Preliminary Evaluation of Antimicrobial Activity of Diastereomeric *cis*/*trans*-3-Aryl(Heteroaryl)-3,4-dihydroisocoumarin-4-carboxylic Acids

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Preliminary differentiating screening of the antibacterial and antifungal activity of a series of diastereomeric *cis*/*trans*-3-aryl(heteroaryl)-3,4-dihydroisocoumarin-4-carboxylic acids (**3a**–**i**) was performed by the agar diffusion method against twelve microorganism strains of different taxonomic groups. *S. aureus* and *A. niger* were the most sensitive strains to the antibiotic effect of the tested compounds, both inhibited by 10 of 12 compounds. The most potent antibacterial agent was *cis*-3-phenyl-3,4-dihydroisocoumarin-4-carboxylic acid (*cis*-**3a**), exhibiting activity against all seven bacterial test strains.

Key words: Isocoumarin, Antibacterial, Antifungal, Homophthalic Anhydride

Introduction

Isocoumarins and their 3,4-dihydro analogues constitute a class of natural (Hill, 1986) and synthetic (Napolitano, 1997) compounds that exhibit a wide range of biological activities, including immunomodulatory (Matsuda *et al*., 1998), cytotoxic (Whyte *et al*., 1996; Devienne *et al*., 2002), antiallergic (Matsuda *et al*., 1999), anti-inflammatory/antiulcer (Shimojima *et al*., 1985) and antitumour (Patel *et al*., 2003) activities. Moreover, natural isocoumarins are recognized as potent antimicrobial agents (Hussain *et al*., 2003; Devienne *et al*., 2005). For instance oosponol possesses a marked antifungal activity (Nozawa *et al*., 1981a; Kovacs *et al*., 1997). Phyllodulcin, a well-known sweetening agent for diabetics, and other modified compounds of this type also show activity against different bacterial and fungal strains (Nozawa *et al*., 1981b; Yoshikawa *et al*., 1996). Thus, derivatives belonging to the isocoumarins family can be considered as a challenging target for the evaluation of their biological activity. As a part of an ongoing program in our laboratory aimed at the synthesis of heterocyclic compounds with potential biological activity (Bogdanov and Palamareva, 2004; Burdzhiev and Stanoeva, 2006; Kandinska *et al*., 2006), we have become interested in compounds containing the isocoumarin core in their structure (Bogdanov *et al*., 2007). The present study deals with the comparison of the influence of 3-aryl and 3-heteroaryl substituents in a 3,4-dihydroisocoumarin core in context of defining a basic structure which can be further modified to more potent antibacterial or antifungal candidate drugs. In this course, here we describe the preliminary antibiotic screening of analogues of oosponol and phyllodulcin of type **3** (Scheme 1) against twelve microorganism strains of different taxonomic groups.

Materials and Methods

Test compounds

In the present work twelve diastereomeric *cis*-/*trans*-3-aryl(heteroaryl)-3,4-dihydroisocoumarin-4-carboxylic acids were assayed. The acids *cis*/*trans*-**3a**–**f** were available from our previous investigation (Bogdanov and Palamareva, 2004). In addition, three new compounds *trans*-**3h**, **i** and *cis*-**3g**, containing a pyridinyl substituent in position 3 of the dihydroisocoumarin core were synthesized. The structure of all products was characterized by spectral methods and the purity was established by elemental analysis.

General experimental procedures

Melting points were determined on a Kofler microscope Boetius PHMK 0.5 and are uncorrected. The IR spectra were acquired in nujol on a Specord 75 and are reported in reciprocal centi-

meters. The ^{1}H NMR spectra were obtained on a Bruker Avance DRX-250 spectrometer at 250.13 MHz in the corresponding solvent given in parentheses. The chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Elemental analyses were obtained in the relevant laboratory at the Faculty of Chemistry, University of Sofia, Bulgaria. The TLC was done on precoated 0.2 mm Merck silica gel 60F 254 plates.

