Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 603–607
Bioorganic & Medicinal Chemistry Letters

Synthesis and in vitro selective anti-*Helicobacter pylori* activity of pyrazoline derivatives

F. Chimenti,[a] B. Bizzarri,[a,*] F. Manna,[a] A. Bolasco,[a] D. Secci,[a] P. Chimenti,[a] A. Granese,[a] D. Rivanera,[b] D. Lilli,[b] M. M. Scaltrito[c] and M. I. Brenciaglia[b]

[a]*Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università 'La Sapienza', P.le A. Moro 5, 00185 Rome, Italy*
[b]*Dipartimento di Scienze di Sanità Pubblica, Università 'La Sapienza', P.le A. Moro 5, 00185 Rome, Italy*
[c]*Istituto di Microbiologia, Università degli Studi di Milano, via Pascal 36, 20133 Milano, Italy*

Received 28 September 2004; revised 15 November 2004; accepted 17 November 2004
Available online 20 December 2004

Abstract—In order to develop new anti-*Helicobacter pylori* agents, a series of N1-substituted 3,5-diphenyl pyrazolines **P1**–**P13** was prepared and evaluated for their antibacterial activity. All synthesized compounds showed little or no activity against different species of Gram-positive and Gram-negative bacteria of clinical relevance and against various strains of pathogenic fungi. The same derivatives exhibited a significant degree of activity against a range of *H. pylori* strains, including those resistant to the reference compound metronidazole. Among the prepared compounds those with an N1-acetyl group and a 4-methoxy substituent in the 5-phenyl ring showed the best activity against *H. pylori* metronidazole resistant strains in the 1–4 μg/mL MIC range.

1. Introduction

It is well known that *Helicobacter pylori*, an S-shaped spiral microaerophilic, Gram-negative bacterium first isolated in human gastric mucosa in 1982,[1–3] is considered the major causative agent of several gastric pathologies, such as chronic active gastritis, peptic ulcer disease and gastric cancer.[4–6] Clinical evidence shows that the eradication therapy of this microorganism, which is believed to be an important etiological factor in the pathogenesis of certain malignant peptic diseases, can significantly reduce the risk of ulcer relapse and may help prevent mucosa-associated lymphoid tissue (MALT)-type gastric carcinoma and other gastric cancers.[7–9] Hence, the World Health Organization (WHO) has proposed *H. pylori* as a Class 1 carcinogen in humans, since it has been demonstrated that chronic infection is strongly associated with the development of gastric malignant diseases.[10]

Until recently, the most effective treatment regimens have included a combination of antibiotics (β-lactams, macrolides and quinolones), bactericidal agents (bismuth salts) and antiprotozoal agents (metronidazole). Although it is widely recognized that this therapy plays a critical role in improving and/or preventing these gastric pathologies, eradication is not always successful and a few problems have been observed in the use of these drugs, such as the emergence of drug resistance[11,12] and low compliance,[13] related to the occurrence of a number of harmful side effects.[14,15]

As a result, there is a need to develop new, alternative therapeutic agents with highly selective antibacterial activity against *H. pylori*, but without the risk of resistance or other untoward effects.[16]

At the start of this work, we reasoned that known antimicrobial agents may not be an appropriate therapy, since they may favour the emergence of resistant colonies and also present a potential for the disruption of intestinal microbial flora, which is responsible for side effects.

Thus, in order to try and overcome these problems, as a part of a screening program of a number of compounds, we decided to evaluate a series of pyrazoline derivatives.

