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Structural Requirements of Hydroxylated Coumarins for *In Vitro* Anti-*Helicobacter pylori* Activity

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Abstract. *We have previously found that a 7-hydroxycoumarin derivative has potent anti-Helicobacter pylori (H. pylori) activity, comparable with metronidazole. In this report, we describe the structural requirement for the anti-H. pylori activity of several hydroxylated coumarins* ***(1-23)****. It was found that 7-hydroxy-4-methylcoumarin* ***(6)****, 6,7-dihydroxy-4-methylcoumarin* ***(8)****, 6-hydroxy-7-methoxy-4-methylcoumarin* ***(10)*** *and 5,7-dihydroxy-cyclopentanocoumarin* ***(21)*** *showed comparable anti-H. pylori activity with metronidazole. The presence of 7- and/or 6-hydroxyl groups seems to be essential to display higher anti-H. pylori activity. Their activities depended on the number and position of the hydroxyl group on the benzenoid ring of the coumarin system. Methylation of the hydroxy group generally diminished the activity. In hydroxylated coumarins, the methyl group at C-4 position enhanced the activity. The inhibitory activity of coumarins* ***(1-23)*** *against jack bean urease was examined, but no coumarins showed any inhibition at 160 μg/mL.*

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral-shaped, strongly motile bacterium (1) and is a major causative factor of a number of gastric pathologies such as gastritis, peptic ulcers and gastric cancers (2). In fact, *H. pylori* is the first bacterium to be classified as a Group 1 carcinogen and definite cause of gastric cancer in humans by the International Agency of Research on Cancer. *H. pylori* urease is considered to be associated with virulence (3). Therefore, eradication of the bacteria and inhibition of the urease are important for the treatment of patients with gastroduodenal diseases (4). Today, the treatment of this infection is mainly based on the use of triple therapies consisting of a combination of two antibiotics (amoxicillin, clarithromycin and/or metronidazole) with a proton pump inhibitor or bismuth (4). Although these regimens attain high cure rates, an eradication failure rate of 5-20% remains. A number of problems are also associated with multiple therapy, among which are drug resistance, side-effects and non-compliance (5). Therefore, the development of a new class of inhibitors of *H. pylori* is urgent.

In our laboratory, we have initiated the screening of a variety of structurally diverse coumarins to identify a lead coumarin with an anti-*H. pylori* activity. The studies led to the identification of a 7-hydroxycoumarin as a lead molecule with anti-*H. pylori* activity, comparable to the activity of metronidazole (6). In this report, we report a follow-up structure-activity relationship (SAR) study in which the OH-substituents of coumarins were systematically varied to determine the anti-*H. pylori* activity.

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Materials and Methods

General. Melting points were determined on an Electrothermal or a Büchi B-545 instrument. ^{1}H- and ^{13}C-NMR spectra were acquired on a Bruker AC 200 or Avance 200 spectrometer (200 MHz both) in [D_6]DMSO. The chemical shifts refer to TMS (^{1}H-NMR) or to [D_6]DMSO (^{13}C-NMR, δ=39.5 ppm), respectively. Combustion analyses were carried out on "Mikroanalytisches Labor Hein", Moembris, Germany. TLC (Thin layer chromatography) was performed on a Merck Kieselgel 60 F254 (Merck 5549, USA).

Materials. The following chemicals were obtained from each indicated company: coumarin (**1**), 7-hydroxycoumarin (**2**), 6,7-dihydroxycoumarin (**3**), 7-hydroxy-6-methoxycoumarin (**4**), 7-hydroxy-4-methylcoumarin (**6**), 6-hydroxy-4-methylcoumarin (**7**), 6,7-dihydroxy-4-methylcoumarin (**8**), 7-methoxy-4-methyl-coumarin (**12**) and 6-methoxy-4-methyl-coumarin (**13**)(Tokyo Kasei Co., Tokyo, Japan); metronidazole and amoxicillin (Wako Pure Chem. Ind. Ltd., Japan); clarithromycin (Taisho Pharmaceutical Co.,

Tokyo, Japan); Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Biosci., Lenexa, KS, USA).

