A very promising antibiofilm activity against Candida albicans from an in vitro screening for antimicrobial, antibiofilm and antiproliferative activity of new synthesized 4-cinnamamido- and 2-phenoxyacedamido-1H-pyrazol-5-yl)benzamides.

Fabiana Plescia  
University of Palermo: Universita degli Studi di Palermo

Valentina Catania  
University of Palermo: Universita degli Studi di Palermo

Antonella D’Anneo  
University of Palermo: Universita degli Studi di Palermo

Demetrio Raffa (✉ demetrio.raffa@unipa.it)  
University of Palermo: Universita degli Studi di Palermo

Marianna Lauricella  
University of Palermo: Universita degli Studi di Palermo

Domenico Schillaci  
University of Palermo: Universita degli Studi di Palermo

Research Article

Keywords: 4-cinnamamido-1H-pyrazol-5-yl)benzamides, 2-phenoxyacedamido-1H-pyrazol-5-yl)benzamides, antiproliferative activity, antimicrobial activity, antibiofilm activity, biofilm associated infections Candida albicans

Posted Date: December 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2326622/v1

License: ☕ 📧 This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Several new synthesized 4-cinnamamido- and 2-phenoxyaceticamido-1H-pyrazol-5-yl)benzamides were obtained by two multi step different synthetic routes in order to maximize their yield. The new derivatives were screened to determine the antiproliferative, antimicrobial and antibiofilm activity. The biological results showed how, respect to the antiproliferative and antimicrobial activities, the compounds showed a low to missing activity. Different are the results obtained with respect to the antibiofilm activity, especially towards Candida albicans. Most of the synthesized compounds showed a good percentage inhibition of biofilm formation ranging from 60 to 73% with a Biofilm Inhibition Concentration 50% (BIC\textsubscript{50}) from 0.13 to 0.01 µM. Among the synthesized the ethyl 5-(4-(2-(4-chlorophenoxy)acetamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylate (27c) resulted the most active with a BIC\textsubscript{50} of 0.01 µM. According to the result obtained, such compound could be considered a lead subject of further studies to obtain novel and more effective antibiofilm agents against C. albicans infections.

1. Introduction

Acetimidobenzamides are well represented in literature as antimicrobial, antibiofilm and antiproliferative agents. Fusaribenzamide A 1 [1], fusarithioamide B 2 [2], and N-(1-Adamantylcarbamothioyl)benzamides 3 [3] are examples of antimicrobial acetimidobenzamides (Fig. 1). Also, the antibiofilm activity is well represented among acetimidobenzamides such as cationic lipo-benzamides 4 [4] and N-(oxazolylmethyl)-thiazolidinediones 5 [5] (Fig. 1).

For such kind of compounds, the antiproliferative activity is also reported. Examples of antiproliferative acetimidobenzamides are Fusarithioamide B 2 [2] which showed cytotoxic effect against BT-549, MCF-7, SKOV-3, and HCT-116 cell lines, the N-(2-aminophenyl)benzamide acridines 6 [6] endowed with HDAC inhibitory activity and the 3-2-(1H-benzo[d]imidazol-2-ylthio)acetamido)-N-(4-methoxyphenyl)benzamide 7 which showed an IC\textsubscript{50} of 4.12 µM against the HCT116 cells [7] (Fig. 1). Our research group has long been interested in the chemistry and pharmacology of this class of molecules aiming to study their biological activity [8–11]. In Fig. 2, compounds 8–11, the most active among our previously synthesized benzamides, were reported.

Furthermore, among this class of derivatives, the 4-acetamido-N-methylbenzamido is the most common scaffold found in many antimicrobial and antiproliferative agents. Particularly, antimicrobial activity for 4-acetamido thiazolidin-4-ones 12 [12], 4-acetylamino 1H-imidazolylbenzamide 13 [13], 4-benzamido-N-(4-oxo-2-phenylthiazolidin-3-yl)benzamides 14 [14], and 1H-pyrrole-2-carboxamide 15 [15] (Fig. 3) is reported.

Also, the antiproliferative activity is described for 3-aminopyridine-derived amides 16 [16] and 3-(4-(2-methoxybenzamido)benzamido)benzoic acid 17 [17] (Fig. 3).

On the basis of our previously research [8–11] and considering the biological activity of 4-acetamido-N-methylbenzamides and the antibiofilm activity described by us for pyrazole-4-carboxamides [18],
previously synthesized benzamides 8–11 (Fig. 2) have been modified according to Fig. 4.

Particularly, the carboxamide group has moved from the 2 to 4 position to obtain the 4-acetamido-N-methylbenzamido structure and the molecule has been stretched by adding a pyrazole nucleus. The antimicrobial, antibiofilm as well as the antiproliferative activities were evaluated for the new synthesized derivatives.

2. Results And Discussion

2.1. Chemistry

4-Nitrobenzoyl chloride 18 is commercially available. Crude 2-phenoxyacetyl chlorides 24a-c, 33a-d and cinnamoyl chlorides 23a-c were obtained by refluxing the appropriate acids 22a,c, 32a-d and 21a,c with thionyl chloride (schemes 1 and 2).