It is worth noting that some pyrazoles[17] and several 1,3,5-trisubstituted pyrazolines are reported to exhibit

Keywords: Pyrazoline; Antibacterial activity; Anti-*Helicobacter pylori* activity.
* Corresponding author. Tel.: +39 06 49913975; fax: +39 06 49913763; e-mail: bruna.bizzarri@uniroma1.it

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.11.042

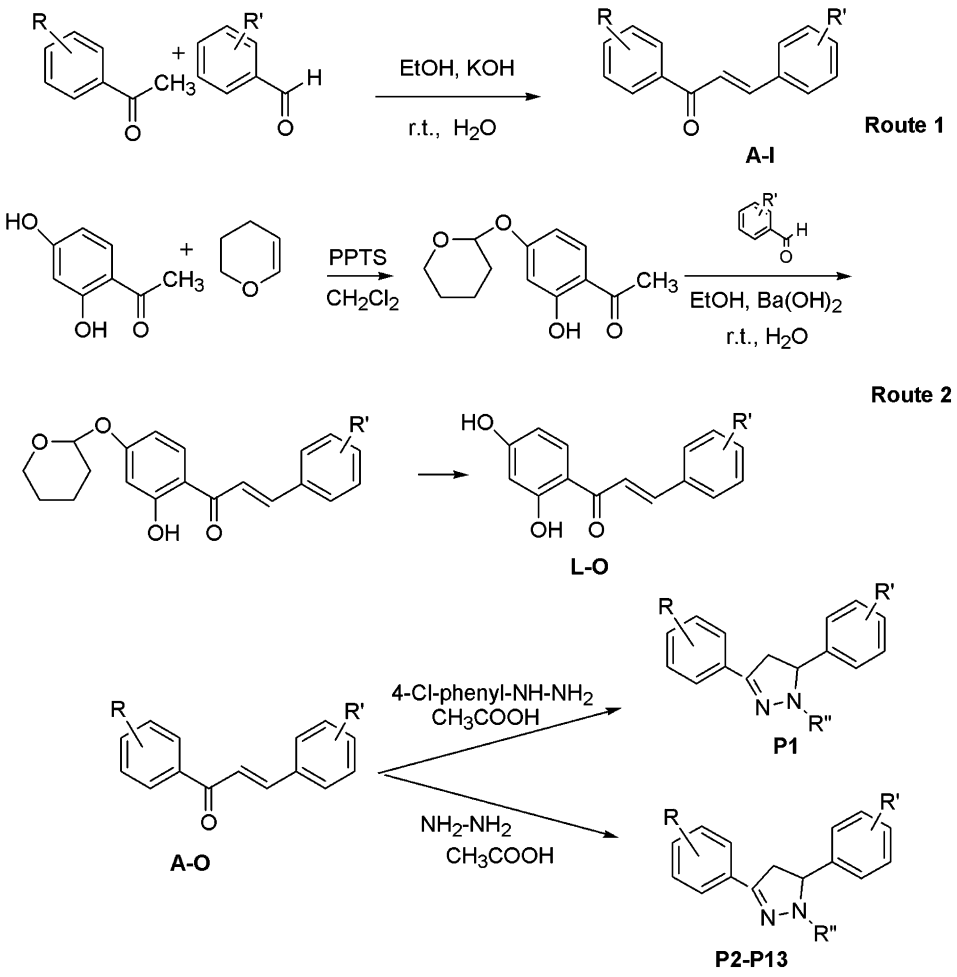
moderate antibacterial and antifungal activity.[18–20] Besides, a number of acetylpyrazoline derivatives were found to possess antibacterial activity.[21–23] More recently, some new classes of pyrazole compounds acting as selective inhibitors of *H. pylori*, with little or no activity towards other Gram-positive and Gram-negative bacteria, have been described.[24,25]

Encouraged by this and pursuing our research,[26–28] we here report on the antimicrobial evaluation of a series of N1-substituted 3,5-diphenyl pyrazoline derivatives against the most common pathogens, both bacterial and fungal, and against *H. pylori*. In previous studies, we developed effective routes to synthesize these compounds; here, we carried out studies first to evaluate the activity of these compounds against Gram-positive and Gram-negative bacteria and then to investigate anti-*H. pylori* activity.

The 1-(4-Cl-phenyl) and 1-acetyl-3,5-diphenyl-4,5-dihydro-(1H)-pyrazole derivatives **P1**–**P13** were prepared by reacting chalcone with appropriate hydrazine in acetic acid[26–28] (Scheme 1 and Table 1).

