Bioorganic & Medicinal Chemistry Letters 19 (2009) 524–527

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Antibacterial alkoxybenzamide inhibitors of the essential bacterial cell division protein FtsZ

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ARTICLE INFO

Article history:
Received 2 October 2008
Revised 7 November 2008
Accepted 10 November 2008
Available online 13 November 2008

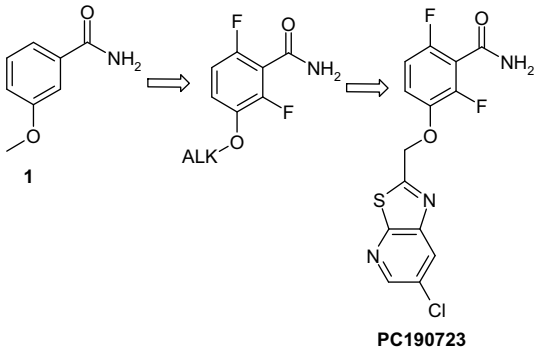
Keywords:
Antibacterial
Alkoxybenzamide
FtsZ
3-Methoxybenzamide
Antibacterial efficiency

ABSTRACT

3-Methoxybenzamide is a weak inhibitor of the essential bacterial cell division protein FtsZ. Exploration of the structure–activity relationships of 3-methoxybenzamide analogues led to the identification of potent anti-staphylococcal compounds.

New antibiotics are urgently needed to treat the increasing number of life-threatening bacterial infections that are resistant to current therapies. In particular, the emergence and spread of drug-resistant staphylococci is of serious concern. Here we report the discovery and characterization of a novel class of small synthetic antibacterials that have potent activity against staphylococci.

The essential bacterial cell division protein FtsZ has been recognized as an attractive but as yet underexploited target for antibacterial drug discovery.[1,2] When bacteria divide, FtsZ undergoes GTP-dependent polymerization at mid-cell to form the Z-ring and then recruits other cell division proteins to synthesize the septum that enables the daughter cells to separate.[3] FtsZ is structurally and functionally homologous to mammalian β-tubulin, which has been successfully exploited for cancer therapy.[4–6] This suggests that FtsZ may also be amenable to inhibitor development. Several compounds have been reported to block bacterial cell division through inhibition of FtsZ.[7–13] We have explored one of these compounds, 3-methoxybenzamide (3-MBA; Compound **1**; Fig. 1)[7], leading to the identification of a potent 3-MBA derivative, PC190723, that inhibits FtsZ activity and kills staphylococci[14] (Fig. 1). Here we present the early structure–activity relationship (SAR) data leading to its synthesis.



Compound 1 3-Methoxybenzamide. *B. subtilis* MIC 4000 μg.ml^{-1}

Figure 1. Design of 3-MBA analogues leading towards PC190723.

3-MBA is an attractive fragment-like starting point for FtsZ inhibitor design. Although it possesses weak on-target antibacterial activity (*Bacillus subtilis* minimum inhibitory concentration

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doi:10.1016/j.bmcl.2008.11.021

(MIC) 4000 μg ml^{-1}), because of its low molecular weight it has a high ligand efficiency.[15] It is able to penetrate bacterial cells, which is often a barrier to novel antibacterial discovery, and it has proven to be an effective starting point for, or has been a key feature in, the discovery of inhibitors in other therapeutic areas.[16–18]

