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Research Article

SYNTHESIS OF BISCOUMARIN DERIVATIVES AS ANTIMICROBIAL AGENTS

SAMIJA MURATOVIĆ[1], KEMAL DURIĆ[2], ELMA VELJOVIĆ[1], AMAR OSMANOVIĆ[1]*, DŽENITA SOFTIĆ[3], DAVORKA ZAVRŠNIK[1]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina,[2]Department of Pharmacognosy, Faculty of Pharmacy, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina,[3]Agency for Medicinal Products and Medical Devices of B&H, Titova 9, 71000 Sarajevo, Bosnia and Herzegovina,
Email: amkooo@hotmail.com

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ABSTRACT

Objective: As a further part of our chemical and biological studies in this field, we describe the preparations of the properly substituted benzylidene-bis-(4-hydroxycoumarin) derivatives **5a-h** and 3-(6-oxo-(1*H*)-benzopyrano[4,3-b]benzopyran-7-yl)-4-hydroxycoumarin derivatives **6a-e**. Methods: The synthesized compounds were screened for their in vitro antimicrobial activity against five strains of bacteria and two fungal strains using disk diffusion assay and dilution method. The way in which the substituent group's physicochemical properties influence the antimicrobial activity is discussed in the paper. Results: The in vitro evaluation of their inhibitory properties towards five strains of Gram-positive and Gram-negative bacteria and two fungal strains indicated that the 4-trifluoromethylbenzylidene derivative of bis-(4-hydroxycoumarin) (compound **5c**) and 3-(6-oxo-(1*H*)-18-bromobenzopyrano[4,3-b]benzopyran-7-yl)-4-hydroxycoumarin derivative (compound **6b**) possess the most potent antibacterial activities, with MIC of 3.9 µg/mL - 7.8 µg/mL against Gram-positive bacteria. Conclusion: The compound **6b** has greater antibacterial activity than the standard chloramfenicol (inhibition zone 26 mm and MIC 1.9 µg/mL) against *Staphyloccocus aureus* and could be considered as leading compound in the future antimicrobial drug development.

Keywords: Benzylidene-bis-(4-hydroxycoumarin), benzopyranocoumarin derivatives, antibacterial assays, antifungal activity.

INTRODUCTION

With the development of new strains of bacteria resistant to many currently available antibiotic treatments, there is increasing interest in the discovery of new antibacterial agents. Antimicrobial resistance refers to microorganism that have developed the ability to inactivate, exclude or block the inhibition or lethal mechanism of the antimicrobial agents [1].

The coumarin (2-oxo-2*H*-chromene) derivatives are quite interesting objects for both synthesis and pharmacological screening. Their reactivity towards nucleophiles provides a useful route to prepare a variety of rearranged products and new heterocyclic systems. A variety of synthesized coumarin derivatives have been experimentally shown to exert pharmacological activities including anticoagulant, antitumor and antiproliferative, anti-inflammatory, antioxidative as well as antiviral activities [2-9]. A number of natural and synthetic coumarin derivatives have been reported to exert notably antimicrobial as well as antifungal activity [10-16].

Three main compounds of the aminocoumarin class of antibiotics, novobiocin, clorobiocin and coumermycin A1, are natural products of soil-dwelling bacteria called *Streptomycetes*. These compounds are potent inhibitors of DNA gyrase, an essential enzyme in bacteria and a validated drug target. However, due to their limited solubility in water, their low penetration into Gram-negative bacteria and their moderate toxicity to humans, they have not been widely applied clinically. Further derivation of novobiocin, clorobiocin, and coumermycin A1 has made the production of new coumarin antibiotics displaying excellent inhibition of DNA supercoiling by DNA gyrase B possible [17, 18].

A number of structurally different natural and synthetic coumarin derivatives have also been reported as the inhibitors of DNA gyrase [19, 20]. As noted, the antimicrobial action of the most of the coumarin compounds was related to inactivation of cellular enzymes, which depended on the substance penetration rate into the cell or caused by membrane permeability changes. Increased membrane permeability is a major factor in the mechanism of antimicrobial action. The introduction of halogens, and in particular bromine, fluorine and fluoroalkyl substituents to coumarin moiety, has led to the enhancement of the lipophilicity. Lipophilicity is an important physicochemical parameter in the development of antibacterial agent because it is known to be closely related to the permeation through a lipid coat of bacteria [21-25]. Studies have also shown that the number and position of substitutions in the coumarin ring influenced the antimicrobial potential.

In connection with our previous work [8, 13] on the synthesis of coumarins and the evaluation of antiviral activity in vitro, our present study is focused on the antimicrobial activity evaluation of the biscoumarins, with variously substituted aryl central linker.