The chalcones **A**–**I** were obtained by direct Claisen-Schmidt condensation between the aromatic aldehydes and the substituted acetophenone, using 20% potassium hydroxide as catalyst in ethanol (Route 1 in Scheme 1). Since the potassium salts precipitated because of the highly alkaline reaction environment due to the synthesis of the 2,4-dihydroxy chalcones **L**–**O**, the hydroxyls had to be protected with 3,4-dihydro-α-pyrane before the condensation reaction. Of the two hydroxyls only the one in position-4 is protected, while the one in position-2 is not available as it is involved in an intramolecular hydrogen bond with the oxygen atom of the carbonyl group. The acetophenone thus protected reacts with the aldehyde to form the protected chalcone, which is subsequently freed by hydrolysis (Route 2 in Scheme 1).

For all the synthesized compounds, except for **P1**, the diagnostic infrared absorptions were 3450–3100 cm^{-1} (OH), 1618 cm^{-1} (C=O) and 1590 cm^{-1} (C=N). As regards ^1H NMR spectra we always detected the following typical peaks for the pyrazoline nucleus: 5.53–5.47 (q, 1H, H_5), 3.89–3.79 (m, 1H, H'_4) and 3.28–3.20 (dd, 1H, H_4). For the dihydroxy derivatives, **P9**–**P13**, two different peaks for the hydroxyl groups were observed as broad singlets at 10.26 and 10.04 ppm, which disappeared on treatment with D_2O. For the 2- or 4-hydroxy derivatives, **P1** and **P3**–**P8**, we only observed a broad



Scheme 1.

Table 1. Chemico-physical data of derivatives **P1**–**P13**

Compd	R	R′	R″	Yield (%)	Mp (°C)
P1	2-OH	4-Cl phenyl	4-OCH_3	66	197–198
P2	H	–C(O)CH_3	4-Cl	74	110–112
P3	2-OH	–C(O)CH_3	4-Cl	66	136–138
P4	2-OH	–C(O)CH_3	3,4-OCH_3	58	160–164
P5	4-OH	–C(O)CH_3	2-Cl	83	214–215
P6	4-OH	–C(O)CH_3	4-Cl	73	259–260
P7	4-OH	–C(O)CH_3	2-OCH_3	93	183–184
P8	4-OH	–C(O)CH_3	4-OCH_3	90	217–218
P9	2,6-OH	–C(O)CH_3	4-CH_3	52	259–260
P10	2,4-OH	–C(O)CH_3	4-CH_3	70	256–258
P11	2,4-OH	–C(O)CH_3	4-Cl	75	253–255
P12	2,4-OH	–C(O)CH_3	4-OCH_3	70	253–255
P13	2,4-OH	–C(O)CH_3	2,4-OCH_3	60	242–244

singlet at 10.20 ppm, which disappears with D_2O. For all compounds, **P1**–**P13**, the aromatic protons appear as multiplets in the 7.60–6.40 ppm range. The methyl group of the N1-acetyl was always observed at 2.03 ppm, while for the mono- and di-methoxy derivatives, **P1**, **P4**, **P7**, **P8**, **P12** and **P13**, a singlet centered at 3.73 ppm was observed. Finally, the methyl substituted compounds, **P9** and **P10**, show an additional peak at 2.35 ppm.

The synthesized compounds **P1**–**P13** were first assayed against different species of Gram-positive and Gram-negative bacteria of clinical relevance and also against various strains of pathogenic fungi in order to identify those with little or no activity as leading compounds.

The data obtained against all the assayed species listed in the Experimental section were in the range 64 to >128 μg/mL range. From these results it was possible to select all the synthesized compounds for subsequent screening towards *H. pylori*.

Comparison of activity of the substances with the reference compound metronidazole was carried out against 17 strains of *H. pylori* including the reference strain NCTC 11637 and the other three metronidazole resistant strains 8, 19 and 20. The MIC ranges and the MIC at which 50% (MIC_{50}) and 90% (MIC_{90}) of the *H. pylori* tested strains are inhibited are presented in Table 2, together with the MIC values of the prepared compounds against the metronidazole resistant strains of *H. pylori*.