Compounds 25a,e, 26a,c,f, 27b,c,d, 28a,e,f, 29a and 34a-d, were obtained by two different synthetic routes in order to maximize their yield as reported in schemes 1 and 2. However, despite all attempts to obtain complete series of derivatives by varying the experimental conditions, we have not been able to isolate some of the foreseen compounds. Particularly, 4-cinnamamidobenzamido)-1H-pyrazoles 25a,e, 26a,c,f, 27b,c,d and 29a were obtained according to scheme 1. By refluxing for 8h in acetonitrile 4-nitrobenzoyl chloride 18 with the appropriate ethyl 5-amino-1-R-1H-pyrazole-4-carboxylate 19a,e, the ethyl 1-R-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylates 20a,e were obtained. By reduction with hydrogen and 10% Pd-C in a Parr apparatus, the parent nitro derivatives 20a,e were transformed in the corresponding key intermediates 25a,e.

By refluxing for 8h in acetonitrile the opportune cinnamoyl or phenoxychloride 23a,e and 24a,e with the intermediates 25a,e, ethyl 5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylates 26a,c,f and ethyl 1-methyl-5-(4-(2-phenoxyacetamido)benzamido)-1H-pyrazole-4-carboxylate 27b,c,d were obtained. Furthermore, hydrolysis of derivatives 26 with a mixture formed by equal volumes of 4% aqueous solution of sodium hydroxide and ethanol, afforded the 5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid 28a,e,f. Finally, by fusion of 5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid 28a it was possible to obtain 4-cinnamamido-N-(1-methyl-1H-pyrazol-5-yl)benzamide 29a.

Finally, regarding the 1-methyl-5-(4-(2-phenoxyacetamido)benzamido)-1H-pyrazole-4-carboxylic acids 34a,e, attempt to obtain them with the same way (scheme 1), failed. Derivatives 34 were therefore obtained to a different synthetic route, according to the scheme 2.

By refluxing for 8h in acetonitrile 4-nitrobenzoyl chloride 18 with the ethyl 5-amino-1-methyl-1H-pyrazole-4-carboxylate 19, the ethyl 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylate 20a were obtained. This last was hydrolyzed with a mixture formed by equal volumes of 8% aqueous solution of sodium hydroxide and ethanol to give 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylic acid 30. The
reduction of the nitro derivative 30 with hydrogen and 10% Pd-C in a Parr apparatus afforded the corresponding 5-(4-aminobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid 31. Finally, by reacting compound 31 with the opportune 2-phenoxyacetyl chlorides 33a-d, the desired 1-methyl-5-(4-(2-phenoxyacetamido)benzamido)-1H-pyrazole-4-carboxylic acids 34a-d were synthesized.

2.2 Biology

2.2.1 Antimicrobial and antibiofilm activity of substances

All the compounds were tested against four relevant bacteria pathogens, Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212) or Gram-negative (*Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 25922), and against the yeast *Candida albicans* ATCC 10231). The results representing the antimicrobial activity are expressed in terms of minimal inhibitory concentration (MIC) in µg/mL. No antibacterial or antifungal activities were detected at the maximum tested concentration of 100µg/mL against tested strains in planktonic (free living) form.

In consideration of the increasing importance of the role of biofilms in chronic and polymicrobial infections, we also evaluated the antibiofilm properties of the compounds in terms of interference with biofilm formation. The inhibition of biofilm formation was tested against bacterial and fungal strains using a screening concentration of substances equal to 100 µg/mL. All samples showed moderate or weak bacterial biofilm inhibition activities against pathogens, *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E.coli*, ranging from 44 to 10% inhibition percentages.

The results of inhibition of biofilm formation of *C. albicans* at the maximum tested concentration is reported in Fig. 5.

As shown in the Fig. 5, all the tested compounds are endowed of a good activity higher than 50% against the biofilm formation of *C. albicans*. Particularly, the best efficacy in inhibiting the biofilm formation was showed by sample 27d with an activity value of 73%. We also evaluated the biofilm inhibition concentration 50% (BIC₅₀), that is the concentration at which the percentage of inhibition of biofilm formation is equal to 50%. As reported in Table 1, all the tested compounds showed an inhibition in the micro or submicromolar concentrations. Among the tested compounds, the sample 27c with a BIC₅₀ of 0.01 µM (6 µg/mL) was the most effective in inhibiting biofilm formation of *C. albicans*. 
Table 1
Values of concentrations of substances at which the percentage of inhibition of biofilm formation is equal to 50% (BIC$_{50}$).

<table>
<thead>
<tr>
<th>BIC$_{50}$ (µg/mL)</th>
<th>C. albicans ATCC 10231</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/mL µM</td>
<td></td>
</tr>
<tr>
<td>26a 32 0.08</td>
<td></td>
</tr>
<tr>
<td>26c 19 0.04</td>
<td></td>
</tr>
<tr>
<td>26f 13 0.02</td>
<td></td>
</tr>
<tr>
<td>27c 6 0.01</td>
<td></td>
</tr>
<tr>
<td>27d 28 0.06</td>
<td></td>
</tr>
<tr>
<td>28a 18 0.05</td>
<td></td>
</tr>
<tr>
<td>29a 53 0.13</td>
<td></td>
</tr>
<tr>
<td>34a 18 0.04</td>
<td></td>
</tr>
<tr>
<td>34b 29 0.06</td>
<td></td>
</tr>
<tr>
<td>34c 29 0.07</td>
<td></td>
</tr>
</tbody>
</table>

Generally speaking, the available data show that the presence of a phenyl group in the 1-pyrazole position (compounds 28e,f) is not favorable to antibiofilm activity. This is probably due to the greater steric hindrance due to the presence of the phenyl group in the N-1 position. In fact, the activity is maintained in the derivative 29a which, while maintaining the phenyl group in the N-1 position to the pyrazole, has a reduced steric hindrance due to the absence of the substituent in the 4-pyrazole position.