The present study has been focused mainly on the investigation of some benzylidene-bis-(4-hydroxycoumarin) derivatives with various substituents on the linker of phenyl ring (R= Br, F, CF_3, SCH_3, NO_2, OH, OCH_3) **5a-h** as well as fused benzopyranocoumarin derivatives **6a-e** for their antimicrobial activity evaluation. The results indicated that the biscoumarin unit linked by a phenyl ring was required for the activity.

MATERIALS AND METHODS

General

Series of benzylidene-bis-(4-hydroxycoumarin) derivatives **5a-h** and fused benzopyranocoumarin derivatives **6a-d** were prepared in moderately good yields by Claisen condensation of 4-hydroxycoumarin and appropriate substituted aromatic aldehydes **2a-h**, all being commercially available [3-bromobenzaldehyde (2a), 4-fluorobenzaldehyde (2b), 4-trifluoromethylbenzaldehyde (2c), 4-nitrobenzaldehyde (2d), 4-methylthiobenzaldehyde (2e), 3,4-dihydroxybenzaldehyde (2f), 2,5-dimethoxybenzaldehyde (2g) and 4-hydroxy-3-methoxy-5-nitrobenzaldehyde (2h)].

Procedures for the preparation of compounds

According to the previously published procedure [8] 4-hydroxycoumarin (0.324 g, 2 mmol) was dissolved in hot ethanol (6

mL), the appropriate aldehyde (1 mmol) was added and the reaction mixture was refluxed for 24 h. After cooling to room temperature, the solid was filtered out and crystallized to give the product benzylidene-bis-(4-hydroxycoumarin) derivatives **5a-h** and 3-(6-oxo-(1*H*)-benzopyrano[4,3-b]benzopyran-7-yl)-4-hydroxycoumarin derivatives **6a-d** (Scheme 1).

Antimicrobial activity evaluation

The synthesized compounds were screened for their in vitro antimicrobial activity against five strains of bacteria and two fungal strains using a disk diffusion assay and dilution method. For antibacterial screening various bacteria, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 were used. Antifungal activity was performed against *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763. Each tested strain was previously grown in Triptic Soy Broth at 25 °C for 24 h, and bacterial and fungi suspensions were adjusted to the turbidity of 0.5 McFarland (1.5 x 10^8 CFU/mL) with a sterile saline solution (0.85% NaCl).

The screening results were compared with chloramphenicol for antibacterial and ketoconazole for antifungal activities respectively and dimethyl sulfoxide treated group served as a control.

In vitro evaluation of antibacterial activity

Mueller Hinton and Sabouraud medium were used for determination of antimicrobial activity (diffusion method). Biscoumarin derivatives disc diffusion method results were presented as the inhibition zones, given in millimeters (mm).

When using the diffusion method, the test samples were dissolved in 30% dimethyl sulfoxide (DMSO) to obtain a 1000 µg/ml stock solution. Bacteria inhibition zones were measured in millimeters at the end of an incubation period of 18 h at 37 °C, and fungi zones after 48 h at 25 °C.

The minimal inhibitory concentration (MIC) of the synthesized compounds was determined in vitro by the Soybean Casein Digest Broth (Triptic Soy Broth) by dilution method.

The solution of the tested compounds was prepared by the dilution method for the analysis, followed by the formation of series of 12 dilutions with liquid nutritious base. The 2.0 mL Casein Soya Bean digest broth was added to the 2.0 mL starting solution of test material and thus the first dilution was formed. Subsequently, 2.0 mL of this solution was diluted with 2.0 mL Casein Soya Bean digest broth to give the second dilution and so on until 12 dilutions were obtained.

After the incubation at 37 °C for 24 h, the first tube without turbidity was determined as the minimal inhibition concentration expressed in µg/mL. The concentrations of the prepared solutions were from 500 µg/mL to 0.244 µg/mL.