All the assayed compounds showed interesting activity against *H. pylori* strains (MIC 1–8 μg/mL) with the exception of derivatives **P1** and **P13**, whose MIC values were in the 32–128 μg/mL range. The pyrazolines **P7**, **P8** and **P12**, bearing a methoxy group in the *ortho*- or *para*-position in the 5-phenyl ring and 4-hydroxy and 2,4-dihydroxy groups in the 3-phenyl ring, showed the lowest MIC against the metronidazole resistant strains, suggesting their importance as substituents. The presence of a second methoxy group in the 5-phenyl ring did not positively affect the activity (see **P4** and **P13** derivatives). An important requisite is the presence of the acetyl group on the N1 of the pyrazoline nucleus, which appears necessary for activity. In fact on comparing the MIC values of compound **P1** and compound **P8**, respectively, with a phenyl group and an acetyl group on the N1 of the pyrazoline, it can be observed that the former

Table 2. Minimal inhibitory concentration (MIC) of compounds **P1**–**P13** and metronidazole (M) against 17 *H. pylori* strains, including four metronidazole resistant strains

				MIC (μg/mL)						
Compd	R	R′	R″	All strains			Metronidazole resistant			
				Range	MIC_{50}	MIC_{90}	NCTC 11637	**8**	**19**	**20**
P1	2-OH	4-Cl phenyl	4-OCH_3	32–128	64	64	128	128	32	128
P2	H	–C(O)CH_3	4-Cl	2–8	4	8	8	8	8	8
P3	2-OH	–C(O)CH_3	4-Cl	2–8	4	8	8	8	2	8
P4	2-OH	–C(O)CH_3	3,4-OCH_3	2–16	8	16	16	8	8	16
P5	4-OH	–C(O)CH_3	2-Cl	1–8	2	8	16	16	16	8
P6	4-OH	–C(O)CH_3	4-Cl	0.25–16	8	16	16	16	8	8
P7	4-OH	–C(O)CH_3	2-OCH_3	4–32	8	16	8	4	2	2
P8	4-OH	–C(O)CH_3	4-OCH_3	2–16	4	8	1	4	4	4
P9	2,6-OH	–C(O)CH_3	4-CH_3	8–128	32	128	64	32	>64	>64
P10	2,4-OH	–C(O)CH_3	4-CH_3	2–16	4	16	4	4	16	16
P11	2,4-OH	–C(O)CH_3	4-Cl	2–8	4	8	4	8	4	8
P12	2,4-OH	–C(O)CH_3	4-OCH_3	4–32	8	8	8	4	4	4
P13	2,4-OH	–C(O)CH_3	2,4-OCH_3	2–32	16	16	16	16	16	32
M				0.125–64	0.5	64	64	64	64	64

is not active, while the latter is one of the most active. The presence in the 5-phenyl ring of an electron-withdrawing group, such as chlorine, or that of a methyl group does not affect activity, except for compound **P3** against the metronidazole resistant strain 19 (Table 2).

2. Experimental[32]

2.1. Synthesis

2.1.1. General procedure for the preparation of chalcones A-I (Route 1). An aqueous solution of potassium hydroxide (20% 10 mL) was added whilst stirring overnight to a solution of the appropriate aryl aldehyde (30 mmol) and appropriate acetophenone (30 mmol) in ethanol 96% (75 mL) at room temperature. The reaction mixture was then poured into water (100 mL) and after neutralization with hydrochloric acid (5%) a yellow solid was recrystallized from ethanol.