Concerning the 1-methyl derivatives, the antibiofilm activity is modulated by the presence of substituents in the phenyl ring of the styryl and phenoxy groups. In principle, the substitution in the para position is favorable to the activity especially when the substituent is a chlorine atom (compounds 26c, 27c, and 34a).

### 2.2.2. Cytotoxic effects

Synthesized benzamido derivatives 28a,e,f, and 34a-d were preliminarily tested in vitro for their anti-tumor activity against the human triple-negative breast cancer MDA-MB231 cells (Fig. 6). Among the tested compounds only 26c and 26f had a weak inhibitory activity against the MDA-MB231 cells with 51 and 70% at 10 µM (48 h).
3. Conclusion

Based on our previously research, considering the biological activity of 4-acetamido-N-methylbenzamides and the antibiofilm activity described by us for pyrazole-4-carboxamides, compounds 26a,c,f, 27b-d, 28a,ef, 29a and 34a-d were synthesized and evaluated for their antimicrobial and antibiofilm activity as well as for their cytotoxic effects. No antibacterial or antifungal activities were detected at the maximum tested concentration of 100µg/mL against tested strains in planktonic (free living) form. The synthesized compounds resulted also inactive as anti-tumor agents being only derivatives 26c and 26f endowed of a weak inhibitory activity against the breast cancer MDA-MB231 cells. The synthesized compounds resulted in active interference with biofilm formation, even if in a moderate or weak way, against bacterial pathogens, S. aureus, E. faecalis, P. aeruginosa and E.coli. Good activity higher than 50% in inhibiting biofilm formation was instead shown against C. albicans being compound 27c the most active. These observations are very encouraging. C. albicans is a very versatile fungal pathogen and its clinical relevance very often depends on its ability to develop as a multilayered community (biofilm) on natural (host tissues) or artificial surfaces (embedded medical devices, including catheters, prostheses, etc.). The biofilms of C. albicans are naturally resistant to conventional antifungal therapies, and new antifungal agents capable of interfering with growth such as biofilms are needed [19]. Based on obtained results, compound 27c can be considered a good candidate as lead, useful for further developments of agent that interfere with biofilm formation of a relevant nosocomial fungal pathogen.

4. Experimental

4.1. Chemistry

4.1.1. General

Reaction progress was monitored by TLC on silica gel plates (Merck 60, F254, 0.2 mm). Organic solutions were dried over Na2SO4. Evaporation refers to the removal of solvent on a rotary evaporator under reduced pressure. All melting points were determined on a Büchi 530 capillary melting point apparatus and are uncorrected. IR spectra were recorded with a Perkin Elmer Spectrum RXI FT-IR System spectrophotometer, with compound as a solid in a KBr disc or nujol. 1H NMR (300 MHz) and APT (75 MHz) spectra were recorded with a Bruker AC-E spectrometer at r.t.; chemical shifts (δ) are expressed as ppm values. Microanalyses data (C, H, N) were obtained by an Elemental Vario EL III apparatus and were within ± 0.4% of the theoretical values. Yields refer to purified products and are not optimized. The names of the compounds were obtained using Chem Draw Ultra 12.0 software (CambridgeSoft).

4.1.2. General procedure for preparation of benzoyl chlorides 18, 23a,e, 24a,e and 33a,d [20].

4-Nitrobenzoyl chloride 18 was commercially available. Substituted benzoyl chlorides 23a,e, 24a,e and 33a,d were obtained by refluxing for 5 h the appropriate acid derivatives 21a,e, 22a,e and 32a,d (0.01
mole) with thionyl chloride (7.25 mL). After evaporation under reduced pressure, the crude liquid residue was used for subsequent reactions without purification.

4.1.3. General procedure for preparation of compounds 20a,e [21, 22].

A solution of ethyl 1-R-5-amino-1H-pyrazole-4-carboxylate 19a,e (0.01 mole) in acetonitrile (50 mL) was heated under reflux with the 4-nitrobenzoyl chloride 18 (0.01 mole) for 7h. The solid which separated was collected then recrystallized from ethanol to give compounds 20a,e which was identical in all respect (mp, mixed mp, Rf, IR, 1H-NMR) with an authentic specimen of ethyl 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylate 20a [21] and ethyl 5-(4-nitrobenzamido)-1-phenyl-1H-pyrazole-4-carboxylate 20e [22].


To a solution of ethyl 1-R-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylate 20a,e (0.013 moles) or 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylic acid 31 (0.017 moles) in warm ethanol (200 mL) 300 mg of 10% Pd-C as catalyst was added. The mixture was left under hydrogenation in a Parr apparatus at 50 psi for 24 h. The suspension was filtered, and the filtrate was concentrated to a small volume. affording a compound which was identical in all respect (mp, mixed mp, Rf, IR, 1H-NMR) with an authentic specimen of ethyl 5-(4-aminobenzamido)-1-methyl-1H-pyrazole-4-carboxylate 25a [23] and ethyl 5-(4-aminobenzamido)-1-phenyl-1H-pyrazole-4-carboxylate 25e [22]. Compound 31 was isolated as white crystalline product.

