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Pulvinones as bacterial cell wall biosynthesis inhibitors

Schuyler Antane,[a,*] Craig E. Caufield,[a] William Hu,[b] David Keeney,[b] Pornpen Labthavikul,[b] Koi Morris,[a] Shaughnessy M. Naughton,[a] Peter J. Petersen,[b] Beth A. Rasmussen,[b] Guy Singh[b] and Youjun Yang[b]

[a]*Medicinal Chemistry, Chemical and Screening Sciences, Wyeth Research, Princeton, NJ 08543, USA*
[b]*Infectious Diseases, Wyeth Research, Pearl River, NY 10965, USA*

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Abstract—Pulvinones were synthesized (>180) in arrays and evaluated as inhibitors of early stage cell wall biosynthesis enzymes MurA–MurD. Several pulvinones inhibited Mur enzymes with IC_{50}'s in the 1–10 μg/mL range and demonstrated antibacterial activity against Gram-positive bacteria including methicillin-resistant *Staphyloccus aureus*, vancomycin-resistant *Enterococcus faecalis*, and penicillin-resistant *Streptococcus pneumoniae*.

Enzymes of the murein pathway mediate bacterial cell wall biosynthesis.[1] The initial enzymes, MurA–MurB, convert uridinediphosphate *N*-acetylglucosamine (UDP-GlcNAc) into uridinediphosphate *N*-acetylmuramic acid (UDP-MurNAc). A pentapeptide chain is appended to the UDP-MurNAc by the amino acid ligases MurC–MurF. The nucleotide of the UDP-MurNAc-peptide unit is replaced with membrane bound lipid phosphate. MurG couples a GlcNAc to the lipid bound MurNAc and the resultant disaccharide is transferred across the membrane to the growing cell wall molecule. Although the early stage enzymes (MurA–MurD) are well validated as antibacterial targets only fosfomycin (a MurA inhibitor) has been used as a clinical product.[2]

Naturally occurring vulpinic acid (Fig. 1) has been reported to possess in vitro activity against Gram-positive bacteria.[3] In-house screening for Mur enzyme inhibitors identified pulvinamide **2**[4] [MurC (IC_{50} = 8 μg/mL)]. Our discovery effort revealed analogues of the des-amido pulvinone **3**[5] also inhibit MurC with an IC_{50} of $\leqslant$8 μg/mL and demonstrated antibacterial activity against Gram-positive bacteria including methicillin-resistant *Staphyloccus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), and penicillin-resistant *Streptococcus pneumoniae* (PRSP).[6]

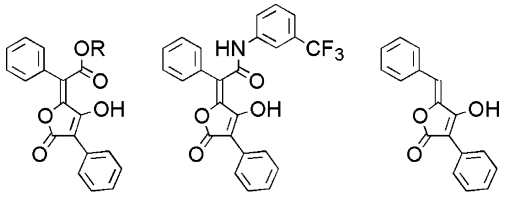

Figure 1. Structures of pulvinic acid **1** (R = H), vulpinic acid **1** (R = Me), pulvinamide derivative **2**, and pulvinone **3**.

Pulvinones (**10**) were prepared in five steps (Scheme 1) starting from commercially available 4-methoxy-2(5*H*)-furanone (**4**). Bromination with *N*-bromosuccinimide yielded **5**.[7] Aldol reaction of various aldehydes with **5** deprotonated by lithium isopropyl cyclohexylamide (LICA)[8] afforded secondary alcohols (**6**). The resultant alcohols were eliminated via in situ mesylate formation to afford the halogenated Suzuki coupling partner **8** in the more thermodynamically stable *Z*-form.[9]

Alternatively, the aldol reaction may be performed first using lithium hydroxide. Reaction of **4** with various aldehydes under these conditions gave **7**.[10] Subsequent bromination in acetonitrile afforded **6** that was eliminated as before to provide the bromo alkylidene furanone

Keywords: Pulvinones; Murein enzyme inhibitors; Antibacterial; Microwave demethylation.
* Corresponding author. E-mail: antanes@wyeth.com