2.1.2. General procedure for the preparation of 2,4-dihydroxy-phenyl chalcones L–O (Route 2). A solution of 3,4-dihydro-α-pyran (89.68 mmol) in methylene chloride (50 mL) was added dropwise to a suspension of 2,4-dihydroxy-acetophenone (30 mmol) and pyridinium *p*-toluene sulfonate (PPTS) (0.72 mmol) in methylene chloride (150 mL) and stirred at room temperature for 12 h. The reaction mixture was washed with water, the organic layer dried (Na_2SO_4) and concentrated in vacuo to obtain the protected acetophenone. A solution of 2,4-dihydroxy-4-(tetrahydropyran-2-yloxy) acetophenone (5 mmol) and the appropriate benzaldehyde (5 mmol) in ethanol (12 mL) was slowly added to a solution of barium hydroxide octahydrate (7.43 mmol) in ethanol (60 mL) and stirred for 24 h at 40 °C. The reaction mixture was concentrated in vacuo, washed with water (100 mL), neutralized with 1 M HCl and extracted with ethyl acetate. The organic layer was dried (Na_2SO_4) and concentrated in vacuo yielding the crude 2,4-dihydroxy-4-(tetrahydropyran-2-yloxy) chalcone. The compound was suspended in ethanol (30 mL) and *p*-toluene sulfonic acid (0.12 mmol) was added. The reaction mixture was stirred for 12 h at room temperature, diluted with water (30 mL), neutralized with Na_2CO_3 and extracted with ethyl acetate. The organic layer was dried (Na_2SO_4) and evaporated in vacuo to give the chalcones **L**–**O**.

2.1.3. General procedure for the preparation of 1-(4-Cl-phenyl) and 1-acetyl-3,5-diphenyl-4,5-dihydro-(1H)-pyrazole derivatives P1–P13. A solution of chalcone **A**–**O** (5 mmol) in 30 mL of acetic acid was added dropwise to 12.5 mmol of the appropriate hydrazine in 10 mL of absolute ethanol and stirred at 120 °C for 24 h. The mixture was then poured in ice water obtaining the crude pyrazole derivatives **P1**–**P13**, which were crystallized from ethanol.

2.2. Antimicrobial Activity

2.2.1. Antibacterial and antifungal activity. All synthesized derivatives were evaluated for their antimicrobial and antifungal activity dissolved in dimethylsulfoxide (DMSO). Organisms from routine clinical Gram-positive (*S. aureus*, *S. epidermidis*, *Staphylococcus* spp., *S. faecium*) and Gram-negative isolates (*E. coli*, *K. pneumoniae*, *E. cloacae*, *P. stuartii*) and four *Candida* species isolates (*C. albicans*, *C. krusei*, *C. sakè* and *C. parapsilosis*) from the respiratory tract were collected from specimens of patients at the 'Azienda Policlinico Umberto I' of Rome 'La Sapienza' University. The isolates were subcultured on qualified medium to ensure purity. Identification of the isolates was performed by conventional methodologies; all isolates were subcultured to ensure optimal growth.

The in vitro antibacterial activities of the compounds were determined by the broth micro dilution method, as recommended by the National Committee for Clinical Laboratory Standards[29] with Müeller-Hinton II broth (BBL Microbiology Systems, Cockeysville, MD). Microtiter plates containing serial dilutions of each antimicrobial agent were inoculated with each organism to yield the appropriate density (10^5/mL) in a 100 μL final volume; each plate included positive controls (bacteria without a compound) and a negative control (medium only). The plates were incubated for 18–22 h at 35 °C. The Minimal Inhibitory Concentration (MIC) for all isolates was defined as the lowest concentration of antimicrobial agent that completely inhibited the growth of the organism, as detected by an unaided eye.

The in vitro antifungal activities of the compounds were determined by the broth micro dilution method with Sabouraud dextrose broth (BBL Microbiology Systems, Cockeysville, MD) as recommended by the NCCLS.[30] Microtiter plates containing serial dilutions of each antimicrobial agent were inoculated with each organism to yield the appropriate density (10^3/mL) in a 100 μL final volume; each plate included positive controls (fungi without a compound) and a negative control (medium only). The plates were incubated for 24 h at 37 °C. The MIC for all isolates was defined as the lowest concentration of antimicrobial agent that completely inhibited growth of the organism as detected by an unaided eye.

