Antibacterial Activity of Coumarins

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The antibacterial activity of coumarin *per se* and other 45 coumarin derivatives was tested against strains of *Bacillus cereus* MIP 96016, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923. The inhibitory effects of coumarins were affected by their substitution patterns. Osthenol (**44**) showed the most effective antibacterial activity against Gram-positive bacteria with MIC values ranging between 125 and 62.5 μg/ml. These results suggested that the prenyl chain of **44** at position 8 and the presence of OH at position 7 of the benzenic ring are required for the antibacterial activity against these strains.

Key words: Coumarins, Osthenol, Antibacterial Activity, Structure-activity Relationships (SAR)

Introduction

Traditional antibacterial therapy is going through a crisis due to the rapidly increasing development of resistance to existing agents (Ojala *et al.*, 2000). However, the plant kingdom constitutes a source of new chemicals, which may be important for their potential use in medicine (Alice *et al.*, 1991). Coumarins are plant secondary metabolites compounds whose biological activity varies according to their substitution patterns. Substituted 4-(1-piperazinyl) coumarins exhibit antiplatelet aggregation activity (Di Braccio *et al.*, 2004), 8-substituted 7-geranyloxycoumarin derivatives (specially the 8-methoxy and 8-acetoxy derivative) have anti-inflammatory activity (Curini *et al.*, 2004) and 8-substituted 7-methoxycoumarins show potent anti-tumor promoting effects (Ito *et al.*, 1999).

The present study reports the evaluation of the antibacterial potency of a series of simple, prenylated, furano- and pyranocoumarins with emphasis on their structure-activity relationships (SAR).

Materials and Methods

Test compounds

During the present work forty-four coumarins provided by Professor Franco Delle Monache (Instituto di Chimica, Univesità Cattolica Del Sacro Cuore, Rome, Italy) and three of a commercial source have been assayed. In addition, two coumarins obtained by simple modification were also tested. The identity of natural and semi-synthetic compounds was proved by comparison of their spectroscopic data (^{1}H and ^{13}C NMR) with literature references (Table I).

Antimicrobial assay

The antibacterial activity of coumarins was investigated by employing a microdilution method. The assay was carried out with four bacterial species, including the Gram-negative bacteria *Escherichia coli* ATCC 25922 (American Type Culture Collection) and *Pseudomonas aeruginosa* ATCC 27853 and the Gram-positive bacteria *Bacillus cereus* MIP 96016 (Departamento de Microbiologia e Parasitologia, UFSC) and *Staphylococcus aureus* ATCC 25923. Mueller-Hinton agar and broth (Difco Laboratories, Detroit, USA) were used for bacterial growth. The inoculum was an overnight culture of each bacterial species in Mueller-Hinton broth diluted in the same media to a final concentration of approx. 10^{8} CFU/ml. The coumarins were dissolved in dimethyl sulfoxide (DMSO) (10% of the final volume) and diluted with Mueller-Hinton broth (Difco Laboratories) to a concentration of 2 mg/ml. Further 1:2 serial dilutions were performed by addition of Mueller-Hinton

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broth to reach a final concentration range 2 to 0.0156 mg/ml. 100 μl of each dilution were distributed in 96-well plates, as well as a sterility control (growth control contained Mueller-Hinton broth plus DMSO, without antimicrobial substance). Each test and growth control well was inoculated with 5 μl of a bacterial suspension (10^8 CFU/ml or 10^5 CFU/well). All experiments were performed in duplicate and the microdilution trays were incubated at 36 °C for 18 h. Bacterial growth was detected first by optical density determination (ELISA reader, CLX800-BioTek Instruments) and later by addition of 20 μl of an alcoholic solution (0.5 mg/ml) of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) (Sigma). The trays were again incubated at 36 °C for 30 min, and in those wells, where bacterial growth occurred, INT changed from yellow to purple. Any remaining yellow color indicated absence of growth. Before the addition of INT, a subculture was made from each well without apparent growth to determine MBC. MIC and MBC values were defined as the lowest concentration of each coumarin, which completely inhibited growth or yielded no viable microorganisms, respectively. The results were expressed in micrograms per millilitre. Penicillin and tetracycline were used to assess the MIC values of the reference strains (Smânia *et al.,* 1995).