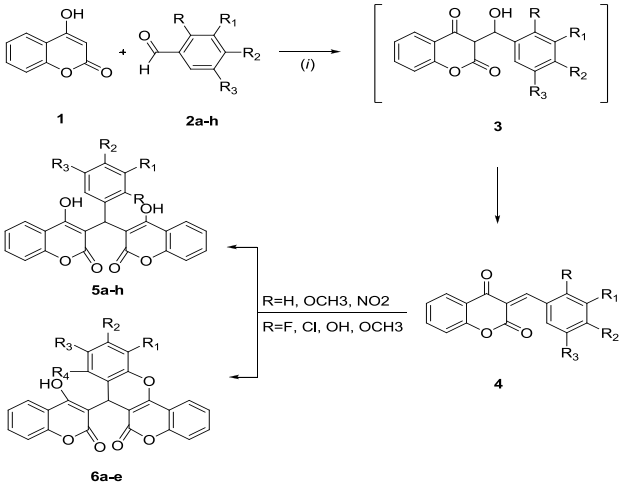
The aim of this method was to determine the lowest concentration of a tested compound resulting in no bacterial growth. This concentration was considered as Minimal Inhibition Concentration (MIC). The Bactericidal Concentration (BC) of each compound, and each bacterial strain, was considered as the lowest concentration of a drug required to kill a particular bacterium.

RESULTS AND DISCUSSION

Chemistry

The benzylidene-bis-(4-hydroxycoumarin) derivatives **5a-h** and fused benzopyranocoumarin derivatives **6a-e** were prepared by a sequence of reactions displayed in the Scheme 1. In the first step of the synthesis the aldol condensation of 4-hydroxycoumarin (**1**) with an appropriately substituted aldehyde (**2**) linker followed by the dehydration of aldol product (**3**) gave a chromone (**4**). Subsequent in situ reaction of the chromon with 4-hydroxycoumarin already excessively present in the reaction mixture gave biscoumarin derivatives **5a-h** bearing an aryl substituent in the central methylene linker. Also, the chromone derivatives containing an ortho substituted phenyl moiety (R = Cl, F, OH, OCH_3) gave under spontaneous cyclization the fused benzopyranocoumarin derivatives **6a-e**. The compound **6i,** described previously [13], was also synthesized for its antimicrobial activity evaluation.

The synthesis and the chemical structure of the synthesized compounds are presented in Scheme 1.



	R	R_1	R_2	R_3
5a	H	Br	H	H
5b	H	H	F	H
5c	H	H	CF_3	H
5d	H	H	NO_2	H
5e	H	H	SCH_3	H
5f	H	OH	OH	H
5g	OCH_3	H	H	OCH_3
5h	H	OCH_3	OH	NO_2

	R_1	R_2	R_3	R_4
6a	H	H	H	H
6b	H	H	Br	H
6c	OCH_3	H	H	H
6d	H	H	NO_2	H
6e	H	OCH_3	H	OCH_3

Scheme 1: The synthesis of biscoumarin derivatives with variously substituted aryl central linkers (5a-h) and fused benzopyranocoumarin derivatives (6a-e). Reagents and conditions: (*i*) EtOH, reflux, rt, 24 h.

Analytical data of synthesized compounds (CHN analysis, IR, $^1H/^{13}C$ NMR, Mass spectrometry, X-ray crystallography) were presented in our previous paper [8].

Bactericidal and bacteriostatic activity

The antimicrobial activities, given by the inhibition zone of the 14 coumarin derivatives are shown in Table 1.

Table 1: Antimicrobial activity of tested biscoumarin derivatives by diffusion method

	Inhibition zones*			
Compound number	***Bacillus subtilis***	***Staphylococcus aureus***	***Candida albicans***	***Saccharomyces cerevisiae***
5a	13	11	9	9
5b	14	18	11	10
5c	19	22	11	9.5
5d	-	-	10	9
5e	14	11	9.5	9
5f	-	-	9	8
5g	17	10	9	9

5h	-	-	11	8
5i**	11	20	11	8
6a	-	17	10	10
6b	19	25	9.5	9.5
6c	11	12	10	10
6d	-	-	9	8
6e	11	16	10	8
Chloramphenicol	25	23	-	-
Ketokonazole	-	-	15	14

***Zone diameter of growth inhibition (mm) after 24 hours, at the concentration 1000 μg/mL in DMSO**

**** 3,3'-(4-Bromobenzylidene)-bis-(4-hydroxycoumarin) [13]**

The compounds which showed the best antimicrobial activity using the diffusion method were further tested by the dilution method. By dilution method we defined Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC). The Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC) were determined by the dilution method (Table 2).

