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A novel class of selective anti-*Helicobacter pylori* agents 2-oxo-2*H*-chromene-3-carboxamide derivatives

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Abstract—A novel class of selective anti-*Helicobacter pylori* agents, 2-oxo-2*H*-chromene-3-carboxamide derivatives, were prepared and evaluated for their anti-bacterial activity. All synthesized compounds showed little or no activity against different species of Gram-positive and Gram-negative bacteria and against various strains of pathogenic fungi. Some of them exhibited a potent and specific inhibitory effect on the growth of *H. pylori*, including metronidazole-resistant strains, in the 0.0039–16 μg/mL MIC range. A cytotoxic screening by the Trypan blue dye exclusion assay was also carried out on the most active compounds as anti-*H. pylori* agents. Among the derivatives examined for their cytotoxic potential, a number of them induced low cytotoxic effects.

For more than 20 years, *Helicobacter pylori*, a spiral-shaped microaerophilic Gram-negative bacterium, has been known to colonize the human gastric mucosa.[1] The persistence of *H. pylori* leads to the development of a subset of gastric pathologies, including chronic active gastritis, peptic ulceration, and the promotion of malignant disorders.[2] Hence since 1994, the World Health Organization (WHO) has classified *H. pylori* as a Class 1 carcinogen responsible for its leading role in the development of gastric neoplasia in humans. Thus the eradication of *H. pylori* can significantly reduce the risk of ulcer relapse and may help prevent the lymphoma of gastric mucosa-associated lymphoid tissue (MALT-lymphoma) and other gastric malignancies.[3]

The guidelines established by several Consensus Conferences held recently in Europe, America, Pacific Asia, Japan, and Canada have proposed, as a first-line choice for the eradication of *H. pylori*, a triple therapy based on a combination treatment with anti-secretory drugs, such as proton-pump inhibitors (PPI) or ranitidine bismuth citrate (RBC), and two antibiotics from among clarithromycin, amoxicillin, and metronidazole for 1–2 weeks.[4]

Furthermore, basic recommendations are given on the choice treatment in the event of failure of a first-line eradicating strategy, suggesting a quadruple therapy based on tetracycline, metronidazole, bismuth salts, and anti-secretory agents (PPI or ranitidine) for at least 7 days, but discordant results have been reported in trials attempting to evaluate its efficacy, with remission rates ranging from 57% to 95%.

Despite this careful approach, the treatment regimens have not always been successful, achieving variable eradication rates of about 70–85%. Multiple drugs, frequent dosing, length of treatment, and occurrence of harmful side effects often contribute to poor patient compliance. In addition, an increasing emergence of drug resistance, which is more frequent and more severe in the case of quadruple therapy,[5] can lead to treatment failure.

