



Synthesis, structure and antibacterial activity of 3-substituted derivatives of 4-hydroxycoumarin

DAVORKA ZAVRŠNIK¹
SELMA ŠPIRTOVIĆ-HALILOVIĆ¹
DŽENITA SOFTIĆ²

¹Department of Pharmaceutical Chemistry
Faculty of Pharmacy, Čekaluša 90,
71000 Sarajevo, Bosnia and Herzegovina

²Agency for Medicinal Products and
Medical Devices of Bosnia and Herzegovina
The Control Laboratory of the Agency
Titova 9, Sarajevo, Bosnia and Herzegovina

Correspondence:

Davorka Završnik
Department of Pharmaceutical Chemistry
Faculty of Pharmacy, Čekaluša 90,
71000 Sarajevo, Bosnia and Herzegovina

Abstract

Background and purpose: Having in mind the results of our previous work, which suggested antimicrobial activity of some 3-cinnamoyl-4-hydroxycoumarins, we carried out the synthesis of several new derivatives of this group. The microbiological activity of compounds was tested by the diffusion and dilution methods on various species of bacteria. The aim of the study was to evaluate the influence of the substituents on antimicrobial activity.

Material and methods: A series of new 3-cinnamoyl-4-hydroxycoumarins was prepared by the reaction of nucleophilic addition from 3-acetyl-4-hydroxycoumarin acting on appropriate aromatic aldehydes. The microbiological activity of compounds was tested by the diffusion and dilution methods on species of bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium*, *Bordetella bronchiseptica*, *Bacillus subtilis*, and *Staphylococcus aureus*.

Results: The synthesis of new 3-cinnamoyl-4-hydroxycoumarins was carried out. The elementary content of the synthesized compounds was confirmed by elementary analysis, and structures were confirmed with IR-spectrophotometry and ¹H-NMR spectrophotometry.

The compounds that have halogens showed the best microbiological activity. Compounds 5, and 6 were the most effective against *Bacillus subtilis* (MBC = 0.0039 mg/mL, and MIC = of 0.0010 mg/mL. Compound 6 was the most effective against *Staphylococcus aureus* (MBC = 0.0156 mg/mL, and MIC = of 0.0019 mg/mL).

Conclusion: All synthesized compounds have larger or smaller growth inhibition zones when it comes to Gram-positive aerobic bacteria *Bacillus subtilis* and *Staphylococcus aureus*. The tested compounds showed resistance to Gram-negative types of bacteria. The compounds that have halogens, as substituents (compound 5 and 6) showed the best microbiological activity.

INTRODUCTION

Permanent need for synthesis of new antimicrobial compounds has resulted in synthesis of a great number of derivatives of 4-hydroxycoumarin with antibacterial activity.

The synthesis and pharmacological investigation of coumarins and their derivatives are still actual, because these compounds have shown broad spectra of activity. A great number of synthesized derivatives are biologically active, and many of them are applied in therapy as antico-

agulant, antibacterial and antifungal agents (1–4). Some of these compounds, such as novobiocin, chlorobiocin, cumermycin A₁ and vanillobiocin, are used in therapy or show very good activity against *Staphylococcus aureus* (5–6).

Our previous results showed that some 3-cinnamoyl-4-hydroxycoumarins were found to have good antibacterial activity (inhibition zones against *Staphylococcus aureus* are from 16 to 27 mm) (5).

We have synthesized a series of new derivatives of 3-cinnamoyl-4-hydroxycoumarin. We have confirmed the structures of the synthesized compounds by elementary analysis, IR-spectrophotometry and ¹H-NMR spectrophotometry. Using the methods of diffusion and dilution, the synthesized derivatives of 3-cinnamoyl-4-hydroxycoumarin have been tested on antimicrobial activity.

