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# Synthesis, selective anti-*Helicobacter pylori* activity, and cytotoxicity of novel N-substituted-2-oxo-2*H*-1-benzopyran-3-carboxamides

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ABSTRACT

N-substituted-3-carboxamido-coumarin derivatives were prepared and evaluated for selective antibacterial activity against 20 isolates of *Helicobacter pylori* clinical strains, including five metronidazole resistant ones. Some of them possessed the best activity against *H. pylori* metronidazole resistant strains with MIC values lower than the drug reference (metronidazole). Furthermore, anti-inflammatory activity through the inhibition of the IL-8 production was investigated.



*Helicobacter pylori* are spiral-shaped Gram-negative bacteria with polar flagella that live near the surface of the human gastric mucosa. They have evolved intricate mechanisms to avoid the bactericidal acid environment in the gastric lumen and to survive near, to attach to, and to communicate with the human gastric epithelium and host immune system. This interaction sometimes results in severe gastric pathology. *H. pylori* infection is the strongest known risk factor for the development of gastroduodenal ulcers, with infection being present in 60–80% of duodenal ulcers.[1]

*Helicobacter pylori* is also the first bacterium to be classified as a definite carcinogen by the World Health Organization's International Agency for Research on Cancer because of its epidemiologic relationship to gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma.[2] Most *H. pylori* transmission occurs in childhood, and, in some countries, up to 90% of children become infected by age 10 years, with reports of infection as early as the first months of life.[3]

From the medical point of view, *H. pylori* is a formidable pathogen responsible for much morbidity and mortality worldwide. However, *H. pylori* infection occurs in approximately half of the world population, with disease being the exception rather than the rule. To avoid the bactericidal activity of acid, *H. pylori* generates large quantities of cytosolic and cell surface-associated urease, an enzyme capable of transiently buffering the acidic environment by the breakdown of urea to generate ammonia and carbon dioxide. This enzymatic activity is highly conserved in different *H. pylori* strains and therefore useful for diagnostic purposes.[4,5] To remain in the mucus layer, *H. pylori* need to utilize their polar flagella for motility. Both the ability to swim with flagellar motion and the ability to control the direction of movement by chemotactic responses are essential for *H. pylori* colonization.[6,7] *H. pylori* causes its damage by initiating chronic inflammation in the gastric mucosa. This inflammation is mediated by an array of pro- and anti-inflammatory cytokines. For this reason, anti-inflammatory activity through the inhibition of the IL-8 production was also investigated.

*Helicobacter pylori* infections are difficult to cure and successful treatment generally requires the administration of several antibacterial agents simultaneously. Duration of therapy is also important and depends upon whether resistance is present. Antibiotic resistance has resulted in unsatisfactory eradication with dual and now triple therapy in many countries. Newer antibiotics and changes in dosing and duration of therapy may overcome resistant strains but may only provide limited improvement in eradication rates. Sequential therapy with amoxicillin and a proton pump inhibitor given for 5 days followed by clarithromycin and tinidazole for 5 days is now a first-line therapy for *H. pylori* in some countries. This work is aimed at trying to overcome these problems as well as evaluating a series of N-substituted-2-oxo-2*H*-1-benzopyran-3-carboxamides as a part of a screening program.

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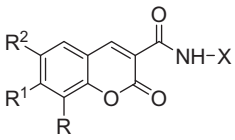
**Table 1**
Chemical and physical data of derivatives **1**–**20**



| Compound | R | $R^1$ | $R^2$ | X |
|---|---|---|---|---|
| **1** | H | OH | H | $(C_6H_4)$-4′-COOEt |
| **2** | H | H | $NO_2$ | $(C_6H_4)$-4′-COOEt |
| **3** | H | H | $CH_3$ | $(C_6H_4)$-4′-COOEt |
| **4** | Cl | H | Br | $(C_6H_4)$-4′-COOEt |
| **5** | Br | H | Br | $(C_6H_4)$-4′-COOEt |
| **6** | OH | H | H | $(C_6H_4)$-4′-COOEt |
| **7** | H | H | OH | $(C_6H_4)$-4′-COOEt |
| **8**[a] | H | $OCH_3$ | H | $(C_6H_4)$-4′-COOEt |
| **9** | H | H | $OCH_3$ | $(C_6H_4)$-4′-COOEt |
| **10** | H | OH | H | $(C_6H_4)$-4′-COOH |
| **11** | H | H | $NO_2$ | $(C_6H_4)$-4′-COOH |
| **12** | H | H | $CH_3$ | $(C_6H_4)$-4′-COOH |
| **13** | Cl | H | Br | $(C_6H_4)$-4′-COOH |
| **14** | Br | H | Br | $(C_6H_4)$-4′-COOH |
| **15** | OH | H | H | $(C_6H_4)$-4′-COOH |
| **16** | H | H | OH | $(C_6H_4)$-4′-COOH |
| **17**[a] | H | $OCH_3$ | H | $(C_6H_4)$-4′-COOH |
| **18** | H | H | $OCH_3$ | $(C_6H_4)$-4′-COOH |
| **19** | H | H | H | $(C_5H_9N)$-1′-COOEt |
| **20** | H | H | Br | $(C_5H_9N)$-1′-COOEt |

