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Synthesis and biological profiling of novel isocoumarin derivatives and related compounds

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Abstract: In the continuation of our study of substituted isocoumarins a series of novel 3-azolyl isocoumarin and their thio derivatives, including some related lactone compounds was prepared and biologically profiled against *C. albicans* showing moderate activity with *MIC* values in range of 4–60 μg mL^{-1}, in general. The additional characterisation of selected compounds was carried out by exploring their activity on CYP3A4 and CYP2D6 enzymes, while experiments on mutagenicity were performed by AMES test. The representative isocoumarins **3b**, **4a** and **4b** showed lower inhibitory activity on CYP enzymes, when compared to the reference inhibitors, ketoconazole and quinidine. Compound **4a** showed a higher mutagenic potential than the other two compounds. Further characterization included cytotoxicity profiling against normal MRC5 cells.

Keywords: antifungal compounds; isocoumarins; *Candida albicans*; CYP enzymes; AMES.

INTRODUCTION

Isocoumarin derivatives have been intensively studied in recent decades.[1–3] In synthetic chemistry, they have been widely explored as starting compounds for the preparation of other heterocyclic derivatives.[4,5] Furthermore, these compounds have been subject of detailed research in medicinal chemistry due to their promising general biological potential.[6–8] Isocoumarin skeleton can be con-

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sidered as a privileged structure, as it is frequently encountered in naturally occurring compounds and also in plethora of synthetic derivatives exhibiting whole array of physiological properties. Natural isocoumarins may or may not have C(3) double bond but are rarely unsubstituted. They possess various substituents diversely positioned around the core, although most frequent substitution pattern, represented by structures in Fig. 1, is characterized by the oxygenated aromatic ring and functionality at C(3).

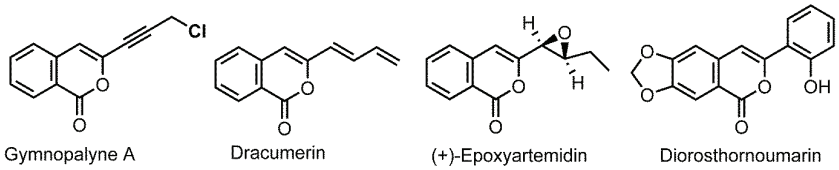


Fig. 1. Structures of some natural isocoumarins.

The C(3)-derived isocoumarins, either natural or synthetic, are known to have broad range of biological activities, such as anti-inflammatory, antimalarial, antimicrobial, antifungal, cytotoxic, antiangiogenic.[6–8] We have disclosed earlier a novel class of these derivatives possessing a heterocyclic ring at C(3).[9] Those compounds showed excellent antifungal properties, in some instances even better than voriconazole, which was used as a standard. Some derivatives demonstrated activity against azole resistant strains such as *Candida krusei* and *Candida parapsilosis*. Brief exploration of the mechanism of action suggested that the selected compounds did not cause any degradation or any interactions with *Candida albicans* DNA, implying that the origin of the biological profile of studied isocoumarins is not related to structural modifications of DNA molecules.

In order to explore further the structure–activity relationship (SAR) for this class of compounds and perform wider biological characterisation, a small series of additional isocoumarin derivatives and related compounds were synthesised and tested for antifungal activity on *C. albicans.* Further biological profiling was carried out by exploring cytotoxicity on healthy human MRC5 cells, inhibitory potential on cytochrome P450 (CYP) enzymes, and mutagenicity using AMES assay.

EXPERIMENTAL

General

The NMR spectra were recorded on Bruker Ascend 400 (400 MHz) and Bruker Avance III (500 MHz) spectrometers. Chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as the internal standard. Deuterochloroform was used as a solvent, unless otherwise stated. Mass spectral data were recorded using Agilent Techologies 6520 Q-TOF spectrometer coupled with Agilent 1200 HPLC or Agilent Technologies 5975C MS coupled with Agilent Technologies 6890N GC. IR spectra were recorded on IR Termo Scientific Nicolet iS10 (4950) spectrometer. Melting points were determined using Boetius PHMK 05 apparatus without correction. Flash chromatography was carried out using silica gel 60

(230–400 mesh) while thin layer chromatography was carried out using alumina plates with 0.25 mm silica layer (Kieselgel 60 F_{254}, Merck). Compounds were seen by staining with potassium permanganate solution and Dragendorff reagent. The solvents were purified by distillation before use.