MATERIAL AND METHODS

The synthesis of 3-cinnamoyl-4-hydroxycoumarins

The synthesis of 3-cinnamoyl-4-hydroxycoumarins was carried out. In the first step a mixture of 4-Hydroxycoumarin (1 g), 4 mL acetic acid and 1 mL phosphoryl chloride was refluxed for 35 min., which resulted in obtaining 3-acetyl-4-hydroxycoumarin. After the reaction was completed, the reaction mixture was cooled to room temperature. The precipitate was filtrated and recrystallized from ethanol. In the second step nucleophilic addition from 3-acetyl-4-hydroxycoumarin (0.0049 mol) acting on appropriate aromatic aldehydes (0.0045 mol) with pyridine (0.25 mL) and piperidine (0.25 mL) as catalysts, the 3-cinnamoyl-4-hydroxycoumarin was prepared. After the reaction was completed, the reaction mixture was cooled to room temperature. The precipitate was filtrated, washed (ether and absolute ethanol) and recrystallized from EtOH-CH₃COCH₃.

The course of the reaction is presented in Figure 1.

Microanalyses for C, H and N were performed on a Perkin Elmer 2400 Series II elementary analyzer. IR spectra were recorded on Perkin Elmer FT-IR 1000, in KBr discs. Vibrational transition frequencies are reported in wave numbers (cm⁻¹).

NMR analysis

Proton nuclear magnetic resonance (¹H NMR) spectra were recorded at 300.75 MHz, in CDCl₃ and DMSO on NMR Spectrometer, Varian Unity Plus 500 MHz and Bruker Advance DPX 300 MHz. Chemical shifts ex-

pressed as δ (parts per million) values with TMS as internal standard. Multiplicities of proton resonance are designated as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m).

The antimicrobial activity of 3-substituted derivatives of 4-hydroxycoumarin

The microbiological activity of compounds was tested by the diffusion and dilution methods (7) on species of bacteria *Pseudomonas aeruginosa* (ATCC (American Type Culture Collection) No. 9027), *Echerichia coli* (ATCC No. 8739), *Salmonella typhimurium* (ATCC No. 1402), *Bordatella bronchiseptica* (ATCC No. 4617), *Bacillus subtilis* (ATCC No. 6633) and *Staphylococcus aureus* (ATCC No. 6538P).

For determination of antimicrobial activity (diffusion method) Müller-Hinton and nutritious bases A, B, F were used. Casein soybean digest broth (Tryptic soybean bouillon) was used in the dilution method.

The diffusion method is based on monitoring the growth inhibition of a specific microorganism caused by a certain concentration of the tested specimen. The results of the tests are shown as inhibition zones (I) expressed in mm.

When using the diffusion method, the test samples were dissolved in dimethyl sulfoxide (99.5 % DMSO) to obtain a 1 mg/mL stock solution. The inhibition zones for bacteria were measured in millimeters at the end of an 18-hour incubation period at 37 °C.

For analysis by the dilution method, solution of the each compound was prepared, which was then followed by preparation of a series of 12 dilutions with liquid nutritious base. The starting solution of the test material (2.0 mL) was added 2.0 mL of casein soybean digest broth, thus forming the first dilution. Subsequently, 2.0 mL of this solution was diluted with 2.0 mL casein soybean digest broth to give the second dilution and so on until 12 dilutions were obtained. After a 24-hour incubation, the last tube with no growth of microorganisms was taken to represent the MIC (minimum inhibitory concentration) expressed in mg/mL.

MBC (minimum bactericidal concentration) is determined by subculturing the solution of substances with no visible opacity onto the culture medium.

The concentration of the prepared solutions was as follows: 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.625 × 10⁻¹ mg/mL, 0.3125 × 10⁻¹ mg/mL, 0.156 × 10⁻¹ mg/mL, 0.78 mg/mL × 10⁻², 0.39 × 10⁻² mg/mL, 0.195 × 10⁻²

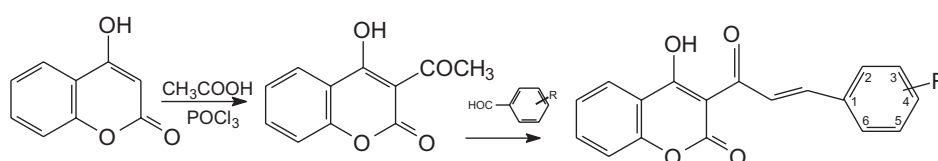


Figure 1. Synthesis of 3-cinnamoyl-4-hydroxycoumarin derivatives.

mg/mL, 0.97×10^3 mg/mL, 0.48×10^{-3} mg/mL and 0.24×10^{-3} mg/mL.