5-(4-aminobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid (31): yields: 68%; mp 248 – 50°C. I.R. (cm\(^{-1}\)): 3470 – 2593 (NH\(_2\), NH, OH); 1680 (CO); 1672 (CO). 1H NMR (DMSO-d6) (δ): 3.63 (3H, s, CH\(_3\)); 5.89 (2H, s, exchangeable, NH2); 6.60–7.81 (5H, set of signals, C\(_6\)H\(_4\) and pyrazole H-3); 9.85 (1H, s, exchangeable, NH); 12.27 (1H, broad, exchangeable, OH); 13C NMR (DMSO-d6) (δ): 36.54, 108.26, 113.01, 119.50, 130.30, 140.09, 140.20, 153.28, 163.91, 166.10. Anal. Calc. for C\(_{12}\)H\(_{12}\)N\(_4\)O\(_3\): C, 55.38%; H, 4.65%; N, 21.53%. Found: C, 55.40%; H, 4.67%; N, 21.40.

4.1.6. General procedure for preparation of 1-Methyl-5-(4-(3-phenylpropanamido)benzamido)-1H-pyrazole-4-carboxylic acid 26a,c,f and ethyl 5-(4-(2-(2-R\(_2\)-4-R\(_1\)-phenoxy)acetamido)benzamido)-1-R-1H-pyrazole-4-carboxylate 27b,c,d.

A solution of 5-(4-aminobenzamido)-1-R-1H-pyrazole-4-carboxylate 25a,e (4 mmol) and the appropriate cinnamoyl chlorides 23a,e (4 mmol) in acetonitrile (20 mL) was refluxed for 8h. The solvent was partially evaporated under reduced pressure until a product precipitates. The residue was collected and
recrystallized from ethanol to give pure 26a,c,f. Compounds 27b,c,d were obtained with the same procedure using 5-(4-aminobenzamido)-1-R-1H-pyrazole-4-carboxylate 25a,e (1.74 mmol) and the appropriate 2-phenoxyacetyl chlorides 24a,e (4 mmol) in acetonitrile (20 mL).

**Ethyl 5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylate (26a):** yields 82%, mp 195–200°C; I.R (Nujol) cm$^{-1}$ 3389 − 3273 (NH), 1694 (CO) 1660 (CO), 1H NMR (CHCl$_3$) $\delta$: 1.32 (t, 3H, CH$_3$); 3.88 (s, 3H, CH$_3$); 4.276 (q, 2H, CH$_2$); 6.597 (d, 1H, J = 15.9 Hz, olefinic CH); 7.26–7.99 (m, 15H, ArH and olefinic CH); 8.23 (s, 1H, pyrazole H3); 9.29 (s, 1H, exchangeable, NH). 13C NMR( $\delta$ (CDCl$_3$) 14.35, 38.56 60,46, 104.40, 119.56, 120.35, 127.71, 128.08, 128.97, 129.11, 130.32, 134.32, 139.49, 140.66, 142.46, 143.31, 164.08, 164.37, 165.07. Anal. Calc. for C$_{23}$H$_{22}$N$_4$O$_4$: C, 66.02%; H, 5.30%; N, 13.39%. Found: C, 65.68%; H, 5.01%; N, 13.05.

**Ethyl 5-(4-(3-(4-chlorophenyl)acrylamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylate 26c:** yields 103.5%, mp 205–207°C; I.R (Nujol) cm$^{-1}$ 3558 − 3254 (NH), 1708-1891-1655 (CO), 1H NMR (DMSO) $\delta$: 1.14 (s, 3H, CH$_3$); 4.13 (q, 2H, CH$_2$); 6.87 (d, 1H, J = 15.9 Hz, olefinic CH); 7.51–7.90 (m, 15H, ArH and olefinic CH); 8.01 (s, 1H, pyrazole H3); 10.33 (s, 1H, exchangeable, NH); 10.56 (s, 1H, exchangeable, NH). 13C NMR( $\delta$ (CDCl$_3$) 14.59, 36.46, 39.17, 39.45, 39.73., 40.01, 40.29, 40.56, 40.84, 108.09, 119.04, 123.18, 127.86, 129.53, 129.58, 130.00, 134.02, 134.89, 139.42, 140.03, 143.27, 162.19, 164.23, 165.85. Anal. Calc. for C$_{23}$H$_{21}$ClN$_4$O$_4$: C, 61.00%; H, 4.67%; N, 12.37%. Found: C, 60.69%; H, 4.70%; N, 12.32.