Analytical and spectral data, as well as additional experimental details are given in the Supplementary material to this paper.

General procedure for synthesis of thioisocoumarins

Thioisocoumarins were synthesised following literature procedure.[10] To a solution of isocoumarin (0.12 mmol) in dry toluene (2 mL) Lawesson's reagent (48.5 mg, 0.12 mmol) was added under nitrogen atmosphere and the mixture was refluxed for 12 h. After the reaction was complete, as indicated by TLC, the mixture was cooled to room temperature and solvent was removed under reduced pressure. The residue was purified by flash chromatography in order to get the clean the product.

General procedure for synthesis of azolyl-methylisocoumarins

Azolyl-methylisocoumarins were synthesised from 3-bromomethylisocoumarin and corresponding azoles following literature procedure.[11,12]

General procedure for synthesis of 3-azolyl-phthalides

Starting compound, 3-bromophthalide (**7**) was prepared from phthalide and NBS.[13] 3-Azolyl-phthalides **8a**–**c** were synthesised from 3-bromophthalide and corresponding azoles following the procedure from literature.[14]

Biology

Antifungal activity. Standard disc diffusion assay was done for the preliminary screen using 0.25 mg of test compounds per disc. Briefly, late stationary phase cells of *C. albicans* ATCC 10231 were spread on potato dextrose agar plates (HiMedia Laboratories, Mumbai, India) and sterile cellulose discs (6 mm diameter) were applied to the surface (HiMedia Laboratories, Mumbai, India). Plates were incubated at 37 °C and zones of inhibition were measured. As a control, voriconazole (0.25 and 0.025 mg/disc) was also included in disc diffusion assay. Broth microdilution assays were carried out in RPMI medium (Sigma Aldrich, Germany) according to the standard NCCLS M27-A2 with the highest concentration of 0.5 mg mL^{-1} applied.[15,16]

Cytotoxicity. Cytotoxicity of the compounds was assessed against human lung fibroblast MRC5 cell line obtained from the American Type Culture Collection (ATCC) using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay.[17] The highest concentration of the compounds used was 0.25 mg mL^{-1}. The MTT assay was performed after 48 h treatment with compounds three times in three replicates and the IC_{50} values (concentrations at which 50 % cell growth inhibition occurred) were calculated in comparison to control (untreated cells) that were arbitrarily set to 100 %.

In vitro *inhibition of CYP3A4 and CYP2D6 enzymes*. The study was performed using commercial tests CYP3A4 P450-Glo™ and CYP2D6 P450-Glo™ provided by Promega (Madison, WI, USA). These assays use the luminescent measurement of the potential inhibition effect on the conversion of the beetle D-luciferin derivative into D-luciferin by respective CYP isoform.[18] The tests were performed in white polystyrene, flat-bottom Nunc™ MicroWell™ 96-well microplates (Thermo Scientific, Waltham, MA, USA). The bioluminescence signal was measured with the microplate reader Perkin Elmer (Waltham, MA, USA) in luminescence mode. Ketoconazole (KE) and quinidine (QD) used as the positive controls were obtained

