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Derivatives of aryl-4-guanidinomethylbenzoate and *N*-aryl-4-guanidinomethylbenzamide as new antibacterial agents: synthesis and bioactivity[1]

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Key words

antibacterial agents; drug-resistant bacteria; Gram-positive bacteria

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Abstract

Aim: The aim of the present study was to design, synthesize, and evaluate novel antibacterial agents, derivatives of aryl-4-guanidinomethylbenzoate and *N*-aryl-4-guanidinomethylbenzamide. **Methods:** A total of 44 derivatives of aryl-4-guanidinomethylbenzoate (series A) and *N*-aryl-4-guanidinomethylbenzamide (series B) were synthesized and their antibacterial activities were assessed *in vitro* against a variety of Gram-positive and Gram-negative bacteria by an agar dilution method. **Results:** Twelve compounds showed potent bactericidal effects against a panel of Gram-positive germs, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *enterococci* (VRE), vancomycin-intermediate *Staphylococcus aureus* (VISA), and methicillin-resistant coagulase-negative *staphylococci* (MRCNS), with minimum inhibitory concentrations (MIC) ranging between 0.5 and 8 μg/mL, which were comparable to the MIC values of several marketed antibiotics. They exhibited weak or no activity on the Gram-negative bacteria tested. In addition, these compounds displayed high inhibitory activities towards oligopeptidase B of bacterial origin. **Conclusion:** In comparison with the previously reported MIC values of several known antibiotics, the derivatives of aryl-4-guanidinomethylbenzoate and *N*-aryl-4-guanidinomethylbenzamide showed comparable *in vitro* bactericidal activities against VRE and VISA as linezolid. Their growth inhibitory effects on MRSA were similar to vancomycin, but were less potent than linezolid and vancomycin against MRCNS. This class of compounds may have the potential to be developed into narrow spectrum antibacterial agents against certain drug-resistant strains of bacteria.

Introduction

The emergence of bacterial resistance to different classes of antibacterial agents, such as β-lactams, quinolones, and macrolides, is an alarming problem that seriously affects human health[1]. To combat this situation, numerous efforts have been made in the development of new approaches to treat bacterial infections, particularly for therapeutics with novel mechanisms of action and little or no cross-resistance[2–4]. As a result, new antibacterial agents against hospital-acquired Gram-positive bacterial pathogens[5], especially against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE), have become the center of attention in this highlighted research field[6,7].

The guanidino group has long been recognized as a ubiquitous moiety present in numerous therapeutic agents, including cardiovascular, antihistamine, anti-inflammatory, antidiabetic, antibacterial, antihypertensive, antiviral, and antineoplastic drugs[8]. Many natural products also contain

guanidino moieties[9–12], and their strong cationic nature is often associated with biological activities. Of particular interest is the presence of guanidine in several peptidic antibiotics, such as capreomycin[13], viomycin[14], tuberac-tinomycin[15], and neomycin[16].

A class of guanidine-containing antibacterial agents that targets a proteinase of microbial origin was identified as competitive trypsin inhibitors *in vitro* and consists of various aromatic esters of *trans*-4-guanidinomethylcyclohexanecarboxylic acid (GMCHA)[17–20]. For instance, *trans*-4-guanidinomethyl cyclohexane carboxylic acid (4-[4-{4-methylbenzyloxy carbonyl}phenyl] phenylester) (TG44), a derivative of GMCHA[21], and 4-guanidinomethyl benzoic acid (4-[4-{4-methylbenzyloxy carbonyl}phenyl] phenylester) (NE-2001; Figure 1) are both selective synthetic antibiotic agents directed against *Helicobacter pylori* (*H pylori*)[22]. TG44, which is undergoing clinical development, possesses rapid bactericidal activity and is useful for eradicating not only the antibiotic-susceptible, but also the antibiotic-resistant strains of *H pylori* by monotherapy[21]. As a drug candidate, NE-2001 displays superior anti-*H pylori* efficacy on clinical isolates either resistant to metronidazole or resistant to both metronidazole and clarithromycin[22].

The activity of NE-2001 as a selective antibacterial agent against *H pylori* prompted us to conduct structural modifications of this core molecule. In this report, we describe the design, synthesis, and biological evaluation of novel guanidine derivatives of aryl-4-guanidinomethylbenzoate (series A) and *N*-aryl-4-guanidinomethylbenzamide (series B). Twelve of these compounds demonstrated potent antibacterial activities against Gram-positive microorganisms, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, but did not display or showed little or no activity against Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and so on. The results suggest that these compounds may have the potential to be developed into narrow spectrum antibacterial agents to help control the wide spreading of drug resistance.