*Preparation of coumarins (**5, 9-11 and 14-23**).* The following coumarins were prepared by the Pechmann reaction (7), unless otherwise noted. One equivalent of the (2-substituted) β-ketoester was added dropwise to a solution of the appropriate phenol in sulfuric acid (75% in water) at 0 to 25°C. The mixture was kept at room temperature for at least 30 minutes and was then placed on ice or diluted with ice/water. The product was filtered off and recrystallized.

7-Hydroxy-5,6-dimethoxy-2*H*-1-benzopyran-2-one (**5**): mp 145-146°C (EtOAc/petrol ether). Preparation according to Ahluwalia (8). The yield of the last step was 45%.

5,7-Dihydroxy-4-methyl-2*H*-1-benzopyran-2-one (**9**): mp 290-292°C (EtOH). Yield 72%. ^{1}H-NMR (DMSO-d_6) δ: 2.50 (d, 3H, *J*=0.9 Hz), 5.85 (q, 1H, *J*=1.0 Hz), 6.18 (d, 1H, *J*=2.4 Hz), 6.27 (d, 1H, *J*=2.4 Hz), 10.31 (s, 1H), 10.53 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ: 23.4 (CH_3), 94.5 (CH), 99.1 (CH), 102.1 (C), 108.8 (CH), 155.0 (C), 156.5 (C), 157.9 (C), 160.1 (C), 161.1 (C). *Anal.* Calcd for $C_{10}H_8O_4$: C, 62.50; H, 4.20. Found: C, 61.94; H, 4.10.

6-Hydroxy-7-methoxy-4-methyl-2*H*-1-benzopyran-2-one (**10**): mp 212-215°C (2-propanol)(9). Yield 79%.

7-Hydroxy-6-methoxy-4-methyl-2*H*-1-benzopyran-2-one (**11**): mp 217-217.5°C (EtOH)(9). Yield 66%.

7-Hydroxy-3-methyl-2*H*-1-benzopyran-2-one (**14**): 109 mmol methyl formate was added dropwise at 13-15°C to a suspension of 200 mmol sodium methanolate in 300 mmol propionic acid ethylester. After 3.5 hours at room temperature, the solidified mixture was diluted with 20 mL methanol. To this solution were added 91 mmol resorcin, dissolved in 50 mL methanol, keeping the reaction temperature between 14 and 17°C. The mixture was stirred at room temperature for 40 hours, acidified with 50 mL 2M sulfuric acid (cooling with ice) and diluted with 600 mL water. The precipitated product was filtered off, dried and recrystallized; mp 226-229°C (EtOAc) (10). Yield 31%. ^{1}H-NMR (DMSO-d_6) δ: 2.04 (d, 3 H, *J*=1.2 Hz), 6.71 (d, 1 H, *J*=2.4 Hz), 6.77 (dd, 1 H, *J*=8.4, 2.3 Hz), 7.43 (d, 1 H, *J*=8.4 Hz), 7.74 (s, 1 H), 10.40 (s, 1 H). ^{13}C-NMR (DMSO-d_6) δ: 16.4 (CH_3), 101.8 (CH), 111.7 (C), 112.9 (CH), 119.9 (C), 128.6 (CH), 140.0 (CH), 154.4 (C), 160.1 (C), 161.6 (C).

7-Hydroxy-3,4-dimethyl-2*H*-1-benzopyran-2-one (**15**): mp 265-270°C (2-propanol/EtOH)(11). Yield 82%.

6,7-Dihydroxy-3,4-dimethyl-2*H*-1-benzopyran-2-one (**16**): starting material 1,2,4-triacetoxybenzene; mp 267.5-268°C (EtOH/TBME)(12). Yield 93%.

6-Hydroxy-7-methoxy-3,4-dimethyl-2*H*-1-benzopyran-2-one (**17**): mp 224-226°C (1-propanol/H_2O, 3/1)(13). Yield 71%.

7-Hydroxy-6-methoxy-3,4-dimethyl-2*H*-1-benzopyran-2-one (**18**): mp 198-200°C (EtOH). Yield 68%. ^{1}H-NMR (DMSO-d_6) δ: 2.05 (s, 3H), 2.36 (s, 3H), 3.86 (s, 3H), 6.74 (s, 1H), 7.13 (s, 1H), 10.09 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ: 12.9 (CH_3), 15.1 (CH_3), 56.2 (CH_3), 102.6 (CH), 106.9 (CH), 111.8 (C), 117.1 (C), 145.1 (C), 147.0 (C x 2), 149.9 (C), 161.5 (C). *Anal.* Calcd for $C_{12}H_{12}O_4$: C, 65.45; H, 5.49. Found: C, 65.30; H, 5.48.