**Ethyl 5-(4-(3-(4-chlorophenyl)acrylamido)benzamido)-1-phenyl-1H-pyrazole-4-carboxylate 26f:** yields 60%, mp 250–255°C; I.R (Nujol) cm$^{-1}$ 3583-3356-3212 (NH), 1894 (CO) 1698 (CO), 1H NMR (DMSO) $\delta$: 1.14 (s, 3H, CH$_3$); 4.18 (q, 2H, CH$_2$); 6.88 (d, 1H, J = 15.9 Hz, olefinic CH); 7.40–7.94 (m, 15H, ArH and olefinic CH); 8.19 (s, 1H, pyrazole H3); 10.41 (s, 1H, exchangeable, NH); 10.56 (s, 1H, exchangeable, NH). 13C NMR( $\delta$ (DMSO) 14.56, 39.16, 39.44, 39.71, 39.99., 40.27, 40.55, 40.83, 110.63, 119.09, 123.16, 124.21, 127.88, 128.88, 129.37, 129.57, 129.73, 129.99, 134.01, 134.90, 138.41, 139.19, 140.03, 141.77, 143.27,162.04, 164.22, 166.44. Anal. Calc. for C$_{28}$H$_{23}$ClN$_4$O$_4$: C, 65.31%; H, 4.50%; N, 10.88%. Found: C, 65.55%; H, 4.84%; N, 11.22.

**Ethyl 1-methyl-5-(4-(2-(o-tolyloxy)acetamido)benzamido)-1H-pyrazole-4-carboxylate 27b:** yields 40%; mp. 183 – 85°C. I.R. (cm$^{-1}$): 3330 (NH); 3210 (NH); 1715(CO); 1664 (CO). 1 H NMR (DMSO-d$_6$) (δ): 1.20 (3H, t, CH$_3$); 2.18 (3H, s, CH$_3$); 3.67 (3H, s, CH$_3$); 4.20 (2H, q, CH$_2$); 4.70 (2H, s, CH$_2$); 6.85–8.01 (m, 9H, 2 x C$_6$H$_4$ and pyrazole H3); 10.30 (1H, s, exchangeable, NH); 10.38 (1H, s, exchangeable, NH). Anal. Calc. for C$_{23}$H$_{24}$N$_4$O$_5$: C, 63.29%; H, 5.54%; N, 12.84%. Found: C, 63.09%; H, 5.15%; N, 12.57.

**Ethyl 5-(4-(2-(4-chlorophenoxy)acetamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylate 27c:** yields 44%; mp 168 − 70°C. I.R. (cm$^{-1}$): 3341 (NH); 3228 (NH); 1716(CO); 1664 (CO). 1 H NMR (DMSO-d$_6$) (δ): 1.13 (3H, t, CH$_3$); 3.69 (3H, s, CH$_3$); 4.12 (2H, q, CH$_2$); 4.77 (2H, s, CH$_2$); 7.03–8.02 (9 H, m, 2 x C$_6$H$_4$ and pyrazole H3); 10.33 (1H, s, exchangeable, NH); 10.43 (1H, s, exchangeable, NH). Anal. Calc. for C$_{22}$H$_{21}$ClN$_4$O$_5$: C, 57.84%; H, 4.36%; N, 12.26%. Found: C, 57.66%; H, 4.98%; N, 12.52.
Ethyl 5-(4-(2-(2,4-dichlorophenoxy)acetamido)benzamido)-1-methyl-1H-pyrazole-4-carboxylate 27d: yields 37%; mp 188 – 90°C. I.R. (cm⁻¹): 3386 (NH); 1712 (CO); 1681 (CO). ¹H NMR (DMSO-d₆) (δ): 1.12 (3H, t, CH₃); 3.68 (3H, s, CH₃); 4.12 (2H, q, CH₂); 4.92 (2H, s, CH₂); 7.12–8.01 (8 H, m, C₆H₃, C₆H₄ and pyrazole H3); 10.33 (1H, s, exchangeable, NH); 10.51 (1H, s, exchangeable, NH). ¹³C NMR(δ) (DMSO) 14.59, 36.46, 59.92, 68.21, 108.10, 115.83, 119.17, 122.96, 125.56, 128.17, 128.52, 129.98, 139.39, 140.05, 142.45, 153.11, 162.19, 165.81, 166.76. Anal. Calc. for C₂₂H₂₀Cl₂N₄O₅: C, 53.78%; H, 4.10%; N, 11.40%. Found: C, 54.09%; H, 3.81%; N, 11.02.

4.1.6. General procedure for preparation of 1-Methyl-5-(4-(3-phenylpropanamido)benzamido)-1H-pyrazole-4-carboxylic acid 28a,e,f.

To a solution of ethyl 1-R-1H-pyrazole-4-carboxylates 26a,c,f (3.2 mmoles) in ethanol (18.75 ml), a solution aqueous 4% of NaOH (22.5 ml) was added. The reaction mixture is heated under reflux for 15’, then left at room temperature for 12 h.

After this time, the ethanol was removed under reduced pressure and the remaining aqueous solution was acidified with 1M HCl until complete precipitation of the acids. Finally, the precipitate was filtered and crystallized with ethanol to give compounds 28a,e,f.

5-(4-cinnamamidobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid (28a): yields 80% mp 228–232°C; I.R (Nujol) cm⁻¹ 3254 (NH) 1698 (CO); ¹H NMR (DMSO) δ: 3.68 (d, 1H, J = 15.9 Hz, olefinic CH); 7.05–7.94 (m, 15H, ArH and olefinic CH); 8.05 (s, 1H, pyrazole H3); 10.35 (s, 1H, exchangeable, NH); 10.38 (s, 1H, exchangeable, NH); 12.35 (s, 1H, broad, exchangeable, OH). ¹³C NMR(δ) (DMSO) 36.50, 108.77, 118.91, 122.72, 127.64, 128.28, 129.50, 130.39, 135.14, 139.37, 140.32, 141.01,143.57, 163.71, 164.56, 165.84. Anal. Calc. for C₂₁H₁₈N₄O₄: C, 64.61%; H, 4.65%; N, 14.35%. Found: C, 64.74%; H, 4.45%; N, 14.43.

