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4-Alkyl and 4,4′-dialkyl 1,2-bis(4-chlorophenyl)pyrazolidine-3,5-dione derivatives as new inhibitors of bacterial cell wall biosynthesis

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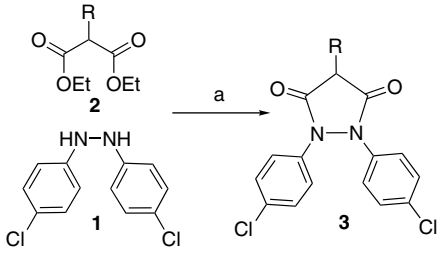
Abstract—Over 195 4-alkyl and 4,4-dialkyl 1,2-bis(4-chlorophenyl)pyrazolidine-3,5-dione derivatives were synthesized, utilizing microwave accelerated synthesis, for evaluation as new inhibitors of bacterial cell wall biosynthesis. Many of them demonstrated good activity against MurB in vitro and low MIC values against Gram-positive bacteria, particularly penicillin-resistant *Streptococcus pneumoniae* (PRSP). Derivative **7l** demonstrated antibacterial activity against both Gram-positive and Gram-negative bacteria. Derivatives **7f** and **10a** also demonstrated potent nanomolar K_d values in their binding to MurB.

Scientific effort for the design and synthesis of novel antibacterials has focused mainly on modifications within the same class of antibiotics already on the market. Due to the ease with which bacteria can exchange genetic material and develop resistance to the preexisting antibiotic classes, our focus has been to develop antibacterials with a novel mode of action. As peptidoglycan is an essential cell wall component of most bacteria, cytosolic enzymes MurA-F at early stages of peptidoglycan biosynthesis are unique and selective target for antibiotic action.[1] Furthermore, the MurA-F cascade is ubiquitous to both Gram-positive and Gram-negative bacteria, but have no mammalian homologue.[1] Inhibition of any of these essential enzymes leads to loss of cell shape and integrity followed by bacterial death.[2,3] From screening of a focused set of the corporate compound collection, the pyrazolidinedione scaffold was identified as a potential inhibitor of MurB. Here we report the synthesis and antimicrobial activity of 4-alkyl and 4,4-bis-alkyl pyrazolidinedione derivatives as new inhibitors of MurA and MurB.

Pyrazolidinediones are conventionally synthesized through the condensation of diethylmalonates with hydrazine using in situ generated sodium ethoxide as the base and heating at elevated temperatures for extended time periods.[4] In order to generate large numbers of compounds, we developed a simple, fast, high yielding, one step method for the synthesis of 4-alkyl-pyrazolidinediones **3** via microwave heating of alkyl-diethylmalonates **2** with diarylhydrazine **1** using commercially available sodium ethoxide (Scheme 1).[5]

Diarylhydrazine derivative **1** was prepared in ~50% overall yield by oxidative dimerization of 2 equiv of 4-chloroaniline (**4**) in the presence of manganese dioxide,



Scheme 1. Reagents and conditions: (a) NaOEt, microwave, 4 min.

Keywords: Microwave-assisted synthesis; Pyrazolidinediones; MurB; MurA; Antibacterial activity.
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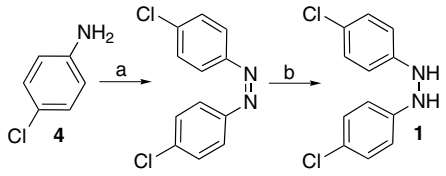
followed by reduction in the presence of zinc dust and ammonium chloride[6] (Scheme 2).

