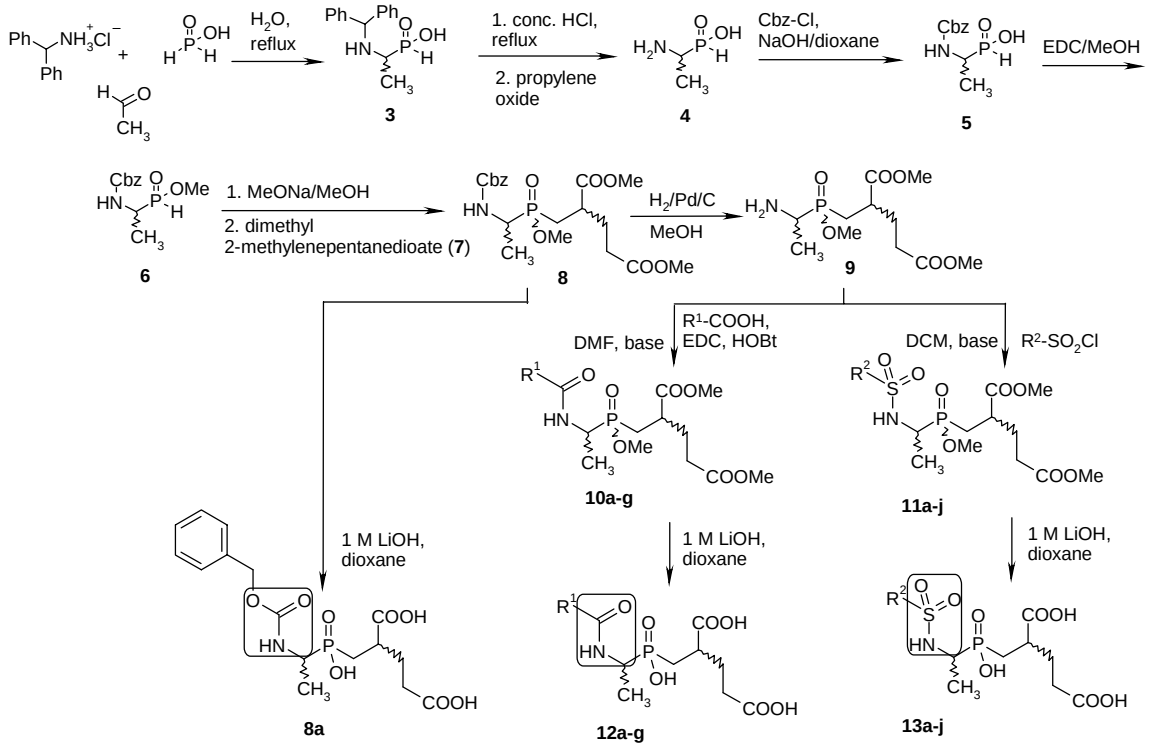


Figure 1. Transition state of the reaction catalyzed by MurD (**1**) and the structure of the potent phosphinate inhibitor **2** (IC_{50} = 20 nM). Structural features of the compounds synthesized are mentioned on the right-hand side.



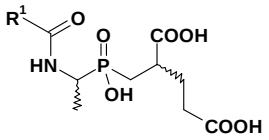
Scheme 1. Synthesis of phosphinate inhibitors **8a**, **12a**–**g** and **13a**–**j**.