5-(4-cinnamamidobenzamido)-1-phenyl-1H-pyrazole-4-carboxylic acid (28e): yields 80% mp 230–232°C; I.R (Nujol) cm⁻¹ 3308 (NH) 1764, 1681, 1655 (CO); ¹H NMR (DMSO) δ: 3.68 (d, 1H, J = 15.9 Hz, olefinic CH); 7.41–7.91 (m, 15H, ArH and olefinic CH); 8.15 (s, 1H, pyrazole H3); 10.37 (s, 1H, NH); 10.54 (s, 1H, exchangeable, OH). ¹³C NMR(δ) (CDCl₃) 39.14, 39.42, 39.70, 39.98, 40.26, 40.53, 40.81, 111.36, 119.08, 122.34, 124.15, 127.87, 128.31, 128.74, 129.37, 129.53, 129.67, 130.47, 135.04, 138.60, 141.46, 142.09, 143.31, 163.57, 164.42, 166.33. Anal. Calc. for C₂₆H₂₀N₄O₄: C, 69.02%; H, 4.46%; N, 12.38%. Found: C, C, 68.79%; H, 4.27%; N, 12.60.

5-(4-(3-(4-chlorophenyl)acrylamido)benzamido)-1-phenyl-1H-pyrazole-4-carboxylic acid 28f: yields 70% mp 155–160°C; I.R (Nujol) cm⁻¹ 3579, 3185 (NH) 1693, 1625 (CO); ¹H NMR (DMSO) δ: 3.90 (d, 1H, J = 15.9 Hz, olefinic CH); 7.40–7.92 (m, 15H, ArH e olefinic CH); 8.14 (s, 1H, pyrazole H3); 10.35 (s, 1H, NH); 10.59 (s, 1H, NH); 12.51 (s, 1H, NH), 12.46 (s, 1H, broad, exchangeable, OH). ¹³C NMR(δ) (DMSO) 119.05, 122.41, 124.14, 125.76, 127.86, 128.30, 128.71, 129.34, 129.52, 129.66, 130.44, 130.92, 135.06, 138.60,
139.09, 141.37, 142.06, 142.32, 163.55, 164.42, 166.32. Anal. Calc. for C₂₆H₁₉ClN₄O₄: C, 64.14%; H, 3.93%; N, 11.51%. Found: C, 64.25%; H, 3.60%; N, 11.73.


L’acido 5-(4-cinnamammido)-1fenil-1H-pirazolo-4-carbossilico 17a è stato decarbossilato tramite fusione per l’ottenimento del corrispondente composto 18a. La miscela di reazione ottenuta dalla fusione è stata cristallizzata da etanolo per dare il derivato 18a come solido puro.

4-cinnamamido-N-(1-phenyl-1H-pyrazol-5-yl)benzamide 29a: yields 70% mp 195–197°C; I.R (Nujol) cm⁻¹ 3585, 3254 (NH), 1741 (CO), 1679 (CO); 1H NMR (DMSO) δ: 6.48 (s, 1H, pyrazole H3); 6.86 (d, 1H, J = 15.9 Hz, olefinic CH); 7.35–7.89 (m, 15H, ArH e olefinic CH, pyrazole H4); 10.29 (s, 1H, NH); 10.53 (s, 1H, exchangeable, NH). 13C NMR(δ) (DMSO) 104.72, 119.01, 122.28, 132.72, 127.78, 128.05, 128.32, 129.28, 129.55, 129.58, 130.49, 135.01, 136.53, 139.39, 140.21, 141.44, 143.14, 164.39, 165.92. Anal. Calc. for C₂₅H₂₀N₄O₄: C, 73.51%; H, 4.94%; N, 13.72%. Found: C, 73.73%; H, 5.03%; N, 13.35.

4.1.8. General procedure for preparation of 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylic acid 30.

Compound 30 is known [24] but was prepared in a different way. To a solution of ethyl 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylate 20a (12.3 mmoles) in ethanol (24 ml), a solution aqueous 8% of NaOH (24 ml) was added. The reaction mixture was left at room temperature for 12 h.

After this time, the ethanol was removed under reduced pressure and the remaining aqueous solution was acidified with 1M HCl until complete precipitation of the acids. Finally, the precipitate was filtered and crystallized with ethanol to give a compound which was identical in all respect (mp, mixed mp, Rf, IR, 1H-NMR) with an authentic specimen of 1-methyl-5-(4-nitrobenzamido)-1H-pyrazole-4-carboxylic acid 30 [24].

4.1.9. General procedure for preparation of 5-(4-(3-(2-R₂-4-R₁-phenyl)acrylamido)benzamido)-1-phenyl-1H-pyrazole-4-carboxylic acids 34a-d.

A suspension of 5-(4-aminobenzamido)-1-methyl-1H-pyrazole-4-carboxylic acid 31 (1.73 mmol) and the appropriate 2-phenoxyacetyl chloride 33a-d (1.73 mmol) in acetonitrile (60 mL) was refluxed for 8h. The reaction mixture was filtered, then the solvent was partially evaporated under reduced pressure until a product precipitates. The residue was collected and recrystallized from ethanol to give pure 34a,c,d. Compound 34b, which separated directly from the reaction mixture, was directly crystallized from ethanol.

