

Antibacterial Activity of Simple Coumarins: Structural Requirements for Biological Activity*

Oliver Kayser and Herbert Kolodziej

Institut für Pharmazie II, Pharmazeutische Biologie, Freie Universität Berlin,
Königin-Luise-Str. 2+4, D-14195 Berlin, Germany

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Coumarins, Antibacterial Activity, Structure-Activity Relationships

The antibacterial activity of a series of simple coumarins was evaluated against 8 microorganisms, including three Gram-positive (*Staphylococcus aureus*, beta-hemolytic *Streptococcus pneumoniae* and *Streptococcus pneumoniae*) and five Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Haemophilus influenzae*), using the microdilution broth method. The coumarins tested showed broad diversity regarding growth inhibitory activity with minimum inhibitory concentrations ranging from 0.9 to >12.4 μM . This study, presenting the first systematic analysis of structure-activity relationships among this group of coumarins, revealed some interesting structural requirements. While coumarins with a methoxy function at C-7 and, if present, an OH group at either the C-6 or C-8 position are invariably effective against the spectrum of tested standard bacteria (Gram-negative microorganisms including the Gram-positive bacterium *Staphylococcus aureus*), the presence of an aromatic dimethoxy arrangement is apparently favourable against those microorganisms which require special growth factors (beta-hemolytic *Streptococcus pneumoniae* and *Haemophilus influenzae*). A combination of these structural features, two methoxy functions and at least one additional phenolic group as reflected by the highly oxygenated coumarins, identify promising candidates with antibacterial broad-spectrum activity.

Introduction

Coumarins constitute a major category of secondary plant products that are widely distributed in the plant kingdom (Murray *et al.*, 1982). They are characterized by a variety of oxygenation patterns on the benzopyrone nucleus (Murray, 1997) and display a remarkable array of biochemical and pharmacological actions (Bourinbaier *et al.*, 1993; Egan *et al.*, 1990; Galabov *et al.*, 1996; Maucher and von Angerer, 1994; Lee *et al.*, 1994; Paya *et al.*, 1994). We have recently reported on some biological activities of a series of simple coumarins including antimutagenic properties with respect to the mutagenic heterocyclic aromatic amine, 2-amino-3-methylimidazo[4,5-f]quinoline (Edenharder *et al.*, 1995), and cytotoxic effects to human tumour cell lines (Kolodziej *et al.*, 1997). Also, our work on medicinally used *Pelargonium* species

clearly demonstrated potential antibacterial activities of some highly oxygenated simple coumarins present in these extracts (Kayser and Kolodziej, 1997). In continuation of our study on biological activities of simple coumarins, we evaluated the antibacterial potency of a series of oxygenated representatives against a panel of bacteria. It should be emphasized that detailed studies on this particular biological activity of simple coumarins are hitherto limited (Bersch and Döpp, 1955; Jurd *et al.*, 1971a and 1971b; Fischer *et al.*, 1976), thus prompting the present more extensive investigation of coumarin derivatives. Attention is given to structure-activity relationships with emphasis on the aromatic oxygenation patterns among this class of secondary metabolites.

Materials and Methods

Test compounds

The compounds 6-hydroxy-7-methoxycoumarin, 7-hydroxy-5,6-dimethoxycoumarin and 6,8-dihydroxy-5,7-dimethoxycoumarin were isolated from *Pelargonium sidoides* DC. (Geraniaceae) accord-

Reprint requests to Prof. Dr. H. Kolodziej.

Fax: +49-30-838-3729

* Dedicated to Prof. Dr. H.-J. Fehrenbach, Robert Koch-Institut, Berlin, Germany on the occasion of his retirement.

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ing to Kayser and Kolodziej (1995), while the remaining coumarins were available as reference samples in the research group of H. K. The identity of the compounds was proven by spectroscopic techniques.

Microorganisms

The above mentioned compounds were tested against a panel of microorganisms including, the Gram-negative bacteria, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* V 6089, *Proteus mirabilis* ATCC 14153, *Pseudomonas aeruginosa* ATCC 27853, *Haemophilus influenzae* ATCC 33379, and the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923, beta-hemolytic *Streptococcus* 1451, DSMZ, Braunschweig, and *Streptococcus pneumoniae* (strain 78), Pasteur Institute, Paris.

Evaluation of antimicrobial activity

For the microdilution assay, standard Mueller Hinton medium was used for most of the microorganisms, with the exception of *H. influenzae*, *S. pneumoniae* and beta-hemolytic *Streptococcus* 1451 (Mueller Hinton containing 50 ml lysed citrate blood/l) (Gersten, 1993). Ethanol (70%) that did not affect the growth of any of the microorganisms in the final sample concentrations, was used as solvent for the test compounds. After 24 h, respectively, 48 h, of incubation at 37 °C and 5% CO₂ (Table II), the antimicrobial effects of samples were quantitated. Minimum inhibitory concentrations (MICs) of the samples were determined for susceptible microorganisms by a standard twofold microdilution broth technique, using penicillin G as reference agent (Vanden Bergh *et al.*, 1991). Inocula were prepared in the same medium by diluting microbial suspensions in Mueller Hinton broth (*vide infra*). Inhibition of growth was judged by comparison with a control culture prepared without any test sample. MIC values were determined as the lowest concentration of the samples completely inhibiting macroscopic growth of microorganisms.