Keywords: Anti-*Helicobacter pylori* activity; Coumarins.
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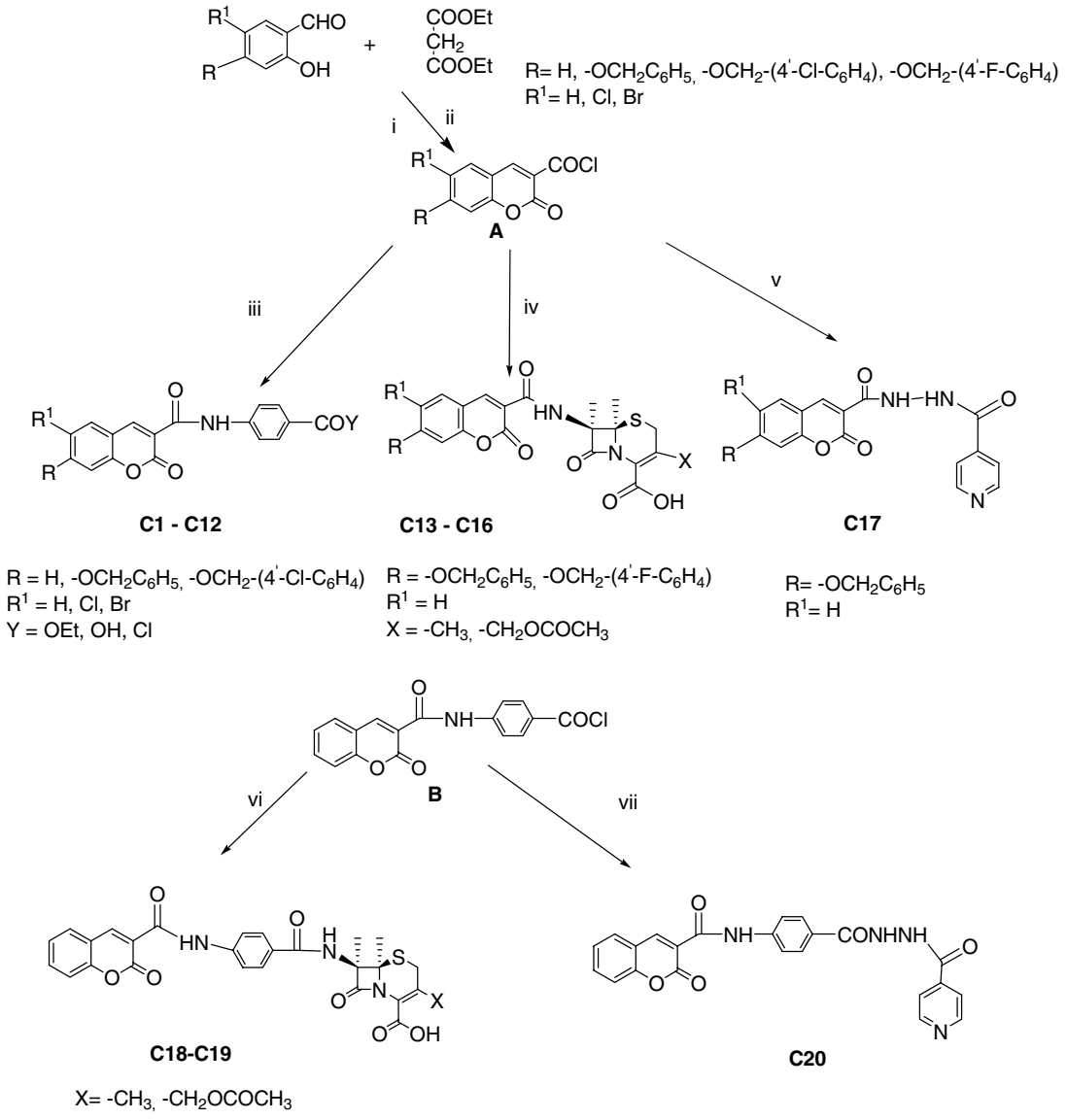
Since known anti-microbial agents may present a potential for the disruption of intestinal microbial flora, which may cause side effects, the development of new alternative therapeutic agents with highly selective anti-bacterial activity against *H. pylori*, but without the risk of resistance or other untoward effects, has become necessary.

This work is aimed at trying to overcome these problems as well as evaluating a series of 2-oxo-2*H*-chromene-3-carboxamide derivatives as part of a screening program.

Naturally occurring coumarins, widely found in plants belonging to the families *Rutaceae*, *Umbelliferae*, and *Compositae*,[6] are endowed with different types of biological applications. It has been reported that coumarin derivatives exhibit an ever-increasing variety of uses, including platelet anti-aggregating activity, anti-inflammatory activity, and anti-tumor activity.[7]

Moreover, coumarin derivatives are well known for their anti-microbial activity toward different microorganisms. Some authors have reported a study on the anti-microbial activity of some coumarin derivatives with reference to anti-*H. pylori* activity.[8] For all assayed coumarins, no activity has been reported except for the derivatives with carboxylic or hydroxyl groups. Based on these results, the authors have pointed out that a carboxylic acid function in the coumarin ring might play an important role leading to activity. In addition, either coumarins derived directly from natural products or synthetic coumarins, both bearing hydroxyl substituents, were identified as potent anti-*H. pylori* agents, comparable to metronidazole.[6]

Moving from these literature indications and pursuing our research in the field,[9,10] in this report we describe the synthesis and anti-microbial evaluation of a new series of coumarin derivatives against the most common



Scheme 1. Reagents: (i) EtOH, piperidine, Δ; (ii) a—NaOH 20%; b—$SOCl_2$; (iii) a—benzocaine, Et_2o; b—NaOH 20%; c—$SOCl_2$; (iv) 7-ACA, 7-ADCA, THF; (v) INH, $CHCl_3$, Et_3N; (vi) 7-ACA, 7-ADCA, THF; (vii) INH, $CHCl_3$, Et_3N.

pathogens, both bacterial and fungal, and against *H. pylori*.