5-{4-[2-(4-chlorophenoxy)acetamido]benzamido}-1-methyl-1H-pyrazole-4-carboxylic acid (34a): yield 14%; mp 230 – 34°C. I.R. (cm⁻¹): 3259 – 2605 multiple bands (NH, OH); 1685 (CO); 1655 (CO). 1H-NMR (DMSO)
(δ): 3.67 (3H, s, CH₃); 4.78 (2H, s, CH₂); 7.03–8.02 (9 H, 2 x C₆H₄ e pyrazole H-3); 10.36 (1H, s, exchangeable NH); 10.44 (1H, s, exchangeable NH); 12.40 (1H, broad, exchangeable OH). 13C-NMR (DMSO-d₆) (δ): 36.39, 67.54, 108.68, 116.93, 119.49, 125.50, 128.15, 129.45, 129.77, 139.12, 140.37, 142.21, 157.00, 163.65, 166.01, 167.38. Anal. Calc. for C₂₀H₁₇ClN₄O₅: C, 56.02%; H, 4.00%; N, 13.07%. Found: C, 55.75%; H, 4.34%; N, 12.76.

5-{4-[2-(2,4-dichlorophenoxy)acetamido]benzamido}-1-methyl-1H-pyrazole-4-carboxylic acid (34b): yields 34%; mp 245 – 47°C. I.R. (cm⁻¹): 3388 – 2671 (multiple bands, NH, OH); 1701 (broad, CO);. 1H NMR (DMSO-d₆) (δ): 3.66 (3H, s, CH₃); 4.92 (2H, s, CH₂); 7.12–8.02 (8 H, m, C₆H₃, C₆H₄ e pyrazole H₃); 10.30 (1H, s, exchangeable, NH); 10.51 (1H, s, exchangeable NH); 12.25 (s, 1H, broad, exchangeable, OH). Anal. Calc. for C₂₀H₁₆Cl₂N₄O₅: C, 51.85%; H, 3.48%; N, 12.09%. Found: C, 52.03%; H, 3.86%; N, 12.00.

1-methyl-5-{4-[2-(2-methylphenoxy)acetamido]benzamido}-1H-pyrazole-4-carboxylic acid (34c): yields 16%; mp 235 – 37°C. I.R. (cm⁻¹): 3220 – 2507 (multiple bands, NH, OH); 1677 (CO); 1659 (CO). 1H NMR (DMSO-d₆) (δ): 2.23 (3H, s, CH₃); 3.66 (3H, s, CH₃); 4.70 (2H, s, CH₂); 6.89–8.02 (9 H, m, 2 x C₆H₄ and pyrazole H₃); 10.30 (1H, s, exchangeable, NH); 10.38 (1H, s, exchangeable, NH); 12.31 (s, 1H, broad, exchangeable, OH). Anal. Calc. for C₂₁H₂₀N₄O₅: C, 61.76%; H, 4.94%; N, 13.72%. Found: C, 61.82%; H, 4.91%; N, 13.41.

1-methyl-5-{4-[2-(4-methylphenoxy)acetamido]benzamido}-1H-pyrazole-4-carboxylic acid (34d): yields 13%; mp 233 – 35°C. I.R. (cm⁻¹): 3223 – 2507 (multiple bands NH, OH); 1712 (CO); 1697 (CO). 1H NMR (DMSO-d₆) (δ): 2.24 (3H, s, CH₃); 3.70 (3H, s, CH₃); 4.75 (2H, s, CH₂); 6.87–8.04 (9 H, m, 2xC₆H₄ and pyrazole H₃); 10.31 (1H, s, exchangeable NH); 10.39 (1H, s, exchangeable NH); 12.29 (s, 1H, broad, exchangeable, OH). Anal. Calc. for C₂₁H₂₀N₄O₅: C, 61.76%; H, 4.94%; N, 13.72%. Found: C, 61.68%; H, 4.56%; N, 14.04.

4.2. Biology

4.2.1 Cell lines and culture conditions

Triple negative breast cancer MDA-MB231 cells, obtained from Istituto Scientifico Tumori (Genoa, Italy), were grown as monolayers in DMEM medium, supplemented with 10% (v/v) fetal bovine serum (FCS), 2 mM glutamine and 1% non-essential amino acids. The cells were grown at 37°C in a humidified atmosphere containing 5% CO₂ as previously reported [25]. For the experiments, cells were plated on 96-well plates, then were allowed to adhere overnight in culture medium before the treatment with chemicals or vehicle only.

4.2.2 Cell viability assay

For these experiments, MDA-MB231 cells were plated in 96-well plate (8×10³/well) in the presence of different concentrations of the compounds. After 48 h cell viability was determined by a colorimetric assay incubating the cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as
reported [10, 11]. MTT is yellow tetrazolium salt that can be reduced to purple formazan by mitochondrial enzymes of living cells. The absorbance of the formazan was measured by a microplate reader (OPSYS MR, Dynex Technologies, Chantilly, VA, USA) at 540 nm with a reference wavelength of 630 nm and cell viability was quantified as the percentage of the optical density (OD) values of treated cells compared with that of untreated control cells. Each experiment was performed in triplicate.