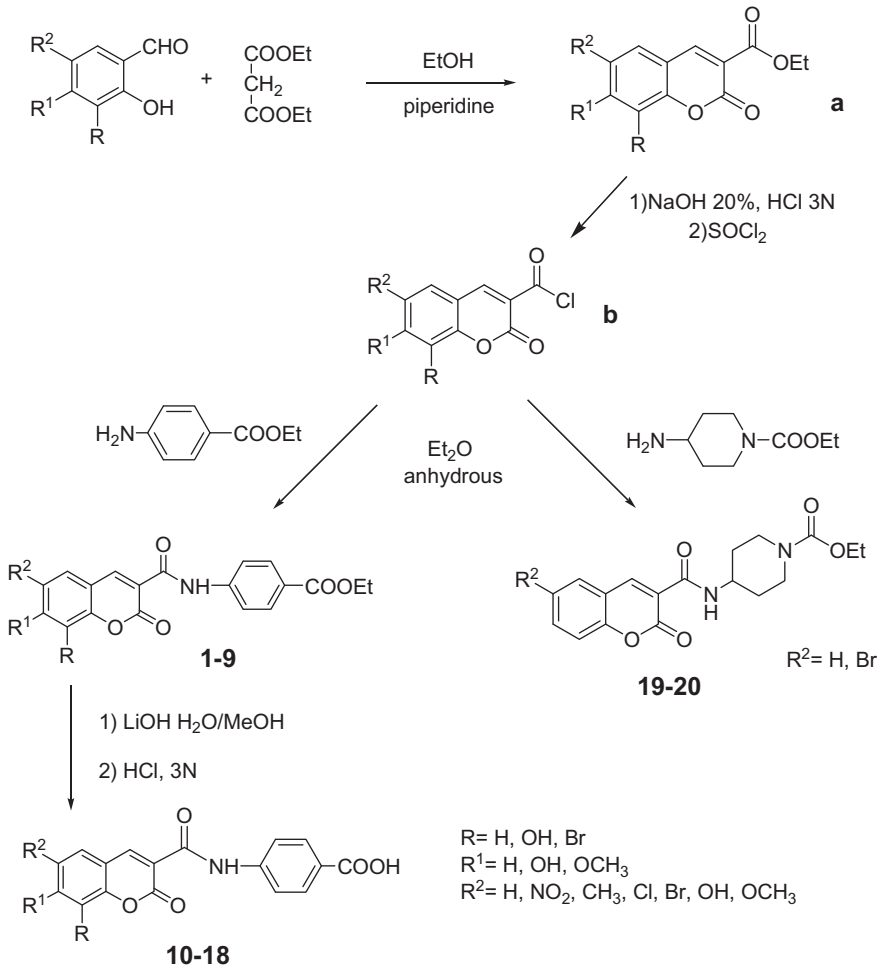
[a] Ref. 14.

In our previous works,[8,9] we have pointed out that a carboxamide function in the coumarin ring might play an important role leading to activity. On the basis of the SAR studies and from the analysis of the structure of natural coumarins reported as potent anti-*H. pylori* agents,[10–12] we have demonstrated that a halogen in the position 6, no bulky substituents in the position 7 and an acyl group on the *p*-position of the *N*-aromatic moiety seemed to be crucial for the activity.

Moving from these indications, in this report we described the synthesis and selective antimicrobial evaluation of a new series of 20 derivatives substituted in the positions 3, 6, 7, and 8 of the coumarin ring (Table 1).

We have synthesized 2-*oxo*-2*H*-chromene-3-carboxylic acid ethyl ester derivatives [coumarins (**a**)] by a Knoevenagel reaction between the appropriate benzaldehyde and diethylmalonate in ethanol by using piperidine as catalyst. They were hydrolyzed with NaOH 20% and HCl 3 N to obtain the corresponding carboxylic acid and treated with thionyl chloride at reflux to give the reactive acyl chlorides (**b**), differently substituted on the 6, 7, and 8 positions of coumarin nucleus. Compounds (**b**) were reacted with 4-amino-benzoic acid ethyl ester and with 4-aminopiperidine-1-carboxylic acid ethyl ester in anhydrous ethyl ether to yield derivatives **1**–**9** and **19**–**20**, respectively, which contain the benzamidic structure important for anti-*H. pylori* activity.