We have focused on the development of a number of coumarin derivatives aimed at identifying features of the structure that could be important for anti-*H. pylori* activity.

In order to establish the cytotoxic activity of this novel series of coumarin 3-carboxamides, 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 was incubated in the presence of compounds **C7**–**C12** which demonstrated to possess the most potent activity against *H. pylori* and the cell viability determined by the Trypan blue dye exclusion assay.

The coumarin moiety of all derivatives is obtained following a Knoevenagel reaction between the appropriate benzaldehydes and diethylmalonate.

In particular, on the basis of previous investigation,[11] starting from the acyl chloride (**A**) we have synthesized coumarins **C1**–**C12** (**C10** is commercially available[12]) which contain the benzamidic structure important for anti-*H. pylori* activity. In addition, we have synthesized compounds **C13**–**C16**, which are made up of coumarins with cephem derivatives as substituents such as 7-aminocephalosporanic acid (7-ACA), 3-deacetyloxy-7-aminocephalosporanic acid (7-ADCA), and also isoniazid (INH) for compound **C17**, according to structure-activity studies.[13] Starting from 4-[(2-oxo-2*H*-chromene-3-carbonyl)-amino]-benzoyl chloride (**B**),[10] we have also synthesized compounds **C18**–**C20**, made up of coumarins with cephem derivatives as substituents such as 7-ACA, 7-ADCA, and also isoniazid (INH) (see Scheme 1).

All synthesized compounds were fully characterized by means of analytical and spectral data as reported in Tables 1 and 2.[14]

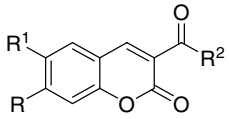
The synthesized compounds were first assayed against several species of Gram-positive and Gram-negative bacteria and against various strains of pathogenic fungi in order to identify those with little or no activity as leading compounds. Ceftazidime and clotrimazole are used as reference compounds.

The data obtained against all the assayed species mostly show a MIC value >128 μg/mL. Based on these results, all synthesized compounds can be submitted to subsequent screening toward *H. pylori*.[15]

The activity of the substances is compared with the reference compound metronidazole against 16 strains of *H. pylori*, including the reference strain NCTC 11637 and two other metronidazole-resistant strains.

The MIC ranges and the MIC at which 50% (MIC_{50}) and 90% (MIC_{90}) of the *H. pylori* tested strains are inhibited by compounds **C1**–**C20** are shown in Table 3, together with the MIC values of the synthesized

Table 1. Chemical and physical data of derivatives **C1**–**C20**



Compound	R	R^1	R^2	mp (°C)	Yield (%)
C1	$-OCH_2C_6H_5$	H	–NH–(4′-COOEt–C_6H_4)	263–265	83
C2	$-OCH_2C_6H_5$	H	–NH–(4′-COOH–C_6H_4)	200–203	32
C3	$-OCH_2C_6H_5$	H	–NH–(4′-COCl–C_6H_4)	153–155	45
C4	$-OCH_2$–(4′-Cl–C_6H_4)	H	–NH–(4′-COOEt–C_6H_4)	260–262	87
C5	$-OCH_2$–(4′-Cl–C_6H_4)	H	–NH–(4′-COOH–C_6H_4)	220–225	95
C6	$-OCH_2$–(4′-Cl–C_6H_4)	H	–NH–(4′-COCl–C_6H_4)	130–135	82
C7	H	Cl	–NH–(4′-COOEt–C_6H_4)	279–281	94
C8	H	Cl	–NH–(4′-COOH–C_6H_4)	>290	56
C9	H	Cl	–NH–(4′-COCl–C_6H_4)	>290	67
C10[12]	H	Br	–NH–(4′-COOEt–C_6H_4)	260–264	96
C11	H	Br	–NH–(4′-COOH–C_6H_4)	>280	54
C12	H	Br	–NH–(4′-COCl–C_6H_4)	>290	70
C13	$-OCH_2$–(4′-F–C_6H_4)	H	7-ACA[a]	135–138	30
C14	$-OCH_2$–(4′-F–C_6H_4)	H	7-ADCA[b]	162–166	70
C15	$-OCH_2C_6H_5$	H	7-ACA	156–160	73
C16	$-OCH_2C_6H_5$	H	7-ADCA	210–213	82
C17	$-OCH_2C_6H_5$	H	INH[c]	260–263	30
C18	H	H	$-NHC_6H_4$–4′-CO–7-ACA	240–242	34
C19	H	H	$-NHC_6H_4$–4′-CO–7-ADCA	229–232	69
C20	H	H	$-NHC_6H_4$–4′-CO–INH	186–200	30