8-Hydroxy-7-methoxy-3,4-dimethyl-2*H*-1-benzopyran-2-one (**19**): mp 229-230°C (EtOH/H_2O, 9/1). Yield 66%. ^{1}H-NMR (DMSO-d_6) δ: 2.07 (s, 3H), 2.33 (s, 3H), 3.88 (s, 3H), 6.99 (d, 1H, *J*=8.9 Hz), 7.19 (d, 1H, *J*=8.9 Hz), 9.35 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ: 12.9 (CH_3), 14.9 (CH_3), 56.2 (CH_3), 108.1 (CH), 114.6 (C), 114.7 (CH), 117.9 (C), 133.1 (C), 141.0 (C), 147.0 (C), 149.5 (C), 161.1 (C). *Anal.* Calcd for $C_{12}H_{12}O_4$: C, 65.45; H, 5.49. Found: C, 65.26; H, 5.19.

2,3-Dihydro-7,8-dihydroxycyclopenta[*c*][1]benzopyran-4(1*H*)-one (**20**): starting material 1,2,4-triacetoxybenzene; mp 260°C (dec., 2-propanol/EtOH). Yield 64%. ^{1}H-NMR (DMSO-d_6) δ: 2.07 (quint, 2H, *J*=7.4 Hz), 2.69 (t, 2H, *J*=7.4 Hz), 2.96 (t, 2H, *J*=7.6 Hz), 6.77 (s, 1H), 6.84 (s, 1H), 9.70 (br s, 2H). ^{13}C-NMR (DMSO-d_6) δ: 22.0 (CH_2), 30.0 (CH_2), 31.6 (CH_2), 102.6 (CH), 109.3 (CH), 110.1 (C), 122.5 (C), 142.6 (C), 148.2 (C), 149.4 (C), 156.4 (C), 159.6 (C). *Anal.* Calcd for $C_{12}H_{10}O_4$: C, 66.05; H, 4.62. Found: C, 65.93; H, 4.56.

2,3-Dihydro-7,9-dihydroxycyclopenta[*c*][1]benzopyran-4(1*H*)-one (**21**): mp 270-275°C (EtOH/TBME). Yield 96%. ^{1}H-NMR (DMSO-d_6) δ: 1.99 (quint, 2H, *J*=7.6 Hz), 2.60 (t, 2H, *J*=7.6 Hz), 3.22 (t, 2H, *J*=7.6 Hz), 6.20 (d, 1H, *J*=2.2 Hz), 6.25 (d, 1H, *J*=2.2 Hz), 10.17 (s, 1H), 10.37 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ: 22.2 (CH_2), 28.9 (CH_2), 35.6 (CH_2), 94.1 (CH), 98.5 (CH), 101.3 (C), 120.1 (C), 156.3 (C), 156,4 (C), 156.5 (C), 159.4 (C), 160.6 (C). *Anal.* Calcd for $C_{12}H_{10}O_4$: C, 66.05; H, 4.62. Found: C, 64.13; H, 4.73.

2,3-Dihydro-8-hydroxy-7-methoxycyclopenta[*c*][1]benzopyran-4(1*H*)-one (**22**): mp 254-255°C (2-propanol). Yield 71%. ^{1}H-NMR (DMSO-d_6) δ: 2.07 (quint, 2H, *J*=7.4 Hz), 2.69 (t, 2H, *J*=7.3 Hz), 2.95 (t, 2H, *J*=7.5 Hz), 3.87 (s, 3H), 6.83 (s, 1H), 6.98 (s, 1H), 9.32 (br s, 1H). ^{13}C-NMR (DMSO-d_6) δ: 21.9 (CH_2), 30.0 (CH_2), 31.5 (CH_2), 55.9 (CH_3), 99.8 (CH), 108.9 (CH), 110.8 (C), 123.4 (C), 143.4 (C), 148.1 (C), 150.9 (C), 156.2 (C), 159.5 (C). *Anal.* Calcd for $C_{13}H_{12}O_4$: C, 67.23; H, 5.21. Found: C, 67.30: H, 5.26.