Compounds **1**–**9** were submitted to mild alkaline hydrolysis with LiOH in $H_2O$/MeOH and treated with HCl 3 N to obtain derivatives **10**–**18** (Scheme 1). All synthesized compounds (which possessed




**Scheme 1.** General synthesis of derivatives **1**–**20**.

**Table 2**
Minimal inhibitory concentration of tested compounds and metronidazole (M) against 20 clinical strains of *Helicobacter pylori*

| Compound | MIC (μg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | All strains | | | Metronidazole resistant strains | | | | |
| | Range | $MIC_{50}$ | $MIC_{90}$ | NCTC 11637 | **1** | **8** | **18** | **20** |
| **1** | 2–8 | 8 | 8 | 8 | 8 | 4 | 8 | 2 |
| **2** | ND | ND | ND | ND | ND | ND | ND | ND |
| **3** | 1–32 | 4 | 8 | 4 | 8 | 8 | 2 | 2 |
| **4** | 4–32 | 8 | 16 | 16 | 8 | 4 | 8 | 8 |
| **5** | 32–128 | 64 | 64 | 64 | 64 | 64 | 64 | 64 |
| **6** | 8 to >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 |
| **7** | 2–8 | 4 | 8 | 8 | 4 | 2 | 8 | 2 |
| **8** | ND | ND | ND | ND | ND | ND | ND | ND |
| **9** | 2–16 | 4 | 8 | 4 | 8 | 4 | 4 | 4 |
| **10** | 4–16 | 8 | 16 | 8 | 8 | 4 | 8 | 8 |
| **11** | 32–128 | 128 | 128 | 128 | 128 | 64 | 128 | 64 |
| **12** | ND | ND | ND | ND | ND | ND | ND | ND |
| **13** | 2–32 | 8 | 32 | 32 | 16 | 32 | 16 | 32 |
| **14** | 2–32 | 16 | 32 | 16 | 16 | 16 | 16 | 16 |
| **15** | 4 to >128 | >128 | >128 | 4 | >128 | >128 | >128 | >128 |
| **16** | 4 to >128 | 16 | >128 | 16 | 4 | 16 | 16 | 4 |
| **17** | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 |
| **18** | 2–16 | 4 | 8 | 4 | 8 | 4 | 4 | 4 |
| **19** | 128 to >128 | >128 | >128 | >128 | 128 | >128 | 128 | >128 |
| **20** | 32–64 | 64 | 64 | 64 | 32 | 64 | 32 | 32 |
| M | 0.5–128 | 2 | 128 | 128 | 64 | 64 | 128 | 128 |

ND, not determined (slightly soluble in DMSO).

calculated log *P* values less than 5) were fully characterized by means of analytical and spectral data.[13]

The synthesized compounds were first assayed against several species of Gram-positive and Gram-negative bacteria and against various strains of pathogenic fungi in order to identify those with little or no activity as leading compounds. Ceftazidime and clotrimazole were used as reference compounds. The data obtained against all the assayed species mostly showed a MIC value >128 μg/mL. Based on these results, all synthesized derivatives could be submitted to subsequent screening toward *H. pylori*.[15] The activity of the substances was compared with the reference compound metronidazole against 20 clinical strains of *H. pylori* including five metronidazole resistant ones (Table 2). We reported on the biological results only for those compounds which were soluble enough in DMSO. Compounds that have been demonstrated to achieve the strongest anti-*H. pylori* activity (**1**, **3**, **4**, **7**, **9**, **10**, and **18**) were analyzed to estimate their cytotoxicity on cultured cell lines in two steps. First, we performed Trypan blue test on an immortalized hybrid cell line displaying an endothelial phenotype, EAhy, derived from the fusion of human umbilical vein endothelial cells (HUVEC) with lung carcinoma cells. In a second moment, the MTT test was performed on a human gastric epithelial cell line AGS, to better quantify the cytotoxic activity showed by Trypan blue test. The results were reported in Table 3.[16] IL-8 release by the same human gastric epithelial cell line AGS was also analyzed.[17]