*General procedure for the synthesis of acids **3h, 3i***

Equivalent quantities of homophthalic anhydride (**1**) and the corresponding aldehyde **2** in dry pyridine were stirred for 30–60 min at room temperature. At the end of the reaction (TLC) the solvent was evaporated under reduced pressure and the products were obtained by crystallization of the residue.

*trans-(±)-1-Oxo-3-pyridin-3-yl-isochroman-4-carboxylic acid (**3h**)*

This compound was obtained from 0.6 g (0.0037 mol) homophthalic anhydride (**1**) and 0.41 ml (0.0048 mol) pyridine-3-carbaldehyde (**2h**). The residue crystallized as white crystals from ethyl acetate. – Yield: 0.53 g (53%), m.p. 203–205 °C (from ethyl acetate). – IR (nujol): (CO) 1720, 1730 cm^{-1}. – ^{1}H NMR (dimethyl sulfoxide-d6): δ = 4.74 (1H, d, J = 7.3 Hz, H-4), 6.05 (1H, d, J = 7.3 Hz, H-3), 7.19–7.22 (1H, m, Ph–H), 7.39–7.48 (2H, m, Pyr–H), 7.72 (1H, dt, J = 1.5, 7.5 Hz, Ph–H), 7.87 (1H, dt, J = 1.7, 8.0 Hz, Ph–H), 7.99 (1H, dd, J = 1.5, 7.8 Hz, Ph–H), 8.55 (1H, dd, J = 1.2, 4.4 Hz, Pyr–H), 8.63 (1H, d, J = 1.7 Hz, Pyr–H). – $C_{15}H_{11}NO_4$: calcd. C 66.93%, H 4.07%; found C 67.13%, H 3.95%.

*trans-(±)-1-Oxo-3-pyridin-4-yl-isochroman-4-carboxylic acid (**3i**)*

This compound was obtained from 1.81 g (0.011 mol) homophthalic anhydride (**1**) and 1.16 ml (0.012 mol) pyridine-4-carbaldehyde (**2i**). The residue crystallized as white crystals from ethyl acetate. – Yield: 2.2 g (74%), m.p. 187–189 °C (from methanol). – IR (nujol): (CO) 1725, 1730 cm^{-1}. – ^{1}H NMR (dimethyl sulfoxide-d6): δ = 4.69 (1H, d, J = 5.0 Hz, H-4), 6.13 (1H, d, J = 5.0 Hz, H-3), 7.12 (1H, d, J = 5.9 Hz, Ph–H), 7.37–7.49 (2H, m, Pyr–H), 7.73 (1H, t, J = 7.6 Hz, Ph–H), 7.85 (1H, dt, J = 1.3, 7.5 Hz, Ph–H), 7.94 (1H, dt, J = 7.5 Hz, Ph–H), 8.37 (1H, d, J = 5.0 Hz, Pyr–H), 8.55 (1H, d, J = 5.0 Hz, Pyr–H). – $C_{15}H_{11}NO_4$: calcd. C 66.93%, H 4.07%; found C 66.94%, H 4.08%.

*Procedure for the synthesis of cis-(±)-1-oxo-3-pyridin-2-yl-isochroman-4-carboxylic acid (**3g**)*