2,3-Dihydro-7-hydroxy-8-methoxycyclopenta[*c*][1]benzopyran-4(1*H*)-one (**23**): mp 226.5-227.5°C (EtOH/H_2O). Yield 52%. ^{1}H-NMR (DMSO-d_6) δ: 2.07 (quint, 2H, *J*=7.3 Hz), 2.70 (t, 2H, *J*=7.4 Hz), 3.00 (t, 2H, *J*=7.6 Hz), 3.83 (s, 3H), 6.79 (s, 1H), 6.93 (s, 1H), 10.14 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ: 22.0 (CH_2), 30.0 (CH_2), 31.7 (CH_2), 56.1 (CH_3), 102.7 (CH), 106.6 (CH), 109.9 (C), 122.6 (C), 145.1 (C), 149.2 (C), 150.3 (C), 156.6 (C), 159.5 (C). *Anal.* Calcd for $C_{13}H_{12}O_4$: C, 67.23; H, 5.21. Found: C, 67.31; H, 5.22.

Bacteria. H. pylori (ATCC43504) was purchased from the American Type Culture Collection (Rockville, MD, USA). Jack bean urease was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Measurement of anti-H. pylori activity. The micro-dilution broth method was used to determine the minimum inhibitory concentration (MIC). Brain Heart Infusion (BHI) broth containing 10% fetal bovine serum (FBS) (Biofluid, Inc., Rockville, MD, USA) and 0.1% glucose was used as the medium, and was cultured in a jar conditioned with AnaeroPack (Campylo)(Mitsubishi Gas Chemical Co., Inc.). Briefly, *H. pylori* was inoculated in the medium and cultured at 37°C for 2 days. The collected bacterial colonies were diluted to about 10^7 colony forming unit (CFU)/mL with the medium. The fractions were dissolved in DMSO and then diluted with the medium. To the solution of the fractions, a suspension of bacteria was added to make 10^6 CFU/150 μL/well. The mixture was incubated at 37°C for 5 days. The MIC_{50} values of the fractions were determined by observation (14).

Measurement of urease. Although the jack bean urease differs somewhat from *H. pylori* urease (15), this plant source was used

for the experiment because of its ready availability. The assay mixture, containing 25 μL (4U) of jack bean urease and 25 μL (100 μg) of the test compound, was preincubated for 30 minutes at room temperature in a 96-well assay plate. After preincubation, 0.2 mL of 100 mM phosphate buffer pH 6.8 containing 500 mM urea and 0.002% phenol red were added and incubated at room temperature. The reaction time was measured by micro plate reader (570 nm), which was the time required for enough ammonium carbonate to form to raise the pH of a phosphate buffer from 6.8 to 7.7 (16).

Results and Discussion

Among 23 coumarin derivatives (see structural formulae in Table I) which had various numbers of hydroxyl and/or methoxyl groups at different positions of the benzenoid ring of the coumarin system, four compounds (**6, 8, 10** and **21**) display potent anti-*H. pylori* activity, ranging from MIC_{50} 23.0-32.6 μg/mL. The susceptibility of *H. pylori* to these four coumarins were comparable with that of metronidazole.

Although the anti-*H. pylori* activity of the hydroxylated 4-methylcoumarins did not exhibit any rigid, uniform trend, their activies were in the following order; 6,7-dihydroxy = 7-hydroxy > 6-hydroxy >> 5,7-dihydroxy derivatives. This suggests that the hydroxy groups at C-6 and C-7 positions are important determinants for the anti-*H. pylori* activity.

Methylation of the hydroxy group generally diminished the activity, as seen in the following cases of : 6,7-dihydroxy (**3**) *vs.* 7-hydroxy-6-methoxy (**4**); 7-hydroxy-4-methyl (**6**) *vs.* 7-methoxy-4-methyl (**12**); 6-hydroxy-4-methyl (**7**) *vs.* 6-methoxy-4-methyl (**13**); 6,7-dihydroxy-4-methyl (**8**) *vs.* 6-hydroxy-7-methoxy-4-methyl (**10**) or 7-hydroxy-6-methoxy-4-methyl (**11**) and 6,7-dihydroxy-3,4-dimethyl (**16**) *vs.* 6-hydroxy-7-methoxy-3,4-dimethyl (**17**) or 7-hydroxy-6-methoxy-3,4-dimethyl (**18**). These results further support the possibility that the hydroxy group is necessary for the high potency.