For comparison, standard antibiotic drug tablets of penicillin, gentamycin and erythromycin were used, containing 6 µg, 30 µg, and 15 µg, respectively.

RESULTS

Structure and chemical names of 3-cinnamoyl-4-hydroxycoumarin derivatives are presented in Table 1.

The results of the elemental analysis and spectral data of the prepared compounds are presented in Table 2.

TABLE 1

Structure and chemical names of 3-cinnamoyl-4-hydroxycoumarin derivatives.

Number of compound	Name of compound	R
1	4-Hydroxy-3-(3-(2-hydroxy-5-bromo-nitrophenyl)prop-2-enoyl)-2H-1-benzopyran-2-one	2-OH; 5-Br
2	4-Hydroxy-3-(3-(4-methylphenyl)prop-2-enoyl)-2H-1-benzopyran-2-one	4-CH ₃
3	4-Hydroxy-3-(3-(4-methoxyphenyl)prop-2-enoyl)-2H-1-benzopyran-2-one	4-OCH ₃
4	4-Hydroxy-3-(3-(4-hydroxyphenyl)prop-2-enoyl)-2H-1-benzopyran-2-one	4-OH
5	4-Hydroxy-3-(3-(4-bromophenyl)prop-2-enoyl)-2H-1-benzopyran-2-one	4-Br
6	4-Hydroxy-3-(3-(4-chlorophenyl)prop-2-enoyl)-2H-1-benzopyran-2-one	4-Cl
7	4-Hydroxy-3-(3-(2-fluorophenyl)prop-2-enoyl)-2H-1-benzopyran-2-one	2-F
8	4-Hydroxy-3-(3-(2-nitrophenyl)prop-2-enoyl)-2H-1-benzopyran-2-one	2-NO ₂

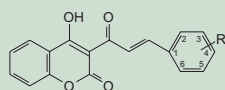


TABLE 2

Spectral characteristics and elemental analysis of 3-cinnamoyl-4-hydroxycoumarin derivatives.

N ^o	IR (KBr) cm ⁻¹	NMR (DMSO-d ₆) δ ppm	%C cal'd/found	%H cal'd/found	%N cal'd/found
1	1698 (C=O lactone) 1610 (C(=O)CH=CH)	3.34(C ₁₃ -s-OH); 8.41–8.46(H ₅ -d); 8.11–8.17(H ₁₅ -d); 8.04–8.07(H ₁₄ -d); 7.64(H ₁₇ -s); 7.44–7.47(H ₆ -t); 7.81–7.87(H ₇ -t); 7.49–7.50(H ₁₅ -d); 7.42–7.43(H ₁₀ -d); 6.91–6.94(H ₁₁ -d); 10.97 (H ₄ -s-OH);	55.84/55.80	2.86/2.36	0.00/0.08
2	1734 (C=O lactone) 1610 (C(=O)CH=CH)	8.25–8.30(H ₅ -d); 8.01–8.07(H ₇ -t); 7.81–7.86(H ₆ -t); 7.68–7.71 (H ₈ -d); 7.46–7.47(H ₁₃ -d); 2.38 (H ₁₅ -s-3H, CH ₃);	74.50/73.40	4.61/4.00	0.00/0.09
3	1734 (C=O lactone) 1612(C(=O)CH=CH)	8.15–8.20(d); 8.00–8.05(t); 87.82–7.82(d); 7.77–7.80 (t); 7.71–7.75(d); 7.37–7.40(d); 7.41–7.44 (d); 7.04–7.08 (d); 3.85 (s, 3H, OCH ₃);	70.80/70.65	4.38/4.52	0.00/0.04
4	1696 (C=O lactone) 1596(C(=O)CH=CH)	8.13–8.19 (H ₅ -d); 8.01–8.07 (t); 7.84–7.85 (d); 7.81(d); 7.78–7.79(d); 7.65–7.68(d); 7.40–7.45(T); 6.89–6.92(d); 3.57(s 1H, OH); 10.43 (s, 1H, OH);	70.1/69.70	3.9/3.5	0.00/0.00
5	1720 (C=O lactone) 1610 (C(=O)CH=CH)	8.25–8.30(H ₅ -d); 7.96–8.04(H ₇ -t); 7.81–7.86(H ₆ -t);	58.25/58.68	2.99/2.74	0.00/0.08
6	718 (C=O lactone) 1612 (C(=O)CH=CH)	8.23–8.28(d); 7.96–8.06(t); 7.84–7.85(d); 7.76–7.82 (t); 7.53–7.57 (d); 7.40–7.41 (d); 7.43–7.46(d)	–	–	–
7	1732 (C=O lactone) 1612 (C(=O)CH=CH)	8.37–8.42(H ₅ -d); 7.99–8.07(H ₇ -t); 7.96–7.97(H ₈ -d); 7.81–7.88 (H ₆ -t); 7.77–7.79(H ₁₄ -d); 7.59–7.61(H ₁₇ -d); 7.54–7.57(H ₁₀ -d); 7.42–7.48(H ₁₅ -t); 7.40–7.41(H ₁₁ -d); 7.25–7.38 (H ₁₆ -t).	69.68/69.66	3.57/3.13	0.00/0.23
8	171 (C=O lactone) 1612 (C(=O)CH=CH)	8.20–8.28(t); 8.13–8.16(d); 8.04–8.05(d); 8.06–8.07(d); 7.88–7.93(t); 7.82–7.85 (d); 7.74–7.78(t); 7.71–7.72(d); 7.45–7.48(t); 7.43–7.44(d)	64.10/63.39	3.29/2.94	4.15/4.01