**Table 3**
Cytotoxic effect of the most active compounds tested on EAhy 926 cells after 24 h of incubation at 37 °C, using Trypan blue exclusion test (a), and MTT assay (b), expressed as cell survival fraction (%)[a]

| Compound (**a**) | Concentration[b] (μg/mL) | | | |
|---|---|---|---|---|
| | 50 | 5 | 0.5 | 0.05 |
| **1** | 64 ± 1.6 | 92 ± 3.5 | 95 ± 4.1 | 96 ± 3.8 |
| **3** | 14 ± 4.9 | 59 ± 4.6 | 77 ± 4.6 | 80 ± 5.2 |
| **4** | 58 ± 7.1 | 71 ± 6.5 | 86 ± 4.5 | 94 ± 2.3 |
| **7** | 77 ± 6.1 | 80 ± 4.1 | 83 ± 7.5 | 93 ± 6.6 |
| **9** | 36 ± 7.8 | 42 ± 1.5 | 67 ± 4.3 | 72 ± 4.4 |
| **10** | 82 ± 1.0 | 92 ± 1.7 | 93 ± 2.5 | 98 ± 2.6 |
| **18** | 62 ± 2.6 | 69 ± 1.7 | 77 ± 5.4 | 85 ± 4.2 |
| Compound (**b**) | Concentration[b] (μg/mL) | | | |
| | 50 | 5 | 0.5 | |
| **1** | 78 ± 2.8 | 82 ± 4.3 | 99 ± 0.2 | |
| **3** | 63 ± 1.2 | 81 ± 2.7 | 99 ± 0.1 | |
| **4** | 64 ± 1.1 | 75 ± 3.1 | 98 ± 0.6 | |
| **7** | 82 ± 4.3 | 99 ± 0.3 | 99 ± 0.2 | |
| **9** | 41 ± 3.8 | 58 ± 1.6 | 98 ± 0.6 | |
| **10** | 77 ± 4.3 | 99 ± 0.3 | 99 ± 0.1 | |
| **18** | 77 ± 4.6 | 99 ± 0.2 | 99 ± 0.1 | |

[a] Cells incubated with culture medium alone represented the controls and the cell viability was always greater than 99.80%.
[b] Data represent arithmetic means ± SD of at least three independent experiments.

A series of new coumarin derivatives were prepared, characterized, and evaluated for selective antimicrobial activity. Most of the assayed compounds showed interesting activity against *H. pylori* clinical strains (MIC 1–32 μg/mL). The modifications on the position 6 of the coumarin nucleus (as just revealed by the introduction of Br or Cl in our previous work) brought good inhibitory activity toward *H. pylori* and, in particular, compounds **3**, **7**, and **9** (bearing $R^2$ as a methyl, hydroxy, and methoxy group) showed the lowest MIC value, so they might be considered lead compounds for further development in this field.

Furthermore, these results have stated that the disubstitution in the 6 and 8 positions with halogens (Br and Cl) led to moderately

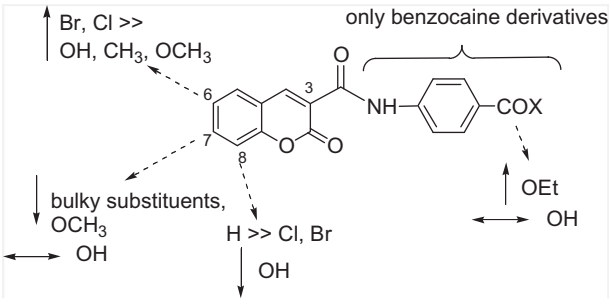



**Figure 1.** SAR studies of substituents on coumarin nucleus against *Helicobacter pylori*.

active derivatives (but weaker than the monohalogenated ones), and no bulky groups in the 7 position (as a polar hydroxy group better than a methoxy one) of the coumarin ring affected positively the biological activity. As regards the acyl function on the *N*-aromatic moiety, the ester derivatives showed better activity with respect to the corresponding carboxylic acids. The substitution of benzocaine linked to the C3 position with a bioisosteric group led to inactive compounds, thus demonstrating the importance of this nucleus to display a strong inhibition activity, as shown in Figure 1 which is a resume of all the obtained results of our performed studies on this scaffold (SAR studies).

All tested compounds possessed the best selectivity against the metronidazole resistant strains with MIC values lower than the drug reference. That could be useful in overcoming the increasing emergence of drug resistance to the conventional therapy.

In general, most of the derivatives presented a discrete cytotoxic profile at concentration between 0.5 and 5 μg/mL, especially in relation to their MIC values. In the current study, we also investigated a possible anti-inflammatory effect of new synthesized compounds in *H. pylori*-induced gastric inflammation. None of the compounds tested was able to significantly reduce IL-8 production, thus demonstrating a different mechanism of action for this scaffold of derivatives.