Materials and methods

Chemistry General synthetic routes towards aryl-4-guanidinomethylbenzoate (A1–24) and *N*-aryl-4-guanidinomethylbenzamide derivatives (B1–20) are illustrated in Figure 2[23]. The target compound A6 was synthesized by condensation of 4-*N*-Boc-aminophenol and 4-guanidinomethylbenzoic acid (intermediate 1), followed by cleavage of the Boc group under acidic conditions[24]. The target compound B5 was synthesized by condensation of 4-benzyloxyaniline and intermediate 1, followed by cleavage of the benzyl group catalyzed with Pd-C[25]. Compounds A8–10 and B10–12 were prepared by condensation of the intermediate 1 with intermediates 4-halo-4'-hydroxybenzophenone or 4-amino-4'-halobenzophenone, respectively[26,27]. Compounds A12–14 and B14–16 were prepared by condensation of the intermediate 1 with intermediate 4-halo-4'-hydroxy-

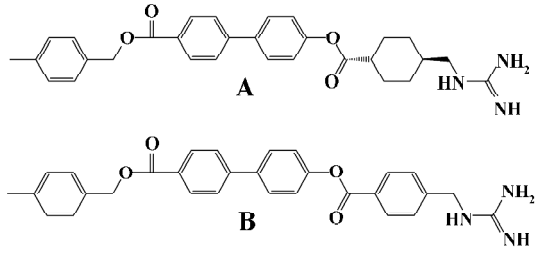


Figure 1. Structures of TG44 (A) and NE-2001 (B).

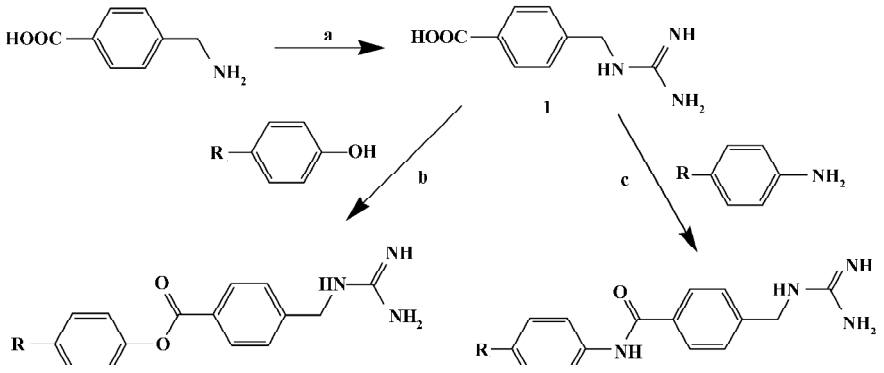


Figure 2. Reagents and conditions. (a) *O*-methylisourea hemisulfate, NaOH, H_2O; (b,c) DCC, dimethylformamide (DMF), pyridine.

diphenyl or 4-amino-4'-halodiphenyl, respectively[28–30]. Substituted benzoyl guanidines (C1–4) and substituted phenyl guanidines (D1–D4) were synthesized according to the literature[31,32]. 4-Guanidinobenzoic acid and 4-guanidinophenylacetic acid were synthesized by the reaction of aminoiminomethanesulfonic acid with 4-aminobenzoic acid and 4-aminophenylacetic acid, respectively[33], followed by condensation with 4-phenylphenol using dicyclohexylcarbodiimide (DCC) to give 4-phenylphenyl 4-guanidinobenzoate (E1) and 4-phenylphenyl 4-guanidinophenylacetate (E2)[23]. All of the compounds were converted to their corresponding hydrochloride salts for biological evaluation.

Antibacterial activity assay Antimicrobial activity was determined by an agar dilution method according to the Clinical and Laboratory Standard Institute (CLSI, formerly National Committee for Clinical Laboratory Standards, NCCLS) guidelines[34]. The test compounds were dissolved in DMSO and diluted at 1:2 serially in distilled water to produce various concentrations. The solutions were loaded onto plates and blundered with quantitative Mueller-Hinton agar medium. Inocula were prepared with overnight cultures and inoculated with a multipoint inoculator (MIT-P, Sakuma, Tokyo, Japan). Final inocula contained 1×10^4 colony-forming units (CFU)/spot. The plates were incubated at 35 °C for 18–24 h depending on the culture requirement. All the bacteria strains used in this study were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Meropenem dissolved in distilled water (Sigma-Aldrich, St Louis, MO, USA) was used as a positive control. Minimum inhibitory concentration (MIC) values were determined as the lowest concentration of the compound that inhibited visible growth.

Enzyme inhibition assay The oligopeptidase B (OpdB) expression vector pET28a-OpdB was provided by Dr Xueyuan JIANG, Department of Biochemistry, Nanjing University (Nanjing, China). Expression, purification, and the enzymatic inhibition assay were carried out as described previously[35] with minor modifications. The reaction volume was reduced to 100 μL from 2 mL in which the amount of enzyme, substrate, and test compounds decreased, but their concentrations remained unchanged. Boc-Glu-Lys-Lys-MCA (Peptide Institute, Osaka, Japan) was used as the substrate for OpdB with a final concentration in the reaction mixture of 10 μmol/L. The liberated 4-methylcoumaryl-7-amide (MCA) was measured fluorometrically with the EnVision 2101 multilabel reader (PerkinElmer, Boston, MA, USA). The excitation and emission wavelengths were 355 and 460 nm, respectively.

