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European Journal of Medicinal Chemistry 41 (2006) 208–212
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://france.elsevier.com/direct/ejmech

Original article

Synthesis and in vitro selective anti-*Helicobacter pylori* activity of N-substituted-2-oxo-*2H*-1-benzopyran-3-carboxamides

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Received 14 July 2005; received in revised form 2 November 2005; accepted 3 November 2005
Available online 10 January 2006

Abstract

In order to develop new anti-*Helicobacter pylori* agents, five new and three already known N-substituted-2-oxo-*2H*-1-benzopyran-3-carboxamides (coumarin-3-carboxamides) were 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. Among the prepared compounds those with a 4-acyl-phenyl group showed the best activity against *H. pylori* metronidazole resistant strains in the 0.25–1 μg/ml MIC range, indicating the presence of an acyl function as an important feature for activity.

Keywords: Coumarin; Antibacterial activity; Anti-*Helicobacter pylori* activity

1. Introduction

It is now recognized that *Helicobacter pylori*, an S-shaped spiral microaerophilic Gram-negative bacterium first isolated in human gastric mucosa in 1982 [1–3], is a pathogenic factor of chronic active gastritis, peptic ulcer disease, and gastric cancer [4–6] and its eradication 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 malignant gastric diseases [10].

The guidelines established by several International Consensus Conferences suggest the use of a first-line therapy based on two antibiotics, clarithromycin (500 mg d.b.) and amoxicillin (1g b.d.) or nitroimidazole (500 mg d.b.) together with a proton pump inhibitor (b.d.) for 7 days. The eradications rate of this scheme is variable and ranges from 70% to 85%. Patients' compliance and bacterial resistance are important factors involved in treatments failure. Thus alternative therapeutic agents with highly selective antibacterial activity against *H. pylori*, but without the risk of resistance or other untoward effects [11], have become necessary.

With this in mind at the start of this work, we reasoned that known antimicrobial agents may not be an appropriate therapy, since they may favor 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 N-substituted-2-oxo-*2H*-1-benzopyran-3-carboxamides (coumarin-3-carboxamides).

Naturally occurring coumarins, widely found in plants belonging to the families Rutaceae, Umbelliferae, and Composi-

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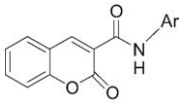
doi:10.1016/j.ejmech.2005.11.001

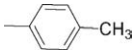
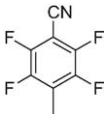
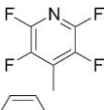
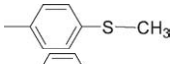
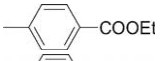
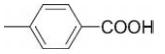
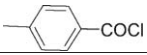
tae [12–14], are endowed with different types of biological applications. It has been reported that coumarin derivatives exhibit an ever-increasing variety of uses including platelet antiaggregating activity [15] anti-inflammatory activity [16] and antitumor activity [17–19]. Moreover, coumarin derivatives are well known for their antimicrobial activity towards different microorganisms [20–23].

Some authors have reported a study on the antimicrobial activity of some coumarin derivatives with reference to anti-*H. pylori* activity [24]. For all the assayed coumarins no activity has been reported except for two 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 be important for the activity. Moving from these literature indications and pursuing our research in the field [25], we here report on the synthesis and the antimicrobial evaluation of a new series of coumarin derivatives against the most common pathogens, both bacterial and fungal, and against *H. pylori*.

In particular, as a preliminary screening, we have decided to study the anti-*H. pylori* activity of some coumarin-3 carboxamides, some of which not already reported in the literature, with the aim of identifying some features of the structure that could be important for the activity. Our choice was addressed to the coumarin-3-carboxamides **C1–C8** whose carboxamide function bears an aryl group substituted with fluorine or carboxylic or methyl or thiomethyl groups as listed in Table 1.