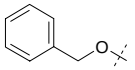
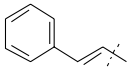
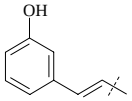
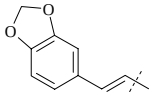
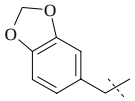
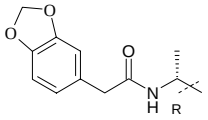
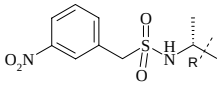
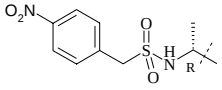
hypophosphorous acid were condensed into diphenylmethylaminophosphonous acid **3**, which was converted in two steps into Cbz N-protected α-aminoalkylphosphonous acid **5**.[15] In our hands, the preparation of the methyl ester of N-protected α-aminoalkylphosphonous acids (e.g., **6**) initially caused much trouble. From the literature it is known that α-aminoalkylphosphonous acids are prone to oxidation,[16] but we found that compound **6** decomposed back to the starting phosphinic acid **5**. For this reason, the formation of the methyl ester was carried out using EDC and dry MeOH in an argon atmosphere, and the product was used immediately in the next reaction step. From intermediates **8** and **9** we obtained carbamate **8a**, amides **12a**–**g** and sulfonamides **13a**–**j**. Alkaline hydrolysis of trimethyl ester **8** afforded compound **8a**. From the crucial intermediate amine **9** the synthesis proceeded in two directions: the amino group was substituted by different moieties mimicking the Mur*N*Ac residue of lead compound **2** via either an amide (**10a**–**g**) or a sulfonamide linkage (**11a**–**j**). The resulting trimethyl esters **10a**–**g** and **11a**–**j** were converted, by alkaline hydrolysis, into the target compounds **12a**–**g** and **13a**–**j**, respectively.

These compounds were tested for their inhibitory activity on MurD from *Escherichia coli*.[17–19] Results are presented as residual activity (RA) of the enzyme in the presence of 1 mM inhibitor (Table 1). For the most active compounds, IC_{50} values have also been determined.[17]

The results for compounds **8a** and **12a**–**g** are shown in Table 1. Compound **8a** had previously been prepared

Table 1. MurD inhibitory activity of carbamate- and amide-substituted phosphinates **8a** and **12a–g**



Compound	R^1	RA (%)[a]
8a		41 ± 2
12a		28 ± 2 (432 ± 28 μM)
12b		23 ± 2
12c		17 ± 1 (95 ± 15 μM)
12d		36 ± 1
12e		44 ± 1
12f		19 ± 3
12g		8 ± 1 (78 ± 19 μM)

[a] IC_{50} values are shown in parentheses.

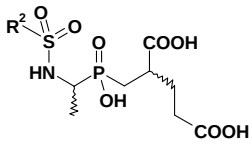
and evaluated as an inhibitor of MurD from *Streptococcus pneumoniae* with IC_{50} 100 μM.[12] From our RA value we estimate that the IC_{50} of this compound for the enzyme from *E. coli* is almost one order of magnitude greater. This relatively large difference could be explained by different assay conditions as well as by differences in the active sites of MurD from *E. coli* and *S. pneumoniae*.

The introduction of the *trans*-cinnamoyl moiety in compound **12a** resulted in a higher inhibitory activity than in compound **8a**. This trend was also observed in compound **12b**, which possesses a 3-hydroxy group on the *trans*-cinammoyl residue. Further improvement was achieved with compound **12c**, in which the *trans*-cinnamoyl moiety was further substituted by the 3,4-methylenedioxy ring. Since this 1,3-benzodioxolyl fragment proved to be a promising pharmacophore, we prepared two new compounds in which the same pharmacophore was connected to the phosphinodipeptide by two different linkers. For compound **12d**, the shorter, flexible methylene group was used. For compound **12e**, the D-alanyl residue was introduced in order to mimic the D-lactoyl residue of inhibitor **2**. However, both compounds were found to be less effective inhibitors (Table 1).

In spite of these results, compounds **12f** and **12g**, in which the nitrobenzylsulfonyl moiety is linked to the D-alanyl residue, were prepared. The latter, with a nitro group at the para position, exhibited the strongest inhibitory activity (IC_{50} = 78 μM). Its meta analogue, **12f**, inhibited the enzyme about 2 to 3 times less strongly (Table 1).