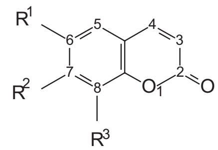
Results and Discussion

In the present study, a series of 45 coumarin derivatives and the parent coumarin (Table I) were tested for their antibacterial activity against 4 strains of bacteria: two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). Bacterial susceptibility to coumarins was evaluated by determining the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and those that were active exhibited MIC values ranging from 62.5 to 2000 μg/ml (Table II). The results indicated that each compound showed more or less pronounced antibacterial potencies, affecting both Gram-positive and Gram-negative microorganisms. Among the active compounds, osthenol (**44**) showed the most potent activity with MIC of 62.5 μg/ml.

A closer structure-activity relationship (SAR) was obtained from the careful examination of the series of coumarins tested. Coumarin *per se* (**1**) exhibited broad antibacterial activity against all strains tested, but was slightly less active against *B. cereus.* Kayser and Kolodziej (1999) suggested that fairly high antibacterial activity of coumarin *per se* is due to both its lipophilic character and planar molecular structure, which contribute in penetration through bacterial cell membrane or cell walls. Within the group of mono-oxygenated coumarins, the addition of a methyl or *O*-methyl group at position C6 or C7 (compounds **2**, **5**, **13**, **14**) to the aromatic nucleus of the coumarin *per se* core structure maintained the antibacterial activity against Gram-negative bacteria, but diminished against Gram-positive strains when compared to the parent coumarin. On the other hand, substitution of the less polar functions (OMe, Me) at C6 by an OH function (compound **3**) reduced the antibacterial activity against all of the tested microorganisms (Jurd *et al.*, 1971). Kayser and Kolodziej (1999) reported that the addition of an OH group at C7 of the coumarin *per se* significantly reduced the antibacterial activity against all the tested bacteria. These findings suggested that the antibacterial activity of oxygenated coumarins apparently depended on the position of polar (OH) and less polar (OMe, Me) functions at the aromatic nucleus of the coumarin structure. On the other hand, the addition of varied substitution patterns as 6- and 7-*O*-acetyl groups (compounds **4** and **15**), halogen groups at positions C6 and C7 (compounds **6**, **7** and **16**), a 6-amino group (compound **8**), a 6-carboxyl function (compound **9**), a 6-cyano group (compound **10**), as well as a 6- and 7-nitro group (compounds **12** and **17**) and the addition of a 6-aldehyde group (compound **11**) significantly reduced the antibacterial activity against all of the tested microorganisms when compared with the parent coumarin with MIC values ranging from 1000 up to 2000 μg/ml.

Within the group of disubstituted coumarins, the addition of two *ortho* OH functions at C6 and C7, esculetin (compound **19**), displayed fairly high antibacterial activity, similar to coumarin *per se* in relation to Gram-positive bacteria (MIC = 500 μg/ml), which is probably due to the facilitated interaction with the peptidoglycan found in the cell wall. Tegos *et al*. (2002) reported that scopoletin displays a better antibacterial activity against Gram-positive bacteria rather than Gram-negative bacteria, perhaps because of Gram-negative bacteria efficient efflux pumps. Scopoletin (OMe at

Table I. Chemical structures of coumarins studied in this work.