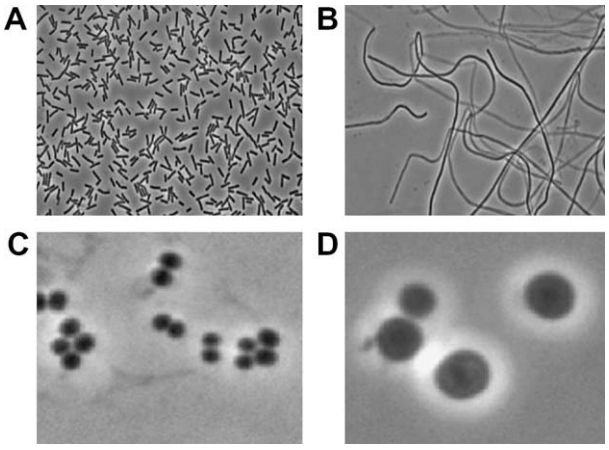
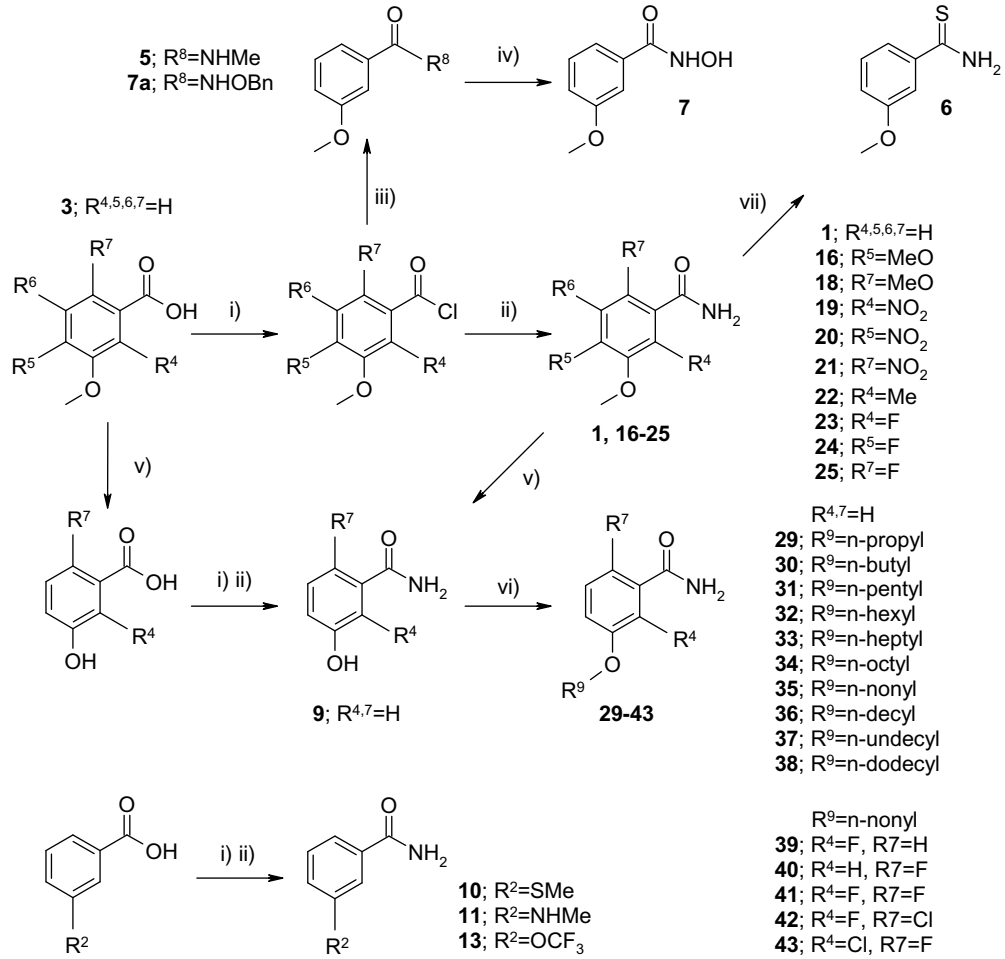


Figure 2. Cells of *B. subtilis* 168 (A and B) or *S. aureus* ATCC 29213 (C and D) were cultured (4 h) in the absence (A and C) or presence of 8 μg ml^{-1} compound **32** (B and D) and analysed by phase-contrast microscopy. *B. subtilis* filaments and *S. aureus* balloons in response to contact with cell division inhibitors.

Our objective was to increase the potency of 3-MBA against bacterial cells while retaining the on-target inhibition of cell division. The biological activities of the compounds were characterized by measuring their MICs against *B. subtilis* 168 and by morphometric analysis to determine on-target activity as expressed by filamentation of the bacilli due to continued short-term growth in the absence of cell division (Fig. 2).[11]

Selected compounds were also tested against *Staphylococcus aureus* ATCC 29213 to determine both potency and on-target activity, expressed by ballooning of the cocci in this species (Fig. 2).[14,19] Genetic studies were used to confirm that selected compounds in the series inhibited cell division through their interaction with FtsZ.[7,14]