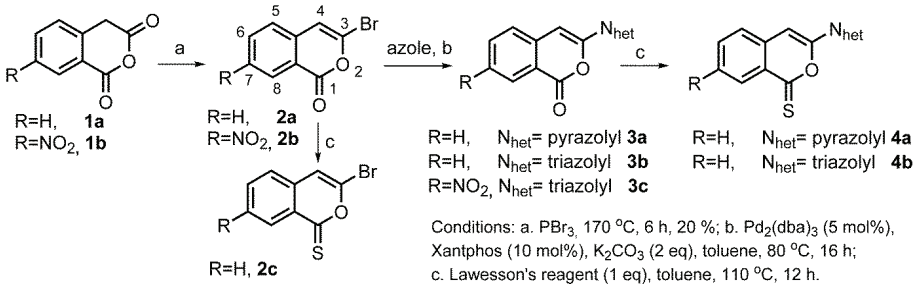
from Sigma–Aldrich. The isocoumarin derivatives were tested in triplicate in concentration range 0.01–25 μM, whereas reference inhibitors 0.0001–0.1 (QD) and 0.01–10 μM (KE), respectively.

AMES test. Ames microplate fluctuation protocol (MPF) assay was performed with *Salmonella typhimurium* TA100 strain, enabling the detection of base substitution mutations.[19] The bacterial strain, as well as exposure and indicator medium, were purchased from Xenometrix AG (Allschwil, Switzerland). *S. typhimurium* TA100 bacteria were exposed to 2 concentrations (1 and 10 μM) of a test agent, the reference was mutagen nonyl-4-hydroxyquinoline-*N*-oxide (4-NQNO, 0.5 μM) and a negative control (1 % DMSO) for 90 min in the medium containing sufficient histidine to support approximately two cell divisions. After 90 min, the exposure cultures were diluted in pH indicator medium lacking histidine, and aliquoted into 48 wells of a 384-well plate. Within two days, cells which have undergone the reversion to histidine prototrophy (revertants) reduced the pH of the indicator medium. The colour change (from purple to yellow) was detected visually by the microplate reader EnSpire Perkin Elmer (Waltham, MA, USA) at 420 nm. The number of wells containing revertants were counted for each dose and compared to the medium control baseline, which is an average of mean revertants from negative control (1 vol.% DMSO) plus the standard deviation. The two-fold increase in number of positive wells, over the medium control baseline indicated the mutagenicity borderline.

RESULTS AND DISCUSSION

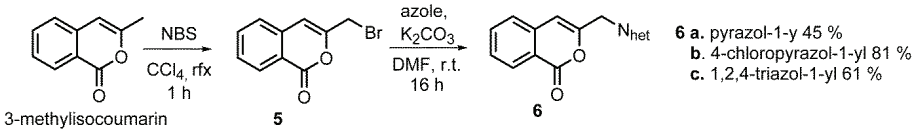
Chemistry

Various synthetic methods for the preparation of isocoumarin derivatives have been reported in the literature.[20–24] Scheme 1 shows our general approach to the synthesis of these compounds.[9] We employed the general Pd-catalysed processes for the functionalisation of the C(3) position of isocoumarin core.[25–27] The common intermediates, which provided access to several classes of compounds, were bromo derivatives **2a/b** (Scheme 1), synthesised from homophthalic anhydride.[28,29] The sulphur-containing derivatives such as **2c** and **4a/b** were prepared by the reaction of appropriate starting material and Lawesson's reagent under typical conditions (Scheme 1). The heterocyclic derivatives were synthesized *via* well-established Pd-promoted aminations, combining bromoisocoumarin scaffold and a distinctive antifungal pharmacophores such as the various azole rings (Scheme 1).[30–33]



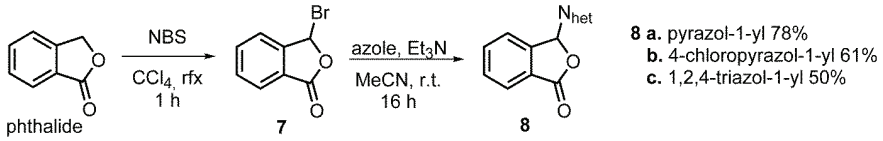
Scheme 1. Synthesis of 3-substituted isocoumarins.

For further structure–activity studies (SAR), several compounds possessing the methylene linker between the isocoumarin scaffold and the azole moiety were prepared using bromination/nucleophilic substitution sequence following the literature procedure (Scheme 2).[12]



Scheme 2. Synthesis of 3-azolylmethylisocoumarins.