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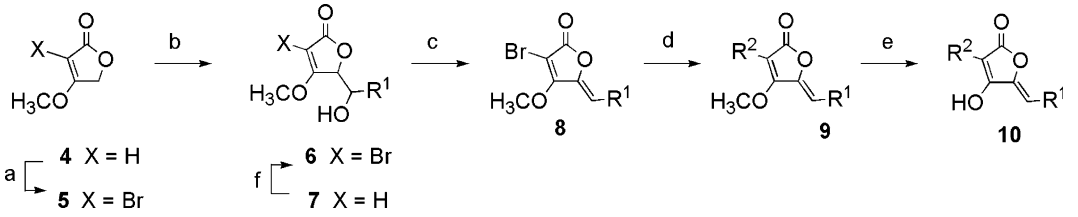


Scheme 1. Synthesis of pulvinones. Reagents and conditions: (a) NBS, CCl_4, reflux; (b) LICA, THF, R^1CHO, −78 °C or LiOH, CH_3CN, R^1CHO, rt; (c) MsCl, TEA, CH_2Cl_2, 0 °C, 90 min; (d) $Pd(PPh_3)_4$, K_3PO_4, $R^2B(OH)_2$ dioxane, Synthewave® 402 microwave (Prolabo), 15% pwr, 5 min or 100 °C, 90 min; (e) LiBr, DMF, Synthewave® 402 microwave (Prolabo), 30% pwr, 4 min or heat 150 °C, 15 min; (f) Br_2, CH_3CN, rt.

8. Suzuki coupling of **8** with various boronic acid derivatives yielded the penultimate methoxy furanones (**9**), which were demethylated with lithium bromide to afford the final pulvinone products (**10**). The last two steps were accelerated by microwaves.[11]

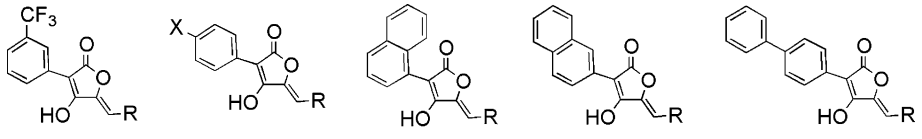
Pulvinones (**10**) were initially prepared in a matrix of five arrays (**11**–**15**). MurA–MurD inhibition was assayed sequentially unlike the efficient combinatorial enzymology approach utilized at Merck.[13] The methoxy precursors (**9**) were devoid of significant activity. Representative final products from each array are shown in Table 1. Overall these pulvinones appeared to consistently inhibit MurC. In addition, the pulvinones displayed varying MurB inhibition and inhibition of MurA to a lesser extent.

The naphthylidene derivatives (**11a**,**b**) were nearly identical in activity. The cyclohexylidene derivatives (**11c** and **14c**) appeared to show a slight preference for MurC and were highly variable in MIC effectiveness. *para*-Chloro substitution (**12a**,**c**) was slightly better than *para*-methoxy substitution (**12b**,**d**) in MurB–MurC inhibition and activity versus VRE.

The phenyl-*m*-trifluoromethylbenzylidene (**12a**) was equipotent with naphthylidene derivatives (**11a**,**b**). The overall antibacterial activity of the 1-,2-naphthyl-*m*-trifluoromethylbenzylidenes (**13a** and **14a**) was slightly enhanced by replacement with biphenylmethylidene (**13b** and **14b**).

Biphenylmethylidene (**14b**) appeared to show a slight preference for MurB. A double Suzuki[14] coupling reaction of dibromo *o*,*m*,*p*-isomers **16** (Scheme 2) provided additional arrays of biphenylmethylidenes **18**. Biphenyl directly bonded to the furanone core **15a** demonstrated the best overall Gram-positive

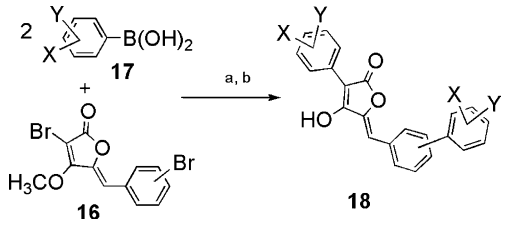
Table 1. Minimal inhibitory concentration (MIC, μg/mL) and in vitro activity against MurA–MurD enzymes (IC_{50}, μg/mL)[12]