Purified OpdA (powder) was also supplied by Dr JIANG and the assay procedure to measure OpdA activity was similar to that in the literature[35], except that the reaction volume was reduced to 100 μL. Enzymatic activities were determined fluorometrically with a Shimadzu RF-5301 spectrofluorometer (Shimadzu Corporation, Kyoto, Japan).

Data analysis Data were analyzed using GraphPad Prism software (San Diego, CA, USA).

Results

Growth inhibitory activities against Gram-positive and negative bacteria We first observed moderate antibacterial activities to certain Gram-positive bacteria, and very little effect on some Gram-negative bacteria in 6 of the series A and B compounds (Table 1). This phenomenon was abolished when substituted benzoyl guanidines (C1–4) and substituted phenyl guanidines (D1–4) were tested (MIC>256 μg/mL; Figure 3). Subsequent structural modifications were directed towards a variety of substitutions on the phenyl group, which led to the synthesis of 2 series of compounds, aryl-4-guanidinomethylbenzoate derivatives (series A) and *N*-aryl-4-guanidinomethylbenzamide derivatives (series B; Table 2).

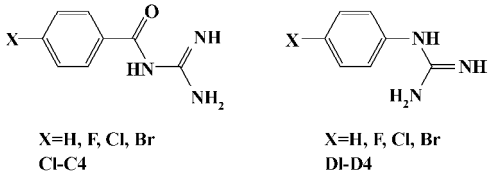
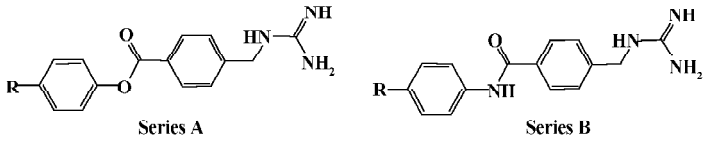


Figure 3. Substituted benzoyl guanidines (C1–4) and substituted phenyl guanidines (D1–4).

It was noted that many of the compounds (series A in particular) were highly selective against most Gram-positive microorganisms tested (eg *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12228), while their inhibitory effects on a range of Gram-negative bacteria were minimal (eg *Escherichia* coli ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Enterobacter cloacae* NICPBP45301, *Salmonella typhi* NICPBP50097, *Acinetobacter anitratus* NICPBP25001, *Proteus morganli* NICPBP49086, *Proteus rettgeri* NICPBP49006, *Shigella dysenteriae*, and *Shigella flexneri*). This lack of efficacy may be attributed to their poor ability to penetrate the additional outer membrane barrier of Gram-negative bacteria[36].

In addition to the Gram-positive and -negative bacteria tested, these compounds also exhibited bactericidal proper-

Table 1. MIC values (μg/mL) of the 6 compounds tested initially.



	Bacterium	Meropenem	GMCHA-OPh^{t}Bu	A1 H	A2 F	A3 Cl	B1 H	B2 F	B3 Cl
G+	*S. aureus* ATCC29213	0.25	8	32	16	8	128	128	32
	S. aureus ATCC25923	0.125	8	32	16-32	4-16	256	128	64
	S. epiderm ATCC12228	0.125	4	32	16	4-8	128	64	16
	WHO-2 methicillin-resistant *S. aureus* MRSA)	>1	4	32	32	8	256	128	32
	Methicillin-susceptible coagulase-negative *staphylococci* (MSCNS) 207096	0.0625	64	256	>256	128	>256	>256	256
	Methicillin-resistant coagulase-negative *staphylococci* (MRCNS) 208165	>1	16	256	128	32	>256	>256	64
	WHO-6 vancomycin-intermediate *Staphylococci* (VISA)	0.5	8	32	16	8	64	64	16
	S. aureus: clinical isolated 210888	>1	4	32	32	8	128	128	32
	WHO-3 vancomycin-resistant *Enterococcus faecium* (VRE)	>1	4	32	32	8	>256	256	32
G-	*Escherichia coli* ATCC25922	0.0625	64	256	256	128	>256	>256	256
	Clinical isolate of *Escherichia coli*: 43073	0.03125	64	256	256	128	>256	>256	256
	Pseudomonas aeruginosa ATCC27853	1	128	>256	>256	128	>256	>256	256
	WHO-8 *Pseudomonas aeruginosa* resistant to fluoroquinolone	>1	128	>256	256	128	>256	>256	256
	Enterobacter cloacae NICPBP45301	>1	256	>256	>256	128	>256	>256	>256
	WHO-5 AmpC-producing standard strain of *Enterobacter cloacae*	0.25	256	>256	>256	256	>256	>256	>256
	Acinetobacter anitratus NICPBP25001	>1	256	>256	>256	128	>256	>256	>256
	Salmonella typhi NICPBP50097	0.03125	64	128	128	32	>256	256	128
	Proteus morganli NICPBP49086	0.125	256	>256	>256	128	>256	>256	>256
	Proteus rettgeri NICPBP49006	0.25	>256	>256	>256	>256	>256	>256	>256
	KPN 1316 *Klebsiella pneumonia*	0.0625	128	>256	>256	256	>256	>256	>256
	Extended spectrum β-lactamase-producing (ESBL) *Klebsiella pneumoniae* ATCC700603	0.0625	256	>256	>256	>256	>256	>256	>256
	CLogP	/	3.14	1.74	2.03	2.60	0.77	1.17	1.74

MIC values shown are representative of at least two independent tests. CLogP, calculated partition coefficient.

ties against *Bacillus cereus* (MIC=4–32 μg/mL), *Bacillus subtilis* (MIC=2–16 μg/mL), *Mycobacterium phlei* (MIC=4–32 μg/mL), and *Candida albicans* (MIC =8–32 μg/mL).