## Acknowledgment

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13. The chemicals, solvents for synthesis and spectral grade solvents were purchased from Sigma–Aldrich (Italy) and used without further purification. Melting points (uncorrected) were determined automatically on a FP62 apparatus (Mettler–Toledo). $^1$H NMR spectra were recorded at 400 MHz on a Bruker spectrometer using DMSO-$d_6$ as solvent. Chemical shifts are expressed as $\delta$ units (parts per million) relative to the solvent peak. Coupling constants *J* are valued in Hertz (Hz). Elemental analysis for C, H, and N were recorded on a Perkin-Elmer 240 B microanalyzer and the analytical results were within ± 0.4% of the theoretical values for all compounds.
*General procedure for the synthesis of derivatives* **1**–**20**. The appropriate salicylaldehyde (5 g) in ethanol (50 mL) is refluxed under magnetic stirring with diethyl malonate and catalytic amounts of piperidine for 24 h. After cooling to room temperature, the suspension is filtered to give the desired ethyl ester of 3-coumarin carboxylic acid (**a**). Hydrolysis with 10% NaOH (100 mL) and addition of HCl 3 N led to the desired product. Following filtration, the corresponding carboxylic acid was refluxed at 100 °C with thionyl chloride for 2 h to obtain the reactive acyl chloride (**b**). Then, it was condensed with the appropriate amine in anhydrous diethyl ether to give the crude products in high yields (**1**–**9** and **19**–**20**). Subsequently, ester derivatives were transformed into carboxylic acids by alkaline hydrolysis for 2 h at room temperature with equimolar quantities of LiOH in water/methanol (1:4; v/v) (**10**–**18**).
*Ethyl 4-(7-hydroxy-2-oxo-2H-chromene-3-carboxamido)benzoate* (**1**). Mp 282–283 °C, 71% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.39–1.42 (t, 3H, $CH_3$), 4.20–4.21 (m, 2H, $CH_2$), 6.70–6.77 (m, 3H, Ar), 7.78–7.91 (m, 4H, Ar), 8.88 (s, 1H, CH=), 10.90 (s, 1H, NH, $D_2O$ exch.), 11.25 (br s, 1H, OH, $D_2O$ exch.).
*Ethyl 4-(6-nitro-2-oxo-2H-chromene-3-carboxamido)benzoate* (**2**). Mp 225–226 °C, 79% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.41–1.44 (t, 3H, $CH_3$), 4.21–4.25 (m, 2H, $CH_2$), 7.68–7.72 (m, 4H, Ar), 8.35–8.39 (m, 3H, Ar), 8.80 (s, 1H, CH=), 10.49 (s, 1H, NH, $D_2O$ exch.).
*Ethyl 4-(6-methyl-2-oxo-2H-chromene-3-carboxamido)benzoate* (**3**). Mp 227–228 °C, 70% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.40–1.42 (t, 3H, $CH_3$), 2.47 (s, 3H, $ArCH_3$), 4.40–4.42 (m, 2H, $CH_2$), 7.26 (s, 1H, Ar), 7.51–7.53 (m, 2H, Ar), 7.83 (s, 2H, Ar), 8.04 (s, 1H, Ar), 8.99 (s, 1H, CH=), 11.09 (s, 1H, NH, $D_2O$ exch.).
*Ethyl 4-(6-bromo-8-chloro-2-oxo-2H-chromene-3-carboxamido)benzoate* (**4**). Mp >290 °C, 79% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.29–1.32 (t, 3H, $CH_3$), 4.29–4.30 (m, 2H, $CH_2$), 7.82–7.83 (m, 2H, Ar), 7.97–7.99 (m, 2H, Ar), 8.14 (s, 1H, Ar), 8.19 (s, 1H, Ar), 8.77 (s, 1H, CH=), 10.78 (br s, 1H, NH, $D_2O$ exch.).