11 **12** **13** **14** **15**

	11a[d]	11b[e]	11c[f]	12a[g,i]	12b[h,i]	12c[d,g]	12d[d,h]	13a[i]	13b[j]	14a[i]	14b[j]	14c[f]	15a[i]	15b[k]
S. aureus GC 1131[a]	8	8	32	16	1	8	32	32	8	8	16	>128	4	16
S. aureus GC 4543	8	8	>128	8	128	8	64	64	4	8	4	16	4	16
E. faecalis GC 4555	16	16	64	16	128	16	>128	>128	32	32	8	32	8	8
E. faecalis GC 2242[b]	8	4	32	8	64	8	32	32	8	8	4	8	4	4
S. pneumoniae GC 1894[c]	0.5	0.25	⩽0.12	1	2	0.5	8	8	4	8	2	⩽0.12	0.5	1
MurA	>25	>25	>25	14	>25	>25	>25	>25	16	20	>25	>25	17	>25
MurB	8	11	>25	9	>25	10	>25	>25	>25	10	2	>25	5	—
MurC	6	6	10	6	14	7	16	13	9	6	10	9	8	16
MurD	>25	>25	>25	>25	25	>25	>25	>25	>25	>25	>25	>25	>25	>25

[a] MRSA.
[b] VRE.
[c] PRSP.
[d] R = 1-naphthyl.
[e] R = 2-naphthyl.
[f] R = cyclohexyl.
[g] X = Cl.
[h] X = OMe.
[i] R = 3-CF_3Ph.
[j] R = biphenyl.
[k] R = 4-ClPh.



Scheme 2. Double Suzuki. Reagents: (a) $Pd(PPh_3)_4$, K_3PO_4, dioxane; (b) LiBr, DMF.

antibacterial activity of the first matrix with MIC's in the range 0.5–8 μg/mL.

Inspired by the oxazolidinone antibiotic linezolid[15] and its suggestive 6-6-5-framework similarity to **15**, we replaced the biphenyl of **15** with an *ortho*-fluorophenylmorpholine to generate **19** (Fig. 2). The preparation of **19** necessitated the synthesis of the boronate derivative **22** from 4-bromo-2-fluoroaniline

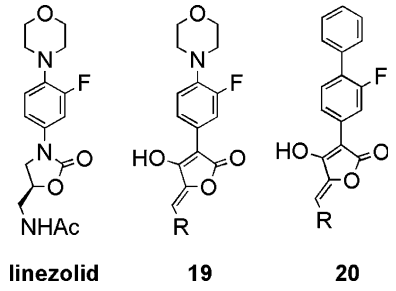
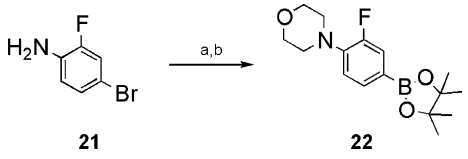
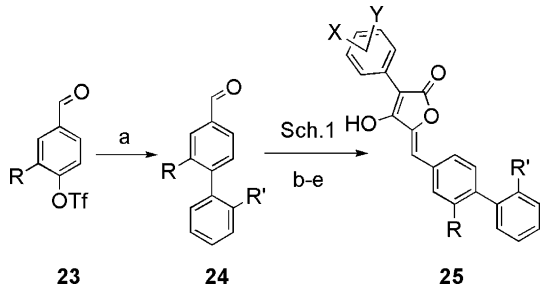


Figure 2. Substructure graft of linezolid onto pulvinones.



Scheme 3. Synthesis of 3-fluoro-4-morpholinophenylboronic acid pinacol ester. Reagents: (a) $(ClCH_2CH_2)_2O$, NaI, K_2CO_3, DMF; (b) pinacolborane, $PdCl_2(PPh_3)_2$, dioxane, TEA.



Scheme 4. Synthesis of 2,2′-disubstituted biphenylmethylidene pulvinones. Reagents and conditions: (a) 2-*R*′PhB(OH)$_2$, $Pd(PPh_3)_4$, K_3PO_4, dioxane, 100 °C, overnight.

(Scheme 3). For comparative purposes we prepared **20** from the commercially available 2-fluoro-4-biphenyl boronic acid.

Polychlorinated pulvinone derivatives **18** evoked toxicity comparisons to the persistent environmental pollutant PCBs.[16] Prudently we reduced the number of halogens in a follow-up array (Scheme 4). Suzuki reaction of *ortho*-substituted boronic acids with *p*-bromo or *p*-triflate aldehydes represented by **23** yielded 2,2′-disubstituted biphenyl aldehydes **24.** These aldehydes were reacted with the bromofuranone **5** as before (Scheme 1, steps b–e) to produce the less halogenated pulvinones (**25**).

