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Design, synthesis and antibacterial study of new potent and selective coumarin–chalcone derivatives for the treatment of tenacibaculosis

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ABSTRACT

With the aim of finding new chemical entities selective for fish pathogens to avoid drug resistance in humans, a series of coumarin–chalcone hybrid compounds with different patterns of substitution were designed and synthesized. Their antibacterial activity was evaluated against important types of human bacteria strains (*Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) and against a fourteen strains of the marine pathogen *Tenacibaculum maritimum*, responsible for tenacibaculosis in fish, which is an important disease that causes great economical loss in the aquaculture industry. All the amino derivatives **5**–**12** presented high activity against different strains of *T. maritimum*, no activity against any of the three human pathogenic bacteria strains and no toxicity. Compounds **6**, **7** and **11** were the most promising molecules. The most sensitive strains to these compounds were LL01 8.3.8 and LL01 8.3.1, being compound **11** up to 20 times more active than enrofloxacin. Therefore these scaffolds are good candidates for aquaculture treatments, avoiding possible drug resistance problems in humans.

1. Introduction

Aquaculture is an emerging industrial sector that requires continued research with scientific and technical developments and innovation and contributes in 40.1% to the world total fish production, and almost all the seaweeds production. World aquaculture production continues to grow, albeit at a slowing rate. According to the latest available statistics collected globally by FAO, world aquaculture production attained another all-time high of 90.4 million tones (live weight equivalent) in 2012 (US$144.4 billion), including 66.6 million tones of food fish (US$137.7 billion) and 23.8 million tones of aquatic algae (mostly seaweeds, US$6.4 billion).[1] Nowadays, the estimated value of farmed food fish is USD 130 billion.

Tenacibaculum maritimum (*T. maritimum*), formerly known as *Flexibacter maritimus*,[2] and other members of the genus *Tenacibaculum* (*T. ovolyticum*, *T. soleae*, *T. discolor* and *T. gallaicum*) are Gram-negative and filamentous bacterium, responsible for tenacibaculosis in marine fish. The pathology of the disease caused by this marine organism has mainly been associated with characteristic gross lesions on the body surface of fish such as ulcers, necrosis, eroded mouth, frayed fins and tail rots, and sometimes necrosis on the gills and eyes.[3–7]

Even though tenacibaculosis has long been recognized as a disease of fish in Japan, the infection was not considered of economical importance until serious mortalities affecting Dover sole *Solea solea* in Scotland were reported.[3,8] To date, tenacibaculosis is one of the most threatening bacterial infections limiting the culture of many species of commercial value in distinct geographical areas of the world.[9] Until recently, no vaccines were available to prevent the disease, but a flexibacteriosis vaccine (FM 95) has been patented by one of our collaborating groups at the University of Santiago (Spain) and is the only vaccine currently in the market to prevent mortalities caused by *T. maritimum*.[10] Therefore there is an urgent need to develop new agents that can be used in aquaculture to treat this fish disease not only in turbot but also in other fish species.

Despite the significance of *T. maritimum* an other *Tenacibaculum* spp. in the aquaculture industry, relatively little is known about the pathogenicity of this bacteria. In vitro studies on the susceptibility of *T. maritimum* to various chemotherapeutic agents indicate

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that bacterial strains isolated from different host species and geographical regions exhibit a similar pattern, with respect to susceptibility to nitrofurans, penicillins, erythromycin, tetracyclines, chloramphenicol, trimethoprim, potentiated sulfonamides and fluoroquinolones, and resistance to colistin, kanamycin, neomycin and the quinolones, oxolinic acid and flumequine.[11]

Among the drugs used in the past few years in turbot and sole cultures (tetracycline, enrofloxacin, flumequine and potentiated sulfonamides), enrofloxacin proved to be the most useful compound for controlling *T. maritimum* outbreaks, although the rapid appearance of resistant strains has already been described (Fig. 1).[12] However, during the last few years, enrofloxacin, a member of the 4-quinolone group with broad spectrum activity, has received growing attention for its potential in fish therapy against infections caused by bacterial pathogens.[13,14]

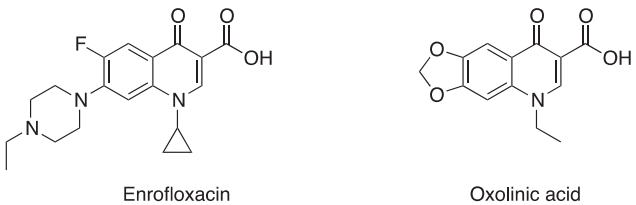
Benzopyrone derivatives could be considered as structurally similar quinolone drugs, widely used in clinic and with a broad antimicrobial spectrum, including many encountered pathogens.[15] There are important natural occurring antibiotics containing the benzopyrone moiety, such as novobiocin and coumermycin A_1 (Fig. 2). This fact makes the study of quinolone-like compounds an interesting topic on this research area.

Coumarins are a large and well-known family of compounds of natural and synthetic origin with great structural variety responsible for a wide range of pharmacological activities.[16,17] Recent studies pay special attention to their anti-inflammatory,[18,19] antioxidative,[20,21] cardioprotective,[22] antitumor,[23,24] antimicrobial[25,26] and enzymatic inhibition[27,28] properties. Special attention can be paid to aminocoumarins, which represents a class of antimicrobial agents that act by the inhibition of the DNA gyrase enzyme involved in the cell division in bacteria by binding tightly to its B subunit, thereby inhibiting this essential enzyme.[29]

On the other hand, chalcones are prominent secondary metabolites precursors of flavonoids and isoflavonoids in plants. Chemically, they consist of two aromatic rings linked by a three-carbon α,β-unsaturated systems that are known to exhibit an impressive array of biological properties.[30] Among them, it is remarkable to mention their antibacterial,[31,32] antimalarial,[33,34] antifungal,[35] antiviral[36,37] and anti-inflammatory[38,39] properties.