*Ethyl 4-(6,8-dibromo-2-oxo-2H-chromene-3-carboxamido)benzoate* (**5**). Mp 223–224 °C, 70% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.23–1.28 (t, 3H, $CH_3$), 4.16–4.20 (m, 2H, $CH_2$), 6.55–6.58 (m, 2H, Ar), 7.62–7.64 (m, 2H, Ar), 8.29 (s, 1H, Ar), 8.44 (s, 1H, Ar), 8.62 (s, 1H, CH=), 10.70 (br s, 1H, NH, $D_2O$ exch.).
*Ethyl 4-(8-hydroxy-2-oxo-2H-chromene-3-carboxamido)benzoate* (**6**). Mp 280–281 °C, 77% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.24–1.27 (t, 3H, $CH_3$), 4.16-4.22 (m, 2H, $CH_2$), 6.62–6.64 (m, 2H, Ar), 7.20–7.21 (m, 2H, Ar), 7.31–7.32 (m, 1H, Ar), 7.65–7.67 (m, 2H, Ar), 8.70 (s, 1H, CH=), 10.50 (s, 1H, NH, $D_2O$ exch.), 11.20 (br s, 1H, OH, $D_2O$ exch.).
*Ethyl 4-(6-hydroxy-2-oxo-2H-chromene-3-carboxamido)benzoate* (**7**). Mp 210–211 °C, 95% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.30–1.31 (t, 3H, $CH_3$), 4.29–4.31 (m, 2H, $CH_2$), 7.29–7.40 (m, 3H, Ar), 7.88–7.97 (m, 4H, Ar), 8.89 (s, 1H, CH=), 10.99 (s, 1H, NH, $D_2O$ exch.), 11.99 (br s, 1H, OH, $D_2O$ exch.).
*Ethyl 4-(6-methoxy-2-oxo-2H-chromene-3-carboxamido)benzoate* (**9**). Mp 209–210 °C, 71% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.30–1.32 (t, 3H, $CH_3$), 3.82 (s, 3H, $OCH_3$), 4.27–4.29 (m, 2H, $CH_2$), 7.39–7.49 (m, 4H, Ar), 7.80–7.92 (m, 3H, Ar), 8.64 (s, 1H, CH=), 10.99 (s, 1H, NH, $D_2O$ exch.).
*4-(7-Hydroxy-2-oxo-2H-chromene-3-carboxamido)benzoic acid* (**10**). Mp 242–243 °C, 76% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 6.73 (s, 2H, Ar), 6.82–6.85 (m, 2H, Ar), 7.72–7.74 (m, 3H, Ar), 8.65 (s, 1H, CH=), 10.80 (s, 1H, NH, $D_2O$ exch.), 11.25 (br s, 1H, OH, $D_2O$ exch.), 13.01 (br s, 1H, COOH, $D_2O$ exch.).
*4-(6-Nitro-2-oxo-2H-chromene-3-carboxamido)benzoic acid* (**11**). Mp 224–225 °C, 72% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.71–7.74 (m, 4H, Ar), 8.39–8.43 (m, 3H, Ar), 8.86 (s, 1H, CH=), 10.59 (s, 1H, NH, $D_2O$ exch.), 12.93 (br s, 1H, COOH, $D_2O$ exch.).
*4-(6-Methyl-2-oxo-2H-chromene-3-carboxamido)benzoic acid* (**12**). Mp >290 °C, 77%; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 2.50 (s, 3H, $ArCH_3$), 7.36 (s, 1H, Ar), 7.56–7.65 (m, 4H, Ar), 7.93 (s, 1H, Ar), 8.44 (s, 1H, Ar), 8.99 (s, 1H, CH=), 11.09 (s, 1H, NH, $D_2O$ exch.), 12.96 (br s, 1H, COOH, $D_2O$ exch.).
*4-(6-Bromo-8-chloro-2-oxo-2H-chromene-3-carboxamido)benzoic acid* (**13**). Mp >290 °C, 84% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.79–7.82 (m, 2H, Ar), 7.95–7.98 (m, 2H, Ar), 8.29 (s, 1H, Ar), 8.40 (s, 1H, Ar), 8.67 (s, 1H, CH=), 10.81 (s, 1H, NH, $D_2O$ exch.), 13.02 (br s, 1H, COOH, $D_2O$ exch.).
*4-(6,8-Dibromo-2-oxo-2H-chromene-3-carboxamido)benzoic acid* (**14**). Mp 275–276 °C, 82% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.80–7.82 (d, $J_o$ = 8.0 Hz, 2H, Ar), 7.94–7.96 (d, $J_o$ = 8.0 Hz, 2H, Ar), 8.27 (s, 1H, Ar), 8.58 (s, 1H, Ar), 8.70 (s, 1H, CH=), 10.79 (s, 1H, NH, $D_2O$ exch.), 13.01 (br s, 1H, COOH, $D_2O$ exch.).