I. Simple coumarins	R^1	R^2	R^3	Source
Monosubstituted				
Coumarin (**1**)	H	H	H	Gottlieb *et al.*, 1979
6-Methylcoumarin (**2**)	CH_3	H	H	Gottlieb *et al.*, 1979
6-Hydroxycoumarin (**3**)	OH	H	H	Gottlieb *et al.*, 1979
6-*O*-Acetylcoumarin (**4**)	$O-C_2H_3O$	H	H	Gottlieb *et al.*, 1979
6-Methoxycoumarin (**5**)	$O-CH_3$	H	H	Gottlieb *et al.*, 1979
6-Chlorocoumarin (**6**)	Cl	H	H	Gottlieb *et al.*, 1979
6-Iodocoumarin (**7**)	I	H	H	Gottlieb *et al.*, 1979
6-Aminocoumarin (**8**)	NH_2	H	H	Gottlieb *et al.*, 1979
6-Carboxycoumarin (**9**)	COOH	H	H	Gottlieb *et al.*, 1979
6-Cyanocoumarin (**10**)	CN	H	H	Gottlieb *et al.*, 1979
6-Aldehydocoumarin (**11**)	CHO	H	H	Gottlieb *et al.*, 1979
6-Nitrocoumarin (**12**)	NO_2	H	H	Gottlieb *et al.*, 1979
7-Methoxycoumarin (**13**) (Herniarin)	H	$O-CH_3$	H	Sarsynthese*
7-Methylcoumarin (**14**)	H	CH_3	H	Gottlieb *et al.*, 1979
7-*O*-Acetylcoumarin (**15**)	H	$O-C_2H_3O$	H	Gottlieb *et al.*, 1979
7-Chlorocoumarin (**16**)	H	Cl	H	Gottlieb *et al.*, 1979
7-Nitrocoumarin (**17**)	H	NO_2	H	Gottlieb *et al.*, 1979
Bisubstituted				
Scopoletin (**18**)	$O-CH_3$	OH	H	Torres *et al.*, 1979
Esculetin (**19**)	OH	OH	H	Fluka**
Di-*O*-Methyl esculetin (**20**)	$O-CH_3$	$O-CH_3$	H	Esculetin methylation
Di-*O*-Methyl daphnetin (**21**)	H	$O-CH_3$	$O-CH_3$	Daphnetin methylation
Trisubstituted				
Fraxetin (**22**)	$O-CH_3$	OH	OH	Sarsynthese

* Sarsynthese Co., Genay, France.
** Fluka, Buchs, Switzerland.

C6 and OH at C7, **18**) also showed antibacterial activity with MIC = 1000 μg/ml for all tested bacteria. On the other hand, the addition of two OMe groups either at C6 and C7 (compound **20**) or either at C7 and C8 (compound **21**) decreases the antibacterial activity if compared to coumarin *per se*. Kayser and Kolodziej (1999) demonstrated that, in general, the introduction of an additional radical to monosubstituted coumarins does not necessarily result in a dramatic enhancement in potency. Based on these results, the addition of one or two OMe groups at the aromatic nucleus of disubstituted coumarins at positions C6, C7 or/and C8 decreased the antibacterial activity if compared to coumarin *per se*. These results suggest that an OMe group at C6 reduced the antibacterial activity of coumarin against the tested bacteria as well as two OMe groups at C7/C8.

Fraxetin (6-methoxy-7,8-dihydroxycoumarin, **22**) showed weak antibacterial activity to all tested bacteria, possibly because it lacks an additional OMe group. Kayser and Kolodziej (1999) studied trisubstituted and tetrasubstituted derivatives and the most active analogues were those with two OMe functions at positions C5/C6, C6/C7 and C5/C7 (MIC values ranging from 200 to 500 μg/ml for standard bacteria).

Within the group of furanocoumarins, psoralene (**29**) presented antibacterial activity (MIC = 1000 μg/ml) against *E. coli* but was ineffective against *S. aureus*. Nonetheless, (–)-heraclenol (**23**) displayed similar antibacterial activity against

Table I. (cont.)

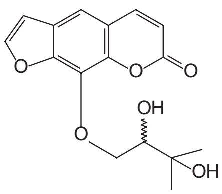
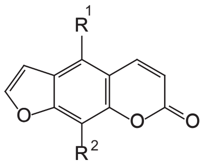
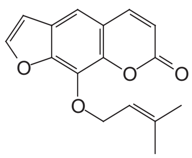
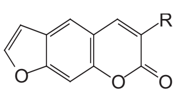
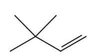
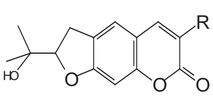
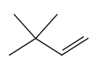
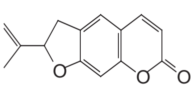
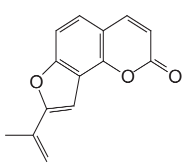
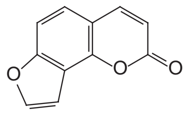
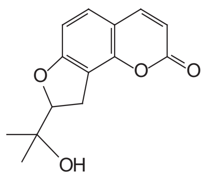
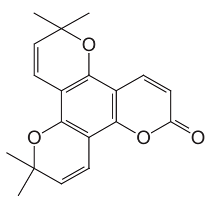
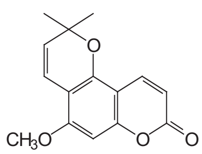
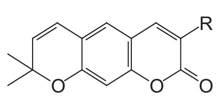
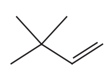
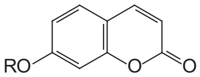
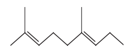
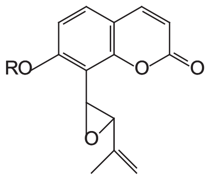
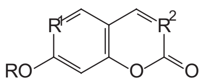
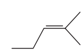
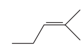
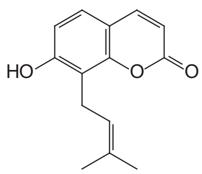
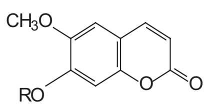
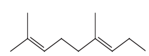
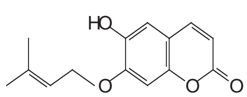
II. Furano- and dihydrofuranocoumarins	R	R[1]	R[2]	Source
 (–)-Heraclenol (**23**)				Trani *et al.*, 1997
				