[a] 7-ACA, 7-aminocephalosporanic acid.
[b] 7-ADCA, 3-deacetiloxy-7-aminocephalosporanic acid.
[c] INH, isoniazid.

Table 2. ^{1}H NMR data of derivatives **C1**–**C20**

Compound	^{1}H NMR δ (ppm)
C1[a]	1.39–1.42 (t, 3H, CH_3); 4.37–4.39 (q, 2H, CH_2); 5.20 (s, 2H, CH_2); 6.98–7.17 (m, 2H, Ar); 7.43–7.45 (m, 5H, Ar); 7.64–7.66 (d, 1H, Ar, J = 8.8 Hz); 7.81–7.83 (d, 2H, Ar, J = 8.6 Hz); 8.06–8.08 (d, 2H, Ar, J = 8.6 Hz); 8.95 (s, 1H, CH=); 11.00 (s, 1H, NH, D_2O exch)
C2[a]	5.20 (s, 2H, CH_2); 7.00 (s, 2H, Ar); 7.08–7.10 (d, 2H, Ar, J = 8.6 Hz); 7.39–7.43 (m, 6H, Ar); 7.64–7.66 (d, 2H, Ar, J = 8.9 Hz); 8.86 (s, 1H, CH=); 11.50 (br s, 1H, NH, D_2O exch)
C3[b]	5.25–5.26 (d, 2H, CH_2, J = 3.4 Hz); 7.15–7.25 (m, 4H, Ar); 7.35–7.41 (m, 4H, Ar); 7.46–7.48 (m, 4H, Ar); 8.71–8.72 (d, 1H, CH=, J = 3.3 Hz); 11.32 (br s, 1H, NH, D_2O exch)
C4[b]	1.55–1.57 (t, 3H, CH_3); 4.50–4.51 (q, 2H, CH_2); 5.30 (s, 2H, CH_2); 7.10–7.19 (m, 5H, Ar); 7.30–7.60 (m, 6H, Ar); 8.86 (s, 1H, CH=); 10.88 (s, 1H, NH, D_2O exch)
C5[b]	5.25 (s, 2H, CH_2); 7.05–7.11 (m, 3H, Ar); 7.42–7.51 (m, 6H, Ar); 7.75–7.81 (m, 2H, Ar); 8.60 (s, 1H, CH=); 10.95 (s, 1H, NH, D_2O exch)
C6[b]	5.30 (s, 2H, CH_2); 7.03–7.12 (m, 3H, Ar); 7.47–7.53 (m, 6H, Ar); 7.80–7.87 (m, 2H, Ar); 8.72 (s, 1H, CH=); 10.89 (s, 1H, NH, D_2O exch)
C7[a]	1.38–1.42 (t, 3H, CH_3); 4.33–4.39 (q, 2H, CH_2); 7.26–7.28 (d, 1H, Ar, J = 8.6 Hz); 7.72–7.81 (m, 4H, Ar); 8.03–8.05 (d, 2H, Ar, J = 8.6 Hz); 9.05 (s, 1H, CH=); 10.85 (s, 1H, NH, D_2O exch)
C8[b]	7.58–7.61 (dd, 1H, Ar, J = 8.7 Hz); 7.80–7.85 (m, 3H, Ar); 7.94–7.97 (m, 2H, Ar); 8.14–8.15 (t, 1H, Ar); 8.84–8.85 (s, 1H, CH=); 10.88 (s, 1H, NH, D_2O exch); 13.85 (s, 1H, COOH, D_2O exch)
C9[b]	7.52–7.54 (m, 1H, Ar); 7.77–7.80 (m, 3H, Ar); 7.90–7.92 (m, 3H, Ar); 8.10–8.12 (m, 1H, Ar); 8.82 (s, 1H, CH=); 10.96 (s, 1H, NH, D_2O exch)
C11[b]	7.51–7.53 (d, 1H, Ar, J = 7.8 Hz); 7.82–7.84 (d, 2H, Ar, J = 7.9 Hz); 7.90–7.96 (m, 3H, Ar); 8.26 (s, 1H, Ar); 8.86 (s, 1H, CH=); 10.90 (s, 1H, NH, D_2O exch); 12.82 (s, 1H, COOH, D_2O exch)