Interestingly, the 2 series of compounds were highly efficacious in suppressing the growth of drug-resistant strains of Gram-positive bacteria, including MRSA, VRE, vancomycin-intermediate *Staphylococci* (VISA), and methicillin-resistant coagulase-negative *Staphylococci* (MRCNS; Table 3). The effects on the 4 strains of drug-resistant bacteria were similar to that observed with drug-susceptible strains of bacteria such as *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* ATCC12228 (Table 2).

The above results (Tables 1,2) allowed us to draw some conclusions. The introduction of halogen atoms resulted in a reasonable inhibitory activities, whereas compounds lacking substitutes (A1 and B1) are less active. We also attempted to increase the aqueous solubility of the compounds

Table 2. MIC values (μg/mL) of aryl-4-guanidinomethylbenzoate derivatives (series A), *N*-aryl-4-guanidinomethylbenzamide derivatives (series B), E1 and E2.

	Bacterium	A4 -Br	A5 -OH	A6 -NH_2	A7 -NO_2	A8 4-FPh OC-	A9 4-ClPh OC-	A10 4-BrPh OC-	A11 Ph-	A12 4-F Ph-	A13 4-Cl Ph-	A14 4-Br Ph-	A15 4-MeO-3-Cl Ph-	A16 4-FPhCOO-	A17 4-ClPhCOO-	A18 4-ClPhOC-	A19 4-(4-FPh)PhCOO-	A20[a] /	A21[b] /	A22[c] /	A23 *t*-Bu-	A24 
G^+	*S. aureus* ATCC29213	4	32	>256	>256	8-16	8	4	1	2	1	1	2	32	64	32	128	4	4	32	32	4
	S. aureus ATCC25923	4	64	>256	>256	16	8	8	1	2	1	2	4-8	32	128	32	>256	4	4	32	32	4
	S. epiderm ATCC12228	2	32	>256	>256	8	2	2	1	2	1	1	2-4	8	8	8	128	2	1	16	16	2
	WHO-2 methicillin-resistant *S. aureus* (MRSA)	4	32	>256	>256	16	8	4	1	2	1	2	2	32	64	32	128	4	2	32	16	4
	Methicillin-susceptible coagulase-negative *staphylococci* (MSCNS) 207096	128	128	>256	>256	128	256	>256	64	>256	>256	>256	>256	>256	128	>256	>256	64	32	128	64	128
	Methicillin-resistant coagulase-negative *staphylococci* (MRCNS) 208165	16	32	>256	>256	64	16	16	4	8	4	4	8	32	64	32	>256	8	8	64	32	16
	WHO-6 vancomycin-intermediate *Staphylococci* (VISA)	2	32	>256	>256	8	4	2	0.5	2	1	1	4	8	16	8	128	2	2	16	16	2
	S. aureus: clinical isolated 210888	4	32	>256	256	16	8	4	1	1	1	1	2	32	16	32	128	4	2	32	16	4
	WHO-3 vancomycin-resistant *Enterococcus faecium* (VRE)	4	128	>256	>256	16	8	4	1	1	1	2	2	32	64	32	128	4	2	32	64	4
G^-	*Escherichia coli* ATCC25922	64	64	>256	>256	128	>256	>256	64	>256	>256	>256	>256	>256	64	>256	>256	32	32	128	128	64
	Clinical isolate of *Escherichia coli*: 43073	64	64	>256	>256	128	>256	>256	64	>256	>256	>256	>256	>256	64	>256	>256	64	32	128	128	64
	Pseudomonas aeruginosa ATCC27853	256	>256	>256	>256	256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	64	64	64	128	256
	WHO-8 *Pseudomonas aeruginosa* resistant to fluoroquinolone	128	>256	>256	>256	256	>256	>256	128	>256	>256	>256	>256	>256	>256	>256	>256	64	128	128	128	128
	Enterobacter cloacae NICPBP45301	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	128	>256	>256	>256	>256
	WHO-5 AmpC-producing standard strain of *Enterobacter cloacae*	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	128	>256	>256	256	>256
	Acinetobacter anitratus NICPBP25001	256	>256	>256	>256	128	128	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	64	128	>256	64	256
	Salmonella typhi NICPBP50097	128	32	>256	>256	256	>256	>256	64	128	>256	>256	>256	>256	32	>256	>256	32	32	64	64	128
	Proteus morganli NICPBP49086	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	128	128	128	>256	>256
	Proteus rettgeri NICPBP49006	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
	KPN 1316 *Klebsiella pneumonia*	256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	256	256
	Extended spectrum β-lactamase-producing (ESBL) *Klebsiella pneumoniae* ATCC700603	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	256	>256
	CLogP	2.75	1.26	0.69	1.79	3.27	3.84	3.99	3.63	3.80	4.37	4.52	4.19	3.53	4.10	4.33	5.42	3.77	4.30	2.96	3.57	2.65