4.2.3. Microbial Strains

The reference strains Staphylococcus aureus ATCC 25923, Enterococcus faecalis ATCC 29212, Pseudomonas aeruginosa ATCC 15442, Escherichia coli ATCC 25922 and Candida albicans ATCC 10231 were used in the determination of Minimum Inhibitory Concentrations (MICs), and Inhibition of Biofilm Formation (IBF) tests. The bacterial strains were cultured aerobically in Muller-Hinton broth (MHB) or tryptic soy agar (TSA) [26]. Fungal C. albicans strain was cultured aerobically on Sabouraud (BS) broth or agar medium [27].

4.2.4 Determination of Minimum Inhibitory Concentrations (MICs)

MICs were determined by a microdilution method. Briefly, a series of solutions were prepared with a range of concentrations from 100 to 1.5 µg/mL (obtained by two-fold serial dilution). The serial dilutions were made in Mueller–Hinton broth (MH) (Sigma Aldrich) in a 96-wells plate, starting from a stock solution of 100 µg/mL in MH [28]. To each well, 10 µL of a bacterial suspension from a 24 h culture grown at 37°C for 24 hours on Tryptic Soy Agar (TSA), containing ~ 10^6 cfu/mL was added. A growth control and negative control, consisting respectively of bacterial strains in the medium without tested substances, and the medium without both substance and inoculum were also included in the 96-wells plate [29]. A substance control, consisting only of the substance solution in the medium without bacterial inoculum were added to evaluate the absorbance of substance at the tested concentrations. The plate was incubated at 37 °C for 24 h, the MICs were determined by a microplate reader (Glomax Multidetection System TM297 Promega, Milano Italy) as the lowest concentration of compound whose OD, read at 570 nm, was comparable with the negative control wells (broth only, without inoculum), [26]. Antifungal activity against C. albicans ATCC 10231 was evaluated by using a micro-method described above, using Sabouraud broth (BS) (Sigma-Aldrich) as growth medium.

4.2.5 Inhibition of Biofilm Formation (Crystal Violet Method)

Compounds 26a,c,f, 27b-d, 28a,e,f, 29a and 34a-d were tested for their ability to interfere with biofilm formation of C. albicans ATCC 10231 and above mentioned bacterial strains. The yeast was grown in Sabouraud broth (BS) containing 2% (w/v) glucose overnight at 37°C. After the incubation time, 2.5 µL of fungal suspension (containing ~ 10^6 cfu/mL) was placed into each well of a sterile flat-bottom 96-well loaded with 200 µL of BS with 2% glucose, supplemented with a screening concentration of 100µg/mL of each substance [30]. The plates were incubated at 37 °C for 24 h; after this incubation time, the medium was removed, the plates were washed twice with sterile NaCl 0.9%, air-dried and then each well was filled
with 100 µL of crystal violet solution (0.1%) for 15 min. The plate was then washed three times with water, and the crystal violet was dissolved in 200 µl of ethanol by pipetting up and down. Each assay was performed in triplicate and repeated at least twice. The plate was read at 570 nm using a microplate reader (Glomax Multidetection System TM297 Promega, Milano, Italy). Inhibition percentages at screening concentration (or at lower concentrations in the case of activity higher than 50% of each sample) were obtained by comparing the OD of control wells with that of the sample wells, by using the following formula:

Inhibition (%) = (OD growth control/OD sample)/OD growth control) x 100.

BIC$_{50}$ (the concentration at which the percentage of inhibition of biofilm formation is equal to 50%) was calculated using AAT Bioquest, Inc. Quest Graph™ IC50 Calculator (v.1), retrieved from https://www.aatbio.com/tools/ic50-calculator-v1.

Inhibition of bacterial biofilms was determined by using the method described above, using Tryptose broth (TS) (Sigma-Aldrich) enriched with 2% w/v of glucose as growth and test medium [31].

**Declarations**

**Acknowledgement**

Financial support from “Fondo di Finanziamento della Ricerca di Ateneo (ex 60%)” is gratefully acknowledged.

**Compliance with Ethical Standards**

Conflict of interest. The authors declare no competing interests.

**References**


**Scheme**

Scheme 1 and 2 are available in Supplementary Files section.

**Figures**
Figure 1

Examples of antimicrobial, antibiofilm and antiproliferative acetamidobenzamides.
The most active benzamides previously synthesized by us.

Figure 2
Figure 3

Examples of some antimicrobial and antiproliferative agents bearing the 4-acetamido-N-methylbenzamido scaffold.
Figure 4

New structural modification starting from previously synthesized benzamides.
**Figure 5**

Inhibition of biofilm formation, data are the mean ± SD of three independent experiments, each performed at least in quadruplicate, and expressed as inhibition percentage respect to the growth control. Data were considered significant at $P < 0.05$.

**Figure 6**

Cytotoxic effects of compounds on MDA-MB231 breast cancer cells. (A) Dose and time dependence of FVPEO effect on cell viability. MDA-MB231 cells ($8 \times 10^3$) were incubated with various concentrations of compounds (5-25 μM) for 48h. Then, cell viability was evaluated by using MTT assay as reported in methods. Values are reported as the mean ± SE. **P<0.01, ***P<0.001 and ****P<0.0001 vs. the control (CTR).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstrac.tif
- Scheme1.tif
- Scheme2.tif
- supplementarymaterial.docx