IR (KBr) cm⁻¹ – IR spectra in KBr discs. Vibrational transition frequencies are reported in wave numbers (cm⁻¹).

NMR (DMSO-d₆) δ ppm – Proton nuclear magnetic resonance (1H NMR) spectra in DMSO

DMSO – Dimethyl sulphonyde

The diffusion method showed that compounds have larger or smaller growth inhibition zones when it comes to Gram-positive aerobic bacteria *B. subtilis* (I (mm) = 9–20.3) and *S. aureus* (I (mm) = 11–24.5). The tested compounds did not show activity against Gram-negative types of bacteria *P. aeruginosa*, *E. coli*, *S. typhimurium*, *B. bronchiseptica*.

The compounds having halogens, chlorine and bromine, as substituents (compounds 1, 5, 6), showed the best antimicrobial activity (I (mm) = 16.95–24.5). Among the derivatives with halogen substituent, 3-(4-bromophenylcinnamoyl)-4-hydroxycoumarin (compound 5) had the best activity. The type *S. aureus* showed the greatest sensitivity to compound 5 (I (mm) = 24.5), while *B. subtilis* had slightly poorer sensitivity (I (mm) = 20.3).

The compounds that showed the best antimicrobial activity using the diffusion method were analyzed by the dilution method. Using the dilution method, we examined MIC and MBC for *S. aureus* and *B. subtilis* of compounds 1,2,3,5,6,7.

TABLE 3

Antimicrobial activity of tested 3-cinnamoyl-4-hydroxycoumarin derivatives expressed as the inhibition zone I (mm).

No. of compound	<i>Staphylococcus aureus</i> *	<i>Bacillus subtilis</i> **
1	24.5	12.5
2	15.5	15.3
3	12.7	9.0
4	–	–
5	24.5	20.3
6	24.35	20.0
7	15.8	11.45
8	11.0	–

*The inhibition zones I (mm) for standards of antibiotics: erythromycin 20.0; penicillin 26.0; gentamycin 31.0

**The inhibition zones I (mm) for standards of antibiotics: erythromycin 27.0; penicillin 17.9.0; gentamycin 22.5

Compound 5 and compound 6 were the most effective against *B. subtilis* (MBC = 0.0039 mg/mL, and MIC = of 0.0010 mg/mL). Compound 6 was the most effective against *S. aureus* ATCC 6538P (MBC = 0.0156 mg/mL, and MIC = of 0.0019 mg/mL). The MIC and MBC of compounds 5 and 6 with halogen substituent are of a relatively low concentration, and these compounds, as expected, are quite effective against *B. subtilis* and *S. aureus*.