Bactericidal kinetic assay

The bactericidal kinetic assay for *E. coli* was performed in Mueller Hinton medium containing

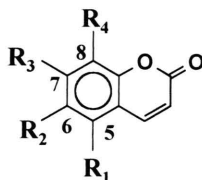
7-hydroxy-5,6-dimethoxycoumarin and 6,8-dihydroxy-5,7-dimethoxy-coumarin, respectively, in concentrations of 0.9–2.2 µM. The initial inoculum was approximately 2 x 10⁵ colony forming units/ml. Samples were taken after 0, 2, 4, 8, and 24 h of incubation, followed by serial 10-fold dilutions and plating on Mueller Hinton agar. After 24 h of incubation at 37 °C, the growth of the bacterium was determined by turbidimetric analysis.

Results and Discussion

Although coumarins are known to possess antibacterial activity, the effect of oxygenation patterns on the potency has not yet been investigated systematically. In the present study, a series of 14 simple coumarins (for structures see Table I) was tested against a panel of microorganisms, including three Gram-positive (*Staphylococcus aureus*, beta-hemolytic *Streptococcus*, and *Streptococcus pneumoniae*) and five Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Haemophilus influenzae*). Bacterial susceptibility to coumarins was evaluated by determining the minimal growth inhibitory concentration using the microdilution broth method. In Table II, the minimum inhibitory concentrations (MIC) of the test compounds against the listed bacteria are displayed. All samples exhibited antibacterial activity with MICs ranging from 0.9 to >12.4 µM. The results indicated that each compound showed more or less pronounced antibacterial potencies, affecting both Gram-positive and Gram-negative pathogens. However, the latter group of bacteria appeared to be more sensitive for most of the coumarins tested, irrespective of the oxygenation pattern. Among the active or potentially active compounds, the highly oxygenated 7-hydroxy-5,6-dimethoxycoumarin (umckalin) and 6,8-dihydroxy-5,7-dimethoxycoumarin represented the most potent candidates with MICs of 0.9–2.1 µM. This first observation suggested that the antibacterial activity of coumarins may be correlated to the number of oxygen substituents, with highly functionalized representatives as the most potent inhibitory agents. However, the trioxxygenated coumarin, 5,6,7-trimethoxycoumarin, was found to be significantly less effective than the highly oxygenated coumarins 7-hydroxy-5,6-dimethoxy-

Table I. Chemical structures of the coumarins used in this study.

Compound	Trivial name	R ₁	R ₂	R ₃	R ₄
Coumarin		H	H	H	H
7-Hydroxycoumarin	umbelliferone	H	H	OH	H
7-Methoxycoumarin	herniarin	H	H	OCH ₃	H
8-Hydroxycoumarin		H	H	H	OH
6,7-Dihydroxycoumarin	esculetin	H	OH	OH	H
6-Hydroxy-7-methoxycoumarin	scopoletin	H	OH	OCH ₃	H
5,7-Dihydroxycoumarin		OH	H	OH	H
6,7-Dimethoxycoumarin		H	OCH ₃	OCH ₃	H
7-Hydroxy-5-methoxycoumarin		OCH ₃	H	OH	H
7,8-Dihydroxycoumarin		H	H	OH	OH
8-Hydroxy-6,7-dimethoxycoumarin	tomentin	H	OCH ₃	OCH ₃	OH
7-Hydroxy-5,6-dimethoxycoumarin	umckalin	OCH ₃	OCH ₃	OH	H
5,6,7-Trimethoxycoumarin		OCH ₃	OCH ₃	OCH ₃	H
6,8-Dihydroxy-5,7-dimethoxycoumarin		OCH ₃	OH	OCH ₃	OH

Table II. Antibacterial activity of simple coumarins using the microdilution method (MIC values of duplicates in μM ; $\mu\text{g/ml}$ in parentheses).