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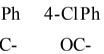
	Bacterium	B4 -Br	B5 -OH	B6 $-NH_2$	B7 $-NO_2$	B8 $PhCH_2O$-	B9 *t*-Bu-	B10 4-FPhOC-	B11 4-ClPhOC-	B12 4-BrPhOC-	B13 Ph-	B14 4-FPh-	B15 4-ClPh-	B16 4-BrPh-	B17 4-MeO-3-Cl Ph-	B18[d] /	B19 (4-Ph) Ph-	B20 	E1 /	E2 /
G+	*S. aureus* ATCC29213	32	>256	>256	>256	8–16	16	16	8	8	4	4	8	4	4	8	>256	32	4	16
	S. aureus ATCC25923	32–64	>256	>256	>256	16	16–32	8–16	8	8	8	8	4	4	8	8	>256	32	4	16
	S. epiderm ATCC12228	16	256	256	>256	16	8	8	4	2–4	4	4	8	4	2	2	>256	16	4	8
	WHO-2 methicillin-resistant *S.aureus* (MRSA)	32	>256	>256	>256	16	16	16	16	8	4	4	8	4	8	8	>256	64	4	16
	Methicillin-susceptible coagulase-negative *staphylococci* (MSCNS) 207096	128	>256	>256	>256	64	32	64	32	64	16	32	32	>256	64	128	>256	256	64	256
	Methicillin-resistant coagulase-negative *staphylococci* (MRCNS) 208165	64	>256	>256	>256	32	32	64	16	16	8	4	8	4	8	16	>256	64	4	16
	WHO-6 vancomycin-intermediate Staphylococci (VISA)	16	128	256	>256	8	16	8	4	2	4	4	8	4	4	2	>256	16	4	16
	S. aureus: clinical isolated 210888	32	128	256	>256	16	16	16	8	8	4	4	8	4	8	8	>256	64	4	16
	WHO-3 vancomycin-resistant *Enterococcus faecium* (VRE)	64	>256	>256	>256	16	16	32	16	8	4	4	8	4	8	8	>256	256	4	32
G-	*Escherichia coli* ATCC25922	256	>256	>256	>256	64	32	128	64	32	16	16	16	>256	64	64	>256	256	64	256
	Clinical isolate of *Escherichia coli*: 43073	256	>256	>256	>256	64	32	128	64	64	16	32	32	>256	32	64	>256	>256	64	256
	Pseudomonas aeruginosa ATCC27853	256	>256	>256	>256	>256	32	128	64	128	32	32	32	>256	64	128	>256	256	64	>256
	WHO-8 *Pseudomonas aeruginosa* resistant to fluoroquinolone	>256	>256	>256	>256	256	32	128	>256	128	32	32	128	>256	128	128	>256	>256	256	>256
	Enterobacter cloacae NICPBP45301	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
	WHO-5 AmpC-producing standard strain of *Enterobacter cloacae*	>256	>256	>256	>256	>256	256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
	Acinetobacter anitratus NICPBP25001	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	32	128
	Salmonella typhi NICPBP50097	64	>256	>256	>256	>256	32	64	64	128	32	32	256	>256	128	64	>256	128	64	256
	Proteus morganli NICPBP49086	>256	>256	>256	>256	>256	128	256	128	>256	128	128	>256	>256	>256	256	>256	>256	>256	>256
	Proteus rettgeri NICPBP49006	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
	KPN 1316 *Klebsiella pneumonia*	>256	>256	>256	>256	64	128	>256	>256	>256	>256	>256	>256	>256	64	>256	>256	>256	>256	>256
	Extended spectrum β-lactamase-producing(ESBL) *Klebsiella pneumoniae* ATCC700603	>256	>256	>256	>256	256	64	>256	256	128	32	256	>256	>256	256	>256	>256	>256	256	>256
	CLogP	1.89	0.11	-0.46	1.07	2.62	2.60	2.50	3.07	3.22	2.66	2.86	3.43	3.58	3.23	2.92	4.55	1.57	4.43	3.60

[a]A20, 4-chloro-1-naphthyl 4-guanidinomethylbenzoate; [b]A21, 2,4-dichloro-1-naphthyl 4-guanidinomethylbenzoate; [c]A22, 7-methoxy-2-naphthyl 4-guanidinomethylbenzoate; [d]B18, 4-guanidinomethylbenzoic acid 4-chloronaphthylamide. MIC values shown are representative of at least two independent tests. CLogP, calculated partition coefficient.

Table 3. Comparison of selected series A and B compounds with antibacterial agents on the market or under development (MIC values in μg/mL).