C12[b]	7.51–7.54 (m, 1H, Ar); 7.82–7.85 (m, 2H, Ar); 7.90–7.97 (m, 3H, Ar); 8.26–8.27 (m, 2H, Ar); 8.82–8.83 (s, 1H, CH=); 10.92–10.93 (d, 1H, NH, D_2O exch J = 2.6 Hz)
C13[b]	2.05 (s, 3H, CH_3); 3.75 (m, 2H, CH_2); 4.90 (m, 2H, CH_2); 5.10 (m, 2H, CH_2); 5.30 (d, 1H, CH, J = 4.3); 6.05 (m, 1H, CH); 7.20–8.00 (m, 7H, Ar); 9.00 (s, 1H, CH=); 9.30 (s, 1H, NH, D_2O exch); 13.10 (s, 1H, COOH, D_2O exch)
C14[b]	2.00 (s, 3H, CH_3); 3.70 (s, 2H, CH_2); 5.20 (s, 2H, CH_2); 5.25 (d, 1H, CH, J = 4.3); 5.90 (m, 1H, CH); 7.30–8.00 (m, 7H, Ar); 8.90 (s, 1H, CH=); 9.30 (s, 1H, NH, D_2O exch); 13.15 (s, 1H, COOH, D_2O exch)
C15[a]	2.15 (s, 3H, CH_3); 3.60 (s, 2H, CH_2); 5.05 (s, 2H, CH_2); 5.10 (s, 2H, CH_2); 5.20 (d, 1H, CH, J = 4.3); 6.20 (m, 1H, CH); 7.00–7.70 (m, 8H, Ar); 8.90 (s, 1H, CH=); 9.60 (s, 1H, NH, D_2O exch); 13.10 (s, 1H, COOH, D_2O exch)
C16[b]	2.05 (s, 3H, CH_3); 3.55 (s, 2H, CH_2); 5.25 (s, 2H, CH_2); 5.30 (d, 1H, CH, J = 4.3); 6.00 (m, 1H, CH); 7.20–8.00 (m, 8H, Ar); 8.95 (s, 1H, CH=); 9.35 (s, 1H, NH, D_2O exch); 13.20 (s, 1H, COOH, D_2O exch)
C17[b]	5.30 (s, 2H, CH_2); 7.10–7.95 (m, 8H, Ar and 2H, Pyr); 8.75 (m, 2H, Pyr); 8.95 (s, 1H, CH=); 10.55 (s, 1H, NH, D_2O exch); 11.35 (s, 1H, NH, D_2O exch)
C18[b]	2.00 (s, 3H, CH_3); 3.70 (s, 2H, CH_2); 4.80 (s, 2H, CH_2); 5.20 (d, 1H, CH, J = 4.3); 5.90 (m, 1H, CH); 7.50–8.00 (m, 8H, Ar); 8.95 (s, 1H, CH=); 9.40 (s, 1H, NH, D_2O exch); 10.95 (s, 1H, NH, D_2O exch); 13.2 (s, 1H, COOH, D_2O exch)
C19[b]	1.95 (s, 3H, CH_3); 3.60 (s, 2H, CH_2); 5.10 (d, 1H, CH, J = 4.3); 5.80 (m, 1H, CH); 7.45–8.00 (m, 8H, Ar); 8.90 (s, 1H, CH=); 9.30 (s, 1H, NH, D_2O exch); 10.85 (s, 1H, NH, D_2O exch); 13.2 (s, 1H, COOH, D_2O exch)
C20[b]	7.45–8.00 (m, 8H, Ar and 2H, Pyr); 8.75 (m, 2H, Pyr); 8.95 (s, 1H, CH=); 10.60 (s, 1H, NH, D_2O exch); 10.80 (s, 1H, NH, D_2O exch); 10.90 (s, 1H, NH, D_2O exch)

[a] $CDCl_3$.
[b] DMSO-d_6.

compounds against the metronidazole-resistant strains of *H. pylori*.