Anti-H. pylori activity: Sixteen clinical *H. pylori* isolates and the reference strain NCTC 11637 were used. Four of these strains were metronidazole resistant.

They were maintained at −80 °C in Wilkins Chalgren with 10% (v/v) horse serum (Seromed) and 20 % (v/v) glycerol (Merck) until they were used for the experiments. The bacteria were grown on Columbia agar base (Difco Laboratories) supplemented with 10% horse serum (Seromed) and 0.25% Bacto yeast extract (Difco) incubated for 72 h at 37 °C under microaerobic conditions (10% CO_2) in a gas incubator (Haereus). Before use the media were always preincubated under the same microaerobic conditions for a minimum of 2 h to allow equilibration and none of the cultures were kept in air for more than 15 minutes.

The MIC were determined by the agar dilution standard method[31] incubating the bacteria in microaerobic conditions.

By serial double dilutions, the testing compounds were diluted in agar medium in order to have concentrations ranging from 128 to 0.0039 μg/mL.

The plates of Columbia agar with horse serum and yeast extract containing antimicrobial agents were prepared on the day they were used. The inoculum was prepared as follows: a suspension of 72 h growth of each strain on agar plates was made in Wilkins Chalgren broth (Difco) at a turbidity equivalent to the 0.5 McFarland standard. The plates were inoculated using a multipoint inoculator (Denley A 400 PBI) dispensing 5 μL and incubated at 37 °C for 72 h under microaerobic conditions (10% CO_2 in a gas incubator). The MIC was defined as the lowest concentration capable of inhibiting any visible bacterial growth.

Acknowledgements

This work was supported by grants from MURST.