The preliminary SAR exploration of compound **1** started with purchasable close analogues and extended to those that could be synthesized from commercial building blocks in one to four steps as illustrated in Scheme 1. Conversion of the carboxylic acids to the corresponding primary amides was achieved via the acyl chlorides and subsequent reaction with aqueous ammonia. In the case of compounds **5** and **7a**, methylamine and *O*-benzyl-hydroxylamine were used, respectively, while catalytic hydrogenation of **7a** afforded compound **7**. Thiobenzamide **6** was prepared from **1** in one-step using Lawesson's reagent. The deprotection of the methoxy group in **1** was performed with boron tribromide to provide **9**. Compounds **29**–**43** were synthesized by alkylation of the phenol with alkyl halides in the presence of catalytic sodium iodide under modified Finkelstein conditions. The chemical structures of



Scheme 1. Reagents and conditions: (i) $SOCl_2$, toluene, reflux; (ii) THF, aq NH_3; (iii) $MeNH_2$ or $BnONH_2$; (iv) H_2 10% Pd/C; (v) BBr_3, CH_2Cl_2, rt; (vi) RBr or RCl, K_2CO_3, NaI, DMF, 60 °C; (vii) Lawesson's reagent. Compounds **2**, **4**, **8**, **12**, **14**, **15**, **17**, **26**, **27**, **28** are not shown on the scheme and were purchased.

the 3-MBA derivatives obtained were confirmed by ^{1}H NMR and mass spectrometry and the purity was demonstrated by HPLC analysis.

The amide and 3-ether substituents of compound **1** appeared to be critical for inhibition of cell division (Table 1). Only 3-ethoxybenzamide **12** improved antibacterial activity.

Preliminary SAR indicated that few substitutions of the benzamide ring were tolerated (Table 2). R^4 and R^7 substitution with small halogens was preferred leading to compounds **23** and **26**, which demonstrated greater potency and on-target activity than compound **1**.

As options for optimization of the benzamide to improve potency appeared to be limited, and encouraged by the biological activity of compound **12** we focused on the modification of the 3-methoxy substituent of compound **1** as shown in Table 3.

Extension of the 3-alkyloxy substituent resulted in a substantial improvement in antibacterial activity and led to the identification of compound **35** with on-target activity and MICs of 0.5 and 2 μg ml^{-1} against *B. subtilis* and *S. aureus*, respectively. The SAR indicates that extension beyond the optimal nonyl alkyl chain results in a reduction of activity.

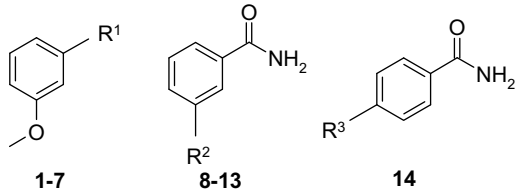
The effect of the active halogen substitutions (compounds **23–28**) in combination with the 3-nonyloxy substituent of **35** was explored (Table 4). Halogenation at R^4 was associated with further improvements in antibacterial activity. Compounds **39** and **41** are >10,000 times more potent than **1** and retain on-target inhibition of cell division in bacterial cells. We believe these compounds are the most potent antibacterial cell division inhibitors described to date.

Genetic studies confirmed that selected compounds exerted their cell division inhibitory activity through their interaction with FtsZ. Spontaneous compound **32** resistant mutants of *S. aureus* ATCC 601055 were isolated at a frequency of 4×10^{-7} at 4× MIC. The FtsZ genes were sequenced and point mutations M218I and P300R were identified (Table 5). The FtsZ mutations conferred resistance to compounds **1**, **32** and **35**. Furthermore, in *B. subtilis* a site-directed FtsZ mutation V307R[14] increased the MIC of compound **32** from 16 to >128 μg ml^{-1}.

Compounds **1** and **32** were effective against four methicillin-resistant *S. aureus* (MRSA), three methicillin-sensitive *S. aureus* (MSSA) strains and one strain of *Staphylococcus epidermidis* (Table 6). There was no evidence that methicillin-resistance altered sensitivity to the alkyloxybenzamide cell division inhibitors.