*4-(8-Hydroxy-2-oxo-2H-chromene-3-carboxamido)benzoic acid* (**15**). Mp 206–207 °C, 86% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.15–7.20 (m, 4H, Ar), 7.30–7.32 (m, 3H, Ar), 8.67 (s, 1H, CH=), 10.35 (s, 1H, NH, $D_2O$ exch.), 11.30 (br s, 1H, OH, $D_2O$ exch.), 13.11 (br s, 1H, COOH, $D_2O$ exch.).
*4-(6-Hydroxy-2-oxo-2H-chromene-3-carboxamido)benzoic acid* (**16**). Mp 204–205 °C, 74% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.29–7.41 (m, 4H, Ar), 7.78–7.89 (m, 3H, Ar), 8.64 (s, 1H, CH=), 10.98 (s, 1H, NH, $D_2O$ exch.), 11.19 (br s, 1H, OH, $D_2O$ exch.), 13.04 (br s, 1H, COOH, $D_2O$ exch.).
*4-(6-Methoxy-2-oxo-2H-chromene-3-carboxamido)benzoic acid* (**18**). Mp >290 °C, 78% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 3.81 (s, 3H, $OCH_3$), 7.37–7.48 (m, 4H, Ar), 7.84–7.96 (m, 3H, Ar), 8.69 (s, 1H, CH=), 10.97 (s, 1H, NH, $D_2O$ exch.), 12.98 (br s, 1H, COOH, $D_2O$ exch.).
*Ethyl 4-(2-oxo-2H-chromene-3-carboxamido)piperidine-1-carboxylate* (**19**). Mp 220–221 °C, 85% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.42–1.44 (t, 3H, $CH_3$), 1.52–1.55 (m, 4H, 2× $CH_2$), 2.77–2.81 (m, 4H, 2× $CH_2$), 2.92–2.95 (m, 1H, CH), 4.14–4.18 (m, 2H, $CH_2$), 7.19–7.27 (m, 3H, Ar), 7.93–7.97 (m, 1H, Ar), 8.89 (s, 1H, CH=), 10.80 (s, 1H, NH, $D_2O$ exch.).
*Ethyl 4-(6-bromo-2-oxo-2H-chromene-3-carboxamido)piperidine-1-carboxylate* (**20**). Mp 201–202 °C, 76% yield; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.29–1.32 (t, 3H, $CH_3$), 1.53–1.55 (m, 4H, 2× $CH_2$), 2.78–2.82 (m, 4H, 2× $CH_2$), 2.91–2.93 (m, 1H, CH), 4.29–4.30 (m, 2H, $CH_2$), 7.82–7.83 (m, 2H, Ar), 8.19 (s, 1H, Ar), 8.77 (s, 1H, CH=), 10.78 (br s, 1H, NH, $D_2O$ exch.).
14. Shiping, X.; Xiaoguang, C.; Song, X.; Lanmin, L.; Longfei, X.; Hongyan, L.; Yan, L.; Guifang, C. WO 2004/050082.
15. *Antibacterial and antifungal activity*. All synthesized derivatives were evaluated for their antimicrobial and antifungal activity when dissolved in DMSO. Organisms, from routine clinical Gram-positive (*S. aureus*, *S. epidermidis*, *S. hominis*, *S. α-hemolyticus*, *S. faecalis*), Gram-negative (*E. coli*, *K. pneumoniae*, *K. oxytoca*, *Enterobacter* spp., *E. aerogenes*, *C. freundii*, *P. vulgaris*), and *Candida* strain isolates (*C. albicans*, *C. sakè*, *C. krusei*) from the respiratory tract, were collected from specimens of patients at the Hospital 'Azienda Policlinico Umberto I°' of Rome 'La Sapienza' University. The isolates were subcultured on a qualified medium to ensure purity and an optimal growth. The isolates were identified by conventional methodologies. The in vitro antibacterial activities of the compounds were determined with the broth micro dilution method, as recommended by the National Committee for Clinical Laboratory Standards[18] with Mueller–Hinton II broth (BBL Microbiology Systems, Cockeysville, MD). Microtiter plates containing serial dilutions of each compound ranging from