The best activity (MIC values ranging between 0.0039 and 8 μg/mL) was observed for derivatives **C7**–**C12** bearing an halogen (chlorine or bromine) in the 6-position, a 3-benzamidic group and hydrogen atom in the 7-position of the coumarin moiety. All these compounds show good activities against all strains and very good activities against the metronidazole-resistant strains. These values are very much lower in comparison with those of metronidazole.

Compounds **C1**–**C6**, 7-benzyloxy substituted, show lower activity, except for **C4** derivative whose MIC was of 0.031 μg/mL against the NCTC 11637 strain. Compounds **C13**–**C20** show no activity.

From these results we can point out that anti-*H. pylori* activity strongly depends on the presence of an acyl function like acid, ester or acyl chloride in the 3-benzamidic group and of an halogen in the 6-position of the coumarin ring. The choice of the acyl functions and particularly of the potentially unstable acyl chloride as substituents in the 3-position of the coumarin ring

Table 3. MIC of compounds **C1–C20** and metronidazole (M) against 16 *H. pylori* strains, including three metronidazole-resistant strains

Compound	Range	All strains		Metronidazole resistant		
		MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	NCTC 11637	5	14
C1	0.5–64	8	64	4	64	32
C2	0.5–4	2	4	2	4	4
C3	0.5–4	2	4	2	4	4
C4	<0.0039–32	4	32	0.031	32	16
C5	2–16	8	16	8	4	8
C6	1–8	4	8	4	4	8
C7	<0.0039–16	0.25	8	<0.0039	8	<0.0039
C8	<0.0039–2	0.0039	0.062	<0.0039	0.062	0.015
C9	<0.0039–0.25	0.0078	0.062	<0.0039	0.0078	0.015
C10	<0.0039–8	0.5	4	0.031	2	0.5
C11	<0.0039–0.125	0.015	0.125	<0.0039	0.062	0.015
C12	<0.0039–0.125	0.0039	0.031	<0.0039	0.015	<0.0039
C13	32–128	64	128	64	128	128
C14	16–128	64	128	32	128	64
C15	16–128	64	128	64	128	64
C16	32–128	64	128	32	128	64
C17	32–>128	128	>128	>128	>128	>128
C18	8–128	32	64	32	64	32
C19	4–>128	128	>128	32	>128	>128
C20	16–>128	128	>128	>128	>128	>128
M	0.125–64	0.25	64	64	64	64

Table 4. Cytotoxic effect of compounds **C7–C12** tested on EAhy 926 cells after 24 h of incubation at 37 °C, using Trypan blue exclusion test, expressed as cell survival fraction (%)[a]

Compound	Concentration (μg/mL)[b]				
	50	5	0.5	0.05	0.005
C7	10 ± 0.5	30 ± 1.2	60 ± 2.0	77 ± 4.4	80 ± 5.5
C8	10 ± 0,8	12 ± 0,8	45 ± 3.0	68 ± 3.4	79 ± 6.2
C9	15 ± 0.5	22 ± 1.4	58 ± 4.0	80 ± 6.4	78 ± 5.8
C10	30 ± 2,2	70 ± 2.3	70 ± 2.8	87 ± 3.5	92 ± 4.0
C11	20 ± 1.6	37 ± 3.2	70 ± 3.8	90 ± 5.2	90 ± 3.8
C12	10 ± 1.1	31 ± 1.4	56 ± 4.2	70 ± 4.4	85 ± 5.6

[a] Cells incubated with culture medium alone represented the controls and the cell viability was always greater than 97%.
[b] Data represent the arithmetic mean ± SD of at least three independent experiments.

was admissible on the basis of our previous studies done on the stability of similar compounds in the assay conditions by ^{1}H NMR and HPLC techniques.[16,17]

If a benzyloxy group is present in the 7-position the activity decreases. Finally, the presence of bulky substituents like 7-ACA, 7-ADCA or INH leads to inactive compounds.