With the intention to explore more chemical space defined by the above mentioned isocoumarins, an additional related class of compounds possessing phthalide moiety, was also prepared. Some phthalides previously demonstrated to have antifungal activity against plant pathogens.[34] Therefore we chose for a small series of azole containing phthalides in order to supplement SAR information (Scheme 3). [14,35]



Scheme 3. Synthesis of 3-azolylphthalides.

Biology

Antifungal activity and cytotoxicity. The antifungal and cytotoxic properties of isocoumarins from our current study and three compounds from our previous work for comparison are presented in Table I. The preliminary biological characterisation was carried out against *C. albicans* using disc diffusion assay with 250 μg of compound per disc.[15,16] For the determination of minimum inhibitory concentration (*MIC*) of the active compounds, in comparison with the clinically used voriconazole, the broth dilution method was applied. *MIC* was not determined for the compounds that showed poor activity against *C. albicans* in the disc diffusion assay. Together with the antifungal properties the cytotoxicity was also determined using human MRC5 cells and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay,[17] which is also presented in Table I. The difference between the activities of nitro derivatives **2b** and **3c** (entries 2 and 6, Table I) and the corresponding compounds without this capability **2a** and **3b** (entries 1 and 5, Table I) is notable. The effect of thiocarbonyl group in place of carbonyl on the biological profile was also briefly explored. This structural alteration was expected to be important if the lacton moiety was involved in the shaping of biological character of this class of compounds. Indeed, the derivat-

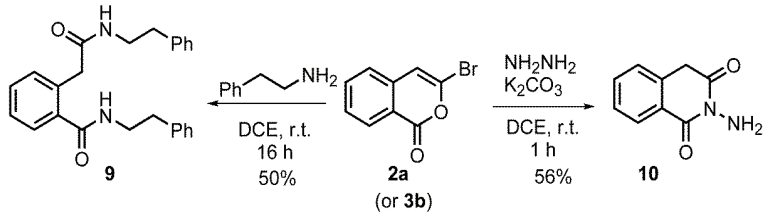
ives of this type **2c**, **4a**, **4b** (entries 3, 7, 8, Table I) showed good activity, in particular **2c**, suggesting that this structural features should be considered in designing novel isocoumarin antifungals. It should be also noted that azolyl-thio-isocoumarins, although showing comparable antifungal activity against *C. albicans*, seem to have slightly higher toxicity against normal cells when compared to the parent *O*-analogues. Unfortunately, none of derivatives possessing more flexible azole moiety such as **6a**–**c** (entries 9–11, Table I) showed any activity against *C. albicans.* Further explored class, 3-azolyl phthalides, compounds **8a**–**c**, have also shown that they were completely inactive (entries 12–14, Table I) in this study. Analysing the structural properties of the active compounds, in particular comparing the isocoumarin and phthalide derivatives, but also isocoumarins with and without directly attached azoles, it seems that the activity might be related to the electrophilicity of the lactone carbonyl.

Table I. Antifungal and cytotoxic properties of isocoumarins and derivatives; –: *MIC* was not determined; *: compounds synthesised in our previous work

Entry	Compound	*MIC* / μg mL^{-1} (*C. albicans*)	IC_{50} / μg mL^{-1} (MRC5)
1	**2a***	15.6	20
2	**2b**	60.0	100
3	**2c**	4.0	7
4	**3a***	31.2	30
5	**3b***	20	40
6	**3c**	>500	70
7	**4a**	20	5
8	**4b**	20	15
9	**6a**	-	-
10	**6b**	-	-
11	**6c**	-	-
12	**8a**	>400	>100
13	**8b**	>400	>100
14	**8c**	>400	>100
15	**9**	>500	20
16	**10**	40	90
17	Voriconazole	7.8	40

Therefore, the susceptibilities of active compounds **2a** and **3b** towards nucleophilic species[36] such as hydrazine and 2-phenylethylamine (Scheme 4) were studied. Both compounds underwent ring opening reaction under mild conditions at room temperature. Although the SAR suggested this possibility it was not a pleasing outcome, as it suggests possible mode of action of these compounds, because it would make them promiscuous towards various biological targets and might also support the compounds toxicity against MRC5 cell lines. However, the differences in *C. albicans*/MRC5 activities for some of the tested compounds suggest the involvement of more specific mechanism of action as well. The ring

opening products were also tested for antifungal activity against *C. albicans* and while the compound **9** did not show any antifungal activity, the compound **10** was moderately active (entries 15 and 16, Table I).