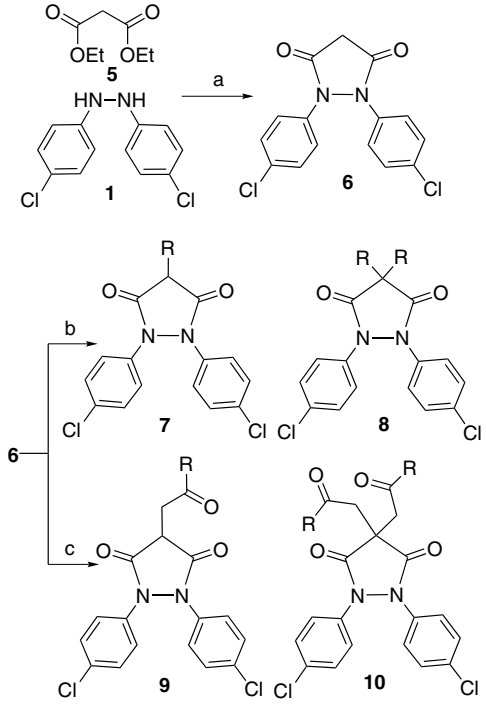
Since limited numbers of diethyl alkylmalonates are available, techniques for the alkylation of pyrazolidinedione itself were also developed utilizing microwave heating (Scheme 3). First unsubstituted pyrazolidinedione **6** was prepared in 95% yield by the microwave-assisted condensation of 1,2-di(4-chlorophenyl)hydrazine **1** with diethylmalonate **5**. Next **6** was reacted with alkyl bromides or alkyl chlorides and lithium carbonate under microwave heating to produce a mixture of 4-alkyated (**7**) and 4,4′-dialkylated products (**8**).[7] Of other bases attempted, potassium carbonate produced somewhat more dialkylated product and no alkylation resulted when triethylamine, pyridine or *N*,*N*-diisopropylethylamine were used. Higher boiling *N*,*N*-dimethylformamide was used as the solvent rather than acetone to prevent evaporation and subsequent charring of reactions in open vessels. For the addition of more reactive 2-bromoacetophenones to unsubstituted pyrazolidinedione **6** *N*,*N*-diisopropylethylamine was utilized as a base to yield a mixture of mono (**9**) and bis (**10**) products.



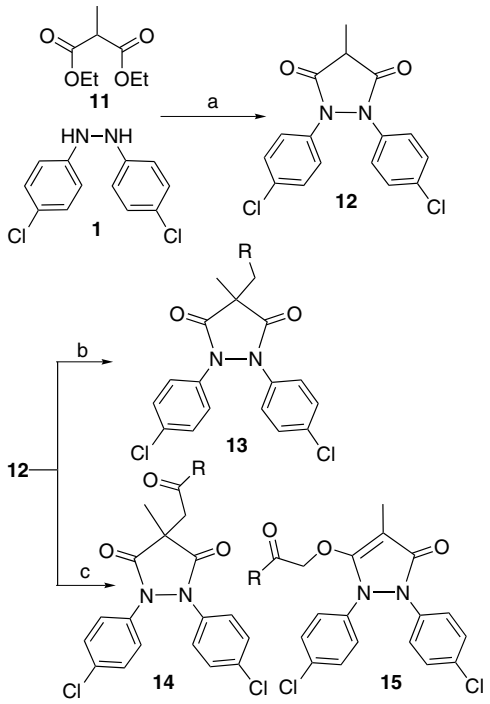
Scheme 2. Reagents and conditions: (a) MnO_2, benzene, reflux, 1 h; (b) Zn dust, NH_4Cl, acetone, 1 h.

4,4′-Differentially alkylated products, Scheme 4, were obtained in a manner similar to Scheme 3. 4-Methyl 1,2-bis(4-chlorophenyl)pyrazolidine-3,5-dione **12** was prepared in 85% yield by the microwave-assisted condensation of diarylhydrazine **1** with methyl diethylmalonate **11**. Next **12** was reacted with alkyl bromides or alkyl chlorides and lithium carbonate under microwave heating (900 W) to produce 4′alkyl-4-methyl-pyrazolidine-3,5-dione products (**13**). Similarly, 2-bromoacetophenones were also added to yield a mixture of 4′-alkyl-4-methyl-pyrazolidine-3,5-dione (**14**) and 4-methyl-5-alkyloxy-pyrazol-3-one (**15**) products.

According to molecular modeling studies, the 4-position of the pyrazolidinedione ring points into a long and large lipophilic pocket. Hence, using Schemes 1, 3 and 4 over 195 4-alkyl or 4,4′-alkyl derivatives were synthesized to probe the lipophilic pocket and submitted for evaluation[8–10] as new inhibitors of bacterial cell wall biosynthesis. SAR (structure activity relationship) trends were established and some are highlighted in Tables 1–3. In terms of activity against MurB and Gram-positive bacteria, particularly *Streptococcus pneumoniae* (PRSP), lipophilic 4-alkyl groups were preferred over somewhat more hydrophilic groups (**3b** vs **3f**, **7i** vs **3e**). Potency was improved against MurB by incorporating an aromatic ring in the lipophilic chain (Table 1, **3a**, **b** compared to **7a**–**d**). Longer chain length (Table 1, **7a**–**d** vs **3c**, **d**) was also a factor in improving IC_{50} values against MurB ($n = 1$ $IC_{50} > 50$ μM, $n = 6$ $IC_{50} \sim 9$ μM) and MIC (minimum inhibitory concentration) values for *Streptococcus pneumoniae* bacteria strains ($n = 1$, 200 μM and $n = 6$, 6.25 μM). Derivative **7d** (chain length