The sulfonamides **13a–j** consist of key phosphinodipeptide Ala-Ψ(PO_2-CH_2)-Glu substituted by several functionalized phenylsulfonyl and benzylsulfonyl moieties (Table 2). The most active compounds in this series were found to be **13i** and **13h**, with the *m*-nitrobenzylsulfonyl

Table 2. MurD inhibitory activity of sulfonamide-substituted phosphinates **13a–j**



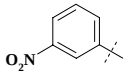
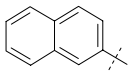
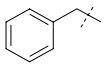
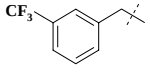
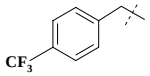
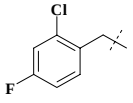
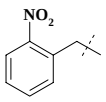
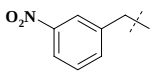
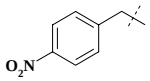
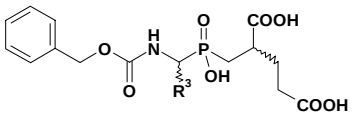
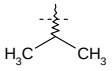
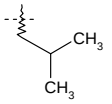
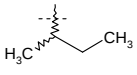
Compound	R^2	RA (%)
13a		54 ± 5
13b		52 ± 2
13c		55 ± 1
13d		40 ± 1
13e		49 ± 2
13f		50 ± 2
13g		37 ± 2
13h		30 ± 5
13i		22 ± 2
13j		47 ± 2

Table 3. MurD inhibitory activity of phosphinates **14**–**16** with branched side chains and comparison with phosphinoalanine derivative **8a**



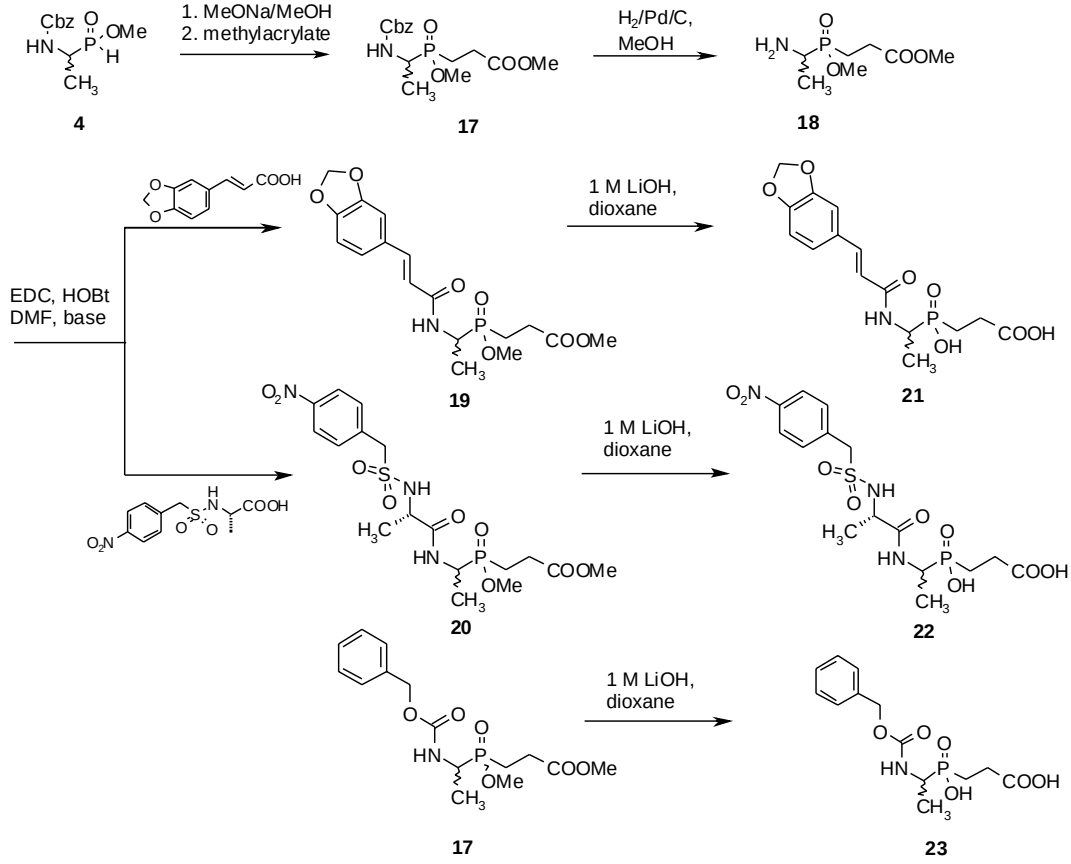
Compound	R^3	RA (%)
8a		41 ± 2
14		66 ± 1
15		65 ± 1
16		83 ± 3