Compound	<i>E. coli</i> 24 h	<i>K. pneumoniae</i> 24 h	<i>St. aureus</i> 24 h	<i>P. aeruginosae</i> 24 h	<i>Prot. mirabilis</i> 24 h	β -hem. Strept. 48 h	<i>St. pneumoniae</i> 48 h	<i>H. influenzae</i> 48 h
Coumarin	2.7 (400)	2.7 (400)	3.4 (500)	3.4 (500)	2.7 (400)	1.7 (250)	1.7 (250)	1.7 (250)
<i>Monosubstituted Derivatives</i>								
7-Hydroxycoumarin (umbelliferon)	6.2 (1000)	>12.4 (>2000)	>12.4 (>2000)	3.1 (500)	>12.4 (>2000)	>12.4 (>2000)	>12.4 (>2000)	>12.4 (>2000)
7-Methoxycoumarin (herniarin)	1.4 (250)	1.9 (330)	1.9 (330)	1.9 (330)	1.9 (330)	5.7 (1000)	5.7 (1000)	5.7 (1000)
8-Hydroxycoumarin	2.3 (375)	2.3 (375)	4.6 (750)	2.3 (375)	4.6 (750)	4.6 (750)	6.2 (1000)	4.6 (750)
<i>Disubstituted Derivatives</i>								
6,7-Dihydroxycoumarin (esculetin)	2.3 (400)	2.8 (500)	2.8 (500)	2.3 (400)	2.8 (500)	11.2 (2000)	11.2 (2000)	11.2 (2000)
6-Hydroxy-7-methoxycoumarin (scopoletin)	2.6 (500)	2.6 (500)	2.6 (500)	2.1 (400)	2.1 (400)	5.2 (1000)	5.2 (1000)	5.2 (1000)
5,7-Dihydroxycoumarin	1.4 (250)	2.8 (500)	2.8 (500)	2.8 (500)	2.8 (500)	5.6 (1000)	2.8 (500)	5.6 (1000)
6,7-Dimethoxycoumarin	4.8 (1000)	4.8 (1000)	2.4 (500)	4.8 (1000)	2.4 (500)	2.4 (500)	2.4 (500)	2.4 (500)
7-Hydroxy-5-methoxycoumarin	1.3 (250)	5.2 (1000)	5.2 (1000)	5.2 (1000)	5.2 (1000)	5.2 (1000)	5.2 (1000)	5.2 (1000)
7,8-Dihydroxycoumarin	>5.6 (>1000)	>5.6 (>1000)	>5.6 (>1000)	>5.6 (>1000)	>5.6 (>1000)	>5.6 (>1000)	>5.6 (>1000)	>5.6 (>1000)
<i>Trisubstituted Derivatives</i>								
8-Hydroxy-6,7-dimethoxycoumarin (tomentin)	1.0 (220)	2.2 (500)	2.2 (500)	2.2 (500)	4.5 (1000)	2.2 (500)	2.2 (500)	2.2 (500)
7-Hydroxy-5,6-dimethoxycoumarin (umckalin)	0.9 (200)	0.9 (200)	0.9 (200)	0.9 (200)	0.9 (200)	2.2 (500)	2.2 (500)	2.2 (500)
5,6,7-Trimethoxycoumarin	4.2 (1000)	4.2 (1000)	4.2 (1000)	4.2 (1000)	4.2 (1000)	4.2 (1000)	2.1 (500)	2.1 (500)
<i>Tetrasubstituted Derivatives</i>								
6,8-Dihydroxy-5,7-dimethoxycoumarin	0.9 (220)	1.1 (250)	0.9 (220)	0.9 (220)	0.9 (220)	2.1 (500)	2.1 (500)	2.1 (500)
Penicillin G	0.4 (125)	0.2 (62)	0.2 (62)	0.4 (125)	0.2 (62)	0.1 (32)	0.05 (16)	0.05 (16)

coumarin and 6,8-dihydroxy-5,7-dimethoxycoumarin (Table II). This finding implied that the degree of substitution is one important factor, while polarity appears to be another one.

A closer structure-activity relationship was obtained from the careful examination of the series of coumarins tested. The parent coumarin itself was found to exhibit fairly high antibacterial activities which may be indicative of passive diffusion, facilitated by both its lipophilic character and planar molecular structure. It has been suggested that simplicity of aromatic substitution and avoidance of bulky side chains aid in penetration through bacterial cell walls (Rauckman *et al.*, 1989). Therefore, it is likely that the mode of action is attributable, at least in part, to interactions with the cell membrane. Note that coumarin proved to be slightly less active on Gram-negative bacteria, due possibly to its particular physical properties.

Within the group of mono-oxygenated coumarins, the antibacterial activity varied with the position and nature of the functional group. For example, the presence of an hydroxyl group at C-7 (umbelliferone) significantly reduced the antibacterial activity against all of the tested microorganisms when compared with the parent coumarin. On the other hand, substitution of the 7-OH group by a methoxy function (herniarin) or its presence at C-8 resulted in similarly active compounds. This finding suggested that the antibacterial activity of oxygenated coumarins apparently strongly depended on the position of polar (OH) and less polar (OMe) functions on the aromatic nucleus of the coumarin core structure.