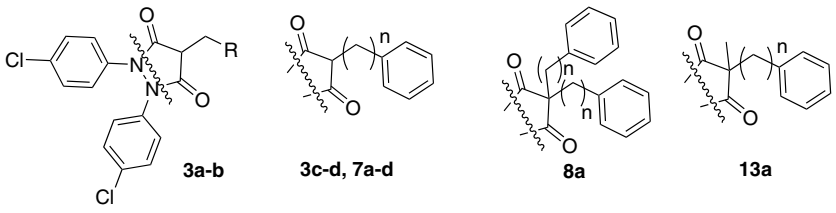


Scheme 3. Reagents and conditions: (a) NaOEt, microwave, 2 min; (b) R-Br, Li_2CO_3, DMF, microwave, 2–4 min; (c) R-C(O)–CH_2–Br, DIPEA, toluene, microwave, 1–3 min.



Scheme 4. Reagents and conditions: (a) NaOEt, microwave, 4 min; (b) R-Br, Li_2CO_3, DMF, microwave, 2–4 min; (c) R-C(O)–CH_2–Br, DIPEA, toluene, microwave, 1–3 min.

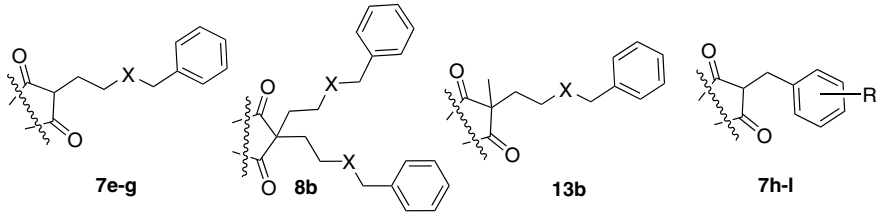
Table 1. Antimicrobial activities (μM) and IC_{50} values of selected 4-alkyl and 4,4′-dialkyl-1,2-bis(4-chlorophenyl)pyrazolidine-3,5-diones



	3a $CH(CH_2CH_3)_2$	**3b** $(CH_2)_5CH_3$	**3c** 1	**3d** 2	**7a** 3	**7b** 4	**7c** 5	**7d** 6	**8a** 6	**13a** 6
S. aureus GC 1131 (MRSA)	100	100	200	200	200	200	200	25	>200	>200
S. aureus GC 4543 (MSSA)	100	100	200	200	200	200	100	25	200	>200
E. faecalis GC 4555 (ATCC)	200	200	>200	>200	>200	200	100	50	>200	200
E. faecalis GC 2242 (VRE)	100	100	>200	200	200	100	200	50	200	100
S. pneumo GC1894 (PRSP)	25	6.25	200	50	25	25	25	6.25	100	0.78
S. pneumo GC1894 (PRSP)[a]	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
MSCNS GC 646	200	200	>200	200	>200	200	>200	100	>200	>200
E. coli GC 4559	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
E. coli GC 4560	200	>200	>200	200	200	>200	>200	200	>200	200
MurA, *E. coli* (IC_{50}, μM)	>50	>50	>50	>50	44.3	23.8	42.3	34.9	9.9	>50
MurB, *E. coli* (IC_{50}, μM)	50	42	>50	35	10	5.6	10	8.9	7.2	>50
MurB, *S. aureus* (IC_{50}, μM)	—	—	—	—	—	6.8	32	12	8.2	>50

R = $CH(CH_2CH_3)_2$ or $(CH_2)_5CH_3$; n = 1–6.
[a] Tested in the presence of 4% bovine serum albumin.