Scheme 4. Ring opening of bioactive isocoumarins.

Interaction with CYP3A4 and CYP2D6. The profiling of novel biologically active compounds against CYP enzymes is very important due to the fact that these enzymes have in metabolism of xenobiotics. The most active compounds surround the azole pharmacophore, a potential ligand for Fe^{3+}, containing the cytochrome P450 family of enzymes, prompted the study in this direction. Majority of drugs are metabolised by only few of CYP enzymes, amongst which CYP3A4 and CYP2D6 are perhaps the most important. Studying *in vitro* properties of the synthesised novel compounds as the inhibitors of these two isoforms in particular is a good basis for predicting their interactions *in vivo*.[37,38] Two compounds from our current (**4a**, **b)**, and one (**3b)** from our previous work, were selected for this investigation because they showed the same antifungal activity on *C. albicans*, but also various cytotoxicity. The inhibition potential of compounds was established using the luminescent assay in comparison to ketoconazole for CYP3A4 and quinidine for CYP2D6. The results outlined in Fig. 2 suggest that all three compounds, **3b** and **4a** and **b**, have significantly lower inhibition capacity towards CYP3A4 than ketoconazole. While ketoconazole at 0.1 μM concentration has strong inhibitory effect, the tested compounds, in particular **4b**, are significantly less potent. The calculated IC_{50} values supported this observation (IC_{50}: **4a** > 25 μM, **4b**, 16.8 μM, **3b**, 16.4 μM, ketoconazole, 0.14 μM).

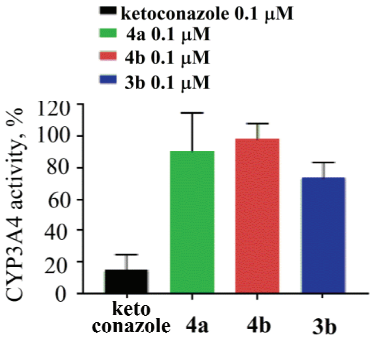


Fig. 2. Inhibition of CYP3A4 enzyme by selected compounds and standard inhibitor.

Further study of our compounds related to CYP2D6 resulted in similar outcome, Fig. 3. The comparison of inhibition effect of **4a** and **b** and **3b** with quinidine revealed very mild influence of our compounds on CYP2D6 activity. While quinidine at 0.1 μM concentration reduced activity of CYP2D6 for >80 %, all other tested compounds showed almost negligible effect. IC_{50} values found for all tested compounds **4a** and **b** and **3b** were >25 μM, whereas for quinidine were 0.01 μM).

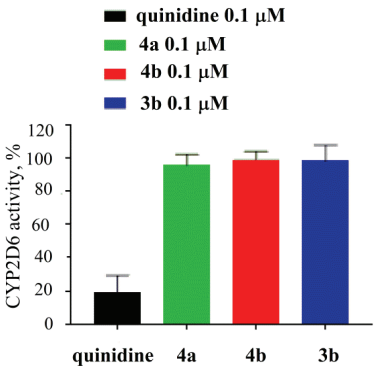


Fig. 3. Inhibition of CYP2D6 enzyme by selected compounds and standard inhibitor.