Bergaptene (**24**)		O–CH_3	H	Compagnone *et al.*, 1993
Xanthotoxin (**25**)		H	O–CH_3	Compagnone *et al.*, 1993
Isopimpinellin (**26**)		O–CH_3	O–CH_3	Trani *et al.*, 2004
 Imperatorin (**27**)				Trani *et al.*, 1997
				
Clausindine (**28**)				Delle Monache *et al.*, 1977
Psoralene (**29**)	**H**			Erazo *et al.*, 1990
Dimethyl allyl psoralene (**30**)				Delle Monache *et al.*, 1976
				
Marmesin (**31**)	**H**			Delle Monache *et al.*, 1989
Chalepin (**32**)				Delle Monache *et al.*, 1977
 Isoangenomalin (**33**)				Delle Monache *et al.*, 1977
 Oroselone (**34**)				Murray, 1978

Table I. (cont.)

II. Furano- and dihydrofuranocoumarins	R	R^1	R^2	Source
 Angelicin (**35**)				Erazo *et al.,* 1990
 Columbianetin (**36**)				Cuca-Suarez *et al.,* 1998
III. Pyranocoumarins				
 Hortiline (**37**)				Delle Monache *et al.,* 1976
 Alloxanthoxyletin (**38**)				Cuca-Suarez *et al.,* 2002
 Xanthyletin (**39**)	H			Delle Monache *et al.,* 1976
Dimethyl allyl xanthyletin (**40**)				Delle Monache *et al.,* 1976

Gram-positive bacteria, but decreased activity against Gram-negative bacteria when compared to parent coumarin *per se* possibly due to oxidation of the isoprenylic chain at C8 of the furanocoumarin. *O*-Methylation at C5 (compound **24**) or C8 (xanthotoxin, **25**) also displayed the same pattern of activity for all tested strains. However, two OMe groups at C5/C8 (isopimpinellin, **26**) decreased the activity of furanocoumarins against Gram-negative and Gram-positive bacteria when compared to the parent coumarin. A prenyloxy chain at C8 (imperatorin, **27**) diminished the antibacterial potency against all tested bacteria, as well as substitutions at C3 as prenyl chains (compounds **28** and **30**), substitutions at C7 as prenyl or hydroxyprenyl chain (compounds **31** and **33**, respectively). It is worth noting, that chalepin (**32**) did not show improved antibacterial activity with the addition of an extra prenyl chain at C3 against all tested strains.

Table I. (cont.)

IV. Prenylated coumarins	R	R^1	R^2	Source
 Auraptene (**41**)				Delle Monache *et al.,* 1995
 Phebalosin (**42**)	CH_3			Cuca-Suarez and Delle Monache, 1991
 Balsamiferone (**43**)	H			Cuca-Suarez, personal communication
 Osthenol (**44**)				Cuca-Suarez *et al.,* 1998
 7-*O*-Geranyl esculetin (**45**)				Torres *et al.,* 1979
 Prenyletin (**46**)				Murray, 1978

Pyranocoumarins (compounds **37**–**40**) were found to be one of the two groups of coumarin analogues that proved to be significantly less effective than all the other series of coumarins derivatives against all tested microorganisms, suggesting that the pyrano ring is not required for enhancing of antibacterial activity of the coumarin *per se.*