Table 2. Antimicrobial activities (μM) and IC_{50} values of selected 4-alkyl and 4,4′-dialkyl 1,2-bis(4-chlorophenyl)pyrazolidine-3,5-diones



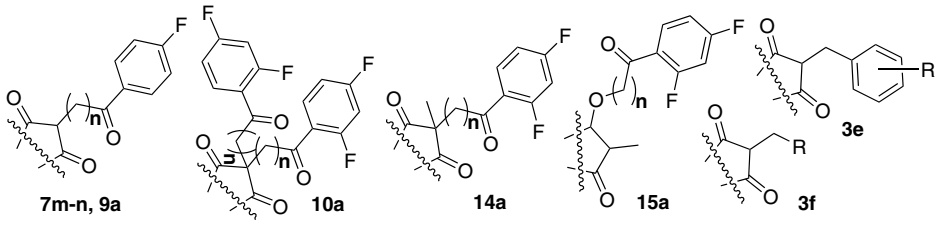
	7e NEt	**7f** S	**7g** O	**8b** O	**13b** O	**7h** 2-Cl	**7i** 3-CF_3	**7j** 2,4-DiCl	**7k** 3,5-$DiCF_3$	**7l** 4-(1-CN-Phenyl)
S. aureus GC 1131 (MRSA)	200	25	100	100	200	>200	>200	50	25	12.5
S. aureus GC 4543 (MSSA)	>200	25	200	100	>200	>200	>200	50	25	6.25
E. faecalis GC 4555 (ATCC)	>200	100	200	25	>200	>200	>200	100	50	25
E. faecalis GC 2242 (VRE)	200	50	100	50	>200	>200	>200	50	25	12.5
S. pneumo GC1894 (PRSP)	100	6.25	25	12.5	100	12.5	50	12.5	6.25	3.12
S. pneumo GC1894 (PRSP)[a]	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
MSCNS GC 646	>200	50	200	200	>200	>200	>200	50	50	6.25
E. coli GC 4559	>200	>200	>200	>200	>200	>200	>200	>200	50	>200
E. coli GC 4560	>200	200	200	200	>200	>200	>200	100	>200	25
MurA, *E. coli* (IC_{50}, μM)	>50	9.8	33.4	20.1	>50	>50	>50	>50	>50	>50
MurB, *E. coli* (IC_{50}, μM)	>50	5.2	11	7.2	20	>50	18	11	12	11
MurB, *S. aureus* (IC_{50}, μM)	>50	5.1	11	7.5	—	>50	—	29	—	12

X = NEt, S or O; R = 2-Cl, 3-CF_3, 2,4-diCl, 3,5-diCF_3, or 4-(1-CN-Phenyl).
[a] Tested in the presence of 4% bovine serum albumin.

n = 6) also demonstrated good activity against other Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *S. aureus* (MSSA), vancomycin-susceptible *Enterococcus faecalis* (ATCC) and vancomycin-resistant *E. faecalis* (VRE) with MICs in the range of 25–50 μM. The 4,4′ bis-alkyl derivative of **7d**, **8a**, showed no change in MurB activity but almost complete loss of antibacterial activity against PRSP. Contrarily, the 4′-methyl derivative **13a** showed complete loss of MurB activity and a significant improvement of PRSP MIC to 0.78 μM, indicating that for this derivative bacterial inhibition was not operating through inhibition of MurB.

In general, the addition of more polar heteroatoms into the chains spanning between the pyrazolidinedione and phenyl ring resulted in a decrease in MurB IC_{50} (**7b** vs **7e** and **7g**) and also a 4-fold decrease in MIC for **7e**. On the other hand, addition of a sulfur atom in the chain (**7f**) lead to a maintenance of MurB IC_{50}, a

Table 3. Antimicrobial activities (μM) and IC_{50} values of selected 4-alkyl and 4,4′-dialkyl 1,2-bis(4-chlorophenyl)pyrazolidine-3,5-diones