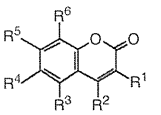
In hydroxylated coumarins, the methyl group at C-4 position enhanced the activity. In four 7-hydroxycoumarins, the order of potency is 4-methyl (**6**) > 3,4-dimethyl (**15**) > none (**2**) > 3-methyl (**14**). In four 6,7-dihydroxycoumarins, the order is 4-methyl (**8**) > 3,4-dimethyl (**16**) > none (**3**) >> cyclopenta (**20**). In four 7-hydroxy-6-methoxycoumarins, the order is 4-methyl (**11**) > none (**4**) >> 3,4-dimethyl (**18**), cyclopenta (**23**). In three 6-hydroxy-7-methoxycoumarins, the order is 4-methyl (**10**) >> 3,4-dimethyl (**17**), cyclopenta (**22**).

The inhibitory activity of coumarins (**1-23**) against jack bean urease was examined, but no coumarins showed any inhibition at 160 μg/mL, in contrast to acetohydroxamic acid (IC_{50}: 3.5 μg/mL).

In conclusion, we synthesized and evaluated a series of hydroxylated coumarin derivatives. It was found that several hydroxylated coumarins showed potent anti-*H. pylori* activity (MIC_{50}=20-30 μg/mL), comparable with metronidazole (MIC_{50}=27.2 μg/mL). It is suggested that coumarins generally possess moderate anti-*H. pylori* activity and their activity strongly depends on the number and position of the hydroxyl group on the benzenoid ring of the coumarin system. However, these compounds did not inhibit the jack bean urease, suggesting a different mode of their actions from the urease inhibitors, omeprazole and rabeprazole (17).

Some epidemiological reports showed that a high intake of Allium vegetables including garlic reduces the risk of gastric cancer (18). Coumarin derivatives are widely distributed in plants and are present in notable amounts in citrus fruits and vegetables such as celeriac, parsnip, egg plant, fennel leaves, parsley and tomatoes (19). Therefore, ingestion of such food could have a protective effect against *H. pylori*-associated gastroduodenal disease.

Table I. *Evaluation of anti-H. pylori activity of coumarins.*



1-23

Compound	R^1	R^2	R^3	R^4	R^5	R^6	MIC_{50} (μg/mL)
1	H	H	H	H	H	H	>100
2	H	H	H	H	OH	H	57.7
3	H	H	H	OH	OH	H	65.8
4	H	H	H	OCH_3	OH	H	79.7
5	H	H	OCH_3	OCH_3	OH	H	69.1
6	H	CH_3	H	H	OH	H	28.3
7	H	CH_3	H	OH	H	H	42.0
8	H	CH_3	H	OH	OH	H	23.0
9	H	CH_3	OH	H	OH	H	>100
10	H	CH_3	H	OH	OCH_3	H	32.6
11	H	CH_3	H	OCH_3	OH	H	66.7
12	H	CH_3	H	H	OCH_3	H	>100
13	H	CH_3	H	OCH_3	H	H	>100
14	CH_3	H	H	H	OH	H	70.2
15	CH_3	CH_3	H	H	OH	H	51.5
16	CH_3	CH_3	H	OH	OH	H	55.0
17	CH_3	CH_3	H	OH	OCH_3	H	>100
18	CH_3	CH_3	H	OCH_3	OH	H	>100
19	CH_3	CH_3	H	H	OCH_3	OH	>100
20	$-(CH_2)_3-$		H	OH	OH	H	>100
21	$-(CH_2)_3-$		OH	H	OH	H	28.0
22	$-(CH_2)_3-$		H	OH	OCH_3	H	>100
23	$-(CH_2)_3-$		H	OCH_3	OH	H	>100
Metronidazole							27.2
Amoxicillin							1.7×10^{-4}
Clarithromycin							1.2×10^{-3}

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