To a mixture of 1.62 g (0.01 mol) homophthalic anhydride (**1**) and 1.15 ml (0.012 mol) pyridine-2-cabaldehyde (**2g**) in 15 ml dry benzene 1.38 g (0.013 mol) powdered sodium carbonate was added. The reaction mixture was stirred for 30 min at room temperature. At the end of the reaction (TLC) the reaction mixture was diluted with 100 ml ethyl acetate and was extracted with 10% Na_2CO_3. The alkaline water layer was acidified with 10% HCl and extracted with ethyl acetate. The organic layer was washed with water (pH 7), dried with Na_2SO_4 and the solvent was then evaporated under reduced pressure giving an oil. The latter afforded white crystals from ethyl acetate. – Yield: 2.15 g (80 %), m.p. 165–167 °C (from methanol). – IR (nujol): (CO) 1710, 1740 cm^{-1}. – ^{1}H NMR (dimethyl sulfoxide-d6): δ = 4.52 (1H, d, J = 3.5 Hz, H-4), 5.98 (1H, d, J = 3.5 Hz, H-3), 7.37–7.42 (1H, m, Ph–H), 7.58–7.63 (3H, m, Pyr–H), 7.73 (1H, dt, J = 1.5, 7.5 Hz, Ph–H), 7.92 (1H, dt, J = 1.7, 7.8 Hz, Ph–H), 8.04 (1H, d, J = 7.6 Hz, Ph–H), 8.61 (1H, ddd, J = 0.9, 1.7, 4.7 Hz, Pyr–H). – $C_{15}H_{11}NO_4$: calcd. C 66.93%, H 4.07%; found C 66.80%, H 4.14%.

In vitro antimicrobial assay

The microbial assay was done by the agar diffusion method. The compounds were tested both at 20 and 200 μg/disc and the activity was evaluated by the diameter of the inhibition zone in mm. Penicillin G was used as a standard drug for comparison of the antibacterial activity. A solvent control was kept. The results obtained are summarized in Table I.

Agar diffusion method

A methanol solution was prepared for each chemical compound. The pure culture suspensions of the studied microorganisms at a concentration 108 CFU/ml were inoculated into 12 ml molten and tempered to 45 °C nutrient agar (Difco) for the bacteria, beer agar for the yeast and moulds and potato dextrose agar (Oxoid) for the phytopathogenic bacteria. The inoculated media were poured into sterile petri dishes. After deposition, 6 holes were cut in each plate using a heat-sterilized cork borer. A corresponding quantity of the

methanol solutions of each chemical compound was spilled into the holes. These solutions diffused into the agar medium while leaving the petri dishes for 2 h in a refrigerator at 4 °C. Afterwards they were put into a thermostat for 1 to 5 d at appropriate temperature to grow the test microorganisms. Antimicrobial activity was determined from the diameter (in millimeters) of the zone of inhibition obtained after incubation.

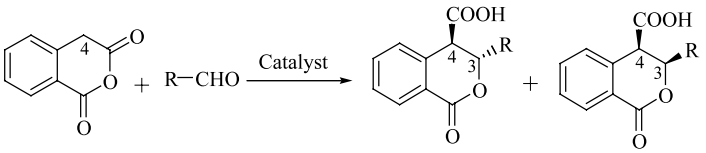
Test microorganisms

The test microorganism strains used for the microbiological assays were taken from National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC), Bulgaria, except for *S. lutea* and *P. notatum* which belong to the Department of General and Industrial Microbiology, Biological Faculty, Sofia University, Bulgaria. The antibacterial activity was tested against the bacterial strains *Bacillus subtilis* (1049 NBIMCC), *Staphylococcus aureus* (744 NBIMCC), *Sarcina lutea* (Gram-positive); *Escherichia coli* (1752 NBIMCC), *Proteus vulgaris* (1393 NBIMCC) and the phytopathogenic bacteria *Erwinia amylovora* (2331 NBIMCC) and *Pseudomonas syringae patovar siringae* (2420 NBIMCC) (Gram-negative). Antifungal activity was tested against *Candida albicans* (72 NBIMCC), *Aspergillus orizae* (118 NBIMCC), *Aspergillus niger* (1107 NBIMCC), *Fusarium oxysporum* (124 NBIMCC) and *Penicillium notatum*.