An apocrystal X-ray structure of the *B. subtilis* FtsZ protein (2vxy) has been used to develop a docking model that is consistent

Table 1
B. subtilis MIC and cell division inhibitory activity for compounds **1–14**

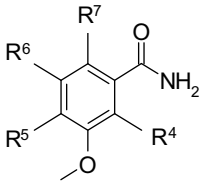


1-7 **8-13** **14**

Compound	R-group	*B. subtilis* MIC (μg ml^{-1})	Cell division inhibition[a] (μg ml^{-1})
1	–$CONH_2$	4000	500
2	–CH_2NH_2	250	O.T.
3	–COOH	>4000	W.T.
4	–CH_2CONH_2	>4000	W.T.
5	–CONHMe	>4000	W.T.
6	–$CSNH_2$	2000	W.T.
7	–CONHOH	4000	W.T.
8	–CH_3	>2000	W.T.
9	–OH	>4000	3000
10	–SMe	>4000	W.T.
11	–NHMe	>4000	W.T.
12	–OEt	2000	500
13	–OCF_3	>4000	W.T.
14	–OCH_3	>4000	W.T.

[a] Lowest concentration at which filamentation of *B. subtilis* is observed indicating on-target activity. W.T. No effect on morphology. O.T. Off-target activity observed, for example cell death without filamentation.

Table 2
B. subtilis MIC and cell division inhibitory activity for compounds **15–28**



Compound	R^4	R^5	R^6	R^7	*B. subtilis* MIC (μg ml^{-1})	Cell division inhibition[a] (μg ml^{-1})
15	MeO	H	H	H	>4000	W.T.
16	H	MeO	H	H	>4000	W.T.
17	H	H	MeO	H	>4000	W.T.
18	H	H	H	MeO	>4000	W.T.
19	NO_2	H	H	H	>4000	W.T.
20	H	NO_2	H	H	>4000	W.T.
21	H	H	H	NO_2	>4000	W.T.
22	Me	H	H	H	>4000	W.T.
23	F	H	H	H	512	128
24	H	F	H	H	>4000	W.T.
25	H	H	H	F	800	W.T.
26	F	H	H	F	512	256
27	F	H	H	Cl	1024	256
28	Cl	H	H	F	>4000	2048

[a] Lowest concentration at which filamentation of *B. subtilis* is observed indicating on-target activity. W.T. No effect on morphology.

Table 3
B. subtilis and *S. aureus* MICs and cell division inhibitory activity for compounds **29–38**

Compound	R^2 (Table 1)	MIC (μg ml^{-1})		*B. subtilis* cell division inhibition[a] (μg ml^{-1})
		B. subtilis	*S. aureus*	
29	Propyloxy	500	256	375
30	Butyloxy	128	128	24
31	Pentyloxy	32	32	24
32	Hexyloxy	16	16	8
33	Heptyloxy	4	8	1.5
34	Octyloxy	1	4	0.37
35	Nonyloxy	0.5	2	0.18
36	Decyloxy	1	64	0.5
37	Undecyloxy	4	>256	1
38	Dodecyloxy	>256	>256	>128

[a] Lowest concentration at which filamentation of *B. subtilis* is observed indicating on-target activity.

Table 4
B. subtilis and *S. aureus* MICs and cell division inhibitory activity for halogenated 3-nonyloxybenzamide compounds **39–43**

Compound	R^4	R^7	MIC (μg ml^{-1})		Cell division inhibition[a] (μg ml^{-1})	
			B. subtilis	*S. aureus*	*B. subtilis*	*S. aureus*
39	F	H	0.125	0.5	0.125	0.5
40	H	F	1	8	1	2
41	F	F	0.125	0.5	0.125	0.25
42	F	Cl	0.5	1	0.25	0.5
43	Cl	F	0.5	2	0.5	1

[a] Lowest concentration at which filamentation of *B. subtilis* or ballooning of *S. aureus* is observed indicating on-target activity.