and *o*-nitrobenzylsulfonyl substituents, respectively. Their positional isomer **13j** inhibited the enzyme to a lesser extent, as did the homologue of **13i** (*m*-nitrophenyl substituted derivative **13b**). However, all of them were less potent than compound **12g**.

In order to study the importance of the phosphinoalanine residue, we prepared analogues of **8a** in which it was replaced by phosphinovaline (**14**), phosphinoleucine (**15**) and phosphinoisoleucine (**16**). These compounds were synthesized by a procedure similar to that described in Scheme 1, using an appropriate starting aldehyde, but with some improvements which will be published elsewhere. All compounds inhibited MurD less than the parent compound **8a** (Table 3). Thus, replacement of the phosphinoalanine side chain by bulkier ones decreases the inhibitory activity, presumably due to the small volume of the L-alanine-binding subsite.

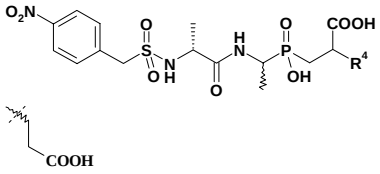
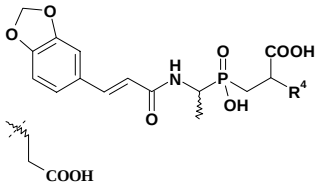
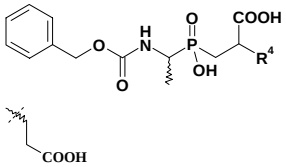
In order to determine the importance of the γ-carboxylate group of the phosphinodipeptide glutamate residue, we prepared compounds **21, 22** and **23** as analogues of **12c**, **12g** and **8a**, respectively, but lacking the glutamic acid side chain. The synthesis of these truncated analogues is similar to that presented in Scheme 1, using methyl acrylate instead of dimethyl 2-methylenepentanedioate **7** (Scheme 2). Compounds **21**–**23** did not inhibit MurD (Table 4), confirming our previous hypothesis[9] that the presence of the glutamate residue, or its appropriate mimetic, in this type of inhibitor is essential for strong inhibition.

In the complex of MurD with its product UDP-Mur*N*Ac-L-Ala-D-Glu (MurD · UMAG, pdb entry 4UAG),[20] the α-carboxylic acid of D-glutamic acid is H-bonded to Lys348 and Thr321, the γ-carboxylate is



Scheme 2. Synthesis of the truncated phosphinates **21**–**23**.

Table 4. MurD inhibitory activity of truncated compounds **21**–**23** and comparison with that of their non-truncated analogues

Compound	R^4	RA (%)[a]
12g		8 ± 1 (78 ± 19 μM)
22		100 ± 5
12c		17 ± 1 (95 ± 15 μM)
21		91 ± 1
8a		40 ± 2
23		92 ± 1

[a] IC_{50} values are shown in parentheses.

held in place by hydrogen bonds with Ser415 and Phe 422, and the amide bond between lactoyl moiety and L-Ala interacts with the side chain of Asn138.[20] The absence of inhibition by truncated analogues **21**–**23** is consistent with the loss of binding energy of the two hydrogen bonds provided by the γ-carboxylate group. We can thus expect that the best phosphinate inhibitors found here (**12c**, **12f** and **12g**) bind the enzyme in a similar manner as UMAG. It is possible that they utilize their substituted aromatic rings to interact also with Leu15, Thr16 or Gln162, which are the residues that bind the phospho-sugar part of UMAG.