Compound	MRSA	VRE	MRCNS	VISA
A11	1	1	4	0.5
A12	2	1	8	2
A13	1	1	4	1
A14	2	2	4	1
A15	2	2	8	4
A20	4	4	8	2
A21	2	2	8	2
A24	4	4	16	2
B13	4	4	8	4
B14	4	4	4	4
B15	8	8	8	8
B16	4	4	4	4
B17	8	8	8	4
B18	8	8	16	2
Meropenem	1	>1	>1	1
Linezolid[40,41]	2	2	2	1–2
Eperezolid[40]	2	2	1	n.r.
Vancomycin[40,42]	1	>64	1	8
Ciprofloxacin[43,44]	>32	64	4–16	n.r.
Levofloxacin[42,43]	4–8	16–32	4–8	n.r.
Gemifloxacin[43,44]	2	0.5	0.25	n.r.
Quinupristin-dalfopristin[40,41]	0.25	0.5	0.5	1
Teicoplanin[42,43]	0.5	32	1	n.r.

n.r., not reported.

by introducing amino and hydroxyl groups on the phenyl group, as shown with compounds A5–6 and B5–6, but we observed a drastic decrease in their antibacterial effects. However, substitution by the hydrophobic groups seemed to improve the *in vitro* MIC values. The benzyloxy group (B8) brought about moderate activity.

Further introduction of a benzoyl group to the phenyl group, giving benzophenone derivatives, elicited better *in vitro* MIC values for compounds A8–10 and B10–12. Substitution by the naphthyl group also improved activity (A20–21 and B18). The modification of the phenyl ring to a diphenyl ring (A11–15 and B13–17) demonstrated the most potent antibacterial activity against drug-resistant Gram-positive bacteria with MIC values of 0.5–8 μg/mL. This may be attributed to the hydrophobic nature of the diphenyl ring compared to the benzophenone derivatives. This also indicates that it is favorable to have the hydrophobic group at the phenyl site, and bulky hydrophobic groups increase the activity. Compounds A11 and B13, which were substituted by a phenyl group, displayed more than a 32-fold increase in bioactivity in comparison with A1 and B1. A12–14 and B14–16 with 4-halophenyl substitutions demonstrated a 4- to 16-fold increase in antibacterial effects as opposed to halogen-substituted phenyl-4-guanidinomethylbenzoate derivatives. Series A compounds possessed much better bactericidal efficacy and selectivity against Gram-positive strains than their counterparts in series B substituted with the same group. We also synthesized 2 guanidinophenyl derivatives (E1–2; Table 2), but their activities were reduced compared to A11, indicating that the methylene group was required in the series A core structure.

Inhibitory activity against OpdA and OpdB We next evaluated the inhibitory effects of designed compounds on 2 trypsin-like proteinases, oligopeptidase A (OpdA) and OpdB isolated from *Escherichia coli*[35]. Table 4 summarizes the inhibitory percentage and the 50% inhibition concentration (IC_{50}) values against OpdA and OpdB of a majority of the synthesized compounds with MIC values (against *Staphylococcus aureus* ATCC25923) lower than 256 μg/mL. It is remarkable that many compounds showed high inhibitory effects (low IC_{50} values) against OpdB. We further studied the correlation between the MIC values and the inhibitory effects on OpdB of the series A and B compounds with basically similar substitutions. A general correlation could be established between the antibacterial activities (MIC_{50} values of the compounds against *Staphylococcus aureus* ATCC25923) and inhibitory effects on OpdB (IC_{50}; Figures 4, 5). However, we found that the benzophenone derivatives (A8–10) did not display this tendency (Figure 4) while possessing fairly low IC_{50} values (Table 4). The reason of this

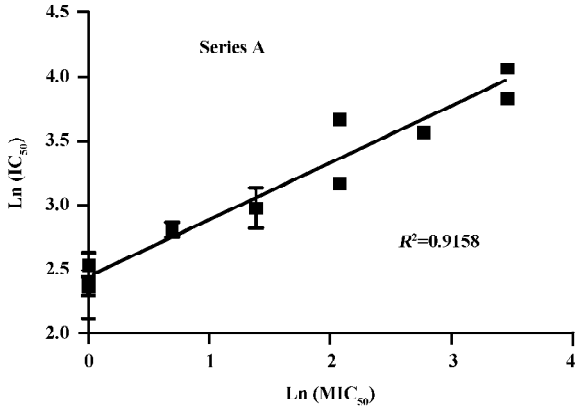


Figure 4. Correlation between the antibacterial activities and the inhibitory effects on OpdB produced by series A compounds. Nonlinear regression analysis was performed to generate a second order polynomial curve between the log value of the antibacterial activities (MIC) and inhibitory effects on OpdB (IC_{50}).

Table 4. Effect of series A and B compounds on enzymatic activities of OpdA and OpdB.