The group of prenylated coumarins also showed weak antibacterial potency compared to coumarin *per se.* Apart from compounds **41**, **42**, **43**, **45** and **46**, which proved to be inactive against all strains, osthenol (**44**) showed the most prominent activity against Gram-positive bacteria (MIC = 62.5 μg/ml). Osthenol is a compound with prenylation at C8 and an OH group at C7, suggesting that those groups are required for good antibacterial activity mostly against Gram-positive bacteria. The results above suggest that an OH group at C7 is an ineffective function for antimicrobial activity, while the substitution pattern at C8 is noteworthy. However, two prenyl chains at C3/C6 reduced antibac-

Table II. Antibacterial activity of coumarins against four bacterial strains.

Coumarins	*E. coli*		*P. aeruginosa*		*S. aureus*		*B. cereus*	
	MIC[a]	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Monosubstituted coumarins								
Coumarin (**1**)	500 (3.42)	1000	500 (3.42)	1000	500 (3.42)	2000	1000 (6.84)	2000
6-Methylcoumarin (**2**)	500 (3.12)	2000	500 (3.12)	> 2000	1000 (6.24)	> 2000	1000 (6.24)	> 2000
6-Methoxycoumarin (**5**)	500 (2.84)	1000	500 (2.84)	> 2000	> 2000 (>11.35)	> 2000	2000 (11.35)	> 2000
6-Aminocoumarin (**8**)	1000 (6.21)	2000	2000 (12.41)	> 2000	> 2000 (> 12.41)	> 2000	2000 (12.41)	> 2000
7-Methoxycoumarin **(13)** (Herniarin)	500 (2.84)	1000	500 (2.84)	> 2000	1000 (5.68)	> 2000	1000 (5.68)	> 2000
7-Methylcoumarin (**14**)	500 (3.12)	2000	500 (3.12)	> 2000	2000 (12.49)	> 2000	1000 (6.24)	> 2000
7-*O*-Acetylcoumarin (**15**)	1000 (4.90)	1000	1000 (4.90)	> 2000	1000 (4.90)	> 2000	1000 (4.90)	> 2000
Disubstituted coumarins								
Scopoletin (**18**)	1000 (5.20)	1000	1000 (5.20)	> 2000	1000 (5.20)	> 2000	1000 (5.20)	2000
Esculetin (**19**)	1000 (5.61)	1000	1000 (5.61)	2000	500 (2.81)	1000	500 (2.81)	2000
Furano- and dihydrofurano-coumarins								
(−)-Heraclenol (**23**)	NT[b] (NT[b])	NT	2000 (6.57)	> 2000	NT (NT)	NT	500 (1.64)	2000
Xanthotoxin (**25**)	2000 (9.25)	2000	2000 (9.25)	> 2000	1000 (4.63)	2000	500 (2.31)	1000
Chalepin (**32**)	2000 (6.36)	> 2000	NT (NT)	NT	1000 (3.18)	> 2000	NT (NT)	NT
Oroselone (**34**)	2000 (8.84)	> 2000	2000 (8.84)	> 2000	1000 (4.42)	2000	500 (2.21)	1000
Angelicin (**35**)	250 (1.34)	1000	NT (NT)	NT	2000 (10.74)	> 2000	NT (NT)	NT
Prenylated coumarins								
Osthenol (**44**)	2000 (8.69)	> 2000	2000 (8.69)	> 2000	62.5 (0.27)	125	62.5 (0.27)	125

[a] MIC and MBC expressed in μg/ml (MIC also expressed in mM).
[b] NT, not tested.
For each observation: deviation from the mean $d = \pm 250$ μg/ml for compound **1** (*E. coli*), **13** (*P. aeruginosa*), **14** (*E. coli* and *S. aureus*), **32** (*E. coli*) and **35** (*E.coli*); for the remaining compounds $d = \pm 0$ μg/ml.

terial activity as well as the presence of OMe at C7 and an α,β-epoxidation of C-8 prenyl group (compound **42**). These findings suggest that a prenyl group or prenyl chain or even a prenyloxy chain at C7 may contribute for the reduction of antibacterial activity of coumarin *per se* (compounds **46**, **45** and **41**, respectively) as well as the addition of OH or OMe groups at C6.

Approx. 2000 MIC values have been obtained with all compounds (of Table I) except for those which are shown by the MIC values in Table II.

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