Table 5
Potency of compounds **1**, **32** and **35** against wild-type *S. aureus* ATCC 601055 and strains with spontaneous FtsZ point mutations M218I and P300R

Compound	MIC (μg ml^{-1})		
	S. aureus ATCC 601055	FtsZ M218I	FtsZ P300R
1	4000	>8000	>8000
32	32	>128	>128
35	2	>128	>128

Table 6
Potency of compounds **1** and **32** against isolates of *S. aureus* and *S. epidermidis*

Strain	MIC (μg ml^{-1})	
	Compound **1**	Compound **32**
S. aureus ATCC 601055 (MSSA)	4096	32
S. aureus ATCC 607004 (MRSA)	4096	32
S. aureus ATCC 700698 (MRSA)	4096	32
S. aureus ATCC 25923 (MSSA)	4096	16
S. aureus ATCC 29213 (MRSA)	2048	16
S. aureus ATCC 43300 (MRSA)	4096	16
S. aureus ATCC 19636 (MSSA)	2048	16
S. epidermidis ATCC 12228	4096	32

with the SAR of this alkyloxybenzamide series of FtsZ inhibitors.[14] The series did not convincingly dock into the GTPase site of FtsZ but did dock into an adjacent cleft. The SAR of the series correlates well with the model, which predicts an optimal hydrophobic alkyl substituent equivalent in length to 9–10 carbons with little space for substitutions off the benzamide group. This is in agreement with our experimental findings (Tables 2 and 3).

The concept and use of ligand efficiency[15] provides a means of normalizing the potency and molecular weight of a compound to enable useful comparison of compounds within a series. Due to difficulties associated with biochemical analysis of FtsZ we have not been able to measure the ligand efficiencies of these compounds directly. Instead we propose to use antibacterial efficiency to provide a means to do this. We define antibacterial efficiency in Eq. 1, as a logarithmic function of the MIC in mg ml^{-1} per non-hydrogen atom (Table 7). This metric describes the ability of the compound to penetrate the cell and to interact with its target to kill the cell as a function of molecular weight.

$$\text{Antibacterial efficiency} = -\ln \text{MIC}_{(\text{mg ml}^{-1})}/N_{\text{non-hydrogen atoms}} \quad (1)$$

The antibacterial efficiencies of clinically approved lower molecular weight compounds as opposed to higher molecular weight natural products, are in the 0.25 to 0.32 mg ml^{-1} per non-hydrogen atom range (Table 7). Compound **1** was much less efficient at −0.065 but extension of the alkoxy substitution significantly improved antibacterial efficiency to >0.3, comparable with marketed drugs. The fluorinated benzamide analogues demonstrated an improvement in antibacterial efficiency compared to non-fluorinated compounds.

Antibacterial efficiency may be a useful tool to direct fragment-based antibacterial optimization. It reduces reliance on the MIC and enables decision making to focus on the efficiency of the ligand. This may lead to lower molecular weight Leads and Drug Candidates.

Our exploration of 3-MBA (**1**) SAR has led to the identification of potent inhibitors of FtsZ that could form the basis of a new targeted treatment for staphylococcal infection. Further exploration and optimization of the compound series, particularly with regard to replacing the long alkyl substituent with more drug-like alternatives, while retaining antibacterial efficiency, is ongoing and will be reported in due course.

Table 7
Antibacterial efficiency of selected compounds

Compound	Mol wt (Da)[a]	Number of non-hydrogen atoms	*S. aureus* MIC (μg ml^{-1})	Antibacterial efficiency (mg ml^{-1} $N_{\text{non-hydrogen atom}}^{-1}$)
1	151	11	2048	−0.065
12	165	12	1024	−0.002
23	169	12	512	0.056
26	187	13	512	0.051
29	179	13	256	0.105
30	193	14	128	0.147
31	207	15	32	0.229
32	221	16	16	0.258
33	235	17	8	0.284
34	249	18	4	0.307
35	263	19	2	0.327
39	281	20	0.5	0.380
41	299	21	0.5	0.362
Ciprofloxacin	331	24	0.5	0.317
Chloramphenicol	323	20	4.0	0.276
Linezolid	337	24	2.0	0.258
Mupirocin	500	35	0.12	0.257
Fusidic acid	516	37	0.06	0.261

[a] Rounded to nearest whole Da.

Acknowledgments

We thank various colleagues including Mike Marriott, Geoff Lawton, Ian Skidmore, Jeff Errington and Steve Ruston for assistance, advice and support. This work was funded by investments from East Hill Management (Boston, USA), a LINK grant in Applied Genomics (AppGen55) from the UK Biotechnology and Biological Sciences Research Council and the UK Department of Trade and Industry. Compounds are the subject of patent application PCT/GB2007/001012. The Prolysis authors declare financial interests.

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