	7m 3	7n 2	9a 1	10a 1	14a 1	15a 1	3e 3-OMe	3f $CH_2N(CH_2CH_3)_2$
S. aureus GC 1131 (MRSA)	50	100	25	12.5	>200	>200	>200	100
S. aureus GC 4543 (MSSA)	50	100	25	12.5	>200	>200	>200	>200
E. faecalis GC 4555 (ATCC)	50	100	50	12.5	200	>200	>200	200
E. faecalis GC 2242 (VRE)	50	100	50	12.5	100	200	>200	200
S. pneumo GC1894 (PRSP)	12.5	50	25	3.12	>200	>200	200	100
S. pneumo GC1894 (PRSP)[a]	>200	>200	>200	>200	>200	>200	>200	>200
MSCNS GC 646	100	200	>200	25	>200	>200	200	200
E. coli GC 4559	>200	>200	>200	>200	>200	>200	>200	>200
E. coli GC 4560	200	200	200	100	>200	>200	>200	>200
MurA, *E. coli* (IC_{50}, μM)	32.4	46.9	8.4	9.6	>50	>50	>50	>50
MurB, *E. coli* (IC_{50}, μM)	10	13	5.4	5.8	49	>50	50	>50
MurB, *S. aureus* (IC_{50}, μM)	11	18	5.2	5.2	>50	>50	—	—

R = 3-OMe, $CH_2N(CH_2CH_3)_2$; n = 1–3.

[a] Tested in the presence of 4% bovine serum albumin.

significant increase in MurA activity, and a 4-fold improvement in PRSP MIC. In contrast to the previous 4,4′-dialkyl case (**7d** vs **8a**) the 4,4′-dialkyl with a heteroatom in the chains causes a increase in both MurB and MIC activity (**8b**). The 4′-methyl derivative **13b**, however, resulted in a loss of MurB activity and antimicrobial activity.

Investigation of non-polar substituents on the terminal phenyl ring (Table 2, **7h–l**) indicated that increased lipophilic bulk improves MurB activity as well as antimicrobial activity against Gram-positive bacteria. Derivative **7l** was the most promising compound with activity against Gram-positive bacteria in the range between 3.12 and 12.5 μM and Gram-negative MIC for *Escherichia coli GC 4560* being 25 μM.

When a carbonyl (Table 3, derivatives **7m**,**n** and **9a**) is placed in the chain, the chain length effect reverses with the shorter chain, **9a**, showing the best potency. The dialkyl derivative, **10a**, and methylalkyl derivative, **14a**, however, show the same trends of increased potency for the dialkyl and loss of potency for the methyl-alkyl, as was noted previously for other derivatives. The interesting O-alkyl side product **15a** obtained in this case showed absolutely no activity.

In summary, microwave heating has proven to be efficient in many steps in the synthesis of 4-alkyl and 4,4′-dialkyl pyrazolidinediones. Alkyl pyrazolidinedione derivatives were identified as a new class of inhibitors in bacterial cell wall biosynthesis. In general they demonstrated good activity against MurB and to a lesser degree against MurA and in some cases good activity against Gram-positive bacteria, particularly PRSP. However, when they were tested in the presence of 4% bovine serum albumin, their MIC values increased to greater than 200 μM against PRSP. Activity against Gram-negative bacteria was not detected with the exception of derivative **7l** with a MIC of 25 μM. Derivatives **7f** (K_d = 400 nM) and **10a** (K_d = 710 nM) were also shown to display potent nanomolar K_d values in their binding to MurB. Since many derivatives, **7d**, **7f**, **7k**, **7l** and **10a** show good selectivity against MurB as well as good activity against Gram-positive penicillin resistant bacteria, they are good leads for further investigation.

Acknowledgements

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5. Representative procedure for microwave assisted pyrazolidine-3,5-dione synthesis: Reactions were carried out in open glass vials using a CEM Corporation Mars 5 commercial multimode microwave at a setting of 900 W

(note: temperature setting was not used since these reactions were run to dryness given that the driving force of the reaction is the elimination of ethanol). To 1,2-bis(4-chlorophenyl)hydrazine **1** (2.53 g, 10 mmol) was added 10 mL of sodium ethoxide (21 wt% in ethanol) and diethyl malonate (5 mL) and subjected to microwave heating for 2 min. The residue was dissolved in water (50 mL), acidified with 1 N hydrochloric acid, and extracted with dichloromethane (100 mL). The organic extracts were concentrated to give 3.05 g of 1,2-bis(4-chlorophenyl)pyrazolidine-3,5-dione **6**. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.78 (s, 2H), 7.32 (m, 4H), 7.42 (m, 4H), MS [(−)ESI, *m*/*z*]: 319.1 [M−H].