Compound	% Inhibition		IC_{50} (μmol/L)
	OpdA	OpdB	OpdB (mean±SEM)
GMCHA-OPh^{t}Bu	26.58	89.19	7.53±0.79
A1	5.55	39.03	57.70
A2	3.20	76.08	35.68
A3	9.41	80.89	20.08±3.14 (2)
A4	17.77	83.11	23.96
A5	3.93	46.71	46.46
A8	16.02	99.24	1.35±0.22 (2)
A9	40.20	95.78	1.52
A10	29.24	96.33	1.33
A11	94.70	91.19	12.69
A12	71.76	88.83	16.77±0.99 (2)
A13	62.60	88.69	10.85±0.78 (3)
A14	71.31	89.58	11.25±2.81 (2)
A15	37.68	58.33	39.39
A16	40.19	81.92	18.00±3.80 (2)
A17	81.47	93.91	19.90±0.24 (2)
A18	83.29	90.05	11.02±1.72 (2)
A19	96.84	92.18	6.69±1.32 (2)
A20	57.63	90.47	6.69
A21	99.25	99.10	5.59±0.81 (2)
A22	n.d.	7.12	178.20
A24	5.29	80.27	3.76±0.85 (2)
B1	2.22	6.70	1725.00±593.00 (2)
B2	12.93	5.80	1204.00±106.00 (2)
B3	8.36	13.51	678.45±225.35 (2)
B4	10.58	16.40	845.20±303.80 (2)
B8	14.02	20.82	142.65±13.15 (2)
B9	5.12	2.48	580.25±35.85 (2)
B10	26.90	37.10	87.03±1.56 (2)
B11	47.24	24.80	135.40±46.00 (2)
B12	57.18	48.22	62.88±7.99 (2)
B13	46.39	22.79	86.98±1.56 (2)
B14	30.44	23.58	110.68±11.73 (3)
B15	41.47	66.57	33.21±0.85 (2)
B16	55.78	81.59	29.86±6.16 (2)
B17	44.42	46.48	37.70±3.42 (2)
B18	n.d.	90.42	8.51
B20	87.13	99.65	3.60±0.93 (2)

The concentration of the test compounds was 90 μmol/L for OpdA and 45 μmol/L for OpdB. Figures in the parenthesis indicate the number of experiments performed. n.d.: not determined.

apparent discrepancy between bactericidal activity and the inhibition on OpdB remains to be investigated. However, there was a good correlation between the MIC and IC_{50} values for all the Series B compounds studied (Figure 5). No similar trend was found with OpdA.

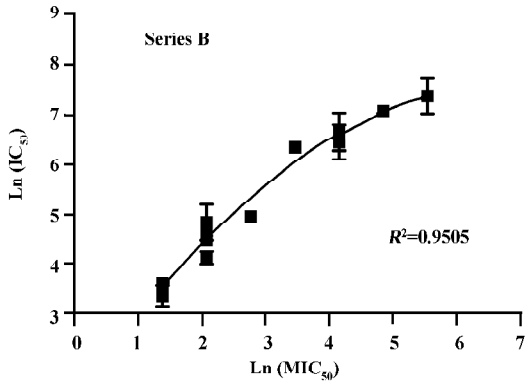


Figure 5. Correlation between the antibacterial activities and the inhibitory effects on OpdB produced by series B compounds. Non-linear regression analysis was performed to generate a second order polynomial curve between the log value of the antibacterial activities (MIC) and inhibitory effects on OpdB (IC_{50}).

Discussion

The growing incidence and frequency of bacterial resistance to current therapeutic agents remains a huge challenge for infectious disease specialists and pharmaceutical companies. In order to keep ahead of this growing issue, novel compounds working by new mechanisms of action are required. Topping the list of infections of most concern are MRSA and VRE. As an example, using the baseline data from 1997, it is shown that in 2002 *Staphylococcus aureus* was the most frequently diagnosed pathogen and exhibited significantly less susceptibility to a variety of antibacterial agents, clearly indicating the need for continued efforts to expand the arsenal of drugs available[37].

It was reported previously that 4-guanidinomethylbenzoic acid arylamides had potent inhibitory effects on gastric lesions[38]. However, their bactericidal effects were not examined. In this study, we describe a novel class of synthetic antimicrobial agents based on a similar structural core (aryl-4-guanidinomethylbenzoate and *N*-aryl-4-guanidinomethylbenzamide derivatives) with a narrow spectrum against Gram-positive bacteria, including several drug-resistant strains of clinical importance.

A preliminary structure-activity relationship (SAR) analysis was performed to identify the active groups on both series A and B compounds. Substitution by the hydrophobic groups on the phenyl ring increases the antibacterial activity, and bulky hydrophobic groups further contribute to this effect. Series A compounds possessed much better bacteri-

cidal efficacy and selectivity against Gram-positive strains than their counterparts in series B.

Linezolid, vancomycin, ciprofloxacin, levofloxacin, gemifloxacin, quinupristin-dalfopristin, and teicoplanin are common antimicrobial agents used in clinics. Linezolid resistance (MIC=16–32 μg/mL) was reported recently in clonally vancomycin-susceptible and -resistant *Enterococcus faecium* isolated from a patient after only 12 days of therapy[39], which indicates the urgency of finding alternative solutions. Table 3 compares the activities of 14 compounds (8 from series A and 6 from series B) described in this study with a number of antibiotic agents reported in the literature[40–44]. The antibacterial activities of A11–15 and A21 are superior to that of ciprofloxacin against MRSA and VRE. All series A and B compounds listed display comparable or better efficacy than ciprofloxacin and levo-floxacin against MRSA, VRE, and MRCNS, and they are sensitive and effective on VISA. With reference to the 4 drug-resistant strains, gemifloxacin shows relatively higher potency than most series A and B compounds. A11–15 and A21 (i) have comparable efficacies as eperezolid, but are slightly less active than quinupristin-dalfopristin; (ii) exhibit an equal potency as linezolid on MRSA, VRE, and VISA; (iii) show similar MIC values as vancomycin and teicoplanin against MRSA, and are more effective than vancomycin and teicoplanin on VRE; and (iv) demonstrate lower potency to MRCNS than most of the antibiotics compared. However, B13–18 are in general less efficacious than A11–15 and A21 (MIC=4–16 μg/mL).