Table 1
Chemical–physical data for derivatives **C1–C8**



Compound	Ar	m.p. (°C)	Yield (%)	*m/z*
C1		157–160	65	355
C2		230–232 (235) [33]	55	279
C3		211–212	50	362
C4		182–187	58	338
C5		205–207	75	311
C6		253–255 (246–247) [34]	80	337
C7		277–279 (275–276) [34]	92	309
C8		275–276	66	327

2. Chemistry

Coumarin **C1–C6** were conveniently achieved by the heterocyclization reaction of benzaldimines and carbon suboxide, according to a previously reported method [26]. This route was preferred to afford multigram scale synthesis and easy purification compared to other syntheses.

The acyl chloride **C8** was obtained by treatment of **C7** with thionyl chloride.

All the synthesized compounds were fully characterized by means of analytical and spectral data. In particular for the new compounds **C1**, **C3–C5** and **C8**, we detected by ^{1}H NMR the amidic proton as a broad singlet ranging between 10.20 and 11.00 ppm and the H_4 proton at 8.90 ppm. In the mass spectra the most abundant fragment ion at $m/z = 173$, corresponding to the 3-acyl coumarin structure was always observed. Compounds **C2**, **C6** and **C7** were identified by comparison with authentic samples.

Since we were dealing with a potentially reactive compound, we tested the chemical stability of the acyl chloride **C8.** The compound exactly weighted was dissolved in dimethylsulfoxide (DMSO) and held for 3 h at 40 °C. The ^{1}H NMR spectrum of the quantitatively isolated sample was made in DMSO-d_6 in order to look for the appearance of a peak at 13.27 ppm relating to the formation of the corresponding acid. No peak was detected indicating that, in the assay conditions, the acyl chloride is stable.

3. Pharmacology

The synthesized compounds were first assayed against different 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.

The data obtained against all the assayed species as listed in Section 5 were in the 64– > 128 μg/ml range. From these results it was possible to select all the synthesized compounds for subsequent screening towards *H. pylori*.

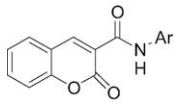
A comparison of the activity of the substances with the reference compound metronidazole was made against 18 strains of *H. pylori*, including the reference strain NCTC 11637 and two other metronidazole resistant strains.

4. Results and discussion

The MIC (minimal inhibitory concentration) ranges and the MIC at which 50% (MIC_{50}) and 90% (MIC_{90}) of the *H. pylori* tested strains were inhibited by compounds **C1–C8** are shown in Table 2, together with the MIC values of the prepared compounds against the metronidazole resistant strains of *H. pylori*.

Compounds **C1–C5**, in which the 3-amidic function is substituted with a phenyl bearing fluorine, methyl, and cyano groups, showed very low or no activity at all against all strains. The 3-[(4-acyl-phenyl)carboxamido]-coumarins **C6–C8** displayed a potent anti-*H. pylori* activity against all strains with MIC values of 0.25–1 μg/ml, much lower than those of the

Table 2
MIC of compounds **C1–C8** and metronidazole (M) against 18 *H. pylori* strains, including three metronidazole resistant strains



Compound	MIC (μg/ml)					
	All strains			Metronidazole resistant		
	Range	MIC_{50}	MIC_{90}	NCTC 11637	7	14
C1	8– > 128	64	> 128	128	> 128	64
C2	64– > 128	> 128	> 128	> 128	> 128	> 128
C3	128 > 128	> 128	> 128	> 128	> 128	> 128
C4	64– > 128	> 128	> 128	> 128	> 128	> 128
C5	> 128– > 128	> 128	> 128	> 128	> 128	> 128
C6	0.25–1	0.5	1	0.5	1	0.5
C7	0.25–2	0.5	1	0.25	1	0.5
C8	0.062–1	0.5	1	0.5	1	0.5
M	0.25–128	1	128	128	128	128

reference compound metronidazole. The best result was obtained with the acid **C7** against the metronidazole resistant strain NCTC 11637 with MIC 0.25 μg/ml.