Table 2: Antimicrobial activity of the biscoumarin compounds tested by dilution method

Compound number	*Bacillus subtilis*		*Staphylococcus aureus*	
	MBC (μg/mL)	**MIC (μg/mL)**	**MBC (μg/mL)**	**MIC (μg/mL)**
5a	62.5	31.25	62.5	31.25
5b	31.25	15.6	15.6	7.8
5c	15.6	7.8	15.6	3.9
5e	62.5	15.6	62.5	31.25
5g	31.25	15.6	62.5	31.2
5i	31.25	7.8	15.6	7.8
6a	62.5	31.25	31.25	15.6
6b	7.8	1.95	7.8	3.9
6c	62.5	31.25	62.5	31.25
6e	62.5	31.25	31.25	15.6
Chloramphenicol	7.8	3.9	7.8	3.9

As a result of our previous work, the antimicrobial activity of some benzylidene-bis-(4-hydroxycoumarin) derivatives against certain Gram-positive bacteria was proved [13]. The result of the present study showed a broad range of antimicrobial activity. All of the coumarins exhibited activity against Gram-positive bacteria and fungi, and few of them were selectively active against one or two strains, whilst they displayed no activity against Gram-negative bacteria. Because of the permeability barrier provided by the outer membrane, Gram-negative bacteria are resistant to all of the tested compounds.

The data found in the literature matching our research claims that the compounds with halogen substituent are the most efficient against Gram-positive bacteria, particularly against *S. aureus* [23, 24]. Fluoro and trifluoromethylated coumarin compounds (**5b**, **5c**) are of a particular interest since the strong electron-withdrawing effect of fluorine and trifluoromethyl groups contributes to molecule's biological properties. The isosteric substitution of hydrogen by fluorine in coumarin compounds increases the lipophilicity and thus enhances the rate of cell penetration, which is a very important feature in drug efficiency.

Compound **5c** with trifluoromethyl substituent at the C-4 position on the linker of phenyl ring showed the best activity with MIC = 3.9 μg/mL against *S. aureus,* and with MIC = 7.8 μg/mL against *Bacillus subtilis.* Compound **5b** with fluorine at the C-4 position showed very strong activity with MIC = 7.8 μg/mL against *S. aureus*, and strong activity with MIC = 15.6 μg/mL against *B. subtilis.*

Compounds **5a, 5b, 5c, 5e, 5i** and **6b** with the range of MIC **=** 3.9 - 62.5 μg/mL showed antimicrobial activity against *S. aureus* at a relatively low concentration.

Bromine at the C-4 (**5i**) and C-5 (**6b**) positions of phenyl ring significantly increases activity against *S. aureus*, while the bromine at the position C-3 (**6a**) of phenyl ring reduces the activity. Comparing their MICs with chloramphenicol, compound **5i** showed similar level of activity and **6b** showed higher level of activity when compared with standard drug. Of all the benzopyranocoumarin derivatives, **6b** showed the best activity with MIC = 1.9 μg/mL against *S. aureus.*

Five evaluated compounds (**5b, 5c, 5e, 5g** and **6b**) with MICs in the range MIC = 7.8 - 31.25 μg/mL showed relatively strong inhibitory activity against *B. subtilis.* The best activity was shown again by compounds with halogen substitents, **5c** and **6b**, with MIC = 7.8 μg/mL.

Moderate activity against *B. subtilis* was shown by the other compounds with methoxy (**5g**), fluorine (**5b**) and thiomethyl group (**5e**) on the linker of the phenyl ring, with MIC = 15.6 μg/mL, MIC = 15.6 μg/mL and MIC = 31.25 μg/mL, respectively.

Methoxy derivative (**6h**) possesses moderate bioactivities against both Gram-positive bacteria. The hydroxyl derivative **5f** showed no expected activity. Compound with hydroxy and nitro substituents (**5d** and **6d**) proved to be completely inactive.

All compounds were also evaluated for their antifungal activities against *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763. No specific antifungal effects were noted for any of the evaluated compounds**.**

CONCLUSIONS

A series of the benzylidene-bis-(4-hydroxycoumarin) derivatives (**5a-e**) and fused benzopyranocoumarin derivatives (**6a-h**) were synthesized and evaluated for their antimicrobial activities against five strains of Gram-positive and Gram-negative bacteria, and two strains of fungi.

Synthesized coumarin derivatives generally possess strong or moderate activity against Gram-positive bacteria, *S. aureus* and *B. subtillis*, and show no activity to Gram-negative bacteria. The MIC results indicated that the antimicrobial activity of the investigated compounds was influenced by the physicochemical properties of the type and position of substituents on the linker of phenyl ring. Among the tested compounds, two structurally similar groups of derivatives, **5a-e** and **6a-h**, compounds **5c** and **6b** demonstrated enhanced activity against Gram-positive bacteria *S. aureus* and *B. subtilis*, with MICs in the range of 1.9 - 7.8 μg/mL, respectively, due to the influence of the bromine and trifluoromethyl substituents.

Results of this research could be considered as a useful base for the future development of potent antimicrobial agents against Gram-positive bacteria, especially against *Staphyloccocus aureus*.

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