The core structure of both series A and B compounds were designed based on NE-2001, whose putative target may involve a proteinase of microbial origin[22]. OpdA (EC 3.4.24.70) is the major soluble enzyme in *Escherichia coli* capable of hydrolyzing the free lipoprotein signal peptide *in vitro*[45]. As a member of the Zn metalloprotease subfamily[46], it is required for the normal development of phage P22[47] and can degrade the cleaved *lpp* signal peptide *in vitro*[48]. OpdB (EC 3.4.21.83) is a member of the prolyl oligopeptidase family of serine peptidases belonging to clan SC, family S9. It is a trypsin-like proteinase commonly found in ancient eukaryotic unicellular organisms, Gram-negative bacteria, and spirochetes[49]. Similar enzymes also exist in some plants and higher organisms[50,51] and have been implicated in the pathogenicity of certain bacteria since the enzyme is involved in host cell invasion by acting as an important virulence factor[49,52]. OpdB from *Trypanosoma brucei* has been identified as a target of several drugs used to treat African trypanosomiasis. The role of OpdB in the pathogenesis of several parasitic diseases and the possibility that OpdB represents a novel target for antimicrobial chemotherapy prompted an analysis of OpdB homologues from bacterial pathogens[53]. Although OpdB was not found in Gram-positive bacteria, the possibility exists that a surrogate molecule may exert similar functions. It is known that OpdB generates a calcium-signaling factor that interacts with a receptor on the mammalian cell surface, mobilizing Ca^{2+} from intracellular pools and promoting invasion by *Trypanosoma cruzi*. The targeted deletion of OpdB in *Trypanosoma cruzi* causes significant impairment of their ability to infect mammalian cells[54,55]. Therefore, this enzyme has the potential of becoming a therapeutic target. Indeed, the trypanocidal action of several drugs is highly correlated with their inhibitory effects on OpdB of a suramin analogue[56].

The results presented in Table 4 demonstrate that, similar to 4-tert-butylphenyl ester of GMCHA (GMCHA-OPh^{t}Bu; Figure 6)[35], a synthetic trypsin inhibitor, both series A and B compounds were capable of inhibiting enzymatic activities of OpdA and OpdB to various extents, but they were in general more selective towards OpdB. When comparing the antibacterial effects (MIC values) of both series A and B compounds with their ability to inhibit OpdB, a clear correlation could be established (Figures 4, 5). This trend was particularly evident with series A and B compounds substituted by small groups, such as halo- (A2–4 and B2–4), hydroxyl- (A5), *t*-butyl- (B9) and halophenyl- (A12–A15 and B14–17) groups. When substitutions were made with larger or complex groups, such as 4-halobenzoyloxy (A16 and A17), 4-chlorophenyloxycarbonyl (A18), and 4-(4-fluorophenyl)benzoyloxy (A19), the correlation between MIC and OpdB inhibition was diminished. The analysis leads to our speculation that derivatives of aryl-4-guanidinomethylbenzoate and *N*-aryl-4-guanidinomethylbenzamide may target a putative OpdB-like molecule in Gram-positive germs to exert antibacterial actions, although an alternative possibility, that is, the compounds are acting as detergents and their activities are due to lysis of the cell membrane of the Gram-positive bacteria, cannot be ruled out at present.

In summary, we have designed, synthesized, and evaluated the antibacterial activities of aryl-4-guanidinomethyl-benzoate and *N*-aryl-4-guanidinomethylbenzamide derivatives. Of

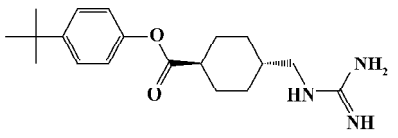


Figure 6. Structure of 4-tert-butylphenyl ester of GMCHA (GMCHA-OPh^{t}Bu).

the compounds tested, A11–15, A20, and A21 displayed consistent and superior antibacterial effects against a spectrum of Gram-positive microorganisms, but showed little or no inhibitory activity against Gram-negative bacteria. Most of these compounds had comparable *in vitro* bactericidal activities against VRE and VISA as linezolid: their growth inhibitory effects on MRSA were similar to vancomycin, but were less potent than linezolid and vancomycin against MRCNS. The *N*-aryl-4'-guanidinomethylbenzamide derivatives B13–17 exhibited similar properties, but were less selective and efficacious. A preliminary SAR analysis was performed to identify the active groups on these compounds, and the mechanism of actions may involve the inhibition of a putative OpdB-like molecule in Gram-positive bacteria.

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