Results and Discussion

Synthesis of compounds

The synthesis of compounds **3a**–**i** is shortly presented in Scheme 1. In one step, homophthalic anhydride (**1**) and an aldehyde cyclize in the presence of a base to the target dihydroisocoumarin-4-carboxylic acids **3** as the main reaction. Both diastereomers (*cis* and *trans*) of **3** can be generally obtained because carbon atoms C-3 and C-4 are stereogenic centres. Compounds **3a**, **c**, **e** were isolated both as *cis*- and *trans*-isomers, compound **3b** was a mixture of both isomers and compounds **3d**, **f** had *trans*-configuration. Trying to perform for the first time the reaction between homophthalic anhydride (**1**) and 2-, 3- and 4-pyridine carbaldehydes (**2g**–**i**), we first used cyclization conditions similar to those applied by Girotra and Wendler (1976) for the preparation of zearalenone, namely pyridine was used both as solvent and a catalyst of the reaction. The consumption of **1** was monitored by TLC. Using this strategy 3-pyridin-4-yl-3,4-dihydroisocoumarin-4-carboxylic acid (**3i**) was obtained in good yield (74%), 3-pyridin-3-yl-3,4-dihydroisocoumarin-4-carboxylic acid (**3h**) was obtained in moderate yield (53%), whereas the reaction between **1** and pyridine-2-carbaldehyde (**2g**), under the same reaction conditions, gave a complex reaction mixture (TLC), and 3-pyridin-2-yl-3,4-dihydroisocoumarin-4-carboxylic acid (**3g**) could not be isolated. Thus, the position of the nitrogen atom in the pyridine ring is of significant importance for the course of the reaction in the presence of pyridine. Alternatively, the later reaction was carried out in the presence of powdered sodium carbonate as a catalyst in dry benzene (Kaji *et al.*, 1986). After work-up of the reaction mixture the 3-pyridine-2-yl acid *cis*-**3g** was isolated in 80% yield. The structure of all products was characterized by spectral methods (IR and ^{1}H NMR) and elemental analysis. The configuration



1 **2a-i** *trans*-**3a-f,h,i** *cis*-**3a-c,e,g**

	R = Ar
a	Phenyl
b	Benzo[1,3]dioxol-5-yl
c	4-Nitro-phenyl
d	N-methyl-carbazol-3-yl

	R = HetAr
e	Thiophene-2-yl
f	Furan-2-yl
g	Pyridin-2-yl
h	Pyridin-3-yl
i	Pyridin-4-yl

Scheme 1.

of **3** was determined on the basis of the vicinal coupling constant $J_{3,4}$ in ^{1}H NMR spectra (Bogdanov *et al.*, 2004; Kamienska-Trela and Wojcik, 2006). *trans*-Configuration was attributed to the isomers with the greater $J_{3,4}$ ($J_{3,4} > 5$ Hz) and *cis*-configuration was attributed to the isomer with the smaller $J_{3,4}$ ($J_{3,4} < 4$ Hz) value.

Antimicrobial activity

As it can see in Table I, acids **3a**–**i** showed a higher antibacterial than antifungal activity. *S. aureus* was the most sensitive strain concerning the antibacterial effect of the tested compounds (10 of 12 compounds active) followed by *B. subtilis* (9 compounds active), *P. vulgaris* (8 compounds active), *S. lutea* (7 compounds active), *E. coli* and *E. amylovora* (6 compounds active), whereas *P. syringae* was the least sensitive microorganism (1 compound active). It is worth noting that 8 compounds have MIC ≤ 20 μg/ml against *P. vulgaris*. It is clear that **3a**–**i** are active both against Gram-negative and Gram-positive bacteria except for phytophathogenic *P. syringae*. Consequenly, the investigation of the antibacterial activity of other derivatives of **3** against these microorganisms should be further considered. The most potent antibacterial agent among the tested compounds was the acid *cis*-**3a**, exhibiting activity against all seven bacterial test strains, followed by *trans*-**3c** and *trans*-**3e** (active against 6 strains), *cis*/*trans*-**3b**, *cis*-**3e** and *trans*-**3a**, **f** (active against 5 strains), *cis*-**3c** (active against 4 strains), whereas *N*-containing *trans*-**3d**, **h**, **i** were the least active compounds, being active against 2 or 1 strains, respectively. *trans*-**3g** was active against none of the bacterial strains. The results obtained showed that the antibacterial activity did not depend on the configuration of the compounds in the cases studied. Thus, the antibacterial effect of acids *cis*-**3a**, **c**, **e** was similar to that of the relevant *trans*-isomers. *N*-containing substituents at position 3 in the isocoumarin core did not evoke activity and compounds **3d**, **g**–**i** were not active against most of the bacterial strains. On the other hand, 3-thiophen-2-yl- and 3-furan-2-yl-substituted compounds **3e**, **f** were more active than the other compounds containing a heterocycle at position 3, showing activities similar to those of the aryl-substituted compounds **3a**, **b**, **c**. Thus, in the majority of the cases, the presence of heteroaryl group instead of the phenyl substituent did not evoke antibacterial activity against the strains tested. It is worth noting that none of the tested