References and notes

1. Marshall, B. J.; Warren, J. R. *Lancet* **1984**, *1*, 1311–1315.
2. Marshall, B. J. *Am. J. Gastroenterol.* **1994**, *89*(8), S116–S128.
3. Lee, A.; O'Rourke, J. In *Biology and Clinical Practice*; Goodwin, C. S., Worsley, B. W., Eds.; CRC: London, 1993; pp 15–35.
4. Nomura, A.; Stemmermann, G. N.; Chyou, P. H.; Perez-Perez, G. I.; Blaser, M. J. *Ann. Intern. Med.* **1994**, *120*(12), 977–981.
5. Sipponen, P.; Hyvarinen, H. *Scand. J. Gastroenterol.* **1993** (Suppl. 196), 3–6.
6. Labenz, J.; Borsch, G. *Gut* **1994**, *35*(1), 19–22.
7. Parsonnet, J.; Friedman, G. D.; Vandersteen, D. P.; Chang, Y.; Vogelman, J. H.; Orentreich, N.; Sibley, R. K. *New. Engl. J. Med.* **1991**, *325*(16), 1127–1131.
8. NIH Consensus Development Panel on *Helicobacter pylori* in peptic ulcer disease. *J. Am. Med. Assoc.* **1994**, *272*, 65–69.
9. Webb, P. M.; Forman, D. *Bailliere's Clin. Gastroenterol.* **1995**, *9*(3), 563–582.
10. Logan, R. P. *Lancet* **1994**, *344*(8929), 1078–1079.
11. Adamek, R. J.; Suerbaum, S.; Pfaffenbach, B.; Opferkuch, W. *Am. J. Gastroenterol.* **1998**, *93*(3), 386–389.
12. Alarcon, T.; Domingo, D.; Prieto, N.; Lopez-Brea, M. *J. Antimicrob. Chemother.* **2000**, *46*(4), 613–616.
13. Graham, D. Y.; Lew, G. M.; Malaty, H. M.; Evans, D. G., Jr.; Evans, D. J., Jr.; Klein, P. D.; Alpert, L. C.; Genta, R. M. *Gastroenterology* **1992**, *102*(2), 493–496.
14. Penston, J. G. *Aliment. Pharmacol. Therap.* **1994**, *8*(4), 369–389.
15. Moayyedi, P.; Sahay, P.; Tompkins, D. S.; Axon, A. T. *Eur. J. Gastroeterol. Hepatol.* **1995**, *7*(9), 835–840.
16. Muri, E. M. F.; Williamson, J. S. *Mini-rev. Med. Chem.* **2004**, *4*, 201–206.
17. Brown, R. A.; Fischer, R.; Blunk, J.; Durham, N. N.; Remalingam, K.; Wong, L. F.; Berlin, K. D. *J. Med. Chem.* **1977**, *20*(5), 664–669.
18. Soliman, R.; Habib, N. S.; Ashour, F. A.; El-Taiebi, M. *Boll. Chim. Farm.* **2001**, *3*, 140–148.
19. Kadu, M. V.; Jamode, V. S.; Tambekar, D. H. *Asian J. Chem.* **1999**, *11*(3), 1064–1066.
20. Deshmukh, M. S.; Rajput, P. R.; Chincholkar, M. M. *Asian J. Chem.* **1997**, *9*(4), 848–850.
21. Hiroshi, N.; Akira, S.; Masumi, I. *Chem. Abstr.* **1970**, *73*, 25457z.
22. Fahmy, A. M.; Hassa, K. M.; Khalaf, A. A.; Ahmed, R. A. *Indian J. Chem.* **1987**, *26B*(9), 884–887.
23. Holla, B.; Shivarama; Akberali, P. M.; Shivananda, M. K. *Il Farmaco* **2000**, *55*, 256–263.
24. Copeland, R. A.; Marcinkeviciene, J.; Haque, T. S.; Kopcho, L. M.; Jiang, W.; Wang, K.; Ecret, L. D.; Sizemore, C.; Amsler, K. A.; Foster, L.; Tadesse, S.; Combs, A. P.; Stern, A. M.; Trainor, G. L.; Slee, A.; Rogers, M. J.; Hobbs, F. *J. Biol. Chem.* **2000**, *275*(43), 33373–33378.
25. Haque, T. S.; Tadesse, S.; Marcinkeviciene, J.; Rogers, M. J.; Sizemore, C.; Kopcho, L. M.; Amsler, K. A.; Ecret, L. D.; Zhan, D. L.; Hobbs, F.; Slee, A.; Trainor, G. L.; Stern, A. M.; Copeland, R. A.; Combs, A. P. *J. Med. Chem.* **2002**, *45*, 4669–4678.
26. Manna, F.; Chimenti, F.; Bolasco, A.; Bizzarri, B.; Befani, O.; Pietrangeli, P.; Mondovì, B.; Turini, P. *J. Enzyme Inhib.* **1998**, *13*, 207–216.
27. Manna, F.; Chimenti, F.; Bolasco, A.; Secci, D. Bizzarri, B.; Befani, O.; Mondovì, B.; Turini, P.; Alcaro, S.; Tafi, A. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3629–3633.
28. Chimenti, F.; Bolasco, A.; Manna, F.; Secci, D.; Chimenti, P.; Befani, O.; Turini, P.; Giovannini, V.; Mondovì, B.; Cirilli, R.; La Torre, F. *J. Med. Chem.* **2004**, *47*(8), 2071–2074.
29. National Committee for Clinical Laboratory Standards ***2002/2003***. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*. Approved standard M7-A6. National Committee for Clinical Laboratory Standards, Wayne, PA.
30. National Committee for Clinical Laboratory Standards ***2003***. *Performance standards for antimicrobial susceptibility testing*. Supplement M100-S13. National Committee for Clinical Laboratory Standards, Wayne, PA.
31. National Committee for Clinical Laboratory Standards ***2004***. *Methods for Antimicrobial Susceptibility Testing of Anaerobic bacteria*. Approved standard M11-A6. 6th Ed. National Committee for Clinical Laboratory Standards, Villanova, PA.
32. Satisfactory analyses (±0.4%) were obtained for all compounds.