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7. Representative procedure for microwave assisted alkylation of pyrazolidine-3,5-diones: Reactions were carried out in open glass vials using a CEM Corporation Mars 5 commercial multimode microwave at a setting of 200 °C and a maximum of 900 W or in closed glass vessels using a Personal Chemistry Smith Workstation single mode microwave at 200 °C. To a solution of 1,2-bis(4-chlorophenyl)pyrazolidine-3,5-dione (0.064 g, 0.2 mmol) in *N*,*N*-dimethylformamide (1 mL) was added lithium carbonate (0.044 g, 0.6 mmol) and benzyl 2-bromoethyl ether (0.043 g, 0.2 mmol). The reaction was subjected to microwave heating for 3.5 min, and then concentrated. The residue was dissolved in water (1.0 mL), acidified with 1 N hydrochloric acid, and extracted with dichloromethane (4.0 mL). The organic extract was concentrated, taken up in acetonitrile and purified by semi-preparative RP-HPLC (Gilson Semi-Preparative HPLC system with Unipoint Software v. 1.71, Phenomenex C_{18} Luna column, 21.6 × 100 mm, 5 μ particle size, water–acetonitrile solvent system with added 0.02% TFA buffer, at 22.5 mL/min) to give **7g** (0.012 g) and **8b** (0.004 g) [LC/MS (Hewlett Packard 1100 MSD with ChemStation Software, xterra C_{18} 2.1 mm × 30 mm column, 3.5 μ particle size, at 50 °C, water–acetonitrile solvent system with added 0.02% TFA buffer at 1.0 mL/min flow rate, 254 nm DAD detection; API-ES scanning mode, fragmentor 140 mV): **7g** *m*/*z* 455.0 (M+H); retention time 3.279 min, **8b** *m*/*z* 589.1 (M+H); retention time 3.622 min].
8. MurA inhibition studies: The MurA reaction followed the method of Marquardt et al. (*J. Bacteriol.* **1992**, *174(17)*, 5748) and consisted of 150 nM MurA, 200 μM PEP, and 200 μM UDP-*N*-acetylglucosamine (UDP-GlcNAc) in 25 μL of 25 mM Tris, pH 8. The reaction was incubated for 30 min at 37 °C and was terminated by the addition of 210 μL of malachite green reagent (0.03% malachite green, 1.4% ammonium molybdate, and 1.3 N HCl). Inhibition of MurA was performed by preincubating of the enzyme and compound for 10 min at 37 °C before addition of the substrates. The amount of phosphate released during the reaction was detected spectrometrically at 660 nm using a Molecular Devices Spectra Max 250 with SoftMax software. The results were compared to the inhibition of the enzyme by fosfomycin (commercially available MurA inhibitor). The results are presented as % inhibition at a compound concentration of 25 μg/mL. The calculation follows the formula: % inhibition = 100 − (OD for sample)/(OD mean of control) × 100. IC_{50}s were then estimated by linear regression analysis using the percent inhibition data bracketing 50% inhibition.
9. MurB inhibition studies: The inhibition of MurB enzymatic activity by pyrazolidinedione derivatives was determined using substrates NADPH (Sigma), and biochemically synthesized, HPLC purified sub EP-UNAG following the method of Dhalla et al. (*Biochemistry* **1995**, *34*, 5390). The reaction was determined following an initial 20 min preincubation of 20 nM of the enzyme with the inhibitor. The substrate mixture was then added to the enzyme-inhibitor mixture, to a final concentration of 50 μM EP-UNAG and 100 μM NADPH. MurB activity was monitored using Molecular Devices Spectra Max 250 with SoftMax software. At least six concentrations of inhibitor, between 1.5 and 50 μM, were used for each compound tested. The IC_{50} values were derived using data analysis function of Microsoft Excel program (Sigmoid Curve Hill analysis 0–100).
10. MICs: The in vitro determination of the MICs against aerobic bacteria was performed by the microdilution broth method as recommended by the National Committee for Clinical Laboratory Standards (*Approved Standards M7 - A3*, **1997**). Mueller–Hinton Broth was used for the *Staphylococci*, and *Enterococci*. *Streptococci* were tested in Mueller–Hinton Broth supplemented with 5% sheep blood. Microtiter plates containing 50 μL sheep blood per well of 2-fold serial dilutions of the antibacterial agents in the appropriate broth were inoculated with 50 μL of inoculum to yield a final density of 1–5 × 105 CFU/mL. The MICs were determined after 18–22 h of incubation at 35 °C in ambient air.