128 to 0.5 μg/mL 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 antibacterial agent that completely inhibited the growth of the organism, as detected by the unaided eye.

The in vitro antifungal activity of the compounds was determined with the broth micro dilution method with Sabouraud dextrose broth (BBL Microbiology Systems, Cockeysville, MD) as recommended by the NCCLS.[19] Microtiter plates containing serial dilutions of each compound 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 antifungal agent that completely inhibited the growth of the organism, as detected by the unaided eye.

*Anti-H. pylori activity*. Twenty clinical strains of *H. pylori* isolated from patients with duodenal ulcer or gastritis were tested. Four of these strains had known metronidazole resistance. *H. pylori* ATCC 43504 was used as control in each assay session.They were maintained at −80 °C in Wilkins Chalgren broth with 10% (v/v) horse serum (Seromed) and 20% (v/v) glycerol (Merck) until required for the experiments. Before being used the bacteria were subcultured twice on Columbia agar base (Difco Laboratories) supplemented with 10% horse serum and 0.25% Bacto yeast extract (Difco). Plates were incubated for 72 h at 37 °C in an atmosphere of 10% $CO_2$ in a gas incubator.

Antimicrobial activity against *H. pylori* was determined by the agar dilution standard method.[20] The strains were inoculated onto Columbia agar base (Difco) supplemented with 10% horse serum and 0.25% bacto yeast extract (Difco), and were incubated for 72 h at 37 °C in an atmosphere of 10% $CO_2$ in a gas incubator. Colonies were suspended in Wilkins Chalgren broth to achieve a turbidity equivalent to 0.5 Mc Farland. Columbia agar plates with 10% horse serum were prepared by using twofold dilutions of the compounds (128–0.0039 μg/mL), and metronidazole (256–0.0039 μg/mL), as control. The inoculum was delivered to the surface of the agar plates with a Steer's replicator in order to obtain approximately $5 \times 10^5$ CFU per spot. Growth control plates without antibiotics were inoculated in each series of tests. All plates were incubated at 37 °C for 72 h under conditions (10% $CO_2$ in a gas incubator). The minimal inhibitory concentration was defined as the lowest concentration of drug inhibiting visible bacterial growth.

16. *In vitro cytotoxicity*. EAhy human cell line has been obtained from a hybridoma between HUVEC cells and epithelial cells from a lung carcinoma, AGS cell line (gastric adenocarcinoma, ATCC CRL 1739) was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in complete medium, consisting of D-MEM for EAhy cells and F-12K medium for AGS ones, supplemented with 10% fetal bovine serum, 1% glutamine, 100 U/mL penicillin G and 100 μg/mL streptomycin. Cell cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. After attaining confluence the cells were subcultured following trypsinization with 0.25% trypsin–EDTA solution.

Trypan blue dye exclusion assay was applied on EAhy cell cultures. Cells were maintained as adherent type cultures under humidified atmosphere in 5% $CO_2$ at 37 °C, Dulbecco's modified Eagle's culture medium (high glucose) supplemented with 2 mM L-glutamine, HAT supplement and containing antibiotic mixture. Experiments were performed in cells grown to 60–70% confluency.[21] The stock solutions of the investigated compounds were prepared in sterile DMSO and successive dilutions were made in culture medium; the DMSO percent present in culture medium never exceeded 0.5%. EAhy cells in the exponential phase of growth ($1 \times 10^5$/mL) were seeded into 24-well microplate and incubated for 24 h with four different concentrations of the compounds (50–0.05 μg/mL). Some plates containing cells alone or cells and DMSO represented the negative controls, whereas cells incubated with 1 mM natrium nitroprusside represented the positive one. After the incubation period, cells were mechanically scraped off from the plates and an aliquot was diluted (1:1) with a solution 0.4% Trypan blue stain. After few minutes at room temperature, cells were counted under an optical microscope in a Thoma hemocytometer chamber by two different operators. On the basis that Trypan blue is a vital dye[22] and can enter and interact with the cells unless the plasmatic membrane is damaged, blue stained cells were considered as having died. Values are expressed as % of viable cells. Cell viability in control samples was always 97–98%.

MTT staining (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was applied on AGS cell cultures. Cells were seeded in triplicate in 96-well plates at the density of $5 \times 10^4$ cell/well in complete medium and incubated with selected compounds at concentration of 50, 5, and 0.5 μg/mL for 24 h. Then, 20 μl of a 5 mg/mL solution of MTT in phosphate-buffered saline was added, and incubation continued for an additional 3 h at 37 °C. The plates were then centrifuged, the supernatants were discarded, and the dark blue formazan crystals were dissolved using 100 mL of lysing buffer consisting of a solution of 20% (w/v) sodium dodecyl sulfate and 40% *N*,*N*-dimethylformamide in $H_2O$ at pH 4.7 adjusted with 80% acetic acid. The plates were then read on a microplate reader (Molecular Devices Co., Menlo Park, CA) using a test wavelength of 550 nm and a reference wavelength of 650 nm. The optical density at 650 nm ($OD_{650}$) was subtracted from the $OD_{550}$ to eliminate nonspecific background.

17. *IL-8 assessment*. AGS cells ($1 \times 10^5$ mL) were seeded in 24-well culture plates for 24 h and then preincubated for 1 h with selected compounds at concentration of 5 and 0.5 μg/mL. After that, AGS cells were incubated with *H. pylori* according to literature.[23]

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