In humans, antibiotic resistance is a worldwide problem and adds considerable and avoidable costs to the healthcare system. The use of antibiotics is the single most important factor leading to antibiotic resistance around the world. One of the major factors in the growth of antibiotic resistance is spread of the resistant strains of bacteria from person to person, or from the non-human sources in the environment, including food.

Based on our experience on 3-substituted coumarin derivatives,[40–43] in previous results of our group with aminocoumarins as antibacterial agents[44,45] and taking into account the economic importance of the marine fish diseases in the aquaculture industry, we have designed and synthesized novel coumarin–chalcone hybrid compounds. These series of compounds were tested in vitro for their antibacterial activity against three human pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) and different species of marine pathogens of the *Tenacibaculum* genus. The increasing need of finding new drug candidates for the treatment of tenacibaculosis and the antibiotic-resistance problems in humans makes this topic an interesting research area.



Enrofloxacin Oxolinic acid

Figure 1. Quinolone derivatives as antibacterial drugs.

2. Materials and methods

2.1. Chemistry

Melting points were determined using a Reichert Koflerthermopan or in capillary tubes on a Büchi 510 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300 and 75.47 MHz, respectively, using TMS as internal standard (chemical shifts in δ values, J in Hz). Mass spectra were obtained using a Hewlett-Packard 5988A spectrometer. Elemental analyses were performed using a Perkin–Elmer 240B microanalyser and were within ±0.4% of calculated values in all cases. Silica gel (Merck 60, 230–00 mesh) was used for flash chromatography (FC). Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm).

2.1.1. General procedure for the synthesis of 3-benzoylcoumarins 1–4

To a solution of the appropriate β-ketoester (1 mmol) and the corresponding salicylaldehyde (1 mmol) in ethanol (3 mL) was added piperidine in catalytic amount. The mixture was refluxed for 2–5 h and after completion (followed by TLC), the reaction was cooled and the precipitated was filtered and washed with cold ethanol and ether to afford the desired compound. Compounds were further recrystallized from MeOH/CH_2Cl_2.

Compounds **1**[46] and **2**[47] were previously described.

3-Benzoyl-8-ethoxycoumarin (**3**): Yield (60%), mp 141–143 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.89 (s, 1H, H-4), 7.71 (d, J = 8.3 Hz, 2H, H-2′, H-6′), 7.44 (t, J = 8.1 Hz, 1H, H-4′), 7.36–7.23 (m, 2H, H-3′, H-5′), 7.14–6.91 (m, 3H, H-5, H-6, H-7), 4.05 (q, J = 7.0 Hz, 2H, CH_2), 1.35 (t, J = 7.0 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 192.15, 158.47, 147.00, 146.06, 145.06, 136.57, 134.16, 130.00, 128.93, 127.61, 125.18, 120.68, 119.27, 116.82, 65.50, 15.12. MS EI m/z (%): 295 ([M+1]$^+$, 28), 294 ([M]$^+$, 78), 266 (43), 237 (35), 105 (100), 77 (77). Anal. Calcd for $C_{18}H_{14}O_4$: C, 73.46; H, 4.79. Found: C, 73.06; H, 4.45.

6-Bromo-3-benzoylcoumarin (**4**): Yield (58%), mp 173–175 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.01 (s, 1H, H-4), 7.90 (d, J = 7.5 Hz, 2H, H-2′, H-6), 7.81–7.72 (m, 2H, H-5, H-7), 7.67 (t, J = 7.5 Hz, 1H, H-4′), 7.52 (t, J = 7.5 Hz, 2H, H-3′, H-5′), 7.33 (d, J = 9.5 Hz, 1H, H-8). ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.48, 158.09, 153.91, 144.10, 136.64, 136.27, 134.43, 131.64, 129.98, 129.07, 128.51, 121.67, 120.03, 119.04, 117.91, 108.26. MS EI m/z (%): 331 ([M+2]$^+$, 26), 330 ([M+1]$^+$, 6), 329 ([M]$^+$, 27), 105 (100), 77 (87). Anal. Calcd for $C_{16}H_9BrO_3$: C, 56.85; H, 3.09. Found: C, 56.51; H, 3.02.

2.1.2. General procedure for the synthesis of amino-3-benzoylcoumarins 5–12

Following the same procedure described for the synthesis of derivatives **1**–**4**, the nitro-3-benzoylcoumarin derivatives were prepared and were used without further purification. To a solution of the corresponding nitro-3-benzoylcoumarin (1 mmol) in pure ethanol (5 mL) was treated with $SnCl_2{\cdot}2H_2O$ and the resulting mixture was left under reflux for 3–7 h. After completion of reaction monitored by TLC, water was poured over the reaction mixture (15 mL), and the solution was further neutralized with a 5% $NaHCO_3$ solution. Extraction was carried out with EtOAc (3 × 20 mL), the combined organic layers were dried over anhydrous Na_2SO_4 and solvent was completely removed. The crude