The results of the antimicrobial activity are presented in Table 3 and Table 4.

DISCUSSION

Our previous results showed that some of 3-cinnamoyl-4-hydroxycoumarin was found to have good antibacterial activity. We have prepared a series of new 3-cinnamoyl-4-hydroxycoumarin, by the reaction of nucleophilic addition from 3-acetyl-4-hydroxycoumarin acting on appropriate aromatic aldehydes with pyridine and piperidine as catalysts. Elementary contents and structures of compounds were confirmed by elementary analysis and with NMR and IR analysis. The newly-prepared derivatives have different substituents, and accordingly they can exhibit antimicrobial activity. Therefore, the antimicrobial activity of these derivatives was tested on various species of bacteria. The aim of the study was to evaluate the influence of the substituents on antimicrobial activity.

Using the diffusion method, the synthesized derivatives of 3-cinnamoyl-4-hydroxycoumarin were tested on antimicrobial activity. Namely, the test included six types of bacteria (*P. aeruginosa*, *E. coli*, *S. typhimurium*, *B. bronchiseptica*, *B. subtilis*, and *S. aureus*).

The tested compounds did not show activity against Gram-negative types of bacteria *P. aeruginosa*, *E. coli*, *S. typhimurium*, *B. bronchiseptica*.

The compounds with halogens, chlorine and bromine as substituents showed the best antimicrobial activity. It is known that Gram-negative bacteria are much more resistant to antimicrobial agents, than Gram-positive bacteria. Based on the fact that Gram-negative bacteria also have more lipophilic membrane than Gram-positive bacteria, it was expected that compounds which

TABLE 4

MBC and MIC of synthesized compounds against *Staphylococcus aureus* and *Bacillus subtilis*.

No. of compound	<i>Staphylococcus aureus</i>			<i>Bacillus subtilis</i>		
	MBC (mg/mL)	MIC (mg/mL)	C ^b mmol/L	MBC (mg/mL)	MIC (mg/mL)	C ^b mmol/L
1	0.25	0.03125	0.081	0.0156	0.00781	0.020
2	0.25	0.03125	0.102	0.0625	0.03125	0.102
3	0.25	0.03125	0.097	0.5	0.125	0.388
5	0.0078	0.0039	0.010	0.0039	0.0010	0.003
6	0.0156	0.0019	0.006	0.0039	0.0010	0.003
7	0.25	0.03125	0.101	0.25	0.0625	0.201

^a value of MIC expressed as molar concentration mmol/L

are hydrophylic in their nature (like compound 4), would not penetrate the cell membranes of these bacteria.

The compounds that showed the best antimicrobial activity using the diffusion method were analyzed by the dilution method. Using the dilution method, we examined MIC and MBC for *S. aureus* and *B. subtilis* of compound 1,2,3,5,6,7.

As the latest research shows, the most probable mechanism of action of compounds of similar structures is the addition on C₄ atom of coumarin ring. It is clear that the compound with bromine as substituent in para position will be more active than the compound 1 containing bromine and hydroxylic group. The presence of bromine contributes most to the increase of lipophilicity which additionally contributes to better activity in the tested compounds. It is clear that the hydroxylic group reduces lipophilicity of compound 1 and decreases its activity.

Besides which, bromine, in comparison to fluor and chlorine, is a less electronegative atom, which affects the stabilisation of coumarin structure. Compound 7 with fluorine as atom with the highest electronegative characteristics and located in ortho position, probably destabilises coumarin, and shows the lowest activity of the tested group. The MIC values of these halogen derivatives show enhanced antimicrobial activity, when compared to that of similar coumarin compounds found in the scientific literature (8).

CONCLUSION

All synthesized compounds have larger or smaller growth inhibition zones when considering Gram-posi-

tive aerobic bacteria *Bacillus subtilis* and *Staphylococcus aureus*. The tested compounds showed resistance to Gram-negative types of bacteria. The compounds having halogens as substituents (compound 5 and 6) showed the best microbiological activity.

The notable antimicrobial effect of certain compounds confirms that these are a good basis for the production of a number of new, possibly physiologically active coumarin derivatives.

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