The picture which emerged from examining the series of disubstituted analogs not only supports this conjecture, but also clearly demonstrated that introduction of an additional functional group did not necessarily result in a dramatic enhancement in potency, as reflected by a similar diverse spectrum of antibacterial activities with MICs ranging from weak (11.2 μM) to fairly high potencies (1.3 μM) (Table II). Thus, while esculetin, characterized by the presence of a 6,7-*ortho* dihydroxyl arrangement, showed considerably enhanced antibacterial activity against all microorganisms that do not require special growth factors (*E. coli*, *K. pneumoniae*, *St. aureus*, *P. aeruginosae* and *Prot. mirabilis*) as compared with umbelliferone, the dihydroxylated analog, 7,8-dihydroxycoumarin, was

found to be negligible active. Although 7-hydroxy-6-methoxycoumarin was not available, the results obtained with 6-hydroxy-7-methoxycoumarin and 6,7-dimethoxycoumarin clearly indicate that a methoxy function at C-6 in the coumarin skeleton is less favourably against cultivated standard microorganisms, but apparently contributes to moderately increased inhibitory activity against the spectrum of the growth factor demanding bacteria (*vide supra*).

It should be noted that 5,7-dihydroxycoumarin proved to be similarly active as 6,7-dihydroxycoumarin. Here, replacement of the hydroxy group at C-5 by a methoxy function greatly decreased the antibacterial activity of the resulting compound, 7-hydroxy-5-methoxycoumarin. This observation, however, does not imply the structural requirement of a 5-OH group for reasonable antibacterial activities of simple coumarins as evident from the MIC data of respective coumarin derivatives (Table II).

Apart from 5,6,7-trimethoxycoumarin, which proved to be very moderately active, all the tested tri- and tetra-oxygenated coumarins exhibited pronounced antibacterial activity against the panel of bacteria with MICs ranging from 0.9–2.2 μM . The results indicated that a high degree of aromatic functionalization is an important determinant for antibacterial broad-spectrum activity. This poses the question for necessary structural requirements and tolerated oxygenation patterns regarding appreciable antibacterial activities of this group of coumarins.

Despite obvious differences in inhibitory effects on the growth of bacteria there is a remarkably strong consensus in the optimal pattern of oxygenation that gives rise to broad-spectrum (all test microorganisms) or selective (standard bacteria, 24 h; problem bacteria in cultivation, 48 h) but pronounced antibacterial activity (Table II). Besides the lipophilic character and a planar structure, as concluded from marked antibacterial activity of coumarin itself, several structural requirements emerged from this study. Thus, coumarins with a methoxy function at C-7 and, if present, an OH group at either the C-6 or C-8 position are invariably effective against the spectrum of tested standard bacteria (*vide supra*). However, when the hydroxy group at C-6 is replaced by a methoxy function (6,7-dimethoxycoumarin) the antibacte-

rial activity showed a tendency to increase against those microorganisms which require special growth factors. Also, the presence of a methoxy function in position 5 may enhance inhibitory activity provided the remaining pattern of hydroxylation is favourable (7-hydroxy-5,6-dimethoxycoumarin, 6,8-dihydroxy-5,7-dimethoxycoumarin and 5,6,7-trimethoxycoumarin vs. 7-hydroxy-5-methoxycoumarin). This finding suggests that the presence of two methoxy groups is a major contributing factor towards antibacterial activity against this group of bacteria. The above observation is in accordance with recent reports in that increased lipophilicity of compounds may be associated with a greater ease of penetration into Gram-positive bacteria, but other factors such as shapes and bulkiness have to be considered as well (Rauckman *et al.*, 1989).

The relatively high potency of the tri- and tetra-oxygenated members against the total spectrum of test bacteria may be rationalized by a combination of structural characteristics discussed above and, hence, is displayed only by derivatives with (i) two

methoxy groups such as the 6,7- or 5,7-dimethoxy arrangement and (ii) at least one additional phenolic group located either at C-6, C-7 or C-8. It should be noted that the pathogens, which require special growth factors, are generally less susceptible when compared to standard microorganisms (Table I). Although the difference in susceptibility can not simply be correlated to various cell membrane and cell wall characteristics, Gram-positive and distinctively encapsulated microorganisms proved to be conspicuously less susceptible than the remaining pathogens. Here, the more lipophilic derivatives show slightly greater inhibitory activity. With MICs ranging from 0.9–2.2 μM , the potentially active highly oxygenated simple coumarins examined were generally only moderately active, when compared with the MICs (0.05–0.4 μM) of the medically used antibiotic, penicillin G.

The bactericidal kinetic assay, using *E. coli* and the potentially active candidates, 7-hydroxy-5,6-dimethoxycoumarin and 6,8-dihydroxy-5,7-dimethoxycoumarin, demonstrated the antibacterial activity of the coumarins tested to be bacteriostatic.

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