Potent antibacterial activities were observed for these biphenyl arrays versus Gram-positive organisms: *S. aureus* including MRSA, *E. faecalis* including VRE, and penicillin-resistant *S. pneumoniae* (Table 2). Clearly the most potent were the isomeric tetrachlorobiphenylmethylidenes **18a**–**c**. The dichloro analogue **18d** inhibited MurA–Mur D but exhibited less antibacterial activity.

The morpholine-substituted pulvinone **19** was ineffective, despite showing modest MurB inhibition. Replacement of the morpholine with a phenyl ring (**20c**) increased inhibition of MurA–MurD. The wider spectrum and enhanced potency of Mur enzyme inhibition exhibited by these pulvinones were difficult to explain. It seemed less probable for the pulvinones to mimic the common phosphate motifs in the natural substrates and co-factors than it was to occupy an allosteric inhibitory site, perhaps located in a solvent exposed region.[17]

The 2,2′-disubstituted biphenylmethylidenes (**25a**,**b**,**d**, and **e**) displayed primarily MurA and MurB inhibition, except for a dimethoxy analogue (**25c**) that also inhibited MurC. For the most part, the Gram-positive (*S. aureus*) enzyme data seemed to be in agreement with the Gram-negative (*Escherichia coli*) enzyme data (Table 2). However, concerning MurD enzyme data, it has been noted that Gram-negative is more restrictive than Gram-positive.[18]

Pulvinones may be considered decarboxylated analogues of pulvinic acids. To summarize, we have found that the carboxyl group of pulvinic acid derivatives is not essential for murein enzyme inhibition. Therefore, pulvinones are novel inhibitors of bacterial cell wall biosynthesis enzymes. Matrix production of pulvinone arrays, some expedited by microwave-assisted Suzuki and demethylation reactions, hastened the identification of several derivatives that possessed antibacterial activity against Gram-positive organisms: *S. aureus* including MRSA, *E. faecalis* including VRE, and penicillin-resistant *S. pneumoniae*. Finally, the ability of selected pulvinones to block multiple Mur enzymes may be preferred since bacterial resistance might require genetic mutations at multiple targets.[19]

Table 2. Minimal inhibitory concentration (MIC, μg/mL) and in vitro activity against MurA–MurD enzymes (IC_{50}, μg/mL) of select pulvinones[12]

	18a[e,n]	**18b**[f,o]	**18c**[e,p]	**18d**[g,n]	**19**[h]	**20a**[i]	**20b**[j]	**20c**[h]	**20d**[k]	**25a**[e,l]	**25b**[e,m]	**25c**[e,q]	**25d**[e,r]	**25e**[g,r]
S. aureus GC 1131[a]	0.25	0.5	4	>128	64	2	2	1	1	0.5	0.5	2	0.5	2
S. aureus GC 4543	0.25	0.25	0.5	1	128	2	2	1	1	0.5	0.5	2	0.5	1
E. faecalis GC 4555	1	1	1	8	128	1	2	4	2	2	2	8	2	4
E. faecalis GC 2242[b]	0.5	0.5	0.5	4	128	0.25	2	1	0.5	1	0.5	8	1	2
S. pneumoniae GC 1894[c]	0.25	⩽0.12	⩽0.12	1	16	0.25	0.5	0.25	0.25	0.5	⩽0.12	0.5	0.5	⩽0.12
MurA[d]	2	3	1	5	>25	10	1	3	1	4	2	17	(2)	4
MurB	3	2	3	4	10	12	1	1	1	2	1	6	3	3
MurC[d]	11	(18)	17	5	(>25)	8	2	5	10	(20)	(23)	6	(>25)	(15)
MurD[d]	(6)	(5)	6	6	(>25)	>25	6	9	16	(17)	(9)	>25	(24)	(12)

[a] MRSA.
[b] VRE.
[c] PRSP.
[d] Values in parentheses are *S. aureus* enzymes, all others are for *E. coli.*
[e] X,Y = 3,5-diCl.
[f] X,Y = 3,4-diCl.
[g] X = 3-Cl, Y = H.
[h] R = 2-Cl,5-CF_3-Ph.
[i] R = 3-CF_3Ph.
[j] R = 2-F,5-CF_3-Ph.
[k] R = 2-Cl,3-CF_3-Ph.
[l] R = OMe, R′ = CF_3.
[m] R = R′ = CH_3.
[n] *meta*-biPh.
[o] *ortho*-biPh.
[p] *para*-biPh.
[q] R = R′ = OMe.
[r] R = CH_3, R′ = CF_3.