Table I. *In vitro* antimicrobial activity. Diameter of inhibition zone (mm) as a criterion for the antibacterial activity[a] of the tested compounds.

Compound	Microorganisms											
	Bacterial strains							Fungal strains				
	Gram-positive			Gram-negative								
						Phytopathogens						
	B. subtilis	*S. aureus*	*S. lutea*	*E. coli*	*P. vulgaris*	*E. amylovora*	*P. syringae*	*C. albicans*	*A. orizae*	*A. niger*	*F. oxysporum*	*P. notatum*
cis-**3a**	11	11	10	12	16[b]	11	10	10	–	11	–	–
trans-**3a**	12	9	10	–	18[b]	12	–	9	–	10	–	11
cis-/*trans*-**3b**	12	12	9	10	16[b]	–	–	–	–	10	–	–
cis-**3c**	11	13[b]	9	–	–	11	–	9	–	9	–	–
trans-**3c**	13[b]	12	9	11	13[b]	10	–	–	–	–	–	–
trans-**3d**	–	11	–	–	11	–	–	–	9	10	–	–
cis-**3e**	11	12	–	13[b]	13[b]	13[b]	–	–	–	9	–	–
trans-**3e**	12	12	10	13[b]	16[b]	11	–	9	–	12	–	10
trans-**3f**	12	12	10	12	16[b]	–	–	–	10	8	–	10
cis-**3g**	–	–	–	–	–	–	–	–	10	9	–	–
trans-**3h**	11	–	–	–	–	–	–	10	–	9	–	–
trans-**3i**	–	10	–	–	–	–	–	–	–	–	–	–
Penicillin G	32	55	57	20	21	13	nd[c]	–	–	–	–	–

[a] Minimum inhibitory concentration (MIC) ≤ 200 μg/ml if not stated otherwise.
[b] MIC ≤ 20 μg/ml (see text).
[c] Not determined.

compounds showed a superior activity of the standard drug, except for the 3-thiophen-2-yl-substituted acid *cis*-**3e** showing the same zone of inhibition like Penicillin G against the gram-negative phytopathogenic bacterium *E. amylovora*.

A. niger was the most sensitive fungal test strain which was inhibited by 10 of 12 compounds. The activity decreased for the following strains: *C. albicans* (5 compounds active), *A. orizae* and *P. notatum* (3 compounds active), wereas *F. oxysporum* was the least sensitive fungal strain inhibited by 0 compounds. It is worth noting that none of the tested compounds showed MIC ≤ 20 μg/ml against the fungal strains. Thus, the antifungal activity of **3a**–**i** is limited. As we mentioned above, oosponol having an isocoumarin structure shows a remarkable high antifungal activity. Consequently, the difference in the structure of this compound relative to the structure of the tested compounds is of importance.

In conclusion, a series of *cis*/*trans*-3-substituted-aryl 3,4-dihydroisocoumarin-4-carboxylic acids were evaluated for their antibacterial and antifungal activity against twelve microorganism strains. The tested compounds were more active against some bacteria than the fungi, but none of them showed superior activity in comparison of the standard drug used. Nevertheless the moderate activity, some hints for further development of more potent antibacterial and antifungal candidate drugs with a dihydroisocoumarin core in their structure were obtained.

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