Mutagenicity (AMES test). Further profiling of the previously selected compounds **4a** and **b** and **3b** involved the determination of their mutagenic potential by the AMES mutagenicity assay with *Salmonella typhimurium* TA100 strain, which allows for the detection of mutagens that cause base-pair substitution and nonyl-4-hydroxyquinoline-*N*-oxide (NQNO), as a standard compound. This test developed few decades ago shows bacterial mutagenicity, and, consequently, may indicate carcinogenic properties of studied compounds. The profiling results summarised in Fig. 4 suggest that compounds **3b** and **4b** do not possess mutagenic properties either at 1 or 10 μM concentration (red dotted line shows muta-

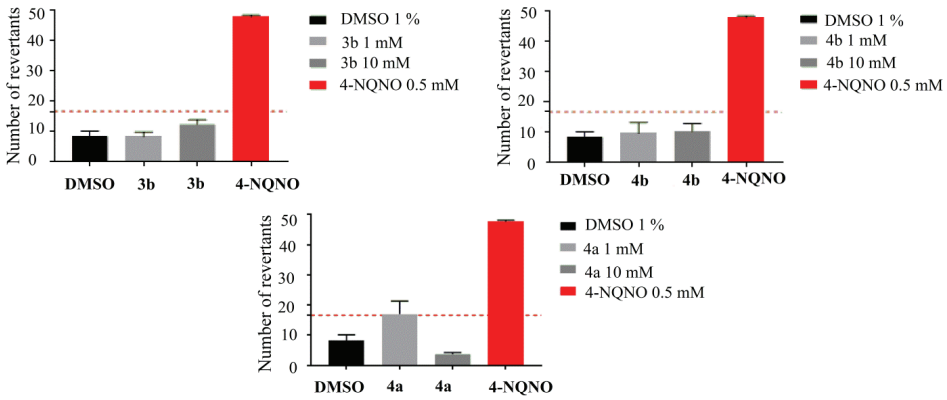


Fig. 4. Evaluation of mutagenic potential of selected compounds by AMES test. DMSO (1 vol.% in growth medium) was used as negative control.

genicity borderline).

Contrary to these, compound **4a** at 1 μM concentration showed mutagenicity, while at 10 μM the number of revertants observed were below the control level of DMSO and thus may indicate the toxic effect of this compound on *S. typhimurium* strain.

CONCLUSION

As an addition to our previous research, a series of azolyl-isocoumarins and related compounds were synthesised and tested against *C. albicans*. Novel azolyl-thioisocoumarin derivatives showed moderate antifungal activity, while azolyl-isocoumarins with the methylene linkers, andazolylphthalides, were inactive in this assay. Further profiling showed that the isocoumarin derivatives **4a** and **b** and **3b** demonstrated lower inhibitory activity on CYP3A4 and CYP2D6 enzymes when compared to ketoconazole and quinidine as reference inhibitors. The same compounds were tested by AMES assay and only **4a** showed activity at the border of mutagenicity. The presented results will be useful in further modification of isocoumarins in order to search for more efficient antifungal derivatives of this class of compounds.

SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of journal website:.https://www.shd-pub.org.rs/index.php/JSCS/index, or from the corresponding author on request.

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ИЗВОД

СИНТЕЗА И БИОЛОШКО ПРОФИЛИСАЊЕ НОВИХ ИЗОКУМАРИНСКИХ ДЕРИВАТА И СЛИЧНИХ ЈЕДИЊЕЊА

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Синтетисана је серија нових 3-азолил-изокумарина и сличних лактонских деривата и евалуирана је њихова антифунгална активност на *Candida albicans*, где су показали умерену активност (*MIC* 4–60 μg mL^{-1}). Испитана је и интеракција одабраних изокумаринских деривата са хуманим CYP3A4 и CYP2D6 ензимима помоћу луминисцентног теста, док им је мутагени потенцијал одређен AMES тестом. Испитивани изокумарини **3b**, **4a** и **4b** не показују значајну интеракцију са наведеним CYP ензимима у поређењу са

референтним инхибиторима. Једињење **4a** показује већи мутагени потенцијал у односу на друга два. Додатна биолошка карактеризација је укључила одређивање цитотоксичности према нормалним MRC5 ћелијама.

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