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11. Typical microwave Suzuki procedure: in a 2.5 mL Wheaton Reacti-Vial was placed 0.2 mmol bromofuranone **8** (Scheme 1), 0.25 mmol aryl boronic acid, 0.7 mmol potassium phosphate, and 5 mol% tetrakis(triphenyl-phosphine)-palladium. The mixture was diluted with dioxane to 0.1 M and irradiated at 15% power for 5 min in a Synthewave® 402 microwave (Prolabo). The vials were centrifuged and decanted onto silica gel in order to purify by parallel chromatography (Biotage Quad 3). Typical microwave demethylation procedure: a 20 × 150 mm test tube was charged with 1 mmol of methoxy furanone **9** (Scheme 1) and 1.1 mmol lithium bromide. The mixture is diluted with 1.5 mL DMF and irradiated in a Synthewave® 402 microwave (Prolabo) at 30% power for 4 min. Upon cooling, crude material was poured over 2 M sulfuric acid and extracted with ethylacetate. The crude lithium tetronates were purified by parallel chromatography (Biotage Quad3) and re-acidified to the tetronic acids **10** by washing with 2 N hydrochloric acid.
12. MICs: The quantitative in vitro determination of minimum inhibitory concentration (MICs) of novel compounds against 20 strains of Gram-positive aerobic bacteria was performed by microdilution broth method of testing as recommended by the standards approved by the National Committee for Clinical Laboratory Standards (NCCLS), *Methods for Dilution Antimicrobial Susceptibility Test for Bacteria that Grow Aerobically*–5th edition. Approved Standard Document M7-A5 Vol. 20. No 2, NCCLS, Wayne, Pa.MurA–MurD enzyme biochemical assays: novel compounds were tested for inhibition of murein enzymes in buffered solutions. Optical densities (OD) due to the release of phosphate during MurA, MurC or MurD enzymatic reactions were measured by a SPECTRAmax®250 (Molecular Devices) 660 nm. The OD measurement in the Mur B assay, however, directly detected the appearance of nicotinamide-adenine dinucleotide phosphate (NADP) at 340 nm. The difference in readings between control conditions (buffered solutions of natural substrate(s), enzyme, and co-factors) and in the presence of novel compounds determines the percent inhibition. IC_{50}'s are then calculated by linear regression analysis using SoftMax® software (Molecular Devices). The MurA assay detected the quantity of inorganic phosphate cleaved from phosphoenolpyruvate (PEP) and consisted of 25 mM Tris–HCl buffer, pH 8, 150 nM MurA, 200 μM PEP, and 200 μM UDP-GlcNAc in final volume 25 μL. The procedure is

similar to that described by Marquardt, Walsh, and others (*Biochemistry* **1994**, *33*, 10646). The second enzyme assay, MurB, followed the appearance of NADP upon reduction of enolpyruvate (EP) via direct readings taken at 340 nm and contained 50 mM Tris–HCl, pH 8.0, 10 mM KCl, 100 μM NADPH, and 50 μM EP-UDP-GlcNAc, in a total volume of 200 μL. This procedure was similar to that described by Dhalla and others (*Biochemistry* **1995**, *34*, 5390). The amino acid ligase MurC assay detected the quantity of inorganic phosphate released (OD at 660 nm) from the adenosinetriphosphate (ATP) hydrolysis and contained 83 mM Tris–HCl buffer, pH 8.5, 200 nM MurC, 50 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 2 mM ATP, 1.275 mM UDP-MurNAc, and 2 mM L-alanine in a final volume of 25 μL. The amino acid ligase MurD assay detected the quantity of inorganic phosphate released (OD at 660 nm) from ATP hydrolysis and consisted of 83 mM Tris–HCl buffer, pH 8.5, 68 nM MurD, 50 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 2 mM ATP, 0.375 mM UDP-MurNAc-L-Ala, and 2 mM D-glutamate (final volume = 25 μL). These reactions were initiated by the addition of appropriate UDP-linked substrate and incubated at 37°C for 30 min. The reactions were terminated by the addition of 210 μL of malachite green reagent (0.03% malachite green, 1.4% ammonium molybdate, and 1.3 N HCl). In order to minimize the acid hydrolysis of ATP in MurC and MurD reactions, 50 μL of 34% (v/w) sodium citrate was added to the reaction after the malachite green reagent. This procedure is similar to that described by Lanzetta and others (*Anal. Biochem.* **1979**, *100*, 95).

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