The cytotoxic profile of the compounds **C7–C12**, the most active agents against *H. pylori*, (Table 4) showed that the derivatives **C10** and **C11** were less toxic at concentrations below 0.5 μg/mL with percentage of viable cells remaining of 87 and 90, respectively.[18]

Acknowledgments

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14. *General procedure for the preparation of 2-oxo-2H-chromene-3-acyl chloride* (**A**). The appropriate salicylaldehyde (5 g) in EtOH (90 mL) is refluxed under magnetic stirring with diethyl malonate and catalytic amounts of piperidine for 24 h. After cooling to room temperature, the solution is filtered to give the desired 2-oxo-2*H*-chromene-3-carboxylic acid ethyl ester. After hydrolysis with NaOH 20% (100 mL) and addition of hydrochloric acid 4 N, the solution is filtered to obtain the desired 2-oxo-2*H*-chromene-3-carboxylic acid. The 2-oxo-2*H*-chromene-3-carboxylic acid (0.05 mol) is refluxed for 3 h at 100 °C under magnetic stirring with thionyl chloride (0.5 mol). After cooling to room temperature, the solution is filtered to give the desired compound **A**.
General procedure for the synthesis of 2-oxo-2H-chromene-3-carboxamide derivatives **C1**–**C20**. A solution of the appropriate amine derivatives (0.0072 mol) in the suitable solvent (30 mL) is added dropwise to a solution of the appropriate 2-oxo-2*H*-chromene-3-acyl chloride (**A**) (0.0072 mol) or 4-[(2-oxo-2*H*-chromene-3-carbonyl)-amino]-benzoyl chloride (**B**) (0.0072 mol) in the same solvent (30 mL) and triethylamine (1 mL). The reaction mixture is stirred for 96 h at reflux, then cooled to room temperature and filtered off to remove triethylammonium salt. The organic layer is concentrated under vacuum, cooled to 4 °C to give the desired compounds. Chemical and physical data of synthesized compounds and ^{1}H NMR spectra are reported in Tables 1 and 2.
15. *Anti-bacterial and anti-fungal activity*. All synthesized derivatives **C1**–**C20** were evaluated for their anti-microbial and anti-fungal activity dissolved in dimethylsulfoxide (DMSO). Organisms from routine clinical Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus warneri*, *Streptococcus α-hemolyiticus*, *Streptococcus faecalis*) and Gram-negative isolates (*Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter* spp., *Enterobacter aerogenes*, *Citrobacter freundii*, *Proteus vulgaris*) and four *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. The isolates were identified by conventional methodologies; all isolates were subcultured to ensure optimal growth. 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[19] 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 to 22 h at 35 °C. The Minimal Inhibitory Concentration (MIC) for all isolates was defined as the lowest concentration of anti-bacterial agent that completely inhibited the growth of the organism, as detected by the unaided eye. The in vitro anti-fungal activities of the compounds were determined with the broth micro dilution method with Sabouraud dextrose broth (BBL Microbiology Systems, Cockeysville, MD) as recommended by the NCCLS.[20] 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 anti-fungal agent that completely inhibited growth of the organism, as detected by the unaided eye.
Anti-H. pylori activity. Fifteen clinical *H. pylori* strain isolates and the reference strain NCTC 11637 were used. Three 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 could be 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 2 h to allow equilibration, and none of the cultures were kept in the air for more than 15 min. The MICs were determined by the agar dilution standard method[21] incubating the bacteria at microaerobic conditions. By serial double dilutions, they were diluted in agar medium to give 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 72 h growth of each strain on agar plates was suspended 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.

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18. *In vitro cytotoxicity.* The cytotoxicity of the newly synthesized compounds under investigation was tested against EAhy, human cell line obtained from a hybridoma between HUVEC cells and epithelial cells from a lung carcinoma. The viability of cells exposed to test compounds was estimated by the Trypan blue dye exclusion assay. Cell lines 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.[22] The stock solutions of the investigated compounds were prepared in sterile dimethylsulfoxide (DMSO) and the 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

(1×10^5/mL) were seeded into 24-well microplate and incubated for 24 h with five different concentrations of the compounds (50–0.005 μg/mL). Some plates containing cells alone or cells and DMSO represented the controls. 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 rt 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[23] and can enter and interact with the cells unless the plasmatic membrane is damaged, blue stained cells were considered as died. Values are expressed as % of viable cells. Cell viability in control samples was always 97–98%.

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