From the results of this preliminary study we can highlight that anti-*H. pylori* activity strongly depends on the presence of a 4-acyl-phenyl group in the coumarin moiety. Comparing the activity of coumarins **C6**–**C8** with those reported by the previously cited authors [24], in which molecules with an acyl function like acid or ester in the 3-position of the coumarin nucleus showed low activity, we can also indicate that the presence of the acyl function not directly linked to the coumarin moiety but located in a phenyl-substituted -3-carboxamide could be another important requisite for anti-*H. pylori* activity.

In conclusion, in this preliminary work we intended to explore the activity of some coumarins against *H. pylori*, on the basis of a very little literature on the matter. The obtained results evidenced a very interesting activity for the acyl substituted compounds **C6**–**C8** and for **C7** in particular, compared to the other substituents in the phenyl ring and compared to the only coumarin derivatives reported in literature [24] (best data: 49–59 μM).

Based on our results we can indicate **C7** derivative as lead compound that needs to be modified to improve its anti-*H. pylori* activity.

5. Experimental protocols

5.1. Chemistry

Melting points were determined with a Büchi capillary apparatus and are uncorrected. NMR spectra were recorded on a Brucker 400 MHz spectrometer using DMSO-d_6 as the solvent. Chemical shifts are reported in ppm relative to the solvent peak. Mass spectra (EI) were recorded with a Fisons QMD 1000 mass spectrometer (70 eV, 200 μA, ion source temperature 200 °C). The samples were introduced directly into the ion source. Elemental analyses for C, H, and N were performed on a Perkin-Elmer 240 B microanalyzer, and the analytical results were within ± 0.4% of the theoretical values.

5.1.1. *N*-substituted-2-oxo-2H-1-benzopyran-3-carboxamides ***C1, C3–C5*** *and* ***C8***

5.1.1.1. General procedure. The appropriate azomethines (0.0016 mol), prepared according to literature procedures [27,28] were reacted with carbon suboxide (0.0016 mol) in dry diethyl ether [29]. The mixture was stirred at 0 °C for 5 h then kept at r.t. for 48 h under stirring. The precipitate was filtered and crystallized from ethanol to give derivatives **C1** and **C3**–**C5**.

5.1.2. 4-[[(2-Oxo-2H-1-benzopyran-3-yl)carbonyl]amino]-benzoyl chloride ***C8***

In a flask equipped with a reflux condenser, 4-[[(2-oxo-2*H*-1-benzopyran-3-yl)carbonyl]amino]-benzoic acid **C7** and thionyl chloride were refluxed and kept under stirring for 3 h. The mixture was then cooled and filtered and the crude acyl chloride **C8** crystallized from diethyl ether.

5.2. Antimicrobial activity

5.2.1. Antibacterial and antifungal activity

All synthesized derivatives **C1–C8**, dissolved in DMSO, were evaluated for their antimicrobial and antifungal activity. Organisms from routine clinical Gram-positive (*S. aureus*, *S. epidermidis*, *S. hominis*, *Enterococcus* sp., *E. faecalis*) and Gram-negative isolates (*E. coli*, *K. pneumoniae*, *K. oxytoca, E. cloacae*, *P. aeruginosa*) and four *Candida* strains (*C. albicans* and *C. tropicalis*) isolates from 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. The isolates were identified by conventional methodologies; all isolates were subcultured to ensure optimal growth. The in vitro anti-

microbial activities of the compounds were determined with the broth micro dilution method, as recommended by the National Committee for Clinical Laboratory Standards [30] with Mueller–Hinton II broth (BBL Microbiology Systems, Cockeysville, Md.). Microtiter plates containing serial dilutions of each compound agent 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 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 with the broth micro dilution method with Sabouraud dextrose broth (BBL Microbiology Systems, Cockeysville, MD) as recommended by the NCCLS [31]. Microtiter plates containing serial dilutions of each compound 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 antifungal agent that completely inhibited growth of the organism, as detected by an unaided eye.

5.2.2. Anti-H. pylori activity

Seventeen 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 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 min.

The MIC values were determined by the agar dilution standard method [32] incubating the bacteria in 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 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.

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