In the present paper, we reported the synthesis and activity of a series of new phosphinate inhibitors of MurD from *E. coli*. A common feature of all the active compounds is the N-substituted phosphinodipeptide Ala-ψ(PO_2-CH_2)-Glu, both alanine and glutamate residues being essential in this series. Compounds **12c** and **12g** had IC_{50} values near 100 μM. Since they consist of mixtures of four diastereoisomers, the actual IC_{50} for the one most closely mimicking the L-Ala-D-Glu stereochemistry is presumably lower than that observed. These compounds constitute promising starting points for further structural modifications.

Acknowledgments

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17. Enzymatic assays were performed as described[8] with slight modifications. The compounds were tested for their ability to inhibit the addition of D-[^{14}C]Glu to UDP-Mur*N*Ac-L-Ala in a mixture (final volume: 50 μl) containing 0.1 M Tris–HCl, pH 8.6, 5 mM $MgCl_2$, 25 μM UDP-Mur*N*Ac-L-Ala, 25 μM D-[^{14}C]Glu (50,000 cpm), 5% (v/v) DMSO, and purified MurD[18] (diluted with 20 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol and 1 mg/ml BSA) and 1 mM tested compound (all compounds were soluble in the assay mixture containing 5% DMSO). The mixture was incubated for 30 min at 37 °C, and the reaction was stopped by adding 10 μl glacial acetic acid. The mixture was lyophilized and taken up in the HPLC elution buffer. The radioactive substrate and product were separated by reverse-phase HPLC with a Nucleosil $5C_{18}$ column (150 × 4.6 mm) as stationary phase, and isocratic elution at a flow rate of 0.6 ml/min with 50 mM ammonium formate, pH 4.7. Compounds were detected and quantified with an HPLC radioactivity monitor LB 506 C-1 (Berthold France, Thoiry, France) using Quickszint Flow 2 scintillator (Zinsser Analytic, Maidenhead, UK) at 0.6 ml/min. Residual activity was calculated with respect to a similar assay without inhibitor. Values are expressed as means ± standard deviations of duplicate determinations. IC_{50} values were determined from a range of inhibitor concentrations; values ± standard deviations at 95% of confidence were calculated from the fitted regression equations using the logit-log plot.
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19. All the compounds gave satisfactory spectroscopic data and elemental analyses. Representative results for compound **12c** are given: mp 82–84 °C; IR (KBr): *v* 3427, 1717, 1653, 1449, 1254, 1036, 970, 807 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.14–1.28 (m, 3H, –C*H*$_3$), 1.53–2.33 (m, 6H, –C*H*$_2$–CH(COOH)–C*H*$_2$–C*H*$_2$–COOH), 2.57–2.70 (m, 1H, –CH$_2$–C*H*(COOH)–CH$_2$), 4.07–4.23 (m, 1H, –PC*H*), 6.06 (s, 2H, –O–C*H*$_2$–O–), 6.59 (d, $^3J_{H-H}(E)$ = 15.1 Hz, 1H, –HC=C*H*–CONH), 6.95 (d, J_{ortho} = 7.9 Hz, 1H, Ar*H*-5), 7.07 (d, J_{ortho} = 7.9 Hz, 1H, Ar*H*-6), 7.11 (s, 1H, Ar*H*-2), 7.36 (d, $^3J_{H-H}(E)$ = 15.4 Hz, 1H, Ar-*H*C=CH–). ^{31}P NMR (DMSO-d_6): δ 45.65 (1P), 45.27 (1P). MS (FAB): *m*/*z* 428 $(M+H)^+$; Anal. Calcd for $C_{18}H_{22}NO_9P \times H_2O$: C, 48.54; H, 5.43; N, 3.14. Found: C, 48.75; H, 5.42; N, 2.99.
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